Silybum marianum

Images Copyright Nick Burgess

Frontispiece: *Silybum marianum:* **Courtesy Nick Burgess**

THE HEP573 STUDY:

A randomised, double-blind, placebo-controlled clinical trial of silymarin alone, and silymarin combined with antioxidants in chronic hepatitis C

Sarah (Ses) Jane Salmond

BA. (MU, NZ), N.D., D.B.M., D.H., D.N.

A thesis submitted for the Degree of Doctor of Philosophy

School of Medicine and Public Health

University of Newcastle

NSW Australia

November 2012

STATEMENT OF ORIGINALITY

The thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. I give consent to the final version of my thesis being made available worldwide when deposited in the University's Digital Repository, subject to the provisions of the Copyright Act 1968.

STATEMENT OF COLLOBORATION

Experts were consulted when necessary in the design and analysis stages as specified in the acknowledgments section and in Chapter 3 Methodology.

. . . .

Acknowledgment of Authorship
The Study protocol design and coordination, analysis and reporting of the results were all undertaken by the author.

. . . .

Date. . . . .

DEDICATION

This work is dedicated to the late Mr Robert John Salmond, my father, who taught me reverence for life, the art of perseverance and the wonder of humour. It is also dedicated to the loving memory of Ms Riwia Whaanga, Dr Lisa MacDonald, Ms Julie Velthuys and Mr Sam Richardson (who all died during the writing of this dissertation).

This also honours all those pioneers, ahead of their time in their respective fields, who willingly embraced other paradigms but, in so doing, needed to weather the attitude of their colleagues until a change in the dominant mindset prevailed.

ACKNOWLEDGMENTS

CONTENTS

AWARD, PUBLICATIONS AND PRESENTATIONS FROM THIS STUDY

AWARD

Douglas Piper Young Investigator Award Clinical Science, 22 October 2010
Gastroenterological Society of Australia

RESEARCH PUBLICATIONS AND PRESENTATIONS

Publications

Peer-reviewed journal articles

Batey RG, Salmond SJ, Bensoussan A. Complementary and alternative medicine in the treatment of chronic liver disease. Curr Gastroenterol Rep. 2005;7(1):63-70.

Conference proceedings

Salmond SJ, George J, Strasser SI, Byth KB, Batey RG. The Hep573 Study – a randomised, double-blind, placebo-controlled trial of silymarin alone or combined with antioxidants in chronic hepatitis C [paper abstract]. In: Proceedings of Australian Gastroenterology Week; 2010 Oct 20-23; Gold Coast. J Gastroenterol Hepatol. 2010;25(Suppl 3):A123-A124. Oral presentation.

Salmond SJ, George J, Strasser SI, Batey RG.Hep573 Study – a randomised, double-blind, placebo-controlled trial of silymarin alone or combined with antioxidants in chronic hepatitis C [paper abstract]. In: Proceedings of Digestive Diseases Week, AASLD; 2010 May 1-5; New Orleans. Gastroenterology. 2010;138(5 Suppl 1):S789-S790. Oral presentation.

Salmond SJ, George J, Strasser SI, Batey RG. Hep573 Study of Complementary medicine in the treatment of chronic hepatitis C [poster abstract]. In: 12th International Symposium on Viral Hepatitis and Liver Disease; 2006 Jul 1-5; Paris, Palais des Congrès. J Clin Virol. 2006;36(Suppl 2):S134-5. Poster presentation.

Salmond SJ, George J, Strasser SI, Batey RG. Hep573 Study of alternative therapy for chronic hepatitis C [paper abstract]. In: Proceedings of the Shanghai-Hong Kong International Liver Congress; 2006 March 25-28; Shanghai, China. J Gastroenterol Hepatol. 2006;21(Suppl 2):A156. Oral presentation.

Book chapters

Salmond SJ. The Hepatobiliary System. In: Hechtman L, editor. Clinical Naturopathic Medicine. Sydney: Elsevier; 2012. p. 210-279.

Salmond SJ, Bensoussan A. Natural therapies and hepatitis C. In: Dore G, Temple-Smith M, Lloyd A, Editors. Hepatitis C: an expanding perspective. Melbourne: IP Communications; 2009. p. 211-229.

Salmond SJ, Batey RG. Complementary therapies and hepatitis B. In: Mathews G, Robotin M, Editors. B Positive- All you wanted to know about hepatitis B: A guide for primary health care providers. Sydney: Australasian Society for HIV Medicine (ASHM); 2008. p. 96-101.

(For complete list see Appendix A.)

LIST OF ABBREVIATIONS

ALA/LA	alpha lipoic acid/lipoic acid/thioctic acid
ALD	alcoholic liver disease
ALT	alanine aminotransferase
AP-1	activator protein-1
ARE	antioxidant response element
AST	aspartate aminotransferase
ATP	adenosine triphosphate
CAM	complementary and alternative medicine
CAT	catalase
CHC	chronic hepatitis C
CLD	chronic liver disease
CM	complementary medicine
C of A	certificate of analysis
CTL	cytotoxic T lymphocyte
CR	calorie restriction
DNA	deoxyribonucleic acid
ECM	extracellular matrix
ERK	extracellular signal-regulated protein kinase
ESLD	end-stage liver disease
EVR	early virological response
F	fibrosis staging on liver biopsy
FM	fibrosis markers
GC-MS	gas chromatography coupled to mass spectrometry
GGT	gamma-glutamyltranspeptidase
GPx	glutathione peroxidase
GSH	glutathione (reduced)
GSSG	glutathione (oxidised)
GST	glutathione transferase
HA	hyaluronic acid
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
HCV RNA	hepatitis C virus ribonucleic acid
HLA	human leukocyte antigen
HPLC	high performance liquid chromatography

HQLQ™	hepatitis quality of life questionnaire
HRQoL	health related quality of life
HSC	hepatic stellate cell
IFN	interferon
IL	interleukin
INN	International Nonproprietary Name
ISO	F_2-Isoprostanes
IVDU	intravenous drug use
KIR	killer cell immunoglobulin-like receptor
MCP	monocyte chemoattractant protein
MDA	malondialdehyde
MF	myofibroblast
MIP	macrophage inflammatory protein
NAC	N-acetyl cysteine
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
NAFLD	non-alcoholic fatty liver disease
NF-κB	nuclear factor kappa B
NK	natural killer cell
Nrf2	nuclear erythroid factor-2
NSW	New South Wales, an Australian State
OR	odds ratio
OS	oxidative stress
P	placebo
P	probability value
P53	tumour protein 53
PCD	programmed cell death
PDGF	platelet-derived growth factor
PNAL	persistently normal ALT level
PPARγ	peroxisome proliferator-activated receptor gamma
RCT	randomised controlled trial
RDBPCT	randomised, double-blind, placebo-controlled trial
RNA	ribonucleic acid
RNS	reactive nitrogen species
ROS	reactive oxygen species
RVR	rapid virological response

S	silymarin
SOD	superoxide dismutase
SOX	silymarin and antioxidant
STAT	signal transducer and activator of transcription
SVR	sustained virological response
TLC	thin-layer chromatography
TLRs	toll-like receptors
TM	traditional medicine
TGF-β	transforming growth factor beta
TNF-α	tumour necrosis factor alpha
Treg	T regulatory cell
TRX, Trx	Thioredoxin
UV/VIS	ultra-violet and visible spectroscopy
WBC	white blood cell
WHO	World Health Organization

GLOSSARY

Antioxidant is a substance that markedly slows or prevents oxidation of a substrate, when the substance is in low concentrations compared to that substrate.[1]

Complementary medicine is an approach to health-care delivery that incorporates disease diagnosis, treatment and/or prevention and adds to conventional medicine by satisfying unmet demand or by broadening orthodox medicine's theoretical structures.[2]

Compensated hepatitis C is an early phase of end-stage liver disease characterised by asymptomatic cirrhosis.[3] It is reflected in a low level of complications from cirrhosis (e.g., jaundice, ascites, coagulopathy, and encephalopathy) as is characterised by a Child-Pugh Score of less than 7.

Decompensated hepatitis C is the advanced phase of end-stage liver disease characterised by portal hypertension and or liver dysfunction[3] (jaundice, ascites, or hepatic encephalopathy).[4] The Child-Pugh Score is greater than 7.

Free radicals are molecules with an outer (valence) shell that contains an unpaired electron.[5]

International Nonproprietary Names (INN) help to identify pharmaceuticals or their active ingredients. Each INN provides a unique name that is public property and recognisable globally. A nonproprietary name is otherwise known as a generic name.[6]

Karyorrhexis is the fragmentation of the nucleus.[7,8]

Oxidative damage refers to the biomolecular harm when a reactive species attacks during oxidative stress.[9]

Oxidative stress (OS) is an imbalance between oxidants (reactive species production[9], radical generating activity[10]) and antioxidants (antioxidant defence,[9] radical scavenging activity[10]) in favour of the oxidants, potentially leading to (tissue) damage.[5]

Powdered extract (P.E.) refers to a dried extract. Fresh or dried plant material may be extracted in water, methanol, ethanol or other solvents to produce a liquid extract. This extract is then typically spray-dried to produce a dry or powdered extract. The powdered extract ratio is required for calculating the corresponding crude drug (plant) amounts e.g. P.E. 5:1 indicates that 5 kg of dried plant material was used to produce 1 kg of dried extract. The powdered extract ratio is synonymous with the drug-to-extract ratio.[11]

Reactive oxygen species (ROS) is a collective term for oxygen radicals, such as superoxide anion (O_2^-), hydroxyl radical (OH), hydroperoxyl (HO_2), peroxyl (RO_2), alkoxyl (RO) and carbon dioxide (CO_2). It also includes some non radicals which are oxidising agents and/or are easily converted into radicals, such as hydrogen peroxide (H_2O_2), ozone (O_3), singlet oxygen (O_2^1), organic peroxides (ROOH), and peroxynitrite (ONOO).[9]

Reactive nitrogen species (RNS) collectively refers to radicals of nitric oxide (NO) and nitrogen dioxide (NO_2). It also includes some non radicals such as nitrous acid (HNO_2), dinitrogen tetroxide (N_2O_4) and peroxynitrite ($ONOO^-$).[9]

Redox status refers to the ratio of reduced (GSH) glutathione to oxidised (GSSG) glutathione.[12]

Social determinants of health are conditions (including health systems) influenced by the distribution of resources, money and power locally, nationally and globally. These, in turn, are influenced by policy decisions. Social determinants of health are the main causes of health inequities within and between countries.[13]

Standardisation means uniformity of all required manufacturing steps, from the crude drug to the final extract, in order to achieve a defined product standard (specification). Herbal extracts are typically standardised to a particular marker compound which may in some cases also be considered the active compound.[11]

Traditional medicine comprises all knowledge, skills and practices which derive from indigenous ideas, beliefs and experiences of different cultures in order to maintain health, and to prevent, diagnose, treat, or improve physical and mental illnesses.[14]

LIST OF APPENDICES

LIST OF FIGURES

LIST OF TABLES

PREFACE

Chapter 1 Introduction: outlines the scientific aims of the Study.

Chapter 2 Literature Review: contains an overview of the hepatitis C virus (HCV) infection; epidemiology, virology, natural history, causes of liver injury including viral, immune, oxidative stress (OS) and the pathobiology of the disease. It also examines the clinical implications of chronic HCV infection and the current management strategies for the chronic hepatitis C (CHC) patients within naturopathic and allopathic paradigms.

Chapter 3 Methodology: outlines the Study design, procedures, quality control, outcome measures and statistical analyses.

Chapter 4 Results: reports on the results achieved in the Hep573 Study.

Chapter 5 Discussion: presents the findings, strengths, limitations and implications of the Study and outlines future research directions.

Chapter 6 Conclusion: offers some concluding remarks.

The Appendices: include all approved, supporting documentation related to the conduct of the Study.

Throughout this dissertation, the research undertaken will be referred to as the 'Hep573 Study' or the 'Study'.

ABSTRACT

Oxidative stress (OS) is a key mechanism by which liver injury occurs in chronic hepatitis C (CHC) virus infection. For this Study, it was hypothesised the use of antioxidant compounds would reduce OS, hepatic necroinflammation and hepatic fibrosis in CHC patients. To test this hypothesis, a randomised, double-blind, placebo-controlled clinical trial (termed the 'Hep573 Study') was conducted in three Australian teaching hospitals in New South Wales.

One hundred and eighteen participants were recruited through the liver outpatient clinics at the hospitals from July, 2003 to March, 2006. They were randomised to treatment in blocks of six to one of three groups: placebo; silymarin (720 mg silybin/day); and silymarin with antioxidants (720 mg silybin plus 13 other ingredients).

Study duration was 48 weeks: 24 weeks on active treatment or placebo, and 24 weeks follow-up post treatment.

The primary outcome measure was the proportion of patients with alanine aminotransferase (ALT) normalisation at Week 24 (Fisher's exact test). Secondary outcome measures were the percentage change from baseline to Week 24 in F_2-isoprostanes, and to Week 24 and Week 48 in ALT, HCV viral load (HCV RNA) and FibroTest (Linear Mixed Effects). Results were analysed on an intention-to-treat basis.

In patients with compensated CHC, the use of silymarin and antioxidant compounds achieved a higher rate of ALT normalisation than placebo (P=0.02) or silymarin (P=0.003) at Week 24. This result could not be attributed to alcohol, diet or caffeine, as intake across the groups did not change throughout the Study. In addition, there was a significant improvement in the overall mental-health score (Mental Component Summary), *QualityMetric Hepatitis Quality of Life Questionnaire™ (HQLQ)* in the silymarin and antioxidant (SOX) group (P=0.002).

This novel randomised, double-blind, placebo-controlled trial of oral silymarin and oral antioxidants has shown a reduction in hepatic necroinflammation and an improvement in overall mental-health status in a specific CHC population.

CHAPTER 1

INTRODUCTION

The focus of this thesis was a randomised, double-blind, placebo-controlled clinical trial testing the safety and efficacy of silymarin alone, or silymarin in combination with antioxidants in chronic hepatitis C (CHC) patients. This trial was conducted in three Australian teaching hospitals (John Hunter Hospital, Newcastle; Royal Prince Alfred and Westmead Hospitals, Sydney).

The understanding in the literature at the commencement of this clinical trial (*c.* 2002/03) was that antioxidants could ameliorate disease progression in CHC patients by reducing oxidative stress (OS), a key mechanism contributing to liver inflammation and hepatic fibrosis. The causes and consequences of OS became the focus of the research and this is represented diagrammatically in Figure 1.1.
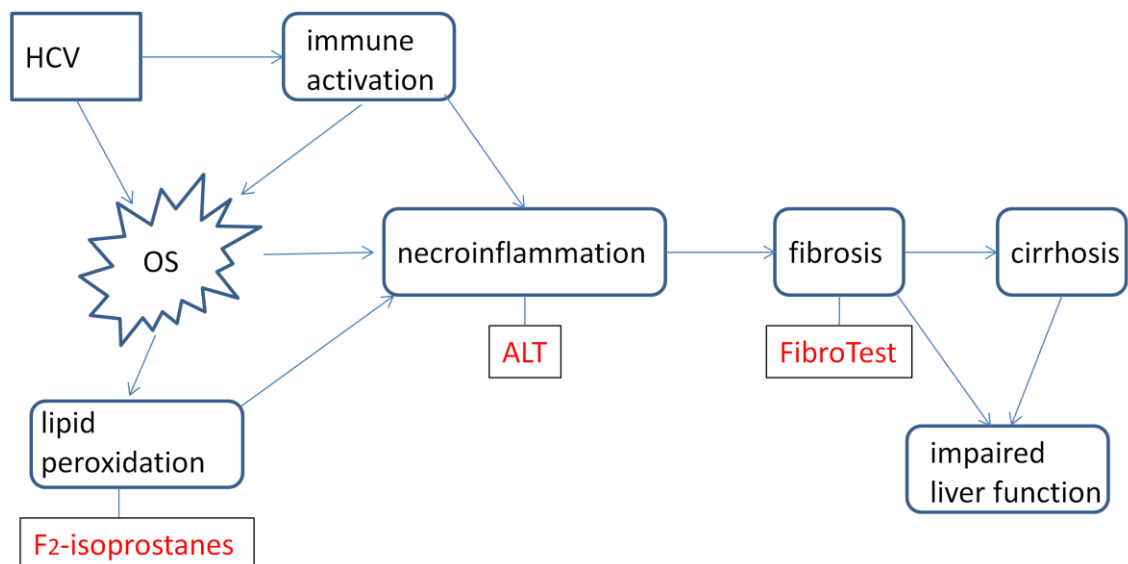
Figure 1.1: Diagramatic representation of pathogenesis of HCV relevant to this thesis.

Oxidative stress and the inflammatory process (Figure 1.1) generated by the HCV infection combined with the host immune response to HCV infection, drive further liver injury and therefore, disease progression in some CHC patients.

Phytochemical-based antioxidants were chosen in this original research to influence OS pathways and enhance the endogenous antioxidant defences and thereby modify liver disease progression.

Two key papers Jain*, et al.,*[15] and Yadav, *et al.,*[16] influenced the direction of this research. Jain*, et al.,*[15] found the antioxidants glutathione, selenium, and vitamins A, C and E were all significantly reduced in CHC patients (N=42), the lipid peroxidation marker (8-isoprostane) was significantly elevated ($P<0.001$) and the ratio of oxidised (GSSG) to reduced (GSH) glutathione (redox status) was also significantly elevated ($P=0.006$). The presence of OS in this population was confirmed. In CHC patients, the striking reduction in total whole blood glutathione (GSH plus GSSG) ($P=0.001$) and total plasma glutathione ($P=0.001$), suggested that both intracellular and intrahepatic reserves of glutathione were compromised. There was a positive correlation between the urinary 8-isoprostane and type III procollagen peptide and the fibrosis score. Jain*, et al.,*[15] concluded that a spectrum of antioxidants should be investigated as they may halt or slow progression to cirrhosis.

These findings were also supported in the same year by Yadav, *et al.,*[16] who found OS present in CHC patients. Micronutrient antioxidants were severely depleted in serum and liver tissue of CHC patients. They also found increasing levels of fibrosis were associated with decreased liver antioxidant levels and suggested that severe liver disease may be a consequence of antioxidant depletion, or decreased liver storage resulting from the fibrosis.[16] The two papers by Jain*, et al.,*[15] and Yadav, *et al.,*[16] were the basis of the testing hypothesis in this Hep573 Study. i.e., the use of antioxidants would modify disease progression.

Experimental models of liver disease (common bile duct ligation,[17] carbon tetrachloride (CCl_4[18]) and cultured hepatic stellate cells[19]) have shown the administration of antioxidants can halt or retard the progression to liver fibrosis. The literature is lacking in clinical translational research that is scientifically rigorous and robust in its design. Consequently, this research would add to the literature by exploring whether antioxidants could reduce liver disease progression in CHC patients.

SCIENTIFIC AIMS OF THE TRIAL

The aim of this trial was to evaluate the safety and effectiveness of silymarin alone, or in combination with other herbal and/or vitamin antioxidant interventions in the treatment of chronic hepatitis C. The Study aimed to investigate in detail whether traditional medicines/antioxidants would:

(1) lower liver enzymes which would indicate a reduction in hepatic necroinflammation;

(2) alter the OS processes which are now recognised to be critical in the inflammatory and fibrotic process of chronic liver disease;

(3) reduce HCV RNA (viral load);

(4) reduce hepatic fibrosis which would reduce the risk for liver related outcomes;

(5) be effective and safe in the treatment of CHC;

(6) be best used as single agents *Silybum marianum* (milk thistle), or in combination with other agents targeting OS and liver inflammation;

(7) be more effective in one HCV genotype compared to another; and

(8) improve quality of life for CHC patients; and

(9) the Study also aimed to evaluate the literature base for such a trial.

A Complementary Medicine Clinical Trial in a Medical Setting?

Both the medical model and the traditional medicine (primarily plant-based) model have their rightful place in primary health-care provision and offer the broadest choice to the patient. Each paradigm has inherent strengths and weaknesses but the intent of both paradigms is to treat the patient without harm. The strength of the medical model lies in its diagnostic capabilities, and logical and scientifically validated approach. The strength of traditional medicine lies in wholism (treating the whole person), enhancing vitality, health promotion and prevention. These two paradigms are not mutually exclusive but rather need to be inclusive of each other; as evidenced in the term: 'complementary medicine'. This Study is an attempt to contribute to the marriage of the two approaches which, until recently, have tended to be seen as incompatible.

How is the Hep573 Study Original and Unique?

There is a strong theoretical basis that oxidative stress contributes to disease progression in CHC patients.[15,16,20] In 2003, no randomised controlled trials had included oral silymarin with a broad range of other oral antioxidants in chronic hepatitis C patients.

The Hep573 Study used a standardised *Silybum marianum* extract (720 mg silybin) combined with oral antioxidants in a well-defined CHC population for 12 months, inclusive of a six-month follow-up period. The outcome measures were ALT normalisation, oxidative stress, hepatic fibrosis, HCV RNA viral load and quality of life.

The Significance of This Research

Chronic HCV infection is a significant health issue, as 1.3% of the Australian population have such an infection, and this figure is expected to double in the next 20 years.[21] Only 50% of those with HCV genotype 1 (the most prevalent genotype) will obtain a sustained virological response (SVR) on current standard antiviral therapy.[22]

This research addresses a gap in the literature regarding clinical research on the use of oral silymarin and oral antioxidants in a well-defined CHC population.

CHAPTER 2

LITERATURE REVIEW

This chapter contains an overview of hepatitis C virus (HCV) infection-epidemiology; virology; the natural history; the causes of liver injury including viral, immune, oxidative stress (OS); and the pathobiology of the disease. It also reviews the clinical implications of chronic HCV infection and the current management strategies for the chronic hepatitis C (CHC) patient. Because quality of life (QOL) is impaired in CHC patients, many turn to complementary medicine in an attempt to modify their well-being; consequently, the philosophy and use of complementary medicine in the management of CHC patients is outlined.

This thesis reports a randomised, double-blind, placebo-controlled clinical trial of silymarin alone and silymarin in combination with antioxidants in CHC participants in a context of treatment options available in 2003. At this time, standard therapy was pegylated interferon and ribavirin, with well-documented shortcomings.

Hepatitis C virus infection invokes an immune response in an attempt to eradicate the virus, and this leads to cell death (to eradicate infected hepatocytes) and fibrosis as the inflammation persists. The initiation of the immune response, a normal physiological process, generates oxidative stress and physiological inflammation. Once the infection becomes chronic, inflammation becomes pathological, as the increased oxidative stress driven by accelerated cell death and fibrosis causes persistent inflammation (pathological inflammation), affecting all cell signalling pathways that are redox-regulated. (The description above will be referred to as the 'inflammatory process'.)

Silymarin and antioxidant interventions were chosen to modify oxidative stress, which, if left unchecked, causes persistent inflammation, necrosis and liver disease progression in CHC patients.

5

EPIDEMIOLOGY

General Background on Hepatitis C Infection

The hepatitis C virus was discovered in 1989[23] and enzyme-linked immunosorbent assays (ELISA)[24] were developed, which revealed HCV as the major causative agent for blood-borne non-A, non-B hepatitis and post-transfusion hepatitis[25] (the latter identified in 1975).[26] The viral genome was subsequently defined.[27] The worldwide distribution of chronic hepatitis C was recognised and Australia, while less affected than many countries, has not been spared from the epidemic.

World Figures and Geographical Distribution

Hepatitis C affects 170 million people worldwide with a total prevalence rate of 3.1 per cent. World Health Organization (WHO) regional data show prevalence rates of: Africa 5.3 per cent, the Americas 1.7 per cent, Eastern Mediterranean 4.6 per cent, Europe 1.03 per cent, South East Asia 2.15 per cent and the Western Pacific 3.9 per cent.[28]

HCV Genotype Distribution

The variation in the hepatitis C virus genomic structure has allowed its classification by phylogenetic methods into six main HCV genotypes (1-6)[29,30] worlwide and many quasi-species.[31] (Figure 2.1)
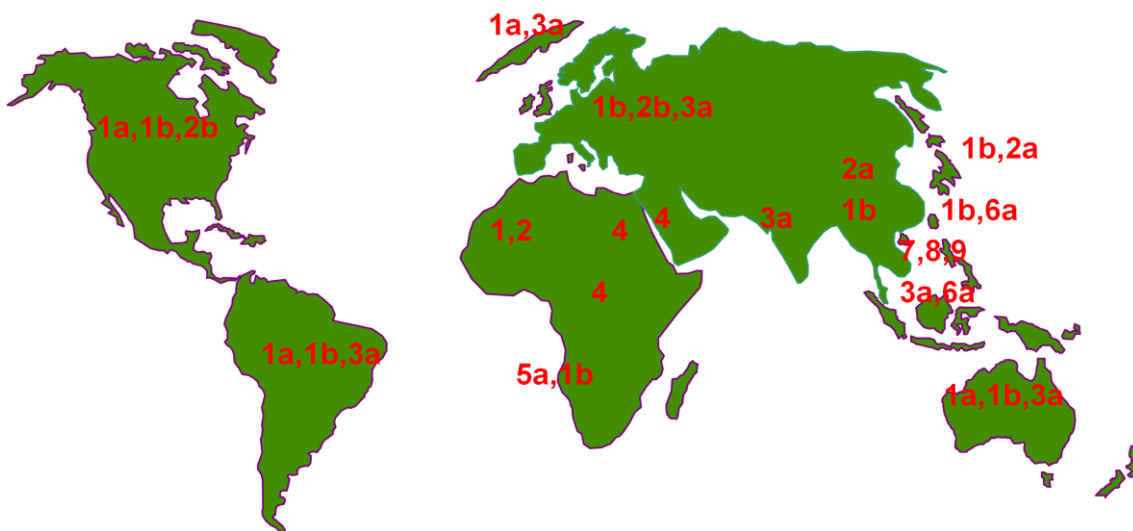
Figure 2.1: Hepatitis C virus genotypes – world view.[32]

Genotype 1a is most prevalent in the United States of America and

Northern Europe, genotype 2 (Japan and Italy), genotype 3 (Indian subcontinent, USA, Europe), genotype 4 (Africa and Middle East), genotype 5 (South Africa) and genotype 6 (Hong Kong, South East Asia).[33] HCV genotypes formally classified as 10a, 7b, 11a, 9a and 8b have been reassigned identities of 3k, 6d, 6g, 6h and 6k respectively.[30]

According to Simmonds,[34] those infected with HCV infection through injecting drug use are more likely to be infected with genotypes 1a and 3a. Genotype 4a has a high prevalence in the Middle East, particularly Egypt, because of widespread use of unsterilised needles for Bilharzia treatment in the 1950s and 1960s.[34] The most prevalent HCV genotype in Australia is genotype 1 (55%), followed by genotype 3 (33%), genotype 2 (8%), genotype 4 (3%), and other genotypes (1%).[35]

HCV genotype and viral load do not appear to exert any influence on hepatitis C disease progression.[36,37] Unlike hepatitis B viral infection, the genotypes do not appear to predict disease severity or risk of hepatocellular carcinoma (HCC).[38,39,37]

The genotype does however; predict response to treatment which determines the duration of therapy needed with the antiviral agents, pegylated interferon and ribavirin. (See Clinical Implications for the Management of the CHC Patient.)

Australian Data

The 2009 Annual Surveillance Report[40] estimated that in December 2008, 284,000 Australians (lower and upper limits of 218,000–348,000) had been exposed to HCV infection. Of the 22.2 million people in Australia in 2009,[41] 1.3 per cent was estimated to be infected with the hepatitis C virus. Of these, 72,100 (55,000–88,000) (25%) had been exposed to HCV and had cleared the virus while the remaining 75 per cent developed chronic hepatitis C virus (HCV) infection.[42] Some 162,000 (124,000–200,000) (57%) had chronic hepatitis C infection and mild liver disease (fibrosis stage (F), F0 or F1). Forty-four thousand (35,000–52,000) (15%) had stage F2/3 moderate liver disease and 5700 (4100–7100) (2%) had cirrhosis (F4). During 2008 in Australia, 229 chronic hepatitis C (CHC) patients developed hepatitis C-related liver failure and 115 progressed to hepatitis C-related hepatocellular carcinoma.[40]

In Australia, HCV-related mortality once cirrhosis is established is estimated at 2 per cent per annum.[43] The NSW Central Cancer Registry Data (one Australian

State) from 1990-2002 indicated that 12.9 per cent of all hepatocellular carcinoma cases were linked to hepatitis C infection.[44] Dore*, et al.,*[35] report the complications associated with this disease, such as liver failure and cancer, are expected to double over the next decade. This will place a considerable burden on the public health system.[35] A proportion of these patients will require a liver transplant, and hepatitis C remains the leading indication for liver transplantation in Australia. Thirty of 119 people (25.2%) who required a liver transplant in 2007 had chronic hepatitis C virus infection.[45] In 2008, the figure was 27.7 per cent.[40]

There are an estimated 10,000 new HCV infections occurring annually.[46] This figure is remarkably consistent with National Notifiable Diseases Surveillance System (2008) data of 10,666 notifications of new HCV infections at 7 December, 2008.[45] Of these new infections, 88.7 per cent are estimated to occur from injecting drug use, the predominant source of HCV transmission in Australia.[47]

CLINICAL IMPLICATIONS FOR THE MANAGEMENT OF THE CHC PATIENT

Australian guidelines in 2003 suggest a coordinated approach to the management of patients with chronic hepatitis C. This involves the general practitioner, liver specialist, other health-care specialists and community support services.[48] Other allied health-care specialists include social workers, psychologists, nutritional experts, dental practitioners and CM practitioners.

The management strategies for the CHC patient require cognisance of the natural history of chronic hepatitis C virus infection.[34] Treatment decisions for hepatitis C are based on disease presentation, genotype, laboratory values, coinfection with HIV or HBV and other comorbidities.[49] Effective therapy must break the HCV replication cycle in the liver,[50] thus the treatment progress is monitored by HCV RNA clearance and ALT normalisation. Thus the primary therapeutic aim is a sustained virological response (SVR) to treatment, with no HCV RNA detected in serum for at least six months after therapy ends, in order to interfere with the disease process and avoid end-stage liver disease (ESLD).[51] Successful therapeutic outcomes have been accompanied by improved liver histopathology[51] and, in some cases, reversal of cirrhosis.[52]

The decision to place a patient on antiviral therapy is dependent on the patient's desire to undertake treatment and their understanding of the complexity and risks of antiviral therapy matched with their psychological stability and social

support networks to cope with treatment. In addition, the disease process itself is influential in the decision-making process.

A decade ago, treatment decisions were based on biopsy evidence of advancing disease. If the disease was advanced F3 or F4 (Metavir), or likely to progress as indicated by abnormal biochemical results, the patient was strongly encouraged to begin antiviral therapy. However, if the patient was alcohol-dependent and did not want treatment, they would be strongly encouraged to reduce their alcohol intake, keep an alcohol diary and have their liver-function tests monitored.

Antiviral treatment should be made available to patients at medium to high risk of disease progression.[48] Treatment could be offered to patients at low to medium risk of progression (i.e. with less than 20 years infection and with abnormal liver tests, minimal fibrosis or inflammation and impaired QOL). If the patient has mild liver disease, genotypes 2 or 3, is well supported and wants treatment, it would be offered.

Roberts, *et al.*, discovered that seven out of 10 HCV genotype 1 patients may be cured if treatment starts before liver damage has occurred.[53] This carries a very important treatment message: 'treat early if HCV genotype 1'.[53] This may substantially increase the SVR in this genotype population to > 50%. Whilst this study involved 896 patients, it is worthwhile reconsidering antiviral therapy early in the disease process.

In the last few years, the term 'warehousing' has superseded the 'monitor, wait and see' approach. This is in anticipation of less toxic treatments, interferon-free treatments[54] and higher viral clearance rates.

Antiviral Therapy

Buti*, et al.,*[55] outlined three ways to define a response to standard therapy of pegylated interferon and ribavirin: sustained virological response (SVR), relapser (R) and non-responder (NR). A sustained virological response (SVR) defined patients who had no HCV RNA detected during treatment and follow-up. A relapser (R) was defined as a patient who was HCV RNA negative when treatment ended but was positive when followed up after 24 weeks. Non-responders continued to have HCV RNA detected during and at the end of their treatment.[55]

The response to antiviral therapy evaluated through viral kinetics has allowed researchers to detect prompt virological response (PVR) (a drop in HCV RNA ≥ 1 $\log_{10}$ in the first 48 hours),[56] rapid virological response (RVR) (serum HCV

RNA undetectable after 4 weeks), early virological response, (EVR) (serum HCV RNA undetectable or a drop in HCV RNA ≥ 2 $\log_{10}$ after 12 weeks)[57,58] and delayed virological response (DVR) (serum HCV RNA undetectable after 24 weeks).[58]

Treatment options, outcomes and side effects.

Hadzyiannis and colleagues[22] provide confidence intervals for treatment success rates with pegylated interferon and ribavirin according to genotype infection. The current antiviral therapy can lead to a sustained virological response in 84% (CI, 77%-92%) of hepatitis C patients with genotypes 2 and 3 (24 weeks of treatment) and 52% (CI, 46%-58%) in genotype 1 (48 weeks).[22] Data with confidence intervals for genotypes 4, 5 and 6 were not reported in this study.[22] The 2008 SVR rates for standard of care treatment are comparable to 2004.[59-61] Current testing protocols using RVR and EVR data are allowing a more accurate assessment than previous protocols of who will achieve a SVR, and allowing futile treatments to be terminated earlier than they would have otherwise.

Retreatment with pegylated interferon and/or ribavirin at varying doses and time lengths has helped some previous nonresponders.[62] Low-dose interferon (IFN) maintenance studies have generally been ineffective. Long-term maintenance pegylated interferon therapy C is associated with excess overall mortality in patients with advanced chronic hepatitis.[63]

The overall SVR declines considerably in patients with HCV liver cirrhosis (33.3%, CI 30.6%-36.2%) and is significantly lower in genotype 1 and 4 (N=692) (21.7%, CI 18.7%-25%) compared to genotypes 2 and 3 (N=422), (55.4%, CI 50.7%-60.1%), *P*<0.0001.[64]

Numbers treated in Australia annually. Numbers of people treated with drugs for hepatitis C infection through the highly specialised (S100) program annually in Australia were 1831, 1847, 2847, 3539, 3562, 3969 and 3760 in 2004-2010 respectively.[40,65,66]

It is predicted that by 2015 there will be an increase of about 38% in the numbers of people living with CHC and more advanced liver disease or cirrhosis.[46] In the light of these projections, the current level of treatment in Australia is not adequate to prevent an increase in the number of patients with ESLD and HCC[46,47] Some factors influencing low-treatment numbers are: interferon-related toxicity, lack of HCV treatment infrastructure along with competing patient health and social priorities.[67]

New antiviral treatments.

An increased understanding of HCV replication and its basic virology has led to the development of a range of new antiviral agents targeting HCV replication and infectivity.[68-76] Two new drugs are now approved for use in the United States of America (USA) and Europe, and the Australian Therapeutic Goods Administration (TGA) is considering these agents for approval.

The addition of the protease inhibitor, Telaprevir (specific to HCV non-structural (NS) 3/4A serine protease) to standard antiviral therapy has increased SVR rates in HCV genotype 1 patients by 51% (Telaprevir for 12 weeks) ($P<0.001$), 53% (Telaprevir for 24 weeks) ($P<0.001$) and 24% (Telaprevir and pegylated interferon for 24 weeks, no ribavirin) ($P=0.02$) compared to standard therapy.[77] Fifty-one per cent of patients developed a rash due to the addition of Telaprevir and 15% discontinued the study drugs compared to 4% on standard therapy.[77]

Another protease inhibitor, Boceprevir (NS3 protease), given for eight weeks in conjunction with pegylated interferon and ribavirin for 48 weeks, increased SVR to 68% in the non-black cohort and to 53% in the black cohort. Sixteen per cent had discontinuation and 35% had dose modifications due to adverse events. The concern with both protease inhibitors is the development of drug resistance. In this phase III clinical trial pegylated interferon and ribavirin was given four weeks prior to the commencement of Boceprevir (40 weeks) to reduce drug resistance.[78]

Table 2.1: The side effect profile of the standard of care treatment plus the two most promising protease inhibitors.

Interferon	Ribavirin	Boceprevir	Telaprevir
Anaemia[79] (19-29%)[78] Anxiety & Depression (26%)[79] Influenza-like syndromes e.g. myalgias, fever, malaise[80] (51%)[79] Neutropenia[79,80] Leucopenia[79] Thrombocytopenia[79] Autoimmune thyroid disease[80] (20%) Cardiac side effects Interstitial nephritis Vision & hearing disturbances Irritability[80] Severe fatigue[80] Apathy[80]	Anaemia[80,81] (19-29%)[78] Lymphopenia[49] Gout[49] Sinusitis[49] Hepatic iron accumulation[49] Cholelithiasis[49] Retinal changes[49] Teratogenic[49,82,83]	Anaemia[84] (49%)[78] Dysegusia[84] Drug-resistant mutations[85]	Anaemia[86] (37%)[78] Rash[81,86] (37%)[78] Pruritis[81,86] (50%)[78] Nausea[86] (43%)[78] Diarrhoea[86] (28%)[78] Retinal detachment & scotoma[86] Depression[86] Decreased haemoglobin levels[86]

The two new protease inhibitors have more than doubled the incidence of anaemia occurring in the combined pegylated interferon and ribavirin group (Table 2.1). Given the level of treatment discontinuance (15-16%) on Telaprevir and Boceprevir respectively, the increased incidence of anaemia (37-49%), development of severe rash (Telaprevir, 37%) and development of drug resistance, it is important to explore other treatment options for hepatitis C patients.

At current treatment rates in Australia, the USA and Europe, less than 10% of those infected with HCV have been treated and now interferon-free treatments are being actively explored[87] while the direct acting antivirals are in phase III clinical trial development.[54]

The side-effect profile of antiviral therapies still necessitates the exploration of alternative treatments. Treatment with complementary medicine has the potential to modify the disease process, rather than kill the virus, while new antiviral treatments and protocols are being perfected.

QUALITY OF LIFE IN HEPATITIS C PATIENTS

Quality of life (QOL) is encompassed in the WHO determinants of social health,[13] and reinforces the concept that no human is an island and their well-being is not defined by the functioning of an individual organ (liver). Quality of life is impacted by infection, host response, environmental factors (social, political and economic), diagnosis of HCV infection, disease severity, antiviral treatment, comorbidities and complementary medicine treatment.

Profoundly impaired QOL in CHC patients has been well documented.[88,89,90,91,92] A variety of questionnaires have been used to measure health-related QOL. One of the most common questionnaires is the Medical Outcomes Trust, *SF-36® Health Survey* (*QualityMetric™*, *HRQoL* Version 1, 1999) that groups the QOL questions into the following health domain scales:

(1) bodily pain (BP);
(2) general health perception (GH);
(3) mental health (MH);
(4) physical functioning (PF);
(5) role limits, emotional (RE);
(6) role limits, physical (RP);

(7) social functioning (SF); and

(8) vitality (V).

These scales are summarised into either the physical component summary (PCS) or the mental component summary (MCS) score with their corresponding correlations in brackets. The PCS score summarises: PF (.85), RP (.81), BP (.76) and GH (.69) and the MCS score includes the remaining subscales: MH (.87), RE (.78), SF (.67) and V (.65).

In the Hep573 Study, a subset of the *HRQoL,* was used. This subset has an additional liver-disease specific section and is known as the *Hepatitis Quality of Life Questionnaire* (*HQLQ™*).[91] *HQLQ™* measures health distress and limitations specific to chronic hepatitis:[93]

(1) health distress (HD);

(2) positive well being (PWB);

(3) hepatitis-specific limitations (HLIM); and

(4) hepatitis-specific health distress (HHD).

HQLQ™ has been used in a range of liver-clinic settings in chronic hepatitis C patients: treatment naïve,[94] interferon monotherapy,[95] interferon relapsers,[96,93] pegylated interferon and ribavirin therapy,[97] advanced fibrosis[97] and orthotropic liver transplantation.[98]

While there are other liver-disease, specific health-related QOL questionnaires such as the *Chronic Liver Disease Questionnaire* (CLDQ), the *Liver Disease Quality of Life Questionnaire* (LDQOL) and the *Liver Disease Symptom Index 2.0 (LDSI 2.0);* the *HQLQ™* is the most validated and reliable questionnaire[91,93] and for this reason, it was used to measure QOL in this Study.

Symptoms Reported by Those Living With Hepatitis C in Australia

Globally, the symptoms associated with HCV-related impaired QOL are: neurocognitive impairment,[88,92,99,100] depression,[88,101,102,103,104] anxiety,[94] fatigue,[88,90,103,92,105,106] emotional distress[94,104] and musculoskeletal problems.[103,105,107]

An Australian study of patients with chronic hepatitis C reported the following symptoms: physical tiredness (86%), irritability (75%), depression (70%), mental tiredness (70%), abdominal pain (68%), forgetfulness (65%), joint pain (64%), poor concentration (62%) and general body pain (57%). Sixty-two per cent

reported their symptoms tended to occur simultaneously, calling them 'hep C attacks'. The Study's social researchers coined the term 'clustering of symptoms.' [103] Another study found that 67% of patients with chronic HCV infection reported joint pain, which may be associated with the presence of a rheumatoid factor and cryoglobulinaemia.[107]

Another Australian study calculated that HCV-related morbidity during 2005 accounted for 37,800 lost-quality adjusted life years mainly occuring in people with stage 0/1 (77% lost) or stage 2/3 (19% lost) chronic HCV infection.[46]

Quality of Life Pre and Post Diagnosis with HCV Infection

Those who were unaware they had HCV infection reported a symptom-free life; however, once notified of their HCV serostatus, many developed symptoms.[108] Those who were aware of their HCV serostatus produced significantly lower scores on seven out of eight SF-36 scales (role limits-physical, bodily pain, general health, vitality, social functioning, role limits-emotional and mental health) compared to those who were unaware of their HCV serostatus. Those who were unaware had lower scores in only three scales (general health, vitality and mental health). Therefore, negative stigmatisation and being labelled with a chronic disease may have contributed to the reduced QOL reported in those diagnosed with HCV infection.[108]

Quality of Life and Comorbidities

There is debate in the literature as to how, and in what proportion, the hepatitis C virus, the host response and the social, political and economic situation (WHO) contribute to impaired QOL.[109,110]CHC patients have lower health-related QOL scores on all eight SF-36 scales at baseline compared to healthy controls ($P<0.001$).[95] Neither the SF-36 nor Hospital Anxiety Depression Scales (HADS) correlated with HCV RNA, suggesting that host and environmental factors rather than the hepatitis C virus impact on QOL.[110]

In contrast, assuming similar socioeconomic and political situations among veterans, then lower mental (MCS, $P<0.001$) and physical (PCS, $P<0.07$) component scores found in veterans suggest hepatitis C virus adversely affects QOL.[111]

In a study of environmental factors, Helbling*, et al.,*[110] reported age and diabetes were associated with a low physical summary score and intravenous drug

use (IVDU) linked with a low mental summary-score.[112] It is possible all the above factors contribute to impaired QOL.

Quality of Life and Disease Severity

Recently, severity of liver disease in chronic HCV infection has been correlated with impaired QOL, particularly in those with decompensated cirrhosis who exhibited the lowest scores in all eight SF-36® domains as well as in the summary scores, PCS and MCS ($P<0.001$).[89] Teuber, *et al.,*[90] found that the PCS was significantly lower in patients with severe fibrosis or cirrhosis compared with mild to moderate fibrosis ($P<0.001$).

Linear multiple regression analysis showed that fibrosis ($P=0.016$) and age ($P=0.001$) were significant independent predictors for the PCS.

Quality of Life and Antiviral Therapy

Both PCS ($P=0.047$) and MCS ($P=0.014$) scales worsened in CHC patients on pegylated interferon and ribavirin therapy.[113,114]

Those for whom treatment failed had lower scores on the eight SF-36 domains ($P<0.01$), lower scores on the hepatitis-specific domains ($P<0.0001$) and lower PCS and MCS scores ($P<0.01$).[115] Interferon (IFN) relapsers (N=324) were found to have impaired QOL as measured by *HQLQ™* in five of the eight domains: physical functioning, role limits-physical, general health, vitality and social functioning.

Those with SVR had improved QOL (vitality, social functioning and health distress).[93] In a post-treatment follow-up study, those with SVR had significantly improved QOL scores in the four of the eight domains: role limits-physical, general health, vitality and role limits-emotional.[97] Patients on IFN who achieved SVR had marked improvements in their quality of life.[95] CHC patients have elevated depression scores and exhibited general symptoms of emotional distress when not receiving interferon therapy.[101]

Quality of Life and Complementary Medicine

Many patients with HCV infection cannot be successfully treated with conventional treatment,[116] and about 80% of patients experience side effects whilst receiving pegylated interferon and ribavirin combination therapy,[117] and in some cases, treatment is not financially feasible.[51] Therefore some patients turn to CM for cost-effective alternatives to either treat their disease, manage their symptoms or to

alleviate the side effects of conventional treatment. Enhanced QOL and symptom management are often cited by patients as the primary motivating factors in using complementary medicine in chronic hepatitis C. According to Rambaldi,[118] many patients have tried complementary and alternative medicines in the belief that they are less toxic and more effective than conventional treatments. Such complementary and alternative medicines include those falling under the umbrella of 'naturopathy', whose main core treatments are herbal and nutritional medicines. Seeff[119] concurs with Rambaldi[118] but adds that CHC patients often use herbal medicine products, particularly silymarin, in order to boost the modest response to antiviral therapy and to minimise side effects of such treatment.

In the HALT C trial,[119] users of silymarin experience significantly less fatigue, nausea, liver pain, anorexia, muscle and joint pain, as well as improvements in general health over non-users (adjusting for age, race, education, alcohol consumption, exercise, body mass index and smoking).[119]

The concept of vitalism is a fundamental principle underlying naturopathic and herbal medicine paradigms. It is this concept of vitality that is often reflected in the QOL and well-being of the patient, and will be discussed in the principles of naturopathic and herbal medicine practice that follows some introductory comments on complementary medicine.

It is generally recognised when CM patients take an active role in their health, they feel enpowered, autonomous and in control.[120,121,122]

COMPLEMENTARY MEDICINE

Use of and Expenditure on Complementary Medicine

The percentage of the population using complementary medicine (CM) has remained constant in recent history. CM use in Australia was found to be 50% in 1993, 52.1% in 2000 and 52.2% in 2004.[123] However, the cost increased by 120% between 1993 and 2000. In 2000, an estimated $AU 2.3 billion was spent on CM, $1.67 billion on CM products, and $616 million on CM therapists, nearly four times the amount people paid for all pharmaceuticals.[124] The increase in total spending on CM between 1993 and 2000 may represent an increased use of CM products and services by those individuals who were already using CM, or an increase in price for existing products and services. In 2004, the total expenditure on CM

products compared to 2000 reduced to $1.3 billion and expenditure on CM therapists fell to $494 million. This may indicate treatment costs have recently been reducing, and CM is becoming more accessible and cost-effective for the individual than it was previously due to increased demand. It could also be that people are limiting their use of CM products and services. It is also possible the Pan Pharmaceutical recall of some CM products may have potentially caused a decline in CM use.

On the other hand, a study conducted in 2005 estimated that 69% (95% CI: 66.1%-71.7%) of the adult Australian population had used CM. The estimated total expenditure on CM products by the Australian adult population was $1.86 billion, whereas the total national expenditure on CM (including visits to CM practitioners and the use of CM products) was $4.3 billion.[125] The differences between these estimates are because the studies conducted in 1993-2004[123,124] were conducted in one Australian State, South Australia, and the 2005 study[125] was nationwide. However, the latter study had a low participation rate and this may have artificially inflated the CM figures.[125]

The Numbers of People Using Complementary Medicine in Liver Clinics

In 2001, 41% of those attending hospital outpatient liver clinics in the USA had used complementary and alternative medicine in the preceding month.[126]

In 2008, 44% of the participants in the Hepatitis C Antiviral Long Term Treatment against cirrhosis trial (HALT-C) carried out in the USA, had previously taken, or were currently taking, complementary medicines. Of those currently taking complementary medicines, 195/269 (72%) were ingesting silymarin.[119]

Thirty-five per cent (113/319) of chronic hepatitis C patients in a tertiary hospital in Spain had used, or were using CM, 63 (20%) because of chronic hepatitis, and 17% (50/319) for other reasons, reported in a self-administered questionnaire.[127] This overall figure matches research conducted at the liver clinics in Royal Prince Alfred Hospital and Westmead Hospital Outpatient Department, NSW, Australia. This showed that 35% of patients were taking complementary medicines for their hepatitis C.[128] From the John Hunter Hospital, the third participating hospital, 30% were taking complementary medicines. (See patient information sheet, version 16, 21st March, 2005, Appendix B).

The demographics of the US population taking herbal medicines in liver clinics are predominantly male, white and well educated.[119] There is a different gender demographic in Australia and Spain. The primary consumers of

complementary medicine in Australia are middle-aged (35-54 years), white, well-educated, married women.[124] However, an Australian study in 2005 found the same demographics with the exception of age (18-34 years).[125] In Spain, the users of complementary medicine are also women and well educated; however, their marital status differs from Australia data, as divorced and widowed women feature highly.[127]

The Philosophy of Herbal Medicine

Western herbal medicine refers to an eclectic practice of herbal medicine drawing on plant-based medicines from around the globe, e.g., Europe, North America, the Far East and the Middle East. Herbal medicine has been common to all cultures throughout the ages, and in 80% of the population in some countries (Asia and Africa), rely on traditional medicine.[14] The World Health Organization (WHO) refers to the use of primarily plant-based medicines as traditional medicine (see Glossary).

According to Mills,

herbalism is any system of medicine or health care that relies on plants as the source of remedies; as seen principally: (1) in almost all cultures prior to the Industrial Revolution, mostly as self help therapy but exemplified in the hands of locally trained rural practitioners (see traditional medicine) and (2) as a substantial modern alternative to conventional drug based medicine. In most countries traditional and modern usage coexist; thus herbalism today is based on remedies and techniques tried and tested through generations of use, but increasingly re-evaluated in the light of modern day medical refinements. A key feature of herbalism is that remedies are used to support and modify disturbed body functions, rather than directly attack the symptoms of disease.[129]

Many modern drugs were discovered and derived from plant-based medicines, e.g., *Digitalis species* (spp) gave rise to the cardiac glycosides. The pain medications, morphine and codeine, were derived originally from *Papaver somniferum* (opium poppy) and Aspirin™ was named and derived from *Filipendula ulmaria* (meadowsweet) previously called *Spirea ulmaria.*

Herbal medicines include herbs, materials and preparations derived from herbs, and finished products which contain plant materials as active ingredients.[14] A Western herbal medicine practitioner is defined as:

A health practitioner who engages in the extemporaneous (prescribed as part of a consultation for an individual patient) compounding of herbs for therapeutic purposes to individuals under his or her care, and who has satisfied the core training requirements in herbal medicine principles,

philosophy and practice as defined by the National Health Training Package (Australian National Training Authority 2002) for Western Herbal Medicine.[130]

The knowledge of a plant's traditional use underpins the practice of herbal medicine. According to the Therapeutic Goods Act 1989, 'traditional use' is defined as:

> use of a designated active ingredient that is well-documented, or otherwise established, according to the accumulated experience of many traditional healthcare practitioners over an extended period; and accords with well-established procedures of preparation, application and dosage.[131]

The major guiding principles of plant based medicine are:

(1) Vitalism, support the life-force;

(2) Wholism, treat the whole person;

(3) Synergy, the whole parts of the plant are used as they are the sum of constituents in that part; and

(4) Individualised treatment, the herbal prescription is tailored to the needs of the individual.[132]

In 1990, one UK medical herbalist-practitioner summarised the principles of herbal medicine, cited as follows:

(1) Treats people rather than diseases, causes rather than symptoms, individuals rather than stereotypes;

(2) Uses medication and advice to support the patient's own vital energy and self-healing potential; and

(3) Prescribes essentially non-toxic herbal treatments derived from the whole plant and not isolated from isolated or synthetic ingredients.[132]

The concept of vitalism is apparent in other disciplines of herbal medicine. Despite different cultures, different continents and different words, there is a universal principle underlying the primary purpose of traditional plant-based medicines of supporting the life-force of the patient: vitality (naturopathic medicine, North America, Western and European herbal medicine), *prana* (Ayurveda, India), *qi* (traditional Chinese medicine, China, Asia) and *tabiat* (Unani medicine, Greece, Persia, India). Vital force/life force was regarded as a creative power for self-regulating and self-healing[133] that directs the processes of metabolism, growth, reproduction, adaptation and interaction in the body.[134]

Trickey states that:

> All traditional medicine, which is based on the belief in vitalistic concepts, strives to maintain and support the individual's inherent vital energy, qi or prana. Central to this theme is that medicines should do no harm, and that correctly administered, they will assist the conservation of, or return to, a state of health.[135]

Mills summarised the integration of physiological science with traditional concepts of health and disease as follows:

> the human is a willful vibrant idiosyncratic wonderful being, not to be divided into compartments, whether these are of 'body', 'mind' or 'spirit', or separate functional fragments; all living beings are inherently self-regulating; and in health their functions are totally integrated and barely identifiable; disorders, however, manifest as patterns of dysfunction that can be recognized, charted and interpreted, to the benefit of any healing intervention.[136]

The fourth guiding principle of herbal medicine–individualised treatment is instigated after a thorough consultation following naturopathic guidelines. The relevant pharmacological/ biological actions of the herbal medicines are combined in a herbal prescription tailored to meet the needs of a patient. This does not readily lend itself easily to the rigorous examination of a randomised double-blind, placebo-controlled trial (RDBPCT). There have been some exceptions, such as the elegant study design (a RDBPCT), of a traditional Chinese medicine intervention that allowed a treatment arm to individualise the treatment in IBS after a consultation with a traditional Chinese medicine practitioner.[137]

The Philosophy of Naturopathy

Naturopathy (an umbrella term for therapy which includes herbal medicine and nutritional medicine as the two core-treatment modalities in Australia)[138] is based on six principles (maxims): the healing power of nature (*vis mediatrix naturae*), identify and treat the cause of disease (*tolle causam*), treat the whole person (*tolle totum*), first do no harm (*primum non nocere*), educate/teach the patient (*docere*) and disease prevention (*preventare*).[138]

Naturopathic treatment follows a therapeutic order which recognises and prioritises the principles of naturopathic medicine. An adapted Australian-specific therapeutic order which follows Zeff, *et al.* in the US[139] comprises the subsequent steps:

(1) restore the foundation of health by reducing 'obstacles to cure' and encouraging opportunities for healing;

(2) identify potential barriers to health and support/treat appropriately;

a. hereditary/genetic factors, including parental preconception health, genetic and epigenetic variations;

　　　　b. socioeconomic, spiritual, environmental and lifestyle factors, including hygiene, exercise, interpersonal relationships, stress and relaxation;

　　　　c. previous history of illness and treatment; and

　　　　d. dietary intake and digestion;

　(3) enhance the body's inherent ability to heal by focussing on the cause of disease;

　(4) modulate body systems, strengthen weakened organs and tonify overactive organs. The focus is on the following aims:

　　　　a. strengthen or modulate the immune system;

　　　　b. remove toxins;

　　　　c. reduce inflammation;

　　　　d. tonify (supplement energy available in a part or system of the body) the nervous system;

　　　　e. balance metabolism and hormonal activity (correct nutritional deficiencies); and

　　　　f. strengthen and tone other body systems where necessary.

　(5) attend to structural problems (if necessary, referral to an osteopath or massage therapist);

　(6) address specific pathological or other conditions and prescribe appropriately (e.g., antivirals for viruses) after the cause has been identified and treated; and

　(7) refer for suppression of disease activity (e.g., cortisone treatment) or surgical intervention if necessary.[138,139]

Advances in pharmacology, pharmacognosy and pathobiology have strengthened knowledge and understanding of traditional medicines and provided a deep understanding of the mechanisms underpinning disease states. This now informs the treatment protocol of the modern naturopath. Multifactorial disease mechanisms in hepatitis C, demand a multifactorial naturopathic treatment protocol.

The Naturopathic Protocol in the CHC Patient

In 1995, Batchelder*, et al.,* suggested a naturopathic treatment protocol for viral hepatitis A-E should: boost the immune system, prevent necrosis, support and encourage regeneration (liver assumed, but unspecified), promote bile flow, waste

elimination and detoxification, address addictions, e.g., alcohol consumption, and address hygiene.[140] Scalzo, in the same year, suggested hepatocyte restoration and immune enhancement as the primary elements in a therapeutic botanical protocol in viral hepatitis.[141]

The Study Coordinator in 2011 suggested the following (recently published in a peer-reviewed Clinical Naturopathic Medicine textbook) as a naturopathic protocol for the treatment of the CHC patient.

(1) reduce hepatic inflammation by decreasing the influence of inflammatory mediators and inhibiting inflammatory pathways (anti-inflammatory);

(2) reduce oxidative stress, enhance endogenous antioxidants and improve mitochondrial function (antioxidant);

(3) support the immune system and balance T helper 1 lymphocyte/ T helper 2 lymphocyte (Th1/Th2) (immune-modulator);

(4) reduce insulin resistance (antioxidant, glycaemic modulator);

(5) reduce hepatic steatosis (antioxidant,hypolipidaemic);

(6) reduce hepatic fibrosis (antifibrotic, antioxidant);

(7) promote antiviral activity or support antiviral activity of conventional protocol (antiviral);

(8) improve overall organ (liver) function (hepatoprotective, hepatorestorative);

(9) improve digestion (promote the release of digestive enzymes and improve carbohydrate, fat and protein metabolism in the liver, pancreas, stomach, jejunum) and reduce any digestive disturbances associated with hepatitis (such as abdominal pain, nausea, fullness or bloating) (bitter);

(10) reduce fatigue, depression and irritability associated with chronic disease (adaptogen); and

(11) improve vitality and quality of life (adaptogen).[142]

According to the above protocol, an ideal naturopathic medicinal prescription would address and target all aspects of the disease mechanism in hepatitis C (outlined above). A complete list of the overlapping pharmacological actions of the Hep573 trial interventions appear at the end of this chapter.

Although a naturopath would individualise the treatment according to each patient's presentation and emphasise certain pharmacological actions accordingly, the question asked in this Study is whether such a generic treatment approach would bear evidence of efficacy.

NATURAL HISTORY

Only a minority of people can eradicate HCV infection (25%, range 15-50%).[33,37,143] Viral clearance and viral persistence in the hepatitis C population has garnered much interest as to the exact host response characteristics that allow some people to clear the virus while the majority develop chronic infection.

Acute Hepatitis C

HCV RNA appears in serum seven to 10 days after initial exposure and infection.[144,145] The median time between exposure to HCV infection and seroconversion (development of HCV specific antibodies) is 50 days,[146,145] with a range of 3-12 weeks.[33] Many patients are unaware they have been exposed to HCV infection until many years later, as in most cases, hepatitis is mild during the early acute phase. The majority (75%) of cases are anicteric and asymptomatic.[37] Acute HCV symptoms only occur in 25% of affected people and include influenza-like symptoms, fever, jaundice, dark urine, fatigue, nausea, vomiting, anorexia and abdominal pain.[143] There is some discussion that symptomatic infection may predispose the individual to viral clearance. Acute hepatitis C rarely results in fulminant (decompensated) hepatitis.[144,145]

Chronic Hepatitis C

Of those infected with HCV, 75% (range, 50-85) will progress to chronic infection.[144,33,145,147,37] Chronic hepatitis C can result in hepatic fibrosis, cirrhosis, hepatocellular carcinoma and end-stage liver disease in some patients.[145,147,33] Complications of cirrhosis include portal hypertension, ascites, hepatorenal syndrome and variceal bleeding.[148]

Clinical symptoms include: fatigue (most common[149]), malaise, nausea, anorexia, pruritis, weight loss,[150] arthralgia,[149] musculoskeletal pain, night sweats, dry eyes (Sicca syndrome), right upper quadrant pain or liver discomfort.[151] Extrahepatic manifestations of chronic hepatitis C include: mixed cryoglobulinaemia, glomerulonephritis, porphyria cutanea tarda, low-grade malignant lymphoma, autoimmune thyroiditis, lichen planus, Sjogren's syndrome, aplastic anaemia, polyarteritis nodosa, erythema nodosum, idiopathic pulmonary fibrosis,[150] and diabetes mellitus.[151]

Factors Influencing the Natural History

Valuable contributions to an understanding of the natural history of CHC have been made by many, but in particular by Poynard, *et al.,*[152,153] who identified a temporal progression relating to age of acquisition of HCV infection along with Missiha, *et al.,*[36] by identifying modifiable and nonmodifiable factors in liver-disease progression.

Poynard, *et al.,*[152] using both single (N=2235) and paired (N=170) liver biopsy specimens, found the average estimated duration of HCV infection for progression to cirrhosis was 30 years; this reduced to 13 years for men infected with HCV after the age of 40. It increased to 42 years in women who were infected with HCV before 40 years of age and were abstinent from alcohol.[152]

The cirrhosis incidence after 20 years of HCV infection is dependent on the person's age at infection: 2% of those infected prior to the age of 20 years developed cirrhosis compared to 6% of those infected between the age of 21–30 years, 10% (31-40 years), 37% (41-50 years) and 63% of those who contracted HCV infection over the age of 50 years.[153]

Poynard, *et al.,*[152] concluded that the identified host factors were stronger than the virological factors in promoting disease progression. A possible reason for the faster progression of HCV infection in those who have acquired the infection at an older age (>40 years) may be associated with host defence mechanisms weakening in older people.[152] Missiha, *et al.,*[36] suggests changes in immune function, decreased hepatic blood flow and declining mitochondrial capacity with age may be mechanisms by which age influences fibrosis progression.

Uni- and multivariate analysis have shown the factors associated with the progression to liver fibrosis are: age at infection[154,155,153] duration of infection,[156] necroinflammatory score on initial biopsy,[36,155] presence of fibrosis on the index biopsy (P=0.014),[157] HIV coinfection,[158] consumption of more than 50 g alcohol per day, male gender,[156,153] CD4 count lower than 200/mL and body mass index (BMI) ≥25[159] to ≥30[159,160] and/or diabetes, metabolic syndrome with insulin resistance and/or worsening steatosis[156,37,161] together with serum ALT levels ≥1.5 x[159] and ≥5 x upper limit of normal (ULN).[155] It is interesting to note that Massard (based in Europe) did not include hepatitis B[37,162] as a risk factor in fibrosis progression which is an issue in the Asia-Pacific region.

Modifiable and nonmodifable factors in fibrosis progression.

The modifiable and nonmodifiable factors which have been associated with hepatic fibrosis progression in CHC patients are now summarised in Table 2.2 and Table 2.3.

Table 2.2: Nonmodifiable factors in progression to hepatic fibrosis.

Factor	Evidence
Age at acquisition of HCV infection	[36,153,157,163,164] Ind RF[153]
Age at acquisition >40 years	[152,153]
Age at biopsy	Ind RF[157]
Duration of infection	[36]
Male gender	[36,152,164,165] Ind RF[153]
Host genetic factors	[36]

Key:
Ind RF = independent risk factor
RS = retrospective study
ULN = upper limit of normal reference range.

The non-modifiable factors in progression to hepatic fibrosis are: age (acquisition of HCV infection, biopsy), gender (male), duration of infection and genetics (immune, interferon sensitivity). The independent risk factors associated with hepatic fibrosis are: age at acquisition of HCV infection, age and necrosis stage at liver biopsy, male gender, ALT levels ranging from ≥1.5-5x ULN, ALT≥ 70IU/L, daily consumption of >50 g alcohol, and cannabis use. One area of controversy is the role that ALT levels play in fibrosis progression. (This will be discussed in the normal ALT and histology section.)

Table 2.3: Modifiable factors in progression to hepatic fibrosis.

Factor	Evidence
Necroinflammatory score (index biopsy)	[36,155]
Necrosis stage (index biopsy)	Ind RF[155]
Fibrosis stage (index biopsy)	[157,166]
ALT levels ≥ 1.5 x ULN	Ind RF[159]
ALT levels ≥ 5 x ULN	Ind RF[155]
ALT levels ≥70IU/L	Ind RF[167]
Co-infection: HIV, HBV	HIV[36] HBV
BMI >25-30	[159]
Steatosis	[36,166,168]
Insulin resistance	[36,169]
High serum glucose	[165]
Alcohol	>60g alcohol daily (men) >40g alcohol daily (women) for >5 years Ind RF[170] >50g alcohol daily[156,171,172] Ind RF[153]
Tobacco smoking	[173-175]
Daily cannabis use	[36,175-177] Ind RF, RS[178] Ind RF, PS[179]

Key:

Ind RF = independent risk factor
PS = prospective study
RS = retrospective study
ULN = upper limit of normal reference range.

Overwhelmingly, the factors promoting hepatic fibrosis are modifiable:
necroinflammatory score and stage on biopsy, fibrosis stage on biopsy, ALT levels,
comorbidities (HIV, HBV, BMI >25-30), steatosis, insulin resistance, and lifestyle
issues of alcohol, tobacco and cannabis intake.

There is consensus in the literature on the non-modifiable factors associated
with progression to hepatic fibrosis, particularly age. The comorbidities that affect
resolution or progression such as hepatic steatosis, insulin resistance, diabetes and
BMI >25-30 are all inflammatory conditions caused by oxidative stress and
therefore, potentially modifiable with the use of antioxidants, weight loss and other
lifestyle interventions.

Biological effects of reactive oxygen species (ROS)/reactive nitrogen species (RNS) in hepatitis C.

Choi J , James Ou J Am J Physiol Gastrointest Liver Physiol 2006;290:G847-G851
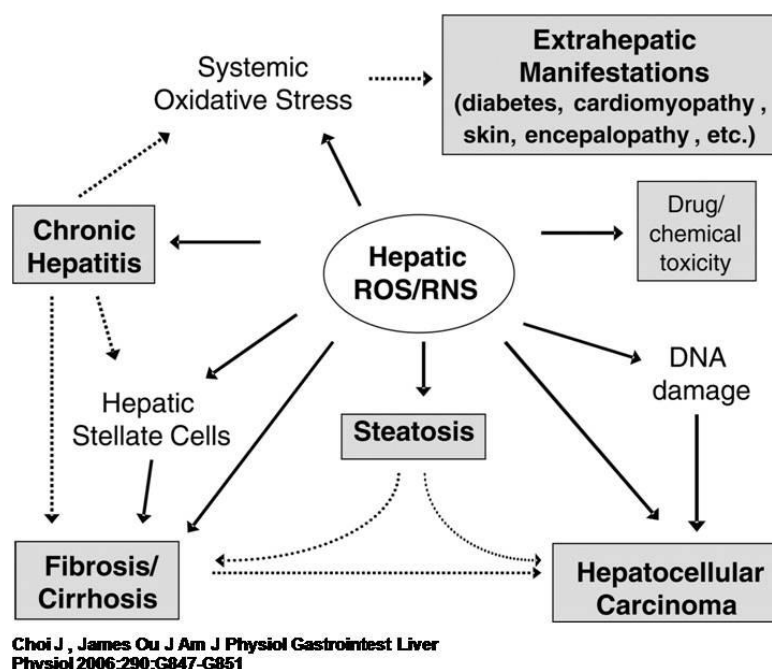
Figure 2.2: Biological effects of reactive oxygen species (ROS)/reactive nitrogen species (RNS) in hepatitis C.
(Reprinted by permission from The American Physiological Society,[180] 2006.)

Figure 2.2 illustrates the complex interplay between ROS/RNS and liver diease progression.

The most useful contribution to the natural history literature has been the characterisation of modifiable risk factors that, if addressed, may slow the rate of hepatic fibrosis progression in CHC patients. The reduction or avoidance of alcohol, weight loss (if overweight), body weight and waist circumference, along with good glycaemic control have all been shown to favourably modify disease progression. This can be empowering for hepatitis C patients as it provides an opportunity to take responsibility for their ongoing health maintenance and general well-being. (This is strongly endorsed by naturopathic principles of treatment discussed previously).

Hepatic steatosis, insulin resistance and liver injury.

Hepatic steatosis in varying degrees is present in about 70% of people with CHC. A complex relationship exists between hepatic lipid turnover, insulin resistance, hepatic inflammation, HCV viral replication, and most importantly, the clinical outcomes of CHC (fibrosis progression and possibly liver cancer). Data

indicate that, apart from adiposity, viral genotype-specific mechanisms exist for hepatic steatosis[181,182,183,184] and CHC is associated with insulin resistance, the precursor to Type 2 Diabetes.[169] Research has estimated that 30% to 70% of hepatitis C patients demonstrate some level of insulin resistance.[185]

Genotype 3 has a direct cytopathic effect on the liver, brought about by the induction of fatty changes. Although it is not yet fully understood; the mechanism of hepatitis-C-induced hepatic steatosis by HCV genotype 3 may be interference with triglyceride secretion.[186] There is strong evidence that the HCV RNA viral load determines the severity of steatosis which can be resolved after HCV antiviral treatment.[187]

Recent studies (including a meta-analysis of 3068 subjects) indicate steatosis and insulin resistance are independently associated with hepatic fibrosis, most likely through intermediates involving cellular oxidative stress and cytokine release.[188,189]

The development of steatosis has not been associated with either autoimmune hepatitis or hepatitis B infection.[190] Steatosis is often referred to as the 'first hit' or substrate for HCV-related oxidative stress, with cytokine release acknowledged as the 'second hit' to induce necroinflammation, apoptosis and hepatic fibrosis.[191] Steatosis plays a role in the progression of liver injury in CHC by regulating death receptors and activating NF-κB.[192]

Insulin resistance is mostly associated with HCV genotype 1 infection.[193] The HCV core protein has a direct effect on hepatocyte insulin signalling,[194,195] particularly the insulin receptor substrates,[193] that leads to the production of cytokines such as TNF-α and IL-6, increasing both the inflammatory response and oxidative stress.[193,194]

Diabetes.

Approximately one-third of CHC patients develop Type 2 diabetes mellitus.[196,197] Diabetes and elevated serum glucose are independent risk factors for hepatic fibrosis.[198] Gopaul, *et al.,* reported that F_2-isoprostanes were elevated three-fold in Type 2 diabetes compared to healthy individuals, confirming that OS is a factor in diabetes.[199] Factors found to promote OS in diabetes included antioxidant deficiencies, increased production of ROS and the process of glycation and glycol oxidation. Insulin resistance and diabetes also reduce the effectiveness of antiviral therapy[193,195] and accelerate the histological and clinical progression of chronic hepatitis C.[195]

Alcohol and chronic hepatitis C.

Regular daily alcohol consumption has been associated with liver injury (elevated transaminases),[200,201] liver fibrosis,[202,203,152] liver cirrhosis,[204,205] higher serum HCV RNA,[206,207,208] increased oxidative stress,[209,210,211] immune suppression,[212] reduced response to interferon monotherapy,[209,213,214,215] and combination therapy in CHC patients[216] and also to mortality in CHC.[217,204,218]

Excess ethanol (40-80g/day)[219] can produce three different pathological disorders in the liver: fatty liver (alcohol-associated hepatic steatosis), alcoholic hepatitis and cirrhosis.[211] Both alcohol and HCV infection can replicate four hallmarks of liver disease: steatosis, steatohepatitis, fibrosis and hepatocellular carcinoma.[220]

While it is well established alcohol consumption causes liver injury and cirrhosis; the daily alcohol intake and duration preceding these outcomes remains controversial. Ostapowicz, *et al.,*[171] found patients with HCV cirrhosis had a greater total alcohol consumption over their lifetime (*P*=0.018) and duration of HCV infection (*P*=0.02) compared to those with non cirrhotic chronic hepatitis. Seeff, *et al.,*[218] observed that two-thirds of CHC patients who died from end-stage liver disease were chronic consumers of alcohol. Corrao, *et al.,*[205] also found alcohol accelerated the progression to cirrhosis in the CHC patient. Vento, *et al.,*[221] summarised this research by reporting the relative risk of developing cirrhosis in the HCV-negative drinkers >50g alcohol/day was 15, the risk in HCV-positive non drinkers was 9 and the risk in HCV-positive drinkers was 147.

Poynard, *et al.,*[152] showed regardless of age or duration of HCV infection, patients who drank > 50g alcohol daily had a 34% increased rate of progression to fibrosis over non-drinkers. Recently, even moderate alcohol consumption has been implicated in disease progression in CHC. The proportion of hepatitis C patients with moderate (F2), marked fibrosis (F3) or cirrhosis (F4) more than doubled (67.6%) if they consumed 31-50 g alcohol per day compared to 29% in the abstinent group (*P*<0.001). The daily amount of alcohol aggravating liver disease progression was lower in women (21-50 g/day) compared to men (31-50 g/day).[222]

Alcohol influences response to pegylated interferon and ribavirin treatment and a consumption of less than 30 g daily is a predictor.[216] The SVR in interferon mono-therapy fell from 33% in non-drinkers to 20% in mild-drinkers (25-50 g alcohol/day) to 9% in heavy drinkers (≥75 g alcohol/day).[213]

Six months abstinence from alcohol before treatment is insufficient to counteract the negative effect of the lifetime intake of alcohol.[213] This may be due to the severity of the liver damage caused by alcohol. An alcohol intake of ≥40 g alcohol/day monitored over five years caused elevations in alanine aminotransferase levels by 1.5 (Odds ratio:1.2-1.8) ($P<0.05$) and aspartate aminotransferase levels by 2.5 (Odds ratio:1.9-3.5)($P<0.01$) in chronic HCV liver disease patients.[200] Serum alanine aminotransferase and aspartate aminotransferase levels are well correlated with self-reported drinking.[223]

The AUDIT-C questionnaire[224,225] an effective and validated screening tool for alcohol consumption, was used to assess alcohol intake throughout the Study duration.

P450 isoenzymes in ROS induction.

Metabolism of ethanol cytochrome CYP2E1 and alcohol dehydrogenase generates acetalaldehyde and ROS. Ethanol also potentiates HCV-induced oxidative stress and lipid peroxidation.[226]
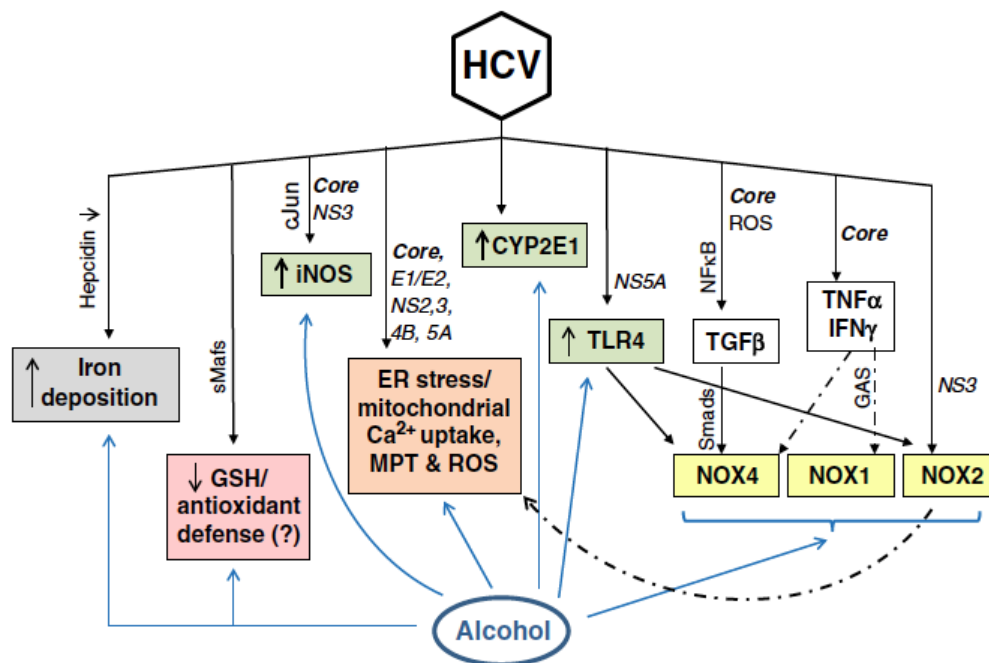
Figure 2.3: Sources of reactive species during HCV infection and possible combined effects of alcohol.
(Reprinted from Free Radic Biol Med, [226] Copyright (2012), with permission from Elsevier.)

In Figure 2.3, HCV factors involved in the generation of OS are italicised. Black dotted lines indicate events, that are probable but have not yet directly demonstrated to have occurred in the presence of HCV.[226]

Antioxidant Systems

Antioxidant systems are an important modifiable factor in the natural history of HCV infection and may attenuate the potential damaging effects of OS and disease progression. Antioxidants work:

(1) directly in the body as part of redox reactions[227];

(2) indirectly, by inhibiting the activity of free radical producing enzymes[227]; or

(3) indirectly, by upregulating endogenous, antioxidant, enzyme systems. [227]

Hepatocytes are armed with both nonenzymatic and enzymatic antioxidant defences to maintain a balanced redox state inside the hepatocyte.[228] During the inflammatory process, hepatocytes are exposed to large amounts of ROS but an efficient antioxidant system to neutralise free radicals, protects liver function.[229]

Nonenzymatic defences.

The nonenzymatic defence systems depend directly on antioxidant nutrients, ascorbic acid[180,230] alpha-tocopherol,[231,228] vitamin A,[228] flavonoids and carotenoids.[230] Herbal medicines such as *Silybum marianum*[232] and *Camellia sinensis* are rich sources of flavonoids[233,234] and tomatoes and carrots are a rich source of carotenoids lycopene and betacarotene respectively.[235,236]

Enzymatic defences.

The three main enzyme controlled intracellular antioxidants are superoxide dismutase, glutathione and catalase.[237,231] Superoxide dismutase is found in the cytosol and mitochondria of the liver and can reduce superoxide anion to hydrogen peroxide and water.[231,228] Glutathione is located in the mitochondria and the cytosol, and eliminates the bulk of the hydrogen peroxide; catalase, found in the peroxisomes, removes any remaining hydrogen peroxide.[231,228]

These antioxidants can also be categorised into sulphur-containing (thiol) and non-sulphur-containing (non thiol) compounds. The two main thiol-containing compounds, glutathione and thioredoxin[238] are particularly important to the redox status of the liver.[239,240]

Thiol. A thiol group is a sulphydryl group attached to a carbon atom group (CH_2-SH). Thiol groups are redox sensers through the sulphydryl (SH) side chain, the active site for electron flow.[241,242] Thiol molecules can be either oxidised or reduced to assist the body in maintaining oxidative homeostasis. In the body, the main source of thiol is the amino acid cysteine and its disulphide cystine. This ability to exist in a reduced or oxidised state and to convert between those two

states forms three thiol-based, redox system couples: cysteine/cystine, GSH/GSSG and TrxSH/TrxSS, discussed below.[243,239]

Glutathione. GSH is the major endogenous antioxidant in hepatocytes.[240] Glutathione is a water-soluble tripeptide comprised of the amino acids glutamine, cysteine and glycine (gamma-L-glutamyl-L-cysteinylglycine).[242] Glutathione may be: free (GSHt), reduced (GSH), oxidised (GSSG), or bound to proteins (RSSG).[244,245] Oxidised glutathione (GSSG) can be converted to reduced glutathione (GSH) and vice versa.[229,246] Glutathione is present in cells mainly in its reduced form.

Hepatic GSH is maintained at a relatively constant concentration, through *de novo* synthesis and turnover,[240,247] controlled by homeostatic mechanisms.[248] GSH can be synthesised *de novo* from the precursor amino acids by a two-step process involving two ATP-dependent cytosolic reactions.[240,249] The first step is catalysed by the enzyme γ-glutamyl cysteine ligase (γ-GCL) which adds glutamic acid, [240,249] the second step is catalysed by glutathione synthase which adds glycine[23,247] to a cysteine residue forming a new glutathione molecule. In this process, (γ-GCL) is a rate-limiting factor.[249,250,238]

Mitochondrial GSH, which comprises 10% of the hepatic glutathione, is considered more important than the 90% present in the cytosol as it supports mitochondrial function that is essential to physiological apoptosis.[250]
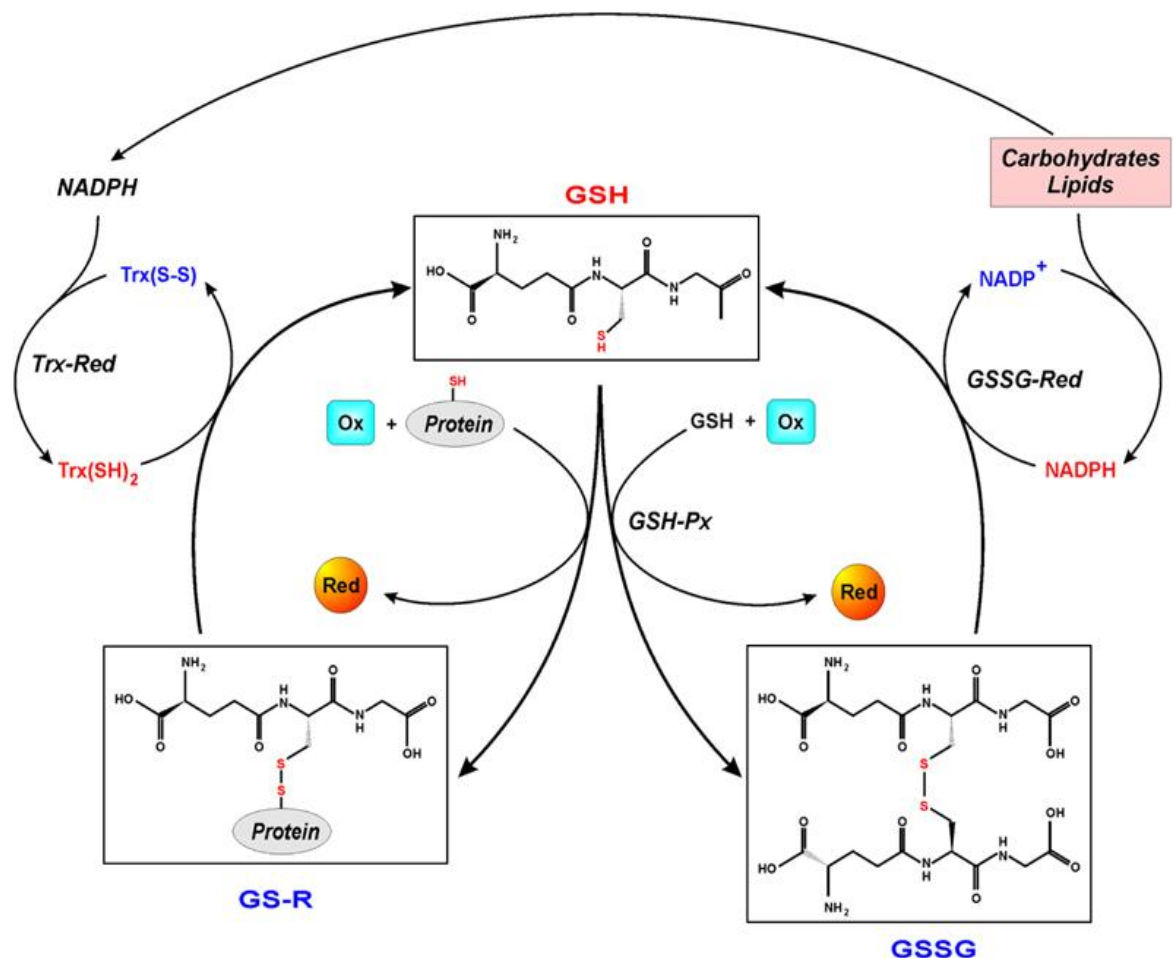
Figure 2.4: The glutathione redox cycle.
(Reprinted by permission from Macmillan Publishers Ltd: Cell Death and Differentiation,[238] Copyright (2005).)

Key:
GSH = reduced glutathione
GSSG = oxidised form of glutathione
GS-R = oxidised form of glutathione
GSSG-Red = GSSG reductase
Trx = thioredoxin
GSH-Px = glutathione peroxidise

Figure 2.4 illustrates the glutathione oxidation and reduction cycle. SS-R and GSSG are reduced by either thioredoxin reductase or GSSG reductase to glutathione. NADPH is an upstream supplier in both cases of reducing equivalents.[238] The GSH/GSSG couple is the most commonly found redox couple of the cell.[251] GSH/GSSG ratio is often used to determine the redox state of the system.[251,252,253]

Nrf2/ARE Pathway. There is currently considerable focus on the nuclear factor E2-related factor-2 (Nrf2) /antioxidant response element (ARE) pathway which mediates phase II detoxification and antioxidant defence systems (Figure 2.5).[254] Nrf2 binds the ARE, activating detoxification and antioxidant defences.[254]

The Nrf2/ARE pathway is activated by pathways such as protein kinase C (PKC), c-jun N-terminal kinase (JNK), p38, extracellular signal-regulated protein kinase (ERK) and phosphatidylinositol-3-kinase (PI3K).[254]
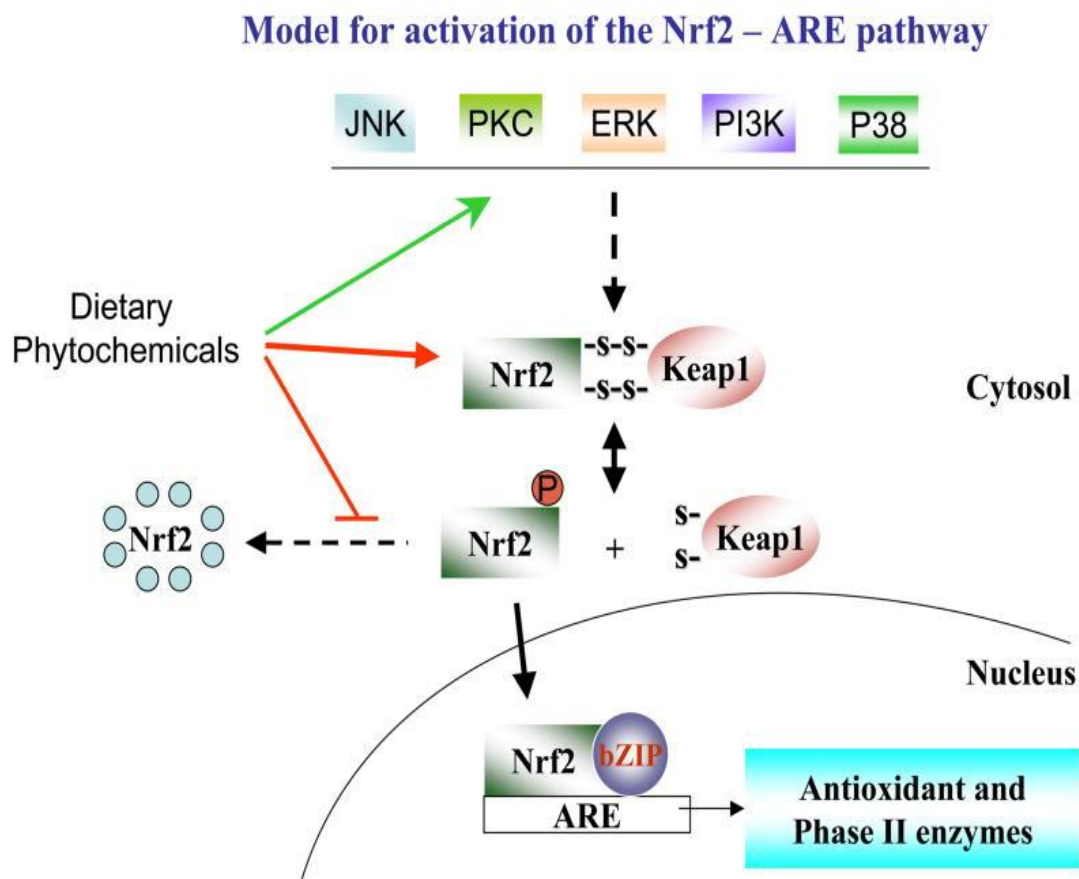
Figure 2.5: Model of activation of Nrf2-mediated ARE pathway by phytochemicals.
(Reprinted with kind permission from Springer Science and Business Media: NeuroMolecular Medicine,[254] (2008).)

Figure 2.5 models the activation of Nrf2-ARE pathway by phytochemicals, directly (red arrow) or indirectly (green arrow) via upstream kinases (PI3K, p38, ERK, PKC, JNK). Activated Nrf2 translocates into the nucleus, interacts with the antioxidant response element (ARE), causing the transcription of target genes to synthesise antioxidant and phase II enzymes.[254]

PATHOGENESIS OF HCV INFECTION AND OXIDATIVE STRESS

The interaction of the virus with antioxidant systems and the generation of oxidative stress are presented below.

Virology

HCV is an enveloped[255] single-stranded RNA virus in the hepacivirus genus of the Flaviviridae family.[256] The core-glycosylated HCV envelope membrane proteins (E1 and E2) bind to the CD81 receptor. This allows the entry of HCV into the hepatocyte.[255,257] There it undergoes an RNA-dependent replication cycle in the cytoplasm.[255] Transcription of the viral genome produces a large polyprotein that is later processed into 10 viral proteins consisting of three structural proteins (core, Envelope 1 (E1), Envelope 2 (E2), six nonstructural (NS) proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) and an ion channel protein p7.[258-259] The hepatitis C virus can produce up to 10^{12} virions per day[50] in the infected host. HCV infection is detected by ELISA which identifies immunoglobulin G (IgG) antibodies against epitopes produced from the core, NS3, NS4 and NS5B antigens.[260] Results are verified by testing for HCV RNA, which has been available in Australia since 2002.

The HCV nonstructural proteins assist HCV replication in the following ways:

(1) NS2/3 protein is a protease that causes the cleavage of the viral peptide between NS proteins NS2 and NS3[261];

(2) NS3 contains three enzymes required in HCV viral replication: a protease, an RNA helicase and a nucleotide triphosphatase (NTPase)[262];

(3) NS3 upregulates and induces tumour necrosis factor alpha (TNF-α, a pro-inflammatory cytokine) production in the liver[263];

(4) NS4A is a cofactor for protease activity[255];

(5) NS4B is an integral membrane protein and is part of the HCV RNA replication complex[264];

(6) NS5A is responsible for interferon sensitivity of HCV[265]; and

(7) NS5B is an error-prone RNA-dependent RNA polymerase that generates minor viral variants which can evade immune recognition.[146] This poor proof-reading capacity of the RNA polymerase leads to random base substitutions into the HCV viral genome and the generation of viral variants or 'quasispecies'.[266,259,267] This occurs in the hypervariable region of the E2 protein of the viral genome.[267]

P7, a structural HCV protein operates as an ion channel,[268,269] and is necessary for the assembly and production of infectious viral particles.

HCV replicates mainly in the liver.[270,271,272] Extrahepatic HCV replication occurs in the dendritic cells, B cells,[271] peripheral blood mononuclear cells

35

(PBMC)[262,58] lymph nodes, spleen, pancreas, adrenal glands, thyroid,[272,273] bone marrow, oral mucosa,[274] as well as epithelial cells of gut and the central nervous system.[255]

The HCV life cycle involves receptor binding and endocytosis, entry into the hepatocyte, fusion and uncoating, translation and polyprotein processing, followed by RNA replication and virion assembly before transport and release from the cell.[255]

Oxidative stress in HCV Infection

Oxidative stress (OS) is an imbalance between oxidants and antioxidants[9-10,275,276,277] Oxidative stress occurs when the metabolic generation[247] of reactive oxygen species (ROS) and reactive nitrogen species (RNS)[278] exceeds the capacity of the homeostatic antioxidant defence systems to neutralise them.[247,278]

OS is the underpinning cause of damage to cells in multiple diseases[279] including chronic hepatitis C,[15,16,20,128,180,280-283] haemochromatosis,[5,284] alcoholic liver disease,[276,285] autoimmune hepatitis,[286] diabetes,[287] and cancer,[288] and it may also influence healthy aging.[247,279]

The HCV infection is a direct cause of oxidative stress (OS). The immune response to infection exacerbates oxidative stress, drives the inflammatory process and correlates with the severity of the disease.[20,16,15,128,180,282,283] The excess and persistent production of ROS/RNS becomes pathological, subsequently depleting antioxidant defences.[279,289] The consequence of this OS is damage to cellular proteins, lipids and DNA causing mitochondrial dysfunction, endoplasmic reticulum stress and modifications to signalling pathways resulting in an ineffective immune response and driving inflammation (Figure 2.6) via necrosis and fibrosis.[238,279,289,283,290]
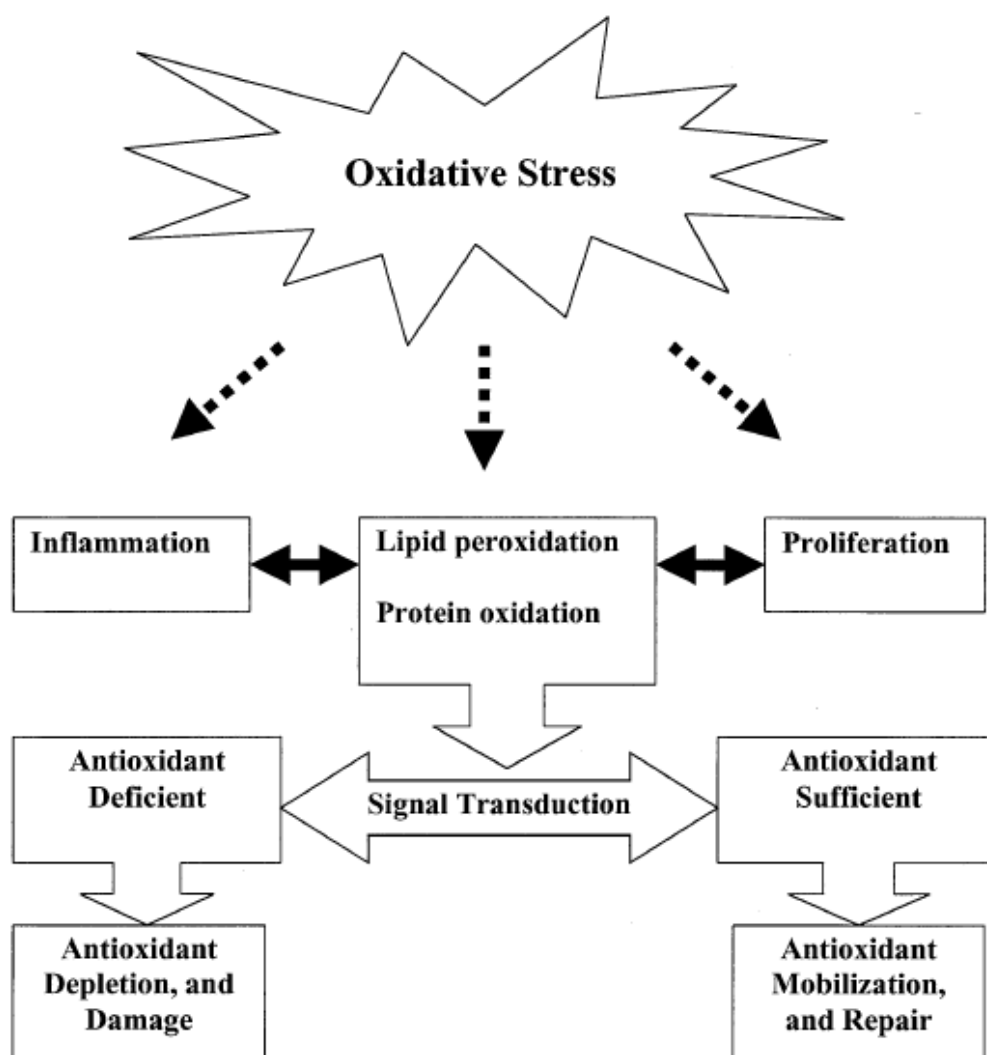
Figure 2.6: Flow chart showing the events leading to antioxidant mobilisation in response to oxidative stress.
(Reprinted from Nutrition,[291].Copyright (2001), with permission from Elsevier)

Figure 2.6 illustrates the physiological or pathological consequences of OS in HCV infection. Oxidative stress is extremely pervasive in its downstream effects. OS affects the messengers, receptors, signalling pathways and intermediary proteins, directly affecting transcription factors which control gene expression, protein synthesis and cell function.[279,282,238,250] Therefore OS is acting at all levels: intracellular and extracellular, as well as intrinsic and extrinsic pathways as illustrated in cell proliferation, differentiation and cell survival.[240,250,247,250,240,238]

That HCV infection leads to OS is supported by the results of clinical trials demonstrating that the OS may be significantly reduced by the eradication of the HCV by interferon and ribavirin therapy.[292,293]ROS levels in human hepatoma cells

infected with HCV JFH-1 have been found to be 30 - 60-fold higher than uninfected cells.[294]

Measures of plasma anti-oxidant capacity and markers of lipid peroxidation have demonstrated utility in monitoring the progression of liver damage and the response to antiviral therapy in CHC.[295] Yadav, *et al.*,[16], Duygy *et al.*,[296] and Chuma, *et al.*,[297] all confirmed a correlation between OS and disease progression. This was confirmed by a negative association between high whole blood glutathione and vitamin C levels and reduced hepatic inflammation (P=0.02) and fibrosis (P=0.02) in 247 CHC patients.[128] Further studies have correlated low antioxidant levels[298,180,16,298,299] with increased fibrosis, elevated malondialdehyde[16,300,] and elevated F_2-isoprostanes.[15] Glutathione, in particular, was reported to be significantly depleted in the liver, blood, and lymph in CHC patients.[301,302]

Some of the direct and indirect causes and consequences of OS follow:
(1) direct HCV infection as described in immune response to HCV infection;
(2) increased cellular metabolism due to:
   a. viral replication;
   b. immune response; and
   c. DNA repair.
(3) mitochondrial dysfunction;
(4) endoplasmic reticulum stress[283,290];
(5) iron overload[220]; and
(6) modulation of antioxidant systems.[226]

Immune Response to HCV Infection

The immune response is complex and multifactorial. The majority (75%) of infected individuals are unable to completely eradicate the HCV virus, as the HCV virus usually has found ways to circumvent the host's immune response. Successful viral clearance is associated with an immediate, persistent and strong HCV-specific CD4+ T-cell[303-304] and a vigorous multispecific cytotoxic T lymphocyte (CTL) response[305,306,146,304] against multiple HCV epitopes.[306] If CD4+T-cell responses do not occur or are not sustained, HCV persistence results, indicating that an HCV-specific loss of T-cell help is essential for immune evasion.[146,307-308]

The failure of the cellular immune system to eradicate the HCV virus could be explained by several different mechanisms, such as T-cell mutations,[309]

abnormal T-cell differentiation and the suppression of virus-specific T-cell responses.[304,308]

OS at the early stage of HCV infection may impair the immune-mediated clearance of infected cells, as has been shown in HCV-infected individuals where there is an association between depleted lymphocytic stores of reduced glutathione and decreased peripheral mononuclear cytotoxicity.[310]

Th1 helper cytokine profile in viral clearance.

Th1 and Th2 cells have different functions. Th1 cells enhance cellular immune responses and secrete IFN-γ, IL-2 and TNF-α. Th2 cells secrete IL-4, IL-5 and IL-10 amongst other cytokines, and promote humoral immune reactions.[311,312] Viral clearance is more likely to occur in acute hepatitis C patients displaying a Th1 profile than a Th2 profile.[313,314,315]

However, persistent Th1 stimulation which fails to clear the virus results in liver injury primarily induced by CTLs, natural killer (NK) cells and macrophages. Research at RPA Hospital, Sydney, demonstrated that significant increases in intra-hepatic macrophage numbers, inflammatory cytokines[316] and intrahepatic Th1 phenotype correlated with portal inflammation and fibrosis.[315] Mahrouf-Yorgov[317] demonstrated that the main factor involved in susceptibility to fibrosis with aging was an increased inflammatory reaction mainly comprised of CD4+ lymphocytes and macrophages expressing Th2 cytokines rather than an acute Th1 response.

In summary, a rapid Th1 dominant response can clear acute HCV infection. A Th2 response will not clear HCV infection and may promote fibrosis. However, persistent Th1 response without viral clearance is inflammatory. In essence, ineffectual and delayed immune responses to chronic HCV infection will drive the inflammatory process.

Host defences, how the host tries to combat HCV infection.

Demonstrated ways the host protects itself from viral attack are to:

(1) mount an effective CD4+ T cell and multispecific cytotoxic T lymphocyte response[146,304-306] which is associated with resolution of acute hepatitis C;

(2) attempt to stop viral entry into the hepatocyte by activating the cytotoxic T cells and natural killer cells[318,319];

(3) release IFN-γ to suppress HCV replication[320];

(4) enhance apoptotic (cell death) pathways to eliminate infected hepatocytes[321,322];

(5) ellicit a 'wound healing' response of the liver to injury caused by the immune-mediated response to chronic hepatitis C infection[147,323,324]; and

(6) activate antioxidant defence systems to counter the oxidative stress caused by the virus.[228]

Viral defences, how HCV proteins alter the host immune response.

Several HCV proteins have been found to alter the immune response as follows:

(1) alter protein kinase cell signalling[325];

The activation of protein kinases, crucial for the initiation of type I interferon signalling, is disrupted by **HCV NS proteins** at many levels: receptor, adaptor protein, transcription and gene expression;

(2) disrupt interferon signalling via interferon regulatory factor 3 (IRF-3) and nuclear factor kappa B (NF-κB)[326];

Two key pathogen-associated molecular pattern (PAMP) receptors are toll like receptor 3 (TLR3) and retinoic acid inducible gene 1 (RIG-1). Both are involved in the recognition of single and double-stranded RNA viruses. RIG-1 is the dominant PAMP receptor involved in HCV RNA recognition. RIG-1 and TLR3 are disrupted when **NS3/4A protein** blocks the activation of two transcription factors, IRF-3 and NF-κB, which inhibits the expression of IFN-α and interferon stimulated genes (ISGs)[327];

(3) disrupt interferon signalling via janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway;

HCV core protein induces degradation of STAT 1, thereby blocking a pathway leading to ISG expression and induces suppressors of cytokine signalling (SOCS), which can further inhibit the JAK-STAT pathway.[328] Inhibition of the JAK-STAT pathway, due to interferon suppression can cause viral persistence and treatment resistance to occur[328,329];

(4) modulate apoptotic pathways[330]

HCV core protein may have a regulatory role in apoptosis by either enhancing it or inhibiting it.[321] Inhibiting apoptosis may be advantageous for HCV infection because host hepatocytes can survive apoptosis, which results in sustained infection.[321] It has been reported that **HCV core protein** raised basal levels of ROS in Huh-7/191-20 cells by a factor of three (P<0.001), increased lipid peroxidation products, raised antioxidant gene

expression threefold to 65-fold, and increased cytosolic cytochrome *c* (*P*<0.01) which may trigger the downstream effects of apoptosis[20];

(5) increase oxygen radical production in phagocytes;

HCV NS3 protein causes an extended release of oxygen radicals from mononuclear and polymorphnuclear phagocytes[331] through activation of reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. NS3 activated phagocytes trigger oxygen radical formation which contributes to dysfunction and apoptosis of the lymphocytes in HCV infected liver tissue[331,332];

(6) escape from T- and B-cell immune surveillance;

HCV NS4 protein changes the immature dendritic cell phenotype and subsequently reduces the antigen-specific T-cell responses and Th1 cytokine concentrations[333] and reduces the possibility of viral clearance. **E2/HCV core proteins** also have an inhibitory effect on T cell function.[334,335] The absence of adequate CD4+ T-cell in impaired memory CD8+ T-cells leads to mutations in Class I HLA restricted epitopes.[336]

Genetic profiles and HCV clearance.

The genetics of the host and the virus interact to influence pathogenesis and viral clearance.

IL28B polymorphism and response to treatment. IL28B is very important in treatment and spontaneous clearance in HCV infection. Six studies[337-342] showed an association with single nucleotide polymorphisms (SNPs) and spontaneous treatment clearance. In HCV genotype 1 patients a significant association was found between these polymorphic genetic variants upstream of IL28B on chromosome 19, encoding interferon-λ-3 (IFN-λ-3) and improved viral clearance rates (SVR) in treatment with pegylated interferon and ribavirin treatment.[341,343] Within the IL28B polymorphism, there is a doubling of the SVR rate in the C/C compared with the T/T genotype.[341] As the C/C genotype is present in greater numbers in those with European compared to African-American ancestry, this offers an explanation for the differing response rates between these two populations.[341]

In addition to improved SVR rates, the IL28B polymorphism and the C/C genotype were found in greater frequency in those who spontaneously cleared HCV infection (N=388) compared to those with persistent infection (N=620).[344] The

C allele frequency in the clearance group was 80.3% compared to 66.7% in the persistent infection group (European ancestry) compared to 56.2% versus 37% respectively (African ancestry) (This warrants further investigation.).[344]

HLA Class II alleles and viral clearance. Data on the role of HLA and hepatitis C is highly controversial. Some studies indicate the HLA DRB1 class II alleles are associated with viral clearance, but the best data on HLA have shown that the HLA class I alleles and killer cell immunoglobulin-like receptors (KIR), such as KIR2DL3, lead to spontaneous clearance and HLA C2C2 genotype is associated with chronicity.[338]

Histological Damage

The types of liver cells involved in liver injury are summarised here, and the inflammatory processes and stages of histological damage of apoptosis, necrosis, fibrosis and cirrhosis are defined. Of special focus will be how these inflammatory processes generate OS and are influenced by ROS.

Liver cells and liver injury.

The major biologically important liver cells governing liver function are the hepatocytes, Kupffer cells (KC), liver sinusoidal endothelial lining cells (LSEC), hepatic stellate cells (HSC),[345] the liver-associated lymphocytes (LAL)[345] and dendritic cells.[346,347] The hepatocyte is the cell primarily affected in HCV infection. This process activates a variety of other hepatic cells to respond to the insult. Kupffer cells play a central role in the liver's homeostatic response to injury because they respond immediately to insult, releasing mediators that regulate inflammation and repair.[345]

Liver sinusoidal endothelial lining cells (LSEC) have a 'fenestrated endothelium', and are important in the clearance of waste products from the blood,[345] vascular tone, immunity,[348] lipid homeostasis[349] and as barriers and modulators of host defence responses.[350]

Hepatic stellate cells (HSC) synthesise, secrete and degrade components of the perisinusoidal extracellular matrix (ECM). In normal liver, stellate cells exist in their quiescent state and store fat[345] and approximately 80% of total body vitamin A.[351,352]

Liver-associated lymphocytes (LALs) reside in the liver sinusoid and have a superior level of natural killer cell activity compared to the peripheral blood lymphocytes.[353] Dendritic cells are now regarded as the primary antigen presenting cells, however, their volume in the liver is exceptionally low.[346,347]

Kupffer cells, hepatocytes, HSCs, natural killer cells, lymphocytes, platelets and dendritic cells in the liver all produce paracrine and autocrine, inflammatory cytokines and growth factors in response to injury (infection and oxidative stress) which activate HSCs.[354,355-357,358]

Cell death.

The recommendations from the Nomenclature Committee on Cell Death (NCCD) in 2009[7] have classified cell death according to a number of distinct criteria: functional aspects (such as programmed accidental, physiological or pathological); morphological appearance (such as apoptotic, necrotic, autophagic or associated with mitosis); enzymological criteria; and immunological characteristics.[7]

Apoptosis and necrosis are the focus of this discussion. They are the result of a dynamic and complex interaction of ligands, receptors, signalling pathways, proteins, enzymes and the environment rather than, as was previously believed, two mutually exclusive pathways.[359] Cells which at first undergo apoptosis can then become necrotic in situations of increased oxidative stress due to disruption of cellular respiration and also if the number of apoptotic bodies is greater than the phagocytic ability.[360]

Apoptosis and necrosis. The expression 'apoptosis' was coined by Kerr*, et al.,*[361] and defined by the NCCD as follows:

> Apoptosis is accompanied by rounding up of the cell, retraction of the pseudopodes, reduction of cellular and nuclear volume (pyknosis), nuclear fragmentation (karyorrhexis), slight modification of cytoplasmic organelles, plasma membrane blebbing (but the maintenance of its integrity until the final stages of the process) and engulfment by resident phagocytes (*in vivo*).[7]

The NCCD define necrotic cell death or necrosis as:

> morphologically characterised by a gain in cell volume (oncosis), swelling of the organelles, plasma membrane rupture and subsequent release of cellular components (cytolysis)[7] which elicits an inflammatory response in the surrounding tissue.[321]

Necrosis is characterised by the failure of cellular respiration, loss of membrane integrity and the rupture of cell contents. During apoptosis, ATP-consuming processes are minimised, whereas in necrosis, ATP consuming-processes, such as DNA repair continue, which with mitochondrial dysfunction, leads to ATP depletion and respiratory failure. Respiratory failure with the loss of

cell and mitochondrial membrane integrity leads to increased Ca^{2+} levels, oncosis and the rupture of cell contents which mediates an inflammatory,[362-364] fibrotic response[364] rather than a silent response.[365] In HCV the HCV NS5A protein directly disturbs intracellular calcium levels.[366]

OS accelerates necrosis, and this process creates additional OS which causes further damage to proteins, DNA, lipids and sensitises redox-regulated necrotic cell signalling pathways, affecting gene expression[230] and causing mitochondrial dysfunction.[367]

In apoptosis, the outer mitochondrial membrane (OMM) becomes permeable and mitochondrial apoptogens, such as cytochrome *c,* which activate the mitochondrial apoptotic pathway are released. In necrosis, a channel is opened in the inner mitochondrial membrane (IMM) which is called the mitochondrial permeability transition pore (MPTP). Water and solutes enter into the matrix normalising the osmotic and electrical gradient between the two membranes causing oncosis. This disrupts ATP production, as the energy gradient between the IMM and the OMM is necessary for the conversion of ADP to ATP. Oncosis can lead to the rupture of the mitochondria and release of apoptogens which may contribute to cell death.[367]

ROS mediated DNA damage activates Poly (ADP-ribose) polymerase (PARP-1) to repair DNA and regulate DNA transcription. Overreaction of PARP-1 depletes cellular NAD+, increases mitochondrial production of ROS and can trigger the release of apoptosis-inducing factor (AIF) from the mitochondria. AIF, in conjunction with an endonuclease, carries out large-scale DNA cleavage. NAD+ is essential to the Krebs cycle and so the rate of glycolysis decreases, depleting ATP, and ATP depletion is exacerbated by mitochondrial dysfunction.[367]

Pias*, et al.,*[368,369] consistently found that an imbalance in GSH/GSSG redox status towards the oxidised state (GSSG) preceded the loss of mitochondrial integrity.[370] Low glutathione inhibits the Caspase 8 deactivation of receptor-interacting proteins RIP1 and RIP3.[371] RIP1 and RIP3 can increase catabolic reactions, increasing ROS, reducing mitochondrial integrity.

Thioredoxin (Trx), binds to apoptosis signalling kinase 1 (ASK1), an upstream activator of both JNK and p38 and under normal conditions inhibits its activity. OS causes disassociation of Trx-ASK1 which promotes autophosphorylation and activation of the downstream JNK pathway. Nuclear translocation of activated JNK promotes expression of proapoptotic TNF-α, FasL

and Bak, mitochondrial translocation of activated JNK promotes cytochrome *c* release and mitochondrial mediated apoptosis.[239]

Apoptosis in the liver allows for the physiological turnover of cells and the efficient removal of unwanted cells such as virus-infected cells and is not associated with inflammation, whereas necrotic cell death, accelerated by increased ROS and decreased antioxidants is pathological and inflammatory.[355,372,373]

Inflammation.

Inflammation is the result of a protective tissue response to injury, such as infection which leads to the destruction of tissues and is characterised macroscopically by redness (rubor), heat (calor), swelling (tumor/oncosis), pain (dolor) and loss of function (functio laesa).[374]

The inflammatory process in hepatitis C virus infection occurs as a result of direct and indirect OS[375] which regulates the expression of both pro- and anti-inflammatory mediators, affecting cell proliferation, differentiation and survival.[376,375]

Hepatic necroinflammation is used to describe the inflammatory destruction of the hepatocytes, described in the histological damage section. A high grade of hepatic necroinflammation is commonly associated with a high stage of fibrosis in CHC patients (N=290).[377] There is strong evidence that reducing OS-induced hepatic necroinflammation may reduce hepatic fibrosis in many patients.[378,379,380]

Hepatic fibrosis.

Hepatic fibrosis is a reversible, healing response to parenchymal injury.[357,381] It is characterised by the activation of HSCs[345] leading to the proliferation of contractile, migratory,[357] fibrogenic myofibroblasts (MFs), and the subsequent accumulation of extracellular matrix (ECM) driven primarily by inflammation.[357]

Friedman[357,382] describes the activation of HSCs to MFs as consisting, conceptually of two stages. The first, preinflammatory initiation stage, is due to paracrine stimuli from damaged parenchymal cells. The second perpetuation stage, is regulated by autocrine and paracrine stimuli and involves at least six changes in HSC function. This includes proliferation, contraction, migration, retinoid loss, the release of inflammatory, fibrogenic and mitogenic cytokines as well as the increased deposition of ECM. [357,382]

Both the increase of ECM in the space of Disse and the changes in structure and metabolic activity of the ECM, produced as a result of liver injury, lead

to capillarisation of the sinusoids. This reduces metabolic exchange between hepatocytes and the bloodstream leading to reduced function.

In fibrosis there is disequilibrium between fibrolytic and fibrogenic processes.[357,383] Some key cytokines and cell signalling pathways which inhibit fibrogenesis are:

(1) STAT1 pathway which reduces fibrosis by inhibition of profibrogenic signalling paths and is activated by IFN-γ, IFN-α, IFN-β;[357]

(2) IFN-γ acts with NK cells to increase apoptosis and reduce fibrogenesis;[358]

(3) Adiponectin, an adipokine, inhibits hepatic fibrosis by inhibiting the use of ATP for non essential processes via the nuclear receptors (PPAR);[357,358]

(4) Adiponectin opposes the fibrogenic actions of leptin. The increase in leptin and decrease in adiponectin in metabolic syndrome and obesity is linked to the fibrosis of NAFLD.[358] Leptin is secreted, due to translation of the obese (ob) gene, by adipocytes and to some degree macrophages, fibroblasts and monocytes. Leptin activates HSCs to MFs and stimulates Kupffer cells, macrophages and endothelial cells to produce TGF-β; and

(5) Ghrelin may also reduce fibrosis.[384]

HCV infection enhances hepatic fibrosis progression through generation of ROS which activates redox-regulated signalling pathways to produce fibrogenic cytokines.[385] Of the fibrogenic cytokines, platelet derived growth factor (PDGF) and the induction of TGF-β are the most important.[357] Strategies have merit which minimise fibrogenesis by limiting the viral induction of OS.

Previously hepatic fibrosis was thought to be irreversible, but recent human clinical trials in certain chronic liver diseases have shown that fibrosis can be reversed if the injurious insult is removed, e.g., hepatitis C virus clearance by pegylated interferon and ribavirin.[382]

Fibrosis resolution consists of degradation of the ECM and an increase in MF apotosis.[357,386] NF-κB, one of the transcription factors regulating inflammation, immunity, healing and cell survival, is maintained at high levels during HSC activation. NF-κB suppresses apoptopic pathways via Bcl-2 and induces inflammatory cytokines.[357] Reduction in ROS will reduce levels of NF-κB, which in turn reduces profibrogenic cytokines and increases apoptosis.[385] An alternative pathway for resolution of fibrosis is MF senescence,[357] which is promoted by p21, p16 and p53 proteins.[357]

Two new single nucleotide polymorphisms (SNPs), rs16851720 and rs4374383 which regulate apoptosis and inflammatory responses respectively, may predict fibrosis progression in CHC patients.[387]

Hepatic cirrhosis.

Cirrhosis is the result of ongoing necroinflammation and progressive fibrosis. This is characterised by the development of regenerative nodules[147] surrounded by fibrous bands which block the sinusoids. This stimulates angiogenesis as well as the creation of vascular, fibrous portosystemic shunts which further distort the liver architecture.[357] This can lead to raised intrahepatic resistance (portal hypertension), synthetic dysfunction, ascites, impaired metabolic capacity,[357,382] end-stage liver disease[388] and hepatocellular carcinoma.[388]

Measures of Oxidative Stress

Oxidative stress can be detected by the direct measurement of the ROS/RNS; however, as these species are inherently unstable, such measurements may be unreliable. OS may also be detected by measuring the resulting damage to DNA, proteins and lipids.[389] A number of compounds have been studied as markers of OS; some of the most commonly used include:

(1) oxidative damage to DNA is 8-hydroxy-2´deoxyguanosine (8-OHdG), or the corresponding base 8-hydroxyguanine (8-OH-G).[390] Another marker for oxidative DNA damage is thymidine glycol that is produced when thymidine is damaged by hydroxyl radicals;

(2) oxidation of amino acid residues on proteins forms protein carbonyls;[391,392]

(3) lipid peroxidation include: malondialdehyde (MDA),[393] 4-hydroxynonenal (4-HNE) and F_2-isoprostanes (ISO);[389,393,394] and

(4) antioxidants and other redox molecules, including catalase and superoxide dismutase.

Lipid peroxidation.

Lipid peroxidation is a major characteristic of oxidant stress. Lipid peroxidation usually arises from interactions between ROS or other free radicals with polyunsaturated fatty acids and is catalysed by the presence of divalent metal ions.[278] Lipid peroxidation destroys polyunsaturated fatty acid components of cellular membranes, which produce toxic and reactive aldehyde metabolites such as malondialdehyde (MDA),[394] 4-hydroxynonenal (4-HNE)[395] (alkoxyl radicals) and F_2-isoprostanes (peroxyl radical).[394,394,396]

The HCV-core protein directly results in an increase in ROS as well as indirectly through interference with mitochondrial function.[20,395,397,398] Induction of HCV-core protein expression has been reported to result in a twofold increase (in both *in vitro* and *in vivo*) in lipid peroxide products (4-HNE, MDA), in both Huh-7/191-20 and HeLa 191-14 cells *(P<0.05)*, as well as in total hepatic lipid peroxidation products in transgenic mice.[20] In addition, HCV-core protein expression caused a threefold increase in the basal ROS content of Huh-7 cells *(P<0.001)*.[20]

Total cellular lipid peroxidation products provide a way to measure the effects of OS.[20] Also fatty acid synthetase (FASN) which catalyses *de novo* lipogenesis and is upregulated following HCV infection directly links viral infection with lipid metabolic disorders.[399] GSH depletion, morphological changes in the mitochondria and the presence of lipid peroxide adducts have been found in liver tissue sampled from HCV-infected patients.[20] Increased lipid peroxidation products in plasma and raised superoxide dismutase activity in peripheral blood mononuclear cells in CHC patients also indicate a response to increased oxidative stress.[20]

Consistent with the literature that OS is implicated in lipid peroxidation and disease progression in CHC patients, cirrhotic patients have significantly higher levels of F_2-isoprostanes, indicating OS damage of lipids, compared to controls *(P<0.0001)*.[400]

Malondialdehyde.

Malondialdehyde (MDA) is commonly utilised in the literature as a marker of lipid peroxidation in CHC patients, but arguably, it is no longer the best marker of oxidative stress.[389,394,401,393,402] The most commonly used method to analyse MDA is the thiobarbituric acid (TBA) reaction expressed as TBA reactive substances (TBARS). Concerns regarding the specificity of MDA using TBA reactants have been expressed,[389,394,403] as up to 98% of MDA that reacts with TBA is artefact formed during the assay.[403] *In vivo*, MDA is rapidly metabolised and altered.[403]

MDA forms a stable pink chromophore with TBA with an absorption maximum at 535 nm. However, chromogens are formed with many aldehydes other than MDA in the presence of carbohydrates, antibodies, antifungals and DNA.[394]

MDA is not specific to lipid peroxidation as it is also a byproduct of thromboxane synthesis in the cyclo-oxygenase cascade.[394,403,389] This presents a

substantial problem in blood samples (serum or plasma) since there will be varying degrees of platelet activation and thromboxane generation.

Increased MDA is a much less sensitive measure of lipid peroxidation compared with measuring the increase in esterified F_2-isoprostanes in the liver (2.7- vs 80-fold increase respectively).[389]

F_2-isoprostanes.

F_2-isoprostanes are prostaglandin-like compounds generated *in vivo* independently of the cyclo-oxygenase enzyme by free-radical-driven peroxidation of arachidonic acid.[394] F_2-isoprostanes are regarded as the gold standard to determine oxidative injury *in vivo* generated by lipid peroxidation by a free radical pathway [389,393,394,401,403,404] and are a reliable marker to assess the efficacy of an antioxidant intervention.[404]

F_2-isoprostanes are easily detected in free forms in all biological fluids and in esterified forms in all tissues.[394] The measurement of F_2-isoprostane levels is not increased by the dietary lipid content[389,405] or by freezing the samples appropriately.[389]

Recently, F_2-isoprostanes levels have been found to correlate with collagen deposition or fibrosis, making it a very valuable marker in hepatitis C patients. In an animal model of CCl_4 hepatotoxicty, F_2-isoprostanes were found to mediate hepatic stellate cell proliferation and collagen production.[406,407] These findings strengthen the link between oxidative stress, lipid peroxidation and hepatic fibrosis.[408]

Independent support for choice of F_2-isoprostanes.

The decision to measure F_2-isoprostanes in this Study was supported by a National Institute of Health (NIH) commissioned reviews of *in vivo* oxidative-stress markers and their respective methodologies published in 2005.[401,393] Kadiiska, *et al.*, evaluated various measures of OS in the CCl_4 induced hepatotoxicity model (rats) and confirmed that plasma or urine samples of F_2-isoprostanes (measured by GC-MS, LC/MS/MS, radioimmunoassay and enzyme immunoassays), malondialdehyde (MDA) (measured by GC-MS, not its common method) and 8-OHdG in urine were the best general biomarkers to assess OS *in vivo*.[401,393]

The use of F_2-isoprostanes and the method (GC-MS) were both independently verified as the gold standard.[393] This is in contrast to MDA, where the validated method for measurement of MDA (GC-MS) is not routinely used in many hepatitis C studies.[293,391,409,410]

In summary, the F_2-isoprostanes clearly have advantages over the other widely available measures for testing oxidative stress such as TBARS, MDA, lipid hydroperoxides, exhaled alkanes (ethane, pentane) or conjugated dienes[389,394] as artefaction is rare if there is strict adherence to sample preparation guidelines.

MEASURES OF LIVER INFLAMMATION

The main serum measures of liver inflammation are alanine aminotransferase, aspartate aminotransferase (routine laboratory measures), cytokines and apoptosis/caspases (laboratory/research tests).

Alanine Aminotransferase

Alanine aminotransferase (ALT) is a hepatic enzyme, found primarily in the cytosol of the hepatocyte[411] and released from damaged hepatocytes into the blood after hepatocellular injury or death.[412] Given its predominant hepatic origin, ALT is regarded as a specific marker of liver injury,[413] and serum elevations are considered the hallmark of hepatocyte necrosis.[414-416] In addition, ALT is inexpensive, accessible, widely used and to date, the most reliable and sensitive serum marker available for screening[413,411,417] diagnosis[411,412,414] and evaluation of inflammatory liver disease.[411,412]

All tests have advantages and disadvantages. Aspartate aminotransferase (AST) is found in a range of metabolically active cells and is used in some clinical settings. It has similar benefits to ALT but is a less specific marker than ALT in liver injury. Liver biopsy is considered the standard test for liver fibrosis; however, a biopsy is invasive, costly and can have serious complications with poor patient compliance which makes it ill-suited to frequent sampling.[418,268] Liver biopsies can also be inaccurate due to sampling variations.[418]

ALT levels fluctuate during the day with ALT activity 45% higher in the afternoon compared to the early morning.[412] In the Hep573 Study, participant tests were taken at the same time of day for consistency where possible. It is recognised that men and women have differing ALT levels, with higher values in men than compared to women.[411] Also, ALT levels vary according to age; age produces an inverted-U pattern that shows the significant impact on ALT levels.[419] Studies also show that ALT levels can be influenced by alcohol consumption, BMI and non-alcoholic fatty liver.[418]

Debate about the upper limit of normal (ULN) for ALT levels started in earnest after this Study had commenced. In the Hep573 Study, the abnormal ALT cut-offs of the reference laboratories at each of the three participating hospitals were used, a practice employed in most multicentre and internationally conducted clinical trials.

Kariv, *et al.,* in 2006, suggested that the new ULN for ALT represented by the 95th percentile of the healthy population was 37.5 U/L in contrast to the manufacturer guidelines of 52 U/L[420] in one branded assay. In 2007, van der Poorten, *et al.,* studied juvenile male offenders (12-19 years) with chronic HCV infection (N=439) and calculated the ULN (95th percentile) for ALT as 28 IU/L.[419] Their study revealed that different ranges of ULN values are required for adolescents compared to adults.[419] In 2009, Ruhl, *et al.,* employed the reference laboratory levels that define abnormal ALT as >40 U/L for men and > 31 U/L for women in their study. They investigated possible links between elevated ALT and GGT levels and increased risk of mortality (all-cause and disease-specific). However in their conclusion, they used lower ALT reference levels, i.e., >30 U/L for men or >19 U/L in women to define ALT levels as abnormal.[413] Puoti, *et al.,* in 2002, divided PNAL patients with HCV into two groups based on ALT at baseline.[421] ULN ALT value was defined as 40 U/L.[421] Patients with ALT less than 50% of ULN were classified as 'low normal ALT': mean 16 U/L, range 12-20 U/L (N=405) and patients with ALT greater than 50% of ULN 'high normal ALT': mean 34 U/L, range 23-40 U/L (N=475).[421] They also found no difference in the histological activity across these two groups and concluded that the present definition of ULN is sufficient in chronic hepatitis C to identify a homologous group that does not require modification.[421]

Normal ALT and liver histology.

A discussion around normal ALT and liver histology usually involves the term 'persistently normal ALT' (now defined in this section). Serum ALT concentrations within the normal range on three separate occasions over a six-month[422] to 12 month period[423] are now coined: 'persistently normal' ALT levels (PNAL).[424] Puoti describes two types of HCV patients with PNAL, one type with wide fluctuations of ALT over time but for extended periods still within normal range and true carriers with biochemistry that persistently yields normal ALT values; however, Puoti states it is not known if disease progression is different between these two groups.[418] He also states that due to these potential flare-ups, the testing

period should be extended to 18 months with ALT tested every two-three months to establish PNAL.[418] The percentage of the HCV infected population with PNAL varies from 8-33%.[424,421]

There is debate as to whether ALT is a true measure and predictor of disease progression. Evidence suggests that disease progression is less likely in patients with persistently normal ALT levels[425,426,153,166,414,421,423,427] than in those with persistently elevated ALT levels. Puoti reveals that progression of liver disease in patients with normal ALT still advances, but at about half the rate of patients with elevated ALT.[418]

A small (N=49) paired biopsy study of chronic hepatitis B and C patients compared ALT levels with the annual rate of fibrosis progression.[167] The study concluded ALT was an independent variable that correlated with liver fibrosis.[167] Fibrosis progression occurred mainly in patients with persistently elevated ALT levels even over a relatively short period. The study also found that patients whose median ALT levels was ≥70 IU/L might experience progression of liver fibrosis by one stage within an average of four to five years of follow-up.[167]

An Italian prospective study of HCV patients with PNAL (N=691), found that while 17% had no liver damage, 34% had minimal chronic hepatitis, 44% had mild hepatitis, 4% had moderate to severe hepatitis, and 1% had cirrhosis.[421] The majority were females (72%) with a genotype 2 infection (52%). The predominance of females in this cohort may account for the observed mild disease progression.[421]

In a comparison of data from three randomised phase III trials of peginterferon alfa-2a with CHC patients, Shiffman, *et al.,*[426] concluded patients with PNAL had less liver disease compared to those with persistently elevated ALT. ALT levels alone were not sufficient basis on which to decide treatment as there was still risk of some patients developing significant liver disease.[426] Definitions of PNAL varied slightly across the three studies. Necroinflammatory activity scores less than five for 65% of the patients with normal ALT levels compared with 11% of patients with raised ALT levels (P=0.01).[426] In the patients with PNAL, while 27% had no fibrosis (F0), 64% had portal fibrosis (F1) and 10% had bridging fibrosis (F3), while none had cirrhosis (F4). In the patients with elevated ALT, 5% had no fibrosis (F0), 71% had portal fibrosis (F1), 24% had bridging fibrosis (F3) and none had cirrhosis (F4).[426]

Pradat, *et al.,* found in CHC patients with PNAL, 35% (23/66) had no fibrosis (F0), 52% (34/66) had mild fibrosis (F1), 12% (8/66) had moderate fibrosis (F2) and

1.5% (1/66) had cirrhosis (F4). In comparison, those with elevated ALT levels, 1% (6/798) had no fibrosis, 24% (189/798) had mild liver disease (F1), whilst 51% (403/798) had moderate fibrosis (F2) ,17% (136/798) had advanced fibrosis (F3) and 8% (64/798) had cirrhosis (METAVIR).[414] PNAL was defined according to the reference standard at each European laboratory participating in this study. The above correlations between PNAL and fibrosis staging are summarised in Table 2.4 and Table 2.5 as percentages followed by elevated ALT and fibrosis staging.

Table 2.4: PNAL and fibrosis staging represented as percentages.

Study	Fibrosis 0	F1	F2	F3	F4
Pradat, et al., [414] N=66	35%	52%	12%		1.5%
Puoti, et al., [421] N=691	17%	34%	44%	4%	1%
Shiffman, et al., [426] N=475	27%	64%		10%	0%
Ranges	**17-35%**	**34-64%**	**12-44%**	**4-10%**	**1-1.5%**

Table 2.5: Elevated ALT and fibrosis staging represented as percentages.

Study	Fibrosis 0	F1	F2	F3	F4
Pradat, et al., [414] N=98	1%	24%	51%	17%	8%
Shiffman, et al., [426] N=419	5%	71%		24%	0%
Ranges	**1-5%**	**24-71%**	**0-51%**	**17-24%**	**0-8%**

The above Tables demonstrate those with PNAL have less hepatic fibrosis and milder disease progression than those with persistent ALT elevation.

Ortiz, et al.,[159] recruited 114 CHC patients into a prospective study (N=97, single biopsy, N=17 paired liver biopsies, five-year interval between liver biopsies) to examine how obesity may affect fibrosis progression in patients with raised and normal ALT levels.[159] The study concluded that the severity of disease and the rate of fibrosis progression was highly related to levels of ALT, 14/53 (26.5%) patients with significantly raised ALT levels were rapid progressors (>0.2 FU/year), compared to 5/30 (17%) with minimally elevated ALT levels and 3/31 (10%) with normal ALT levels (P=0.05).[159] The study also concluded that obesity in HCV infection may speed up disease progression and that weight loss may reduce fibrosis progression.[159]

Ghany, et al.,[155] examined fibrosis progression in a retrospective-prospective analysis of 123 CHC patients who opted for no treatment with two liver biopsy specimens taken at a mean interval of about 4 years.[155] Ghany, et al., concluded that the two independent risk factors for fibrosis progression were ALT levels ≥5xULN and the degree of periportal necrosis on the initial biopsy.[155] The

upper limit of normal for ALT in their study was 42 U/L.[155] The Trent HCV Study Group[157] did not confirm the Ghany, et al., conclusion that ALT or several other factors (gender, alcohol consumption, HCV genotype, steatosis and siderosis on index-biopsy) predicted progression but confirmed that age at first biopsy and fibrosis on first biopsy did.[157] The Trent HCV Study Group is the largest prospective study undertaken to date with paired liver biopsies and a well characterised study population.[157] The discrepancies between Ghany, et al., and the Trent study may be related to the biopsy interval Ghany, et al., (~4 years) and Trent study (2.5 years).[155,157]

Ghany, et al.,[428] build on their early work with analysis of the data collected in the Hepatitis C, Antiviral, Long-term, Treatment against Cirrhosis (HALT-C) study, a randomised, controlled trial designed to evaluate the safety and efficacy of pegylated interferon in the treatment of chronic hepatitis patients with advanced fibrosis or compensated cirrhosis (N=1050).[428] Ghany, et al., conclude that compared to others, the best model to predict clinical outcomes and decompensation in severe liver disease consists of four variables: $\log_{10}$ AST/ALT ratio, platelet count, total bilirubin and albumin. The model to predict histological severity included baseline BMI, platelet count and hepatic steatosis.[428]

In a study to assess clinical predictors of fibrosis in patients with chronic liver disease, sequential liver biopsies in 96 patients approximately four years apart, showed that significant fibrosis at the baseline liver biopsy (Metavir ≥2) and elevated liver enzymes (ALT, 111.67 ±83.38, P=0.028) were independently associated with fibrosis progression in CLD (N=72, HCV; N=12, HBV; N=12, NAFLD).[429]

In a prospective study by Collier, et al., to assess and identify factors predictive of change in CHC patients, 105 patients with paired liver-biopsy specimens (mean interval, 41 months, range 5-183) were selected. Fourteen per cent had persistently normal ALT levels and no fibrosis progression (P=0.038).[166] The remaining patients had elevated ALT. 5% of the total developed worsening fibrosis by more than two stages, compared to 43%, where no change in fibrosis stage was found.[166] Current excessive alcohol intake (112 g per week in women and 168 g per week in men) (P=0.037), baseline fibrosis and fibrosis at second biopsy (P=0.01) also predicted rate of progressive fibrosis (P=0.0002); this agrees with Ghany[155] and Trent.[157,166]

In the literature, there is much evidence to show that ALT is a reasonable and convenient indicator of liver disease. Definitions of PNAL vary, as do opinions of what the most appropriate ULN level should be. Even if patients have PNAL, they need to be monitored; decisions about treatment should not be based on ALT levels alone although the literature consistently shows PNAL is indicative of reduced liver disease severity.

Fas ligand.

In chronic hepatitis-C patients, the Fas-ligand induced-death pathway appears to be gathering momentum as an important marker of liver injury. In liver injury that is Fas-receptor ligation-dependent there firstly occurs mitochondrial cytochrome *c* release, activation of the caspase cascade, DNA fragmentation and morphological changes that are characteristic of apoptosis followed by secondary necrosis, with release of hepatocellular enzymes as well as inflammatory changes.[430]

There is a positive correlation between caspase activation and levels of Fas ligand in inflammatory liver damage[322] and chronic hepatitis C progression.[321] However, Fas ligand is not routinely measured as yet and additional studies are required to confirm this association and the value of this measure in comparison to ALT measurement.

Fibrosis and Cirrhosis Measures

The current fibrosis markers (serum and tissue) are fraught with limitations. The histological scoring systems that include Knodell, Scheuer, Ishak and Metavir[431] are also based on inflammatory activity (grade) and fibrosis (stage). There are a range of validated serum fibrosis measures with the Fibrotest and Hepascore consistently scoring well in both sensitivity and specificity. Hyaluronic acid has also achieved recognition as a worthwhile serum marker of the ECM. This Hep573 Study chose the latter three measures of fibrosis.

A liver biopsy was not included as an outcome measure in the Hep573 Study as Ethics Committee approval would not have been granted for such a procedure in a CM study. In addition, this requirement would have adversely affected participation rates.

Fibrotest is the most validated marker of hepatic fibrosis and combines serum concentrations of $\alpha2$ macroglobulin, apolipoprotein A1, bilirubin, γ-glutamyl

transferase, haptoglobin,[432] age and gender.[433] A score of > 0.5 had a sensitivity of 85% and a specificity of 74% [434] in measuring advanced fibrosis.

Hepascore is a validated serum-fibrosis marker model comprising the following: age, α_2 macroglobulin, bilirubin, γ-glutamyl transferase, hyaluronic acid and gender.[433,435] A score of ≥0.5 yielded a specificity and sensitivity for significant fibrosis of 89% and 63% respectively, whereas scores <0.5 had a 74% specificity and 88% sensitivity for excluding advanced fibrosis.[435] A cut-off score of 0.84 was was used to detect cirrhosis (F4) as it provided 71% (63.2%-79.6%) sensitivity and 84% (76.9-90.3%) specificity.[435]

Bourlière[433] found that a cut-off point of 0.5 for ≥F2 diagnosis provided a specificity of 86% (81-90%) and a sensitivity of 63%, whereas a cut-off point of 0.84 for F4 prediction provided 88% specificity (85-91%) and 71% (57-83%) sensitivity. He compared Hepascore with other non-invasive hepatic fibrosis markers and found that Fibrotest and Hepascore displayed similar accuracy in the diagnosis of significant hepatic fibrosis and fibrosis staging concordance with liver biopsy. He also independently validated Hepascore as a fibrosis marker and concluded additional studies on blood donor and larger populations were required before Hepascore could routinely be used in practice. However, legitimacy was given to the use of APRI in addition to either Hepascore or Fibrotest as, in conjunction with APRI, as both offered more diagnostic accuracy in determining ≥F2 than APRI alone, and may minimise the need for liver biopsy.[433]

Schuppan suggested a correlation with fibrosis stage could be shown for some serum markers derived from components of the extracellular matrix. A correlation with fibrosis stage on histology was demonstrated for hyaluronic acid, TIMP-1, the amino terminal propeptide of pro-collagen III (PIIIP), collagen, laminin and their combinations.[517] Hyaluronic acid (HA) is produced by the stellate cells (HSC) within the liver and serum levels are found to be low in the normal liver.[517] Hyaluranon also reflects a compromised sinusoidal-endothelial function. Schuppan[517] reported that the serum marker hyaluronic acid on its own demonstrated 80% specificity[517] for excluding significant fibrosis.

McHutchinson, *et al.,*[436] found that a serum hyaluronic acid concentration of <60 ug/L was an accurate measure to exclude the presence of cirrhosis or significant fibrosis in chronic hepatitis C patients, having a predictive value of 99% and 93% respectively. Significantly higher serum HA values (382±31) were found in CHC patients with cirrhosis compared with non-cirrhotic patients (110±9 ug/L)

(P<0.001). Similarly, significantly higher mean serum HA values (179±11ug/L) were found in patients with fibrosis compared to patients without fibrosis (62±20 ug/L) (P<0.001).

SILYMARIN IN LIVER DISEASE INCLUDING CHRONIC HEPATITIS C

The medicinal plant, *Silybum marianum* (milk thistle, also known as St Mary's thistle) has been used for centuries for the treatment of a long list of liver diseases,[437] and recently in chronic hepatitis C populations to suppress HCV RNA replication.[438,439,440]

A typical milk thistle extract made from the crushed seeds, contains silymarin and fatty acids, e.g., linoleic acid. Silymarin is a complex of at least seven flavonolignans, one flavonoid taxifolin[441,442] and a bioflavonoid quercetin.[441] In both the European Pharmacopoeia and the World Health Organization International Non-Proprietary Names of Pharmaceutical Substances, these flavonolignans are called silibinin A, silibinin B, isosilibinin A, isosilibinin B, silichristin, isosilichristin and silidianin.[441,443,444] The US Pharmacopoeia uses the following terms respectively: silybin A, silybin B, isosilybin A, isosilybin B, silychristin, isosilychristin and silydianin.[441] (Silibinin rather than silybin will be the preferred term used here.)

The most biologically active of the above flavonolignans are silibinin A and B, which are 1:1 diastereoisomers of silibinin.[443,444] The current literature is mainly based on milk-thistle products which have been standardised to contain 65-80% of the flavonolignans and 20-35% fatty acids.[444]

Purified silibinin has been used historically to treat death-cap mushroom (*Amanita phalloides*) poisoning. According to a study by Hruby (at the poison information centre, in Vienna, Austria), if silibinin is administered within 48 hours of poisoning, only mild to moderate liver injury would be expected (N=18).[445] Severe liver damage, coagulation disorders and coma are likely to occur after 48 hours if left untreated. Four divided doses of silibinin were administered intravenously, each dose consisting of 20-50 mg/kg body weight/day; the dose varied on the severity of intoxication.[445]

Silymarin protects against the *Amanita phalloides* toxins, α-amanita and phalloidin, by inhibiting the toxins binding to cell receptors as well as their uptake and interaction with cell components. Silymarin prevents α-amanita binding with nuclear receptors which can inhibit protein synthesis and therefore prevent cell

repair.[446,446] As silymarin reduces the uptake of toxins into cells, the earlier it is administered after exposure, the better the outcome for liver injury and death.

Pharmacological Actions of Silymarin

Silymarin has confirmed antioxidant [447,448,449,450,437,] antifibrotic[451,19,452,] anti-inflammatory,[447,442,453,51] hepatoprotective,[454,455,446] anti-hypercholesterolaemic,[456] anti-hyperglycaemic[450], immunomodulatory[453] and antiviral[441,457,438,453] pharmacological actions *in vitro, in vivo* and in human studies.

Silymarin Has Direct Anti-HCV Activity (*in vitro*)

Studies discussed below have confirmed the antiviral action of silymarin *in vitro*.[453,458,459] Two main hypotheses explored to explain this effect are: (1) improved cellular response and (2) inhibition of viral function.

In 2007 Polyak*, et al.,* demonstrated a dose-dependent, improved cellular response to HCV infection with silymarin (proprietary extract MK-001) through antiviral, immunomodulatory and anti-inflammatory actions.[453] MK-001 has a high concentration of standardised silymarin at 92%.[453]

The study with MK-001 demonstrated inhibition of an established JFH-1 (genotype 2a) infection in Huh7.5.1 cells *in vitro* over 72-hours (MK-001, 10-20 μg/mL), as well as prophylactic protection over 48 hours (MK-001, 20 μg/mL). MK-001 at concentrations of 20 μg/mL had a similar antiviral potency to IFN-α (100 U/mL) in Huh7 and Huh7.5.1 cells.[453]

Polyak*, et al.,* investigated the possible antiviral mechanisms of MK-001 and demonstrated enhanced JAK-STAT signalling and the induction of IFN-α in Huh7 and Huh7.5.1 cells infected with JFH-1 (MK-001, 20 μg/mL).[453] They investigated further, and demonstrated that silymarin (MK-001, 20 μg/mL) and IFN-α (500 U/mL) together had a greater effect on the phosphorylation of STAT1 and STAT2 than IFN-α alone.[453] This synergism was confirmed in an experiment where silymarin (MK-001, 20 μg/mL) and IFN-α (0,1,10,100 U/mL) together had a greater effect on the inhibition of HCV replication than alone.[453]

Polyak*, et al.,* discuss the possible mechanisms for the up regulation of IFN-α, including changes to cell membranes, changes to IFN-α receptors, or interference with the negative regulation of the JAK-STAT pathway.[453] Experiments with fractions of MK-001 identified that only one fraction directly induced STAT1; therefore, Polyak, *et al*., suggest that the effect of MK-001 on IFN-α is more likely due to other antiviral mechanisms than silymarin's ability to directly affect the JAK-

STAT pathways.[453] HCV core protein is a negative regulator of IFN-α pathway. It is possible the synergistic action observed *in vitro* and *in vivo* when IFN is combined with silymarin is due to the ability of silymarin to reduce viral replication and therefore reduce the negative regulators of the IFN pathway. [458]

Bonifaz, *et al.,*[458] investigated this further than Polyak*, et al.,*[453] and demonstrated that silymarin does not directly affect the JAK-STAT pathway by comparing STAT1 levels in non infected, CNS3 and 9-13 cell lines, with silymarin, IFN and a control.[456-459]Bonifaz, *et al.,* interestingly achieved different antiviral effect with different cell lines, which may also explain the different STAT1 result to those of Polyak *et al.*[453]. In CNS3 cells, silymarin significantly down regulated HCV core mRNA (20%-36%) and protein (30%-60%). However in 9-13 cells, silymarin did not reduce the expression of HCV NS5A mRNA or protein.[458]

Polyak*, et al.,* also demonstrated silymarin's anti-inflammatory effects via the inhibition of TNF-α induced NF-κB and CXCL-8 in Huh7 cells and the inhibition of TNF-α in peripheral blood mononuclear cells (PBMC). They suggest because NF-κB is redox sensitive, silymarin achieves this anti-inflammatory effect via its antioxidant activity.[453]

Bonifaz*, et al.,* demonstrated potential, dose-dependent, antioxidant effects via increases in haem oxygenase-1 mRNA (HO-1), (60%-400%, at 100 μm and 200 μm) in Huh-7, CNS3 and 9-13 cells. HO-1 is an endogenous anti-oxidant and HCV infection is linked with increased OS. Possible mechanisms for the up regulation of HO-1 are via nuclear factor erythroid-2-related factor2 (Nrf2) and BTB and CNC homology 1 (BACH1), DNA binding proteins which activate Antioxidant Response Elements (ARE). However, they found that Nrf2 and BACH1 mRNA levels did not correlate with increased HO-1 mRNA.[458]

Polyak*, et al.,* in a later study, demonstrated OS post infection and showed that different proprietary extracts of silymarin (MK-001, USP and Legalon), eight silymarin pure compounds and IFN all reduced OS in a Huh7.5.1 and JFH-1 replicon model. They suggest the antioxidant affects are achieved via antiviral as well as by direct antioxidant function.[232]

Wagoner*, et al.,* demonstrated improved cellular response in various hepatitis C virus cultures (HCVcc) and pseudo particle assays (HCVpp). This showed that although silymarin did not prevent virus binding (40 μM), it inhibited virus entry (40 μM), fusion with liposomes (IC50 5 μM), HCV RNA and protein synthesis (40 μM), and reduced virus transmission (40 μM).[459]

Their study also investigated the inhibition of viral function and although some inhibition of viral function was shown, it was at levels five to 10 times higher than those required for anti-HCVcc effects.[459] Silymarin inhibited JFH-1, NS5B RNA-dependent RNA polymerase (RdRp) activity *in vitro* (IC50 300 μM) though silibinin had minimal effects (IC50>400 μM).[459] Silymarin tested on the RdRp from genotype 1b BK and four patient-derived 1b genotypes were poorly inhibited (IC50 27.7 μM to 162 μM). [459]

Silymarin did not inhibit viral activity of replicon lines which did not produce infectious virus.[459] This lack of antiviral activity and the weak interference with viral function suggests the main antiviral activity of silymarin in the HCVcc system was through improved cellular response rather than inhibition of viral function. Possible mechanisms include the inhibition of virus entry due to the stabilisation of cell membranes and the inhibition of virus transmission because of alterations to lipid metabolism and transport mechanisms.[459]

In cells, HCV accumulates at lipid droplets and the virus may use apolipoprotein assembly and secretion to 'hitch a ride' out of the cell. [459-460] Wagoner*, et al.,* demonstrated that silymarin inhibited microsomal triglyceride transfer protein (MTP) dependent production of apolipoprotein B (apoB) in a dose-dependent manner with a corresponding decrease in all virus transmission, including direct cell to cell.[459]

Ahmed-Belkacem*, et al.,* conducted experiments *in vitro*, to demonstrate which of the principal isomers of silymarin inhibit HCV replication and to explore the mechanisms of inhibition. This is particularly relevant work in light of new drugs which target HCV enzyme function, particularly inhibitors of NS3/4A serine protease and HCV RdRp polymerase.[441]

Their study showed that silibinin A and silibinin B inhibited JFH1 (genotype 2a) replication in Huh7 cells (EC50s 20-40 μm), HCV genotype 1b Replicon replication in Huh7 cells (EC50s, 1μm) and HCV RdRp (IC50s 75-100 μm) in a dose-dependent manner. Isosilibinin A and isosilibinin B were effective in inhibiting HCV genotype 1b Replicon replication in Huh7 cells but two to three times less potent in inhibiting HCV RdRp than silibinin. Silichristin and silidianin had no effect in any of the models. Silymarin isomers had no effect on HCV NS3/4A protease.[441]

These four studies confirmed the antiviral effects of silymarin *in vitro*. The reduction in cell replication was achieved at much lower doses of silymarin than the inhibition of viral function. This suggests silymarin acts by improving the host

response to infection rather than interfering with viral function, through antioxidant, anti inflammatory and immunomodulatory actions, affecting membrane stability, lipid metabolism and cell signalling pathways.[441,453,459]

Silymarin Has Direct Anti-HCV Activity (Intravenously)

Ferenci, *et al.,* treated patients who had failed to respond to pegylated interferon and ribavirin with increasing doses of intravenous silibinin (Legalon,SIL;Madaus).[438] A dose-dependent reduction of HCV RNA was achieved. Patients were given 15 or 20 mg/kg/day IV silibinin for 14 days, and 280 mg oral silymarin three times per day combined with pegylated interferon and ribavirin therapy from Day eight. At Week 12, 7/14 patients who had been previously classed as non responders, had undetectable HCV RNA.[438]

Although there was no control group and the numbers were small, this was the first time in the literature that silibinin (intravenously) had shown a direct anti-HCV activity in chronic hepatitis C patients.[438] It identifies a new pharmacological action for silibinin in humans and provides useful information about effective doses of intravenous silibinin and oral silymarin. Clinically, it is significant because adding pegylated interferon and ribavirin to intravenous silibinin showed greater efficacy than silibinin or pegylated interferon and ribavirin alone. Patients with both genotypes were involved in the trial but no information was provided on the genotype of those with undetectable HCV RNA at Week 12.

The recent use of intravenous silibinin at a dose of 1400 mg daily for 14 days prevented HCV RNA reinfection after orthotopic liver transplantation.[457] Neumann and colleagues[457] started silibinin infusions eight hours after orthotopic liver transplantation, when HCV RNA levels measured 182 IU/mL and after three days HCV RNA became undetectable (<15 IU/mL) and remained so 168 days later. No data were provided past 168 days. This 1400 mg intravenous silibinin dose equated to 20 mg/kg and was based on the original study by Ferenci, *et al.,*[438] who used intravenous silibinin in chronic hepatitis C patients. This clinical case supports the use of silymarin as an antiviral in the treatment of hepatitis C.

Previous Silymarin Dosing Regimens in Alcoholic Liver Disease

Bunout used oral silymarin at varying doses from 280 mg/day for 15 months in a randomised, controlled study of 70 patients with alcoholic liver disease.[461] Trinchet used 420 mg/day for three months in a randomised double-blind trial of 58 patients with histologically proven alcoholic hepatitis. [462] Pares used 450 mg/day

(sourced by Madaus) for two years in a randomised, double-blind study of 200 alcoholic patients with liver cirrhosis. [463] Results for these studies showed no clinically relevant effect.

The studies had several limitations, the dose used by Bunout[461] was low and the duration of the Trinchet[462] study was short. In the study by Pares,[463] although other aetiologies for liver cirrhosis were excluded, the patients were not tested for HCV until after the trial. After the trial, 75 patients were tested for HCV and 39% tested positive. According to Hawke, *et al.,*[464] liver damage, especially in chronic inflammation may affect the bioavailability of the different components of silymarin and therefore the efficacy. A reduction in the absorption of silymarin correlated with increased plasma levels of caspase indicative of increased inflammation.[464,443]

Silymarin achieved clinically relevant and significant results in the following studies. Ferenci, in a randomised, controlled study used 420 mg/day for two years and significantly reduced mortality in those with alcoholic cirrhosis ($P=0.01$).[446] Feher used 420 mg/day for six months in a double blind, clinical trial and showed it to be effective in alcoholic liver disease (normalisation of bilirubin, ALT, AST, histological improvement).[465] Velussi used 600 mg/day for 12 months in a randomised, open, controlled study with diabetic patients with cirrhosis and significantly reduced malondiadehyde, ALT, AST, fasting and mean glucose levels (HbA1c) ($P<0.01$).[450] These last three studies successfully demonstrated the effectiveness of silymarin in cirrhosis and alcoholic liver disease and used silymarin marketed under the brand name Legalon (provided by Madaus).

In the randomised, controlled trial in patients with cirrhosis by Ferenci and colleagues (mentioned above), 87 patients with cirrhosis (alcoholic, N=46, non-alcoholic, N=41) took 420 mg silymarin daily for two years.[446] Unlike Pares, Ferenci identified the aetiology of the liver cirrhosis.[463,446] A similar group of 83 patients with cirrhosis (alcoholic, N=45, non-alcoholic, N=38), were given a placebo preparation for the same period. The four-year survival rate in the silymarin-treated group was 58% compared to 39% in the control group ($P=0.036$).[446]

The analysis showed silymarin was more effective among alcoholic cirrhotics ($P= 0.01$) than non alcoholic cirrhotics and particularly in alcoholic cirrhotics with Child's Class A ($P= 0.03$). This suggested silymarin is particularly protective against the toxic effects of alcohol on the liver and that early treatment is

effective.[446] The research by Ferenci was methodologically sound and one of the early reports of the efficacy of silymarin in chronic alcohol induced liver disease.[446]

Saller reviewed 36 papers and concluded the available evidence suggested that silymarin may have a role in the treatment of liver cirrhosis, especially alcoholic cirrhosis. In five trials with a total of 602 patients with liver cirrhosis a significant but modest (7%) reduction of liver-related mortality (not corrected for study duration) was achieved on silymarin.[466] These findings were confirmed in a recent meta-analysis by him.[467]

All these studies[446,450,461,462,463,465] provide mixed results. However, they indicate that the higher the dose, the sooner administered and the longer the study, the more likely a protective effect is found. This, and the reviews by Saller,[466,467] are of significance to individuals with heavy alcohol use, as high levels of long-term dosing of silymarin may prolong survival in cirrhotics.

Cytochrome P450 2E1 (CYP2E1) is a phase I enzyme in the liver and is the major hepatic alcohol metabolising enzyme. There are differences of opinion as to whether silymarin inhibits phase I detoxification or not. Inhibiting phase I could protect against alcohol and toxins such as *A. Phalloides* as phase I activates certain toxins*. Alternatively, silymarin may protect against these bioactivated toxins through its broad antioxidant capabilities. Dixit*, et al.,*[468] suggests that silymarin enhances liver detoxification via the inhibition of phase I detoxification.

Song*, et al.,*[469] investigated the effects and mechanisms of action of silymarin in rats with ethanol-induced liver disease. Silymarin demonstrated a wide range of hepatoprotective actions. It also reduced inflammation via the inhibition of TNF-α, reduced oxidative stress by maintaining glutathione levels, prevented an increase in ALT levels, reduced the level of microvesicular steatosis, prevented necrosis and reduced triglyceride accumulation in the liver. They also demonstrated this effect was not due to modulation of enzyme CYP2E1 and phase I.[469] Miguez*, et al.,*[470] agree that silymarin reduces alcohol toxicity, associated lipid peroxidation and GSH depletion (0.01-0.5 mM), but not via cytochrome CYP2E1.

Oral Silymarin in Chronic Hepatitis C

Huber performed a retrospective, observational study using a standardised silymarin extract (Legalon) in 40 hepatitis C patients with HCV genotype 1 who were ineligible for antiviral therapy.[471] The patients were dosed in three groups, group 1 at the recommended dose of 420 mg silymarin day (N=13), group 2 at 840 mg/day (N=20) and group 3 at 1260 mg/day (N=7). The mean duration of silymarin

treatment was 125±78 days. If there was no reduction of ALT level after three months, the treatment was stopped. The silymarin doses had no significant impact on any of the biochemical markers of liver inflammation compared to baseline. While this study is valuable in terms of providing information about dosage and adverse events, the robustness of any conclusion based on the results is weakened because of the limitations of the study design (no randomisation, no control group, no double-blinding of patients, short duration and small numbers) and by the fact that biochemical markers of liver inflammation were the only outcome measures used in this study.[471]

Huber's study used doses that were two and three times higher than the recommended daily dose of silymarin in order to discount inadequate dosing as an explanation for the lack of efficacy of silymarin in viral hepatitis.[471] Hawke has since demonstrated that even at 700 mg three times per day, oral dosing may not be sufficient to exert an antiviral effect.[464]

A further randomised, double-blind, placebo-controlled, crossover pilot study using a different standardised extract of silymarin (Indena, 80% silymarin flavonolignans) found that *Silybum marianum* at similar doses as the Huber study (600 mg and 1200 mg silymarin per day for 12 weeks) had no effect on ALT levels, quality of life, or HCV RNA amongst 24 individuals with chronic HCV infection.[472] While this was a well-designed study, the short duration of therapy of 12 weeks and small population (only 17 completed) may explain the failure to observe a benefit in chronic HCV infection. The conclusion of the pilot study was the suggestion that in the future, clinical studies of *S. marianum* in CHC should combine *S. marianum* with other herbal therapies in order to identify clinical and laboratory indicators that are appropriate and reliable in this condition.

The Hepatitis C, Antiviral, Long-term, Treatment against Cirrhosis (HALT-C) trial was a randomised, controlled trial designed to evaluate the safety and efficacy of pegylated interferon in the treatment of chronic hepatitis patients. Of the 1145 patients enrolled, 1050 patients were randomised, all of whom had failed to respond to previous interferon therapy.[473,119] The aim of the HALT-C Trial was to determine if prolonged interferon therapy changed histological and clinical outcomes for patients who had failed to clear the hepatitis C virus after previous interferon treatment.[473,119]

Seeff reported that among 1145 HALT-C study participants, 56% had never taken herbal medicines, 21% admitted past use and 23% were using herbs at

enrolment.[119] Silymarin constituted 72% of the 60 herbal medicines used at enrolment. Sixty-seven per cent of the participants had never used silymarin, 16% had used it previously and 17% used it at the beginning of the study.[119] He did not find any statistical difference in the biochemical markers (liver inflammation) and virological markers (HCV RNA) between those using silymarin or not (non silymarin users). However silymarin users reported fewer symptoms and slightly better quality-of-life scores. Use and dosing of silymarin in HALT-C was self-directed and not controlled by the researchers. Seeff reported that the low efficacy of silymarin in viral hepatitis studies may be due to the lack of standardisation, inadequate dosing and patients who were non responders to standard therapy, consequently difficult to cure.[119]

Freedman reported on the HALT-C trial that the continuous use of silymarin for about 3.5 years was associated with less disease progression from fibrosis to cirrhosis but had no statistically significant impact on clinical outcomes.[473] The silymarin users had significantly less collagen in their baseline liver biopsies. He hypothesised that silymarin efficacy is increased if used early in the disease progression process.[473]

In a randomised study by El-Zayadi, et al.,[51] designed to find cost-effective alternatives to IFN treatment, 170 chronic hepatitis patients were randomly assigned to receive 600-800 mg ribavirin, amantadine 200 mg and ursodeoxycholic acid (UDCA) 500 mg daily or silymarin 450 mg/day for 24 weeks.[51] ALT normalisation at the end of treatment was 58.5% for the ribavirin-based therapy compared to 15.3% in the silymarin group (P<0.001).[51] Although silymarin was less effective than a novel combination of oral agents for HCV treatment, silymarin achieved ALT normalisation of 15% and is an affordable alternative to IFN with limited side effects. The randomised trial design and study duration of 24 weeks are strengths of this study.[51] The study was limited by failing to document the process of random allocation and did not include an intention-to-treat analysis.[51]

In essence, the study by Huber was of short duration and poor design,[471] while Gordon's study was a good quality study but of short duration and involved only a small population.[472] The primary purpose of the HALT-C study was to evaluate the efficacy and safety of long-term pegylated interferon, not the efficacy of silymarin.[473,119] The HALT-C study produced some interesting results which highlight the need for long-term, standardised dosing. All these studies were of short duration and in some, non-standardised silymarin or low doses were used.

These were issues the Hep573 Study sought to address with a RDBPCT, a standardised high oral dose of silibinin and longer study duration (24 weeks rather than four to 12 weeks). El Zayadi explored alternatives to interferon and assessed the cost effectiveness of silymarin and these drug alternatives on SVR and ALT normalisation.[51] The Hep573 Study also explored silymarin as an alternative treatment in some chronic hepatitis C patients and used ALT normalisation as an outcome measure. Freedman's suggestion that silymarin used early on in the disease process, increases efficacy is interesting since studies on pharmacokinetics suggest the bioavailability of silymarin is significantly reduced in chronic inflammatory disease.[443,473] This information could inform future treatment protocols.

Silymarin and Pharmacokinetics

The optimal dosage of silymarin has been controversial and pharmacokinetic studies are an attempt to provide clarity on this issue. Two recent studies have sought to define optimal dosing regimens. [443,464]

Hawke*, et al.,*[464] clearly demonstrated through their oral silymarin dose acceleration and pharmacokinetic study, that the low bioavailability associated with usual doses of silymarin may be remedied with silymarin doses above 700 mg three times per day. In 32 CHC patients, all previous non responders to interferon-based therapies, the only flavonolignans detected in the plasma at silymarin doses from 140 mg to 560 mg were silibinin A and silibinin B. In contrast, six silymarin flavonolignans were detected in the plasma when a 700 mg silymarin dose was administered.

Hawke*, et al.,* also showed that oral doses up to 2100 mg oral silymarin were non toxic.[464] However at the oral dose of 700 mg silymarin three times per day, a steady-state peak plasma concentration of silibinin A and silibinin B was still one to two orders of magnitude lower than the concentrations demonstrated to inhibit HCV viral replication *in vitro*; the trough concentrations of silymarin were approximately 25 times below the peak concentrations.[464]

A reduction in bioavailabilty with oral silymarin was correlated with caspase levels as a measure of liver inflammation.[464] Loguercio, *et al.*, suggests that liver damage, especially chronic inflammation may affect the bioavailability of silymarin, explaining low efficacy in patients with chronic hepatitis C.[443] This supports the results and comments from the HALT-C trial,[473] Ferenci's study[446] and *A.*

Phalloides poisoning;[445] that early administration of silymarin will improve efficacy through hepatoprotection and greater bioavailability.

The reported clinical case by Neumann*, et al.,* describing the effective use of intravenous silibinin in post orthotopic liver transplantation helps confirm that silibinin can achieve remarkable antiviral results in patients.[457]

Silymarin Summary

Research prior to Ferenci*, et al.,*[438] consistently showed *Silybum marianum* had no effect on HCV RNA in chronic hepatitis C patients. However, *in vitro* and intravenous studies on silymarin and/or purified silibinin showed a direct anti-HCV activity. Oral dosages of 420 mg/day demonstrated therapeutic benefit with good tolerance if used to treat alcoholic cirrhosis and to reduce liver inflammation (ALT).[474] Oral dosages of 700 mg are required for six flavonolignans to be detected in plasma as opposed to only two flavolignans at doses of less than 560 mg in CHC patients.[464]

In the literature, the studies show the dose, preparation of silymarin administered, duration of treatment, patient's diagnosed condition and any comorbidities, the stage in the disease process as indicated by inflammation,[443] the genotype of the virus,[471] and the *in vitro* replicon model [441,453,459,458] all influence the effectiveness of silymarin as a treatment in liver disease. In summary:

(1) to impact hepatic necroinflammation: 450 mg/day silymarin for 12 months achieved ALT normalisation in 15% of the CHC patients in a randomised study[51];

(2) to improve liver histology: 420 mg/day silymarin for 41 months (mean) is effective in alcoholic cirrhosis (Child-Pugh A, 5-7)[446,465];

(3) to elicit antioxidant effects: 600 mg/day silymarin for 12 months reduced malondialdehyde in diabetic cirrhotics[450]; and

(4) to elicit direct anti-HCV activity: 1400 mg/day intravenous silibinin for 14 days is required.[438,439,457.]

Therefore, the 720 mg/day silymarin (calculated as silibinin) administered in the Hep573 Study to examine the effects of antioxidants on hepatic necroinflammation, OS and hepatic fibrosis in CHC patients was sufficient to examine the testing hypothesis. The Hep573 Study silymarin dose was applied at the upper end of the dose range shown to have some effect on ALT levels (240-800 mg silymarin, circa 2003) and confirmed by the data reported above.

The emerging application of intravenous silibinin (1400 mg/day) to significantly reduce HCV replication[438] means that the dose used in the Hep573 Study was unlikely to be sufficient for a direct anti-HCV effect.

CLINICAL TRIALS USING ANTIOXIDANTS

In this Study the implication of OS in hepatitis C disease progression provided the rationale for the use of antioxidants in CHC patients. Firstly, it is necessary to address some of the controversies that have arisen from the use of antioxidants in diseases other than hepatitis C before reviewing the literature on hepatitis C specifically.

Antioxidant Therapy in Conditions Other than HCV

There is controversy over the use of antioxidants and whether they are beneficial or harmful to health. It is known the creation of free radicals is a normal physiological process; however, excess free radicals can drive pathological processes, and it is this delicate homeostasis that is the key to the controversy.

Some of the major studies that have contributed to this controversy will now be examined placing particular emphasis on the type of antioxidant and dose administered and health status of the individual, which are considered key components in this debate.

Pro-oxidative stress can happen when antioxidants involved in a redox reaction become unstable oxidants themselves.[227] Antioxidant-induced stress[475,476] is the suppression of physiological levels of oxidative stress that are essential to certain biochemical pathways.

The effects of OS and antioxidants are dose dependent. H_2O_2 on Jurkat T-cells, 0.7 µM resulted in cell proliferation, 1.0-1.3 µM resulted in apoptosis and >3 µM resulted in necrosis.[477] Beta-carotene protected DNA from damage at 1-3 µM but oxidised DNA at 4-10 µM.[477] Poljsak. *et al.*,[476] also state the body maintains antioxidant homoeostasis and that exogenous antioxidants may suppress endogenous antioxidant systems and a change in one antioxidant will result in a compensatory change in another resulting in no change in the cells antioxidant potential.[476,478]

A synergistic network of interacting antioxidants is required to prevent the creation of pro-oxidants (Figure 2.7).[477,475,479] The higher the redox potential of the

antioxidant, the more reactive the antioxidant may become. The redox potentials in volts (V) of some antioxidants are: 0.65 V for beta-carotene, 0.50 V for Vitamin E, 0.25–0.50 V for flavonoids, 0.25 V for uric acid and 0.01 V for Vitamin C. [477]

Also some antioxidants become pro-oxidants in the presence of transition metals.[284] Ascorbic acid becomes an ascorbyl radical, and retinals and carotenoids become radicals in the presence of oxygen and ferrous iron.[477,180,476]
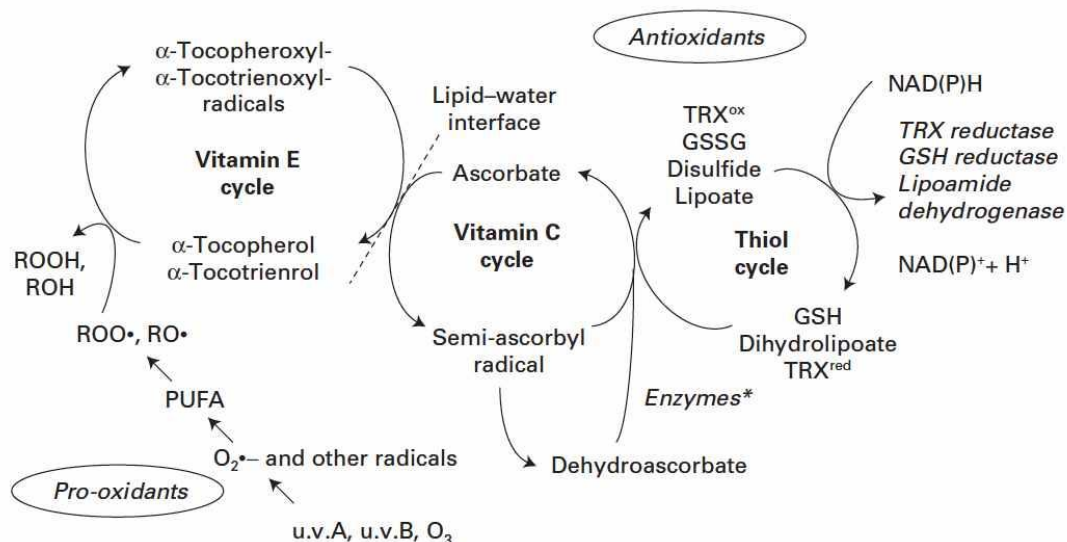
Fig. 2. The antioxidant network showing the interaction among vitamin E, vitamin C and thiol redox cycles. PUFA, polyunsaturated fatty acids; GSH, glutathione; TRX, thioredoxin; TRX^{ox}, TRX^{red}, oxidised and reduced forms of TRX respectively. * Thiol transferase (glutaredoxin), GSH-dependent dehydroascorbate reductase, protein disulfide isomerase, TRX reductase. (From Packet *et al.* 2001.)

Figure 2.7: The antioxidant network.
(Reprinted by permission Am J Clin Nutr[479] (2001;131:369S-373S), American Society for Nutrition.)

There is agreement that dietary levels of natural antioxidants are beneficial to health.[480,481,482,483,484] Epidemiological studies have specifically identified that plasma levels of beta-carotene and a diet containing foods rich in beta-carotene are linked with a reduced risk of lung and certain types of cancer.[485] This led to a series of studies to evaluate supplementing the diet with beta-carotene which showed that more is not necessarily better, and highlighted the need to supplement at the appropriate dose with multiple synergistic natural antioxidants.

One of the largest systematic reviews and meta-analyses of the use of antioxidants concludes that: 'Treatment with beta-carotene, vitamin A, and vitamin E may increase mortality'.[483] The findings of that particular review are not particularly relevant to the use of a broad range of natural, phytochemical-based,

antioxidants in the Hep573 Study because all the antioxidants in the studies were synthetic, and in 38 of the 68 trials, antioxidants were given singly.[483]

Vitamin E consists of multiple natural isoforms which are R,R,R stereoisomers, whereas synthetic tocopherols are R or S racemic stereoisomers.[486] These sterioisomers are not bioequivalent, or therapeutically equivalent.[487] Tests in rats showed highly different biological activities for different steriosomers.[488] It is suggested that synthetic antioxidants behave differently from natural ones, in that they do not interact with other antioxidants and endogenous antioxidant systems in the same way, becoming pro-oxidants and forming toxic metabolites.[227]

The Alpha-Tocopherol, Beta-carotene Cancer Prevention Study (ATBC),[489] assessed the effect of supplements on the incidence of lung and other cancers. Participants were randomly assigned to four groups: (1) alpha-tocopherol 50 mg; (2) beta-carotene 20 mg; (3) alpha-tocopherol 50 mg plus beta-carotene 20 mg; or (4) placebo. The study agents were synthetic dl-α-tocopheryl acetate and synthetic beta-carotene.[489] Participants who received beta-carotene had an 18% (95% CI, 3%-36%) higher incidence of lung cancer and 8% (95% CI, 1%-16%) and higher total mortality. There was no increase among the group receiving alpha tocopherol alone, or alpha tocopherol combined with beta-carotene. Further analyses showed the adverse effects of supplemental beta-carotene in the ATBC study may be associated with heavy smoking and high alcohol intake.[490,491]

Similar results were obtained in the Beta-carotene and Retinol Efficacy Trial (CARET).[492,493] The group receiving beta-carotene and high dose retinol supplementation had a 28% (95% CI, 4%-57%) higher lung cancer incidence and 17% (95% CI, 3%-33%) higher total mortality compared with those who received the placebo.[494] Again, there were associations of the increased lung cancer incidence with the highest quartile of alcohol intake and with large-cell histology.[492]

The results of the ATBC Study and CARET suggest that beta-carotene hastens the progression of lung cancer in a highly oxidative environment when abnormal cells and/or latent tumors are already present.[494] Poljsak, *et al.,* suggest antioxidants may increase the survival of altered cells and their proliferation as seen in the CARET, ATBC trials and studies with epithelial cell malignancy.[476,495]

Smoking decreases plasma levels of beta-carotene, ascorbic acid [477,496] and alpha tocopherol,[496] which is important for the recycling of antioxidants and preventing the formation of pro-oxidants. Beta-carotene can produce highly reactive, pro-oxidant, carotenoid cleavage or breakdown products (CCPs or CBPs)

in the lungs of smokers.[494,497,498] A series of experiments on ferrets' lung tissue exposed to cigarette smoke, demonstrated low dose beta-carotene was more protective than single high dose beta-carotene[499] and that beta-carotene should be given with other antioxidants. Alpha tocopherol and ascorbic acid improved the retinoic acid content in the lungs,[500] and were synergistic in preventing oxidative cleavage of beta-carotene.[496]

Studies on rats between 2002[494] and 2009[498] showed that in highly oxidative environments such as in the lungs of smokers, in people with asbestosis and in tissue proximal to active white blood cells, beta-carotene is oxidised into CBPs.[498] These CBPs were found to inhibit mitochondrial respiration in the rat lung, brain and liver,[497] to stimulate apoptosis in neutrophils,[497] oxidise DNA[498] and upregulate advanced glycation endproducts.[501] CBPs also decreased glutathione and elevated malondialdehyde (MDA).[498] The 2009 study concluded complementary antioxidants could reduce mitochondrion toxicity.[498]

In contrast to the adverse results experienced in the CARET and the ATBC studies, the Nutrition Intervention Trial conducted in Linxian, China,[502] found a 21% (95% confidence interval [CI], 1%-36%) drop in stomach cancer mortality and a 13% (95% CI, 0%-25%) reduction in total cancer mortality after receiving a combination of vitamin E, beta-carotene and selenium over five years.[502] The major differences in this trial were that a combination of antioxidants was given to participants suspected of having chronic nutrient deficiencies.[502]

There is significant evidence from published studies that dietary antioxidants[480-482] are not only beneficial to health but also show conclusively that large doses of single synthetic antioxidants are damaging to health especially in conditions of high oxidative stress.[476,477,490,491,493,494,497,498,503]

The literature also found that certain levels of OS are necessary for physiological processes but excess OS drives pathology.[475] Therefore in chronic pathology with high oxidative stress, a broad range of natural antioxidants is recommended.[477,495,504,505]

Poljsak, *et al.*,[476] suggest that effective antioxidant treatment reduces the damage caused by OS because it prevents free radicals forming in the first place without disrupting the integrated antioxidant systems.[506] One of the main targets for this action is the stabilisation of mitochondria.

Although some phytochemicals at high concentrations can scavenge free radicals, in lower, dietary amounts phytochemicals may activate adaptive cellular

stress response pathways which can improve stress tolerance and life expectancy.[254]

In 1956, Harman hypothesised that the metabolic rate was related to the production of ROS, and consequently calorie restriction (CR) resulted in reduced metabolism and reduced ROS and therefore reduced cellular damage. This theory became known as the Free Radical Theory of Aging (FRTA).[504] However, it has been found CR increases the metabolic rate as measured by oxygen consumption and heat generation. Only glucose can be metabolised anaerobically, by glycolysis outside the mitochondria without the production of ROS. Therefore in CR, as mitochondrial respiration increases so does the production of ROS. Increased ROS is the mechanism which upregulates adaptive responses in CR, exercise and impaired glucose metabolism. This results in a net reduction in ROS due to the stimulation of antioxidant defences and detoxification.[504] This adaptation or preconditioning of the mitochondria has been named mitochondrial hormesis or mitohormesis.[505,504,477] Mitochondrial hormesis may delay aging and age-related diseases.[504] In studies with nematodes, restricting dietary glucose resulted in increased life span.[507] Supplemental antioxidants reduced the OS and negated the extension of life span.[507]

Similar to the preconditioning effect of exercise and glucose restriction, Son, et al., postulate low doses of phytochemicals act as toxins which increase the cells adaptive response to stress.[254] These hormetic pathways, activated by phytochemicals, include the sirtuin-FOXO pathway, the NF-kappaB pathway and the Nrf-2/ARE pathway.[254] Surh[508] terms this exogenous form of hormesis, xenohormesis.[254,509]

The literature suggests that antioxidants are only beneficial when the body's antioxidant systems are depleted.[502,503] In healthy people with adequate nutrition, supplemental antioxidants may reduce the benefit of preconditioning in exercise, and CR may not increase cellular levels of antioxidants as the body's antioxidant homeostatic mechanisms will merely compensate. Phytochemical-based antioxidants may provide a preconditioning, protective effect by being mildly toxic. The generation of reactive oxygen and nitrogen species and antioxidants are hormetic and in small doses they promote health and longevity but in larger doses may be damaging. [254]

The relevance of this section goes to the heart of the Hep573 antioxidant interventions, which are a broad range of non-synthetic, predominantly

phytochemical antioxidants, in doses that support adaptive antioxidant defences rather than generating a pro-oxidant effect. It shows the importance of monitoring both tobacco and alcohol use in the Hep573 Study as both lifestyle factors exacerbate oxidative stress, and can be counterproductive in single synthetic antioxidant therapy. Chronic hepatitis C patients are deficient in a range of antioxidants including glutathione,[310] trace elements and minerals.[510] The literature[480-482] indicates that in cases of nutritional deficiency, antioxidants are more efficacious than in those who are well nourished.

Summary of Antioxidant Research in CHC

By reducing OS in CHC, antioxidants can alleviate hepatic damage, protect vital immune cell function as well as promote apoptosis and therefore improve the chances of eliminating the virus.[332]

Evidence for antioxidants and herbal medicines inhibiting inflammation,[511,512] hepatic fibrosis[147,233,382,513,514] and carcinogenesis[231] is expanding.

Friedman [382,357] and others identify that the administration of antioxidants in CHC patients, reduces hepatic necroinflammation[147,382,515,516,51,511,512,517,518] and inhibits HSC activation,[233,382,514,519-521] thereby reducing progression to hepatic fibrosis. OS in CHC patients is associated with non response to treatment[522] and those with high serum thioredoxin levels exhibit resistance to antiviral therapy.[298] Antioxidants increase SVR in those treated with pegylated interferon and ribavirin[293,438] and also reduce recurrence of HCV reinfection after liver transplantation.[457]

HCV infected patients have been found to have an imbalance of toxic and essential trace metals. A small study of patients who were anti-HCV antibody-positive found significantly lower concentrations of plasma zinc (Zn), higher copper (Cu), iron (Fe), lead (Pb), cadmium (Cd), and aluminum (Al), lower activities of erythrocyte antioxidant enzymes glutathione peroxidase and catalase, and raised superoxide dismutase activity.[510] The patients were also found to have significantly increased levels of lipid peroxidation malondialdehyde (MDA), and inflammatory markers including alanine aminotransferase (ALT), high sensitivity C-reactive protein (hs-CRP), ferritin, and Cu/Zn ratios, as well as decreased albumin and high density lipoprotein (HDL). The increase in lipid peroxidation malondialdehyde could be correlated with the interactions between toxic (e.g., Pb, Cd, and Al) and essential metals (e.g., Zn, Cu, Fe).[510] Such trace element imbalance may

exacerbate oxidative stress, inflammation and hepatic damage in individuals with HCV infection.

Clinical trials using antioxidants in CHC.

Antioxidants were tested in chronic hepatitis C patients who were non responders to interferon monotherapy. N-acetylcysteine (NAC) was used on its own; [523,524] in combination with vitamin E,[525] and in combination with selenium and vitamin E.[392] In each of these studies, the listed antioxidants were found to increase the efficacy of interferon monotherapy and led to further research in the efficacy and mechanism of action of antioxidants in CHC.

Prior to the commencement of the Hep573 Study, a small randomised controlled trial examined the impact of vitamin E and antioxidants on IFN response in chronic HCV infection.[392] Twenty-four study participants were randomised to three groups receiving IFN-α (4.5 million units thrice weekly subcutaneously), IFN only, IFN plus NAC (1,800 mg/day) plus sodium selenite (400 mcg/day), or IFN, N-acetyl cysteine, sodium selenite and vitamin E (544 IU/day). The study group receiving vitamin E had a greater reduction in HCV RNA (viral load) (P=0.028) than the control group. However, the sustained virological response was similar in all groups.[392] The authors suggested the trends towards greater virological response warranted further evaluation of the role of vitamin E as a supplement to IFN-based therapy. However, the weaknesses of this study are the small numbers and the fact that SVR was not affected.

A randomised double-blind, placebo-controlled, clinical trial using the Chinese herbal medicine preparation CH100 was conducted in people with chronic HCV infection at John Hunter Hospital, Newcastle, Australia. There was a significant reduction in ALT levels at the end of the six-month study period (P<0.03).[526] The CH100 consisted of 18 phytochemical based ingredients, 12 of which had antioxidant effects.[527-536] The study did not have a follow-up period and this is a limitation. In a CH100 follow-up study (N=100), there were no significant differences in either ALT levels or HCV RNA over a six-month treatment period (intention-to-treat analysis). However in per protocol analysis, a significant decline in mean ALT (as measured at Weeks 4 and 24) was demonstrated. These changes reverted to baseline scores in the post-treatment follow-up period of 24 weeks.[537] This study informed the choice of the Hep573 Study Coordinator to select a number of phytochemical-based interventions with antioxidant pharmacological actions. Further animal studies identified anti-inflammatory and antifibrotic effects of the

CH100 interventions on liver pathology.[538,539] These findings may be due to the antioxidant nature of many of the CH100 ingredients reducing OS and inflammation.

In an open labelled, one centre, non-randomised clinical trial, 50 people with chronic HCV infection were treated orally for 20 weeks with glycyrrhizin (1000 mg), schisandra (1500 mg), silymarin (750 mg), ascorbic acid (6000 mg), lipoic acid (300 mg), L-glutathione (300 mg) and d-α-tocopherol (800 IU) daily.[511] Intravenous preparations of glycyrrhizin (120 mg), ascorbic acid (10,000 mg), L-glutathione (750 mg) and vitamin B complex (1 mL) were also administered daily for the first 10 weeks. After treatment, participants then entered a follow-up period of 20 weeks.[511]

In this trial, ALT normalisation (defined as a single normal result by testing laboratory standards) was reported in 32.3% (11/34) of the participants with elevated ALT, while HCV viral load (≥1 log) decreased in 25% of participants and QOL improved in 58%.[511] However, the study design was weak (non-randomised) [511] and would need to be reproduced in a randomised controlled trial.

In a double-blind, placebo-controlled study of 100 interferon non responders, 50 were assigned to intravenous and oral antioxidants (N=25) or placebo (N=25) and 50 were given oral antioxidants only (N=25) or placebo (N=25) for 24 weeks.[512] The interventions were identical to the study[511] listed above. In the intravenous and oral antioxidant group, ALT levels reduced in 52% (13/25) of participants on active treatment compared to 20% (5/25) in the placebo group (P=0.05).[512] The oral antioxidant group did not achieve any significant changes in the designated outcome measures. The study was well designed with appropriate outcome measures of liver inflammation, histology activity index and HCV RNA levels. The daily dose of silymarin was 750 mg. This is similar to the 720 mg silibinin daily dose used in the Hep573 Study. These two studies showed that combined oral and intravenous, broad spectrum antioxidants formulations may prove effective in CHC patients.[511,512]

Groenbaek, *et al.,* followed 23 CHC patients in a randomised, controlled trial for 24 weeks. There were 12 patients in the antioxidant group and 11 in the placebo group. The daily dose of the antioxidants was: ascorbic acid (500 mg), d-alpha tocopherol (945 IU) and selenium (200 μg). They found no effect on oxidative-stress markers, lipid peroxidation (MDA) or protein oxidation (2-amino-adipic semialdehyde (2-AAS)) or ALT and HCV RNA.[540] However, it is hard to draw a conclusion of no benefit from these three antioxidants in CHC patients when there

was no published sample size calculation on the primary-outcome measure,[540] and the study was not able to deliver a result.

Thirty HCV patients who were non-responders to, or ineligible for IFN/RBV treatment took mitoquinone (co-enzyme Q10) at either 40 mg or 80 mg per day or placebo in a randomised, double-blind parallel design study for 28 days. Both doses of mitoquinone led to significant reductions in absolute and total/mean percentage changes in ALT from baseline to Day 28 ($P<0.05$). The primary endpoint was a percentage change in ALT at Day 28 compared to baseline on the two treatment arms compared to the placebo.[518]

Choi, *et al.*,[226] identified some antioxidant formulations used without concurrent IFN therapy that had favorable effects on ALT whereas other antioxidant formulations such as NAC, vitamin E, or vitamin E plus NAC, ascorbic acid and/or selenite, were disappointing when used in combination with IFN. Choi, *et al.*, list possible reasons for such varied outcomes as: possible low bioavailability and other nonspecific effects of antioxidants, disturbance of healthy (i.e., 'good') functions of ROS/RNS, presence of high oxidative/nitrosative stress, and problems in study design (e.g., small sample size).[226]

The aim of the Hep573 Study was to test the hypothesis that antioxidants could influence disease progression in CHC patients by reducing oxidative stress (OS). In a review of 17 clinical trials from 1997-2010[226] using antioxidants in HCV infection alone or with interferon therapy, the majority (12/17) had less than 50 participants. This lack of quality studies and inconsistent results informed the design of the Hep573 Study. Some of the ways the Hep573 Study sought to redress a gap in current antioxidant research in CHC patients are: the randomised placebo-controlled study design in the Hep573 Study, the enrolment of 118 participants, a 24-week treatment period and a 24-week follow-up period, the utilisation of the well respected outcome measures of ALT, HCV RNA, F_2-isoprostanes and the use of Fibrotest in the examination of an oral 'antioxidant intervention'.

HEP573 STUDY INTERVENTIONS

The Study design (detailed in the methodology section) included a targeted oral 'antioxidant intervention'. The rationale, research and link to pathology of the Hep573 interventions are examined here.

Silymarin

Silymarin was chosen as the comparison arm in this Study as it has a proven track record in ameliorating liver disease. In addition, silymarin attenuates liver injury provoked by alcohol, insulin resistance, hepatic steatosis and OS; all of which are implicated in hepatitis C disease progression.[19,51,437,438,446,447,452,453,455]

Antioxidant Intervention

Oxidative stress occurs as a direct and indirect response to the virus and the immune response. OS damages lipids, proteins and DNA, affecting cell proliferation, differentiation and cell survival[240,250,247,250,240] resulting in necrosis, inflammation and fibrosis. Antioxidants reduce OS by scavenging free radicals, enhancing the synthesis of adaptive endogenous antioxdants and also by inhibiting the activity of oxidant producing enzymes.[227]

The interventions in the Hep573 Study include a broad range of non-synthetic, predominantly phytochemical antioxidants.

Hep573 Study Pharmacology

The pathogenesis of HCV infection has been previously outlined in detail. The pharmacology and specific mechanisms of action of the interventions on liver disease will now be reviewed.

The next section reviews the evidence supporting the selection of the Hep573 ingredients (Table 2.6), and is divided into two parts. The first part, reviews the evidence at the time the Study commenced, pre-2003 (Table 2.7). The second part examines the evidence, post-2003 (Table 2.8) to the present. At the end of the pre- and post-2003 Tables the salient points are summarised.

The evidence in Table 2.7 and Table 2.8 is reported in terms of the pharmacology and mechanism of action as follows:

(1) antioxidant: scavenging and upregulating enzymatic defences[233,514,541-545] and inhibiting free radical producing enzymes[180,230,231,228];

(2) anti-inflammatory: affecting cell signalling in redox sensitive pathways such as NF-κB[453];

(3) apoptotic[546] and antinecrotic[547] stabilisation of mitochondria, cell membranes and redox regulated pathways;

(4) antifibrotic: inhibiting profibrogenic gene expression,[548,549] HSC activation[550] and ECM deposition[233];

(5) immune-modulator: modulation of gene expression and endogenous production of IFN-γ and other cytokines[551];

(6) antiviral: improved host response to viral infection[441,459,552-556]; and

(7) hepatoprotective: maintaining liver function, e.g., protecting the liver mitochondria and microsomes from lipid peroxidation through 'membrane stabilising action'.[546]

EVIDENCE OF PHARMACOLOGY AND MECHANISMS OF ACTION

Table 2.6: Hep573 trial interventions, evidence pre- and post-2003

Hep573 SOX Trial Ingredients	Standardised to specified daily amount	Studies Pre-2003	Studies Post-2003
Alpha lipoic acid 200 mg/d		Han, et al., 1997[541]	Castro, et al., 2012[557] Min, et al., 2010[549] Hultberg, et al., 2006[558] Petersen Shay, et al., 2008[559]
Andrographis paniculata 3g/d	Andrographolide 34.8 mg	Chiou, et al., 2000[560] Shen, et al., 2002[561]	Chang, et al., 2008[562] Chen, et al., 2011[563] Hidalgo, et al., 2005[564] Lee, et al., 2012[565] Verma, et al., 2008[544] Ye, et al., 2011[566]
Astragalus membranaceus 3g/d		Zhang, et al., 1990[567]	Gui, et al., 2006[545] Jia, et al., 2012[568] Li, et al.,2012[569]
Camellia sinensis 4g/d		Skrzydlewska, et al., 2002[542]	Calland, et al.,2012[554] Chen, et al.,2012[555] Ciesek, et al.,2011[552] Fukazawa, et al.,2012[556] Inami, et al., 2007[234] Park, et al., 2012[570] Yumei, et al., 2006[233] Zhen, et al., 2007[571]
Curcuma longa 8g/d	Curcuminoids 280 mg	Naito, et al., 2002[572] Deeb, et al., 2003[573]	Ak, et al., 2008[574] Bruck, et al., 2007[547] Chen, et al., 2008[550] Zheng, et al., 2007[514]
Eleutherococcus senticosus 3g/d	syringaresinol diglucosides 1.2 mg	Glatthaar-Saalmuller, et al., 2001[575]	Chen, et al., 2008[576] Park, et al.,2004[577]
Hypericum perforatum 1.5g/d	Hypericin 0.8 mg	Hunt, et al., 2001[578]	Orcic et al., 2011[579]
Lycopene 80 mg/d		Porrini, et al., 2000[580]	Bahcecioglu, et al., 2010[581] Kim, et al., 2004[582]

Table 2.6: Hep573 trial interventions, evidence pre- and post-2003

Hep573 SOX Trial Ingredients	Standardised to specified daily amount	Studies Pre-2003	Studies Post-2003
Phyllanthus amarus 3g/d		Kiemer, *et al.,* 2003[583]	Harikumar, *et al.,* 2004[584] Ravikumar, *et al.,* 2011[585]
Selenomethionine 40 mg/d	elemental selenium 200 mcg	Navarro-Alarcon, *et al.,* 2002[586]	Aboul-Soud *et al.,* 2011[587] Clarke, *et al.,* 2010[588] Himoto, *et al.,* 2011[589] Teodor, *et al.,* 2011[590] Viezeliene *et al.,* 2011[591] Youn, *et al.,* 2008[592]
Silybum marianum 60 grams/day (g/d)	silibinin 720 mg	Bindoli, *et al.,* 1977[546] Jia, *et al.,* 2001[548] Schumann, *et al.,* 2003[593] Tager, *et al.,* 2001[543] Valenzuela, *et al.,* 1989[594]	Ahmed-Belkacem, *et al.,* 2010[441] Alidoost, *et al.,* 2006[595] Das, *et al.,* 2012[596] Ferenci, *et al.,*2008[438] Fried, *et al,* 2012[597] Guedj *et al.,* 2012[553] Kim *et al.,* 2012[598] Polyak, *et al.,* 2010[232] Trappoliere, *et al.,* 2009[19] Tzeng, *et al.,*2012[599] Wagoner,*et al.,*2010[459] Wallace, *et al.,* 2008[600]
Vitamin C 400 mg/d	calcium ascorbate 400 mg	Bowie *et el* 2000[601] Maellaro, *et al.,* 1994[602]	Abhilash, *et al.,*2012a[603] Abhilash, *et al.,*2012b[604] Hong, *et al.,*2012[605] Weyers, *et al.,* 2008[606]
Vitis vinifera 12g/d	procyanidins 80 mg	Maffei Facino, *et al.,* 1994[607] Nair, *et al.,* 2002[551] Vigna, *et al.,*2003[608]	Dulundu, *et al.,* 2007[609]
Zinc amino acid chelate 20% 250 mg/d	elemental zinc 50 mg	Camps, *et al.,* 1992[610]	Farias *et al.*2012[611] Somi, *et al.,* 2012[612] von Bulow, *et al.,* 2007[613]

Key to Table 2.6: Pre- and post-2003 Tables:

AA = ascorbic acid, ALA=alpha lipoic acid, BDL=bile duct ligation, BDO=bile duct occlusion,

BHT = butylated hydroxytoluene, BID=pro-apoptotic protein,

CAF = crude astragaloside fraction, CAPD=continuous ambulatory peritoneal dialysis,

CCl_4 = carbon tetrachloride, COX-2=cycloxygenase-2,

CTGF = connective tissue growth factor, DC=dendritic cell, EC=epicatechin,

EGC = epigallocatechin, EGCG=epigallocatechin gallate, ECM=extracellular collagen matrix,

ERK = extracellular signal-regulated kinase, fMLP=N-formyl-methionyl-leucyl-phenyalanine,

FRAP = ferric reducing antioxidant potential, GPx=glutathione peroxidase

GSE = grape seed extract, GSH=glutathione, GSSG reductase =glutathione reductase,

4-HNE = 4-hydroxynonenal, HCVcc=HCV cell culture, HSC=hepatic stellate cell,

Huh = human hepatoma cells, IC= inhibitory concentration,

ICAM-1 = intracellular adhesion molecule-1, IFN-γ=interferon-γ, IL-10=interleukin-10, IL-1β=interleukin-1beta,

I-κB = inhibitory kappa B, iNOS=inducible nitric oxide synthase, IRF=interferon regulatory factor,

JFH-1 = Japanese fulminant hepatitis-1, JNK=Jun N-terminal kinase, LDL=low density lipoproteins,

LNCaP = a cell line established from a metastatic lesion of human prostatic adenocarcinoma,

LOOH = lipid hydroperoxides, LPS=lipopolysaccharide (LPS), Mac-1=macrophage adhesion molecule-1,

MAPK = mitogen activated protein kinase, MDA=malondialdehyde, MEK=MAP kinase kinase,

MIP = macrophage inflammatory protein, mRNA=messenger RNA,

MyD88 = myeloid differential factor 88, NF-κB=nuclear factor kappa B,

OxLDL = oxidised low density lipoproteins,

PAF = platelet activating factor, PCL=phosphatidylcholine liposomes,

PDGF = platelet derived growth factor,

PKC = protein kinase C, PMBC=polymorphonuclear cells,

PPAR = peroxisome proliferator-activated receptor

ROS = reactive oxygen species, TAS=total antioxidant status,

TBARS = thiobarbituric acid reactive substances, Tβ-R1=T beta receptor 1, TCR=T cell receptor,

TGFβ1 = transforming growth factor beta1, TIMP-1=tissue inhibitor of metalloproteinase-1,

TLR3 = toll like receptor 3, TNF-α=tumour necrosis factor alpha,

TRAIL = tumour necrosis factor realted apoptosis inducing ligand,

TRIF = toll interleukin-1 receptor domain containing adaptor inducing interferon,

VCAM = vascular cell adhesion molecule-1.

Evidence of Mechanisms of Action Pre-2003

Table 2.7: Evidence of mechanisms of action of Hep573 interventions pre-2003.

Study	Formulation	Study design	Mechanism	Pharmacological Action
Alpha lipoic acid				
Han, *et al.,* 1997 Effect of ALA on total cellular thiols and uptake of cystine[541]	Alpha lipoic acid (ALA) (100 µM)	Flow cytometric assay of human peripheral blood lymphocytes incubated with ALA for 48 hours	ALA increased glutathione in peripheral blood lymphocytes by 48% compared to controls ($P<0.05$). Enhanced *de novo* synthesis of GSH. ALA helped maintain redox regulation, restore cellular glutathione and improve immune and antioxidant response.	Antioxidant (endogenous) Immune-modulator
Andrographis paniculata				
Chiou, *et al.,* 2000 Effect of andrographolide on inducible NO synthase mRNA expression[560]	*Andrographis paniculata*	LPS/IFN-γ stimulated raw 264.7 macrophages	Suppression of pro-inflammatory gene expression TNF-α, IL-6, macrophage inflammatory protein 2 (MIP-2).	Anti-inflammatory Immune-modulator
Shen, *et al.*, 2002 Mechanisms involved in anti-inflammatory effect of andrographolide[561]	Andrographolide (0.1-10 µM)	Adhesion & transmigration of isolated peripheral human neutrophils	Andrographolide decreased ROS production (hydrogen peroxide and superoxide anion radicals) ($P<0.05$) by modulation of protein kinase C (PKC) dependent pathway & down-regulation of Mac-1(CD11b/CD18) expression critical for neutrophil adhesion and transmigration.	Anti-inflammatory Antioxidant Immune-modulator
Astragalus membranaceus				
Zhang *et al.*, 1990 Effect of *Astragalus membranaceus* on stilbenemidine (SBM) - induced liver injury[567]	*A. membranaceus* root extract (ARE) 3 g/kg for 7 days	Adult male and female mice	ALT was significantly reduced in the SBM and ARE group compared to the SBM group ($P<0.01$).	Anti-inflammatory Hepatoprotective

Table 2.7: Evidence of mechanisms of action of Hep573 interventions pre-2003.

Study	Formulation	Study design	Mechanism	Pharmacological Action
Camellia sinensis				
Skrzydlewska*, et al.,* 2002 Effect of green tea on antioxidant enzymes and lipid peroxidation products[542]	*Camellia sinensis* (Green tea) EGCG (337 mg/L) EGC (268 mg/L) EC(90 mg/L) ECG(60 mg/L)	Rats (5-10 weeks ad libitum)	Increased glutathione peroxidase (GPx) by 33%, and GSSG reductase by 38% in rat liver, ($P<0.001$) compared to controls. Lipid peroxidation products were reduced as follows: LOOH by 13%,($P<0.05$), 4-HNE by 10% ($P<0.05$) and MDA by 33% ($P<0.001$) in liver tissue and by 10%, 16% and 22% (all, $P<0.001$) in serum in green tea drinking rats compared to controls.	Antioxidant (endogenous)
Curcuma longa				
Naito, *et al.,* 2002 Evaluate the antioxidative activity of THC[572]	Tetrahydrocurcumin (THC) *Curcuma longa*	*In vitro,* human LDL *In Vivo,* rabbits	Inhibition of oxidation of human LDL in vitro. In diets containing 1% cholesterol with or without 0.5% THC. THC inhibited TBARS formation.	Antioxidant
Deeb, *et al.,* 2003 Effect of curcumin and TRAIL on LNCaP cell death[573]	10 µM curcumin and 20 ng/ml TRAIL	*In vitro* LNCaP cell lines	Combined TRAIL and curcumin activated both the extrinsic (receptor-mediated) and intrinsic (chemical-induced) pathways of apoptosis through induced cleavage of procaspase-3, procaspase-8, and procaspase-9, truncation of Bid, and release of cytochrome *c* from the mitochondria.	Apoptotic
Eleutherococcus senticosus				
Glatthaar-Saalmuller *et al.*, 2001 Effect of *Eleutheroccus senticosus* on virus replication[575]	*E. senticosus* liquid extract, 33% (v/v) ethanol	Plaque reduction assays in cell cultures infected with DNA and RNA viruses	*E. senticosus* in various dilutions led to: 1:80, 62% and 64% reduction in human rhinovirus (HRV) and influenza A virus respectively. At this dilution it had a comparable effect to 5 µg/mL amantadine 1:640, ~100% reduction in respiratory syncytial virus (RSV) 1:2240, showed similar antiviral activity to 5 µg/mL ribavirin on RSV No activity against DNA viruses.	Antiviral

Table 2.7: Evidence of mechanisms of action of Hep573 interventions pre-2003.

Study	Formulation	Study design	Mechanism	Pharmacological Action
Hypericum perforatum				
Hunt, *et al.,* 2001 Evaluation of the antioxidant properties of *H. perforatum*[578]	*Hypericum perforatum* standardised extracts Nature Plus® Movana®	Cell-free system Human vascular tissue	Standardised plant extracts of 1:2.5 & 1:5 concentrations of *H. perforatum* markedly reduced superoxide production vs controls ($P<0.05$) demonstrating significant free radical scavenging properties. 1:20 standardised hypericin extract had greater antioxidant properties ($P<0.0001$) than a standardised hyperforin extract ($P<0.001$) indicating hypericin is the main ingredient required for free radical scavenging.	Antioxidant (scavenger)
Lycopene				
Porrini *et al.,* 2000 The effect of lycopene on plasma and lymphocyte carotenoid concentration[580]	25 g tomato puree (7 mg carotene, 0.3 mg β-carotene) given to 11 healthy female patients for 14 days	Open study design	Plasma and lymphocyte lycopene concentrations significantly increased ($P<0.001$, $P<0.005$) respectively 50% reduction in lymphocyte DNA damage ($P<0.001$) Inverse relationships were found between plasma lycopene concentration and oxidative DNA damage (r=-0.82, $P<0.0001$) and between lymphocyte lycopene concentration and oxidative DNA damage (r=-0.62, $P<0.01$).	Antioxidant
Phyllanthus amarus				
Kiemer, *et al.,* 2003 Galactosamine/LPS model for acute toxic hepatitis[583]	*Phyllanthus amarus*	Rat kupffer cells, RAW 264.7 macrophages, Human whole blood, Mice	Inhibition of LPS-induced TNF-α in RAW 264.7 macrophages & human whole blood via NF-κB. *P. amarus* inhibited IL-1β, IFN-γ & IL-10 ($P<0.05$) in human whole blood. Inhibited induction of iNOS and COX-2.	Anti-inflammatory Antioxidant Immune-modulator

Table 2.7: Evidence of mechanisms of action of Hep573 interventions pre-2003.

Study	Formulation	Study design	Mechanism	Pharmacological Action
Selenium				
Navarro-Alarcon *et al.*, 2001 The influence of type of liver disease, sex and age on serum selenium concentrations[586]	Total serum selenium measurements	Open study (N=50 liver disease patients) compared to healthy and elderly controls Cirrhosis (N=12) Hepatitis (N=38)	In hepatic patients serum total cholesterol was positively correlated with serum selenium concentrations (r=0.912, P<0.05) There was an inverse relationship between GGT levels and serum selenium levels (r=-0.083, P<0.05), e.g., as hepatic injury increased, serum selenium levels decreased significantly.	Antioxidant Hepatoprotective
Silybum marianum				
Bindoli*, et al.,*1977 Evaluating the hepatoprotective 'membrane stabilising action' of silymarin in lipid peroxidation[546]	Silymarin (10-50 μM)	Rat liver mitochondria & microsomes	Silymarin protected liver mitochondria & microsomes from lipid peroxide formation. In the liver mitochondria, silymarin inhibited Fe^{2+}-ascorbate induced swelling, oxygen uptake and MDA formation (all expressions of lipid peroxide formation) Antiperoxide action of silymarin was superior to α-tocopherol by 10 fold.	Anti-inflammatory Antioxidant Apoptotic
Jia*, et al.,* 2001 To examine antifibrotic mechanism of silymarin[548]	Silymarin 50 mg/kg/day 6 weeks	Bile duct occlusion (BDO) (rats)	Silymarin downregulated procollagen α1(I), TIMP-I, TGFβ1 mRNA levels by 40-60% (P<0.01) compared to BDO alone (controls) Silymarin downregulates profibrogenic mRNAs and reduces total and relative collagen accumulation.	Antifibrotic
Schumann*, et al.,* 2003 Immune-response in T cell-dependent hepatitis[593]	Silibinin 25 mg/Kg	Mouse model of concanavalin A (ConA)	Inhibited intrahepatic expression of TNF-α, NF-kB, IFN-γ, IL-2, IL-4, iNOS and augmented synthesis of IL-10 (P<0.05). Silibinin significantly inhibited ConA-induced liver disease through immune-response modification.	Anti-inflammatory Immune-modulator

Table 2.7: Evidence of mechanisms of action of Hep573 interventions pre-2003.

Study	Formulation	Study design	Mechanism	Pharmacological Action
Silybum marianum				
Tager, *et al.,* 2001 Antioxidant effects of silymarin and silibinin on cellular thiol status[543]	70 µg/mL silibinin 84 µg/mL silymarin	Peritoneal macrophages from dialysis fluid of CAPD patients (N=30)	Intracellular thiol content increased 3.5 fold ($P<0.01$) in silibinin and silymarin groups compared to controls after 20 days of culture. Silibinin and silymarin reduced protein oxidation and ROS induced cell signalling.[543]	Antioxidant (endogenous)
Valenzuela, *et al.,* 1989 Effect of silymarin on GSH content in rat tissue[594]	Silymarin (200 mg/kg) Intraperitoneal injection	Male Wistar rats	Silymarin increased total glutathione content (GSHT=GSH+2GSSG) by >50% in rat liver and intestine compared to controls ($P<0.05$).	Antioxidant (endogenous)
Vitamin C				
Bowie, *et al.,* 2000 To examine the effect of vitamin C on NF-κB activation[601]	Vitamin C	Endothelial cell line (ECV 304)	Dose dependently (20 mM) blocked IL-1 & TNF mediated degradation & phosphorylation of IκBα due to inhibition of I-κB kinase. This effect was mediated by p38 MAPK Vitamin C inhibited TNF-mediated NF-κB activation.	Anti-inflammatory Antioxidant
Maellaro, *et al.,* 1994 The ability of Vitamin C to protect rat hepatocytes from prooxidant-induced liver injury[602]	Vitamin C as ascorbic acid (AA) 0.6 mM-4.8 mM ascorbate	Rat hepatocytes Allyl alcohol Diethyl maleate CCl_4 menadione	Ascorbic acid was able to protect hepatocytes from lipid peroxidation induced by allyl alcohol & diethyl maleate. AA has a direct ability to scavenge ROS.	Antioxidant (scavenger) Hepatoprotective
Vitis vinifera				
Maffei Facino, *et al.,* 1994 Effect of procyanidins on lipid peroxidation[607]	Procyanidins from *Vitis vinifera* (seed)	Ultrasound irradiation induced lipid peroxidation of phosphatidylcholine liposomes (PCL) membrane	Procyanindins prevented both the induction (IC50=0.1µmol/L) by 52% and propagation (IC=0.5µmol/L) phases by 55% of conjugated dienes.	Antioxidant

Table 2.7: Evidence of mechanisms of action of Hep573 interventions pre-2003.

Study	Formulation	Study design	Mechanism	Pharmacological Action
Vitis vinifera				
Nair*, et al.,* 2002 Effect of GSE on Th1 (IFN-γ) & Th2 (IL-6) derived cytokine production[551]	*Vitis vinifera* GSE 0.1 and 0.5 mg/mL	Humans Peripheral blood mononuclear cells	Upregulated: - IFN-γ gene expression ($P<0.001$, 0.1 mg/mL and $P<0.02$, 0.5 mg/mL) by PBMC compared to untreated controls - number of IFN-γ positive cells ($P<0.001$, 0.1 mg/mL and $P<0.007$, 0.5 mg/mL) - endogenous production of IFN-γ ($P<0.001$, 0.1 mg/mL and $P<0.0001$, 0.5 mg/mL).	Antiviral Immune-modulator
Vigna*, et al.,* 2003 Effect of GSE & phosphatidylcholine on lipoproteins and antioxidant defense[608]	*Vitis vinifera* 75 mg procyanindin	Randomised double blind crossover study of male cigarette smokers (N=24)	The FRAP test showed a significant difference from baseline in the GSE group compared to placebo at week 4 (N=12, $P<0.001$) TBARS concentration significantly reduced in GSE group compared to placebo ($P<0.01$) over the study duration of 14 weeks.	Antioxidant
Zinc				
Camps *et al.*, 1992 Effect of zinc on lipid peroxidation and hepatic fibrosis[610]	227 mg of $ZnSO_4$/l	Male Wistar rats CCl_4-induced hepatic fibrosis	Zinc significantly reduced lipid peroxidation by 51% ($P<0.05$), collagen deposition by 32% ($P<0.01$) and proline hydroxylase activity by 30% ($P<0.05$) at week 18 in the CCl_4-treated rats.	Antifibrotic Antioxidant

Evidence of Mechanisms of Action Post-2003

Table 2.8: Evidence of mechanisms of action of Hep573 interventions post-2003.

Study	Formulation	Study design	Mechanism	Pharmacological Action
Alpha lipoic acid				
Castro, *et al*, 2012 Evaluated co-administration of R/S-alpha-lipoic acid in the prevention oxidative stress and metabolic changes induced by a fructose-rich diet[557]	Standard diet (control) and fructose without or with R/S-alpha-lipoic acid	*In vivo*, mice	(1) Prevention of hyperinsulinemia, hypertriglyceridemia and insulin resistance (2) Improved hepatic insulin sensitivity and glucose tolerance (3) Decreased liver oxidative stress and increased antioxidant capacity and expression of antioxidant enzymes (4) Decreased expression of uncoupling protein 2 and PPARδ protein and increased PPARγ levels, (5) Restored basal gene expression of PPARδ, SREBP-1c and the lipogenic genes fatty acid synthase and glycerol-3-phosphate acyltransferase (6) Decreased fructose-mediated enhancement of glucokinase activity.	Antioxidant
Hultberg, *et al.,* 2006 Effect of ALA on GSH turnover against different levels of H_2O_2 (24 hours) [558]	Alpha lipoic acid 100 μM & 500 μM	HeLa cell culture (cancer cell line) and hepatoma cell culture	Increased total glutathione (*P*<0.05) in both cell cultures.	Antioxidant (endogenous)
Min*, et al.,* 2010 Determine whether ALA prevents hepatic fibrosis[549]	Alpha lipoic acid 100 mg/kg/d intravenous	*In vivo,* mice, bile duct ligation *in vitro*	Inhibits hepatic PAI-1 expression through inhibition of TGF-β induced molecular mediators, Smad3, AP1, and Sp1.	Antifibrotic

Table 2.8: Evidence of mechanisms of action of Hep573 interventions post-2003.

Study	Formulation	Study design	Mechanism	Pharmacological Action
Alpha lipoic acid				
Petersen Shay, *et al.,* 2008 Oxidant scavenger *in vivo* or induction of stress-activated signalling mechanisms[559]	Alpha lipoic acid	*In vitro, in vivo* (rats)	*In vitro* direct scavenger of ROS *In vivo*-induces transcription of genes via Nrf2/ARE that synthesise glutathione. Reduction of cystine to cysteine Increases cysteine uptake for glutathione synthesis. Affects thiol redox sensitive pathways – Inhibits TNF-α induced NF-κB inflammation. Stabilise the cysteine residues in the insulin signalling pathway Modification of B/Akt, induction of P13K. Reduction of diabetic polyneuropathies, improves glucose uptake via upregulation of GLUT4 receptors.	Anti-diabetic Anti-inflammatory Antioxidant (scavenger and endogenous) Apoptotic
Andrographis paniculata				
Chang, *et al.,* 2008 Effect of andrographolide on glutathione-S-transferase expression[562]	Andrographolide (10-20 μM), active constituent in *Andrographis paniculata*	Primary hepatocytes (rat)	Both the whole plant extract and andrographolide significantly increased glutathione-S-transferase activity compared to controls ($P<0.05$) Andrographolide increased GST activity implying that it has chemopreventive potential.	Antioxidant Chemo-preventive
Chen, *et al.,* 2011 Effect of down-regulation of the PI3K/Akt signaling pathway on TNF-alpha-induced Inflammation[563]	Isolated andrographolide	Expression of HL-60 cells and adhesion onto human umbilical vein endothelial cells (HUVEC)	Inhibition of PI3K/Akt pathway and NF-κB activation: (1) Decreased TNF-α-induced intercellular adhesion molecule-1 (ICAM-1) expression and adhesion of HL-60 cells (2) Inhibition of TNF-α-induced Akt phosphorylation (3) Blockade of TNF-α-induced IkappaB-alpha.	Anti-inflammatory

Table 2.8: Evidence of mechanisms of action of Hep573 interventions post-2003.

Study	Formulation	Study design	Mechanism	Pharmacological Action
Andrographis paniculata				
Hidalgo, *et al.*,2005 Effect of andrographolide on activation of NF-κB induced by PAF & fMLP[564]	*Andrographis paniculata* 5 and 50 μM	HL-60 derived neutrophils	Inhibited NF-κB luciferase activity induced by PAF Reduced DNA binding to NF-κB.	Anti-inflammatory
Lee, *et al.,* 2012 Suppression of matrix metalloproteinase-9 expression by andrographolide[565]	Isolated andrographolide	Human monocytic THP-1 cells	(1) Inhibition of MMP-9 activation, induced by either TNF-α, or lipopolysaccharide (2) Suppression of expression of MMP-9 messenger RNA. (3) Inhibition of the degradation of inhibitor-kappaB-α induced by TNF-α. (4) Inhibition of NF-κB signaling, anti-translocation and anti-activation.	Antifibrotic Anti-inflammatory
Verma*, et al.,* 2008 Effect of Andrographis on antioxidant defense system in liver[544]	*Andrographis paniculata* extract	Lymphoma bearing AKR mice	Increased levels of glutathione S transferase, catalase and superoxide dismutase enzymes.	Antioxidant (endogenous)
Ye, *et al.,* 2011 Effect of andrographolide against carbon tetrachloride-induced acute liver injury in mice[566]	Isolated andrographolide	CCl_4-induced acute liver injury in mice.	Pretreatment with andrographolide significantly reduced CCl_4-induced hepatotoxicity by : significant reduction of serum ALT, AST levels and hepatic MDA activity, increase in hepatic GSH content.	Anti-inflammatory Antioxidant (endogenous)

Table 2.8: Evidence of mechanisms of action of Hep573 interventions post-2003.

Study	Formulation	Study design	Mechanism	Pharmacological Action
Astragalus membranaceus				
Gui, *et al.,* 2006 The effect of crude astragaloside fractions from *Astragalus membranaceus* on rat fibrosis and possible mechanisms.[545]	Crude astragaloside fraction) (CAF)(I-IV) (10, 20 & 40 mg/kg),	CCl_4 induced hepatic fibrosis (rats)	CAF increased liver total antioxidant capacity (superoxide dismutase (SOD), ($P<0.01$) glutathione peroxidase (GPx), ($P<0.05$) CAF markedly reduced TGF-β1 in Kupffer cells ($P<0.05$, 20 mg/kg, ($P<0.01$, 40 mg/kg). CAF significantly decreased hydroxyproline content in liver tissue & serum hyaluronic acid $P<0.05$, 20 mg/kg, $P<0.01$, 40 mg/kg, & procollagen III $P<0.01$, both doses The inhibitory effect of CAF may be related to its free radical scavenging activity and its ability to inhibit production of TNF-α & TGF-β from activated Kupffer cells CAF significantly reduced plasma ALT & AST levels at 20 and 40 mg/kg compared to the control group ($P<0.01$).	Antifibrotic Anti-inflammatory Antioxidant (endogenous & scavenger) Hepatoprotective
Jia, *et al.*, 2012 Hepatoprotective and antioxidant effects of Astragalus[568]	Astragalus polysaccharides (200, 400 and 800 μg/ml)	*In vitro* and *in vivo* hepatocyte damage induced by carbon tetrachloride in common carp.	*In vitro* Significantly improved cell viability and inhibition of the elevation of SGPT, SGOT, LDH, MDA, and increased SOD *In vivo* The elevated activities of SGPT, SGOT and LDH were significantly reduced, and the reduced levels of total protein and albumin in the serum were increased after pre-treatment.	Antioxidant Hepatoprotective
Li, *et al.*, 2012 Inhibiting effect on the functions of CD4+CD25 cells[569]	Astragalus polysaccharides	Tumor microenvironment of human hepatocellular carcinoma	Inhibition of the growth and proliferation of CD4+CD25+Treg cells in vitro Restoration of the cytokine imbalance and reducing the expression of FOXp3 in local hepatocellular carcinoma microenvironments.	Immune-modulator

Table 2.8: Evidence of mechanisms of action of Hep573 interventions post-2003.

Study	Formulation	Study design	Mechanism	Pharmacological Action
Camellia sinensis				
Calland, *et al.,* 2012 Effect on hepatitis C virus entry[554]	(-)-Epigallocatechin-3-gallate	HCV cell culture	Inhibition of hepatitis C virus entry. HCV infectivity was inhibited by more than 90% when administered at a concentration of 50 µM of EGCG in an early step of the viral life cycle (most likely the entry step). Viral replication and virion secretion were not affected by EGCG.	Antiviral
Chen, *et al.*, 2012a Effect on the the replication cycle of hepatitis C virus[555]	(-)-Epigallocatechin-3-gallate	HCV cell culture and JFH1-GFP chimeric virus (50 % effective concentration (EC(50)) of 17.9 µM).	Inhibition of replication cycle of HCV by suppressing HCV entry and RNA replication. Insignificant effect by EGCG on translation directed by the viral internal ribosome entry site.	Antiviral
Ciesek, *et al.,* 2011 Effect on hepatitis C virus entry[552]	(-)-Epigallocatechin-3-gallate	Cell-culture-derived HCV	Inhibition of entry into hepatoma cell lines and primary human hepatocytes by HCV derived by cell culture.. Effect not due to differences in HCV genotype , and inhibition effects observed in cells infected by extracellular virions and in cell-to-cell spread. No effect on HCV RNA replication, assembly, or release of progeny virions.	Antiviral
Fukazawa, *et al.*, 2012 Effect on inhibition of HCV[556]	Green tea constituents	*In vitro*, HCV-JFH1, Huh7.5.1 cells	7,8-benzoflavone and green tea gallate catechins inhibit the hepatitis C virus. EGCG apparently acted mainly on HCV entry, although it may also block other steps. In contrast, 7,8-benzoflavone was presumed to inhibit the HCV life cycle at later stages.	Antiviral

Table 2.8: Evidence of mechanisms of action of Hep573 interventions post-2003.

Study	Formulation	Study design	Mechanism	Pharmacological Action
Camellia sinensis				
Inami*, et al.,* 2007 Effect of catechin intake on lipid profile and plasma oxidised LDL cholesterol[234]	Catechin (500 mg) ~6/7 cups green tea daily for 4 weeks	Randomised controlled study using anti-oxidised phosphatidylcholine monoclonal antibody N=29 catechin group N=11 control group	Catechin reduced plasma oxidized LDL by 19% (reduced from 9.56±9.2 to 7.76±7.7 U/mL, *P*=0.005) and may be useful in hypercholesterolemia and coronary artery disease.	Antioxidant
Park*, et al.,* 2012 Effect on diet-induced nonalcoholic steatohepatitis[570]	Low fat or high fat diet with or without green tea extract.	Model of diet-induced obesity	NFκB activation and inflammatory responses suppressed by green tea in diet-induced obese rats with nonalcoholic steatohepatitis ALT and hepatic lipid levels lowered by green tea.	Anti-inflammatory
Yumei*, et al.,* 2006 Examines inhibition by EGCG on ECM gene expression[233]	EGCG (purity>95%) Sigma	Passaged hepatic stellate cells (rat)	EGCG attenuates oxidative stress by promoting the *de novo* synthesis of intracellular GSH. This interrupts CTGF mediated production of TGF-β induced ECM in activated HSC Increased *de novo* synthesis of GSH and interrupted pro-fibrogenic TGF-β signalling by suppressing gene expression of Tβ-RI and Tβ-RII (*P*<0.05) versus no treatment Interruption of TGF-β signalling by EGCG suppressed gene expression of CTGF and ECM and inactivated HSC, thereby exerting an antifibrotic effect.	Antifibrotic Antioxidant (endogenous)

Table 2.8: Evidence of mechanisms of action of Hep573 interventions post-2003.

Study	Formulation	Study design	Mechanism	Pharmacological Action
Camellia sinensis				
Zhen*, et al.,* 2007 Examine the effects of EGCG on CCl_4-induced hepatic fibrogenesis and mechanisms involved[571]	Green tea polyphenol EGCG Epigallocatechin gallate	CCl_4-induced hepatic fibrosis (rats)	Hepatic GSH levels were significantly increased by 51% from $3.5\pm0.1\mu mol/g$ to $5.3\pm0.2\mu mol/g$ following treatment with EGCG ($P<0.05$) EGCG attenuates oxidative stress and restores the redox state in the hepatocyte by raisingGSH levels in cultured HSCs Fibrotic area was significantly reduced compared to controls (Knodell) as were hepatic hydroxyproline content & activity of MMP2 in liver ($P<0.05$) EGCG prevents the development of hepatic fibrosis in a rat model of CCl_4 induced hepatic fibrosis and suppressed α-SMA-positive cells, indicating EGCG might attenuate HSC activation Serum ALT levels were significantly reduced after EGCG treatment ($P<0.05$).	Antifibrotic Anti-inflammatory Antioxidant (endogenous)
Curcuma longa				
Ak*, et al.,* 2008 To elucidate the role of curcumin in antioxidant, radical scavenging and metal chelating[574]	Curcumin (15 μg/mL or 20 mM)	Various *in vitro* antioxidant assays Linoleic acid emulsion	Curcumin had a hydrogen-peroxide scavenging-activity of 28% ($P<0.0031$) and superoxide scavenging-activity of 43% ($P<0.013$) compared to α-tocopherol and BHT. Curcumin (15µg/mL concentration) inhibited 97.3% lipid peroxidation of linoleic acid emulsion.	Antioxidant (scavenger)
Bruck*, et al.,* 2007 To examine ability of curcumin to prevent thioacetamide-induced cirrhosis, and mechanism of its effect on hepatic fibrosis[547]	Curcumin 300 mg/kg/day	Thioacetamide induced cirrhosis (rats)	Less nodule formation, fibrotic septa ($P<0.001$), inflammatory infiltration & hepatocyte apoptosis & necrosis ($P<0.01$) Curcumin reduced liver injury through down-regulation of inflammatory processes.	Antifibrotic Anti-inflammatory Anti-necrotic Apoptotic

Table 2.8: Evidence of mechanisms of action of Hep573 interventions post-2003.

Study	Formulation	Study design	Mechanism	Pharmacological Action
Curcuma longa				
Chen*, et al.,* 2008 To evaluate signal transduction pathways during curcumin suppression of CTGF expression in HSC[550]	Curcumin 20 µm (purity>94%), Sigma	HSC *in vitro* male Sprague–Dawley rats. Transfection assays	CTGF gene suppression resulted in activated HSC (*P*<0.05), because of interruption to NF-kB (via TLR4) and ERK signalling.	Antifibrotic
Zheng*, et al.,* 2007 To elucidate the underlying mechanisms whereby curcumin inhibits HSC activation[514]	Curcumin (20 µM) Incubated for 24 hours	Activated hepatic stellate cells	*In vitro*, curcumin dose and time dependently increased *de novo* synthesis of GSH by stimulating gene expression of glutamatcysteine ligase (GCL) in activated HSC. Curcumin requires *de novo* synthesis of GSH to inhibit HSC activation, as it impedes cell proliferation, induces apoptosis and suppresses the production of αl (I) collagen*.*	Antifibrotic Antioxidant (endogenous)
Eleutherococcus senticosus				
Chen*, et al.,* 2008 Investigate antioxidant properties of three adaptogens[576]	*E. senticosus*	Chemiluminescent analysis	*E senticosus* displayed antioxidant activity against singlet oxygen, hypochlorite and hydrogen peroxide.	Antioxidant

Table 2.8: Evidence of mechanisms of action of Hep573 interventions post-2003.

Study	Formulation	Study design	Mechanism	Pharmacological Action
Eleutherococcus senticosus				
Park, et al, 2004 Effect hepatic failure induced by D-galactosamine and lipopolysaccharide[577]	70% ethanol extract, water extract, or purified polysaccharides, IP (300 mg/kg and 50 mg/kg) or oral (300 mg/kg)	*In vivo*, injection of trial medications in mice 12 hrs and 1 hr before injection with D-galactosamine/ lipopolysaccharide	IP and oral water extract and polysaccharide significantly lowered serum levels of TNF-α, AST, ALT; improved the histologic changes in liver, inhibited hepatocyte apoptosis and suppressed the lethality induced by D-galactosamine/lipopolysaccharide. No protective effect by ethanol extract (70%) and ethanol-soluble part of the water extract when treated either intraperitoneally or orally.	Hepatoprotective
Hypericum perforatum				
Orcic *et al.,* 2011 Antioxidant activity of the phenolic compounds in *Hypericum perforatum*[579]	Phenolic compound fractions from *Hypericum perforatum*	LC-MS separated & identified flavonoids, napthodiathrones and phloroglucinols	Confirmed significant antioxidant activity by DPPH assay (lowest IC_{50} of 0.52 μg/mL) NO scavenging (6.11 μg/mL), superoxide scavenging (1.86 μg/mL), lipid peroxidation (0.0079 μg/mL) and FRAP (highest reduction capacity of 104 mg Fe equivalents/g) assays. Majority of the fractions had higher antioxidant activity than the synthetic antioxidants.	Antioxidant
Lycopene				
Bahcecioglu, *et al.,* 2010 Effect on nonalcoholic steatohepatitis-induced by high-fat diet[581]	Lycopene, 2 or 4 mg/kg body weight	High fat diet with our without lycopene, Sprague-Dawley rats, 6 weeks	Serum MDA and TNF-α levels reduced and liver GSH level raised (P<0.001) by lycopene. Lycopene supplements appear to reduce high-fat diet-induced oxidative stress in cells.	Antioxidant
Kim*, et al.,* 2004 Mechanism of action of lycopene in murine bone marrow derived dendritic cells (DC)[582]	Lycopene 10 μM	Murine bone marrow derived (DC) (LPS induced DC maturation)	Downregulated CD80 & CD86 & major histocompatibility complex (MHC) molecules. Inhibition of MAPK such as ERK1/2, p38 &JNK & NF-κB inhibited DC production of pro-inflammatory cytokine IL-12	Anti-inflammatory Immune-modulator

Table 2.8: Evidence of mechanisms of action of Hep573 interventions post-2003.

Study	Formulation	Study design	Mechanism	Pharmacological Action
Phyllanthus amarus				
Harikumar*, et al.,* 2004 Radioprotective effect[584]	*Phyllanthus amarus* 740 mg/kg & 250 mg/kg oral	BALB mice	Increased white blood cell count, increased endogenous antioxidant activity with a significant increase in glutathione. Reduced lipid peroxidation	Antioxidant (endogenous) Immune-modulator
Ravikumar, *et al.,*2011 Effect on inhibiton of hepatitis C virus replication[585]	*P. amarus* leaf extract	Screening for inhibitory effect of different plant extracts against the NS3 and NS5B enzymes of hepatitis C virus.	*In vitro,* NS5B was observed to be inhibited. In HCV cell culture, HCV monocistronic replicon RNA and HCV H77S viral RNA were observed.	Antiviral
Selenium				
Aboul-Soud, *et al.,* 2011 Effect of selenium on OS-induced liver injury[587]	Selenium (0.1 mg kg(-1) b.w) with alpha-tocopherol (100 mg kg(-1) body weight, b.w.)	Malathion (MTN)-induced oxidative stress and hepatic injuries in experimental rats	Selenium with alpha-tocopherol partially protected against MTN-induced hepatic oxidative stress and injuries.	Antioxidant Hepatoprotective
Clarke*, et al.,* 2010 Oleate-treated human hepatoblastoma (C3A) cells were used in the cell culture method of fat loading[588]	Selenium Selenite (50 nM)	Human hepatoblastoma (C3A) cells	Induced GPx activity (70 fold) and thioredoxin reductase activity (2 fold) Fat loading and severe selenium deficiency in cultured human hepatocytes induced TGF-β1 gene expression and increased procollagen synthesis Selenium reduced the expression of pro-inflammatory and pro-fibrogenic cytokines.	Anti-inflammatory Anti-fibrotic Antioxidant (endogenous)

Table 2.8: Evidence of mechanisms of action of Hep573 interventions post-2003.

Study	Formulation	Study design	Mechanism	Pharmacological Action
Selenium				
Himoto, *et al.*, 2011 To examine selenium deficiency associated with insulin resistance in hepatitis C virus-related chronic liver disease[589]		HCV-related CLD patients (N=52) Some patients had chronic hepatitis and liver cirrhosis	Serum Se levels: (1) reduced significantly, and proportionately to the severity of hepatic fibrosis. Serum albumin and zinc concentrations were positively correlated. (2) linked to serum glutathione peroxidase activities. (3) inversely correlated with the homeostasis model for assessing insulin resistance (4) independent of HCV genotype and HCV-RNA load.	Antifibrotic
Teodor, *et al.*, 2011 [590]	Sodium selenite	Selenium effects in protecting rat liver from acrylamide toxicity, and on oxidative stress.	Selenium significantly increased GSH and GPx levels and decreased MDA compared to group which received only acrylamide.	Antioxidant Hepatoprotective
Viezeliene, *et al.*, 2011 [591]	1.25 mg Selenium per kg of body mass	*In vivo,* protective effect of selenium on mouse liver from aluminium-induced oxidative stress.	No protective effect observed on aluminium-linked liver toxicity, but selenium improved glutathione-mediated process of intracellular oxidative stress.	Antioxidant Hepatoprotective
Youn*, et al.,* 2008 To identify the molecular target of selenium in TRIF-dependent signalling pathways of TLRs[592]	Selenium Sodium selenite 20 µM, 50 µM	RAW 264.7 macrophages & 293T human embryonic kidney cells	Modulated MyD88 & TRIF pathways of TLR3 & 4 ($P<0.01$) Inhibited IRF3 activation ($P<0.05$) Reduced inflammatory cytokine gene expression.	Anti-inflammatory Apoptotic Immune-modulator

Table 2.8: Evidence of mechanisms of action of Hep573 interventions post-2003.

Study	Formulation	Study design	Mechanism	Pharmacological Action
Silybum marianum				
Ahmed-Belkacem, *et al.,*2010[441]	Silybin 75-100 μM	*In vitro,* hepatocarcinoma cell line Huh7	Silibinin inhibits HCV genotype 1b and 2a strain JFH1 replication in cell culture system by inhibiting polymerase activity.	Antiviral (inhibition of replication)
Alidoost, *et al.,* 2006 Effect of silymarin on GSH level & proliferation of PBMC[595]	Silymarin 5,10,20 μg/mL	β-thalassemia major patients (N=28) Controls (N=28)	Increased intracellular GSH levels of PBMC ($P<0.001$).	Antioxidant (endogenous)
Das, *et al.*, 2012 The effect of Silymarin on ethanol-induced OS[596]	Silibinin (250 mg/kg body weight)	Ethanol induced oxidative damage in 8- to 10-week-old male BALB/c mice	In whole blood hemolyzate, silymarin normalised increases in thiobarbituric acid reactive substance (TBARS) and nitrite levels in addition to glutathione-S-transferase (GST) activity. It also normalized decreases in reduced glutathione (GSH) levels and activitiy of superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), and glutathione peroxidase (GPx). Silymarin significantly inhibited the ability of ethanol to raise, activity of interleukin (IL)-10, tumor necrosis factor (TNF)-α, γ interferon (IFN-γ), vascular endothelial growth factor (VEGF)-A, and transforming growth factor (TGF)-β1, as well as to lower IL-4 activity.	Antioxidant Anti-inflammatory
Ferenci, *et al.,*2008 Silibinin dose and administration in non-responders[438]	5-20 mg/kg/day IV silibinin	Dose comparison study (N=20) in non-responders to Peg-IFN plus ribavirin	15 or 20 mg/kg/day IV silibinin for 14 days and 280 mg oral silymarin three times a day combined with pegylated interferon and ribavirin therapy from day eight. At Week 12, 7/14 patients, who had been previously classed as non-responders, had undetectable HCV RNA.	Antiviral

Table 2.8: Evidence of mechanisms of action of Hep573 interventions post-2003.

Study	Formulation	Study design	Mechanism	Pharmacological Action
Silybum marianum				
Fried, *et al.*, 2012 Effect of silymarin on liver disease activity in CHC patients[597]	420 mg or 700 mg of silymarin or placebo 3 times daily	Multicentre, double-blind, randomised, placebo-controlled trial with IFN non-responders with chronic HCV infection (N=154)	Higher than the usual doses of Silymarin produced no significant reduction in serum ALT levels compared with placebo.	Negative result
Guedj, *et al.*, 2012 To investigate mode of action of silibinin[553]	10, 15 or 20 mg/kg/daily Legalon Sil (IV)	Viral kinetic modeling in 25 patients with HCV infection, IFN-non-responders	Silibinin prevented viral infection and viral production and/or release, and dose effects were mainly on prevention of viral production and or release.	Antiviral

Table 2.8: Evidence of mechanisms of action of Hep573 interventions post-2003.

Study	Formulation	Study design	Mechanism	Pharmacological Action
Kim *et al.*, 2012 The effect of Silymarin on hepatic stellate cells and fibrosis in experimentially induced-NASH[598]	Oral silymarin	Non-alcoholic steatohepatitis induced by methionine- and choline-deficient diet fed to insulin-resistant rats	Silymarin reduced steatohepatitis, raised nuclear translocation of nuclear factor erythroid 2-related factor 2 (Nrf2), & reduced tumor necrosis factor (TNF)-α mRNA expression in the liver.	Anti-inflammatory Antioxidant Hepatoprotective
Polyak*, et al.,* 2010 Identification of hepatoprotective actions of flavonolignans from silymarin[232]	Silymarin (MK-001, USP, and Legalon)	HCVcc (Huh7,JFH-1) T cells Assays	Dose dependent effects: Antiviral - prevented virus entry, fusion, and production. *In vitro* and at high concentrations, prevented HCV NS5B polymerase activity. Antioxidant - prevented oxidative strees induced by JFH-1 virus, including compounds that lacked antiviral activity. Immunomodulatory – in T cells, Inhibited TCR–mediated proliferation and cytokine production. Anti-inflammatory - Reduced TNF-α, NF-κB and inflammatory cytokines in Huh7 and PBMC via antioxidant stabilisation of redox pathways.	Anti-inflammatory Antioxidant Antiviral Hepatoprotective Immune-modulator

Table 2.8: Evidence of mechanisms of action of Hep573 interventions post-2003.

Study	Formulation	Study design	Mechanism	Pharmacological Action
Silybum marianum				
Trappoliere*, et al.,* 2009 To examine mechanisms that regulate silybin's anti-fibrogenic and anti-inflammatory action[19]	Silibin 25-50 µM	Human hepatic stellate cells (HSC)	Dose dependently inhibited: Cell proliferation induced by growth factor ($P < 0.001$), cell motility ($P < 0.001$), extracellular matrix components ($P < 0.05$), IL-1-induced synthesis of MCP-1 ($P < 0.01$) and IL-8 ($P < 0.01$), ERK, MEK and Raf phosphorylation, with reduction inNHE1 activation (Na+/H+ exchanger, $P < 0.05$) and IkBα phosphorylation. Silibinin inhibited TGF-β induced *de novo* synthesis of pro-collagen I ($P<0.05$) directly, by reducing PDGF-induced cell proliferation, and indirectly, by *de novo* reduction of TGF-β induced synthesis of collagen type 1.	Antifibrotic Anti-inflammatory Antioxidant
Tzeng*, et al.,*2012 [599]	Oral administration of silymarin (200 mg/kg, three times daily)	Rat model of hepatic fibrosis induced by carbon tetrachloride	Decrease in plasma SGOT and SGPT, reduced fibrosis, hepatic hydroxyproline and connective tissue growth factor.	Antifibrotic
Wagoner*, et al.,*2010 Antiviral activity of silymarin[459]	Silymarin 40 µM	*In vitro*, hepatocarcinoma cell line Huh7	Inhibition of virus entry, fusion with liposomes, HCV RNA and protein synthesis and reduced virus transmission.	Antiviral
Wallace*, et al.,* 2008 To examine inhibitory effect of silymarin on oxidised low-density lipoprotein (OxLDL), & on monocyte adherence to OxLDL mediated by scavenger receptor[600]	Silymarin 38,75,150, 300 µM	TBARS assay LDL treated with DSMO	Silibinin (300 µM) reduced OxLDL by 60%. Whole milk thistle extract inhibited OxLDL generation by 93% Silibinin (38-150 µM) inhibited monocyte adhesion by 77-95% Silymarin reduces TNF-α induced production ICAM-1 & VCAM-1 involved in cell adhesion, and blocks the interaction between endothelial cells and monocytes thereby preventing the inflammatory cascade.	Anti-inflammatory Antioxidant Immune-modulator

Table 2.8: Evidence of mechanisms of action of Hep573 interventions post-2003.

Study	Formulation	Study design	Mechanism	Pharmacological Action
Vitamin C				
Abhilash, *et al*, 2012a Effect on reduced glutathione content in the regression of alcohol-induced hepatotoxicity[603]	Ascorbic acid (25 mg/100 g bodyweight daily).	Alcoholic guniea pigs	Ascorbic acid causes faster improvement in reduced glutathione content during reduction of alcohol-induced hepatotoxicity in male guinea pigs.	Hepatoprotective
Abhilash, *et al.*, 2012b Effect on alcohol-induced oxidative stress[604]	Ascorbic acid	Alcohol-induced hepatic fibrosis in male guinea pigs (N = 36).	Reduces: activity of toxicity markers and levels of products from lipid and protein peroxidation; alpha-SMA production; caspase-3 activity; and mRNA levels of CYP2E1, TGF-beta(1), TNF-alpha and alpha(1)(I) collagen in liver.	Antioxidant Antifibrotic
Hong, *et al.*, 2012 Effect on aging of hepatic stellate cells[605]	Ascorbic acid	Age-related histological changes in the liver, using a senescence marker in protein knockout mice. These mice are more sensitive to apoptotic reagents and have a shorter life span.	Aging of hepatic stellate cells with up-regulation of PPARγ is hastened by ascorbic acid deficiency.	Hepatoprotective
Weyers, *et al.,* 2008 Examine antioxidant capacity of vitamin C in mouse liver tissue[606]	Vitamin C Roche, 200 mg/mL	Mice liver Ciprofloxacin-induced lipid peroxidation after 15 days on vitamin C	Pre-treatment with vitamin C reduced nmol of LOOH in liver tissue from 145±15 g to 45±11 g (P<0.01) Pre-treatment with vitamin C may promote oxidative balance in the liver.	Antioxidant

Table 2.8: Evidence of mechanisms of action of Hep573 interventions post-2003.

Study	Formulation	Study design	Mechanism	Pharmacological Action
Vitis vinifera				
Dulundu*, et al.,* 2007 To examine ability of grape seed extract (GSE) to protect against oxidative liver injury and fibrosis[609]	*Vitis vinifera* (seed) GSE 50 mg/kg for 28 days	Bile duct ligated rats	GSE reduced luminol (hydrogen peroxide, hydroxyl radicals, lipid peroxyl radicals) ($P<0.001$) and lucigenin (superoxide radical) ($P<0.01$), MDA ($P<0.05$), MPO activity ($P<0.05$) and hepatic collagen content ($P<0.01$) and increased hepatic GSH levels ($P<0.01$). GSE protects liver (in rats) from oxidative damage after BDL.	Antifibrotic Antioxidant (endogenous and scavenging)
Zinc				
Farias, *et al.*, 2012 The effect of antioxidant supplementation on oxidative stress in chronic hepatitis C patients[611]	Daily antioxidant supplementation (vitamin E 800 mg, C 500 mg and zinc 40 mg) for 6 months	Evaluate the antioxidant status in the blood of HCV-infected patients treated or not with standard therapy before and after supplementation of vitamins E, C and zinc	Both untreated patients and patients treated with antivirals have oxidative stress. Antioxidant supplementation may reduce oxidative stress in both groups.	Antioxidant
Somi, *et al.*, 2012 To examine effects of low dose zinc on biochemical markers in non-alcoholic cirrhosis[612]	50 mg elemental Zn sulfate daily	Double-blind, placebo-controlled, randomised clinical trial (N=60 cirrhotic patients)	Zn supplementation in non-alcoholic cirrhotic patients was able to clinically prevent deterioration of cirrhosis and excess Cu accumulation. Zn has metabolic effects and indicates some ability to improve liver function, hepatic encephalopathy, and nutritional status.	Hepatoprotective
von Bulow*, et al.,* 2007 Molecular mechanism by which zinc affects TNF-α production in LPS stimulated monocytes[613]	Zinc 45 µM	LPS activated stimulated monocytes	Inhibited Raf-1/IKKβ/NF-κB pathway Zinc inhibited NF-κB translocation and TNF-α production.	Anti-inflammatory Immune-modulator

Summary of Evidence of Mechanisms of Action

The majority of studies pre-2003 were *in vitro*, as pharmacological actions were confirmed and interest grew there was an increase *in vivo* (animal studies) work post-2003.

Pre-2003 evidence (Table 2.7) illustrated the selection of interventions and the specific mechanisms by which disease progresssion can be ameliorated in HCV. Post-2003 evidence (Table 2.8) confirmed the original selection of interventions.

The evidence pre-2003 showed that herbal interventions increased glutathione. This was confirmed four-fold post-2003. Research showed the majority of trial ingredients were antioxidants and specifically enhanced glutathione[249]: alpha lipoic acid,[541,558,559] *Andrographis paniculata* (andrographolide),[544,562,566] *Astragalus membranaceus*,[545] *Camellia sinensis* (green tea, epicatechin, epigallocatechin gallate),[233,571] *Curcuma longa* (turmeric, curcumin),[514] lycopene,[581] *Phyllanthus amarus*,[584] selenium[588,590] and *Silybum marianum* (milk thistle, silymarin, silibinin),[594,595,596] vitamin C[604] as well as *Vitis vinifera* (grape seed extract).[609]

Antioxidants also prevent damage to lipids, proteins and DNA, affecting cell proliferation, differentiation and cell survival with wide ranging anti-inflammatory effects. [240,247,250]

Post-2003 evidence now attributes antiviral activity to three phytochemical based antioxidants (all polyphenolic compounds) used in Hep573 Study: *Phyllanthus amarus*,[585] *Camellia sinensis,* [554,552,555,556] *and Silybum marianum.* [232,459] The latter two have shown inhibition of HCV entry at the fusion stage. Each of these phytochemicals also influence lipid metabolism[584,234,600] leading to speculation that interference with lipids may affect viral entry.[459]

Therefore, most interventions had multiple overlapping pharmacological actions; the most common presentations consisted of antioxidant, antiviral, anti-inflammatory and antifibrotic.[459,233,542,574,614,615,519,616] This information informed both the testing hypothesis and the selection of the trial interventions.

These multiple overlapping pharmacological actions show the complexity of phytochemical-based interventions making them particularly suitable to address the multifactorial aspects of the pathobiology of HCV infection. (This wholism is congruent with the naturopathic treatment protocol previously outlined (page 20).

The emerging data on intravenous antioxidants would inform future research in terms of effective administration and dosage.

The next chapter outlines the methods employed to test oral silymarin and oral antioxidants in CHC patients in a randomised, double-blind, placebo-controlled clinical trial.

.

CHAPTER 3

METHODOLOGY

STUDY DESIGN

The Hep573 Study was a randomised double-blind, placebo-controlled clinical trial testing the safety and efficacy of two different herbal medicines and/or vitamin formulations in the treatment of patients with chronic hepatitis C.

ETHICS COMMITTEE APPROVAL

The Hep573 Study was approved by four Human Research Ethics Committees (the three participating hospitals and the University Human Research Ethic Committees). The corresponding approval numbers were:

(1) Hunter New England Area Research Ethics Committee: 01/02/14/3.07;

(2) University of Newcastle Human Research Ethics Committee: H-342-0602;

(3) Central Sydney Area Health Service Ethics Review Committee (RPAH zone): X02-0071; and

(4) Sydney West Area Health Service Human Research Ethics Committee: 2003/2/4.4 (1583).

THERAPEUTIC GOODS ADMINISTRATION (TGA)

In accordance with Australian regulatory requirements for the conduct of clinical trials, a clinical trial notification was submitted to the Therapeutic Goods Administration, Commonwealth Department of Health, Housing, Local Government and Community Services, Canberra, from the individual hospital sites (sponsors) as they received Human Research Ethics Committee approval (TGA 2002/319).

PARTICIPANT RECRUITMENT

The three participating hospital trial centres were: John Hunter, Newcastle, Royal Prince Alfred and Westmead Hospitals, Sydney, New South Wales, Australia. Participants in the trial were recruited from these hospital outpatient liver clinics from July, 2003 to March, 2006 using the following series of media campaigns. Media releases were distributed to local radio stations, local newspapers and television stations in the hospital catchment areas. Advertisements were placed in the *Medical Observer, Australian Doctor, Sunday Telegraph (Body and Soul Supplement), The Australian, Sydney Morning Herald, Newcastle Sun, Newcastle Herald, Cumberland Newspaper* and the Hepatitis C Council of NSW Quarterly Magazine (*Hepatitis C Review*). Radio and local television interviews were conducted on request. All advertising material was cleared for publication and distribution by the human research ethics committees. (Appendix C includes a copy of the approved advertising material.)

Participant Selection

The following inclusion and exclusion criteria were applied to participant recruitment.

Inclusion criteria:

(1) able to give informed consent;

(2) aged between 18 and 75 years;

(3) hepatitis C antibody and HCV RNA positive;

(4) abnormal liver tests on at least three occasions in the past two years;

(5) prepared to stop Western and Chinese herbs, vitamins and nutritional supplements used in the Study for the study duration and for a wash out period of 12 weeks prior to trial entry;

(6) prepared to visit the hospital site monthly for blood tests and to complete questionnaires;

(7) stable on their methadone dose and less than 100 mg daily dose; and

(8) women were prepared to practice two methods of contraception during the Study period.

Exclusion criteria:

(1) Alcohol-related liver disease;

(2) alpha 1-antitrypsin deficiency;

(3) autoimmune hepatitis;

(4) drug-induced liver disease;

(5) haemochromatosis;

(6) hepatitis B and D;

(7) decompensated cirrhosis (Child-Pugh Score > 7);

(8) human immunodeficiency virus (HIV);

(9) non-alcoholic steatohepatitis;

(10) antiviral therapy (pegylated interferon and ribavirin) in the past six
    months;

(11) platelet count $\leq$50 x 10^9/L;

(12) alcohol intake >70 grams per week;

(13) methadone >100 mg/day or unstable on methadone dose;

(14) non prescription or recreational drugs >3-4 times per week;

(15) pregnant or lactating females;

(16) potential drug-herb interactions, i.e., cyclosporin, warfarin, digoxin,
    selective serotonin reuptake inhibitor (SSRI), theophylline; and

(17) normal alanine aminotransferase (ALT) levels.

The inclusion and exclusion criteria applied to the Hep573 Study were
consistent with the standard applied to pegylated interferon and ribavirin trials in
chronic hepatitis C populations[22] with the exception of the extended wash-out
period from herbal medicines.

Complementary medicines (apart from the trial interventions) could be taken
during the course of the Study if the participant had taken that medicine for a
condition other than liver disease continuously for the previous 12 months, for
example, glucosamine for osteoarthritis. (See Appendix D, Complementary
medicine exclusions during the Study.)

PARTICIPANT SCREENING PROCEDURES

Screening procedures of potential participants included informed consent, medical
history, a physical examination performed by a gastroenterologist/hepatologist,
general observations (height, weight, blood pressure and pulse rate) and laboratory
investigations. Screening information obtained from participants included: history of
HCV infection, source of exposure to HCV infection, country of birth, ethnicity, past
medical treatment and complementary treatment for hepatitis C, symptoms,

concurrent medications, caffeine intake, diet, symptoms, alcohol and other drug intake.

The Alcohol Use Disorders Identification Test for alcohol consumption (AUDIT-C) questionnaire[224,225] was used as a screening tool to ensure that participants were not consuming more than 70 g alcohol per week. A full drug history including substance used, route of administration, dose and frequency, years of using and the date the substance was last used was recorded at the screening visit.

At this screening visit, the gastroenterologist/hepatologist specialists assessed the severity of the hepatitis-C-related liver disease and determined whether the potential participant should be referred for screening for pegylated interferon and ribavirin, or whether they were suitable for the herbal Study (Hep573).

Once entered into the Study, the participants were committed to 13 monthly visits to the hospital. Blood (20-30ml) was collected at these appointments (Refer to Table 3.3 which identifies the laboratory investigations taken at the screen visit, subsequent visits and the personnel involved in the Study conduct.) The trial participants were required to fast for 12 hours prior to all research-blood collections on four occasions (Weeks 0, 12, 24 and 48).

A gastroenterologist/hepatologist also reviewed the participants and conducted physical examination at Weeks 12 and 24 of treatment.

The Hep573 Study questionnaires were handed to the participants on arrival at the hospital liver-clinic outpatient reception desk. Monthly questionnaires were on the subject of alcohol and other drugs, caffeine intake, diet and symptoms, lifestyle practices and concurrent medications. The *HQLQ™v1* was administered quarterly. (Questionnaires not in the public domain, or have been amended will appear in Appendix E, Alcohol, Drugs, Diet and Symptoms Questionnaires.)

Initial Characteristics of the Participants at Baseline

The following baseline parameters were measured on all participants: age, gender, HCV genotype, years of infection, ALT, AST, GGT, ALP, albumin, bilirubin, globulins, F_2-isoprostanes (ISO), HCV RNA viral copies, Hepascore, hyaluronic acid (HA), FibroTest (FT), alpha-2 macroglobulin, haptoglobin, lipid profile including cholesterol, triglycerides and apolipoprotein A1 (ApoA1); BMI, ferritin, platelets (Plt), coagulation studies including prothrombin time (PT) and international

normalised ratio (INR); and thyroid stimulating hormone (TSH) and white cell count (WCC).

Interventions Administered to the Participants

Participants were randomised to the three treatment arms: 1. silymarin and antioxidant (SOX), 2. silymarin (S) and 3. placebo (P). The full list of interventions used in the SOX arm appear in Table 3.1 following.

Table 3.1: Full List of Hep573 silymarin and antioxidant (SOX) trial interventions.

Hep573 SOX Trial interventions	Standardised to specified daily amount
Silybum marianum 60 grams/day (g/d)	720 mg silybin
Astragalus membranaceus 3 g/d	
Andrographis paniculata 3 g/d	34.8 mg andrographolide
Camellia sinensis 4 g/d	
Curcuma longa 8 g/d	280 mg curcuminoids
Eleutherococcus senticosus 3 g/d	1.2 mg syringaresinol diglucosides
Hypericum perforatum 1.5 g/d	0.8 mg hypericin
Vitis vinifera 12 g/d	80 mg procyanidins
Vitamin C 400 mg/d	calcium ascorbate 400 mg
Zinc amino acid chelate 20% 250 mg/d	elemental zinc 50 mg
Selenomethionine 40 mg/d	elemental selenium 200 mcg
Alpha lipoic acid 200 mg/d	
Lycopene 80 mg/d	

The silymarin was equivalent to 60 grams of *Silybum marianum,* standardised to contain 720 mg silybin per day as outlined in Table 3.1. The full list of ingredients seen in Table 3.1 was dispensed to the participants in three separate bottles (120 tablets in each) labelled 'Immuhep', 'Hepavir' and 'Antioxidant compound' from the hospital outpatient pharmacy. They were distributed across the three formulations in the following manner.

Hepavir

- *Silybum marianum* (milk thistle) 70:1 seed extract (standardised) 14,000 mg;
- *Andrographis paniculata* (andrographis) 19:1 herb extract (standardised) 750 mg;
- *Phyllanthus amarus* (phyllanthus) 5:1 herb extract 750 mg; and

- *Hypericum perforatum* (Saint John's wort) 6:1 herb extract (standardised) 375 mg.

Immuhep

- *Astragalus membranaceus* (astragalus) 5:1 extract 750 mg;
- *Eleutherococcus senticosus* (Siberian ginseng) 10:1 root extract (standardised) 750 mg;
- Vitamin C as calcium ascorbate 100 mg;
- Zinc amino acid chelate 20% 62.5 mg equivalent to elemental zinc 12.5 mg; and
- Alpha lipoic acid 50 mg.

Antioxidant Compound

- *Vitis vinifera* (grape) 120:1 seed extract (standardised) 3000 mg;
- *Silybum marianum* (milk thistle) seed 70:1 extract (standardised) 1000 mg;
- *Camellia sinensis* (green tea) 5.5:1 leaf extract 1000 mg;
- *Curcuma longa* (turmeric) 25:1 rhizome extract (standardised) 2000 mg;
- Lycopene 5% 100 mg equivalent to 20 mg (tablet grade); and
- Selenomethionine 10 mg equivalent to elemental selenium 50 mcg.

Table 3.2 following shows how the participants' dose was titrated over the first week of administration so that by Day 7, the participants in the SOX arm were receiving the specified total daily amount of the ingredients outlined in Table 3.1.

Table 3.2: Titration of the Hep573 Study dose over first Week of administration.

Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Morning	**Morning**	**Morning**	**Morning**	**Morning**	**Morning**	**Morning**
1 x Hepavir	1 x Hepavir	1 x Hepavir	2 x Hepavir	2 x Hepavir	2 x Hepavir	2 x Hepavir
1 x Immuhep	1 x Immuhep	1 x Immuhep	2 x Immuhep	2 x Immuhep	2 x Immuhep	2 x Immnhep
1 x Antioxid	1 x Antioxid	1 x Antioxid	2 x Antioxid	2 x Antioxid	2 x Antioxid	2 x Antioxid
Night	**Night**	**Night**	**Night**	**Night**	**Night**	**Night**
1 x Hepavir	1 x Hepavir	1 x Hepavir	1 x Hepavir	1 x Hepavir	1 x Hepavir	2 x Hepavir
1 x Immuhep	1 x Immuhep	1 x Immuhep	1 x Immuhep	1 x Immuhep	1 x Immuhep	2 x Immuhep
1 x Antioxid	1 x Antioxid	1 x Antioxid	1 x Antioxid	1 x Antioxid	1 x Antioxid	2 x Antioxid

By the end of Week 1, participants were taking two tablets twice daily with meals from each of the three containers, i.e., a total of 12 tablets daily.

STUDY DURATION

The Study duration was 48 weeks, comprised of a 24-week treatment-period and a 24-week, follow-up period post-treatment. A 12-week wash-out period from specified herbs and vitamins (see Appendix D) was required prior to trial entry. The schedule of participant procedures required at each hospital visit, identified personnel and timeframes are outlined in Table 3.3.

Table 3.3: Hep573 timeline and events schedule.

Study Personnel GP, N, PH, SC, SS	Screen	Wk 0	Wk 4 Rx1	Wk 8 Rx2	Wk 12 Rx3	Wk 16 Rx4	Wk 20 Rx5	Wk 24 Rx6	Wk 28 FU1	Wk 32 FU2	Wk 36 FU3	Wk 40 FU4	Wk 44 FU5	Wk 48 FU6
Informed consent (N, SC, SS)	■													
Medical History (N,GP,SS)	■													
Physical examination (SS)	■				■			■						
General Observations – BP, PR, Wt (N,SC,SS)	■	■	■	■	■	■	■	■	■	■	■			■
HIV/HBV/HCV Serology	■													
PCR HCV RNA genotype	■													
PCR HCV RNA Qualitative Test	■							■						■
PCR HCV RNA Quantitative Test		■			■			■						■
Pregnancy Test (β-HCG)	■	■	■	■	■	■	■	■						
Haematology, including full blood count (FBC)	■	■	■	■	■	■	■	■	■	■	■			■
Liver function test (LFT)	■	■	■	■	■	■	■	■	■	■	■			■
Urea, creatinine and electrolytes	■	■	■	■	■	■	■	■						
Prothrombin time/INR	■				■			■						■
Haematinics/ ferritin	■				■			■						
Thyroid function (TSH,T4)	■				■			■						
Serum lipids:HDL, LDL & total cholesterol, Triglycerides & Apolipoprotein A1		■			■			■						■
Oxidative stress marker: F_2-isoprostanes (ISO), malondialdehyde (MDA		■			■			■						■

Table 3.3: Hep573 timeline and events schedule.

Study Personnel GP, N, PH, SC, SS	Screen	Wk 0	Wk 4 Rx1	Wk 8 Rx2	Wk 12 Rx3	Wk 16 Rx4	Wk 20 Rx5	Wk 24 Rx6	Wk 28 FU1	Wk 32 FU2	Wk 36 FU3	Wk 40 FU4	Wk 44 FU5	Wk 48 FU6
Antioxidant activity: glutathione (GSH), total lipid soluble antioxidant assay		X			X			X						X
Fibrosis markers: haptoglobin, alpha 2 macroglobulin, hyaluranon		X			X			X						X
Dispense study medications (PH)		X	X	X	X	X	X							X
Record study medication compliance (N, PH, SC)		X	X	X	X	X	X	X						X
Record adverse events (GP, N, SC, SS)		X	X	X	X	X	X	X	X	X	X	X	X	X
Record concurrent medications (GP, N, SC, SS)	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Record alcohol intake (N, SC, SS)	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Diet and Symptom questionnaire	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Caffeine intake questionnaire	X	X	X	X	X	X	X	X	X	X	X	X	X	X
HQLQ™		X			X			X			X			X

Key:

Wk	= Study week,
Rx	= active treatment or placebo treatment,
FU	= follow-up period,
GP	= general practitioner,
N	= hepatitis C nurse consultant,
P	= Clinical trials pharmacist,
SC	= Study Coordinator,
SS	= Staff specialist,
HQLQ™	= *QualityMetric™ Hepatitis Quality of Life Questionnaire*
Blue highlight	= activity performed

METHODS USED TO VERIFY AND QUANTIFY THE TRIAL INTERVENTIONS

The ingredients were verified and authenticated at input by thin layer chromatography (TLC) and high performance liquid chromatography (HPLC), which was the TGA standard at the commencement of the Hep573 Study. In addition ultra-violet and visible spectroscopy (UV/VIS) was utilised. All three techniques were important in the quality control of the Hep573 trial interventions.

TLC, a chromatographic technique used for separating organic compounds and checking the purity of products,[617,618] was used for plant identification. HPLC was used to determine the authenticity of raw material supplied against a reference sample (herbarium specimen or Pharmacopoeiac standard) and to quantify the standardised ingredients. UV/VIS was used to determine the presence of certain compounds by their colour (light absorption), wavelength and frequency.

The results from these chemical analyses are recorded on a certificate of analysis (C of A) that is supplied by the manufacturer to confirm authenticity and the quantity of the known standardised ingredient with specified lower and upper limits of the listed substance. The source of the raw material and the certificate of analysis from the manufacturer for all the Hep573 trial interventions were as follows:

Alpha lipoic acid was sourced by Trans Chem Pty Ltd (Sydney, Australia) and manufactured by Newsmart (Shanghai) International Trading (China) (Batch Number 020428). The content of alpha lipoic acid was confirmed by HPLC to be 99.9%.

Andrographis paniculata (andrographis) (Powdered extract, P.E. 19:1). The dried leaf, plant extract was supplied by Pathway International Pty Ltd (Sydney, Australia) and manufactured by Natural Remedies (Bangalore, India) (Batch number AP/02007). Phytochemical analysis confirmed the presence of andrographolide (20.8% w/w) and total andrographolides (27.8% w/w) respectively. The autoscaled chromatogram documented andrographolide (RT 25.299, Area 2404933, Height 169532, Amount 0.205, 20.53%). The lower limit for andrographolide is 20% w/w and the upper limit is 24% w/w.

Astragalus membranaceus (astragalus) (P.E. 5:1). The plant extract was sourced from R & T Australia Pty Ltd (Sydney, Australia) and manufactured by CZ. Medipro Botanical Laboratories Pty (Changzhou, China) (Batch number CZI-0909AME). While the active ingredient is recorded as polysaccharides, this was not

quantified. The solvent used for the extraction of the plant material was 75% (v/v) ethanol and the excipient was 20% maltodextrin.

Camellia sinensis (green tea) (P.E. 5.5:1). The dried leaf of the powdered plant extract was supplied by Trans Chem Pty Ltd (Sydney, Australia) and manufactured by Flachsmann (Wadenswill, Switzerland) in two batches (Batch number 3028871, 3003556). The extraction medium was 80% (m/m) ethanol and 0.0025% (m/m) ascorbic acid in a carrier of 0–20% maltodextrin USP. The polyphenol content calculated as epicatechin by HPLC (lower limit 47.5, upper limit 52.5) was 51.3% w/w in the first batch (3028871) and 48.4% w/w in the second batch (3003556). The polyphenol content calculated as caffeine by HPLC (lower limit 5 and upper limit 10) was 7.7% w/w in the first batch (3028871) and 7.37% w/w in the second batch (3003556).

Curcuma longa (turmeric) (P.E. 25:1). The rhizome extract was supplied by Pathway International Pty Ltd (Sydney, Australia) and manufactured by Natural Remedies (Bangalore, India) (Batch number CL/02001). The HPLC testing revealed that the turmeric rhizome extract contained 93.7% w/w total curcuminoids. The specific curcuminoids tested were curcumin (RT 13.705, Area 9913181, 17.65%), demethoxycurcumin (RT 14.400, Area 2763557, 4.92%) and bis-demethoxycurcumin (RT 15.042, Area 1415379, 2.42%).

Eleutherococcus senticosus (Siberian ginseng) (P.E. 10:1). The standardised root extract was sourced from Pathway International Pty Ltd (Sydney, Australia) and manufactured by Indena (Tours, France) (Batch number 20005). The HPLC analysis of the dried extract confirmed the presence of eleutheroside E at the following levels: (RT 11.380, Area 9115660, Height 639852, 1.08%). The lower and upper limits for eleutheroside E are 0.5% and 0.68% respectively.

Hypericum perforatum (Saint John's wort) (P.E. 6:1).The dried aerial parts extract was sourced from Trans Chem Pty Ltd (Sydney, Australia) and manufactured by Phytopharm, Kleka SA (Nowe Miastro N. Warta, Poland) (Batch number 010399). The total hypericins calculated as hypericin by UV/VIS was 0.38% (lower limit 0.30 and upper limit 0.36).

Lycopene 5% was supplied and manufactured by Roche Vitamins Australia Pty Ltd (Batch number UT02101028). The certificate of analysis (C of A) provided by Roche reported that the lycopene content was 5.6% w/w. The lycopene content of 5.6% w/w was confirmed by Tabco in their C of A.

Phyllanthus amarus (phyllanthus) (P.E. 5:1).The whole plant extract was sourced from Pathway International Pty Ltd (Sydney, Australia), manufactured by Natural Remedies (Bangalore, India) (Batch Number PA01002). The total phyllanthin and hypophyllanthin by HPLC was 1.01% w/w. The lower limit for both is 1.0.

Selenomethionine > 1.25%. This was supplied by Trans Chem Pty Ltd (Sydney, Australia) and manufactured by Sabinsa Corporation (New Jersey, USA) (Batch number: U21912081). UV maxima of selenomethionine was 220nm (lower limit), the elemental selenium in selenomethionine was 074 ppm.

Silybum marianum (milk thistle) (P.E. 70:1). The plant extract of the seeds used to produce the Phytomedicine *Silymarin 15000* supplied for the trial was manufactured by the Italian company, Indena, purchased from their Australian distributor, Pathyway International Pty Ltd. The tablet manufacturer, Tabco, standardised the product *Silymarin 15000*, to contain 80-88% silymarin according to the product specification.

Five different batches of the Indena milk thistle powdered extract were used in the production of the milk thistle product used in the trial (Batch numbers: 27405/M5, 27691/M4 (x2), 27691/M1, 2786/M5 and 28503/M2 (x2)).

There were seven separate lot numbers assigned to the raw material of the seeds of *Silybum marianum* as it was formulated into the two separate treatment arms (identified above) and required for replacement stock. The lot number 23986 (BN: 27405/M5) of *Silybum marianum* P.E. 70:1 tested by UV/VIS detected a silymarin content calculated as silybin of 82.6 mg (lower limit 80 mg and upper limit 88 mg). Lot number 24143 (BN: 27691/M4) contained a silymarin content of 80.1 mg. Lot number 24235 (BN: 27691/M4) was 82.1 mg silybin. Lot number 24342 (BN: 27691/M1) contained 85.8 mg silybin. Lot number 24593 (BN: 2786/M5) revealed a silymarin content calculated as silybin of 89.7 mg. The latter was just above the upper limit and was used as replacement stock during the Study. Lot number 27802 (BN: 28503/M2) revealed that the silymarin content of 86.3 mg and lot number 27951 (BN: 28503/M2) of 85.6mg silybin.

According to the overlay fingerprint report provided by Pathway International for Lot number 24593, the peaks for the following constituents became visible at identified minutes: silychristin 20.900, silybin A 28.511, silybin B 29.464, isosilybin A 31.299 and isosilybin 30.211.

The Study dose administered to both the silymarin only arm (Group 2) and the silymarin and antioxidants arm (Group 3) was 857 mg of a 70:1 *Silybum marianum* extract of the seed standardised to contain, on average, 720 mg silybin daily. The specific composition of the silymarin content was not further investigated or documented.

Vitamin C (calcium ascorbate). This was supplied and manufactured by Roche Vitamins Australia Pty Ltd. The certificate of analysis was missing from the documentation and numerous requests to Phytomedicine Pty Ltd to secure this certificate of analysis failed. The HPLC fingerprint 340nm for the finished product (Immuhep) identified the vitamin C content as 112.2 mg per tablet (lower limit 106.4 and upper limit 123.6 mg).[619]

Vitis vinifera (grape) (P.E. 120:1). The seed of *Vitis vinifera* was sourced from Phytamedica Laboratories P/L (Sydney, Australia) and manufactured by Indena (Batch number P00280). HPLC testing revealed that the content of procyanidins in grape seed extract was 83.6% w/w. The seed was also sourced from R & T (Australia) Pty Ltd and manufactured by C.Z. Medipro-Botanical Laboratories Pty (Changzhou, China) Batch number:20020508. The HPLC testing revealed that the procyanidins were 83.7% w/w (lower limit 76.5 and upper limit 93.5).

Zinc amino acid chelate 20%. This was supplied by Trans Chem Pty Ltd (Sydney, Australia) and manufactured by Kelatron Corporation (Utah, USA) (Batch number G301201). The C of A from Kelatron reported the zinc content as 19.9% (lower limit 19.0 and upper limit 21%). Tabco's C of A specified the elemental zinc by amino acid assay as 20.4% (lower limit 19.5 and upper limit 21.0).

Stability and Verification of Trial Interventions

The issue of stability of trial preparations and verification of the standardised components is of crucial importance in determining the efficacy or otherwise of all CAM preparations. All the raw ingredients for the trial formulations for Hep573 were sourced by Phytomedicine Pty Ltd and manufactured by Tabco, TGA Licence 31683. Tabco has since closed down but had an excellent reputation as a tablet manufacturer, and was certified by the Australian Therapeutic Goods Administration to manufacture therapeutic goods under Good Manufacturing Practice standards.

Stability testing of Phytomedicine *Silymarin 15000* and Phytomedicine Antioxidant compound had been commenced but not completed. The Study

Coordinator requested verification of stability and was only provided with certificates of analysis based on the physical characteristics of the tablets. The tablets provided by Phytomedicine Pty Ltd were found to maintain their integrity over the duration of the study as evidenced by physical examination showing no deterioration of the tablets during the Study. However, since the tablets contained standardised powdered extracts that are generally quite stable for a number of years, it can be assumed the level of active compounds did not significantly deteriorate over the course of the Study period.

Phytomedicine Pty Ltd developed placebo and active tablet formulations which were identical. The ingredients used in the placebo tablets were as follows: Calcium hydrogen phosphate, cellulose microcyrstalline, sodium starch glycollate, magnesium stearate, hypromellose, coating colour. The placebo ingredients were the same for each tablet, with the exception of the coating colour which was product dependent. The tablets were coated using a coloured blackout film based on a hypromellose solution. The tablet colour was matched to the active tablet colour for each product. This blackout coat imparted a neutral taste to the tablet when swallowed.

The coating of the silymarin needed to be redone as the size of the tablet did not match the other trial formulations. Following instructions from the Study Coordinator, Phytomedicine Pty Ltd double-coated the silymarin tablets again so the tablets were identical in colour and size. The extra coating is unlikely to have affected bioavailability as it was designed to be easily digested in the stomach preparing for the release of the herbal material in the small intestine.

STUDY CONDUCT, OUTCOME MEASURES AND STATISTICAL ANALYSIS

This section outlines the sample size calculation, randomisation, participant accountability, safety, dose compliance, primary and secondary outcome measures and statistical analyses.

Sample Size Calculation

A sample size of 57 per arm was calculated to have 80% power to detect a significant increase in the ALT normalisation rate from 12% in the control group to ≥36% in the treated group (continuity corrected chi-square test, two tailed, 5% significance level). If a drop-out rate of 20% is assumed, a total of 72 participants

were required per arm. It was expected 10 subjects would be recruited each month with a total sample of 216 participants.

Randomisation Method

Participants were randomised to treatment in blocks of six. In every six participants, two were allocated at random to the placebo group, two to silymarin and two to the SOX group. This randomisation method was designed by Adrienne Kirby from the National Health and Medical Research Centre (NHMRC) Clinical Trials Centre at the University of Sydney. Thirty nine participants were randomised to both the placebo and SOX group, and 40 participants to the silymarin group.

Participant Accountability

One part of the CONSORT Guidelines 2001[620] for reporting randomised trials is a participant flowchart that tracks the flow of all participants throughout the Study. Accordingly,
Figure 4.1 Hep573 Study Participant Flowchart (Chapter 4 Results), represents the numbers screened, enrolled, randomised to each treatment arm and lost in between each of these steps, the numbers completing both the treatment and follow-up phases of the treatment arms, the reasons for stopping therapy, and the numbers lost to follow-up.

Intention-to-Treat

Response to treatment was assessed on an intention-to-treat basis.

Analysis of Safety Endpoints

Toxicity and safety data were summarised at each Study visit by treatment received. Therapy-related adverse events were reported to the relevant human research ethics committees and the Therapeutic Goods Administration.

Independent Safety Review

An independent safety review was conducted by Professor Ian Whyte, Pharmacology Department, University of Newcastle in 2006 on all the blinded data, non-serious and serious adverse-event reports. He recommended to the lead Human Research Ethics Committee (Hunter New England Area Health Service) that the trial be continued.

Compliance

Dose compliance (number of tablets remaining) was measured by tablet count of the returns at each visit by the hepatitis C nurse consultants or the Study Coordinator at each of the hospital sites as well as by audits of hospital pharmacy records. The hospital pharmacy record was seen as the primary source document for the calculation of dose/tablet compliance. Where there were missing data, the information on dose compliance in the case-report form-folders, or the hospital medical record was used.

Compliance to the Study protocol was monitored at each monthly visit by documenting alcohol intake, concurrent medications and CAM use. In the event of a Study protocol violation, the participant was discontinued.

OUTCOME MEASURES

Statistical Analyses of the Outcome Measures

Karen Byth (PhD, DIC, C Stat RSS, Biomedical Statistician at Westmead Millennium Institute, NHMRC Clinical Trials Centre, University of Sydney) conducted all statistical analyses of the outcome measures in the Hep573 Study in consultation with the Study Coordinator.

Primary Outcome Measure

The primary endpoint and efficacy measurement was the proportion of participants who had ALT normalisation from baseline to Week 24. Chi-squared or Fisher's exact test[621] (as appropriate) were used to test for differences in the proportions. ALT normalisation was defined as a single ALT reading within the normal laboratory range during the treatment period of 24 weeks.

Secondary Outcome Measures

Secondary outcome measures included the change over time in: ALT, HCV RNA Viral Load (VL), plasma F_2-isoprostanes (ISO), glutathione (GSH) and fibrosis markers (Fibrotest, Hepascore) (FM) and QOL. The statistical packages SPSS Version 17 (SPSS Inc, Chicago, IL) and S_PLUS Version 8 (Insightful Corp., Seattle, Washington) were used to analyse the data. The Medical Outcomes Trust *Hepatitis Quality of Life Questionnaire* (*HQLQ*™) (*QualityMetric*™, Version 1, 1999) were analysed at Weeks 0, 12, 24, 36 and 48 according to published

guidelines[622,93,623] and their scoring software. The change in *HQLQ™v1* was the difference from baseline at Weeks 24 and Weeks 48.

Table 3.4 shows both the schedule for the Hep573 Study primary and secondary outcome measures.

Table 3.4: Schedule for the Hep573 Study outcome measures.

	ACTIVE							FOLLOW UP			
Week	**0**	**4**	**8**	**12**	**16**	**20**	**24**	**28**	**32**	**36**	**48**
ALT	◆	◆	◆	◆	◆	◆	◆	◆	◆	◆	◆
ISO	◆			◆			◆				
VL	◆						◆				◆
GSH	◆			◆			◆				◆
HQLQ™	◆			◆			◆			◆	◆
FM	◆			◆			◆				◆

Key:
ALT   = alanine aminotransferase
ISO   = F_2-isoprostanes
VL   = HCV RNA Viral Load (Quantitative)
GSH   = Glutathione
HQLQ™ = *QualityMetric™ Hepatitis Quality of Life Questionnaire*
FM   = Fibrosis markers, FibroTest, Hepascore and hyaluronic acid

The ALT, F_2-isoprostanes, HCV RNA viral load and hyaluronic acid values were log transformed to approximate normality prior to statistical analysis. Linear Mixed Effects Models (LMEs) were used to investigate differences between groups in the within patient changes observed over time. These models take account of the correlation between repeated measurements on the same patient. Two-tailed tests with a significance level of 5% were used throughout. In the models, treatment was considered as a fixed effect, time as either a fixed factor or covariate, with participant identifier and time as random effects. The time-by-treatment interaction term in the models, tests for differences between groups in the within participant changes. Absolute changes from baseline on the log scale were back transformed and reported in the text as percentage changes from baseline. The FibroTest and Hepascore values did not require log transformation and, along with *HQLQ™v1*, were analysed as actual changes from baseline.

Spearman's Rank Correlation was used to quantify the extent of association between within participant changes, in the secondary outcome measures, in ALT, HCV RNA or FibroTest and the within participant change in F_2-isoprostanes from Weeks 0-24.

The terms 'within participant change' and "homogeneity (interaction) are now defined:

- Within participant change represents the change in a particular participant's score/outcome from one time point to another time point, i.e., change is measured within groups of participants by taking repeated measurements of an independent variable under different conditions. Therefore, every participant acts as their own control for individual difference, and fewer participants are needed for collecting the data required.[624]
- Homogeneity (interaction) represents the participant change from one time point to another and quantifies the effect of time. By testing whether this effect differs between treatment groups, this essentially tests for an interaction between the effects of time and treatment on the outcome of interest.

The baseline scores (mean plus standard error) across all treatment groups from the *HQLQ™v1* data from the Study were compared to the SF-36 age and sex adjusted Australian healthy population norms from the Australian Bureau of Statistics (ABS) National Health Survey for the Australian and NSW populations and for people with one serious medical condition. These comparisons were made to ascertain the level of impairment in QOL related to chronic hepatitis C infection in the Study participants.

Linear Mixed Effects models (LMEs) were used to examine the within participant changes over time in the five symptoms clusters (neuropsychiatric, neurological, gastrointestinal, algesic and general) and to test for associations between these changes and treatment.

One way ANOVA or the nonparametric equivalent Kruskal-Wallis Test, as appropriate, was used to test for differences in the distribution of continuous outcomes by treatment in caffeine and alcohol consumption.

McNemar tests were used to assess within participant changes in dichotomous (yes/no) outcomes (diet and symptoms). Wilcoxon tests were used to assess the significance of within participant changes from baseline for diet or symptom severity at 24 and 48 Weeks.

<h1 style="text-align:center">RESEARCH BLOODS AND OUTCOME MEASURES</h1>

Alanine Aminotransferase (ALT)

The normal reference ranges for ALT varied across the three hospitals. At John Hunter Hospital, the normal reference range for ALT was 0-40 U/L, at Royal Prince Alfred Hospital (5-55 U/L) and at Westmead Hospital (10-47 U/L, men) and (7-33 U/L, women). However, as the analyses examined the individual's response to treatment, the impact on the outcome of normalisation of varying ALT levels was considered negligible.

HCV RNA Viral Load (Quantitative)

The polymerase chain reaction (PCR) HCV RNA Quantitative samples were analysed in three batches by the South Eastern Area Laboratory Services (SEALS) at Prince of Wales Hospital by Professor Bill Rawlinson and colleagues. The method used was the Bayer Versant HCV RNA 3.0bDNA assay with a specificity of 99.5% and a cut-off of 3200 viral copies per ml. These samples were analysed in batches from 2004 to 2007.

F_2-Isoprostanes

F_2-isoprostanes blood collection method.

The blood collection, processing and storage protocol for the F_2-isoprostanes was provided by Dr Manohar Garg and Richard Blake at the University of Newcastle and is reported below.

The Study Coordinator (the researcher) preweighed 4 mg of reduced glutathione and prepared the cryogenic tube with butylated hydroxytoluene (BHT) according to the protocol. She delivered the prepared isoprostane tubes to the three participating hospital sites on the morning of the blood collection where it was stored at 4 degrees Celsius until immediately prior to blood collection.

Immediately after blood collection, the preweighed reduced glutathione (GSH, 4 mg) was added to a chilled 4 ml plasma EDTA tube by tapping the powder into the whole blood. The tube was recapped and mixed by inverting, and the EDTA tube was then kept on ice. The eppendorf tube was kept for reweighing after the powder had been removed.

Plasma samples were centrifuged at 4800 rpm for 10 minutes at 4 degrees Celsius; then 1.1 ml of the EDTA plasma containing the reduced glutathione was added into 9 μL of BHT already present in the cryogenic tube. A Gilson pipette was

used to remove 1 ml and then 100 μL of plasma was added to make the total volume of 1.1 ml (mentioned above).

The F$_2$-isoprostane samples were stored at -80 degrees Celsius until they were shipped to Perth for analysis. Specimens were taken three times during the 24 Week treatment-period at baseline, Week 12 and Week 24. The three samples per participant were processed together to reduce batch-to-batch variation in the analysis.

A Beckman Coulter GS-15R centrifuge at Storr Liver Unit Hepatitis Laboratory at Westmead Hospital was used to spin all research blood samples at that site. The technician at the Royal Prince Alfred Hospital site processed the research bloods except when on leave. The Study Coordinator completed the task at this site at those times. Hunter Area Pathology Services staff processed these samples at the John Hunter Hospital site (JHH).

A variation to the above blood collection protocol occurred at JHH because this was the only site without an allocated laboratory within the Gastroenterology Department. This presented a problem regarding the immediate inclusion of the reduced glutathione powder into the chilled EDTA for the F$_2$-isoprostanes analysis and an occupational health and safety issue as JHH would not permit the transfer of the reduced glutathione at the blood collection station.

The Study Coordinator canvassed the manufacturer of the EDTA tubes (BD Vacutainers) to determine whether reduced glutathione could be added into the EDTA tubes and then vacuum sealed prior to blood collection. However, as there were potential risks to the participant with this alternative, this was immediately discounted. The Study Coordinator negotiated to place the EDTA tube into a glove and immediately positioned it on ice. She immediately took the sample to the Hunter Area Pathology Services (HAPS) laboratory so the reduced glutathione could be incorporated into the blood within two minutes of collection by HAPS-designated personnel. If the HAPS staff member was held up, the Study Coordinator ensured the powder was included into the blood in a timely manner.

The L-glutathione (reduced) minimum 98% powder was purchased from Sigma-Aldrich Laboratories, and received 11 September, 2003 (G4251-5G 033K1528 EC 200-725-4). A new reduced glutathione batch was purchased due to a cool storage malfunction because the storage requirement of 2-8 degrees Celsius could not be guaranteed. The L-glutathione (reduced), minimum 99% powder was purchased from Sigma-Aldrich Laboratories, and received 5 April, 2005 (G4251-5G

114KO571 EC 200-725-4). As all other parameters were identical, the 1% variation among the batches was not ideal, but considered negligible. The BHT was purchased from Sigma-Aldrich (Batch number 123K0041) and the stock prepared every 10 weeks.

The method for the analysis of F_2-isopostanes.

The accepted 'gold standard' for analysis of isoprostane samples is mass spectrometry and gas chromatography used by Dr Trevor Mori and his colleagues from the University of Western Australia.[393,401] They analysed the F_2-isoprostanes for the Hep573 Study population. The F_2-isoprostanes were measured using a method previously published[625] with minor modifications. In brief, 15-F_{2t}-IsoP-d_4 and 8-F_{2t}-IsoP-d_4 (5ng) were added as internal standards to plasma (250 μl). Samples were purified by chromatography on a Certify II column (Varian), derived to the trimethylsilyl, pentaflurobenzyesters and analysed by gas chromatography-mass spectrometry on an Agilent 6890 gas chromatograph coupled to Agilent 5973 mass-selective detector using electron capture negative ionisation. F_2-isoprostanes were detected by SIM monitoring m/z 569 and m/z 573 for 15-F_{2t}-IsoP, and 15-F_{2t}-IsoP-d_4 and 8-F_{2t}-IsoP-d_4, respectively.

Whole Blood Glutathione

Samples to assay for whole blood glutathione were collected at baseline, Week 12, Week 24 and Week 48. The method for the collection of the whole blood glutathione was provided by Dr Priyanka Bandara, Storr Liver Unit, Westmead Hospital in September, 2002. Metaphosphoric acid (MPA) crystals (7.2 g) were dissolved in 10 ml (total volume) of sterile water to make the MPA stock.

On the morning of blood collection, 160 μL of MPA stock was added to 640 μL of sterile water and placed into an eppendorf tube and mixed by inverting the tube several times. This was stored at 4 degrees Celsius until blood collection.

Within five minutes of blood collection, 200 μL of whole blood from a chilled 10 ml plasma EDTA tube was added to the MPA-treated eppendorf tube, producing 1 ml total volume. This was stored on ice for 10 minutes before centrifuging. After 10 minutes, the sample was centrifuged at maximum speed, (1300 rpm) for two minutes. The supernatant was removed from the MPA-treated tube into a fresh tube and stored at -80 degrees Celsius. The remaining pellet was discarded.

The metaphosphoric acid (MPA) crystals (239275) were purchased through Sigma-Aldrich laboratories (batch numbers 01816DA, 04316BE, 06923JC). The MPA stock was freshly prepared at 10-12 week intervals. Originally, the stock was made every three months until the consistency of the stock changed (white when previously clear). The technician at RPAH Gastroenterology and Liver Centre advised the viability is best if new MPA stock is prepared every 10 weeks. In 2005, this protocol was identified, the stock preparation protocol was amended, and a new MPA batch was purchased at Westmead Hospital.

The Study Coordinator prepared the tubes, centrifuged the research bloods at Westmead Hospital in the Storr Liver Unit Hepatology laboratory and performed this task when the technician from Royal Prince Alfred Hospital was on leave.

Fibrosis markers

Despite recognised limitations,[626] two composite fibrosis markers were used in this Study: FibroTest and Hepascore. The frozen serum samples were shipped to PathWest Laboratory Medicine, Department of Health, Government of Western Australia, Perth, Australia (formerly the Faculty of Medicine, Dentistry and Health Sciences (Meddent), University of Western Australia) and analysed using the following methods.

Hepascore.

The calculation to determine Hepascore values was as follows: Hepascore = y/ (1+y) with y = exp(-4.185818 - (0.0249 x age) + (0.7464 x 1 if male, 0 if female gender) + (1.0039 x α2 macroglobulin) + (0.0302 x hyaluronate) = (0.0691 x bilirubin) – (0.0012 x γ-glutamyl transferase).[432,435]

Alpha-2 macroglobulin was measured by nephelometry (BN II; Dade-Behring, Marburg, Germany). Hyaluronic acid was measured by an enzyme-linked protein binding assay, on a 96-well microplate colorimetic reader (Corgenix Inc, Denver, Colorado, USA).[435]

PathWest Laboratory Medicine in-house analytical coefficients of variation (CVs) were 2.8% at a α2-macroglobulin concentration of 2.5 g/L, 3.5% at a hyaluronic acid concentration of 50 μg/L, 1.7% at a bilirubin concentration of 16 μmol/L and 2.7% at a GGT activity of 33 U/L.[435]

FibroTest.

As this is a patented model, there are no details of the model reproduced in the literature. However as directed by Bourliere*, et al.,*[433] the following FibroTest formula

is outlined on the US Patents Office website (http://www.uspto.gov Patent number 6,631,330). For transparency of the model used in this thesis only and for no wider distribution, it appears below:

Logistic function of 5 markers and age and sex: 4.467 .times. LogAlpha-2 macroglobulin (g/l) - 1.357 .times. LogHaptoglobin (g/l) + 1.017 .times. LogGGT (IU/1) + 0.0281 .times. Age (in years) + 1.737 .times. LogBilirubin (umol/l) -1.184 .times. ApoA1 (g/l) + 0.301 .times. Sex (female = 0, male = 1) - 5.540. ND: Not determined R.sup.2 = R squared.[627]

Haptoglobin and apolipoprotein A1 were measured by nephelometry (Immage; Beckman Coulter[435]) and α_2-macroglobulin was measured as described above. Bilirubin and gamma glutamyltransferase were measured on an automated analyser (Hitachi 917.[628])

PathWest Laboratory Medicine in-house analytical coefficients of variation (CVs) were 5.6% at a haptoglobin concentration of 0.69 g/L, 3.7% at a haptoglobin concentration of 1.27 g/L, 4.6% at an apolipoprotein concentration of 0.79 g/L and 5.8% at a concentration of 2.64 g/L (Rossi E, email communication, 2010). The other inhouse analytical CVs for the other biochemical analytes are described above in the Hepascore method.

The FibroTest score was calculated by entering the participant's age, sex and results for the biochemical analytes of haptoglobin, α2-macroglobulin, apolipoprotein A1, γ-glutamyltransferase and total bilirubin into the algorithm published in the patent.[433]

QUESTIONNAIRES

Hepatitis Quality of Life Questionnaire (*HQLQ*™)

Permission was granted from *QualityMetric*™ Incorporated for the Hep573 Study to use *Hepatitis Quality of Life Questionnaire* (*HQLQ*™), Version 1, 1999. The questionnaire was interpreted according to the Medical Outcomes Trust Guidelines[622,93,623] and accompanying software scoring program.

The Hep573 Study administered the validated (*HQLQ*™)[623] as used by Ware*, et al.,*[93] at baseline, 12 and 24 Weeks during treatment and at 12 and 24 Weeks post-treatment to assess changes in QOL during the Study. While this questionnaire has been validated for monthly information retrieval, the researchers decided to reduce the demands on the participants in the Study and collect the

information every three months on the understanding the participants would complete it on the basis of their QOL in the preceding month. (It is noted one of the originators of the SF-36 used the *HQLQ™* according to the above timeframe in a validation study in a chronic hepatitis C population.[93])

The raw *HQLQ™* data from the 118 participants at the five measured timepoints (Weeks 0, 12, 24, 36 and 48) were entered into a Microsoft Office Excel 2007 spreadsheet and imported into the *QualityMetric™ HQLQ™* Scoring Program. The hepatitis-specific questions were scored according to *QualityMetric™* algorithms. The raw score was transformed to a 0 to 100 scale, with 100 representing the most favourable score in each of the following four items: least amount of health distress, highest level of psychological well-being, least amount of hepatitis-specific limitations and least amount of hepatitis-related health distress. The positive well being scores were reversed, so that a low score for any outcome was considered poor QOL and a high score improved quality of life. (The summary measures of PCS and MCS have been described in Chapter 2.)

Diet and Symptoms Questionnaire

Dr Tim Sladden kindly gave permission for the Hep573 Study to use a questionnaire he had designed for the original CH100 study conducted in 1996-1998.[526] The extent of information about diet, physical and emotional symptoms experienced by hepatitis C patients was included as this information was considered complementary to the *Hepatitis Quality of Life Questionnaire v1*.[526,537] The data collected in the symptoms questionnaire were analysed using nonparametric tests: the McNemar Test for prevalence and frequency and the Wilcoxon Signed Ranks test for the severity associated with the diet or symptom. Given the enormous amount of data, the 34 symptoms were grouped according to Australian symptom-clustering data in chronic hepatitis C patients[103] categories of neuropsychiatric, algesic and gastrointestinal symptom clusters. Two additional categories of symptom-clustering, neurological and general-symptom clustering emerged from a review of the 34 listed symptoms in the Study diet and symptoms questionnaire. A linear mixed effects model was then used to analyse the within participant change in symptom-clustering over time in the Hep573 Study participants.

The diet questionnaire included 20 food items which were condensed into five categories. Three categories: protein, fat and carbohydrate were generated using the Food Standards Australia New Zealand (FSANZ) Nutrient Tables

(NUTTAB) 2010 online searchable database. Each food item was mapped against the NUTTAB database for protein, fat and carbohydrate content per 100 grams. The maximum content of these three components determined the food group for the item in the diet questionnaire. Honey, coffee and alcohol were the only items that were specified per 100 mL. The latter two, coffee and alcohol, were considered as separate groups because the literature attests to their differing effects on liver health.

Caffeine Questionnaire

The caffeine intake questionnaire used in the Hep573 Study was designed for a National Institute of Health study on the natural history of chronic hepatitis C which was conducted at Westmead and Royal Prince Alfred Hospitals, NSW.

The different types of caffeine beverages quantified were: decaffeinated coffee, instant coffee, brewed coffee, tea and caffeinated soft drinks (Coke™, Pepsi™). There were nine frequency response categories, ranging from 'never' or 'less than once a month' to 'more than six beverages per day'. The numbers in this Study were too small to analyse these multiple subsections of frequency by treatment group according to the McNemar test at Weeks 0, 24 and 48.

Therefore, the Study data were converted to one category, total weekly caffeine intake per participant from the frequency data collected in the caffeine intake questionnaire.

The determination of caffeine intake per beverage was taken from the NUTTAB 2010 online searchable database and the relevant sections appear in the following table (Table 3.5).

Table 3.5: Caffeine content of beverages from NUTTAB 2010 database.

Beverage	Calculated caffeine content Mg/100 mL	Caffeine per average serve

Average decaffeinated coffee:
    Instant coffee: 1   mg/100mL
    Ground coffee: 12 mg/100mL
	6.5	16.25 mg/250 mL

Instant coffee	31.0	77.50 mg/250 mL

Average brewed coffee:
    Cappuccino: 64 mg/100mL
    Long black :   101 mg/100mL
	82.5	206.25 mg/250 mL

Tea	19.0	47.50 mg/250 mL
Coca Cola	10.0	60.00 mg/600 mL

The caffeine content analysed in the NUTTAB 2010 database was measured in milligrams per 100 mL. For the decaffeinated coffee, the NUTTAB 2010 entries for instant coffee and ground coffee were 1 mg/100 mL and 12

mg/100 mL respectively. Therefore, a midpoint between the two decaffeinated types was taken as 6.5 mg/100 mL. The same process applied to brewed coffee and the NUTTAB entries of cappuccino (64 mg/100 mL) and long black (101 mg/100 mL) were identified as reflective of brewed coffee. The midpoint between these two types of brewed coffee was 82.5 mg/100 mL. The entries for mg caffeine per 100 mL were converted to an appropriate serving size-250 mL cup size or 600 mL bottle size. The total caffeine content per beverage per average serve was determined in this manner is as follows: decaffeinated coffee (16.25 mg), instant coffee (77.5 mg), brewed coffee (206.25 mg), tea (47.5 mg) and coke (60 mg per 600 mL bottle).

The nine caffeine consumption frequency categories were converted to a weekly frequency and then multiplied by the caffeine content to give a total weekly caffeine intake per participant per treatment group at Weeks 0, 24 and 48. In order to analyse the change in caffeine consumption across the Study duration, the data were categorised into three categories of caffeine consumption, where low caffeine consumption was regarded as 500 mg caffeine per week, approximately equivalent to one instant coffee per day, medium caffeine consumption group was defined as between 501-2500 mg caffeine per week and the high caffeine intake group was defined as greater than 2501 mg caffeine per week (about five cups of instant coffee per day).

Alcohol and Other Drugs Questionnaire

The Study incorporated the three alcohol consumption items from the Alcohol Use Disorders Identification Test-consumption (AUDIT-C), into the alcohol and other drugs questionnaire. These are also the first three out of 10 questions in the full AUDIT questionnaire.

The AUDIT-C has demonstrated comparable accuracy to the full AUDIT questionnaire.[629] AUDIT and AUDIT-C are well validated screening instruments for hazardous alcohol intake and identification of binge drinking. Both tests have a high degree of internal consistency and test and retest reliability, including monthly recall.[629] Alcohol intake was coded to reflect the quantity of alcohol consumed in the preceding month which was converted into daily alcohol consumption measured in grams.

The Hep573 Study also incorporated daily tobacco consumption and recreational drug use in the preceding month. Tobacco consumption was recorded as part of the alcohol and other drugs questionnaire which was administered on a

monthly basis as tobacco may have interfered with the antioxidant action of the herbal treatments. In addition, other recreational drug use was recorded monthly.

CHAPTER 4

RESULTS

One hundred and eighteen participants (80 males, 38 females) were recruited into a randomised, double-blind, placebo-controlled, clinical trial in three New South Wales teaching hospitals from July 2003–March 2006. The three treatment groups were: placebo (P, N=39), silymarin (S, N=40) and silymarin with antioxidants (SOX, N=39).

STUDY POPULATION

According to CONSORT Guidelines,[630] the numbers screened for the Hep573 Study were: (N=190), enrolled in the Study (N=118), and randomised to each treatment arm (placebo, N=39, silymarin, N=40, and silymarin with antioxidant, N=39). Four of the 118 participants who were randomised did not enter the Study because of the following: work commitments (silymarin, N=1, placebo, N=1), surgery required (silymarin with antioxidant (SOX), N=1), and ingestion of vitamins included in the trial preparations (SOX, N=1); hence, N=114 entered the Study.

Figure 4.1 provides a flowchart of the Hep573 Study participants from randomisation. Each of the boxes in Figure 4.1 report on the reasons given and the numbers lost in each treatment group at each of the major time-points. They appear in the preceding timeline box as follows: those lost between randomisation and baseline appear in randomisation, between baseline and Week 12 (in baseline), between Weeks 12 and 24 (in Week 12), between Weeks 24 and 48 (in Week 24).
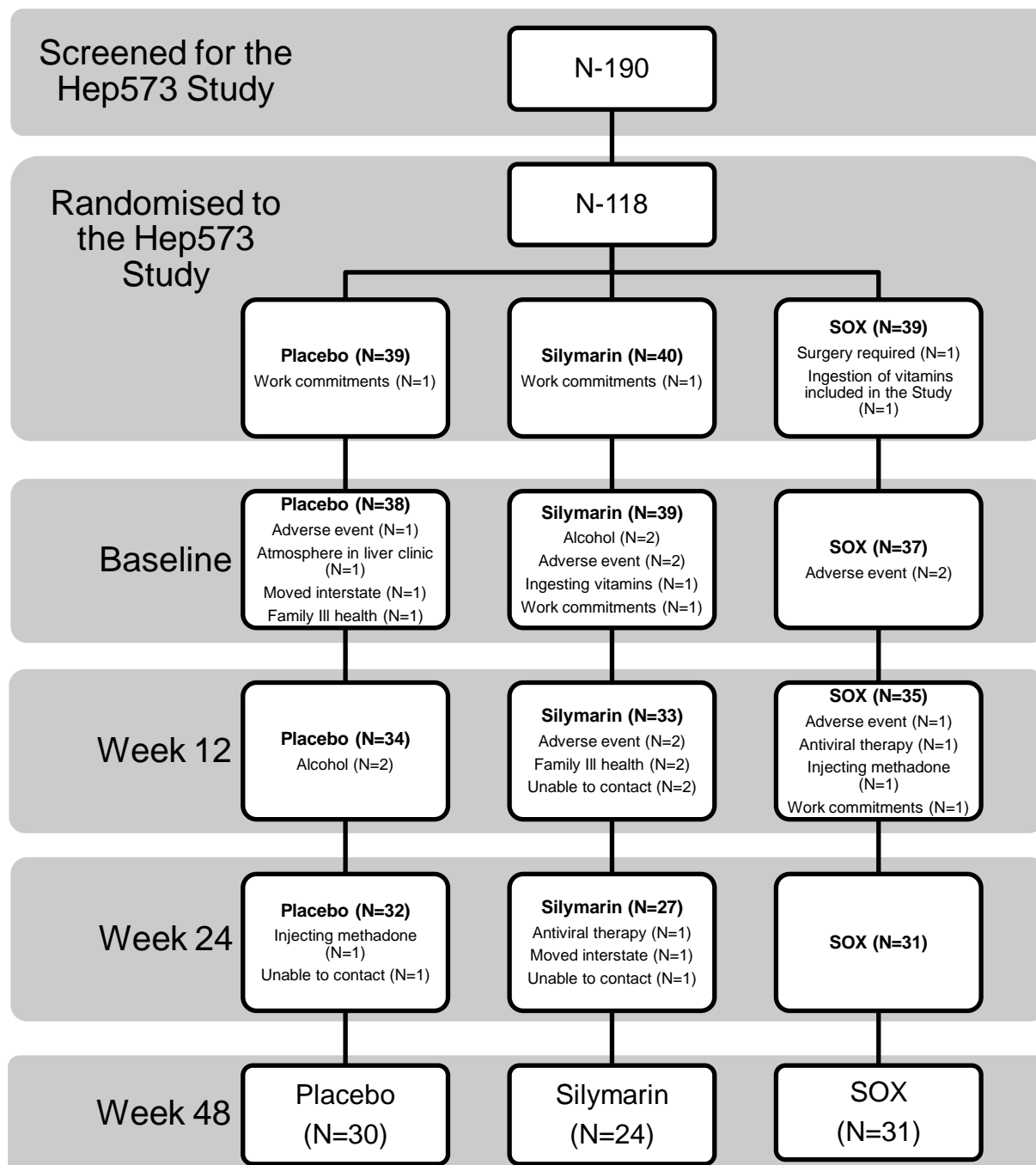
Figure 4.1: Hep573 Study participant flowchart

While 102 out of 118 (86%) participants completed 12 weeks of active treatment, 90/118 (76%) completed the full 24 weeks of active treatment, and 85/118 (72%) completed the 24 weeks follow-up post-treatment. The largest drop-out rate occurred in the silymarin group, with 13/40 (33%) failing to complete 24

weeks of treatment. Once the participants reached 24 weeks, the retention rates were stable.

Twenty-four out of 114 participants (21%) who entered the Study stopped therapy during the treatment phase. On an intention-to-treat basis, when the four participants who were randomised but did not enter the Study are added to 24 participants (28/118), there was a 23.7% drop-out rate amongst the participants during the 24-week treatment phase of the Study.

Only five participants who entered the Study and completed up to 24 weeks of treatment did not complete follow-up (N=2, placebo, N=3 silymarin). Twenty-nine out of 114 participants failed to complete treatment or follow-up (25.4%) compared to 33/118 (27.9%) of all randomised participants.

The initial characteristics of the Study population: age, gender, HCV genotype, duration of infection (years), ALT, AST, GGT, ALP, albumin, bilirubin, globulins, F_2-isoprostanes, HCV RNA viral copies, Hepascore, hyaluronic acid, FibroTest, alpha-2 macroglobulin, haptoglobin, lipid profile including apolipoprotein A1, BMI, ferritin, platelets, prothrombin time, INR, TSH, WCC are shown in Table 4.1 by the treatment group along with the median and interquartile ranges.

Table 4.1: Initial characteristics by treatment group (median and interquartile ranges).

Variable	Placebo	Silymarin	SOX	*P*-value
Participant Numbers (N)	39	40	39	-
Age	47 (37-53)	48 (42-52)	50 (44-54)	*P*=0.337
Gender (M,F)	(30,9)	(23,17)	(27,12)	
Genotype (n)	1,4,6 (26) 2,3 (13)	1,4,6 (25) 2,3 (15)	1,4,6 (31) 2,3 (8)	*P*=0.234
Duration of infection	21.0 (12.5-26)	25.5 (20-30)	23 (20-29.5)	*P*=0.011
ALT	87 U/L (56-128)	100 U/L (60-133)	76 U/L (57-112)	*P*=0.372
AST	50 U/L (40-79)	65 U/L (45–88)	50 U/L (43–69)	*P*=0.217
GGT	42 U/L (23-64)	58 U/L (36-98)	45 U/L (22-79)	*P*=0.150
ALP	74 U/L (59–85)	74 U/L (66–95)	73 U/L (63–95)	*P*=0.638
Albumin	43 g/L (40–45)	41 g/L (39–44)	43 g/L (41–44)	*P*=0.491
Bilirubin	13 umol/ L (10-17)	10 umol/L (8-17)	12 umol/L (9-18)	*P*=0.361
Globulins	33 g/L (29–37)	37 g/L (32–40)	33 g/L (31–40)	*P*=0.014
F_2-isoprostanes (ISO)	2118 pmol/L (1605-2698)	2410 pmol/L (1829-3646)	2141 pmol/L (1768-2696)	*P*=0.262

Table 4.1: Initial characteristics by treatment group (median and interquartile ranges).

Variable	Placebo	Silymarin	SOX	P-value
HCV RNA 10^5	32.1 bDNA copies/ml (10.6–83.6)	79.3 copies/ml (30.4–132.2)	46.8 copies/ml (24.0-117.7)	P=0.047
Hepascore	0.52 (0.32–0.84)	0.74 (0.31–0.88)	0.52 (0.29–0.90)	P=0.688
Hyaluronic acid (HA)	30 ug/L (18–51)	43 ug/L (19–85)	30 ug/L (14–104)	P=0.451
Fibrotest (FT)	0.54 (0.26–0.71)	0.64 (0.36–0.85)	0.55 (0.38–0.78)	P=0.455
Alpha-2 macroglobulin	3.02 (2.22–3.79)	3.88 (2.48–4.34)	2.87 (2.47–4.10)	P=0.247
Haptoglobin	0.98 (0.84–1.18)	1.14 (0.60–1.48)	0.94 (0.48–1.26)	P=0.399
Apolipoprotein A1 (ApoA1)	1.40 (1.24–1.62)	1.37 (1.20–1.53)	1.36 (1.19–1.47)	P=0.567
Triglycerides	0.83 mmol/L (.57–1.12)	1.00 mmol/L (.70–1.20)	0.97 mmol/L (.79–1.30)	P=0.087
Total cholesterol	4.3 mmol/L (3.6–4.7)	3.9 mmol/L (3.3–4.7)	4.6 mmol/L (3.9–5.5)	P=0.066
HDL cholesterol	1.30 mmol/L (1.04–1.78)	1.27 mmol/L (1.07–1.52)	1.26 mmol/L (1.10–1.60)	P=0.626
LDL cholesterol	2.31 mmol/L (1.92–2.82)	2.05 mmol/L (1.45–3.15)	2.90 mmol/L (2.16–3.69)	P=0.023
BMI	25.7 (22.8–28.0)	27.6 (23.1–32.9)	28.2 (23.7–30.7)	P=0.198
Ferritin	131.3 ug/L (78.0–324.2)	124.6 ug/L (80.5–352.0)	128.7 ug/L 82.5–215.8)	P=0.707
Platelets	202 10^9/L (170–249)	201 10^9/L (158–261)	221 10^9/L (165–269)	P=0.703
Prothrombin time	12 secs (11.0–13.3)	12 secs (11.0–14.0)	12 secs (11.0–13.2)	P=0.982
INR	1.0 (0.9–1.1)	1.0 (0.9–1.1)	1.0 (0.9–1.1)	P=0.621
Thyroid stimulating hormone (TSH)	1.265 mIU/L (.925–1.730)	1.480 mIU/L (.960–2.020)	1.110 mIU/L (.820–1.790)	P=0.498
WCC	6.0 10^9/L (5.0–7.7)	5.9 10^9/L (4.9–8.0)	6.0 10^9/L (5.1–7.3)	P=0.934

The initial characteristics were similar between the three treatment groups. The average age of the participants was 48.3 years and the average duration of infection was 23.2 years. The silymarin group had a longer duration of infection (P=0.01), higher globulins (P=0.014) and a higher HCV RNA viral load (P=0.047) compared to the placebo and SOX groups. The silymarin and antioxidant (SOX) group had higher LDL cholesterol (P=0.023) compared to the placebo or silymarin groups.

General Information on the Study Population

While 48% (57/118) of the Hep573 Study participants had used complementary medicine for hepatitis C prior to trial entry, 52% (61/118) had never tried complementary medicine.

Forty-one per cent (48/118) of the Study population had previously received interferon with/without ribavirin, and 59% (70/118) were treatment-naïve.

The primary sources of HCV infection were injecting drug use (58%) and blood transfusion (20%).

Fifteen per cent (18/118) of the Study population were cirrhotic (as determined by clinical examination, blood tests, ultrasound or biopsy) and 85% (100/118) were non-cirrhotic. Twenty-eight per cent (33/118) were regular cannabis users and 72% (85/118) were not. Forty-two per cent (50/118) were tobacco smokers and 58% (68/118) were ex- or non-smokers.

Dose Compliance

The compliance ratios within each group demonstrated some departure from normality. A comparison of the compliance rates using a Kruskal-Wallis non parametric test[631] one way analysis of variance showed there was no significant difference between the groups ($P= 0.497$).

The Mann-Whitney test[632] was used for pair-wise comparisons of compliance rates: placebo was compared to silymarin ($P=0.272$), and to silymarin and antioxidant (SOX) group ($P=0.341$), and silymarin was compared to silymarin with antioxidant ($P= 0.956$).

The standard recommendation for encouraging compliance with pegylated interferon and ribavirin therapy is the 80% rule: participants who take 80% of the dose, 80% of the time achieve an optimal outcome.[633] The Study data sets were examined to see how well they matched with the 80% compliance recommendation.

Table 4.2 shows the cross tabulation of treatment compliance.

Table 4.2: Cross tabulation of treatment compliance >80%.

| | | | Compliance >80% | | |
			Noncompliant	Compliant	Total
Treatment	Placebo	Count	1	34	35
		% within Treatment	2.9%	97.1%	100.0%
	Silymarin	Count	5	31	36
		% within Treatment	13.9%	86.1%	100.0%
	SOX	Count	6	31	37
		% within Treatment	16.2%	83.8%	100.0%
Total		Count	12	96	108
		% within Treatment	11.1%	88.9%	100.0%

	Value	Monte Carlo Sig. (2-sided) Sig.
Fisher's Exact Test	3.906	.145

From Table 4.2, it can be seen there is no evidence of a difference in compliance across the three treatment groups (P=0.145) (Fisher's exact test). The Table also shows that the compliance rate of the Study population in per-protocol analysis (96/108) was 88.9% and above the 80% recommendation for interferon/ribavirin. When an intention-to-treat analysis was applied (96/118), a dose compliance rate of 81.4% was obtained.

PRIMARY OUTCOME

The primary outcome measure was the proportion of participants experiencing ALT normalisation from baseline to Week 24.

ALT Normalisation From Baseline to Week 24

The proportion of participants experiencing ALT normalisation from baseline to Week 24 differed significantly by treatment (P=0.002) (Fisher's exact test) in the intention-to-treat analysis as shown in Table 4.3.

Table 4.3: ALT normalisation from baseline at Week 24 (intention-to-treat analysis).

ALT normalisation	Placebo	Silymarin	SOX	*P* value
N	2/39	1/40	10/39	0.002
%	5% *	2.5% **	26%	

The ALT level in the SOX group (10/39) (26%) normalised in significantly more participants compared to (2/39) (5%*) on placebo (P=0.02) and (1/40)

138

(2.5%**) on silymarin alone (*P*=0.003).

Table 4.4 shows the individuals who achieved ALT normalisation at Week 24 in the SOX Group.

Table 4.4: ALT normalisation from baseline at Week 24 in the SOX Group

Participant ID	Week 0 ALT U/L	Week 24 ALT U/L	Percentage fall in ALT
106	89	43	-51.7%
114	53	42	-20.7%
129	58	27	-53.4%
201	109	55	-49.5%
214	150	39	-74.0%
227	73	48	-34.2%
319	72	31	-56.9%
329	51	34	-33.3%
332	45	32	-28.9%
347	58	40	-31.0%
Total N=10			Average % fall In ALT= -43.4%

There was a 43.4% reduction in the ALT by Week 24 in those participants in the SOX group who normalised their ALT.

Nine out of 10 of those who had ALT normalisation in the SOX group had HCV genotype 1. There was no significant difference between treatment and ALT normalisation rates in the non genotype 1 group (*P*=0.440) compared to significant differences in the ALT normalisation rates amongst genotype 1 groups (*P*=0.008) (Fisher's exact test).

Table 4.5 shows ALT normalisation from Weeks 0-24 per-protocol analysis.

Table 4.5: ALT normalisation from baseline at Week 24 per-protocol analysis.

ALT normalisation	Placebo	Silymarin	SOX	*P* value
N	2/32	1/27	10/31	0.003
%	6.25%	3.7%	32.25%	

The proportion of participants with ALT normalisation from Weeks 0-24 differed significantly by treatment (*P*=0.003) (Fisher's exact test) in per-protocol analysis. The ALT normalisation rates were comparable in the placebo and silymarin groups (4-6%) and significantly higher in the SOX group (32%) (*P*<0.05).

If the current normal values for ALT of ≤30U/L for men and ≤19U/L for women are utilised,[413,634] none of the participants in the Study normalised. If upper limits for ALT of ≤40U/L for men and ≤30 U/L for women are used,[413,634] then 4/39 in the SOX group and 1/40 in the silymarin group normalised as shown in Table 4.6 and Table 4.7.

Table 4.6: Normalisation rate with revised ALT range (≤ 40UL for male and ≤30 for female) from baseline at Week 24 (intention-to-treat analysis).

ALT normalisation	Placebo	Silymarin	SOX	*P* value
N	0/39	1/40	4/39	0.081
%		2.5%	10.3%	

The proportion of participants with ALT normalisation levels ((≤ 40UL for male and ≤30 for female) did not differ significantly by treatment (*P*=0.081).

Table 4.7: Normalisation rate with revised ALT range (≤ 40UL for male and ≤30 for female) from baseline at Week 24 (per protocol analysis).

ALT normalisation	Placebo	Silymarin	SOX	*P* value
N	0/32	1/27	4/31	0.053
%		3.7%	12.9%	

The proportion of participants with ALT normalisation (≤ 40UL for male and ≤30 for female) did not differ significantly by treatment (*P*=0.053). However there was a marginally significant difference in the proportion with ALT normalisation in the SOX group (13%) compared to none in the placebo group *(P*=0.053) (Fisher's exact test).

SECONDARY OUTCOMES WEEKS 0-24

The secondary outcome measures were the percentage change in ALT, F_2-isoprostanes (ISO), HCV RNA, Fibrotest, Hepascore and hyaluronic acid from baseline at Week 24.

Table 4.8: Percentage change from baseline in ALT, F_2-isoprostanes, HCV RNA, FibroTest at Week 24 together with 95% Confidence interval (CI), *P*-value and overall test of homogeneity.

Outcome Measure	Treatment group	Percentage change from baseline	95% confidence interval (CI)	*P*-value	Homogeneity (Interaction) *P*-value
ALT	Placebo	-5.0	(-18.0, +9.9)	0.490	0.113
	Silymarin	+8.6	(-7.0, +26.6)	0.291	
	SOX	-13.1	(-25.0, +0.3)	0.055	
ISO	Placebo	-7.0	(-14.5, +0.4)	0.066	0.071
	Silymarin	+2.0	(-10.0, +7.0)	0.666	
	SOX	-12.0	(-18.4, -4.3)	0.003	
HCV RNA	Placebo	+13.0	(-21.3, +63.1)	0.504	0.542
	Silymarin	-9.0	(-37.1, +32.5)	0.633	
	SOX	+21.0	(-15.5, +73.6)	0.300	
FibroTest	Placebo	+19.7	(-3.9, +43.4)	0.105	0.674
	Silymarin	+6.4	(-15.0, +27.8)	0.560	
	SOX	+5.4	(-16.2,+26.9)	0.626	

In Table 4.8, a negative percentage change implies a decrease and a positive percentage change denotes an increase in relevant outcome measure. The *P*-value column relates to the percentage change from baseline in the outcome measure of the listed treatment group. The homogeneity *P*-value compares the three treatment groups and was calculated using ANOVA.

Figure 4.2 shows the percentage change in ALT from baseline at Week 24 and corresponding *P*-value. There was no statistically significant interaction between treatment and the within participant change over time in ALT ($P=0.113$). However, there was a marginally significant fall in ALT in the SOX group ($P=0.055$), but not in the placebo group ($P=0.490$). There was an increase in ALT in the silymarin group ($P=0.291$).
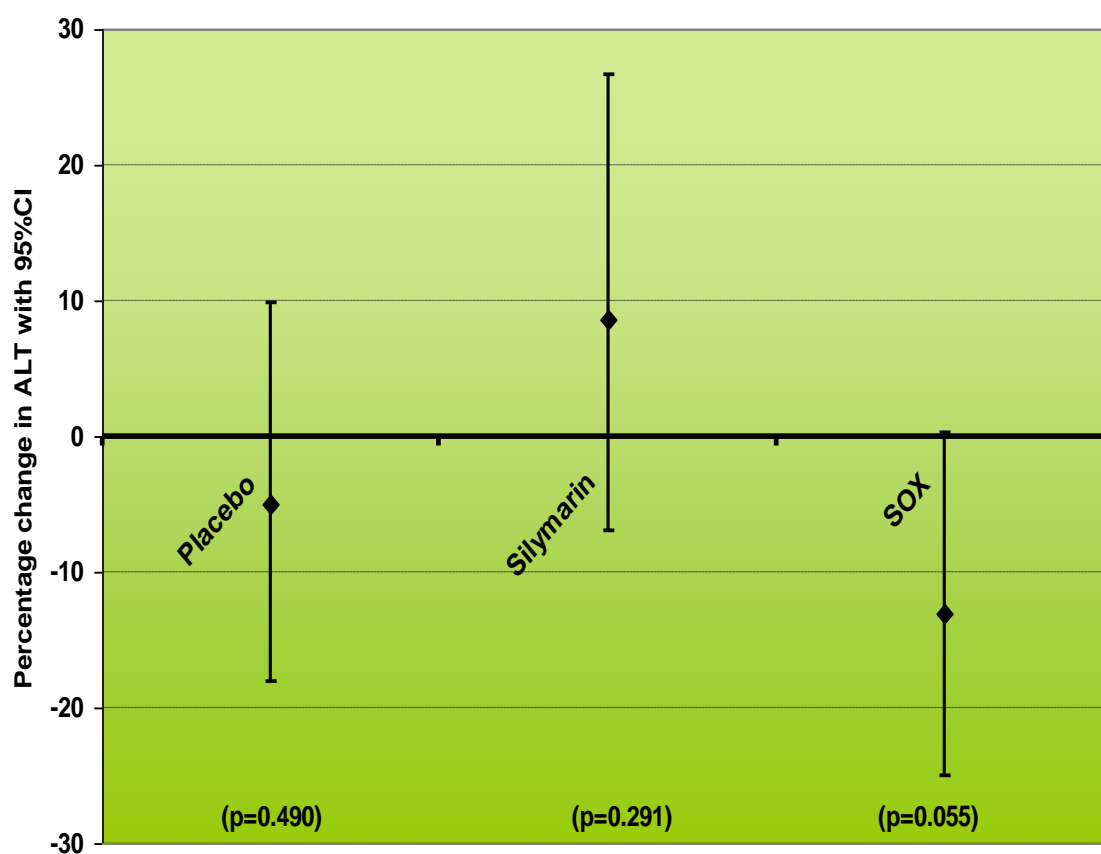
Figure 4.2: Percentage change in ALT from baseline at Week 24 and corresponding *P*-value.

F_2-Isoprostanes

F_2-isoprostanes (ISO) values were similar in all three groups at baseline ($P=0.262$). They were measured twice during the treatment period, i.e., at Weeks 12 and 24, but not in the 24-week, follow-up period. There was no statistically

significant interaction between treatment and the within participant change over time in log (ISO) ($P=0.071$). There was a statistically significant fall in F_2-isoprostanes (ISO) in the SOX group ($P=0.003$) but not in the placebo ($P=0.066$) or silymarin groups ($P=0.666$) (Figure 4.3).
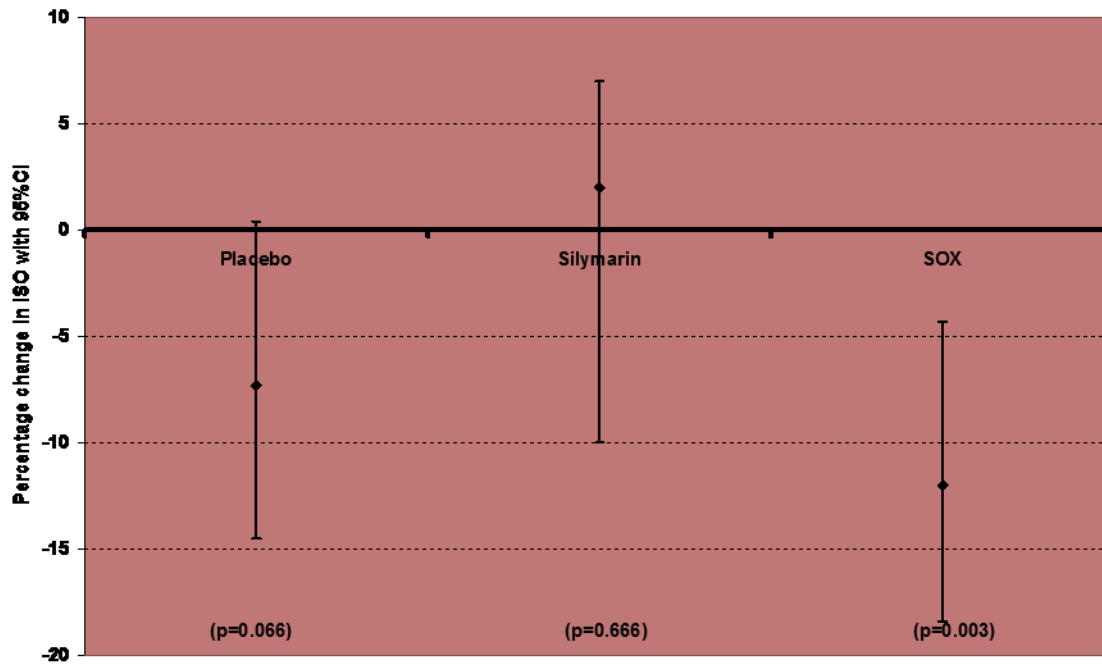
Figure 4.3: Percentage change in F_2-isoprostanes from baseline at Week 24 and corresponding *P*-value.

Viral Load (HCV RNA Quantitative Test)

The HCV viral load at baseline was almost twice as high in the silymarin group compared to the placebo or the SOX group. There was no significant interaction between treatment and the within participant change over time in log HCV RNA ($P=0.542$). In an alternative approach, analysis of covariance (ANCOVA) of the logHCV RNA viral load at Week 24 showed no significant differences between the treatment groups ($P=0.454$) after adjusting for logHCV RNA viral load at baseline.

There was a 13% increase in HCV RNA at Week 24 in the placebo group ($P=0.504$), a 9% fall in HCV RNA in the silymarin group ($P=0.633$), and a 21% increase in HCV RNA at Week 24 in the SOX group compared to baseline ($P=0.300$) (Figure 4.4).
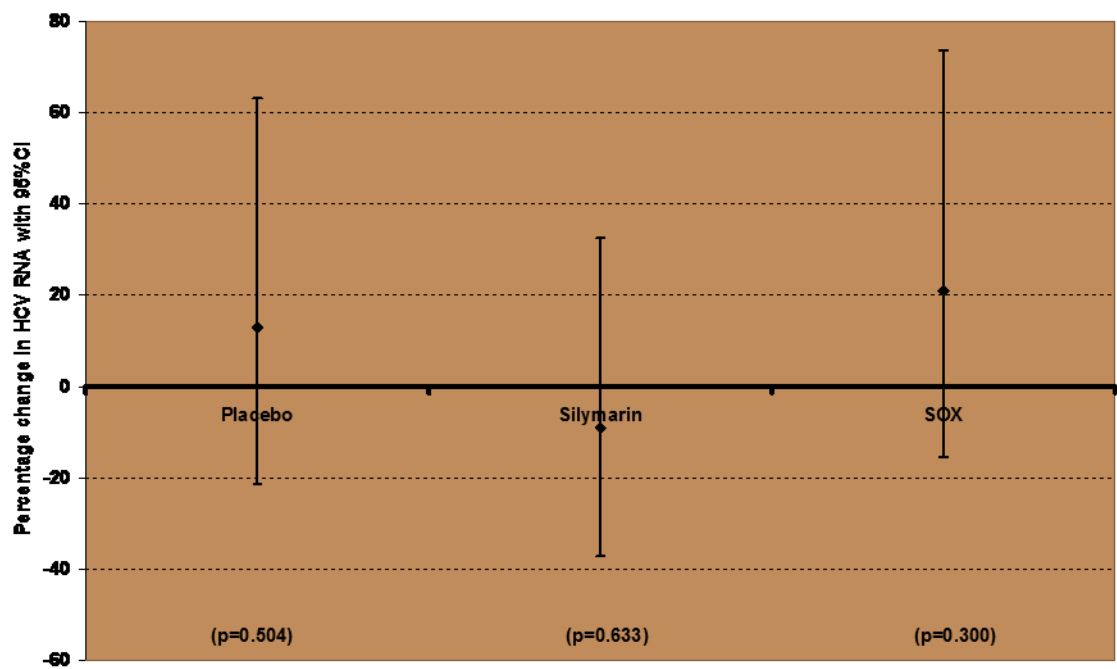
Figure 4.4: Percentage change in HCV RNA from baseline at Week 24 and corresponding *P*-value.

FibroTest

There was no statistically significant interaction between treatment and the within participant change over time in FibroTest (*P*=0.674) (Figure 4.5).
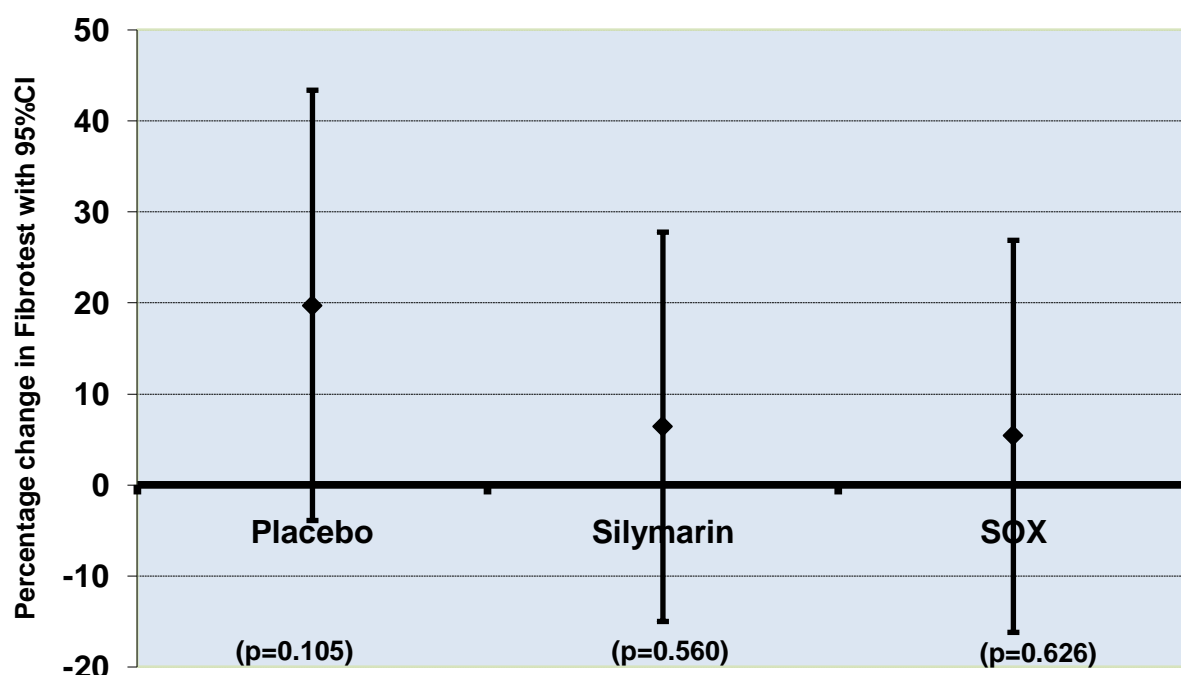
Figure 4.5: Percentage change in FibroTest from baseline to Week 24 and corresponding *P*-value.

Table 4.9: Percentage change from baseline in LFTs at Week 24 together with 95% confidence interval (CI), *P*-value and overall test of homogeneity.

Outcome measure	Treatment group	Percentage change from baseline	95% confidence interval (CI)	*P*-value	Homogeneity (Interaction) *P*-value
ALB	Placebo	+0.6	(-1.3, +2.5)	0.552	0.403
	Silymarin	-1.2	(-3.2, +0.9)	0.263	
	SOX	+0.5	(-0.1, +2.4)	0.645	
ALP	Placebo	-5.5	(-9.8, -0.9)	0.019	0.113
	Silymarin	-5.0	(-9.2, +0.3)	0.066	
	SOX	-11.0	(-15.0, -6.5)	<0.0001	
AST	Placebo	-3.7	(-14.6, +8.6)	0.536	0.078
	Silymarin	+10.2	(-3.0, +25.1)	0.135	
	SOX	-10.0	(-19.7, +1.8)	0.096	
BIL	Placebo	-1.5	(-11.3, +9.3)	0.772	0.148
	Silymarin	+4.0	(-7.0, +16.3)	0.492	
	SOX	-10.4	(-19.2, -0.6)	0.038	
GGT	Placebo	+9.1	(-3.1,+22.7)	0.149	0.152
	Silymarin	+3.4	(-8.7,+17.0)	0.601	
	SOX	-7.1	(-17.3,+4.3)	0.211	

The data in Table 4.9 show there was no statistically significant interaction between treatment and the within participant change over time in any of the above liver enzymes, ALP (*P*=0.113), AST (*P*=0.078) and GGT (*P*=0.152) or markers of liver function, ALB (*P*=0.403) and BIL (*P*=0.148). However, there were significant reductions in ALP in the placebo group (5.5%, *P*=0.019) and in the SOX group

144

(11%, P=0.0001). The reduction in the silymarin group (5%) was comparable to the placebo group reduction and did not reach statistical significance (P=0.066). In addition, there was a significant reduction in bilirubin, (10.4%) in the SOX group (P=0.038), which was not evident in the other two groups.

Correlations Between Secondary Outcome Measures

Spearman's Rank Correlation was used to quantify the extent of association between within participant changes in the secondary outcome measures, in ALT, HCV RNA or FibroTest and the within participant change in F_2-isoprostanes from Weeks 0-24.

The Spearman's Rank Correlation was used to overcome some extreme values or outliers. Relative changes were also examined and, as similar results to the absolute values were obtained, the absolute values are shown. The two significant correlations appear in Figure 4.6 and Figure 4.7.
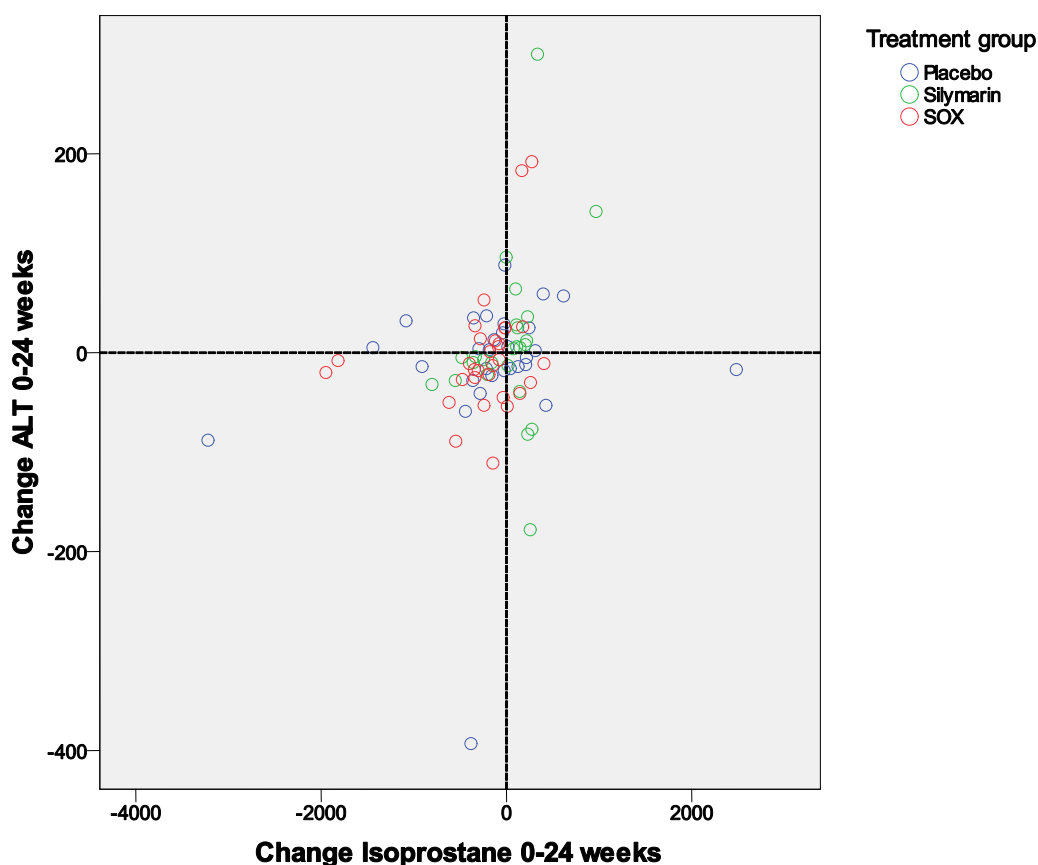
Figure 4.6: Scatterplot of the within participant change in ALT versus the within participant change in F_2-isoprostanes from Week 0 at Week 24.

The dotted lines represent the area of no change between the variables.

145

The within participant change in ALT is weakly positively associated with the change in F_2-isoprostanes from Week 0 at Week 24 (Spearman's rho =0.285, P=0.008) (Spearman's Rank Correlation).
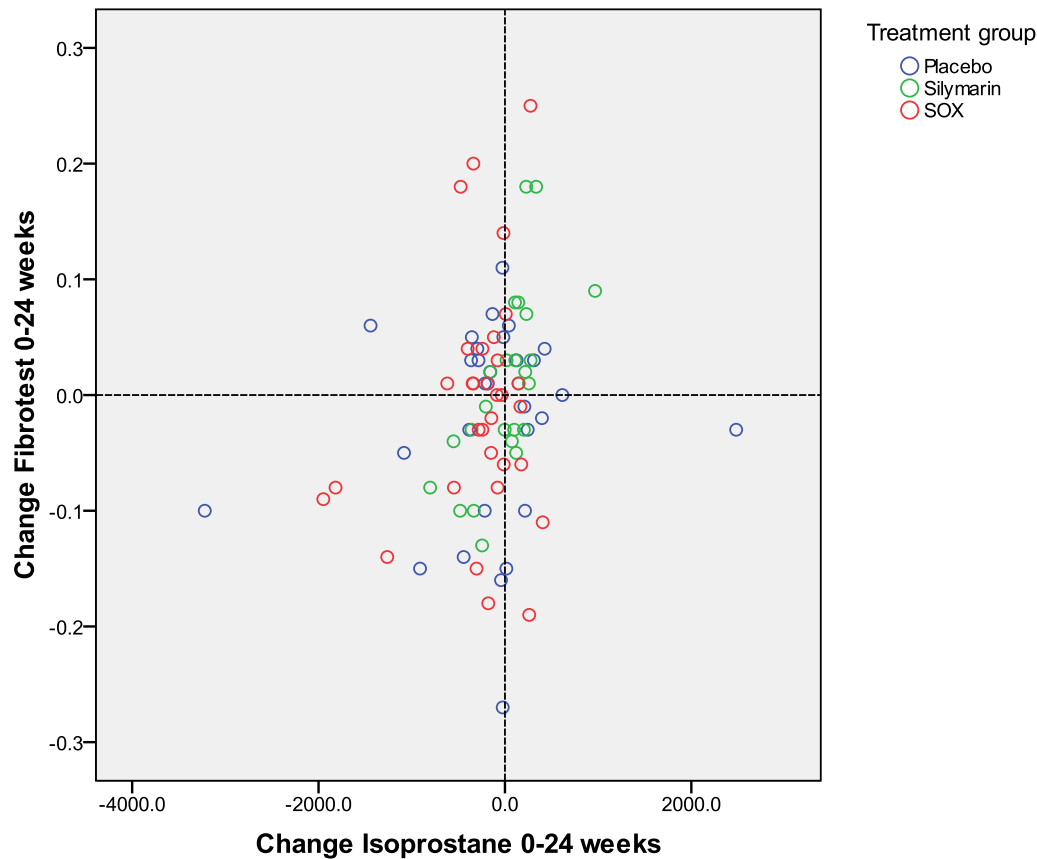
Figure 4.7: Scatterplot of the within participant change in FibroTest versus the within participant change in F_2-isoprostanes from Week 0 at Week 24.

The within participant change in FibroTest is weakly positively associated with the change in F_2-isoprostanes from Week 0 to Week 24 (Spearman's rho =0.252, P=0.019) (Spearman's Rank Correlation).

SECONDARY OUTCOMES WEEKS 24-48

The secondary outcome measures were the percentage change in ALT, HCV RNA, Fibrotest, liver function tests (LFTs) Hepascore and hyaluronic acid from Week 24 at Week 48.

Table 4.10: Percentage change in ALT, HCV RNA and FibroTest from Week 24 at Week 48 together with 95% confidence interval (CI), *P*-value and overall test of homogeneity.

Outcome measure	Treatment group	Percentage change from baseline	95% confidence interval (CI)	*P*-value	Homogeneity (Interaction) *P*-value
ALT	Placebo	+5.0	(-10.4, +23.0)	0.549	0.810
	Silymarin	+11.8	(-4.4, +30.7)	0.162	
	SOX	+12.3	(-5.5, +33.4)	0.189	
HCV RNA	Placebo	+2.3	(-37.1, +66.4)	0.926	0.100
	Silymarin	-35.1	(-61.4, +9.3)	0.108	
	SOX	-28.0	(-55.2, +15.7)	0.178	
FibroTest	Placebo	8.1	(-20.9, +37.1)	0.587	0.906
	Silymarin	5.3	(-22.9, +33.6)	0.713	
	SOX	0.8	(-26.9, +28.5)	0.954	

In the follow-up period from Weeks 24-48, irrespective of group, the ALT increased over time (*P*=0.051). On average, there was a 9.6% increase in ALT in the six-month follow-up period. There was no evidence that the ALT at Week 48 differed significantly between the groups (*P*=0.810) contrary to the finding at the end of treatment at Week 24.

There was no significant difference in the HCV RNA levels across the three treatment groups at Week 48 (*P*=0.100) (Figure 4.8).
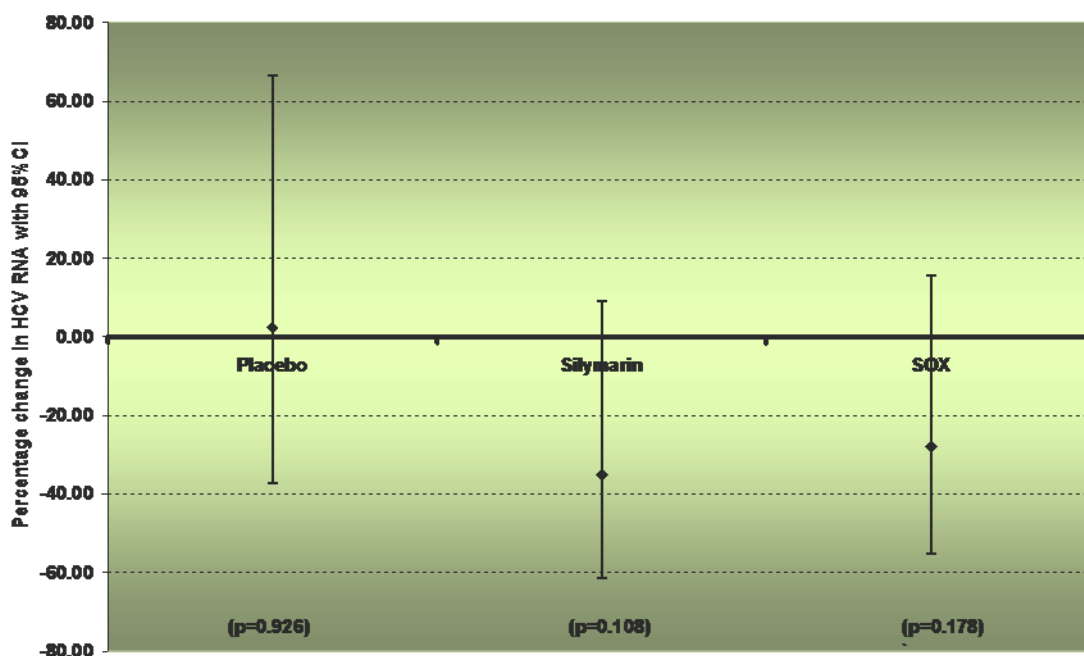
Figure 4.8: Percentage change in HCV RNA in the follow-up (Weeks 24-48 inclusive) and corresponding *P*-value.

While there was a small increase in the FibroTest scores in each of the groups to Week 48, there was no significant interaction between treatment group

and the within participant change over time in the FibroTest score in the follow-up period (*P*=0.906).

Table 4.11: Percentage change in LFTs from Week 24 to Week 48 together with 95% confidence interval (CI), *P*-value and overall test of homogeneity.

Outcome measure	Treatment group	Percentage change from baseline	95% confidence interval (CI)	*P*-value	Homogeneity (Interaction) *P*-value
ALB	Placebo	+0.2	(-1.8,+2.3)	0.847	0.238
	Silymarin	+0.4	(-1.7,+2.7)	0.693	
	SOX	-1.8	(-3.8, 0.2)	0.074	
ALP	Placebo	+6.7	(+1.5,+12.3)	0.011	0.996
	Silymarin	+6.8	(+1.2,+12.8)	0.017	
	SOX	+7.0	(+1.8, +12.6)	0.008	
AST	Placebo	+8.6	(-6.3,+25.9)	0.271	0.926
	Silymarin	+12.8	(-3.7,+32.2)	0.137	
	SOX	+8.5	(-6.4,+25.9)	0.279	
BIL	Placebo	+13.0	(-0.5,+28.2)	0.061	0.277
	Silymarin	+20.0	(+4.4, 37.0)	0.010	
	SOX	+3.0	(-9.4,+17.0)	0.658	
GGT	Placebo	+8.6	(-5.5,+25.0)	0.245	0.898
	Silymarin	+7.6	(-7.4,+25.1)	0.339	
	SOX	+12.6	(-2.0,+29.4)	0.096	

Table 4.11, shows there were no significant interactions between treatment and the within participant change over time in the follow-up period (Weeks 24-48 inclusive) in liver enzymes: ALP (*P*=0.996), AST (*P*=0.926) and GGT (*P*=0.898) and in the markers of liver function: ALB (*P*=0.238) and BIL (*P*=0.277). However, on average, there was a 6.8% increase in ALP levels in all the treatment groups, placebo (*P*=0.011), silymarin (*P*=0.017) and SOX (*P*=0.008). There was a small non-significant increase in bilirubin in the SOX group (3%, *P*=0.658) and placebo group (13%, *P*=0.061) with a significant increase in bilirubin in the silymarin group (20%, *P*=0.010) in the follow-up period. (LMEs)

SECONDARY OUTCOMES WEEKS 0-48

The secondary outcome measures were the percentage change in ALT, HCV RNA, Fibrotest, Hepascore and hyaluronic acid from baseline at Week 48.

Table 4.12: Percentage change in ALT, HCV RNA and FibroTest from baseline at Week 48 together with 95% confidence interval (CI), *P*-value and overall test of homogeneity.

Outcome Measure	Treatment group	Percentage change from baseline	95% confidence interval (CI)	*P*-value	Homogeneity (Interaction) *P*-value
ALT	Placebo	-7.00	(-23.4, +13.0)	0.468	0.408
	Silymarin	+11.7	(-8.8, +36.8)	0.289	
	SOX	-4.0	(-20.5, +16.8)	0.705	
HCV RNA	Placebo	+12.2	(-35.1, +93.8)	0.682	0.281
	Silymarin	-41.0	(-66.9, +5.46)	0.079	
	SOX	-8.0	(-46.3, +57.8)	0.764	
FibroTest	Placebo	+30.2	(-3.8, +64.1)	0.085	0.419
	Silymarin	+3.1	(-30.0, +36.2)	0.855	
	SOX	+0.1	(-30.8, +31.1)	0.993	

At Week 48, there was a 12% increase in HCV RNA in the placebo group (*P*=0.682), an 8% decrease in HCV RNA in the SOX group (*P*=0.764) and a 41% decrease in HCV RNA in the silymarin group (*P*=0.079) compared to their baseline scores. One participant in the silymarin group had a two log drop in HCV RNA at Week 48 compared to their baseline score. Another participant in the silymarin with antioxidant group had a three log drop in their HCV RNA at Week 48 compared to their baseline score.

In the placebo group, there was one participant who cleared the virus, and one with a three log increase in HCV RNA at Week 48 compared to baseline.

There was no significant interaction over time in the FibroTest score in any of the three treatment groups (*P*=0.419) at Week 48 compared to baseline.

Table 4.13: Percentage change in LFTs from baseline at Week 48 together with 95% confidence interval (CI), *P*-value and overall test of homogeneity.

Outcome measure	Treatment group	Percentage change from baseline	95% confidence interval (CI)	*P*-value	Homogeneity (Interaction) *P*-value
ALB	Placebo	-0.2	(-2.6,+2.3)	0.884	0.429
	Silymarin	-1.6	(-4.0,+0.9)	0.226	
	SOX	+0.8	(-1.7,+3.3)	0.539	
ALP	Placebo	+2.7	(-3.4,+9.1)	0.400	0.444
	Silymarin	+2.7	(-3.5,+9.4)	0.403	
	SOX	-2.3	(-8.1,+4.0)	0.469	
AST	Placebo	+0.3	(-16.2,+20.2)	0.970	0.314
	Silymarin	+18.9	(-1.3,+43.1)	0.071	
	SOX	-1.1	(-17.9,+19.1)	0.909	
BIL	Placebo	-2.0	(-14.5,+12.3)	0.769	0.285
	Silymarin	+9.4	(-5.0,+25.8)	0.215	
	SOX	-6.5	(-18.6,+7.4)	0.345	

Table 4.13: Percentage change in LFTs from baseline at Week 48 together with 95% confidence interval (CI), *P*-value and overall test of homogeneity.

Outcome measure	Treatment group	Percentage change from baseline	95% confidence interval (CI)	*P*-value	Homogeneity (Interaction) *P*-value
GGT	Placebo	+7.9	(-8.7,+27.5)	0.374	0.447
	Silymarin	+15.9	(-2.4,+37.8)	0.096	
	SOX	-0.9	(1114.1, -91.9)	0.915	

From Table 4.13, it can be seen there was no significant interaction between treatment and the within participant change from Week 0 at Week 48 in liver enzymes: ALP (*P*=0.444), AST (*P*=0.314), GGT (*P*=0.447) and markers of liver function: ALB (*P*=0.429) and BIL (*P*=0.285). However, in the silymarin group, there was a trend towards an elevated AST (18.9% increase, *P*=0.071) from Week 0 at Week 48.

SECONDARY OUTCOMES

Hepascore

There was no significant interaction between the within participant differences over time in the Hepascore model (age, sex, GGT, bilirubin, hyaluronic acid and alpha-2 macroglobulin) using the McNemar test at Weeks 24 and 48 compared to Week 0.

Hyaluronic Acid

The Kruskall-Wallis Test[631] of non-parametric independent samples for hyaluronic acid at two groups (<60 ug/L and >60 ug/L) and four groups according to the McHutchison Study[436] showed no change in the mean scores over time and treatment group from baseline at Weeks 24 and 48 compared to baseline.

QUALITY OF LIFE

Hepatitis C Quality of Life Questionnaire (*HQLQ*™)

Table 4.14 to Table 4.16 following show the absolute changes in *HQLQ*™, from baseline to Week 24, from Week 24 to Week 48 and from Week 0 to Week 48.

Table 4.14: Absolute change from baseline at Week 24 in *HQLQ™v1*, together with 95% confidence interval (CI), associated *P-* value and overall test of homogeneity.

SF-36 Outcome	Absolute change	Placebo	Silymarin	SOX	Homogeneity *P*-value
PF	Change	+3.7	+0.4	+1.0	0.129
	CI	(+1.3, +6.0)	(-1.9, +2.8)	(-2.2, +4.3)	
	P-value	0.003	0.715	0.382	
RP	Change	+1.6	+2.1	+0.6	0.869
	CI	(-2.5, +5.7)	(-2.0, +6.3)	(-3.4, +4.6)	
	P-value	0.435	0.314	0.767	
BP	Change	+0.9	+1.0	+0.6	0.982
	CI	(-2. 5, +4.3)	(-2.4, +4.4)	(-2.7, +3.9)	
	P-value	0.593	0.560	0.730	
GH	Change	+0.2	+1.0	+2.6	0.359
	CI	(-2.3, +2.6)	(-1.5, +3.5)	(0.2, +5.0)	
	P-value	0.901	0.416	0.034	
VT	Change	+1.0	+1.6	+5.6	0.112
	CI	(-2.4, +4.3)	(-1.8, +5.0)	(+2.3, +8.9)	
	P-value	0.564	0.351	0.001	
SF	Change	+3.1	-1.8	+2.2	0.142
	CI	(-0.5, +6.7)	(-5.4, +1.9)	(-1.3, +5.8)	
	P-value	0.090	0.346	0.213	
RE	Change	+1.4	-1.1	+4.4	0.261
	CI	(-3.2, +6.1)	(-5.8, +3.6)	(-0.2, +9.0)	
	P-value	0.540	0.652	0.061	
MH	Change	+1.5	-0.5	+3.6	0.137
	CI	(-1.4, +4.4)	(-3.4, +2.4)	(+0.8, +6.5)	
	P-value	0.307	0.736	0.012	
PCS	Change	+1.9	+1.9	+0.0	0.498
	CI	(-0.8, +4.5)	(-0.7, +4.6)	(-2.6, +2.6)	
	P-value	0.161	0.152	0.997	
MCS	Change	+1.3	-1.4	+5.2	0.024
	CI	(-2.0, +4.6)	(-4.8, +2.0)	(+2.0, +8.4)	
	P-value	0.443	0.422	0.002	

Key:
PF   =Physical Functioning scale
RP   =Role Physical scale
BP   =Bodily Pain scale
GH   =General Health scale
VT   =Vitality scale
SF   =Social Functioning scale
RE   =Role Emotional scale
MH   =Mental Health scale
PCS   =Physical Component Summary scale
MCS   =Mental Component Summary scale

The absolute change in MCS score differed significantly by treatment (P=0.024) (LMEs). A positive score represented an improvement in overall mental-health status and a negative score reflected the opposite. There was an absolute change from Week 0 at Week 24 of +5.2 (+2.0, +8.4) in the MCS score in the SOX group (P=0.002) compared to +1.3 (-2.0, +4.6) in the placebo (P=0.443) and -1.4 (-4.8, +2.0) in silymarin (P=0.422) groups. In this Study, mental-health status overall significantly improved in the SOX group.

While there was no significant interaction between treatment and the individual SF-36 domains, there was a significant difference (improvement) in the scores for vitality (*P*=0.001), general health (*P*=0.034) and mental health (*P*=0.012) in the SOX group. There was a trend towards improvement in emotional health (*P*=0.061) in the SOX groups, which was not evident in the placebo or silymarin groups. There was also a significant improvement in the physical functioning (*P*=0.003) in the placebo group, which was not seen in the silymarin or silymarin and antioxidant groups.

Table 4.15: Absolute change from Week 24 at Week 48 in *HQLQ™v1*, together with 95% confidence interval (CI), associated *P*-value and overall test of homogeneity.

SF-36 Outcome	Absolute change	Placebo	Silymarin	SOX	Homogeneity P-value
PF	Change	-3.7	+0.0	-2.0	0.147
	CI	(-6.3, -1.2)	(-2.7, +2.7)	(-4.5, +0.5)	
	P value	0.005	0.993	0.122	
RP	Change	-1.2	-0.5	-2.0	0.888
	CI	(-5.5, +3.1)	(-5.0, +4.0)	(-6.2, +2.2)	
	P value	0.580	0.830	0.350	
BP	Change	-2.0	-0.1	-0.7	0.653
	CI	(-5.0, +0.9)	(-3.2, +3.0)	(-3.5, +2.2)	
	P value	0.174	0.941	0.654	
GH	Change	-0.2	+0.6	-3.8	0.064
	CI	(-3.0, +2.6)	(-2.3, +3.5)	(-6.6, -1.1)	
	P value	0.901	0.416	0.034	
VT	Change	+0.2	-0.9	-3.1	0.278
	CI	(-2.8, +3.2)	(-4.0, +2.2)	(-6.0, -0.2)	
	P value	0.888	0.577	0.038	
SF	Change	-2.3	+2.3	-3.4	0.056
	CI	(5.7, +1.1)	(-1.3, +5.8)	(-6.7, -0.1)	
	P value	0.180	0.208	0.043	
RE	Change	-1.2	-0.5	-2.0	0.888
	CI	(-5.5, +3.1)	(-5.0, +4.0)	(-6.2, +2.2)	
	P value	0.580	0.830	0.350	
MH	Change	-2.3	+2.7	-4.1	0.005
	CI	(-5.1, +0.5)	(-0.3, +5.6)	(-6.9, -1.3)	
	P value	0.111	0.079	0.004	
PCS	Change	-2.5	-0.8	-1.5	0.658
	CI	(-5.0,+0.0)	(-3.5, +1.8)	(-3.9, +1.0)	
	P value	0.051	0.525	0.242	
MCS	Change	+0.5	+2.1	-4.0	0.042
	CI	(-2.9, +3.9)	(-1.5, +5.7)	(-7.3, -0.6)	
	P value	0.787	0.251	0.021	

Key:
PF   =Physical Functioning scale
RP   =Role Physical scale
BP   =Bodily Pain scale
GH   =General Health scale
VT   =Vitality scale
SF   =Social Functioning scale
RE   =Role Emotional scale
MH   =Mental Health scale
PCS   =Physical Component Summary scale, MCS   =Mental Component Summary scale

In the follow-up period from Week 24 (stopping treatment) to Week 48 (end of Study), there were two significant interactions between stopping treatment and an absolute negative change in both the mental health scale (P=0.005) and in the MCS score (P=0.042) from Weeks 24 to 48 in the SOX group, with absolute negative changes of -4.1 in mental health (P=0.004) and -4.0 in the MCS (P=0.021).

There were two marginally significant interactions between treatment cessation and the SF-36 general health scale (P=0.064) and social functioning scale (P=0.056). Significant absolute negative changes in the general health (P=0.034) and in social functioning (P=0.043) occurred in the SOX group and not in the placebo or silymarin groups.

While there was no significant interaction between treatment and physical functioning (PF) from Week 24 at Week 48 (P=0.147), there was a deterioration in physical functioning in the placebo group (P=0.005) which was not evident in the silymarin or SOX group.

There was no significant interaction between treatment and absolute change in vitality from Weeks 24-48 (P=0.278). However, there was a significant fall in vitality in the SOX group (P=0.038) in the follow-up period.

While there was no significant interaction between treatment and change in PCS (P=0.658), there was a marginally significant reduction in PCS in the placebo group (P=0.051), not seen in the silymarin or SOX groups.

Table 4.16: Absolute change from baseline at Week 48 in *HQLQ™v1* together with 95% confidence interval (CI), associated *P-v*alue and overall test of homogeneity.

SF-36 Outcome	Absolute change	Placebo	Silymarin	SOX	Homogeneity P-value
PF	Change	-0.5	+0.5	-1.0	0.728
	CI	(-3.2, -2.2)	(-2.4, -0.5)	(-3.7, -1.0)	
	P value	0.717	0.710	0.449	
RP	Change	+0.0	+1.2	-1.6	0.675
	CI	(-4.4, +4.5)	(-3.4, +5.9)	(-6.0, +2.8)	
	P value	0.984	0.604	0.466	
BP	Change	-1.0	+0.2	+1.2	0.577
	CI	(-3.9, +1.9)	(-2.9, +3.3)	(-1.7, +4.0)	
	P value	0.494	0.888	0.428	
GH	Change	-0.4	+1.2	-1.4	0.481
	CI	(-3.3, +2.5)	(-1.9, +4.3)	(-4.2, +1.5)	
	P value	0.793	0.446	0.343	
VT	Change	+0.3	+1.0	+2.9	0.520
	CI	(-2.9,+3.6)	(-2.5, +4.4)	(-0.3, +6.2)	
	P value	0.838	0.575	0.079	
SF	Change	+0.8	+1.1	-0.5	0.800
	CI	(-2.9, +4.5)	(-2.8, +5.1)	(-4.2, +3.1)	

**Table 4.16: Absolute change from baseline at Week 48 in *HQLQ™v1*
together with 95% confidence interval (CI), associated *P-value*
and overall test of homogeneity.**

SF-36 Outcome	Absolute change	Placebo	Silymarin	SOX	Homogeneity *P*-value
	P value	0.664	0.564	0.771	
RE	Change	+3.3	-0.0	+1.5	0.636
	CI	(-1.5, +8.1)	(-5.0, +5.0)	(-3.2, +6.2)	
	P value	0.173	0.995	0.526	
MH	Change	-2.1	+2.4	-0.4	0.197
	CI	(-5.4, +1.3)	(-1.2, +6.0)	(-3.7, +2.9)	
	P value	0.494	0.888	0.428	
PCS	Change	-0. 8	+0.6	-1.1	0.722
	CI	(-3.8, +2.2)	(-2.6, +1.9)	(-4.0, +1.9)	
	P value	0.615	0.710	0.467	
MCS	Change	+0.9	+1.2	+1.3	0.991
	CI	(-2.8, +4.7)	(-2.8, +5.2)	(-2.5, +5.0)	
	P value	0.622	0.543	0.497	

From Table 4.16, there were no significant interactions between treatment and the SF-36 domains from Week 0 at Week 48.

In Table 4.17, Table 4.18 and Figure 4.9, the mean scores and standard error (SE) of the combined Hep573 Study population at baseline, or as specified, are compared against the Australian Bureau of Statistics National Health Survey (ABS NHS) SF-36 Populations Norms 1997[635] for Australian and NSW populations and one illness populations respectively.

**Table 4.17: Comparisons of the mean scores plus standard error (SE) of SF-
36 scales in Australian, NSW and one illness and Hep573 Study
populations.**

SF36	Mean Aust popn	SE Aust popn	Mean NSW popn	SE NSW popn	Mean One Illness	SE One Illness	Mean Hep573 Study	SE Hep573 Study
PF	82.5	0.2	82.0	0.6	83.2	0.3	79.1	2.1
RP	79.8	0.4	80.0	0.9	79.8	0.7	61.6	3.8
BP	76.8	0.3	76.6	0.7	75.6	0.5	26.8	2.2
GH	71.6	0.2	71.5	0.5	70.4	0.4	55.7	1.2
V	64.5	0.2	64.7	0.5	63.5	0.4	55.0	1.2
SF	84.9	0.2	84.9	0.6	84.3	0.5	49.5	0.9
RE	82.8	0.3	82.1	0.9	82.6	0.7	63.2	3.6
MH	75.9	0.2	75.3	0.5	75.0	0.4	62.2	0.8
PCS	49.7	0.1	49.7	0.2	49.8	0.2	43.0	0.4
MCS	50.1	0.1	49.9	0.3	49.6	0.2	42.4	0.6

Table 4.18 shows the difference in mean scores of the SF-36 scales of the NSW population, one illness population and the Hep573 Study population at baseline compared to the Australian population.

Table 4.18: Comparative differences in designated study populations compared to the Australian ABS NHS population data.

SF36	Mean Australian population	Difference in mean of Australian population compared to:		
		NSW population	One illness population	Hep573 Study at week 0 population
PF	82.5	-0.5	+0.7	-3.4
RP	79.8	+0.2	0.0	-18.2
BP	76.8	-0.2	-1.2	-50.0
GH	71.6	-0.1	-1.2	-15.9
V	64.5	+0.2	-1.0	-9.5
SF	84.9	0.0	-0.6	-35.4
RE	82.8	-0.7	-0.2	-19.6
MH	75.9	-0.6	-0.9	-13.7
PCS	49.7	0.0	+0.1	-6.7
MCS	50.1	+0.2	-0.5	-7.7
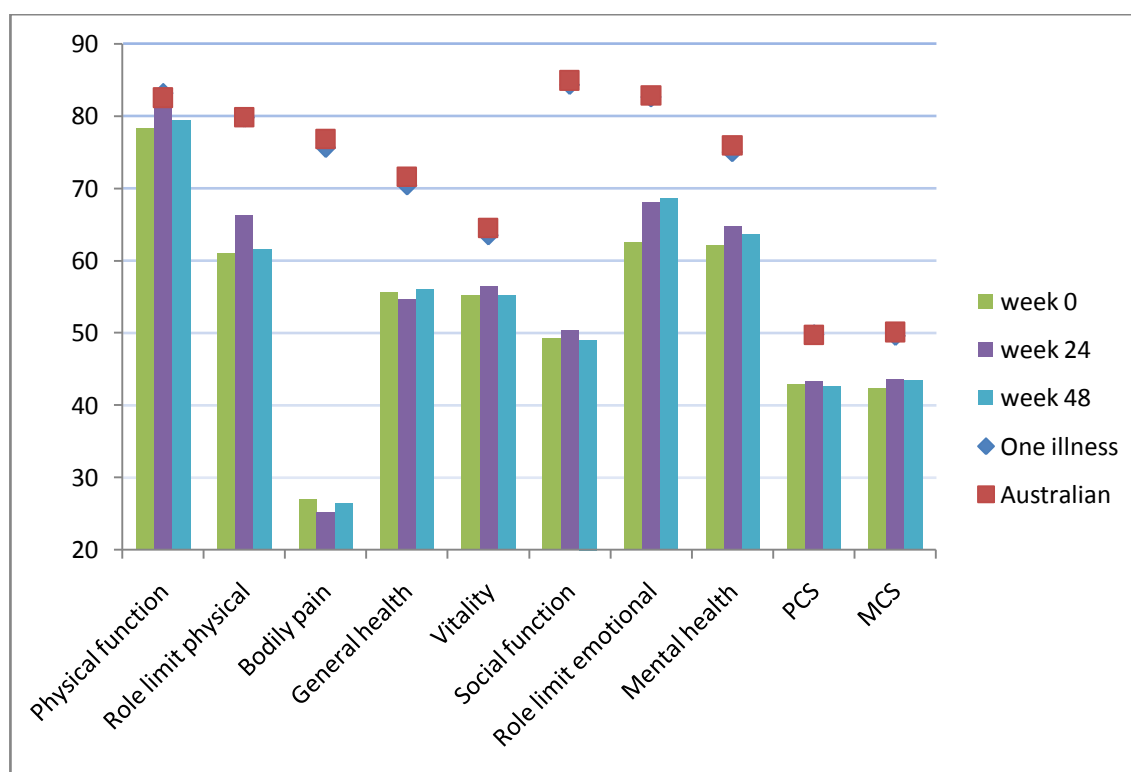
Figure 4.9: Comparisons of the total Hep573 Study population mean scores in SF-36 scales at Weeks 0, 24 and 48 against the Australian population and the one illness population.

Key: Week 0, 24 & 48=combined Hep573 Study population
One illness=ABS NHS SF-36 population norms 1997, one illness mean scores
Australian=ABS NHS SF-36 population norms 1997, Australian population mean scores.

As there are only marginal differences between the Australian and NSW populations as indicated in Table 4.18 and these are often indistinguishable on a graph, only the Australian population figures were included in Figure 4.9.

In Figure 4.9 it can be seen the total Hep573 Study population overall is well below the Australian and one illness normative data (range 3-50% lower). The only

occasion when the Hep573 population closely matched (83.0) the one illness data
(83.2) is at Week 24 for physical function.

The following Table 4.19-Table 4.21 display the results from the hepatitis-
specific questions in the *HQLQ™*.

Table 4.19: Absolute change in the hepatitis-specific items in *HQLQ™v1*, from baseline at Week 24 together with 95% confidence intervals (CI), associated *P-v*alue and overall test of homogeneity.

HQLQ™	Absolute change	Placebo	Silymarin	SOX	Homogeneity *P*-value
HD	Change	+4.0	+1.6	+4.2	0.899
	CI	(-4.5,+12.5)	(-6.9,+10.2)	(-4.2, +12.5)	
	P value	0.353	0.704	0.328	
PWB	change	+4.3	+0.6	+6.8	0.384
	CI	(-2.0, +10.6)	(-5.8, +7.0)	(-0.6, +13.0)	
	P value	0.182	0.860	0.032	
HLIM	change	+6.1	+0.1	+5.4	0.562
	CI	(-2.4, +14.5)	(-8.4, +8.6)	(-2.9, +13.7)	
	P value	0.159	0.979	0.202	
HHD	change	+6.9	+2.4	+11.3	0.287
	CI	(-1.1, +14.8)	(-5.6, +10.4)	(+3.5, +19.1)	
	P value	0.090	0.554	0.005	

Key:
HD   =Health Distress (Generic)
PWB   =Positive Well-being
HLIM =Hepatitis-specific Limitations
HHD   =Hepatitis-specific Health Distress

As shown in Table 4.19, there was no significant interaction between
treatment and the health distress (generic) (P=0.899), personal well-being
(P=0.384) hepatitis-specific limitations (P=0.562) and hepatitis-specific health
distress (P=0.287) from Weeks 0-24. However, the positive well-being (P=0.032)
and the hepatitis-specific health distress (P=0.005) improved in the SOX group
compared to the placebo and silymarin groups (LMEs).

Table 4.20: Absolute change in the hepatitis-specific Items in *HQLQ™v1* from Week 24 at Week 48 together with 95% confidence intervals (CI), associated *P*-value and overall test of homogeneity.

HQLQ™	Absolute change	Placebo	Silymarin	SOX	Homogeneity *P*-value
HD	Change	-0.9	+2.0	-5.0	0.357
	CI	(-7.7, +5.8)	(-5.1, +9.1)	(-11.7, +1.6)	
	P value	0.790	0.582	0.137	
PWB	Change	-1.8	+1.9	-11.5	0.004
	CI	(-7.4, +3.8)	(-4.0, +7.8)	(-17.0, -6.0)	
	P value	0.536	0.528	0.0001	
HLIM	Change	-0.4	+10.1	-1.8	0.012
	CI	(-6.3, +5.4)	(+4.0, +16.3)	(-7.6, +4.0)	
	P value	0.880	0.002	0.529	
HHD	Change	-3.2	+2.9	-7.7	0.078
	CI	(-9.5, +3.2)	(-3.8, +9.6)	(-13.9, -1.4)	
	P value	0.324	0.395	0.017	

Key:
HD =Health Distress (Generic)
PWB =Positive Well-being
HLIM =Hepatitis-specific Limitations
HHD =Hepatitis-specific Health Distress

Table 4.20 shows there was a significant interaction between treatment cessation and the hepatitis specific personal well-being score from Weeks 24-48 (*P*=0.004), with a highly significant deterioration in personal well-being found in the SOX group (*P*=0.0001) in the follow-up period.

There was a significant interaction between treatment and hepatitis-C-related limitations (*P*=0.012) from Weeks 24-48, with the silymarin group appearing far less limited by their hepatitis C in the follow-up period (*P*=0.002) compared to the placebo or SOX groups.

While there was no significant interaction between treatment and hepatitis-specific health distress (*P*=0.078), there was an increase in health distress specific to hepatitis C experienced by the SOX group (*P*=0.017) in the follow-up period.

Table 4.21: Absolute change in the hepatitis-specific items in *HQLQ™v1* from baseline at Week 48 together with 95% confidence intervals (CI), associated *P*-value and overall test of homogeneity.

HQLQ™	Absolute change	Placebo	Silymarin	SOX	Homogeneity *P*-value
HD	Change	+1.3	+2.0	+2.4	0.980
	CI	(-6.8, +9.3)	(-6.4,+10.4)	(-5.5, +10.3)	
	P value	0.756	0.640	0.552	
PWB	Change	+0.2	+3.2	-3.7	0.284
	CI	(-5.8, +6.1)	(-3.1, +9.4)	(-9.6, +2.2)	
	P value	0.953	0.215	0.317	
HLIM	Change	+7.6	+9.2	+4.6	0.006
	CI	(-1.0, +16.3)	(+0.3, +18.1)	(-4.0, +13.2)	
	P value	0.083	0.043	0.287	
HHD	Change	+3.1	+3.4	+4.5	0.976
	CI	(-6.1, +12.3)	(-6.2, +12.9)	(-4.6, +13.5)	
	P value	0.502	0.486	0.331	

Key:
HD =Health Distress (Generic)
PWB =Positive Well-being
HLIM =Hepatitis-specific Limitations
HHD =Hepatitis-specific Health Distress

From Table 4.21, it can be seen there was a significant interaction between treatment and the absolute change in the hepatitis C-specific health limitations from Week 0 to Week 48 (*P*=0.006). The silymarin group registered significantly less hepatitis C related limitations at Week 48 compared to their baseline scores (*P*=0.043) and the other two treatment groups.

SYMPTOM STATUS, FREQUENCY AND SEVERITY

Table 4.22 following indicates Hep573 cohort symptom prevalence at baseline represented by number of participants and percentage of the Study population.

Table 4.22: Hep573 Cohort Symptom Prevalence at Baseline

Symptom	Total number (N)	Percentage
Tiredness	91	79.8
Wake up tired	84	73.7
Poor sleep	71	71.0
Irritability	69	60.5
Liver pain	66	57.9
Tired aching muscles	66	57.9
Depression	59	51.8
Gas after meals	58	50.9
Back pain	57	50.0
Poor concentration	57	50.0
Mood swings	56	49.2
Frequent urination	56	49.2
Joint pain	53	46.5

Table 4.22: Hep573 Cohort Symptom Prevalence at Baseline

Symptom	Total number (N)	Percentage
Dull liver pain	51	44.7
Headaches	51	44.7
Gas anytime	49	43.0
Dry eyes	47	41.2
Vision problems	43	37.7
Skin rash	43	37.7
Nausea	40	35.1
Constipation	37	32.5
Sore throat	36	31.6
Dark urine	36	31.6
Diarrhoea	33	28.9
Night sweats	33	28.9
Poor appetite	33	28.9
Indigestion	33	28.9
Dizziness	30	26.3
Sharp liver pain	28	24.6
Bleeding gums	23	20.2
Swollen ankles	21	18.4
Pale stools	18	15.8
Fevers	12	10.5
Vomiting	7	6.1

Table 4.22 shows the prevalence of 34 symptoms reported in the Hep573 Study symptom questionnaire. The 10 most prevalent symptoms at Week 0 were: tiredness (80%), waking up tired (74%), poor sleep (71%), irritability (61%), liver pain (58%), tired aching muscles (58%), depression (52%), gas after meals (51%), back pain (50%) and poor concentration (50%). The above percentages were calculated on the 114 participants who entered the Study at Week 0.

Table 4.23: Hep573 Symptom Prevalence and Gender Comparison at Baseline and *P*-value of homogeneity.

Hep573 symptom prevalence at baseline	Gender		
	male	female	
	Mean	Mean	*P*-value
Indigestion	19.2%	50.0%	0.001
Headache	35.9%	63.9%	0.005
Wake up tired	66.7%	88.9%	0.012
Tired muscles	50.0%	75.0%	0.012
Sharp liver pain	17.9%	38.9%	0.016
Poor concentration	42.3%	66.7%	0.016
Constipation	25.6%	47.2%	0.022
Nausea	28.2%	50.0%	0.023
Tiredness	74.4%	91.7%	0.032
Liver pain	51.3%	72.2%	0.035
Back pain	43.6%	63.9%	0.044
Gas after meals	44.9%	63.9%	0.059
Swollen ankles	14.1%	27.8%	0.080

Table 4.23: Hep573 Symptom Prevalence and Gender Comparison at Baseline and *P*-value of homogeneity.

Hep573 symptom	Gender		
prevalence at	male	female	
baseline	Mean	Mean	*P*-value
Joint Pain	41.0%	58.3%	0.085
Sore throat	26.9%	41.7%	0.115
Bleeding gums	16.7%	27.8%	0.169
Diarrhoea	25.6%	36.1%	0.252
Poor sleep	67.9%	77.8%	0.282
Gas anytime	39.7%	50.0%	0.304
Moodswings	46.2%	55.6%	0.351
Irritability	57.7%	66.7%	0.362
Dry Eyes	38.5%	47.2%	0.377
Poor appetite	26.9%	33.3%	0.483
Dizziness	28.2%	22.2%	0.500
Vomiting	5.1%	8.3%	0.508
Skin problems	39.7%	33.3%	0.512
Vision problems	35.9%	41.7%	0.555
Dull liver pain	43.6%	47.2%	0.717
Dark urine	30.8%	33.3%	0.784
Night sweats	28.2%	30.6%	0.797
Pale stools	15.4%	16.7%	0.861
Depression	51.3%	52.8%	0.882
Fevers	10.3%	11.1%	0.890
Frequent urination	48.7%	50.0%	0.899

Key: Yellow highlighting identifies the significant *P*-values

The prevalence of the following symptoms in women were significantly different compared to men: indigestion (50% vs 19%, *P*=0.001), headaches (64% vs 36%, *P*=0.005), waking up tired (89% vs 67%, *P*=0.012), tired aching muscles (75% vs 50%, *P*=0.012), sharp liver pain (39% vs 18%, *P*=0.016), poor concentration (67% vs 42%, *P*=0.016), constipation (47% vs 26%, *P*=0.022), nausea (50% vs 28%, *P*=0.023), tiredness (92% vs 74%, *P*=0.032), liver pain (72% vs 51%, *P*=0.035), back pain (64% vs 44%, *P*=0.044).

Table 4.24 shows the number of the symptoms (from the list itemised in Table 4.22) reported by each participant.

Table 4.24: Hep573 symptoms per participant at baseline.

Number of symptoms per participant	Number of Participants	Percentage
Asymptomatic (0)	4	3.5
1-5 symptoms	16	14.0
6-10 symptoms	29	25.4
11-15 symptoms	14	12.3
16-20 symptoms	23	20.2
21-25 symptoms	19	16.7
26-30 symptoms	9	7.9

The majority of the Study population experienced numerous symptoms concurrently. Four participants (3.5%) were asymptomatic, 59 (51.7%) experienced between one and 15 symptoms and 51 (44.8%) experienced 16 to 30 symptoms.

Because of the huge amount of data generated from the *Hep573 Symptom Questionnaire*, and the possibility of false positives from the multiple statistical analyses with a *P*-value of 0.05 across the three treatment groups and 34 symptoms (Table 4.22), the symptoms were grouped into the following five categories of symptoms clusters: 1. neuropsychiatric, 2. neurological, 3. gastrointestinal, 4. algesic and 5. general.

(1) Neuropsychiatric symptoms include: tiredness, poor sleep, depression, poor concentration, irritability, wake up tired and mood swings.

(2) Neurological symptoms include: headache, dizziness, vision problems and dry eyes.

(3) Gastrointestinal symptoms include: liver pain, sharp liver pain, dull liver pain, nausea, vomiting, diarrhoea, constipation, poor appetite, indigestion, gas after meals, gas anytime and pale stools.

(4) Algesic symptoms include: tired aching muscles, back pain and joint pain and

(5) General symptoms include: skin problems, bleeding gums, fevers, swollen ankles, frequent urination, night sweats and dark urine.

These groupings were influenced by the work of Lang*, et al.,* [103] and adapted for an additional 13 questions compared to 21 questions used by those authors.

Table 4.25: _P_-value for homogeneity of the within participant change from Weeks 0-24, Weeks 24-48 and from Weeks 0-48 by treatment group (obtained from LMEs)

Variable	Weeks 0-24	Weeks 24-48	Weeks 0-48
Number of symptoms			
Neuropsychiatric symptoms	0.298	0.472	0.869
Neurological symptoms	0.097	0.031	0.313
Gastrointestinal symptoms	0.347	0.923	0.277
Algesic Symptoms	0.701	0.134	0.886
General Symptoms	0.427	0.033	0.797
Symptom frequency			
Neuropsychiatric frequency	0.044	0.101	0.445
Neurological frequency	0.028	0.001	0.195
Gastrointestinal frequency	0.218	0.609	0.438
Algesic frequency	0.400	0.207	0.871
General frequency	0.100	0.005	0.482
Symptom severity			
Neuropsychiatric severity	0.343	0.137	0.654
Neurological severity	0.142	0.017	0.152
Gastrointestinal severity	0.175	0.746	0.664
Algesic severity	0.111	0.755	0.617
General severity	0.590	0.007	0.792

Key: yellow highlighting shows _P_-values <0.05

For each of the five major symptom clusters groupings, there was no statistically significant difference between treatment in the within participant change in the number of symptoms in the relevant symptom clusters observed from Weeks 0-24. There were statistically significant differences between treatment in the within participant change over time at Week 24 compared to baseline in symptom frequency of two symptom clusters (Table 4.26, Table 4.27, Figure 4.10 and Figure 4.11). The within participant changes from Weeks 0-24 are reported separately for each treatment group in the following section. For reasons of clarity in the Figures, no _P_-values for the separate within participant changes over time are shown. If the entire confidence interval for the change lies below zero, there has been a statistically significant decrease over time for this group.
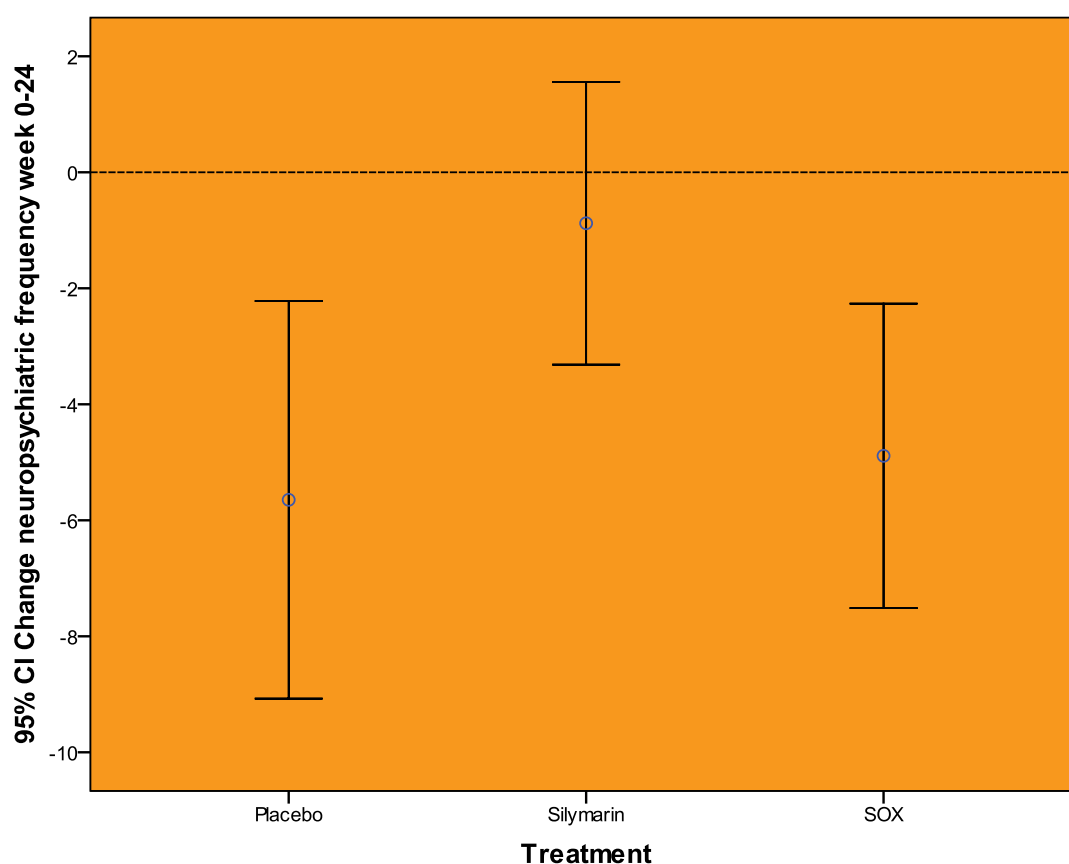
Figure 4.10: Change in the frequency of neuropsychiatric symptom clusters at Week 0-24 by treatment group with 95% confidence interval.

Table 4.26: Pairwise comparisons between treatment groups of the change in the frequency of neuropsychiatric symptom clusters from Weeks 0-24.

						95% Confidence Interval	
Dependent Variable	(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.	Lower Bound	Upper Bound
Change neuropsychiatric frequency week 0-24	Placebo	Silymarin	-4.7683*	2.0152	.020	-8.766	-.770
		SOX	-.7582	1.9721	.701	-4.671	3.155
	Silymarin	Placebo	4.7683*	2.0152	.020	.770	8.766
		SOX	4.0101*	1.9875	.046	.067	7.953
	SOX	Placebo	.7582	1.9721	.701	-3.155	4.671
		Silymarin	-4.0101*	1.9875	.046	-7.953	-.067

Pairwise Comparisons / LSD

*. The mean difference is significant at the 0.05 level.

As shown in Figure 4.10 and Table 4.26, the statistically significant decreases in the frequency of neuropsychiatric symptoms from Weeks 0 at Weeks 24 were comparable in the placebo and SOX groups (P=0.701), and significantly

163

larger than the non significant change in the silymarin group (*P*=0.020 and
P=0.046) respectively.
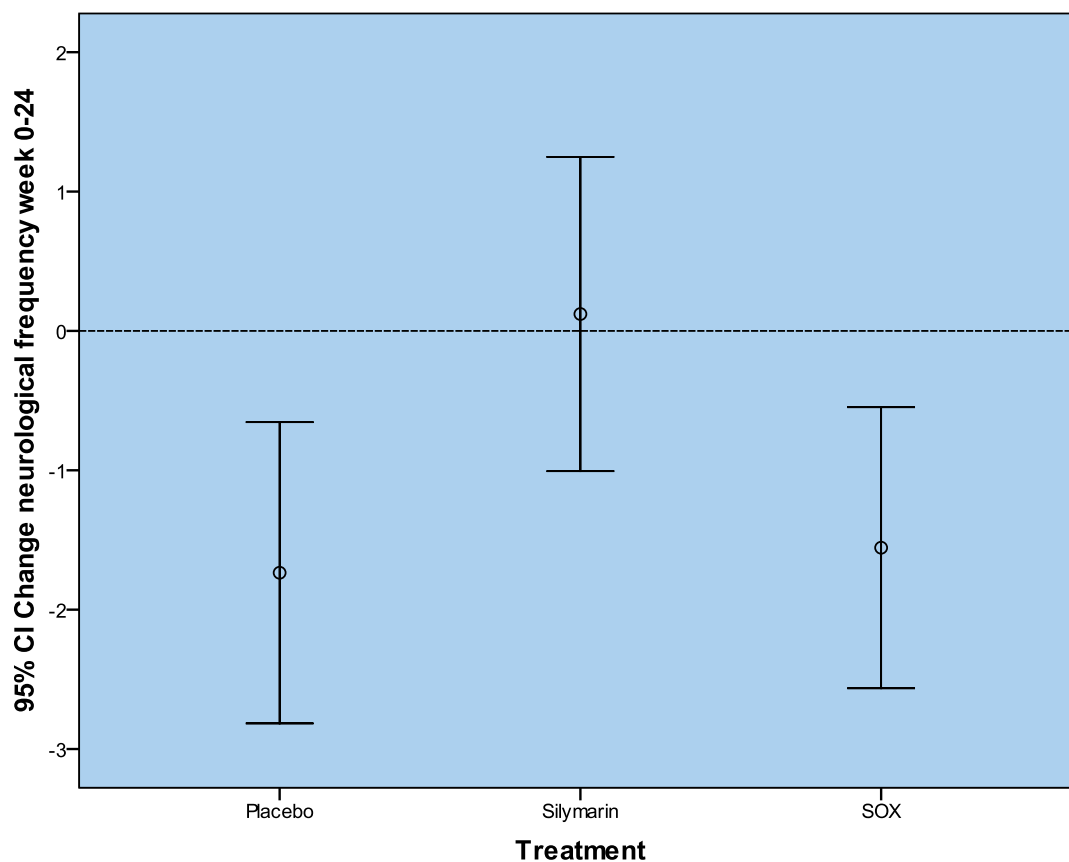
**Figure 4.11: Change in the frequency of neurological symptom clusters from
Weeks 0-24 by treatment group with 95% confidence interval (CI).**

**Table 4.27: Pairwise comparisons between treatment groups of the change in
frequency of neurological symptom clusters from Weeks 0-24.**

Pairwise Comparisons							
LSD							
						95% Confidence Interval	
			Mean Difference (I-J)	Std. Error	Sig.	Lower Bound	Upper Bound
Dependent Variable	(I) Treatment	(J) Treatment					
Change neurological frequency week 0-24	Placebo	Silymarin	-1.85651*	.75359	.015	-3.3516	-.3614
		SOX	-.17974	.73749	.808	-1.6429	1.2834
	Silymarin	Placebo	1.85651*	.75359	.015	.3614	3.3516
		SOX	1.67677*	.74321	.026	.2023	3.1513
	SOX	Placebo	.17974	.73749	.808	-1.2834	1.6429
		Silymarin	-1.67677*	.74321	.026	-3.1513	-.2023
*. The mean difference is significant at the 0.05 level.							

As shown in Figure 4.11 and Table 4.27, the statistically significant decreases in the frequency of the neurological symptom clusters from Weeks 0- 24 were comparable in the placebo and the SOX groups (P=0.808) and significantly larger than the non significant change observed in the silymarin group (P=0.015, P=0.026) respectively.

The next section of Figures and Tables refers to the within participant changes from Weeks 24-48 (follow-up period) where the statistically significant differences between treatments in symptom clusters will be reported separately for each treatment group. (Figure 4.12-Figure 4.17 and Table 4.28-Table 4.29).
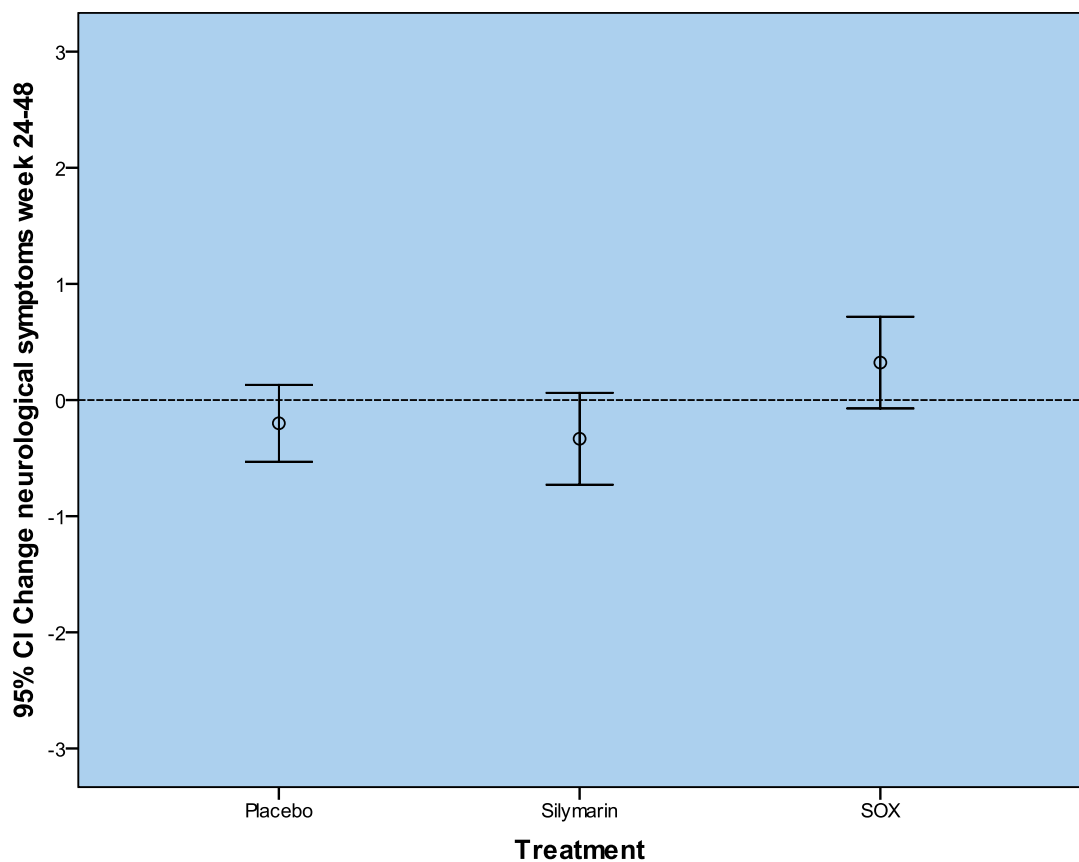
Figure 4.12: Change in neurological symptom clusters from Weeks 24-48 by treatment group with 95% confidence interval (CI).
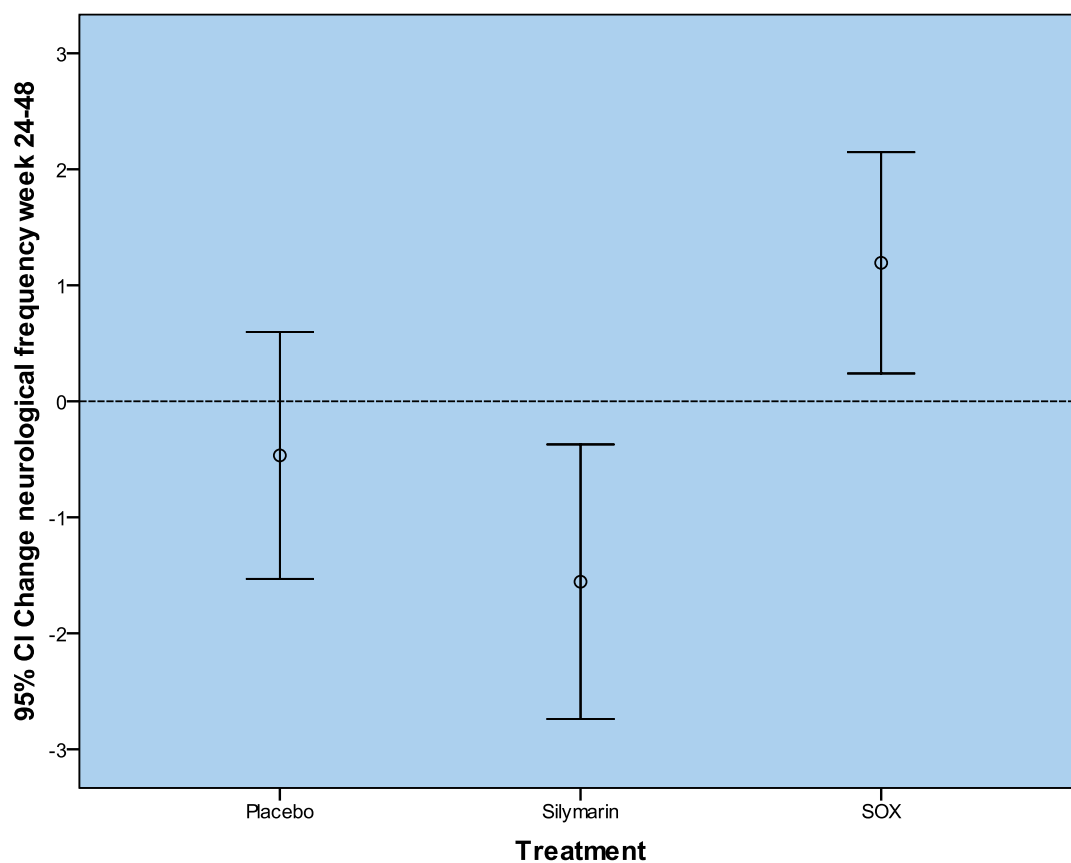
Figure 4.13: Change in the frequency of neurological symptom clusters from Weeks 24-48 by treatment group with 95% confidence interval (CI).
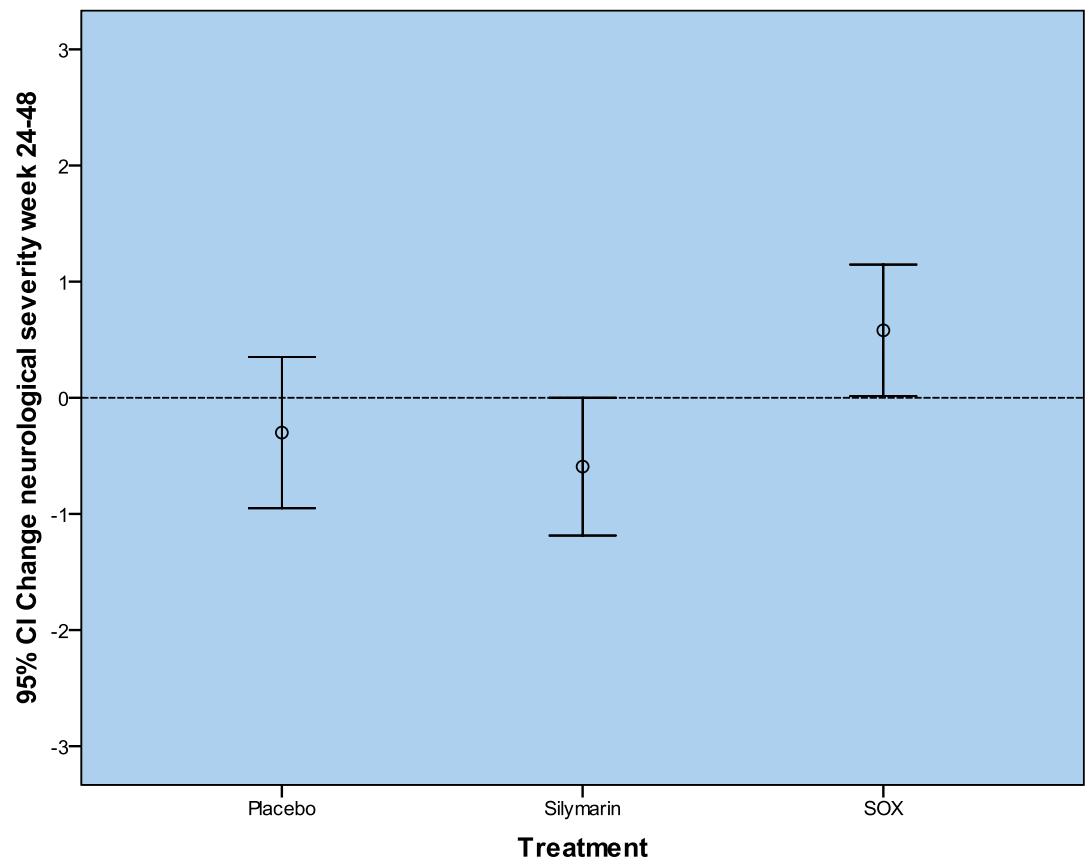
Figure 4.14: Change in neurological symptoms clusters severity from Weeks 24-48 by treatment group with 95% confidence interval (CI).

Table 4.28: Pairwise comparisons between treatment groups and neurological symptom clusters at Weeks 24-48.

Dependent Variable	(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
Pairwise Comparisons							
LSD							
Change neurological symptoms Week 24-48	Placebo	Silymarin	.13333	.26304	.614	-.9389	.0565
		SOX	-.52258*	.25395	.043	-.4282	.5459
	Silymarin	Placebo	-.13333	.26304	.614	-.0565	.9389
		SOX	-.65591*	.26102	.014	.0092	.9908
	SOX	Placebo	.52258*	.25395	.043	-.5459	.4282
		Silymarin	.65591*	.26102	.014	-.9908	-.0092
Change neurological frequency Week 24-48	Placebo	Silymarin	1.08889	.74531	.148	-3.3516	-.3614
		SOX	-1.66022*	.71956	.023	-1.6429	1.2834
	Silymarin	Placebo	-1.08889	.74531	.148	.3614	3.3516
		SOX	-2.74910*	.73960	.000	.2023	3.1513
	SOX	Placebo	1.66022*	.71956	.023	-1.2834	1.6429
		Silymarin	2.74910*	.73960	.000	-3.1513	-.2023
Change neurological severity Week 24-48	Placebo	Silymarin	.29259	.42519	.493	-1.6368	.0521
		SOX	-.88065*	.41049	.035	-.9571	.6957
	Silymarin	Placebo	-.29259	.42519	.493	-.0521	1.6368
		SOX	-1.17324*	.42192	.007	-.1712	1.4944
	SOX	Placebo	.88065*	.41049	.035	-.6957	.9571
		Silymarin	1.17324*	.42192	.007	-1.4944	.1712

*. The mean difference is significant at the 0.05 level.

As shown in Figure 4.12 to Figure 4.14 and Table 4.28 above, the decreases in the number of neurological symptom clusters in the follow-up period (Weeks 24-48) was comparable in the placebo and silymarin groups (P=0.614), and significantly larger in these groups than the increase observed in the SOX group (P=0.043, P=0.014) respectively.

The decrease in the frequency of the neurological symptom clusters in the follow-up period was comparable in the placebo and silymarin groups (P=0.148), and significantly larger than the significant increase in frequency observed in the SOX group (P=0.023, P<0.001) respectively.

The decrease in the severity of the neurological symptom clusters in the follow-up period was comparable in the placebo and silymarin groups (P=0.493), and significantly larger in these groups than the significant increase in severity observed in the SOX group (P=0.035, P=0.007) respectively.
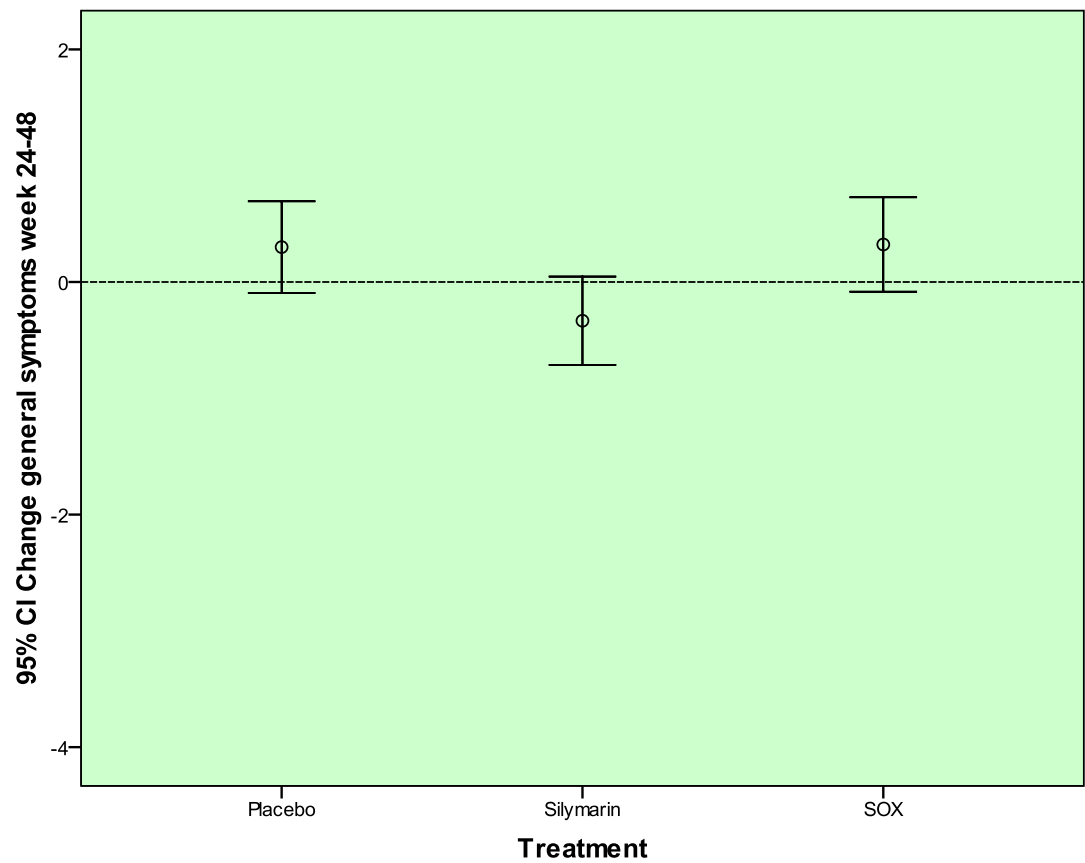
Figure 4.15: Change in general symptoms clusters from Weeks 24-48 by treatment group with 95% confidence interval (CI).
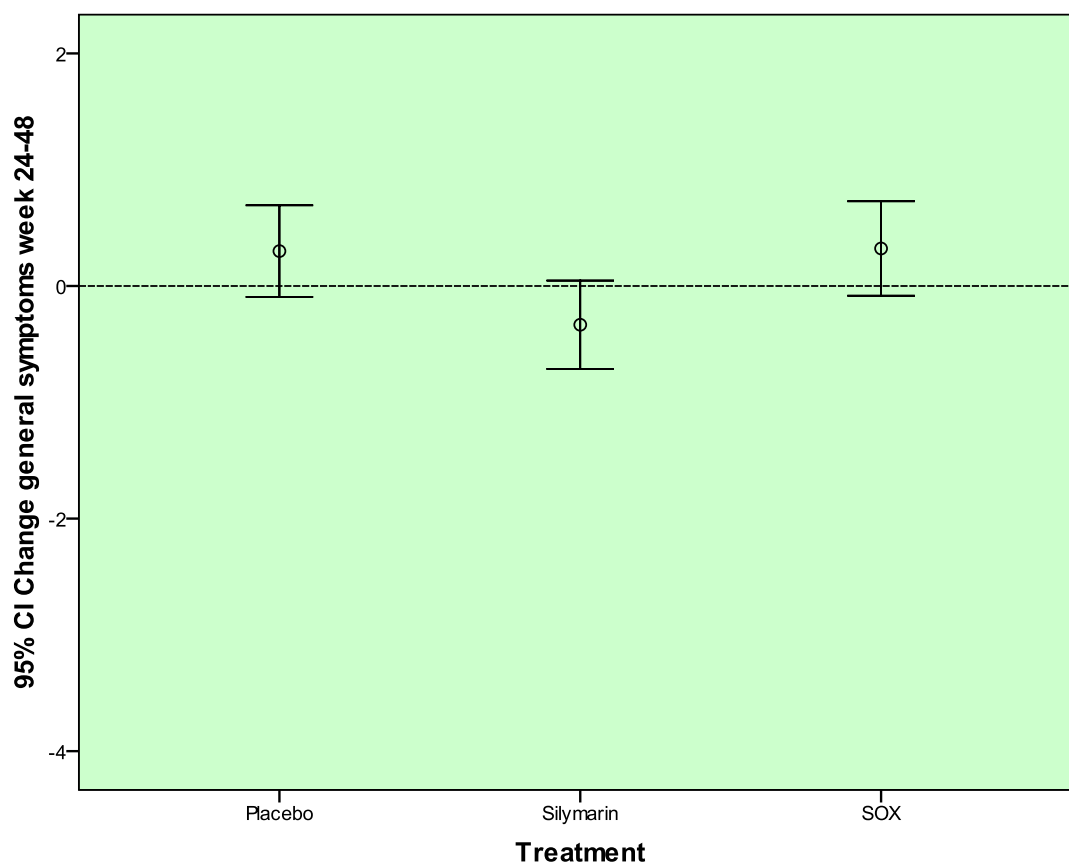
Figure 4.16: Change in the frequency of general symptom clusters from Weeks 24-48 by treatment group with 95% confidence interval (CI).
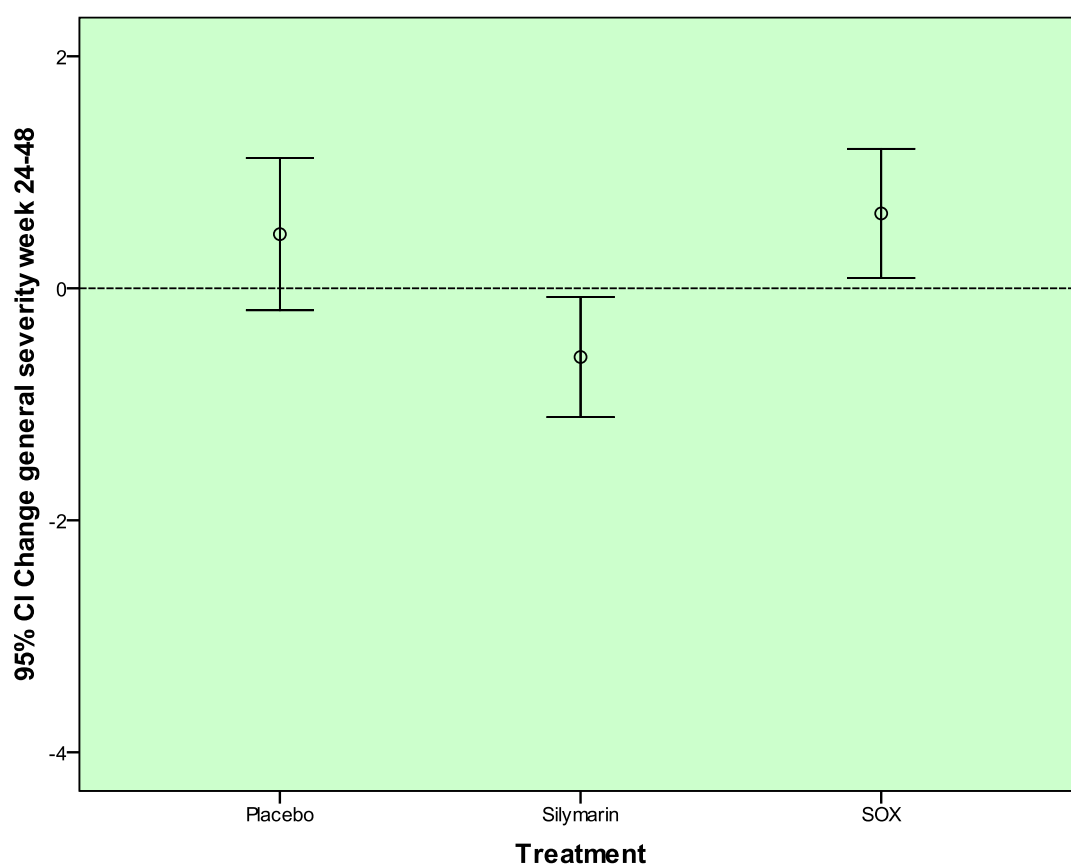
Figure 4.17: Change in general symptoms clusters severity from Weeks 24-48 by treatment group with 95% confidence interval (CI).

Table 4.29: Pairwise comparisons between treatment groups of the change in general symptom clusters at Weeks 24-48.

Multiple Comparisons							
LSD							
Dependent Variable	(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	Upper Bound
Change general symptoms Week 24-48	Placebo	Silymarin	.63333*	.27759	.025	.0814	1.1853
		SOX	-.02258	.26800	.933	-.5554	.5103
	Silymarin	Placebo	-.63333*	.27759	.025	-1.1853	-.0814
		SOX	-.65591*	.27546	.019	-1.2036	-.1082
	SOX	Placebo	.02258	.26800	.933	-.5103	.5554
		Silymarin	.65591*	.27546	.019	.1082	1.2036
Change general frequency Week 24-48	Placebo	Silymarin	2.81852*	.95113	.004	.9274	4.7096
		SOX	.08495	.91827	.927	-1.7408	1.9107
	Silymarin	Placebo	-2.81852*	.95113	.004	-4.7096	-.9274
		SOX	-2.73357*	.94383	.005	-4.6102	-.8570
	SOX	Placebo	-.08495	.91827	.927	-1.9107	1.7408
		Silymarin	2.73357*	.94383	.005	.8570	4.6102

Table 4.29: Pairwise comparisons between treatment groups of the change in general symptom clusters at Weeks 24-48.

Multiple Comparisons							
LSD							
Dependent Variable	(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
Change general severity Week 24-48	Placebo	Silymarin	1.05926*	.41004	.011	.2440	1.8745
		SOX	-.17849	.39587	.653	-.9656	.6086
	Silymarin	Placebo	-1.05926*	.41004	.011	-1.8745	-.2440
		SOX	-1.23775*	.40689	.003	-2.0468	-.4287
	SOX	Placebo	.17849	.39587	.653	-.6086	.9656
		Silymarin	1.23775*	.40689	.003	.4287	2.0468
*. The mean difference is significant at the 0.05 level.							

As shown in Figure 4.15-Figure 4.17 and Table 4.29, the increase in the number of general symptoms clusters in the follow-up period was comparable in the placebo and the SOX groups (*P*=0.933), and significantly larger in these groups than the decrease observed in the silymarin group (*P*=0.025, *P*=0.019) respectively.

The increases in the frequency of the general symptoms clusters in the follow-up period was comparable in the placebo and the SOX groups (*P*=0.927), and significantly larger than the significant decrease in frequency observed in the silymarin group (*P*=0.004, *P*=0.005), respectively.

The increase in the severity of the general symptoms clusters in the follow-up period was comparable in the placebo and the SOX groups (*P*=0.653), and significantly larger than the significant decrease observed in the silymarin group (*P*=0.011, (*P*=0.003) respectively.

CONFOUNDING FACTORS

Alcohol and Other Drugs Intake By Trial Participants

Table 4.30 documents difference in tobacco consumption, average alcohol intake, number of standard alcoholic drinks consumed per day, consumption of more than six standard alcoholic drinks in one drinking session and recreational drug intake at baseline between the treatment groups.

Table 4.30: Alcohol and drug intake at baseline with the associated *P*-value.

Outcome	Tobacco	Average alcohol intake	Standard drinks (SD) per day	Consumed > 6 SD on one occasion	Recreation drug use
P-value	0.536	0.686	0.880	0.642	0.077

From Table 4.30, it can be seen that there was no statistically significant evidence of differences between the groups with respect to tobacco, alcohol and recreational drug consumption at baseline (Kruskal-Wallis test).[631] There was also no statistically significant evidence of differences between the groups with respect to the within participant changes in these outcomes from Weeks 0-24, Weeks 24-48 and Weeks 0-48.

Table 4.31-Table 4.33 following show the daily alcohol intake per treatment group at baseline, Weeks 24 and 48 respectively.

Table 4.31: Daily alcohol intake (grams) per treatment group at baseline with median and interquartile ranges.

		Alcohol per day Week 0		
		Median	Percentile 25	Percentile 75
Treatment	Placebo	.33	.00	1.97
	Silymarin	.66	.00	3.45
	SOX	.66	.00	3.45

From Table 4.31, there was no statistically significant evidence of a difference in daily alcohol consumption at baseline across the three treatment groups. (*P*=0.809) (Kruskal-Wallis).

Table 4.32: Daily alcohol intake (grams) per treatment group at Week 24 with median and interquartile ranges.

		Alcohol per day Week 24		
		Median	Percentile 25	Percentile 75
Treatment	Placebo	.00	.00	1.97
	Silymarin	.33	.00	5.42
	SOX	.00	.00	1.48

There was no statistically significant evidence of a difference in daily alcohol consumption at Week 24 across the three treatment groups (*P*=0.664) (Kruskal-Wallis).

Table 4.33: Daily alcohol intake (grams) per treatment group at Week 48 with median and interquartile ranges.

		Alcohol per day Week 48		
		Median	Percentile 25	Percentile 75
Treatment	Placebo	.00	.00	1.97
	Silymarin	.66	.00	3.94
	SOX	.00	.00	1.97

There was no statistically significant evidence of a difference in daily alcohol consumption at Week 48 across the three treatment groups (*P*=0.245) (Kruskal-Wallis).

Body Mass Index (BMI)

There was a significant interaction between treatment groups and BMI at Week 24 compared to Week 0 (*P*=0.034). The silymarin group compared to the placebo group had a reduction of 0.4 in BMI during the treatment period of 24 weeks (*P*=0.010). This change in BMI is shown in Table 4.34.

Table 4.34: Change in BMI at Week 24 compared to baseline together with 95% Confidence Interval (CI), interquartile ranges and *P*-value.

		Mean Difference			95% Confidence Interval	
(I) Treatment	(J) Treatment	(I-J)	Std. Error	Sig.	Lower Bound	Upper Bound
Placebo	Silymarin	.65798*	.25074	.010	.1605	1.1554
	SOX	.25792	.24538	.296	-.2289	.7448
Silymarin	Placebo	-.65798*	.25074	.010	-1.1554	-.1605
	SOX	-.40006	.24728	.109	-.8907	.0905
SOX	Placebo	-.25792	.24538	.296	-.7448	.2289
	Silymarin	.40006	.24728	.109	-.0905	.8907
* The mean difference is significant at the 0.05 level.						

Diet Questionnaire

The *Diet Questionnaire* was administered monthly to the Study participants. The central question posed in the statistical analysis of the results was whether there was a change in food consumption in the identified items over time from Weeks 0-24, Weeks 24-48 and from Weeks 0-48. McNemar tests were used to assess whether participants systematically changed the presence or absence of a particular food consumed from one time to another. The percentage experiencing any ill feeling from consuming a particular food is shown. For those who consumed a particular food type at the two time-points, Wilcoxon Signed Ranked tests[636] were

used to test for changes in severity score (0-3 scale) over time due to the food consumed.

Table 4.35 shows the proportions of participants consuming each food type and if consumed, the percentage experiencing ill effects for each treatment group at Weeks 0, 24 and 48.

Table 4.35: Percentage of Hep573 Study population consuming specified food groups, any ill effect associated with its consumption across the three Study treatment groups at Weeks 0, 24 and 48.

	Treatment					
	Placebo		Silymarin		SOX	
	%	Valid N	%	Valid N	%	Valid N
Any protein wk 0	92	38	97	39	95	37
Any protein wk 24	88	34	100	33	100	36
Any protein wk 48	81	31	93	27	97	31
Any fat wk 0	100	38	95	39	100	37
Any fat wk 24	97	34	100	33	94	36
Any fat wk 48	97	31	100	27	100	31
Any carbohydrate wk 0	100	38	100	39	100	37
Any carbohydrate wk 24	97	34	100	33	100	36
Any carbohydrate wk 48	97	31	100	27	100	31
Any alcohol wk 0	47	38	56	39	51	37
Any alcohol wk 24	44	34	48	33	39	36
Any alcohol wk 48	42	31	56	27	32	31
Any coffee wk 0	66	38	77	39	62	37
Any coffee wk 24	79	34	70	33	53	36
Any coffee wk 48	77	31	70	27	58	31
If protein, made sick wk 0	66	35	74	38	69	35
If protein, made sick wk 24	63	30	70	33	58	36
If protein, made sick wk 48	76	25	72	25	73	30
If fat, made sick wk 0	40	38	27	37	24	37
If fat, made sick wk 24	21	33	21	33	18	34
If fat, made sick wk 48	27	30	22	27	26	31
If carbohydrate, made sick wk 0	40	38	36	39	38	37
If carbohydrate, made sick wk 24	33	33	30	33	33	36
If carbohydrate, made sick wk 48	33	30	33	27	42	31

Table 4.35: Percentage of Hep573 Study population consuming specified food groups, any ill effect associated with its consumption across the three Study treatment groups at Weeks 0, 24 and 48.

	Treatment					
	Placebo		Silymarin		SOX	
	%	Valid N	%	Valid N	%	Valid N
If alcohol, made sick wk 0	33	18	23	22	37	19
If alcohol, made sick wk 24	20	15	06	16	21	14
If alcohol, made sick wk 48	08	13	13	15	20	10
If coffee, made sick wk 0	36	25	10	30	22	23
If coffee, made sick wk 24	19	27	09	23	16	19
If coffee, made sick wk 48	17	24	05	19	22	18

Key:
%   =percentage,
N   =number,
Wk   =week

Table 4.36: Change in severity (how sick) over time across the treatment groups with corresponding *P*-value of homogeneity

Change in severity over time	Homogeneity *P*-value
Change protein, how sick wk 0-24	0.805
Change protein, how sick wk 24-48	0.294
Change protein, how sick wk 0-48	0.543
Change fat, how sick wk 0-24	0.293
Change fat, how sick wk 24-48	0.741
Change fat, how sick wk 0-48	0.338
Change carbohydrate, how sick wk 0-24	0.799
Change carbohydrate, how sick wk 24-48	0.219
Change carbohydrate, how sick wk 0-48	0.806
Change alcohol, how sick wk 0-24	0.255
Change alcohol, how sick wk 24-48	0.256
Change alcohol, how sick wk 0-48	0.439
Change coffee, how sick wk 0-24	0.257
Change coffee, how sick wk 24-48	0.875
Change coffee, how sick wk 0-48	0.055

Table 4.36 shows that there was no interaction between the size of the change in the severity score (how sick) from food consumption over time by treatment group (Kruskal-Wallis ANOVA test).

Caffeine Questionnaire

Table 4.37, shows the within participant change in caffeine intake across the three treatment groups at Weeks 0-24, 24-48 and 0-48.

Table 4.37: Summary statistics for caffeine intake and within patient changes in intake over time (mean and standard deviation).

	Treatment					
	Placebo		Silymarin		SOX	
	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation
Caffeine Week 0	1754	1606.9	1979.7	1735.4	1724.3	1423.7
Caffeine Week 24	1495.4	1494.7	1748.9	1421.8	1756.1	1577.5
Caffeine Week 48	1459.3	1336.6	1802.5	1712.1	2167.4	1746
Change caffeine Weeks 0-24	-315.4	1192	-239.1	1368.4	31.8	1075.7
Change caffeine Weeks 24-48	-72.3	1027.2	-49.3	615.4	233.6	956.1
Change caffeine Weeks 0-48	-354.1	960.1	-56.5	1218.7	284.6	1435

Table 4.37 shows that the average weekly caffeine intake at Week 0 per participant was 1819 mg caffeine, compared to 1667 mg at Week 24 and 1810 mg at Week 48.

Table 4.38 shows the difference in caffeine intake changes by treatment group at Weeks 0-24, 24-48 and 0-48.

Table 4.38: Oneway ANOVA to test for difference in caffeine intake changes by treatment group.

		F	Sig.
Change caffeine Weeks 0-24	Between Groups	.809	.448
	Within Groups		
	Total		
Change caffeine Weeks 24-48	Between Groups	1.104	.336
	Within Groups		
	Total		
Change caffeine Weeks 0-48	Between Groups	2.126	.125
	Within Groups		
	Total		

There were no statistically significant differences in the within participant change in caffeine intake between the three treatment groups from Week 0 at Week 24 (P=0.448), from Week 24 at Week 48 (P=0.336) and from Week 0 at Week 48 (P=0.125).

ADVERSE EVENTS

The following adverse events presented in Table 4.39 were reported to the relevant Human Research Ethics Committees and the Therapeutic Goods Administration (with the exception of those marked with an asterisk).

Table 4.39: Occurrence of adverse events by treatment group.

Placebo	Silymarin	SOX
Plane crash (103)	Chest pain (125)	Diverticular abscess (106)
Irritability, Liver pain (203)*	Respiratory Tract infection (310)	Irritability (209)*
Right-sided abdominal pain (344)	Chest pain, headache, blurred vision (341)	Suicide (334)
	Fatigue, rash (223)*	

From Table 4.39, the only adverse event related to the trial preparations was an idiosyncratic reaction to silymarin which included: dry mouth, blurred vision, headache and 'hot feeling on upper chest'. This reaction stopped when the product was no longer taken.

CHAPTER 5

DISCUSSION

FINDINGS OF THE STUDY

This Study has shown that the use of silymarin and antioxidants (SOX) has a beneficial effect in normalising ALT levels in participants with chronic hepatitis C over 24 weeks. This was achieved in participants predominantly infected with HCV genotype 1. The SOX intervention also significantly increased QOL. By 24 weeks off treatment, all treatment differences were lost, suggesting the benefit in reducing hepatic necroinflammation and improving QOL was due to active treatment.

The data relating to OS showed a trend towards significance. A new trial using optimal doses of SOX as informed by current research could prove the link between ALT normalisation and a reduction of oxidative stress.

Alanine Aminotransferase (ALT) Normalisation

In this cohort of CHC participants with Child-Pugh ≤7, ALT normalisation was dependent on treatment. The SOX group achieved a higher rate of ALT normalisation (10/39, P=0.002) compared to placebo (2/39, P=0.02), or silymarin (1/40, P=0.003) at Week 24.

In the subset analysis, the ALT normalisation was most evident in HCV genotype 1 (P=0.008), which is often refractory to pegylated interferon and ribavirin therapy. Those with ALT normalisation in the SOX group had a mean 43.4% reduction in ALT levels at Week 24, compared to Week 0. Two had persistently normal ALT levels over the 24 weeks, while two had one normal ALT during treatment and again at Week 24; the remaining six normalised at Week 24; suggesting that long-term administration is required for ALT normalisation.

An elevated ALT is an indication of hepatocyte necrosis and inflammation. Hepatic necroinflamation drives liver disease progression leading to advanced fibrosis and eventually to liver cirrhosis and HCC.[377,155,159,167] A reduction in ALT is

associated with reduced liver disease progression, a positive outcome for patients with active HCV infection.

This positive finding of ALT normalisation in HCV genotype 1 participants is clinically interesting. It suggests there may be HCV genotype-specific interactions with oxidative stress[302,637] with glutathione (GSH) depletion more marked in genotype 1 CHC patients compared to genotype 2 or 3 CHC patients.[302]

Decreased GSH levels may be involved in liver disease progession as this depletion sensitises the hepatocyte to a necrotic death rather than an apoptotic one. A previous study confirmed that reduced concentrations of glutathione in the liver, plasma and PBMC in CHC patients correlated with ALT elevations ($P<0.001$) and fibrosis scores ($P<0.001$).[310] This may mean interventions which maintain glutathione levels may have specific relevance to genotype 1 patients. This warrants further examination.

F_2-Isoprostanes

Whilst the reduction in F_2-isoprostanes from Weeks 0-24 differed by treatment, as there was a significant significant fall in the F_2-isoprostanes in the SOX group ($P=0.003$), but not in the placebo ($P=0.066$) or silymarin groups ($P=0.666$); it failed to reach statistical significance ($P=0.071$) overall (interaction) (LMEs). This is indicative of a trend towards SOX treatment lowering F_2-isoprostanes.

HCV RNA Viral Load

The within participant change in HCV RNA observed during the Study period did not differ between the treatment groups.

At the commencement of this Study in 2003, there was no supporting literature to show that oral herbal medicines or vitamins could influence HCV RNA; however, recent work has shown that an intravenous dose of silibinin (1400 mg/day) can significantly reduce HCV RNA levels.[438]

Extrapolating from bioavailability figures,[468,455] the oral daily dose of silibinin (720 mg) used in the Hep573 Study was between 3.8 to 7.7 times lower than the dose Ferenci, *et al.,*[438] identified as being required to substantially reduce HCV RNA in CHC patients. This Study may have found additional response if a higher oral dose of silibinin had been used than 720 mg per day.

FibroTest and Hepascore

There was no interaction between treatment and a change in FibroTest, Hepascore or hyaluronic acid during the Study period. This was not unexpected, given the short treatment duration of 24 weeks.

However, it was observed that from Week 0 to Week 48, the FibroTest score increased by 30.2% in the placebo group (P=0.085), by 3.1% in the silymarin group (P=0.855) and by 0.1% in the SOX group (P=0.993).

While these results are not statistically significant, the 30% increase in the FibroTest score in the placebo group, compared to the silymarin and SOX groups is of potential clinical significance. Extended treatment with antioxidants for 2–5 years, could positively influence the natural history of hepatitis C and liver disease progression.

Association between F_2-isoprostanes and ALT and FibroTest

There was a weak positive correlation between the within participant change in both ALT and FibroTest and the within participant change in the F_2-isoprostanes at Week 24. This correlation was as expected as F_2-isoprostanes are measuring the consequence of OS in one pathway (lipid peroxidation) and the ALT and FibroTest reflect multiple events in the liver (necrosis, inflammation, fibrosis) not just OS.

Mental Component Summary Score and Vitality

The overall improvement in QOL, MCS score in the SOX group at Week 24 may have been influenced by the statistically significant improvements in two of its four components, mental health (P=0.012) and vitality (P=0.001).

The systematic review by Speigel, *et al.,* identified that the vitality scale was one of the key domains most affected by HCV and therefore, the most important predictor of QOL status in HCV infection.[638]

There was an absolute positive change in the vitality scale of +5.6 in the SOX group (P=0.001) which was higher than the +1.6 achieved in the silymarin group (P=0.351) and +1.0 in the placebo group (P=0.564) at Week 24 compared to baseline. The extra percentage weighting placed on the vitality scale within the MCS measure in the *HQLQ™v1*,[88] increases the clinical significance of this finding and is also reflective of the naturopathic treatment goal to restore vitality in the patient.

The Study population had significantly lower baseline scores for QOL compared to the Australian population indicating that these CHC participants felt their symptoms keenly. PCS and MCS scores were 13% and 15% respectively lower than the Australian population. Improving QOL is a significant achievement, as improved QOL leads to improved life choices.

The cessation of treatment was associated with a significant decline back to baseline levels in MCS and in mental-health status in the SOX group with similar absolute negative changes of -4.0 in MCS (P=0.021) and -4.1 in mental health (P=0.004). A return to baseline levels is expected on cessation of treatment.

Hepatitis-specific items in *HQLQ*™

The hepatitis-specific items in the *HQLQ*™ *v1,* for the SOX group in the follow-up period showed a similar return to baseline as reported above. There was a significant decline in personal well-being with an absolute negative change of -11.5 (P=0.0001) in the SOX group compared to a -1.8 change in the placebo group (P=0.536) and a +1.9 improvement in the silymarin group (P=0.528). This further strengthens the notion that the benefit seen in the SOX group was a treatment effect which declined post-treatment.

However, contrary to the findings in the SOX group, the silymarin group improved in one of the hepatitis-specific items post-treatment. There was a significant improvement in hepatitis-specific limitations in the silymarin group, with absolute positive changes of +10.1 from Weeks 24-48 (P=0.002) and of +9.2 from baseline to Week 48 (P=0.043). The participants in the silymarin group were far less limited in their activities due to the presence of HCV infection in both the follow-up period and from baseline to Week 48 compared to placebo group and the SOX group.

The effects of silymarin are many and it is known as a trophorestorative to the liver. This Study focussed on the viral, oxidative stress and inflammatory processes; other measures may have identified the direct cause for this improvement.

SF-36 Scales and Australian Population Norms

The mean scores for the SF-36 scales at Week 0 for the entire Hep573 Study population were substantially lower than Australian Bureau of Statistics National Health Survey SF-36 Population Norms 1997 for the Australian population, NSW population and one illness population.[639]

The significant improvements in QOL reported from the ingestion of SOX for six months are clinically relevant given the severe impairment in each of the subscales at baseline in this hepatitis C Study population compared to the Australian healthy population.

Body Mass Index

There was a significant association between treatment and BMI at Week 24 compared to Week 0 (P=0.034). The silymarin group had a reduction of 0.4 in BMI from Weeks 0-24 (P=0.010) compared to the placebo. The anti-diabetic and hypoglycaemic properties of silymarin may have contributed to this finding.[640,641]

Symptom Prevalence

Fatigue is defined as a feeling of weariness, tiredness or lack of energy.[642] The present Study found the most prevalent symptom at baseline was 'tiredness' (91/118, 80%). This percentage is substantially higher than 45-62% experiencing fatigue reported by other studies.[643-646] The second and third most frequently reported symptoms in the Hep573 Study were 'waking up tired' (84/118, 74%) and 'poor sleep' (71/118, 71%).

The remaining seven symptoms in the 10 most prevalent symptoms were: irritability (69/118, 60.5%), liver pain (66/118, 58%), tired aching muscles (66/118, 58%), depression (59/118, 52%), gas after meals (58/118, 51%), back pain (57/118, 50%) and poor concentration (57/118, 50%). Women were significantly overrepresented in six out of 10 of these symptoms compared to men in the Study population. (These gender differences will be discussed in relation to the Lang*, et al.,*[103] study in the section entitled: 'Comparisons with other studies'.)

Symptom Clusters in the Study Population

The SOX intervention significantly reduced the frequency of the neurological symptom clusters from baseline to Week 24 and then showed a corresponding rebound increase back to baseline levels in the follow-up period (treatment effect).

During the follow-up period, the silymarin group consistently showed a significant decrease in the number, frequency and severity of the general symptom clusters, compared to increases in both the placebo and SOX groups. The silymarin group showed sustained improvement in general symptoms, the measures to elucidate the causes for this were not taken.

Alcohol

The fact there were no significant differences between the treatment groups in alcohol intake during the entire Study period is encouraging and interesting. Overall, this group had a low alcohol intake and the fact that it did not change supports the conclusion that improved liver function was related to treatment rather than altered alcohol intake.

Diet

There were no interactions detected between diet and treatment throughout the Study duration, again supporting a treatment effect of the SOX intervention.

Caffeine

There were no statistically significant differences in the within participant change during the entire Study in caffeine intake between the treatment groups. Reporting this non significant finding in caffeine intake is necessary to accommodate the conflicting views surrounding caffeine intake between traditional naturopathic teaching and medicine. Previous naturopathic doctrine considered coffee was harmful to the liver and not associated with a healthy lifestyle. Recent scientific literature throws into question this traditional naturopathic teaching, as studies have found drinking more than three cups of coffee per day improved SVR to pegylated interferon and ribavirin[647] and reduced the relative risk to 0.47 (or by 53%) for liver disease progression in CHC participants with bridging fibrosis or cirrhosis at baseline.[648]

In addition, there is a growing body of evidence that both tea[649,650] and coffee[651] contain antioxidant properties. Therefore, the monitoring of caffeine intake throughout the Study was an important aspect of its design, given that antioxidant interventions were being examined. The finding of no statistically significant differences between the groups in caffeine intake over time, strengthens the value of the SOX intervention regardless of the antioxidant content in the caffeine-containing tea and coffee beverages.

ADVERSE EVENTS

Safety of the Trial Interventions

Similar numbers of participants experienced adverse effects in the three treatment groups (placebo, N=3; silymarin, N=4; SOX, N=3). The only adverse effect directly linked to the trial preparations was an idiosyncratic reaction in one participant to silymarin which resolved immediately when the dose was stopped. (Refer also to Chapter 4 'Results', page 178.)

STRENGTHS OF THE STUDY

Design Strengths of the Study

The Hep573 Study was a randomised, double-blind, placebo-controlled trial of CMs in the treatment of chronic hepatitis C. It utilised standard inclusion and exclusion criteria and post-treatment follow-up, similar to pegylated interferon and ribavirin trials. Outcome measures which had not been routinely used in other randomised controlled CM studies in chronic hepatitis C participants at the time of initiating this Study, such as F_2-isoprostanes, HCV RNA Quantitation (Viral Load), FibroTest and *HQLQ™v1* were all used.

The possibility of confounding factors such as alcohol, caffeine, tobacco, recreational drugs and diet were all examined. There were no statistically significant changes in consumption in these factors, strengthening the likelihood that the positive results in the SOX group were due directly to the active interventions used.

The research team was comprised of members with specialist knowledge of hepatitis C virus infection, complementary medicine and research methodologies: hepatologists, hepatitis C nurse consultants, a biomedical statistician and a CM practitioner as the Study Coordinator.

Rigour and Sample Selection.

The Study cohort was generally reflective of the Australian HCV genotype distribution with genotypes 1 and 3 being the most common. This Study cohort is reflective of the worldwide predominance of genotype 1[652] (except in Egypt and Japan). See Table 5.1 following.

Table 5.1: Genotype distribution comparison between Australian and Hep573 data

HCV Genotype	Australia wide	Hep573 cohort
1	55%	67%
2	8%	2.5%
3	33%	28%
4	4%	2%

The previous complementary medicine intake (48%) amongst the Hep573 Study participants was similar to the extrapolated national average of complementary medicine intake of 52% in 2000.[124] Thus a good representative sample of chronic hepatitis C participants across the HCV genotypes and their relationship to complementary medicine was obtained in the present Study.

Validating the Study Aims.

Five of the eight scientific aims of this Study (outlined on page 3) were substantiated by this trial:

(1) The primary efficacy outcome of ALT normalisation at Week 24 was dependent on treatment (P=0.002) with silymarin and antioxidants (SOX). ALT levels were lowered by 43.4% (on average) in this group and this is known to be associated with reduction in hepatic necroinflammation. (aim (1)).

(2) The safety of the trial interventions was independently reviewed in this Study and the products were declared safe in this cohort of chronic hepatitis C participants. (aim (5)).

(3) This Study demonstrated that in the setting of chronic HCV infection, oral *Silybum marianum* in combination with other antioxidants targeting OS and liver inflammation was more effective in normalising ALT levels than *Silybum marianum* alone. Its contribution to the result achieved in the SOX group cannot be quantified. (aim (6)).

(4) ALT normalisation occurred in HCV genotype 1 participants (P=0.008) significantly more frequently than in non genotype 1 participants. This suggests that SOX treatment may be more effective in genotype 1 participants. It had been assumed that those participants with HCV genotypes 2 and 3 would be more responsive to naturopathic treatment ((Chen JJ, oral communication, 2002), also observed in traditional Chinese medicine) as is the case with pegylated interferon and ribavirin. This finding needs independent verification. It requires a study with equal numbers of

186

genotype 1 vs genotype 3 (the two most common HCV genotypes in Australia) and a power calculation based on the genotype differential as the primary outcome measure. (aim (7)).

(5) An improvement in QOL was dependent on treatment (P=0.024) as the MCS score improved significantly in SOX group at the end of the 24-weeks treatment period (P=0.002). (aim (8)).

The following three study aims were not substantiated in this research and future research directions are suggested at the end of this chapter:

(1) There was a trend towards SOX altering OS processes (P=0.071).
In addition, there was a weak positive association between F_2-isoprostanes and both, ALT and FibroTest at Week 24. A larger sample size than that used here, may help determine this association. (aim 2)).

(2) There was no significant reduction in HCV RNA (viral load). The issue of HCV RNA viral load appears dependent on dose and route of administration. (aim (3)).

(3) There was no significant reduction in hepatic fibrosis. The fibrosis outcome may need a longer time frame, e.g., two-five years to assess the effectiveness of an antioxidant intervention. (aim (4)).

A thorough evaluation of the literature was conducted to establish the need for the Hep573 Study. This review has confirmed the selection of interventions and has informed some future research directions.

Secondary Strengths of the Study

The Hep573 Study provided the vehicle for a group of people to gain access to hospital liver outpatient clinics as some, unknown to them, had advanced liver disease and required urgent medical attention. The hospital liver clinic had an alternative treatment protocol to offer hepatitis C participants who were ineligible to participate, or chose not to participate in standard antiviral therapy.

The combination of herbal medicines and vitamins used in the Hep573 Study reflected standard naturopathic protocol in Australia by targeting the multifactorial disease mechanisms of chronic HCV infection.

- As oxidative stress is a major pathway contributing to liver injury, interventions with primarily antioxidant pharmacological actions were chosen.

- Other mechanisms of the hepatitis C disease process were also targeted in the choice of trial interventions. Mechanisms including antifibrotic, anti-inflammatory, hepatoprotective, immune-modulating, as highlighted in the naturopathic protocol in Chapter 2 Literature Review and Table 2.7 and Table 2.8.

LIMITATIONS OF THE STUDY

The calculated sample size of 171 (57 per treatment arm) was not reached, and only 118 participants were recruited. The trial was stopped before sample size was reached because the trial preparations had passed their expiry date and their stability could not be guaranteed. Even though the sample size in this Study was not reached, it still achieved findings of statistical significance primarily in the SOX group.

Liver biopsies were not undertaken to assess whether a change in liver fibrosis stage had occurred as the participating researchers did not anticipate gaining Human Research Ethics Committee clearance. Biopsies may also have deterred some participants from joining the Study. This latter point is supported by the fact that on 1 April, 2006, the Australian Federal Government removed the mandatory requirement of a liver biopsy prior to receiving antiviral therapy. This was an attempt to encourage more Australians with chronic hepatitis C to take up antiviral therapy, than the 2847 who did in 2006;[40] also in light of the HALT-C study[119,473] a measurable change in biopsies would not have been evident in the HEP573 study time frame.

Serum hepatic fibrosis markers were measured in the Hep573 Study, it was acknowledged from the outset that fibrotic changes do not evolve quickly. The HALT-C study[119,473] highlighted the need for long-term treatment to affect fibrosis. This is confirmed by a recent RBPCT study by Fried *et al.*[597] of 154 non responders with HCV infection with an ALT of > 65 U/L. The study concluded that oral silymarin (legalon) at doses of 420 mg TDS and 700mg TDS over a 24 week period did not significantly alter biochemical or virological markers of disease progression or

quality of life scores. The duration of therapy in the Hep573 study was appropriate for monitoring inflammatory activity and the impact of therapy. The failure to show any change in fibrosis markers is not surprising but the measurement of the markers allowed the study to detect change if it had occurred.

Recruitment into the Study was conducted over three years as the enrolments were much slower than expected. The stipulated wash-out period of 12 weeks from any complementary medicines used in the Hep573 Study hindered recruitment. In hindsight, this period was deemed to be excessive and a four-week wash-out period would have sufficed based on an understanding of the half life of phytochemicals used in this Study.

The duration of HCV infection in those recruited was significantly different between groups at baseline (P=0.011). The placebo group had a shorter duration of infection, 21 years (12.5-26), compared to the silymarin group, 25.5 years (20-30) and the silymarin and antioxidant group 23 years (20-29.5). As age of HCV acquisition[152,153] is considered a more reliable prognostic factor than duration of infection in disease progression,[158] the small variation in duration of HCV infection (≤4.5 years) is not considered clinically significant in this Study. [35,158,653]

There was a significant difference in serum globulin levels between the groups at baseline (P=0.014). The silymarin group had higher levels of globulins 37g/L (32-40), compared to both the placebo 33g/L (29-37) and SOX groups 33g/L (32-40). The significance of this is unclear, but it is unlikely the higher level in the silymarin group reflects cirrhosis in this population, as the entire Study population only included 18 participants with cirrhosis (as determined by clinical examination, blood tests, ultrasound or biopsy). In addition, none of the other markers of advanced liver disease such as thrombocytopenia, coagulopathy (INR) as well as the Hepascore and FibroTest data were present at baseline.

There was a significant difference in HCV RNA viral load at baseline across the groups (P=0.047) with silymarin (79.3 x 10^5 viral copies per mL) almost double the levels of the other two: placebo (32.1 x 10^5 copies) and SOX (46.8 x 10^5 copies). Given that any viral load over 600,000 viral copies is regarded as high viral load, the issue of viral load will not be discussed further.

There was a significant difference at baseline in LDL cholesterol (P=0.023). SOX had higher LDL cholesterol at baseline (2.90 mmol/L) compared to placebo (2.31 mmol/L) or silymarin groups (2.05 mmol/L). LDL cholesterol and F_2-isoprostanes were found to be independent predictors of RVR with antiviral

therapy.[522] Higher LD lipoprotein cholesterol levels predict SVR to pegylated interferon and ribavirin in HIV/Hep C coinfected patients.[654] The literature concerning LDL cholesterol allows speculation that the higher LDL cholesterol in the SOX group at baseline may be a factor that contributed to the ALT normalisation and improved QOL in this group. Studies examining this speculative association in CM interventions could prove illuminating.

Whole blood glutathione was not measured because funding for inclusion of this measure unexpectedly fell through once the Study was underway and alternative funding could not be found. This was a limitation of the Study as serum glutathione levels are generally reflective of liver-tissue levels. This set of data would have provided information as to whether the trial ingredients increased the *de novo* synthesis of glutathione, thus elucidating a possible mechanism of action of herbal medicines and vitamins.

The Study used ALT normalisation as its primary outcome measure. The link between ALT and liver histology and the current debate on normal ranges for ALT in men and women is covered in detail in the section 'Normal ALT and liver histology' in Chapter 2. Evidence suggests that disease progression is less likely in patients with persistently normal ALT levels.[425,426,153,166,414,421,423,427] Puoti goes so far as to state that in patients with PNAL, disease progression is halved compared to those with elevated ALT.[418]

The strength of the ALT normalisation achieved in this Study may need to be reevaluated. Using previously reported laboratory standards, 10/39 participants in the SOX group achieved ALT normalisation at Week 24. If the current normal values for ALT (≤30 U/L for men, ≤19 U/L for women) are applied[413,634] then none of participants achieved normalised ALT levels. Applying the most frequently reported normal ALT values in the literature (≤40 U/L for men and ≤31 U/L for women),[413] four out of 10 participants achieved ALT normalisation. Two out of these four participants achieved PNAL within the six-month Study period.

The inclusion of *Hypericum perforatum* (Saint John's wort) was shown to be a limitation to participant recruitment as this excluded many potential participants who were already on antidepressants. It excluded others because of potential drug-herb interactions. Ironically, this herbal medicine along with *Eleutherococcus senticosus* (Siberian ginseng) may have contributed to the improved mental-health status found in the silymarin and antioxidant group. However, this cannot be proved.

The standard formulations used in the Hep573 Study did not reflect the herbal and naturopathic medicine practice in Australia of prescribing treatments tailored to a patient's needs. While it is a strength of this Study that the formulations were standardised across participants (thereby permitting comparisons to be made), it was also a limitation. This did not permit tailoring of treatment to specific participant presentations as would be undertaken in a clinical context, thereby perhaps limiting treatment effectiveness for some participants.

Twenty eight of 118 randomised participants (23.7%) stopped therapy during the treatment phase (in the first 24 weeks). This percentage is slightly higher than the assumed drop-out rate of 20% in the sample-size calculation. Thirty three participants did not finish the full 48-week Study period (27.9%). There were only five participants lost in the follow-up period from Weeks 24-48, but the Study design did not allow for an interpretation of why the drop-out decreased after treatment ended. The drop-out rate in the Hep573 Study was comparable to a recent NAFLD study of CM (41/180, 22.7%).[655]

It was a limitation of this Study that vitamin E was not included in the Hep573 trial preparations as emerging data has now shown vitamin E (800IU)[656] improves liver histology in nonalcoholic steatothepatitis (NASH) patients.[656,657] Hepatic steatosis is a common histologic feature present in 40-86% of CHC participants.[383] Of those with NASH, 15-25% will develop cirrhosis.[383] Both hepatic fibrosis and cirrhosis are the result of repeated liver injury and extracellular matrix deposition which are the recognised endpoints of most liver disease.[383] Prior to this Study, findings about vitamin E were equivocal and, according to Singal, *et al.,*[658] they still are in relation to HCV infection.

As outlined in the methods sections of this thesis, the Hep573 Study comprised of three intervention groups:

(1) Placebo;

(2) Silymarin; and

(3) SOX (silymarin plus antioxidants)

Silymarin was chosen as the intervention arm due to its known hepatoprotective effects. Silymarin is also a confirmed antioxidant[659] and the combined effect of silymarin with antioxidants was determined to be another important intervention arm. The omission of an antioxidant only group is a limitation in this Study as it could have provided important information on the benefit of antioxidants alone.

The standard proprietary product chosen for the SOX arm contained 1000 mg of *S. marianum*, there was no affordable proprietary product available that could have provided a suitable antioxidant only intervention. Also in hindsight, a further division of the participants, given limited recruitment numbers due to a lack of resources, would have reduced the statistical power of the results. Therefore the limitations were driven by a pragmatic response to logistics. As previously stated despite the logistics, the lack of an antioxidant only intervention is a limitation.

COMPARISONS WITH OTHER STUDIES

This is the first randomised double-blind placebo-controlled Study that administered oral silymarin (720 mg silibinin) and oral antioxidants for six months to chronic hepatitis C participants. There are very few sound randomised double-blind studies suitable for comparison with the Hep573 Study. (This has been explored in detail in the literature review.)

The oral silymarin and oral antioxidants in Hep573 Study achieved a similar ALT normalisation rate to a non-randomised oral and intravenous antioxidant combination.[511] Considering that intravenous antioxidants have much greater bioavailability than oral antioxidants, this strengthens the dose and selection of oral antioxidants in the Hep573 Study.

Gordon*, et al.,*[472] found no improvement in ALT, HCV RNA or QOL in their cohort of CHC participants who ingested oral silymarin (600 mg or 1200 mg) for 12 weeks. The extra 12 weeks duration in the Hep573 Study, and the addition of antioxidants combined with silymarin may have contributed to the positive findings in QOL and ALT in this Study.

The original contribution to the literature made by Lang*, et al.,*[103] regarding both the prevalence and clustering of symptoms reported by CHC participants added a further layer of understanding to the complexity of the symptom experience and the deleterious impact of this on the QOL and sense of well-being in this population.

The most prevalent symptoms in the Hep573 Study were compared to the Lang*, et al.,*[103] study; and found to be similar, i.e., poor sleep (71% vs 65%), depression (51.8% vs 70%), irritability (60.5% vs 75%), and poor concentration (50% vs 62%).[103] The two studies[103] had slightly different categories for tiredness. The Hep573 Study included the symptoms 'tiredness' (80%) and 'wake-up tired'

(74%) whereas Lang*, et al.,*[103] included 'physical tiredness' (86%) and 'mental tiredness' (70%). When both categories are combined into percentages, tiredness becomes comparable between the two studies, i.e., 76.7% vs 78% respectively.

The gender comparisons between the two studies highlight the fact that women are more vocal or detailed in their symptom reporting than men. Headaches (63.9% vs 76%), poor concentration (66.7% vs 71%) and nausea (50% vs 65%) tiredness percentages were identical (90%).

IMPLICATIONS OF THE HEP573 STUDY

Two very important achievements in this Study were ALT normalisation which, can be associated with a reduction in hepatic necroinflammation (see the section on "Normal ALT and liver histology" in Chapter 2 for discussion), and an improvement in mental well-being (MCS) in chronic hepatitis C participants (with the administration of oral silymarin and oral antioxidants). A reduction in liver inflammation may reduce the impact and progression of chronic HCV infection. Improved mental well-being is vital to improved QOL in CHC participants. People with improved mental well-being might be content to take CM treatment while waiting for more aggressive/efficacious medical treatment. Improvements in mental-health and vitality may enable and motivate patients to improve self care through positive lifestyle choices. It may also reduce social isolation through greater participation in community activities, further improving clinical outcomes.

The findings may assist a treatment disadvantaged group of CHC participants as those with HCV genotype 1-as ALT normalisation significantly occurred in this group.

Roberts, *et al.*, have shown that commencing treatment early in the disease process can lead to improved SVRs and improved clinical outcomes.[53] They found that HCV genotype 1 patients with early liver disease and minimal fibrosis were more likely to respond to current standard antiviral therapy than those with advanced liver disease.

This idea of improved clinical outcomes if treated early in disease process also applied to silymarin,[446,473,660] and therefore possibly to antioxidants and CM generally. It is postulated the genotype 1 patients in the Hep573 Study who had ALT normalisation, had early liver disease (Child-Pugh <7), and this may be a

contributing factor to their responsiveness to the SOX treatment. This requires independent verification.

At the time of setting up the design of the Hep573 Study, oral administration of silibinin was considered best practice (and still is in Australia). Since then, however, research overseas has extended to intravenous use of silibinin and the results are promising. Polyak*, et al.,* first identified that silymarin had direct anti-HCV activity *in vitro.* [453,661] At the cellular level, silymarin blocks HCV protein and RNA expression (inhibits HCV RNA dependent RNA polymerase)[441] and also blocks virus entry, fusion and transmission.[232,459] The findings of Polyak, *et al.,* [453,661] led Ferenci, *et al.,*[438] to experiment with the intravenous use of silibinin. The first intravenous study of silibinin (1400 mg[438]) in CHC participants (non-responders) sparked a flurry of research,[441,459,458,439,440] as this route of administration showed direct anti-HCV activity and synergy with pegylated interferon and ribavirin. The clinical implications of intravenous silibinin are profound: increased SVR in previous non-responders with pegylated interferon and ribavirin[438,662] and prevention of HCV-reinfection after liver transplantation.[457,663] As the numbers treated in Europe are small, rescue treatment to pegylated interferon and ribavirin non responders (N=27),[662] (N=11),[439] (N=20)[664] and prevention of HCV reinfection after liver transplantation (N=4),[663] it would be important to conduct intravenous silibinin trials in Australia to confirm the promising results achieved in Europe. Given some potentially significant and clinically relevant findings from these preliminary studies of intravenous administration of silibinin, perhaps it would be worthwhile researching the applicability of silibinin administration to the chronic hepatitis C patient in hospital liver outpatient-clinics in Australia.

At this stage, it seems relevant to explore whether a considerably higher dose of oral silibinin than was used in the Hep573 Study (720 mg) might affect viral load in a similar way to that attained by intravenous administration. Perhaps an oral dose equivalent to 2800 mg to 5600 mgs may be found effective as an alternative to intravenous treatment. Unfortunately there may be drawbacks to this, due to practicality and compliance issues of taking 16-32 tablets, based on 720 mg silibinin per tablet.

COMPLEXITIES IN THE HEP573 CLINICAL TRIAL CONDUCT

The Hep573 Study encountered substantial delays because of the length of time it took to obtain Human Research Ethics Committee approval at the various institutions, e.g., 16 months (Hunter New England Research Ethics Committee, University of Newcastle Human Research Ethics Committee) and three and four months respectively at Sydney West Area Health Service Human Research Ethics Committee and Central Sydney Area Health Service Ethics Review Committee (RPAH zone).

Recruitment of participants took longer than anticipated despite the intense interest generated by the CM Study. Nursing-staff shortages at one hospital meant that interest did not translate into corresponding enrolments. Some keen participants from that particular understaffed hospital enrolled at the other two sites in order to participate in the Study.

As previously stated, the complementary medicine paradigm of wholism in case-taking, individualised herbal-medicine prescription and tailored treatment is frequently at odds with the medical model of identifying a single agent responsible for the end result. The standard practice of prescribing herbal liquid extracts matching the identified pharmacological actions required to restore the body to homeostasis was not adopted in this Study as this concept was not approved by the lead research Ethics Committee. Other difficulties related to creating a liquid placebo as all herbal medicines possess therapeutic actions. The issue of possible breakage of the glass amber bottles by participants and the convenience of administration to the participants influenced the final choice of tablets instead of liquids across the three groups.

The ownership of Phytomedicine Pty Ltd changed hands three times during the Study. This became problematic once the Study was completed as the SOX intervention was found to be more effective than the placebo or silymarin. After the Study the ethical obligation was to provide the placebo group with six-months supply of the active preparations used in the Hep573 Study.

The original formulation was not able to be reproduced in the third handover as the Phytomedicine brand was discontinued and the antioxidant compound was no longer produced. Despite these difficulties, the following formulations were provided to the placebo group from Integria HealthCare Pty Ltd (the company that purchased Phytomedicine Pty Ltd): *Phytomedicine Silymarin* (identical formulation

to the Study formulation) and *Nutrimedicine Antiox BioComplex* (with additional ingredients to those studied in the trial). This was by no means ideal, but meant the placebo group received an equivalent formulation for their participation in the Study.

Due to budget constraints, the outsourcing of the Hep573 Study research bloods for processing meant they were analysed by those with expertise in their relevant field; however, this process was subject to lengthy delays (nine months on each occasion) before the results of HCV RNA, Fibrotest and Hepascore were received. This meant the staff at Phytomedicine Pty Ltd were not informed of the Study's results in a timely manner in response to their investment.

A prior understanding of the lengthy time required for recruitment in clinical trials would have allowed the Study Coordinator to negotiate the production of additional stock until the sample size was attained. The inexperience of both the staff at Phytomedicine Pty Ltd and the Study Coordinator in the conduct of a clinical trial meant that voucher specimens of the trial ingredients were not kept.

It was a mammoth undertaking to conduct a complementary medicine trial across three hospital sites without a research assistant or access to the infrastructure and resources of companies involved in clinical research to ensure the independence of the Study research.

FUTURE RESEARCH DIRECTIONS

Based on the Hep573 Study, the following areas have been identified where further research would inform the clinical management of chronic HCV infection.

Oral Silibinin-dose Finding Study

An oral dose of 720 mg silibinin per day administered for six months to CHC participants in the Hep573 Study led to a 41% fall in HCV RNA at Week 48 compared to baseline.

Ferenci*, et al.,*[438] discovered that 1400 mg silibinin daily administered intravenously influenced HCV RNA. *In vitro* data have shown that various commercial extracts of oral silibinin impact HCV RNA replication, fusion and virus entry.[661]

Therefore the current gap in the literature is whether an oral silibinin dose in humans can significantly reduce HCV RNA and this would be the purpose of a

dose-finding oral silibinin study. The extrapolation from the intravenous dose to an oral equivalent dose would appear the logical place to commence an oral dose escalation study.

The oral silibinin-dosing study would involve an escalating dose range from 2800 mg to 5600 mg silibinin daily for 12 months with HCV RNA measured at baseline, Weeks 4, 12, 24, 36 and 48 in the treatment period and at Weeks 60, 72 and 96 in the follow-up period. Other measures would support a pharmacokinetic study assessing the oral bioavailability of silibinin. In addition, plasma glutathione, both reduced and oxidised, would be measured in conjunction with proinflammatory cytokines to ascertain whether silibinin can increase the *de novo* synthesis of glutathione and reduce the pro-inflammatory cytokines thereby attenuating oxidative stress and the cytokine immune-mediated liver injury. The level of ALT normalisation and its percentage change from baseline would also be examined as would the oxidative stress marker, F_2-isoprostanes. FibroTest would also be examined at baseline, 6, 12, 18 and 24 months. If the change in Fibrotest was shown to be significant, there would be justification for a histological study.

The control group would be given an oral dose of 720 mg silibinin, which is a suboptimal dose for HCV RNA reduction. The SF-36 Quality of Life questionnaire would investigate any health-related QOL changes due to the intervention.

Glutathione Enhancement: Nutritional and Silibinin Protocol

Glutathione depletion has been linked to hepatic fibrosis and disease progression in chronic hepatitis C patients.[128] While results with glutathione replenishment have been conflicting,[658] it would be useful to track both plasma glutathione levels and fibrosis markers to ascertain whether disease progression can be modified with interventions that enhance glutathione.

Given the hypothesis that OS is linked to necrosis, inflammation and fibrosis, the addition of nutrients to protect the mitochondria could reduce necrosis and the consequent inflammation and promote cell repair and apoptosis. Silibinin would be an essential ingredient in this proposed study as it plays a paramount role in glutathione restoration in the hepatocyte. A literature review would ascertain the most effective ingredients and dose for mitochondrial support such as co-enzyme Q10, alpha lipoic acid and acetyl-L-carnitine.[665] Recent studies have shown that phytochemicals improve mitochondrial function through hormesis.[254]

The oral silibinin from the above dose-finding study, or if ineffective, then an intravenous 1400 mg silibinin dose for 14 days would be combined with oral antioxidants for 12 months as follows:

(1) glutathione enhancement

    a. silibinin[594,595,596];

    b. L-Glutathione; and

    c. cysteine

(2) mitochondrial support

    a. Co-enzyme Q10[518];

    b. alpha lipoic[541,558,559]; and

    c. acetyl-L-carnitine[665]

(3) antioxidants

    a. Vitamin E succinate (800IU) or RRR tocopherol as per recent NASH study[657];

    b. calcium ascorbate[604];

    c. zinc; and

    d. selenomethionine.[588,590]

The aforementioned outcome measures would be applied to this group.

Glutathione Enhancement: Herbal Medicine Protocol

Phytochemical-based antioxidants at dietary levels[480,481,482,483,484] applied in situations of nutritional deficiency[502] or OS[502,503] are beneficial to health and are involved in mitochondrial hormesis. In addition to oral silibinin or intravenous silibinin, the following phytocemical-based antioxidants (herbal medicines) would be administered to CHC participants: *Andrographis paniculata,*[544,562,566] *Astragalus membranaceus,*[545] *Bupleurum falcatum,*[666] *Camellia sinensis,*[233,571] *Curcuma longa,*[514] *Phyllanthus amarus,*[667] *Schisandra chinensis*[668,669] and *Vitis vinfera.*[609] All these herbal medicines increase *de novo* synthesis of glutathione.

The study design and outcome measures outlined in oral silibinin dose-finding study will be incorporated in this protocol. In both glutathione protocols, the sample-size calculation would be based on the genotype differential as the primary outcome measure. This would confirm whether those with genotype 1 are deficient in glutathione and whether effective treatment depends on genotype.

CHAPTER 6

CONCLUSION

This is the first clinical trial to find that oral silymarin and oral antioxidants normalised ALT in participants with compensated chronic hepatitis C (Child-Pugh ≤7) over 24 weeks. This intervention also significantly improved QOL, specifically psychological well-being (MCS). The predominant phytochemical nature of the antioxidants (broad range and non-synthetic) used in this Study may explain the successful outcomes achieved here compared to other antioxidant trials.

The improvement in QOL and particularly the responsiveness of HCV genotype 1, indicate that silymarin and antioxidants have an important role in future clinical treatment. Also, it is clinically important that oral silymarin was more effective when combined with oral antioxidants than when used alone. SOX interventions could be used as adjuncts in CHC patients and in other diseases where oxidative stress is present such as NAFLD and metabolic syndrome.

Dose and administration of silibinin have emerged as crucial issues in hepatitis C management since the initiation of the Hep573 Study. It is postulated on the basis of bioavailability data in dose comparison studies of CHC patients with cirrhosis, that oral silibinin and oral antioxidants may be more effective if they are introduced earlier rather than later in the disease process. It is also hypothesised that oral daily doses in the range of 2800 mg to 5600 mg of silibinin from 70:1 *Silybum marianum* extract may be required to exert direct anti-HCV activity comparable to intravenous administration.

Further investigation of the trial interventions, along with the testing of intravenous silibinin in CHC patients in Australia need to be independently evaluated in a large multicentre study. This would have important clinical implications if validated.

The checklist for conducting complementary medicine clinical trials (Appendix F) which has arisen out of the Hep573 Study has equipped the Study Coordinator to be involved in the establishment of intravenous silibinin clinical trials

in Australia or, at the very least, this tool will assist other CM practitioners embarking on clinical research.

The original motivation for the Study was to improve clinical treatment for CHC patients and to test the hypothesis that antioxidants may alter liver disease progression in these patients. The ALT normalisation in the SOX intervention represented a reduction in hepatic necroinflammation. The trend towards a reduction in oxidative stress (F_2-isoprostanes) was related to treatment (SOX intervention). Also the direct association, in the Study, between F_2-isoprostanes and both ALT and FibroTest at Week 24 means that antioxidants may alter disease progression. This association needs to be independently validated.

Thus silymarin with antioxidants can be said to offer a rational and scientific approach to counteracting the liver injury caused by oxidative stress in CHC patients and consequently may offer a clinically relevant and cost-effective treatment option compared to that currently on offer.

REFERENCES

1.   Halliwell B. How to characterize a biological antioxidant. Free Radic Res Commun. 1990;9(1):1-32.
2.   Ernst E, Resch KL, Mills S, Hill R, Mitchell A, Willoughby M, et al. Complementary medicine - a definition. Br J Gen Pract. 1995;45(398):506.
3.   D'Amico G, Garcia-Tsao G, Pagliaro L. Natural history and prognostic indicators of survival in cirrhosis: a systematic review of 118 studies. J Hepatol. 2006;44(1):217-31.
4.   Alazawi W, Cunningham M, Dearden J, Foster GR. Systematic review: outcome of compensated cirrhosis due to chronic hepatitis C infection. Aliment Pharmacol Ther. 2010;32(3):344-55.
5.   Sies H. Oxidative stress: oxidants and antioxidants. Exp Physiol. 1997;82(2):291-5.
6.   World Health Organization. International Nonproprietary Names. [Internet] 2012; Available from: http://www.who.int/medicine/services/inn/en.
7.   Kroemer G, Galluzzi L, Vandenabeele P, Abrams J, Alnemri ES, Baehrecke EH, et al. Classification of cell death: recommendations of the Nomenclature Committee on Cell Death 2009. Cell Death Differ. 2009;16(1):3-11.
8.   Malhi H, Gores GJ. Cellular and molecular mechanisms of liver injury. Gastroenterology. 2008;134(6):1641-54.
9.   Halliwell B, Whiteman M. Measuring reactive species and oxidative damage in vivo and in cell culture: how should you do it and what do the results mean? Br J Pharmacol. 2004;142(2):231-55.
10.   Guha IN, Moore K. F2-isoprostanes and the liver. Prostaglandins Other Lipid Mediat. 2003;72(1-2):73-84.
11.   Wichtl M, editor. Herbal drugs and phytopharmaceuticals. A handbook for practice on a scientific basis. 3rd ed. Stuttgart: Medpharm, CRC Press; 2001.
12.   Franco R, Schoneveld OJ, Pappa A, Panayiotidis MI. The central role of glutathione in the pathophysiology of human diseases. Arch Physiol Biochem. 2007;113(4-5):234-58.
13.   WHO. Social determinants of health. 2011 [cited 2011 10/04/2011]; Available from: http://www.who.int/social_determinants/en/.
14.   WHO. Traditional Medicine Fact Sheet 134. 2008 [cited 2009 06/05]; Available from: http://www.who.int/mediacentre/factsheets/fs134/en.
15.   Jain SK, Pemberton PW, Smith A, McMahon RF, Burrows PC, Aboutwerat A, et al. Oxidative stress in chronic hepatitis C: not just a feature of late stage disease. J Hepatol. 2002;36(6):805-11.
16.   Yadav D, Hertan HI, Schweitzer P, Norkus EP, Pitchumoni CS. Serum and liver micronutrient antioxidants and serum oxidative stress in patients with chronic hepatitis C. Am J Gastroenterol. 2002;97(10):2634-9.
17.   Soylu AR, Aydogdu N, Basaran UN, Altaner S, Tarcin O, Gedik N, et al. Antioxidants vitamin E and C attenuate hepatic fibrosis in biliary-obstructed rats. World J Gastroenterol. 2006;12(42):6835-41.
18.   Mourelle M, Franco MT. Erythrocyte defects precede the onset of CCl4-induced liver cirrhosis. Protection by silymarin. Life Sci. 1991;48(11):1083-90.
19.   Trappoliere M, Caligiuri A, Schmid M, Bertolani C, Failli P, Vizzutti F, et al. Silybin, a component of silymarin, exerts anti-inflammatory and anti-fibrogenic effects on human hepatic stellate cells. J Hepatol. 2009;50(6):1102-11.
20.   Okuda M, Li K, Beard MR, Showalter LA, Scholle F, Lemon SM, et al. Mitochondrial injury, oxidative stress, and antioxidant gene expression are induced by hepatitis C virus core protein. Gastroenterology. 2002;122(2):366-75.

21.   Law MG, Dore GJ, Bath N, Thompson S, Crofts N, Dolan K, et al. Modelling hepatitis C virus incidence, prevalence and long-term sequelae in Australia, 2001. Int J Epidemiol. 2003;32(5):717-24.

22.   Hadziyannis SJ, Sette H, Jr., Morgan TR, Balan V, Diago M, Marcellin P, et al. Peginterferon-alpha2a and ribavirin combination therapy in chronic hepatitis C: a randomized study of treatment duration and ribavirin dose. Ann Intern Med. 2004;140(5):346-55.

23.   Choo QL, Kuo G, Weiner AJ, Overby LR, Bradley DW, Houghton M. Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. Science. 1989;244(4902):359-62.

24.   Kuo G, Choo QL, Alter HJ, Gitnick GL, Redeker AG, Purcell RH, et al. An assay for circulating antibodies to a major etiologic virus of human non-A, non-B hepatitis. Science. 1989;244(4902):362-4.

25.   Miyakawa Y, Okamoto H, Mayumi M. Classifying hepatitis C virus genotypes. Mol Med Today. 1995;1(1):20-5.

26.   Feinstone SM, Kapikian AZ, Purcell RH, Alter HJ, Holland PV. Transfusion-associated hepatitis not due to viral hepatitis type A or B. N Engl J Med. 1975;292(15):767-70.

27.   Tedeschi V, Seeff LB. Diagnostic tests for hepatitis C: where are we now? Ann Intern Med. 1995;123(5):383-5.

28.   WHO. Hepatitis C. 2000 [23/11/2008]; Available from: http://www.who.int/mediacentre/factsheets/fs164en.

29.   Webster G, Barnes E, Brown D, Dusheiko G. HCV genotypes--role in pathogenesis of disease and response to therapy. Baillières Best Pract Res Clin Gastroenterol. 2000;14(2):229-40.

30.   Simmonds P, Bukh J, Combet C, Deléage G, Enomoto N, Feinstone S, et al. Consensus proposals for a unified system of nomenclature of hepatitis C virus genotypes. Hepatology. 2005;42(4):962-73.

31.   Farci P, Bukh J, Purcell RH. The quasispecies of hepatitis C virus and the host immune response. Springer Semin Immunopathol. 1997;19(1):5-26.

32.   Department of Health and Ageing. National hepatitis C resource manual. Second ed. Canberra: Commonwealth of Australia; 2008.

33.   Hoofnagle JH. Course and outcome of hepatitis C. Hepatology. 2002;36(5 Suppl 1):S21-9.

34.   Simmonds P. The origin and evolution of hepatitis viruses in humans. J Gen Virol. 2001;82(Pt 4):693-712.

35.   Dore GJ, Law M, MacDonald M, Kaldor JM. Epidemiology of hepatitis C virus infection in Australia. J Clin Virol. 2003;26(2):171-84.

36.   Missiha SB, Ostrowski M, Heathcote EJ. Disease progression in chronic hepatitis C: modifiable and nonmodifiable factors. Gastroenterology. 2008;134(6):1699-714.

37.   Alberti A, Vario A, Ferrari A, Pistis R. Review article: chronic hepatitis C--natural history and cofactors. Aliment Pharmacol Ther. 2005;22 Suppl 2:74-8.

38.   Fattovich G, Bortolotti F, Donato F. Natural history of chronic hepatitis B: special emphasis on disease progression and prognostic factors. J Hepatol. 2008;48(2):335-52.

39.   Bowden DS, Berzsenyi MD. Chronic hepatitis C virus infection: genotyping and its clinical role. Future Microbiol. 2006;1:103-12.

40.   NCHECR. HIV/AIDS, viral hepatitis and sexually transmissible infection in Australia Annual Surveillance Report 2009. National Centre in HIV Epidemiology and Clinical Research and The University of NSW; 2009.

41.   Australian Bureau of Statistics. 3101.0 - Australian Demographic Statistics, December 2009. 2009 [cited 2010 29/08]; Available from: http://www.abs.gov.au/austats/abs@nsf/mf/3101.0.

42.   The Global Burden of Hepatitis C Working Group. Global Burden of Disease (GBD) for Hepatitis C. J Clin Pharmacol. 2004;44(1):20-9.

43. Razali K, Thein HH, Bell J, Cooper-Stanbury M, Dolan K, Dore G, et al. Modelling the hepatitis C virus epidemic in Australia. Drug Alcohol Depend. 2007;91(2-3):228-35.

44. Amin J, O'Connell D, Bartlett M, Tracey E, Kaldor J, Law M, et al. Liver cancer and hepatitis B and C in New South Wales, 1990-2002: a linkage study. Aust N Z J Public Health. 2007;31(5):475-82.

45. NCHECR. HIV/AIDS, viral hepatitis and sexually transmissible infection in Australia Annual Surveillance Report 2008. National Centre in HIV Epidemiology and Clinical Research and the University of NSW; 2008.

46. UNSW. Ministerial Committee on AIDS and Hepatitis C HCV Projections Working Group Report. In: NCHECR, editor.2006.

47. Razali K, Amin J, Dore GJ, Law MG, H. C. V. Projections Working Group. Modelling and calibration of the hepatitis C epidemic in Australia. Stat Methods Med Res. 2009;18(3):253-70.

48. Australian National Council on AIDS Hepatitis C and Related Diseases. A model of care for the management of hepatitis C infection in adults. Canberra: Commonwealth of Australia; 2003.

49. Modi AA, Liang TJ. Hepatitis C: a clinical review. Oral Dis. 2008;14(1):10-4.

50. Simmonds P. Genetic diversity and evolution of hepatitis C virus--15 years on. J Gen Virol. 2004;85(Pt 11):3173-88.

51. El-Zayadi AR, Attia M, Badran HM, El-Tawil A, Zalata K, Barakat E, et al. Non-interferon-based therapy: an option for amelioration of necro-inflammation in hepatitis C patients who cannot afford interferon therapy. Liver Int. 2005;25(4):746-51.

52. Poynard T, Colombo M, Bruix J, Schiff E, Terg R, Flamm S, et al. Peginterferon alfa-2b and ribavirin: effective in patients with hepatitis C who failed interferon alfa/ribavirin therapy. Gastroenterology. 2009;136(5):1618-28.e2.

53. Roberts SK, Weltman MD, Crawford DH, McCaughan GW, Sievert W, Cheng WS, et al. Impact of high-dose peginterferon alfa-2A on virological response rates in patients with hepatitis C genotype 1: a randomized controlled trial. Hepatology. 2009;50(4):1045-55.

54. Gane E. Future hepatitis C virus treatment: interferon-sparing combinations. Liver Int. 2011;31 Suppl 1:62-7.

55. Buti M, Sanchez-Avila F, Lurie Y, Stalgis C, Valdes A, Martell M, et al. Viral kinetics in genotype 1 chronic hepatitis C patients during therapy with 2 different doses of peginterferon alfa-2b plus ribavirin. Hepatology. 2002;35(4):930-6.

56. Akuta N, Suzuki F, Kawamura Y, Yatsuji H, Sezaki H, Suzuki Y, et al. Prediction of response to pegylated interferon and ribavirin in hepatitis C by polymorphisms in the viral core protein and very early dynamics of viremia. Intervirology. 2007;50(5):361-8.

57. Yu JW, Wang GQ, Sun LJ, Li XG, Li SC. Predictive value of rapid virological response and early virological response on sustained virological response in HCV patients treated with pegylated interferon alpha-2a and ribavirin. J Gastroenterol Hepatol. 2007;22(6):832-6.

58. Craxi A. EASL clinical practice guidelines: management of hepatitis C virus infection. J Hepatol. 2011;55:245-64.

59. Cross TJ, Antoniades CG, Harrison PM. Current and future management of chronic hepatitis C infection. Postgrad Med J. 2008;84(990):172-6.

60. Hügle T, Cerny A. Current therapy and new molecular approaches to antiviral treatment and prevention of hepatitis C. Rev Med Virol. 2003;13(6):361-71.

61. McHutchison JG, Fried MW. Current therapy for hepatitis C: pegylated interferon and ribavirin. Clin Liver Dis. 2003;7(1):149-61.

62. Jacobson IM. Treatment options for patients with chronic hepatitis C not responding to initial antiviral therapy. Clin Gastroenterol Hepatol. 2009;7(9):921-30.

63. Di Bisceglie AM, Stoddard AM, Dienstag JL, Shiffman ML, Seeff LB, Bonkovsky HL, et al. Excess mortality in patients with advanced chronic hepatitis C treated with long-term peginterferon. Hepatology.53(4):1100-8.

64.   Bota S, Sporea I, Popescu A, Sirli R, Neghina AM, Danila M, et al. Response to standard of care antiviral treatment in patients with HCV liver cirrhosis - a systematic review. J Gastrointestin Liver Dis.20(3):293-8.

65.   NCHECR. HIV, viral hepatitis and sexually transmissible infections in Australia, Annual Surveillance Report 2010. In: National Centre for HIV Epidemiology and Clinical Research, editor. Sydney: The University of New South Wales; 2010.

66.   The Kirby Institute. HIV, viral hepatitis and sexually transmissible infections in Australia Annual Surveillance Report 2011. Sydney: The Kirby Institute, The Univeristy of NSW; 2011.

67.   Grebely J, Dore GJ. What is killing people with hepatitis C virus infection? Semin Liver Dis. 2011;31(4):331-9.

68.   McGovern BH, Abu Dayyeh BK, Chung RT. Avoiding therapeutic pitfalls: the rational use of specifically targeted agents against hepatitis C infection. Hepatology. 2008;48(5):1700-12.

69.   Asselah T, Benhamou Y, Marcellin P. Protease and polymerase inhibitors for the treatment of hepatitis C. Liver Int. 2009;29 Suppl 1:57-67.

70.   Thompson A, Patel K, Tillman H, McHutchison JG. Directly acting antivirals for the treatment of patients with hepatitis C infection: a clinical development update addressing key future challenges. J Hepatol. 2009;50(1):184-94.

71.   Flisiak R, Parfieniuk A. Investigational drugs for hepatitis C. Expert Opin Investig Drugs. 2010;19(1):63-75.

72.   Lange CM, Sarrazin C, Zeuzem S. Review article: specifically targeted anti-viral therapy for hepatitis C - a new era in therapy. Aliment Pharmacol Ther. 2010;32(1):14-28.

73.   Pockros PJ. New direct-acting antivirals in the development for hepatitis C virus infection. Therap Adv Gastroenterol. 2010;3(3):191-202.

74.   Powdrill MH, Bernatchez JA, Götte M. Inhibitors of the Hepatitis C Virus RNA-Dependent RNA Polymerase NS5B. Viruses. 2010;2(10):2169-95.

75.   Ciesek S, Manns MP. Hepatitis in 2010: the dawn of a new era in HCV therapy. Nat Rev Gastroenterol Hepatol. 2011;8(2):69-71.

76.   Kwong AD, Kauffman RS, Hurter P, Mueller P. Discovery and development of telaprevir: an NS3-4A protease inhibitor for treating genotype 1 chronic hepatitis C virus. Nat Biotechnol. 2011;29(11):993-1003.

77.   McHutchison JG, Manns MP, Muir AJ, Terrault NA, Jacobson IM, Afdhal NH, et al. Telaprevir for previously treated chronic HCV infection. N Engl J Med. 2010;362(14):1292-303.

78.   Pawlotsky JM. The results of Phase III clinical trials with telaprevir and boceprevir presented at the Liver Meeting 2010: a new standard of care for hepatitis C virus genotype 1 infection, but with issues still pending. Gastroenterology. 2011;140(3):746-54.

79.   Matthews SJ, McCoy C. Peginterferon alfa-2a: a review of approved and investigational uses. Clin Ther. 2004;26(7):991-1025.

80.   Manns MP, Wedemeyer H, Cornberg M. Treating viral hepatitis C: efficacy, side effects, and complications. Gut. 2006;55(9):1350-9.

81.   Gentile I, Carleo MA, Borgia F, Castaldo G, Borgia G. The efficacy and safety of telaprevir - a new protease inhibitor against hepatitis C virus. Expert Opin Investig Drugs. 2010;19(1):151-9.

82.   Pianko S, McHutchison JG. Treatment of hepatitis C with interferon and ribavirin. J Gastroenterol Hepatol. 2000;15(6):581-6.

83.   Scott LJ, Perry CM. Interferon-alpha-2b plus ribavirin: a review of its use in the management of chronic hepatitis C. Drugs. 2002;62(3):507-56.

84.   Sarrazin C, Zeuzem S. Resistance to direct antiviral agents in patients with hepatitis C virus infection. Gastroenterology. 2010;138(2):447-62.

85.   Berman K, Kwo PY. Boceprevir, an NS3 protease inhibitor of HCV. Clin Liver Dis. 2009;13(3):429-39.

86.   McHutchison JG, Everson GT, Gordon SC, Jacobson IM, Sulkowski M, Kauffman R, et al. Telaprevir with peginterferon and ribavirin for chronic HCV genotype 1 infection. N Engl J Med. 2009;360(18):1827-38.

87.   Poordad F, Chee GM. Interferon free hepatitis C treatment regimens: the beginning of another era. Curr Gastroenterol Rep. 2012;14(1):74-7.

88.   Foster GR. Quality of life considerations for patients with chronic hepatitis C. J Viral Hepat. 2009;16(9):605-11.

89.   Bjornsson E, Verbaan H, Oksanen A, Fryden A, Johansson J, Friberg S, et al. Health-related quality of life in patients with different stages of liver disease induced by hepatitis C. Scand J Gastroenterol. 2009;44(7):878-87.

90.   Teuber G, Schafer A, Rimpel J, Paul K, Keicher C, Scheurlen M, et al. Deterioration of health-related quality of life and fatigue in patients with chronic hepatitis C: Association with demographic factors, inflammatory activity, and degree of fibrosis. J Hepatol. 2008;49(6):923-9.

91.   Gutteling JJ, de Man RA, Busschbach JJ, Darlington AS. Overview of research on health-related quality of life in patients with chronic liver disease. Neth J Med. 2007;65(7):227-34.

92.   Weissenborn K, Ennen JC, Bokemeyer M, Ahl B, Wurster U, Tillmann H, et al. Monoaminergic neurotransmission is altered in hepatitis C virus infected patients with chronic fatigue and cognitive impairment. Gut. 2006;55(11):1624-30.

93.   Ware JE, Jr., Bayliss MS, Mannocchia M, Davis GL. Health-related quality of life in chronic hepatitis C: impact of disease and treatment response. The Interventional Therapy Group. Hepatology. 1999;30(2):550-5.

94.   Fontana RJ, Hussain KB, Schwartz SM, Moyer CA, Su GL, Lok AS. Emotional distress in chronic hepatitis C patients not receiving antiviral therapy. J Hepatol. 2002;36(3):401-7.

95.   Bonkovsky HL, Woolley JM. Reduction of health-related quality of life in chronic hepatitis C and improvement with interferon therapy. The Consensus Interferon Study Group. Hepatology. 1999;29(1):264-70.

96.   Neary MP, Cort S, Bayliss MS, Ware JE, Jr. Sustained virologic response is associated with improved health-related quality of life in relapsed chronic hepatitis C patients. Semin Liver Dis. 1999;19 Suppl 1:77-85.

97.   Bonkovsky HL, Snow KK, Malet PF, Back-Madruga C, Fontana RJ, Sterling RK, et al. Health-related quality of life in patients with chronic hepatitis C and advanced fibrosis. J Hepatol. 2007;46(3):420-31.

98.   Feurer ID, Wright JK, Payne JL, Kain AC, Wise PE, Hale P, et al. Effects of hepatitis C virus infection and its recurrence after liver transplantation on functional performance and health-related quality of life. J Gastrointest Surg. 2002;6(1):108-15.

99.   Kramer L, Hofer H, Bauer E, Funk G, Formann E, Steindl-Munda P, et al. Relative impact of fatigue and subclinical cognitive brain dysfunction on health-related quality of life in chronic hepatitis C infection. AIDS. 2005;19 Suppl 3:S85-92.

100.   Kramer L, Bauer E, Funk G, Hofer H, Jessner W, Steindl-Munda P, et al. Subclinical impairment of brain function in chronic hepatitis C infection. J Hepatol. 2002;37(3):349-54.

101.   Erim Y, Tagay S, Beckmann M, Bein S, Cicinnati V, Beckebaum S, et al. Depression and protective factors of mental health in people with hepatitis C: a questionnaire survey. Int J Nurs Stud. 2010;47(3):342-9.

102.   Bieliauskas LA, Back-Madruga C, Lindsay KL, Snow KK, Kronfol Z, Lok AS, et al. Clinical relevance of cognitive scores in hepatitis C patients with advanced fibrosis. J Clin Exp Neuropsychol. 2006;28(8):1346-61.

103.   Lang CA, Conrad S, Garrett L, Battistutta D, Cooksley WG, Dunne MP, et al. Symptom prevalence and clustering of symptoms in people living with chronic hepatitis C infection. J Pain Symptom Manage. 2006;31(4):335-44.

104.   Gallegos-Orozco JF, Fuentes AP, Gerardo Argueta J, Perez-Pruna C, Hinojosa-Becerril C, Sixtos-Alonso MS, et al. Health-related quality of life and depression in patients with chronic hepatitis C. Arch Med Res. 2003;34(2):124-9.

105.	Poynard T, Cacoub P, Ratziu V, Myers RP, Dezailles MH, Mercadier A, et al. Fatigue in patients with chronic hepatitis C. J Viral Hepat. 2002;9(4):295-303.

106.	Goh J, Coughlan B, Quinn J, O'Keane JC, Crowe J. Fatigue does not correlate with the degree of hepatitis or the presence of autoimmune disorders in chronic hepatitis C infection. Eur J Gastroenterol Hepatol. 1999;11(8):833-8.

107.	Banks SE, Riley TR, 3rd, Naides SJ. Musculoskeletal complaints and serum autoantibodies associated with chronic hepatitis C and nonalcoholic fatty liver disease. Dig Dis Sci. 2007;52(5):1177-82.

108.	Rodger AJ, Jolley D, Thompson SC, Lanigan A, Crofts N. The impact of diagnosis of hepatitis C virus on quality of life. Hepatology. 1999;30(5):1299-301.

109.	Abdo AA. Hepatitis C and poor quality of life: is it the virus or the patient? Saudi J Gastroenterol. 2008;14(3):109-13.

110.	Helbling B, Overbeck K, Gonvers JJ, Malinverni R, Dufour JF, Borovicka J, et al. Host- rather than virus-related factors reduce health-related quality of life in hepatitis C virus infection. Gut. 2008;57(11):1597-603.

111.	Kwan JW, Cronkite RC, Yiu A, Goldstein MK, Kazis L, Cheung RC. The impact of chronic hepatitis C and co-morbid illnesses on health-related quality of life. Qual Life Res. 2008;17(5):715-24.

112.	Forton DM, Taylor-Robinson SD, Thomas HC. Reduced quality of life in hepatitis C-is it all in the head? J Hepatol. 2002;36(3):435-8.

113.	Younossi Z, Kallman J, Kincaid J. The effects of HCV infection and management on health-related quality of life. Hepatology. 2007;45(3):806-16.

114.	Falasca K, Mancino P, Ucciferri C, Dalessandro M, Manzoli L, Pizzigallo E, et al. Quality of life, depression, and cytokine patterns in patients with chronic hepatitis C treated with antiviral therapy. Clin Invest Med. 2009;32(3):E212-8.

115.	John-Baptiste AA, Tomlinson G, Hsu PC, Krajden M, Heathcote EJ, Laporte A, et al. Sustained responders have better quality of life and productivity compared with treatment failures long after antiviral therapy for hepatitis C. Am J Gastroenterol. 2009;104(10):2439-48.

116.	Fried MW, Jensen DM, Rodriguez-Torres M, Nyberg LM, Di Bisceglie AM, Morgan TR, et al. Improved outcomes in patients with hepatitis C with difficult-to-treat characteristics: randomized study of higher doses of peginterferon alpha-2a and ribavirin. Hepatology. 2008;48(4):1033-43.

117.	Calvaruso V, Mazza M, Almasio PL. Pegylated-interferon-alpha(2a) in clinical practice: how to manage patients suffering from side effects. Expert Opin Drug Saf. 2011;10(3):429-35.

118.	Rambaldi A, Jacobs BP, Gluud C. Milk thistle for alcoholic and/or hepatitis B or C virus liver diseases. Cochrane Database Syst Rev. 2007(4):CD003620.

119.	Seeff LB, Curto TM, Szabo G, Everson GT, Bonkovsky HL, Dienstag JL, et al. Herbal product use by persons enrolled in the hepatitis C Antiviral Long-Term Treatment Against Cirrhosis (HALT-C) Trial. Hepatology. 2008;47(2):605-12.

120.	Carboon I. Rethinking the evidence imperative: why patients choose complementary and alternative medicine. Leuk Lymphoma. 2008;49(2):181-2.

121.	Ritenbaugh C, Nichter M, Nichter MA, Kelly KL, Sims CM, Bell IR, et al. Developing a patient-centered outcome measure for complementary and alternative medicine therapies I: defining content and format. BMC Complement Altern Med. 2011;11:135.

122.	Bates A, Wilkinson J. Perceptions of patients with cancer attending a natural health retreat. JATMS. 2009;l5(3):153-9.

123.	MacLennan AH, Myers SP, Taylor AW. The continuing use of complementary and alternative medicine in South Australia: costs and beliefs in 2004. Med J Aust. 2006;184(1):27-31.

124.	MacLennan AH, Wilson DH, Taylor AW. The escalating cost and prevalence of alternative medicine. Prev Med. 2002;35(2):166-73.

125.	Xue CC, Zhang AL, Lin V, Da Costa C, Story DF. Complementary and alternative medicine use in Australia: a national population-based survey. J Altern Complement Med. 2007;13(6):643-50.

126.   Seeff LB, Lindsay KL, Bacon BR, Kresina TF, Hoofnagle JH. Complementary and alternative medicine in chronic liver disease. Hepatology. 2001;34(3):595-603.
127.   Bruguera M, Barrera JM, Ampurdanés S, Forns X, Sánchez Tapias JM. [Use of complementary and alternative medicine in patients with chronic hepatitis C]. Med Clin (Barc). 2004;122(9):334-5.
128.   Bandara P, George J, McCaughan G, Naidoo D, Lux O, Salonikas C, et al. Antioxidant levels in peripheral blood, disease activity and fibrotic stage in chronic hepatitis C. Liver Int. 2005;25(3):518-26.
129.   Mills SY. The complete guide to modern herbalism. Third ed. London: Thorsons; 1994.
130.   Lin V, Bensoussan A, Myers SP, McCabe P, Cohen M, Hill S, et al. The Practice and Regulatory Requirements of Naturopathy and Western Herbal Medicine. In: Funded by Department of Human Services Victoria, editor. Melbourne: LaTrobe University School of Public Health; 2005.
131.   Department of Health and Aging. Acronyms & glossary. Canberra: Commonweath of Australia; 2011.
132.   Evans M. A guide to herbal remedies: Wigmore Publications; 1990.
133.   Evans S. Changing the knowledge base in Western herbal medicine. Soc Sci Med. 2008:1-9.
134.   Kirschner M, Gerhart J, Mitchison T. Molecular "vitalism". Cell. 2000;100(1):79-88.
135.   Trickey R. Women, hormones and the menstrual cycle. Herbal and medical solutions from adolesence to menopause. Second ed. Sydney: Allen and Unwin; 2003.
136.   Mills SY. The essential book of herbal medicine. Second ed. London: Arkana; 1993.
137.   Bensoussan A, Talley NJ, Hing M, Menzies R, Guo A, Ngu M. Treatment of irritable bowel syndrome with Chinese herbal medicine: a randomized controlled trial. JAMA. 1998;280(18):1585-9.
138.   Seaton K. Naturopathic philosophy. In: Hechtman L, editor. Clinical Naturopathic Medicine. Sydney: Elsevier; 2011. p. 2-13.
139.   Zeff J, Snider P, Pizzorno J. Philosophy of Natural Medicine. The Textbook of Natural Medicine. St Loius: Churchill Livingstone, Elsevier; 2006.
140.   Batchelder H.J., Hudson T. Naturopathic Specific Condition Review Treatment: Viral Hepatitis. Protocol J Bot Med. 1995;1(2):138-40.
141.   Scalzo R. Therapeutic Botanical Protocol for Viral Hepatitis. Protocol J Bot Med. 1995;1(2):159.
142.   Salmond SJ. The hepatobiliary system. In: Hechtman L, editor. Clinical Naturopathic Medicine. Sydney: Elsevier; 2011. p. 210-79.
143.   Kamal SM. Acute hepatitis C: a systematic review. Am J Gastroenterol. 2008;103(5):1283-97; quiz 98.
144.   Vogel W. Treatment of acute hepatitis C virus infection. J Hepatol. 1999;31 Suppl 1:189-92.
145.   Thomas DL, Seeff LB. Natural history of hepatitis C. Clin Liver Dis. 2005;9(3):383-98, vi.
146.   Bowen DG, Walker CM. Adaptive immune responses in acute and chronic hepatitis C virus infection. Nature. 2005;436(7053):946-52.
147.   Schuppan D, Krebs A, Bauer M, Hahn EG. Hepatitis C and liver fibrosis. Cell Death Differ. 2003;10 Suppl 1:S59-67.
148.   Albanis E, Friedman SL. Diagnosis of hepatic fibrosis in patients with chronic hepatitis C. Clin Liver Dis. 2006;10(4):821-33.
149.   Barrett S, Goh J, Coughlan B, Ryan E, Stewart S, Cockram A, et al. The natural course of hepatitis C virus infection after 22 years in a unique homogenous cohort: spontaneous viral clearance and chronic HCV infection. Gut. 2001;49(3):423-30.
150.   Marcellin P. Hepatitis C: the clinical spectrum of the disease. J Hepatol. 1999;31 Suppl 1:9-16.
151.   Farrell GC. Chronic viral hepatitis. Med J Aust. 1998;168(12):619-26.

152.	Poynard T, Bedossa P, Opolon P. Natural history of liver fibrosis progression in patients with chronic hepatitis C. The OBSVIRC, METAVIR, CLINIVIR, and DOSVIRC groups. Lancet. 1997;349(9055):825-32.
153.	Poynard T, Ratziu V, Charlotte F, Goodman Z, McHutchison J, Albrecht J. Rates and risk factors of liver fibrosis progression in patients with chronic hepatitis C. J Hepatol. 2001;34(5):730-9.
154.	Lawson A, Ryder SD. Progression of hepatic fibrosis in chronic hepatitis C and the need for treatment in mild disease. Eur J Gastroenterol Hepatol. 2006;18(4):343-7.
155.	Ghany MG, Kleiner DE, Alter H, Doo E, Khokar F, Promrat K, et al. Progression of fibrosis in chronic hepatitis C. Gastroenterology. 2003;124(1):97-104.
156.	Massard J, Ratziu V, Thabut D, Moussalli J, Lebray P, Benhamou Y, et al. Natural history and predictors of disease severity in chronic hepatitis C. J Hepatol. 2006;44(1 Suppl):S19-24.
157.	Ryder SD, Irving WL, Jones DA, Neal KR, Underwood JC. Progression of hepatic fibrosis in patients with hepatitis C: a prospective repeat liver biopsy study. Gut. 2004;53(3):451-5.
158.	Thein HH, Yi Q, Dore GJ, Krahn MD. Estimation of stage-specific fibrosis progression rates in chronic hepatitis C virus infection: a meta-analysis and meta-regression. Hepatology. 2008;48(2):418-31.
159.	Ortiz V, Berenguer M, Rayon JM, Carrasco D, Berenguer J. Contribution of obesity to hepatitis C-related fibrosis progression. Am J Gastroenterol. 2002;97(9):2408-14.
160.	Friedenberg F, Pungpapong S, Zaeri N, Braitman LE. The impact of diabetes and obesity on liver histology in patients with hepatitis C. Diabetes Obes Metab. 2003;5(3):150-5.
161.	Castéra L, Pawlotsky JM, Dhumeaux D. Worsening of steatosis and fibrosis progression in hepatitis C. Gut. 2003;52(10):1531.
162.	Cheruvu S, Marks K, Talal AH. Understanding the pathogenesis and management of hepatitis B/HIV and hepatitis B/hepatitis C virus coinfection. Clin Liver Dis. 2007;11(4):917-43, ix-x.
163.	Minola E, Prati D, Suter F, Maggiolo F, Caprioli F, Sonzogni A, et al. Age at infection affects the long-term outcome of transfusion-associated chronic hepatitis C. Blood. 2002;99(12):4588-91.
164.	Wright M, Goldin R, Fabre A, Lloyd J, Thomas H, Trepo C, et al. Measurement and determinants of the natural history of liver fibrosis in hepatitis C virus infection: a cross sectional and longitudinal study. Gut. 2003;52(4):574-9.
165.	Ratziu V, Munteanu M, Charlotte F, Bonyhay L, Poynard T. Fibrogenic impact of high serum glucose in chronic hepatitis C. J Hepatol. 2003;39(6):1049-55.
166.	Collier JD, Woodall T, Wight DG, Shore S, Gimson AE, Alexander GJ. Predicting progressive hepatic fibrosis stage on subsequent liver biopsy in chronic hepatitis C virus infection. J Viral Hepat. 2005;12(1):74-80.
167.	Fujiwara A, Sakaguchi K, Fujioka S, Iwasaki Y, Senoh T, Nishimura M, et al. Fibrosis progression rates between chronic hepatitis B and C patients with elevated alanine aminotransferase levels. J Gastroenterol. 2008;43(6):484-91.
168.	Fartoux L, Chazouilleres O, Wendum D, Poupon R, Serfaty L. Impact of steatosis on progression of fibrosis in patients with mild hepatitis C. Hepatology. 2005;41(1):82-7.
169.	Hui JM, Sud A, Farrell GC, Bandara P, Byth K, Kench JG, et al. Insulin resistance is associated with chronic hepatitis C virus infection and fibrosis progression [corrected]. Gastroenterology. 2003;125(6):1695-704.
170.	Wiley TE, McCarthy M, Breidi L, Layden TJ. Impact of alcohol on the histological and clinical progression of hepatitis C infection. Hepatology. 1998;28(3):805-9.
171.	Ostapowicz G, Watson KJ, Locarnini SA, Desmond PV. Role of alcohol in the progression of liver disease caused by hepatitis C virus infection. Hepatology. 1998;27(6):1730-5.

172.   Harris DR, Gonin R, Alter HJ, Wright EC, Buskell ZJ, Hollinger FB, et al. The relationship of acute transfusion-associated hepatitis to the development of cirrhosis in the presence of alcohol abuse. Ann Intern Med. 2001;134(2):120-4.
173.   Mori M, Hara M, Wada I, Hara T, Yamamoto K, Honda M, et al. Prospective study of hepatitis B and C viral infections, cigarette smoking, alcohol consumption, and other factors associated with hepatocellular carcinoma risk in Japan. Am J Epidemiol. 2000;151(2):131-9.
174.   Pessione F, Ramond MJ, Njapoum C, Duchatelle V, Degott C, Erlinger S, et al. Cigarette smoking and hepatic lesions in patients with chronic hepatitis C. Hepatology. 2001;34(1):121-5.
175.   Hezode C, Lonjon I, Roudot-Thoraval F, Mavier JP, Pawlotsky JM, Zafrani ES, et al. Impact of smoking on histological liver lesions in chronic hepatitis C. Gut. 2003;52(1):126-9.
176.   Teixeira-Clerc F, Julien B, Grenard P, Tran Van Nhieu J, Deveaux V, Li L, et al. CB1 cannabinoid receptor antagonism: a new strategy for the treatment of liver fibrosis. Nat Med. 2006;12(6):671-6.
177.   Sylvestre DL, Clements BJ, Malibu Y. Cannabis use improves retention and virological outcomes in patients treated for hepatitis C. Eur J Gastroenterol Hepatol. 2006;18(10):1057-63.
178.   Hezode C, Zafrani ES, Roudot-Thoraval F, Costentin C, Hessami A, Bouvier-Alias M, et al. Daily cannabis use: a novel risk factor of steatosis severity in patients with chronic hepatitis C. Gastroenterology. 2008;134(2):432-9.
179.   Ishida JH, Peters MG, Jin C, Louie K, Tan V, Bacchetti P, et al. Influence of cannabis use on severity of hepatitis C disease. Clin Gastroenterol Hepatol. 2008;6(1):69-75.
180.   Choi J, Ou JH. Mechanisms of liver injury. III. Oxidative stress in the pathogenesis of hepatitis C virus. Am J Physiol Gastrointest Liver Physiol. 2006;290(5):G847-51.
181.   Hui JM, Kench J, Farrell GC, Lin R, Samarasinghe D, Liddle C, et al. Genotype-specific mechanisms for hepatic steatosis in chronic hepatitis C infection. J Gastroenterol Hepatol. 2002;17(8):873-81.
182.   Kumar D, Farrell GC, Fung C, George J. Hepatitis C virus genotype 3 is cytopathic to hepatocytes: Reversal of hepatic steatosis after sustained therapeutic response. Hepatology. 2002;36(5):1266-72.
183.   Chitturi S, George J. Hepatitis C and overweight. Hot Topics in Viral Hepatitis. 2006;2:15-21.
184.   Bugianesi E, Marchesini G, Gentilcore E, Cua IH, Vanni E, Rizzetto M, et al. Fibrosis in genotype 3 chronic hepatitis C and nonalcoholic fatty liver disease: Role of insulin resistance and hepatic steatosis. Hepatology. 2006;44(6):1648-55.
185.   Harrison SA. Insulin resistance among patients with chronic hepatitis C: etiology and impact on treatment. Clin Gastroenterol Hepatol. 2008;6(8):864-76.
186.   Cheung O, Sanyal AJ. Hepatitis C infection and nonalcoholic fatty liver disease. Clin Liver Dis. 2008;12(3):573-85, viii-ix.
187.   Castéra L, Hézode C, Roudot-Thoraval F, Lonjon I, Zafrani ES, Pawlotsky JM, et al. Effect of antiviral treatment on evolution of liver steatosis in patients with chronic hepatitis C: indirect evidence of a role of hepatitis C virus genotype 3 in steatosis. Gut. 2004;53(3):420-4.
188.   Sud A, Hui JM, Farrell GC, Bandara P, Kench JG, Fung C, et al. Improved prediction of fibrosis in chronic hepatitis C using measures of insulin resistance in a probability index. Hepatology. 2004;39(5):1239-47.
189.   Leandro G, Mangia A, Hui J, Fabris P, Rubbia-Brandt L, Colloredo G, et al. Relationship between steatosis, inflammation, and fibrosis in chronic hepatitis C: a meta-analysis of individual patient data. Gastroenterology. 2006;130(6):1636-42.
190.   Björnsson E, Angulo P. Hepatitis C and steatosis. Arch Med Res. 2007;38(6):621-7.

191.   Lonardo A, Adinolfi LE, Loria P, Carulli N, Ruggiero G, Day CP. Steatosis and hepatitis C virus: mechanisms and significance for hepatic and extrahepatic disease. Gastroenterology. 2004;126(2):586-97.

192.   Hung CH, Lee CM, Kuo FY, Jiang SR, Hu TH, Chen CH, et al. Steatosis correlates with hepatic expression of death receptors and activation of nuclear factor-kappaB in chronic hepatitis C. Liver Int. 2008;28(3):339-46.

193.   Douglas MW, George J. Molecular mechanisms of insulin resistance in chronic hepatitis C. World J Gastroenterol. 2009;15(35):4356-64.

194.   Hung CH, Lee CM, Lu SN. Hepatitis C virus-associated insulin resistance: pathogenic mechanisms and clinical implications. Expert Rev Anti Infect Ther. 2011;9(5):525-33.

195.   Kaddai V, Negro F. Current understanding of insulin resistance in hepatitis C. Expert Rev Gastroenterol Hepatol. 2011;5(4):503-16.

196.   Mehta SH, Brancati FL, Sulkowski MS, Strathdee SA, Szklo M, Thomas DL. Prevalence of type 2 diabetes mellitus among persons with hepatitis C virus infection in the United States. Ann Intern Med. 2000;133(8):592-9.

197.   Bahtiyar G, Shin JJ, Aytaman A, Sowers JR, McFarlane SI. Association of diabetes and hepatitis C infection: epidemiologic evidence and pathophysiologic insights. Curr Diab Rep. 2004;4(3):194-8.

198.   Ratziu V, Trabut JB, Poynard T. Fat, diabetes, and liver injury in chronic hepatitis C. Curr Gastroenterol Rep. 2004;6(1):22-9.

199.   Gopaul NK, Anggård EE, Mallet AI, Betteridge DJ, Wolff SP, Nourooz-Zadeh J. Plasma 8-epi-PGF2 alpha levels are elevated in individuals with non-insulin dependent diabetes mellitus. FEBS Lett. 1995;368(2):225-9.

200.   Suzuki A, Angulo P, St Sauver J, Muto A, Okada T, Lindor K. Light to moderate alcohol consumption is associated with lower frequency of hypertransaminasemia. Am J Gastroenterol. 2007;102(9):1912-9.

201.   Schiff ER. Hepatitis C and alcohol. Hepatology. 1997;26(3 Suppl 1):39S-42S.

202.   Monto A, Patel K, Bostrom A, Pianko S, Pockros P, McHutchison JG, et al. Risks of a range of alcohol intake on hepatitis C-related fibrosis. Hepatology. 2004;39(3):826-34.

203.   Khan MH, Thomas L, Byth K, Kench J, Weltman M, George J, et al. How much does alcohol contribute to the variability of hepatic fibrosis in chronic hepatitis C? J Gastroenterol Hepatol. 1998;13(4):419-26.

204.   Lederer SL, Walters KA, Proll S, Paeper B, Robinzon S, Boix L, et al. Distinct cellular responses differentiating alcohol- and hepatitis C virus-induced liver cirrhosis. Virol J. 2006;3:98.

205.   Corrao G, Torchio P, Zambon A, Ferrari P, Arico S, di Orio F. Exploring the combined action of lifetime alcohol intake and chronic hepatotropic virus infections on the risk of symptomatic liver cirrhosis. Collaborative Groups for the Study of Liver Diseases in Italy. Eur J Epidemiol. 1998;14(5):447-56.

206.   Romero-Gómez M, Grande L, Nogales MC, Fernández M, Chavez M, Castro M. Intrahepatic hepatitis C virus replication is increased in patients with regular alcohol consumption. Dig Liver Dis. 2001;33(8):698-702.

207.   Pessione F, Degos F, Marcellin P, Duchatelle V, Njapoum C, Martinot-Peignoux M, et al. Effect of alcohol consumption on serum hepatitis C virus RNA and histological lesions in chronic hepatitis C. Hepatology. 1998;27(6):1717-22.

208.   Cromie SL, Jenkins PJ, Bowden DS, Dudley FJ. Chronic hepatitis C: effect of alcohol on hepatitic activity and viral titre. J Hepatol. 1996;25(6):821-6.

209.   McCartney EM, Beard MR. Impact of alcohol on hepatitis C virus replication and interferon signaling. World J Gastroenterol. 2010;16(11):1337-43.

210.   Vidali M, Occhino G, Ivaldi A, Rigamonti C, Sartori M, Albano E. Combination of oxidative stress and steatosis is a risk factor for fibrosis in alcohol-drinking patients with chronic hepatitis C. Am J Gastroenterol. 2008;103(1):147-53.

211.   Wang JH, Batey RG, George J. Role of ethanol in the regulation of hepatic stellate cell function. World J Gastroenterol. 2006;12(43):6926-32.

212.   Szabo G, Dolganiuc A, Mandrekar P, White B. Inhibition of antigen-presenting cell functions by alcohol: implications for hepatitis C virus infection. Alcohol. 2004;33(3):241-9.

213.   Tabone M, Sidoli L, Laudi C, Pellegrino S, Rocca G, Della Monica P, et al. Alcohol abstinence does not offset the strong negative effect of lifetime alcohol consumption on the outcome of interferon therapy. J Viral Hepat. 2002;9(4):288-94.

214.   Loguercio C, Di Pierro M, Di Marino MP, Federico A, Disalvo D, Crafa E, et al. Drinking habits of subjects with hepatitis C virus-related chronic liver disease: prevalence and effect on clinical, virological and pathological aspects. Alcohol Alcohol. 2000;35(3):296-301.

215.   Okazaki T, Yoshihara H, Suzuki K, Yamada Y, Tsujimura T, Kawano K, et al. Efficacy of interferon therapy in patients with chronic hepatitis C. Comparison between non-drinkers and drinkers. Scand J Gastroenterol. 1994;29(11):1039-43.

216.   Chang A, Skole K, Gautam M, Schmutz J, Black M, Thomas R, et al. The impact of past alcohol use on treatment response rates in patients with chronic hepatitis C. Aliment Pharmacol Ther. 2005;22(8):701-6.

217.   Chen CM, Yoon YH, Yi HY, Lucas DL. Alcohol and hepatitis C mortality among males and females in the United States: a life table analysis. Alcohol Clin Exp Res. 2007;31(2):285-92.

218.   Seeff LB, Buskell-Bales Z, Wright EC, Durako SJ, Alter HJ, Iber FL, et al. Long-term mortality after transfusion-associated non-A, non-B hepatitis. The National Heart, Lung, and Blood Institute Study Group. N Engl J Med. 1992;327(27):1906-11.

219.   Peters MG, Terrault NA. Alcohol use and hepatitis C. Hepatology. 2002;36(5 Suppl 1):S220-5.

220.   Mas VR, Fassnacht R, Archer KJ, Maluf D. Molecular mechanisms involved in the interaction effects of alcohol and hepatitis C virus in liver cirrhosis. Mol Med. 2010;16(7-8):287-97.

221.   Vento S, Cainelli F. Does hepatitis C virus cause severe liver disease only in people who drink alcohol? Lancet Infect Dis. 2002;2(5):303-9.

222.   Hezode C, Lonjon I, Roudot-Thoraval F, Pawlotsky JM, Zafrani ES, Dhumeaux D. Impact of moderate alcohol consumption on histological activity and fibrosis in patients with chronic hepatitis C, and specific influence of steatosis: a prospective study. Aliment Pharmacol Ther. 2003;17(8):1031-7.

223.   Liangpunsakul S, Lai X, Ringham HN, Crabb DW, Witzmann FA. Serum Proteomic Profiles In Subjects with Heavy Alcohol Abuse. J Proteomics Bioinform. 2009;2:236-43.

224.   Bradley KA, DeBenedetti AF, Volk RJ, Williams EC, Frank D, Kivlahan DR. AUDIT-C as a brief screen for alcohol misuse in primary care. Alcohol Clin Exp Res. 2007;31(7):1208-17.

225.   Bush K, Kivlahan DR, McDonell MB, Fihn SD, Bradley KA. The AUDIT alcohol consumption questions (AUDIT-C): an effective brief screening test for problem drinking. Ambulatory Care Quality Improvement Project (ACQUIP). Alcohol Use Disorders Identification Test. Arch Intern Med. 1998;158(16):1789-95.

226.   Choi J. Oxidative stress, endogenous antioxidants, alcohol, and hepatitis C: pathogenic interactions and therapeutic considerations. Free Radic Biol Med. 2012; 52(7):1135-50. Available from: http://www.sciencedirect.com/science/article/pii/S0891584912000366.

227.   Lu JM, Lin PH, Yao Q, Chen C. Chemical and molecular mechanisms of antioxidants: experimental approaches and model systems. J Cell Mol Med. 2010;14(4):840-60.

228.   Conde de la Rosa L, Schoemaker MH, Vrenken TE, Buist-Homan M, Havinga R, Jansen PL, et al. Superoxide anions and hydrogen peroxide induce hepatocyte death by different mechanisms: involvement of JNK and ERK MAP kinases. J Hepatol. 2006;44(5):918-29.

229.   Boya P, de la Pena A, Beloqui O, Larrea E, Conchillo M, Castelruiz Y, et al. Antioxidant status and glutathione metabolism in peripheral blood mononuclear cells from patients with chronic hepatitis C. J Hepatol. 1999;31(5):808-14.

230.   Finkel T, Holbrook NJ. Oxidants, oxidative stress and the biology of ageing. Nature. 2000;408(6809):239-47.

231.   Sasaki Y. Does oxidative stress participate in the development of hepatocellular carcinoma? J Gastroenterol. 2006;41(12):1135-48.

232.   Polyak SJ, Morishima C, Lohmann V, Pal S, Lee DY, Liu Y, et al. Identification of hepatoprotective flavonolignans from silymarin. Proc Natl Acad Sci USA. 2010;107(13):5995-9.

233.   Yumei F, Zhou Y, Zheng S, Chen A. The antifibrogenic effect of (-)-epigallocatechin gallate results from the induction of de novo synthesis of glutathione in passaged rat hepatic stellate cells. Lab Invest. 2006;86(7):697-709.

234.   Inami S, Takano M, Yamamoto M, Murakami D, Tajika K, Yodogawa K, et al. Tea catechin consumption reduces circulating oxidized low-density lipoprotein. Int Heart J. 2007;48(6):725-32.

235.   Khachik F, Carvalho L, Bernstein PS, Muir GJ, Zhao DY, Katz NB. Chemistry, distribution, and metabolism of tomato carotenoids and their impact on human health. Exp Biol Med (Maywood). 2002;227(10):845-51.

236.   Zaini RG, Brandt K, Clench MR, Le Maitre CL. Effects of bioactive compounds from carrots (Daucus carota L.), polyacetylenes, beta-carotene and lutein on human lymphoid leukaemia cells. Anticancer Agents Med Chem. 2012;12(6):640-52.

237.   Adhikari S, Indira Priyadarsini K, Mukherjee T. Physico-chemical studies on the evaluation of the antioxidant activity of herbal extracts and active principles of some Indian medicinal plants. J Clin Biochem Nutr. 2007;40(3):174-83.

238.   Filomeni G, Rotilio G, Ciriolo MR. Disulfide relays and phosphorylative cascades: partners in redox-mediated signaling pathways. Cell Death Differ. 2005;12(12):1555-63.

239.   Circu ML, Aw TY. Reactive oxygen species, cellular redox systems, and apoptosis. Free Radic Biol Med. 2010;48(6):749-62.

240.   Yuan L, Kaplowitz N. Glutathione in liver diseases and hepatotoxicity. Mol Aspects Med. 2009;30(1-2):29-41.

241.   Moran LK, Gutteridge JM, Quinlan GJ. Thiols in cellular redox signalling and control. Curr Med Chem. 2001;8(7):763-72.

242.   Townsend DM, Tew KD, Tapiero H. The importance of glutathione in human disease. Biomed Pharmacother. 2003;57(3-4):145-55.

243.   Hansen JM, Zhang H, Jones DP. Differential oxidation of thioredoxin-1, thioredoxin-2, and glutathione by metal ions. Free Radic Biol Med. 2006;40(1):138-45.

244.   Michelet F, Gueguen R, Leroy P, Wellman M, Nicolas A, Siest G. Blood and plasma glutathione measured in healthy subjects by HPLC: relation to sex, aging, biological variables, and life habits. Clin Chem. 1995;41(10):1509-17.

245.   Peters WH, van Schaik A, Peters JH, van Goor H. Oxidised- and total non-protein bound glutathione and related thiols in gallbladder bile of patients with various gastrointestinal disorders. BMC Gastroenterol. 2007;7:7.

246.   Cabre M, Camps J, Paternain JL, Ferre N, Joven J. Time-course of changes in hepatic lipid peroxidation and glutathione metabolism in rats with carbon tetrachloride-induced cirrhosis. Clin Exp Pharmacol Physiol. 2000;27(9):694-9.

247.   Mari M, Colell A, Morales A, von Montfort C, Garcia-Ruiz C, Fernandez-Checa JC. Redox control of liver function in health and disease. Antioxid Redox Signal. 2010;12(11):1295-331.

248.   Kaplowitz N. The importance and regulation of hepatic glutathione. Yale J Biol Med. 1981;54(6):497-502.

249.   Dickinson DA, Moellering DR, Iles KE, Patel RP, Levonen AL, Wigley A, et al. Cytoprotection against oxidative stress and the regulation of glutathione synthesis. Biol Chem. 2003;384(4):527-37.

250.   Han D, Hanawa N, Saberi B, Kaplowitz N. Mechanisms of liver injury. III. Role of glutathione redox status in liver injury. Am J Physiol Gastrointest Liver Physiol. 2006;291(1):G1-7.

251.   Schafer FQ, Buettner GR. Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. Free Radic Biol Med. 2001;30(11):1191-212.

252.   Valko M, Leibfritz D, Moncol J, Cronin MT, Mazur M, Telser J. Free radicals and antioxidants in normal physiological functions and human disease. Int J Biochem Cell Biol. 2007;39(1):44-84.

253.   Lu SC. Regulation of hepatic glutathione synthesis: current concepts and controversies. FASEB J. 1999;13(10):1169-83.

254.   Son TG, Camandola S, Mattson MP. Hormetic dietary phytochemicals. Neuromolecular Med. 2008;10(4):236-46.

255.   Lindenbach BD, Rice CM. Unravelling hepatitis C virus replication from genome to function. Nature. 2005;436(7053):933-8.

256.   Penin F, Dubuisson J, Rey FA, Moradpour D, Pawlotsky JM. Structural biology of hepatitis C virus. Hepatology. 2004;39(1):5-19.

257.   Zeisel MB, Fofana I, Fafi-Kremer S, Baumert TF. Hepatitis C virus entry into hepatocytes: molecular mechanisms and targets for antiviral therapies. J Hepatol. 2011;54(3):566-76.

258.   Post J, Ratnarajah S, Lloyd AR. Immunological determinants of the outcomes from primary hepatitis C infection. Cell Mol Life Sci. 2009;66(5):733-56.

259.   Boonstra A, van der Laan LJ, Vanwolleghem T, Janssen HL. Experimental models for hepatitis C viral infection. Hepatology. 2009;50(5):1646-55.

260.   Liang SL. An overview of current practice in hepatitis C testing. MLO Med Lab Obs. 2008;40(6):14-6, 8-9.

261.   Welbourn S, Pause A. The hepatitis C virus NS2/3 protease. Curr Issues Mol Biol. 2007;9(1):63-9.

262.   Ishii S, Koziel MJ. Immune responses during acute and chronic infection with hepatitis C virus. Clin Immunol. 2008;128(2):133-47.

263.   Hassan M, Selimovic D, Ghozlan H, Abdel-Kader O. Induction of high-molecular-weight (HMW) tumor necrosis factor(TNF) alpha by hepatitis C virus (HCV) non-structural protein 3 (NS3) in liver cells is AP-1 and NF-kappaB-dependent activation. Cell Signal. 2007;19(2):301-11.

264.   Egger D, Wolk B, Gosert R, Bianchi L, Blum HE, Moradpour D, et al. Expression of hepatitis C virus proteins induces distinct membrane alterations including a candidate viral replication complex. J Virol. 2002;76(12):5974-84.

265.   Enomoto N, Sakuma I, Asahina Y, Kurosaki M, Murakami T, Yamamoto C, et al. Mutations in the nonstructural protein 5A gene and response to interferon in patients with chronic hepatitis C virus 1b infection. N Engl J Med. 1996;334(2):77-81.

266.   Bowen DG, Walker CM. The origin of quasispecies: cause or consequence of chronic hepatitis C viral infection? J Hepatol. 2005;42(3):408-17.

267.   Domingo E, Gomez J. Quasispecies and its impact on viral hepatitis. Virus Res. 2007;127(2):131-50.

268.   Griffin SD, Beales LP, Clarke DS, Worsfold O, Evans SD, Jaeger J, et al. The p7 protein of hepatitis C virus forms an ion channel that is blocked by the antiviral drug, Amantadine. FEBS Lett. 2003;535(1-3):34-8.

269.   Pavlovic D, Neville DC, Argaud O, Blumberg B, Dwek RA, Fischer WB, et al. The hepatitis C virus p7 protein forms an ion channel that is inhibited by long-alkyl-chain iminosugar derivatives. Proc Natl Acad Sci USA. 2003;100(10):6104-8.

270.   Davis GL, Krawczynski K, Szabo G. Hepatitis C virus infection--pathobiology and implications for new therapeutic options. Dig Dis Sci. 2007;52(4):857-75.

271.   Gremion C, Cerny A. Hepatitis C virus and the immune system: a concise review. Rev Med Virol. 2005;15(4):235-68.

272.   Blackard JT, Kemmer N, Sherman KE. Extrahepatic replication of HCV: insights into clinical manifestations and biological consequences. Hepatology. 2006;44(1):15-22.

273.   Freeman AJ, Marinos G, Ffrench RA, Lloyd AR. Immunopathogenesis of hepatitis C virus infection. Immunol Cell Biol. 2001;79(6):515-36.

274.   Carrozzo M, Quadri R, Latorre P, Pentenero M, Paganin S, Bertolusso G, et al. Molecular evidence that the hepatitis C virus replicates in the oral mucosa. J Hepatol. 2002;37(3):364-9.

275.   Sies H. Oxidative stress:oxidants and antioxidants. London: Academic Press; 1991.

276.   Albano E. Alcohol, oxidative stress and free radical damage. Proc Nutr Soc. 2006;65(3):278-90.

277.   Medina J, Moreno-Otero R. Pathophysiological basis for antioxidant therapy in chronic liver disease. Drugs. 2005;65(17):2445-61.

278.   Novo E, Parola M. Redox mechanisms in hepatic chronic wound healing and fibrogenesis. Fibrogenesis Tissue Repair. 2008;1(1):5.

279.   Finkel T. Signal transduction by reactive oxygen species. J Cell Biol. 2011;194(1):7-15.

280.   Valgimigli M, Valgimigli L, Trere D, Gaiani S, Pedulli GF, Gramantieri L, et al. Oxidative stress EPR measurement in human liver by radical-probe technique. Correlation with etiology, histology and cell proliferation. Free Radic Res. 2002;36(9):939-48.

281.   Beard MR, Jones BE. Hepatitis C virus and oxidative stress: a dangerous liaison Future Virol. 2006;1(2):223-32.

282.   Koike K, Miyoshi H. Oxidative stress and hepatitis C viral infection. Hepatol Res. 2006;34(2):65-73.

283.   Wang T, Weinman SA. Causes and consequences of mitochondrial reactive oxygen species generation in hepatitis C. J Gastroenterol Hepatol. 2006;21 Suppl 3:S34-7.

284.   Houglum K, Ramm GA, Crawford DH, Witztum JL, Powell LW, Chojkier M. Excess iron induces hepatic oxidative stress and transforming growth factor beta1 in genetic hemochromatosis. Hepatology. 1997;26(3):605-10.

285.   Wu D, Cederbaum AI. Oxidative stress and alcoholic liver disease. Semin Liver Dis. 2009;29(2):141-54.

286.   Pemberton PW, Aboutwerat A, Smith A, Burrows PC, McMahon RF, Warnes TW. Oxidant stress in type I autoimmune hepatitis: the link between necroinflammation and fibrogenesis? Biochim Biophys Acta. 2004;1689(3):182-9.

287.   Davi G, Falco A, Patrono C. Lipid peroxidation in diabetes mellitus. Antioxid Redox Signal. 2005;7(1-2):256-68.

288.   Waris G, Ahsan H. Reactive oxygen species: role in the development of cancer and various chronic conditions. J Carcinog. 2006;5:14.

289.   Apte M. Oxidative stress: does it 'initiate' hepatic stellate cell activation or only 'perpetuate' the process? J Gastroenterol Hepatol. 2002;17(10):1045-8.

290.   Machida K, Cheng KT, Lai CK, Jeng KS, Sung VM, Lai MM. Hepatitis C virus triggers mitochondrial permeability transition with production of reactive oxygen species, leading to DNA damage and STAT3 activation. J Virol. 2006;80(14):7199-207.

291.   Elsayed NM. Antioxidant mobilization in response to oxidative stress: a dynamic environmental-nutritional interaction. Nutrition. 2001; 17(10):828-34. Available from: http://www.sciencedirect.com/science/article/pii/S0899900701006463.

292.   Serejo F, Emerit I, Filipe PM, Fernandes AC, Costa MA, Freitas JP, et al. Oxidative stress in chronic hepatitis C: the effect of interferon therapy and correlation with pathological features. Canadian journal of gastroenterology = Journal canadien de gastroenterologie. 2003;17(11):644-50.

293.   Levent G, Ali A, Ahmet A, Polat EC, Aytaç C, Ayşe E, et al. Oxidative stress and antioxidant defense in patients with chronic hepatitis C patients before and after pegylated interferon alfa-2b plus ribavirin therapy. J Transl Med. 2006;4:25.

294.   Pal S, Polyak SJ, Bano N, Qiu WC, Carithers RL, Shuhart M, et al. Hepatitis C virus induces oxidative stress, DNA damage and modulates the DNA repair enzyme NEIL1. J Gastroenterol Hepatol. 2010;25(3):627-34.

295.   Loguercio C, Federico A. Oxidative stress in viral and alcoholic hepatitis. Free Radic Biol Med. 2003;34(1):1-10.

296.   Duygu F, Koruk ST, Karsen H, Aksoy N, Taskin A, Hamidanoglu M. Prolidase and oxidative stress in chronic hepatitis C. J Clin Lab Anal. 2012;26(4):232-7.
297.   Chuma M, Hige S, Nakanishi M, Ogawa K, Natsuizaka M, Yamamoto Y, et al. 8-Hydroxy-2'-deoxy-guanosine is a risk factor for development of hepatocellular carcinoma in patients with chronic hepatitis C virus infection. J Gastroenterol Hepatol. 2008;23(9):1431-6.
298.   Sumida Y, Nakashima T, Yoh T, Nakajima Y, Ishikawa H, Mitsuyoshi H, et al. Serum thioredoxin levels as an indicator of oxidative stress in patients with hepatitis C virus infection. J Hepatol. 2000;33(4):616-22.
299.   Nakashima T, Sumida Y, Yoh T, Kakisaka Y, Nakajima Y, Ishikawa H, et al. Thioredoxin levels in the sera of untreated viral hepatitis patients and those treated with glycyrrhizin or ursodeoxycholic acid. Antioxid Redox Signal. 2000;2(4):687-94.
300.   Ko WS, Guo CH, Yeh MS, Lin LY, Hsu GS, Chen PC, et al. Blood micronutrient, oxidative stress, and viral load in patients with chronic hepatitis C. World J Gastroenterol. 2005;11(30):4697-702.
301.   Seronello S, Sheikh MY, Choi J. Redox regulation of hepatitis C in nonalcoholic and alcoholic liver. Free Radic Biol Med. 2007;43(6):869-82.
302.   Barbaro G, Di Lorenzo G, Asti A, Ribersani M, Belloni G, Grisorio B, et al. Hepatocellular mitochondrial alterations in patients with chronic hepatitis C: ultrastructural and biochemical findings. Am J Gastroenterol. 1999;94(8):2198-205.
303.   Missale G, Bertoni R, Lamonaca V, Valli A, Massari M, Mori C, et al. Different clinical behaviors of acute hepatitis C virus infection are associated with different vigor of the anti-viral cell-mediated immune response. J Clin Invest. 1996;98(3):706-14.
304.   Semmo N, Klenerman P. CD4+ T cell responses in hepatitis C virus infection. World J Gastroenterol. 2007;13(36):4831-8.
305.   Cooper S, Erickson AL, Adams EJ, Kansopon J, Weiner AJ, Chien DY, et al. Analysis of a successful immune response against hepatitis C virus. Immunity. 1999;10(4):439-49.
306.   Lechner F, Wong DK, Dunbar PR, Chapman R, Chung RT, Dohrenwend P, et al. Analysis of successful immune responses in persons infected with hepatitis C virus. J Exp Med. 2000;191(9):1499-512.
307.   Dustin LB, Rice CM. Flying under the radar: the immunobiology of hepatitis C. Annu Rev Immunol. 2007;25:71-99.
308.   Billerbeck E, Thimme R. CD8+ regulatory T cells in persistent human viral infections. Hum Immunol. 2008;69(11):771-5.
309.   Chang KM, Rehermann B, McHutchison JG, Pasquinelli C, Southwood S, Sette A, et al. Immunological significance of cytotoxic T lymphocyte epitope variants in patients chronically infected by the hepatitis C virus. J Clin Invest. 1997;100(9):2376-85.
310.   Barbaro G, Di Lorenzo G, Soldini M, Parrotto S, Bellomo G, Belloni G, et al. Hepatic glutathione deficiency in chronic hepatitis C: quantitative evaluation in patients who are HIV positive and HIV negative and correlations with plasmatic and lymphocytic concentrations and with the activity of the liver disease. Am J Gastroenterol. 1996;91(12):2569-73.
311.   Lucey DR, Clerici M, Shearer GM. Type 1 and type 2 cytokine dysregulation in human infectious, neoplastic, and inflammatory diseases. Clin Microbiol Rev. 1996;9(4):532-62.
312.   Semmo N, Krashias G, Willberg C, Klenerman P. Analysis of the relationship between cytokine secretion and proliferative capacity in hepatitis C virus infection. J Viral Hepat. 2007;14(7):492-502.
313.   Tsai SL, Liaw YF, Chen MH, Huang CY, Kuo GC. Detection of type 2-like T-helper cells in hepatitis C virus infection: implications for hepatitis C virus chronicity. Hepatology. 1997;25(2):449-58.
314.   McGuinness PH, Bishop GA, Painter DM, Chan R, McCaughan GW. Intrahepatic hepatitis C RNA levels do not correlate with degree of liver injury in patients with chronic hepatitis C. Hepatology. 1996;23(4):676-87.

315.   Napoli J, Bishop GA, McGuinness PH, Painter DM, McCaughan GW. Progressive liver injury in chronic hepatitis C infection correlates with increased intrahepatic expression of Th1-associated cytokines. Hepatology. 1996;24(4):759-65.
316.   McGuinness PH, Painter D, Davies S, McCaughan GW. Increases in intrahepatic CD68 positive cells, MAC387 positive cells, and proinflammatory cytokines (particularly interleukin 18) in chronic hepatitis C infection. Gut. 2000;46(2):260-9.
317.   Mahrouf-Yorgov M, de L'hortet A C, Cosson C, Slama A, Abdoun E, Guidotti JE, et al. Increased susceptibility to liver fibrosis with age is correlated with an altered inflammatory response. Rejuvenation Res. 2011;14(4):353-63.
318.   Hilleman MR. Strategies and mechanisms for host and pathogen survival in acute and persistent viral infections. Proc Natl Acad Sci USA. 2004;101 Suppl 2:14560-6.
319.   Ahlenstiel G, Edlich B, Hogdal LJ, Rotman Y, Noureddin M, Feld JJ, et al. Early changes in natural killer cell function indicate virologic response to interferon therapy for hepatitis C. Gastroenterology. 2011;141(4):1231-9, 9.e1-2.
320.   Guidotti LG, Chisari FV. Noncytolytic control of viral infections by the innate and adaptive immune response. Annu Rev Immunol. 2001;19:65-91.
321.   Kountouras J, Zavos C, Chatzopoulos D. Apoptosis in hepatitis C. J Viral Hepat. 2003;10(5):335-42.
322.   Bantel H, Schulze-Osthoff K. Apoptosis in hepatitis C virus infection. Cell Death Differ. 2003;10 Suppl 1:S48-58.
323.   Guo J, Friedman SL. Hepatic fibrogenesis. Semin Liver Dis. 2007;27(4):413-26.
324.   Rockey DC. Hepatic fibrosis, stellate cells, and portal hypertension. Clin Liver Dis. 2006;10(3):459-79, vii-viii.
325.   Macdonald A, Crowder K, Street A, McCormick C, Harris M. The hepatitis C virus NS5A protein binds to members of the Src family of tyrosine kinases and regulates kinase activity. J Gen Virol. 2004;85(Pt 3):721-9.
326.   Lin W, Kim SS, Yeung E, Kamegaya Y, Blackard JT, Kim KA, et al. Hepatitis C virus core protein blocks interferon signaling by interaction with the STAT1 SH2 domain. J Virol. 2006;80(18):9226-35.
327.   Horner SM, Gale M, Jr. Intracellular innate immune cascades and interferon defenses that control hepatitis C virus. J Interferon Cytokine Res. 2009;29(9):489-98.
328.   Heim MH, Moradpour D, Blum HE. Expression of hepatitis C virus proteins inhibits signal transduction through the Jak-STAT pathway. J Virol. 1999;73(10):8469-75.
329.   Blindenbacher A, Duong FH, Hunziker L, Stutvoet ST, Wang X, Terracciano L, et al. Expression of hepatitis c virus proteins inhibits interferon alpha signaling in the liver of transgenic mice. Gastroenterology. 2003;124(5):1465-75.
330.   Galluzzi L, Brenner C, Morselli E, Touat Z, Kroemer G. Viral control of mitochondrial apoptosis. PLoS Pathog. 2008;4(5):e1000018.
331.   Bureau C, Bernad J, Chaouche N, Orfila C, Béraud M, Gonindard C, et al. Nonstructural 3 protein of hepatitis C virus triggers an oxidative burst in human monocytes via activation of NADPH oxidase. J Biol Chem. 2001;276(25):23077-83.
332.   Thorén F, Romero A, Lindh M, Dahlgren C, Hellstrand K. A hepatitis C virus-encoded, nonstructural protein (NS3) triggers dysfunction and apoptosis in lymphocytes: role of NADPH oxidase-derived oxygen radicals. J Biol Chem. 2004;76(6):1180-6.
333.   Takaki A, Tatsukawa M, Iwasaki Y, Koike K, Noguchi Y, Shiraha H, et al. Hepatitis C virus NS4 protein impairs the Th1 polarization of immature dendritic cells. J Viral Hepat. 2010;17(8):555-62.
334.   Sklan EH, Charuworn P, Pang PS, Glenn JS. Mechanisms of HCV survival in the host. Nat Rev Gastroenterol Hepatol. 2009;6(4):217-27.
335.   Yao ZQ, Waggoner SN, Cruise MW, Hall C, Xie X, Oldach DW, et al. SOCS1 and SOCS3 are targeted by hepatitis C virus core/gC1qR ligation to inhibit T-cell function. J Virol. 2005;79(24):15417-29.

336.	Grakoui A, Shoukry NH, Woollard DJ, Han JH, Hanson HL, Ghrayeb J, et al. HCV persistence and immune evasion in the absence of memory T cell help. Science. 2003;302(5645):659-62.

337.	Beinhardt S, Aberle JH, Strasser M, Dulic-Lakovic E, Maieron A, Kreil A, et al. Serum level of IP-10 increases predictive value of IL28B polymorphisms for spontaneous clearance of acute HCV infection. Gastroenterology. 2012;142(1):78-85 e2.

338.	Suppiah V, Gaudieri S, Armstrong NJ, O'Connor KS, Berg T, Weltman M, et al. IL28B, HLA-C, and KIR variants additively predict response to therapy in chronic hepatitis C virus infection in a European Cohort: a cross-sectional study. PLoS Med. 2011;8(9):e1001092.

339.	Smith KR, Suppiah V, O'Connor K, Berg T, Weltman M, Abate ML, et al. Identification of improved IL28B SNPs and haplotypes for prediction of drug response in treatment of hepatitis C using massively parallel sequencing in a cross-sectional European cohort. Genome Med. 2011;3(8):57.

340.	Rauch A, Kutalik Z, Descombes P, Cai T, Di Iulio J, Mueller T, et al. Genetic variation in IL28B is associated with chronic hepatitis C and treatment failure: a genome-wide association study. Gastroenterology. 2010;138(4):1338-45, 45 e1-7.

341.	Ge D, Fellay J, Thompson AJ, Simon JS, Shianna KV, Urban TJ, et al. Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. Nature. 2009;461(7262):399-401.

342.	Tanaka Y, Nishida N, Sugiyama M, Kurosaki M, Matsuura K, Sakamoto N, et al. Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. Nat Genet. 2009;41(10):1105-9.

343.	Suppiah V, Moldovan M, Ahlenstiel G, Berg T, Weltman M, Abate ML, et al. IL28B is associated with response to chronic hepatitis C interferon-alpha and ribavirin therapy. Nat Genet. 2009;41(10):1100-4.

344.	Thomas DL, Thio CL, Martin MP, Qi Y, Ge D, O'Huigin C, et al. Genetic variation in IL28B and spontaneous clearance of hepatitis C virus. Nature. 2009;461(7265):798-801.

345.	Malarkey DE, Johnson K, Ryan L, Boorman G, Maronpot RR. New insights into functional aspects of liver morphology. Toxicol Pathol. 2005;33(1):27-34.

346.	Doherty DG, O'Farrelly C. Innate and adaptive lymphoid cells in the human liver. Immunol Rev. 2000;174:5-20.

347.	Parker GA, Picut CA. Liver immunobiology. Toxicol Pathol. 2005;33(1):52-62.

348.	Huebert RC, Jagavelu K, Liebl AF, Huang BQ, Splinter PL, LaRusso NF, et al. Immortalized liver endothelial cells: a cell culture model for studies of motility and angiogenesis. Lab Invest. 2010;90(12):1770-81.

349.	Oie CI, Appa RS, Hilden I, Petersen HH, Gruhler A, Smedsrod B, et al. Rat Liver Sinusoidal Endothelial Cells (LSECs) express functional Low Density Lipoprotein Receptor-Related Protein-1 (LRP-1). J Hepatol. 2011;55:1346-52.

350.	Faust DM, Markiewicz JM, Danckaert A, Soubigou G, Guillen N. Human liver sinusoidal endothelial cells respond to interaction with Entamoeba histolytica by changes in morphology, integrin signalling and cell death. Cell Microbiol. 2011;13(7):1091-106.

351.	Senoo H, Yoshikawa K, Morii M, Miura M, Imai K, Mezaki Y. Hepatic stellate cell (vitamin A-storing cell) and its relative--past, present and future. Cell Biol Int. 2010;34(12):1247-72.

352.	Maschmeyer P, Flach M, Winau F. Seven steps to stellate cells. J Vis Exp. 2011;10(51):pii 2710.

353.	Winnock M, Garcia Barcina M, Lukomska B, Huet S, Saric J, Balabaud C, et al. Human liver-associated lymphocytes: an overview. J Gastroenterol Hepatol. 1995;10 Suppl 1:S43-6.

354.	Novo E, Marra F, Zamara E, Valfrè di Bonzo L, Monitillo L, Cannito S, et al. Overexpression of Bcl-2 by activated human hepatic stellate cells: resistance to apoptosis as a mechanism of progressive hepatic fibrogenesis in humans. Gut. 2006;55(8):1174-82.

355.   Canbay A, Friedman S, Gores GJ. Apoptosis: the nexus of liver injury and fibrosis. Hepatology. 2004;39(2):273-8.
356.   Canbay A, Higuchi H, Bronk SF, Taniai M, Sebo TJ, Gores GJ. Fas enhances fibrogenesis in the bile duct ligated mouse: a link between apoptosis and fibrosis. Gastroenterology. 2002;123(4):1323-30.
357.   Hernandez-Gea V, Friedman SL. Pathogenesis of liver fibrosis. Annu Rev Pathol. 2011;6:425-56.
358.   Mann DA, Marra F. Fibrogenic signalling in hepatic stellate cells. J Hepatol. 2010;52(6):949-50.
359.   Los M, Mozoluk M, Ferrari D, Stepczynska A, Stroh C, Renz A, et al. Activation and caspase-mediated inhibition of PARP: a molecular switch between fibroblast necrosis and apoptosis in death receptor signaling. Mol Biol Cell. 2002;13(3):978-88.
360.   Schulze-Bergkamen H, Schuchmann M, Fleischer B, Galle PR. The role of apoptosis versus oncotic necrosis in liver injury: facts or faith? J Hepatol. 2006;44(5):984-93.
361.   Kerr JF, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer. 1972;26(4):239-57.
362.   Waris G, Turkson J, Hassanein T, Siddiqui A. Hepatitis C virus (HCV) constitutively activates STAT-3 via oxidative stress: role of STAT-3 in HCV replication. J Virol. 2005;79(3):1569-80.
363.   Qadri I, Iwahashi M, Capasso JM, Hopken MW, Flores S, Schaack J, et al. Induced oxidative stress and activated expression of manganese superoxide dismutase during hepatitis C virus replication: role of JNK, p38 MAPK and AP-1. Biochem J. 2004;378(Pt 3):919-28.
364.   Tardif KD, Waris G, Siddiqui A. Hepatitis C virus, ER stress, and oxidative stress. Trends Microbiol. 2005;13(4):159-63.
365.   Benedetti A, Marucci L. The significance of apoptosis in the liver. Liver. 1999;19(6):453-63.
366.   Dionisio N, Garcia-Mediavilla MV, Sanchez-Campos S, Majano PL, Benedicto I, Rosado JA, et al. Hepatitis C virus NS5A and core proteins induce oxidative stress-mediated calcium signalling alterations in hepatocytes. J Hepatol. 2009;50(5):872-82.
367.   Kung G, Konstantinidis K, Kitsis RN. Programmed necrosis, not apoptosis, in the heart. Circ Res. 2011;108(8):1017-36.
368.   Pias EK, Aw TY. Early redox imbalance mediates hydroperoxide-induced apoptosis in mitotic competent undifferentiated PC-12 cells. Cell Death Differ. 2002;9(9):1007-16.
369.   Pias EK, Ekshyyan OY, Rhoads CA, Fuseler J, Harrison L, Aw TY. Differential effects of superoxide dismutase isoform expression on hydroperoxide-induced apoptosis in PC-12 cells. J Biol Chem. 2003;278(15):13294-301.
370.   Circu ML, Aw TY. Glutathione and apoptosis. Free Radic Res. 2008;42(8):689-706.
371.   Hentze H, Latta M, Künstle G, Lucas R, Wendel A. Redox control of hepatic cell death. Toxicol Lett. 2003;139(2-3):111-8.
372.   Guicciardi ME, Gores GJ. Apoptosis as a mechanism for liver disease progression. Semin Liver Dis. 2010;30(4):402-10.
373.   Torchinsky MB, Garaude J, Blander JM. Infection and apoptosis as a combined inflammatory trigger. Curr Opin Immunol. 2010;22(1):55-62.
374.   Rather LJ. Disturbance of function (functio laesa): the legendary fifth cardinal sign of inflammation, added by Galen to the four cardinal signs of Celsus. Bull N Y Acad Med. 1971;47(3):303-22.
375.   Spengler U, Nattermann J. Immunopathogenesis in hepatitis C virus cirrhosis. Clin Sci (Lond). 2007;112(3):141-55.
376.   Lawrence T. The nuclear factor NF-kappaB pathway in inflammation. Cold Spring Harb Perspect Biol. 2009;1(6):a001651.
377.   Asselah T, Boyer N, Guimont MC, Cazals-Hatem D, Tubach F, Nahon K, et al. Liver fibrosis is not associated with steatosis but with necroinflammation in French patients with chronic hepatitis C. Gut. 2003;52(11):1638-43.

378. Wilson LE, Torbenson M, Astemborski J, Faruki H, Spoler C, Rai R, et al. Progression of liver fibrosis among injection drug users with chronic hepatitis C. Hepatology. 2006;43(4):788-95.

379. Wali M, Lewis S, Hubscher S, Harrison R, Ahmed M, Elias E, et al. Histological progression during short-term follow-up of patients with chronic hepatitis C virus infection. J Viral Hepat. 1999;6(6):445-52.

380. Bruck R, Schey R, Aeed H, Hochman A, Genina O, Pines M. A protective effect of pyrrolidine dithiocarbamate in a rat model of liver cirrhosis. Liver Int. 2004;24(2):169-76.

381. Iredale JP. Models of liver fibrosis: exploring the dynamic nature of inflammation and repair in a solid organ. J Clin Invest. 2007;117(3):539-48.

382. Friedman SL. Hepatic fibrosis -- overview. Toxicology. 2008;254(3):120-9.

383. van der Poorten D, George J. Disease-specific mechanisms of fibrosis: hepatitis C virus and nonalcoholic steatohepatitis. Clin Liver Dis. 2008;12(4):805-24, ix.

384. Fried MW. Hepatitis C infection with normal liver chemistry tests. Clin Gastroenterol Hepatol. 2008;6(5):503-5.

385. Lin W, Tsai WL, Shao RX, Wu G, Peng LF, Barlow LL, et al. Hepatitis C virus regulates transforming growth factor beta1 production through the generation of reactive oxygen species in a nuclear factor kappaB-dependent manner. Gastroenterology. 2010;138(7):2509-18, 18.e1.

386. Higuchi H, Gores GJ. Mechanisms of liver injury: an overview. Curr Mol Med. 2003;3(6):483-90.

387. Patin E, Kutalik Z, Guergnon J, Bibert S, Nalpas B, Jouanguy E, et al. Genome-wide association study identifies variants associated with progression of liver fibrosis from HCV infection. Gastroenterology. 2012;143(5):1244-52 e12.

388. Schuppan D, Afdhal NH. Liver cirrhosis. Lancet. 2008;371(9615):838-51.

389. Basu S. F2-isoprostanes in human health and diseases: from molecular mechanisms to clinical implications. Antioxid Redox Signal. 2008;10(8):1405-34.

390. Guetens G, De Boeck G, Highley M, van Oosterom AT, de Bruijn EA. Oxidative DNA damage: biological significance and methods of analysis. Crit Rev Clin Lab Sci. 2002;39(4-5):331-457.

391. Köken T, Serteser M, Kahraman A, Gökçe C. Oxidative stress markers in hepatitis C infected hemodialysis patients. J Nephrol. 2002;15(3):302-7.

392. Look MP, Gerard A, Rao GS, Sudhop T, Fischer HP, Sauerbruch T, et al. Interferon/antioxidant combination therapy for chronic hepatitis C--a controlled pilot trial. Antiviral Res. 1999;43(2):113-22.

393. Kadiiska MB, Gladen BC, Baird DD, Graham LB, Parker CE, Ames BN, et al. Biomarkers of oxidative stress study III. Effects of the nonsteroidal anti-inflammatory agents indomethacin and meclofenamic acid on measurements of oxidative products of lipids in CCl4 poisoning. Free Radic Biol Med. 2005;38(6):711-8.

394. Moore K, Roberts LJ, 2nd. Measurement of lipid peroxidation. Free Radic Res. 1998;28(6):659-71.

395. Paradis V, Mathurin P, Kollinger M, Imbert-Bismut F, Charlotte F, Piton A, et al. In situ detection of lipid peroxidation in chronic hepatitis C: correlation with pathological features. J Clin Pathol. 1997;50(5):401-6.

396. Basu S. Isoprostanes: novel bioactive products of lipid peroxidation. Free Radic Res. 2004;38(2):105-22.

397. Lai MM. Hepatitis C virus proteins: direct link to hepatic oxidative stress, steatosis, carcinogenesis and more. Gastroenterology. 2002;122(2):568-71.

398. Vidali M, Tripodi MF, Ivaldi A, Zampino R, Occhino G, Restivo L, et al. Interplay between oxidative stress and hepatic steatosis in the progression of chronic hepatitis C. J Hepatol. 2008;48(3):399-406.

399. Yang W, Hood BL, Chadwick SL, Liu S, Watkins SC, Luo G, et al. Fatty acid synthase is up-regulated during hepatitis C virus infection and regulates hepatitis C virus entry and production. Hepatology. 2008;48(5):1396-403.

400. Pratico D, Iuliano L, Basili S, Ferro D, Camastra C, Cordova C, et al. Enhanced lipid peroxidation in hepatic cirrhosis. J Investig Med. 1998;46(2):51-7.

401.   Kadiiska MB, Gladen BC, Baird DD, Germolec D, Graham LB, Parker CE, et al. Biomarkers of oxidative stress study II: are oxidation products of lipids, proteins, and DNA markers of CCl4 poisoning? Free Radic Biol Med. 2005;38(6):698-710.
402.   Longmire AW, Swift LL, Roberts LJ, 2nd, Awad JA, Burk RF, Morrow JD. Effect of oxygen tension on the generation of F2-isoprostanes and malondialdehyde in peroxidizing rat liver microsomes. Biochem Pharmacol. 1994;47(7):1173-7.
403.   Morrow JD, Frei B, Longmire AW, Gaziano JM, Lynch SM, Shyr Y, et al. Increase in circulating products of lipid peroxidation (F2-isoprostanes) in smokers. Smoking as a cause of oxidative damage. N Engl J Med. 1995;332(18):1198-203.
404.   Milne GL, Yin H, Morrow JD. Human biochemistry of the isoprostane pathway. J Biol Chem. 2008;283(23):15533-7.
405.   Gopaul NK, Halliwell B, Anggård EE. Measurement of plasma F2-isoprostanes as an index of lipid peroxidation does not appear to be confounded by diet. Free Radic Res. 2000;33(2):115-27.
406.   Comporti M, Arezzini B, Signorini C, Sgherri C, Monaco B, Gardi C. F2-isoprostanes stimulate collagen synthesis in activated hepatic stellate cells: a link with liver fibrosis? Lab Invest. 2005;85(11):1381-91.
407.   Comporti M, Signorini C, Arezzini B, Vecchio D, Monaco B, Gardi C. Isoprostanes and hepatic fibrosis. Mol Aspects Med. 2008;29(1-2):43-9.
408.   Tsen KT, Tsen SW, Kiang JG. Lycopene is more potent than beta carotene in the neutralization of singlet oxygen: role of energy transfer probed by ultrafast Raman spectroscopy. J Biomed Opt. 2006;11(6):064025.
409.   Osman HG, Gabr OM, Lotfy S, Gabr S. Serum levels of bcl-2 and cellular oxidative stress in patients with viral hepatitis. Indian J Med Microbiol. 2007;25(4):323-9.
410.   Fierbinteanu-Braticevici C, Mohora M, Cretoiu D, Cretoiu S, Petrisor A, Usvat R, et al. Role of oxidative stress in the pathogenesis of chronic hepatitis C (CHC). Rom J Morphol Embryol. 2009;50(3):407-12.
411.   Kim WR, Flamm SL, Di Bisceglie AM, Bodenheimer Jr HC, Public Policy Committee of the American Association for the Study of Liver Disease. Serum activity of alanine aminotransferase (ALT) as an indicator of health and disease. Hepatology. 2008;47(4):1363-70.
412.   Green RM, Flamm S. AGA Technical Review on the Evaluation of Liver Chemistry Tests. Gastroenterology. 2002;123(4):1367-84.
413.   Ruhl CE, Everhart JE. Elevated serum alanine aminotransferase and gamma-glutamyltransferase and mortality in the United States population. Gastroenterology. 2009;136(2):477-85 e11.
414.   Pradat P, Alberti A, Poynard T, Esteban JI, Weiland O, Marcellin P, et al. Predictive value of ALT levels for histologic findings in chronic hepatitis C: a European collaborative study. Hepatology. 2002;36(4 Pt 1):973-7.
415.   Gopal DV, Rosen HR. Abnormal findings on liver function tests. Interpreting results to narrow the diagnosis and establish a prognosis. Postgrad Med. 2000;107(2):100-2, 5-9, 13-4.
416.   Hanada K, Tanaka Y, Mizokami M, Gojobori T, Alter HJ. A reduction in selective immune pressure during the course of chronic hepatitis C correlates with diminished biochemical evidence of hepatic inflammation. Virology. 2007;361(1):27-33.
417.   Kaplan MM. Alanine aminotransferase levels: what's normal? Ann Intern Med. 2002;137(1):49-51.
418.   Puoti C, Bellis L, Guarisco R, Dell' Unto O, Spilabotti L, Costanza OM. HCV carriers with normal alanine aminotransferase levels: healthy persons or severely ill patients? Dealing with an everyday clinical problem. Eur J Intern Med. 2010;21(2):57-61.
419.   van der Poorten D, Kenny DT, Butler T, George J. Liver disease in adolescents: A cohort study of high-risk individuals. Hepatology. 2007;46(6):1750-8.
420.   Kariv R, Leshno M, Anat Beth-Or A, Strul H, Blendis L, Ehud Kokia E, et al. Re-evaluation of serum alanine aminotransferase upper normal limit and its modulating factors in a large-scale population study. Liver Int. 2006;26(4):445-50.

421.   Puoti C, Castellacci R, Montagnese F, Zaltron S, Stornaiuolo G, Bergami N, et al. Histological and virological features and follow-up of hepatitis C virus carriers with normal aminotransferase levels: the Italian prospective study of the asymptomatic C carriers (ISACC). J Hepatol. 2002;37(1):117-23.
422.   Marcellin P, Lévy S, Erlinger S. Therapy of hepatitis C: patients with normal aminotransferase levels. Hepatology. 1997;26(3 Suppl 1):133S-6S.
423.   Persico M, Persico E, Suozzo R, Conte S, De Seta M, Coppola L, et al. Natural history of hepatitis C virus carriers with persistently normal aminotransferase levels. Gastroenterology. 2000;118(4):760-64.
424.   Ahmed A, Keeffe EB. Chronic hepatitis C with normal aminotransferase levels. Gastroenterology. 2004;126(5):1409-15.
425.   Dienstag JL, McHutchison JG. American Gastroenterological Association technical review on the management of hepatitis C. Gastroenterology. 2006;130(1):231-64; quiz 14-7.
426.   Shiffman ML, Diago M, Tran A, Pockros P, Reindollar R, Prati D, et al. Chronic hepatitis C in patients with persistently normal alanine transaminase levels. Clin Gastroenterol Hepatol. 2006;4(5):645-52.
427.   Martinot-Peignoux M, Boyer N, Cazals-Hatem D, Pham BN, Gervais A, Le Breton V, et al. Prospective study on anti-hepatitis C virus-positive patients with persistently normal serum alanine transaminase with or without detectable serum hepatitis C virus RNA. Hepatology. 2001;34(5):1000-5.
428.   Ghany MG, Lok AS, Everhart JE, Everson GT, Lee WM, Curto TM, et al. Predicting clinical and histologic outcomes based on standard laboratory tests in advanced chronic hepatitis C. Gastroenterology. 2010;138(1):136-46.
429.   Stepanova M, Aquino R, Alsheddi A, Gupta R, Fang Y, Younossi Z. Clinical predictors of fibrosis in patients with chronic liver disease. Aliment Pharmacol Ther. 2010;31(10):1085-94.
430.   Jaeschke H, Lemasters JJ. Apoptosis versus oncotic necrosis in hepatic ischemia/reperfusion injury. Gastroenterology. 2003;125(4):1246-57.
431.   Sempoux C, Rahier J. Histological scoring of chronic hepatitis. Acta Gastroenterol Belg. 2004;67(3):290-3.
432.   Leroy V, Hilleret MN, Sturm N, Trocme C, Renversez JC, Faure P, et al. Prospective comparison of six non-invasive scores for the diagnosis of liver fibrosis in chronic hepatitis C. J Hepatol. 2007;46(5):775-82.
433.   Bourlière M, Penaranda G, Ouzan D, Renou C, Botta-Fridlund D, Tran A, et al. Optimized stepwise combination algorithms of non-invasive liver fibrosis scores including Hepascore in hepatitis C virus patients. Aliment Pharmacol Ther. 2008;28(4):458-67.
434.   Poynard T, Morra R, Ingiliz P, Imbert-Bismut F, Thabut D, Messous D, et al. Biomarkers of liver fibrosis. Adv Clin Chem. 2008;46:131-60.
435.   Adams LA, Bulsara M, Rossi E, DeBoer B, Speers D, George J, et al. Hepascore: an accurate validated predictor of liver fibrosis in chronic hepatitis C infection. Clin Chem. 2005;51(10):1867-73.
436.   McHutchison JG, Blatt LM, de Medina M, Craig JR, Conrad A, Schiff ER, et al. Measurement of serum hyaluronic acid in patients with chronic hepatitis C and its relationship to liver histology. Consensus Interferon Study Group. J Gastroenterol Hepatol. 2000;15(8):945-51.
437.   Morazzoni P, Bombardelli E. Silybum marianum (Carduus marianus). Fitoterapia. 1995;66(1):3-42.
438.   Ferenci P, Scherzer TM, Kerschner H, Rutter K, Beinhardt S, Hofer H, et al. Silibinin is a potent antiviral agent in patients with chronic hepatitis C not responding to pegylated interferon/ribavirin therapy. Gastroenterology. 2008;135(5):1561-7.
439.   Biermer M, Stoehr L, Schlosser B, Fülöp B, van Bömmel F, Berg T. Silibinin as a rescue treatment for HCV-infected patients showing suboptimal virologic response to standard combination therapy. J Hepatol. 2010;52(Suppl 1):S16.
440.   Biermer M, Berg T. Rapid suppression of hepatitis C viremia induced by intravenous silibinin plus ribavirin. Gastroenterology. 2009;137(1):390-1.

441. Ahmed-Belkacem A, Ahnou N, Barbotte L, Wychowski C, Pallier C, Brillet R, et al. Silibinin and related compounds are direct inhibitors of hepatitis C virus RNA-dependent RNA polymerase. Gastroenterology. 2010;138(3):1112-22.

442. Morishima C, Shuhart MC, Wang CC, Paschal DM, Apodaca MC, Liu Y, et al. Silymarin inhibits in vitro T-cell proliferation and cytokine production in hepatitis C virus infection. Gastroenterology. 2010;138(2):671-81, 81.e1-2.

443. Loguercio C, Festi D. Silybin and the liver: from basic research to clinical practice. World J Gastroenterol. 2011;17(18):2288-301.

444. Kroll DJ, Shaw HS, Oberlies NH. Milk thistle nomenclature: why it matters in cancer research and pharmacokinetic studies. Integr Cancer Ther. 2007;6(2):110-9.

445. Hruby K, Csomos G, Fuhrmann M, Thaler H. Chemotherapy of Amanita phalloides poisoning with intravenous silibinin. Hum Toxicol. 1983;2(2):183-95.

446. Ferenci P, Dragosics B, Dittrich H, Frank H, Benda L, Lochs H, et al. Randomized controlled trial of silymarin treatment in patients with cirrhosis of the liver. J Hepatol. 1989;9(1):105-13.

447. Aghazadeh S, Amini R, Yazdanparast R, Ghaffari SH. Anti-apoptotic and anti-inflammatory effects of Silybum marianum in treatment of experimental steatohepatitis. Exp Toxicol Pathol. 2010;63(6):569-74.

448. Gazák R, Purchartová K, Marhol P, Zivná L, Sedmera P, Valentová K, et al. Antioxidant and antiviral activities of silybin fatty acid conjugates. Eur J Med Chem. 2010;45(3):1059-67.

449. Shaker E, Mahmoud H, Mnaa S. Silymarin, the antioxidant component and Silybum marianum extracts prevent liver damage. Food Chem Toxicol. 2010;48(3):803-6.

450. Velussi M, Cernigoi AM, De Monte A, Dapas F, Caffau C, Zilli M. Long-term (12 months) treatment with an anti-oxidant drug (silymarin) is effective on hyperinsulinemia, exogenous insulin need and malondialdehyde levels in cirrhotic diabetic patients. J Hepatol. 1997;26(4):871-9.

451. Basiglio CL, Sanchez Pozzi EJ, Mottino AD, Roma MG. Differential effects of silymarin and its active component silibinin on plasma membrane stability and hepatocellular lysis. Chem Biol Interact. 2009;179(2-3):297-303.

452. Schuppan D, Jia JD, Brinkhaus B, Hahn EG. Herbal products for liver diseases: a therapeutic challenge for the new millennium. Hepatology. 1999;30(4):1099-104.

453. Polyak SJ, Morishima C, Shuhart MC, Wang CC, Liu Y, Lee DY. Inhibition of T-cell inflammatory cytokines, hepatocyte NF-kappaB signaling, and HCV infection by standardized Silymarin. Gastroenterology. 2007;132(5):1925-36.

454. Lieber CS, Leo MA, Cao Q, Ren C, DeCarli LM. Silymarin retards the progression of alcohol-induced hepatic fibrosis in baboons. J Clin Gastroenterol. 2003;37(4):336-9.

455. Fraschini F, Demartini G, Esposti D. Pharmacology of Silymarin. Clinical Drug Investigation. 2002;22:51-65.

456. Krecman V, Skottova N, Walterova D, Ulrichova J, Simanek V. Silymarin inhibits the development of diet-induced hypercholesterolemia in rats. Planta Med. 1998;64(2):138-42.

457. Neumann UP, Biermer M, Eurich D, Neuhaus P, Berg T. Successful prevention of hepatitis C virus (HCV) liver graft reinfection by silibinin mono-therapy. J Hepatol. 2010;52(6):951-2.

458. Bonifaz V, Shan Y, Lambrecht RW, Donohue SE, Moschenross D, Bonkovsky HL. Effects of silymarin on hepatitis C virus and haem oxygenase-1 gene expression in human hepatoma cells. Liver Int. 2009;29(3):366-73.

459. Wagoner J, Negash A, Kane OJ, Martinez LE, Nahmias Y, Bourne N, et al. Multiple effects of silymarin on the hepatitis C virus lifecycle. Hepatology. 2010;51(6):1912-21.

460. Polyak SJ. The Molecular Virology of Hepatitis C Virus [Internet]. Seattle (WA): University of Washington, Department of Laboratory Medicine; 2012 [updated 2012 Mar 29]; Available from:
http://www.courses.washington.edu/conj504/slides/PolyakSlides.ppt.

461.   Bunout D, Hirsch S, Petermann M, de la Maza MP, Silva G, Kelly M, et al. [Controlled study of the effect of silymarin on alcoholic liver disease]. Rev Med Chil. 1992;120(12):1370-5.

462.   Trinchet JC, Coste T, Lévy VG, Vivet F, Duchatelle V, Legendre C, et al. [Treatment of alcoholic hepatitis with silymarin. A double-blind comparative study in 116 patients]. Gastroenterol Clin Biol. 1989;13(2):120-4.

463.   Parés A, Planas R, Torres M, Caballeria J, Viver JM, Acero D, et al. Effects of silymarin in alcoholic patients with cirrhosis of the liver: results of a controlled, double-blind, randomized and multicenter trial. J Hepatol. 1998;28(4):615-21.

464.   Hawke RL, Schrieber SJ, Soule TA, Wen Z, Smith PC, Reddy KR, et al. Silymarin ascending multiple oral dosing phase I study in noncirrhotic patients with chronic hepatitis C. J Clin Pharmacol. 2010;50(4):434-49.

465.   Fehér J, Deák G, Müzes G, Láng I, Niederland V, Nékám K, et al. [Liver-protective action of silymarin therapy in chronic alcoholic liver diseases]. Orv Hetil. 1989;130(51):2723-7.

466.   Saller R, Meier R, Brignoli R. The use of silymarin in the treatment of liver diseases. Drugs. 2001;61(14):2035-63.

467.   Saller R, Brignoli R, Melzer J, Meier R. An updated systematic review with meta-analysis for the clinical evidence of silymarin. Forsch Komplementmed. 2008;15(1):9-20.

468.   Dixit N, Baboota S, Kohli K, Ahmad S, Ali J. Silymarin: A review of pharmacological aspects and bioavailability enhancement approaches. Indian J Pharmacol. 2007;39:172-9.

469.   Song Z, Deaciuc I, Song M, Lee DY, Liu Y, Ji X, et al. Silymarin protects against acute ethanol-induced hepatotoxicity in mice. Alcohol Clin Exp Res. 2006;30(3):407-13.

470.   Miguez MP, Anundi I, Sainz-Pardo LA, Lindros KO. Hepatoprotective mechanism of silymarin: no evidence for involvement of cytochrome P450 2E1. Chem Biol Interact. 1994;91(1):51-63.

471.   Huber R, Futter I, Lüdtke R. Oral silymarin for chronic hepatitis C - a retrospective analysis comparing three dose regimens. Eur J Med Res. 2005;10(2):68-70.

472.   Gordon A, Hobbs DA, Bowden DS, Bailey MJ, Mitchell J, Francis AJ, et al. Effects of Silybum marianum on serum hepatitis C virus RNA, alanine aminotransferase levels and well-being in patients with chronic hepatitis C. J Gastroenterol Hepatol. 2006;21(1 Pt 2):275-80.

473.   Freedman ND, Curto TM, Morishima C, Seeff LB, Goodman ZD, Wright EC, et al. Silymarin use and liver disease progression in the Hepatitis C Antiviral Long-Term Treatment against Cirrhosis trial. Aliment Pharmacol Ther. 2011;33(1):127-37.

474.   Wellington K, Jarvis B. Silymarin: a review of its clinical properties in the management of hepatic disorders. BioDrugs. 2001;15(7):465-89.

475.   Dündar Y. AR. Antioxidant stress. Eastern J Med. 2000;5:45–7.

476.   Poljsak B, Milisav I. The neglected significance of "antioxidative stress". Oxid Med Cell Longev. 2012;2012:480895.

477.   Villanueva C, Kross RD. Antioxidant-induced stress. Int J Mol Sci. 2012;13(2):2091-109.

478.   Augustyniak A, Bartosz G, Cipak A, Duburs G, Horáková L, Luczaj W, et al. Natural and synthetic antioxidants: an updated overview. Free Radic Res. 2010;44(10):1216-62.

479.   Packer L, Weber SU, Rimbach G. Molecular aspects of alpha-tocotrienol antioxidant action and cell signalling. J Nutr. 2001;131(2):369S-73S.

480.   Tavani A, La Vecchia C. Fruit and vegetable consumption and cancer risk in a Mediterranean population. Am J Clin Nutr. 1995;61(6 Suppl):1374S-7S.

481.   Riboli E, Norat T. Epidemiologic evidence of the protective effect of fruit and vegetables on cancer risk. Am J Clin Nutr. 2003;78(3 Suppl):559S-69S.

482.   Steinmetz KA, Potter JD. Vegetables, fruit, and cancer. I. Epidemiology. Cancer causes & control. 1991;2(5):325-57.

483.	Bjelakovic G, Nikolova D, Gluud LL, Simonetti RG, Gluud C. Mortality in randomized trials of antioxidant supplements for primary and secondary prevention: systematic review and meta-analysis. JAMA. 2007;297(8):842-57.
484.	Bobe G, Weinstein SJ, Albanes D, Hirvonen T, Ashby J, Taylor PR, et al. Flavonoid intake and risk of pancreatic cancer in male smokers (Finland). Cancer Epidemiol Biomarkers Prev. 2008;17(3):553-62.
485.	Donaldson MS. A carotenoid health index based on plasma carotenoids and health outcomes. Nutrients. 2011;3(12):1003-22.
486.	McCary CA, Abdala-Valencia H, Berdnikovs S, Cook-Mills JM. Supplemental and highly elevated tocopherol doses differentially regulate allergic inflammation: reversibility of alpha-tocopherol and gamma-tocopherol's effects. J Immunol. 2011;186(6):3674-85.
487.	Blatt DH, Pryor WA. High-dosage vitamin E supplementation and all-cause mortality. Ann Intern Med. 2005;143(2):150-1; author reply 6-8.
488.	Brigelius-Flohe R, Traber MG. Vitamin E: function and metabolism. FASEB J. 1999;13(10):1145-55.
489.	ATBC Cancer Prevention Study Group. The alpha-tocopherol, beta-carotene lung cancer prevention study: design, methods, participant characteristics, and compliance. Ann Epidemiol. 1994;4(1):1-10.
490.	Greenwald P. Beta-carotene and lung cancer: a lesson for future chemoprevention investigations? J Natl Cancer Inst. 2003;95(1):E1.
491.	Albanes D, Heinonen OP, Taylor PR, Virtamo J, Edwards BK, Rautalahti M, et al. Alpha-Tocopherol and beta-carotene supplements and lung cancer incidence in the alpha-tocopherol, beta-carotene cancer prevention study: effects of base-line characteristics and study compliance. J Natl Cancer Inst. 1996;88(21):1560-70.
492.	Omenn GS, Goodman GE, Thornquist MD, Balmes J, Cullen MR, Glass A, et al. Risk factors for lung cancer and for intervention effects in CARET, the Beta-Carotene and Retinol Efficacy Trial. J Natl Cancer Inst. 1996;88(21):1550-9.
493.	Omenn GS, Goodman GE, Thornquist MD, Balmes J, Cullen MR, Glass A, et al. Effects of a combination of beta carotene and vitamin A on lung cancer and cardiovascular disease. N Engl J Med. 1996;334(18):1150-5.
494.	Siems W, Sommerburg O, Schild L, Augustin W, Langhans CD, Wiswedel I. Beta-carotene cleavage products induce oxidative stress in vitro by impairing mitochondrial respiration. FASEB J. 2002;16(10):1289-91.
495.	Schafer ZT, Grassian AR, Song L, Jiang Z, Gerhart-Hines Z, Irie HY, et al. Antioxidant and oncogene rescue of metabolic defects caused by loss of matrix attachment. Nature. 2009;461(7260):109-13.
496.	Liu C, Russell RM, Wang XD. Alpha-tocopherol and ascorbic acid decrease the production of beta-apo-carotenals and increase the formation of retinoids from beta-carotene in the lung tissues of cigarette smoke-exposed ferrets in vitro. J Nutr. 2004;134(2):426-30.
497.	Siems W, Wiswedel I, Salerno C, Crifo C, Augustin W, Schild L, et al. Beta-carotene breakdown products may impair mitochondrial functions--potential side effects of high-dose beta-carotene supplementation. J Nutr Biochem. 2005;16(7):385-97.
498.	Siems W, Salerno C, Crifo C, Sommerburg O, Wiswedel I. Beta-carotene degradation products - formation, toxicity and prevention of toxicity. Forum Nutr. 2009;61:75-86.
499.	Liu C, Russell RM, Wang XD. Low dose beta-carotene supplementation of ferrets attenuates smoke-induced lung phosphorylation of JNK, p38 MAPK, and p53 proteins. J Nutr. 2004;134(10):2705-10.
500.	Kim Y, Chongviriyaphan N, Liu C, Russell RM, Wang XD. Combined alpha-tocopherol and ascorbic acid protects against smoke-induced lung squamous metaplasia in ferrets. Lung Cancer. 2012;75(1):15-23.
501.	Yeum KJ, Aldini G, Russell RM, Krinsky NI. In Carotenoids. Basel, Switzerland: Birkhäuser Verlag; 2009.
502.	Blot WJ, Li JY, Taylor PR, Guo W, Dawsey S, Wang GQ, et al. Nutrition intervention trials in Linxian, China: supplementation with specific vitamin/mineral

combinations, cancer incidence, and disease-specific mortality in the general population. J Natl Cancer Inst. 1993;85(18):1483-92.

503.   Cook NR, Le IM, Manson JE, Buring JE, Hennekens CH. Effects of beta-carotene supplementation on cancer incidence by baseline characteristics in the Physicians' Health Study (United States). Cancer causes & control. 2000;11(7):617-26.

504.   Ristow M, Schmeisser S. Extending life span by increasing oxidative stress. Free Radic Biol Med. 2011;51(2):327-36.

505.   Ristow M, Zarse K. How increased oxidative stress promotes longevity and metabolic health: The concept of mitochondrial hormesis (mitohormesis). Exp Gerontol. 2010;45(6):410-8.

506.   Poljsak B. Strategies for reducing or preventing the generation of oxidative stress. Oxid Med Cell Longev. 2011;2011:194586.

507.   Schulz TJ, Zarse K, Voigt A, Urban N, Birringer M, Ristow M. Glucose restriction extends Caenorhabditis elegans life span by inducing mitochondrial respiration and increasing oxidative stress. Cell Metab. 2007;6(4):280-93.

508.   Surh YJ. Xenohormesis mechanisms underlying chemopreventive effects of some dietary phytochemicals. Ann N Y Acad Sci. 2011;1229:1-6.

509.   Kim J, Cha YN, Surh YJ. A protective role of nuclear factor-erythroid 2-related factor-2 (Nrf2) in inflammatory disorders. Mutat Res. 2010;690(1-2):12-23.

510.   Guo CH, Chen PC, Lin KP, Shih MY, Ko WS, Duygu F, et al. Trace metal imbalance associated with oxidative stress and inflammatory status in anti-hepatitis C virus antibody positive subjects. Environ Toxicol Pharmacol. 2012;33(2):288-96.

511.   Melhem A, Stern M, Shibolet O, Israeli E, Ackerman Z, Pappo O, et al. Treatment of chronic hepatitis C virus infection via antioxidants: results of a phase I clinical trial. J Clin Gastroenterol. 2005;39(8):737-42.

512.   Gabbay E, Zigmond E, Pappo O, Hemed N, Rowe M, Zabrecky G, et al. Antioxidant therapy for chronic hepatitis C after failure of interferon: results of phase II randomized, double-blind placebo controlled clinical trial. World J Gastroenterol. 2007;13(40):5317-23.

513.   Shimizu I. Antifibrogenic therapies in chronic HCV infection. Curr Drug Targets Infect Disord. 2001;1(2):227-40.

514.   Zheng S, Yumei F, Chen A. De novo synthesis of glutathione is a prerequisite for curcumin to inhibit hepatic stellate cell (HSC) activation. Free Radic Biol Med. 2007;43(3):444-53.

515.   Schuppan D. Diagnosis and treatment of liver fibrosis. A Collection of Papers of the First International Symposium on Liver Diseases with Chinese Integrative Medicine; 2005 Sept 23-25; Shanghai, China. [place unknown]: [publisher unknown]; 2005. p. 19-28.

516.   Houglum K, Venkataramani A, Lyche K, Chojkier M. A pilot study of the effects of d-alpha-tocopherol on hepatic stellate cell activation in chronic hepatitis C. Gastroenterology. 1997;113(4):1069-73.

517.   Halim AB, el-Ahmady O, Hassab-Allah S, Abdel-Galil F, Hafez Y, Darwish A. Biochemical effect of antioxidants on lipids and liver function in experimentally-induced liver damage. Ann Clin Biochem. 1997;34 (Pt 6):656-63.

518.   Gane EJ, Weilert F, Orr DW, Keogh GF, Gibson M, Lockhart MM, et al. The mitochondria-targeted anti-oxidant mitoquinone decreases liver damage in a phase II study of hepatitis C patients. Liver Int. 2010;30(7):1019-26.

519.   Cohen-Naftaly M, Friedman SL. Current status of novel antifibrotic therapies in patients with chronic liver disease. Therap Adv Gastroenterol. 2011;4(6):391-417.

520.   Fu Y, Zheng S, Lu SC, Chen A. Epigallocatechin-3-gallate inhibits growth of activated hepatic stellate cells by enhancing the capacity of glutathione synthesis. Mol Pharmacol. 2008;73(5):1465-73.

521.   Lin J, Zheng S, Chen A. Curcumin attenuates the effects of insulin on stimulating hepatic stellate cell activation by interrupting insulin signaling and attenuating oxidative stress. Lab Invest. 2009;89(12):1397-409.

522.   Angelico F, Francioso S, Del Ben M, Feole K, Carbone M, Pignatelli P, et al. Clinical trial: low plasma cholesterol and oxidative stress predict rapid virological

response to standard therapy with peginterferon and ribavirin in HCV patients. Aliment Pharmacol Ther. 2009;30(5):444-51.

523.   Grant PR, Black A, Garcia N, Prieto J, Garson JA. Combination therapy with interferon-alpha plus N-acetyl cysteine for chronic hepatitis C: a placebo controlled double-blind multicentre study. J Med Virol. 2000;61(4):439-42.

524.   Beloqui O, Prieto J, Suárez M, Gil B, Qian CH, Garcia N, et al. N-acetyl cysteine enhances the response to interferon-alpha in chronic hepatitis C: a pilot study. J Interferon Res. 1993;13(4):279-82.

525.   Idéo G, Bellobuono A, Tempini S, Mondazzi L, Airoldi A, Benetti G, et al. Antioxidant drugs combined with alpha-interferon in chronic hepatitis C not responsive to alpha-interferon alone: a randomized, multicentre study. Eur J Gastroenterol Hepatol. 1999;11(11):1203-7.

526.   Batey RG, Bensoussan A, Fan YY, Bollipo S, Hossain MA. Preliminary report of a randomized, double-blind placebo-controlled trial of a Chinese herbal medicine preparation CH-100 in the treatment of chronic hepatitis C. J Gastroenterol Hepatol. 1998;13(3):244-7.

527.   Liao H, Banbury LK, Leach DN. Antioxidant activity of 45 Chinese herbs and the relationship with their TCM characteristics. Evid Based Complement Alternat Med. 2008;5(4):429-34.

528.   Ghanim H, Sia CL, Abuaysheh S, Korzeniewski K, Patnaik P, Marumganti A, et al. An antiinflammatory and reactive oxygen species suppressive effects of an extract of Polygonum cuspidatum containing resveratrol. J Clin Endocrinol Metab. 2010;95(9):E1-8.

529.   Chan JY, Koon JC, Leung PC, Che CT, Fung KP. Suppression of low-density lipoprotein oxidation, vascular smooth muscle cell proliferation and migration by a herbal extract of Radix Astragali, Radix Codonopsis and Cortex Lycii. BMC Complement Altern Med. 2011;11:32.

530.   Kim SH, Lee MK, Lee KY, Sung SH, Kim J, Kim YC. Chemical constituents isolated from Paeonia lactiflora roots and their neuroprotective activity against oxidative stress in vitro. J Enzyme Inhib Med Chem. 2009;24(5):1138-40.

531.   Kim TH, Kim JS, Kim ZH, Huang RB, Wang RS. Khz (Fusion of Ganoderma lucidum and Polyporus umbellatus Mycelia) Induces Apoptosis by Increasing Intracellular Calcium Levels and Activating JNK and NADPH Oxidase-Dependent Generation of Reactive Oxygen Species. PLoS One. 2012;7(10):e46208.

532.   Shan X, Zhou J, Ma T, Chai Q. Lycium barbarum Polysaccharides Reduce Exercise-Induced Oxidative Stress. Int J Mol Sci. 2011;12(2):1081-8.

533.   Jiang Y, Zhang Y, Wark L, Ortiz E, Lim S, He H, et al. Wolfberry Water Soluble Phytochemicals Down-Regulate ER Stress Biomarkers and Modulate Multiple Signaling Pathways Leading To Inhibition of Proliferation and Induction of Apoptosis in Jurkat Cells. J Nutr Food Sci. 2012;S2.

534.   Wu TY, Khor TO, Saw CL, Loh SC, Chen AI, Lim SS, et al. Anti-inflammatory/Anti-oxidative stress activities and differential regulation of Nrf2-mediated genes by non-polar fractions of tea Chrysanthemum zawadskii and licorice Glycyrrhiza uralensis. AAPS J. 2011;13(1):1-13.

535.   Huo HZ, Wang B, Liang YK, Bao YY, Gu Y. Hepatoprotective and Antioxidant Effects of Licorice Extract against CCl(4)-Induced Oxidative Damage in Rats. Int J Mol Sci. 2011;12(10):6529-43.

536.   Wang AY, Lian LH, Jiang YZ, Wu YL, Nan JX. *Gentiana manshurica* Kitagawa prevents acetaminophen-induced acute hepatic injury in mice via inhibiting JNK/ERK MAPK pathway. World J Gastroenterol. 2010;16(3):384-91.

537.   Mollison L, Totten L, Flexman J, Beaman M, Batey R. Randomized double blind placebo-controlled trial of a Chinese herbal therapy (CH100) in chronic hepatitis C. J Gastroenterol Hepatol. 2006;21(7):1184-8.

538.   Batey R, Cao Q, Pang G, Clancy RL. Effects of CH-100, a chinese herbal medicine, on acute concanavalin A-mediated hepatitis in control and alcohol-fed rats. Alcohol Clin Exp Res. 2000;24(6):852-8.

539.	Li MY, Ryan P, Batey RG. Traditional Chinese medicine prevents inflammation in CCl4-related liver injury in mice. Am J Chin Med. 2003;31(1):119-27.

540.	Groenbaek K, Friis H, Hansen M, Ring-Larsen H, Krarup HB. The effect of antioxidant supplementation on hepatitis C viral load, transaminases and oxidative status: a randomized trial among chronic hepatitis C virus-infected patients. Eur J Gastroenterol Hepatol. 2006;18(9):985-9.

541.	Han D, Handelman G, Marcocci L, Sen CK, Roy S, Kobuchi H, et al. Lipoic acid increases de novo synthesis of cellular glutathione by improving cystine utilization. Biofactors. 1997;6(3):321-38.

542.	Skrzydlewska E, Ostrowska J, Farbiszewski R, Michalak K. Protective effect of green tea against lipid peroxidation in the rat liver, blood serum and the brain. Phytomedicine. 2002;9(3):232-8.

543.	Tager M, Dietzmann J, Thiel U, Hinrich Neumann K, Ansorge S. Restoration of the cellular thiol status of peritoneal macrophages from CAPD patients by the flavonoids silibinin and silymarin. Free Radic Res. 2001;34(2):137-51.

544.	Verma N, Vinayak M. Antioxidant action of Andrographis paniculata on lymphoma. Mol Biol Rep. 2008;35(4):535-40.

545.	Gui SY, Wei W, Wang H, Wu L, Sun WY, Chen WB, et al. Effects and mechanisms of crude astragalosides fraction on liver fibrosis in rats. J Ethnopharmacol. 2006;103(2):154-9.

546.	Bindoli A, Cavallini L, Siliprandi N. Inhibitory action of silymarin of lipid peroxide formation in rat liver mitochondria and microsomes. Biochem Pharmacol. 1977;26(24):2405-9.

547.	Bruck R, Ashkenazi M, Weiss S, Goldiner I, Shapiro H, Aeed H, et al. Prevention of liver cirrhosis in rats by curcumin. Liver Int. 2007;27(3):373-83.

548.	Jia JD, Bauer M, Cho JJ, Ruehl M, Milani S, Boigk G, et al. Antifibrotic effect of silymarin in rat secondary biliary fibrosis is mediated by downregulation of procollagen alpha1(I) and TIMP-1. J Hepatol. 2001;35(3):392-8.

549.	Min AK, Kim MK, Seo HY, Kim HS, Jang BK, Hwang JS, et al. Alpha-lipoic acid inhibits hepatic PAI-1 expression and fibrosis by inhibiting the TGF-beta signaling pathway. Biochem Biophys Res Commun. 2010;393(3):536-41.

550.	Chen A, Zheng S. Curcumin inhibits connective tissue growth factor gene expression in activated hepatic stellate cells in vitro by blocking NF-kappaB and ERK signalling. Br J Pharmacol. 2008;153(3):557-67.

551.	Nair N, Mahajan S, Chawda R, Kandaswami C, Shanahan TC, Schwartz SA. Grape seed extract activates Th1 cells in vitro. Clin Diagn Lab Immunol. 2002;9(2):470-6.

552.	Ciesek S, von Hahn T, Colpitts CC, Schang LM, Friesland M, Steinmann J, et al. The green tea polyphenol, epigallocatechin-3-gallate, inhibits hepatitis C virus entry. Hepatology. 2011;54(6):1947-55.

553.	Guedj J, Dahari H, Pohl RT, Ferenci P, Perelson AS. Understanding silibinin's modes of action against HCV using viral kinetic modeling. J Hepatol. 2012;56(5):1019-24.

554.	Calland N, Albecka A, Belouzard S, Wychowski C, Duverlie G, Descamps V, et al. (-)-Epigallocatechin-3-gallate is a new inhibitor of hepatitis C virus entry. Hepatology. 2012;55(3):720-9.

555.	Chen C, Qiu H, Gong J, Liu Q, Xiao H, Chen XW, et al. (-)-Epigallocatechin-3-gallate inhibits the replication cycle of hepatitis C virus. Archives of virology. 2012;157(7):1301-12.

556.	Fukazawa H, Suzuki T, Wakita T, Murakami Y. A cell-based, microplate colorimetric screen identifies 7,8-benzoflavone and green tea gallate catechins as inhibitors of the hepatitis C virus. Biological & pharmaceutical bulletin. 2012;35(8):1320-7.

557.	Castro MC, Massa ML, Schinella G, Gagliardino JJ, Francini F. Lipoic acid prevents liver metabolic changes induced by administration of a fructose-rich diet. Biochim Biophys Acta. 2012.

558.	Hultberg M, Hultberg B. The effect of different antioxidants on glutathione turnover in human cell lines and their interaction with hydrogen peroxide. Chem Biol Interact. 2006;163(3):192-8.

559.	Petersen Shay K, Moreau RF, Smith EJ, Hagen TM. Is alpha-lipoic acid a scavenger of reactive oxygen species in vivo? Evidence for its initiation of stress signaling pathways that promote endogenous antioxidant capacity. IUBMB Life. 2008;60(6):362-7.

560.	Chiou WF, Chen CF, Lin JJ. Mechanisms of suppression of inducible nitric oxide synthase (iNOS) expression in RAW 264.7 cells by andrographolide. Br J Pharmacol. 2000;129(8):1553-60.

561.	Shen YC, Chen CF, Chiou WF. Andrographolide prevents oxygen radical production by human neutrophils: possible mechanism(s) involved in its anti-inflammatory effect. Br J Pharmacol. 2002;135(2):399-406.

562.	Chang KT, Lii CK, Tsai CW, Yang AJ, Chen HW. Modulation of the expression of the pi class of glutathione S-transferase by Andrographis paniculata extracts and andrographolide. Food Chem Toxicol. 2008;46(3):1079-88.

563.	Chen HW, Lin AH, Chu HC, Li CC, Tsai CW, Chao CY, et al. Inhibition of TNF-alpha-Induced Inflammation by andrographolide via down-regulation of the PI3K/Akt signaling pathway. J Nat Prod. 2011;74(11):2408-13.

564.	Hidalgo MA, Romero A, Figueroa J, Cortés P, Concha, II, Hancke JL, et al. Andrographolide interferes with binding of nuclear factor-kappaB to DNA in HL-60-derived neutrophilic cells. Br J Pharmacol. 2005;144(5):680-6.

565.	Lee WR, Chung CL, Hsiao CJ, Chou YC, Hsueh PJ, Yang PC, et al. Suppression of matrix metalloproteinase-9 expression by andrographolide in human monocytic THP-1 cells via inhibition of NF-kappaB activation. Phytomedicine. 2012;19(3-4):270-7.

566.	Ye JF, Zhu H, Zhou ZF, Xiong RB, Wang XW, Su LX, et al. Protective mechanism of andrographolide against carbon tetrachloride-induced acute liver injury in mice. Biological & pharmaceutical bulletin. 2011;34(11):1666-70.

567.	Zhang ZL, Wen QZ, Liu CX. Hepatoprotective effects of astraglus root. J Ethnopharmacol. 1990;30(2):145-9.

568.	Jia R, Cao L, Xu P, Jeney G, Yin G. In vitro and in vivo hepatoprotective and antioxidant effects of Astragalus polysaccharides against carbon tetrachloride-induced hepatocyte damage in common carp (Cyprinus carpio). Fish Physiol Biochem. 2012;38(3):871-81.

569.	Li Q, Bao JM, Li XL, Zhang T, Shen XH. Inhibiting effect of Astragalus polysaccharides on the functions of CD4+CD25 highTreg cells in the tumor microenvironment of human hepatocellular carcinoma. Chin Med J (Engl). 2012;125(5):786-93.

570.	Park HJ, Lee JY, Chung MY, Park YK, Bower AM, Koo SI, et al. Green tea extract suppresses NFkappaB activation and inflammatory responses in diet-induced obese rats with nonalcoholic steatohepatitis. J Nutr. 2012;142(1):57-63.

571.	Zhen MC, Wang Q, Huang XH, Cao LQ, Chen XL, Sun K, et al. Green tea polyphenol epigallocatechin-3-gallate inhibits oxidative damage and preventive effects on carbon tetrachloride-induced hepatic fibrosis. J Nutr Biochem. 2007;18(12):795-805.

572.	Naito M, Wu X, Nomura H, Kodama M, Kato Y, Osawa T. The protective effects of tetrahydrocurcumin on oxidative stress in cholesterol-fed rabbits. J Atheroscler Thromb. 2002;9(5):243-50.

573.	Deeb D, Xu YX, Jiang H, Gao X, Janakiraman N, Chapman RA, et al. Curcumin (diferuloyl-methane) enhances tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis in LNCaP prostate cancer cells. Mol Cancer Ther. 2003;2(1):95-103.

574.	Ak T, Gülçin I. Antioxidant and radical scavenging properties of curcumin. Chem Biol Interact. 2008;174(1):27-37.

575.	Glatthaar-Saalmuller B, Sacher F, Esperester A. Antiviral activity of an extract derived from roots of Eleutherococcus senticosus. Antiviral Res. 2001;50(3):223-8.

576.	Chen TS, Liou SY, Chang YL. Antioxidant evaluation of three adaptogen extracts. Am J Chin Med. 2008;36(6):1209-17.

577.   Park EJ, Nan JX, Zhao YZ, Lee SH, Kim YH, Nam JB, et al. Water-soluble polysaccharide from Eleutherococcus senticosus stems attenuates fulminant hepatic failure induced by D-galactosamine and lipopolysaccharide in mice. Basic & clinical pharmacology & toxicology. 2004;94(6):298-304.

578.   Hunt EJ, Lester CE, Lester EA, Tackett RL. Effect of St. John's wort on free radical production. Life Sci. 2001;69(2):181-90.

579.   Orčić DZ, Mimica-Dukić NM, Francišković MM, Petrović SS, Jovin ED. Antioxidant activity relationship of phenolic compounds in Hypericum perforatum L. Chem Cent J. 2011;5:34.

580.   Porrini M, Riso P. Lymphocyte lycopene concentration and DNA protection from oxidative damage is increased in women after a short period of tomato consumption. J Nutr. 2000;130(2):189-92.

581.   Bahcecioglu IH, Kuzu N, Metin K, Ozercan IH, Ustundag B, Sahin K, et al. Lycopene prevents development of steatohepatitis in experimental nonalcoholic steatohepatitis model induced by high-fat diet. Veterinary medicine international. 2010:262179.

582.   Kim GY, Kim JH, Ahn SC, Lee HJ, Moon DO, Lee CM, et al. Lycopene suppresses the lipopolysaccharide-induced phenotypic and functional maturation of murine dendritic cells through inhibition of mitogen-activated protein kinases and nuclear factor-kappaB. Immunology. 2004;113(2):203-11.

583.   Kiemer AK, Hartung T, Huber C, Vollmar AM. Phyllanthus amarus has anti-inflammatory potential by inhibition of iNOS, COX-2, and cytokines via the NF-kappaB pathway. J Hepatol. 2003;38(3):289-97.

584.   Harikumar KB, Kuttan R. Protective effect of an extract of *Phyllanthus amarus* against radiation-induced damage in mice. J Radiat Res. 2004;45(1):133-9.

585.   Ravikumar YS, Ray U, Nandhitha M, Perween A, Raja Naika H, Khanna N, et al. Inhibition of hepatitis C virus replication by herbal extract: Phyllanthus amarus as potent natural source. Virus Res. 2011;158(1-2):89-97.

586.   Navarro-Alarcon M, Lopez-Ga de la Serrana H, Perez-Valero V, Lopez-Martinez MC. Selenium concentrations in serum of individuals with liver diseases (cirrhosis or hepatitis): relationship with some nutritional and biochemical markers. Sci Total Environ. 2002;291(1-3):135-41.

587.   Aboul-Soud MA, Al-Othman AM, El-Desoky GE, Al-Othman ZA, Yusuf K, Ahmad J, et al. Hepatoprotective effects of vitamin E/selenium against malathion-induced injuries on the antioxidant status and apoptosis-related gene expression in rats. J Toxicol Sci. 2011;36(3):285-96.

588.   Clarke C, Baghdadi H, Howie AF, Mason JI, Walker SW, Beckett GJ. Selenium supplementation attenuates procollagen-1 and interleukin-8 production in fat-loaded human C3A hepatoblastoma cells treated with TGFbeta1. Biochim Biophys Acta. 2010;1800(6):611-8.

589.   Himoto T, Yoneyama H, Kurokohchi K, Inukai M, Masugata H, Goda F, et al. Selenium deficiency is associated with insulin resistance in patients with hepatitis C virus-related chronic liver disease. Nutrition research. 2011;31(11):829-35.

590.   Teodor V, Cuciureanu M, Filip C, Zamosteanu N, Cuciureanu R. Protective effects of selenium on acrylamide toxicity in the liver of the rat. Effects on the oxidative stress. Rev Med Chir Soc Med Nat Iasi. 2011;115(2):612-8.

591.   Viezeliene D, Jansen E, Rodovicius H, Kasauskas A, Ivanov L. Protective effect of selenium on aluminium-induced oxidative stress in mouse liver in vivo. Environ Toxicol Pharmacol. 2011;31(2):302-6.

592.   Youn HS, Lim HJ, Choi YJ, Lee JY, Lee MY, Ryu JH. Selenium suppresses the activation of transcription factor NF-kappa B and IRF3 induced by TLR3 or TLR4 agonists. Int Immunopharmacol. 2008;8(3):495-501.

593.   Schümann J, Prockl J, Kiemer AK, Vollmar AM, Bang R, Tiegs G. Silibinin protects mice from T cell-dependent liver injury. J Hepatol. 2003;39(3):333-40.

594.   Valenzuela A, Aspillaga M, Vial S, Guerra R. Selectivity of silymarin on the increase of the glutathione content in different tissues of the rat. Planta Med. 1989;55(5):420-2.

595. Alidoost F, Gharagozloo M, Bagherpour B, Jafarian A, Sajjadi SE, Hourfar H, et al. Effects of silymarin on the proliferation and glutathione levels of peripheral blood mononuclear cells from beta-thalassemia major patients. Int Immunopharmacol. 2006;6(8):1305-10.

596. Das SK, Mukherjee S. Biochemical and immunological basis of silymarin effect, a milk thistle (*Silybum marianum*) against ethanol-induced oxidative damage. Toxicol Mech Methods. 2012;22(5):409-13.

597. Fried MW, Navarro VJ, Afdhal N, Belle SH, Wahed AS, Hawke RL, et al. Effect of silymarin (milk thistle) on liver disease in patients with chronic hepatitis C unsuccessfully treated with interferon therapy: a randomized controlled trial. JAMA. 2012;308(3):274-82.

598. Kim M, Yang SG, Kim JM, Lee JW, Kim YS, Lee JI. Silymarin suppresses hepatic stellate cell activation in a dietary rat model of non-alcoholic steatohepatitis: analysis of isolated hepatic stellate cells. Int J Mol Med. 2012;30(3):473-9.

599. Tzeng JI, Chen MF, Chung HH, Cheng JT. Silymarin Decreases Connective Tissue Growth Factor to Improve Liver Fibrosis in Rats Treated with Carbon Tetrachloride. Phytother Res. 2012.

600. Wallace S, Vaughn K, Stewart BW, Viswanathan T, Clausen E, Nagarajan S, et al. Milk thistle extracts inhibit the oxidation of low-density lipoprotein (LDL) and subsequent scavenger receptor-dependent monocyte adhesion. J Agric Food Chem. 2008;56(11):3966-72.

601. Bowie AG, O'Neill LA. Vitamin C inhibits NF-kappa B activation by TNF via the activation of p38 mitogen-activated protein kinase. J Immunol. 2000;165(12):7180-8.

602. Maellaro E, Del Bello B, Sugherini L, Pompella A, Casini AF, Comporti M. Protection by ascorbic acid against oxidative injury of isolated hepatocytes. Xenobiotica. 1994;24(3):281-9.

603. Abhilash PA, Harikrishnan R, Indira M. Ascorbic acid supplementation causes faster restoration of reduced glutathione content in the regression of alcohol-induced hepatotoxicity in male guinea pigs. Redox Rep. 2012;17(2):72-9.

604. Abhilash PA, Harikrishnan R, Indira M. Ascorbic acid supplementation down-regulates the alcohol induced oxidative stress, hepatic stellate cell activation, cytotoxicity and mRNA levels of selected fibrotic genes in guinea pigs. Redox Rep. 2012;46(2):204-13.

605. Hong IH, Han JY, Goo MJ, Hwa SY, Ki MR, Park JK, et al. Ascorbic acid deficiency accelerates aging of hepatic stellate cells with up-regulation of PPARgamma. Histology and histopathology. 2012;27(2):171-9.

606. Weyers A, Ugnia LI, Ovando HG, Gorla NB. Antioxidant capacity of vitamin C in mouse liver and kidney tissues. Biocell. 2008;32(1):27-31.

607. Maffei Facino R, Carini M, Aldini G, Bombardelli E, Morazzoni P, Morelli R. Free radicals scavenging action and anti-enzyme activities of procyanidines from Vitis vinifera. A mechanism for their capillary protective action. Arzneimittelforschung. 1994;44(5):592-601.

608. Vigna GB, Costantini F, Aldini G, Carini M, Catapano A, Schena F, et al. Effect of a standardized grape seed extract on low-density lipoprotein susceptibility to oxidation in heavy smokers. Metabolism. 2003;52(10):1250-7.

609. Dulundu E, Ozel Y, Topaloglu U, Toklu H, Ercan F, Gedik N, et al. Grape seed extract reduces oxidative stress and fibrosis in experimental biliary obstruction. J Gastroenterol Hepatol. 2007;22(6):885-92.

610. Camps J, Bargallo T, Gimenez A, Alie S, Caballeria J, Pares A, et al. Relationship between hepatic lipid peroxidation and fibrogenesis in carbon tetrachloride-treated rats: effect of zinc administration. Clin Sci (Lond). 1992;83(6):695-700.

611. Farias MS, Budni P, Ribeiro CM, Parisotto EB, Santos CE, Dias JF, et al. Antioxidant supplementation attenuates oxidative stress in chronic hepatitis C patients. Gastroenterologia y hepatologia. 2012;35(6):386-94.

612.   Somi MH, Rezaeifar P, Ostad Rahimi A, Moshrefi B. Effects of low dose zinc supplementation on biochemical markers in non-alcoholic cirrhosis: a randomized clinical trial. Archives of Iranian medicine. 2012;15(8):472-6.
613.   von Bulow V, Dubben S, Engelhardt G, Hebel S, Plümäkers B, Heine H, et al. Zinc-dependent suppression of TNF-alpha production is mediated by protein kinase A-induced inhibition of Raf-1, I kappa B kinase beta, and NF-kappa B. J Immunol. 2007;179(6):4180-6.
614.   Holt AP, Salmon M, Buckley CD, Adams DH. Immune interactions in hepatic fibrosis. Clin Liver Dis. 2008;12(4):861-82.
615.   Marra F. Hepatic stellate cells and the regulation of liver inflammation. J Hepatol. 1999;31(6):1120-30.
616.   Schuppan D, Popov Y. Rationale and targets for antifibrotic therapies. Gastroenterol Clin Biol. 2009;33(10-11):949-57.
617.   Poole CF. Thin-layer chromatography: challenges and opportunities. J Chromatogr A. 2003;1000(1-2):963-84.
618.   Poole CF, Dias NC. Practitioner's guide to method development in thin-layer chromatography. J Chromatogr A. 2000;892(1-2):123-42.
619.   Sood SP, Sartori LE, Wittmer DP, Haney WG. High-pressure liquid chromatographic determination of ascorbic acid in selected foods and multivitamin products. Anal Chem. 1976;48(6):796-8.
620.   Altman DG, Schulz KF, Moher D, Egger M, Davidoff F, Elbourne D, et al. The revised CONSORT statement for reporting randomized trials: explanation and elaboration. Ann Intern Med. 2001;134(8):663-94.
621.   Wacholder S, Weinberg CR. Paired versus two-sample design for a clinical trial of treatments with dichotomous outcome: power considerations. Biometrics. 1982;38(3):801-12.
622.   Ware JE, Kosinski M. Interpreting SF-36 summary health measures: a response. Qual Life Res. 2001;10(5):405-13; discussion 15-20.
623.   Bayliss MS, Gandek B, Bungay KM, Sugano D, Hsu MA, Ware JE, Jr. A questionnaire to assess the generic and disease-specific health outcomes of patients with chronic hepatitis C. Qual Life Res. 1998;7(1):39-55.
624.   Gould JE. Concise handbook of experimental methods for the behavioral and biological sciences: CRC Press; 2001.
625.   Mori TA, Croft KD, Puddey IB, Beilin LJ. An improved method for the measurement of urinary and plasma F2-isoprostanes using gas chromatography-mass spectrometry. Anal Biochem. 1999;268(1):117-25.
626.   Leroy V, Halfon P, Bacq Y, Boursier J, Rousselet MC, Bourlière M, et al. Diagnostic accuracy, reproducibility and robustness of fibrosis blood tests in chronic hepatitis C: a meta-analysis with individual data. Clin Biochem. 2008;41(16-17):1368-76.
627.   Poynard T, inventor Assistance Publique-Hopitaux de Paris, assignee. Diagnosis method of inflammatory fibrotic or cancerous disease using biochemical markers. France2003.
628.   Rossi E, Adams L, Prins A, Bulsara M, de Boer B, Garas G, et al. Validation of the FibroTest biochemical markers score in assessing liver fibrosis in hepatitis C patients. Clin Chem. 2003;49(3):450-4.
629.   Reinert DF, Allen JP. The alcohol use disorders identification test: an update of research findings. Alcohol Clin Exp Res. 2007;31(2):185-99.
630.   Moher D, Schulz KF, Altman DG. The CONSORT statement: revised recommendations for improving the quality of reports of parallel-group randomized trials. Ann Intern Med. 2001;134(8):657-62.
631.   Mutapi F, Roddam A. p values for pathogens: statistical inference from infectious-disease data. Lancet Infect Dis. 2002;2(4):219-30.
632.   Applegate KE, Tello R, Ying J. Hypothesis testing III: counts and medians. Radiology. 2003;228(3):603-8.

633.	McHutchison JG, Manns M, Patel K, Poynard T, Lindsay KL, Trepo C, et al. Adherence to combination therapy enhances sustained response in genotype-1-infected patients with chronic hepatitis C. Gastroenterology. 2002;123(4):1061-9.
634.	Prati D, Taioli E, Zanella A, Della Torre E, Butelli S, Del Vecchio E, et al. Updated definitions of healthy ranges for serum alanine aminotransferase levels. Ann Intern Med. 2002;137(1):1-10.
635.	Australian Bureau of Statistics. National Health Survey SF-36 Population Norms. Canberra: Australian Bureau of Statistics; 1995.
636.	Krauth J. The interpretation of significance tests for independent and dependent samples. J Neurosci Methods. 1983;9(4):269-81.
637.	Jahan S, Khaliq S, Ijaz B, Ahmad W, Hassan S. Role of HCV Core gene of genotype 1a and 3a and host gene Cox-2 in HCV-induced pathogenesis. Virol J. 2011;8:155.
638.	Spiegel BM, Younossi ZM, Hays RD, Revicki D, Robbins S, Kanwal F. Impact of hepatitis C on health related quality of life: a systematic review and quantitative assessment. Hepatology. 2005;41(4):790-800.
639.	Australian Bureau of Statistics. National Health Survey SF-36 Population Norms, Australia, 1995 Canberra: Australian Bureau of Statistics; 1997.
640.	Maghrani M, Zeggwagh NA, Lemhadri A, El Amraoui M, Michel JB, Eddouks M. Study of the hypoglycaemic activity of Fraxinus excelsior and Silybum marianum in an animal model of type 1 diabetes mellitus. J Ethnopharmacol. 2004;91(2-3):309-16.
641.	Huseini HF, Larijani B, Heshmat R, Fakhrzadeh H, Radjabipour B, Toliat T, et al. The efficacy of Silybum marianum (L.) Gaertn. (silymarin) in the treatment of type II diabetes: a randomized, double-blind, placebo-controlled, clinical trial. Phytother Res. 2006;20(12):1036-9.
642.	Dugdale DC. Fatigue. [Internet] Bethesda: Department of Health and Human Services National Institute of Health; 2011 [updated 5/29/2011]; Available from: http://www.nlm.nih.gov/medlineplus/enc/article/003088.htm.
643.	Lee DH, Jamal H, Regenstein FG, Perrillo RP. Morbidity of chronic hepatitis C as seen in a tertiary care medical center. Dig Dis Sci. 1997;42(1):186-91.
644.	Tong MJ, el-Farra NS. Clinical sequelae of hepatitis C acquired from injection drug use. West J Med. 1996;164(5):399-404.
645.	Barkhuizen A, Rosen HR, Wolf S, Flora K, Benner K, Bennett RM. Musculoskeletal pain and fatigue are associated with chronic hepatitis C: a report of 239 hepatology clinic patients. Am J Gastroenterol. 1999;94(5):1355-60.
646.	Hoofnagle JH. Hepatitis C: the clinical spectrum of disease. Hepatology. 1997;26(3 Suppl 1):15S-20S.
647.	Freedman ND, Curto TM, Lindsay KL, Wright EC, Sinha R, Everhart JE. Coffee consumption is associated with response to peginterferon and ribavirin therapy in patients with chronic hepatitis C. Gastroenterology. 2011;140(7):1961-9.
648.	Freedman ND, Everhart JE, Lindsay KL, Ghany MG, Curto TM, Shiffman ML, et al. Coffee intake is associated with lower rates of liver disease progression in chronic hepatitis C. Hepatology. 2009;50(5):1360-9.
649.	Rietveld A, Wiseman S. Antioxidant effects of tea: evidence from human clinical trials. J Nutr. 2003;133(10):3285S-92S.
650.	Unachukwu UJ, Ahmed S, Kavalier A, Lyles JT, Kennelly EJ. White and green teas (Camellia sinensis var. sinensis): variation in phenolic, methylxanthine, and antioxidant profiles. J Food Sci. 2010;75(6):C541-8.
651.	Natella F, Nardini M, Giannetti I, Dattilo C, Scaccini C. Coffee drinking influences plasma antioxidant capacity in humans. J Agric Food Chem. 2002;50(21):6211-6.
652.	World Health Organization. Hepatitis C fact sheet No 164. 2004; Available from: http://www.who.int/csr/disease/hepatitis/whocdscsrlyo2003/en/index2.html#HCV.
653.	Dore GJ, Freeman AJ, Law M, Kaldor JM. Is severe liver disease a common outcome for people with chronic hepatitis C? J Gastroenterol Hepatol. 2002;17(4):423-30.

654.   del Valle J, Mira JA, de los Santos I, Lopez-Cortes LF, Merino D, Rivero A, et al. Baseline serum low-density lipoprotein cholesterol levels predict response to hepatitis C virus therapy in HIV/hepatitis C virus coinfected patients. AIDS. 2008;22(8):923-30.

655.   Loguercio C, Andreone P, Brisc C, Brisc MC, Bugianesi E, Chiaramonte M, et al. Silybin combined with phosphatidylcholine and vitamin E in patients with nonalcoholic fatty liver disease: a randomized controlled trial. Free Radic Biol Med. 2012;52(9):1658-65.

656.   Sanyal AJ, Chalasani N, Kowdley KV, McCullough A, Diehl AM, Bass NM, et al. Pioglitazone, vitamin E, or placebo for nonalcoholic steatohepatitis. N Engl J Med. 2010;362(18):1675-85.

657.   Bell LN, Wang J, Muralidharan S, Chalasani S, Fullenkamp AM, Wilson LA, et al. Relationship between adipose tissue insulin resistance and liver histology in NASH: a PIVENS follow-up study. Hepatology. 2012.

658.   Singal AK, Jampana SC, Weinman SA. Antioxidants as therapeutic agents for liver disease. Liver Int. 2011;31(10):1432-48.

659.   Trouillas P, Marsal P, Svobodova A, Vostalova J, Gazak R, Hrbac J, et al. Mechanism of the antioxidant action of silybin and 2,3-dehydrosilybin flavonolignans: a joint experimental and theoretical study. The journal of physical chemistry A. 2008;112(5):1054-63.

660.   Tanamly MD, Tadros F, Labeeb S, Makld H, Shehata M, Mikhail N, et al. Randomised double-blinded trial evaluating silymarin for chronic hepatitis C in an Egyptian village: study description and 12-month results. Dig Liver Dis. 2004;36(11):752-9.

661.   Polyak SJ, Morishima C, Hawke R. Antiviral effects of silymarin against hepatitis C: the jury is still out. Hepatology. 2008;48(1):345-6.

662.   Rutter K, Scherzer TM, Beinhardt S, Kerschner H, Stattermayer AF, Hofer H, et al. Intravenous silibinin as 'rescue treatment' for on-treatment non-responders to pegylated interferon/ribavirin combination therapy. Antivir Ther. 2011;16(8):1327-33.

663.   Eurich D, Bahra M, Berg T, Boas-Knoop S, Biermer M, Neuhaus R, et al. Treatment of hepatitis C-virus-reinfection after liver transplant with silibinin in nonresponders to pegylated interferon-based therapy. Exp Clin Transplant. 2011;9(1):1-6.

664.   Biermer M, Schlosser B, Fulop B, van Bommel F, Brodzinski A, Heyne R, et al. High-dose silibinin rescue treatment for HCV-infected patients showing suboptimal virologic response to standard combination therapy. J Viral Hepat. 2012;19(8):547-53.

665.   Xia Y, Li Q, Zhong W, Dong J, Wang Z, Wang C. L-carnitine ameliorated fatty liver in high-calorie diet/STZ-induced type 2 diabetic mice by improving mitochondrial function. Diabetol Metab Syndr. 2011;3:31.

666.   Kim SM, Kim SC, Chung IK, Cheon WH, Ku SK. Antioxidant and Protective Effects of Bupleurum falcatum on the L-Thyroxine-Induced Hyperthyroidism in Rats. Evid Based Complement Alternat Med. 2012;2012:578497.

667.   Karuna R, Reddy SS, Baskar R, Saralakumari D. Antioxidant potential of aqueous extract of Phyllanthus amarus in rats. Indian J Pharmacol. 2009;41(2):64-7.

668.   Lam PY, Ko KM. Schisandrin B as a hormetic agent for preventing age-related neurodegenerative diseases. Oxid Med Cell Longev. 2012;2012:250825.

669.   Pu HJ, Cao YF, He RR, Zhao ZL, Song JH, Jiang B, et al. Correlation between Antistress and Hepatoprotective Effects of Schisandra Lignans Was Related with Its Antioxidative Actions in Liver Cells. Evid Based Complement Alternat Med. 2012;2012:161062.

APPENDIX A

AWARDS, RESEARCH PUBLICATIONS AND PRESENTATIONS

Appendix A comprises the awards gained, publications and presentations throughout the duration of the dissertation.

AWARDS, PUBLICATIONS AND PRESENTATIONS FROM 2001–2011

SECTION A

Awards

Douglas Piper Young Investigator Award Clinical Science, 2010 Oct 22, Gastroenterological Society of Australia.

SECTION B

Publications

B1.   Peer-reviewed ournal articles

Batey RG, Salmond SJ, Bensoussan A. Complementary and alternative medicine in the treatment of chronic liver disease. Curr Gastroenterol Rep. 2005;7(1):63-70.

B2.   Conference proceedings

Salmond SJ, George J, Strasser SI, Byth K, Batey RG. The Hep573 Study--a randomized double blind placebo controlled trial of silymarin alone or combined with antioxidants in chronic hepatitis C [abstract]. In Proceedings of Australian Gastroenterology Week, 2010 Oct 20-23; Gold Coast. J Gastroenterol Hepatol; 2010;25(Suppl 3):A123-A124.

Salmond SJ, George J, Strasser SI, Batey RG. Hep573 study--a randomised double-blind placebo-controlled trial of silymarin alone or combined with antioxidants in chronic hepatitis C [abstract]. In Proceedings of Digestive Diseases Week, AASLD; 2010 May 1-5; New Orleans. Gastroenterology; 2010;138(5 Suppl 1):S789-S790.

Salmond SJ, George J, Strasser SI, Batey RG. Hep573 Study--a randomised controlled trial of herbal medicines in chronic hepatitis C [poster abstract]. In Third International Congress on Complementary Medicine Research; 2008 Mar 29-31; Sydney, Australia. 2008:p. 87-88.

Salmond SJ, George J, Strasser SI, Batey RG. Hep573 Study of complementary medicine in the treatment of chronic hepatitis C [poster abstract]. In 12th International Symposium on Viral Hepatitis and Liver Disease; 2006 Jul 1-5; Paris, Palais des Congrès. J Clin Virol; 2006;36(Suppl 2):S134-5.

Salmond SJ, George J, Strasser SI, Batey RG. Hep573 Study of alternative therapy for chronic hepatitis C [abstract]. In Proceedings of the Shanghai-Hong Kong International Liver Congress; 2006 March 25-28; Shanghai, China. J Gastroenterol Hepatol; 2006;21(Suppl 2):A156.

Salmond SJ, Batey R. Hep573 Study of alternative medicine in the treatment of chronic hepatitis C [abstract]. In Fifth Australasian Conference on Viral Hepatitis; 2006 Feb 20-22; Sydney, Australia. [place unknown]: [publisher unknown]; 2006:111

Batey R, Salmond S. Hep573 Study of alternative therapy for chronic hepatitis C [abstract]. In A Collection of Papers of the First International Symposium on Liver Diseases with Chinese Integrative Medicine; 2005 Sept 23-25; Shanghai, China. [place unknown]: [publisher unknown]; 2005:66-67.

B3.   Book chapters

Salmond SJ. The Hepatobiliary System. In: Hechtman L, editor. Clinical Naturopathic Medicine. Sydney: Elsevier; 2012. p. 210-279.

Salmond SJ, Bensoussan A. Natural therapies and hepatitis C. In: Dore G, Temple-Smith M, Lloyd A, Editors. Hepatitis C: an expanding perspective. Melbourne: IP Communications; 2009. p. 211-229.

Salmond SJ, Batey RG. Complementary therapies and hepatitis B. In: Mathews G, Robotin M, Editors. B Positive- All you wanted to know about hepatitis B: A guide for primary health care providers. Sydney: Australasian Society for HIV Medicine (ASHM); 2008. p. 96-101.

SECTION C

Unpublished presentations

C1.   Oral presentations as sole author

Salmond S. Complementary therapies and hepatitis C. Justice Health; 2011 May 4; Long Bay Correctional Facility Education Centre, Sydney, Australia.

Salmond S. Complementary therapies and hepatitis C. Justice Health; 2010 Jun 11; Long Bay Correctional Facility Education Centre, Sydney, Australia.

Salmond S. Looking after your liver. RPAH Koori Women's Group; 2010 Apr 19; Leichhardt Women's Community Health Centre (LWCHC), Leichhardt, Australia.

Salmond S. Complementary therapies and hepatitis C. Hepatitis Support Group; 2009 Nov 17; Hepatitis C Council of NSW, Sydney, Australia.

Salmond S. Herbal and naturopathic protocol in chronic hepatitis C patients. North Coast Area Health Service (NCAHS) Liver Clinic Nurses, Southern Cross University Naturopathy students, and local naturopaths; 2009 Sept 17; Ballina, Australia.

Salmond S. Complementary medicine and hepatitis C: North Coast Area Health Service (NCAHS) Liver Clinic Workshop for Liver Clinic Nurses; 2009 Sept 17; Ballina, Australia.

Salmond S. Complementary therapies and hepatitis C. Justice Health; 2009 Jun 19; Long Bay Correctional Facility Education Centre, Sydney, Australia.

Salmond S. The role of complementary medicine in hepatitis C. Department of Justice; 2008 Jun 20; Bankstown Sports Club, Bankstown, Australia.

Salmond S. Hep573 Study--a randomised controlled trial of herbal medicines in chronic hepatitis C. New Zealand Association of Medical Herbalists Annual Conference and AGM; 2008 May 23-24; Novotel, Auckland, New Zealand.

Salmond S. The role of complementary medicine in hepatitis C. Department of Justice; 2007 Jun 8; Corrective Services Academy, Sydney, Australia.

Salmond S. Questions answered on complementary therapies and hepatitis C. Transfusion Related and other Infectious Diseases Service (TRAIDS); 2006 Oct 5; Parramatta Community Health Centre, Sydney, Australia.

Salmond S. Nutritional aspects of hepatitis C. Westmead Hospital; 2006 May 16; Sydney, Australia.

Salmond S. Complementary therapies and interferon and ribavirin in hepatitis C. Heplink Forum; 2006 Mar 21; Royal Prince Alfred Hospital, Sydney, Australia.

Salmond S. Hep573 Study of herbal medicines in the treatment of chronic hepatitis C. Australian Hepatitis Council Hepatitis C Research Forum; 2006 Feb 23; Sydney, Australia.

Salmond S. How to manage the side effects of antiviral therapy. Clinic 16, Royal North Shore Hospital; 2006 Feb 8; Sydney, Australia.

Salmond S. Herbal therapies and hepatitis C--how to manage the side-effects of antiviral therapy. Hepatitis C Council of NSW AGM; 2005 Nov 11; Sydney, Australia.

Salmond S. Complementary therapies in hepatitis C and HIV. Sydney West Area Health Service; 2005 May 19; Westmead Hospital, Sydney, Australia.

Salmond S. The Hep573 Study and research on complementary therapies and hepatitis C. Liver Unit/TRAIDS Hepatitis C Information Night; 2005 May 10; Westmead Hospital, Sydney, Australia.

Salmond S. Hep573 Study. Research Seminars for Post Graduate Students; 2004 Sept 9; University of Newcastle, Ourimbah campus, Australia.

Salmond S. Hep573 Study. Central Sydney Area Health Service (CSAHS) Hepatitis C Treatment Options Forum; 2004 Aug 11; Canterbury Hospital, Sydney, Australia.

Salmond S. Hep5673 Study. CSAHS Health Promotion Services, GPs, sexual health workers and nurses; 2004 Jul 12; Royal Prince Alfred Hospital, Sydney, Australia.

Salmond S. Liver health. Phytomedicine Practice in Focus Teleconference; 2004 Jun 24; Brisbane, Australia.

Salmond S. Liver health. Phytomedicine Practice in Focus Teleconference; 2004 Jun 22; Melbourne, Australia.

Salmond S. Hep573 Study. Central Sydney Area Health Service (CSAHS) Hepatitis C Treatment Options Forum; 2004 Jun 16; Newtown Neighbourhood Centre, Sydney, Australia.

Salmond S. Liver health. Phytomedicine Practice in Focus; 2004 Jun 2; Terrigal, Australia.

Salmond S. Hep573 Study. Hepatitis C Information Night; 2004 Apr 6; Westmead Hospital, Sydney, Australia.

Salmond S. Hep573 Study testing herbal medicines and vitamins in the treatment of chronic hepatitis C. Third Year Bachelor of Herbal Therapies; 2004 Apr 1; University of Newcastle, Ourimbah campus, Australia.

Salmond S. Case studies on the herbal management of hepatitis C. Third Year Bachelor of Herbal Therapies; 2004 Apr 1; University of Newcastle, Ourimbah campus, Australia.

Salmond S. Hep573 Study information for potential participants. Royal Prince Alfred Hospital; 2003 Jun 24; Sydney, Australia.

Salmond S. Nutrition, self-help and complementary therapies. Paper presented at: ASHM HCV Pilot Program for General Practitioners; 2003 Mar 15; Sydney, Australia.

Salmond S. Complementary therapies and hepatitis C. Paper presented at: Phytomedicine Conference on Infection, Inflammation and Immunity; 2003 Mar 1; Sydney, Australia.

Salmond S. Complementary therapies in hepatitis C and non-pharmacological measures in drug and alcohol withdrawal. Department of Juvenile Justice; 2001 Oct; Sydney, Australia.

Salmond S. Complementary therapies in hepatitis C and drug and alcohol withdrawal. Phoebe House; 2001 Sept; Sydney, Australia.

Salmond S. Herbal medicine and hepatitis C. Hepatitis C Nurse Consultants Meeting; 2001 Aug; Royal North Shore Hospital, Sydney, Australia.

Salmond S. Hep573 Study: pilot data and trial protocol. Paper presented at: John Hunter Hospital Gastroenterology Research Meeting; 2001 Jun; Newcastle, Australia.

Salmond S. Testing herbal medicines in the treatment of chronic hepatitis C. Paper presented at: National Herbalists Association of Australia (NHAA) Fourth International Phytotherapeutics Conference; 2001 Feb; Meroo, Australia.

C2.   Oral presentations with others

Salmond S, Batey RG. Evaluating herbal hepatotoxicity, concurrent use of herbal medicines & pegylated interferon and ribavirin and Hep573 Study results. NCAHS liver specialists and nurses; 2009 Sept 18; Ballina, Australia.

Salmond S, George J, Strasser S, Byth K, Batey R. Hep573 Study--a randomised controlled trial in chronic hepatitis C patients. Paper presented at: Australian Hepatology Association Annual Summit; 2009 Mar 23; Sydney, Australia.

Salmond S, George J, Strasser S, Byth K, Batey R. Hep573 Study--preliminary results. Paper presented to: Clinical Nurse Consultants at Australian Gastroenterology Week; 2008 Oct 23; Brisbane Convention Centre, Brisbane, Australia

Batey R, Salmond S. Traditional Chinese medicine (TCM) and hepatitis in a western liver clinic experience. International Society for Chinese Medicine International Conference on Chinese Medicine and Pre-Conference Symposium on Evidence-Based Chinese Medicine; 2005 Apr; Macau, China.

Salmond S, Strasser SI, Batey RG. What is the evidence for the use of anti-oxidant nutrients or antioxidant herbal medicines in the treatment of chronic hepatitis C? Paper presented at: Third Australasian Conference on Hepatitis C; 2002 Mar 25-27; Melbourne, Australia.

SECTION D

Other publications

D1. Fact sheets

Salmond S. Stress and the liver [Fact Sheet]. Canberra, Australia: Australian Injecting and Illicit Drug Users League (AIVL); 2007 Jan.

APPENDIX B

PATIENT INFORMATION SHEET AND INFORMED CONSENT

Appendix B comprises the patient information sheet, the informed consent form and two attachments: the formulae used in the Hep573 Study and the patient safety information sheet.

INFORMATION SHEET

Study Title: Double-blind, randomised, placebo-controlled clinical trial to compare the efficacy and safety of Hep573 (two different herbal &/or vitamin formulations) in patients with chronic hepatitis C.

Short title: Herbal treatment (Hep573) in chronic hepatitis C

Protocol:	Hep573
Principal Investigator:	Professor Robert Batey (Director, Drug and Alcohol Clinical Services, Hunter New England Health Services)
Coinvestigators:	Ses Salmond (PhD student), Dr Jon Watson (Staff Specialist, Department of Gastroenterology, Hunter New England Health Services)
Institution:	University of Newcastle/ John Hunter Hospital

Background

This research Study is a joint venture between the University of Newcastle and the John Hunter Hospital.

You are being invited to take part in a human research Study entitled 'Hep573 in chronic hepatitis C.'

Herbal medicine can be used for the treatment and management of the symptoms of the hepatitis C virus infection and there is some evidence that herbal medicine may reduce liver inflammation and scarring (liver fibrosis) and fatigue.

You have been diagnosed as having hepatitis C, a chronic, progressive liver disease that can cause varying degrees of liver damage. The prognosis of hepatitis C in patients with persistently normal ALT levels (most important liver enzyme in hepatitis C) is good. There is very low risk of progressive liver injury or of the complications of liver disease with or without treatment. If ALT is elevated, hepatitis C may progress to an advanced stage in 15-20% of patients over a period of 10-30 years from the date of infection. The severity of the injury varies from patient to patient, with only 15-20% ever progressing to cirrhosis. One of the main options for treating chronic hepatitis C is the use of combination therapy with interferon and ribavirin.

Treatment with interferon/ribavirin can result in a "cure" of the infection in 40-50% of patients. This is associated with a marked reduction in liver damage and an improved prognosis. However, this treatment is not without significant side effects. Many of those attending the John Hunter Hospital Liver Clinic choose not to take this form of treatment (approx. 30%) with some instead choosing herbal medicine.

Purpose of the Study

The purpose of this clinical Study (called Hep573) is to compare the effectiveness and safety of two different active herbal and/or vitamin formulations and to investigate the actions of these regimens in the treatment of hepatitis C. We wish to identify the most effective combination of alternative treatments defined by ALT outcome and well being scores. More details are in Appendix A of the consent form.

Procedures

If you enter the trial you will receive one of these two active formulations or a placebo for up to six months and then you will be followed up for a further six months off

treatment. You have a 66% chance of getting an active formulation. A placebo preparation looks and tastes like the active preparation, but it contains no active preparation. This allows us to test the true effects of the treatment.

Neither you nor your clinician will know which you are receiving until the twelve months trial period is complete (six months' treatment and six months' follow-up).

If you are eligible to be involved, you will be asked to take up to six (6) tablets twice daily with meals for six months. Your dose will be gradually increased over a period of one week, starting at three (3) tablets twice daily. This trial will require monthly visits (thirteen in total) to the John Hunter Hospital in order to monitor your progress with blood tests. These blood tests will measure liver function and include other routine tests normally part of our follow up of hepatitis C patients. The side effects of the blood tests are minimal. In a few cases participants may experience bleeding and/or bruising at the injection site and possible dizziness or fainting. If this has happened to you before please advise the medical practitioner before you have your blood taken.

We will need you to fast for 12 hours (eat or drink nothing except water) prior to your blood tests on Week 0, Week 12, Week 24 and Week 48. This is because the food you eat may contain certain types of nutrients (antioxidants) that may interfere with the accuracy of the blood test. Please let us know if there is any reason why you would find this hard to do.

At the completion of the twelve months Study if you were not in the group that received the most promising active formulation, you will then be offered six months supply of the most active formulation. In the unanticipated event that the recipients of the placebo show a better clinical response (e.g., lowered viral load and normalisation of liver enzymes), you will not be offered placebo at the end of the Study. The chart below details the procedures at each of your visits.

Screen	12 week wash out period	Visit 1 Week 0	Visit 2 Wk 4	Visit 3 Wk 8	Visit 4 Wk 12	Visit 5 Wk 16	Visit 6 Wk 20	Visit 7 Wk 24
Sign Consent form to participate in the Study	Wait 12 weeks if you have taken herbs &/or vitamins	BT Fast PE Full medical history	BT PE	BT PE	BT Fast PE	BT PE	BT PE	BT Fast PE
		Genotype						
		Viral load						Viral load
		Viral detection test (DT)						Viral DT
		QOLQ			QOLQ			QOLQ
		DSQ	DSQ	DSQ	DSQ	DSQ	DSQ	DSQ
		HA	HA	HA	HA	HA	HA	HA

	Visit 8 Wk 28	Visit 9 Wk 32	Visit 10 Wk 36	Visit 11 Wk 40	Visit 12 Wk 44	Visit 13 Wk 48
	BT PE	BT PE	BT PE	PE	PE	BT Fast PE
			QOLQ			QOLQ
	DSQ	DSQ	DSQ	DSQ	DSQ	DSQ
	HA					

Key to the chart:
BT - Blood test (including liver enzymes, platelets & thyroid function tests)
Fast - nothing to eat or drink (except water) for 12 hours prior to the blood test
PE - Physical examination including your blood pressure being taken
Genotype - This test tells us which of the nine different types of hepatitis C virus you have. Some of the different types are more responsive to treatment with antiviral therapy.
Viral load - measures the number of viral particles in a mL of blood.
Viral DT - Viral detection test - is the virus detectable (positive) or not (negative - cleared the virus)
QOLQ - Quality of Life Questionnaire
DSQ - Diet & Symptom Questionnaire
HA - Herbal Assessment - assessing your progress and making any necessary adjustments to dosage.

Eligibility
If you are aged between 18 and 75 years you are eligible to enter this Study.

Washout period
Once you have been accepted as a participant in the trial, it is essential that you discontinue all herbs and vitamins for a period of twelve weeks so that these can clear out of your body before you start the Study.

Exclusions
You may not take part in this Study if you are:
- Pregnant or intending to become pregnant
- Breastfeeding
- Have high blood pressure (> 140/90)
- On certain medications (listed below)
- Drink more than seven (7) standard drinks per week
- Have severe cirrhosis (decompensated cirrhosis)
- Have other liver diseases other than hepatitis C
- On an unstable methadone dose or your methadone dosage > 100 mg per day
- Using recreational drugs or non-prescription drugs > 3-4 times per week.

The reasons for these exclusions are provided below.

- Pregnancy and breastfeeding:
  If you are a woman and are contemplating becoming or hope to become pregnant during the course of the trial you will not offer yourself as a participant in this trial.

  I) One of the herbs used in this Study *Andrographis paniculata* (andrographis) is not to be taken in pregnancy as it can cause miscarriage.

2) The full effects of herbal medicine on the developing fetus is unknown
3) As herbs can be absorbed from breast milk some of the Study herbs may be transferred to an infant and these may cause gastrointestinal upsets.

Female participants must use two forms of effective contraception during the course of the Study and for a period of two weeks after completion of the Study because of the above risks.
If you do become pregnant whilst on the Study, you should advise your treating doctor **immediately**, who will withdraw you from the Study and the pregnancy will be managed by whoever you choose to manage your pregnancy.

- Other medications: one herb used in our preparations, *Hypericum perforatum* (Saint John's wort) can interact with medications such as antidepressants, HIV medications such as indinavir, immune suppressants, heart medications such as digoxin, pharmaceutical anticoagulants, such as warfarin and the asthma medication theophylline.

- Alcohol intake: if you drink more than seven (7) standard drinks per week you are not eligible to offer yourself as a participant in this trial. Alcohol can cause the hepatitis C virus to replicate more quickly. If your current alcohol intake is less than or equal to seven standard drinks per week you are asked not to vary your alcohol intake during the course of the trial in order to standardise Study conditions.

- Severe cirrhosis: if you have severe cirrhosis often referred to as decompensated cirrhosis, then it is necessary for you to be under close medical care by your liver specialist.

- Other liver diseases, apart from hepatitis C: if you have other liver diseases you are not eligible as the Study is to gain information on the impact of herbal medicines on hepatitis C only.

- Methadone dose and stability: if your methadone dose is under adjustment or your dosage is greater than 100 mg per day you will not be eligible to join the trial as it will be difficult to differentiate whether your symptoms are related to your methadone dosage or the trial preparations.

The researchers will complete a medication checklist with you in a face-to-face interview. Please bring either a list or samples of medications you are currently taking to this interview, as there may be drug/herb interactions, which may exclude your participation in this Study.

Side effects
Anyone taking part in the trial who experiences any major symptoms (e.g. headache, nausea, vomiting, reflux, diarrhoea. chest or abdominal pain, light-headedness, garlic odour to your breath or sweat) after taking the tablets should cease taking the tablets and immediately contact Professor Robert Batey on 0419 481 546 (24 hour number). Please refer to the extra Patient Safety Information Sheet attached.

Confidentiality
Your personal information is strictly confidential and will not be publicly available in any form. No names will be identified with the data collected during the Study, although your medical records or any information gained during the Study may be inspected by the Australian Government's Therapeutic Goods Administration, the Office of Complementary Medicine and representatives from Phytomedicine; all due care will be taken to ensure your confidentiality.

Voluntary nature of participation and withdrawal

Your participation in this Study is completely voluntary. If you do not wish to take part, you are under no obligation to do so. If you decide to take part but later change your mind, you are free to withdraw from the Study at any stage. Your decision whether to take part or not to take part, or to withdraw, will not affect your routine medical treatment or your relationship with those treating you or your relationship with the hospital. While we ask you to endeavour to complete the course of treatment, you are free to withdraw from the Study at any time.

Persons to contact

If you would like more information about the Study or if you have any questions, do not hesitate to ask one of the researchers or one of the Study doctors treating you. People you can ask include:

Professor Robert Batey	Ses Salmond
John Hunter Hospital	University of Newcastle
Principal Investigator	Study Co-ordinator
Ph: (02) 4924 6484	Ph: (02) 9560 3011

This Study has been approved by the Hunter Area Research Ethics Committee and the University of Newcastle Human Research Ethics Committee. If you have any questions or concerns about your rights as a participant in this Study or complaints about how the Study is being run and you wish to speak to an independent person, please contact:

Dr Nicole Gerrand	or	Sue O'Connor
Professional Officer		Human Research Ethics Officer
Hunter Area Research Ethics		Research Branch
Committee		The Chancellery
Locked Bag No.l		University of Newcastle
New Lambton NSW 2305		Callaghan NSW 2308
Ph: (02) 4921 4950 Fax: (02) 4921 4818		Ph: (02) 4921 6333 Fax: (02) 4921 7164

Professor Robert Batey	Ms Ses Salmond
Gastroenterologist/Hepatologist	PhD Student
John Hunter Hospital	University of Newcastle

Dr Jon Watson
Staff Specialist
Department of Gastroenterology
John Hunter Hospital

INFORMED CONSENT
JOHN HUNTER HOSPITAL/UNIVERSITY OF NEWCASTLE
Study Title: Hep573, a herbal treatment in chronic hepatitis C.

By signing this form, I agree that:

1. My participation in this Study is voluntary and I may withdraw from the Study at any time without prejudice or loss of benefits to which I am otherwise entitled.
2. I have informed the investigator of all other clinical studies that I am currently participating in and any other medications I am taking.
3. I have fully read or have had read and explained to me in my native language this form and the information sheet detailing the research project in a way that I fully understand, and have had the opportunity of having my questions answered to my satisfaction.
4. The general purposes, methods and demands, and possible risks/side effects, inconveniences and discomforts that may occur during the trial have been made well known to me.
5. I will be allocated at random to receive one of the two active Hep573 herbal medicine and/or vitamin preparations or a placebo preparation. Neither I nor my clinician will know which of the three preparations I am receiving until after completion of the Study, which will take twelve months. If I experience an adverse reaction, I will report this to one of the researchers. If these effects continue on a reduced dose I understand that my anonymity code will be broken in order to investigate the contents of the preparation I was receiving and I will withdraw from the trial.
6. I understand that during the Study I will have to come to the Medical Outpatients Department at the John Hunter Hospital at the beginning and at 1,2,3,4,5,6,7,8,9,10,11,12 months thereafter (13 visits).
7. I will be asked to provide blood samples at these times. I will be asked to fast for 12 hours once every three months before certain blood tests. I will be available for these visits, and I understand that I may be withdrawn from the trial if I fail to attend for these visits, and I may not receive further supplies of the Hep573 tablets.
8. If I drink more than seven (7) standard alcoholic drinks per week 1 will not offer myself as a participant in this trial. If my current alcohol intake is equal to or less than seven (7) standard alcoholic drinks per week 1 should not vary my alcohol intake during the trial period of twelve months in order to standardise Study conditions.
9. I understand that I should not take any other alternative or complementary treatments or therapies (including other herbal medications) during the Study. I understand that I will need to wait 12 weeks before entering the Study if I have been taking herbs and or vitamins so these clear out of my body.
10. If I am a woman and I am contemplating becoming or hope to become pregnant during the course of the trial I will not offer myself as a participant in this trial. I understand that there are unknown effects of the trial preparations on the developing fetus and thus I understand the importance of contraception during the Study period of twelve months and for an added six months should I be a member of the placebo group. If during the trial, pregnancy becomes an issue I will withdraw from the trial without prejudice or loss of benefits to which I am entitled.
11. If I have severe cirrhosis I will not offer myself as a trial participant as close liver specialist follow up is the medical recommendation for my care.

12. If I have any other liver disease other than hepatitis C, I will not offer myself as a participant as I understand it will be hard to determine the impact of the trial preparations on hepatitis C alone.
13. If my methadone dosage is undergoing adjustment or my daily dosage is greater than 100 mg per day I will not offer myself as a trial participant as my methadone management may be affected by the trial preparations.
14. If I use recreational drugs or non-prescription drugs more than 3-4 times per week I will not offer myself as a participant as it is uncertain how these drugs will interact with the trial preparations.
15. I have had sufficient time to consider my participation in this Study and have asked any questions I need.
16. I have received a copy of this consent form and patient information sheet and I am aware that the investigator at the John Hunter Hospital will also retain a copy for his or her files.
17. By signing this form, I consent to participate in the Study.

_____
Name of Participant (In CAPITAL LETTERS)

_____
Signature of Participant   Date

_____
Name of Researcher (In CAPITAL LETTERS)

_____
Signature of Researcher   Date

The researchers in this Study are Professor Robert Batey, Principal Investigator, Drug and Alcohol Clinical Services, Hunter New England Health Sevices, Newcastle 2310, Ses Salmond, Study Coordinator, School of Medical Practice, University of Newcastle 2308 and Dr Jon Watson, Coinvestigator, Department of Gastroenterology, Hunter New England Health Sevices, Newcastle 2310.

HEP573 Study

Attachment one to the Patient Information Sheet and Consent form (Appendix A) detailing the formulae to be used in the Hep573 Trial.

Randomised, placebo-controlled, double-blind clinical trial
Trial preparations will be in tablet form for ease of compliance and for reproducibility of results

Daily Dose Chart of Trial Formulations
Group 1: Silymarin, Herbs and Vitamins
Group 1: Silymarin, Herbs and Vitamins delivered in tablet form as Antioxidant Compound, Hepavir and Immuhep tablets

Antioxidant compound (Silvmarin. Herbs and Vitamins)
Camellia sinensis (green tea) (equivalent to dry leaf)	4000 mg
Curcuma longa (turmeric) (equivalent to dry rhizome)	8000 mg
standardised to contain curcuminoids	280 mg
Silybum marianum (Saint Mary's thistle) (equivalent to dry fruit)	4000 mg
standardised to contain flavonolignans calculated as silybins	48 mg
Vitis vinifera (grape seed) equivalent to dry seed	12000 mg
standardised to contain procyanidins	80 mg
Selenomethionine	40 mg
Elemental selenium	200 mcg
Lycopene	80 mg

Hepavir tablets (Silvmarin and herbs)
Silybum marianum (Saint Mary's thistle) (equivalent to dried *Silybum marianum* fruits) (approx 14 grams standardised to 70% silymarin calculated as silybin 180 mg) calculated as silybins	672 mg
Andrographis paniculata (andrographis) standardised to contain 34.8 mg andrographolide	3000 mg
Hypericum perforatum (Saint John's wort) standardised to contain 0.8 mg hypericin	1500 mg
Phyllanthus amarus (phyllanthus)	3000 mg

Immuhep tablets (Herbs and Vitamins)

Astragalus memhranaceus (astragalus) equivalent to dry root	3000 mg

Eleutherococcus senticosus (Siberian ginseng)   3000 mg
standardised to contain 1.2 mg Syringaresinol diglucosides (eleutherosides)

Alpha lipoic acid   200 mg

Calcium ascorbate (Buffered Vitamin C)   400 mg

Zinc Amino Acid Chelate 20% 250 mg, elemental zinc   50 mg

Group 2: Silymarin only group

Silymarin
Silybum marianum (equivalent to dried *Silybum marianum* fruits).
(approx 15 grams standardised to 70% silymarin
calculated as silybin 180 mg) calculated as silybins   720 mg

Placebo Herbs
Placebo Vitamins

Group 3: Placebo group.

Placebo Silymarin
Placebo Herbs
Placebo Vitamins

Dose: Six tablets (two of each compound) twice daily with meals.

All the above are listable items with the Therapeutic Goods Administration.

**Attachment two to the Information Sheet and Consent Form (Appendix A.1)
Patient Safety Information Sheet for the HEP573 Study**

The herbs and vitamins you are being prescribed are safe and within the recommended daily doses. Individuals vary in how much of a given dose they absorb and also in how they excrete the substances prescribed and thus their side effects. For this reason we would like you to be aware of the known major adverse effects that might be attributable to certain components of the Study preparations are listed below:

Alpha lipoic acid may lead to light-headedness, tiredness, irritability, ravenous hunger if blood sugar levels are unstable. It may improve blood sugar control in diabetes.

Andrographis paniculata (andrographis) reduces fertility in men and women and must not be taken during pregnancy as it can cause miscarriage. High doses may cause gastric discomfort, lack of appetite, vomiting and hives. This is not expected on the dose you will be given in the Study. A study of the isolated ingredient andrographolide from the above herb led to anaphylaxis, a severe allergic reaction. In this Study we are using the whole medicinal parts of the plant and this is not an expected outcome.

Astragalus membranaceus (astragalus) is a powerful immune stimulant. If you are on any immune suppressing drugs such as cyclosporine, azathioprine and methotrexate, Astragalus may cause the opposite effect to your prescribed medication.

Camellia sinensis (green tea) contains small amounts of caffeine. This may affect those individuals who are sensitive to caffeine. The Study dose of green tea is equivalent to 4 grams, so this means you will be receiving approximately 75 mg of caffeine per day.

Curcuma longa (turmeric) may also thin the blood so high doses (>5 grams per day) and should not be given to patients taking antiplatelet or anticoagulant drugs such as warfarin. The dose in the active preparation in the trial is 8 grams, so it should not be given to patients taking these drugs.

Hypericum perforatum (Saint John's wort) can interact with medications such as antidepressants, immune suppressants, medications for HIV (Indinavir), heart (Digoxin), asthma (Theophylline), blood thinners (Warfarin). Saint John's Wort can cause abdominal symptoms and tiredness and in rare cases can cause photosensitivity (sensitivity to the sun).

Selenium is present in onc of the formulations. Garlic odour of the breath and sweat is a sign of selenium toxicity. If this occurs, please stop taking the tablets immediately. This is not expected on the dose you will be given in the Study.

Silvbum marianum (Saint Mary's thistle) is generally well tolerated but can occasionally cause diarrhoea

Unbuffered Vitamin C as ascorbic acid may cause diarrhoea, intestinal distension or gas. Buffered forms of vitamin C do not have this effect. You will be taking a buffered form of this vitamin (Calcium ascorbate).

Zinc is present in one of the formulations. If zinc is taken on an empty stomach it can result in gastrointestinal upset and nausea, so make sure you take it with food.

It's important for you to let the researchers know about all your prescribed medications, as well as over-the-counter medications to ensure there are no potential drug/herb interactions. Your safety while taking the herbs and vitamin preparations in this Study is paramount and will be managed by the two researchers (Professor Robert Batey and Ses Salrnond) and will also be independently monitored by the Clinical Pharmacology Department at the University of Newcastle. Please report any concerns or side effects from the Study preparations to the researchers immediately so that a prompt assessment and readjustment (if necessary) can be made.

The 24-hour numbers of the researchers are as follows:
Professor Robert Batey 0419 481 546   Ses Salmond 0414 453 243

APPENDIX C

ADVERTISING MATERIAL

Appendix C comprises the approved advertising material which appeared in the Australian Doctor and Medical Observer in 2004 and 2006, an advertisement in Sunday Telegraph and a poster in the Hep C Review in 2005. Flyers for participants and health professionals were also approved in 2004 and 2006.

Advertisement for the Australian Doctor, Version 4, 20 February 2006

Hepatitis C Herbal Medicine Clinical Trial (Hep573 Study)

This Study is to test the safety and effectiveness of herbs and vitamins in the treatment of hepatitis C.

The Hep573 Study is under way at John Hunter Hospital, Royal Prince Alfred Hospital and Westmead Hospital. The trial duration will be six months on treatment or placebo and six months follow-up. Participants will receive six months supply of the trial preparations and regular 'check ups'.

The trial is being conducted by Ses Salmond, PhD student at the University of Newcastle under the supervision of Professor Robert Batey. Participants should be aged between 18 and 75 years. If you would like an information pack for General Practitioners to be forwarded to you, please contact Ses on 0414 453 243.

Approval for the protocol has been granted by the Human Research Ethics Committees of the University of Newcastle, Hunter New England Area Health Service, Central Sydney Area Health Service and Sydney West Area Health Service.

Enrolments in this Study close 31 March 2006.

Hepatitis C Herbal Medicine Clinical Trial (Hep573 Study)

This study is to test the safety and effectiveness of herbs and vitamins in the treatment of hepatitis C.

The Hep573 Study is under way at John Hunter Hospital, Royal Prince Alfred Hospital and Westmead Hospital. The trial duration will be six months on treatment or placebo and six months follow-up after treatment. Participants should be aged between 18 and 75 years and will receive six months supply of the trial preparations and regular 'check ups'.

The trial is being conducted by Ses Salmond, PhD student at the University of Newcastle under the supervision of Professor Robert Batey. If you would like an information pack for General Practitioners to be forwarded to you, please contact Ses on 0414 453 243.

Approval for the protocol has been granted by the Human Research Ethics Committees of the University of Newcastle, Hunter New England Area Health Service, Central Sydney Area Health Service and Sydney West Area Health Service.

Enrolments in this Study close 31 March 2006.

Hepatitis C – Have you considered herbal medicine?

Clinical trial with a difference

Clinical trial for hepatitis C – Herbal Medicine

The Hep573 Study, a scientifically based clinical trial is under way and will help us better understand the safety and effectiveness of herbs and vitamins in the treatment of hepatitis C.

Do you have hepatitis C, three raised liver function tests (ALT), no other severe liver diseases and are able to come to the hospital each month for 12 months for blood tests and to complete questionnaires?

If so we would appreciate hearing from you in order to assess if you can help us in this Study.

The trial duration will be six months on treatment or placebo and six months follow-up after treatment.

If you participate in the study you will receive six months supply of the trial preparations free and will receive regular check ups and ongoing support

There are three trial sites: John Hunter Hospital, Newcastle; Royal Prince Alfred Hospital and Westmead Hospital, Sydney.

This Study is being conducted as part of a PhD program at the University of Newcastle by Ses Salmond under the supervision of Professor Robert Batey and Associate Professor Alan Bensoussan.

Professor Robert Batey, Dr Simone Strasser and Professor Jacob George are the gastroenterologist specialists associated with John Hunter Hospital, Royal Prince Alfred Hospital and Westmead Hospital respectively.

Approval has been granted from the relevant ethics committees.

Please contact:

If you would like to join the study please contact Ses Salmond on 0414 453 243 or ask your doctor or health professional for more information.

Enrolments close on 31 March 2006.

NEWCASTLE | CENTRAL COAST | PORT MACQUARIE | SINGAPORE

The University of Newcastle   enquirycentre@newcastle.edu.au   T +61 2 4921 5000
Callaghan NSW 2308 Australia   CRICOS Provider Number: 00109J   www.newcastle.edu.au

261

Flyer for health professionals, Version 2, 20 February 2006

New hepatitis C herbal medicine trial has commenced.

A significant proportion of people with hepatitis C take herbal preparations. The effect of these herbs is not fully understood therefore a randomised, double-blind, placebo-controlled clinical trial testing the safety and efficacy of herbal medicine in the treatment of chronic hepatitis C has commenced at three NSW hospitals.

Personnel involved: Professor Robert Batey, John Hunter Hospital; Dr Simone Strasser Royal Prince Alfred Hospital; Professor Jacob George, Westmead Hospital and Ses Salmond, University of Newcastle.

Details of the Study are listed below:

Three arms to the study

Full treatment:–Saint Mary's thistle, Herbs and Vitamins
Partial treatment:–Saint Mary's thistle only
No treatment:–placebo
The placebo group at the end of the Study will receive six months supply of the most promising formulation.

Study duration
Six months on treatment, six months follow-up

Number to be recruited
216 patients across three hospitals

Trial sites

John Hunter Hospital, Newcastle
Royal Prince Alfred Hospital, Sydney
Westmead Hospital, Sydney

Inclusion criteria	*Exclusion criteria*
Able to give informed consent	Hepatitis B or Hepatitis D or HIV
Aged between 18 and 75 years	Severe liver disease
Hepatitis C antibody positive	Prior combination therapy in last 6 months
Abnormal ALT/AST on 3 occasions in the past 6 months–2 years	Pregnant or lactating females
Prepared to attend monthly clinics for tests, procedures	Unstable on methadone dose
Adequate renal and haematological function	>70 g alcohol per week

Contact numbers

Please register your interest in participating in this trial by phoning Ses Salmond on 0414 453 243. Enrolments close on 31 March 2006.

Outcome measures

ALT levels

PCR HCV RNA Quantitative tests (Viral load)

PCR HCV RNA Qualitiative tests (Viral detection)

Antioxidant activity

Markers of oxidative stress and lipid peroxidation

Fibrosis markers

Hepatitis C Herbal Medicine Clinical Trial (Hep573 Study)

This study is to test the safety and effectiveness of herbs and vitamins in the treatment of hepatitis C.

The Hep573 Study is underway at John Hunter Hospital, Royal Prince Alfred Hospital and Westmead Hospital. The trial duration will be six months on treatment or placebo and six months follow up. Participants aged between 18 and 65 years will receive six months supply of the trial preparations and regular 'check ups'.

The trial is being conducted by Ses Salmond, PhD student at the University of Newcastle under the supervision of Professor Robert Batey. You could contact Ses on 0414 453 243 for an information package or alternatively you could forward this advertisement to your patients.

Approval for the protocol has been granted by Hunter Area Research Ethics Committee, University of Newcastle Human Research Ethics Committee, Central Sydney Area Health Service Ethics Committee and Western Sydney Area Health Service Ethics Committee.

Enrolments in this Study close March 2005

Hepatitis C Herbal Medicine Clinical Trial (Hep573 Study)

This study is to test the safety and effectiveness of herbs and vitamins in the treatment of hepatitis C.

The Hep573 Study is underway at John Hunter Hospital, Royal Prince Alfred Hospital and Westmead Hospital. The trial duration will be six months on treatment or placebo and six months follow-up after treatment. Participants aged between 18 and 65 years will receive six months supply of the trial preparations and regular 'check ups'.

The trial is being conducted by Ses Salmond, PhD student at the University of Newcastle under the supervision of Professor Robert Batey. If you would like an information pack for General Practitioners to be forwarded to you, please contact Ses on 0414 453 243.

Approval for the protocol has been granted by Hunter Area Research Ethics Committee, University of Newcastle Human Research Ethics Committee, Central Sydney Area Health Service Ethics Committee and Western Sydney Area Health Service Ethics Committee.

Enrolments in this Study close March 2005.

Hepatitis C Herbal Medicine Clinical Trial (Hep573 Study)

This study is to test the safety and effectiveness of herbs and vitamins in the treatment of hepatitis C.

The Hep573 Study is underway at John Hunter Hospital, Royal Prince Alfred Hospital and Westmead Hospital. The trial duration is six months on treatment or placebo and six months follow-up after treatment. Participants will receive six months supply of the trial preparations and regular 'check ups'.

The trial is being conducted by Ses Salmond, PhD student at the University of Newcastle under the supervision of Professor Robert Batey. If you have hepatitis C and are aged between 18 and 75 years and are interested in participating in this herbal study contact Ses on 0414 453 243.

Approval has been granted by the relevant ethics committees

Enrolments close on 31 March 2005.

Phytomedicine – a natural medicines company is proudly supporting this research.

Poster, Version 3, 10 February 2005

Front page of the poster

GET HEP!

Graphic: man holding Milk thistle

Join our Groovy Milk Thistle Hep C Trial

Phone 1800 803 990 for more info

Page 2 of the poster

216 people with hep C to trial herbal treatment

Would you like to help test whether Milk thistle and other ingredients are effective for hep C, without the side effects of interferon or ribavirin? This trial has the support of the Hep C Council of NSW and if you have Hep C you could be one of the participants.

Where will the trial be run?

The trial will be run from Royal Prince Alfred Hospital and Westmead Hospital in Sydney and John Hunter Hospital in Newcastle.

Here's what an independent expert says about Milk thistle

"Many people want to try natural medicines (things like plant extracts) to see if their liver function improves. Some people with chronic hepatitis C find that Milk thistle does improve liver tests, but there is no definite proof this is an effective treatment against a virus or to protect the liver. It is important that trials like this be conducted to find out reliably whether people could take preparations like this against hepatitis C".

Professor Geoff Farrell, Professor of Hepatic Medicine, Storr Liver Unit, University of Sydney at Westmead Hospital, NSW.

When do I start?

Screening of candidates is now underway so contact us by phoning this week.
Don't put it off. Get HEP!

Like more info?

To find out more details about how you can participate in the 'Milk thistle' Hep C trial call the helpline at the Hep C Council of NSW on 1800 803 990.

Flyer for participants, Version 1, 13 October 2004

Hepatitis C – Have you considered herbal medicine?

Clinical trial with a difference

Clinical trial for hepatitis C – Herbal Medicine

The Hep573 Study, a scientifically based clinical trial is under way and will help us better understand the safety and effectiveness of herbs and vitamins in the treatment of hepatitis C.

Do you have hepatitis C, three raised liver function tests (ALT), no other severe liver diseases and are able to come to the hospital each month for 12 months for blood tests and to complete questionnaires?

If so we would appreciate hearing from you in order to assess if you can help us in this study.

The trial duration will be six months on treatment or placebo and six months follow-up after treatment.

If you participate in the study you will receive six months supply of the trial preparations free and will receive regular check-ups and ongoing support

There are three trial sites: John Hunter Hospital, Newcastle; Royal Prince Alfred Hospital and Westmead Hospital, Sydney.

This Study is being conducted as part of a PhD program at the University of Newcastle by Ses Salmond under the supervision of Professor Robert Batey and Associate Professor Alan Bensoussan.

Professor Robert Batey, Dr Simone Strasser and Professor Geoff Farrell are the gastroenterologist specialists associated with John Hunter Hospital, Royal Prince Alfred Hospital and Westmead Hospital respectively.

Approval has been granted from the relevant ethics committees.

Please contact:

If you would like to join the Study please contact Ses Salmond on 0414 453 243 or ask your doctor or health professional for more information.

Flyer for health professionals, Version 1, dated 13 October 2004

New hepatitis C herbal medicine trial has commenced.

A significant proportion of people with hepatitis C take herbal preparations. The effect of these herbs is not fully understood therefore a randomised, double-blind, placebo-controlled clinical trial testing the safety and efficacy of herbal medicine in the treatment of chronic hepatitis C has commenced at three NSW hospitals. Personnel involved: Professor Robert Batey, John Hunter Hospital; Dr Simone Strasser Royal Prince Alfred Hospital; Professor Geoff Farrell, Westmead Hospital and Ses Salmond, University of Newcastle.

Details of the Study are listed below:

Three arms to the study

Full treatment:–Saint Mary's thistle, Herbs and Vitamins

Partial treatment:–Saint Mary's thistle only

No treatment:–placebo

The placebo group at the end of the Study will receive six months supply of the most promising formulation.

Study duration

Six months on treatment, six months follow-up

Number to be recruited

216 patients across three hospitals

Trial sites

John Hunter Hospital, Newcastle

Royal Prince Alfred Hospital, Sydney

Westmead Hospital, Sydney

Inclusion criteria   *Exclusion criteria*

Inclusion criteria	Exclusion criteria
Able to give informed consent	Hepatitis B or Hepatitis D or HIV
Aged between 18 and 65 years	Severe liver disease
Hepatitis C antibody positive	Prior combination therapy in last 6 months
Abnormal ALT/AST on 3 occasions in the past 6 months–2 years	Pregnant or lactating females
Prepared to attend monthly clinics for tests, procedures	Unstable on methadone dose
Adequate renal and haematological function	>70 g alcohol per week

NEWCASTLE | CENTRAL COAST | PORT MACQUARIE | SINGAPORE

The University of Newcastle   enquirycentre@newcastle.edu.au   T +61 2 4921 5000
Callaghan NSW 2308 Australia   CRICOS Provider Number: 00109J   www.newcastle.edu.au

269

Contact numbers

Please register your interest in participating in this trial by phoning Ses Salmond on 0414 453 243. Recruitment has begun. Places are still available.

Outcome measures

ALT levels

PCR HCV RNA Quantitative tests (Viral load)

PCR HCV RNA Qualitiative tests (Viral detection)

Antioxidant activity

Markers of oxidative stress and lipid peroxidation

Fibrosis markers

APPENDIX D

COMPLEMENTARY MEDICINES EXCLUSIONS

Appendix D comprises the list of complementary medicines that were excluded whilst on the Hep573 Study.

Complementary Medicines Exclusions 10 September 2003 for the Hep573 Study

1. Any alternative chemical treatment (nutritional supplement, herb, vitamin, mineral etc)

Unless the alternative chemical treatment has been treating a separate condition (other than hepatitis C) for over 12 months, in which case the supplement and the condition are duly noted. e.g glucosamine sulphate for osteoarthritis.

The definition of alternative chemical treatment includes: Traditional Chinese Medicine, Western herbal medicine, Ayurvedic Medicine, Kampo medicine (Japanese herbs) and Naturopathy (western herbs, vitamins, minerals and nutritional supplements).

The best way to achieve this is to have the cleanest baseline measurement possible by stopping all herbs and vitamins prior to trial entry, unless someone is a recovering alcoholic and is on thiamine.

However if the product used for a separate condition contains anyone of the ingredients below it is excluded and requires a wash out period of 12 weeks prior to trial entry.

Any supplement that contains any of the following trial ingredients is inadmissible
Alpha lipoic acid
Andrographis
Astragalus
Grapeseed extract
Green tea
Phyllanthus
Turmeric
St John's wort (Hypericum)
Selenium
Siberian ginseng (Eleutherococcus)
Vitamin C
Zinc

Although not a trial ingredient Vitamin B12 may help stall HCV replication. Any supplements that include vitamin B12 are excluded whilst on the.trial unless prescribed for another condition for greater than 12 months.

2. Physical therapies

The following physical therapies are permitted while on the trial:
Osteopathy, Chiropracty, Reflexology, Rolfing, Deep tissue massage, Remedial massage, Aromatherapy massage, Swedish massage, Physiotherapy, Acupuncture, Shiatsu.
It is requested that these physical therapies remain stable during the Study period and are recorded.

3. Tea, Coffee, Herbal tea

No specifications will be made regarding tea, coffee and herbal tea consumption except that the consumption of Green tea, St John's wort tea are prohibited as they are trial ingredients. However accurate recording of the participant's intake of these substances must be taken.

4. The following lifestyle practices are permissible while on the trial:

Qi Gong, Tai Chi, Yoga, Pilates, Feldenkrais, Alexander technique, Bowen Technique (Therapy), Kinesiology, Meditation and exercise. It is also requested that these remain stable during the study period. Accurate recording of lifestyle practices would be appreciated.

APPENDIX E

ALCOHOL, DRUGS, DIET AND SYMPTOMS QUESTIONAIRES

Appendix E contains questionnaires used in the Hep573 study to assess possible confounding factors.

Name of the Instrument: **ALCOHOL AND OTHER DRUGS SCREENING QUESTIONNAIRE**

To be completed by: Trial participant in consultation with one of the research team

Hep573:   Randomised, double-blind, placebo-controlled clinical trial to
compare the efficacy and safety of two different herbal and/or
vitamin formulations in patients with chronic hepatitis C.

Site Number	Subject's Initials	Date of Birth	Visit Date	Screen
☐☐	☐☐☐	☐☐/☐☐/☐☐☐☐	☐☐/☐☐/☐☐☐☐	Patient Identification Number ☐☐☐

TOBACCO HISTORY

Q1. Please tick the most correct answer

Non-smoker   ☐

Ex-smoker   ☐

Current smoker   ☐

Years smoked _____ No. per day/week (e.g. 15/d or 15/wk) _____ Strength in mg _____

Quit how long ago _____ No. of quit attempts _____ Weak ☐ Strong ☐

ALCOHOL HISTORY

The following diagram shows what is referred to as a standard drink.
Use the following diagram to help you measure the amount of alcohol (per standard drink size) you drink.

Light Beer (schooner) 425 ml   OR   Heavy Beer (middy) 285 ml   OR   Wine 100 ml   OR   Fortified Wine 60 ml   OR   Spirits Liqueurs 30 ml

Q2. When was the last time you drank alcohol, (please specify day, week ago etc) _____

and how many standard drinks did you drink (tick below) over what time period (hours, day) _____

► 20 or more standard drinks per day   ☐

► 15-19 standard drinks   ☐

► 10-14 standard drinks   ☐

► 5-9 standard drinks   ☐

► 3-4 standard drinks   ☐

► 2 standard drinks   ☐

► 1 standard drink   ☐

► Other amount e.g. 1/2 glass of wine   ☐   Please specify _____

► Never   ☐   Go to (Q7)

Q3. When was the second last time you had a drink (please specify the time between drinks e.g. 1 week ago, the day before)_____

and how many standard drinks did you drink (tick below) over what time period? _____

_____

► 20 or more standard drinks per day ☐

► 15-19 standard drinks ☐

► 10-14 standard drinks ☐

► 5-9 standard drinks ☐

► 3-4 standard drinks ☐

► 2 standard drinks ☐

► 1 standard drink ☐

► Other amount ☐ Please specify _____

Q4. When was the time before that, that you had a drink _____

and how many standard drinks did you drink (tick below) over what time period? _____

_____

► 20 or more standard drinks per day ☐

► 15-19 standard drinks ☐

► 10-14 standard drinks ☐

► 5-9 standard drinks ☐

► 3-4 standard drinks ☐

► 2 standard drinks ☐

► 1 standard drink ☐

► Other amount ☐ Please specify _____

Q5. Has this been your normal drinking pattern during the past three months? Yes ☐ No ☐

Q6. If not, how has your drinking pattern changed? Please specify _____

_____

_____

_____

Q7. On average, how often do you have a drink containing alcohol?

| Never ☐ | Monthly ☐ or less | Once per week ☐ or less | 2-4 times ☐ a week | 5 or more ☐ a week |

Q8. How many standard drinks do you have on a typical day when you are drinking?

1 ☐   2 ☐   3 or 4 ☐   5 or 6 ☐   7–9 ☐   10–14 ☐   15–19 ☐   20 or more ☐

Q9. How often do you have 6 or more standard drinks on one occasion?

| Never ☐ | Less than ☐ monthly | Monthly ☐ | Weekly ☐ | Daily ☐ |

Q10. Have you changed your alcohol use since your diagnosis of hepatitis C?   Yes ☐ No ☐

Year of diagnosis of Hep C (Fill in)

Please tick the applicable box/es

☐　　Stopped drinking

☐　　Cut down drinking. By how much? _____

☐　　Changed type of alcohol. What change? _____

☐　　Increased drinking. By how much? _____

☐　　No change

☐　　Other, please specify _____

Q11. Other drug history (Specifically recreational and non-prescription drugs)

Substance	Route of administration	Dose/Frequency E.g. 2 tabs daily	How long using the substance?	Date last used
☐　Heroin				
☐　Methadone				
☐　Cocaine				
☐　Speed/ Amphetamines				
☐　Cannabis				
☐　Solvents				
☐　Prescription medicine type				
☐　Hallucinogens e.g. acid				
☐　Ecstasy/ Party drugs/type				
☐　Other				

Name of the Instrument: **ALCOHOL AND OTHER DRUGS FOLLOW-UP QUESTIONNAIRE**

To be completed by: Trial participant in consultation with one of the research team

Hep573:   Randomised, double-blind, placebo-controlled clinical trial to compare the efficacy and safety of two different herbal and/or vitamin formulations in patients with chronic hepatitis C.

Site Number	Subject's Initials	Date of Birth	Visit Date	Screen
☐☐	☐☐☐	☐☐/☐☐/☐☐☐☐	☐☐/☐☐/☐☐☐☐	Patient Identification Number ☐☐☐

Cigarette smoking in the past month

If you are a non-smoker go to Q2. Non-smoker ☐

Q1. On average, how many cigarettes/rollies have you smoked in the last month?

No. per day/week (e.g 15/d or 15/wk) _____ Strength in mg _____

Comment: _____

_____

Alcohol consumption in the past month

If you never drink alcohol go to Q5. Non-drinker ☐

Q2. On average, how often do you have a drink containing alcohol?

Never ☐   Monthly ☐ or less   Once per week ☐ or less   2-4 times ☐ a week   5 or more ☐ a week

Q3. How many standard drinks did you have in the past month on a typical day when you were drinking?

Light Beer (schooner) 425 ml   OR   Heavy Beer (middy) 285 ml   OR   Wine 100 ml   OR   Fortified Wine 60 ml   OR   Spirits Liqueurs 30 ml

1 ☐   2 ☐   3 or 4 ☐   5 or 6 ☐   7–9 ☐   10–14 ☐   15–19 ☐   20 or more ☐

Q4. How often do you have 6 or more standard drinks on one occasion in the past month?

Never ☐   Less than ☐ monthly   Monthly ☐   Weekly ☐   Daily ☐

Comment: _____

_____

Recreational drug use in the past month

Q5 On average. how often in the last month have you used a recreational drug?

Never ☐   Monthly ☐ or less   Once per week ☐ or less   2-4 times ☐ a week   5 or more ☐ a week

Comment: _____

_____

Diet And Symptoms Checklist

Confidential Hep573 ☐☐☐

Visit Month: _____ Date completed: (dd/mm/yyyy) ___ / ___ / 20 ___

• We would like to know about your recent diet and health.

• First, mark which of the foods or drinks listed below you have had in the *past month*. For those you have had, mark if they made you feel sick or ill, and if they did, how sick or ill you felt .

• Then overleaf, mark which of the list of symptoms you have had in the *past month*. For each symptom you have had, show how much of the month it has troubled you, and how bad it has been.

PLEASE COMPLETE THIS CHECKLIST _BEFORE_ YOUR NEXT DOCTOR'S APPOINTMENT AND GIVE IT TO YOUR DOCTOR WHEN YOU GO TO SEE THEM
THANK YOU

In the past month have you eaten or drunk any of the following:	Foods Eaten	Did it make you feel sick or ill?	How sick did you feel?		
Food list	**No \ Yes** (Circle)	**No \ Yes** (Circle)	✓the correct answer		
			Mild	Moderate	Severe
Dairy foods, full fat (e.g. milk, cheese etc)	No \ yes, If yes = →	No \ yes, If yes = →	[ ]	[ ]	[ ]
Dairy foods, reduced/low fat or "light"	No \ yes, If yes = →	No \ yes, If yes = →	[ ]	[ ]	[ ]
Butter, margarine, fats or oils	No \ yes, If yes = →	No \ yes, If yes = →	[ ]	[ ]	[ ]
Fatty meats (e.g. mince, sausage, bacon etc)	No \ yes, If yes = →	No \ yes, If yes = →	[ ]	[ ]	[ ]
Roast meat or vegetables	No \ yes, If yes = →	No \ yes, If yes = →	[ ]	[ ]	[ ]
Fried foods (e.g. chips, fish/chicken, spring rolls)	No \ yes, If yes = →	No \ yes, If yes = →	[ ]	[ ]	[ ]
Takeaways (e.g. pies, pasties, sausage rolls)	No \ yes, If yes = →	No \ yes, If yes = →	[ ]	[ ]	[ ]
Soft/fizzy drinks (with sugar)	No \ yes, If yes = →	No \ yes, If yes = →	[ ]	[ ]	[ ]
Soft/fizzy drinks (with artificial sweeteners)	No \ yes, If yes = →	No \ yes, If yes = →	[ ]	[ ]	[ ]
Spicy hot foods (e.g. curries, chilis etc)	No \ yes, If yes = →	No \ yes, If yes = →	[ ]	[ ]	[ ]
Seafoods (e.g. prawns, oysters, marinara etc)	No \ yes, If yes = →	No \ yes, If yes = →	[ ]	[ ]	[ ]
Nuts or peanut butter	No \ yes, If yes = →	No \ yes, If yes = →	[ ]	[ ]	[ ]
Canned fruit (sweetened)	No \ yes, If yes = →	No \ yes, If yes = →	[ ]	[ ]	[ ]
Sugar, honey or syrup	No \ yes, If yes = →	No \ yes, If yes = →	[ ]	[ ]	[ ]
Lollies, sweets, fudge, caramel etc	No \ yes, If yes = →	No \ yes, If yes = →	[ ]	[ ]	[ ]
Chocolate, chocolate bars	No \ yes, If yes = →	No \ yes, If yes = →	[ ]	[ ]	[ ]
Cakes, biscuits, pastries	No \ yes, If yes = →	No \ yes, If yes = →	[ ]	[ ]	[ ]
Coffee	No \ yes, If yes = →	No \ yes, If yes = →	[ ]	[ ]	[ ]
Alcohol	No \ yes, If yes = →	No \ yes, If yes = →	[ ]	[ ]	[ ]
Bread/Gluten	No \ yes, If yes = →	No \ yes, If yes = →	[ ]	[ ]	[ ]

SYMPTOM CHECKLIST (✓ the correct answer)

In the past month have you been troubled by:		How much of the last month has this occurred?						How bad has it has been?		
Symptom		Less than 1 week	About 1 week	About 2 weeks	About 3 weeks	Most/ all of the month		Mild	Moderate	Severe
Pain in liver area (upper right belly)	No \ yes, If yes = →	[ ]	[ ]	[ ]	[ ]	[ ]	And = →	[ ]	[ ]	[ ]
-sharp stabbing pain	No \ yes, If yes = →	[ ]	[ ]	[ ]	[ ]	[ ]	And = →	[ ]	[ ]	[ ]
-dull ache	No \ yes, If yes = →	[ ]	[ ]	[ ]	[ ]	[ ]	And = →	[ ]	[ ]	[ ]
Tiredness/fatigue	No \ yes, If yes = →	[ ]	[ ]	[ ]	[ ]	[ ]	And = →	[ ]	[ ]	[ ]
Poor, interrupted or broken sleep	No \ yes, If yes = →	[ ]	[ ]	[ ]	[ ]	[ ]	And = →	[ ]	[ ]	[ ]
Night sweats	No \ yes, If yes = →	[ ]	[ ]	[ ]	[ ]	[ ]	And = →	[ ]	[ ]	[ ]
Wake up feeling tired	No \ yes, If yes = →	[ ]	[ ]	[ ]	[ ]	[ ]	And = →	[ ]	[ ]	[ ]
Tired/aching muscles	No \ yes, If yes = →	[ ]	[ ]	[ ]	[ ]	[ ]	And = →	[ ]	[ ]	[ ]
Poor appetite	No \ yes, If yes = →	[ ]	[ ]	[ ]	[ ]	[ ]	And = →	[ ]	[ ]	[ ]
Bleeding gums	No \ yes, If yes = →	[ ]	[ ]	[ ]	[ ]	[ ]	And = →	[ ]	[ ]	[ ]
Sore, dry or uncomfortable throat	No \ yes, If yes = →	[ ]	[ ]	[ ]	[ ]	[ ]	And = →	[ ]	[ ]	[ ]
Nausea	No \ yes, If yes = →	[ ]	[ ]	[ ]	[ ]	[ ]	And = →	[ ]	[ ]	[ ]
Vomiting	No \ yes, If yes = →	[ ]	[ ]	[ ]	[ ]	[ ]	And = →	[ ]	[ ]	[ ]
Indigestion	No \ yes, If yes = →	[ ]	[ ]	[ ]	[ ]	[ ]	And = →	[ ]	[ ]	[ ]
Gas or bloating after meals	No \ yes, If yes = →	[ ]	[ ]	[ ]	[ ]	[ ]	And = →	[ ]	[ ]	[ ]
Gas or bloating anytime	No \ yes, If yes = →	[ ]	[ ]	[ ]	[ ]	[ ]	And = →	[ ]	[ ]	[ ]
Diarrhoea	No \ yes, If yes = →	[ ]	[ ]	[ ]	[ ]	[ ]	And = →	[ ]	[ ]	[ ]
Constipation	No \ yes, If yes = →	[ ]	[ ]	[ ]	[ ]	[ ]	And = →	[ ]	[ ]	[ ]
Pale stools/bowel motions	No \ yes, If yes = →	[ ]	[ ]	[ ]	[ ]	[ ]	And = →	[ ]	[ ]	[ ]
Frequent urination (often at night)	No \ yes, If yes = →	[ ]	[ ]	[ ]	[ ]	[ ]	And = →	[ ]	[ ]	[ ]
Dark urine	No \ yes, If yes = →	[ ]	[ ]	[ ]	[ ]	[ ]	And = →	[ ]	[ ]	[ ]
Skin rash, itchy skin	No \ yes, If yes = →	[ ]	[ ]	[ ]	[ ]	[ ]	And = →	[ ]	[ ]	[ ]
Swollen ankles (fluid retention)	No \ yes, If yes = →	[ ]	[ ]	[ ]	[ ]	[ ]	And = →	[ ]	[ ]	[ ]
Fevers	No \ yes, If yes = →	[ ]	[ ]	[ ]	[ ]	[ ]	And = →	[ ]	[ ]	[ ]
Back pain	No \ yes, If yes = →	[ ]	[ ]	[ ]	[ ]	[ ]	And = →	[ ]	[ ]	[ ]
Joint pain	No \ yes, If yes = →	[ ]	[ ]	[ ]	[ ]	[ ]	And = →	[ ]	[ ]	[ ]
Dry or itchy eyes	No \ yes, If yes = →	[ ]	[ ]	[ ]	[ ]	[ ]	And = →	[ ]	[ ]	[ ]
Vision problems	No \ yes, If yes = →	[ ]	[ ]	[ ]	[ ]	[ ]	And = →	[ ]	[ ]	[ ]
Dizziness	No \ yes, If yes = →	[ ]	[ ]	[ ]	[ ]	[ ]	And = →	[ ]	[ ]	[ ]
Headache	No \ yes, If yes = →	[ ]	[ ]	[ ]	[ ]	[ ]	And = →	[ ]	[ ]	[ ]
Poor concentration	No \ yes, If yes = →	[ ]	[ ]	[ ]	[ ]	[ ]	And = →	[ ]	[ ]	[ ]
Irritable or cranky	No \ yes, If yes = →	[ ]	[ ]	[ ]	[ ]	[ ]	And = →	[ ]	[ ]	[ ]
Mood swings	No \ yes, If yes = →	[ ]	[ ]	[ ]	[ ]	[ ]	And = →	[ ]	[ ]	[ ]
Unhappy/depressed	No \ yes, If yes = →	[ ]	[ ]	[ ]	[ ]	[ ]	And = →	[ ]	[ ]	[ ]
Other (optional):	No \ yes, If yes = →	[ ]	[ ]	[ ]	[ ]	[ ]	And = →	[ ]	[ ]	[ ]

In the past month have you: [ ] stayed the same weight   [ ] gained weight   [ ] lost weight

FOR WOMEN ONLY: Do you have?

Pre-menstrual tension	No \ yes, If yes = →	[ ] stayed the same	[ ] improved	[ ] worsened
Period pain	No \ yes, If yes = →	[ ] stayed the same	[ ] improved	[ ] worsened

APPENDIX F

CONDUCTING A COMPLEMENTARY MEDICINE TRIAL, A CHECKLIST

Appendix F comprises a checklist of lessons learnt from the Hep573 Study and a list of possible administration issues.

CONDUCT OF COMPLEMENTARY MEDICINE RESEARCH CHECKLIST

The following checklist for the conduct of complementary medicine research imparts the lessons learnt and valuable experience gained by the Study Coordinator which may benefit future complementary medicine (CM) colleagues in the planning and conduct of clinical trials. It is suggested that CM colleagues:

- Canvass widely with colleagues and peers re the study design to ensure that the primary and secondary outcome measures are relevant to investigative questions and are reflective of both the current literature and the paradigm of complementary medicine.

- Establish a research team, comprising specialists in their particular field of research attached to institution/s conversant in and with a track record in research, along with CM colleagues who have conducted research in complementary medicine.

- Involve a biomedical statistician in the study design, sample-size calculation on the primary endpoint, the randomisation method to ensure wherever possible that the groups are evenly distributed across predesignated primary baseline characteristics and provide the method outline for the statistical analysis of the results.

- Obtain human research ethics committee approval for the study protocol, participant information sheet and all advertising material simultaneously.

- Register the trial with the Australian Therapeutics Goods Administration (Note: each site requires a separate application fee).

- Register the trial with the Australian New Zealand Clinical Trials Register (ANZCTR).

- Request the CM manufacturer that they archive voucher specimens, provide certificates of analysis and stability data as soon as the study has obtained ethics committee approval. Obtain a commitment from the manufacturer for the provision of replacement stock as well as active preparations for placebo (for effective intervention) and provisions for possible company changes in direction.

- Allow the maximum time possible for recruitment of participants.

- Intention-to-treat analysis means ensuring there are data on each randomised person, therefore it is better to keep all participants in the study regardless of protocol violations (which can be duly noted and addressed in subanalyses of the data).

- Ask for help from colleagues from both medical and CM disciplines as they are both generous in their support and stewardship of solid research.

OTHER ADMINISTRATIVE SUGGESTIONS

- Secure newspaper articles or television spots to generate interest in the study and arrange personnel to field the subsequent phone calls.
- Design a form for each site to track every call of interest, along with age, sex, postcode, reason for ineligibility or eligibility of potential participants and referral to relevant site for screening.
- Provide the sites with possible scenarios of allowable complementary medicines or possible drug-herb interactions.
- Establish a list of participants, contact details, date study commenced, research blood results.
- Allocate time to monitor the records of each site, ideally on a weekly basis as bloods are kept by laboratories for one week and a missing blood may be able to be added, to minimise missing data wherever possible.
- Label cryogenic tubes for the study with permanent-marker pen rather than using labels which can deteriorate over time in freezer storage.
- Store the frozen bloods in specimen boxes with study name, study week, serum or plasma clearly identified as this provides the easiest and most systematic retrieval of samples compared to random stacks of samples. Paper labels in one hospital meant retrieval was difficult and time intensive. A corresponding entry for each participant, date of collection and despatch of samples for analysis in a separate folder or book is essential.
- Establish data model and processing procedures to aid statistical analyses.
- Enter and validate the data as they become available.