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Hepcidin regulation in chronic kidney disease

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Hepcidin Regulation in Chronic Kidney Disease

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PhD Supervisor: Professor Iain Macdougall

A thesis submitted for the degree of

Doctor of Philosophy

June 2019

Declaration

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Abstract

Iron homeostasis in man is tightly regulated by a 25-amino-acide peptide hormone (hepcidin) that is produced in the liver in response to inflammatory stimuli and iron loading. Its main role is thought to be part of the innate immune system, restricting iron delivery to infectious microorganisms, and as a consequence, reducing iron availability for erythropoiesis in the host. The anaemia associated with chronic kidney disease (CKD) is known to be in part due to relative erythropoietin deficiency, and we also now know that excess hepcidin is one of the reasons that dietary iron is less well absorbed in these patients, necessitating the administration of intravenous iron. We know that haemodialysis patients in particular have elevated hepcidin levels, although the precise mechanisms remain unclear.

Characterisation of hepcidin levels in a number of CKD cohorts, including non-dialysis and dialysis-CKD patients, confirm that patients receiving haemodialysis have the highest levels, and that a considerable degree of day-to-day variability exists that is not completely explained by the regular administration of intravenous iron. An approach designed to measure hepcidin production using *HAMP* gene expression levels in circulating peripheral blood mononuclear cells found that patients with the highest circulating hepcidin levels had the lowest degree of *HAMP* expression, suggesting the presence of a negative feedback mechanism.

Although bacterial sepsis is an extremely powerful driver of hepcidin production, haemodialysis patients do not appear to be able to mount an increased hepcidin response under such conditions, raising the possibility that their hepcidin burden is maximised. It is therefore possible that the role of inflammation in this context may not be as significant as the role of reduced hepcidin clearance, or the role of iron loading. In contrast, inflammation is probably a major driver of hepcidin induction in those who develop an acute kidney as a result of ANCA-associated vasculitis or a crescentic glomerulonephritis. Hepcidin expression was suppressed following the use of dexamethasone in a cellular hepatocyte model, and this was partly IL-6 dependent. In a pilot study examining the aforementioned patient groups, hepcidin levels were elevated at presentation and were markedly reduced following treatment with intravenous methylprednisolone. The glucocorticoid-induced suppression of hepcidin in both *in vitro* and *in vivo* settings suggests this as a pathway for the improvement in anaemia seen in inflammatory conditions following steroid therapy.

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This work would not have been possible without the support and encouragement of many of my colleagues, friends, and family. Firstly, I would like to express my absolute gratitude to Professor Iain Macdougall for his generosity and guidance throughout. It has been a real pleasure working with Iain and his research team, and I have benefited enormously. There were additional clinical studies undertaken during my time as his clinical research fellow that have not graced the pages of this thesis, but the work will undoubtedly stand me in good stead as a practicing clinician in the years to come. I will particularly remember our discussions concerning grammar and syntax with great fondness, and I can only hope that some of his natural flair for scientific writing has somehow rubbed off on me!

I have learnt a tremendous amount from Professors Claire Sharpe, Bruce Hendry, Andrew McKie and Paul Sharp, and I thank them for their deliberations and input. However, the character that perhaps deserves the most thanks for his day-to-day running of the laboratory is Mr Mazhar Noor, who taught me many of the basic science techniques included in this thesis. Special thanks are reserved for Dr Andrew Armitage from Oxford, who took time out from his busy schedule to show me how to extract RNA from peripheral blood nuclear cells, and Dr Nnenna Kanu from UCL, for her help with my irksome Western blots. There have been other PhD students who have helped me during my journey, including Drs Sujit Saha, Lucy Newbury, and Andrea Cove-Smith. I was fortunate to share an office with Dr Pantelis Sarafidis, from whom I learnt about clinical studies and statistics/SPSS. I am very grateful to Dr Stefan Brincat who started the work, and from whom I took over.

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Writing this thesis over the last two years has been laborious for all concerned, and my wife has shouldered the burden of responsibility for non-thesis-related matters. I would like to wholeheartedly thank my family for their continued love and support. I thank Mariam(S.E.) for being by my side through it all, and for her patience and endless optimism, without which I would not have made it this far.

I dedicate this work to my wife, my mum and dad.

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Abbreviations

%HRC	percentage of hypochromic red blood cells
ACD	anaemia of chronic disease
ACR	albumin:creatinine ratio
AKI	acute kidney injury
ANCA	anti-neutrophil cytoplasmic antibody
AVF	arterio-venous fistula
AVG	arterio-venous graft
BMP	bone morphogenetic protein
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
CKD	chronic kidney disease
CRP	C-reactive protein
CV_1	coefficient of variance
DCYTB	duodenal cytochrome B
DMEM	Dulbecco's modified Eagle medium
DMSO	dimethyl sulfoxide
DMT1	divalent metal transporter 1
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
eGFR	estimated glomerular filtration rate
ELISA	enzyme-linked immunosorbent assay
EPO	erythropoietin
ESA	erythropoiesis-stimulating agent
ESR	erythrocyte sedimentation rate
ESRF	end-stage renal failure
EtOH	ethanol
FBS	fetal bovine serum
FPN	ferroportin
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
HAMP	hepcidin antimicrobial peptide
Hb	haemoglobin
HD	haemodialysis
HD-CKD	haemodialysis - CKD
HIF	hypoxia-inducible factor
HJV	hemojuvelin
hsCRP	high-sensitivity C-reactive protein
IL-6	interleukin-6
INT	iodonitro-tetrazolium violet
IRIDA	iron-refractory iron deficiency anaemia
KDIGO	kidney disease improving global outcomes
KDOQI	kidney disease outcomes quality initiative
LDH	lactate dehydrogenase
LEAP-1	liver expressed antimicrobial peptide-1
MCV	mean cell volume

mRNA	messenger ribonucleic acid
MWF	Monday/Wednesday/Friday
NAD	nicotinamide adenine dinucleotide
ND-CKD	non-dialysis CKD
NICE	The National Institute for Health and Clinical Excellence
PBMC	peripheral blood mononuclear cell
PBS	phosphate-buffered saline
PBST	phosphate-buffered saline with 0.1% tween
PCR	polymerase chain reaction
PEG	polyethylene glycol
PSA	polysialic acid
PTH	parathyroid hormone
qPCR	quantitative PCR
RBC	red blood cells
rHuEpo	recombinant human erythropoietin
RNA	ribonucleic acid
RPM	revolutions per minute
RRT	renal replacement therapy
RT-QPCR	reverse transcriptase quantitative polymerase chain reaction
RTx	renal transplant
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SMAD	sma and mothers against decapentaplegic homologue
SOCS-3	suppressor of cytokine signalling-3
STAT	signal transducer and activator of transcription
TAE buffer	Tris-acetate-EDTA buffer
TBS	Tris buffered saline
TBST	Tris buffered saline with 0.1% tween
Tf	transferrin
TfR	transferrin receptor
TIBC	total iron-binding capacity
TMPRSS6	transmembrane protease serine 6
TSAT	transferrin saturation (serum iron ÷ TIBC)
TTS	Tuesday/Thursday/Saturday
UKRR	United Kingdom Renal Registry
WBC	white blood cells
WHO	World Health Organisation

1 Introduction

The association between kidney disease and anaemia was initially recognised by Richard Bright, who described characteristic features in his case reports published in 1836 during his time working at Guy's Hospital (Feehally, McIntyre, and Cameron 2013). It was, however, his contemporary, the forensic chemist and physician, Christison, whose meticulous observations signalled the first robust link between advanced kidney disease and the development of anaemia (Cameron 1999). At the turn of the 20th century, the French scientists Carnot and Deflandre were convinced of the existence of a humoral factor that controlled erythropoiesis (Carnot and Deflandre 1906), for which they coined the term hemopoietine. This factor was later renamed erythropoietin, although it took over a hundred years from Christison's original observations before its eventual detection. It was only in 1957 that the role of the kidney in erythropoiesis was finally discovered (Jacobson et al. 1957), and this provided a possible reason to explain why patients with end-stage renal disease often developed profound blood transfusion-dependent anaemia. Two decades elapsed before human erythropoietin was purified from the urine of patients with aplastic anaemia (Miyake et al, 1977, Goldwasser 1977), and it took a further eight years before the human erythropoietin gene was cloned and expressed (Miyake, Kung, and Goldwasser 1977; Lin et al. 1985; Jacobs et al. 1985), leading to the first clinical use of recombinant human erythropoietin (rhuEpo) in anaemic dialysis patients in 1986 (Winearls et al. 1986). The following year, Eschbach and colleagues published their phase I and II clinical trial, which showed that the administration of rhuEpo to another cohort of anaemic haemodialysis patients not only treated the anaemia (and in some cases abolished the need for recurrent transfusions of packed red cells), but subsequently exposed the patients to a state of relative iron deficiency (Eschbach et al. 1987). Up until this point, researchers had largely been concerned with solving the conundrum of erythropoietin deficiency in renal failure. It only became apparent after the clinical trials of erythropoietin in the late-1980s, that the use of erythropoietin had to be combined with the use of iron replacement therapy in order to optimally treat the anaemia of advanced CKD (Macdougall et al. 1989; Fishbane, Frei, and Maesaka 1995).

Although it had long been known that erythropoiesis depended on the availability of iron for the production of red blood cells in the bone marrow, it was only in the 1970s that the first reports of the use of intravenous iron in haemodialysis patients emerged

(Edwards, Pegrum, and Curtis 1970). Reports of severe anaphylaxis in a few patients receiving parenteral iron undoubtedly led to its limited use over the next two decades, amidst these obvious safety concerns (Becker et al. 1966). At the time, there was perhaps less impetus to study the use of parenteral iron in end-stage renal failure, given the inconsistent data examining the difference between the effect of parenteral and oral iron on various outcomes in haemodialysis patients (Brozovich et al. 1971; Rossi et al. 1976; Winney et al. 1977). These data, along with the findings from studies that implied that gastrointestinal absorption of iron in haemodialysis patients was normal (Eschbach, Cook, and Finch 1970), reinforced the prevalent view that blood loss via dialyser connections, frequent blood sampling, and increased gastrointestinal losses, were the principal reasons that haemodialysis patients became iron deficient, and in need of iron replacement therapy (Milman and Larsen 1976). In contrast, experimental animal models examining iron absorption from the duodenum of uraemic rats showed that there was a reduction in iron absorption in the uraemic group (Delano, Manis, and Manis 1977). The gastrointestinal absorption of iron was not really examined in any great depth, until after the work of Eschbach and colleagues.

Around the turn of the millennium, the discovery of the factor controlling the absorption of iron from the gastrointestinal tract occurred somewhat by chance, whilst scientists were engaged in the search for cysteine-rich peptides in the ultrafiltrate from human blood that had antimicrobial activity (Krause et al. 2000). This factor, which was initially labelled LEAP-1 (liver expressed antimicrobial peptide-1), and we now know as hepcidin, was also isolated from the urine of healthy donors by a second group, and their findings were again presented as a novel hepatic antimicrobial peptide (Park et al. 2001). The extent to which these discoveries would revolutionise our understanding of the regulation of iron metabolism only became apparent after the publication of two French papers, which suggested that hepcidin had a role to play in iron homeostasis (Nicolas et al. 2001; Pigeon et al. 2001). We now know that hepcidin is not only critical in this process, but is indeed the master-regulator of iron homeostasis in the body, exerting its action by binding to ferroportin, the sole mammalian exporter of iron (Nemeth, Tuttle, et al. 2004). The major consequence of increased hepcidin (thereby leading to a decrease in ferroportin) is a reduction in iron availability to the bone marrow for erythropoiesis. In recent times, it has become evident that this process is linked to the pathogenesis of the anaemia associated with chronic kidney disease, which in turn has a similitude with the anaemia of chronic disease. This has opened the

door to the development of treatment strategies that target hepcidin (and other mediators involved in the pathogenesis of renal anaemia), particularly since we now know that safety concerns surround the use of erythropoietin. The question of potential harm as a result of the liberal use of intravenous iron was addressed by the PIVOTAL study; there was no difference in adverse events (cardiovascular outcomes or sepsis) between the haemodialysis group receiving high dose iron versus the group receiving the lower dose (Macdougall et al. 2019).

1.1 Chronic Kidney Disease

1.1.1 Definition

Chronic kidney disease (CKD) is a progressive, irreversible, and (at least in the early stages of the disease) often asymptomatic condition that results from a substantial loss of nephron function, and is typified in its later stages by a clinical syndrome that includes a progressive decline in glomerular filtration, systemic hypertension, oedema, and proteinuria. Characteristic histological findings on renal biopsy specimens from patients with advanced CKD include progressive tubulointerstitial fibrosis, tubular atrophy, and loss of peritubular capillaries resulting from hypoxia (Eddy 2005).

The diagnosis of CKD is relatively simple; in the vast majority of cases, it requires two blood tests (that estimate the glomerular filtration rate (GFR) to be less than 60 ml/min/1.73 m^2) separated by at least 90 days. The diagnosis can also be made in the presence of a structural or functional defect, if this has also been shown to persist for more than 90 days (Table 1.1).

Decreased GFR	GFR <60 ml/min/1.73 m^2
Markers of kidney damage (≥1)	Albuminuria (ACR ≥ 3mg/mmol) Abnormal urinary sediment Evidence of tubular dysfunction Renal histological abnormality Structural abnormality History of kidney transplantation

Table 1.1 Diagnostic criteria for CKD
One or more of the above must be present for greater than 90 days in order to make a diagnosis of CKD (adapted from (Kidney Disease: Improving Global Outcomes (KDIGO) CKD Work Group 2013)).

Once the diagnosis of CKD has been made, the severity of the condition is stratified according to glomerular filtration (G) and urinary protein excretion (A), and the category within which an individual falls, has implications for the likelihood of that person progressing from CKD to end-stage renal failure (Figure 1.1). There are, however, a number of factors other than proteinuria, which are associated with this path to end-stage renal failure, including age, gender, ethnicity, diabetes, hypertension, cardiovascular comorbidities (including heart failure), body mass index, smoking, low socioeconomic status, and a history of one or more episodes of acute kidney injury (Lo et al. 2009; Collister et al. 2016).

Prognosis of CKD by GFR and Albuminuria Categories: KDIGO 2012				Persistent albuminuria categories Description and range		
				A1	A2	A3
				Normal to mildly increased	Moderately increased	Severely increased
				<3 mg/mmol	3-30 mg/mmol	>30 mg/mmol
GFR categories (ml/min/ 1.73 m^2) Description and range	G1	Normal or high	≥90	low risk	med risk	high risk
	G2	Mildly decreased	60-89	low risk	med risk	high risk
	G3a	Mildly to moderately decreased	45-59	med risk	high risk	very high risk
	G3b	Moderately to severely decreased	30-44	high risk	very high risk	very high risk
	G4	Severely decreased	15-29	very high risk	very high risk	very high risk
	G5	Kidney failure	<15	very high risk	very high risk	very high risk

Figure 1.1 Classification of CKD
Risk of progressing to end-stage renal failure according to GFR (G1 to G5) category and degree of proteinuria (A1 to A3) (reproduced from (Kidney Disease: Improving Global Outcomes (KDIGO) CKD Work Group 2013))

1.1.2 Epidemiology in the UK

Chronic kidney disease is a growing problem in the United Kingdom, and is thought to affect an estimated 1.8 million people, accounting for approximately 1.5% of the entire National Health Service budget (£95.6 billion for 2013/14). In addition to this colossal figure, there may be as many as a further one million people with undiagnosed CKD in the population (Kerr et al. 2012). The most recent data from the UK Renal Registry (UKRR) are available to the year ending 2014, and these showed that the incidence of adults requiring renal replacement therapy (RRT) in the UK had increased to a mean of 115 new patients per million people of the population. The incidence rate had plateaued in the decade preceding this annual iteration of the report (Figure 1.2).

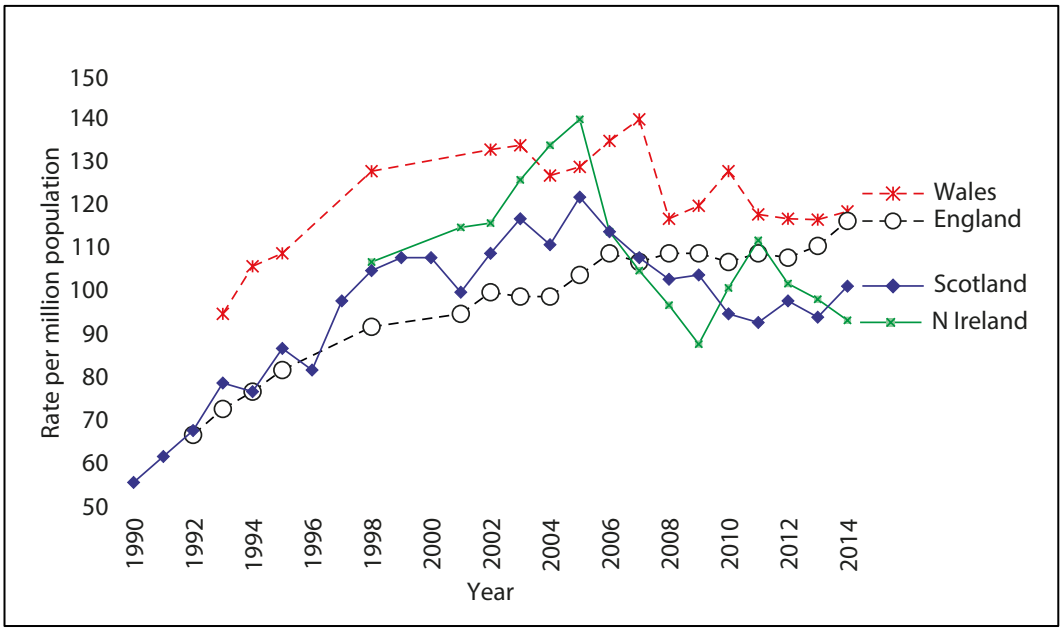

Figure 1.2 Incidence of renal replacement therapy in the UK
Reproduced from (Gilg, Caskey, and Fogarty 2016) outlining the number of patients commencing renal replacement therapy on an annual basis from 1990 to 2014, in the countries of the United Kingdom

As in previous years, the leading cause of renal disease in these patients developing end-stage renal failure was diabetes, which accounted for over a quarter of all of the new cases seen. The other leading causes of renal disease in the UK included glomerulonephritis, and renal vascular disease (Table 1.2). Chronic kidney disease is a condition that mainly affects those in the latter stages of their lives, and the median age of patients commencing RRT in the UK in 2014 was 64.8 years, a figure that has remained virtually unchanged since 2007. Patients who were white had a median age at start of RRT of 66.4 years, whereas the median age at start of RRT for non-white patients was considerably lower at 58.7 years (Gilg, Caskey, and Fogarty 2016).

Diagnosis	England	N Ireland	Scotland	Wales	UK
Diabetes	27.4	21.2	30.1	30.4	27.6
Glomerulonephritis	13.5	7.6	15.3	19.4	13.8
Pyelonephritis	5.9	6.0	4.7	8.1	5.9
Hypertension	7.3	6.5	3.9	1.9	6.7
Polycystic kidney	6.6	9.2	9.7	8.1	7.0
Renal vascular disease	6.1	4.3	7.9	12.9	6.5
Other	19.0	17.9	20.2	16.2	19.0
Uncertain aetiology	16.7	19.0	9.5	17.8	16.2
Data not available	14.8	1.6	0.0	3.9	12.6
All	**117**	**93**	**101**	**119**	**115**

Table 1.2 Primary renal diagnosis incidence rates in the UK
Reproduced from (Gilg, Caskey, and Fogarty 2016) outlining the primary renal diagnosis in the patients commencing renal replacement therapy in the countries of the United Kingdom in 2014

This difference is likely a reflection of the older age distribution of whites versus non-whites (5.3% of non-whites were over the age of 65 years old, compared to 18.3% of whites, UK Census 2011), and also the differences in the disease processes underlying their respective paths towards end-stage kidney disease.

The prevalence of adult patients receiving RRT has been increasing every year since the data have been collected, and in 2014, was 913 per million population of the UK (Figure 1.3). This is nearly 75% higher than the reported figure from the year 2000, when the prevalence of RRT in UK adults was 523 per million population. The general age of the RRT population has been increasing over time; the median age of prevalent patients in 2014 was 59 years, and nearly one-sixth of these patients were over the age of 75 years. As expected, the prevalence rate in men was greater than for women in all age categories. In contrast to the incident RRT population, the most common primary renal diagnosis in the 2014 prevalent cohort was glomerulonephritis (18.9%) followed by diabetes (16.1%) (MacNeill et al. 2016).

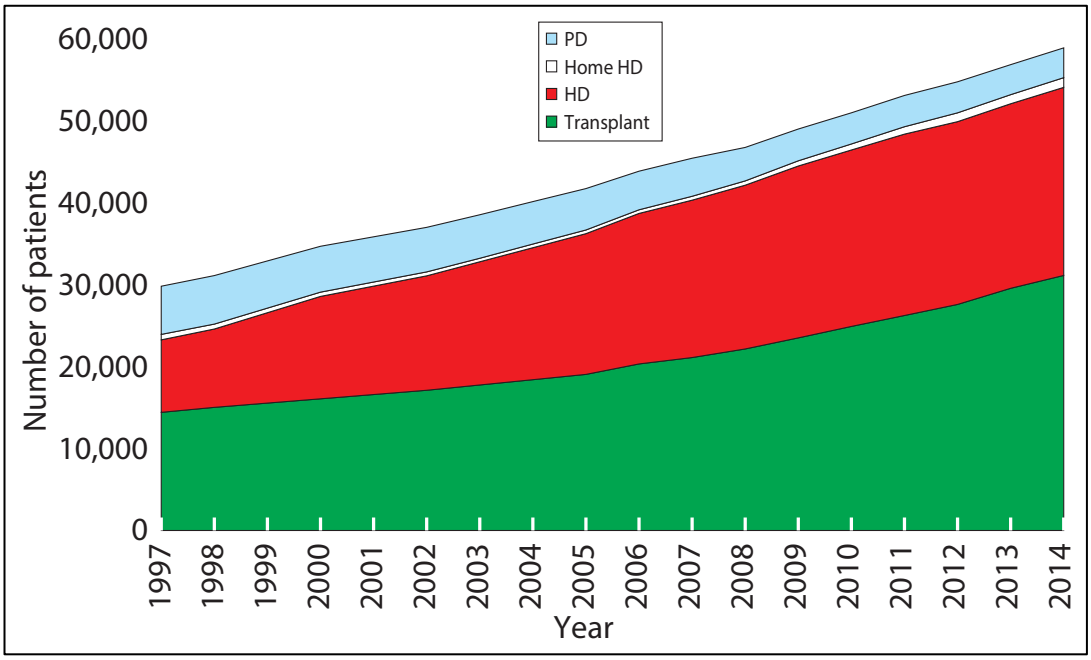

Figure 1.3 Prevalence of renal replacement therapy in the UK
The prevalence of patients in receipt of RRT in the UK from 1997 to 2014, categorised by modality (peritoneal dialysis (PD), haemodialysis (HD), or transplant) (reproduced from (MacNeill et al. 2016)

As with cardiovascular disease, men are more likely to require RRT than women, irrespective of whichever variable is statistically corrected for (i.e. gender, ethnicity etc.). Men are also far more likely to present late to their local renal services, within 90

days of starting RRT (which in turn is far more likely to be haemodialysis as opposed to peritoneal dialysis) (Gilg, Caskey, and Fogarty 2016).

The most recent data for the UK population estimates a life expectancy from birth of 79.1 years for males, and 82.8 years for females; this is a figure that has been rising by approximately 10 and 13 weeks per year for males and females, respectively, since 1980 (Office for National Statistics 2014). A diagnosis of end-stage renal failure decidedly reduces life expectancy across all groups (Hallan et al. 2012), and is influenced by a number of factors including age at diagnosis, the presence of other comorbidities, and the choice of RRT modality (Turin et al. 2012). In 2013, the overall one-year (after 90 day survival) unadjusted mortality rate in the UK incident RRT population was approximately 12%, and 15% in the prevalent dialysis population (Steenkamp, Rao, and Fraser 2016). Epidemiological evidence suggests that cardiovascular disease is by far the largest contributor to the excess mortality seen in patients with chronic kidney disease, and this is inclusive of an increase in cardiac failure and valvular heart disease (Go et al. 2004; Thompson et al. 2015). This is supported by data from the 2016 UKRR report, which showed that cardiac disease was the primary cause of death in all groups of RRT patients that were examined, except for transplanted patients (malignancy, then infections) (Steenkamp, Rao, and Fraser 2016).

1.1.3 Progression of CKD

A slow decline in excretory renal function is a natural consequence of aging (Stevens et al. 2006), but the trajectory is often such that a person will reach the end of his/her natural life, before ever progressing to end-stage renal failure. There are a number of risk factors that affect this trajectory, some of which are modifiable. The two risk factors that nephrologists seek to address before any other are proteinuria and hypertension. The link between (even modest amounts of) proteinuria and progression of CKD is well established, and inhibition of the renin-angiotensin-aldosterone system (RAAS) has been shown to slow the progression of CKD, independent of their effects on blood pressure (Collister et al. 2016). The necessity for controlling blood pressure to retard the progression of CKD is an essential component of all national (The National Institute for Health and Care Excellence 2015a) and international guidelines (Kidney Disease: Improving Global Outcomes (KDIGO) CKD Work Group 2013) on the treatment of CKD, but whether the target blood pressure should be lower than stipulated by the

current guidelines remains a source of discussion (Wright et al. 2015). Correction of metabolic acidosis (de Brito-Ashurst et al. 2009), hyperuricaemia (Goicoechea et al. 2015), and lowering the intake of dietary protein in certain situations (Levey et al. 2006) have also been shown to slow the rate of progression to end-stage renal failure. Although there have been data suggesting that treating asymptomatic hyperuricaemia may be of benefit in CKD, a randomised controlled trial examining the use of febuxostat in stage 3 CKD showed no benefit versus placebo (Kimura et al. 2018). Other treatments for hyperphosphataemia, hyperparathyroidism, dyslipidaemia, and smoking cessation do not have the same level of evidence when it comes to slowing the decline in renal function, but are nonetheless utilised due to the benefits in terms of cardiovascular risk reduction (Agarwal, Haddad, and Hebert 2008). The non-modifiable elements that influence the development and progression of CKD include male gender, African-American ethnicity, and a genetic predisposition associated with sequence variants in the apolipoprotein L1 gene, as well as the various gene mutations associated with the cystic diseases of the kidney (Collister et al. 2016).

1.1.4 Sequelae of CKD

Other than hypertension and proteinuria, which can present in early CKD (stages 1/2), the major clinical manifestations of CKD tend to become apparent from stage 3a onwards (i.e. eGFR 45-59 ml/min/1.73m^2) (Table 1.3).

Hypertension	Malnutrition
Anaemia	Sodium and fluid overload
Dyslipidaemia	Endocrine abnormalities
Bone and mineral disease	Immune dysregulation
Metabolic acidosis	Psychological effects

Table 1.3 Clinical manifestations of CKD

As well as being a cause of CKD, hypertension frequently develops as a result of CKD, and it is reported that between 50-75% of patients with CKD stage 3a onwards will have hypertension (defined as a systolic BP ≥140 mm Hg, or diastolic BP ≥90 mm Hg). As the kidney is responsible for hydroxylation of 25-hydroxycholecalciferol, CKD will also bring about changes in bone and mineral metabolism. The combination of a deficiency in 1,25-dihydroxycholecalciferol and hyperphosphataemia (brought about by a reduction in renal phosphate clearance) contribute to secondary hyperparathyroidism, and if left unchecked, eventually results in the development of

renal bone disease. Likewise, a relative deficiency of the renal hormone, erythropoietin, induces anaemia due to insufficient red cell production by the bone marrow. The anaemia of renal disease becomes more prevalent from CKD stage 3a onwards and will be discussed in greater depth later in this chapter (from page 30). Tubular dysregulation in the renal handling of sodium in the later stages of CKD (stages 4/5) eventually lead to sodium and water retention, and subsequently to increased intravascular volume expansion, resulting in peripheral and/or pulmonary oedema. This no doubt plays a significant role in the pathogenesis of hypertension in CKD. The tubules (via reduction in excretion of hydrogen ions +/- loss of alkali) are also responsible for the metabolic acidosis seen in CKD stages 4/5, although the clinical manifestations of acidosis are often seen late in stage 5. Chronic kidney disease is an inflammatory state, and this, along with anorexia, metabolic acidosis, and oxidative stress lead to malnourishment in its very advanced stages. Endocrine abnormalities, such as hypothyroidism, insulin resistance, hyperprolactinaemia, and decreased levels of circulating growth hormone and testosterone are also commonly seen in very advanced renal disease. An altered hormonal milieu in CKD increases the risk of infertility, but this risk can be mitigated by renal transplantation (Wheeler 2010).

1.1.5 Immunity and Inflammation

After cardiovascular disease, infection is the second most common cause of death in patients with end-stage renal failure. Immunity is impaired in these patients through a number of different mechanisms; both T-cell and neutrophil responses are defective, meaning that patients are often susceptible to bacterial infections. The frequent use of tunnelled haemodialysis and peritoneal dialysis catheters in these functionally immunodeficient patients present possible routes of entry for opportunistic pathogens (Wheeler 2010).

Frequent infective episodes in dialysis patients, such as catheter-related, and access site infections, in addition to thrombotic episodes involving arterio-venous fistulae and grafts, provide a rich source of cytokines and stimuli that are amongst the factors that form the pro-inflammatory environment that is typical of the patient with end-stage renal failure. Other stimuli include a higher incidence of periodontal disease, metabolic acidosis, deficiency of vitamin D, and other dialysis-related factors (Figure 1.4). Chronic inflammation, especially in the dialysis patient, is associated with increased morbidity and mortality, particularly cardiovascular death. Mortality has been found to

be associated with a number of biomarkers such as serum albumin, C-reactive protein, interleukin-1 and -6, and tumour necrosis factor alpha. The strong association of hypoalbuminaemia with increased cardiac mortality has been shown to be a result of inflammation, as opposed to malnutrition (Kalantar-Zadeh et al. 2005; Akchurin and Kaskel 2015). Highly relevant to this thesis is the fact that inflammation in CKD is also one of the main contributors to the anaemia of renal disease, and is a cause of resistance to erythropoietin therapy (Kovesdy 2013).

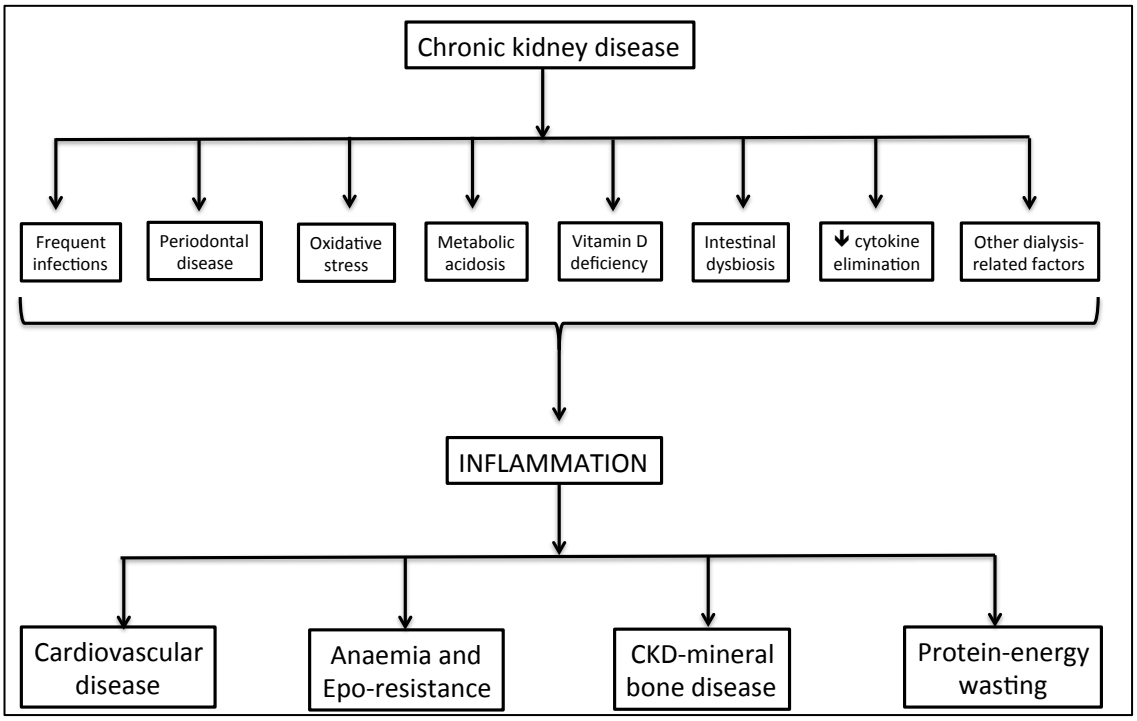

Figure 1.4 Causes and consequences of inflammation in CKD
CKD-associated inflammation results in increased cardiovascular disease and renal anaemia (adapted from (Akchurin and Kaskel 2015))

1.2 Renal Anaemia

1.2.1 Definition of Anaemia

Anaemia is defined as a reduction in the number of circulating red blood cells (RBCs), and is determined by comparing the haemoglobin concentration (Hb) against standard laboratory reference values, which were initially determined in the 1960s by researchers working for the World Health Organisation (WHO). The recommendations for adults residing at sea level are that males should have an Hb ≥130 g/L, non-pregnant females should have an Hb ≥120 g/L, and pregnant females should have an Hb ≥110 g/L (Blanc, Finch, and Hallberg 1968). However, residential elevation above sea level and cigarette smoking are known to increase Hb levels, and thus adjustments should be made in these circumstances (World Health Organisation 2011). It should be noted that the original 1968 WHO study was conducted using very few data, and also using methods that suggest that the authors of the original paper did not intend for their work to be the definitive article on the subject. The WHO data has since been validated for Caucasian men and women in large epidemiological studies, such as the National Health and Nutritional Examination Survey (Hollowell et al. 2005).

Men tend to have higher Hb levels than women, and this is very likely due to two factors; that men have higher levels of testosterone, and that women have regular menstrual blood losses. The link between androgens and erythropoiesis has long been recognised, with testosterone stimulating RBC production (Carrero et al. 2012). In general, the main causes of anaemia are due to blood loss, decreased production of RBCs, increased destruction, and shortened survival (Table 1.5). Age is another factor that affects Hb concentrations (Cheng et al. 2004) but it remains a matter of debate whether the WHO values are applicable in those who are over 65 years of age, as there are no currently accepted definitions of anaemia in the elderly. This is complicated by the difficulty in finding or even defining a “healthy” cohort of elderly patients in which to sample Hb levels. Beutler and colleagues have made attempts to address this issue by mining the NHANES III database and removing those with iron deficiency (defined as a serum ferritin level <10 µg/L, or a transferrin saturation (TSAT) <16%), inflammation (elevated C-reactive protein (CRP) or erythrocyte sedimentation rate (ESR)), renal impairment (creatinine > 106 µmol/L), and pregnancy, to derive Hb levels that are representative of those younger (age 20-59 for men; 20-49 for women), and older white and black adults. As can be seen in Table 1.4, the actual 5th centile Hb values for African-American patients are significantly lower than the corresponding cut-

off WHO values, so it is highly probable that anaemia is more likely to be over-diagnosed and over-investigated in the black population compared to their white counterparts (Beutler and Waalen 2006).

Gender	Age (y)	Number of patients sampled	Hb concentration (5^{th} centile) (g/L)	Hb cut-off (g/L) (WHO)
White men	20-59	1456	138	130
	≥60	934	128	130
White women	20-49	1045	122	120
	≥50	1495	120	120
Black men	20-59	1253	128	130
	≥60	235	118	130
Black women	20-49	904	113	120
	≥50	442	113	120

Table 1.4 Reassigning normal haemoglobin values following NHANES III analysis
Lower limits (5^{th} centile) of normal for haemoglobin concentration (g/L) of younger (age 20-59 for men; 20-49 for women) and older white and black adults (adapted from (Beutler and Waalen 2006))

1.2.2 Definition of Renal Anaemia

Renal anaemia is a chronic condition that is defined as a reduction in haemoglobin concentration occurring primarily as a result of renal disease. A gradual decrease in the relative concentration of circulating erythropoietin through reduced EPO production by the peritubular interstitial cells in the kidney is the main causative mechanism for the development of the normochromic, normocytic anaemia of CKD (Shanks et al. 1996). There are, however, a number of other contributory factors, which will be expanded upon further in section 1.2.4.

1.2.3 Epidemiology

Much of the current epidemiological evidence that we possess outlining the association between kidney disease and anaemia comes from the Third National Health and Nutrition Survey (NHANES III), wherein 15419 U.S. participants' data were examined (Astor et al. 2002). In contrast to the WHO recommendations, the definition of anaemia in this study was defined as an Hb <120g/L and <110 g/L in men and women, respectively. Anaemia did not tend to be a feature of CKD until the eGFR was approximately 30 ml/min/1.73m^2. The prevalence of anaemia was 9% at the start of stage 4 CKD, and 33% at the start of stage 5 (Figure 1.5). The one clinical situation where renal anaemia is often seen earlier is diabetic kidney disease, when it is not uncommon to see a decline in the Hb concentration with a higher eGFR threshold of (below) 45/ml/min/1.73m^2. There are several reasons for this that include loss of

endogenous EPO in those with severe proteinuria, reduced production of EPO, and chronic inflammation (Deray et al. 2004; Loutradis et al. 2016).

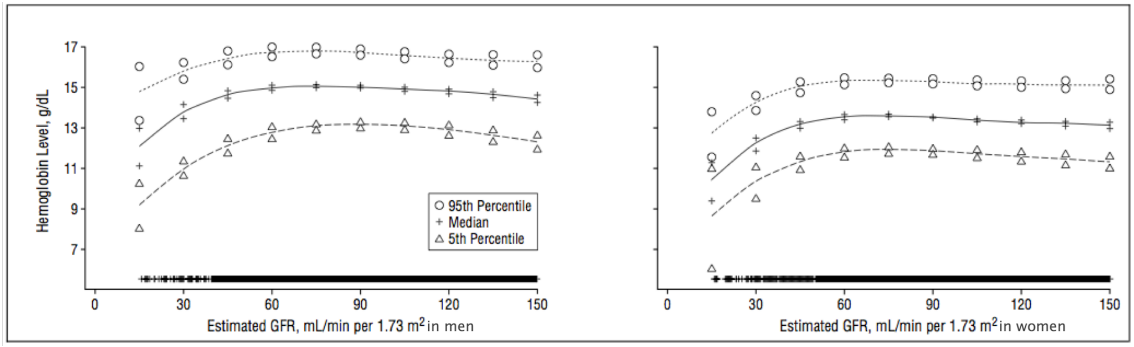

Figure 1.5 NHANES III epidemiological data – eGFR and haemoglobin levels
Median, 5th and 95th percentiles of haemoglobin levels in men and women aged 20 and over, at different estimated glomerular filtration rates (reproduced from (Astor et al. 2002))

The 2016 UKRR report showed that the median Hb level in incident HD patients from the 2013 cohort was 97 g/L (IQR 87 – 106 g/L), and 108 g/L (IQR 100 – 117 g/L) in those starting on PD. The Hb levels would have been even lower, had it not been that approximately 60-80% of these patients were receiving an erythropoiesis-stimulating agent (ESA) by the time they started dialysis. Initiation of ESA therapy in individual patients requires regular monitoring in order to guide Hb levels into the current target range of 100-120 g/L. Unsurprisingly, patients who saw a nephrologist only within 90 days of starting dialysis ("late-presenters") generally had lower Hb levels than those who had a smoother transition from CKD to RRT (Gilg, Rao, and Williams 2016).

1.2.4 Pathogenesis

Under normal circumstances, the anaemic state should produce a compensatory increase in circulating blood levels of EPO and therefore increase red cell production in the bone marrow, with a consequent increase in Hb concentration. This physiological adaptation occurs in healthy individuals, independent of age (de Klerk et al. 1981; Mori et al. 1988). Serum EPO levels in patients with CKD are actually comparable to the levels seen in non-anaemic healthy individuals, but the inability of the kidney to boost EPO production in response to a decreased Hb concentration is the main reason that renal anaemia develops in advanced CKD (Figure 1.6). Renal anaemia as a result of kidney disease is not absolute; patients with benign or malignant cystic kidney disease, or renal artery stenosis, may not suffer the same degree of anaemia, and might even see an increase in Hb concentrations (Chandra et al. 1985).

The possibility that renal anaemia is actually a protective mechanism in end-stage renal failure is lent some credence with the observation that patients with kidney disease possess the ability to boost EPO production under conditions of hypoxic stress. Blumberg and colleagues examined the effect of altitude on 6 HD patients and showed that plasma EPO levels were significantly increased, although not at the same level as in healthy controls (Blumberg, Keller, and Marti 1973). Chandra and colleagues also showed significant upregulation of EPO levels in 6 HD patients suffering episodes of hypoxia (pulmonary oedema, congestive cardiac failure and hypotension as a result of sepsis), from baseline EPO levels of approximately 15 i.u./mL, to levels between 50 – 600 i.u./mL (Chandra, Clemons, and McVicar 1988).

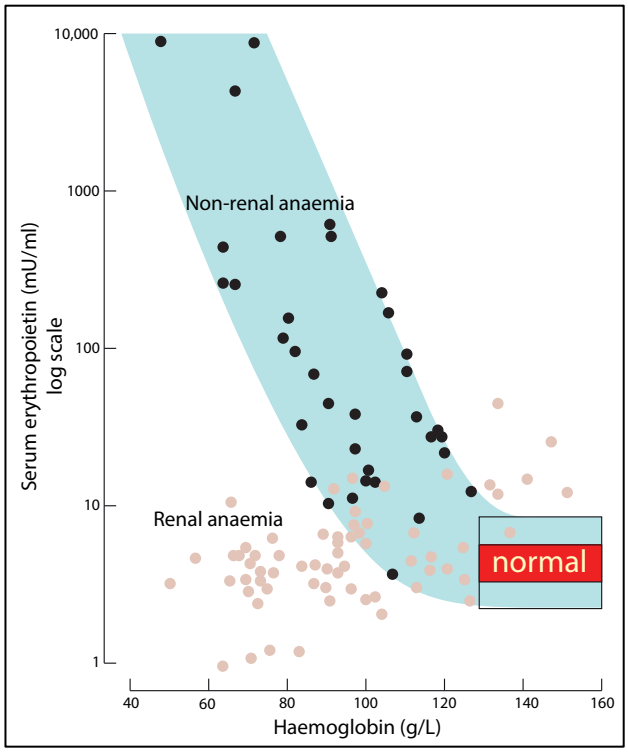

Figure 1.6 Relationship between serum erythropoietin levels and haemoglobin
The normal ranges of erythropoietin (EPO) and haemoglobin (Hb) levels for non-anaemic healthy individuals are indicated on the graph as 'normal'. As non-renal anaemia develops, there is an exponential increase in serum EPO levels, whereas in renal anaemia, the serum EPO levels remain relatively unchanged (reproduced from (Macdougall 2011))

Bosman and colleagues undertook a similar study comparing EPO levels in two groups of anaemic patients (early diabetic nephropathy versus non-dialysis stage 5 CKD) whilst exposing them to 6 hours of hypoxia (inspired oxygen concentration 11.6-12.6%). EPO levels were increased from baseline in both groups, although the diabetic

patients had a greater EPO response, comparable to the rise in EPO levels seen in hypoxic healthy controls (Bosman et al. 2002).

Following the discovery of the hypoxia-inducible factors (HIF) by Semenza and colleagues, it is now accepted that hypoxia is the most important element in the regulation of the HIF transcriptional cascade and consequently of the EPO gene. The discovery of this group of transcription factors further advanced the science of EPO from the foundations laid by Miyake, Lin, Jacobs and their colleagues (see page 19), using the availability of the then newly-cloned EPO gene, and hepatoma-derived cells that produced EPO in an oxygen-sensitive manner under laboratory conditions (Semenza and Wang 1992). We now know that there are three HIF-α subunits; HIF-1α and HIF-2α appear to be the principal elements in regulating cellular hypoxic responses. In the presence of oxygen, prolyl hydroxylation of HIF-α in the cytoplasm eventually results in its degradation by the von Hippel-Lindau (VHL)-E3 ubiquitin ligase complex, thereby halting its effect. The degradation of HIF-α does not occur in the hypoxic state, however, and the transcription factor subsequently translocates to the nucleus and binds to specific regulatory elements within the EPO gene (Figure 1.7).

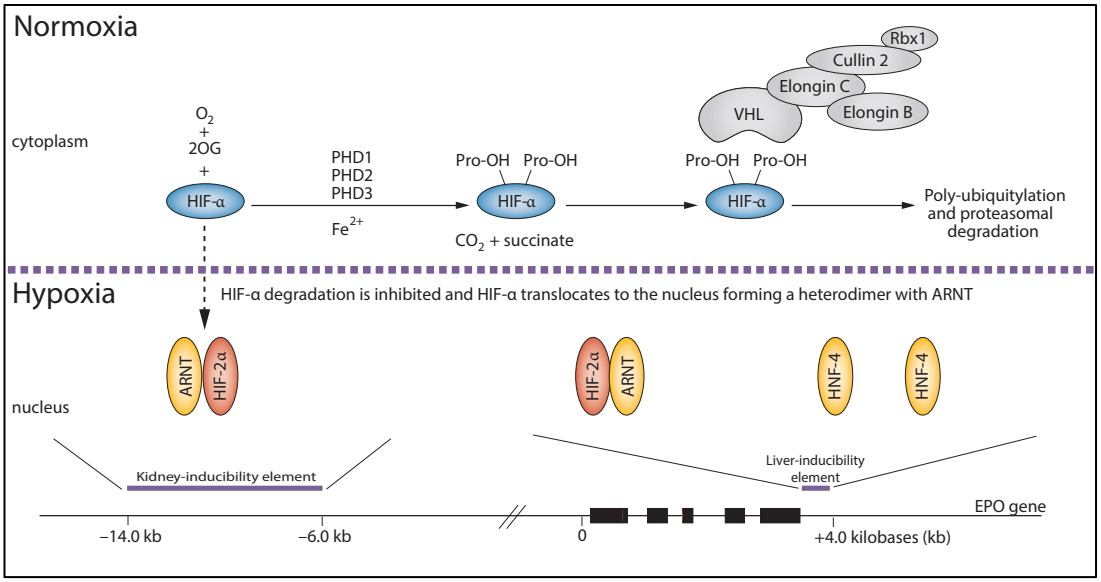

Figure 1.7 Regulation of HIF in normoxia and hypoxia
Abbreviations: 2OG, 2-oxoglutarate; PHD, prolyl-4-hydroxylase domain; ARNT, aryl hydrocarbon receptor nuclear translocator; Pro-OH, proline residue hydroxylation; VHL, von Hippel-Lindau; HNF-4, hepatocyte nuclear factor 4 (reproduced from (Koury and Haase 2015))

In normoxia, HIF-α is degraded and therefore unable to upregulate EPO gene expression. This process is dependent on the presence of 2-oxoglutarate and the prolyl

hydroxylase enzymes. However, this feature of EPO gene regulatory function is not sufficient to explain the low-level physiological expression of the EPO promoter that is also witnessed in normoxic conditions. Tsuchiya and colleagues demonstrated that in this scenario, GATA-2 functioned as a concomitant negative regulator of the EPO gene in order to prevent erythrocytosis, and act as a protective mechanism (Tsuchiya et al. 1997).

More recently, processes have been described that occur independent of HIF (and precede the effects of hypoxia on subsequent transcriptional events) by inducing histone hypermethylation of key enzymes (methyl transferases), and these processes require neither a change in enzymes that modify histones or increases in the physiological inhibitors of dioxygenases (Batie et al. 2019; Chakraborty et al. 2019).

In much the same way that serum EPO levels are inappropriately low for the level of anaemia seen in CKD, the same phenomenon is also witnessed in patients with chronic inflammatory conditions such as in persistently active inflammatory bowel disease or rheumatoid arthritis. The pro-inflammatory cytokines, IL-1 and TNF-α have been implicated in the pathogenesis of the anaemia of chronic disease, as both directly downregulate EPO gene expression under experimental conditions (Jelkmann 1998). A blunted response to the action of rHuEpo is also seen in inflamed patients; *ex vivo* data appears to show IFN-γ, IL-10 and IL-13 as the responsible cytokines (Cooper et al. 2003). Other regulators of EPO include angiotensin II, which increases EPO levels via the action of the angiotensin II subtype I receptor (Gossmann et al. 2001). It remains unknown whether this is a direct effect or not.

There are other factors that contribute to the anaemia of CKD, and these include inflammation and iron deficiency, and their respective interactions with hepcidin, disorders of MBD (which can also be tangentially linked with iron metabolism, and therefore hepcidin), decreased RBC survival in CKD, and the so-called uraemic inhibitors of erythropoiesis.

The normal life-span of RBCs is in the region of 120 days, but increased osmotic fragility markedly reduces this to 60-90 days in advanced renal disease. Even with the advent of treatments for anaemia in well-dialysed patients, the RBC life-span remains shortened (Ly, Marticorena, and Donnelly 2004). The exact mechanism is yet to be

elucidated, although we know that blood transfusions from uraemic patients into the bloodstream of healthy individuals lead to the normalisation of RBC survival. Conversely, when blood was transfused from normal donors into the uraemic milieu, RBC survival is again shortened (Joske, McAlister, and Prankerd 1956; Loge, Lange, and Moore 1958). It therefore seems likely that factors within the uraemic environment are responsible for reduced RBC survival.

There has been great interest in the accumulation (and removal) of small and middle molecules (delineated by a cut-off around a molecular weight of 500 Da), and protein-bound compounds in renal failure, and the possible effects of these uraemic toxins in exacerbating renal anaemia. It is thought that some of these factors create an environment such that the erythroid precursors become relatively insensitive to the effects of EPO. The polyamines, parathyroid hormone and cytokines have all been postulated as candidate toxins (Macdougall 2001). The European Uremic Toxin Work Group has now created a database of more than 140 uraemic toxins, but no outstanding contender has emerged (European Work Group on Uremic toxins (EUTox) 2016).

There are a number of other factors that play a role in the pathogenesis of renal anaemia such as functional iron deficiency related to high hepcidin levels, and the effects of inflammation/cytokines and hyperparathyroidism that will be covered in 1.4 - Hepcidin And Its Uses In CKD.

1.2.5 Clinical Features

The presence of anaemia in CKD is associated with a number of co-morbidities. There are a host of subjective clinical features associated with chronic anaemia, including lassitude, dyspnoea, angina, palpitations, reduced attention span, poor memory, reduced libido, and depression. Objectively, anaemia is associated with reduced maximum oxygen consumption and anaerobic threshold (Mayer, Thum, and Graf 1989). Severe anaemia can affect the cardiovascular system, and is associated with high-output cardiac failure subsequent to an increase in stroke volume and heart rate. Inflammation is likely to be the unifying precursor that explains the complex interplay between heart failure, CKD, and anaemia (O'Meara et al. 2014). Anaemia is also an independent risk factor for the development of left ventricular hypertrophy (LVH). Levin and colleagues found that even a small decrement in Hb concentration of just 5 g/L in

the context of renal disease conferred a 32% increase in risk of developing LVH (Levin et al. 1999). Despite the abundance of observational data detailing the existence of a link between anaemia and LVH, it is still uncertain whether anaemia causes an increase in cardiac mortality. In one example of a large observational trial in this field, Foley and colleagues followed 432 dialysis patients, and showed that the presence of anaemia in this patient cohort was independently associated with an 14% increased mortality risk for every 10 g/L decrement in Hb concentration (Foley et al. 1996). Whether or not causality is eventually proven, it is clear that anaemia in the context of kidney disease confers a poor prognosis.

1.2.6 Evaluation

The evaluation of renal anaemia relies on a diagnosis of CKD (see 1.1.1), and elimination of all other possible causes of anaemia. It is therefore a diagnosis of exclusion. In CKD, the mean cell volume (MCV) is typically within the normal range, and therefore, MCV values that are outside of the normal range should increase the suspicion for other contributory causes. Microcytosis is suggestive of iron deficiency or a haemoglobinopathy such as alpha thalassaemia, whereas macrocytosis is suggestive of vitamin B12 or folate deficiency, or hypothyroidism. Several drugs such as azathioprine or mycophenolate may also induce a macrocytosis.

Much of the literature concerned with the evaluation of anaemia in CKD and dialysis has focussed on the accurate assessment of iron stores using serum ferritin and/or transferrin saturation (TSAT), which are the favoured biomarkers in the most recent KDIGO anaemia guidelines (Kidney Disease: Improving Global Outcomes (KDIGO) CKD Work Group 2012). Both ferritin and TSAT, however, are heavily influenced by the inflammatory state, which is highly prevalent in the end-stage renal failure population (see 1.1.5.). Ferritin, an iron storage protein, is a positive acute phase reactant, whereas transferrin, the main iron transport protein, is a negative acute phase reactant (Gabay and Kushner 1999). Aside from the influence of inflammation, both of these biomarkers are subject to external factors that may affect their levels, including gender difference, but not circadian variation (Ridefelt et al. 2010). Despite their widespread use, ferritin and TSAT do not perform particularly well in detecting iron insufficiency in dialysis patients. TSAT performs better than ferritin, with sensitivities for iron insufficiency that range from 59% to 88% (Fishbane et al. 1996; Kalantar-Zadeh et al. 1995; Tessitore et al. 2001). Multiple studies have shown that the sensitivity of serum ferritin is less than 50%, irrespective of whether a cut-off value of 100 µg/L or

200 µg/L is used (Fishbane et al. 1996; Tessitore et al. 2001). The one clinical scenario where absolute iron insufficiency (reduction in total body iron stores) can definitively be diagnosed occurs when the serum ferritin level is <20 ng/mL (Macdougall 1994). In contrast to absolute iron deficiency, functional iron deficiency is a condition in which total body iron stores are normal (or increased), but the iron cannot be mobilised effectively for the purposes of erythropoiesis. This occurs either as a result of inflammation (this will be covered in greater detail in the chapter on hepcidin physiology (1.3)), or the use of ESA therapy in 'over-driving' the bone marrow so that the demand for iron outstrips the ability of circulating transferrin to provide an adequate amount of iron for erythropoiesis. In this situation, the TSAT will often be low (<20%), as iron will be rapidly shifted from transferrin for RBC production, but not replenished in a timely fashion from the iron stores. As a consequence, serum ferritin levels may be normal or high.

At the other end of the spectrum, the utility of serum ferritin as a marker of iron overload in dialysis patients is also uncertain. The significant threat of iron overload exists in the dialysis population, given the systematic use of intravenous iron in virtually all HD patients. According to the guidance issued by KDIGO, a serum ferritin level of 500 ng/mL is considered the threshold above which intravenous iron should not be administered to dialysis patients due to the risk of inadvertently causing systemic iron overload (Kidney Disease: Improving Global Outcomes (KDIGO) CKD Work Group 2012). Rostoker and colleagues illustrated this by using magnetic resonance imaging (MRI) to examine the liver iron content of 119 HD patients, and found that 100 from the 119 had excessive hepatic iron content. The mean serum ferritin level across the entire patient cohort was less than 500 ng/mL, and this was the case even in the group that was designated as having "severe" iron overload (Rostoker et al. 2012). However, it should be noted that the measurement of hepatic iron content by MRI to identify patients with iron overload remains a matter of debate (Sarigianni et al. 2015) since this technique cannot differentiate between iron accumulation in Kupffer cells (which are physiologically designed to store iron), and hepatocytes (where iron accumulation could be toxic).

Despite the reservations surrounding the use of serum ferritin and TSAT, both KDIGO and the European Renal Best Practice Working Group continue to endorse both laboratory measures as the biomarkers of choice for managing anaemia in CKD

(Locatelli et al. 2013). This is juxtaposed against the most recent guidance on the treatment of anaemia in CKD published by NICE in 2015, which recommended the use of percentage hypochromic red blood cells (%HRC) as the biomarker of choice in managing anaemia, referencing the results of an Italian study published 14 years earlier (Tessitore et al. 2001). This change in biomarker affiliation, as well as the other salient points from the NICE guideline are summarised as follows:

- An Hb concentration of ≤110 g/L is the trigger for investigating and treating anaemia when the eGFR <60 ml/min/1.73m^2
- The target Hb should be 100-120 g/L, but treatment should be individualised
- The tests for investigating anaemia in CKD:
  - Percentage of hypochromic red blood cells (%HRC) >6%
  - Reticulocyte Hb content (CHr) <29 pg, or equivalent tests
  - TSAT < 20% and serum ferritin <100 µg/L
- The assessment of iron status should not rely solely on TSAT or serum ferritin measurements alone, nor should erythropoietin levels be measured (The National Institute for Health and Care Excellence 2015b)

1.2.7 Treatment

The first step is to ensure that alternative causes for anaemia are excluded (Table 1.5). The mainstay of current treatment usually involves the use of iron replacement therapy in the first instance, followed by the use of ESAs once the patient is deemed iron-replete. Blood transfusions are now used to support the Hb only if the above measures prove unsuccessful and the patient remains anaemic, or in an emergency situation such as acute blood loss.

Blood loss	Decreased RBC production	Increased RBC destruction
Gastrointestinal	Thalassaemia	Congenital
Trauma	Haematinic deficiency	- haemoglobinopathy
Vaginal	Bone marrow infiltration	
	Aplasia	Acquired
	Endocrine deficiency	- drug-induced, autoimmune, mechanical
	Chronic disease	

Table 1.5 Causes of anaemia by mechanism

1.2.7.1 Red Cell Transfusions

Prior to the widespread use of recombinant human erythropoietin in the early 1990s, the only option available to treat anaemia in the dialysis patient was the regular transfusion of red cells (as well as the use of androgen therapy (Eschbach and Adamson 1985)). This led to a significant proportion of patients suffering with iron overload, and also exposed patients to the risks of blood-borne viruses as well as the increased likelihood of allosensitisation. There are also rare side effects such as transfusion-related acute lung injury (TRALI). Blood transfusions are also more costly than ESAs or iron therapy. One of the many impacts of rHuEpo was to significantly decrease the transfusion requirements of patients with end-stage renal failure (Popovsky and Ransil 1996).

1.2.7.2 Erythropoiesis-Stimulating Agents

Recombinant human erythropoietin revolutionised the treatment of anaemia in patients with CKD and has been widely available for over 25 years. The principal cause of renal anaemia is a relative lack of EPO, and therefore ESAs treat the underlying pathophysiology, and often reverse the clinical manifestations of the condition. Several publications have outlined the truly magnificent work that has taken us from Christison's observation connecting anaemia to kidney disease in 1839, through to Erslev and Jacobson's discovery in the 1950s that the kidney is the main repository of EPO production, concluding with the purification of erythropoietin (Miyake, Kung, and Goldwasser 1977) and cloning and expression of the erythropoietin gene (Lin et al. 1985) – a wonderful account of the essence of "bench-to-bedside" medicine (Jelkmann 1986; Fisher 2010).

Erythropoietin is glycoprotein containing a backbone of 165 amino acids (34 KDa) and the recombinant human form is available in a number of different preparations (Table 1.6). Modifications in the glycosylation pattern account for differences between the endogenous hormone and the various preparations; they are orally inactive and thus have to be administered parenterally (often intravenously in the haemodialysis population, and subcutaneously in non-haemodialysis patients), with the 1st generation ESAs (the epoetins) having a half-life in the region of 6-8 hours, which is longer if given subcutaneously. The 1st generation ESAs are administered 2-3 times per week, in contrast to the 2nd generation ESA (darbepoetin alfa), which has a longer half-life and is given weekly/fortnightly. The 3rd generation ESA (CERA) has an even longer half-life

(approximately 130 hours) due to the integration of a large pegylated side-chain, and this ESA can be administered fortnightly/monthly. Following expiration of the patents of the first-generation ESAs, a number of biosimilar epoetins have been developed, which are often less costly than the reference products. These biosimilars have an almost identical structure to the originator epoetin and show pharmacological bioequivalence, being tightly regulated by the medicines regulatory agencies in Europe, the US, and elsewhere. The theoretical risks of increased immunogenicity highlighted a few years ago (Covic et al. 2008) do not appear to have been borne out in reality.

The EPO mimetic peginesatide, on the other hand, has no structural similarity with epoetin, but has comparable pharmacological activity, and this was demonstrated in two parallel trials that confirmed its non-inferiority versus epoetin in non-dialysis CKD patients, and in haemodialysis patients, respectively (Macdougall et al. 2013; Fishbane et al. 2013). Since there is no cross-reactivity between this drug and anti-erythropoietin antibodies, peginesatide has also successfully been used as a rescue therapy in some patients who developed antibody-mediated pure-red cell aplasia (Macdougall et al. 2009). However, the drug was withdrawn from the market in February 2013 following reports of fatal hypersensitivity reactions in some recipients.

Generic erythropoiesis-stimulating agent	**Trade names**
epoetin alfa	Epogen®, Eprex®
epoetin beta	NeoRecormon®
epoetin zeta	Retacrit®, Silapo®
epoetin alfa (biosimilar)	Binocrit®, Abseamed®, Hexal®
epoetin theta	Eporatio®
darbepoetin alfa	Aranesp®
methoxypolyethylene glycol epoetin beta (CERA)	Mircera®
peginesatide	Omontys® (Hematide®)

Table 1.6 Preparations of erythropoiesis-stimulating agents
CERA; continuous erythropoietin receptor activator

In 1998, Besarab and colleagues published the first major randomised controlled trial evaluating whether correction of the haematocrit into the normal range (42%), with the use of an ESA (Epogen®) in haemodialysis patients, could reduce mortality and the risk of heart attacks in this patient population.

All-cause death was significantly higher in the normal haematocrit group (Figure 1.8), but the topic of optimal haematocrit (later to become Hb) targets in CKD and dialysis patients remained a matter of considerable debate for nearly a decade until the publication of the TREAT trial. Pfeffer and colleagues then showed similar negative findings to those of Besarab et al, with full correction of renal anaemia increasing the risk of cardiovascular morbidity compared to partial correction (Pfeffer et al. 2009). More specifically, the TREAT study showed an increased risk of stroke and venous thromboembolism, as well as an increased risk of cancer-related mortality (in those with a prior history of cancer), reinforcing the case against full correction of renal anaemia.

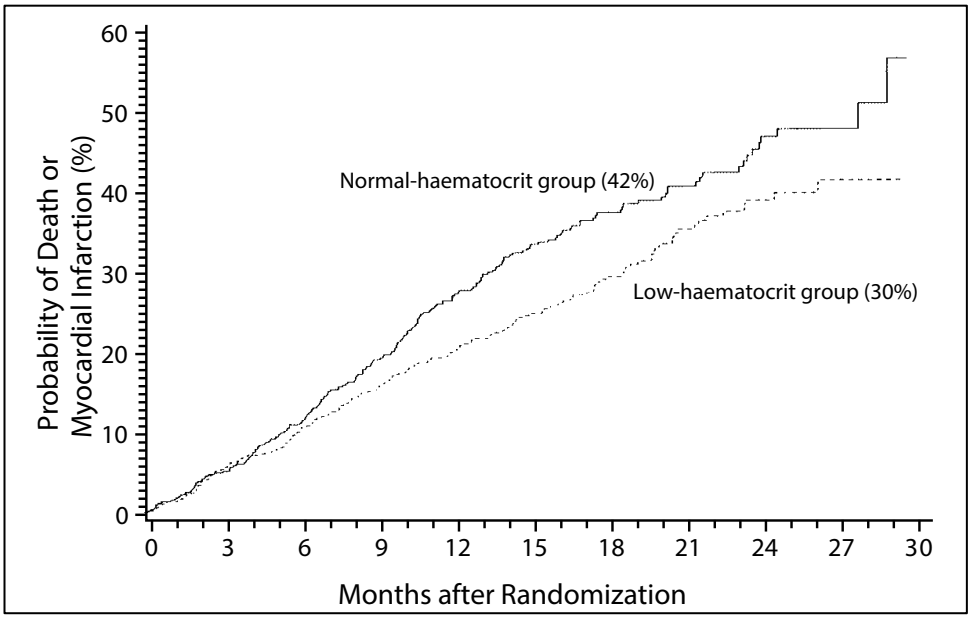

Figure 1.8 Death and cardiac morbidity in the Normal Hematocrit Trial
Probability of death or non-fatal myocardial infarction was higher in the normal haematocrit group (reproduced from (Besarab et al. 1998))

ESAs are usually very well tolerated with the main side effect being related to hypertension. A rare and serious complication is PRCA, which arises as a consequence of anti-erythropoietin antibodies that are formed following treatment with exogenous EPO. The white cell and platelet cell lines are unaffected, differentiating this condition from aplastic anaemia. Immunosuppression is the mainstay of treatment for PRCA, although peginesatide can be used in lieu of the offending ESA to rescue these patients from their severe transfusion-dependent anaemia.

ESAs are not universally successful in raising the Hb concentration in the desired manner, as some patients are resistant to their effects. The current UK NICE guidance defines ESA-resistance as being unable to achieve an Hb concentration in the aspirational range despite treatment with ≥300 i.u./kg/week epoetin (subcutaneously), ≥450 i.u./kg/week epoetin (intravenously) or ≥1.5 μg/kg/week darbepoetin (The National Institute for Health and Care Excellence 2015b). ESA-resistance can occur as a result of iron deficiency, inflammation, severe hyperparathyroidism, folate deficiency, or PRCA.

1.2.7.3 Iron Therapies

Iron deficiency is common in patients with CKD for a variety of reasons including poor dietary intake, increased gastrointestinal losses, regular phlebotomy, and decreased absorption as a result of hepcidin upregulation (Table 1.7). Iron deficiency in the haemodialysis population was recognised over 50 years ago and 2g/year of iron replacement was the suggested regimen at that time (Edwards, Pegrum, and Curtis 1970). Although we now know that gastrointestinal iron absorption is impaired in CKD, studies undertaken in the 1970s concluded that iron absorption was in fact normal, and that oral iron supplementation was likely to be as effective as intravenous preparations (Eschbach, Cook, and Finch 1970; Baker et al. 1975; Milman and Larsen 1976). The use of ESA and iron therapy is synergistic, as iron deficiency can be caused by the use of ESAs, and ESA resistance is caused by iron deficiency. Therefore the use of iron will reduce the amount of ESA therapy required to correct anaemia in CKD (Fishbane, Frei, and Maesaka 1995) and intravenous iron is superior in this regard to oral iron in both the dialysis (Macdougall et al. 1996) and CKD populations (Macdougall et al. 2014).

Both routes of administration are associated with significant side effects; the oral preparations lead to gastrointestinal disturbance in approximately 30-40% of patients due to the Fenton reaction, but are generally considered to be safe, whereas the intravenous preparations can occasionally cause hypersensitivity reactions (Bailie et al. 2005). A large retrospective analysis of a Medicare database containing 688,183 non-dialysis patients between 2003 and 2013 was analysed to examine the risk of anaphylaxis in incident users of iron dextran, gluconate, sucrose and ferumoxytol. Wang and colleagues concluded that the risk of anaphylaxis was highest for those initially exposed to high molecular weight iron dextran, whilst it was lowest for iron sucrose (Wang et al. 2015). The authors included the administration of

diphenhydramine as one of the ways to define anaphylaxis, and this flawed analysis led to the probable overestimate of fatal events. After the supplementary data were re-analysed, it was found that deaths were actually higher in those receiving non-dextran irons (DeLoughery and Auerbach 2016; Kalra and Bhandari 2016).

Systemic reactions to intravenous iron occur, as unbound circulating iron is highly volatile and potentially toxic due to its tendency to induce reactive oxygen species. In order to circumvent this problem, all iron preparations consist of a carbohydrate shell that shields elemental iron from its environs, allowing its slow release and subsequent uptake by either transferrin or ferritin, hence limiting exposure and reducing the risk of hypersensitivity reactions.

Route of administration	Iron preparation
Oral	Ferrous sulphate Ferrous fumarate Ferrous gluconate
Intravenous	Iron dextran Iron sucrose Ferric gluconate Ferumoxytol Ferric carboxymaltose Iron isomaltoside

Table 1.7 Preparations of iron replacement therapies

Iron is a critical element for both host and pathogen and as a result, iron therapy has traditionally been withheld from renal patients suffering from an acute bacterial infection. Although there are few clinical data to support this stance at the present time (Avni et al. 2015), there is a surfeit of pre-clinical data demonstrating that excess iron may cause microbial proliferation and simultaneously depress T-cell and B-cell function (Marx 2002).

1.2.7.4 Novel Treatments

The mainstay of renal anaemia management over the past 25 years has been ESA therapy and iron supplementation, consequently giving us the significant benefit of long-term efficacy data. As previously mentioned, both these classes of therapeutic agents have question marks against their long-term safety. In the event of resistance to

ESAs and/or iron therapies, there are few treatments in the armoury left, and clinicians have no recourse other than to utilise red cell transfusions in order to treat ESA-resistant anaemia. The scientific advances in the molecular biology underpinning the regulation of EPO and iron metabolism over the last decade have led to the emergence of a number of potential therapeutic targets and investigational drugs, with the HIF-stabilisers furthest along in their development.

1.2.7.5 Strategies That Indirectly Stimulate Erythropoiesis

The HIF-stabilisers inhibit prolyl hydroxylase (and compete with 2-oxoglutarate), with their primary function being to prevent the degradation of HIF-α, in effect mimicking the hypoxia-driven expression of endogenous erythropoietin in the kidney. The main advantage of the HIF-stabilisers, in contrast to the ESAs, is that they are orally active, making them a much more attractive proposition. The drawback of these therapeutic agents is that HIF transcription factors are not limited to the pathway mentioned above (Figure 1.7), but are also involved in the regulation of a number of other biological processes. Manipulation of HIF thus possesses the clear potential for a number of off-target effects, the most worrying of which is the promotion of angiogenesis, and potentially tumour growth (Ratcliffe 2013). There are now at least 6 prolyl hydroxylase inhibitors under clinical development, some of which are in phase 3 trials (Malyszko and Malyszko 2016).

The activin traps, like HIF-stabilisers, have the objective of treating renal anaemia by indirectly targeting the EPO receptor. Activins belong to the transforming growth factor β (TGFβ) superfamily, and regulate erythropoiesis by their action on erythroid progenitor, precursor, and/or the bone marrow accessory cells (Maguer-Satta et al. 2003; Jelkmann 2015). Enhanced erythropoiesis as a result of the sequestration of activin was a surprise finding in a phase 1 trial investigating the effect of ACE-011 on bone biomarkers in post-menopausal women (Ruckle et al. 2009), paving the way for the current trials exploring their use in renal anaemia. However, their exact mechanism of action is yet to be determined, given that these molecules have been shown to both stimulate erythropoiesis and inhibit erythroid differentiation (Bonomini et al. 2016).

1.2.7.6 Strategies That Directly Stimulate Erythropoiesis

Novel second generation ESAs, using the addition of polysialic acid (PSA) instead of polyethylene glycol (PEG) polymer chains to EPO, considerably extend the half-life of

EPO and are currently in phase 2-3 trials. The PSA-EPO construct is an attractive biological alternative, given that the PSA moiety is completely biodegradable (Smirnov et al. 2015). EPO fusion molecules, which are created by the synthesis of EPO mimetic peptides onto human IgG-based constructs, are currently under evaluation in a phase 2 trial (Bonomini et al. 2016). It is unclear whether these two strategies provide pharmacological advantages over the more established ESAs.

Synthetic EPO mimetic peptides, which have no structural homology with the endogenous hormone, have the ability to directly activate the EPO receptor. As previously mentioned, peginesatide has now been withdrawn, but there are newer strategies under phase 1 development, such as pegolsihematide (Yu 2016).
Other strategies that have pre-clinical data but are not yet in clinical development include antibodies that are agonists to the EPO receptor, and inducers of dimerisation of the EPO receptor intracellular domain (Bonomini et al. 2016).

1.2.7.7 Novel Iron Strategies

Soluble ferric pyrophosphate is a compound that was first studied in 1999 in comparison with intravenous iron dextran (Gupta et al. 1999), and is currently the subject of a number of phase 2 and 3 trials. Oral ferric citrate hydrate, originally studied as an iron-containing phosphate binder, presented investigators with an unintended, but positive outcome in terms of enhanced iron supplementation, thereby reducing the need for intravenous iron (Umanath et al. 2015). Despite the theoretical risks of aluminium toxicity (Gupta 2014), further studies are required to test its efficacy and safety profile. Other iron products under recent investigation include ferric maltol, which is not affected by gastric pH, and therefore has superior bioavailability compared to ferrous iron, and possibly a better side-effect profile (Kelsey et al. 1991; Reffitt et al. 2000; Barrand et al. 1991; Stallmach and Buning 2015). Strategies that target hepcidin or its associated regulatory pathways will be discussed in detail later in this chapter in section 1.4.3.

1.3 Hepcidin Physiology

1.3.1 Background

Iron is a critical trace element that is required by all living organisms but is nevertheless toxic in its non-bound form and thus, its uptake, transport, storage, and export are very tightly regulated. It was only in the year 2000, when hepcidin was isolated from human sera, that the elusive iron regulatory peptide was finally discovered (Krause et al. 2000). There have been some fortunate occurrences along the way that have led us to our current model of iron regulation; Nicolas and colleagues were attempting to investigate glucose dependent gene regulation in the liver, and during the course of their work, they created a $Usf^{-/-}$ murine knockout model. They subsequently noted that the knockout mice displayed features consistent with severe iron overload, and it transpired that the *Usf2* gene was contiguous with the gene responsible for hepcidin production (*HAMP*) (Nicolas et al. 2001). Now called hepcidin, the peptide was initially known as liver expressed antimicrobial peptide-1 (LEAP-1), and was considered an anti-microbial (Park et al. 2001). However, there now appears to be an overall consensus that hepcidin-25 has a much more important role and is considered the master regulator of iron homeostasis.

1.3.2 Iron Homeostasis

Iron is the essential ingredient of haem, facilitating the transport of oxygen in the blood to all distal cellular components in biological systems. In healthy adult humans, total body iron amounts to approximately 55mg/kg body weight, which is equivalent to 4g in the typical adult male (Andrews 1999). The daily requirement of iron in healthy individuals is in the region of 25mg/day, of which 20mg is reserved for erythropoiesis. The circulating plasma pool of iron consists of approximately 3mg, and the remainder is recovered from senescent red blood cells. Iron excretion occurs in a completely unregulated fashion via the gastrointestinal (GI) tract. GI losses of iron in healthy individuals are in the order of 1mg/day, but their counterparts on haemodialysis can lose up to 5 times that amount, providing one explanation for why dialysis patients are comparatively deficient in total body iron (Zumbrennen-Bullough and Babitt 2014). Since there is no excretory mechanism for iron, the rate at which iron absorption occurs is tightly regulated. Dietary iron is absorbed in the duodenum, and can either be bound to haem, or not, in which case it is known as non-haem bound iron (NHB). The acidic milieu of the stomach allows NHB iron to be dissociated from food; NHB iron is maintained in the ferric (Fe^{3+}) state, and is reduced to ferrous iron (Fe^{2+}) by

ferrireductases, such as duodenal cytochrome B (DCYTB), which are located on the apical brush border of the duodenal enterocytes (Figure 1.9). Ferric reduction in man occurs with the assistance of ascorbic acid, which is only available via the diet (McKie et al. 2001).

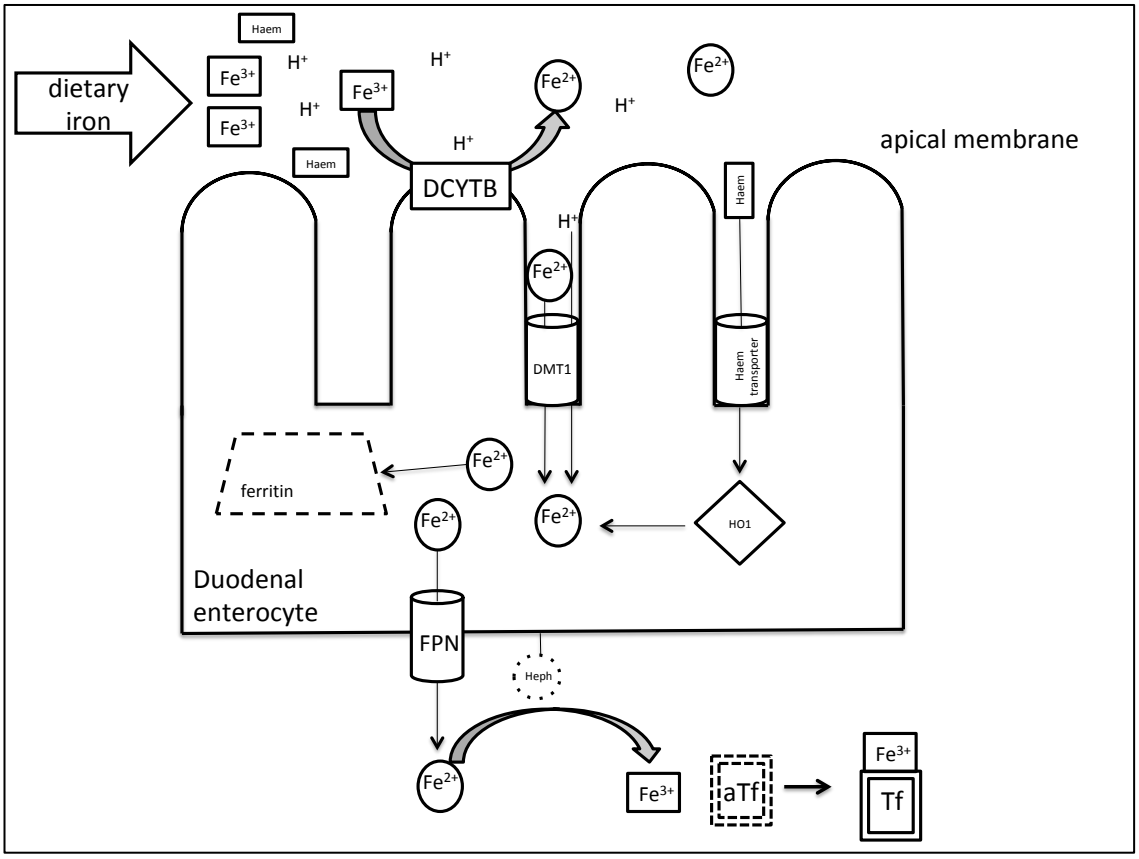

Figure 1.9 Schematic representation of dietary iron absorption via duodenal enterocyte
(DCYTB – duodenal cytochrome B; DMT1 – divalent metal transporter 1; HO1 – haem oxygenase 1; FPN – ferroportin; Heph – hephaestin; aTf – apotransferrin; Tf – transferrin)

The symporter, divalent metal transporter 1 (DMT1), which is located on the apical surface of the enterocyte, transports ferrous iron in association with ionised hydrogen, and the iron is then retained within the cellular storage system (ferritin) or allowed to exit the cell on the basolateral surface into the portal circulation via the membrane-bound protein, ferroportin (McKie et al. 2000). Haem-bound iron is transported across the apical membrane via a haem transporter, known as haem carrier protein 1, which is now known to also transport folate (Nakai et al. 2007). Once in the intracellular space, haem-bound iron subsequently interacts with haem oxygenase 1, which releases ferrous iron for cellular storage or export (Poss and Tonegawa 1997). Once ferrous

iron is in the plasma pool, it is oxidised back to its original ferric form via the membrane-bound ferroxidases, hephaestin and ceruloplasmin, and is then loaded onto apotransferrin, which is the iron-free form of transferrin, for distribution to distal cells including the bone marrow (for erythropoiesis) (Roeser et al. 1970; Vulpe et al. 1999).

Cellular uptake of iron is dependent upon the presence of the transferrin receptors (TfR), which are expressed at the cell surface allowing the entry of the iron-transferrin complex via endocytosis. TfR1 is ubiquitously expressed, with the exception of mature erythrocytes, whereas TfR2 is confined to the liver. During endocytosis, the intravesicular acidic environment allows the decoupling of ferric iron from transferrin, and whilst iron is then utilised for intracellular functions (or simply stored in the liver), the transferrin receptor is then recycled into the plasma (as soluble transferrin receptor), to return to the enterocytes and continue its endless cycle of iron transportation (Hentze et al. 2010). The average 70kg man has approximately 5-6L of blood; given that each litre of blood contains approximately 150g of haemoglobin, and that each gram of haemoglobin contains approximately 2.5mg of iron, it can be estimated that mature erythrocytes contain nearly 70% of total body iron stores. However, this iron is not wasted; there is a highly efficient system designed to recover this iron, which is enabled by macrophages that phagocytose and digest old erythrocytes, known as erythrophagocyosis. Haem oxygenase 1 degrades haemoglobin thereby releasing iron, which is then exported out of the phagosome by DMT1 and subsequently stored in ferritin, or exported from the cell altogether (Ganz 2007).

1.3.3 The Ferroportin-Hepcidin Axis

The only iron cellular export protein currently identified is ferroportin, and the concentration of functional membrane-associated ferroportin is controlled by its ligand, hepcidin. Three groups independently discovered ferroportin (also known in the scientific literature as SLC40A1, MTP1 and IREG1) and its role in iron export. Interestingly, ferroportin appears to be unrelated to any other mammalian transporter proteins and is well conserved with orthologs in plants and worms (Abboud and Haile 2000; Donovan et al. 2000; McKie et al. 2000). Ferroportin is highly expressed by hepatocytes, duodenal enterocytes, and macrophages, which are all associated with the trafficking of iron. Donovan and colleagues inactivated the FPN gene in mice, both globally and selectively, and found that $FPN^{-/-}$ mice did not survive the embryonic

stage. The selective deletion of intestinal FPN in adult mice resulted in the mice developing anaemia, showing that FPN was essential for the intestinal absorption of iron. However, the parenteral administration of iron was able to override this process and abrogate the anaemia (Donovan et al. 2005).

Cellular ferroportin expression is not static and is dependent on intracellular concentrations of iron. In iron deplete conditions, there is downregulation of ferroportin mRNA that is mediated by the 5′ iron response element (IRE) binding to iron-regulatory proteins (IRP) (Lymboussaki et al. 2003). In the circumstance of elevated levels of intracellular iron, the presence of haem leads to the increase in both haem oxygenase 1 and ferroportin expression (Delaby et al. 2008). Despite our understanding of ferroportin regulation, the molecular mechanism by which intracellular iron is delivered to ferroportin, or the manner in which ferroportin actually transports intracellular iron is less well understood (Drakesmith, Nemeth, and Ganz 2015).

As has been well documented, Nemeth and colleagues demonstrated that the external regulation of ferroportin was controlled by hepcidin, which, as its ligand, was able to bind to its receptor, resulting in the endocytosis and proteolysis of ferroportin. This interaction has the net result of restricting iron to the intracellular compartment of hepatocytes or macrophages (Nemeth, Tuttle, et al. 2004). The importance of the hepcidin-ferroportin axis can be highlighted by the clinical conditions that are characterised by hepcidin excess or deficiency. Iron-refractory iron deficiency anaemia (IRIDA) is an autosomal recessive disorder that results from mutations in the gene encoding for *TMPRSS6*, leading to elevated hepcidin levels. As the name of the disease implies, patients remain extremely anaemic despite attempts to correct it with iron therapy (Finberg et al. 2008). In contrast, mutations causing a profound reduction in hepcidin levels have been localised to the *HAMP* gene (although many other genes have also been implicated), and these can lead to conditions characterised by iron overload, such as juvenile haemochromatosis (Rideau et al. 2007).

1.3.4 Structure and Kinetics of Hepcidin

Bioactive hepcidin is a 25 amino acid polypeptide hormone that is principally synthesised by hepatocytes, but macrophages and other cell types (including adipocytes) are also able to generate small amounts. The *HAMP* gene is located on chromosome 19q13.1 and encodes an initial prepropeptide that consists of 84 amino

acids that then undergoes a series of enzymatic cleavages to produce prohepcidin (60-64 amino acids) (Figure 1.10). Prohepcidin undergoes further post-translational modifications, resulting in the establishment of the bioactive form (hepcidin-25) and other forms (hepcidin-20, hepcidin-22) whose role remains uncertain (Valore and Ganz 2008). Recent work from within our institution has shed light on the existence of another isoform, hepcidin-24 but again, its role remains unclear (Handley et al. 2017).

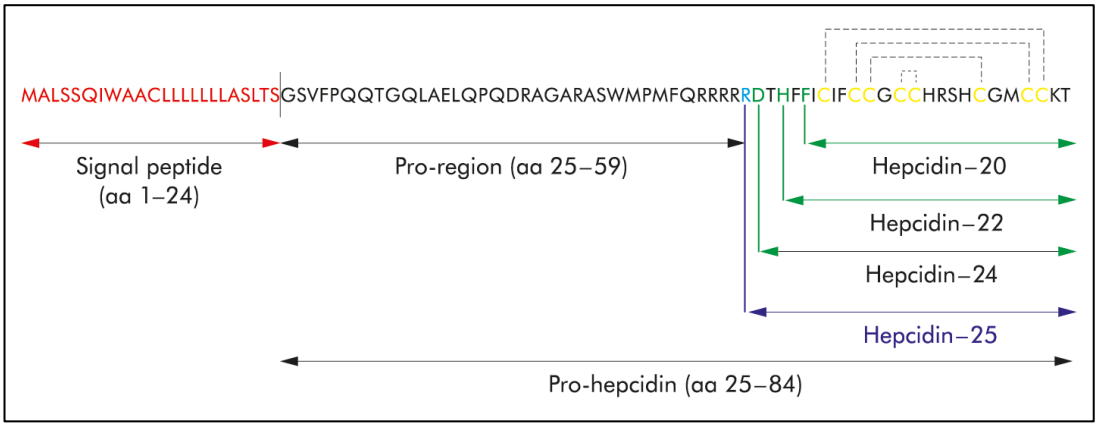

Figure 1.10 Amino acid sequence of prohepcidin, hepcidin-25, -24, -22, and -20
Cysteine residues indicated in yellow with 4 disulphide bonds (dashed lines). Hepcidin-25 indicated in blue. Other hepcidin isoforms are indicated in green. Adapted from (Kulaksiz et al. 2004)

Circulating hepcidin can be bound to carrier proteins such as α2-macroglobulin, and to a lesser degree, albumin, preventing its glomerular clearance under these conditions. When hepcidin attaches to its receptor (ferroportin), a proteolytic process occurs, and it is thought that this contributes to the clearance of hepcidin, with terminal excretion via the kidneys. However, the extent to which hepcidin is protein bound appears to be relatively small, and is estimated to be between 3 and 11% (Peslova et al. 2009; Itkonen et al. 2012). Given its size, unbound hepcidin is freely filtered at the glomerulus and mechanisms exist in the proximal tubular apparatus to facilitate its reabsorption. Thus, there is a relatively low fractional excretion of hepcidin, which has been estimated to be in the region of 5% (Peters et al. 2013; Kroot et al. 2011).

Multiple studies have been undertaken to determine the utility of urinary hepcidin as a biomarker in a variety of clinical settings (various haematological conditions and AKI following cardiac surgery) but as yet, there is little prospect of this particular biomarker being adopted in the day-to-day practice of clinicians. Probably the most promising study to date was conducted by Prowle and colleagues, who showed that urinary hepcidin was associated with the development of AKI in the first 5 days following

cardiopulmonary bypass surgery, although the source of the urinary hepcidin is not well understood (Prowle et al. 2012). A possible mechanism that might contribute to the AKI seen in relation to cardiac surgery may be explained by increased haemolysis and the subsequent increased intravascular consumption of nitric oxide (Vermeulen Windsant et al. 2014). Van Swelm and colleagues showed that hepcidin was broken down in the kidney and reabsorbed via megalin, and that administered hepcidin was protective against early haemoglobin-mediated injury in mice (van Swelm et al. 2016). Administered hepcidin has also been used in pre-clinical models of renal ischaemia reperfusion injury (IRI). IRI induces changes in renal iron homeostasis, and hepcidin-deficient mice sustain a more severe renal injury. Scindia and colleagues demonstrated that a murine intra-peritoneal injection of hepcidin 24 hours prior to IRI was protective against renal injury (lower plasma creatinine levels) and this was strongly corroborated by histological evidence, which showed far fewer tubular injuries (Scindia et al. 2015).

When it comes to the other truncated forms of hepcidin (hepcidin-20, -22, and -24), little is known about their protein binding, plasma levels or excretion. These isoforms do not appear to have any activity towards ferroportin and are therefore unlikely to play a significant role in iron metabolism (Laarakkers et al. 2013; Nemeth et al. 2006).

1.3.5 Regulation of Hepcidin 1: Iron and Inflammation

A wide range of external and internal stimuli regulates hepcidin expression in the liver. The two most potent upregulators of hepcidin are body iron status (Figure 1.11) and acute inflammation, whereas iron deficiency (anaemia), hypoxia and erythropoiesis tend to downregulate hepcidin.

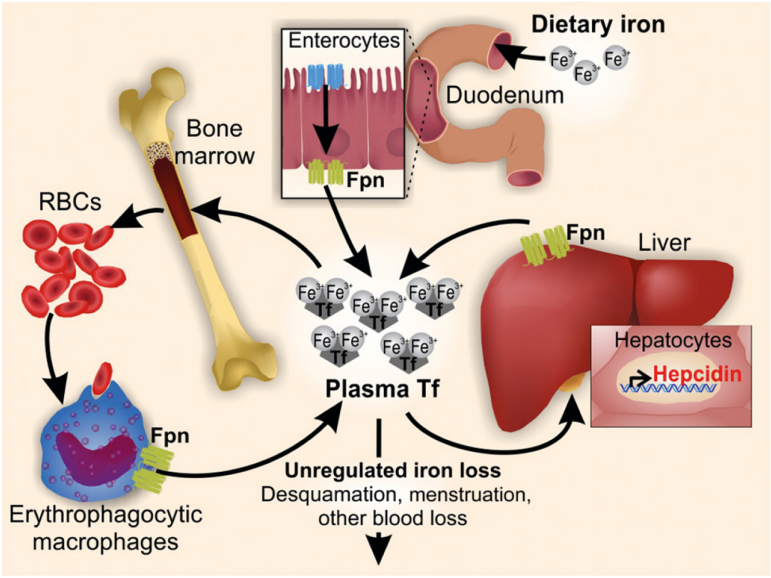

Figure 1.11 Schematic overview of the iron cycle and hepcidin regulation
The hormone hepcidin regulates plasma iron concentrations by controlling ferroportin concentrations on iron-exporting cells, including duodenal enterocytes, macrophages, and hepatocytes. Fpn – ferroportin; Tf – transferrin. Reproduced from (Sangkhae and Nemeth 2017)

As has been mentioned previously, hepcidin excretion occurs via the kidney, and there is a body of evidence supporting the relationship between chronic kidney disease and elevated hepcidin levels. Other factors, such as vitamin D, the sex steroids, and heparin, have also been shown to influence hepcidin. The following sections will be devoted to reviewing the various stimuli and signalling pathways that are involved in the regulation of hepcidin (Figure 1.12 Intracellular regulation of hepcidin).

1.3.5.1 Iron

Hepcidin, being the hormone responsible for iron homeostasis, is exquisitely responsive to iron loading, and increases the expression of hepcidin via the BMP-HJV-SMAD pathway in order to limit the amount of iron that is available to the circulation (Pigeon et al. 2001; Nicolas et al. 2002). In the iron-deficient state, hepcidin production is minimised in order to maximise the amount of iron available to the circulation, which is ultimately reserved for erythropoiesis (Hentze et al. 2010).

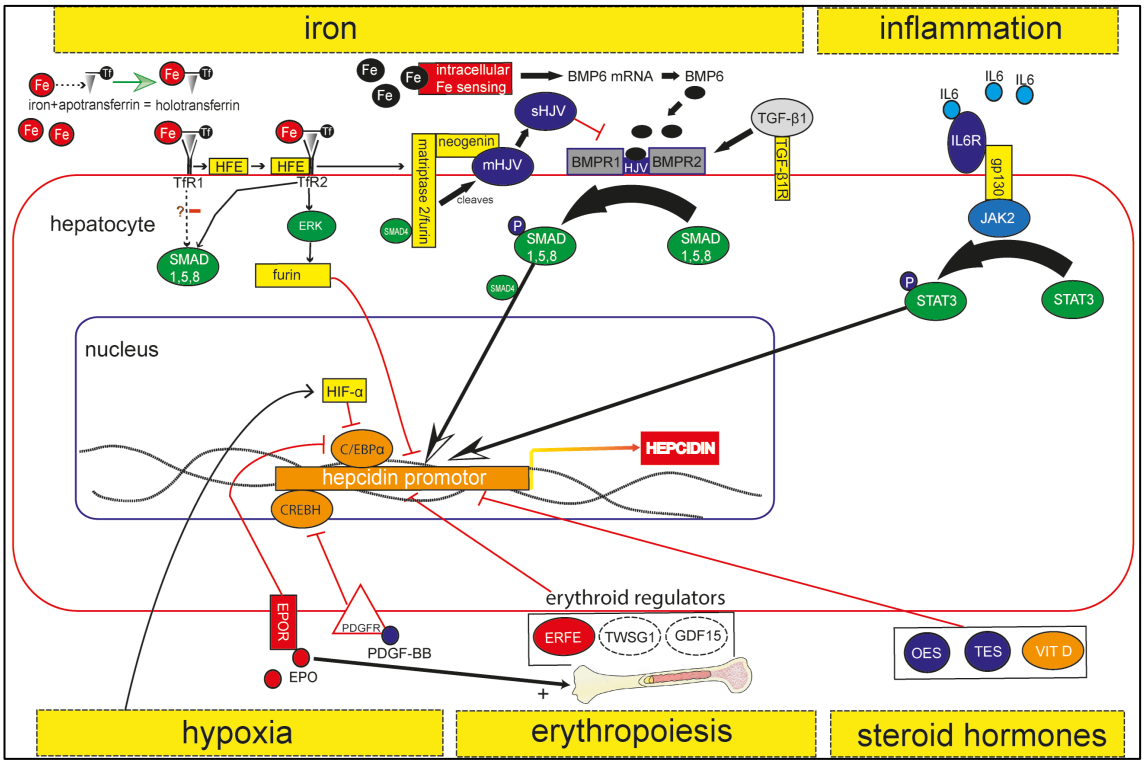

Figure 1.12 Intracellular regulation of hepcidin
The positive regulators of hepcidin are sited at the top of the figure (yellow boxes), whereas the negative regulators are situated in yellow boxes at the bottom of the figure. Abbreviations: BMP6 – bone morphogenetic protein 6; BMPR – bone morphogenetic protein receptor; C/EBPα – CCAAT enhancer binding protein α; CREB/H – cAMP response-element binding protein/H; EPO – erythropoietin; EPOR – erythropoietin receptor; ERFE – erythroferrone; ERK – extracellular signal–regulated kinases; GDF15 – growth differentiation factor 15; HFE – hemochromatosis protein; HIF – hypoxia-inducible factor; HJV – hemojuvelin (membrane and soluble); IL6 – interleukin 6; IL-6R – interleukin 6 receptor; JAK2 – Janus kinase; PDGF-BB – platelet-derived growth factor-BB; PDGFR – platelet-derived growth factor receptor; SMAD – sma and mothers against decapentaplegic homologue 1/5/8 complex; STAT3 – signal transducer and activator of transcription 3; TFR – transferrin receptor 1 or 2; TGFβ – transforming growth factor β; TGFβR – transforming growth factor β receptor; TWSG1 – twisted gastrulation BMP signalling modulator 1; OES – oestrogen; TES – testosterone; VIT D – vitamin D [diagram adapted from (Rishi, Wallace, and Subramaniam 2015; Ueda and Takasawa 2017)]

The bone morphogenetic proteins play a pivotal role in the pathways of iron-regulated hepcidin, which are part of the TGF-β superfamily of ligands. As the name would imply, they were originally found to have a role in the development of bone and cartilage, but we now know that their functions are far more widespread and they appear to be involved in an extensive array of biological functions ranging from embryological development to the formation of teeth. At the last count, there were approximately 20 BMPs related to human biological activity (Carreira et al. 2015). In the specific arena of iron regulation, a number of these BMPs have been found to possess the capacity to stimulate hepcidin transcription *in vitro,* including BMP-2, BMP-4 to BMP-7, and BMP-9 (Truksa et al. 2006; Babitt et al. 2007). Of the aforementioned bone morphogenetic

proteins, BMP-6 appears to be the most important ligand involved in the regulation of hepcidin (Andriopoulos et al. 2009).

There is ample evidence in the literature in both *in vivo* and *in vitro* studies to show that iron loading leads to an increase in BMP-6 mRNA and protein levels, and this ultimately leads to an upregulation in *HAMP* transcription and the production of hepcidin in the liver (the liver is comprised of parenchymal cells, or hepatocytes (70%), and non-parenchymal cells, such as Kuppfer cells (stellate macrophages), hepatic stellate cells (involved in liver fibrosis), and liver sinusoidal epithelial cells (act as the interface between blood cells and hepatocytes/hepatic stellate cells)). The mechanism by which iron leads to an increase in BMP-6 remains largely unknown, but it has been shown that both parenchymal and non-parenchymal hepatic cells are able to produce BMP-6, albeit under different conditions, and that both canonical and non-canonical pathways may be involved (Enns et al. 2013; Arndt et al. 2010; Kautz et al. 2009; Kautz et al. 2011).

The current understanding is that high levels of iron are sensed by hepatocytes, and this then stimulates the production of BMP-6. The BMP-6 ligand then binds to BMP receptors 1 and 2, and the co-receptor hemojuvelin (HJV) stimulates phosphorylation of the SMAD 1/5/8 intracellular signalling proteins, which subsequently activate the SMAD4 downstream signalling cascade. The phosphorylated SMAD1/5/8/4 complex then induces hepcidin transcription by binding to the hepcidin promotor on the target gene (Babitt et al. 2006; Wang et al. 2005).

Our understanding of the downstream mechanisms of BMP signalling is relatively well known, but the same cannot be said of the upstream mechanisms. It is still not entirely clear how iron leads to activation of the BMP-SMAD signalling system (Figure 1.13). There is a growing body of evidence suggesting that rather complex mechanisms exist in this area of iron regulation, and a number of means of 'iron-sensing' have been postulated, from the presence of intracellular or tissue iron sensors, detection of transferrin saturation, through to activation of transferrin receptor 2, and activation of hypoxia-inducible factor (HIF). It is highly likely that the sensing of iron is due to a number of factors and is not confined to a single governing mechanism (Ramos et al. 2011).

The transferrin receptors and HFE may play a key role here; it has been suggested that in response to a low transferrin saturation, TfR1, which is upregulated in iron deficiency, may lead to its association with the HFE protein, and it is this complex that ultimately leads to a net downregulation of hepcidin expression. The biology of the TfR1 system has been well characterised, but TfR2 biology remains less so.

Unlike TfR1, which is ubiquitous, TfR2 is restricted to the hepatocytes and some erythroid cells and is able to bind iron-loaded transferrin (holotransferrin). The HFE protein possesses the ability to shuttle between the two TfR homologues, and the activated TfR2 receptor associates with HFE, leading to activation of furin, with a net increase in BMP-SMAD signalling. Rishi and colleagues reviewed this subject matter and proposed that TfR2 initiated the BMP signalling cascade that resulted in the formation of BMP-6, but the BMP-6 protein could only bind with its receptor (BMPR type 1), which was allowed to localise to the cell surface with the aid of HFE (Rishi, Wallace, and Subramaniam 2015).

Probably the area of BMP-SMAD signalling that has the most evidence to support it is hemojuvelin, which is also known in the scientific literature as HFE2. Hemojuvelin is of great clinical significance, as genetic mutations in the gene encoding hemojuvelin lead to a severe juvenile onset form of hereditary haemochromatosis (an iron overload condition that is a heterogeneous genetic disorder, and can be caused by mutations in several genes including those that encode for hepcidin, ferroportin, hemojuvelin, HFE, TfR2) and the phenotype is similar to that seen in mutations of hepcidin itself (Roetto et al. 2003; Papanikolaou et al. 2004; Babitt and Lin 2011).

The action of hemojuvelin greatly depends on whether it is bound to the cellular membrane, or in its soluble form. TMPRSS6 (also known as matriptase-2) is activated in part by iron-loaded transferrin and cleaves cell-associated hemojuvelin to form a soluble form. Membrane-bound hemojuvelin is critical to the BMP-SMAD signalling cascade, as it acts as a co-receptor for the BMP-receptor-BMP-6 complex and ultimately upregulates hepcidin expression, whereas the soluble form actually inhibits its activity (Babitt et al. 2006; Lin, Goldberg, and Ganz 2005). TMPRSS6 is not the only protein that can cleave hemojuvelin; furin is also thought to be able to do this, and if hemojuvelin interacts with neogenin, then it is perhaps even more susceptible to cleavage (Silvestri et al. 2008; Zhang et al. 2009).

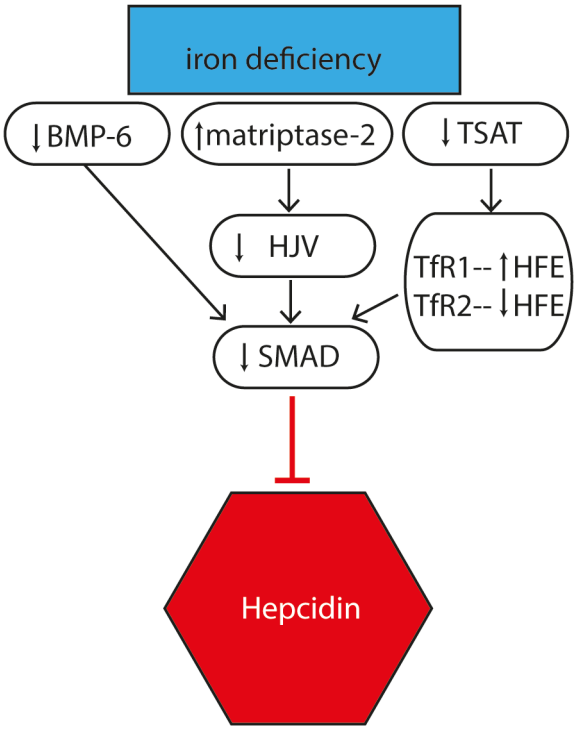

Figure 1.13 Putative mechanisms of iron deficiency leading to reduced hepcidin expression
Abbreviations: BMP6 – bone morphogenetic protein 6; TSAT – transferrin saturation; HFE – hemochromatosis protein; HJV – hemojuvelin; TfR – transferrin receptor 1 or 2; SMAD – sma and mothers against decapentaplegic homologue

Although there are still some gaps in our understanding of basic hepcidin biology, there is a fairly robust relationship between iron and hepcidin in the clinical setting. There are ample studies showing that iron loading, in both the oral and intravenous forms, leads to a rise in hepcidin levels. A secondary analysis of the randomised FIND-CKD trial showed that both oral and intravenous iron led to a rise in patient hepcidin levels, but that the patients' baseline hepcidin levels did not allow the extent of the rise to be predicted (Gaillard et al. 2016). In contrast, hepcidin levels in iron deficient cohorts tend to have low hepcidin levels. As a result of monthly iron losses of between 10 and 40mg, pre-menopausal females have been found to have lower hepcidin levels than males, but this gender difference is negated once the females become menopausal (Galesloot et al. 2011).

1.3.5.2 Inflammation

In contrast to the preceding section, our understanding of the relationship between acute inflammatory stimuli and hepcidin upregulation is clearer, and our knowledge in this area has less uncertainty compared to iron regulation and hepcidin (Figure 1.15).

The role of hepcidin in inflammation resulting from infectious stimuli is perhaps unsurprising, since it was originally called Liver-Expressed Antimicrobial Peptide 1 (LEAP-1) prior to the identification of its pivotal role in iron regulation (Park et al. 2001; Krause et al. 2000).

Iron is crucial for the function of all biological systems including microbes, and under normal conditions is extremely tightly regulated. In acute infection, hepcidin upregulation not only restricts iron availability to the host, but also diminishes the invading organisms' access to iron, thereby creating one of the first lines of defence of the innate immune system, and consequently resulting in the hypoferraemia of infection, which was described over 80 years ago (Cartwright et al. 1946). There is evidence that hepcidin is an ancient defence mechanism, as its structure and transcriptional machinery appears to be conserved amongst vertebrate mammals and fish (Figure 1.14). There is even homology with a protein found in drosophila species, since there is a striking resemblance between hepcidin and drosomyin, which is a 4-disulfide defensin (like hepcidin) that is synthesised in response to infections (Krause et al. 2000; Lombardi et al. 2015).

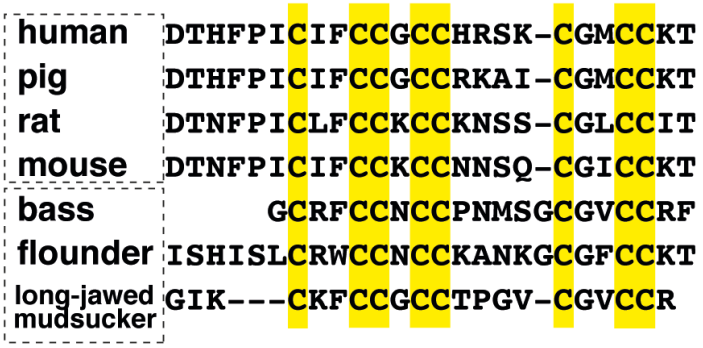

Figure 1.14 Sequences of mammalian and fish hepcidins
The yellow boxes indicate the cysteine residues that are conserved across the species (reproduced from (Ganz 2005))

There is a veritable body of evidence supporting cytokine-induced hyperhepcidinaemia that was initiated by Nemeth and colleagues, who described both the direct and indirect impact of IL-6 on hepcidin levels. Serum iron levels fell in wild-type mice that were injected with turpentine, but this effect was not seen in IL-6 knockout mice. They went on to infuse recombinant IL-6 into healthy human subjects, and saw that these patients' urinary hepcidin excretion peaked 2 hours after the end of the infusion with a concomitant fall in their serum iron levels (Nemeth, Rivera, et al. 2004). Other researchers, perhaps spurred on by this study, gained ethical approval to infuse

lipopolysaccharide into healthy human subjects (medical students) in order to examine the impact of an inflammatory activator that was upstream of IL-6; similar results were obtained (Kemna et al. 2005).

Interleukin-6 is typically considered to be pro-inflammatory but like some other cytokines, it has a degree of functional pleiotropy meaning that under certain conditions, it can actually act in an anti-inflammatory manner. Cells that are responsive to the *classical signalling* system require those cells to express both the membrane-bound IL-6 receptor (IL-6R) and gp130, before binding to the IL-6 ligand, thereby forming the IL-6R complex with ensuing downstream activation of the JAK-STAT, ERK and PI3K transduction pathways. It has been shown that the corollary of STAT-3 activation in this manner leads to a net reduction in inflammation. In the context of cells that express gp130 alone, they can be activated by the circulating IL-6/IL-6R complex that requires IL-6 to associate with soluble IL-6R (*trans-signalling*). This eventually leads to activation of the immune system and a pro-inflammatory environment (Scheller et al. 2011).

Hepcidin induction occurs with inflammatory stimuli regardless of the type of infectious organism. Armitage and colleagues showed that bacterial, viral, and fungal toll-like receptor agonists were able to induce hepcidin transcription in human PBMCs in an IL-6 dependent manner. However, they discovered that hepcidin upregulation by *candida albicans* occurred independent of IL-6, instead relying on TGF-β, suggesting that the type of organism encountered by the innate immune system influenced the pathway that was involved in the downstream activation of hepcidin (Armitage et al. 2011). The same group of researchers have been very prolific in the area of infection-induced hepcidin induction, going on to show that HIV-1 infection, but not hepatitis B or C induce hepcidin, and that typhoid strongly induces hepcidin in the early phase of infection (Armitage et al. 2011; Armitage et al. 2014).

The signalling pathway that mediates IL-6 induction of hepcidin is shown in Figure 1.15. IL-6 activates the IL-6 receptor (which interacts with gp130) that causes activation of Janus Kinase (JAK), which leads to the phosphorylation of the STAT3 protein, and this translocates to the nucleus and binds to the *HAMP* gene promotor and ultimately leads to hepcidin transcription (Verga Falzacappa et al. 2007; Wrighting and Andrews 2006).

There remain some areas, however, that do not fit neatly into the paradigm described above and suggest that there is interaction between the BMP-SMAD system, and the JAK-STAT signalling pathways. Examples of these include SMAD4$^{-/-}$ mice treated with IL-6 that did not induce hepcidin, and LPS-treated mice increased phosphorylated SMAD in both wild-type and BMP6$^{-/-}$ mice, suggesting that upregulation of SMAD can occur independently of the usual BMP signalling pathways, possibly at the level of the hepcidin promotor (Wang et al. 2005; Besson-Fournier et al. 2012). Another example of crosstalk between the "iron-side" and the "inflammation-side" of the hepcidin divide can be seen by the influence of inflammation on TMPRSS6, causing its downregulation and this in turn leads to increased hepcidin expression (Meynard et al. 2013).

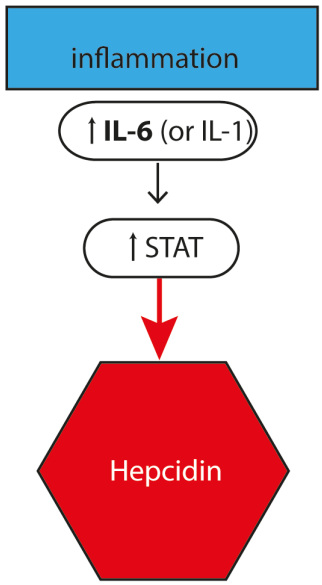

Figure 1.15 Mechanism of hepcidin upregulation in acute inflammation
Abbreviations: IL – interleukin; STAT – signal transducer and activator of transcription

There is a distinction to be drawn between acute inflammation (or anaemia of critical illness), such as mediated by an infectious stimulus, and chronic inflammation, seen in some non-infectious conditions such as rheumatoid arthritis and inflammatory bowel diseases. Both acute and chronic inflammation lead to disorders of iron processing but perhaps manifest in slightly different ways. Patients with an acute insult often develop the hypoferraemia of inflammation, which can develop within a few days of a critical illness, but that does not necessarily translate to a reduction in blood haemoglobin or mean cell volume (Cunietti et al. 2004). Patients with chronic inflammatory states, including the anaemia of CKD, tend to exhibit anaemia with a normocytic picture and

EPO levels within the normal range. It is thought that this is due to a functional iron deficiency and a degree of EPO resistance (Barany 2001).

The distinction between acute and chronic inflammation can also be inferred from data gathered in the pre-clinical setting. Armitage and colleagues showed that hepcidin production in *ex vivo* PBMCs were refractory to repeated stimulation by IL-6 (but not TGFβ) raising the possibility of a difference in the cellular handing of an acute inflammatory/infectious stimulus versus a persistent, chronic inflammatory stimulus. (Armitage et al. 2011)

1.3.6 Regulation of Hepcidin 2: Other Regulators

1.3.6.1 Erythropoiesis

The *raison d' être* of iron *in vivo* is the production of red blood cells, and an increase in erythropoietic activity, via an erythroid regulator, is therefore a potent suppressor of hepcidin in order to prevent polycythaemia. The search for the elusive erythroid regulator was a long-standing one, and was believed to be a molecule that could be activated by anaemia and EPO, but also allowed an increase in iron supply to the bone marrow (which we now know equates to the ability to suppress hepcidin production) (Finch 1994). Both Growth Differentiation Factor 15 (GDF15) and Twisted Gastrulation (TWSG1) were considered possible candidates for the role, but in recent times, the molecule erythroferrone has come to the fore as being able to satisfy the criteria of an erythroid regulator (Figure 1.12). Kautz and colleagues showed that erythroferrone was produced by erythroblasts in response to EPO, and it was able to suppress hepcidin (Kautz, Jung, Valore, et al. 2014). They were also able to show that erythroferrone suppressed hepcidin and thus contributed to the recovery from an induced inflammatory anaemia in mice (Kautz, Jung, Nemeth, et al. 2014).

1.3.6.2 Hypoxia

The link between hypoxia and erythropoiesis has been known for more than a century and it is this relationship that forms the basis for athletes training at altitude, in order to seek improved athletic performance (Berglund 1992). Hypoxia mediates an increase in EPO levels that is orchestrated by the hypoxia-inducible factors (HIF) and this has been reviewed in great detail by Haase (Haase 2013).

In brief, it is the transcriptional regulator HIF that possesses an O_2-sensitive α subunit, and under normoxic conditions, HIF is hydroxylated by prolyl dehydrogenase (PHD), ultimately leading to the proteasomal degradation of HIF by von Hippel-Lindau factor. However, in hypoxic conditions, the PHDs are inactivated thus allowing HIF to target the promotor of the target gene, which in the case of the EPO gene causes increased transcription, whereas it leads to the eventual suppression of hepcidin transcription probably secondary to erythropoiesis (via an interaction with C/EBPα) (Pinto et al. 2008). Inhibition of the prolyl hydroxylases is a strategy that is currently being investigated in order to seek new treatments to treat CKD-anaemia (Haase 2017).

It is now widely accepted that hypoxia leads to decreased hepcidin levels, and this was shown in a simple experiment subjecting 23 healthy volunteers to hypoxia in a hypobaric chamber for 6 hours, which allowed their mean pO_2 to fall from 96.3 mmHg to 45.8 mmHg. Various parameters were longitudinally assessed, including serum EPO (increased), serum hepcidin (decreased), and serum platelet-derived growth factor (PDGF)-BB (increased). The same group also provided evidence that in murine models, PDGF-BB likely played a role in the hypoxia-driven inhibition of hepcidin (Sonnweber et al. 2014).

1.3.6.3 Endocrine Suppressors of Hepcidin

The main suppressors of hepcidin are hypoxia, anaemia, and erythropoiesis, but there are a range of other molecules that are capable of hepcidin suppression, including growth factors, steroid hormones and other endocrine factors. It has long been known that pituitary dysfunction can lead to anaemia resulting from a deficiency in testosterone, and this possibility ought to be considered amongst the differential diagnoses of iron-deficiency anaemia in men when more common diagnoses have been eliminated (Watkinson, Mc, and Evans 1947). It had been thought that testosterone possessed the ability to stimulate erythropoiesis via a direct effect on the bone marrow, but as far back as 1967, Haurani and colleagues showed that testosterone caused a ‘reutilisation’ of iron, and posterity has proven their work to be correct (Haurani and Green 1967). Through both clinical and pre-clinical work, we have now learnt that the most likely mechanism for testosterone’s action is by suppressing hepcidin through an effect on epidermal growth factor receptor signalling, thus increasing iron availability to the bone marrow, even in the presence of EPO-neutralising antibodies (Guo, Bachman, et al. 2013; Bachman et al. 2010; Bachman et

al. 2014; Latour et al. 2014). In separate experiments, both epidermal growth factor receptor and hepatocyte growth factor were shown to suppress hepcidin synthesis by interfering with the SMAD pathway (Goodnough et al. 2012).

Hepcidin suppression is not the preserve of testosterone alone, given that the female sex hormones were also thought to play a role in haematopoiesis, and it has now been shown that 17β-oestradiol and oestrogen suppress hepcidin, albeit via a different mechanism (targeting the oestrogen response element in the hepcidin promotor) (Yang et al. 2012; Hou et al. 2012). It has also been shown that murine hepcidin levels have a negative correlation with bone loss and that iron levels affect osteoblastic and osteoclastic function, suggesting a link between the pathways regulating iron and mineral bone metabolism (Zhang et al. 2018).

This hypothesis is further strengthened by the work of Bacchetta and colleagues, who were able to show that the steroid hormone, vitamin D, possessed the ability to downregulate hepcidin transcription in HepG2 cell culture experiments (both 25-D and 1,25-D), as well as reducing plasma hepcidin in human experiments. Seven healthy human subjects were dosed with 100,000 i.u. oral vitamin D_2 and their mean hepcidin levels fell by almost 50% within 24 hours and remained at this level for at least 72 hours. Their mechanistic work suggested direct transcriptional suppression at the level of the *HAMP* gene (Bacchetta et al. 2014). This work has been replicated by another American group, who have showed similar *in vitro* results in THP-1 cells, and have produced underpowered data suggesting that vitamin D supplementation in patients with CKD stage 2/3 may reduce hepcidin levels (Zughaier et al. 2014).

The same group, perhaps encouraged by the findings of their 2014 work, undertook a double-blind placebo-controlled randomised trial that strengthened the assertion that vitamin D suppressed hepcidin levels. Their trial included 2 groups of 14 patients who were well matched for age, gender, ethnicity, vitamin D deficiency and inflammatory status, and who were randomised to receive one dose of 250,000 i.u. of vitamin D_2, or placebo. The patients in the study arm had approximately a 70% reduction in their hepcidin levels after one week, with no reduction in markers of inflammation, supporting the results that vitamin D directly suppresses hepcidin rather than via the inflammatory cellular signalling pathway(s) (Smith et al. 2017). These data do not appear to translate to patients with established CKD. Randomised trials in both

paediatric and adult CKD populations show that vitamin D does not reduce hepcidin levels in these groups (Atkinson et al. 2017; Panwar et al. 2018). Atkinson and colleagues treated two small groups (n=17) with either 400 i.u. or 4000 i.u. of vitamin D_2 and did not show a reduction in hepcidin levels. Panwar and colleagues used vitamin D_3 for 6 weeks and also showed no reduction in hepcidin. This may be explained by the fact that hepcidin elevation in moderate/severe CKD has numerous contributory factors and that attenuating one factor has little or no overt impact on the total levels. They used vitamin D_3 whilst the original study by Bacchetta used vitamin D_2. This is supported by the data generated by Zughaier and colleagues, who used vitamin D_2 versus placebo in 19 patients with early CKD and did show a correlation between the change in hepcidin and change in 25(OH) vitamin D levels (Zughaier et al. 2014).

Our current knowledge of hepcidin places it directly at the centre of iron homeostasis, but its regulation is highly complex and influenced by a number of factors. The main pathways linking hepcidin to iron, inflammation, hypoxia/erythropoiesis and hormonal regulation have been decoded, but there are likely to be a number of other pathways that will be uncovered, given the pivotal role of iron in a wide range of biological processes.

1.4 Hepcidin And Its Uses In CKD

Some of the causes of anaemia associated with renal disease, such as EPO dysfunction, have been covered in section 1.2.4, and the impact that both iron and inflammation have on hepcidin has been covered in detail in section 1.3.5. Both iron and inflammation are two factors that play a major role in the disordered iron regulation in CKD. Chronic kidney disease has long been considered a pro-inflammatory state, particularly marked in patients receiving chronic haemodialysis, and can be due to the underlying disease process leading to kidney dysfunction, reduced renal clearance of cytokines, susceptibility to bloodstream infections (e.g. endocarditis or discitis), sub-clinical infections (e.g. periodontal disease), and/or dialysis-related factors (Jofre et al. 2006; Lukaszyk et al. 2015; Malyszko et al. 2012).

Patients with CKD can be in net negative iron balance leading to reduced total body iron stores (true iron deficiency), as they may absorb less iron via the duodenal enterocytes, due to excess circulating hepcidin, and suffer increased losses of iron from the gastrointestinal tract as a result of uraemic dysfunction. The near-ubiquitous use of phosphate binders in the CKD-HD patient population may cause binding of dietary iron in addition to their intended target, and antacids and drugs intended to suppress the production of gastric acid may also reduce the absorption of iron. Another factor that may lead to iron deficiency is frequent phlebotomy, and those on haemodialysis may also suffer blood losses in the haemodialysis circuit. Counter-intuitively, the administration of ESAs themselves lead to the rapid utilisation of available iron stores and contribute to a relative iron deficiency (Babitt and Lin 2010).
In contrast to true iron deficiency, functional iron deficiency is characterised by the presence of adequate total body iron stores, but the depleted plasma iron pool is denied access to the iron as it is detained in the iron-storage cells (hepatocytes and splenic macrophages) by hepcidin. Many groups have shown that the renal clearance of hepcidin declines as the eGFR declines, including some of the work contained within this thesis, and this is one of the main contributory factors to elevated hepcidin levels in CKD (Tomosugi et al. 2006; Zaritsky et al. 2009; Ashby et al. 2009; Weiss et al. 2009).

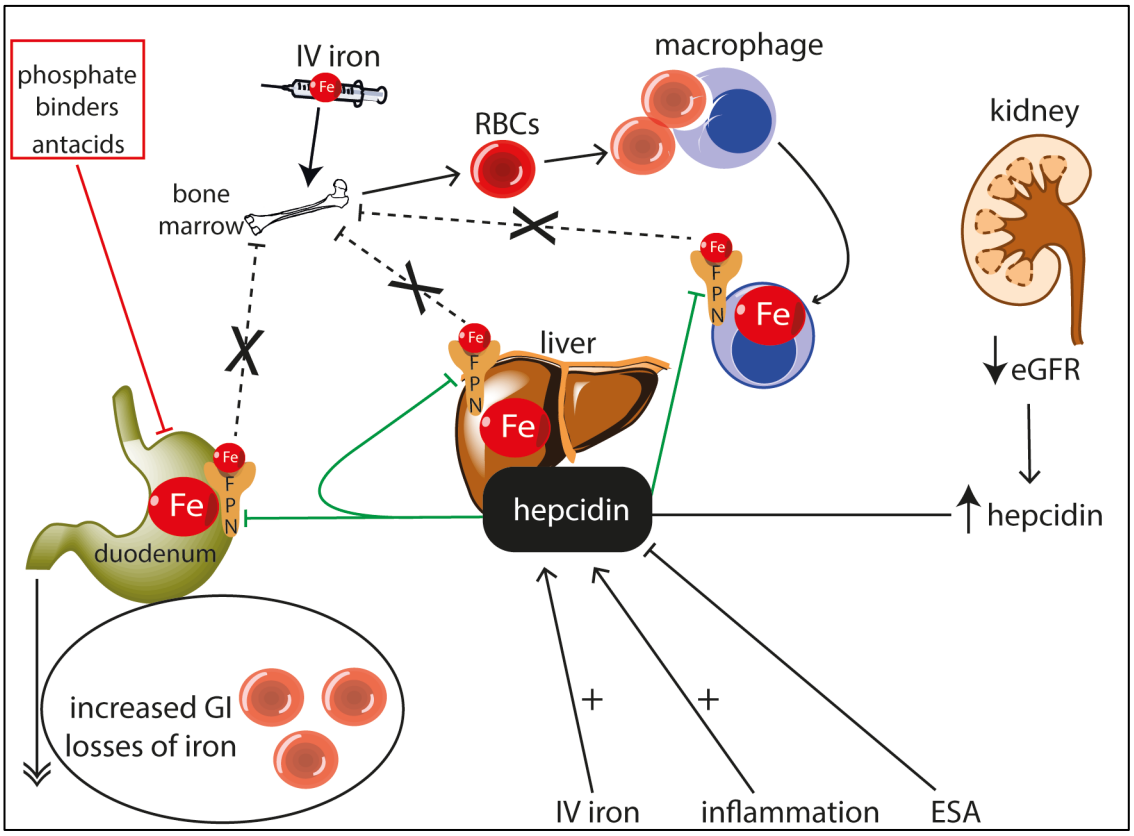

Figure 1.16 Regulation of iron homeostasis by hepcidin in CKD
(Abbreviations: FPN – ferroportin; ESA – erythropoiesis-stimulating agent)

The main factors that cause an elevation of hepcidin in CKD are reduced renal clearance, inflammation, and the use of intravenous iron, allowing increased hepcidin binding to its receptor (ferroportin) in the liver, duodenum and macrophages, preventing iron delivery to the bone marrow. In order to maintain red cell production, intravenous iron is used to bypass the problems of gastrointestinal iron absorption, and ESAs are administered (which causes hepcidin inhibition). Once the red cells reach the end of their lifespan, splenic macrophages are on hand to recover iron through erythrophagocytosis (Figure 1.16).

Other potential factors that can influence hepcidin include obesity, vitamin D deficiency (see 1.3.6.3), and hyperparathyroidism. Obesity has been associated with iron-deficiency anaemia and elevated hepcidin levels, possibly as a consequence of increased inflammatory cytokines, but the effect is offset in haemodialysis patients, where no difference is seen between obese and non-obese haemodialysis patients (Amato et al. 2010; Sarafidis et al. 2012).

Although there are data to suggest an improvement in ESA hyporesponsiveness following surgical parathyroidectomy for tertiary hyperparathyroidism, there is no direct evidence supporting hyperparathyroidism as a cause of ESA hyporesponsiveness (Bamgbola 2011). Currently, there is no evidence connecting hyperparathyroidism with hepcidin regulation.

Linking small molecules such as hepcidin or fibroblast growth factor 23, and ESA hyporesponsiveness, is problematic due to the multifactorial nature of this condition. There is evidence that the CKD-MBD and iron regulatory pathways are not discrete entities but may actually be interdependent in a way that has not yet been fully elucidated. Examples of the interplay between the two pathways include vitamin D, which has a clear role in the CKD-MBD pathway and yet is a direct transcriptional suppressor of hepcidin (Bacchetta et al. 2014). Iron-induced renal phosphate wasting, via FGF23 induction, is another phenomenon suggesting a link between the 2 pathways (Schouten et al. 2009).

1.4.1 Measuring Hepcidin

Despite the sterling efforts of clinical biochemists in the field to address this issue, the determination of hepcidin levels remains an area in which there is little standardisation, and the normal reference ranges vary markedly from laboratory to laboratory (Kroot et al. 2009). There are currently 4 main types of assays that are available for use; mass spectrometry, competitive ELISA, radioimmunoassays, and ligand binding assays, but it is the first two that have chiefly been employed in the measurement of hepcidin.

As bioactive hepcidin is only 25 amino acids in length, generating a specific antibody has proved challenging, as the antibodies employed in ELISAs are often subject to non-specific antigen binding. Ganz and colleagues developed an ELISA with a biotinylated hepcidin as a tracer, which had no reported cross-reactivity with hepcidin-20 or -22, but they nevertheless reported reference values that were nearly 7-fold higher than measurements with mass spectrometry assays. Levels of the non-bioactive hepcidin isoforms are demonstrably higher in renal failure, and it may be that antibody cross-reactivity particularly affects measurements in CKD (Macdougall et al. 2010; Ganz et al. 2008).

Mass spectrometry works by determining the elemental composition and structure of peptides, by means of molecular fragmentation and the generation of charged species.

Measurement of the mass-to-charge ratio is enabled when positive ions hit a charged end plate and generates a signal, which can then be deciphered (Figure 1.17).

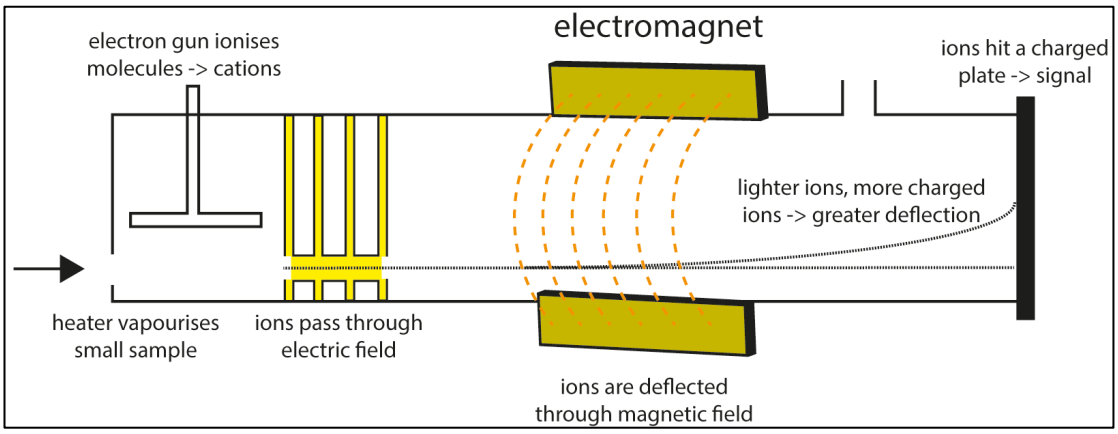

Figure 1.17 Schematic representation of mass spectrometric analysis

There are a number of mass spectrometry assays that have been deployed to detect hepcidin-25, including MALDI (Matrix-Assisted Laser Desorption Ionisation) and SELDI (Surface Enhanced Laser Desorption Ionisation), with or without the addition of TOF (Time-of-Flight) measurements, and Liquid Chromatography MS/MS. The differences between the various assays have been extensively reviewed elsewhere (Macdougall et al. 2010).

Mass spectrometry is considered superior to ELISAs and relies on expensive equipment, is labour intensive, and few laboratories possess the required technical expertise to ensure reliable results are obtained. Laboratories measuring hepcidin using both mass spectrometry and ELISA participated in a 'round-robin' exercise designed to harmonise laboratory processes with the intention of agreeing methods. Although the individual assays were generally found to have a low within-sample variance, there was a high degree of non-concordance between assays, which was somewhat mitigated following the development of an algorithm using the results of the analysis. Some of the reasons cited were the difference in hepcidin internal standards and a lack of a reference measurement procedure (RMP) given that reference materials for hepcidin do not exist (Kroot et al. 2012). A more recent attempt to navigate this issue has led to the identification of a commutable secondary reference material, which is an encouraging development and will hopefully allow standardisation of hepcidin measurement results in the future (van der Vorm et al. 2016).

There are a number of factors including age, gender, and circadian rhythm that influence hepcidin levels and there is little doubt that these will have an impact on any future reference tables that are produced. Hepcidin levels are similar in childhood, with no difference between boys and girls, but levels vary following the transition into adulthood, with adult females having lower hepcidin (and ferritin) levels compared to adult males. The literature appears split, with Zaritsky and colleagues finding nearly double the hepcidin level in adults versus children, whereas Sdoguo and colleagues found higher levels in children (Zaritsky et al. 2009; Sdogou et al. 2015). The disparity may be explained by possible differences in iron handling, illustrated by the linear relationship between ferritin and hepcidin in virtually all the published adult literature, which is in stark contrast to the childhood data, where no correlation exists between the two biomarkers. However, there is agreement that hepcidin is subject to a diurnal variation, except in end-stage renal failure (Sdogou et al. 2015; Galesloot et al. 2011; Ashby et al. 2009).

1.4.2 Hepcidin As A Biomarker

As discussed in section 1.2.6, the current biomarkers that are at our disposal for iron status evaluation are limited, as they are unable to reliably estimate total body iron stores or predict the response to iron therapy (Coyne et al. 2007; Singh et al. 2007). The discovery of hepcidin led to renewed hopes of the emergence of a biomarker that was fit for purpose, but alas, these have not been realised. In all the published adult data on hepcidin levels to date, the extremely strong linear correlation with ferritin levels remains the only constant finding, leaving the distinct possibility that hepcidin is no better than ferritin as a marker of iron stores (Ashby et al. 2009; Zaritsky et al. 2009; Galesloot et al. 2011). Additionally, there are a number of confounding factors that impact on hepcidin levels (Figure 1.16), and there is also no convincing evidence that hepcidin can be used to reliably predict a patient's response to iron therapy.

Tessitore and colleagues examined the merits of percentage of hypochromic red cells (%hypo), reticulocyte haemoglobin content (CHr), hepcidin-25 (hep-25), and hepcidin-20 (hep-20), as markers of iron responsiveness in a cohort of 56 haemodialysis patients receiving intravenous iron therapy, and found that the best performing biomarker was the percentage of hypochromic red cells (Figure 1.18). In contrast, both hepcidin isoforms were comparatively poor predictors of iron responsiveness, and only performed marginally better than the flipping of a coin (Tessitore et al. 2010). Weiss

and colleagues also produced data in 20 haemodialysis patients that showed intravenous iron did not cause a rise in hepcidin levels; both studies used mass spectrometry assays (Weiss et al. 2009).

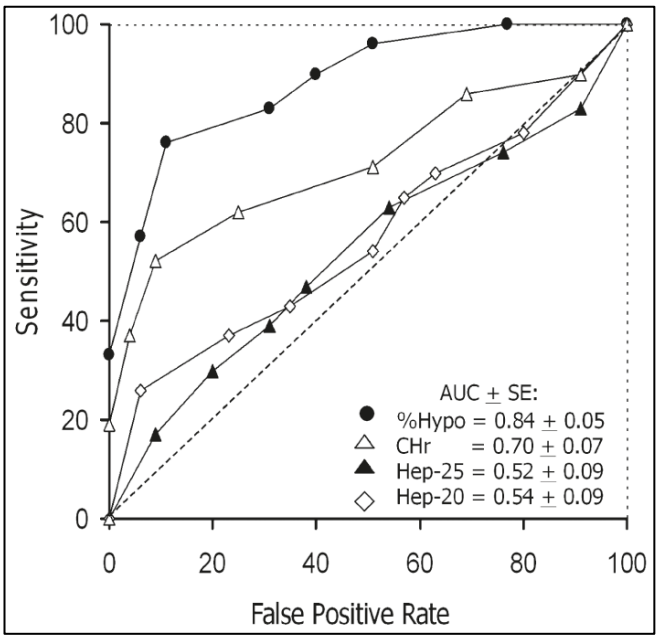

Figure 1.18 Diagnostic accuracy for iron responsiveness by ROC curve analysis
Abbreviations: %Hypo – percentage of hypochromic red cells; CHr – reticulocyte haemoglobin content; Hep – hepcidin; AUC – area under the curve (reproduced from (Tessitore et al. 2010))

In contrast, there are other research groups that have produced data showing that intravenous iron leads to a rise in hepcidin; Tomosugi and colleagues used mass spectrometry but only reported on the effects in 2 patients, whilst Malyszko and colleagues had more patients (12), but used an ELISA assay (DRG), which may have been a confounder (Tomosugi et al. 2006; Malyszko, Malyszko, and Mysliwiec 2009). A subset of non-HD CKD patients were assessed as part of the FIND-CKD trial, and the administration of iron elicited a positive hepcidin response, but neither the baseline hepcidin levels nor *delta* hepcidin levels were predictors of the responsiveness to iron therapy (Gaillard et al. 2016).

In the non-renal population where hepcidin levels are not confounded by reduced glomerular clearance, Bregman and colleagues showed that hepcidin possessed the ability to predict non-responsiveness to oral iron therapy in those with iron-deficiency anaemia (Bregman et al. 2013).

Therefore, in the arena of iron management in CKD, there is no evidence that hepcidin offers an advantage over the currently available biomarkers. Other markers that have

generated interest since the discovery of hepcidin include soluble hemojuvelin (sHJV), and growth differentiation factor 15 (GDF15). Soluble HJV levels have been shown to fall following iron therapy, and levels are increased in iron deficiency, but studies examining its use as a biomarker are still required (Brasse-Lagnel et al. 2010; Chen et al. 2013). GDF15 plays a role in the erythropoietic regulation of hepcidin (Figure 1.12) but like hepcidin, it is influenced by iron, inflammation and CKD, which may limit its usefulness as a ferric biomarker (Theurl et al. 2010).

Attempting to find a biomarker that helps us refine patient care to the level being discussed may require a shift from cellular biology to genomics, and there has been a distinct shift in the U.K. towards 'personalised medicine', following in the wake of the 100,000 genomes project (Turnbull et al. 2018). A gene currently attracting interest is TMPRSS6, which encodes matriptase-2 ultimately resulting in the decreased expression of hepcidin, by cleaving membrane-bound hemojuvelin. A rare, loss-of-function germline mutation is implicated in IRIDA (see section 1.3.3), but more subtle genetic variants may theoretically help predict the response to iron therapy in CKD.

Pelusi and colleagues evaluated polymorphisms in the TMPRSS6 (A736V) and HFE (C282Y, H63D) genes in 183 haemodialysis patients (compared to 188 controls) and sought to determine if there were any relationships with hepcidin and in markers of erythropoiesis and iron status. They found that patients with HFE mutations had lower hepcidin levels. They concluded that patients with the A736V TMPRSS6 (V/V variant) genotype had higher hepcidin levels and may possibly impact on anaemia management in haemodialysis patients. A prospective analysis will be necessary to see if it possesses any utility in this area (Pelusi et al. 2013). Other genome-wide association studies involving TMPRSS6 reveal that women suffering with menorrhagia who are RS855791 C homozygotes are less susceptible to iron-deficiency anaemia (Pei et al. 2014). Although this area of investigation is in its relative infancy, it clearly has the potential to revolutionise the assessment and treatment of patients with CKD-anaemia.

1.4.3 Hepcidin As A Therapeutic Target In CKD

Hepcidin excess is thought to play a significant role in the pathogenesis of renal anaemia, and a number of therapeutic strategies have been pursued in order to antagonise hepcidin with the potential of new treatments emerging. These strategies

seek to block either the ligand (hepcidin) or its action on the receptor (ferroportin), or by interfering with the intracellular signalling pathways (Figure 1.19).

1.4.3.1 Dialysis Clearance

But perhaps the simplest method available to modulate hepcidin in the haemodialysis population is by increasing dialysis adequacy. It is well known that increasing clearance of small and medium-sized molecules leads to an ensuing reduction in ESA dosing, and it is possible that this is in part mediated by increased clearance of hepcidin (Ifudu, Feldman, and Friedman 1996). There are multiple studies confirming that hepcidin is cleared by dialysis, although some of them report that hepcidin levels rebound to pre-dialysis levels very quickly after the end of the session (Weiss et al. 2009; Zaritsky et al. 2010; Campostrini et al. 2010; Peters et al. 2010; Kuragano et al. 2010). The efficiency of hepcidin removal may differ according to the type of haemodialysis membrane used. For instance, high-volume online haemodiafiltration had been shown to be superior to standard low-flux bicarbonate haemodialysis following 6 months of treatment in both reducing hepcidin levels and improving the ESA resistance index (Panichi et al. 2015).

1.4.3.2 Hepcidin/*HAMP* Antagonism

Monoclonal antibodies raised against hepcidin have been successfully used to treat anaemia in mice and non-human-primates, and the molecule LY2787106 is in early-phase trials to treat cancer-related anaemia (Sasu et al. 2010; Cooke et al. 2013; Vadhan-Raj et al. 2017). Other small proteins such as anticalins and spiegelmers are able to target and bind to specific ligands. In the sphere of direct anti-hepcidin agents, Pieris Pharmaceuticals have engineered PRS-080, an anticalin technology that has been developed to exhibit sub-nanomolar affinity for hepcidin. It has passed through a successful trial in primates in lowering hepcidin and raising iron levels and will next be tested in phase 1 human trials (Hohlbaum et al. 2018). Anticalins are based on naturally occurring lipocalins, whereas spiegelmers are synthetic compounds that potentially bind to their targets in a manner similar to antibodies. Lexaptepid pegol (NOX-H94) has been shown to be safe in healthy human subjects, and is currently in phase 2 clinical trials (Boyce et al. 2016). The company that have produced the spiegelmer technology (Noxxon Pharma) have another 2 candidate molecules for use in CLL and diabetic nephropathy.

Agents that inhibit *HAMP* transcription include vitamin D, testosterone, and oestrogen, and molecules involved in erythropoiesis, and have been discussed in section 1.3.6.3. Another molecule, K7174, was also shown to indirectly downregulate *HAMP* transcription by induction of GDF15, but does not appear to have been taken forward to clinical trials (Fujiwara et al. 2013).

1.4.3.3 Ferroportin-Hepcidin Interference

Ross and colleagues generated fully human anti-human antibodies against the ferroportin receptor *in vitro* with the potential for translation trials, and produced a thorough review of the current data (Ross et al. 2017). One other such molecule has already passed through the phase 2 clinical trial stage of development. LY2928057 is a humanized immunoglobulin (IgG4) monoclonal antibody, which binds to ferroportin and prevents the action of hepcidin and the degradation of ferroportin without affecting iron efflux. It has been shown to be safe in CKD patients, and although the expected changes in iron parameters were seen, this did not translate into a rise in haemoglobin (Barrington et al. 2016). Fursultiamine is a thiamine derivative that was shown to bind to ferroportin (inhibiting the action of hepcidin) *in vitro*, but did not perform well in *in vivo* studies (Fung et al. 2013).

1.4.3.4 BMP-Hemojuvelin-SMAD Pathway Inhibitors

Most of the strategies that have been developed to date target the BMP signalling system, either by disrupting the ligand (BMP) or the adjacent molecular structures including the BMP receptors (I,II), or by modulating the BMP co-receptors.

Hemojuvelin is the main co-receptor for the BMP receptors, and it is cleaved leading to the formation of soluble hemojuvelin (sHJV), which is a negative regulator of BMPs. There was much interest in the action of sHJV, and an sHJV.Fc fusion protein was created by the fusion of the extracellular domain of the BMP co-receptor HJV to the Fc portion of human immunoglobulin G (Babitt et al. 2007). Although the fusion protein was successful in reducing hepcidin levels and correcting anaemia in an inflammatory rodent model, clinical trials of FMX-8 (Ferrumax Pharmaceuticals) have been discontinued due to lack of recruitment (Theurl et al. 2011; ClinicalTrials.gov. Bethesda (MD): National Library of Medicine (US) 2016). Another potent inhibitor of BMPs is heparin, but its anticoagulant properties preclude the use of high doses in man. Non-anticoagulant heparins (SST0001/Roneparstat) have been designed to circumvent this,

and there are encouraging signs that this might provide a useful line of treatment in clinical settings (Poli, Asperti, Naggi, et al. 2014; Galli et al. 2018). There are other molecules that antagonise BMPs including BMPER and SOSTDC1 but as yet, have not been investigated in clinical settings (Patel et al. 2012; Tesfay et al. 2015).

Agents that suppress hepcidin synthesis by way of BMP-receptor I inhibition include the dorsomorphin derivative, LDN-193189, which successfully ameliorated an inflammatory anaemia in a murine model, and plans are afoot to prepare the drug for testing in humans (Mayeur et al. 2015; National Institutes of Health Bethesda (MD) 2017). Another molecule, TP-0184, modulates hepcidin levels in a similar manner to LDN-193189, and is slightly further ahead in its drug development, but recruitment to the first-in-man clinical trial has yet to commence (Peterson et al. 2017; ClinicalTrials.gov. Bethesda (MD): National Library of Medicine (US) 2018).

As the main co-receptor to the BMP receptors, there has been a huge focus on hemojuvelin neutralisation, as it leads to a suppression of hepcidin synthesis. At least 2 antibodies (ABT-207 and h5F9-AM8) have been raised against hemojuvelin; both have been efficacious in pre-clinical rodent and simian studies, and are being further developed by Abbvie (Boser et al. 2015; Kovac et al. 2016).

Alnylam Pharmaceuticals developed gene silencing technology using small interfering RNAs (siRNAs), which have been used to knock down gene expression targeting a number of genes including *HAMP*, *HJV*, and *HFE*. They were shown to be effective in a host of animal models of inflammatory anaemia (Akinc et al. 2011). The company do not appear to have taken this therapeutic approach forward in CKD-anaemia but are working on other strategies targeting iron overload conditions.

1.4.3.5 Disruption of IL-6/JAK/STAT Pathway

The hepcidin “inflammatory pathway” appears to have garnered the most success in terms of therapeutics that have gained regulatory approvals. Toclizumab is a humanised anti-IL-6 receptor antibody that is used in patients with rheumatoid arthtitis and idiopathic juvenile arthritis, and it has been shown to reduce hepcidin in patients with Castleman disease (Song et al. 2010). Siltuximab is a murine-human chimeric monoclonal antibody directed against IL-6 and is approved for use in Castleman

disease. Retrospective data suggests that it too downregulates hepcidin levels when used in multiple myeloma or Castleman disease (Casper et al. 2015).

Downstream of the IL-6 receptor, there are 3 molecules that inhibit the JAK-STAT intracellular signalling pathway. AG490 disrupts JAK, and PpYLKTL and curcumin disrupt STAT, respectively. AG490 has been shown to have some *in vivo* activity against hepcidin (Zhang et al. 2011). PpYLKTL has also been shown to have *in vivo* effects against hepcidin, whereas curcumin, which is a naturally occurring compound found in turmeric that is liberally used in South Asian cooking, reduces hepcidin *in vitro*, but conversely has also been shown to be an iron chelator (Fatih et al. 2010; Jiao et al. 2009). Currently, none of these have progressed to clinical development.

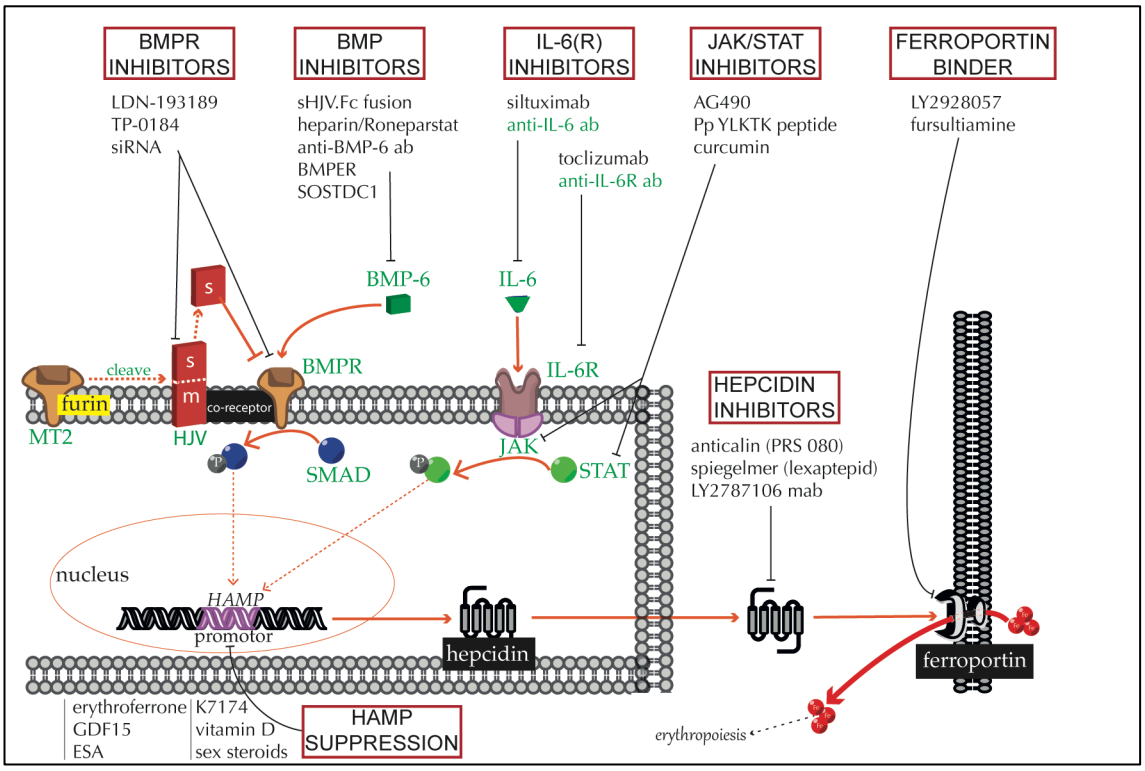

Figure 1.19 Overview of hepcidin antagonists
Abbreviations: BMP6 – bone morphogenetic protein 6; BMPR – bone morphogenetic protein receptor; ESA – erythropoiesis-stimulating agent; GDF15 – growth differentiation factor 15; HJV – hemojuvelin (**m**embrane and **s**oluble); IL6 – interleukin 6; IL-6R – interleukin 6 receptor; JAK – Janus kinase; MT2 – matriptase 2; SMAD – sma and mothers against decapentaplegic; STAT – signal transducer and activator of transcription

1.5 Aims And Objectives Of Thesis

In conclusion, this is an expanding field of knowledge and research, although there remain huge gaps, especially in the basic science regulation of hepcidin. It is an area of discovery that is highly relevant to CKD-anaemia, and hepcidin antagonism is an aspect that is being exploited in order to bring new potential anaemia treatments to the therapeutic arena.

The aim of this thesis was initially developed from the following two primary research questions: is hepcidin elevated in CKD/haemodialysis patients (chapter 3) and is this a result of increased production of hepcidin/HAMP transcription (in PBMC monocytes) (chapter 4)? The main finding from chapter 4 was the downregulation of *HAMP* mRNA in PBMC monocytes in haemodialysis patients (compared to healthy controls) with approximately 10 times the serum hepcidin levels. In chapter 5, I set out to answer whether there was a circulating factor within the plasma of these dialysis patients that was impacting on *HAMP* by incubating human hepatocytes in plasma. Through the serendipitous use of heparin to prevent coagulation in cell culture, I realised that heparin was in fact a potent inhibitor of *HAMP* transcription. The project then evolved to look for ways that hepcidin could be reduced in CKD patients, and I then set out to examine whether it was the use of heparin in haemodialysis patients that led to the downregulated *HAMP* (from chapter 4) by measuring *HAMP* expression in heparin-naïve patients following the administration of heparin (chapter 6). I sought to examine other ways of inhibiting hepcidin production through the use of corticosteroids (chapter 7), given that the steroid hormone vitamin D had been shown to do this.

The objectives were implemented by the following means:

1. Use of a case-control design to match patients from the haemodialysis, transplant and CKD stage 4/5 populations.
2. Use of PBMCs cultivated *ex vivo* from haemodialysis patients as a means of assessing hepcidin production in this patient population.
3. Use of HepG2 cells for *in vitro* work to assess the effect of cytokines (interleukin-6).
4. Use of dexamethasone and heparin administration on *HAMP* mRNA in HepG2 cells.
5. Use of a patient model of systemic (including renal) inflammation to study the effect of methylprednisolone on hepcidin levels to ascertain for the first time whether this corticosteroid could modulate hepcidin levels as has been shown for other steroid hormones.

2 Materials and Methods

Dr Sukhi Bansal, Reader in Pharmaceutical Chemistry, performed the mass spectrometry measurements of hepcidin-25 levels in the Franklin-Wilkins Building, Waterloo Campus, King's College London, and the methodology is available elsewhere (Bansal et al. 2010). All other methodologies including cell culture, RNA extraction and reverse transcription, RT-qPCR, protein extraction and immunoblotting were performed by this investigator, in the Renal Laboratory at the Rayne Institute, Denmark Hill, King's College London.

2.1 Cell Culture

2.1.1 HepG2 Cells

Professor Andrew McKie (King's College London) gifted HepG2 cells that were used during the experiments undertaken in this thesis. HepG2 cells are human hepatoma cells that were derived from a 15-year-old Caucasian male with hepatocellular carcinoma and have been used in many studies that have investigated the cellular regulation of hepcidin.

The cells were incubated in a Techne incubator in a humidified atmosphere containing 5% CO_2, set to a temperature of 37°C. HepG2 cells were routinely cultured in Dulbecco's modified Eagle Medium (DMEM), which was supplemented with 'high' glucose, and L-glutamine, with the further addition of two antimicrobial agents, penicillin and streptomycin. The common working concentration for penicillin was 100 U/mL, and 100 µg/mL for Streptomycin (both from ThermoFisher Scientific, see Table 2.2). Fetal bovine serum (10%, Sigma) was added to the culture medium to provide a rich variety of proteins in order to help the cells grow and divide. When the cell confluence reached 70-80%, the cells were prepared for experimental use and were simultaneously subcultured to extend the cell lineage. Cells that had been passaged (a maximum of) 5 times or fewer were used for experimental purposes.

This adherent cell line was grown in 75cm^2 flasks (431464, Corning, UK) and at the time of passaging, the cells were twice thoroughly washed with PBS (D8537, Sigma-Aldrich) prior to the addition of 2mL trypsin EDTA (ThermoFisher Scientific) in order to detach the cells from the flask. The trypsin-bathed cells were incubated for a maximum of 5 minutes before 18mL culture medium was added to dilute the trypsin and inactivate it. The cells were washed with PBS and centrifuged (Heraeus Labofuge 400)

at 15000G for 5 minutes in order to completely remove all the trypsin in the supernatant. Following the removal of the supernatant, the cells were washed with PBS, and re-suspended in 20mL of warmed medium. The cell pellet was suspended in 37°C medium to allow cell counts (by way of a haemocytometer), and cell viability tests (using trypan blue) to be performed prior to plating in experimental dishes measuring either 35 or 60cm in diameter (Greiner Bio-one 627 160 or 627 160, respectively).

2.1.2 Peripheral Blood Mononuclear Cells

Primary peripheral blood mononuclear cells (PBMCs) were isolated following the donation of blood from volunteers and patients within the Renal Unit. Donor blood was collected in EDTA tubes, and gently layered onto Ficoll® Paque Plus density gradient media (GE Healthcare) to allow a sharp delineation to form between the two media (Figure 2.1). The separation protocol was undertaken using either a 15mL or 50mL Falcon conical flask, depending on whether 10mL or 30-35mL whole blood was donated. The remainder was made up by Ficoll® Paque Plus.

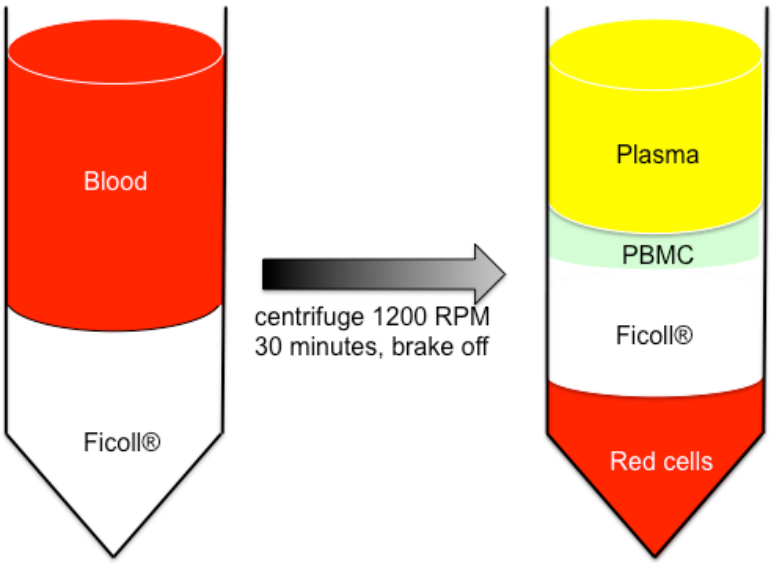

Figure 2.1 Separation of blood using density gradient media

The Falcon tubes were then centrifuged (Heraeus Labofuge 400) at 1200 RPM at 4°C with no brake for 30 minutes, to prevent mixing and to allow separation of the various blood components (plasma, PBMCs, and blood cells). The plasma was siphoned off and aliquoted (500µL), and then the PBMCs were isolated. The PBMC layer was transferred to a fresh 50mL Falcon tube and the cells were washed twice with buffer solution (PBS and 2.5mM EDTA) by centrifugation at 1800 RPM for 8 minutes. Cell

counts and viability testing were then performed once the PBMCs had been re-suspended in RPMI-1640 (ThermoFisher Scientific).

The amount of blood obtained from individuals determined the number of PBMCs that could be isolated. On average, 2.5mL whole blood would yield in the region of $5x10^6$ PBMCs. The PBMCs were then immediately placed through the RNA extraction protocol, or re-suspended in RPMI-1640 and plated at a concentration of $5x10^6$ cells/mL at 37°C in 5% CO_2 for work that involved cytokine stimulation.

2.2 Recombinant Peptide/Drug Treatments

Recombinant peptides were purchased from R&D Systems (USA) and Life Technologies (USA), and were reconstituted according to the manufacturers' instructions (Table 2.1). Controls were created for all the treatments listed, depending on the excipient specified by the relevant manufacturer. A number of drugs were used *in vitro* and were obtained from the companies listed in Table 2.2.

Recombinant peptide	**Company**	**Catalogue number**
BMP-6 (20µg)	R&D systems	507-BP-020
IL-6 (10µg)	Life Technologies	PHC0065

Table 2.1 List of recombinant peptides used *in vitro*

Drug	**Company**	**Catalogue number**
Dexamethasone	Sigma-Aldrich	D4902
Enoxaparin (Clexane®)	Sanofi-Aventis	N/A
Heparin sodium	LEO Pharma	N/A
Penicillin	ThermoFisher Scientific	15070-063
Streptomycin	ThermoFisher Scientific	15070-063

Table 2.2 List of drugs used *in vitro*

2.3 RNA Extraction

All work surfaces were cleaned with detergents and industrial methylated spirits prior to RNA extraction. An RNase decontamination solution (AM9780, Invitrogen) was used for added security against RNases. The QIAshredder (79654, Qiagen) and RNeasy Mini Kits (74104, Qiagen) were the kits used in the extraction of RNA from cellular

material, and used according to the manufacturers' instructions, and all the steps took approximately 23-30 minutes in total to complete, depending on the number of samples that were processed. Ethanol and beta-mercaptoethanol were not provided in the kit, but necessary for RNA extraction. All RNA that was obtained was aliquoted and then immediately stored at -80°C for reverse transcription.

2.4 Reverse Transcription

Reverse transcription from RNA to cDNA was undertaken using the High Capacity RNA-to-cDNA Kit (4387406, Applied Biosystems), according to the manufacturer's instructions. An 8-well NanoDrop spectrophotometer (ThermoFisher Scientific) was utilised to determine the quantity and purity of the RNA prior to reverse transcription. Samples were then pipetted into 96-well reaction plates (N801-0560, Applied Biosystems) combined with the reagents supplied by the manufacturer, to a total volume of 20µL. This kit contained random octamers and oligo dT-16 primers, which have a high specificity for mRNA. The samples were heated in a cyclical manner (Veriti Thermal Cycler, Applied Biosystems) for 65 minutes, consisting of 60 minutes at 37°C and 5 minutes at 94°C, before being cooled to 4°C; 80µL of RNase-free water (129112, Qiagen) was added to each sample to bring the reaction to a complete halt. Samples were immediately stored at -20°C. All samples had a non-reverse transcriptase control created for use in qPCR.

2.5 Quantitative PCR

Real time PCR was conducted using the cDNA obtained from section 2.4. TaqMan Gene Expression Assays (Applied Biosystems, USA) were used for the detection of:

1. HAMP mRNA (Hs00221783_m1) as the gene of interest
2. GAPDH mRNA (Hs02758991_g1) as the housekeeping gene

The primer-probes were combined with TaqMan Universal PCR Master Mix (4324018, Applied Biosystems), RNase-free water and the reverse-transcribed cDNA (from section 2.4). Volumes of 10µL per sample, which were tested in triplicate, were placed in a 384-well plate (4309849, Applied Biosystems), and a QuantStudio 7 Flex Real Time PCR System (Applied Biosystems) was used to perform the analysis that would determine the expression of the target gene relative to the housekeeping gene. For each experimental run, all the samples were defrosted and subsequently kept on ice. Each sample tested was made up to a final volume of 10µL, and this consisted of

Master Mix (5µL), prime probe (0.5µL), water (2.5µL), and cDNA (2µL). The total number of samples was calculated in advance and a mixture of all the elements listed above (except the cDNA) was made, and 8µL pipetted into each well along with the cDNA. The TaqMan assays are optimised to run as follows; 50°C for 2 minutes, 95°C for 10 minutes, and a final stage that cycles 40 times between 95°C (15 seconds) and 60°C (60 seconds).

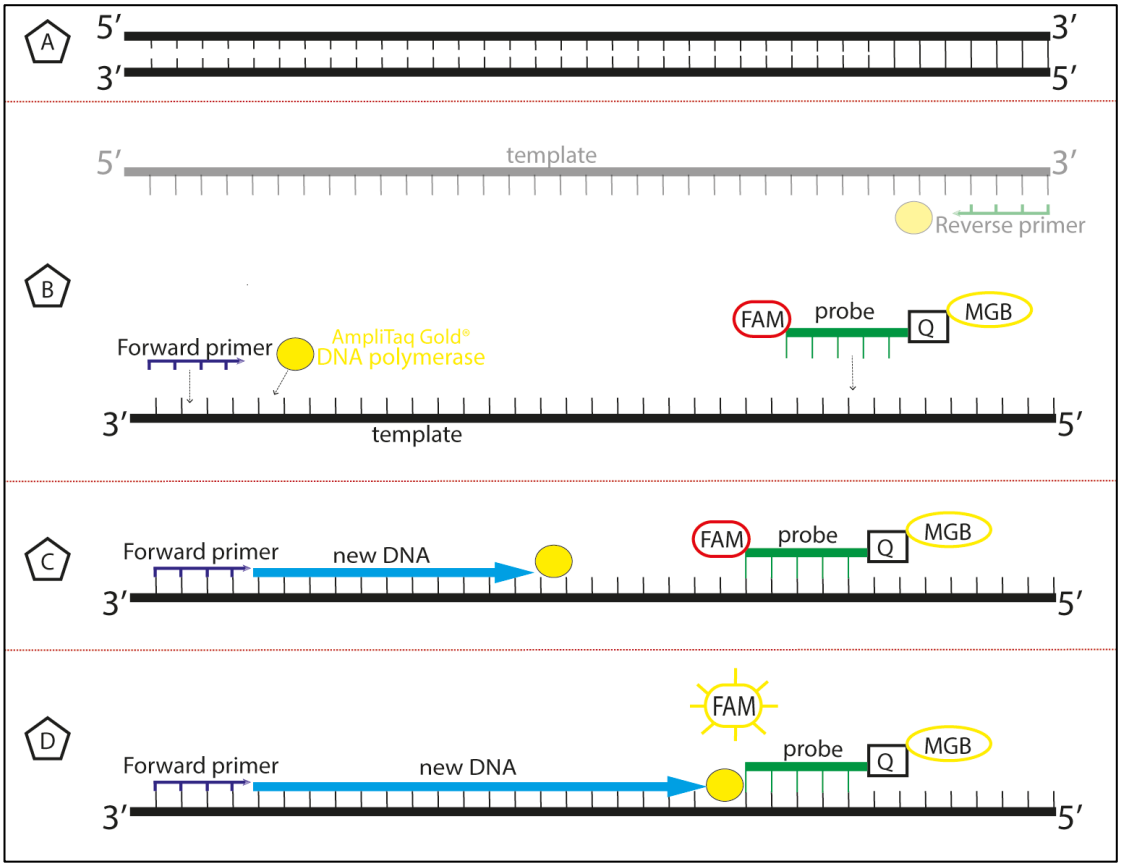

Figure 2.2 Schematic representation of the TaqMan gene expression assay process
(Abbreviations: FAM – dye; Q – non-fluorescent quencher; MGB – minor groove binder)

TaqMan expression assays comprise a pair of primers, a TaqMan probe with a FAM™ dye label on the 5' end and a non-fluorescent quencher on the 3' end, and TaqMan DNA polymerase. The initial heating allows the cDNA (Figure 2.2, A) to denature into single strands, and the temperature is lowered, thus allowing the primer and probe to anneal to their targets (Figure 2.2, B). Taq DNA polymerase allows the synthesis of new strands (Figure 2.2, C) and upon reaching the TaqMan probe, its endogenous nuclease activity cleaves the probe causing the FAM™ dye to separate from the probe and fluoresce (Figure 2.2, D). The fluorescence intensity is proportional to the amount

of amplicon synthesised, and reflective of the quantity of the target gene (ThermoFisher Scientific 2018). The cycling process allows the accumulation of a fluorescent signal during amplification. Control samples included non-template controls, serving as a general control for extraneous nucleic acid contamination.

TaqMan assays are highly sensitive and allow the detection of differences as little as two-fold in gene expression. Each sample is assessed for the gene expression of the target gene, and the housekeeping gene, and a relative comparison is made to quantify the amount of the gene present. The cycle time (Ct) value is the number of cycles at which the fluorescence reaches a pre-determined threshold set/optimsed by the machine, and values are given for all the genes for a particular sample. The earlier a sample reaches the threshold, the lower the Ct value and the higher the amount of target gene.

The method of calculating relative gene expression (*HAMP vs GAPDH)* was performed as follows:

$$\Delta Ct = Ct^{GAPDH} - Ct^{HAMP}$$

$$2^{\Delta Ct}$$

This would then be normalised to the reference sample

2.6 Protein Extraction

2.6.1 Cell Lysates

Total cell lysates were extracted from HepG2 cells by the addition of 200µL of a solution that was formed from PBSTDS and inhibitors, made firstly from the following:

Concentration	**Volume**
PDSTDS	20mL
Leupeptin 0.5 µg/ml	2µL
Pepstatin 1.0 µg/ml	4µL
EDTA 1.0 mM	40µL
PMSF 0.2 mM	40µL

To allow for the detection of phosphorylated proteins, phosphatase inhibitors (1mM sodium orthovanadate, 25mM sodium fluoride) were added to 5mL of the above solution to create the protein extraction buffer. The cells were washed twice with ice cold PBS and harvested in the buffer and left for 45 minutes on ice, before the cells were scraped and homogenised using a 1mL syringe and a 22G needle (Terumo) to mechanically disrupt the cells. The homogenates were centrifuged at 4°C at 14000 RPM for 10 minutes (Mikro 22R, Hettich), to separate cellular debris from the protein-rich supernatant.

2.6.2 Protein Quantification

The supernatants from the samples were collected in labelled eppendorf tubes and the protein content determined using a spectrophotometric method detecting colorimetric absorbance at a wavelength of 562nm. Quantification of protein within the study samples was calculated using the Pierce™ BCA Protein Assay Kit (23225, ThermoScienific), and compared to pre-prepared protein standards with known concentrations of protein containing bovine serum albumin (BSA). The BSA concentrations were diluted to form a standard curve (0 mg/mL to 2mg/mL), against which the experimental samples were compared, in order that their protein concentrations were ascertained. 10µL of homogenate had been diluted up to 8-fold and pipetted into a 96-well plate.

The assay is a two-component, high precision assay reagent set, and instructions were followed as detailed by the manufacturer. Briefly, this included the sequential addition of 2 separate reagents at a ratio of 40:1, resulting in a green solution. 200µL of the mixture of the reagents was subsequently added to each well. The presence of protein transformed the colour of the mixture from green to purple. The plate was then incubated at 37°C for 20 minutes and the optical densities of each well were then measured using a plate reader set to a wavelength of 562nm. The samples were normalised to a protein concentration of 1-2µg/µL, and 10µL of the sample was added to 2µL of betamercaptoethanol and 3µL of Western Sample Buffer in preparation for immunoblotting. These samples were heated to 100°C for 5 minutes to denature the proteins.

2.7 Immunoblotting

2.7.1 Preparation Of Polyacrylamide Gel

Gels were made of a stacking and running gel, to allow the proteins to cluster before running through the running gel that is achieved through the application of voltage across the gel. The stacking gel has a low concentration of acrylamide and the running gel a higher concentration capable of slowing the movement of the proteins.

10mL stacking gels were prepared using a combination of water (6.8mL), 30% acrylamide mix (1.7mL), 10% SDS (0.1mL), 1.0M Tris (pH 6.8) 1.25mL, 10% ammonium persulphate (0.1mL), and Temed (0.01mL).

20 mL running (also known as resolving gels, 12%) were prepared using a combination of water (6.6mL), 30% acrylamide mix (8.0mL), 10% SDS (0.2mL), 1.5M Tris (pH 8.8) 5.0mL, 10% ammonium persulphate (0.2mL), and Temed (0.008mL).

2.7.2 Gel Electrophoresis

The Bio Rad Trans-Blot® Cell system was used according to the manufacturer's instructions. The polyacrylamide gel was placed into the gel holder and electrophoresis buffer was added to the system. Five litres of this was prepared using the following ingredients: 60.4g Tris, 288g glycine, 20g SDS; q.s. as 5L with distilled deionised water made up to 1 litre. The protein samples were loaded onto the gel and 200 volts was applied for 1-2 hours in order to obtain the widest possible separation of the samples.

The samples were then transferred from the gel to a nitrocellulose membrane using the same system as above, with Towbin transfer buffer. The expression of ferroportin, SMAD5, STAT3, and phosphorylated SMAD 1/5 and STAT3 proteins were determined by immunoblotting. The samples were prepared by adding loading buffer consisting of β-mercaptoethanol (βME), bromophenol blue, EDTA, glycerol, SDS, and Tris HCL (pH 6.8). The samples were heated at 100°C in order to denature the proteins.

Total cell extracts (10-20μg) were separated on 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels that were made with 30% acrylamide, 10% ammonium persulphate, 10% SDS, TEMED, 1.5M Tris, and water, before being electro-blotted onto a nitrocellulose membrane. Ponceau S solution (Sigma Aldrich, 6226-79-5) was used to detect the successful transfer of proteins to the

membrane. The membrane was then washed and blocked in 5% dried skimmed milk with PBST at room temperature, followed by an overnight incubation with primary antibodies against ferroportin, SMAD5, STAT3, and phosphorylated SMAD and STAT proteins, in blocking solution at 4°C (Table 2.3). For phosphorylated proteins, 5% BSA was used as the blocking solution due to the high presence of phosphoproteins in milk. This was followed by 3 washes (10 minutes each) with PBST at room temperature after which, the membranes were incubated with HRP-conjugated secondary antibodies (1:1000) were applied at room temperature. A further three 10-minutes washes were performed, and the protein products were then visualised using the ECL Prime System (GE Healthcare Life Sciences) on Hyperfilm ECL (GE Healthcare). Image J software (NIH, USA) was used to quantify the intensities of the protein bands relative to GAPDH.

Antibody	**Company**	**Catalogue number**	**Dilution**
Ferroportin	Novus Biological	21502SS	1:1000
Ferroportin	Santa Cruz	SC-49668	1:2000
Ferroportin	Abcam	AB70866	1:1000
SMAD5	Cell Signalling	9517	1:1000
STAT3	Cell Signalling	4904	1:4000
Phospho-SMAD1/5	Cell Signalling	9516	1:4000
Phospho-STAT3	Cell Signalling	9134	1:6000
GAPDH	Millipore	MAB374	1:5000

Table 2.3 List of antibodies used in Western blot analysis
(Dilutions listed after optimisation)

2.8 Buffers and Reagents

The following reagents and buffers were used:

Buffer	Ingredients
PBS (X10)	80g sodium chloride, 2g potassium chloride, 14.4g disodium hydrogenphosphate, 2.4g potassium dihydrogen phosphate. Titrate to pH 7.4 with 1M HCL
PBST 0.1%	PBS X1, 0.1% Tween 20 (Bio Rad, UK)
PBSTDS lysis buffer	PBS X1, 1% Triton-X 100, 0.5% sodium deoxycholate, 0.1% SDS, leupeptin 0.5 µg/ml, pepstatin 1.0 µg/ml, EDTA 1.0 mM, PMSF 0.2 mM
Towbin transfer buffer (1L)	200mL methanol, 14,4g glycine, 3.03g Tris base; q.s. as 1L with deionised water
TBS	50mM Tris-HCL, pH 7.5, 150mM NaCl
TBST 0.1% (1L)	1L TBS, 1mL Tween 20
Western blot electrophoresis buffer	0.025M Tris, 0.192M glycine, 0.1% SDS, pH 8.3. For 5L, 60.4g Tris, 288g glycine, 20g SDS; q.s. as 5L with deionised water
Western sample buffer (6X)	0.5M Tris, pH 6.8, 0.35M SDS, 30% glycerol, 0.6M dithiothreitol, 0.175M bromophenol blue (Bio Rad, UK)

Reagent	Company	Catalogue number
Beta-mercaptoethanol	Sigma-Aldrich	M7522
Dulbecco's modified Eagle Medium	ThermoFisher Scientific	41966029
EDTA	ThermoFisher Scientific	118432500
Ethanol	Haymankimia	F200236
Fetal bovine serum	Sigma-Aldrich	F7524
Ficoll-Paque	GE Healthcare	17144003
Phosphate-buffered saline	Sigma-Aldrich	D8537
RPMI-1640	ThermoFisher Scientific	28175034
Trypsin EDTA	ThermoFisher Scientific	25300

Standards/ladders	Company, catalogue number
Biotinylated protein ladder detection pack	Cell signalling, 7727S
ColorBurst electrophoresis marker	Sigma-Aldrich C1992

2.9 Lactate Dehydrogenase Cytotoxicity Assay

HepG2 cell viability during experimental conditions was assessed using a lactate dehydrogenase (LDH) calorimetric method assay (Cytotox 96 non-radioactive cytotoxicity, G1780, Promega, UK). LDH is a stable enzyme ($t_{½}$=9 hours) found in the cytosol and is released when there is cellular damage. This assay uses a calorimetric method that relies on:

1. The eventual conversion of released LDH into a red formazan product that uses lactate, NAD^+, and INT as substrates in the presence of diaphorase (Figure 2.3)
2. The amount of colour product being proportional to the number of damaged/lysed cells

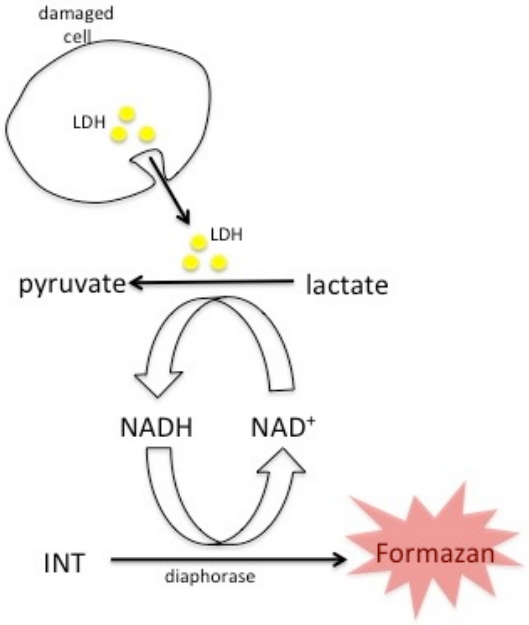

Figure 2.3 Generation of red formazan product from LDH released by lysed cells in the cytotoxicity assay
Abbreviations: LDH – lactacte dehydrogenase; INT – iodonitro-tetrazolium violet; NAD+/NADH - nicotinamide adenine dinucleotide, oxidised form/reduced form, respectively

2.10 Statistics

Differences between study groups were evaluated in pairs with the independent Student's t test or the Mann-Whitney U test, depending of the normality of the distribution of the relevant variable. Frequencies of baseline categorical variables were calculated using Chi-squared tests. Relationships between various parameters under study were also looked at; simple linear coefficients (r) were calculated using the Pearson's product formula, and stepwise multiple linear regression analyses were also performed. P values calculated to a level of <0.05 (two-tailed) were considered

statistically significant. Data were expressed as mean (±SD) or median (inter-quartile range, IQR) where appropriate. The Shapiro–Wilks test was used to test for normality of distribution. Data that were not normally distributed were log-transformed.

Comparisons between groups were performed using either a paired Student's t-test or Wilcoxon rank-sum test for normally and non-normally distributed variables, respectively. Bivariate correlation coefficients were calculated using the Pearson's product formula. A P-value of <0.05 was considered statistically significant, although Tukey's method (in conjunction with ANOVA) was used to correct for multiple comparisons. Analyses were performed using Statistical Package for Social Science version 17.0 for Windows XP (SPSS Inc., Chicago, IL) or version 19.0, and GraphPad Prism version 6.0 for Macintosh, GraphPad Software, La Jolla California USA.

3 Hepcidin Levels in Chronic Kidney Disease

3.1 Introduction and Aims

Since the discovery of hepcidin as the principal hormone regulating iron homeostasis, there has been interest in investigating the potential role in the pathogenesis of renal anaemia, above and beyond the role of (relative) Epo deficiency. Several studies, using ELISA or mass spectrometry, have demonstrated that pro-hepcidin and hepcidin-25 are both elevated in chronic haemodialysis (HD-CKD) patient (Tomosugi et al. 2006; Kato et al. 2008; Ashby et al. 2009; Costa et al. 2009; Weiss et al. 2009; Zaritsky et al. 2009; Campostrini et al. 2010; Peters et al. 2010). At the time of writing, only one study had been conducted assessing the impact of dialysis on the variability of hepcidin levels, the conclusion of which precluded the routine adoption of hepcidin as an iron marker in the HD-CKD population (Ford et al. 2010). This study was undertaken using an ELISA immunoassay (La Jolla, California) with a stated intra-assay CV_1 of 5-19% and inter-assay CV_1 of 0-44% (Ganz et al. 2008). Ford and colleagues found that using the same assay, the median 2-week intra-individual variability of serum hepcidin and ferritin was 19.0% [IQR 18.2-39.7] and 8.6% [5.2-13.7], respectively. Over a 6-week period, the median CV_1 was even higher; 22.9% [IQR 13.0-25.2] for serum hepcidin, and 11.5% [7.3-17.8] for serum ferritin. There are many factors that influence hepcidin levels in the typical HD-CKD setting. The regular administration of intravenous iron and the inflammatory milieu to which most HD-CKD patients are exposed will upregulate hepcidin. On the other hand, the anaemic state and ESA use, to name a few examples, will downregulate hepcidin (Girelli, Nemeth, and Swinkels 2016). Dialysis itself removes hepcidin from the circulation although the levels tend to rebound to pre-dialysis levels within an hour after the end of the session (Weiss et al. 2009; Kuragano et al. 2010). Ford and colleagues' use of an ELISA may have captured total hepcidin rather than just the bioactive isoform (hepcidin-25), and this may have confounded the results seen. We decided to extend the observations using a validated liquid chromatography mass spectrometry assay and to compare those results to an ELISA (Bachem, UK) in order to assess the variability of hepcidin longitudinally in a stable chronic haemodialysis cohort.

The aim of the work in this chapter was two-fold:

1. Cross-sectional study - characterise hepcidin-25 levels in three cohorts of patients with CKD (chronic haemodialysis, renal transplantation and non-dialysis chronic kidney disease) within the population of kidney patients treated at King's College Hospital, matched for age and gender.
2. Longitudinal study - assess variability of hepcidin in a stable chronic haemodialysis cohort using a validated mass spectrometry assay and ELISA, and to ascertain whether hepcidin levels were influenced by the length of time between dialysis sessions.

3.2 Subjects and Methods

3.2.1 Cross-sectional Study

3.2.1.1 Subjects

Subjects were drawn from various areas of the Department of Renal Medicine at King's College Hospital. There were 3 cohorts of CKD patients that were selected from various departments from within the renal unit that were included in this study:

1. Haemodialysis (HD-CKD) patients from the Main Haemodialysis Unit
2. CKD (non-HD CKD) patients that attended the Renal Outpatients' Clinic
3. Renal transplant patients that attended the Renal Transplant Clinic

3.2.1.2 Study Design

This was an exploratory study using a case-control design. Of the 3 CKD patient groups that were enlisted into the study, the total numbers of HD-CKD patients at KCH were fewest (compared to the numbers of HD-CKD and RTx patients) and on that basis were the first patient cohort to be collated. Thus, for the purposes of this study, the HD-CKD cohort would be considered the "control group". As this was an exploratory study, there were no statistical power calculations and a list of 40 HD-CKD patients was generated using broad pre-specified inclusion and exclusion criteria (Table 3.1). Compared to the satellite unit HD-CKD patients served by KCH, the Main KCH HD Unit, which had a capacity of approximately 120 patients, was considered to have the clinically less well patients (cf 'stable on dialysis' in the inclusion criteria below). For this reason, the goal of recruiting 40 patients was considered a practical target in the absence of any formal statistical power calculation.

INCLUSION CRITERIA	EXCLUSION CRITERIA
Dialysis patient in KCH Main Unit	Signs of acute/occult bleeding
≥18 years of age	Hospital admission within 4 weeks
Stable on dialysis for ≥3 months	History of haematological dyscrasia
	History of liver disease
	Active malignancy

Table 3.1 Inclusion and exclusion criteria (cross-sectional study)

Patients were considered to be stable according to the clinical judgement of the investigator. Patients were considered clinically unstable if, for example, they exhibited any overt signs of infection or were on antimicrobial therapy.

Exclusion criteria included a history of haematological dyscrasias, defined as any disease process that necessitated the patient having visited the Haematology department at any time for treatment or follow-up (except for iron-deficiency anaemia).
A list of patients matching the criteria was generated, and patients were invited to participate until a group of 40 haemodialysis patients was achieved. Following the formation of the initial cohort, an independent researcher blinded to the initial HD-CKD group data was tasked with interrogating the King's College Hospital RenalWare database in order to compile two further groups that comprised non-HD-CKD patients (with any aetiology), obtained from the general nephrology outpatient population, and renal transplant patients with functioning allografts. The 2 subsequent groups were age- and gender-matched with the HD-CKD cohort. Despite trying to apply the criteria listed above to these groups, it became clear that 9 HD patients could not be suitably matched with non-HD CKD and RTx cohorts and thus, the final patient population consisted of three groups of 31 patients. A single blood sample was required, and this would allow the measurement of hepcidin levels, and markers of inflammation including high-sensitivity CRP (hs-CRP), interleukin-6 (IL-6) and serum albumin. The timing of the HD-CKD blood draws was influenced by reports of the effect of dialysis on hepcidin levels (and other analytes) (Zaritsky et al. 2010). Consequently, single 10mL blood samples were taken prior to the commencement of dialysis. The patients in the other two groups had an extra 10mL of blood drawn along with the standard blood tests taken as part of their routine clinical care.

3.2.1.3 Demographics and Baseline Laboratory Data

A total of 93 subjects were included in this study, comprising 3 groups of 31 patients.

1. HD-CKD patients or the "control" group.
2. Non-HD CKD stages 2-4, irrespective of aetiology.
3. RTx patients who had received a renal transplant at least 3 months prior to the commencement of the study, with stable renal function.

The baseline demographic characteristics of the participants in all 3 groups are summarised in Table 3.2. *A priori*, there were no differences in the age and male-to-female ratio across the groups. No statistically significant differences were observed in the primary cause of kidney disease across the three groups, and furthermore, there was no statistical difference in the prevalence of hypertension between cohorts. However, fewer patients in the non-CKD group (12.9%) had diabetes compared to the RTx (22.6%) and HD-CKD (32.3%) groups. There was no statistical difference between

the prevalence of diabetes in the HD-CKD group versus the non-HD CKD group (p=0.068, see discussion 3.4). The only statistically significant difference was seen in the renal function of the non-dialysis subjects, which was better in the transplant cohort; the creatinine in the non-HD CKD group was 218 ± 161 μmol/L compared to 127 ± 55 μmol/L in the RTx group (p=0.003). The mean eGFR in the non-HD CKD group was 40.2 ± 24.9 ml/min compared to 54.3 ± 18.4 ml/min in the RTx group (p=0.014).

	Haemodialysis patients	CKD patients	Transplant patients	HD vs. CKD p value	HD vs. transplant p value	CKD vs. transplant p value
number of patients	31	31	31	–	–	–
Age, years	55.0+/-14.8	55.3+/-14.8	53.7+/-14.9	0.939	0.714	0.658
Gender, male/female	15/16	15/16	15/16	1	1	1
Primary kidney disease, n						
Diabetes	7	2	6	0.365	0.524	0.080
Hypertension	8	7	8			
Glomerulonephritis	5	6	2			
Vasculitis	3	7	1			
PKD	1	3	6			
Obstruction	1	1	1			
Other	4	5	4			
Unknown	2	–	3			
Diabetes, n	10	4	7	0.068	0.393	0.319
Hypertension, n	25	22	23	0.374	0.544	0.776
Urea, mmol/l	–	13.5+/-7.9	10.4+/-3.9	–	–	0.053
Creatinine, μmol/l	–	218+/-161	127+/-35	–	–	0.003
eGFR, ml/min/1.73 m^2	–	40.2+/-24.9	54.3+/-18.4	–	–	0.014

Table 3.2 Demographics and baseline laboratory data (cross-sectional study)
Adapted from (Rumjon et al. 2012) (PKD – polycystic kidney disease)

Other baseline data included; the mean dialysis adequacy (Kt/V) of the HD-CKD group was calculated to be 1.14 ± 0.28; the mean level of proteinuria in the non-HD CKD group was 210.9 ± 207.0 mg/mmol but was not available for the RTx group.

3.2.2 Longitudinal Study

3.2.2.1 Subjects

Subjects were patients drawn from the Main Haemodialysis Unit who consistently attended for their treatments three times per week.

3.2.2.2 Study Design

Patients from the King's College Hospital Main Haemodialysis Unit who fulfilled the inclusion and exclusion criteria listed in Table 3.3 were invited to participate in this exploratory, prospective, longitudinal study. Of approximately 120 patients in the dialysis unit, only 20 patients fulfilled the criteria and gave their informed consent to participate. Blood samples (10mL) were drawn before the commencement of each dialysis session for 3 weeks (9 samples) for measurement of hepcidin, and high-sensitivity C-reactive protein (hs-CRP). No alteration to the patients' pre-existing iron replacement regimen or ESA dose was permitted during the duration of this short study unless there were patients safety concerns. Baseline blood samples were taken as part of the routine clinical care of the patients. The same definitions for 'stable on dialysis' and 'haematological dyscrasia' applied to this study, as for the cross-sectional study (chapter 3.2.1.2, above).

INCLUSION CRITERIA	EXCLUSION CRITERIA
Dialysis patient in KCH Main Unit	Signs of acute/occult bleeding
≥18 years of age	Hospital admission within 4 weeks
Stable on dialysis for ≥3 months	History of haematological dyscrasia
Intravenous iron dose stable for ≥2 weeks	History of liver disease
ESA dose stable for ≥4 weeks	Active malignancy
Regular dialysis 3 times/week	

Table 3.3 Inclusion and exclusion criteria (longitudinal study)

Typical dialysis schedules involve patients attending three times per week for their treatments. As Sunday is the traditional rest day, dialysis schedules involve patients attending for their treatments on Monday, Wednesday and Fridays (MWF), or Tuesday, Thursday and Saturdays (TTS). The gap between sessions varies from 2 to 3 days, with the longer inter-dialytic gap (**c**, in Figure 3.1) inevitably occurring over the course of the weekend.

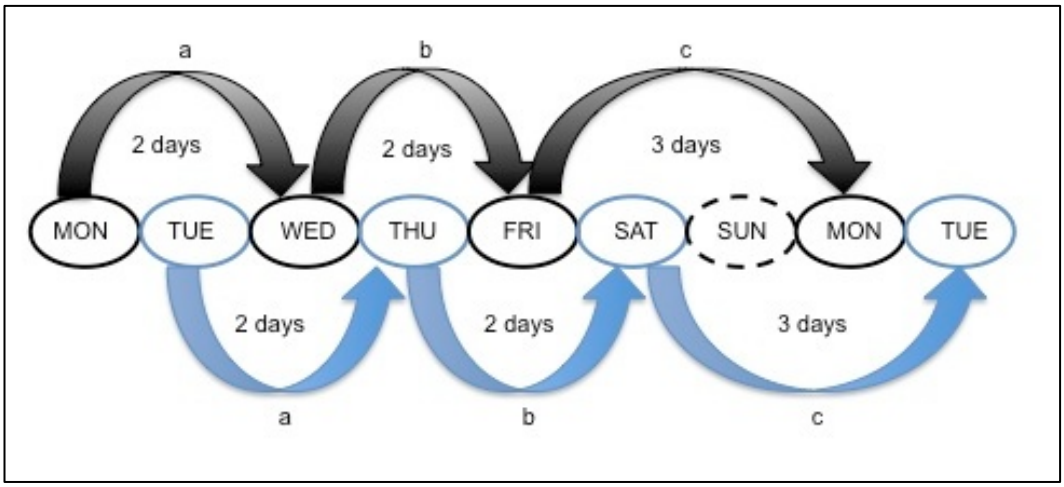

Figure 3.1 Maintenance haemodialysis scheduling – time between sessions
(**a** and **b** indicate a 2-day interval between successive dialysis sessions; **c** indicates a 3-day interval between dialysis sessions, occurring across the weekend)

As has previously been discussed, up to 50% of hepcidin is removed by dialysis although levels normalise shortly after the end of the session (Kuragano et al. 2010; Zaritsky et al. 2010). The importance of the day of the week on which samples were drawn, and amount of dialysis that each individual received were also taken into consideration. It was therefore reasonable to assume that the hepcidin levels in samples drawn on a Monday or Tuesday (following a three-day inter-dialytic gap) may have differed from samples drawn on other days of the week (following a two-day inter-dialytic gap) due to the nature of dialysis scheduling (Figure 3.1).

3.2.2.3 Demographics and Baseline Laboratory Data

A total of 20 patients were recruited to this study; 10 had their haemodialysis treatments on the MWF schedule, and 10 that had their treatments on the TTS schedule. The baseline demographic characteristics and laboratory data of the 20 participants in the longitudinal study are summarised in Table 3.4.

Age, years (mean ± SD)	63.0 ± 15.9
Males (n (%))	14 (70)
BMI (kg/m^2) (mean ± SD)	26.1 ± 4.8
Afro-Caribbean (n (%))	11 (55)
History of malignancy (n (%))	1 (5)
Haemoglobin (g/L) (median, IQR)	109 (98 - 118)
ESA use (n (%))	19 (95)
Epoetin dose (i.u./week) (median, IQR)	6000 (4000 – 9000)
IV iron supplementation (n (%))	12 (60)
IV iron dose (mg/week) (median, IQR)	100 (100 – 150)
Serum ferritin (ng/mL) (median, IQR)	591.5 (321.9 – 861.1)
C-reactive protein (mg/L) (median, IQR)	5.4 (0.8 – 18.9)
AV fistula/AV graft/tunnelled dialysis catheter (n)	8/5/7
Dialysis vintage (years)	4.1 ± 15.9
Dialysis adequacy (Kt/V)	1.07 ± 0.19

Table 3.4 Demographics and baseline laboratory data (longitudinal study)
(BMI – body mass index; ESA – erythropoiesis-stimulating agent; AV – arterio-venous)

Twenty HD-CKD patients were randomly selected to take part in this study and had been receiving dialysis for a mean of 4.1 (± 4.6) years. The patients were aged between 35 and 85, and 14 of the 20 patients were male. All patients were receiving dialysis thrice weekly, and all had been stable for a period of at least 3 months. All patients, bar one, were dialysed in the afternoon (between midday and 4 p.m.). None of the patients had been in receipt of antimicrobial therapy within the month preceding the commencement of the study, and similarly, none of the patients had a history of liver disease. Of the 20 participants, 95% had been on an ESA with a stable dose for greater than 4 weeks. Fewer patients were receiving IV iron; only 60% of the enlisted participants had IV iron administered within the 4 weeks prior to the commencement of the study, and this position was maintained for the duration of the study.

3.2.3 Healthy Volunteers

Staff working in the Renal Department were approached at random and asked to volunteer blood samples, which would be analysed for serum hepcidin. Twenty-three members of staff agreed to donate 10mL of whole blood, which was immediately processed. None amongst the 23 had a significant medical history and on that basis were assumed to be completely healthy. Thus, no other laboratory tests were conducted.

3.2.4 Laboratory Methods

All samples taken from arterio-venous fistulae and grafts were obtained immediately after the needles had been placed but before connection to the haemodialysis circuit. Patients with tunnelled dialysis catheters had 5mL of blood taken from each lumen (and discarded) before having 10mL of blood taken that was placed into Vacutainer® biochemistry tubes (containing silica and a serum separating gel with clot activator). The patients were then connected to the haemodialysis circuit. The study samples were allowed to rest at room temperature for 30 minutes, before being centrifuged at 10000 RPM for 10 minutes. Serum was decanted and aliquotted into cryotubes that were immediately transferred into the -80° Celsius freezer. Following the completion of the longitudinal study, these samples were couriered to two laboratories (King's College London, UK (for measurement of serum hepcidin using mass spectrometry), and the University of Bialystok, Poland (for measurement of serum hepcidin using ELISA). Two different operators processed the samples in a single run in order to minimise intra-assay variability. The methods (serum hepcidin measurement by mass spectrometry) employed in this study have been described previously ((Bansal et al. 2010)). The same samples were analysed by ELISA (Bialystok, Poland) using a commercially available kit (Bachem, UK). The reported normal range for this assay is 0.02–25 ng/mL, and both the calculated intra- and inter-assay variations were below 10%. High sensitivity CRP levels were determined using turbidemitry (P.Z. Cormay, Lublin, Poland).

In the cross-sectional study, the sample obtained from each of the 93 patients was divided into two and in addition to the samples sent for assessment of hepcidin levels, the second sample was sent to the central laboratory at King’s College Hospital for measurement of standard biochemical and haematological laboratory parameters. Serum ferritin was measured using a two-site immunoassay using a direct

chemiluminometric technique (Siemens Healthcare Diagnostics Ltd, UK). C-reactive protein was measured with ELISA (Wako Chemicals, Germany). Interleukin-6 was also measured with a commercially available ELISA kit (R & D, Abingdon, UK).

3.2.5 Statistical Analyses

Differences between study groups were evaluated in pairs with the independent Student's t test or the Mann-Whitney U test, depending of the normality of the distribution of the relevant variable. Frequencies of baseline categorical variables were calculated using Chi-squared tests. Relationships between various parameters under study were also looked at; simple linear coefficients (r) were calculated using the Pearson's product formula, and stepwise multiple linear regression analyses were also performed. $P < 0.05$ (two-tailed) were considered statistically significant. Data that were non-Gaussian were log-transformed if required for the purposes of linear correlation. Comparisons between 3 or more groups were undertaken using a one-way analysis of variance (ANOVA) with Tukey's corrections for multiple analyses.

The coefficient of variance (CV_1) for every individual was calculated from all nine hepcidin values acquired for each patient in the longitudinal study. The CV_1 was calculated using the standard deviation divided by the mean. Data were expressed as mean (±SD) or median (inter-quartile range, IQR) where appropriate. The Shapiro–Wilks test was used to test for normality of distribution. Comparisons between groups were performed using either a paired Student's t-test or Wilcoxon rank-sum test for normally and non-normally distributed variables, respectively. Bivariate correlation coefficients (r) were calculated using the Pearson's product formula. A P-value of <0.05 was considered statistically significant. Analyses were performed using Statistical Package for Social Science version 17.0 for Windows XP (SPSS Inc., Chicago, IL) and version 19.0 for the longitudinal study and the cross-sectional study, respectively.

3.2.6 Ethics and Regulatory Approvals

All patients gave informed consent for participation in the studies, which were conducted in accordance with the Declaration of Helsinki and approved by the London Research Ethics Committee 1 (LREC 09/H0718/034). Patient information leaflets were provided to all participants who gave their written informed consent.

3.3 Results

3.3.1 Hepcidin Levels in CKD Patient Groups and Healthy Volunteers

Serum hepcidin levels in the HD-CKD group were nearly double the level (139.9 ± 48.1 ng/mL) compared to either the non-HD CKD (62.9 ± 40.5 ng/mL) or the RTx (69.3 ± 35.0 ng/mL) groups ($p<0.001$ for both comparisons). The biggest difference was seen between the HD-CKD group and the healthy volunteers ($p<0.0001$). The mean hepcidin level of the 23 healthy volunteers was 44 ± 22 ng/mL (no other data available); this was significantly different compared to the RTx cohort ($p=0.004$), but not statistically different from the non-HD CKD group ($p=0.06$) (Figure 3.2).

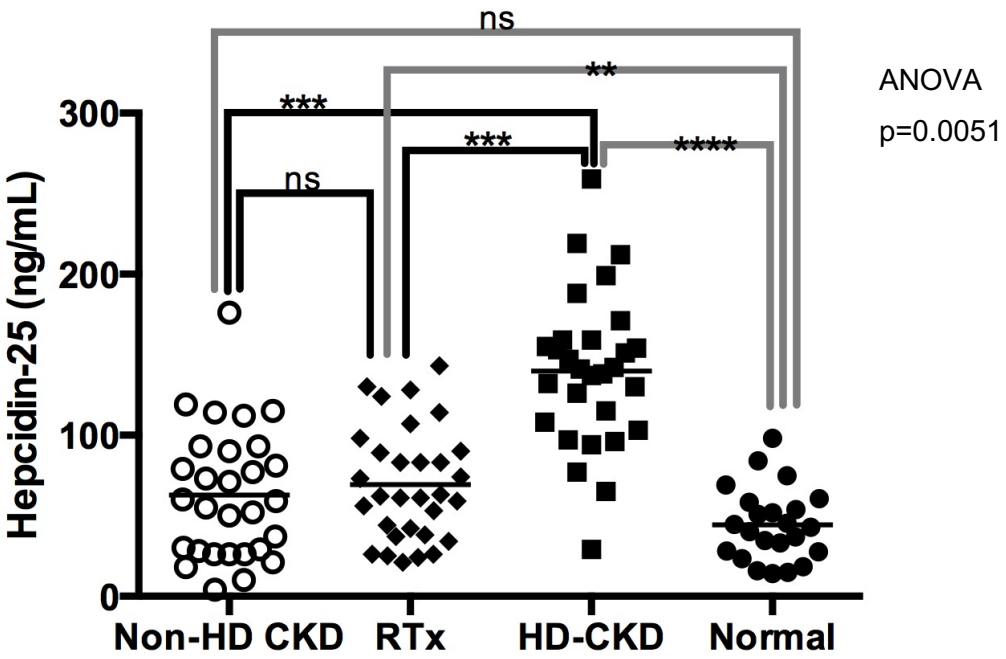

Figure 3.2 Mean hepcidin levels in 3 patient cohorts and healthy volunteers
(ns – not significant; * <0.05; ** <0.01; *** <0.001; **** <0.0001; black lines are comparisons within the 3 CKD cohorts; grey lines are comparisons between healthy volunteers and the 3 CKD cohorts)

There were no differences in the measured haematological indices between the non-HD CKD and RTx patients (Table 3.5). The mean haemoglobin level was 111 ± 11 g/L in the HD-CKD patients, which was lower than both the non-HD CKD patients (124 ± 14 g/L) and RTx recipients (125 ± 16 g/L) ($p<0.001$ for both comparisons). Serum ferritin levels were unsurprisingly higher in the haemodialysis group (558.8 ± 215.4 μg/L), given the protocolised administration of intravenous iron in this cohort of patients, compared to the non-HD CKD (126.9 ± 96.6 μg/L) and RTx groups (181.3 ± 147.7 μg/L) ($p<0.001$ for both comparisons). No significant difference was found

between the CRP levels in the three groups. In contrast, IL-6 levels were significantly higher in the haemodialysis group (8.39 ± 6.56 pg/mL) compared to the non-HD CKD group (4.77 ± 5.67, p=0.027) and the RTx group (4.80 ± 6.44, p=0.039). Albumin levels, which are of course another marker of inflammation, were lower in the HD-CKD patient group (38.7 ± 2.3 g/L), in comparison to the other two groups (non-HD CKD (42.5 ± 5.1 g/L) and RTx (41.5 ± 2.2 g/L) (p<0.001 for both comparisons)) (Table 3.5).

	Haemodialysis patients	CKD patients	Transplant patients	HD vs. CKD p value	HD vs. transplant p value	CKD vs. transplant p value
Haemoglobin, g/l	111 +/-11	124 +/-14	125 +/-16	<0.001	<0.001	0.731
Hypochromia, %	4.93+/-5.43	2.45+/-3.25	2.39+/-2.74	0.033	0.023	0.930
Reticulocyte count	77.2+/-34.4	83.2+/-31.8	79.2+/-31.7	0.483	0.816	0.624
Ferritin, μg/l	558.8+/-215.4	126.9+/-96.6	181.3+/-147.7	<0.001	<0.001	0.092
Hepcidin, ng/ml	139.9+/-48.1	62.9+/-40.5	69.3+/-35.0	<0.001	<0.001	0.515
CRP, mg/l	15.8+/-15.6	19.8+/-19.0	21.7+/-36.4	0.584	0.622	0.883
Interleukin-6, pg/ml	8.39+/-6.56	4.77+/-5.67	4.80+/-6.44	0.027	0.039	0.981
Albumin, g/l	38.7+/-2.3	42.5+/-5.1	41.5+/-2.2	<0.001	<0.001	0.318

Table 3.5 Biochemical and haematological results (cross-sectional study
Adapted from (Rumjon et al. 2012) (hepcidin measured by LS-LS tandem mass spectrometry)

3.3.2 Correlates of Serum Hepcidin With Other Variables

Simple linear regression analyses were undertaken in order to examine potential associations between the hepcidin level and relevant variables thought to impact on hepcidin levels. As eGFR levels were not required in the HD-CKD group, analyses to detect a correlation between renal function and hepcidin were undertaken. Serum hepcidin was not significantly associated with eGFR (r= -0.047, p=0.723). Total population analyses assessing the impact of inflammation on found no association - CRP (r= -0.254, p=0.175); IL-6 (r=0.132, p=0.227) (Figure 3.3). There was a significant inverse correlation seen with haemoglobin (r= -0.282, p=0.007), and a significant positive correlation seen with serum ferritin levels (r=0.649, p<0.001) (Table 3.6).

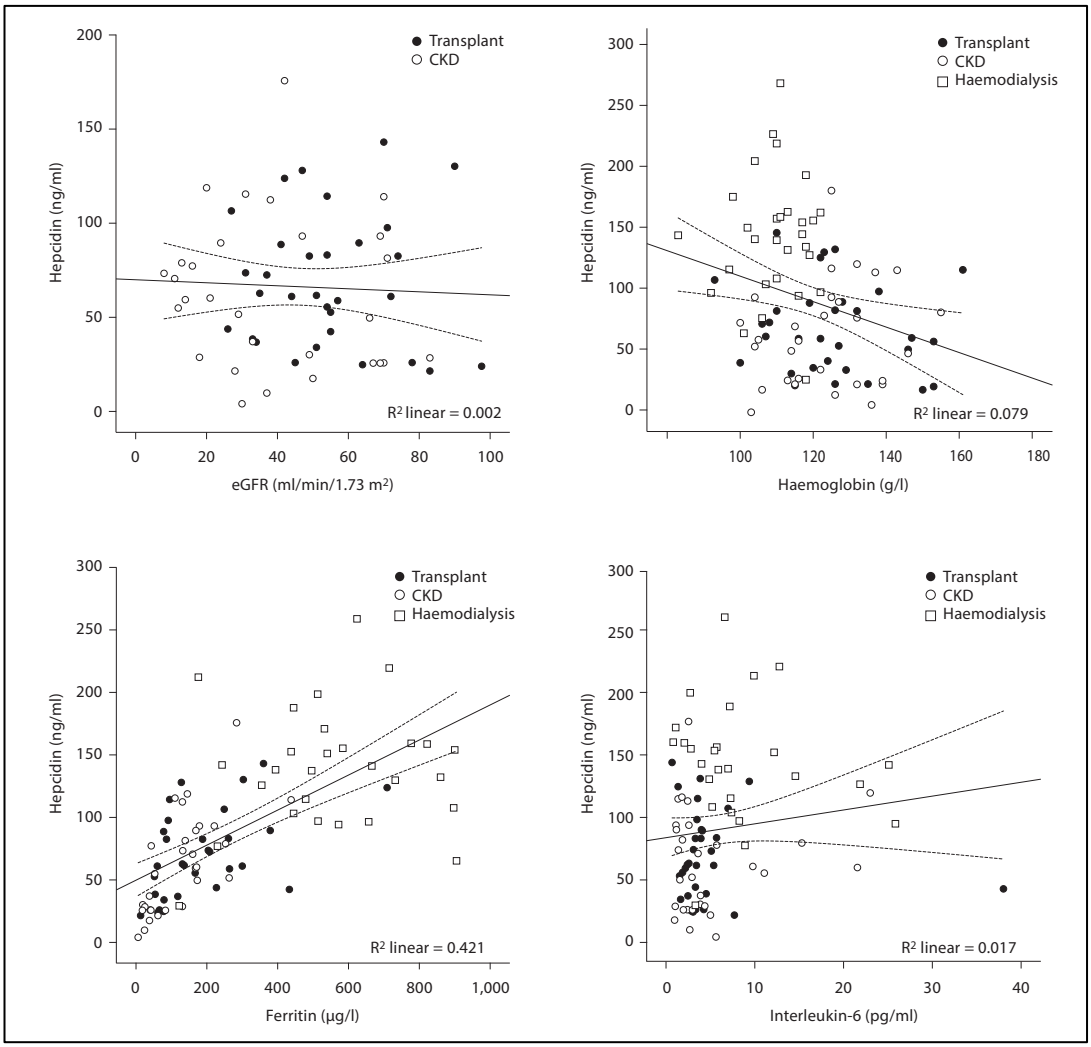

Figure 3.3 Correlations between serum hepcidin levels and eGFR, haemoglobin, ferritin and interleukin-6
Adapted from (Rumjon et al. 2012). Dashed lines indicate 95% confidence intervals.

To identify independent predictors of hepcidin variability, stepwise multiple linear regression analysis was performed in the total population studied, with hepcidin as the dependent variable and albumin, haemoglobin, ferritin, and IL-6 as independent variables in the model. As shown in Table 3.7, ferritin was the only variable that displayed an independent strong association with hepcidin levels.

	eGFR (mL/min/1.73m^2)	Haemoglobin (g/L)	Ferritin (µg/L)	CRP (mg/L)	Interleukin-6 (pg/mL)	Albumin (g/L)
All patients (n=93)*						
Hepcidin (ng/mL)	R^2= 0.002 *p*=0.724	R^2= 0.079 ***p*=0.007**	R^2= 0.421 ***p*<0.001**	R^2= 0.065 *p*=0.175	R^2= 0.017 *p*=0.227	R^2= 0.096 ***p*=0.003**
Haemodialysis patients (n=31)§						
Hepcidin (ng/mL)	----	R^2= 0.001 *p*=0.873	R^2=0.010 *p*=0.597	R^2= 0.048 *p*=0.494	R^2= 0.016 *p*=0.538	R^2= 4x10^{-6} *p*=0.993
CKD patients (n=31)						
Hepcidin (ng/mL)	R^2= 0.015 *p*=0.531	R^2= 0.035 *p*=0.331	R^2= 0.458 ***p*<0.001**	R^2= 0.030 *p*=0.607	R^2= 0.013 *p*=0.554	R^2= 0.056 *p*=0.216
Transplant patients (n=31)						
Hepcidin (ng/mL)	R^2= 0.0009 *p*=0.874	R^2= 0.026 *p*=0.383	R^2= 0.250 ***p*=0.004**	R^2= 0.496 *p*=0.077	R^2= 0.012 *p*=0.562	R^2= 0.043 *p*=0.261

Table 3.6 Pearson's correlation coefficients between hepcidin and the main biochemical and haematological parameters
(§HD-CKD patients were not included in correlational analyses of eGFR; p<0.05 emboldened)

Independent variables	beta	P value
Ferritin	0.691	<0.001
Albumin	-0.124	0.134
Haemoglobin	-0.012	0.887
Interleukin-6	-0.038	0.647
R^2	0.48	<0.001

Table 3.7 Coefficients of determination (beta) in multiple linear regression analysis including ferritin, albumin, haemoglobin, and IL-6 as independent variables and hepcidin as the dependent variable

3.3.3 Intra-Individual Variability in HD-CKD

Two assays (mass spectrometry and ELISA) were used to assess the variability of serum hepcidin levels in this study. Irrespective of the methodology used, both assays displayed a significant degree of biological variability in this patient cohort (Figure 3.4). The median CV_1 for the mass spectrometry assay was 23.0% (16.8 – 27.9), compared to a median of 23.3% (17.1 – 38.9) for the ELISA assay. There appeared to be no correlation with the assay used and the calculated CV_1. At the lower end of the range for calculated CV_1 levels, both assays performed equally as well. 40% of the patients with a CV_1 of less than 20% was seen in the use of both assays. A CV_1 between 20-25% was seen in 15% of patients using both assays. At greater CV_1 ranges, however, both assays performed equally well; at a CV_1 of 25-30%, the mass spectrometry assay accounted for 25% of the patients, compared to 10% with the ELISA. The situation was

reversed when examining CV_1 levels of greater than 30%; the ELISA accounted for 35% of the patients' results, compared to 20% with mass spectrometry. There was no statistical difference between the median CV_1 levels calculated by mass spectrometry compared to the ELISA assay.

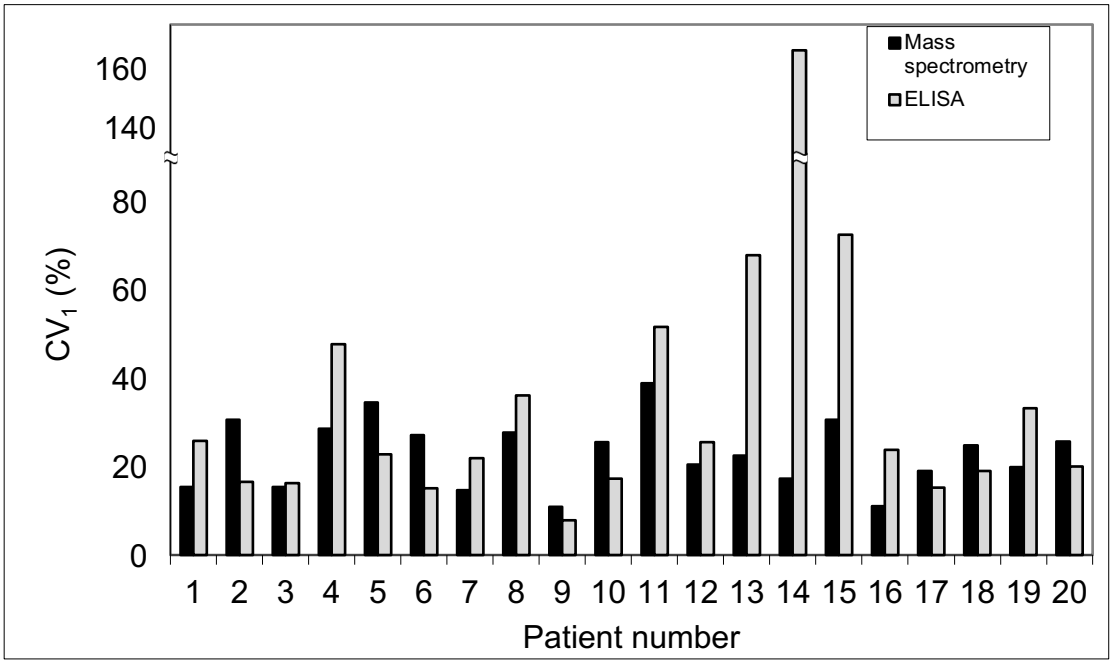

Figure 3.4 The coefficient of variance (CV_1) of serum hepcidin in 20 haemodialysis patients, using MS and ELISA

3.3.4 Inter-method Difference in Hepcidin Values

In contrast to the CV_1 results listed above, there was a statistical difference seen in the absolute hepcidin levels according to the type of assay used. The median hepcidin level in this haemodialysis cohort assessed by mass spectrometry was 168.2 (127.9 – 217.6) compared to 170.0 (107.0 – 238.8) when using the ELISA assay ($p = 0.03$). Two outlying ELISA hepcidin values of 4256.4 and 1372.1 ng/ml were obtained from a single patient, and these were confirmed on repeat testing (the mean hepcidin level of this patient's other 7 samples was 251.7 ± 170.9 ng/ml).

3.3.5 The Effect of IV Iron Administration on CV_1 and Hepcidin Levels

The CV_1 did not appear to be affected by the administration of IV iron. In the 12 patients receiving IV iron, the median CV_1 for hepcidin was 22.9% (19.7 – 31.5) and 23.3% (17.4 – 56.8) using mass spectrometry and ELISA, respectively. This compared to a median CV_1 of 25.9% (18.6 – 27.9) (MS) and 22.8% (15.4 – 34.0) (ELISA) in the remaining patients who did not receive IV iron. No statistically significant differences were observed in the CV_1 results of the patients in relation to whether they received IV iron or not, irrespective of the assay used (Table 3.8).

	Patients receiving IV Iron	Patients not receiving Iron replacement	p-value
n (%)	12 (60)	8 (40)	
Median CV_1 (MS)	22.9% (19.7, 31.5)	25.9% (18.6, 27.9)	0.77
Median CV_1(ELISA)	23.3% (17.4,56.8)	22.8% (15.4, 34.0)	0.29

Table 3.8 Effect of intravenous iron on the variability of serum hepcidin levels

Of the 12 patients receiving IV iron supplementation, 11 were having iron administered on a weekly basis across haemodialysis. This allowed hepcidin levels to be compared across three time-points: immediately before the administration of IV iron, and immediately prior to the subsequent two dialysis sessions. There was a trend towards slightly higher mean hepcidin levels measured at the beginning of the first subsequent dialysis session after the administration of IV iron, but overall there was no obvious impact of IV iron on serum hepcidin (Figure 3.5). The iron administration protocol in the dialysis unit necessitated the administration of thrice-weekly iron in those patients in whom serum ferritin levels were found to be below 100 ng/mL. This affected only one patient, and his hepcidin CV_1 values were similar to those of the other patients: 25.6% (MS) and 20.0% (ELISA).

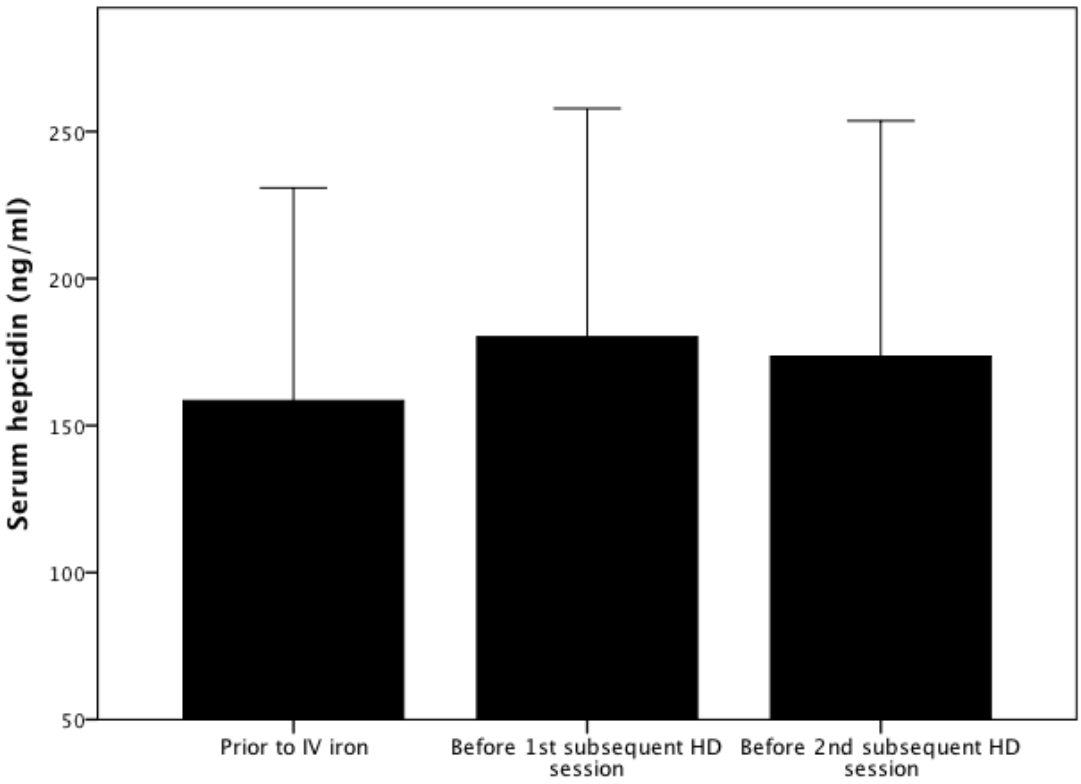

Figure 3.5 Mean hepcidin levels taken immediately before a dialysis session during which 100mg of IV iron sucrose was administered, and the subsequent two dialysis sessions (p=0.55)

3.3.6 Effect of Inflammatory Status on CV_1 and Hepcidin Levels

All samples of patient sera that had hepcidin measurements were also assessed for high-sensitivity CRP (hsCRP). The median hsCRP level was 6.1 (1.1 – 18.9) mg/L across the entire cohort over the three weeks of the study. The linear correlation between hepcidin (assessed by mass spectrometry) and hsCRP in this analysis was weak (r=0.15; p=0.04) (Figure 3.6). Mean serum hepcidin levels were assessed according to tertiles of hsCRP, and were slightly higher in the highest hsCRP tertile compared to the lowest hsCRP tertile [190.4±91.1 mg/L versus 161.1±50.7 mg/L, respectively (p=0.04)]. There was no correlation between the CV_1 for hsCRP and the CV_1 for hepcidin. Using the mass spectrometry assay, the Pearson's correlation between CV_1 hepcidin and CV_1 hsCRP was r=0.18 (p=0.10). For the ELISA assay, there was actually an inverse correlation between the CV_1 of hepcidin and hsCRP (r= -0.18, p=0.04).

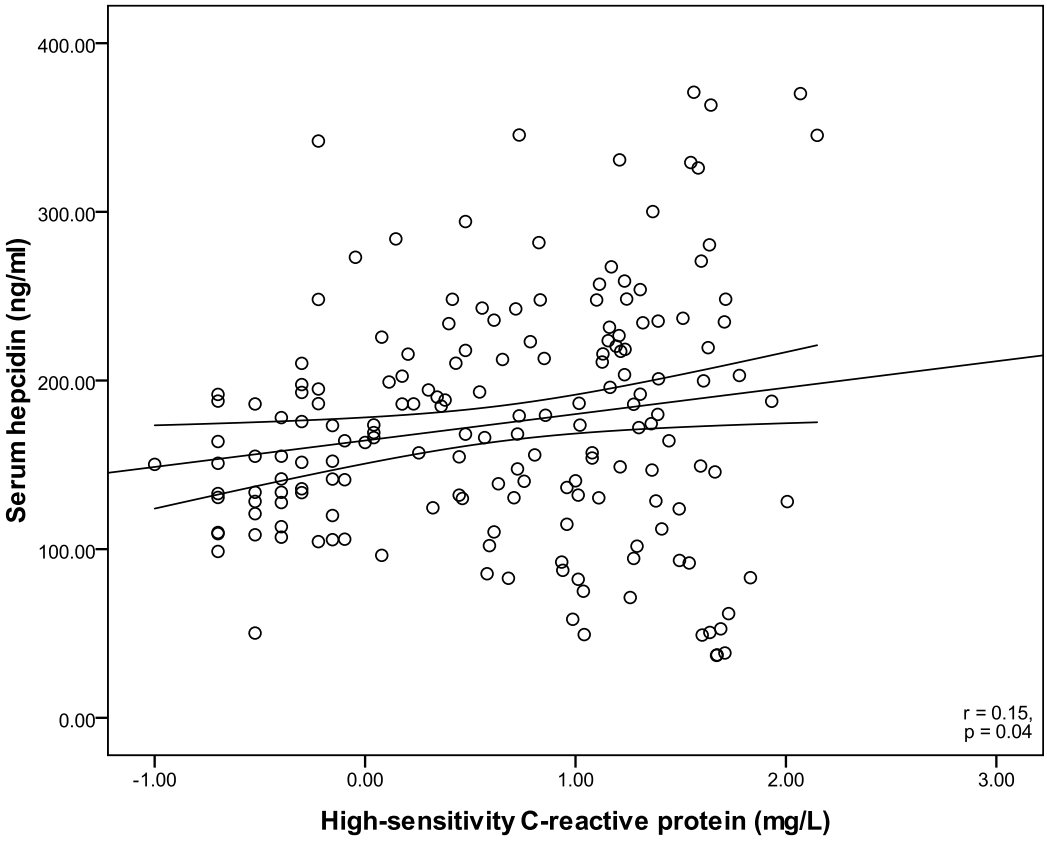

Figure 3.6 Correlation between hepcidin (MS assay) and high sensitivity CRP (log-transformed)

3.3.7 Effect of the Inter-dialytic Interval on Serum Hepcidin

As described earlier, three haemodialysis sessions per week inevitably leads two 2-day intervals and one 3-day interval between dialysis sessions (the latter following the weekend) (Figure 3.1). Hepcidin levels were found to be higher following a 3-day inter-dialytic interval compared to hepcidin levels following a 2-day inter-dialytic interval (Figure 3.7) (p=0.02, ANOVA). Median hepcidin levels on a Monday or Tuesday (the first dialysis session after the weekend/3-day inter-dialytic interval) were 182.8 (138.8 – 235.0) and 184.1 (111.9 – 267.7) ng/mL with mass spectrometry and ELISA, respectively. No associations were found between serum hepcidin, haemoglobin, dosage of epoetin or serum ferritin.

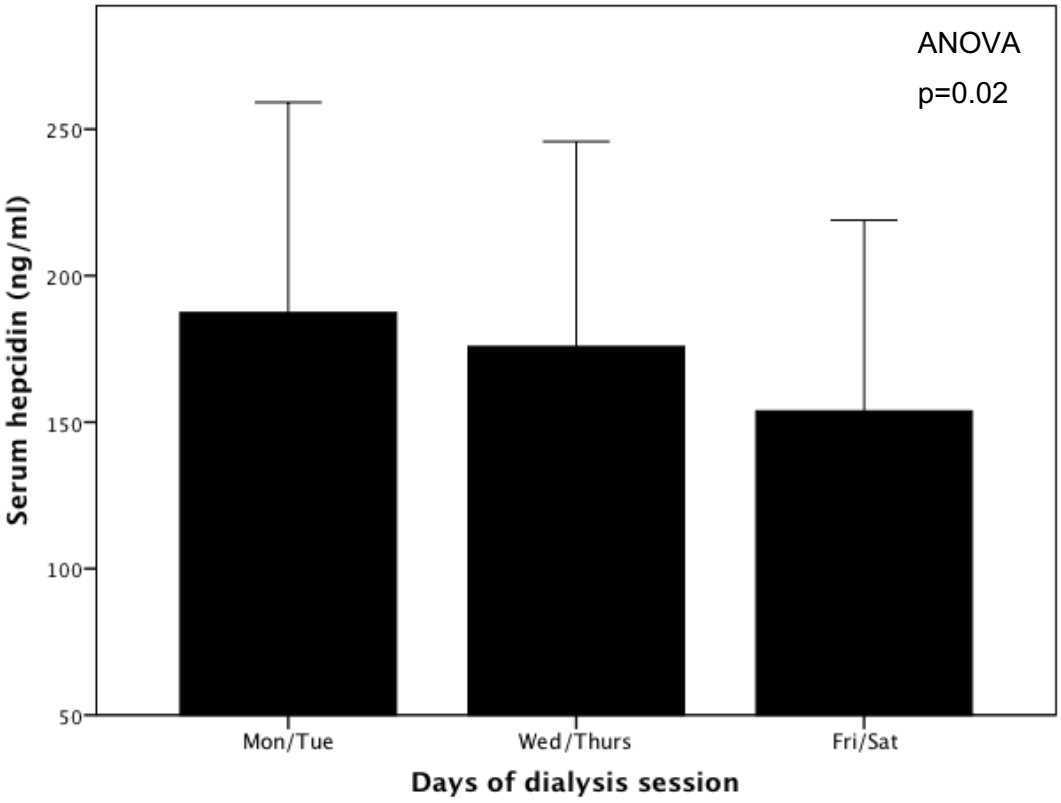

Figure 3.7 Mean hepcidin levels categorised by day of dialysis session

Baseline dialysis adequacy was obtained but was not available for each individual haemodialysis session. Two of the 20 participants had haemodiafiltration (one in each dialysis cohort), whilst the rest had standard haemodialysis. All participants used standard FX dialysers (Fresenius®) but the size of the dialysers was not captured. There was no statistical difference between the dialysis quantity (in litres processed during haemodialysis) between the dialysis administered between the two groups over the 3 combined sessions (ANOVA p=0.82).

3.4 Conclusions and Discussion

The main findings from the studies in this chapter were as follows;

1. Hepcidin levels were highest in the HD-CKD population, significantly higher than in non-dialysis CKD/transplant patients and healthy volunteers.
2. Hepcidin levels were increased in non-HD CKD and renal transplant patients. The transplant patients had significantly higher hepcidin levels compared to healthy volunteers.
3. In a small, clinically stable HD-CKD cohort, there appeared to be no impact of inflammation on hepcidin levels.
4. There was high variability of hepcidin levels in HD-CKD patients, irrespective of whether measured by mass spectrometry or ELISA.
5. In HD-CKD patients, pre-dialysis hepcidin levels were highest after the weekend interval.
6. The administration of 100mg iron sucrose appeared to have no significant effect on hepcidin levels in HD-CKD patients

In a well-matched cohort study, the highest serum hepcidin levels were found in the HD-CKD group (139.9±48.1 ng/mL), with a mean figure that was more than double that found in either the non-HD CKD or renal transplant groups. This was approximately 4 times the level found in a group of healthy volunteers garnered from within the renal unit. These findings are in keeping with the current literature on the subject. A number of previous studies have examined the levels of serum hepcidin in their respective HD-CKD patients, and with one exception, have results consistent with the findings from this cross-sectional study (Table 3.9).

Investigator	HD patients: n of subjects age (years)	Healthy controls: n of subjects, age (years)	Age and sex matching (HD vs. controls)	Method for hepcidin quantification	Hepcidin results
Tomosugi	40 Age n.a.	16 Age n.a.	n.a.	MS-based (semiquantitative)	Higher in HD
Zaritsky	33 60.3±20.7	24 28.4±6.6	Sex matched; Age unmatched	Competitive ELISA	Higher in HD (total hepcidin)
Campostrini	54 67±14	57 35±15	Sex matched; Age unmatched	MS-based (quantitative)	Higher in HD
Valenti	65 65.5±12	57 35±15	Sex matched; Age unmatched	MS-based (quantitative)	Higher in HD
Ashby	94 64.6 (39.2–83.0)	64 Age n.a.	Sex and age unmatched	Radioimmunoassay (RIA)	Higher in HD (total hepcidin)
Peters	48 61±15	24 39±12	Sex and age unmatched	MS-based (quantitative)	Higher in HD
Tessitore	56 67±14	57 35±15	Sex matched; Age unmatched	MS-based (quantitative)	Higher in HD
Ghoti	21 63.4±12.9	63 63.4±12.9	Sex and age matched	MS-based (quantitative)	Higher in HD
Costa	33 59.5±17.6	17 Age n.a.	n.a.	MS-based (quantitative)	Higher in HD
Kurugano	198 58±11	33 34±8	Sex and age unmatched	MS-based (quantitative)	Higher in HD
Pelusi	155 64.3±14	188 60±17	Sex and age matched	MS-based (quantitative)	Similar in HD and controls

HD, chronic haemodialysis ; MS, mass spectrometry

Table 3.9 Studies comparing serum hepcidin-25 levels (unless stated) between adult haemodialysis patients and healthy controls
Adapted from ((Valenti et al. 2014))

Of the studies referred to in Table 3.9, Pelusi and colleagues performed the only study with results that contradict ours. One of the major strengths of the Pelusi study was the attention to detail in matching for age and gender between the control subjects and the HD-CKD patient cohort. Control subjects who had a serum ferritin level lower than 30/40ng/mL (females and males, respectively) were excluded. This enabled patients with sub-clinical iron-deficiency (a strong upregulator of hepcidin) to be excluded from their study. They also included HD-CKD subjects with a median serum ferritin that was approximately half the levels seen in the HD-CKD patients from the cross-sectional cohort (265ng/mL [155-411] vs 558.8±215.4ng/mL, respectively). In using patients from a haemodialysis unit favouring a policy of relatively low iron supplementation, they included patients who were less likely to be iron overloaded. Pelusi and colleagues also sampled hepcidin from their haemodialysis patients after a 3-day inter-dialytic period in order to minimise the clearance effect of dialysis on hepcidin levels (citing the evidence from the longitudinal study in this chapter). They concluded that there was no

substantive difference in hepcidin levels between well-matched HD-CKD patients and healthy controls, but in essence they showed that there was no difference in iron-replete healthy controls and relatively iron-deplete HD-CKD patients (compared to our HD-CKD patients) (Pelusi et al. 2013). The difference between the Pelusi study and the data from this study may be explained by the difference in ferritin levels alone.

There was a very strong association seen in this study between ferritin and hepcidin in the non-HD CKD and the renal transplant patients, but this was not seen in the HD-CKD patients (Table 3.6). Therefore, based on these data, and extrapolating the study methods of Pelusi and colleagues to the cross-sectional study population, exclusion of low-ferritin healthy controls would likely adjust the mean hepcidin levels of the healthy controls upwards. This would not necessarily be the case in the HD-CKD population, however, as the correlation between serum ferritin and hepcidin in the cross-sectional haemodialysis group had an r-value of 0.104 ($p=0.597$); thus any adjustment or selection of patients based on ferritin levels, should in theory, leave hepcidin levels unaffected.

One limitation with respect to the healthy controls used in this study is that they were assumed to be healthy. This was a reasonable assumption in light of the fact that the hepcidin levels in this group were in keeping with previous control data, but a more robust control group would have been from healthy kidney donors.

In contrast to the HD-CKD population, there have been fewer studies examining hepcidin levels in renal transplant recipients and in non-HD CKD patients. The transplant and non-HD CKD groups in the cross-sectional study had patients with a mean eGFR of 54.3±18.4, and 40.2±24.9 mL/min, respectively, but there was no statistical difference in the mean hepcidin levels between these two groups. Despite this, there was an interesting difference comparing hepcidin levels in the two non-HD cohorts against healthy volunteers. The non-HD CKD cohort had hepcidin levels that were comparable to healthy volunteers ($p=0.06$). However, the transplant group had a level that was higher in comparison ($p=0.004$). As hepcidin is largely excreted via the kidneys, this observation could be considered counterintuitive, but studies examining the relationship between eGFR and hepcidin have not conclusively proven a direct relationship between the two (Peters et al. 2010). What is not known is the extent to which hepcidin is renally excreted in chronic kidney disease. Peters and colleagues

showed that in healthy individuals, the fractional excretion of urinary hepcidin is in the region of 2% and there is a 10-15-fold increase in the hours following coronary artery bypass surgery. They also showed a 4-fold increase in the fractional excretion of urinary hepcidin in moderate kidney disease (CKD stage 3a, non-proteinuric patients with a mean eGFR of 57mL/min) and it is conceivable that the upregulation of urinary hepcidin excretion may account for the non-linear relationship between eGFR and hepcidin, although data examining urinary hepcidin in advanced CKD is currently lacking (Peters et al. 2013).

A possible confounder was the difference in prevalence of diabetes in the 3 cohorts in the cross-sectional study. Although there was no statistically significant difference between the groups (a possible type 2 statistical error), the actual percentage of diabetic patients in the HD-CKD group was 32.3%, which was nearly 50% higher than the number in the RTx group. There were nearly double the number of diabetic patients in the RTx group (22.6%) compared to the non-HD CKD group (12.9%) and this may have contributed to the higher hepcidin level seen in the RTx group. The relationship between diabetes and hepcidin is complex, as there are conflicting reports suggesting both positive and negative associations between diabetes and levels of hepcidin in patient cohorts. It must also be considered that excessive iron loading is linked with the development of diabetes. Vela and colleagues have recently reviewed this and concluded that in type 2 diabetes mellitus, there are 2 distinct phenotypes seen; one subset of patients has low levels of hepcidin linked with insulin resistance, whilst the other subset has high levels of hepcidin, linked with chronic inflammation, morbid obesity, and of course, renal impairment (Vela, Sopi, and Mladenov 2018). This is a potentially significant confounder and in retrospect, should have been corrected for, but these data were not known/published at the outset of the studies in this chapter. Although the 3 groups were age- and gender-matched, the use of a propensity matching algorithm would have potentially reduced the confounding variables.

The results of the longitudinal study suggest that there is significant intra-individual variability of serum hepcidin in chronic haemodialysis patients. This is consistent with a previous report by Ford and colleagues (Ford et al. 2010). One limitation of their study was the use of an ELISA to measure hepcidin. This is known to cross-react with hepcidin fragments (hepcidin-20 and hepcidin-22), and the contribution of this lack of specificity to their findings of hepcidin variability was impossible to ascertain. In the

longitudinal study, however, we utilized a mass spectrometry assay for biologically active hepcidin-25 and confirmed significant hepcidin variability. Our study design differed from that of Ford and colleague in that serum hepcidin was measured before every dialysis session, we also attempted to examine factors that could potentially influence hepcidin levels (IV iron administration and the degree of inflammation), as well as controlling for epoetin and iron administration. With the exception of an early drop-out (no data obtained) we were fortunate that no patient encountered any intercurrent morbid events such as infection, surgery, hospitalisation, or acute cardiovascular events during this 3-week period.

The hepcidin results obtained using MS were generally lower than those obtained using the ELISA. This is likely to be due to the fact that the ELISA detects the smaller isomers of hepcidin as well as biologically active hepcidin-25. Nevertheless, as in previous studies, hepcidin levels in haemodialysis patients were consistently elevated compared to normal healthy individuals. Both assays showed a similar degree of hepcidin variability, and the median CV_1 values were almost identical for MS versus ELISA (Table 3.8).

Higher hepcidin levels were seen following a 3-day inter-dialytic interval versus a 2-day interval, possibly due to greater generation of hepcidin during the longer time period between dialysis sessions. No difference existed in the amount of dialysis (in terms of litres processed) each patient received, and it would appear that the time difference between dialysis sessions had a minor impact on variability. In order to further verify the impact of dialysis and the time between dialysis sessions on hepcidin levels, the analyses could have been strengthened by taking into account the patients' residual renal function (using inter-dialytic urine collection and pre- and post-dialysis blood sampling) as well as the extent to which hepcidin was actually cleared by measuring dialysate hepcidin. Malyszko and colleagues produced data suggesting that the presence of residual renal function influenced hepcidin levels in both peritoneal and haemodialysis patients (Malyszko et al. 2009). If the patients in our cohort were uric, then the measurement of urinary hepcidin (excretion) may have determined whether residual renal function was a contributor to increased hepcidin variability.

The use of a cohort of haemodialysis patients was considered on the basis that this group of patients were readily available thrice-weekly allowing regular blood sampling

as required. The use peritoneal dialysis patients may have been a better group to study, as they have fewer interventions, the process of dialysis is less pro-inflammatory, and they have regular peritoneal equilibration testing (and potentially easier access to testing effluent hepcidin levels). In a very recent study by Lim and colleagues, both incident haemodialysis and peritoneal dialysis patients were prospectively evaluated for a period of 6 months. There were no significant differences between the baseline data between the 2 groups. After 6 months' follow-up, a number of differences emerged between the 2 groups; in the HD group, the use of CERA and IV iron was higher, whilst the use of diuretics was higher in the PD group. Haemoglobin levels were significantly higher in the PD group (106 ± 10 g/L vs 100 ± 10 g/L, p=0.01) as was hepcidin (115.9 ± 26.9 vs 101.7 ± 24.4, p=0.008). Both AST and ALT were higher in the PD patients whilst serum albumin was higher in the HD group. Aside from the iron biomarkers (ferritin, TSAT) that unsurprisingly correlated well with hepcidin levels, urinary production in early haemodialysis patients was an independent predictor of hepcidin levels (Lim et al. 2019). Hepcidin levels were highest in PD patients and this was also observed by Zaritsky and Niikura and colleagues. Comparing hepcidin in the HD dialysate and PD effluent may have strengthened these studies and would have also added to the studies undertaken in this chapter (Zaritsky et al. 2009; Niikura et al. 2018).

It has previously been demonstrated that inflammation (acute and chronic), IV iron, and epoetin administration influence hepcidin levels (Ashby et al. 2010). As in our parallel study by Peters and colleagues (Peters et al. 2012), we were unable to demonstrate a convincing association between CRP and hepcidin levels, in contrast to the findings of Ford and the multivariate analyses by Zaritsky (Zaritsky et al. 2009). We found only a very weak correlation with hsCRP in this study, excluding a major impact of inflammation on hepcidin variability. Interestingly, using a different mass spectrometry assay and a different study design, Peters and colleagues found very similar CV_1 values (26%, 17-48) to those seen in our study (24%, 17-28) (Peters et al. 2012).
In general, the age- and gender-matching between the 3 CKD groups in the cross-sectional study were well carried out. The longitudinal study was conducted prospectively, and nearly half of the patients did not receive IV iron (previously reported as a major confounder in this patient population). Moreover, this cohort was observed closely for intercurrent morbid events that could impact on variability – no overt events were seen. The studies were not without limitations, however. The lack of

characterisation of the healthy control group did not allow for matching of subjects as performed by Pelusi and colleagues. The small sample size (n = 20) and the relatively short duration of assessment (3 weeks) in the longitudinal study were its main limitation. Most of the subjects dialysed in the afternoon shift; it is not clear whether the data reported here would be applicable to patients dialysing in the morning or evening, although Ashby and colleagues did not find a diurnal variation of hepcidin levels in HD-CKD patients (Ashby et al. 2010).

In conclusion, and in concordance with the results by Peters and colleagues, we have demonstrated that there is considerable variability in serum levels of hepcidin-25 in a clinically stable cohort of haemodialysis patients, using a mass spectrometry assay. No major impact of IV iron or inflammatory status was observed, although a minor effect of length of intra-dialytic interval was seen. These data have implications for studies examining factors affecting hepcidin levels, and also for studies investigating the utility of serum hepcidin as a biomarker of iron status in haemodialysis patients.

4 HAMP mRNA in PBMCs From CKD Patients

4.1 Introduction and Aims

Serum hepcidin levels in CKD patients are increased compared to normal healthy controls and (as shown in the previous chapter) even within the spectrum of CKD, significant differences are seen. Thus, patients on regular haemodialysis have several-fold higher hepcidin levels compared to non-dialysis CKD and transplant recipients. The regulation of hepcidin is complex with a number of factors known to increase hepcidin, while other factors have been shown to decrease hepcidin levels. At a molecular level, this is mediated through its interaction with ferroportin, the major iron exporter in mammalian cells (Nemeth, Tuttle, et al. 2004). The intracellular signalling mechanisms that regulate *HAMP* expression (the gene controlling hepcidin production) vary according to the stimulus. Iron increases BMP-6, which in turn activates the hepcidin promotor via SMAD 1/5/8 (Andriopoulos et al. 2009). Inflammation, and specifically IL-6, activate the IL-6 receptor that in turn acts on the JAK2/STAT3 pathway to increase hepcidin expression (Nemeth, Rivera, et al. 2004).

The clinical conditions known to increase hepcidin levels include administration of blood transfusions and iron, as well as acute and chronic inflammation. Those in which hepcidin levels are decreased include anaemia, and ESA therapy (mediated by erythroferrone), hepatic viral infections, and administration of vitamin D or testosterone therapy. Several of these clinical states may be applicable to chronic haemodialysis patients, particularly iron and ESA therapy as well as chronic inflammation (Girelli, Nemeth, and Swinkels 2016).

Haemodialysis patients are known to be chronically inflamed. The primary disease process leading to kidney dysfunction may be a factor (e.g. autoimmune diseases, vasculitis or amyloidosis), and comorbidities such as diabetes or peripheral vascular disease may also act as a substrate for inflammation (Jaar et al. 2000). Other factors that contribute to the inflammatory milieu the haemodialysis population include reduced renal clearance of cytokines, susceptibility to bloodstream infections (e.g. endocarditis or discitis), sub-clinical infections (e.g. periodontal disease), and dialysis-related factors (Jofre et al. 2006). Dialysis may induce the release of pro-inflammatory cytokines during the treatment and reactive oxygen species/oxidative stress (Spittle et al. 2001; Tarakcioglu et al. 2003). The link between inflammation and hepcidin was seen when subjects injected with lipopolysaccharide mounted a rapid inflammatory response in

concert with a rise in hepcidin levels , likely mediated by interleukin 6 via STAT3 intracellular signalling (Kemna et al. 2005; Wrighting and Andrews 2006). In the haemodialysis setting, researchers have previously shown that interleukin-6 is higher than in healthy controls, but was not necessarily influenced by hepcidin (Kuragano et al. 2010).

The administration of iron in both oral and intravenous forms has been shown to increase hepcidin levels in the non-dialysis dependent CKD population, although the greatest response was seen in patients receiving a high dose intravenous iron preparation. Baseline hepcidin levels did not appear to predict the patients' response to iron therapy (Gaillard et al. 2016). In the haemodialysis setting, there is an extremely strong positive relationship between hepcidin and ferritin levels, which has been demonstrated on a number of occasions as well as in the previous chapter of this thesis (Weiss et al. 2009).

Being a small peptide, hepcidin is freely filtered via the glomerulus with greater than 95% reabsorption by the proximal tubule (Swinkels et al. 2008). Thus, an increase in hepcidin levels could also be mediated via reduced clearance of the peptide as for other similarly sized molecules (e.g. inulin). Early studies using ELISA did indeed show an inverse correlation between eGFR and hepcidin (Troutt, Butterfield, and Konrad 2013). Later studies using mass spectrometry showed that hepcidin-25 levels were actually independent of eGFR, although other hepcidin isoforms such as hepcidin-20 were shown to accumulate in renal failure (Peters et al. 2010). To further explore the impact of GFR on hepcidin levels, it was good to study non-HD/HD CKD patients who have low clearance and to investigate whether it was possible to use a surrogate for hepatic production of *HAMP* mRNA. The expression of the gene (*HAMP*) responsible for hepcidin production in circulating macrophages was one mechanism that researchers in Oxford employed to determine that Plasmodium falciparum infected erythrocytes induced hepcidin production (Armitage et al. 2009). We therefore employed this methodology as a surrogate marker of hepcidin production in our CKD population.

The aim of the work in this chapter was to therefore measure *HAMP* mRNA in PBMCs isolated ex vivo from CKD patients to see whether there was any correlation with serum hepcidin levels.

4.2 Subjects and Methods

4.2.1 Subjects

Subjects were selected from various areas of the Department of Renal Medicine at King's College Hospital. These included 3 cohorts of CKD patients, as follows:

Cohort 1 - CKD (non-HD CKD) patients who attended the Renal Outpatient Clinic

Cohort 2 - Haemodialysis (HD-CKD) patients from the Main Haemodialysis Unit

Cohort 3 - Septic haemodialysis patients that were emergency admissions to the ward

In addition, a random group of healthy volunteers working within the department provided control samples (cohort 4).

4.2.2 Study Design

This was an exploratory study using a prospective, cross-sectional design. The data from cohorts 1 and 2 (CKD, both non-HD and HD, respectively) would be compared to data obtained from the healthy controls. Cohort 3 HD patients were assessed at baseline presenting with presumed sepsis and were then followed up prospectively with a blood sample taken after 28 days, at a time when there was a reasonable expectation that they had recovered from the initial septic insult (Figure 4.1). Being an exploratory study, there was no formal pre-specified statistical plan was employed, and no power calculation conducted. To obtain meaningful data a minimum of 6 patients were to be recruited to each arm of the study. Blood samples were analysed for serum hepcidin, and other standard laboratory variables, and PBMCs were extracted to allow measurement of *HAMP* mRNA.

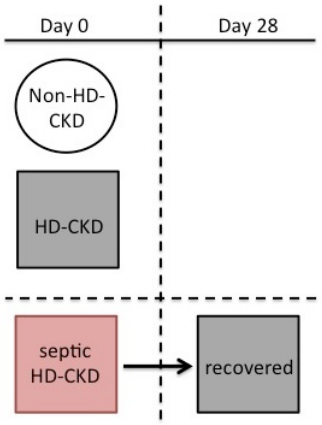

Figure 4.1 Study design – time points for data collection

4.2.3 Cohort 1 - Non-dialysis CKD Patients

Non-dialysis CKD patients were randomly approached prior to their routine general nephrology outpatients' appointment. The laboratory inclusion criteria were identical to the HD-CKD patients in cohort 2, whereas the exclusion criteria differed only in respect of previous ESA and IV iron use (Table 4.1). Non-dialysis CKD patients had an extra 10mL of blood taken for study purposes at the end of their routine outpatient clinic visit in addition to any routine blood samples that had been requested by their clinician.

INCLUSION CRITERIA	EXCLUSION CRITERIA
Haemoglobin >100g/L	History of ESA use
Ferritin 200-500 ng/mL	History of IV iron use
C-reactive protein <20mg/L	Signs of acute/occult bleeding
	Hospital admission within 4 weeks
	History of haematological dyscrasia
	History of liver disease
	Active history of malignancy

Table 4.1 Inclusion and exclusion criteria for cohort 1

4.2.4 Cohort 2 - Haemodialysis CKD Patients

The KCH RenalWare database was interrogated to find patients who matched the inclusion criteria, and the patient notes were examined to filter out those patients who had a number of pre-specified exclusion criteria (Table 4.2). Patients were considered to be stable according to the clinical judgement of the investigator. Exclusion criteria included a history of haematological dyscrasias, defined as any disease process that necessitated the patient having visited the Haematology department at any time for treatment or follow-up (except for iron-deficiency anaemia). Examples of disease conditions commonly encountered in the typical renal patient population included myeloma, MGUS, and the myelodysplastic syndromes. Patients who had been hospitalised for any reason within 4 weeks of the commencement of the study were excluded, but all haemodialysis patients who participated had been stable on dialysis for considerably longer than 4 weeks. Potentially eligible patients were approached to assess their willingness to participate in the study and if so, then full written consent was obtained. A single extra blood sample (10mL) was then taken at the beginning of their dialysis session, following insertion of the dialysis needles, but prior to connection to the haemodialysis circuit.

INCLUSION CRITERIA	EXCLUSION CRITERIA
Dialysis patient in KCH Main Unit	Signs of acute/occult bleeding
Haemoglobin >100g/L	Hospital admission within last 4 weeks
Ferritin 200-500 ng/mL	History of haematological dyscrasia
C-reactive protein <20mg/L	History of liver disease
ESA and iron dosage stable for ≥1 month	Active history of malignancy
Definitive dialysis vascular access	

Table 4.2 Inclusion and exclusion criteria for cohort 2

4.2.5 Cohort 3 - Septic Haemodialysis CKD Patients

Unlike the groups outlined in 4.2.4 and 4.2.3 above, the patients included in this cohort were not pre-selected, but were considered eligible following an emergency admission to the Renal Ward with presumed bacterial sepsis. Only then were they approached for inclusion into the study following review of the pre-specified criteria outlined below (Table 4.3). Patients in this cohort were not eligible for the study if they had a medical history that included a chronic inflammatory state such as inflammatory bowel disease, or rheumatoid arthritis.

INCLUSION CRITERIA	EXCLUSION CRITERIA
Dialysis vintage >3 months	Blood transfusion within 1 month
C-reactive protein >100 mg/L	Other known chronic inflammatory condition
Presenting fever of ≥ 37.8 °C	Active hepatitis/HIV infection
	Active liver disease
	Active history of malignancy

Table 4.3 Inclusion and exclusion criteria for cohort 3

4.2.6 Cohort 4 – Healthy Volunteers

Staff working in the Renal Department were approached at random and asked to volunteer blood samples, which would be analysed for serum hepcidin and *HAMP* mRNA. Nine members of staff agreed to donate 10mL of whole blood, which was immediately processed. None amongst the 9 had a significant medical history and on that basis were assumed to be completely healthy. Thus, no other laboratory tests were conducted.

4.2.7 Demographics and Baseline Laboratory Data

The baseline demographic characteristics and laboratory data of cohorts 1, 2 and 4 are summarised in Table 4.4.

	Cohort 1 Non-HD CKD	**Cohort 2 HD-CKD**	**Cohort 4 Controls**
Number of participants	10	7	9
Age (years)	62.9 ±19.2	55.5 ±19.5	44.0 ± 15.0
Males (n)(%)	6 (60.0)	5 (71.4)	5 (55.5)
Haemoglobin (g/L)	125 ± 22	113 ± 8	
eGFR (ml/min)	32.9 ± 13.8		
CRP (mg/L)	5.3 ± 4.1	10.4 ± 5	
Albumin (g/L)	43.3 ± 1.7	39.7 ± 1.8	
Ferritin (ng/mL) **(med/IQR)**	322 ± 359 132 (74-430)	367 ± 90	
Hepcidin (ng/mL)	32.9 ± 31.7	149.1 ± 27.3	26.9 ± 8.6
Weekly IV iron dose (med/IQR)		100 (0-100)	
ESA dose (iu/kg/week) **(med/IQR)**		46.2 (18.9-114.6)	

Table 4.4 Baseline demographic and laboratory data for cohorts 1, 2, and 4
(data expressed as mean ± standard deviation, unless otherwise specified. Grey boxes indicate that data were not available/applicable)

A total of 17 patients were recruited to cohorts 1 and 2, as well as 9 healthy controls. Cohort 1 (non-HD CKD) were slightly older with a mean age of 62.9 ±19.2, compared to a mean age of 55.5 ±19.5 in cohort 2 (HD-CKD). Amongst the healthy volunteers in cohort 4, the youngest volunteer was 24 and the eldest was 70. Despite this wide range, the mean age of this cohort was lower than for the other two cohorts, at 44.0 ± 15.0 (ANOVA, $P<0.001$). The representative mean eGFR for the non-HD CKD patients was 32.9 ± 31.7, equivalent to CKD stage 3b. The mean haemoglobin in this cohort was 125 ± 22 g/L – not supported by iron or ESA therapy – which could be considered to be within the normal range. Notwithstanding the relatively few patients in this study, the baseline biochemical data from cohorts 1 and 2 were largely in keeping with that obtained from the larger CKD cohorts, reported in chapter 3 (page 99). As had previously been observed, the mean haemoglobin and albumin levels were lower in the HD-CKD group than in the non-HD CKD group ($P<0.001$ for both comparisons), whilst

the mean CRP and hepcidin levels were higher in the HD-CKD group than in the non-HD CKD group (P<0.001 for both comparisons) (Figure 4.2). The only observed difference in the baseline biochemical variables between these cohorts and the cohorts in chapter 3 was in the mean ferritin levels between the two CKD groups. The distribution of serum ferritin levels amongst the 10 non-dialysis CKD patients was non-Gaussian and heavily influenced by the presence of 2 outliers, and hence no statistical difference was observed (Figure 4.2).

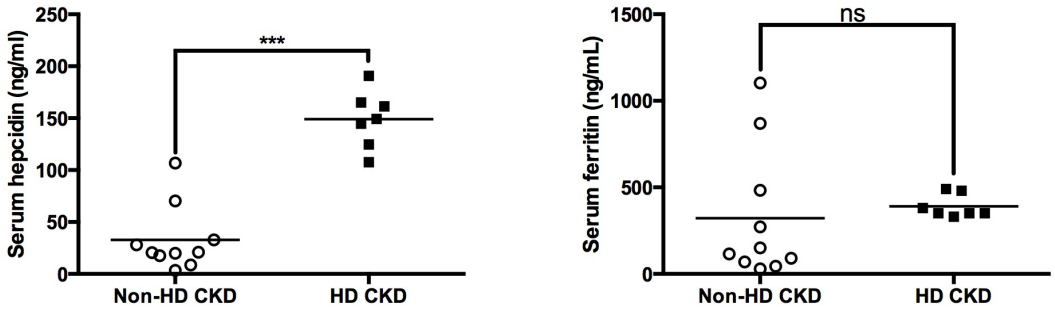

Figure 4.2 Baseline serum hepcidin and ferritin levels in non-HD (cohort 1) and HD (cohort 2) CKD patients

The baseline demographic characteristics and laboratory data of the septic HD-CKD participants (cohort 3) are summarised in Table 4.5.

Number of participants	10
Age (years)	61.1 ± 11.9
Males (n)(%)	6 (60)
Haemoglobin (g/L)	100 ± 19.5
CRP (mg/dL)	221.5 ± 116.9
Albumin (g/L)	34.9 ± 2.3
Ferritin (ng/mL)	1702 ± 1271
Hepcidin (ng/mL)	95.0 ± 55.5
Weekly IV iron dose (med/IQR)	100 (0-100)
ESA dose (iu/kg/week) (med/IQR)	56.3 (30.4 – 76.2)

Table 4.5 - Baseline demographic and laboratory data for septic HD patients (cohort 3)
(data expressed as mean ± standard deviation, unless otherwise specified)

In total, 10 HD-CKD patients were admitted to the renal ward and fulfilled the broad inclusion and exclusion criteria outlined in Table 4.3. Of these, 6 were male and the mean age of the cohort was 61.1 ± 11.9 years. Being septic, the mean CRP of the cohort was very high and this no doubt influenced the serum ferritin levels, the mean of which was more than 3 times greater than that seen in the (non-septic) HD-CKD patients in cohort 2. This observation did not extend to hepcidin, however, which did not seem to be as responsive to sepsis as ferritin. The mean hepcidin level was 95.0 ± 55.5 ng/mL and the mean albumin level was 34.9 ± 2.3 g/L (Figure 4.3).

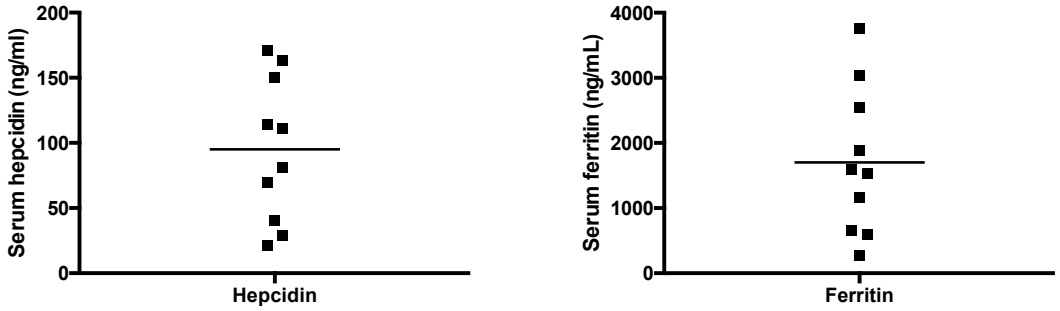

Figure 4.3 Baseline serum hepcidin and ferritin levels in septic HD patients (cohort 3)

4.2.8 Laboratory Methods

The methods of hepcidin measurement by mass spectrometry have been discussed in detail elsewhere (Bansal et al. 2010). Routine patient blood samples for were processed in the King's College Hospital central laboratory. The method for *HAMP* mRNA measurement will be briefly revisited but is covered comprehensively on page 79.

***HAMP* mRNA measurement**

- 10mL blood drawn (and divided into two) and separated by Ficoll-Hypaque Plus (GE Healthcare) centrifugation
- Plasma was obtained post-centrifugation and immediately stored in 500μL aliquots
- Two sets of $5x10^6$ peripheral blood mononuclear cells were isolated for each participant
- Total cellular RNA was subsequently extracted (RNeasy, Qiagen) and immediately stored at -80°C

- 500ng RNA was reverse transcribed (High Capacity RNA-to-cDNA Kit, Applied Biosystems)
- All cDNA samples were processed in a single run to eliminate inter-experimental variability
- Amplification reactions were performed using Taqman *HAMP* and GAPDH primer-probes in triplicate
- Quantitative RT-PCR was performed using the 7900 HAT Fast Real-Time system (Applied Biosystems)

4.2.9 Statistical Analyses

Differences between the haemodialysis, non-dialysis CKD and healthy control groups were determined with the independent Student's t test. Relationships between various parameters under study were also examined; simple linear coefficients (r) were calculated using the Pearson's product formula. P-values <0.05 (two-tailed) were considered statistically significant. Analyses were performed using GraphPad Prism version 6.0 for Macintosh, GraphPad Software, La Jolla California USA.

4.2.10 Ethics and Regulatory Approvals

The London Research Ethics Committee 1 (LREC 09/H0718/034) granted ethical approval. Local R&D approval was obtained to undertake this study, which was conducted in accordance with the principles outlined in the Declaration of Helsinki. Patient information leaflets were provided to all participants prior to obtaining their written informed consent.

4.3 Results

4.3.1 *HAMP* mRNA Levels in Non-HD and HD CKD Patients

Despite haemodialysis patients having markedly higher circulating hepcidin levels, *HAMP* mRNA levels were considerably lower in cohort 2 (HD-CKD patients) compared to cohorts 1 (non-HD CKD patients) and 4 (healthy controls). The distribution of the *HAMP* mRNA results was greatest in the non-HD cohort; this was in contrast to the HD cohort, where the distribution of results was much tighter. It was noted that the participant with the highest *HAMP* mRNA level in cohort 2, had a level that was lower than the mean *HAMP* mRNA in either of the other two cohorts. In contrast to what might have been predicted, *HAMP* mRNA was significantly lower in the HD patients in cohort 2 (p=0.026). Despite the graphical representation below (fold-change as opposed to absolute values), there was no difference in *HAMP* mRNA levels between the non-HD and HD cohorts of CKD patients (p=0.4) (Figure 4.4).

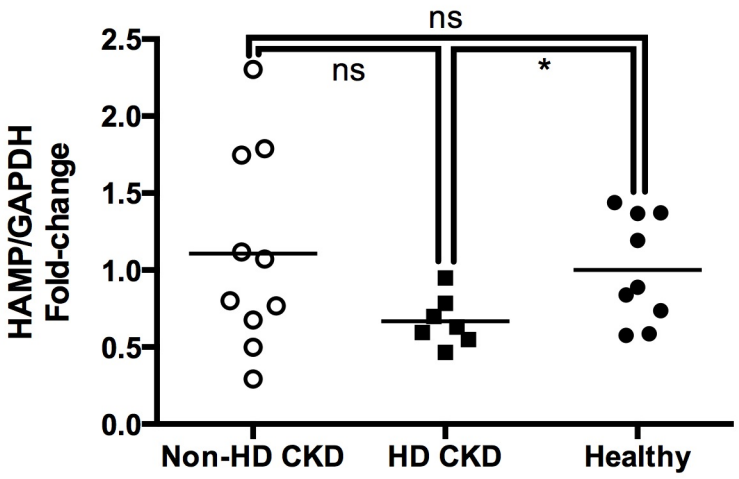

Figure 4.4 *HAMP* mRNA levels in CKD cohorts and healthy volunteers
(* Comparison between HD CKD cohort and healthy volunteers, p=0.026)

4.3.2 Effect of Hepcidin on *HAMP* mRNA

There was no correlation between the total circulating levels of hepcidin with *HAMP* mRNA levels in either the haemodialysis group or the non-HD CKD group separately (Figure 4.5a, Figure 4.5b) or when both were combined (Figure 4.5c).

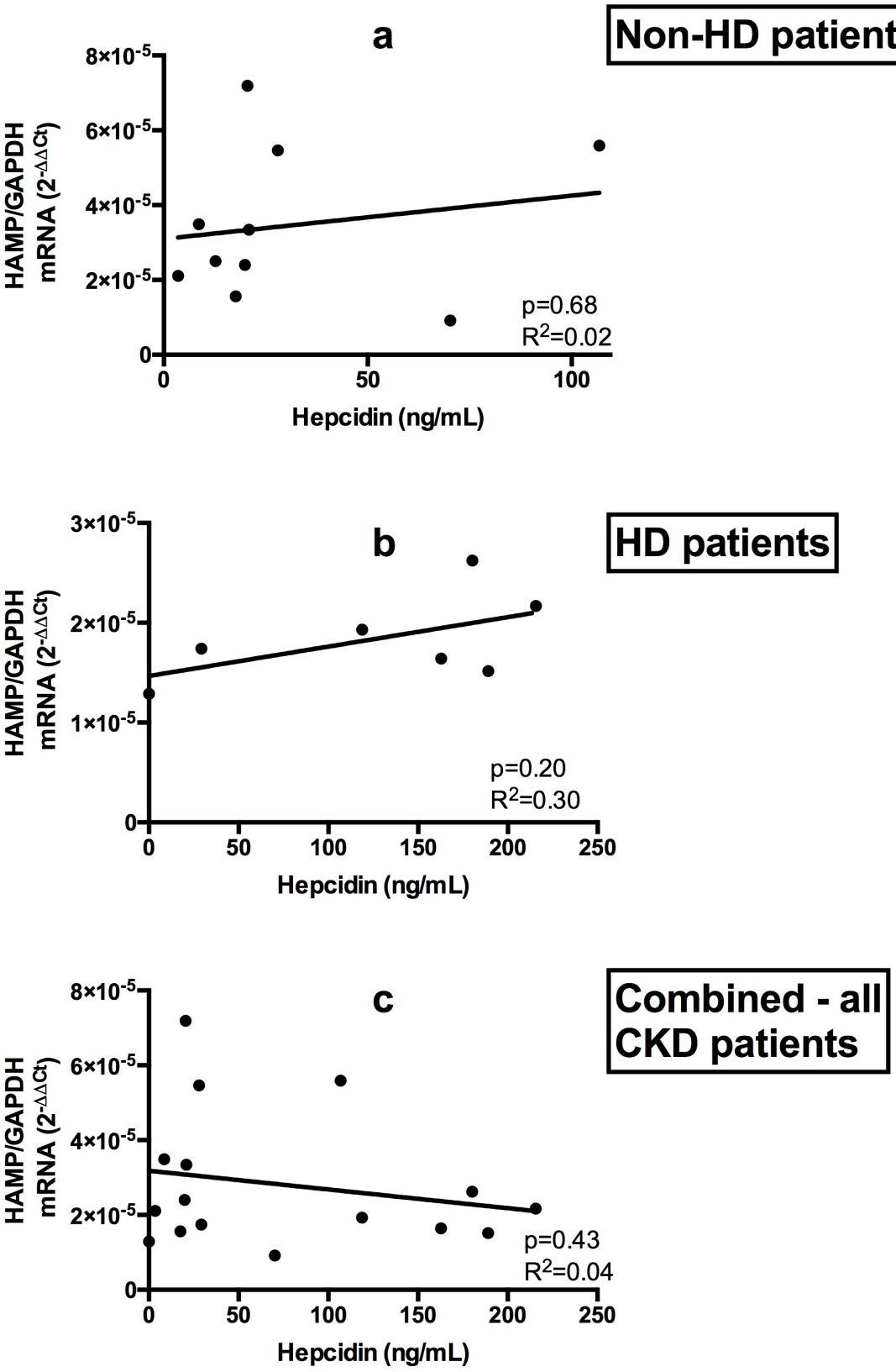

Figure 4.5 Correlations between hepcidin and *HAMP* mRNA
a – cohort 1; b – cohort 2; c – cohorts 1 and 2 combined

4.3.3 Effect of Ferritin on *HAMP* mRNA

A similar pattern was seen when examining the effect of ferritin on *HAMP* mRNA. No correlation was seen between serum ferritin levels and *HAMP* mRNA levels in either the HD-CKD group or the non-HD CKD group separately (Figure 4.6a, Figure 4.6b) or when both were combined (Figure 4.6c).

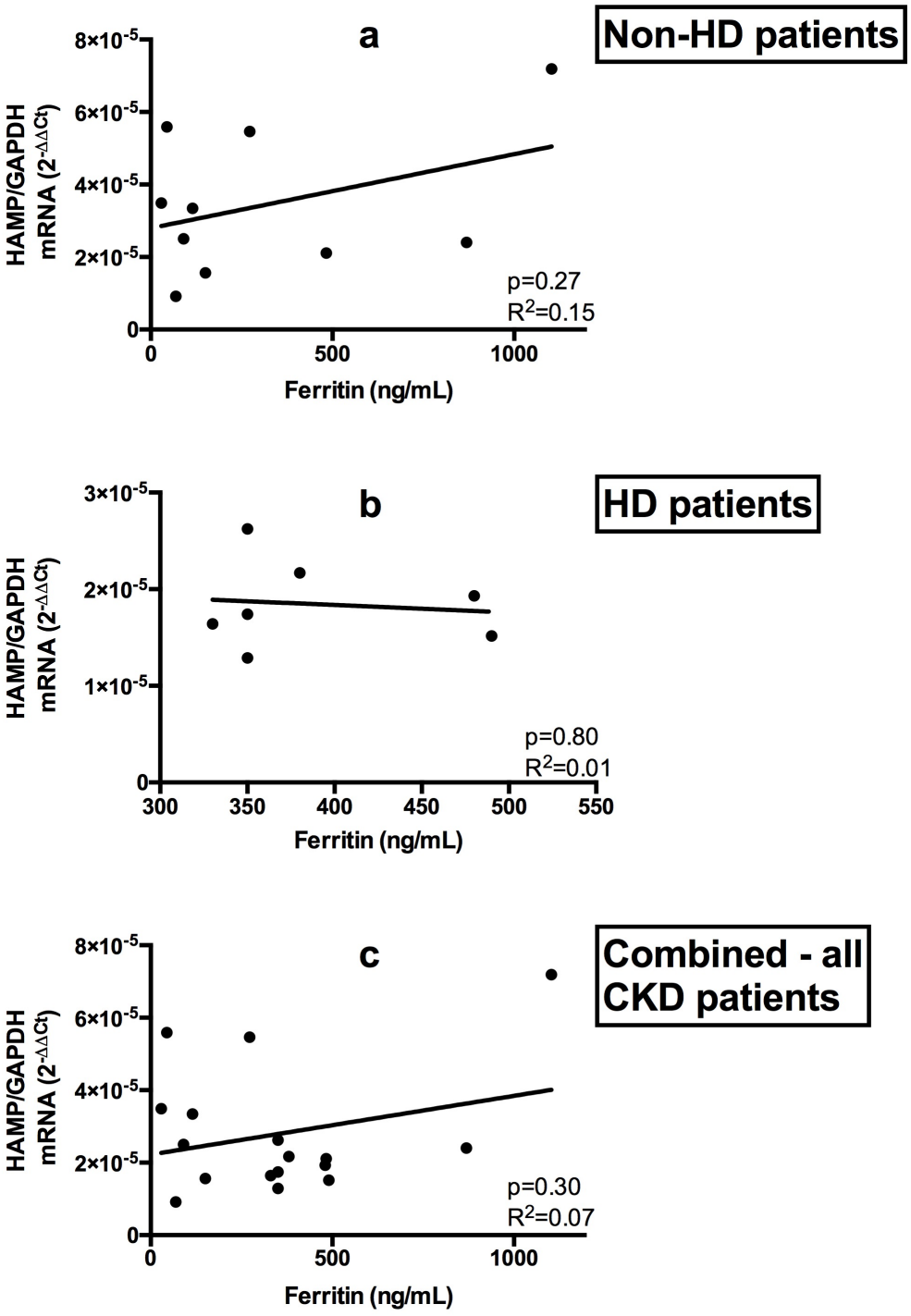

Figure 4.6 Correlations between ferritin and *HAMP* mRNA
a – cohort 1; b – cohort 2; c – cohorts 1 and 2 combined

4.3.4 Does Sepsis Influence Hepcidin Levels in HD-CKD Patients?

Blood samples were taken within 12 hours of the patients' admission to the renal ward and were processed immediately. A second sample was taken after 28 days, and the results from these two time-points can be seen in Table 4.6.

	Baseline	Recovery (28 days)	p-value
Haemoglobin (g/L)	100 ± 19.5	102 ± 8.6	0.80
CRP (mg/L)	221.5 ± 116.9	32.4 ± 22.2	<0.001***
Albumin (g/L)	34.9 ± 2.3	36.4 ± 3.1	0.23
Ferritin (ng/mL)	1702 ± 1271	919.3 ± 442.6	0.03*
Hepcidin (ng/mL)	95.0 ± 55.5	76.9 ± 58.5	0.09

Table 4.6 Laboratory results at baseline and recovery

As expected in patients with bacterial sepsis, there was a dramatic fall in the CRP and ferritin levels (Figure 4.7 a,b), In contrast, there was no change in the mean hepcidin levels (Figure 4.7c) between baseline and after 28 days. Four patients appeared to have a fall in hepcidin levels, but 5 showed a slight increase or no change (Figure 4.7d). There was no significant change in haemoglobin or albumin levels.

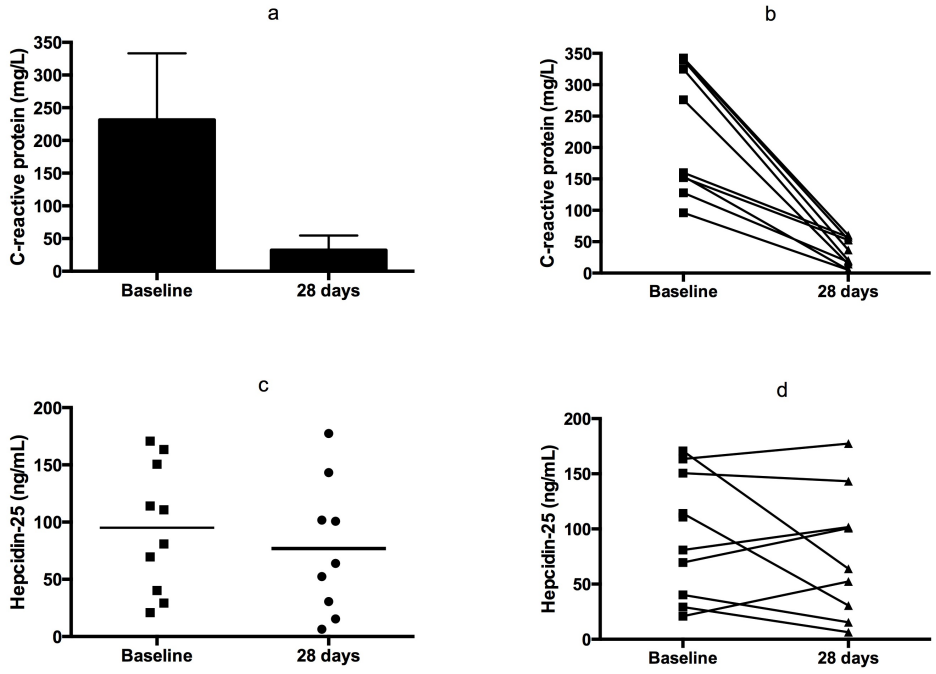

Figure 4.7 CRP and hepcidin levels at baseline and recovery
(a – mean CRP level; b – individual data; c – mean hepcidin level; d – individual data)

4.3.5 Correlation Between Hepcidin and CRP

At baseline when patients were septic, there was no correlation seen between hepcidin and CRP levels (Figure 4.8a). In contrast, after 28 days, a correlation between hepcidin and CRP levels emerged (R^2 0.62, p=0.01) (Figure 4.8b).

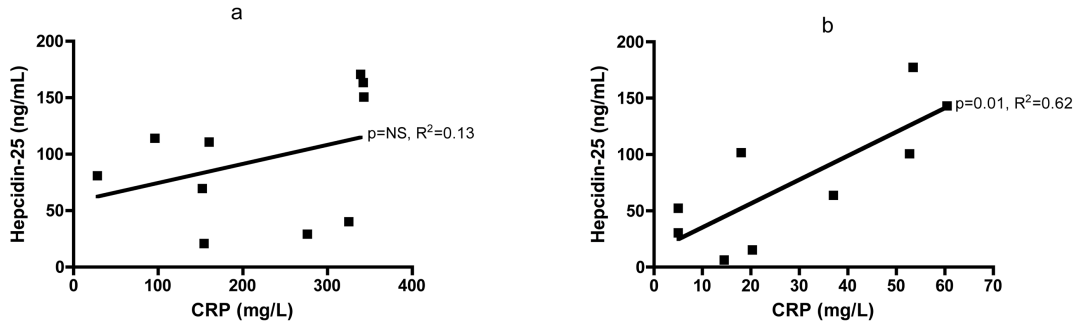

Figure 4.8 Correlation between hepcidin and CRP at (a) baseline and (b) 28 days

4.3.6 *HAMP* mRNA Levels – Septic HD Patients

Unfortunately, due to a combination of time factors and experimental methodological failure, *HAMP* mRNA levels were available in 7 patient samples at baseline and only 4 patient samples at 28 days. Technical failure in extracting PBMCs occurred in 3 of the baseline samples. At the 28-day time-point, all the patients had been relocated to their satellite units. For 3 of the patients, the time taken from blood sampling to PBMC extraction exceeded 2 hours, which is associated with unstable *HAMP* expression (5.3.1), hence the data were unreliable. Technical failure in extracting PBMCs occurred in 3 samples. Since only 4 *HAMP* mRNA levels were available at 28 days, it was impossible to draw meaningful comparisons with the baseline data Figure 4.9.

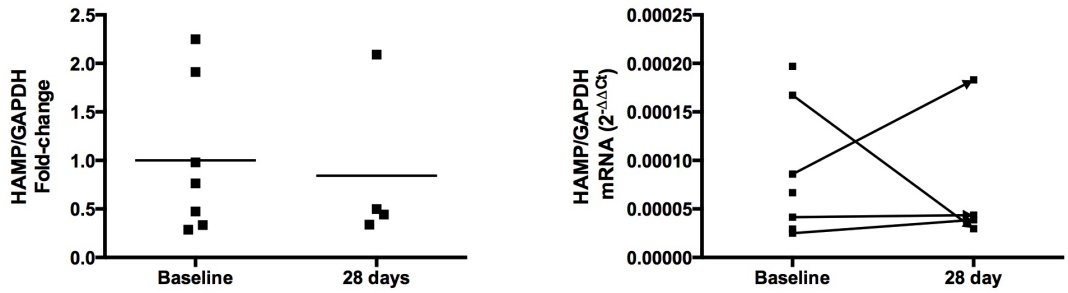

Figure 4.9 *HAMP* mRNA in septic HD patients at baseline and 28 days
(mean data (left) and individual data points (right))

The baseline data were compared to *HAMP* mRNA from HD-CKD patients and healthy volunteers (Figure 4.10).

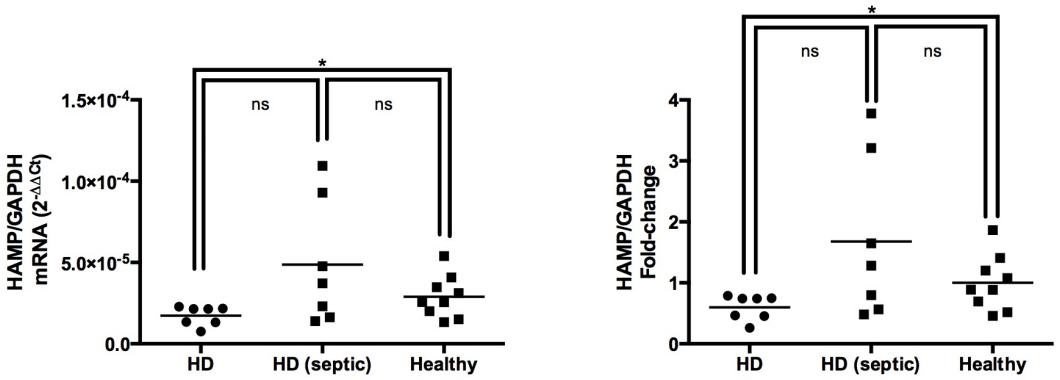

Figure 4.10 *HAMP* mRNA levels in HD patients (septic and stable) and healthy individuals
(absolute values (left) and fold-change (right); *comparison between stable HD patients and healthy individuals, p=0.046; comparison between septic HD and stable HD patients, p=0.06)

The mean *HAMP* mRNA level in the septic HD cohort was higher than the other two cohorts, but the comparisons did not reach statistical significance. There was a trend towards higher *HAMP* mRNA levels in the septic HD cohort at baseline compared to their non-septic counterparts (p=0.06) and healthy controls.

4.4 Conclusions and Discussion

The main aim of the work presented in this chapter was to elucidate possible mechanisms of increased hepcidin levels in HD patients using a previously published methodology of *HAMP* mRNA production in PBMCs as a surrogate for increased hepcidin production. Ideally, hepatocytes would have been used but this was not possible in the clinical setting. The secondary aim was to determine whether high inflammatory states affected hepcidin levels in these patients, who already had a very high basal level of circulating hepcidin.

The following conclusions can be made:

1. Serum hepcidin levels are generally higher in HD-CKD patients compared to non-HD CKD patients and healthy controls.
2. The data of *HAMP* mRNA do not support increased production of hepcidin.
3. There is no correlation between *HAMP* mRNA and hepcidin suggesting that the PBMC ex vivo model may be not sensitive or robust enough to detect increased hepcidin. Clearly, increased *HAMP* mRNA production and generation in HD could still be present in the liver, but this would be difficult to determine in a human model.
4. A trend towards higher *HAMP* mRNA in septic HD patients at baseline suggests possible upregulation of hepcidin production in PBMCs after a bacterial insult.
5. No striking effect or a reduction in hepcidin levels after sepsis in HD patients as is the case for CRP and ferritin. This may be because hepcidin levels in HD patients are already at their maximum, and/or that hepcidin kinetics are different from CRP and ferritin in sepsis. In contrast, *HAMP* mRNA levels were significantly reduced compared to non-HD CKD and healthy controls. Whether this suggests a negative feedback mechanism on *HAMP* mRNA production is impossible to prove but remains a possibility.

The finding was even more striking for the fact that this occurred in few subjects with markedly high levels of circulating hepcidin, suggesting a possible negative feedback mechanism to account for this (Figure 8.1).

Chronic haemodialysis patients are continually exposed to a milieu of low-level inflammation and this is one of the mechanisms thought to contribute to the elevated levels of hepcidin in this group of patients. They are also susceptible to bacterial sepsis

and so it was interesting to see that despite having extremely high markers of inflammation, the hepcidin levels in septic haemodialysis patients were no higher than their basal levels once they had recovered from their respective septic episodes. This suggests that inflammation not playing a particularly major role in the high hepcidin levels seen in haemodialysis. The *HAMP* mRNA levels in the septic haemodialysis group were marginally higher than the uninflamed group that were used as a control, but certainly not dramatic.

There are no previous studies on the effect of sepsis of hepcidin in HD-CKD, though there are two that examine this effect in non-CKD patients. A Dutch group studied 92 adult patients (13 of whom had chronic kidney disease, which was not defined further) with bacterial sepsis. Hepcidin was highest on the day of admission, and had fallen by half by the third day (van Eijk et al. 2011). A neonatal study was undertaken in very low birth weight babies and a similar pattern was found. Hepcidin was highest at admission, and had fallen to normal levels (similar to non-septic neonates) after 2 weeks (Wu et al. 2013).

One possible unifying explanation for the findings in this chapter is that circulating hepcidin levels in haemodialysis patients are at their maximum level, and there is limited capacity for additional hepcidin production, even when an acute inflammatory/infective state supervenes. The PBMC *HAMP* data also support this hypothesis, as there is suppression at the mRNA level. Confirmation of this data would require a greater number of subjects, and possibly more stringent inclusion and exclusion criteria to eliminate as many confounders as possible e.g. the temporary cessation of ESA and iron therapy.

The data presented here are not without limitations. The very use of haemodialysis patients as an experimental model introduced a number of possible confounders that were difficult to control for, especially with the increasing number of factors that are now known to positively and negatively affect hepcidin production (Langer and Ginzburg 2017). This was an exploratory study, and the number of patients may have been too small to detect certain effects, such as differences in *HAMP* mRNA levels in the septic haemodialysis group. It was also unfortunate that methodological issues resulted in sub-optimal numbers of samples being obtained from cohort 3 in their recovery phase for *HAMP* mRNA analysis. The use of circulating macrophages as a

surrogate marker for hepcidin production may be a practical approach to this issue but is clearly less than ideal given that the main cells producing hepcidin are hepatocytes, and there is no known data on PBMC *HAMP* mRNA in the renal setting. Obtaining hepatocytes from haemodialysis patients was briefly considered but the idea was rapidly rejected given the potential safety and ethical issues. The logic for selecting PBMC *HAMP* as a surrogate for liver *HAMP* was based on hepcidin upregulation being an integral mechanism in innate immunity and the importance of PBMCs as an essential component of the innate immune system's response to infection, and so, assessing PBMC responses to IL-6 stimulation/inflammation was considered apposite. This approach is consistent with other researchers in the arena of hepcidin regulation, who have used also PBMC methodology as a surrogate marker for liver *HAMP* in the absence of access to primary *in vivo* hepatocytes. I am not aware of the existence of any published pre-clinical models/studies that have been produced, which prove the correlation between neutrophil and hepatic cellular *HAMP* responses to infectious/iron stimuli.

5 Cytokine Regulation of *HAMP* in Hepatoma Cells and PBMCs

5.1 Introduction and Aims

Mammals do not possess regulatory processes that allow iron to be excreted from the body, and thus, iron homeostasis is very tightly controlled via a process of absorption, recruitment and recycling of existing iron stores. Cellular iron flow is completely controlled by membrane-bound ferroportin, which in turn is under the (probably exclusive) control of its ligand, hepcidin. In the presence of increased hepcidin, ferroportin is degraded and iron transit into the plasma ceases, thus maintaining intracellular stores and inhibiting erythropoiesis (Nemeth, Tuttle, et al. 2004). Although the liver is the predominant site of hepcidin, and ferroportin is fundamentally a hepatocyte-expressed protein, ferroportin is also expressed in a number of other cell types, including enterocytes, macrophages and erythroid precursors.

The import of iron via enterocytes has been well characterised, with the most important apical protein being divalent metal transporter 1 (DMT1) (Illing et al. 2012). However, the actual contribution of dietary iron to total body iron stores is very limited. Reticuloendothelial macrophages play a far greater role in regulating total body iron stores, as they are principally responsible for the recovery and recycling of iron from senescent red blood cells. In contrast to enterocytes, which predominantly interact with ferrous iron (Fe^{2+}), splenic and hepatic (Kuppfer) macrophages are able to phagocytose terminal age whole red cells and extract heme and then iron from them (Beaumont and Delaby 2009).

Studying hepcidin and ferroportin regulation has been greatly facilitated by the use of commercial hepatoma cell lines, which have been essential in determining many of the basic cellular and signalling mechanisms involved. Access to primary human hepatocytes is difficult, but hepatoma cell lines, such as HepG2 and HuH-7 cells, have been shown to be well suited to studies examining the transcriptional regulation of hepcidin (Jacolot, Ferec, and Mura 2008; Kanamori et al. 2014). Using these in vitro models, we now know that exogenous IL-6 causes increased transcription of *HAMP* (which then translates to hepcidin) via the janus kinase-2 (JAK2) STAT3 pathway (Wrighting and Andrews 2006; Fein et al. 2007; Verga Falzacappa et al. 2007).

We also know that macrophages export iron in a similar fashion to hepatocytes, using a series of elegant experiments demonstrating that macrophages overexpressing ferroportin allowed the release of greater amounts of ^{59}Fe-labelled rat erythrocytes than control cells, after erythrophagocytosis. This effect was significantly diminished following treatment with hepcidin, confirming the parallel iron export mechanisms that exist between hepatocytes and macrophages (Knutson et al. 2005).

The mechanisms of hepcidin regulation in monocytes and macrophages have been elucidated predominantly using well-characterised models such as the THP-1 commercial monocyte cell line, which was derived from a 1-year-old patient with acute myeloid leukaemia. THP-1 cells are grown in suspension, and possess the ability to differentiate into macrophages when exposed to phorbol 12-myristate 13-acetate (Jacolot, Ferec, and Mura 2008). These cells have been studied independently, or in cross-culture with hepatoma cells in order to establish the cell-to-cell interaction that exists.

The first report of the use of THP-1 cells being co-cultured with hepatoma cells originated from Andriopoulos and colleagues, who showed that hepcidin released from IL-6-stimulated HuH-7 cells significantly decreased the efflux of ^{59}Fe from co-cultured THP-1 cells (Andriopoulos and Pantopoulos 2006). Although both hepatocytes and macrophages respond to the same stimuli, the speed of their respective responses differ; macrophages appear to be more sensitive and respond more acutely to hepcidin than intestinal epithelial cells, perhaps indicative of the macrophage's central position in maintaining body iron homeostasis (Chaston et al. 2008).

However, macrophages do not necessarily require liver-derived hepcidin for their activation and possess the ability to act in an autocrine manner (Theurl et al. 2008; Sasaki et al. 2014). Consequently, there is very likely great utility in measuring *HAMP* mRNA from human peripheral blood mononuclear cells (PBMCs) in order to determine the cells' intrinsic production of *HAMP*, and therefore hepcidin (Ryan et al. 2012). Both THP-1 cells and PBMCs express significant amounts of *HAMP* mRNA (although much lower amounts than that expressed in hepatocytes), and are activated by IL-6 and BMP-6 in the same manner as hepatocytes (Armitage et al. 2011). Accordingly, researchers have made use of PBMC *HAMP* mRNA measurements in a variety of clinical scenarios (Table 5.1).

Authors, year	PBMC *HAMP* mRNA induction agents and/or findings from clinical studies
(Theurl et al. 2008)	Anaemia of chronic disease patients had approximately double the expression of PBMC *HAMP* mRNA than control patients
(Armitage et al. 2009)	Plasmodium falciparum infected RBCs induced *HAMP* production in co-cultured PBMCs
(Armitage et al. 2011)	A variety of infectious and inflammatory stimuli induced *HAMP*
(Sow et al. 2011)	Mycobacterium induction of *HAMP*
(Ryan et al. 2012)	Reduction of *HAMP* within 12 hours of PEG-IFNα treatment in hepatitis C patients. **Correlated with serum hepcidin**
(Wu et al. 2012)	3-fold increase in *HAMP* in patients with severe sepsis compared to controls
(Andrews Guzman and Arredondo Olguin 2014)	*HAMP* mRNA was highest in obese diabetic and obese non-diabetics compared to control subjects

Table 5.1 A selection of studies utilising measurement of PBMC *HAMP* mRNA

Over the years, nephrologists appear to have extensively utilised PBMCs as a tool with which to study disease processes in the haemodialysis population, with hundreds of citations in the literature referencing their use. They have been used in a variety of scientific studies, mainly as a gauge of immune function and oxidative stress, or as a surrogate marker of cytokine activation or production.

The aim of the work presented in this chapter is to:

1. Replicate laboratory work in the Oxford laboratory of Dr Drakesmith, in order to validate these techniques
2. Verify results from within the scientific literature with reference to *HAMP* induction in HepG2 cells and peripheral blood mononuclear cells
3. Determine the ideal concentrations of cytokines for stimulating *HAMP* upregulation in HepG2 cells and ex vivo peripheral blood mononuclear cells, for use in downstream experiments

5.2 Methods

All the experimental work undertaken in this chapter was carried out in the Renal Sciences Laboratory at the Rayne Institute, Denmark Hill, King's College London.

5.2.1 Cell Culture and Reagents

Cell culture experiments were carried out using commercial HepG2 cells (ATCC), and human peripheral blood mononuclear cells donated by healthy volunteers from within the laboratory and from the Renal Unit at King's College Hospital. A list of the reagents is listed in Table 5.2. A total of 3 separate experiments were performed unless otherwise stated.

5.2.2 Stability of Peripheral Blood Mononuclear Cells

Peripheral blood mononuclear cells (PBMCs) are circulating cells that possess a single nucleus. Examples of these cells include monocytes, macrophages and lymphocytes. In contrast, neutrophils possess multiple nuclei, whilst red blood cells and platelets have no nuclei at all. In healthy individuals, monocytes typically form 20-30% of the PBMC population, with lymphocytes comprising the rest. Using the varying properties of these cell types, PBMCs can be reliably separated from the other blood cells using density gradient media. Prior to obtaining blood from the volunteers, 50mL Falcon® conical centrifuge tubes were prepared with 50µL of heparin-sodium (1000 iu/mL) in to which the blood, obtained via venepuncture was transferred in order to prevent coagulation. PBS supplemented with sterile 2.5mM EDTA solution was used to perform the cell washes and additional 50mL conical tubes were prepared with 15mL Ficoll solution. The entire protocol typically took 25-30 minutes to perform.

Blood (35mL) was carefully layered onto the Ficoll to ensure a sharp separation between the two layers. The tubes were then centrifuged at 1200 RPM at 4°C for 30 minutes with no brake applied. The plasma layer was aspirated and stored at -80°C. The PBMC layer was aspirated and transferred to a 50mL conical tube and washed twice with the PBS-EDTA solution. The cell pellet was re-suspended in RPMI 1640 and divided into two fractions, for assessment and cell seeding. A haemocytometer was used to count the number of cells, and trypan blue was used to assess cell viability. The cells were re-suspended in RPMI 1640 to a concentration of 1 x 10^6 PBMCs/mL, and 1 x 10^6 PBMCs were seeded into 12-well plates (665180, Greiner Bio-one) and incubated at 37°C in 5% CO_2 for 1 hour, prior to experimental use.

In preparation for the mRNA work to be undertaken during this thesis, and due to the possibilities of time delays between bloods samples being drawn from subjects, to processing of these samples in the laboratory, it was necessary to ascertain the stability of PBMCs *ex vivo*. One of the prime objectives of this exercise was to ascertain whether blood samples obtained from patients in satellite haemodialysis could be used for experimentation.

In order to test this, healthy volunteers donated 35mL of blood, and the blood was immediately transferred into 7 heparinised 15mL conical tubes (5mL in each tube). One reference sample was immediately processed, and the remaining samples were rested at room temperature or on ice. The samples were processed on an hourly basis, for up to 3 hours, as this was the maximum time delay that was anticipated (Figure 5.1).

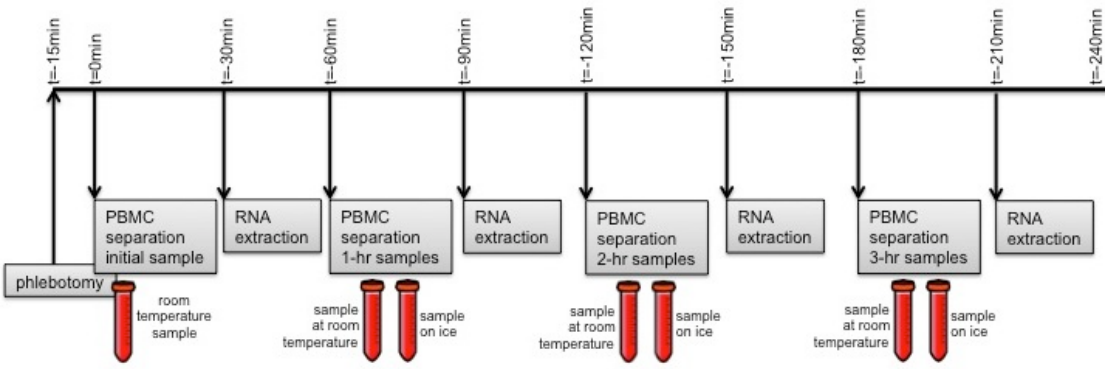

Figure 5.1 Study schedule outlining the time of blood processing in relation to the initial blood draw
(Blood samples were Ficoll® separated on a hourly basis and RNA immediately extracted)

5.2.3 Laboratory Methods

5.2.3.1 HepG2 Cells

HepG2 cells are human hepatoma cells that were derived from a 15-year-old Caucasian male with hepatocellular carcinoma. Cell culture involves supplementation with 10% fetal bovine serum (FBS), and treatment with 100U/mL penicillin and 100µg/mL streptomycin. The flasks were incubated in a Techne incubator at a temperature of 37°C, in a humidified atmosphere containing 5% CO_2. When the cells were approximately 80% confluent, the growth medium was removed and the cells were twice-washed with phosphate-buffered saline (PBS) (D8537, Sigma-Aldrich) to remove any trace of serum-supplemented medium, which may have rendered the trypsinisation process ineffective.

Trypsin EDTA (2mL) was added to allow complete coverage of the cells, and the flasks were incubated for 5 minutes to allow complete detachment of the adherent cells from the flask. FBS-supplemented medium was then added to neutralise the trypsin. In order to completely remove the trypsin, the cells were centrifuged at 15000G for 5 minutes, with subsequent removal of the supernatant; the cells were then washed with PBS, and 20mL of warmed medium was used to suspend the pellet. The cells were counted and seeded into 35mm dishes (627160, Greiner Bio-one) at a concentration of 8 x 10^5 cells/dish with a total of 2mL DMEM. The cells reached 70% confluence after a 48-hour incubation period. The cells were serum-starved overnight, for experimental use the following morning.

Reagent	Company	Catalogue number
Beta-mercaptoethanol	Sigma-Aldrich	M7522
BMP-6	R&D systems	507-BP-020
Dulbecco's modified Eagle Medium	ThermoFisher Scientific	41966029
EDTA	ThermoFisher Scientific	118432500
Ethanol	Haymankimia	F200236
Fetal bovine serum	Sigma-Aldrich	F7524
Ficoll-Paque	GE Healthcare	17144003
Heparin sodium	LEO Pharma	N/A
IL-6	Life Technologies	PHC0065
Penicillin	ThermoFisher Scientific	15070-063
Phosphate-buffered saline	Sigma-Aldrich	D8537
RPMI-1640	ThermoFisher Scientific	28175034
Streptomycin	ThermoFisher Scientific	15070-063
Trypsin EDTA	ThermoFisher Scientific	25300

Table 5.2 Reagents used in experimental cell culture

5.2.3.2 RNA Extraction

All work surfaces were cleaned with detergents and industrial methylated spirits prior to RNA extraction. An RNase decontamination solution (AM9780, Invitrogen) was used for added security against RNases. The QIAshredder (79654, Qiagen) and RNeasy Mini Kits (74104, Qiagen) were the principal kits used in the extraction of RNA from cellular material, and used according to the manufacturers' instructions, and all the steps took approximately 25-30 minutes in total to complete, depending on the number

of samples that were processed. Ethanol and beta-mercaptoethanol were not provided in the kit, but necessary for RNA extraction. All RNA that was obtained was immediately stored at -80°C for reverse transcription.

5.2.3.3 Reverse Transcription

Reverse transcription from RNA to cDNA was undertaken using the High Capacity RNA-to-cDNA Kit (4387406, Applied Biosystems), according to the manufacturer's instructions. An 8-well NanoDrop spectrophotometer (ThermoFisher Scientific) was utilised to determine the quantity and purity of the RNA prior to reverse transcription. Samples were then pipetted into 96-well reaction plates (N801-0560, Applied Biosystems) combined with the reagents supplied by the manufacturer, to a total volume of 20µL. The samples were heated in a cyclical manner (Veriti Thermal Cycler, Applied Biosystems) for 65 minutes, before being cooled to 4°C; 80µL of RNase-free water (129112, Qiagen) was added to each sample to bring the reaction to a complete halt. Samples were immediately stored at -20°C.

5.2.3.4 Quantitative PCR

TaqMan (Applied Biosystems) primer-probes were used for the detection of *HAMP* mRNA (Hs00221783_m1) and *GAPDH* mRNA (Hs02758991_g1). The primer-probes were combined with TaqMan Universal PCR Master Mix (4324018, Applied Biosystems), RNase-free water and the reverse-transcribed cDNA. Volumes of 10µL per sample, which were tested in triplicate, were placed in a 384-well plate (4309849, Applied Biosystems), and a QuantStudio 7 Flex Real Time PCR System (Applied Biosystems) was used to perform the analysis that would determine the expression of the target gene relative to the housekeeping gene.

5.2.3.5 Cytokines

IL-6 (PH C0065, Life Technologies), and BMP-6 (507-BP, R&D Systems) were reconstituted according to the manufacturers' instructions. Experiments were performed to examine the effect of repeated IL-6 stimulation on PBMCs and HepG2 cells. Treatments were applied at baseline (0 hours) and after 3 hours, the cells were washed, and fresh medium was applied. Following this, a second treatment was applied, and RNA was extracted after a further one hour.

5.2.3.6 Human Plasma

Frozen plasma from haemodialysis patients and healthy subjects obtained in chapter 3 was used in the incubation medium at varying concentrations (0.5-10%) in order to assess the effect on *HAMP* mRNA expression in HepG2 cells.

5.3 Results

Results are expressed as means ± standard deviations, unless otherwise specified.

5.3.1 Stability of *Ex Vivo* PBMC *HAMP* and *GAPDH*

Three volunteers donated blood samples for use in this experiment. There was no significant difference in *HAMP* mRNA levels between the samples that were immediately processed, and the samples that were rested at room temperature or on ice, for up to 2 hours after the initial blood draw. The standard deviation of the reference samples was extremely tight, as was the standard deviation for the samples resting for 1 hour on ice.

After 3 hours rest, there was a marked increase in *HAMP* mRNA, in samples kept at both at room temperature (p=0.07) and on ice (p=0.009) (Figure 5.2).

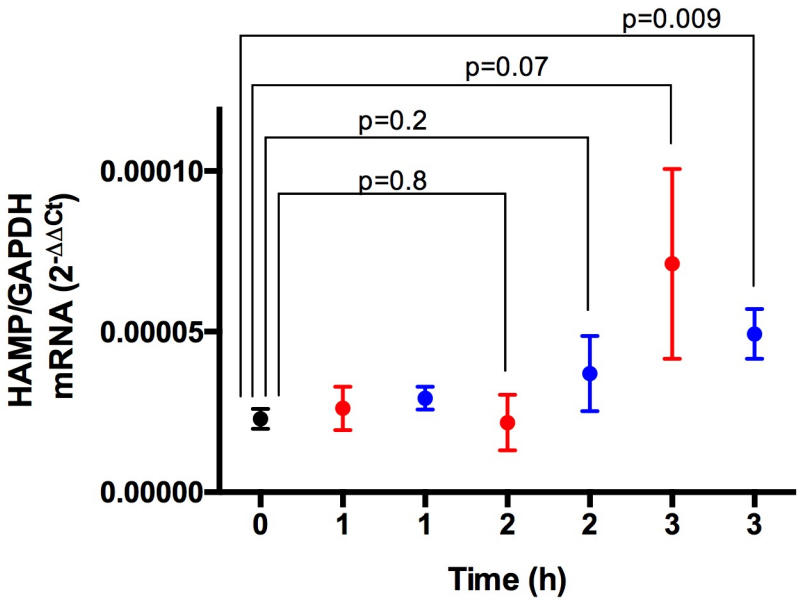

Figure 5.2 RNA extracted from PBMCs resting at room temperature or on ice
(Black data plot indicates the reference sample; red data plots indicate samples resting at room temperature; blue data plots indicate samples resting on ice; comparison between reference sample and samples indicated with p-values)

When the data were pooled together at the 3 time points, there was no difference seen between PBMC *HAMP* mRNA levels after 1 and 2 hours, compared to the reference time point. After 3 hours, significant *HAMP*/*GAPDH* instability was demonstrated (p=0.0477) (Figure 5.3); however, this could not be attributed to the instability of the housekeeping *GAPDH* gene.

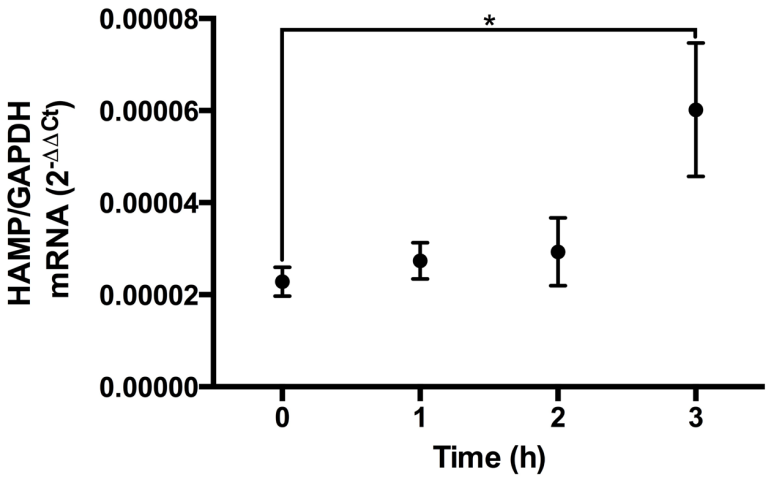

Figure 5.3 RNA extracted from PBMCs – pooled data
(**HAMP*/*GAPDH* mRNA at 3 hours versus 0 hours, p=0.0477)

To assess the suitability of *GAPDH* as the housekeeping gene, a separate experiment was conducted to determine the stability of *GAPDH* up until 24 hours post-venepuncture. Three people donated 30mL of blood, which was separated into 6 conical tubes. The first sample was processed immediately, and the other 5 samples were rested at room temperature for 1, 2, 3, 6, and 24 hours, respectively. *GAPDH* was robustly stable for 3 hours, but after this time, the ΔCt (i.e. maximum Ct – minimum Ct) values rose outside the bounds of acceptability (Ct ≥1) after 24 hours (Figure 5.4).

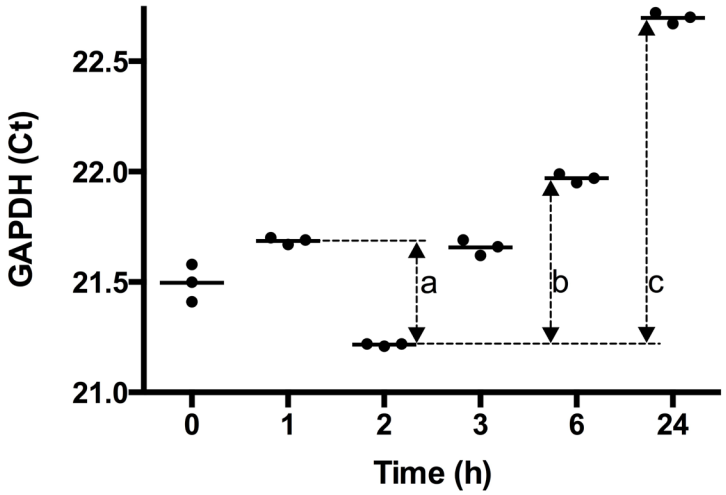

Figure 5.4 *GAPDH* Ct values from PBMCs rested at room temperature up to 24 hours
(Arrow 'a' denotes a *GAPDH* difference (ΔCt) of 0.47 between the samples with the minimum and maximum Ct values within the first 3 hours; 'b' represents a ΔCt of 0.75, and 'c' represents a ΔCt of 1.48 between their respective samples, and the sample with the minimum Ct value)

5.3.2 PBMC Stimulation Using IL-6

Stimulation of PBMCs was performed with IL-6. Serial dilutions of stock concentrations of IL-6 (10ng/μL) were performed to make a range of concentrations, which were applied for 2 hours. The doses used were 0.39, 0.78, 1.56, 3.13, 6.25, 12.5, and 25ng/mL.

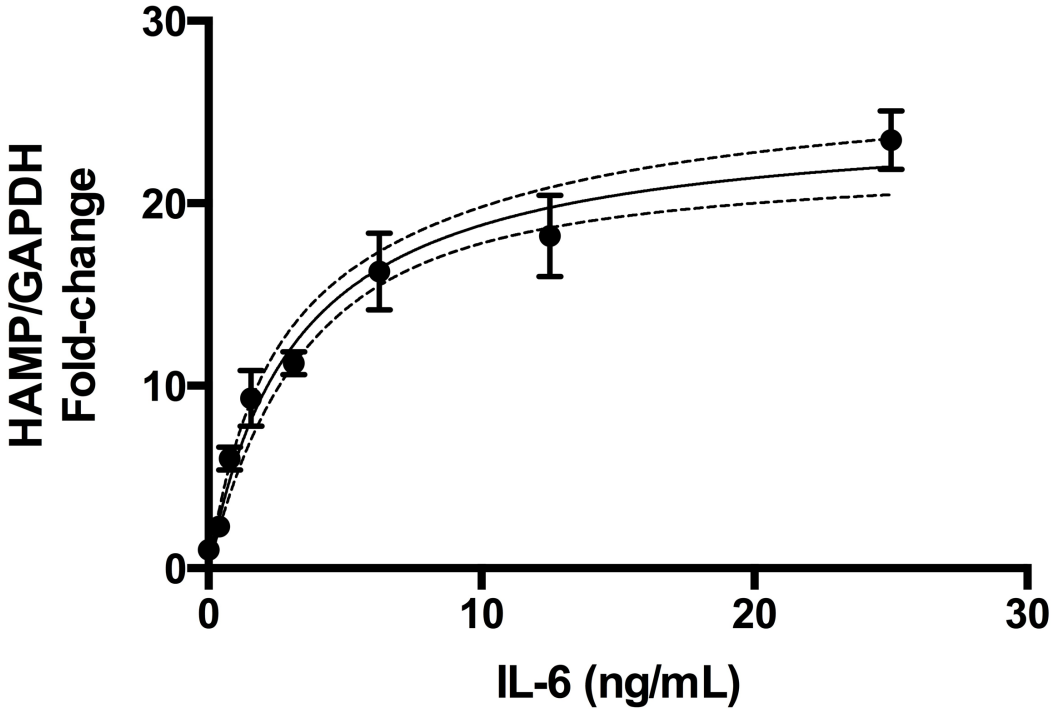

Figure 5.5 Dose-response curve for IL-6 stimulation of PBMCs
(IL-6 concentrations started from 25 ng/mL diluted down to 0.39ng/mL; hyperbolic model with 95% confidence intervals shown – R2 = 0.9563; results from 3 independent experiments)

There was a clear dose-response curve of IL-6 at the lower concentrations, which then reached a plateau using doses ≥12.5ng/mL (Figure 5.5). On the basis of these results, a dose of 12.5ng/mL of IL-6 was selected for the subsequent set of experiments, examining the impact of various time delays on RNA extraction. For this experiment, *HAMP* mRNA relative to the housekeeping gene was assessed at 30-minute intervals up to 180 minutes (Figure 5.6).

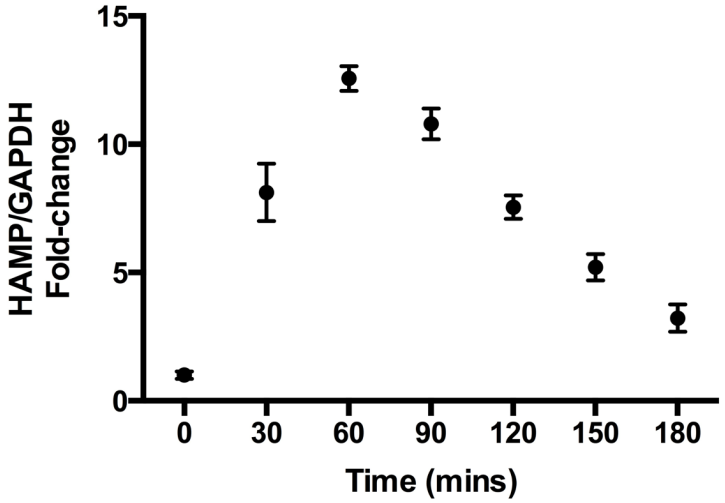

Figure 5.6 PBMC time-response curve using 12.5ng/mL of IL-6
(Results from 3 independent experiments)

The maximum response to this dose was witnessed at 60 minutes, and the response to IL-6 fell significantly after 180 minutes but was not back to baseline and would have taken approximately a further 30-60 minutes to do so if the data was extrapolated and the descent remained linear.

5.3.3 HepG2 Stimulation Using IL-6

Similar experiments were undertaken using HepG2 cells. Serial dilutions from a stock concentration of 10ng/μL IL-6 were undertaken resulting in the following concentrations being applied for 2 hours: 3.13, 6.25, 12.5, 25, 50, and 100 ng/mL.

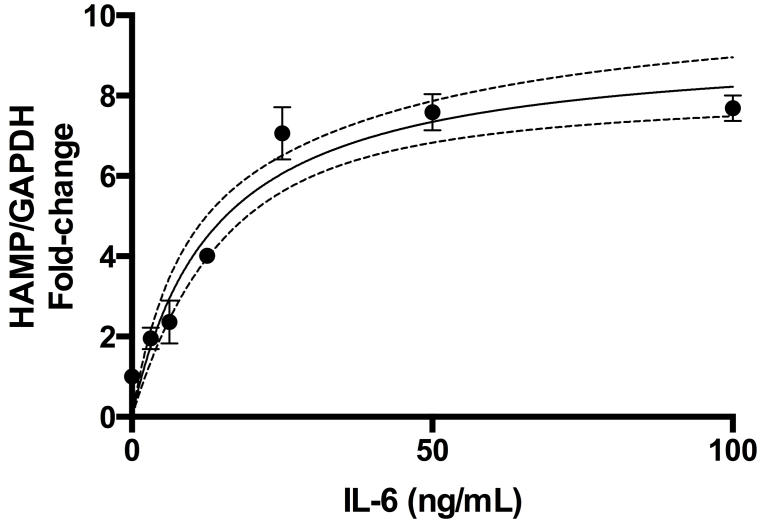

Figure 5.7 Dose-response curve for IL-6 stimulation of HepG2 cells
(IL-6 concentrations started from 100ng/mL diluted down to 3.125ng/mL; hyperbolic model with 95% confidence intervals shown – $R^2 = 0.9267$; results from 3 independent experiments)

Again, the dose-response relationship to IL-6 plateaued, but this time at a concentration of 25ng/mL (Figure 5.7). On the basis of these results, a dose of 25ng/mL of IL-6 was selected to create a time-response curve extracting RNA at 30-minute intervals.

The response of the HepG2 cells to IL-6 differed somewhat to the PBMCs; the maximum attainable response of these cells occurred at 120 minutes after the initial treatment, with a 50% reduction in the response 60 minutes after the peak response. The variance in responses from 120 minutes onwards (as was judged by the standard deviations) were greater compared to those seen within the first 90 minutes (Figure 5.8).

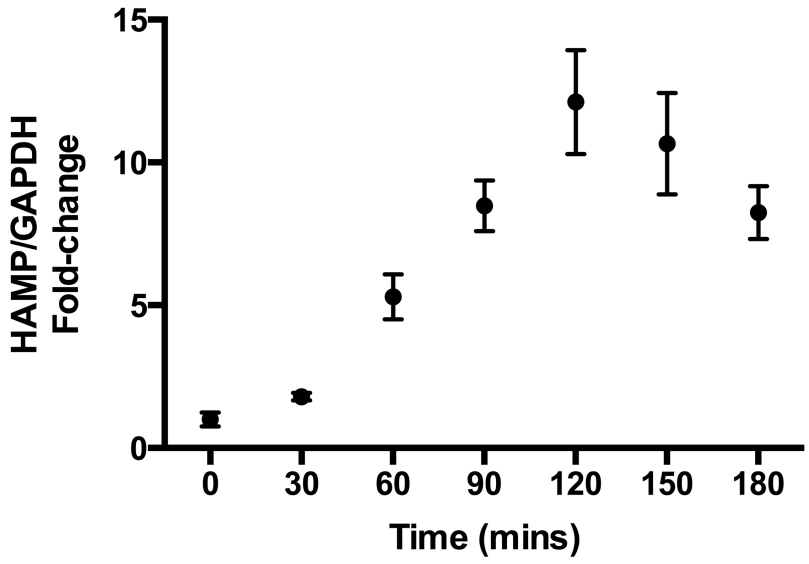

Figure 5.8 HepG2 time-response curve using 25ng/mL IL-6
(Results from 3 independent experiments)

5.3.4 HepG2 Stimulation Using BMP-6

Stimulation of HepG2 cells was performed with BMP-6. Serial dilutions of stock concentrations of BMP-6 (20ng/µL) were performed to create a range of concentrations, which were applied for 2 hours. The doses used were 1.56, 3.13, 6.25, 12.5, 25, 50, and 100 ng/mL.

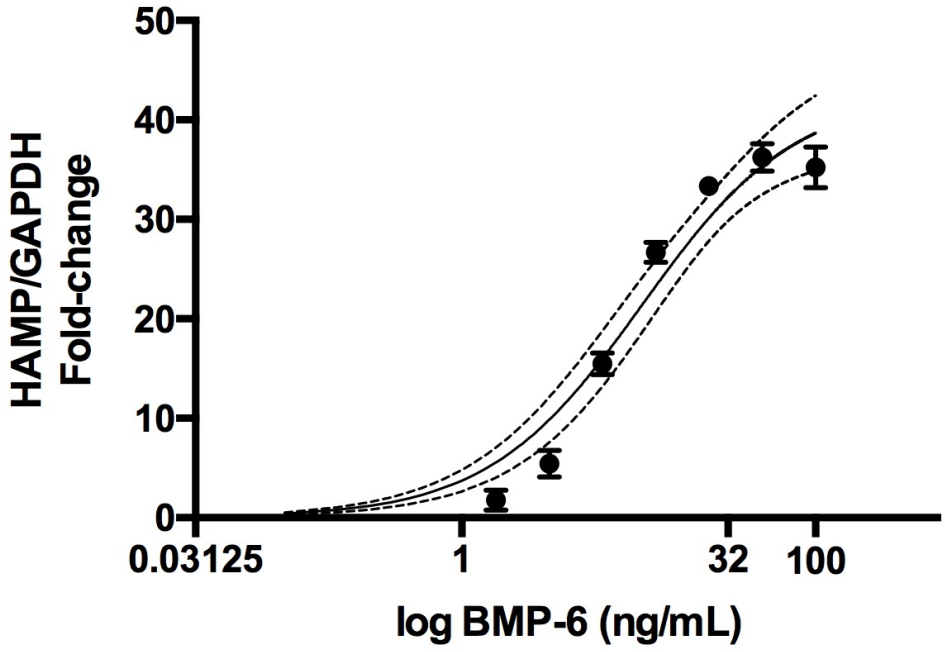

Figure 5.9 Dose-response curve for BMP-6 stimulation of HepG2 cells
(BMP-6 concentrations started from 100ng/mL diluted down to 1.5625ng/mL; hyperbolic model with 95% confidence intervals shown – R^2 = 0.9507; results from 3 independent experiments)

The maximal attainable response elicited with BMP-6 was much higher than the responses seen with IL-6. IL-6 stimulation elicited a maximal *HAMP/GAPDH* fold-change rise in the region of 10-15, whereas 30- to 40-fold-change rises were witnessed with BMP-6. The *HAMP*/*GAPDH* mRNA level plateaued after a dose of 25ng/mL, with no additional responses seen, even with doses as high as 100ng/mL (Figure 5.9).

5.3.5 Are PBMCs and HepG2 Cells Refractory to Repeated IL-6 Stimuli?

These data from this set of experiments suggest that PBMCs and HepG2 cells behave differently to repeated stimulation of IL-6. The same experiment was undertaken in both cell types, with treatments applied at 0 hours and again at 2 hours. The cells were washed prior to the second treatment. RNA was extracted 1 hour later, a total of 3 hours after the initial treatment.

When PBMCs were treated with 12.5ng/mL of IL-6 followed by vehicle, there was no difference seen to PBMCs that were treated twice with IL-6 (Figure 5.10).

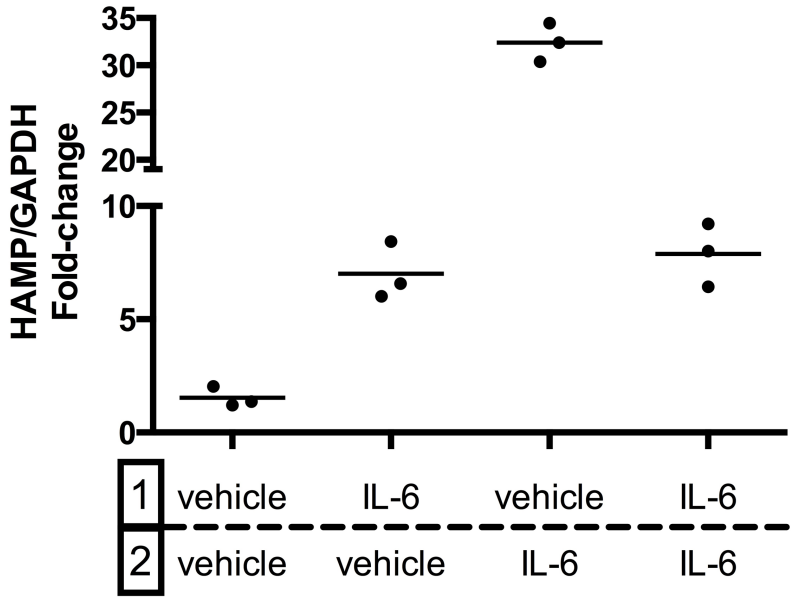

Figure 5.10 Repeated treatments of IL-6 or vehicle applied to PBMCs
(Treatments 1 and 2 indicated above; IL-6 dose was 12.5ng/mL; cells treated twice with IL-6 showed the same response compared to cells treated with IL-6 followed by a vehicle treatment; results from 3 independent experiments)

In contrast, HepG2 cells treated with 25ng/mL of IL-6 had a greater *HAMP* mRNA response with a second IL-6 treatment, compared to cells that had only had a baseline IL-6 treatment (Figure 5.11).

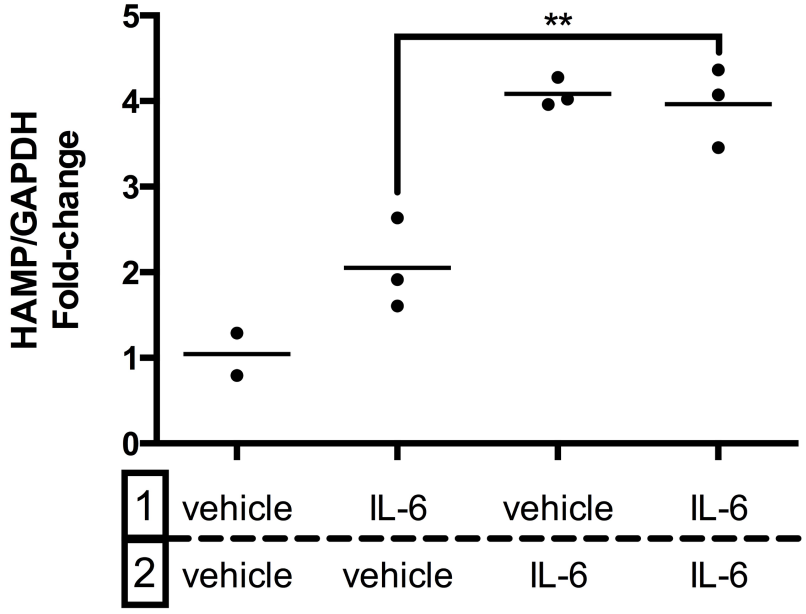

Figure 5.11 Repeated treatments of IL-6 or vehicle applied to HepG2 cells
(Treatments 1 and 2 indicated above; IL-6 dose was 25ng/mL; two treatments with IL-6 produced a significantly increased response compared to IL-6 treatment followed by vehicle, p=0.009; results from 3 independent experiments)

5.3.6 Is HepG2 *HAMP* Expression Induced By Human Plasma?

As was shown earlier in this thesis, PBMC *HAMP* expression was lower in clinically stable and relatively uninflamed haemodialysis patients compared to healthy controls (4.3.1). In order to assess whether a circulating factor was possibly responsible for this, plasma from 6 haemodialysis patients and from 4 healthy individuals was incubated with HepG2 cells. A plasma concentration of 10% was used initially, followed in successive experiments by 5%, 2.5%, 1%, and 0.5% plasma, and these cells were incubated for 24 hours prior to RNA extraction. Irrespective of the plasma concentration used to incubate the cells, coagulation of the culture medium was witnessed, even with a plasma concentration as low as 0.5%. To circumvent this problem, heparin-sodium (10µL of 1000 iu/mL), as an anticoagulant, was added to the culture medium. No difference was seen in *HAMP* mRNA expression between HepG2 cells that had been incubated with patient plasma, and cells incubated with plasma from healthy individuals.

The experiment was repeated with plasma-free HepG2 cells, and plasma-free cells with heparin alone; rather unexpectedly, this showed considerable (>95%) downregulation of *HAMP* expression in the plasma-free cells treated with heparin, compared to the treated cells treated with heparin ($p<0.0001$) (Figure 5.12).

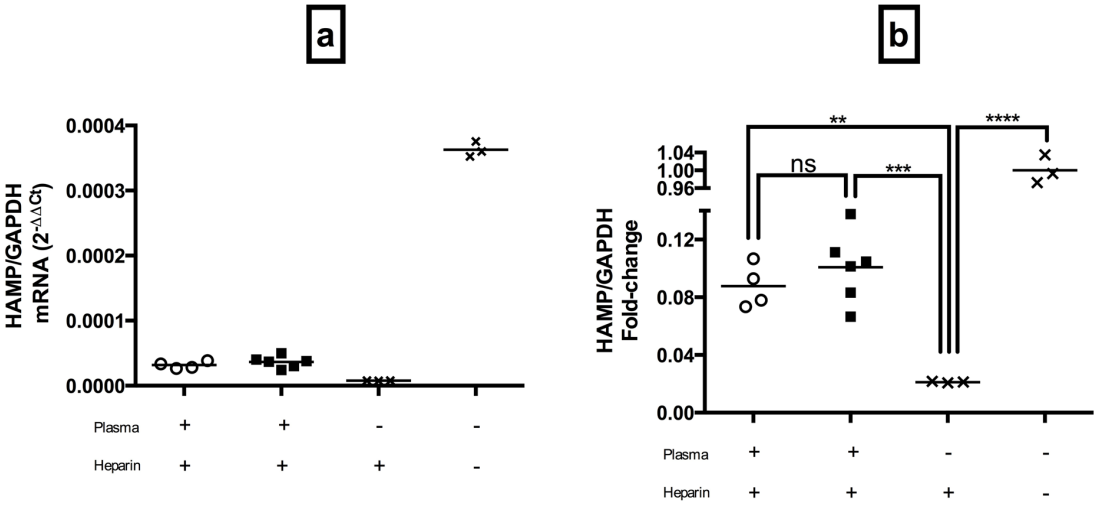

Figure 5.12 HepG2 cells incubated with plasma and treated with heparin represented as (a) absolute mRNA values, and (b) fold-change values
(Control plasma indicated by open circles; black squares indicate plasma from HD patients; crosses indicate that cells were free of plasma; heparin dose equivalent to 10 i.u.; plasma-free, heparin treated cells v plasma-free, heparin-free cells, $p<0.0001$;***$p=0.0009$; **$p=0.0018$)

There was no difference in *HAMP* expression between the cells treated with patient plasma, and the cells treated with control plasma from healthy individuals. *HAMP* expression was on average 90-92% lower in the plasma-treated cells, when compared to the cells that were treated with neither plasma nor heparin (Figure 5.12b).

5.4 Conclusions and Discussion

The main findings from the work undertaken in this chapter were as follows;

1. *HAMP* expression was upregulated in both PBMCs and HepG2 cells following treatment by IL-6 and BMP-6
2. In PBMCs, *HAMP* expression peaked at 60 minutes following treatment with IL-6; in HepG2 cells it peaked at 120 minutes
3. *HAMP* induction in HepG2 cells was considerably more pronounced with BMP-6 than with IL-6
4. PBMCs that were treated with an initial dose of IL-6 appeared to be refractory to a second IL-6 treatment, whereas in HepG2 cells, a second dose of IL-6 produced an additional effect
5. The addition of heparin to the culture medium caused >90% *HAMP* downregulation in HepG2 cells

The mechanism by which IL-6 upregulates *HAMP* expression in PBMC and HepG2 cells occurs via activation of the IL-6 receptor with downstream JAK-STAT signalling causing increased *HAMP* transcription. In this model, STAT3 signalling only fully works in the presence of a functional SMAD pathway (Verga Falzacappa et al. 2007; Wang et al. 2005). The suppressive action of heparin on *HAMP* occurs via a completely separate pathway to IL-6; HepG2 cells express endogenous BMPs, which (in an autocrine fashion) eventually leads to *HAMP* transcription via the SMAD intracellular signalling system, and heparin is an enormously potent BMP binder (Poli et al. 2011).

The HepG2 cell line was selected to study *HAMP* regulation in this chapter of work as it had been used by many researchers previously, including some of those that published seminal works in field of hepcidin regulation. The results, in terms of IL-6 stimulation, have been robustly consistent throughout the literature. The other human hepatic cell lines that have been used to study cellular hepcidin regulation are the Hep3B and Huh-7 cell lines, which were all originally derived from patients with a form of hepatocellular carcinoma. Cancer is a well-known cause of anaemia affecting up to one-third of patients who are not on anti-neoplastic therapy (Gascon and Barrett-Lee 2006). There has been some interest in the possible role of cancer in hepcidin regulation. Vela and Vela-Gaxha produced an extensive review of the known mechanisms of hepcidin regulation in cancer, which appears to be mainly driven by the bone morphogenetic

proteins. Although BMP-6 is of primacy in iron/hepcidin regulation, some of the other BMPs are linked with hepcidin upregulation and are specifically linked with different tumour types e.g. BMP-7 in prostate and breast cancer, and BMP-2 in lung cancer. The expression of interleukin-6 is frequently increased in cancer and thus related to elevated hepcidin levels. Local hepcidin expression appears to be increased in most tumour types (except colon and hepatocellular carcinoma). Tumour hepcidin overexpression may be part of a tumour's strategy to increase cellular iron (i.e. prevent its export from the cell) and this might represent an adaptive process to increase tumourigenesis. It is interesting that local hepatocellular carcinoma hepcidin levels are lower compared to controls given that the liver is the principal reservoir of hepcidin, and that relatively low hepcidin allows hepatocytes to become the preferential site of iron storage allowing the uninterrupted inward flow of iron (cf. enterocytes, macrophages) in order to increase cellular iron levels (Vela and Vela-Gaxha 2018).

The results from the work in this chapter replicated some of the results published by Armitage and colleagues, both validating their work and confirming the ability of this investigator to replicate the methodology (Armitage et al. 2011). In the experiments to determine whether PBMCs were refractory to successive IL-6 stimulation, there was a slight difference in methodology used. Armitage and colleagues did not change the PBMC culture medium or wash the cells prior to the second treatment; nonetheless, both sets of results indicated that PBMCs were refractory to repeated IL-6 stimulation, thought to be a result of feedback inhibition on IL-6 signalling by SOCS3. Inhibition of IL-6 signalling is not a phenomenon witnessed in HepG2 cells, suggesting that the intra-cellular signalling mechanisms differ between the two cells types, perhaps underlining the difference that they play in innate immunity (Bode et al. 1999; Armitage et al. 2011).

The most striking finding in this chapter was the profound suppression of *HAMP* expression, seen with heparin. This was a chance finding, arising from an attempt to investigate the effect of circulating factors in the plasma of haemodialysis patients upon HepG2 cells. Heparin has been shown to inhibit BMP-6, and an Italian group have previously demonstrated the suppressive effect of heparin on *HAMP* mRNA that it is mediated via the BMP-SMAD signalling system (Zhao et al. 2006; Poli et al. 2011).

Unfractionated heparin-sodium is part of the standard anticoagulation regimen administered to haemodialysis patients at King's College Hospital, and given its apparent substantial effect *in vitro*, the question then arose as to whether it was in fact administered heparin that caused the reduction in PBMC *HAMP* expression rather than a feedback inhibitor. This will be further explored in the next chapter of work.

6 Heparin and Hepcidin

6.1 Introduction and Aims

For decades, heparin has been used as a therapeutic anticoagulant in the management and thromboprophylaxis of conditions such as atrial fibrillation and venous thromboembolism. Interestingly, this glycosaminoglycan was originally discovered by a 2nd year medical student following extraction and purification from canine livers (McLean 1916; Howell and Holt 1918). Specifically, in the field of nephrology, heparin has, and still is used as an anticoagulant in the extracorporeal blood circuits of patients undergoing chronic haemodialysis. Under normal physiological conditions, heparin is produced by mast cells and since it is found in only trace amounts in the body, possesses no appreciable anticoagulant properties. However, when manufactured heparin is administered via the intravenous or subcutaneous route, it exerts a potent anticoagulant effect by combining with and increasing the activity of circulating antithrombin III causing neutralisation of thrombin and other factors found within the coagulation cascade (Guyton 1991).

Although the initial discovery and work on heparin was undertaken by McLean, it was Howell who deduced that heparin was a carbohydrate and contained no phosphate residues (Howell 1928). Jorpes and others continued working on determining the structure of heparin and discovered that it contained a high degree of sulfate and carboxyl groups, rendering heparin both highly acidic and giving it possibly the highest negative charge of any known biological macromolecule (Capila and Linhardt 2002). It is these qualities that permit the promiscuous interaction of heparin with a number of positively charged endothelial and plasma-bound proteins (Table 6.1).

Chemokines	**Growth Factors**
Platelet factor 4	Basic fibroblast growth factor
NAP-1 (IL-8)	Acidic fibroblast growth factor
NAP-2	Kaposi sarcoma fibroblast growth factor
MIP-1α, 1β	Platelet-derived growth factor
ENA-78	Transforming growth factor β
I-309	Vascular endothelial growth factor
RANTES	
MCP-1	
Extracellular Matrix Proteins	**Enzymes**
Laminin	Superoxide dismutase
Thrombospondin	Cathepsin G
Collagens	Elastase
Fibronectin	
Vitronectin	

Table 6.1 Selection of proteins that bind to heparin
(Adapted from (Tyrell, Kilfeather, and Page 1995))

While heparin is in widespread use as an anticoagulant and is generally considered safe, it has a wide range of biological activity that is responsible for the varied side-effect profile that is witnessed outside the sphere of the coagulation systems. Up to 5% of those receiving long-term unfractionated heparin are in danger of developing osteoporosis, but hepatotoxicity, heparin-induced skin lesions, and hypoaldosteronism have also been associated with its use (Hirsh et al. 2001). At least 18 bone morphogenetic proteins (BMP) have been identified, which are part of the transforming growth factor-β superfamily of proteins, and the BMP signalling pathway is implicated in many of the body's biological functions (Figure 6.1); the interaction between heparin and BMP-2 is thought to be one of the factors that contributes to the risk of osteoporosis (Simann et al. 2015).

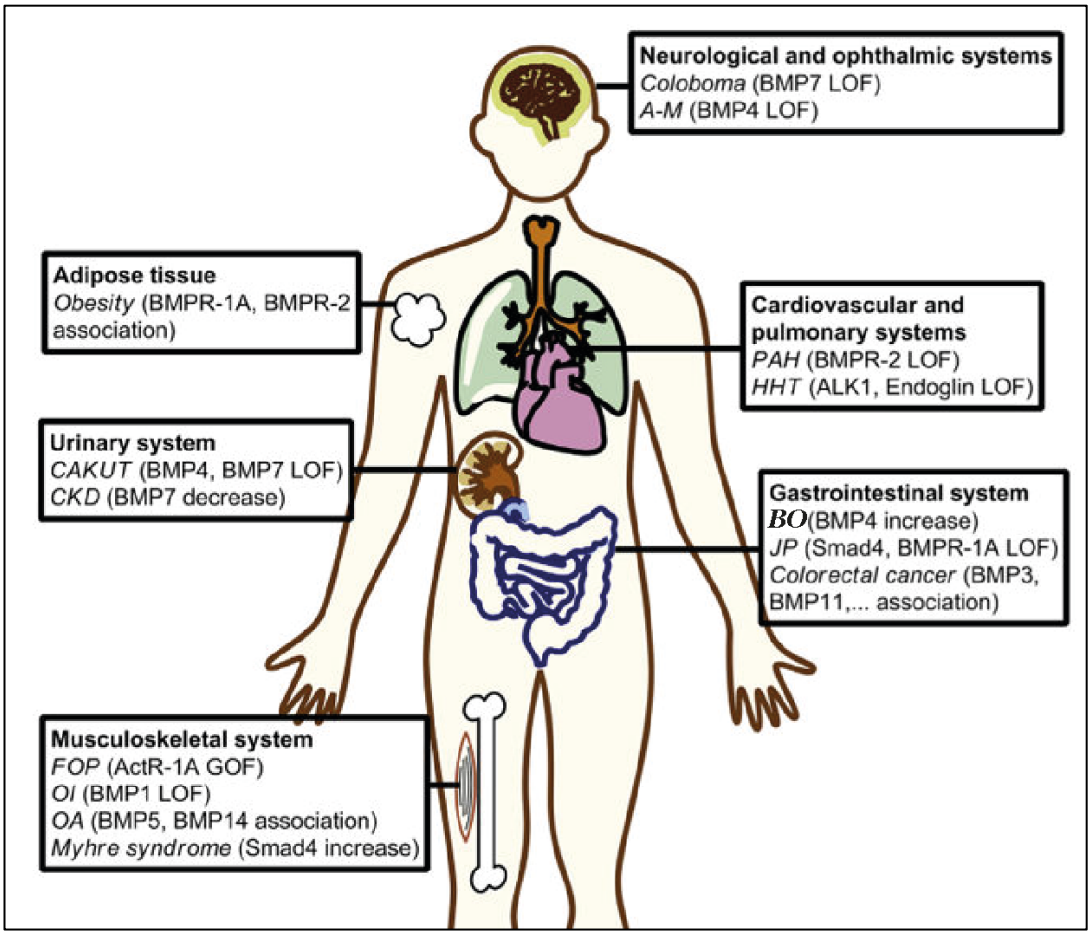

Figure 6.1 Representative members of the BMP signalling pathway that have been demonstrated to cause or be associated with human diseases
Abbreviations: BMP(R) – bone morphogenetic protein (receptor); LOF, loss-of-function; GOF: gain-of-function; CAKUT, congenital anomalies of the kidney and urinary tract; CKD, chronic kidney disease; FOP, fibrodysplasia ossificans progressiva; OI, osteogenesis imperfecta; OA, osteoarthritis; A–M, anophthalmia–microphthalmia; PAH, pulmonary arterial hypertension; HHT, hereditary haemorrhagic telangiectasia; BO, Barrett's oesophagus; JP, juvenile polyposis (reproduced from (Wang et al. 2014))

Contained within the list of BMP-mediated biological functions is iron metabolism and the control of the main iron regulatory hormone, hepcidin. Babitt and colleagues established the importance of the BMP signalling system in the regulation of hepcidin, and furthermore showed that hemojuvelin (HJV) was an important co-receptor in the signalling pathway (Babitt et al. 2006). Both circulating and hepatic iron regulate the activity of hepcidin by modulating the BMP-6-SMAD signalling system, although these occur through different mechanisms (Ramos et al. 2011; Corradini et al. 2011).

As far as can be ascertained, until Poli and colleagues showed that heparin was a potent inhibitor of hepcidin both in vitro and in vivo, there had been only one other (German) study that examined the use of heparin and demonstrated derangements in iron metabolism and availability (Braunsteiner, Sailer, and Weippl 1959). Poli *et al* used cultured hepatocyte (HepG2) cells to show that concentrations of unfractionated heparin of 4μg/mL and above were sufficient to reduce HAMP expression to <5% of basal levels, and that this was enacted through a reduction in phosphorylated SMAD 1/5/8 proteins via BMP-6 (Poli et al. 2011). Other research groups have also confirmed that heparin strongly binds BMP-6 (Brkljacic et al. 2013). Similar effects were witnessed with low-molecular weight heparin and a synthetic selective factor Xa inhibitor, although the inhibition of HAMP expression was less marked than that achieved with unfractionated heparin. The reduction of hepcidin and subsequent increase in serum iron concentrations were confirmed in murine models, cementing the relationship between heparin and hepcidin. The conclusion from their patient data was weaker, however, with the authors tentatively suggesting that heparin may induce an increase in serum iron concentrations as well as a reduction in CRP levels in 5 inflamed patients (Poli et al. 2011).

There have been recent attempts to capitalise on heparin's powerful negative regulatory effect on hepcidin and harness it as a potential therapeutic. Poli and colleagues have investigated the non-anticoagulant heparins and found that glycol-split non-anticoagulant heparins as well as over-sulfated low molecular weight heparins inhibit hepcidin both *in vitro* and *in vivo* (Poli, Asperti, Naggi, et al. 2014; Poli, Asperti, Ruzzenenti, et al. 2014). Heparins that are able to maximally antagonise hepcidin are both highly sulfated and possess an N-acetylation degree of approximately 14% (Asperti et al. 2015).

To date, there has been one published clinical study examining the impact of low-molecular weight heparin on hepcidin levels in a paediatric cohort presenting with acute ischaemic strokes, in which the authors showed that use of subcutaneous enoxaparin sodium (Sanofi-Aventis) was associated with a fall in serum hepcidin levels (Azab et al. 2016).

As was shown in 4.3.1, monocyte HAMP expression is reduced in chronic haemodialysis patient in spite of having very high plasma hepcidin levels. It is possible that this may be a result of feedback inhibition, but dialysis patients also receive intravenous heparin with each dialysis session. Given the potent negative effect of heparin on HAMP expression, it was important to determine whether administered heparin was responsible for reduced PBMC HAMP expression.

The aim of the work presented in this chapter is to:

1. Examine the effect of unfractionated heparin and low-molecular weight heparin on in vitro HAMP expression
2. Investigate whether human PBMC HAMP is affected by the administration of unfractionated heparin to renal patients that are new to haemodialysis and are heparin-naïve
3. Assess the impact of heparin on hepcidin levels in response to iron therapy

6.2 Methods

6.2.1 In Vitro Experiments

The in vitro experiments presented in this chapter of work were undertaken using HepG2 cells that were gifted by Professor Andrew McKie (King's College London). All experiments were carried out in the Renal Sciences Laboratory at the Rayne Institute, Denmark Hill, King's College London.

6.2.1.1 Cell Culture and Reagents

HepG2 cells are an adherent human hepatoma cell line that are commonly used in studies investigating hepcidin regulation, and were used by Poli and colleagues to examine the effect of heparin on HAMP expression (Poli et al. 2011). The standard culture medium included Dulbecco's modified Eagle medium (DMEM) (11965092, Gibco, ThermoFisher Scientific, Paisley, UK) supplemented with 10% fetal bovine serum (FBS) (F7524, Sigma-Aldrich), which was then treated with 100U/mL penicillin and 100µg/mL streptomycin (15070-063, ThermoFisher Scientific). The cells were routinely grown and passaged in rectangular 75cm^2 flasks (431464, Corning, UK) and incubated in a Techne incubator at a temperature of 37°C, in a humidified atmosphere containing 5% CO_2. Passaging of the cells was permitted by the use of phosphate-buffered saline (PBS) (D8537, Sigma-Aldrich) to wash the cells, and trypsin-EDTA (25300, ThermoFisher Scientific) to separate the cells from the flask. The cells were centrifuged (Heraeus Labofuge 400) at 15000G for 5 minutes; following the removal of the supernatant, the cells were washed with PBS, and re-suspended in 20mL of warmed medium. The cells were seeded into 35mm dishes (627 160, Greiner Bio-one) at a concentration of 8 x 10^5 cells/dish with a total of 2mL DMEM. After the cells reached 70% confluence, usually after a 48-hour incubation period, they were serum-starved overnight, for experimental use the following morning.

Heparin sodium and enoxaparin was obtained from the Renal Ward supply and were applied for 18 hours, after which time RNA was extracted. The concentrations of heparin sodium (i.u./mL) that were applied were as follows:

0.48, 0.98, 1.95, 3.90, 7.81, 12.50, 15.63, 25.00, 31.25, 50.00, 62.50, 100.00

The concentrations that are underlined were serial dilutions made with DMEM culture medium from an initial stock concentration of 1000 i.u./mL. The other (non-underlined)

concentrations were made from an initial stock concentration of 100 i.u./mL, again using DMEM culture mediums to make the dilutions

The concentrations of enoxaparin (µg/mL) that were applied were as follows:

0.32, 1.60, 8.00, 40.00, 200.00, 400.00

Serial dilutions were made from an initial stock concentration of 400 µg/mL, again using DMEM culture medium. All the reagents used during the in vitro experiments are listed in Table 6.2.

Reagent	Company	Catalogue number
Dulbecco's modified Eagle Medium	ThermoFisher Scientific	41966029
Enoxaparin (Clexane®)	Sanofi-Aventis	N/A
Ethanol	Haymankimia	F200236
Fetal bovine serum	Sigma-Aldrich	F7524
Heparin sodium	LEO Pharma	N/A
Penicillin	ThermoFisher Scientific	15070-063
Phosphate-buffered saline	Sigma-Aldrich	D8537
Streptomycin	ThermoFisher Scientific	15070-063
Trypsin EDTA	ThermoFisher Scientific	25300

Table 6.2 Reagents used in experimental cell culture

6.2.1.2 RNA Extraction

All work surfaces were cleaned with detergents and industrial methylated spirits prior to RNA extraction. An RNase decontamination solution (AM9780, Invitrogen) was used for added security against RNases. The QIAshredder (79654, Qiagen) and RNeasy Mini Kits (74104, Qiagen) were the principal kits used in the extraction of RNA from cellular material and used according to the manufacturers' instructions. All RNA that was obtained was immediately stored at -80°C for reverse transcription on a future occasion.

6.2.1.3 Reverse Transcription

Reverse transcription from RNA to cDNA was undertaken using the High Capacity RNA-to-cDNA Kit (4387406, Applied Biosystems), according to the manufacturer's instructions. An 8-well NanoDrop spectrophotometer (ThermoFisher Scientific) was

utilised to determine the quantity and purity of the RNA prior to reverse transcription. Samples were then pipetted into 96-well reaction plates (N801-0560, Applied Biosystems) combined with the reagents supplied by the manufacturer, to a total volume of 20μL. The samples were heated in a cyclical manner (Veriti Thermal Cycler, Applied Biosystems) for 65 minutes, before being cooled to 4°C; 80μL of RNase-free water (129112, Qiagen) was added to each sample to bring the reaction to a complete halt. Samples were immediately stored at -20°C.

6.2.1.4 Quantitative PCR

Quantitative PCR is used to measure the amplification of DNA, and this methodology can detect even minor changes in gene expression. TaqMan (Applied Biosystems) primer-probes were used for the detection of HAMP mRNA (Hs00221783_m1) and GAPDH mRNA (Hs02758991_g1). The primer-probes were combined with TaqMan Universal PCR Master Mix (4324018, Applied Biosystems), RNase-free water and the reverse-transcribed cDNA. Volumes of 10μL per sample, which were tested in triplicate, were placed in a 384-well plate (4309849, Applied Biosystems), and a QuantStudio 7 Flex Real Time PCR System (Applied Biosystems) was used to perform the analysis that would determine the expression of the target gene relative to the housekeeping gene.

6.2.2 Clinical Study

The clinical element of the work presented in this chapter was carried out using samples obtained from patients who were dialysed on the Main Haemodialysis Unit, King's College Hospital, London. The aim of this study was to assess the response of hepcidin levels in patients who were newly receiving heparin therapy, over 6 haemodialysis sessions.

6.2.2.1 Subjects

Subjects were adult patients who were tracked in the Low Clearance Clinic and were approached by this investigator prior to their planned start to haemodialysis. The decision to commence dialysis was taken by the patients' renal physician, during the weekly Low Clearance Results meeting, when the criteria for haemodialysis were considered. Biochemical data, patient symptoms, pulmonary/peripheral oedema, and electrolyte disturbances were the main criteria that were taken into consideration.

6.2.2.2 Study Design

This was an exploratory, prospective, longitudinal cohort study. Once patients had been identified in the Low Clearance Clinic weekly meeting, they were approached and given a patient information sheet. If they agreed to participate in the study and provided a signed consent form, they were considered eligible for the study. It was imperative that the patients had been embedded within the Low Clearance service for 3 months or longer, and that the transition to haemodialysis was deliberately planned. Patients who had been consented but were admitted as an emergency to the Accident and Emergency department and who commenced haemodialysis in an emergency unplanned fashion were not included in the study. Patients who were formally anticoagulated with vitamin K antagonists, or who had received any form of heparin within the 4 previous weeks were deemed ineligible. The other inclusion and exclusion criteria can be seen in Table 6.3. The exclusion criteria included a history of haematological dyscrasias, defined as any disease process that necessitated the patient having visited the Haematology department at any time for treatment or follow-up (except for iron-deficiency anaemia).

INCLUSION CRITERIA	EXCLUSION CRITERIA
≥18 years of age	Active malignancy
Low Clearance patient ≥3 months	Anticoagulation within 4 weeks
Stage 5 CKD-ND	Blood transfusion within 4 weeks
	Chronic inflammatory condition
	History of haematological dyscrasia
	History of liver disease
	Hospital admission within 4 weeks
	Signs of acute/occult bleeding

Table 6.3 Inclusion and exclusion criteria for heparin study

The King's acute haemodialysis protocol for patients newly commenced on haemodialysis has specific recommendations for the commencement of anticoagulation, and this is summarised in Figure 6.2. Only the first two haemodialysis sessions are undertaken without anticoagulation, with unfractionated heparin being administered thereafter, as a bolus of 1000 i.u. and a subsequent infusion of 1000 i.u.

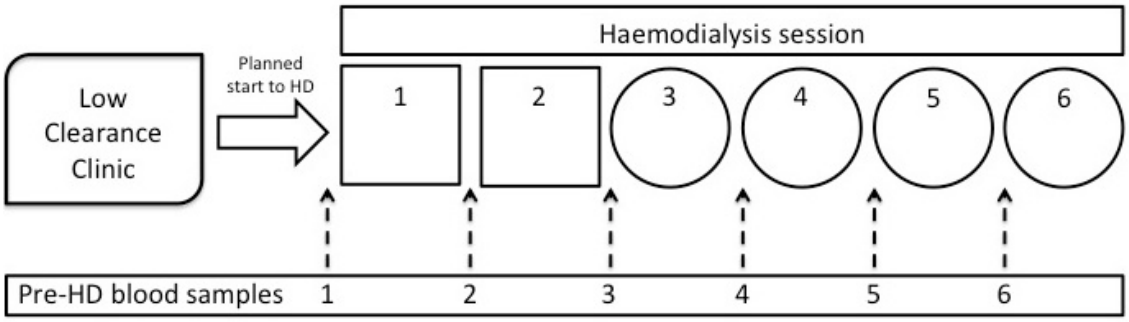

Figure 6.2 Blood sampling schedule
(Squares indicate haemodialysis (HD) sessions that were heparin-free; circles indicate HD sessions where a total of 2000 i.u. unfractionated heparin was administered)

Baseline blood samples were obtained prior to the start of the first session of haemodialysis as part of routine clinical care. Study samples were taken prior to the start of the first 6 HD sessions (Figure 6.2).

6.2.2.3 Demographics and Baseline Data

A total of 8 patients, who satisfied the inclusion and exclusion criteria, were recruited for this study. Of the 8 patients, 6 started haemodialysis in a planned fashion and their data were analysed. The 2 other patients were recruited but commenced dialysis in an unplanned fashion and were admitted to the Renal Ward for emergency care.

Age (years)	56.3±17.0
Males (n (%))	5 (83.3)
Caucasian (n (%))	4 (66.7)
Diabetes (n (%))	2 (33.3)
Hypertension (n (%))	4 (66.7)
eGFR (mL/min/1.73m^2)	8.2±1.0
Haemoglobin (g/L)	100.2±14.7
Ferritin (ng/mL)	207.5±62.8
Albumin (g/L)	38.8±3.1

Table 6.4 Demographics and baseline laboratory data

The mean age of the patients who were recruited into this study was 56.3±17.0 years of age. The patients were anaemic, at the lower end of the acceptable range for stage 5 CKD patients, with a mean haemoglobin of 100.2 g/L and ferritin of 207.5 ng/mL (Table 6.4). None of the patients had been commenced on an ESA prior to the commencement of dialysis. The mean eGFR at the start of dialysis was 8.2

mL/min/1.73m^2, and the commonest reason for the commencement of haemodialysis was the development of uraemic symptoms, in 4 patients (Figure 6.3).

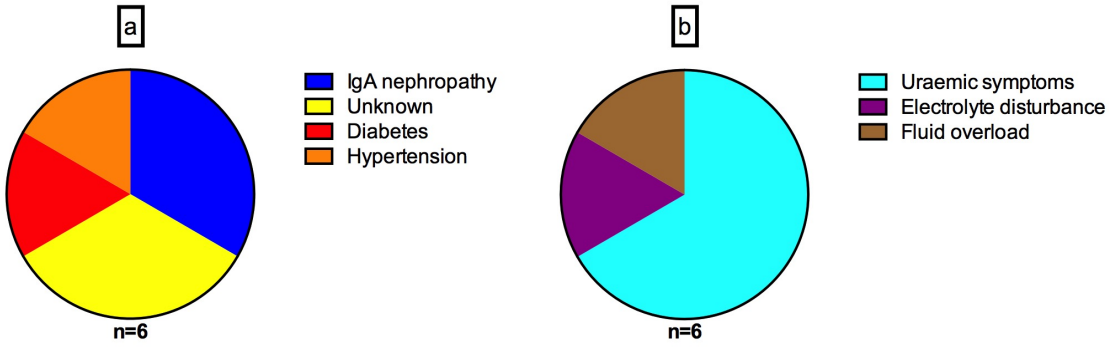

Figure 6.3 Schematic representation of numbers of subjects with their (a) cause of end-stage renal failure (b) reasons for commencement of haemodialysis

6.2.2.4 Methods

The methods of hepcidin measurement by mass spectrometry have been discussed in detail previously (1.4.1). Routine patient blood samples were processed in the King's College Hospital central laboratory. The method for HAMP mRNA measurement will be briefly revisited but is covered comprehensively on page 80.

HAMP mRNA measurement

- 10mL blood drawn (and divided into two) and separated by Ficoll-Hypaque Plus (GE Healthcare) centrifugation
- Plasma was obtained post-centrifugation and immediately stored in 500μL aliquots
- Two sets of 5x10^6 peripheral blood mononuclear cells were isolated for each participant
- Total cellular RNA was subsequently extracted (RNeasy, Qiagen) and immediately stored at -80°C
- 500ng RNA was reverse transcribed (High Capacity RNA-to-cDNA Kit, Applied Biosystems)
- All cDNA samples were processed in a single run to eliminate inter-experimental variability
- Amplification reactions were performed using Taqman HAMP and GAPDH primer-probes in triplicate
- Quantitative RT-PCR was performed using the 7900 HAT Fast Real-Time system (Applied Biosystems)

6.2.2.5 Statistical Analyses

Differences between the means of normally distributed test statistics were determined with the independent Student's t test. Relationships between various parameters under study were also examined; simple linear coefficients (r) were calculated using the Pearson's product formula. P-values <0.05 (two-tailed) were considered statistically significant. Analyses were performed using GraphPad Prism version 6.0 for Macintosh, GraphPad Software, La Jolla California USA.

6.2.2.6 Ethics and Regulatory Approvals

The London Research Ethics Committee 1 (LREC 09/H0718/034) granted ethical approval. Local R&D approval was obtained to undertake this study, which was conducted in accordance with the principles outlined in the Declaration of Helsinki. Patient information leaflets were provided to all participants who gave their written informed consent.

6.3 Results

Results are expressed at means ± standard deviation, unless otherwise specified, of three separate experiments.

6.3.1 Unfractionated Heparin Dose-Response

HepG2 cells were incubated with increasing doses of unfractionated heparin from 0.48 i.u./mL to 100 i.u./mL for 18 hours.

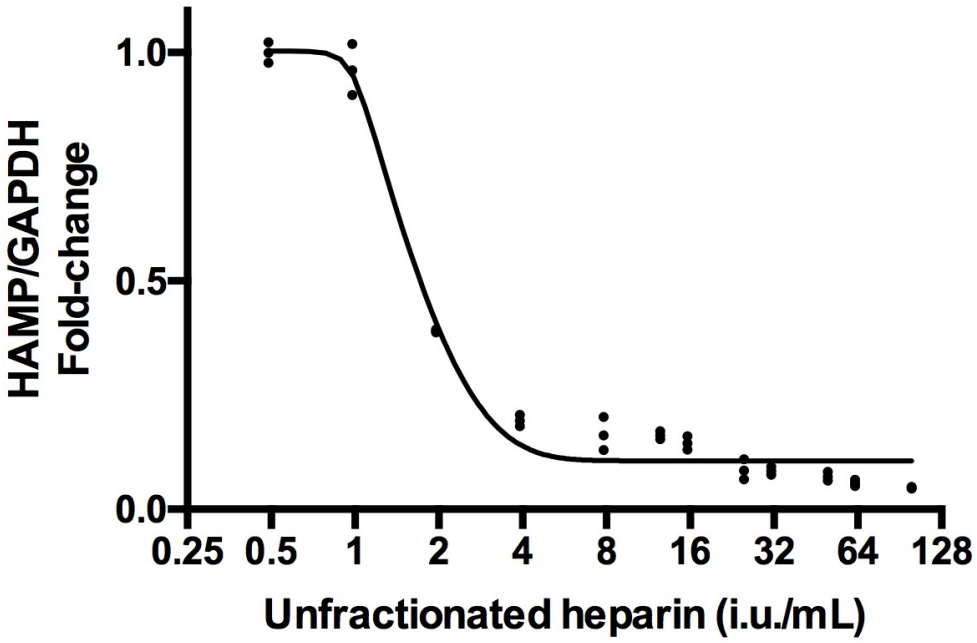

Figure 6.4 Log dose-response curve relative to basal levels: unfractionated heparin concentrations 0.48 to 100 i.u./mL

At concentrations of unfractionated heparin below 1 i.u./mL, there was no reduction in HAMP expression relative to basal levels. However, there was a steep dose-response relationship seen between 1 and 4 i.u./mL, with HAMP expression levels suppressed to approximately 20% of basal levels.

Between 4 and 100 i.u./mL, there continued to be a dose-dependent suppression of *HAMP* expression, with levels down to 5% with suppression of HAMP expression down to approximately 5% of the basal level seen with the maximum concentration tested (100 i.u./mL) (Figure 6.4).

6.3.2 Low Molecular Weight Heparin Dose-Response

HepG2 cells were incubated with increasing doses of low molecular weight heparin, enoxaparin, from 0.32 µg/mL to 400 µg/mL for 18 hours.

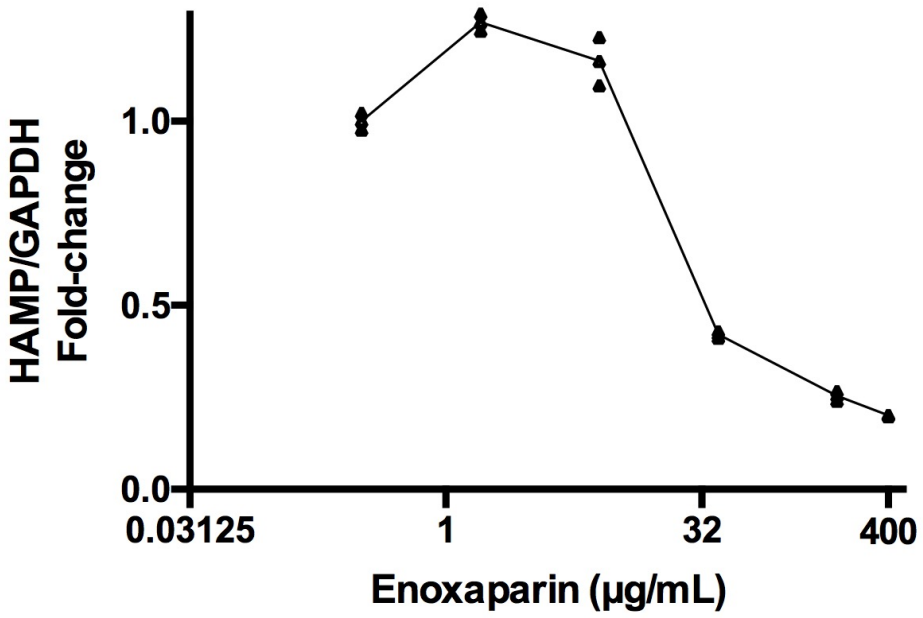

Figure 6.5 Log dose-response curve relative to basal levels: low-molecular weight heparin concentrations 0.32 to 400 µg/mL

In contrast to unfractionated heparin, the low molecular weight heparin appeared to induce *HAMP* expression at the lower concentrations of enoxaparin, of 1.6 µg/mL and 8 µg/mL. At a concentration of 1.6 µg/mL, HAMP expression was approximately 30% higher compared to basal levels, while at 8 µg/mL, levels were 10% higher than at basal.

However, with higher concentrations of enoxaparin, HAMP expression was significantly reduced. At 40 µg/mL, HAMP expression was approximately 40% of basal levels; this fell to approximately 25% of basal levels at 200 µg/mL, and approximately 20% of basal levels at 400 µg/mL (Figure 6.5).

6.3.3 Hepcidin and HAMP Levels – Individual Data

Blood samples were taken at the beginning of each haemodialysis session for 6 patients who transitioned from the Low Clearance Service. All patients received no heparin during their first two haemodialysis sessions, and then went on to receive heparin during their subsequent sessions. All patients were prescribed intravenous iron

and were given 100mg iron sucrose weekly, thereby receiving two doses during the course of the study.

As can be seen in Figure 6.6, Patient A received 2 doses of intravenous iron, which was administered during their second and fifth haemodialysis session (as indicated by the solid arrows in the figure). There was a rise in hepcidin in response to the first dose, but this was not seen following the second dose. HAMP expression appeared to fall consistently from the third haemodialysis session onwards, with no overt response to systemic iron.

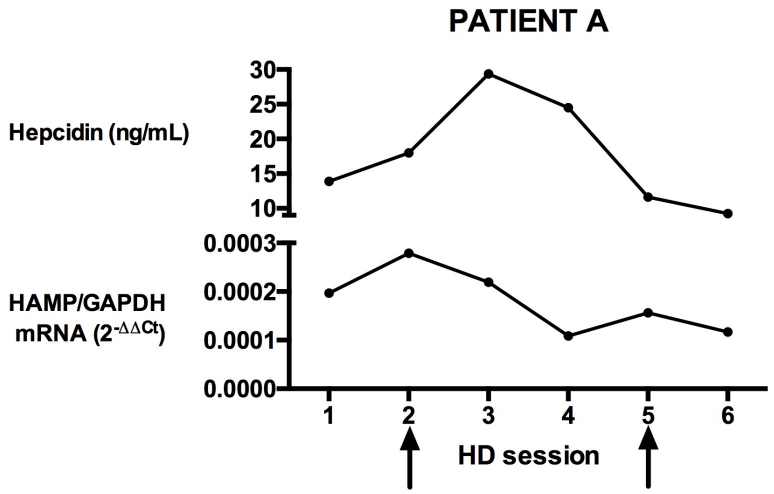

Figure 6.6 Hepcidin and HAMP expression in Patient A following their first 6 haemodialysis sessions
(Arrows indicate the HD session wherein iron sucrose was administered (100mg))

Hepcidin and *HAMP* data for all 6 patients (A to F) is shown in Figure 6.7. All of the patients had a rise in hepcidin levels following the administration of the first dose of IV iron. Following the second dose of iron, 3 patients had fall in hepcidin levels (patients A, B, and F), and 3 patients had a rise in iron (C and E), whilst patient F had a dose of iron at the end of the 2 weeks and the hepcidin response to this dose was not assessed. There was an enormous degree of variability in the hepcidin levels across the 2 weeks among the 6 patients, and no pattern emerged related to the timing of the administration of IV iron. The *HAMP* response to IV iron also appeared indiscriminate. Four of the patients (B, C, D, and E) had a rise in *HAMP* mRNA levels following the first dose of IV iron. As was seen for hepcidin, fewer patients (only 2; patients B and C) had a rise in *HAMP* mRNA levels following the second IV iron dose. Again, *HAMP* mRNA levels were highly variable and there was no pattern between the dosing of the iron and the subsequent measured levels (Figure 6.7, overleaf).

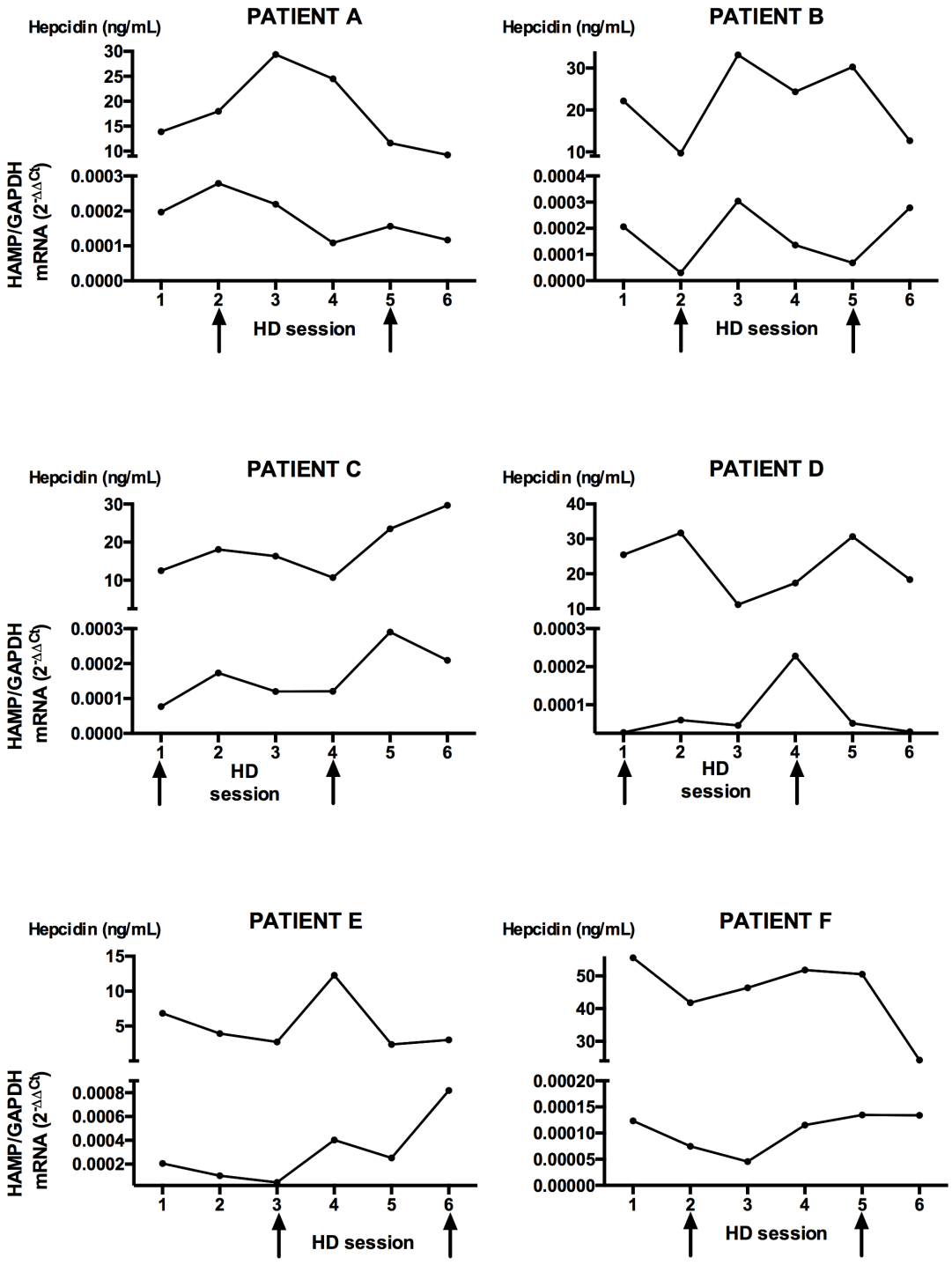

Figure 6.7 Hepcidin and HAMP expression in all six patients following their first 6 haemodialysis sessions

(Bold arrows indicate the HD session wherein iron sucrose was administered (100mg); upper panels represent hepcidin levels, and lower panels represent *HAMP* mRNA levels)

6.3.4 Hepcidin and HAMP Expression Levels – Group Data

There was no difference in the mean hepcidin levels categorised by haemodialysis session (Figure 6.8).

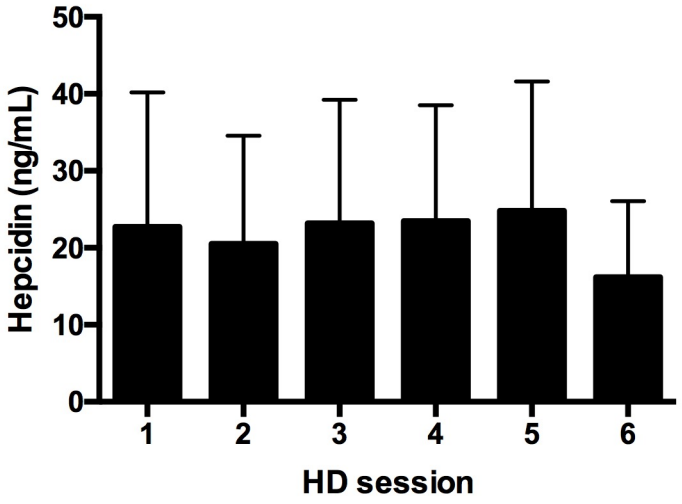

Figure 6.8 Mean hepcidin levels categorised per haemodialysis session

Although there was a trend towards greater mean HAMP expression levels in the samples taken prior to the sixth haemodialysis session, this was not statistically different from the levels seen at any of the other time points (Figure 6.9).

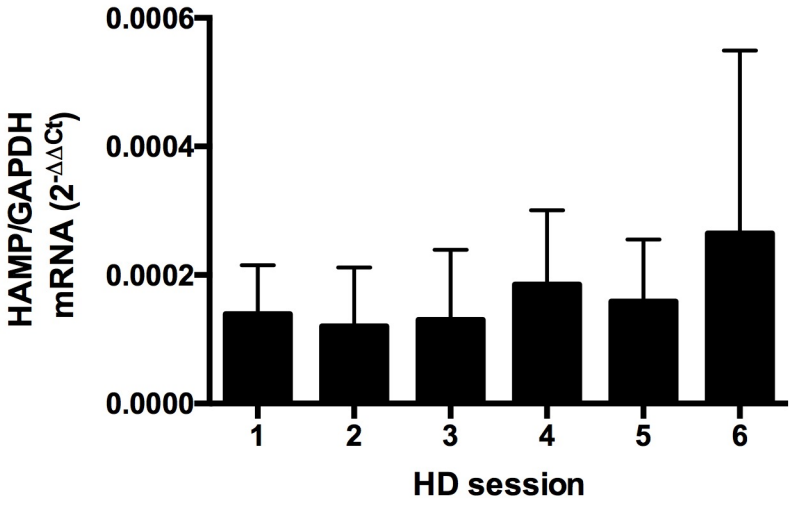

Figure 6.9 Mean HAMP levels categorised per haemodialysis session

There was also no correlation seen (R^2=0.07) between absolute HAMP expression and hepcidin levels (Figure 6.10).

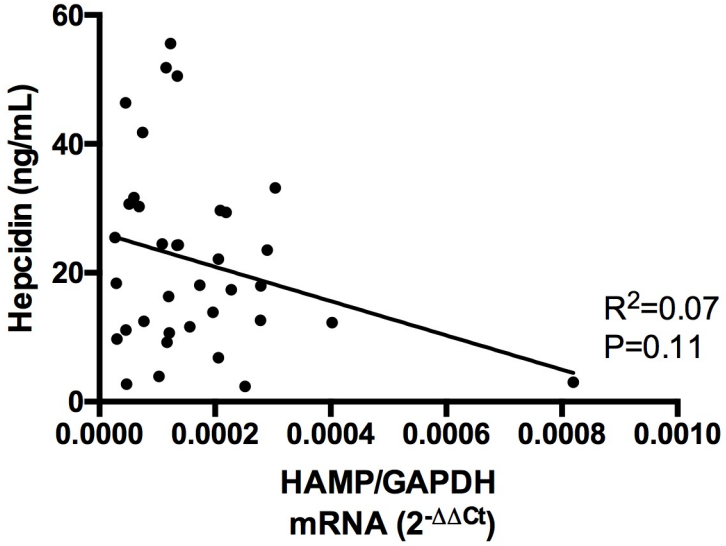

Figure 6.10 Correlation between hepcidin and HAMP expression

6.3.5 Does Heparin Affect Hepcidin or HAMP Expression Following Iron Therapy?

All 6 patients received their first dose of intravenous iron therapy within the first two haemodialysis session; during this time, no heparin was administered. Their second dose of intravenous iron was administered one week after their respective first doses, and all the patients were receiving heparin by this point.

Without heparin, there was a trend towards greater mean HAMP expression and hepcidin levels post-iron therapy compared to the pre-iron therapy levels, although the differences were not statistically significant (HAMP mRNA, p=0.13; hepcidin, p=0.2) (Figure 6.11). The impact of haemodialysis on the levels was negligible, given that the mean dialysis dose delivered during the sessions within which the first dose of intravenous iron was administered was 31.1±8.1 litres processed.

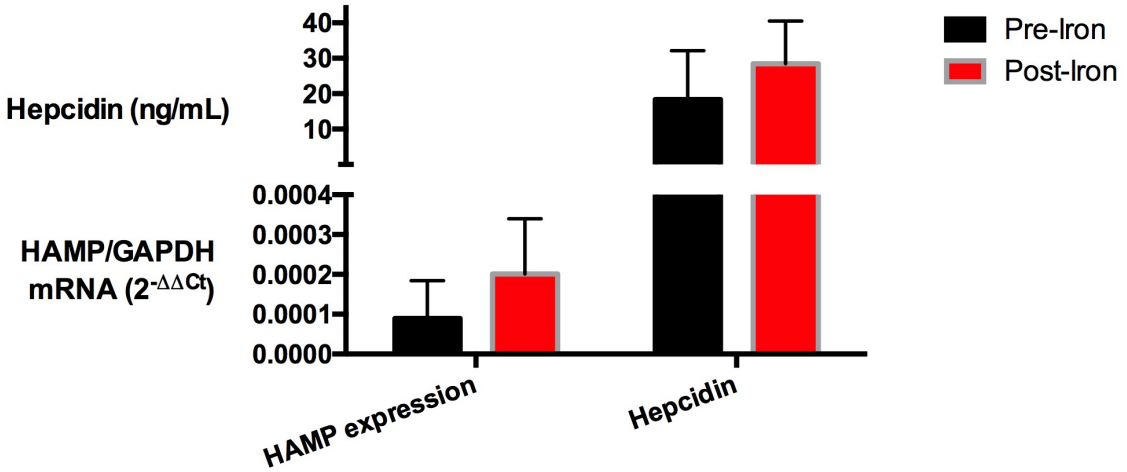

Figure 6.11 Hepcidin and HAMP expression before and after the first dose of intravenous iron
(HAMP mRNA expression, comparison p=0.13; hepcidin comparison, p=0.2)

In contrast, mean hepcidin levels following the second dose of intravenous iron were slightly lower following iron therapy, compared to the pre-iron level, but were not statistically different (p=0.6) (Figure 6.12). HAMP expression followed the same trajectory as seen in the pre-heparin comparison (p=0.6). The dialysis dose delivered during the sessions within which the second dose of intravenous iron was administered was 34.7±9.2 litres processed, which was similar to the pre-heparin group (p=0.7).

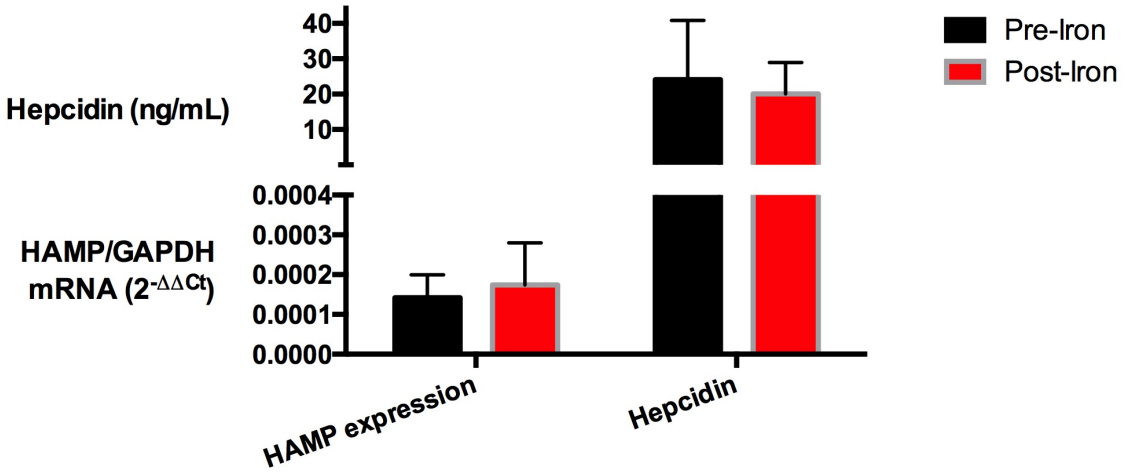

Figure 6.12 Hepcidin and HAMP expression before and after the second dose of intravenous iron

6.4 Conclusions and Discussion

The main findings from the work undertaken in this chapter were as follows;

1. Unfractionated heparin suppresses *HAMP* expression in HepG2 cells in a dose-dependent manner, with maximal suppression occurring above 4 i.u./mL
2. Low molecular weight heparin also suppresses *HAMP* expression, but appears to induce *HAMP* at lower concentrations
3. Patients who were heparin-naïve and started haemodialysis in a planned fashion had a rise in hepcidin levels following a dose of IV iron
4. Once these patients received heparin, 50% of them had a fall in hepcidin levels following a second dose of IV iron
5. No correlation was seen between hepcidin and *HAMP* mRNA

The *in vitro* data presented in this chapter are consistent with the data from Poli and colleagues, confirming that heparin is a potent suppressor of hepcidin expression in hepatocytes (Poli et al. 2011). Unfractionated heparin suppressed hepcidin in a dose-dependent fashion, with near-maximal suppression from 4 i.u./mL in this chapter, whereas Poli and colleagues showed maximal suppression at slightly higher concentrations. Interestingly, HAMP suppression was also demonstrated with the low-molecular weight heparin, but HAMP stimulation was observed at the lower concentrations. The ability of low molecular weight heparins to simultaneously stimulate and suppression HAMP may be due to competitive inhibition with endogenous heparin sulfates or the interaction with BMP antagonists, although the exact mechanism for this remains unknown, and requires elucidation. Similar phenomena have been previously reported in relation to the bone morphogenetic proteins (Takada et al. 2003; Miyazono, Kamiya, and Morikawa 2010).

This is the first attempt to examine the effect of heparin on hepcidin levels in a haemodialysis cohort. This exploratory study was undertaken in a small patient cohort and despite the small numbers, there was a suggestion that those patients who were new to haemodialysis and received IV iron had a rise in hepcidin levels in response to the IV iron. However, this observation was not replicated in patients in whom heparin was administered. None of the patients were on an ESA at the start of the study, but all of the patients were prescribed Neorecormon® 2000 i.u. thrice-weekly as part of routine clinical care, and had a dose administered by at least the second session of haemodialysis. ESAs are a potential confounder as they reduce *HAMP* expression, but

all the patients had a rise in hepcidin levels, whist 4 out of 6 patients had a rise in *HAMP* mRNA following the first dose of iron. Following administration of the second dose of iron, half of the patients had a fall in hepcidin levels and *HAMP* mRNA.

The effect of dialysis on hepcidin levels has been well described, but in this study, the dialysis dose (in terms of litres processed) administered to each patient was less than half the recommended dose. Ashby and colleagues have shown that hepcidin is correlated with both dialysis clearance and ESA dose, although the authors' interpretation of the data was that increased hepcidin clearance led to a reduced erythropoietin requirement, rather than ESAs leading to reduced hepcidin levels (Ashby et al. 2017). If this is true, then we are left with the only other significant modifier being the addition of heparin. However, there was no statistically significant difference between mean hepcidin and *HAMP* levels, before and after the administration of iron.

The dose of heparin administered to the haemodialysis patients was 2000 i.u., which equates to approximately 0.35 i.u./mL in the typical patient with a circulating volume of 5-6L. In the *in vitro* study, this concentration had no discernible effect, and perhaps this may explain the lack of effect at this dose. In light of this, it is possible that the reduction in hepcidin levels in heparin-naïve patients was simply a chance finding and would be better tested in a larger cohort of patients who are ESA-naïve. These patients could be split into 2 groups; one group in whom iron is administered, and a control group. It is theoretically possible that higher concentrations of heparin might suppress hepcidin to a larger degree than seen in this study, but the administration of such doses would not be possible in clinical practice. There are no published data on whether the effect of heparin on *HAMP* differs when cells are in plasma or serum.

The obvious limitation of the study was the patient numbers, which were too small for a definitive effect to be seen, and the study was conducted within the constraints of routine clinical care, which did not allow for the correction for factors such as time between dialysis or dialysis dose (which were incidentally equivalent between the two groups), or the withholding of ESA therapy.

7 Glucocorticoids and Hepcidin

7.1 Introduction and Aims

Given the principal role that hepcidin plays in the metabolism of iron, it is perhaps not surprising that much of the recent renal literature concerning hepcidin has been devoted to its potential role either as a biomarker (to stratify the severity of anaemia or to guide iron therapy), or as a therapeutic target to treat anaemia. There has been less focus on the role that inflammation plays in the renal setting, which in part prompted the work presented in this chapter. As has previously been discussed, the two main drivers of hepcidin production are inflammation, which is largely mediated by IL-6, and iron, which is predominantly mediated by BMP-6 (1.3.5.1). In addition to IL-6, IL-1β and lipopolysaccharide have both been shown to upregulate HAMP expression via the JAK/STAT intracellular signalling pathway (Lee et al. 2005). There is emerging evidence that the two pathways are not completely separate and that there may be crosstalk between the two sides of the hepcidin divide. TMPRSS6 (also known as matriptase-2) inhibits hepcidin expression and is crucial in regulating iron homeostasis (Guo, Casu, et al. 2013). However, it has now been shown that inflammation is also a downregulator of TMPRSS6 expression, acting independently of the BMP-SMAD pathway, occurring instead through a reduction in STAT5 phosphorylation (Figure 7.1) (Meynard et al. 2013).

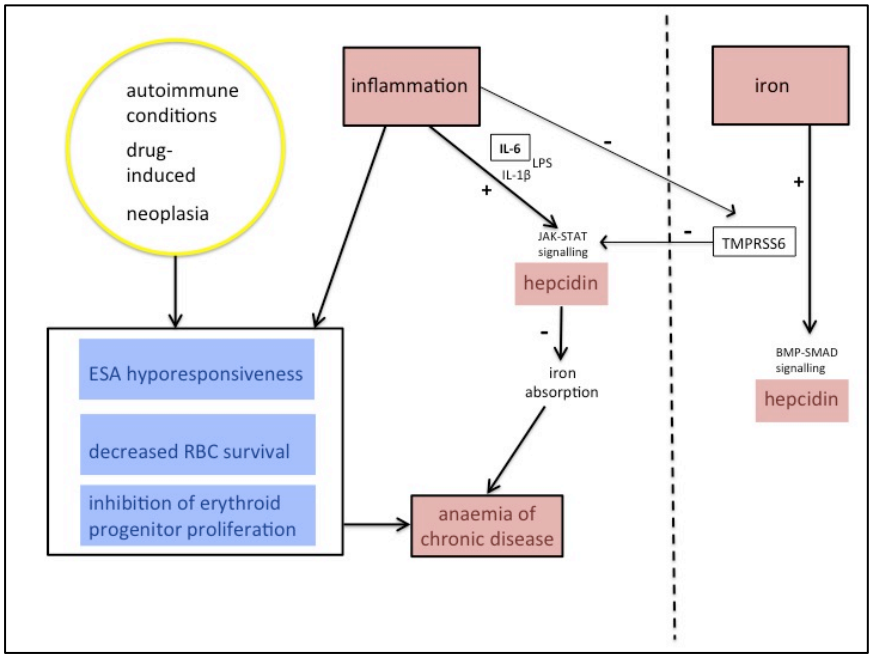

Figure 7.1 Schematic representation of the role of inflammation and hepcidin in the anaemia of chronic disease

It has long been recognised that inflammatory cytokines cause functional iron deficiency by locking iron stores in the reticuloendothelial system, but the effector mechanism was not known until the discovery of hepcidin, providing increased clarity in the understanding of the aetiology of the anaemia of chronic disease (ACD). Hepcidin is crucial for maintaining iron homeostasis, but in the presence of increased hepcidin levels, the cellular iron export protein, ferroportin, is degraded and iron is locked within the reticuloendothelial system, incapable of being used for erythropoiesis (Nemeth, Tuttle, et al. 2004). Thus, inflammatory cytokines ostensibly lead to iron deficiency, or at least an inability to utilise iron efficiently, but they also instigate other effects that result in an anaemic state. This of course is not recent information; the link between inflammation, disordered iron metabolism and anaemia was reported in the 1940s by researchers who injected animals with bacteria and then measured their subsequent capacity to utilise iron (for Hb synthesis) using radioactive tracers (Wintrobe, Greenberg, and et al. 1947). Twenty-seven years later in the same journal, Zucker and colleagues added to this earlier work by describing the link between inflammation and the blunting of EPO responsiveness. They showed that an inflammatory anaemia could be overcome by the use of erythropoietin (Zucker, Friedman, and Lysik 1974). We now know the identity of some of the over-expressed cytokines that are involved in the anaemia of chronic disease, including IL-1, IL6, IL-10, LPS, TNF-α, and IFN-γ (Weiss and Goodnough 2005). Other factors that contribute to the anaemia of chronic disease include direct bone marrow suppression, and decreased survival of red blood cells. Their sites of action are summarised below in Table 7.1.

Cytokine(s)	Site of action
IL-6, LPS	Hepcidin induction
IFN-γ	FPN degradation
IFN-γ, LPS	Increase expression of DMT-1 stimulating iron uptake
IL-1, IL-6, IL-10, TNF-α	Increase ferritin, leading to macrophage iron storage
IL-1, TNF-α, IFN-γ	Inhibit differentiation/proliferation of erythroid progenitor cells
TNF-α, IFN-γ	Decreases renal EPO production
IL-22	Hepcidin induction (animal models)

Table 7.1 Sites of action of cytokines in the development of anaemia
Abbreviations: IL – interleukin; LPS – lipopolysaccharide; IFN – interferon; TNF – tumour necrosis factor; DMT-1 – divalent metal transporter 1; EPO – erythropoietin; FPN – ferroportin; TfR – transferrin receptor; TBI – transferrin bound iron

Many of the treatment options, available for decades prior to the discovery of hepcidin, are still in use, including epoetin, iron, and red cell transfusions (Weiss 2002). Predictably, there is a quest to develop anti-hepcidin strategies, as they provide an attractive addition to the ACD armamentarium, theoretically unlocking hitherto inaccessible iron stores for the purpose of erythropoiesis. These and other novel agents are at various stages in the drug development cycle.

The main drivers of hepcidin production in non-renal chronic inflammatory conditions, such as inflammatory bowel disease or rheumatoid arthritis, are related to the production of monocyte-derived IL-6 and IL-1 that subsequently activate production of interferon β and γ (Raj 2009; Voulgari et al. 1999). To further highlight the integral part that IL-6 plays in inflammatory anaemias, the use of a monoclonal antibody specific for IL-6 (tocilizumab) has been very successful in the treatment of juvenile rheumatoid arthritis, significantly improving the associated anaemia (De Benedetti et al. 2012). In renal disease, many of the pathophysiological mechanisms described above apply to the pathogenesis of anaemia in CKD. The vast majority of studies to date have examined the role of inflammatory cytokines in CKD-anaemia. There is, however, also some interest in the role of IL-22, which is simultaneously implicated in AKI and also able to induce hepcidin in pre-clinical models (Wallace and Subramaniam 2015). The inflammatory milieu of renal disease has consequences aside from anaemia, including renal fibrosis and changes in the renal tubule that control sodium transport, thereby allowing sodium retention in the face of an inflammatory stimulus (Meng, Nikolic-Paterson, and Lan 2014; Norlander and Madhur 2017).

Along with autoimmune-driven disease processes, inflammation is one of the most important pathophysiological mechanisms in nephrology, given the fact that anti-inflammatory drugs, in the form of glucocorticoids, play an essential role in the treatment of a host of renal diseases (Kidney Disease: Improving Global Outcomes (KDIGO) Glomerulonephritis Work Group 2012). These include many of the glomerulonephritides and vasculitides that are encountered during everyday practice. In end-stage kidney disease, corticosteroids are one of the three pillars of current transplant immunosuppression practice along with the anti-proliferative agents and calcineurin inhibitors.

Zucker and colleagues showed that erythropoietin could overcome an inflammatory anaemia without a concomitant rise in the concentration in serum iron, but this was not the only hormone known to have this effect. A clinical study in the 1960s showed that testosterone could also mobilise iron from the reticuloendothelial system, although the mechanism was not understood (Haurani and Green 1967). We now know that steroid hormones such as testosterone, oestradiol and vitamin D have negative regulatory effects on hepcidin, thereby providing an explanation for Zucker's observations (Dhindsa et al. 2016; Yang et al. 2012; Bacchetta et al. 2014). In contrast to the sex steroids, there are very limited data published on the role of glucocorticoids in human hepcidin regulation (Table 7.2).

Author, year	Study/subjects	Steroid – finding
(Salas-Leiton et al. 2012)	Sole (fish)	Dexamethasone – HAMP1 mRNA increased after 48h, but fell significantly after 2 weeks
(Nie et al. 2014)	Case report	Methylprednisolone – hepcidin fell with concomitant rise in Hb, in a patient with IRIDA
(Eisenga et al. 2017)	Observational; RTx patients	Hepcidin inversely associated with urinary prednisolone excretion

Table 7.2 Studies examining the relationship between hepcidin and glucocorticoids
Abbreviations: HAMP – hepcidin antimicrobial peptide; IRIDA – iron-refractory iron deficiency anaemia; RTx – renal transplant

There is a single case report of a glucocorticoid (methylprednisolone) being used as a therapeutic agent in a 10-year-old girl with IRIDA (iron-refractory iron deficiency anaemia, resulting from a mutation in the gene encoding for TMPRSS6). Despite recurrent red cell transfusions, she remained profoundly anaemic. TMPRSS6 works by cleaving membrane-bound HJV, with the net effect of preventing BMP-SMAD activation, thus preventing hepcidin upregulation (Finberg et al. 2008).

In IRIDA, the TMPRSS6 mutation leads to unregulated hyperhepcidinaemia, explaining why this patient remained anaemic despite iron and EPO therapy. Methylprednisolone (0.8mg/kg/day) was administered daily for one month; her hepcidin level fell 3-fold into the normal range, whilst simultaneously experiencing a rise in her Hb, which more than doubled from 58g/L to 124g/L (Figure 7.2).

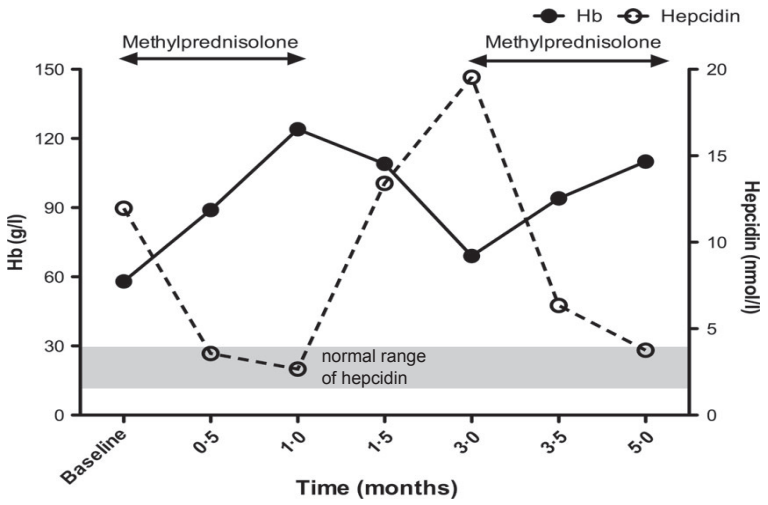

Figure 7.2 The effect of methylprednisolone on hepcidin and haemoglobin levels in a patient with IRIDA
(Reprinted from (Nie et al. 2014))

Upon cessation of the methylprednisolone, the patient's hepcidin levels once again dramatically increased and her Hb returned to its pre-treatment level. Following a re-challenge with methylprednisolone, her hepcidin levels fell and her Hb once again increased (Nie et al. 2014).

Given the paucity of data in the literature examining the interaction of glucocorticoids and hepcidin, the aim of the work presented in this chapter is to:

1. Use an in vitro model to investigate the effect of glucocorticoids on HAMP mRNA in a human hepatoma cell line
2. Explore the effect of methylprednisolone on hepcidin levels in a cohort of vasculitis and glomerulonephritis patients presenting acutely to the renal unit at King's College Hospital

7.2 Subjects and Methods

7.2.1 In Vitro Experiments

The in vitro experiments presented in this chapter were undertaken using cells that were gifted by Professor Andrew McKie (Nutritional Sciences, King's College London). All experiments were carried out in the Renal Laboratory at the Rayne Institute, Denmark Hill, King's College London. Cell culture experiments were carried out using HepG2 cells (ATCC). The reagents used are listed in Table 7.3.

7.2.1.1 Cell Culture and Reagents

HepG2 cells are human hepatoma cells that were derived from a 15-year-old Caucasian male with hepatocellular carcinoma. They have been used in many studies investigating hepcidin regulation and are adherent in cell culture. They were routinely grown and passaged in rectangular 75cm^2 flasks (431464, Corning, UK) containing Dulbecco's modified Eagle medium (DMEM) (11965092, Gibco, ThermoFisher Scientific, Paisley, UK) supplemented with 10% fetal bovine serum (FBS) (F7524, Sigma-Aldrich). The medium was treated with 100U/mL penicillin and 100µg/mL streptomycin (15070-063, ThermoFisher Scientific), and the flasks were incubated in a Techne incubator at a temperature of 37°C, in a humidified atmosphere containing 5% CO_2. When the cells were approximately 80% confluent, the growth medium was removed and the cells were twice-washed with phosphate-buffered saline (PBS) (D8537, Sigma-Aldrich) to remove any trace of serum-supplemented medium, which may have rendered the trypsinisation process ineffective.

The smallest amount (2mL) of trypsin EDTA (25300, ThermoFisher Scientific) was added to allow complete coverage of the cells, and the flasks were placed in the incubator for 5 minutes to allow complete detachment of the cells from the flask. FBS-supplemented medium was then added to neutralise the trypsin. In order to completely remove the trypsin, the cells were centrifuged (Heraeus Labofuge 400) at 15000G for 5 minutes, with subsequent removal of the supernatant; the cells were then washed with PBS, and 20mL of warmed medium was used to suspend the pellet. The cells were counted and seeded into 35mm dishes (627 160, Greiner Bio-one) at a concentration of 8 x 10^5 cells/dish with a total of 2mL DMEM, reaching 70% confluence after a 48-hour incubation period. The cells were pre-treated with dexamethasone (D4902, Sigma-Aldrich) for up to 18 hours, and serum-starved overnight, for experimental use the following morning.

Reagent	Company	Catalogue number
BMP-6	R&D systems	507-BP-020
Dexamethasone	Sigma-Aldrich	D4902
Dulbecco's modified Eagle Medium	ThermoFisher Scientific	41966029
Ethanol	Haymankimia	F200236
Fetal bovine serum	Sigma-Aldrich	F7524
IL-6	Life Technologies	PHC0065
Penicillin	ThermoFisher Scientific	15070-063
Phosphate-buffered saline	Sigma-Aldrich	D8537
Streptomycin	ThermoFisher Scientific	15070-063
Trypsin EDTA	ThermoFisher Scientific	25300

Table 7.3 Reagents used in experimental cell culture

Dexamethasone (10mg) was reconstituted with ethanol (F200236, Haymankimia) and DMEM, as per the manufacturer's instructions, to a stock solution of 20 µg/mL, and serial dilutions were then made in order to achieve a final concentration of 1.22 ng/mL using DMEM growth medium (Table 7.4). IL-6 (PH C0065, Life Technologies) and BMP-6 (507-BP, R&D Systems) were also reconstituted according to the manufacturers' instructions.

Dilution	Concentration
0	20 µg/mL
1	10 µg/mL
2	5 µg/mL
3	2.5 µg/mL
4	1.25 µg/mL
5	625 ng/mL
6	312.5 ng/mL
7	156.25 ng/mL
8	78.12 ng/mL
9	39.06 ng/mL
10	19.53 ng/mL
11	9.77 ng/mL
12	4.88 ng/mL
13	2.44 ng/mL
14	1.22 ng/mL

Table 7.4 Serial dilutions of 20µg/mL stock solution of dexamethasone
(Shaded concentrations used in 7.3.1)

7.2.1.2 Cell Viability Assay

HepG2 cell viability during experimental conditions was assessed using a lactate dehydrogenase (LDH) assay (Cytotox 96 non-radioactive cytotoxicity, G1780,

Promega, UK). LDH is a stable enzyme ($t_{½}$=9 hours) found in the cytosol and is released when there is cellular damage.

5 x 10^3 HepG2 cells were suspended in 200µL DMEM, seeded into a sterile 96-well plate (3596, Corning), and incubated in an environment containing 5% CO_2 at 37°C. The experimental schema comprised cells that were untreated, cells with the addition of dexamethasone (a dose of 20ng/mL was selected following the dose-response treatment curves), and cells with vehicle alone (2% ethanol). The cells were incubated for 48 hours before being serum-starved overnight (DMEM) and were subsequently treated with dexamethasone for 18 hours. LDH activity was assessed according to the manufacturer's instructions, and absorbance was read on a 96-well spectrophotometer. Absorbance, at a wavelength of 490nm, was measured in each well.

7.2.1.3 RNA Extraction

All work surfaces were cleaned with detergents and industrial methylated spirits prior to RNA extraction. An RNase decontamination solution (AM9780, Invitrogen) was used for added security against RNases. The QIAshredder (79654, Qiagen) and RNeasy Mini Kits (74104, Qiagen) were the principal apparatus used in the extraction of RNA from cellular material and used according to the manufacturers' instructions. All RNA that was obtained was immediately stored at -80°C for reverse transcription at a future occasion.

7.2.1.4 Reverse Transcription

Reverse transcription from RNA to cDNA was undertaken using the High Capacity RNA-to-cDNA Kit (4387406, Applied Biosystems), according to the manufacturer's instructions. An 8-well NanoDrop spectrophotometer (ThermoFisher Scientific) was utilised to determine the quantity and purity of the RNA prior to reverse transcription. Samples were then pipetted into 96-well reaction plates (N801-0560, Applied Biosystems) combined with the reagents supplied by the manufacturer, to a total volume of 20µL. The samples were heated in a cyclical manner (Veriti Thermal Cycler, Applied Biosystems) for 65 minutes, before being cooled to 4°C; 80µL of RNase-free water (129112, Qiagen) was added to each sample to bring the reaction to a complete halt. Samples were immediately stored at -20°C.

7.2.1.5 Quantitative PCR

Quantitative PCR is used to measure the amplification of DNA, and this methodology can detect even minor changes in gene expression. TaqMan (Applied Biosystems) primer-probes were used for the detection of HAMP mRNA (Hs00221783_m1) and GAPDH mRNA (Hs02758991_g1). The primer-probes were combined with TaqMan Universal PCR Master Mix (4324018, Applied Biosystems), RNase-free water and the reverse-transcribed cDNA. Volumes of 10µL per sample, which were tested in triplicate, were placed in a 384-well plate (4309849, Applied Biosystems), and a QuantStudio 7 Flex Real Time PCR System (Applied Biosystems) was used to perform the analysis that would determine the expression of the target gene (*HAMP)* relative to the housekeeping gene (*GAPDH)*.

7.2.1.6 Protein Extraction

1 x 10^6 HepG2 cells were plated in 60mm dishes with 5mL culture medium containing DMEM supplemented with 10% FBS, penicillin and streptomycin as described in 6.2.1.1 (page 157). The dishes were incubated until the cells were approximately 70% confluent; cells were serum-starved prior to the application of experimental treatments/vehicle. The dishes were placed on ice and the cells washed thoroughly (twice) with PBS. Isolation of cellular protein occurred via disruption of the cell membranes using a PBSTDS lysis buffer with the addition of protease inhibitors (1x PBS, 1% Triton-X 100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), leupeptin 0.5 µg/ml, pepstatin 1.0 µg/ml, EDTA 1.0 mM, PMSF 0.2 mM). To allow for the detection of phosphorylated proteins, phosphatase inhibitors (1mM sodium orthovanadate, 25mM sodium fluoride) were added. The dishes were rested on ice for 45 minutes and the contents were subsequently transferred to eppendorf tubes. A 1mL syringe and 22G needle (Terumo) was used to mechanically disrupt the cells to allow complete homogenisation of the samples, and the tubes were centrifuged at 4°C at 14000 RPM for 10 minutes (Mikro 22R, Hettich), to separate cellular debris from the protein-rich supernatant.

7.2.1.7 Protein Quantification

The supernatants from the samples were collected in labelled eppendorf tubes and the protein content determined using a spectrophotometric method detecting colorimetric absorbance at a wavelength of 562nm. Quantification of protein within the study samples was calculated using the Pierce™ BCA Protein Assay Kit (23225,

ThermoScienific), and compared to pre-prepared protein standards with known concentrations of protein containing bovine serum albumin (BSA).

7.2.1.8 Protein Analysis

Expression of ferroportin, SMAD5, STAT3, and phosphorylated SMAD 1/5 and STAT3 proteins were determined by immunoblotting. The samples were prepared by adding loading buffer consisting of β-mercaptoethanol (βME), bromophenol blue, EDTA, glycerol, SDS, and Tris HCL (pH 6.8). The samples were heated at 100°C in order to denature the proteins.

Total cell extracts (10-20μg) were separated on 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels that were made with 30% acrylamide, 10% ammonium persulphate, 10% SDS, TEMED, 1.5M Tris, and water, before being electro-blotted onto a nitrocellulose membrane. Ponceau S solution (Sigma Aldrich) was used to detect the successful transfer of proteins to the membrane. The membrane was then washed and blocked in overnight at 4°C in 5% BSA, before being probed with primary antibodies against ferroportin, SMAD5, STAT3, and phosphorylated SMAD and STAT proteins (Table 7.5). HRP-conjugated secondary antibodies (**REF**) were applied at room temperature and the protein products were then visualised using the ECL Prime System (GE Healthcare Life Sciences).

Antibody	Company	Catalogue number
Ferroportin	Novus Biological	21502SS
Ferroportin	Santa Cruz	SC-49668
Ferroportin	Abcam	AB70866
SMAD5	Cell Signalling	9517
STAT3	Cell Signalling	4904
Phospho-SMAD1/5	Cell Signalling	9516
Phospho-STAT3	Cell Signalling	9134
GAPDH	Millipore	MAB374

Table 7.5 Antibodies used in immunoblotting experiments

7.2.2 Clinical Study

The aim of this study was to assess the response of hepcidin levels in patients receiving systemic glucocorticoid therapy, over a 4-day period.

7.2.2.1 Subjects

Subjects were adult patients who were admitted to the Renal Unit at King's College Hospital to receive glucocorticoid therapy in the form of methylprednisolone. We chose to include patients suspected of renal vasculitis or glomerulonephritis, as they were a group of patients who were relatively frequently encountered who were to receive pulsed steroid therapy as part of routine clinical care.

7.2.2.2 Study Design

This was an exploratory, prospective, longitudinal cohort study. Patients with existing end-stage kidney disease or presenting with an acute kidney injury requiring dialysis (before the baseline study sample) were excluded from the study due to the impact of dialysis on hepcidin levels. The inclusion criteria (Table 7.6) were extremely broad. We excluded anyone who had received steroid therapy within 4 weeks of admission, based on the case report described in Table 7.2. In effect, this excluded the vast majority of renal transplant patients, who were taking prednisolone as part of their transplant immunosuppression regimen, and who may otherwise have been considered if they were admitted for treatment for acute transplant rejection. Exclusion criteria included a history of haematological dyscrasias, defined as any disease process that necessitated the patient having visited the Haematology department at any time for treatment or follow-up (except for iron-deficiency anaemia).

INCLUSION CRITERIA	EXCLUSION CRITERIA
≥18 years of age	Active malignancy
Suspected renal vasculitis or glomerulonephritis	Acute kidney injury requiring dialysis
	Blood transfusion within 4 weeks
	Chronic inflammatory condition
	End-stage kidney disease
	History of haematological dyscrasia
	History of liver disease
	Hospital admission within 4 weeks
	Signs of acute/occult bleeding
	Steroid therapy within 4 weeks

Table 7.6 Inclusion and exclusion criteria for glucocorticoid study

Patients identified as having an acute kidney injury as a result of an ANCA-associated vasculitis or crescentic glomerulonephritis were transferred into the Renal Unit from other wards within King's College Hospital, or peripheral hospitals allied to KCH, with a

plan to treat with steroids. Initial treatment for these conditions was 3 intravenous pulses of methylprednisolone (500mg).

Once the patients had been identified, they were approached and given a patient information sheet. If they agreed to participate in the study and provided written informed consent, baseline blood samples were taken.

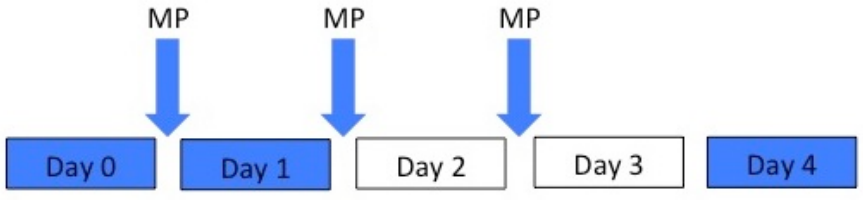

Figure 7.3 Schematic representation of the blood sampling schedule in relation to the dosing of methylprednisolone
(Abbreviation: MP – methylprednisolone; blood samples taken on days 0, 1, and 4)

A baseline sample (Day 0) was obtained prior to the first administration of methylprednisolone. A second sample (Day 1) was taken after the first dose of methylprednisolone but before the second dose, and a final sample was taken (Day 4) 48 hours after the third and final dose of methylprednisolone (Figure 7.3). It was felt that samples taken after this time would have been confounded by the likely addition of further immunosuppressive therapies.

7.2.2.3 Demographics and Baseline Data

A total of 6 patients who satisfied the inclusion and exclusion criteria were recruited for this study. Other patients were eligible (n=3) but were considered unable to provide consent by the investigator. All the patients were admitted with a plan to treat with steroids; 5 patients had a suspected ANCA-associated vasculitis and the 6th had a suspected crescentic glomerulonephritis. The mean age of the 6 patients was 57.5 ± 20.0 years. Five of the subjects were male, and 4 were Caucasian. The patients presented with advanced kidney dysfunction; the mean presenting creatinine was markedly elevated at 412.3 ± 186.3 µmol/L. As would be expected in this patient cohort, there was laboratory evidence of inflammation (mean CRP was 79.2 ± 54.2 mg/L, and mean albumin was 28.5 ± 5.4 g/L). The patients were also markedly anaemic – the mean haemoglobin was 98.9 ± 16.2 g/L – with a normocytic picture (Table 7.7).

Age (years)	57.5 ± 20.0
Males (n (%))	5 (83.3)
ANCA, myeloperoxidase/proteinase-3	4/1
Caucasian (n (%))	4 (66.7)
Diabetes (n (%))	1 (16.6)
Hypertension (n (%))	1 (16.6)
Creatinine (μmol/L)	412.3 ± 186.3
Haemoglobin (g/L)	98.9 ± 16.2
Mean cell volume (10^{-15}/L)	89.6 ± 4.8
CRP (mg/L)	79.2 ± 54.2
WBC/neutrophils/lymphocytes (10^9/L)	11.5 ± 1.5 / 9.0 ± 1.5 / 1.4 ± 0.4
Albumim (g/L)	28.5 ± 5.4

Table 7.7 Demographics and baseline laboratory data

The patients had biochemical features that would be expected to induce an increase in hepcidin levels up (inflammation, renal impairment), with anaemia as the only factor expected to lower hepcidin.

7.2.2.4 Methods

The methods of hepcidin measurement by mass spectrometry have been discussed in detail previously (1.4.1). Routine patient blood samples were processed in the King's College Hospital central laboratory. The method for HAMP mRNA measurement will be briefly revisited but is covered comprehensively on page 80.

HAMP mRNA measurement

- 10mL blood drawn (and divided into two) and separated by Ficoll-Hypaque Plus (GE Healthcare) centrifugation
- Plasma was obtained post-centrifugation and immediately stored in 500μL aliquots
- Two sets of $5x10^6$ peripheral blood mononuclear cells were isolated for each participant
- Total cellular RNA was subsequently extracted (RNeasy, Qiagen) and immediately stored at -80°C
- 500ng RNA was reverse transcribed (High Capacity RNA-to-cDNA Kit, Applied Biosystems)

- All cDNA samples were processed in a single run to eliminate inter-experimental variability
- Amplification reactions were performed using Taqman HAMP and GAPDH primer-probes in triplicate
- Quantitative RT-PCR was performed using the 7900 HAT Fast Real-Time system (Applied Biosystems)

7.2.2.5 Statistical Analyses

Differences were determined with the independent Student's t test. Relationships between various parameters under study were also examined; simple linear coefficients (r) were calculated using the Pearson's product formula. P-values <0.05 (two-tailed) were considered statistically significant. ImageJ (NIH, version May 2018) was used for the optical density evaluation of the immunoblots, and the statistical analyses incorporated the Tukey's method in conjunction with ANOVA to correct for multiple comparisons. Analyses were performed using GraphPad Prism version 6.0 for Macintosh, GraphPad Software, La Jolla California USA.

7.2.2.6 Ethics and Regulatory Approvals

The London Research Ethics Committee 1 (LREC 09/H0718/034) granted ethical approval. Local R&D approval was obtained to undertake this study, which was conducted in accordance with the principles outlined in the Declaration of Helsinki. Patient information leaflets were provided to all participants who gave their written informed consent.

7.3 Results

Results are expressed at means ± standard deviations, unless otherwise specified.

7.3.1 Dexamethasone Dose-Response

HepG2 cells were incubated in increasing doses of dexamethasone, ranging from 1.2 ng/mL to 78.1 ng/mL for 18 hours. The control dishes contained vehicle (0.5% ethanol).

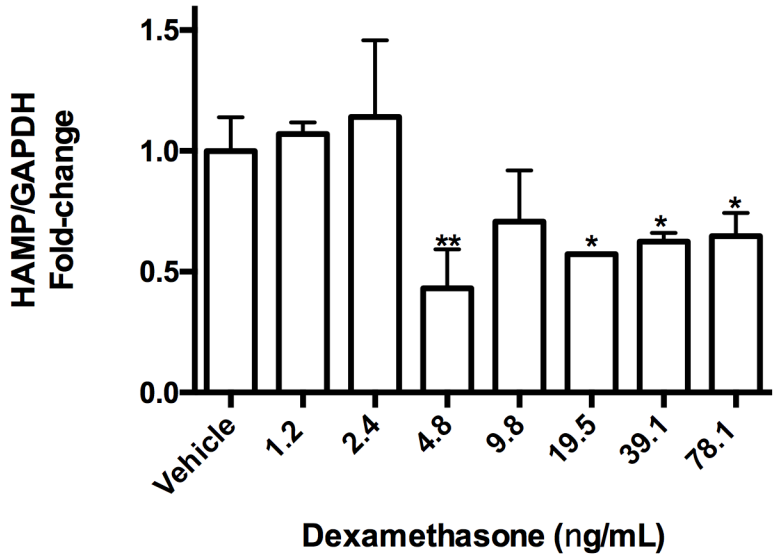

Figure 7.4 Dose-response curve: dexamethasone concentrations 1.2 to 78.1 ng/mL
(Comparisons were between varying doses of dexamethasone and vehicle)

There was a sustained decrease in HAMP mRNA levels with doses of dexamethasone that were greater than 2.4 ng/mL, although the results did not appear consistent. At a concentration of 4.8 ng/mL, there was a significant decrease in *HAMP* mRNA ($p=0.009$) compared with vehicle, and this trend was maintained at all higher doses with significantly reduced levels at 19.5, 39.1, and 78.1 ng/mL (all $p<0.05$) (Figure 7.4).

The experiment was repeated with fewer serial dilutions (7 versus 14) of the initial stock solution of dexamethasone. There was a sustained decrease in HAMP mRNA seen with doses of dexamethasone as small as 0.3 ng/mL ($p=0.0043$), which was sustained through to 20 ng/mL, with no apparent dose dependent response above 0.3 ng/mL (Figure 7.5).

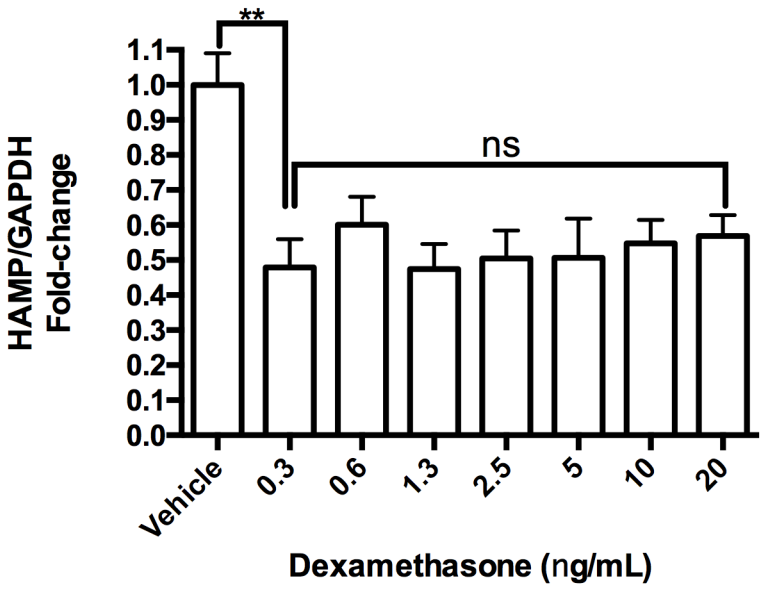

Figure 7.5 Dose-response curve: dexamethasone concentrations 0.3 to 20 ng/mL
(Comparison between vehicle and 0.3 ng/mL, p=0.0043)

7.3.2 Effect of Dexamethasone on HepG2 Viability

The solvent concentration of ethanol 2% did not reduce cell viability. A dose of 20ng/mL of dexamethasone was selected to test HepG2 cell viability, and no effect on cell viability was observed (Figure 7.6).

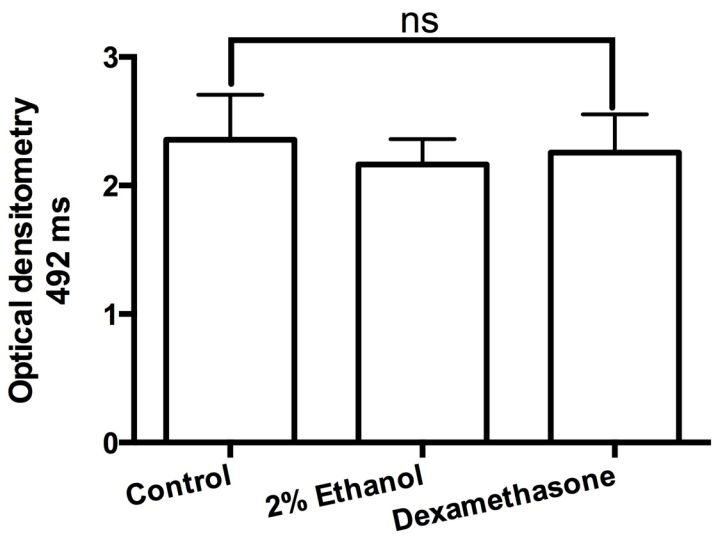

Figure 7.6 – Effect of 20ng/mL dexamethasone upon HepG2 cells

7.3.3 Effect of IL-6 Stimulation on Dexamethasone Treated Cells

Dexamethasone treated HepG2 cells showed a reduction in HAMP mRNA with increasing concentrations of corticosteroid. Dexamethasone doses of 10ng/mL and above diminished the IL-6-induced upregulation of HAMP mRNA, which was clearly witnessed in vehicle treated cells, and in cells treated with 0.01, 0.1, and 1.0 ng/mL of dexamethasone (Figure 7.7).

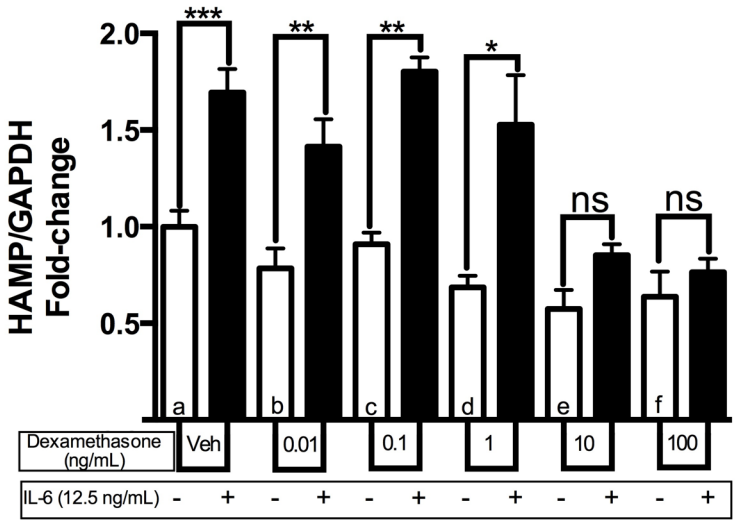

Figure 7.7 Effect of IL-6 stimulation (12.5ng/mL for 1 hour) upon dexamethasone pre-treated HepG2 cells (20ng/mL for 18 hours)
(Black bars represent IL-6 treated cells; white bars (a-f) are vehicle (IL-6) treated cells; asterisks represent comparisons between IL-6 and vehicle treated cells at increasing concentrations of dexamethasone; *** $p<0.0001$, ** $p<0.01$, * $p<0.05$)

All differences between the vehicle treated control sample (labelled 'a' in Figure 7.7) and the other samples (labelled b-f) were significant, except sample c (Table 7.8).

Sample	p-value
b	0.047
c	0.198
d	0.005
e	0.0045
f	0.015

Table 7.8 Comparison of reference sample (a) with other non-IL-6 treated samples (b-f) in Figure 7.7

Even when HepG2 cells were exposed to a higher dose of IL-6 (25ng/mL) for a longer period of time (2 hours), a similar dose dependent relationship was observed. At

1ng/mL and 6.25ng/mL of dexamethasone, the effect of IL-6 appeared to be diminished, which varied slightly from the previous experiment (Figure 7.8).

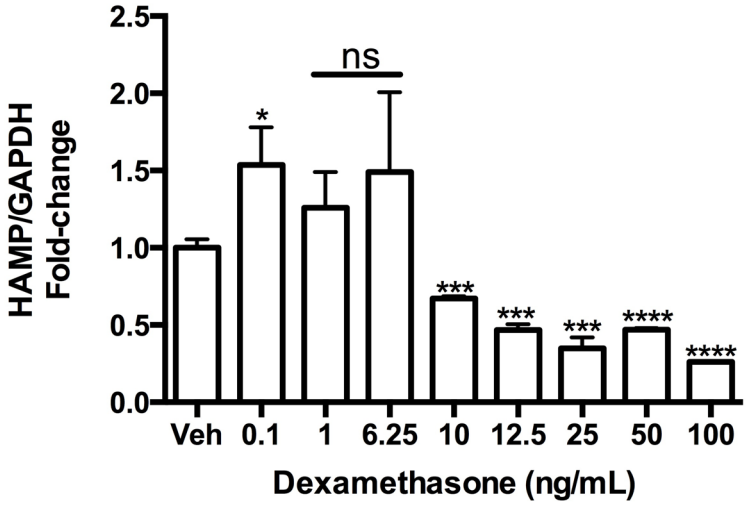

Figure 7.8 Effect of IL-6 stimulation (25ng/mL for 2 hours) upon dexamethasone pre-treated HepG2 cells (16 hours)
(Comparisons: the vehicle treated cells act as the reference experiment)

However, the abolition of the robust IL-6 upregulation on HAMP mRNA in HepG2 cells occurred at dexamethasone concentrations greater than 10ng/mL in both experiments.

7.3.4 Effect of BMP-6 Stimulation on Dexamethasone Treated Cells

In contrast to IL-6, dexamethasone had no evident ability to reduce BMP-6 induced upregulation of HAMP.

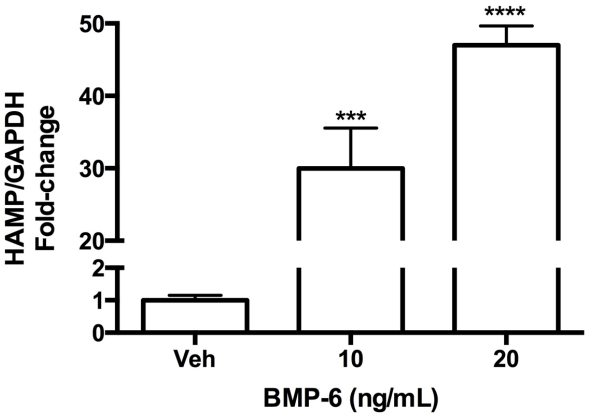

Figure 7.9 Effect of BMP-6 stimulation (applied for 2 hours) upon dexamethasone pre-treated HepG2 cells (18 hours)

HepG2 cells were incubated with 20ng/mL of dexamethasone for 18 hours and stimulated with BMP-6 at two separate concentrations for 2 hours. HAMP was nearly 30-fold higher (p=0.0008) following the addition of 10ng/mL BMP-6, and nearly 45 to 50-fold higher following a 20ng/mL treatment (p<0.00001) (Figure 7.9). These results were in keeping with previous results (5.3.4).

7.3.5 Ferroportin and Dexamethasone

HepG2 cells were treated with 20ng/mL dexamethasone for 18 hours, and subsequently treated with IL-6 (25ng/ml), BMP-6 (20ng/mL), or vehicle for 2 hours. Immunoblotting was undertaken to assess the potential in vitro effect of dexamethasone upon ferroportin. Two ferroportin antibodies demonstrated marked non-specific binding (Santa Cruz and Novus Biological). A third antibody was employed (Abcam) that produced blots that allowed interpretation of the results (Figure 7.10).

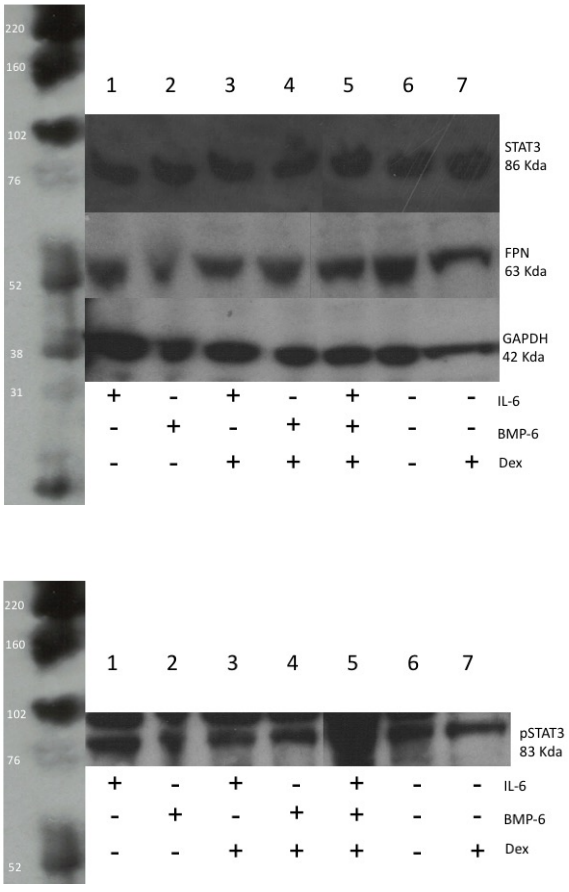

Figure 7.10 Western blots of GAPDH, ferroportin, STAT3, and pSTAT3
(Dexamethasone 20ng/mL treatment applied for 18 hours; IL-6 25ng/mL, and BMP-6 20ng/mL applied for 2 hours; blots representative of 2 independent experiments)

The BMP-6 control (blue line) reduced ferroportin expression, as was expected. The IL-6 control (lane 1) did not produce meaningful data and was thus excluded from further analyses. Dexamethasone-treated cells had higher ferroportin expression (lane 7) than untreated cells (lane 6) (p=0.092), although statistical significance was lost once Tukey's correction was employed to correct for multiple analyses. Dexamethasone-treated cells that were then stimulated with cytokines (IL-6 or BMP-6, lanes 3 and 4, respectively) had lower FPN expression that cells treated with dexamethasone alone (lane 7). Cells that were stimulated with BMP-6, regardless of whether they had been pre-treated with dexamethasone, had lower ferroportin expression than the reference cells. The IL-6 stimulated cells that were treated with dexamethasone (lane 3) and the reference cells had similar levels of ferroportin expression (Figure 7.11).

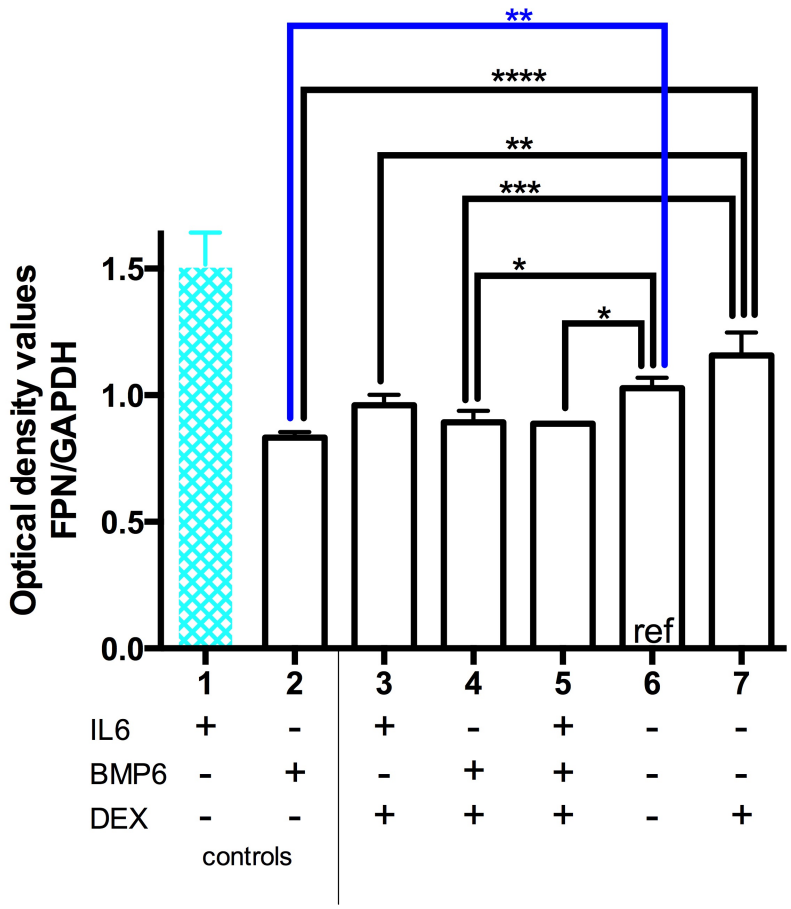

Figure 7.11 Optical density of FPN normalised to GAPDH
Reference protein (ref) in lane 6; control in lane 1 (IL-6 stimulated) was not used in the analyses. BMP-6 negative control (blue line).

7.3.6 Immunoblotting For STAT and SMAD Proteins

There was no difference in STAT3 expression across any of the experimental conditions. Lanes 4 (dexamethasone and BMP-6) and 7 (dexamethasone alone) appeared to have levels of STAT3 expression that appeared lower than the others, but this was not borne out in the statistical analyses (Figure 7.12).

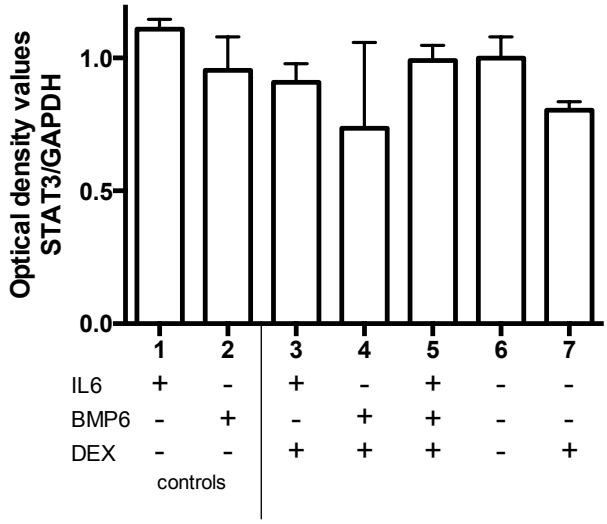

Figure 7.12 Optical density of STAT3 normalised to GAPDH

Blotting for phosphorylated proteins proved very difficult and the optical density analysis was such that it did not allow for meaningful interpretation (Figure 7.13).

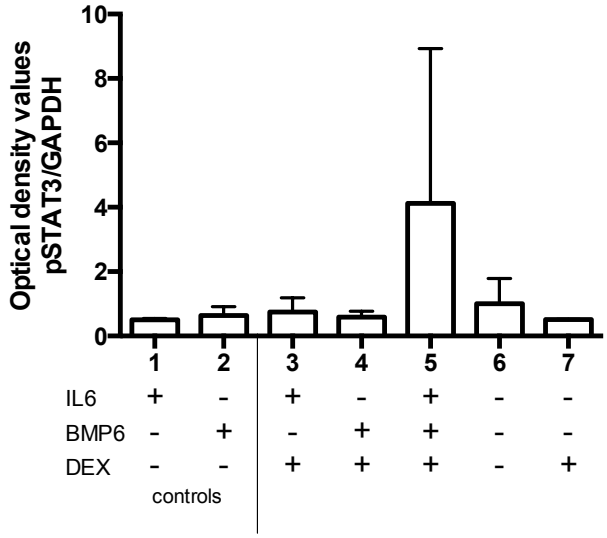

Figure 7.13 Optical density of phosphoSTAT3 normalised to GAPDH

Multiple blots were also undertaken for SMAD proteins to analyse the effects of BMP-6 stimulation in their role as a control (Figure 7.14). Despite multiple attempts to run the experiments in their entirety, and immunoblotting undertaken by 2 operators in different laboratories, the blots were not analysed due to their appearance.

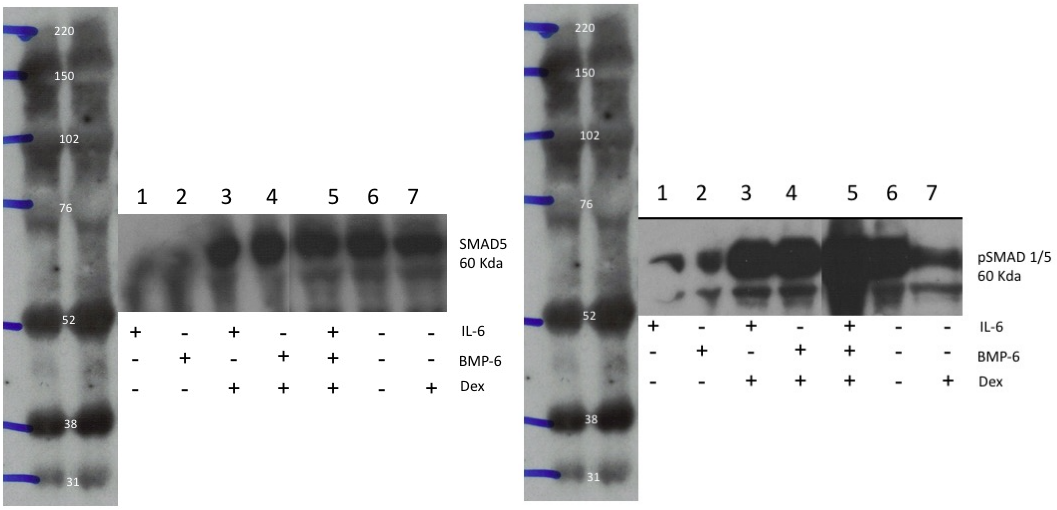

Figure 7.14 Western blots of SMAD5 and pSMAD 1/5
(Dexamethasone 20ng/mL treatment applied for 18 hours; IL-6 25ng/mL, and BMP-6 20ng/mL applied for 2 hours; blots representative of 2 independent experiments; results did not allow for meaningful interpretation)

7.3.7 Does Glucocorticoid Steroid Therapy Reduce Hepcidin In Vivo?

Hepcidin levels were markedly increased at baseline in 5 of the 6 patients recruited to this study. There was a significant decrease in hepcidin levels following the first dose of 500mg intravenous methylprednisolone in all the study subjects (p=0.0486) (Figure 7.15).

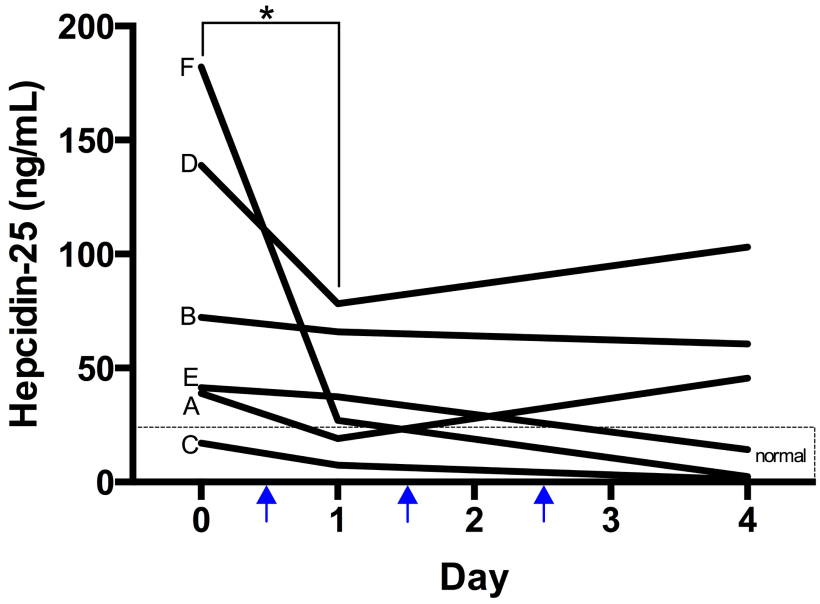

Figure 7.15 Hepcidin levels in AKI patients before and after receiving 3 pulses of methylprednisolone
(Individual patient data plots labelled A-F; blue arrows denote timing of methylprednisolone administration; normal range for hepcidin-25 shown by dashed box; hepcidin levels were lower on day 1 post-methylprednisolone compared to baseline (p=0.0486))

The decrease in hepcidin levels from day 1 to day 4 was seen in 4 out of the 6 subjects, with 3 of the subjects having hepcidin levels within the normal range by the final time point. Two of the subjects (patients A and D) experienced a rise in hepcidin levels from day 1 to day 4. These two patients had blood transfusions after day 2 and 3, respectively. Patient D had plasma exchange due to a presenting creatinine of 737µmol/L and a session of haemodialysis after day 2. Patient F had a session of haemodialysis on day 3, but this did not appear to have an influence on their hepcidin levels (Figure 7.16).

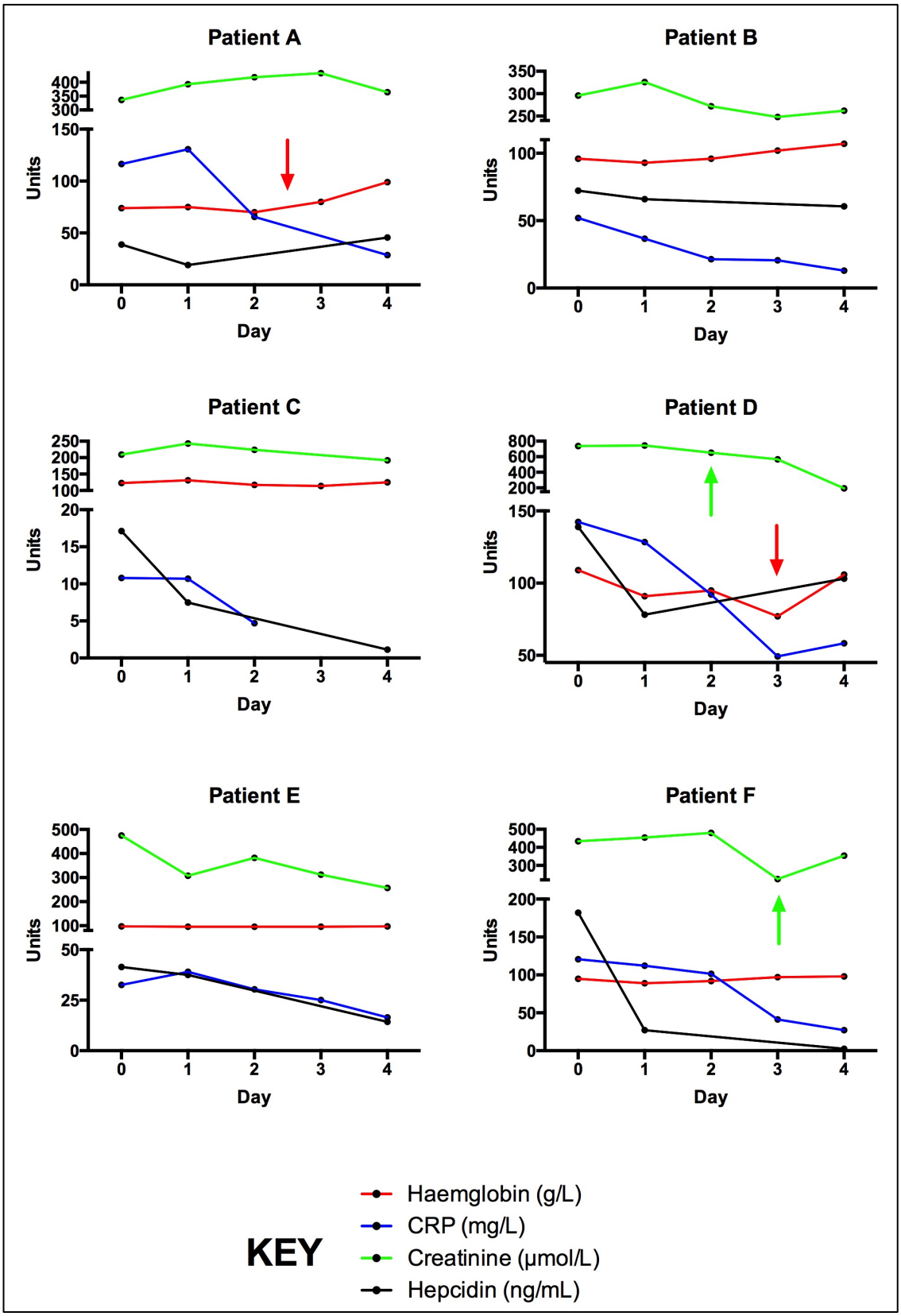

Figure 7.16 – Individual patient data outlining haemoglobin, CRP, creatinine and hepcidin levels
(Red arrows denote transfusion of pack red cells; green arrows denote commencement of haemodialysis)

7.3.8 Does Glucocorticoid Steroid Therapy Impact Upon PBMC HAMP?

In contrast to the protein levels, there was no significant difference between PBMC HAMP levels before and after the administration of methylprednisolone. HAMP levels at both the day 1 and day 4 time points were numerically greater than at baseline (Figure 7.17).

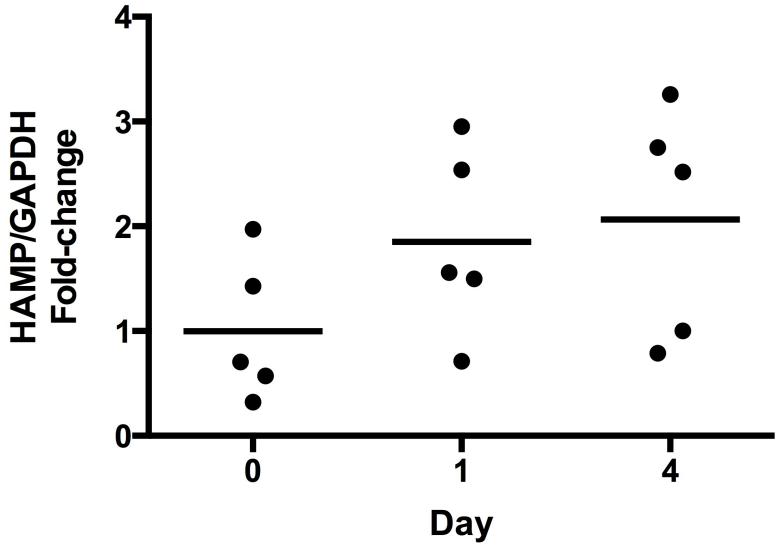

Figure 7.17 HAMP mRNA at baseline, and at days 1 and 4 after initiation of methylprednisolone therapy

There were 5 samples (instead of 6) analysed at each of the three time points. This was due to the poor quality of the RNA extracted from patient A at baseline, and at day 4, and from patient E, at day 1. The variability in results obtained in this small number of patients precluded any meaningful conclusion to be made.

7.3.9 Hepcidin and Inflammation

The CRP levels in all the participants were lower at the end of the study period than their corresponding baseline levels (p=0.0142) (Figure 7.18). Patient D experienced an upturn in CRP between day 3 and day 4, whilst all the other study subjects had CRP trends that were progressively falling. Patient C had near-normal baseline CRP levels and further measurements were not deemed clinically necessary.

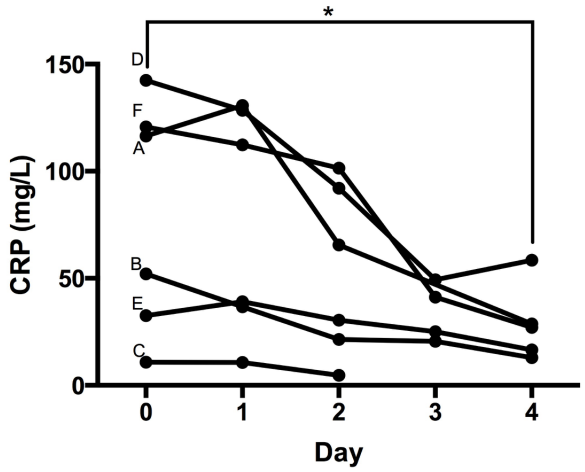

Figure 7.18 CRP levels in AKI patients before and after receiving 3 pulses of methylprednisolone
(Individual patient data plots labelled A-F; comparison between day 0 and day 4: p=0.0142)

There were 17 (of a possible 18) available data points that allowed a comparison between CRP and hepcidin. There was a positive correlation (R^2=0.2557, p=0.038) noted between the two variables (Figure 7.19).

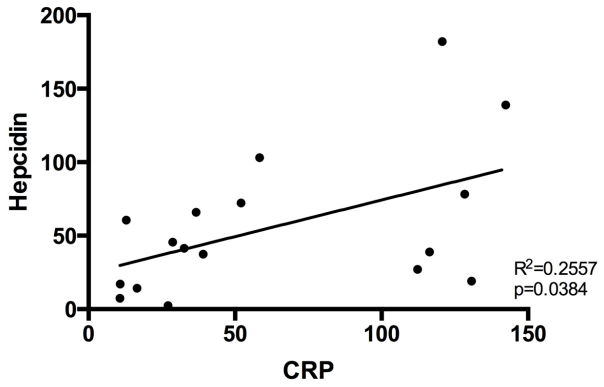

Figure 7.19 Correlation between CRP and hepcidin

7.3.10 The Impact of Renal Clearance and Anaemia on Hepcidin

There was a positive correlation between hepcidin and creatinine that was approaching statistical significance when the data were analysed in their entirety (Figure 7.20a). After a creatinine data point was removed from two patients (patients D and F) that were confounded by the use of haemodialysis, the correlation was significantly stronger (R^2 0.3360, p=0.0186) (Figure 7.20b).

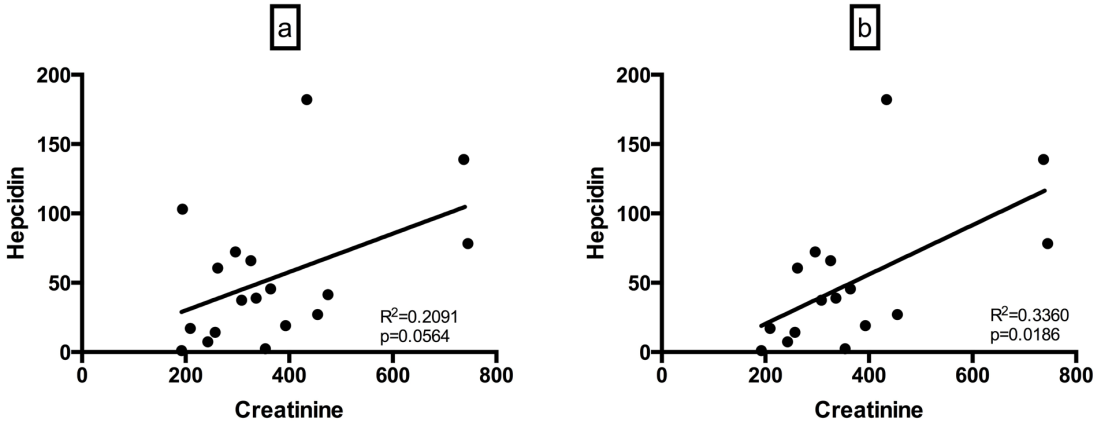

Figure 7.20 Correlation between hepcidin and creatinine (a) with all data points, and (b) with data excluded confounded by haemodialysis

No such correlation, however, was noted between hepcidin and haemoglobin levels, even when data, confounded by the use of blood transfusions, were excluded (Figure 7.21).

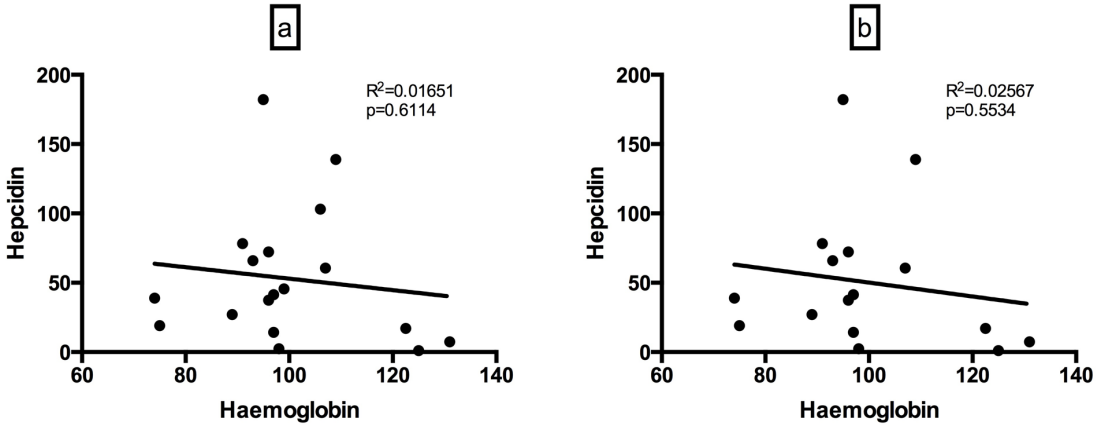

Figure 7.21 Correlation between hepcidin and haemoglobin (a) with all data points, and (b) with data excluded confounded by blood transfusion

7.4 Conclusions and Discussion

The main findings from the work undertaken in this chapter were as follows;

1. Methylprednisolone reduced hepcidin levels in a highly inflamed patient group with severe kidney dysfunction.
2. A similar effect was also seen in dexamethasone-treated HepG2 cells, where there was a significant reduction in HAMP gene expression consistent at concentrations of 20ng/mL and above
3. Hepcidin levels were correlated to levels of renal function and inflammation, but not to the degree of anaemia.
4. Ferroportin expression was increased in dexamethasone-treated cells.
5. Dexamethasone-induced HAMP downregulation was IL-6 dependent.
6. PMBC HAMP mRNA was not affected in patients given methylprednisolone despite a significant reduction in hepcidin levels.

To the best of my knowledge, this is the first clinical study examining the effect of glucocorticoids in the regulation of hepcidin. In a prospective study involving a small clinical cohort, there was a striking reduction in hepcidin levels within 24 hours of the administration of a large dose of intravenous methylprednisolone. There is a high degree of certainty that the observed effect was due to steroid therapy, as it was witnessed in all of the patients, and the effect occurred relatively rapidly, without the aid of any other therapeutic interventions, and with little change in the patients' clinical or biochemical status.

Four of the 6 patients experienced a steady fall in their hepcidin levels, with 3 having normal hepcidin levels by day 4. Two patients saw a rise in their day 4 hepcidin levels (cf day 1) but they had both received transfusions of packed red cells. By virtue of having systemic kidney disease, the patients were, of course, highly inflamed (baseline CRP 79.2 ± 54.2 mg/L). Both CRP and creatinine were correlated with hepcidin levels and were highly likely to be responsible for the high hepcidin levels witnessed in this patient group. The effect of steroids in dampening inflammation provides a logical explanation for the witnessed effect.

The ability of a blood transfusion to abrogate the hepcidin-lowering effect of methylprednisolone in this patient cohort is in accordance with current scientific

knowledge. The effect of packed red cells and administration of iron upon hepcidin are governed by a separate intracellular signalling pathway (BMP-SMAD), whereas inflammation is mediated via the JAK-STAT pathway. It also lends weight to the hypothesis that iron has a greater ability to induce hepcidin than inflammation and can overcome potent anti-inflammatories.

A similar effect was seen with HepG2 cells - HAMP mRNA was suppressed to approximately 50% of basal levels when incubating the cells with dexamethasone concentrations of 20ng/mL and greater, without affecting cell viability. In separate experiments, increasing concentrations of dexamethasone abolished the effect of IL-6 at concentrations of 10ng/mL and greater. However, dexamethasone did not have a similar effect on BMP-6. There was a reasonable degree of consistency among the results in the mRNA experiments.

Dexamethasone is over 5 times as potent as methylprednisolone for the equivalent dose (British National Formulary 2018). It has been reported that following a single 40mg IV bolus injection, peak methylprednisolone levels reached 42-47μg/100ml in 6 adult male volunteers, which corresponds to a dexamethasone concentration of approximately 80ng/mL (Electronic Medicines Compendium (eMC)). This is 4-times higher than the dose of dexamethasone that was used in the protein extraction experiments and may be one of the factors that led to the inconsistent results seen in the Western blots. The effect of decreased HAMP mRNA expression is the increase in ferroportin expression, and there was partial confirmation of this in the in vitro work in section 7.3.5. Ferroportin expression was higher in dexamethasone-treated cells than in any of the other treatment groups, but confidence in this effect would have been higher if there was a consistency of results across the entirety of the experimental protein work in this chapter. The failure of the IL-6 positive control (Figure 7.11) remains difficult to explain.

Unlike plasma hepcidin, which decreased after 1 day following steroid therapy, *ex vivo* PBMCs probed for mean HAMP mRNA levels showed highly variable results precluding meaningful conclusions. The effect of ANCA-associated vasculitis upon macrophages and monocytes perhaps confounds their use as a surrogate marker for hepcidin production. Thus, the composition and activation of the white cells is markedly different in a group of ANCA-associated vasculitis patients, compared to healthy

individuals (O'Brien et al. 2015), or possibly a group of relatively uninflamed haemodialysis patients (see 4.3.1).

Finally, the small number of samples analysed exacerbated our inability to draw meaningful conclusions, particularly since there were additional confounders, such as blood transfusions and dialysis. The major limitation of the work in this chapter was due to an inability to produce meaningful Western blots. The method of Western blotting required cell culture, protein extraction, followed by electroblotting on gels that were made from base ingredients. The cell culture techniques were identical for both the mRNA and protein extraction work, and there is confidence that there were no issues with the cell work *per se*. A possible explanation for the inconsistent results likely lies with the techniques employed in the other areas such as gel making and protein analysis, which perhaps could have been overcome by using commercial gels and seeking expert help earlier.

The major strength of this study is the consistency of both the in vitro cellular work and the in vivo clinical study showing suppression of HAMP expression and a reduction in hepcidin levels. The in vitro work suggests that this is IL-6 mediated. The clinical data show a highly consistent reduction in serum hepcidin levels within 24 hours of administering IV methylprednisolone to patients with an acute insult caused by ANCA-associated vasculitis or a crescentic glomerulonephritis.

Having shown that corticosteroids reduce hepcidin levels in an IL-6 dependent manner, the exact mechanism by which this occurs is yet to be determined and may either be driven by genomic or non-genomic mechanisms. Direct inhibition of the JAK/STAT signalling pathway mediated by IL-6 is one potential non-genomic mechanism at the intracellular protein level. Direct inhibition of transcription factors involved in *HAMP* expression may be an alternative mechanism at the genomic level. Bacchetta and colleagues examined the mechanism by which vitamin D caused reduced *HAMP* transcription using chromatin immunoprecipitation precipitation (in PBMCs, THP-1 monocytes and HepG2 cells) and a similar method could be employed to investigate the same with corticosteroids given that both are steroid hormones.

A potential experiment to investigate this further could involve a comparative analysis between *HAMP* gene expression pre- and post-corticosteroid treatment in patients

using RNA sequencing of PBMCs. This would also enable an assessment of altered expression of transcription factors involved in regulating *HAMP* expression. In order to navigate the potential issue of activated white cells in a pro-inflammatory state, flow cytometry with markers selective for PBMCs on manually derived PBMCs from whole blood can be used that may help enrich for true PBMCs prior to RNA extraction and subsequent sequencing.

8 Discussion and Conclusion

The novel data presented in this thesis (chapter 7) demonstrate that glucocorticoids possess the ability to reduce hepcidin levels in patients presenting with ANCA-associated vasculitis or crescentic glomerulonephritis and do so by dampening the inflammatory response. This was corroborated by *in vitro* work that studied the effect of dexamethasone on HepG2 cells, resulting in the marked downregulation of *HAMP* transcription. It was striking that hepcidin levels decreased in all 6 patients within 24 hours of administration of methylprednisolone, despite the presence of advanced kidney dysfunction. There was approximately a 50% reduction in the mean hepcidin level, falling from 81.8 ± 64.9 mg/L (pre-treatment), to 39.2 ± 27.6 mg/L 24 hours after IV methylprednisolone therapy (p=0.0486). There were no changes in any of the other parameters, including renal function. The mean pre-treatment creatinine was 412 ± 186 μmol/L, compared to 412 ± 179 μmol/L, 24 hours later (p=0.44), suggesting that in this clinical context, inflammation is a more important factor than renal clearance in modulating hepcidin levels.

Clearly, the major limitation of the glucocorticoid study was the small sample size of the clinical cohort. However, the consistency of the results in all patients along with the concordance seen between the *in vitro* cellular work (reduction in *HAMP* gene expression) and the *in vivo* clinical data (reduction in hepcidin levels) suggest that this was a real effect, hitherto not previously reported. The *in vitro* results suggest that the effect is IL-6 mediated. Taken as a whole, our data suggest that glucocorticoids suppress hepcidin production, which has important implications for the anaemia associated with inflammation/CKD. Ideas for future clinical work include the investigation of a greater number of patients prospectively for 12-24 months, with the intention of examining whether a fall in hepcidin levels is a prelude to the recovery of anaemia in this patient group, which would perhaps provide some insight into the pathogenesis of anaemia in ANCA-associated vasculitis. Although *HAMP* transcription was demonstrably lowered in HepG2 cells by dexamethasone, further work examining whether ferroportin levels are affected is an important line of inquiry, with additional work looking at other mediators in the IL-6/inflammatory pathway, and also whether genomic or non-genomic factors are responsible for the corticosteroid reduction in hepcidin.

Glucocorticoids are very useful anti-inflammatory treatments and are the mainstay of treatment in conditions such as ANCA-associated vasculitis and crescentic glomerulonephritis. However, they have many side effects, and studies are currently being undertaken to investigate the possibility of limiting their use in these conditions (Walsh et al. 2018); as such, it is not a feasible treatment for hyperhepcidinaemia. Heparin is also a potent suppressor of hepcidin and used in the anticoagulation of haemodialysis circuits. As discussed earlier (chapter 6), it is unlikely that the doses of heparin that are currently administered across haemodialysis have a significant effect on hepcidin or iron regulation.

The case-control study (chapter 3) confirmed that hepcidin levels were markedly higher in haemodialysis patients than in other CKD patients, with no evidence of higher levels of inflammation. However, the major confounder in the haemodialysis patients was the weekly administration of IV iron, which undoubtedly stimulates hepcidin production. Therefore, the unique finding of reduced *HAMP* PBMC (chapter 4) in the haemodialysis cohort was unexpected, and tentatively points to the possibility that a feedback mechanism exists to downregulate *HAMP* in the face of hyperhepcidinaemia. Pre-clinical evidence to support this theory has been provided by Laftah and colleagues, who injected mice with synthetic hepcidin and observed a subsequent reduction in hepatic *HAMP* mRNA (Laftah et al. 2004). There are few animal models investigating hepcidin regulation in renal failure; one such study was of a rat model using 5/6th nephrectomies that was undertaken by Srai and colleagues; they found that there was no difference in hepatic hepcidin expression between sham and nephrectomised animals. However, there did not appear to be any measurement of protein levels or inflammation in this study, which was principally designed to investigate the effect of erythropoietin on intestinal iron absorption, and thus could have been confounded (Srai et al. 2010). Further pre-clinical work could include the use of an animal model of renal failure designed to see whether reduced glomerular clearance leads to a reduction in *HAMP* expression and whether there is concordance between PBMC *HAMP*, and hepatic *HAMP* expression. If a feedback mechanism were established, elucidating the underlying mechanism would be an interesting line of enquiry.

In my view, the data with the most impact regarding the regulation of hepcidin in chronic kidney disease was gleaned from the haemodialysis patients presenting with acute bacterial sepsis (chapter 4) Despite a very substantial increase in CRP levels,

they had no apparent change in hepcidin levels, suggesting an inability of hepcidin upregulation in this group, possibly due to *HAMP* downregulation. As this was an exploratory study, a more detailed clinical study should be undertaken with larger numbers and an increased blood-sampling plan in order to properly identify the kinetics of CRP (and other inflammatory cytokines) and hepcidin.

Conclusion

Hepcidin regulation in chronic kidney disease is highly complex and subject to a number of competing factors, including clearance (via the glomerulus or dialysis membrane), inflammation, and administration of drug treatments (erythropoietin, iron, red cell transfusions, and possibly heparin).

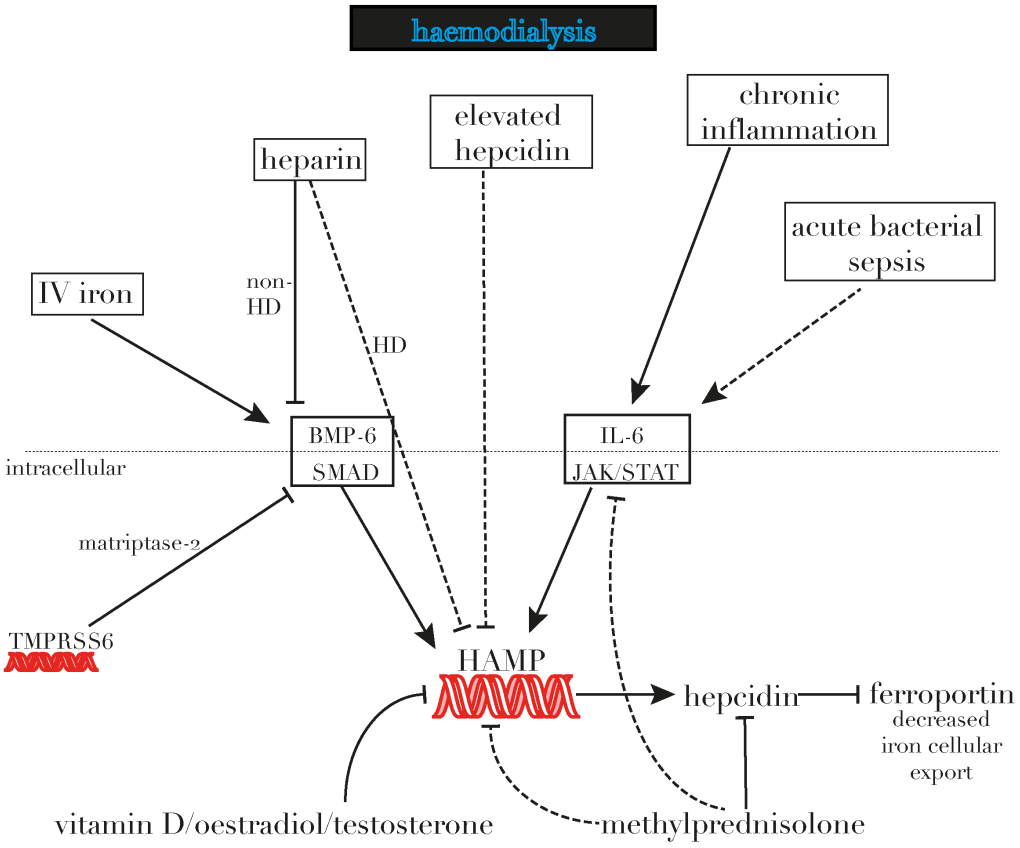

Figure 8.1 Proposed schema of factors impacting on hepcidin levels in haemodialysis
(dashed line indicates possible mechanism; full lines indicate known mechanism)

Hepcidin levels are undoubtedly highest in haemodialysis patients but *HAMP* levels are low, suggesting a negative feedback mechanism. The use of IV iron plus the chronic inflammatory milieu and the lack of renal clearance all play a role in elevating hepcidin, but when stable, chronic haemodialysis patients suffer a superadded bacterial infection, hepcidin levels do not appear to be further able to increase, suggesting that hepcidin is already at its maximal level.

The use of the potent hepcidin inhibitor, heparin, in dialysis patients may play a role in dampening *HAMP* expression but it is likely that this is not the case due to the doses that are used. The steroid hormones oestradiol, testosterone and vitamin D are known to inhibit *HAMP* and we now know that methylprednisolone reduces hepcidin *in vivo* and dexamethasone does the same *in vitro* in an IL-6 dependent manner. The mechanism, however, remains to be clarified (Figure 8.1).

9 Appendix

9.1 List of Papers and Abstracts Published During This PhD

9.1.1 Primary Research Articles

1. Serum hemojuvelin and hepcidin levels in chronic kidney disease. Am J Nephrol. 2012;35(3):295-304
2. Intra-individual variability of serum hepcidin-25 in haemodialysis patients using mass spectrometry and ELISA. Nephrol Dial Transplant. 2012 Oct;27(10):3923-9
3. Obesity does not influence hepcidin and hemojuvelin levels in hemodialysis patients. Nephron Clin Prac. 2013; 124:159-66

9.1.2 Review Articles

1. Obesity and iron deficiency in chronic kidney disease: the putative role of hepcidin. Nephrol Dial Transplant. 2012 Jan;27(1):50-7

9.1.3 Abstracts and Presentations

1. The relationship between obesity and hepcidin levels in haemodialysis patients [Abstract]. CKJ 27 (Suppl 2), 2012: SAP513
2. Does hepcidin-25 correlate with LV mass in paediatric CKD patients? [Abstract]. CKJ 27 (Suppl 2), 2012: SAP717
3. Hepcidin-25 and markers of mineral metabolism in children with pre-dialysis CKD stages 3-5 [Abstract]. CKJ 27 (Suppl 2), 2012: SAP 694
4. Hepcidin-25 and markers of mineral metabolism in children with pre-dialysis CKD stages 3-5 (***oral presentation at the U.K. Renal Association***)
5. Is production of hepcidin-25 increased in haemodialysis patients? (U.K. Renal Association and BioIron) [Abstract]. Am J of Haematology 88(5): E115-116
6. Is production of hepcidin-25 increased in patients with CKD? [Abstract]. JASN 24, 2013:216A
7. Heparin May Reduce Hepcidin Levels in Dialysis Patients by Inhibiting HAMP mRNA. [Abstract]. JASN 25, 2014:304A
8. Glucocorticoids may inhibit HAMP (hepcidin) mRNA in HepG2 cells [Abstract]. JASN 25, 2014:304A
9. Pharmacokinetics and pharmacodynamics of lexaptepid, a novel anti-hepcidin molecule in ESA-resistant haemodialysis patients [Abstract] NDT (2015) 30 (suppl 3):294-5
10. Glucocorticoids inhibit HAMP mRNA in HEPG2 cells and in CKD patients with vasculitis [Abstract]. JASN 27, 2016:875

9.2 Ethical Permission

NHS

National Research Ethics Service

NRES Committee London - Central
Level 7N019, Maternity Block
Northwick Park Hospital
Watford Road
Harrow
Middx
HA1 3UJ

Telephone: 020 8869 3775
Facsimile: 020 8869 5222

13 June 2011

Dr Iain C MacDougall
Renal Admin Portakabin B
Kings College Hospital NHS Foundation Trust
Denmark Hill
London
SE5 9RS

Dear Dr MacDougall

Study Title:	**MEASUREMENT OF SERUM HEPCIDIN IN PATIENTS WITH CHRONIC KIDNEY DISEASE**
REC reference number:	**09/H0718/34**
Protocol number:	**NA**
Amendment number:	**Amendment 2.0 (number of sites)**
Amendment date:	**26 April 2011**

Thank you for submitting the above amendment, which was received on 31 May 2011.

Research Site	Principal Investigator / Local Collaborator
Guy's and St Thomas' Hospitals	Dr Paramit Chowdhury

The amendment relates solely to the addition of new site(s) and/or investigator(s) within the National Health Service (NHS) or Health and Social Care (HSC) in Northern Ireland. The site-specific assessment for the site(s) will therefore form part of the research governance review. The Site-Specific Information (SSI) Form for the site should be included with the application for R&D approval.

On behalf of the Committee, I am pleased to confirm the extension of the favourable opinion to the new site(s) and/or investigator(s), subject to management permission being given by the relevant NHS/HSC R&D office(s) prior to the study starting at the site.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

09/H0718/34	**Please quote this number on all correspondence**

Yours sincerely

pp **Ms Julie Kidd**
Committee Co-ordinator

Email: julie.kidd@nwlh.nhs.uk

This Research Ethics Committee is an advisory committee to London Strategic Health Authority
The National Research Ethics Service (NRES) represents the NRES Directorate within the National Patient Safety Agency and Research Ethics Committees in England

9.3 Material Transfer Agreements

9.3.1 Bialystock

KCH 10 -001

MATERIAL TRANSFER AGREEMENT

THIS AGREEMENT is made on the 08/04 ...2010.

BETWEEN:

(1) **KING'S COLLEGE HOSPITAL NHS FOUNDATION TRUST,** a National Health Service Trust of Denmark Hill, London SE5 9RS ("**KCH**")

(2) **MEDICAL UNIVERSITY OF BIALYSTOCK**, with registered offices at 15–089 Bialystock, Klinskiego 1, Poland (the "**Recipient**")

hereinafter each a "Party" and collectively the "Parties"

RECITALS:

(A) KCH is the owner of clinical information, samples and other specimens relating to patients who have attended KCH.

(B) KCH has agreed to provide to the Recipient with the Materials being clinical information and materials relating to samples and other specimens (as further defined in Schedule 1) for use by the Recipient for the approved Study (as further defined in Schedule 2).

(C) The Recipient has agreed to accept such Materials in accordance with the terms and conditions of this Agreement.

NOW IT IS HEREBY AGREED as follows:

1. **DEFINITIONS AND INTERPRETATION**

1.1 In this Agreement (except where the context otherwise requires) the following words shall have the following meanings:

"Clinical Data"	means data relating to a patient or Sample; provided by KCH to the Recipient.
"Commencement Date"	means ....2010.
"Foreground Information"	means all information, know-how, results, designs, inventions, specimens, samples, DNA sequencing data and/or other matter capable of being the subject of Intellectual Property Rights, which is created, conceived, first achieved, first confirmed, first reduced to practice or writing or developed in whole or in substantial part in the course of the Study using the Materials.
"Intellectual Property Rights"	means any patent, design right, copyright, database right, trade mark, service mark, trade secret, Know-How, right in unpatented know-how, right of confidence and any other intellectual or industrial property right of any nature whatsoever in any part of the world (whether or not registered, registerable or otherwise, and including applications for any of those rights) for the full duration of those rights (including any extensions or renewals).
"Materials"	means the Clinical Data and/or Samples provided by KCH to the Recipient as more particularly described in Schedule 1.
"Principal Investigator"	means the person leading and co-ordinating the Study.
"Recipient Principal Investigator"	means Professor Jolanta Malyszko.
"Samples"	means tissues, blood, serum, patient samples, other biological specimens and all derivatives thereof provided to the Recipient by KCH under this Agreement, and including all and any parts or cultures grown from such

9.3.2 King's College London

MATERIAL TRANSFER AGREEMENT

THIS AGREEMENT is made on the 19/4/10 ~~of March 2010~~ ("Commencement Date").

BETWEEN:

(1) **KING'S COLLEGE HOSPITAL NHS FOUNDATION TRUST,** a National Health Service Trust of King's College Hospital, Denmark Hill, London SE5 9RS ("**KCH**")

(2) **KING'S COLLEGE LONDON**, a university incorporated by Royal Charter, of Strand, London WC2R 2LS (the "**Recipient**")

hereinafter each a "Party" and collectively the "Parties"

RECITALS:

(A) KCH is the owner of clinical information, samples and other specimens relating to patients who have attended KCH.

(B) KCH has agreed to provide to the Recipient with the Materials being clinical information and materials relating to samples and other specimens (as further defined in Schedule 1) for use by the Recipient for the approved Study (as further defined in Schedule 2).

(C) The Recipient has agreed to accept such Materials in accordance with the terms and conditions of this Agreement.

NOW IT IS HEREBY AGREED as follows:

1. **DEFINITIONS AND INTERPRETATION**

1.1 In this Agreement (except where the context otherwise requires) the following words shall have the following meanings:

"Clinical Data"	means data relating to a patient or Sample; provided by KCH to the Recipient.
"Foreground Information"	means all information, know-how, results, designs, inventions, specimens, samples, DNA sequencing data and/or other matter capable of being the subject of Intellectual Property Rights, which is created, conceived, first achieved, first confirmed, first reduced to practice or writing or developed in whole or in substantial part in the course of the Study using the Materials.
"Intellectual Property Rights"	means any patent, design right, copyright, database right, trade mark, service mark, trade secret, Know-How, right in unpatented know-how, right of confidence and any other intellectual or industrial property right of any nature whatsoever in any part of the world (whether or not registered, registerable or otherwise, and including applications for any of those rights) for the full duration of those rights (including any extensions or renewals).
"Materials"	means the Clinical Data and/or Samples provided by KCH to the Recipient as more particularly described in Schedule 1.
"KCH Principal Investigator"	means Dr Stephan Brincat.
"Recipient Principal Investigator"	means Dr Sukhvinder Bansal.
"Samples"	means tissues, blood, serum, patient samples, other biological specimens and all derivatives thereof provided to the Recipient by KCH under this Agreement, and including all and any parts or cultures grown from such whether or not mixed with any other substances, and including all derivatives, portions, progeny,

9.4 Consent Form - Patients

KING'S HEALTH PARTNERS
Pioneering better health for all

King's College Hospital NHS
NHS Foundation Trust
Kings College Hospital
Denmark Hill, London SE5 9RS
Tel: 020 3299 9000
Fax: 020 3299 6472

THE RENAL UNIT
Iain C Macdougall BSc, MD, FRCP
Consultant Nephrologist

Centre Number:
Patient Identification Number:
Study Number:

CONSENT FORM

Title of Project: Regulation of hepcidin in chronic kidney disease

Name of Investigator: Prof. Iain Macdougall

Please initial box

1. I confirm that I have read and understand the information sheet dated 26th June, 2013 (version No.1.0) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected. ☐

3. I understand that relevant sections of my medical notes and data collected during the study may be looked at by individuals from regulatory authorities or from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. ☐

4. I will be asked to donate **1 2 3 4 5** samples (circle as appropriate) ☐

5. I agree to take part in the above study. ☐

_____	_____	_____
Name of Patient	Date	Signature
_____	_____	_____
Name of Person taking consent (if different from investigator)	Date	Signature
_____	_____	_____
Investigator	Date	Signature

[Completed forms: 1 for participant; 1 for Investigator; 1 (original) to be kept in medical notes]

9.5 Patient Information Sheet

King's College Hospital NHS
NHS Foundation Trust

THE REGULATION OF HEPCIDIN IN CHRONIC KIDNEY DISEASE

Patient Information Sheet

We would like to invite you to take part in our research study. Before you decide, we would like you to understand why the research is being done and what it would involve for you. **One of our team will go through the information sheet with you and answer any questions you have.** Please take time to read the information carefully and discuss it with friends/relatives if you wish. You may also ask us if there is anything that is not clear.

What is the purpose of the Study?

Hepcidin is a recently discovered substance that is produced by the liver, and which circulates in the bloodstream. Some of our previous research has shown that levels of hepcidin in patients with kidney disease are extremely high, and we believe that this is a major contributor to the development of anaemia in such patients. What is not known, however, is why and how having kidney disease leads to these high levels.

The purpose of this preliminary study is to understand what is happening regarding production of hepcidin in patients who suffer from chronic kidney disease, and the factors that influence it. Of particular interest are those patients who do not respond to the usual treatments for anaemia.

Why have I been invited?

You are being invited to take part because you have chronic kidney disease, but it is up to you to decide whether to join the study. Participation is entirely voluntary. If you agree to take part, we will then ask you to sign a consent form and give you a copy of this information sheet and the consent form to keep.

What will I be asked to do if I take part?

We are asking if you would be prepared to donate one or more blood samples (up to a maximum of five) for this study. The volume of blood will be a maximum of 50 ml (5 tablespoons) at any one time, and you will be asked for no more than 50 ml of blood over a period of one month. These blood samples will be performed at the same time as your routine blood tests either in the dialysis unit/clinic/ward. The blood samples will be processed immediately, and separated into serum, white and red blood cells (the red cells will be discarded). RNA will immediately be extracted from the white cells, and will be stored in a secure location along with the serum for analysis at a later date. The samples will be completely anonymised (you can not be identified). All material will be destroyed at the end of the study (01/04/ 2018).

What are the possible side effects of taking part?

The research study consists of taking blood samples. Minor bruising/pain at the site of blood taking would be the main side-effect, unless you are a haemodialysis, in which case, we will take the sample as you are being put on the dialysis machine.

What are the possible benefits in taking part?

The information we get from this study may help us evaluate better the treatment of anaemia in patients with chronic kidney disease and you would hopefully gain some satisfaction from knowing

you had helped us with research. Otherwise, we do not expect there to be any benefits for you in taking part.

Will my taking part in this study be kept confidential?

All information which is collected about you during the course of the research will be kept strictly confidential, and any information about you which leaves the hospital will have your name and address removed so that you cannot be recognised.

What will happen if I don't want to carry on?

You are free to withdraw at any time, without giving a reason. This would not affect the standard of care you receive. Information collected up till that point will still be used.

What if there is a problem?

If you have a concern about any aspect of this study, you should ask to speak to the researchers who will do their best to answer your questions [*Tel No: 0207 848 0431*]. If you remain unhappy and wish to complain formally, you can do this by calling the Patient Advice and Liaison Service on telephone number 0203 299 3601 or write to PALS, Ground Floor, Hambledon Wing, King's College Hospital, Denmark Hill, London, SE5 9RS.

Who has reviewed this study?

This study has been reviewed by the Renal Research Governance Board, and has been given a favourable opinion by the _____Research Ethics Committee.

What will happen to the results of the research study?

The results may be reported in professional publications or meetings but you will not be identified by name.

Who is funding this study?

This study is funded by the King's College Hospital Charity.

What do I do now?

Thank you for considering taking part in this research. One of our team members will contact you in a few days. You can ask the questions you have and let him/her know whether you would like to take part.

Prof Iain Macdougall, Consultant Nephrologist
Dr Adam Rumjon, Clinical Research Fellow
Ms Leela Goldstein, Clinical Research Manager (Renal)
Tel No: 0207 848 0431

9.6 Consent Form And Patient Information – Healthy Controls

King's College Hospital NHS
NHS Foundation Trust

Kings College Hospital
Denmark Hill, London SE5 9RS
Tel: 020 3299 9000
Fax: 020 3299 6472

THE RENAL UNIT
Iain C Macdougall BSc, MD, FRCP
Consultant Nephrologist

Centre Number:
Patient Identification Number:
Study Number:

CONSENT FORM

Title of Project: Measurement of blood hepcidin in patients with chronic kidney disease

Name of Investigator: Dr Iain Macdougall

Please initial box

1. I confirm that I have read and understand the information sheet dated 22nd of March, 2010 (version No.2.0) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐

2. I understand that my participation is voluntary. ☐

3. I understand that data collected (age, gender and ethnicity) during the study may be looked at by individuals from regulatory authorities or from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access this information. ☐

4. I agree to take part in the above study. ☐

Name of Patient	Date	Signature
Name of Person taking consent (if different from investigator)	Date	Signature
Investigator	Date	Signature

[Completed forms: 1 for participant; 1 for Investigator]

King's College Hospital NHS
NHS Foundation Trust

MEASUREMENT OF BLOOD HEPCIDIN LEVELS IN PATIENTS WITH CHRONIC KIDNEY DISEASE

Participant Information Sheet

We would like to invite you to take part in our research study. Before you decide we would like you to understand why the research is being done and what it would involve for you. **One of our team will go through the information sheet with you and answer any questions you have.** Please take time to read the information carefully. You may also ask us if there is anything that is not clear.

What is the purpose of the Study?

Hepcidin is a newly-discovered substance which is produced by the liver, and which circulates in the bloodstream. It is critical in controlling how iron is absorbed from the gut and how the body stores and uses it. Iron is important in the production of red blood cells and lack of it, results in anaemia. Scientists at King's College, London have developed a reliable and sophisticated way to measure hepcidin in the blood. We expect that hepcidin will be a useful marker to help us interpret better iron levels in blood in patients suffering from chronic kidney disease.

Why have I been invited?

You are being invited to take part as a healthy volunteer. This would allow us to compare serum hepcidin levels in normal individuals with those obtained from patients suffering from chronic kidney disease.

Do I have to take part?

It is up to you to decide to join the study. Participation is entirely voluntary. If you agree to take part, we will then ask you to sign a consent form and give you a copy of this information sheet and the consent form to keep.

What will I be asked to do if I take part?

We are asking you if you would be prepared to donate one blood sample for this study. The blood sample provided by you will be taken to the laboratory and analysed for hepcidin levels. The volume of blood will be only 5ml of blood (1 teaspoonful). After analysis, your blood sample will be disposed of.

How many blood tests will I have?

Only one sample is required.

What are the possible side effects of taking part?

Minor bruising/pain at the site of blood taking would be the main side-effect.

What are the possible benefits in taking part?

You would hopefully gain some satisfaction from knowing you had helped us with our research. Otherwise, we do not expect there to be any benefits for you in taking part.

Will my taking part in this study be kept confidential?

All information (age, gender and ethnicity) which is collected about you during the course of the research will be kept strictly confidential, and any information about you which leaves the hospital will be encoded so that you cannot be recognised.

What will happen if I don't want to carry on?

You are free to withdraw at any time, without giving a reason. Information collected up until that point will still be used. We will **NOT** be storing any blood samples of our participants.

What if there is a problem?

If you have a concern about any aspect of this study, you should ask to speak to the researchers who will do their best to answer your questions [*Tel No: 0207 848 0431*]. If you remain unhappy and wish to complain formally, you can do this by calling the Patient Advice and Liaison Service on telephone number 0203 299 3601 or write to PALS, Ground Floor, Hambledon Wing, King's College Hospital, Denmark Hill, London, SE5 9RS.

Who has reviewed this study?

This study has been reviewed and has been given a favourable opinion by the London Research Ethics Committee.

What will happen to the results of the research study?

The results may be reported in professional publications or meetings but you will not be identified by name.

Who is funding this study?

This study is funded by a grant from King's College Hospital NHS Foundation Trust, London.

What do I do now?

Thank you for considering taking part in this research. One of our team members will contact you in a few days. You can ask the questions you have and let him/her know whether you would like to take part.

Dr Iain Macdougall, Consultant Nephrologist
Dr Stephan Brincat, Clinical Research Fellow
Dr Beatriz Tucker, Associate Specialist
Ms Leela Goldstein, Anaemia Research Sister
Department of Nephrology, King's College Hospital

Tel No: 0207 848 0431

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