THE UNIVERSITY *of* EDINBURGH

The effect of autologous macrophage therapy in cirrhosis in response to individual immune reparative pathways: developing a novel therapy.

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Table of Contents

Declaration

This thesis represent my own work and it has been written by myself.

The trial original design was completed by Prof Forbes and collaborators including myself and amendments were undertaken by myself in collaboration with other clinical scientists, statisticians and sponsors involved in the trial. The experiments performed in this work were undertaken by myself in collaboration with other scientists and declared in the text. The magnetic resonance analysis was performed in collaboration with physicists of the University of Edinburgh as declared in the text.

Chapter 2 describes the outcome of the phase 1 clinical trial MATCH. This works has been previously published in Nature Medicine published “Safety profile of autologous macrophage therapy for liver cirrhosis” (doi: 10.1038/s41591-019-0599-). I was first author in this paper and all the collaborators are aware of the use of data in this thesis and follows Nature guidance on reuse of the material (https://www.nature.com/nature-portfolio/reprints-and-permissions). Copy of the paper is included in the Appendix.

This work has not been submitted for any other degree or professional qualification.

Acknowledgments

I would like to sincerely thank Prof S Forbes for the support, guidance and opportunity to learn and work in his team. He has been and will be a mentor to me and I cannot express enough my gratitude for the patience showed.

Equally I have to thank Prof J Fallowfield for the invaluable and personal input to this work. I would not have made it here without it.

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Dr Morris and Dr Sample have been instrumental in allowing me to understand the principle of MRI and helped me interpreting the data.

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And my eternal gratitude goes to my partner Colin who gave up his work to look after our wonderful but extremely disable child to allow me to achieve my dreams.

ABSTRACT

Background: Liver cirrhosis is the end stage of any injury process to the liver. Once established it inevitably progresses to complications such as portal hypertension,

cancer and death. There is not cure for liver cirrhosis besides liver transplant. We face an unmet demand for treatment of this condition. The role of macrophages in fibrosis development and resolution in the liver has been extensively investigated. Prof Forbes group invested in the development of autologous macrophage product to promote fibrosis resolution hence cirrhosis regression. This has demonstrated its efficacy and safety in animal models. From these encouraging pre-clinic data a phase 1 first in human clinical trial of autologous activated macrophage product for cirrhotic patients was developed.

Methods: Using an established 3+3 dose escalation model we enrolled a total of 9 subject in the phase 1 trial reaching a maximum achieved and safe dose of 1x10^9 macrophages. In addition to adverse events, dose toxicity and macrophage activation syndrome (MAS) parameter, we evaluated a varied range of circulating cytokines and chemokine pre and post treatment using a commercial kit. Moreover we developed a protocol for P13- magnetic resonance spectrometry (MRS) for the analysis of the metabolically active liver parenchyma. Data from the phase 1 trial were used to improve the autologous cellular produce and phase 2 randomised controlled trial.

Results: The autologous activated macrophage produce is demonstrated not to cause any toxicity in this first in human study of cirrhotic population of different aetiology. Cytokine and chemokine analysis supports these findings and specifically demonstrates low levels of IL-8, which represent cardinal feature of MAS. Other interesting cytokine signals may support extra cellular matrix remodelling effect of the autologous macrophage product infusion. In addition we demonstrated a reproducible protocol for MRS in liver disease.

Discussion: Autologous activated macrophage infusion did not result in any toxicity in cirrhotic subjects taking part in this study and shows preliminary signs of efficacy in fibrosis resolution both clinically and biochemically. This work places the basis of development of cellular products for treatment of cirrhosis and fibrosis and provides invaluable insight in immune response to cellular treatment.

LAY SUMMARY

Cirrhosis is the scaring in the liver after it has been injured for a long time. We have no cure for cirrhosis besides liver transplant. In animal experiments we saw that cells part of the immune system can help breaking down scaring in the liver and reverse liver cirrhosis.

In this work we demonstrate that it is safe to use cells called macrophages taken from an individual, matured in the laboratories and reinfused in the same individual to treat cirrhosis.

This opens new options for treatment of liver disease.

LIST OF ABBREVIATIONS

UK	United kingdom of Great Britain and Northern Ireland
NAFLD	Non-alcoholic fatty liver disease
ARLD	Alcohol related liver disease
HCV	Hepatitis C virus
DAA	Direct antiviral agents
SVR	Sustained viral response
HIV	Human immunodeficiency virus
AIH	Autoimmune hepatitis
LFTS	Liver function tests
MMF	Mycophenolate mofitil
NASH	Non-alcoholic steatohepatitis
T2DM	Type 2 diabetes mellitus
GLP-1 RA	Glucagon-like peptide 1 receptor agonist
DPP-IV	dipeptidyl-peptidase 4
RCT	Randomised controlled trial
US	United States
ALT	Alanine aminotransferase
ASK1	Apoptosis Signal-Regulating Kinase 1
MAPK	Mitogen Activated Protein Kinase
PPAR/PPR	Peroxisome Proliferator-Activated Receptor/
NADPH	Nicotinamide adenine dinucleotide phosphate
NOX	NADPH oxidase
DALY	Disability adjusted life years

HCC	Hepatocellular carcinoma
ARFI	Acoustic radiation force impulse
USS	Ultrasound
BMI	Body mass index
RR	Risk ratio
MRI	Magnetic resonance images
MRE	Magnetic resonance elastography
TE	Transient elastography
PDFF	proton density fat fraction
ELF	Enhanced Liver Fibrosis
ELISA	Enzyme-linked immunosorbent assay
HA	Hyaluronic acid
PPV	Positive predictive value
NPV	Negative predictive value
ECM	Extracellular matrix
MMP	Matrix Metallo-proteinase
CTP	Child-Turcotte-Pugh
HE	Hepatic encephalopathy
ALF	Acute liver failure
ICU	Intensive care unit
MELD	Model for End Stage Liver Disease
UKELD	United Kingdom model for End Stage Liver Disease
NA	Sodium

TIPSS	Transjugular porto-systemic shunt
OLT	Orthoptic liver transplant
INR	International normalized ratio
SBP	Spontaneous bacterial peritonitis
HBV	Hepatitis B virus
ESPEN	European Society of Parenteral and Enteral Nutrition
ASPEN	American Society of Parenteral and Enteral Nutrition
NF-KB	nuclear factor – kappa light chain enhancer for B cells
HSC	Hepatic stellate cells
TIMP1	Tissue Inhibitor of Metalloproteases 1
EGFR	epithelial growth factor receptor
FXR	Farnesoid X receptor
ACE	Angiotensin-covering enzymes
TRTGF	Transforming growth factor
LOXL2	Lysyl oxidase homolog 2
PSC	Primary Sclerosing cholangitis
CCR2	C-C chemokine receptor type 2
CCL2	C-C motif chemokine ligand 2
HLC	hepatocyte-like cells
IPSCS	Induced pluripotent stem cells
ESC	Embryonic stem cells
HPC	Hepatocytes progenitor cells
FLEPC	Foetal liver epithelial progenitor cells

HBTSC	Human biliary tree stem cells
HPSC	Hematopoietic stem cells
AFP	Alpha foetal protein
G-CSF	Granulocyte colony-stimulating factor
MSC	Mesenchymal Stem Cell
DAMP	damage-associated molecular patterns
PAMP	pathogen-associated molecular patterns
LPS	Lipopolysaccharide
TLR	toll-like receptor
KC	Kupffer cells
DR	Ductular reaction
MCP	Monocyte chemoattractant protein
IL	interleukin
TNF	Tumor necrosis factor
PDGF	platelets derived growth factor
INF	Interferon
DC	Dendritic cells
CCL4	Carbon tetrachloride
NK	Natural Killer
TH	T helper
MAIT	mucosal associated invariant T
TCR	T cell receptor
PV	Portal vein
TV	Tail vein

CTIMP	Clinical trial of an investigational medical product
MFI	mean fluorescence intensity
IMP	Investigational medical product
MATCH	Macrophage therapy for liver cirrhosis
GCP	Good clinical practice
GDPR	General Data Protection Regulation
HTA	Human Tissue Act
REC	Research ethics committee
NHS	National health service
R&D	Research and Development
CRF	Clinical research facilities
GMP	Good Manufacturing Practice
MRC	Medical Research Council
ACCORD	Academic and Clinical Central Office for Research and Development
MHRA	Medicines & Healthcare products Regulatory Agency
PIRSC	Phase I Review Study Committee
EUDRACT	European Clinical Trials Database
HRA	Health Research Authority
CI	Chief investigator
PI	Principal investigator
QA	Quality assurance
DMC	Data monitoring committee
RIE	Royal Infirmary of Edinburgh

SIV	Site initiation visit
SATO	Sponsor agreement to open trial
TSC	Trail steering committee
PCRF	Paper case reporting forms
ECTU	Edinburgh Clinical Trial Unit
PIS	Patient information sheet
GP	General practitioner
IB	Investigator brochure
IMPD	Investigational medicinal product dossier
IRAS	Integrated research application system
CESP	Common European Submission Portal
NS	Non substantial
SA	Substantial amendment
DPFS	Development pathway funding
DSUR	Development Safety Update Report
AE	Adverse events
SAE	Serious adverse events
AR	Adverse reactions
SUSAR	Serious unexpected adverse reaction
SAR	Serious adverse reaction
QC	Quality control
CTCAE	Common Terminology Criteria for Adverse Events
DLT	Dose limit toxicity
MAS	Macrophage activation syndrome

CLDQ	Chronic liver disease questionnaire
PBS/EDTA	phosphate buffered saline/ ethylenediamine tetra-acetic acid
SNBTS	Scottish national blood transfusion service
TBV	Total body weight
CT	Computer tomography
HRQL	Health related quality of life
M	Medium
XL	Extra large
WCC	White cell count
PLT	Platelets
HAS	Human albumin solution
KDA	kilo Dalton
LOQ	Limit of quantification
GM-CSF	Granulocytes-macrophages colony-stimulating factor
VEGF	Vascular Endothelial Growth Factors
TSLP	Thymic Stromal Lymphopoietin
PIGF	Placenta Growth Factor
MIP	Macrophage inflammatory protein
CRP	C reactive protein
ICAM	Intercellular Adhesion Molecule
SAA	Serum Amyloid A
VCAM	Vascular cell adhesion molecule
RNA	Ribonucleic acid
IBD	Inflammatory bowel disease

CP	Child-Pugh score
MRS	Magnetic resonance spectrometry
ATP	Adenosine triphosphate
PI	Phosphorus inorganic
PME	Membrane phosphate
PDE	Phosphate di-esterase
SNR	Signal to noise ratio
SVS	Single voxel signal
CSI	Chemical Shift Images
PRESS	Point Resolved Excitation Spin-echo Sequence
STEAM	STimulated Echo Acquisition Mode
D-GAIN	D-galactosamine
TAA	Thioacetamide induced
CBDL	Common bile duct ligation
PCR	phosphocreatine
LCAR	liver cell to area ratio
HV	Healthy volunteer
PEP	phosphoenolpyruvate
NADH	Nicotinamide adenine dinucleotide
CRM	Centre for regenerative medicine
CAU	Clinical apheresis unit
QC	Quality control
MORE II	Medicine In Cirrhotics II

1. Chapter 1: Introduction

Table of Contents

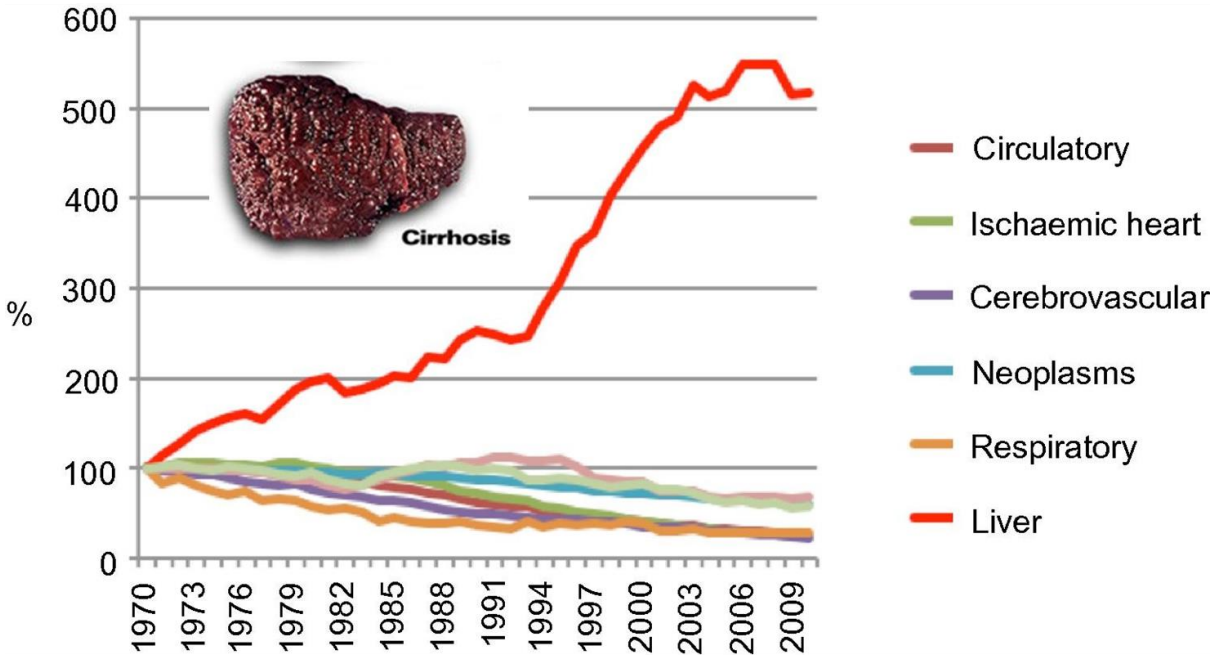
Figure and Tables content

1.1 Liver cirrhosis

1.1.1 Definition and epidemiology

Cirrhosis of the liver is the end stage of prolonged injury of any aetiology to the liver. Data available on the prevalence and incidence of cirrhosis around the world are sparse. In Europe deaths caused by cirrhosis are estimated to be around 1.8% of all deaths with 170000 deaths per year. (1) Cirrhosis-related deaths accounted for over a million deaths globally in 2010 with mortality rates increasing substantially in the UK. (2, 3) According to the UK government birth and death statistics, mortality for liver disease has tripled in the past 3 decades. In 2017 liver disease was respectively the second and third cause of death in females and males in the age group 30-34. It continues to be between 2nd and 4th cause of death until the age of 65, only to be preceded by ischaemic heart disease and neoplasia of the lung and breast (4). In the UK, chronic liver disease accounts for the majority of liver transplantations. (5) Liver disease is the only major cause of death in UK that continues to increase year by year. (6)

Figure 1.1: Main mortality causes in UK over years.



From: National plan for liver services 2009 http://www.yhln.org.uk/data/documents/Useful%20Documents%20area/National%20Liver%20Plan%202009.pdf . This figure shows percentage of death (y axes) by major disease contributors in UK by year (x axes). Liver disease (red line) is the only major cause of death that continues to increase each year while all other causes of death are steadily declining.

1.1.2 Aetiology of liver disease

Cirrhosis of the liver is the end process of prolonged injury to the liver due to multiple causes. Independently from the aetiology of liver disease, once damage has occurred and fibrosis is established the liver becomes irreversibly cirrhotic. The commonest causes of liver diseases in UK are Non-Alcoholic fatty liver disease (NAFLD), Alcohol Related liver disease (ArLD) and Hepatitis C (HCV).(7) Despite the relative success of therapeutic interventions for specific aetiologies (e.g. novel antiviral therapy for hepatitis C virus infection, alcohol abstinence for alcoholic liver disease and weight loss for non-alcoholic steatohepatitis), patients often present to medical attention late when cirrhosis and related complications have already occurred. It is also important to recognise the hurdle of life-style modification interventions (for example weight loss is often not-sustained or unsuccessful in patient with NAFLD) and therefore of

suboptimal efficacy.(8) Government driven campaigns to tackle the obesity and alcohol burden in UK have been explored(7) and in 2018 Scotland became the first nation to have introduced minimum alcohol pricing.(9) Nevertheless end stage liver disease continue to remain an unmet clinical need.

1.2 Treatments for different liver disease aetiologies

1.2.1 Hepatitis C Virus

Treatment of chronic hepatitis C virus has been revolutionised by the advent of numerous oral direct antiviral agents (DAA) in the last few years. While in the past efficacy and tolerability of interferon based treatment was far from ideal and limited by genotype (sustained viral response [SVR] <50%), the new oral direct antiviral agents are efficacious for all genotypes and very well tolerated with SVR of around 90%. (10) These interferon-free therapies have also shown improved safety in difficult to treat groups like HIV-HCV coinfection, decompensated cirrhosis, and above all in advanced liver disease and can be used in patient on the waiting list for liver transplantation.(11-13) This results in very high rates of SVR across the board with overall prospects of vanquish HCV infection worldwide. However, on a more practical note, the availability of DAA is limited worldwide by socio-economic aspects and market rules thus the prospects of a HVC-free world remain distant.

It has been previously demonstrated that in patients achieving SVR, interferon and ribavirin based regimens have an effect in resolution of fibrosis that correlates with improvement of necro-inflammation.(14) There is also increasing evidence that DAA have effects on fibrosis. When this is measured using transient elastography, a non-invasive method to evaluate liver fibrosis measuring liver stiffness, it shows improvement in patients who achieve SVR at 24 weeks in interferon-free therapy.(15) Despite SVR being an independent factor for improvement of liver stiffness a significant proportion of patients continue to demonstrate persistent liver damage.(15) Moreover published data also suggests that improvement in liver stiffness in SVR patients is related to a reduction of inflammation rather than resolution of fibrosis.(16) It is important to highlight that non-invasive measurement of fibrosis of the liver like liver stiffness is current clinical practice and often outcomes measured in clinical trials.

1.2.2 Autoimmune hepatitis

Autoimmune hepatitis (AIH) is a rare inflammatory disease of the liver parenchyma that responds well to steroids and immunosuppressant therapy.(17) It can present with an insidious clinical course in asymptomatic patients with derange fluctuating liver function tests (LFTs) or even with fulminant liver failure.(18) Standard therapy is based on corticosteroids and Azathioprine and this can induce remission in 80-90% of cases.(19) Second line therapies (like mycophenolate mofitil (MMF) or cyclosporine or tacrolimus) can be used for patients who do not respond or are intolerant to first line treatments with the majority of efficacy data available for MMF.(17) Despite the proven efficacy of currently available treatment options, it is not unusual to see individuals affected by AIH presenting for the first time to medical attention with established fibrosis or cirrhosis. This is probably a combination of the usually asymptomatic nature of this condition and the typically poor compliance of the mostly affected age groups (children, young adults or elderly) causing persistent hepatic inflammation leading to fibrosis. (20, 21)

1.2.3 Non-alcoholic fatty liver disease (NAFLD)

Non-alcoholic fatty liver disease (NAFLD) is the most common liver disease in the western world with prevalence between 17% and 46%.(22) It is defined by the presence of at least 5% of fat in hepatocytes (detected either by biopsy or magnetic resonance). Histologically steatosis can present as non-alcoholic steatohepatitis (NASH) or steatosis without hepatitis. NASH is characterised by lobular inflammation, ballooning and degeneration of hepatocyte while steatosis without inflammation can be macro or micro-vesicular. The two histological conditions can carry different prognosis but can both lead to fibrosis and cirrhosis.(23) Nevertheless both NASH and steatosis can be considered a dynamic state of the same pathological process. The diagnosis of NAFLD is made by the exclusion of both: (i) other aetiologies of liver disease and (ii) daily alcohol consumption >30g for men and >20g for women. (24) However, increased alcohol consumption does not completely exclude NAFLD, especially in subjects for which metabolic risks factor are significant.(25) The relationship between the development of NAFLD and metabolic syndrome is well described as well as the impact of insulin resistance in hepatic fat accumulation. Hence steatosis in subjects with metabolic syndrome or type 2 Diabetes Mellitus (T2DM) who consume excessive alcohol is likely the results of both NAFLD and

alcohol related liver disease.(26) The main treatment of NAFLD is weight loss: 5-7% of total body weight reduction can resolve NASH, while >10% can improve fibrosis.(23) Weight loss is mainly reached by dietary modification and exercise. Compliance with life style changes and long term weight goals is very poor, leading to overall low efficacy of this treatment. (27)

Thiazolidinedione (e.g. pioglitazone) have been extensively explored as a potential therapy for NAFLD because of their effect on improving peripheral and hepatic insulin sensitivity and on lowering tissue adiponectin (and hepatic inflammation), all of which are directly linked to the pathogenesis of NAFLD. (28) A recent meta-analysis showed that pioglitazone use improves advanced fibrosis in NASH, even in patients without diabetes.(29) Unfortunately, the clinical use of pioglitazone is limited by the risk of adverse events, including weight gain, heart failure, bone fractures, macular oedema, and possibly bladder cancer. Metformin is a well-known hypoglycaemic oral agents that improves peripheral and hepatic insulin resistance by altering glucose homeostasis. It does not cause weight gain in T2DM, however metformin does not perform efficiently in NASH studies. (30) Fat lowering medications and statins have been considered for NAFLD but clinical results are inconsistent.(31) Various studies demonstrate the effect of Vitamin E in improving liver function tests and oxidative stress in NAFLD but a meta-analysis involving high dose Vitamin E supplements revealed an increased all-cause mortality in treated subjects. (32) There is robust evidence that coffee has a protective role in the development of NASH and liver fibrosis of potentially any aetiology: coffee drinkers have a significantly reduced risk to develop steatohepatitis, fibrosis and hepatocellular carcinoma.(33-35) Glucagon-like peptide 1 receptor agonist (GLP-1 RA) is proven to be effective in the treatment of T2DM as it lowers circulating glucose levels and weight by increasing insulin secretion. Similarly dipeptidyl-peptidase 4 (DPP-IV) inhibitor reduces glucose and glucagon. Both GLP-1RA and DPP-IV inhibitor have a safe profile in NAFLD and showed potential benefit in randomised controlled trials (RCTs). A GLP-1 agonist (Liraglutide) improves glucose homeostasis, drives weight loss and suppresses appetite improving NASH scores, but it does not have an effect on fibrosis resolution, on the contrary, in a proportion of subjects, liver fibrosis worsen upon treatment.(36) Similar data are available for DPP-IV inhibitors which suggest a positive effect on inflammation at the expenses of increased liver fibrosis.(37)

There are no currently solid data in the literature on NAFLD incidence, however it can be estimated that there are around 25-85/1000 cases per year in United Stated (US) and it is often referred to as a “NAFLD pandemic”. (38) This is undoubtedly a powerful drive to the exploration of new compounds to treat NAFLD. Thus there are numerous drugs in pre-clinical and initial clinical studies that show favourable outcomes: caspase inhibitors seem to have effects in improving alanine aminotransferase (ALT) in preliminary data from a RCT(39); Apoptosis Signal-Regulating Kinase 1 (ASK1) inhibitors have shown a reduction of hepatic steatosis and fibrosis and they are currently being tested in a clinical trial(40); p38 Mitogen Activated Protein Kinase (MAPK) inhibitors have a theoretic therapeutic benefit in NAFLD as p38 protein is overexpressed in obesity and induce oxidative stress and inflammation(41); Peroxisome Proliferator-Activated Receptor (PPAR) α and δ agonists (Elafibranor) failed to meet expectation of reducing inflammation and fibrosis in NASH in a recent RCT(42); nicotinamide adenine dinucleotide phosphate (NADPH) Oxidase (NOX) 1-4 inhibitors has been found to reduce inflammation and fibrosis in NASH murine models(43); Galectin-3 antagonist have demonstrated signals of efficacy in fibrosis resolution in a phase I trial for NAFLD patients with advanced fibrosis and a RCT is ongoing(44). These are only few examples among the currently tested molecules.

1.2.4 Alcohol Related Liver disease (ALD)

Harmful alcohol consumption constitutes a significant health burden worldwide with 6.5% of all deaths due to alcohol in Europe. Liver cirrhosis attributable to alcohol caused 22.2million disability adjusted life years (DALYs) in 2016 world-wide.(45) The only available cure for ArLD is abstinence which is often difficult to achieve in subjects with psychological and physical dependence. Acute liver injury due to alcohol (alcoholic hepatitis) can be a life threatening condition not only due to potential liver failure but also because it generates a systemic inflammatory reaction. Corticosteroids have a role in the treatment of alcoholic hepatitis in a selected subgroup. The STOPAH trial demonstrated that corticosteroids reduce 28days morality if used in subjects free from infections and gastrointestinal bleeds with favourable combinations of static and dynamic score (Glasgow alcoholic hepatitis score >8 and Lille score at 7 days <0.45) and lymphocytes to neutrophil ratio 5-8. (46, 47)A positive role of antioxidants and pentoxyfilline have not been demonstrated in alcohol induced liver injury.(48) Nutrition

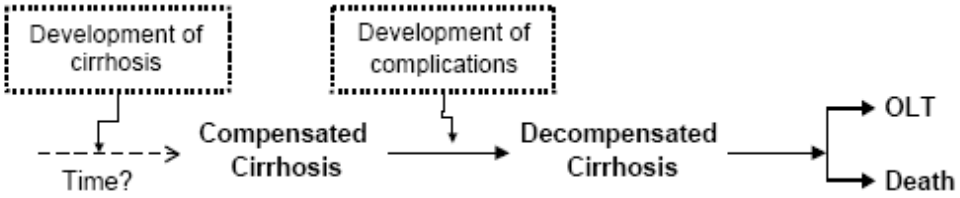
also plays an important part in survival in chronic and acute alcohol related liver disease.

1.3 Natural history of cirrhosis

Despite the development of aetiology specific treatments the efficacy of those therapies in reversing cirrhosis is not proven. Furthermore, patients often present to medical attention late when complication of cirrhosis (portal hypertension) have already developed.

The natural history of the disease is characterised by a period of “compensation”, when the patient has no symptoms related to cirrhosis and “decompensation”, when symptoms occur.(49) As the disease progresses portal hypertension increases and liver function deteriorates, leading to the development of varices, ascites, jaundice and coagulopathy.(50)

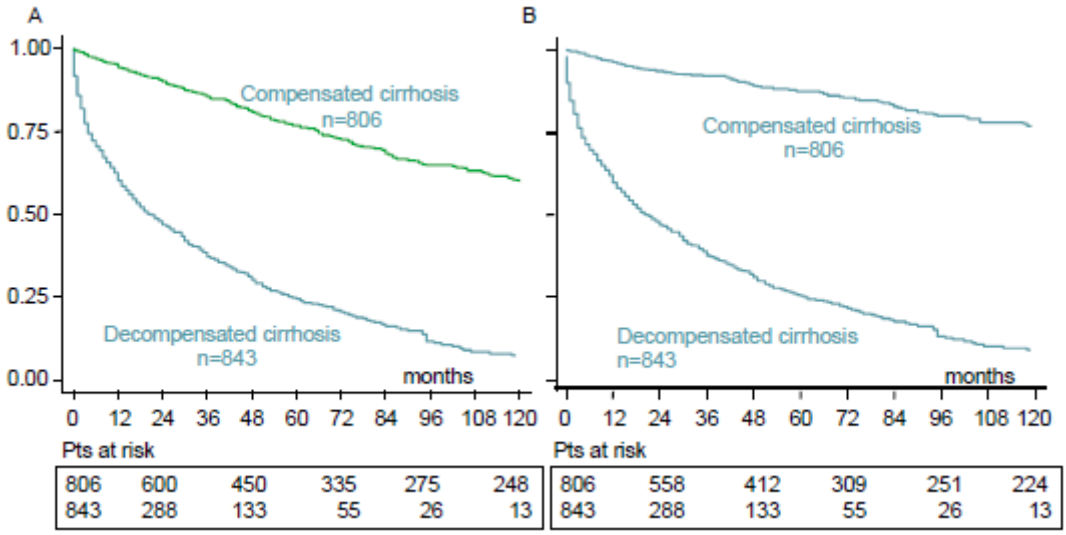
Figure 1.2: Natural history of cirrhosis



From D’Amico et al J Hepatol 2006;44:217-231. This figure explain natural history of liver cirrhosis over time. Once cirrhosis is established, which time frame can be variable according to aetiology and subject’s specific variabilities, a state of “compensation” will progress to “decompensation” with the development of complications. Decompensated cirrhosis can only be resolved with transplantation or results in death.

These complications occur at the rate of 5-7% per year and have a devastating implication for prognosis.(49) Patients with compensated cirrhosis have a 1-3.4% risk of death at one year (median survival 12 years) and in those with decompensated cirrhosis this rises dramatically to 20-57% risk of death at one year (median survival 2 years).

Figure 1.3: Survival according to state of compensation or decompensation



From D'Amico et al J Hepatol 2006;44:217-231. Graph A shows the proportion of cirrhotic patients (y axes) surviving over months from diagnosis (x axes) if in a state of compensation (green line) or decompensation (blue line). Graph B shows survival if remaining in compensated or developing decompensation. This data is collegiate from two perspective studies.

Once decompensation has developed, cirrhosis becomes a systemic disease which is mainly the consequence of haemodynamic changes leading to portal hypertension. Vasodilation of systemic circulation leads to reduced effective blood volume.(51) This activates the renin-angiotensin system, the sympathetic nervous system and affects the secretion of arginine-vasopressin. The direct effect of these haemodynamic adaptations leads to sodium and water retention, hepato-renal syndrome and the formation of ascites. Although the above statements are correct, more recent advances in the understanding of the pathophysiology of vasodilation in decompensated liver disease highlight the significant effects of the persistent inflammatory environment (raised pro-inflammatory cytokines and chemokines) which may be caused by bacterial infections. (52) Patients with decompensated cirrhosis have increased susceptibility to infections, while they show innate and acquired immunity dysregulation.(53) Infections critically increase mortality in this population. (54) It is possible that the source of these bacteria is the microbiome translocating through the disrupted mucosal barriers of the colon. (53) To add to the risk of decompensation, patients with cirrhosis develop hepatocellular carcinoma (HCC) at a constant rate of 3% per year. (49) Incidence of HCC in UK is estimated around 4.7-5.8 100.000 person-year while mortality reaches 4.3-5.3 100.000 person-year. HCC

is the most common cause of liver cancer and ranks fourth as cancer-related cause of death.(55)

1.4 Assessment of cirrhosis

According to the natural history of cirrhosis, prognosis completely changes once complications related to portal hypertension and decompensation occur. When portal pressure exceeds 10mmHg, varices develop. However, it is not until ascites accumulates that mortality shifts from 3.4% to 20% in 1 year. (49) It is therefore important to effectively assess the degree of liver fibrosis and evaluate its severity before symptoms occur and prognosis worsen. Liver fibrosis is therefore used as a surrogate for clinical outcome.

Liver biopsy is an established method to assess fibrosis/cirrhosis, but there are now clinically adopted non-invasive solutions to evaluate liver fibrosis. Despite being an irreversible process, cirrhosis is a dynamic state, and it is useful to adopt easy to calculate and non-invasive assessment tools to guide interventions and measure the efficacy of therapies.

1.4.1 Biopsy

Liver biopsy remains the gold standard to stage fibrosis in the liver and offers direct histological assessment of the liver parenchyma as well as information on aetiology. It heavily depends on the quality of the sample, which relies on the experience of the operator. Variability in the liver tissue can affect the outcome as the sample selected may not be representative of the entire organ's fibrosis stage. The interpretation of the histological finding is also subjected to the reader eye and experience and there can be a degree of disagreement among pathologists. (56) Liver biopsy is also a costly and invasive procedure that carries risks and complications, including pain, bleeding, infection and even death (approximately 1 in 10,000 cases). (57) Therefore there has been a recent shift towards less invasive methods to assess liver fibrosis.

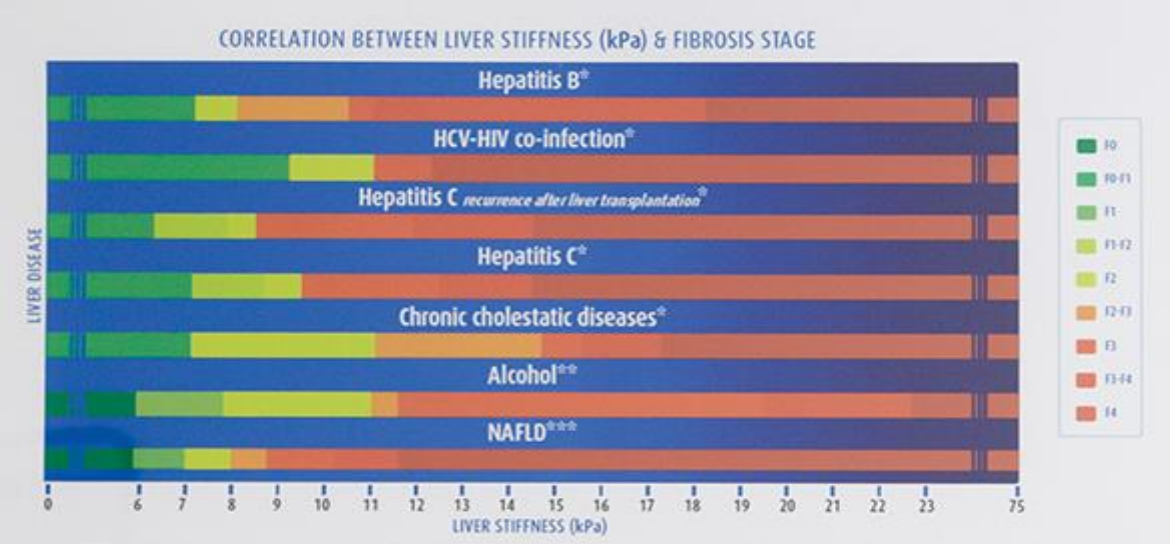
1.4.2 Non-invasive markers of fibrosis – Elastography

1.4.2.a 1D transient elastography: Fibroscan®

Fibroscan® is a validated and widely used method in clinical practice to evaluate liver stiffness via ultrasound waves and transient elastography (TE). An external vibration impulse is sent to the liver where shear waves are generated and tracked. Their propagation speed is converted into a value of liver stiffness expressed in kPa. (58)

Value cut offs have been established to quantify fibrosis in the different aetiologies of liver disease. (59)

Figure 1.4: Fibrosis stratification according to Fibroscan® value per aetiology of liver disease



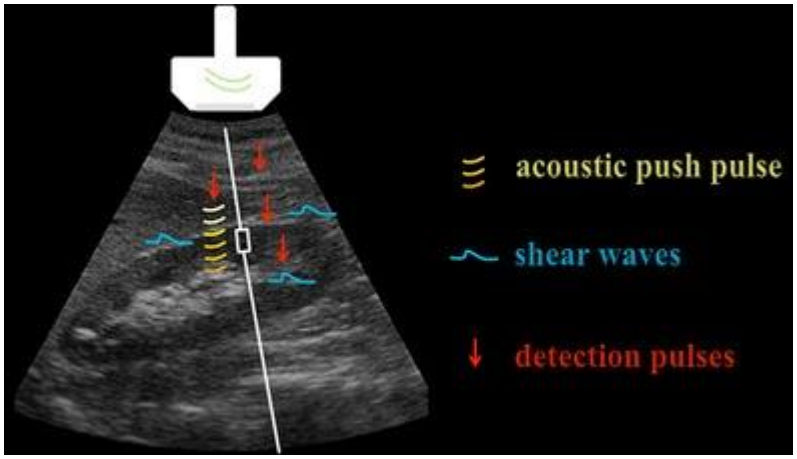
From http://liverscanaustralia.com.au/service/fibroscan-502-touch/ This figure stratifies fibrosis grade by aetiology of liver disease according to Fibroscan® results. For example a liver stiffness of 10kPa represents fibrosis grade F3-4 in NAFLD or HCV infection, while in ARLD or cholestatic liver disease the same value corresponds to F2. (See colour coding as per legend table on the right side of the figure)

Fibroscan® is also used in combination with platelets count to risk stratify patients who may benefit of non-selective beta-blockade because of oesophageal varices, thus avoiding endoscopy as per the recommendation made in the consensus statement of Baveno VI. (60) While Fibroscan® is very well validated to assess fibrosis, once the subject is in the cirrhotic range it is very difficult to interpret changes in the kPa scores. It should also be noted that the Fibroscan® machines used in clinical practice have a maximum reading of 75kPa- meaning they lose sensitivity in the higher ranges.

1.4.2.b Focal point shear-wave ultrasound elastography: ARFI (acoustic radiation force impulse)

A high intensity acoustic impulse is applied in a discrete area of the liver and generates mechanical excitement of the tissue. This is expressed in mechanical shear-waves and it can be mapped in 2D using ultrasound (USS) tracking. The tissue's displacement is then measured and used as indirect measurement of fibrosis.(61)

Figure 1.5: Focal Point shear-wave USS elastography - ARFI



Bruno C, et al. ARFI: from basic principles to clinical applications in diffuse chronic disease-a review. Insights Imaging. 2016 Oct;7(5):735-46. doi: 10.1007/s13244-016-0514-5. Epub 2016 Aug 23. This figure illustrates ARFI USS technology to indirectly measure liver fibrosis. The USS probe pulses acoustic inputs (yellow markers) directed towards the structure needed to be analysed (red arrow). The acoustic pulse excites the tissue which is expressed in shear waves (blue markers).

Both ARFI and Fibroscan® are operator dependent and in obese subjects (body mass index BMI>30) have relatively high failure rate (Fibroscan® medium probe: risk ratio (RR) 0.24, 95% CI 0.14-0.38 and extra-large probe: RR 0.16, 95% CI 0.08-0.32) and unreliable results (both probes: RR 0.76, 95% CI 0.50-1.16).(62)

1.4.2.c MRE (MR elastography)

Magnetic Resonance Elastography (MRE) uses the same concept of external vibration as USS TE but in MRE vibrations are constant. MRI has the advantage of not being operator nor subject dependent unlike USS TE or ARFI. Moreover it samples the whole liver and has a lower failure rate in obese subjects. Published data confirm that MRE has higher diagnostic accuracy than 1D-TE or focal point shear-wave US elastography (ARFI) for the detection of fibrosis.(61) But it is not yet known whether it is sufficiently sensitive or dynamic for the longitudinal monitoring of fibrosis progression/regression.

1.4.2.d Multiparametric MRI (LiverMultiScan™)

Liver*MultiScan*™ (Perspectum Diagnostics, Oxford, UK) uses non-contrast multiparametric MRI to quantify hepatic fibro-inflammatory injury (iron-corrected T1

mapping), steatosis (proton density fat fraction, PDFF) and iron content (T2* mapping). In patients, Liver*MultiScan*™ can accurately quantify liver disease (63) (64-66), may predict clinical outcomes (65), and is now being adopted into clinical trial protocols as a surrogate endpoint.

1.4.3 Non-invasive markers of fibrosis – serum based

1.4.3.a ELF

Enhanced Liver Fibrosis (ELF) is a validated panel of highly sensitive enzyme-linked immunosorbent assay (ELISA) measuring matrix components and enzymes involved in their turnover: Hyaluronic Acid, Tissue Inhibitor of Matrix Metalloproteinase 1, and Procollagen Type III. The values for each of these markers is combined in an algorithm which produces a discriminant score (the ELF score) related to the level of liver fibrosis.(67) ELF has been shown to be as accurate to predict liver-related complications and death as liver biopsy. (68) ELF has also been proven to be comparable to other fibrosis markers in different aetiologies of liver disease (69) When *Parkes et al* compared the prediction of clinical outcomes at 6 years between ELF and MELD they found an area under the curve of respectively 0.88 and 0.74 highlighting the high performance of ELF. (67)

Hyaluronic acid (HA), the major component of the ELF algorithm, can be used alone to determine fibrosis in different aetiologies of liver disease. With HA, a cut-off of ≥150 ng/mL gives a 72% sensitivity, 79% specificity, 77% PPV and 69% NPV for detecting cirrhosis.(70)

1.4.3.b Nordic Bioscience Fingerprint biomarkers™

Nordic Bioscience has developed a suite of assays for the non-invasive detection of fibrogenesis and fibrolysis. These circulating markers have been shown to have utility in staging fibrosis, detecting portal hypertension, and monitoring the response to anti-fibrotic therapy. (71, 72) In advance stages of liver disease, the liver contains over 6 times more extracellular matrix (ECM) proteins then normal parenchyma. Matrix metallo-proteinase (MMP) 2 and 9 are involved in the remodelling of the ECM proteins creating circulating disease-specific protein fragments. These fragments can be measured using markers (MMP degraded collagen - C1M C3M, C4M,C5M, C6M, MMP degraded elastin – ELM, collagen formation markers (pro-C3) and others.(73) Lemming et al. evaluated the correlation between these protein biomarkers and

degree of portal hypertension and fibrosis in patients with liver disease and confirmed a direct correlation with a strong association between degree of fibrosis and pro-C3 in particular.(74)

Commercially (Protein Fingerprint™ technology) and in research programs, the most frequently used markers are ProC3 and C3M. As described above, C3M is a marker of collagen degradation and pro-C3 is a marked of fibrinogenesis.

1.4.4 Scoring systems

1.4.4.a Child-Turcotte-Pugh score (CTP)

The Child-Turcotte-Pugh score is generated using 5 variables: 2 subjective evaluations (hepatic encephalopathy and ascites) and 3 blood parameters (albumin, INR and bilirubin).

We define hepatic encephalopathy (HE) as a neuro-psychiatric condition characterized by different degrees of impaired higher brain function that range from subtle abnormalities only detectable by psychometric testing to confusion, stupor or coma. HE has been described both in acute liver failure (ALF) and cirrhosis. It is postulated that the abnormal behaviour observer in HE may be caused by increasing level of ammonia. In chronic liver damage Urea synthesis is impaired and the brain astrocytes act as ammonia detoxification. In the process there is an excessive accumulation of glutamine in the cytosol of astrocytes which collects water for osmosis causing oedema. However inflammation and infection may also play an important role.(75) We classify HE in grades: Grade 0 (or minimal HE) only detectable using psychometric tests, Grade 1 (or minimal HE) mild confusion, disturbed sleep pattern, slurred speech; Grade 2 (or moderate HE) moderate confusion and lethargy; Grade 3 (or severe HE) incoherent speech, stupor; Grade 4 (or hepatic coma) unresponsiveness.

Child-Pugh score was created to assess mortality in cirrhotic patients following portal-caval shunt operations for variceal bleeding. (76) According to the score, patients can be separated into 3 categories with worsening prognosis: A, B and C with survival at 1 year of 100%, 80%, 45% respectively.(76) Despite its limitations it has a role in assessing the severity of cirrhosis and above all to predict mortality during admission in Intensive care unit (ICU) or following surgery.

1.4.4.b MELD and UKELD

MELD (Model for End Stage Liver Disease) and UKELD (United Kingdom model for End Stage Liver Disease) are scores designed to estimate the severity of liver disease, predict mortality and determine prognosis. (77, 78) They are based on blood results including INR, Creatinine, and Bilirubin. UKELD also incorporates sodium (Na) in the equation. MELD has been demonstrated to be better at predicting survival than the CTP score. (79) The formulas for MELD and UKELD are given below:

Equation 1: MELD score

***MELD**=10*[(0.957*ln(Creat(umol/l)*0.011312217))+(0.378*ln(Bil(umol/l)*0.058479532))+(1.12*ln(INR))]+6.43*

Equation 2: UKELD score

***UKELD**=[(5.395*ln(INR))+(1.485*ln(Creat)+(3.130*ln(Bil))-(81.565*ln(Na))]+435*

MELD was initially created to assess survival post trans jugular intrahepatic portosystemic shunt (TIPSS) in patients with portal hypertension. However it has been extensively validated in patients with cirrhosis to predict survival after infections, variceal bleeding etc. (80) MELD and UKELD are used to prioritize cases on the transplant waiting list, in the US and UK respectively. In 2018 the UK adopted an allocation system based on *survival benefit,* while the USA continues to use MELD as it provides a simple and effective measure of survival benefit post-transplant. (81). UKELD score of 49 predicts 9% mortality at 1 year without orthoptic liver transplant (OLT).(82)

Both scoring systems have limitations. For example, MELD estimates renal function using Creatinine alone, both scores use blood parameters that have significant variability among laboratories (international normalized ratio INR and Na) and exclude important complications of cirrhosis like hepatic encephalopathy. Therefore MELD variations (see below) have been proposed to mitigate these problems but none of these has been currently widely adopted.

MELD and serum Sodium (Na) incorporation

- MELD-Na
- Integrated MELD score (iMELD)
- MESO

MELD-XI (without INR)

MELD-gender

Re-weighted MELD / re-Fit-MELDNa

ΔMELD (changes in MELD over time)

1.5 Treatment options for liver cirrhosis

There are currently no available treatment to reverse cirrhosis of the liver. However there are several treatments that targeting specific pathogenic mechanism that have an effect on the progression of disease. Rifaximin (a non-absorbable antibiotic) is efficacious in preventing HE by altering the gut microbiota and reducing the number of nitrogenous compounds producing bacteria. (83) Emerging data suggest the effect of Rifaximin extends to the prevention of Spontaneous Bacterial Peritonitis (SBP).(84) SBP is defined as an infection of the ascitic fluid without any evidence of intra-abdominal source. Eliminating alcohol injury prevents developing decompensation in patients with alcohol related liver cirrhosis and can in some patients slowly re-establish a compensated state. However in a group of subjects with cirrhosis of the liver due to alcohol, the disease continues to progress despite abstinence. (85) The same statement is valid for HBV and HCV cirrhosis treated with antiviral therapies. (86, 87)

1.5.1 Nutrition

Malnutrition is very common in liver cirrhosis with prevalence reported between 50 and 90% in decompensated disease and 20% in compensated disease. The rate of malnutrition increases with the Child Pugh score. Malnutrition is associated with increased mortality and rates of complications due to cirrhosis, while patients with adequate nutritional intake have overall better outcomes at the same stage of liver disease. (88, 89) A large observational study in critically ill patients in intensive care demonstrated that optimising patients nutritional state with appropriate calorie intake improves all outcomes including mortality and quality of life.(90) This is true for patients with liver disease. European Society of Parenteral and Enteral Nutrition (ESPEN) and American Society of Parenteral and Enteral Nutrition (ASPEN) guidelines therefore recommend early nutritional intervention with the aim to achieve 30-35 kcal/kg/day in patients with cirrhosis and a protein intake of 1.3-1.5g/kg/day.(91) Although nutrition plays a very important role in the management of patients with cirrhosis of the liver and can influence the natural history of the disease it has no proven effect on fibrosis.

1.5.2 Milk thistle

Sylibum marianum or milk thistle is the most studies plant for the treatment of liver cirrhosis. The active principle is a lipophilic extract from its seeds. It contains 3 isomers flavonoligrans (silybin, silydianin and silychristin) known as silymarin. (92)

Silymarin is an antioxidant. It reduces free radical production and lipid periossidation. In vitro it reduces fibrosis and acts like a toxin blockade to hepatocytes membrane receptors.(92) A pivotal step towards formation of fibrosis in the liver is the conversion of stellate cells into myofribrosblast. Silymarin inhibits NF-kB (nuclear factor – kappa light chain enhancer for B cells) which has a crucial role in mammalian cells immune-regulation. NF-kB is also involved in cell apoptosis and proliferation. Silymarin also retards hepatic stellate cells (HSC) activation justifying its antifibrotic activity.(93) Most of the evidence of milk thistle antifibrotic effect derives from animal model studies. In CCl4 cirrhotic rats treated with colchicine or silymarin the collagen content reduced of 50%.(94) Reduction of collagen and pro-collagen III is observed in a rat model of biliary cirrhosis when administered silymarin at 50mg/kg/d, but not at a dose of 25mg/kg/d. (95) Silymarin administered for 3 years delayed the development of hepatic fibrosis in baboons with alcohol related liver disease. (96)

Interestingly all pre-clinical studies are published from the same group and moreover a recent Cochrane literature review highlighted that any possible beneficial effects of milk thistle on viral and alcohol related liver disease are cancelled when only high quality clinical trials are taken into consideration. (97) Quality clinical trials to generate valuable data in this field are needed thus currently we do not have enough evidence to support the use of Sylimarin as treatment for cirrhosis of the liver.

1.5.3 Coffee

Robust evidence is emerging in the scientific literature on the beneficial effect of coffee consumption in preventing liver disease. (98) Coffee is a combination of many different compounds including caffeine, chlorogenic acid, melanoids and the pentacyclic diterpenes, kahweol and cafestol. The well-known effects of coffee on the central nervous system is generated by caffeine but coffee has also anti-oxidant, anti-carcinogenic and anti-inflammatory actions. (99) Animal studies, observational human studies and a RCT in HCV induced liver injury highlighted that coffee consumption reduces the risk of deranged liver function tests, fibrosis, and cirrhosis and HCC

development. (34, 35, 100, 101) A metanalysis including 5 cohort studies and 4 case-control studies confirmed the protective effect of coffee use on cirrhosis development: increasing daily intake by 2 cups of coffee can half the risk of developing liver cirrhosis in male and female adults with deranged LFTs.(98) The risk of developing HCC is also reduced by coffee intake as demonstrated in a separate metanalysis. (35)

While coffee has roles in disease prevention and development, there is no evidence it has any effect on reversing cirrhosis. It is also very difficult to correct for confounders when evaluating a dietary compound and therefore narrowing the effect to single element may be impossible.

1.5.4 Anti-fibrotic targets

While liver fibrinogenesis and ECM deposition are a dynamic process, targeting molecular pathways involved in their development and progression could reverse disease. Most of the experimental therapies are still at in vitro or model stage and showed various degree of success.

Table 1.1: Anti-fibrotic targets

Target mechanism	**Pharmaceutical targets/molecule**	**Drug / trial**
Apoptosis of hepatic myofibroblast	Sulphasalazine and analogues	Rodent model
	CB1 antagonist	Rodent model
	Tissue Inhibitor of Metalloproteases 1 (TIMP1) blockade via si-RNA	In vitro experiments
	anti- epithelial growth factor receptor (EGFR) single chain fragment variable antibody-TRAIL fusion protein	In vitro experiments

	vitamin A-coupled liposomes to deliver siRNA against a collagen-specific chaperone	Rodent model
	ASK1	Selonsertib - RCT
Senescence of hepatic myofibroblast	atorvastatin	Cohort study on HCV veterans Proof of concept human study
Reversion of hepatic myofibroblast	PPR-γ	Elafibranor - RCT
	Farsenoid X receptor (FXR) agonist	Rodent model
Inhibit hepatic myofibroblast proliferation	Multikinase inhibitors	Imatinib, Nilotinib, Sorafenib – rodent model
Fibrogenesis	Angiotensin-covering enzymes (ACE) inhibitors, angiotensin II receptor antagonists	RCT and cohort studies
	Transforming growth Factor (TGF)-β inhibitors	Rodent model
	Pirfenidone	Rodent model
Contractility	Relaxin	Serelaxin – rodent models Human RCT to lower portal pressure
	Statins	RCT and cohort studies

	Endothelin-1 antagonists	Rodent models
Scaring degradation	MMP gene therapy	Rodent model
	Halofuginone	Rodent model
	Lysyl oxidase homolog 2 (LOXL2) inhibitors	Simtuzumab – RCT in Primary sclerosing cholangitis (PSC) and NAFLD; HCV open label trial;
	TIMP1 blockade via si-RNA	Rodent model
Immune response	C-C motif chemokine ligand 2 / C-C chemokine receptor type 2 (CCL2/CCR2)	Cenicriviroc – phase 1 and 2b
	Efferocytosis of macrophages	Delivery of drug in rodent models
	Chemokine receptors CXCL9/CXCR3 axis	Pre-clinical in vitro experiments

This table illustrates a summary of the trailed targets and the stage of research for each of them.

A multicentre RCT in subjects with NAFLD and bridging fibrosis or early cirrhosis evaluated the effect of Selonsertib alone or in combination with Simtuzumab in fibrosis resolution. Results showed potential improvement in fibrosis category in the Selonsertib only group. On the other hand Simtuzumab failed to improve fibrosis. (102) Elafibranor has been studied in a multicentre, double-blinded, RCT in population with NASH fibrosis. While it showed improvement of NASH with no evidence of worsening fibrosis, it failed to show fibrosis resolution. (42) A Phase 3 trial is undergoing. (103)

Somewhat results of drug therapy trails for these molecules targeting liver fibrosis have been underwhelming and different strategies need to be explored.

1.5.4 Cellular therapies

Cellular therapies can promote the recovery of diseased and injured tissue. Different cells have been used in liver disease, both in pre-clinical studies and human trials. Hepatocytes transplantation gave the first evidence of cellular therapy having efficacy when used for metabolic liver disease (104-106). Allogenic hepatocytes were transplanted intra-splenically in acute liver failure patients with hepatic encephalopathy grade 3-4 in 2 trials and this resulted in a minimal improvement in survival. (107) When tested in patients in the transplant list, hepatocyte transplantation improved cardiovascular performance but did not improve liver function.(108) It appears that hepatocytes transplantation is beneficial only as a bridge to transplant. Hepatocytes engraftment via the portal vein causes thrombosis and ischaemia-reperfusion injury in mouse models.(109) However, although the approach via the spleen is safer, hepatocytes seed in the liver in a very low number because around 90 % of transplanted cells are phagocytosed by Kuppfer Cells.(110)

Another issue relevant to clinical implementation is that adult hepatocytes have limited availability, thus there is a drive to generate hepatocyte-like cells (HLCs) from pluripotent stem cells that have features and function comparable to adult hepatocytes.(111) (112) HLCs can be derived in vitro from induced pluripotent stem cells (iPSCs) or embryonic stem cells (ESCs). (113, 114) Autologous HLCs can be derived from iPSC from individual patients- which may have the theoretical advantage of autologous use - however the costs and technical issues associates with deriving such cells for individual patients is prohibitive. (115, 116) At present human hepatocytes transplantation has been trailed in a single early clinical trial of few cirrhotic individual without success. (117) . To overcome engraftment and function issues foetal hepatocytes have been explored as alternative. Foetal hepatocyte have been shown to improve metabolic function in rodents' pre-clinical models (118, 119). In a clinical trial of acute liver failure, allogenic foetal hepatocytes were transplanted through the peritoneum of to seven patients leading to partial recovery of liver function in 3 subjects (120).

Hepatocyte therapy fails as an effective therapeutic option for liver disease because of the risk of thrombosis, *ex vivo* phenotypic instability, and poor engraftment (109, 117, 121). Thus different cells like hepatocyte progenitor cells (HPCs) have been tested as sources of cells for therapy. In adult livers HPCs are located in the canal of Hering. Injury to the liver activates them and stimulates differentiation in functional hepatocytes or biliary cells to replenish the cellular death.(122) HPCs are small (5-15μm) and this reduces the risk of embolism, facilitates cryopreservation, improves ischaemia tolerability, and lower immunogenicity. (122) We can only find very small number of HPCs in adult liver, and liver engraftment is inefficient, however recent studies suggest that coating the cells with hyaluronic acid can improve engraftment in mice. (123) It remains still to be demonstrated whether human HPCs can differentiate in mature hepatocytes. Foetal liver epithelial progenitor cells (FLEC) are closely related but distinct from HPCs.(124) Transplanted FLECs can express, in animal models, either an unipotent or pluripotent phenotype according to their lineage and to the host liver. (125) Interestingly these transplanted cells at 2 weeks from engrafting will express phenotype of mature hepatocytes in zone 2 and 3 of liver while they show characteristics of mature bile ducts cells in zone 1.(125)

High numbers of HPCs can be found in foetal livers and they can be easily isolated and cultured. For this reason foetal human biliary tree stem cells (hBTSCs) have a role in the development of HPC therapy (126). hBTSCs have been safely transplanted via the hepatic artery in two cirrhotic patients as reported in a case report. However the improvements of the liver function was only transitory (127).

Hematopoietic stem cells (HpSCs) don’t replicated well ex-vivo mainly because their expansion is regulated by extrinsic modulators provided by the niche. HpSCs are able to differentiate into immature hepatocytes expressing high alpha feto protein (AFP) and low albumin signals and not into mature hepatocytes. (128) (129-131). Nevertheless human-cord HpSCs can be transplanted in mouse livers and differentiate in hepatocytes like cells without cell fusion(132) and repeated infusions of purified HpSC can reduce fibrosis in mice model with chronic liver disease by promoting recruitment of neutrophils and macrophages.(133) HSC-like bone-marrow derived mononuclear cell have been injected in a RCT of 30 cirrhotic (15 each arm) patients on the transplant waiting list demonstrating transient but not statistically significant improvement of albumin in the treatment arm while Child-Pugh score did

not improve. (134) A recently published multicentre RCT showed that CD-133+ HSC cell therapy with Granulocyte colony-stimulating factor (G-CSF) does not improve MELD score in compensated cirrhosis (135).

Other potentially therapeutic multipotent cells are Mesenchymal Stem Cells (MSCs). When isolated from adult bone marrow or umbilical cord MSCs can expand and differentiate into several lineages including hepatocytes-like cells(136-138). In different animal liver injury models, MSCs have shown immunomodulatory functions, reduction of inflammation and hepatocyte apoptosis (139-143). MSCs have been used as therapy in cirrhotic patients with small improvements in liver synthetic function and MELD score. Data available so far do not support the use of MSCs in cirrhosis therefore larger clinical trials with pre-defined primary end points are necessary. (144-148).

As described above there have been several different approaches to cellular therapy in liver disease but most are not yet ready to be used in clinical practice as positive data from properly designed and powered randomised controlled trials are absent. The injured liver appears to be a significant obstacle to engraftment to these cells. Also the mechanism of action in modulating injury and promoting regeneration of these cells requires elucidations before designing phase 1 clinical trials to test safety and efficacy. The available data from clinical trials are still at an early stage and have only showed limited success in few cases (147, 149-151)

1.5.6 Growth factors

G-CSF is the most potent cytokine able to mobilize hematopoietic stem cells from the bone marrow. While G-CSF increases mobilization of bone marrow derived stem cells, hepatocyte growth factors and hepatocytes proliferation in decompensated cirrhosis, it does not improve MELD score. (152, 153) A double-blind RCT on the use of G-CSF in combination with Erythropoietin in decompensated cirrhotics proved the safety of this approach and an improvement at 1 year survival and MELD score in the treated group compared to placebo.(154) However more recently Newsome et al. demonstrated that G-CSF with or without CD13+ autologous stem cell does not improve liver function in compensated cirrhosis. (135) Results in the use of growth factors are overall inconsistent and more recent data would not support their beneficial effect in cirrhotics.

1.6 Mechanism of cirrhosis progression

1.6.1 Cell death - inflammation – fibrosis – epithelial regeneration

The commonest injury to the liver is mediated by inflammation. Independently from the insult (virus, fat deposition from metabolic disturbance or alcohol, autoantibodies mediated), persistent damage to the liver results in protracted inflammatory changes leading to cellular death and fibrosis. (155)

Cell death contributes to organ homeostasis of the healthy liver with very low turn-over of hepatocytes (0.05%), this is classically programmed cell death via apoptosis. On the other hand, injury mediated death can cause a large loss of hepatocytes and initiate hepatocytes regeneration. (156) Cell death in the acute setting mainly results in necrosis and, as a consequence, release of inflammatory signals. There is increasing evidence of the direct role of damage-associated molecular patterns (DAMPs), released by dying cells upon hepatocytes in acute liver injury. The effect of DAMPs upon other cellular types contributing to fibrinogenesis in the chronic liver injury model is emerging. (157) Also cell death by apoptosis can promote fibrosis as demonstrated in mouse models with selective increases of hepatocyte apoptosis by hepatocyte-specific deletion of Nemo, Mcl-1, or Bcl-xl. (158)

Cell damage plays a fundamental role in perpetuating inflammation and extracellular matrix deposition in chronic liver injury. Steatosis induced by NAFLD is a well studies model: saturated free fatty acids are directly cytotoxic and can induce formation of lysophosphatidylcholine which triggers apoptotic signals and increase TRIAL-R2 (apoptosis mediator). Injured hepatocytes release a pro-inflammatory cytokines and chemokines like TNF-α, IL-6 and CCL2. (159) This inflammatory signalling is enhanced by gut microbiota translocation which contributes to an inflammatory cascade in chronic liver disease - in this setting pathogen-associated molecular patterns (PAMPs – proteins expressed by the microbiome metabolisms) activate the toll-like receptor (TLR) 4 pathway.(160) TLR4 are transmembrane receptors of innate immune system cells like dendritic cells. The best recognised activation of TLR4 is by bacteria’s Lipopolysaccharide (LPS), which induces release of pro-inflammatory cytokines required for the immune response. In the human liver TLR4 is expressed by a variety of parenchymal and non-parenchymal cells but primarily by niche cells with immunity role: resident macrophages (KC), T cells and dendritic cells. When KC’s TLR4 pathway is activated by circulating PAMPs or DAMPs a cascade of pro-

inflammatory and pro-fibrotic signalling is released. This not only contributes to pathology and disease progression but also worsen portal hypertension, gut permeability and therefore translocation of gut microbiome, creating a vicious circle of persistent TLR4 activation. (161)

After injury the liver can manifest extraordinary regenerative abilities via hepatocyte regeneration. This is only possible with expansion of the extracellular matrix and collagen, the “scaffolding” to provide a stable structure for the hepatocytes to expand within. However during prolonged injury the balance between cell death and restoration of functioning liver becomes maladaptive with excessive matrix deposition.

During chronic injury ductular reaction (DR) appears in the periportal regions. DR refers to epithelial cells and inflammatory surrounding cells, mainly macrophages and myofibroblasts (termed the ductular niche). While mature hepatocytes are the main contributor of parenchyma regeneration in the healthy organ or in acute injury models, during chronic damage and cirrhosis hepatocytes lose the ability to do so and become senescent. Hepatocytes regeneration in the context of hepatocyte senescence and ductular reactions is believed to be in part perpetuated by hepatocytes progenitor cells located in the Canal of Hering: these bipotential stem cell-like cells can differentiate both in hepatocytes or biliary cells. (162) Following chronic liver injury it is thought that Wnt signalling from niche macrophages adjacent to the DR promotes hepatocytes differentiation without significant ECM expansion. Following biliary injury it is thought that Notch signalling activates biliary epithelial differentiation and myofibroblast expansion. During biliary activated DR the HPC are surrounded by myofibroblast producing Collagen I separating HPCs from macrophages direct contact and thus diminishing the Wnt pathways influence. (163) The extent of fibrosis is reflective of the magnitude of the DR across a broad range of liver diseases. (164)

Overall the excessive expansion of ECM and collagen deposition in chronic liver disease, substituting functioning liver parenchyma, generates cirrhosis. This eventually leads to portal hypertension and its related clinical complications.

1.7 Cell types that link inflammation to fibrosis

1.7.1 Monocytes

Monocytes are component of the leukocytes pool and constitute about the 5-10% of the total circulating leukocytes in all vertebrate animals. They are produced in the bone

marrow by haematopoietic precursors and circulate in blood, spleen and enter tissues. Monocytes are considered the precursors of both dendritic cells and macrophages. (165, 166) Monocytes are driven into the liver parenchyma from circulation following injury and inflammation and are a crucial mediator and perpetuators of injury in acute and chronic settings. (167) The macrophages resident in the liver are replaced by monocytes infiltrating the liver tissue during injury.(168) In liver injury, monocyte derived macrophages release inflammatory cytokines that induce hepatic stellate cells to differentiate in collagen producing myofibroblasts. (166) Circulating monocytes are recognised by the expression of CD14. Simplistically we can split monocytes in two categories: "classic" monocytes $CD14^{+}CD16^{-}$ ($Ly6C^{hi}$ in mice) and "non-classic" $CD14^{+}CD16^{+}$ ($Ly6C^{lo}$ in mice).(169) Those subsets express different adhesion molecules and chemokine receptors (CCR): classic monocytes express very high level of CCR2 compared to the non-classic; CCR2 (and CCR8 in mice models of liver disease) drives monocytes response to inflammation by promoting release of $Ly6C^{hi}$ monocytes from the bone marrow and infiltration in injured tissues where they promote proliferation of pro-inflammatory macrophages.(170) High level of CCR2 in the liver activates hepatic stellate cells releasing monocyte chemoattractant protein MCP-1 promoting collagen deposition and fibrosis. As shown in the previous table CCR2/CCL2 have been target in phase 1 studies as anti-fibrotic therapy. Infiltrated monocytes also express CCR1/MIP1α and MIP1β that have been proven to promote liver fibrosis. (171)

The dynamism between monocytes and macrophages has a role in fibrosis formation during injury and fibrosis resolution during tissue repair.(172)

1.7.2 Macrophages

Macrophages play a key regulatory role in liver fibrosis and repair. Macrophages are a heterogeneous population of cells that can either differentiate from bone-marrow derived precursors or are found as a subset of tissue resident cells, known in the liver as Kupffer cells. Tissue-resident macrophages, including KCs, develop from embryonic precursors with the capacity to proliferate and self-renew (173, 174). Macrophages are capable of phenotype's adaptation in response to stimuli from the microenvironment. Simplistically we can define the macrophages phenotypes as 'M1' (classical-activation with enhanced bactericidal properties), or 'M2' (alternative-activation with enhanced tissue remodelling properties) (175). Classically activated

‘pro-inflammatory’ macrophages promote inflammation by releasing signals that recruit inflammatory cell and activate HSCs. Activated HSCs produce extracellular matrix proteins that are the main component of the scaring of the liver in response to injury. On the other hand, alternatively activated ‘pro-resolution’ macrophages release matrix metalloproteases (MMP) that degrade the scar, and reduce inflammation of the liver. Pro-inflammatory macrophages can switch to fibrosis-resolving macrophages which occurs at the time of modulation of the expression of Ly-6C from high to low.(176) During the time that this cohort of macrophages proliferates, pro-inflammatory cytokines and chemokines are down regulated. Pro-fibrotic macrophages express transforming growth factor Beta (TGFβ), platelets derived growth factor (PDGF), Interleukin (IL) 1β and tumour necrosis factor Alpha (TNFα), while anti fibrotic macrophages produce mostly MMPs. When in “fibrosis resolution mode” macrophages also show increase gene expression of lysosomes, endocytosis, scavenger receptors and antigen presentation pathways, which are all implicated in phagocytosis. This finding support the concept that macrophages around the DR can have enhanced phagocytic activity, increasing their crucial role in degradation of extracellular matrix via MMP13 and therefore are involved in the remodelling of scar tissue.(177)

Macrophages can be polarised in vitro using for example Interferon Gamma (INFγ) or IL4/13, in this way their phenotypic stability improves. Efficient efferocytosis of necrotic material is necessary to stop the inflammatory cascade in acute liver injury. Alternatively activated macrophages have enhanced phagocytosis when compared to classically activated macrophages.(178-180). Tissue-resident macrophages express unique gene expression profiles which differ from other macrophage populations (181). Thus it is not surprising that, while macrophages share a common set of functions, we do observe some tissue-specific functions, like bone reabsorption by osteoclasts or the supportive function provided to neurons by the microglia (182, 183). In the liver, Kupffer cells are specialised in clearing damaged erythrocytes from the circulation (184). Kupffer cells express genes of the lipid and iron metabolism pathway (scavenger receptors) (185). Kupffer cells also have an essential role as a barrier to prevent bacteraemia and systemic inflammation. When resident macrophages are lost during injury, circulating inflammatory monocytes infiltrate the liver and differentiate into short-lived monocyte-derived macrophages, as described above (176). Large

afflux of monocytes in the liver parenchyma can results in peripheral blood monocytopenia and this phenomenon is associated with poor prognosis in patients with acute liver failure (186). Under specific conditions, for example when in mice model the niche is depleted artificially of KCs, monocyte-derived macrophages can repopulate the liver and become self-renewing. (185).

In advanced liver disease (cirrhosis) hepatocytes are characterized by significant senescence, thus are unable to efficiently contribute to regeneration of the liver. The hepatic progenitor cells' (HPCs) destiny appears to be set by the cells surrounding HPCs in the Ductular Reaction (DR). When hepatocyte's regeneration occurs, the HPCs appear to be in tight contact with macrophages which express Wnt3. Wnt3 expression on macrophages surface occurs following phagocytosis of tissue's debris by the macrophages. These data support the idea that macrophages have also a pivotal role in promoting hepatocytes regeneration. (163) Bird et al. identified a direct correlation between macrophages' paracrine activity and amplification of the DR. They established that macrophage derived TWEAK 14 signalling to bile ducts expressing the Fn14 receptor leads to expansion of HPCs with resulting hepatocyte regenerative function. (187)

Injections of exogenous macrophage or expansion of endogenous populations with cytokine stimuli can offer clinically-relevant therapeutic strategies that have potential to increase hepatic innate immunity, reduce liver inflammation and fibrosis and stimulate liver regeneration after liver injury.

1.7.3 Dendritic cells

Dendritic cells (DC) are the main antigen presenting cells and they are found in the liver near the portal tract and scattered throughout the parenchyma. They play a protective role in hepatic inflammation. In genetic mice models with depletion of DCs, during the recovery phase of Carbon tetrachloride (CCl^4) induced fibrosis, ECM degradation is reduced implying a role of DCs in fibrosis resolution through production of MMP9.(188) DCs influence over liver fibrosis is also expressed by regulating the homeostasis of other key players in fibrinogenesis like Natural Killer (NK) cells. (189) TNF-producing DCs proliferate when circulating monocytes enter injured tissues.(190)

1.7.4 Neutrophils

Neutrophils are recruited into the liver parenchyma by inflammation. This occurs by different modalities and the mechanisms controlling this are not well understood. For example neutrophils home the liver following intravascular chemokine gradients or formyl-peptide signals released by injured cells. The recruitment of neutrophils also occurs when hyalurons expressed by liver sinusoids interact with neutrophils' surface antigen CD44. (159)

1.7.5 T lymphocytes

The development of inflammation in the liver is primarily mediated by innate immunity and therefore Thelper17 (Th17), NK and mucosal associated invariant T (MAIT) cells are important contributors. Th17 produce IL-17 which has been proven to promote liver inflammation and fibrosis. IL-17 stimulates Kupffer cells to release IL-6, IL-1β, TNFα and TGF-β1 and HSCs to differentiate in myofibroblasts.(191) MAIT cells are characterised by the invariant T cell receptor (TCR) chain and are found in large numbers in the peri-portal and peri-biliary areas of human livers and are avid producers of IL-17. MAIT appear reduced in number in liver and blood in some chronic liver disease but increased in conditions like NAFLD. (192) NK cells have an antifibrotic function via INFγ and cause apoptosis of HSCs though TRAIL and FasL pathways, however this mechanism is suppressed in advance liver fibrosis by TGFβ. (193)

1.8 Macrophages injection as treatment of liver fibrosis in animal models

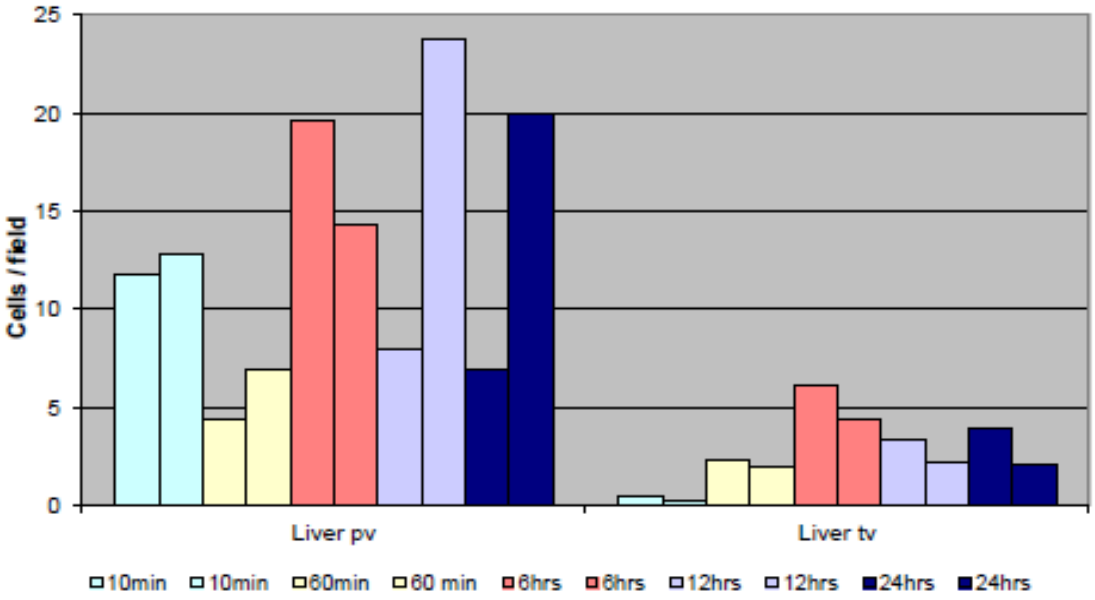
The paragraph above highlights the *scaring-resolution* role of macrophages in liver fibrosis, suggesting their potential therapeutic role. Prof Forbes group previously published encouraging therapeutic results of macrophages infusion in animal models of liver cirrhosis. (194) CCl4 liver injury mice-models receiving injections of mature macrophages (and not monocytes) showed improvement in liver function markers like albumin and early chemokine up-regulation leading to hepatic recruitment of endogenous macrophages, increase in anti-inflammatory cytokines, and decrease in hepatic myofibroblasts. (194)

This section explores two main hurdles that can be overcomed in the use of macrophage cellular therapy for liver fibrosis: homing to the liver and phenotype switch between pro-inflammatory and anti-inflammatory.

1.8.1 Liver engraftment

Pre-clinical studies in CCl4 injured murine models by Prof Forbes' group pursued injection of macrophages both in the portal vein (PV) or tail vein (TV). These studies demonstrate that injected macrophages home the liver.(194)

Figure 1.6: Macrophages homing the liver following injections in animal models



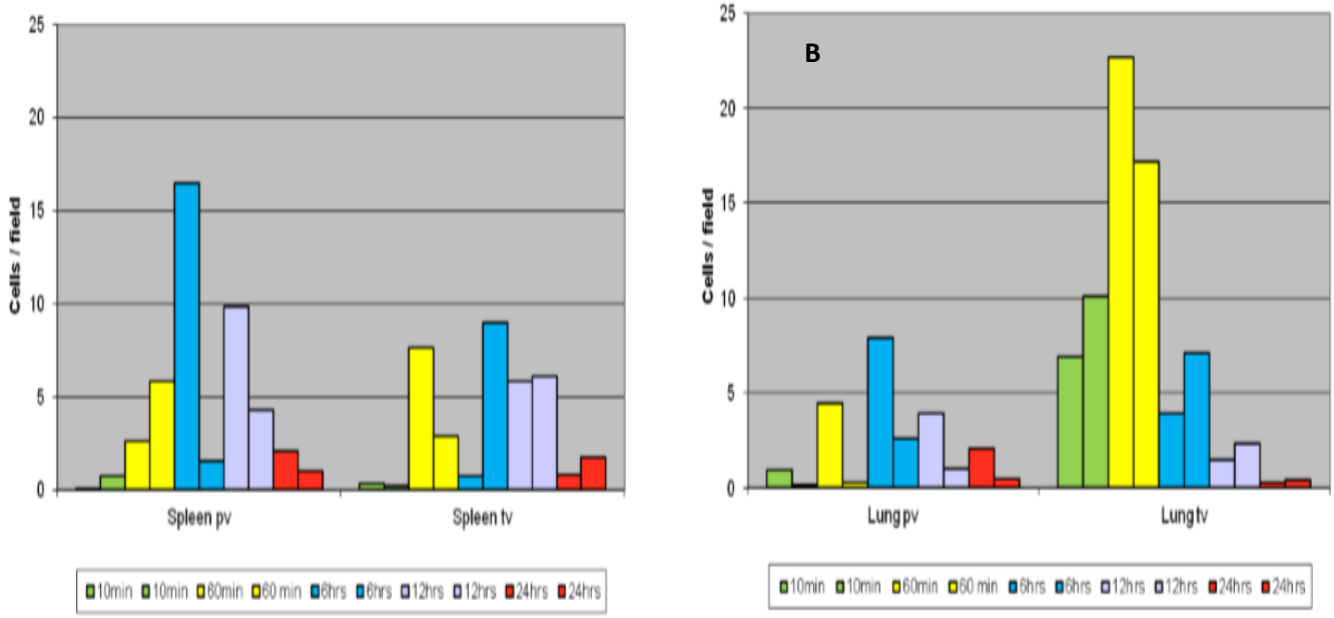
This figures is taken with permission from Prof Forbes' group and illustrate the number of macrophages per field (y axes) which have homed the liver at time points (x axes) when injected via the portal vein (PV) or the tail vein (TV) in rodent models of cirrhosis. Measures were taken sampling the organ at 10minutes (green), 60 minutes (yellow), 6 hours (pink), 12 hours (light blue) and 24 hours (blue).

While peripheral injection of macrophages via the tail vein results in less engrafted cells in the end organ (as shown above), it is also associated with less complication (for example PV thrombosis) and mortality in mice models. When translating to humans with cirrhosis PV injection is considered not be a safe option. Complications associated with the procedure, its invasive nature, coagulopathy and thrombocytopenia (common consequence of advance liver cirrhosis) are among the reasons why.

Macrophages are found in the liver with peak of concentration at 6h post injection (both PV and TV). These macrophages persist in the liver at 24h from injection, while after

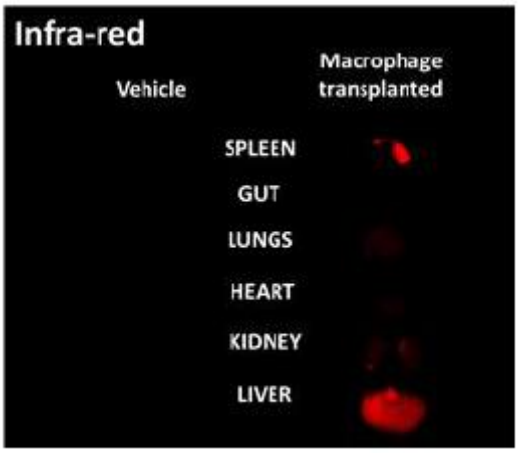
an initial pass (with peak at 60minutes), macrophages disappear from the lungs by 12h after administration. Infused macrophages seem to home the spleen too but they do not persist in the organ at 24h.

Figure 1.7: Macrophages pass through spleen and lung following injections in animal models



This figure is taken from Prof Forbes' group with permission and illustrate the number of macrophages per field (Y axes) reaching the spleen (figure A) or the lung (figure B) at set time points (colour columns) if injected via the portal vein (PV) or the tail vein (TV).

Figure 1.8: Infra-red fluorescence of macrophages

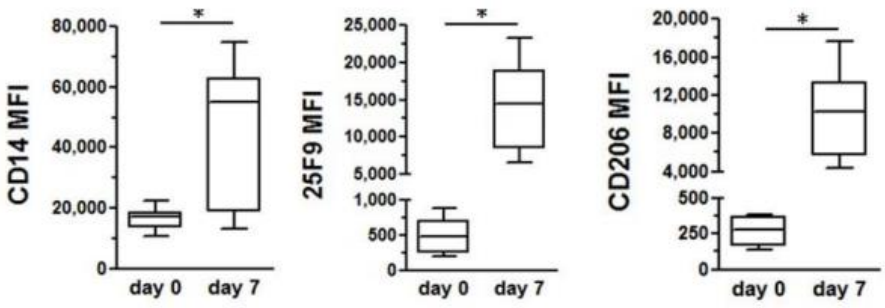


This figure is taken with permission from Prof Forbes' group and it demonstrates via infra-red fluorescence the presence of Macrophages 5 hours after 5x10^6 macrophage infusion via the tail vein in mice (n=2) without liver injury

1.8.2 Phenotype of Macrophages for therapeutic use

The Scottish Blood Transfusion research team in collaboration with Prof Forbes' lab developed and ameliorated the selection process of donor's monocytes. Following a standard apheresis to a target of 2.5 blood volumes, leukapheresis product undergoes checks including count and characterization using an antibody cocktail specific for monocytes phenotype (CD45-VB, CD14-PE, CD15-FITC and DRAQ7 to assess viability). Dilution may be required to comply with specification of the Mylteny CliniMACS® Prodigy. A CD14+ selection is applied to the CliniMACS® Prodigy. Once the selected CD14+ monocyte product is ready, it is matured in culture bags for 7 day with Macrophage-colony stimulating Factor. Matured macrophages express, beside CD14+, increase 25F9 and CD206 in keeping with a regenerative phenotype. (see Figure 9)

Figure 1.9: Characteristics of ex-vivo matured macrophages



This figure is taken with permission from the Edinburgh Scottish Blood Transfusion Laboratory Group and it shows the characteristics of macrophages following 7 days of maturation. Variation are expressed as mean fluorescence intensity (MFI).

Although not part of the release criteria, the extended panel also highlights other properties of our matured ex-vivo macrophages showing characteristics like phagocytosis, scavenger receptors and migration to inflammation. All these support a repair-regeneration phenotype.

1.9 Characteristics of macrophages matured ex-vivo from cirrhotic patients

The experiments above proven that peripheral monocytes can be successfully matured in regenerative-type macrophages ex-vivo. However to be able to translate this cellular therapy to clinical trial and to clinical use, Prof Forbes group had to demonstrate that autologous product from subjects aim to received it as treatment had same characteristics. Therefore monocytes were collected from compensated cirrhotic individuals and matured using the process describe above.

Moore et al. (195) demonstrated that leukapheresis is safe and well tolerated in cirrhotics in a clinical trial. Moreover the trial confirmed that macrophages matured from peripheral monocytes collected from subjects with cirrhosis are comparable to macrophages matured from monocytes of healthy volunteers.

1.10 Conclusions

This chapter systematically illustrates the need for successful therapy to reverse liver fibrosis. Liver injury and disease progression to fibrosis and then cirrhosis is a complex phenomenon which is mainly regulated by injury and injury resolution. A key component of this pathway is the macrophage. Bone marrow derived macrophages have the ability to replenish the niche when KC drop following injury and self-sustain. In animal model peripheral injections of activated macrophages reach the liver and can modulate fibrosis resolution. This leads the way to phase 1 first in human study to assess the safety and maximum achievable dose of autologous macrophages for the treatment of liver cirrhosis.

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Chapter 2: Autologous Macrophage Therapy for Liver Cirrhosis (MATCH)

Table of Contents

Figure and table content

2.1 Introduction

Translational medicine is the “bench to bed-side” leap necessary for clinical medicine to advance. Clinical trials are defined as clinical research studies in human subjects designed to prove the efficacy of a specific intervention.

Clinical trials can investigate a medicinal product, these are called clinical trial of an investigational medicinal product (CTIMP), or other interventions like diagnostic tools, questionnaires, equipment. Classically CTIMP progress via a step wise approach through different phases to evaluate efficacy and safety of the IMP in order to be adopted in clinical practice.

Phase 1 studies are first in human studies set up to ascertain safety and best dose of an IMP which has never been tested in human before. They are also designed to assess side effects and efficacy signals. Phase I studies are usually conducted in a small number of subjects and all are exposed to the IMP.

Phase 2 studies involve a larger number of participants and they are designed to prove efficacy of the IMP. Phase II clinical trials are typically randomised controlled trials (RCT) where the new treatment’s efficacy is evaluated against placebo or standard practice. Participants are randomly assigned to the IMP or placebo and investigators can be blinded to the randomization results to avoid bias.

Phase 3 clinical trials are usually large multicentre studies involving different countries and aiming to evaluate the efficacy of the IMP in a large population. They are usually randomized. Phase 4 studies are designed to assess use of the new treatment in “real life”.

This chapter will focus on the design and conduction of an investigator led, single centre, single arm dose escalation phase 1 clinical trial (Macrophage therapy for liver cirrhosis MATCH) evaluating the safety and maximum tolerated dose of monocytes derived autologous macrophage infusion in cirrhotic patients. In this chapter I will

discuss the safety and efficacy results of the phase 1 trial and review some of the safety results of the first 3 patients in the ongoing RCT.

2.2 Regulatory Frame Work for Clinical Trials

All clinical trial should comply with local, national and international legislations, regulations and standards.

Table 2.2: Legal framework for clinical trials

	Legislation & guidance	Approvals
CTIMP	GCP Data Protection Act and GDPR 2018 Freedom of Information Act 2000 HTA Medicine for Human Use Regulations	MHRA REC NHS R&D offices Local CRF agreements

This table offers an overlook on required approvals and observed legislations required for Clinical Trials of Investigatory Medicinal Product (CTIMP).

2.2.1 Good Clinical Practice GCP

All clinical trial must be conducted under Good Clinical Practice (GCP) Regulations. These regulations are set against international ethical and scientific quality standards to ensure safety of human subjects involved in clinical trials and to guarantee the design, the conduct, the auditing, the recording and the analysis involved in the trial are of high quality. All investigators involved in human trials have to demonstrate GCP training.

2.2.2 Data Protection Act and General Data Protection Regulation (GDPR) 2018

Clinical trials have not been exempted from the changes in Data Protection inforce by the GDPR act on 25 May 2018. The reviews law offers clarity to subjects (and indeed general public) on the use of their personal data once in clinical trials (or indeed navigating the internet, etc). GDPR was agreed as Europe wide law and aims to

reform existing law to take into account fast pace changes in cyber data collection. The act of law contains 99 articles but there are 8 core rights for individuals which allow easier access to the data held and obligation to consent to data collection. These changes occurred while our trial was ongoing and adaptations had to be put in place as well as education for the trial team.

2.2.3 Freedom of Information Act 2000 / Freedom of Information (Scotland) Act 2002

This is an act of the Parliament of the United Kingdom which allows public to access data held by public authorities. This act is interlinked to the GDPR 2018, in specific, to section 40 “personal information”.

2.2.4 Human Tissue Act (HTA) 2002/ Human Tissue Act (Scotland) 2006

The human tissue act (HTA) regulates all the activities involved in human tissues (which include human cells) and cover specifically 3 areas: donation, removal and retention and disposal. This refers both to the clinical use but also to the educational or research purpose. (1) In the MATCH trial all subjects are considered tissue donors.

2.3 MATCH set up of trial

2.3.1 Prove of concept

Preclinical studies on the use of autologous macrophages for the treatment of liver fibrosis illustrated in chapter 1 set out the field for a first in human safety and feasibility study.

Briefly, previous work by the Forbes group has demonstrated that mature macrophages (but not undifferentiated monocytes) can lead to early chemokine up-regulation, thus leading to recruitment of endogenous macrophages, an increase in anti-inflammatory cytokines, a decrease in hepatic myofibroblasts and fibrosis and an overall improvement in clinically relevant parameters such as albumin in the CCl4 murine liver injury model. (2)

Published studies have shown the safety of apheresis in cirrhotic participants (3-5) and Moore et al. also demonstrated that macrophages matured under Good Manufacturing Practice (GMP) conditions from circulating monocytes have comparable characteristics to macrophages from healthy volunteers. (3) Therefore, a clinical trial of the use of autologous monocyte-derived macrophages infusion in the treatment of liver cirrhosis was proposed.

2.3.2 Funding

With supporting preclinical data an application to the Medical Research Council (MRC) for funding was submitted and a grant was awarded in November 2014.

2.3.4 Sponsorship

The role of the sponsor is defined by the UK health departments' research governance framework and the UK clinical trials regulations. It involves confirming that arrangements are in place to initiate, manage and report the study, it ensures conduct of the study operates within scientifically sounds boundaries, reporting is respected, finance is sustainable and all regulatory agreement are met. The MRC also states that sponsors must ensure that appropriate indemnity is in place before research begins to cover potential liability due to the study conduct, design or management. It is expected that the sponsor is the principal employer of the chief investigator in the trial.

The MATCH trial is sponsored by The Academic and Clinical Central Office for Research and Development (ACCORD). ACCORD is a partnership between the University of Edinburgh and NHS Lothian Health Board and it is underpinned by the first joint Research Framework Agreement in Scotland.

2.4 Approvals

All clinical trials require approvals. CTIMP's like the MATCH studies require all the below tabled approvals. These can be sought in parallel, however the formal agreement from specific bodies may require approvals from other bodies to be in places (i.e. Research and Development will formally approve a study only after Ethics approval)

Table 2.3: Approvals required for clinical trials

Approval/ Registration	All research	CTIMP
Research Ethics	YES	YES
Medicines & Healthcare products Regulatory Agency (MHRA)		YES

Medicines & Healthcare products Regulatory Agency (MHRA) innovation office		YES*
Research and Development (R&D)	YES	YES
Phase 1 committee (PISRC)		YES**
European Clinical Trials Database (EudraCT)		YES
Registration in public registry	YES	YES

**for IMP classified as Advanced therapy medicinal product (ATIMP). These are: gene therapy; somatic cell therapy; tissue engineered product*
***phase 1 studies only (before R&D)*

2.4.1 Medicines & Healthcare products Regulatory Agency (MHRA)

The Medicine and healthcare products regulatory agency (MHRA) is a government body funded in 2003 by the fusion of the Medicine Control Agency and the Medical Device Agency. The MHRA ensures all medicines (including now blood product – in collaboration with blood transfusion) and medical devices are safe to use in clinical practice. All clinical trials testing medications need to be approved by MHRA and have to meet specific criteria.

MHRA are required, under European law, to inspect CTIMPs. Inspections of clinical trials conducted by MHRA assess compliance with relevant legislation and guidance.

The MATCH trial received approval from MHRA in January 2016 and in May 2018 MATCH trial underwent a very successful inspection from the MHRA.

2.4.2 Research Ethics Committee (REC)

Research and Ethics Committee is one of the fundamental functions of the Health Research Authority (HRA). REC are formed by individuals (member of the public as well as experts in the field addressed by the trial in review) and have the duty to review clinical trials proposals and amendments to guarantee they respect the dignity, rights, safety and well-being of the people who take part in them.

REC Scotland – A is the committee assigned to the MATCH trial. The original approval from REC for the study was granted in January 2016.

2.4.3 Research and Development (R&D)

The local Research and Development offices are within NHS institutions and provide support to the chief investigator (CI) and principal investigator (PI) to run clinical trials smoothly. R&D reviews local available resources and ensure funding are distributed appropriately. R&D also facilitates agreements between the trial institution and laboratories and radiology in the NHS, for example. R&D approval is necessary prior to commence a study and follows REC approval. MATCH was approved by R&D in July 2016.

2.4.4 Phase I Study Review Committee (PISRC)

There are considerations to take when we deal specifically with phase 1 trials. Scientific review and clinical risk assessment from a committee of experts (clinicians – both adult and paediatrics, pharmacologist, toxicologist, statistician, CRF manager, pharmacist, quality assurance (QA), etc) has to be obtained before the trial can become active. Issues addressed in the formal review from phase I study review committee (PISRC) are: expertise of the Principal Investigator and Research Team in the conduct of early phase trials, trial design, pre-clinical and clinical work already undertaken, dose escalation strategy and risk mitigation.

Specifically for MATCH, the PISRC risk assessment focused on dose escalation plans to ensure maximum safety for subjects enrolled in the study and contingency plans within the trial team and the acute care unit in the Royal Infirmary. Specifically the PISRC highlighted the need for independent pharmacology review, before reporting to the Data Monitoring Committee (DMC). The DMC had the authority to advise on the dose escalation, the arrangements necessary to be in place with ICU clinical team in the event of an acute reaction to the product, and the required monitoring period for subjects exposed to the IMP.

2.4.5 Clinical Research Facility (CRF)

The CRF is a dedicated area in which trained research staff (admin, nursing and management) arrange trial visits (clinic rooms), trial procedures (non-radiation images, sample collection and processing, biometrics, administration of IMPs), trial administration and QA.

The CRF at the RIE is situated in within the premises of the main hospital and it has a long standing expertise in running clinical trials including phase 1 studies. Its location within the hospital's ground it is a necessary feature to obtain Phase 1 accreditation.

2.4.6 Site Initiation visit (SIV) and Sponsor Agreement to Open Trial (SATO)

Once all approvals and agreements are in place the core trial team (PI, trial manager) and the monitoring team (provided by the sponsor) has to organise a Site Initiation Visit (SIV). During this visit the extended trial team (including CRF staff and all other involved in different aspects of the trial) attend to receive specific trial education. The SIV should cover trial purpose, design, conduction and management as well as all trial specific procedures. The SIV should also describe timelines in terms of recruitment and reporting. For the MATCH trial, the SIV was conducted on 14 Jun 2016 and the sponsor provided the agreement to open the trial on 03 Aug 2016.

2.5 Managing the trial

MATCH trial represented a challenge in terms of management not only because it was a phase 1 trial but also because it required coordinating different teams involved in different, but equally fundamental, parts of the study.

2.5.1 Data Monitoring Committee (DMC)

Dose escalation decisions and the overall safety of the study had to be reviewed by a Data Monitoring Committee (DMC). DMC is composed by experts who have a full understanding of clinical research and of the condition/IMP. Members of the DMC have to be independent from the trial team.

In MATCH the DMC is made by 3 independent members (2 medical experts in the field and 1 statistician). The DMC role and commitments are outlined in the DMC charter. This was written by myself following guidance of the MHRA *Good Clinical Practice Guide* and approved by all DMC members.

Briefly, the DMC met following the first infusion of the IMP. The trial's statistician circulated a safety report to the DMC and PI prior to the meeting, thus clarification or additional data could be addressed in advance. The meeting occurred via teleconference. Until the DMC was satisfied with the safety of the IMP no other subject could be treated in the trial. Following this first meeting, the DMC met after the 14 days safety visits of the last subject in each dose group. These meetings occurred in the same format as the one described above. At the end of the meeting the DMC members

would decide if the data provided could support a safe decision to escalate the dose. The DMC chair provided a letter to the sponsor, CI and the trial steering committee chair with the decision. During the dose escalation phase of the MATCH study we received approval to escalate at every dose group review. Until this decision was formally communicated by the DMC chair no patient was administered any dose of the IMP. The DMC also met at the end of the dose escalation to review the available safety data and deliberated on decision to progress to the RCT. Ad hoc meetings could be arranged if deemed necessary to discuss safety issues.

2.5.2 Trial Steering Committee (TSC)

The DMC provides advice on safety of the trial to the Trial Steering Committee (TSC). The TSC is a mix group of independent individuals and people on the trial team that provide guidance about the conduct of the trial. The TSC for MATCH trial counts a statistician, a local clinician expert in clinical trials (both phase1 and RCT), one of the trial monitors, the trial manager, the trial physician, the PI and a layperson. TSC duties are listed in the TSC charter. The TSC meets shortly after the DMC to act on its recommendations. Therefore in the dose escalation for MATCH the TSC met after the first infusion and at any dose escalation decision's point and before progressing to the RCT. Ad hoc meetings could be organised as necessary to discuss management of the trial or arising issues.

2.5.3 Paper Case Report Forms (pCRF) and electronic database

The trial team together with the trial statistician and the CRF staff designed paper case report forms (pCRF) that had the sole purpose to record anonymised data for the trial. During the phase 1 study specific safety check were inserted in the pCRF to ensure adherence to PISRC recommendations. The pCRF were used as a model to build an electronic database build by the Edinburgh Clinical Trial Unit (ECTU) with my support.

2.5.4 Amendments

All amendments to the trial protocol or documents relative to the trial (patient information sheet (PIS), consent, GP letter, Investigator brochure (IB), investigational medicinal product dossier (IMPD), product labels) have to be assessed by the sponsor via a tracked version with justification of changes. The sponsor provides a classification letter that assess the type of amendment: non-substantial (requiring R&D approval only), Substantial (requiring REC approval or REC and MHRA approval as

well). All amendments are submitted via Integrated Research Application System (IRAS) and Common European Submission Portal (CESP) if to the MHRA. During the phase 1 study, MATCH underwent 4 amendments of which one was substantial to REC only (see table below)

Table 2.4: List of Amendments for phase 1 MATCH trial

Sponsor Amendment number	NS 02	SA02	NS03	NS04
Sponsor classification	Non Substantial	Substantial	Non Substantial	Non Substantial
Date of classification by Sponsor	25-Jul-16	12-Aug-16	18-Oct-16	19-Jan-17
Amendment sent to	R&D	REC R&D	Sent to REC for information R&D	R&D
Date of REC approval	N/A	12-Sep-16	Acknowledged by email 20 Oct 2016	N/A
Date of MHRA approval	N/A	N/A	N/A	N/A

Date of R&D approval	Initial approval 01 Aug 2016, approval recalled to await phase 1 committee review re-issued 10 Aug 2016	14-Oct-16	Acknowledged by email 04 Nov 2016	24-Feb-17
Documents Approved	Protocol V4 PIS V3 (DE & RCT)	Protocol V5 Doctors poster V1 Patient poster V1	DMC charter V3	Protocol V6 IMPD V4 IB V4

This table summarises all the amendments from the Phase 1 MATCH trial. These have been classified as non-substantial (NS) or substantial amendment (SA), the latest required approval not only from Research and Development (R&D) but also from Research Ethic Committee (REC). A summary of the documents amended and version is also offered

2.5.5 Reporting

2.5.5.a DMC reporting

The trial statistician provided a safety report to the DMC before each meeting.

2.5.5.b Quarterly Development pathway funding (DPFS) reporting

As illustrated above the trial team would provide a quarterly report to the funder illustrating spending and trial progress.

2.5.5.c Development Safety Update Report (DSUR)

Every year a development safety report has to be filed to the MHRA highlighting AEs, SAEs, SARs, and SUSARs. This report also provides a comprehensive literature review of any new published (paper or abstracts) data using the same IMP.

2.5.5.d REC safety report

REC requires an update on safety every year. This report briefly outlines recruitment against target and is submitted together with the DSUR.

2.5.5.e PIRSC end of phase 1 report and additional safety report

A full report including primary and secondary outcome data was provided to the PIRSC at the end of the phase 1 study. Recommendations were provided to the trial team how to mitigate risk going forward to the RCT. The PIRSC requested a further safety report once the first 3 subjects in the RCT completed the safety visits following the administration of the IMP.

2.5.5.f Quality Control, Monitoring and Audit

In the phase 1 study all pCRF had to comply with 100% quality control (QC). Therefore all the pCRF were checked for completeness in a timely manner prior to the subsequent visit. Following QC all the visits were monitored against source data by the trial's monitors assigned by the sponsors in a timely manner but before the administration of the IMP. Following the monitoring visits and once the actions raised in the visit were completed, the data could be entered into the database. The electronic data were again checked against source data and pCRF by a delegated member of the trial team. Only at this stage could the data be used to produce any reports. The sponsor also provided full audit of the processes described above.

In the RCT 100% QC is not a requirements. Monitoring visits still occurs but every quarter. The process of validating the database entries continues as described above.

2.5.5.g Adverse Events (AEs), Adverse Reactions (ARs), Serious Adverse Events (SAEs), Serious Adverse Reactions (SARs) and Serious Unexpected Adverse Reaction (SUSARs)

Adverse events (AE) and Adverse Reactions were reported through the trial following the CTCAE grading and Medra coding. Causality and Expectedness were addressed against the Investigator Brochure (IB).

A monthly AEs listing is provided by the trial monitors to the wider trial team.

Serious Adverse Events (SAE) were classified as defined in the protocol and assess for severity. All SAE were reported in 24h from occurrence by the PI to the sponsor and the SNBTS team. The DMC was expect to meet and discuss any Serious Adverse

Events when occurred. The trial physician would provide the DMC and the TSC chair with a clinical summary (excluding any patient's identifiable). This would include time and dose of IMP infusion and assessment of causality, severity and expectedness. The trial would hold further recruitment until the SAE were reviewed and decision about continuing with the trial was taken.

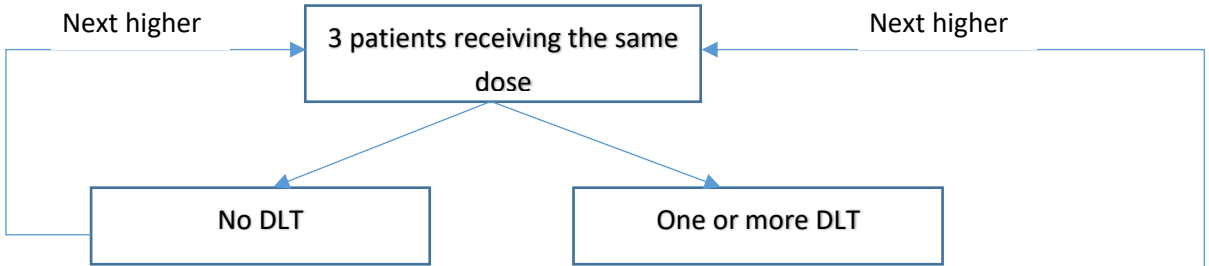
During the phase 1 study we did not record any SUSARs or SARs.

2.6 Trial Design

This phase 1 trial was designed as a classic 3+3 dose escalation model. Respectively each dose group received 10^7, 10^8 and up to 10^9 autologous macrophages. This model allow us to test safety, and tolerability of the maximum achievable dose.

If in the first dose group there are no toxicity recorded, the trial will progress to the next higher dose again if no toxicity are recorded the trial will progress to the next higher dose. If one toxicity occurs in a dose group of 3 subjects then the same dose should be repeated in another group of 3 subjects. If in this dose group (n=6) a total of 1 toxicity occurred then the dose can be escalated to the higher group of 3 subjects. If more than 1 toxicity occurred then escalation should stop.

Figure 2.10: Phase 1 dose escalation model



This figure illustrates a classical model of dose escalation of phase 1 trials. The IMP dose can be escalated to the next higher level only when 3 patients who have received the same dose show no Dose-Limiting Toxicity (DLT). If one or more DLT is recorded in a dose group further 3 patients should receive the same dose. If one DLT is detected the 6 patients' group then dose can be escalated to the next higher level. However if 2 or more DLT are recorded in the 6 patients' cohort then escalation should stop.

Because we are infusing an autologous cellular product, the total number of mononuclear cells collected from the subjects is variable. This affects ultimately the dose produced of mature macrophages. This is only evident in the highest dose group when participants respectively received 0.7, 0.6 and 0.8 x 10^9 cells. For the lower dose group this issues did not present as all participant has a cell collection permitting the manufacture of the set dose of 10^7 and 10^8 macrophages.

2.6.1 Limitation of 3+3 dose escalation design

The 3+3 dose escalation model is a popular first in human trial design, described initially in 1940s (7) then again in 1989 (8). It is a very well structured design and easy to adopt without employing statistical equations and probably this is why remains

popular. However in most recent years questions have been raised about its limitations.

Firstly, size cohort is fixed as well as dose escalations steps. This limits the flexibility of this design. This may not be a significant issue in the past but in more recent years it has become usual to assess in first in human trial complex medicinal products such as gene therapy, immunomodulatory treatments or, like in our case, engineered cellular products. These new therapies may have a spectrum of side effects and toxicity not easily assessed in 3+3 classical design where patients are followed up for a very short period of time. The number of subjects treated at the same dose is also very small (max 6). This will limit the wider understanding of the drug in use, including clinically relevant toxicities. This is becoming more important now that signals of efficacy are sought in phase 1 studies. These are better evaluated in larger cohorts. (9)

With this design is likely that more patients are treated with sub therapeutic doses, exposing those to potential toxicities and no benefit.(10) Equally in has been demonstrated that only around 30% of 3+3 model studies reached a true maximum tolerated dose (11).

For the above reasons are models have bene explored (toxicity based escalations, larger number of patients in each escalation cohort, Bayesian designs, etc). (9)

2.7 Patients and Methods

2.7.1 Inclusion and exclusion criteria

Table 2.5: Inclusion and Exclusion criteria for MATCH

Inclusion criteria	Exclusion criteria
Age 16-75 included	Refusal or inability to give informed consent to participate in the study
Diagnosis of cirrhosis - Biopsy OR - Radiology OR	Other cause of chronic liver disease / cirrhosis not included in listed aetiologies

- Fibroscan >15 kPa	
Aetiology of liver disease - Alcohol Related Liver Disease (ALD) - Primary Biliary cirrhosis (PBC) - Non-Alcoholic Fatty Liver Disease (NAFLD) - Cryptogenic cirrhosis - Haemochromatosis - Alpha-1-Antitrypsin deficiency - Treated (sustained viral response) hepatitis C	Portal Hypertensive Bleeding; active episode of bleeding requiring hospitalisation in the last 3 months where varices have not been eradicated by banding
MELD 10-16 included	Ascites unless, in the opinion of the investigator, the ascites is minimal and well controlled with no increase to diuretic therapy in last 3 months
	Encephalopathy; current or requiring hospitalisation for treatment in last 3 months

	Hepatocellular Carcinoma – uncertain cases to be discussed at local hepatobiliary Multidisciplinary meeting, dysplastic or indeterminate nodules to be excluded, regenerative or other nodules to be included at discretion of MDM
	Previous diagnosis of Hepatocellular Carcinoma
	Previous organ Transplant or previous recipient of tissue
	Listed for Liver Transplantation
	Any situation that in the Investigators opinion may interfere with optimal study participation
	Presence of clinically relevant acute illness
	Presence or history of cancer within past 5 years
	Pregnancy or Breastfeeding
	Allergy to steroids
	Active infection on the mandatory microbiology blood tests
	Immunosuppressant eg Azathioprine
	Current enrolment in an interventional study

Trial schedule

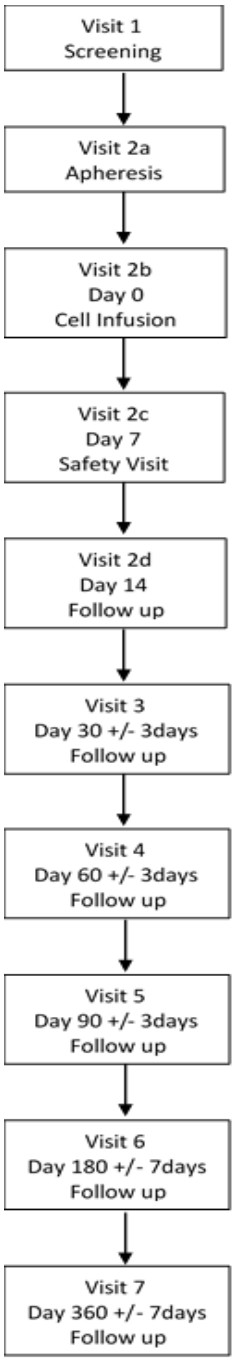


Figure2.11: flow chart of patients' visit schedule

Patients interested in the study attended for a screening visit to ensure eligibility 7 ± 4 days before scheduled leukapheresis. Leukapheresis occurred one week before infusion. On the day of the infusion, we excluded any active infection by physical examination and laboratory investigations (CRP <10, WCC <11 and Temperature <38). Before the infusion of macrophages, the subject received intravenously for safety measures 10 mg of chlorphenamine and 100 mg hydrocortisone. During the infusion, patients were monitored closely with observations (including BP, Hr, T°C, RR and Sat %) every 5 minutes to exclude acute transfusion reactions. The following 2 hours from the end of the infusion, observations as listed above, were performed every 30 minutes, thereafter hourly. All subjects were observed overnight in the Clinical Research Facility (CRF). According to recommendations from the PIRSC, the trial team informed the local ICU, with whom we had special prior arrangements in the event of a severe reaction, of scheduled infusion. The following morning full blood count, renal function, electrolytes, liver function tests, triglycerides and ferritin were checked prior to discharge to exclude toxicity, including Macrophage Activation Syndrome (MAS).

The first two follow-up visits (day 7 and day 14 after IMP infusion – defined as safety visits) we assessed AEs, dose-limiting toxicity (DLT) and the presence of MAS, both described in details in paragraph 2.7.

Thereafter, subjects were followed up at day 30, 60, 90, 180 and 360 after IMP infusion with routine and biomarker blood tests, abdominal ultrasound, transient elastography and health related quality of life assessment at specific time points (full details are provided in the table below).

Table 2.6: Trial Visit Schedule

Trial Schedule Dose Escalation										
	Screening	Apheresis	Cell Infusion	Safety Visit	Follow-Up	Follow-Up	Follow-Up	Follow-Up	Follow-Up	Follow-Up
	Visit 1	Visit 2a (within 7 days of visit 1)	Visit 2b (7 days after apheresis)	Visit 2c (Day 7)	Visit 2d (Day 14 days)	Visit 3 (Day 30±*3* days)	Visit 4 (Day 60±*3* days)	Visit 5 (Day 90±*3* days)	Visit 6 (Day 180±*7* days)	Visit 7 (Day 360±*7* days)
Informed consent	x									
Clinical Assessment	x	x	x	x	x	x	x	x	x	x
Vital Signs	x	x	x	x	x	x	x	x	x	x

Screening Blood Tests	x										
ECG	x										
Standard Blood Tests	x		x	x	x	x	x	x	x	x	
Mandatory Microbiology	x										
Pregnancy test	x*		x*	x**		x*				x*	
Abdominal USS	x								x	x	
Fibroscan	x							x	x	x	
ELF Panel	x					x	x	x	x	x	
Protein biomarkers	x					x		x			
Fingerprint™											
CLDQ	x							x	x	x	
Apheresis		x									

Macrophage Infusion and observation			x							
Adverse Events	x	x	x	x	x	x	x	x	x	x
Clinical Events			x	x	x	x	x	x	x	x
Concomitant Medication	x		x	x	x	x	x	x	x	x

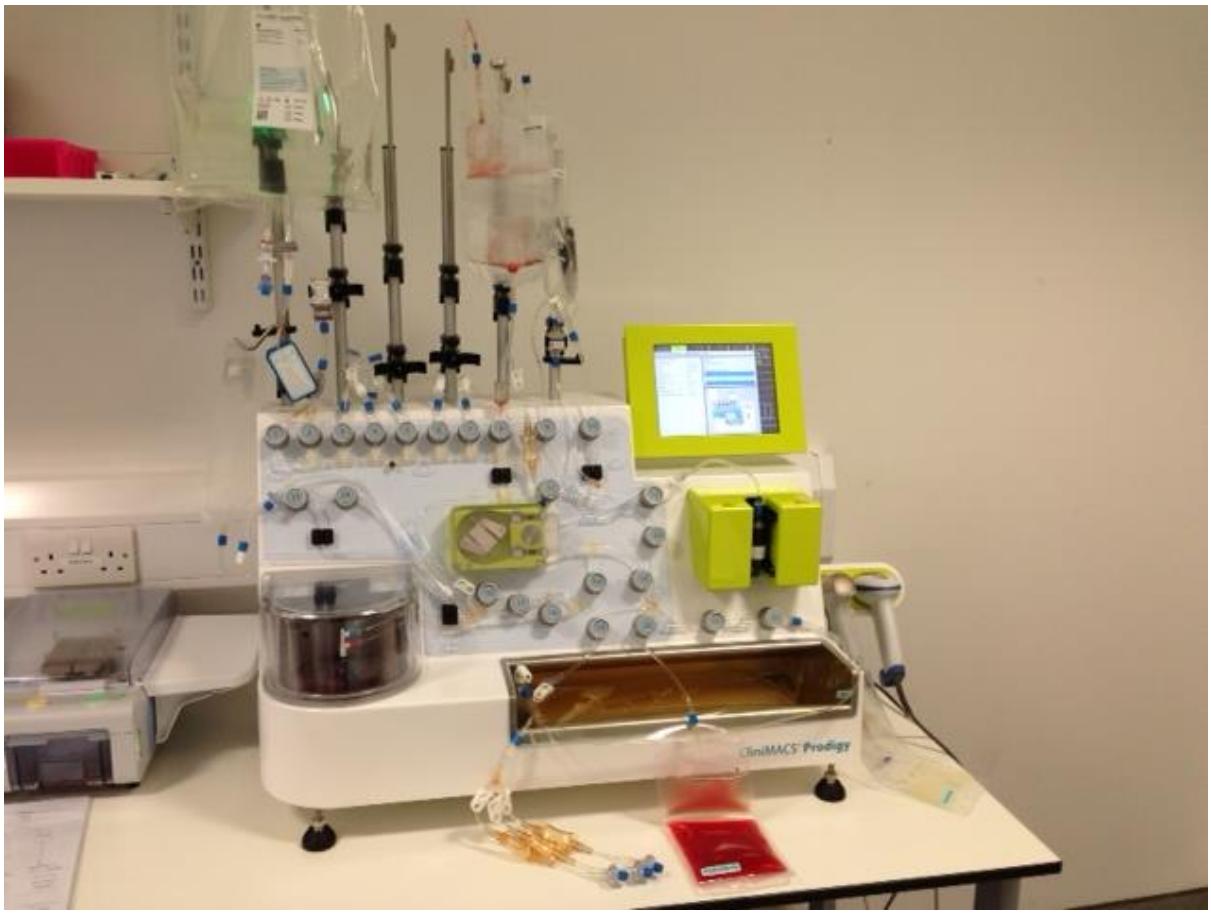
**women of child bearing age only, ** If test not carried out at previous visit.*

2.7.2 Method of cell production

Monocyte-derived autologous macrophages production has been described before (6). Fundamental development took place in the blood transfusion facilities in the Scottish Centre for Regenerative Medicine to comply with GMP regulation to be able to deliver our IMP.

Briefly, a leukapheresis was obtained using the standard Optia apheresis system using a standard collection programme for mononuclear cells (processing around 2.5 total blood volumes). The total numbers of monocytes in the preparation were determined using flow cytometer, and sufficient leukapheresis collection to contain ≤ $3.5x10^9$ monocytes allocated for monocyte selection. Monocytes were isolated using the CliniMACS Prodigy cell Processor with CliniMACS CD14 Reagent, TS510 tubing set and LP-14 program (all Miltenyi Biotec, Surrey, UK), using low adhesion culture bags and closed system for media replenishment.

Figure 2.12: CliniMACS Prodigy Cell Processor



This figure was kindly borrowed with agreement from Dr Chloe Pass and it illustrates the CliniMACS Prodigy Cell Processor by Miltenyi Biotech. It allows cell enrichment, depletion, centrifugation and cultivation in a single automated system.

The leukapheresis product is further purified with selectin of CD14 positive monocytes using a CliniMACS automated separation device, a closed-system procedure where the product is incubated with CD14 labelled magnetic beads, allowing separation of CD14 positive cells when passed over a magnetic column. CD14 positive monocytes are washed and re-suspended in PBS/EDTA buffer containing 0.5% Human Albumin Solution (HAS). Once counted the CD14 monocytes are re-suspended in differentiation medium containing 100ng/mL Macrophage Colony-Stimulating Factor (M-CSF). Monocytes are cultured at $2x10^6$ cells per cm^2 and per mL in culture bags (MACS GMP differentiation bags, Miltenyi) with GMP-grade TexMACS (Miltenyi) and 100ng/ml GMP-compliant recombinant human M-CSF (R&D Systems, Abingdon, UK). Cells were cultured in a humidified atmosphere at 37°C, with 5% CO2 for 7 days. A 50% volume media and cytokine replenishment is carried out twice during culture (day 2 and 4). Media replenishment is carried out twice during culture (typically days 3 and 5), using differentiation media supplemented with 100ng/mL M-CSF. Macrophages are harvested from the culture bags at day 7 using PBS/EDTA buffer (CliniMACS buffer, Miltenyi) containing pharmaceutical grade 0.5% human albumin from serum (HAS, Alburex). Harvested cells are re-suspended in excipient composed of 0.9% saline for infusion (Baxter) with 0.5% human albumin (Alburex) at the appropriate dose required, then stored at 18-24°C before administration to patients.

Day 0 freshly isolated monocytes, and final macrophage products, were characterized according to validated flow cytometry criteria (6). Cell counts were determined using Trucount tunes (BD Biosciences) and viability determined using DRAQ-7 (Biostatus UK) dye exclusion. Macrophages were determined as CD14+ cells having mean fluorescence intensity (MFI) five times higher than the level expressed on day 0 by the freshly isolated monocytes for the cell surface markers 25F9 and CD206. 25F9 is an antibody from mature macrophages and CD206 is only by alternatively activate (M2) macrophages, both are expressed at a very low level in monocytes. This macrophage product is a very terminally differentiated cell product and shows little cellular plasticity beyond the capacity to be polarised.

The leukapheresis product from all patients contained a very large percentage of CD14+ cells, as noted previously(3, 6), and the initial collections required to be adjusted to not breach the limits of the CD14 CliniMACS reagent, in all but 2 cases. CD14+ monocytes were successfully isolated from all patients, with a mean purity of 98.3% +/- 0.7% and a mean yield of 55.25 +/- 5.4%. A therapeutic macrophage product was successfully manufactured and administered for all patients.

The production of therapeutic macrophages for infusion requires collaboration between Scottish National Blood Transfusion Service (SNBTS) and the Forbes group. To reach the stage described above with a product safe to be use in human studies the product has gone through significant development.

Development	Outcome
Optimization of blood volume at apheresis	Increased cell yield when apheresis collection processed 2.5 total body weight (TBV) compared to 1.5 TBV
GMP grading medium	TexMACS medium improved cell yield, produced bigger macrophages that adhered less to the culture bag (resulting in reduced cell loss) compared to the other 2 medium tested.
M-CSF medium	A fully GMP compliant medium is used at a concentration of 100ng/ml which consistently had a higher conversion rate compared to 50ng/ml (that has the benefit of less protein)

Table 2.7: Manufacturing developments of the autologous macrophage product

The SNBTS team together with the Forbes group and the MATCH team is continuing to develop the production process to improve quality, yield and efficiency of the macrophage product. This aspect will be discussed in further details in Chapter 5.

2.7.3 Statistical analysis plan

A plan was agreed with the trial statistician before the trial was opened to outline the analysis required of the primary and secondary outcomes. The statistical plan also described the content of the DMC reports

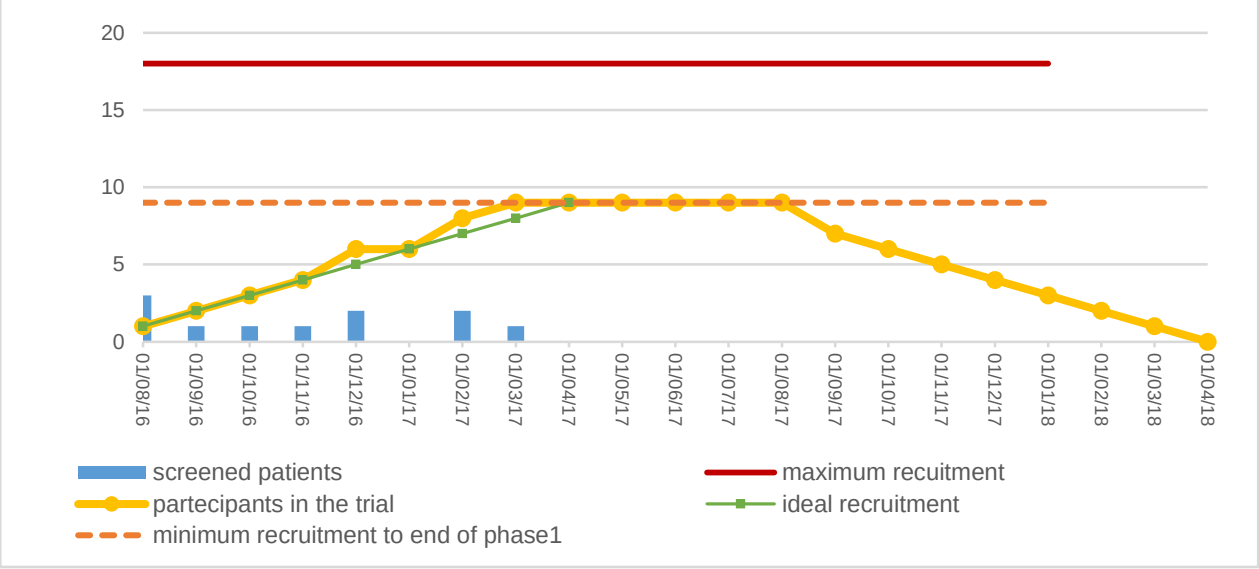
Primary outcome of safety and tolerability are presented as descriptive static analysis only.

There was 100% quality control of the data collected, with no missing data other than a single collagen biomarker sample at day 60 post-infusion. All the adverse events (AEs) are reported by dose.

2.7.4 Recruitment

During the DE phase recruitment proceed as expected.

Figure 2.13: Recruitment

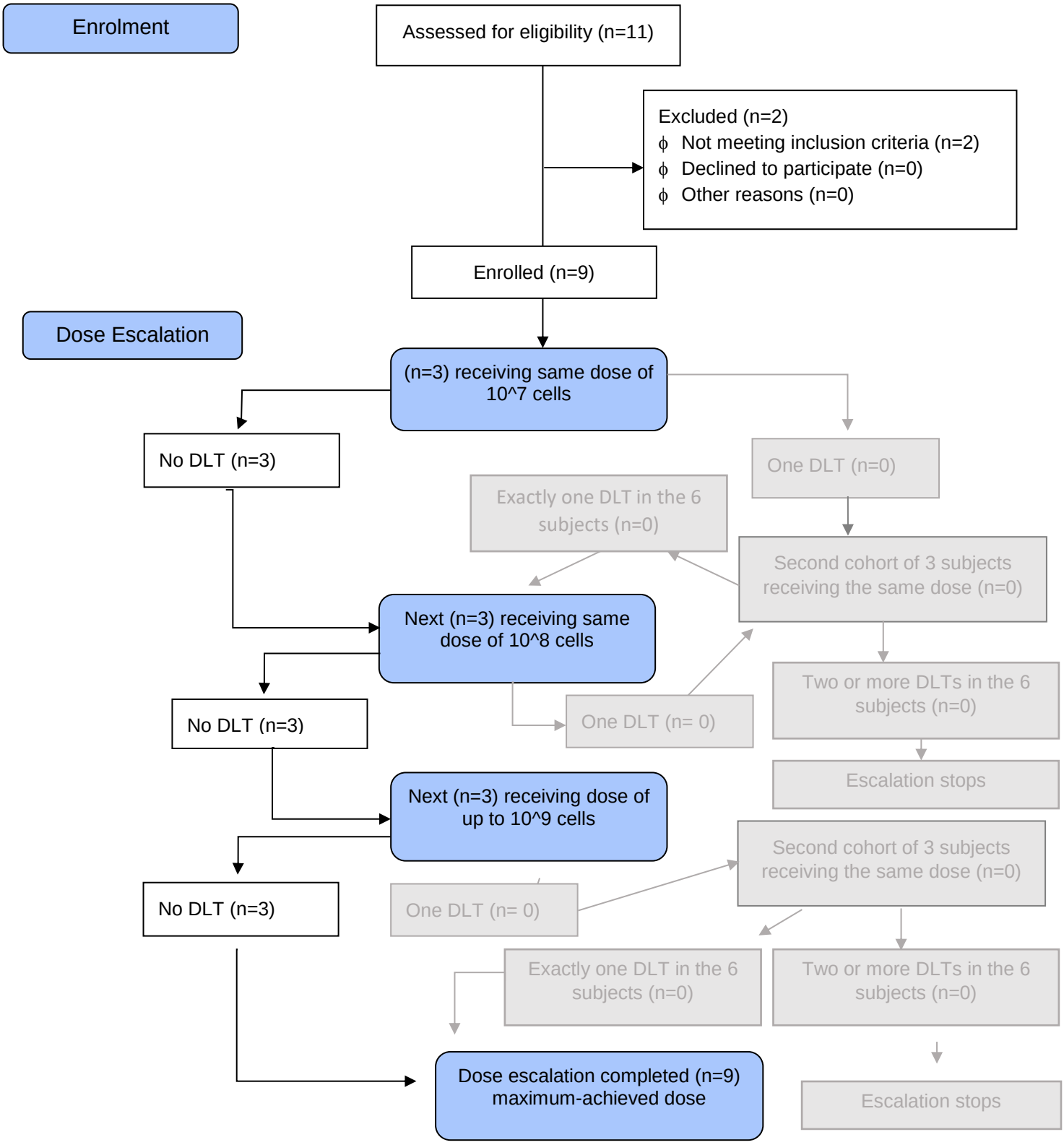


This figure illustrates the actual recruitment (yellow line) against the ideal recruitment (green line). The lines overlap demonstrating recruitment targets were met on schedule.

During the phase 1 trial I screened 11 subjects, 2 failed screening (both due to exclusion criteria which were an undetermined nodule on USS and computer tomography (CT), and a MELD score of <10). Nine subjected entered the trial as illustrated in the Consort diagram below.

The dose escalation proceeded without complications. There were no toxicity recorded in any of the dose groups and therefore DMC decision was to continue escalate following the safety assessment after the 3rd patient in each dose group.

Figure2.14: Consort Diagram



This figure shows the actual escalation process during the MATCH phase 1 trial. No DLT were identified in any of the dose groups hence escalation occurred as expected. The part of the flow chart illustrating events in case of DLT has been blurred as this did not actually occur.

2.7.4.a Patients' characteristics

Table 2.8: Patients' characteristics

	Screening failures		10^7 cells			10^8 cells			Up to 10^9 cells		
									0.6x10^9	0.8x10^9	0.7x10^9
Subject	*001*	*002*	*003*	*004*	*005*	*006*	*007*	*008*	*009*	*010*	*011*
Demographics											
MEAN Age (n=2 screen fails; N=3 others)	63.00 ± 5.66		59.33 ±8.50			55.67 ±6.35			57.67± 2.88		
BMI	32.1	28.2	24.7	29.6	35.6	26	27.8	27.8	33.6	27.6	29
SEX (M:F)	2:0		1:2			3:0			1:2		
Ethnicity	All Caucasian		All Caucasian			All Caucasian			All Caucasian		

Aetiology of liver disease											
ALD	1		2			2			2		
NAFLD	1		0			0			1		
HCV (SVR)	0		0			1			0		
PBC	0		1			0			0		
Duration of cirrhosis											
years	8	6	1.5	11	1	3.5	9	12	1	4	4
Severity of cirrhosis											
MELD	9	11	13	11	14	13	10	13	10	13	11
Mean MELD (n=3)			12.37±1.51			11.90±1.48			11.36±1.62		
UKELD	45	51	50	50	50	51	51	51	48	51	47
CP SCORE	6	6	6	5	7	6	6	8	5	9	9
CP class	A	A	A	A	B	A	A	B	A	B	B
Liver disease complications											
Ascites	x		x				X	x		x	x
SBP											
Variceal bleed			x				X	x		x	x
HE										x	x

This table describes patients' characteristics at baseline. These include demographics, aetiology and severity of liver disease and previous decompensation events. Text colour identify dose group (blue 10^7, red 10^8, and green up to 10^9)

2.7.5 Study assessments

2.7.5.a Safety

Safety has been assessed against toxicity criteria, MAS criteria, transfusion reaction criteria and AE assessment.

Dose Limit Toxicity (DLT)

The definition of DLT was formulated using accepted criteria for acute kidney injury, drug induced liver injury, acute transfusion reaction.(12, 13) We define DLT in this study as

Serum creatinine ≥ 1.5-fold from baseline

Haemoglobin 1.5-fold ≤ baseline

Platelets < 2-fold from baseline

Total white cell count <2.0 x 10^9

Alanine aminotransferase (ALT) >3-fold from baseline

Total bilirubin > 3-fold from baseline

MELD score >4 points from baseline.

Macrophage Activation Syndrome (MAS)

MAS is defined as: (14)

Fever

Peripheral cytopoenia in 2 or more lineages

- Hb <10.0 g/dl (or 9.0 g/dl in infants <4 weeks)
- Platelets,100x 109/ l
- Neutrophils <1.0x 109/ l

Hypertriglyceridemia or hypofibrinogenemia

- Triglycerides >265 mg/dl
- Fibrinogen <150 mg/dl

Ferritin >500 g/dl

Macrophage activation syndrome is a haemophagocytic condition that is characterised by a cytokine storm (in particularly IL8). It is life threatening and usually occurs as a rare complication of some rheumatic disease like adult still disease. Because it is believed to be cause by overproduction of T cells or mature macrophages it represents a theoretical risk in following the administration of our IMP.

Transfusion reaction

Transfusion reaction is defined by the British committee for standard in haematology guideline as below(15)

- Temperature ≥38ºC or raise of 2ºC
- Heart rate>100
- Fall in BP of ≥30mmHg
- Collapse
- Pain (chest or abdominal)
- Dysponea or Sat<94% in room air
- Stridor
- Mucosal swelling
- New rash
- Diathesis haemorrhagica

2.7.5.b Efficacy

This has been assessed using scores of liver function like Model for End Stage Liver Disease (MELD), UK Model for End Stage Liver Disease (UKELD), Albumin, Non-

invasive markers of Fibrosis like Fibroscan®, Health Related Quality of Life (HRQL) measured using the Chronic liver disease questionnaire and serum biomarkers (ELF panel and PRO-C3 and C3M).

HRQL

CLDQ was developed by Younossi et al. in 2009 as a specific health-related quality of life instrument for cirrhosis. Several validation studies throughout the world are available in literature confirming the performance of this questionnaire in different severity and aetiology of liver disease. The CLDQ is a 29-item self –reported questionnaire addressing 6 domains (fatigue, activity, emotional function, abdominal pain, systemic symptoms and worry) that when combined give a composite score. Score is calculated by the patient's response options from 1 to 7 (where 1 corresponds to "worse outcome" and 7 "best outcome") for each item. CLDQ has been translated in several languages and validated in cirrhotic as well as non-cirrhotic patients. (16) (17)

2.8 Results

2.8.1 Safety

All subjects underwent a complete apheresis with expected self-limiting side effects of hypocalcaemia. All infusion were completed without clinically significant adverse events. In one single subject, the Finger Pulse Oximetry Saturation % reached 94% transiently. The baseline recording just before infusion was 95% and the subject did not show any clinical signs of distress (normal Respiratory Rate, normal Heart Rate, and normal blood pressure). This was likely positional as saturation improved as soon as the subject changed position. In the 12h of observation following the infusion there were no recorded adverse events of significance or related to the infusion.

In total, we recorded 70 Adverse Events in the duration of the phase 1 study among all subjects. The table below classifies the AEs by dose, using Medical Dictionary for Regulatory Activities (MedDRA) coding version 20.0. All AEs listed were defined as grade 1 or 2 according to the Common Terminology Criteria for Adverse Events (CTCAE) version 5.0.

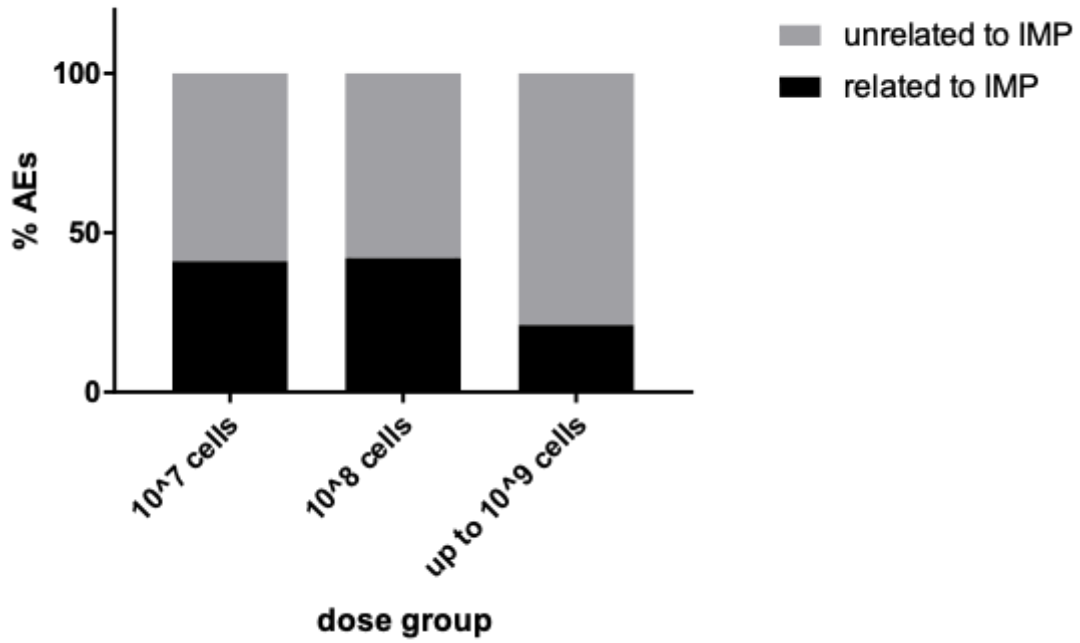
Table2. 9: Type of adverse Events

Type of Adverse Event	*10^7 dose*	*10^8 dose*	*Up to 10^9 dose*
Nausea	1	0	0
Abdominal pain	0	2	3
Anorexia	0	1	0
Light-headedness	1	2	2
Fatigue	1	1	3
Chest pain	4	6	0
Joint pain/malaise	2	2	3
Rash	2	0	3
Hypocalcaemia symptoms (leukapheresis)	1	2	3
Ascites	0	1	0
Anaemia	1	1	0
Infective	3	0	2
Others	5	1	10
TOTAL	22	19	29
Number of probably related AEs	9(41%)	8(42%)	6(21%)

During the trial period 3 subjects reported an episode of chest pain relatively close (within 4 weeks) to infusion of macrophages. Macrophages infused in peripheral vein pass through the lungs before homing the liver and spleen. According to pre-clinical studies macrophages do not engraft the lung parenchyma and the organ is virtually free of the infused cells by 4h. However, the theoretical risk of pulmonary embolism exists and therefore these episodes were investigated thoroughly. In one case the

event was clearly related to occupational muscle-skeletal injury and resolved with simple analgesia. Another subject had a history of inflammatory pleural effusion 10 years previously, which resolved at the time without the need for thoracoscopy. Because of the pleuritic nature of the pain and her previous history a chest radiograph was obtained and was reported as normal. During the visits the subject continued to maintain normal saturation % and respiratory rate. A d-Dimer investigation was also obtained in the occasion (it has to be noted this specific subject had normal liver synthetic function and fibrinogen at all stages during the trial) and this excluded venous thromboembolism. Symptoms resolved in about 7 days and it was attributed to a mild exacerbation of chronic obstructive pulmonary disease (a pre-existing condition). For the last subject who experienced chest pain we were unable to identify a cause, however the pain was focal to “finger pointing” and very short lasting (not longer than few minutes). There were no other recurrent adverse events temporary close to the infusion of the IMP.

Figure2.15: Related and Unrelated Adverse Events per dose group



This figure documents the absence of dose related phenomenon with comparable numbers of potentially related and unrelated AEs in the 3 dose groups. Percentage of AE related (black) and unrelated (grey) to IMP (y axes) are displayed for each dose group (x axes)

We recorded three SAE in the duration of the trial. All three were unrelated to the IMP and resolved without sequelae for the subjects.

The table below illustrate the type of event and the temporal relation to the infusion of macrophages.

Table 2.10: Duration and occurrence of SAE in relation to IMP

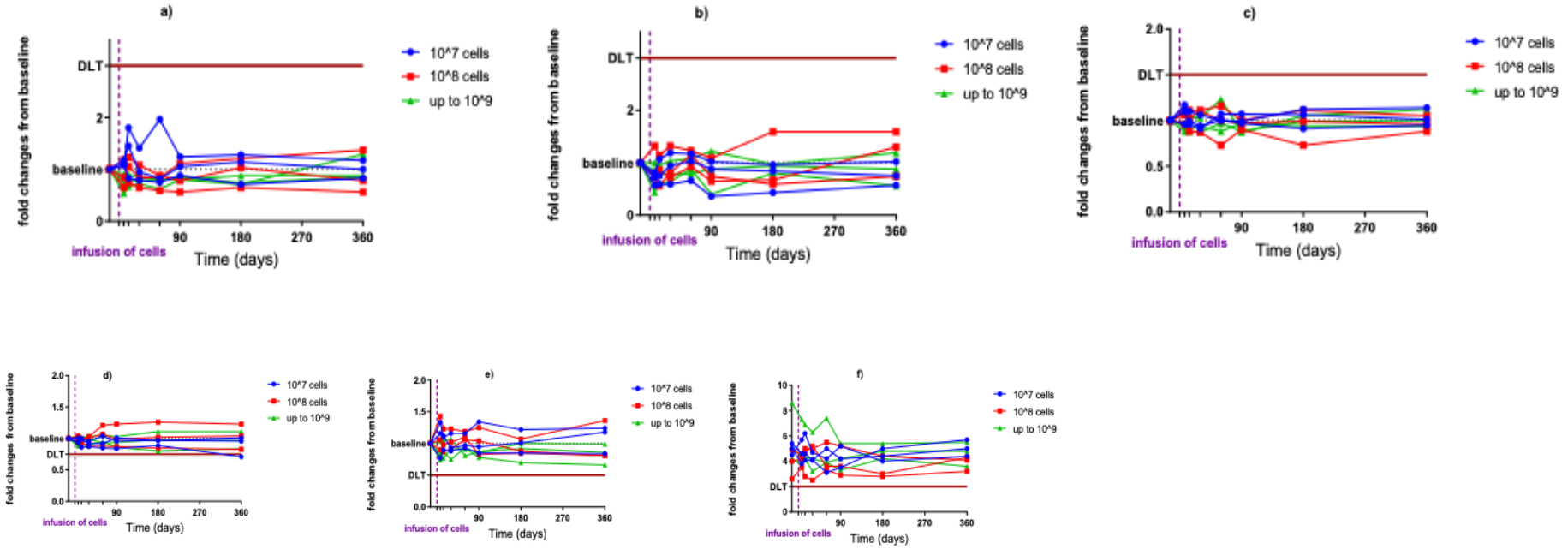
Type of Adverse Event	*Dose*	*Duration*	*Time in relation to IMP*
Abdominal pain required hospital admission – investigation confirmed constipation	Up to 10^9 dose	24h	2 months
Incidental finding of benign breast lesion at planned mammogram requiring lumpectomy	10^7	30days	5 months
Abdominal pain and loose motions required hospital admission – investigation confirmed constipation	Up to 10^9 dose	24h	6 months

During infusion of macrophages, we did not witness any clinically significant transfusion reactions and all subjects tolerated the infusion very well

Upon assessment of the dose limit toxicity (DLT) we did not record values dropping below the threshold set for Hb drop. However, subject 005 developed clinically relevant microcytic anaemia at the last follow up visit (360 days after infusion of macrophages). When the anaemia was investigated it became apparent this was due

to iron deficiency and endoscopic investigation revealed florid portal hypertensive gastropathy as the cause.

Figure 2.16: Dose Limiting Toxicity



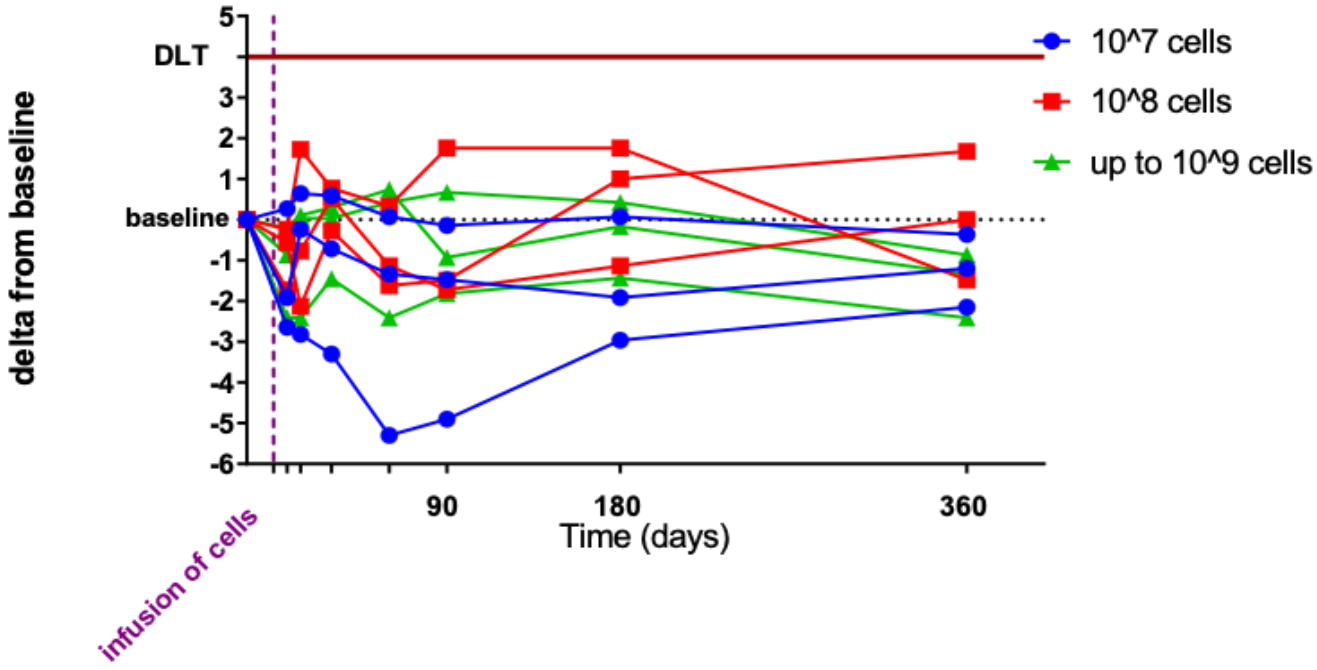
This figure illustrates Dose limiting toxicity, by dose of cells infused, expressed as change from baseline over time. DLT = dose limiting toxicity. A) Fold-change in serum alanine aminotransferase (ALT); DLT defined as >3-fold. B) Fold-change in serum total bilirubin; DLT defined as >3-fold. C) Fold-change in serum creatinine; DLT defined as ≥1.5-fold. D) Fold-change in haemoglobin; DLT defined as >-1.5 fold. One subject in 10^7 cell dose group developed anaemia at 360 day follow-up visit. This was confirmed, after the trial was

completed, to be related to florid portal hypertensive gastropathy. E) Fold-change in platelets; DLT defined as >-2 fold. F) Total white cells count absolute numbers; DLT defined as < 2.0 x10^{9}/µL.

Serum ALT and bilirubin changes at 90 days were respectively 0.88±0.21 and 0.80±0.30 fold from baseline. Fluctuation in platelet count is common in patients with cirrhosis and portal hypertension, but we did not observe a reduction in platelets to lower than 30% from baseline or clinically significant thrombocytopenia. The baseline total white cell count varied in this patient's group. As expected, total circulating leukocyte counts were affected by leukapheresis, but returned to baseline prior to infusion (7 days after leukapheresis). In some subjects we noted a small and transient increase in white cell count following infusion of IMP which did not persist beyond 7 days post-infusion.

As part of our safety profile we also assessed changes in MELD score: as above we described as toxicity a MELD score >4 points from baseline.

Figure 2.17: MELD score



This figure shows the changes in MELD score for each subject by dose in relation to the threshold identified as DLT.

During the trial we also recorded "clinical events", these were defined in the protocol as adverse events related to decompensation of liver disease like hepatic encephalopathy, ascites or variceal bleeding, as well as the need for liver

transplantation. In the whole duration of the trial we recorded a single clinical event: this was minimal ascites noted in USS only and not detectable clinically. It was successfully treated with a very small dose of a potassium sparing diuretic. Resolution was confirmed at subsequent USS.

2.8.2 Efficacy

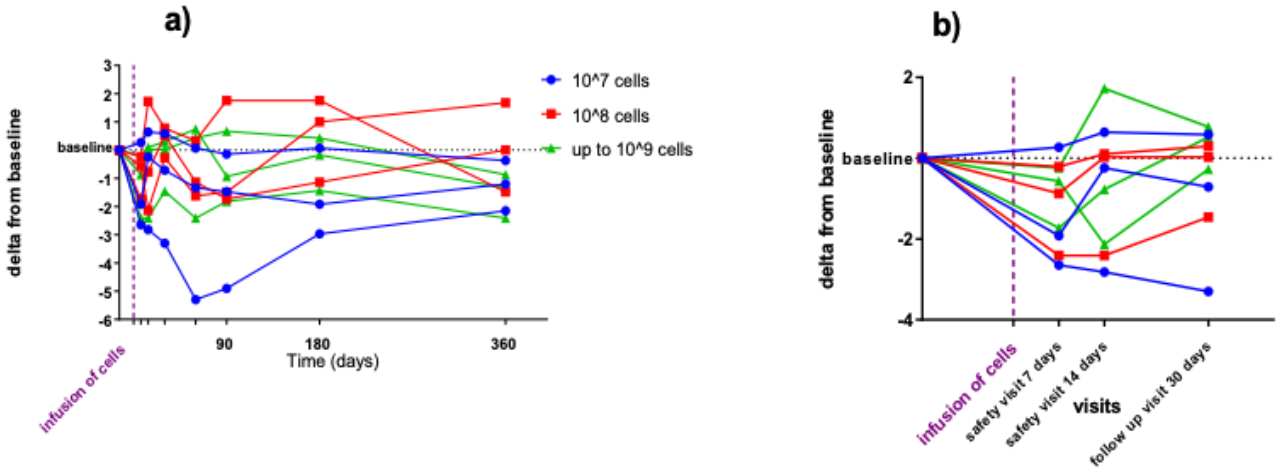
This phase 1 study had no control group and so we could not truly assess efficacy. However, we were able to assess markers of liver function and other markers against baseline.

2.8.2.a MELD, UKELD and Albumin

We assessed the "efficacy" of the macrophage infusion by assessing improvement in liver function. We used MELD score and UKELD score as surrogate measure of liver health and Albumin (g/dl) as a surrogate measure for liver synthetic function.

As illustrated in the graph above showing safety over MELD score changes, we observed an improvement in MELD score from baseline.

Figure 2.18: Changes in MELD score from baseline after IMP infusion



This figure illustrates the change in MELD score from baseline (y axes) over time (x axes). In graph a) we show changes over 1 year period and in graph b) for the first month after cell infusion. Graph a) Individual patient data, classified by cell dose group (n=3 per group), expressed as the delta-MELD from baseline (dotted black line) over

time. Time-points indicate the time of macrophage infusion (black line; approximately 14 days from baseline) and study-specific follow-up visits in the trial. Primary and secondary outcomes were measured at day-90 post-infusion. Graph b) Individual patient data by cell dose expressed over initial safety and follow-up visits up to 30 days after infusion of macrophages. Graph b) indicates MELD changes closer to infusion time-point.

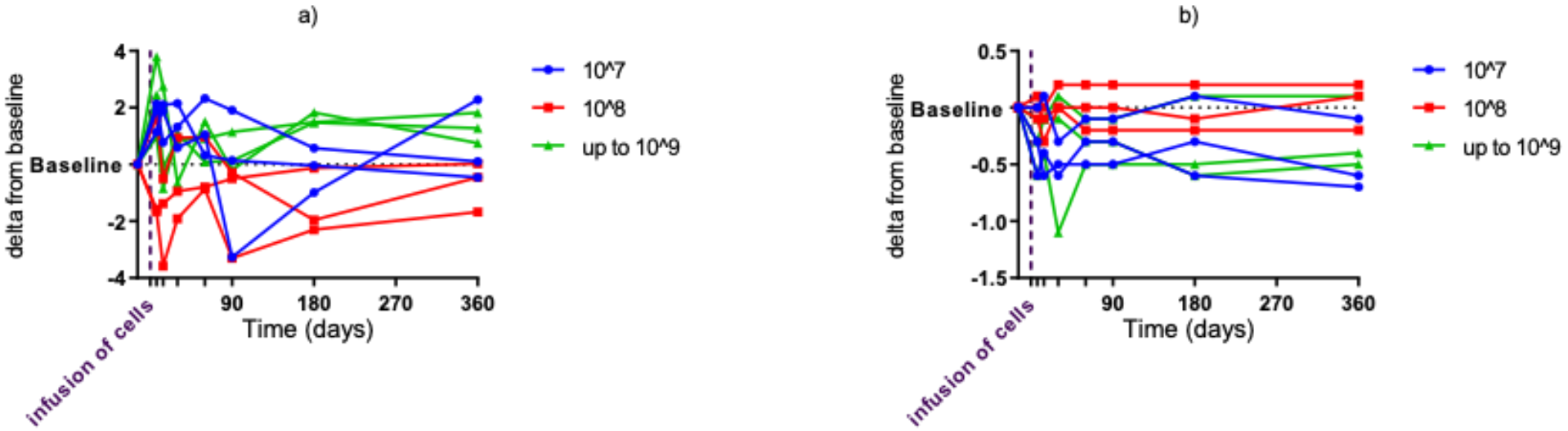
Efficacy was evaluated by delta-MELD at day 90 post-infusion. Six out of 9 subjects showed an improvement in MELD score. The MELD at baseline was 11.87±1.40 (range 9.90 to 13.87) with a mean delta-MELD at 90 days of -1.14±1.83. At 1-year follow-up MELD improved in 7 out of 9 subjects, among these subjects the mean delta-MELD at 1 year was -1.40±0.79. At 360 days 7 subjects improved MELD score with mean improvement among all subjects of -0.91 ± 1.24. Time point of 90 days post infusion was chosen in the study to assess efficacy based on the half-life of the IMP (around 2 weeks according to pre-clinical studies). In our study population the changes in MELD score are primarily due to Bilirubin and INR while Creatinine remained very stable in all subjects through the study as illustrated in the safety figure above.

Table 2.11: MELD variables' contribution to score change at time point expressed as mean.

	Creatinine	Bilirubin	INR
Baseline	0.00	0.00	0.00
Day 7	0.02	-0.79	-0.37
Day 14	0.00	-0.53	-0.13
Day 30	0.00	-0.10	-0.29
Day 60	0.00	-0.84	-0.29
Day 90	0.00	-0.73	-0.38
Day 180	0.00	-0.31	-0.18
Day 360	0.00	-0.34	-0.56

Pre-clinical studies suggested Albumin improves 4 weeks following macrophage injection in CCl4 murine models with cirrhosis (46.0 ±2.6 g/l versus 39.9±0.9 g/l in controls). In this phase 1 study we did not observe the same level of albumin improvement. At 90 days post infusion of macrophages Albumin drops by mean of -2.0 ± 2.29 g/L.

Figure 2.19: UKELD score and Albumin changes from baseline following IMP infusion



This figure demonstrates changes in liver function, by dose of infused cells, expressed as changes from baseline (y axes) over time (x axes). A) Changes in United Kingdom End-Stage Liver Disease (UKELD) score from baseline (arbitrary units). B) Changes in serum albumin (g/dL) from baseline. At 90 days after infusion of macrophages the mean delta UKELD score was -0.47 ± 1.77.

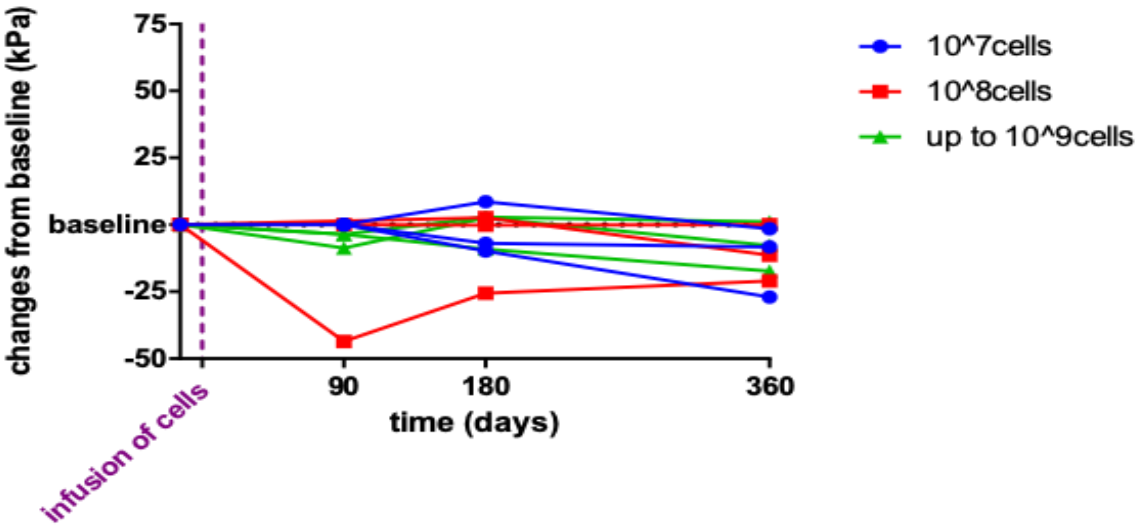
2.8.2.b Non-invasive markers of fibrosis

Fibrosis markers

Macrophages have been proven in murine models of liver fibrosis to promote hepatic scar's resolution by upregulating MMP and leading to extracellular matrix degradation. We therefore hypothesized that fibrosis may reduce following infusion of the IMP in our trial. To assess this we used non-invasive assessments for fibrosis instead of biopsy. Whilst biopsy would potentially have been informative, it has risks in cirrhotic patients, a repeat biopsy would have been required and there is an issue of sampling error where a biopsy may be unrepresentative of the overall liver fibrosis. Based upon these considerations non-invasive measures of liver fibrosis were preferred.

One-dimensional transient elastography was performed in fasted subjects using FibroScan® (Echosens, Paris, France) using either an M or XL probe to obtain ten valid readings, with a success rate of at least 60% and IQR <30% of the median result. Three results did not meet the manufacturer's recommended validity criteria and were therefore removed (baseline measure for sub 004 and sub 005 and 90 days measure for sub 008), giving a technical success rate of 91.66% in the study. Baseline liver stiffness measurements (LSM) were consistent with cirrhosis (mean 57.44±24.01 kPa). In 5/9 patients LSM decreased by >6 kPa at 1-year of follow-up (with a mean reduction of -10.36±9.79 kPa).

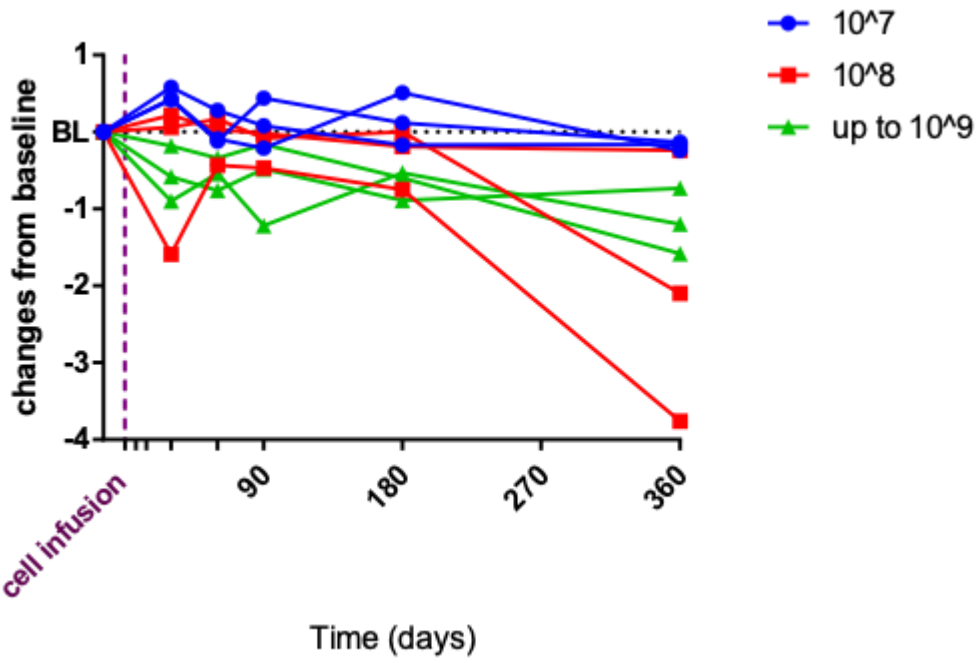
Figure 2.20: Liver Stiffness changes from baseline following IMP infusion



This figure shows the transient elastography (Fibroscan®) results (kPa), by dose of infused cells, expressed as changes from baseline over time.

We also used serum based assessments for fibrosis. The ELF panel highlighted reduction of fibrosis markers following injection of the IMP.

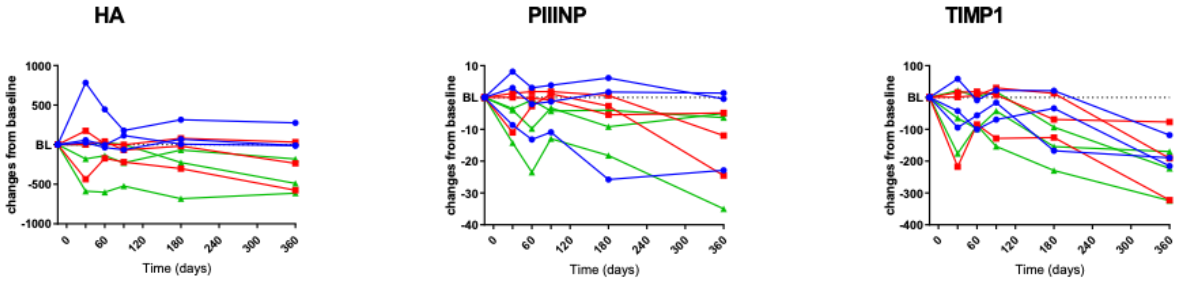
Figure 2.21: ELF changes from baseline following IMP infusion



This figure illustrates the changes of ELF score from baseline (y axes) over time (x axes) following autologous macrophages infusion.

The mean ELF score at baseline was 12.43±0.94 with mean delta-ELF at 90 days of -0.24±0.46 and at 1 year of -1.13±1.21. Reduction of ELF appears to be dose dependant.

Figure 2.22: Changes of ELF components from baseline following IMP infusion

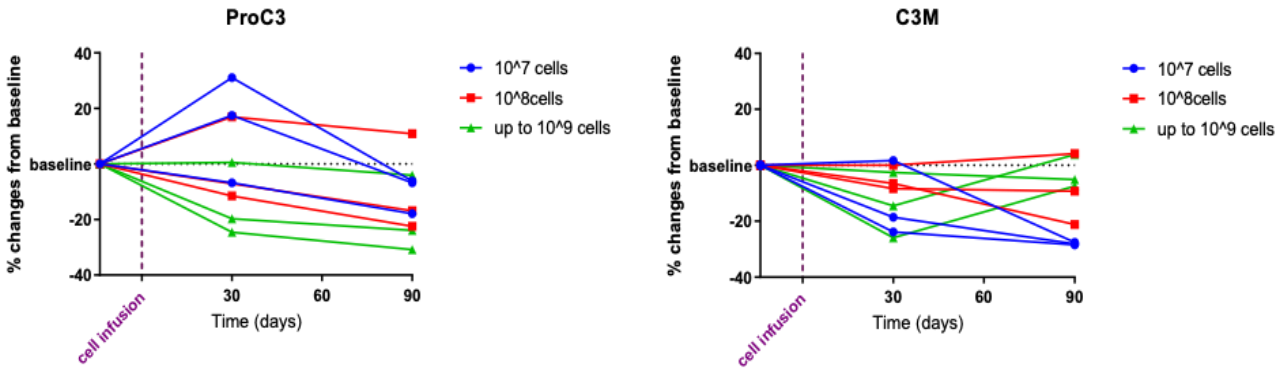


This figure shows the single components of ELF (HA, PIIINP, TIMP1) changes from baseline (y axes) over time (x axes) following autologous macrophages infusion.

At 90 days after infusion of macrophages mean HA, PIINP and TIMP1 are respectively -89.27±210.57, -2.94±5.67, -36.77±67.58 while at 1 year they are -200.80±305.84, -12.17±12.495 and -203.28±82.09. At 1 year time point TIMP-1 shows a mean reduction of over 44% while PIIINP reduces by 33% and HA by 22%.

The anti-fibrotic role of the macrophages, in this uncontrolled study, is potentially suggested by the results we observed in two biomarkers of collagen-III turnover ProC3 and C3M. The mean % changes of PRO-C3 and C3M at 90 days were of -13.09±12.77 and -10.95±13.37 ng/mL at 90 days after infusion of macrophages.

Figure 2.23: Fibrosis Markers ProC3 and C3M changes from baseline following IMP infusion



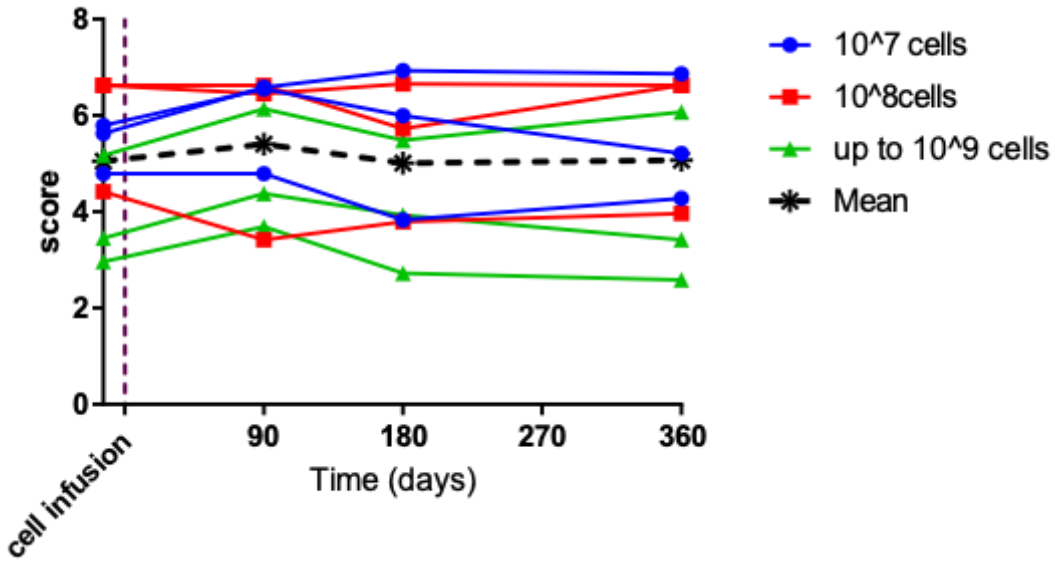
This figure shows fibrosis serum markers ProC3 and C3M changes from baseline (y axes) over time (x axes) following autologous macrophages infusion.

Quality of life assessment

We assessed HRQL with the validated self-assessment CLDQ at baseline before infusion of the cells and at 3 other time points: 90 days, 180 days and 360 days after infusion.

The baseline scores among the participants varied from a minimum of 2.97 to a maximum 6.67 (where the worse possible score is 1 and the best possible score is 7) with mean of 5.05±1.28.

. *Figure 2.24: quality of life score during trial period*

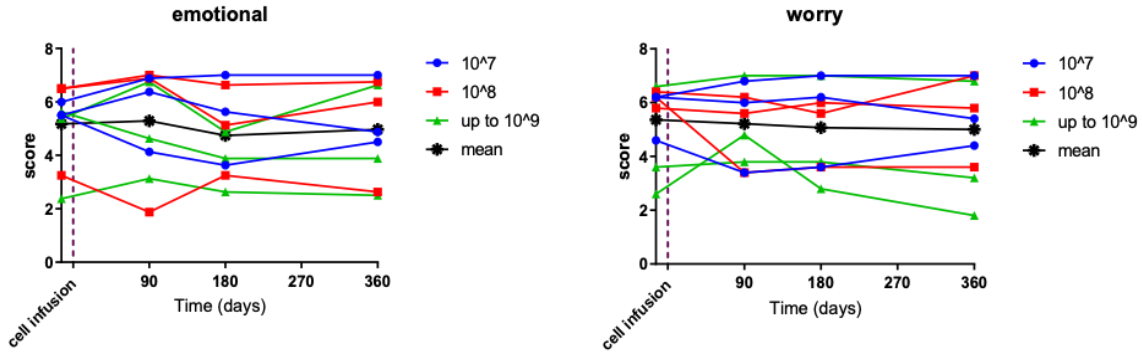


This figure illustrates the quality of life score measures using the CLDQ questionnaire (y axes) over time following autologous macrophages infusion (y axes). Because of the significant spread of scores among the participants we show the score at each time point for each subject classified as per dose of cell infused (coloured lines) and the mean (black dotted line)

The mean score at 90 days post infusion of macrophages improved of 0.35 points with only 2 subjects worsening their baseline score following infusion of cells and one subjects with unchanged HRQL score.

When we deal with chronic diseases, it is arguable that psychological factors have as much impact on the quality of life as the chronic physical symptoms.

Figure 2.225: Quality of life score in "emotional" and "worry" domains



This figure shows the scores in the emotional and worry domains of the CLDQ at time points for each subject classified as per dose of cell infused (coloured lines) and the mean (black dotted line).

The mean change from baseline in the “emotional” domain at 90 day following infusion of macrophages showed an improvement of 0.11 points while for the “worry” domain the score worsened of 0.13 points.

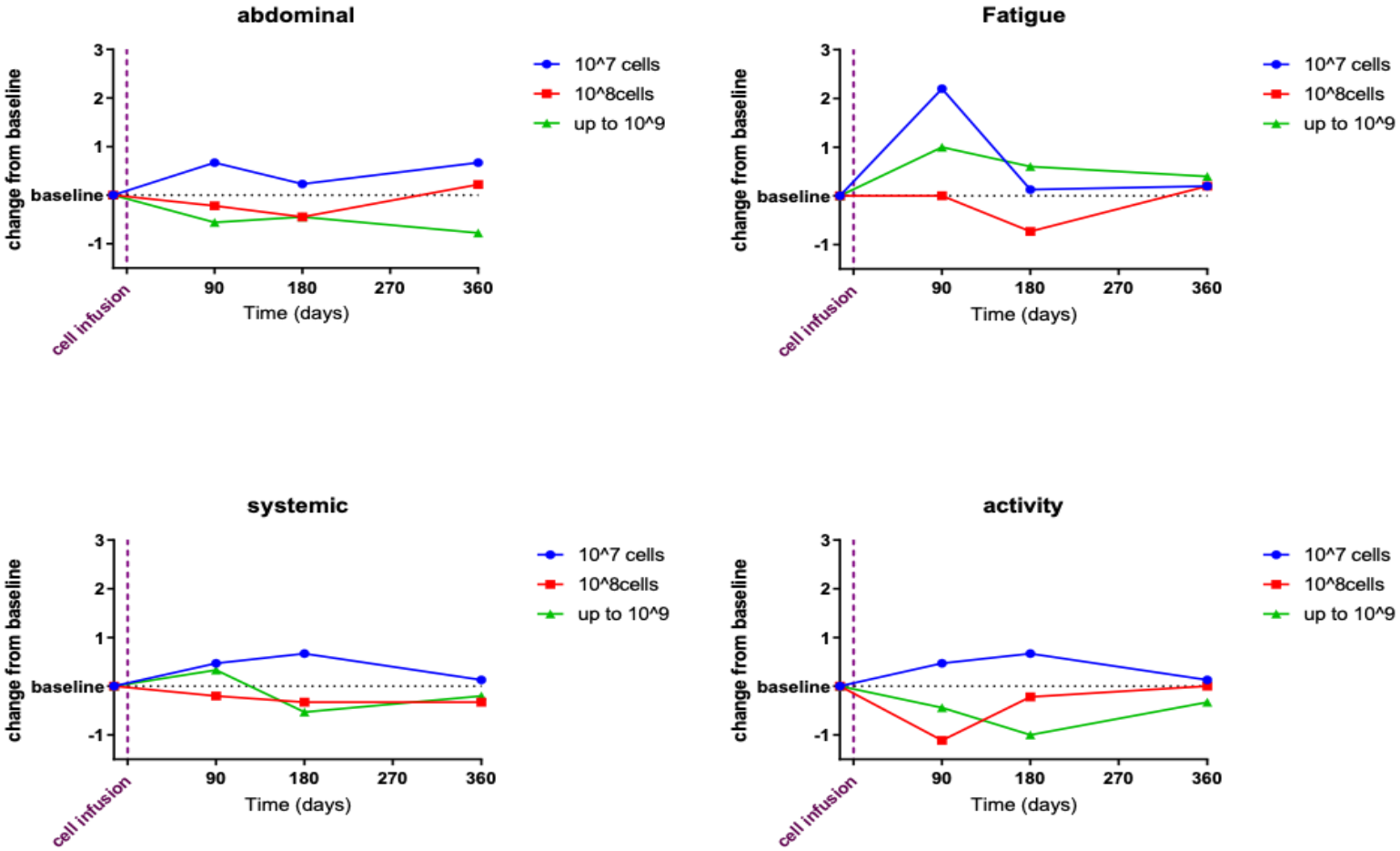
Table 2.12: CLDQ score changes by single domain per dose group

Domains	10^7 cells				10^8 cells				Up to10^9 cells			
	Baseline	*90 days*	*180 days*	*360 days*	*Baseline*	*90 days*	*180 days*	*360 days*	*Baseline*	*90 days*	*180 days*	*360 days*
Abdominal pain	5.33±0.00	6.00±1.20	5.56±1.02	6.00±1.00	6.56±0.77	6.33±0.67	6.11±0.84	6.78±0.39	5.11±0.51	4.55±0.39	4.67±1.20	4.33±0.67
Fatigue	5.20±0.87	7.40±1.22	5.33±2.08	5.40±1.71	5.20±2.78	5.20±2.43	4.47±2.40	5.40±1.91	2.47±0.61	3.47±0.70	3.07±0.31	2.87±0.99
Systemic symptoms	5.00±0.92	5.47±1.33	5.67±1.33	5.13±0.95	6.27±0.76	6.07±1.10	5.93±1.17	5.93±1.36	4.47±1.36	4.80±1.97	3.93±1.81	4.27±2.04

Activity	5.44±0.51	5.89±1.65	6.22±1.35	5.56±1.50	6.78±0.39	5.67±1.15	6.56±0.77	6.78±0.39	4.89±2.22	4.44±1.39	3.89±2.17	4.55±2.12
Emotional function	5.67±0.29	5.80±1.46	5.42±1.69	5.46±1.35	5.42±1.88	5.25±2.92	5.00±1.69	5.13±2.19	4.46±1.81	4.84±1.82	3.80±1.13	4.34±2.10
Worry	5.67±0.92	5.40±1.78	5.60±1.78	5.60±1.31	6.13±0.31	5.07±1.47	5.07±1.29	5.47±1.72	4.27±2.08	5.20±1.64	4.53±2.19	3.93±2.58

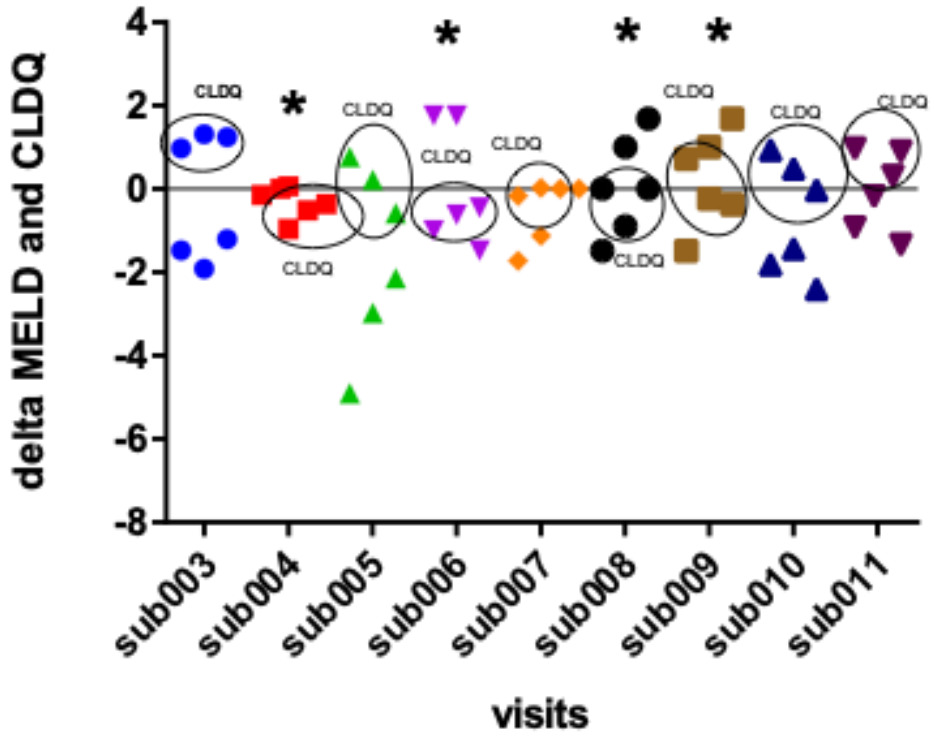
This table summarises the scores expressed as mean ± standard deviation classified by dose of cell infused for all domains of the CLDQ score at different time points.

Figure 2.26: Changes in CLDQ score domains as delta mean by dose group



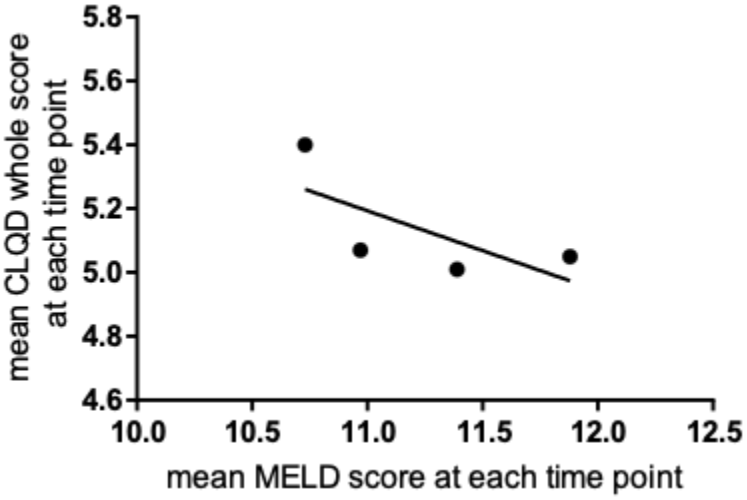
This figure shows the mean delta changes for dose group. There does not appear to be a dose effect on the improvement of the CLDQ scores. It is interesting to note that the 10^8 dose group which has the highest baseline scores in all domains but "emotional" is also the group that fails to improve the scores following the infusion of the IMP. Anecdotally the majority of patients reported increase level of energy in the week following the infusion of macrophages and it is reflected in the improvement of fatigue with very significant mean improvement in the both the lowest and highest dose group with respectively 2.20 and 1 point increase.

Figure 2.27: Correlation between delta MEDL and delta CLDQ



*This figure illustrates changes in MELD (coloured shapes) and changes in CLDQ (circled coloured shapes) (y axes) for each subject (x axes). For each subjects we represent 3 MELD delta scores and 3 CLDQ delta scores (as these were recorded at 80, 180 and 360 days post infusion). Subjects who fail to improve MELD also fail to improve CLQD (highlighted in the figure by *).*

Figure 2.28: Correlation between mean CLDQ



This figure shows the correlation between mean CLDQ (y axes) and mean MELD (x axes) scores at each time points during the trial. Because a negative delta represents improvement in MELD while the same is true for positive delta for CLQD the 2 scores are inversely correlated.

2.9 Randomised Controlled Trial (Phase 2)

Following approval from both the DMC and the Phase 1 committee upon review of the safety data report, which included all AEs and safety variables as illustrated above, we were allowed to proceed to the phase 2 part of the study, which is currently ongoing.

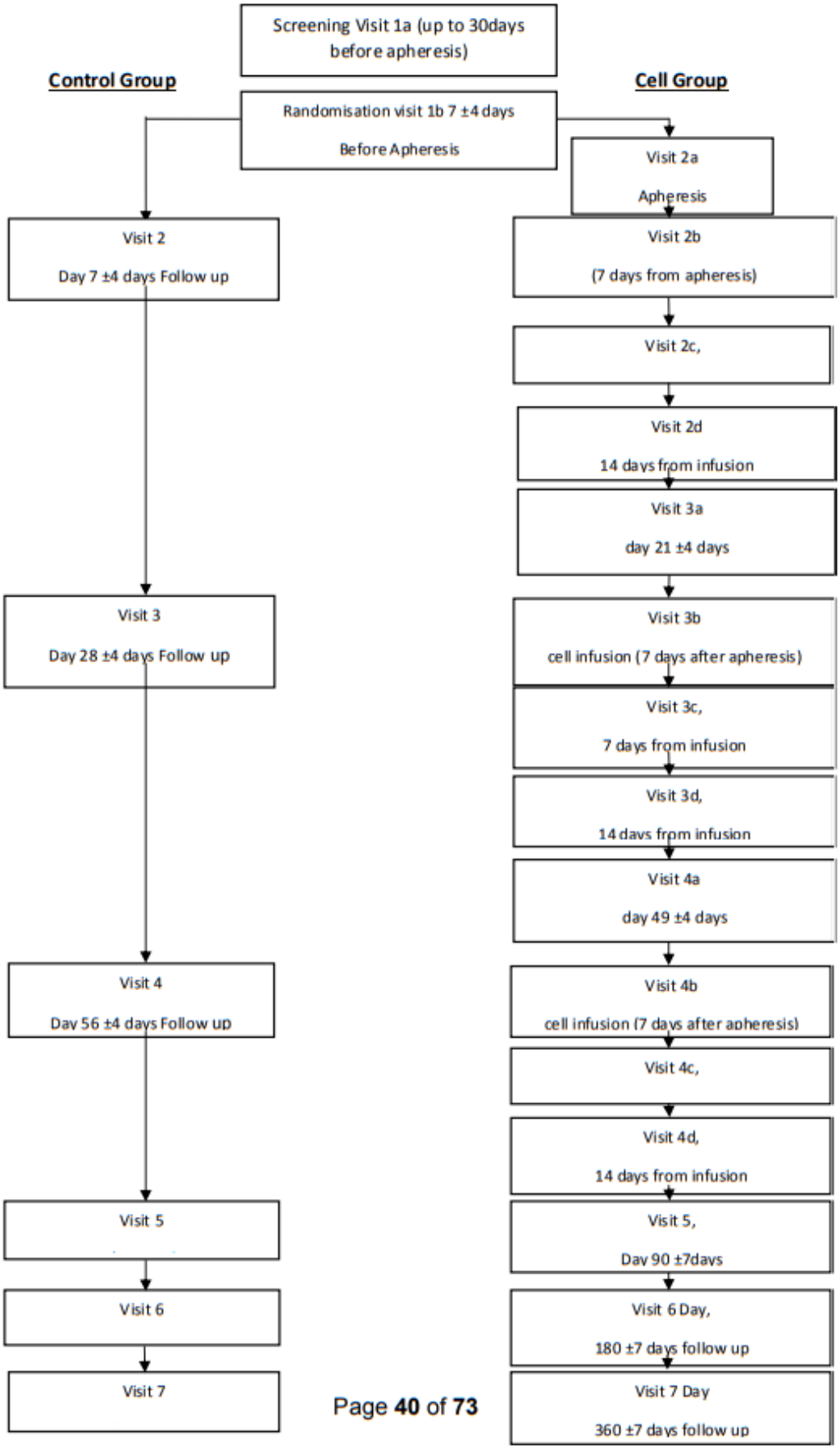
However both the DMC and phase 1 committee requested additional safety data because the maximum dose was not met in the top dose group. Here the production team were not able to reach the highest set dose of $1x10^9$ cells. Therefore, a true maximum tolerated dose was not established. It was agreed the dose infused to be communicated in real time to the DMC at time of infusion and a safety report to be filed for review.

2.9.1 Phase 2 trial design

The Randomised controlled trial (RCT) is designed to demonstrate efficacy. The study is powered using preclinical data. In view of the short life of the macrophage product

(around 2 weeks) it is postulated that repetitive doses may be of benefit to ensure maximum effect of the IMP. The RCT resembles the phase 1 design besides in the treatment arm the subject receives 3 infusions of IMP following the safety visit. In the control arm the subject receives standard of care only with visit schedule parallel to the treatment arm. A placebo arm is not feasible in this study as subjects should undergo leukopheresis and the leukapheresis product should be disposed of. This is not considered ethical. Standard of in this case is considered direct contact with physician, blood and USS HCC surveillance. It also includes adjustment of therapy and consideration for transplant if the subject should be considered to benefit from it. Standard of care interventions are offered to the subjects both arms.

Figure 2.29: visit schedule for RCT


Screening Visit 1a (up to 30days before apheresis)
Control Group
Randomisation visit 1b 7 ±4 days Before Apheresis
Cell Group
Visit 2a Apheresis
Visit 2 Day 7 ±4 days Follow up
Visit 2b (7 days from apheresis)
Visit 2c,
Visit 2d 14 days from infusion
Visit 3a day 21 ±4 days
Visit 3 Day 28 ±4 days Follow up
Visit 3b cell infusion (7 days after apheresis)
Visit 3c, 7 days from infusion
Visit 3d, 14 days from infusion
Visit 4a day 49 ±4 days
Visit 4 Day 56 ±4 days Follow up
Visit 4b cell infusion (7 days after apheresis)
Visit 4c,
Visit 4d, 14 days from infusion
Visit 5
Visit 5, Day 90 ±7days
Visit 6
Visit 6 Day, 180 ±7 days follow up
Visit 7
Visit 7 Day 360 ±7 days follow up

While the trial is still recruiting in the following paragraph I will discuss exclusively the safety results for the first three subjects randomised to cell arm. These are the only data analysed for the RCT.

2.9.2 Safety results

Table 2.13: RCT first 3 subjects demographics

Subj	Age	Gender	Aetiology of liver disease	Macrophages infusions					
				Dose	Date	Dose	Date	Dose	Date
012	51	M	ARLD	0.7x10^9	03/10/17	0.8x10^9	31/10/17	0.8x10^9	28/11/17
015	67	F	NAFLD	0.4x10^9	18/01/18	0.5x10^9	15/02/18	0.4x10^9	15/03/18
021	56	M	ARLD	0.7x10^9	09/07/18	0.8x10^9	06/08/18	0.9x10^9	03/09/18

2.9.2.a Adverse events

We counted overall 43 adverse events in the first 3 infused patients in the RCT, respectively 14 in sub 012, 22 in subject 015 and 7 in subject 021.

Table 2.14: Adverse Events among first 3 subjects of RCT

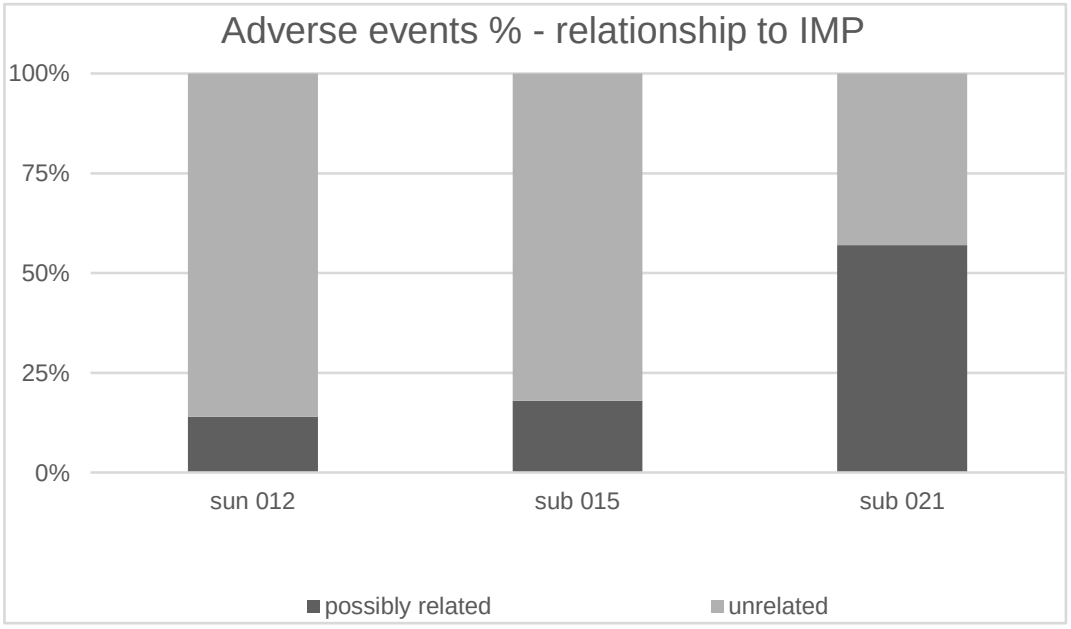
	Subj	Type of AE	Infusion date	start	IMP related	NCICTC grade	duration	resolved
1	012	Hypocalcaemia	03/10/17	26/09/17	No	2	<1day	yes
2		fatigue		04/10/17	No	1	1 day	yes
3		Fatigue		11/10/17	Possibly	1	10days	yes
4		Hypocalcaemia	31/10/18	24/10/17	No	2	<1 day	yes
5		Constipation		25/10/17	No	2	1 day	yes
6		Fatigue	28/11/17	22/11/17	No	1	1 day	yes
7		Fatigue		29/11/17	Possibly	1	4 days	yes
8		Sore throat		06/12/17	No	1	3 days	Yes
9		Sinusitis		20/12/17	No	1	7 days	yes
10		R sided rib pain		28/01/18	No	1	>6months	no
11		Light-headedness		25/07/18	No	1	5 days	yes
12		Worsened back pain		May 2018	No	1	3 months	no
13		Hypertension		03/03/18	No	2	5 months	yes

14		R leg paraesthesia		20/08/18	No	1	1month	no
15	15	Hypocalcaemia	18/01/18	11/01/18	No	2	<1day	yes
16		Poor sleep at night		19/01/18	No	1	5 days	yes
17		Bruising L antecubital fossa		18/01/18	No	1	6 days	yes
18		Worsened lower back pain		30/01/18	No	2	2 days	yes
19		In-growing toe nail		29/01/18	No	2	10 days	yes
20		Abdominal pain		31/01/18	No	2	1 day	yes
21		Crampy abdominal pain		01/02/18	No	2	6 days	yes
22		Fatigue	15/02/18	08/02/18	No	2	2 days	yes
23		Hypocalcaemia	15/03/18	08/03/18	No	2	<1day	yes
24		Fatigue		09/03/18	No	1	4 days	no
25		Dark, soft bowel motion		09/03/18	No	1	<1 day	Yes
26		Headache		20/03/18	Possibly	1	<1 day	Yes

27		Dark bowel motion		21/03/18	No	1	<1 day	Yes
28		Urine frequency		15/03/18	Possibly	1	1 day	Yes
29		Headache		01/04/18	Possibly	1	9 days	Yes
30		Fatigue		15/04/18	Possibly	1	2 days	Yes
31		Nose bleed		13/06/18	No	2	<1 day	Yes
32		Palpitations		11/06/18	No	2	2 days	Yes
33		Palpitations		25/06/18	No	2	<1 day	Yes
34		Light-headedness and nausea		10/10/18	No	1	<1 day	Yes
35		Unexplained vocalization		Aug 2018	No	1	4 months	No (improved)
36		Cramps of hands		05/10/18	No	1	<1 day	yes
37	021	R eye stye	09/07/18	11/07/18	Possibly	1	27days	Yes
38		Puffy eyes		11/07/18	Possibly	1	1 day	Yes
39		R ankle swelling		12/07/18	Possibly	1	1day	Yes
40		Swollen feet	06/08/18	12/08/18	Possibly	1	1 day	Yes

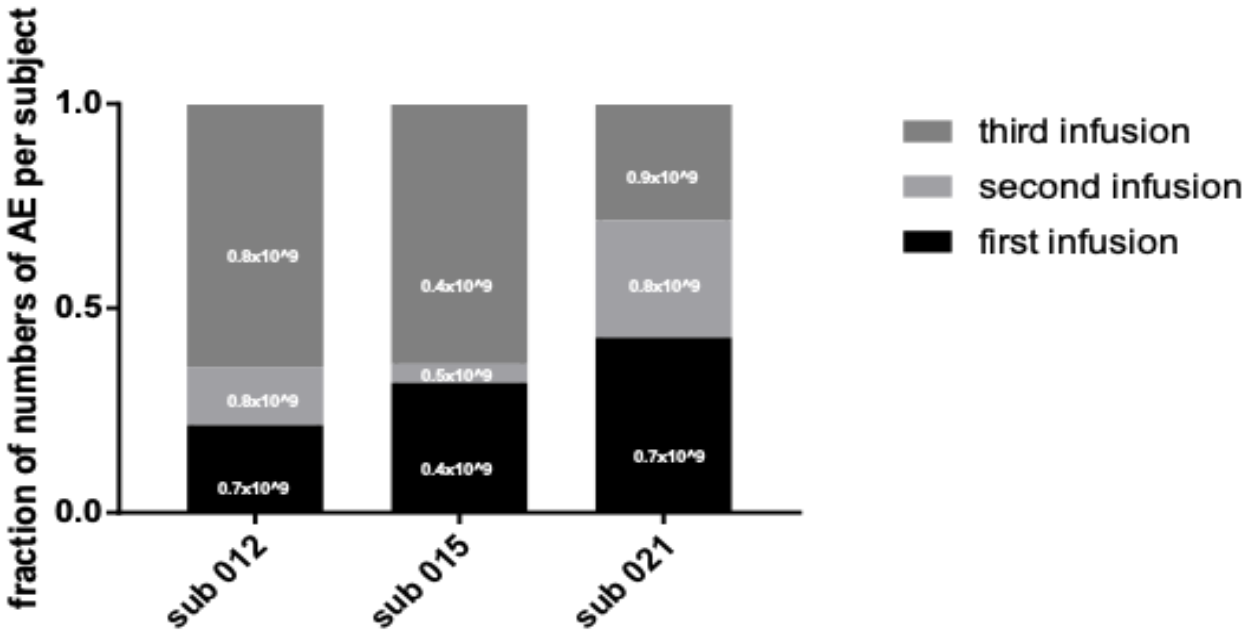
41		Abdominal bloating and loose stools		26/08/18	No	1	2 days	Yes
42		L antecubital fossa haematoma	03/09/18	05/09/18	No	1	10 days	Yes
43		Reduction in WCC		03/09/18	Possibly	1	6 days	Yes

Figure 2.30: Adverse Events related and non-related to IMP



This figure shows the percentage of AE related (black) and unrelated (grey) to IMP (y axes) per each subject (x axes)

Figure 2.31: AE in first 3 subjects of RCT distributed by infusion



This figure illustrates the percentage of AEs (y axes) by subjects (x axes) per infusion (colour block). Although there is a tendency in the first 2 subjects at increasing number of AE in the last infusion, this is not associated with higher dose of cells.

The three infusions were well tolerated however subjects reported to be onerous: both because of the commitment to the trial schedule and because physically demanding.

In a single occasion: 3rd infusion of 3rd patient the WCC count dropped below 2.0 10^9, set as toxicity target. The subject felt very well and was discharged home as per protocol after 4h observation period following infusion of macrophages and before the blood results were available. Once results were reviewed the patient was contacted and offered the possibility to attend the clinical research facility to carry out repeated blood test and physical examination. The participant declined the offer as feeling very well. Upon repeated blood tests at 7 days as per trial schedule the differential and total leukocyte count completely restored without consequences for the patient. We didn't record any other DLT in the safety period up to 14 days after infusion of IMP. The table below illustrate all safety results for the 3 subjects who underwent 3 infusion.

Table 2.15: Safety parameters by subject for each infusion

	Sub 012			Sub 015			Sub 021			MAS
Infusion day										
infusion	1	2	3	1	2	3	1	2	3	
T°C	No	No	No	No	No	No	No	No	No	>38
Hb	154	149	156	138	138	137	120	119	118	<100
WCC	6.8	4.2	4.2	7.7	2.6	2.8	5.7	2.3	1.8	<1.0
PLT	92	90	91	53	40	43	28	26	19	<100
Trigly	0.8	0.9	0.8	1	1.2	1.5	1.2	2.7	2.2	>265
Fibrin	3	2.9	3.3	1.7	1.8	1.8	1.4	1.3	1.2	<1.5
Ferritin	244	230	224	168	138	144	637	751	856	>500
Day 7 after infusion - safety visit										
T°C	No	No	No	No	No	No	No	No	No	>38
Hb	154	151	156	140	140	138	122	119	125	<10
WCC	3.5	4	4	3	3.3	3.1	3.6	3.5	4	<1.0
PLT	91	100	92	43	56	47	30	25	23	<100
Trigly	0.6	0.6	0.8	1.6	1.3	1.2	2.2	1.9	2.4	>265
Fibrin	3	3.1	3.4	1.4	1.7	1.8	1.4	1.3	1.2	<1.5
Ferritin	253	241	228	157	135	104	597	729	832	>500

2.10 Discussion

The data illustrated in the section above confirm the safety and feasibility of infusion of autologous macrophages in a population of compensated cirrhotics of different aetiology. As previously demonstrated by our group, leukapheresis is well tolerated by cirrhotic patients and in this trial reiterated these findings. All subjects reported minimal side effects even in the case of multiple infusions. Peripheral administration of macrophages is safe, with no clinically significant adverse reactions recorded during the infusion or in the immediate post-infusion period. This is also true for multiple infusion however, the data analysed is limited to 14 days after infusion.

The 3+3 trial dose-escalation model is designed to define a maximum-tolerated dose. Due to monocyte isolation and production limitations, we were only able to generate a dose of up to 10^9 cells (specifically 0.8×10^9 cells) in the phase 1 study- the "maximum-achieved dose". During the trial period we continued to improve the manufacturing process this included reducing the feeding and washing from 2 during the maturation week to 1. This improved cell yield by limiting cell handling thus cell loss. It is also true that as this is an autologous cellular product, the dose infused is also dependent by the subject's characteristics such as number of circulating monocytes and leukapheresis success (which can be affected for example by vein capacity or other individual factors). Because we did not reach the predicted maximum dose of 1×10^9 cells in any subjects during the phase 1 trial, we collected further safety data for the first 3 subjects in the RCT. In the third subject we reached a dose of 0.9×10^9 cells. It is possible that with continue development in the production and subject characteristics allowing, we will reach a dose of 10^9 macrophages in the RCT.

Subjects in this study have advanced cirrhosis and significant co-morbidities, therefore we were not surprised to observe a large number of AEs throughout the study. However, most of the AEs recorded were exacerbations of existing conditions or minor self-limiting events. The 3 SAEs were considered mild and unrelated to the IMP. Among AEs possibly related to the IMP, none had CTCAE severity grading over 2. We did not highlight any dose-related phenomena. We had no withdrawals from the trial and all patients reached 360 days of follow-up. One subject developed ascites while in the trial: this was identified on ultrasound and resolved with diuretics. This was the

only clinical event recorded in the trial period. All other patients remained well compensated.

We did not label the infused macrophages during the trial period but I would postulate that the majority of macrophages homed the liver. This is based upon pre-clinical data and case reports that suggest that macrophages infused peripherally will transiently pass through the lungs, before engrafting within the liver and spleen. (18-20) Respiratory rate or oxygen saturation did not change to a clinical significant level at any point during infusion or 12-hour follow-up period. Overall peripherally infused macrophages appeared safe during administration and the extended follow-up period of 360 days.

Although phase 1 studies are neither powered nor designed to demonstrate efficacy, the studies may be scrutinised for signals to guide further phase 2 studies. Thus from our data, 6 of the 9 participants had an improvement in MELD score at 90 days, mainly driven by the reduction of serum bilirubin. This is in contrasts with a recent published RCT of autologous CD133-positive stem cells in cirrhotic patients of comparable severity to this study. This study showed no improvement in MELD score.(21) In one subject in the phase1 MATCH study, MELD raised at 360 day follow up following infusion, this was due to elevated total bilirubin. However when explored this further it was demonstrated that the 85% of the total bilirubin was actually unconjugated, representing haemolysis. This subject had cirrhosis secondary to treated HCV. It is possible the haemolysis was due to cold agglutinins.

We used different non-invasive assessments to evaluate liver fibrosis, the majority of these improved following macrophage infusion, including transient elastography, ELF score and the collagen turnover markers PRO-C3 and C3M. Whilst uncontrolled, these results can tentatively support the anti-fibrotic effect of autologous monocyte-derived macrophage infusion demonstrated in pre-clinical studies and support the further testing in phase 2 trials.

For example, the reduction of TIMP-1 is an interesting finding in view of previously published preclinical data from our group. We demonstrate that MMP-12 protein can control degradation of elastin but in progressive liver fibrosis is mostly bound to TIMP-1. MMP-12 in liver fibrosis is most exclusively produced by macrophages.(22) This

potentially confirms the crucial role of macrophages in the “fibrosis production versus fibrosis degradation” balance in the injured liver. Furthermore, the largest percentage decrease in PRO-C3 may be consistent with a predominant decrease in fibroblast activity following infusion of macrophages. Despite the very low numbers in each dose group it appears that the PROC3 decrease may be dose dependant. This is not reproduced in the C3M results, where a more consistent percentage reduction (even if lower compared to PROC3) seems to be represented in all subjects independent of the dose group. This would suggest an increased macrophage and MMP activity, which would confirm the TIMP-1 results described above.

HRQL is often impaired in patients with advanced cirrhosis, like in the majority of chronic diseases, and data in literature show that HRQL scores improve following liver transplantation.(23) A change of 0.5 on the 1 to 7 scale represents an important difference in CLDQ score. In our population 5 of 9 patients exhibited improvement in overall HRQL score at day 90 post-infusion. For the other patients, the composite CLDQ scores were unchanged (n=2) or worse (n=2) at 90 days. We noted an inverse association between delta-MELD and CLDQ scores. More importantly, subjects that failed to improve MELD also showed no amelioration of the HRQL score.

In the three subjects who received multiple infusions we noted a decrease in whole leukocytes numbers immediately after infusion. This was driven by a transient reduction in all forms of white cells. This effect of the infusion was only appreciated after the second and third infusions. During the dose escalation study and for the first infusion of the first 3 subjects of the RCT, all subjects spent the night under observation in the clinical research facility. Therefore safety bloods were obtained in the morning of the following day, 16h post infusion. While for the 2nd and 3rd infusion in the RCT, bloods were obtained at discharge time (4h post infusion). Because of the short time frame between infusion and blood check, it is possible that leukocytes are sequestrated in the organs as a consequence of the macrophage infusion. Subject 012 attended the hospital the morning after the 2nd infusion and at that time point (18h after infusion) the number of white cells and the differential count was restored (as observed in the phase 1 subjects). We do believe this is therefore a short lasting and self-limiting event that has at to date caused no clinical impact on the subjects.

In conclusion, this first-in-human study confirmed safety, feasibility and established the maximum-achievable dose of autologous macrophages in patients with advanced cirrhosis. The efficacy results are intriguing but should be treated with caution in a small phase 1 study with no control group. The phase 2 study currently underway will provide more solid efficacy data.

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Chapter 3. Peripheral cytokine and chemokine analysis following infusion of macrophages in cirrhotic patients with cirrhosis.

Table of Content

Figures and Tables

3.1 Introduction

Cytokines are low molecular weight proteins secreted by cells of the immune system and are key factors in the innate immunity, facilitating communication between cells. Cytokines can initiate, mediate, regulate and amplify processes around systemic acute phase responses, local inflammation and tissues repair. Cytokines bind to specific cells' receptors: this usually initiates cell signalling regulating specific cell's functions, for example the secretion of other cytokines or the upregulation of surface receptors for other signals or even the suppression of the cytokine own effect. Cells produce different cytokines in response to the environment and different stimuli. Generally speaking cytokines act in an autocrine or paracrine fashion thus their action is respectively on the cells that secretes them or in their vicinity. Different cytokines can have the same functions and a cytokine can act on different cell types.

Chemokines are small molecules (typically 8-10kDa) with the function to promote the collection of immune cells in areas of infection. They can be divided in 4 subgroups: CAC, CC, CXC and C based on the cysteine residue at the N-terminal of the molecule. They are secreted in response to a signal (usually pro-inflammatory cytokines). They bind G proteins coupled receptors. They can be classified as inflammatory (with the function of recruiting leukocytes to the site of inflammation) or homeostatic.

Macrophages, as discussed extensively in previous chapters, are cells of immune system and secrete an array of different cytokines. These can broadly be separated in inflammatory (secreted by the classically activated macrophages) and anti-inflammatory cytokines (secreted by alternative active or "repair" macrophages). Pro-

inflammatory cytokines are tumour necrosis factor (TNF), IL-1, IL-6, IL8 and IL-12 and although they are primarily produced by classically activated macrophages and monocytes, they can be secreted by lymphocytes and endothelial cells. While inflammatory cytokines are necessary for the physiological immune response, if produced in excessive amounts, they can precipitate pathology, systemic inflammatory response and even multi-organ failure. Anti-inflammatory cytokines, on the other hand, are expressed by alternatively activated macrophages and are typically: IL-10 and TGF-β.

Although it is helpful to differentiate macrophages in the two aforementioned subcategories, macrophages show plasticity and they can switch phenotype according to the environment (including cytokines) that they are exposed too.

Thus in this chapter we analyse the changes in circulating cytokines following peripheral injection of high dose of alternatively activated macrophages at different time points. This analysis adds to the safety profile of the autologous macrophage product of the MATCH trial.

3.2 Method used for Cytokine analysis

We analysed serum cytokines using a V-PLEX Human Biomarker 54-Plex kit on a MESO Quickplex SQ 120 according to the manufacturers' instructions (Meso Scale Discovery) I am grateful to Bend Dwyer (Forbes group) for running the cytokine plates. The V-PLEX assay has been validated by the manufacturer to detect both physiological and clinically relevant pathological level of cytokines. The assay also provides a lower limit of detection determined by the manufacturer, this corresponds to a signal 2.5 standard deviation above the background level. Moreover each cytokine assay shows the functional limits of quantification (LOQ): the range of the assay, for which the results is reliable, lies between the lower LOQ and the upper LOQ. These are illustrated as standard curve for each assay in the results section of this chapter.

Serum blood was collected from each subject at baseline (at screening) and from safety visit 2b (7 days after infusion) during every follow up visit. Serum was separated and frozen in 2ml aliquots at -80°C. All samples were batch tested on the same day at the end of the follow up period.

V-PLEX kit uses multi-array assay technique with 7 0r 10 spot per well.

Figure 32: multi array assay technique with 10 spots per well.

This figure show a 10spot well kit used in this trial for analysis

3.3 Cytokine and Chemokines description

The V-PLEX Human Biomarker 54-Plex kit analyses a series of human chemokines and cytokines implicated in inflammation, cancer and infections.

Chemokine panel 1 includes: Eotaxin, Eotaxin-3, IL-8, IL-8 (HA), IP-10, MCP-1, MCP-4, MDC, MIP-1α, MIP-1β, TARC. In this panel we analyse both pro-inflammatory (inducing immune cell migration to the infection) and homeostatic chemokines (maintaining and developing tissues).

Pro-inflammatory panel 1 includes: IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, TNF-α.

Cytokine panel 1 includes: GM-CSF, IL-1α, IL-5, IL-7, IL-12/IL-23p40, IL-15, IL-16, IL-17A, TNF-β, VEGF-A.

These panels focus on cytokines fundamental in the inflammatory response and immune-regulation.

Cytokines panel 2 counts IL-1RA, IL-3, IL-9, IL-17A/F, IL-17B, IL-17C, IL-17D, TSLP. This panel shows cytokines relevant in autoimmune and inflammatory conditions.

Th17 panel is constituted by 7 cytokine and chemokines employed in the bridge between innate and adaptive immunity. It includes: IL-17A, IL-21, IL-22, IL-23, IL-27, IL-31, MIP-3α.

Angiogenesis panel 1 evaluate the process of neo-angiogenesis both in physiological or pathological conditions. Angiogenesis is modulated by inflammatory signals and

FGF (basic), PlGF, Tie-2, VEGF-A, VEGF-C, VEGF-D, and VEGFR-1/Flt-1 are molecules in the panel that regulate initiation or inhibition of angiogenesis.

The vascular injury panel 2 includes CRP, ICAM-1, SAA, and VCAM-1.

Eotaxin

It is a chemoattractant of eosinophils and it is largely expressed in the gastrointestinal tract. (1) Eotaxin has been recently linked in vitro to migration of CD34+ cells from bone marrow to the circulation via CCR3. (2) Eotaxin is expressed in epithelial cells, endothelial cells, T cells, macrophages, and eosinophils. Eotaxin RNA expression is upregulated by inflammatory cytokines like TNFα and IL-1. Th2 cytokine (like IL-4 and IL13) can also be very potent stimulation for Eotaxin. (3)

Eotaxin-3

As Eotaxin, Eotaxin-3 is a chemokine that attracts eosinophils, but also basophils and Th2 lymphocytes. Most of its activity is through CCR3 but Eotaxin-3 also binds the CCR2 on monocytes. In contrast MCP-1 Eotaxin acts like a natural antagonist to CCR2 and has a repulsive effect on monocytes. (4)

IL-8

Interleukin 8 is part of the chemokines family with chemoattractant properties of leukocytes to the inflammation. It is released by endothelial and epithelial cells as well as macrophages and neutrophils. (5, 6) TNFα and IL-1β promote release of IL-8 which has a potent chemotaxis effect on neutrophils. It has been studied as a relevant predictor marker in septic shock and data suggest that higher level of IL8 in ICU admission predict mortality in children with septic shock.(7) It has also effects on mobilization of haemopoietic stem cells. Besides granulocytes it is secreted by cancer cells and it has a role in angiogenesis. (8)

IP-10

IFNγ inducible protein -10 (IP-10) is a monokine involved in NK cells recruitment. It is produced primarily by monocytes but also by endothelial cells and fibroblasts. It is a member of the CXC family and it is a pro-inflammatory and anti-angiogenic signal. It is also released by dendritic cells in the pathway of T-cell activation. Neutrophils produce IL-10 when they are exposed to IFNγ and bacterial lipopolysaccharide while IL-4 and IL-10 antagonise this process. (9)

MCP-1

Monocytes chemoattractant protein 1 (MCP-1) is also known as CCL2 and it is a key regulator of the monocyte/macrophage migration and/or infiltration. It belongs to the CC family. Besides its role in recruiting monocytes into the site of inflammation, CCL2 also affects migration of NK cells and T lymphocytes. CCL2 expression is associated with expansion of Th2 lynphocytes suggesting that MCP-1 has role in T-cell immunity response. In contrary to other chemokine of CC family that promote Th1 cells, MCP-1 switches Th0 to Th2 via direct upregulation of IL-4 and it is found in abundance in conditions like asthma. (10)

MIP-1α and MIP-1β

Macrophage inflammatory protein 1α and 1β are two forms of human macrophage inflammatory chemokines (also known as CCL2 and CCL4, respectively). As per their nam, they are produced primarily by macrophages (but also dendritic cells). They commonly use CCR5 as a receptor. This is expressed on the surface of macrophages, NK cells and a activated Th1. MIP-1α and MIP-1β work as chemoattractant for monocytes/macrophages, NK cells and subpopulation of T cells. CCL2 and 4 stimulate the release of inflammatory cytokines like IL1 and IL6 and TNFα. (11)

TARC

Thymus and Activated Reactive Chemokine (TARC) is also known as CCL17. Its expression is physiological in the thymus, dendritic cells, keratokines, and fibroblasts. It binds the CCR4 and it is a strong chemoattractant of Th2 cells. It is located on chromosome 16. (12)

IFN-γ

Interferon gamma is a Th1 signature and major inflammatory cytokine. It is secreted from activate T lymphocytes and NK cells. Besides its antiviral activity; interferon-γ has a regulatory functions for macrophages, T and B lymphocytes, and granulocytes. IFN-γ stimulates the production of major histocompatibility (MHC) class I and II antigen in the macrophages. It also induces secretion of inflammatory cytokines like IL1, IL6 and TNFα. IFNγ has a role in many inflammatory condition like Lupus or Tuberculosis and interestingly plays a fundamental role in the development of Graft Versus Host Disease after hematopoietic cell transplantation. (13)

IL-1β, IL-1α, IL-1RA

Interleukin 1 family shares some of its characteristics with the Toll-like receptor family (TLR). They are the main cytokines of the innate immunity. Innate immunity is based on inflammatory response independent from T and B cells. Inflammation is the host defence but if it is uncontrolled leads to the opposite effect and compromises survival. Both IL1 family and TLR share the Toll-IL-1 receptor domain. IL-1 family's innate immune response is non-specific and in response to injury IL-1 family triggers inflammatory cascade like production of chemo and cytokines, induction of cyclo-oxygenase and synthesis of Nitric Oxide.

IL-1α is a dual-cytokine as it has a receptor in the nucleus and in the cytoplasm. Its secretion increases IL-8 production. On the contrary to IL-1β, which is released only in pathological conditions, IL-1α is produced constitutively by endothelial and mesenchymal cells in health.

IL-1RA Interleukin 1 Receptor Antagonist blocks the activity of IL-1a and IL1b. Its purified form from E.coli is used in the treatment of auto-inflammatory conditions (that differ from autoimmune conditions where T lymphocytes immunity is not involved) like Familial Mediterranean Fever and pericarditis. (14)

IL-17A, IL-17A/F, IL-17B, IL-17C, IL-17D

Interleukin 17 cytokines are pro-inflammatory cytokines produced by T helper 17 cells and other lymphocytes. The best known IL17 are A and F and they share similar structure and features. They play a role in the adaptive immunity. IL-17 A and F have a role in the defence against certain extracellular bacteria like Staphylococcus Aureus and Klebsiella Pneumoniae as well as fungal infections. While IL17 has a protective role in infections, when dysregulated it can induce autoimmune disease like Rheumatoid Arthritis. (15)

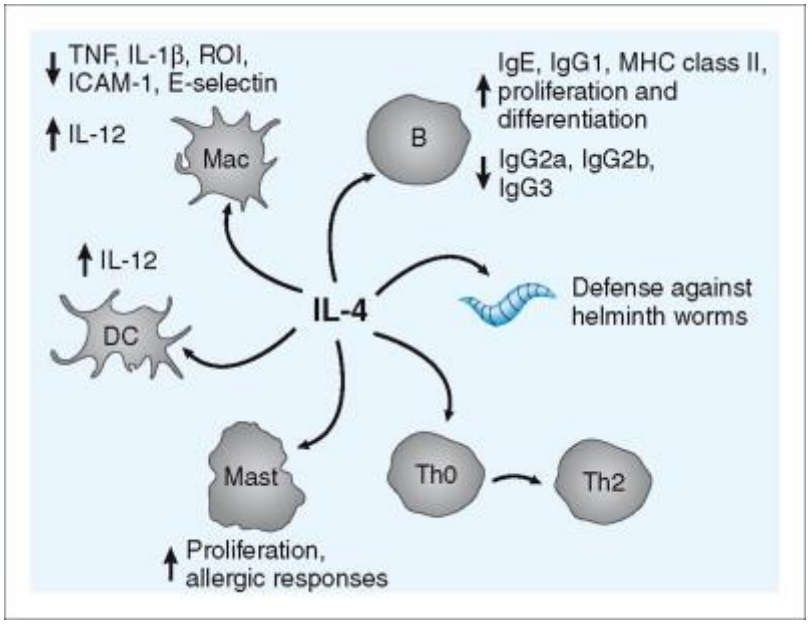
IL-2

Interleukin 2 plays a fundamental role in the maturation and expansion of T lymphocytes and it is produced by activated T Lymphocytes. It has a role in the cell-mediated immune response and in allograft rejection. (16)

IL4

Interleukin 4 is important in the humoral immunity against extracellular pathogens. It is one of the most widely diffuse Interleukins in the body and it influences a large variety of cell types. Th2 are dependant to IL4 for development. IL4 is secreted in large quantities by NK cells and activated mast cells and basophils. IL4 has an antagonistic function to INFγ and promotes switch in B cell to IgG to IgE and therefore plays a major role in allergy development and helminth. IL4 is classified as anti-inflammatory cytokine as inhibits the secretion of TNF and IL1β.(17)

Figure 33: Interaction between IL-4 and cells in the immune system



This figure uses an image from https://ars.els-cdn.com/content/image/3-s2.0-B9780120884513500193-f17-10-9780120884513.jpg and it illustrates the relationship between IL-4 and immune cells such as macrophages (mac), dendritic cells (DC), B cells (B), Mast cells (Mast) and T helper 0 and 2 cells (Th0 and Th2).

IL-6

Interleukin 6 is secreted by monocytes, macrophages and T cells in response to trauma and infection. IL6 is very elevated in the initial phase of inflammation and it is an acute phase response signal. It plays a synergistic role in the inflammation with TNF and IL1. (18)

IL-10

Interleukin 10 is a potent anti-inflammatory cytokine that inhibits inflammatory signals in both adaptive and innate immunity. It inhibits the effects of TNFα, IL1β, IL8 and adhesion molecules. (19)

IL-12 and IL23

Interleukin 12 is a potent pro-inflammatory cytokine that enhances the cytotoxic activity of T lymphocytes. It promotes differentiation of Th1 to T lymphocytes and the secretion of IFNγ. IL-12p70 is the bioactive form of IL12 secreted by the T lymphocytes after stimuli of IL4 or IFNγ. (20).

IL-12 and IL-23 are heterodimers and share the p40 subunit and therefore have similar effect on the differentiation of the Th1 and Th17. P40 unit is a target for anti-inflammatory drugs like Ustikinumab. (21)

IL23 promotes secretion of IFNγ from CD14+ T memory cells but not naïve T cells in contrast to IL12. IL23 helps generate Th17 and non-functional variants IL23 receptors protects from the development of IBD. (22)

IL13

It is primarily produced by Th2 and has anti-inflammatory effects by inhibiting production of pro-inflammatory cytokines by macrophages and monocytes. It plays a role in IgE mediated allergic reactions. (23)

TNF-α and TNFβ

Tumour necrosis factor alpha is perhaps one of the best studied pro-inflammatory cytokines associated with sepsis. Among its effects it enhances cell adhesion and induction of nitrous oxide (NO). Despite the proven influence of TNFα on sepsis its blockage failed to be efficacious in treating sepsis. Anti-TNFα therapy is used in clinical practice to treat autoimmune inflammatory conditions with success (i.e. Rheumatoid Arthritis and IBD).

Tumour necrosis factor beta shares most of features with its homotrimer alpha: it is a potent immune-mediator and drives inflammatory responses.

GM-CSF

Granulocytes-macrophages colony stimulating factor (GM-CSF), despite its name it is not essential as a myelopoietic stimulating factor in physiological state but has a

significant role in the inflammation and as a mediator between phagocytic cells and tissue invading lymphocytes.(24)

IL-5

Interleukin 5 is a cytokine and growth factor for B cell and especially for eosinophils. Its levels are elevated in allergic reaction mediated by eosinophils (like allergic rhinitis). (25)

IL-7

Interleukin 7 is a non-redundant cytokine that plays a part in the lymphocytes T and B differentiation and maturation. It is also known for its role within the gut mucosal layers and the pathogenesis of Ulcerative Colitis as well as the regulation of bone metabolism. (26)

IL15

Interleukin 15 is part of the IL2 superfamily and shares most of its functions on proliferation of B lymphocytes and immunoglobulin production. It also plays a part in NK cells and T cells expansion.(27)

IL 16

Interleukin 16 has its only receptor in CD4 molecules and its exclusive function is to recruit T cells CD4+. It is secreted by T cells, eosinophils and mast cells. (28)

VEGF-A, VEGF-C and VEGF-D

Vascular Endothelial Growth Factors are cysteine-knot grow factors. They only bind to endothelial receptors and promote angiogenesis in physiological and pathological conditions. These growth factors can activate the *Notch* pathway among other pathways involving cell migration and endothelial permeability.(29)

Flt / VEGFR 1

Vascular endothelial receptor 1 is necessary as a negative modulator of the angiogenic effect of VEGFs via Notch signalling. (30)

IL-3

Interleukin 3 acts like a haemopoietic growth factor for primitive line of bone marrow cells. It has a role in the proliferation of multiple cell lines. Together with IL5 and GM-CSF stimulate differentiation and migration of eosinophils and mast cells in allergic

reactions. It has been found to be in elevated levels in some forms of hematologic malignancies. (31)

IL9

Interleukin 9 is one of the understudied cytokines. It is strongly associated with T cells and especially with Th2. There is some emerging evidence the production of IL9 may be associated to a specific subset of T cells. IL4 and TNFβ stimulate secretion of IL9 and it has effects on inflammation and proliferation of several immune cell lines. (32)

TSLP

Thymic Stromal Lymphopoietin is a cytokine IL-7 like produced by epithelial and stromal cells. It contributes to the hematopoiesis of basophils and has a role in the differentiation of Th2 and in allergic reaction like in atopic dermatitis. (33)

IL-21

Interleukin 21 is secreted mainly by CD4+ T helper cells and contributes to the differentiation and expansion of memory and plasma B cells as well as the switch of antibodies production. Its activity touches the differentiation process of T helper cells in subtypes of Th17. It has an implication in allergic reactions, immunity and cancer. (34)

IL-22

Interleukin 22 is of the IL-10 family and secreted mainly by Th17. Its fundamental role is of mucosal barrier but also has an active function on thyme regeneration after injury. (35)

IL-27

Interleukin 27 has a wide effect on diverse functions of the immune system including CD4+ and CD8+ T cells, IL10 secretion and regulatory T cells responses via IL2 limitation. IL27 affects, among others, infection diseases, autoimmune conditions and mucosal barriers and therefore it has been explored as a therapy for several condition including IBD.(36)

IL31

Interleukin 31 is produced principally by Th2 cells. It mediates allergic reaction especially in skin where its receptor is constitutively expressed on keratinocytes and epithelial cells.

MIP-3α

Macrophage inflammatory protein 3 alpha is a chemokine of the CC family and it is predominantly expressed in lymph nodes and appendix. It binds it receptor MIP-3α/CCL20 on dendritic cells and may have a role on the pathogenesis of chronic obstructive pulmonary disease. (37)

PlGF

The placental growth factor is a part of the endothelial growth factor family like (VEGF). It is involved in neo-angiogenesis both in health and in disease. It affects a large array of different cells involved in the process during inflammation and not only endothelial cells. It influences the faith of macrophages (recruitment and activation), stromal cells (proliferation and contractility), fibroblasts (proliferation), neuronal cells (proliferation) and hepatic stellate cells (chemotaxis and proliferation). (38)

Tie-2

Tie-2 has an angiogenetic function in creating the vascular network. Its function is regulated by the Angiopoitin 1 and 2 that bind Tie-2 and respectively stimulates or inhibits its function .

ICAM-1

Intercellular Adhesion Molecule 1 expression is stimulated in the endothelium and keratinocytes by high levels of IL1, TNF and IFN. It mediates the interaction between these tissues and leucocytes.

VCAM-1

Vascular Cell Adhesion Molecule 1 is a glycoprotein expressed in endothelium activated by cytokines (as described in ICAM – 1 above). It mediates interactions between monocytes and eosinophils. .As well as ICAM-1, VCAM-1 allows migration of lymphocytes through the endothelium in inflammation sites.

SAA

Serum Amyloid A is a phase reaction protein and it is mainly produced in the liver. Its expression is regulated by inflammatory cytokines (IL1α, TNFα and IL17A). Its role is to maintain an inflammatory environment, activate innate immunity and Th1 recruitment.

3.4 Results

Below I illustrate the cytokines and chemokines results in graphs expressed as fold changes from baselines at time points the analysis was performed after infusion of macrophages. Baseline is considered the time point prior to infusion at screening day or apheresis day (respectively -14 or -7 days from infusion). For convenience in the graphs the baseline has been shown as -14 days. Time 0 in the graphs represents the infusion of the cellular product. Each line in the graphs represent a subject and they have been separated by colour as per dose group (10^7, 10^8, and up to 10^9 cells infused).

Cytokines and chemokines are illustrated in graphs as per the manufacturer panels followed by illustration of each parameter standard curve. These compare the curves, with lower and upper LOQ, to the results in our population. Results falling out of the reliable limits, in the specific for our results below the lower LOQ, or un-detectable results are not showed in the graphs.

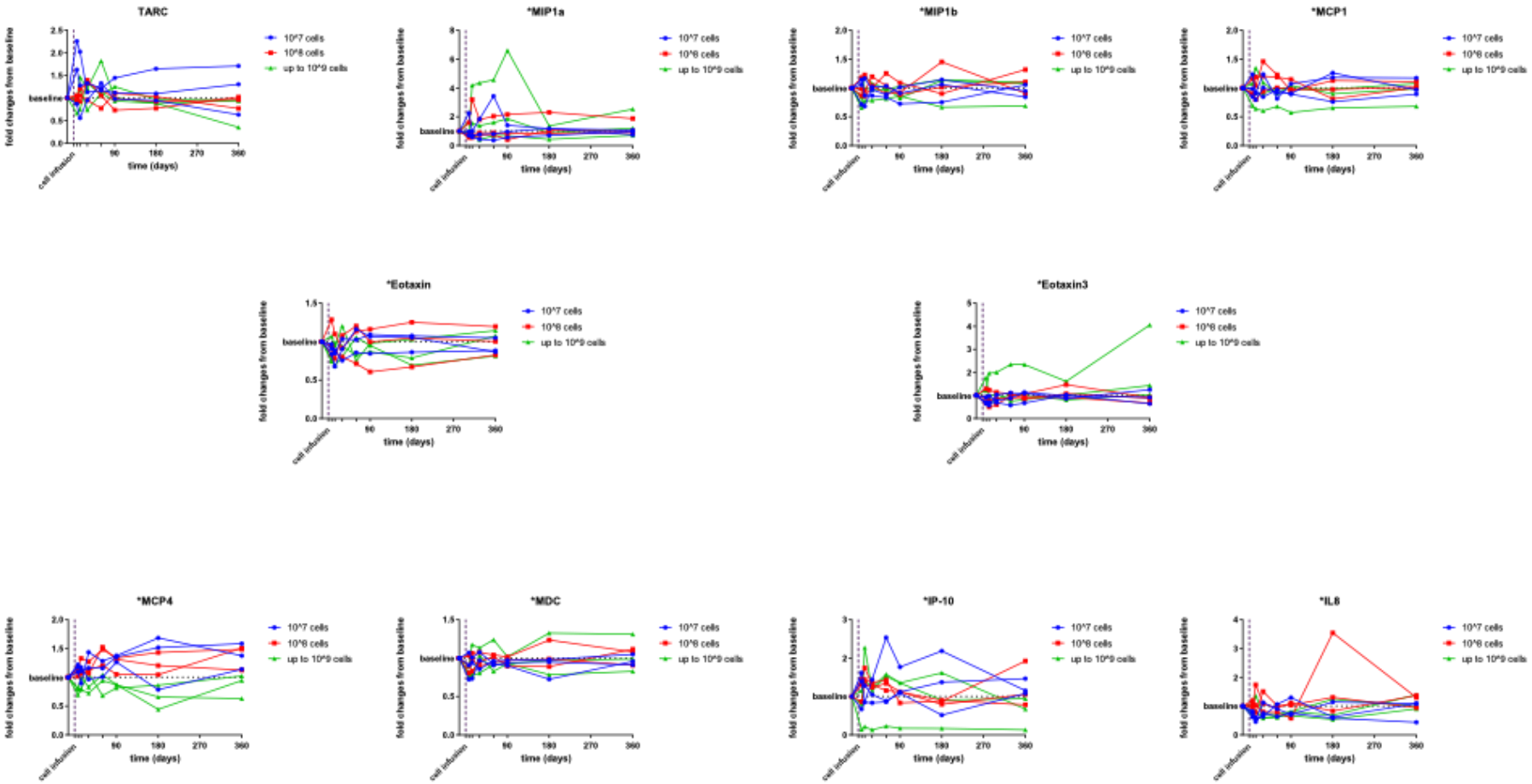
Overall we did not observe significant shift of cytokine or chemokine levels following the infusion of macrophages.

Inflammatory cytokines like IL1β, IL6, TNFα and PIGF showed minimal changes from the baseline with respectively mean fold ± SD of 3.11 ± 3.57, 1.81 ± 0.73, 0.93 ± 0.13 and 1 ± 0.15 at 7 days after cell infusion and 0.72 ± 0.58, 1.11 ± 0.29, 0.94 ± 0.12 and 0.97 ± 0.16 respectively at 90 days after infusion of macrophages. There is a very slight propensity in reduction of IL1β at 90 days following 3 fold raise in the short term after infusion at day 7.

While anti-inflammatory cytokines like IL10 showed again no significant changes from the BL but with transient increased with mean ± SD of 1.4 ± 0.97 at 7 days after treatment that resolved by day 90 with mean of 0.75±0.29.

MCP1 levels are stable although with a marginal tendency to reduce with a mean fold of 0.97 ± 0.17 at 7 days and 0.95 ± 0.16 at 90 day after macrophages injection while IL4 levels are undetectable throughout the study in all subjects.

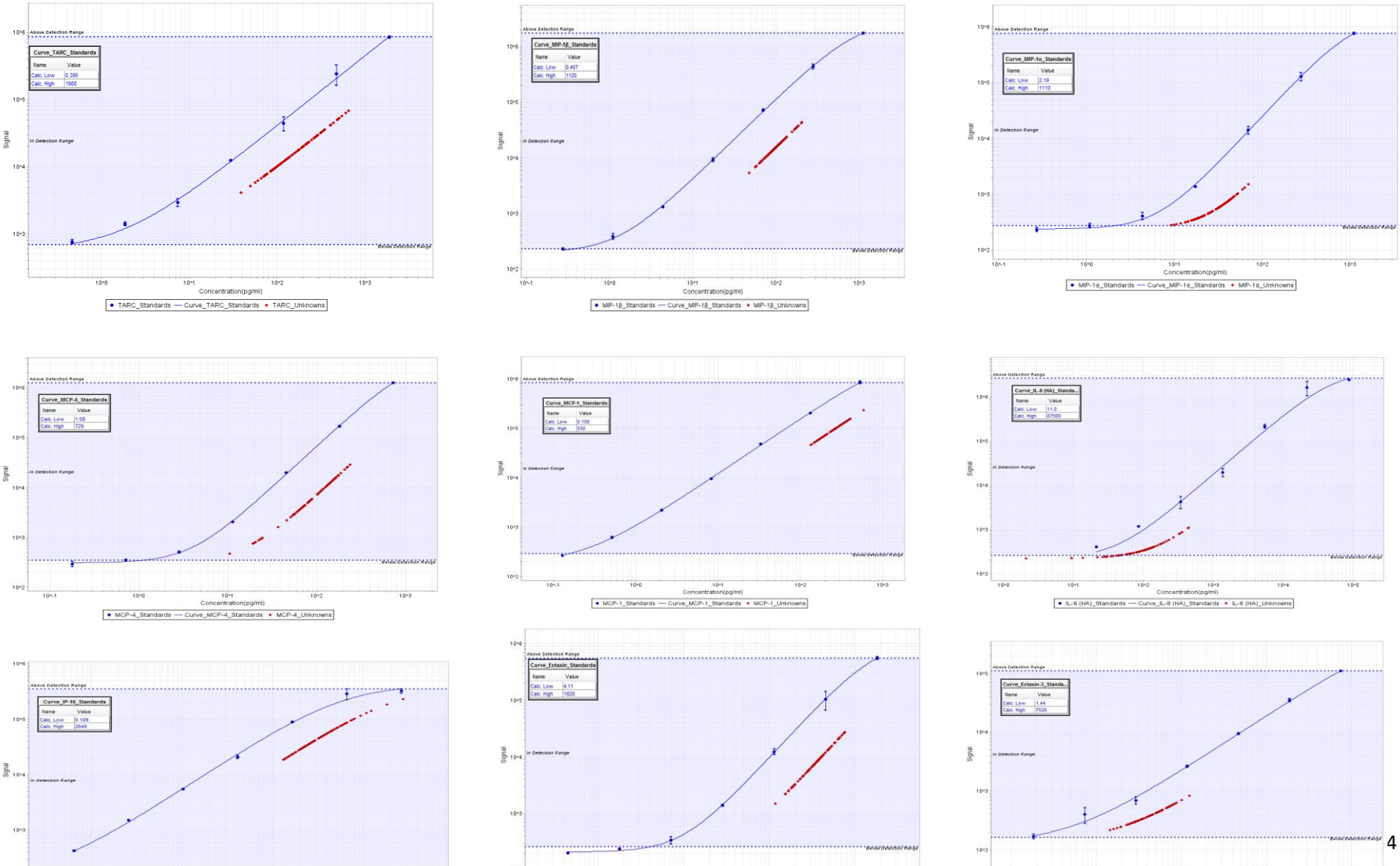
Figure 34: Chemotaxis panel 1



This figure shows changes in cytokines expressed as fold changes (y axes) from baseline over time expressed in days (x axes).

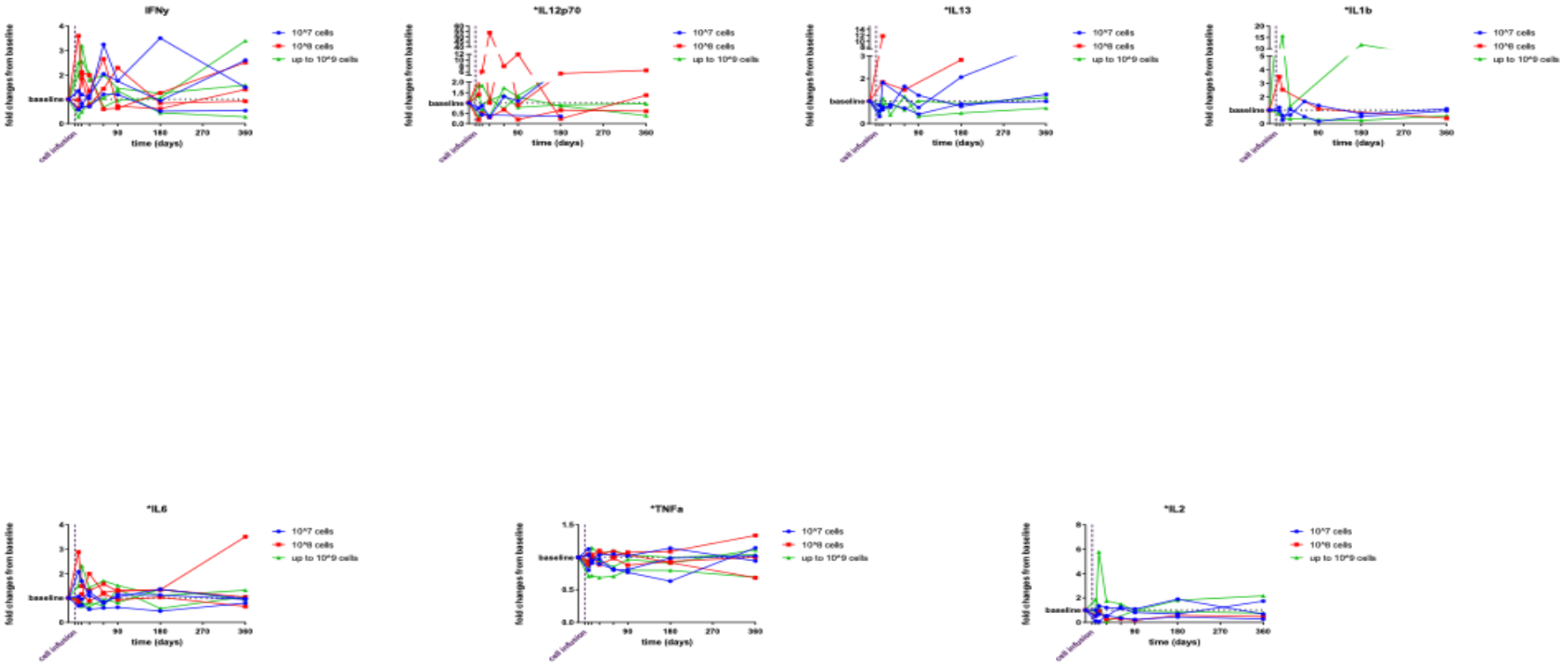
Baseline defined as level prior to Macrophages infusion (noted as vertical broken line). Each line corresponds to a subject in the study. Colours represent different dose groups as per legend.

Figure 35: Standard curve for Chemotaxis panel



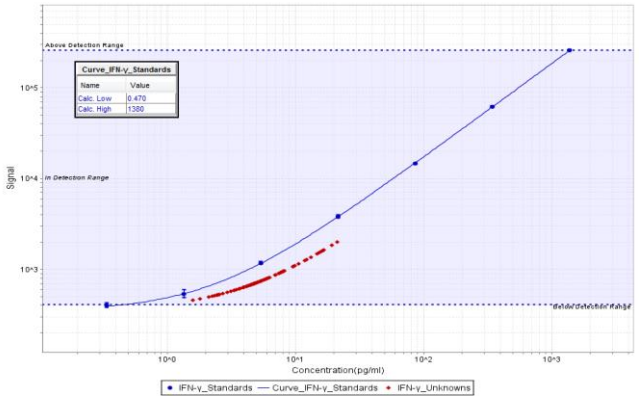
This figure shows the standard curve (blue line) of each cytokine in the Chemotaxis panel kit and their signal in our population. These are expressed as concentration (pg/ml) over signal. Cytokines with signals above or below the detection range should not be used for analysis.

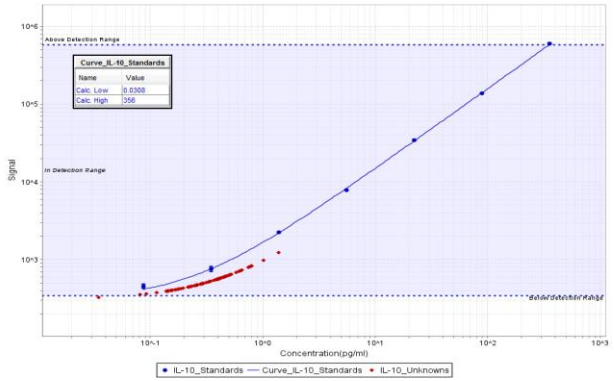
Figure 36: Pro-Inflammatory panel 1

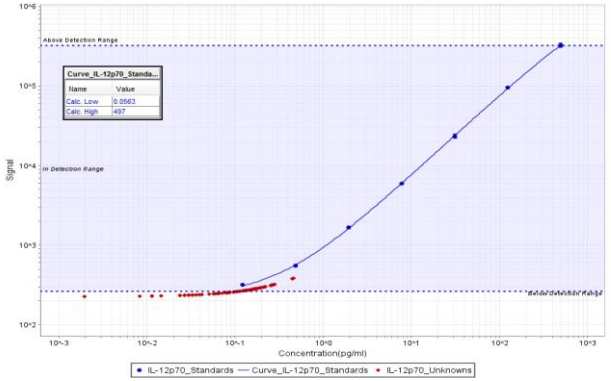


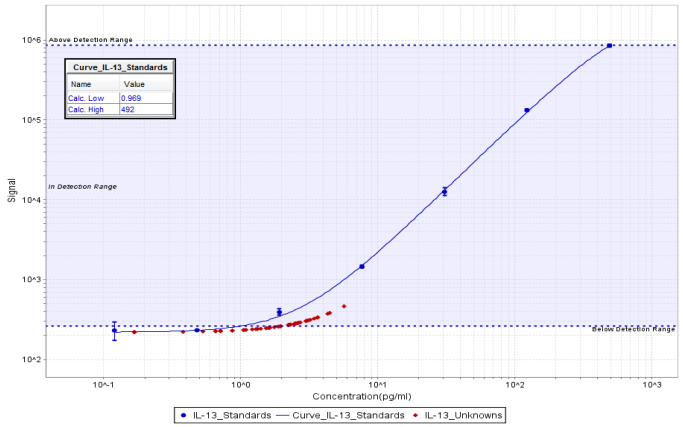
This figure shows changes in cytokines expressed as fold changes (y axes) from baseline over time expressed as days (x axes). Baseline defined as level prior to Macrophages infusion (noted as vertical broken line). Each line corresponds to a subject in the study. Colours represent different dose groups as per legend.

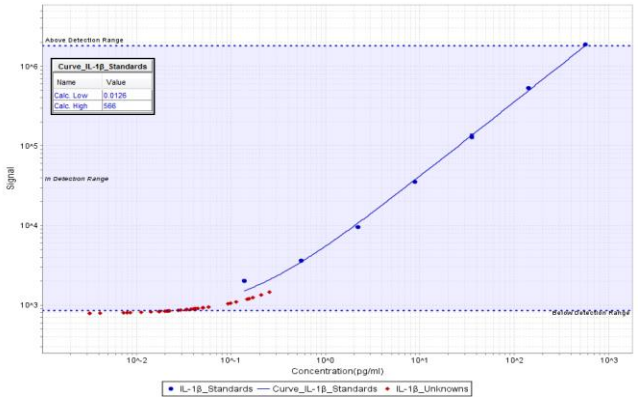
Figure 37: Standard curves for pro-inflammatory panel 1

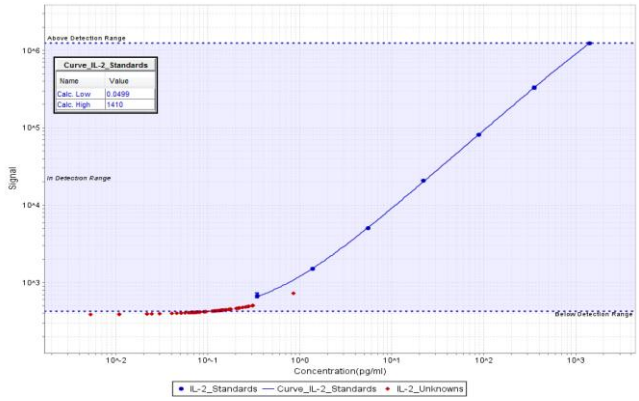


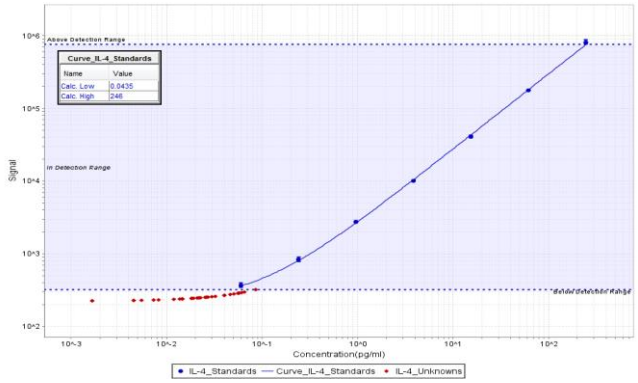


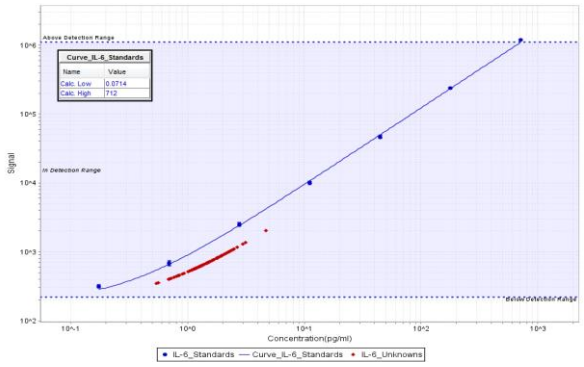


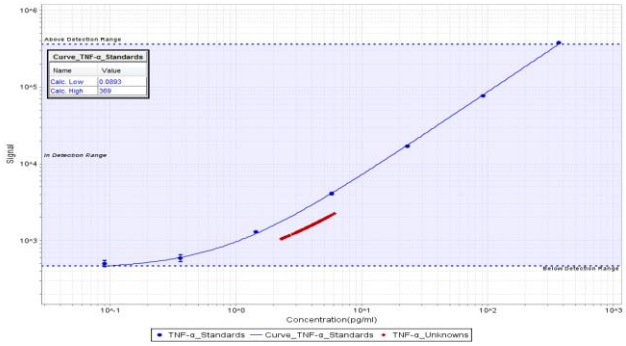






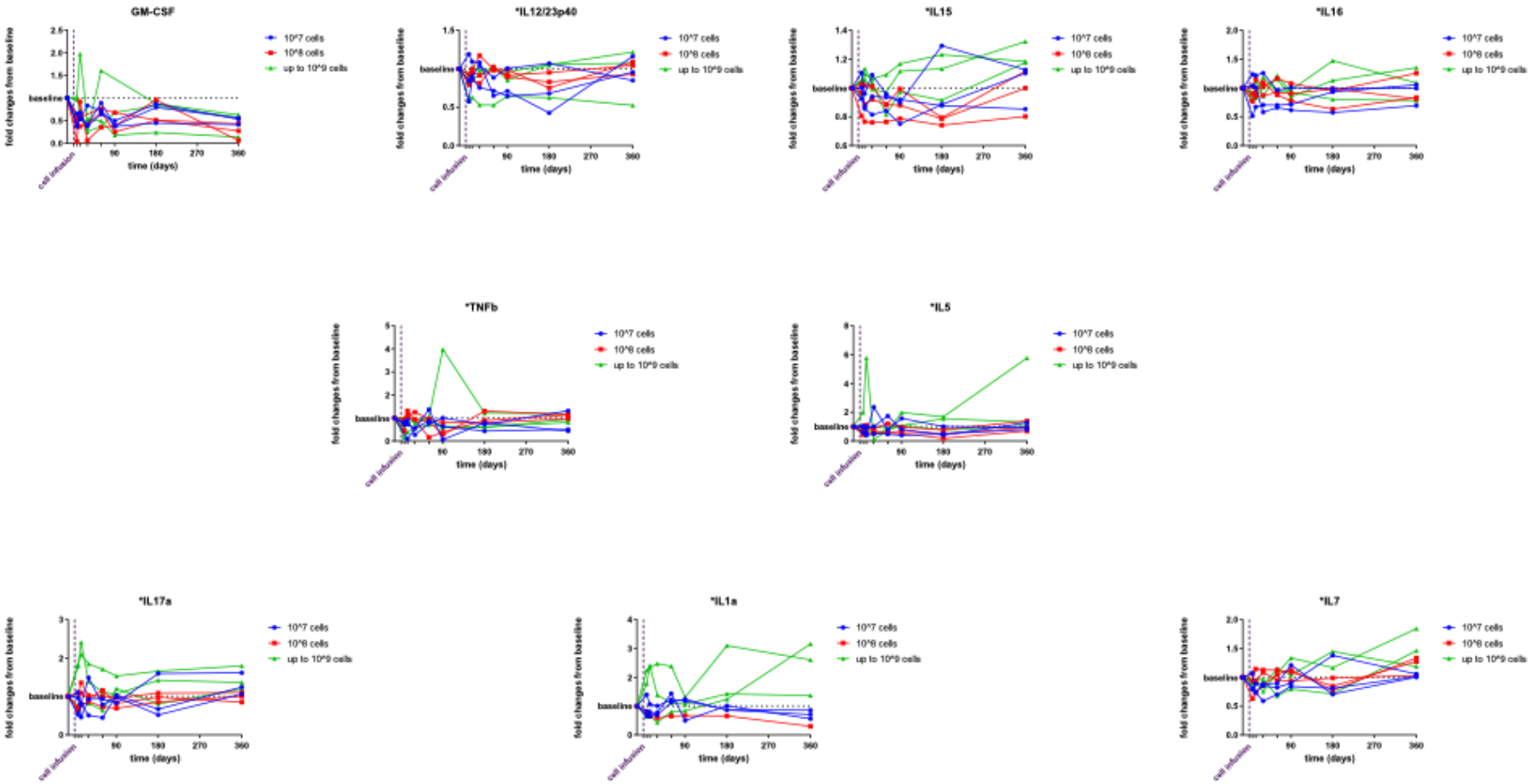






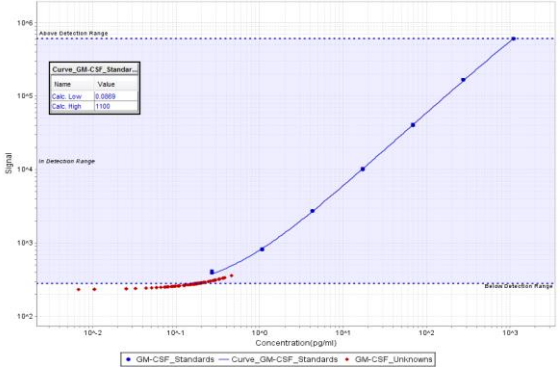
This figure illustrates the standard curve (blue line) of each cytokine in the pro inflammatory panel1 kit and their signal in our population. These are expressed as concentration (pg/ml) over signal. Cytokines with signals above or below the detection range should not be used for analysis.

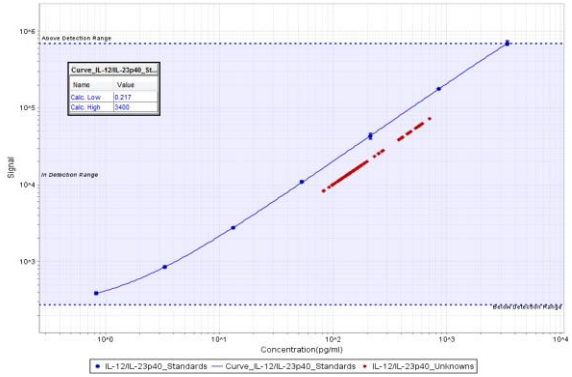
Figure 38: Cytokine panel 1

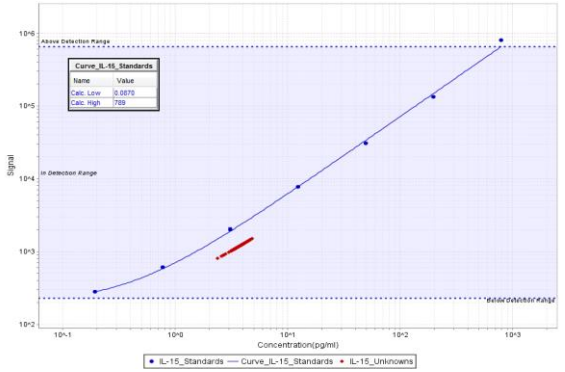


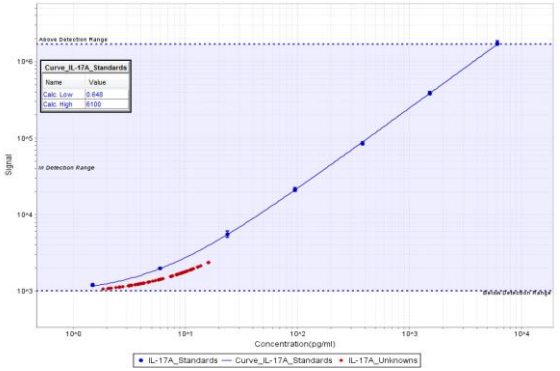
This figure illustrates changes in cytokines expressed as fold changes from baseline (y axes) over time expressed in days(x axes). Baseline defined as level prior to Macrophages infusion (noted as vertical broken line). Each line corresponds to a subject in the study. Colours represent different dose groups as per legend.

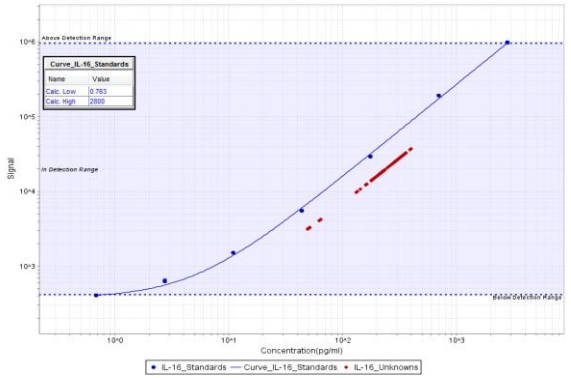
Figure 39: Standard curves for cytokine panel 1.

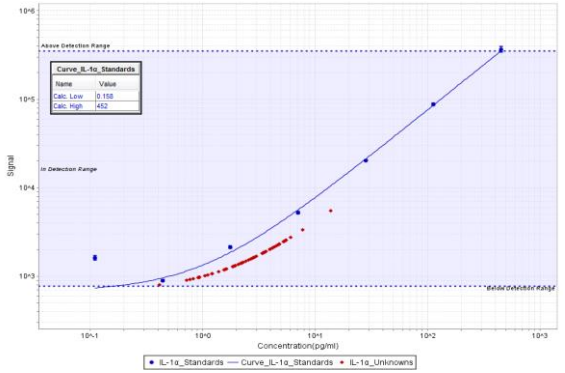


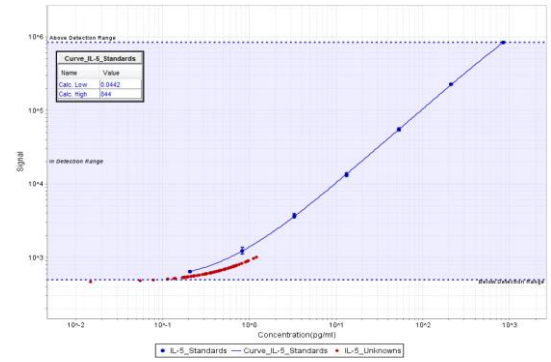


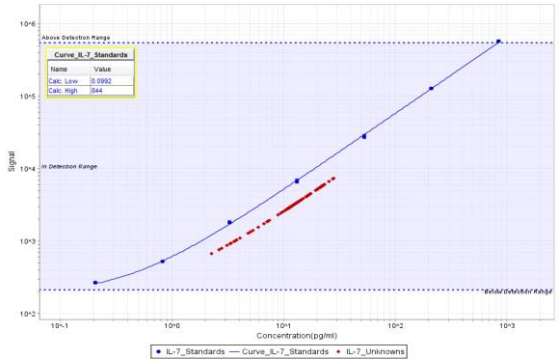


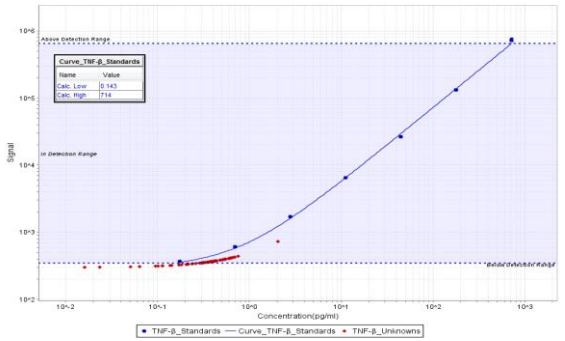






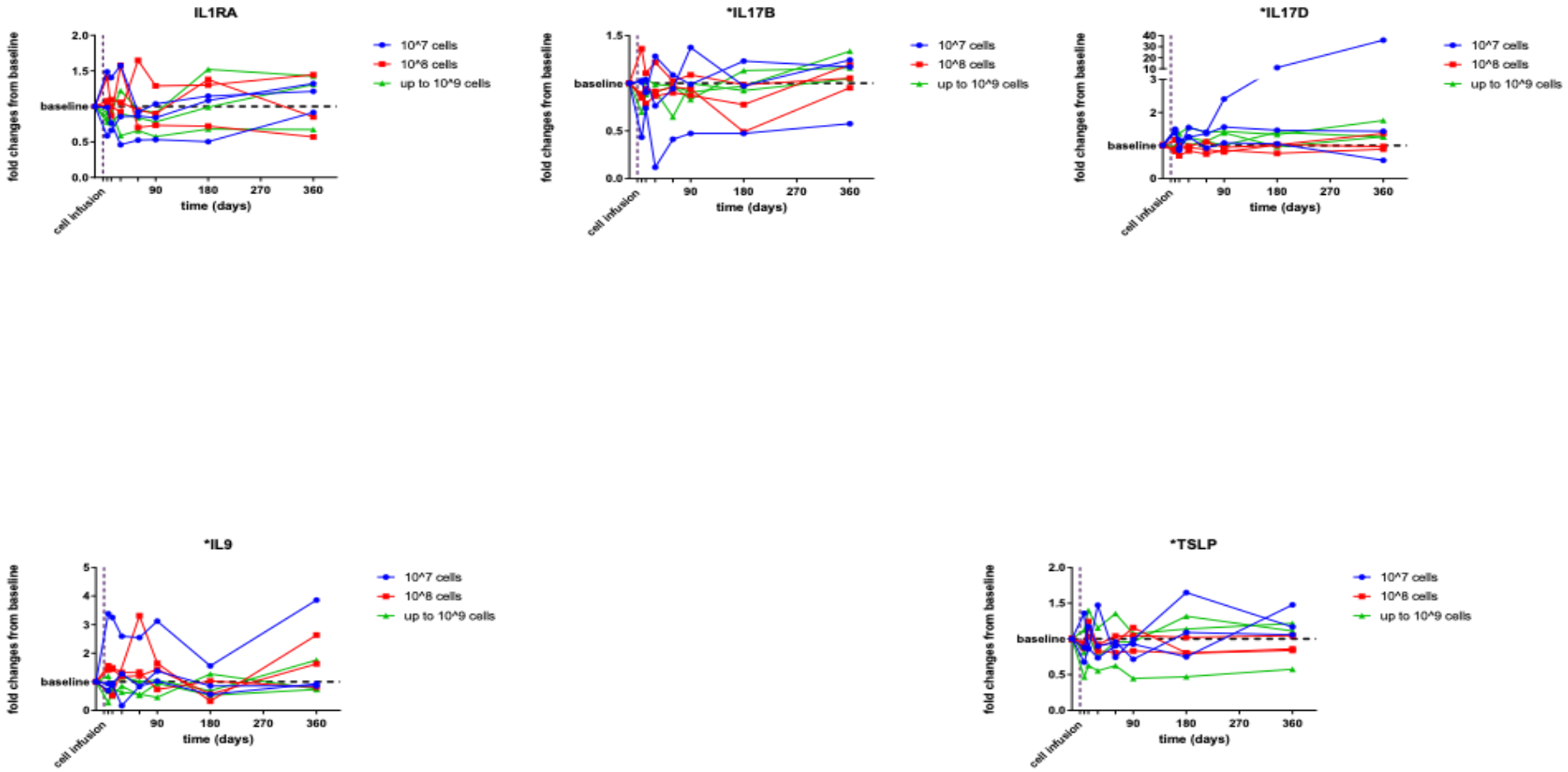






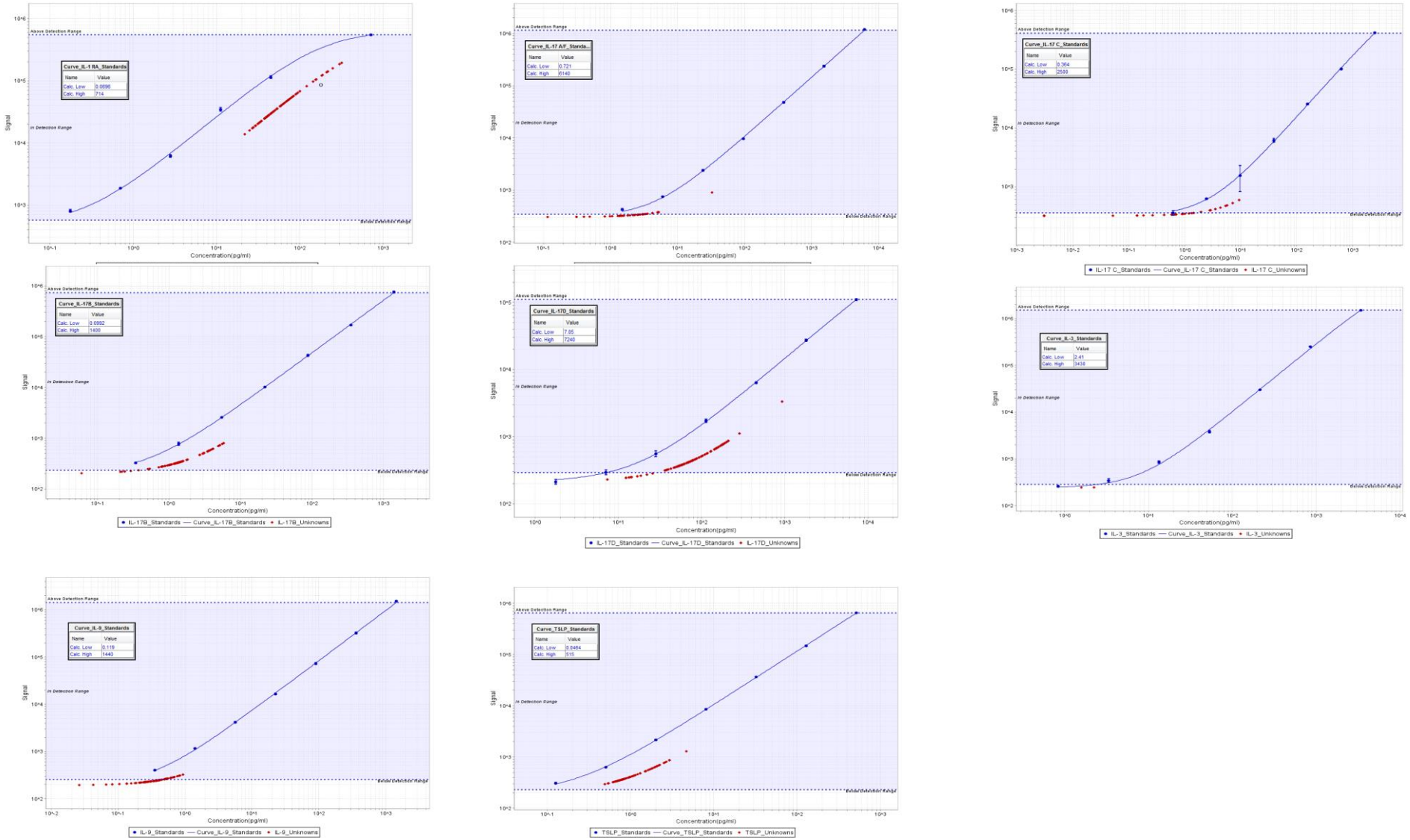
This figure shows the standard curve (blue line) of each cytokine in the Cytokine panel 1 kit and their signal in our population. These are expressed as concentration (pg/ml) over signal. Cytokines with signals above or below the detection range should not be used for analysis.

Figure 40: Cytokine panel 2



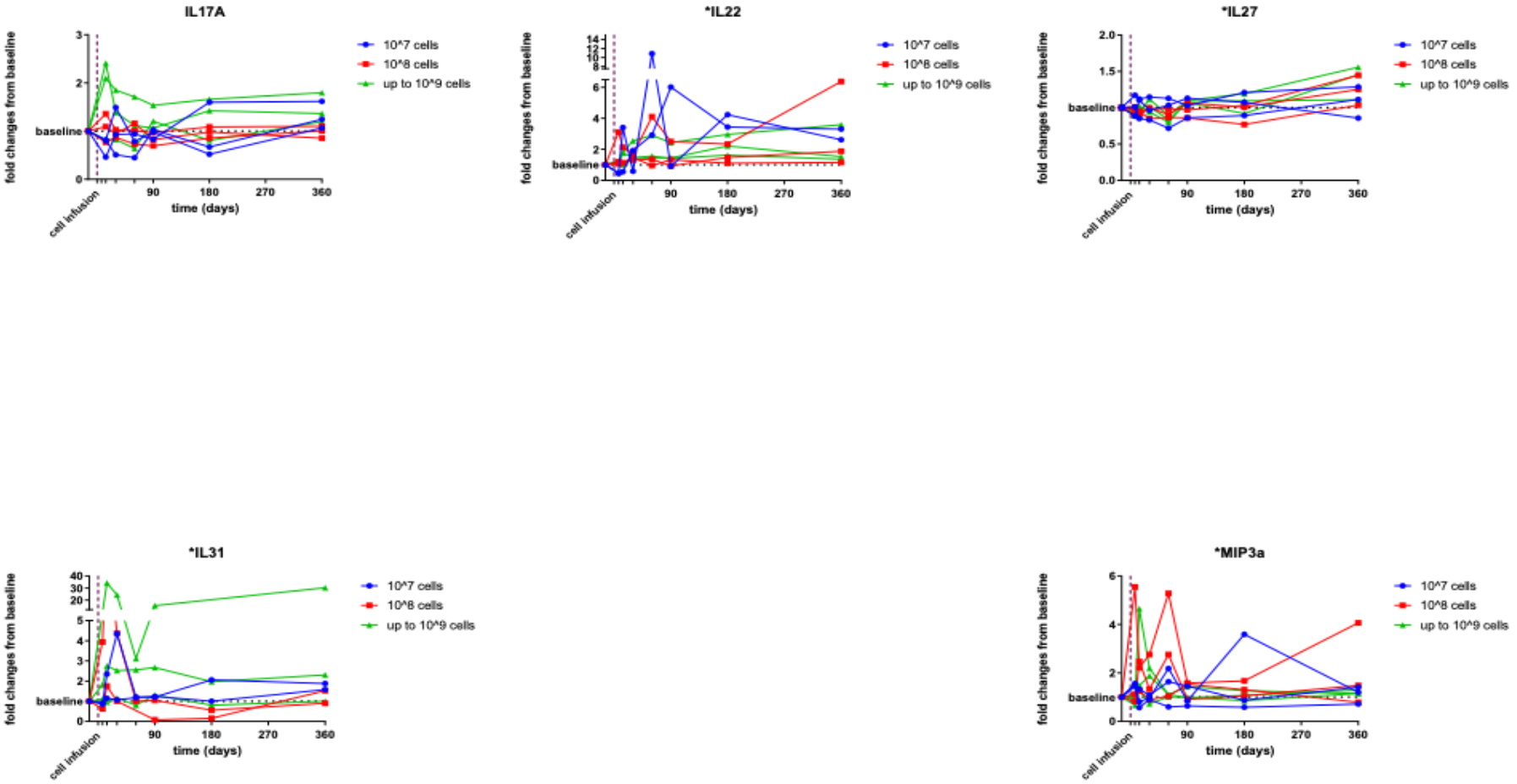
This figure shows changes in cytokines expressed as fold changes from baseline (y axes) over time expressed in days(x axes). Baseline defined as level prior to Macrophages infusion (noted as vertical broken line). Each line corresponds to a subject in the study. Colours represent different dose groups as per legend.

Figure 41: Standard curves for Cytokine Panel 2.



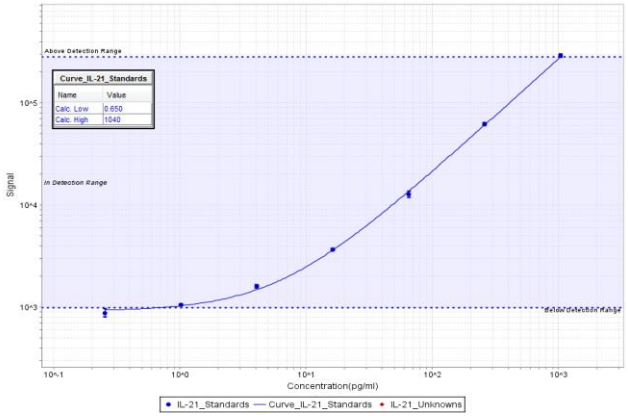
This figure illustrates the standard curve (blue line) of each cytokine in the cytokine panel 2 kit and their signal in our population. These are expressed as concentration (pg/ml) over signal. Cytokines with signals above or below the detection range should not be used for analysis.

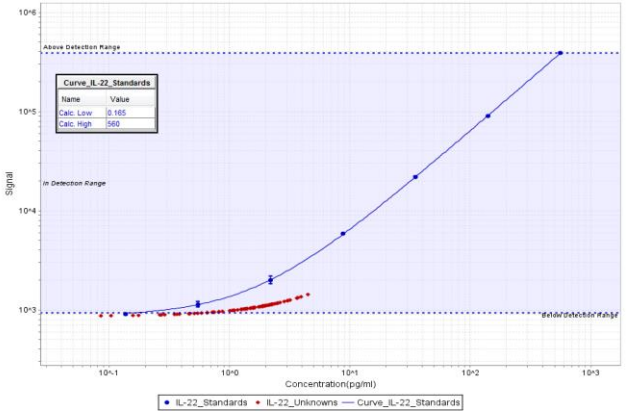
Figure 42: TH17 panel

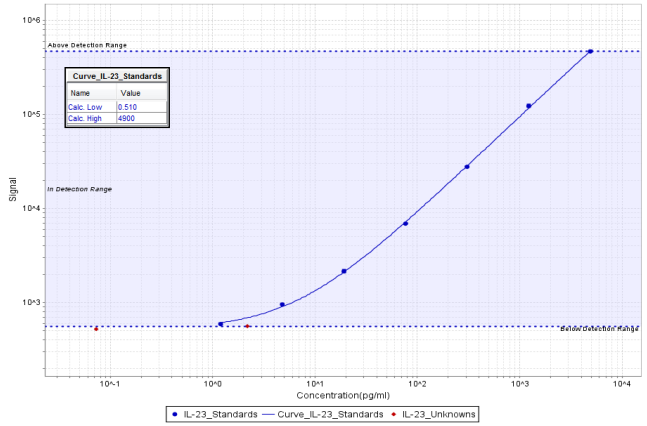


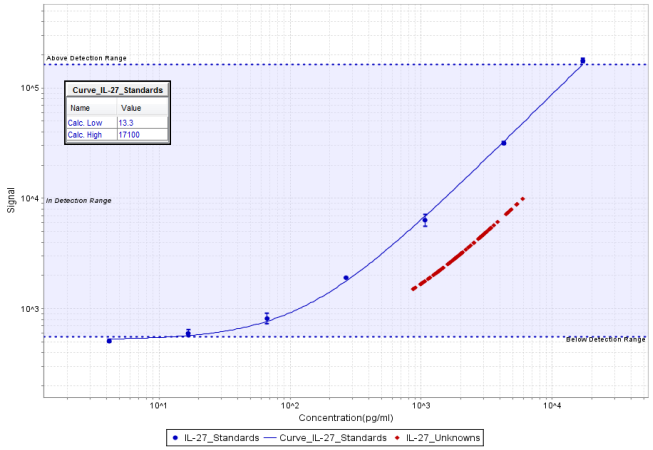
This figure shows changes in cytokines expressed as fold changes from baseline (y axes) over time expressed in days (x axes). Baseline defined as level prior to Macrophages infusion (noted as vertical broken line). Each line corresponds to a subject in the study. Colours represent different dose groups as per legend.

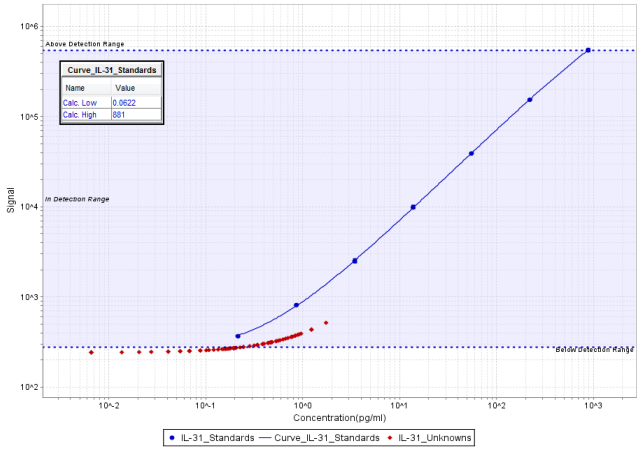
Figure 43: Standard curve for TH17 panel.

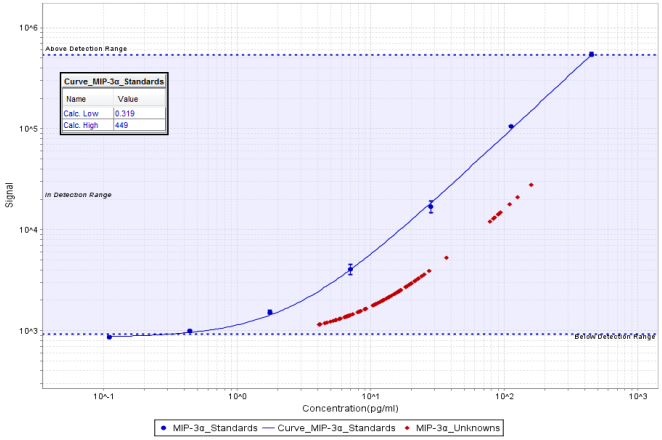






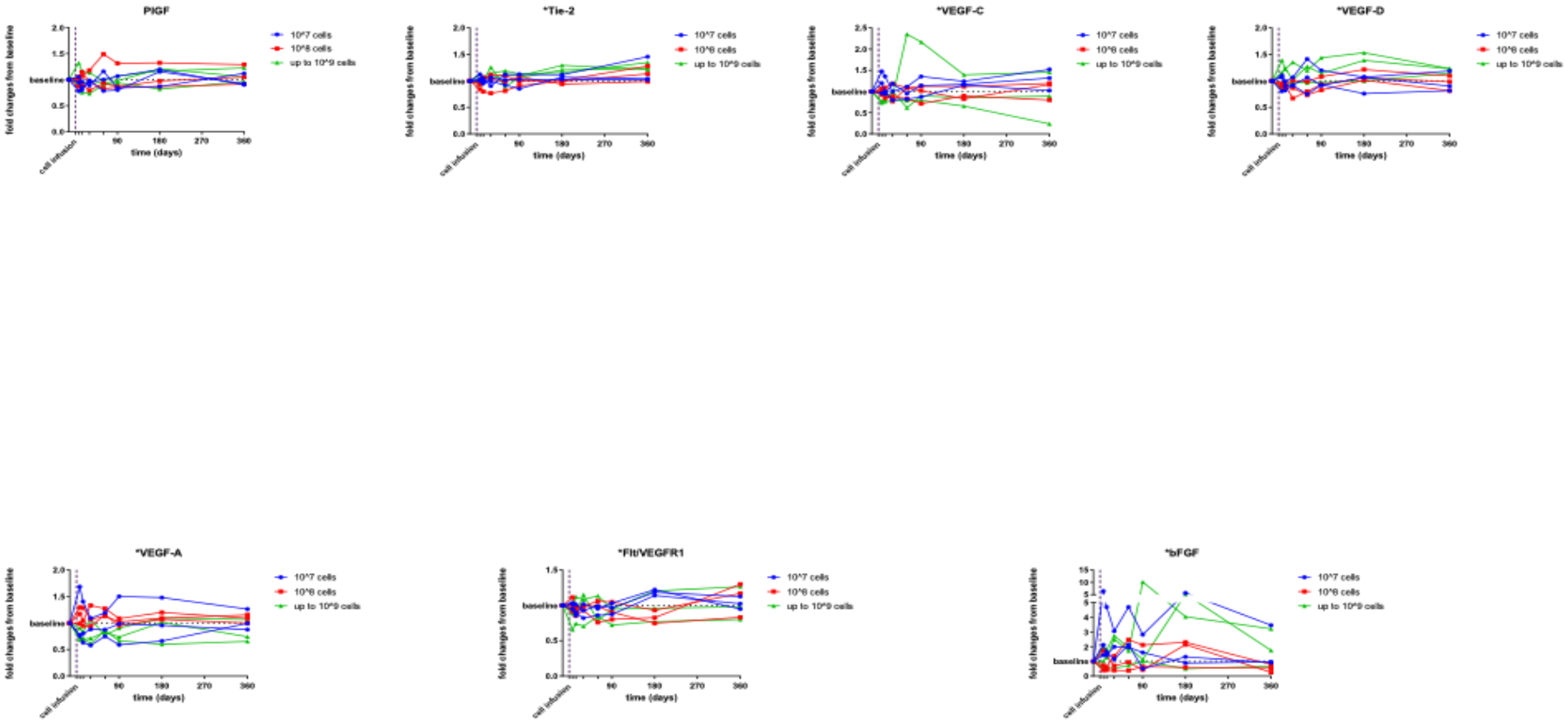






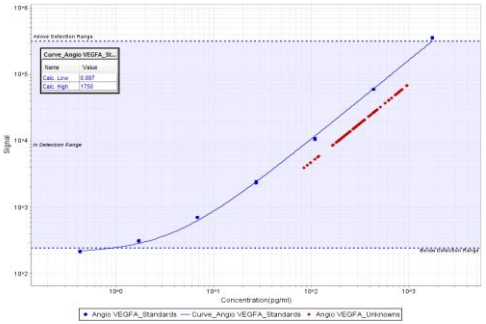
This figure shows the standard curve (blue line) of each cytokine in the TH17 panel kit and their signal in our population. These are expressed as concentration (pg/ml) over signal. Cytokines with signals above or below the detection range should not be used for analysis.

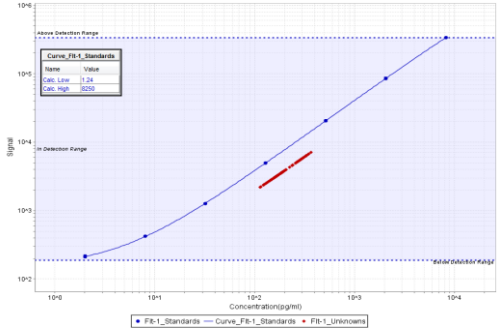
Figure 44: Angiogenesis panel

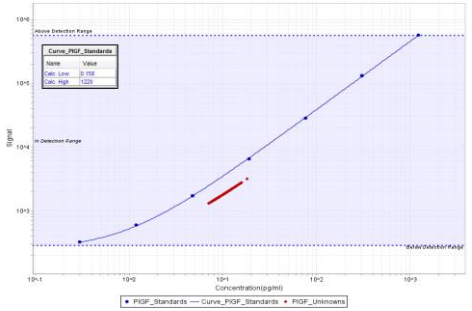


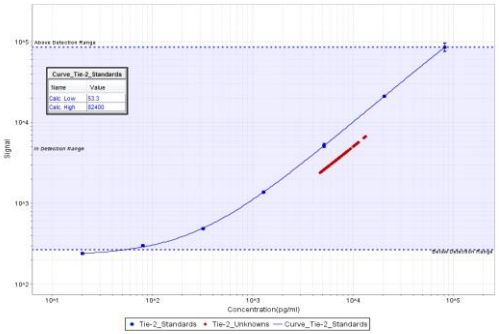
This figure shows changes in cytokines expressed as fold changes from baseline (y axes) over time expressed as days(x axes). Baseline defined as level prior to Macrophages infusion (noted as vertical broken line). Each line corresponds to a subject in the study. Colours represent different dose groups as per legend.

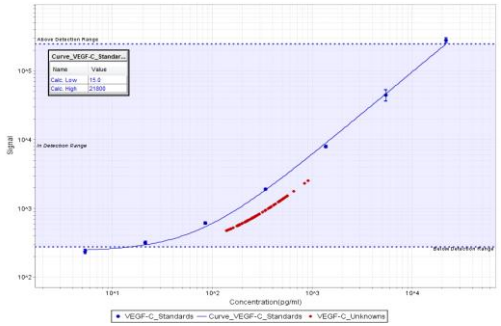
Figure 45: Standard curves for angiogenesis panel.

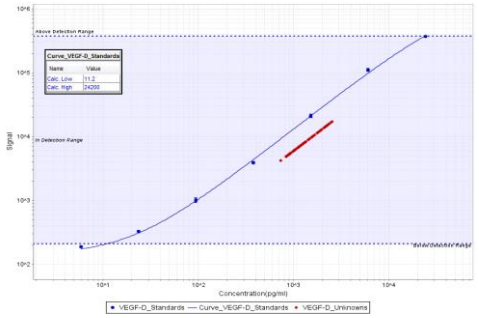


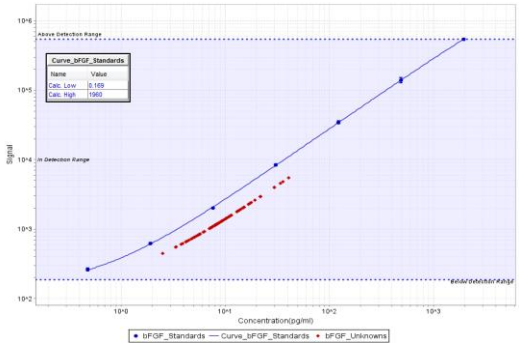






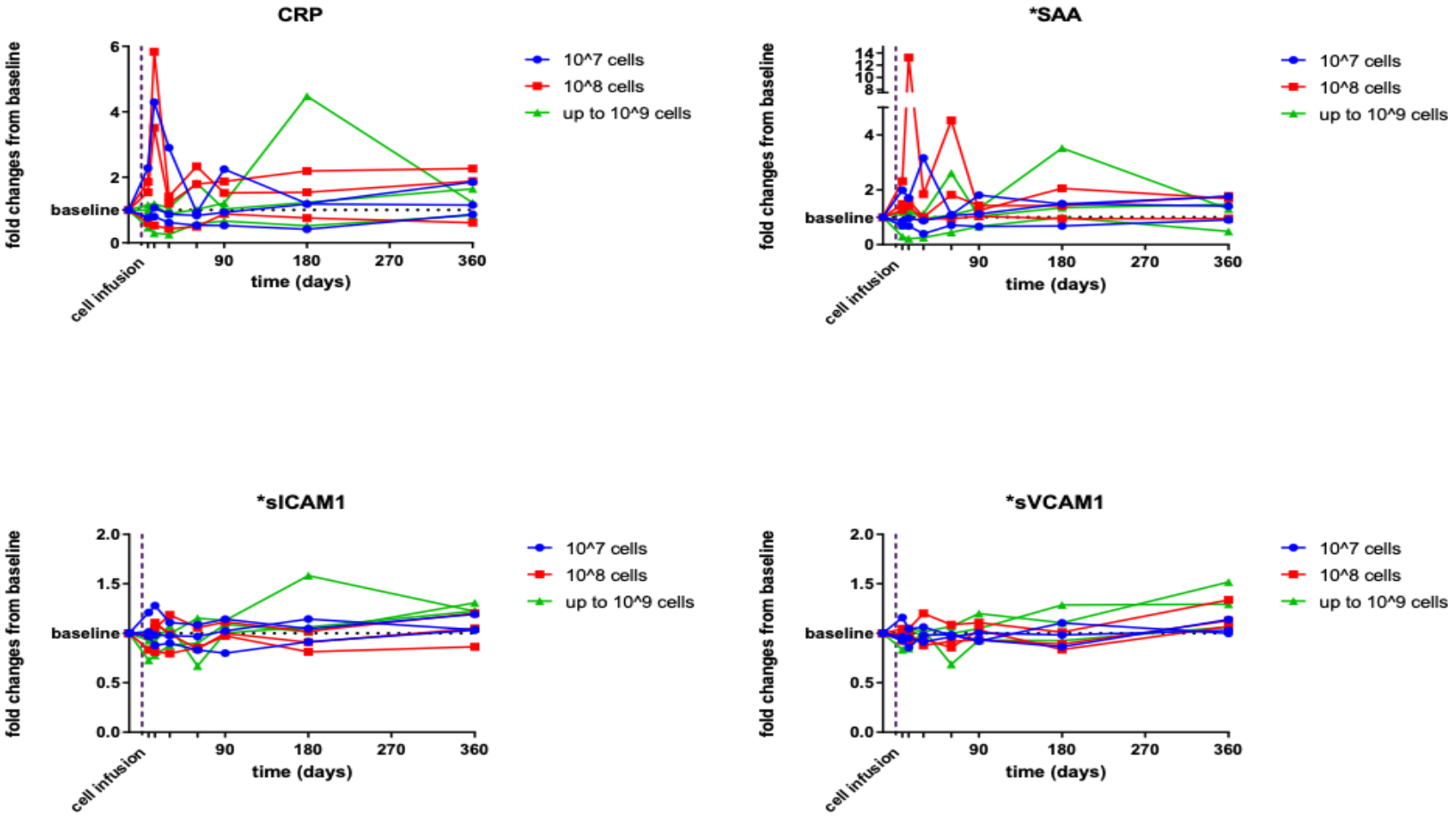






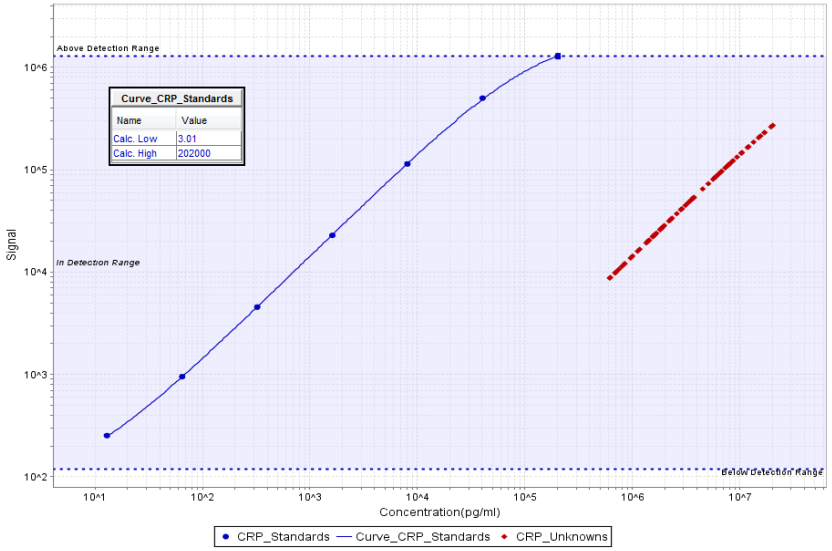
This figure shows the standard curve (blue line) of each cytokine in the Angiogenesis panel kit and their signal in our population. These are expressed as concentration (pg/ml) over signal. Cytokines with signals above or below the detection range should not be used for analysis.

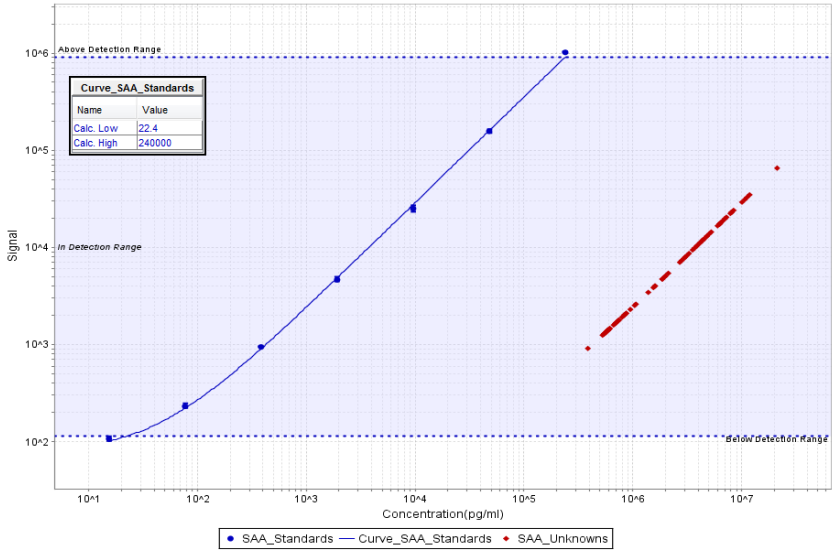
Figure 46: Vascular panel

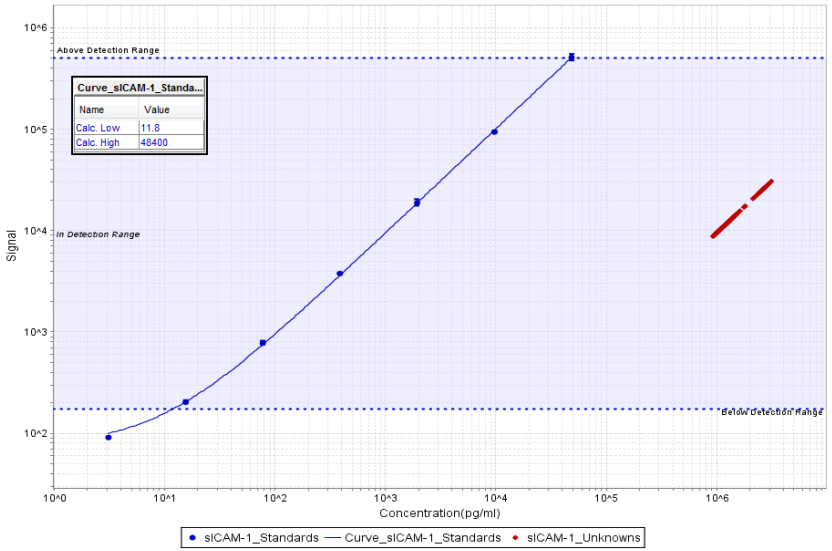


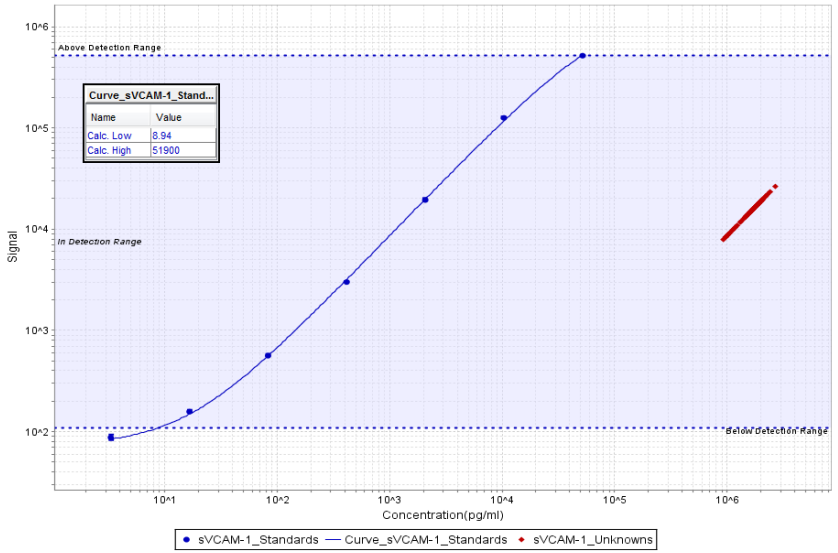
This figure illustrates changes in cytokines expressed as fold changes from baseline (y axes) over time expressed in days(x axes). Baseline defined as level prior to Macrophages infusion (noted as vertical broken line). Each line corresponds to a subject in the study. Colours represent different dose groups as per legend.

Figure 47: Standard curves vascular panel.









This figure shows the standard curve (blue line) of each cytokine in the Vascular panel kit and their signal in our population. These are expressed as concentration (pg/ml) over signal. Cytokines with signals above or below the detection range should not be used for analysis.

3.5 Conclusion

The overall results of the chemo-cytokines panel analysed for this study support the safety profile of our IMP when infused a single dose in cirrhotic patients with different aetiologies. We didn't observe significant changes from baseline and above all we did not witness a "cytokine storm" following infusion of macrophages.

Some crucial inflammatory cytokines like IL1β showed an increase following the infusion of the cell product, however this was transitory (with complete resolution at 90 days). While other inflammatory cytokines like TNFα appeared to remain stable after treatment with autologous macrophages or with minimal propensity to reduce. In 3/9 subjects IL2 levels were undetectable.

IL8 is one of the pathognomonic cytokines of MAS. Mean ± SD fold changes from baseline in during this study showed a drop in IL8 levels after infusion of macrophages with mean fold change of 0.88±0.20. This adds to the safety profile of the IMP. Dose related analysis revealed no pathological increase of IL8 associated to dose with means of 0.74, 1.08 and 0.80 at 10^7, 10^8 and up to 10^9 cells, respectively (see Chemotaxin panel 1).

Hepatic IFNγ levels have been associated with liver dysfunction and directly correlate to the degree of fibrosis independently by the aetiology. However elevated IFNγ levels in the liver are also inducers of apoptosis of hepatic stellate cells and this activity has been associated with remodelling of the ECM and inhibition of fibrosis progression. (39) In our population circulating IFNy level were raised, however marginally, with mean of 1.47 fold ± 1.07 and 1.47 ± 0.56 at 7 and 90 days after infusion of macrophages (see Pro-inflammatory panel 1), this is potentially related to the remodelling for the ECM either due to the macrophage infusion or as part of the natural history of the disease. This will be further elucidated in the phase 2 study.

Interestingly upon analysis of the angiogenesis panel levels of VEGF-A,C,D remain unchanged both at short term and at 3 months after infusion of macrophages in all subjects with mean fold changes around 0.95 ± 0.2 (see Angiogenesis panel).

Previous studies demonstrated very low levels of circulating VEGF in cirrhotic patients suggesting a low regenerative rate in the cirrhotic liver. (40)

However, previous published data suggest that bFGF may have a more central role compared to VEGF in the angiogenesis in the liver. In patient with HCC and in rat model following liver injury, levels of bFGF rise, respectively in serum and liver. (41, 42) In the rat model this phenomenon was associated with repair mechanisms. Our data demonstrated a 1.81 ± 1.88 and 2.27 ± 3.06 fold increase of bFGF respectively at day 7 and day 90 after infusion of macrophages. (see Angiogenesis panel) These results may support the regenerative/repair effect on liver injury induced by the macrophages therapy.

MCP-1 levels remains almost unchanged. This could be interpreted along with the unremarkable changes of the inflammatory cytokines as a sign of reduced inflammation in the liver parenchyma rather than a consequence of the MCP-1 binding the CCR2 receptor on the infused macrophages as they have a hugely reduced expression of it (as previously demonstrated during the GMP product development). (43)

As demonstrated by Fraser et al., the autologous macrophage product showed a secretory profile characterised by low IL1RA, IL10 and VEGF. This was switched by exposure to TNFα which stimulates secretion of all the above mentioned cytokines but not IL2. It is interesting how IL2 is not detectable in 2/9 subjects in our study population.(43) In our cytokine analysis IL1RA levels dropped minimally at 90 days after infusion of macrophages with a mean of 0.84 ± 0.24 fold change (see Cytokine panel 2)) while remain unchanged at day 7 with mean fold change of 1.08 ± 0.22. IL10 level show a similar pattern to IL1RA with a slight increase at day 7 and a drop at day 90 suggesting a possible switch to an anti-inflammatory environment immediately after the infusion of macrophages.

It is reassuring to note that the changes in circulating cytokines remained static following macrophage infusion. The systemic environment's stability and lack of cytokine changes immediately following infusion of macrophages could be a supporting factor for the stability of the infused macrophages' phenotype itself.

Although these results may be regarded as preliminary as they are collected from a small sample of subjects, they offer supporting evidence for the safety of the

autologous macrophage infusion. While MAS could have constituted a significant risk to the subjects infused with a large dose of macrophages, this risk remained theoretical as demonstrated by the very safe levels of cytokines (especially IL8). Whilst uncontrolled, some of the data showed above may support a shift into a “repair environment” triggered by the infusion of macrophages.

Results from the phase 2 trial will offer a more in-depth overview of the circulating cytokines in cirrhotic following infusion of macrophages compared to control subjects.

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Chapter 4. MRI Spectrometry to assess functioning liver parenchyma

With the contribution of Dr David Morris and Dr Scott Sample

Table of Contents

Figure and Table content

41 Introduction

There is a pressing clinical need to develop non-invasive techniques to evaluate liver function. Currently gold standard to assess progression of liver disease is liver biopsy, which is not only invasive but also carries significant potential side effects. It also has anatomical limitations: fibrosis can present as an inhomogeneous process and the needle biopsy could not be a representative sample. Serum markers discussed in the previous chapters have a prognostication value in terms of fibrosis stage. However currently to assess liver function we are limited by the use of blood tests (like albumin) or composite scoring systems (MELD, UKELD or CP score). These are only indirect measurements of the actual liver function. MR Spectroscopy on the other hand can assess the metabolic function of tissues and can provide insight into liver function via energy metabolism.

In this chapter we evaluate the use of ^{31}PMRS in human liver of healthy volunteers and cirrhotic subjects.

4.2 MR Spectroscopy (MRS)

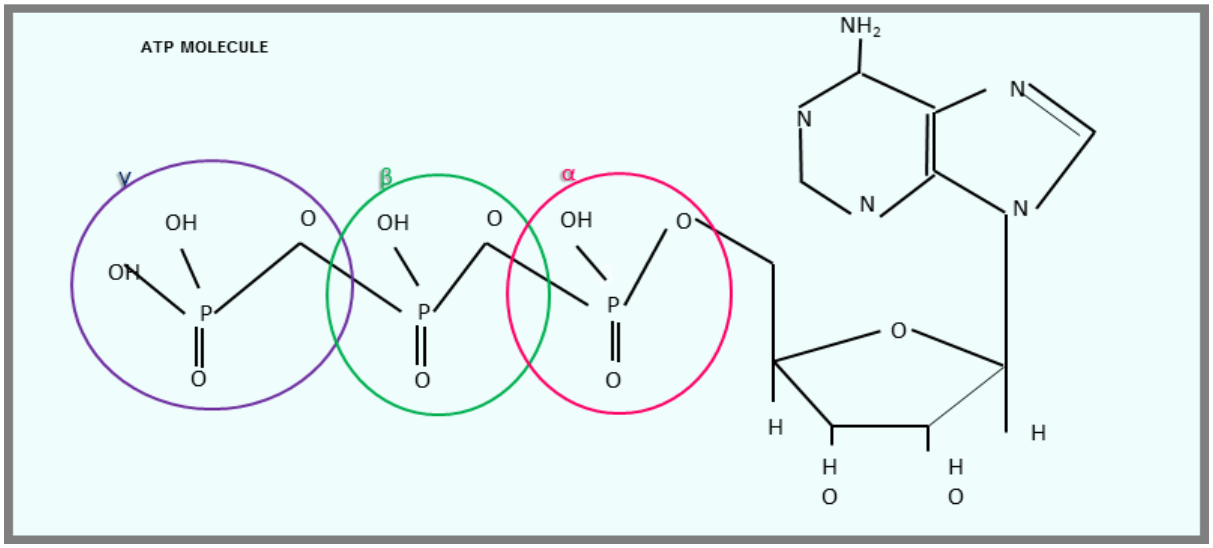
While clinical MRI can identify anatomy, MR Spectroscopy (MRS) is able to identify the metabolic composition of tissues and compare normal to abnormal functioning structures.

Hydrogen is extremely abundant in human tissues thus Hydrogen Spectroscopy ^{1}H-MRS has very high sensitivity but its spectrum is limited to compounds containing H. (1) On the other hand due to very low density of phosphate in human tissues multiple averages are required, taking a long time forcing at present Phosphorus Spectroscopy ^{31}P-MRS to remain a modality limited to research purposes. The main functional difference in ^{31}P-MRS compared to ^{1}H-MRS is the frequency required to mobilize the Phosphate molecules and the tuning of the receiver coils to match this frequency.

4.3 Phosphorus MRS (^{31}P- MRS)

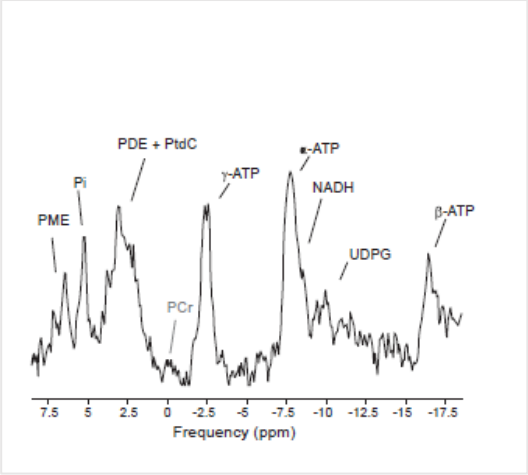
Phosphorus MRS (^{31}P- MRS) measures the cytoplasmatic phosphorus contained in energy compounds (like ATP), inorganic phosphorus (Pi), the membrane phospholipid metabolism (PME, a cell membrane precursor) and phosphodiester (PDE – cell membrane degradation).Therefore it measures indirectly, as a ratio between these peaks, the energy metabolism in tissues . ATP molecules contain 3 phosphate groups (α- ; β- ; γ-): these have different resonance frequency and have distinct signals in MRS.(2)

Figure 48: ATP molecule



This figure identify the ATP molecule structure and its 3 phosphate groups (α – pink; β – green and γ- purple).

Figure 49: In-vivo 31P-MRS of liver



This figure is an image from L Valkovic et al illustrating the In-vivo ^{31}P-MRS of liver. MRI spectrometry is a way for non-invasive assessment of their metabolism. This image show the spectra detected in liver.

Due to the low signal of ^{31}P-MRS, the signal to noise ratio (SNR= signal/noise) is low despite multiple averages. Signal is low because of the low phosphorus density and noise may be increased by patient motion. This can render the data unusable. However, allowing longer scanning time, more acquisitions and averages can be obtained . This increases the SNR at the expenses of the patient comfort, which increases the chance of motion. Moreover to overcome the low sensitivity of MRS, high sensitivity coils, applied in the proximity of the organ imaged, can be used. ^{31}P-MRS can be recorded with single (SVS) or multiple voxels techniques (i.e. Chemical Shift Images - CSI). While SVS have a higher SNR and therefore it is more reproducible and accurate, it is limited by the selection of a single volume of the

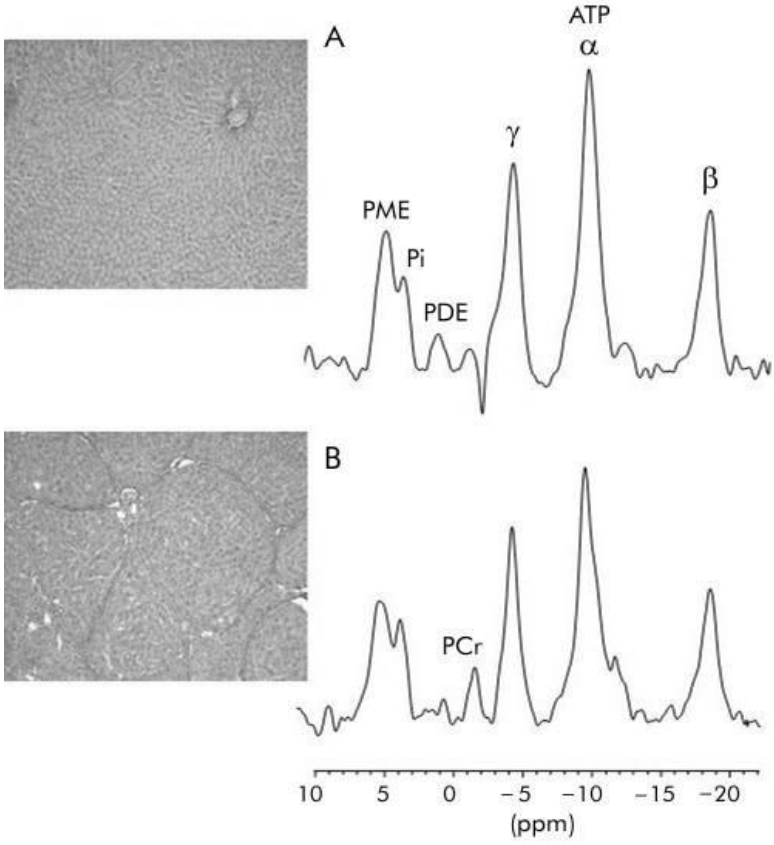
tissue that may not be representative, particularly if motion occurs during the acquisition. This selection of sample error can be avoided in the multi-voxel technique as a much larger area is covered and provides the option to select the most representative voxel (i.e. avoid the voxel showing signal from overlying muscle if trying to image the liver). However this is dependent on the size of the area that is getting sampled and the voxel size, i.e. if the voxel is large in relation to the region being sampled, the choice of voxel is limited. Point Resolved Excitation Spin-echo Sequence (PRESS) and the STimulated Echo Acquisition Mode (STEAM) technique are widely use methods of MRS.

4.4 Liver 31P-MRS: Preclinical studies

Previous studies have demonstrated that levels of ATP in liver tissues correlated with hepatic dysfunction following hepatic resection, acute liver injury and chronic liver disease mice models. In D-galactosamine (D-galN)-induced rodent model of acute liver failure the concentration of hepatic phosphorylated metabolites decreased in proportion to the severity of liver injury after 48 hours from injection of D-galN. A correlation was demonstrated between ATP and hepatic function and histological severity of liver injury. Moreover ^{31}P-MRS was able to accurately predict rats with clinically severe liver injury before the liver damage reached its peak so that at risk population could be stratified in advance to target therapy.(3)

Similarly in the chronic liver disease phosphate metabolites (ATP, PME, Pi) are affected by liver fibrosis. In a study by Corbin et al., the authors performed serial ^{31}P-MRS in different mice cirrhosis models: thioacetamide induced (TAA), carbon tetrachloride induced (CCL4) and common bile duct ligation (CBDL). (4) This approach allows to investigate if different injury modalities (hepatocellular vs biliary for example) can affect the results of MRS. In all mice models, phosphate metabolites progressively reduce with increase in liver fibrosis. In TAA models ATP levels and Pi levels show significant reduction once fibrosis reaches stage 4. Similarly in CCL4 model ATP levels lowered to a statistically significant level when fibrosis reached stage 4. PME and Pi level showed a down trend too. CBDL models showed similar results as the TAA model. The strongest correlation between stage of fibrosis and ATP level was displayed in the CCL4 cirrhosis model. These differences could be due to the distribution and pattern of liver damage: CCL4 mice model develop progressive fibrosis into cirrhosis with the classical progressive features of bridging fibrosis to micro and macro nodular cirrhosis.(4)

Figure 50: Hepatic MRS in rat models of chronic liver disease



This figure is taken from Corbin et al. Hepatic 31P MRS in rat models of chronic liver disease: assessing the extent and progression of disease. Gut. 2003 Jul; 52(7): 1046–1053 and it shows histological section of liver and corresponding hepatic 31PMRS from (A) a control rat and (B) CCL4 cirrhotic rats. PME, phosphomonoesters; Pi, inorganic phosphate; PDE, phosphodiesters; ATP, adenosine triphosphate. PCr, phosphocreatine resonance arising from abdominal wall muscle.

It is noted that the β peak of ATP (used to quantify the total ATP) is reduced in cirrhosis compared to normal liver. Also there is a down trend in PME and PDE, while Pi is unchanged. Despite histological correlation the authors failed to demonstrate a consistent correlation in the 3 models with serological markers of liver function and injury (respectively albumin and ALT). On the other hand liver cell to area ratio (LCAR) showed a statistically significant correlation with ATP level in the 3 models. This may suggests that loss of functioning hepatocytes may play a significant role in the decrease ATP levels in the liver.

4.5 Liver 31P-MRS: Clinical Studies

Energy metabolism is affected by pathological changes in the liver parenchyma: ATP is reduced in cirrhosis(5), fatty liver in type 2 diabetes mellitus and obesity.(6) This phenomenon has been attributed to “impaired ATP homeostasis” in diseased liver tissue. Other studies demonstrated reduction of PME/ATP and PDE/ATP ratio in autoimmune and viral hepatitis, suggesting an impairment of membrane function. (7) Liver ATP is also influenced by metabolic challenges, nutrition and exercise. Nevertheless assessing liver ATP and phosphate metabolites ration could provide useful information on the “energy state” of the liver.

Laufs et al used a Clinical 3 tesla scanner to validate a single liver voxel method in 85 healthy volunteers. The reproducibility of the method has been assessed taking in consideration intra and inter observer variability as well as intra and inter-day variability.(8) Moreover

Hakkarainen et al. assess the effect of diet and exercise on the ^{31}P-MRS liver spectra in healthy volunteers. Both meals and exercise affect the ratio between total phosphorus and γATP with respectively increase and reduction when subject was exposed to these conditions. (2) Therefore clinical liver ^{31}P-MRS should be performed at standard conditions: at rest and fasting.

When ^{31}P-MRS is used to assess fibrosis and liver function using energy metabolism in subjects with NAFLD a statistically significant correlation between PE/(PME+PDE), PME/ATP, PE/ATP and PE/TP ratios (markers of cell membrane turnover) is demonstrated. Ratio appears lower in subjects with no/mild fibrosis (F0-2) compared to advanced fibrosis F3-4. On the other hand GPC/ (PME+PDE) ratios (cell membrane breakdown products) appear higher in F0-F2 versus F3 and F4. As expected from pre-clinical studies γATP/TP ratio (marker of energy metabolism) is reduced with progression of fibrosis. (9)

Cardiac ^{31}P-MRS protocol published by Rodgers et al.(10) represents a good model for the application of liver ^{31}P-MRS in clinical setting. A body coil is used to initially identify the anatomy and position the ^{31}P spectroscopy coil over the interventricular septum. The ^{31}P coils are adjusted using RF sweeper. The ^{31}P MR spectra are then acquired with a short repetition times and multiple averages to increase SNR. Routinely for ^{31}P-MRS a 3D CSI protocol is used.

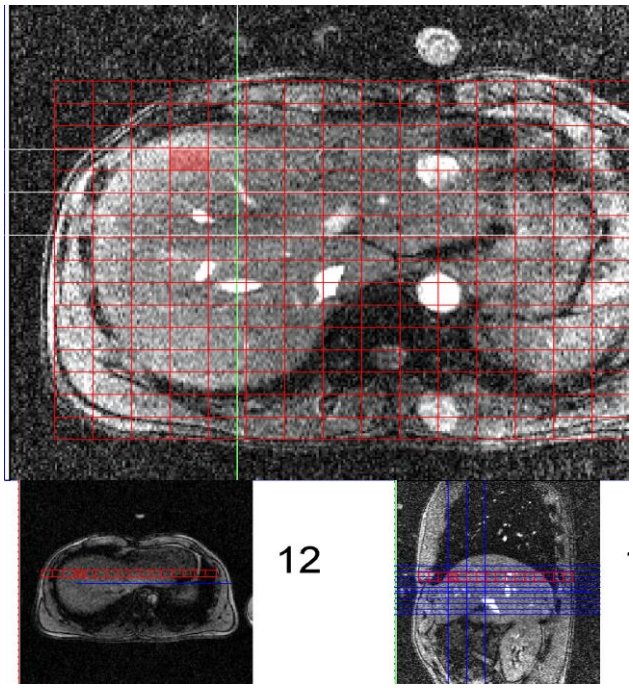
4.6 Liver 31P-MRS – protocol developed at University of Edinburgh Clinical Research Images Centre

The protocol adopted by the Clinical Research Images Centre (CRIC) of the University of Edinburgh was based upon the protocol designed by Rodgers et al.(10) using a multi element surface coil. The protocol was validated with images obtained from 5 healthy volunteers (HV). This was thereafter adopted in the subjects entering the phase 2, RCT of MATCH trail with paired 31P-MRS.

All subjects scanned were fasted prior to the scan. All scans occurred in the morning with minimum fasting time of 4h.

We collected a total of 80 readings. As a method of quality assurance all values with error >100% were removed from the analysis: thus 12/80 values had to be discarded because of above, 3 values from patients and 9 from HV.

Figure 51: 31P- MRS picture from a HV.

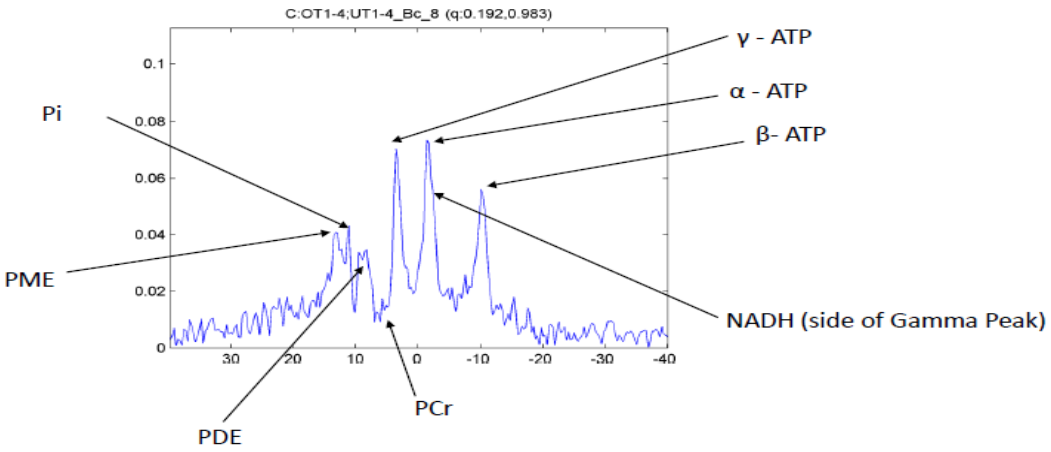


This figure shows a section of MRI with voxel panels. The analysis was performed on a selected voxel in the liver as illustrated from the picture. The voxel was chosen per quality of data and consistency.

While the data collected is small, no meaningful statistical analysis could be performed but I will describe below the results found and compare HV versus patients.

For all the individuals scanned we recorded selecting a single voxel the peaks of αATP, βATP, γATP, PCr (phosphorus creatinine), Pi (inorganic phosphate), PDE (phosphate di-esterase), PME (Membrane phosphate), NADH (Nicotinamide adenine dinucleotide), PEP (phosphoenolpyruvate).

Figure 52: Phosphorus liver spectra from single voxel



This figure shows an example of a patient scanned in the Clinical Research Images Centre (CRIC), University of Edinburgh.

The results will be presented as peak ratios: NADH/total esters, PME/PDE, PME/ATP and αATP/total phosphorus. Previous data on subjects with diffuse liver disease suggest an increase in PME/PDE compared to HV.

4.7 HV data

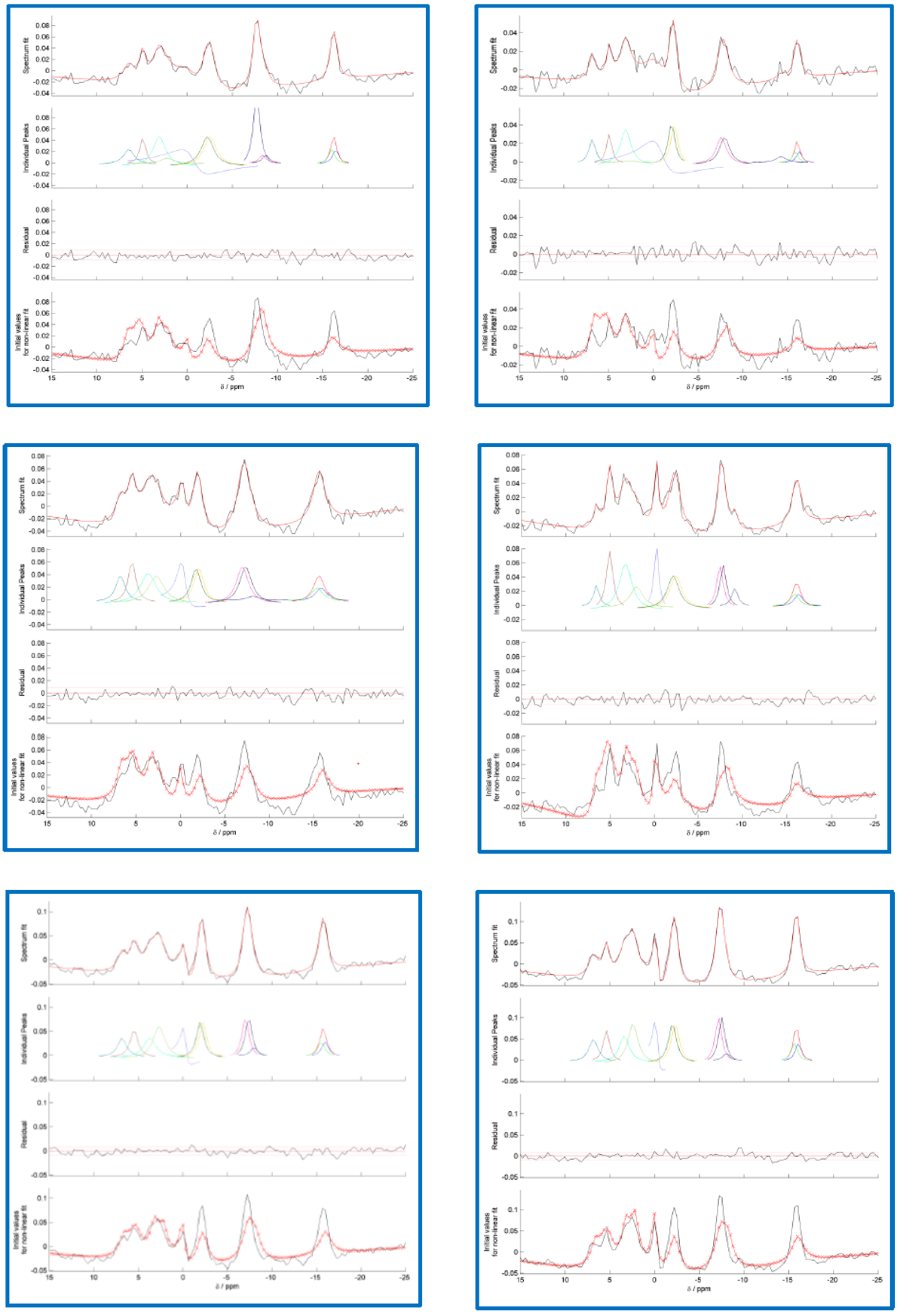
4 HV were scanned twice in comparable conditions, in one volunteer the scans could not be used .. Measurements that did not meet the QA criteria were removed from the analysis.

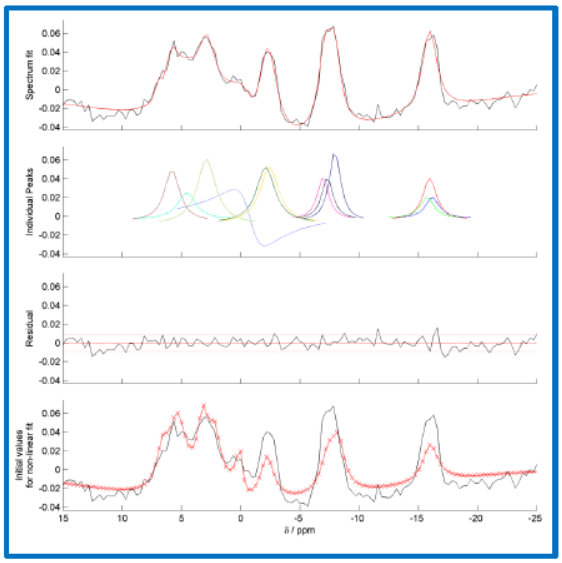
Table 16: 31P-MRS results from 4 HV - the ratio are represented as mean of the peak area ratio

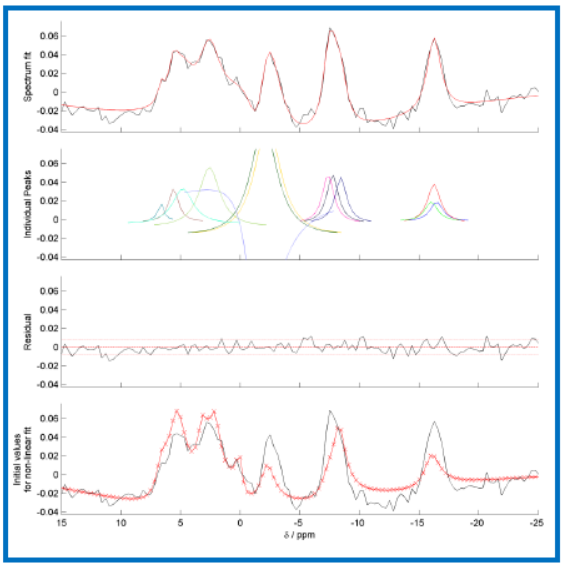
HV	NADH/tot ester	PME/PDE	PME/ATP	αATP/tot phosp
HV 1-1		0.28	0.45	0.26
HV 1-2	0.10	0.10	0.29	0.14
HV 2-1	0.75	0.30	0.46	0.06
HV 2-2			0.44	
HV 3-1	0.12	0.49	0.38	0.29
HV 3-2	0.08	0.44	0.46	0.26
HV 4-1	1.03	0.08	0.05	0.20
HV 4-2	0.47	0.09		
HV 5	0.40			0.18

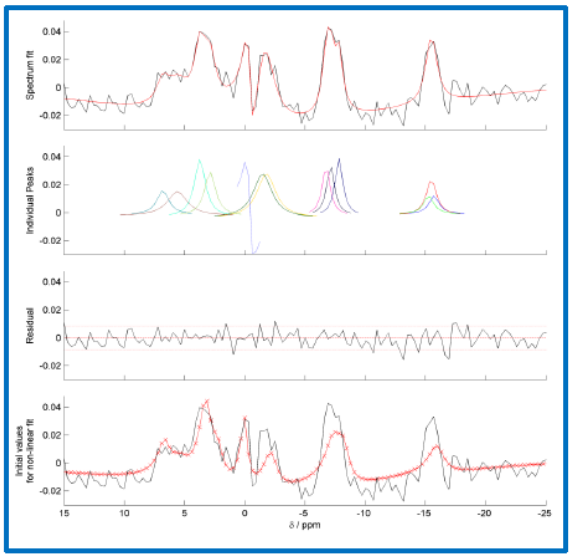
The mean±SD of NADH/tot ester is 0.42 ± 0.38; PME/PDE 0.28±0.22; PME/ATP 0.30±0.22; αATP/tot phosph 0.23±0.55.

Figure 53: Liver Spectra of HV









This image represents spectra of 5 HV with 9 scans. Peaks from left to right represent respectively: PME, Pi, PDE, PCr, γATP, αATP, NADH, βATP.

4.8 Cirrhotic patients from phase 2 MATCH study

In this paragraph I will summarise the data gathered from 5 subjects enrolled in the phase 2 MATCH trial. The baseline 31PMRS only have been analysed here and not the paired scans after macrophage infusion or at 90 days in the control group.

Data are expressed as above for the HV. Once again measurements that did not meet the QA criteria were removed from the analysis.

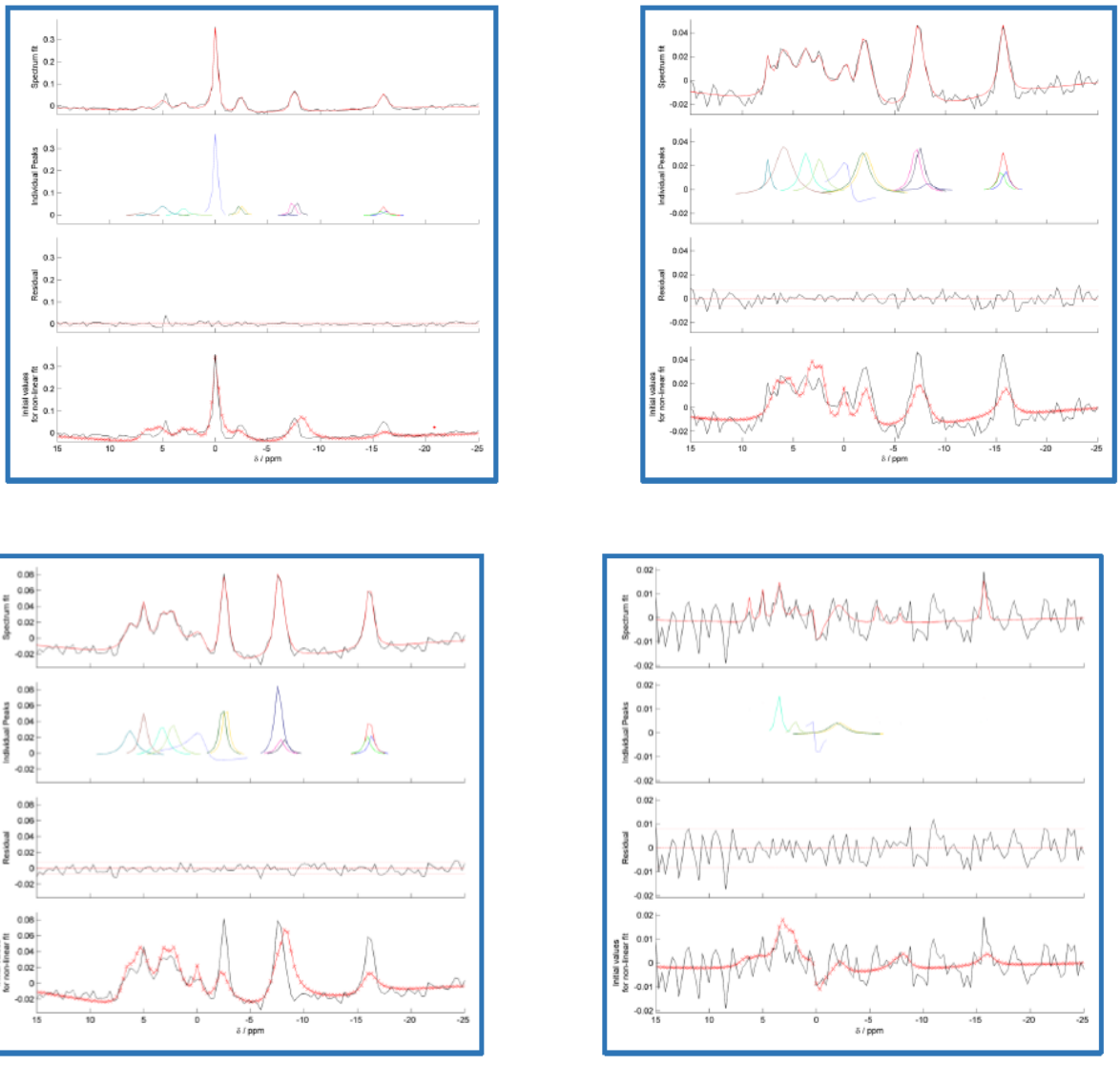
Table 17: Results from MATCH phase2 scans.

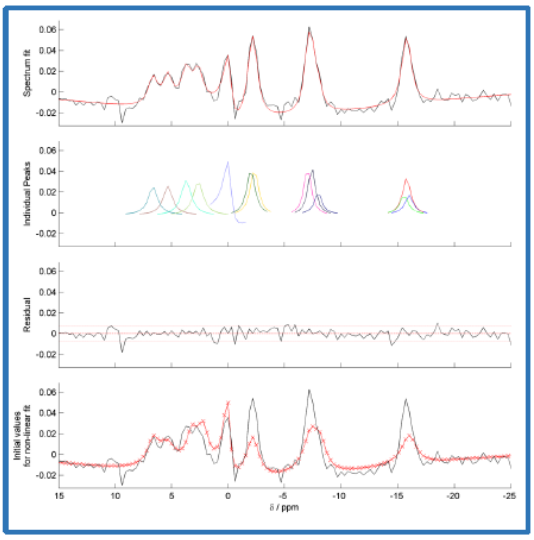
subjects	NADH/tot ester	PME/PDE	PME/ATP	αATP/tot phosp
Sub 012		1.31	1.08	0.29
Sub 015		0.17	0.15	0.21

Sub 016	0.76	0.62	0.83	0.10
Sub 018	0.09	0.24	0.35	0.09
Sub 021	0.22	0.57	0.40	0.27

The mean±SD of NADH/tot ester is 0.36 ± 0.35; PME/PDE 0.58 ± 0.45; PME/ATP 0.56 ± 0.38; αATP/tot phosph 0.19 ± 0.09.

Figure 54: Spectra of 5 subjects in phase 2 MATCH study.





Peaks from left to right represent respectively: PME, Pi, PDE, PCr, γATP, αATP, NADH, βATP.

4.9 Conclusion

While the data shown here is very preliminary, they illustrate a reproducible and reliable technique that can be used to assess liver disease.

The MRI physicists evaluated both single voxel and whole liver spectrometry and noted that when choosing a “good” voxel, this would provide good quality data reducing the scanning time. Cirrhotic patients tolerated the MRI very well.

Comparable to available literature our HV and cirrhotic subject data suggest that PME/PDE ratio increases in liver disease (with increase of 48.27% in cirrhotic patients).

We are looking forward to the paired 31PMRS results from the MATCH RCT.

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Chapter 5. Future work and conclusions

Table of Content

5.1 Introduction

The rationale articulated in this thesis highlights the clinical need to develop alternative strategies to treat liver disease. Furthermore, there is a requirement to identify reliable non-invasive methods to assess the severity of liver disease and the response to treatment.

Despite the significant progress made in translating “bench work” to “bed-side” these are still early steps towards more concrete and widely adoptable results in clinical practice.

During my time as research fellow undertaking the work described in this thesis, I also had the opportunity to facilitate the transition from a phase 1/first-in-human study into the phase 2/randomised controlled trial. Initially, the phase 2 trial posed significant

recruitment challenges. In this chapter I will describe the strategies I developed to mitigate the risk of insufficient recruitment.

The encouraging results of the phase 1 trial opened the way to macrophages as a safe therapy for the treatment of liver disease. However, the autologous macrophage infusion produced as described in chapter 2 also presents challenges that may limit widespread adoption.

In this chapter I will describe future and ongoing work related to the MATCH trial and to the development of macrophages as a potential therapeutic option for patients with liver disease.

5.2 IMP development

The Scottish National Blood Transfusion Services (SNBTS) team continues to improve the efficiency of the production line not only to optimise the process but also to ensure maximization of the cell yield whilst maintaining the same quality characteristics of the end product. The published data (1)describe the original GMP approved process. During the process the cells were washed and the media was changed twice: at day 2 and day 4 with endotoxin testing. This had implications for the trial schedule as cell collection could only occur on Mondays to avoid weekend media change. It also had a more direct effect on the macrophage end product's yield as the cells were manipulated twice resulting in inevitable cell loss during the process. Accordingly, the SNBTS production team validated a single manipulation (wash and media change) at day 3 or 4 with equal quality and safety results and improved yield. This also allowed cell collection on other days than Wednesday providing more flexibility for the trial schedule.

5.3 Recruitment challenges and strategy for MATCH RCT

5.3.1. Analysis of recruitment issues

The MATCH RCT was designed (as described in 2.9) as a multiple infusions trial. Participants randomised to the cell product arm of the trial were planned to receive 3 infusions of autologous macrophages at time intervals of around 28 days. Because the IMP has to be used fresh, the participants in the treatment arm had to undergo 3 apheresis procedures (once a month) to collect the required monocytes for the macrophage infusion production. This of course multiplied the risks of adverse events

related to the apheresis. It has to be highlighted that due to production and safety reasons the visit schedule during the 3 months of treatment (apheresis x3, infusion of macrophages x3 and 2 safety visits x3) is fixed with a calendar agreed in advance by the SNBTS production team. The lack of flexibility is a logistical challenge for the research team, but an understandable consequence of complex trials such as MATCH. This degree of required monitoring in the trial squedule, combined to create a situation where the subjects found attending the study slots difficult.

Therefore, in the first 8 months of the RCT we encountered significant difficulties in recruitment. Of the people contacted in the aforementioned period, 8 out of 15 who were approached declined to participate because the trial appeared too onerous in terms of visits and time commitment. As mentioned, participants randomised to the cell treatment arm have to attend the hospital once weekly on fixed days for 12 weeks. This presented a barrier to recruitment and skewing of the age/demographic towards an older, “non-working” population.

5.3.2 Strategies to improve recruitment

Once the reasons behind slow recruitment were analysed, strategies could be formulated to ameliorate rate of participants’ enrolment. The core trial team coordinated a meeting with the Sponsor and Monitors, as well as the TSC chair. Here, the proposal of amending the trial protocol was discussed in detail.

The two strategies proposed by the trial team were as follows:

1. Reduction in the number of cell infusions from 3 to 1
2. Expanding the trial to include additional sites

Reduction in number of infusions: this solution stemmed directly from feedback received from the potential participants that we approached. The rigidity of the study design and the number of weekly visits was deemed too onerous for most of the individuals who declined to participate.

The decision to set to 3 the number of infusions in the RCT was taken to maximise the therapeutic effect of macrophages. In the pre-clinical models of mouse liver fibrosis macrophage cell therapy was given as a single injection and so the move to a single macrophage infusion seemed justifiable. (2, 3)

It is important to highlight this as once both the Sponsor and Monitors and the TSC chair agreed on supporting the amendment proposal, the trial statistician was asked to repeat a power calculation for the study based on a single infusion design. While the phase 1 study offered some preliminary data, these couldn't be used as efficacy analysis in the power calculation. The recommendation was to maintain the same power excluding the 3 participants who received 3 infusions of macrophages. These 3 individuals will be included in the final reporting, but not presented in the primary endpoint analysis.

A protocol amendment for the change in study design to a single infusion was submitted and the Sponsor recommended waiting for this to be approved before considering expanding the trial to other centres.

Multicentre trial: it became apparent that to ensure a constant flux of patients in the trial so that the recruitment milestones could be met, the study had to be expanded to other centres. Planning around a multicentre amendment required significant forward thinking.

Firstly, the SNBTS facility at Centre for Regenerative Medicine (CRM) was the only accredited cell production centre. This has 3 main implications: a) only a single product can be manufactured at any time in the facility; b) the cell collection via apheresis has to reach the production centre on the same day to be purified and placed into culture; c) transportation of the IMP from the production centre to the infusion location needed to be validated. A solution to overcome these limitations is to open other production centres across the country. However, this requires significant planning and training from the SNBTS team. Even once appropriate production centres affiliated to hospital facilities with expert hepatology groups were identified and approached, the timescale to establish local production of the IMP appeared to be too long for practical use in the trial schedule. Indeed, the MHRA requires evidence that the macrophage product released in Edinburgh by the CRM SNBTS team is equivalent to the one produced in other centres.

While the work around the expansion of the cell production to other centres in UK started, the trial team felt the need for a more timely solution to the recruitment issues. Therefore, we suggested to open the trial to other centres in Scotland located at a distance which allowed participants to travel to Edinburgh. We proposed therefore to

maintain the production centre in Edinburgh and, to avoid any other issues around the transportation of the apheresis collection or the IMP, we suggested the subjects recruited in other centres to attend the Edinburgh facility for the cell collection and the IMP infusion visits. Effectively the other centres participating in the study will be "recruiting" centres leaving the most challenging visits to the lead centre and the more experienced core trial team. The centres which met the characteristics required are Ninewells Hospital (Dundee) and Glasgow Royal Infirmary (Glasgow).

After preliminary contact with local teams and the identification of a PI in both centres, myself and the trial manager arranged feasibility visits and discussed the day-to-day activities involved in the trial. These visits were designed to highlight potential site-specific obstacles to the smooth running of the trial. The main issues that were identified were:

1. Patient travel: we agreed pre-booked and paid taxis would be preferable for long journeys compared to the re-imbursement approach we had in place for the trial at the time

2. Patient overnight stay: we discussed the possibility for Dundee patients (who would need to travel the farthest) to spend the night in a paid hotel room in Edinburgh. This option remained opened but was not favoured by the Dundee team who know their patient population well.

3. Mandatory quality-assured screening for transfusion-transmissible infections screening tests as dictated by Humant Tissue Act (HTA): at screening visit a series of tissue trace blood testing (mandatory microbiology) has to be obtained as per HTA rules. These blood samples have to reach Edinburgh and Glasgow to be analysed and recorded. Donor centres and apheresis units in Dundee and Glasgow respectively have daily transport to the SNBTS lab testing facility. We agreed this transport method should be used to transport our blood samples.

4. Apheresis consent and vein assessment: During screening visits patients are consented for apheresis and their veins are assessed by trained staff in the Cell Apheresis Unit (CAU) in Edinburgh. This arrangement could not operate in other recruiting centres. We agreed the best strategy was to educate the investigators and study nurses at the sites to perform this tasks. Consent could be reconfirmed on the apheresis day by the staff in Edinburgh CAU.

5. Study-specific research samples: during every follow up visit 15 ml of anticoagulated blood (citrate) and 4.9 ml of serum are collected from each subject. While serum can be separated and easily frozen at -80C, the anticoagulated blood has to be processed fresh to isolate monocytes. These samples should reach the Forbes laboratory at the CRM facility in Edinburgh within 8 hours of collection. This requires an *ad hoc* taxi service. While this is a possibility, we are aware these samples do not contribute to primary or secondary endpoints, although their analysis will support observations about safety and mechanism of action.

6. MRI/MRS: while the MRI/MR spectroscopy provides exploratory biomarker/mechanistic data, these do not contribute to the primary or secondary endpoints of the trial. The MRS requires specific coils and software that the other recruiting centres do not have in place. We therefore decided that only participants recruited in Edinburgh will undergo MRI.

7. Quality checks: following screening and randomization visits the paper case report forms (pCRF) requires quality checks (QC) to confirm eligibility before the patient is randomised and potentially undergo apheresis. While the first 2 visits are undertaken at the recruiting centre, apheresis and infusion occur in Edinburgh. QC has to occur in a timely manner in the recruiting centre and eligibility has to be confirmed with the Edinburgh site. Both recruiting sites agreed on performing QC checks.

8. Cell product release's calendar: as described above there is currently a single production centre. Therefore, only a single collection product can be transported to the study sites at a time (i.e. if a subject in centre A is randomised to cell arm, centre B and C cannot randomise until another slot for cell release is available). Although seemingly straightforward, this requires tight planning and efficient communication between centres. We therefore created a dedicated NHS.NET email address to share study visits' calendar and for the 3 centres to inform each other's on patients screening.

5.3 Further development of the autologous macrophage cell product for cirrhosis treatment

While the opening of neighbouring Scottish centres helped recruitment, we are still working in expanding to other UK centres while maintaining production centralised in Edinburgh. At the same time, validating other production facilities will facilitate the schedule flexibility and potentially recruitment pace. These developments should consolidate two important aspects: continue improvement in recruitment for the trial with the aim of achieving the primary endpoint within the proposed timeframe and to prove that this IMP could be used clinically across the country with additional production centres geographically distributed so that the cell product could be delivered to all areas.

Transporting the final macrophage cell product to a centre a few hours away from the production facilities could be easily validated by a few test runs that check the product stability and quality and safety characteristics once it has arrived at the destination, while kept in a constant environment during transit (i.e. medibox in a van). However, the leukapheresis product needs to reach the production facility on the same day of collection and sorted fresh. Leukapheresis takes 4-5 hours. Routinely the collected monocytes are not available to the SNBTS production team before 2pm. It takes several hours to separate, purify and culture the monocytes and often the scientists at SNBTS work a number of extra hours to complete the process. If we have to add the time to transport the cell collection to the production facilities this will potentially result in the team having to work overnight to complete selection and place cells in the culture bags. A solution to this problem would be to hold the leukapheresis cell collection overnight and transport it/ sort it on the following day.

5.3.1 Macrophages For Regenerative Medicine In Cirrhotics II (MORE2)

To validate a new SNBTS production facility and the overnight hold of the cell collection we set up another clinical study called Macrophages For Regenerative Medicine In Cirrhotics II (MORE2) which aims to collect monocytes from patients with cirrhosis and use the cells to address the two issues discussed above, rather than for therapeutic use. The study received REC and R&D approval on 18/01/19 and 30/01/19 respectively. The full trial protocol is available in the appendix of this manuscript.

MORE2 study also allows for some of the monocytes collected from patients with cirrhosis to be used by the Forbes research team to improve the macrophage product. The autologous cell product requires collection from an individual well enough to undergo leukapheresis. At present the IMP had to be used fresh on the day of release (or it could be held for up to 48h in stable conditions). Thus, the scientists in the Forbes laboratory have been studying cryopreservation of the macrophage product. They are also investigating ways of improving the therapeutic properties of the macrophage by utilising different technologies including genetic manipulation. There is also a significant drive to continue optimising the production process. Experiments are ongoing looking at changing the culture time, culture media and other environmental characteristics to enhance the therapeutic properties of the macrophage end product.

5.4 Macrophage cell therapy for acute liver failure

As discussed in Chapter 1 macrophage cell therapy has also been used in experimental rodent models of acute liver failure with varying degrees of success. (4)

Macrophages express cell surface receptors (Mer, phosphatidylserine receptors, lectins, and scavenger receptors) that recognise dead or dying cells and facilitate their uptake and degradation.(5-8) Macrophage-mediated removal of dead cells is known as efferocytosis. For example, following paracetamol induced liver injury, early and widespread hepatocyte necrosis is observed. If treatment with N-acetylcysteine is not provided in a timely manner, liver necrosis can result in over 50% of hepatocyte loss, generating a massive inflammatory response mediated by DAMPs.(9) At the peak of liver injury, a loss of KCs is also observed, causing a reduction in barrier and immune function against translocation of gut lumen bacteria via the portal system into the liver. This reduction of KCs compromises the innate immune function of the mononuclear phagocyte system of the liver, predisposing to bacterial and fungal infection. Moreover, bacterial and fungal infections are leading causes of death in patients with acute liver failure despite antimicrobial use.(10) Therefore stimulating macrophages' activity or proliferation could represent a strategy to overcome fatal hepatotoxicity and systemic inflammation and infections following paracetamol overdose.

Because of its scavenger, immunomodulatory and hepatothrophic function, the macrophage represents an intuitive target to attempt the modulation of immune response in the liver during the acute phase of liver injury. Pre-clinical data support

this. In murine models of acute liver failure, the administration of colony stimulating factor 1 (which promotes survival, proliferation and differentiation of macrophages) leads to improvement of ALT.(11) In these models both resident and infiltrating macrophages migrate to areas of necrosis. Equally in ALF mice models ablated of Kupffer cells or lacking of infiltrating monocytes-derived macrophages, necrosis continues to develop translating into raising ALT. (12-14)The paracrine functions of macrophages contribute to resolution of inflammation in the injured liver: IL10 has hepato-protective activity; VEGF promotes neoangiogenesis and liver sinusoidal endothelial cells proliferation; WNT signals enhance liver proliferation. (15-19)

As mentioned several times in this thesis macrophages exhibit different phenotypes with different roles in controlling inflammation. These phenotypes are influenced by the surrounding microenvironment.

The Forbes's group are establishing pre-clinical models to evaluate a macrophage cell product aimed to ameliorating hepatic injury in acute liver failure caused by paracetamol. The macrophage cell product's characteristics have to be different from those described here in the MATCH study for chronic liver disease. While cirrhotic patients may have a prolonged period of clinical stability allowing manufacturing of an autologous cell product (via apheresis collection of circulating monocytes), patients with acute liver failure are by definition very unwell and unstable and would not tolerate apheresis. Significant effort is currently being invested in producing an allogeneic macrophage product that can be cryopreserved and used at the point of need. Also, careful analysis of the therapeutic window is necessary to ensure that infused macrophages of the desired phenotype reach the liver parenchyma and reverse liver injury and promote healing, rather than exacerbating the inflammatory cascade.

5.5 Conclusions

This chapter describes exciting recent developments and ongoing work in the field of translational medicine in liver cirrhosis, and acute liver failure, where macrophages have been identified in mouse models as important cells in fibrosis regression(20) and liver regeneration (19, 21). They have been tested as a cell therapy in animal models (2, 3) and shown to have efficacy. Based upon these pre-clinical data, they have been taken through a process of "GMPisation" and now tested in a phase1 and 2 study. The phase 1 data showed clearly the safety of these cell products in patients with cirrhosis.

The phase 2 data will either confer or refute the potential signals of efficacy seen in the phase 1 study.

Results of the MATCH phase 2 trial will evaluate the efficacy of autologous macrophage cell therapy and will pave the way for further studies to explore either molecular targets or development of a more readily available allogeneic cryopreserved product.

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Appendix

Table of Content

1. Appendix 1. Publication of safety results of MATCH phase 1 trial

Safety profile of autologous macrophage therapy for liver cirrhosis

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ABSTRACT

Therapies to reduce liver fibrosis and stimulate organ regeneration are urgently needed. We conducted a first-in-human, phase 1 dose-escalation trial of autologous macrophage therapy in 9 adults with cirrhosis and Model for End-Stage Liver Disease (MELD) score of 10-16 (ISRCTN10368050). Groups of 3 participants received a single peripheral infusion of 10^7, 10^8, or up to 10^9 cells. Leukapheresis and macrophage infusion was well-tolerated with no transfusion reactions, dose-limiting toxicities or macrophage activation syndrome. All participants were alive and transplant-free at 1 year, with only 1 clinical event recorded, the occurrence of minimal ascites. The primary outcomes of safety and feasibility were met. This study informs and provides a rationale for efficacy studies in cirrhosis and other fibrotic diseases.

BACKGROUND

Globally, liver cirrhosis currently causes 1.16 million deaths every year. In the US, among people aged 45–64 years, chronic liver disease is the 4th leading cause of death.[1] Cause-specific interventions are effective, but patients often present with advanced liver disease and cirrhosis. No curative options are available for cirrhosis except for organ transplantation which requires major surgery and lifelong immunosuppression. Donor organ availability also restricts access to transplantation.[2] Alternative therapies to treat cirrhosis are therefore being developed including cell therapies.[3,4]

The macrophage is a cellular regulator of liver fibrosis deposition and resolution.[5] During disease progression macrophages release signals which drive inflammatory cell recruitment and activation of hepatic stellate cells to produce extracellular matrix (ECM). Following cessation of injury, macrophages release matrix metalloproteinases (MMPs) that promote fibrotic ECM degradation, and factors that dampen the inflammatory response[6-8,9] and drive liver regeneration.[7,10]

In mouse models of liver fibrosis, macrophages injected via a peripheral vein home to the liver, express MMPs, and recruit host immune cells to liver scar via chemokine expression, ameliorating liver fibrosis, stimulating liver regeneration and improving function.[10] Circulating $CD14^+$ monocytes can be isolated from cirrhotic patient mononuclear cell (MNC) leukapheresis products with high yield and purity and can be differentiated using Good Manufacturing Practice (GMP)-compliant processes into macrophages with a comparable phenotype to those from healthy volunteers.[11,12] These macrophages can also resolve liver fibrosis in mouse models.[12] These data prompted us to conduct a first-in-human, phase 1, single-arm, dose-escalation clinical trial in people with cirrhosis evaluating maximum-tolerated dose and safety of peripheral infusion of *ex vivo* matured autologous monocyte-derived macrophages.

RESULTS

Trial population, baseline and treatment characteristics

11 participants (4 female and 7 male, mean age 58.54±5.85) with compensated liver cirrhosis and MELD score between 10 and 16 attended a single centre (Royal Infirmary of Edinburgh, UK) for screening between 08 August 2016 and 27 March 2017 (Fig. 1). Two individuals did not meet screening criteria. Nine participants were enrolled in the trial and were followed-up for 1 year to 06 April 2018. Demographic and baseline characteristics of study participants are shown in Table 1. The mean duration of cirrhosis was 5.22±4.22 years. All participants were abstinent from alcohol at the time of recruitment except for one individual who had a history of intermittent low-level alcohol consumption (1-10 units per week). A week before the planned treatment, participants underwent a standard leukapheresis to collect circulating monocytes. Monocytes were isolated from MNC and the Investigational Medical Product (IMP) produced in a GMP-accredited facility (Extended Data 1).

Each group of 3 participants (9 in total) received a single infusion of autologous macrophages at 10^7, 10^8 or up to 10^9 cells, respectively in a dose-escalation manner. All participants were successfully evaluated for safety, feasibility and maximum-achieved safe dose of autologous macrophages. We also measured changes in: markers of liver fibrosis (serum Enhanced Liver Fibrosis (ELF™) test (Siemens Healthineers, UK), serum PRO-C3 and C3M (Nordic Bioscience, Denmark) and transient elastography (Fibroscan®, Echosens, France)); liver function (MELD and UKELD scores); health-related quality of life (HRQL) using the Chronic Liver Disease Questionnaire (CLDQ) instrument; transplant-free survival and number of clinical events related to decompensation of cirrhosis.

Safety outcomes

All participants completed 1-year of follow-up after macrophage infusion. No participants withdrew from the study and none developed acute transfusion reactions during macrophage infusion or in the 12h post-infusion observation period. A total of 3 serious adverse events were recorded; these were assessed as mild in severity, unrelated to the IMP and there were no sequelae (Table 2). There were 70 adverse events documented in the reporting period (Table 2). A single clinical event occurred, described as a small volume of ascites around the liver on ultrasound. 9/22 (41%), 8/19 (42%) and 6/29 (21%) adverse events were considered possibly related to the IMP in the 10^7, 10^8 and up to 10^9 cell dose groups, respectively. Overall, 56% of adverse events were considered unrelated to the IMP. No dose-toxicity relationships were identified. At the end of the study period all 9 participants were alive and transplant-free.

Serum ALT and bilirubin changes at 90-days were respectively 0.88±0.21 and 0.80±0.30-fold from baseline. Fluctuation in platelet count is common in patients with cirrhosis and portal hypertension, but we did not observe a reduction in platelets to lower than 30% from baseline or clinically significant thrombocytopenia. The baseline total white cell count varied in this study population. As expected, total circulating leukocyte counts were affected by leukapheresis, but returned to baseline prior to infusion (7 days after leukapheresis). In some individuals we noted a small and transient increase in white cell count following infusion of macrophages which did not persist beyond 7 days post-infusion (Extended Data 2). Serum cytokines (including IL1a, IL6, IL8, IL10, TNFa and IFNγ) did not change significantly from baseline (Extended Data 3). Specifically, levels of IL8 (which correlate with risk of macrophage

activation syndrome (MAS)) decreased transiently after macrophage infusion, with a delta of -8.23±14.39 pg/mL at 30 days and of -1.58±13.54 pg/mL at 90 days.

Secondary outcomes

At day 90 following macrophage infusion, six out of 9 participants showed a decrease in MELD score (Fig. 2 and Extended Data 4). For all patients, the MELD at baseline was 11.88±1.40 (range 9.90 to 13.87) with a mean Δ-MELD at 90 days of -1.12±1.87 (range -4.90 to 1.76). (Fig. 2 and Extended data 4). At 1-year follow-up MELD decreased in 7 out of 9 participants; with a mean Δ-MELD for all patients at 1 year of -0.910±1.24 (range -2.41 to 1.68). Overall, we did not observe a clear dose-related response; however, in the highest cell group the MELD scores all followed a similar downward trajectory over the period of follow up (Fig. 2). The mean Δ-UKELD score for all participants at 90 days was -0.42±2.27. Serum albumin levels at 90 days showed little change from baseline in all participants with mean Δ-albumin of -0.20±.0.23 g/dL, with range +0.2 to -0.5 (Extended Data 5). Similarly, INR was unaffected in all participants by macrophage infusion, with mean ±SD change from baseline of -0.04±0.09 and -0.06±0.09 at 90 days and 360 days respectively.

To detect a change in fibrosis, a range of non-invasive markers of liver fibrosis were quantified. The technical success rate of transient elastography was 91.66%. Data not meeting the quality specification as per manufacturer recommendation were removed (2 baseline and 1 90-day measurements). Baseline liver stiffness measurements were consistent with cirrhosis (mean 57.44±24.01 kPa). In 5 out of 9 participants liver stiffness measurements decreased by >6 kPa at 1-year of follow-up, with an overall mean reduction of -11.91±10.55 kPa (Extended Data 6). While a change of 6 kPa

might be considered meaningful in the context of pre-cirrhotic liver fibrosis,[13] the importance of this change in established cirrhosis is uncertain. There was a downward trend in ELF scores following macrophage infusion (Fig. 3a). The mean ELF score at baseline was 12.43±0.94 with mean delta-ELF at 90 days of -0.24±0.46 and at 1 year of -1.13±1.21 (Extended Data 7). There was a similar change in serological markers of type-III collagen turnover, with mean % change of PRO-C3 of -14.86±14.50 and % change of C3M of -10.95±13.37 ng/mL at day 90 (Fig. 3b-c). The larger % decrease in PRO-C3 could indicate a predominant decrease in fibrogenic activity following infusion of macrophages. Longitudinal of health-related quality of life scores (HRQL) assessment showed relatively small variations in composite Chronic Liver Disease Questionnaire (CLDQ) scores over time, but 5 out of 9 participants showed an improvement in overall HRQL at day 90 post-macrophage infusion (Fig. 3d and Extended Data 8). Individual domain scores are shown in Extended Data Table 1.

DISCUSSION

This first-in-human trial confirmed the safety and feasibility of a single peripheral infusion of autologous macrophages in participants with compensated liver cirrhosis of differing aetiology. Leukapheresis was well-tolerated by all participants with minimal side effects. Administration of macrophages was safe, with no clinically relevant adverse reactions recorded during the infusion or in the immediate post-infusion period. The 3+3 trial dose-escalation model is designed to define a maximum-tolerated dose. Due to monocyte isolation and production limitations, we were able to generate a “maximum-achieved dose” of up to 10^9 cells (specifically 0.8 x 10^9 cells), for which we sought to determine the safety and feasibility.

As expected, in a study population with advanced cirrhosis and other co-morbidities, we observed adverse events throughout the study. One participant had a previous history of intermittent low-level alcohol consumption, but serial gamma-glutamyl transpeptidase (GGT) levels (a biochemical marker of alcohol consumption) remained static at all follow-up visits, suggesting that this did not influence the measured outcomes for this patient. Most of the adverse events recorded in the study were exacerbations of existing conditions or minor self-limiting events. The 3 serious adverse events were considered mild and unrelated to the IMP. Among AEs possibly related to the IMP, none had Common Terminology Criteria for Adverse Events (CTCAE) severity grading over 2. There were no dose-related phenomena. All participants reached 360 days of follow-up and were transplant-free. We listed a single clinical event (worsening ascites) during the whole follow-up period. This was identified on ultrasound and resolved with diuretics. All other participants remained well compensated.

Although we did not label the infused macrophages, previous animal models and human case reports[14] suggest that macrophages infused via peripheral or central veins will transiently pass through the lungs, before engrafting in the liver and spleen.[10,15,16] While this does not prove that the cell product used in our study reached the liver, these observations are supportive. We did not record any clinically meaningful changes in respiratory rate or oxygen saturation at any point during infusion or 12-hour follow-up period. Overall the IMP appeared safe during administration and the extended follow-up period of 360 days.

This single-arm phase 1 study was not designed or powered to demonstrate statistically significant changes in efficacy measures following macrophage therapy. However, in 6 of 9 participants reductions in MELD score were observed at 90 days,

largely due to a decrease in serum bilirubin. This contrasts with a recent RCT using autologous CD133+ stem cells in adults with cirrhosis of comparable severity to this study which showed no improvement in MELD score.[17] In one individual, total bilirubin and MELD score were higher at 360 days of follow-up compared to baseline; however, over 85% of the total bilirubin was unconjugated, representing haemolysis likely due to cold agglutinins (the patient had treated hepatitis C with sustained viral response). Other parameters of liver function did not change in response to cell infusion, including UKELD score and serum albumin. Overall, no robust dose-dependent treatment effects were observed in secondary outcomes.

The macrophages manufactured using GMP-compliant processes have been comprehensively characterised and demonstrate a mature phenotype (CD14+ / high 25F9 expression), plus retention of high levels of markers associated with tissue repair and inflammation resolution (CD206, CD163 and CD169).[11]

A number of non-invasive measures of liver fibrosis improved following macrophage infusion including transient elastography, serum ELF score and the collagen turnover markers PRO-C3 and C3M, highlighting the potential antifibrotic effect of autologous monocyte-derived macrophage infusion in cirrhosis.

There was variability in measured responses to macrophage infusion, even in participants treated with the same cell dose. This likely reflects the multiple factors that could determine the effect of macrophage infusion in an individual with cirrhosis such as duration and aetiology of liver disease, other comorbidities, or engraftment and survival of the infused macrophages in the liver. The influence of these variables will be better addressed in a larger randomised controlled phase 2 trial.

Impairment of HRQL is reported by most patients with advanced cirrhosis and HRQL scores improve significantly following liver transplantation.[18] Given that a change of 0.5 on the 1 to 7 scale represents an important difference in CLDQ score, 5 of 9 participants exhibited an improvement in overall HRQL score at day 90 post-infusion.[19] In the remaining participants, composite CLDQ scores were either unchanged (n=2) or worse (n=2) at 90 days. Interestingly, there was an improvement in most participants in the emotional domain at day 90 post-infusion. We noted an inverse association between delta-MELD and CLDQ scores. Moreover, in the 4 individuals in

	Screen Failure (n=2)	10^7 Cells (n=3)	10^8 Cells (n=3)	Up to 10^9 Cells (n=3)
				$0.6x10^9$ $0.8x10^9$ $0.7x10^9$
Participant ID	*001* *002*	*003* *004* *005*	*006* *007* *008*	*009* *010* *011*
DEMOGRAPHICS				
Mean Age	63.00 ±5.66	59.33 ±8.50	55.67 ±6.35	57.67± 2.88
Body Mass Index	32.1 28.2	24.7 29.6 35.6	26 27.8 27.8	33.6 27.6 29
Sex (Male:Female)	2:0	1:2	3:0	1:2
Ethnicity	All Caucasian	All Caucasian	All Caucasian	All Caucasian
AETIOLOGY OF LIVER DISEASE				
ALD (n)	1	2	2	2
NAFLD (n)	1	0	0	1
HCV (SVR) (n)	0	0	1	0
PBC (n)	0	1	0	0
SEVERITY OF CIRRHOSIS				
MELD score		13 11 14	13 10 13	10 13 11

Mean MELD score		12.37±1.51			11.90±1.48			11.36±1.62		
UKELD score		50	50	50	51	51	51	48	51	47
Child-Pugh score		6	5	7	6	6	8	5	9	9
Child-Pugh class		A	A	B	A	A	B	A	B	B
LIVER DISEASE COMPLICATIONS										
Ascites	x	x				x	x		x	x
SBP										
Variceal bleeding		x				x	x		x	x
Hepatic encephalopathy									x	x

whom MELD failed to decrease or worsened, we observed no improvement in HRQL.[19]

This first-in-human study confirmed the safety, feasibility and maximum-achievable dose of autologous macrophages and facilitate future efficacy studies in cirrhosis and other fibrotic diseases. The effects of macrophage therapy upon efficacy measures including transplant-free survival, MELD and UKELD score, fibrosis markers and HRQL will be evaluated in an ongoing phase 2 randomised controlled trial (ISRCTN 10368050).

Table 1. Baseline characteristics of trial participants classified by cell dose group. ALD, alcohol-related liver disease; NAFLD, non-alcoholic fatty liver disease; HCV, hepatitis C virus; SVR, sustained viral response (> 6 months); PBC, primary biliary cholangitis; MELD, Model for End-Stage Liver Disease; UKELD, United Kingdom Model for End-Stage Liver Disease; SBP, spontaneous bacterial peritonitis.

Adverse Event	**10^7 cell dose**	**10^8 cell dose**	**Up to 10^9 cell dose**
Nausea	1	0	0
Abdominal pain	0	2	3
Anorexia	0	1	0
Light-headedness	1	2	2
Fatigue	1	1	3
Chest pain	4	6	0
Joint pain/malaise	2	2	3
Rash	2	0	3
Hypocalcaemia symptoms (leukapheresis)	1	2	3
Ascites	0	1	0
Anaemia	1	1	0
Infective	3	0	2

Others	5	1	10
TOTAL	**22**	**19**	**29**
Number of probably related AEs	**9 (41%)**	**8 (42%)**	**6 (21%)**
Type of Serious Adverse Event			
Abdominal pain and constipation			2
Papillary lesion of breast	1		

Table 2. Recorded adverse events and serious adverse events during the study period. Adverse events (AEs) and serious adverse events (SAEs) classified by dose, using Medical Dictionary for Regulatory Activities (MedDRA) coding version 20.0. All AEs listed were defined as grade 1 or 2 according to the Common Terminology Criteria for Adverse Events version 5.0. All the SAE were considered unrelated to the macrophage infusion. Two, although rated of mild severity, resulted in overnight admission to hospital. The SAE relative to the incidental finding of a papillary lesion of breast through screening mammogram led to surgical excision hence was considered moderate in severity.

Fig. 1. Trial profile**.** A 3+3 model for dose escalation was used. During the study, there was no dose-limiting toxicity (DLT); therefore, only 9 participants were needed to complete the dose-escalation phase.

Fig. 2. MELD score over time per cell dose group. Each line represents a participant in the trial. Time-points indicate the time of macrophage infusion (purple line; approximately 14 days from baseline) and study-specific follow-up visits in the trial. Primary and secondary outcomes were measured at day-90 post-infusion. **a)** 10^7 cells; **b)** 10^8 cells; **c)** 10^9 cells.

Fig. 3. Secondary outcomes a) Individual participant ELF score changes from baseline (BL) over time (delta-ELF). **b)** Individual participant PRO-C3 level changes from baseline over time (% changes of PRO-C3). **c)** Individual participant C3M level changes from baseline over time (% changes of C3M). **d**) Individual self-reported health related quality of life (HRQL) measures over time, expressed as the composite Chronic Liver Disease Questionnaire (CLDQ) score and not delta changes to highlight the significant variability in baseline HRQL composite score in this population. All data are shown by dose group (n=3).

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METHODS

Study oversight

The MATCH 0.1 trial is an investigator-led study, funded by the Medical Research Council (Reference: MR/M007588/1) and sponsored by ACCORD (Academic and Clinical Central Office for Research and Development for NHS Lothian/University of Edinburgh). All study-related documents were designed by the trial team with input from ACCORD, an independent statistician and the Scottish National Blood Transfusion Service (SNBTS) team. The trial was approved by Scotland A Research Ethics Committee (Reference: 15/SS/0121), NHS Lothian Research and Development department and the Medicine and Health Care Regulatory Agency (MHRA-UK). The trial was registered in the International Standard Randomized Controlled Trial registry (ISRCTN10368050) and the European Clinical Trial Database (Reference: 2015-000963-15). All participants enrolled in the study gave informed consent and the trial was conducted under Good Clinical Practice regulations.

Study design

A phase 1 first-in-human trial using a standard 3+3 dose-escalation design was conducted in a single centre (Royal Infirmary of Edinburgh, Edinburgh, UK).[20] Due to limitations in production and cell selection, the maximum number of cells that could be produced for infusion was 10^9; this study was therefore designed to ascertain the tolerability of the maximum-achievable dose and not the maximum-tolerated dose. This approach was approved by the appropriate oversight bodies (Phase I/First in Human Study Review Committee, Data Monitoring Committee and Trial Steering Committee). Escalation decisions were taken by an independent Data Monitoring Committee and recommendations discussed within the Trial Steering Committee and acted upon before each dose-escalation.

Study participants

All participants were recruited through the hospital outpatient service in NHS Lothian between 08 August 2016 and 06 April 2018. 9 adult participants with liver cirrhosis of different aetiologies and a MELD score between 10 and 16 were enrolled. To confirm eligibility only, we used a MELD calculator adopted by the transplant coordinators within our unit; this rounds MELD score to the nearest integer. Full inclusion and exclusion criteria are detailed in the protocol in the Extended Data. Inclusion criteria included: age 18-75; MELD score 10-16 (inclusive); liver disease aetiology of alcohol-related liver disease, primary biliary cholangitis, non-alcoholic fatty liver disease, cryptogenic cirrhosis, haemochromotosis, alpha-1 antitrypsin deficiency or treated chronic hepatitis C (sustained viral response); liver cirrhosis (diagnosed by at least one of: liver biopsy, Fibroscan™ median liver stiffness measurement >15 kPa, or clinical and radiological evidence consistent with cirrhosis). Exclusion criteria included:

history of decompensated cirrhosis in the previous 3 months (portal hypertensive bleeding, ascites requiring medical treatment or hepatic encephalopathy requiring hospitalisation); hepatocellular carcinoma or undetermined liver nodules; cancer in the previous 5 years (excluding adequately treated and localised skin cancer or carcinoma-in-situ of the cervix); previous organ or tissue transplantation; listed for liver transplant; pregnancy and breastfeeding; presence of acute illness that may compromise safety of the patient in the trial. No active alcohol misuse ≥6 calendar months prior to screening was permitted. Individuals attended for a screening visit to ensure eligibility 7±4 days before scheduled leukapheresis. Participants underwent leukapheresis a week before infusions. The Investigational Medical Product (IMP) was produced in a GMP-accredited facility. On the day of infusion, active infection was excluded by physical examination and laboratory investigations. Prior to infusion, 10 mg i.v. chlorphenamine and 100 mg i.v. hydrocortisone was administered. Each group of 3 participants received a single infusion given over 30 +/- 5 minutes of 10^7, 10^8 and up to 10^9 cells, respectively.

Study Assessments

During infusion, participants were monitored closely and observed overnight in the RIE Clinical Research Facility (CRF). Special arrangements were in place with the intensive care unit in the event of a severe reaction. The following morning full blood count, renal function, electrolytes, liver function tests, triglycerides and ferritin were checked prior to discharge to exclude toxicity, including Macrophage Activation Syndrome (MAS).

During the first two follow-up visits (day 7 and day 14 after IMP infusion) safety, dose-limiting toxicity (DLT) and the presence of MAS were assessed. The definition of DLT was formulated using accepted criteria[21-24]. serum creatinine

≥ 1.5-fold from baseline, haemoglobin 1.5-fold ≤ baseline, platelets < 2-fold from baseline, total white cell count < 2.0 x 10^9, alanine aminotransferase (ALT) > 3-fold from baseline, total bilirubin > 3-fold from baseline, MELD score > 4 points from baseline. Thereafter, participants were followed up at day 30, 60, 90, 180 and 360 after IMP infusion with routine and biomarker blood tests, abdominal ultrasound, transient elastography and health-related quality of life (HRQL) assessment (full details are provided in the Protocol in the Extended Data).

Transient elastography (Fibroscan®, Echosens, France) is a well-validated non-invasive test to quantify liver fibrosis. It records the velocity of a sound wave passing through the liver and then converts that measurement into a liver stiffness value (expressed in kilopascals (kPa)).[13]

A range of serological biomarker tests are available for assessment of liver fibrosis. We used the Enhanced Liver Fibrosis (ELF™ test (Siemens Healthineers, UK)), a biochemical panel comprising serum markers that are indicators of ECM metabolism (hyaluronic acid, procollagen-III N-terminal pro-peptide (PIIINP), and tissue inhibitor of matrix metalloproteinase-1 (TIMP-1)). The composite ELF score has been validated for detection of liver fibrosis and for prognostication in chronic liver disease.[25,26] By serological assessment of specific ECM fragments it may be possible to separate tissue formation from tissue degradation.[27] We also measured PRO-C3 and C3M (Nordic Bioscience Protein Fingerprint™ technology) which are two markers derived from type-III collagen remodeling, i.e. N-terminal pro-peptide and MMP-9 degraded collagen fragment from the helix region, respectively,[28,29] with utility for staging liver fibrosis and monitoring response to antifibrotic therapy[30,31].

Liver function was assessed by the MELD and the United Kingdom Model for End-Stage Liver Disease (UKELD). These are established clinical scores

calculated from objective variables (serum bilirubin, creatinine, International Normalized Ratio (INR) and sodium) that are used to estimate the severity of liver disease, determine prognosis and prioritize patients for transplantation.[32,33]

The Chronic Liver Disease Questionnaire (CLDQ) is a 29-item self-reported disease-specific instrument, measuring HRQL in the following domains: fatigue, activity, emotional function, abdominal symptoms, systemic symptoms, and worry. A composite score is calculated by the patient's response options in each domain using seven-point scales, ranging from the worst (1) to the best (7) possible function. The CLDQ is reliable, responsive and correlates with the severity of liver disease.[19,34]

Serum cytokines were analysed using a V-PLEX Human Biomarker 54-Plex kit on a MESO Quickplex SQ 120 according to the manufacturers' instructions (Meso Scale Discovery). We selected a set of 6 safety-related cytokines associated with 'cytokine storm' in MAS. These were IL8 (pivotal in the pathogenesis of MAS), IL1a, IL6, TNFa, IFNγ and IL10.

Method of cell production

The monocyte-derived macrophages were manufactured as previously described.[11] Briefly, steady-state leukapheresis was collected from each patient (standard MNC program, 2.5 blood volume). Monocytes were isolated using a CliniMACS Prodigy® cell processor, programme LP14, tubing set TS510 with CliniMACS CD14 Reagent (all Miltenyi). Up to $3.5x10^{10}$ TNC containing $4x10^{9}$ CD14+ cells were processed in a single operation. Mean CD14+ cell purity was 98.3%±0.7% and the mean selection yield of 55.25%±5.4%. A total of $2x10^{9}$ CD14+ cells were cultured in 4x gas-permeable plastic bags (MACS GMP cell differentiation bag 500, Miltenyi Biotec) at $1x10^{6}$ cells per ml in TexMACS GMP

(phenol red-free) medium supplemented with 100 ng/mL M-CSF (GMP-grade, RND systems). Medium was replenished by removing 50% spent medium and replacing with 50% fresh medium supplemented with 200ng/mL M-CSF after 48 and 96 hours of culture. After 7 days, macrophages were harvested, counted and formulated into saline for injection supplemented to 0.5% Alburex (CSL Behring UK). Macrophages were characterized as viable, CD45+, CD14+, 25F9+ cells as previously described.[11] $CD14^+$ monocytes were successfully isolated from all participants. A macrophage product was successfully manufactured and administered for all participants.

Statistics

A descriptive analysis of the primary outcome of safety and tolerability is presented. Secondary outcomes are presented graphically by dose and as changes from baseline. Unless stated, numerical data is expressed as mean±standard deviation (SD). A safety report was produced to review the day 14 results of the first participant, thereafter DMC reports were produced following the day 14 safety blood samples of each escalation group of 3 participants at each dose level or as required by serious adverse events. Any additional analysis was performed at the end of the trial once the electronic database was locked following quality checks (QC). There was 100% QC of the data collected, with no missing data other than a single collagen biomarker sample at day 60 post-infusion. We report all adverse events by dose.

Data availability

Data in the published article (and its Supplementary Information files) has been presented where possible in aggregated form. Any data presented to illustrate individual patient performance has been de-identified and only includes

analysis of performance within the trial (such as MELD score). The datasets generated during and/or analysed during the current study are available from the corresponding author (SJF) upon reasonable request, although restrictions may apply due to patient privacy and General Data Protection Regulation.

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Author Contributions

Conceptualization and design of the work were carried out by SJF, CP, LR, LB, DM, AL, SD, EH, ARF, MLT, JDMC, NWAM, JB, JKM, PCH, JAF; the acquisition, analysis, and interpretation of data were performed by SJF, JAF, FM, BD, CG, DJL, MJN, KM; trial delivery and administration were carried out by FM, AG; the original draft of the manuscript was written by FM; the draft was reviewed and edited by all the authors.

Competing interests

JAF reports personal fees from Novartis, Ferring Pharmaceuticals, Galecto Biotech, Caldan Therapeutics, Gilde Healthcare, Arix Bioscience, Guidepoint and grants from GlaxoSmithKline, Novartis and Intercept Pharmaceuticals, outside the submitted work. SJF has a grant from Syncona to develop macrophages as a therapy. DJL, KM, MJN are full-time employees at Nordic Bioscience. DJL, MK and MJN are among the original inventors and patent holders of C3M and PRO-C3. DJL holds stock in Nordic Bioscience. PCH is an advisor for AbbVie, BMS, Eisai Ltd, Falk, Ferring, Gilead, Gore, Janssen, Lundbeck, MSD, Norgine, Novartis, ONO Pharmaceuticals, Pfizer and Roche, outside the submitted work.

2. Appendix 2. MORE2 protocol V3

Non-CTIMP Study Protocol

MACROPHAGE FOR REGENERATIVE MEDICINE IN CIRRHOTICS – II

Macrophage fOr Regenerative mEdicine in cirrhotics – II (MORE-2)

	The University of Edinburgh and/or Lothian Health Board ACCORD The Queen's Medical Research Institute 47 Little France Crescent Edinburgh EH16 4TJ
Protocol authors	**Chloë Pass, Francesca Moroni**
Funder	**Medical Research Council**
Funding Reference Number	**MR/M007588/1**
Chief Investigator	**Professor Stuart Forbes, Director CRM**
Sponsor number	**AC18140**
REC Number	**19/WS/0006**
Version Number and Date	**v3 05 June 2019**

LIST OF ABBREVIATIONS

ACCORD	**Academic and Clinical Central Office for Research & Development - Joint office for The University of Edinburgh and Lothian Health Board**
AE	Adverse Event
AFP	Alpha Feto Protein
BMI	Body Mass Index
BP	Blood Pressure
CAU	Clinical Apheresis Unit
CI	Chief Investigator
CRF	Case Report Form
CTIMP	Clinical Trial of Investigational Medicinal Product
CV	Curriculum Vita
CVA	Cerebrovascular Accident
ECG	Electrocardiogram
FBC	FBC Full Blood Count
GCP	Good Clinical Practice
GMP	Good Manufacturing Practice

GP	General Practictioner
HBV	Hepatitis B Virus
HCV	Hepatitis C Virus
HEV	Hepatitis E Virus
HIV	Human Immunodeficiency Virus
HR	Heart Rate
HTLV	Human T-Lymphotrophic Virus
ICH	International Conference on Harmonisation
IMP	Investigational Medicinal Product
INR	International Normalised Ration
ISF	Investigator Site File
JCC	Jack Copland Centre
kPa	KiloPascal
LFT	Liver Function Tests
LPS	Lypopolysaccarides
MATCH	Macrophage Therapy for liver Cirrhosis
M-CSF	Macrophages Colony Stimulating Factor

MDMs	Monocyte Derived Macrophages
MI	Myocardial Infarction
MIA(IMP)	Manufacturer's Authorisations for IMPs
mL	Microliters
MMP	matrix metalloproteinase
MNC	Mononuclear Cells
NCI CTCAE	National Cancer Institute common terminology for classification of Adverse Events
NHS	National Healthcare System
PCR	Polymerase Chain Reaction
QA	Quality Assurance
R&D	Research and Development
REC	Research Ethics Committee
RNA	Ribonucleic Acid
SAE	Serious Adverse Event
SBP	Spontaneous Bacterial Peritonitis
(S)CRM	(Scottish) Centre for Regenerative Medicine
SNBTS	Scottish National Blood Transfusion Service

SOP	Standard Operating Procedure
U&E	Urea, Creatinine and Electrolytes

INTRODUCTION

2.1. BACKGROUND

Liver disease is a rapidly rising cause of morbidity and mortality in the western world, being the fifth highest cause of death in the UK and the only one of the top five that continues to rise (Source: Office for National Statistics). Liver transplantation is the only curative option for end stage liver disease and increased demand is not being matched by increased supply of donor organs. There is an urgent need to find alternative options.

The pathophysiological process of cirrhosis is common to all causes of chronic liver disease and results in (i) a disordered scarred hepatic architecture with associated intrahepatic resistance (portal hypertension) leading to the clinical manifestations of varices, ascites and encephalopathy and (ii) loss of hepatocyte mass resulting in failure of hepatic synthetic function. Liver transplantation is currently the only curative treatment available for end stage liver disease.

There is a rising demand for transplantation, which has not been matched by an increased supply of donor organs, resulting in significant mortality and morbidity whilst on the waiting list. Transplantation requires lifelong immunosuppression with associated side effects including renal failure, cardiovascular complications and increased risk of malignancy.

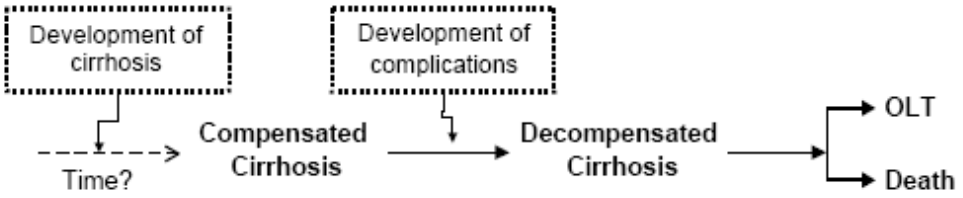
Source: British Liver Trust/Office for National Statistics

Animal data suggests that an infusion of macrophages reduces hepatic fibrosis and improves clinically relevant parameters such as albumin [1]. A previous clinical trial [15] demonstrated that monocytes collected from cirrhotic patients via apheresis can differentiate into macrophages with therapeutic potential, with identical characteristics as macrophages generated from healthy volunteers [16].

A phase 1 first in human safety dose escalation study (MATCH Study: ISRCTN10368050) has been concluded and demonstrated safety of peripheral infusion of autologous macrophages in cirrhotic patients.

2.2. RATIONALE FOR STUDY

Patients develop cirrhosis at varying rates depending upon the underlying aetiology of the liver disease. Patients with compensated chronic liver disease have advanced fibrosis or cirrhosis but have not yet developed the clinical complications of ascites, varices or encephalopathy.



From D'Amico et al J Hepatol 2006;44:217-231

These complications occur at the rate of 5-7% per year and have devastating implications for prognosis [2]. Patients with compensated cirrhosis have a 1-3.4% risk of death at one year (median survival 12 years) and in those with decompensated cirrhosis this rises dramatically to 20-57% risk of death at one year (median survival 2 years).

Furthermore, patients with cirrhosis develop Hepatocellular Carcinoma at a constant rate of around 3% per year [2].

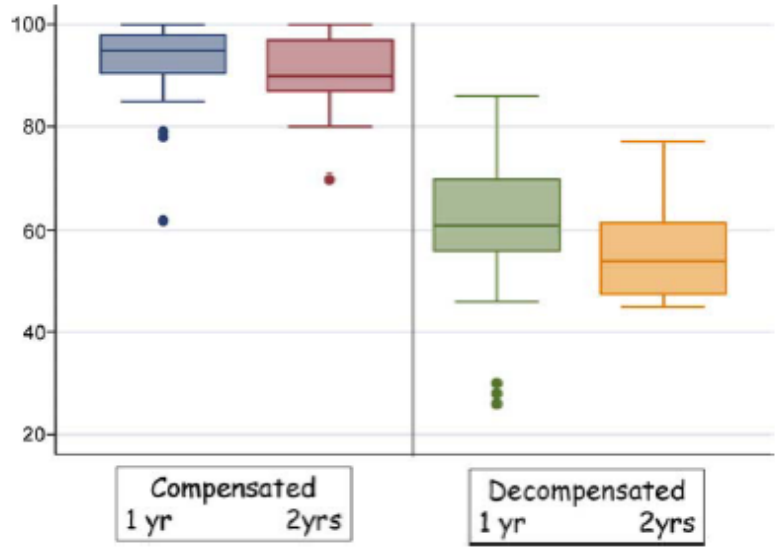


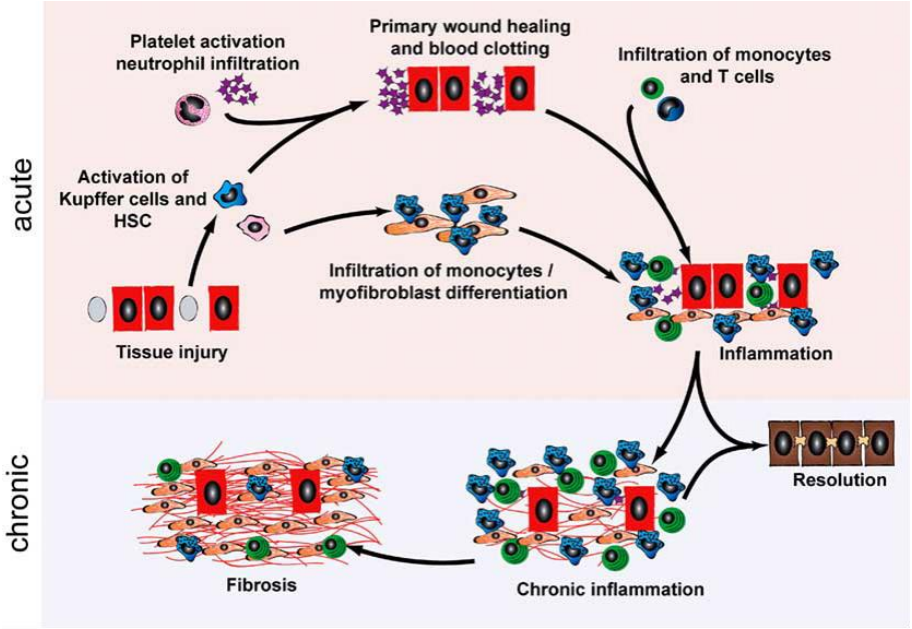
Fig. 7. Box plots of one and two-year survival rate in studies including only compensated ($n = 18$ studies) or decompensated ($n = 23$ studies) patients.

From: D'Amico et al J Hepatol 2006;44:217-231

Published studies have showed the safety of apheresis in cirrhotic patients [3-5] and our group has validated the feasibility of differentiating human monocytes from healthy volunteers into macrophages in vitro under GMP conditions (unpublished data).

This is a feasibility study with the aim of improving our current methodology of GMP differentiation of macrophages from monocytes for the MATCH clinical trial to enable processing at multiple centres. If this is successful then the ultimate aim would be to extend the manufacturing for the current clinical trial (MATCH) to other centres in the UK, whereby monocytes are collected from cirrhotic patients via apheresis, differentiated into macrophages and then infused back to these patients as a treatment to help improve morbidity and mortality. This study is therefore an important milestone in this overall aim.

Inflammation is a key factor in the initiation and maintenance of fibrotic processes within the liver [6, 7]. Macrophages have a major role in the resolution of the resultant damage by clearing up tissue debris and releasing cytokines and chemokines which promote repair [8-11]. As our group has shown previously, they also mediate hepatic scar remodelling through local matrix metalloproteinase (MMP) expression [12, 13] and macrophage cell therapy itself improves clinically relevant parameters in experimental chronic liver injury. [1]



From: Heymann et al, Inflammation and Allergy – Drug Targets, 2009, 8, 307-318 [14].
Diagram illustrating the role of Macrophages in the inflammatory process

3. STUDY OBJECTIVES

3.1. OBJECTIVES

3.1.1. Primary Objective

This study aim is to improve the current production protocol for monocytes-derived autologous activated macrophages product. We will evaluate the feasibility of storing the apheresis product used for differentiation of monocytes overnight prior to GMP processing and validating a shorter differentiation protocol. This will allow more patients to have access to this innovative product.

Secondary Objectives

To introduce a second SNBTS manufacturing site for the MATCH trial – The Jack Copland Centre (JCC).

3.2. ENDPOINTS

3.2.1. Primary Endpoint

Validation of extended storage of the apheresis product prior to manufacturing measured as stability availability of product characteristics following overnight hold. Successful differentiation and comparable end product characteristic to current standard protocol (16)

3.2.2. Secondary Endpoints

Regulatory audit to gain MIA (IMP) license at SNBTS Jack Copland Centre (JCC).

4. STUDY DESIGN

Pilot feasibility cohort and laboratory study taking place over 12 months.

This study will consist of four stages:

- Stage 1 Screening visit and enrolment
- Stage 2 Apheresis visit (within 30 days of screening)
- Stage 3 Differentiation of monocytes (7 day GMP manufacture)
- Stage 4 Development of macrophage product

A telephone follow-up consultation will be performed 48hrs after apheresis

5. STUDY POPULATION

5.1. NUMBER OF PARTICIPANTS

Up to 15 patients with liver Cirrhosis will be recruited into this feasibility cohort study, allowing for 2 to withdraw prior to completion. Recruitment will take place over an 18 month period at one site (the Royal Infirmary of Edinburgh).

5.2. INCLUSION CRITERIA

1) 18≥AGE≤75

2) Cirrhosis, defined as one of:

   - Previous liver biopsy confirming histological features of cirrhosis
   - Transient elastography (Fibroscan) > 15kPa
   - Clinical and radiological features that in the opinion of the investigator correlate with a diagnosis of cirrhosis

5.3. EXCLUSION CRITERIA

- Refusal or inability to give informed consent to participate in the study

- Patients with active viral hepatitis
- Average alcohol ingestion >14units/week
- Ascites: unless, in the opinion of the investigator, the ascites is minimal and well controlled with diuretic therapy in last 3 months
- Encephalopathy requiring hospitalisation for treatment in last 3 months.
- Portal hypertensive bleeding: active episode of bleeding requiring hospitalisation in the last 3 months
- History of Hepatocellular carcinoma
- Previous liver transplant
- Listed for liver transplantation
- Any situation that in the investigators opinion may interfere with optimal study participation such as alcohol or drug abuse, domicile too distant from study site, potential non-compliance or inability to co-operate
- Presence of clinically relevant acute illness that in the opinion of the investigator might compromise the patient's safe participation in the study
- Presence or history of cancer within past 5 years with exception of adequately treated localised basal cell carcinoma of the skin, in situ cervical cancer or solid malignancy surgically excised in total without recurrence for 5 years
- Pregnancy or breastfeeding

5.4. CO-ENROLMENT

Participants who are active in the interventional phase of a non-CTIMP can be co-enrolled to a CTIMP provided the CTIMP-nonCTIMP Co-enrolment Checklist (POL008-F02) is be completed by the Sponsor Representative(s) in conjunction with the CI prior to the co-enrolment proceeding. Arrangements for co-enrolment with

another interventional non-CTIMP will be permitted in compliance with the study protocol. Written agreement in the form of email communication is required from both CIs and must include a statement on the impact on participant burden and study outcomes as a minimum. Co-enrolment between non-interventional research (e.g. sample only, questionnaire studies) will not typically require any formal documentation or authorisation from the Sponsor. (Please refer to https://www.accord.scot/research-access-resources-researchers/policies POL008 co-enrolment v1.0 PDF)

6. PARTICIPANT SELECTION AND ENROLMENT

6.1. IDENTIFYING PARTICIPANTS

- Permission will be sought from potential participants (by a member of the direct healthcare team) for the researcher to approach them face to face or over the phone.
- The study will be introduced and explained to the potential participant, written participant information will be provided at least 24 hours prior to consent to the study and there will be an opportunity for the potential participant to ask questions.
- The potential participant will be asked to read the provided information. They will have the opportunity to discuss their participation with family and/or friends if they wish to do so.

6.2. CONSENTING PARTICIPANTS

- The Investigator (or designated co-investigator as documented on the Signature and Delegation log) will obtain written informed consent for each participant prior to performing any study related procedure. A Participant Information Sheet will be provided to facilitate this process. The Investigator will ensure that they adequately explain the aim, anticipated benefits and potential hazards of taking part in the study to the participant, including the risks and potential benefits of undergoing

apheresis. The Investigator should also stress that the participant is completely free to refuse to take part or withdraw from the study at any time.

- The participant will be given ample time (greater than 24 hours) to read the Participant Information Sheet and to discuss their participation with others outside of the research team. The participant will be given an opportunity to ask questions which should be answered to their satisfaction. The right of the participant to refuse to participate in the study without giving a reason will be respected.
- If the participant expresses an interest in participating in the study they should be asked to sign and date the latest version of the Informed Consent Form. The Investigator (or designated representative) will then sign and date the form. A copy of the Informed Consent Form will be offered to the participant, a copy should be filed in the hospital notes, a copy should be retained by SNBTS, one copy should be given to the clinical Apheresis unit and the original placed in the Investigator Site File (ISF).
- Details of the informed consent discussions should be recorded in the participant's medical notes. This should include date of and information regarding the initial discussion. Throughout the study the participant should have the opportunity to ask questions and any new information that may be relevant to the participant's continued participation should be shared with them in a timely manner. On occasion it may be necessary to re-consent the participant in which case the process above should be followed and the participant's right to withdraw from the study respected.
- With the participant's prior consent, their General Practitioner (GP) should also be informed that they are taking part in the study if they are recruited. A GP Letter is provided for this purpose.

6.2.1. Withdrawal of Study Participants

Participants are free to withdraw from the study at any point or a participant can be withdrawn by the Investigator. If withdrawal occurs, the primary reason for withdrawal will be documented in the participant's case report form, if possible. The participant will have the option of withdrawal from all aspects of the trial and continued use of data collected up to that point

(i) with removal of previously collected and stored participant samples.

(ii) with continuous use of previously collected and stored participant samples.

The following are justifiable reasons for the Investigator to withdraw a participant from study:

Unforeseen events

Any event which in the judgement of the Investigator makes further participation inadvisable

Withdrawal of consent

Participants may withdraw consent at any time during the study. The details of withdrawal should be clearly documented. The participant should not be pressured in any way to give a reason for withdrawal if he/she does not wish to supply this information.

Participant withdrawals will be replaced. All participants will be included in the analysis unless they have withdrawn consent to remain in the study in which case participants will be included in the analysis up to the point they withdraw consent.

7. STUDY ASSESSMENTS

7.1. STUDY ASSESSMENTS

Screening Visit

Note: If participants have undergone the investigations listed below within 30 days of the apheresis visit they will not be repeated.

No trial specific tests or interventions which don’t form part of routine standard practice should be conducted prior to the participant providing written informed consent.

- Review with participant all information pertaining to the aims and methods of the trial, including potential risks and benefits
- If valid informed consent is obtained, (written consent form signed and dated by participant (see section 5.2)) the following screening investigations will take place:

- Participant demographics recorded and participant registered
- Full medical history taken including:
    - date of diagnosis of liver disease
    - Alcohol history (confirmed by family member if possible)
    - Current / recent (90 days) medications and illnesses
    - Current / previous liver related complications (including but not limited to ascites/SBP, encephalopathy, variceal bleeding)
    - Previous Cardiovascular events (eg MI, CVA)
- Full clinical examination
- Record HR, BP
- Measure height, weight – calculate BMI
- Obtain blood:
    - Baseline investigations:

      FBC; INR; U&E; LFT; AFP; Calcium; Magnesium

    - Mandatory microbiology pre apheresis. As part of the screening procedure all participants will require mandatory testing for blood borne infectious agents as per National Blood Service requirements for screening of blood products prior to processing and storage. These tests must be performed within 30 days of Leukopheresis and consist of serological testing for HIV 1/2, Hepatitis B virus (HBV), HB core, Hepatitis C virus (HCV), Human T-Lymphotrophic Virus (HTLV), syphilis and PCR testing for HIV, HCV, HBV and HEV. Appropriate pre-test counselling will be available and in the event of an unexpected positive result, the investigator will provide initial

counselling and referral to the appropriate specialist service. The General Practitioner will also be informed.

- Obtain 12 lead ECG
- Perform urinary pregnancy test in Women of child bearing potential
- Vein assessment at CAU for suitability for apheresis

Apheresis visit

Conducted as per clinical standard by CAU team. Participant will be seen by delegated member of trial team prior to the apheresis procedure to ensure they are fit to proceed.

Isolation of Monocytes and Differentiation

A single leukopheresis will be performed using the Optia apheresis system using a sterile connection technique. Leukopheresis will take place within 30 days of screening. A standard programme for collection of mononuclear cells (MNC) will be employed. Two blood volumes will be processed where circumstances permit. Isolation of monocytes and differentiation into macrophages will be done following the established GMP procedures currently employed for the MATCH clinical trial. Briefly, monocytes will be purified from the resultant product using clinical grade CD14 MicroBeads and an autoMACS separation apparatus (Miltenyi). Our data suggest approximately $1x10^8$ – $1x10^9$ CD14+ve monocytes can be isolated using this procedure. The purity of the separation will be assessed using a panel of antibodies against human leucocytes. CD14+ve monocytes will be cultured under low adhesion conditions in the presence of 100ng/ml human recombinant M-CSF. After 7 days in culture macrophages will be phenotyped again using a panel of antibodies. It is expected that the mature macrophage markers (25F9 and CD206) will be expressed.

The data produced will be compared to data gathered from the ongoing MATCH clinical trial.

Macrophage Further Development

Further some of the macrophages may be used for cryopreservation or genetic modification studies. Any leftover CD14+ monocyte material will be used to validate a shorter production protocol. Monocytes will be cultured under low adhesion conditions in the presence of 100ng/ml human recombinant M-CSF for 5 days without feeding to develop macrophages. This protocol will be compared to the standard macrophage conditions as described above (7 days culture with one feed). Purity and differentiation of monocytes and macrophages will be performed using a panel of antibodies (including 25F9 and CD206) as described above. In addition, we may collect conditioned medium for analysis of secreted proteins, perform in vitro functional assays (such as assessment of phagocytosis), isolate RNA to assess gene expression, test the ability of macrophages to respond to polarising stimuli (e.g. cytokines, LPS et.), test the ability to cryopreserve macrophages, overexpress or knockdown genes introduced by electroporation and also assess therapeutic function *in vivo* in mouse models of liver disease.

7.2. LONG TERM FOLLOW UP ASSESSMENTS

Participants will return to the treating physician's care. If unexpected results are revealed at any stage, such as positive virology, the participant will be referred to the relevant speciality and their direct healthcare team informed as well as the participant.

A telephone follow-up consultation will be performed 48hrs after the collection however, by the trial team, to ensure that the participant has not experienced any unexpected side effects from undergoing apheresis.

7.3. STORAGE AND ANALYSIS OF SAMPLES

Apheresis product (50-200mL) will be used to manufacture monocyte derived macrophages (MDMs). MDMs will be stored for 48hr stability analysis, following which they will either be disposed of as clinical waste or may be used to validate cryopreservation or genetic modification protocols. Genetic material to overexpress or knockdown genes involved in macrophage functions (e.g. phagocytosis, cytokine

secretion, migration etc.) is introduced into the cells by electroporation. Cells for cryopreservation may be frozen and stored for up to two years to test longevity of cryopreservation protocols after which time they will be disposed of as clinical waste.

Approximately 40mL of blood samples will be taken at each visit for testing (refer to section 6.1). One sample (7mL) will be archived and retained by SNBTS in accordance with regulatory requirements

Details of samples taken are covered in patient consent for leukapheresis.

8. DATA COLLECTION

The Case Report Form (CRF) will be completed at set time points and will correlate appropriately to the trial:

Form	**Summary of data recorded**
Screening	Check of Inclusion and Exclusion Criteria, Relevant Examination medical history, Vital Signs, Baseline blood results Medications at Start of Study,
Apheresis	Changes during Study, Concomitant Medication, vital signs, adverse events. Blood results Record of Adverse Events

Adverse Event	Dates, Severity, Management and Outcomes

Ad hoc forms

Withdrawal form

Serious Adverse Event Form

The CRF will be completed by the Investigator or an authorised member of the research team (as delegated on the Site Signature and Delegation Log). The exception is the SAE Form which must be signed by the Investigator. See Adverse Event reporting section 9 for further details.

9.

9.1. Source Data Documentation

The Case Report Form (CRF) will compromise the following forms:

1) Eligibility checklist (check of inclusion and exclusion criteria)
2) Registration form (Participant demographics)
3) Initial Assessment (history, examination, vital signs, blood tests)
4) Medications

A formal letter confirming suitability for apheresis to include full history, examination findings, outcome of vein assessment conducted by trained staff and relevant blood results will be sent to the Clinical Apheresis Unit, the Haematology Registrar covering the Clinical Apheresis Unit and the Scottish National Blood Transfusion Service (SNBTS) by the Clinical Research Fellow.

Entries on the CRF should be made in ballpoint pen, in blue or black ink, and must be legible. Any errors should be crossed out with a single stroke, the correction inserted

and the change initialled and dated. If it is not obvious why a change has been made, an explanation should be written next to the change.

9.2. Case Report Forms

Detailed in 7.1.

9. STATISTICS AND DATA ANALYSIS

9.1. SAMPLE SIZE CALCULATION

A total of up to 15 participants will be recruited, up to 6 will be used to complete the validation of overnight hold of the leukapheresis product and validation of GMP manufacturing at the JCC site. Up to 4 will be used to perform additional stability analysis, which the final product from each run split into several stability samples to analyse different densities and temperatures of storage. These will be written up as validation protocols using the SNBTS quality management system. Any leftover apheresis product will be used to validate the shorter production protocol and cryopreservation. If no sufficient leftover product will be available for the aforementioned analysis additional participants (up to 5) will recruited to complete the trial.

9.2. PROPOSED ANALYSES

Results will be analysed using the established release criteria for the MATCH clinical study. Data will be analysed as a mean from 3 validation runs, and compared against existing data from the MATCH clinical trial. All data will be written up in validation protocols, with updated procedures included in clinical trial amendments and/or incorporated into future clinical trials as appropriate.

Cells produced using the shorter production protocol will be compared to cells produced using the standard 7 day protocol.

We will analyse the established release criteria for the MATCH clinical study, as well as an extended panel of activation markers and receptors using flow cytometry (e.g. CCR2, CD163, CD169, CD86, HLA-DR/DP/DQ) to determine whether cells generated

using the shorter protocol are phenotypically equivalent to cells produced using the established protocol.

To determine functional equivalence of cells generated with these protocols we may perform the following experiments:

Proteins secreted into the cell culture medium by macrophages may be assessed using commercially available multi-spot ELISA kits.

Gene expression may be assessed by isolating RNA and performing real-time PCR.

Phagocytosis may be assessed by the ability to take up pHRodo Beads and assessing bead fluorescence using live cell imaging or flow cytometry.

Cells may be exposed to polarising cytokines or other molecules to assess their ability to external stimuli and assessed by the above assays.

Any excess material generated that has not been used for in vitro validation of day 5 versus day 7 products, or leftover from overnight hold material may be used to develop conditions to further improve the therapeutic efficacy of clinical products and may include:

- Introduction of exogenous DNA or RNA may be performed using electroporation and functional consequences of genetic modification assessed by the above assays, or in vivo assays.
- The ability of these cells to be cryogenically frozen for long-term cold storage may also be assessed
- The therapeutic efficacy of these cells may be tested by their ability to alter liver damage dynamics in acute (e.g. paracetamol overdose) or chronic (e.g. carbon tetrachloride-induced fibrosis) models in mice as has been previously described (15).

10. ADVERSE EVENTS

All AEs will be recorded from the time a participant signs the consent form to take part in the study until completion of follow up at day 48 hrs post leukapheresis. Any AE events still present at that time will be confirmed and recorded as ONGOING in the Case Report Form. If appropriate, these should be handed over to the participants General Practitioner or usual care team.

All serious adverse events (SAEs) will be reported from the date of consent until day 48hrs post leukapheresis.

If a participant is withdrawn from the study prior to leukapheresis all SAEs will be reported until the date of withdrawal. Any SAE events with a start date that is the same as, or prior to the participant being withdrawn from study will be followed until resolution.

Participants will be asked about the occurrence of AEs/SAEs at every visit during the study. Open-ended and non-leading verbal questioning of the participant will be used to enquire about AE/SAE occurrence. Participants will also be asked if they have been admitted to hospital, had any accidents, used any new medicines or changed concomitant medication regimens. If there is any doubt as to whether a clinical observation is an AE, the event will be recorded.

All adverse events will be evaluated by the investigator and recorded. This includes an evaluation of the seriousness and causality between the treatment and the adverse event.

All events should be graded according to the NCI CTCAE toxicity Criteria (version 4.0).

The reporting period for AEs and Serious Adverse Events (SAEs) be recorded from the time a participant signs the consent form to take part in the study until completion of follow up.

Adverse events should be recorded on the appropriate form in the Clinical Apheresis Unit during apheresis.

Staff should take all therapeutic measures necessary for resolution of any adverse event.

For events not listed in the toxicity table, severity should be recorded as:

Mild Subject is aware of the event or symptom, but the event or symptom is easily tolerated.

Moderate Subject experiences sufficient discomfort to interfere with or reduce their usual level of activity.

Severe Significant impairment of functioning, subject is unable to carry out usual activities.

Life threatening Risk of death, organ damage or disability

11. OVERSIGHT ARRANGEMENTS

11.1. INSPECTION OF RECORDS

Investigators and institutions involved in the study will permit trial related monitoring and audits on behalf of the sponsor, REC review, and regulatory inspection(s). In the

event of audit or monitoring, the Investigator agrees to allow the representatives of the sponsor direct access to all study records and source documentation. In the event of regulatory inspection, the Investigator agrees to allow inspectors direct access to all study records and source documentation.

11.2. RISK ASSESSMENT

A study specific risk assessment will be performed by representatives of the sponsors, ACCORD monitors and the QA group, in accordance with ACCORD governance and sponsorship SOPs. Input will be sought from the Chief Investigator or designee. The outcomes of the risk assessment will form the basis of the monitoring plans and audit plans. The risk assessment outcomes will also indicate which risk adaptions (delete if no adaptations were possible) could be incorporated into to trial design.

11.3. STUDY MONITORING AND AUDIT

The ACCORD Sponsor Representative will assess the study to determine if an independent risk assessment is required. If required, the independent risk assessment will be carried out by the ACCORD Quality Assurance Group to determine if an audit should be performed before/during/after the study and, if so, at what frequency.

Risk assessment, if required, will determine if audit by the ACCORD QA group is required. Should audit be required, details will be captured in an audit plan. Audit of Investigator sites, study management activities and study collaborative units, facilities and 3rd parties may be performed.

12. GOOD CLINICAL PRACTICE

12.1. ETHICAL CONDUCT

The study will be conducted in accordance with the principles of the International Conference on Harmonisation Tripartite Guideline for Good Clinical Practice (ICH GCP).

Before the study can commence, all required approvals will be obtained and any conditions of approvals will be met.

12.2. INVESTIGATOR RESPONSIBILITIES

The Investigator is responsible for the overall conduct of the study at the site and compliance with the protocol and any protocol amendments. In accordance with the principles of ICH GCP, the following areas listed in this section are also the responsibility of the Investigator. Responsibilities may be delegated to an appropriate member of study site staff as documented in the Delegation Log.

12.2.1. Informed Consent

The Investigator is responsible for ensuring informed consent is obtained before any protocol specific procedures are carried out. The decision of a participant to participate in clinical research is voluntary and should be based on a clear understanding of what is involved.

Participants must receive adequate oral and written information – appropriate Participant Information and Informed Consent Forms will be provided. The oral explanation to the participant will be performed by the Investigator or qualified delegated person, and must cover all the elements specified in the Participant Information Sheet and Consent Form.

The participant must be given every opportunity to clarify any points they do not understand and, if necessary, ask for more information. The participant must be given sufficient time to consider the information provided. It should be emphasised that the participant may withdraw their consent to participate at any time without loss of benefits to which they otherwise would be entitled.

The participant will be informed and agree to their medical records being inspected by regulatory authorities and representatives of the sponsor(s).

The Investigator or delegated member of the trial team and the participant will sign and date the Informed Consent Form(s) to confirm that consent has been obtained. The participant will receive a copy of this document and a copy filed in the Investigator Site File (ISF) and participant's medical notes (if applicable) and a copy sent to the Clinical Apheresis Unit.

12.2.2. Study Site Staff

The Investigator must be familiar with the protocol and the study requirements. It is the Investigator's responsibility to ensure that all staff assisting with the study are adequately informed about the protocol and their trial related duties.

12.2.3. Data Recording

The Principal Investigator is responsible for the quality of the data recorded in the CRF at each Investigator Site.

12.2.4. Investigator Documentation

- The Principal Investigator will ensure that the required documentation is available in local Investigator Site files ISFs.

12.2.5. GCP Training

All researchers are encouraged to undertake GCP training in order to understand the principles of GCP. However, this is not a mandatory requirement unless deemed so by the sponsor. GCP training status for all investigators should be indicated in their respective CVs.

12.2.6. Confidentiality

All laboratory specimens, evaluation forms, reports, and other records must be identified in a manner designed to maintain participant confidentiality. All records must be kept in a secure storage area with limited access. Clinical information will not be released without the written permission of the participant. The Investigator and study site staff involved with this study may not disclose or use for any purpose other than performance of the study, any data, record, or other unpublished, confidential information disclosed to those individuals for the purpose of the study. Prior written agreement from the sponsor or its designee must be obtained for the disclosure of any said confidential information to other parties.

12.3. DATA MANAGEMENT

12.3.1. Personal Data

The following personal data will be collected as part of the research:

DOB and Initials.

12.3.2. Personal data will be stored by the research team in a locked cabinet within a locked office at The University of Edinburgh SCRM building, only delegated members of the trial team will have access to these files.

Personal data will be stored for three year after last visit of last patient.

12.3.3. Transfer of Data

All data collected or generated by the study (including personal data) will not be transferred to any external individuals or organisations outside of the Sponsoring organisation(s).

Data generated by this research study may be shared with third parties as described in the participant information sheets. This excludes all participant personal data.

12.3.4. Data Controller

The data controller is University of Edinburgh

All Investigators and study site staff involved with this study must comply with the requirements of the appropriate data protection legislation (including the General Data Protection Regulation and Data Protection Act) with regard to the collection, storage, processing and disclosure of personal information. Access to collated identifiable participant data will be restricted to individuals from the research team treating the participants, representatives of the sponsor(s) and representatives of regulatory authorities.

Computers used to collate the data will have limited access measures via user names and passwords

13. STUDY CONDUCT RESPONSIBILITIES

13.1. PROTOCOL AMENDMENTS

Any changes in research activity, except those necessary to remove an apparent, immediate hazard to the participant in the case of an urgent safety measure, must be reviewed and approved by the Chief Investigator.

Amendments will be submitted to a sponsor representative for review and authorisation before being submitted in writing to the appropriate REC, and local R&D for approval prior to participants being enrolled into an amended protocol.

13.2. MANAGEMENT OF PROTOCOL NON COMPLIANCE

Prospective protocol deviations, i.e. protocol waivers, will not be approved by the sponsors and therefore will not be implemented, except where necessary to eliminate an immediate hazard to study participants. If this necessitates a subsequent protocol amendment, this should be submitted to the REC, and local R&D for review and approval if appropriate.

Protocol deviations will be recorded in a protocol deviation log and logs will be submitted to the sponsors every 3 months. Each protocol violation will be reported to the sponsor within 3 days of becoming aware of the violation. All protocol deviation logs and violation forms should be emailed to QA@accord.scot

Deviations and violations are non-compliance events discovered after the event has occurred. Deviation logs will be maintained for each site in multi-centre studies. An alternative frequency of deviation log submission to the sponsors may be agreed in writing with the sponsors.

13.3. SERIOUS BREACH REQUIREMENTS

A serious breach is a breach which is likely to effect to a significant degree:

(a) the safety or physical or mental integrity of the participants of the trial; or

(b) the scientific value of the trial.

If a potential serious breach is identified by the Chief investigator, Principal Investigator or delegates, the co-sponsors (seriousbreach@accord.scot) must be notified within 24 hours. It is the responsibility of the co-sponsors to assess the impact of the breach on the scientific value of the trial, to determine whether the incident constitutes a serious breach and report to research ethics committees as necessary.

13.4. STUDY RECORD RETENTION

All study documentation will be kept for a minimum of 3 years from the protocol defined end of study point. When the minimum retention period has elapsed, study documentation will not be destroyed without permission from the sponsor.

13.5. END OF STUDY

The end of study is defined as the last participant's last visit.

The Investigators or the co-sponsor(s) have the right at any time to terminate the study for clinical or administrative reasons.

The end of the study will be reported to the REC, and R+D Office(s) and co-sponsors within 90 days, or 15 days if the study is terminated prematurely. The Investigators will inform participants of the premature study closure and ensure that the appropriate follow up is arranged for all participants involved. End of study notification will be reported to the co-sponsors via email to resgov@accord.scot.

A summary report of the study will be provided to the REC within 1 year of the end of the study.

13.6. CONTINUATION OF TREATMENT FOLLOWING THE END OF STUDY

No study treatment will be administered during this study

13.7. INSURANCE AND INDEMNITY

The co-sponsors are responsible for ensuring proper provision has been made for insurance or indemnity to cover their liability and the liability of the Chief Investigator and staff.

The following arrangements are in place to fulfil the co-sponsors' responsibilities:

- The Protocol has been designed by the Chief Investigator and researchers employed by the University and collaborators. The University has insurance in place (which includes no-fault compensation) for negligent harm caused by

poor protocol design by the Chief Investigator and researchers employed by the University.

- Sites participating in the study will be liable for clinical negligence and other negligent harm to individuals taking part in the study and covered by the duty of care owed to them by the sites concerned. The co-sponsors require individual sites participating in the study to arrange for their own insurance or indemnity in respect of these liabilities.
- Sites which are part of the United Kingdom's National Health Service will have the benefit of NHS Indemnity.
- Sites out with the United Kingdom will be responsible for arranging their own indemnity or insurance for their participation in the study, as well as for compliance with local law applicable to their participation in the study.

14. REPORTING, PUBLICATIONS AND NOTIFICATION OF RESULTS

14.1. AUTHORSHIP POLICY

Ownership of the data arising from this study resides with the study team.

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