

# **Neutrophil chemotaxis in liver failure**

By  
**Dr. Hazem Helmy Mohamed**

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**Doctor of Philosophy**  
**(Ph.D.)**

**Liver Unit, Internal Medicine,  
Department of Clinical and Surgical Sciences, Faculty  
of Medicine, Edinburgh University.**

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DEDICATION

FOR MY PARENTS,  
MY WIFE, AND CHILDREN,  
SHADY, ALIAA, AND HADY

## **DECLARATION**

I hereby declare that the thesis is based on the results of my own experiments and that the thesis is exclusively of my own composition. This work was undertaken at the University of Edinburgh, Liver Research Laboratories, Department of Medicine (Clinical and Surgical Sciences), Royal Infirmary of Edinburgh. The data presented in this thesis has not been submitted previously for any other degree.

Hazem Helmy Mohamed  
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## **ABSTRACT**

**Background** Infection is a serious complication in patients with acute and chronic liver failure. Neutrophil chemotaxis is an important body defense mechanism against infection, which has been reported to be defective in such patients. However, the newly described family of potent chemoattractant cytokines (chemokines) and their relationship to neutrophil chemotaxis has not been previously studied in patients with acute and chronic liver failure.

**Aim of the thesis** To identify the presence of and characterise the neutrophil chemotactic defect to chemokines in patients with acute and chronic liver failure.

**Patients and methods** Neutrophils were isolated from patients with acute and chronic liver failure, and controls. Chemotaxis was determined using the CXC chemokines Interleukin-8 (IL-8) and Growth related oncogen- $\alpha$  (Gro- $\alpha$ ) in a 48 well-modified Boyden chamber assay. Serum chemokine concentrations were measured using ELISA and neutrophil CXC chemokine receptors expression by flow cytometry.

**Results** Neutrophil chemotaxis to IL-8 and Gro- $\alpha$  was reduced in patients with acute and chronic liver failure either due to alcoholic liver disease or hepatitis C compared with controls. This impairment was correlated with the severity of the disease. A partial correction in chemotaxis of patients' neutrophils was observed after cross incubation with the control sera and vice versa using control neutrophils and patients' sera. Neutrophil chemotaxis in patients with chronic liver failure is further reduced 2 hours after oral administration of an amino acid solution, which simulates human blood. Neutrophils isolated from the portal venous blood had similar chemotactic defects to neutrophils isolated from peripheral venous blood. However, chemotaxis was significantly reduced in neutrophils isolated from hepatic venous blood compared to neutrophils isolated from portal or peripheral blood. In cross over studies, portal neutrophil chemotaxis was significantly reduced after incubation with hepatic venous serum and vice versa. The CXC chemokines IL-8, IFN- $\gamma$ -inducible protein (IP-10), and Monokine Induced by Interferon- $\gamma$  (Mig) were significantly elevated in patients with both acute and chronic liver failure compared with controls. There was no significant

difference in neutrophil expression of both CXCR1 and CXCR2 chemokine receptors in patients with either acute or chronic liver failure compared with the controls.

**Conclusion** Neutrophil chemotaxis to CXC chemokines is impaired in patients with either acute or chronic liver failure. There is a functional defect in both CXCR1 and CXCR2 chemokine receptors. Impaired neutrophil chemotaxis may contribute to the increased risk of infection in these patients. This impairment in chemotaxis may be due to CXC receptor desensitisation caused by circulating humeral factor/s plus an intrinsic defect of the neutrophils.



## **LIST OF ABBREVIATIONS**

AFLP,	acute fatty liver of pregnancy
ALD,	alcoholic liver disease.
ALF,	acute liver failure.
ALT,	Alanine transferase.
ANOVA,	analysis of variance.
ARDS,	adult respiratory distress syndrome.
BCA-1,	B cell attracting chemokine-1.
BLC,	B-lymphocyte chemoattractant.
CAH,	chronic active hepatitis.
CCL4,	carbon tetrachloride.
CFI,	chemotactic factor inactivator.
CLF,	chronic liver failure.
CMV,	cytomegalovirus.
DAG,	diacylglycerol.
DC,	dendritic cells.
DIC,	disseminated intravascular coagulopathy.
DNA,	deoxy ribonucleic acid.
E coli,	escherichia coli.
ELISA,	enzyme linked immuno sorbent assay.
ELR,	glutamine, leucine, and arginine.
ENA-78,	epithelial cell-derived neutrophil attractant-78.
ET,	endotoxin.
FACs,	fluorescence-activated cell sorting.
FCA,	fetal calf albumin.
FHF,	fulminant hepatic failure.
FiTC,	fluorescein isothiocyanate.
FMLP,	formylmethionyl-leucyl-phenylalamine.
FSCs,	fat storing cells.
GCP-2,	granulocyte chemotactic protein-2.
Gro,	growth related oncogene.
HAV,	hepatitis A virus.
HBcAg,	hepatitis B core antigen.
HBsAg,	hepatitis B surface antigen.
HBV,	hepatitis B virus.
HCC,	hepatocellular Carcinoma.
HCV,	hepatitis C virus.
HDV,	hepatitis D virus.
HE,	hepatic encephalopathy.
HELLP,	haemolysis, elevated liver enzymes and low platelets.
HEV,	hepatitis E virus.
HIV,	human immune deficiency virus.

HPF,	high power field.
hr,	human recombinant.
HSV-1,	herpes simplex virus type 1.
ICAM-1,	intracellular adhesion molecule-1.
IFN- $\gamma$ ,	interferon-gamma.
Ig,	immunoglobulin.
IL,	interleukin.
IP-10,	interferon $\gamma$ inducible protein-10.
KDa,	kilo dalton.
LARC,	liver and activation regulated chemokine.
LPS,	lipopolysaccharides.
LTB <sub>4</sub> ,	leukotriene B <sub>4</sub> .
Mac-1,	macrophage-1 antigen.
MAPK,	mitogen associated protein kinases.
MCP,	monocyte chemotactic protein.
Mig,	monokine induced by interferon $\gamma$ .
MIP-1 $\alpha$ ,	macrophage-inflammatory protein-1 $\alpha$ .
MMP,	matrix metalloproteinases.
MO,	microorganism.
NAC,	N-acetylcysteine.
NANBNC,	non A non B non C.
NAP-2,	neutrophil-activating peptide-2.
NAPQL,	N-acetyl-p-benzoquinoneimine.
NFKB,	nuclear factor-Kappa B.
NK cells,	natural killer cells.
ORFs,	open reading frames.
PBC,	primary biliary cirrhosis.
PBS,	phosphate buffer saline.
PBSF,	pre-B-cell stimulatory factor.
PF-4,	platelet factor-4.
PKC,	protein kinase subtype C.
PMNL,	polymorphonuclear leukocytes.
POD,	paracetamol overdose.
PT,	Prothrombin time.
PVP,	polyvinylpyrrolidone.
RANTES, secreted.	regulated upon activation, normal T cell expressed and
RNA,	ribonucleic acid.
SDF-1,	stromal cell-derived factor-1.
STAT,	signal transduction and activator of transcription.
TARC,	thymus and activation regulated chemokine.
TECK,	thymus-expressed chemokine.
TFA,	trifluoroacetyl.
TGF-1 $\beta$ ),	Tumour Growth Factor-1 $\beta$ .

TIMP, tissue inhibitor of matrix metalloproteinases.  
TIPSS, transjugular intrahepatic portosystemic stent shunt.  
TNF- $\alpha$ , tumour necrosis factor-alpha.  
VOD, veno occlusive disease.

## **AIMS OF THE THESIS**

The aims of the thesis were:

① To identify the presence of and characterise the neutrophil chemotactic defect induced by the potent CXC chemokines IL-8 and Gro- $\alpha$  in patients with acute and chronic liver failure compared with control subjects. The effects of different aetiologies and severity of liver diseases on neutrophil chemotaxis were also investigated. ② To determine the effect of one of the important chronic complications of liver disease, namely upper gastrointestinal bleeding on neutrophil chemotaxis. ③ The circulatory concentrations of the CXC chemokines were also studied. The studies also investigated the possibility of differential change in neutrophil chemotaxis and chemokine concentrations across the liver.

## **CHAPTER 1**

### **GENERAL INTRODUCTION**

## **1.1 LIVER**

The liver is one of the largest organs in the body weighting 1200-1500g. It has a dual blood supply via portal vein and hepatic artery. Both vessels enter the liver at the porta hepatis accompanied by the bile duct. The right, middle and left hepatic veins drain blood into the inferior vena cava just before its entry to the right atrium of the heart. The hepatic lobule is the basic unit of the liver, which is described as a pyramidal lobule with central branch of hepatic vein and a peripheral portal tract. Each portal tract contains branches of bile duct, portal vein and hepatic artery. Connecting the portal tract and central vein are single columns of hepatocytes with intervening biliary canaliculi and capillary sinusoids {Kiernan 1833 and Sherlock 1993}. Rappaport described the functional liver acini and divided each of these acini into 3 zones. Each liver acinus contains central portal triad (zone 1) interdigitated mostly perpendicularly with terminal hepatic veins of the adjacent acini (zone 3). The area in between is considered as zone 2 (Figure 1.1) {Rappaport 1976}. The differential metabolic functions of each zone are demonstrated in table 1.1 {Gumucio and Miller 1981}. The cellular components of the liver include hepatocytes and sinusoidal cells, which consist of endothelial cells, Kupffer cells (the largest tissue macrophage population in the body), fat storing or Stellate cells, and natural killer cells, which may be positive or negative (NK+ and NK-) for T cell markers {Wisse et al 1985}. The functions of each type of cells are included in Table 1.2 {Finlayson and Bouchier 1995}. This anatomical situation and cellular make-up allows the liver to play a central role in the bodies response to infections.

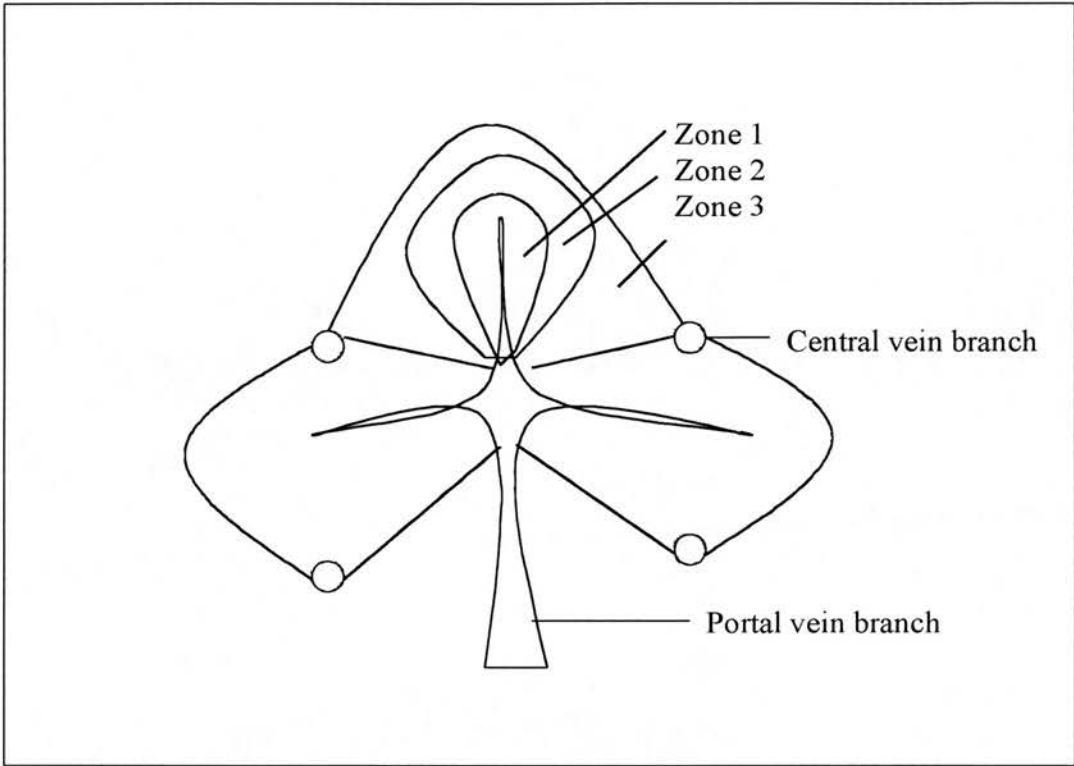


Figure 1.1: Functional acinus of the liver demonstrating the zones (adapted from Sherlock 1993).

Table 1.1: Zonal metabolic functions of the liver.

	Zone 1 (peri-portal zone)	Zone 3 (central zone)
Carbohydrates	Gluconeogenesis	Glycolysis
Proteins	Synthesis of albumin and fibrinogen	Synthesis of albumin and fibrinogen
Glutathione	++	-
Oxygen supply	+++	+
Bile formation		
Bile salt dependent	++	-
Non-bile salt dependent	-	++
Sinusoids	Small and highly anastomotic	Straight and radial.

Table 1.2: Liver cells and their functions.

Cell type	Functions
Hepatocytes	These are the main type of cells of the liver that are concerned with metabolism (carbohydrates, protein and fat), protein synthesis (albumin, fibrinogen and prothrombin), bile formation and cytokine production {Sherlock 1993 and Knolle and Gerken 2000}.
Kupffer cells	Phagocytosis of large particles such as aged cells, tumour cells, bacteria, endotoxin and viruses, potent sources of prostaglandins {Brouwer et al 1988} and cytokines {Andus et al 1991}.
Endothelial cells	Control the exchange of fluid and particles to and from space of Disse and the hepatocytes, and play an important role in defense against viral infection {Wisse et al 1985}.
Stellate cells	Store vitamin A and the other fat soluble vitamins {Hendriks et al 1987}, secrete collagen types 1, 3, 4 and laminin, regulate the sinusoidal blood flow {Bhathal et al 1985}, and cytokine production.
NK cells	Mobile killer lymphocytes, forming an important component of the innate immune response {Shi 2001}.



## **1.2 LIVER FAILURE**

Hepatocellular failure can complicate most of the diseases of the liver. It may complicate viral hepatitis, drugs, alcohol and chronic cholestasis. Although the clinical manifestations may differ according to the aetiology of the disease, the overall picture and treatment are similar. Changes in hepatic pathology, especially necrosis, are not consistent in patients with liver failure and so, it is generally considered to be a functional syndrome. The syndrome of liver failure may comprise some or all of the clinical features shown in table 1.3 reflecting defective liver functions {O'Grady and Williams 1990} and {Shakil et al 2000}. Liver failure is generally sub-classified into acute liver failure in which hepatic encephalopathy develops as a consequence of extensive liver damage in a previously normal liver within 8 weeks of the onset of symptoms, and chronic liver failure in which decompensation in the liver function occurs in a previously diseased cirrhotic liver.

### **1.2.1 Acute Liver Failure (ALF)**

Acute liver failure is a relatively uncommon but dramatic clinical syndrome. The duration between the onset of symptoms and hepatic encephalopathy sub-classifies the syndrome into acute liver failure if it is < 8 weeks and subacute liver failure if this duration is 8 - 26 weeks {Trey and Davidson 1970 and Gimson et al 1982}. The mortality is high and may approach 50-90% of cases depending on the aetiology of the disease and the timing of medical intervention {Trey 1972, and Plevris 1998}.

#### **1.2.1.1 Aetiology**

The causes of acute liver failure are shown in table 1.4. Worldwide, the main cause of ALF is viral hepatitis especially hepatitis B. The incidence of the other causes varies from country to country such as in case of paracetamol (acetaminophen) overdose-induced ALF that predominates in UK and USA. Population studies of the aetiological distribution of ALF in different countries are summarised in Table 1.5 {Lee and Schiodt 1999}.

Table 1.3: The main clinical features of liver failure.

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Jaundice
Fetor hepaticus
Hepatic encephalopathy
Ascites
Skin changes (Spider telangiectasis, palmer erythema and white nails)
Endocrine changes (Hypogonadism, gynaecomastia and feminisation)
Hypoglycaemia
Coagulopathy

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Table 1.4: Causes of acute liver failure.

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Common causes	
Viral causes	Hepatitis A, B, NANBNC, Delta or E viruses, Cytomegalo-virus, Epstein-Barr, and other rare viruses such as Herpes simplex and Yellow fever viruses.
Drug overdose	Paracetamol in UK and USA.
Idiosyncratic drug reaction	Halothane, isoniazid, valproic acid, rifampicin, ecstasy (amphetamine like action) in UK and others.
Other toxicity	Mushroom ( <i>Amanita phalloids</i> )
Metabolic	Wilson's disease.
Pregnancy related	HELLP syndrome and acute fatty liver of pregnancy.
Rare causes	
	Reye's syndrome in children 5-15 years old.
	Budd-Chiari syndrome.
	Sepsis, ischaemic hepatitis, spontaneous rupture of the liver and malignant infiltration.

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*NANBNC*=non A non B non C,

*HELLP* = haemolysis, elevated liver enzymes and low platelets.

Table 1.5: Aetiological distribution of FHF in different countries represented as frequency of cases (per cent).

	USA <sup>1</sup>	UK <sup>2</sup>	France <sup>3</sup>	India <sup>4</sup>
Acetaminophen	20	73	3	-
Drug reaction	13	3	17	6
Non A non B	15	8	20	66
Hepatitis B	10	3	32	29
Hepatitis A	6	3	4	3
Delta virus	-	-	-	13
Wilson's disease	5	3	3	3
Pregnancy	1	2	-	-

1) Schiodt et al 1997.

2) Williams 1997.

3) Chow et al 1989.

4) Fagan and Williams 1990.

#### **1.2.1.1.1 Hepatitis A (HAV)**

HAV is a highly infectious ribonucleic acid (RNA) enterovirus (picorna virus), which is directly cytopathic for liver cells. The mode of transmission of HAV is by the faeco-oral route. ALF due to hepatitis A is more common in elderly patients than children and young adults. The survival rate for HAV induced ALF is more than 50%, but the mortality is increased in elderly patients {Fagan and Williams 1990}. There is no carrier state. Infection and hence ALF can be prevented by active immunization with inactivated virus {Binn et al 1986}.

#### **1.2.1.1.2 Hepatitis B (HBV)**

HBV is a hepadna virus that comprises a capsule, a core of deoxy ribonucleic acid (DNA), and DNA polymerase enzyme {Okamoto 1990}. It is transmitted parenterally, either by blood transfusions, shared contaminated needles or sexual contact. Unlike HAV, HBV is not directly cytopathic for hepatocytes, but the liver injury is induced by the host's immune system attacking viral-infected hepatocytes {Brecht et al 1984}. Acute hepatitis B is also seen with activation of latent chronic hepatitis B after withdrawal of chemotherapy. HBV mutations in the precore region of the genome, termed HbeAg negative mutant can also cause acute hepatitis B as this mutation can preclude secretion of HbeAg {Sato et al 1995}. The prevalence and the patterns of HBV infection in the world are shown in Table 1.6 {Margolis et al 1991 and Raakow et al 1994}. Both active and passive vaccinations against HBV are available and can prevent development of ALF with the recombinant DNA vaccine that provides active immunization with 95% success rate {Hilleman 1985}.

Table 1.6: The prevalence and pattern of HBV infection.

Prevalence	High	Intermediate	Low
Carrier state	10-20%	3-5%	0.1-2%
Geographical distribution	Southeast Asia, China, Pacific islands, Sub-Saharan Africa, and Alaska.	Mediterranean basin, Eastern Europe, Central Asia, Japan, Latin and South America, Middle East.	USA, Canada, West Europe, Australia, New Zealand.
Predominant age affected	Perinatal and early childhood	Early childhood	Adults

#### **1.2.1.1.3 Hepatitis D (HDV)**

HDV is a defective RNA virus dependent for replication and infection on helper functions provided by the HBV {Papaevangelou and Tassopoulos 1986}. HDV infection is located in countries with high local prevalence of HBV infection. The mode of transmission is similar to that of the HBV. Superinfection is more likely to induce ALF compared with co-infection. HDV can cause severe acute hepatitis and acute liver failure, which is limited by recovery from HBV infection {Rizzetto and Smedile 1999}. Vaccination against HBV prevents HDV infection {Da Villa et al 1995}.

#### **1.2.1.1.4 Hepatitis E (HEV)**

HEV is a RNA-containing virus that is transmitted by the faeco-oral route. In endemic areas, prevalence of HEV infection may reach 5% of children less than 10 years of age and 10-40% in adults older than 25 years of age. Acute HEV infection predominantly exists in developing countries. ALF is more common in pregnant women and is associated with a 25% mortality rate {Lok et al 1992 and Arankalle et al 1992}. No vaccination is available yet for HEV infection.

#### **1.2.1.1.5 Drug induced acute liver failure**

The liver is the main organ responsible for metabolism of most drugs. Many drugs are implicated in development of ALF and the overall incidence may reach up to 15% of cases in certain countries. A few drugs can induce acute liver cell damage in a dose dependent manner such as paracetamol over-dose, but the majority induces such damage in a non-dose dependent manner i.e. an idiosyncratic drug reactions {Lee 1995}.

#### ***Dose dependent drugs***

Paracetamol is a common widely used analgesic and is safe if used in the recommended doses of < 4 g/d. Ingestion of as little as 10 grams of paracetamol may induce severe hepatocellular necrosis. Hepatotoxicity is related to the unstable metabolite of paracetamol, N-acetyl-p-benzoquinoneimine (NAPQI), which is inactivated by

glutathione. After depletion of glutathione stores, NAPQI accumulates and leads to lipid peroxidation and cell damage {Muriel et al 1993}. Paracetamol-induced ALF occurs with reduced doses in individuals who have chronic induction of the P450 system such as in patients treated with anticonvulsants and those with low hepatic glutathione stores such as in fasting conditions and malnutrition {Zimmerman and Maddrey 1995}. N-acetylcysteine (NAC) is the available antidote that acts as a substrate for repletion of glutathione and helps to improve the patients' survival {Makin et al 1995 and O'Grady 1997}. Carbon tetrachloride (CCL<sub>4</sub>) via induction of perivenous zonal necrosis {Lee et al 1993} and yellow phosphorus via inactivation of cytochrome P450 {Dahl and Hodgson 1978} also cause dose dependent hepatotoxicity.

#### ***Non-dose dependent (idiosyncratic) hepatotoxic drugs***

These drugs induce idiosyncratic reactions only in susceptible individuals. Drugs in this group combine with cellular proteins to form drug-protein complexes that act as neoantigens. Individuals at risk of these severe drug reactions are the rapid acetylators, alcoholics and those individuals with genetic variants of the cytochrome P450 isoenzymes {Lee and Dinsdale 1995}. Examples of these drugs are valproic acid {Devictot et al 1992}, cocaine {Mallat and Dhumeaux 1991}, halothane, diclofenac {Jones et al 1998} and isoniazid especially if given with rifampicin as in anti-tuberculous treatment {Pessayre and Larrey, 1991}. Some studies of halothane-induced hepatitis identified specific IgG antibodies against hepatic endoplasmic reticulum proteins such as carboxylesterase. This protein has been found modified by the trifluoroacetyl (TFA) group, which is derived from halothane {Kenna et al 1992 and 1993}.

#### **1.2.1.1.6 Other causes of acute liver failure**

Severe liver diseases may occur during pregnancy e.g. acute fatty liver of pregnancy (AFLP), HELLP syndrome, hepatic rupture, or viral hepatitis especially HEV as mentioned before in section 1.2.1.1.4. Most of these diseases are associated with

preeclampsia and predominate in the second or third trimester except viral hepatitis in which there is no association with preeclampsia with no trimester preference. The maternal mortality rate ranges from 1-3% in HELLP, 15% in AFLP, and up to 60% in hepatic rupture. The foetal mortality is similar to the maternal mortality except it is higher and may reach up to 35% in HELLP syndrome {Lee and Schiodt 1999}.

Inborn errors of metabolism may induce acute liver failure in the first year of life such as galactosaemia, fructose intolerance, tyrosinaemia, and neonatal haemochromatosis. Wilson's disease and alpha-1-antitrypsin deficiency may cause ALF in older children and adults {Balistreri 1997}. Without transplantation, acute Wilson's disease is severe and almost always fatal. Patients with acute Wilson's disease mostly show previously recognised underlying cirrhosis. This may create some argument about the fulfilment of the criteria for definition of acute liver failure. The clinical features of acute Wilson's disease may include severe hyperbilirubinaemia due to copper-induced haemolysis, and low alkaline phosphatase concentration {Schilsky et al 1994}.

Other rare causes of acute liver failure are listed in Table 1.4 and include Budd-Chiari syndrome, veno-occlusive disease, and autoimmune hepatitis. Budd-Chiari syndrome is characterized by occlusion of the hepatic veins, with severe upper abdominal pain, ascites, and clotting abnormalities {Langnas and Sorrell 1993}. Veno-occlusive disease is a frequent complication in bone marrow-transplanted patients occurring in 54% of cases and is associated with a high mortality (39%). Patients with VOD have hepatomegaly, ascites, and abnormal liver function in the early post-transplant period {McDonald et al 1993}. Malignancy via massive hepatic infiltration from metastasis or lymphoma may induce acute liver failure. Rarely, liver transplantation is indicated for treatment in some of these cases, which are almost always fatal {Krauss et al 1979 and Woolf et al 1994}.



### **1.2.1.2. Pathogenesis**

The mechanisms leading to acute liver failure may involve Kupffer cells {Peltekian et al 1997} and the cytokine network {Andus et al 1991}. The initial stimulus may induce multiple inflammatory cascades that may start with secretion of proinflammatory mediators such as IL-1, IL-6, TNF- $\alpha$  and endotoxin followed by upregulation of other mediators such as adhesion molecules {Essani et al 1995} and nitric oxide {Nathan 1992}. Neutrophils can be recruited into the liver by these inflammatory mediators such as TNF- $\alpha$ , activated complement factors {Jaeschke et al 1993}, and Kupffer cell-induced oxidant stress and injury {Jaeschke and Farhood 1991}. Neutrophils accumulate in sinusoids and hepatic venules then migrate out of the vessels and injure parenchymal cells inducing hepatocellular injury {Jaeschke et al 1991}. The process of neutrophil transendothelial extravasation is dependent on neutrophil  $\beta_2$  integrins and intracellular adhesion molecule-1 (ICAM-1) receptor on endothelial cells {Smith 1992}. In acute liver failure, serum IL-1, IL-6 and TNF- $\alpha$  concentrations are increased {Sheron et al 1990}. These mediators stimulate the liver to produce acute phase proteins such as C reactive protein and the protease inhibitor  $\alpha$ -antitrypsin, and may play a role in the pathogenesis of multiorgan failure {Izumi et al 1994}.

### **1.2.1.3. Clinical presentation and complications of ALF**

The clinical presentation of ALF is dependent on the aetiology. Symptoms may be nonspecific such as nausea, vomiting, abdominal pain and dehydration. With loss of liver functions, hypoglycaemia, coagulopathy, and hepatic coma occur {Lee and Schiødt 1999}. The important clinical features of acute liver failure are shown in Table 1.7 {Schiff et al 1999}.

The mechanism of hepatic encephalopathy in liver failure is still unclear. H.E. is classified into mild impairment (grades 1 and 2) and severe impairment in the level of consciousness (grades 3 and 4), which is associated with higher mortality. Cerebral oedema occurs in 80% of patients with H.E. grade 4 {Lee 1993} and often leads to

increased intracranial pressure with possible risk of herniation of the uncus and subsequent cerebral ischaemic injury {Bernuau et al 1986}. Cerebral complications may be partly due to raised endogenous benzodiazepines concentration {Grimm et al 1988}. Hypoglycaemia is a common metabolic complication caused by impaired gluconeogenesis, and decreased glycogen production by the failing liver. Acid-base imbalance, either a respiratory alkalosis due to centrally induced hyperventilation, or metabolic acidosis in case of paracetamol toxicity or hyperlactataemia are common {O'Grady et al 1989 and Bihari et al 1985}. Decreased synthesis of clotting factors I, IV, VII, and IX, leads to marked prolongation of prothrombin time and activated partial thromboplastin time. Disseminated intravascular coagulopathy (DIC) can also complicate patients with ALF {Pernambuco, et al 1993}. Renal failure including oliguria, electrolyte imbalance, and raised serum creatinine is more common in patients with grade IV encephalopathy and following paracetamol overdose.

Renal failure may be due to pre-renal causes, acute tubular necrosis, hepatorenal syndrome, or a combination of these causes {Bihari et al 1986}. Continuous dialysis is the ideal support as it gives less vascular instability than haemodialysis and so is preferable. The cardiovascular manifestations of ALF include hypotension (20%), dysrhythmia, increased cardiac output, low systemic vascular resistance with abnormal oxygen transport and utilization. Pulmonary oedema is a frequent complication seen in up to 40% of patients with ALF, especially those with by cerebral oedema. Adult respiratory distress syndrome (ARDS) is less frequently seen, but associated with an increase in the mortality rate as it may contraindicate transplantation {Trewby et al 1978 and Bihari et al 1986}. The infective complications will be discussed later.

Table 1.7: Clinical manifestations of acute liver failure:

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Cerebral manifestations	Hepatic encephalopathy, Cerebral oedema.
Metabolic manifestations	Hypoglycaemia, respiratory alkalosis or metabolic acidosis, Coagulopathy, and hypophosphataemia especially in paracetamol overdose.
Hyperdynamic syndrome	High cardiac output, Low systemic vascular resistance.
Tissue hypoxia	Hyperlactataemia.
Infections	Bacterial and fungal infections.
Renal	Acute tubular necrosis, especially in paracetamol overdose.
Pulmonary	Adult respiratory distress syndrome and pulmonary oedema.

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## **1.2.2. Chronic Liver Failure**

Chronic liver failure can complicate chronic liver disease of any cause. Chronic hepatitis and cirrhosis are the two main forms of chronic liver disease. These two forms are closely related to each other, as cirrhosis may be the end stage of many forms of chronic hepatitis. Progression of liver cirrhosis may lead eventually to decompensation of liver function and chronic liver failure.

### **1.2.2.1. Aetiology**

There are many causes of chronic liver diseases that may lead to liver failure (Table 1.8). The most common aetiologies are HBV and HCV infections, alcohol, autoimmune hepatitis and primary biliary cirrhosis, which vary in frequency in different countries. HBV is one of the most common causes of chronic liver diseases in endemic areas as shown previously in Table 1.5 and 1.6, while alcoholic liver diseases are more common in Western and American countries {Finlayson and Bouchier 1995}.

#### **1.2.2.1.1. Hepatitis C (HCV)**

HCV is a rapidly replicating RNA flavivirus that has a high degree of genetic diversity. This diversity allows it to escape effective detection by the host's humoral and cellular responses. The mode of transmission is similar to HBV. Previously, blood transfusion was the most common route of transmission that accounted to about 90% of HCV infection {Alter 1995}. Nowadays, blood transfusion accounts for only 15% of HCV transmission but intravenous drug abuse and sharing needles are now considered the major route of transmission of HCV in several countries, especially in the West {Stauber 2000}. The rate of HCV infection is widely variable from being very rare (0.1%) as in Europe and North America {Lee 1993 and Kuwada et al 1994} to a very high rate of up to 16% in isolated areas of Japan {Suou et al 1992}. Most cases of acute HCV infection progress to chronicity {Mosmann and Coffman 1989, and Clerici and Shearer 1994}. Neither active nor passive vaccination is available.

#### **1.2.2.1.2. Alcoholic liver diseases (ALD)**

Alcohol is the most common cause of chronic liver diseases in the Western countries. The actual mechanism of alcohol induced liver injury is still unclear, but the toxic metabolites of alcohol, altered immune reaction to liver cells, and the genetic predisposition of some individuals have a role {Finlayson and Bouchier 1995}. 90-100% of heavy drinkers show evidence of fatty liver but only 10-35% develop alcoholic hepatitis and 8-20% develop cirrhosis {Mezey 1982}. Consistent heavy drinking for a period of 5-10 years can lead to liver cirrhosis. Women tend to develop severe ALD quicker and at lower levels of alcohol consumption than men {Tuyns and Pequignot 1984}. This increase in susceptibility of females may be related to sex-dependent differences in hepatic alcohol metabolism {Teschke and Wiese 1982}, cytokine production {Lynch et al 1994} and gastric metabolism of alcohol {Frezza et al 1990}. Some studies proposed that countries with high intakes of saturated fatty acids have a lower incidence of alcoholic cirrhosis {Nanji and French 1986}. Genetic predisposition has a role in development of ALD. Some studies reported an increased frequency of homozygosity of ADH2\*1 gene, encoding the beta 1 subunit of ADH isoenzyme in patients with alcoholic cirrhosis compared with controls {Lumeng and Crabb 1994} and {Day et al 1991}. Serologic surveys have found an increased prevalence of HCV and HBV in patients with ALD {Koff and Dienstag 1995}. This association may induce more severe form of liver injury than alcohol alone {Mendenhall et al 1993}.

#### **1.2.2.2. Pathogenesis**

The variability in the clinical manifestations and liver function in cirrhotic patients is dependent on the aetiology. Diseases such as chronic alcoholism and viral hepatitis allow persistent and continuous damage to the liver. The immunological response of the host may be triggered and continue to induce liver cell injury and alteration of liver blood flow. This alteration in blood flow deprives the liver parenchymal cells from nutrition as in case of intrahepatic shunts that created by the process of bridging necrosis {Schaffner 1969}.

Table 1.8: Causes of chronic liver failure.

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Infections	Hepatitis B, C, and D viruses.
Toxins	Alcohol and drugs.
Biliary obstruction	Primary and secondary biliary cirrhosis, and primary sclerosing cholangitis.
Metabolic diseases	Haemochromatosis, Wilson's disease, and $\alpha$ 1-antitrypsin deficiency.
Fibrocystic disease	
Hepatic congestion	Budd-Chiari syndrome, veno-occlusive disease, and cardiac failure.
Unknown causes	Autoimmune hepatitis, and cryptogenic cirrhosis.

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The participation of matrix metalloproteinases (MMP) and their specific inhibitors, the tissue inhibitors of matrix metalloproteinases (TIMP), in both the formation and recovery processes of liver fibrosis have been recently reviewed. It has also been reported that activated stellate cells can produce extracellular matrix components, including Type I collagen. In response to liver injury, the hepatic stellate cells change from a quiescent to an activated phenotype {Brenner et al 2000}. This activation process includes a phenotypic change to a myofibroblast-like cell, increased proliferation rate, loss of retinoid stores, increased production of extracellular matrix proteins, chemokines, and cytokines, and contractility. Hepatic stellate cells are activated via the expression of the oncogene subunit c-myc, and nuclear factor-kappaB (NFkappaB), which is induced by oxidative stress, and inhibited by antioxidants such as 1-alpha-tocopherol and butylated hydroxytoluene. The relationship between the activation mechanism of stellate cells and the production and secretion of MMP and TIMP in the formation and recovery process of hepatic fibrosis is still unclear {Okazaki et al 2000}.

#### **1.2.2.3. Clinical manifestation of chronic liver failure**

The clinical manifestations are variable and may include any combination of signs shown in table 1.9 {Finlayson and Bouchier 1995}.

Table 1.9: Manifestations of chronic liver failure.

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General manifestations	Pigmentation, finger clubbing, and low grade fever
Jaundice	
Ascites	
Hepatosplenomegaly	
Circulatory changes	Palmer erythema, cyanosis and spider telangiactasia.
Portal hypertension	Collateral circulation, fetor hepaticus and variceal bleeding.
Endocrine manifestations	Loss of libido and hair loss. Gynaecomastia, testicular atrophy and impotence in men and breast atrophy, irregular menses and amenorrhoea in women.
Hepatic encephalopathy	
Infections	Bacterial and fungal infection, Spontaneous bacterial peritonitis.
Renal manifestations	Hepatorenal syndrome.
Haemorrhagic tendency	Bruises, purpura, epistaxis and menorrhagia.

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### **1.3. INFECTIONS IN LIVER FAILURE**

Infection is a common complication in patients with both acute and chronic liver failure. In such patients, the normal body defence mechanisms can be affected in many ways.

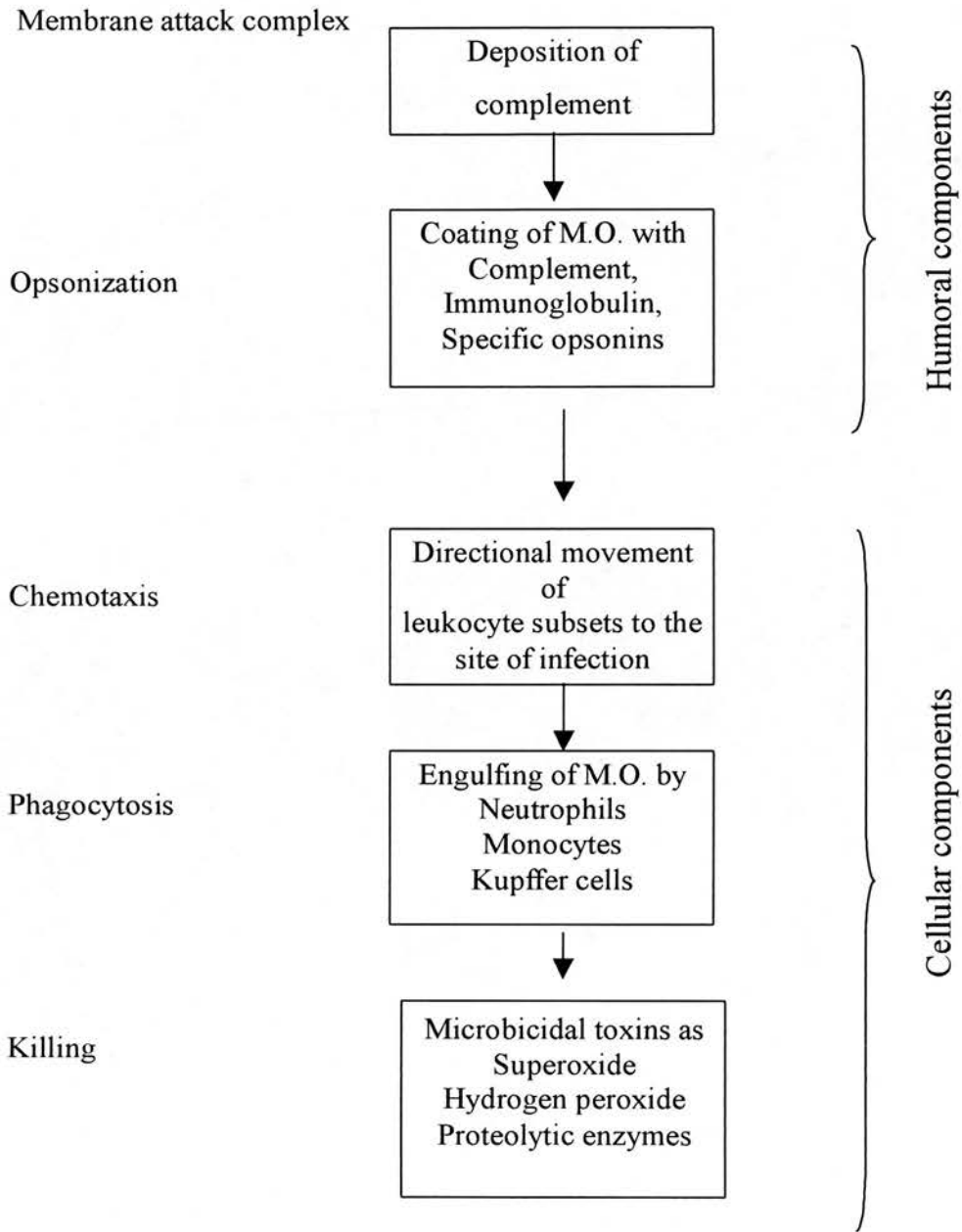
#### **1.3.1. Normal Body Defence Against Infection**

The body has evolved numerous defence mechanisms against infection, including the immune system. The immune system is a complex network, which detects and eliminates foreign material such as bacteria or altered host cells. The immune system is sub-classified into the innate and adaptive immune systems (Table 1.10). Innate immunity exists from birth and may be influenced by species, race, age and sex. It may also be suppressed by malnutrition and alcohol. Once activated, the innate system acts directly and non-specifically in the same manner regardless of the type of the stimulus. Examples of this system are the skin and mucous membranes, which act as a physical barrier against intruding microorganisms; tears and urine flow, which act by sweeping of foreign bodies or microorganisms, and acidity of the stomach, which provides an unsuitable environment for the microorganisms. Activation cascade of complement system, neutrophil and macrophage phagocytosis are components of the innate immune system. The cascade of processes involved in the innate immune system is shown in figure 1.2.

Adaptive immunity comprises a group of acquired specific reactions that are activated after host exposure to certain stimuli; the main cellular component of this type of immunity is the T lymphocyte. This type of immunity takes some time to be activated and induces specific immune products such as antibodies, B lymphocytes and antigen-specific T lymphocytes. The adaptive immune response is sub-classified into active, passive, and adoptive. The active process is normally developed in immune competent individuals against foreign intruders, which may be either true infection or vaccination with an attenuated organism.

Table 1.10: Comparison between the two types of the immune system.

<b>Innate Immunity</b>	<b>Adaptive Immunity</b>
<ul style="list-style-type: none"><li>• Non-specific</li><li>• From birth</li><li>• Immediate response whatever the stimulus</li><li>• The first defense mechanism against exogenous stimuli</li><li>• No discrimination in reactions to different stimuli</li><li>• The cellular components are mainly macrophages and neutrophils</li><li>• The humoral components are complement, lysozymes and chemokines</li></ul>	<ul style="list-style-type: none"><li>• Specific</li><li>• Acquired</li><li>• Latent response depends on, is it the first exposure or re-exposure to the stimulus</li><li>• It may follow the innate system if insufficient or as a complementary to it.</li><li>• Different reactions to different stimuli</li><li>• The cellular component is mainly T lymphocytes</li><li>• The humoral components are antibodies and cytokines</li></ul>



M.O. = microorganism

Figure 1.2: Defence mechanism against infection.

The passive adaptive immunity comprises an administration of immune products such as specific immunoglobulins to make it immediately available in the emergency situations such as accidental exposure to hepatitis B virus. The adoptive process consists of transplantation of immune competent cells or tissue into immune deficient or immunocompromised patients {Parslow and Bainton 1997}.

B-lymphocytes participate in both the innate and the adaptive immunity via production of immunoglobulins that act as opsonins of the innate immune system and contribute to antibody-mediated cell lysis in the adaptive immune system. Natural Killer (NK) cells are large granular lymphocytes, which can activate phagocytes and secrete cytokines. NK cells are readily available cells that have the ability to kill target cells especially virally infected cells via their cytoplasmic granules containing proteolytic enzymes such as perforins and granzyme. NK cells constitute 15% of the peripheral blood lymphocytes and 4% of splenic lymphocytes {Trinchieri 1989 and Yokoyama 1995}, and are commonly found in the liver.

### **1.3.2. Incidence of infection in liver disease**

The incidence of bacterial infection in patients with acute and chronic liver failure is very high, reaching 80% and 50% of cases respectively {Rolando et al 1990} and {Navasa et al 1999}. Infection may be directly responsible for death of 10-60% of patients with acute liver failure {Lee and Schiodt 1999} and {Rolando et al 2000}. Infection was directly responsible for death in 29-44% of patients with chronic liver failure {Toledo et al 1994} and {Rosa et al 2000}. The most common infections are bacteraemia, bacterial peritonitis, pneumonia, urinary tract infections, and infective endocarditis {Jones et al 1967 and Wyke et al 1982}. Infection of skin and plastic devices such as venous catheters are more common in patients with acute liver failure {Iber 1999}. The common causal organisms include *Escherichia coli*, *Pseudomonas aeruginosa*, and *Klebsiella* species, *Staphylococci aureus* and *epidermidis*, and *Streptococci* such as *Pneumococci* {Rolando et al 1990}. Fungal infection is common

and usually due to *Candida albicans*, which occurs in up to one third of patients with acute and chronic liver failure {Lee and Schiodt 1999}.

### **1.3.3. Immune Defects in Acute Liver Failure**

The defects of the immune system reported in patients with acute liver failure are numerous. These defects include defective synthesis or increased consumption of serum complement factors such as C3, C4, C5, the alternative pathway activity, factor B and D activity {Fox et al 1971 and Larcher et al 1982}. Neutrophil bactericidal activity is defective in patients with acute liver failure due to viral hepatitis, but not drug causes. The causes of such defect have been suggested to be due to an intrinsic neutrophil defects, the virus itself {Saunders et al 1978}, or the existence of serum inhibitory factor/s {Bailey et al 1976}. Impaired neutrophil chemotactic activity has also been reported in patients with acute liver failure. This defect was explained by either the existence of inhibitory chemotactic factors {Larcher et al 1981} or serum factors that inhibit the metabolic activity of the leukocyte hexose monophosphate shunt. These factors were further characterised as heat-stable, low molecular weight, and water-soluble substances, which are dialyzable and adsorbable by charcoal {Bailey et al 1976}. Neutrophil adhesion is one of the important primary steps of chemotaxis and is also defective in acute liver failure {Altin et al 1983}. Deficiency of chemotactic factors such as complement factors especially C3 was also considered as one of the causes of the defective neutrophil chemotaxis in patients with acute liver failure. Other defects reported include defective opsonisation of bacteria and yeast, reduced fibronectin {Clark et al 1979 and Naveau et al 1985}, and severely impaired Kupffer cell function {Canalese et al 1981}. Normalisation or reversibility of these defects may occur with the recovery of patients, which was found correlated with improvement in serum concentration of complement factors {Larcher et al 1981}.

### **1.3.4. Immune Defects in chronic liver diseases**

Many immune defects have also been reported in patients with chronic liver diseases and specific defects may vary according to the aetiology of the disease. Portosystemic shunts that develop in patients with cirrhosis may cause spill over of enteric organisms from the portal into systemic circulation allowing bypass of the macrophages in the liver {Mills and Scheuer 1985}. Defects may also include complement factor deficiency such as C3, C4, C5 and C3 PA {DeMeo et al 1971}, occur in nearly 33% of patients with chronic liver diseases {Akalin et al 1985}. A defect in the C3b receptor may lead to defective phagocytic activity of Kupffer cells and has been considered one of the major predisposing factors for infections in patients with liver diseases {Jaffe et al 1978}. Impaired bactericidal function of serum IgM against some types of E. coli was reported in 80% of cirrhotic patients {Fierer et al 1979}. Defective monocyte spreading, chemotaxis, bacterial phagocytosis and bacterial killing are detected in patients with chronic liver failure possibly because of serum inhibitors against chemoattractant factors or reduced synthesis of monocyte lysosomal enzymes {Holdstock et al 1982}. The antimicrobial activities of ascitic fluid, such as opsonisation are also impaired {Fromkes et al 1977 and 1982}.

The specific immune defects may affect different causes of liver disease. For example, in patients with chronic alcoholic liver disease, an intrinsic neutrophil locomotive defect may result from a specific recognition abnormality or defective binding of the serum chemotactic factor C5a {Rajkovic 1984}. Immunoglobulins (Igs), particularly IgA and IgM concentrations are either normal or elevated in patients with alcoholic cirrhosis and portal hypertension {DeMeo et al 1971 and Triger et al 1973}. Depletion of intracellular reduced glutathione and granule enzymes may also lead to defects in phagocytosis and bactericidal activity of neutrophils {Rajkovic et al 1984} in patients with ALD. A direct inhibitory effect of ethanol on leukocyte migration, phagocytosis and intracellular killing has also been reported. Ethanol may also inhibit synthesis of complement factors and cause bone marrow depression leading to granulocytopenia {Brayton et al 1970 and Wyke 1989}. A general reduction in the bactericidal capacity of the tissues was found in

patients with ALD {Rajkovic and Williams 1986}. The elevated serum and tissue ammonia concentration observed in patients with advanced alcoholic liver diseases was found to have an anticomplement effect {Klerx et al 1985}. Malnutrition may have a secondary role in depression of the immune system and predisposition of infection {Wyke 1989}. Histologically, Mills and Scheuer (1985) have also found a decrease in number of hepatic sinusoidal macrophages in proportion to the severity of alcoholic liver disease.

Patients with autoimmune chronic active hepatitis (CAH) have several immunological defects, including defective opsonisation of *Candida albicans* by patients' sera {Wyke et al 1983}, a complement deficiency (minor effect), the immunosuppressive effect of anti-inflammatory steroids and immunosuppressive treatment of on neutrophil adherence, aggregation, degranulation and oxygen radical production {Clark et al 1979}.

Infection is a less frequent complication in patients with primary biliary cirrhosis (PBC), most likely due to the relatively few or minor defects in comparison with other forms of chronic liver diseases. These defects include defective opsonisation in 23% of patients {Wyke et al 1983} and an increase in catabolism of some complement factors {Finlayson et al 1972 and Potter et al 1976} with generation of active fragments. These complement fragments were found to have inhibitory effect on phagocytosis of neutrophils {Tagami et al 1987}. Defective monocyte and neutrophil Fc receptor-mediated phagocytosis have been also detected in some patients with PBC {Loof et al 1987}.

Patients with primary hepatocellular carcinoma (HCC) have impaired chemoattractant activity due to the presence of inhibitory factors against chemotaxis secreted by the tumour cells {Brozna and Ward 1979}, and defective leukocyte production of oxygen-derived free radicals {Uehara et al 1994}.

## **1.4. CHEMOKINES**

Neutrophils chemotaxis in the previous studies was stimulated by different substances such as zymosan, activated autologous plasma, complement factors and immune complexes {Campbell et al 1981} prior to the discovery of the more potent and specific leukocyte chemoattractants, the chemokine group of cytokines. Chemokines are a recently described group of small molecular weight proteins (8-10 KDa), which share 20-90% homology in their amino acid sequences and have the ability to stimulate chemotaxis and activate leukocytes. Chemokines play an important role in the innate and adaptive immune response {Oppenheim et al 1991}.

### **1.4.1. Classification of chemokines**

Chemokines are classified according to the primary amino acid sequence around the first two conserved cysteine residues. The first two cysteines are separated by one amino acid in CXC or  $\alpha$  chemokines and are adjacent to each other in CC or  $\beta$  chemokines. Lymphotactin is the only example of C or  $\gamma$  chemokine in which the first and third of the four cysteines are missing. The CX3C or  $\delta$  chemokines have the first two cysteines separated by three amino acid residues as in Fractalkine/neurotactin {Rollins 1997}.

The CXC chemokines are subclassified into two groups. The first group has a characteristic three amino acids sequence of glutamine, leucine, and arginine, or ELR motif (ELR+ve) at the N-terminal side of the CXC sequence. In the second group, such a motif is lacking, and are described as ELR-ve CXC chemokines. Only the ELR+ve chemokines have specific chemotactic activity for neutrophils {Horuk 1994}.

The CXC chemokine genes are clustered on the human chromosome 4q except the human Pre-B-Cell Stimulatory Factor/Stromal cell-derived Factor-1 (PBSF/SDF-1) gene, which is located on chromosome 10q. The IL-8 gene consists of four exons and three introns and contains NF $\kappa$ B, NF-IL-6, AP-1, AP-2, and AP-3 transcription activation sites within the 5' untranslated region {Mukaida and Matsushima 1992} The 5' untranslated region for Growth related oncogene (Gro- $\alpha$ ,  $\beta$ ,  $\gamma$ ) genes are homologous



to the IL-8 gene up to the position -136 that contain the binding sites for NF $\kappa$ B and AP-3 {Geiser et al 1993}. The CC chemokine genes are located on human chromosome 17q {Shirozu et al and Kennedy et al 1995} and differ from the CXC chemokine genes in that they have three exons and two introns. The Lymphotactin gene is localised to chromosome 1 and the Fractalkine gene is localized to human chromosome 16q {Rollins 1997}.

### **1.4.2. Chemokine receptors**

The cellular effects of chemokines are mediated by binding to their specific receptors on the target cell surface. These receptors are structurally related to the G-protein coupled seven transmembrane domain, which are called the serpentine receptors.

#### **1.4.2.1. CXC chemokine receptors**

At present, five CXC chemokine receptors have been characterised. The CXCR-1 and CXCR-2 share approximately 77% amino acid sequence identity, and only neutrophils express both receptors {Murphy and Tiffany 1991, Lee et al 1992, and Holmes et al 1991}. CXCR-1 binds most avidly with IL-8, whereas CXCR-2 binds to IL-8 and the other ELR+ve CXC chemokines such as Gro, Neutrophil-activating peptide-2 (NAP-2), and Epithelial cell-derived neutrophil attractant-78 (ENA-78). CXCR-3 binds the ELR-ve CXC chemokines Platelet Factor-4 (PF-4), Interferon  $\gamma$  inducible protein-10 (IP-10) and Monokine induced by interferon  $\gamma$  (Mig). CXCR-3 has 40% protein sequence identity with CXCR-1 and CXCR-2 and 35.5% of amino acid sequence identity with the CC chemokine receptors {Loetscher et al 1996}. CXCR-4 has been identified as a necessary cofactor for entry of T cell-tropic HIV into CD4+ cells {Deng et al 1996 and Moore et al 1997}. PBSF/SDF-1 is the ligand for CXCR4, and was found to be a powerful inhibitor of infection by the T cell-tropic HIV-1 strain {Bleul et al 1996 and Oberlin et al 1996}. CXCR5 was cloned on "B" lymphocytes and Burkitt's lymphoma cells, and binds the ELR-ve CXC chemokines B-lymphocyte chemoattractant/B cell attracting chemokine-1 (BLC/BCA-1) {Legler et al 1998}.

#### **1.4.2.2. CC chemokine receptors**

There are at least nine different CC chemokine receptors characterised that bind the CC chemokines. CCR-1 is expressed on monocytes, neutrophils and eosinophils, binding macrophage-inflammatory protein-1 $\alpha$  (MIP-1 $\alpha$ ), Regulated Upon Activation, Normal T Cell Expressed and Secreted (RANTES), and monocyte chemotactic protein-3 (MCP-3) with high affinity and to MIP-1 $\beta$  and MCP-1 with a lower affinity {Neote et al 1993 and Gao et al 1993}. There are two subtypes of CCR-2, CCR-2a and CCR-2b, which have a different protein sequence at the carboxy-terminus. Both receptor subtypes are expressed by monocytes and specifically bind MIP-1 and MCP-3 respectively {Charo et al 1994}. CCR-3 is expressed exclusively on eosinophils with high affinity for eotaxin-1 and 2, and MCP-3 {Kitaura et al 1996}. CCR-4 is expressed on T cells and IL-5-primed basophils. It mediates the biological activities of RANTES, MIP-1 $\alpha$ , Thymus and activation regulated chemokine (TARC), and MCP-1 {Power et al 1995}. CCR-5 is expressed on primary adherent monocytes, and binds MIP-1 $\alpha$ , MIP-1 $\beta$ , and RANTES {Combadiere et al 1995}. CCR-5 is also acts as a fusion cofactor for macrophage-tropic HIV-1 strains {Alkhatib et al 1996}. CCR-6 and its ligand MIP-3 $\alpha$ / Liver and activation regulated chemokine (LARC) were detected in normal pancreatic tissues and over-expressed in the cancerous human pancreatic tissues and the infiltrating macrophages and lymphocytes {Kleeff et al 1999, Tanaka et al 1999}. CCR-7 is expressed on dendritic cells (DCs) and may have a role in tumour cell apoptosis and migration of DCs to the regional lymph nodes {Hirao et al 2000}. Binding of CCR-8 to its ligand I-309 was found to induce a migratory function on leukocytes and similarly, binding of the CCR-9 to its ligand Thymus-expressed chemokine (TECK) was found to induce migration of lymphocytes into intestinal lamina {Papadakis et al 2000}.

#### **1.4.2.3. Erythrocyte chemokine receptors**

Erythrocytes express a chemokine receptor characterised as the Duffy antigen or DARC. It has the ability to bind CXC chemokines such as IL-8, Gro, and NAP-2, and CC chemokines such as MCP-1 and RANTES, but not to MIP-1 $\alpha$  or MIP-1 $\beta$  {Neote et al

1994}. This receptor is localised on the surface of erythrocytes, and recently an isoform of this receptor have been localised on the endothelial cells of the postcapillary venule in the kidney {Hadley et al 1994}. The exact function of this receptor is not known, but it is likely to maintain low plasma concentrations of chemokines to avoid desensitisation of the circulating leukocytes. Table 1.11 shows the recently suggested nomenclature of chemokines, their receptors and ligands.

#### **1.4.2.4. Viral encoding chemokine receptors**

There are viral open reading frames (ORFs) that have been found to encode several chemokine and receptor homologues {Gao et al 1994 and Ahuja et al 1993}. The ORF US28 of human cytomegalovirus (CMV) and the ORF ECRF3 of herpes saimiri virus encoded proteins have a 30% amino acid sequence identity with CCR-1 and 30% amino acid sequence identity with the IL-8 receptors respectively. The CMV chemokine receptor can bind to the CC chemokines MIP-1 $\alpha$ , MIP-1 $\beta$ , MCP-1, and RANTES. The herpes saimiri chemokine receptor binds to the CXC chemokines IL-8, Gro- $\alpha$ , and NAP-2. The role of the viral chemokine receptors in the pathogenesis of viral infection is still to be elucidated. Some viruses are also found to have the ability to synthesise chemokines. Kaposi's sarcoma-associated herpes virus encodes the viral chemokines vMIP-1 and vMIP-11, which have a higher binding affinity to both CXC and CC chemokine receptors than the human chemokines. In contrast to other chemokines, the vMIP-11 was found to inhibit Ca<sup>++</sup> mobilisation induced by the endogenous chemokines and therefore can act as a potent antagonist of chemotaxis. Interestingly, vMIP-11 was also found to block the HIV-1 infection of CD4<sup>+</sup> cell line and peripheral blood mononuclear cells {Kledal et al and Boshoff et al 1997}.

#### **1.4.3. Intracellular signalling**

Binding of chemokines to their receptors stimulates phospholipase C hydrolysis of phosphatidyl inositol 4,5-biphosphate into 4,5-triphosphate (IP3) and diacylglycerol (DAG) synthesis. IP3 can activate both calcium influx and calmodulin dependent protein

kinases, but DAG can stimulate serine/threonine protein kinase C. The chemokine receptors can also stimulate adenylate cyclase, cGMP phosphodiesterases, Ras-GTPase, mitogen associated protein kinases (MAPK), and signal transduction and activator of transcription (STAT) proteins. Stimulation of the different intracellular pathways is likely to determine the specificity of the different members of chemokines {Ahmad and Goldstein 1997}.

#### **1.4.4. Hepatic chemokine synthesis**

The liver consists of several different cell types, each of which may produce chemokines following the appropriate stimuli. In response to TNF- $\alpha$ , IL-1 $\beta$  or carbon tetrachloride (CCL4) primary hepatocyte cultures and hepatoma cell lines can produce both CXC such as IL-8, Gro- $\alpha$ ,  $\beta$ ,  $\gamma$ , ENA-78 and CC chemokines such as RANTES {Rowell et al 1997}. Incubation of rat hepatocytes with ethanol can induce the expression of IL-8 and Gro {Shiratori et al 1994}. Endotoxin and lipopolysaccharide can stimulate Kupffer cells to express TNF- $\alpha$  and/or IL-1, which subsequently induces IL-8 expression in hepatocytes. This is an example of cell-cell (cytokine) communication networks between parenchymal and non-parenchymal cells that may occur within the whole tissue {Thornton et al 1991}. Expression of ENA-78 is induced by the ischaemia-reperfusion reaction and TNF- $\alpha$  secreted by Kupffer cells. The normal fat storing cells (FSCs) or Stellate cells can express MCP-1 in response to TNF- $\alpha$ , IL-1, tumour growth factor-1 $\beta$  (TGF-1 $\beta$ ), or oxygen free radicals. These cells become hypersensitive to these stimuli in the disease states, such as CCL4-induced cirrhosis and fulminant hepatic failure {Czaja et al 1994}. MIP-1 $\alpha$  and MIP-1 $\beta$  are expressed by biliary epithelium during allograft liver rejection in which the biliary epithelium is considered as a target for T cell mediated graft injury. Biliary epithelial cells in culture are also found to secrete IL-8 and MCP-1 in response to pro-inflammatory cytokines {Scholmerich and Holstege 1990}. MIP-1 $\beta$  can also be expressed on the vascular and sinusoidal endothelium of liver grafts undergoing rejection along with MIP-1 $\alpha$  {Adams et al 1996}.

Table 1.11: New nomenclature of human chemokines, receptors and their ligands.  
 {Homey and Zlotnik 1999}.

<b>CXC chemokine</b>	<b>Human ligand</b>	<b>Chemokine receptor</b>
CXCL1	GRO-1, GRO, MGSA-	CXCR2 > CXCR1
CXCL2	GRO2, GRO, MIP-2, MGSA-	CXCR2
CXCL3	GRO3, GRO, MIP-2	CXCR2
CXCL4	PF4	Unknown
CXCL5	ENA-78	CXCR2
CXCL6	GCP-2	
CXCL7	NAP-2	CXCR2
CXCL8	IL-8, MDNCF, NAP-1, NCF	CXCR1, CXCR2
CXCL9	Mig, Humig	CXCR3
CXCL10	IP-10	CXCR3
CXCL11	I-TAC, H174, b-R1	CXCR3
CXCL12	SDF-1 $\alpha$ , SDF-1 $\beta$ , PBSF	CXCR4
CXCL13	BLC, BCA-1	CXCR5
CXCL14	BRAK/bolekine	Unknown
CXCL15	Unknown	Unknown
<b>CC chemokine</b>		
CCL1	I-309	CCR8
CCL2	MCP-1, MCAF	CCR2
CCL3	MIP-1, LD78, LD78, AT464.1	CCR1, CCR5
CCL4	MIP-1, AT744.1, AT744.2, Act-2, G-26	CCR5
CCL5	RANTES	CCR1, CCR3, CCR5
CCL6	Unknown	Unknown
CCL7	MCP-3	CCR1, CCR2, CCR3
CCL8	MCP-2, HC14	CCR2, CCR3
CCL9, CCL10	Unknown	Unknown
CCL11	Eotaxin	CCR3
CCL12	Unknown	CCR2
CCL13	MCP-4, NCC-1, CK-10	CCR2, CCR3
CCL14	HCC-1, HCC-3, NCC-2	CCR1
CCL15	HCC-2, MIP-1, NCC-3, MIP-5, LKn-1	CCR1, CCR3
CCL16	HCC-4, NCC-4, LEC, LMC	CCR1
CCL17	TARC, dendrokine	CCR4
<b>C chemokine</b>		
XCL1	Lymphotactin, SCM-1 $\alpha$ , ATAC	XCR1
XCL2	SCM-1 $\beta$	XCR1
<b>CX3C chemokine</b>		
CX3CL1	Fractalkine, neurotactin	CX3CR1

#### **1.4.5. Leukocyte migration and activation by chemokines**

Leukocyte chemotaxis occurs in five steps. The first step is the primary adhesion through leukocyte adhesion molecules, called selectins, which cause the initial transient contact between leukocytes and endothelial cells. This initial adhesion allows interaction with endothelial expressed chemokines, and further strong secondary adhesion or triggering, mediated by integrins. This step is followed by arrest of leukocytes on the endothelial cell surface with spreading and migration through the endothelial wall into the tissues (Figure 1.3). Leukocyte migration then occurs along a chemokine concentration gradient to the site of inflammation, and is followed by phagocytosis and killing of microorganisms and damaged cells {Butcher and Picker 1996}. Leukocyte adhesion and killing of bacteria is also activated by chemokines {Taub et al 1996}.

#### **1.4.6. Some other biological effects of chemokines**

Chemokines not only control inflammatory cell migration, but are also involved in a number of other biological processes. Generation of new blood vessels (angiogenesis) is vital in the process of wound healing as well as in the pathogenesis of chronic inflammatory conditions. Angiogenesis is also important in tumour growth and metastasis. This process is antagonised by another process, known as angiostasis. CXC chemokines can act as angiogenic and angiostatic factors according to presence of the ELR motif. The ELR<sup>+</sup>ve CXC chemokines such as IL-8 are potent angiogenic factors, in contrast the ELR<sup>-</sup>ve CXC chemokines such as IP-10 are angiostatic. Imbalance between angiostatic and angiogenic chemokines may be responsible for tumour growth and the progression of some inflammatory diseases {Strieter et al 1995}. An example of this imbalance is the study which showed that lung tissue from patients with interstitial pulmonary fibrosis express more IL-8 and less IP-10 compared with control subjects {Keane et al 1997}. This suggests that the angiogenic drive overrides the angiostatic drive that resulted in fibroplasia and extracellular matrix deposition leading to progressive fibrosis and loss of pulmonary function. Another study demonstrated that the squamous cell carcinoma (a subtype of non small cell lung cancer), which is known for its better patient survival, less neovascularisation, and lower incidence of metastasis

showed higher IP-10 expression (angiostatic) than the other subtype; the adenocarcinoma, which is known by its worse prognosis, high vascularisation and metastasis potential {Luan et al 1997}. It is clear that the balance between the two opposing actions that controlling tumour growth and metastasis could be controlled by shifting the chemokine expression from ELR+ve to ELR-ve chemokines and vice versa. Chemokines are also involved in specific migration of tumour cells to certain organs according to the chemokines and chemokine receptors expressed by these target organs {Youngs et al 1997}. Cellular infiltrate is a characteristic finding responsible for the inflammatory reactions in chronic inflammatory and infectious diseases. This cellular recruitment and infiltration to the affected organs or tissues are also believed to be the result of chemokine activity. It is well established that there are antagonistic actions between Th1 cells as a proinflammatory and inflammatory mediators, and Th2 cells as a protective or anti-inflammatory mediators. These antagonistic cellular activities are found to have a close relation with the antagonistic activities of chemokines. It was reported that the chemokines induced by proinflammatory cytokines (Th1 cytokines) such as IFN- $\alpha$ , IL-2, and IL-12, are linked to Th1 cellular infiltration of the inflamed tissues. On the other hand, chemokines induced by anti-inflammatory cytokines (Th2 cytokines) such as IL-4, IL-10, and IL-13 are linked to Th2 responses in monocytes and the other cells {Zlotnik and Rossi 2000}. These findings are also specific to the chemokine receptors. CXCR-3 and CCR-5 are associated with Th1 response and the CCR-3, 4, and 8 are associated with Th2 response {Annunziato et al 1998}. It is still unclear whether this pattern of chemokine receptor expression is only useful for migration of Th1 or Th2 cells into tissues or potentially assists in the differentiation of these cells.

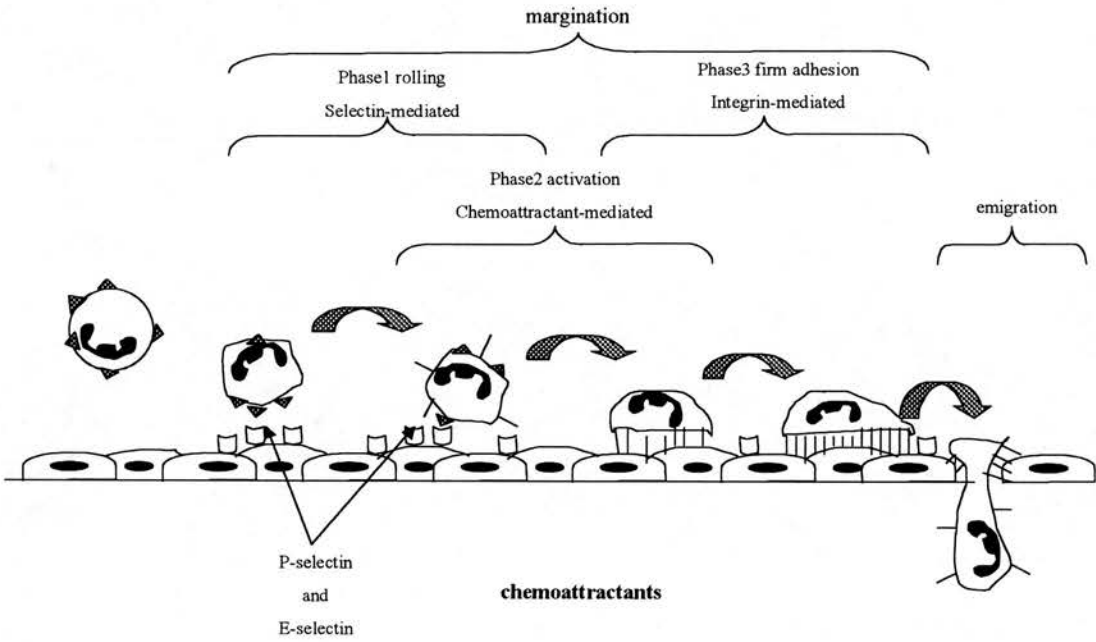


Figure 1.3: Steps of neutrophil chemotaxis (adapted from Springer TA, Annu Rev Physiol 1995;57:827).



Some other chemokines are strongly expressed in normal tissues and in serum without any apparent stimuli or inflammation such as the Human CC Chemokine-1 (HCC-1). These chemokines may act as homeostatic factors and help in maturation of certain types of cells or tissues. TECK is an example of the organ-specific chemokine as it is only found in large quantities in the thymus and was found to play a role in T cell maturation {Schulz-Knappe et al 1996}. Fractalkine is an example of a mixed function chemokine that found to have both inflammatory and homeostatic actions {Bazan et al 1997}. Theoretically, any chemokine capable of inducing the migration of T, NK cells, dendritic cells, and/or macrophages could promote the regression or eradication of a tumour mass by boosting the immune response against the tumour. Recently, several studies demonstrated the potential benefit of some chemokines as adjuvants in antitumour therapy such as IP-10, Mig, MCP-1, MCP-3 and others. Either expression or inoculation of IP-10 or Mig intratumourally results in tumour regression, accompanied by extensive vascular damage and necrosis {Sgadari et al 1996 and 1997}.

**CHAPTER 2**

**MEASUREMENT OF NEUTROPHIL**

**CHEMOTAXIS**

## **2.1 INTRODUCTION AND AIMS**

Neutrophil chemotaxis is impaired in patients with acute {Larcher et al 1981} and chronic liver failure {Rajkovic 1984}. However, in most of the studies, which measured chemotaxis, neutrophils were stimulated by non-specific chemoattractants such as immune complex-activated serum {DeMeo and Andersen 1972} and zymosan activated plasma {Campbell et al 1981}. More recently a large family of small molecular weight chemoattractant proteins have been characterised that act via specific G-protein coupled 7-transmembrane receptors. This family of proteins, called chemokines, has been extensively studied for their ability to activate and induce chemotaxis in many different cell types. The chemokine family can be sub-classified according to their primary amino acid structure, which also has functional significance. Interleukin-8 (IL-8), the prototypical CXC chemokine, acts via the chemokine receptors CXCR1 and CXCR2 expressed on a number of cells and is probably most widely known as a neutrophil chemoattractant. Other CXC chemokines include growth related oncogene (Gro- $\alpha$ ,  $\beta$ , and  $\gamma$ ), and neutrophil activating peptide-2 (NAP-2). These chemokines have a sequence of amino acids; Glutamine-Leucine-Arginine- or ELR-motif, at the N-terminal side of the CXC sequence, which is an absolute requirement for specific receptor binding and neutrophil activation {Clark-Lewis et al 1994}. This ELR motif is a characteristic feature of CXC chemokines that act via CXCR1 or CXCR2 {Baggiolini et al 1994}.

Neutrophils express two types of CXC chemokine receptors, CXCR1 and CXCR2. These receptors share 77% amino acid homology and their genes are co-localised on chromosome 2q35 {Ahuja et al 1992}. Both IL-8 and Gro- $\alpha$  contain the ELR-motif, but there are characteristic differences in receptor binding capacity between these two chemokines {Ludwig et al 1997}. CXCR1 and CXCR2 receptors bind IL-8 with high affinity {Jones et al 1995}, but only CXCR2 binds Gro- $\alpha$  with high affinity {Lee et al 1992}.

This chapter describes the methodology adapted to isolate neutrophils and measure chemotaxis using the CXC chemokines IL-8 and Gro- $\alpha$ , the former acting via CXCR1 and CXCR2 and the latter acting via CXCR2.

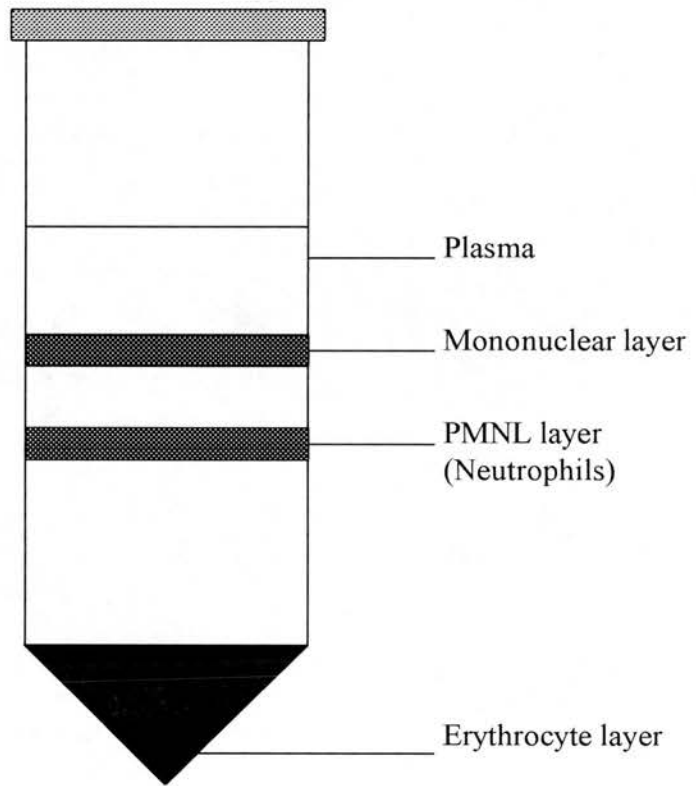
## **2.2 SUBJECTS**

12 healthy members of the laboratory staff, 7 males and 5 females, mean age 35.8 years (SEM  $\pm$  1.4, range 29-45 years) were studied. Repeated measurements of CXC chemokine-stimulated neutrophil chemotaxis in 5 individuals, 3 males and 2 females, mean age 36.2 years (SEM  $\pm$  2.3, range 32-45 years) over time (between 3-10 times) were used to determine the individual variation in neutrophil chemotaxis. Chemotaxis of neutrophils isolated from 36, 30, and 32 years old, healthy control males were measured repeatedly at a single time point and under the same conditions to test the reproducibility of the results of the same control subjects.

## **2.3 MATERIALS AND METHODS**

### **2.3.1 Neutrophil isolation**

3.5-5 ml of venous blood was taken into a heparinised syringe and layered over 5 ml of Polymorphprep<sup>TM</sup> solution (Nycomed Pharma AS, Oslo-Norway). After centrifugation at 500g for 35 minutes at 20°C, two leucocyte bands were visible. The upper band contained the mononuclear cells and the lower one contained the neutrophils. The erythrocytes were pelleted at the bottom of the tube (Figure 2.1). Pasteur pipettes were used to collect the neutrophil band and one volume of 0.45% NaCl solution was added to restore normal osmolality. The cell suspension was washed twice before being resuspended in 10 ml of phosphate buffered saline (PBS, Sigma Chemical Company, UK). The cell suspension was then adjusted to a constant concentration of  $2 \times 10^6$  neutrophil / ml PBS.



PMNL = polymorphonuclear leukocyte.

Figure 2.1: Neutrophil isolation.

### **2.3.2 Trypan blue viability test**

Neutrophil viability was determined by mixing 0.5 ml of trypan blue (Sigma Chemical Company, UK), with 0.3 ml PBS and 0.2ml of the neutrophil suspension. After incubation of the mixture for 10 minutes at room temperature, the viable cells (non-stained) and nonviable cells (stained) were counted. The viability of the neutrophils was constantly  $\geq 97\%$ .

### **2.3.3 Neutrophil Chemotaxis**

Neutrophil chemotaxis was measured using a modified 48 well Boyden chamber method (Figure 2.2). This method is widely used to measure leucocyte chemotaxis due to its relative simplicity compared with the old chambers such as the open-well and blind well chambers {Bignold 1989}. 5  $\mu\text{m}$  thick polycarbonated, polyvinylpyrrolidone (PVP)-free filters (Poretics<sup>®</sup> Products, Livemore-USA) were used which prevent drop-off of cells from the lower surface of the filter into lower compartment of the chamber {Harvath et al 1980, Koedel et al 1999}. The CXC chemokines, interleukin-8 (IL-8) and growth-regulated oncogene- $\alpha$  (Gro- $\alpha$ ), (R & D Laboratories, Abingdon, Oxon-UK) were reconstituted in 1ml of PBS and stored at  $-70^{\circ}\text{C}$  in small aliquots using 10 ng/ml concentrations as a stock solution. Each chemokine was serially diluted on the day of the study to test neutrophil chemotaxis towards 10ng/ml, 1ng/ml, 100pg/ml and 10pg/ml chemokine concentration. The lower compartment of the Boyden chambers was loaded with 28  $\mu\text{l}$  of the chemokine or PBS as a negative control in duplicate wells. Care was taken to create a good meniscus. The filter was carefully placed over the wells to avoid any air bubbles. A silicon gasket was placed over the filter followed by the upper compartment and then fixed into place. 50  $\mu\text{l}$  of neutrophil suspension was added to the upper wells in duplicates and the chamber was incubated for one hour at  $37^{\circ}\text{C}$  in an incubator supplied with 5%  $\text{CO}_2$  and 95%  $\text{O}_2$ . After incubation, the cell (upper most) side of the filter was washed 3 times in PBS and then wiped with a firm rubber wiper to remove any attached cells to this side of the filter.

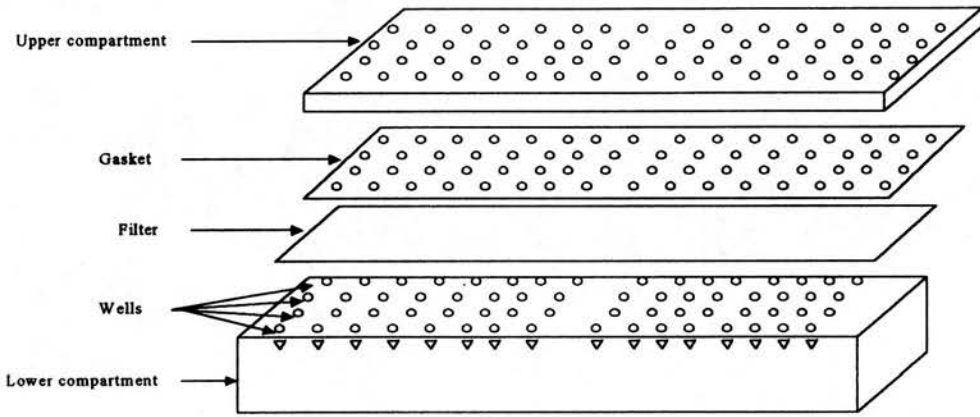


Figure 2.2: Boyden chamber.

The filter was fixed in methanol for 10 minutes, left to air dry and then stained with 1/10 diluted Gentian violet for 10 minutes. After further washing, the filter was placed on a broad slide with the migrated cell (lower most) side facing up and the migrated cells counted in 2 high power fields in each duplicate well (100x). The net result of neutrophil chemotaxis was counted by subtraction of the results of the neutrophils migrating in response to PBS from the results of the neutrophils migrating in response to the CXC chemokines to exclude the random neutrophil movement {Zaslaver et al 2001}.

#### **2.3.4 Statistical analysis**

In the following Chapters of this thesis, the variables were tested for distribution (normal or skewed) by using Kolmogorov-Smirnov (exploration) test. Statistical testing was done accordingly. Parametric tests were used in case of normally distributed variables and non-parametric tests were used if the variables were skewed. Unpaired t-test was used to compare 2 groups if the variables were normally distributed and Mann-Whitney rank sum test if they were skewed. Paired t-test was used to compare the same group before and after the procedure if the variables were normally distributed and Wilcoxon Signed Ranked test was used if they were skewed. One-way analysis of variance (ANOVA) was used to compare between more than 2 groups. Pearson's and Spearman's correlation tests were used to correlate between normally and skewedly distributed variables respectively.

In this Chapter, the Student t-Test or ANOVA test was used to compare means between individuals as appropriate. Pearson's correlation test was used to test the relationship between neutrophil chemotaxis with age and sex of the control subjects.

## **2.4 RESULTS**

Neutrophil chemotaxis in 12 different control individuals towards serially diluted IL-8 and Gro- $\alpha$  concentrations is shown in Figure 2.3 and 2.4. Neutrophil chemotaxis stimulated with 10ng/ml IL-8 ( $46.1 \pm 1.5$  neutrophils/high power field, mean  $\pm$  SEM,



n=12), was significantly higher compared with 1ng/ml ( $34.3 \pm 0.9$ ,  $P < 0.0001$ ), with 100 pg/ml ( $27.2 \pm 0.9$ ,  $P < 0.0001$ ), and with 10 pg/ml IL-8 ( $19.5 \pm 0.6$ ,  $P < 0.0001$ , Figure 2.3). Neutrophil chemotaxis stimulated by 10ng/ml Gro- $\alpha$  ( $48.9 \pm 1.2$  neutrophils/high power field, mean  $\pm$  SEM, n=12) was significantly higher compared with 1ng/ml ( $38.5 \pm 0.9$ ,  $P < 0.0001$ ), with 100 pg/ml ( $30.9 \pm 1.2$ ,  $P < 0.0001$ ), and with 10 pg/ml Gro- $\alpha$  ( $23.3 \pm 0.7$ ,  $P < 0.0001$ , Figure 2.3).

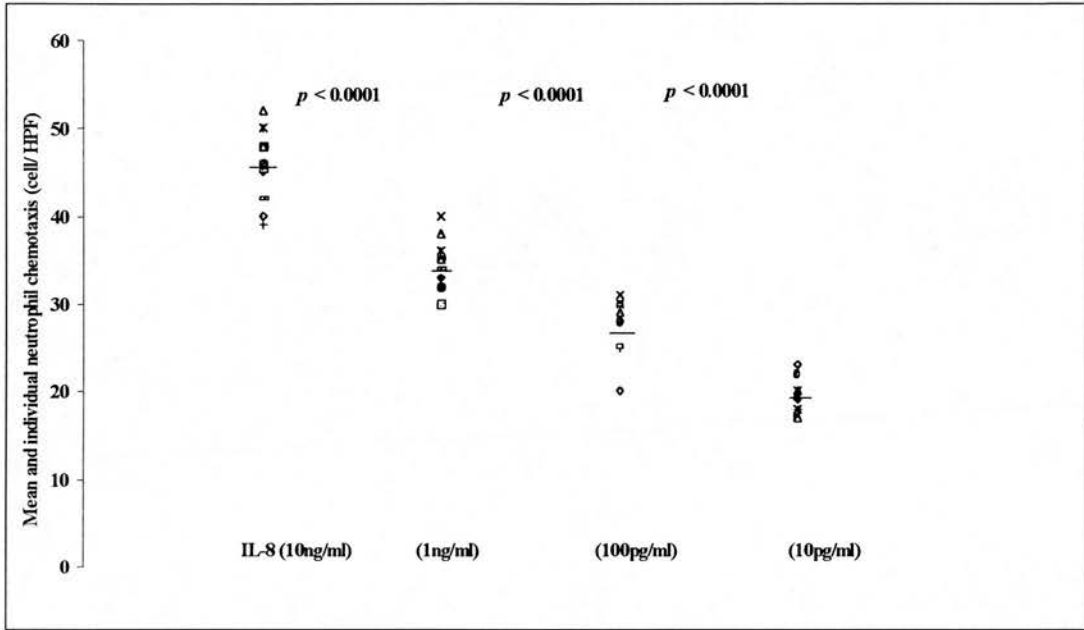


Figure 2.3: Mean and individual neutrophil chemotaxis stimulated with serial dilutions of IL-8 in 12 controls.

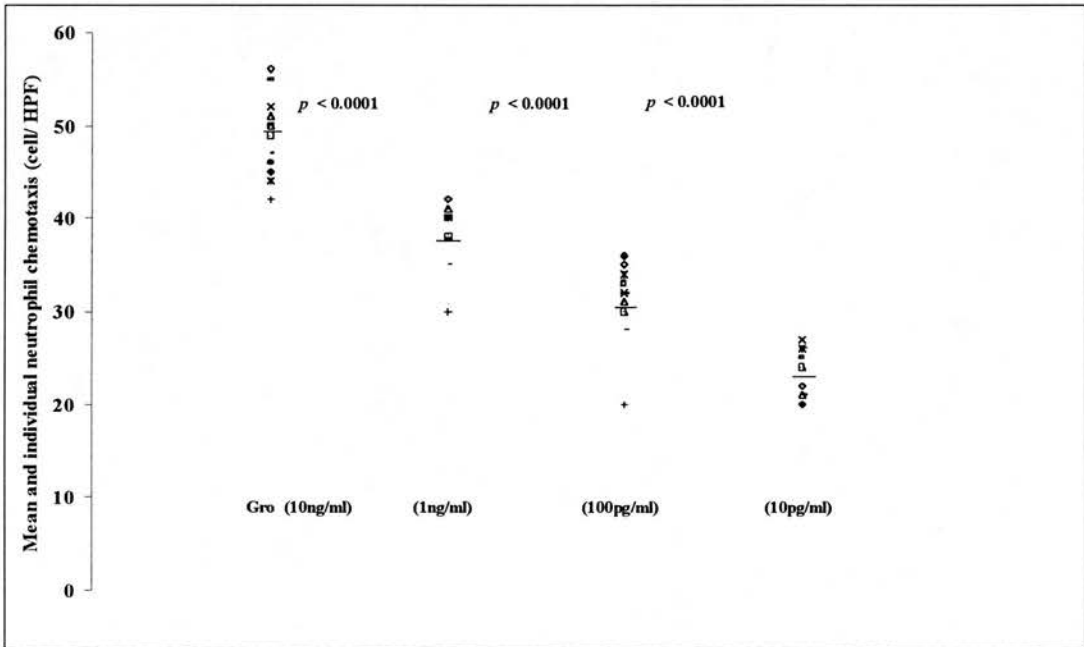


Figure 2.4: Mean and individual neutrophil chemotaxis stimulated with serial dilutions of Gro- $\alpha$  in 12 controls.

Neutrophil chemotaxis in same controls (n=5) repeated between 2-10 times at different time points towards serially diluted IL-8 and Gro- $\alpha$  concentrations are shown in Figure 2.5 and 2.6 respectively. No significant changes were detected between neutrophil chemotaxis repeated at different time points for each of these control subjects and for each chemokine concentration ( $p = 0.2$ ) or between members of this group for each chemokine concentration ( $p = 0.3$ ). Neutrophil chemotaxis results were similar when stimulated with 10ng/ml IL-8 ( $48.8 \pm 0.9$ ,  $46.2 \pm 1.3$ ,  $45.75 \pm 0.6$ ,  $46.75 \pm 0.7$ , and  $46.5 \pm 1.5$  cell/high power field), with 1ng/ml ( $35.6 \pm 0.87$ ,  $35.3 \pm 0.76$ ,  $36.2 \pm 0.95$ ,  $35.2 \pm 0.48$ , and  $36 \pm 1$ ), with 100pg/ml ( $26.8 \pm 0.8$ ,  $25.2 \pm 0.7$ ,  $26.7 \pm 0.5$ ,  $25.2 \pm 1.3$ , and  $19.5 \pm 0.5$ ), or with 10pg/ml ( $18.4 \pm 0.6$ ,  $16 \pm 0.6$ ,  $14.7 \pm 1.5$ ,  $18.7 \pm 0.6$ , and  $15.5 \pm 0.5$ ) for control subjects 1-5 respectively (Figure 2.5).

Neutrophil chemotaxis results was similar when stimulated with 10ng/ml Gro- $\alpha$  ( $49.7 \pm 1.2$ ,  $48.2 \pm 0.7$ ,  $46.7 \pm 1$ ,  $44.5 \pm 0.9$ , and  $46.5 \pm 0.5$  cell/high power field), with 1ng/ml ( $39 \pm 1$ ,  $33.2 \pm 1$ ,  $34 \pm 0.9$ ,  $32.7 \pm 0.7$ , and  $38.5 \pm 0.5$ ), with 100pg/ml ( $32.2 \pm 1.3$ ,  $29 \pm 1.1$ ,  $32.2 \pm 1$ ,  $29.5 \pm 1$ , and  $29 \pm 0.0$ ), or with 10pg/ml ( $24 \pm 1.5$ ,  $21.2 \pm 0.6$ ,  $25 \pm 1.1$ ,  $26.2 \pm 0.6$ , and  $22 \pm 0.0$ ) for control subjects 1-5 respectively (Figure 2.6).

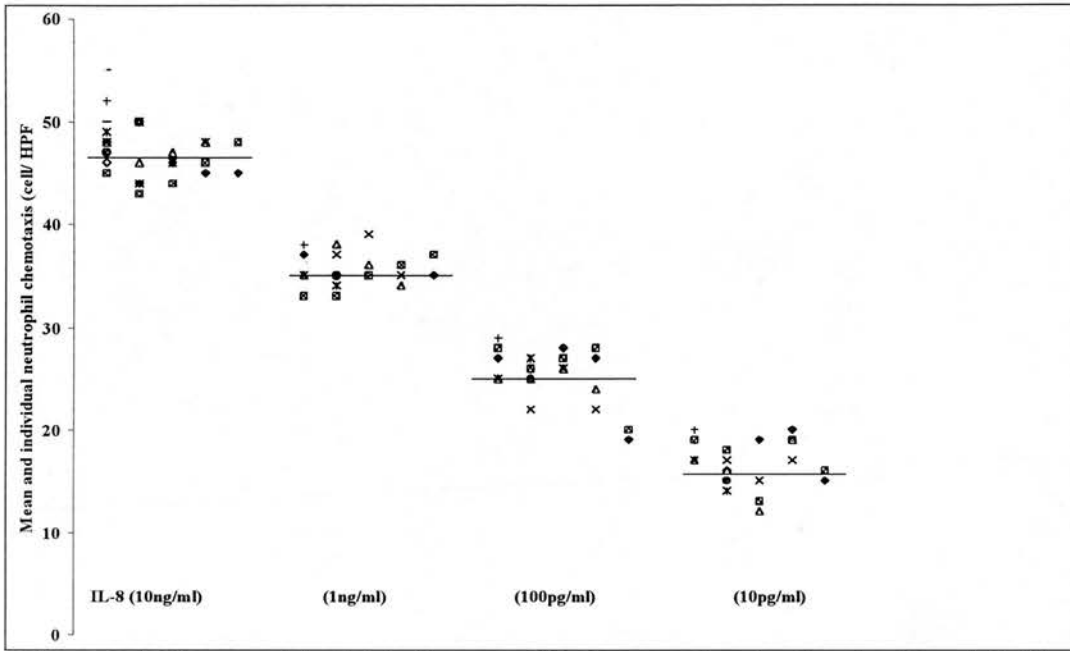


Figure 2.5: Mean and individual neutrophil chemotaxis stimulated with serial dilution of IL-8 of 5 control subjects on 2-10 different occasions.

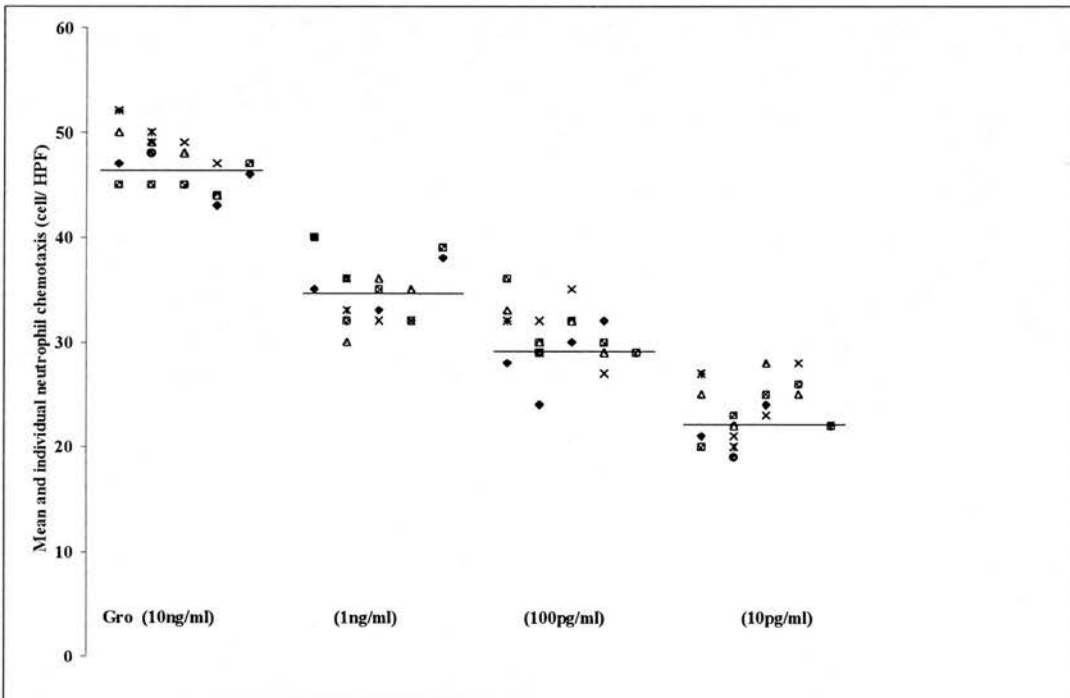


Figure 2.6: Mean and individual neutrophil chemotaxis stimulated with serial dilution of Gro- $\alpha$  of 5 control subjects on 2-10 different occasions.

Measurement of neutrophil chemotaxis in the same individuals was also performed repeatedly. No significant differences between repeated measurements of neutrophil chemotaxis stimulated with 10ng/ml of either IL-8 or Gro- $\alpha$  for each individual of the three tested control subjects. There were also no significant differences between neutrophil chemotaxis for the first control (IL-8  $49 \pm 1$ , Gro- $\alpha$   $51 \pm 1.3$  cell/high power field, mean  $\pm$  SEM), second control (IL-8  $46 \pm 0.7$ , Gro- $\alpha$   $49.5 \pm 0.5$ ), or third control (IL-8  $48 \pm 0.5$ , Gro- $\alpha$   $47 \pm 0.4$ , respectively,  $P = 0.3$ , Figure 2.7).

There were no statistical differences between the results of different controls, repeated testing of the same controls at different time intervals, and repeated testing of the same control at the same time and under the same test settings ( $p$  value  $> 0.05$ ).

There was no significant correlation between IL-8-induced- and Gro- $\alpha$ -induced-neutrophil chemotaxis in all of the studied groups ( $r$  values were ranged between -0.04 and 0.8 and  $p$  values ranged between 0.9 and 0.06).

There was no statistical correlation between IL-8-induced chemotaxis and age ( $r = 0.3$ ,  $p = 0.3$ , Figure 2.8). However, there appeared to be an inverse correlation between Gro- $\alpha$ -induced chemotaxis and age ( $r = -0.6$ ,  $p = 0.04$ , Figure 2.9). There was no difference in either IL-8 or Gro-induced chemotaxis between males and females (Figure 2.10).

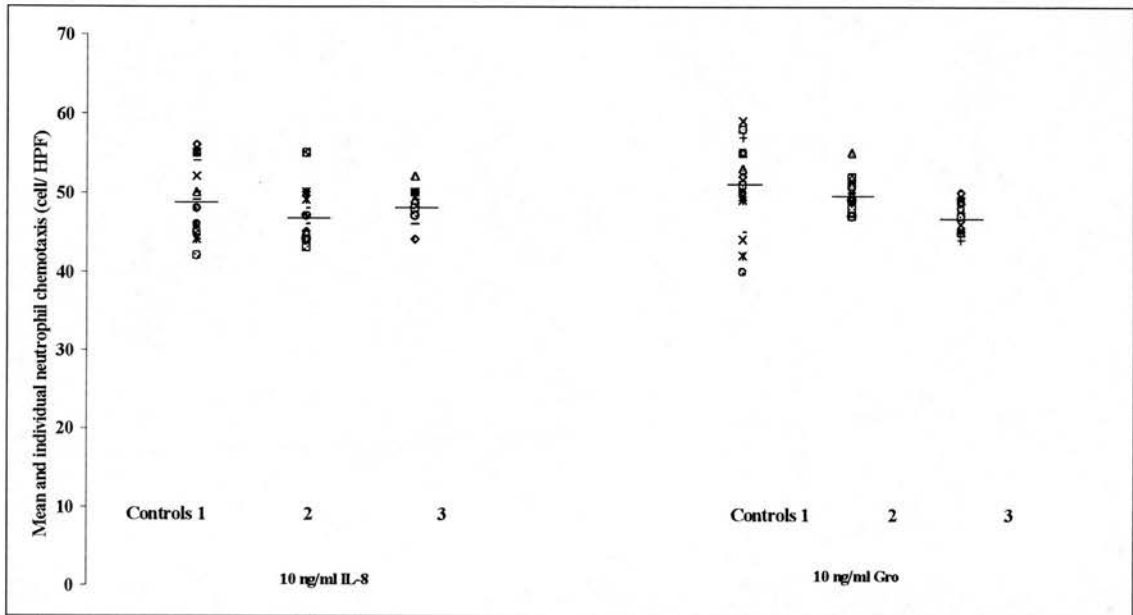


Figure 2.7: Mean and individual neutrophil chemotaxis of the same controls stimulated with 10ng/ml of IL-8 and Gro- $\alpha$ , duplicated in 20 well at the same time and under the same test settings.

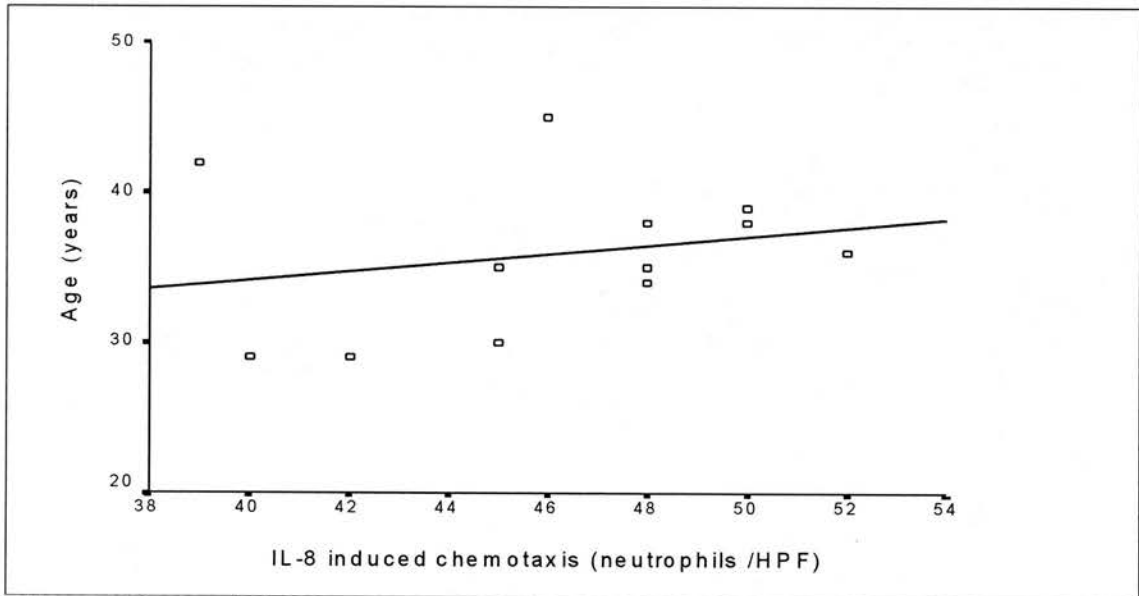


Figure 2.8: correlation between IL-8-induced neutrophil chemotaxis and age.

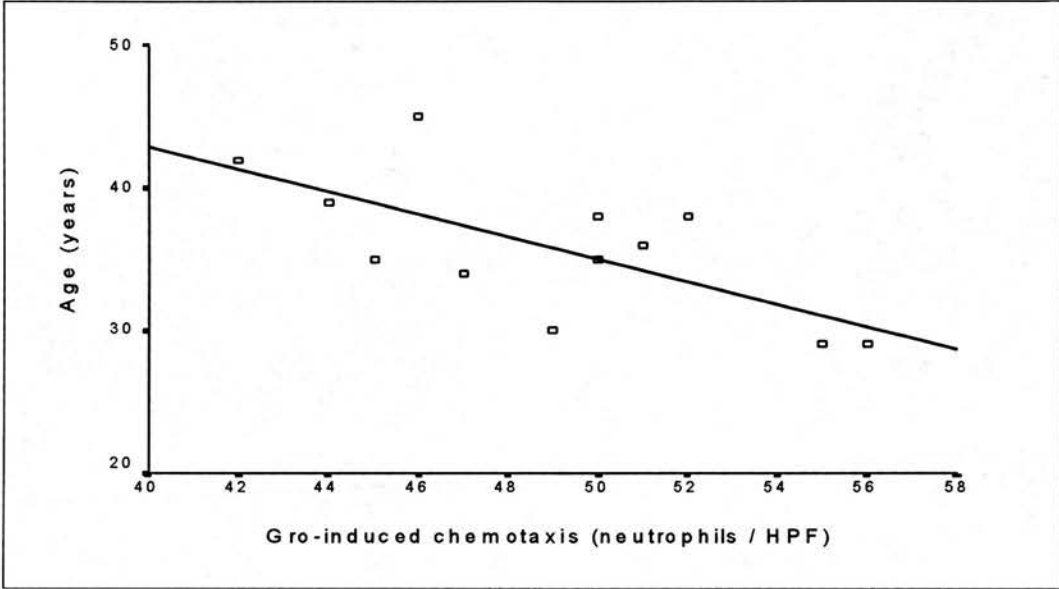


Figure 2.9: Correlation between Gro-induced neutrophil chemotaxis and age.

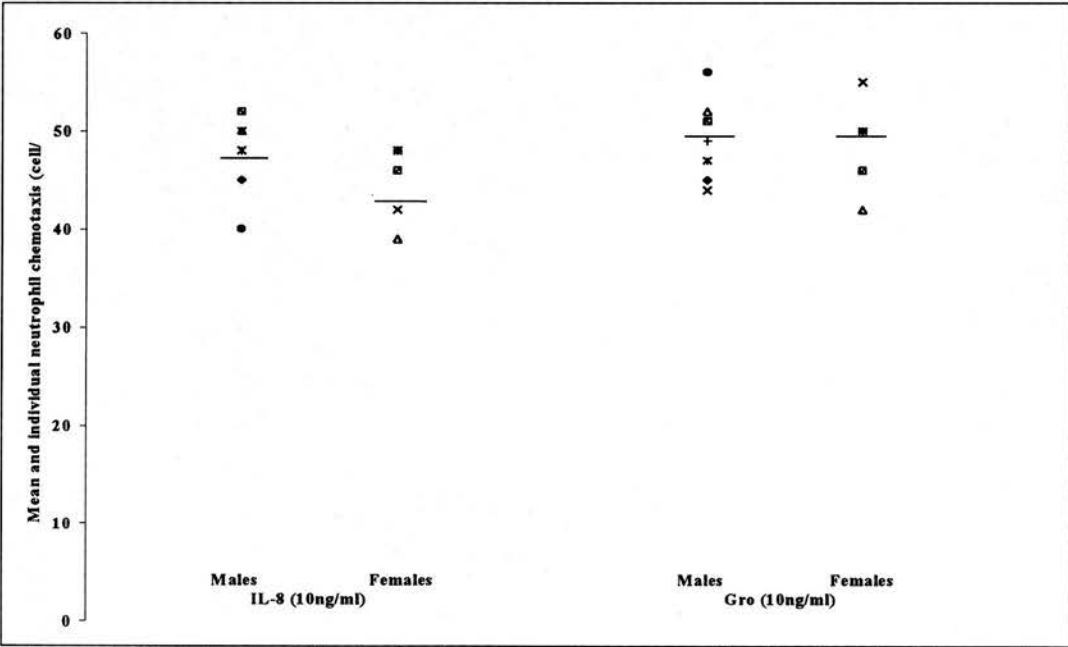


Figure 2.10: Mean and individual neutrophil chemotaxis stimulated with 10ng/ml of IL-8 and Gro- $\alpha$  in male versus female control subjects.

## **2.5 DISCUSSION**

In this chapter the methodology of neutrophil isolation and chemotaxis was adapted and optimised. The method of neutrophil isolation used allowed rapid preparation of neutrophils of high viability. Neutrophil chemotaxis stimulated with serially diluted CXC chemokines showed a dose-dependent response i.e. lower chemokine concentration was associated with a lower chemotactic response as expected. The characteristic bell-shaped curve of chemotactic response was not observed, most likely because higher chemokine concentrations than 10ng/ml were not studied. Interestingly there were similar chemotactic responses to IL-8 and Gro in the controls. The chemotactic response to both chemokines showed little variability over a time period or within the assay. Although Gro-induced chemotaxis appeared inversely correlated with age, the chemotactic response to IL8 was not related to age and the response to either chemokine was not affected by gender.

The modified Boyden chamber has been widely used to measure chemotaxis of the different subsets of leucocytes. Comparative studies of neutrophil chemotaxis using the modified Boyden chamber method and other open-well or blind-well chambers have shown the most satisfactory and reproducible results were obtained with the modified Boyden chamber method. This method provides easy and accurate filling of the lower compartment, lack of distortion of the filter and reliability of the seals around edges of the filter. Neutrophil drop off from the lower surface of the filter during incubation period is also minimal {Bignold 1989}.

Using the modified Boyden chamber method neutrophil chemotaxis induced by a variety of chemoattractants has been studied in different species including human, monkey, dog, rabbit, hamster, rat, and mouse, {Sugawara et al 1995}. Interestingly, although neutrophils of all animal species so far studied respond to human recombinant IL-8, the sensitivity of human and monkey neutrophils to IL-8 is highest. Similar methods have been used to study neutrophil and other leucocyte subset chemotaxis in a large variety of



the human disease states {Langner et al 1983}. A variety of chemoattractants have been used for example culture supernatants from immortalised corneal epithelial cells and HIV-1 infected keratocytes induce neutrophil chemotaxis in a modified Boyden chamber assay. Even the aqueous humor of the eye has been used to study neutrophil chemotaxis in patients with anterior uveitis {Rosenbaum et al 1987}. Animal studies have also utilised the modified Boyden chamber technique to measure neutrophil chemotaxis. {Galligan and Coomber 2000}.

In these studies the ELR+ CXC chemokines, IL-8 and Gro- $\alpha$  were used as neutrophil chemoattractants for two reasons. Firstly, IL-8 and Gro- $\alpha$  are considered the potent neutrophil chemoattractants chemotaxis studies {Geiser et al 1993}. Secondly, neutrophils express both CXCR1 and CXCR2 surface chemokine receptors. Blocking antibody studies indicated that the IL8 chemotactic response is mediated mainly via CXCR1, while chemotaxis stimulated by Gro- $\alpha$  is solely via CXCR2 {Hammond et al 1995}. Therefore measuring both IL8 and Gro-induced chemotaxis allows identification of defects in either both receptors or localises the defect to CXCR2. For example if IL-8-induced neutrophil chemotaxis is reduced, this may involve impairment or down-regulation of CXCR1 and/or CXCR2, but reduction of Gro- $\alpha$ -induced chemotaxis indicates involvement of only CXCR2.

Several reports have suggested differences in the chemotactic response to IL8 mediated by CXCR1 and CXCR2. CXCR2 is reported to be more responsive than CXCR1 to low concentrations of IL-8. Based on the ability to undergo internalisation and recycling, it was suggested that CXCR1 mediates IL-8-induced chemotaxis at the site of inflammation, where the IL-8 concentration is high. In contrast, CXCR2 can initiate neutrophil migration at sites distant from inflammation, where the IL-8 concentration is low {Chuntharapai and Kim 1995}. Furthermore, stimulation of CXCR1 can down-regulate CXCR2 and in contrast, stimulation of CXCR2 up-regulates CXCR1 {Hauser et al 1999}.

In summary this chapter describes the technique adapted to measure IL-8 and Gro- $\alpha$  stimulated neutrophil chemotaxis in healthy controls. The method is rapid and reproducible and shows little variation over time. No significant variations were detected in neutrophil chemotaxis in relation to gender or age, except with Gro- $\alpha$ -stimulated chemotaxis, which showed an inverse correlation with age. The modified Boyden chamber method has been widely used to study neutrophil chemotaxis in a variety of disease states. However, there are no studies relating to chemokine induced neutrophil chemotaxis in human liver diseases. Such studies are described in the following chapters of this thesis.

**CHAPTER 3**

**NEUTROPHIL CHEMOTAXIS IN ACUTE AND  
CHRONIC LIVER FAILURE**

### **3.1 INTRODUCTION**

Infection is a common complication in patients with acute and chronic liver failure {Rolando et al 1990 and Navasa et al 1999}, and is associated with significant mortality. The normal body defence mechanisms in such patients can be affected in many ways. Intact neutrophil function is important in the innate immune response and defective neutrophil chemotaxis contributes to increased risk of infection in patients with liver failure {Wyke et al 1983, and Yousif-Kadaru et al 1984}. Previous reports used a wide variety of non-specific stimulants and test neutrophil chemotaxis. These substances include zymosan-activated autologous plasma {Campbell et al 1981}, the complement factors such as C3, C5 and C567 {Ward 1996}, and serum activated by immune complexes composed of 10% fresh serum, 40% ovalbumin-rabbit-antiovalbumin and 50% Hank's solution {DeMeo and Andersen 1972}. No data is available regarding neutrophil chemotaxis stimulated by the more recently described family of potent neutrophil chemoattractants; the chemokines.

In the previously published data, the relation between neutrophil chemotaxis and severity of the liver disease was not clear. Some studies have found a good correlation between certain neutrophil functions and the severity of liver disease. For example, reduced neutrophil peroxidase production in patients with hepatitis B infection has been reported, which correlates with the severity of disease and returned to normal with recovery {Vasil'ev 1984}. Another study showed a reduction in neutrophil production of reactive O<sub>2</sub> species in patients with liver cirrhosis. This reduction was found to correlate with the severity of liver cirrhosis {Itoh et al 1993}.

The relationship between the aetiology of liver disease and neutrophil chemotaxis is also unclear. Alcohol has an *in-vivo* direct inhibitory effect on leukocyte migration. This was observed in a control group after ethyl alcohol either intravenously or orally. As little as 0.1mg/% alcohol *in-vitro* can also inhibit leukocyte chemotaxis {Brayton et al 1970}. Alcohol also has indirect inhibitory effects on the bone marrow leading to

granulocytopenia and an inhibitory effect on synthesis of serum factors needed for leukocyte chemotaxis such as complement factors {Wyke 1989}. Previous studies have shown significantly impaired neutrophil chemotaxis in patients with alcoholic liver disease and to lesser extent in patients with cryptogenic cirrhosis, compared with controls {Campbell et al 1981}. Infection is believed to be an infrequent complication in patients with Primary Biliary Cirrhosis (PBC) due to relatively few or minor immune defects in comparison with the other chronic liver diseases, but neutrophil chemotaxis in such patients has not been studied {Wyke et al 1983}.

These studies were designed to test the hypothesis that neutrophil chemotaxis stimulated by chemokines is impaired in patients with acute and chronic liver failure and to determine if there was a relationship with disease aetiology or severity.

### **3.2 SUBJECTS**

Patients were divided into 4 groups; 24 patients with paracetamol-induced acute liver failure; 13 patients with alcoholic liver cirrhosis; 12 patients with hepatitis C cirrhosis, and 10 patients with primary biliary cirrhosis. 38 healthy medical and laboratory staff served as controls. The patients and controls' characteristics are shown in Table 3.1 For the purposes of analysis the patients with paracetamol overdose (POD) were the only patients to represent the acute liver failure group (n=24), with alcoholics, hepatitis C and primary biliary cirrhosis were grouped together and defined as having chronic liver failure (n=21).

As an indicator of severity in patients with POD, these patients were divided according to the grade of encephalopathy they had into, patients without encephalopathy (group 0, n=4), with grades 1 or 2 (group1, n=9), and patients with grades 3 and 4 (group2, n=11).

Table 3.1: Patient characteristics presented as mean  $\pm$ SEM.

	ALF	CLF			Controls
	POD	ALD	Hepatitis C	PBC	
Age: (yr)	37 $\pm$ 2	50 $\pm$ 3	45 $\pm$ 3	63 $\pm$ 5	36 $\pm$ 1
Number:	24	13	12	10	38
Sex: F:M	9:15	5:8	4:8	10:0	11:27
Child-Pugh grade: A:B:C		3:3:7	4:4:4	7:3:0	
Child-Pugh score		8.2 $\pm$ 2.1	7.2 $\pm$ 1.2	6.1 $\pm$ 0.2	
Serum bilirubin: ( $\mu$ mol.L <sup>-1</sup> )	98 $\pm$ 12	145 $\pm$ 40	39 $\pm$ 4.5	128 $\pm$ 14	
Serum albumin: (g.L <sup>-1</sup> )	39 $\pm$ 0.9	28 $\pm$ 2.1	33 $\pm$ 1.7	41 $\pm$ 1.4	
Prothrombin time: (s)	50.5 $\pm$ 4	12.4 $\pm$ 0.6	14.7 $\pm$ 0.8	10.7 $\pm$ 0.6	
Blood urea: (mmol.L <sup>-1</sup> )	11.6 $\pm$ 2	5 $\pm$ 1.6	4.5 $\pm$ 0.6	6 $\pm$ 0.5	
Serum creatinine: ( $\mu$ mol.L <sup>-1</sup> )	186 $\pm$ 21	90.9 $\pm$ 9	68 $\pm$ 7.5	80 $\pm$ 3.8	

ALF= Acute liver failure.

CLF= chronic liver failure.

POD=Paracetamol overdose.

ALD= alcoholic liver disease.

PBC= primary biliary cirrhosis.

M= male. F= female.

The severity of liver disease in the chronic liver failure group was defined using the widely used Child-Pugh score {Conn 1981 and Pugh et al 1983}. These patients were divided into 14 patients with grade A, 10 patients with grade B, and 11 patients with grade C.

### **3.3 METHODS**

To reduce potential variability in a day-to-day testing, the neutrophil chemotaxis of each patient was compared directly with a control performed at the same time.

#### **3.3.1 Neutrophil chemotaxis**

Neutrophil isolation and chemotaxis were performed in all subjects as described in Chapter 2 (sections 2.3.1 and 2.3.3).

#### **3.3.2 Statistical analysis**

Results were examined as number of neutrophils counted/high power field (HPF) in 2 fields of each of the duplicate wells for each sample. The Mann-Whitney rank sum test was used to compare the different groups as discussed in Chapter 2 (section 2.3.4).

### **3.4 RESULTS**

#### **3.4.1 Neutrophil chemotaxis in acute and chronic liver failure**

Neutrophil chemotaxis induced by IL-8 (10ng/ml) was significantly impaired in patients with acute liver failure ( $19.5 \pm 0.9$  neutrophils / high power field, mean  $\pm$  SEM,  $n = 24$ ) and chronic liver failure ( $11.2 \pm 2.3$ ,  $n = 21$ ) compared with controls ( $46 \pm 1$ ,  $n = 38$ ,  $p < 0.0001$ , Figure 3.1). Furthermore, neutrophil chemotaxis induced by IL-8 was significantly more impaired in patients with chronic liver failure compared with patients with acute liver failure ( $p < 0.001$ , Figure 3.1).

Neutrophil chemotaxis induced by Gro- $\alpha$  (10ng/ml) was significantly impaired in patients with acute liver failure ( $18.6 \pm 0.8$ ,  $n = 24$ ) and chronic liver failure ( $13.6 \pm 1.8$ ,  $n = 21$ ) compared with controls ( $49 \pm 1$ ,  $n = 38$ ,  $p < 0.0001$ , Figure 3.2). Gro- $\alpha$ -induced chemotaxis was also significantly more impaired in patients with chronic liver failure compared with patients with acute liver failure ( $p < 0.02$ , Figure 3.2).



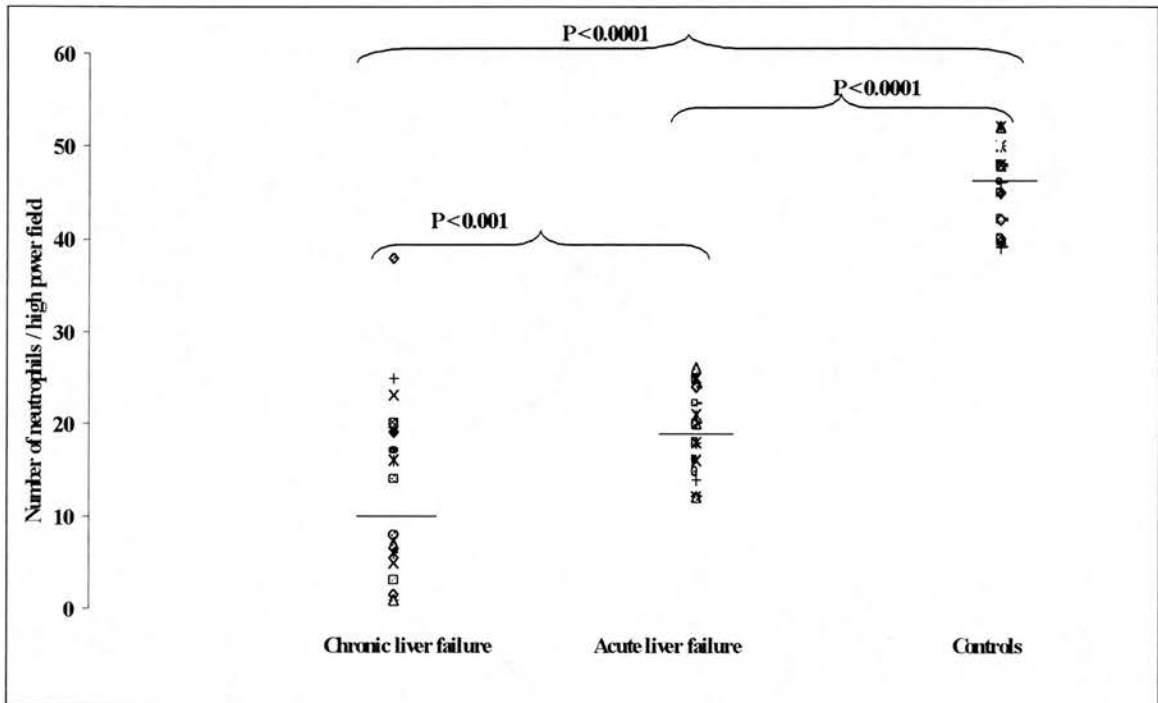


Figure 3.1: Neutrophil chemotaxis in patients with chronic liver failure, acute liver failure, and controls stimulated with 10 ng/ml IL-8.

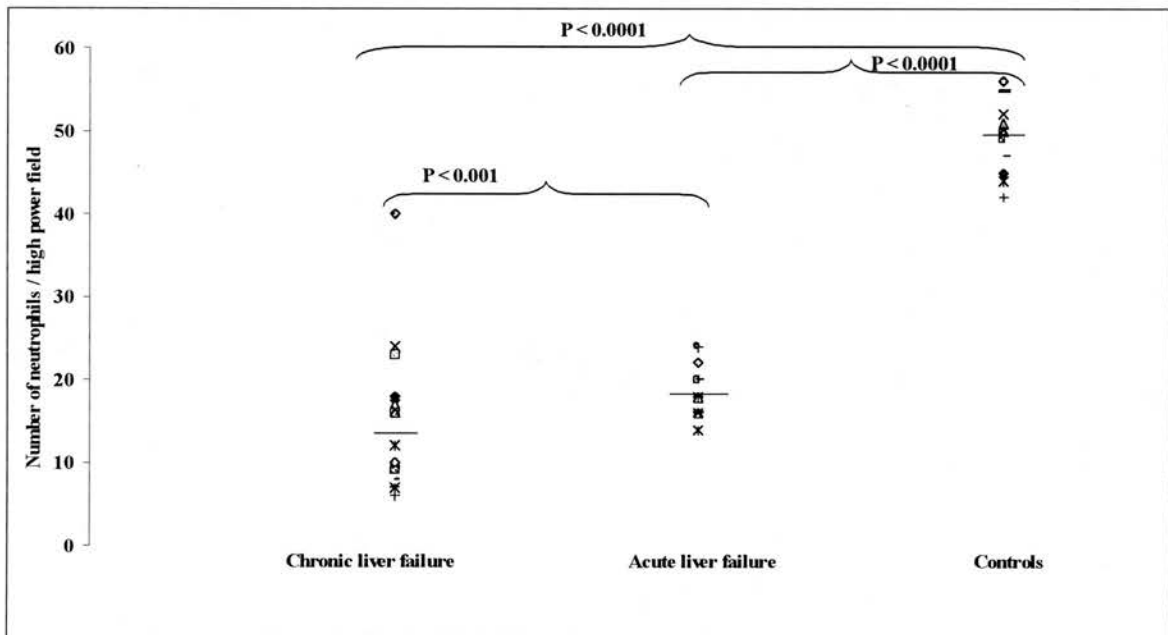


Figure 3.2: Neutrophil chemotaxis in patients with chronic liver failure, acute liver failure, and controls stimulated with 10 ng/ml Gro- $\alpha$ .

### **3.4.2 Neutrophil chemotaxis: effect of aetiology**

Neutrophil chemotaxis induced by IL-8 (10ng/ml) was similar in patients with ALD ( $9.8 \pm 2.8$  neutrophils / high power field, mean  $\pm$  SEM,  $n = 13$ ) compared with patients with hepatitis C cirrhosis ( $15.7 \pm 2.9$ ,  $n = 12$ ,  $p < 0.2$ , Figure 3.3). However, neutrophil chemotaxis induced by IL-8 was significantly impaired in patients with ALD compared with patients with POD ( $19.5 \pm 0.9$ ,  $n = 24$ ,  $p = 0.002$ ) and patients with PBC ( $36.8 \pm 4.7$ ,  $n = 10$ ,  $p < 0.0001$ ). Neutrophil chemotaxis induced by IL-8 was similar in patients with hepatitis C compared with patients with POD ( $p = 0.2$ ), but was significantly impaired compared with patients with PBC ( $36.8 \pm 4.7$ ,  $n = 10$ ,  $p = 0.003$ ). Neutrophil chemotaxis induced by IL-8 was significantly impaired in patients with POD compared with patients with PBC ( $p = 0.002$ ). Neutrophil chemotaxis induced by IL-8 in all sub-groups of patients was significantly impaired compared with the controls ( $46 \pm 1$ ,  $n = 38$ ,  $p < 0.0001$ ). As shown in Figure 3.3, neutrophil chemotaxis to IL-8 was lowest in patients with alcoholic liver disease, followed by hepatitis C cirrhosis, POD, and PBC.

Neutrophil chemotaxis induced by Gro- $\alpha$  (10ng/ml) was similar in patients with ALD ( $13.6 \pm 2.8$  neutrophils / high power field, mean  $\pm$  SEM,  $n = 13$ ) compared with patients with hepatitis C cirrhosis ( $18 \pm 2$ ,  $n = 12$ ,  $p = 0.09$ , Figure 3.4) and patients with POD ( $18.6 \pm 0.8$ ,  $n = 16$ ,  $p = 0.05$ ). However, neutrophil chemotaxis induced by Gro- $\alpha$  was significantly impaired in patients with ALD compared with patients with PBC ( $35 \pm 3$ ,  $n = 10$ ,  $p = 0.002$ ). Neutrophil chemotaxis induced by Gro- $\alpha$  was similar in patients with hepatitis C compared with patients with POD ( $p < 0.4$ ), but was significantly impaired compared with patients with PBC ( $p = 0.003$ ). Neutrophil chemotaxis induced by Gro- $\alpha$  was significantly impaired in patients with POD compared with patients with PBC ( $p < 0.0004$ ). Neutrophil chemotaxis induced by Gro- $\alpha$  in all sub-groups of patients was significantly impaired compared with the controls ( $49 \pm 1$ ,  $n = 38$ ,  $p < 0.0001$ ). As shown in Figure 3.4, neutrophil chemotaxis to Gro- $\alpha$  was similar to IL-8, lowest in patients with alcoholic liver disease and hepatitis C cirrhosis, followed by POD, and PBC.

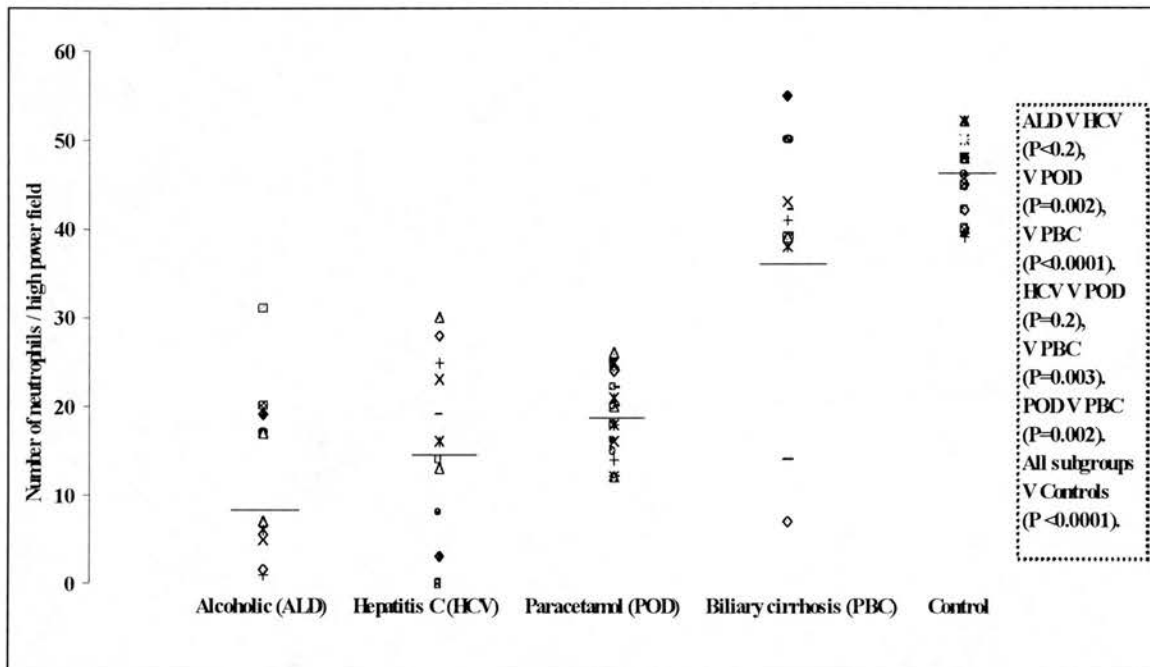


Figure 3.3: Neutrophil chemotaxis in different groups of patients and controls stimulated with 10 ng/ml IL-8.

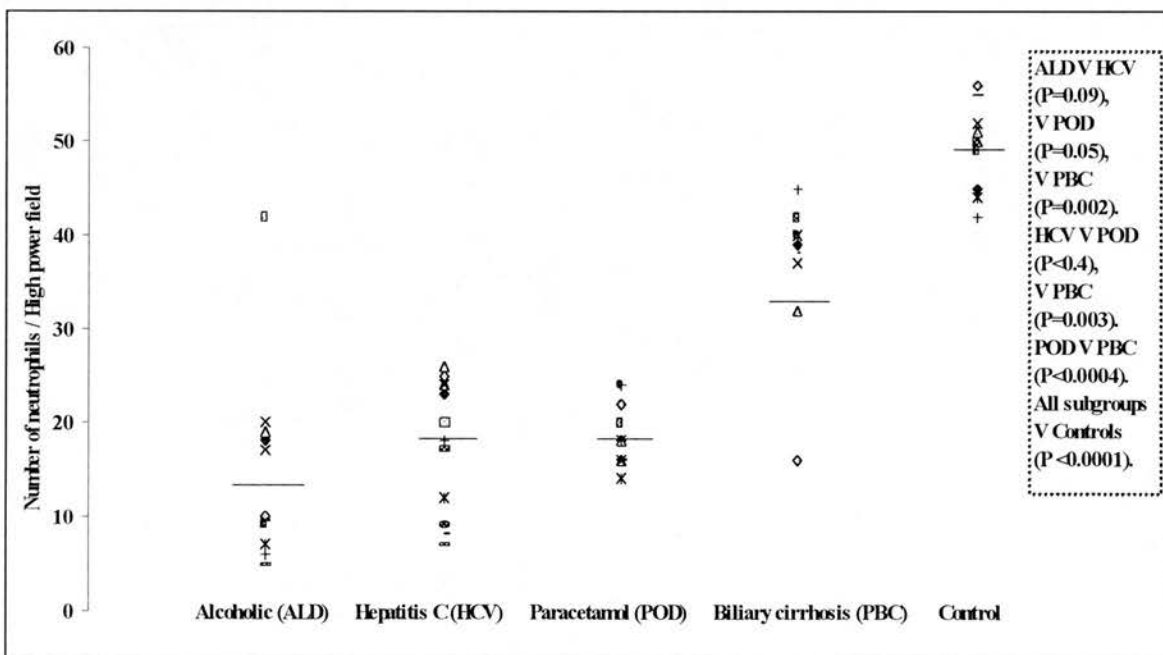


Figure 3.4: Neutrophil chemotaxis in different groups of patients and controls stimulated with 10 ng/ml Gro- $\alpha$ .

### **3.4.3 Neutrophil chemotaxis: relationship with disease severity in ALF**

Only 3 patients with POD died making statistical comparison between those that lived and those that died invalid. The degree of hepatic encephalopathy in patients with acute liver failure induced by paracetamol poisoning is correlated with disease severity. Figure 3.5 shows that neutrophil chemotaxis induced by IL-8 (10ng/ml) was similar in patients with POD irrespective of the encephalopathy grade. Neutrophil chemotaxis was similar in patients with grade 0 encephalopathy ( $21 \pm 2$ , mean  $\pm$  SEM,  $n = 4$ ) compared with patients with grades 1 or 2 ( $21.4 \pm 1$ ,  $n = 9$ ,  $p = 0.9$ ) and grades 3 or 4 ( $17.5 \pm 1.5$ ,  $n = 11$ ,  $p < 0.2$ ). Neutrophil chemotaxis induced by IL-8 was also similar in patients with grades 1 or 2 encephalopathy compared with patients with grades 3 or 4 ( $p = 0.6$ ).

Figure 3.6 shows that neutrophil chemotaxis induced by Gro- $\alpha$  (10ng/ml) was similar in patients with POD despite the encephalopathy grades of the patients. Neutrophil chemotaxis was similar in patients with grade 0 encephalopathy ( $18 \pm 2$ , mean  $\pm$  SEM,  $n = 2$ ) compared with patients with grades 1 or 2 ( $18.7 \pm 1.3$ ,  $n = 6$ ,  $p = 0.9$ ) and grades 3 or 4 ( $18.8 \pm 1.2$ ,  $n = 8$ ,  $p < 0.8$ ). Neutrophil chemotaxis induced by Gro- $\alpha$  was also similar in patients with grades 1 or 2 encephalopathy compared with patients with grades 3 or 4 ( $p = 0.9$ ).

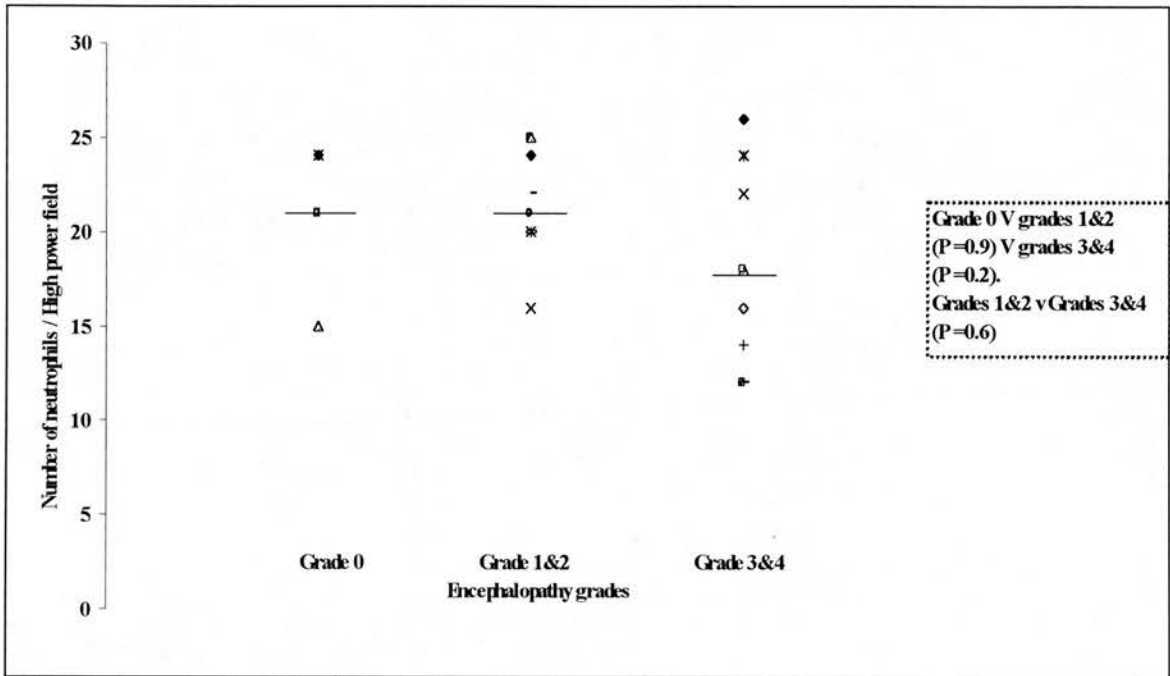


Figure 3.5: Neutrophil chemotaxis stimulated with 10 ng/ml IL-8 in patients with POD according to encephalopathy grades.

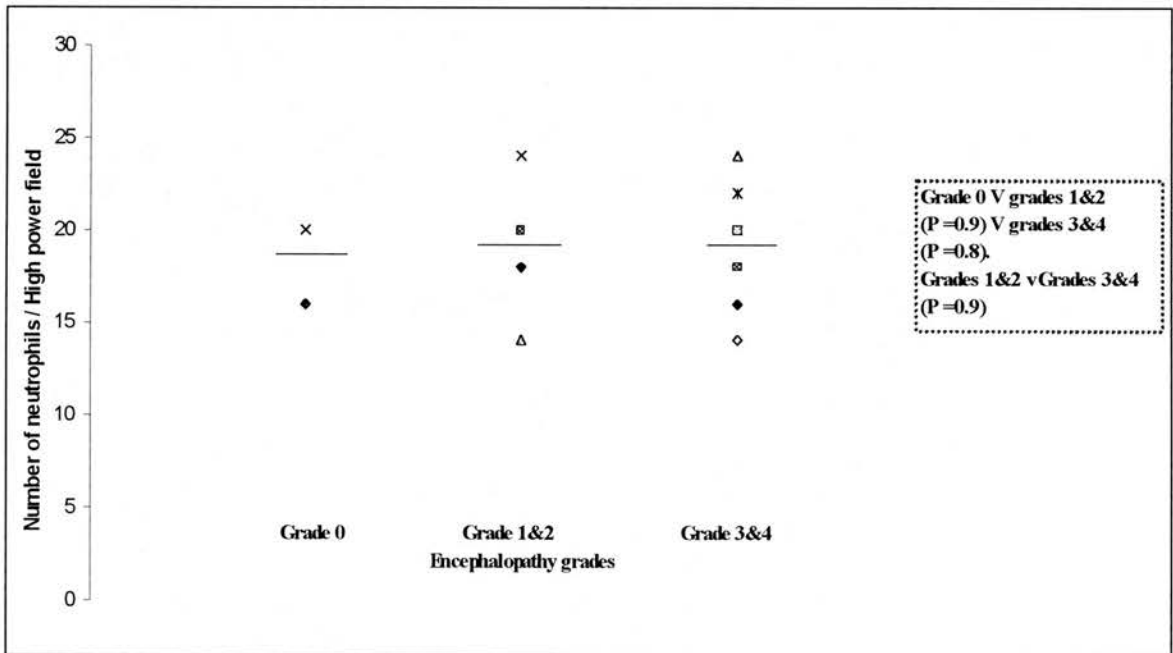


Figure 3.6: Neutrophil chemotaxis stimulated with 10 ng/ml Gro-α in patients with POD according to encephalopathy grades.

### **3.4.4 Neutrophil chemotaxis: relationship with disease severity in CLF**

The Child-Pugh score is a widely used scoring system to determine the severity of chronic liver disease and is related to prognosis. Figure 3.7 shows that the patients with the most severe chronic liver failure had the most impaired neutrophil chemotaxis induced by IL-8. Neutrophil chemotaxis induced by IL-8 (10ng/ml) was significantly reduced in patients with Child grade C cirrhosis ( $4.6 \pm 2.4$ , mean  $\pm$  SEM,  $n = 11$ ) compared with patients with Child grade B cirrhosis ( $18.2 \pm 3.1$ ,  $n = 10$ ,  $p = 0.003$ ) and Child grade A cirrhosis ( $32 \pm 4$ ,  $n = 14$ ,  $p < 0.0001$ ). Neutrophil chemotaxis induced by IL-8 was also significantly impaired in patients with Child grade B cirrhosis compared with patients with Child grade A cirrhosis ( $p = 0.03$ ).

Figure 3.8 shows that neutrophil chemotaxis induced by Gro- $\alpha$  was also most impaired in patients with the most severe chronic liver failure. Neutrophil chemotaxis induced by Gro- $\alpha$  (10ng/ml) was significantly impaired in patients with Child grade C cirrhosis ( $9.2 \pm 1.4$ , mean  $\pm$  SEM,  $n = 11$ ) compared with patients with Child grade B cirrhosis ( $18.5 \pm 2.9$ ,  $n = 10$ ,  $p = 0.005$ ) and Child grade A cirrhosis ( $32.1 \pm 2.5$ ,  $n = 14$ ,  $p < 0.0001$ ). Neutrophil chemotaxis induced by Gro- $\alpha$  (10ng/ml) was also significantly impaired in patients with Child grade B cirrhosis compared with patients with Child grade A cirrhosis ( $p = 0.001$ ). Neutrophil chemotaxis stimulated with IL-8 or Gro- $\alpha$  in each group of patients with Child A were significantly reduced compared with control subjects ( $p < 0.0001$ ).

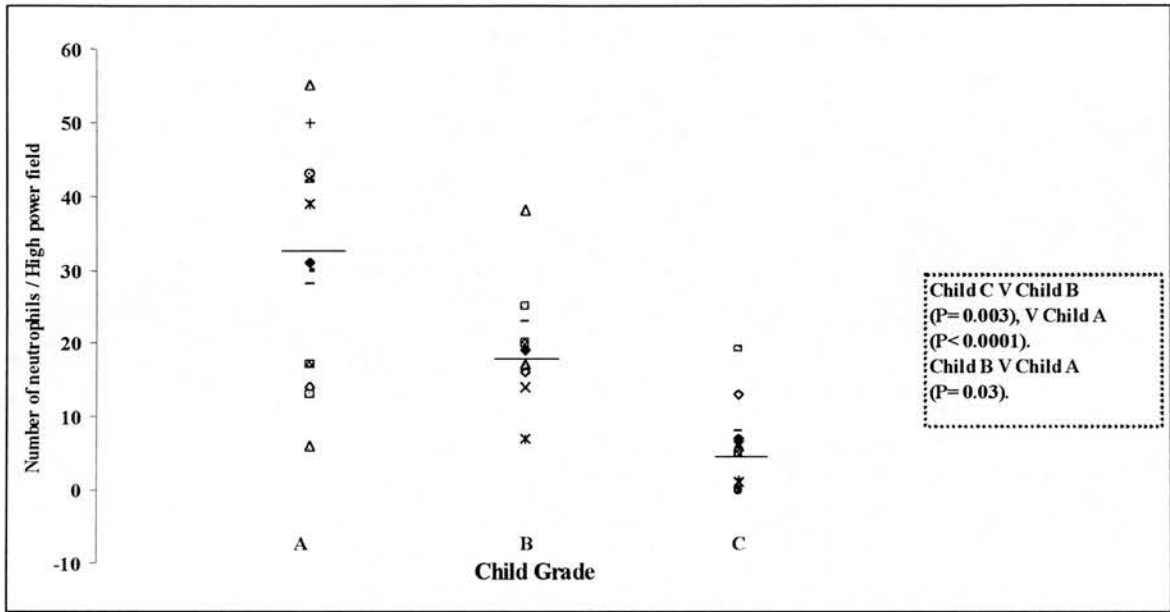


Figure 3.7: Neutrophil chemotaxis stimulated with 10 ng/ml IL-8 in patients with chronic liver diseases according to Child-Pugh grades.

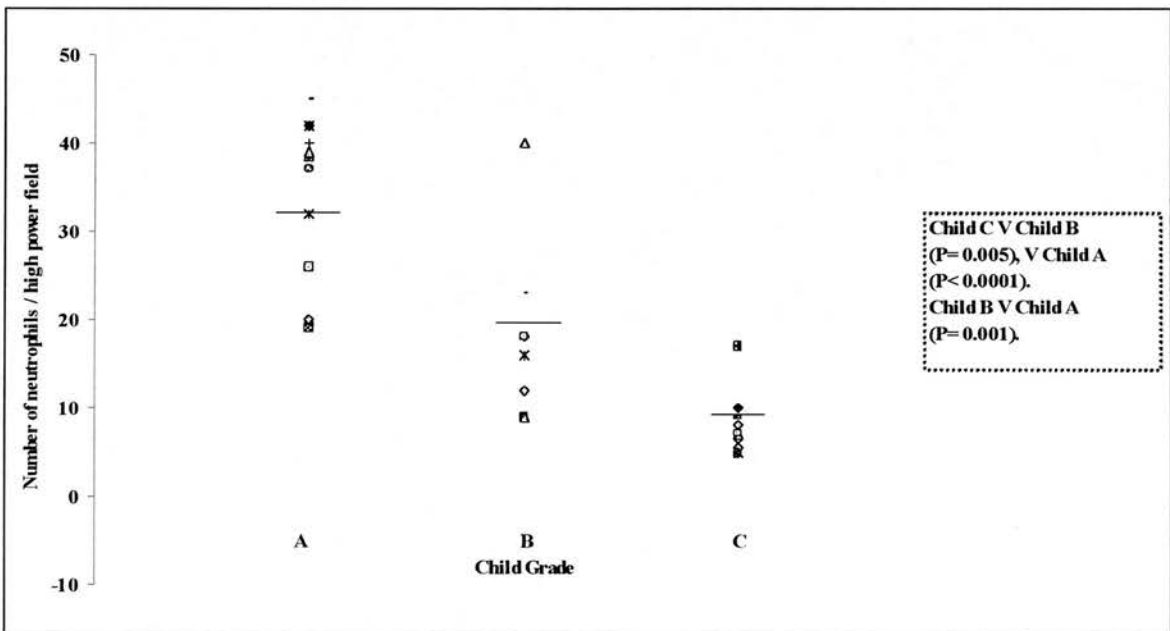


Figure 3.8: Neutrophil chemotaxis stimulated with 10 ng/ml Gro- $\alpha$  in patients with chronic liver diseases according to Child-Pugh grades.

### **3.5. DISCUSSION**

In this chapter neutrophil chemotaxis was measured in patients with acute or chronic liver failure, and controls using the potent neutrophil chemokines IL-8 and Gro- $\alpha$ . Subgroup analysis was performed to determine the effect of aetiology and severity of liver disease on chemokine-stimulated chemotaxis. The results of these studies clearly show that CXC chemokine stimulated (IL-8 and Gro) neutrophil chemotaxis is significantly impaired in patients with both acute and chronic liver failure compared with healthy controls and that comparing chronic liver failure with acute liver failure showed that the former have significantly reduced neutrophil chemotaxis. Patients with alcoholic liver disease and those with the most severe liver failure have the most impaired neutrophil chemotaxis.

As regard the effect of different aetiologies of liver disease, our studies show that neutrophil chemotaxis is similarly reduced in patients with alcoholic liver cirrhosis and patients with hepatitis C cirrhosis, but significantly reduced compared with patients with the other aetiologies. Neutrophil chemotaxis was least reduced in patients with primary biliary cirrhosis despite of their age difference compared with the other patient' groups. Neutrophil chemotaxis was significantly impaired in all groups of patients compared with the control group. In DeMeo and Andersen's study neutrophil chemotaxis stimulated with immune complex-activated serum was measured in a group of patients with alcoholic liver cirrhosis and healthy controls, and found reduced in the alcoholic group. They explained this reduction in neutrophil chemotaxis by a serum defect possibly hypocomplementaemia {DeMeo and Andersen 1972}. Others reported a similar significant reduction in neutrophil chemotaxis stimulated by zymosan-activated plasma in relatively well-compensated patients with alcoholic liver cirrhosis compared with controls and suggested that the low serum levels of complement factors especially C4 may be the cause of this defect {Campbell et al 1981}. Other neutrophil functions not including chemotaxis have been tested in patients with acute as well as chronic liver failure. For example, defective opsonisation was detected in patients with paracetamol-



and viral hepatitis- induced acute liver failure {Wyke et al 1980}. This defect in opsonisation was also detected in patients with either alcoholic liver cirrhosis or chronic active hepatitis compared with controls. This defect in opsonisation has also been associated with low serum complement level {Wyke et al 1983}. Abnormal neutrophil adherence occurs in patients with paracetamol-induced acute liver failure, alcoholic liver cirrhosis, chronic active hepatitis and primary biliary cirrhosis. Adherence is an important step in chemotaxis that enables the neutrophils to stick to the endothelium before extravasation outside the vasculature {Altin et al 1983}. Neutrophil phagocytic function and bacterial killing of staphylococcus aureus was impaired in patients with alcoholic liver cirrhosis, chronic active hepatitis, and primary biliary cirrhosis {De Fernandez et al 1987}. All these studies did not test neutrophil chemotaxis to the CXC chemokines, or compare the difference in neutrophil function in patients with acute and chronic liver failure.

In the literature, the relation between the aetiology of liver disease and neutrophil chemotaxis stimulated with non-specific or specific chemoattractants is still unclear or does not exist. However, our results showed clearly that there is a relationship between the different aetiologies of the liver disease and the reduction in neutrophil chemotaxis. The least impairment in neutrophil chemotaxis was in patients with primary biliary cirrhosis. Previous studies showed similar findings when studying other neutrophil functions such as neutrophil adherence, which showed minimal defects in patients with primary biliary cirrhosis compared with other aetiologies especially patients with alcoholic liver cirrhosis {Altin et al 1983}. In the same study, there was no statistical relationship between age or sex of the patients studied suggesting that the difference between PBC and the other groups in our study are not related to the differences in either age or sex (chapter 2, Figure 2.8-2.10). Brayton et al measured *in-vivo* neutrophil chemotaxis in a healthy control group divided according to their age into below and above 65 years old and found no difference in chemotaxis between both groups {Brayton et al 1970}. Some studies have shown the incidence of infection in patients with PBC was lower than the other disease aetiologies {Wyke et al 1983}. Others

suggested that there is different mechanism controlling serum complement in patients with primary biliary cirrhosis, and so both infection and the potential serum defect are less affects in such patients {Meyer and Buschenfede 1977}. Most previous studies testing neutrophil functions in patients with liver diseases found that the most affected group was the alcoholic liver cirrhosis. These patients have significantly more impaired opsonisation, serum complement defect or defective neutrophil chemotaxis, phagocytosis or killing {Wyke et al 1983, Yousif-Kadaru et al 1984, and De Fernandez et al 1987}. These findings are similar to those reported in this chapter, which shows that CXC chemokine induced chemotaxis is most impaired in the group of alcoholic cirrhosis.

Our studies also clearly show that neutrophil chemotaxis was similar in patients with paracetamol-overdose despite of the difference in encephalopathy grade they had, but in contrast was significantly reduced in patients with Child grade C and B cirrhosis compared with patients with grade A cirrhosis. The relationship between impairment in neutrophil chemotaxis and severity of the liver disease has not previously been fully explored. No studies have correlated neutrophil functions including neutrophil chemotaxis in patients with POD with the disease severity such as encephalopathy grade. Previous studies in patients with chronic liver disease have found that the reduction of neutrophil chemotaxis, phagocytosis and killing were not correlated with the severity of the disease as determined by the Child score {Campbell et al 1981}. Other studies have tested the correlation between other parameters of neutrophil function such as opsonisation, and neutrophil adherence, with the disease severity with contrasting results. In one study no relationship between the defects in opsonisation or complement serum level with the parameters of the disease severity such as prothrombin time was observed {Wyke et al 1983}. In contrast, the defective neutrophil adherence and complement serum levels measured by others were found to be dependent on the stage and severity of liver disease {Altin et al 1983}. A good correlation between neutrophil peroxidase activity {Vasil'ev 1984}, and defective neutrophil production of active O<sub>2</sub> species with the disease severity has also been reported {Itoh et al 1993}.

According to the results in this chapter, it is clear that the severity of the disease is more important than the aetiology, especially in patients with chronic liver failure. A more significant reduction in neutrophil chemotaxis was observed in patients with Child C grade than Child A and B. Most of the patients with PBC were Child A grade, which may explain why they had the least reduction in neutrophil chemotaxis. Proper multivariate analysis may be of help to detect the importance of either the severity or the aetiology of the disease as regard neutrophil chemotaxis.

Neutrophils express two CXC chemokine receptor types, CXCR1 and CXCR2 {Ahuja et al 1992}. Both types of receptors bind IL-8 {Jones et al 1995} while only CXCR2 binds Gro- $\alpha$  with high affinity {Lee et al 1992}. Defective Gro- $\alpha$ -induced neutrophil chemotaxis may result from only CXCR2 down-regulation. In contrast, down-regulation of both types of receptors is required to reduce IL-8-induced chemotaxis.

In conclusion, neutrophil chemotaxis is reduced in patients with acute and chronic liver failure. This reduction in neutrophil chemotaxis is well correlated with the severity of the liver disease, but may also be dependent to a lesser extent on the aetiology of the liver disease.

**CHAPTER 4**  
**IN VIVO AND IN VITRO MODULATION OF**  
**NEUTROPHIL CHEMOTAXIS**

## **4.1 INTRODUCTION**

Portal hypertension is a common complication of cirrhosis observed in 30-60% of patients at the time of their diagnosis {GarciaTsao et al 1985}. Upper gastrointestinal bleeding from oesophageal or gastric varices is a recognised major and sometimes fatal complication of portal hypertension. Potential risk factors for variceal haemorrhage include continued alcohol abuse {Dagradi 1972}, advanced liver disease {Pugh et al 1973}, portal pressure gradient greater than 12 mmHg {Lebrec et al 1980}, large varices, and red spots on the varices during endoscopy {Beppu et al 1981}. The mortality rate from the first variceal bleeding may be as high as 50% of cases, especially in patients with decompensated cirrhosis {Pagliaro et al 1992} and untreated re-bleeding from varices occurs in about 60% of surviving patients within 10 days {Graham and Smith 1981}.

Infection is a recognised complication occurring in patients following variceal haemorrhage {Lee and Schiodt 1999}. Bacterial infections occur in 35-66% of cirrhotic patients with bleeding oesophageal varices. Most of these infections are diagnosed on admission or during the first few days {Bernard et al 1996}. Gram-negative bacilli of enteric origin are the most common organisms. Bacterial peritonitis commonly complicates patients with ascites who present with bleeding varices {Rimola et al 1985 and Barns et al 1988}. Bacterial infection is an independent risk factor for early re-bleeding (within 5 days of admission) following variceal bleeding {Goulis et al 1998}, and an independent predictor factor of mortality in such patients {Piqueras et al 2001}.

Several factors are likely to be involved in the increased risk of infection observed in cirrhotic patients with variceal bleeding, including blood transfusion, invasive cardiovascular monitoring and instrumentation such as endoscopy. The acute gastrointestinal mucosal lesions detected in 70% of infected patients compared with 19% of non-infected patients following variceal haemorrhage were considered secondary rather than the portal of entry of infection in these patients {Bleichner et al 1986}. The

study of changes in neutrophil chemotaxis in this clinical situation is difficult because of the multiple factors occurring in patients during and subsequent to a variceal haemorrhage. Therefore an amino acid solution identical in composition to haemoglobin was used to simulate blood in the gastrointestinal tract and the changes in neutrophil chemotaxis following administration of this solution studied in this chapter. In addition cross over incubation with normal or cirrhotic serum was used to assess the presence of a circulatory inhibitor of neutrophil chemotaxis.

## **4.2 SUBJECTS**

### **4.2.1 Simulated bleeding studies**

20 patients with biopsy-proven alcoholic liver cirrhosis (15 males and 5 females, mean age 49.2 years, SEM 1.5) were studied. The patients' characteristics are shown in Table 4.1.

### **4.2.2 Cross over incubation study**

14 patients with chronic alcoholic liver disease were compared with 8 healthy controls, and 8 patients with paracetamol-induced acute liver failure compared with another 8 healthy controls. The patients and controls characteristics are shown in Table 4.2.

## **4.3 METHODS**

### **4.3.1 Simulated bleeding solution**

The simulated bleeding solution was prepared by dissolving 75 grams of amino acid mixture (Nutricia, Cuijk, Netherlands) in 200ml of sterile water. The simulated bleeding powder is a tailor-made mixture of amino acids that mimics the amino acid composition of haemoglobin, which lacks the essential amino acid isoleucine.

Table 4.1: Patients characteristics presented as mean  $\pm$  SEM.

Child score	Urea	Creatinine	Bilirubin	ALT	Albumin	PT
8.3 $\pm$ 0.3	5 $\pm$ 0.6	66.5 $\pm$ 5.1	65.5 $\pm$ 4.5	67.8 $\pm$ 4.2	31.2 $\pm$ 1	14.8 $\pm$ 0.5

*ALT= Alanine transferase.*

*PT= Prothrombin time.*

Table 4.2: Patient characteristics presented as mean  $\pm$  SEM.

Subjects	CLF study		ALF study	
	ALD	Controls	POD	Controls
Age: (yr)	48.6 $\pm$ 3	37.6 $\pm$ 1.5	33 $\pm$ 2	34.5 $\pm$ 1.7
Number:	14	8	8	8
Sex: F:M	5:9	2:6	3:5	3:5
Child-Pugh score	9 $\pm$ 0.5	–	–	–
Serum bilirubin: ( $\mu$ mol.L <sup>-1</sup> )	148 $\pm$ 40		81 $\pm$ 10	
Serum albumin: (g.L <sup>-1</sup> )	29 $\pm$ 1.3		39 $\pm$ 1.6	
Prothrombin time: (s)	16.6 $\pm$ 1.5		43.3 $\pm$ 8.4	
Blood urea: (mmol.L <sup>-1</sup> )	6.3 $\pm$ 1.4		12.6 $\pm$ 2.8	
Serum creatinine: ( $\mu$ mol.L <sup>-1</sup> )	96 $\pm$ 15		195 $\pm$ 28	

*CLF= chronic liver failure.*

*ALF= Acute liver failure.*

*ALD= alcoholic liver disease.*

*POD=Paracetamol overdose.*

*M= male. F= female.*

Administration of this solution orally raises the plasma ammonia concentration and induces hypoisoleucinaemia and has been used previously to mimic the systemic biochemical effects of gastrointestinal haemorrhage {Olde Damink et al 1998}. Gum (Sigma, St Louis-USA) was used as placebo after dissolving in a similar amount of sterile water as before.

### **4.3.2 Effect of the simulated bleeding on neutrophil chemotaxis**

In the initial ten patients neutrophil chemotaxis and plasma ammonia concentration were measured at time 0 and 2 hours after ingestion of the simulated bleeding solution. Subsequently, a further 10 patients were randomly allocated to receive either the simulated bleeding solution or the placebo solution (using the closed envelope method). The researcher was blinded as to the group to which the patient was allocated. Peripheral venous blood was collected for determination of neutrophil chemotaxis as described in Chapter 2 (sections 2.3.1 and 2.3.3). Neutrophil chemotaxis was studied at time 0 and 2 hours after oral administration of either the placebo or the simulated bleeding solution.

### **4.3.3 Plasma ammonia concentration**

Blood collected into lithium heparin tubes were kept on ice and within half an hour, the plasma recovered by centrifugation at 3000 rpm for 20 minutes at 4°C. The plasma ammonia concentration was measured directly with automatic clinical analyzer (Hitachi model 717; Boehringer, Mannheim, Germany) using a specific enzymatic assay (Da Fonseca-Wollheim 1992 and Lukkarinen et al 2000).

### **4.3.4 Cross over incubation study**

The neutrophils were prepared from patients and controls as described in Chapter 2 (section 2.3.1). Serum was retrieved by centrifugation from 10 ml of clotted peripheral



venous blood at 3000 rpm for 20 minutes at 4°C. Neutrophil chemotaxis was measured as described in Chapter 2 (section 2.3.2), before and after incubation of 0.5 ml of patients neutrophils ( $1 \times 10^6$  cells) with 20  $\mu$ l of control subjects serum (or vice versa) for 30 minutes at 37°C. Neutrophils were washed and resuspended in PBS before measuring chemotaxis.

### **4.3.5 Statistical analysis**

Because the data was not normally distributed, the Wilcoxon Signed Ranked Test was used to compare the results at time 0 and 2 hours, and before and after cross over incubation. Spearman's correlation test was used to detect correlations of the results of neutrophil chemotaxis and plasma ammonia concentrations.

## **4.4 RESULTS**

### **4.4.1 The simulated bleeding (S.B.) study in the first ten patients**

IL-8 (10ng/ml) stimulated neutrophil chemotaxis was significantly reduced at 2 hours ( $6.3 \pm 4.9$ , mean  $\pm$  SEM, n=10) following administration of the simulated bleeding solution compared with pre-administration ( $24.8 \pm 4.6$ ,  $p < 0.0001$ ). Gro- $\alpha$  (10ng/ml) stimulated chemotaxis was also significantly reduced at 2 hours ( $7.6 \pm 1.8$ , n=10) following the stimulated bleeding compared with pre-administration ( $30 \pm 1.8$ ,  $p < 0.0001$ ). Figure 4.1 shows both IL-8- and Gro- $\alpha$ -stimulated chemotaxis at time 0 and 2 hours following administration of the simulated bleeding solution. Plasma ammonia concentration was increased at time 0 ( $75.1 \pm 4.2$ , mean  $\pm$  SEM, n=10) compared with a normal range of plasma ammonia concentration in healthy controls as measured in previous studies (10-35  $\mu$ mol/L) {Van Buuren et al 1982}. The level rose significantly 2 hours following administration of the simulated bleeding solution ( $124.6 \pm 8.5$ , n=10,  $p < 0.01$ , Figure 4.2).

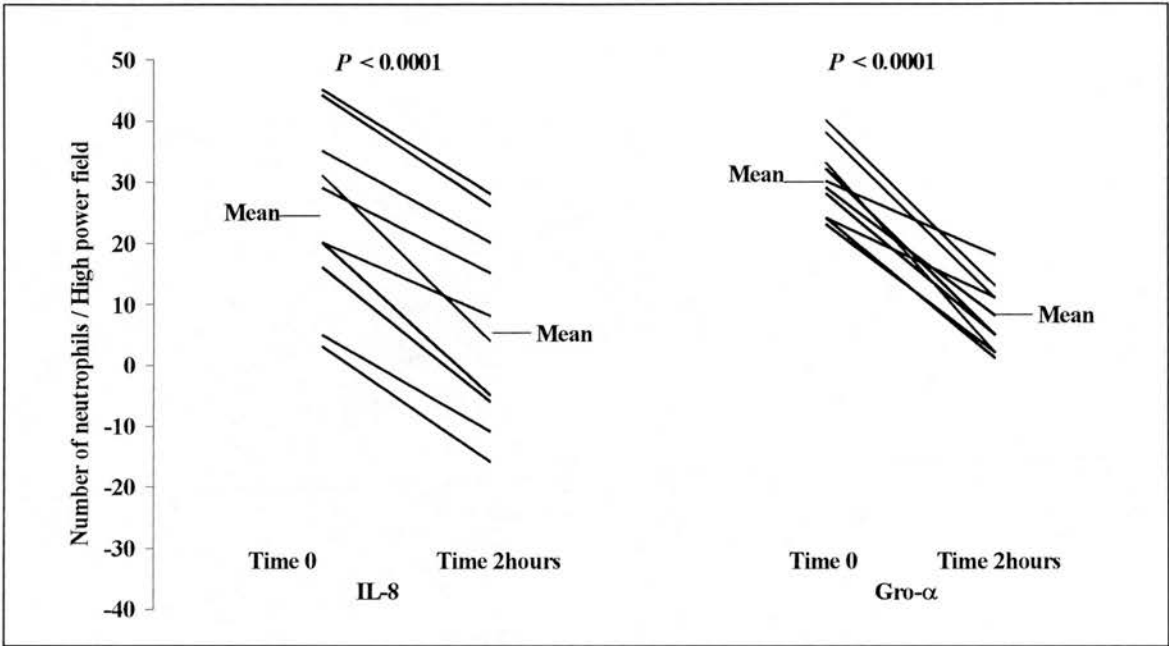


Figure 4.1: Individual and mean neutrophil chemotaxis stimulated with 10ng/ml IL-8 and Gro in patients with alcoholic liver cirrhosis at time0 and 2 hours after the simulated bleeding.

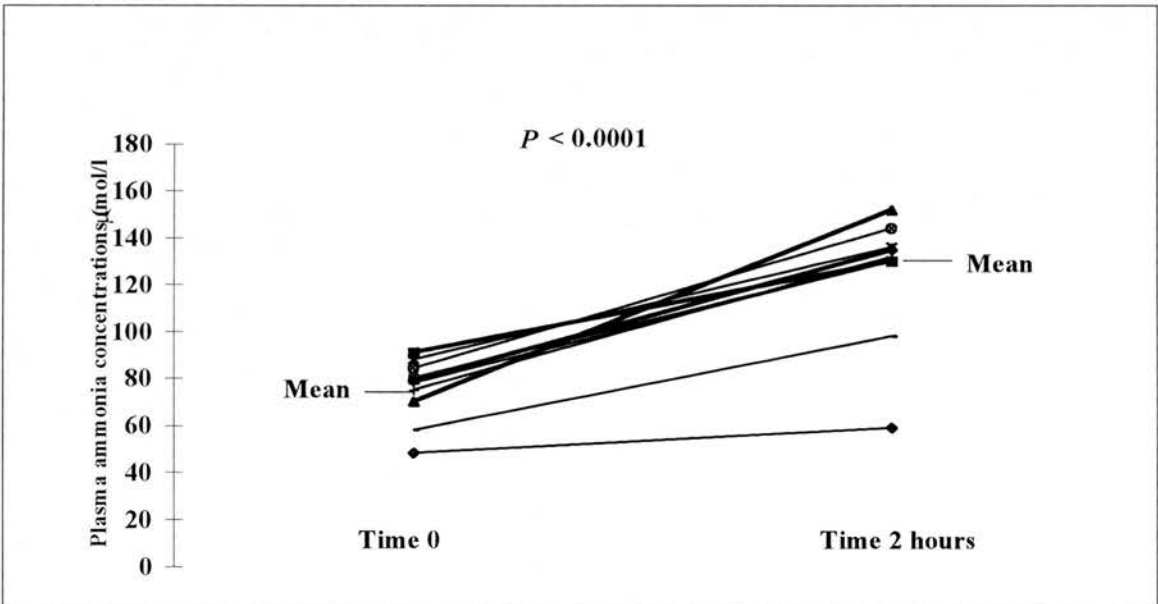


Figure 4.2: Individual and mean plasma ammonia concentrations in patients with alcoholic liver cirrhosis at time 0 and 2 hours after the simulated bleeding.

There was no correlation between the percentage changes in both IL-8- and Gro- $\alpha$ -induced neutrophil chemotaxis and plasma ammonia concentration between time 0 and time 2 hours following administration of the simulated bleeding solution (Figures 4.3 and 4.4).

#### **4.4.2 Randomised simulated bleeding study**

IL-8 (10ng/ml) stimulated neutrophil chemotaxis was significantly reduced at 2 hours ( $7.2 \pm 6.4$ , mean  $\pm$  SEM, n=5) following administration of the simulated bleeding solution compared with pre-administration ( $28.2 \pm 5$ ,  $p < 0.0001$ ). Gro- $\alpha$  (10ng/ml)-stimulated chemotaxis was also significantly reduced at 2 hours ( $4.4 \pm 1.9$ , n=5) following the stimulated bleeding compared with pre-administration ( $26.8 \pm 1.8$ ,  $p < 0.0001$ ). Figure 4.5 shows both IL-8- and Gro- $\alpha$ -stimulated chemotaxis at time 0 and 2 hours following administration of the simulated bleeding solution. There was no change in IL-8-stimulated neutrophil chemotaxis between pre-administration ( $19.8 \pm 3.9$ , n=5) compared with 2 hours following administration of placebo ( $21.6 \pm 5.4$ ,  $p < 0.5$ ). Gro-stimulated neutrophil chemotaxis also showed no change between pre-administration ( $20.4 \pm 3$ , n=5) compared with 2 hours following administration of placebo ( $21.3 \pm 4.9$ ,  $p < 0.5$ ). Figure 4.6 shows both IL-8- and Gro- $\alpha$ -stimulated chemotaxis at time 0 and 2 hours following administration of the placebo solution. Plasma ammonia concentration was elevated at time 0 ( $84 \pm 2.5$ , n=5) compared with the normal range of plasma ammonia concentration in healthy controls, and rose significantly 2 hours following administration of the simulated bleeding solution ( $135 \pm 2.6$ , n=5,  $p < 0.001$ , Figure 4.7). However, there was no change in plasma ammonia concentration between time 0 and 2 hours in the placebo group (Time 0:  $86 \pm 2$ , n=5, and Time 2 hours:  $89 \pm 1$ , n=5,  $p < 0.5$ , Figure 4.7).

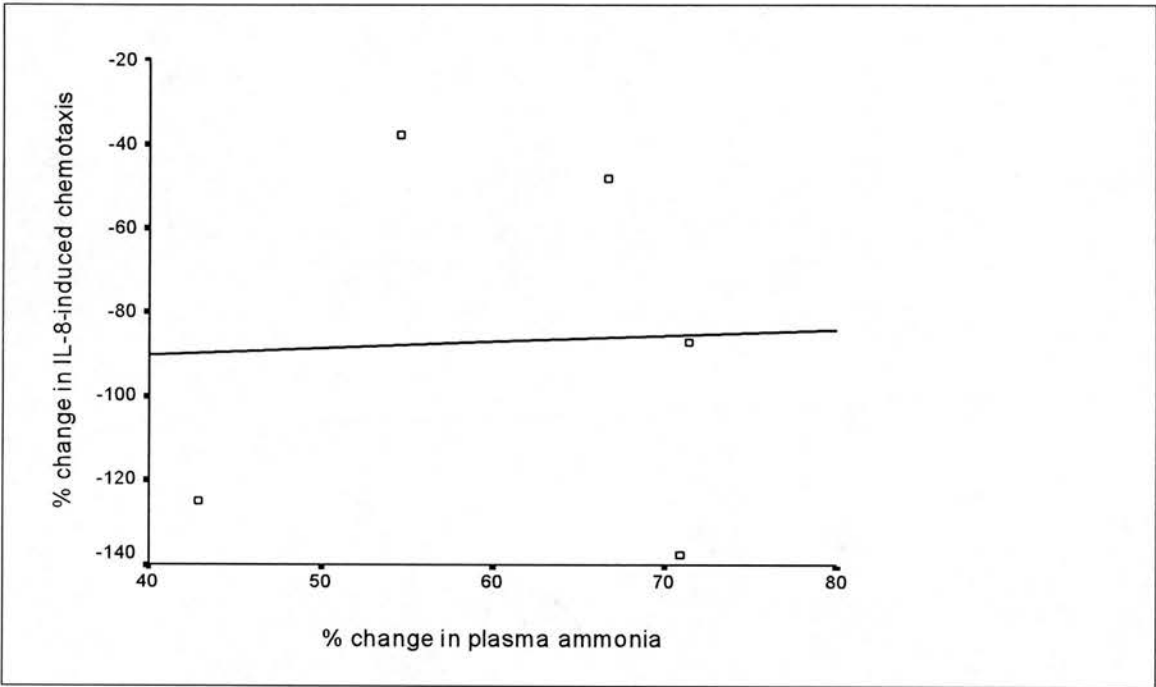


Figure 4.3: The correlation between the percentage changes in IL-8 induced neutrophil chemotaxis and plasma ammonia concentrations.

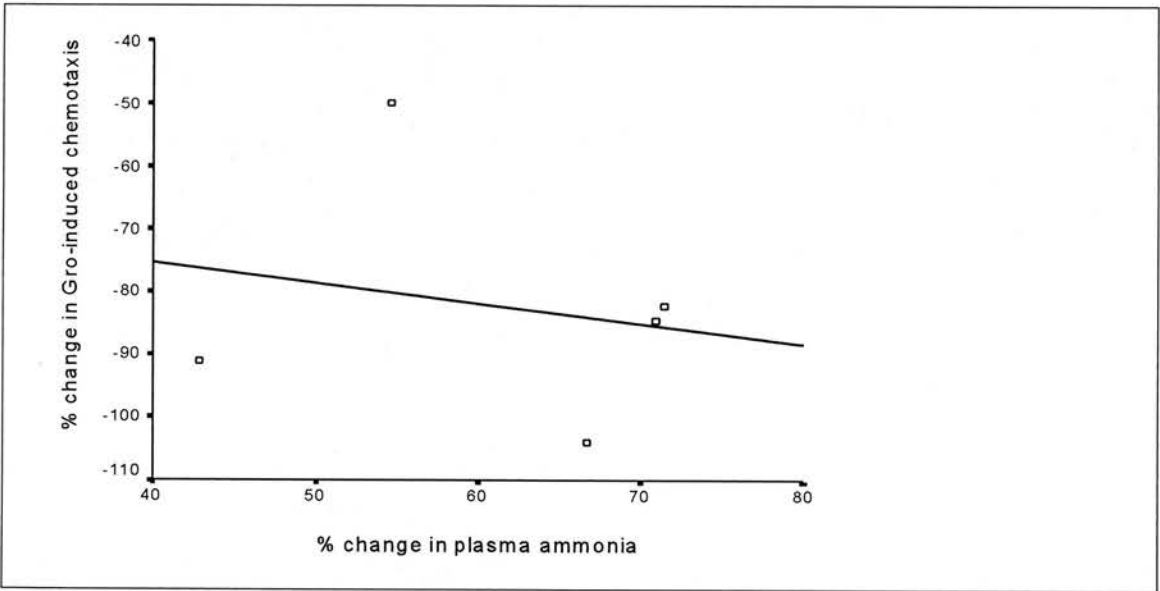


Figure 4.4: The correlation between the percentage changes in Gro- $\alpha$ -induced neutrophil chemotaxis and plasma ammonia concentrations.

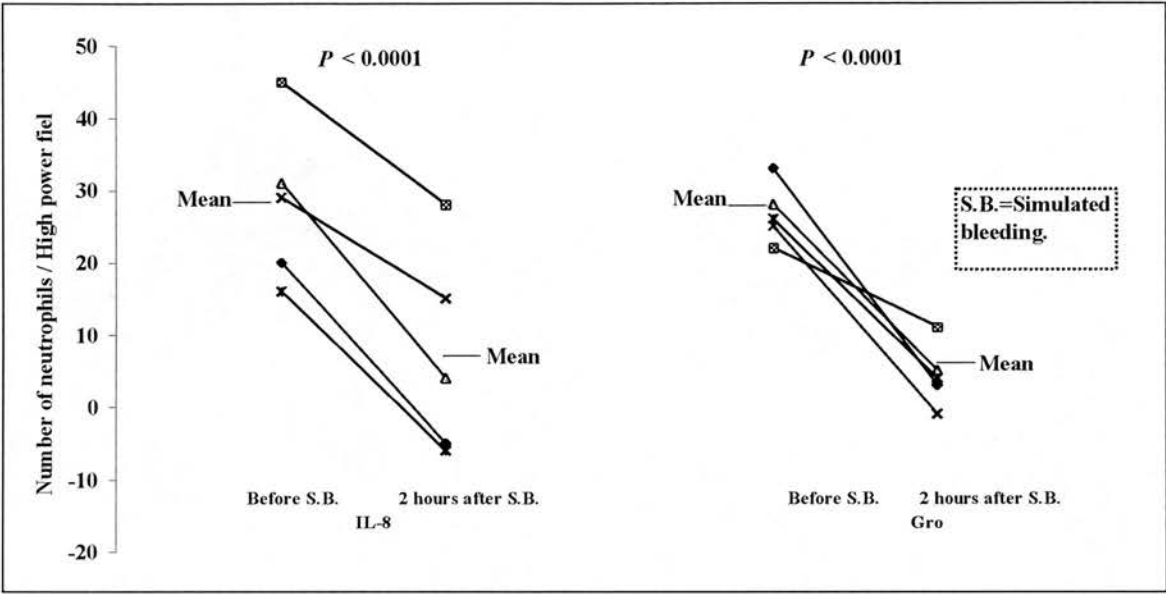


Figure 4.5: Individual and mean neutrophil chemotaxis stimulated with 10 ng/ml of IL-8 and Gro- $\alpha$  in patients with alcoholic liver cirrhosis before and 2 hours after the simulated bleeding.

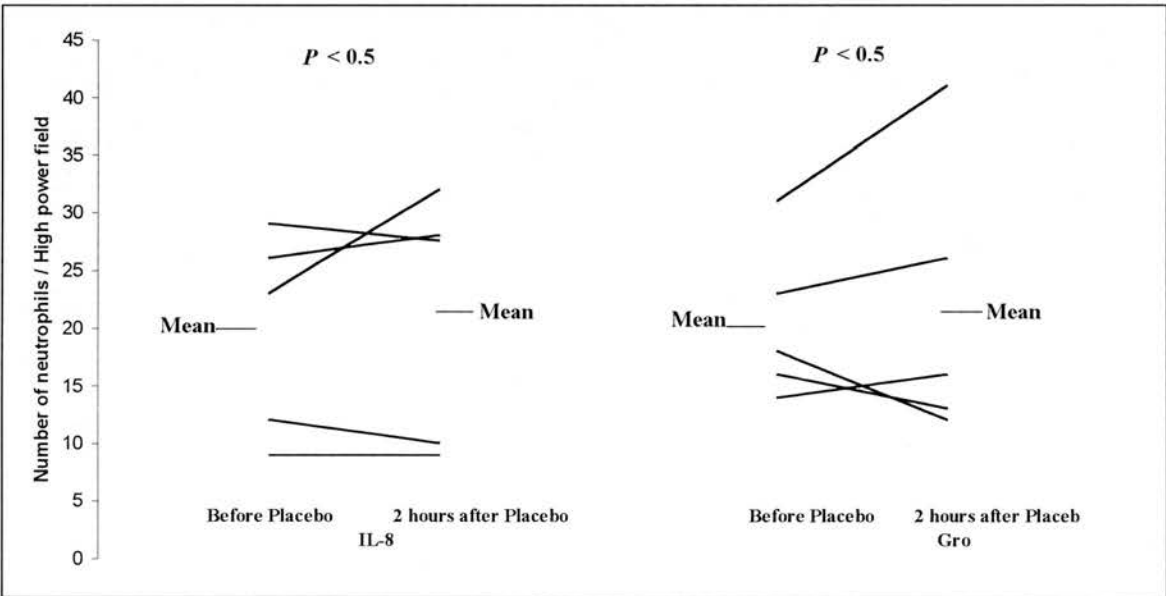


Figure 4.6: Individual and mean neutrophil chemotaxis stimulated with 10 ng / ml of IL-8 and Gro- $\alpha$  in patients with alcoholic liver cirrhosis before and 2 hours after placebo.

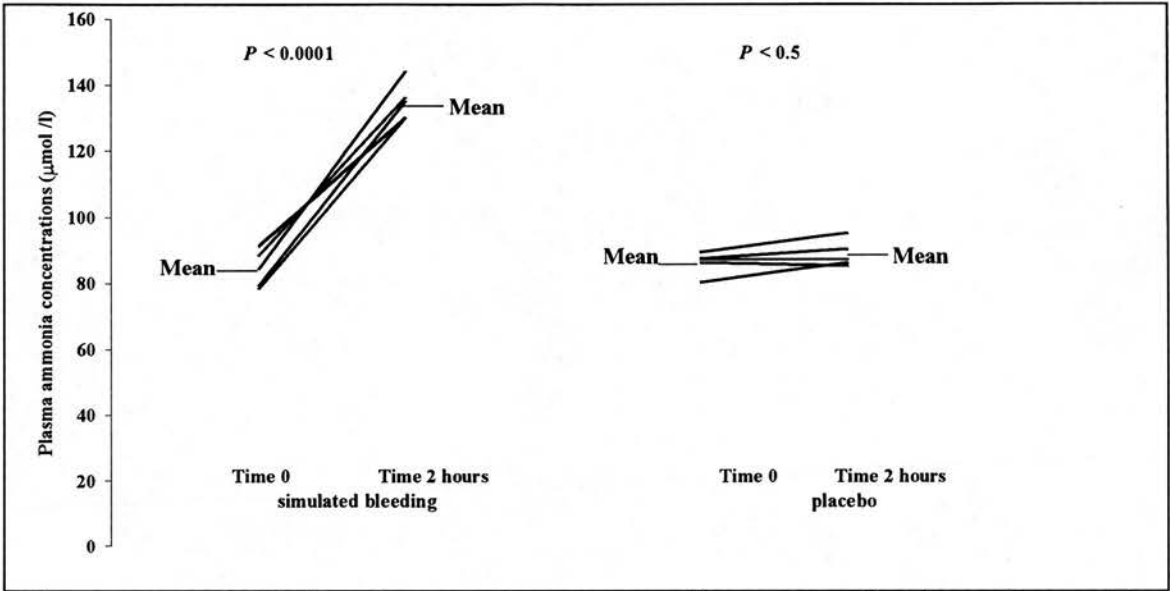


Figure 4.7: Individual and mean plasma ammonia concentration in patients with alcoholic liver cirrhosis at time 0 and time 2 hours after the simulated bleeding and placebo.

#### **4.4.3 Effect of cross incubation in chronic liver failure**

Neutrophil chemotaxis stimulated with IL-8 (10ng/ml) was significantly improved following incubation with controls subjects' serum (before  $14.1 \pm 0.8$ , after  $26.1 \pm 0.8$  neutrophils / high power field, mean  $\pm$  SEM,  $n=14$ ,  $p < 0.0001$ , Figure 4.8). In contrast, chemotaxis of neutrophils prepared from control subjects was significantly reduced after incubation with patients' serum (before  $48 \pm 1.1$ , after  $28 \pm 0.8$ ,  $n=8$ ,  $p < 0.0001$ , Figure 4.9).

#### **4.4.4 Effect of cross incubation in acute liver failure**

Neutrophil chemotaxis was significantly improved following incubation with control subjects' serum (before  $17.6 \pm 1.2$ , after  $32.3 \pm 1.2$ ,  $n=8$ ,  $p < 0.0001$ , Figure 4.10). In contrast, chemotaxis of neutrophils prepared from control subjects was significantly reduced after incubation with patients' serum (before  $51.6 \pm 1$ , after  $30.1 \pm 1.6$ ,  $n=8$ ,  $p < 0.0001$ , Figure 4.11).

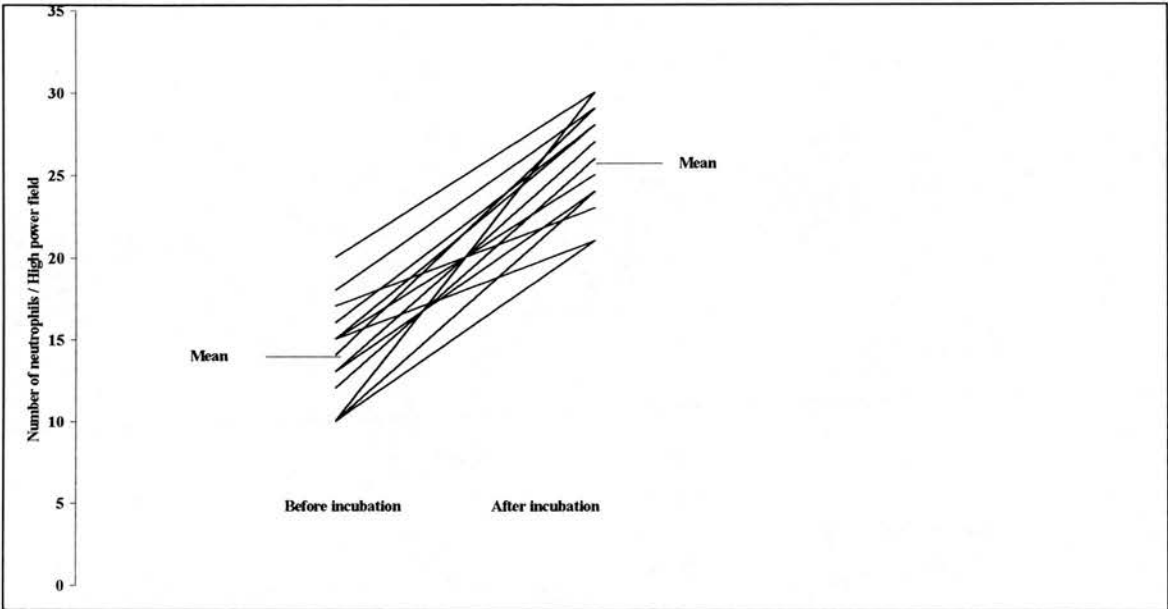


Figure 4.8: Individual and mean (bar) neutrophil chemotaxis stimulated with 10ng/ml of IL-8 in patients with alcoholic liver cirrhosis before and after incubation with control subjects serum.

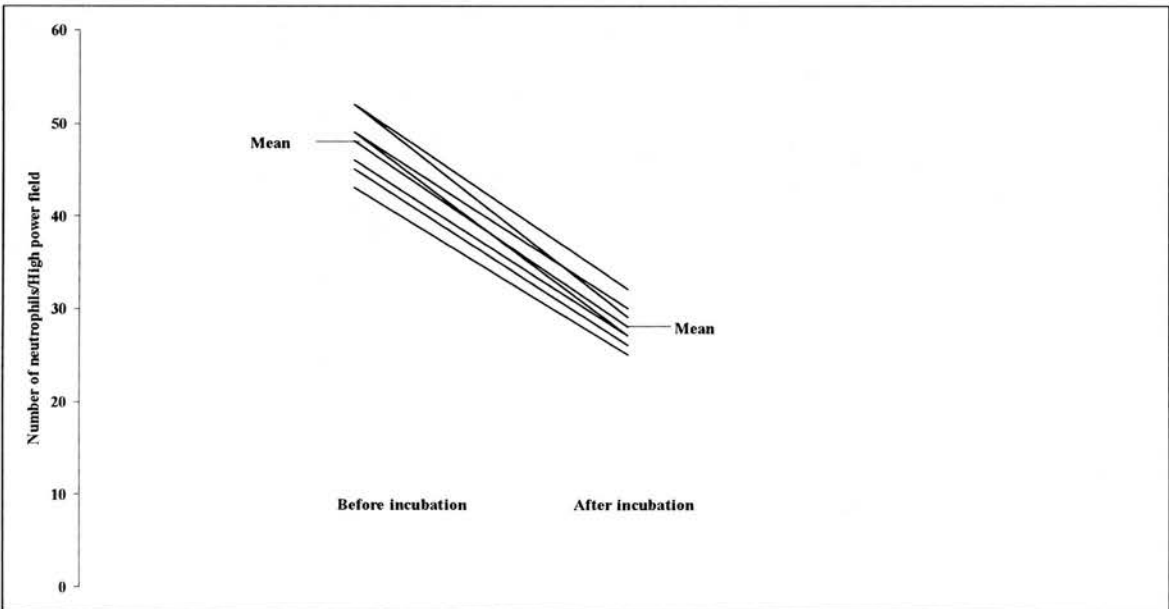


Figure 4.9: Individual and mean (bar) neutrophil chemotaxis stimulated with 10ng/ml of IL-8 in controls before and after incubation with alcoholic liver cirrhosis patients' serum.



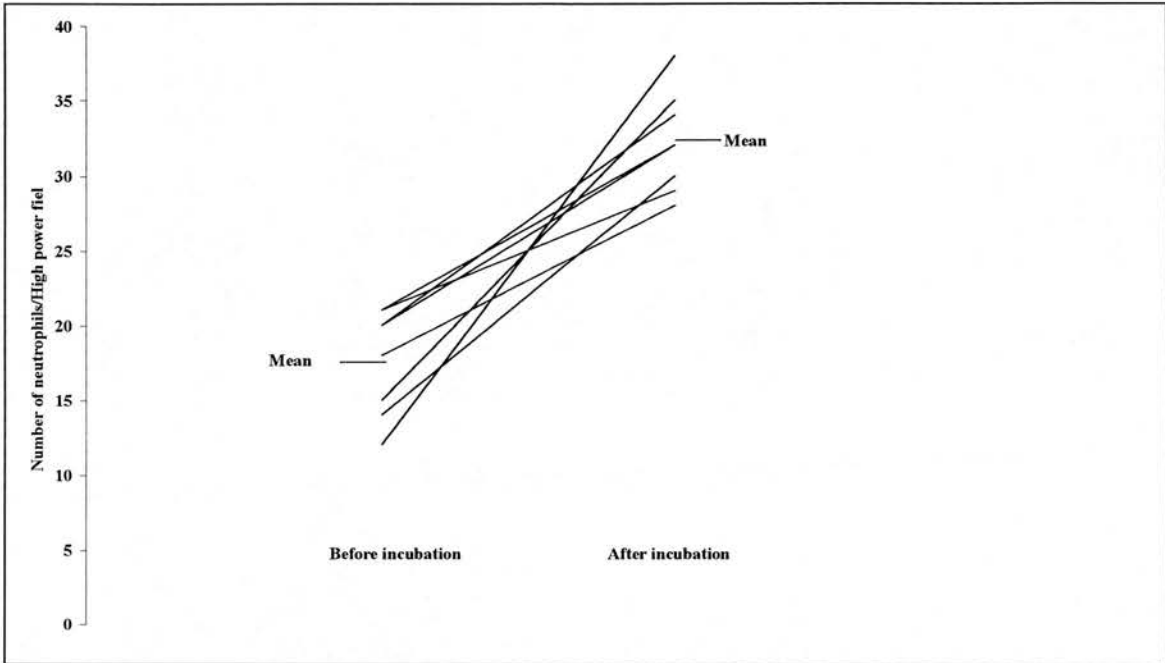


Figure 4.10: Individual and mean (bar) neutrophil chemotaxis stimulated with 10ng/ml of IL-8 in patients with POD-induced acute liver failure before and after incubation with control subjects serum.

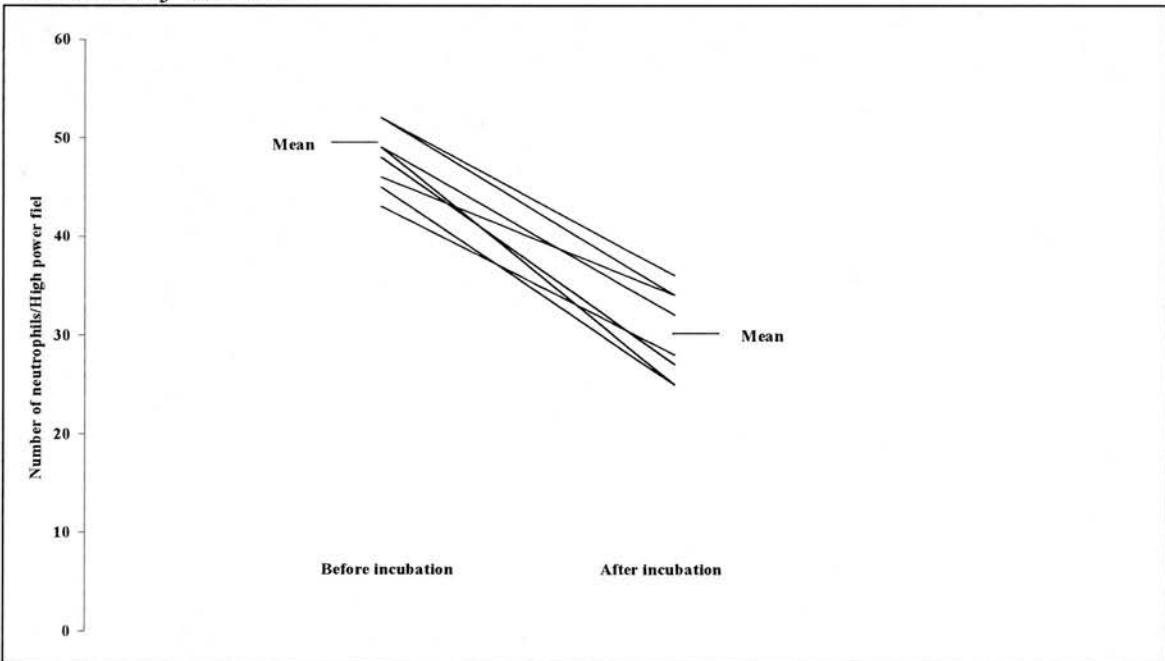


Figure 4.11: Individual and mean neutrophil chemotaxis stimulated with 10ng/ml of IL-8 in controls before and after incubation with POD-induced acute liver failure patients' serum.

## **4.5. DISCUSSION**

In this chapter neutrophil chemotaxis and plasma ammonia concentrations were measured in patients with alcoholic liver cirrhosis before and 2 hours after administration of either a simulated bleeding or placebo solution. The simulated bleeding solution was used in these studies instead of actual upper gastrointestinal bleeding to avoid confounding factors, such as transfusion, that may make the results of neutrophil chemotaxis difficult to interpret. The simulated bleeding solution mimics the amino acid composition of haemoglobin and lacks the essential amino acid isoleucine. This solution induces the same biochemical changes induced by the actual gastrointestinal bleeding such as hypoisoleucinaemia and hyperammonaemia {Olde Damink et al 1998}. Previous studies have used this solution in animal and human studies and have shown the resulting hyperammonaemia following simulated bleeding or actual upper gastrointestinal bleeding is similar {Klerx et al 1985 and Dejong et al 1996}.

These data presented in this chapter clearly show that IL-8 and Gro- $\alpha$  stimulated neutrophil chemotaxis is significantly reduced in patients with alcoholic liver cirrhosis 2 hours after oral administration of the simulated bleeding solution in both the non-randomised and randomised studies. The reduction in neutrophil chemotaxis was associated with significant elevation in plasma ammonia concentration. However, there was no significant correlation between neutrophil chemotaxis and plasma ammonia concentration. Other studies have suggested increased ammonia concentration may affect neutrophil chemotaxis. Hyperammonaemia can reduce cellular cytosolic glutaminase activity, and therefore reduce glutamine utilisation by neutrophils. Glutamine and glucose are major substrate for energy production in neutrophils {Curi et al 1997}. Disruption of neutrophil glutamine metabolism by hyperammonaemia may affect the cellular bioenergies and limit neutrophil chemotaxis. Hyperammonaemia can also reduce the affinity of neutrophil CXC chemokine receptors for their ligands {Coppi

and Niederman 1989}. However no correlation was noted between ammonia and neutrophil chemotaxis in the studies reported in this chapter. In contrast the hypoisoleucine state that also occurs with gastrointestinal bleeding and administration of the simulated bleeding solution can adversely affect cellular functions, which could also include neutrophil functions such as chemotaxis.

In this chapter the presence of a circulating chemotactic inhibitory factor/s was tested with a cross over incubation study. Such studies have been utilised previously to demonstrate the presence of a circulating inhibitor of platelet function in patients with cirrhosis {Forrest et al 1996}. The results in this chapter demonstrate the inhibitory effect of serum from both patients with acute or chronic liver failure on chemotaxis of control subjects neutrophils. In contrast there was partial recovery of patients neutrophil chemotaxis following incubation with controls. The results suggest the presence of a circulating chemotactic inhibitory factor/s in patients with acute and chronic liver failure. Because the patients defect is only partially reversible, this suggests that there is also an intrinsic neutrophil defect.

Previous studies suggested presence of chemotaxis inhibitory factor/s in serum prepared from either patients with acute liver failure {Bailey et al 1976 and Yousif-Kadaru et al 1984} or chronic liver failure {DeMeo and Andersen 1972}. In patients with acute liver failure, an intrinsic leucocyte abnormality or hypocomplementaemia was not observed as a cause for the limited neutrophil chemotaxis. However a low molecular weight, water soluble, dialyzable, and adsorbable factor, that inhibits the metabolic activity of leucocyte hexose monophosphate shunt was observed (Bailey et al 1976). Defective neutrophil chemotactic activity occurs in patients with chronic liver failure possibly due to hypocomplementaemia and/or a serum chemotactic inhibitory factor/s {DeMeo and Andersen 1972, Yousif-Kadaru et al 1984}. Other studies have also detected the existence of a chemotaxis inhibitory factor (CIF) in patients with chronic alcoholic liver disease {Robbins 1987 and MacGregor 1990}.

The exact nature of the circulating chemotactic inhibitor remains to be characterised. Previous studies used a variety of non-specific chemoattractants. The studies reported in this thesis used the CXC chemokines IL-8 and Gro. Circulating IL-8 is not detectable in normal subjects but has been reported at high concentration in patients with alcoholic hepatitis especially those who died. Increased circulating IL8 was also reported in other liver disorders and were correlated with markers of disease severity such as bilirubin and serum albumin {Sheron et al 1993}. Similar results have been shown in another study which reported higher mortality rates in patients with alcoholic diseases (hepatitis, cirrhosis and fatty changes) with IL-8 concentration more than 479pg/ml {Huang et al 1996}. High CXC chemokine concentrations persisted until death of the non-survivor group, but declined towards the recovery in the survivors {Fujimoto et al 2000}. Increased hepatic expression of chemokines has also been reported in a variety of liver diseases. The increased circulating chemokine concentrations reported in patients with liver disease may therefore induce neutrophil CXC receptor down-regulation and hence impair neutrophil chemotaxis.

Before testing this hypothesis, and in common with previous studies (Forrest et al 1996), we also sought to measure chemokine-induced Cytosolic  $Ca^{++}$  influx; an important step in intracellular signalling mechanism in neutrophil activation and chemotaxis was investigated. FURA-2AM (Sigma-Aldrich Company LTD.) was used to load neutrophils in order to test fluorescence using the Knotron SFM 25 spectrofluorimeter. Fluorescence was measured before and after stimulation with chemokines IL-8. Unfortunately, the maximum fluorescence {using 0.8% Triton X-100 (Sigma-Aldrich Company LTD.)} showed similar or lower  $Ca^{++}$  concentration to minimal fluorescence {using 10 mmol/l EDTA and 30 mmol/l TRIS buffer}. We changed the culture media used, check cell viability, increased the FURA concentration, took serial readings and tried different type of cells such as monocytes (using chemokine MIP-1 $\alpha$  as a stimulant). Later, we pre-chilled the cells at 4°C to prevent dye sequestration, added probenecid to avoid dye leakage from the cells, added calcium chloride solution before the maximum

fluorescence reading and Pluronic-F127 to improve dye loading. Unfortunately, none of these manipulations gave consistent meaningful results for calcium flux and so this technique was abandoned and future studies focused on changes in chemotaxis and other hypothesis of chemokine receptor down-regulation in Chapter 7.

## **CHAPTER 5**

# **SERUM CHEMOKINE CONCENTRATIONS**

## **5.1 INTRODUCTION**

In the previous chapter the presence of a circulatory inhibitor of neutrophil chemotaxis was implicated in the chemotactic defect identified in patients with acute and chronic liver failure. Previous studies have shown increased circulatory IL-8 in patients with severe alcoholic hepatitis {Sheron, 1993} as well as in patients with chronic alcoholic liver cirrhosis and alcoholic hepatitis {Huang et al 1996}. In this chapter serum concentrations of IL-8 were measured. In addition the relation with disease severity was investigated. The circulating concentrations of the CXCR3 ligands, MIG and IP10, were also measured.

## **5.2 SUBJECTS**

These studies included 55 patients with paracetamol-induced acute liver failure, 28 patients with chronic alcoholic liver disease, and 10 healthy laboratory staff as controls. The patients and controls characteristics are shown in Table 5.1.

## **5.3 METHODS**

### **5.3.1 Serum isolation**

Peripheral venous blood was collected from patients and controls and serum prepared as described previously. Serum was stored at  $-70^{\circ}\text{C}$  prior to assay.

### **5.3.2 Serum CXC chemokine concentrations**

A modified double-ligand enzyme linked immunosorbent assay was used to measure serum CXC chemokine concentrations {Koch et al 1995 Elner et al 1998}. Briefly, Paisley flat-bottom 96 well microplates (Nunc immuno-plate I 96-F, Kamstrup, Denmark) were coated with 50  $\mu\text{l}$  of polyclonal rabbit anti IL-8, IP-10, or Mig antibodies (1ng/ $\mu\text{l}$  in 0.6 mmol/L of NaCl, 0.26 mmol/L of  $\text{H}_3\text{BO}_4$ , and 0.08 NaOH; pH 9.6) for 24 hours at  $4^{\circ}\text{C}$ , followed by washing with PBS. Polyclonal anti-human IL-8, IP-10, and Mig were produced by immunization of rabbits with rIL-8, IP-10, or Mig

with fetal calf albumin (FCA). These antibodies did not cross-react with cytokines TNF- $\alpha$ , IL-1, IL-2, IL-4, IL-6, or IFN- $\gamma$ . In addition, the antibodies did not cross-react with either CXC chemokines such as Gro- $\alpha$ , -B, - $\gamma$ , neutrophil activating peptide-2 (NAP-2), or granulocyte chemotactic protein-2 (GCP-2), or CC chemokines such as monocyte chemoattractant protein-1 (MIP)-1, -2, -3, or macrophage inflammatory protein-1 (MIP-1)- $\alpha$  and - $\beta$ .



Table 5.1: Patient characteristics presented as mean  $\pm$ SEM.

	POD	ALD	Controls
Age: (yr)	36 $\pm$ 1.9	58.7 $\pm$ 2	36 $\pm$ 2.2
Number:	55	28	10
Sex: F:M	24:31	10:18	2:8
Child-Pugh score		8.3 $\pm$ 0.4	-
Child grade A:B:C		9:6:13	-
Serum bilirubin: ( $\mu\text{mol.L}^{-1}$ )	134 $\pm$ 18	132 $\pm$ 32	-
Serum albumin: ( $\text{g.L}^{-1}$ )	37 $\pm$ 0.8	32 $\pm$ 1.8	-
Prothrombin time: (s)	54.3 $\pm$ 4	15.1 $\pm$ 1.3	-
Blood urea: ( $\text{mmol.L}^{-1}$ )	23.6 $\pm$ 15	6.5 $\pm$ 1	-
Serum creatinine: ( $\mu\text{mol.L}^{-1}$ )	214 $\pm$ 28	104.3 $\pm$ 9.8	-

*POD=Paracetamol overdose.*

*ALD= alcoholic liver disease.*

*M= Male. F= Female.*

Non-specific binding was blocked with 2 % bovine serum albumin in PBS and incubated for 1 hour at 37°C. After washing 3 times with PBS, samples were added in duplicates (50 µl /well), and incubated for further 1 hour at 37°C. After 3 washings, 50 µl/well of biotinylated polyclonal rabbit anti-chemokine antibodies (3.5 ng/µl in PBS at pH 7.5; 0.05% Tween 20; 2% fetal calf serum) were added, and the plates incubated for 45 minutes at 37°C. After washing, streptavidin-peroxidase conjugate (Bio-Rad labs, Richmond, California) was added and incubated for further 30 minutes at 37°C. The colour was developed with chromogen substrate (Bio-Rad labs.) and the reaction terminated with 50 µl/well of 3 mmol/l of H<sub>2</sub>SO<sub>4</sub> solution / well. Plates were read at 490 nm in an ELISA reader. Standards were 0.5-log dilutions of recombinant IL-8, IP-10, or Mig (R&D systems, Minneapolis, Minnesota) from 100ng to 1 pg/ml (50 µl /well). The ELISA consistently detected chemokine concentrations > 30pg/ml.

### **5.3.3 Statistical tests**

Initial statistical testing showed the data to be normally distributed. Statistical analysis was therefore performed using a one-way analysis of variance (ANOVA) and Student's t- test. Correlation coefficients were determined using a Pearson's correlation test.

## **5.4 RESULTS**

### **5.4.1 Patients with acute liver failure**

Serum chemokine concentrations were significantly higher in patients (IL-8  $51.2 \pm 7.6$  pg/ml, IP-10  $0.55 \pm 0.19$  ng/ml, and Mig  $2.29 \pm 0.3$ , mean  $\pm$  SEM, n=55) compared with controls ( $3 \pm 3$  pg/ml,  $0.04 \pm 0.04$  ng/ml,  $0.2 \pm 0.1$ , n=10,  $p = 0.0001$ , 0.01. and 0.0001 respectively, Figure 5.1).

#### **5.4.1.1 Relationship with disease severity**

The patients were subdivided into survivors or patients who either died or were transplanted (Tx). Serum IL-8 was higher in patients who died or were transplanted ( $95.9 \pm 16.2$  pg/ml,  $n=15$ ) compared with those who survived ( $34.5 \pm 7$  pg/ml,  $n=40$ ,  $p < 0.0001$ ). However, there was no significant difference in serum IP-10 or Mig between patients who died or were transplanted (IP-10;  $0.61 \pm 0.19$ , MIG;  $3.2 \pm 0.9$  ng/ml) and those who survived (IP-10;  $0.52 \pm 0.3$  MIG;  $1.9 \pm 0.3$  ng/ml,  $p < 0.8$  and  $0.1$  respectively, Figure 5.2).

As an alternative marker of severity of the acute liver failure patients were subdivided into those without hepatic encephalopathy (but with severe liver injury), those with grade 1 or 2 encephalopathy, and those with grade 3 or 4 encephalopathy. Serum chemokine concentrations were significantly higher in patients with encephalopathy grades 3 or 4 (IL-8;  $92.2 \pm 25.3$ , IP-10;  $1.8 \pm 1.02$ , MIG;  $3.4 \pm 1.01$ ,  $n=9$ ) compared with patients without encephalopathy (IL-8;  $33.4 \pm 7.3$ , IP-10;  $0.2 \pm 0.06$ , MIG;  $1.8 \pm 0.3$ ,  $n=30$ ,  $p < 0.004$ ,  $0.006$ , and  $0.04$  respectively). There was no significant difference in serum chemokines between patients with grades 1 or 2 (IL-8;  $63.5 \pm 15.6$ , IP-10;  $0.4 \pm 0.2$ , MIG;  $2.7 \pm 0.8$ ,  $n=15$ ) and patients without encephalopathy ( $p = 0.05$ ,  $0.3$ , and  $0.2$  respectively) or patients with encephalopathy grades 3 or 4 ( $p = 0.3$ ,  $0.1$ , and  $0.6$  respectively, Figure 5.3). Both serum IL-8 and IP-10 concentrations were correlated significantly with progression of encephalopathy grade from grade 0 to grades 3 and 4 ( $p < 0.01$  for both), with no significant correlation as regard serum MIG ( $p < 0.2$ ).

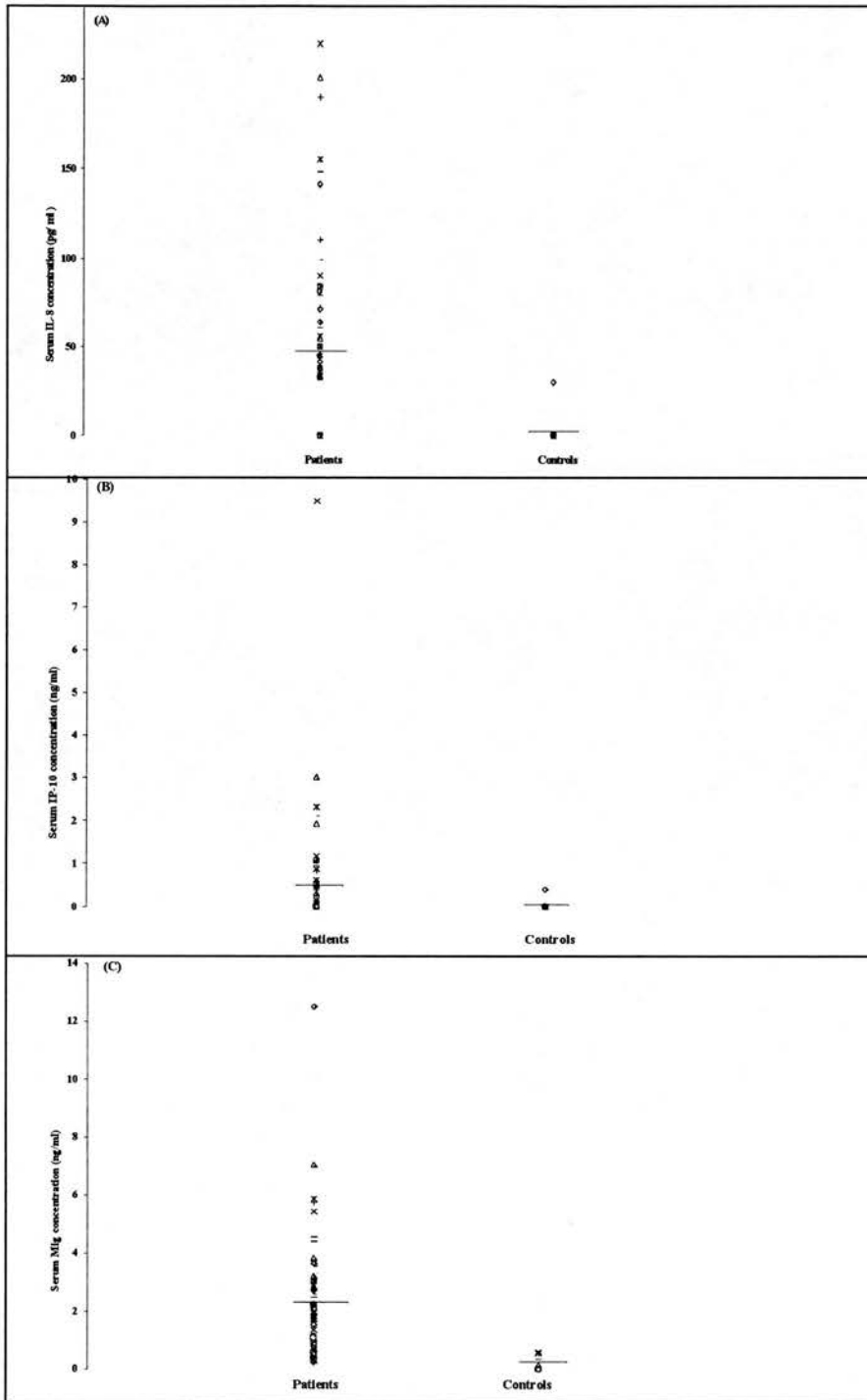


Figure 5.1: Individual and mean (bar) serum (A) IL-8, (B) IP-10, and (C) Mig concentrations in patients with POD-induced acute hepatic failure versus controls.

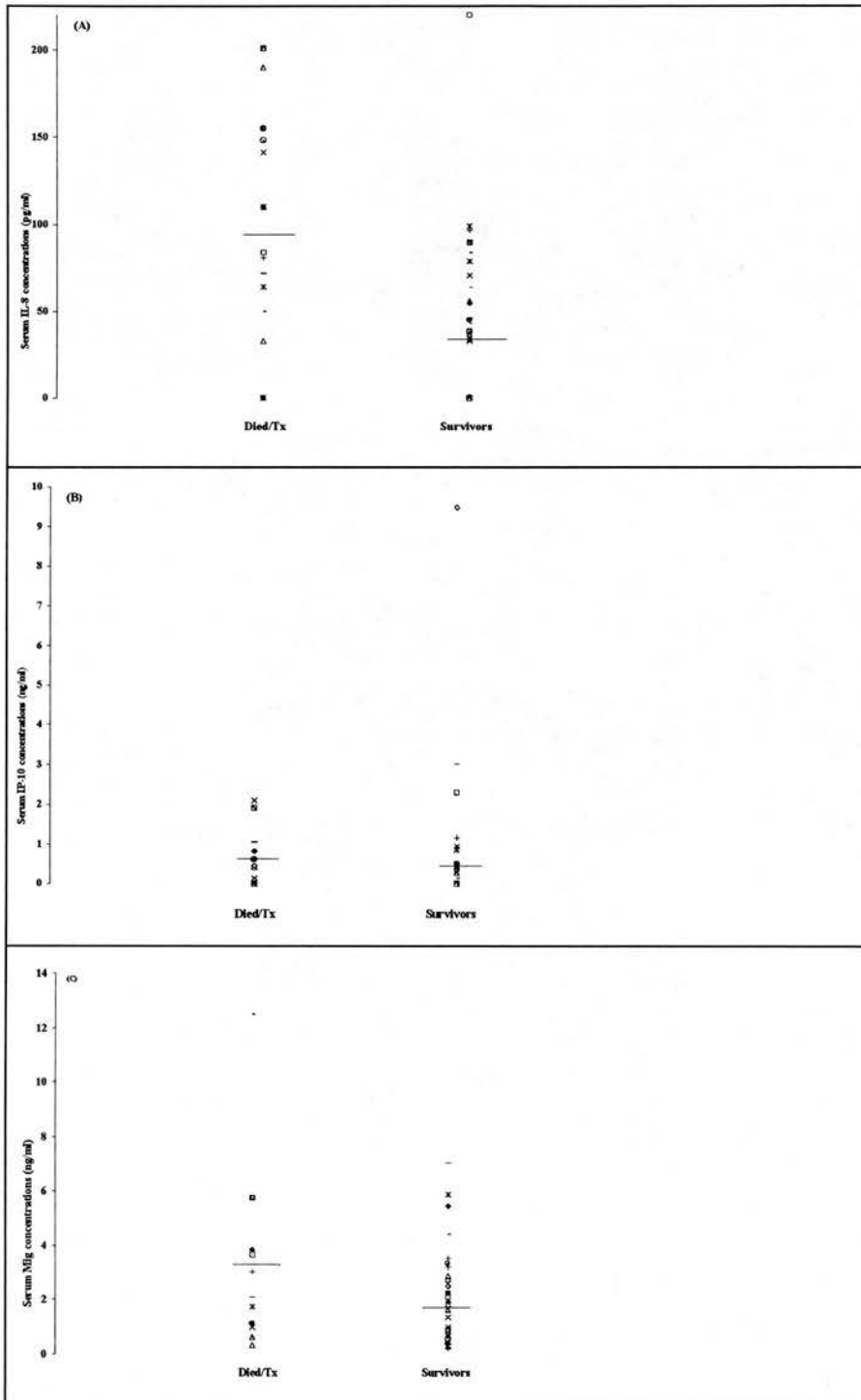


Figure 5.2: Individual and mean (bar) serum (A) IL-8, (B) IP-10, and (C) Mig concentrations in both dead/transplanted versus lived patients with POD-induced fulminant hepatic failure.

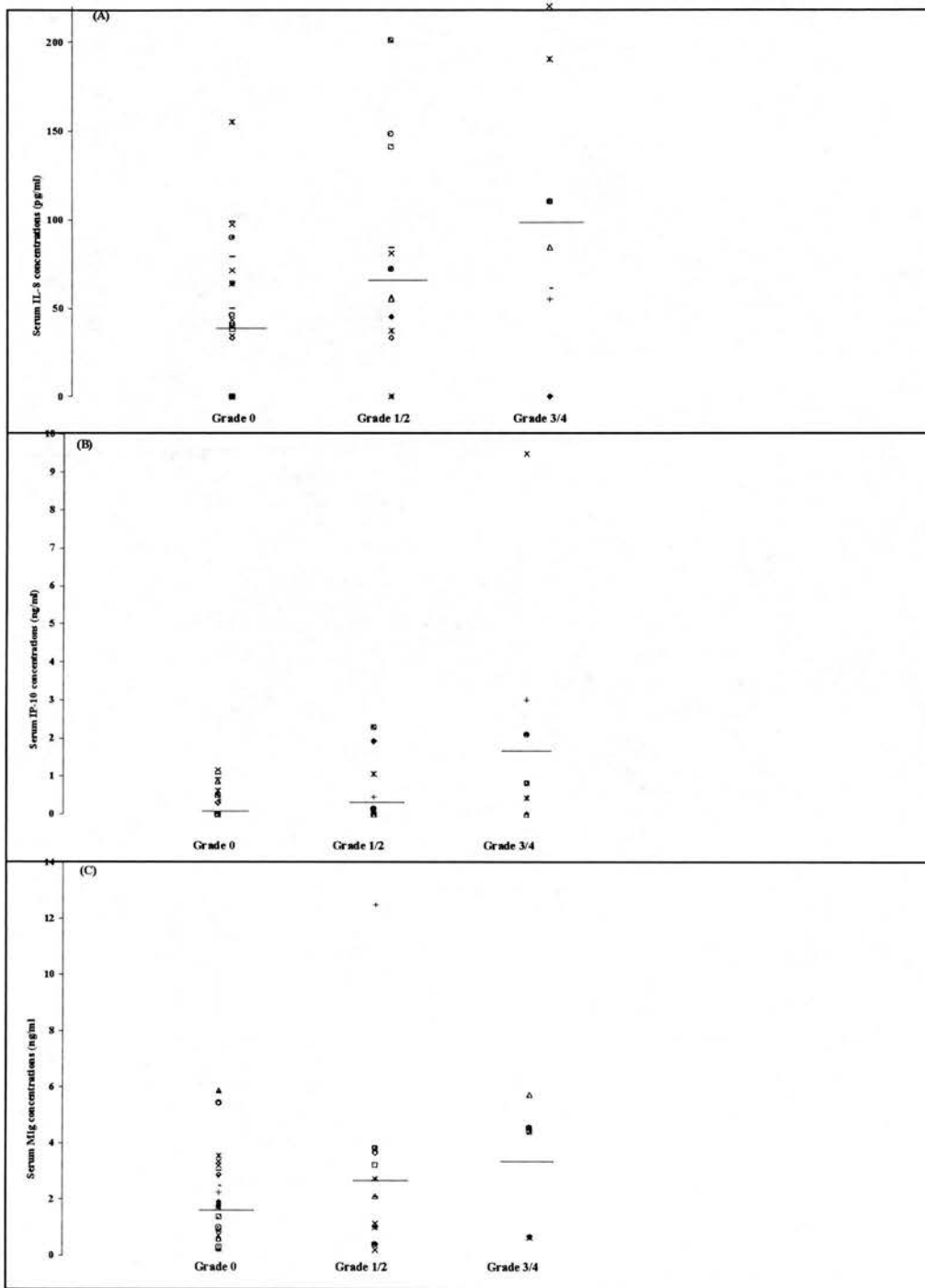


Figure 5.3: Individual and mean (bar) serum (A) IL-8, (B) IP-10, and (C) Mig concentrations in patients with POD-induced fulminant hepatic failure with no encephalopathy as (grade 0), grade 1/2 and grade 3/4 encephalopathy.

#### **5.4.1.2 Correlation with biochemical markers**

Serum IL-8 was significantly correlated with bilirubin ( $r = 0.2$ ,  $p = 0.01$ ), and was inversely correlated with serum  $\gamma$ -glutamyl transferase concentrations ( $r = -0.2$ ,  $p = 0.04$ , Figure 5.4). Serum IL-8 was also significantly correlated with prothrombin time ( $r = 0.3$ ,  $p = 0.001$ , Figure 5.4), other chemokines (IP-10  $r = 0.2$ ,  $p = 0.04$  and Mig  $r = 0.3$ ,  $p = 0.002$ , Figure 5.5), and with total white blood count ( $r = 0.3$ ,  $p = 0.0003$ , Figure 5.6). Serum IP-10 and Mig were correlated significantly with serum bilirubin concentration ( $r = 0.3$ ,  $p = 0.03$  and  $r = 0.2$ ,  $p = 0.01$  respectively, Figure 5.7).

Paracetamol poisoning can induce a severe metabolic acidosis, which has prognostic significance. We subdivided the patients into those with low or normal serum bicarbonate (i.e. normal range, 21-28 mmol/L). However, there was no significant difference in serum chemokine concentrations between patients with normal range of serum bicarbonate (IL-8;  $61.1 \pm 11.8$  pg/ml, IP-10;  $0.78 \pm 0.39$  ng/ml, MIG;  $1.8 \pm 0.3$  ng/ml,  $n=27$ ) and those with low serum bicarbonate ( $41.8 \pm 9.6$  pg/ml,  $0.34 \pm 0.01$  ng/ml, and  $2.7 \pm 0.5$ ,  $n=28$ , respectively,  $p = 0.2$  for all chemokines). No significant correlations were detected between serum chemokines and bicarbonate concentrations, or other biochemical parameters such as serum albumin, amino transferases and alkaline phosphate.

In POD, renal failure also has prognostic significance. A serum creatinine greater than 300  $\mu\text{mol/L}$  in the presence of other clinical and biochemical features is associated with mortality of more than 90%. Serum IL-8 level was significantly higher in patients who needed renal support ( $97.8 \pm 16.1$  pg/ml,  $n=15$ ) compared with those who did not ( $33.8 \pm 6.9$  pg/ml,  $n=40$ ,  $p = 0.002$ , Figure 5.8). However, there was no difference in serum IP-10 or Mig concentration between patients who needed renal support ( $0.96 \pm 0.7$  and  $2.8 \pm 0.4$  ng/ml) and those who did not ( $0.4 \pm 0.1$  and  $2.3 \pm 0.4$  ng/ml,  $p = 0.2$ , and  $0.3$  respectively). There was also no correlation between serum chemokines and urea or creatinine concentrations.

As sepsis is a frequent complication in patients with acute liver failure, we subdivided patients depending on whether sepsis complicated their clinical condition. Serum IL-8 and IP-10 concentrations were significantly higher in patients with subsequent sepsis ( $64.3 \pm 12$  pg/ml and  $0.84 \pm 0.36$  ng/ml,  $n = 30$ ) compared with those patients without sepsis ( $35.6 \pm 7.6$  pg/ml and  $0.22 \pm 0.07$  ng/ml,  $n = 25$ , respectively,  $p = 0.02$  for both chemokines). No significant difference was detected as regard Mig (sepsis  $2.5 \pm 0.5$ , no sepsis  $2 \pm 0.3$  ng/ml,  $p = 0.2$ , Figure 5.9).



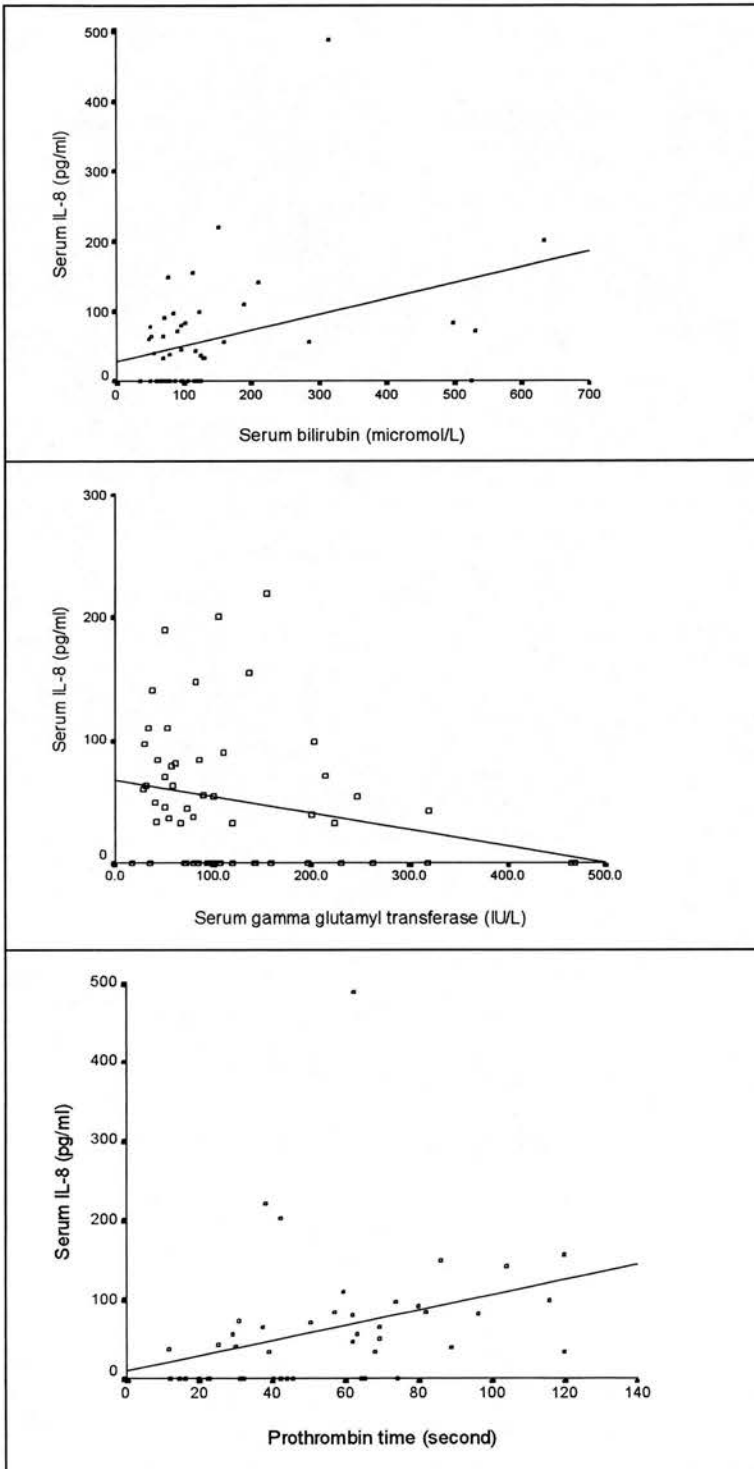


Figure 5.4: Correlations between serum IL-8 and biochemical parameters (bilirubin and  $\gamma$ -glutamyl transferase), and prothrombin time.

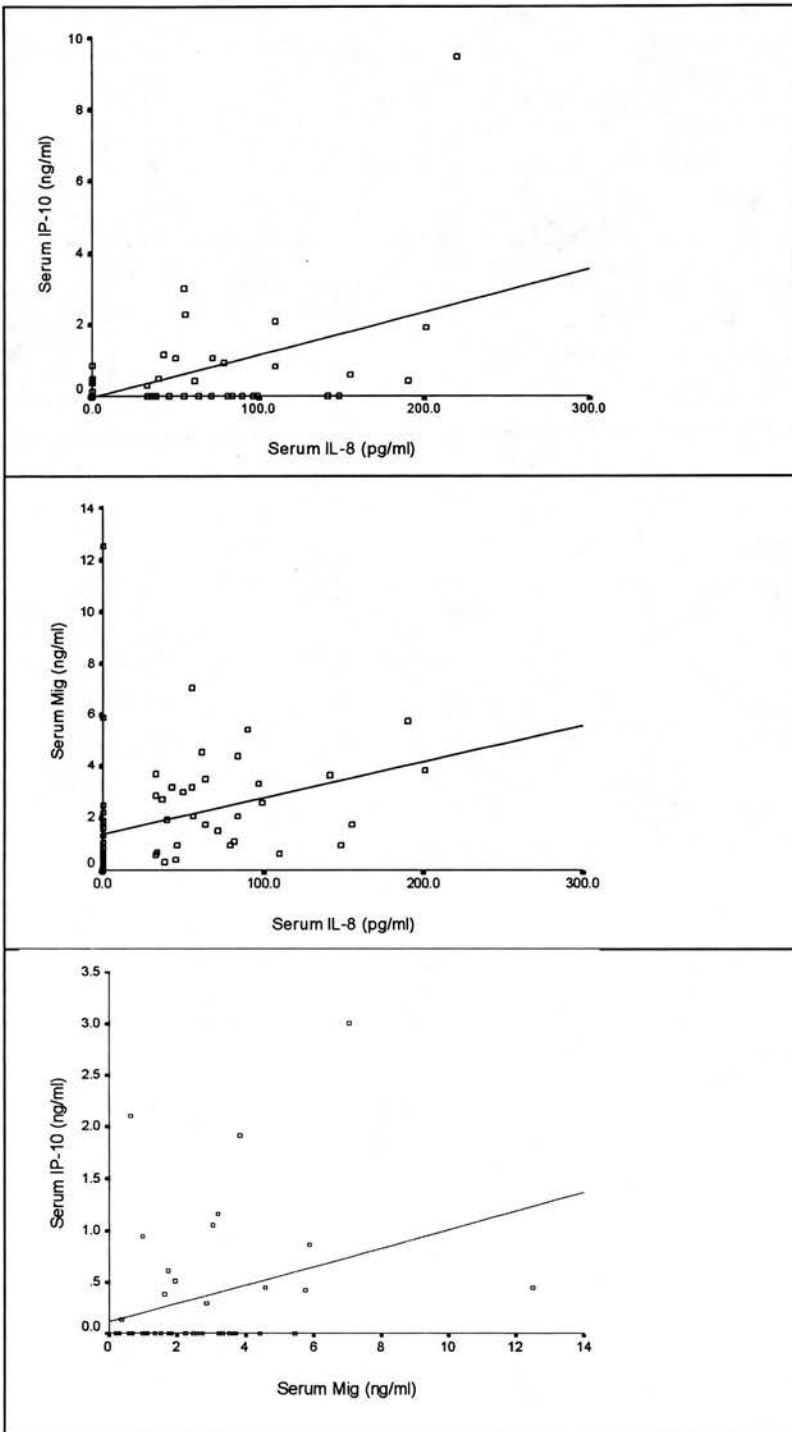


Figure 5.5: Correlations between serum chemokine concentrations.

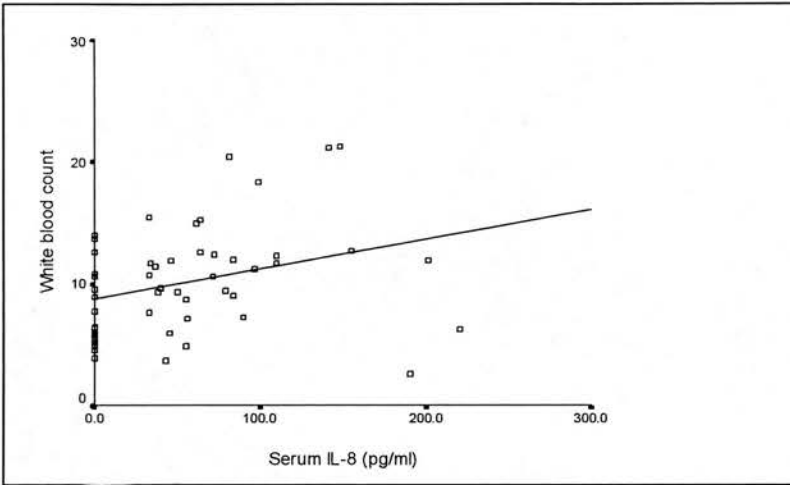


Figure 5.6: Correlation between serum IL-8 concentration and total white blood count.

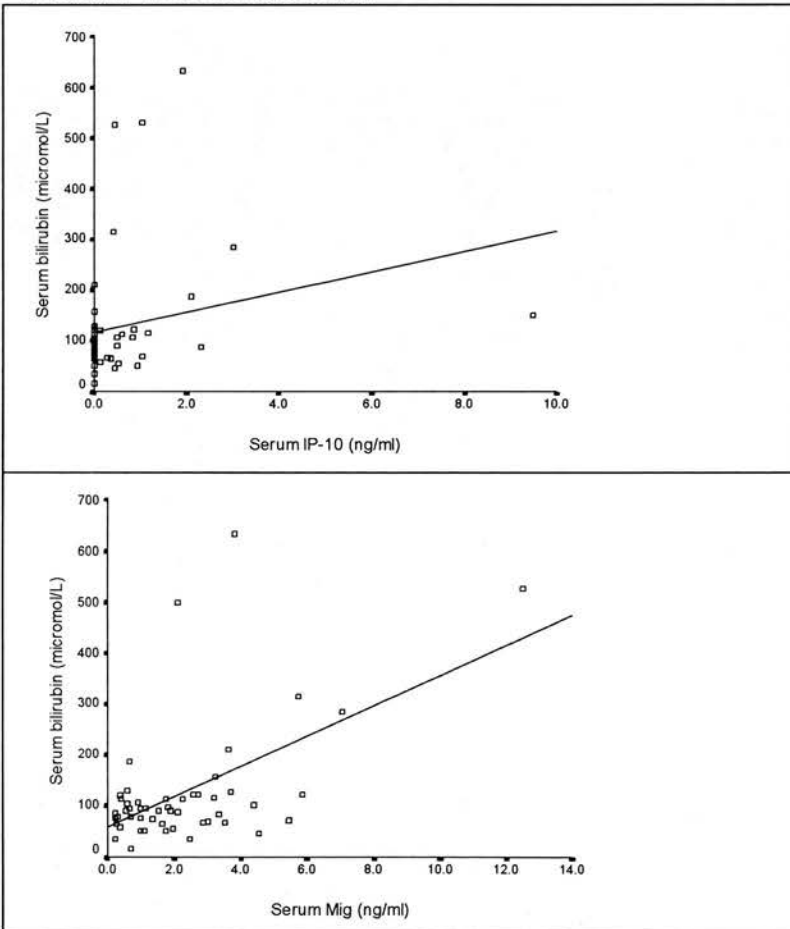


Figure 5.7: Correlations between serum IP-10, Mig, and bilirubin concentrations.

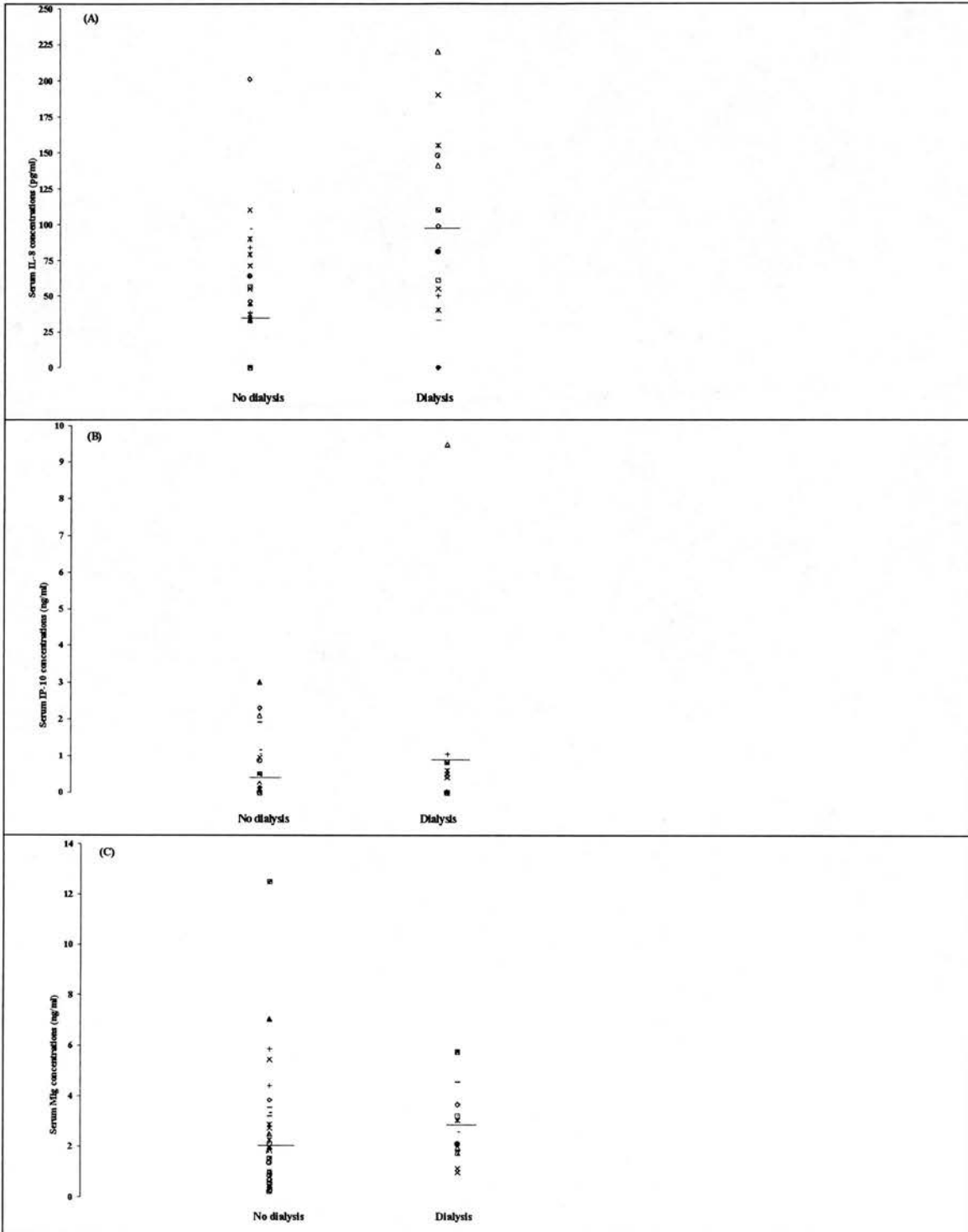


Figure 5.8: Individual and mean (bar) serum (A) IL-8, (B) IP-10, and (C) Mig concentrations in patients with POD-induced fulminant hepatic failure with and without renal support.

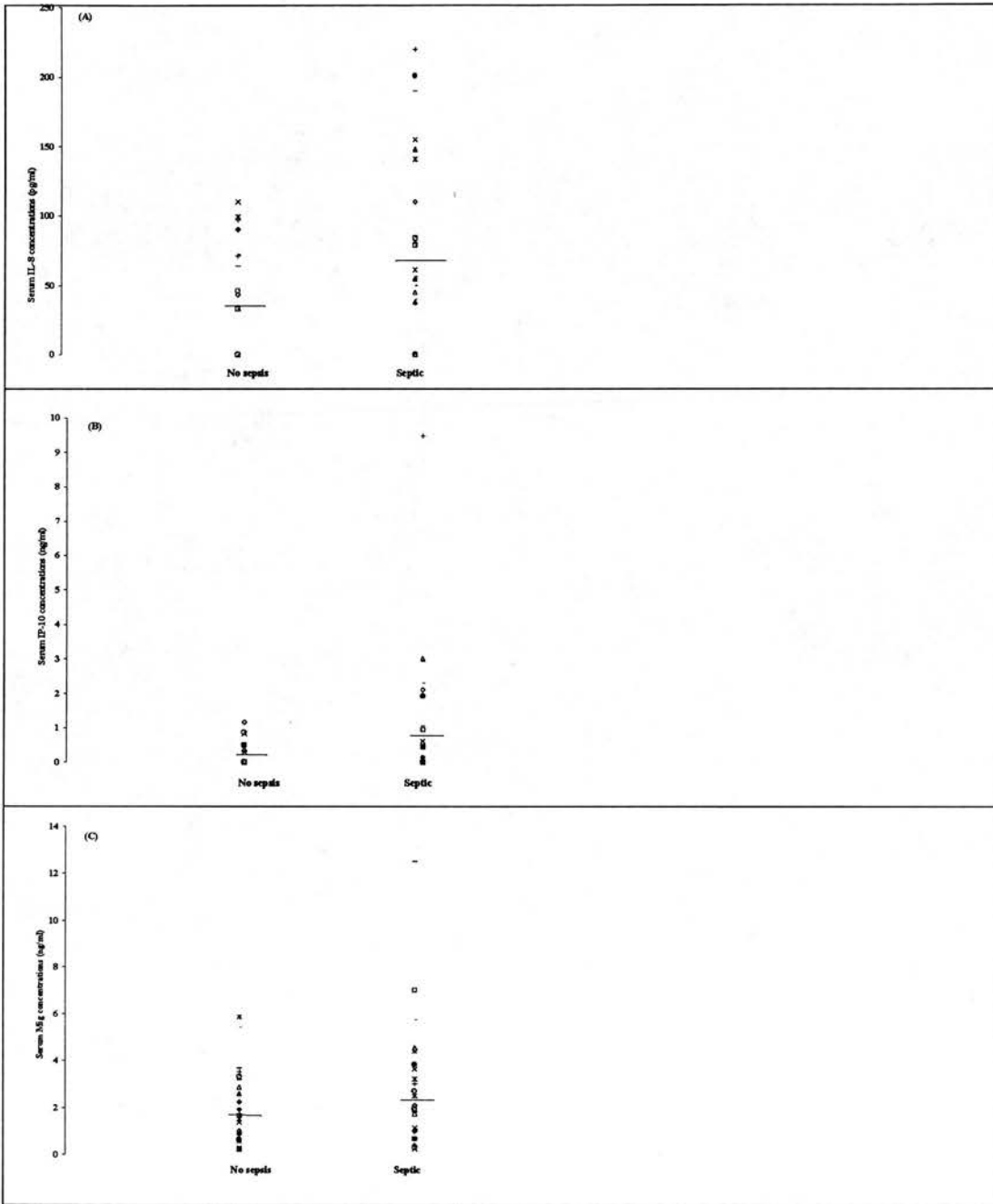


Figure 5.9: Individual and mean (bar) serum (A) IL-8, (B) IP-10, and (C) Mig concentrations in patients with POD-induced fulminant hepatic failure with and without sepsis.

## **5.4.2 Patients with chronic alcoholic liver disease**

Serum IL-8 and Mig concentrations were significantly higher in patients (IL-8;  $157.5 \pm 20.7$  pg/ml, MIG;  $1.27 \pm 0.4$ , mean  $\pm$  SEM,  $n=28$ ) compared with controls (IL-8;  $3 \pm 3$  pg/ml, MIG;  $0.17 \pm 0.1$ ,  $n=10$ ,  $p < 0.0001$  for both chemokines, Figure 5.10). There was no significant difference in serum IP-10 between patients ( $0.04 \pm 0.03$  ng/ml) and controls ( $0.04 \pm 0.04$  ng/ml,  $p = 0.2$ , Figure 5.10).

### **5.4.2.1 Relationship with disease severity**

The severity of chronic liver disease is estimated by calculating the Child-Pugh score. Because prognostically the most important difference is between Child-Pugh A and Child-Pugh B, we subdivided patients into Childs A and Childs B/C groups. Serum chemokine concentrations were higher in patients with Childs B/C cirrhosis (IL-8;  $214.2 \pm 26$  pg/ml, IP-10;  $0.06 \pm 0.05$  ng/ml, MIG;  $1.58 \pm 0.58$  ng/ml,  $n = 19$ ) compared with patients with Childs A cirrhosis ( $37.8 \pm 20.7$  pg/ml,  $0.0 \pm 0.0$ ,  $0.6 \pm 0.2$  ng/ml,  $n = 9$ ,  $p < 0.04$ ,  $0.0001$ ,  $0.0001$  respectively, Figure 5.11).

There were no significant differences in serum chemokine concentrations between patients who had ascites (IL-8;  $183 \pm 25$  pg/ml, IP-10;  $0.09 \pm 0.07$  ng/ml, MIG;  $1.55 \pm 0.79$  ng/ml,  $n = 15$ ), encephalopathy (IL-8;  $186 \pm 41$  pg/ml, IP-10;  $0.1 \pm 0.1$  ng/ml, MIG;  $2.0 \pm 1.0$  ng/ml,  $n= 11$ ), or sepsis (IL-8;  $170 \pm 31$  pg/ml, IP-10;  $0.08 \pm 0.08$  ng/ml, MIG;  $1.67 \pm 1.0$  ng/ml,  $n = 11$ ) and patients who did not have ascites (IL-8;  $135 \pm 40$  pg/ml, IP-10;  $0.0 \pm 0.0$  ng/ml, MIG;  $1.0 \pm 0.24$  ng/ml,  $n = 13$ ), encephalopathy (IL-8;  $246 \pm 41$  pg/ml, IP-10;  $0.0 \pm 0.0$  ng/ml, MIG;  $1.1 \pm 0.2$  ng/ml,  $n = 17$ ), or sepsis (IL-8;  $151 \pm 35$  pg/ml, IP-10;  $0.02 \pm 0.02$  ng/ml, MIG;  $1.05 \pm 0.2$  ng/ml,  $n = 17$ ).

According to the outcome of patients, we divided patients into those who died and those who survived. There were no significant differences in serum chemokines between patients who died (IL-8;  $124.3 \pm 36$  pg/ml, IP-10;  $0.12 \pm 0.12$  ng/ml, MIG;  $1.89 \pm 1.49$

ng/ml,  $n = 7$ ) and patients who survived (IL-8;  $169 \pm 30$  pg/ml, IP-10;  $0.01 \pm 0.01$  ng/ml, MIG;  $1.07 \pm 0.19$  ng/ml,  $n = 21$ ,  $p = 0.4$ ,  $0.4$ , and  $0.6$  respectively, Figure 5.12).

#### **5.4.2.2 Correlation with biochemical markers**

There were no significant correlations between serum chemokines and any biochemical markers such as serum bilirubin, amino transferases, albumin, urea, creatinine, or prothrombin time.

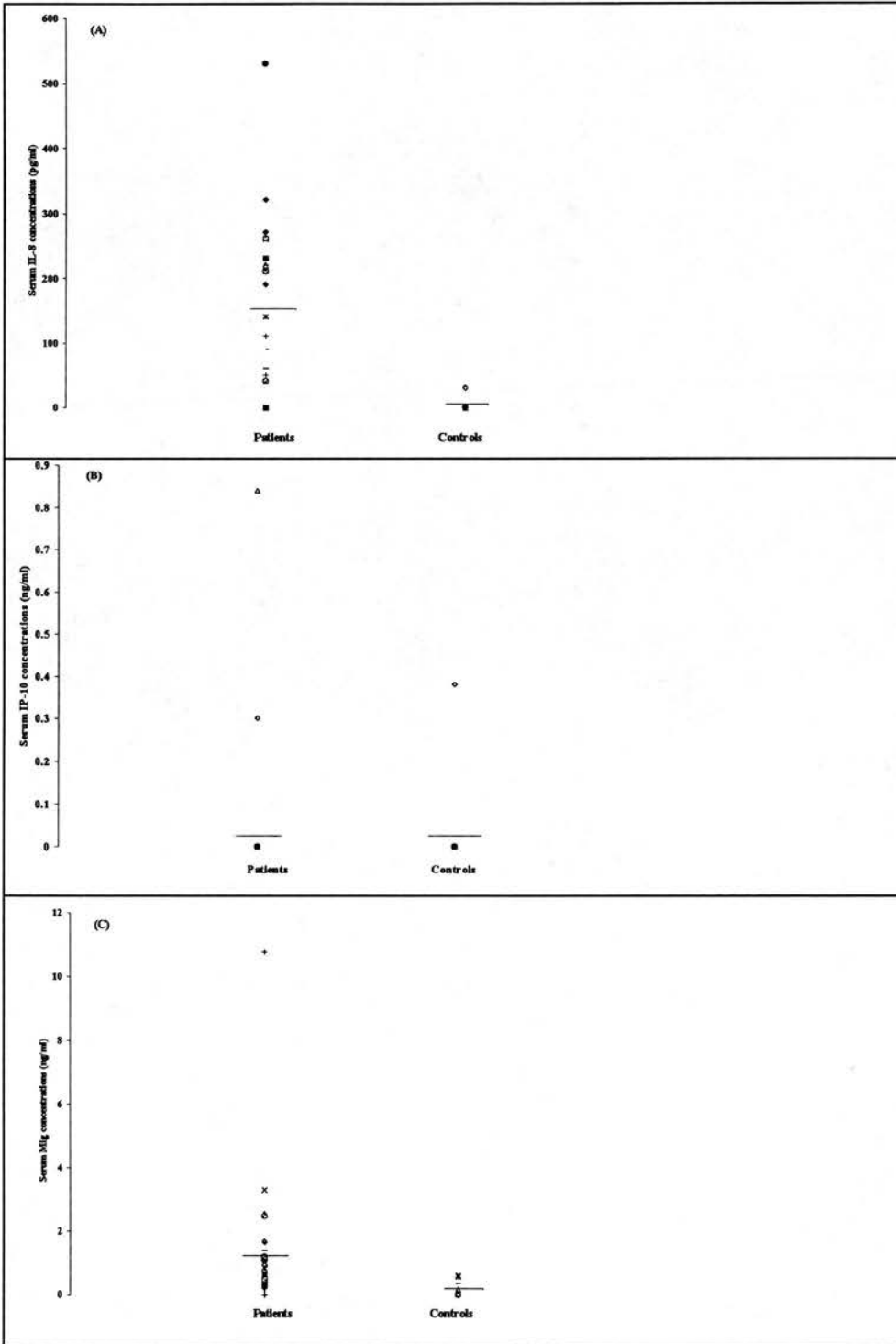


Figure 5.10: Individual and mean (bar) serum (A) IL-8, (B) IP-10, and (C) Mig concentrations in patients with alcoholic liver cirrhosis versus controls.



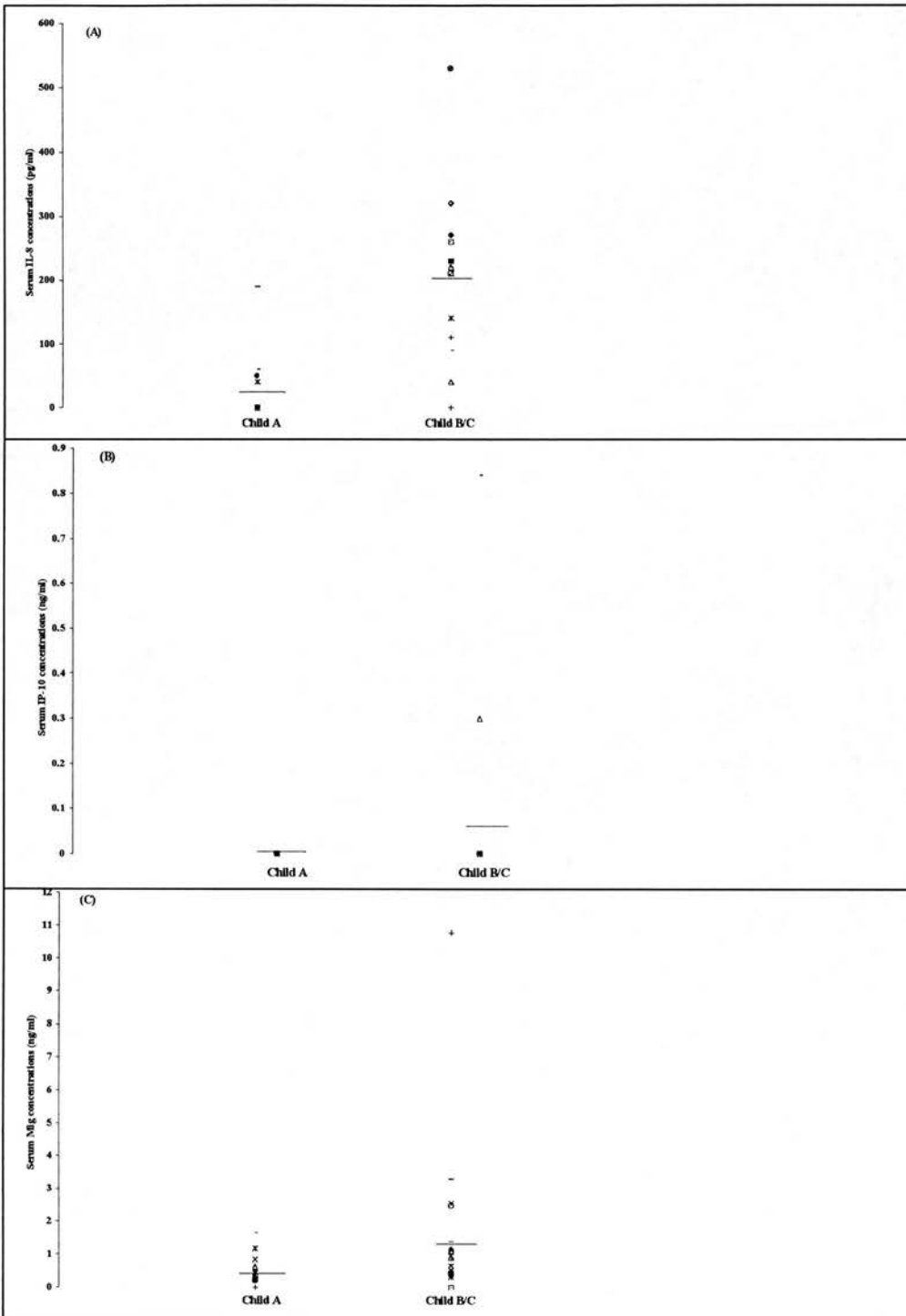


Figure 5.11: Individual and mean (bar) serum (A) IL-8, (B) IP-10, and (C) Mig concentrations in patients with alcoholic liver cirrhosis according to Child grade.

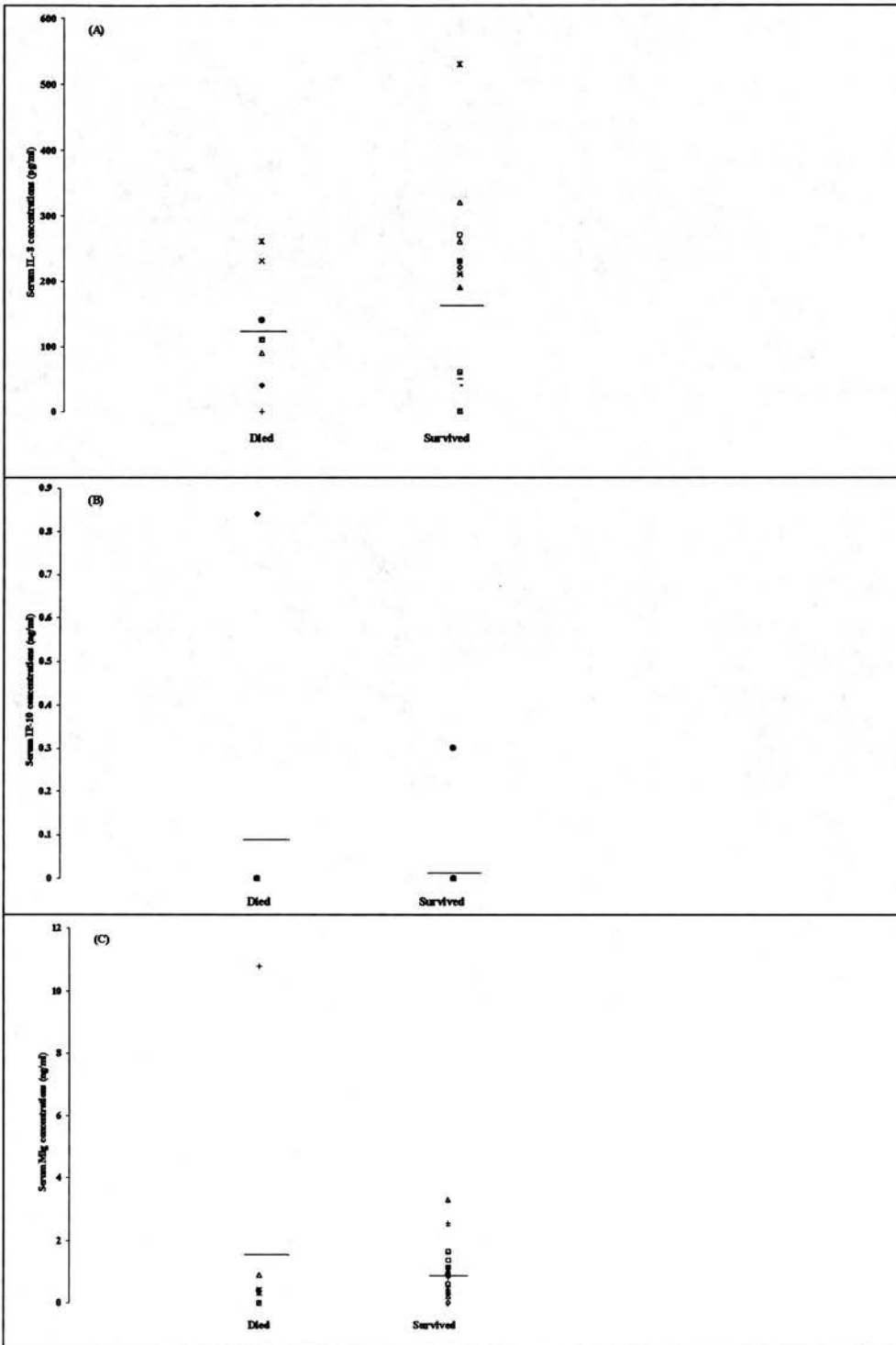


Figure 5.12: Individual and mean (bar) serum (A) IL-8, (B) IP-10, and (C) Mig concentrations in patients with alcoholic liver cirrhosis according to the outcome.

## **5.5 DISCUSSION**

In this chapter serum CXC chemokine IL-8, IP-10, and MIG concentrations were measured in patients with acute and chronic liver failure, and controls using an “in-house” ELISA technique. Subgroup analysis was performed to determine the effect of severity of liver disease on these chemokine concentrations. Serum concentrations of IL-8, IP-10, and MIG were significantly higher in patients with POD-induced acute liver failure compared with healthy controls. The three chemokines were significantly elevated in patients with advanced encephalopathy (grade III or IV) compared with patients with no (grade 0) or early encephalopathy (grade I or II). Only serum IL-8 was significantly higher in patients who died or were transplanted compared with those who survived. All of the three chemokines showed a significant correlation with serum bilirubin concentration. Only serum IL-8 concentration was significantly correlated with other markers of disease severity such as prothrombin time and those patients that required renal support.

In patients with chronic liver failure studies only serum IL-8 and MIG were significantly higher in patients compared with healthy controls. However, all chemokines measured were significantly higher in patients with advanced chronic liver disease (Child grade B and C) compared with patients with less severe or relatively early chronic liver disease (Child grade A). In chronic liver failure there were no correlations between serum chemokines and the other biochemical markers such as bilirubin, amino transferases, and albumin concentrations or urea and creatinine.

These data confirm and expand previous reports of elevated circulating chemokine concentrations in patients with liver disease. Most studies have measured circulating concentrations of chemokines in patients with chronic liver failure. A single study has reported high plasma IL-8 concentration in patients with fulminant hepatic failure, but the association with disease severity was not explored (Sheron et al 1993}. In contrast, several studies have measured circulating IL-8 and other chemokine concentrations in

peripheral blood in patients with alcoholic hepatitis and chronic liver diseases. In alcoholic hepatitis both circulating and hepatic IL-8 concentrations were correlated with the degree of neutrophil infiltration and it was suggested that the local production of IL-8 directed the neutrophil infiltration of the liver in such cases {Sheron et al 1993}. Increased circulating CC chemokine concentrations have also been reported in patients with alcoholic liver disease and increased hepatic production may direct the monocytic infiltration also observed in this disorder {Fisher et al 1999}.

The results relating circulating IL-8 concentrations in patients with acute liver failure with markers of disease severity are reminiscent of the relationships reported in patients with severe alcoholic hepatitis. Concentrations of circulating chemokines, such as IL-8 and Gro- $\alpha$ , were correlated with biochemical markers of disease severity, such as bilirubin, prothrombin ratio and white blood cell count (WBC) in patients with alcoholic hepatitis and levels were higher in those who died compared with survivors {Sheron et al 1993 and Maltby et al 1996}. In acute liver failure the three chemokines measured were significantly higher in patients with hepatic encephalopathy grades 3 or 4 compared with patients without encephalopathy. However, only the IL-8 concentration was related to other markers of disease severity such as prothrombin time and need for renal support. IL-8 was also significantly higher in patients who died or were transplanted compared with survivors. This relationship between circulating IL-8 and prognosis is not easy to explain. Many patients with severe alcoholic hepatitis or acute liver failure die from infection. It is interesting to speculate that the increased IL-8 concentrations are associated with more severe immunocompromise and hence greater risk of infection. Serum IL-8, IP-10 and Mig concentrations were significantly higher in patients with Child grades C compared with less severe chronic liver failure groups. Others have also reported a positive correlation between serum IL-8 concentrations and markers of severe disease in patients with chronic liver disease {Huang et al 1996}.

The source of the elevated circulating chemokine concentrations reported in this chapter remains to be defined. Increased blood levels may reflect increased hepatic production

or defective clearance of these chemokines by the injured liver. Previous studies have also reported high concentrations of proinflammatory cytokines such as TNF- $\alpha$  and IL-1 {Bird et al 1990 and Khoruts et al 1991} in such patients. These proinflammatory cytokines are major stimulants of chemokine synthesis in a wide variety of cell types and therefore extra-hepatic chemokine production may also have a role in the elevated circulating chemokine concentration observed. Because neutrophils are able to produce IL-8, IP-10 and MIG, activated neutrophils may also contribute to the elevated circulating chemokine concentration.

Although CXCR3 ligands, such as IP-10 and MIG can inhibit the biological effects of the ELR-positive CXC chemokines, neutrophils have not been reported to express CXCR3. However, increased circulating IL-8 concentrations may lead to down-regulation of the neutrophil chemokine receptor expression of CXCR1 and CXCR2. Having confirmed the elevated circulating IL-8 in the patient populations studied throughout this thesis, this hypothesis was studied further in the subsequent chapters.

**CHAPTER 6**

**TRANSHEPATIC NEUTROPHIL CHEMOTAXIS**  
**AND CHEMOKINE CONCENTRATIONS**

## **6.1 INTRODUCTION**

The liver is an important organ of synthesis as well as clearance of several cytokines and chemokines {Tilg et al 1992}. These inflammatory mediators may induce liver injury either directly or by stimulating liver infiltration with inflammatory cells, such as in alcoholic hepatitis {Shiratori et al 1989}. The CXC chemokine IL-8 is a potent stimulant of neutrophil recruitment and activation. Some studies found very high levels of IL-8 {Sheron et al 1993} and Gro- $\alpha$  {Maltby et al 1996} in both serum and liver tissue in patients with alcoholic hepatitis. Moderate elevation of these chemokines were also detected in other alcohol related liver diseases such as cirrhosis and fatty liver, with less marked elevation in non alcoholic liver disease such as chronic active hepatitis {Sheron et al 1993}. These chemokine levels were correlated with markers of disease severity and were higher in non-survivors and in patients with hepatic encephalopathy. In addition to neutrophil infiltration of the liver, peripheral neutrophilia was also detected in such patients in the absence of any detectable infection {Bird et al 1990}.

Reported in the previous chapters of this thesis were the findings that neutrophil chemotaxis is reduced in patients with either acute or chronic liver failure and that serum IL-8, IP-10, and Mig concentrations are increased in such patients. The cross over studies suggested an inhibitory factor for chemotaxis in serum of these patients, possibly related to the increased chemokine concentration itself. These studies in this chapter investigate the transhepatic differences in neutrophil chemotaxis and in the concentrations of serum IL-8, IP-10, and Mig. A cross over study was also performed to test whether the liver may produce a chemotactic inhibitory factor.

## **6.2 SUBJECTS**

Neutrophil chemotaxis and plasma ammonia concentration were measured in 15 patients with chronic alcoholic liver disease (12 males and 3 females, mean age 48.2 years (SEM 3.4)). The patients' characteristics are shown in table 6.1. The study also included 12 healthy individuals (8 males and 4 females, mean age 37 (SEM 2.2)) as controls.

Table 6.1: Patients characteristics presented as mean  $\pm$  SEM.

Child score	Urea	Creatinine	Bilirubin	ALT	Albumin	PT
8.8 $\pm$ 0.5	5 $\pm$ 0.8	70.5 $\pm$ 8.1	101 $\pm$ 22	63.5 $\pm$ 5	31 $\pm$ 1.3	16.3 $\pm$ 1.1

*ALT= Alanine transferase.*

*PT= Prothrombin time.*



## **6.3 METHODS**

### **6.3.1 Neutrophil chemotaxis**

Transjugular Intrahepatic Porto Systemic Shunt (TIPSS) is currently a treatment modality used following failure of endoscopic treatment of bleeding oesophageal varices in patients with chronic liver failure. TIPSS check is a follow up scheme to test the patency of the shunt and pressure gradients across the shunt {Gatta et al 1999}. This procedure was our access for blood sampling from the portal vein. Hepatic venous blood was sampled from a hepatic vein radical separate from that to which the TIPSS was draining. This was done to avoid sampling of a mixture of portal and hepatic venous blood. Neutrophils were isolated from portal, hepatic and peripheral veins during TIPSS check and chemotaxis stimulated by IL-8 measured as described in Chapter 2.

### **6.3.2 CXC chemokine assay**

Serum was recovered from portal and hepatic venous samples as described in Chapter 4 for chemokine assays of IL-8, IP-10, and Mig by ELISA as described in chapter 5.

### **6.3.3 Cross over neutrophil chemotaxis**

A 0.5 ml of portal and hepatic neutrophil suspension ( $2 \times 10^6$  cell/ml) was incubated with 20  $\mu$ l of hepatic and portal serum respectively, for 30 minutes at 37°C. Then the neutrophils were washed and resuspended in PBS before measuring chemotaxis.

### **5.3.3 Statistical tests**

Initial statistical testing showed the data to be normally distributed. Statistical analysis was therefore performed using Student's t- test. Correlation coefficients were determined using a Pearson's correlation test.

## **6.4 RESULTS**

### **6.4.1 Neutrophil chemotaxis**

Chemotaxis of neutrophils isolated from portal venous blood ( $21.8 \pm 2.5$  neutrophils / high power field, mean  $\pm$  SEM,  $n=15$ ) was similar to chemotaxis of neutrophils isolated from peripheral venous blood ( $19.1 \pm 2$ ,  $n=15$ ). However, chemotaxis of hepatic venous neutrophils was significantly reduced ( $2.7 \pm 0.8$ ,  $n=15$ ,  $p < 0.0001$ ) compared with peripheral or portal venous neutrophils. Chemotaxis of neutrophils prepared from patients hepatic, portal, or peripheral veins were significantly reduced compared with neutrophils prepared from controls peripheral venous blood ( $48.7 \pm 1.4$ , Figure 6.1).

### **6.4.2 Chemokine assay**

IL-8 and Mig concentrations from serum prepared from hepatic venous blood (IL-8;  $310 \pm 35$  pg/ml, Mig;  $3.1 \pm 0.8$  ng/ml, mean  $\pm$  SEM,  $n=4$ ) were significantly higher compared with serum prepared from portal venous blood (IL-8;  $130 \pm 53$  pg/ml, MIG;  $0.7 \pm 0.2$ ,  $n=4$ ,  $p < 0.02$  and  $0.03$  respectively). No significant change in IP-10 concentrations in serum prepared from hepatic venous blood ( $0.07 \pm 0.07$  ng/l,  $n=4$ ) compared with undetectable levels in portal venous blood ( $p = 0.2$ ). Figure 6.2 shows the local changes in serum chemokine concentrations.

### **6.4.3 Cross over neutrophil chemotaxis**

Incubation of portal venous neutrophils with serum isolated from hepatic venous blood significantly reduced IL-8-stimulated chemotaxis (before incubation  $29.2 \pm 2.7$ , after incubation  $6.3 \pm 1.6$  neutrophils/high power field, mean  $\pm$  SEM,  $n = 6$ ,  $p < 0.0001$ , Figure 6.3). In contrast, a significant but partial improvement in hepatic venous neutrophil chemotaxis occurred following incubation with portal venous serum (before incubation  $2.8 \pm 1.0$ , after incubation  $23 \pm 3.9$  neutrophils/high power field,  $p < 0.0001$ ).

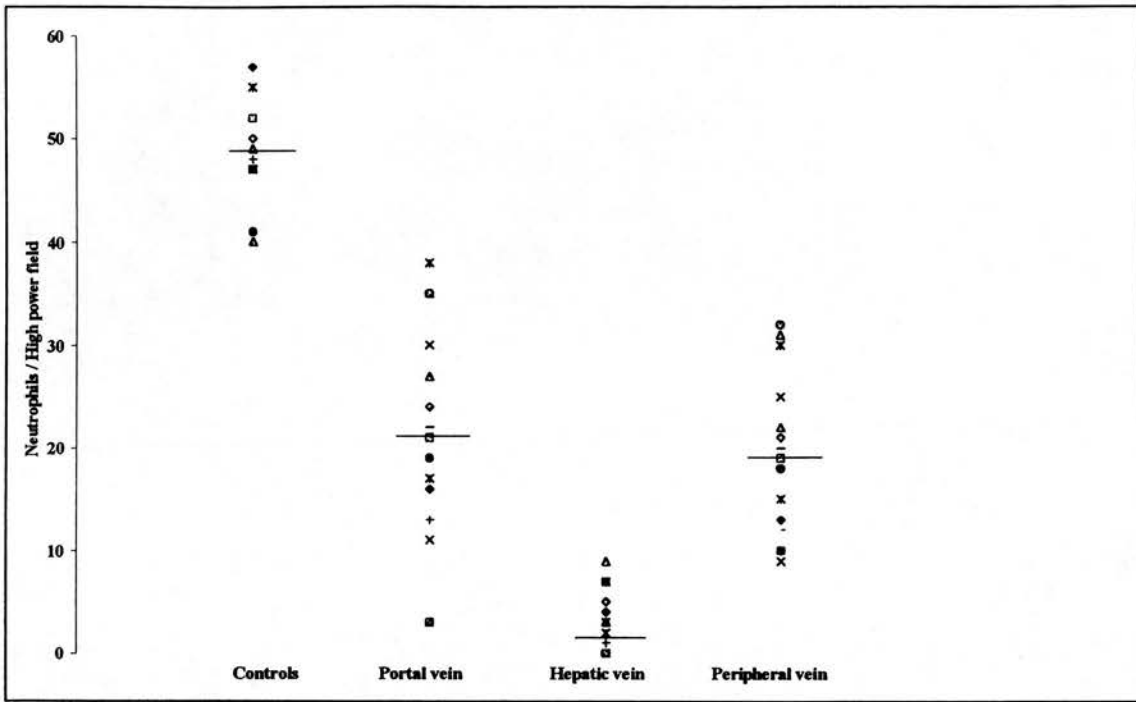


Figure 6.1: Mean neutrophil chemotaxis to 10 ng/ml of IL-8 in portal, hepatic and peripheral veins in patients with alcoholic liver cirrhosis during TIPSS check and controls.

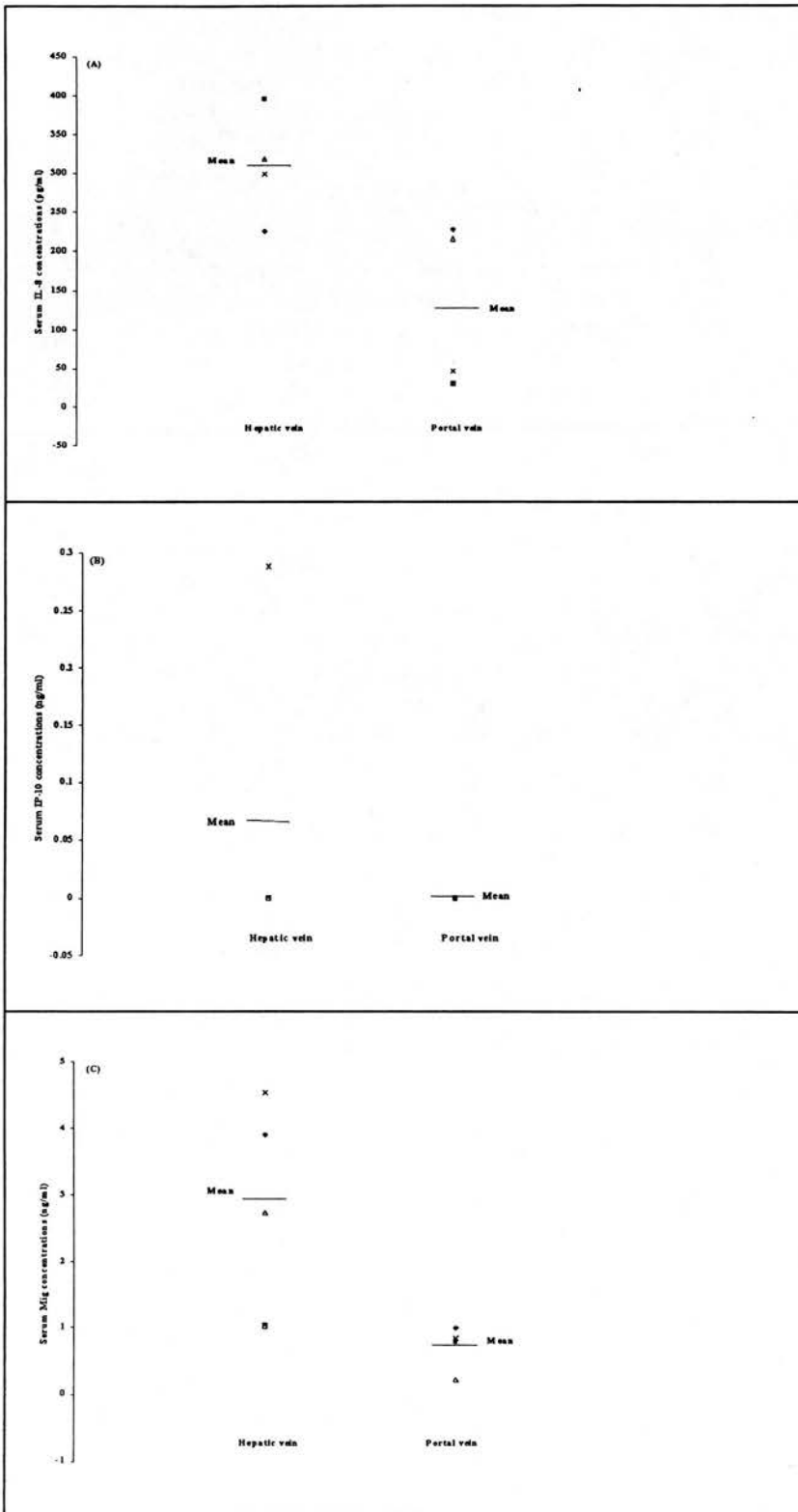


Figure 6.2: Individual and mean serum IL-8 (A), IP-10 (B), and Mig concentrations in hepatic and portal venous blood in patients with alcoholic liver cirrhosis.

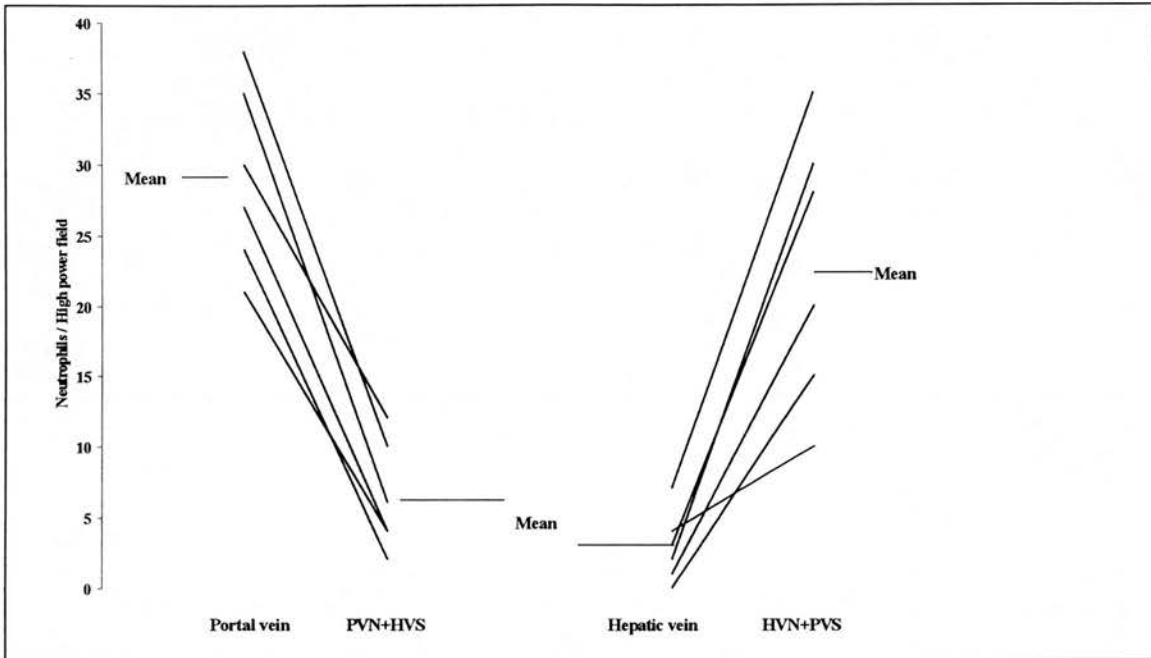


Figure 6.3: Individual and mean neutrophil chemotaxis stimulated with IL-8 (10ng/ml) in portal and hepatic veins of patients with alcoholic liver cirrhosis before and after cross over incubation (PVN= portal venous neutrophils, HVS= hepatic venous sera, HVN= hepatic venous neutrophils, PVS= portal venous sera).

## **6.5 DISCUSSION**

In this chapter we measured transhepatic differences in neutrophil chemotaxis and chemokine concentrations in patients with chronic liver failure. The results clearly showed a significant reduction in the chemotaxis of hepatic venous neutrophils compared with portal venous neutrophils of patients with alcoholic liver cirrhosis. Although the numbers were small, a significantly higher concentration of IL-8 and Mig were found in hepatic compared with portal venous blood. A cross over study was also performed; chemotaxis of portal neutrophils was reduced following incubation of neutrophils with hepatic venous serum, and vice versa.

The liver is not only an important organ of synthesis of cytokines but also an important site for clearance of several cytokines {Tilg et al 1992}. However, local or transhepatic changes in neutrophil chemotaxis and chemokine concentrations in patients with liver failure are unknown. In liver diseases the hepatocytes and other cellular components of the liver such as kupffer cells are the main source of liver cytokines and chemokines, perhaps in response to endotoxaemia and high level of Tumour necrosis factor- (TNF)- $\alpha$  that occurs in such patients {Thornton et al 1990}. In animal studies, the activity of hepatic macrophages in producing cytokines is markedly increased when exposed to endotoxin {Crofton et al 1978 and Greig et al 1989}. A previous study measured transhepatic levels of TNF- $\alpha$ , IL-6 and endotoxin (ET) in the portal and hepatic veins after hepatic reperfusion in 13 consecutive patients who underwent orthotopic liver transplantation {Steininger et al 1994}. This study showed that the viability of the graft was closely related to transhepatic TNF- $\alpha$  concentrations. Patients with primary non-function or dysfunction had a significantly higher hepatic venous concentration of TNF- $\alpha$  compared with portal venous concentration, with completely undetectable difference in patients with good graft function. The local changes of IL-6, IL-8 and IL-1 receptor antagonist concentrations were studied in hepatic, portal veins, and radial artery after liver resection in 13 patients {Ueda et al 2000}. All of the studied cytokines were significantly higher during the surgery. The levels of IL-8 and IL-1 receptor antagonist were significantly higher in hepatic vein than the artery, but IL-6 level was lower in

hepatic than radial artery or portal vein. These results regarding a higher IL-8 concentration in hepatic versus portal blood are similar to those reported in this chapter.

Previously, transhepatic MCP-1 concentrations were studied using TIPSS shunts as described in this chapter {Fisher et al 1999}. In this study, a significantly higher MCP-1 concentration was observed in hepatic veins compared with peripheral or portal vein, suggesting local hepatic synthesis of this chemokine. Our results are similar in regard to IL-8 and Mig. It is interesting to speculate that the reduced chemotaxis in hepatic venous neutrophils compared with portal venous neutrophils is secondary to the differential chemokine concentrations across the liver. Alternatively, exposure to increased hepatic chemokine concentrations as the neutrophils traverse the liver may limit their chemotaxis.

This Chapter describes the differences in neutrophil chemotaxis and chemokine concentrations across the liver. Neutrophil chemotaxis was reduced in hepatic versus portal veins with higher concentrations of chemokines in the former. The mechanism of this reduction in neutrophil chemotaxis may be secondary to down-regulation of neutrophil CXC chemokine receptors and this hypothesis is examined further in the following Chapter.

**CHAPTER 7**  
**CXC CHEMOKINE RECEPTOR**  
**EXPRESSIONS**



## **7.1 INTRODUCTION**

Chemokines bind to and activate cell surface expressed receptors in order to induce their biological actions. Neutrophils express two CXC chemokine receptor types, CXCR1 and CXCR2. Both receptors share 77% amino acid homology and their genes are co-localised on chromosome 2q35 {Ahuja et al 1992}. CXCR1 and CXCR2 are members of the G protein-coupled receptor (GPCR) superfamily, with seven transmembrane domains. Although IL-8 and Gro- $\alpha$  are ELR+ve chemokines, their receptor binding capacities are different {Ludwig et al 1997}. Both CXCR1 and CXCR2 have been reported to bind IL-8 with high affinity {Jones et al 1995}. While CXCR1 binds Gro- $\alpha$  with low affinity, CXCR2 is reported to bind it with high affinity {Lee et al 1992}.

The regulatory mechanisms determining cellular expression of the CXC chemokine receptors are numerous. More than 90% of IL-8-receptor complex are endocytosed within 10 minutes of binding {Samanta et al 1990}. *In vitro* studies have reported preferential loss of CXCR2 expression and function in response to IL-8, fMLP, and TNF- $\alpha$  {Quaid et al 1999}. Pretreatment of neutrophils with TNF- $\alpha$  was also reported to decrease CXCR2 receptor levels, which showed partial recovery at 120 minutes. Alternatively, CXCR1 showed a sharp decline at 15 minutes and persisted up to 120 minutes {Jawa et al 1999}. All leukocyte subsets express the cell surface glycoprotein CD45 that has intrinsic intracellular protein tyrosine phosphatase activity. In addition to its regulatory role in activation-induced signalling in lymphocytes, it has been reported to also act to modulate neutrophil responses to chemokines via up-regulation of CXCR1 and CXCR2 receptors. CD45 inhibition with anti-CD45RB antibody (Bra11) down-regulate both receptor expressions up to 47% of their respective controls {Suria et al 1999}.

Like other members of the G protein-coupled receptor superfamily, the functions of CXC receptors are determined by the phosphorylation state {Mueller et al 1997}. Agonist administration enhances receptor phosphorylation by protein kinases, GPCR

kinases and protein kinase C, which leads to desensitisation of the receptors {Aragay et al 1998}. The phosphorylated receptor is then internalised through clathrin-coated pits into endosomes and subsequently dephosphorylated by intracellular protein phosphatases {Cheng et al 2000}. Such dephosphorylated receptors might be either recycled via sorting endosomes back to the cell surface or transported to the lysosomes for degradation {Chuntharapai and Kim 1995}. Therefore, chemokine binding to their specific receptors leads to rapid internalisation of the ligand-receptor complex and recycling or degradation of the receptors. Hence, increased chemokine concentrations may induce desensitisation of these receptors via the process of down-regulation.

The previous chapters reported a reduction in neutrophil chemotaxis in patients with either acute or chronic liver failure. The mechanism of this reduction may be related to circulatory chemotaxis inhibitory factor/s, as indicated by the cross over studies. Increased concentrations of serum CXC chemokines in these patients was also observed. These high chemokine concentrations may explain the previously detected reduction in neutrophil chemotaxis via desensitisation and down-regulation of the neutrophil CXC receptor expressions. The experiments in this chapter were designed to investigate neutrophil expression of the CXCR1 and CXCR2 chemokine receptors in patients with either acute or chronic liver failure.

## **7.2 SUBJECTS**

These studies included 7 patients with paracetamol-induced acute liver failure, 15 patients with chronic alcoholic liver disease, and 16 healthy laboratory staff served as controls. The patients and controls characteristics are shown in Table 7.1.

Table 7.1: Patient and controls characteristics presented as mean  $\pm$ SEM.

	POD	ALD	Controls
Age: (yr)	44 $\pm$ 4.9	51.3 $\pm$ 1.9	35 $\pm$ 4.3
Sex: F:M	2:5	5:10	4:12
Child-Pugh score		8 $\pm$ 0.5	-
Serum bilirubin: ( $\mu\text{mol.L}^{-1}$ )	181 $\pm$ 70	129 $\pm$ 33	-
Serum albumin: ( $\text{g.L}^{-1}$ )	37 $\pm$ 2.3	28.9 $\pm$ 1.9	-
Prothrombin time: (s)	43.1 $\pm$ 15	17.7 $\pm$ 1.2	-
Blood urea: ( $\text{mmol.L}^{-1}$ )	8.1 $\pm$ 1.9	6.9 $\pm$ 1	-
Serum creatinine: ( $\mu\text{mol.L}^{-1}$ )	174 $\pm$ 33	86.7 $\pm$ 11	-

*POD=Paracetamol overdose.*

*ALD= alcoholic liver disease.*

*M= Male. F= Female.*

## **7.3 METHODS**

### **7.3.1 Neutrophil isolation and chemotaxis**

Neutrophils were isolated from patients and controls, and chemotaxis stimulated by IL-8 and Gro- $\alpha$  was measured as described in Chapter 2 (sections 2.3.1 and 2.3.3 respectively).

### **7.2.2 Neutrophil CXC chemokine receptor expression**

50  $\mu$ l of neutrophil suspension was added to 50 $\mu$ l of anti-CXCR1 and CXCR2 monoclonal antibodies (BD Pharmingen, Oxford-UK), agitated for few seconds and incubated at room temperature for 30 minutes. 1ml of PBS buffer was then added and the cells then washed twice. 50 $\mu$ l of sheep anti-mouse FiTC labeled secondary antibody was added (Sigma-Aldrich CO LTD, Dorset-UK) and the cells were incubated at room temperature for 30 minutes and washed twice again. The cell pellet then resuspended in 1ml of FACS fixative (1% formaldehyde in PBS). All samples were analysed by a Coulter EPICS flow cytometry, and the mean fluorescence was determined from 5,000 neutrophils after proper gating of the cells by forward and sideward scatter parameters.

### **7.3.3 Statistical tests**

As the initial statistical analysis showed that the data is normally distributed, neutrophil chemotaxis and expression of CXCR1 and CXCR2 in patients and controls were compared using Student's unpaired t-test with unequal variance. Correlation coefficients were determined using Pearson's correlation test.

## **7.4 RESULTS**

### **7.4.1 Neutrophil chemotaxis**

Neutrophil chemotaxis induced by IL-8 and Gro- $\alpha$  (10ng/ml) was significantly impaired in patients with acute liver failure (IL-8;  $17.9 \pm 1.7$  neutrophils / high power field and Gro;  $16.6 \pm 1.4$ , mean  $\pm$  SEM, n = 7) and chronic liver failure (IL-8;  $23 \pm 2$  and Gro;  $24 \pm 1$ , n = 12) compared with controls (IL-8;  $46 \pm 1.6$  and Gro;  $46 \pm 1.7$ , n = 8,  $p < 0.0001$  for both chemokines).

### **7.4.2 Neutrophil CXCR1 and CXCR2 receptor expression**

No significant change in neutrophil CXCR1 or CXCR2 expression (expressed as mean fluorescence intensity) was observed in patients with acute liver failure (CXCR1  $3.3 \pm 0.26$  arbitrary units, CXCR2  $0.64 \pm 0.07$ , mean  $\pm$  SEM, n = 7) compared with controls ( $3.6 \pm 0.15$ ,  $0.68 \pm 0.03$ , n = 16,  $p = 0.3$  and  $0.06$ , Figure 7.1). No significant change in CXCR1 or CXCR2 expression was also detected in patients with chronic liver failure ( $3.2 \pm 0.20$ ,  $0.65 \pm 0.04$ , n = 15) compared with controls ( $p = 0.15$  and  $0.09$  respectively, Figure 7.2). Figures 7.3 and 7.4 show the flow charts of FACs analysis of neutrophil CXCR1 and CXCR2 respectively, in patients with POD-induced acute liver failure, chronic liver failure, and in the control subjects. No significant correlations were detected between both neutrophil CXC chemokine receptor expression and chemotaxis towards IL-8 or Gro- $\alpha$  (Figures 7.5 and 7.6).

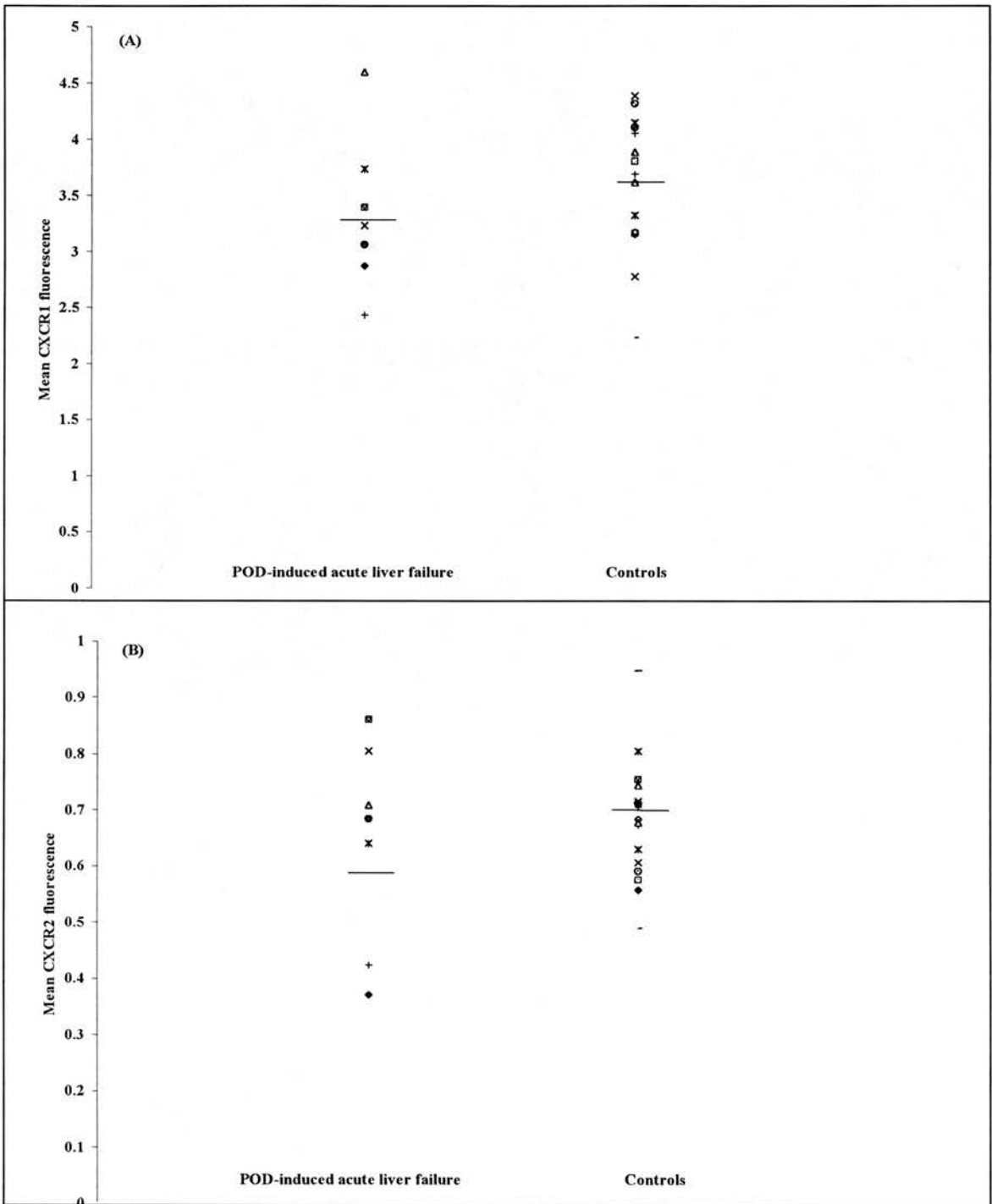


Figure 7.1: Individual and mean (bar) fluorescence of neutrophil CXCR1 (A) and CXCR2 (B) in patients with POD- induced acute liver failure compared with controls.

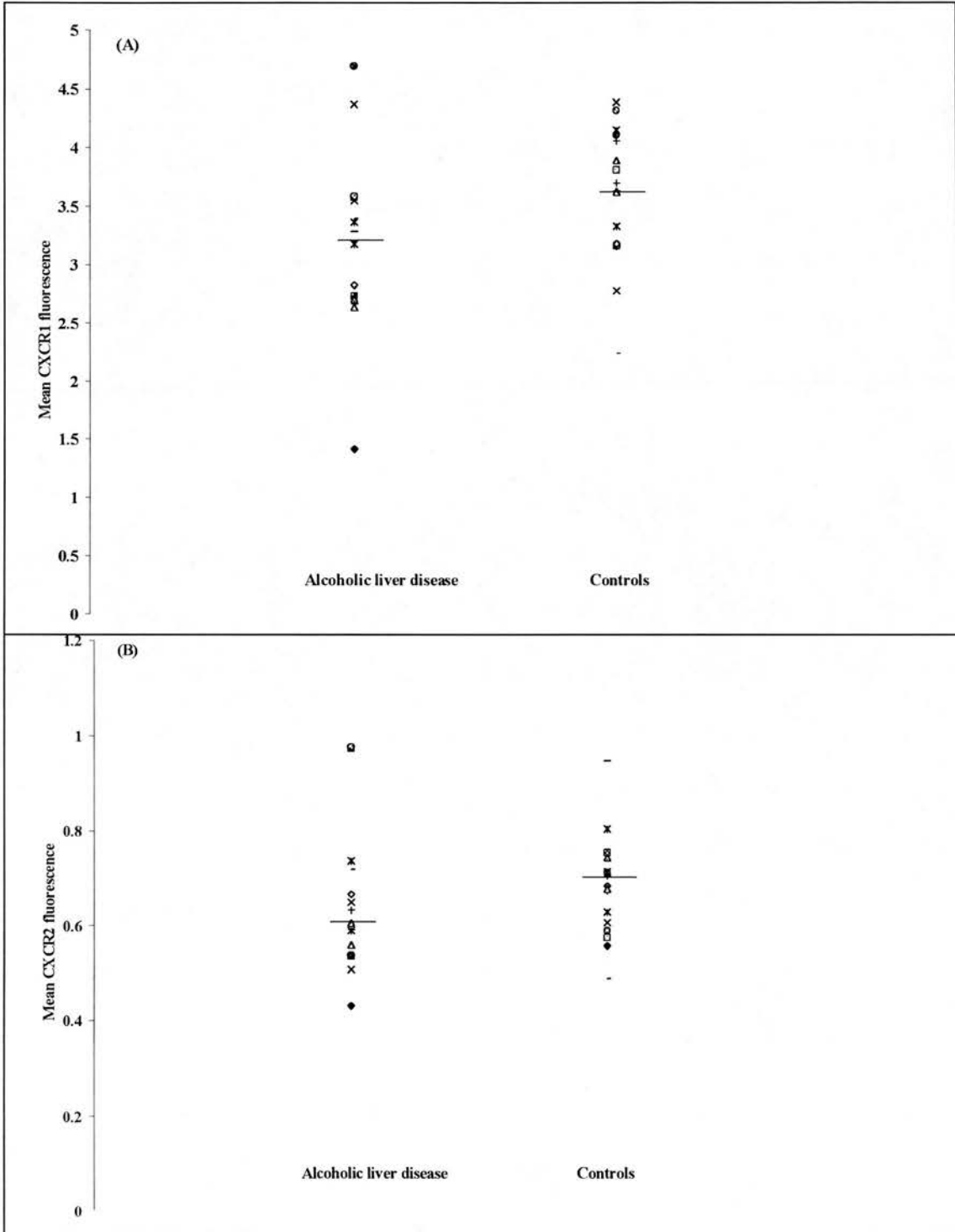


Figure 7.2: Individual and mean (bar) fluorescence of neutrophil CXCR1 (A) and CXCR2 (B) in patients with alcoholic liver cirrhosis compared with controls.

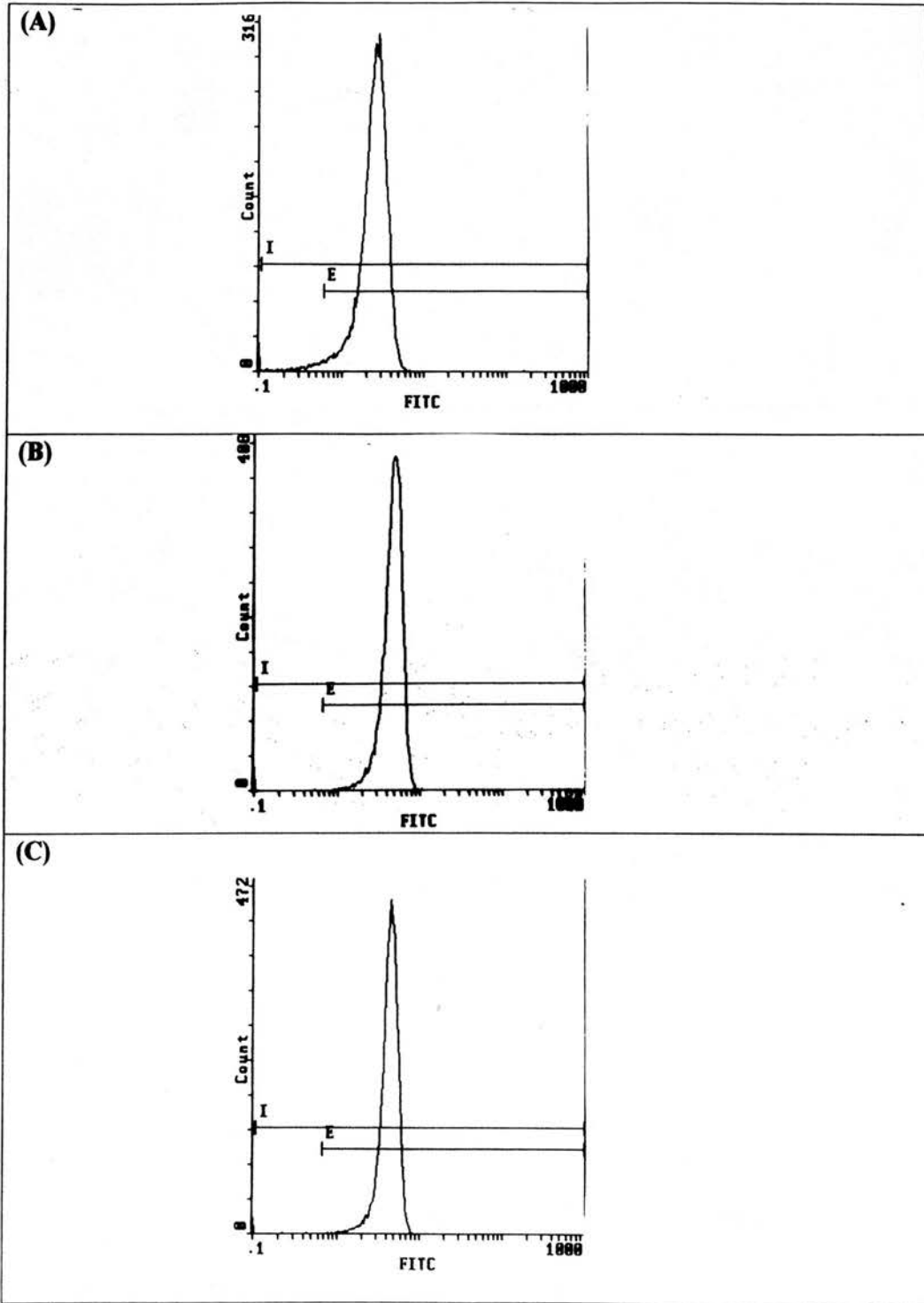


Figure 7.3: Flow cytometer chart of FACS analysis of neutrophil CXCR1 expression in patients with POD-acute liver failure (A), chronic liver failure (B) and controls (C).



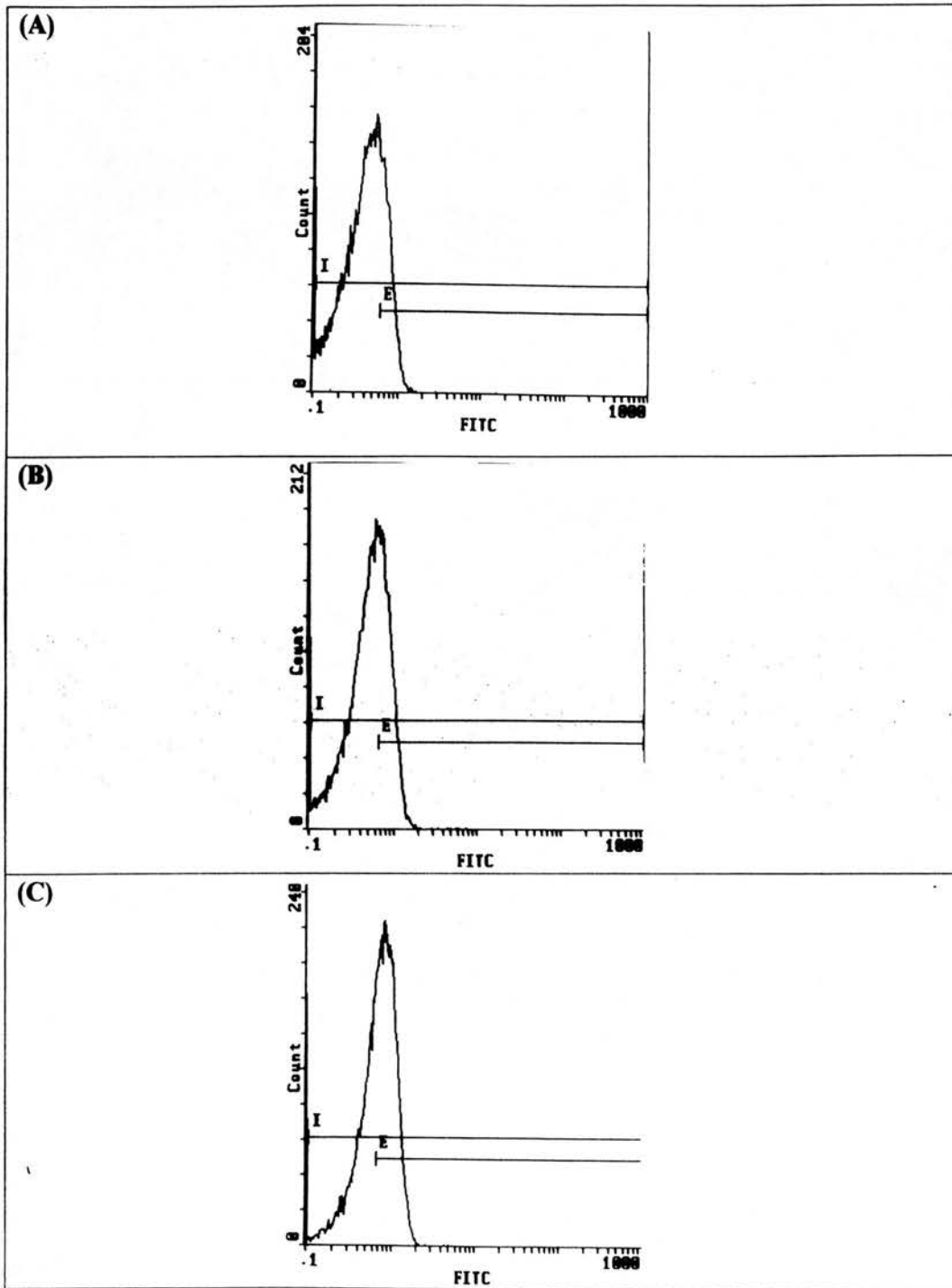


Figure 7.4: Flow cytometer chart of FACS analysis of neutrophil CXCR2 expression in patients with POD-acute liver failure (A), chronic liver failure (B) and controls (C).

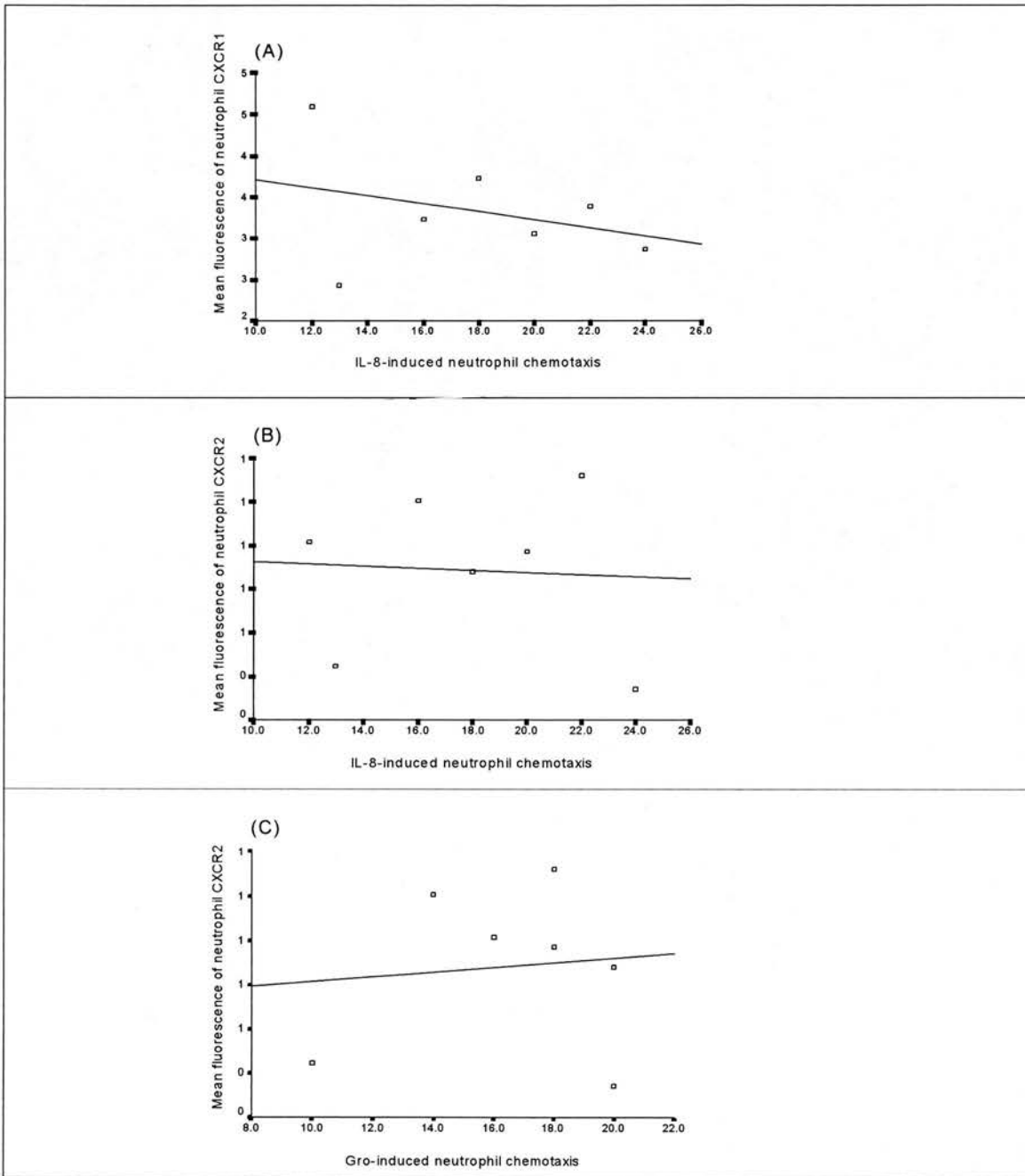


Figure 7.5: Correlation between IL-8-induced neutrophil chemotaxis (number of cell/high power field) and mean fluorescence of neutrophil CXCR1 (A) and CXCR2 (B), and Gro- $\alpha$ -induced neutrophil chemotaxis with mean fluorescence of CXCR2 (C) in patients POD-induced acute liver failure.

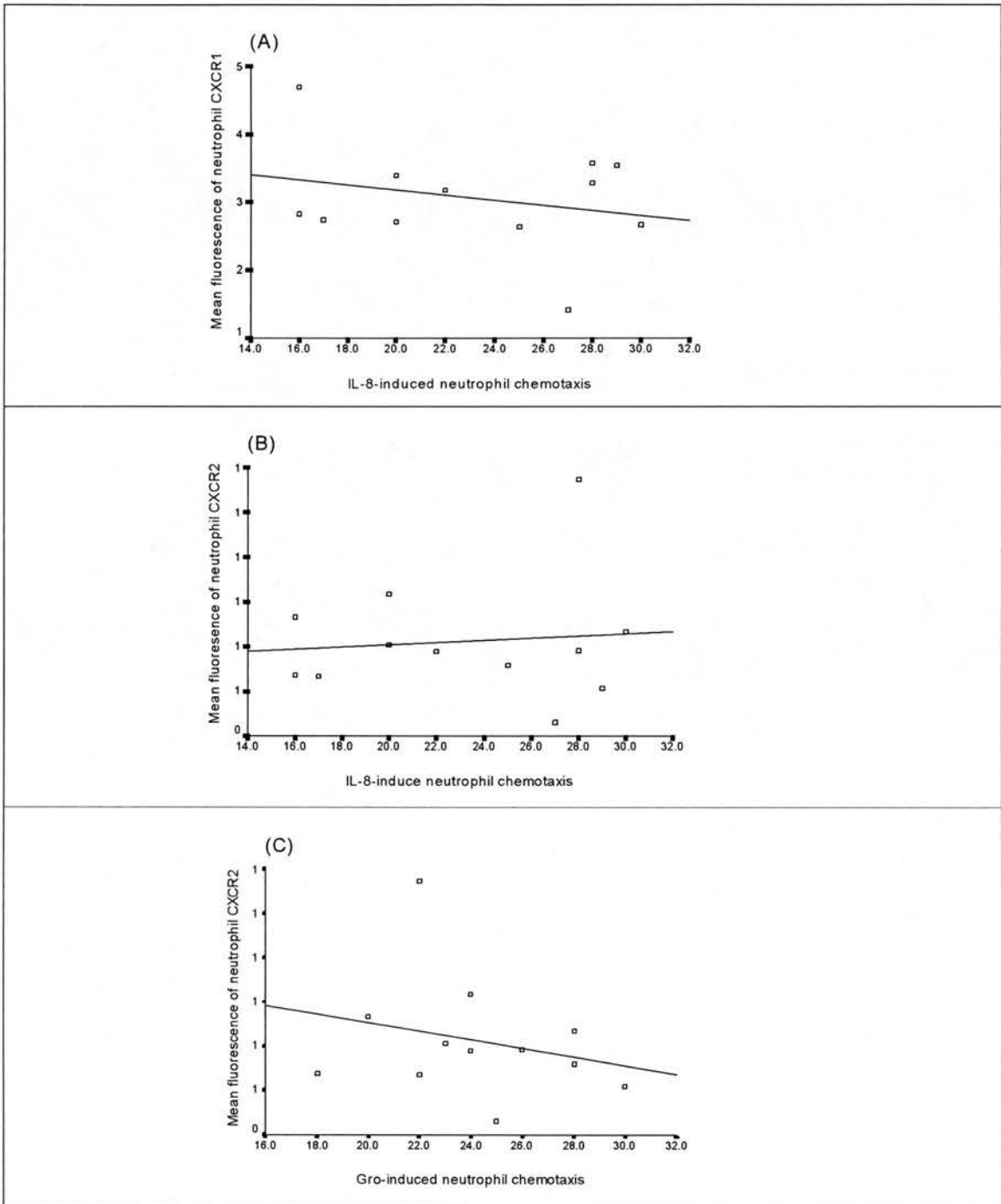


Figure 7.6: Correlation between IL-8-induced neutrophil chemotaxis (number of cell/high power field) and mean fluorescence of neutrophil CXCR1 (A) and CXCR2 (B), and Gro- $\alpha$ -induced neutrophil chemotaxis with mean fluorescence of CXCR2 (C) in patients with alcoholic liver cirrhosis.

## **7.5 DISCUSSION**

In this chapter neutrophil chemotaxis and chemokine receptors CXCR1 and CXCR2 neutrophil expression in patients with POD-induced-acute liver failure and patients with alcohol-induced chronic liver failure were compared with a group of healthy controls. Both IL-8- and Gro-stimulated neutrophil chemotaxis was reduced in patients with acute or chronic liver failure compared with healthy controls. However, no significant difference in expression of CXCR1 and CXCR2 was observed in patients with either acute or chronic liver failure compared with healthy controls.

Previous studies showed a rapid internalisation and degradation of the ligand-receptor complex by lysosomal endocytosis following binding of IL-8 to its receptors. Endocytosis of more than 90% of IL-8-receptor complex was detected within 10 minutes of binding. But, a rapid receptor recycling, as indicated by re-expression on the cell surface was also detected {Samanta et al 1990}. A previous study showed a dose dependent down-regulation of CXC receptors following incubation of neutrophils with increasing concentration of IL-8 or NAP-2 {Ludwig et al 1997}. In the studies reported in this chapter the effect of high circulatory IL-8 concentrations may have been diminishing with time during neutrophil isolation and preparation for FACs analysis. This allows the neutrophils to recycle and re-express their surface receptors again back to normal. However, the same neutrophils have reduced chemotaxis in response to IL-8 and Gro suggesting an alternative explanation.

Previous studies have shown that regulation of CXCR1 and CXCR2 expression is an important mechanism for controlling neutrophil activation by chemokines. This regulatory mechanism involves agonist-dependent down-regulation of these receptors in response to chemokines {Khandaker et al 1998}. The carboxyl terminal domain of CXCR1 and CXCR2 is important in IL-8-mediated receptor desensitisation, signalling, and internalisation {Prado et al 1996}. Truncation of C-terminus of CXCR2 in transfected 293 cells resulted in loss of IL-8-dependent migration {Ben-Baruch et al

1995} and mutation of multiple serine residues limits ligand-induced receptor desensitisation {Muller et al 1997}.

Bacterial endotoxin or LPS is a major component of the outer most membrane of Gram-negative bacteria {Lynn et al 1992}. Previous studies have shown high concentrations of endotoxin in both systemic and portal circulations in patients with chronic liver failure {Guarner et al 1993}. Such endotoxaemia results from increased translocation of gut-derived endotoxin into the portal circulation. The reduction in the phagocytic function of the reticuloendothelial system and portosystemic shunting of blood predisposes to systemic endotoxaemia {Ziegler et al 1982 and Goulis et al 1999}. In case of sepsis, LPS is able to induce down-regulation of CXCRs, rapid degradation of CXCR mRNA, and inhibition of CXCR1 and CXCR2 transcription in PMNLs {Llyod et al 1995}. In contrast with IL-8, the LPS-induced chemokine receptor down-regulation is independent and distinct from agonist-mediated internalisation. IL-8 induces a uniform attenuation of CXCR expression among the total neutrophil population compared with a non-uniform-mediated down-regulation of the receptors induced by LPS. Recently, metalloproteases have been found to be involved in the down-regulation of several receptors such as CD16 {Middelhoven et al 1994}, CD43 and CD44 {Bazil and Strominger 1994}. In addition, it has been suggested that a  $\text{Ca}^{++}$  amino peptidase is also involved in the LPS-mediated inhibition of IL-8 binding to its receptor {Bhattacharya et al 1997}, which may be dependent on tyrosine kinase activity {Khandaker et al 1998}.

However, LPS can desensitise the receptors at receptor phosphorylation level distal to the receptor/G protein interaction {Blackwood et al 1996 and Sabroe et al 1997}. LPS can also deactivate neutrophil intracellular signalling pathways stimulated by chemokines {Laffi et al 1993}. The protein kinase inhibitor staurosporine inhibits desensitisation of IL-8 receptors without affecting the number of binding sites or receptor internalisation and therefore, desensitisation can occur independently of reduction in receptor number and internalisation {Johnston et al 1994}. These

mechanisms may explain the defective CXC chemokine stimulated chemotaxis in patients with liver failure without affecting the CXC receptor expression.

A previous study reported that the neutrophil chemotactic defect was much greater than the decline in cell surface receptors {Jawa et al 1999}. Another study suggested that in addition to receptor down-regulation, other mechanisms might play a role in the neutrophil chemotactic defect in similar diseases {Quaid et al 1999}. These possible mechanisms include impaired chemokine receptor signal transduction due to defective secondary messengers or defective ligand-receptor binding due to circulating inhibitory factor/s or antibodies against CXC chemokines.

## **CHAPTER 8**

### **SUMMARY AND CONCLUSIONS**

Patients with acute or chronic liver failure are at increased risk of infection during the course of their disease. Infection in such patients is frequently leads to complications such as severe encephalopathy, upper gastrointestinal haemorrhage, and even death. Neutrophil functions including chemotaxis are important components of the immune system and body defense mechanisms against infection.

Previously, neutrophil chemotaxis was studied in liver diseases using several non-specific chemoattractant substances such as zymosan-activated plasma, immune complexes-activated serum, and components of complement factors {DeMeo and Andersen 1972, and Campbell et al 1981}. Subsequently the chemokine family of potent neutrophil chemoattractants were described. This thesis details studies designed to investigate if in patients with liver failure, neutrophil chemotaxis was also deficient to chemokines.

Chapter 2 describes and optimises the methodology adapted to isolate neutrophils and measure chemotaxis using the CXC chemokines IL-8 and Gro- $\alpha$ . Using the modified Boyden chamber gave us satisfactory and reproducible results. Neutrophil chemotaxis stimulated with serially diluted CXC chemokines showed a dose-dependent response as expected, with similar chemotactic responses to IL-8 and Gro in the controls. The chemotactic response to both chemokines showed little variability over a time period or within the assay.

Chapter 3 studied both IL-8- and Gro- $\alpha$ -induced neutrophil chemotaxis in patients with paracetamol-induced acute liver failure and patients with chronic liver failure, which was found reduced in both groups of patients, compared with the control healthy subjects. The reduction in neutrophil chemotaxis was more significant in patients with chronic compared with acute liver failure. According to the aetiology, the greatest reduction in chemotaxis was in the patients with alcoholic liver disease, followed by chronic HCV cirrhosis, and paracetamol-induced acute liver failure. The least affected group were patients with PBC. In POD-induced acute liver failure hepatic



encephalopathy is considered as a marker of severity of the disease. However, no significant change was detected in both IL-8- and Gro- $\alpha$ -induced neutrophil chemotaxis in patients with or without encephalopathy. In chronic liver failure, the reduction in neutrophil chemotaxis was significantly correlated with the disease severity as indicated by the Child-Pugh score. These findings may explain the increased liability of patients with acute and chronic liver failure to infection.

In chapter 4, we simulated the effect of upper gastrointestinal bleeding on IL-8- and Gro- $\alpha$ -induced-neutrophil chemotaxis in patients with chronic alcoholic liver disease using a tailor-made protein mixture that gives the same biochemical effect of blood when ingested or present in the upper gastrointestinal tract. Both IL-8- and Gro- $\alpha$ -induced neutrophil chemotaxis was significantly reduced 2 hours after oral administration of the simulated bleeding solution and was associated with a significant rise in the plasma ammonia concentration. However, no significant correlation between changes in neutrophil chemotaxis and plasma ammonia concentration was detected. In the second part of this chapter, cross over studies showed that incubation of neutrophils isolated from patients with acute or chronic liver failure with controls' serum resulted in partial, but significant improvement of neutrophil chemotaxis. In contrast, a significant reduction in chemotaxis of controls neutrophils was observed following incubation with patients' serum. These results indicated presence of chemotactic inhibitory factor/s circulating in blood of patients with either acute or chronic liver failure and that the defect in neutrophil chemotaxis is at least partially reversible. Also *in vivo* neutrophil chemotaxis may change in a dynamic fashion following a common complication of cirrhosis, namely variceal bleeding.

Chapter 5 studied serum IL-8, IP-10, and Mig concentrations in patients with POD-induced acute liver failure as well as patients with chronic liver failure. All the chemokines studied were significantly high in concentration in patients with POD-induced acute liver failure especially in patients with advanced encephalopathy (grades 3 or 4) compared with patients with mild or no encephalopathy and control subjects.

Serum IL-8 and IP-10 concentrations were significantly higher in patients with sepsis and only serum IL-8 was significantly high in patients who died or were transplanted and in patients who needed renal support compared with survivors and patients did not need renal support respectively. IL-8 was also significantly correlated with prothrombin time and total white blood count. As regard patients with chronic liver failure, both serum IL-8 and Mig concentrations were significantly high in patients compared with controls, while serum IP-10 was undetectable. Serum chemokine concentrations were significantly high in patients with advanced disease (Child grades B and C) compared with milder form of the disease (grade A), with no other significant differences according to presence or absence of ascites, encephalopathy, sepsis, or the patients' outcome. There were also no significant correlations between serum chemokine concentrations and biochemical markers of liver disease such as bilirubin, and albumin. These studies may indicate either increased chemokine production by either the injured liver or other extrahepatic cells or organs. Alternatively, defective clearance of these proteins by the injured liver may occur.

Chapter 6 studied the possible variation in neutrophil chemotaxis and chemokine concentrations across the liver during TIPSS check in patients with chronic liver disease. Neutrophil chemotaxis was significantly reduced in hepatic compared with portal or peripheral veins. IL-8 and Mig concentrations in hepatic venous blood were significantly higher compared with portal venous blood. Moreover, incubation of neutrophils isolated from hepatic venous blood with serum isolated from portal venous blood resulted in partial, but significant improvement in chemotaxis and vice versa. These results suggest that the liver has an important inhibitory effect on neutrophil chemotaxis possibly as a result of chemokine release by the injured liver.

In chapter 7 no significant changes in neutrophil expression of both CXC chemokine receptors CXCR1 or CXCR2 in patients with either acute or chronic liver failure was observed compared with controls. These findings suggest that other mechanisms

independent of receptor expression lead to reduction in neutrophil chemotaxis in patients with either acute or chronic liver failure.

In summary, this thesis has reported data that expands the current knowledge regarding neutrophil chemotaxis in patients with acute and chronic liver failure. Defective CXC chemokine stimulated neutrophil chemotaxis may explain the increased risk of infection in such patients. In addition, the data confirm the presence of circulating chemotactic inhibitory factor/s in patients with acute and chronic liver failure. The high circulating chemokine concentrations may act themselves as inhibitors of chemotaxis. However, CXC chemokine-induced down-regulation of the neutrophil CXCR1 or CXCR2 receptor expression is not the explanation for the chemotactic defect. This will need further studies to investigate the possible role of post-receptor signal transduction and kinase systems, which may lead to such reduction in neutrophil chemotaxis. The *in vivo* studies show that neutrophil chemotaxis may be regularly modulated, suggesting that alternative strategies may improve the chemotactic defect in patients with liver failure.

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