Modulation of inflammation in the reduction of intra hepatic resistance

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I, Vikram Sharma, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

Modulation of inflammation in the reduction of intra hepatic resistance

In cirrhosis, there is a dichotomy in the haemodynamic abnormality in the intra hepatic and splanchnic circulation, as a result of an imbalance between vasoactive agents. In the intra-hepatic circulation, there is vasoconstriction secondary to endothelial dysfunction and vasodilatation in the splanchnic circulation which is provoked by a multitude of mechanisms. Inflammation has been also implicated in propagation of the haemodynamic abnormalities of portal hypertension in patients with acute on chronic liver failure (ACLF).

The studies conducted in this thesis aim to understand how modulating inflammation leads to the reduction of intra hepatic resistance. In the first part of the thesis, I validated the bile duct ligated rat model for my studies. I establish ADRA2a adrenergic receptor as the target receptor in this model to treat portal hypertension. I was able to show that a specific ADRA2a adrenergic receptor antagonist was able to improve the haemodynamic and inflammatory parameters. This strategy was also able to improve the function of the immune cells in this model. This provided a proof-of-concept for ADRA 2a adrenergic receptor as a target for intervention in ACLF to reduce inflammation and thereby reduce portal pressure.

In the second section of the thesis, I studied the role of arginine in the modulation of portal hypertension in a bile duct ligated rat model of cirrhosis. Thereafter, I showed a significant lowering of portal pressure in a BDL model of cirrhosis by L-Arginine supplementation via modulation of the NO-ADMA-DDAH and inflammatory pathway. The findings from this study provides the basis for novel agents for the treatment of portal hypertension.

.The studies conducted within the remit of this thesis provides valuable insight into the pathophysiology of portal hypertension and alludes to an alternative management strategy for portal hypertension.

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Role of various contributors

I have been intimately involved in the studies carried out at the UCL Institute of Hepatology and towards the later stages at the Institute of Liver and Digestive Health, UCL, Royal Free Campus. This included design of the study, carrying out all the rodents experiment and subsequent laboratory and data analyses. Due to the nature of the complex experimental designs for my studies, it was inevitable that I was helped by various people within and outside my research group. Dr. Nathan Davies, Senior Lecturer within the group was instrumental in facilitating the various experiments and guiding me through the various laboratory experiments. He also helped me in operating the FACS Canto II machine for the cytokine analysis and helped me in carrying out the haemodynamic measurement in the rodent experiments, while Abeba Habtesion and Naina Shah helped me with bile duct ligation operation on the rats to establish the model. Dr. Karla Lee, Veterinary Surgeon from the Royal Veterinary College helped me with the haemodynamic experiments on the rodents. Dr. Maria Jover-Cobos, post-doctoral fellow, Dr. Rita Garcia Martinez , Dr. Jane Macnaughtan, PhD student helped me collecting the tissue and plasma samples and undertaking some of the bench work. Dr. Gautam Mehta, Dr. Fausto Areobela, Francesco Chiari, PhD student within the group helped me with the collection of tissues for RNA extraction and with doing the PCR for the cell experiments. Dr. Junpei Sonada, Post-Doctoral Fellow from Dr. Jude Oben's group helped and guided me through the extraction of the Kupffer cells and PBMC cells and did the FACS analyses for the phagocytosis and ROS assay on the Kupffer cells population. Dr. Helen Jones PhD, post-doctoral fellow in our group helped me significantly in the later part of my

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Abbreviations

ALD	Alcoholic liver disease
NAD	Nicotinamide adenine dinucleotide
NFκB	Nuclear factor-kappa B
TNFα	Tumour necrosis factor alpha
BH_4	Tetrahydrobiopterin
NADPH	Nicotinamide adenine dinucleotide phosphate
HVPG	Hepatic venous pressure gradient
WHVP	Wedged hepatic venous pressure
FHVP	Free hepatic venous pressure
IHR	Intra hepatic resistance
MAP	Mean Arterial Pressure
NO	Nitric oxide
cGMP	Cyclic guanosine-3’,5-monophosphate
NOS	Nitric oxide synthethase
BDL	Bile duct ligation
ADMA	Asymmetrical Dimethyl Arginine
SDMA	Symmetrical Dimethyl Arginine
DDAH	Dimethylarginine Dimethylminohydrolase
PRMT	Protein methyl transferase

PPARγ	Peroxisome Proliferator-activated receptor gamma
HSC	Hepatic stellate cells
ARG	Arginine
PCR	Polymerase Chain Reaction
RNA	Ribo nucleic acid
ED	Endothelial Dysfunction
PAMPS	Pathogen associated molecular patterns
TLR	Toll like receptor
PAMP	Pathogen associated molecular pattern
DAMPS	Damage associated molecular pattern
HMGB1	High Mobility Group Box 1
NA	Noradrenaline

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For Rashmi, Vihaan and Vaanya

Chapter 1

Modulation of inflammation in the reduction of intra hepatic resistance

1.1 Introduction

The first published description of cirrhosis of the liver was in 1817 by Samuel Black of Newry, Ireland. The caption of this paper is: “Dissections of Two Habitual Drunkards ”. The case was observed in 1808 and the paper read in 1816. It has been published in the “Transactions of the Association of Fellows and Licentiates of the King’s and Queen’s College of Physicians in Ireland”, Dublin (1817), Vol. I, pp. 56-62. Wherein, he describes the post mortem findings of the liver of a journeyman baker, who had a significant alcohol history. He states that the liver was shrunken to about a third of the size, was hard and rigid and dark in colour. He commented that there were numerous tubercle with variable size and this was also found on the liver parenchyma.

From this early description of cirrhosis, it has become the eighteenth most common cause of death in the world and seventh in Europe (WHO data).In data published by the Office of National Statistics, cirrhosis of the liver is the second most common cause of death in male patients aged between 34 to 55 years accounting for about ten percent of deaths. And even more significantly, the second most common cause of death in female patients in the age group of 24 to 55 years is cirrhosis of the liver accounting up to eight percent of the total deaths.

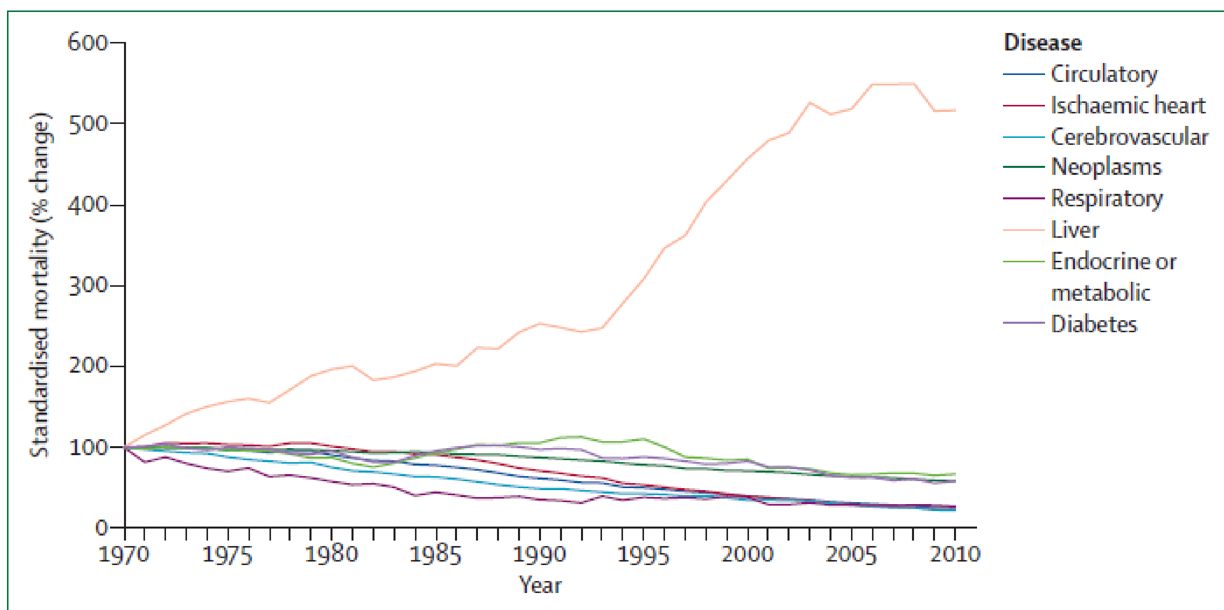

Fig 1: National Audit Office data: Standardized mortality rate for chronic disease in UK

In another set of data published by the National Audit office, it is clear that while most other cause of death is going down in the United Kingdom, the death rate from cirrhosis has gone up significantly as shown in Figure 1. As a consequence of this increase in mortality and morbidity associated with cirrhosis in the UK the increase in chronic liver disease poses particular problems with respect significant costs to the public both in terms of treatment and in loss of productivity. The impact of increase incidence notably alcohol intake, obesity and type 2 diabetes both here in the UK and globally implies that these problems are likely to increase in the future.

The incidence of cirrhosis has been estimated by several studies but all these studies calculated the numbers from the death certification figures. These figures may therefore

over-estimate the actual number of patients with cirrhosis. In a recent study from Nottingham, the incidence of cirrhosis was calculated after extrapolation of data from the general practice research database.

It is estimated in these studies estimate 76 per 100,000 people were living with cirrhosis in 2001 and there is a 45% increase in the incidence of cirrhosis in the last decade, majority of them attributable to alcohol. Disease progression with cirrhosis among this mainly ambulatory population was rapid with a rate of decompensation in people with compensated disease of 5% per year and 1 in 10 dying in the first year following diagnosis. This figure increased to 25% of people dying within one year for those with decompensated disease. Mortality in subjects with compensated and decompensated cirrhosis was 93.4 and 178.0 per 1000 person years compared with only 19.2 per 1000 person years in the general control population. Following adjustment for age and sex people with compensated and decompensated disease were respectively 5 and 10 times more likely to die than the general population (1). The statistical data presented above implies that the burden of liver disease will continue to challenge us to find strategies to improve the outcome in the next century.

1.2 The Pathophysiology of Portal hypertension

Portal hypertension is the most common haemodynamic abnormality caused by cirrhosis of the liver in the western world (2). The onset of portal hypertension heralds the beginning of complications such as variceal bleeding, ascites, hepatorenal syndrome, spontaneous bacterial peritonitis and hepatic encephalopathy, which in turn results in increased morbidity and mortality. It is defined as the pathological increase of portal venous pressure to above 10-12 mmHg from the normal value of 5 mmHg. The portal venous pressure is measured by assessing the gradient between the wedged hepatic pressures to the free hepatic venous pressure via a transjugular route as the portal vein is inaccessible. The formulae used to calculate the hepatic venous pressure gradient is (3)

HVPG = WHVP - FHVP

The correction is required to adjust the increased intra-abdominal pressure to provide an accurate measurement of the portal venous pressure. The actual pressure in the portal vein is measured by the principles governing any pressure difference between two points, by applying the Ohm's Law:

$$\Delta P = Q \times R$$

Where the delta change in pressure between any two points in the portal vein is determined by the rate of blood flow and the resistance applied by the wall of the vein in that section.

Although it is a surrogate measurement of portal venous pressure, it has been shown to have excellent clinical co-relation. A reduction of hepatic venous pressure gradient below

12 mmHG or a reduction of less than 20% from the baseline has been shown to reduce the incidence of variceal bleeding to less than 10% (4), (5). Recently, there has been a suggestion that there may be other benefits of reduction of portal pressure other than just lowering portal pressure, which improves the survival of patients with portal hypertension (6). Although there has been a great improvement in the understanding of the pathophysiology of portal hypertension, there is still an unmet need for the development of a treatment strategy for portal hypertension.

The classical view of portal hypertension suggested that the increased resistance to portal blood flow to the liver is the main determinant in the propagation and maintenance of portal hypertension. This has been the basis of the treatment modalities available up to now. This includes non-selective beta blocker (propanolol, nadolol) for the prevention of primary (7) and secondary episodes of variceal bleeding (8) and short acting intravenous drugs like somastatin and its analogues and terlipressin (9) for the treatment of acute variceal bleeding which effectively reduced the splanchnic blood flow. However, recent evidence suggest that reduction of the splanchnic blood flow may not be the most appropriate treatment for certain stages of refractory ascites and can even have deleterious outcome (10).

There has been a gradual paradigm shift in the understanding of the pathophysiology of portal hypertension. Clinicians and scientists are beginning to arrive at a consensus that intrahepatic resistance to portal blood flow is the initiating factor in the development of portal hypertension. Correction of this aspect is more likely to improve the severe

haemodynamic abnormality and prognosis associated with clinically significant portal hypertension.

1.2.1 Increased portal blood flow resistance:

In portal hypertension, there is an increase in the resistance to the portal blood flow. This can be due to the increase in intrahepatic resistance as well as resistance generated by the co-lateral porto-systemic circulation.

1.2.1.1 Intra hepatic resistance:

The normal liver is a very compliant vascular organ whereby it can accommodate increase in portal blood blow, without any detrimental effect. The intra hepatic vascular tree is able to dilate appropriately in response to the increased blood flow as seen in the context of postprandial increase in portal blood flow. The main site of resistance in a normal liver has been suggested to be at the hepatic sinusoids, terminal hepatic venules and portal venules. However as the portal pressure remains within the normal level of 4-8 mmHg, there is no real significance to the resistance (11).

However, this compliance is not seen in portal hypertension. Intrahepatic resistance (IHR) was classically thought to be as a result of the mechanical effects of cirrhosis including fibrosis, thrombosis and nodule formation which leads to abnormal architecture of the sinusoidal space and hence distortion to the laminar flow of blood (12). The exact site of intra hepatic resistance is variable and can change according to etiological factors and disease process. Chronic hepatitis appears to have both pre-sinusoidal and sinusoidal

vascular abnormalities which contribute towards the increased resistance (13) where as there is abnormal architecture in the sinusoidal and post sinusoidal site (14), when liver injury is caused by ischaemia. As the disease progresses, there is terminal vein fibrosis, reduction in sinusoidal space by enlarging hepatocyte and deposition of collagen in the space of disse, all of which contribute to the increased vascular resistance. Thus, at the beginning of research into this area, the importance was given to the anatomical abnormalities as the most important cause of portal hypertension. There is some emerging evidence that anatomical architectural deformities can be corrected by treatment of underlying condition like treatment of Hepatitis C (15)

The seminal paper by Mittal and Groszmann in 1984 gave the first indication into the presence of a modifiable component in the intra hepatic vasculature (16). Bhathal and Grossman in 1985 confirmed this perspective and led to the theory of presence of a pharmacologically modifiable vascular element in addition to the mechanical factors that influence intra hepatic resistance (17). A significant body of research has evolved since then to understand and apply this principle to modify intra hepatic resistance as a treatment of portal hypertension.

It has been estimated that this dynamic and reversible component of intrahepatic resistance may account for about 30-40% of the total resistance in the cirrhotic liver. These include vascular smooth muscle cells present in the intra hepatic vasculature (18) activated hepatic stellate cells and hepatic myofibroblasts (19, 20) that may distort the regenerative nodule within the fibrous septa. In addition to these factors, there is also an imbalance between the intrahepatic vasoactive agents. Angiotensin II, endothelin I,

adrenergic stimulation are some of the agents which propagate the vasoconstriction of the intra hepatic microcirculation (21), (22). On the other hand, Nitric oxide, CO, H_2S are some of the vasodilators present in the intra hepatic circulation. Normally, vascular tone is maintained by a delicate balance between the vasoconstrictors and vasodilators. Endothelial dysfunction (ED) implicate a loss of function of numerous activities of the endothelium (23), mainly characterised by impairment of the production and release of endothelium driven vasodilatory factors including NO (24), (25). Endothelial dysfunction is thought to be a key event in the development of distinct human vascular diseases, including liver cirrhosis, hypertension, diabetes and atherosclerosis. Reduced NO bioavailability makes a major contribution to endothelial dysfunction and is mainly due to reduced NO production or increased NO breakdown due to the chemical reaction with oxidant radicals (26). The hepatic vascular bed of cirrhotic liver exhibits ED and is now considered to play a key role in the initiation and advancement of liver cirrhosis.

As a result, there is an abundance of vasoconstrictors and a lack of vasodilators especially nitric oxide (NO) ((27), (25),(28) , (29)) which leads to intrahepatic vasoconstriction and increased resistance. NO, in particular has been shown to have a potent vasodilator function and plays an important role in maintaining the vascular tone in normal livers (16). The deficiency of NO is due to the loss of activity of endothelial Nitric Oxide Synthetase, which is the rate-limiting enzyme in the synthesis of NO. Although the protein expression of eNOS remains same or increases, there is loss of activity, which suggest that there is a post translational modification in the eNOS protein, hence leading to its inactivation (25). Among the culprits for this modification that may influence hepatic eNOS activity is reduction in the akt mediated phosphorylation of eNOS and increased

expression of caveolin I (30), (28). Furthermore, eNOS may be unable to synthesize NO in the presence of oxidative stress which depletes the essential co-factor like tetrahydrobiopterin which are essential for the oxidation process to take place for NO synthesis (31) leading to uncoupling of the eNOS protein. There has been recent interest in the elevated levels of the endogenous inhibitor Asymmetrical Dimethyl Arginine (ADMA), which leads to inhibition of eNOS, thus leading to decrease NO production (32). In addition, there is scavenging of the produced NO by reactive oxygen species which can be prevented by the use of anti-oxidants (33) (34)

The hepatic stellate cells located in the sinusoids with perisinusoidal and inter cellular branching acquire contractile properties and modulate intra hepatic resistance dynamically (35). They synthesize collagen and form a myofibrobalst like matrix around the sinusoids and terminal hepatic venules in cirrhotic livers and have been shown to have an enhanced response to endothelins (36). The endocannabinoid system has also been implicated in the activation of hepatic stellate cells and cannabinoid receptor CB1 upregulation is associated with fibrogenesis of the liver and some associated metabolic effects like insulin resistance and dyslipidaemia (37).

Thus, in summary, there is a combination of reversible dynamic component and irreversible components, which lead to increase in intra hepatic resistance.

1.2.1.2 Portosystemic collateral resistance

Portosystemic collaterals are formed as a result of portal hypertension as a means to decompress the portal circulation by diverting blood to the systemic circulation by

opening up of unopened and new blood vessels. However, the resistances present in these vessels are still more than normal vascular resistance, therefore, they contribute to the overall resistance of blood flow.

1.2.2 Splanchnic vasodilatation

The other important phenomenon occurs in the systemic circulation in cirrhosis is splanchnic vasodilatation. This is associated with an increase flow in the splanchnic circulation, which contributes to the propagation and maintenance of portal hypertension (38). This is manifested by the classical phenomenon of vascular hyporeactivity to vaso constrictors which can be corrected by removal of the endothelium (39) and overproduction of NO. The importance of this overproduction of NO is implicated by the fact that a non-specific inhibition of NOS leads to the correction of the haemodynamic abnormalities in the splanchnic circulation (40). The main enzyme responsible for this increased NO production is endothelial Nitric Oxide Synthetase (eNOS). This is proven by the increased protein expression (41) and activity of eNOS (42) and excessive production of NO by the pulsatile flow and shear stress generated activation of the eNOS enzyme (43). Although there is good evidence to suggest that eNOS is the main enzyme responsible for the NO overproduction (44) there is evidence that the upregulation of eNOS prior to onset of the hyperdynamic circulation suggesting that there may be other factors involved in the increased production of NO (45). In 1991, Moncada and colleagues showed that endotoxemia present in liver cirrhosis model caused upregulation of inducible Nitric Oxide Synthetase (iNOS) which acted as a source of increased NO production and resulting in the hyperdynamic circulation (46). Subsequently it has been

shown that selective inhibition of iNOS does not abrogate the hyperdynamic circulation and vascular hyporesponsiveness to vasoconstrictors (47), thus suggesting that iNOS production may not have a direct effect on splanchnic vascular regulation. Furthermore, it has been shown in several studies that there is a causal relationship between endotoxemia and NO production (48) (49). There is also suggestion that neuronal NOS (nNOS) normally found in the neurons and vascular smooth muscle cells is upregulated in the mesenteric arteries (50)and aorta and takes part in the progression and maintenance of hyperdynamic circulation of cirrhosis (51).Several other factors have been implicated in the upregulation of the eNOS enzyme and among them, the presence of co-factors like Calcium, Calmodulin and tetrahydyobiopterin, which is increased by circulating endotoxin are important (52). Among the positive regulators, Heat Shock Protein 90 (Hsp90) (53) and akt phosphorylation contributes to the splanchnic eNOS activation (54). The portal pressure can also dictate the amount of splanchnic dilatation as a rise in portal pressure increases the shear stress which leads to increase phosphorylation of eNOS via the akt/protein kinase B pathway (44).Vascular Endothelial Growth Factor (VEGF) is one of the most important potent inducer of angiogenesis and eNOS phosphorylation via the akt pathway and it helps to propagate and maintain the hyperdynamic circulation in cirrhosis(55). There is also some suggestion from the literature that the alteration of the eNOS protein from its normal sub-cellular location near the golgi body to the plasma membrane make it more able to produce NO and may be related to the endothelial dysfunction in the splanchnic vasculature noted in cirrhosis (56). Furthermore, recently it has been shown that eNOS is negatively regulated by S-nitrosylated, which means addition of a NO group to a thiol side chain to a cystiene residue (57)

Among other molecules that have been implicated in the splanchnic vasodilation in cirrhosis is carbon monoxide (CO), which is a byproduct of the Heme Oxygenase (HO) pathway through which there is activation of soluble Guanylate Cyclase (sGC) in the vascular smooth muscle cells leading to vasodilatation and hyperdynamic circulation (58). Prostacylin I, derived from the cycloxygenase pathway has been found be increase in cirrhosis and acts on the adenyl cyclase to induce vasodilatation (59). There is also a suggestion of involvement of the endocannabinoid system in the splanchnic vasodilatation as it already known that anandamide, a cannabinoid receptor 1 (CB1) receptor agonist is elevated in cirrhosis and participates in vasodilatation of the mesenteric vessels but there is an ongoing controversy whether its mechanism involves NO or not (60). A growing body of evidence also suggests the involvement of Hydrogen Sulphide (H_2S) in the vasodilatation of the aorta and mesentery via the opening of the kATP channel and independent of the cGMP pathway (61), (62). Another molecule that has been shown to be involved in the propagation of the hyperdynamic circulation is the pro-inflammatory cytokine TNFα, although there is still some controversy about the exact mechanism involved. The proof that it is implicated in this process is given by the study using thalidomide, an anti-TNFα agent in portal hypertensive rats which abrogates the hyperdynamic circulation (63). It has been suggested that TNFα induces synthesis of tetrahydrobiopterin, which is an essential co-factor of eNOS in the synthesis of NO (64) and it is also implicated in the induction of iNOS in the aorta to play a part in the maintenance of the hyperdynamic circulation (65).Lastly, another molecule that has been found to be increased in cirrhosis is adrenomedullin, which is an endogenous potent

vasodilatory peptide, which is thought to be working through the cGMP and the akt phosphorylation pathways (66, 67)

In addition to arterial hypotension, there is also volume expansion, which helps to maintain the hyperdynamic circulation. Peripheral vasodilatation leads to activation of the baroreceptors and volume receptors which in turn activate the neurohumoral system and the renin angiotensin system. The consequence is absorption of salt and water from the kidneys leading to increased plasma volume. This increased plasma volume and peripheral vasodilatation leads to increased splanchnic blood flow. In cirrhosis, the increased blood flow leads to aggravation of the intra hepatic resistance, which is already increased as result of intra hepatic endothelial dysfunction.

In summary, the intra hepatic resistance is the initiator of portal hypertension and the splanchnic vasodilatation and consequent increased blood flow to the liver maintains and propagates portal hypertension. There is an imbalance between the vasodilators and vasoconstrictors in the intra hepatic and splanchnic circulation with opposing effects. There has been multiple factors which have been implicated in the pathogenesis of portal hypertension, nitric oxide being one of the most important factor influencing this process. The modulation of nitric oxide remains an important strategy to decrease intra hepatic resistance and various strategies have already been applied to increase the availability of NO in the intra hepatic circulation.

1.3 The impact of Inflammation on portal hypertension

The onset of portal hypertension is the defining event in the clinical course of a cirrhotic patient (68). It is associated with the development of complication from portal hypertension which include variceal bleeding, ascites, spontaneous bacterial peritonitis and hepatic encephalopathy. All of these complications are associated with poor outcome. Portal hypertension in cirrhosis is propagated by two components. Splanchnic vasodilatation is caused by reduced systemic vascular resistance and results in increased blood flow to the liver. The primary haemodynamic change is recognised to be the increased intra hepatic resistance (IHR). IHR has two important component. One is termed as the fixed component, which is as a result of architechtural distortion due to nodule formation and microthrombi. However, it is being increasingly recognised that the intra hepatic vascular tone and the contractility are a significant component of IHR. The splanchnic vasodilatation and hyperdynamic circulation associated with portal hypertension in cirrhosis is thought to be a compensatory mechanism as a consequence of the increased intra hepatic resistance.

Pro inflammatory cytokines have been shown to be elevated in liver cirrhosis (69). Lemmers et al showed that progressive elevation of IL-6 levels were found in patients with cirrhosis and alcoholic hepatitis as compared to normal controls. The levels of pro inflammatory cytokines have been shown to have been increased in alcoholic hepatitis (70). In a more recent study by our group, we have shown that TNFα is an important mediator of portal and systemic haemodynamic derangement in alcoholic hepatitis. In this study, Dr.Mookerjee shows that the plasma TNFα level was directly related to the severity

of liver disease i.e. it was highest in the group of patients with alcoholic hepatitis as compared to the cirrhotic patients. In the same study, an anti TNFα agent (Infliximab) was used in all ten patients as a one off bolus dose and the patients were assessed at 24 hours and on the day of discharge. The portal pressure was significantly reduced after 24 hours of administration of infliximab and it remained low at the time of discharge. The systemic vascular resistance was increased and there was improvement in the hepatic and renal blood flow. This study provides proof of the role of TNFα as a pro inflammatory cytokine in the propagation of portal hypertension (71).

Acute on chronic liver failure is a recently described clinical syndrome wherein there is acute deterioration of liver function precipitated by superimposed liver injury or superadded infection. The definition and natural history of this clinical syndrome has been established in the recently concluded European consortium study CANONIC study where the ACLF patients were differentiated from patients with acute decompensation by the presence of one or more organ failure, presence of high systemic inflammatory response and high mortality within 28 days of admission. In fact the high systemic inflammatory response was identified as the key precipitating factor in the pathogenesis of this syndrome. (72).Studies in patients with acute severe alcoholic hepatitis have shown that they have a higher portal pressure as compared to decompensated cirrhosis (73).

1.3.1 The impact of inflammation on intra hepatic resistance

Cirrhosis is characterized by architectural distortion resulting in resistance to sinusoidal blood flow. This is primarily caused by nodule formation and scarring of the liver.

However increasingly increased vascular tone because of an imbalance between the vasoactive agents is being implicated as a more important aspect of increase intra hepatic resistance in portal hypertension. Nitric oxide (NO) has been demonstrated to be a key regulator of intrahepatic vascular tone, and NO production from endothelial nitric oxide synthase (eNOS) in the sinusoidal endothelial cell (SEC) is decreased in cirrhosis (16), (27).NO modulates the vascular tone by acting on the smooth muscle cells. Intra hepatic resistance is also regulated by activated hepatic stellate cells, which adapt a myofibroblastic phenotype upon activation. Activated HSC also played a key role in the development of angiogenesis leading to intra hepatic shunting and vascular collateral formation.

Hepatic innate immune signaling also has a role in the propagation of increased intra hepatic resistance. It plays a part in both increased fibrosis and vascular tone. The role of Pathogen associated molecular pattern (PAMP) in the progression of fibrosis has been shown in rodent models where in various knockout of the Toll Like Receptor (TLR) pathway such as CD14, Liposaccharide Binding Protein (LBP), MYd88, TIR-domain-containing adapter-inducing interferon-β (TRIF) have shown reduction in fibrosis in Bile duct ligated (BDL) and Carbon tetrachloride (CCL4) model ((74), (75)). These pathways have been demonstrated as Kupffer Cell (KC) independent processes. However, in advanced liver disease, hepatic stellate cells (HSC) play a more active role in the development of hepatic inflammation and oxidative stress, which in turn increases the intra hepatic vascular tone. The upregulation of the TLR signaling pathway promotes the production of pro inflammatory cytokines which include TNFα , IL6 and IL8, initiating

both hepatic and systemic inflammation (76). Another downstream effect of HSC activation is production of Reactive Oxygen Species (ROS). Oxidative stress leads to impaired production of NO via multiple pathways. ROS directly reacts with NO to produce peroxynitrate and other reactive nitrogen species ROS leads to direct eNOS dysfunction by ‘uncoupling’ and inhibition of phosphorylation and increase in the formation of eNOS inhibitors. (77).Plasma level of NOS inhibitor ADMA are raised in cirrhosis and further raised in patients with Alcoholic Hepatitis (78).In addition, hepatic expression of eNOS inhibitor caveolin-1 and eNOS trafficking protein NOSTRIN are increased in alcoholic hepatitis as compared to decompensated cirrhotic patients. These proteins will reduce eNOS activity and in turn NO production (79)

Hepatocye cell death through oxidative injury will activate local innate immune pathways by way of Damage Associated Molecular Pattern (DAMPs).These protein are responsible for the introduction of ‘sterile inflammation’ through the TLR pathway as well (80).There is cross talk between the PAMPs and the DAMPs as bacterial LPS stimulates DAMP proteins like HMGB1 which activate both pathways (81). Therefore, local liver injury propagated by bacterial translocation and activation of the innate immune pathway through the TLR pathway leads to further inflammation and oxidative stress. This leads to significant intra hepatic vascular dysfunction and thereby propagates the progression of portal hypertension.

1.3.2 The impact of inflammation in the splanchnic circulation

The forward theory of portal hypertension suggests that there is splanchnic vasodilatation and reduction in the systemic vascular resistance with resultant increased portal venous inflow to the liver. As opposed to the intra hepatic circulation, there is excess NO in the splanchnic circulation and reduced responsiveness of the mesenteric circulation to vasoconstrictors. In a recent study, it has been shown that peri vascular cells, which are present in the adventitial layer of mesenteric vessels have increased number of activated macrophages expressing inducible NOS. Thus paracrine effect of inflammatory cells producing NO may augment portal hypertension (82).The relative contribution of the various NOS isoforms are still being debated. In rodent models of pre sinusoidal portal hypertension using the portal vein ligation (PVL) model, it is clear from various studies, that endothelial NOS (eNOS) is the predominant contributor of NO production the mesenteric circulation (83).However in the studies using Bile Duct Ligation (BDL) model inducing biliary cirrhosis and portal hypertension, there is evidence to show that there is increased expression of aortic iNOS after administration of LPS (84).

The role of the gut liver axis in propagating splanchnic vasodilatation has been investigated. Patients with advanced cirrhosis produces increased systemic NO and endotoxemia post TIPSS and there is also evidence to show increased in iNOS activity in these group of patients (85). Flouroquinolone antibiotics like norfloxacin had been used in cirrhotic patients to show there is reduction in the endotoxin load, improvement in the mean arterial pressure, and systemic vascular resistance as well as reduction in NO mediated mid arm vasodilatation (86), (87). In cirrhotic rats, pro inflammatory cytokines

like TNFa and IL6 are reduced by down regulation of eNOS phosphorylation by Akt upon treatment with norfloxacin (88)

Studies from iNOS and eNOS knockout studies have suggested there are agents other than NO that are implicated in the pathogenesis of splanchnic vasodilatation in cirrhosis (83). Microparticles (MP), membrane vesicles that can affect the vascular and inflammatory signaling pathways have been found to be increased in cirrhotic patients and their levels co-relate to disease severity and inflammation. They have also been shown to impair the vasoconstrictor effect on cultured rat aorta rings (89).

All the above factors are implicated in the modulation of inflammatory pathways in the regulation of splanchnic vascular tone.

1.4 Bacterial Translocation and Portal Hypertension

Due to the unique anatomical location the liver and its vascular blood supply, there is significant exposure to intestinal bacteria and bacterial products in the context of cirrhosis. The role of gut microflora in the pathogenesis of complications of cirrhosis including spontaneous bacterial peritonitis and hepatic encephalopathy is well recognised. Recently there has been renewed interest in the concept of bacterial translocation in the pathogenesis of portal hypertension and complication of cirrhosis.

Bacterial Translocation (BT) is defined as the passage of viable and non-viable products of microbes and microbial particles from the intestine into the mesenteric lymphnodes and other organs.BT has become an increasingly interesting focus for many complication

of cirrhosis. Bacterial cultures are positive in mesenteric lymph nodes in 30.8% of Child C cirrhotic patients as compared to 6.8% of non cirrhotic patients (90). Similarly the surrogate marker of BT, lipopolysaccharide (LPS)-binding protein (LBP), was observed to be increased in 42% of cirrhotic patients (86). It has also been recognized that bacterial infections are associated with a poorer prognosis from variceal haemorrhage (VH) (91)). BT is associated with other portal hypertension-related complications, such as hepatic encephalopathy and spontaneous bacterial peritonitis (SBP) (92).

The downstream mechanism of BT is complex but the most preserved pathway of innate immunity signals is via the exposure to microbial products or pathogen associated molecular patterns (PAMP) leading to activation of toll like receptors (TLR), which are most commonly expressed in Kupffer Cells (KC) although they are expressed in all types of parenchymal and non parenchymal cells. The kupffer cells in turn become activated by the TLR pathway and adopt a pro inflammatory phenotype releasing cytokines like TNFa, IL 6 and IL 2 (93). This pro-inflammatory cytokine response to BT is associated with severe portal hypertension in cirrhosis. Serum bacterial DNA levels, as a surrogate marker of BT, are correlated with severity of inflammation and portal hypertension in cirrhosis (94). Moreover, in patients with SBP, elevated levels of catecholamines and TNFa are associated with higher HVPG (95).In rodent studies, it has been shown that injection of LPS increases the portal pressure (96) and antibiotics have been used to reduce the occurrence of bacterial translocation (97). In humans, use of antibiotics have been shown to improve the haemodynamic abnormalities in the cirrhotic patient proving the notion that reduction of the bacterial translocation has a beneficial effect on the portal pressure (87). Direct inhibition of TNFa in patients with severe alcoholic hepatitis showed

significant reduction in portal pressure both immediately and also sustained till day of discharge of patients. This was associated with improvement in the hepatic and renal blood flow. This study suggest that targeting inflammation may be an important target to reduce portal pressure and improve outcome of patients (71).Several pro inflammatory cytokines like CRP and IL 6 have been shown to be raised in patients with cirrhosis and the level of the cytokine load co-relates with the portal pressure (98) (99). Therefore, bacterial translocation is a major mediator of inflammation induced portal hypertension in advanced cirrhosis.

1.5 The link between inflammation, sympathetic nervous system and portal hypertension

The relationship between sympathetic nervous system and inflammation has been known for a long time. In the mid-1980s, it was well recognised that secondary lymphoid tissues is highly innervated by sympathetic nervous fibres in close proximity to immune cells (100). These immune cells express adrenoreceptors which are functional and translate neuronal signals into immune cell signal (101).The activation of the SNS in the context of an active immune system results in release of sympathetic neurotransmitters. Sympathetic influence on immune cells can be direct via adrenergic receptors on immune cells or via regulating blood or lymph flow (102).One of its main function is to modulate the release of pro inflammatory peptides (103)

High levels of catecholamines in the portal venous plasma of patients with cirrhosis were reported by Shaldon et al. in 1961 (104). Patients with a high Child score, more pronounced portal hypertension, avid fluid-sodium retention, and the hepatorenal

syndrome have the highest plasma levels of NA and adrenaline. Selective catheterisation techniques and tracer kinetic methods have disclosed that the increased circulating noradrenaline in patients with cirrhosis is caused by sympathetic nervous overactivity in a number of organs (105). The very high noradrenaline from the kidney accords with the conception of a preferential renal sympathetic nervous over activity in patients with advanced liver disease (106)

Several investigators have found a direct relation between the size of the portal/sinusoidal pressure and the circulating level of noradrenaline. This may suggest that sympathetic over-activity is involved in the pathogenesis of the portal hypertension in such patients.

There is a large body of evidence to suggest that a decrease in the central and arterial blood volume of patients with cirrhosis (107).Moreover, it is already known that inverse relationship between the size of the central and arterial blood volume (i.e. the volume in the heart cavities, lungs, and central arterial tree) on the one hand and arterial and renal venous NA on the other (108). This suggests to us that central and arterial hypovolaemia is a significant afferent stimulus to overall and renal sympathetic over activity. Moreover, the increased arterial level of noradrenaline is directly related to the increased portal venous pressure and azygos blood flow. Various experiments have been done to block central and peripheral adrenoreceptors which lowers systemic and portal pressures. These findings suggest that the sympathetic nervous system plays a role in the genesis and perpetuation of portal hypertension (109). A close relation has been demonstrated between circulating noradrenaline and survival, and the level of plasma noradrenaline is an independent prognostic factor in cirrhosis.

In the recently published prospective CANONIC study, acute on chronic liver failure patients can be differentiated from acute decompensation of cirrhosis by the presence of organ failure, a marked systemic inflammatory response and higher short term mortality. Systemic inflammation appears to the most important prognostic factor as it rises with the progression of disease. However, the key precipitants for acute decompensation and acute on chronic liver failure were similar, suggesting that the major determinant for the development of ACLF is host response to injury rather the nature of the precipitant (72).

There is increasing evidence that norepinephrine modulates the activation of kupffer cells by pro inflammatory cytokines via the adrenergic receptors (110). Gut derived bacteria or bacterial products activate pathogen associated molecular patterns which secrete pro inflammatory cytokines to activate kupffer cells. NE is thought to upregulate TNFα production in KC through the adrenergic receptor pathway (111). Extra hepatic tissue and cells also have been shown to secrete catecholamine in an acute inflammatory environment via the alpha 2a receptors (112)

The close relationship between noradrenaline levels, SIRS and intrahepatic resistance leads to the hypothesis that modulation of the sympathetic nervous system will reduce intra hepatic resistance and provides a valid target for the management of portal hypertension.

1.6 Adreno-receptors in the hepatic vasculature

The adreno receptors are a group of transmembrane receptor in the class of G-protein coupled receptors that are targeted by catecholamine. A significant number of cells possess these receptors and stimulation by catecholamine will provoke the activation of downstream pathways and stimulation of the sympathetic nervous system.

Up to now, several different adreno-receptors have been identified. They have been divided into two main groups – α and β adrenergic receptors. They have been further sub divided into α1 and α2 and the β adrenergic receptor has been sub divided into β1, β2 and more recently β3.The α adrenergic receptors are further divided into sub groups. Alpha 1 adrenergic receptors are have three highly homologous subtypes –α1a, α1b, α1d. Alpha 2 adrenergic receptors are also further sub divided into three sub types. They are α2a, α2b and α2c. These are homologous subtypes with main action being inhibition of insulin release and induction of glucagon release and more importantly giving pre-synaptic negative feedback for the release of noradrenaline.

Adrenaline or noradrenaline are receptor ligands to either α_1, α_2 or β-adrenergic receptors. α_1 couples to G_q, which results in increased intracellular Ca^{2+} and subsequent smooth muscle contraction. α_2, on the other hand, couples to G_i, which causes a decrease in neurotransmitter release, as well as a decrease of cAMP activity resulting in smooth muscle contraction. β receptors couple to G_s, and increases intracellular cAMP activity, resulting in e.g. heart muscle contraction, smooth muscle relaxation and glycogenolysis.

There is presence of adrenergic receptors in the liver, the predominant ones being α1 and β2 adrenergic receptors. The α1 adrenergic receptor is primarily responsible for the vasoconstriction in the resistance vessels which includes the venules whereas the β2 adrenergic receptors are primarily responsible for vasodilataion of the arterial side of the hepatic vasculature. The evidence for the presence of α2 adrenergic receptor and in particular α2a adrenergic receptor is scarce, especially in the normal liver. However, there is definite proof of its presence in kupffer cells (111) and other migratory cells (113). This makes it an attractive target for modulation in the diseased state.

1.7 Arginine metabolism in liver disease

The previous section of the thesis focused on targeting the sympathetic nervous system in order to modulate inflammation and thereby reduce portal hypertension. Another aspect of modulation of the intra and extra hepatic vascular tone is the vasoactive substance nitric oxide. Inflammation is both directly and indirectly involved in the metabolism of nitric oxide in the hepatic vasculature. L-Arginine is the sole substrate for the production of nitric oxide and had immunological properties that merit further investigation in the context of liver cirrhosis and portal hypertension. In order to understand the metabolic pathways that are implicated in this process, I have started this section of the thesis with a literature review and have attempted to highlight the rational of studying this pathway in more detail as my alternative strategy for the modulation of inflammation for the reduction of intra hepatic resistance in portal hypertension.

Arginine is a semi essential amino acid which acts a sole substrate of enzyme eNOS for oxidation into NO and citrulline (114). As discussed previously, NO has become an important vasoactive agent both in the intra and extra hepatic circulation which modulates vascular tone. There is a growing body of research into modulation of NO in order to reduce intra hepatic resistance but very little effort has gone into the understanding of the metabolism of L-Arginine in liver disease. Most of the studies related to arginine supplementation in humans have been done as part of immunonutrition in intensive care patients and therefore the outcomes are confounded by the presence of other amino acid constituents in the packet.

Moreover, therapeutic interventions based on both stimulation and inhibition of arginine metabolism has given contradictory results. The metabolism of L-arginine in normal and disease state are influenced by many factors and therefore it will be important to understand these pathways before a rational strategy can be applied to modulate inflammation in order to lower portal pressure.

1.7.1 Normal arginine metabolism

Muscle derived glutamine starts the pathway for arginine synthesis by converting to citrulline in the small intestines via the glutamine- ornithine pathway (115). Most of the released citrulline (83%) in turn is absorbed by the kidney (116). Arginine is synthesised proximal renal tubules in the kidney from the citrulline (117) (118).About 10-15% of whole body arginine is produced by this pathway. Alternative pathways for arginine

availability include protein breakdown and diet intake with the jejunum as the major site of intestinal absorption (119).

There are four major metabolic pathways of arginine. Firstly, arginine is metabolised by the enzyme Arginase. There are two isoforms of Arginase, Arginase I being the cytosolic enzyme which is expressed in the liver and Arginase II which is present in the mitochondria and present in the intestines, kidney and brain. Arginase I mainly involved in the metabolism of ammonia and urea whereas arginase II is used up in the synthesis of ornithine, proline and glutamine (120). Arginase II plays an important role in the degradation of intestinally absorbed arginine (121).

Second major pathway for arginine metabolism is by the synthesis of NO. NO is produced by the oxidation of L-arginine by the enzyme NOS (Nitric oxide synthetase). There are three isoforms of the enzyme which are endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS).All the three isoforms produce NO which have different functions (119).

Besides these two important pathways, a large portion of L-arginine is used up in the synthesis of proteins and agmatine. L-arginine is also utilised in the biosynthesis of creatine which is the pre cursor of creatinine (122).

1.7.2 Arginine metabolism in disease state

There is a paucity of data for arginine metabolism in liver disease itself but a surrogate to this would be to understand the metabolism of arginine in sepsis, which is

pathophysiologically akin to liver disease. Plasma arginine concentration have been found to be consistently low in the humans and animal models when they are under stress or septic and the prognosis was significant worse in patients with low arginine levels along with low levels of other amino acids (123).The whole body plasma level of arginine , which is the total volume of arginine synthesised from protein breakdown and de novo arginine production via the intestinal-renal axis remains the same in the septic patients and non septic intensive care patients (124). The main impetus for maintenance is on the de novo arginine synthesis of arginine through the intestinal-renal axis (125), (126).

However, in critically ill patients the net amount of plasma arginine may be reduced because of impaired intestinal function, which is the main site for production of citrulline (119).Therefore, de novo arginine production is the main pathway for the maintenance of the balance of plasma arginine levels.

The reduction of plasma arginine levels may suggest that there is a shift of arginine to the intra cellular compartment. In order to facilitate transport, there are certain conditions that have to be met like availability of a transport system of which there are many. The most important transport system is the y+ is the most important and high affinity transport system. These transport system are associated with transport proteins called Cationic Amino Acid Transporters (CAT), of which there are three, namely CAT1, CAT2 (B) and CAT3. The uniqueness of these transport system is that they may be co-localised with a NOS enzyme in the plasma membrane (127). More specifically, eNOS is co localised with the CAT1, which helps to regulate the flux of arginine inhibitors into the cell.

Furthermore, some endogenous inhibitors like ADMA also compete for the same transport system, therefore may be able to regulate the arginine flux across the cells (128).

1.7.3 Catabolism of Arginine

1.7.3.1 Arginase Pathway

Arginase is a binuclear manganese mettaloenzyme, which is an integral part of the urea cycle in the liver and is the main enzyme for the catabolism of L-Arginine into ornithine and urea. There are two isoforms of arginase Arginase I and Arginase II, which share 60% homology (129).Arginase I is a cytosolic enzyme which is present primarily in the liver and Arginase II is a mitochondrial enzyme present in the extra-hepatic tissue including the vascular smooth muscle cells (120). It is suggested that Arginase will compete with NOS for the substrate L-Arginase but on consideration of their biochemical properties, it is evident that the L-arginine has greater affinity for the purified NOS than arginase although there is a suggestion that there is similar rate of substrate utilisation at physiological levels of L-Arginine (122). However, early studies on activated macrophages reveal that majority of substrate are being consumed by the arginase pathway to produce urea and inhibition of arginase (130), (131). Apart from depleting L-arginine as a substrate for NO production, arginase also inhibit the expression of iNOS repressing the translation and stability of the protein (132) and by production of urea (133).On the other hand, the intermediate product of L-Arginine by the NOS pathway, N-Hydroxy-L-Arginine is a potent inhibitor of arginase and therefore there is presence of a reciprocal inhibition pathway (134). Apart from depleting the substrate L-Arginine,

arginase also sensitise the endothelial cells to the endogenous NOS inhibitor ADMA, which will prevent the formation of NO (135).There is also suggestion in the literature that depletion of arginine results in uncoupling of the eNOS protein leading to the formation of oxygen free radical, which can directly inactivate any NO formed. Thus indirectly increased arginase activity can have a negative impact on NO generation. In addition, superoxide in combination with NO forms peroxynitrite, which causes increased vascular reactivity and endothelial damage (136).

Apart from causing endothelial dysfunction as a result of depletion of substrate L-Arginine for the production of NO by various mechanisms mentioned above, there is also growing evidence that increased arginase activity causes vascular smooth muscle dysfunction. Urea produced by the arginase pathway is eliminated via the kidney, however ornithine is used up in the formation of polyamine and L-proline, which are important component for the proliferation of vascular smooth muscle and essential for the formation of several structural proteins including collagen (137), (138).Pharmacological inhibition of arginase leads to reduction in vascular smooth muscle proliferation. Increased arginase activity can directly stimulate smooth muscle cell proliferation and polyamine synthesis and can be associated with the decrease in NO as it is a known inhibitor of smooth muscle cell proliferation (138).In the context of cirrhosis, recent data suggest that both isoforms of Arginase is regulated differently as there is increased expression of arginase II and decrease expression of arginase I, which suggest that they are involved in different processes in cirrhosis. Arginase I being utilised by the urea cycle in the liver and the arginase II being used up in the synthesis of ornithine, polyamine and L-proline which lead to proliferation of vascular smooth muscle cells (139)

In conclusion, arginase has established itself as a major regular of NO in the vasculature. It can cause both endothelial and vascular dysfunction by modulation the intra cellular metabolism of L-arginine. More specifically, arginase compete with NOS enzyme for the substrate

L-arginine, depleting it significantly by various mechanisms and causing reduced NO production. In the vasculature arginase depletes NO generation by directing the utilisation of L-arginine in the production of ornithine and polyamine, which is responsible for smooth muscle cell proliferation and causing vascular dysfunction.

1.7.3.2 Arginine - NO Pathway

L-Arginine is the sole substrate for the production of NO and citrulline by NOS (140). This is the second most important pathway of arginine metabolism after the arginase pathway. There are three types of NOS that have been identified. Endothelial NOS (eNOS or Type 3) which is the major NOS involved in the vasodilation and neuronal NOS (nNOS or Type 2) found in the nerve endings have been designated as constitutive NOS and inducible NOS (iNOS or Type 2) is the other NOS which is induced by cytokines and endotoxins (141).In order for the reaction to take place, NOS enzyme requires NADPH and O_2 in stoichiometric quantities. The NOS enzyme also requires important co-factors including Flavin-adenine dinudestide (FAD), flavin mononucleotide (FMN), tetrahydrobiopterin and glutathione (142). In addition electron transfer to the haeme group is mediated by calmodulin and calcium is required for the calmodullin binding in the constitutive NOS enzymes although it is not a necessary condition for the iNOS reaction to take place (143).

Various alterations in the availability of these co-factors will impact on the reaction of these NOS enzymes. The NOS enzymatic reaction is also regulated by arginase by multiple mechanism as detailed above and by methylarginine, which will be detailed in a separate section.

1.7.3.3 ADMA pathway

There is a dichotomy in the amount of NO present in the circulation. In the splanchnic and peripheral circulation, there is an abundance of NO which may be due to the continuous induction of iNOS through endotoxins, which have been translocated through the gut barrier. However in the intra hepatic circulation, there is a deficit in NO as a consequence of endothelial dysfunction. This deficiency in NO may be due to arginine depletion as a consequence of presence of large amount of arginase and Asymmetrical Dimethyl Arginine in the liver, which will compete with the same transport system as arginine and therefore leads to its intracellular depletion and hence reduction on NO production (144),(145). From the sepsis literature, it is clear that ADMA is an independent risk factor for intensive care mortality and has been shown to be associated with hepatic dysfunction (146). This increased ADMA level will alter the Arginine to ADMA ratio in the intra cellular compartment, therefore provide a rationale to supplement arginine to alter this ratio.

1.7.4 ADMA metabolism

Asymmetrical Dimethyl Arginine (ADMA) are methylated L-arginine residue in protein produced during proteolysis that freely circulate in the plasma and excreted in the urine

and present in cells and tissues (147).It is the most important endogenous inhibitor of endothelial nitric oxide synthesise and therefore has been implicated in endothelial dysfunction (145)

ADMA is synthesised when arginine residue of protein are methylated by the enzyme protein arginine methyltransferase (PRMT) Type I. Type II PRMT methylate protein to form symmetrical dimethyl arginine (SDMA), whereas both Type I and type II can monomethylate the arginine residue to form N-monomethyl L-arginine (L-NMMA). Both ADMA and L-NMMA can inhibit NOS but SDMA does not (148).PRMT type I are expressed in the heart, smooth muscle cells and the endothelium. The expression of PRMT Type I can be increased due to shear stress and inhibited by suppression of the IκB kinase or by using a peroxisome proliferated-activator receptor gamma(PPARγ) activator, troglitazone, suggesting that the ADMA levels can be regulated by modulating the PRMT Type I receptor expression (149)

The most important target for ADMA is the NOS enzyme and it can inhibit all three isoforms of NOS with equal potency. There is a suggestion that ADMA can also cause uncoupling of the NOS enzyme however it is still unclear whether this is a true effect (150). Another target for ADMA would be the y+ transport system , wherein it would compete with L-Arginine for its transport to the intra-cellular compartment but this occurs when there is a significant rise in the ADMA level which almost become physiologically irrelevant(151)

The major metabolic pathway for degradation of ADMA is by Dimethylaginine Dimethylamino Hydrolase (DDAH) to citrulline and dimethylamine (152)There are two

isoforms of DDAH that have been identified with different tissue localisation. DDAH I has been encoded from the genes expressed in Chromosome 1 and DDAH II in chromosome 6 (153). Both the enzymes are expressed abundantly in the vascular tissues but with a preponderance to DDAH II (154).

The concern of an increased plasma level of ADMA relates to its concentration to the plasma arginine concentration. In physiological states the level of ADMA is about 0.45µmol/L to 0.65µmol/L and in diseased states, it can go from 0.69µmol/L to 0.85µmol/L. In comparison, the arginine levels are in the range of 30-100µmol/L in the plasma and 1-2mmol/L in the intra-cellular compartment (155) (122).

As such, it is logical to assume that that the vast amount of arginine outcompetes with the cellular ADMA, however there is evidence that in disease states such as cirrhosis, there is a relative increase in ADMA which is disproportionate to the normal arginine to ADMA ratio and hence can inhibit the NOS enzyme. This phenomenon is known as the arginine paradox.

We and others have shown that the plasma ADMA level rises significantly in cirrhosis in humans and rats (156) (32). The rise in the level of ADMA and the relative deficiency of arginine confirms the presence of the 'arginine paradox' which implies that although the total amount of arginine is greater than the plasma ADMA level, the increase in ADMA level alters the ratio. This paradox provides a rational for the supplementation of L-arginine to reverse the ratio and benefit from the effects of increased nitric oxide production, especially in the intra-hepatic circulation.

1.7.5 Protein synthesis and energy source

Arginine is necessary for the production of various proteins. In sepsis, there is an increase in the level of acute phase proteins and antibodies (157). There is also some evidence of increased muscle protein synthesis. In children with sepsis, there is an increase in arginine oxidation, which suggests that arginine is also needed for energy production.

1.7.6 Arginine and Immune Function

Evidence from studies in sepsis and endotoxaemia suggest that this is a L-arginine deficiency state (158). The arginine deficiency is as a result of decreased uptake, reduced de novo production of L-arginine and rapid metabolism of L-arginine by arginase both in the liver and in other organs in sepsis. This is inspite of the fact that there is more arginine production from protein breakdown. This turns arginine into a semi-essential amino acid. Arginine deficiency has been noted in endotoxaemic murine models and patients admitted to ITU ((159), (160). Decreased nitric oxide production during sepsis is probably due to the reduced activity of the Nitric oxide synthetase 3. This reduction in nitric oxide results in endothelial dysfunction. There is also evidence that the cationic amino acid transporter, which is the main transporter for arginine is altered and CAT2b is upregulated. This transporter is particularly important for activated macrophage which imports large amount of L-arginine for the production of nitric oxide (161). Besides the observed deficiency of L-arginine and nitric oxide in sepsis, there is also documented deficiency of citrulline, which in turn worsens arginine availability and nitric oxide production (162). However,

the main pathway for the metabolism of L-arginine in the context of sepsis remains via the arginase pathway.

During inflammatory conditions both arginase I and arginase II activity are induced by specific cytokines with preferential activation of Arginase I ((119), (163), (164). Replenishing the plasma arginine level would be important to maintain all the cellular function including its immune function. This could be achieved by either replacing L-arginine directly or indirectly by replacing citrulline. All these evidence suggest that there is an overall inverse relationship of L-arginine with sepsis and other inflammatory condition.

1.7.7 Discussion

Therefore, in conclusion, arginine concentration is significantly reduced in the context of inflammation and sepsis. The deficiency is determined by the regulation of the various metabolic pathways in sepsis. Arginase metabolic pathway is the most important pathway for the degradation of arginine. The plasma ADMA levels are high relative to the plasma arginine concentration in inflammatory or sepsis therefore providing an imbalance in the arginine: ADMA ratio. This provides an opportunity to test a hypothesis that supplementation of L-arginine would alter the ratio and also act as a regulator of inflammation in liver diseases

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1.8 Hypothesis

In cirrhosis, portal hypertension is propagated by systemic inflammation and upregulation of the sympathetic nervous system. Modulation of the sympathetic nervous system will reduce inflammation which thereby reduces the intra hepatic resistance and portal pressure.

Aim of the studies:

1. To define the relationship between inflammatory mediators and the sympathetic nervous system and haemodynamic abnormality in a rodent model of portal hypertension.
2. To antagonise the alpha 2a receptor in a rodent model of biliary cirrhosis and portal hypertension and to assess its effect on inflammatory parameters and haemodynamic abnormalities.
3. To explore the importance of arginine in liver failure and portal hypertension and to understand the implication of arginine supplement as an anti-inflammatory strategy in the treatment of portal hypertension.

Chapter 2

Materials and Methods

2.1 Animals

All animal experiments were conducted according to the Home Office guidelines under the UK Animals in Scientific Procedures Act 1986. This study was performed in male Sprague-Dawley (SD) rats (Charles-rivers), weighing 220–250gms were obtained from the Comparative Biological Unit at University College London. All rats were housed in the respective unit and given free access (*ad libitum*) to standard rodent chow and water, with a light/dark cycle of 12 hours (the dark phase extended from 1900–0700 hours), at a temperature of 22–23°C and humidity of approximately 50%.

2.1.1. Bile-duct ligation (BDL):

The bile duct ligation of the rats were done as described. Following induction anesthesia (1L/min oxygen containing 5% isoflorane), anesthesia was maintained with 2% isoflorane in air, delivered at 0.5L/min to a facemask. Rectal temperature was maintained at 36±1oC using a heating pad. A midline laparotomy was performed and the common bile duct (CBD) was isolated by blunt dissection. The CBD tied off with triple ligatures (4-0 silk), before transecting the CBD between the distal two ligatures. 4 weeks BDL was allowed for the development of secondary biliary cirrhosis often with observed hepatic decompensation (e.g. ascites).

2.1.2 Sham operation:

These operations as described were performed in the same manner as described in Section 2.1.1 but no ties were placed and the duct was not ligated. The surgery was completed within 15 min, and rats were returned to individual cages after cessation of anesthesia where feeding and water ingestion resumed.

2.2 Study design

Male Sprague Dawley rats weighing 220-250 g were randomly assigned to receive sham or bile duct ligation (BDL) surgery as described above and previously (165). Four weeks later haemodynamic measurements were performed and samples collected for analysis. For the BRL44408 study, treatment with either 10 mg/kg BRL44408 maleate (Sigma-Aldrich Company Ltd., Dorset, UK) dissolved in sterile water or saline (control, equal volume) by subcutaneous (s.c.) injection was given on the last 2 days of the study. In the second part of the experiments, the rats were injected for the last 10 days of the experiment using the same protocol. Previous pre clinical studies have shown that the sub cutaneous route as the best route at the dose of 10mg/kg (166).

For the L-arginine supplementation studies, treatment with either 30gm/kg L-Arginine (Damor Pharma, Italy) dissolved in sterile water or saline (control, equal volume) by oral gavage was given on the last 7 days of the study. The dose regime and route was chosen from previous studies (167). Rats were randomly assigned to the different treatment groups.

All rats were exsanguinated under terminal anesthesia (2% isofluorane). Within seconds blood was withdrawn from the descending aorta and immediately put into ice cold heparin/EDTA containing tubes (until full exsanguination), centrifuged at 3000 rpm and 4°C for 10 min, and the plasma collected and stored at -80°C until assayed. Liver tissue was also removed immediately harvested and snap frozen for storage at -80°C until analyzed. Liver tissues were also fixed with 10% formalin for further histological analysis.

2.3 Haemodynamic measurements

Haemodynamics were measured under anesthesia (2% isofluorane in air). Mean arterial pressure (MAP) was measured pre-laparotomy by direct cannulation of the right carotid artery. Portal pressure (PP) was measured by direct cannulation of the portal vein post laparotomy. Pressures were transduced to a Powerlab 4SP data acquisition device (AD Instruments Ltd., Oxford, UK) analysed using BioPac Student Lab v4 software (BioPac Systems Inc., Goleta CA, USA). Hepatic arterial and portal venous blood flow were measured using a doppler flow probe connected to a TS420 transit-time perivascular flowmeter (Transonic Systems Inc., Ithica NY, USA). Intrahepatic resistance was calculated by dividing the portal pressure by the sum of the hepatic arterial and portal venous flows.

2.4 Echocardiography

Rats are placed in the supine position and transthoracic echocardiography performed under 1.5% anesthesia using a commercial echocardiography machine (Vivid 7

Dimension™, GE Healthcare, Bedford, UK) with a 14 MHz probe recording at a depth of 0-2 cm. End diastolic and systolic diameters were determined from a parasternal long-axis view using two-dimensional B-mode with the diameter measured from the apex to the aortic. Left ventricular dimensions during diastole and systole were also determined from a parasternal short-axis view using time-motion (M)-mode. Measurements were taken of the internal dimension of the cavity, the anterior and posterior wall. Stroke volume was determined as the product of the velocity time integral (VTI) and the vessel cross-sectional area (π x [0.5 x diameter] 2). Prior studies in rats of this age showed the aortic diameter to be 0.26 cm, thus a cross sectional area of (0.13)2 x π was assumed for all animals studied. As heart rate ranges from 350-500 beats per minute and respiration rate from 60-100 breathes per minute, the theoretical number of cycles needed to account for the variation in flow with respiration ranges from 3.5 (350/100) to 8.3 (500/60). In practice, an average of 5-6 consecutive cycles was enough to account for this variability. Heart rate was determined by measuring the time between 5-6 consecutive cycles from the start of each Doppler trace. Cardiac output was calculated as the product of stroke volume and heart rate.

2.5 Measurement of plasma biochemistry

All basic chemical reagents were purchased from Fisher Scientific (Loughborough, UK), Sigma-Aldrich Company Ltd. (Dorset, UK), or VWR International Ltd. (Lutterworth, UK).

All biochemical parameters (ALT, ALP, albumin, total protein, ammonia, bilirubin, urea and creatinine, lactate) were analyzed using 200µl of respective plasma samples using a

Cobas Integra 400 multianalyzer with the appropriate kits (Roche-diagnostics, Burgess Hill, West Sussex, UK).

2.6 Plasma and liver tissues cytokines

Snap frozen (-80°C) and stored plasma and liver samples were later analyzed for cytokine profiles. Prior to analysis, 100µg of liver tissues was homogenized and deproteinized in 300µl of ice-cold cell lysis buffer solution. After centrifugation at 12,000 x g for 10 minutes at 4°C, the supernatants were collected for processing. Following protein concentration quantification of equilibrated liver protein samples and plasma supernatants (50µl) were analyzed for TNF-α cytokine levels (pg/ml) and IL-6 levels (pg/ml) by flow cytometry using the Becton Dickinson (BD™ biosciences, San Diego, CA) rat inflammation cytometric bead array (CBA) kit as described by the manufacturer's instructions. Samples were analysed by measuring the fluorescence produced by the CBA beads on a 'FACS Canto™ II flow cytometry system (BD™ Sciences) and the data analyzed with BD™ CTA software.

2.7 Western blot analysis

Frozen liver was homogenised in RIPA buffer (Pierce Biotechnology Inc., Rockford IL, USA) with complete ultra mini protease inhibitors and phostop phosphatase inhibitors (Roche Diagnostics Ltd., Burgess Hill, UK) using 7mm steel beads with a tissue lyser (Qiagen Ltd., Manchester, UK). Proteins were extracted by incubating for 2 hours at 4 degC with continuous rotation and insoluble cell debris was removed by centrifugation at 16,000rpm x for 20 mins, 4 degC. Protein concentration was measured using a BCA assay

kit (Pierce Biotechnology Inc., Rockford IL, USA) for liver lysates. Equal amounts of protein were separated on 4–12% NuPAGE Bis-Tris gel under reduced denaturing conditions using either MES or MOPS running buffer (Life Technologies, Renfrew, UK) depending on the molecular weight of the protein of interest. The molecular weight markers, Novex Sharp pre-stained protein standards and MagicMark XP Western protein standards (Life Technologies, Renfrew, UK), were combined in 1 lane. Separated proteins were transferred on to PVDF membranes (Invitrogen, Paisley, UK) and successful blotting was confirmed with Ponceau S stain (Sigma-Aldrich Company Ltd., Dorset, UK). Following transfer, membranes were blocked for 1 hr at room temperature in TBS/0.1% Tween-20/5% BSA then incubated overnight at 4 deg C in the appropriate primary antibody diluted in TBS/0.1% Tween-20/5% BSA. The primary antibodies used were specific to the protein of interest. The primary antibodies used were:rabbit anti Adra2a (1:1000 dilution, PA1-048, Pierce Biotechnology Inc., Rockford IL, USA), mouse anti eNOS (1:1000 dilution, 610297, BD Biosciences, Oxford, UK), rabbit anti phospho-eNOS (Ser1177) (1: 1000, #9571, Cell Signalling Technology, Inc., Danvers MA, USA), and mouse anti GAPDH (1:1000 dilution, ab6046, Abcam plc., Cambridge, UK). Secondary detection was performed by incubating for 1 hour at room temperature with the appropriate HRP-conjugated secondary antibody diluted in TBS/0.1% Tween-20/5% BSA. The secondary antibodies used were donkey anti mouse IgG (human and rat IgG cross-adsorbed, 1:10,000 dilution, A16017, Life Technologies, Renfrew, UK), goat anti Rabbit IgG (human and rat IgG cross-adsorbed, 1:10,000 dilution, sc-2054, Santa Cruz Biotechnology, Inc., Dallas TX, USA. The bands were visualized using an enhanced chemiluminescence detection kit and quantified by densitometry. Loading accuracy was

evaluated via membrane rehybridization with antibodies against mouse and rabbit anti-α tubulin (1:1000; Upstate Biotechnology, Albany, NY) or mouse anti GAPDH (1:1000 dilution, ab6046, Abcam plc., Cambridge, UK). Densitometry was measured using Alphaview SA v3.4.0 software (ProteinSimple, San Jose CA, USA).

2.8 Histology

Formalin fixed liver tissue was processed through graded alcohol and xylene and embedded in paraffin. 3-5mm sections were dewaxed in xylene, stained with hematoxylin and eosin (for assessment of routine liver histology) or picrosirius red (for assessment of collagen deposition), and mounted in DPX. Liver tissue was processed in accordance with standard protocol and Haematoxylin and Eosin together with Sirius Red staining was performed. Histological staging was conducted by a consultant histopathologist using a 14 point secondary biliary cirrhosis scoring system. Sirius red staining was quantified using computer assisted digital image analysis. A circular polarising filter was used for picrosirius red stained slides. Collagen proportionate area was determined using Zeiss KS300 image analysis software.

2.9 Radiometric analysis of hepatic NOS activity

The conversion of ^{14}C L-arginine to ^{14}C L-citrulline was used as an index of NOS activity. Snap frozen liver tissue was homogenised and measured for protein content as above. For NOS activity determination, 5µl of supernatant was incubated with 40µl of a reaction medium [Tris-HCL buffer (50mM, pH 7.4); NADPH (1.25mM); ^{14}C-arginine (10µCi/ml, Amersham, UK); norvaline (5mM); and either $CaCl_2$ (400µM) or EGTA (600µM)] at

$30^{o}C$ for 30 minutes, and the reaction stopped with 500ml of ice-cold citrate buffer (50mM pH5) containing EDTA (1mM). The arginine:citrulline ratio were determined by separating the amino acid components using thin layer chromatography on silica plates (Kieselgel 60, Merck, Darmstadt, Germany). Non-tritiated amino acids were added to aid spot detection, and the components separated using a running mixture of $CHCl_3:MeOH:NH_4OH:H_2O$ (ratio of 10:45:20:10). The individual spots were removed and the scintillation activity measured as for DDAH activity assay above. The activity was expressed as μmoles citrulline made/ mg protein/ min. The difference in the measured NOS activities between the calcium present / calcium chelated (EGTA present- for iNOS activity determination) assays described above, were interpreted as representing eNOS activity.

2.10 Plasma Renin Activity

Plasma renin activity was assessed using a FRET assay specific for rat renin (SensoLyte 520 Rat renin assay kit; AnaSpec, EGT Group, Seraing, Belgium). Briefly, plasma samples (EDTA as anticoagulant) were incubated with a 5-FAM/QXL520 FRET peptide corresponding to a part of rat angiotensinogen. Kinetic measurements (every 5 minutes for 1 hour, 37degC) of fluorescence intensity at ex/em 490 nm/520 nm were taken using a fluostar omega plate reader (BMG Labtech Ltd., Aylesbury, UK) with excitation and emission filters of 485/12 nm and 520/20 nm. The initial velocity (V0) in RFU/min was calculated from the linear portion of the kinetic curves using Omega MARS data analysis software v11.31277.0 (BMG Labtech Ltd., Aylesbury, UK)

2.11 Plasma Noradrenaline Levels

Plasma noradrenaline concentration was measured using a competitive ELISA (LDN Labor Diagnostika Nord GmbH & Co. KG, Nordhorn, Germany). Briefly, noradrenaline was extracted from plasma samples (EDTA as anticoagulant), standards, and controls using a boronate affinity gel coated plate. The extracted noradrenaline was acylated and enzymatically derivatised. The concentration of derivatised noradrenaline was measured using a competitive ELISA with a HRP-conjugated secondary antibody and TMB as substrate. The absorbance spectrum from 350 to 850 nm was measured using a Fluostar Omega plate reader (BMG Labtech Ltd., Aylesbury, UK). The absorbance at 450nm for the standards was used to generate a 4-parameter non-linear regression (y linear, x logarithmic) from which the concentrations of the samples was calculated using Omega MARS data analysis software v11.31277.0 (BMG Labtech Ltd., Aylesbury, UK).

2.12 Cyclic AMP ELISA

96 well plates pre-coated with mouse monoclonal anti-rabbit IgG and blocked with a proprietary formulation of proteins (Cayman Chemical,AnnArbor,USA). To prepare the plasma, to 500 µl plasma 2 ml ice cold ethanol is added and vortexed. The supernatant obtained after centrifugation at 1,500 x g for 10 minutes is transferred to a clean 10 ml test tube. The supernatant is dried then resuspended in 500 µl of ELISA Buffer. Supernatants of these cultures were applied to the ELISA plates in 3 step dilutions and incubated for 3 hrs at room temperature. Plates were washed and incubated with an HRP coupled secondary goat anti-mouse Ig antibody Southern Biotechnology) for one hour at room temperature. Thereafter Ellman's reagent is reconstituted and 200µl pipetted into

each well. The plate is then incubated for 90 minutes in a orbital shaker. The plate is read in a colour absorbance wavelength range between 405-420nm in a sphectrophotometer using the Endoscan V software.

2.13 Cell culture

Rat hepatic stellate cells were a kind gift from Jonathan Fallowfield. Rat HSCs were cultured in DMEM (high glucose, with sodium pyruvate & glutamax) with 10% FBS (Life Technologies, Renfrew, UK). All treatments with agonist or antagonist were performed in serum free medium.

2.14 Gel contraction

Rat hepatic stellate cells were cultured within 3D collagen gels (Fibroblast-populated collagen lattices; FPCL). 24 well cell culture plates were pre-coated with sterile 2% BSA (Sigma) in PBS (Sigma). Ice cold neutral collagen solution was made by mixing 1 part 0.2M HEPES pH 8.0 (life tech), 4 parts >2mg/ml rat tail collagen type I (First Link (UK) Ltd., Wolverhampton, UK), and 5 parts DMEM (high glucose, with sodium pyruvate and glutamax; Life tech). For FPCL, equal volumes of the neutral collagen solution and hepatic stellate cells resuspended at 10cells/ml in DMEM were mixed. 1ml of the FPCL was added to each well and allowed to polymerise for 1 hour at 37degC 5% CO2. After polymerisation, 1ml of serum free medium with or without 50uM guanfacine was added causing detachment of the FPCL from the plate. After 6 hours the contracted gels were imaged using a Fluorchem M ccd (Protein simple) with white light transillumination.

2.15 Isolation of Liver Non-Parenchymal Cells and circulating cells.

The isolation of Liver non parenchymal cells was performed in the following manner. Perfused liver tissue was dissected with a scalpel and homogenized in Hanks balanced salt solution (with calcium and magnesium + collagenase 0.01% and DNAse I (0.01%). The homogenate was then transferred to a 50ml Falcon tube and incubated at 37'C prior to filtration through a 100mcm cell strainer.

Thereafter it was centrifuged at 500rpm for 5 minutes at 4'C and the supernatant subsequently centrifuged at 2000rpm for 10 minutes at 4'C. The supernatant was discarded and the pellet resuspended in PF4 (HBSS with no calcium or magnesium, DNAse I 0.01%, bovine serum albumin (0.25%) and centrifuged at 2000rpm for 10 minutes at 4'C. The pellet was then resuspended in 3.9ml of RPMI 1640 and mixed gently with 2.1ml (RPMI and optiprep 22%). RPMI was then layered on top followed by 25 minute centrifugation (2800rpm without brake at 4'C). The non-parenchymal cells were then isolated from the interface, resuspended in an equivalent volume of PF4 and centrifuged at 2000'C at 4'C for 10 minutes. 10×10^6cells were used in all subsequent assays.

The peripheral circulating cells were suspended over a polymorphoprep solution and centrifuged at 500rpm for 10 mins at 4'C . The layer of interest (neutrophils at the bottom layer and the monocyte at the middle layer) are pipetted out and resuspended in a 10ml HBSS solution without Ca^+/Mg^+. A further centrifuge at 350rpm for 10 mins produced a pellet. A red cell lysis buffer is added to the pellet and resuspended with vortex. After

removing the supernatant, 250 µl of HBSS is added to the solution and cell count is made by FACS after appropriate gating.

2.16 Phagocytic function of Non parenchymal and circulating cells

The phagocytic function of non parenchymal and circulating cells was assessed by the following method. The neutrophils and monocytes were isolated by the method described above in Section 2.15. The cells were centrifuged at 2000rpm for 5 minutes at 4ºC and the supernatant discarded. 200ul of latex beads containing media were added to the pellet and incubated at 37’C in the dark for 20 minutes. 5ml of ice cold PBS was then added and centrifuged at 2000rpm for 5 minutes at 4ºC. The pellet was then washed with 5ml of cold PBS and centrifuged. Fc blocker was then added and incubated for 10 minutes at 4’C. Cell surface markers for neutrophils (Anti CD32 antibody) and kupffer cells (Anti-CD163 antibody) was then added and incubated for 30minutes at 4’C in the dark. The phagocytic function was assessed with the help of the FACS canto machine after the cells were washed with 1ml of FACS buffer, centrifuged and resuspended in 100ml FACS buffer solution.

2.17 Kupffer cell ROS production

To assess Kupffer cell ROS production, 20ug/ml of *E.coli* endotoxin was added to 1x 10^6 non-parenchymal cells sample and incubated for 30minutes at 37’C. ROS inducer at a final concentration of 200-500uM was used for the positive control. The samples were

then centrifuged at 500g for 5 minutes and the supernatant discarded. The cells were then resuspended in 5ml of wash buffer, centrifuged at 500g for 5minutes and the supernatant removed. The cells were re-suspended in 500ul of ROS detection solution and incubated for 30 minutes at 37'C in the dark. Following centrifugation, the cells were resuspended in 100ul of FACS buffer, Fc blocker added (1:25) and incubated for 10minutes at 4'C. Anti-CD163 antibody was added and the cells incubated for 30minutes at 4'C in the dark. The cells were then washed with 1ml of FACS buffer, centrifuged and resuspended in 100mcl FACS buffer solution.

2.18 Limulus amoebocyte Lysate (LAL) endotoxin assay

The chromogenic limulus amoebocyte lysate kinetic assay (Charles River Laboratories) was used for the detection of endotoxin. Portal venous plasma (100mcl) was diluted 1:10 with endotoxin-free water and incubated at 75'C for 30 minutes. 100mcl of sample and 100mcl of LAL reagent were mixed in a 96-well plate and analysed at 405nm with spectrophotometer using the Endoscan V software. Results are expressed as EU/ml.

2.19 Brain water Measurement

Brain water content was measured 24 hour post surgery. At the time of sacrifice, the rat brain was immediately removed and the right frontal lobe was excised with a sharp scalpel and inserted into a plastic tube and weighed in a electronic digital weighing machine. They are placed in an oven at 105 degrees for 48 hours. The percentage of water content was calculated as [(wet weight-dry weight)/wet weight] x 100%.

2.20 Arginase activity assay

Liver tissue was suspended in a TRIS/HCL buffer containing a protease inhibitor cocktail (Sigma-Aldrich) and PMSF, and manually macerated with a glass mortar and pestle. The suspension was then centrifuged, and the clear homogenate was collected leaving the precipitate behind. 20 µl liver homogenate or serum was then combined with 20µl MnCl (10 mM), 150 µl Tris Buffer (pH 7.4, 50 mM), and 100µl of l-arginine (50 mM; Sigma-Aldrich) for 30 min at 37° C. Arginine hydrolysis was stopped with 0.5 ml Tungstic acid solution (150 mM) containing concentrated hydrochloric and sulphuric acid (100 mM each). Unwanted particulate matter was pelleted by centrifuging at 5,000 RPM for 10 min at 4° C. 200µl of clear solution was used for detection of urea on a COBAS INTEGRA 400 using a UREAL kit assay (Roche). To subtract background serum urea levels, each experiment was duplicated substituting the l-arginine substrate with 100 µl water. 1 U of arginase activity is defined as the amount of enzyme required to catalyze formation of 1µM urea per minute. To avoid any bias in arginase activity attributable to baseline differences in protein content of the liver sections, protein content for each sample was determined by the Biuret test. This allowed arginase activity to be standardized against protein content, expressed as IU/liter serum or IU/mg protein.

2.21 RNA isolation and qRT-PCR

Frozen liver was homogenized in TRI reagent (Sigma-Aldrich Company Ltd., Dorset, UK) using 7mm steel beads with a tissue lyser (Qiagen Ltd., Manchester, UK). After extraction with chloroform total RNA was isolated using an RNeasy mini kit (Qiagen Ltd., Manchester, UK). RNA concentration was measured using a nanodrop (Labtech International Ltd., Uckfield, UK). Reverse transcription was performed on 1ug RNA per sample with combined polyT and random hexamer primers using a Quantitect reverse transcription kit (Qiagen Ltd., Manchester, UK).

qPCR was performed using hydrolysis probe assays (Integrated DNA Technologies, BVBA., Leuven, Belgium) and Taqman Fast Advanced marmix (Life Technologies, Renfrew, UK) on a 7500 Fast qPCR machine (Life Technologies, Renfrew, UK)

2.22 Statistics

Data are expressed as mean ± SEM or median [range] as appropriate. Significance of difference was tested with student T-test or Mann-Witney comparisons test; $p<0.05$ was taken to be statistically significant. Comparison between multiple groups was by analysis of variance (ANOVA) with a Bonferroni correction for multiple comparisons. Software used included Microsoft Excel 2007 (Microsoft Corp., Redmond, WA) and GraphPad Prism 4.0 (GraphPad Software, Inc., San Diego, CA)

Chapter 3

Characterisation of the Bile duct ligated rat as a model of Portal Hypertension and biliary cirrhosis

3.1 Introduction

The effect on hepatic morphology after traditional bile duct ligation has been studied extensively since the original publication by Cameron and Oakley et al in 1932.The timing of development of cirrhosis varied between various studies. Some reported evidence of cirrhosis in 3-5 weeks post bile duct ligation. Biliary obstruction for 15 days or more led to cirrhosis and after 4 weeks , this was found to be the majority of the rats. It was associated with extra hepatic cholestasis , with a narrow zone of oedema and ductular proliferation at the junction of the parenchyma and the septa which is characteristic of biliary cirrhosis in man (168)

Systemic and splanchnic haemodynamics of the chronic bile duct-ligated rat were previously characterized by radioactive microspheres. The conscious bile duct ligated rats had a significantly more hyperdynamic circulation as compared to sham operated animals with increased cardiac output and portal venous blood flow (169).The chronology of evolution of portal hypertension , sodium handling and hyperreninaemia in a bile duct ligated rat model was studied by Poo et al (170). They showed that the portal pressure increases at week 1 and the mean arterial pressure remains the same until week 4, when it drops in the bile duct ligated group as compared to the sham operated animal. A significant reduction in sodium excretion and hyperreninaemia was also noted in the bile duct ligated rats as compared with control rats. However there is a suggestion that the

characteristic hyperdynamic circulation and increase portal pressure in the bile duct ligated rats occur earlier than the reduction in the sodium excretion and hyperreninaemia , suggesting that these are secondary phenomenon (171) In bile duct ligated rats, increased levels of pro inflammatory cytokines were found as compared to the control rats (171). BDL-induced cirrhosis was corroborated by the elevated liver damage markers and histopathological analysis. Chronic BDL significantly increased most of plasma and hepatic cytokine levels and diminished the hepatic IFN-gamma amount (172)

When bile duct ligated rats and sham operated are challenged with lipopolysaccharide (LPS), there was increased sensitivity in the bile duct ligated rats as compared to the sham operated rats. This was associated with increased cytokine release and plasma nitrate/nitrite levels. It also was associated with increased liver injury and mortality in the bile duct ligated rats (165)

The aim of our study was to establish that the bile duct ligated model with or without lipopolysaccharide had all the necessary vascular and biochemical parameters to test my hypothesis.

3.2 Animals

The animals were cared for as per standard protocol as described in section 2.1

3.2.1. Animal models: Physical appearance

Male Sprague Dawley rats (Charles River, UK) were housed in a temperature and light controlled (12 hours light/dark cycle) facility at the Comparative Biology Unit, UCL. Rats received standard chow and water ad libitum. All procedures were performed in accordance with UK Home Office Animals (Scientific Procedures) Act 1986.

3.2.2 Weight Changes

	Sham	**BDL**	**BDL + BRL44408**
Weight gain (%)	43.0 (21.6)	25.8 (14.8)	20.4 (10.3)
Liver weight (g)	14.6 (1.62)	27.9 (3.84)	24.5 (5.29)

Table 3.1 : Weight Changes in the three groups of animals.

All the animals studied were male Sprague Dawley rats supplied by Charles Rivers. Their initial weights were between 220-250 gms. Both Sham operated rats and BDL rats gained weight albiet at different rates of gain. The chart above denotes the percentage change in their weight from baseline up to the time of exsanguination. The liver weights were also

calculated in each group. I have included the intervention arm in this table as it shows the liver tends to gain weight upon treatment with an ADRA2a adrenergic receptor antagonist.

3.2.3 Biochemistry Profile

	Sham	BDL	BDL + LPS
Albumin (g/L)	32.4±0.76	24.41±0.93***	24.80±0.80
Bilirubin (µmol/L)	1.00±0.62	183.80±21.02	190.1±10.20
ALT (IU/L)	70.27±9.07	100.10±13.26*	130.3±24.40
AST (IU/L)	131.70±17.37	444.50±58.3***	587.5±56.48
Lactate (mg/dL)	2.74±0.52	7.05±0.42	10.23±0.24
Creatinine (mmol/L)	31.50±1.75	33.47±1.63	40.90±1.96

Table 3.2: Shows the plasma biochemistry parameters in the three groups, Sham, BDL and BDL + LPS. These parameters include serum albumin, alanine transaminase, Aspartate transaminases, Lactate and Creatinine levels. (* p<0.0001; * p<0.01)**

The serum biochemistry was measured as per method described in section 2.5.The serum albumin was significantly different between the sham operated with saline and BDL animals treated with saline (32.4 v 24.4 g/L,$p<0.0001$) . There was no significant difference between BDL animals treated with saline or LPS.

Similarly, there was a significant increase in the serum bilirubin the BDL rats as compared with the sham operated rats (183.80±21.02 v 1.00±0.62, $p<0.0001$). But there was no difference between the BDL animals treated with saline or LPS.

There was a non statistical increase in the BDL rats as compared with the sham operated rats in the alanine aminotransferase (ALT) levels (100.10±13.26 v 70.27±9.07). There was a further increase in the ALT levels in the BDL rats treated with LPS as compared with saline, however, this was not statistically significant (130.30±24.46 v 100.10±13.26). There was a significant increase in the plasma AST level in the BDL rats treated with saline as compared to the sham operated rats treated with saline (444.50±58.3 v 131.70±131.70, $p<0.05$). Although there was a further increase in the BDL rats treated with LPS as compared to the BDL rats treated with saline, it was statistically not significant.

Moreover, the plasma lactate was also significantly higher in the BDL animals treated with saline as compared to the Sham operated rats (7.65± 0.96 v 2.74± 0.52, $p<0.05$) and serum creatinine was not significantly raised in the BDL rats treated with saline as compared with the sham operated rat.

3.2.4. The Mean Arterial Pressure

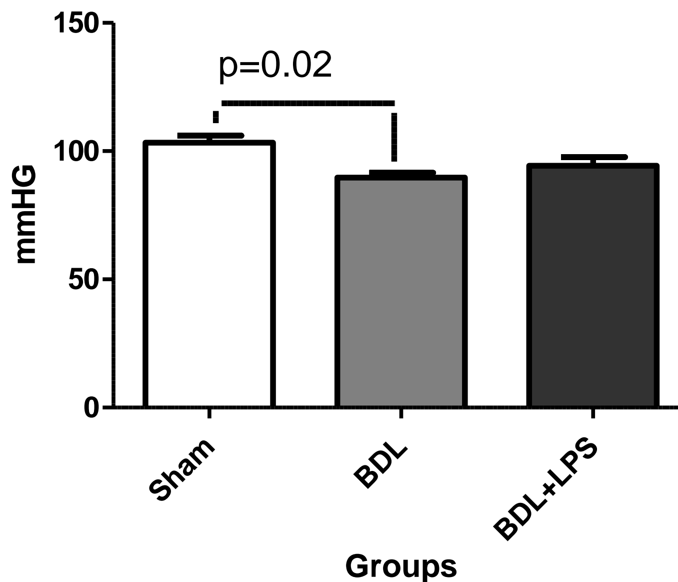


Fig 3.1: Mean Arterial Pressure (MAP) in the three groups (Sham , BDL and BDL rats treated with LPS). It shows that the MAP is reduced significantly in the BDL rat treated with saline as compared to the Sham operated rat (p=0.02).There was no further decrease in the MAP in the BDL rats treated with LPS. (Sham=16, BDL=20, BDL+LPS=6)

The mean arterial pressure was measured in all the three groups as described previously in section 2.3. (Sham, BDL, BDL+LPS). There was significant reduction in the MAP in the BDL group as compared to the Sham operated rats (89.63 ± 1.91 v 103.3 ± 2.75, p=0.02) . There was however no significant difference in the MAP between the BDL rats with or without LPS (99.02 ± 3.4 v 89.63 ± 1.91).

3.2.5 Plasma Renin Activity

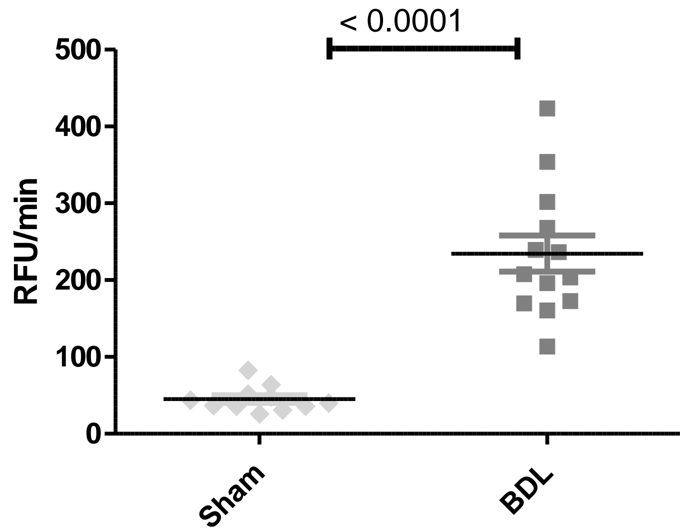


Fig 3.2: Plasma Renin Activity in Sham and BDL animals. This shows that there is a significant elevation of PRA in the BDL rats as compared to the Sham animals (p<0.0001).

The plasma renin activity is measured by an ELISA test in sham and BDL rats as described in section 2.10. There is a significant increase in the plasma renin activity in the BDL rats as compared to the sham rats (234.4 ± 23.39 v 45.5 ± 11.7, p<0.001).

3.2.6 Portal Pressure measurements

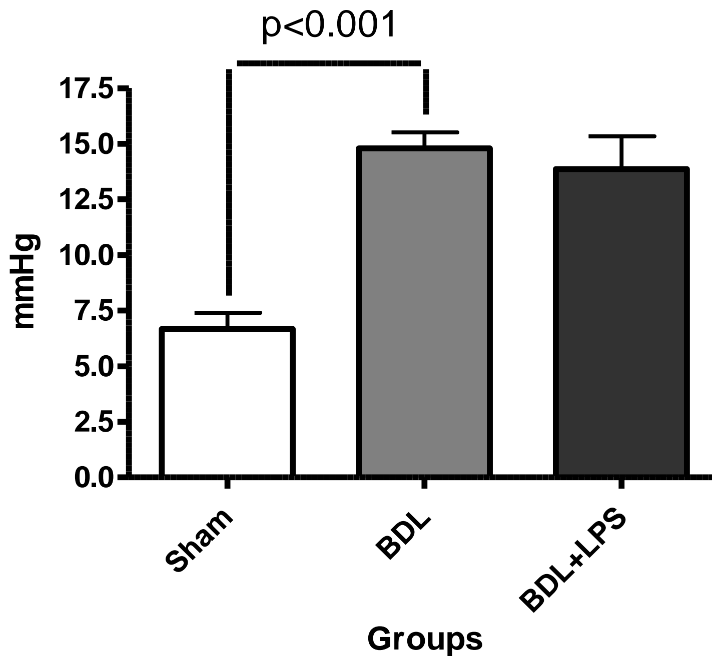


Fig 3.3: Portal pressure measurement in the three groups (Sham, BDL and BDL animals treated with LPS). This shows that there is a significant increase in the portal pressure in the BDL rats as compared to the Sham animals (p<0.001).However, there was no difference in the portal pressure in the BDL rats treated with LPS as compared to BDL rats treated with saline. (Sham=16, BDL=21, BDL+LPS =6)

The portal pressure was measured in all the three groups (Sham, BDL and BDL + LPS) as described in section 2.3. There was significant increase in the portal pressure in the BDL group as compared to the Sham operated rats (14.79± 0.72. v 6.67±0.72). However on injection of LPS to the BDL rats, there was no difference in their portal pressure (14.79± 0.72 v 13.86 ± 1.47)

3.2.7: The relation between the portal pressure and plasma noradrenaline levels.

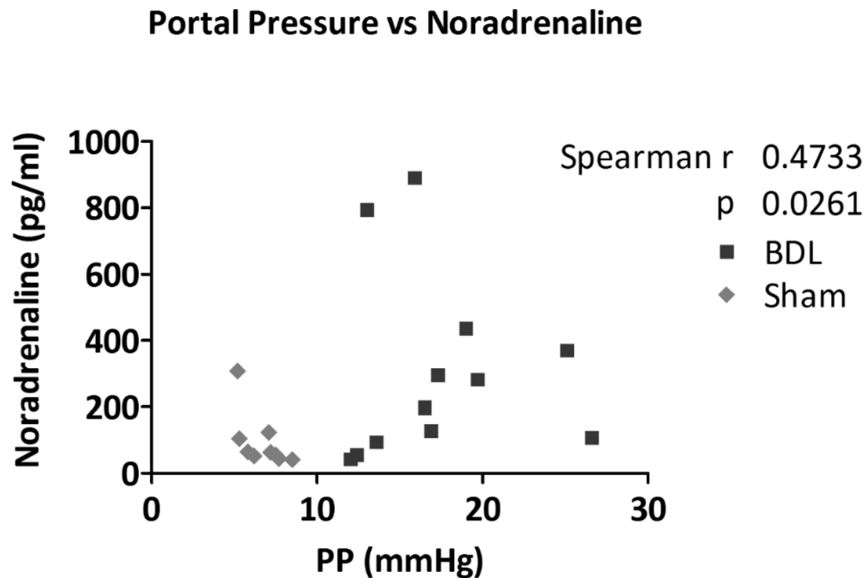


Fig: 3.4: Relationship between the portal pressure and plasma noradrenaline levels.Shows a scatter plot diagram of the Noradrenaline levels in relation to the Portal pressure between the Sham and the BDL group.It shows that there is a significant linear co-relation between the two variables (p=0.02). (Sham=9, BDL=12)

The plasma noradrenaline levels was measured by ELISA in sham and BDL rat plasma as described in section 2.11. There is a significant difference between the plasma noradrenaline levels in the sham and BDL animals. The BDL animals had significantly more noradrenaline levels as compared to the Sham operated animals. On plotting the noradrenaline levels to the portal pressure, there is a direct co relation between the plasma noradrenaline levels and the portal pressure.

3.2.8 Hepatic tissue TNFα levels

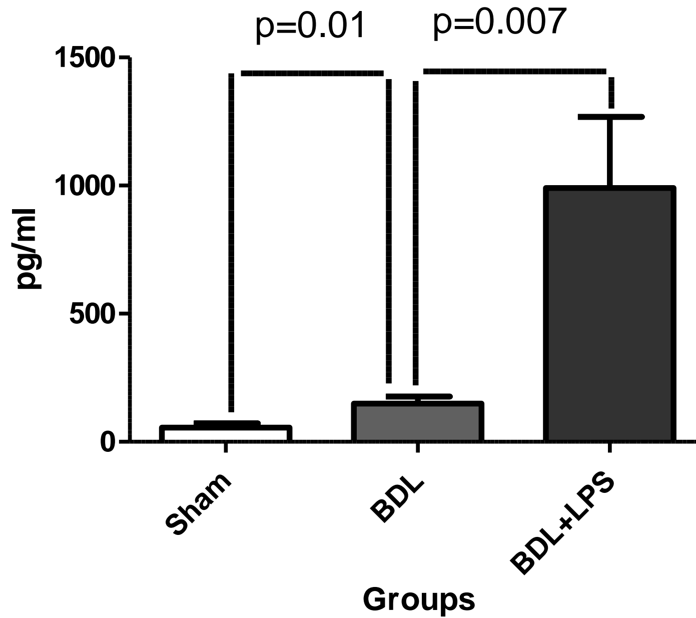


Fig 3.5 : Liver tissue TNFα levels in Sham, BDL and BDL rats treated with LPS. The FAC cytokine bead array test shows that the TNFα levels is significant increase in the BDL rats as compared to the sham rats (p=0.01). And it rises significantly in BDL rats treated with LPS as compared to BDL rats treated with saline (p=0.007) (Sham=8,BDL=7, BDL+LPS = 6)

The liver tissue TNFalpha cytokine levels were measured by FAC cytokine bead array as described in section 2.6. The TNFalpha levels was significantly increased in the bile duct ligated animals as compared to the sham operated animals (73.23±31.26 v 49.41±22.31, p=0.01). However on injecting LPS to the BDL rat, there is a further significant rise in the TNFα levels as compared to the BDL group without LPS (990.6±227.7 v 73.23±31.23, p=0.007).

3.2.9 Plasma TNFα levels

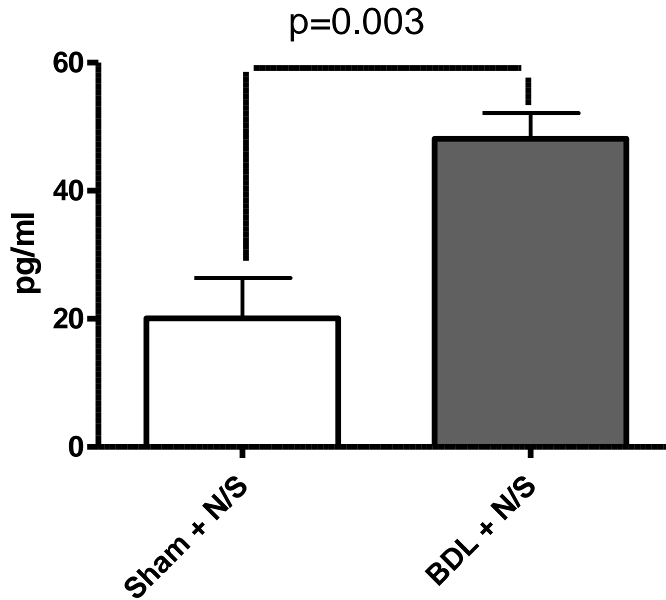


Fig 3.6 The plasma TNFα in Sham and BDL animals. This shows that there is a significant inthe plasma level of TNFα in the BDL rats as compared to the Sham operated animals (p=0.003) (Sham+N/S =6, BDL+N/S=6)

The plasma level of cytokine TNFα is measured by an ELISA test as described in section 2.6. This shows that the plasma TNFα level increases significantly in the BDL rats as compared to the Sham operated rats (19.5±7.84 v 55.26±16.80, p=0.003).

3.2.10 Presence of the ADRA 2a receptor in the liver tissue

To analyse the protein expression of alpha 2a receptor in the liver, western blot analysis were undertaken on liver homogenate. This showed a significantly increased protein expression in the bile duct ligated rats as compared with the sham rats (p=0.01,fig 1a). Alpha 2a receptor protein was detected at 68 kilodalton. GAPDH was used to correct for protein loading.

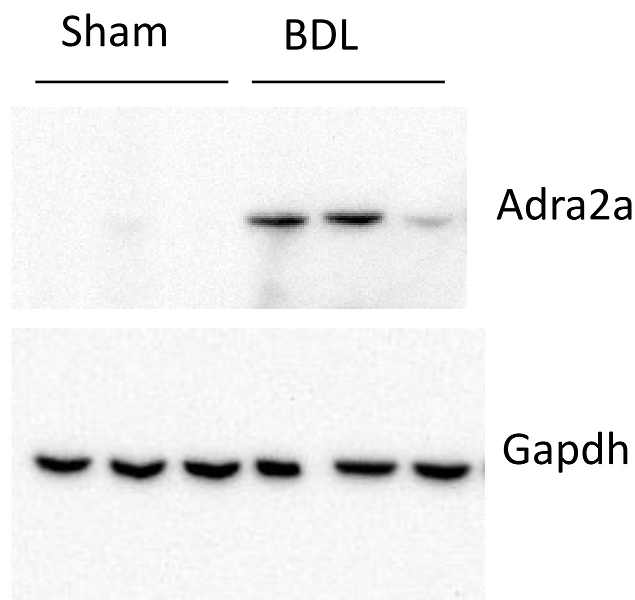


Fig 3.7: Protein expression of alpha 2a adrenoreceptor showing a significant increase expression in the BDL rat as compared to the Sham operated rats (p<0.01,t-test)

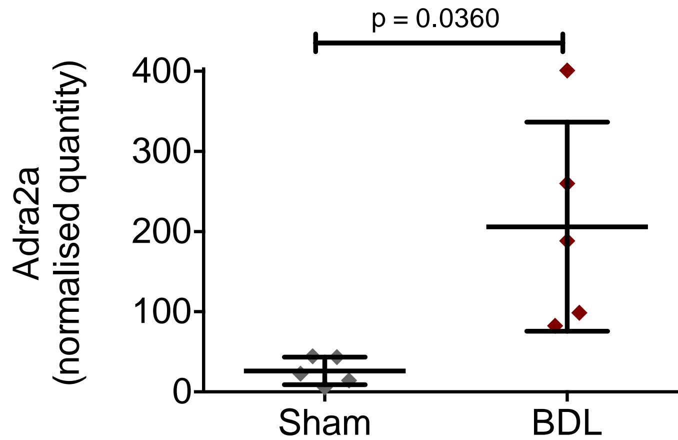


Fig 3.8: mRNA expression of ADRA 2a receptors in Sham, BDL and BDL animals There is a significant difference in the number of ADRA 2a receptors in the two groups with a significant number which is more in the BDL rats as compared to the sham operated rats (p=0.03)

The receptor density in the liver is shown in the figure above as quantified by qPCR. The method of isolation of mRNA was undertaken as per the method described in section 2.20. There is a significant difference in the number of receptors in the BDL rats as compared to the Sham operated animals.

3.3 The effect of inflammation on structure

The initial elements of the study were to determine the impact of inflammation on the structure and function in a rodent model of bile duct ligated rats with reference to liver structure and measureable variables of biochemical function and inflammatory parameters.

3.3.1 Structural changes in the liver due to inflammation

Histological presentation of the bile duct ligated rat:

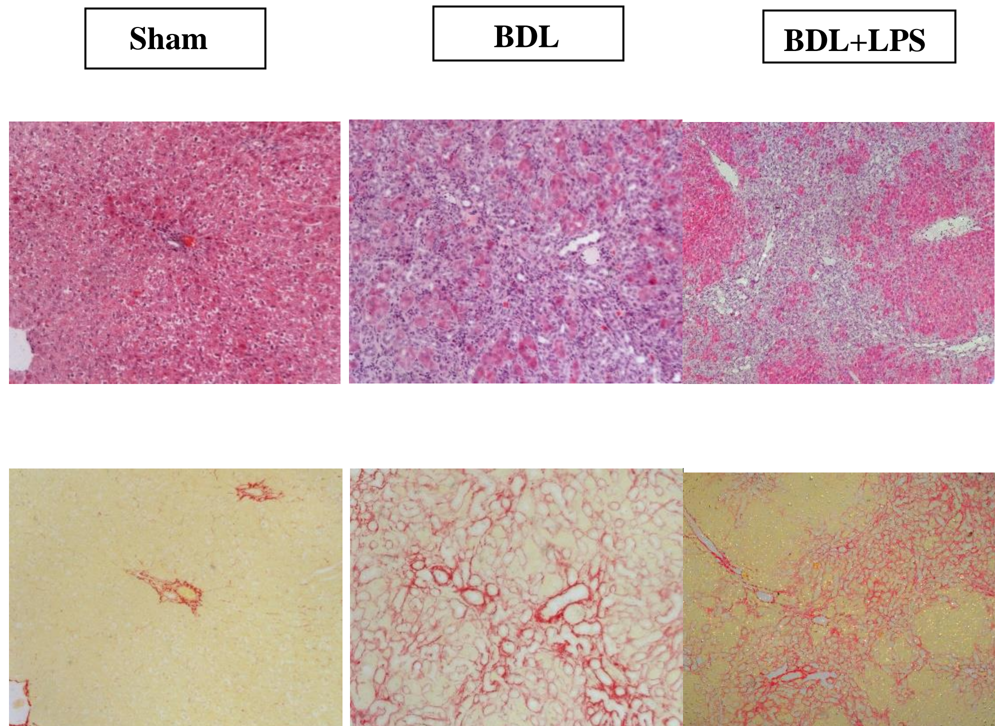


Fig 3.9: Histological assessment in the three groups. The top panel shows the Haematoxylin and Eosin stain in the Sham, BDL and BDL+LPS in the top panel. The bottom panel shows the Pico Sirius Red staining in the three groups –Sham ,BDL and BDL+LPS.

The histological assessment of the rat liver tissue were prepared as described in section 2.8. As seen in patients with cirrhosis, BDL rats undergo significant loss of the normal liver architecture .The sham operated rats show that the normal lobular architecture is intact. In bile duct ligated rats, there is increasing loss of defined portal tracts. There is a significant increase in the proliferation of cholangiocytes. There is loss of liver architecture with early nodule formation. This distortion and loss of liver architecture is further augmented by the addition of lipopolysaccharide (LPS), as an inflammatory stimulus to BDL animals with greater parenchymal loss. With the Pico Sirius Red staining, it is evident that there is preservation of normal architecture in the sham rats , whereas there is increased collagen deposition in the in the BDL rats and the BDL rats treated with LPS. There is no clear difference in the level of collagen deposition between the BDL and the BDL rats treated with LPS.

3.4 Discussion

Improved understanding of the pathogenesis of chronic liver disease and development of novel diagnostic, prognostic and therapeutic tools depend on development of appropriate reproducible animal models. However, no single animal model has been deemed to be perfect for the study of a diverse range of pathological processes leading to acute and chronic liver disease. Cholestatic liver injury is one of the major causative factors for the development of liver fibrosis and cirrhosis in patients with chronic liver disease. Therefore, experimental models have been generated that mimic various aspects of the complex mechanisms that lead to hepatic inflammation, fibrosis and cirrhosis. (173). Surgical BDL is one of the most widespread experimental models that is used to induce obstructive cholestatic injury in mice and rats. (174). We and others have already used this model in experiments involving inflammation and portal hypertension. However, there are discrepancies in the acceptance of the optimum time for the establishment of fibrosis in this model. In a recent study by Tarcin et al, it was demonstrated that collagen formation was optimum at week 3 and thereafter there is reduction in the amount of collagen content (175).This implied that the fibrosis in this model is at its peak at week 3 post bile duct ligation and thereafter there is gradual reduction in fibrosis. Therefore it was important to validate this model to justify using this model to study this area.

There can be several important conclusions that can be made from the results of this study. We have been able to show that there is a significant difference in the biochemical characteristics between the sham operated rat and the bile duct rats. The sham operated rats have a significantly higher albumin than the BDL rats, however, the bilirubin is

significantly higher in the BDL rats as compared to the sham operated animals. There was significant increase in aspartate transaminase in the BDL rats as compared to the sham operated rats and there was a trend towards increases in the serum alanine transaminase in the BDL rats as compared to the sham operated rats. There was no different in the results when the BDL rats were treated with Lipopolysaccharide. From the literature review, it is clear that in chronic hepatocellular injury, ALT is more commonly elevated than AST; however, as fibrosis progresses, ALT activities typically decline, and the ratio of AST to ALT gradually increases, so that by the time cirrhosis is present, AST is often higher than ALT (176) (177). In my BDL model, at the end of 4 weeks we already note that the liver architecture has changed significantly with evidence of evolving fibrosis. This may help to explain why there is no significant difference in the ALT levels between the sham and BDL rats in my study.

The histological slides provide further proof of the progressive nature of liver injury that is seen in the bile duct ligated rat model as compared to the sham rats. This is characterised by the increased proliferation of cholangiocyte and septa formation in the BDL rats as compared to absence of these features in the sham operated rats. On assessment of the Sirius red staining of each of the group, it reveals that there is a significant increase in the deposition of collagen in the liver disease as compared to the sham operated animals. There is no increase in the collagen deposition by the BDL rats treated with LPS as compared to untreated BDL rats.

This is further supported by the finding of the significantly increased pro inflammatory cytokine TNFα in the liver tissue of the BDL rats treated with LPS as compared to the BDL rats. There was also significant increase in the TNFα level both in the plasma and liver tissue in the BDL rats as compared to the sham operated rats.. This indicates that the inflammation is propagated significantly by the addition of LPS. This supports the finding of increased cytokine release and plasma nitrate/nitrite levels associated with increased liver injury and mortality in the bile duct ligated rats noted by Harry et al. (165). The timing of 3 hours post LPS injection and the dose of 0.33mg/Kg via intra peritoneal route has a major impact on the study. In a recent study by Chou et al, it has been demonstrated that there is maximum stimulation of the pro-inflammatory cytokines at 3 hours both in the liver tissue and plasma when they used a 0.5mg/kg dosage regime given by a tunnelled biliary line(178). There is significant increase in the TNFα in the plasma however there was reduction in the TNFα level in the liver tissue in this study. In our study, we used a 4 week old BDL model with a lower dose of LPS (0.33mg/Kg) given via the intra peritoneal route. The bioavailability of LPS may be variable because of this choice of route and probably has led to the finding of no significant difference in the Sham and BDL groups. Although we have demonstrated that there is change in the cytokine profile in the BDL rats as compared to the Sham rats, this dose and route may not have been adequate to effect a haemodynamic change.

The other significant finding is the upregulation of the ADRA 2a receptor protein in the BDL rats as compared to the sham operated animals. There is no evidence of presence of ADRA2a receptors in the normal livers. There is increased protein expression of the ADRA 2a receptor in the BDL rats. I am able to show this difference between the sham

and BDL both by protein expression and mRNA. In the context of a bile duct ligated rat model, the addition of LPS worsens the pro inflammatory cytokine load. This predisposes the migrating macrophages to be activated and translocated to the liver.

In this characterised BDL model we are able to show that the portal pressure is significantly raised as compared to the sham operated rat. There is no further augmentation of the portal pressure in the BDL rats treated with LPS. The study design consisted of giving one dose of LPS by the intra peritoneal route three hours before exsanguination. This probably impacted on the fact there was no significant difference in the portal pressure in the BDL rats treated with LPS as compared to the untreated BDL ones. In clinical practice the secondary insult is usually over a longer period of time and therefore is able to influence the portal pressure in a more significant way. We have also shown that the noradrenaline levels in the BDL rats were significantly increased as compared to the sham operated animals. It is well known that production of noradrenaline may be from a multitude of sources including the adrenal glands and the circulating immune cells which have been shown to have the mechanism to produce noradrenaline on its own in a paracrine effect. Intestinal translocation of bacteria and its products stimulate the adrenergic receptors in the migrating immune cells. These cells in turn stimulate the transmembrane receptors present in each cell to produce the increased amount of noradrenaline that is observed in this study.

There is a reduction of the mean arterial pressure in the BDL rats as compared with the sham operated rats. There is no difference in the MAP when LPS is injected to the BDL rats. The same early effect of the LPS effect is probably in play as a cause for the non

difference in the haemodynamic outcome post injection of LPS. As a compensatory mechanism, we are also able to show that the plasma renin activity is increased in response to the low MAP found in the BDL rats as compared to the sham operated animals.

In conclusion, This model provides me with all the necessary haemodynamic, biochemical and cytokine characteristics that will enable me to carry out the studies that I have planned for. The increased protein expression of the ADRA 2a receptor is an important finding in the BDL rat. The fact that ADRA2a receptor was not found in the sham operated rat suggest that is produced by the migrating immune cells provide a rational target to reduce intra hepatic and systemic inflammation and thereby mitigate the haemodynamic dysfunction. The next phase of the study will attempt to reduce the high inflammatory load in this model by using an ADRA2a adrenergic receptor antagonist and thereby reduce intra hepatic resistance and portal hypertension.

Chapter 4

The effect of ADRA2a adrenergic receptor antagonism in hepatic and systemic haemodynamics in a BDL rats

4.1 Introduction.

Portal hypertension is the clinical syndrome which is implicated in the complication of liver cirrhosis. The onset of clinically significant portal hypertension defines disease progression. It is responsible for a spectrum of consequences which lead to acute decompensation of the liver by way of ascites, variceal bleeding, hepatic encephalopathy and hepato-renal syndrome (68). Portal hypertension is characterized by both increased intra hepatic resistance and splanchnic vasodilatation. The primary haemodynamic change that occurs in portal hypertension is increasing being accepted as increased intra hepatic resistance. This increased intra hepatic resistance was originally thought to be as a consequence of established fibrosis and nodule formation. However, increasingly it is being recognized that there is a reversible component to intra hepatic resistance (179). This consist of the vascular tone and activation and contractility of hepatic stellate cells. The splanchnic and systemic haemodynamic changes are thought to be a consequence of the increased intra hepatic resistance (180)

It is well established in the literature that the sympathetic system is upregulated in portal hypertension. It has been shown in several studies that the plasma norepinehrine is elevated in patients with high Childs-Pugh score and severe portal hypertension, with the level co relating to disease severity (181-183). Catecholamine are thought to play a role

in the pathogenesis of portal hypertension as in experimental models of cirrhosis, they have been shown to increase intra hepatic resistance (184).

Adrenergic receptors have been studied in great detail in the control of vascular regulation (185). The main strategy for treatment of portal hypertension is currently a non selective beta blocker (186).It is poorly tolerated in about 30% of patients due to side effects and is effective in another 30% of patients only. Various other adrenergic receptors have been investigated for the treatment of portal hypertension. However, most of them have not been used in routine clinical practice because of concerns about systemic hypotension. ADRA2a adrenergic receptor is a specific receptor, which is subtype of G-protein related peptide which are present as transmembrane protein receptors. It is present in various densities in the central and peripheral nervous system and its mechanism of vascular regulation has been studied in detail (187). In the sepsis literature, it has been used as a target to reduce the inflammatory cytokines produced by the migrating macrophages including the kupffer cells (110). Although there is no evidence of significant receptor density in the liver, the migrating macrophages and the resistance vessels present a valid target in liver disease to assess the modulation of the sympathetic receptor in improvement of intrahepatic resistance in the context of cirrhosis of the liver

The aim of this study is to assess whether that antagonism of the ADRA2a adrenergic receptor reduces the intra hepatic resistance and thereby decrease portal pressure. This study will also investigate on the possible mechanisms by which the antagonism of the ADRA2a adrenergic receptor modulates intra hepatic resistance.

4.2 Methods:

4.2.1 Study design

Male Sprague Dawley rats weighing 220-250 g were randomly assigned to receive sham or bile duct ligation (BDL) surgery as described previously (165). Four weeks later haemodynamic measurements were performed and samples collected for analysis. For the BRL44408 study, treatment with either 10 mg/kg BRL44408 maleate (Sigma-Aldrich Company Ltd., Dorset, UK) dissolved in sterile water or saline (control, equal volume) by subcutaneous (s.c.) injection was given on the last 2 days of the study

Rats were randomly assigned to the following treatment groups:

i. Sham group: sham surgery, s.c. saline
ii. Sham+BRL group, s.c BRL 44408
iii. BDL group: BDL surgery, s.c. saline
iv. BDL+BRL group: BDL surgery, s.c. BRL44408

All rats were exsanguinated under terminal anesthesia (2% isofluorane). Within seconds blood was withdrawn from the descending aorta and immediately put into ice cold heparin/EDTA containing tubes (until full exsanguination), centrifuged at 3000 rpm and 4ºC for 10 min, and the plasma collected and stored at -80°C until assayed. Liver tissue was also removed immediately harvested and snap frozen for storage at -80°C until analyzed. Liver tissues were also fixed with 10% formalin for further histological analysis.

All the four groups were used for the haemodynamic assessment. However, in subsequent analysis, only the Sham+Saline, BDL+Saline, BDL+BRL were only used. The rationale for omitting the Sham+BRL group was that as there was no haemodynamic difference in the Sham animal treated with BRL as compared with the Sham animal treated with normal saline, it was assumed that it would not have an impact on the results of the other experiments. The focus of this set of experiment was to assess the impact of the proposed treatment on the diseased animals, therefore the analysis was based on the effect of treatment on diseased animal as compared to non treated diseased animals.

4.2.2 Haemodynamic measurement

All haemodynamic measurements were undertaken exactly as described previously in section 2.3.

4.2.3. Biochemical Parameters

All biochemical parameters (ALT, ALP, albumin, total protein, ammonia, bilirubin, urea and creatinine) were analyzed using 200µl of respective plasma samples using a Cobas Integra 400 multianalyzer with the appropriate kits (Roche-diagnostics, Burgess Hill, West Sussex, UK).

4.2.4 Plasma and tissue Cytokine Assays

All cytokine assays were undertaken in a similar method as described previously in section 2.6.

4.2.5 Western Blot Analysis

All the western blot analysis was undertaken in exactly the same technique as previously described in section 2.7. The primary antibody that have been used in this study are Adra2a (1:1000 dilution, PA1-048, Pierce Biotechnology Inc., Rockford IL, USA),), mouse anti eNOS (1:1000 dilution, 610297, BD Biosciences, Oxford, UK), rabbit anti phospho-eNOS (Ser1177) (1: 1000, #9571, Cell Signalling Technology, Inc., Danvers MA, USA), mouse anti ACTA2 (alpha-SMA; 1: dilution, M0851, Dako UK Ltd., Ely, UK) and mouse anti GAPDH (1:1000 dilution, ab6046, Abcam plc., Cambridge, UK), mouse anti DDAH1 (1:1000 dilution ab2231,abcam plc, Cambridge,UK) mouse anti NFkB p65 (1:1000, dilution, 2A12A7,Invitrogen,UK). Loading accuracy was evaluated via membrane rehybridization with antibodies against mouse and rabbit anti-α tubulin (1:1000; Upstate Biotechnology, Albany, NY) or mouse anti GAPDH (1:1000 dilution, ab6046, Abcam plc., Cambridge, UK).

4.3 Results.

Following surgery all animals continued to gain weight. From the final body weight taken before termination, cirrhotic rats were found to be significantly lower than that of sham rats (350 ± 5g vs. 472 ± 9g, respectively; $P < 0.001$).

4.3.1 Biochemical Profile

Table 4.1 Biochemical Profile

	Sham+Saline	**BDL+Saline**	**BDL + BRL**
Albumin (g/L)	**33.05±0.84**	**26.06±0.93*** **	**25.88±1.29**
Bilirubin (µmol/L)	**44.16±15.46**	**132.20± 21.02*** **	**123.40 ± 11.61**
ALT (IU/L)	**60.75±8.81**	**90.73±5.49***	**94.73±4.90**
AST (IU/L)	**131.70±17.37**	**564.30±59.64*** **	**589.30±56.32**

Fig 4.1 Shows the biochemical parameters in the three groups (Sham, BDL and BDL+BRL) (* p<0.001, *p<0.01)**

The serum albumin was no different between the sham operated with saline or BRL 44408. There was a significant reduction in the serum albumin levels in the BDL rats as

compared to the sham operated rats (26.06± 0.93 v 33.05±0.84,p<0.001) . This did not alter after treatment with BRL 44408 (26.06±0.93 v 25.88±1.29)

Similarly, the serum bilirubin was not difference between the sham operated animals treated with saline and BRL44408.There was a significant increase in the serum bilirubin the BDL rats as compared with the sham operated rats (132.2±2.02 v 44.16±15.46, p<0.001). However there was no difference between the BDL rats treated with saline or BRL 44408 (123.40±11.61 v 132.20±21.02).

There was a significant increase in the plasma ALT levels in the BDL rats as compared with the sham operated rats (90.73±5.49 v 60.75±8.81, p<0.001). There was a trend towards a reduction in the plasma ALT levels in the BDL rats treated with BRL as compared to the BDL rats treated with saline (94.73±4.90 v 90.73±5.49).

There was a significant increase in the plasma AST level in the BDL rats treated with saline as compared to the sham operated rats treated with saline (564.30±59.69 v 131.70±17.37,p<0.001). However, there was no reduction in the plasma AST level in the BDL rats treated with BRL 44408 as compared with the BDL rats treated with saline (589.30±56.32 v 564.30±59.64).

4.3.2 Mean Arterial Pressure Measurement

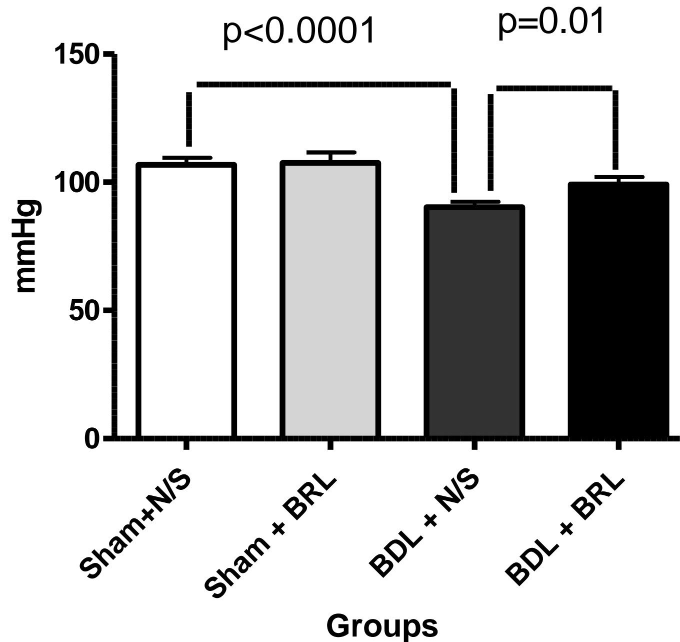


Fig 4.2 : Mean arterial pressure measurement of all four groups There is a significant increase in the MAP in the BDL rats treated with BRL 44408 as compared to the BDL rats treated with saline (p=0.01) (Sham + N/S =16, Sham + BRL =9, BDL+N/S = 24, BDL+BRL=21)

The mean arterial pressure (MAP) was measured in all the groups as described in the methods section 2.3. The MAP in the bile duct ligated rats is significantly lower than sham operated rats treated with saline (93.28±3.45 mmHg vs 109.9±3 mmHg.,p<0.0001).However on treatment with BRL 44408, there is a significant increase in the MAP in the treated group as compared with BDL rats (93.28±3.45 mmHg vs 99.67±4.38 mmHg, p=0.05, Fig 4.3).

4.3.3 Portal Haemodynamic outcome

4.3.3.1 Portal Pressure Measurement

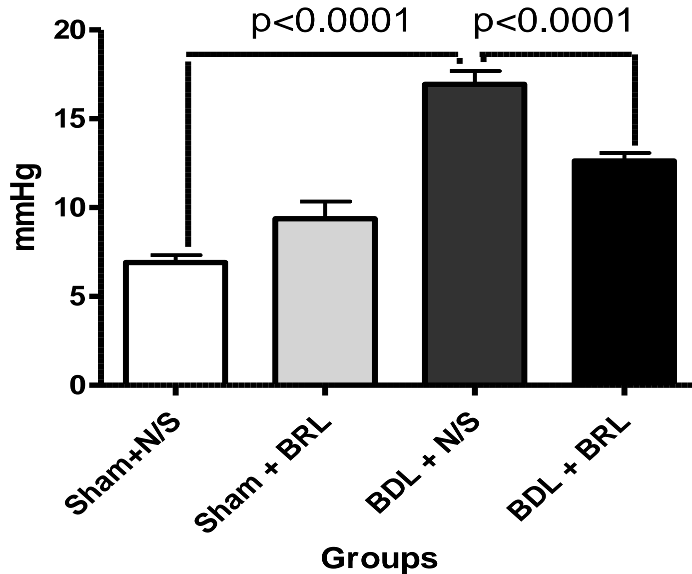


Fig 4.3: Portal pressure measurement of all four groups. The portal pressure was significantly increased in the BDL rats treated with saline as compared to the Sham operated rats treated with saline (p<0.001).The portal pressure is significantly reduced in the BDL rats treated with BRL 44408 as compared with the BDL rats treated with saline (p<0.001). (Sham + N/S =16, Sham + BRL =9, BDL+N/S = 24, BDL+BRL=21)

The portal pressure was measured in all the groups as described in section 2.3. The portal pressure was significantly increased in the bile duct ligated rat as compared to the sham operated rats (18.24±1.0 vs 7.6±0.6 mmHg,p<0.0001).The portal pressure was significantly reduced in the BDL rats treated with BRL 44408 as compared to the BDL treated with saline (11.61±0.9 vs 18.24±1.0 mmHg, p<0.0001 Fig 4.4).

4.3.3.2: Hepatic Blood Flow

A.

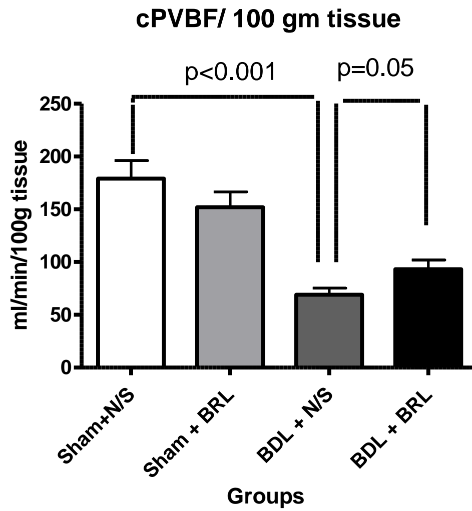


Fig 4.4(A): Portal Vein Blood Flow

B.

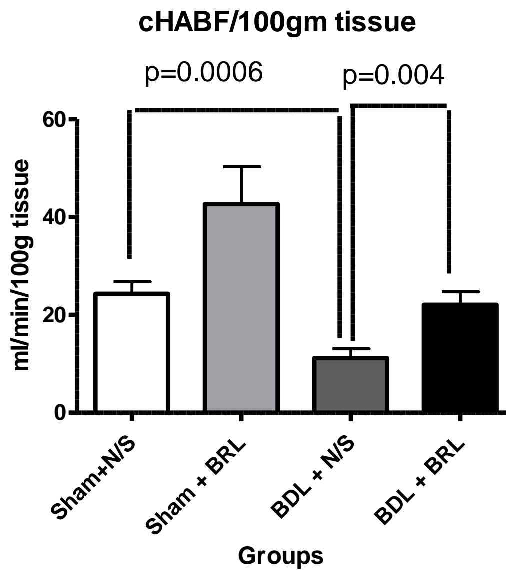


Fig4.4(B): Hepatic Artery Blood Flow

Fig 4.4(A) and Fig 4.4(B): Hepatic blood flow in all the four groups). The portal vein blood flow reduces significantly in the BDL rat as compared to the sham operated rat ($p<0.001$), whereas there is a significant increase portal vein blood flow in the BDL rats treated with BRL 44408 as compared to the BDL rats treated with saline ($p=0.05$). A similar pattern was observed in the hepatic artery blood flow with reduced blood flow in the BDL rat as compared to the sham operated rat ($p=0.0006$).However the blood flow goes up on treatment of the BDL rats with BRL as compared to BDL rats ($p=0.004$) (Sham +N/S=12, Sham+BRL=3, BDL+N/S=9, BDL+BRL=12)

The portal vein in the BDL rats showed a significant drop in the blood flow as compared to the sham operated rats (68.98 ± 19.23 vs 179.1 ± 59.08,$p<0.001$). The blood flow in the portal vein was increased significantly in the BDL rats treated with BRL 44408 as compared to the BDL rats treated with saline alone (93.16 ± 30.31 vs 68.98 ± 19.23, $p=0.05$). The blood flow in the hepatic artery (HABF) was also decreased in the BDL rats as compared to the sham operated rats (24.35 ± 8.04 vs 11.18 ± 5.63,$p<0.001$), however the blood flow increased significantly in the hepatic artery in the BDL rats treated with BRL 44408 as compared with the BDL rats treated with saline (11.18 ± 5.63 vs 22.09 ± 8.29, $p<0.01$)

4.3.4. Cardiac Haemodynamic outcomes:

4.3.4.1 Cardiac Output

A. Cardiac Output

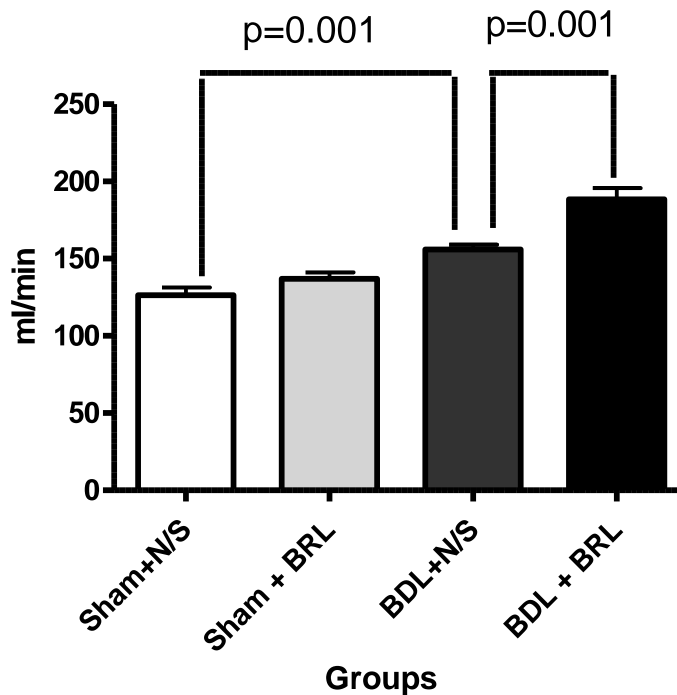


Fig 4.5(A): Cardiac output data in all the four groups. The cardiac output increases significantly in the BDL rats treated with saline as compared to the sham operated rats treated with saline (p=0.001).This was further increased in the BDL rats treated with BRL as compared to the BDL rats treated with saline (p=0.001). (Sham+N/S=3, Sham+BRL=3, BDL+N/S=6, BDL+BRL=6)

4.3.4.2 Stroke Volume

B. Stroke Volume

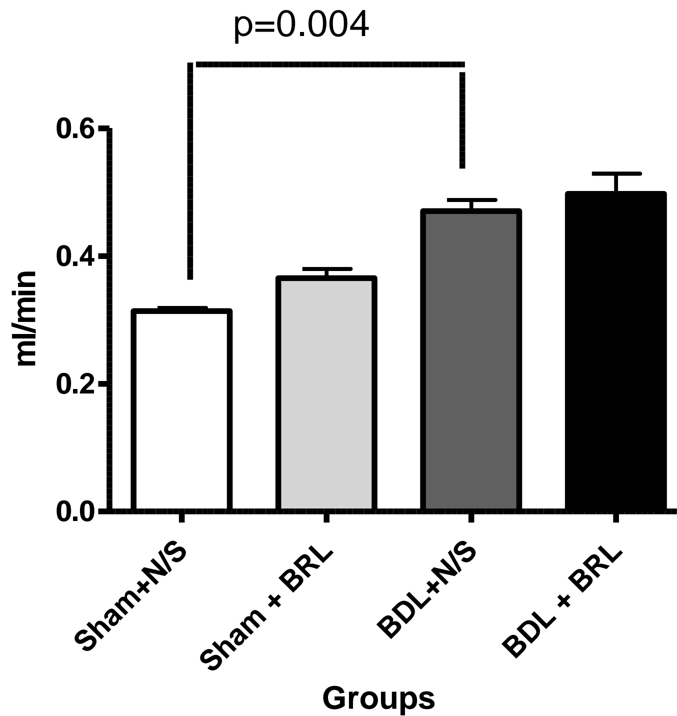


Fig 4.5(B): Stroke volume data in all the four groups. The stroke volume increases significantly in the BDL rats treated with saline as compared to the sham operated rats treated with saline (p=0.004).There was no further increased in the stroke volume in the BDL rats treated with BRL as compared to the BDL rats treated with saline. (Sham+N/S=3, Sham+BRL=3, BDL+N/S=6, BDL+BRL=6)

4.3.4.3 Heart Rate

C. Heart Rate

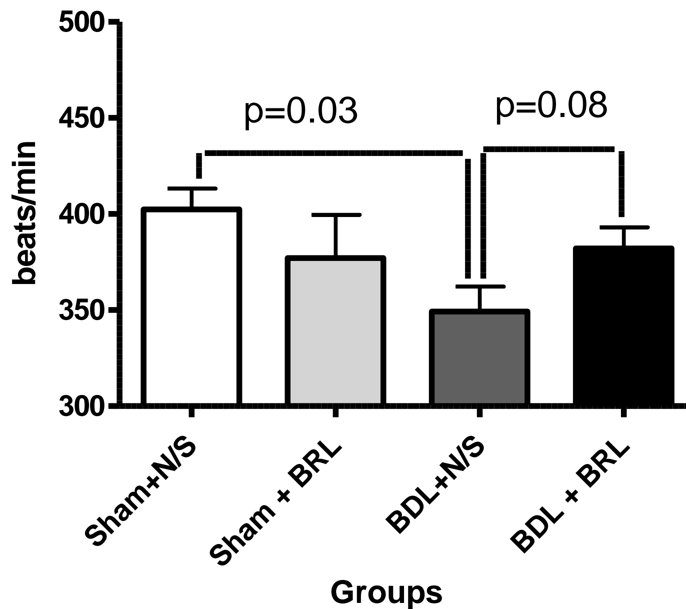


Fig 4.5(C): Heart rate data in all the four groups. The heart rate reduces significantly in the BDL rats treated with saline as compared to the sham operated rats treated with saline (p=0.03).There was a non significant increase in the heart rate in the BDL rats treated with BRL as compared to the BDL rats treated with saline (p=0.08). (Sham+N/S=3, Sham+BRL=3, BDL+N/S=6, BDL+BRL=6)

The echocardiogram was undertaken in all the groups of animals as described in section 2.4. There was no difference between the cardiac output in the sham operated group treated with either saline or BRL 44408. However the cardiac output increased significantly in the BDL rats as compared to the sham operated rats (156.0 ± 3.055 v 126.3

± 5.044, p<0.001). . Further, on treatment with BRL 44408, the BDL rats increased their cardiac output significantly as compared to the BDL rats treated with saline (188.5 ± 7.154 v 156.0 ± 3.055, p<0.001). The stroke volume was increased significant in the BDL rats as compared to the sham operated rats. However, the heart rate was significantly reduced in the BDL rats as compared to the sham operated rats. This was improved significantly in the BDL rats treated upon treatment with BRL 44408. Overall, the cardiac output is increased in the BDL rats treated with BRL 44408 as compared to the BDL rats treated with saline. However, the increased output is more significantly determined by the heart rate and not by the stroke volume. This indicates that actual cardiac output is not increased.

4.3.5 Reduction of Intra Hepatic Resistance :

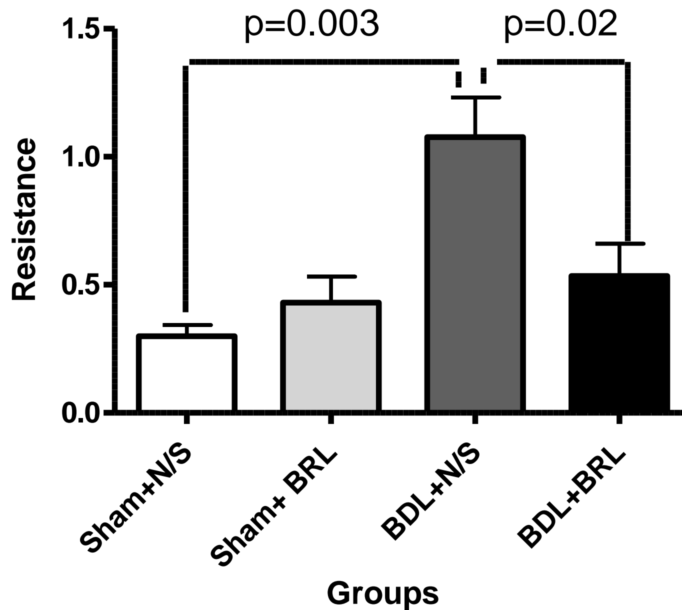


Fig 4.6: The calculated intra hepatic resistance for all the four groups. The intra hepatic resistance in the BDL rats was significantly greater as compared to sham operated rats (p=0.003). This was significantly reduced in the BDL rats treated with BRL 44408 as compared to the BDL rats treated with saline (p=0.02)

The intra hepatic resistance is calculated by the dividing the portal pressure by the total hepatic blood flow. In our experiment, there is no significant difference in the intra hepatic resistance between the sham operated animals treated with saline or BRL 44408. However, there is a significant increase in the intra hepatic resistance in the BDL rats treated with saline as compared to the sham operated animals treated with saline. This increased resistance is in the BDL rats treated with saline is significantly reduced with the treatment of the BDL rats with BRL 44408.

4.3.6 Plasma renin activity levels

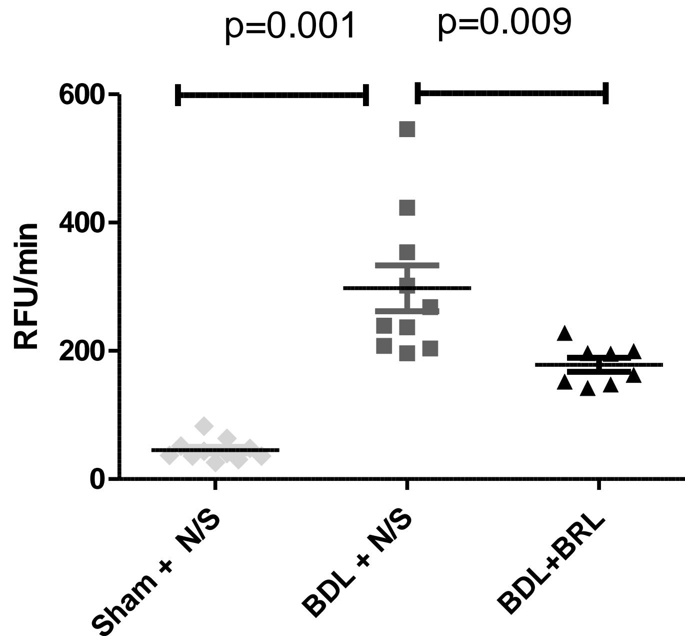


Fig 4.7: Plasma renin activity in the three groups. The plasma renin activity was significantly increased in the BDL rats as compared to the Sham rats (p=0.001).However, the renin activity goes down significantly in the BDL rats treated with BRL 44408 as compared to BDL rats treated with saline.(Sham+N/S =11, BDL+N/S =10, BDL+BRL=8)

The plasma renin activity measured by ELISA as described in the methods chapter 2.10 was compared between the sham operated, BDL rats treated with saline and BDL rats treated with BRL.The plasma renin activity was significantly increased in the BDL rats as compared to the sham operated rats (297.6±4.89 v 45.15±4.89, p=0.001). However after treatment with BRL 44408 , BDL rats reduced their plasma renin activity significantly as compared to the BDL rats treated with saline (178.2±11.0 v297.6±4.89 , p=0.009)

4.3.7 Plasma Noradrenaline Levels in the three groups

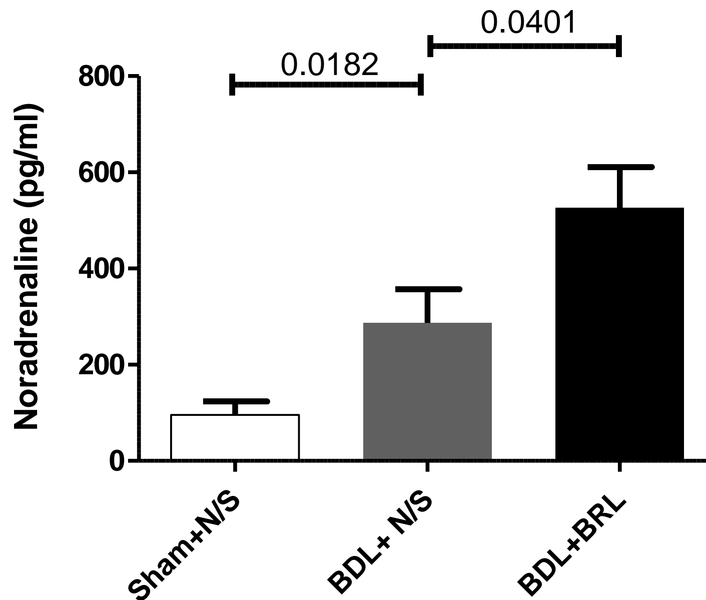


Fig 4.8: Plasma noradrenaline levels in the three groups. This shows that the plasma noradrenaline is significantly increased in the BDL rats as compared to Sham operated rats (p=0.01). This was further increased in the BDL rats treated with BRL 44408 (p=0.04) (Sham+N/S =9, BDL+N/S =14, BDL+BRL=11).

The plasma Noradrenaline level was measured by an ELISA assay as described in section 2.11. This shows that the plasma noradrenaline level goes up in the BDL rats as compared to the sham operated rats (286.8±70.40 v 95.40±28.22, p=0.01). This was further augmented by treatment of the BDL rat with BRL 44408 (526.22 ±84.69 v 286.8±70.40, p=0.04)

4.3.8 Reduction of inflammation in BDL rats with BRL 44408 treatment.

4.3.8.1 Liver Tissue TNFα level

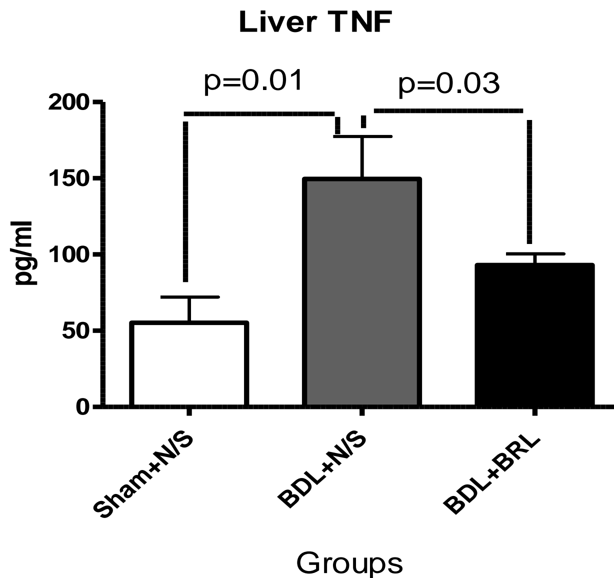


Fig 4.9(A):Liver tissue TNFa levels in the three groups (Sham, BDL and BDL+BRL).The TNFα level goes up significantly in the BDL rats as compared to the sham rats (p=0.01). However, on treatment of the BDL rats with BRL 44408, there is significant reduction in the liver tissue TNFα level (p=0.03) (Sham+N/S =8, BDL+N/S =7, BDL+BRL=5)

The liver tissue TNFalpha cytokine levels were measured by FAC cytokine bead array as described previously in section 2.6. The TNFalpha levels was increased significantly in the bile duct ligated animals as compared to the sham operated animals(149.50±73.67 v 72.80±5.56,p=0.01) However, on treatment with BRL 44408 , the liver tissue TNFalpha cytokine levels were significantly reduced as compared with the saline treated BDL rats (93.11±16.32 v 149.50±73.67,p=0.03).

4.3.8.2: Plasma TNFa levels in the three groups

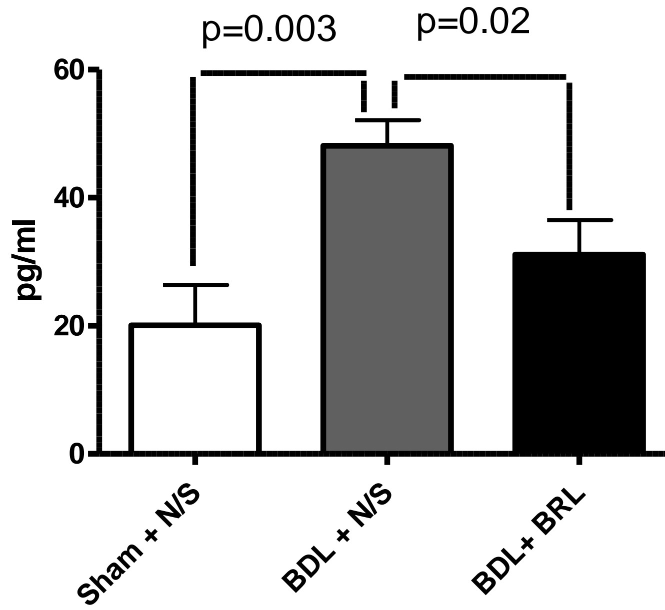


Fig 4.9(B): Plasma TNFα level for the three groups (Sham, BDL and BDL+BRL).The plasma TNFα level was significantly raised in the BDL rat as compared to the sham rats (p=0.003) and the level was significantly reduced with treatment of the BDL animal with BRL 44408 (p=0.02). (Sham+N/S =6, BDL+N/S =7, BDL+BRL=5)

The plasma TNFalpha cytokine levels were measured by FAC cytokine bead array as described previously in section 2.6. The plasma TNFalpha levels was increased significantly in the BDL rats treated with saline as compared to the sham operated rats (48.12±4.08 v 20.06±6.06, p=0.003).However, the TNFalpha was significantly reduced in the BDL rats treated with BRL 44408 as compared to the BDL rats treated with saline (31.11 ±6.08 v 48.12±4.08, p=0.02)

4.3.8.3 Liver Tissue NFkB protein expression

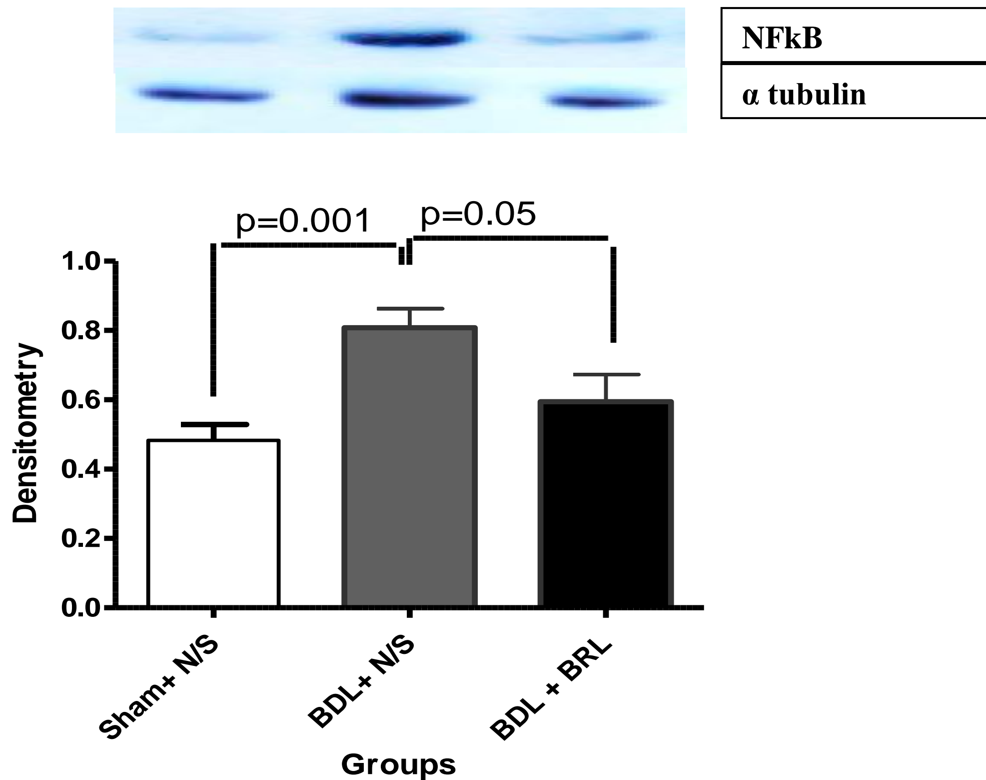


Fig 4.10: NFkB levels in liver tissue in the three groups). It shows that the NFkB levels go up in the liver tissue in the BDL rats as compared to the sham animals (p=0.001). This level goes down when BDL rats are treated with BRL 44408 as compared to the BDL rats treated with saline (p=0.05)

The nuclear factor kappa B is a transcription factor which acts a final common pathway for a multitude of inflammatory mediators. The western blot analysis of the protein expression of the nuclear factor kappa B shows that the expression goes up significantly in the BDL rats as compared to the sham operated rats (0.4826 ± 0.04642 v 0.8082 ± 0.05441,p=0.001).This protein expression goes down in the BDL rats with treatment with BRL 44408 as compared with the BDL rats treated with saline (0.5946 ± 0.07793 v 0.8082 ± 0.05441, p=0.05)

4.3.9 Pathways that have been implicated in the improvement in portal pressure.

4.3.9.1 Cyclic AMP activity after treatment with BRL 44408.

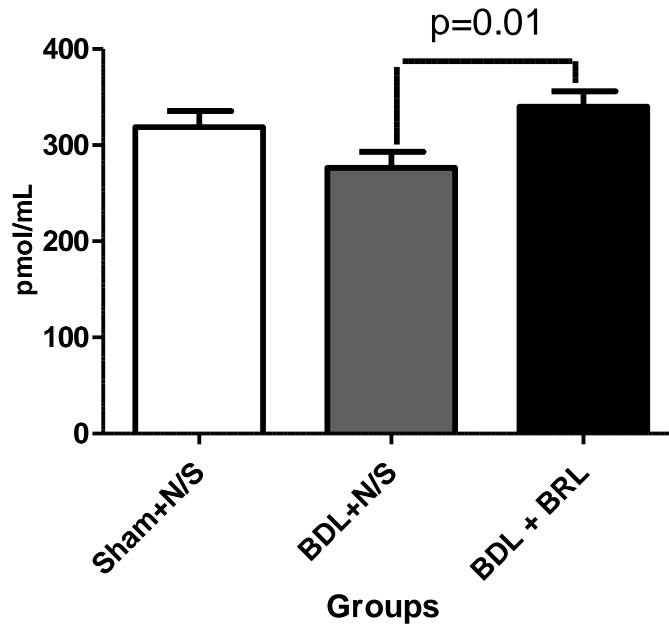


Fig 4.11: Cyclic AMP levels measured in the three groups. The cAMP levels in the BDL rats was reduced in the BDL rats as compared to the Sham operated rats. However, upon treatment with BRL , the BDL rats have increased cAMP activity (p=0.01). (Sham+N/S=7, BDL+N/S=6, BDL+BRL=7)

The cAMP activity was measured from the liver homogenate by a ELISA method as described section 2.12. The cAMP activity was non significantly increased in the BDL rats as compared with the sham operated rats (276.6 ± 16.48 v 318.7 ± 16.88,p=0.10).The cAMP activity was however reduced significantly in the BDL rats treated with BRL 44408 (340.3 ± 15.78 v 276.6 ± 16.48, p=0.01)

4.3.9.2 Hepatic Stellate Cell Activation:

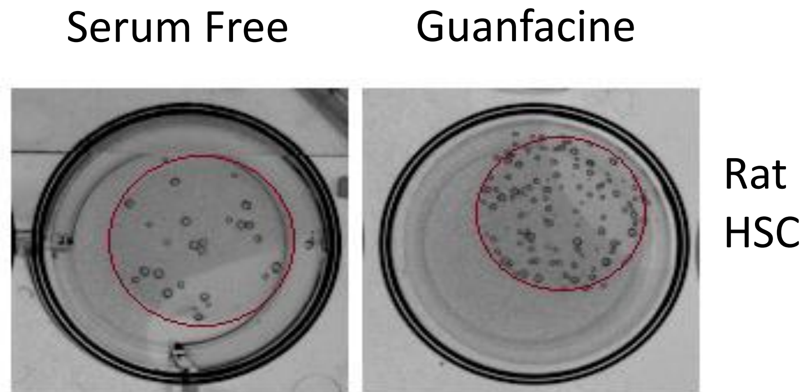


Fig 4.12: Rat hepatic stellate cells contraction assay. Rat hepatic stellate cells grown in the presence of 5uM Guanfacine show greater contraction in a 3D collagen gel than cells grown in the absence of guanfacine

Gel contraction assay was undertaken as per method detailed in section 2.14. I sought to determine whether adrenergic signalling could enhance hepatic stellate cell activation to a myofibroblast phenotype. Rat hepatic stellate cells (a gift from Jonathan Fallowfield) were seeded into serum-free 3D collagen gels. They were incubated in serum-free medium or in the presence of 5uM Guanfacine (an ADRA2a adrenergic receptor agonist). Stimulation with Guanfacine caused an increase in the contraction of the 3D collagen gel compared to serum free medium.

Subsequent experiments beyond the remit of this thesis has confirmed this finding in human hepatic stellate cells , wherein experiments conducted in this laboratory showed that the human stellate cells grown in 5uM of guanfacine showed greater contraction as compared to serum free medium. However, on addition of BRL 44408 to the Hepatic Stellate Cells and Guanfacine, there was definitive relaxation. Downstream signalling

pathway was also analysed to confirm that this pathway was involved in the contraction. Western blot analysis of erk-1/2 phosphorylation was increased in guanfacine stimulated cells. Although , a numerical assessment of the number of cells was not done, the above experiments confirm the involvement in the contraction of the gel by way of reversibility of contraction by addition of the ADRA2a antagonist.

4.3.9.3 Alpha Smooth Muscle Actin protein expression in the three groups

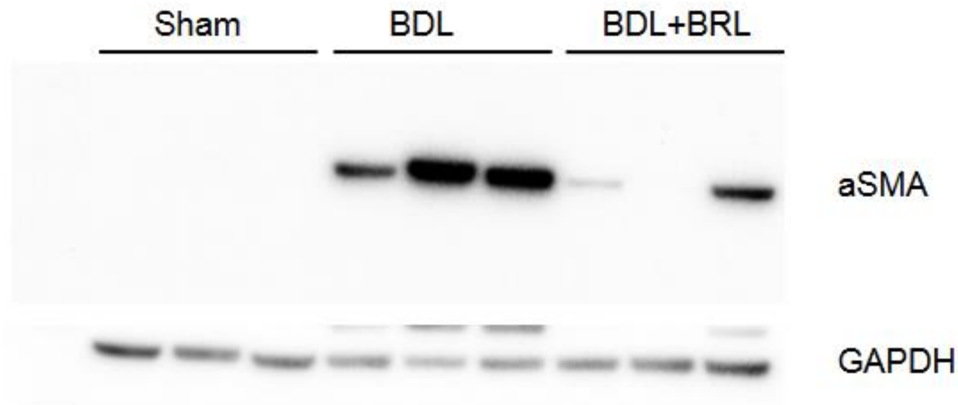


Fig 4.13: αSMA protein expression in Sham, BDL and BDL rats treated with BRL 44408. This shows that the protein expression of αSMA is significantly increased in the BDL liver as compared to the BDL rat liver. However, this expression was significantly reduced in the BDL treated with ADRA2a as compared to BDL rats treated with saline. GAPDH was used as a control for the western blot.

The Alpha Smooth Muscle Actin (αSMA) is a surrogate marker for Hepatic Stellate Cell activation. The protein expression in this study suggest that there is significant increase in the HSC activation in the BDL rats as compared to the sham operated animals as evidenced by the increased protein expression of αSMA in the BDL rats . However, this protein expression was significantly reduced in the BDL rats treated with BRL 44408 suggesting that there is reduction in HSC activation with ADRA2a antagonism.

It is well established that there is increased production in the cytokine production in the BDL rats as compared to the sham animal. These are propagated by increased bacterial translocation from the gut. These cytokines in turn activate the hepatic stellate cells as

they reach the liver through the portal circulation. The alpha smooth muscle actin protein expression is upregulated by the activated hepatic stellate cells. However, on treatment with the ADRA2a antagonist, there is reduction in the cytokine production in the liver. This in turn deactivates the hepatic stellate cells and thereby indirectly reduces the protein expression of the alpha smooth muscle actin.

4.3.9.4 Endothelial Nitric oxide Synthetase protein expression in the three groups

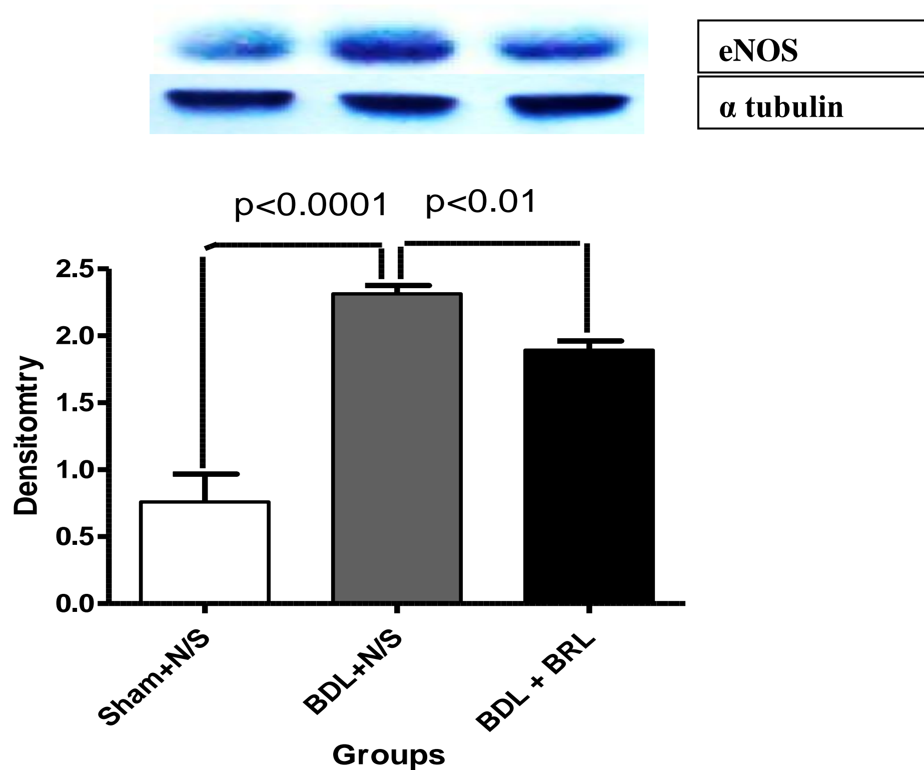


Fig 4.14: eNOS protein expression in the three groups of animals. This shows that the eNOS protein expression is increased significantly in the BDL rats as compared to the sham operated rats (p<0.0001) . However, the protein expression of eNOS is down regulated in the BDL rats treated with BRL as compared with the BDL rats treated with saline (p<0.01)

The protein expression of eNOS protein reveal that the expression goes up in the BDL animal as compared to the Sham operated rat (2.313 ± 0.06395 v 0.7588 ± 0.2095, p<0.0001) .However, there is reduced protein expression of eNOS in the BDL rats treated with BRL 44408 as compared to the BDL rats treated with BDL(1.892 ± 0.07007 v 2.313 ± 0.06395, p<0.01)

4.3.9.5 Phosphorylated endothelial Nitric oxide synthetase protein expression in the three groups

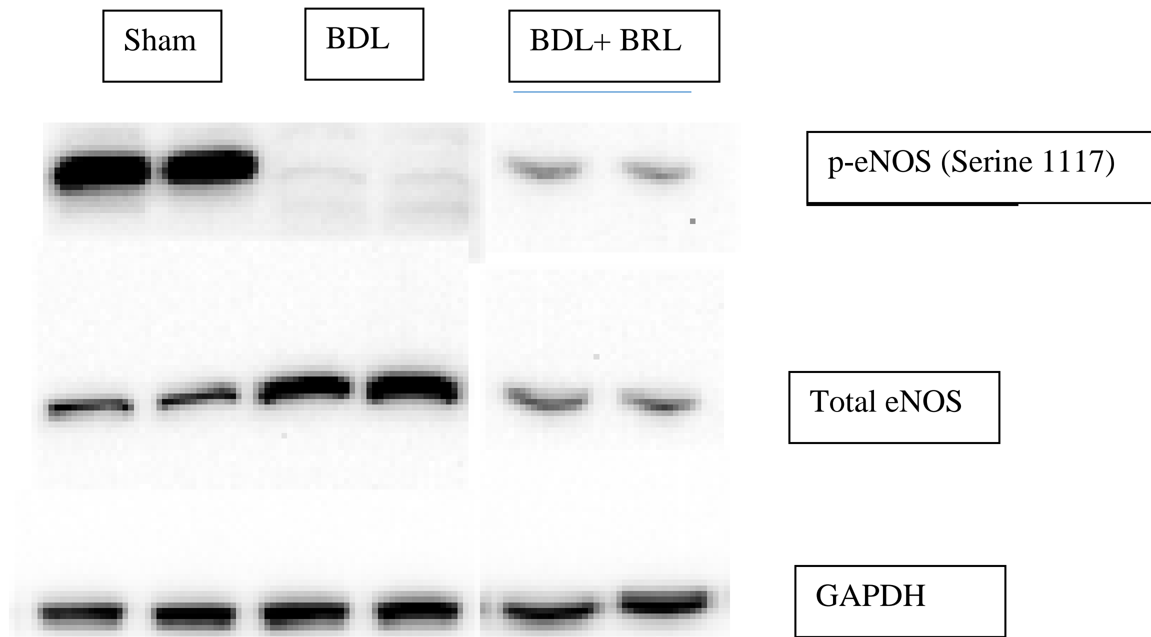


Fig 4.15: Phosphorylated eNOS and total eNOS protein expression in all the three groups. The expression of total eNOS protein is increased in BDL rats compared to sham operated rats whereas the level of eNOS phosphorylation is reduced. Treatment with BRL44408 reduces the expression of total eNOS protein and increases the level of eNOS phosphorylation.

The expression of total eNOS protein was increased in BDL rats compared to sham operated rats but despite this the level of eNOS phosphorylation was greatly reduced. Treatment with BRL44408 both reduced the expression of total eNOS protein and increased the level of eNOS phosphorylation.

4.3.9.6 DDAH 1 levels in the three groups of animals

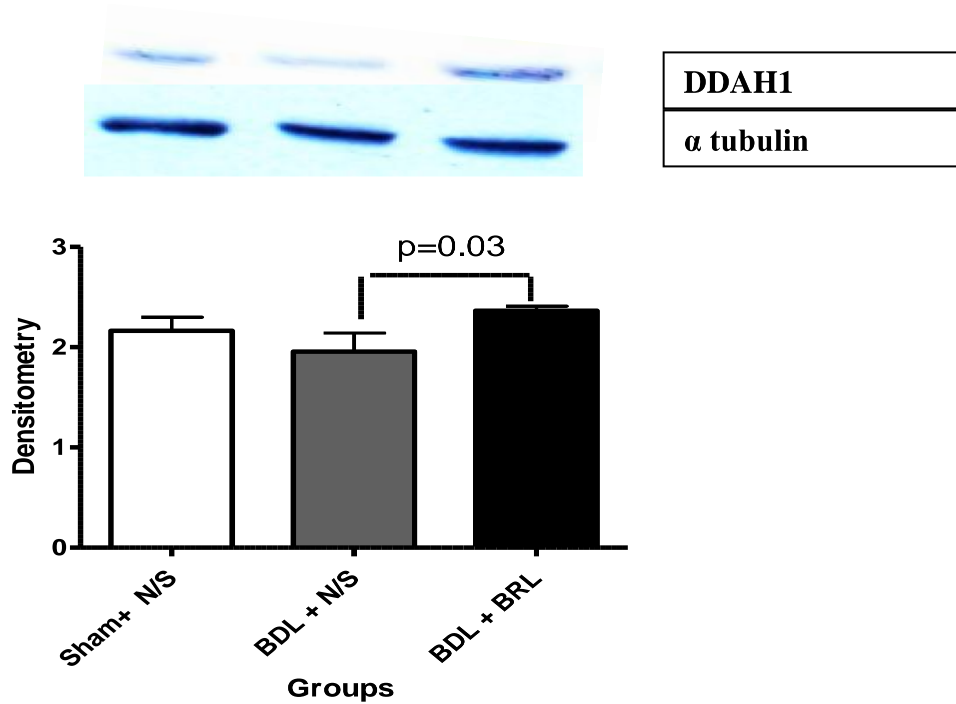


Fig 4.16: DDAH1 protein expression in the three groups. This shows that the protein expression of DDAH1 in the liver tissue is decreased non significantly in the BDL rats as compared to the sham operated rats. However, on treatment of the BDL rats with BRL 44408, there is a significant increase in the protein expression of DDAH1 in the liver tissue as compared to the BDL rats treated with saline (p=0.03)

The DDAH1 protein expression in the liver tissue shows there is non significant reduction in its expression in the BDL rats as compared to the sham operated rats (1.955 ± 0.1854 v 2.164 ± 0.1353). However, on treatment with BRL 44408, the BDL rats had significantly increased DDAH1 protein expression (2.363 ± 0.04553 v 1.955 ± 0.1854, p=0.03). This signifies that there is less inhibition of eNOS enzyme, which in turn lead to increase NO production in the BRL 44408 treated animals.

4.3.10 Histological assessment in the three groups

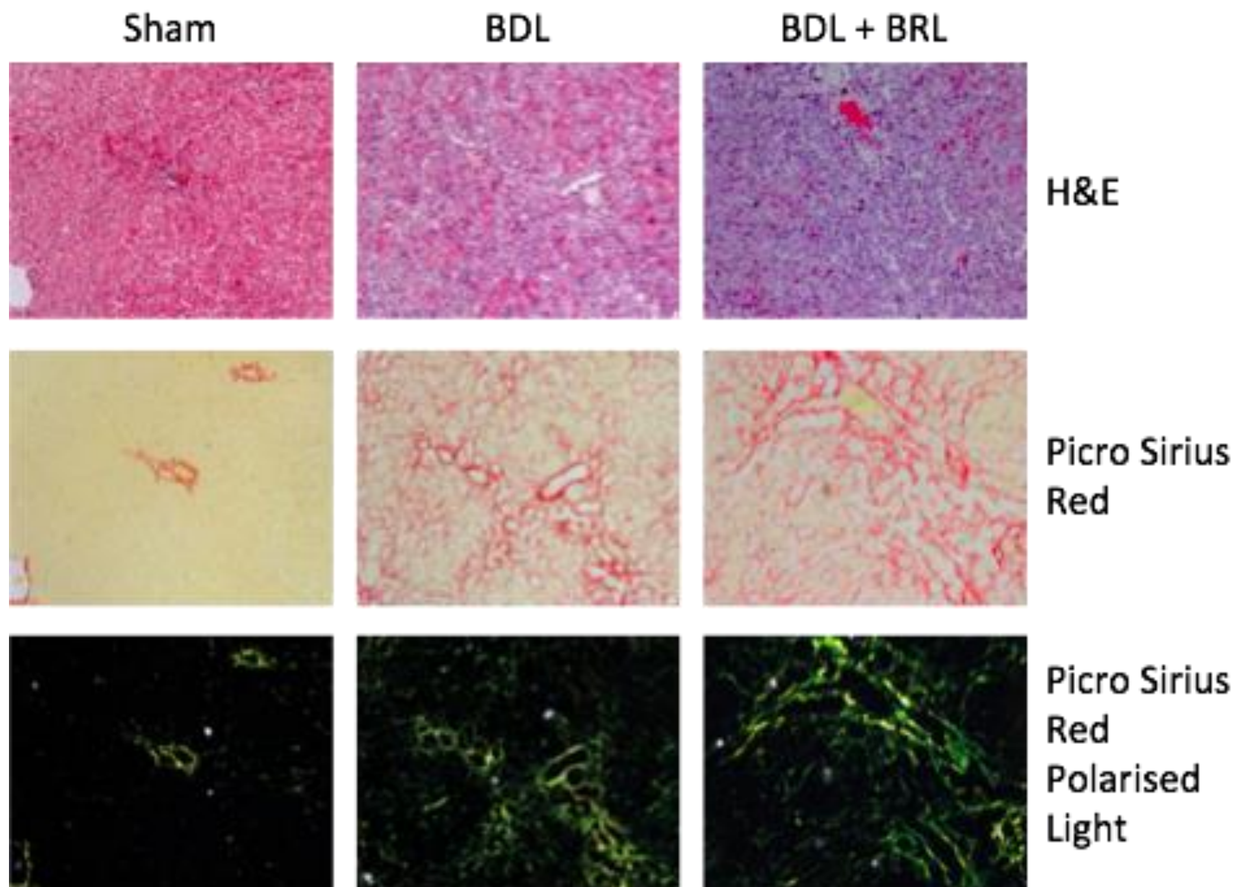


Fig 4.17: Histological assessment of all the three groups. Liver architecture and fibrosis in Sham, BDL, and BDL + BRL rats was assessed by H&E and picrosirius red (PSR) staining, respectively.

Sham rats demonstrate a normal liver architecture. BDL rats show a loss of acinar structure and number of hepatocytes, along with the development of fibrous bands, significant cholangiocyte proliferation, and some cholestasis. BDL rats treated with BRL show the same features with no visible reduction in the level of injury. Representative images of the same region of liver stained with picrosirius red imaged by both bright field and circular polarised light microscopy are also shown. Under brightfield microscopy, sham livers show strong collagen staining surrounding vessels but very little visible throughout the parenchyma. BDL livers show a large amount of collagen staining

throughout the whole section coinciding with the ductular proliferation. In sham livers, polarised light microscopy reveals yellow staining (indicating medium thickness collagen fibres) surrounding vessels with some green staining (indicating thin collagen fibres) within the parenchyma. BDL livers show both yellow and green staining (suggesting medium thickness maturing fibres and thin newly synthesized fibres) throughout the section. BDL rats treated with BRL show the same types of collagen fibres without any visible reduction in fibrosis

4.3.11 Other Organ involvement:

4.3.11.1 Reduction in brain water

A. Brain Water

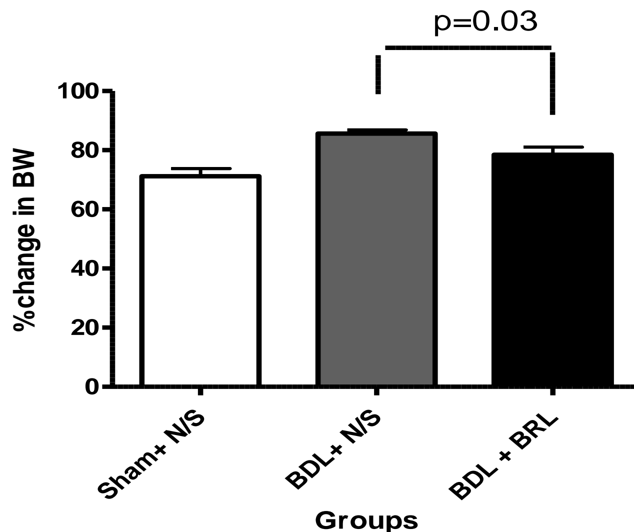


Fig 4.18(A): Brain water changes in all the three groups. Shows the percentage change in the brain water in the three groups. The brain water percentage change is significant in the BDL rats as compared to the Sham rats (p=0.005). There is significant reduction in the brain water content in the BDL rats treated with BRL 44408 as compared to the BDL rats treated with saline (p=0.03). (Sham+N/S =5, BDL+N/S =7, BDL+BRL=7)

The brain water content is a surrogate marker for cerebral inflammation that occurs in cirrhosis. The brain water content was measured as described in section 2.19. The brain water content is increased in the BDL rat as compared to the sham operated rat (85.64 ± 1.235 v 71.20 ± 2.605, p=0.005). However, on treatment with BRL 44408, the brain water

content in the BDL rats reduces significantly as compared to the BDL rats treated with saline (78.47 ± 2.540 v 85.64 ± 1.235,p=0.03)

.4.3.11.2 Plasma Lactate levels

B.Plasma Lactate Levels

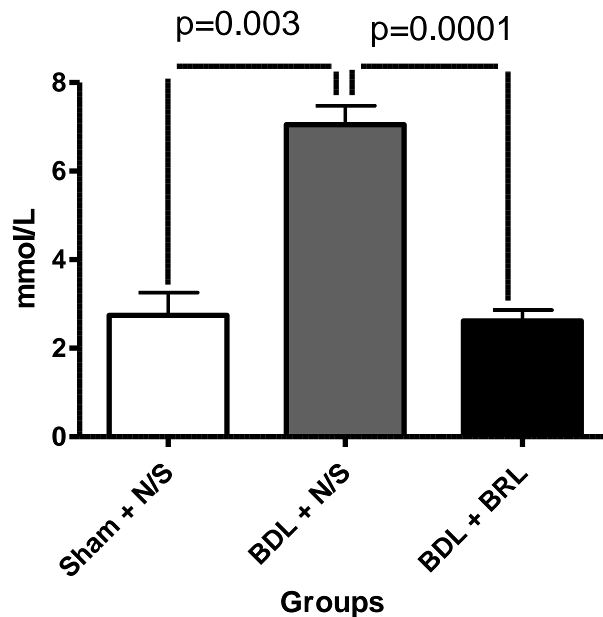


Fig 4.18(B):Plasma lactate levels in the three groups. The Plasma lactate level goes up significantly in the BDL rats as compared to the sham operated rats(p=0.003). However, upon treatment with BRL 44408, there was significant reduction in the plasma lactate levels as compared to the BDL rats treated with saline (p=0.02). (Sham+N/S=5, BDL+N/S=7, BDL+BRL=7)

The plasma lactate level is a surrogate marker for peripheral ischemia, which in turn is a marker for adequacy of systemic circulation. In this model, the plasma lactate level was significantly increased in the BDL rats as compared to the sham operated animals (7.053 ± 0.4239 vs 2.74 ± 0.52,p=0.003) However,upon treatment with BRL 44408 , the plasma lactate level decreased

significantly in the BDL rats as compared to the BDL rats treated with saline (2.61 ± 0.24 vs 7.053 ± 0.4239, p=0.02)

4.3.11.3 Renal Function

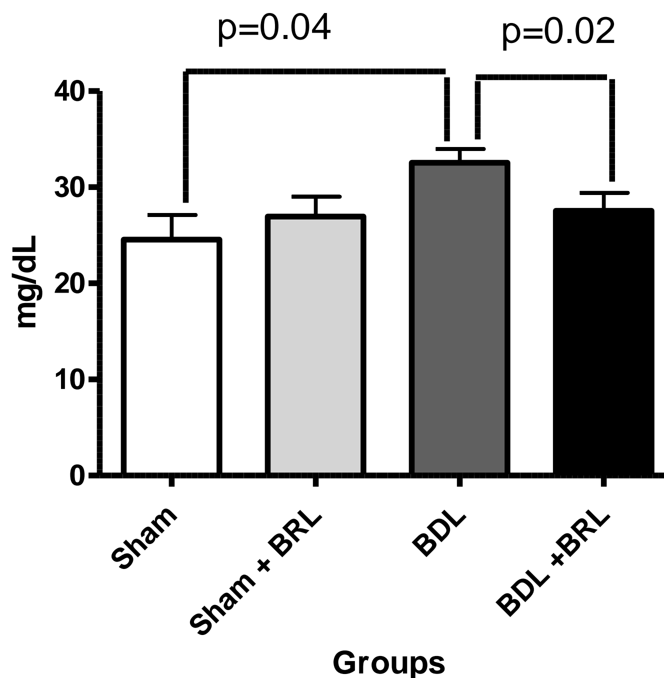


Fig 4.18(C): Serum creatinine levels in the four groups. This shows that the serum creatinine increases in the BDL rats as compared to the sham operated rats (p=0.04). This level goes down with treatment of the BDL rats with BRL 44408 as compared to the BDL rats treated with saline (p=0.02). (Sham+N/S=8, Sham+BRL = 3,BDL+N/S=7, BDL+BRL=5)

The serum creatinine is a marker for renal function. The level gives an indication whether there is renal impairment. In this study, the serum creatinine did significantly rise as

compared to the sham operated animal (32.55±1.46 v 24.55 ± 2.573, p=0.04). However this reduced significantly in the BDL rats treated with BRL 44408 as compared to the BDL rats treated with saline (27.55±1.76 v 32.55±1.45, p=0.02)

4.4 Discussion

I was able to make several observations based on the results of this study. Firstly, the results show that treatment of the bile duct ligated rats with BRL 44408, an ADRA2a adrenergic receptor antagonist, results in significant improvement in the haemodynamic abnormalities. In particular, there is significant reduction in the portal pressure in the BDL rats treated with BRL 44408 as compared to the BDL rats treated with saline. In addition, there is an increase in the mean arterial pressure and increased portal and hepatic blood flow in the BRL 44408 treated rats as compared to the BDL rats treated with normal saline. Secondly, the results show that there is also a significant reduction in the inflammatory markers including plasma and tissue TNFα level and Nuclear Factor Kappa B protein expression in the BDL rats treated with BRL 44408 as compared to the BDL rats treated with saline. Thirdly, the results also show various pathways that are implicated in the reduction of intra hepatic resistance. This includes the liver tissue cAMP activity which is increased in the BRL 44408 treated rats as compared to the BDL rats treated with saline, implying that there is relaxation of vasomotor tone thereby improved hepatic blood flow. The Hepatic stellate cells (HSC) displays increased contractility with an ADRA2a agonist suggesting that the antagonism of this receptor will induce HSC relaxation thereby facilitating intra hepatic vasodilatation. Lastly, ADRA2a antagonism improves

endothelial dysfunction as evidenced by improvement in the pNOS protein expression, indicating increased nitric oxide production in the intra hepatic circulation.

Intrahepatic resistance (IHR) was classically thought to be as a result of the mechanical effects of cirrhosis including fibrosis, thrombosis and nodule formation which leads to abnormal architecture of the sinusoidal space and hence distortion to the laminar flow of blood (12). The exact site of intra hepatic resistance is variable and can change according to etiological factors and disease process. Chronic hepatitis appears to have both pre-sinusoidal and sinusoidal vascular abnormalities which contribute towards the increased resistance (13) where as there is abnormal architecture in the sinusoidal and post sinusoidal site (14), when liver disease is caused by injury. As the disease progresses, there is terminal vein fibrosis, reduction in sinusoidal space by enlarging hepatocyte and deposition of collagen in the space of disse, all of which contribute to the increased vascular resistance. However, the seminal paper by Mittal and Groszmann in 1984 gave the first indication into the presence of a modifiable component in the intra hepatic vasculature (16).Bhathal and Grossman in 1985 confirmed this perspective and led to the theory of presence of a pharmacologically modifiable vascular element in addition to the mechanical factors that influence intra hepatic resistance (17). A significant body of research has been undertaken and published since then to understand and apply this principle to modify intra hepatic resistance as a treatment of portal hypertension.

It is clear from the literature that reduction of portal pressure to below 12 mmHg or 20% below the basal reading has shown to have clear benefit in patients outcome by reducing the risk of first bleeding episode to less than 10% (5). In our study, we show that the portal

pressure rises to 16.45±3.49 mmHg in the bile duct ligated rats as compared to the portal pressure in the sham operated rats which have a portal pressure of 7.36±1.31 mmHg. However on treatment with BRL 44408, the BDL rat dropped the portal pressure to 12.05±2.74 mmHg. This shows that the BRL 44408, a ADRA2a adrenergic receptor antagonist lowers portal pressure significantly (27%) from the baseline. The portal blood flow was increased in the BDL treated with ADRA2a antagonist as compared to the BDL rats treated with saline. This implies that the liver is able to accommodate increased blood volume. These results are even more significant in the context of an increased mean arterial pressure in the BDL rats treated with BRL 44408. This was complimented by the results of the plasma renin activity results which shows that treatment with ADRA2a antagonist reduces the plasma renin activity, suggesting increased systemic vascular tone. Overall, all the haemodynamic findings in this study suggest that there is significant reduction in the intra hepatic resistance in the BDL rats treated with ADRA2a antagonist as compared to the BDL rats treated with saline.

It is well established that the mean arterial pressure and the peripheral resistance is lower in rats with established portal hypertension as compared with controls (188)and this has been noted in human studies as well(189, 190) . The various treatment strategies that have been applied in the clinical trials settings have had a major issue with the systemic blood pressure as the drugs which are used to vasodilate the intra hepatic circulation also dilates the systemic vasculature and hence causes a deleterious effect on the clinical outcome due to significant systemic hypotension. This is due to the presence of a dichotomy between the blood vessel caliber in the intrahepatic and systemic and splanchnic circulation. In the intrahepatic circulation there is vasoconstriction, whereas in the splanchnic circulation,

there is significant vasodilatation as a result of an imbalance between the vasoactive substances in the different vasculature beds(191). The observed increase in the mean arterial pressure is obviously desirable in the setting of cirrhosis and portal hypertension. The cardiac haemodynamic results in this study demonstrate that the BDL rats have a hyperdynamic circulation with higher cardiac output as compared to the sham rats. However, although the cardiac output was increased in the BDL rats treated with BRL 44408, the actual increase was due to a non significant increase in the heart rate, which may be due to the action of BRL 44408 itself.

These observations are further supported by the increased noradrenaline levels that have been found in my experiments. The plasma noradrenaline is significantly higher in the BDL rats as compared to the sham operated rats. The noradrenaline level remains at a high level in the BDL rat treated with BRL 44408. The fact that the noradrenaline remains high even on treatment of BRL 44408 into the BDL rats suggest that the free noradrenaline are being not able to bind to their receptors as they have been saturated by the receptor antagonist. We can speculate that the mechanism of action for this observation is the presence of high density ADRA1 adrenoreceptors, which in turn are stimulated by the increased noradrenaline level that is produced by the ADRA2a adrenergic receptor antagonist (BRL 44408) used in this study. This causes vasoconstriction of the large vessels and thus increases the mean arterial pressure. We have not measured the ADRA1 adrenoreceptor density in the aorta in this study but it has been suggested in other reviews. (192). .

The other significant observation made in our study was significant increased hepatic blood flow after treatment of the BDL rats with BRL 44408 in comparison to the BDL rats treated with saline. The forward flow theory of portal hypertension hypotheses' that the splanchnic vasodilatation results in increased blood to the liver with the hepatic arterial buffering for the decreased blood flow to the liver. The net effect is an increased hepatic blood flow which helps to propagate and maintain the portal hypertension. The above findings are more relevant taking into account that the portal pressure was significantly reduced in the BRL 44408 treated BDL rats as compared to the BDL rats treated with normal saline. The fact that the cirrhotic liver is able to accommodate an increased blood flow is evidence of modulation of the reversible component of the intra hepatic resistance. The vascular tone forms a significant part of this reversible part of the intrahepatic resistance. We show in our study that the cyclic AMP activity in the liver tissue is significantly increased in the BDL rats treated with BRL 44408 as compared to the BDL rats treated with saline. This implies that there is an increase in the calcium flux into the myosin which helps to relax the vascular smooth muscle cells which in turn help to vasodilate the intrahepatic vasculature leading to reduction in intra hepatic resistance.

One of the most important observation in this study is the significant increase in the ADRA2a receptor density in the liver of the BDL rats as compared to the Sham operated rats. From the sepsis literature , it is clear that the ADRA2a receptor has got significant increased expression in the migrating macrophages in the ceacal ligation and puncture rat model and this theory might help to explain the increased receptor expression in the BDL rats as compared to the Sham operated rats (193). To compliment this finding, there is also increased inflammatory markers including tissue and plasma TNFα and Nuclear

Factor in the BDL rats as compared to the sham operated rats. These markers show a significant reduction in the level of TNFα, both in the tissue and plasma and also in the protein expression of NF kappa B in the BDL rats upon treatment with BRL 44408.It is probable that the migrating macrophages from the portal circulation in the BDL rats have increased cytokine load and therefore provoke an inflammatory response with the rise of TNFα in the plasma and liver tissue. This pro-inflammatory cytokine drive is ameliorated by treatment with BRL 44408, demonstrating its anti-inflammatory properties.

In order to interrogate the possible pathways that are implicated in the reduction of intrahepatic resistance with the ADRA2a antagonism, I also assessed the cyclicAMP levels in the three goups of animals. There is significant reduction in the cyclicAMP levels in the ADRA2a antagonist treated BDL rats as compared to the BDL rats treated with saline. This suggests that this pathway is involved in the vasodilatation process in the intra hepatic vasculature. cyclicAMP is a second messenger in the adenyl cyclase pathway which is a G-protein receptor dependent signaling cascade which is used in cell communication. When a GPCR is activated by its extracellular ligand, a conformational change is induced in the receptor that is transmitted to an attached intracellular heterotrimeric G protein complex. This in turn stimulate the cyclic nucleoside gated calcium channel, which is necessary in the steps for the vasomotor relaxation which in the case of intra hepatic circulation reduces intra hepatic resistance. Till date the various treatment strategies have targeted this increased blood flow towards the liver with an aim of reducing the total hepatic blood flow. However, there is some conflicting evidence that in cirrhosis with refractory ascites, there may be an adverse effect by adopting this strategy

as there is already an existent intense vasoconstriction of the intrahepatic vasculature and further restriction of blood flow has a deleterious clinical outcome (10).

The hepatic stellate cells are contractile cells located in the peri-sinusoidal space with elongated protrusion which encircle more than one sinusoid. The stellate cells become contractile in the face of chronic liver injury and manifest as fibrotic band of collagen which regulate the pre-sinusoidal blood flow. They also express various receptors which endothelin 1, angiotensin 1 which induce generation of contractile force by contractile cell types (194, 195). In my study, I demonstrate that upon incubation of rat hepatic stellate cells in a serum free medium as compared to a medium containing guanfacine (ADRA2a agonist), there is increased contractility in the guanfacine containing gel plate. This suggest that the hepatic stellate cells also express ADRA2a receptor and provides another target cell to reduce the contractility of these cells in order to improve hepatic blood flow.

Alpha Smooth Muscle Actin (αSMA) is a marker of hepatic stellate cells activation. This indicates that the HSC modify to form myofibroblasts which impact on the contractibility of the sinusoidal cells. In my study, I looked at the protein expression for the αSMA protein in the three groups within the experiments. The results reveal that there is a significant expression of αSMA in the BDL rats as compared to the sham operated rats. However, on treatment with BRL 44408, there is significant reduction in the αSMA expression. This suggest that there is deactivation of the HSC upon treatment of the BDL rats treated with BRL 44408. This leads to reduction in the sinusoidal contraction, in turn reducing intra hepatic resistance in the treated group.

Based on the haemodynamic observations. I have elicited mechanistic pathways that may be involved in these observations. Previous studies from our research group has shown that the endothelial nitric oxide protein expression is increased in the BDL rats as compared to the sham operated rat. In this study, I have been able to validate that observation. Further to that, following treatment with BRL 44408, the ADRA2a adrenergic receptor antagonist, the protein expression of eNOS is significantly reduced. In contrast, the phosphorylated endothelial nitric oxide which is a marker for the enzyme activity is reduced in the BDL rats as compared to the sham operated rats. This is increased significantly in the BDL rats treated with BRL 44408. This implies that there is increased production of intra hepatic nitric oxide, which facilitates reduced vascular tone and thereby reduce intra hepatic resistance in the BDL rats treated with BRL 44408.

Although the treatment protocol for this set of experiment was over two days, I have made a histological assessment of the liver tissues from the rats involved in this experiment. I have shown that there is significant architectural changes noted in the BDL rats as compared to the Sham operated rats. This was evidenced by increased number of cholangiocytes in the BDL rats with architectural distortion of the hepatic lobule in the BDL rat as compared to the Sham operated animal. This changes are similar to previous finding during the establishment of the model in my laboratory and therefore data is not shown. However, there was no difference between the BDL rats treated with BRL 44408 as compared to the BDL rats treated with saline. This indicates that there was no structural influence on the haemodynamic changes that is observed in the experiments.

Apart from these results related to the effect of ADRA2a antagonism on the porto-systemic vasculature, I also show some preliminary data on improvement of perfusion to other organs including the brain and the kidney. First of all, I show that there is significant improvement in the plasma lactate levels in the ADRA2a antagonist treated BDL rats as compared to BDL rats treated with saline. This indicates that there is improvement in the perfusion of end organs with ADRA2a antagonism. In the brain, there is significant reduction in the brain water in the ADRA2a antagonist treated rats indicating that there is less brain swelling in this group of animal as compared to the saline treated BDL rats. As brain swelling can initiate transcription of cytokines, it is also possible that reduction in brain water following treatment with ADRA2a led to a reduction in brain inflammation. Studies from our lab have shown that using an anti-inflammatory strategy, there is reduction of neuroinflammation and restoration eNOS activity (196). I also show that there is significant improvement in the serum creatinine in the BDL rats treated with ADRA2a antagonist as compared to the BDL rats treated with saline. I accept that serum creatinine does not accurately reflect renal function but it highlights an important signal to justify further investigation of the renal effect of ADRA2a antagonism.

In conclusion, in this study I was able to show an alternative strategy to reduce portal pressure. The beneficial effect of an increased mean arterial pressure and increased hepatic blood are both desirable in the setting of cirrhosis and portal hypertension. These data provide a rationale for considering ADRA2a adrenergic receptor antagonism as a legitimate strategy for lowering portal pressure.

Taking into account all the above findings, it is clear that the intra hepatic resistance is propagated by a combination of the architectural distortion as a result of nodule formation and micro thrombi and a reversible vascular component. This reversible vascular component is modifiable with various intervention. Inflammation, mainly propagated by the gut-liver axis has been given its due importance as a main proponent of progression of intra hepatic resistance. The migratory immune cells from the gut to the liver have been implicated in the production of inflammatory cytokines. There has been some suggestion in the literature, especially from the sepsis literature about targeting these circulating immune cells as a strategy to reduce the inflammatory burden (197). It poses an interesting question as to whether targeting these set of circulating immune cells in the context of liver disease will have a positive effect on portal hypertension. The next section of studies will attempt to answer some of these questions.

Chapter 5

The modulation of the immune system by ADRA2a adrenergic receptor antagonism

5.1 Introduction

The relationship between inflammation and portal hypertension has now been shown in several studies. Mortensen et.al. have shown that there is a close linear relationship between the highly sensitive C-Reactive Protein and the portal pressure (99). Pro-Inflammatory cytokines like IL-6 have been found to be increased in patient with portal hypertension and alcoholic hepatitis (98). We have previously shown that on treatment with an anti-TNF agent, the portal pressure improves significantly in patients with alcoholic hepatitis, suggesting that targeting the inflammatory cytokines are a valid target to reduce portal pressure (198), albeit their clinical translation has been limited by serious adverse effects.

The understanding of the pathophysiology of gut derived catecholamine release has improved significantly in the last decade. Many inflammatory molecules have been identified and these have been noted to be increased in a septic model. There is evidence to suggest that the central nervous system receives messages from the immune system and vice versa. Messages from the brain modulate immune function in health and disease (199). There is also evidence that noradrenaline levels are released by the sympathetic nervous system and modulate the immune function (200). It is already

known that the sympathetic nervous activity is increased in sepsis and this leads to increased release of noradrenaline.. Previous studies have shown that the level of NE release is directly co-related to the level of cytokine (TNFα) and other cytokine release (201).

Similarly, intra-portal infusion of NE in vivo or in isolated livers increased TNFα synthesis and release which was inhibited by co-infusion of yohimbine. Furthermore, the increased cellular levels of TNFα in Kupffer cells after in vivo administration of NE was also blocked by yohimbine (ADRA2 adrenergic receptor antagonist). These results, taken together, suggest that gut-derived NE upregulates TNF-α production in Kupffer cells through an α2 adrenergic pathway, which appears to be responsible at least in part for the increased levels of circulating TNF-α observed during early sepsis (111)

Further experiments shows that the enhanced pro inflammatory cytokine release in early sepsis in kupffer cells is via the ADRA2a adrenoreceptors. More specifically, it has been found that the NE increased the release of TNF-a in cultured KCs, which was specifically inhibited by the ADRA2a antagonist BRL-44408.This suggest that ADRA2a adrenergic receptor provides a viable target for the treatment of sepsis (193)

Therefore, the above studies have provided evidence that in the context of sepsis, gut derived NE produces TNFα from the kupffer cells and liver derived macrophages causes liver dysfunction. This phenomenon is reversible by antagonism of the ADRA2a adrenergic receptor. The ADRA2a adrenergic receptor has been shown to have a significant role in the propagation of this inflammatory cascade. Therefore the ADRA2a adrenergic receptor would be an ideal target to explore in the context of liver disease and portal hypertension

The aim of this study was to assess the relevance of the ADRA2a adrenergic receptor in the modulation of inflammation in the circulating immune cells and to address the hypothesis that the ADRA2a receptor is a valid target for reduction of portal pressure by ameliorating inflammation.

5.2 Methods and Materials:

5.2.1 Study design

Four weeks after bile duct-ligation or Sham-operation, rats were randomized in to six groups. The treatment regime for both placebo (N/Saline) and BRL 44408 (ADRA2a antagonist) included daily subcutaneous injection at the rate of 10mg/kg. These injections were given once a day from day 18 till day 28 when the haemodynamic experiments were carried out. Lipopolysaccharide (E.coli,Sigma Aldrich) was injected at 0.33mg/kg per rat as a intra peritoneally 3 hours prior to start of the haemodynamic measurement. The rats were monitored in a temperature controlled incubator for three hours and then were exsanginated as per prescribed method. The groups that were set up were:

Sham +N/S (n=16)
Sham+LPS (n=3)
Sham +BRL (n=3)
BDL+ N/S (n=20)
BDL+LPS (n=9)
BDL+BRL (n=21)
BDL+LPS+BRL (n=7)

5.2.2 Haemodynamic measurement

All haemodynamic measurements were undertaken exactly as described previously in section 2.3.

5.2.3. Biochemical Parameters

All biochemical parameters (ALT, ALP, albumin, total protein, ammonia, bilirubin, urea and creatinine) were analyzed using 200µl of respective plasma samples using a Cobas Integra 400 multianalyzer with the appropriate kits (Roche-diagnostics, Burgess Hill, West Sussex, UK).

5.2.4 Tissue Cytokine Assays

All cytokine assays were undertaken in a similar method as described previously in section 2.6.

5.2.5 FACS analysis

Whole liver tissue was taken and homogenized as per protocol and population studies and functional studies on various cell types were conducted after cell isolation. The details of the methods used for the FACS analysis in various cell types are described in section 2.15 till 2.17.

5.2.6 Endotoxin Assay

The chromogenic limulus amoebocyte lysate kinetic assay (Charles River Laboratories) was used for the detection of endotoxin. The method used is described in detail in section 2.18.

5.3 Results.

Following surgery all animals continued to gain weight. From the final body weight taken before termination, cirrhotic rats were found to be significantly lower than that of sham rats (350 ± 5g vs. 472 ± 9g, respectively; $P < 0.001$).

5.3.1 Biochemical paramenters in the different groups

Six groups of animals were studied namely Sham operated (Sham), Sham + BRL, BDL and BDL+BRL, BDL+LPS and BDL+LPS+BRL. Bilirubin, creatinine, albumin and plasma aminotransferase were measured using standard kits on a Cobas Integra analyser (Roche Diagnostics Ltd, West Sussex, UK).

Table 5.1 Biochemical pattern in all the groups

	Sham+N/S	Sham+LPS	BDL+N/S	BDL+LPS	BDL+BRL	BDL+LPS+BRL
ALT (IU/L)	70.27±9.07	80.20±7.23	100.1±13.26*	130.3±24.46	82.12±6.26	69.10±5.19
AST (IU/L)	131.3±17.3	157.5±11.75	444.5±58.24*	587.5±52.48	406.2±64.82	488.4±8.36
Bil (µmol/L)	44.16±15.4	57.88±7.25	132.2±21.02*	136.2±20.11	123.4±11.61	130.2±16.14
Albumin (gm/L)	32.40±0.76	37.65±3.04	24.41±0.94	24.80±0.80	25.74±1.02	26.61±1.02

Table 5.1: Shows the biochemical parameters in the six groups (Sham+N/S, Sham+LPS, BDL+N/S, BDL+LPS,BDL+BRL and BDL+LPS+BRL) (*p<0.01)

The alanine aminotransferase (ALT) level was no different between the sham operated rats treated with the saline or BRL 44408. However there was a significant increase in the plasma ALT levels in the BDL rats as compared with the sham operated rats (100.1 ± 13.26 v 70.27 ± 9.07 , p<0.01). There was a trend towards a reduction in the plasma ALT levels in the BDL rats treated with BRL and with or without LPS as compared to the BDL rats treated with saline or LPS.

The aspartate aminotransferase (AST) level was also no different between the sham operated rats treated with saline and BRL. There was a significant increase in the plasma AST level in the BDL rats treated with saline as compared to the sham operated rats treated with saline (444.5± 58.24 v 131.3±17.3, p<0.01). However, there was no reduction in the plasma AST level in the BDL rats treated with BRL 44408 with or without LPS as compared with the BDL rats treated with saline or LPS.

Similarly, the serum bilirubin was not difference between the sham operated animals treated with saline and BRL44408.There was a significant increase in the serum bilirubin the BDL rats as compared with the sham operated rats (132.2±2.02 v 44.16±15.46, $p<0.01$). However there was no difference between the BDL rats treated with saline or BRL 44408 or LPS.

The serum albumin was no different between the sham operated with saline or BRL 44408.There was a significant reduction in the serum albumin levels in the BDL rats as compared to the sham operated rats (24.41.06 ± 0.94 v 32.40 ± 0.76 ,p0.01) . This did not alter after treatment with BRL and or LPS.

5.3.2 Mean Arterial Pressure

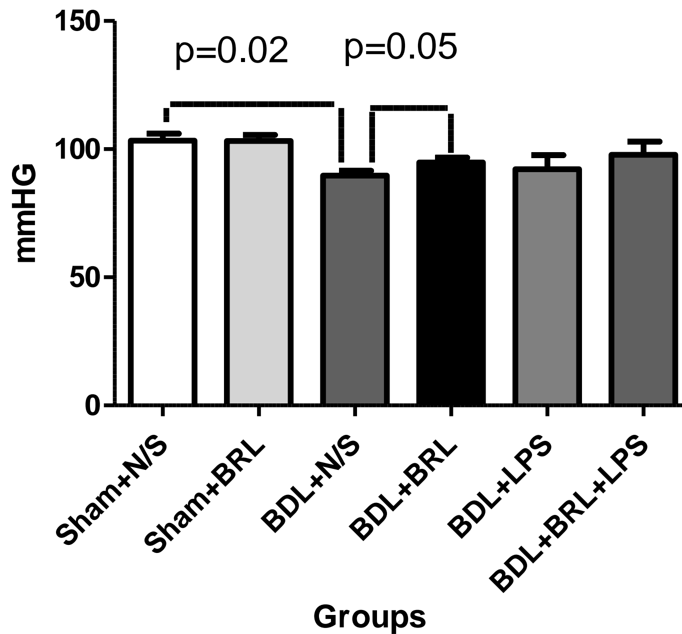


Fig 5.1: Mean arterial pressure measurement of all six groups (Sham + N/S, Sham + BRL, BDL + N/S and BDL +BRL,BDL+LPS,BDL+LPS+BRL).There is a significant increase in the MAP in the BDL rats treated with BRL 44408 as compared to the BDL rats treated with saline (p=0.01). (Sham+N/S=16, Sham+BRL=3, BDL+N/S = 21, BDL+BRL=20, BDL+LPS=8, BDL+BRL+LPS=7)

The mean arterial pressure (MAP) was measured in all the groups as described in section 2.3.The MAP in the bile duct ligated rats is significantly lower than sham operated rats (89.64±1.91 mmHg v 103.3±2.75 mmHg, p=0.02).However on treatment with BRL 44408, there is a significant increase in the MAP in the BDL treated group treated with BRL as compared to BDL rats treated with saline (89.64±1.91 mmHg v 94.84±1.8 mmHg, p=0.05). There is no difference in the MAP between the rats treated with an additional dose of LPS.

5.3.3 Portal Pressure Measurements:

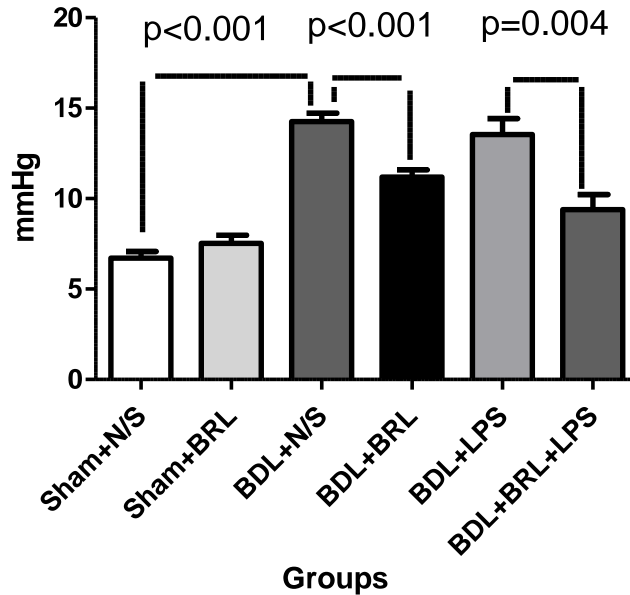


Fig 5.2: Portal pressure measurement of all six groups (Sham + N/S, Sham + BRL, BDL + N/S and BDL +BRL, BDL+LPS and BDL+BRL+LPS).The portal pressure is significantly reduced in the BDL rats treated with BRL 44408 as compared with the BDL rats treated with saline (p<0.001) .The portal pressure was reduced in the BDL rats treated with BRL and LPS as compared to the BDL rats treated with LPS alone (p=0.04). There was no difference between the portal pressure in the sham operated rats. (Sham+ N/S=16, Sham+BRL=3, BDL+ N/S= 21, BDL+BRL=20, BDL+LPS=8, BDL+BRL+LPS=7)

The portal pressure was significantly increased in the bile duct ligated rat as compared to the sham operated rats (14.21±0.43 mmHg v 6.71±0.36 mmHg, p<0.001).The portal pressure was significantly reduced in the BDL rats treated with BRL 44408 as compared to the BDL treated with normal saline (11.19±0.40 mmHg v 14.20±0.47 mmHg, p<0.001) in the study. Furthermore, there was a significant reduction the portal pressure of BDL rats treated with LPS and BRL 44408 as compared to the BDL rats treated with LPS alone (13.55±0.87 v 9.39±0.82).

5.3.4 Endotoxin Assay:

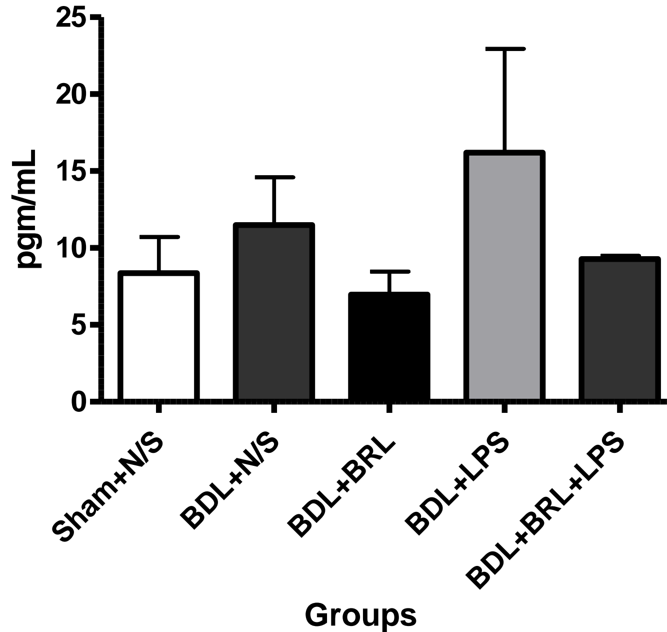
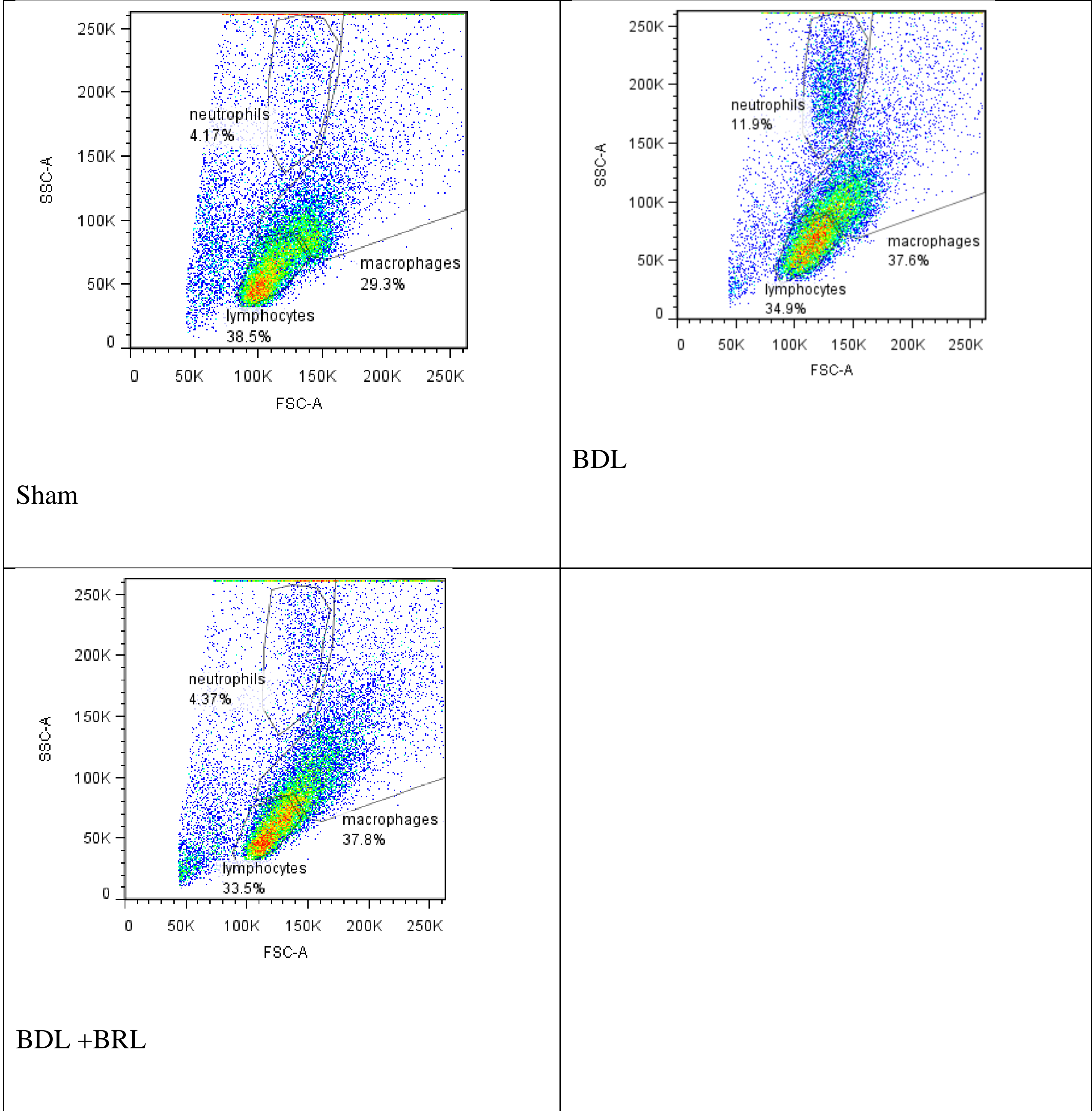


Fig 5.3: Limulus Amebocyte Lysate Assay of the various groups of rats (Sham, BDL, BDL+BRL, BDL+LPS and BDL+BRL+LPS). The endotoxin shows a non-significant trend towards the improvement of the endotoxin load in the BDL rats treated with BRL with or without LPS. (Sham+N/S=4, BDL+N/S=4, BDL+BRL=4, BDL+LPS=3, BDL+LPS+BRL=3)

The Limulus Amebocyte Lysate Assay is an assay which reacts with bacterial endotoxin and lipopolysaccharide (LPS) which is a membrane protein in gram negative bacteria. This is used for the quantification of bacterial endotoxin load. In this set of experiments, the endotoxin load showed a non statistically significant trend towards the lowering of the endotoxin load in the BDL rats treated with LPS and BRL 44408 treated rats as compared to the BDL rats treated with LPS alone (p=0.06). The assay results described here do not show a significant trend. This may be due to the variability within the assay itself and may have been improved if there was increased numbers of rats per group. Therefore the results from this experiment have to be interpreted with caution.

5.3.5 Population studies

A. FACS data on cell population in the portal circulation



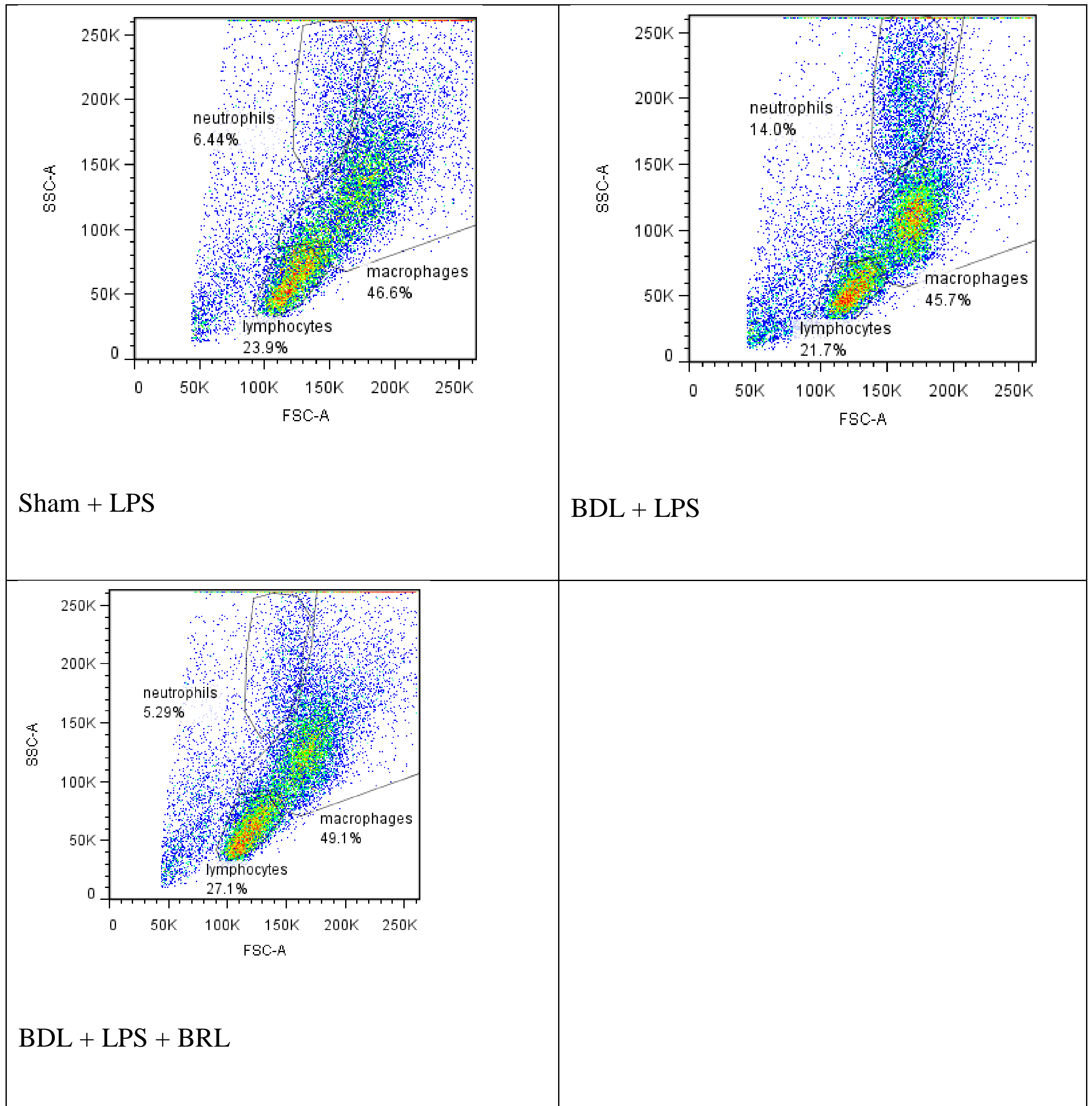


Fig 5.4 : Population studies in the various groups. Gated Population study of the various cell types. The gated population in the different groups Sham, BDL, BDL+BRL, Sham+LPS, BDL+LPS and BDL+LPS+BRL.

The population studies were done as described in method section 2.15. The Neutrophil count is increased in the BDL rats treated with Saline and LPS as compared to the Sham rats treated with Saline and LPS respectively. This was significantly reduced in the Sham and BDL rats treated with BRL 44408 with or without LPS.

5.3.5.1 The neutrophil population in the portal circulation

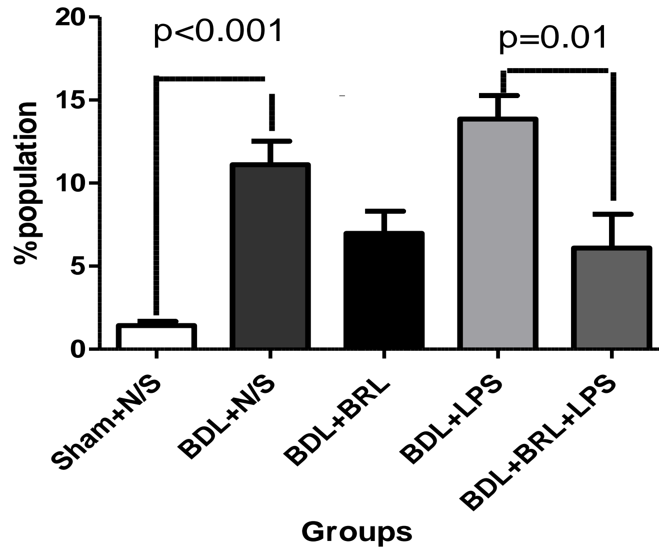


Fig 5.5(A): Neutrophil population in the portal vein in various groups (Sham, BDL, BDL+BRL, BDL+LPS, BDL+LPS+BRL). The neutrophil count is elevated significantly in the BDL rats as compared to the sham operated animals (p<0.001). The neutrophil count is reduced non-significantly in BDL rats treated with BRL 44408. However, in BDL rats treated with LPS, BRL 44408 reduces the neutrophil count significantly (p=0.01). (Sham+N/S=7, BDL+N/S=5, BDL+BRL=5, BDL+LPS=5, BDL+LPS+BRL=5)

The neutrophil population in the portal vein is shown in all the groups. The neutrophil population in the portal circulation was calculated by FACS analysis by a method described in section 2.15. It shows that there is significant increase in the neutrophil count in the BDL as compared with Sham operated animals 11.12±1.41 v 1.42±0.24, p<0.001). However, after treatment with BRL, the neutrophil count is significantly reduced in the BDL animals with (6.08±2.03 v 13.86±1.41, p=0.01) or without LPS 6.98±1.32 v 11.12±1.41, p=0.01)

5.3.5.2 Neutrophil Population in the systemic circulation:

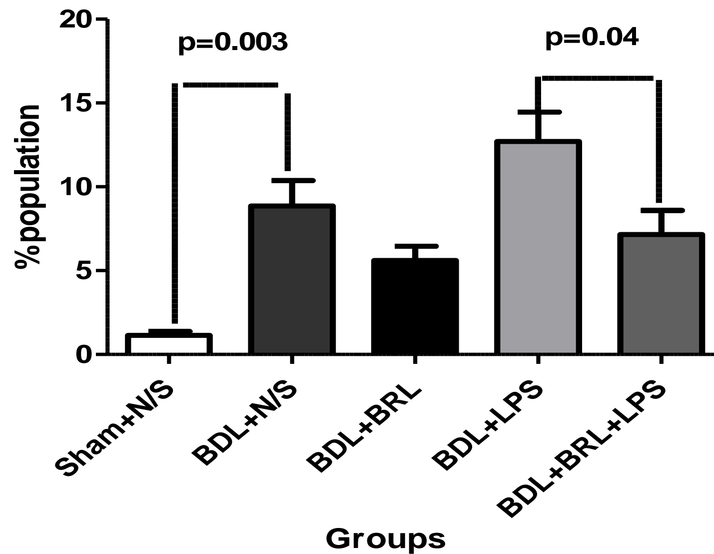


Fig 5.5(B): Neutrophil population in the systemic circulation in the various groups (Sham, BDL, BDL+BRL, BDL+LPS, BDL+LPS+BRL). The neutrophil count increases significantly in the BDL rats as compared to the Sham operated rats. There is a non- significant trend towards reduction in the BDL rats treated with BRL 44408. However, in the BDL rats treated with LPS, BRL 44408 reduces the neutrophil counts significantly (p=0.04). (Sham+N/S =7, BDL+N/S =5, BDL+BRL=5, BDL+LPS=5, BDL+LPS+BRL=5)

The neutrophil population in the systemic circulation is calculated in the various groups. The neutrophil population in the systemic circulation was calculated by FACS analysis by a method described in section 2.15. It shows that the neutrophil count significantly increases in the BDL animals as compared to the sham operated animals (8.86±1.51 v 1.13±0.23, p=0.003) .However, The BDL rats treated with BRL 44408, there is a significant reduction in the rats with (7.16±1.43 v 12.71±1.74, p=0.04) or without LPS (5.60±0.85 v 8.86±1.51, p=0.04).

5.3.6 Phagocytic capacity of circulating cells:

5.3.6.1 Phagocytic capacity of circulating neutrophils

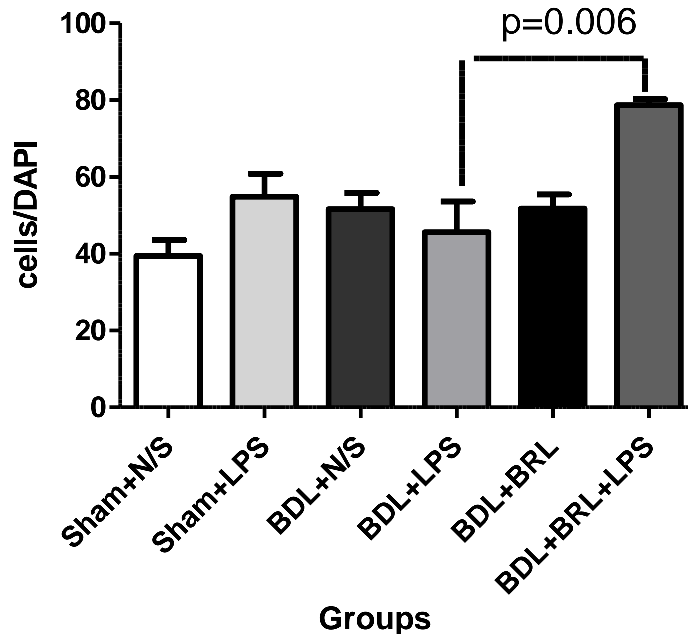


Fig 5.6(A): Phagocytic capacity of circulating neutrophils. The phagocytic capacity increases in the BDL+LPS group treated with BRL44408 as compared to the rats not treated with BRL (P=0.006). (Sham+N/S=7, Sham+LPS=5, BDL+N/S=5, BDL+BRL=5, BDL+LPS=5, BDL+LPS+BRL=5)

The phagocytic capacity of the neutrophils were assessed for all the six groups of rats. The phagocytic capacity of neutrophils was assessed by the method described in Section 2.16. Although there was no significant difference in the animals treated with saline, there was a significant increase in the phagocytic capacity of the neutrophils in the BDL rats treated with BRL 44408 and LPS as compared to the BDL rats treated with LPS alone (78.70±1.5 v 45.60±8.06, p=0.006)

5.3.6.2 Phagocytic capacity of monocytes:

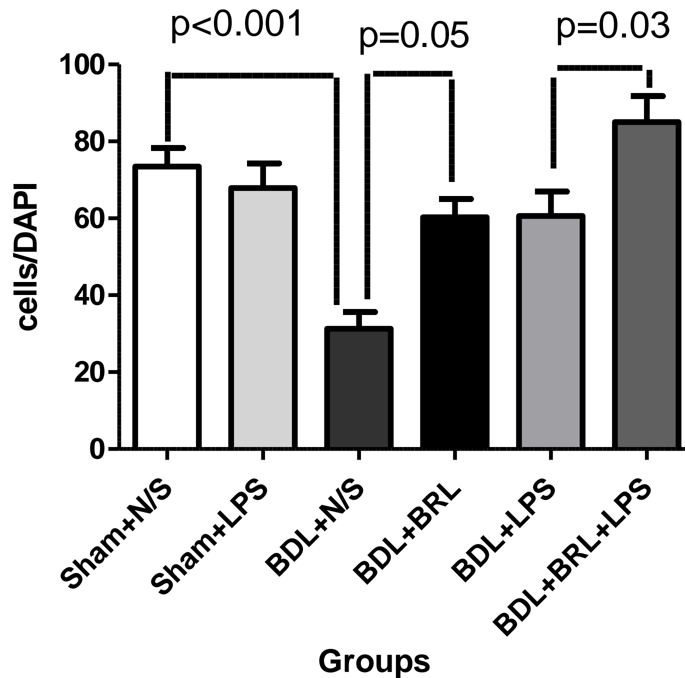


Fig 5.6(B): Phagocytic capacity of circulating monocytes (Sham, Sham+LPS, BDL, BDL+BRL, BDL+LPS, BDL+LPS+BRL). There is reduction in the phagocytic capacity of the BDL as compared to the Sham animals (p<0.001), whereas upon treatment with BRL 44408, there is significant improvement in the phagocytic capacity in BDL rats treated with (p=0.03) or without LPS (p=0.05) (Sham+N/S=7, Sham + LPS=5, BDL+N/S=5, BDL+BRL=5, BDL+LPS=5, BDL+LPS+BRL=5)

The phagocytic capacity of the monocytes in the different groups of rats are shown above. The monocyte phagocytic capacity was assessed by the method described in Section 2.16. The monocyte phagocytic capacity is reduced in the BDL rats as compared with Sham animals (31.26± 4.35 v 73.43±4.82, p<0.001) However, this is increased in the BDL rats after treatment with BRL44408 with (85.07±6.67 v 60.65±6.33p=0.03), or without LPS (60.32±4.68 v 31.26±4.35, p=0.05).

5.3.7 Activation of Kupffer Cells:

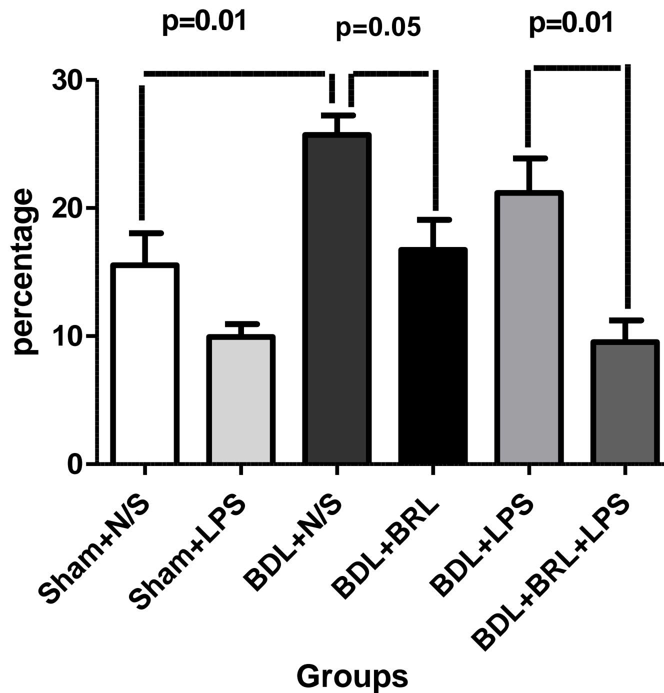


Fig 5.7: Activated Kupffer cells in the various groups. The activated Kupffer cells are gated by the CD68 cell marker against the CD163 positive cells in all the groups (Sham, Sham+LPS, BDL, BDL+BRL, BDL+LPS, BDL+LPS+BRL). The kupffer cells are activated significant in the BDL rats as compared to the Sham operated rats (p=0.01). (Sham+N/S=7, Sham + LPS=5, BDL+N/S=5, BDL+BRL=5, BDL+LPS=5, BDL+LPS+BRL=5)

The activated kupffer cells are gated by the specific markers in all the groups. It shows that the kupffer cells are activated in the BDL animals as compared to the Sham operated animals (25.71±1.52 v 15.53±2.49, p=0.01). The BDL rats treated with BRL 44408 showed a significantly reduced activated Kupffer cells in the rats treated with (9.54±1.69 v 21.19±2.69, p=0.01) or without LPS (16.73±2.36 v 25.71±1.52, p=0.05)

5.3.8 Reduction of Reactive Oxygen Species in Kupffer Cells:

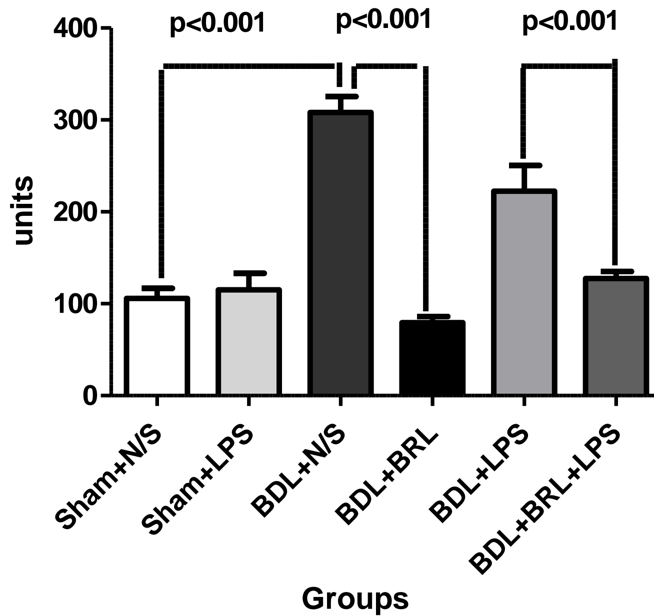


Fig 5.8:Reactive Oxygen Species in Kupffer Cells in various groups. The CD163 gated ROS levels in all six groups (Sham, Sham+LPS, BDL, BDL+ BRL, BDL+LPS, BDL+BRL+LPS) . The ROS level is significantly increased in the BDL rats as compared to the Sham operated rats (p<0.001). The ROS levels are reduced significantly in the BDL rats treated with BRL with (p<0.001) or without LPS (p<0.001). (Sham+N/S=7, Sham + LPS=5, BDL+N/S=5, BDL+BRL=5, BDL+LPS=5, BDL+LPS+BRL=5)

The Reactive Oxidative Species (ROS) levels are done in all the six groups. It shows that the ROS level increases significantly in the BDL rats as compared to the sham operated rats (308.0±17.32 v 105.8±11.01, p<0.001). However, upon treatment with BRL 44408, the BDL rats have significantly reduced level of ROS in the rats treated with (127.5±15.56 v 222.5±28.06, p<0.001) or without LPS (79.56±6.49 v 308±17.32, p<0.001)

5.3.9 Reduction in Inflammatory cytokines with ADRA2a antagonism

5.3.9.1 Liver Tissue TNFα level

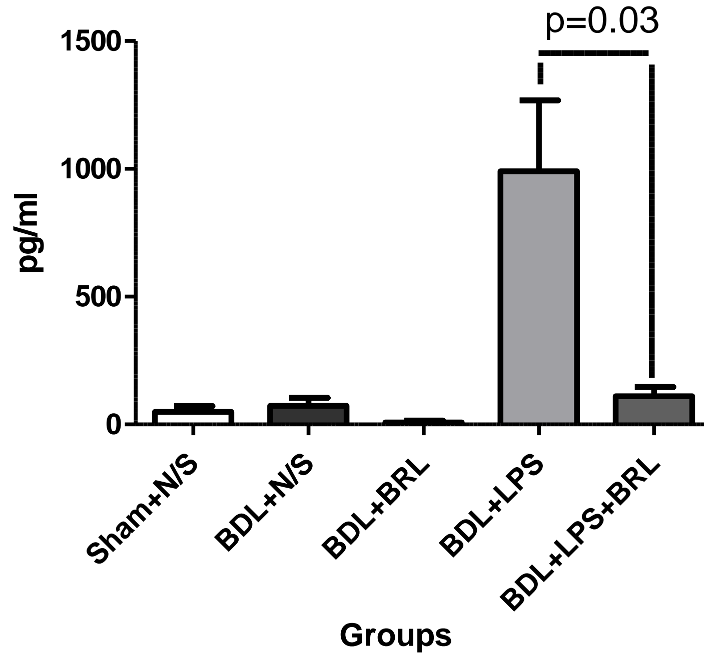


Fig 5.9(A): Liver tissue TNF levels in the various groups. The liver tissue TNFα levels were increased after administration of LPS (0.33mg/kg) , however, it was reduced significantly after treatment with BRL 44408 (p=0.03).There was no significant difference in the tissue TNFα levels in all the other groups. (Sham+N/S=7, BDL+N/S=6, BDL+BRL=5, BDL+LPS=6, BDL+LPS+BRL=6)

The liver tissue TNFa levels were measured by cytokine bead array test done on the FACS machine as described in section 2.6. The liver tissue TNFa was shown to be significantly elevated in the BDL rats treated with LPS. However when treated with BRL 44408, there was significant reduction in the TNFa levels in the BDL rats treated with LPS (111.1±35.32 v 990.6±277.7, p=0.03).

5.3.9.2 Liver Tissue IL-6 level

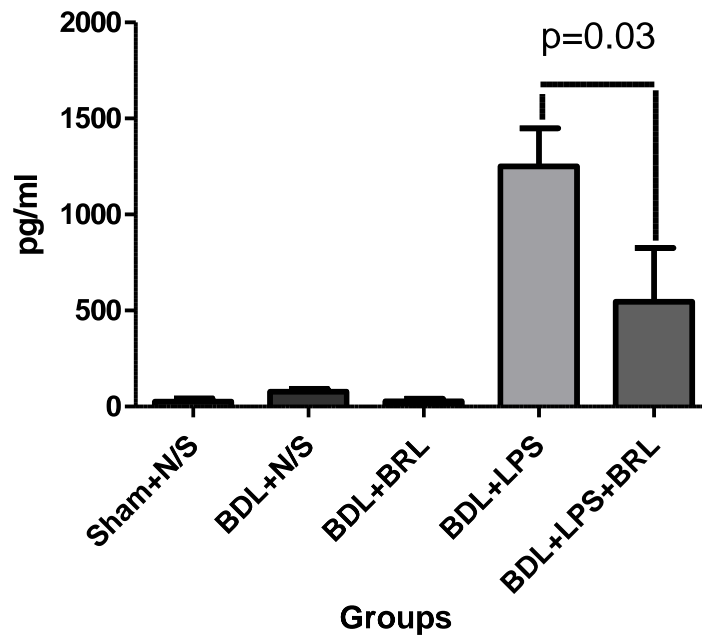


Fig 5.9(B):Liver tissue IL-6 levels in various groups. The IL-6 level is highest in BDL rats treated with LPS and this was reduced significantly after treatment with BRL 44408 (p=0.03). (Sham+N/S=7, BDL+N/S=6, BDL+BRL=5, BDL+LPS=6, BDL+LPS+BRL=6)

The liver tissue IL-6 levels is calculated in all the groups of animals. It shows that the liver tissue IL-6 level goes up in the BDL rats treated with LPS. However, on treatment with BRL 44408, there is significant reduction in the BDL rats treated with LPS (546.1±279.4 v 1251±198.5, p=0.03).There was no statistical difference in the level in the other groups.

5.4 Discussion:

From the data presented I was able to conclude that the modulation of the innate immune system by blocking the ADRA2a adrenergic receptor improves the immune function and reduces the oxidative stress and inflammatory cytokines. This leads to reduction of the portal pressure in the bile duct ligated rats treated with or without lipopolysaccharide.

The gut liver axis has been being accepted as an important pathophysiological pathway in the propagation of liver dysfunction in cirrhosis. It is well documented that there is increased bacterial translocation in cirrhosis and this in turn leads to an increased cytokine load in the portal circulation(202). The inflammatory reflex controls innate immune responses by a mechanism that targets the regulatory transcription factor nuclear factor-κB (NF-κB). Ligand–receptor interactions activate innate immune responses and induce the secretion of pro-inflammatory cytokines. Molecular products of infection activate macrophages, monocytes and dendritic cells that interact with Toll-like receptors (TLRs), which transduce intracellular signals, ultimately leading to the activation of NF-κB and the increased release of pro-inflammatory cytokines (203)

The results of the recently published CANONIC study shows that inflammation plays an important part in the pathophysiology and propagation of acute on chronic liver failure (72). In this study, I show that there is a trend towards increase in the endotoxin load in the circulation of the bile duct ligated rats. This was further augmented trend upon treatment with LPS. This is complemented by the increase in the liver tissue pro – inflammatory cytokine (TNFα, IL-6). However, treatment with the ADRA 2a receptor

antagonist BRL 44408, there is trend towards reduction in the endotoxin levels in the BDL rats treated with LPS.

From the sepsis literature, there is clear evidence that the pro-inflammatory cytokines in the portal circulation activate the migrating macrophages, which in turn produce more pro-inflammatory cytokines and reactive oxygen species. In this study I show that the macrophages (kupffer cells) gated by the CD163 shows an activated state (CD163 gated CD68 cells) in the BDL rats treated with saline. This is also noted to be significantly increased in the BDL rats treated by the addition of LPS. This was complimented by the significant increased production of Reactive Oxygen Species in the BDL rats and further increased in the BDL rats treated with LPS. This was again significantly reduced with treatment with BRL 44408.

Rat lymphocytes and human peripheral blood mononuclear cell (PBMC) both have been shown to have increased level of expression of enzymes which results in increased production of dopamine, noradrenaline when stimulated(204). Thus, it now is becoming clear that lymphocytes and phagocytes not only possess the ability to produce, store, release, and re- uptake catecholamines *de novo*, but that these cells also are capable of exquisitely regulating their catecholamine-synthesis in response to various extracellular stimuli. (113).

In this study, I have assessed the function of the circulating neutrophils and macrophages. I show that the functional capacity of phagocytosis of these cells are significantly increased in the BDL rats treated with LPS and BRL 44408 as compared to the BDL rats treated with LPS only. . Moreover, I also show that the absolute number of neutrophils in the portal and systemic circulation are significantly increased in the BDL

rats treated with or without LPS. This was reduced by treatment with BRL 44408 in BDL rats treated with or without the LPS. This implies that neutrophil migration to the liver that is noted in a setting of acute on chronic liver failure such as alcoholic hepatitis can be modified by this specific ADRA2a adrenergic receptor blockade. There is evidence of neutrophil dysfunction in cirrhosis and a study from our lab has previously shown that the neutrophils present in the setting of alcoholic hepatitis loses its function capacity to phagocytose the increased bacteria and bacterial products that are being translocated from the gut wall (71).

It is already known that the sympathetic nervous activity is increased in sepsis and this leads to increased release of norepinephrine. Yang et al has shown that the level of NE was lower in pre enterectomised rats where in sepsis was induced by ceacal ligation and puncture as compared to the non pre enterectomised rats. They also show that the level of pro-inflammatory cytokines TNFα and IL-6 were reduced and attenuated liver dysfunction in the pre enterectomised group (110). Furthermore, the increased cellular levels of TNF-α in Kupffer cells after in vivo administration of NE was also blocked by yohimbine. These results, taken together, suggest that gut-derived NE upregulates TNF-α production in Kupffer cells through an α_2-adrenergic pathway, which appears to be responsible at least in part for the increased levels of circulating TNF-α observed during early sepsis (111). In my study, I have demonstrated that the Kupffer cells are activated as evidenced by the increased percentage of CD163 gated CD68 cells. This activated state also produced significantly more Reactive Oxygen Species in the BDL rats treated with or without LPS. However on treatment with BRL 44408, the percentage of activated kupffer cells were significantly reduced as well the amount of ROS production.

This study suggests that the ADRA2a adrenergic receptor plays an important role in the production of pro-inflammatory cytokines and elevation of portal pressure. By using a specific ADRA 2a adrenergic receptor antagonist we are able to demonstrate that it improves neutrophil and macrophage function, reduces inflammation, reduces activation of kupffer cells and its consequent ROS production and thereby reduces portal pressure. Based on these results, it can be postulated that ADRA2a adrenergic receptor may be a target for the modulation of inflammation and sympathetic tone in order to reduce portal pressure. However, further studies are required to validate our findings before clinical translation can be considered.

In conclusion for this section of this research, I have been able to show that the ADRA2a adrenergic receptor is present in the BDL rat liver more significantly than in sham operated rats. By antagonising this particular receptor, I have been able to reduce the portal pressure in the BDL rats whilst maintaining their mean arterial pressure. These results are accompanied by the significant reduction of the pro-inflammatory cytokines. The mechanism of action was also explored and the results show that there is improvement in the cyclic AMP levels suggesting a possible vasorelaxing mechanism. There is also improvement in the eNOS activity suggesting that there is an improvement of endothelial dysfunction. I have also shown that the stellate cell contraction is also influenced by the ADRA2a adrenergic receptor pathway and its antagonism will relax the contractility of the stellate cells. While all these phenomenon are going on in the liver, I also show evidence of reduction of neutrophils from the portal circulation and

improvement in the functional capacity of the neutrophils and monocyte with treatment with an ADRA2a adrenergic receptor antagonist. This strategy will also reduce the activation of the kupffer cells and its consequent ROS production. Overall, these results prove that ADRA2a adrenergic receptor antagonism may be an important alternative strategy to reduce portal hypertension.

Chapter 6

Role of L-Arginine supplementation in the modulation of intra hepatic resistance

6.1 Introduction

Over the past few decades, several studies have provided evidence that suggest supplementation of L-arginine was considered a logical therapeutic option to restore the decreased arginine levels in septic and critical ill patients, as increased arginine concentrations may restore the important physiologic processes, including organ perfusion, immune function, protein synthesis and wound healing (205), (206).Arginine supplementation between three to eight grams per day is very well tolerated but above nine milligrams causes patients to have side effects including diarrhoea and vomiting. There have been several meta analyses and reviews on this subject of arginine replacement in critically ill patients as part of their immune nutrition (207),(208). The results have been conflicting and may be compounded by the fact the diets are mixed with other compounds such as Omega 3 which will have an impact on the outcome.

NO is formed by the oxidation of sole substrate L-arginine, a semi essential amino acid by the enzyme Nitric oxide synthetase (NOS) (140). NO has been found be the main modulator of intra hepatic vascular tone (16) Since then a growing number of studies have proven that there is presence of endothelial dysfunction and low NO levels in cirrhosis (25) and that liver sinusoidal endothelial cells modulate NO levels (209).Therefore, it has been reasonable strategy to use vasodilators and NO donors to improve the intra hepatic

tone. However, most of them have not been accepted in routine clinical practice because of the systemic hypotensive effect.

L-Arginine plays an important role in the modulation of intrahepatic tone by supplying NO, which acts as the most important vasodilator in the intra hepatic circulation. It has been proven that the increased intra hepatic vascular tone induced by L-NAME, a non-specific NO inhibitor is reversed when L-arginine is supplemented (16). In cirrhosis and various other diseased states, arginine levels have been found to be high or low ((210), (211) and therefore supplementation of L-arginine have been reported with contradictory outcomes ((212), (213)

L-Arginine has several metabolic pathways which may have an impact on the availability of substrate for NO synthesis (119).Among them Asymmetrical Dimethyl Arginine, (ADMA), an endogenous inhibitor of endothelial nitric oxide synthetase (147) and Arginase (214) which is an enzyme which converts L-arginine to urea and ornithine as part of the urea cycle has been mostly implicated in the regulation of L-arginine. We have previously shown in a devascularised porcine model of acute liver failure there is increased plasma arginase activity and ADMA levels. However, the arginine deficiency in this model was related to arginase related arginine clearance inspite of de novo arginine production (215). Therefore, in order to overcome the relative arginine deficiency because of increased ADMA levels and arginase activity in diseased states including cirrhosis, it was rational to supplement L-arginine exogenously to assess its effect on portal hypertension.

The aim of this study is to assess whether supplementation of L-arginine in the bile duct ligated rats lower their portal pressure. The secondary aim would be to investigation the underlying pathway involved in this process and whether there was an anti-inflammatory effect.

6.2 Methods and Materials

All animal experiments were conducted according to the Home Office guidelines under the UK Animals in Scientific Procedures Act 1986. This study was performed in male Sprague-Dawley (SD) rats (Charles-rivers), weighing 220–250g, were obtained from the Comparative Biological Unit at University College London. All rats were housed in the respective unit and given free access (*ad libitum*) to standard rodent chow and water, with a light/dark cycle of 12 hours (the dark phase extended from 1900–0700 hours), at a temperature of 22–23°C and humidity of approximately 50%.

The rats underwent Bile duct ligation and sham operation as described in section 2.1.1 and 2.1.2 respectively.

6.2.1 Study design:

Male Sprague Dawley rats weighing 220-250 g were randomly assigned to receive sham or bile duct ligation (BDL) surgery as described previously. Four weeks later hemodynamic measurements were performed and samples collected for analysis. For this arginine supplementation studies, treatment with either 30gm/kg L-Arginine (Damor

Pharma, Italy) dissolved in sterile water or saline (control, equal volume) by oral gavage was given on the last 7 days of the study.

Rats were randomly assigned to the following treatment groups:

1.Sham + N/Saline group: n = 8

2.Sham+ L-Arginine group: n = 11

3.BDL + N/Saline group: n =12

4.BDL+ L-Arginine group: n = 13

All rats were exsanguinated under terminal anaesthesia (2% isofluorane). Within seconds blood was withdrawn from the descending aorta and immediately put into ice cold heparin/EDTA containing tubes (until full exsanguination), centrifuged at 3000 rpm and 4ºC for 10 min, and the plasma collected and stored at -80°C until assayed. Liver tissue was also removed immediately harvested and snap frozen for storage at -80°C until analyzed. Liver tissues were also fixed with 10% formalin for further histological analysis.

6.2.2 Haemodynamic measurement

All haemodynamic measurements were undertaken exactly as described previously in section 2.3.

6.2.3 Biochemical Parameters

All biochemical parameters (ALT, AST, albumin, bilirubin,) were analyzed using 200µl of respective plasma samples using a Cobas Integra 400 multianalyzer with the appropriate kits (Roche-diagnostics, Burgess Hill, West Sussex, UK).

6.2.4 Plasma and tissue Cytokine Assays

All cytokine assays were undertaken in a similar method as described previously in section 2.6.

6.2.5 Western Blot

Proteins were isolated from snap frozen liver tissue using standard techniques as described in section 2.7. Equal amounts of protein extract were denatured and separated on 4-12% NuPAGE Bis-Tris Gels and transferred on to PVDF membranes (Lifetech, UK), The membranes were then incubated with different primary antibodies including a goat anti DDAH 1 (1:1,000; in-house preparation), mouse anti-ADMA (1:1,000; Acris Antibodies, Herford, Germany), mouse anti-eNOS (1:1000, Cell Signaling Technology, Beverly, MA), mouse anti NFkB p65 (1:1000, dilution, 2A12A7,Invitrogen,UK). The bands were visualized using an enhanced chemiluminescence detection kit and quantified by densitometry. Loading accuracy was evaluated via membrane rehybridization with antibodies against mouse and rabbit anti-α tubulin (1:1,000; Upstate Biotechnology, Albany, NY).

6.3 Results

6.3.1 Biochemical parameters

	Sham (Mean±SEM)	BDL (Mean±SEM)	BDL + Arg (Mean±SEM)	Significance *p<0.01, **p<0.001
ALT	42.08±3.49	118.1±15.47**	81.83±5.54*	** Sham v BDL *BDL v BDL+Arg
AST	76.76±9.45	500.7±81.58**	492.4±39.02	** Sham v BDL
Bilirubin	1.75±1.077	118.3±14.61**	117.8±6.43	** Sham v BDL
Albumin	32.93±3.10	21.59±1.27**	20.52±1.18	** Sham v BDL

Table 6.1: Plasma levels of ALT, AST, Bilirubin andAlbuminin the various groups. (p<0.001, *p<0.01)**

The results of the biochemical analysis shows that apart from a significant reduction in the serum Alanine Amino Transferase level in the rats treated with L-arginine treated BDL rats, there was no significant difference between the groups.

6.3.2 Effect of L-Arginine supplementation on the Mean Arterial Pressure

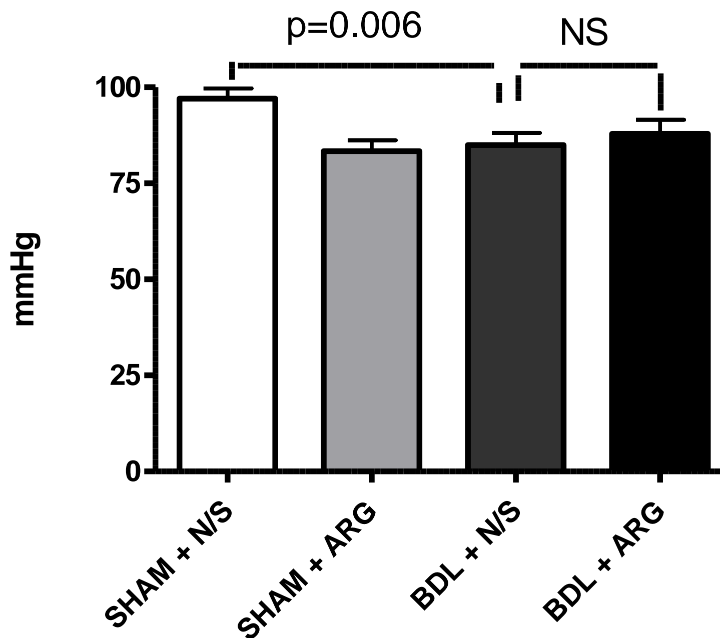


Fig 6.1: Mean Arterial Pressure in the four Groups of Animals. This shows that the Mean arterial pressure is significantly reduced in the BDL rats as compared to the sham rats (p=0.006).There was no change in the MAP after treatment of the BLD ratswithL-Arginine.(SHAM+N/S=14,SHAM+ARG=9,BDL+N/S=14, BDL+ARG=13)

The results show that there was a significant reduction in the mean arterial pressure in the BDL rats treated with L-Arginine as compared to the sham operated rats (84.96±2.87 v 97.03±2.67, p=0.006). However there was no significant difference between BDL rats treated with saline as compared to the BDL rats treated with Arginine.

6.3.3 Effect of L-Arginine supplementation on the portal pressure

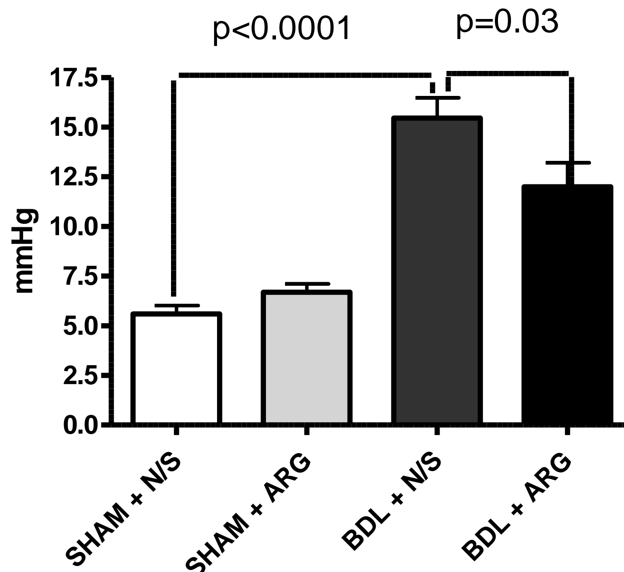


Fig 6.2: Portal Pressure in the four groups of animals. This shows that the portal pressure is significantly raised in the BDL rats as compared to the sham operated ones (p<0.001).There was reduction in the portal pressure in the BDL rats treated with arginine as compared to BDL rats treated with saline (p=0.03). (SHAM+N/S=14, SHAM+ARG=9, BDL+N/S=14, BDL+ARG=13)

The portal pressure was measured on the day of the sacrifice after seven days of Arginine or equivalent amount of Normal Saline in sham and BDL rats. The results show that there was no significant difference between the sham animals treated with L-arginine or normal saline (6.6 ± 0.4 vs. 7.4 ± 0.7 p = 0.4). There was a significant increase in the portal pressure in the BDL rats as compared to Sham operated rats treated with saline (5.59 ± 0.42 vs 15.25 ± 1.02, p <0.0001) However, treatment of the BDL rats with L-arginine significantly lowered the portal pressure as compared to the BDL rats treated with normal saline (12 ±1.2 vs. 15.8 ± 1.2, P<0.05).

6.3.4 Endothelial NOS protein expression:

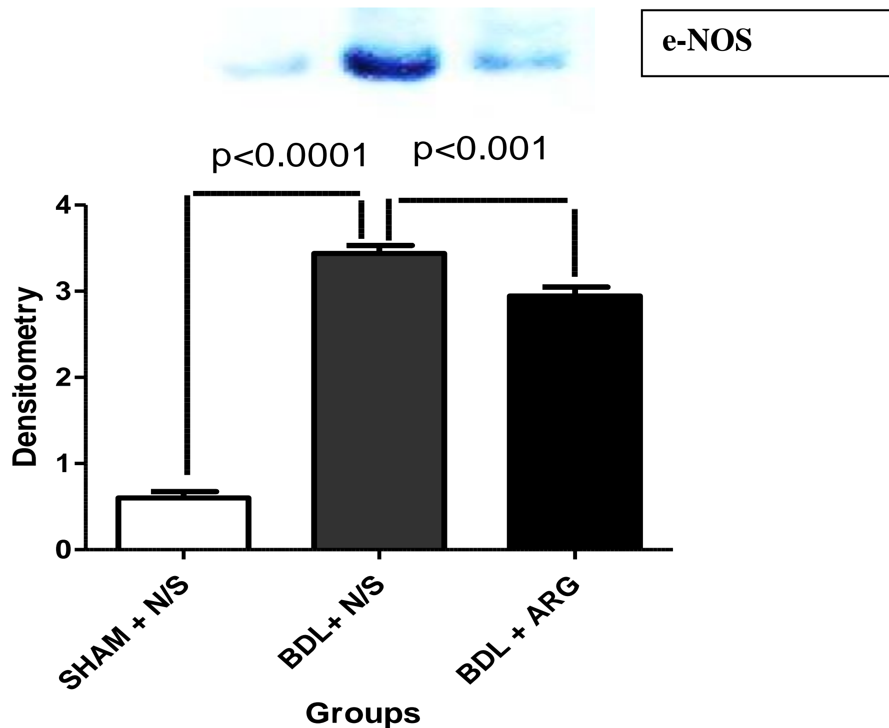


Fig 6.3: eNOS protein expression in the three groups. This shows that the eNOS protein expression is significantly raised in the BDL rats as compared to the sham operated ones (p<0.001).There was reduction in the eNOS protein expression in the BDL rats treated with arginine as compared to BDL rats treated with saline (p<0.001). (SHAM+N/S=5, BDL+N/S=6, BDL+ARG=6)

Western blotting technique was used for the protein expression of eNOS in the various groups of animals. The results show that that there is a significant increase in the eNOS protein expression in the BDL group (p<0.0001) as compared to the sham group and it significantly goes down on treatment with L-arginine ((p<0.001) in the BDL group as compared to the BDL group treated with saline.

6.3.5 Endothelial NOS protein activity

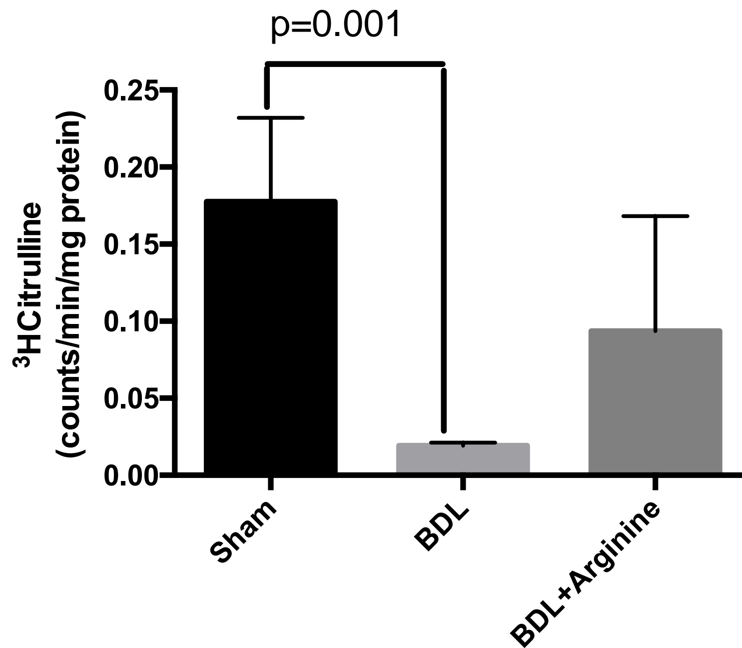


Fig 6.4: eNOS protein activity in all the three groups (detected by the radioactive method). This shows that there is significant reduction in the eNOS activity in the BDL rats as compared to the sham rats (p=0.001). There was a trend towards increase in the eNOS activity in the BDL rats treated with L-arginine as compared to BDL rats treated with saline. (SHAM+N/S=5, BDL+N/S=6, BDL+ARG=5)

The eNOS activity was measured by the radiometric analysis of hepatic eNOS activity as described in section 2.9. This shows that the BDL group has significant reduction in eNOS activity as compared to the sham. However upon treatment with L-arginine, the BDL rats will regain some of the activity of the enzyme, although this is not statistical significant. This was calculated by the radioactive method of conversion from citrulline to arginine as described in section 2.9.

6.3.6 Protein expression for ADMA

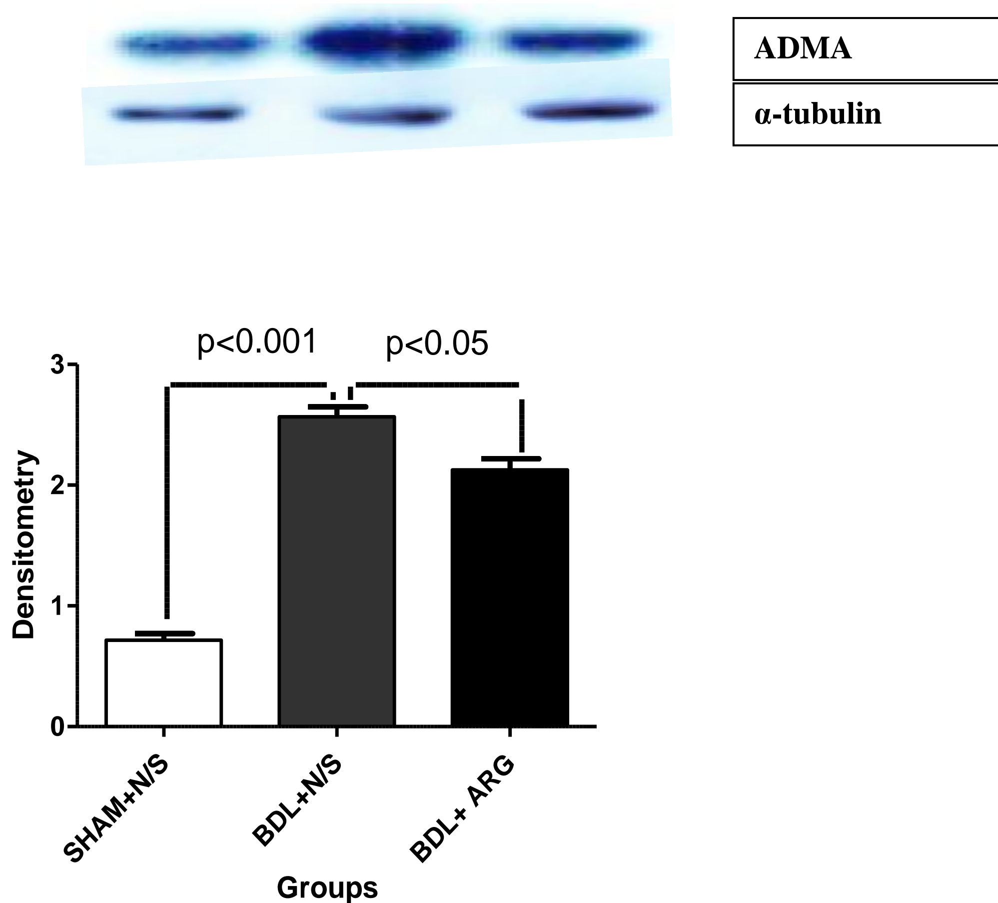


Fig 6.5: ADMA protein expression in all the three groups. The ADMA protein expression goes up significantly in the BDL rats as compared to the sham operated animal. This expression was significantly reduced in the BDL rats treated with L-arginine. (Sham+N/S=5, , BDL+N/S=6, BDL+ARG=6)

Western blot technique was used to elicit the protein expression of ADMA. The results show that there is a significant increase in the protein expression of ADMA in the BDL group ($p<0.001$) as compared to the sham group. However, on treatment with L-Arginine in the BDL group there is a significant reduction of the expression of ADMA as compared to BDL rats treated with saline ($p<0.05$).

6.3.7 Protein expression of DDAH1:

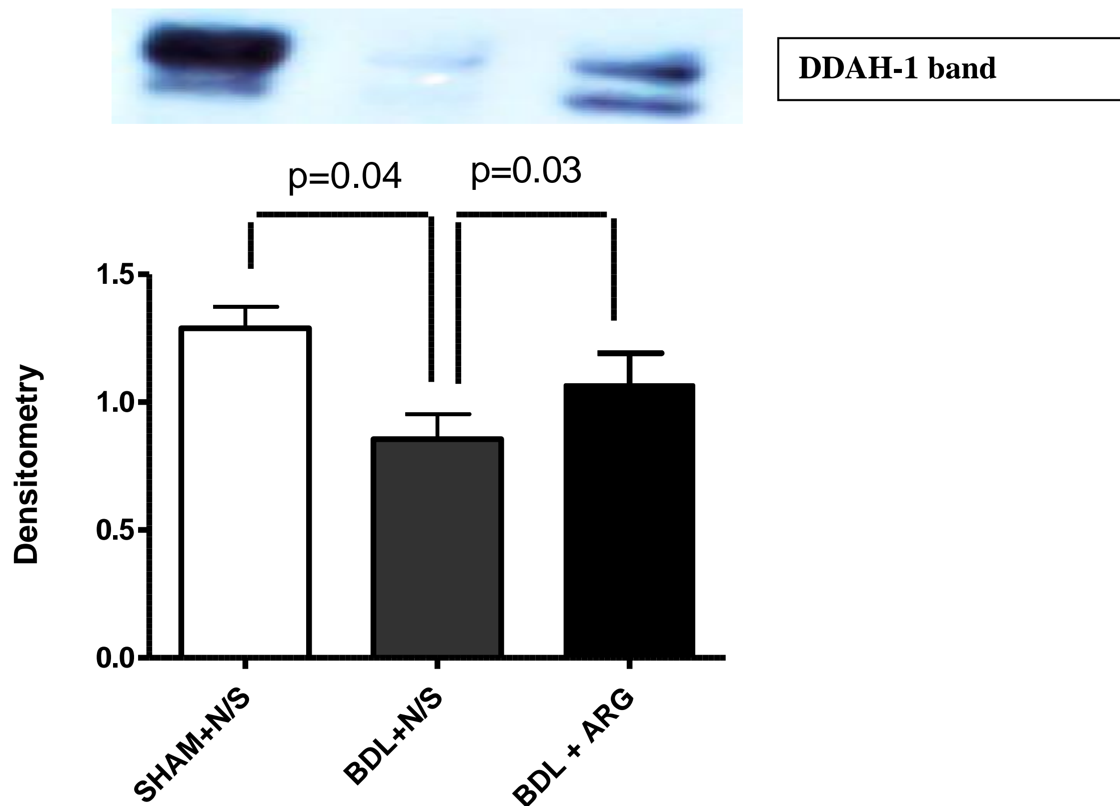


Fig 6.6: DDAH protein expression in all the groups. The DDAH1 protein expression is reduced in the BDL ratsa s compared to the sham operated rats. However, this expression is significantly increased in the BDL rats treated with arginine as compared to the ones treated with placebo. (SHAM+N/S=5,BDL+N/S=6, BDL+ARG=6)

The results show that there is a significant decrease in the DDAH1 protein expression in the BDL group as compared to the Sham group treated with saline (p=0.04) and on treatment with L-Arginine, the protein expression increases significantly in the BDL rats as compared to the BDL rats treated with saline(p=0.03).

6.3.8 Protein expression of Nuclear Factor kappa B (NFkB):

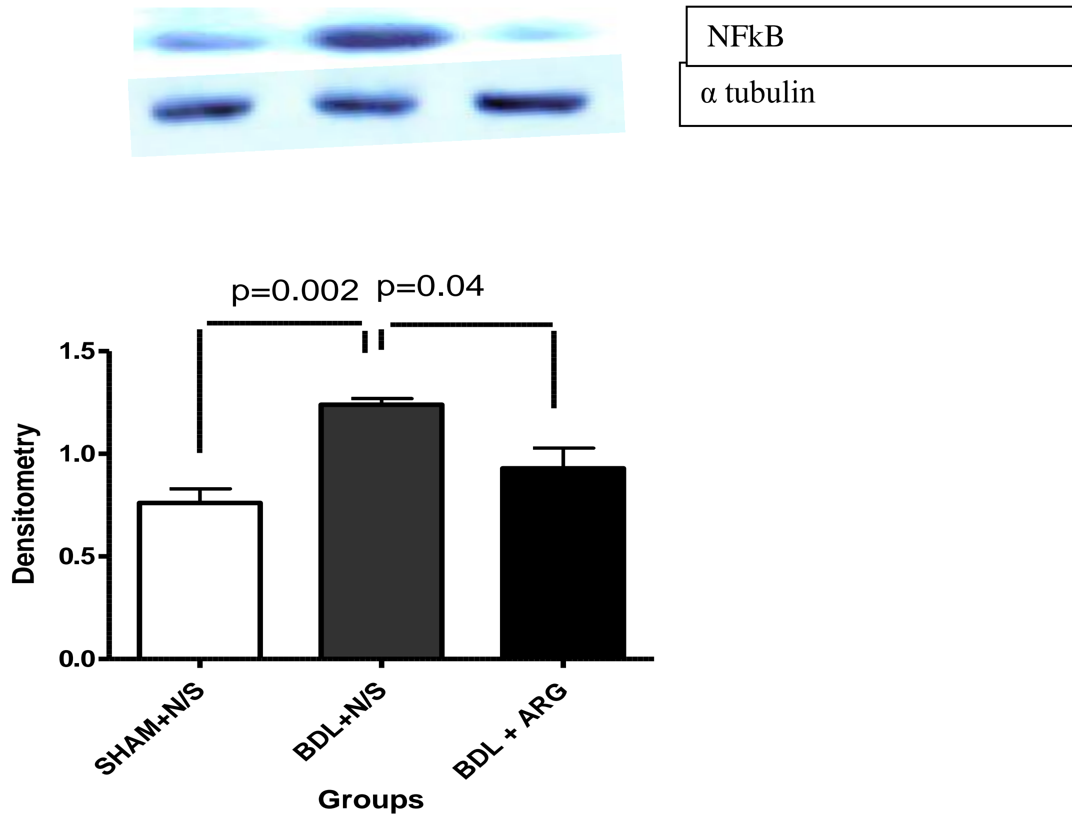


Fig 6.7: NFkB protein expression in all the three groups. The NFkB protein expression goes up in the BDL rats as compared to the Sham operated animal. This was however decreased upon treatment of the BDL rats with L-arginine as compared to the BDL rats treated with placebo. (SHAM+N/S=5,BDL+N/S=6, BDL+ARG=6)

The result shows that there is a significant increase in the protein expression of the NFkB protein in the BDL group as compared to the Sham group treated with saline($p<0.002$). On treatment with L-Arginine, there is a significant reduction in the protein expression of NFkB in the BDL rats as compared to the BDL animals treated with normal saline ($p=0.04$).

6.3.9 Tumour Necrosis Factor alpha (TNFα) cytokine levels

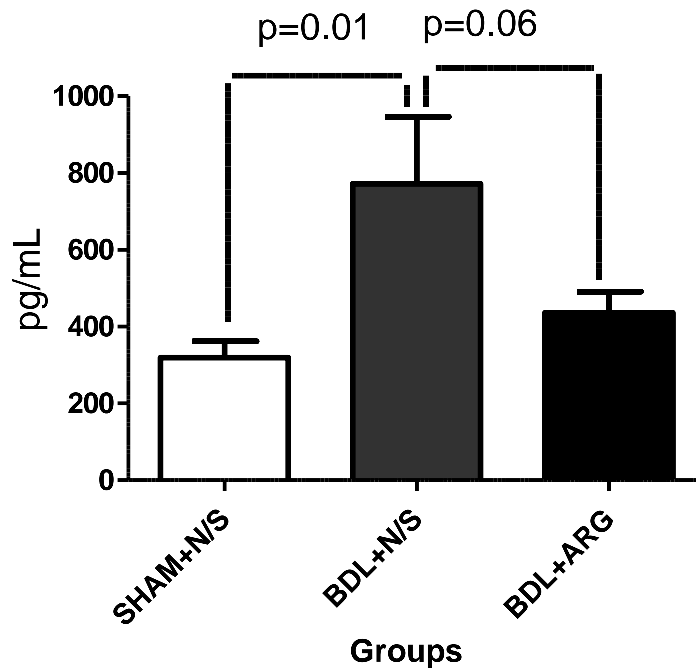


Fig 6.8: TNFα levels in the hepatic tissue in the various groups. The plasma TNFα level is increased significantly in the BDL rats as compared to the sham operated animals however this effect is abrogated by the treatment of the BDL rats with L-arginine as compared to the placebo treated BDL rats. (Sham+N/S=5, , BDL+N/S=6, BDL+ARG=6)

TNFα titre was measured by the BD bioscience Cytometric Bead Assay Kit and analysed in the FACS Canto II machine. The results show that there is a significant increase in the TNFα level in the BDL rats as compared with the Sham rats treated with saline (p=0.01). However the levels of TNFα are reduced in the BDL rats treated with L-Arginine as compared to the BDL rats treated with normal saline (p=0.06).

6.3.10 Liver tissue Arginase Activity:

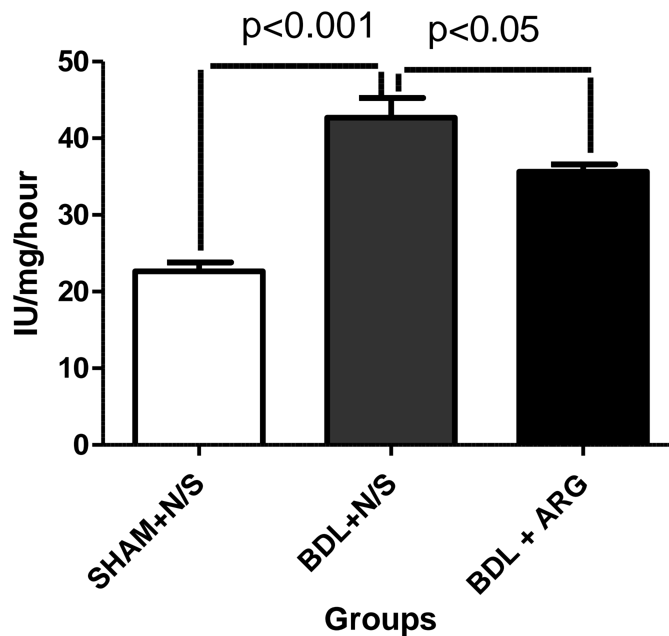


Fig 6.9: Liver tissue arginase activity in all the group. This shows that the arginase activity is significantly increased in the BDL rats as compared to the sham operated rats. This was significantly reduced in the BDL rats treated with arginine as compared to the BDL rats treated with placebo. (Sham+N/S=5,BDL+N/S=8, BDL+ARG=8)

The arginase activity was measured as per section 2.20. The above results show that the liver arginase activity is significantly increased in the BDL rats as compared with the sham operated rats treated with saline ($p<0.001$) and the arginase activity in the liver is decreased significantly in the BDL rats treated with arginine as compared to the BDL rats treated with saline ($p<0.05$).

6.3.11 Plasma Arginine to ADMA ratio:

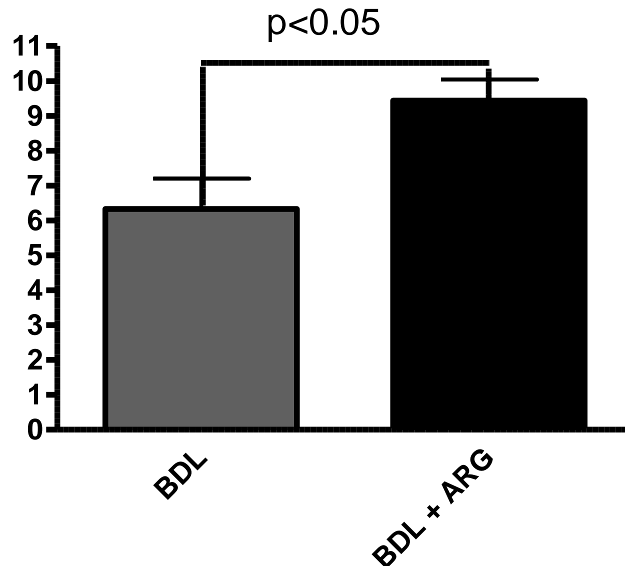


Fig 6.10: Arginine to ADMA ratio between BDL and BDL animals treated with L-Arginine animals. This shows that the ratio is increased in the BDL rats treated with L-arginine as compared to the BDL rats treated with saline ((p<0.05).

The results show that the ratio of Arginine to ADMA is significantly increased in the BDL rats treated with L-Arginine as compared to the BDL rats treated with normal saline(p<0.05). The Arginine to ADMA ratio has been implicated in the synthesis of NO as a reduction in this ratio implies that there is relative increase in the ADMA level which inhibits eNOS and thereby reduces NO production. However on supplementation with L-Arginine, there is an improvement in this ratio implying increase in the forward reaction for NO synthesis.

6.4 Discussion

This result of this study reveals several important observations. Firstly, the results of this study show a significant reduction of portal pressure in the BDL rats treated with L-Arginine as compared to the BDL rats treated with normal saline. This drop in portal pressure is noted in the context of the MAP without any significant difference between the BDL rats treated with L-arginine as compared to the BDL rats treated with saline. Secondly, the results of the protein expression studies reveal the involvement of the ADMA-DDAH pathway in the modulation of portal hypertension. Thirdly, the results of the biochemistry and the cytokine analysis, together with the NFkB protein expression show the involvement of the inflammatory pathway in the modulation of portal hypertension.

Ever since the seminal paper by Mittal and Groszmann about the presence of a reversible component in the pathophysiology of portal hypertension in 1994, there has been a growing body of evidence about the various mechanistic pathways involved in the modulation of portal hypertension {Mittal, 1994 #1892. The focus of these strategies is to reduce intra hepatic resistance by modulating the various vasoactive agents but as there is no specific tools to assess it, portal pressure and portal blood flow can be taken as a surrogate indicators for the change in intra hepatic resistance.

It is clear from the literature that reduction of portal pressure to below 12 mmHg or 20% below the basal reading has shown to have clear benefit in patients outcome by reducing the risk of first bleeding episode to less than 10%. Most of the strategies applied earlier have been to reduce the increased splanchnic inflow of blood to the liver including beta

blockers but recent data emerging suggest that this strategy may not be beneficial to patients in end stages of their disease process (10). Endothelial dysfunction has been the main feature of the pathobiology of intrahepatic resistance and reduction in the intra hepatic NO has been implicated in this process. In this study, I have used L-arginine as a treatment for portal hypertension. L-Arginine is the sole substrate for the production of NO with the help from the enzyme eNOS (140). L-Arginine metabolism is highly regulated and various pathways are compartmentalised (119). The rationale for using exogenous L-Arginine supplementation is to provide adequate substrate for eNOS to synthesis NO and reduce the intra hepatic resistance. Plasma concentration of L-arginine has been shown to have contradictory levels in various studies in humans and animal studies ((210), (216)). This suggests that L-Arginine supplementation has other important impact in the modulation of intrahepatic resistance. The finding from this study of reduction in portal pressure in the L-Arginine treated BDL rats by 18% from the BDL rats treated with normal saline is an important one. This finding is complemented by the finding that there is no difference between the two groups in terms of mean arterial pressure. Many of the drugs that have been tested until now to reduce portal pressure in animal models and clinical trial settings have not translated into routine clinical practice because of concerns with systemic hypotension (217), (218). Although, there is a risk of systemic hypotension with L-arginine supplementation, the studies done previously showed a significant reduction in MAP within the first thirty minutes of an intravenous bolus dose along with a peak plasma level of arginine within the same time. Whereas, after an oral dose, the peak plasma levels were reached at ninety minutes and the

bioavailability was around 70%, hence showed a less acute change in systemic haemodynamics (167)

Endothelial Nitric Oxide Synthetase (eNOS) is the main enzyme involved in the oxidation of L-Arginine to produce NO and citrulline. In this study, I show that the protein expression of eNOS is increased in the BDL rats but is reduced upon treatment with L-arginine. The enzyme eNOS requires cofactors like calcium, calmodulin and tetrahydrobiopterin (BH4) as necessary ingredients to function adequately. Therefore the finding of an increased protein expression of eNOS with a loss of activity in the BDL is not surprising as it suggest that the activity is lost due to a post translational modification. Hepatic eNOS activity regulation is multifactorial and literature suggest that impaired akt mediated phosphorylation and increased expression of caveolin I are implicated (28).The modification that has been shown to be most common is the reduction in the level of BH4 which is reduced by increased oxidative stress that is known to be present in the diseased liver (219).Recently it has been shown that supplementation of BH4 helps to reduce portal pressure by increasing the eNOS activity and thereby increasing NO production (220).

Asymmetrical Dimethyl Arginine is an endogenous inhibitor of eNOS. In normal physiological condition, there is a fine balance in the regulation of NO synthesis as it has about 10% inhibition effect on eNOS. However in the context of vascular injury which is akin to endothelial dysfunction, there is a significant rise in the ADMA levels, which in turn inhibit the eNOS mediated vasorelaxation (221). However, it has already been shown to have an increased level of ADMA in both patients (222)and animal models of cirrhosis(32)and act as marker for poor outcome (78).Elevated ADMA levels inhibit

eNOS and disturbs the ratio between the arginine and ADMA. In this study, I have shown that the ADMA protein expression is significantly increased in the bile duct legated rat but on treatment with L-Arginine, the protein expression is significantly reduced. The reason for the raised level of ADMA in the BDL rat may be multi factorial. ADMA is metabolised by Dimethylarginine Dimethylaminohydrolase (DDAH) in the intra-cellular compartment. In this study, I show that the DDAH protein expression is reduced in the BDL rat liver and goes up with treatment with L-Arginine. The reduced DDAH may play a significant part in its failure to metabolise ADMA in the BDL rats. There is a suggestion from the literature that the regulation of DDAH and L-Arginine may be intricately linked by a negative feedback mechanism and this mechanism may regulate the fine balance between the arginine and ADMA levels (223)

In this study, I have conclusive proved that the inflammatory indices including serum ALT levels, TNFα pro-inflammatory cytokine and NFkB protein expression are all significantly increased in the BDL rats and goes down after L-arginine supplementation. This is further proof of earlier experiments wherein TNFα incubated with cultured endothelial cells showed a reduction in DDAH activity and increased in ADMA (224) and in vivo experiments done in our laboratory previously which showed that using an anti-TNFα agent in a BDL rat decreased the portal pressure and ADMA protein expression in the liver (225). These results could also imply that L-Arginine might have an anti-inflammatory property.

Another important metabolic pathway of L-arginine is via the arginase pathway. In this study, I show that the arginase activity is increased in the BDL rats and goes down

significantly with treatment. There are multiple mechanisms that are involved in the modulation of vascular reactivity. The most important pathway for L-Arginine metabolism is via arginase and arginase also competes with eNOS for substrate although L-arginine has a natural preference of eNOS. Early studies on activated macrophages reveal that majority of L-arginine is consumed by arginase to produce urea and L-ornithine. The NO bioavailability is only increased by either supplementing L-Arginine or inhibiting arginase (130). Depletion of substrate L-Arginine for NO production leads to uncoupling of the eNOS leading to free radical formation, which in turn inactivates NO, thereby indicating that increase arginase activity indirectly leads to decreased NO bioavailability (136) Another alternative mechanism in which arginase works is by sensitising ADMA to eNOS either directly (135) or through the presence of inflammatory cytokines (226).

The effect of an elevated ADMA level signifies an alteration in the arginine to ADMA ratio. In a recent study using a rat model where rats were randomised to receive normal saline as control, ADMA or a combination of arginase and ADMA as infusion shows that the group with the low arginine level with high ADMA had the most significant haemodynamic deterioration (227). In a previous study done from our group, it has been shown that in the presence of inflammation in cirrhosis, there is an increase in the ADMA levels and is associated with worse outcome (78). These result suggest that there is an alteration in the in the physiological balance between Arginine to ADMA which causes a reduction in NO synthesis and consequent haemodynamic abnormalities. The effect of this is found on the transporter system present in the transmembranous region. The cationic acid transporters are protein which transport the arginine into the intracellular

compartment also transport ADMA. When there is an increase in the level of ADMA, though it may be disproportionally small increase, it alters the transport mechanism and facilitates the transport of ADMA to the intra cellular compartment instead of arginine. Therefore, there is a relative deficiency of arginine in the intra cellular space, which in turn leads to reduced production of NO. The phenomenon of altered transport of Arginine and ADMA is known as the arginine paradox. Although there is apparently abundant pool of arginine available, since it is all compartmentalised, not enough arginine may be available for the production of NO. It is for this rationale that exogenous replacement may play a part in the regulation of intra cellular arginine levels and therefore has been used in our experiment.

In conclusion, the results presented in this section of the thesis suggest that exogenous supplementation of L-arginine may be an important alternative therapeutic strategy for the treatment of portal hypertension. This has a significant effect on the inflammation that prevails in the bile duct ligated model and improvement in the endothelial dysfunction. I have been able to prove my hypothesis that L-arginine supplementation can be used an anti-inflammatory strategy for treatment of portal hypertension.

Chapter 7

Summary and Future Directions

The modulation of inflammation in the reduction of portal hypertension

7.1 Summary

The onset of portal hypertension is an important clinical juncture for a patient with cirrhosis. This implies that the patient will be at risk of progression to severe and often fatal complications like ascites, spontaneous bacterial peritonitis, variceal bleeding and hepatic encephalopathy. Their mortality and morbidity with these complications goes up significantly. It has become clear that a decrease in portal pressure is not only protective against the risk of variceal (re)bleeding but is also associated with a lower long-term risk of developing complications and an improved long-term survival. Despite the high mortality associated with portal hypertension, there are a limited number of treatment options and a lack of consensus amongst physicians on their use. Treatment options for portal hypertension remain limited to non selective beta blocker (NSBB). These achieve a target reduction of > 20% reduction in hepatic venous pressure gradient (HVPG) in only 40% of patients. About 20% of patients cannot tolerate the treatment because of easy fatigability and hypotension. Many other drugs have been able to show reduction in portal pressure in the research setting. However, many of these successes have not translated to their use in routine clinical settings. This may be because of the inability of overcoming the dichotomy of vasoconstriction in the intrahepatic circulation and vasodilatation in the splanchnic and systemic circulation.

Inflammation has been highlighted recently to play a major role in the propagation of portal hypertension by increasing intra hepatic resistance. This is mainly by inducing endothelial dysfunction. Anti-inflammatory strategies in portal hypertension have shown improvement in endothelial function and reduction in portal hypertension (228),(71). However, the exact mechanisms of these beneficial effects have not been elucidated in most of these strategies. There is therefore a significant clinical need to understand the impact of inflammatory mechanism on portal hypertension and the benefit of their modulation as a therapeutic target.

The series of studies done as part of this thesis is an attempt to modulate inflammation as a strategy to reduce intra hepatic resistance and thereby portal hypertension. In the first section of the thesis in Chapter 3, I set out to validate the rodent model of bile duct ligated rat as a model suitable for study in this project. Bile duct ligated rats have been used in various studies on portal hypertension and cirrhosis. However, there is heterogeneity in the establishment of the model with some studies suggesting that there is evidence of portal hypertension from end of week 2 post bile duct ligation whereas other studies have shown that portal hypertension is fully established at the end of four weeks.

In my study, this model provides characteristic features of secondary biliary cirrhosis induced by the bile duct ligation at the end of four weeks. This induces a significant architectural change in the liver with evidence of cholangiocyte proliferation and formation of septa, which could be translated to nodule formation in a cirrhotic human liver. Biochemically they show a low albumin and a high bilirubin as compared to the sham operated rat. The aminotransferase enzymes are raised non significantly in the BDL

rats as compared to the sham operated rats. The cytokine profile shows that there is a significant increase in the level of TNFα in the BDL rats as compared to sham operated. However, on injection of Lipopolysaccharide to the BDL rats, there is a further significant increase in the cytokine (TNFα) as well as increase in the aminotransferase levels. This indicates that the second hit with LPS injection provokes a more profound inflammatory reaction in the BDL rats. This is akin to the recently described concept of Acute on Chronic Liver Failure where a superadded infection or inflammatory insult provokes an exaggerated Systemic Inflammatory Response

There was a significant difference in the haemodynamic features between the sham and the BDL rats with the BDL rat having lower mean arterial pressure and higher portal pressure as compared to the sham operated animal. The heterogeneity of the model in our laboratory was less than 10%. These include the failed BDL rats and also variation in the haemodynamic measurements. With all these generated data, it was evident that this model would be a good model to undertake the proposed research project.

Inflammation has been recently recognized as an important precipitant of acute on chronic liver failure. It is associated with significantly increased early mortality of patients. It is a well-established fact that the presence of inflammation in cirrhotic patients is associated with high portal pressure. Recently, we have shown that the presence of inflammation in patients with cirrhosis is directly related to the levels of sympathetic activation in these patients (229). Given the acknowledged reduction in beta-receptors with cirrhosis and evidence from the sepsis literature that suggests the ADRA2a may be a target for reduction of inflammation and liver dysfunction, this lent itself to further study as of

potential mechanistic importance in portal hypertension. In my studies, I used the BDL rat as the portal hypertension model which also demonstrates an increase in inflammatory markers.

In chapter 4, I demonstrated that there was a significant increase in the ADRA2a receptor (mRNA and protein) in the BDL rats as compared to the Sham operated animals. By the use of a specific ADRA2a receptor antagonist, I was able to reduce portal hypertension by a clinically significant level. I also showed an increase in the cyclicAMP level and the endothelial NOS activity. These provide evidence of a link between increased ADRA2a and the development of endothelial dysfunction. In further experiments involving hepatic stellate cells, and following their challenge with Guanfacine, an ADRA2a adrenergic receptor agonist), there was a significant increase in the contractility of the HSC. This implied that antagonism of ADRA2a causes relaxation of the HSC, thereby promoting a reduction in intra hepatic resistance. This set of experiments provided a proof of concept that there was an alternative pathway for the treatment of portal hypertension other than targeting the beta adrenergic preceptor.

The gut-liver axis has been well established as the main pathway for the propagation of inflammation by way of increased gut bacterial translocation. These bacterial products are carried by the circulating immune cells to the liver where in inflammatory cytokines are produced to affect changes to tissue resident macrophages (Kupffer cells), which in turn leads to increased resistance vessels contraction and intra hepatic resistance.

In the next set of experimentation in Chapter 5, I studied immune cells and their function in the setting of a BDL rat treated with or without LPS, thereby mimicking the clinical

picture of ACLF. I was able to demonstrate that there is an increase in the population of neutrophils and monocytes within the liver in the BDL rats treated with or without LPS as compared to sham operated rats. But more importantly, there is a significant improvement of functional capacity of the neutrophils and monocyte as evidenced by the improved phagocytosis and reduced Reactive Oxygen Species generated in the BDL rats treated with an ADRA2a antagonist. These findings are complemented by reduction in the inflammatory cytokine levels and significant reduction in portal pressure in the BDL rats treated with saline or LPS. This supports the contention that targeting the ADRA2a adrenergic receptor may be a valid target for the treatment of portal hypertension , especially in the context of added inflammation.

Another area of interest is the impact of endothelial dysfunction in the pathogenesis of portal hypertension. In physiological conditions, the intact endothelium acts a natural barrier to the underlying vascular tissue and helps to maintain vascular equilibrium. The term endothelial dysfunction refers to impairment of the various functions of the endothelium, the most important amongst them being impaired release of vaso-relaxant like Nitric Oxide. L-arginine , an amino acid, is an essential substrate for the enzyme endothelial nitric oxide synthetase to produce nitric oxide. However, in the context of inflammation in liver cirrhosis, there is evidence of endothelial dysfunction leading to reduced intra hepatic nitric oxide and increased intra hepatic resistance. We have previously described the decrease in total body nitric oxide level in a devascularised porcine model and this may have been contributed by increase production of plasma arginase and ADMA levels.

In the second section of my thesis in Chapter 6, my focus was in the importance of modulating Nitric oxide in maintaining intra hepatic vascular tone, in the context of inflammation. L-Arginine is the sole substrate needed for the production of nitric oxide. However, it is well established in the literature that there are multiple factors which alter the availability of L-Arginine for the production of NO. The two most important factors that have gained importance is that of increased ADMA levels and a more efficient arginase metabolic pathway. Both of these factors have been shown to be most activated in a pro-inflammatory environment and it is already known that l-Arginine has significant anti-inflammatory properties.

Therefore, in this section, I undertook a study of supplementation of L-Arginine in the BDL rats and explored whether this reduced portal hypertension and the potential mechanisms involved. Through this study , I was able to demonstrate that the portal pressure is significantly reduced in the BDL rats treated with L-Arginine as compared to the BDL rats treated with saline. This was not associated with any significant change in the MAP in the two groups. Moreover, there was also a significant drop in the inflammatory markers in the BDL rats treated with L-Arginine as compared to the BDL rats treated with saline. There was also increased eNOS activity in the arginine treated group in the context of decreased liver ADMA demonstrating the proof of concept that supplementation of L-arginine reverses the Arginine:ADMA ratio to a favourable one, and thereby reduces portal pressure in these rats.

Therefore, in conclusion, the studies described in this thesis confirm a clear association between inflammation and vascular dysfunction and more importantly increased portal

pressure. Furthermore, it is established that inflammation is a key factor in the propagation of portal hypertension. This leads to increased production of pro-inflammatory cytokines and significant reduction in eNOS activity. However with these two different anti-inflammatory strategies, I have been able to show significant improvement of hepatic haemodynamics and eNOS activity indicating improvement in endothelial function. Based on the results presented in this thesis, both L-arginine supplementation and antagonism of ADRA2a provide potential new targets to treat portal hypertension.

7.2 Limitations of the studies:

7.2.1. ADRA2a study limitations:

1). It should be acknowledged that although the data presented in this thesis strongly suggest that an anti-inflammatory pathway is definitely implicated in the BDL rats treated with a ADRA2a adrenergic receptor antagonist, there are other adrenergic receptors including the adrenergic receptor 2 subtypes B and C present in the systemic and portal vasculature. . Especially the ADRA2b have opposing physiological vasomotor effect which have not been studied in detail in these experiments. There is a potential that ADRA2a antagonism may have a significant unintended effect on the ADRA2b receptor which has not been accounted for in these studies. ADRA1 is another important receptor which has been antagonized in portal hypertension (230). ADRa2a antagonism increases the level of noradrenaline which will have a potential unintended effect on the ADRA1 receptor which has not been studied as part of this thesis.

Moreover, as part of this thesis, I have demonstrated that there is presence of ADRA2a receptor in the Hepatic stellate cells and endothelial cells. However, there is some indication of modulation of inflammation by ADRA2a antagonism via the migrating immune cells. The exact contribution of each of these cell types have not been studied in detail as part of this thesis.

2). The second most challenging aspect was the technical limitations of reliable ADRA2a adrenergic receptor antibodies from which western blots and Immunohistochemistry analysis could be undertaken. Therefore, these had to be done multiple times in different test conditions and with different suppliers, in order to get an interpretable result. Ultimately, the most reproducible analysis was based on quantitative Polymerase Chain Reaction analysis.

3). There were also a few logistical challenges in having to do several ex vivo experiments including haemodynamic experiments and cell separation assays simultaneously. However, I was fortunate enough to be able to avail the help offered by my co -research fellow colleagues in helping out at various stages of these studies.

7.2.2. L-Arginine study limitations:

1). The biggest limitation in this study was to work out the optimum method for Nitric oxide generation. I have attempted to do the Nox method to quantify generation of Nitric oxide. However, the results were very difficult to replicate. The eNOS activity assay also depends on experimental condition and the integrity of the tissue. The radioactive isotope

method is difficult to access and also operator dependent and therefore, difficult to generate reproducible results.

2). Although, I have demonstrated that exogenous supplementation of L-Arginine alters the Arginine:ADMA ratio, the role of the cationic amino acid transporters have not been explored in this study. It is well established that a number of carrier proteins for amino acids have been identified on the molecular level. They belong to different gene families, exhibit overlapping but distinctive substrate specificities, and can further be distinguished by their requirement for the cotransport or counter-transport of inorganic ions. Uptake of amino acids by these transporters therefore depends largely on the intracellular substrate composition. Hence, there is a complex crosstalk between transporters for cationic and neutral amino acids. The transmembrane transporter Cationic Amino Acid Transporters (CAT) are further divided into subtypes with CAT1, CAT2A/2B and CAT3. The transporting properties depend on several important parameters including the pH and substrate availability. As part of this study, I did not assess the role of the individual sub type of co transporter in the BDL model and therefore it remains unclear as to the contribution it makes to alter the Arginine:ADMA ratio and thereby it's influence in the improvement in endothelial dysfunction.

However, inspite of these challenges, I have been able to present my finding with data to prove the hypothesis and make an assertion that there is value in pursuing these strategies in the near future in the setting of a clinical trial.

7.3 Future Directions:

The two most important finding in the context of the studies presented in this thesis have been the reduction of portal pressure in the BDL rats treated with an ADRA2a receptor antagonist with a maintenance of the mean arterial pressure and the supplementation of L-arginine as an anti inflammatory strategy for the treatment of portal hypertension. I accept that these results have been generated using a single rodent model of secondary biliary cirrhosis with evidence of portal hypertension and inflammation. Therefore, it is important to address whether the observed findings can be reproduced in another model of liver injury such as a Carbon Tetrachloride induced liver injury rodent model. This would increase confidence in the data that the findings are more generalizable to modulation of inflammation in cirrhosis and not unique to the BDL model.

There are several experimental questions that have arisen from the observations that have been made thus far. They are as follows:

1). The experiments in these studies have been designed to specifically look at the role of ADRA2a in the modulation of intra hepatic circulation. It is well established that other end organs, specifically the kidney and brain are critically involved in the evolution of decompensation of cirrhosis with advancing portal hypertension. I have shown some preliminary data on improvement in kidney function and brain inflammation in this BDL model using an ADRA2a antagonist. It has already been shown that the kidney and the brain have got abundance of ADRA2a receptors normally. It would be interesting to assess the effect of ADRA2a antagonism in these organs in the BDL model in more detail.

In order to address this question a BDL model can be used to particularly focus on the kidney and brain. In the kidney, haemodynamic assessment with flow measurements and functional assessment with ioxehol infusion can be measured in both the healthy and diseased (BDL) states. This is in addition to measuring the urine output from collections made in metabolic cages pre and post treatment, plasma creatinine and protein expression and immunohistological assessment of Toll Like Receptor 4 expression as a marker of renal inflammation and the effect of treatment with ADRA2a antagonist.

In the brain, haemodynamic measurements could be assessed with functional MRI techniques which are being validated in animal models in our lab since the time I finished my practical experimentation. The functional study could be assessed by Barnes Maze experiments wherein working memory and learning could be assessed. This is in addition to measurement of brain tissue inflammatory markers and assessment of astrocyte swelling by brain water measurement and glutamine protein expression in pre and post treatment animals. These experiments will further our knowledge in the affected animals of progression of cirrhosis and portal hypertension, and the impact of modulating ADRA2a receptor in these models, in relation to effects on these major end organs.

2). In these sets of experiments, I have studied the effect of ADRA2a antagonism in portal hypertension in the BDL model. I accept that adrenergic antagonism may not completely block the effect of the ADRA2a adrenergic receptor. In order to elaborate further on the role of adrenergic receptor blockade in more detail, it would be interesting to study the impact of hepatic and vascular dysfunction in a heterozygous ADRA2a knockout model

treated with or without LPS. If the injury is non-lethal, it would be important to note if the dysfunction can be reversed by using a ADRA2a agonist.

ADRA2a KO mice are now widely available on a commercial basis and with collaborators. These can be acquired so that further studies can be carried out to assess level of injury and dysfunction. In particular, all haemodynamic , biochemical and organ dysfunction parameters can be collected and analysed in all the groups. More specifically, markers of endothelial dysfunction including eNOS and nitric oxide production, inflammatory markers (TNFa, IL6) and liver architecture in wild type and diseased animals could be assessed. I would expect an abrogation of the inflammatory response and possibly improvement in liver architecture as compared to a wild type BDL rats with or without LPS.

3). In these set of experiments , the design of the study was to only look specifically at the effect of ADRA2a antagonism. As alluded to previously, there will be some unintended effect on the ADRA 2 subtypes including the ADRA2b and ADRA2c adrenergic receptors. ADRA1a is a major sub set of adrenergic receptors that are present in abundance in the systemic vasculature and heart. The question that needs further investigation is the role of the other adrenoreceptors in the progression of liver disease. Does a specific ADRA2a antagonist have reciprocal effect in the other adrenoreceptors in the intra and extra hepatic vascular bed.

By using the same model of BDL rats , the subtypes of ADRA adrenergic receptors can be assessed by protein expression and/or quantative PCR. These analysis could be co-related to the plasma noradrenaline and haemodynamic results obtained from these studies

and specific conclusions regarding the role of these subtype of receptors in this model can be addressed.

4). In order to progress into the next steps of translating the findings from these experiments into clinical trial, there are several steps that needs to be achieved. Currently, the ADRA2a receptor antagonist is only available as a scientific compound in powder form and therefore will not qualify as an Investigational Medical Product under the Good Manufacturing Practice licence agreement. The closest compound that is already in use in the clinical context is Yohimbine (non selective ADRA2 antagonist) which has been produced in a GMP approved facility and therefore eligible to be used in man as part of a clinical trial. Preliminary data from our laboratory have shown a significant drop in the portal pressure in Bile duct ligated rats with preserved mean arterial pressure. These promising results appear to suggest that it might be a suitable alternative to using a specific ADRA2a antagonist.

In the second part of this thesis, I used L-Arginine as an anti-inflammatory agent for lowering portal pressure. It is clearly going to be important to validate these findings in other forms of liver injury. These observations have given rise to further research questions

1). As previously highlighted, the experiments that have been designed as part of these studies focussed in the lowering of intra hepatic resistance by using an anti inflammatory strategy. Therefore , it will be important to understand the effect of treatment with L-arginine on other end organs in this model , more specifically the kidney and brain.

To answer these questions, a similar BDL model of portal hypertension can be used and the experimental design specifically focussed on the kidney and the brain. Kidney and brain tissue can be collected and stored as fresh frozen samples. The haemodynamic parameters in the kidney can be assessed by flow probes and the function can be assessed by ioxehol clearance, as in the ADRA2a experiments, in both treated and untreated groups. Tissue based cytokine assays and immunohistochemical assessment of TLR4 expression can be assessed to probe into the anti-inflammatory properties of L-arginine treatment in the kidney in this model.

Similarly, assessment of haemodynamic parameters and functional activity status of the brain can be assessed with similar experiments as highlighted in the ADRA2a studies. It is interesting to note since the end of my practical experiments, other researchers from my lab have published an paper using an anti inflammatory strategy in the BDL model showing an alteration of the Arginine:ADMA in the brain tissue (196). Therefore, it will important to study the exact mechanism of L-Arginine supplementation which plays a role in the modulation of inflammation in the brain and kidney.

2).It is clear from the published literature that L-arginine supplementation strategies vary widely between studies. The heterogeneity is in relation to route, dose and method of supplementation. This has a bearing on the tissue bioavailability of L-arginine. There is an alternative strategy which would involve replacing citrulline in place of L-arginine, which might be a more effective strategy to increase intra hepatic NO and lower portal pressure as compared to direct L-arginine supplementation.

The rational basis of supplementing citrulline to increase arginine availability is that there is a significant degradation of arginine by way of first pass metabolism whereas the only fate of citrulline is conversion to arginine in the small intestine. Therefore it may be a more efficient strategy to increase plasma arginine levels. A similar experimental model of portal hypertension has to be implemented to test this hypothesis.

3). In order to overcome one of the major issues that I have highlighted in the limitation section is to address the best method of measuring Nitric oxide synthetase activity. The methods that have already been established have each got its own issues in the methodology and the interpretation of the results. I have attempted to do the Nox method to quantify generation of Nitric oxide. However, the results were very difficult to replicate. The eNOS activity assay also depends on experimental condition and the freshness of the tissue. The radioactive isotope method is difficult to access and also operator dependant and therefore, difficult to reproduce the results.

In our lab, we have now used phosphorylated eNOS protein expression by western blot as the surrogate marker for Nitric oxide production. This method has previously been validated in other published studies. In order to assess this further, it would be ideal to use this protein expression study in other animal models to assess if we get the same results.

4). If the availability of L-arginine can be circumvented, then an ideal clinical trial using L-Arginine infusion in cirrhotic patients would be able to be set up to assess if it would drop portal pressure by reduce the inflammatory burden in this cohort of patients. All involved end organs including the kidney and the brain can be assessed in the same study

to understand whether L-arginine supplementation has a beneficial effect in these organs too by way of improved hameodynamics and function.

Overall, in conclusion of this thesis, I have been able to make several important observations by providing an alternative strategy to ameliorate portal hypertension. In pursuit of these answers to my hypothesis, I have been able to gather a significant amount of knowledge and understanding about the pathophysiology of portal hypertension. Moreover, as highlighted in the future directions section, these studies have been able to generate further important questions that needs to be pursued systemically as part of the ongoing studies to develop this area of research. I hope that I will be able to sustain my scientific and clinical inquisitiveness to undertake some of these studies and remain an integral part of this research group and to continue to contribute actively to achieve ground breaking research output.

References

1. Fleming KM, Aithal GP, Solaymani-Dodaran M, Card TR, West J. Incidence and prevalence of cirrhosis in the United Kingdom, 1992-2001: a general population-based study. Journal of hepatology. 2008 Nov;49(5):732-8. PubMed PMID: 18667256.

2. Groszmann RJ, Abraldes JG. Portal hypertension: from bedside to bench. Journal of clinical gastroenterology. 2005 Apr;39(4 Suppl 2):S125-30. PubMed PMID: 15758647. Epub 2005/03/11. eng.

3. Groszmann RJ, Glickman M, Blei AT, Storer E, Conn HO. Wedged and free hepatic venous pressure measured with a balloon catheter. Gastroenterology. 1979 Feb;76(2):253-8. PubMed PMID: 759258. Epub 1979/02/01. eng.

4. Groszmann RJ, Bosch J, Grace ND, Conn HO, Garcia-Tsao G, Navasa M, et al. Hemodynamic events in a prospective randomized trial of propranolol versus placebo in the prevention of a first variceal hemorrhage. Gastroenterology. 1990 Nov;99(5):1401-7. PubMed PMID: 2210246. Epub 1990/11/01. eng.

5. Feu F, Garcia-Pagan JC, Bosch J, Luca A, Teres J, Escorsell A, et al. Relation between portal pressure response to pharmacotherapy and risk of recurrent variceal haemorrhage in patients with cirrhosis. Lancet. 1995 Oct 21;346(8982):1056-9. PubMed PMID: 7564785. Epub 1995/10/21. eng.

6. Thalheimer U, Triantos CK, Samonakis DN, Patch D, Burroughs AK. Infection, coagulation, and variceal bleeding in cirrhosis. Gut. 2005 Apr;54(4):556-63. PubMed PMID: 15753544. Pubmed Central PMCID: 1774431. Epub 2005/03/09. eng.

7. Groszmann RJ, Garcia-Tsao G, Bosch J, Grace ND, Burroughs AK, Planas R, et al. Beta-blockers to prevent gastroesophageal varices in patients with cirrhosis. The New England journal of medicine. 2005 Nov 24;353(21):2254-61. PubMed PMID: 16306522.

8. D'Amico G, Pagliaro L, Bosch J. Pharmacological treatment of portal hypertension: an evidence-based approach. Seminars in liver disease. 1999;19(4):475-505. PubMed PMID: 10643630.

9. Levacher S, Letoumelin P, Pateron D, Blaise M, Lapandry C, Pourriat JL. Early administration of terlipressin plus glyceryl trinitrate to control active upper

gastrointestinal bleeding in cirrhotic patients. Lancet. 1995 Sep 30;346(8979):865-8. PubMed PMID: 7564670.

10. Serste T, Melot C, Francoz C, Durand F, Rautou PE, Valla D, et al. Deleterious effects of beta-blockers on survival in patients with cirrhosis and refractory ascites. Hepatology. 2010 Sep;52(3):1017-22. PubMed PMID: 20583214.

11. Greenway CV, Stark RD. Hepatic vascular bed. Physiol Rev. 1971 Jan;51(1):23-65. PubMed PMID: 5543903. Epub 1971/01/01. eng.

12. Popper H, Zak FG. Pathologic aspects of cirrhosis. Am J Med. 1958 Apr;24(4):593-619. PubMed PMID: 13520759. Epub 1958/04/01. eng.

13. van Leeuwen DJ. Assessment of portal hypertension: understanding will improve treatment. Dig Dis. 1991;9(2):92-105. PubMed PMID: 2036736. Epub 1991/01/01. eng.

14. Lieber CS. Alcoholic fatty liver: its pathogenesis and precursor role for hepatitis and cirrhosis. Panminerva Med. 1976 Sep-Oct;18(9-10):346-58. PubMed PMID: 980533. Epub 1976/09/01. eng.

15. Rincon D, Ripoll C, Lo Iacono O, Salcedo M, Catalina MV, Alvarez E, et al. Antiviral therapy decreases hepatic venous pressure gradient in patients with chronic hepatitis C and advanced fibrosis. The American journal of gastroenterology. 2006 Oct;101(10):2269-74. PubMed PMID: 17032192. Epub 2006/10/13. eng.

16. Mittal MK, Gupta TK, Lee FY, Sieber CC, Groszmann RJ. Nitric oxide modulates hepatic vascular tone in normal rat liver. The American journal of physiology. 1994 Sep;267(3 Pt 1):G416-22. PubMed PMID: 7943239. Epub 1994/09/01. eng.

17. Bhathal PS, Grossman HJ. Reduction of the increased portal vascular resistance of the isolated perfused cirrhotic rat liver by vasodilators. Journal of hepatology. 1985;1(4):325-37. PubMed PMID: 4056346. Epub 1985/01/01. eng.

18. Kaneda K, Ekataksin W, Sogawa M, Matsumura A, Cho A, Kawada N. Endothelin-1-induced vasoconstriction causes a significant increase in portal pressure of rat liver: localized constrictive effect on the distal segment of preterminal portal venules as revealed by light and electron microscopy and serial reconstruction. Hepatology. 1998 Mar;27(3):735-47. PubMed PMID: 9500702. Epub 1998/03/21. eng.

19. Bauer M, Paquette NC, Zhang JX, Bauer I, Pannen BH, Kleeberger SR, et al. Chronic ethanol consumption increases hepatic sinusoidal contractile response to

endothelin-1 in the rat. Hepatology. 1995 Nov;22(5):1565-76. PubMed PMID: 7590677. Epub 1995/11/01. eng.

20. Kawada N, Tran-Thi TA, Klein H, Decker K. The contraction of hepatic stellate (Ito) cells stimulated with vasoactive substances. Possible involvement of endothelin 1 and nitric oxide in the regulation of the sinusoidal tonus. European journal of biochemistry. 1993 Apr 15;213(2):815-23. PubMed PMID: 7682947. Epub 1993/04/15. eng.

21. Arroyo V, Planas R, Gaya J, Deulofeu R, Rimola A, Perez-Ayuso RM, et al. Sympathetic nervous activity, renin-angiotensin system and renal excretion of prostaglandin E2 in cirrhosis. Relationship to functional renal failure and sodium and water excretion. European journal of clinical investigation. 1983 Jun;13(3):271-8. PubMed PMID: 6409652. Epub 1983/06/01. eng.

22. Ballet F, Chretien Y, Rey C, Poupon R. Differential response of normal and cirrhotic liver to vasoactive agents. A study in the isolated perfused rat liver. The Journal of pharmacology and experimental therapeutics. 1988 Jan;244(1):283-9. PubMed PMID: 3336005. Epub 1988/01/01. eng.

23. Galle J, Quaschning T, Seibold S, Wanner C. Endothelial dysfunction and inflammation: what is the link? Kidney international Supplement. 2003 May(84):S45-9. PubMed PMID: 12694307.

24. Ganz P, Vita JA. Testing endothelial vasomotor function: nitric oxide, a multipotent molecule. Circulation. 2003 Oct 28;108(17):2049-53. PubMed PMID: 14581383.

25. Gupta TK, Toruner M, Chung MK, Groszmann RJ. Endothelial dysfunction and decreased production of nitric oxide in the intrahepatic microcirculation of cirrhotic rats. Hepatology. 1998 Oct;28(4):926-31. PubMed PMID: 9755227.

26. Clapp BR, Hingorani AD, Kharbanda RK, Mohamed-Ali V, Stephens JW, Vallance P, et al. Inflammation-induced endothelial dysfunction involves reduced nitric oxide bioavailability and increased oxidant stress. Cardiovascular research. 2004 Oct 1;64(1):172-8. PubMed PMID: 15364625.

27. Rockey DC, Chung JJ. Reduced nitric oxide production by endothelial cells in cirrhotic rat liver: endothelial dysfunction in portal hypertension. Gastroenterology. 1998 Feb;114(2):344-51. PubMed PMID: 9453496. Epub 1998/02/07. eng.

28. Shah V, Toruner M, Haddad F, Cadelina G, Papapetropoulos A, Choo K, et al. Impaired endothelial nitric oxide synthase activity associated with enhanced caveolin binding in experimental cirrhosis in the rat. Gastroenterology. 1999 Nov;117(5):1222-8. PubMed PMID: 10535886. Epub 1999/10/27. eng.

29. Shah V, Cao S, Hendrickson H, Yao J, Katusic ZS. Regulation of hepatic eNOS by caveolin and calmodulin after bile duct ligation in rats. American journal of physiology Gastrointestinal and liver physiology. 2001 Jun;280(6):G1209-16. PubMed PMID: 11352814. Epub 2001/05/16. eng.

30. Morales-Ruiz M, Cejudo-Martin P, Fernandez-Varo G, Tugues S, Ros J, Angeli P, et al. Transduction of the liver with activated Akt normalizes portal pressure in cirrhotic rats. Gastroenterology. 2003 Aug;125(2):522-31. PubMed PMID: 12891555. Epub 2003/08/02. eng.

31. Graupera M, Garcia-Pagan JC, Pares M, Abraldes JG, Rosello J, Bosch J, et al. Cyclooxygenase-1 inhibition corrects endothelial dysfunction in cirrhotic rat livers. Journal of hepatology. 2003 Oct;39(4):515-21. PubMed PMID: 12971960. Epub 2003/09/16. eng.

32. Laleman W, Omasta A, Van de Casteele M, Zeegers M, Vander Elst I, Van Landeghem L, et al. A role for asymmetric dimethylarginine in the pathophysiology of portal hypertension in rats with biliary cirrhosis. Hepatology. 2005 Dec;42(6):1382-90. PubMed PMID: 16317694. Epub 2005/12/01. eng.

33. Gracia-Sancho J, Lavina B, Rodriguez-Vilarrupla A, Garcia-Caldero H, Fernandez M, Bosch J, et al. Increased oxidative stress in cirrhotic rat livers: A potential mechanism contributing to reduced nitric oxide bioavailability. Hepatology. 2008 Apr;47(4):1248-56. PubMed PMID: 18273863. Epub 2008/02/15. eng.

34. Lavina B, Gracia-Sancho J, Rodriguez-Vilarrupla A, Chu Y, Heistad DD, Bosch J, et al. Superoxide dismutase gene transfer reduces portal pressure in CCl4 cirrhotic rats with portal hypertension. Gut. 2009 Jan;58(1):118-25. PubMed PMID: 18829979. Epub 2008/10/03. eng.

35. Pinzani M, Failli P, Ruocco C, Casini A, Milani S, Baldi E, et al. Fat-storing cells as liver-specific pericytes. Spatial dynamics of agonist-stimulated intracellular calcium transients. The Journal of clinical investigation. 1992 Aug;90(2):642-6. PubMed PMID: 1644929. Pubmed Central PMCID: 443145. Epub 1992/08/01. eng.

36. Rockey DC, Weisiger RA. Endothelin induced contractility of stellate cells from normal and cirrhotic rat liver: implications for regulation of portal pressure and resistance. Hepatology. 1996 Jul;24(1):233-40. PubMed PMID: 8707268. Epub 1996/07/01. eng.

37. Tam J, Liu J, Mukhopadhyay B, Cinar R, Godlewski G, Kunos G. Endocannabinoids in liver disease. Hepatology. 2011 Jan;53(1):346-55. PubMed PMID: 21254182. Epub 2011/01/22. eng.

38. Groszmann RJ. Hyperdynamic circulation of liver disease 40 years later: pathophysiology and clinical consequences. Hepatology. 1994 Nov;20(5):1359-63. PubMed PMID: 7927273. Epub 1994/11/01. eng.

39. Atucha NM, Shah V, Garcia-Cardena G, Sessa WE, Groszmann RJ. Role of endothelium in the abnormal response of mesenteric vessels in rats with portal hypertension and liver cirrhosis. Gastroenterology. 1996 Dec;111(6):1627-32. PubMed PMID: 8942743. Epub 1996/12/01. eng.

40. Sieber CC, Lopez-Talavera JC, Groszmann RJ. Role of nitric oxide in the in vitro splanchnic vascular hyporeactivity in ascitic cirrhotic rats. Gastroenterology. 1993 Jun;104(6):1750-4. PubMed PMID: 8388839. Epub 1993/06/01. eng.

41. Martin PY, Ohara M, Gines P, Xu DL, St John J, Niederberger M, et al. Nitric oxide synthase (NOS) inhibition for one week improves renal sodium and water excretion in cirrhotic rats with ascites. The Journal of clinical investigation. 1998 Jan 1;101(1):235-42. PubMed PMID: 9421486. Pubmed Central PMCID: 508560. Epub 1998/02/14. eng.

42. Cahill PA, Redmond EM, Hodges R, Zhang S, Sitzmann JV. Increased endothelial nitric oxide synthase activity in the hyperemic vessels of portal hypertensive rats. Journal of hepatology. 1996 Sep;25(3):370-8. PubMed PMID: 8895017. Epub 1996/09/01. eng.

43. Hori N, Wiest R, Groszmann RJ. Enhanced release of nitric oxide in response to changes in flow and shear stress in the superior mesenteric arteries of portal hypertensive rats. Hepatology. 1998 Dec;28(6):1467-73. PubMed PMID: 9828208. Epub 1998/11/26. eng.

44. Tsai MH, Iwakiri Y, Cadelina G, Sessa WC, Groszmann RJ. Mesenteric vasoconstriction triggers nitric oxide overproduction in the superior mesenteric artery of portal hypertensive rats. Gastroenterology. 2003 Nov;125(5):1452-61. PubMed PMID: 14598261. Epub 2003/11/05. eng.

45. Wiest R, Shah V, Sessa WC, Groszmann RJ. NO overproduction by eNOS precedes hyperdynamic splanchnic circulation in portal hypertensive rats. The American journal of physiology. 1999 Apr;276(4 Pt 1):G1043-51. PubMed PMID: 10198349. Epub 1999/04/13. eng.

46. Vallance P, Moncada S. Hyperdynamic circulation in cirrhosis: a role for nitric oxide? Lancet. 1991 Mar 30;337(8744):776-8. PubMed PMID: 1706450. Epub 1991/03/30. eng.

47. Fernandez M, Garcia-Pagan JC, Casadevall M, Bernadich C, Piera C, Whittle BJ, et al. Evidence against a role for inducible nitric oxide synthase in the hyperdynamic circulation of portal-hypertensive rats. Gastroenterology. 1995 May;108(5):1487-95. PubMed PMID: 7537235. Epub 1995/05/01. eng.

48. Guarner C, Soriano G, Tomas A, Bulbena O, Novella MT, Balanzo J, et al. Increased serum nitrite and nitrate levels in patients with cirrhosis: relationship to endotoxemia. Hepatology. 1993 Nov;18(5):1139-43. PubMed PMID: 8225220. Epub 1993/11/01. eng.

49. Chu CJ, Lee FY, Wang SS, Lu RH, Tsai YT, Lin HC, et al. Hyperdynamic circulation of cirrhotic rats with ascites: role of endotoxin, tumour necrosis factor-alpha and nitric oxide. Clinical science. 1997 Sep;93(3):219-25. PubMed PMID: 9337636. Epub 1997/10/24. eng.

50. Jurzik L, Froh M, Straub RH, Scholmerich J, Wiest R. Up-regulation of nNOS and associated increase in nitrergic vasodilation in superior mesenteric arteries in pre-hepatic portal hypertension. Journal of hepatology. 2005 Aug;43(2):258-65. PubMed PMID: 15963596. Epub 2005/06/21. eng.

51. Xu L, Carter EP, Ohara M, Martin PY, Rogachev B, Morris K, et al. Neuronal nitric oxide synthase and systemic vasodilation in rats with cirrhosis. American journal of physiology Renal physiology. 2000 Dec;279(6):F1110-5. PubMed PMID: 11097630. Epub 2000/11/30. eng.

52. Wiest R, Cadelina G, Milstien S, McCuskey RS, Garcia-Tsao G, Groszmann RJ. Bacterial translocation up-regulates GTP-cyclohydrolase I in mesenteric vasculature of cirrhotic rats. Hepatology. 2003 Dec;38(6):1508-15. PubMed PMID: 14647062. Epub 2003/12/03. eng.

53. Shah V, Wiest R, Garcia-Cardena G, Cadelina G, Groszmann RJ, Sessa WC. Hsp90 regulation of endothelial nitric oxide synthase contributes to vascular control in portal hypertension. The American journal of physiology. 1999 Aug;277(2 Pt 1):G463-8. PubMed PMID: 10444461. Epub 1999/08/13. eng.

54. Iwakiri Y, Tsai MH, McCabe TJ, Gratton JP, Fulton D, Groszmann RJ, et al. Phosphorylation of eNOS initiates excessive NO production in early phases of portal hypertension. American journal of physiology Heart and circulatory physiology. 2002 Jun;282(6):H2084-90. PubMed PMID: 12003815. Epub 2002/05/11. eng.

55. Ackah E, Yu J, Zoellner S, Iwakiri Y, Skurk C, Shibata R, et al. Akt1/protein kinase Balpha is critical for ischemic and VEGF-mediated angiogenesis. The Journal of clinical investigation. 2005 Aug;115(8):2119-27. PubMed PMID: 16075056. Pubmed Central PMCID: 1180542. Epub 2005/08/03. eng.

56. Fulton D, Babbitt R, Zoellner S, Fontana J, Acevedo L, McCabe TJ, et al. Targeting of endothelial nitric-oxide synthase to the cytoplasmic face of the Golgi complex or plasma membrane regulates Akt- versus calcium-dependent mechanisms for nitric oxide release. The Journal of biological chemistry. 2004 Jul 16;279(29):30349-57. PubMed PMID: 15136572. Epub 2004/05/12. eng.

57. Erwin PA, Mitchell DA, Sartoretto J, Marletta MA, Michel T. Subcellular targeting and differential S-nitrosylation of endothelial nitric-oxide synthase. The Journal of biological chemistry. 2006 Jan 6;281(1):151-7. PubMed PMID: 16286475. Epub 2005/11/16. eng.

58. Chen YC, Gines P, Yang J, Summer SN, Falk S, Russell NS, et al. Increased vascular heme oxygenase-1 expression contributes to arterial vasodilation in experimental cirrhosis in rats. Hepatology. 2004 Apr;39(4):1075-87. PubMed PMID: 15057912. Epub 2004/04/02. eng.

59. Sitzmann JV, Campbell K, Wu Y, St Clair C. Prostacyclin production in acute, chronic, and long-term experimental portal hypertension. Surgery. 1994 Mar;115(3):290-4. PubMed PMID: 8128353. Epub 1994/03/01. eng.

60. Batkai S, Jarai Z, Wagner JA, Goparaju SK, Varga K, Liu J, et al. Endocannabinoids acting at vascular CB1 receptors mediate the vasodilated state in advanced liver cirrhosis. Nature medicine. 2001 Jul;7(7):827-32. PubMed PMID: 11433348. Epub 2001/07/04. eng.

61. Zhao W, Zhang J, Lu Y, Wang R. The vasorelaxant effect of H(2)S as a novel endogenous gaseous K(ATP) channel opener. The EMBO journal. 2001 Nov 1;20(21):6008-16. PubMed PMID: 11689441. Pubmed Central PMCID: 125693.

62. Fiorucci S, Antonelli E, Mencarelli A, Orlandi S, Renga B, Rizzo G, et al. The third gas: H2S regulates perfusion pressure in both the isolated and perfused normal rat liver and in cirrhosis. Hepatology. 2005 Sep;42(3):539-48. PubMed PMID: 16108046. Epub 2005/08/19. eng.

63. Lopez-Talavera JC, Merrill WW, Groszmann RJ. Tumor necrosis factor alpha: a major contributor to the hyperdynamic circulation in prehepatic portal-hypertensive rats. Gastroenterology. 1995 Mar;108(3):761-7. PubMed PMID: 7875478. Epub 1995/03/01. eng.

64. Wever RM, van Dam T, van Rijn HJ, de Groot F, Rabelink TJ. Tetrahydrobiopterin regulates superoxide and nitric oxide generation by recombinant endothelial nitric oxide synthase. Biochemical and biophysical research communications. 1997 Aug 18;237(2):340-4. PubMed PMID: 9268712. Epub 1997/08/18. eng.

65. Sztrymf B, Rabiller A, Nunes H, Savale L, Lebrec D, Le Pape A, et al. Prevention of hepatopulmonary syndrome and hyperdynamic state by pentoxifylline in cirrhotic rats. The European respiratory journal. 2004 May;23(5):752-8. PubMed PMID: 15176692. Epub 2004/06/05. eng.

66. Kojima H, Sakurai S, Uemura M, Satoh H, Nakashima T, Minamino N, et al. Adrenomedullin contributes to vascular hyporeactivity in cirrhotic rats with ascites via a release of nitric oxide. Scandinavian journal of gastroenterology. 2004 Jul;39(7):686-93. PubMed PMID: 15370692. Epub 2004/09/17. eng.

67. Nishimatsu H, Suzuki E, Nagata D, Moriyama N, Satonaka H, Walsh K, et al. Adrenomedullin induces endothelium-dependent vasorelaxation via the phosphatidylinositol 3-kinase/Akt-dependent pathway in rat aorta. Circulation research. 2001 Jul 6;89(1):63-70. PubMed PMID: 11440979. Epub 2001/07/07. eng.

68. Ripoll C, Groszmann R, Garcia-Tsao G, Grace N, Burroughs A, Planas R, et al. Hepatic venous pressure gradient predicts clinical decompensation in patients with compensated cirrhosis. Gastroenterology. 2007 Aug;133(2):481-8. PubMed PMID: 17681169.

69. Tilg H, Wilmer A, Vogel W, Herold M, Nolchen B, Judmaier G, et al. Serum levels of cytokines in chronic liver diseases. Gastroenterology. 1992 Jul;103(1):264-74. PubMed PMID: 1612333.

70. Bird GL, Sheron N, Goka AK, Alexander GJ, Williams RS. Increased plasma tumor necrosis factor in severe alcoholic hepatitis. Annals of internal medicine. 1990 Jun 15;112(12):917-20. PubMed PMID: 2339855.

71. Mookerjee RP, Sen S, Davies NA, Hodges SJ, Williams R, Jalan R. Tumour necrosis factor alpha is an important mediator of portal and systemic haemodynamic derangements in alcoholic hepatitis. Gut. 2003 Aug;52(8):1182-7. PubMed PMID: 12865279. Pubmed Central PMCID: 1773767.

72. Moreau R, Jalan R, Gines P, Pavesi M, Angeli P, Cordoba J, et al. Acute-on-chronic liver failure is a distinct syndrome that develops in patients with acute decompensation of cirrhosis. Gastroenterology. 2013 Jun;144(7):1426-37, 37 e1-9. PubMed PMID: 23474284.

73. Rincon D, Lo Iacono O, Ripoll C, Gomez-Camarero J, Salcedo M, Catalina MV, et al. Prognostic value of hepatic venous pressure gradient for in-hospital mortality of patients with severe acute alcoholic hepatitis. Alimentary pharmacology & therapeutics. 2007 Apr 1;25(7):841-8. PubMed PMID: 17373923.

74. Seki E, De Minicis S, Osterreicher CH, Kluwe J, Osawa Y, Brenner DA, et al. TLR4 enhances TGF-beta signaling and hepatic fibrosis. Nature medicine. 2007 Nov;13(11):1324-32. PubMed PMID: 17952090.

75. Jagavelu K, Routray C, Shergill U, O'Hara SP, Faubion W, Shah VH. Endothelial cell toll-like receptor 4 regulates fibrosis-associated angiogenesis in the liver. Hepatology. 2010 Aug;52(2):590-601. PubMed PMID: 20564354. Pubmed Central PMCID: 2916032.
76. Mandrekar P, Szabo G. Signalling pathways in alcohol-induced liver inflammation. Journal of hepatology. 2009 Jun;50(6):1258-66. PubMed PMID: 19398236. Pubmed Central PMCID: 3342816.
77. Karaa A, Kamoun WS, Clemens MG. Oxidative stress disrupts nitric oxide synthase activation in liver endothelial cells. Free radical biology & medicine. 2005 Nov 15;39(10):1320-31. PubMed PMID: 16257641.
78. Mookerjee RP, Malaki M, Davies NA, Hodges SJ, Dalton RN, Turner C, et al. Increasing dimethylarginine levels are associated with adverse clinical outcome in severe alcoholic hepatitis. Hepatology. 2007 Jan;45(1):62-71. PubMed PMID: 17187433.
79. Mookerjee RP, Wiesenthal A, Icking A, Hodges SJ, Davies NA, Schilling K, et al. Increased gene and protein expression of the novel eNOS regulatory protein NOSTRIN and a variant in alcoholic hepatitis. Gastroenterology. 2007 Jun;132(7):2533-41. PubMed PMID: 17570224.
80. Kubes P, Mehal WZ. Sterile inflammation in the liver. Gastroenterology. 2012 Nov;143(5):1158-72. PubMed PMID: 22982943.
81. Kim JB, Lim CM, Yu YM, Lee JK. Induction and subcellular localization of high-mobility group box-1 (HMGB1) in the postischemic rat brain. Journal of neuroscience research. 2008 Apr;86(5):1125-31. PubMed PMID: 17975839.
82. Kajita M, Murata T, Horiguchi K, Iizuka M, Hori M, Ozaki H. iNOS expression in vascular resident macrophages contributes to circulatory dysfunction of splanchnic vascular smooth muscle contractions in portal hypertensive rats. American journal of physiology Heart and circulatory physiology. 2011 Mar;300(3):H1021-31. PubMed PMID: 21193589.
83. Theodorakis NG, Wang YN, Skill NJ, Metz MA, Cahill PA, Redmond EM, et al. The role of nitric oxide synthase isoforms in extrahepatic portal hypertension: studies in gene-knockout mice. Gastroenterology. 2003 May;124(5):1500-8. PubMed PMID: 12730888.

84. Moreau R, Barriere E, Tazi KA, Lardeux B, Dargere D, Urbanowicz W, et al. Terlipressin inhibits in vivo aortic iNOS expression induced by lipopolysaccharide in rats with biliary cirrhosis. Hepatology. 2002 Nov;36(5):1070-8. PubMed PMID: 12395316.
85. Jalan R, Olde Damink SW, Ter Steege JC, Redhead DN, Lee A, Hayes PC, et al. Acute endotoxemia following transjugular intrahepatic stent-shunt insertion is associated with systemic and cerebral vasodilatation with increased whole body nitric oxide production in critically ill cirrhotic patients. Journal of hepatology. 2011 Feb;54(2):265-71. PubMed PMID: 21067839.
86. Albillos A, de la Hera A, Gonzalez M, Moya JL, Calleja JL, Monserrat J, et al. Increased lipopolysaccharide binding protein in cirrhotic patients with marked immune and hemodynamic derangement. Hepatology. 2003 Jan;37(1):208-17. PubMed PMID: 12500206.
87. Rasaratnam B, Kaye D, Jennings G, Dudley F, Chin-Dusting J. The effect of selective intestinal decontamination on the hyperdynamic circulatory state in cirrhosis. A randomized trial. Annals of internal medicine. 2003 Aug 5;139(3):186-93. PubMed PMID: 12899586.
88. Tazi KA, Moreau R, Herve P, Dauvergne A, Cazals-Hatem D, Bert F, et al. Norfloxacin reduces aortic NO synthases and proinflammatory cytokine up-regulation in cirrhotic rats: role of Akt signaling. Gastroenterology. 2005 Jul;129(1):303-14. PubMed PMID: 16012955.
89. Rautou PE, Bresson J, Sainte-Marie Y, Vion AC, Paradis V, Renard JM, et al. Abnormal plasma microparticles impair vasoconstrictor responses in patients with cirrhosis. Gastroenterology. 2012 Jul;143(1):166-76 e6. PubMed PMID: 22465620.
90. Cirera I, Bauer TM, Navasa M, Vila J, Grande L, Taura P, et al. Bacterial translocation of enteric organisms in patients with cirrhosis. Journal of hepatology. 2001 Jan;34(1):32-7. PubMed PMID: 11211904.
91. Goulis J, Armonis A, Patch D, Sabin C, Greenslade L, Burroughs AK. Bacterial infection is independently associated with failure to control bleeding in cirrhotic patients with gastrointestinal hemorrhage. Hepatology. 1998 May;27(5):1207-12. PubMed PMID: 9581672.

92. Wiest R, Garcia-Tsao G. Bacterial translocation (BT) in cirrhosis. Hepatology. 2005 Mar;41(3):422-33. PubMed PMID: 15723320.

93. Schwabe RF, Seki E, Brenner DA. Toll-like receptor signaling in the liver. Gastroenterology. 2006 May;130(6):1886-900. PubMed PMID: 16697751.

94. Bellot P, Garcia-Pagan JC, Frances R, Abraldes JG, Navasa M, Perez-Mateo M, et al. Bacterial DNA translocation is associated with systemic circulatory abnormalities and intrahepatic endothelial dysfunction in patients with cirrhosis. Hepatology. 2010 Dec;52(6):2044-52. PubMed PMID: 20979050.

95. Ruiz-del-Arbol L, Urman J, Fernandez J, Gonzalez M, Navasa M, Monescillo A, et al. Systemic, renal, and hepatic hemodynamic derangement in cirrhotic patients with spontaneous bacterial peritonitis. Hepatology. 2003 Nov;38(5):1210-8. PubMed PMID: 14578859.

96. Steib CJ, Hartmann AC, v Hesler C, Benesic A, Hennenberg M, Bilzer M, et al. Intraperitoneal LPS amplifies portal hypertension in rat liver fibrosis. Laboratory investigation; a journal of technical methods and pathology. 2010 Jul;90(7):1024-32. PubMed PMID: 20212458.

97. Arroyo V, Navasa M, Rimola A. Spontaneous bacterial peritonitis in liver cirrhosis: treatment and prophylaxis. Infection. 1994;22 Suppl 3:S167-75. PubMed PMID: 7843826.

98. Lemmers A, Gustot T, Durnez A, Evrard S, Moreno C, Quertinmont E, et al. An inhibitor of interleukin-6 trans-signalling, sgp130, contributes to impaired acute phase response in human chronic liver disease. Clinical and experimental immunology. 2009 Jun;156(3):518-27. PubMed PMID: 19438606. Pubmed Central PMCID: 2691982.

99. Mortensen C, Andersen O, Krag A, Bendtsen F, Moller S. High-sensitivity C-reactive protein levels predict survival and are related to haemodynamics in alcoholic cirrhosis. European journal of gastroenterology & hepatology. 2012 Jun;24(6):619-26. PubMed PMID: 22441510.

100. Felten DL, Felten SY, Bellinger DL, Carlson SL, Ackerman KD, Madden KS, et al. Noradrenergic sympathetic neural interactions with the immune system: structure and function. Immunological reviews. 1987 Dec;100:225-60. PubMed PMID: 3326822.

101. Carr DJ, De Costa BR, Radesca L, Blalock JE. Functional assessment and partial characterization of [3H](+)-pentazocine binding sites on cells of the immune system. Journal of neuroimmunology. 1991 Dec;35(1-3):153-66. PubMed PMID: 1955564.

102. Howarth D, Burstal R, Hayes C, Lan L, Lantry G. Autonomic regulation of lymphatic flow in the lower extremity demonstrated on lymphoscintigraphy in patients with reflex sympathetic dystrophy. Clinical nuclear medicine. 1999 Jun;24(6):383-7. PubMed PMID: 10361930.

103. Scheiermann C, Kunisaki Y, Lucas D, Chow A, Jang JE, Zhang D, et al. Adrenergic nerves govern circadian leukocyte recruitment to tissues. Immunity. 2012 Aug 24;37(2):290-301. PubMed PMID: 22863835. Pubmed Central PMCID: 3428436.

104. Shaldon C, Peacock JH, Walker RM, Palmer DB, Badrick FE. The portal venous content of adrenaline and noradrenaline in portal hypertension. Lancet. 1961 May 6;1(7184):957-61. PubMed PMID: 13750619.

105. Henriksen JH, Christensen NJ, Ring-Larsen H. Noradrenaline and adrenaline concentrations in various vascular beds in patients with cirrhosis. Relation to haemodynamics. Clinical physiology. 1981 Jun;1(3):293-304. PubMed PMID: 7199989.

106. Henriksen JH, Ring-Larsen H, Kanstrup IL, Christensen NJ. Splanchnic and renal elimination and release of catecholamines in cirrhosis. Evidence of enhanced sympathetic nervous activity in patients with decompensated cirrhosis. Gut. 1984 Oct;25(10):1034-43. PubMed PMID: 6479678. Pubmed Central PMCID: 1432557.

107. Henriksen JH, Bendtsen F, Sorensen TI, Stadeager C, Ring-Larsen H. Reduced central blood volume in cirrhosis. Gastroenterology. 1989 Dec;97(6):1506-13. PubMed PMID: 2583416.

108. Henriksen JH, Bendtsen F, Gerbes AL, Christensen NJ, Ring-Larsen H, Sorensen TI. Estimated central blood volume in cirrhosis: relationship to sympathetic nervous activity, beta-adrenergic blockade and atrial natriuretic factor. Hepatology. 1992 Nov;16(5):1163-70. PubMed PMID: 1358779.

109. Henriksen JH, Ring-Larsen H, Christensen NJ. Aspects of sympathetic nervous system regulation in patients with cirrhosis: a 10-year experience. Clinical physiology. 1991 Jul;11(4):293-306. PubMed PMID: 1914433.

110. Yang S, Koo DJ, Zhou M, Chaudry IH, Wang P. Gut-derived norepinephrine plays a critical role in producing hepatocellular dysfunction during early sepsis. American journal of physiology Gastrointestinal and liver physiology. 2000 Dec;279(6):G1274-81. PubMed PMID: 11093951.

111. Zhou M, Yang S, Koo DJ, Ornan DA, Chaudry IH, Wang P. The role of Kupffer cell alpha(2)-adrenoceptors in norepinephrine-induced TNF-alpha production. Biochimica et biophysica acta. 2001 Jul 27;1537(1):49-57. PubMed PMID: 11476962.

112. Flierl MA, Rittirsch D, Nadeau BA, Sarma JV, Day DE, Lentsch AB, et al. Upregulation of phagocyte-derived catecholamines augments the acute inflammatory response. PloS one. 2009;4(2):e4414. PubMed PMID: 19212441. Pubmed Central PMCID: 2636885.

113. Flierl MA, Rittirsch D, Huber-Lang M, Sarma JV, Ward PA. Catecholamines-crafty weapons in the inflammatory arsenal of immune/inflammatory cells or opening pandora's box? Molecular medicine. 2008 Mar-Apr;14(3-4):195-204. PubMed PMID: 18079995. Pubmed Central PMCID: 2136428.

114. Palmer RM, Ashton DS, Moncada S. Vascular endothelial cells synthesize nitric oxide from L-arginine. Nature. 1988 Jun 16;333(6174):664-6. PubMed PMID: 3131684.

115. Windmueller HG, Spaeth AE. Uptake and metabolism of plasma glutamine by the small intestine. The Journal of biological chemistry. 1974 Aug 25;249(16):5070-9. PubMed PMID: 4605420. Epub 1974/08/25. eng.

116. Windmueller HG, Spaeth AE. Source and fate of circulating citrulline. The American journal of physiology. 1981 Dec;241(6):E473-80. PubMed PMID: 7325229. Epub 1981/12/01. eng.

117. Featherston WR, Rogers QR, Freedland RA. Relative importance of kidney and liver in synthesis of arginine by the rat. The American journal of physiology. 1973 Jan;224(1):127-9. PubMed PMID: 4683290. Epub 1973/01/01. eng.

118. Yu YM, Burke JF, Tompkins RG, Martin R, Young VR. Quantitative aspects of interorgan relationships among arginine and citrulline metabolism. The American journal of physiology. 1996 Dec;271(6 Pt 1):E1098-109. PubMed PMID: 8997231. Epub 1996/12/01. eng.

119. Luiking YC, Poeze M, Dejong CH, Ramsay G, Deutz NE. Sepsis: an arginine deficiency state? Critical care medicine. 2004 Oct;32(10):2135-45. PubMed PMID: 15483426. Epub 2004/10/16. eng.

120. Jenkinson CP, Grody WW, Cederbaum SD. Comparative properties of arginases. Comp Biochem Physiol B Biochem Mol Biol. 1996 May;114(1):107-32. PubMed PMID: 8759304. Epub 1996/05/01. eng.

121. Castillo L, Chapman TE, Yu YM, Ajami A, Burke JF, Young VR. Dietary arginine uptake by the splanchnic region in adult humans. The American journal of physiology. 1993 Oct;265(4 Pt 1):E532-9. PubMed PMID: 8238326. Epub 1993/10/01. eng.

122. Wu G, Morris SM, Jr. Arginine metabolism: nitric oxide and beyond. The Biochemical journal. 1998 Nov 15;336 (Pt 1):1-17. PubMed PMID: 9806879. Pubmed Central PMCID: 1219836. Epub 1998/11/10. eng.

123. Freund H, Atamian S, Holroyde J, Fischer JE. Plasma amino acids as predictors of the severity and outcome of sepsis. Annals of surgery. 1979 Nov;190(5):571-6. PubMed PMID: 389183. Pubmed Central PMCID: 1344534. Epub 1979/11/01. eng.

124. Luiking YC, Hallemeesch MM, Vissers YL, Lamers WH, Deutz NE. In vivo whole body and organ arginine metabolism during endotoxemia (sepsis) is dependent on mouse strain and gender. The Journal of nutrition. 2004 Oct;134(10 Suppl):2768S-74S; discussion 96S-97S. PubMed PMID: 15465783.

125. Evoy D, Lieberman MD, Fahey TJ, 3rd, Daly JM. Immunonutrition: the role of arginine. Nutrition. 1998 Jul-Aug;14(7-8):611-7. PubMed PMID: 9684265. Epub 1998/07/31. eng.

126. van de Poll MC, Soeters PB, Deutz NE, Fearon KC, Dejong CH. Renal metabolism of amino acids: its role in interorgan amino acid exchange. The American journal of clinical nutrition. 2004 Feb;79(2):185-97. PubMed PMID: 14749222. Epub 2004/01/30. eng.

127. McDonald KK, Zharikov S, Block ER, Kilberg MS. A caveolar complex between the cationic amino acid transporter 1 and endothelial nitric-oxide synthase may explain the "arginine paradox". The Journal of biological chemistry. 1997 Dec 12;272(50):31213-6. PubMed PMID: 9395443. Epub 1998/02/11. eng.

128. Cynober LA. Plasma amino acid levels with a note on membrane transport: characteristics, regulation, and metabolic significance. Nutrition. 2002 Sep;18(9):761-6. PubMed PMID: 12297216. Epub 2002/09/26. eng.

129. Dizikes GJ, Grody WW, Kern RM, Cederbaum SD. Isolation of human liver arginase cDNA and demonstration of nonhomology between the two human arginase genes. Biochemical and biophysical research communications. 1986 Nov 26;141(1):53-9. PubMed PMID: 3801008. Epub 1986/11/26. eng.

130. Chang CI, Liao JC, Kuo L. Arginase modulates nitric oxide production in activated macrophages. The American journal of physiology. 1998 Jan;274(1 Pt 2):H342-8. PubMed PMID: 9458885. Epub 1998/02/12. eng.

131. Park KG, Heys SD, Harris CI, Steele RJ, McNurlan MA, Eremin O, et al. Arginine metabolism in benign and malignant disease of breast and colon: evidence for possible inhibition of tumor-infiltrating macrophages. Nutrition. 1991 May-Jun;7(3):185-8. PubMed PMID: 1802207. Epub 1991/05/01. eng.

132. Lee J, Ryu H, Ferrante RJ, Morris SM, Jr., Ratan RR. Translational control of inducible nitric oxide synthase expression by arginine can explain the arginine paradox. Proceedings of the National Academy of Sciences of the United States of America. 2003 Apr 15;100(8):4843-8. PubMed PMID: 12655043. Pubmed Central PMCID: 153643. Epub 2003/03/26. eng.

133. Prabhakar SS, Zeballos GA, Montoya-Zavala M, Leonard C. Urea inhibits inducible nitric oxide synthase in macrophage cell line. The American journal of physiology. 1997 Dec;273(6 Pt 1):C1882-8. PubMed PMID: 9435493. Epub 1998/01/22. eng.

134. Boucher JL, Custot J, Vadon S, Delaforge M, Lepoivre M, Tenu JP, et al. N omega-hydroxyl-L-arginine, an intermediate in the L-arginine to nitric oxide pathway, is a strong inhibitor of liver and macrophage arginase. Biochemical and biophysical research communications. 1994 Sep 30;203(3):1614-21. PubMed PMID: 7945311. Epub 1994/09/30. eng.

135. Boger RH, Bode-Boger SM. Asymmetric dimethylarginine, derangements of the endothelial nitric oxide synthase pathway, and cardiovascular diseases. Semin Thromb Hemost. 2000;26(5):539-45. PubMed PMID: 11129410. Epub 2000/12/29. eng.

136. Berka V, Wu G, Yeh HC, Palmer G, Tsai AL. Three different oxygen-induced radical species in endothelial nitric-oxide synthase oxygenase domain under regulation by L-arginine and tetrahydrobiopterin. The Journal of biological chemistry. 2004 Jul 30;279(31):32243-51. PubMed PMID: 15166218. Epub 2004/05/29. eng.

137. Tabor CW, Tabor H. Polyamines. Annu Rev Biochem. 1984;53:749-90. PubMed PMID: 6206782. Epub 1984/01/01. eng.

138. Durante W. Regulation of L-arginine transport and metabolism in vascular smooth muscle cells. Cell Biochem Biophys. 2001;35(1):19-34. PubMed PMID: 11898853. Epub 2002/03/20. eng.

139. Chrzanowska A, Gajewska B, Baranczyk-Kuzma A. Arginase isoenzymes in human cirrhotic liver. Acta Biochim Pol. 2009;56(3):465-9. PubMed PMID: 19636440. Epub 2009/07/29. eng.

140. Palmer RM, Rees DD, Ashton DS, Moncada S. L-arginine is the physiological precursor for the formation of nitric oxide in endothelium-dependent relaxation. Biochemical and biophysical research communications. 1988 Jun 30;153(3):1251-6. PubMed PMID: 3390182. Epub 1988/06/30. eng.

141. Muriel P. Regulation of nitric oxide synthesis in the liver. Journal of Applied Toxicology. 2000 May-Jun;20(3):189-95. PubMed PMID: 10797471. Epub 2000/05/08. eng.

142. Salter M, Knowles RG, Moncada S. Widespread tissue distribution, species distribution and changes in activity of Ca(2+)-dependent and Ca(2+)-independent nitric oxide synthases. FEBS letters. 1991 Oct 7;291(1):145-9. PubMed PMID: 1718778. Epub 1991/10/07. eng.

143. Abu-Soud HM, Stuehr DJ. Nitric oxide synthases reveal a role for calmodulin in controlling electron transfer. Proceedings of the National Academy of Sciences of the United States of America. 1993 Nov 15;90(22):10769-72. PubMed PMID: 7504282. Pubmed Central PMCID: 47859. Epub 1993/11/15. eng.

144. Leiper J, Vallance P. Biological significance of endogenous methylarginines that inhibit nitric oxide synthases. Cardiovascular research. 1999 Aug 15;43(3):542-8. PubMed PMID: 10690326. Epub 2000/02/26. eng.

145. Cooke JP. Does ADMA cause endothelial dysfunction? Arteriosclerosis, thrombosis, and vascular biology. 2000 Sep;20(9):2032-7. PubMed PMID: 10978245. Epub 2000/09/09. eng.

146. Nijveldt RJ, Teerlink T, van Leeuwen PA. The asymmetrical dimethylarginine (ADMA)-multiple organ failure hypothesis. Clinical nutrition. 2003 Feb;22(1):99-104. PubMed PMID: 12553957. Epub 2003/01/30. eng.

147. Vallance P, Leone A, Calver A, Collier J, Moncada S. Accumulation of an endogenous inhibitor of nitric oxide synthesis in chronic renal failure. Lancet. 1992 Mar 7;339(8793):572-5. PubMed PMID: 1347093. Epub 1992/03/07. eng.

148. McBride AE, Silver PA. State of the arg: protein methylation at arginine comes of age. Cell. 2001 Jul 13;106(1):5-8. PubMed PMID: 11461695. Epub 2001/07/20. eng.

149. Osanai T, Saitoh M, Sasaki S, Tomita H, Matsunaga T, Okumura K. Effect of shear stress on asymmetric dimethylarginine release from vascular endothelial cells. Hypertension. 2003 Nov;42(5):985-90. PubMed PMID: 14557285.

150. Pou S, Keaton L, Surichamorn W, Rosen GM. Mechanism of superoxide generation by neuronal nitric-oxide synthase. The Journal of biological chemistry. 1999 Apr 2;274(14):9573-80. PubMed PMID: 10092643. Epub 1999/03/27. eng.

151. Bogle RG, MacAllister RJ, Whitley GS, Vallance P. Induction of NG-monomethyl-L-arginine uptake: a mechanism for differential inhibition of NO synthases? The American journal of physiology. 1995 Sep;269(3 Pt 1):C750-6. PubMed PMID: 7573406. Epub 1995/09/01. eng.

152. Kimoto M, Whitley GS, Tsuji H, Ogawa T. Detection of NG,NG-dimethylarginine dimethylaminohydrolase in human tissues using a monoclonal antibody. Journal of Biochemistry. 1995 Feb;117(2):237-8. PubMed PMID: 7608105. Epub 1995/02/01. eng.

153. Leiper JM, Santa Maria J, Chubb A, MacAllister RJ, Charles IG, Whitley GS, et al. Identification of two human dimethylarginine dimethylaminohydrolases with distinct tissue distributions and homology with microbial arginine deiminases. The Biochemical journal. 1999 Oct 1;343 Pt 1:209-14. PubMed PMID: 10493931. Pubmed Central PMCID: 1220543. Epub 1999/09/24. eng.

154. Jones LC, Tran CT, Leiper JM, Hingorani AD, Vallance P. Common genetic variation in a basal promoter element alters DDAH2 expression in endothelial cells. Biochemical and biophysical research communications. 2003 Oct 24;310(3):836-43. PubMed PMID: 14550280. Epub 2003/10/11. eng.

155. Schulze F, Lenzen H, Hanefeld C, Bartling A, Osterziel KJ, Goudeva L, et al. Asymmetric dimethylarginine is an independent risk factor for coronary heart disease: results from the multicenter Coronary Artery Risk Determination investigating the Influence of ADMA Concentration (CARDIAC) study. American heart journal. 2006 Sep;152(3):493 e1-8. PubMed PMID: 16923419.

156. Mookerjee RP, Jalan R. ADMA correlates with portal pressure in patients with compensated cirrhosis by Vizzutti et al. Eur J Clin Invest 2007; 37: 509-15. European journal of clinical investigation. 2008 Jan;38(1):67-8; author reply 9-70. PubMed PMID: 18173553.

157. Bruins MJ, Soeters PB, Deutz NE. Endotoxemia affects organ protein metabolism differently during prolonged feeding in pigs. The Journal of nutrition. 2000 Dec;130(12):3003-13. PubMed PMID: 11110860. Epub 2000/12/09. eng.

158. Luiking YC, Poeze M, Ramsay G, Deutz NE. Reduced citrulline production in sepsis is related to diminished de novo arginine and nitric oxide production. The American journal of clinical nutrition. 2009 Jan;89(1):142-52. PubMed PMID: 19056593.

159. Wijnands KA, Vink H, Briede JJ, van Faassen EE, Lamers WH, Buurman WA, et al. Citrulline a more suitable substrate than arginine to restore NO production and the microcirculation during endotoxemia. PloS one. 2012;7(5):e37439. PubMed PMID: 22666356. Pubmed Central PMCID: 3362574.

160. Wijnands KA. Reduced arginine production and arginase activity are independent of the cause of

sepsis. . 2008.

161. Yeramian A, Martin L, Serrat N, Arpa L, Soler C, Bertran J, et al. Arginine transport via cationic amino acid transporter 2 plays a critical regulatory role in classical or alternative activation of macrophages. Journal of immunology. 2006 May 15;176(10):5918-24. PubMed PMID: 16670299.

162. Luiking YC, Poeze M, Deutz NE. Arginine infusion in patients with septic shock increases nitric oxide production without haemodynamic instability. Clinical science. 2015 Jan;128(1):57-67. PubMed PMID: 25036556.
163. Tabuchi S, Gotoh T, Miyanaka K, Tomita K, Mori M. Regulation of genes for inducible nitric oxide synthase and urea cycle enzymes in rat liver in endotoxin shock. Biochemical and biophysical research communications. 2000 Feb 5;268(1):221-4. PubMed PMID: 10652239.
164. Modolell M, Corraliza IM, Link F, Soler G, Eichmann K. Reciprocal regulation of the nitric oxide synthase/arginase balance in mouse bone marrow-derived macrophages by TH1 and TH2 cytokines. European journal of immunology. 1995 Apr;25(4):1101-4. PubMed PMID: 7537672.
165. Harry D, Anand R, Holt S, Davies S, Marley R, Fernando B, et al. Increased sensitivity to endotoxemia in the bile duct-ligated cirrhotic Rat. Hepatology. 1999 Nov;30(5):1198-205. PubMed PMID: 10534341.
166. Dwyer JM, Platt BJ, Rizzo SJ, Pulicicchio CM, Wantuch C, Zhang MY, et al. Preclinical characterization of BRL 44408: antidepressant- and analgesic-like activity through selective alpha2A-adrenoceptor antagonism. The international journal of neuropsychopharmacology. 2010 Oct;13(9):1193-205. PubMed PMID: 20047711.
167. Bode-Boger SM, Boger RH, Galland A, Tsikas D, Frolich JC. L-arginine-induced vasodilation in healthy humans: pharmacokinetic-pharmacodynamic relationship. British journal of clinical pharmacology. 1998 Nov;46(5):489-97. PubMed PMID: 9833603. Pubmed Central PMCID: 1873701.
168. Kountouras J, Billing BH, Scheuer PJ. Prolonged bile duct obstruction: a new experimental model for cirrhosis in the rat. British journal of experimental pathology. 1984 Jun;65(3):305-11. PubMed PMID: 6743531. Pubmed Central PMCID: 2040968.
169. Lebrec D, Girod C. Comparison of the circulation between fed and fasted normal and portal hypertensive rats. Journal of pharmacological methods. 1986 Jul;15(4):359-65. PubMed PMID: 3724204.
170. Poo JL, Estanes A, Pedraza-Chaverri J, Cruz C, Perez C, Huberman A, et al. [Chronology of portal hypertension, decreased sodium excretion, and activation of the renin-angiotensin system in experimental biliary cirrhosis]. Revista de investigacion

clinica; organo del Hospital de Enfermedades de la Nutricion. 1997 Jan-Feb;49(1):15-23. PubMed PMID: 9229751. Cronologia de hipertension portal, disminucion de excrecion de sodio y activacion del sistema renina-angiotensina en cirrosis biliar experimental.

171. Martinez-Prieto C, Ortiz MC, Fortepiani LA, Ruiz-Macia J, Atucha NM, Garcia-Estan J. Haemodynamic and renal evolution of the bile duct-ligated rat. Clinical science. 2000 May;98(5):611-7. PubMed PMID: 10781394.

172. Fernandez-Martinez E, Perez-Alvarez V, Tsutsumi V, Shibayama M, Muriel P. Chronic bile duct obstruction induces changes in plasma and hepatic levels of cytokines and nitric oxide in the rat. Experimental and toxicologic pathology : official journal of the Gesellschaft fur Toxikologische Pathologie. 2006 Aug;58(1):49-58. PubMed PMID: 16617007.

173. Liedtke C, Luedde T, Sauerbruch T, Scholten D, Streetz K, Tacke F, et al. Experimental liver fibrosis research: update on animal models, legal issues and translational aspects. Fibrogenesis & tissue repair. 2013 Oct 01;6(1):19. PubMed PMID: 24274743. Pubmed Central PMCID: 3850878.

174. Weiler-Normann C, Herkel J, Lohse AW. Mouse models of liver fibrosis. Zeitschrift fur Gastroenterologie. 2007 Jan;45(1):43-50. PubMed PMID: 17236120.

175. Tarcin O, Basaranoglu M, Tahan V, Tahan G, Sucullu I, Yilmaz N, et al. Time course of collagen peak in bile duct-ligated rats. BMC gastroenterology. 2011 Apr 28;11:45. PubMed PMID: 21527001. Pubmed Central PMCID: 3117813.

176. Williams AL, Hoofnagle JH. Ratio of serum aspartate to alanine aminotransferase in chronic hepatitis. Relationship to cirrhosis. Gastroenterology. 1988 Sep;95(3):734-9. PubMed PMID: 3135226.

177. Sheth SG, Flamm SL, Gordon FD, Chopra S. AST/ALT ratio predicts cirrhosis in patients with chronic hepatitis C virus infection. The American journal of gastroenterology. 1998 Jan;93(1):44-8. PubMed PMID: 9448172.

178. Chou MH, Chuang JH, Eng HL, Tsai PC, Hsieh CS, Liu HC, et al. Effects of hepatocyte CD14 upregulation during cholestasis on endotoxin sensitivity. PloS one. 2012;7(4):e34903. PubMed PMID: 22511970. Pubmed Central PMCID: 3325271.

179. Wiest R, Groszmann RJ. The paradox of nitric oxide in cirrhosis and portal hypertension: too much, not enough. Hepatology. 2002 Feb;35(2):478-91. PubMed PMID: 11826425.

180. Iwakiri Y, Groszmann RJ. The hyperdynamic circulation of chronic liver diseases: from the patient to the molecule. Hepatology. 2006 Feb;43(2 Suppl 1):S121-31. PubMed PMID: 16447289.

181. Gaudin C, Braillon A, Poo JL, Moreau R, Hadengue A, Lebrec D. Regional sympathetic activity, severity of liver disease and hemodynamics in patients with cirrhosis. Journal of hepatology. 1991 Sep;13(2):161-8. PubMed PMID: 1744420.

182. Bomzon A, Binah O, Blendis LM. Hypotension in experimental cirrhosis. Is loss of vascular responsiveness to norepinephrine the cause of hypotension in chronic bile-duct-ligated dogs? Journal of hepatology. 1993 Jan;17(1):116-23. PubMed PMID: 8445210.

183. Sancho-Bru P, Bataller R, Colmenero J, Gasull X, Moreno M, Arroyo V, et al. Norepinephrine induces calcium spikes and proinflammatory actions in human hepatic stellate cells. American journal of physiology Gastrointestinal and liver physiology. 2006 Nov;291(5):G877-84. PubMed PMID: 16782692.

184. Chan CC, Chang CC, Huang HC, Wang SS, Lee FY, Chang FY, et al. Effects of norepinephrine and acetylcholine on portal-systemic collaterals of common bile duct-ligated cirrhotic rat. Journal of gastroenterology and hepatology. 2005 Dec;20(12):1867-72. PubMed PMID: 16336446.

185. Perez-Rivera AA, Hlavacova A, Rosario-Colon LA, Fink GD, Galligan JJ. Differential contributions of alpha-1 and alpha-2 adrenoceptors to vasoconstriction in mesenteric arteries and veins of normal and hypertensive mice. Vascular pharmacology. 2007 May;46(5):373-82. PubMed PMID: 17329171. Pubmed Central PMCID: 3549429.

186. de Franchis R. Updating consensus in portal hypertension: report of the Baveno III Consensus Workshop on definitions, methodology and therapeutic strategies in portal hypertension. Journal of hepatology. 2000 Nov;33(5):846-52. PubMed PMID: 11097497.

187. Kanagy NL. Alpha(2)-adrenergic receptor signalling in hypertension. Clinical science. 2005 Nov;109(5):431-7. PubMed PMID: 16232127.

188. Groszmann RJ. Hyperdynamic state in chronic liver diseases. Journal of hepatology. 1993;17 Suppl 2:S38-40. PubMed PMID: 8491969.

189. Moller S, Hobolth L, Winkler C, Bendtsen F, Christensen E. Determinants of the hyperdynamic circulation and central hypovolaemia in cirrhosis. Gut. 2011 Sep;60(9):1254-9. PubMed PMID: 21504996.

190. Blendis L, Wong F. The hyperdynamic circulation in cirrhosis: an overview. Pharmacology & therapeutics. 2001 Mar;89(3):221-31. PubMed PMID: 11516477.

191. Siniscalchi A, Aurini L, Spedicato S, Bernardi E, Zanoni A, Dante A, et al. Hyperdynamic circulation in cirrhosis: predictive factors and outcome following liver transplantation. Minerva anestesiologica. 2013 Jan;79(1):15-23. PubMed PMID: 23090103.

192. Li Y, Han CD. [Effects of alpha 1-adrenergic receptor subtypes activation on proliferation of smooth muscle cells in rabbit aorta]. Sheng li xue bao : [Acta physiologica Sinica]. 1995 Oct;47(5):498-504. PubMed PMID: 8711515.

193. Miksa M, Das P, Zhou M, Wu R, Dong W, Ji Y, et al. Pivotal role of the alpha(2A)-adrenoceptor in producing inflammation and organ injury in a rat model of sepsis. PloS one. 2009;4(5):e5504. PubMed PMID: 19430535. Pubmed Central PMCID: 2677660.

194. Pinzani M, Milani S, De Franco R, Grappone C, Caligiuri A, Gentilini A, et al. Endothelin 1 is overexpressed in human cirrhotic liver and exerts multiple effects on activated hepatic stellate cells. Gastroenterology. 1996 Feb;110(2):534-48. PubMed PMID: 8566602.

195. Bataller R, Gines P, Nicolas JM, Gorbig MN, Garcia-Ramallo E, Gasull X, et al. Angiotensin II induces contraction and proliferation of human hepatic stellate cells. Gastroenterology. 2000 Jun;118(6):1149-56. PubMed PMID: 10833490.

196. Balasubramaniyan V, Wright G, Sharma V, Davies NA, Sharifi Y, Habtesion A, et al. Ammonia reduction with ornithine phenylacetate restores brain eNOS activity via the DDAH-ADMA pathway in bile duct-ligated cirrhotic rats. American journal of physiology Gastrointestinal and liver physiology. 2012 Jan 01;302(1):G145-52. PubMed PMID: 21903766.

197. Zhang F, Wu R, Qiang X, Zhou M, Wang P. Antagonism of alpha2A-adrenoceptor: a novel approach to inhibit inflammatory responses in sepsis. Journal of molecular medicine. 2010 Mar;88(3):289-96. PubMed PMID: 19894027. Pubmed Central PMCID: 2837104.

198. Mookerjee RP, Tilg H, Williams R, Jalan R. Infliximab and alcoholic hepatitis. Hepatology. 2004 Aug;40(2):499-500; author reply -1. PubMed PMID: 15368459.

199. Elenkov IJ, Wilder RL, Chrousos GP, Vizi ES. The sympathetic nerve--an integrative interface between two supersystems: the brain and the immune system. Pharmacological reviews. 2000 Dec;52(4):595-638. PubMed PMID: 11121511.

200. Straub RH, Schaller T, Miller LE, von Horsten S, Jessop DS, Falk W, et al. Neuropeptide Y cotransmission with norepinephrine in the sympathetic nerve-macrophage interplay. Journal of neurochemistry. 2000 Dec;75(6):2464-71. PubMed PMID: 11080198.

201. Yang S, Zhou M, Chaudry IH, Wang P. Norepinephrine-induced hepatocellular dysfunction in early sepsis is mediated by activation of alpha2-adrenoceptors. American journal of physiology Gastrointestinal and liver physiology. 2001 Oct;281(4):G1014-21. PubMed PMID: 11557522.

202. Seo YS, Shah VH. The role of gut-liver axis in the pathogenesis of liver cirrhosis and portal hypertension. Clinical and molecular hepatology. 2012 Dec;18(4):337-46. PubMed PMID: 23323248. Pubmed Central PMCID: 3540369.

203. Medzhitov R. Origin and physiological roles of inflammation. Nature. 2008 Jul 24;454(7203):428-35. PubMed PMID: 18650913.

204. Buttarelli FR, Fanciulli A, Pellicano C, Pontieri FE. The dopaminergic system in peripheral blood lymphocytes: from physiology to pharmacology and potential applications to neuropsychiatric disorders. Current neuropharmacology. 2011 Jun;9(2):278-88. PubMed PMID: 22131937. Pubmed Central PMCID: 3131719.

205. Barbul A. Arginine and immune function. Nutrition. 1990 Jan-Feb;6(1):53-8; discussion 9-62. PubMed PMID: 2135757.

206. Kirk SJ, Barbul A. Role of arginine in trauma, sepsis, and immunity. JPEN Journal of parenteral and enteral nutrition. 1990 Sep-Oct;14(5 Suppl):226S-9S. PubMed PMID: 2232107.

207. Wilmore D. Enteral and parenteral arginine supplementation to improve medical outcomes in hospitalized patients. The Journal of nutrition. 2004 Oct;134(10 Suppl):2863S-7S; discussion 95S. PubMed PMID: 15465802.

208. Efron DT, Barbul A. Arginine and immunonutrition: a reevaluation. Nutrition. 2000 Jan;16(1):73-4. PubMed PMID: 10674244.

209. Shah V, Haddad FG, Garcia-Cardena G, Frangos JA, Mennone A, Groszmann RJ, et al. Liver sinusoidal endothelial cells are responsible for nitric oxide modulation of resistance in the hepatic sinusoids. The Journal of clinical investigation. 1997 Dec 1;100(11):2923-30. PubMed PMID: 9389760. Pubmed Central PMCID: 508500. Epub 1998/02/12. eng.

210. Nijveldt RJ, Siroen MP, van der Hoven B, Teerlink T, Prins HA, Girbes AR, et al. High plasma arginine concentrations in critically ill patients suffering from hepatic failure. Eur J Clin Nutr. 2004 Apr;58(4):587-93. PubMed PMID: 15042126. Epub 2004/03/26. eng.

211. Nijveldt RJ, Prins HA, Siroen MP, Rauwerda JA, Teerlink T, van Leeuwen PA. Low arginine plasma levels in patients after thoracoabdominal aortic surgery. Eur J Clin Nutr. 2000 Aug;54(8):615-7. PubMed PMID: 10951509. Epub 2000/08/22. eng.

212. Bower RH, Cerra FB, Bershadsky B, Licari JJ, Hoyt DB, Jensen GL, et al. Early enteral administration of a formula (Impact) supplemented with arginine, nucleotides, and fish oil in intensive care unit patients: results of a multicenter, prospective, randomized, clinical trial. Critical care medicine. 1995 Mar;23(3):436-49. PubMed PMID: 7874893. Epub 1995/03/01. eng.

213. Lorente JA, Landin L, Renes E, De Pablo R, Jorge P, Rodena E, et al. Role of nitric oxide in the hemodynamic changes of sepsis. Critical care medicine. 1993 May;21(5):759-67. PubMed PMID: 8482098. Epub 1993/05/01. eng.

214. Durante W, Johnson FK, Johnson RA. Arginase: a critical regulator of nitric oxide synthesis and vascular function. Clin Exp Pharmacol Physiol. 2007 Sep;34(9):906-11. PubMed PMID: 17645639. Pubmed Central PMCID: 1955221. Epub 2007/07/25. eng.

215. Sharma V, Ten Have GA, Ytrebo L, Sen S, Rose CF, Dalton RN, et al. Nitric oxide and L-arginine metabolism in a devascularized porcine model of acute liver failure.

American journal of physiology Gastrointestinal and liver physiology. 2012 Aug 01;303(3):G435-41. PubMed PMID: 22421619. Pubmed Central PMCID: 3774247.

216. Ytrebo LM, Sen S, Rose C, Davies NA, Nedredal GI, Fuskevaag OM, et al. Systemic and regional hemodynamics in pigs with acute liver failure and the effect of albumin dialysis. Scandinavian journal of gastroenterology. 2006 Nov;41(11):1350-60. PubMed PMID: 17060130. Epub 2006/10/25. eng.

217. Tripathi D, Ferguson JW, Kochar N, Leithead JA, Therapondos G, McAvoy NC, et al. Randomized controlled trial of carvedilol versus variceal band ligation for the prevention of the first variceal bleed. Hepatology. 2009 Sep;50(3):825-33. PubMed PMID: 19610055. Epub 2009/07/18. eng.

218. Bellis L, Berzigotti A, Abraldes JG, Moitinho E, Garcia-Pagan JC, Bosch J, et al. Low doses of isosorbide mononitrate attenuate the postprandial increase in portal pressure in patients with cirrhosis. Hepatology. 2003 Feb;37(2):378-84. PubMed PMID: 12540788. Epub 2003/01/24. eng.

219. Loureiro-Silva MR, Iwakiri Y, Abraldes JG, Haq O, Groszmann RJ. Increased phosphodiesterase-5 expression is involved in the decreased vasodilator response to nitric oxide in cirrhotic rat livers. Journal of hepatology. 2006 May;44(5):886-93. PubMed PMID: 16545481. Epub 2006/03/21. eng.

220. Matei V, Rodriguez-Vilarrupla A, Deulofeu R, Garcia-Caldero H, Fernandez M, Bosch J, et al. Three-day tetrahydrobiopterin therapy increases in vivo hepatic NOS activity and reduces portal pressure in CCl4 cirrhotic rats. Journal of hepatology. 2008 Aug;49(2):192-7. PubMed PMID: 18534709. Epub 2008/06/07. eng.

221. Cardounel AJ, Cui H, Samouilov A, Johnson W, Kearns P, Tsai AL, et al. Evidence for the pathophysiological role of endogenous methylarginines in regulation of endothelial NO production and vascular function. The Journal of biological chemistry. 2007 Jan 12;282(2):879-87. PubMed PMID: 17082183. Epub 2006/11/04. eng.

222. Vizzutti F, Romanelli RG, Arena U, Rega L, Brogi M, Calabresi C, et al. ADMA correlates with portal pressure in patients with compensated cirrhosis. European journal of clinical investigation. 2007 Jun;37(6):509-15. PubMed PMID: 17537159. Epub 2007/06/01. eng.

223. Wang J, Sim AS, Wang XL, Wilcken DE. L-arginine regulates asymmetric dimethylarginine metabolism by inhibiting dimethylarginine dimethylaminohydrolase activity in hepatic (HepG2) cells. Cellular and Molecular Life Sciences. 2006 Dec;63(23):2838-46. PubMed PMID: 17075694. Epub 2006/11/01. eng.

224. Ito A, Tsao PS, Adimoolam S, Kimoto M, Ogawa T, Cooke JP. Novel mechanism for endothelial dysfunction: dysregulation of dimethylarginine dimethylaminohydrolase. Circulation. 1999 Jun 22;99(24):3092-5. PubMed PMID: 10377069. Epub 1999/06/22. eng.

225. Mookerjee RP, Balasubramaniyan V, Mehta G. ADMA and hepatic endothelial dysfunction in cirrhosis--the DDAH isoform is the key. Liver international : official journal of the International Association for the Study of the Liver. 2012 Aug;32(7):1186; author reply 7. PubMed PMID: 22574915.

226. Nelin LD, Nash HE, Chicoine LG. Cytokine treatment increases arginine metabolism and uptake in bovine pulmonary arterial endothelial cells. Am J Physiol Lung Cell Mol Physiol. 2001 Nov;281(5):L1232-9. PubMed PMID: 11597915. Epub 2001/10/13. eng.

227. Richir MC, van Lambalgen AA, Teerlink T, Wisselink W, Bloemena E, Prins HA, et al. Low arginine/asymmetric dimethylarginine ratio deteriorates systemic hemodynamics and organ blood flow in a rat model. Critical care medicine. 2009 Jun;37(6):2010-7. PubMed PMID: 19384222. Epub 2009/04/23. eng.

228. Lopez-Talavera JC, Cadelina G, Olchowski J, Merrill W, Groszmann RJ. Thalidomide inhibits tumor necrosis factor alpha, decreases nitric oxide synthesis, and ameliorates the hyperdynamic circulatory syndrome in portal-hypertensive rats. Hepatology. 1996 Jun;23(6):1616-21. PubMed PMID: 8675185.

229. Mehta G, Mookerjee RP, Sharma V, Jalan R. Systemic inflammation is associated with increased intrahepatic resistance and mortality in alcohol-related acute-on-chronic liver failure. Liver international : official journal of the International Association for the Study of the Liver. 2015 Mar;35(3):724-34. PubMed PMID: 24703488.

230. Roulot D, Braillon A, Gaudin C, Ozier Y, Girod C, Lebrec D. Mechanisms of a clonidine-induced decrease in portal pressure in normal and cirrhotic conscious rats. Hepatology. 1989 Oct;10(4):477-81. PubMed PMID: 2777209.

Appendix

Publications related to the work in this thesis