The Pathogenesis of Oxaliplatin Induced Sinusoidal Obstruction Syndrome

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Declaration

I confirm that the material contained within this thesis is my own work unless otherwise stated within the text. The data contained within this thesis has not been previously submitted for the award of a higher degree at this or any other University.

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Abstract

Oxaliplatin based chemotherapy has demonstrated remarkable efficacy in down staging colorectal liver metastases such that patients initially considered to have inoperable disease are able to undergo a potentially curative resection. In addition the use of neoadjuvant Oxaliplatin based chemotherapy has been shown to improve progression free survival following liver resection. Taken together this means that ever increasing numbers of patients are presenting for liver resection having received multiple cycles of chemotherapy.

Whilst this approach has many advantages the use of pre-operative chemotherapy has been associated with the development of sinusoidal obstruction syndrome (SOS) in the liver parenchyma in up to 40% of patients. It is thought that the presence of SOS significantly increase the risks associated with major liver resection. More recent data also suggests that the presence of SOS within the liver may result in poorer disease specific outcomes in the long term and in particular a higher risk of early intra-hepatic recurrence. At present the pathogenesis of SOS in this context is not understood and no treatment exists to either prevent its development or reverse the histological changes in the liver associated with it.

The aim of the current study was to develop a reproducible *in vivo* experimental model of Oxaliplatin induced SOS and to interrogate this to identify the pathophysiological mechanisms which underpin its development. With this knowledge it was hoped that potential therapeutic strategies could be suggested to ameliorate the development of SOS in patients treated with Oxaliplatin based chemotherapy.

C57BL/6J mice treated with weekly intraperitoneal injections of Oxaliplatin and 5-FU/Leucovorin for 5 weeks develop histological changes of SOS when maintained on a

purified, but not chow, diet. This is associated with increased expression of key matrix remodelling genes within the liver parenchyma such as MMP2, MMP9, TIMP1, TGFβ and Procollagen I. The development of these gene expression changes is accelerated in the presence of tumour within the liver perhaps as a consequence of increased production of inflammatory mediators such as CXCL1.

The presence of SOS is associated with a dramatic increase in expression of the serine protease family member PAI-1 which is involved in a variety of processes including matrix remodelling, thrombus formation and cellular senescence. Immunohistochemistry revealed endothelial cells in areas of sinusoidal injury stained positive for the cell cycle inhibitor $p21^{CIP1/WAF1}$ in keeping with senescence in these cells. This process was associated with depletion of hepatic glutathione and decreased expression of the antioxidant transcription factor NRF2 suggesting a role for oxidative stress in the pathogenesis of SOS.

To explore this further the experiment was repeated but this time using dietary supplementation with either the thiol donor N-Acetylcysteine (NAC) or the NRF2 activator butyrated hydroxyanisole (BHA) alongside FOLFOX treatment. Whilst supplementation with NAC had no effect on the development of SOS its development was completely prevented by supplementation with BHA suggesting that NRF2 activating antioxidants may be a useful therapeutic strategy in preventing the development of SOS.

In conclusion this study has described the first reproducible experimental model of Oxaliplatin induced SOS which accurately reflects the pathogenesis of the disease in humans. Through interrogation of this it has been possible to identify therapeutic strategies which may be of value in preventing the development of SOS in patients with colorectal liver metastases.

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List of Abbreviations

5-FU	5-Fluouracil
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
αSMA	α smooth muscle actin
ARE	Anti-oxidant response element
AST	Aspartate aminotransferase
ATP	Adenosine triphosphate
ATP7A	ATPase 7A
ATP7B	ATPase 7B
BHA	Butyrated hydroxyanisole
CALI	Chemotherapy associated liver injury
CT	Computed Tomography
CTR1	Copper transporter 1
CTR2	Copper transporter 2
DHFU	Dihydrofluorouracil
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid

DPD	Dihydropyrimidine dehydrogenase
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbant assay
FdUMP	Fluorodeoxyuridine monophosphate
FdUTP	Fluorodeoxyuridine triphosphate
FLR	Future Liver Remnant
FUTP	Fluorouridine Triphosphate
GSH	Reduced glutathione
GSSG	Oxidised glutathione
γH2AX	Phosphorylated histone variant H2AX protein
H&E	Haematoxylin and eosin
HGF	Hepatocyte growth factor
IC_{50}	50% Inhibitory Concentration
i.p.	Intraperitoneal
LV	Leucovorin (Folinic acid)
MMLV-RT	Murine leukaemic virus reverse transcriptase
MMP2	Matrix metalloproteinase 2

MMP9	Matrix metalloproteinase 9
MOPS	3-(N-morpholino)propanesulfonic acid
MPA	Metaphosphoric acid
MTT	Thiazoyl blue tetrazolium bromide
NAC	N-Acetylcysteine
NADPH	β-nicotinamide adenine dinucleotide phosphate
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NRF2	Nuclear factor erythroid-2 related factor
NQO1	NAD(P)H dehydrogenase 1
PAI-1	Plasminogen activation inhibitor 1 (SERPINE1)
PBS	Phosphate buffered saline
PVE	Portal Vein Embolisation
RNA	Ribonucleic acid
SOS	Sinusoidal obstruction syndrome
STAT1	Signal transducer and activator of transcription 1
STAT3	Signal transducer and activator of transcription 3
TBS	Tris buffered saline

TGF-β	Transforming growth factor β
TIMP1	Tissue inhibitor of metalloproteinase 1
TLR4	Toll like receptor 4
TNF-α	Tumour necrosis factor α
TP	Thymidine phosphorylase
TS	Thymidilate synthase
TXN1	Thioredoxin1
VEGF-A	Vascular endothelial growth factor-A
VEGFR-1	Vascular endothelial growth factor receptor 1
VEGFR-2	Vascular endothelial growth factor receptor 2
vWF	von Willebrand factor

Chapter 1

Contemporary Management of Colorectal Liver Metastases

1.1 Background

Colorectal cancer is the third most commonly diagnosed malignancy in the UK and in 2008 a new diagnosis of this disease was made in 39,991 patients and was listed as the primary cause of death in 16,259 patients. The incidence of colorectal cancer begins to rise from the age of 50 and peaks between the ages of 70 and 80. (Cancer Research UK 2011) A large population based survey of 13,463 patients presenting with a primary colorectal cancer over a 14 year period demonstrated that 15% of patients had liver metastases present at the time of diagnosis. In the vast majority of these cases (77%) the liver was the only site of metastatic disease.(Manfredi, Lepage et al. 2006) A similar registry based study reported that overall 27% of patients with colorectal cancer will develop liver metastases at some point in the course of their disease with 19% developing this within 6 months of the primary diagnosis.(Leporrier, Maurel et al. 2006)

Untreated the prognosis for patients with liver metastases is extremely poor with the median survival being as low as 7.5 months.(Stangl, Altendorf-Hofmann et al. 1994) As early as 1963 the concept of resecting isolated colorectal liver metastases began to emerge with a series of 25 patients, published by George Woodington and colleagues at the Mayo clinic. These patients had a median survival of 35.1 months.(Woodington and Waugh 1963) A later series from the same centre in 1976 confirmed these positive results in a larger series of 60 patients with a 5 year survival of 28%.(Wilson and Adson 1976) A seminal paper from Stangl et al. published in 1994 firmly established surgical resection of liver metastases as the bench

mark against which other treatments would be judged. In this series of 1099 consecutive patients with colorectal liver metastases 261 underwent surgical resection of all disease, achieving an overall 5 year survival rate of 41% (median survival 41 months). In comparison there were no 5 year survivors in those treated with either systemic chemotherapy (n=71; median survival 11.1 months) or those who received no treatment at all (n=484; median survival 7.5 months).(Stangl, Altendorf-Hofmann et al. 1994)

The five year survival rates for colorectal cancer have improved significantly in the UK over the last few decades being a little over 20% during the period 1971 – 1975 to almost 50% during the period 2001 – 2006.(Cancer Research UK 2011) Whilst advances in the surgical treatment of metastatic disease may account for some of these improvements it is by no means the only factor. Another significant advance in the management of this disease has been the development of effective systemic chemotherapy. The adoption of systemic chemotherapy as a treatment option for metastatic colorectal metastases is highlighted in a study from the Cote D'Or cancer registry. This demonstrated that for those patients presenting with distant metastatic disease during the period 1976 – 1984 only 8% received systemic chemotherapy as compared to 30% of patients during the period 1994 – 2003.(Guyot, Faivre et al. 2005)

In 2009 Kopetz et al. published a retrospective review of survival in a series of 2471 patients with metastatic colorectal cancer treated at either MD Anderson or the Mayo Clinic over a 17 year period from 1990 until 2006. In this study there was a dramatic increase in overall 5 year survival from 9% during the period 1990 – 1997 to 19% during the period 2001 – 2003. Only 20% of patients in this series were able to undergo a curative hepatic resection for their metastatic disease but for those who did, the 5 year survival during the period 1998 –

2006 was 55% as compared to 20% in those who did not. In the group that did not undergo hepatic resection there was a dramatic improvement in overall survival during the period 2004 to 2006 as compared to those patients treated prior to 1998 (Hazard Ratio 0.53, 95% CI 0.45 – 0.62; $p<0.001$). It was noteworthy that this improvement was temporally linked with the widespread adoption of modern systemic chemotherapeutic agents such as Oxaliplatin and Irinotecan.(Kopetz, Chang et al. 2009)

The advances made in systemic chemotherapy for metastatic colorectal cancer have undoubtedly played a significant role in the improvements in 5 year survival that have been observed for this disease. This has lead to an increasing recognition that a multimodal approach optimising the use of both surgery and chemotherapeutic agents together is required in order to achieve long term survival.(Alberts and Poston 2011) In the remainder of this chapter the current trends in the surgical management of colorectal liver metastases and how this is complemented by systemic therapy in patients with both operable and inoperable disease at the time of presentation will be discussed.

1.2 Surgical Resection of Colorectal Liver Metastases

1.2.1 Defining Resectability

As surgical resection of colorectal liver metastases began to emerge as a viable treatment strategy in the 1970's and 80's it became important to define which patient groups would benefit from this treatment option and which would not. In 1986 Ekberg et al. reported an analysis of factors predicting survival in 72 patients undergoing resection of colorectal liver metastases.(Ekberg, Tranberg et al. 1986) On the basis of this study it was concluded that the following should be considered contra-indications to surgical treatment:

1) The presence of 4 or more liver metastases
2) The presence of extra-hepatic disease
3) The inability to obtain a clear hepatic resection margin of at least 1cm

These criteria were subsequently adopted by the surgical community as defining what constituted resectable disease. The difficulty with these criteria is that when they are applied only 10% of patients with colorectal liver metastases will be considered suitable candidates for liver resection.(Poston, Adam et al. 2006)

The concept that tumour number is a limiting factor in determining resectability has been challenged in recent years. In 1995 Scheele reported a series of 469 patients who underwent liver resection for colorectal metastases. The presence of five or more metastases was identified in this series as being an indicator of poor prognosis but if all evidence of other disease was removed then the prognostic effect of this variable was lost.(Scheele, Stang et al. 1995) In a direct challenge to the dogma that 4 or more metastases are a contraindication to surgery Pawlik et al. published a series of 159 patients undergoing surgical resection for colorectal metastases all of whom had a minimum of 4

tumour deposits (median 5). In this series an overall 5 year survival of 51% was achieved and the authors concluded that this group of patients should not be excluded from surgical treatment on the basis of tumour number alone.(Pawlik, Abdalla et al. 2006) Kornprat et al. also published a series of 98 patients with 4 or more colorectal liver metastases undergoing curative intent surgery over an 8 year period in whom an actuarial 5 year survival of 31% was achieved. It is of note that in those patients with fewer than 6 tumours the actuarial 5 year survival was 39% as compared to 19% in those with 6 or more tumours. (Kornprat, Jarnagin et al. 2007)

In 2009 de Jong et al. published a multi-institutional analysis of 1669 patients who had undergone curative intent surgery for colorectal metastases with a specific focus on identifying those factors able to predict recurrence. On multivariate analysis it was identified that the presence of 4 or more tumours at the time of surgery was associated with an increased risk of extra-hepatic recurrence (Hazard Ratio 1.68; p=0.01) but not intra-hepatic recurrence.(de Jong, Pulitano et al. 2009)

The evidence for the effect of tumour number on outcome following resection of colorectal liver metastases is summarised in Table 1 below. In light of these studies it is reasonable to conclude that, provided it is possible to remove all evidence of disease, the number of hepatic metastases per se is not a contra-indication to resection and it can allow local disease control to be obtained. Nonetheless an increasing number of metastases is a marker of poorer tumour biology and as such this group of patients are at an overall greater risk of disease recurrence and are perhaps appropriate for more intense follow up in the early post-operative course.(Gomez, Morris-Stiff et al. 2010)

Study	Summary of Key Findings
Ekberg, Tranberg et al. 1986	No patients with > 4 metastases survived beyond 3 years
Scheele, Stang et al. 1995	The presence of >4 metastases was a negative predictor of resectability but if a radical resection was possible it had no impact on disease free or overall survival
Pawlik, Abdalla et al. 2006	Patients with >4 metastases undergoing liver resection achieved a 5 year overall survival of 51%
Kornprat, Jarnagin et al. 2007	Resection of >4 metastases was associated with an actuarial overall 5 year survival of 33%
de Jong, Pulitano et al. 2009	Resection of >4 metastases was associated with an increased risk of extrahepatic recurrence (HR 1.68; p=0.01)

Table 1 - Summary of evidence for the effect of tumour number on outcome following resection of colorectal liver metastases

It is not particularly surprising that the presence of extra-hepatic metastases is associated with a poorer prognosis in patients undergoing resection of liver metastases, a view upheld in several early case series.(Ekberg, Tranberg et al. 1986; Hughes, Simon et al. 1986) It is increasingly recognised that there are two groups of patients with extra-hepatic disease – those with diffuse multifocal disease and those with localised extra-hepatic disease that might be amenable to surgical resection.

In patients with isolated lung metastases from colorectal cancer it is generally accepted that surgical resection, where possible, offers a good option for disease control with 5 year survival rates in the order of 30 – 45% being reported in the literature.(Zink, Kayser et al.

2001; Saito, Omiya et al. 2002; Inoue, Ohta et al. 2004; Iizasa, Suzuki et al. 2006; Rama, Monteiro et al. 2009; see Table 2) This has led some authors to question whether or not patients presenting with synchronous, but resectable, liver and lung metastases should be offered surgical treatment.

Study	**Summary of Key Findings**
Zink, Kayser et al. 2001	In 110 patients undergoing resection of isolated lung metastases a 5 year overall survival of 33% was achieved
Saito, Omiya et al. 2002	In 165 patients undergoing resection of isolated lung metastases a 5 year overall survival of 39.6% was achieved. The overall survival at 10 years was 37.2%
Inoue, Ohta et al. 2004	In 128 patients undergoing resection of isolated lung metastases a 5 year overall survival of 45% was achieved.
Iizasa, Suzuki et al. 2006	In 75 patients undergoing resection of isolated lung metastases a 5 year overall survival of 41% was achieved.
Rama, Monteiro et al. 2009	In 61 patients undergoing resection of isolated lung metastases a 5 year overall survival of 48% was achieved. The overall survival at 10 years was 11%.

Table 2 - Summary of outcomes from studies reporting outcome of isolated lung metastases from colorectal cancer

Brouquet et al. recently published a consecutive series of 112 patients undergoing liver and lung resection for colorectal metastases over a 14 year period and compared their outcome to 1148 patients who underwent liver only resection. Remarkably they demonstrated that the overall 5 year survival for patients undergoing combined liver and lung resection was

50% as compared to 40% in the patients undergoing liver only resection (p=0.01). It should be noted however that the use of systemic chemotherapy was considerably higher in the former group (69% vs. 57% p=0.01).(Brouquet, Vauthey et al. 2011) In a similar series of 131 patients undergoing resection of both liver and lung metastases from colorectal cancer Miller et al. reported an overall 5 year survival of 31% once both sites of disease had been resected.(Miller, Biernacki et al. 2007) Similar findings have been reported in other published series (Shah, Haddad et al. 2006; Neeff, Horth et al. 2009) suggesting that, in appropriately selected patients, the presence of both pulmonary and hepatic metastases should not be considered a barrier to surgical resection. The key findings of these studies are summarised in Table 3. It is likely that the role of surgery in the management of pulmonary colorectal metastases will be more clearly defined when PulMICC randomised control study, whereby patients with pulmonary metastases are allocated to either surgery or active observation, reports in due course.(Treasure, Fallowfield et al. 2012)

Study	Summary of Key Findings
Miller, Biernacki et al. 2007	131 patients undergoing resection of both liver and lung metastases achieved an overall 5 year survival of 31%
Shah, Haddad et al. 2006	39 patients undergoing resection of both liver and lung metastases achieved an overall 5 year survival of 74%
Neeff, Horth et al. 2009	44 patients undergoing resection of both liver and lung metastases achieved an overall 5 year survival of 42%
Brouquet, Vauthey et al. 2011	112 patients undergoing resection of both liver and lung metastases achieved an overall 5 year survival of 50%

Table 3 - Summary of key findings from studies reporting the outcome of patients undergoing resection of both liver and lung metastases of colorectal cancer

In 1997 Beckurts et al. reported a series of 126 patients who had undergone resection of colorectal liver metastases 28% of whom were identified to have metastatic disease present in lymph nodes of the hepato-duodenal ligament. In those patients with node negative disease the overall 3 and 5 year survival figures were 48% and 22% respectively as compared to 3% and 0% for those with node positive disease.(Beckurts, Holscher et al. 1997) Two more recent prospective series have confirmed the dismal survival in patients undergoing liver resection in the presence of peri-portal lymph node metastases.(Kokudo, Sato et al. 1999; Laurent, Sa Cunha et al. 2004) In 2009 Carpizo et al reported a series of 127 patients who underwent concomitant resection of liver metastases and extra hepatic disease, 27 of whom underwent resection of isolated lymph node metastases at the porta hepatis. It is noteworthy that the 5 year survival of this subgroup of patients was significantly worse (12%) as compared to those who underwent resection of either lung

(28%), peritoneal (30%) or ovarian metastases (51%).(Carpizo, Are et al. 2009) At the present time these data support the view that the presence of overt lymph node metastases in the hepato-duodenal ligament should be regarded as a relative contra-indication to the resection of liver metastases.

Occasionally isolated adrenal metastases of colorectal cancer are encountered and when unilateral are potentially amenable to surgical treatment. De Haas et al. reported a single centre experience of 796 resections for colorectal liver metastases. During follow of this patient cohort they detected adrenal metastases in 14 individuals. Of these 14 patients all received chemotherapy and 10 went on to have a subsequent adrenalectomy. The median overall for patients with adrenal metastases was 23 months irrespective of surgical status.(de Haas, Rahy Martin et al. 2009) The reported series of patients presenting with isolated adrenal metastases and undergoing subsequent surgery are extremely small and therefore make it difficult to reach meaningful conclusions about the role of surgery in this cohort of patients.(Mourra, Hoeffel et al. 2008; Marangos, Kazaryan et al. 2009)

Following on from the paper of Ekberg et al.(Ekberg, Tranberg et al. 1986) in 1986 several case series were published which up held the belief that a clear margin of 1cm was the minimum requirement when undertaking resection of colorectal liver metastases – indeed less than this was demonstrated to be associated with an increased risk of recurrence and poorer overall survival. (Hughes, Simon et al. 1986; Cady, Jenkins et al. 1998) In a multicentre series of 557 patients undergoing resection of colorectal liver metastases, over a 14 year period, Pawlik et al. examined the effect of different resection margin widths on both disease recurrence and overall survival. On univariate analysis they demonstrated that the presence of tumour at the resection margin (i.e. margin <1mm) was associated with an

increased risk of disease recurrence (51.1% vs. 38.6% p = 0.04) and poorer 5 year survival (63.8% vs. 17.1%; p = 0.01). Provided tumour was greater than 1mm from the hepatic transection line no effect on survival or recurrence rates could be demonstrated. It is also noteworthy that on multivariate analysis resection margin status was not an independent predictor of disease recurrence or poorer survival.(Pawlik, Scoggins et al. 2005)

In a similar single centre series of 314 patients undergoing resection of colorectal liver metastases Muratore et al. compared the effect of a margin positive resection (<1mm), a clear margin of less than 1cm and a margin greater than 1cm on long term outcome. Again in this study the width of the surgical margin was not a factor in determining the risk of recurrence or long term survival provided that that the line of transection was free of tumour.(Muratore, Ribero et al. 2010) Other case series have confirmed the view that a less than 1cm resection margin is not an independent predictor of disease recurrence on multivariate analysis.(Bodingbauer, Tamandl et al. 2007; Figueras, Burdio et al. 2007)

It can be seen from the discussion so far that, over the last decade, many of the conventional criteria for defining resectability of colorectal liver metastases have been challenged and, in many cases, found not to hold true. The decision of whether or not to proceed with liver resection for colorectal liver metastases can no longer be based upon these criteria alone. Rather one must also consider the extent of resection necessary to remove the disease and therefore the risks posed to the individual patient in the peri-operative period.

1.2.2 Safety of Liver Surgery for Colorectal Liver Metastases

In a systematic review of studies reporting outcome for patients undergoing resection of colorectal liver metastases Simmonds et al. reported a median 30 day mortality of 2.8% and

a median hospital stay of 9.5 days.(Simmonds, Primrose et al. 2006) Despite being published in 2006 the literature search for this study was performed in 2000 meaning that the majority of included studies were published in the 1980's and 90's. A single centre series of 1803 consecutive liver resections over the decade 1991-2001 revealed an overall operative mortality of 3.1%. When the periods 1992-3 (n=259) and 2000-01 (n=392) were compared a significant reduction in operative mortality was demonstrated (3.5% vs. 1.3%).(Jarnagin, Gonen et al. 2002) Other large series published within the last decade have confirmed a reduction in operative mortality with typical rates being less than 1%.(Imamura, Seyama et al. 2003; Cescon, Vetrone et al. 2009; Kamiyama, Nakanishi et al. 2010) It is unlikely that this improvement is attributable to any one single factor but rather represents a variety of factors including increased surgical expertise, technological advances and improved patient selection.

Perhaps one of the most dreaded complications following liver resection is the development of post-hepatectomy liver failure, which was recently defined in a consensus statement from the International Study Group of Liver Surgery as:

> "...a postoperatively acquired deterioration in the ability of the liver to maintain its synthetic, excretory and detoxifying functions, which are characterised by an increased INR and concomitant hyperbilirubinaemia on or after postoperative day 5."(Rahbari, Garden et al. 2011)

Clearly the difficulty with such a definition is that it will encompass a varied spectrum of disease ranging from little more than deranged blood tests to patient death. The study group therefore went on to further subdivide post-hepatectomy liver failure into 3 grades where grade A required no changes to be made to patient management, grade B required a

change to patient management but no invasive treatment and grade C required invasive treatment.(Rahbari, Garden et al. 2011) To validate this system it was applied retrospectively to a series of 835 patients of whom 65 (11%) developed post-hepatectomy liver failure. Grade A liver failure was present in 8% (5 of 65) of these, grade B in 72% (47 of 65) and grade C in 20% (13 of 65) with the peri-operative mortality in these 3 groups being 0%, 12% and 54% respectively.(Reissfelder, Rahbari et al. 2011) A similar system was applied by Mullen et al. to a series of 1059 patients undergoing liver resection and they demonstrated on multivariate analysis that a bilirubin of 7mg/dl, on or after post-operative day 5, was associated with a markedly increased risk of 90 day mortality (Odds Ratio = 250; $p<0.0001$).(Mullen, Ribero et al. 2007)

A key determinant in predicting the development of post-hepatectomy liver failure is the presence of underlying liver disease. In a series of 747 patients undergoing liver resection over an 8 year period Belghiti et al. reported in those with histological abnormalities in the underlying liver (either cirrhosis or steatosis > 30%, n=253) the operative mortality was 9.5% as compared to only 1% in those without underlying parenchymal disease.(Belghiti, Hiramatsu et al. 2000) A systematic review and meta-analysis of the published literature by De Meijer et al. reported that the presence of steatosis > 30% was associated with an increased risk of both peri-operative complications (Risk Ratio 2.01; $p<0.0001$) and mortality (Risk Ratio 2.79; $p = 0.02$) in patients undergoing major hepatectomy (≥3 Couinaud segments).(de Meijer, Kalish et al. 2010) In support of this rodent studies have demonstrated that liver resection in the context of steatosis is associated with increased hepatocellular injury and a poorer functional recovery.(Vetelainen, Bennink et al. 2007; Vetelainen, van Vliet et al. 2007)

The other key factor in determining the likelihood of post-hepatectomy liver failure is the volume of liver that will remain after resection (known as the future liver remnant or FLR) and this can normally be calculated pre-operatively using techniques such as CT volumetry.(Karlo, Reiner et al. 2010) In a study of patients undergoing hepatic trisectionectomy for colorectal liver metastases Shoup et al. determined that, in those with a FLR of 25% or less, 90% developed post-hepatectomy liver failure as compared to none of those with an FLR greater than 25% ($p<0.0001$).(Shoup, Gonen et al. 2003) In a prospective study of 119 patients undergoing liver resection Ferrero et al. reported that in those patients with a normal hepatic parenchyma who went on to develop post-hepatectomy liver failure the median FLR was 26.9% as compared to 49.1% in those who did not.(Ferrero, Vigano et al. 2007) It is on the basis of evidence such as this that the recommended minimum safe FLR, in patients with an otherwise normal liver, undergoing resection of colorectal metastases is 25%.(Garden, Rees et al. 2006; Pawlik, Schulick et al. 2008) In patients with underlying parenchymal disease the required FLR for safe liver surgery increases significantly and maybe as high as 40%.(Kubota, Makuuchi et al. 1997)

For those patients in whom it is not possible to resect all disease with an adequate FLR it is sometimes necessary to consider alternative strategies, such as pre-operative portal vein embolisation (PVE) or two-stage hepatectomy, in order to minimise the risk of surgical treatment. The role of these strategies, in patients with colorectal liver metastases, is discussed in more detail below.

1.2.3 Pre-Operative Portal Vein Embolisation

Whilst the volume of the individual liver segments (Figure 1) is highly variable data from a series of 102 patients who underwent CT volumetry has suggested that the right lobe

typically constitutes 65% of the total liver volume.(Abdalla, Denys et al. 2004) On occasion, in patients with colorectal liver metastases, it is necessary to perform more extensive procedures than a classical hemi-hepatectomy. For example, in a patient with a tumour in the right lobe of the liver but in close proximity to the middle hepatic vein, it may be necessary to perform a right hepatectomy (segments 5 to 8) alongside resection of segment 4.(Halazun, Al-Mukhtar et al. 2007; Karanjia, Lordan et al. 2009) In this situation the FLR would consist of segments I, II and III which, according to the data of Abdalla et al., would comprise no more than 20% of the original liver volume thereby placing these patients at high risk for post-hepatectomy liver failure.(Abdalla, Denys et al. 2004)

In situations such as these where disease could technically be removed in a single operation but would leave an inadequate FLR, then the technique of PVE is particularly useful. The experimental basis for this technique originates from work in the 1920's, where it was observed that ligation of one branch of the portal vein in a rabbit results in atrophy of the ipsilateral hepatic lobe with subsequent compensatory hypertrophy of the contralateral lobe. (Rous and Larimore 1920) In the clinical situation the portal vein is normally accessed by a percutaneous approach and then embolised using an appropriate material (e.g. coils, cyanoacrylate glue etc).(Abdalla, Hicks et al. 2001) The safety of this approach was demonstrated by Makuuchi et al. in a series of 14 patients with hilar cholangiocarcinoma.(Makuuchi, Thai et al. 1990) In a prospective trial by Farges et al. patients undergoing a right hepatectomy were assigned to receive either right PVE or standard management. In those patients with an otherwise normal liver parenchyma who underwent PVE there was a median 16% increase in the FLR (as assessed by CT Volumetry) as compared to 9% in those with background chronic liver disease.(Farges, Jagot et al. 2002)

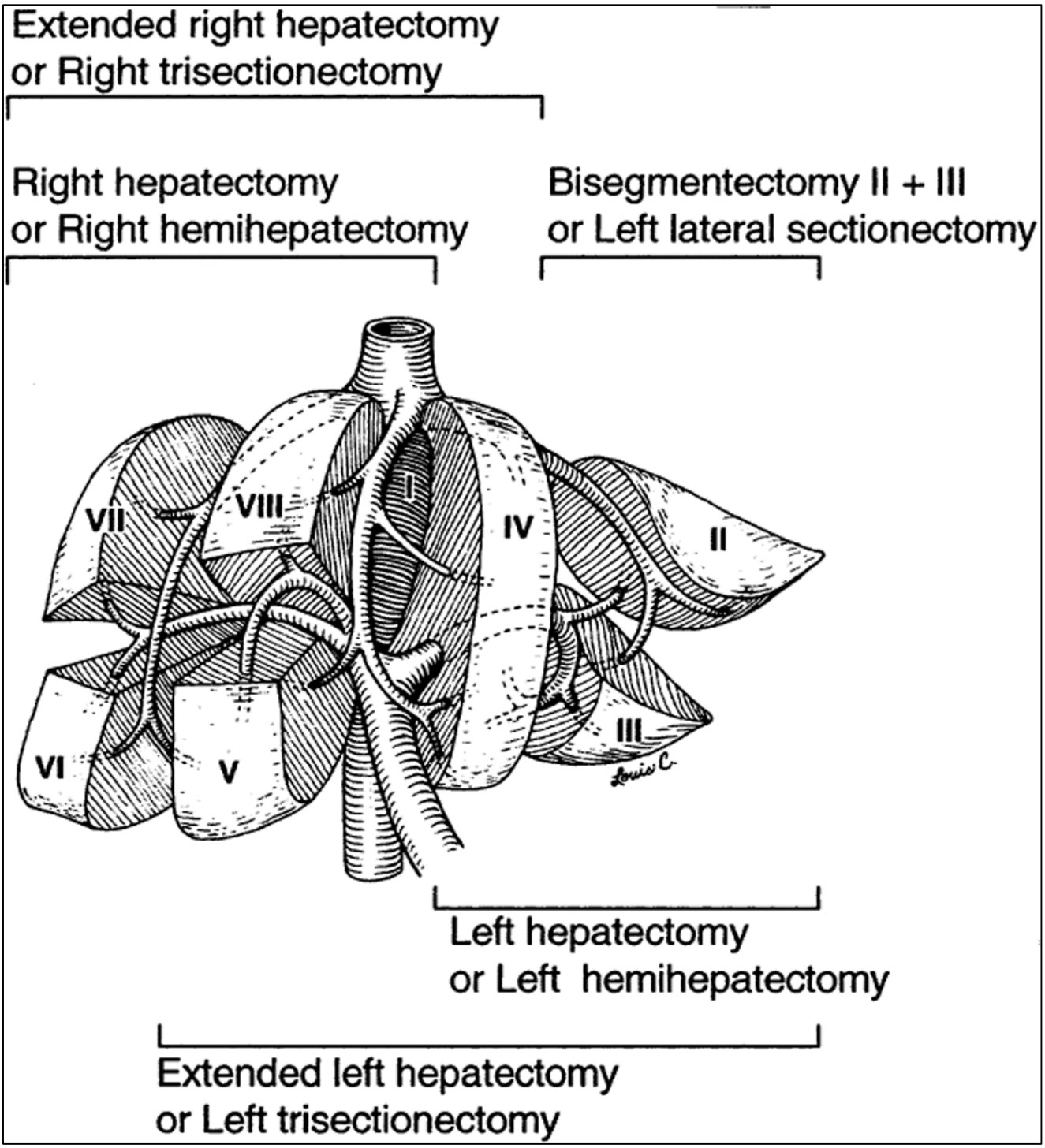

Figure 1 - Pictoral description of hepatic anatomy with common resections labelled. From Abdalla et al. 2004(Abdalla, Denys et al. 2004)

Wicherts et al. retrospectively compared the outcome of 67 patients who had received pre-operative PVE with 297 who did not and subsequently underwent major hepatic resection (≥3 Couinaud segments). They demonstrated that whilst there was no difference in post-operative mortality between the two groups the overall complication rate was significantly higher in those who had PVE performed (55% vs. 41.1%; p=0.035). Interpreting this data is difficult as 54% of patients in the PVE group underwent a right trisectionectomy as compared to only 28% in the control group. Those who underwent pre-operative PVE had poorer 3 year overall survival than controls (44% vs. 61%; p<0.001). It is noteworthy that in 32 patients who underwent PVE embolisation but subsequently didn't proceed to surgery there were no survivors at 3 years suggesting that the combination of PVE and surgery offers a significant survival advantage but is probably disadvantageous if surgery is not performed.(Wicherts, de Haas et al. 2010)

In a similar study Pamecha et al. compared the outcome of 22 patients undergoing either right hepatectomy or right trisectionectomy after PVE with 65 who did not receive PVE. The surgical mortality did not differ between groups but the morbidity was higher in those receiving PVE (36% vs. 20%) however again more patients in the PVE group underwent right trisectionectomy (22% vs. 11%). The 3 year overall survival in the PVE group was slightly lower at 52% as compared to 65% in the control arm although this difference was not statistically significant. Of the original cohort who underwent PVE (n=36) 12 did not progress to surgery and had a median survival of 14 months as compared to 42 months in the PVE and surgery group (p<0.0001).(Pamecha, Glantzounis et al. 2009)

It is striking from both of these series that approximately one third of patients with colorectal liver metastases treated with PVE will not subsequently undergo planned surgery

and, in the majority of cases, this is as a result of disease progression.(Pamecha, Glantzounis et al. 2009; Wicherts, de Haas et al. 2010) In a series of 18 patients undergoing PVE prior to resection of colorectal metastases Kokudo et al. noted there was a 21% increase in tumour mass at 3 weeks following the procedure as measured by CT Volumetry. They also compared the Ki67-index in these patients with that of 29 controls and found this to be significantly increased in the PVE group (46.6% vs. 35.4%; p = 0.013).(Kokudo, Tada et al. 2001) In a similar study Pamecha et al. also confirmed an elevated Ki-67 index and mitotic cell count in the tumours of patients treated by PVE.(Pamecha, Levene et al. 2009)

Following PVE there is increased hepatic production of a variety of growth factors such as HGF, as well as cytokines involved in liver regeneration, and these may play a role in driving metastases progression.(Uemura, Miyazaki et al. 2000; Yokoyama, Nagino et al. 2007) In addition post-PVE the arterial supply to the embolised lobe increases.(Denys, Abehsera et al. 2000) Given that the blood supply of colorectal metastases is predominantly derived from the hepatic artery(Archer and Gray 1989) it is conceivable that this leads to increased oxygen and nutrient supply to the tumour thereby encouraging its growth.

It can be seen from this discussion that whilst PVE is a useful technique for patients who are at risk for having an insufficient FLR it must be remembered that nearly one third of patients in whom this procedure is performed will not progress to surgery as a result of PVE induced tumour progression. This group of patients appear to have a particularly poor prognosis and therefore PVE in patients with colorectal liver metastases must be considered very carefully.

1.2.4 Two-Stage Hepatectomy

In patients presenting with bilobar disease who may require, for example, an extended right hepatectomy and resection of a solitary metastases in segment II then a two-stage

hepatectomy may be preferable to PVE because of the risk of tumour progression in the FLR. In the case of this scenario it would typically consist of an initial procedure to clear the left lateral segment of the liver with a metastatectomy with either intra-operative ligation of the right portal vein or post-operative percutaneous right portal vein embolisation, thereby encouraging hypertrophy of the remnant liver, before undertaking an extended right hepatectomy as a second operation several weeks later.(Jaeck, Oussoultzoglou et al. 2004) On occasion it may not be necessary to undertake the portal vein embolisation if it is felt that the hypertrophy induced by the primary procedure will be enough to give an adequate FLR following the second resection.(Adam, Laurent et al. 2000)

In 2008 Wicherts et al. reported a series of 59 patients who were considered to have non resectable colorectal liver metastases using a single step surgical approach but were felt to be amenable to a two stage procedure. All patients underwent an initial surgical procedure which, in the majority of cases, consisted of a minor hepatic resection (< 3 Couinaud segments) with the aim of clearing the left liver of tumour. Of the 59 patients 17 were unable to go a second procedure because of tumour progression. Of those patients that did undergo a second procedure this took place typically 3 months following the primary procedure and consisted of a major resection (≥3 Couinaud segments) in the majority of cases. The overall 5 year survival in this series was 31% on an intention to treat basis. It is noteworthy that when compared to patients undergoing a single stage procedure over the same time period there was no statistically significant difference in survival between the two groups although there was a trend towards poorer survival in the two-stage group.(Wicherts, Miller et al. 2008)

In another series Narita et al. report the outcome of 79 patients selected to undergo a two stage hepatectomy. Following the primary procedure 75 patients were considered appropriate to undergo a second stage procedure, the majority of whom (92%) underwent portal vein embolisation. Off this cohort 61 underwent a second stage procedure (78% of original patients). The main reasons why patients failed to progress to a second resection were either tumour progression (n=10) or insufficient regeneration following PVE (n=5). In those undergoing a two stage procedure 16% were found to have a de novo metastases present in the FLR which was dealt with at the time of operation. The overall survival 5 year survival in the 61 patients who underwent a completed two-stage hepatectomy was 32%. In addition 11 patients went on to have a subsequent lung resection for metastatic disease but their survival was no different to the 50 patients who did not require this. (Narita, Oussoultzoglou et al. 2011)

Two-stage hepatectomy is clearly a major undertaking for any patient and given the not in-significant risk of failing to complete the treatment plan it is essential that, in a similar manner to PVE, this is only offered to a carefully selected group of patients. Nonetheless it does provide a treatment option for those patients with advanced disease which has the potential to prolong survival.

1.2.5 The New Surgical Paradigm

It is clear that our understanding of the behaviour of metastatic colorectal cancer and the benefits offered by surgical treatment have evolved considerably over the last decade. The strict stipulations set down in the 1980's with regard to what constituted resectable disease have been challenged in recent years and are now of little relevance to modern surgical practice. In addition the advent of techniques such as PVE and two-stage hepatectomy have

meant that patients can undergo more extensive hepatic resections that would previously have not been possible because of the unacceptable risk of post-hepatectomy liver failure. The result of this has been a fundamental change in the mind set of surgeons treating this disease with the question having changed from “Who is resectable?” to “Who is not resectable?”. (Pawlik, Schulick et al. 2008)

This change in outlook was reflected in a recent international consensus conference of the management of colorectal liver metastases which concluded that liver resection should be considered whenever it is possible to achieve a margin negative resection whilst maintaining adequate vascular inflow and biliary/hepatic venous outflow to a liver remnant of sufficient volume to avoid the risk of PHLF. In addition patients with extrahepatic metastatic disease should still be considered for resection provided that disease is amenable to either surgical resection or long term control with other adjuvant treatments. (Adams, Aloia et al. 2013)

Within this context surgical treatment canot be viewed in isolation. Alongside the evolution in thesurgical management of this diseasethere has also been significant progressing in the available systemic treatment options in terms of both conventional chemotherapeutics and biological agents targeting specific pathways implicated in tumour progression.(Robinson, Manas et al. 2009) These advances have important ramifications for the management of this group of patients, not only for those with inoperable disease but also for those amenable to surgical treatment, and this will be discussed in the next section of this chapter.

1.3 Systemic Agents for the Treatment of Colorectal Cancer

For many years the mainstay of chemotherapy for colorectal malignancies consisted solely of fluoropyrimidines (e.g. 5-FU) and these agents still form the backbone of modern chemotherapy regimens. A major transformation in chemotherapy for this disease took place over the early part of the last decade with the widespread introduction of newer agents such as Oxaliplatin and Irinotecan. These agents have revolutionised the treatment options available to patients with metastatic colorectal cancer and the evidence for their use is discussed in more detail below.

1.3.1 Fluoropyrimidines

The fluoropyrimidines are a family of compounds which include the parenterally administered agent 5-FU (Adrucil®, Fluouracil®, Efudex®, Fluoroplex®) and its oral pro-drug Capecitabine (Xeloda®). 5-FU is an analogue of the pyrimidine nucleotide uracil in which one of the hydrogen atoms is replaced by fluorine. After entry to the cell 5-FU is metabolised into several active compounds including:

- Fluorodeoxyuridine monophosphate(FdUMP)
- Fluorodeoxyuridine triphosphate (FdUTP)
- Fluorouridine triphosphate (FUTP)

FdUMP exerts its toxic effects through inhibition of thymidylate synthase which is a key enzyme involved in the generation of pyrimidine nucleotides necessary for DNA synthesis. In addition to this effect FUTP is able to be incorporated into RNA thereby disrupting its function. The generation of these active metabolites of 5-FU is dependent on the enzyme thymidine phosporylase (TP) however an alternative pathway also exists in many cells in which 5-FU is converted into the inactive metabolite dihydrofluorouracil (DHFU) whereby it

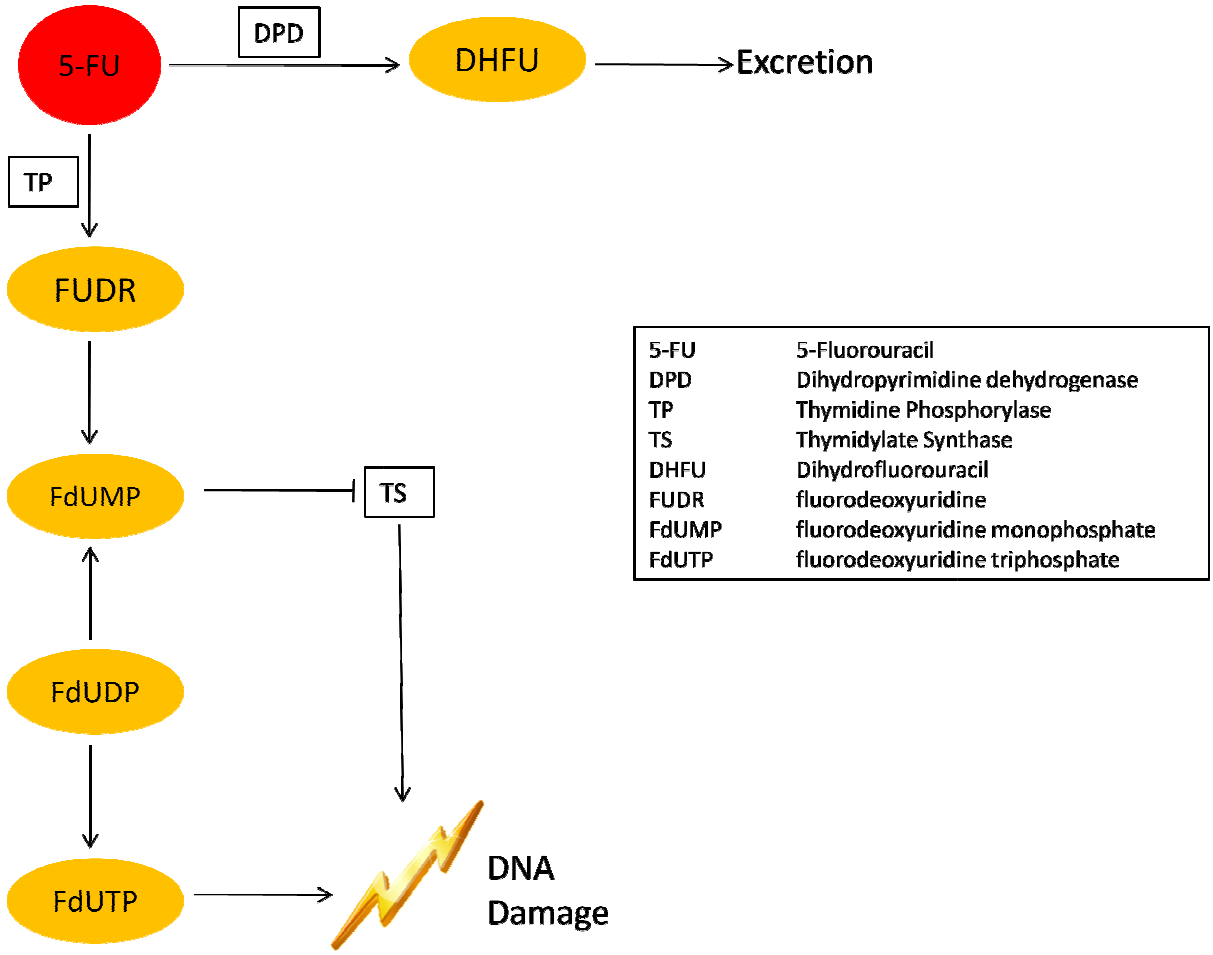

Figure 2 - Summary of 5-FU metabolism. Adapted from Longley et al.(Longley, Harkin et al. 2003)

is excreted, a process facilitated by the enzyme dihydropyrimidine dehydrogenase (DPD).(Longley, Harkin et al. 2003) The metabolism of 5-FU is summarised in Figure 2.

It is standard practice to administer Folinic acid (Leucovorin, LV) alongside fluoropyrimidines as this has been demonstrated to improve response rates when compared to 5-FU administered alone.(Poon, O'Connell et al. 1989)Administration of Folinic acid increases the intracellular levels of reduced folate which is essential for the irreversible binding of FdUMP to TS.(Longley, Harkin et al. 2003) The Mayo regimen consists of bolus injections of 5-FU/LV daily for 5 days in repeated cycles. When compared to 5-FU alone, in patients with advanced colorectal cancer, the addition of LV was found to improve overall median survival by almost 5 months (7.7 months vs. 12.2 months; p = 0.037).(Poon, O'Connell et al. 1989)

The efficacy of the Mayo regimen has been questioned since 5-FU has a very short half life in the plasma. Furthermore it is only active on cells that are in S phase and therefore in theory a more prolonged exposure of tumours to 5-FU would increase response rates. Awareness of this led to the development of the De Gramont regimen in which bolus 5-FU was substituted for a 24 hour infusion.(De Gramont, Krulik et al. 1988) A meta-analysis of 6 randomised controlled trials comparing bolus or continuous infusion of 5-FU, in a total of 1219 patients with metastatic colorectal cancer demonstrated that infusion of 5-FU was associated with an improvement in tumour response rate (22% vs. 14%; p = 0.0002) and a modest improvement in median survival (12.1 months vs. 11.3 months; p = 0.039).(Meta-analysis Group In Cancer 1998) Chronomodulated infusion of 5-FU, whereby the drug infusion is adjusted to match changes in circadian rhythm, has been proposed as a means of improving tumour response rates and minimising toxicity.(Cure, Chevalier et al. 2002)

Clearly the use of a self administered oral chemotherapeutic has many potential advantages to offer, in terms of quality of life for patients with advanced malignancy. In a randomised controlled trial of oral Capecitabine (n = 301) versus the Mayo regimen (n=301) in patients with advanced colorectal cancer the investigators assessed tumour response rates which were significantly greater in patients treated in the Capecitabine arm (26.6% vs. 17.9%; p = 0.013). There was no difference in median survival between the two groups (13.2 months vs. 12.1 months). The incidence of haematological toxicity was lower in those treated with Capecitabine whereas the incidence of cutaneous toxicity was higher.(Van Cutsem, Twelves et al. 2001) These results have been confirmed in a second similarly sized randomised phase III study.(Hoff, Ansari et al. 2001)

1.3.2 Oxaliplatin

Oxaliplatin is a third generation platinum (Pt) compound the structure of which consists of a platinum atom conjugated to diaminocyclohexane and oxalate groups. Following cellular uptake Oxaliplatin undergoes non-enzymatic hydrolysis to form the active drug which results in the generation of DNA-Pt adducts. These adducts cause DNA double strand breaks to occur with subsequent activation of cellular DNA repair mechanisms or, if sufficient DNA damage occurs, the activation of programmed cell death pathways. The active forms of Oxaliplatin are excreted from the cell by conjugation to glutathione and undergo subsequent renal clearance.(Kweekel, Gelderblom et al. 2005)

In 1996 Machover et al. reported two single arm Phase II studies which included a total of 106 patients with advanced colorectal cancer treated with Oxaliplatin, all of whom had experienced disease progression during treatment with a fluoropyrimidine based regimen. The overall tumour response rate for patients in both studies was 10% and the major dose

limiting toxicity was peripheral neuropathy.(Machover, Diaz-Rubio et al. 1996) A second phase II trial, this time using chronomodulated Oxaliplatin, by Levi et al. similarly demonstrated a 10% tumour response rate in patients who had experienced disease progression on fluoropyrimidine therapy.(Levi, Perpoint et al. 1993)

In vitro experiments on cancer cell lines and *in vivo* xenograft tumour models have demonstrated that the combination of Oxaliplatin and 5-FU are synergistic.(Raymond, Buquet-Fagot et al. 1997) This experimental data was confirmed in subsequent phase II trials which demonstrated tumour response rates anywhere between 20 and 50% when the two drugs were used in combination.(Levi, Zidani et al. 1994; Andre, Louvet et al. 1998) In a phase III trial Giacchetti et al. randomised 200 patients with metastatic colorectal cancer to receive chronomodulated 5-FU/LV either alone or in combination with Oxaliplatin. This study demonstrated a dramatic improvement in objective tumour response in the group receiving Oxaliplatin (53% vs. 16%; $p<0.01$) although there was no difference in median survival between the two groups (19.9 months vs. 19.4 months).(Giacchetti, Perpoint et al. 2000) In a second randomised control trial 420 patients, again with metastatic colorectal cancer, were randomised to receive 5-FU/LV either alone or in conjunction with Oxaliplatin. In this study the patients receiving the combined therapy demonstrated again a markedly improved objective response rate (50.7% vs. 22.3%; $p<0.0001$) but with no improvement in overall survival.(de Gramont, Figer et al. 2000)

Despite the lack of improvement in overall survival in these studies the dramatic improvement in objective tumour response suggested that there may be a role for the 5-FU/LV and Oxaliplatin regimen in down staging patients with inoperable metastatic disease and this will be discussed in more detail below. The combination of 5-FU/LV with

Oxaliplatin is now widely known by the acronym FOLFOX and this will be used throughout the rest of this discussion. A variety of different FOLFOX regimes have been reported in the literature e.g. FOLFOX4, FOLFOX 6 etc which all differ slightly in drug dosing regimen and scheduling. There is no direct evidence that one regimen is superior to any other and therefore no distinction is made in this text. In some studies Capecitabine is substituted for 5-FU and in this instance the regimen is referred to by the acronym CAPOX.

1.3.3 Irinotecan

Irinotecan (Campto®), a derivative of Camptothecin, is a pro-drug which upon entry into the cell undergoes metabolism by the enzyme carboxylesterase into its active form SN-38 or alternatively is inactivated by cytochrome CYP3A4. SN-38 binds to DNA/topoisomerase I complexes leading to an inability of the cell to repair DNA single strand breaks and thereby ultimately results in cell death. SN-38 is inactivated by glucurodination, a process facilitated by the enzyme UGT1A1. Polymorphisms in the gene encoding this enzyme lead to excessive accumulation of the active form of SN-38 and consequentially increased drug toxicity.(Kweekel, Guchelaar et al. 2008; Marsh and Hoskins 2010)

In 1998 Rougier et al. randomised 267 patients with advanced colorectal cancer, who had experienced disease progression on a 5-FU based regimen, to either Irinotecan or further infusional 5-FU. The patients in the Irinotecan arm demonstrated an improvement in median progression free survival (4.2months vs. 2.9 months; $p = 0.03$) and overall 1 year survival (45% vs. 32%; $p = 0.035$) as compared to those receiving 5-FU.(Rougier, Van Cutsem et al. 1998) A second randomised trial, published in the same edition of the Lancet, allocated 279 patients with metastatic colorectal cancer and disease progression on 5-FU to receive either Irinotecan or best supportive care. Those in the Irinotecan group

demonstrated a significant improvement in overall 1 year survival as compared to those in the supportive care alone arm (36% vs. 13%; p=0.0001).(Cunningham, Pyrhonen et al. 1998) It is on the basis of these two trials that Irinotecan became established as a treatment option for advanced colorectal cancer.

In subsequent trials Irinotecan was combined with 5-FU/LV in an attempt to improve efficacy (known by the acronym FOLFIRI). Saltz et al. randomised 683 patients with metastatic colorectal cancer, who had not received systemic treatment within the last year, to receive either 5-FU/LV alone, Irinotecan alone or FOLFIRI. They were able to demonstrate that those allocated to the FOLFIRI arm had a superior objective response rate (50% vs. 28%; p<0.001) and longer median survival (14.8 months vs. 12.6 months; p = 0.04) as compared to those receiving 5-FU/LV. There was no difference in either objective response (28% vs. 29%) or median survival (12.6 months vs. 12.0 months) in those receiving either 5-FU/LV or Irinotecan alone.(Saltz, Cox et al. 2000) In a smaller study Maiello et al. randomised 102 patients with either locally advanced or metastatic colorectal cancer to FOLFIRI or 5-FU/LV. Again patients had not received adjuvant systemic therapy within the year prior to study entry. Patients in the FOLFIRI arm demonstrated a significantly improved objective tumour response rate (40% vs. 18%; p = 0.014) although there was no significant difference in median survival (15 months vs. 14 months) between the two groups.

From these trials it seems, in a similar manner to Oxaliplatin, that whilst the addition of Irinotecan to conventional 5-FU may offer, at best, a modest increase in overall patient survival its major advantage is improving the tumour response rate.

1.3.4 Biological Agents

In parallel with the development of conventional chemotherapy biological agents, i.e. antibody based therapies, have emerged which target specific pathways involved in the maintenance of tumour growth and development. In particular agents targeting tumour angiogenesis and tumour proliferation have been the subject of much investigation and are now finding their place in routine clinical practice.

1.3.4.1 Tumour Angiogenesis

For both primary and malignant tumours development there must be an independent blood supply which enables delivery of nutrients and oxygen. The process through which this blood supply evolves, known as angiogenesis, is driven by production of a large number of pro-angiogenic factors by tumour cells.(Carmeliet and Jain 2000; Garcea, Lloyd et al. 2004) One such factor is Vascular Endothelial Growth Factor-A (VEGF-A) which is a member of the VEGF family of growth factors. VEGF-A acts through the VEGF receptors VEGFR-1 and VEGFR-2, expressed on endothelial cells, whereby it stimulates the proliferation, migration and invasion of endothelial cells into the tumour resulting in new blood vessel formation.(Ellis and Hicklin 2008) Bevacizumab is a humanised monoclonal antibody which specifically targets soluble VEGF-A. By binding to VEGF-A Bevacizumab prevents the interaction of the ligand with its receptor and therefore aims to reduce tumour angiogenesis.(Ellis and Hicklin 2008; Geva, Prenen et al. 2010)

In 2003 Kabbinavar et al. published a phase II randomised trial which allocated patients with metastatic colorectal cancer to receive either 5-FU/LV alone or in combination with either 5mg/kg or 10mg/kg Bevacizumab. The key finding from this study was that, when both Bevacizumab arms were pooled, its addition resulted in a 55% reduction in the hazard of

disease progression as compared to 5-FU/LV alone (p=0.02). (Kabbinavar F 2003) In a key phase III trial Hurwitz et al. randomised patients with metastatic colorectal cancer to receive either FOLFIRI alone (n=411) or FOLFIRI & Bevacizumab (n=402). In those allocated to receive Bevacizumab there were significant improvements in median survival (20.3 months vs. 15.6 months; p<0.001), progression free survival (10.6 months vs. 6.2 months; p<0.001) and the measured overall response rate (44.8% vs. 34.8%; p = 0.004).(Hurwitz, Fehrenbacher et al. 2004) Similar results were also demonstrated when Bevacizumab was added to FOLFOX based chemotherapy in the Tree-1 and Tree-2 studies with an 11% improvement in tumour response rates.(Hochster, Hart et al. 2008)

1.3.4.2 Tumour Proliferation

One of the key features of tumour cells is disordered proliferation. Numerous pathways exist to regulate cellular proliferation and the activity of many of these is regulated by a variety of growth factors and their associated receptors. One such pathway is regulated by the epidermal growth factor (EGF) which on binding to its receptor (EGFR) activates a signalling cascade which ultimately results in up regulation of a variety of genes involved in cellular growth, migration, differentiation and adhesion of cells to the extracellular matrix.(Yarden 2001) Over-expression of EGFR is well documented in colorectal cancers and is considered to be a poor prognostic marker.(Lockhart and Berlin 2005; Theodoropoulos, Karafoka et al. 2009; Ljuslinder, Melin et al. 2011) Awareness of this has led to the development of the anti-EGFR antibodies Cetuximab (chimeric IgG1) and Panitumumab (fully humanised IgG2) for use as adjuncts in the management of colorectal cancer.(Geva, Prenen et al. 2010)

The Bond study randomised patients with EGFR expressing metastatic colorectal cancer, who had experienced disease progression on Irinotecan based chemotherapy, to receive either Cetuximab monotherapy (n= 111) or Cetuximab and further Irinotecan (n=218). In this study those receiving the combination of Irinotecan and Cetuximab experienced a higher overall response rate (22.9% vs. 10.8%; p = 0.0007) but no statistically significant difference in overall median survival (8.6% vs. 6.9%; p = 0.48).(Cunningham, Humblet et al. 2004) In the EPIC trial patients with EGFR expressing metastatic colorectal cancer who experienced treatment failure with FOLFOX chemotherapy were randomised to either Irinotecan alone (n=650) or Irinotecan and Cetuximab (n=650). Again the addition of Cetuximab was associated with an improvement in overall response rates (16.4% vs. 4.2%; p = 0.0001) but no effect was seen on median survival (10.7 months vs. 10.0 months; p = 0.71).(Sobrero, Maurel et al. 2008)

A key intermediary in the EGFR signalling pathway is the protein RAS which is encoded by the Kirsten-RAS (K-RAS) oncogene. Mutations in this gene are present in about one third of patients with colorectal cancer and are associated with poorer disease specific and overall survival.(Andreyev, Norman et al. 1998; Esteller, Gonzalez et al. 2001) K-RAS mutations lead to constitutive activation of the signalling pathways downstream of EGFR thereby negating the effect of agents targeting the receptor itself. (Ramos and Tabernero 2008) Consequently this has resulted in the retrospective analysis of K-RAS mutation status in the major randomised trials utilising Cetuximab. The OPUS trial patients randomised patients with EGFR expressing metastatic colorectal cancer to receive either FOLFOX (n=168) or FOLFOX & Cetuximab (n=170). The addition of Cetuximab was associated with a trend towards improved overall tumour response (46% vs. 36%; p = 0.064) although this did not

reach statistical significance. When the analysis was limited to patients with only wild type K-RAS the difference in overall response rate was markedly higher in the Cetuximab group and statistical significance was achieved (61% vs. 37%; p = 0.011).(Bokemeyer, Bondarenko et al. 2009)

In the CRYSTAL study patients with EGFR expressing metastatic colorectal cancer were randomised to receive either FOLFIRI alone (n=599) or FOLFIRI and Cetuximab (n=599). When considering the overall study population the addition of Cetuximab was associated with an improved overall tumour response rate (46.9% vs.. 38.7%; p = 0.0004) but no overall difference in median survival (19.9 months vs. 18.6 months; p=0.31). Wild type K-RAS in particular was associated with an improved tumour response rate when Cetuximab was added (59.3% vs. 43.2%; p = 0.03) whereas mutant K-RAS was associated with a poorer tumour response (36.2% vs. 40.2%).(Van Cutsem, Kohne et al. 2009)

Mutations in oncogenes other than K-RAS have also been linked with tumour responsiveness too Cetuximab. One of these is BRAF which encodes the RAF protein, another downstream intermediary in the EGFR signalling pathway. Mutations in BRAF, which again lead to constitutive activation of this pathway, are independent of K-RAS status and have been reported as predictors of poorer overall and progression free survival in patients treated with both Oxaliplatin and Irinotecan based regimens, as well as with Bevacizumab.(Siena, Sartore-Bianchi et al. 2009; Souglakos, Philips et al. 2009) In addition patients with wild type K-RAS but mutant BRAF have been demonstrated to be unresponsive to either Cetuximab or Panitumumab.(Di Nicolantonio, Martini et al. 2008; Loupakis, Ruzzo et al. 2009)

The MRC Coin trial randomised patients with inoperable advanced colorectal cancer, irrespective of EGFR status, to receive either Oxaliplatin based chemotherapy (CAPOX or FOLFOX; n = 815) or the same regimen with the addition of Cetuximab (n=815). The investigators also performed mutation analysis for K-RAS and BRAF and subgroup analysis was performed based on this. Overall the addition of Cetuximab was associated with a modest increase in overall tumour response rate in patients with wild-type K-RAS only (64% vs. 57%; p = 0.049). No benefit in terms of overall survival was seen with the addition of Cetuximab in any of the three groups.(Maughan, Adams et al. 2011)

Whilst much of the data describing the efficacy of anti-EGFR antibodies in patients with colorectal cancer relates to Cetuximab the reported outcomes for Panitumumab are similar. For example in one trial patients with metastatic colorectal cancer were randomised to receive either FOLFIRI alone (n=595) of FOLFIRI and Panitumumab (n=591). Whilst there was no effect on overall survival the addition of Panitumumab was associated with an improved overall response rate (35% vs. 10%; $p < 0.001$) an effect which was lost in patients with mutant K-RAS.(Peeters, Price et al. 2010) Similar results were also reported when Panitumumab was combined with FOLFOX in a separate randomised trial.(Douillard, Siena et al. 2010)

1.4 Systemic Therapy for Inoperable Colorectal Liver Metastases

It can be seen from the discussion thus far that one of the key attributes of systemic therapy are tumour response rates allowing down-staging of disease. In patients who present with inoperable metastatic disease one of the key goals of therapy is to down-stage in a manner which may enable subsequent surgical resection – an approach which has been labelled "conversion chemotherapy".(Khatri, Chee et al. 2007)

As early as 1996 Bismuth reported a series of 434 patients presenting with colorectal liver metastases, of whom 330 were considered to have inoperable disease and were thus treated with Oxaliplatin based chemotherapy. Of these 330 patients 16% (n=53) were subsequently down-staged in such a manner that they were able to undergo surgery with curative intent. In 46 of these patients all evidence of disease was removed following a primary liver resection however in 7 patients there was residual disease present after the primary procedure. These patients underwent a second course of chemotherapy and all had any remaining disease removed during a second surgical procedure (either with a further liver resection or pulmonary resection). The overall 5 year survival in this series of patients was 40% which the authors state compared favourably to that being obtained in patients with initially operable disease during the same period.(Bismuth, Adam et al. 1996) A updated report from the same unit in 2001 which included 701 patients with initially inoperable disease showed the overall conversion rate to operable disease to be 13.6% with a 5 year actuarial survival of 34%.(Adam, Avisar et al. 2001)

In a non-randomised phase II trial Alberts et al. reported the outcome of 42 patients with initially inoperable colorectal liver metastases who were treated with FOLFOX chemotherapy. 25 (60%) of these patients experienced some degree of tumour response to

chemotherapy and 17 (40%) were deemed to have potentially operable disease and underwent exploratory laparotomy. From this group of patients 14 (33% of the original cohort) were deemed to have had complete resection of their disease. For those patients undergoing a complete resection an overall 3 year survival of 67% was reported compared to a median survival of only 26months for the others. Taken together these findings confirm there is a significant survival advantage for those patients who are able to undergo a complete surgical resection after down-staging chemotherapy despite initially having inoperable disease.(Alberts, Horvath et al. 2005)

Nuzzo et al. reported the outcome of 42 patients with initially inoperable colorectal liver metastases who were treated with FOLFIRI chemotherapy. From this group 18 patients were considered to have been down-staged sufficiently to warrant exploratory laparotomy with 15 patients (35.7%) undergoing curative intent surgery. The outcomes of these 15 patients were compared to a cohort of 60 patients who had undergone primary surgical treatment of operable liver metastases. The median overall survival did not differ between the two groups (46 months vs. 47 months) however those who had received down-staging chemotherapy had a poorer 3 year recurrence free survival (31% vs. 58%; $p = 0.04$).(Nuzzo, Giuliante et al. 2007)

Whilst these studies demonstrate that the concept of down-staging inoperable disease is worthy of pursuit they do not provide any insight into the optimal chemotherapy strategy for obtaining this. In an attempt to address this question the GOIM No 9901 trial randomised patients with either locally advanced or inoperable metastastic disease to receive treatment with either FOLFOX (n=182) or FOLFIRI (n=178). This trial did not detect any difference in objective response rates between either regimen (36% vs. 34%) or in

median overall survival (15 months vs. 14 months). Unfortunately however the study did not report how many patients, if any, were able to progress to surgical treatment.(Colucci, Gebbia et al. 2005)

The GERCOR trial randomised patients with inoperable metastatic colorectal cancer to receive either FOLFIRI until disease progression or unacceptable toxicity and then FOLFOX or the reverse sequence (n=113 per arm). When used as first line therapy there was no difference in progression free survival with FOLFIRI or FOLFOX (8.5 months vs. 8 months). As second line therapy FOLFOX demonstrated an improved progression free survival as compared to FOLFIRI (4.2 months vs. 2.5 months; p = 0.003). There was no difference in overall survival in either arm of the study. In the first line setting FOLFOX demonstrated superiority in down-staging with 24 patients (22%) in this arm undergoing subsequent curative intent surgery as compared to 10 patients (9%) in the first line FOLFIRI arm (p=0.02).(Tournigand, Andre et al. 2004)

More recently the combination of 5-FU/LV, Oxaliplatin and Irinotecan (FOLFOXIRI) has been proposed as a therapeutic strategy in patients with inoperable metastatic colorectal cancer. The GONO group randomised patients with inoperable metastatic colorectal cancer to receive either FOLFIRI or FOLFOXIRI (n=122 per arm). Those in the FOLFOXIRI arm demonstrated an improvement in overall tumour response (66% vs. 41%; p = 0.0002) with a higher proportion of patients going on to have complete resection of their metastatic disease (18% vs. 6%; p = 0.033). If the analysis is limited to patients with liver only metastases then an even greater proportion were able to undergo curative intent surgery after FOLFOXIRI (36% vs. 12%; p = 0.017).(Falcone, Ricci et al. 2007) In a pooled analysis of patients in this and 2 smaller phase II studies who underwent complete resection of their

disease after FOLFOXIRI treatment (37/196; 19%)a 5 year survival of 42% was achieved.(Masi, Loupakis et al. 2009)

In addition to conventional chemotherapy the role of biological agents as adjuncts in disease down-staging has also been explored. The first BEAT trial, an uncontrolled phase IV study, assessed the addition of Bevacizumab to fluoropyrimidine based chemotherapy, given according to the treating physicians' choice, in patients with inoperable metastatic colorectal cancer. Only 16% of patients received a fluoropyrimidine alone whereas 50% of patients received additional Oxaliplatin and 34% of patients received additional Irinotecan. Of the 1965 patients in this study 145 (7.6%) underwent a liver resection with curative intent after treatment with a Bevacizumab containing regimen. In those treated with regimens containing Oxaliplatin the resection rate was 10.4% whereas in those containing Irinotecan the rate was 6.5%. If the analysis was limited to those with inoperable liver only disease at the time of study entry 15.2% of patients overall were able to undergo subsequent liver surgery with curative intent but the individual rate in those receiving Oxaliplatin being 20.3% and for Irinotecan 14.3%. In this study the 2 year overall survival in patients with inoperable liver only disease at the time of study entry was 54% whereas for those who underwent liver resection with curative intent this increased to 89% ($p<0.0001$) again confirming the necessity of surgical intervention in those who are down-staged by systemic treatment regimens.(Okines, Puerto et al. 2009; Van Cutsem, Rivera et al. 2009)

The NO16966 study initially commenced as a randomised trial which allocated patients with inoperable metastatic colorectal cancer to receive either FOLFOX or XELOX. On the basis of the study by Hurwitz et al. (Hurwitz, Fehrenbacher et al. 2004) this was subsequently modified to a 2 x 2 design whereby patients received these regimens either with or without

the addition of Bevacizumab. A total of 1401 patients were randomised on a 1:1 basis. In those patients who received Bevacizumab alongside either FOLOX or XELOX there was an improvement in progression free survival (10.4 months vs. 7.9 months; p<0.0001) but not in overall tumour response rates (49% vs. 47%; p = 0.31). In light of this it is not overly surprising that this study also failed to demonstrate an improvement in the number of patients who underwent potentially curative surgical resection with the addition of Bevacizumab either overall (8.4% vs. 6.1%; p=0.24) or amongst those with liver only disease at the time of study entry (12.3% vs. 11.6%; p = 0.81).(Okines, Puerto et al. 2009)

The BOXER trial was a phase II uncontrolled study which examined the effect of XELOX and Bevacizumab in combination for patients with colorectal liver metastases deemed not suitable for liver resection (n=46). Of those entered into the study an objective response was obtained in 35 patients (78%). 18 patients (40%) proceeded to undergo a liver resection with curative intent and a further 5 patients (11%) had a complete radiological response to treatment and therefore were not offered surgery. One criticism of this study is that the definition of resectability applied was somewhat conservative and excluded patients with >4 metastases and patients with a maximum tumour size of >5cm as well as those in whom it was not thought possible to obtain a clear resection margin or who would have had an insufficient FLR. (Wong, Cunningham et al. 2011)

The CELIM study was a randomised phase II study which allocated patients with inoperable colorectal liver metastases to receive either FOLOX (n=53) or FOLFIRI (n=53) in combination with Cetuximab. Overall an objective tumour response was seen in 66 patients (62%) but there was no statistically significant difference between the two groups. 36 patients (34%) in this study were able to undergo a complete resection of metastatic disease following

treatment. The proportion of patients undergoing resection in the FOLFOX group was marginally higher but this difference did not reach statistical significance (20% vs. 16%).(Folprecht, Gruenberger et al. 2010)

In the previously discussed CRYSTAL trial, which randomised patients with inoperable metastatic colorectal cancer to receive either FOLFIRI alone or in conjunction with Cetuximab, those patients who received Cetuximab and were K-RAS wild type were more likely to be able to undergo complete resection of metastatic disease when compared to those receiving FOLFIRI alone (5.1% vs. 2.0%; p = 0.03).(Van Cutsem, Kohne et al. 2009; Van Cutsem, Kohne et al. 2011) Similarly in a retrospective analysis of the OPUS trial according to K-RAS status, whereby patients were randomised to either FOLFOX or FOLFOX and Cetuximab, it was reported that, for K-RAS wild type patients, those who received pre-operative Cetuximab were more likely to undergo curative intent surgery of metastatic disease (12% vs. 3%; p = 0.02).(Bokemeyer, Bondarenko et al. 2011)

When discussing the merits of aggressive systemic therapy in patients with initially inoperable disease it is important to be aware of what the long term outcome is for those who achieve the goal of subsequent surgical resection. In an attempt to address this Adam et al. reported a series of 184 patients with initially inoperable colorectal liver metastases who underwent hepatectomy with curative intent after down-staging chemotherapy. In this cohort of patients an overall 5 year survival of 33% was obtained and a recurrence free 5 year survival of 19%. 148 patients in this series had complete 5 year follow-up data available and of these patients 24 were considered to have been ‘cured’ i.e. free of recurrent disease for 5 years following the last surgical resection. It is noteworthy that of these patients 16 remained disease free after the initial hepatic resection whereas a further

8 required surgery to remove either residual, or recurrent, metastatic disease before 'cure' was obtained.(Adam, Wicherts et al. 2009) This observation serves to highlight that, at least for a subgroup of patients, two or more surgical procedures often interspersed with chemotherapy are required to achieve long lasting disease control.

It is undoubtedly true that systemic treatment of inoperable metastatic colorectal cancer can, in a proportion of patients, result in down-staging such that a potentially curative resection can be offered and the likelihood of long term survival increased. There appears to be little real difference in terms of conventional chemotherapy regimens to achieve effective down-staging, although on the basis of the GERCOR trial it is more common to use FOLFOX as first line.(Tournigand, Andre et al. 2004; Kelly and Cassidy 2007; Hind, Tappenden et al. 2008) Whilst there is good evidence that Cetuximab, in K-RAS wild type patients, can undoubtedly offer an advantage in terms of down-staging when combined with conventional chemotherapy the evidence for Bevacizumab is less clear. In light of this, and the expensive nature of these agents, at the present time the UK National Institute for Health and Clinical Excellence only permits the use of Cetuximab in patients with inoperable colorectal liver metastases.(National Institute for Health and Clinical Excellence 2009)

1.5 Systemic Therapy for Operable Colorectal Liver Metastases

In those who present with operable disease from the outset the goal of systemic chemotherapy is to improve overall long term outcome and should be distinguished from down-staging chemotherapy. Two chemotherapy strategies are available in this setting i.e. either neoadjuvant or adjuvant although it is not particularly clear from the current evidence which, if any, is superior.

One difficulty in interpreting the evidence relating to neoadjuvant chemotherapy for patients with operable disease is that it has, in many cases, been combined with adjuvant chemotherapy making it extremely difficult to discern which of the two components is of most benefit. Bathe et al. conducted a phase II uncontrolled study to examine the safety and efficacy of peri-operative FOLFIRI in 35 patients with operable liver metastases. Patients were treated with chemotherapy for 12 weeks and then underwent re-staging prior to surgery. Of this cohort 14 (40%) had some degree of response to chemotherapy, 12 had stable disease (36%) and 7 (18%) had evidence of disease progression. 31 patients progressed to surgery and 30 had successful resection of all disease. Of these 22 were then treated with adjuvant chemotherapy with 2 patients refusing, 2 being ineligible because of peri-operative complications and the remainder refused because of disease progression on neoadjuvant chemotherapy. The overall survival for those patients who experienced disease progression on chemotherapy was a median of 39 months whereas the median survival was not reached during the follow-up period for those with responsive disease. (Bathe, Ernst et al. 2009)

The EPOC trial was the first prospective randomised controlled trial to examine the role of peri-operative chemotherapy in patients with resectable liver metastases. Patients were

allocated to receive either surgery alone or peri-operative FOLFOX consisting of 6 cycles prior to surgery and 6 cycles after surgery (n=182 per arm). Of those assigned to peri-operative FOLFOX 143 patients (79%) were able to complete all 6 cycles of whom 67 had some degree of tumour response, 60 had stable disease and 11 had disease progression. In the surgery alone arm 170 patients ultimately underwent exploratory laparotomy with 152 (84%) being resected. Whilst the number proceeding to surgery in the peri-operative chemotherapy arm was smaller (n=159; 87%) a similar proportion underwent a curative intent resection (n=151; 83%). It is noteworthy that only 50% of patients in the peri-operative chemotherapy arm were able to complete all 6 cycles of adjuvant chemotherapy (n=80) suggesting that it was poorly tolerated. On an intention to treat basis there was a trend towards improved 3 year progression free survival in the chemotherapy arm although this did not reach statistical significance (35.4% vs. 28.1%; p = 0.058). If the analysis was limited to those who underwent resection however then statistical significance was reached (42.4% vs. 33.2%; p = 0.025).(Nordlinger, Sorbye et al. 2008)

In an attempt to understand whether all patients with operable disease benefit from peri-operative chemotherapy the LiverMet database was analysed to identify patients who underwent liver resection for solitary, liver only, metachronous metastases. Two groups of patients were analysed, those who underwent surgery alone (n=1302) or those who received at least 3 courses of neoadjuvant chemotherapy which contained either Irinotecan or Oxaliplatin. In this group of select patients there appeared to be no advantage in terms of either overall survival (60% vs. 60%; p = 0.57) or disease free survival (42% vs. 46%; p = 0.09) at 5 years in those receiving neoadjuvant chemotherapy. Multivariate analysis on the entire cohort however identified the use of adjuvant chemotherapy as being associated with

better overall (Relative Risk 1.64; p<0.01) and disease specific survival (Relative Risk 1.46; p<0.01) than surgery alone.(Adam, Bhangui et al. 2010)

The proponents of neoadjuvant chemotherapy argue that it allows an assessment of disease biology prior to resection, with patients being classified as either responders, having stable disease or those experiencing disease progression. Adam et al. performed a retrospective analysis of 131 patients with more than 4 colorectal liver metastases undergoing hepatectomy after systemic chemotherapy and stratified outcome according to tumour response. They noted that in those who had experienced disease progression whilst on chemotherapy the overall 5 year survival was only 8% as compared to 37% in responders and 30% in those with stable disease (p<0.0001).(Adam, Pascal et al. 2004) One criticism of this study however is that the 44% of patients who experienced disease progression were treated with 5-FU/LV alone as compared to only 19% who experienced disease response. Whilst treatment with regimens containing Oxaliplatin or Irinotecan may have changed the response of those with progressive disease it is impossible to know how this might have impacted on their overall long term outcome.

In an attempt to assess the benefit of adjuvant systemic therapy Figueras et al. retrospectively compared the outcome of 99 patients who had undergone complete resection of colorectal liver metastases and received adjuvant chemotherapy (predominantly 5-FU/LV) to that of 81 patients who were treated with surgery alone. They demonstrated that the 5 year actuarial survival of those receiving adjuvant chemotherapy was 53% as compared to 25% in the surgery alone group (p = 0.0008).(Figueras, Valls et al. 2001)

The retrospective nature of this study means it is hard to make any definitive statements regarding the utility of adjuvant chemotherapy. In order to address this two prospective randomised trials were set up however both were underpowered as a result of slow patient accrual and as such were unable to demonstrate any statistically significant difference in survival.(Langer, Bleiberg et al. 2002; Portier, Elias et al. 2006) In an attempt to overcome this the results of these two trials were pooled. It should be noted that this pooled analysis was not limited to patients with liver only metastases but also included a subset of patients who underwent curative resection of lung only metastases who had been included in the study by Langer et al..(Langer, Bleiberg et al. 2002) Nonetheless those treated with surgery alone were found to be at higher risk of early disease recurrence than those who had received adjuvant 5-FU/LV (HR 1.39; p = 0.026) which was reflected in a longer median progression free survival in the adjuvant therapy arm (27.9% vs. 18.8%). Despite this apparent benefit there was no statistically significant difference in overall survival for those receiving adjuvant chemotherapy, although the trend was very much in favour of this (62.2% vs. 47.3%).(Mitry, Fields et al. 2008) One might expect that the results of these trials would be improved by addition of either Oxaliplatin or Irinotecan to the 5-FU/LV regimen however there is, at the present time, no prospective experimental data to support this assertion.

In summary the role of peri-operative chemotherapy in patients with operable colorectal liver metastases is less than clear cut. The EPOC trial certainly demonstrated a survival advantage to patients treated with peri-operative chemotherapy but it is impossible to dissect out whether it is the neoadjuvant or adjuvant components of the regimen that are important. Proponents of neo-adjuvant chemotherapy would argue that its use allows one

to make an assessment of tumour biology but high quality prospective data to back this assertion is lacking. The evidence to date does however seem to suggest that adjuvant chemotherapy is poorly tolerated and this may mean there is a practical advantage in utilising a neoadjuvant strategy. The role of biological agents in this setting has not been evaluated as yet, although it is the subject of investigation in an ongoing EPOC trial (peri-operative FOLFOX vs. peri-operative FOLFOX and Cetuximab).

From the data of Adam et al. it would seem that not all patients with operable disease would necessarily benefit from peri-operative chemotherapy but rather it might be better targeted at those with poor prognostic features such as large number of metastatic deposits or large tumour size.(Fong, Fortner et al. 1999; Adam, Bhangui et al. 2010) In any case it is likely that, as clarity emerges around the optimal target patient group, an ever increasing number of patients with operable metastatic disease will be treated using a combined approach of surgery and peri-operative systemic therapy.

Chapter 2

Chemotherapy Associated Liver Injury – A Systematic Review and Meta-Analysis

2.1 Background

Data from 7764 patients entered into the LiverMet survey over a 22 year period suggests that 32% of patients receive chemotherapy prior to surgery, although it is likely that this proportion has increased significantly in recent years as our understanding of the use of chemotherapy in these patients has become clearer.(Adam, Frilling et al. 2010) As experience of patients receiving pre-operative chemotherapy has grown there has been an increasing recognition that chemotherapy use can be associated with injury to the hepatic parenchyma (chemotherapy associated liver injury, CALI). Two distinctive patterns of chemotherapy associated liver injury have been described i.e. steatosis/steatohepatitis and sinusoidal obstruction syndrome, each of which is discussed in more detail below.(Nordlinger and Benoist 2006)

2.1.1 Hepatic Steatosis/Steatohepatitis

Non-alcoholic fatty liver disease (NAFLD) is increasingly common in the general adult population with the UK prevalence thought to be in the order of 25%.(Anstee, McPherson et al. 2011) It is most commonly associated with the so called metabolic syndrome which is characterised by obesity and insulin resistance. NAFLD does not represent a single entity but rather characterises a spectrum of disease that ranges from simple steatosis through to steatohepatitis (non-alcoholic steatohepatitis, NASH) and at the extreme end of the spectrum cirrhosis.(Anstee, McPherson et al. 2011) The key features that distinguish NASH

from simple steatosis are the presence of lobular inflammation and hepatocellular degeneration characterised by ballooning of hepatocytes.(Kleiner, Brunt et al. 2005)

In an attempt to make a definitive statement on the risk of steatosis in patients undergoing hepatic surgery de Meijer et al. performed a systematic review and meta-analysis of the published data. In this study patients with steatosis were classified as either having <30% steatosis or ≥30% steatosis and a major resection was considered as being resection of at least 3 Couinaud segments. Those patients with steatosis <30% undergoing major hepatectomy were at a moderately increased risk of overall complications (Relative Risk 1.53; $p < 0.001$) but at no greater risk of overall post-operative mortality when compared to those without steatosis. In those with ≥30% steatosis undergoing major hepatectomy there was again an increased risk of overall complications (Relative Risk 2.01; $p<0.001$) but also of peri-operative mortality (Relative Risk 2.79; $p = 0.02$) as compared to those without steatosis.(de Meijer, Kalish et al. 2010)

The association between chemotherapy use and hepatic steatosis has been recognised for almost 15 years. In 1998 Peppercorn et al. reported a series of 21 patients with colorectal liver metastases who were treated with systemic 5-FU/LV. On baseline CT none of these patients had evidence of hepatic steatosis whereas, after 12 cycles of chemotherapy, radiological changes of hepatic steatosis were present in 48% of patients (n=10).(Peppercorn, Reznek et al. 1998) Pawlik et al. reported a series of 212 patients who underwent resection of colorectal liver metastases of whom 153 had received pre-operative chemotherapy. Overall the use of chemotherapy was associated with an increased risk of steatosis ≥ 30% (18.3% vs. 3.4%; $p = 0.004$). When chemotherapy was stratified according to regimen those who received Irinotecan were found to be at highest risk of steatosis ≥

30% as compared to chemotherapy naive controls (27.3% vs. 3.4%; p<0.001) followed by those receiving fluoropyrimidine monotherapy (14.9% vs. 3.4%; p = 0.03) and finally those receiving Oxaliplatin based regimens (9.6% vs. 3.4%; p = 0.04). There was no statistically significant association between chemotherapy use and the development of steatohepatitis.(Pawlik, Olino et al. 2007)

In a separate series of 406 patients undergoing resection of colorectal liver metastases no association could be demonstrated between pre-operative chemotherapy and the development of hepatic steatosis ≥ 30% either overall or when patients were stratified according to the regimens received. In the 94 patients who received Irinotecan based regimens however there was an increased incidence of steatohepatitis when compared to those who were chemotherapy naive (20.2% vs. 4.4%; p = 0.001). Importantly in those patients with steatohepatitis in this series there was an increase in all cause 90 day mortality (14.7% vs. 1.6%; p = 0.001) and specifically death from post-hepatectomy liver failure (5.8% vs. 0.8%; p = 0.01).(Vauthey, Pawlik et al. 2006) In a small study of 37 patients undergoing resection of colorectal liver metastases Fernandez et al. demonstrated that, on multivariate analysis, the use of either Irinotecan or Oxaliplatin based chemotherapy and the presence of a high BMI were independent risk factors for the development of steatohepatitis.(Fernandez, Ritter et al. 2005)

2.1.2 Sinusoidal Obstruction Syndrome

Prior to the development of modern chemotherapeutic agents Sinusoidal Obstruction Syndrome (SOS), previously known as hepatic veno-occlusive disease, was considered to be a rare phenomenon that was primarily related to the ingestion of a group of compounds known as pyrrolizidine alkaloids which are commonly found in plants used in traditional

African herbal remedies.(Willmot and Robertson 1920; DeLeve, Shulman et al. 2002) Latterly SOS has been described in patients receiving myeloablative chemotherapy prior to bone marrow transplantation where the reported incidence is in the order of 27 – 54%.(McDonald, Hinds et al. 1993; Hasegawa, Horibe et al. 1998) Early histological features of SOS are the development of dilatation of the hepatic sinusoids with associated hepatocyte atrophy. In later stage disease these features are accompanied by the development of peri-sinusoidal fibrosis and nodular regenerative hyperplasia. The severity of sinusoidal dilatation is commonly graded according to the method of Rubbia-Brandt (0 = absent; 1 = mild; 2 = moderate; 3 = severe) where an increasing score reflects a more severe sinusoidal injury.(Rubbia-Brandt, Audard et al. 2004)

In 2004 Rubbia-Brandt et al. published a retrospective analysis of histological changes in the non tumour bearing liver of 153 patients who had undergone resection of colorectal liver metastases. In 44 out of 87 patients (51%) who had received pre-operative chemotherapy there were marked centrilobular changes characterised by sinusoidal dilatation and peri-sinusoidal fibrosis in keeping with a diagnosis of SOS. In contrast none of the patients who had undergone surgery alone had these changes. When the patients who had received chemotherapy were stratified according to regimen it was found that SOS was much more common in those who had received Oxaliplatin based treatment as compared to those who had not (79% vs. 23%; $p < 0.001$).(Rubbia-Brandt, Audard et al. 2004)

In the previously described series by Vauthey et al. those patients who had received treatment with Oxaliplatin based regimens were more likely to develop SOS than those patients who were chemotherapy naive (18.9% vs. 1.9%; $p < 0.001$). Those treated with other chemotherapy regimens were not demonstrated to be at increased risk of developing

SOS as compared to those treated with surgery alone. (Vauthey, Pawlik et al. 2006) Aloia et al. reported the effect of chemotherapy on liver histology in 75 patients treated with either pre-operative 5-FU/LV or FOLFOX as compared to 17 patients who underwent surgery alone. It was also demonstrated that those receiving pre-operative chemotherapy were at higher risk of developing vascular lesions in keeping with SOS (52% vs. 18%; p = 0.01) but were at no greater risk of developing hepatic steatosis than controls (12% vs. 13%).(Aloia, Sebagh et al. 2006) In those patients in the EPOC trial who progressed to surgery 48% of those who received pre-operative FOLFOX had changes consistent with SOS as compared to only 11% of controls who received surgery alone.(Julie, Lutz et al. 2007)

The effect of SOS on peri-operative outcomes has been a source of debate in the literature. In the studies by Vauthey et al. and Aloia et al. there was no demonstrable effect on surgical morbidity or mortality although those with SOS had an increased requirement for blood transfusion.(Aloia, Sebagh et al. 2006; Vauthey, Pawlik et al. 2006) In a series of 51 patients undergoing major hepatectomy (3 or more Couinaud segments) after neoadjuvant Oxaliplatin based chemotherapy 75% (n=38) developed SOS as characterised by grade 2 or greater sinusoidal dilatation. The presence of histological changes associated with SOS was associated with an increased incidence of post hepatectomy liver failure (68% vs. 23%; p = 0.004).(Soubrane, Brouquet et al. 2010) In a similar manner Nakano et al. compared the outcome of 20 patients with SOS undergoing major hepatectomy (>3 Couinaud segments) to 16 patients without SOS. The presence of SOS was associated with an increased risk of peri-operative complications (40% vs. 6.3%; p = 0.026) and a prolonged mean hospital stay (17 days vs. 11 days; p = 0.006).(Nakano, Oussoultzoglou et al. 2008)

In addition to the short term effects of SOS on patient outcome a more recent paper by Tamandl et al. has raised concerns about its implications on long term patient survival. In this paper they compared the outcome of 20 patients with SOS to 154 patients without SOS all of whom had undergone curative intent surgery for colorectal liver metastases. Those patients with SOS were found to have a much poorer 3 year progression free as compared to those without (6.7% vs. 22.7%; p = 0.006) and indeed this increased risk for early recurrence was upheld on multivariate analysis (Hazard Ratio 2.20; p = 0.006). In particular patients with SOS seemed to be at higher risk of intra-hepatic recurrence (66.7% vs. 30.5%; p = 0.003). In light of this it is not surprising that the presence of SOS was also associated with an overall increased risk of early death (Hazard Ratio 2.90; p<0.001).(Tamandl, Klinger et al. 2011)

2.1.3 Summary

As can be seen from the discussion thus far the literature with regard to chemotherapy associated liver injury is less than clear with a variety of conflicting reports published. For example whilst the studies or Ryan et al. and Vauthey et al. report a strong association between Irinotecan usage and the development of hepatic steatosis that of Pawlik et al. failed to demonstrate any such association.(Vauthey, Pawlik et al. 2006; Pawlik, Olino et al. 2007; Ryan, Nanji et al. 2010) Similarly whilst Vauthey et al. and Tamandl et al. report a strong link between Oxaliplatin usage and SOS only a very modest association, if any at all, is reported by Makowiec et al. and Ryan et al..(Vauthey, Pawlik et al. 2006; Ryan, Nanji et al. 2010; Makowiec, Mohrle et al. 2011; Tamandl, Klinger et al. 2011) To address this issue of heterogeneity in the literature I elected to perform a systematic review and meta-analysis of published studies in order that the nature and extent of clinical problem can be properly defined.

2.2 Method

I undertook a systematic search for reports of studies published between the dates 1st January 1996 and 31st June 2011 using the online databases Medline, Embase and the Cochrane library. These searches included the key words “liver resection”; “hepatectomy”; “chemotherapy”; “steatosis”; “steatohepatitis”; “sinusoidal obstruction syndrome”. In addition I utilised the medical subject headings (MeSH terms) “Surgical Procedures, Operative”; “Colorectal Neoplasms”; “Hepatectomy”; “Drug-Induced Liver Injury” and “Fatty Liver”. In order to further ensure all applicable reports were included I hand searched the reference lists of relevant review papers on the subject.

In order to screen out irrelevant studies I initially reviewed the titles of reports identified during the search process and rejected case reports, commentaries/editorials, reviews, animal studies, *in vitro* studies and non-English reports. Following this the abstracts of all the remaining studies were retrieved and reviewed for potential relevance, applying the same criteria used for screening the titles. For abstracts identified as being of potential relevance the full text reports were retrieved and selected for inclusion in the review if they met the following criteria:

- Included patients undergoing treatment of colorectal liver metastases only
- Histological data was provided in relation to the non tumour bearing liver parenchyma
- Included a minimum of 10 patients per group

Reports that did not include a control group, that also met the above criteria, were excluded. Studies in the form of a published abstract only were excluded. The process of study selection is summarised in Figure 3.

In order to facilitate the process of data extraction from the identified original reports I designed a standardised proforma and for each study recorded the following details :

- Study design characteristics
- Histological scoring of the liver parenchyma

In addition close attention was paid to the kin relationship of studies, i.e. multiple publications using the same patient cohorts. Where the potential for duplicate publication was identified only that study which provided the largest amount of data to assess a given outcome was utilised.

In order to assess the quality of included studies each was scored according to the Newcastle-Ottawa scoring system. This is a validated tool which aims to assess the quality of non-randomised trials included in systematic reviews. Studies are scored a maximum of 8 points according to the method of patient selection, the comparability of patient cohorts and the quality of outcome measures.(Wells, Shea et al. 2011) The level of evidence provided by each of the included studies was graded using the Oxford Centre for Evidence-based Medicine scale.(OCEBM Levels of Evidence Working Group 2011) All data extraction was performed on two separate occasions and cross referenced to ensure accuracy.

In order to gain an accurate picture of the effect of pre-operative chemotherapy on the hepatic parenchyma, as well as on post-operative morbidity and mortality, a meta-analysis was performed according to the Meta-analysis of Observational Studies in Epidemiology (MOOSE) guidelines.(Stroup, Berlin et al. 2000) The effects of pre-operative chemotherapy on histological scores of liver injury were estimated using a pooled relative risk along with 95% confidence interval using a random-effects model.(DerSimonian and Laird 1986) This

approach was chosen as it was felt it would better accommodate the inherent heterogeneity in outcome measures that exists in non-randomised trials and therefore would avoid the risk of over-estimating effect which might be seen with a fixed-effects model.(Higgins and Green 2011) Studies were weighted according to population size and the overall effect was determined with the Z statistic. Statistical significance was set at a level of $p = 0.05$. Heterogeneity across studies was assessed by inspection of forest plots and utilisation of the I^2 statistic. All analysis was performed using Review Manager (RevMan) software (Version 5.1, The Nordic Cochrane Collaboration, Copenhagen). Data from observational and randomised controlled studies were not included in the same analysis.

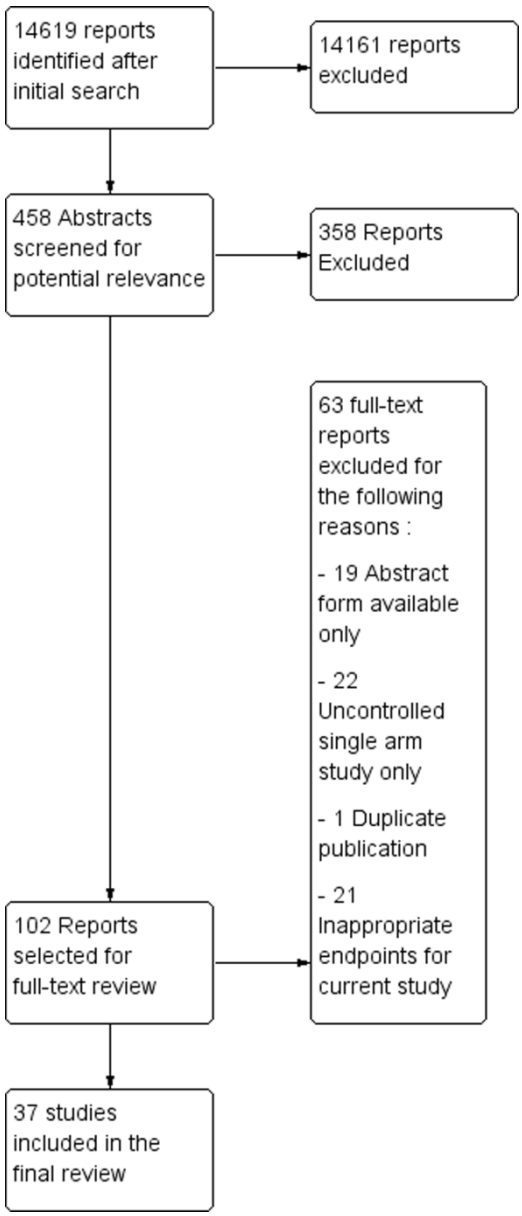

Figure 3 – Flow diagram summarising the process of study selection for the systematic review

2.3 Results

A total of 14,619 studies were identified from the search process of which 37 were eventually considered appropriate for inclusion in the systematic review. The majority of studies were considered to be of evidence level 2b (i.e. retrospective cohort studies) or greater (n=33; 89%). All studies apart from 2 were observational in character. The potential for overlap of patient cohorts in the identified studies was high, existing in 18 out of the 37 studies (49%). The full details of the included studies along with the NOS score, the evidence level and a summary of key findings can be found in Appendix 1 of this thesis.

The effect of pre-operative chemotherapy (all regimens) on the development of hepatic steatosis was assessed in a total of 13 studies which included 799 patients who had received chemotherapy and 709 patients treated with surgery alone. There was no demonstrable association between pre-operative chemotherapy and the presence of hepatic steatosis of all grades (Relative Risk 1.25; 95% Confidence Interval 0.99 – 1.57; p = 0.06). Data regarding the incidence of hepatic steatosis affecting at least 30% of hepatocytes was available for 2040 patients but again no association could be demonstrated (Relative Risk 1.25; 95% Confidence Interval 0.92 – 1.68; p = 0.15; Figure 4A). In order to assess whether the development of hepatic steatosis following chemotherapy was regimen specific analysis was performed for those patients receiving either Oxaliplatin or Irinotecan based regimens.

In the case of Oxaliplatin there was no association with the development of hepatic steatosis which affected at least 30% of hepatocytes (Relative Risk 0.98; 95% Confidence Interval 0.59 – 1.63; p = 0.95; Figure 4B). For Irinotecan again there was no significant association with hepatic steatosis ≥ 30% (Relative Risk 2.51; 95% Confidence Interval 0.59 – 1.63; p = 0.95; Figure 4C) however it can be seen from the funnel plot that there is

significant heterogeneity amongst the included studies (I^2 = 74%; p = 0.01). This arises from the two smaller studies, which contribute only 144 patients between them, demonstrating a positive association (Pawlik, Olino et al. 2007; Gomez-Ramirez, Martin-Perez et al. 2010) whereas the two larger studies, which contribute 504 patients, failed to show this.(Vauthey, Pawlik et al. 2006; Ryan, Nanji et al. 2010)

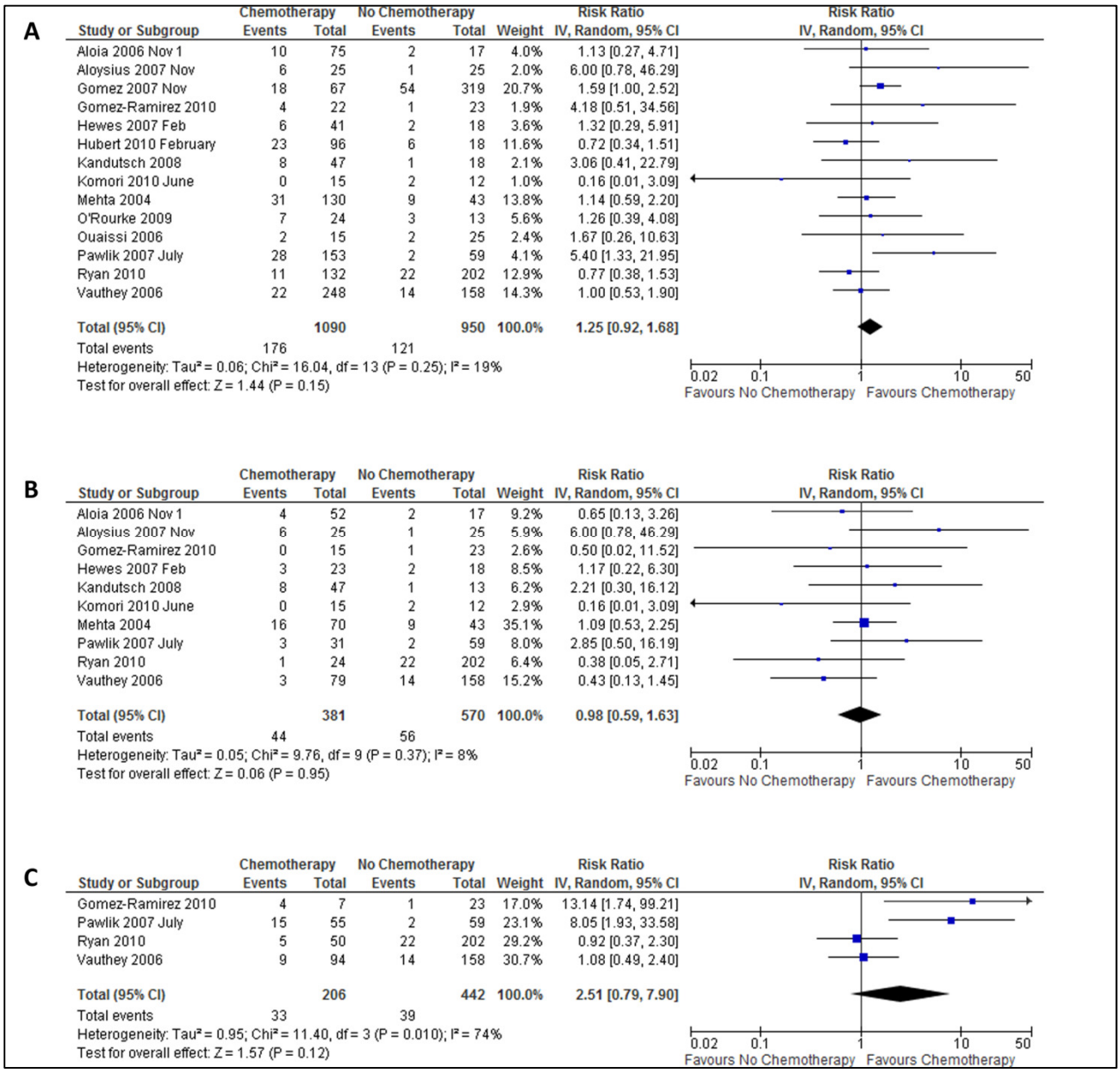

Figure 4 – Pre-operative chemotherapy is not associated with the development of hepatic steatosis either when all regimens are considered together (A). Neither is there evidence of a specific association between Oxaliplatin (B) or Irinotecan (C) and the development of hepatic steatosis.

As already described steatohepatitis represents a more severe form of the NAFLD spectrum and its presence has been linked to an increased risk of post hepatectomy liver failure and subsequent surgical mortality.(Vauthey, Pawlik et al. 2006) Overall the use of pre-operative chemotherapy was associated with a borderline statistically significant trend towards an increased risk of steatohepatitis (Relative Risk 1.89; 95% Confidence Interval 0.99 – 3.63; p = 0.05). When individual regimens were considered there was no demonstrable association between Oxaliplatin usage and steatohepatitis (Relative Risk 1.17; 95% Confidence Interval 0.45 – 3.04; p = 0.75). On the other hand those patients treated with Irinotecan based regimens had a 3.45 fold increased risk of steatohepatitis as compared to those treated by surgery alone (95% Confidence Interval 1.12 – 10.62; p = 0.03; Figure 5). Analysis of the funnel plot reveals a moderate degree of heterogeneity in this analysis (I^2 = 29%; p=0.24) which arises because the study of Ryan et al. which failed to show any association.(Ryan, Nanji et al. 2010) If this study were to be removed from the analysis then the risk of steatohepatitis in patients receiving Irinotecan based regimens would be 5 fold that of patients undergoing surgery alone (95% Confidence Interval 2.31 – 10.79; p < 0.0001).

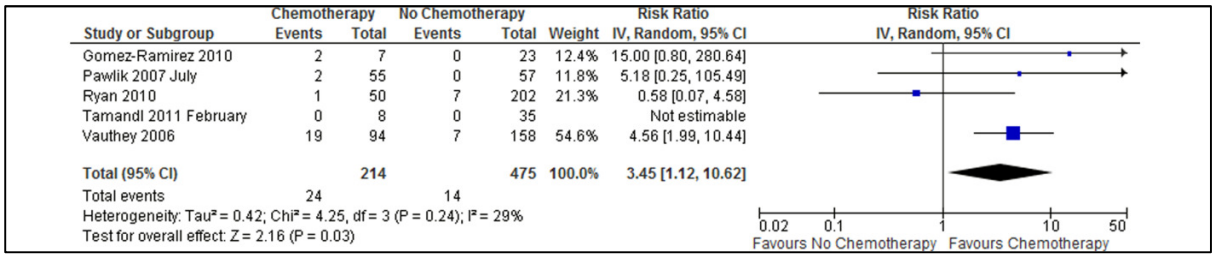

Figure 5 – Irinotecan based pre-operative chemotherapy is associated with a 3.45 fold increased risk of steatohepatitis as compared to surgery alone

The incidence of all grades of sinusoidal dilatation was reported in 633 patients receiving pre-operative chemotherapy (all regimens) as compared to 238 patients who underwent surgery alone. Overall chemotherapy administration was associated with a 1.95 fold increased risk of sinusoidal dilatation (95% Confidence Interval 1.46 – 2.61; $p < 0.00001$). It is broadly accepted that grade 2 or greater sinusoidal dilatation is more representative of SOS.(Rubbia-Brandt, Audard et al. 2004; Rubbia-Brandt 2010) Overall the use of pre-operative chemotherapy was associated with a 2.78 fold increase in the risk of grade 2 or greater sinusoidal dilatation (95% Confidence Interval 1.35 – 5.69; $p = 0.0007$; Figure 6A) although there was significant heterogeneity amongst the included studies ($I^2 = 66\%$; $p = 0.0007$).

In those receiving Oxaliplatin based regimens the risk of grade 2 or greater sinusoidal dilatation was 4.36 fold that of surgery only controls (95% Confidence Interval 1.36 – 13.97; $p = 0.01$; Figure 6B). There is a significant amount of heterogeneity in this analysis ($I^2 = 69\%$; $p = 0.01$) which arises from the small study of Makowiec et al. which appears to be at odds with the rest of the included data.(Makowiec, Mohrle et al. 2011) If this study is excluded from the analysis then the heterogeneity is resolved ($I^2 = 0$; $p = 0.48$) and the risk of grade 2 or greater sinusoidal dilatation increases to 6.19 fold (95% Confidence 3.14 – 12.22; $p<0.00001$). Irinotecan based regimens were not found to be associated with the development of grade 2 or greater sinusoidal dilatation (Relative Risk 1.11; 95% Confidence Interval 0.65 – 1.90; $p = 0.70$; Figure 6C) strongly suggesting that this is a regimen specific phenomenon.

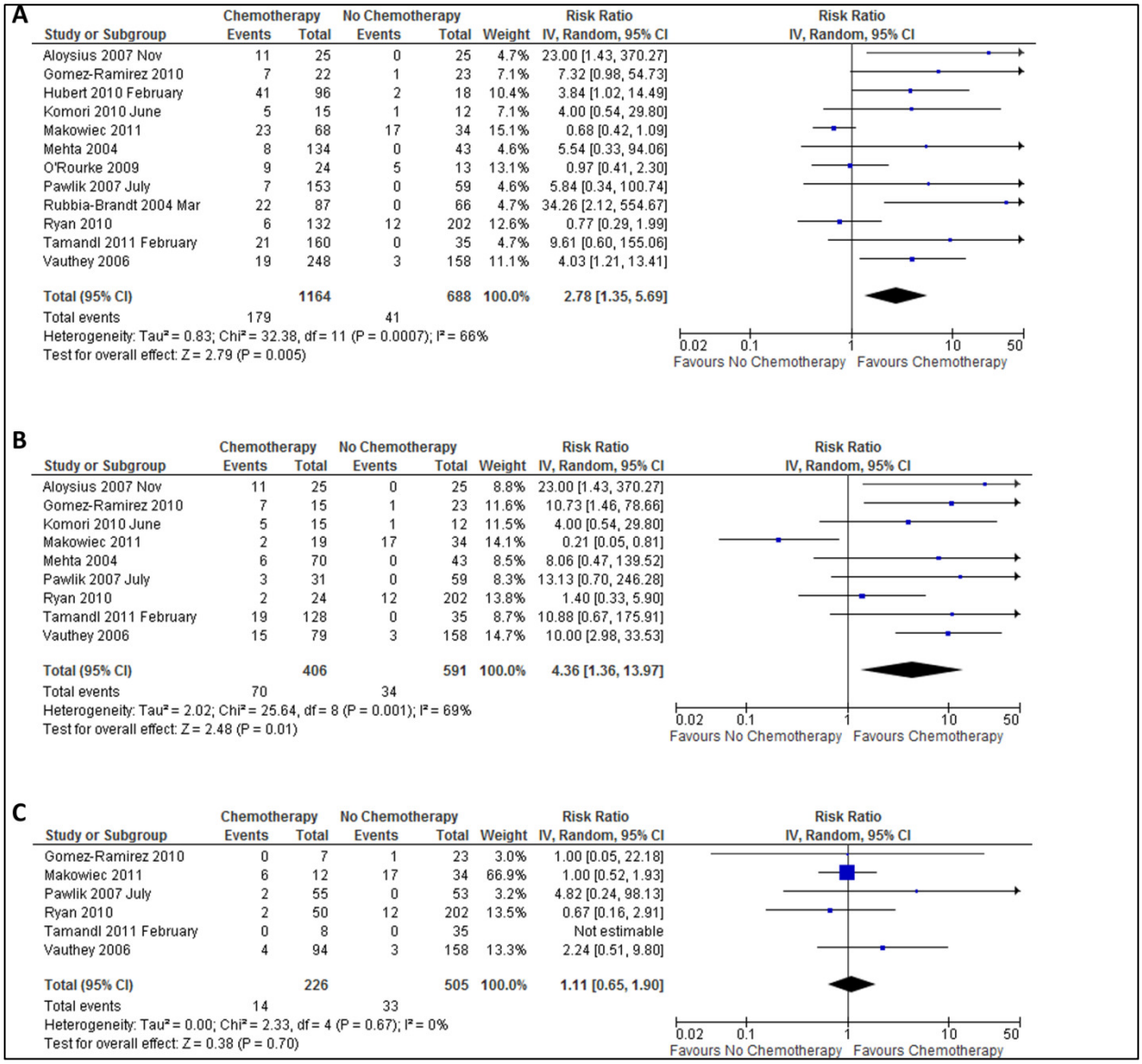

Figure 6 – Overall pre-operative chemotherapy is associated with an increased risk of grade 2 or greater sinusoidal dilatation (A). When broken down by regimen however this seems to be predominantly a feature of Oxaliplatin based regimens (B) whereas Irinotecan based regimens are not associated with sinusoidal dilatation (C).

2.4 Conclusion

In performing this systematic review and meta-analysis of the published literature I have been able to bring a degree of clarity to the discussion surrounding the nature of chemotherapy associated liver injury. Despite commonly held beliefs to the contrary there is insufficient evidence to demonstrate an association between the administration of pre-operative chemotherapy and the development of hepatic steatosis. Furthermore the nature of the liver injury induced by chemotherapy appears to be regimen specific with Irinotecan based regimens being associated with steatohepatitis and Oxaliplatin based regimens being associated with sinusoidal obstruction syndrome.

Using the data presented it is possible to calculate the number needed to treat with pre-operative chemotherapy to cause one case of parenchymal injury. In the case of Irinotecan for every 12 patients treated with this agent pre-operatively 1 would be expected to develop steatohepatitis. In the case of Oxaliplatin however 1 in every 9 patients would be expected to develop grade 2 or greater sinusoidal obstruction. In other words, although the patterns of liver injury with the two regimens are different, liver parenchymal injury as a result of chemotherapy is more likely to occur in those treated with Oxaliplatin based chemotherapy regimens.

Locally the majority of patients with colorectal liver metastases are treated, as first line, with Oxaliplatin based regimens. Taken together with the findings of the meta-analysis that this regimen is more commonly associated with parenchymal injury the remainder of this thesis will concentrate on Oxaliplatin induced liver injury.

Chapter 3

Current Knowledge Regarding the Pathogenesis of Chemotherapy induced Sinusoidal Obstruction Syndrome

At the present time there is no firm understanding of how or why patients with colorectal liver metastases treated with Oxaliplatin containing chemotherapy regimens develop SOS and as such no treatment options exist to either prevent the development of these pathological changes or indeed to reverse them once they have developed. This knowledge is vitally important if we are to try and reduce the morbidity and mortality associated with liver resection in this group of patients.

It is however possible to gain some insight into potential risk factors associated with the development of SOS from published case series. In addition some researchers have explored the pathogenesis of this condition at the molecular level using a rodent model of Monocrotaline induced SOS although the relevance of this to Oxaliplatin induced SOS may be somewhat limited. In this chapter I explore the current literature regarding SOS before setting out the aims and objectives for the experimental work contained within this thesis.

3.1 Risk Factors Associated with the Development of SOS

Intuitively one might expect that the prolonged exposure to Oxaliplatin based chemotherapy would be a risk factor for the development of SOS. In a study of 219 patients undergoing liver resection after pre-operative treatment with FOLFOX Kishi et al. reported that sinusoidal injury (grade 2 or greater) was present in 26% of those who had received 1 to 8 cycles of chemotherapy and in 42% of those who had received 9 or more cycles (p=0.017).(Kishi, Zorzi et al. 2010) Similarly Aloia et al. noted that 50 % of those who received 12 or more cycles of FOLFOX chemotherapy developed hepatic vascular injury as compared to 25% of those receiving 6 – 12 cycles and only 10% of those receiving less than 6 cycles (p=0.01)(Aloia, Sebagh et al. 2006) Nakano et al. performed a multivariate analysis to identify factors associated with development of sinusoidal injury and identified that more than 6 cycles of Oxaliplatin based chemotherapy placed patients at greater risk of developing SOS(Relative Risk 3.198; 95% Confidence Interval 1.01 – 10.12; p = 0.048).(Nakano, Oussoultzoglou et al. 2008) In contrast to this multivariate analysis at least 3 other studies has failed to identify duration of chemotherapy as an independent risk factor for the development of SOS.(Overman, Maru et al. 2010; Soubrane, Brouquet et al. 2010; Tamandl, Klinger et al. 2011)

It has been proposed that tumour related factors might contribute to the development of SOS. Soubrane et al. performed a univariate analysis to identify potential risk factors for the development of SOS in 78 patients who had received pre-operative Oxaliplatin based chemotherapy and found that those with grade 2 or greater sinusoidal dilatation tended to have a larger tumour size (7.8cm vs. 5.2cm; $p < 0.004$) although this was not found to be an independent risk factor on multivariate analysis.(Soubrane, Brouquet et al. 2010) In

contrast to this Tamandl et al. reported that a tumour diameter >5cm was independently associated with an increased risk of SOS on multivariate analysis (Hazard Ratio 4.42; 95% Confidence Interval 1.38 – 13.18; p = 0.012).(Tamandl, Klinger et al. 2011)

Several groups have examined the ability of pre-operative blood tests to predict the presence of SOS. On univariate analysis Soubrane et al. found that those with SOS tended to have a higher AST (53 U/L vs. 37 U/L; p = 0.0009) and ALT (49.8 U/L vs. 32.7 U/L; p = 0.02) but a lower platelet count (167 x 10^3 vs. 244 x 10^3; p < 0.0001). They also demonstrated on multivariate analysis that a higher ratio of AST to platelet count (APRI score) was strongly predictive of the presence of SOS (Odds Ratio 5; 95% Confidence Interval 4 – 1532; p < 0.005).(Soubrane, Brouquet et al. 2010) Nakano et al. also demonstrated, on multivariate analysis, that an AST of greater than 36 U/L was associated with an increased risk of SOS (Relative Risk 3.68; 95% Confidence Interval 1.034 – 13.162; p = 0.044).(Nakano, Oussoultzoglou et al. 2008) Tamandl et al., on the other hand, did not find any association between pre-operative AST or ALT and the development of SOS. They did however demonstrate, on multivariate analysis, that an elevated ALP or γGT were associated with an increased risk of SOS (Hazard Ratio 4; 95% Confidence Interval 1.08 – 14.73; p = 0.038).(Tamandl, Klinger et al. 2011)

In a study of patients receiving either adjuvant FOLFOX (n=96) or fluoropyrimidine alone (n=40) following resection of primary colon cancer (non-metastatic) Overman et al. compared spleen size prior to starting chemotherapy with that 6 weeks after the final cycle. They demonstrated that in those receiving FOLFOX there was a median increase in spleen size of 22% as compared to -3% for those receiving fluoropyrimidine therapy alone (p<0.001) which was also associated with the presence of thrombocytopenia (p<0.003). The

group then performed a multivariate analysis of 63 patients who had received FOLFOX prior to liver resection and demonstrated that a greater than 50% increase in spleen size was an independent risk factor for the presence of SOS ($p<0.003$).(Overman, Maru et al. 2010)

Table 4 presents a summary of the potential risk factors identified on multivariate analysis as being associated with the presence of SOS following Oxaliplatin based chemotherapy however it should be noted that most of these variables are likely to be altered as a consequence of SOS rather than truly causative. From the discussion it can be seen that much of the evidence relating to risk factors is contradictory and this likely reflects both the small numbers of patients in most of these studies and also the different variables entered into the multivariate analyses.

Risk Factors for SOS	**Supporting Studies**
Prolonged Chemotherapy Exposure	Nakano et al. 2008(Nakano, Oussoultzoglou et al. 2008)
Elevated AST	Nakano et al. 2008(Nakano, Oussoultzoglou et al. 2008)
Elevated ALP/γGT	Tamandl et al. 2011(Tamandl, Klinger et al. 2011)
Elevated AST to Platelet Ratio (APRI)	Soubrane et al. 2010(Soubrane, Brouquet et al. 2010)
Larger tumour size	Tamandl et al. 2011(Tamandl, Klinger et al. 2011)
Splenomegaly > 50%	Overman et al. 2010(Overman, Maru et al. 2010)
Female Gender	Nakano et al. 2008(Nakano, Oussoultzoglou et al. 2008)

Table 4 – Summary of risk factors for the presence of SOS identified on multivariate analysis

3.2 The Monocrotaline Model of SOS

Monocrotaline is a pyrrolizidine alkaloid which when administered to rats results in the rapid appearance of SOS like histological changes in the liver (although there is no effect on the liver following administration to mice). The nature of these changes has been well characterised using both light and electron microscopy. Within 24 hours of administration there is evidence of injury to endothelial cells, in the region of the central veins, which on electron microscopy is characterised by a reduced number of endothelial fenestrae and the rounding up of endothelial cells. This rounding up results in the appearance of large gaps between adjacent endothelial cells which, by day 3, is sufficient to allow extravasation of red blood cells into the space of Disse. This process results in dissection of the endothelial lining of the sinusoid which subsequently embolises and obstructs the microcirculation with resultant dilatation of the hepatic sinusoid.(DeLeve, McCuskey et al. 1999; DeLeve, Ito et al. 2003)

At the same time as this process of microvascular obstruction by embolised endothelial cells reaches a peak, on days 3 to 4 post administration, coagulative necrosis of the hepatic parenchymal cells is seen, predominantly in centrilobular regions. These changes persist up until day 6 when there is evidence of the gaps between endothelial cells reducing and resolution of coagulative necrosis.(DeLeve, McCuskey et al. 1999) It is clear that the speed at which the histological changes following Monocrotaline occur is extremely rapid whereas SOS following Oxaliplatin administration seems to occur as a consequence of prolonged repeated drug exposure.

The evolution of these changes seen in animals suggests that the primary insult, following administration of Monocrotaline, is to the sinusoidal endothelium with hepatocyte injury

occurring secondary to this. *In vitro* studies appear to confirm these findings with Monocrotaline being much more toxic, at like for like doses, to cultured sinusoidal endothelial cells than it is to hepatocytes.(DeLeve, Wang et al. 1996) The toxic effects of Monocrotaline on cultured sinusoidal endothelial cells are exacerbated when intracellular glutathione levels are depleted whereas endothelium treated with Monocrotaline in the presence of exogenous glutathione appears to be protected from Monocrotaline toxicity suggesting that oxidative stress may play a role in SOS development.(DeLeve, Wang et al. 1996) Rats treated with Monocrotaline whilst receiving an intra-portal infusion of glutathione do not develop SOS thereby confirming the relevance of this mechanism *in vivo*.(Wang, Kanel et al. 2000)

This mechanism could theoretically have potential relevance for Oxaliplatin induced SOS. As already discussed one of the key mechanisms through which cells are able to remove platinum is through conjugation to glutathione.(Zhang, Mack et al. 1998) One of the major side effects of platinum agents is the development of peripheral neuropathy and it has been demonstrated that variations in intracellular glutathione levels alter the sensitivity of human neural cell lines to platinum induced cytotoxicity.(Iida, Doi et al. 1999) In a randomised controlled trial the neurotoxicity of Oxaliplatin was diminished when co-administered with glutathione.(Cascinu, Catalano et al. 2002) It has been observed that in patients who develop grade 2 – 4 neuropathy there is an increased incidence of sinusoidal injury (86% vs. 23%; $p = 0.002$) suggesting that glutathione depletion may be a common link between these two pathologies.(Julie, Lutz et al. 2007)

In the Monocrotaline model it has been suggested that matrix metalloproteinases such as MMP2 and MMP9 play key roles in the development of SOS. Initial observations regarding

the role of MMPs in microcirculatory liver disturbances were made in allografts subjected to cold preservation. When organs had been stored at $4^{o}C$ for greater than 4 hours it was noted that sinusoidal endothelial cells became swollen and by 8 hours had sloughed off into the lumen of the sinusoid.(McKeown, Edwards et al. 1988) It was demonstrated that this process was driven predominantly by MMP2 and 9 which digested the fibronectin tethering the cells to the extracellular matrix.(Upadhya, Harvey et al. 1997) Whole liver extracts of rats treated with Monocrotaline 48 hours previously demonstrate a 50-fold increase in MMP9 protein levels and a 5-7 fold increase in MMP2 protein levels which appears to be driven at a transcriptional level.(Deleve, Wang et al. 2003) De Leve et al. demonstrated unequivocally that the increase in MMP9 transcript arises specifically in the sinusoidal endothelium and does not occur in Kupffer cells, hepatocytes or stellate cells of Monocrotaline treated rats. Co-administration of the MMP2 and 9 inhibitor doxycyline alongside Monocrotaline prevented the development of an SOS phenotype.(Deleve, Wang et al. 2003)

Whilst Monocrotaline might replicate some of the changes seen in SOS at a histological level it is not clear how faithfully this truly reflects the pathogenesis of the disease in patients with colorectal liver metastases who are treated with Oxaliplatin based chemotherapy. For example a key feature of severe SOS is the development of nodular regenerative hyperplasia which is not described in reports relating to the Monocrotaline model suggesting that it does not occur in this context.(Rubbia-Brandt, Audard et al. 2004) Likewise a prominent feature of Monocrotaline induced SOS is the development of a lobular inflammatory infiltrate, consisting predominantly of neutrophils, which is not described in patients who develop SOS.(Coppell, Brown et al. 2003; Rubbia-Brandt, Audard et al. 2004)

Furthermore the administration of Monocrotaline to rats was initially carried out to generate a model of pulmonary hypertension.(Roth and Reindel 1991; Schultze and Roth 1998) The presence of pathology such as this which can have a profound effect on multi-organ physiology really serves to limit the translational value of the Monocrotaline model for patients with chemotherapy induced SOS.

3.3 Project Aims

The aims of this project are three fold:

1) To develop a reproducible *in vivo* experimental model which accurately reflects the development of SOS in patients treated with Oxaliplatin based chemotherapy regimens
2) To interrogate this model to identify the key pathophysiological processes that underpin the development of SOS in these patients
3) To identify potential therapeutic strategies to ameliorate the development of SOS in patients with colorectal liver metastases

Chapter 4

Materials & Methods

4.1 Animal Models

All animal studies were carried out using the male C57BL/6J mice purchased from a licensed supplier (Harlan Laboratories Ltd, UK). Animals were maintained under standard animal house conditions in filtered cages with 12 hour light/dark cycles. Access to standard chow diet (Special Diets Service, UK; see Appendix 2) and water was provided ad libitum unless otherwise stated.

After shipment animals were allowed to acclimatise for 1 week prior to commencing experimental protocols.

All animal studies were approved by the local research ethics committee and the UK home office.

4.1.1 FOLFOX Administration

All drugs for use *in vivo* studies were prepared using aseptic technique and filter sterilised solutions. Oxaliplatin (Sigma-Aldrich, Dorset, UK) was dissolved in PBS (Lonza, Basel, Switzerland) to a final concentration of 1mg/ml. 100mg of 5-FU was dissolved in 1ml of DMSO (Sigma-Aldrich, Dorset, UK) before making up to a final volume of 10ml in PBS to give a concentration of 10mg/ml. Folinic Acid (Sigma-Aldrich, Dorset, UK) was dissolved in water to a final concentration of 12.5mg/ml. Once prepared drug solutions were aliquoted and stored at -80^{o}C until use. Freeze-thaw cycles were avoided.

The FOLFOX chemotherapy used in this study consisted of 6mg/kg of Oxaliplatin intraperitoneally followed two hours later by 50mg/kg of 5-FU and 90mg/kg of Folinic Acid.

The manner by which this dosing schedule was determined is described in detail in Chapter 5. Prior to chemotherapy administration mice were weighed and drugs administered as per the tables in Appendix 3. Control animals received the equivalent amount of vehicle for each drug according to body weight. Drug administration was repeated weekly for 5 weeks unless stated otherwise. Animals were culled 1 week after the final dose of chemotherapy and organs harvested for subsequent analysis.

4.1.2 Hepatic Tumour Implantation

MCA38 colorectal cancer cells were stably transfected with the pGL4.51 luciferase reporting vector (see section 2.2.2) and harvested using Trypsin EDTA (Life Technologies Ltd, Paisley, UK) prior to centrifugation at 400G for 4 minutes to pellet. The cells were resuspended in HBSS (Life Technologies Ltd, Paisley, UK) and counted using a haemocytometer. After centrifugation a second time the cells were resuspended in HBSS to give a final concentration of 10^7 cells per ml. Cells were then placed on ice until the time of implantation.

10 week old C57BL/6J mice were subjected to a short upper midline laparotomy under isoflurane anaesthesia and buprenorphine analgesia. The falciform ligament was divided and the left lobe of the liver delivered into the wound. A gauze swab was placed between the delivered lobe and the wound edges to minimise the risk of wound contamination from spilt cells. 10μL of the cell solution (i.e. 10^5 cells was then injected under the capsule of the delivered lobe using a 30G needle. After withdrawing the needle manual compression was then applied to the injection site using a cotton bud until haemostasis was achieved. The injection track was then sealed by application of butylcyanoacrylate glue (Indermil, Henkel

Ireland Ltd). The liver was returned to the abdominal cavity and the wound closed in two layers with interrupted 3/0 vicryl sutures.

On the 5th post-operative day the presence of tumour within the liver was confirmed using the IVIS *in vivo* imaging system (Caliper Life Sciences, Hopkinton, USA). Ten minutes prior to imaging mice were injected intraperitoneally with XenoLight D-Luciferin (Caliper Life Sciences, Hopkinton, USA) at a dose of 150mg/kg body weight. Imaging was performed under isoflurane anaesthesia.

If the presence of tumour was confirmed then mice went on to receive chemotherapy as described above.

4.1.3 Dietary Manipulation

To establish hepatic steatosis 4 week old C57BL/6J mice were maintained on either a high fat diet containing 45%kcal fat (D12451, Research Diets Inc, New Brunswick, USA; Appendix 2). Control animals were maintained on a 10% kcal fat (D01060501, Research Diets Inc, New Brunswick, USA; Appendix 2). Chemotherapy was commenced after 6 weeks using the protocol described above and the mice were maintained on their allocated diet throughout.

The addition of either 3% N-Acetylcysteine or 0.7% Butylated Hydroxyanisole to the D01060501 diet was performed by the manufacturer. These diets were started 1 week prior to commencing chemotherapy.

4.2 Cell Culture

The MCA38 colorectal cancer cell line was used in all experiments in this thesis since it was derived in the C57BL/6J mouse and could therefore be used *in vivo* without eliciting an immune response. The cell line was supplied as a gift by Dr Mario Paolo Colombo (Istitutio Tumori, Milan, Italy).

Cells were cultured in 10% Dulbecos Modified Eagles Medium (Life Technologies Ltd, Paisley, UK) supplemented with 10% fetal calf serum, 100u/ml Penicillin, 100μg/ml streptomycin and 2mM L-Glutamine unless otherwise stated.

4.2.1 *In Vitro* FOLFOX Treatment

Prior to chemotherapy treatment cells were plated overnight and allowed to adhere overnight before exchanging it for that supplemented with 10μM Folinic Acid. Cells were then treated with the IC_{50} concentration of Oxaliplatin (7μM) and 2 hours later the IC_{50} concentration of 5-FU (14μM) in the presence of 10μM Folinic Acid as has been previously described in the literature.(Fischel, Formento et al. 2002)

The IC_{50} concentration was determined for both Oxaliplatin and 5-FU using the MTT assay. To do this 2000 cells were plated in 90 wells of a 96 well plate leaving 6 wells to serve as blanks. After being allowed to adhere overnight the media was replaced and supplemented with the drug of interest in a range of concentrations from 1nM to 1mM with control cells being treated with the vehicle alone. There were 6 wells for each condition to act as technical repeats. Cells were cultured for 48 hours before adding 20μL of a 5mg/ml MTT stock solution (Sigma-Aldrich, Dorset, UK) and incubating the cells for a further 4 hours. Plates were then centrifuged at 400G for 5 minutes and the media exchanged for 200μL of DMSO. After incubation at room temperature on a rocker for 30 minutes absorbance was

measured at 570nm and a curve of drug concentration against % cell viability plotted from which the IC_{50} was derived.

4.2.2 Production of Stably Transfected Luciferase Reporting MCA38 Cells

For *in vivo* tumour implantation experiments it was necessary to produce MCA38 cells stably transfected with a luciferase reporting vector. For this purpose the pGL4.51 vector (Promega, Southampton, UK) was chosen which has a CMV promoter and a luciferase 2 gene which when transfected produces a constitutively active luciferase 2 expressing cell (for vector map see Appendix 4).

JM109 competent cells were placed on ice for 30minutes in a buffer containing 10mM MOPS, 10mM Rubidium chloride, 75mM calcium chloride and 15% glycerol w/v (All Sigma-Aldrich, Dorset, UK) in 100μL aliquots with 1μg of vector per aliquot. The aliquots were subsequently placed in a water bath preheated to 42^{o}C for 45 seconds before placing back on ice. Cells were subsequently plated on agar supplemented with 100μg/ml Ampicillin (Sigma-Aldrich, Dorset, UK) and cultured overnight at 37^{o}C.

The next morning colonies were picked and grown in flasks of LB media (Sigma-Aldrich, Dorset, UK) again supplemented with 100μg/ml Ampicillin for a further 24 hours. Plasmid DNA was then extracted from the cells using the QIAprep Spin Miniprep Kit (Qiagen, Crawley, UK) according to the manufacturer's instructions. Isolated DNA was quantified on a spectrophotometer before storage at -20^{o}C.

MCA38 cells were harvested and resuspended in PBS at a concentration of 10^7 cells per ml for transfection by electroporation. To do this 1ml of the cell solution was placed in a 4mm cuvette (Harvard Apparatus Inc, Holliston, USA) with 30μg of the isolated vector before electroporation with 4 pulses each of 1msec duration at 475V using an

ElectroSquarePorator (Harvard Apparatus Inc, Holliston, USA). Cells were then cultured in 10% Dulbecos Modified Eagles Medium supplemented as above with the addition of the antibiotic G418 (Sigma-Aldrich, Dorset, UK) at a concentration of 1mg/ml. Prior to use in the *in vivo* experiments cells were cultured in the absence of G418 supplementation for a minimum of 48 hours.

4.3 Western Blot

Tissue samples were lysed in an appropriate volume of RIPA buffer containing protease inhibitors (Roche Diagnostics Ltd, Burgess Hill, UK) and phosphatase inhibitors (Sigma-Aldrich, Dorset, UK) before incubation at 4^{o}C for 30 minutes. Samples were then passed through a QIAshredder homogenisation column (Qiagen, Crawley, UK) before centrifugation at 13,000rpm at 4^{o}C in a benchtop centrifuge. The supernatant was transferred to a new tube before quantification using a modified Bradford assay according to the manufacturer's instructions (DC Protein Assay, Bio-Rad Laboratories, Hemel Hempstead, UK). Samples were subsequently stored at -80^{o}C prior to use.

For western blot 50μg of each protein sample was diluted to 25μL in RIPA buffer before adding 5μL of sample loading buffer (4% SDS, 10% 2-mercaptoethanol, 20% glycerol, 0.004% bromophenol blue, 0.125M Tris HCl). Samples were heated to 105^{o}C to denature before loading onto 9% Acrylamide gels for separation by SDS-PAGE. Once adequately separated proteins were transferred onto nitrocellulose membrane for 1 hour at room temperature. The adequacy of transfer was confirmed by staining the membrane with Ponceau Red solution (Sigma-Aldrich, Dorset, UK).

After washing in 0.01% Tween-TBS to remove all evidence of Ponceau red membranes were blocked with either 5% skimmed milk or, in the case of phosphorylated proteins, 5% bovine serum albumin (Sigma-Aldrich, Dorset, UK) for 1 hour at room temperature. Primary antibodies were then added as outlined in Table 5 and membranes incubated overnight at 4^{o}C on a rocking platform.

The following morning membranes were washed thoroughly in 0.01% Tween-TBS before incubation for 2 hours with a HRP conjugated secondary antibody appropriate for the

species in which the primary antibody was raised. After further washing with 0.01% Tween-TBS before detecting bands with an enhanced chemo-luminescence system (Thermo-Scientific, Rockford, USA). In the early part of the project β-Actin was used as the loading control before a change in laboratory policy when GAPDH was used.

Target	**Concentration**	**Supplier**
Total p53	1:1000	Abcam
p-p53	1:1000	Cell Signalling Technology
p21	1:1000	Abcam
γH2AX	1:1000	Cell Signalling Technology
NRF2	1:1000	Abcam
Total STAT3	1:1000	Cell Signalling Technology
p-STAT3	1:1000	Cell Signalling Technology
GAPDH	1:2500	Abcam
β-Actin	1:5000	Sigma-Aldrich

Table 5 - Primary antibodies for western blot

4.4 Histology / Immunohistochemistry

All histological analysis was performed on 5μm thick formalin fixed paraffin embedded tissue sections unless otherwise stated.

4.4.1 Haematoxylin and Eosin Staining

Sections were de-waxed by immersion in Xylene (Sigma-Aldrich, Dorset, UK) for 10 minutes followed by rehydration for 2 minutes in 100% ethanol and 2 minutes in 95% ethanol. Sections were then washed thoroughly in tap water before immersion in Mayers Haematoxylin for 5 minutes. After blueing in Scott's water for 30 seconds the sections were washed and immersed in Eosin until adequately counterstained. The sections were then dehydrated by immersion in increasing concentrations of ethanol (50%, 75%, 95% and 100%) before placing in Xylene until clear and mounting with DPX (Cellpath LTD, Powys, UK).

Haematoxylin and eosin stained sections were assessed for the presence of sinusoidal dilatation according to the method of Rubbia-Brandt (Rubbia-Brandt, Audard et al. 2004; Table 6). In addition disruption and loss of the endothelial layer within the hepatic sinusoid was graded as either absent or present. All histological scoring was carried out by Professor Alastair Burt (Dean of Clinical Medicine & Honorary Consultant Histopathology, Newcastle University & The Newcastle upon Tyne Hospitals NHS Trust).

Grade	Description
0	Sinusoidal dilatation absent
1	Sinusoidal dilatation limited to one third of the lobular surface
2	Sinusoidal dilatation extending to two thirds of the lobular surface
3	Sinusoidal dilatation involving the entire lobule

Table 6 - Semi-quantitative scoring system for assessment of sinusoidal dilatation as propsoed by Rubbia-Brandt (Rubbia-Brandt, Audard et al. 2004)

4.4.2 $p21^{CIP1/WAF1}$ Immunohistochemistry

Sections were de-waxed in Clearene (Leica Microsystems Ltd, Milton Keynes, UK) for 10 minutes followed by rehydration in 100% and 70% industrial methylated spirits each for 5 minutes. Endogenous peroxidase activity was then blocked by immersion in methanol with 2% hydrogen peroxide (Sigma-Aldrich, Dorset, UK). Antigen retrieval was performed by immersing sections in sodium citrate solution pH6 (Vector Laboratories, Burlingame, USA) and heated in a microwave on full power for 15minutes before being allowed to cool. Sections were mounted in a sequenza and washed with PBS before inhibiting non-specific binding using an Avidin/Biotin blocking kit (Vector Laboratories, Burlingame, CA). After incubation in 20% swine serum for 20 minutes at room temperature with the primary antibody (ab2961, Abcam, Cambridge, UK) at 4^{o}C overnight.

The following morning the sections were washed with PBS before incubation with an anti-rabbit biotinylated secondary antibody (Dako UK Ltd, Ely, UK) at a concentration of 1:200 for 2 hours at room temperature. After a further PBS wash sections were incubated with a streptavidin biotin-peroxidase complex (Vector Laboratories, Burlingame, USA) for 45 mins

before visualising positive cells with 3,3'-daminobenzidine tetrahydrochloride (DAB) (Vector Laboratories, Burlingame, USA). Counterstaining was performed with Meyers haematoxylin before dehydrating sections in increasing concentrations of industrial methylated spirits and mounting in Pertex(Cellpath LTD, Powys, UK).

4.4.3 γH2AX Immunohistochemistry

The protocol used was identical to that for $p21^{CIP1/WAF1}$ except for the following variations :

- Antigen retrieval was by immersing sections in 1mM EDTA pH 8.0 and microwaving on full power for 15 minutes
- The primary antibody was used at a concentration of 1:100 (#9718, Cell Signalling Technology, Beverley, USA)

4.4.4 p-STAT3 Immunohistochemistry

The protocol used was identical to that for γH2AX except the primary antibody was used at a concentration of 1:400 (#9145, Cell Signalling Technology, Beverley, USA).

4.4.5 Tissue Factor Immunohistochemistry

The protocol used was identical to that for $p21^{CIP1/WAF1}$ except the primary antibody was used at a concentration of 1:100 (ab104513, Abcam, Cambridge, UK).

4.5 Determination of Hepatic Triglyceride Content

A sample of liver tissue was weighed and homogenised in 5% Triton-X100 (Sigma-Aldrich, Devon, UK). The homogenate was then heated to 80^{o}C for 5 minutes before being allowed to cool slowly to room temperature before repeating the heating a second time. The homogenate was centrifuged at 13000 rpm for 5 minutes before recovering the supernatant and diluting this 10 fold with distilled water.

The remainder of this assay was performed with a commercial kit (ab65336, Abcam, Cambridge, UK). In brief a 96 well plate was set up with standards of known triglyceride concentration along with serial dilutions of the experimental samples all diluted in the provided assay buffer. To each sample and standard 2μl of lipase was added along with a further 46μl of assay buffer, 2μl of triglyceride probe and 2μl of triglyceride enzyme mix. The plate was then incubated for 60 minutes at room temperature protected from light before measuring absorbance at 570nm. Using the generated standard curve the triglyceride content in each experimental sample was derived and corrected for the original weight of tissue used in the assay.

4.6 Oxidised/Reduced Glutathione Assay

A known weight of liver tissue was homogenised in RIPA buffer (as described for sample preparation for western blot). The remainder of the assay was performed using a commercial kit (GT40, Oxford Biomedical, Rochester Hills, USA).

To determine the concentration of reduced glutathione (GSH) 50µL of the lysate above was mixed with 350µL of 5% Metaphosphoric acid (MPA) solution by vortexing before centrifugation at 1000g for 10 minutes at 4^{o}C. 25µl of the supernatant was added to 1.5ml of the provided assay buffer and the prepared sample stored on ice until ready to use.

To determine the concentration of oxidised glutathione (GSSG) 130µl of the tissue lysate was incubated at room temperature for 10 minutes before mixing with 270µL of 5% MPA by vortexing before centrifugation at 1000g for 10 minutes. 50µL of the supernatant was then added to 700µL of the provided assay buffer and placed on ice until ready to use.

Standards were prepared as per the manufacturer's instructions and a 96 well plate set up with 50µl of both these and the experimental samples repeated in duplicate. To each well 50µL of lyopholised 5,5'-dithiobis-2-nitrobenzoic acid and 50µl of recombinant glutathione reductase were subsequently added before incubating on a shaker at room temperature for 5 minutes. 50µl of NADPH was then added to each well before measuring absorbance at 412nm every minute for 10 minutes. The net rate of change in absorbance over time was determined for each sample and a standard curve constructed from which the concentration of GSH and GSSG were derived for each experimental sample.

It should be noted that this method only applies to the GSH/GSSG assay described in chapter 5. That presented in chapter 7 was performed by a collaborator (Dr Aphrodite Vasilaki, Faculty of Health & Life Sciences, University of Liverpool).

4.7 PCR

4.7.1 Quantitative Real Time PCR

Total RNA was extracted from both tissue and cell samples using the RNeasy purification system (Qiagen, Crawley, UK) according to the manufacturers instructions and quantified on a spectrophotometer. RNA integrity was assured by only using samples which had an OD 260/280 of greater than 1.8. Samples were stored at -80^{o}C.

All reagents for cDNA synthesis were from Promega, Southampton, UK. 1µg of RNA was diluted to 8µl in nuclease free water before adding 1µl each of DNAse and DNAse buffer and incubating at 37^{o}C for 30 minutes. 1µl of DNAse stop solution was then added to each sample and incubated at room temperature for 2 minutes before adding 0.5µl of a random hexamer primer and a further 2µ of nuclease free water. The samples were heated to 72^{o}C for 5 minutes before placing onto ice. To each sample was then added 0.5µL of RNA synthase inhibitors, 1µl of MMLV-RT, 4µl of MMLV-RT buffer and 1µl of dNTPs. The final mixture was heated for 42^{o}C for 1 hour before diluting to a final volume of 50µL with nuclease free water.

qRT-PCR was performed using SYBR-green reagents (Sigma-Aldrich, Dorset, UK) on an ABPI 7500 fast machine (Applied Biosystems, Foster City, USA). The PCR reaction consisted of 6.5µl of the 2x concentrated SYBR-green reaction master mix, 1µl of the cDNA sample generated above, 1µl each of forward and reverse primers diluted and 3.5µl of nuclease free water. Each experimental sample was run in triplicate. The PCR cycle consisted of a denaturation step at 95^{o}C for 5 seconds, annealing at an appropriate temperature for the primer pair for 30 seconds and elongation at 72^{o}C for 30 seconds. This cycle was repeated 40 times before melt curve analysis was performed to validate the results. GAPDH was used

as the internal control for all PCR experiments and the fold change in mRNA expression calculated using the ΔΔCT method. A list of all primer sequences used in this study is provided in Table 7.

Product	Forward Primer Sequence	Reverse Primer Sequence
PAI-1	gatgctatgggattcaaagtca	tccacctgtttcaccatagtct
$p21^{Cip1}$	atgtccaatcctggtgatgt	tgcagcagggcagaggaagt
TXN1	catgccgaccttccagtttta	tttccttgttagcaccggaga
NQO1	tggccgaacacaagaagctg	tgggaactgaaatatcaccaggt
CXCL1	ggctgggattcacctcaag	gcgaccattcttgagtgtg
CXCL2	ccaaccaccaggctacagg	gcgtcacactcaagctctg
CXCL5	tgccctacggtggaagtca	gtgcattccgcttagctttc
CCL 2	aggtccctgtcatgcttctg	tctggacccattccttcttg
CCL 5	tgctgctttgcctacctctcc	tggcacacacttggcggtttc
αSMA	tcagcgcctccagttcct	aaaaaaaaccacgagtaacaaatcaa
Pro Coll 1	ttcacctacagcacgcttgtg	gatgactgtcttgccccaagtt
TIMP1	tccccagaaatcaacgagacc	cttcactgcggttctgggact
MMP2	ccgatgctgatactgacact	gtcactgtccgccaaataaa
MMP9	tcactttcccttcaccttcg	caaagatgaacgggaacaca
TGF β	cccgaacccccattgctgtcc	aggcgtatcagtgggggtcag
IL-6	gaggataccactcccaacagacc	aagtgcatcatcgttgttcataca
Factor X	gccctaaacaccagcgacagt	gccacccacaatccgaacaag
PAR-1	ggtgctcattggctttttcta	tgccttcttcctggggtgtcc
PAR-2	ctgctgtttgtggttggtgta	cccagttgttgccattgagat
vWF	cgggaagagtgtgatggttgac	agcatctcccacagcattcacc
GAPDH	gcacagtcaaggccgagaat	gccttctccatggtggtgaa

Table 7 - Primer sequences used in qRT-PCR

4.7.2 Real Time PCR

For standard real time PCR cDNA was prepared as described above. The PCR reaction consisted of 12.5µl of PCR Master Mix (Promega, Southampton, UK), 1µl of cDNA, 1µl of each forward and reverse primers and 9.5µl of nuclease free water to give a total reaction volume of 25µl. The PCR reaction was carried out on a bench top thermal cycler using an initiation step of 95^{o}C for 30 seconds followed by 35 cycles of 95^{o}C denaturing for 5 seconds, 30 seconds of the appropriate annealing temperature and 30 seconds at 72^{o}C for

elongation. This was followed by a further final elongation step of 72^{o}C for 7 minutes. The PCR product was subject to electrophoresis on a 1% agarose gel before staining with ethidium bromide and visualisation under an ultraviolet lamp. The sequences of primers used for RT-PCR are listed in Table 8.

Product	**Forward Primer Sequence**	**Reverse Primer Sequence**
CTR1	gatgatgatgcctatgacctt	cgaatgctgacttgagacttt
CTR2	gccgtgcttctctttgatttc	gggtcctattgtctgaagttg
ATP7A	tgctaacccccctccctgtctt	catcccttccactttcatctt
ATP7B	ccgatggagtagaggagaatg	gaacagatgaggcacaggtaa
TS	gaggcattttggagcagagta	tgtaggtgagcagagcatagc
TP	cccgagaactggcaaagatgt	gtttccgcctgtccgctaatc
DPD	ttttggctcggttgggctatt	tatggtccttttgggttctg
β-Actin	tggaatcctgtggcatccat	taaaacgcagctcagtaaca

Table 8 - Primer sequences used in RT-PCR

4.8 Measurement of Extracellular ATP

The extracellular ATP content in the media from FOLFOX treated MCA38 cells was determined using the ENLITEN ATP assay system (Promega, Southampton, UK) using the manufacturer's instructions. In brief 100µl of either experimental sample or standard was placed into a tube to which 100µL of Enliten Luciferase/Luciferin Reagent was added. Emission was measured on a luminometer (average of three readings) and a standard curve constructed from which the ATP concentration in the experimental samples was determined.

4.9 CXCL1 ELISA

This assay was developed using the reagents and antibodies supplied in the R&D Systems CXCL1 ELISA development kit according to the manufacturer's instructions (R&D Systems, Abingdon, UK). In summary a 96 well plate was coated overnight at room temperature with 50µl of the capture antibody. The next morning the plate was washed in 0.05%Tween-PBS before blocking the plate for 1 hour at room temperature with 1% bovine serum albumin in PBS. After washing the plate again 50µl of either standard or experimental sample were added to the plate in triplicate, the plate was covered and incubated for 2 hours at room temperature.

The samples were washed from the plate in 0.05% Tween-PBS and 50µL of the detection antibody added to each well for 2 hours at room temperature. After a further wash 100µl of streptavidin-HRP conjugate was added to each well for 20 minutes at room temperature. 100µl of substrate solution was then added to each well for a further 20 minutes before adding 50µl of 2N H_2SO_4. The absorbance was then measured at 450nm with correction at 540nm and a standard curve constructed from which the CXCL1 concentration in the experimental samples was derived.

4.10 Statistical Analysis

It was assumed that all continuous data were non-parametric and were therefore assessed with a Mann-Whitney U-Test using GraphPad Prism software (GraphPad Software, La Jolla, USA). Statistical significance was set at a p-value of <0.05. Statistical significance is denoted on figures as follows *=<0.05, **=<0.01, ***=<0.001.

Chapter 5

Initial *In Vivo* Models

The first aim of this project was to establish a reproducible *in vivo* experimental model of CALI which accurately reflects events in patients who receive chemotherapy prior to resection of colorectal liver metastases. After careful consideration I elected to establish a murine model for the following reasons:

- Mice are widely utilised in therapeutic and toxicity studies utilising chemotherapeutic agents suggesting that they are sensitive to their toxic effects(Boughattas, Levi et al. 1989)
- The mouse has been widely utilised in other models of liver pathophysiology, e.g. liver fibrosis, and responds in a manner that is broadly similar to the human liver(Starkel and Leclercq 2011)
- Laboratory mouse strains are inbred eradicating the effects of genetic variability and allowing a reduction in the number of animals required to obtain statistical significance(Justice, Siracusa et al. 2011)
- Laboratory reagents such as antibodies are widely available for murine tissues and cells enabling ease of analysis in an experimental study

Furthermore, owing to the large number of knockout strains available, which may be of benefit in the future for interrogating particular pathways involved in the pathophysiology of CALI, I opted to use the C57BL/6J strain of mouse.

5.1 Assessment of Direct Drug Induced Liver Toxicity

Initially I wished to determine the maximum dose of Oxaliplatin which could be administered to a mouse without running the risk of drug induced mortality. Fortunately much of the pre-clinical work to establish toxicity and efficacy of these drugs has been performed in this species and a review of the literature demonstrated that Oxaliplatin doses of up to 10mg/kg, via the intraperitoneal (i.p.) route, were well tolerated whereas doses much in excess are associated with a significant risk of animal death.(Boughattas, Levi et al. 1989; Louvet, Coudray et al. 2000; Cividalli, Ceciarelli et al. 2002; Mathieu, Remmelink et al. 2004) Twice weekly administration of low dose Oxaliplatin for four weeks has been demonstrated to cause peripheral neuropathy in mice, a complication which typically affects 10 – 40% of patients treated with Oxaliplatin based chemotherapy regimens. (Ghirardi, Lo Giudice et al. 2005; Gauchan, Andoh et al. 2009; Melisi, Ossovskaya et al. 2009; Renn, Carozzi et al. 2011) As a consequence it was determined that 4 weeks would be an appropriate initial time course for drug administration in my model.

On the background of these studies I performed an initial pilot experiment whereby mice (n=3 per group) were treated with Oxaliplatin 5mg/kg i.p. twice weekly for four weeks, or vehicle control, and culled 4 days after the final dose. Whilst the mean body weight was broadly similar in the control and treatment groups at the beginning of the experiment (24.8 ± 0.2 vs. 23.97 ± 0.73) the mice treated with Oxaliplatin failed to gain weight over the course of the experiment, unlike those in the control arm (27.53 ± 0.22 vs. 24.07 ± 0.25; Fig 7A) indicating systemic toxicity of the drug regimen. This difference did not reach statistical significance as a result of the small number of animals in each group. Despite this systemic

effect, H&E stained sections of the liver failed to demonstrate any evidence of injury (Fig 7B).

As already discussed Oxaliplatin is rarely, if ever, administered to patients with colorectal liver metastases as mono-therapy but rather is given in combination with a fluoropyrimidine because of the proven increased efficacy of this regimen. (de Gramont, Figer et al. 2000; Giacchetti, Perpoint et al. 2000) After examination of the literature it was decided to administer 5-FU at half of the reported maximum tolerated dose in rodents (i.e. 50mg/kg) since it has been reported that, when co-administered with other chemotherapeutics, doses greater than this result in animal death. (Cao, Frank et al. 1996; Cao and Rustum 2000) 5-FU was administered alongside 90mg/kg of Folinic acid (leucovorin, LV) as this has been demonstrated to enhance both the efficacy and toxicity of 5-FU.(Nadal, van Groeningen et al. 1989; Wright, Dreyfuss et al. 1989) This initial FOLFOX regimen therefore consisted of 5mg/kg Oxaliplatin, 50mg/kg 5-FU and 90 mg/kg Folinic acid all by i.p. injection. It was decided to administer the Oxaliplatin 2 hours prior to 5-FU/LV as the combined volume of the drugs would be excessive for a single i.p. injection.

In order to test the viability of this regimen a further pilot study was set up this time with 5 animals per group treated with the above FOLFOX regimen on a twice weekly basis however this was associated with a 50% mortality by week 2 and as such the experiment was terminated on the grounds of animal welfare.

Following on from this experiment I elected to move to a once weekly drug administration protocol. At this time a paper was published by Keizman et al. which described an animal model of chemotherapy induced steatohepatitis whereby C57BL/6J mice had been treated on a once weekly basis with 6mg/kg Oxaliplatin.(Keizman, Maimon et al. 2010) As a result

the FOLFOX regimen used was amended to a weekly i.p. injection of 6mg/kg Oxaliplatin followed two hours later by 50mg/kg 5-FU and 90mg/kg LV for four weeks. Six mice were treated with this regimen and 6 received vehicle control. The mice in the FOLFOX arm failed to gain weight at the same rate as the control arm mice confirming the systemic toxicity of this regimen (mean total weight gain 2.94±0.41g vs. 1.18±0.35g; $p<0.05$; Fig 8A). This regimen was not associated with the development of hepatic toxicity, as demonstrated by review of H&E stained sections of the liver (Fig 8B).

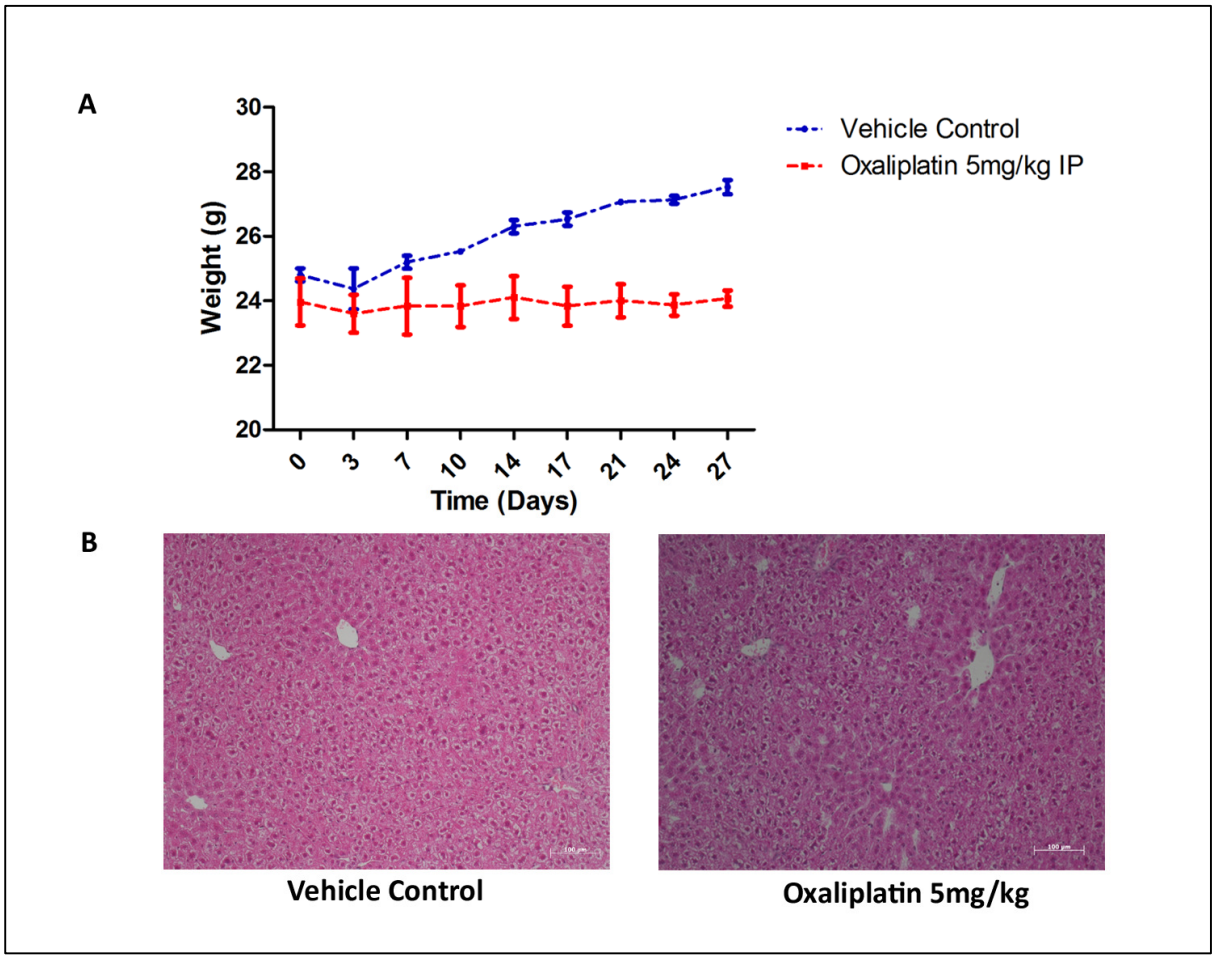

Figure 7 - Chronic Oxaliplatin administration is associated with a failure to gain weight (A) indicating systemic toxicity but there is no evidence of liver injury (i.e. Rubbia-Brandt grade 0) on H&E stained Sections (B; 10x magnification)

In view of the lack of histological changes I wished to determine if these drugs were actually reaching the liver and having any effect at a molecular level. Both 5-FU and Oxaliplatin exert their effects through DNA damage the presence of which is associated with a typical cellular response directed at either repairing the damage, or if the damage is excessive, causing programmed cell death. To facilitate DNA damage repair the chromatin structure at the site of the damaged DNA is modified to allow localisation of repair proteins. A key event in this chromatin modification is phosphorylation of the histone variant H2AX (known as γH2AX) which when present is often considered to be pathognomic of DNA damage. (Polo and Jackson 2011) A key regulator of the DNA damage response is the tumour suppressor p53 which when activated by phosphorylation is able to regulate the transcription of a variety of genes involved in regulation of the cell cycle and apoptosis.(Yoshida and Miki 2010) One such target gene is the negative regulator of the cell cycle $p21^{CIP1/WAF1}$ which plays a role in cell cycle arrest and inhibition of DNA synthesis following DNA damage.(Cazzalini, Scovassi et al. 2010)

In order to determine if there was any evidence of DNA damage in the liver of FOLFOX treated animals whole liver protein extracts were made and Western Blot performed with antibodies directed against γH2AX, the phosphorylated form of p53 and total $p21^{CIP1/WAF1}$ protein. I was able to detect increased expression of both γH2AX and $p21^{CIP1/WAF1}$, but not phosphorylated p53, in the liver of FOLFOX treated animals as compared to vehicle controls (Figure 8C). This confirmed that the chemotherapy was reaching the liver and causing DNA damage but that this alone was not sufficient, in this context, to result in histological changes associated with SOS.

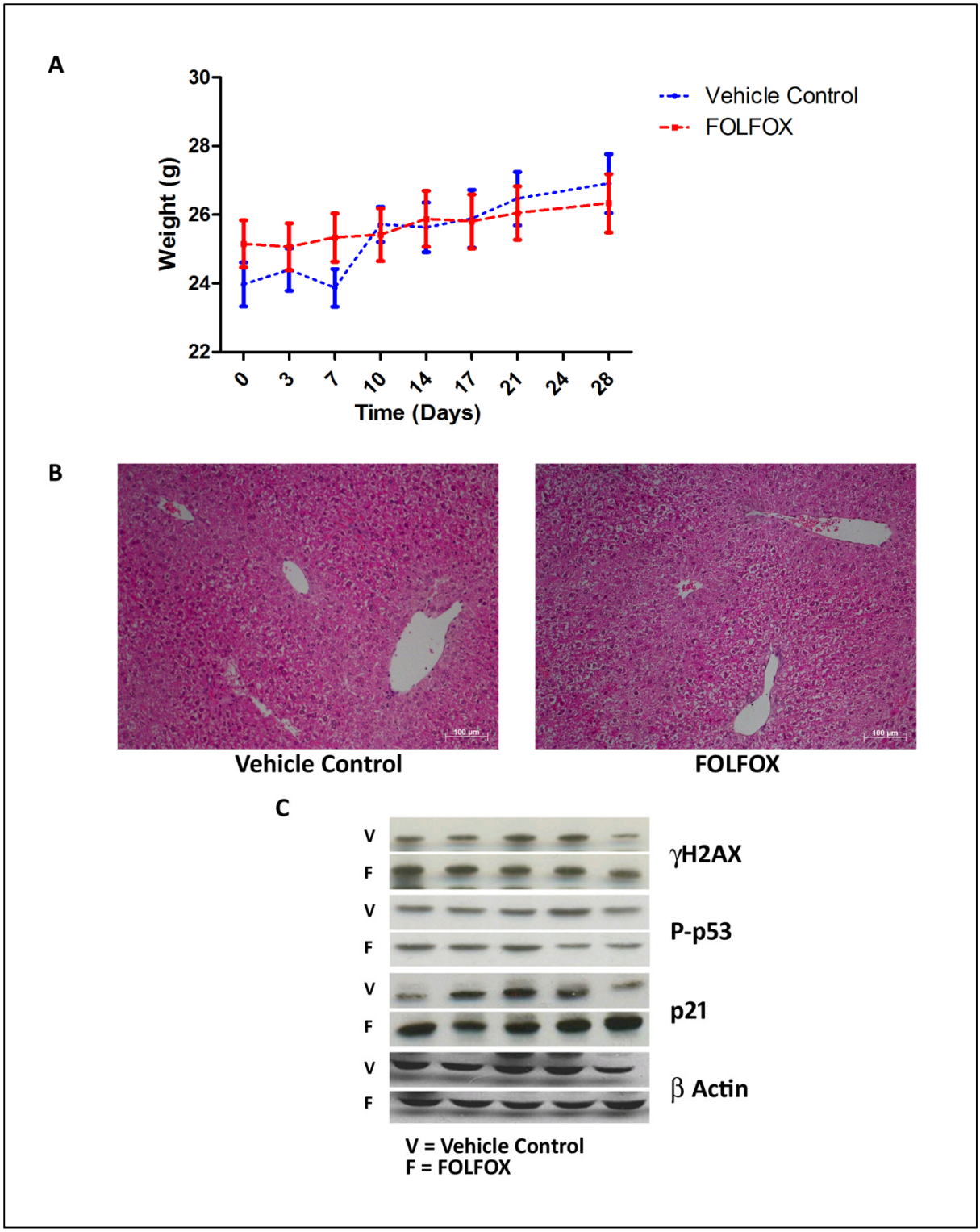

Figure 8 – FOLFOX administration for 4 weeks is associated with a failure to gain weight (A) as compared to vehicle controls. Despite the lack of histological evidence of liver injury (B; Rubbia-Brandt grade 0 all groups) there is evidence of FOLFOX induced DNA damage in whole liver extracts of treated animals (C) with up regulation of γH2AX and p21 but not phos-p53.

Keizman et al. claim that mice treated with Oxaliplatin 6mg/kg weekly for four weeks develop steatohepatitis. Interestingly the authors stated that they could not detect any changes on H&E stained sections (neither fat or inflammatory infiltrates) but rather based this diagnosis on elevated liver total fat content as measured in a biochemical assay which was subsequently demonstrated, on HPLC, to consist predominantly of triglycerides. The authors stated that this change was also reflected in Oil Red O stained sections of the liver but did not formally analyse this as frozen tissue sections were not available for the majority of mice.(Keizman, Maimon et al. 2010) To determine if these findings could be replicated in this model the hepatic triglyceride content, as measured from whole liver extract, was compared between animals treated with FOLFOX or its vehicle control however no difference was detectable (11.96 ± 1.42nM vs. 11.78 ± 1.49nM; p = 0.699; Fig 9A).

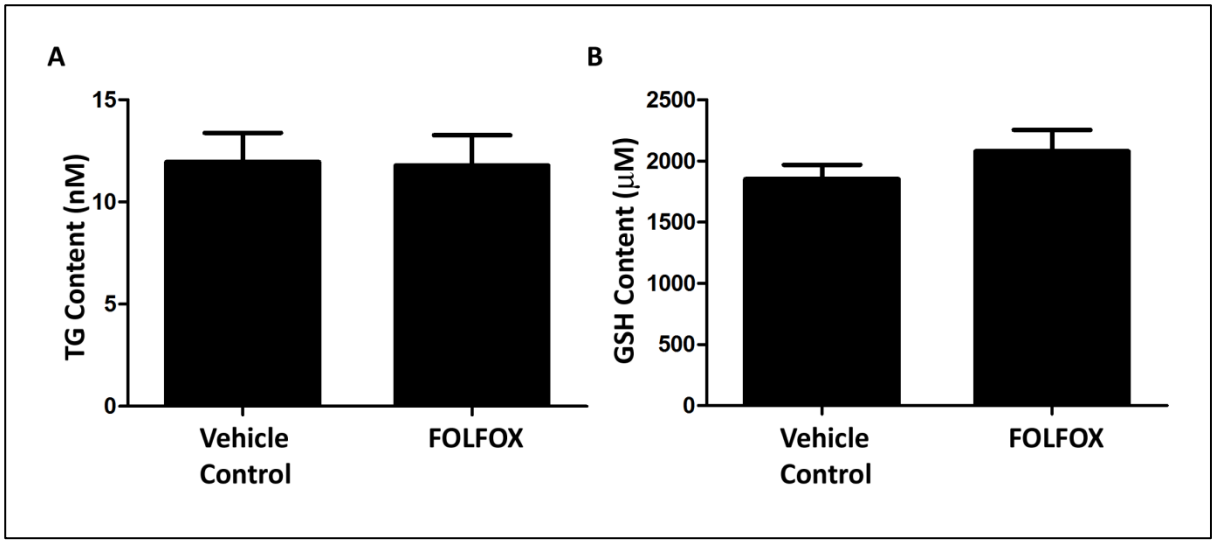

Figure 9 - FOLFOX administration is not associated with changes in either hepatic triglyceride content (A) or glutathione concentration (B)

As mentioned previously Oxaliplatin exposure might be expected to deplete intracellular glutathione (GSH) concentrations.(Zhang, Mack et al. 1998) Indeed this has also been proposed as a mechanism through which Monocrotaline administration results in the development of SOS.(Wang, Kanel et al. 2000) In order to assess if this was occurring in mice treated with FOLFOX the GSH concentration was measured in whole liver extracts however no difference was detected between treatment groups (1851 ± 116.8μM vs. 2079 ± 173.5 μM; p = 0.484; Fig 9B). This does not completely exclude specific depletion of sinusoidal endothelial cell glutathione which may be masked in this assay by the high concentration of glutathione present within hepatocytes. It should be observed that all animals in this study were not culled in a fasted state.

Following systemic administration Oxaliplatin will clearly not only be taken up by the liver but by a wide variety of organs. Boughattas et al. measured the platinum concentration in a variety of tissues 24 hours after the intravenous injection of 17mg/kg Oxaliplatin and demonstrated that the drug was predominantly taken up in the spleen.(Boughattas, Levi et al. 1989) On this basis H&E stained sections of the spleen were examined. There was a striking reduction in the size of the germinal centres within the spleen noted in FOLFOX treated animals with the red pulp making up proportionately more of the tissue mass (Fig 10).

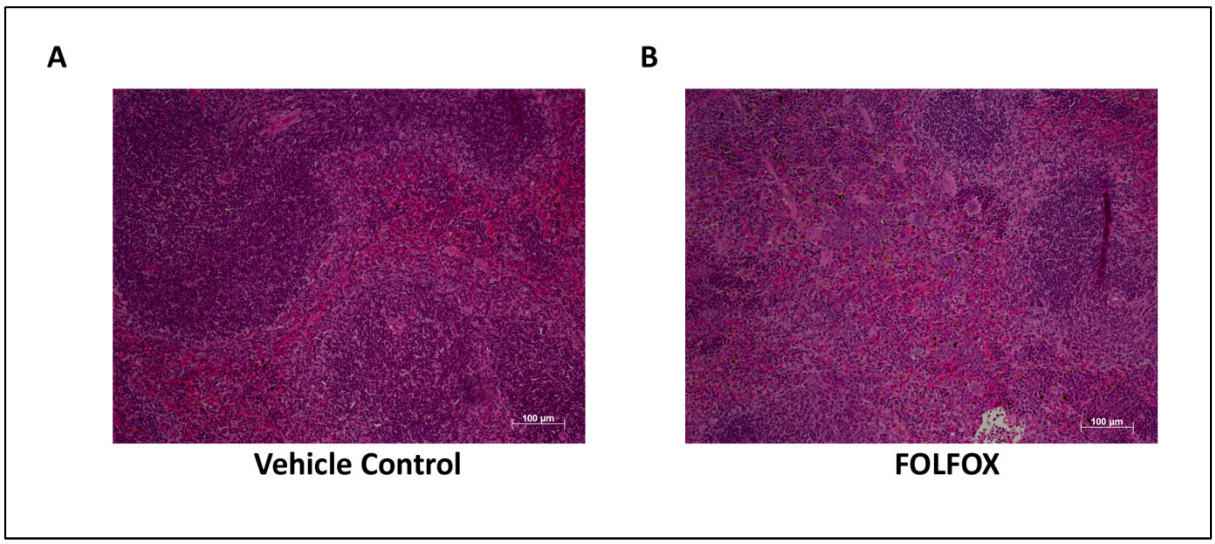

Figure 10 - FOLFOX administration to C57BL/6J mice for 4 weeks results in injury to the spleen

5.2 Is tumour death a silent event?

The lack of liver injury in mice treated with FOLFOX for 4 weeks led to the question of whether other factors, rather than direct drug toxicity alone, might be involved in the development of SOS. In a similar experiment to that described in the first part of this chapter Rickenbacher et al. also failed to demonstrate any evidence of liver injury following administration of high dose chemotherapy to C57BL/6J mice. In the conclusion to their paper they proposed the hypothesis that a "second hit" was required for the development of liver injury although they did not allude to what this hit might consist of.(Rickenbacher, DeOliveira et al. 2011)

The obvious difference between the model used thus far and the human situation is the absence of colorectal liver metastases in the mice. Chemotherapeutics, including Oxaliplatin and 5-FU, exert their effects on tumours by causing sufficient DNA damage to result in apoptotic cell death.(Zitvogel, Casares et al. 2004) Classically the response to apoptotic cells is thought to be immunologically silent which, in part, is mediated by the local release of anti-inflammatory mediators such as TGF-β and prostaglandins.(Chen, Frank et al. 2001; Savill, Dransfield et al. 2002) In addition it has been demonstrated that *in vitro* exposure of phagocytic cells to apoptotic bodies results in down-regulation of the pro-inflammatory cytokines TNF-α, IL-1β and IL-12 and up-regulation of the anti-inflammatory cytokine IL-10.(Voll, Herrmann et al. 1997)

In contrast to this Tesniere et al. reported that Oxaliplatin induced apoptosis of colorectal cancer cells is not immunologically silent and an immune response is indeed required for effective tumour killing. In order to demonstrate this they established a subcutaneous tumour in BALB/C mice using the syngeneic CT26 colorectal cancer cell line. Treatment of

tumours in these mice with Oxaliplatin resulted in significant shrinkage but when this experiment was repeated in immunodeficient nu/nu mice no such effect was seen.(Tesniere, Schlemmer et al. 2009) The authors proposed two mechanisms that might be responsible for mediating this immunogenic cell death :

1) Treatment of colorectal cancer cell lines with Oxaliplatin leads to expression of Calreticulin on the cell surface which then targets the cell for phagocytosis and enables it to interact with Dendritic cells.(Zeng, Aldridge et al. 2006; Obeid, Tesniere et al. 2007) When the exposure of calreticulin is inhibited in CT26 cells then Oxaliplatin loses its *in vivo* efficacy(Tesniere, Schlemmer et al. 2009)
2) Oxaliplatin treatment of CT26 cells results in the release of HMGB1 which is a ligand for the Toll Like Receptor 4 (TLR4) and is capable of initiating an innate immune response.(Park, Gamboni-Robertson et al. 2006) TLR4 knockout mice exposed to apoptotic CT26 cells fail to initiate an immune response unlike mice with intact TLR4 signalling.(Tesniere, Schlemmer et al. 2009)

This evidence led me to question whether tumour cell death in response to FOLFOX may result in the local release of factors which contribute to the development of SOS.

In order to test this hypothesis I elected to establish an *in vivo* model of colorectal liver metastases. Prior to doing this I wished to determine what effect FOLFOX treatment of colorectal cancer cells had on the production of chemokines and other factors implicated in wound healing and inflammation. For this I utilised the murine *MCA38* colorectal cancer cell line which was established from a chemically induced colorectal cancer in C57BL/6J mice thereby making it syngeneic and suitable for use in subsequent *in vivo* experiments without the potential for the development of an allogenic immune response.

Cells were treated *in vitro* with IC_{50} doses of Oxaliplatin and 5-FU, as determined by MTT assay, in the presence of 10μM Folinic Acid. The IC_{50} dose of these agents was based upon treatment for 48 hours. In order to determine the effect of this chemotherapy regimen on the production of chemokines I wished to look at live cells, rather than cells that had already undergone apoptosis, and therefore subsequent experiments were performed on cells treated with FOLFOX for 24 hours.

One of the most striking findings in these experiments was a more than 30 fold increase in the expression of CXCL1 mRNA in FOLFOX treated cells ($p<0.001$; Fig 11A). An ELISA performed on cell culture supernatants confirmed this finding with a 3 fold increase in the secretion of soluble CXCL1 protein ($p<0.001$; Fig 11B). CXCL1 is the murine homologue of the human chemokine CXCL8 (IL-8). Both of these chemokines are highly active on the vascular endothelium and play key roles in vascular angiogenesis and expression of matrix remodelling enzymes such as MMP-2.(Strieter, Burdick et al. 2005) Increased levels of circulating IL-8 have been detected in the serum of bone marrow transplant patients who have gone on to develop SOS.(Remberger and Ringden 1997; Schots, Kaufman et al. 2003) IL-8 has also been demonstrated to play a pivotal role in hepatic vascular injury following insults such as ischaemia reperfusion.(Petreaca, Yao et al. 2007)

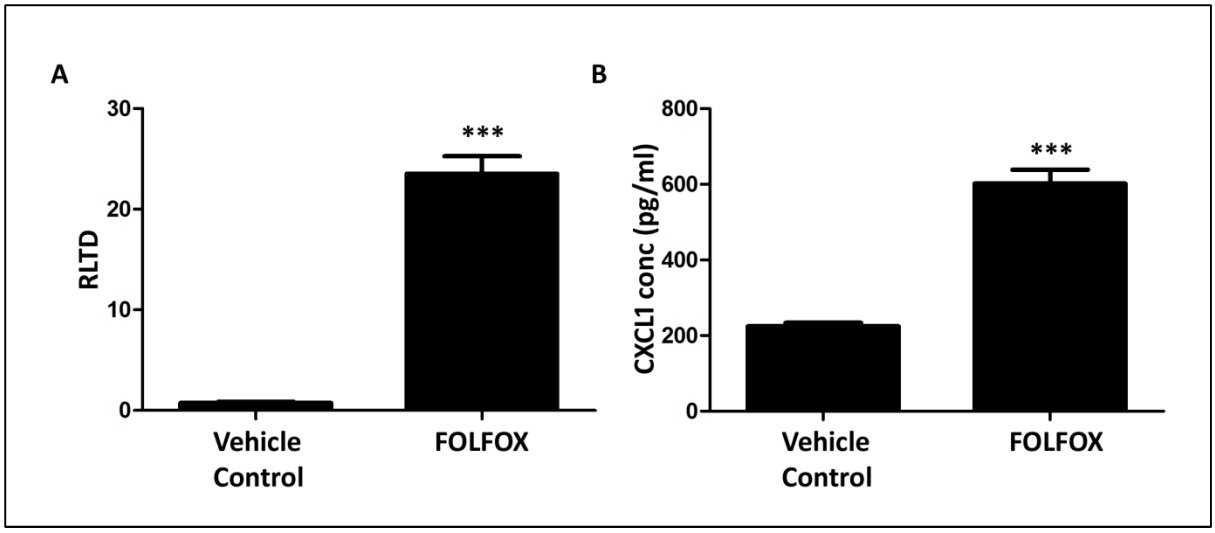

Figure 11 - Treatment of MCA38 with FOLFOX *in vitro* results in increased CXCL1 mRNA expression (A) which is reflected in the levels of secreted CXCL1 (B)

In addition to CXCL1 I was also able to demonstrate up-regulation of other key inflammatory chemokines implicated in the wound healing response including CXCL5, CCL2 and CCL5 (Figure 12A). Martins et al. recently reported that colorectal cancer cell lines treated with a variety of chemotherapy agents, including Oxaliplatin, release extracellular ATP and that this contributes to mediating the immune response directed against dying tumour cells.(Martins, Tesniere et al. 2009) Sinusoidal endothelial cells express high levels of the ectonucleotidase CD39 which is responsible for converting extracellular ATP to adenosine by hydrolysis.(Beldi, Wu et al. 2008) When this system is exposed to high levels of ATP however it becomes overwhelmed and vascular injury can result.(Beldi, Enjyoji et al. 2008) I was able to demonstrate that MCA38 cells also release extracellular ATP after exposure to FOLFOX with a 4 fold increase being detected in the culture supernatant ($p<0.001$; Fig 12B).

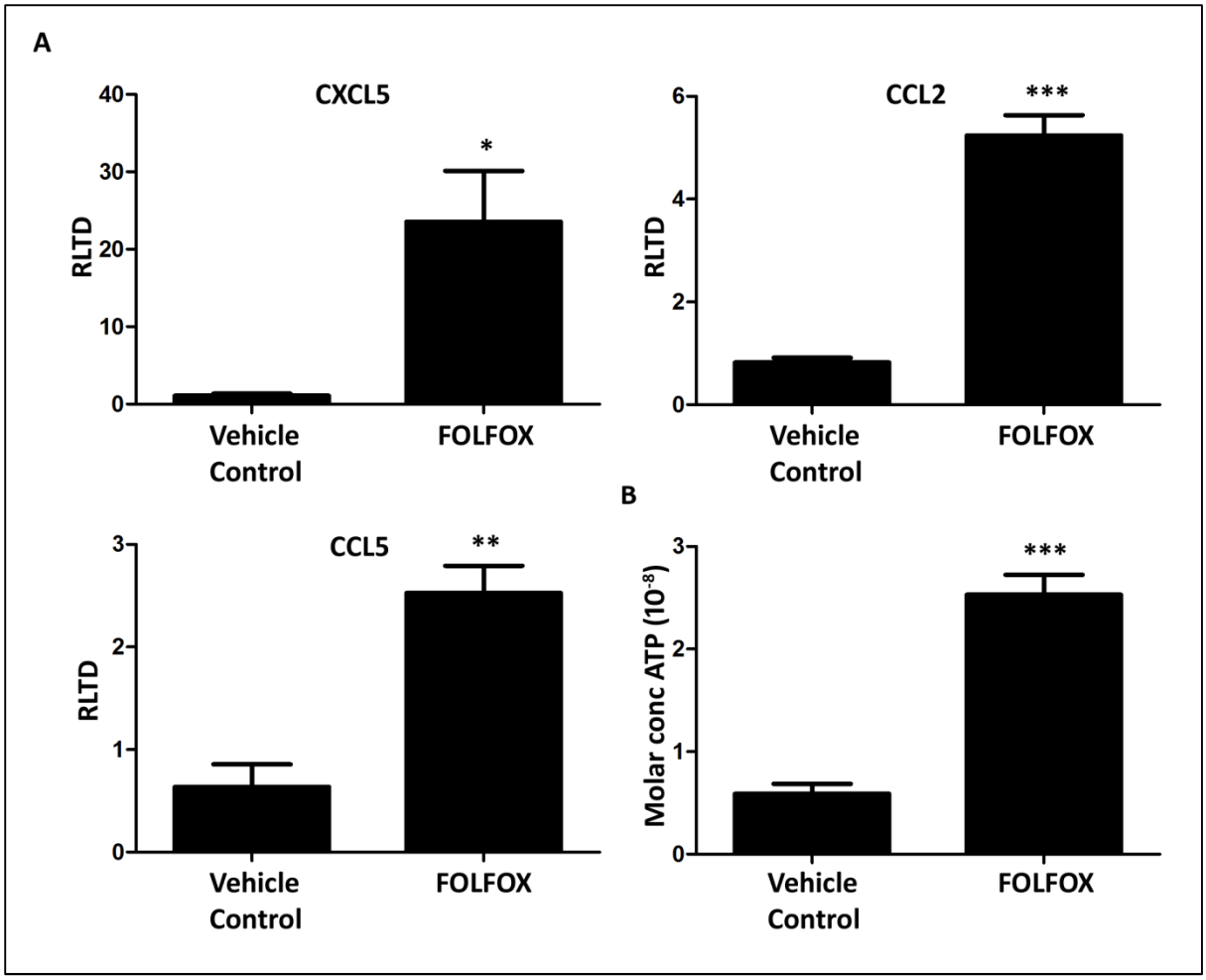

Figure 12 - In response to FOLFOX treatment MCA38 cells up-regulate transcription of CXCL5, CCL2 & CCL5 (A). In addition there is release of extracellular ATP (B)

On the basis of these preliminary *in vitro* experiments I felt confident that FOLFOX treated MCA 38 cells were producing chemokines and other modulators of the extracellular environment and, as such, it was worthwhile to proceed with an *in vivo* model to explore the hypothesis that these tumour related factors may contribute to the development of SOS.

5.3 Does tumour death contribute to the development of SOS?

In order to determine what affect tumour related factors might have on the development of SOS, I developed a model of experimental colorectal liver metastases in C57BL/6J mice. A commonly utilised method for developing experimental metastases is through intraportal or intrasplenic injection of tumour cells however the disadvantage of this technique is that it may result in the rapid development of disseminated metastatic disease. An alternative technique is to implant tumour cells directly into the liver, either through subcapsular injection of a suspension of tumour cells or by implantation of a fragment of solid tumour tissue from another mouse. The advantage of this technique is that it allows for the development of localised metastatic disease but has the disadvantage that it misses out many of the early processes involved in metastasis development such as cell arrest, extravasation and liver colonisation.(Heijstek, Kranenburg et al. 2005)

I elected to use the technique of subcapsular hepatic injection of MCA38 cells to establish experimental metastases, for the following reasons:

1) The intention of this model was not to study the process of metastatic tumour formation but rather the effect of the tumour on the hepatic micro-environment once it was established
2) If the model were to be successful I would consider performing experiments to look at the effect of SOS on liver regeneration, using models such as 70% partial hepatectomy, and therefore a localised tumour would better enable this

In order to be able to monitor the development of tumours MCA38 cells were stably transfected with the pGL4.51 luciferase reporting vector. Prior to *in vivo* experiments the expression of luciferase in these cells was confirmed *in vitro* using a luminometer (Fig 13A).

Initial experiments were performed to determine the optimum number of cells for implantation and the time taken for solid tumour development. Mice were injected with either 10^6, 10^5 or 10^4 cells and tumour development measured at 1 week both by *in vivo* imaging and post-mortem examination. Those animals injected with 10^6 cells all had large tumours present which in the majority of cases had begun to metastasise to the lungs. In contrast those mice injected with 10^4 cells had either failed to develop tumours or they were less than 5mm in diameter. As a result 10^5 cells was selected as the optimum number for injection.

The next pilot study sought to determine the time taken for tumour development in mice injected with 10^5 cells. Following subcapsular injection of cells *in vivo* imaging was performed on a daily basis. Five days following implantation tumour was detectable in 5 out of 6 mice (83%). One week later the mouse with no tumour detectable on day 5 had no measurable tumour either by *in vivo* imaging or on post-mortem examination. It was therefore decided that 5 days following tumour implantation would be the time point for determining if tumour was present and at that point to commence experiments.

To examine the effect of tumour related factors on the development of chemotherapy induced SOS 10^5 MCA 38 cells were injected beneath the liver capsule of 12 C57BL/6J mice via midline laparotomy. A further 12 mice underwent laparotomy alone to form the sham operated control groups. On post-operative day 5 the presence of solid tumour within the liver was confirmed by *in vivo* imaging (Fig 13B). At this point mice were subdivided into four groups as follows:

- Sham Control & Vehicle (n=6)
- Sham Control & FOLFOX (n=6)

- Tumour Implantation & Vehicle (n=6)
- Tumour Implantation & FOLFOX (n=6)

FOLFOX chemotherapy was commenced on day 5 using the previously described protocol. The initial intention was to repeat this for four weeks however due to rapid tumour growth in the tumour bearing animals not receiving chemotherapy it was necessary to terminate the experiment early because of animal welfare. As a consequence mice were culled and organs harvested 24 hours after the 3rd dose of FOLFOX.

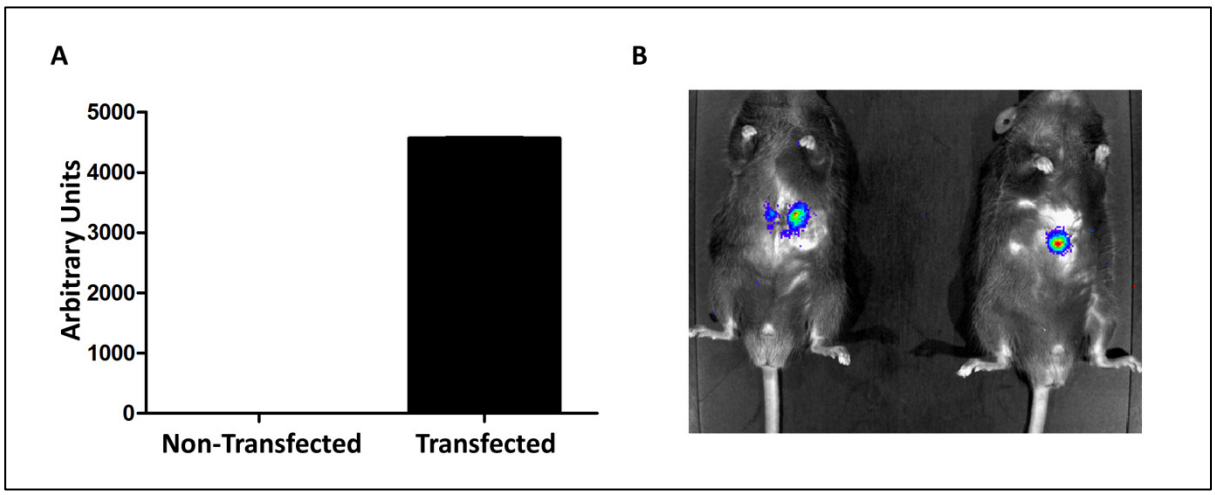

Figure 13 - Luciferase expression in stably transfected MCA38 cells was confirmed in vitro (A). In vivo imaging confirming the presence of tumour 5 days following implantation of 105 MCA38 cells (B)

Both sham operated and tumour bearing animals treated with FOLFOX lost weight as compared to vehicle controls confirming systemic drug toxicity in a similar manner to previous experiments. It can be seen that the mean weight gain over the course of the experiment was much greater in the tumour bearing vehicle controls as compared to the sham operated vehicle controls which is a reflection of the rapid tumour growth in this

group, although this difference did not reach statistical significance (3.78 ± 2.80g vs. 10.33 ± 2.51g; p = 0.132; Fig 14A). In contrast the tumour growth in the FOLFOX treated animals was clearly much less (Fig 14B) and this was reflected by a lower tumour to body weight ratio (0.147 ± 0.042 vs. 0.045 ± 0.010; $p < 0.05$) and tumour volume (5.90 ± 1.85cm^3 vs. 1.86 ± 0.53cm^3; p = 0.062) in this group (Fig 14C). Review of H&E stained sections of the liver did not reveal histological evidence of SOS.(Fig 14D).

Despite the lack of convincing histological changes of SOS on H&E stained sections of the liver I felt it would be appropriate to go ahead and look for surrogate molecular markers in the liver which might be expected during the development of an SOS phenotype. This was done using a qRT-PCR based screen in the first instance as it allows for multiple biological pathways to be interrogated in a time efficient manner. The choice of target genes was guided by a micro-array study performed by Rubbia-Brandt et al. on a small number of patients with Sinusoidal Obstruction Syndrome. Whilst this study was underpowered, and therefore difficult to interpret, it is the only paper which has directly attempted to discover the pathophysiological processes which underpin SOS induced by Oxaliplatin containing chemotherapy.(Rubbia-Brandt, Tauzin et al. 2011)

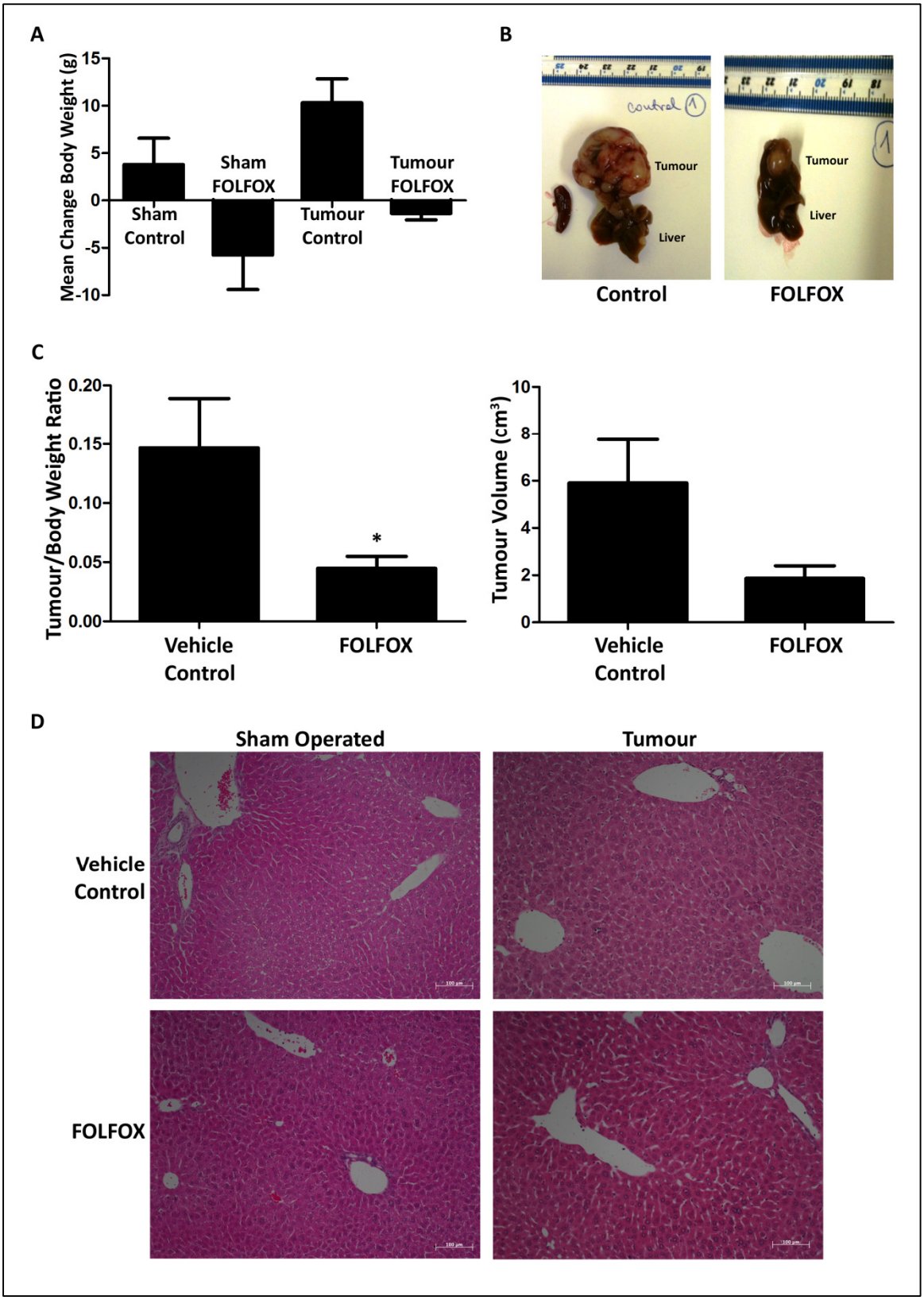

Figure 14 - FOLFOX treatment is associated with weight loss in both sham operated and tumour bearing groups (A). FOLFOX is effective in slowing the rate of tumour growth (B and C). H&E stained sections of the liver fail to demonstrate changes of SOS (D; 10x magnification; Rubbia-Brandt grade 0 all groups)

Remodelling of the extracellular matrix is a key event in the development of SOS following Monocrotaline administration and in keeping with this Rubbia-Brandt et al. demonstrated up-regulation of a variety of genes implicated in this process in patients with Oxaliplatin induced SOS.(Deleve, Wang et al. 2003; Rubbia-Brandt, Tauzin et al. 2011) Following administration of Monocrotaline the extracellular matrix is disrupted through the actions of the gelatinases MMP-2 and MMP-9 thereby allowing extravasation of blood cells into the space of Disse. As already discussed inhibition of MMP's, by the antibiotic doxycycline, prevents SOS developing in this model.(Deleve, Wang et al. 2003) In keeping with these findings I was able to demonstrate that, in the presence of experimental liver metastases, there was a 13 fold up-regulation of MMP2 gene expression ($p<0.01$). This compared to a 5 fold increase in sham operated animals treated with FOLFOX ($p<0.01$). Hepatic MMP2 expression was also up-regulated 5 fold simply by the presence of tumour within the liver ($p<0.01$) suggesting that the large increase in expression in the tumour bearing animals is a result of both drug effect and tumour related factors (Fig 15A). FOLFOX treatment also resulted in a marked up-regulation of MMP9 expression. Whilst this was most marked in sham operated animals the difference between tumour bearing animal groups was not statistically significant ($p=0.126$; Fig 15B).

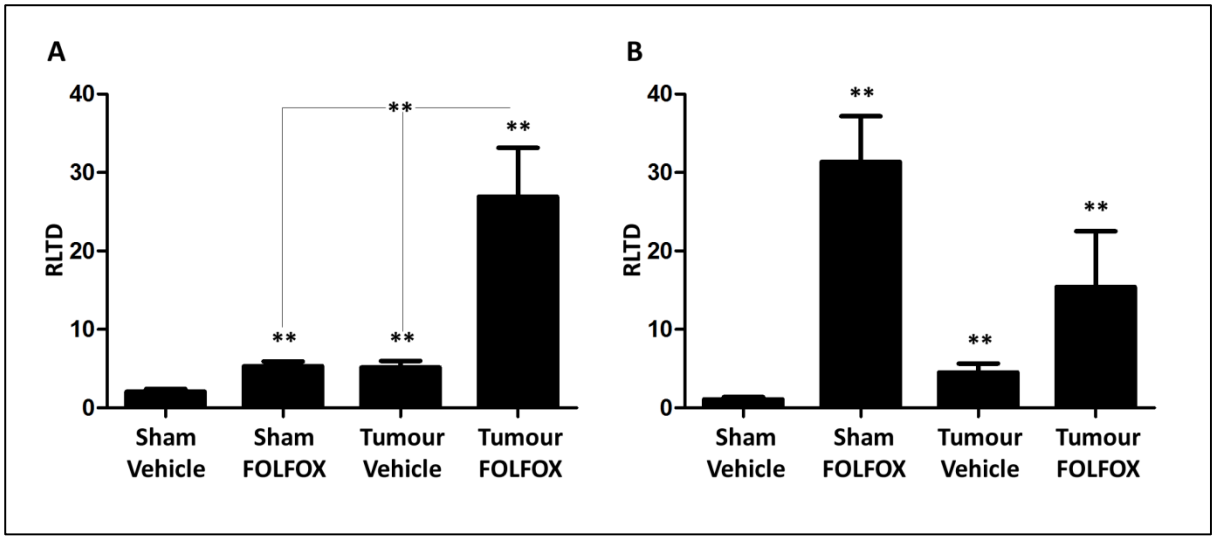

Figure 15 - Both MMP2 (A) and MMP 9 (B) are up-regulated both as an effect of FOLFOX administration and an effect of tumour related factors

The effects of the gelatinases are counteracted by their inhibitors – the tissue inhibitors of metalloproteinases (TIMP) and it is the balance between MMP and TIMP expression that modulate the process of tissue remodelling. The livers of FOLFOX treated tumour bearing animals demonstrated a 67 fold increase in TIMP1 ($p<0.01$) expression which was not seen in sham operated animals treated with the drug combination. Again the presence of tumour alone was associated with a 10 fold increase in the expression of TIMP1 ($p<0.01$) suggesting that tumour related factors may be playing a key role in its regulation (Figure 16A).

One of the key sources of TIMP1 in the liver is the hepatic stellate cell.(Olivie, Lepanto et al. 2007) Hepatic stellate cells are normally present in the hepatic sinusoid in a quiescent state but when activated they take on the phenotype of a myofibroblast and play a pivotal role in matrix remodelling and in particular fibrogenesis.(Friedman 2008) Activation of hepatic stellate cells has been reported in patients with SOS(Rubbia-Brandt, Audard et al. 2004)and

in keeping with this I was able to detect a 9.5 fold increase in αSMA transcript levels in FOLFOX treated tumour bearing animals ($p<0.05$; Fig 16B) which was supported by increased numbers of αSMA positive cells being detected on immunohistochemistry (Fig 16C). One of the key roles of activated stellate cells is to lay down collagen and in support of this there was a 16 fold increase in liver Procollagen I transcript ($p<0.01$; Fig 16D). Transforming growth factor β is regarded as the master regulator of liver fibrosis and indeed it appears that this is playing a role in the process of matrix remodelling in response to FOLFOX with a 4 fold increase in transcript level being detected in tumour bearing animals (Fig 16E).

These findings support the concept that tumour related factors may contribute to the up-regulation of genes involved in the pathogenesis of SOS, in particular those concerned with matrix remodelling and hepatic fibrogenesis. Clearly the balance between the expression of MMP's and TIMP will determine the effect of these enzymes on the liver parenchyma. If the balance was in favour of increased MMP activity then this would favour destruction of the extracellular matrix as has been implicated in the sinusoidal injury seen in SOS. (Deleve, Wang et al. 2003). In contrast if the increased TIMP expression in FOLFOX treated animals resulted in MMP inhibition then this would tend to favour the deposition of extracellular matrix such as occurs in liver fibrosis. (Friedman 2008)

With the benefit of hindsight it would have been desirable to include assays to determine the relative activity of MMPs (e.g. gelatin zymography) in FOLFOX treated tumour bearing animals. Should it have been possible to continue this model for a longer period of time it would seem reasonable to expect there would have been evidence of parenchymal injury although it is not possible to predict, with the current data, the likely nature of this.

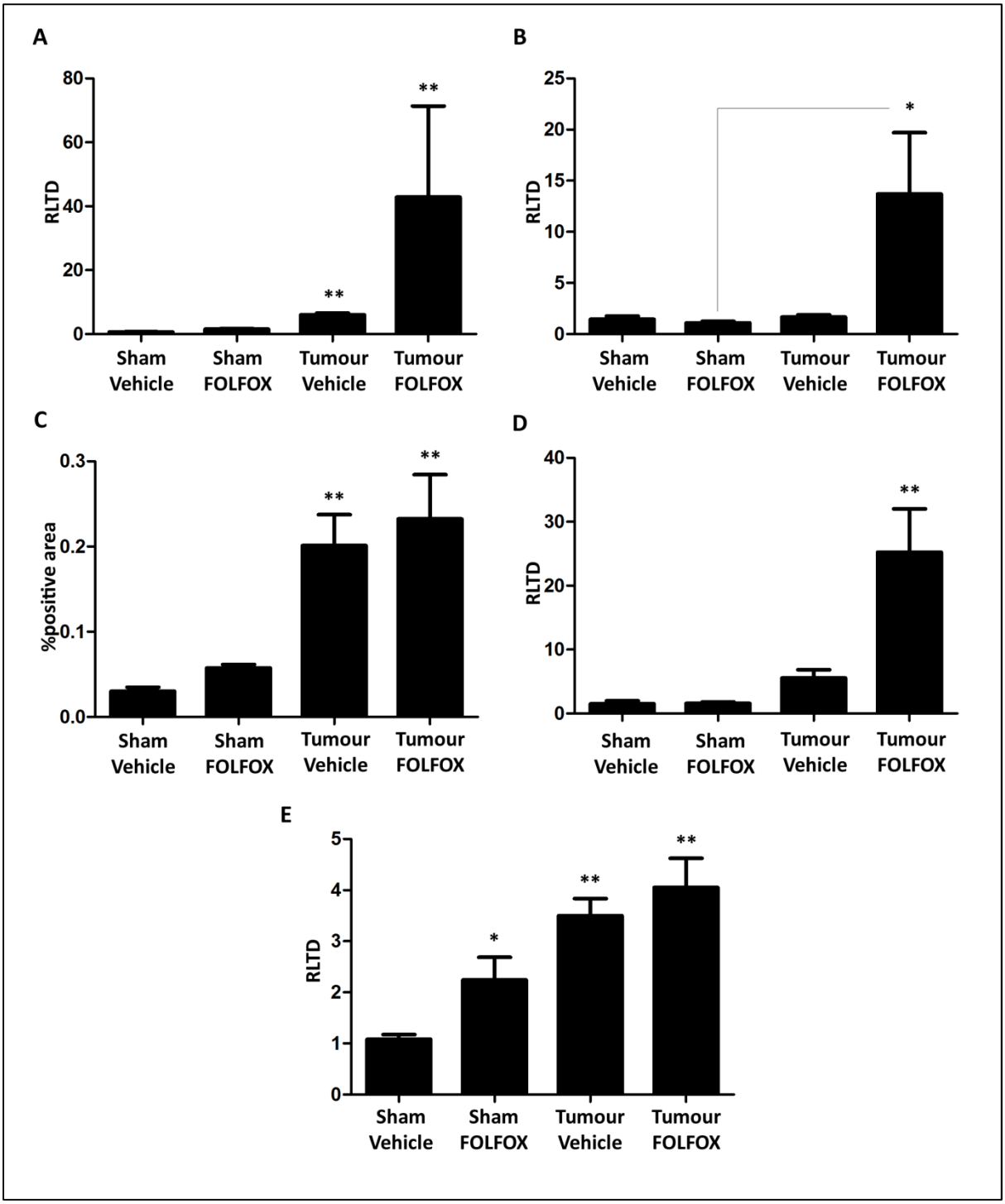

Figure 16 Tumour bearing mice treated with FOLFOX demonstrate up-regulation of the pro-fibrogenic TIMP1 transcript (A). In keeping with this there is increased expression of αSMA at a transcript level (B) and on immunohistochemistry (C). Increased levels of procollagen I transcript support a pro-fibrotic environment (D) and it is likely that this is driven by TGFβ (E).

5.4 Is the Murine Liver Resistant to FOLFOX

The lack of histological changes in the liver following administration of Oxaliplatin, either alone or in combination with 5-FU, leads one to ask if C57BL/6J mice are resistant to the effects of these drugs. Before making further attempts to establish an animal model I elected to answer this question by looking at expression, at an RNA level, of key members of the pathways involved in the handling and metabolism of Oxaliplatin and 5-FU.

5.4.1 Oxaliplatin Metabolism

At a cellular level the uptake and excretion of platinum based drugs is regulated by copper transport proteins. Initial observations linking cellular platinum handling to these proteins were made by Katano et al. who demonstrated that ovarian cancer cell lines selected as being resistant to Cisplatin were cross resistant to high concentrations of copper sulphate.(Katano, Kondo et al. 2002) Subsequently it was shown that the kinetics of influx and efflux of platinum compounds mirrors that of copper.(Safaei, Katano et al. 2004)

Copper transporter 1 (CTR1) is a plasma membrane bound transport protein that regulates the influx of copper into the cell. It has been demonstrated that cell lines which do not express CTR1 are resistant to platinum based chemotherapeutics, even at high doses.(Holzer, Manorek et al. 2006; Larson, Blair et al. 2009) High intracellular platinum (and copper) concentrations lead to the degradation of CTR1 thereby limiting the cytotoxicity of these compounds.(Holzer and Howell 2006) High expression levels of CTR1 have been shown to mediate the sensitivity of dorsal root ganglia, in rats, to the toxic effects of Oxaliplatin whereas ganglia expressing low levels of CTR1 are relatively resistant.(Liu, Jamieson et al. 2009)

Copper transporter 2 (CTR2) is much less well characterised in terms of platinum handling. One study examined the expression of CTR1 and CTR2 in patients with ovarian cancer treated with platinum based chemotherapy. When CTR2 was the predominant copper transporter expressed in the cancer tissue, with low levels of expression of CTR1, then response to chemotherapy was poor.(Lee, Choi et al. 2011) This finding supports the results of *in vitro* studies using ovarian cancer cell lines whereby knockdown of CTR2 resulted in increased sensitivity to platinum based chemotherapy.(Blair, Larson et al. 2009; Blair, Larson et al. 2010)

The key copper efflux transporters are the ATPases ATP7A and ATP7B. It has been demonstrated that both of these transporters play roles in regulating intracellular platinum concentrations. Increased expression of both ATP7A and ATP7B is a key feature of platinum resistant cancer cell lines preventing intracellular accumulation of platinum.(Komatsu, Sumizawa et al. 2000; Katano, Kondo et al. 2002; Samimi, Safaei et al. 2004) The validity of these *in vitro* observations has been confirmed in patients with colorectal cancer treated with Oxaliplatin based chemotherapy in whom high levels of expression of ATP7B are associated with an increased risk of disease progression (Hazard Ratio 3.56; 95% Confidence Interval 1.6 – 7.9; p = 0.002)(Martinez-Balibrea, Martinez-Cardus et al. 2009)

In mice with experimental liver metastases treated with FOLFOX I have demonstrated that the spleen, along with the implanted tumour, are key sites of activity for this drug combination whereas the liver seems to be relatively in-sensitive in so far as no histological changes are seen. In order to confirm this lack of drug effect immunohistochemistry for γH2AX, a marker of DNA damage, was performed. It can be seen that the number of γH2AX

positive cells in the liver is very small when compared to both spleen and tumour following FOLFOX treatment. This suggests that there is, in reality, very little DNA damage in this organ (Fig 17A). On the other hand FOLFOX treatment results in statistically significant increases in the number of γH2AX positive cells in both spleen and tumour tissue (Fig 17 B & C) confirming that they are particularly sensitive to this drug combination.

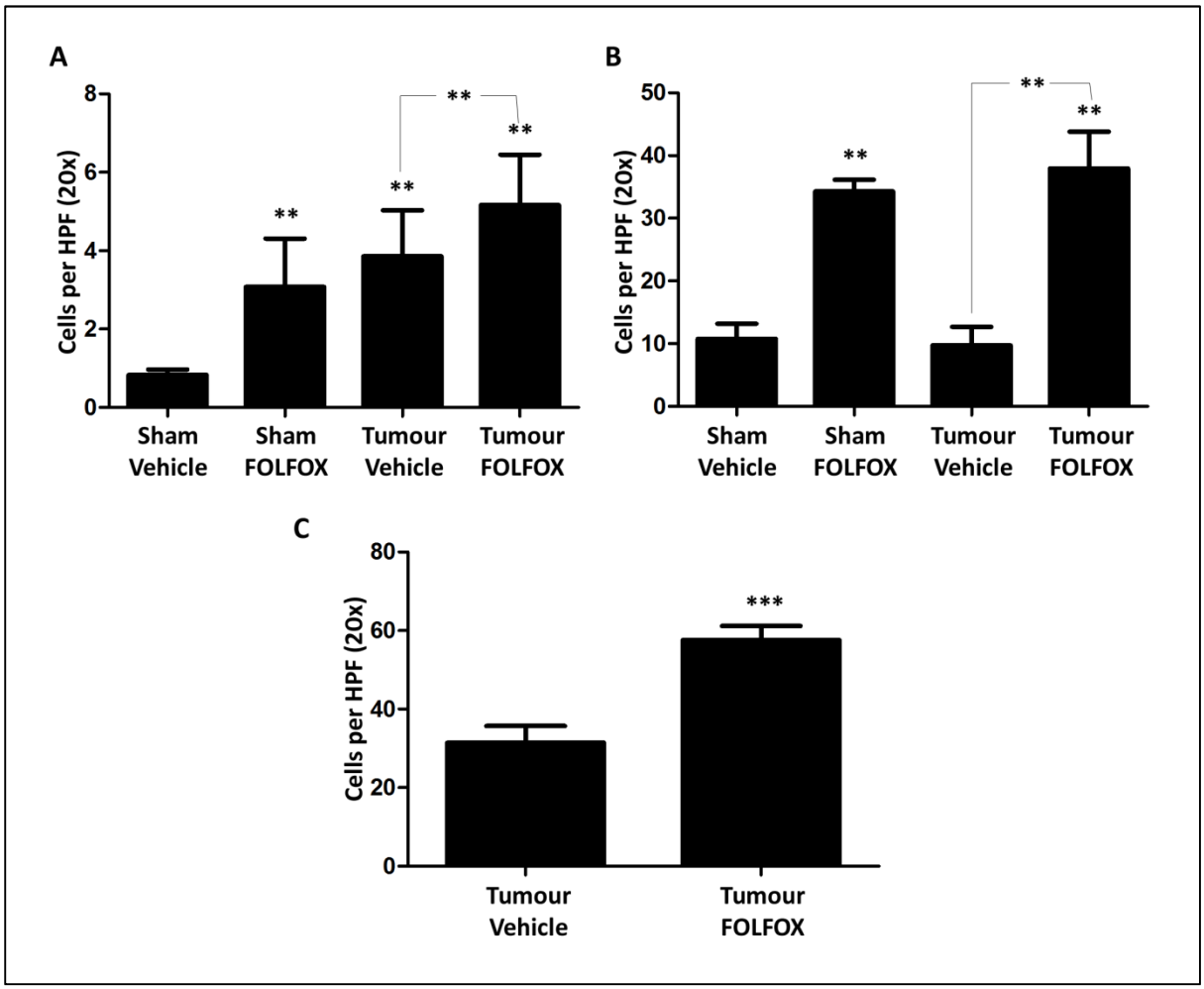

Figure 17 – Counts of γH2AX positive cells in Liver (A), Spleen (B) and Tumour (C) from mice with experimental colorectal liver metastases (or shams) treated with FOLFOX

In order to explore if copper transport proteins may play a role in mediating this resistance to FOLFOX, and in particular Oxaliplatin, RNA was isolated from the liver, spleen and tumour tissue of chemotherapy naive mice. RT-PCR performed was then performed to determine the expression of copper influx (CTR1, CTR2) and efflux (ATP7A, ATP7B) transporters. Whilst it might be argued that it would be better to look for expression of these receptors at a protein level there is a lack of good quality commercially available antibodies available for all of these receptors making this approach impractical.

The expression of the influx transporters CTR1 and CTR2 within the liver follows the pattern that might be expected in Oxaliplatin sensitive tissue i.e. high CTR1 expression and low CTR2 expression. This suggests that the liver is able to take up Oxaliplatin and as such this isn't the cause of the resistance to drug induced injury. On the other hand there is very high expression of the efflux transporter ATP7B, when compared to other tissues, suggesting that the liver is rapidly able to export Oxaliplatin and this may provide a mechanism through which the murine liver is able to limit its exposure to Oxaliplatin and thus minimise drug toxicity.

In the human population genetic polymorphisms leading to loss of function in the ATP7B gene have been linked to the development of Wilsons disease which is characterised by excessive accumulation of copper within the liver. (Huster D 2010) Polymorphisms in the ATP7B appear to be extremely common with the general population, the majority of which do not result in the development of clinically overt Wilsons disease. In one study of 203 cancer patients 61 were found to have polymorphisms in the ATP7B gene although the functional consequences of this were not explored. (Fukushima-Uesaka H, Saito Y et al. 2009). It is interesting to hypothesise that these polymorphisms may be associated with an

increases susceptibility to the toxic effects of Oxaliplatin based chemotherapy. Before exploring this further in a clinical study it would be worthwhile administering FOLFOX to ATP7B gene knockout mice to determine the impact of this gene on the development of liver injury.

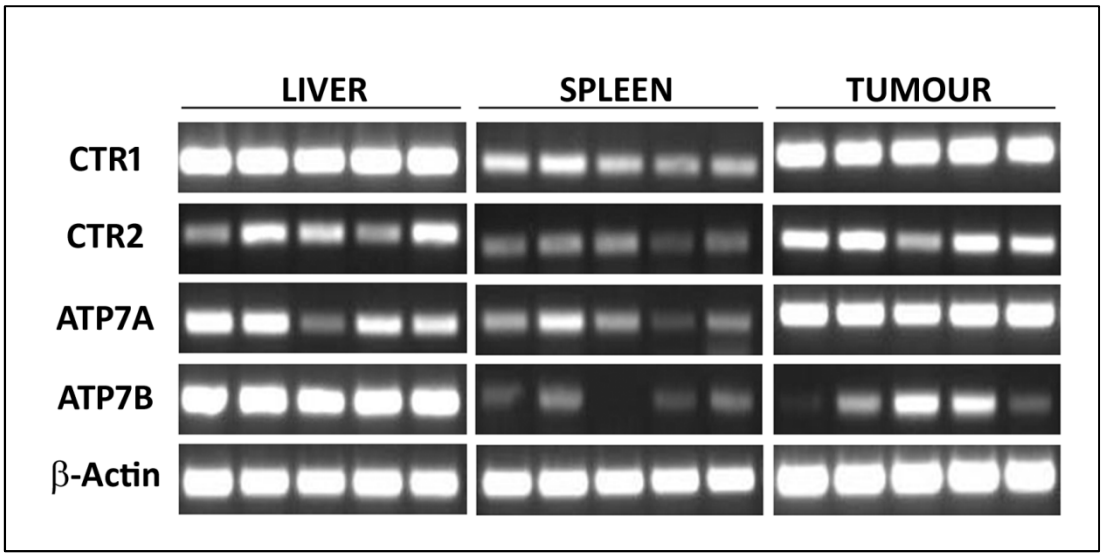

Figure 18 - Expression of copper transporters in liver spleen and tumour tissue of mice with experimental colorectal liver metastases who had not received chemotherapy treatment (n=5 per group). β-Actin served as loading control.

5.4.2 5-FU Metabolism

The intracellular processing of 5-FU has already been summarised in Chapter 1 (see Figure 2). In brief on entry into the cell 5-FU is converted into the active products fluorodeoxyuridine and fluorouridine triphosphate by the enzyme thymidine phosphorylase (TP) resulting in DNA damage through inhibition of thymidilate synthase (TS). Alternatively 5-FU can be converted into the inactive metabolite dihydrofluorouracil by the enzyme dihydropyrimidine dehydrogenase (DPD). High levels of expression of both DPD and TS are

associated with resistance to 5-FU based chemotherapy.(Ciaparrone, Quirino et al. 2006; Ishikawa, Miyauchi et al. 2008; Yamada, Iinuma et al. 2008) In addition low levels of DPD mRNA expression in the liver have been reported in patients with chemotherapy induced injury.(Pilgrim, Brettingham-Moore et al. 2011)

To assess the sensitivity of the liver to 5-FU, the expression of TS, TP and DPD was assessed at the RNA level by RT-PCR (Figure 19). It can be seen that the liver expresses the highest levels of TP suggesting that it should be able to convert 5-FU into its active form. In contrast the expression of DPD is similar to that seen in tumour tissue and TS levels are similar across all three tissues suggesting that resistance to 5-FU within the liver should not be a significant barrier to the development of liver injury in response to FOLFOX in C57BL/6J mice.

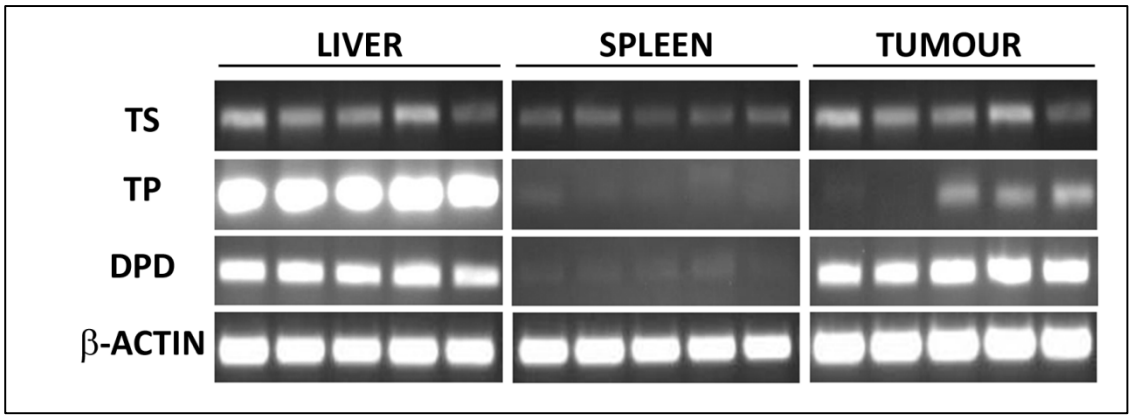

Figure 19 - Expression of enzymes involved in 5-FU activity in liver spleen and tumour tissue of mice with experimental colorectal liver metastases who had not received chemotherapy treatment (n=5 per group). β-Actin served a loading control.

5.5 Summary of Key Findings

- Administration of Oxaliplatin, either alone or in combination with 5-FU/LV, to C57BL/6J mice for 4 weeks leads to systemic drug toxicity but mice fail to develop evidence of liver injury
- Colorectal cancer cells treated with 5-FU/LV and Oxaliplatin *in vitro* produce chemokines and other modulators of the extracellular environment
- Tumour death within the liver may contribute to the up-regulation of genes involved in the development of SOS, especially those concerned with matrix remodelling and hepatic fibrogenesis
- The murine liver expresses the necessary transporters and enzymes for the uptake and intracellular processing of both Oxaliplatin and 5-FU. High levels of ATP7B mRNA might mean that Oxaliplatin is rapidly cleared from the liver making it relatively resistant to Oxaliplatin as compared to other tissues.

Chapter 6

Dietary Factors in the Development of FOLFOX Induced SOS

6.1 Background

The development of SOS following treatment with Oxaliplatin based chemotherapy is not uniform but rather, from the meta-analysis data presented earlier, seems to occur in approximately in 1 in every 9 treated patients. The animal model data presented in the previous chapter would seem to suggest that healthy C57BL/6J mice are resistant to Oxaliplatin based chemotherapy which raised the question of whether pre-existing liver injury may predispose to the development of liver toxicity.

Hepatic steatosis is the most common background abnormality in the liver and is present in up to a quarter of the UK adult population.(Anstee, McPherson et al. 2011) Whilst the presence of steatosis, per se, is not particularly troublesome it is widely acknowledged that it increases the risk of liver injury from a variety of insults.(Powell, Jonsson et al. 2005) In mice with dietary induced steatosis there is up-regulation of TLR4 expression within the liver. This increases the susceptibility of the liver to portal endotoxaemia with a resultant recruitment of leukocytes to the liver.(Shi, Kokoeva et al. 2006; Rivera, Gaskin et al. 2010) The enhanced inflammatory response in patients with steatosis that is thought to be one of the factors determining progression to steatohepatitis in patients with both NAFLD and alcohol induced liver disease.(Nagata, Suzuki et al. 2007; Rivera, Adegboyega et al. 2007; Olleros, Martin et al. 2008)

One of the proposed mechanisms for the development of SOS following treatment with Oxaliplatin is the generation of oxidative stress as a result of glutathione depletion.(DeLeve,

Wang et al. 1996; Zhang, Mack et al. 1998; Wang, Kanel et al. 2000) The presence of hepatic steatosis is associated with an increased susceptibility to oxidative stress and this has been linked to the more rapid progression of liver fibrosis in patients with Hepatitis C virus infection.(Powell, Jonsson et al. 2005; Choi and Ou 2006) This susceptibility to oxidative stress is thought to arise as a result of lipid induced mitochondrial dysfunction and this plays a key role in the progression of NAFLD.(Pessayre, Fromenty et al. 2004; Pessayre 2007) The importance of this mechanism has been confirmed in several models of hepatic steatosis whereby mice treated with a glutathione precursor develop less severe disease.(Baumgardner, Shankar et al. 2008; de Oliveira, de Lima et al. 2008; Sinha-Hikim, Sinha-Hikim et al. 2011)

6.2 Does hepatic steatosis predispose to the development of SOS?

To determine if a background liver injury could act as a predisposing factor for the development of Oxaliplatin induced SOS I elected to establish a model of hepatic steatosis again in C57BL/6J mice. A variety of genetic knock-out models of hepatic steatosis exist, such as the leptin deficient ob/ob mice, but it is not known what impact the loss of these genes may have on the response to chemotherapy and so these were not considered suitable for use. An alternative approach is to establish hepatic steatosis using dietary models. One such model that is widely utilised is the Methionine and Choline deficiency (MCD) diet that leads to the rapid development of hepatic steatosis due to an induced defect in mitochondrial β-Oxidation. This model however has several drawbacks, not least of which is that the animals lose large amounts of weight which may cause significant problems when used with systemic chemotherapy which also affects weight gain.(Koteish and Diehl 2001; Anstee and Goldin 2006)

An alternative dietary approach is to feed mice a high fat diet which results in impaired hepatic glucose tolerance and insulin resistance with the subsequent development of hepatic steatosis.(Fraulob, Ogg-Diamantino et al. 2010; Eccleston, Andringa et al. 2011; Peng, Rideout et al. 2012) As this process most closely replicates the pathogenesis of NAFLD in human subjects I elected to adopt this approach to establish hepatic steatosis in mice before treatment with FOLFOX. To this end 4 week old male C57BL/6J were commenced on either high fat (45%) diet or a control low fat (10%) diet for 10 weeks. Review of H&E stained liver sections from high fat diet fed animals culled at this time point demonstrated pronounced lipid droplets within hepatocytes in keeping with the development of steatosis

confirming that this was an effective model (Figure 20). The manufacturers' analysis of the content of these diets is listed in Appendix 2.

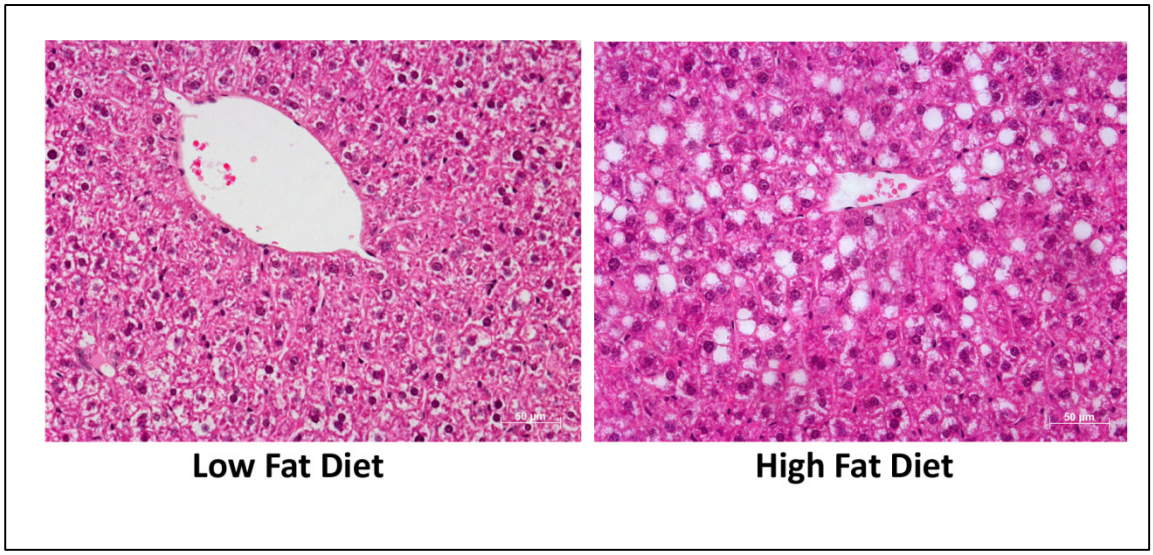

Figure 20 - Mice fed a high (45%) fat diet for 10 weeks show evidence of lipid droplets within hepatocytes when compared to mice fed control (10% fat) diet

Following this pilot to assess diet efficacy mice were allocated to receive either high or low fat diet (n=20 per group) for 6 weeks. At this point these groups were split and mice were treated with either FOLFOX or Vehicle control by intraperitoneal injection on a weekly basis (n=10 per group). Following my earlier experiments which had demonstrated the relative resistance of the murine liver to the effects of Oxaliplatin I wished to treat the mice with chemotherapy for 6 weeks to increase the likelihood of SOS developing. This was not possible due to 3 deaths in the high fat diet fed FOLFOX group in the week following the 5th treatment. The experiment was therefore terminated 1 week following the 5th dose of FOLFOX. There were no deaths in the low fat diet group treated with FOLFOX suggesting the high fat diet was associated with increased systemic drug toxicity.

It can be seen from Figure 21 that body weights between the control diet and high fat diet groups differed significantly at baseline (25.53 ± 1.99 vs. 31.15 ± 2.94g; p <0.0001) as would be expected. Whilst the vehicle only animals in each group continued to gain weight over the course of the experiment those treated with FOLFOX lost weight in keeping with systemic toxicity. This weight loss in response to chemotherapy was more dramatic in those receiving the high fat (7.3 ± 3.82 g) as compared to the low fat diet (1.6 ± 1.75g; p <0.01) again suggesting the high fat diet was associated with greater systemic toxicity.

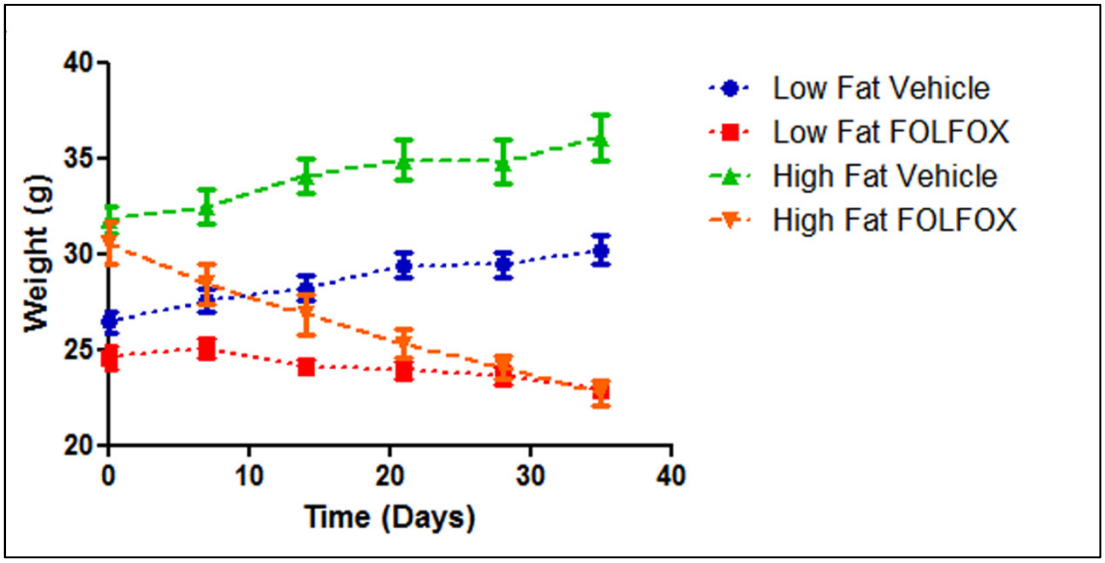

Figure 21 - Body weight over the course of FOLFOX administration demonstrates weight loss in FOLFOX treated animals which was most marked on the high fat diet

Next H&E stained sections of the liver were reviewed in conjunction with a liver pathologist (Professor Alastair Burt) to determine if there was evidence of liver injury. It can be seen in Figure 2 that mice treated with FOLFOX, both in the low and high fat diet groups, had marked sinusoidal dilatation and evidence of atrophy of peri-venular hepatocytes in keeping

with the early stages of SOS. The severity of these changes was, if anything, more modest in those fed the high fat diet. It was striking that those in the high fat group treated with vehicle alone had significant hepatic steatosis evident which was not present in the FOLFOX group confirming the results of the meta-analysis in data presented in chapter 2 that this chemotherapy regimen is not associated with hepatic steatosis or steatohepatitis. Blinded scoring of H&E stained tissue sections from animals maintained on a low fat diet revealed that sinusoidal dilatation was universally of Rubbia-Brandt grade 1 in FOLFOX treated animals as compared to grade 0 in vehicle controls ($p<0.0001$; Table 9). Similarly endothelial disruption was identified in all FOLFOX treated animals but not in controls ($p<0.0001$; Table 9).

	Vehicle (n=9)	**FOLFOX (n=9)**	**p-Value**
Rubbia-Brandt Grade			
0	9	0	
1	0	9	$p < 0.0001$
2	0	0	
3	0	0	
Endothelial Disruption Present	0	9	$p < 0.0001$

Table 9 - Semi-quantitative scoring of H&E stained sections from animals maintained on a low fat diet treated with either FOLFOX or Vehicle Control

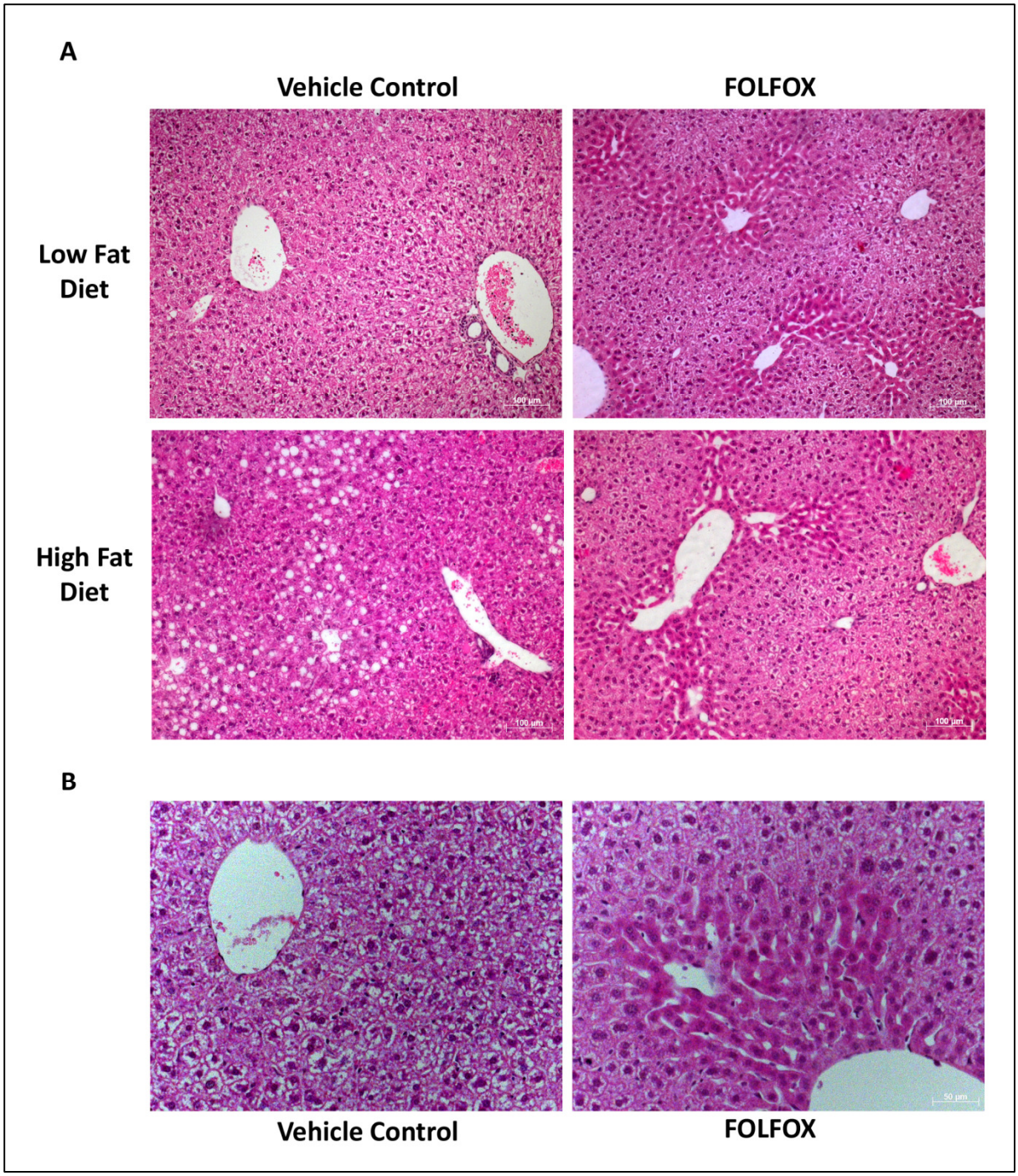

Figure 22 – 10x magnification H&E stained sections of the liver from FOLFOX treated animals demonstrate sinusoidal dilatation and peri-venular hepatocyte atrophy which occurs irrespective of dietary background (A). These changes can be seen more clearly in 20x magnification images from low fat diet animals (B).

Liver enzymes were measured in the serum of mice from all four groups as an alternative marker of injury. It can be seen that, in animals on the low fat diet, FOLFOX administration was associated with an elevated ALT (42.7 ± 4.52 U/L vs. 76.0 ± 14.5 U/L; $p<0.05$; Fig 22A) and AST (225 ±31 U/L vs. 478 ± 77 U/L; $p<0.05$; Fig 22B) but not ALP (72.2 ± 2.66 U/L vs. 68.7 ± 2.93 U/L; Fig 22C) when compared to vehicle controls. It is noteworthy that these changes were not demonstrated in the high fat diet animals treated with FOLFOX suggesting the degree of hepatocellular injury is worse in the low fat diet group. This may be a result of the most severely injured animals in the high fat group dying earlier than expected. In any case it is clear that, in terms of FOLFOX induced liver injury, there is little evidence that background steatosis has any effect on its development.

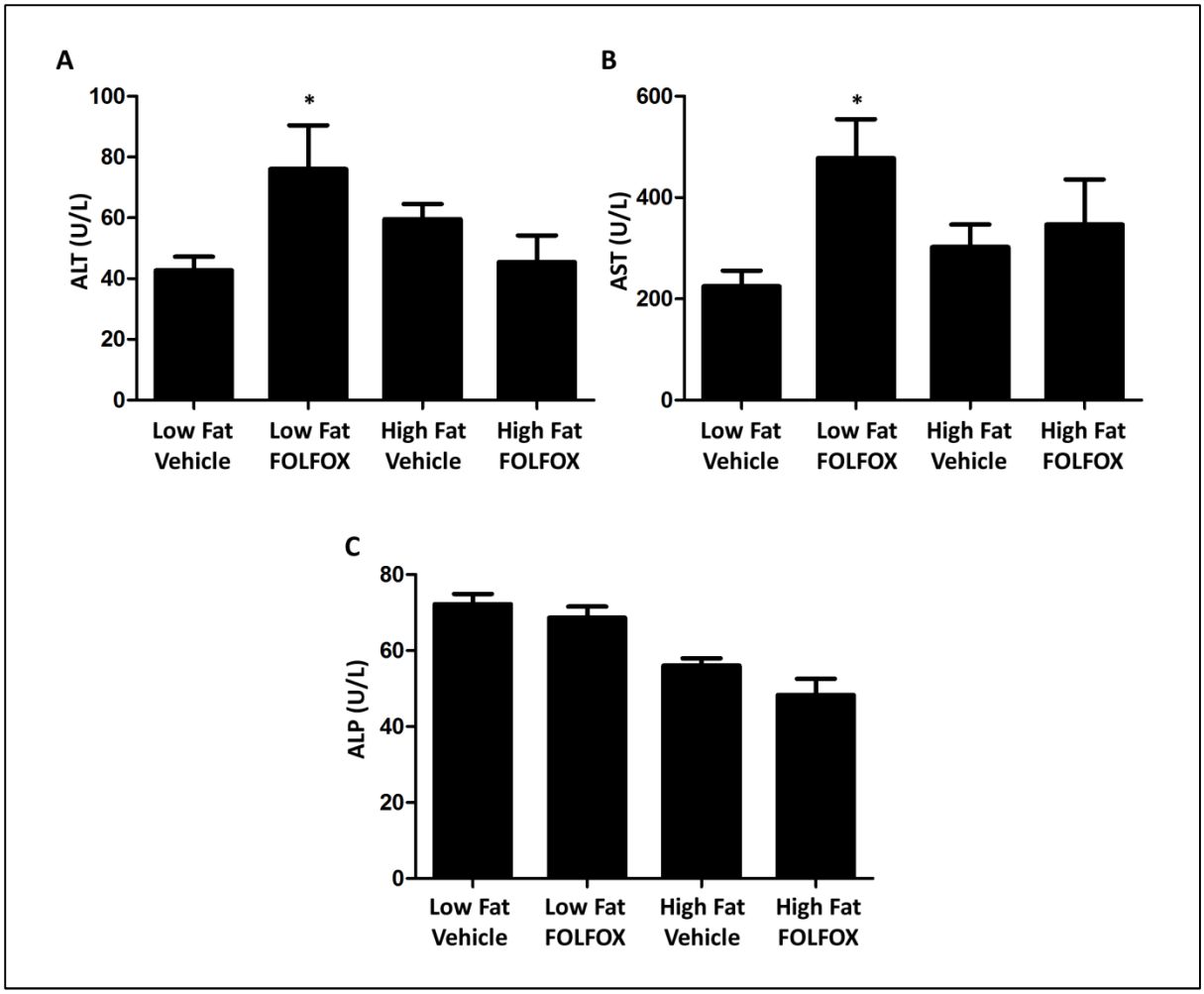

Figure 23 – FOLFOX administration is associated in an elevated ALT (A), AST (B) but not ALP (C) in control diet animals but not those fed a high fat diet

6.3 Validation of Matrix Remodelling Gene Expression in SOS Development

As previously discussed matrix remodelling appears to play a pivotal role in the development of Monocrotaline induced SOS and increased expression of these genes was demonstrated in a micro-array study performed on a small number of patients with Oxaliplatin induced SOS.(Deleve, Wang et al. 2003; Rubbia-Brandt, Tauzin et al. 2011) Given that I now had a model in which there were definitive histological changes of SOS I wished to validate this gene expression pattern for the following reasons :

1) To confirm that increased expression of these matrix remodelling genes is temporally associated with the development of SOS
2) To validate the conclusions made based on this gene expression signature in my previous models

Since the presence of steatosis within the liver had no effect on SOS development I used liver RNA extracts only from those animals who received the low fat diet for this analysis.

In keeping with previous models mice with FOLFOX induced SOS demonstrated a 2.63 fold increase in expression of MMP2 ($p<0.001$; Fig 24A) and an 8.47 fold increase in MMP 9 transcript ($p<0.001$; Fig 24B). This was associated with a 16.3 fold increase in TIMP1 expression ($p<0.001$; Fig 24C) and a 5.84 fold increase in Collagen I expression ($p<0.001$; Fig 24D). As previously discussed it is likely that TGFβ is playing a key role in driving this process of matrix remodelling with a 3.62 fold increase in its expression ($p<0.001$, Fig 24E).

When mice with an experimental colorectal liver metastases were treated with FOLFOX the change in expression of matrix remodelling genes following FOLFOX treatment was

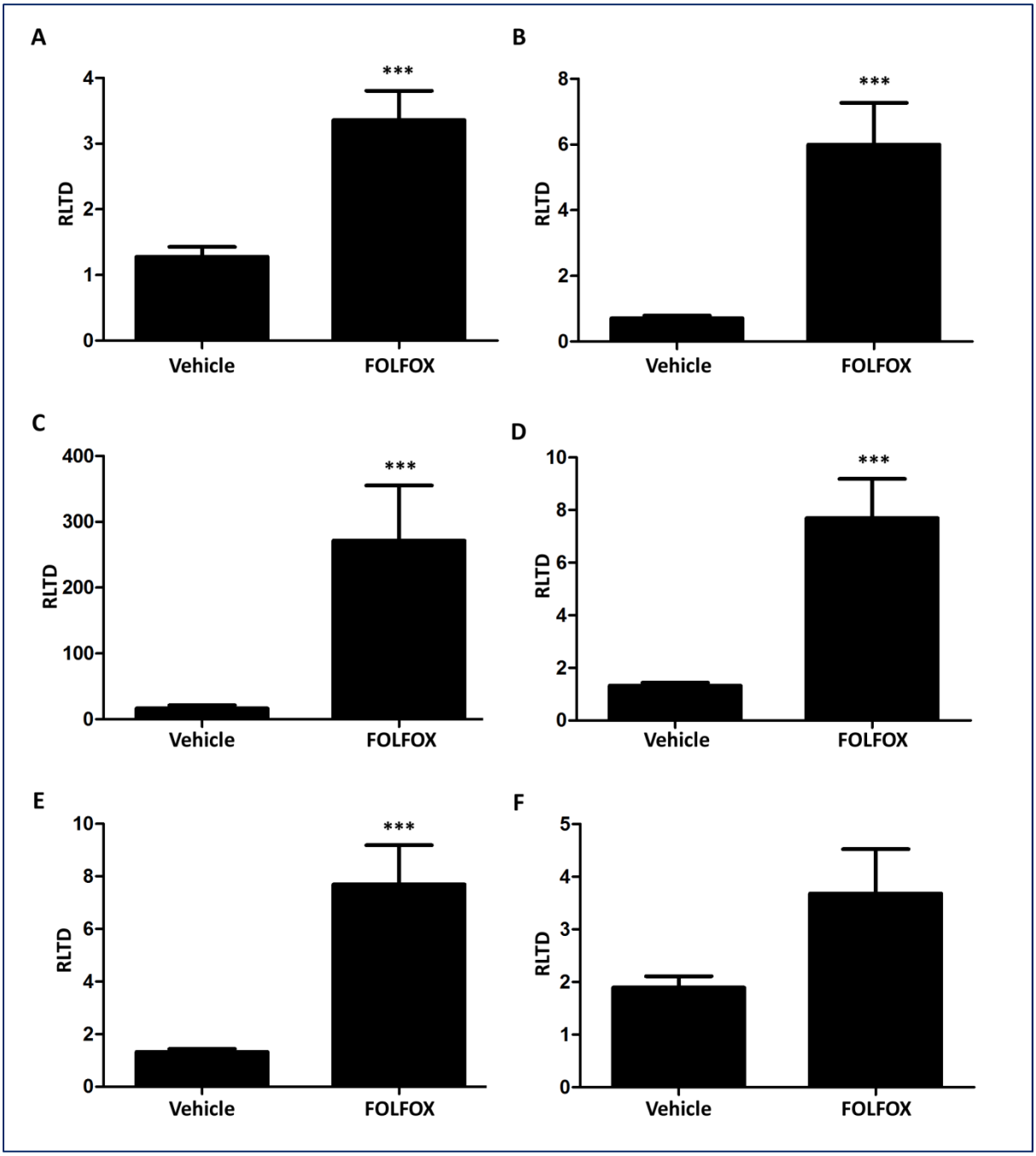

Figure 24 - FOLFOX induced SOS is associated with up-regulation of matrix remodelling genes including MMP2 (A), MMP9 (B), TIMP1 (C), Pro-Collagen I (D) and TGFβ(E). There is no increase in expression of the stellate cell marker αSMA (F) suggesting that these changes are arising predominantly from the sinusoidal endothelium.

associated with 9.5 fold increase in αSMA transcript, suggesting activation of hepatic stellate cells, and this finding was corroborated by immunohistochemistry (Figure 16). In contrast, despite the presence of SOS, I was not able to demonstrate a statistically significant up-regulation of αSMA transcript in FOLFOX treated animals (1.94 fold; p = 0.195; Fig 24F) and nor was there evidence on H&E stained sections of peri-sinusoidal fibrosis which would be in keeping with stellate cell activation (Fig 22). This was a somewhat surprising result as stellate cell activation has been demonstrated to be a feature of human SOS.(Rubbia-Brandt, Audard et al. 2004) There are two possible explanations for these findings :

1) The early events in SOS development are driven by damaged sinusoidal endothelium and stellate cell activation occurs later in the disease process perhaps as an attempt to limit injury to the sinusoid
2) Endothelial injury is a direct consequence of drug induced injury whereas stellate cell activation occurs as a consequence of tumour related factors.

6.4 Are dietary factors protective for the development of SOS in a murine model?

From the results presented thus far in this Chapter it is clear that a high fat diet does not lead to accelerated development of SOS following FOLFOX treatment. It does however seem that mice fed with the low fat diet regimen seem to have an increased susceptibility to SOS development as compared to mice maintained on a standard chow diet. It is important to note that the current experiment differed in several key ways to the experiment presented in Chapter 5 section 1:

- Mice receiving standard animal house care are given sunflower seeds as a treat. It is standard practice when using dietary models to not provide this treat in order that these are not consumed in preference to the experimental diet.
- It is common to maintain mice on sterile water to minimise the risk of exposure to environmental pathogens and this was the case in the original experiment unlike in the current experiment where mice were maintained on standard tap water.
- Mice were treated with FOLFOX for 5 weeks as compared to a 4 week maximum in earlier experiments.

It is plausible that any of these variables, in addition to the diet itself, may have contributed to the development of SOS in the current experiment. To understand further what might be responsible the experimental model outlined in section 1 of this chapter was repeated with stepwise substitution of each of the identified variables to determine which, if any of these, may protect against the development of SOS. This gave 4 groups of animals (n=6 per group) as follows :

- Control Diet, No Sunflower seeds, Standard tap water
- Control Diet, No Sunflower seeds, Sterile water
- Control Diet, Sunflower seeds ad libitum, standard tap water
- Standard chow, No Sunflower seeds, standard tap water.

Mice were treated with FOLFOX IP for 5 weeks and culled one week following the final dose.

On review of H&E stained sections of the liver it became apparent that features of SOS developed in all groups of animals to a similar extent except for those fed with standard chow (Figure 25) which appeared to offer a protective effect.

In order to determine if standard chow completely protected against the development of SOS or merely slowed its development 10 week old C57BL/6J mice, maintained on this diet, were treated with FOLFOX IP (or vehicle control) weekly for either 5 or 7 weeks (n=6 per group). Mice were culled 1 week following the final dose of chemotherapy.

H&E stained sections of the liver were again reviewed to assess for the development of features in keeping with SOS. As already alluded to mice treated with FOLFOX for 5 weeks have very minimal changes of SOS with, at worst, mild sinusoidal dilatation present in a few sinusoids scattered throughout the liver parenchyma. In contrast mice treated with FOLFOX for 7 weeks showed widespread sinusoidal dilatation with associated hepatocyte atrophy in keeping with SOS. The histological changes at this time point are indistinguishable from those in mice treated with FOLFOX on the experimental low fat diet for 5 weeks (Figure 26).

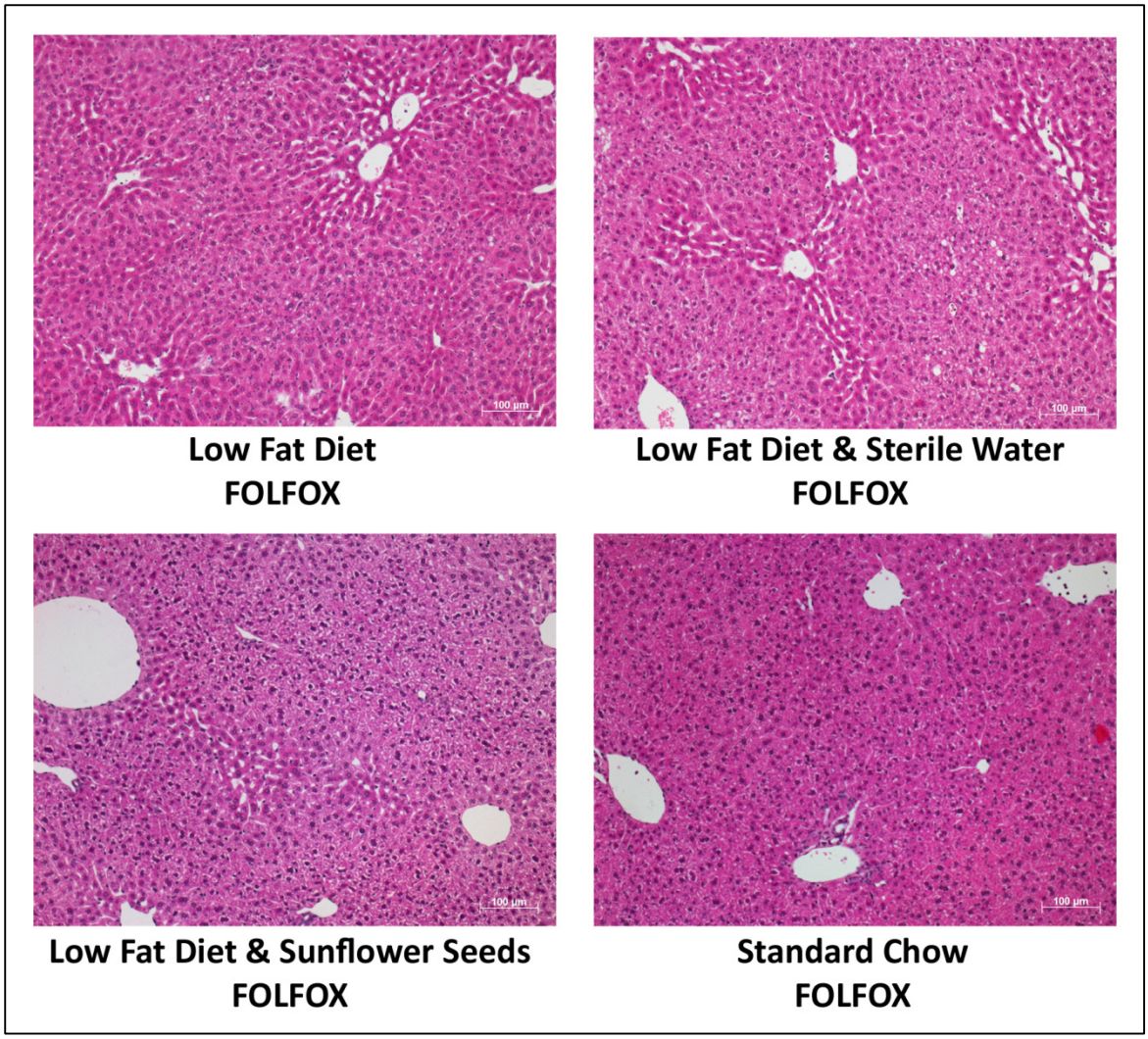

Figure 25 - A Standard Chow diet appears to protect against the development of FOLFOX induced SOS

It is not immediately apparent why a standard chow diet would slow the development of FOLFOX induced SOS although there are several potential explanations for this. A key difference between chow diets and the purified 'low fat' diet is that the former are manufactured from plant materials whereas the latter are manufactured with refined ingredients. The result of this is that chow diets can contain large quantities of phytoestrogens which have been shown to interfere with a large number of biological processes including the development of hepatic steatosis and fibrosis.(Ascencio, Torres et

al. 2004; McCarty, Barroso-Aranda et al. 2009) Other possibilities include differing nutrient levels, e.g. of naturally occurring anti-oxidants, which may have protective effects. Whilst nutritional content sheets are available for both diet types (See Appendices 2&3) it is impossible to directly compare the two since those for chow diets report minimal nutritional content whereas those for purified diets report precise nutrient levels. It would be of interest to determine the mechanism underpinning the protective effects of a chow diet but this is beyond the scope of the current body of work.

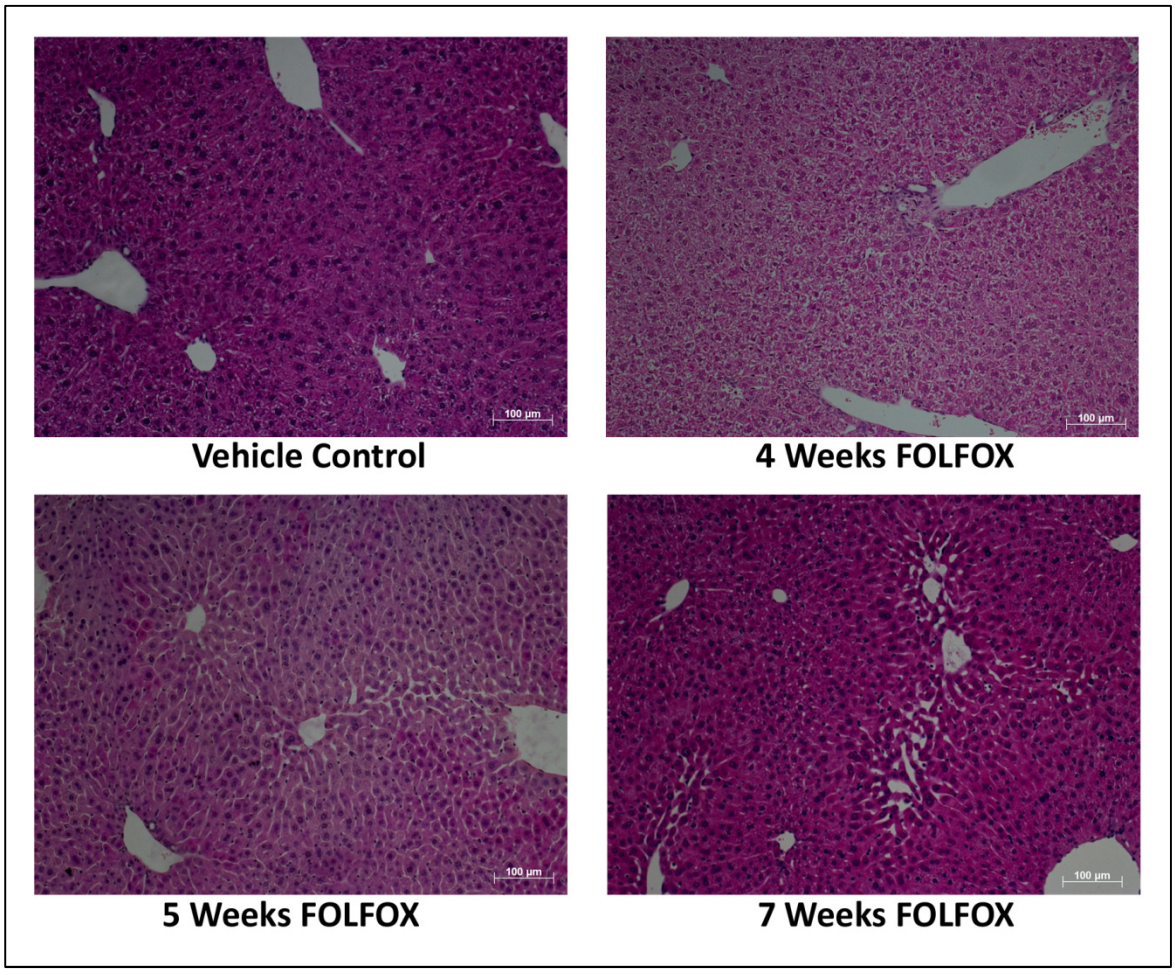

Figure 26 - Standard Chow delays but does not protect from the development of FOLFOX induced SOS with animals treated with 7 weeks developing changes of similar severity to those treated for 5 weeks on the experimental diet

6.5 What is the Natural History of FOLFOX induced SOS?

In order to establish the natural history of the morphological changes in the liver parenchyma following FOLFOX treatment mice on fed with the experimental low fat diet were treated with FOLFOX for 5 weeks and then left to recover for a total of 5 weeks before culling (n=6). To confirm that SOS had developed a further 6 mice were treated FOLFOX for 5 weeks and culled 1 week after the final dose (n=6). Control animals received drug vehicle alone for 5 weeks and were culled 5 weeks after the final dose (n=6). H&E stained sections of the liver confirmed the presence of SOS at 5 weeks following FOLFOX treatment which had completely resolved in mice allowed to recover for 5 weeks with the appearances of the liver being identical to that of those animals which received vehicle alone (Figure 27).

In the current model mice do not develop the more severe changes associated with SOS such as peri-sinusoidal fibrosis and nodular regenerative hyperplasia. In an effort to see if these changes could be reproduced mice, maintained on the experimental diet, were treated with FOLFOX for 6 weeks (with the intention of continuing for longer). Unfortunately in the week following the 6^{th} dose of FOLFOX 3 out of 6 mice were found dead. I suspect these deaths were as a consequence of systemic toxicity, most likely bone marrow failure with subsequent profound anaemia, as reflected by marked peripheral pallor and weight loss in the preceding days. It may be that increasing the time interval between doses of chemotherapy may allow for more prolonged treatment and thus more advanced SOS however time did not allow me to undertake these experiments.

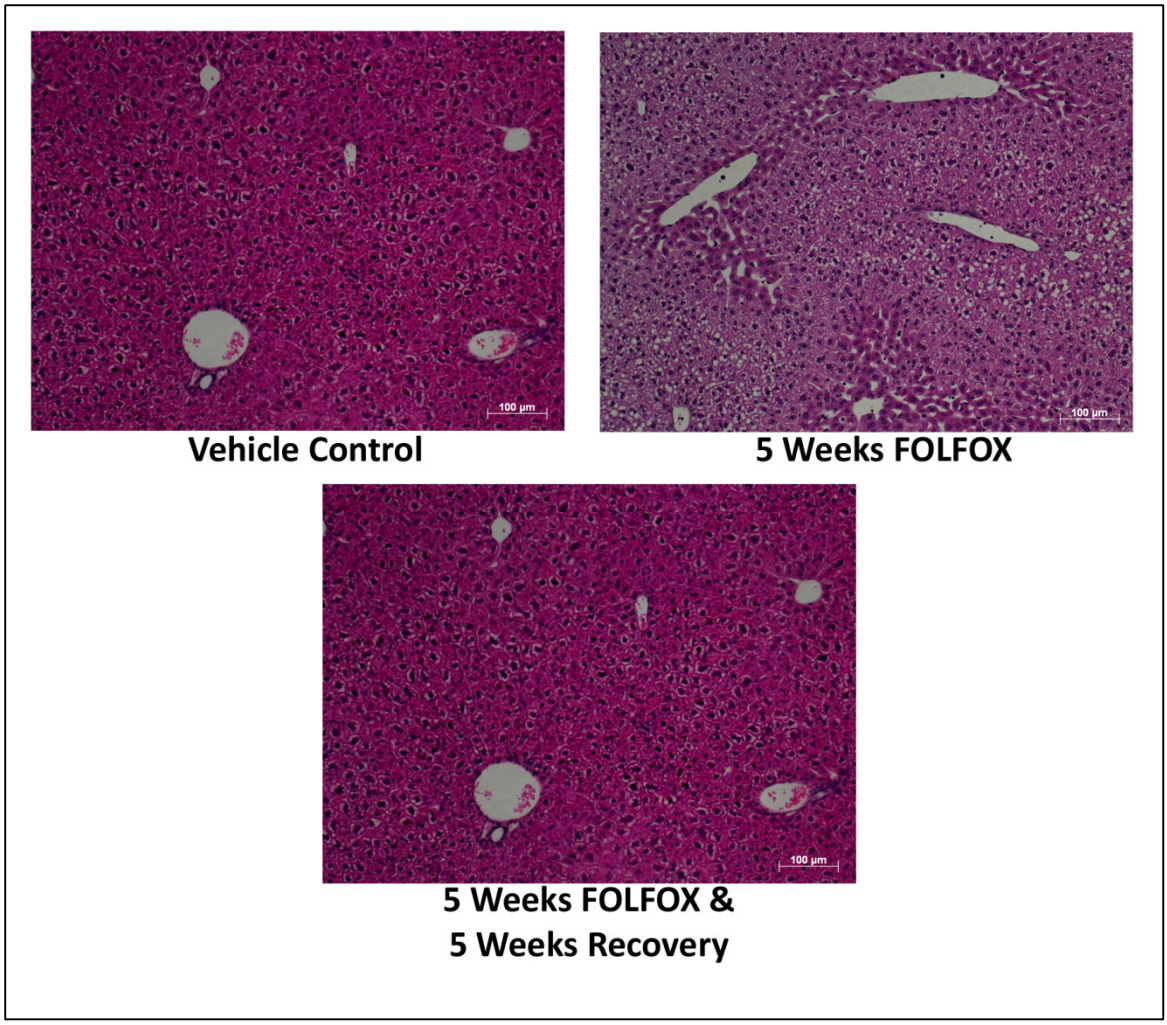

Figure 27 - The features of SOS induced by 5 weeks of FOLFOX treatment can be reversed by allowing mice to recover for 5 weeks

6.6 Summary of Key Findings

- Mice fed a purified, rather than chow, diet develop SOS after 5 weeks treatment with FOLFOX with H&E stained sections of the liver demonstrating sinusoidal dilatation and hepatocyte atrophy.
- The presence of hepatic steatosis is not associated with an increased risk of developing SOS following FOLFOX treatment
- FOLFOX treated mice with histological changes of SOS demonstrate up-regulation of key matrix remodelling genes (MMP2, MMP9, TIMP1, TGFβ, Pro-Collagen 1)
- A standard chow diet protects against, but does not prevent, the development of FOLFOX induced SOS with histological changes occurring after prolonged drug exposure
- FOLFOX induced SOS, at least in its earlier stages, appears to be reversible in mice

Chapter 7

The Pathogenesis of FOLFOX Induced SOS

7.1 A central role for PAI-1?

Having established a reproducible model of FOLFOX induced SOS I was able to begin a search to identify the pathophysiological processes underpinning the development of this condition so as to identify potential therapeutic targets for its prevention. In a micro-array study of changes in gene expression within the liver of patients with SOS one of the most highly up regulated transcripts was PAI-1 (Plasminogen Activation Inhibitor-1; SERPINE1).(Rubbia-Brandt, Tauzin et al. 2011) In response to this observation qRT-PCR was performed on whole liver extracts from mice with FOLFOX induced SOS which demonstrated that this was dramatically up-regulated (2816 fold; $p<0.0001$; Fig 28).

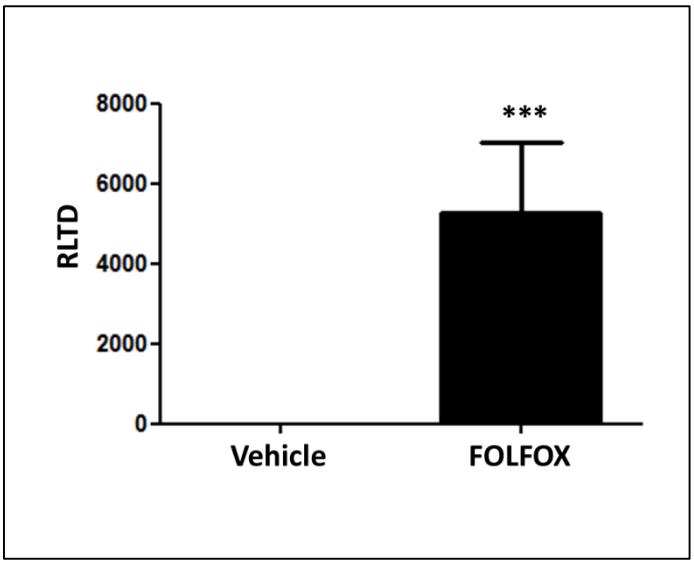

Figure 28 - FOLFOX induced SOS is associated with a 2816 fold increase in liver expression of PAI-1 mRNA ($p<0.0001$)

PAI-1 belongs to the serine protease inhibitor family (serpin) and was initially described as an inhibitor of both tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA). Through inhibition of these enzymes PAI-1 prevents the conversion of plasminogen into plasmin and thus functions as an inhibitor of fibrinolysis and encourages the formation of thrombus.(Andreasen, Egelund et al. 2000; Kietzmann and Andreasen 2008)

More recently it has been recognised that mice deficient in PAI-1 ($PAI\text{-}1^{-/-}$) demonstrate increased activity of the collagenase MMP9 with a subsequent reduction in the development of liver fibrosis in response to bile duct ligation and angiotensin II administration.(Bergheim, Guo et al. 2006; Wang, Zhang et al. 2007; Beier, Kaiser et al. 2011) As already discussed increased MMP 9 activity is thought to play a key role in the pathogenesis of SOS and it may be that an increase in PAI-1 serves to dampen down the activity of this enzyme thereby protecting from SOS development. If this were to be true then one might expect that $PAI^{-/-}$ mice would develop SOS more rapidly following SOS treatment however since these mice were not available to me this hypothesis was not explored further.

In addition to its roles in matrix remodelling and thrombosis PAI-1 has also been reported to play a role in cell cycle regulation and cell death.(Kortlever and Bernards 2006; Balsara and Ploplis 2008) Both cancer cell lines and human umbilical vein endothelial cells have been shown to be resistant to spontaneous and camptothecin induced apoptosis when cultured in the presence of PAI-1.(Kwaan, Wang et al. 2000) In cells subject to DNA damage an alternative response to apoptosis is to enter a state of cell cycle arrest in G1 phase – known

as senescence – a process for which PAI-1 is thought to be essential.(Kortlever, Higgins et al. 2006)

Given the dramatic up-regulation of PAI-1 in mice with FOLFOX induced SOS it seemed logical to explore some of the processes in which this protein is involved, particularly cell cycle regulation and thrombus formation, and the role they may play in the pathogenesis of this condition.

7.2 Endothelial Senescence

Cellular sensecence is a phenomenon which was originally described by Hayflick et al. in 1961 when he observed that normal human fibroblasts maintained in optimal growth conditions lost their proliferative capacity after a finite number of divisions.(Hayflick and Moorhead 1961) The first described cause of senescence was telomere shortening in response to repeated replication however since this time numerous other causes of cellular senescence have since been described including DNA damage, damage to the structure of chromatin, oxidative stress, lack of sufficient nutrients and oncogenic muations.(Ben-Porath and Weinberg 2005)

There are two key independent pathways involved in the generation of a senescence response. The first of these commences with nuclear translocation of the tumour suppressor p53 which results in activation of key transcriptional targets, in particular $p21^{CIP1/WAF1}$. The $p21^{CIP1/WAF1}$ protein interacts with cyclins D and E to function as a negative regulator of G1 to S phase progression in the cell cycle.

The alternative pathway for senescence induction involves up-regulation of the Cyclin D inhibitor $p16^{INK4A}$ which is normally expressed at low levels in healthy tissues. Activation of both the p53/$p21^{CIP1/WAF1}$ and $p16^{INK4A}$ pathways results in increased activity of the retinoblastoma protein which serves to inhibit the activity of the transcription factor E2F leading to diminished transcription of its target genes the majority of which play important roles in cell cycle progression.(Ben-Porath and Weinberg 2005; Evan and d'Adda di Fagagna 2009; Larsson 2011) These pathways are summarised in Figure 29.

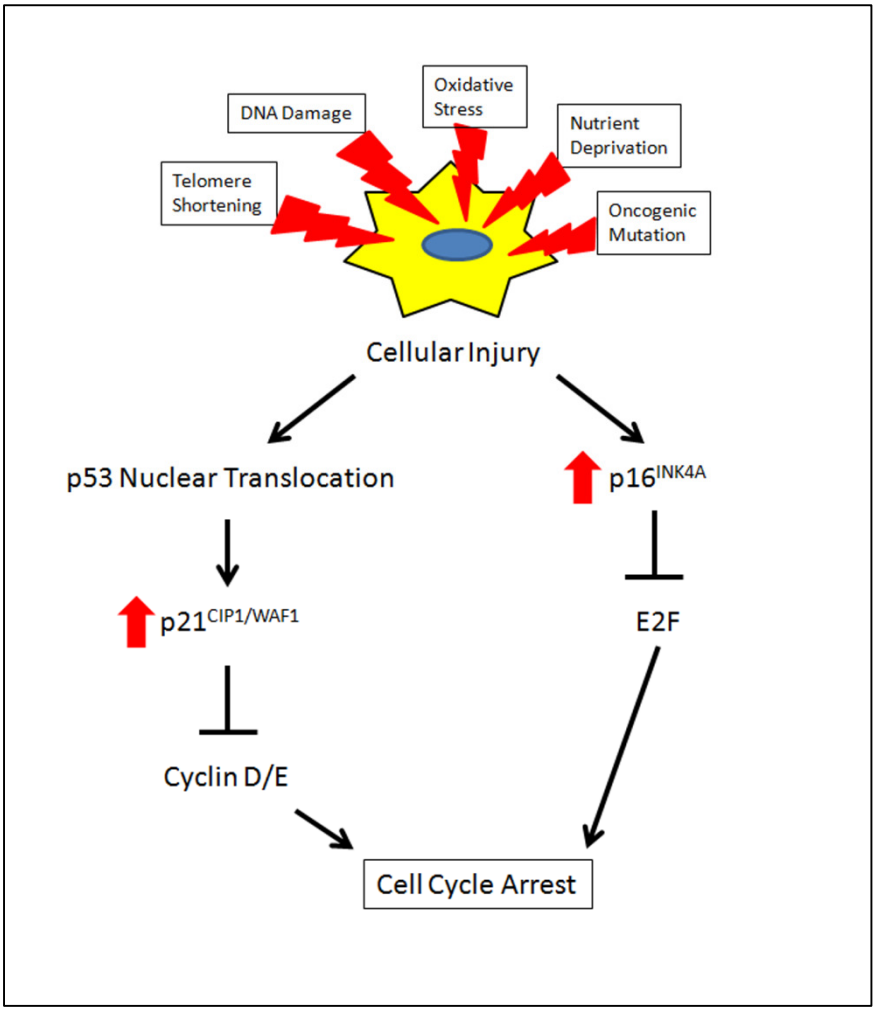

Figure 29 - Summary of the signalling pathways that lead to the induction of cellular sensecence

Both of these pathways may be active in the senescence process or alternatively one pathway may be activated whilst the other is unaffected – a pattern which varies between cell types but also upon the nature of the senescence inducing stimulus. To determine if either of these pathways were activated in the liver of animals with FOLFOX induced SOS qRT-PCR was performed to determine the activity of the $p21^{CIP1/WAF1}$ and $p16^{INK4A}$ genes. This demonstrated that in animals with SOS there was a 21 fold increase in the expression of $p21^{CIP1/WAF1}$ transcript ($p<0.001$; Fig 30A) whereas $p16^{INK4A}$ transcript was not detectable in either control or FOLFOX treated animals.

The increased expression of p21$^{CIP1/WAF1}$ in FOLFOX induced SOS was confirmed by western blot of whole liver protein extracts (Fig 30B). Immunohistochemistry for p21 $^{CIP1/WAF1}$ demonstrated that this increased expression was limited to what appeared to be rounded up endothelial cells in the areas of sinusoidal dilatation (Fig 30C).

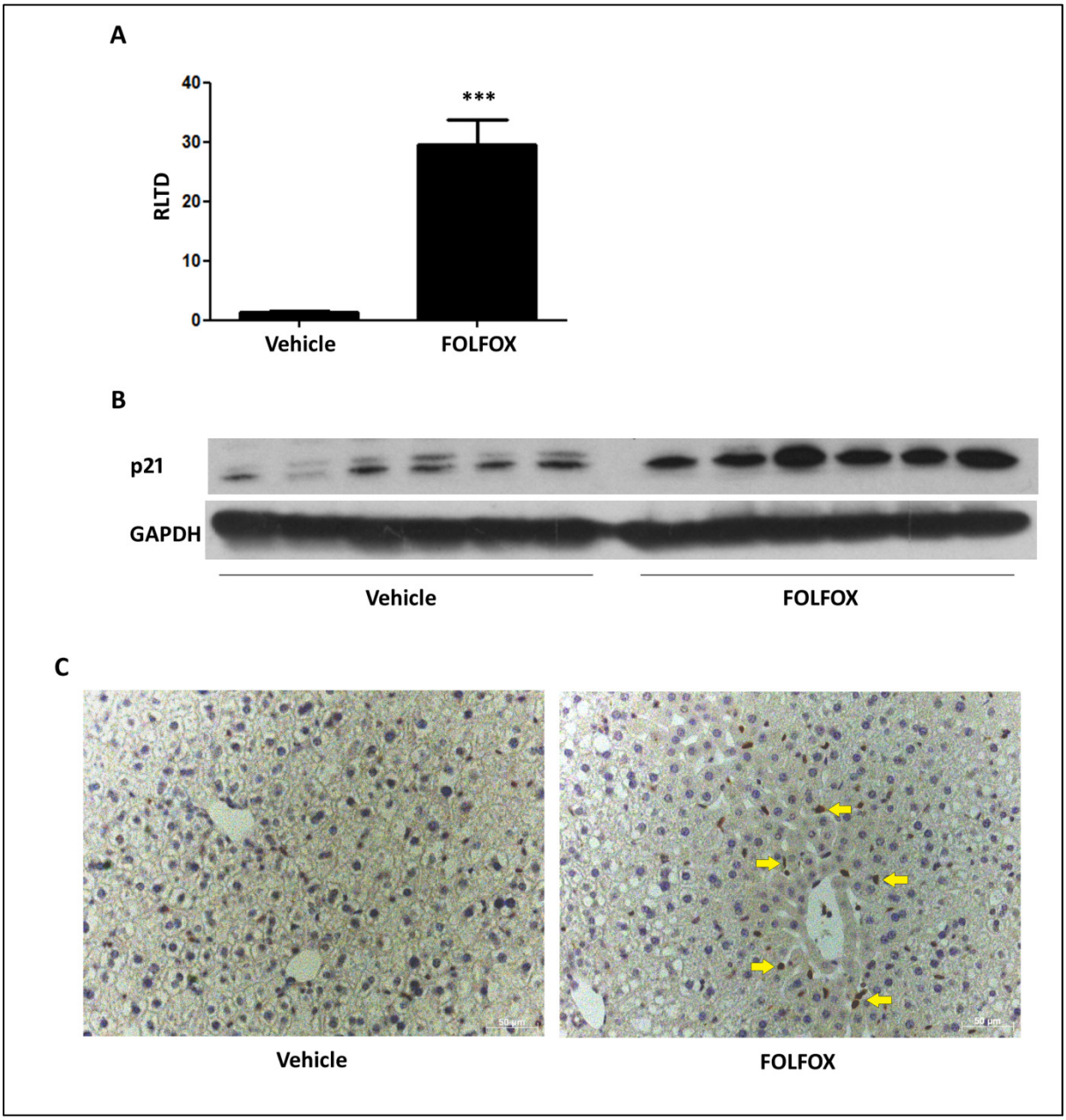

Figure 30 - FOLFOX induced SOS is associated with up regulation of hepatic p21$^{WAF1/CIP1}$ at both a transcript (A) and protein (B) level in whole liver. Immunohistochemistry (C; 20x magnification) demonstrates that this up regulation occurs predominantly in endothelial cells in injured sinusoids (yellow arrows)

As discussed the main mode of action of both 5-FU and Oxaliplatin is to induce DNA damage a process which can lead to phosphorylation of p53 at serine 15. This phosphorylation event is associated with transcriptional up regulation of $p21^{CIP1/WAF1}$ by p53.(Jung, Qian et al. 2010) Western blot of whole liver protein extract from the liver of animals with FOLFOX induced SOS confirmed that this pathway was likely implicated in the regulation of $p21^{CIP1/WAF1}$ in this model (Fig 31A). Immunohistochemistry for γH2AX, a marker of DNA damage, demonstrated increased staining within endothelial cells at the site of sinusoidal injury in the same manner as $p21^{CIP1/WAF1}$ suggesting that drug induced DNA damage was, at least in part, contributing to the induction of senescence in these cells (Fig 31B).

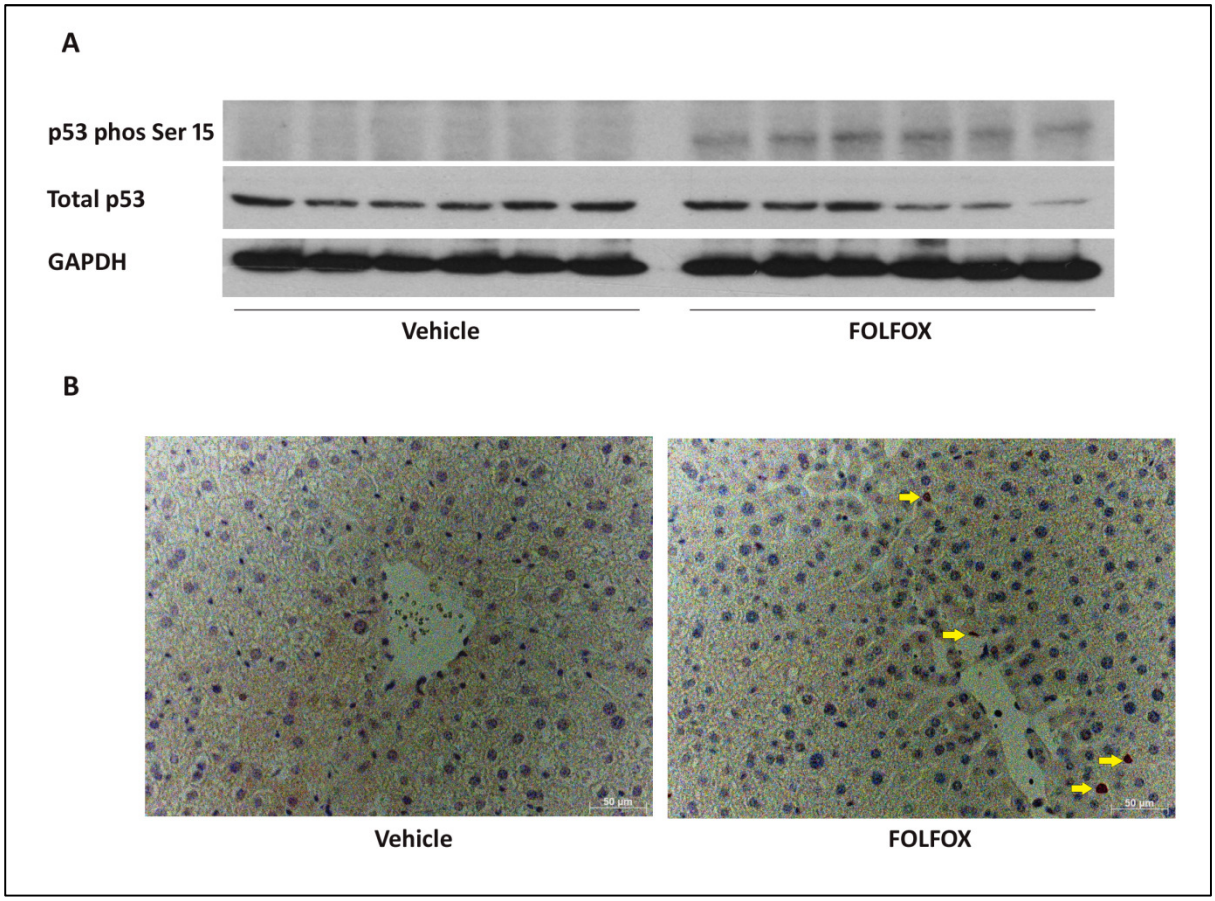

Figure 31 - FOLFOX induced SOS is associated with increased phosphorylation of p53 at serine 15 (A) and positive γ H2AX staining in damaged endothelium (B; 20x magnification) suggesting that DNA damage is at least in part driving senescence in this model (yellow arrows).

The process of cellular senescence has been associated with the production and extracellular secretion of a wide variety of pro-inflammatory cytokines and chemokines which may play important roles in maintaining the senescent phenotype.(Acosta, O'Loghlen et al. 2008; Kuilman, Michaloglou et al. 2008; Novakova, Hubackova et al. 2009) qRT-PCR of whole liver extracts demonstrated up regulation of CXCL1 (6.6 fold; $p<0.001$), CXCL2 (4.2 fold, $p<0.001$), CCL2 (12.5 fold, $p<0.001$) and IL-6 (2.0 fold, $p<0.05$) in animals with FOLFOX induced SOS (Fig 32).

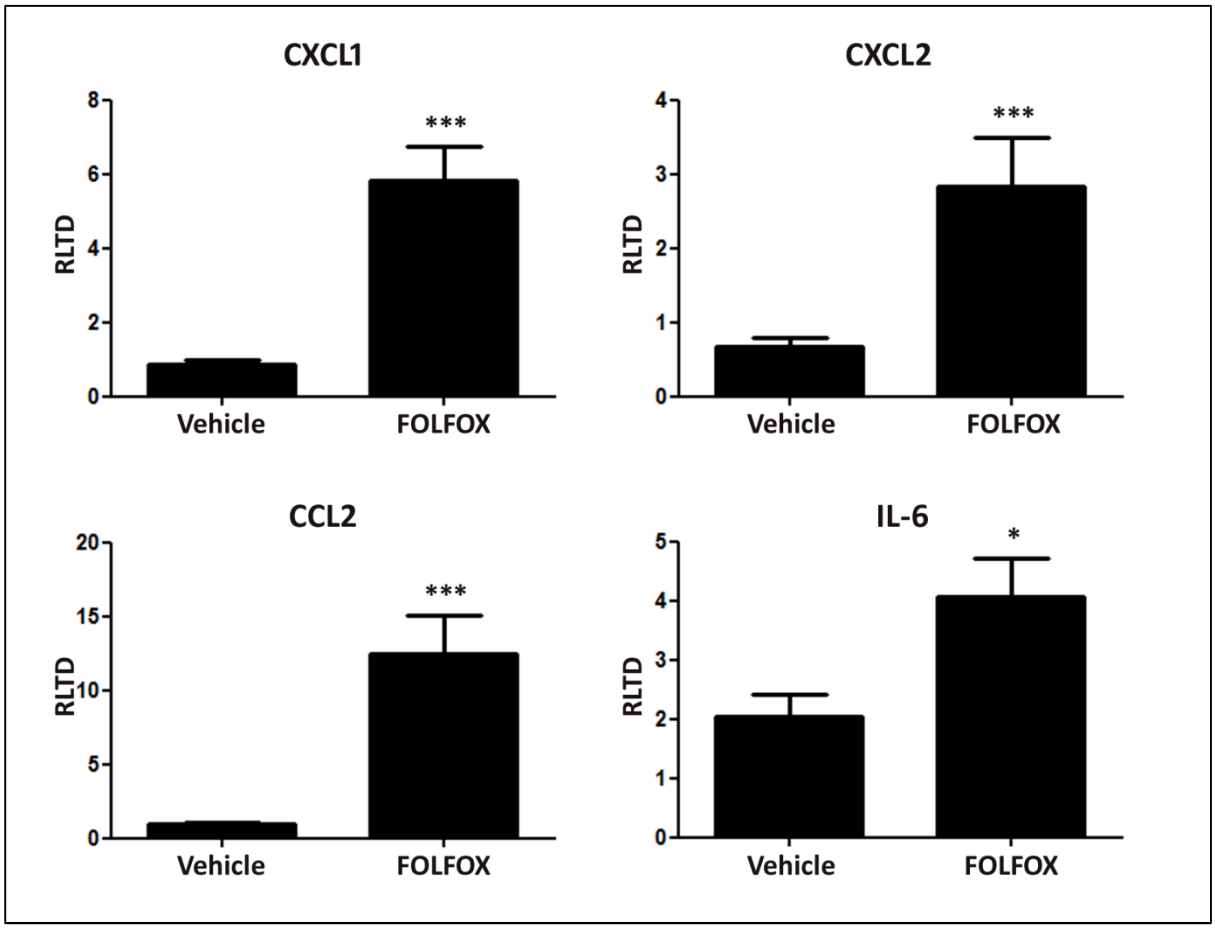

Figure 32 - FOLFOX Induced SOS is associated with up regulation of liver CXLC1, CXCL2, CCL2 and IL-6 transcript expression in keeping with a pro-inflammatory senescence phenotype

It has been suggested that IL-6, signalling through STAT1, may contribute to the maintenance of a senescent phenotype in cancer cell lines subject to genotoxic stress.(Novakova, Hubackova et al. 2009) This is in contrast to the more traditional view that IL-6 is a pro-proliferative cytokine which signals via STAT3 to facilitate increased transcription of a variety of genes involved in cell growth and proliferation. The IL-6/STAT3 pathway has been shown to play a pivotal role in hepatocyte regeneration following liver injury.(Drucker, Gewiese et al. 2009; Wang, Lafdil et al. 2011)

Western blot of whole liver protein extracts from mice with FOLFOX induced SOS demonstrated increased phosphorylation of STAT-3 as compared to those receiving vehicle alone suggesting a role for pro-proliferative IL—6 signalling (Fig 33A). Immunohistochemistry demonstrated that the site of STAT3 phosphorylation was not in the areas of sinusoidal injury but rather in hepatocytes surrounding the portal tracts (Fig 33B). More advanced SOS is associated with the development of nodular regenerative hyperplasia which arises in the peri-portal areas and it seems likely therefore that this is driven by IL-6 mediated activation of STAT3. This hypothesis is supported by the work of Horiguchi et al. who demonstrated increased STAT3 phosphorylation within regenerative nodules of patients with liver cirrhosis.(Horiguchi, Ishac et al. 2007)

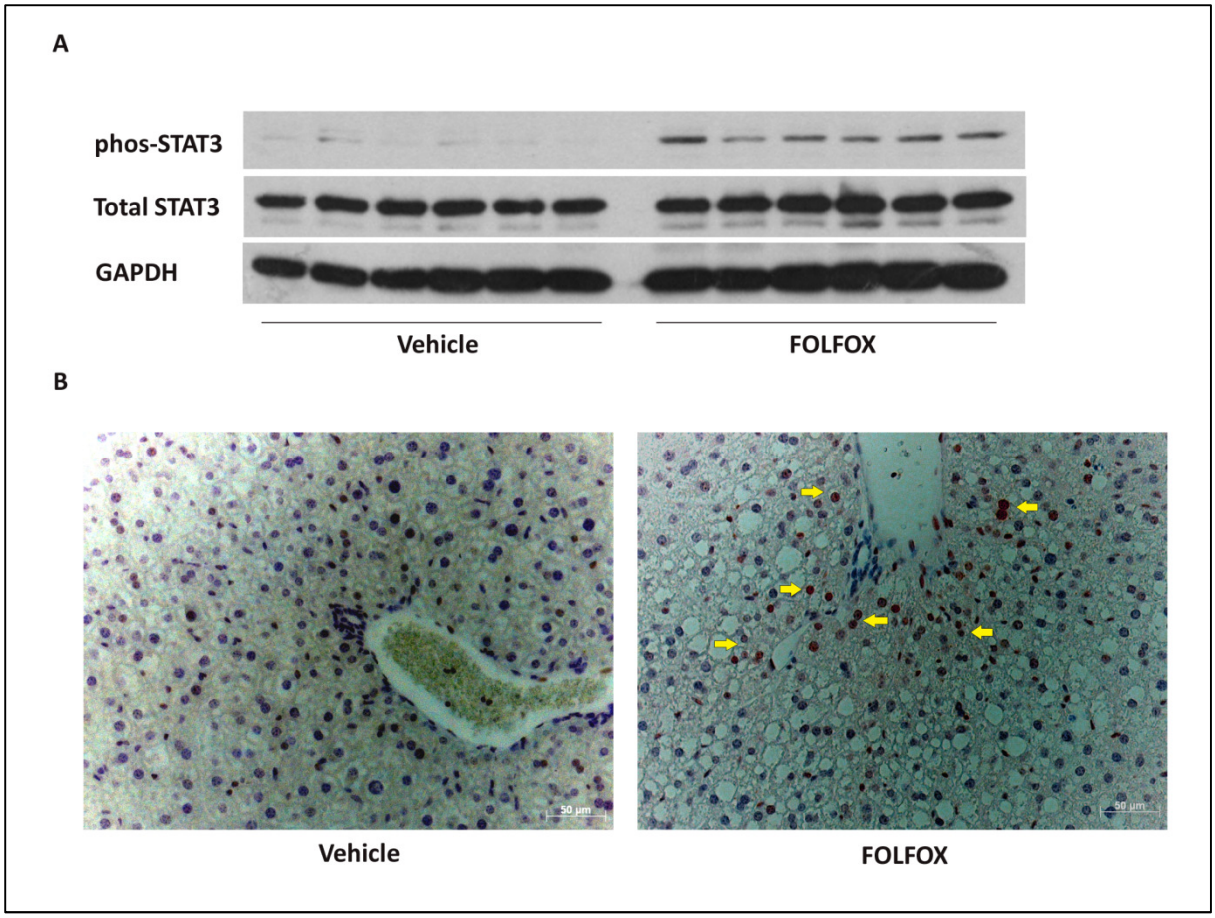

Figure 33 - FOLFOX induced SOS is associated with phosphorylation of the STAT3 transcription factor (A) within the liver. Immunohistochemistry demonstrates that this occurs predominantly in peri-portal hepatocytes (B)

7.3 Oxidative Stress

Whilst the pattern of staining for γH2AX closely mirrors that of p21 it should be noted that only a minority of the p21 positive cells in areas of sinusoidal injury demonstrate evidence of DNA damage suggesting that this is not the only mechanism contributing to cellular senescence. As already discussed an alternative mechanism of senescence induction is through the action of reactive oxygen species (ROS).(Passos, Nelson et al. 2010) It is also known that Oxaliplatin exposure can lead to depletion of intracellular glutathione which, in the Monocrotaline model, is a key mechanism underpinning the development of SOS.(Zhang, Mack et al. 1998; Wang, Kanel et al. 2000) To explore the relevance of this mechanism to FOLFOX induced SOS the total and oxidised glutathione levels were determined in snap frozen liver samples. This demonstrated a statistically significant reduction in total hepatic glutathione (95.4 vs. 76.9 µmoles/g protein; $p<0.05$; Fig 34) but no overall difference in the proportion of oxidised glutathione (1.4% vs. 1.2%; $p=0.132$; Fig 34).

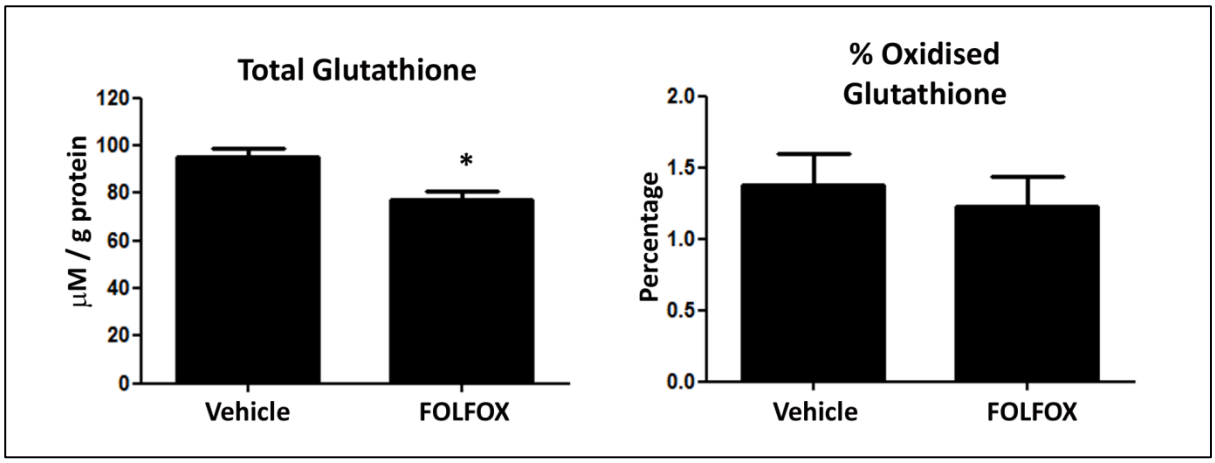

Figure 34 - FOLFOX induced SOS is associated with a reduction in the total liver glutathione concentration but not the proportion of oxidised glutathione (Analysis performed by Dr Aphrodite Vasilaki, Faculty of Health & Life Sciences, University of Liverpool)

Interpretation of this data is difficult. The reduction in total liver glutathione concentration would suggest an increased susceptibility to oxidative stress. In the face of significant oxidative stress one would expect an increase in the proportion of oxidised glutathione which is not seen in these animals. It should be remembered that the major site of drug induced injury appears to be the sinusoidal endothelial cell which makes up only 3% of the liver parenchymal volume and therefore this result more likely reflects what is happening within hepatocytes which make up the bulk of the liver parenchymal volume.(Blouin, Bolender et al. 1977) To assess events in the sinusoidal endothelium individually one would need to isolate these cells however since this process in itself would also induce cellular stress this was not considered a strategy worthy of pursuing.

To assess whether an anti-oxidant strategy may be of value in preventing the development of SOS in patients receiving Oxaliplatin based chemotherapy the current experiment was repeated utilising two different anti-oxidant strategies. The first of these was dietary supplementation with 3% N-Acetylcysteine (NAC). The rate limiting step in the de novo synthesis of glutathione is the availability of the amino acid cysteine.(Davis, Ronai et al. 2001) NAC is a synthetic thiol which serves to increase the intracellular cysteine concentration with subsequent increased glutathione synthesis.(Zafarullah, Li et al. 2003) If endothelial toxicity arises solely as a consequence of glutathione deprivation then one would expect this strategy to be effective.

The second anti-oxidant strategy was dietary supplementation with 0.7% butylated hydroxyanisole (BHA). BHA belongs to a group of compounds known as phenolic anti-oxidants which exerts its effect by increasing the activity of the transcription factor NRF2 (Nuclear factor erythroid 2-related factor).(Higgins and Hayes 2011) NRF2 regulates the

expression of a wide variety of genes implicated in the response to oxidative stress through the binding of antioxidant response elements (AREs) in the promoter region of target genes thereby leading to their transcription.(Reisman, Yeager et al. 2009)

The antioxidant drugs were added to the base 10% fat purified diet used in previous experiments by the manufacturer. Treatment with the drug containing diets was started a week prior to commencing chemotherapy treatment and continued until the time animals were culled. A dietary supplementation strategy was chosen over either parenteral administration or oral gavage since both of these techniques are associated with a degree of morbidity which if carried out on a daily basis for 6 weeks might lead to a considerable risk to the animals. Furthermore a parenteral strategy was not considered appropriate as it is unlikely that this route would be acceptable to patients receiving multiple courses of chemotherapy and therefore any intervention would need to be effective when administered via an oral route.

To determine the effectiveness of these antioxidant strategies H&E stained sections of the liver from FOLFOX treated and vehicle control mice were examined to determine the extent of sinusoidal injury. It can be seen in Figure 35 that NAC treatment had no effect on the development of SOS as compared to animals fed the control diet whereas BHA treatment resulted in a much less severe injury to the hepatic sinusoid with at worst very minor sinusoidal dilatation being present, although in some animals there was no evidence of injury at all. This was reflected in the histological scoring of H&E stained tissue sections with the incidence of endothelial disruption being reduced to only 25% in FOLFOX treated animals receiving BHA treatment ($p<0.05$; Table 10).

The observation that NAC administration does not prevent the development of FOLFOX induced SOS is an unexpected finding that appears to contradict the findings of Wang et al. in the Monocrotaline model.(Wang, Kanel et al. 2000) It should be noted that in this study NAC was administered by intraportal infusion and it may be that the oral route is not sufficient for adequate drug delivery to the target tissue.

	Control Diet		**0.7% BHA**	
	Vehicle (n=5)	**FOLFOX (n=5)**	**Vehicle (n=5)**	**FOLFOX (n=4)**
Rubbia-Brandt Grade				
0	5	0	5	1
1	0	4	0	3
2	0	1	0	0
3	0	0	0	0
Endothelial Disruption Present	0	5	0	1

Table 10 - The impact of BHA treatment on the histological scoring of H&E stained liver sections from FOLFOX treated animals (Scoring by Professor Alastair Burt)

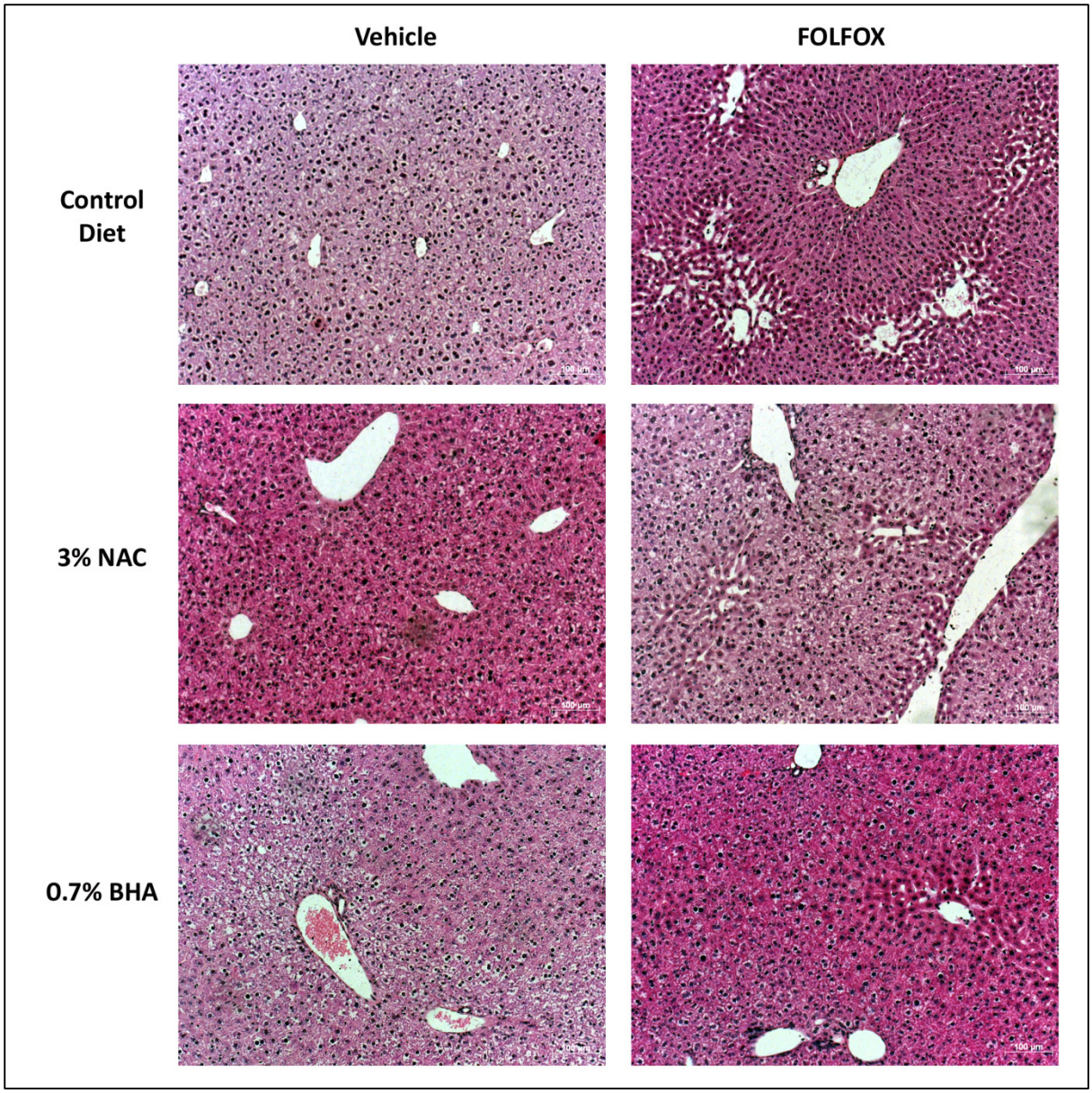

Figure 35 - Representative H&E stained liver sections from FOLFOX or Vehicle control treated animals with antioxidant dietary supplementation

In keeping with reduced sinusoidal injury in animals fed a BHA supplemented diet qRT-PCR of whole liver RNA extracts demonstrated decreased expression of p21 and PAI-1 following FOLFOX treatment (Fig 36).

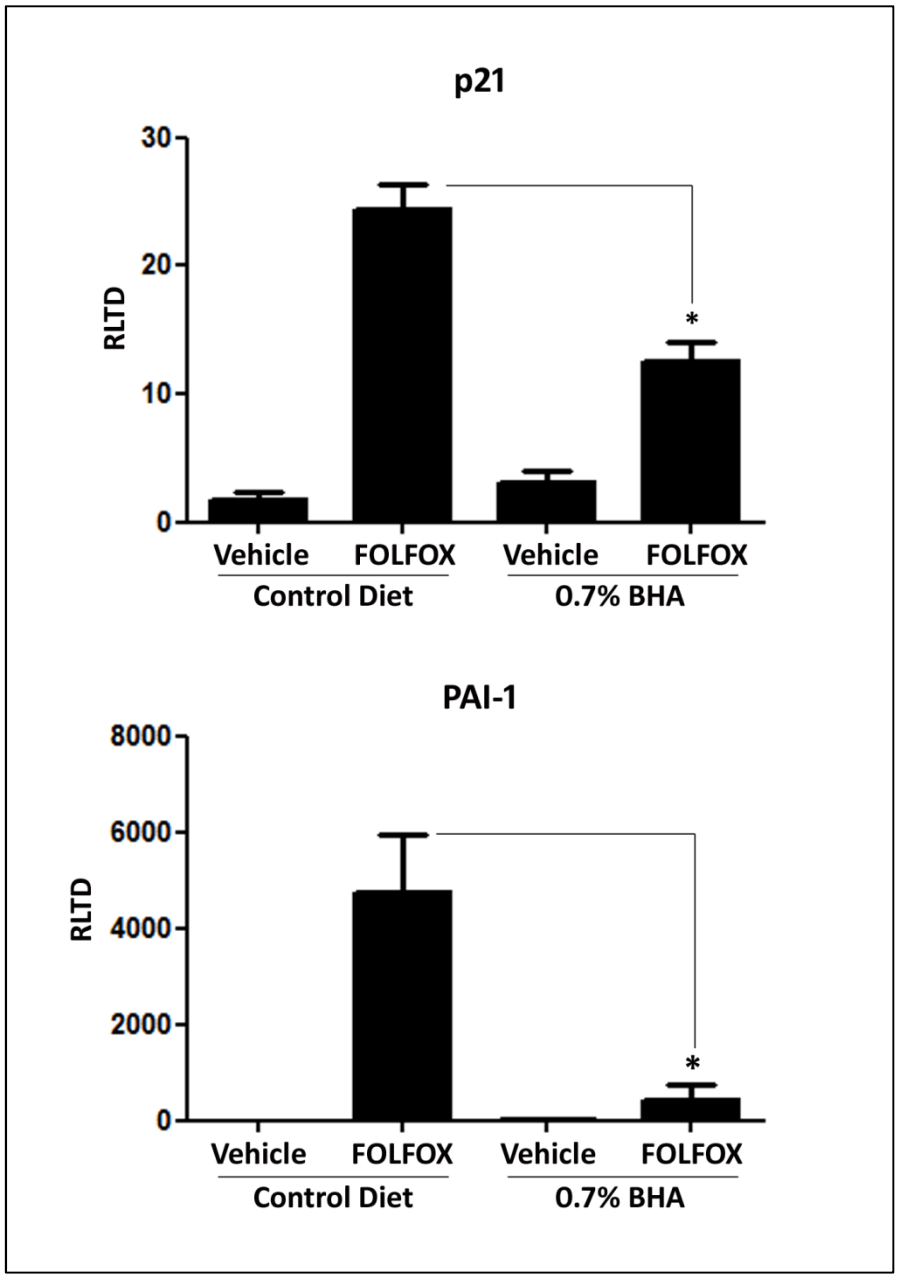

Figure 36 - In accordance with a lesser degree of sinusoidal injury there is a reduction in FOLFOX induced p21 and PAI-1 expression in animals on a BHA supplemented diet

To confirm that BHA was mediating its effects through NRF2 a western blot was initially performed on whole liver extracts to determine changes in protein expression following FOLFOX administration. This assay revealed that the presence of SOS is associated with reduced overall expression of NRF2, a finding which has not been previously reported. This reduction in NRF2 protein expression is prevented in animals maintained on a 0.7% BHA diet (Fig 37A).

To assess the effect of these changes on gene transcription qRT-PCR was performed to determine the expression of TXN1 (Thioredoxin1) and NQO1 (NAD(P)H dehydrogenase 1) both of which play important roles in the elimination of ROS and are classically recognised as being regulated by NRF2.(Taguchi, Motohashi et al. 2011) This demonstrated that SOS was associated with a 3 fold reduction in TXN1 expression ($p<0.01$) an effect that was prevented by feeding with a BHA diet, which in itself led to a 2 fold increase in baseline TXN1 expression ($p<0.01$; Fig 37B). SOS was not associated with a reduction in NQO1 expression however a BHA diet was associated with a 19 fold increase in NQO1 expression ($p<0.01$) suggesting increased protection from ROS in these animals (Fig 37C).

In summary the current data support the notion that oxidative stress is important in the pathogenesis of FOLFOX induced SOS. Anti-oxidant strategies that increase NRF2 transcriptional activity may be particularly useful in trying to prevent this complication of chemotherapy.

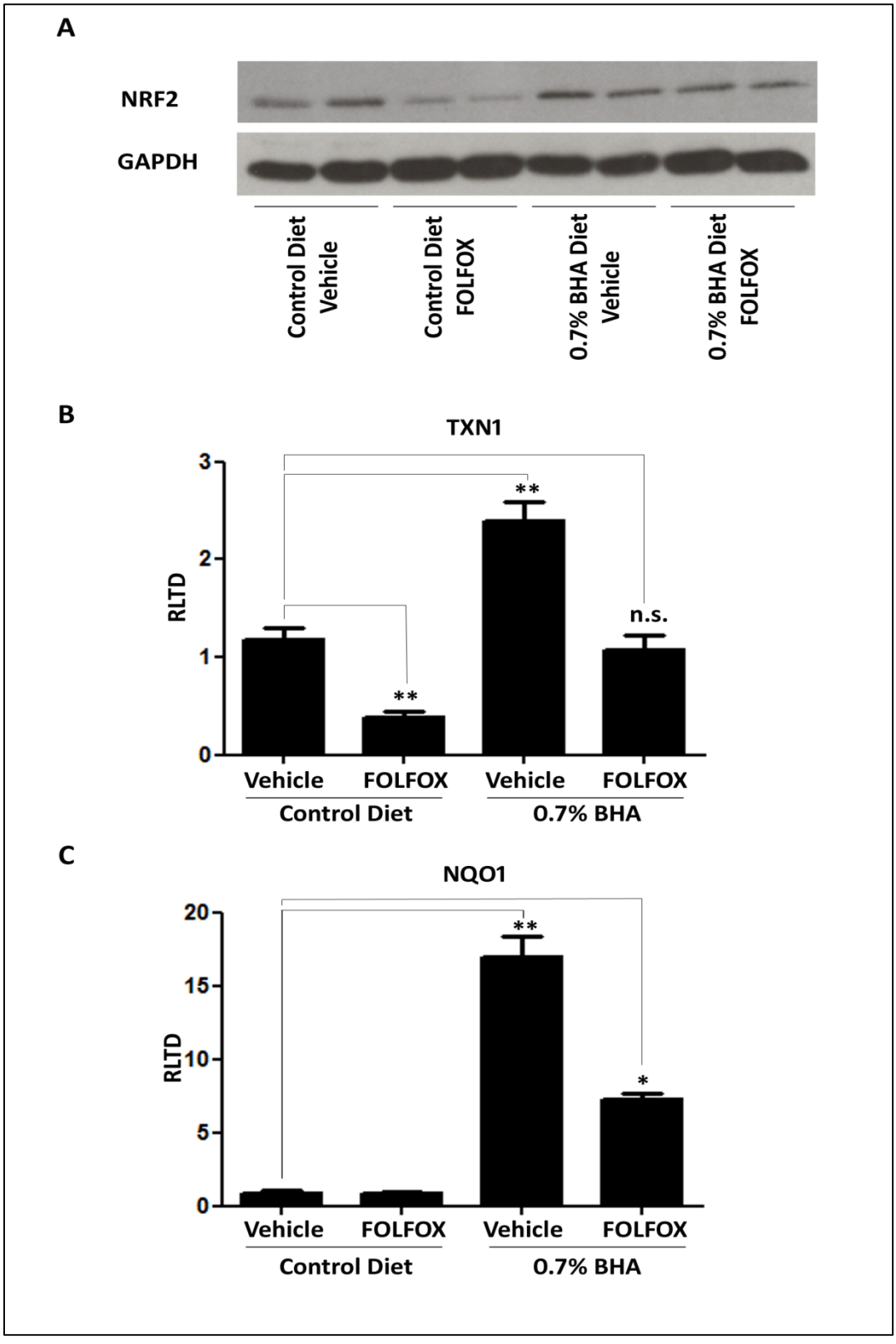

Figure 37 - FOLFOX induced SOS is associated with decreased expression of the transcription factor NRF2 which is prevented by administration of a diet supplemented with 0.7% BHA (A). This prevents the FOLFOX induced reduction in thioredoxin transcription (B) and increased basal NQO1 transcription (C) thereby resulting in increased protection from ROS

7.4 The Coagulation Cascade in SOS Development

There is indirect evidence to suggest that abnormalities in the clotting cascade may contribute to the development of SOS in patients. In a series of 146 patients undergoing resection of colorectal liver metastases Brouquet et al. demonstrated histological features of SOS in 50 of whom the majority had received pre-operative Oxaliplatin based chemotherapy. On multivariate analysis they identified that those taking regular Aspirin had a lower risk for developing SOS (Hazard Ratio 0.44; p=0.03) as compared to those who were not.(Brouquet, Benoist et al. 2009) In patients who develop severe SOS following myeloablative chemotherapy prior to bone marrow transplantation the fibrinolytic agent defibrotide has been reported to be of modest benefit in improving outcome.(Benimetskaya, Wu et al. 2008)

It is widely acknowledged that both platinum based chemotherapeutics and 5-FU have pro-thrombotic effects on vascular endothelium.(Cwikiel, Eskilsson et al. 1996; Nuver, De Haas et al. 2010) In the Monocrotaline model of SOS it has been shown that activation of the clotting cascade with deposition of fibrin along the sinusoid is a key feature.(Copple, Banes et al. 2002) The clotting cascade consists of two separate pathways – the intrinsic and extrinsic pathways – which converge on a common pathway whereby Factor X is cleaved to the active form, Factor Xa, which subsequently cleaves thrombin from its precursor molecule prothrombin. Thrombin is then able to convert fibrinogen to fibrin resulting in the formation of clot.(Ganong 1997)

In support of activation of the clotting cascade there was a 1.9 fold increase in expression of Factor X transcript in the liver of mice with FOLFOX induced SOS ($p<0.001$; Fig 38A). Tissue factor is a key component of the extrinsic coagulation pathway. Upon injury cells express

tissue factor on their surface which is able to come into contact with circulating Factor VII which together form a complex which then activate Factor X.(Ganong 1997; Usui, Kuriyama et al. 2009) Immunohistochemistry demonstrated increased expression of tissue factor within injured hepatic sinusoids (Fig 38B) suggesting this may be a relevant mechanism of clotting cascade activation in SOS.

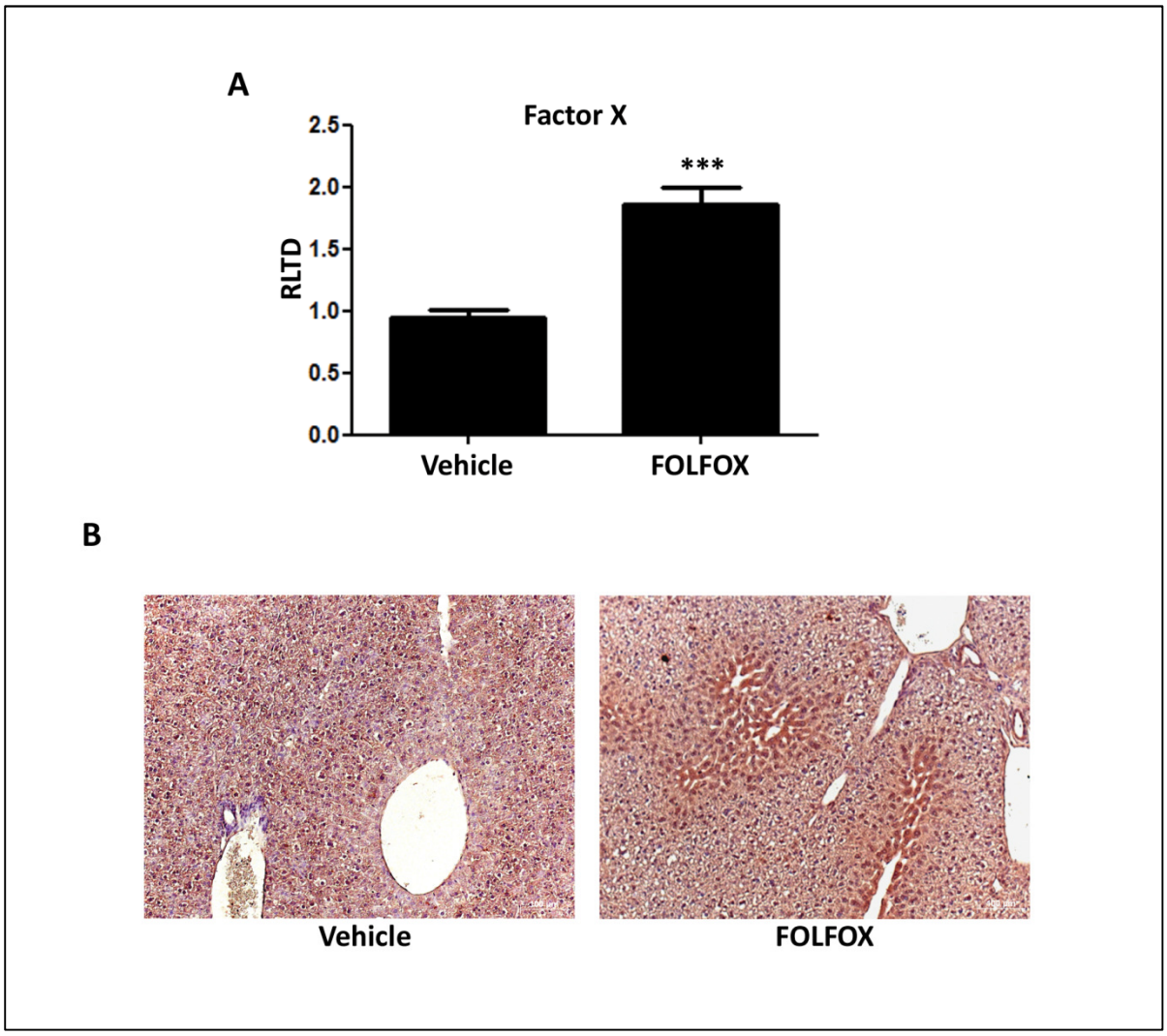

Figure 38 - In support of coagulation cascade activation there is increased expression of Factor X in the liver of mice with FOLFOX induced SOS (A). Coagulation cascade activation is likely occurring via the extrinsic pathway as a consequence of increased tissue factor expression in areas of sinusoidal injury (B)

As already discussed FOLFOX administration is associated with splenic injury in mice (Figure 10; Chapter 3). Further review of H&E stained sections of the spleen in the current model revealed the presence of large clusters of Megakaryocytes in mice with FOLFOX induced SOS (3.8 vs. 1.3 cells per HPF; $p<0.001$; Fig 39 A & B) suggesting that perhaps significant numbers of platelets are being released directly into the portal circulation. This was also associated with a 1.9 fold increase in hepatic von Willebrand Factor (vWF) expression in the liver of these animals suggestive of an increased potential for platelet adhesion and activation ($p<0.01$; Fig 39 C).

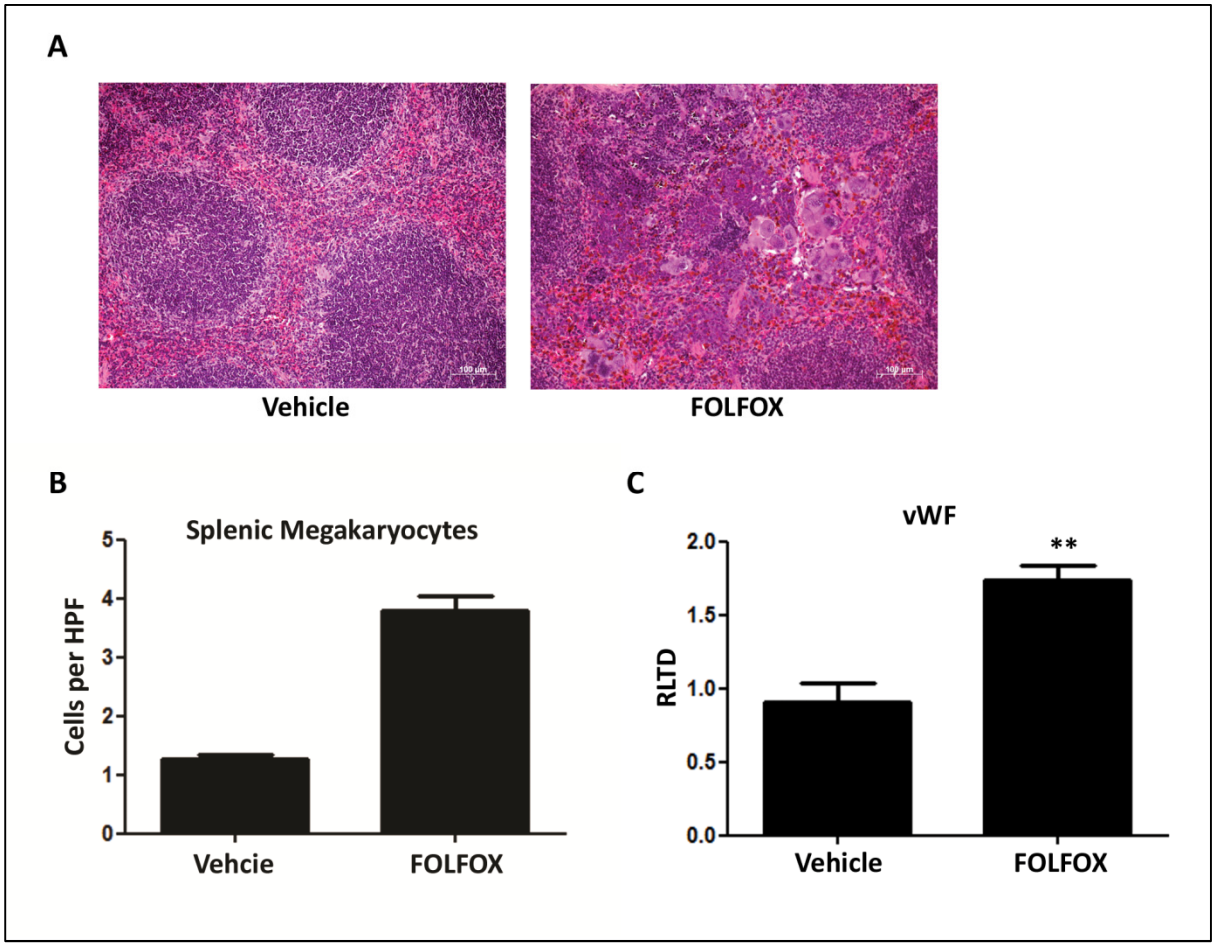

Figure 39 - FOLFOX induced SOS is associated with the presence of large clusters of Megakaryocytes within the spleen (A & B). In addition there is increased hepatic expression of vWF mRNA suggesting an increased propensity of platelet adherence and activation within the liver.

In addition to its roles in thrombus formation Factor X is also able to interact with and activate pathways involved in matrix remodelling which it achieves by signalling through the protease activated receptors PAR1 and PAR2. It is noteworthy that mRNA expression of both of these receptors is increased in the liver of FOLFOX treated mice (Fig 40).

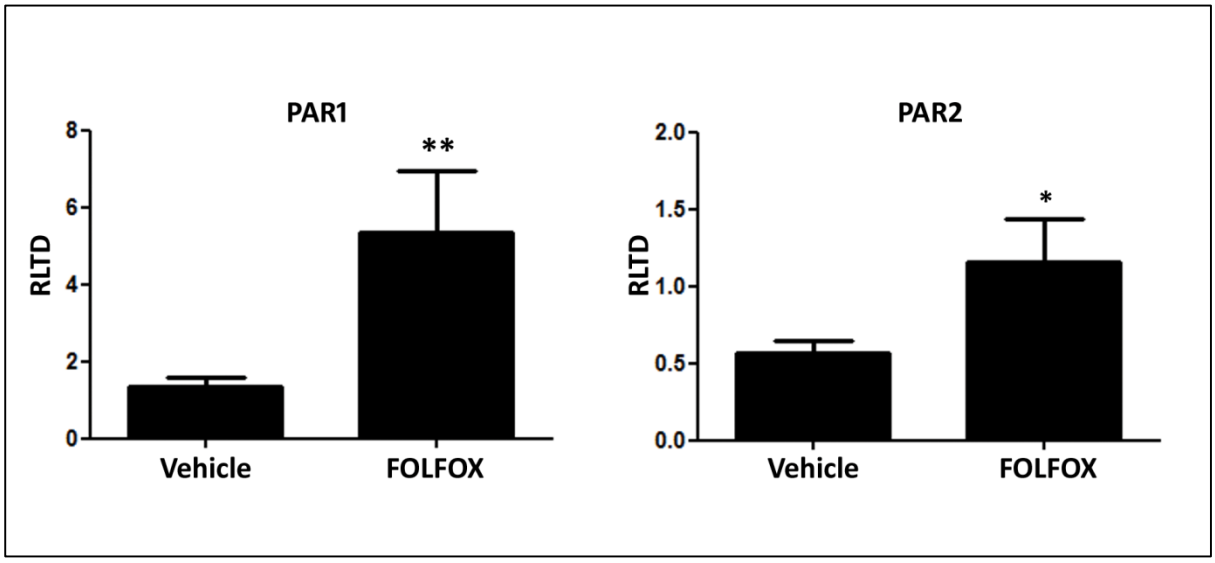

Figure 40 - Increased mRNA expression of the Factor X activated receptors PAR1 and PAR2 in the liver of mice with FOLFOX induced SOS

7.5 Summary of Key Findings

- FOLFOX induced SOS is associated with cellular senescence in the injured sinusoidal endothelium which expresses high levels of the cyclin dependent kinase inhibitor p21. This process seems to be driven by activation of the transcription factor p53.
- This process is associated with increased expression of the cytokine IL-6 which appears to result in increased phosphorylation of the oncogenic transcription factor STAT3 in peri-portal hepatocytes suggesting this is the driver of SOS associated regenerative hyperplasia
- SOS is associated with depletion of intracellular glutathione and diminished expression of the anti-oxidant transcription factor NRF2.
- Treatment of mice with the NRF2 activating anti-oxidant BHA prevents the development of FOLFOX induced SOS
- The development of SOS appears to be associated with a pro-thrombotic environment. Manipulation of this may be beneficial as a therapeutic target to prevent the development of SOS

Chapter 8

Discussion

The primary aim of this study was to establish a reproducible *in vivo* model of SOS induced by Oxaliplatin based chemotherapy. This has been achieved and represents a significant move forward for translational research relating to this condition. The only model in existence prior to this work was that of Monocrotaline administration to rats however for the reasons outlined in the Introduction to this thesis it was felt that this was poorly suited to study the pathogenesis of SOS in the current context and was therefore acting as a barrier to the development of effective therapeutics.

Perhaps the most surprising finding in the current study is that mice maintained on a standard chow diet seem to be relatively protected from developing a sinusoidal injury in response to FOLFOX as compared to those fed a purified diet despite identical drug treatment. Whilst exploring the mechanisms behind this observation is beyond the scope of the current study it is nonetheless interesting to hypothesise as to why this might be.

Purified diets, such as the one used in this study, are manufactured in a factory with refined ingredients of a known quantity and purity. In contrast chow diets are manufactured with plant materials and are designed to meet a minimum nutritional standard although the precise content is not known. The result of this is that chow diets will often contain high levels of phytoestrogens and these are well known to have protective effects on the development of a variety of liver diseases including steatosis and fibrosis.(Ascencio, Torres et al. 2004; McCarty, Barroso-Aranda et al. 2009)

It appears that the murine liver is relatively resistant to developing SOS after FOLFOX treatment since, even in animals fed a purified diet, I was not able to replicate the more advanced histological features seen in patients e.g. nodular regenerative hyperplasia despite high dose drug administration. As already discussed it may be that high expression of the ATP7B transporter, responsible for the cellular export of platinum, is in part responsible for this. The importance of this observation is that it highlights a potential role for pharmacogenomics in determining susceptibility to SOS. A recent study examined the genomic DNA of peripheral blood leukocytes in 203 cancer patients and identified polymorphisms of the ATP7B gene in 61 patients suggesting that these are relatively common in the general population.(Fukushima-Uesaka, Saito et al. 2009) None of these patients were reported to have clinical features of Wilson's disease which occurs as a consequence of specific mutations in this gene.

One of the unanswered questions regarding SOS is why some patients develop severe histological changes after a solitary short course of chemotherapy whilst others who receive multiple prolonged courses of chemotherapy do not. It is possible that pharmacogenomics, in particular the effect of functional polymorphisms in the ATP7B gene, could explain this observation by altering individual susceptibility to Oxaliplatin. This concept is not new, for example it has long been recognised that patients with polymorphisms in the UGT1A1 gene demonstrate increased toxicity to Irinotecan and as such it is common place to reduce the dose of drug given to these patients.(Kweekel, Guchelaar et al. 2008; Marsh and Hoskins 2010) On this basis it would seem appropriate to establish further studies in patients exploring the relationship between ATP7B polymorphisms and SOS development.

An alternative explanation for the varying individual susceptibility to SOS may be found in the tumour response to chemotherapy and indeed in this study I have shown that the presence of tumour within the liver accelerates gene expression changes associated with the development of SOS. The concept that tumour death in response to chemotherapy is immunologically silent with no effect on the external environment has been challenged and it is now acknowledged that a wide variety of chemotherapeutic agents are able to illicit an immune reaction by stimulating an inflammatory response by the tumour.(Tesniere, Apetoh et al. 2008; Martins, Tesniere et al. 2009) In support of the hypothesis for tumour death contributing to the development of SOS it has been shown that patients with a higher tumour burden are more likely to develop SOS independently of the number of chemotherapy cycles received.(Tamandl, Klinger et al. 2011)

A potential explanation of how the tumour response might contribute to the development of SOS is provided by the cytokine CXCL1, the murine IL-8 equivalent. This cytokine is produced in high quantities by FOLFOX treated MCA38 cells as well as being elevated in the serum of patients with SOS.(Schots, Kaufman et al. 2003) Acting through the CXCR2 receptor CXCL1 is able to increase the transcription of genes implicated in matrix remodelling and angiogenesis in SOS such as MMP2 and VEGF-A.(Li, Dubey et al. 2003; Li, Varney et al. 2005) Furthermore IL-8 signalling in human endothelial cells is reported to increase the expression of PAI-1 which this study has demonstrated to be strongly implicated in the pathogenesis of SOS.(Cheng, Li et al. 2008)

The marked elevation in PAI-1 expression in FOLFOX treated mice resulted in the identification of replicative senescence within the damaged sinusoidal endothelium as a potential mechanism involved in SOS pathogenesis. Rather than being a passive marker of

senescence, as was previously thought, it is now recognised that it is essential both for the induction and maintenance of this condition by down regulating signalling in the mitogenic PI3K-PKB pathway which is common to many pro-proliferative growth factors.(Kortlever, Higgins et al. 2006) On the basis of the evidence presented in this study it would seem that endothelial senescence arises as a consequence of both direct drug induced DNA damage and oxidative stress. In reality it would not be plausible to target DNA damage therapeutically to prevent SOS development without having a negative impact on tumour response and therefore oxidative stress was pursued instead.

Working with the Monocrotaline model SOS Wang et al. reported that intra-portal infusion of both NAC and glutathione were effective in preventing the development of SOS.(Wang, Kanel et al. 2000) In contrast to this I was not able to demonstrate any therapeutic benefit to dietary supplementation with 3% NAC. One explanation for this discrepancy may be that the route of administration chosen in the current study was not appropriate to allow sufficient concentrations of NAC to reach the liver however this seems unlikely since it is well recognised that NAC is well absorbed from the gut and directly enters the portal circulation.(Smilkstein, Knapp et al. 1988) It may be that the administration of chemotherapy impairs the absorption of NAC from the gut – it is well recognised that 5-FU administration is associated with mucositis and the subsequent development of diarrhoea in patients.(Petrelli, Cabiddu et al. 2012)

For the first time we have shown that the development of SOS after FOLFOX treatment is associated with down regulation of the antioxidant transcription factor NRF2. Furthermore mice receiving the NRF2 activating antioxidant BHA alongside FOLFOX treatment do not develop histological features of SOS suggesting that targeting this pathway may be of some

translational value. One of the key concerns when developing drugs to prevent the complications of systemic chemotherapy is that one does not have a negative impact on the cytotoxic effect. The NRF2 pathway is particularly attractive in this regard since it has been shown that the Curcumin, an NRF2 activator, can actually increase the cytotoxicity of conventional chemotherapeutics and is currently the subject of several clinical trials in this regard.(Patel, Gupta et al. 2010) To have an agent that could both reduce the incidence of SOS and increase the cytotoxicity of Oxaliplatin would be a major breakthrough and this is an area that should be pursued in more detail in further studies.

An alternative pathological process identified in this study which is of potential relevance to the development of SOS is generation of a pro-thrombotic environment within the liver. There is some evidence from clinical studies to support this hypothesis in that patients treated with Oxaliplatin whilst taking regular Aspirin appear to have a lower incidence of SOS.(Brouquet, Benoist et al. 2009) In addition in patients receiving myeloablative chemotherapy prior to bone marrow transplantation the fibrinolytic agent defibrotide has demonstrated some success in treating SOS.(Benimetskaya, Wu et al. 2008) The most obvious explanation for how a pro-thrombotic environment contributes to the development of SOS would be that it leads to the generation of thrombi within the hepatic sinusoid resulting in microvascular occlusion. It should be noted however that there is significant cross talk between the coagulation system and a variety of cellular processes including matrix remodelling which is mediated via the PAR receptors. For example Factor Xa acting via the PAR1 receptor is able to convert TGFβ from its latent to active form thereby contributing to the development of liver fibrosis.(Fiorucci, Antonelli et al. 2004; Rullier, Gillibert-Duplantier et al. 2008; Scotton, Krupiczojc et al. 2009) Whilst it may be attractive

to consider anticoagulation as a strategy to reduce the incidence of SOS it is possible that this would lead to an increase in tumour related bleeding complications and as such further work should proceed cautiously.

In conclusion the studies presented in this thesis describe a reproducible model of Oxaliplatin induced SOS which more closely replicates events in patients treated with these regimens. Using this model I have identified several pathological processes involved in the development of this condition. In particular through manipulation of the NRF2 pathway I have identified a therapeutic target which may be of value in reducing the incidence of SOS. It is hoped that further work using this model will validate these findings and quickly lead to the first studies in patients.

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Appendix 1

Summary of Studies Included in Systematic Review

Reference	Year	Study Type	Comparisons	n	NOS	Evidence Level	Key Findings	Overlap with Other Studies
Adam et al.(Adam, Bhangui et al. 2010)	2010	CS(R)	Neoadjuvant Chemotherapy vs. Surgery Alone	1471	7	2b	The use of pre-operative chemotherapy does not seem to offer any benefit to patients with a solitary metachronous colorectal liver metastases	Data from LiverMet survey (i.e. multiple centres)
Aloia et al.(Aloia, Sebagh et al. 2006)	2006	CS(R)	Neoadjuvant (OHP based) Chemotherapy vs. Surgery Alone	75	8	2b	The main hepatic injury following OHP based chemotherapy is vascular not steatosis. The risk of complications is related to the duration of chemotherapy.	
Aloysius et al.(Aloysius, Zaitoun et al. 2007)	2007	CC(R)	Neoadjuvant (FOLFOX-4) Chemotherapy vs. Surgery Alone	50	7	3b	The use of neoadjuvant FOLFOX-4 is associated with hepatic steatosis and sinusoidal dilatation	
D'Angelica et al.(D'Angelica, Kornprat et al. 2007)	2006	CC(R)	Neoadjuvant Chemotherapy vs. Neoadjuvant Chemotherapy & Bevacizumab	32	8	3b	The use of pre-operative Bevacizumab is not associated with an increase in peri-operative complications	
Nordlinger et al.(Nordlinger, Sorbye et al. 2008)	2008	RCT	Peri-operative (FOLFOX) Chemotherapy vs. Surgery Alone	364		1b	Preoperative FOLFOX-4 chemotherapy increases the risk of peri-operative complications but improves progression free survival	Multi-centre RCT
Folprecht et al.(Folprecht, Gruenberger et al. 2010)	2010	RCT	Pre-operative FOLOX & Cetuximab vs. FOLFIRI & Cetuximab	45		1b	There is little difference in morbidity between the two groups	Multi-centre RCT
Gomez et al.(Gomez, Malik et al. 2007)	2007	CS(R)	Hepatic Steatosis vs. No Hepatic Steatosis	386	8	2b	Hepatic steatosis increases the morbidity of liver resection	(Malik, Farid et al. 2007)
Gomez-Ramirez et al.	2010	CS(P)	Neoadjuvant Chemotherapy vs. Surgery alone	45	6	2b	Neoadjuvant Irintocean is associated with an increased risk of steatohepatitis	
Hewes et al.(Hewes, Dighe et al. 2007)	2007	CS(R)	Neoadjuvant Chemotherapy vs. Surgery alone	67	8	2b	Neoadjuvant OHP based chemotherapy increases the risk associated with liver resection	
Hubert et al.(Hubert,	2010	CS(R)	Neoadjuvant Chemotherapy	114	8	2b	Neoadjuvant chemotherapy is	

Fervaille et al. 2010)			vs. Surgery alone				associated with sinusoidal congestion but has no impact on peri-operative outcome	
Kandutsch et al.(Kandutsch, Klinger et al. 2008)	2008	CS(R)	Neoadjuvant (OHP based) Chemotherapy vs. Surgery Alone	63	8	2b	Sinusoidal obstruction but not steatohepatitis occurs as a consequence of OHP based chemotherapy	(Klinger, Eipeldauer et al. 2009; Tamandl, Klinger et al. 2011)
Karoui et al.(Karoui, Penna et al. 2006)	2006	CS(R)	Neoadjuvant Chemotherapy vs. Surgery Alone	67	7	2b	Prolonged chemotherapy injures the hepatic parenchyma and increases the morbidity of liver resection when performed under total vascular exclusion	
Kesmodel et al.(Kesmodel, Ellis et al. 2008)	2008	CS(R)	Neoadjuvant Chemotherapy vs. Neoadjuvant Chemotherapy & Bevacizumab	125	8	2b	The use of pre-operative Bevacizumab in conjunction with traditional chemotherapy is not associated with an increase in surgical morbidity	(Vauthey, Pawlik et al. 2006; Ribero, Wang et al. 2007; Kishi, Zorzi et al. 2010; Rubbia-Brandt, Lauwers et al. 2010)
Kishi et al.(Kishi, Zorzi et al. 2010)	2010	CS(R)	Neoadjuvant FOLFOX vs. Neoadjuvant FOLFOX & Bevacizumab	219	8	2b	Extended pre-operative chemotherapy increases the risk of parenchymal injury without improving pathological response	(Vauthey, Pawlik et al. 2006; Ribero, Wang et al. 2007; Kesmodel, Ellis et al. 2008; Rubbia-Brandt, Lauwers et al. 2010)
Klinger et al.(Klinger, Eipeldauer et al. 2009)	2009	CS(R)	Neoadjuvant (OHP based) Chemotherapy vs. Neoadjuvant (OHP) based chemotherapy & Bevacizumab	99	7	2b	Bevacizumab protects against sinusoidal obstruction syndrome but does not improve tumour response to OHP based chemotherapy	(Kandutsch, Klinger et al. 2008; Tamandl, Klinger et al. 2011)
Komori et al.(Komori, Beppu et al. 2010)	2010	CS(R)	Neoadjuvant (FOLFOX) Chemotherapy vs. Surgery alone	27	8	2b	FOLFOX use results in parenchymal injury but has no effect on peri-operative morbidity and mortality	
Konopke et al.(Konopke, Kersting et al. 2009)	2009	CS(R)	Neoadjuvant Chemotherapy vs. Surgery alone	240	8	2b	Preoperative chemotherapy is an independent risk factor for post-operative complications in patients undergoing major liver resection	
Lubezky et al.(Lubezky, Geva et	2009	CS(R)	Neoadjuvant Chemotherapy vs. Adjuvant Chemotherapy	56	8	2b	Post-operative morbidity is higher in patients receiving neoadjuvant	

al. 2009)							chemotherapy	
Mahfud et al.(Mahfud, Breitenstein et al. 2010)	2010	CS(R)	Neoadjuvant Chemotherapy vs. Neoadjvuant Chemotherapy & Bevacizumab	90	8	2b	The addition of Bevacizumab to conventional chemotherapy does not impact on peri-operative outcome	(Nakano, Oussoultzoglou et al. 2008)
Makowiec et al.(Makowiec, Mohrle et al. 2011)	2011	CS(R)	Neoadjuvant Chemotherapy vs. Surgery alone	102	7	2b	Neither pre-operative chemotherapy or the presence of parenchymal injury affect peri-operative outcome	
Malik et al.(Malik, Farid et al. 2007)	2007	CC(R)	Neoadjuvant (OHP Based) Chemotherapy vs. Surgery alone	155	8	3b	Neoadjuvant chemotherapy had no impact on peri-operative outcome	(Gomez, Malik et al. 2007)
Mehta et al.(Mehta, Ravikumar et al. 2008)	2008	CS(R)	Neoadjuvant Chemotherapy vs. Surgery alone	173	6	2b	Oxaliplatin based chemotherapy is associated with a vascular injury to the liver parenchyma but this has no effect on peri-operative outcome	
Nakano et al.(Nakano, Oussoultzoglou et al. 2008)	2008	CS(R)	Neoadjuvant (OHP Based) Chemotherapy vs. Neoadjuvant (Other regimens) Chemotherapy	90	8	2b	OHP based chemotherapy is associated with an increased incidence of sinusoidal injury. Sinusoidal injury is associated with a poorer outcome after major hepatectomy.	(Mahfud, Breitenstein et al. 2010)
O'Rourke et al.(O'Rourke, Welsh et al. 2009)	2009	CS(P)	Neoadjuvant Chemotherapy vs. Surgery alone	37	8	2b	Liver specific MRI can accurately predict the severity of parenchymal injury	(Welsh, Tilney et al. 2007)
Ouaissi et al.(Ouaissi, Moutardier et al. 2006)	2006	CS(R)	Neoadjuvant Chemotherapy vs. Surgery alone	40	6	2b	Pre-operative chemotherapy does not influence the outcome of liver resection	
Pawlik et al.(Pawlik, Olino et al. 2007)	2007	CS(R)	Neoadjuvant Chemotherapy vs. Surgery alone	212	8	2b	Neoadjuvant chemotherapy is associated with parenchymal injury in 20 – 30% of patients. The nature of the injury is regimen specific.	
Reddy et al.(Reddy, Morse et al. 2008)	2008	CS(R)	Neoadjuvant Chemotherapy vs. Neoadjuvant Chemotherapy & Bevacizumab	96	8	2b	The addition of Bevacizumab to conventional chemotherapy does not increase morbidity following liver resection	

Ribero et al.(Ribero, Wang et al. 2007)	2007	CS(R)	Neoadjuvant (OHP Based) Chemotherapy vs. Neoadjuvant (OHP Based) Chemotherapy & Bevacizumab	105	8	2b	The addition of Bevacizumab to OHP based chemotherapy reduces the incidence of sinusoidal injury and increases tumour response to chemotherapy as assessed histologically	(Vauthey, Pawlik et al. 2006; Kesmodel, Ellis et al. 2008; Kishi, Zorzi et al. 2010; Rubbia-Brandt, Lauwers et al. 2010)
Rubbia-Brandt et al.(Rubbia-Brandt, Audard et al. 2004)	2004	CS(R)	Neoadjuvant Chemotherapy vs. Surgery alone	153	6	2b	Neoadjuvant OHP based chemotherapy is associated with sinusoidal obstruction syndrome	(Rubbia-Brandt, Lauwers et al. 2010)
Rubbia-Brandt et al.(Rubbia-Brandt, Lauwers et al. 2010)	2010	CS(R)	Neoadjuvant (OHP Based) chemotherapy vs. Neoadjuvant (OHP Based) chemotherapy & Bevacizumab vs. Surgery alone	385	6	2b	OHP based chemotherapy is associated with sinusoidal obstruction syndrome the incidence of which is reduced if used alongside Bevacizumab	(Rubbia-Brandt, Audard et al. 2004; Vauthey, Pawlik et al. 2006; Ribero, Wang et al. 2007; Kesmodel, Ellis et al. 2008; Kishi, Zorzi et al. 2010)
Ryan et al.(Ryan, Nanji et al. 2010)	2010	CS(R)	Neoadjuvant Chemotherapy vs. Surgery alone	334	8	2b	Neoadjuvant chemotherapy is associated with a vascular injury to the hepatic parenchyma but not steatohepatitis	(Sahajpal, Vollmer et al. 2007)
Sahajpal et al.(Sahajpal, Vollmer et al. 2007)	2007	CS(R)	Neoadjuvant Chemotherapy vs. Surgery alone	96	7	2b	Neoadjuvant chemotherapy does not affect short term outcomes following liver resection	(Ryan, Nanji et al. 2010)
Scoggins et al.(Scoggins, Campbell et al. 2009)	2008	CS(R)	Neoadjuvant Chemotherapy vs. Surgery alone	186	8	2b	Neoadjuvant chemotherapy does not affect the morbidity associated with liver resection	
Tamandl et al.(Tamandl, Klinger et al. 2011)	2011	CS(R)	Neoadjuvant Chemotherapy vs. Surgery alone	196	8	2b	OHP induced sinusoidal obstruction is associated with poorer overall and disease specific survival	(Kandutsch, Klinger et al. 2008; Klinger, Eipeldauer et al. 2009)
Vauthey et al.(Vauthey, Pawlik et al. 2006)	2006	CS(R)	Neoadjuvant Chemotherapy vs. Surgery alone	406	8	2b	Neoadjuvant Irinotecan based chemotherapy is associated with the development of steatohepatitis	(Ribero, Wang et al. 2007; Kesmodel, Ellis et al. 2008; Kishi, Zorzi et al. 2010; Rubbia-Brandt, Lauwers et al. 2010)
Welsh et al.(Welsh, Tilney et al. 2007)	2007	CS(R)	Neoadjuvant Chemotherapy vs. Surgery alone	497	8	2b	Liver resection is safe following neoadjuvant chemotherapy	(O'Rourke, Welsh et al. 2009)
Yebidela et	2005	CC(R)	Neoadjuvant Chemotherapy	64	8	3b	Neoadjuvant chemotherapy does	

al.(Yebidela, Elad et al. 2005)			vs. Surgery alone				not increase surgical morbidity or mortality	

Appendix 2

Dietary Constituents

Product Data

DIO SERIES DIETS

OpenSource DIETS™

The "Original" High Fat Diets for Diet Induced Obesity

Formulas

Product #	D12450B		D12451		D12492	
	gm%	kcal%	gm%	kcal%	gm%	kcal%
Protein	19.2	20	24	20	26.2	20
Carbohydrate	67.3	70	41	35	26.3	20
Fat	4.3	10	24	45	34.9	60
Total		100		100		100
kcal/gm	3.85		4.73		5.24	
Ingredient	**gm**	**kcal**	**gm**	**kcal**	**gm**	**kcal**
Casein, 80 Mesh	200	800	200	800	200	800
L-Cystine	3	12	3	12	3	12
Corn Starch	315	1260	72.8	291	0	0
Maltodextrin 10	35	140	100	400	125	500
Sucrose	350	1400	172.8	691	68.8	275.2
Cellulose, BW200	50	0	50	0	50	0
Soybean Oil	25	225	25	225	25	225
Lard*	20	180	177.5	1598	245	2205
Mineral Mix S10026	10	0	10	0	10	0
DiCalcium Phosphate	13	0	13	0	13	0
Calcium Carbonate	5.5	0	5.5	0	5.5	0
Potassium Citrate, 1 H2O	16.5	0	16.5	0	16.5	0
Vitamin Mix V10001	10	40	10	40	10	40
Choline Bitartrate	2	0	2	0	2	0
FD&C Yellow Dye #5	0.05	0				
FD&C Red Dye #40			0.05	0		
FD&C Blue Dye #1					0.05	0
Total	**1055.05**	**4057**	**858.15**	**4057**	**773.85**	**4057**

Formulated by E. A. Ulman, Ph.D., Research Diets, Inc., 8/26/98 and 3/11/99.

*Typical analysis of cholesterol in lard = 0.95 mg/gram. D12450B -
Cholesterol (mg)/4057 kcal = 19
Cholesterol (mg)/kg = 18
D12451 -
Cholesterol (mg)/4057 kcal = 168.6
Cholesterol (mg)/kg = 196.5
D12492 -
Cholesterol (mg)/4057 kcal = 232.8
Cholesterol (mg)/kg = 300.8

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OpenSource DIETS™ Formula

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D12450B and D01060501

Rodent Diet With 10 kcal% Fat with Corn Starch and Sucrose or Corn Starch Alone

Product #	D12450B		D12451		D01060501		D01060502	
%	gm	*kcal*	gm	*kcal*	gm	*kcal*	gm	*kcal*
Protein	19.2	*20*	23.7	*20*	19.2	*20*	23.7	*20*
Carbohydrate	67.3	*70*	41.4	*35*	67.3	*70*	41.4	*35*
Fat	4.3	*10*	23.6	*45*	4.3	*10*	23.6	*45*
Total	90.8	*100*	88.7	*100*	90.8	*100*	88.7	*100*
kcal/gm	3.85		4.73		3.85		4.73	
Ingredient	**gm**	***kcal***	**gm**	***kcal***	**gm**	***kcal***	**gm**	***kcal***
Casein, Lactic	200	*800*	200	*800*	200	*800*	200	*800*
L-Cystine	3	*12*	3	*12*	3	*12*	3	*12*
Corn Starch	315	*1260*	72.8	*291*	575	*2300*	220.6	*882*
Maltodextrin 10	35	*140*	100	*400*	125	*500*	125	*500*
Sucrose	350	*1400*	172.8	*691*	0	*0*	0	*0*
Cellulose, BW200	50	*0*	50	*0*	50	*0*	50	*0*
Soybean Oil	25	*225*	25	*225*	25	*225*	25	*225*
Lard	20	*180*	177.5	*1598*	20	*180*	177.5	*1598*
Mineral Mix S10026	10	*0*	10	*0*	10	*0*	10	*0*
DiCalcium Phosphate	13	*0*	13	*0*	13	*0*	13	*0*
Calcium Carbonate	5.5	*0*	5.5	*0*	5.5	*0*	5.5	*0*
Potassium Citrate, 1 H2O	16.5	*0*	16.5	*0*	16.5	*0*	16.5	*0*
Vitamin Mix V10001	10	*40*	10	*40*	10	*40*	10	*40*
Choline Bitartrate	2	*0*	2	*0*	2	*0*	2	*0*
FD&C Yellow Dye #5	0.05	*0*	0	*0*	0.025	*0*	0	*0*
FD&C Red Dye #40	0	*0*	0.05	*0*	0.025	*0*	0	*0*
FD&C Blue Dye #1	0	*0*	0	*0*	0	*0*	0.05	*0*
Total	**1055.05**	***4057***	**858.15**	***4057***	**1055.05**	***4057***	**858.15**	***4057***

D01060501.for.xls

See VanHeek et al., J. Clin. Invest. 99:385-390, 1997

E.A. Ulman, Ph.D., Research Diets, Inc, 8/26/98 and 6/4/01.

RESEARCH DIETS™
Standard & Custom Diets for Laboratory Animals

RESEARCH DIETS, INC.
20 Jules Lane, New Brunswick, NJ 08901-3275 USA
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V10001

Vitamin Mix for AIN-76A Rodent Diet

Use at 10 gm/kg diet or
10 gm/4000 kcal digestible energy.

Ingredient	gm	Amount in 10 gm
Vitamin A Palmitate 500,000 IU/gm	0.8	4,000 IU
Vitamin D_3 100,000 IU/gm	1.0	1,000 IU
Vitamin E Acetate 500 IU/gm	10.0	50 IU
Menadione Sodium Bisulfite 62.5% Menadione	0.08	0.5 mg
Biotin, 1.0%	2.0	0.2 mg
Cyancocobalamin, 0.1%	1.0	10 ug
Folic Acid	0.2	2 mg
Nicotinic Acid	3.0	30 mg
Calcium Pantothenate	1.6	16 mg
Pyridoxine-HCl	0.7	7 mg
Riboflavin	0.6	6 mg
Thiamin HCl	0.6	6 mg
Sucrose	978.42	
Total	**1000.**	

J. Nutr. 107:1340-1348, 1977.
J. Nutr. 110:1726, 1980.

V10001.FOR

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S10026B

RD-96 Salt Mix

Use at 50 gm/kg diet or
50 gm/4000 kcal digestible energy.

Ingredient	gm		Amount in 50 gm
Calcium Phosphate, Dibasic 29.5% Ca, 22.8% P	260	Ca	6.0 gm
Calcium Carbonate 39.3% Ca	110	P	3.0 gm
Potassium Citrate, 1 H_2O 36.2% K	330	K	6.0 gm
Sodium Chloride	51.8	Na	1.0 gm
39.3% Na, 60.7% Cl		Cl	1.6 gm
Magnesium Oxide 60.3% Mg	8.38	Mg	0.5 gm
Magnesium Sulfate, 7 H_2O 9.87% Mg, 13.0% S	51.52	S	0.33 gm
Chromium K Sulfate, 12 H_2O, 10.4% Cr	0.385	Cr	2.0 mg
Cupric Carbonate, 57.5% Cu	0.21	Cu	6.0 mg
Sodium Fluoride, 45.2% Fl	0.04	Fl	0.9 mg
Potassium Iodate, 59.3% I	0.007	I	0.2 mg
Ferric Citrate, 21.2% Fe	4.2	Fe	45. mg
Manganous Carbonate, 47.8% Mn	2.45	Mn	59. mg
Ammonium Molybdate, 4 H_2O, 54.3% Mo	0.06	Mo	1.6 mg
Sodium Selenite, 45.7% Se	0.007	Se	0.16 mg
Zinc Carbonate, 52.1% Zn	1.12	Zn	29. mg
Sucrose	179.821		
TOTAL	1000.		

Formulated by E.A. Ulman, Ph.D., Research Diets, Inc., January 18, 1996. When used at 50 gm/kg diet this salt mix adds the same amount of all of the elements added by 35 gm of AIN-76 salts, except calcium is raised from 5.2 gm to 6 gm, phosphorous is lowered from 4 gm to 3 gm, potassium is raised from 3.6 gm to 6 gm and florine and molybdenum are added.

Special Diets Services

Rat and Mouse No.3 Breeding

Expanded, Expanded Short and Expanded Ground

SUITABLE SPECIES AND APPLICATIONS

Rats and mice for breeding, lactation, and growth of young stock.

BENEFITS

- High nutrient levels promote excellent breeding performances and fast growth rates in young stock.
- Expanded diets have improved palatability, suffer less wastage and are microbiologically cleaner due to the high processing temperature.

FEEDING GUIDE

Ad-lib feeding is recommended.

AVAILABLE AS

Diet	Form	Product Code
Standard		
RM3 (E)	*Expanded*	*801066*
RM3 (E) DU	*Expanded Short*	*801080*
RM3 (E) FG	*Expanded Ground*	*801067*
SQC		
RM3 (E) SQC	*Expanded*	*811181*
RM3 (E) FG SQC	*Expanded Ground*	*811182*

- All diets are available irradiated and are available in a range of packaging.
- All Standard diets are available with full analysis on request.

INGREDIENTS

Wheat, Wheatfeed, De-hulled Extracted Toasted Soya, Barley, Fish Meal, Whey Powder, Macro Minerals, Yeast, Soya Oil, Vitamins, Micro Minerals, Amino Acids.

Rat and Mouse No.3 Breeding (E)

Calculated Analysis

NUTRIENTS		Total	Supp (9)
Proximate Analysis			
Moisture (1)	%	10.00	
Crude Oil	%	4.25	
Crude Protein	%	22.39	
Crude Fibre	%	4.21	
Ash	%	7.56	
Nitrogen Free Extract	%	51.20	
Digestibility Co-Efficients (7)			
Digestible Crude Oil	%	3.86	
Digestible Crude Protein	%	20.21	
Carbohydrates, Fibre and Non Starch Polysaccharides (NSP)			
Total Dietary Fibre	%	15.43	
Pectin	%	1.43	
Hemicellulose	%	9.20	
Cellulose	%	3.93	
Lignin	%	1.50	
Starch	%	33.92	
Sugar	%	5.75	
Energy (5)			
Gross Energy	MJ/kg	15.21	
Digestible Energy (15)	MJ/kg	12.42	
Metabolisable Energy (15)	MJ/kg	11.36	
Atwater Fuel Energy (AFE) (8)	MJ/kg	13.90	
AFE from Oil	%	11.50	
AFE from Protein	%	26.93	
AFE from Carbohydrate	%	61.57	
Fatty Acids			
Saturated Fatty Acids			
C12:0 Lauric	%	0.05	
C14:0 Myristic	%	0.20	
C16:0 Palmitic	%	0.36	
C18:0 Stearic	%	0.09	
Monounsaturated Fatty Acids			
C14:1 Myristoleic	%	0.01	
C16:1 Palmitoleic	%	0.13	
C18:1 Oleic	%	1.03	
Polyunsaturated Fatty Acids			
C18:2(ω6) Linoleic	%	1.15	
C18:3(ω3) Linolenic	%	0.17	
C20:4(ω6) Arachidonic	%	0.22	
C22:5(ω3) Clupanodonic	%	0.04	
Amino Acids			
Arginine	%	1.54	
Lysine (6)	%	1.33	0.09
Methionine	%	0.34	
Cystine	%	0.34	
Tryptophan	%	0.27	
Histidine	%	0.57	
Threonine	%	0.86	
Isoleucine	%	0.98	
Leucine	%	1.68	
Phenylalanine	%	1.03	
Valine	%	1.10	
Tyrosine	%	0.80	
Taurine	%		
Glycine	%	1.88	
Aspartic Acid	%	1.43	

NUTRIENTS		Total	Supp (9)
Glutamic Acid	%	4.07	
Proline	%	1.38	
Serine	%	0.97	
Hydroxyproline	%	0.06	
Hydroxylysine	%		
Alanine	%	0.14	
Macro Minerals			
Calcium	%	1.15	0.56
Total Phosphorus	%	0.82	0.09
Phytate Phosphorus	%	0.25	
Available Phosphorus	%	0.58	0.09
Sodium	%	0.32	0.19
Chloride	%	0.43	0.31
Potassium	%	0.81	
Magnesium	%	0.29	0.04
Micro Minerals			
Iron	mg/kg	188.17	82.50
Copper	mg/kg	20.28	8.75
Manganese	mg/kg	102.01	52.70
Zinc	mg/kg	51.34	8.64
Cobalt	µg/kg	617.02	525.00
Iodine	µg/kg	1395.12	775.00
Selenium	µg/kg	497.70	200.00
Fluorine	mg/kg	9.24	
Vitamins			
β-Carotene (2)	mg/kg	0.15	
Retinol (2)	µg/kg	5977.24	5812.50
Vitamin A (2)	iu/kg	19923.60	19375.00
Cholecalciferol (3)	µg/kg	102.22	72.50
Vitamin D (3)	iu/kg	4088.65	2900.00
α-Tocopherol (4)	mg/kg	100.35	81.14
Vitamin E (4)	iu/kg	110.39	89.25
Vitamin B_1 (Thiamine)	mg/kg	27.08	19.11
Vitamin B_2 (Riboflavin)	mg/kg	10.60	7.60
Vitamin B_6 (Pyridoxine)	mg/kg	19.54	14.46
Vitamin B_{12} (Cyanocobalamine)	µg/kg	26.78	17.75
Vitamin C (Ascorbic Acid)	mg/kg	1.33	
Vitamin K (Menadione)	mg/kg	4.15	3.72
Folic Acid (Vitamin B_9)	mg/kg	2.73	0.49
Nicotinic Acid (Vitamin PP) (6)	mg/kg	85.00	19.11
Pantothenic Acid (Vitamin $B_{3/5}$)	mg/kg	40.27	23.80
Choline (Vitamin $B_{4/7}$)	mg/kg	1641.65	366.60
Inositol	mg/kg	1903.20	
Biotin (Vitamin H) (6)	µg/kg	322.87	

Notes

1. All values are calculated using a moisture basis of 10%.
   Typical moisture levels will range between 9.5 - 11.5%.
2. a. Vitamin A includes Retinol and the Retinol equivalents of β-carotene
   b. Retinol includes the Retinol equivalents of β-Carotene.
   c. 0.48 µg Retinol = 1 µg β-carotene = 1.6 iu Vitamin A activity
   d. 1 µg Retinol = 3.33* iu Vitamin A activity
   e. 1 iu Vitamin A = 0.3 µg Retinol = 0.6 µg β-carotene
   f. The standard analysis for Vitamin A does not detect β-carotene
3. 1 µg Cholecalciferol (D_3) = 40.0 iu Vitamin D
4. 1 mg all-*rac*-α-tocopherol = 1.1 iu Vitamin E activity
   1 mg all-*rac*-α-tocopherol acetate = 1.0 iu Vitamin E activity
5. 1 MJ = 239.23 Kcalories = 239.23 Calories = 239,230 calories
6. These nutrients coming from natural raw materials such as cereals may have low availabilities due to the interactions with other compounds.
7. Based on in-vitro digestibility analysis.
8. AF Energy = Atwater Fuel Energy = ((CO%/100)*9000)+((CP%/100)*4000)+((NFE%/100)*4000)/239.23
9. Supplemented nutrients from manufactured and mined sources.
15. Calculated.

Appendix 3

FOLFOX Dosing Schedule

Weight (g)	Dose (ug)	Volume of Stock Solution (uL)
20	120	120
21	126	126
22	132	132
23	138	138
24	144	144
25	150	150
26	156	156
27	162	162
28	168	168
29	174	174
30	180	180

Volume of Oxaliplatin 1mg/ml stock solution to administer according to body weight (6mg/kg dose)

Weight (g)	Dose (ug)	Volume of Stock Solution (uL)
20	1000	100
21	1050	105
22	1100	110
23	1150	115
24	1200	120
25	1250	125
26	1300	130
27	1350	135
28	1400	140
29	1450	145
30	1500	150

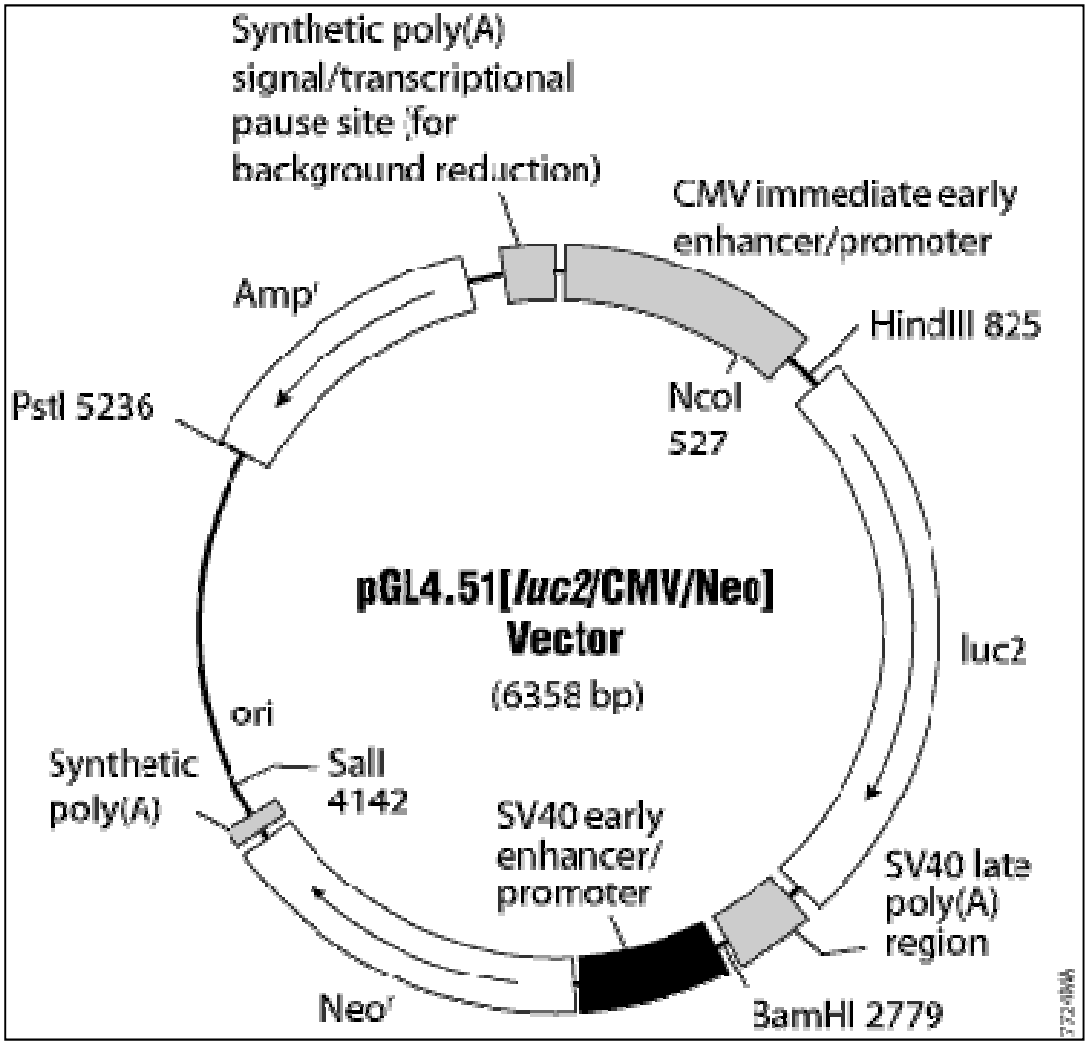
Volume of 5-FU 10mg/ml stock solution according to administer according to body weight (50mg/kg dose)

Weight (g)	Dose (ug)	Volume of Stock Solution (uL)
20	1800	144
21	1890	151.2
22	1980	158.4
23	2070	165.6
24	2160	172.8
25	2250	180
26	2340	187.2
27	2430	194.4
28	2520	202
29	2610	208.8
30	2700	216

Volume of Folinic Acid 12.5mg/ml stock solution to administer according to body weight (90mg/kg dose)

Appendix 4

Vector Map pGL4.51



Map downloaded from Promega On-Line Catologue (www.promega.com)

Appendix 5

Prizes Awarded to Work from this Thesis

The work contained within this thesis has been presented at a variety of regional, national and international meetings (see Appendix 6) where it has been awarded the following prizes:

AUGIS BJS Prize (for best original research presentation)

Digestive Disorders Federation, Liverpool, UK

19 June 2012

Ella Foster Memorial Award (for best original research presentation)

Newcastle upon Tyne Hospitals NHS Trust.

18 February 2011

Appendix 6

Presentations of Work Contained within this Thesis

Work contained within this thesis has been presented at regional, national and international meetings as follows:

1) Robinson SM, Mann J, Manas DM, Mann DA, White SA. An experimental model to investigate the pathogenesis of Oxaliplatin induced liver injury.
   - *ASCO GI Cancers Symposium* San Francisco January 2013 (Poster Presentation)
2) Robinson SM, Mann J, Vasilaki A, Manas DA, Mann DM, White SA. Antioxidant therapy is able to prevent the development of Oxaliplatin induced sinusoidal obstruction syndrome.
   - *Society of Academic and Research Surgeons Annual Scientific Meeting* London, January 2013 (Oral Presentation)
3) Robinson SM, Mann J, Manas DM, Mann DA, White SA. Identification of molecular pathways implicated in the pathogenesis of Oxaliplatin induced sinusoidal obstruction syndrome.
   - *Society of Academic and Research Surgeons Annual Scientific Meeting* London, January 2013 (Oral Presentation)
4) Robinson S, Mann J, Burt A, Manas D, Mann D, White S. A potential role for pharmacogenomics in determining Oxaliplatin induced tissue injury after down staging colorectal liver metastasis. HPB [Abstract] 2012; 14 (Suppl 2):152-3
   - *International Hepato-Pancreato-Biliary Association Biannual Meeting* Paris, July 2012 (Oral Presentation)

5) Robinson S, Wilson C, Burt A, Manas D, White S. Chemotherapy associated liver injury : A systematic review and meta-analysis. HPB [Abstract] 2012; 14 (Suppl 2):403-4

   - *International Hepato-Pancreato-Biliary Association Biannual Meeting*
     Paris, July 2012 (Poster)

6) Robinson S, Mann J, Burt A, Manas D, Mann D, White S. Clinical and experimental investigation of hepatic steatosis after FOLFOX chemotherapy for CRLM. HPB [Abstract] 2012; 14 (Suppl 2):404

   - *International Hepato-Pancreato-Biliary Association Biannual Meeting*
     Paris, July 2012 (Poster)

7) Robinson S, Mann J, Burt A, Manas D, Mann D, White S. Unpicking the pathogenesis of FOLFOX induced sinusoidal obstruction syndrome. HPB [Abstract] 2012; 14 (Suppl 2):404-5

   - *International Hepato-Pancreato-Biliary Association Biannual Meeting*
     Paris, July 2012 (Poster)

8) Robinson S, Mann J, Manas D, Mann D, White S. Gene expression in Oxaliplatin related sinusoidal obstruction syndrome. HPB [Abstract] 2012; 14 (Suppl 2):409

   - *International Hepato-Pancreato-Biliary Association Biannual Meeting*
     Paris, July 2012 (Poster)

9) Robinson S, Wilson C, Burt A, Manas D, White S. Chemotherapy associated liver injury : A systematic review and meta-analysis. Gut [Abstract] 2012; 61(Suppl 2): A357

   - *Digestive Disorders Federation*

     Liverpool, June 2012 (Poster)

10) Robinson S, Mann J, Manas D, Mann D, White S. Gene expression in Oxaliplatin related Sinusoidal Obstruction Syndrome. Gut [Abstract] 2012; 61(Suppl 2):A357

   - *Digestive Disorders Federation*

     Liverpool, June 2012 (Poster)

11) Robinson S, Mann J, Manas D, Burt A, Mann D, White S. An experimental study to determine the pathogenesis of Oxaliplatin induced sinusoidal obstruction syndrome. Gut [Abstract] 2012; 61(Suppl 2): A26

   - *Digestive Disorders Federation*

     Liverpool, June 2012 **(Oral Presentation; Winner of BJS Prize)**

12) Robinson SM, Mann J, Manas DM, Burt AD, Mann DA, White SA. An experimental model of FOLFOX-induced sinusoidal obstruction syndrome.

   - *ASCO GI Cancers Symposium*

     San Francisco, January 2012

13) Robinson SM, Mann J, Burt AD, Manas DM, Mann DA, White SA. Does a Pro-Thrombotic Envrionment Contribute to the Development of Chemotherapy Associated Liver Injury in Patients with Colorectal Liver Metastases.

- *Society of Academic and Research Surgeons Annual Scientific Meeting*

  Nottingham, January 2012.

14) Robinson SM, Mann J, Manas DM, Mann DA, White SA. Expression of the Copper Export Transporter ATPase7B Correlates with Tissue Specific Injury in a Murine Model of Colorectal Liver Metastases.

- *Society of Academic and Research Surgeons Annual Scientific Meeting*

  Nottingham, January 2012.

15) Robinson SM, Koshy A, Knisely A, Manas DM, Burt AD, White SA. Biliary Dysfunction in Patients with Sinusoidal Obstruction Syndrome.

- *Society of Academic and Research Surgeons Annual Scientific Meeting*

  Nottingham, January 2012.

16) Robinson S, Koshy A, Knisely A, Manas D, White S, Burt A. Biliary Dysfunction in Patients with Chemotherapy Induced Sinusoidal Obstruction Syndrome after Liver Resection for Colorectal Liver Metastases. EJSO [Abstract] 2011;37(11):995

- *British Association of Surgical Oncology Annual Meeting*

  London, November 2011(Poster)

17) Robinson S, Manas D, Mann D, White S, Mann J. Tumour cytokine production after FOLFOX chemotherapy is mediated by the NF-κB DNA damage response-an important contribution to chemo induced liver injury. EJSO [Abstract] 2011;37(11):995

- *British Association of Surgical Oncology Annual Meeting*

London, November 2011(Poster)

18) Robinson S, Mann J, Manas D, Mann D, Burt A, White S. An evaluation of hepatic steatosis/steatohepatitis after Oxaliplatin based chemotherapy for colorectal liver metastasis. EJSO [Abstract] 2011;37(11):995

- *British Association of Surgical Oncology Annual Meeting*

  London, November 2011(Poster)

19) Robinson S, Mann D, Burt A, Manas D, Mann J, White S. Mechanism of chemo-associated liver injury prior to liver resection for colorectal liver metastasis – a double hit phenomenon? EJSO [Abstract] 2011;37(11):995

- *British Association of Surgical Oncology Annual Meeting*

  London, November 2011(Poster)

20) Robinson SM, Mann J, Mann D, Manas DM, White SA. Expression of the Copper Export Transporter ATPase 7B Correlates with Tissue Specific Injury in a Murine Model of Colorectal Liver Metastases. EJSO [Abstract] 2011; 37(11):1003

- *British Association of Surgical Oncology Annual Meeting*

  London, November 2011(Poster)

21) Robinson SM, Mann J, Burt AD, Manas DM, Mann DA, White SA. Does a Pro-Thrombotic Envrionment Contribute to the Development of Chemotherapy Associated Liver Injury in Patients with Colorectal Liver Metastases.

- *4th International Conference on Coagulopathy in Liver Disease*

  London, September 2011.

22) Robinson SM, Mann D, Burt A, Manas D, Mann J, White SA. Mechanism of Chemo-Associated Liver Injury Prior to Liver Resection for Colorectal Liver Metastases – A Double Hit Phenomenon? British Journal of Surgery [Abstract] 2011; 98(S7):4

- *Association of Upper Gastrointestinal Surgeons of Great Britain and Ireland Annual Meeting*

  Belfast, September 2011(Oral Presentation)

23) Robinson SM, Manas D, Mann D, White S, Mann J. Tumour Cytokine Production after FOLFOX Chemotherapy is Mediated by the NF-κB DNA damage response-an Important Contribution to Chemo Induced Liver Injury. British Journal of Surgery [Abstract] 2011; 98(S7):4

- *Association of Upper Gastrointestinal Surgeons of Great Britain and Ireland Annual Meeting*

  Belfast, September 2011(Oral Presentation)

24) Robinson SM, Koshy A, Knisely A, Manas D, White S, Burt A. Biliary Dysfunction in Patients with Chemotherapy Induced Sinusoidal Obstruction Syndrome after Liver Resection for Colorectal Liver Metastasis. British Journal of Surgery [Abstract] 2011; 98(S7):17

- *Association of Upper Gastrointestinal Surgeons of Great Britain and Ireland Annual Meeting*

  Belfast, September 2011**(Poster of Distinction)**

25) Robinson S, Mann J, Manas D, Mann D, Burt A, White S. An Evaluation of Hepatic Steatosis/Steatohepatitis after Oxaliplatin Based Chemotherapy for Colorectal Liver Metastases. British Journal of Surgery [Abstract] 2011; 98(S7):46

- *Association of Upper Gastrointestinal Surgeons of Great Britain and Ireland Annual Meeting*

  Belfast, September 2011(Poster)

26) Robinson SM, Mann J, Burt AD, Manas DM, Mann DA, White SA. Pathogenesis of Chemotherapy Associated Liver Injury Prior to Major Hepatic Resection.

- *Society of Academic and Research Surgeons Annual Scientific Meeting*

  Dublin, January 2011

27) Robinson SM, Mann J, Burt AD, Manas DM, Mann DA, White SA. FOLFOX Therapy is not directly toxic to the murine liver. British Journal of Surgery [Abstract] 2010; 97(Supplement 5):16

- *Association of Upper Gastrointestinal Surgeons of Great Britain and Ireland Annual Meeting*

  Oxford, September 2010**(Poster of Distinction)**

28) Robinson S, Mann J, Manas DM, Mann D, White SA. Cytokine Production by Colorectal Tumours in Response to Irinotecan may be linked to the Development of CASH. HPB [Abstract]. 2010;12(Supplement 1):390

- *International Hepato-Pancreato-Biliary Association Biannual Meeting*

  Buenos Aires, April 2010(Poster)

29) Robinson SM, Mann J, Manas DM, Mann DA, White SA. Irinotecan treatment impairs expression of cytokines required for effective liver regeneration as a result of the NF-κB DNA damage response. HPB [Abstract]. 2010;12(Supplement 1):314

- *International Hepato-Pancreato-Biliary Association Biannual Meeting*

  Buenos Aires, April 2010(Poster)

Appendix 7

Publications arising from work contained within this Thesis

The following papers based on the work within this thesis have been either submitted, accepted for publication or published in peer reviewed journals:

1) Robinson SM, Mann J, Vasilaki A, Mathers J, Burt AD, White SA, Mann DA. Pathogensis of FOLFOX induced Sinusoidal Obstruction Syndrome in a Murine Chemotherapy Model. J Hepatology (Accepted for publication)
2) Robinson SM, Mann J, Manas DM, Mann DA, White SA. An experimental study to identify the potential role of pharmacogenomics in Oxaliplatin induced liver injury. HPB (Epub ahead of print)
3) Robinson SM, White SA. Hepatic Sinusoidal Obstruction Syndrome (SOS) reduces the effect of Oxaliplatin in colorectal liver metastases. Histopathology 2012 **61**(6):1247-8
4) Robinson SM, Wilson CH, Burt AD, Manas DM, White SA. Chemotherapy Associated Liver Inury in Patients with Colorectal Liver Metastases : A Systematic Review and Meta-analysis. Ann Surg Onc 2012 **19**(3):4287-99
5) Robinson S, Manas DM, Mann DA, White SA. Systemic Chemotherapy and its Implications for Resection of Colorectal Liver Metastasis Surgical Oncology. Surgical Oncology. 2011 **20**(2):57-72