IRON, HYPOXIA AND CHRONIC DISEASE

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To Adrienne

"Science is not only a disciple of reason but, also, one of romance and passion."

Stephen Hawking

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Abstract

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Iron, Hypoxia and Chronic Disease

The main theme of this thesis is centred around the physiology and pathophysiology of two elements essential for human life: oxygen and iron. The work presented here is focused on the complex regulatory mechanisms, the interactions between iron and oxygen and the perturbations within those pathways underlying various disease conditions. Translational applications are presented in the settings of chronic obstructive pulmonary disease (COPD), critical illness and hypoxic conditioning.

In *Chapter 3*, a randomised, controlled clinical trial investigating the effects of iron augmentation in patients with COPD is presented. In this study, 48 participants received a single dose of either intravenous iron (ferric carboxymaltose) or placebo. Over an eight-week period, oxygenation, exercise performance, symptomatology, health-related quality of life and lung function were assessed. Intravenous iron did not improve peripheral oxygen saturation. However, participants receiving an iron infusion walked farther and were less breathless. Six-minute walk distance improved by 14 ± 8 m after eight weeks following iron administration compared to placebo ($p = 0.025$). Improvements ≥ 40 m were observed in 29.2% of iron-treated and 0% of placebo-treated participants after one week ($p = 0.009$). MRC dyspnoea scores were lower one week after iron treatment, with a larger fraction of participants reporting scores ≤ 1 in the iron group than in the placebo group (66.7% vs. 33.3%, $p = 0.021$). Adverse event rates were similar in both groups except for hypophosphataemia, a common and well-known side effect of ferric carboxymaltose, which occurred more frequently in the iron group (91.7% vs. 8.3%, $p = 0.001$). This decrease in serum phosphate levels remained asymptomatic.

Chapter 4 presents results from the retrospective analysis of iron status in critically ill patients. Inflammation commonly occurs in these patients as highlighted by elevated inflammatory markers C-reactive protein and interleukin-6. Several iron parameters, including serum iron, ferritin, hepcidin, transferrin, and transferrin saturation, strongly correlated with the increase in inflammatory markers. Anaemia, often a consequence of iron sequestration in systemic inflammation, was observed in nearly all patients.

Chapter 5 describes a small study investigating the effects of prolonged exposure to hypoxia in healthy 50–70-year-old volunteers. Using a residential hypoxia facility, eight participants were exposed to mild hypoxia for one week in a crossover study design. The exposure to 15% of inspired oxygen fraction resulted in physiological adaptations, including increased heart rate, respiratory rate, haemoglobin, and haematocrit. Erythropoietin, a marker of hypoxia signalling, and hepcidin, the main regulator of iron homeostasis, were assessed by enzyme-linked immunosorbent assay. While erythropoietin was significantly elevated in response to hypoxia, hepcidin levels decreased. The exposure to hypoxia was well-tolerated by all participants.

A main summary, overall conclusions, and an outlook are provided in *Chapter 6*.

Table of contents

Acknowledgements ..... **iv**

Abstract ..... **v**

Table of contents ..... **vi**

List of figures ..... **xi**

List of tables ..... **xiv**

Abbreviations used in this thesis ..... **xvi**

CHAPTER 1: General introduction **1**

1.1 HUMAN IRON METABOLISM 2

1.1.1 Physiology of iron metabolism 2

1.1.1.1 Iron absorption *3*

1.1.1.2 Iron transport *4*

1.1.1.3 Iron storage *5*

1.1.1.4 Iron excretion and recycling *6*

1.1.2 Regulation of iron homeostasis 6

1.1.2.1 The role of hepcidin in systemic iron homeostasis *6*

1.1.2.2 Intracellular regulation of iron homeostasis *9*

1.1.3 Dysregulations of iron homeostasis 10

1.1.3.1 Iron deficiency *10*

1.1.3.2 Iron overload *11*

1.2 HYPOXIA PHYSIOLOGY 12

1.2.1 Oxygen sensing 12

1.2.2 The hypoxia-inducible factor pathway 14

1.2.3 Physiological responses to hypoxia 16

1.2.3.1 Ventilatory response *17*

1.2.3.2 Cardiac response *17*

1.2.3.3 Pulmonary vascular response *17*

1.2.3.4 Erythropoietic response *18*

1.3 INTERPLAY OF IRON AND OXYGEN HOMEOSTASIS 20

1.3.1 Hypoxia, iron and the pulmonary circulation 21

1.4 SUMMARY AND THESIS OBJECTIVES 23

CHAPTER 2: General methods 25
2.1 PULSE OXIMETRY 26
2.2 PULMONARY FUNCTION TESTING 29
2.2.1 Spirometry 29
2.2.2 Static lung volumes 31
2.2.3 Diffusion capacity 31
2.3 ECHOCARDIOGRAPHY 33
2.4 ASSESSMENTS SPECIFIC TO CHRONIC LUNG DISEASE 37
2.4.1 Functional exercise capacity: Six-minute walk test 37
2.4.2 Symptom, quality of life and prognostic indices 38
2.4.2.1 BODE index 38
2.4.2.2 Respiratory scores 39
2.4.2.3 Dyspnoea indices 40
2.4.2.4 Non-respiratory scores 42
2.5 ENZYME-LINKED IMMUNOSORBENT ASSAY 43
2.6 INTRAVENOUS IRON PREPARATIONS 45
2.6.1 Ferric carboxymaltose 45
2.6.1.1 Chemical and pharmacological properties 45
2.6.1.2 Safety profile 46

CHAPTER 3: Effects of intravenous iron in chronic obstructive pulmonary disease .. 48
3.1 INTRODUCTION 49
3.1.1 Definition 49
3.1.2 Epidemiology and socioeconomic impact 50
3.1.3 Aetiology and pathogenesis 51
3.1.3.1 Hypoxia, pulmonary hypertension and the role of iron 54
3.1.4 Diagnosis and treatment 56
3.1.5 Iron homeostasis in COPD 58
3.1.5.1 Pulmonary iron homeostasis 59
3.1.5.2 Dysregulation of iron homeostasis in COPD 61
3.1.6 Aims and rationale 64
3.2 METHODS 65
3.2.1 Trial overview 65
3.2.2 Participants 65
3.2.3 Interventions 67
3.2.4 Study visits and assessments 67
3.2.4.1 Screening visit 67
3.2.4.2 Baseline visit 68
3.2.4.3 Follow-up visits 71

3.2.5 Outcomes 73
3.2.6 Randomisation and blinding 74
3.2.7 Sample size calculation 75
3.2.8 Safety reporting 75
3.2.9 Venous blood sample analysis 77
3.2.10 Statistical analysis 78
3.3 RESULTS 80
3.3.1 Recruitment and participant flow 80
3.3.2 Participant characteristics 82
3.3.3 Treatment effect on iron status 89
3.3.4 Oxygen saturation measurements 91
3.3.5 Effects on exercise capacity 95
3.3.6 Echocardiographic findings 99
3.3.7 Quality of life and symptom questionnaires 101
3.3.8 Haematology and blood biochemistry parameters 106
3.3.9 Inflammatory markers 111
3.3.10 Blood gas analysis 112
3.3.11 Spirometry 113
3.3.12 Exacerbations 115
3.3.13 Adverse events 116
3.4 DISCUSSION 118
3.4.1 Main study findings 118
3.4.1.1 Primary outcome: Oxygenation 118
3.4.1.2 Improvement in functional outcomes: Exercise capacity and dyspnoea 119
3.4.1.3 Safety of ferric carboxymaltose in COPD 122
3.4.1.4 Other outcome variables 124
3.4.2 Study limitations 125
3.4.3 The problem of defining iron deficiency in COPD 126
3.4.4 Iron supplementation in other chronic disease conditions 128
3.4.5 Conclusions 129

CHAPTER 4: Iron parameters in critical illness 130
4.1 INTRODUCTION 131
4.1.1 Inflammation in critical illness 131
4.1.2 Iron homeostasis during inflammation 133
4.1.3 Anaemia of inflammation 134
4.1.4 Aims and rationale 135

4.2 STUDY DESIGN 136
4.2.1 Overview of original study protocol 136
4.2.2 Source data 136
4.2.3 Sample analysis 137
4.2.4 Statistical analysis 137
4.3 RESULTS 139
4.3.1 Participant characteristics 139
4.3.2 Laboratory parameters on admission 140
4.3.2.1 Haematological parameters 141
4.3.2.2 Inflammatory parameters 142
4.3.2.3 Iron parameters 142
4.3.3 Correlation of inflammation and iron status 145
4.3.4 Survival and iron parameters 148
4.3.5 Temporal trends in laboratory parameters 150
4.4 DISCUSSION 153
4.4.1 Main findings 153
4.4.2 Assessment of iron status in critical illness 155
4.4.3 Iron status and outcomes in critical illness 157
4.4.4 Limitations 158
4.4.5 Conclusions 159

CHAPTER 5: Prolonged hypoxic exposure and markers of iron and oxygen homeostasis 160
5.1 INTRODUCTION 161
5.1.1 Oxygen, hypoxia and HIF 161
5.1.2 Effects of mild hypoxia 162
5.1.3 Implications for perioperative medicine 163
5.1.4 Aims and rationale 164
5.2 STUDY DESIGN 165
5.2.1 Study overview 165
5.2.2 Residential hypoxia facility 165
5.2.3 Participants 168
5.2.4 Study procedures and interventions 169
5.2.5 Outcomes 173
5.2.6 Sample analysis 173
5.2.7 Statistical analysis 174

5.3 RESULTS 175
5.3.1 Participant characteristics 175
5.3.2 Environmental monitoring data 177
5.3.3 Oxygenation and other physiological measurements 178
5.3.4 Haematology parameters 180
5.3.5 Effects on erythropoietin 181
5.3.6 Effects on hepcidin 183
5.3.7 Correlations between oxygenation and iron homeostasis 185
5.4 DISCUSSION 187
5.4.1 Main findings 187
5.4.2 Limitations 189
5.4.3 Context and conclusion 189

CHAPTER 6: Conclusions 191
6.1 FINAL SUMMARY 192
6.2 FUTURE DIRECTIONS 193
6.3 CONCLUDING REMARKS 193

REFERENCES 194

APPENDICES 223
APPENDIX A: VALIDATION OF HEPCIDIN MEASUREMENTS 224
APPENDIX B: CONTRIBUTIONS 226
APPENDIX C: SCIENTIFIC COMMUNICATIONS ASSOCIATED WITH THIS THESIS 228
APPENDIX D: FIGURE PERMISSIONS 229

List of figures

CHAPTER 1

Figure 1.1 Overview of systemic iron homeostasis 3

Figure 1.2 Regulators of hepcidin expression 7

Figure 1.3 Putative mechanism of oxygen sensing in the carotid body 13

Figure 1.4 Hypoxia-inducible factor pathway 16

CHAPTER 2

Figure 2.1 Absorption spectra of oxygenated and deoxygenated haemoglobin 27

Figure 2.2 Overview of light absorbance by a pulse oximeter 28

Figure 2.3 Schematic of respiratory excursions, lung volumes, and lung capacities 30

Figure 2.4 Typical forced spirometry traces 30

Figure 2.5 Visualisation of tricuspid regurgitation by Doppler echocardiography 35

Figure 2.6 Measurement of ΔP_{max} by continuous-wave Doppler echocardiography 36

Figure 2.7 Schematic representation of the structure of ferric carboxymaltose 46

CHAPTER 3

Figure 3.1 Immune cells involved in the inflammatory response in COPD 52

Figure 3.2 Proteins involved in pulmonary iron import and export 60

Figure 3.3 Overview of study protocol 72

Figure 3.4 Participant flow diagram 81

Figure 3.5 Temporal changes in iron parameters 90

Figure 3.6 Primary study outcome: Peripheral oxygen saturation changes 92

Figure 3.7 Daily self-measured oxygen saturations 94

Figure 3.8 Temporal changes in 6-minute walk distance 96
Figure 3.9 Distribution of changes in 6-minute walk distance 98
Figure 3.10 Temporal changes in maximum trans-tricuspid pressure gradient..... 100
Figure 3.11 Relative changes in symptom and quality of life scores 104
Figure 3.12 Proportions of MRC dyspnoea grades at each study visit..... 105
Figure 3.13 Proportions of BODE score quartiles..... 105
Figure 3.14 Main haematological and serological findings..... 109
Figure 3.15 Changes in serum phosphate levels after infusion 110
Figure 3.16 Temporal changes in inflammatory markers..... 111
Figure 3.17 Daily self-measured peak expiratory flow rates (PEFR)..... 114

CHAPTER 4

Figure 4.1 Distribution of haematological parameters on admission 141
Figure 4.2 Distribution of inflammatory parameters on admission..... 143
Figure 4.3 Distribution of iron parameters on admission 144
Figure 4.4 Correlation of C-reactive protein with iron status markers..... 146
Figure 4.5 Correlation of interleukin-6 with iron status markers 147
Figure 4.6 Hospital survival and markers of iron status..... 149
Figure 4.7 Temporal changes in haematological and inflammatory parameters..... 151
Figure 4.8 Temporal changes in iron parameters 152

CHAPTER 5

Figure 5.1 Residential hypoxia facility..... 166
Figure 5.2 Schematic overview of hypoxic air generation by membrane separation... 167
Figure 5.3 Overview of study protocol..... 171
Figure 5.4 Participant flow diagram 175
Figure 5.5 Oxygen levels in the residential hypoxia facility during study weeks..... 177

Figure 5.6 Effects of hypoxic exposure on peripheral oxygen saturation 178

Figure 5.7 Effects of hypoxic exposure on vital signs and Lake Louise Score 179

Figure 5.8 Temporal changes in haemoglobin and haematocrit 180

Figure 5.9 Temporal changes in erythropoietin levels 181

Figure 5.10 Changes in erythropoietin levels by group 182

Figure 5.11 Temporal changes in hepcidin levels 183

Figure 5.12 Changes in hepcidin levels by group 184

Figure 5.13 Correlation of SpO_2 with erythropoietin and hepcidin 185

Figure 5.14 Correlation of erythropoietin with hepcidin 186

Figure 5.15 Correlation of ferritin with hepcidin 188

APPENDIX

Figure A1 Correlation of hepcidin measurements obtained with different assays 225

List of tables

CHAPTER 2

Table 2.1 Components of the BODE index 39

Table 2.2 Modified MRC dyspnoea scale 41

CHAPTER 3

Table 3.1 GOLD classification of airflow limitation in COPD 57

Table 3.2 Baseline participant demographics & characteristics 83

Table 3.3 Severity of airflow obstruction at baseline 84

Table 3.4 Pulmonary function test results 85

Table 3.5 Screening blood results 87

Table 3.6 Iron status at baseline visit 88

Table 3.7 Temporal changes in iron parameters 89

Table 3.8 Overview of peripheral oxygen saturation measurements 93

Table 3.9 Six-minute walk test results 97

Table 3.10 Proportion of participants with improvements in 6MWD of ≥ 40 m 97

Table 3.11 Overview of symptom and quality of life scores 102

Table 3.12 Haematology parameters 107

Table 3.13 Serum biochemistry parameters 108

Table 3.14 Inflammatory markers 111

Table 3.15 Capillary blood gas analysis 112

Table 3.16 Spirometry results 113

Table 3.17 Exacerbations during the study period 115

Table 3.18 Adverse events 117

CHAPTER 4

Table 4.1 Characteristics of participating patients 139

Table 4.2 Blood parameters on ICU admission 140

CHAPTER 5

Table 5.1 Study schedule 172

Table 5.2 Baseline characteristics of study participants 176

Abbreviations used in this thesis

6MWD/T	Six-minute walk distance/test
AHVR	Acute hypoxic ventilatory response
AMS	Acute mountain sickness
APACHE	Acute physiology and chronic health evaluation
BMI	Body mass index
BMP	Bone morphogenic protein
CAT	COPD Assessment Test™
CBP	CREB-binding protein
CO	Carbon monoxide
CO_2	Carbon dioxide
COPD	Chronic obstructive pulmonary disease
CPET	Cardiopulmonary exercise test
CRP	C-reactive protein
DAMP	Damage-associated molecular pattern
Dcytb	Duodenal cytochrome b
DFO	Desferrioxamine
DMT1	Divalent metal transporter 1
DO_2	Oxygen delivery
ELISA	Enzyme-linked immunosorbent assay
EPO	Erythropoietin
ERFE	Erythroferrone
ERV	Expiratory reserve volume
FCM	Ferric carboxymaltose
Fe	Iron
FEV_1	Forced expiratory volume in 1 second

FGF23	Fibroblast growth factor 23
FIH	Factor inhibiting HIF (asparaginyl hydroxylase)
FLVCR1	Feline leukaemia virus subgroup C receptor 1
FPN	Ferroportin
FRC	Functional residual capacity
FVC	Forced vital capacity
H_2S	Hydrogen sulphide
HADS	Hospital Anxiety and Depression Score
Hb	Haemoglobin
HCO_3	Bicarbonate
HCP1	Haem carrier protein 1
HIF	Hypoxia-inducible factor
HJV	Haemojuvilin
HO	Haem oxygenase
HPV	Hypoxic pulmonary vasoconstriction
HRE	Hypoxia response element
HRG1	Haem responsive gene 1
ICU	Intensive care unit
IL	Interleukin
IRE	Iron response element
IRIDA	Iron-refractory iron deficiency anaemia
IRP	Iron-regulatory protein
MCID	Minimal clinically important difference
N_2	Nitrogen
NRAMP1	Natural resistance-associated macrophage protein 1
NTBI	Non-transferrin bound iron
O_2	Oxygen
PAMP	Pathogen-associated molecular pattern
PaO_2	Arterial partial pressure of oxygen

PASP	Pulmonary arterial systolic pressure
PHD	Prolyl hydroxylase domain
P_{max}	Maximum pressure gradient across the tricuspid valve during ventricular systole
PO_2/PCO_2	Partial pressure of oxygen/carbon dioxide
RAP	Right atrial pressure
ROS	Reactive oxygen species
RV	Residual volume
RVP	Right ventricular pressure
SaO_2	Arterial oxygen saturation
SGRQ	St George's Respiratory Questionnaire
SIRS	Systemic inflammatory response syndrome
SO_2	Oxygen saturation
SpO_2	Peripheral oxygen saturation
STEAP3	Six-transmembrane epithelial antigen of the prostate 3
sTfR	Soluble transferrin receptor
TfR	Transferrin receptor
TLC	Total lung capacity
TLCO	Transfer factor for carbon monoxide
TNF-α	Tumour necrosis factor alpha
TR	Tricuspid regurgitation
TSat	Transferrin Saturation
UTR	Untranslated region
$\dot{V}/\dot{Q}$	Ventilation-perfusion ratio
VA	Alveolar volume
VC	Vital capacity
VHL	von-Hippel-Lindau protein
ZIP14	Zrt/Irt-like protein 14

CHAPTER 1:
General introduction

1.1 Human iron metabolism

Iron is an essential element for most forms of life. In the human body it can exist in its ferrous (Fe^{2+}) or ferric (Fe^{3+}) states, which allows iron to participate in essential electron transfer reactions, but is also responsible for its toxicity (Papanikolaou and Pantopoulos 2005). The biological functions of iron and iron-containing proteins are widespread. It is involved in oxygen transport and storage in blood and muscle, energy metabolism in mitochondria, host defence mechanisms, and haematopoiesis (Ganz 2013).

1.1.1 Physiology of iron metabolism

The total iron content in an average adult human adds up to approximately 3–4 g, most of which (approximately 2–3 g) is contained in haemoglobin molecules within erythrocytes (Ganz 2013). Organs with high iron content are the liver and spleen (approximately 20–30% of total body iron), where iron is stored in hepatocytes and reticuloendothelial macrophages, and skeletal muscle tissue, where iron is mainly found in myoglobin (Papanikolaou and Pantopoulos 2005, Ganz 2013). Figure 1.1 shows the major iron-containing sites in the human body as well as the main iron fluxes between them. As will be discussed in more detail, only small amounts (1–2 mg/d) of iron are absorbed and lost each day; most of the daily iron need is met by recycling old erythrocytes in macrophages (20–25 mg/d). In summary, systemic iron homeostasis is characterised by controlled intestinal uptake of iron, uncontrolled losses and continuous recycling.

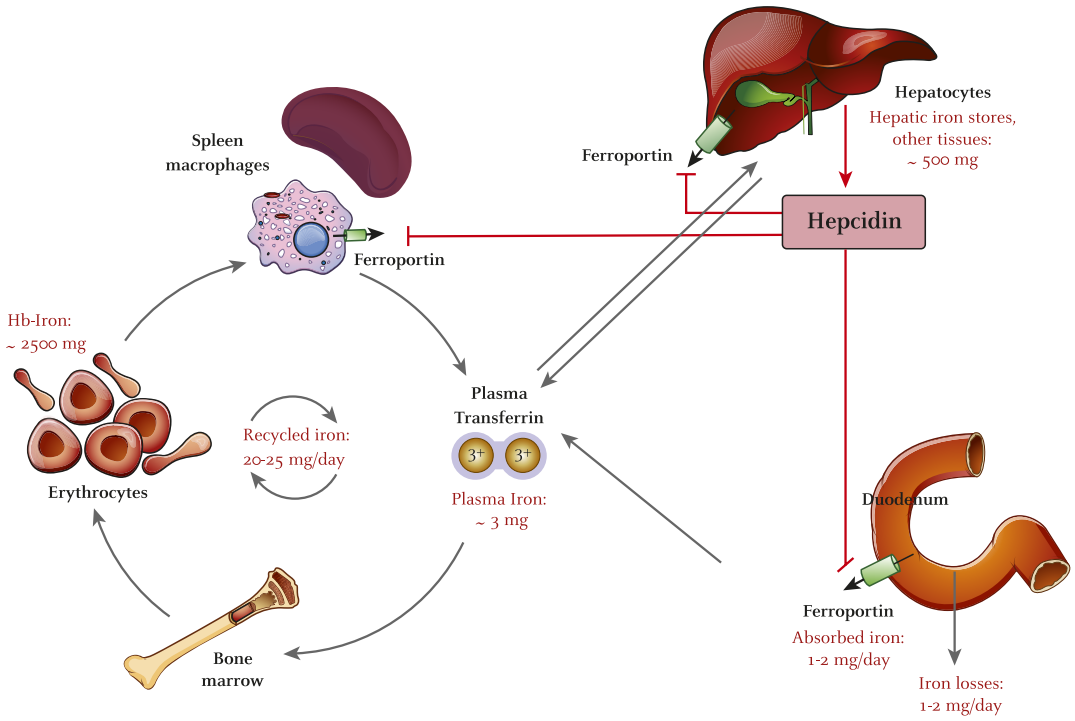


Figure 1.1 Overview of systemic iron homeostasis. Most of the total body iron is used for erythropoiesis and contained in erythrocytes. Liver and spleen are the two major sites of iron storage and recycling. Absorption from the intestine and overall losses of iron are minimal. Distribution of iron between tissues occurs mostly bound to transferrin. The regulation of iron homeostasis by hepcidin and its receptor ferroportin are also shown. Adapted from Evstatiev and Gasche (2012) with permission of BMJ Publishing Group Ltd. Copyright © 2012 BMJ Publishing Group Ltd.

1.1.1.1 *Iron absorption*

The absorption of dietary iron predominantly occurs in the duodenum and proximal parts of the small intestine. The source of dietary iron has a substantial impact on absorption. Animal-derived haem iron (e.g. meat), which is tightly bound in haemoglobin and myoglobin, is absorbed more efficiently than non-haem iron (e.g. plants), the absorption of which is more dependent on other dietary or luminal factors (Anderson and Frazer 2017).

Most ingested iron is ferric non-haem iron, which needs to be reduced to ferrous iron before absorption (Mastrogiannaki *et al.* 2013). This reaction is facilitated by ferric

reductases such as membrane-bound duodenal cytochrome b (Dcytb) (McKie *et al.* 2001). Ferrous iron is then absorbed into the enterocytes by apical transporter proteins. The most important of those proteins in the intestine is the divalent metal transporter 1 (DMT1) (Gunshin *et al.* 1997).

Inside the enterocyte, iron is either stored in the form of ferritin (e.g. in situations of reduced need for iron) or exported at the basolateral surface of the cell through the only known iron exporter ferroportin (Mastrogiannaki *et al.* 2013, McKie *et al.* 2000). Exported iron is then loaded onto the iron carrier transferrin once it has been oxidised back to its ferric form by ferroxidases such as hephaestin and caeruloplasmin (Ganz 2013).

The mechanisms of haem-iron uptake are not fully understood. The folate transporter haem carrier protein 1 (HCP1) has been identified as one candidate (Le Blanc *et al.* 2012). Haem responsive gene 1 (HRG1) is a second protein, which is essential for iron recycling from haem in macrophages and is potentially also involved in intestinal haem-iron uptake (Yanatori *et al.* 2010, White *et al.* 2013). Inside the cell, iron is extracted from haem by haem oxygenase (HO) and subsequently stored or exported as described (Mastrogiannaki *et al.* 2013). Alternatively, feline leukaemia virus subgroup C receptor 1 (FLVCR1) is abundantly expressed in the intestine and has been shown to be an important haem exporter during erythropoiesis (Quigley *et al.* 2004). Whether FLVCR1 actually exports haem in enterocytes as well remains unclear.

1.1.1.2 *Iron transport*

Iron exported from cells binds to the iron carrier transferrin, which can carry up to two ferric iron ions, and is distributed with the bloodstream. Under normal conditions, only approximately 30% of the iron binding sites on transferrin are occupied (i.e. a

transferrin saturation of 30%), which leaves considerable buffering capacity to prevent the accumulation of toxic non-transferrin bound iron (NTBI) (Anderson and Frazer 2017).

Transferrin-bound circulating iron is imported into cells via receptor-mediated endocytosis through binding to transferrin receptor 1 (TfR1) on the cell membrane. The TfR-transferrin complex is internalised and iron is released following acidification of the endosome and conformational change of the TfR-transferrin complex. The released ferric iron is reduced to ferrous iron by six-transmembrane epithelial antigen of the prostate 3 (STEAP3) and exported into the cytosol by DMT1 (Frazer and Anderson 2014). Alternatively, NTBI can also be imported into cells via ion transporters such as Zrt/Irt-like protein 14 (ZIP14) (Jenkitkasemwong *et al.* 2015).

1.1.1.3 *Iron storage*

Depending on the requirement of a cell, the imported iron can either be kept readily available in the labile iron pool by binding to chaperone proteins, or stored for later use. The main intracellular iron storage protein is ferritin. The spherical protein can store up to 4,500 individual iron atoms, providing a large reservoir for iron sequestration (Anderson and Frazer 2017). Ferritin consists of 24 subunits of either heavy (H) or light (L) type (Ganz 2013). The H-subunits act as ferroxidases and convert the cytosolic ferrous iron to ferric iron for storage. Small amounts of ferritin are released from cells and circulate in the bloodstream. This serum ferritin can easily be measured and provides a good indicator of intracellular iron stores (Finch *et al.* 1986, Anderson and Frazer 2017).

1.1.1.4 *Iron excretion and recycling*

No physiological iron excretion mechanism exists. Therefore, the normal daily loss of iron is minimal (1–2 mg/d) and primarily due to desquamation of dead skin cells or enterocytes, sweating, or blood loss (Ganz 2013).

Most systemic iron is derived from recycling damaged or senescent red blood cells. These erythrocytes are engulfed and internalised by macrophages of the reticuloendothelial system in the spleen, liver, and bone marrow and are degraded inside the phagolysosome (Knutson 2017). Haem is transported across the phagolysosomal membrane by HRG1 and degraded by a cytosolic haem oxygenase to release iron, carbon monoxide, and biliverdin (White *et al.* 2013). Alternatively, it has also been proposed that haem may be degraded within the phagolysosome and iron subsequently released into the cytosol via natural resistance-associated macrophage protein 1 (NRAMP1) (Knutson 2017).

1.1.2 Regulation of iron homeostasis

1.1.2.1 *The role of hepcidin in systemic iron homeostasis*

Systemic iron homeostasis is tightly regulated by the liver-derived hormone hepcidin (Ganz 2013), which was discovered only within the last two decades and first described as an antimicrobial peptide (Krause *et al.* 2000). Hepcidin regulates the export of iron from cells by post-translationally modifying its receptor ferroportin. After binding hepcidin, ferroportin is internalised and degraded in lysosomes, which results in a decreased membrane concentration of the protein. Consequently, iron efflux from cells is reduced, which leads to overall reduced systemic iron availability (Nemeth *et al.* 2004b). The central role of hepcidin in regulating iron homeostasis is highlighted in studies of abnormal hepcidin expression. Transgenic overexpression of hepcidin caused

severe iron deficiency in mice (Nicolas *et al.* 2002a), while hepcidin deficiency resulted in severe iron overload in mice and humans (Nicolas *et al.* 2001, Lesbordes-Brion *et al.* 2006, Roetto *et al.* 2003).

Hepcidin production is stimulated by elevated liver or plasma iron as well as by inflammation, and suppressed by iron deficiency, hypoxia, or increased erythropoiesis (Ganz 2013, Rishi *et al.* 2015). An overview of the pathways regulating the expression of hepcidin is shown in Figure 1.2.

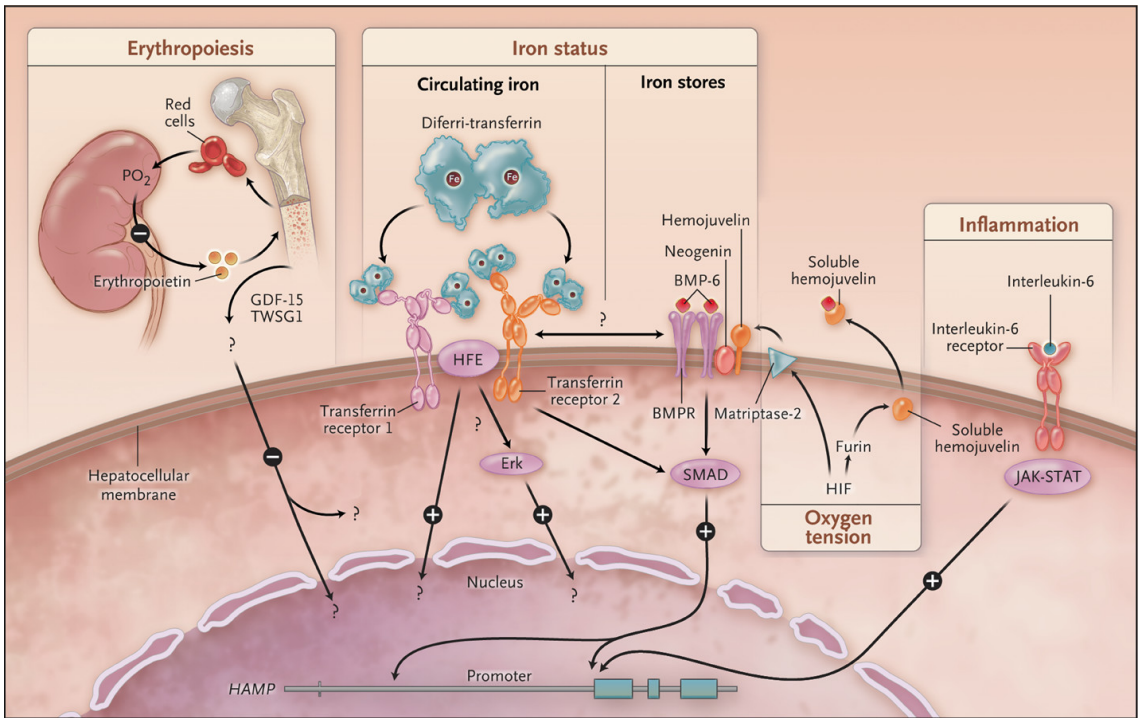


Figure 1.2 Regulators of hepcidin expression. Hepcidin expression is regulated by erythropoiesis, iron status, oxygen tension, and inflammation (see text). Iron status affects hepcidin expression primarily through the BMP/SMAD pathway, which is activated through BMP receptors. Extracellular sensing of circulating iron involves transferrin receptors and HFE. Hypoxia mediates its inhibitory effect on hepcidin production through HIF-controlled expression of matriptase-2 and furin, which interfere with the BMP/SMAD pathway. The proinflammatory cytokine IL-6 activates JAK/STAT signalling resulting in hepcidin expression. The exact mediator translating erythropoietic activity is not fully understood yet. Reproduced with permission from Fleming and Ponka (2012). Copyright © 2012 Massachusetts Medical Society.

The expression of hepcidin is regulated on the transcriptional level, primarily through the bone morphogenic protein (BMP)/SMAD signalling pathway, which responds to changes in iron levels (Ganz 2013). Ligand binding to BMP receptors results in phosphorylation of cytosolic SMAD proteins, which translocate to the nucleus to act as transcription factors. The signalling cascade is further enhanced by the co-receptor haemojuvilin (HJV). The extracellular iron sensor activating the BMP/SMAD pathway is not completely understood, but TfR2 and the haemochromatosis protein HFE are likely to be involved in the process (Nemeth and Ganz 2009, Ganz and Nemeth 2012).

The mechanisms underlying the regulation of hepcidin by erythropoiesis are still a focus of research (Rishi and Subramaniam 2017). Erythroferrone (ERFE), a molecule expressed in erythroblasts, seems to be a promising erythroid regulator. It was recently shown that erythropoietin stimulates the synthesis of ERFE, which leads to suppressed hepcidin expression (Gammella *et al.* 2015).

Hepcidin regulation by hypoxia involves HIF-mediated expression of matriptase-2 and furin. Both enzymes interfere with haemojuvilin and impair its co-receptor function, which results in reduced BMP receptor activity and hence reduced hepcidin expression (Fleming and Ponka 2012, Lakhal *et al.* 2011).

Hepcidin is also upregulated in response to inflammation and infection, which leads to the sequestration of iron commonly seen in inflammatory responses. The synthesis of hepcidin in this setting is primarily mediated by the cytokine interleukin-6 (IL-6), which induces the expression of hepcidin through the JAK-STAT3 pathway (Nemeth *et al.* 2004a).

1.1.2.2 *Intracellular regulation of iron homeostasis*

Regulation of intracellular iron homeostasis occurs primarily post-transcriptionally through iron-regulatory proteins 1 and 2 (IRP1 and IRP2) (Ponka 1999, Rouault 2006, Wilkinson and Pantopoulos 2014). These proteins can modulate the translation of several mRNAs which encode proteins involved in iron metabolism. To achieve this, IRPs bind to iron response elements (IREs) in the 5' or 3' untranslated region (UTR) of the target mRNA (Wilkinson and Pantopoulos 2014).

The activity of IRPs is dependent on the intracellular iron concentration. In conditions of intracellular iron deficiency, IRPs can bind to IREs and exert their effect, which depends on the binding location of the IRP to the target mRNA (Anderson and Frazer 2017). IRP-binding to the 5' UTR suppresses translation of the respective protein, whereas binding to the 3' UTR stabilises the mRNA by protecting it from degradation. In iron-replete conditions, IRP1-binding is inhibited by iron-loading, which converts the protein into cytosolic aconitase, whereas IRP2 is degraded (Hentze *et al.* 2004).

The IRP/IRE system allows cells to adequately respond to varying intracellular iron concentrations (Anderson and Frazer 2017). When iron availability is limited, the binding of IRPs to the 5' UTR of the ferritin mRNA suppresses translation and thereby limits the unnecessary synthesis of the storage protein. Similarly, an IRE in the 5' UTR of the ferroportin mRNA limits iron export from the cell. The mRNA for both TfR1 and DMT1 are stabilised by IRP-binding to the 3' UTR of those transcripts. This leads to increased synthesis of these iron uptake proteins and ensures adequate iron supply to the cell.

1.1.3 Dysregulations of iron homeostasis

1.1.3.1 *Iron deficiency*

Iron deficiency is a global health problem affecting an estimated two billion people worldwide (Zimmermann and Hurrell 2007, von Haehling *et al.* 2015). Infants, children, adolescents, and elderly individuals are most commonly affected. Pregnancy and menstruation further add to the risk of developing iron deficiency, as does the presence of chronic inflammatory diseases (von Haehling *et al.* 2015).

Iron deficiency arises when the body's demands for iron are not met. As mentioned before, iron is needed for several biological functions, but the majority of the body's iron is directed towards erythropoiesis. In conditions of reduced iron availability, erythropoietic activity is prioritised over other iron-dependent functions (von Haehling *et al.* 2015, Andrews 1999). Red blood cell production therefore remains unaffected until iron depletion becomes more severe (Camaschella 2015). In fact, anaemia is often the first symptomatic manifestation of iron deficiency as it is the "easiest recogni[s]able sign of [iron deficiency]" (Camaschella 2017).

It is of pathophysiological importance to differentiate absolute iron deficiency from functional iron deficiency. In the former, total body iron is depleted and insufficient to meet demands, whereas the latter is characterised by an inadequate iron supply in the presence of normal total body iron (Jankowska *et al.* 2013, Camaschella 2017). Absolute iron deficiency is normally caused by reduced dietary intake, poor gastrointestinal absorption, or excessive iron loss, e.g. through bleeding (Jankowska *et al.* 2013). In functional iron deficiency, iron stores are normal or even elevated, but iron is "trapped" inside enterocytes, hepatocytes, and macrophages, and hence not available in circulation (von Haehling *et al.* 2015). This sequestration of iron is primarily caused by inflammation and mediated by the overproduction of hepcidin in response to pro-

inflammatory stimuli and the subsequent degradation of ferroportin (Jankowska *et al.* 2013).

In rare cases, iron deficiency can also occur on the basis of genetic predisposition. In iron-refractory iron deficiency anaemia (IRIDA), low iron levels arise from upregulated hepcidin expression by a mutation in the enzyme TMPRSS6 (Finberg *et al.* 2008).

1.1.3.2 *Iron overload*

While iron deficiency is usually acquired, iron overload often has a genetic aetiology. Primary iron overload syndromes arise on the basis of mutations in iron transport and regulatory proteins (Anderson and Frazer 2017). The most common of these disorders is hereditary haemochromatosis caused by a homozygous mutation in the HFE gene (type 1) (Feder *et al.* 1996, Feder 1999). Less common are alterations in haemojuvilin (type 2A), hepcidin (type 2B), TfR2 (type 3), or FPN1 (type 4) (Brissot *et al.* 2018). With the exception of type 4 haemochromatosis, which results from a gain of activity in ferroportin, all types share inadequately low levels of hepcidin as underlying causative mechanism (Brissot *et al.* 2018). The lack of hepcidin's inhibitory effect on iron uptake leads to excessive iron release into the blood stream and iron loading into several organs. The iron overload causes irreversible tissue damage and leads to hepatic and cardiac dysfunction, diabetes, and arthritis (Anderson 2007). The toxicity of excessive iron is primarily explained by its propensity to induce oxidative stress (Papanikolaou and Pantopoulos 2005).

Secondary iron overload is usually a side effect of repeated blood transfusions, which are administered to treat haematological disorders like β-thalassaemia, sideroblastic anaemia, and chronic haemolytic or hypoplastic anaemias (Anderson 2007).

1.2 Hypoxia physiology

Like iron, oxygen is vital for an organism's survival and proper functioning. Its adequate supply is an indispensable requirement for cellular respiration and energy production. Low levels of oxygen, or hypoxia, can therefore have detrimental effects on cells, tissues, and the entire organism, and are a pathophysiological element in many disease conditions including heart disease, lung disease, stroke, and cancer. Since changes in the availability of oxygen are critical, complex mechanisms have evolved in order to maintain oxygen levels within strict limits and to sense and respond to alterations if necessary (Semenza 2011).

1.2.1 Oxygen sensing

In the absence of any oxygen storage capabilities, hypoxia can have serious consequences within minutes. To prevent these, the human body quickly responds to oxygen deprivation through specialised cells located bilaterally at the bifurcation of the common carotid arteries, the so-called carotid bodies. These oxygen sensing cells can detect even small drops in arterial partial oxygen pressure and initiate systemic countermeasures within the fraction of a second (Prabhakar and Semenza 2015).

Morphologically, the carotid bodies consist of glomeruli, which are in close proximity to a dense capillary network and afferent sensory nerve fibres. The glomeruli are composed of two different cell types: neuron-like glomus (type I) cells, which are more abundant and the main site of oxygen sensing, and glia-like sustentacular (type II) cells, which surround the former (Lopez-Barneo *et al.* 2008).

The response to hypoxia involves sensing the partial pressure of oxygen in the arterial blood and transducing that signal into cell depolarisation, which ultimately results in the release of neurotransmitters (Rakoczy and Wyatt 2018). During hypoxia (as well as

hypercapnia and acidosis), oxygen-sensitive membrane potassium channels are inhibited, resulting in membrane depolarisation, subsequent calcium influx through voltage-gated calcium channels, and lastly, neurotransmitter release (Lopez-Barneo *et al.* 2016b). The exact molecular oxygen sensing mechanism linking the hypoxic signal to the inhibition of potassium channels is still unclear. More recent evidence has implicated the gaseous messengers carbon monoxide (CO) and hydrogen sulphide (H_2S) in the hypoxia sensing mechanism (Prabhakar and Semenza 2015). This putative pathway is schematically depicted in Figure 1.3.

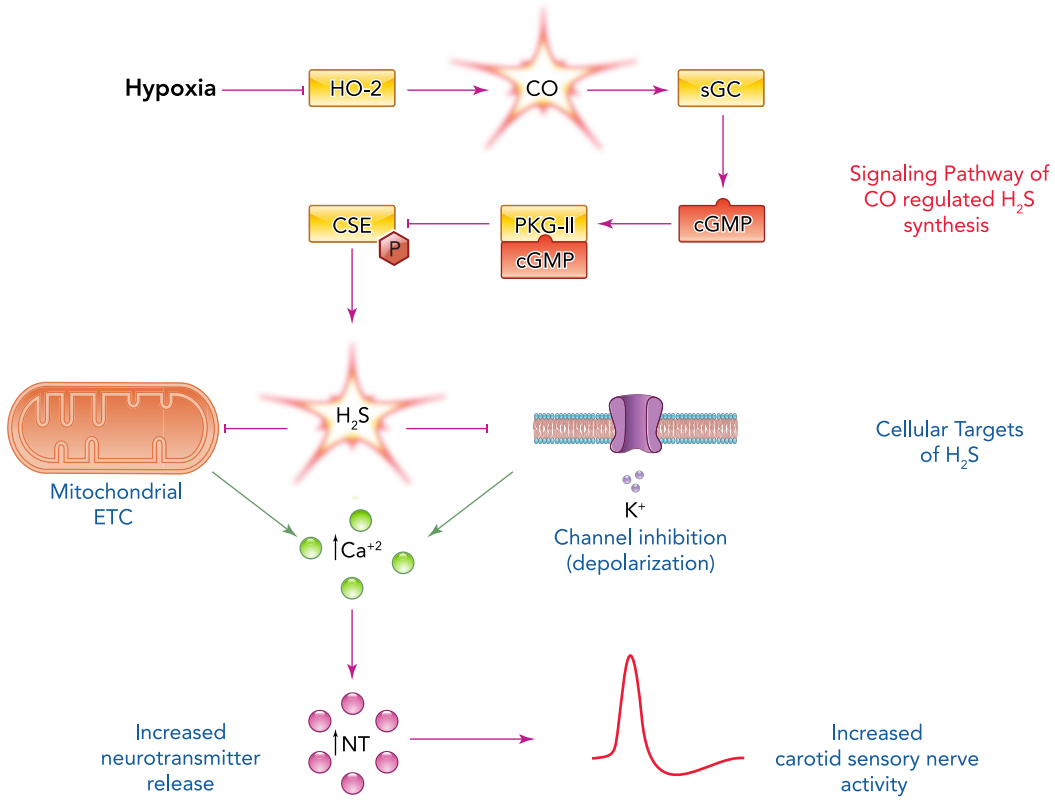


Figure 1.3 Putative mechanism of oxygen sensing in the carotid body. Haem oxygenase 2 (HO-2) requires oxygen to catalyse the degradation of haem, during which carbon monoxide (CO) is produced. CO leads to stimulation of soluble guanylate cyclase (sGC), production of cyclic GMP (cGMP), and activation of protein kinase G (PKG). Synthesis of hydrogen sulphide (H_2S) is usually low due to inhibition of cystathionine-γ-lyase (CSE) by PKG. During hypoxia, this inhibitory influence is reduced, resulting in increased levels of H_2S. This is thought to alter ion channel conductance and affect mitochondria, ultimately leading to calcium influx and neurotransmitter (NT) release. Reprinted from Prabhakar and Semenza (2015) with permission of The American Physiological Society. Copyright © 2015 American Physiological Society.

In brief, hypoxia reduces CO generation in the carotid body by inhibiting the activity of the oxygen-dependent haem oxygenase 2 (HO-2). Reduced CO levels lead to increased H_2S concentrations by disinhibiting cystathionine-γ-lyase, the enzyme generating H_2S in the carotid bodies. H_2S is thought to alter ion channel conductance and potentially also mitochondrial activity, thereby inducing the aforementioned calcium influx and transmitter release (Prabhakar and Semenza 2015). Multiple other mechanisms of oxygen sensing in the carotid bodies have been postulated. Many of those have focused on the mitochondria and suggested the involvement of energy metabolism, redox signalling, mitochondrial complex signalling, or the lactate receptor in transducing the hypoxic stimulus (Lopez-Barneo *et al.* 2016a, Rakoczy and Wyatt 2018).

Even though the carotid bodies represent the central and most important element in the body's acute oxygen-sensing system, other tissues can sense local oxygen levels as well. Examples include the neuroepithelial bodies in the lungs, chromaffin cells of the foetal adrenal gland, and certain arterial smooth muscle cells (Weir *et al.* 2005).

1.2.2 The hypoxia-inducible factor pathway

Apart from oxygen sensing in specialised tissues such as the carotid bodies, every cell has built-in mechanisms to respond to hypoxia. The central element in mediating these responses is the hypoxia-inducible factor (HIF) pathway, which acts as the "master regulator of oxygen homeostasis" at the transcriptional level (Semenza 2004). HIF activates the transcription of a wide range of genes involved in erythropoiesis, angiogenesis, cell growth, apoptosis, and energy metabolism (Schofield and Ratcliffe 2004). It was its role in stimulating erythropoiesis in response to hypoxia by inducing erythropoietin (EPO) that led to the discovery of the HIF protein (Semenza and Wang 1992).

The HIF transcriptional complex consists of two heterodimeric subunits: the HIF-α subunit, which exists in three isoforms (HIF-1α, HIF-2α and HIF-3α) and is regulated in an oxygen dependent manner, and the HIF-β subunit, which is constitutively expressed (Wang *et al.* 1995). Under normoxic conditions, HIF-α is rapidly degraded, a process which involves hydroxylation of proline and asparagine residues on the protein by prolyl hydroxylase domain (PHD) and asparaginyl hydroxylase (FIH, factor inhibiting HIF) enzymes (Figure 1.4) (Semenza 2004). These dioxygenases use oxygen and α-ketoglutarate as substrates and require iron and ascorbate as cofactors. As a consequence, iron and ascorbate availability can modulate dioxygenase activity in addition to oxygen (Knowles *et al.* 2003). HIF-hydroxylation through PHD enzymes (PHD-1, PHD-2 and PHD-3) allows binding of von-Hippel-Lindau (VHL) protein to HIF-α, which initiates the formation of a ubiquitin protein ligase that targets the protein for degradation by the proteasome (Maxwell *et al.* 1999, Ivan *et al.* 2001, Jaakkola *et al.* 2001). Hydroxylation by FIH, on the other hand, inhibits the transactivation domain of HIF-α, which prevents binding of the coactivators p300 and CREB-binding protein (CBP) (Mahon *et al.* 2001).

Under conditions of low cellular oxygen levels, the activity of the dioxygenases is reduced due to the lack of substrate. This prevents proteasomal degradation of HIF-α and leads to the protein's stabilisation and translocation to the nucleus. There, it forms heterodimers with HIF-β and interacts with coactivators like p300 and CBP. This complex then binds to hypoxia response elements (HRE) and initiates the transcription of HIF target genes (Semenza 2004).

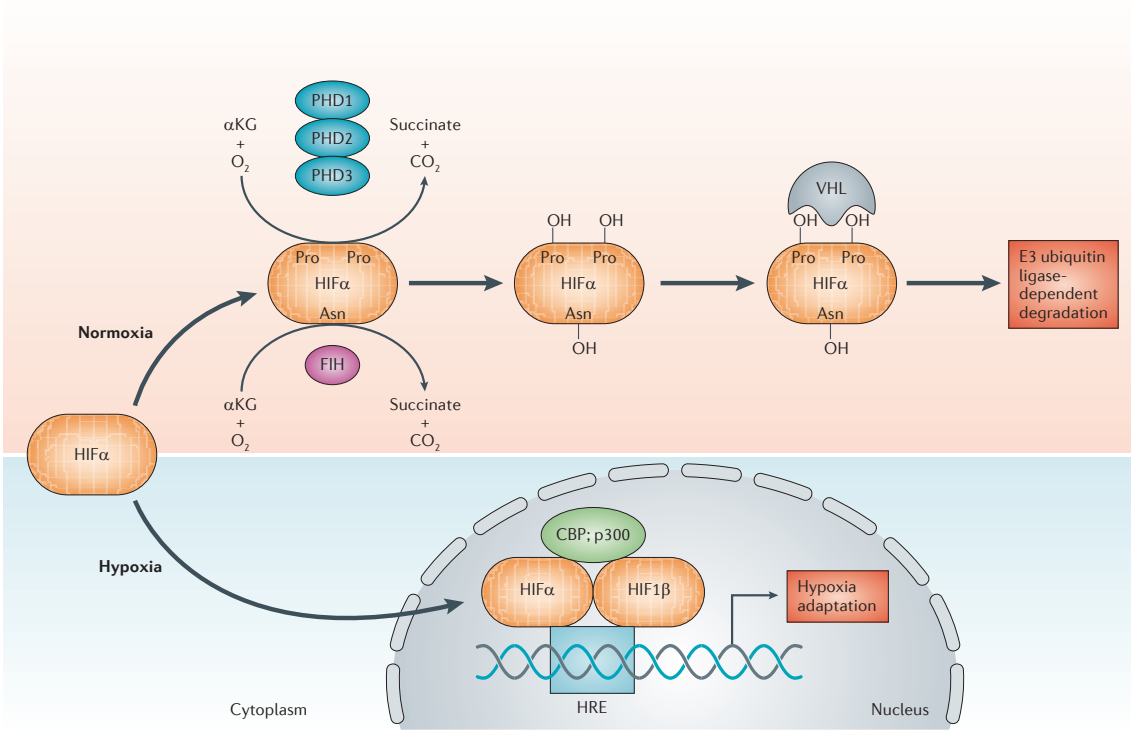


Figure 1.4 Hypoxia-inducible factor pathway. Under normoxic conditions, HIF-α is quickly degraded after oxygen-dependent hydroxylation by prolyl hydroxylase domain (PHD) and asparaginyl hydroxylase (FIH) enzymes. PHD-mediated hydroxylation leads to binding of von-Hippel-Lindau (VHL) protein and targeting of HIF-α for proteasomal degradation. During hypoxia, HIF-α accumulates due to reduced hydroxylase activity. Subsequently, HIF-α subunits translocate to the nucleus and form heterodimers with HIF-β. This complex acts as a transcription factor, binds to hypoxia response elements (HRE) and promotes the transcription of several hundred genes. Adapted from Eltzschig *et al.* (2014) with permission of Springer Nature. Copyright © 2014 Nature Publishing Group.

1.2.3 Physiological responses to hypoxia

The cellular mechanisms described in Chapters 1.2.1 and 1.2.2 initiate local and systemic responses aimed at restoring adequate oxygenation once hypoxia has occurred. In the acute setting, ventilatory and cardiac output adjustments as well as pulmonary vascular responses ensure oxygen uptake and delivery are maximised. Subsequently, increased erythropoietic activity provides additional oxygen carriage capacity to limit the effects of tissue hypoxia (Smith *et al.* 2008b).

1.2.3.1 *Ventilatory response*

The immediate ventilatory response to hypoxia – referred to as acute hypoxic ventilatory response (AHVR) – is mediated by the peripheral chemoreceptors in the carotid bodies (Prabhakar and Semenza 2015, Pamenter and Powell 2016). Ventilation is increased within seconds of arterial oxygen partial pressure dropping (Powell *et al.* 1998). If hypoxia persists for more than a few minutes, minute ventilation paradoxically decreases back to pre-hypoxic levels before increasing again after several hours. This rise in ventilatory activity continues for several days and is referred to as hypoxic ventilatory acclimatisation (Powell *et al.* 1998, Smith *et al.* 2008b). It represents a mechanism of adjustment to prolonged hypoxic exposure (e.g. ascent to and stay at altitude).

1.2.3.2 *Cardiac response*

Another acute response to hypoxia mediated by the autonomic nervous system is a rapid increase in cardiac output in order to minimise tissue hypoxia by increasing organ perfusion (Smith *et al.* 2008b). This is caused primarily by an elevation in heart rate, rather than increased stroke volume (Vogel and Harris 1967).

1.2.3.3 *Pulmonary vascular response*

In the pulmonary vasculature, hypoxia results in localised vasoconstriction, which is the opposite response to what is seen in the remainder of the circulatory system. Hypoxic pulmonary vasoconstriction (HPV), also referred to as Euler-Liljestrand mechanism, represents another physiological attempt at reducing the impact of hypoxia by redirecting pulmonary blood flow away from poorly oxygenated alveoli to areas with higher oxygen partial pressure (Sommer *et al.* 2008). Euler and Liljestrand (1946) first

described this physiological mechanism in anaesthetised cats. It was subsequently demonstrated in humans that hypoxia significantly increases pulmonary vascular resistance and pulmonary arterial pressure (Motley *et al.* 1947). The pulmonary vascular response to hypoxia exhibits two distinct phases: pulmonary vascular resistance increases rapidly on exposure to hypoxia and plateaus within a few minutes before continuing to rise more slowly after 40–45 minutes (Talbot *et al.* 2005). While the exact mechanisms causing HPV are still the subject of research, it is generally accepted that the pulmonary artery smooth muscle cells represent the main site mediating this response (Sylvester *et al.* 2012). The acute phase of HPV seems to involve extracellular calcium influx into smooth muscle cells following membrane depolarisation induced by oxygen-sensitive potassium channels (Sommer *et al.* 2008), whereas the second phase could be caused by altered gene expression mediated by HIF (Shimoda and Laurie 2014). While regional HPV is a beneficial physiological mechanism ensuring ventilation-perfusion ($\dot{V}/\dot{Q}$) matching, sustained global alveolar hypoxia leads to pulmonary vascular remodelling and ultimately pulmonary hypertension (Shimoda and Laurie 2014).

1.2.3.4 *Erythropoietic response*

A final response to be discussed in this context concerns the increased erythropoietic activity in response to hypoxia. Compared to the cardiorespiratory adaptations discussed before, the increase in erythropoiesis is initiated over a considerably longer timespan. This is easily explained by the HIF-dependence of this response: the expression of EPO, which stimulates red cell production, is activated by HIF-binding (Semenza and Wang 1992). This response on the transcriptional level is inevitably slower than acute reactions mediated by the autonomic nervous system or membrane

ion channels. Significant increases in serum EPO levels are detectable as early as 1.5 hours after the start of a hypoxic exposure (Eckardt *et al.* 1989). Within days of continued hypoxic exposure (e.g. at altitude), haemoglobin and haematocrit increases become noticeable (Richalet *et al.* 1994).

1.3 Interplay of iron and oxygen homeostasis

While the previous sections of this chapter gave an overview of iron metabolism and hypoxia physiology, this section will focus on the interactions between iron and oxygen homeostasis in the human body.

The close interplay between the two elements is evident in the HIF signalling pathway activated by low oxygen levels (see Chapter 1.2.2). As highlighted, the PHD enzymes initiating the breakdown of HIF-α not only require oxygen as an essential substrate, but also critically depend on the availability of iron for optimal catalytic activity (Schofield and Ratcliffe 2004, Mole 2010). Iron availability can therefore modulate enzymatic activity in a similar fashion as oxygen availability. Iron chelation with desferrioxamine (DFO), for example, resulted in HIF stabilisation and increased expression of EPO mRNA *in vitro* similarly to hypoxia (Wang and Semenza 1993). These findings were reproduced in human subjects, where infusions of DFO mimicked the hypoxic response and led to increased EPO expression under normoxic conditions (Ren *et al.* 2000).

The transcription of HIF is further regulated in an iron-dependent manner. HIF-2α contains a 5' UTR IRE, which causes translational suppression of HIF-2α through IRP-binding in the presence of iron deficiency (Sanchez *et al.* 2007). This link between oxygen and iron metabolism makes HIF-2α an important regulator of intestinal iron absorption (Mastrogiannaki *et al.* 2013).

While iron availability can modify hypoxia signalling pathways, this does not constitute a unidirectional relationship as highlighted by the fact that iron homeostasis is equally responsive to oxygen availability. Several proteins involved in iron uptake and transport are targets of transcriptional regulation by HIF. As such, genes encoding for Dcytb, DMT1, FPN, transferrin, TfR, and caeruloplasmin all contain HREs in their promoter regions and are upregulated in response to hypoxia (Peyssonnaux *et al.* 2008,

Mastrogiannaki *et al.* 2013). This aspect of the interaction between oxygen and iron homeostasis is easily explained by the increased iron demand during hypoxia-mediated erythropoietic activity.

The expression of the iron-regulatory protein hepcidin has long been known to be downregulated in response to hypoxia (Nicolas *et al.* 2002b). Several underlying mechanisms have been proposed, but it remains unclear whether this suppression is directly mediated by hypoxia or indirectly through the increased erythropoietic drive (Rishi *et al.* 2015). Regulation of hepcidin expression directly by HIF remains controversial: both direct involvement as well as lack thereof have been reported (Peyssonnaux *et al.* 2007, Volke *et al.* 2009).

1.3.1 Hypoxia, iron and the pulmonary circulation

The close interaction between iron and oxygen physiology has been extensively studied in the context of the pulmonary circulation. Due to the iron requirement of the hypoxia-sensing HIF pathway, low levels of iron can mimic similar responses to hypoxia. The pulmonary circulation provides a well-studied example of this relationship.

As described before, pulmonary blood vessels constrict in response to hypoxia, which leads to an increase in pulmonary arterial systolic pressure (PASP). Similar changes in pulmonary arterial pressure are caused by iron deficiency. Infusion of the iron chelator DFO, which induces artificial iron deficiency, was shown to increase PASP in healthy adults breathing normoxic air (Balanos *et al.* 2002). The time course of this rise was very similar to the effect of hypoxia on PASP. Further research revealed that iron chelation had similar effects when applied during hypoxia. In this setting, DFO further augmented hypoxia-mediated PASP increases and sensitised the pulmonary vascular response to acute hypoxia (Smith *et al.* 2008a). On the other hand, infusion of

intravenous iron significantly reduced the hypoxia-driven elevation in pulmonary arterial pressure and the enhanced sensitivity to acute hypoxia (Smith *et al.* 2008a).

In subsequent randomised controlled studies, Smith *et al.* (2009) reproduced these findings in the context of hypoxia due to altitude exposure. Sea-level residents staying at high altitude (elevation > 4,300 metres) exhibited an approximately 40% reduction in the pulmonary hypertensive response to hypoxia after iron administration. On the other hand, high-altitude residents with chronic mountain sickness, who were rendered iron deficient through venesection, showed an approximately 25% increase in PASP.

Further evidence supporting the importance of iron availability in modulating vascular responses to hypoxia in the lungs was obtained from studies of clinically iron deficient, but otherwise healthy, adults (Frise *et al.* 2016). In line with previous studies, iron deficient individuals exhibited a more pronounced increase in PASP when exposed to hypoxia compared to iron-replete control subjects. Intravenous iron attenuated the hypoxic response in both iron-deficient and iron-replete participants, but the effect of administering iron was more pronounced in iron-deficient subjects.

The effects of intravenous iron on hypoxic pulmonary hypertensive responses persist for at least several weeks after a single dose has been administered (Bart *et al.* 2016).

The findings from the aforementioned studies confirm the important role of iron availability in modulating hypoxic responses. The iron-requirement of the HIF-hydroxylases has been implied as the putative underlying mechanism in all of these studies.

1.4 Summary and thesis objectives

Iron and oxygen are both indispensable elements of human life. The physiological processes involved in maintaining iron and oxygen homeostasis are closely linked and alterations in one system lead to changes in the other. Low oxygen levels and low iron levels can, for example, induce similar systemic responses. Disturbances in the regulatory processes involved in iron and oxygen homeostasis occur in a variety of different diseases.

The overarching objective of this thesis was to apply well-established knowledge of iron and oxygen physiology to clinical settings of disease conditions characterised by alterations in these processes. This approach pursued the goal of obtaining new evidence to further understand the pathophysiology of these conditions as well as studying the potential for interventions to modulate these pathways. In the long-term setting, this might potentially improve diagnosis and treatment options for affected patients. In this thesis, three studies are presented, all of which reflect a different aspect of iron and hypoxia interrelatedness.

A randomised controlled clinical trial of administering intravenous iron to patients with chronic obstructive pulmonary disease (COPD) is presented in *Chapter 3*. COPD is characterised by disturbances in both oxygen and iron physiology with hypoxia and iron deficiency occurring frequently. The effects of intervening pharmacologically in this condition are shown. The aim of this study was to establish feasibility of this intervention and obtain data about physiologically important outcome variables.

Chapter 4 describes a retrospective observational analysis of blood samples from critically ill patients. The aim of this project was to establish changes in iron parameters occurring in critical illness and to gain additional insight into suitable parameters to assess iron status in this population.

In *Chapter 5*, a small interventional study of mild hypoxic conditioning in 50–70-year-old participants is presented. This study aimed to establish feasibility and safety of exposure to a long period of mild hypoxia, and to assess the effects on physiological outcomes and biomarkers.

CHAPTER 2:
General methods

2.1 Pulse oximetry

Pulse oximetry is widely used clinically to monitor oxygenation continuously and non-invasively. Due to its ubiquitous use in medicine, oxygen saturation (SO_2) measured by pulse oximetry has long been referred to as the "fifth vital sign" (Neff 1988).

Most oxygen is transported from the lungs to tissues in erythrocytes bound to haemoglobin; only a small fraction (< 3%) of oxygen is dissolved in plasma (Hall 2016). Since this small amount of dissolved oxygen is negligible, arterial oxygenation can be described as haemoglobin oxygen saturation in arterial blood (SaO_2), which is defined as the ratio of oxygen-saturated haemoglobin concentration to total haemoglobin concentration (Nitzan *et al.* 2014):

$$SaO_2 = \frac{[HbO_2]}{[HbO_2] + [Hb]}$$

The percentage of oxygenated haemoglobin is dependent on the arterial partial pressure of oxygen (PaO_2); the relationship between PaO_2 and SaO_2 is defined by the sigmoidal oxygen-haemoglobin dissociation curve (Bohr *et al.* 1904, Hall 2016). The saturation of arterial blood with oxygen can be estimated non-invasively by measuring the peripheral saturation of oxygen (SpO_2) by means of pulse oximetry (Nitzan *et al.* 2014).

The principle underlying pulse oximetry is explained by the fact that oxygenated and deoxygenated haemoglobin exhibit different absorption properties for red and infrared light (Figure 2.1): oxyhaemoglobin absorbs more infrared light (940 nm), while non-oxygenated haemoglobin absorbs more red light (660 nm) (Butterworth 2013). Pulse oximeters exploit this property by emitting light of those two wavelengths from a pair of photodiodes located on one arm of the device and measuring the absorption at the opposite sensor after the light has passed through tissue (e.g. finger or earlobe) (Chan *et al.* 2013).

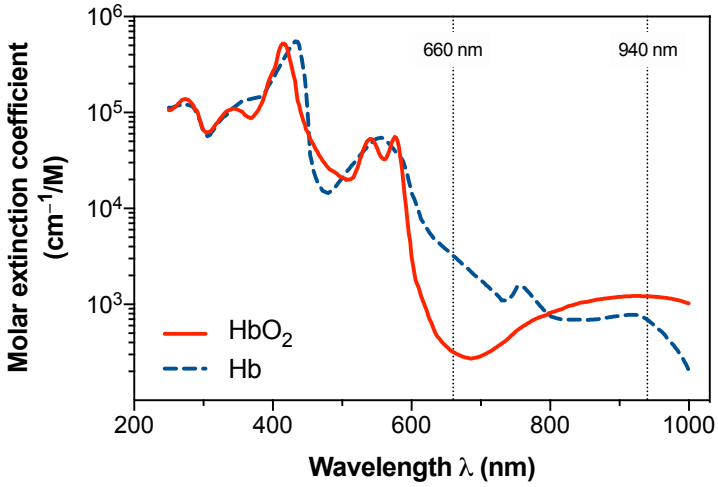


Figure 2.1 Absorption spectra of oxygenated and deoxygenated haemoglobin. Oxygenated (HbO_2) and deoxygenated (Hb) haemoglobin have distinctly different light absorption properties. Hb absorbs more red light, HbO_2 more infrared light (measured at wavelengths of 660 nm and 940 nm, respectively). Based on data from Prahl (1998).

Another principle underlying pulse oximetry is the fluctuation of the absorbed light with the cardiac cycle, which is referred to as photoplethysmography (Nitzan *et al.* 2014). As the arterial blood volume increases during systole, the transmitted light intensity decreases. In contrast, the blood volume in veins and capillaries and the volume of skin and tissue are relatively independent from the cardiac pulsation. This explains the ability of a pulse oximeter to only detect the SpO_2 of arterial blood by measuring a non-pulsatile "direct current (DC)" component and a pulsatile "alternating current (AC)" (Chan *et al.* 2013) (Figure 2.2). Calculating the ratio of the absorbance of pulsatile to non-pulsatile component produces a pulse-added absorbance signal for each wavelength (Hurford and Kratz 2004). The ratio of the two pulse-added absorbance signals can be expressed as:

$$R = \frac{AC_{660}/DC_{660}}{AC_{940}/DC_{940}}$$

This ratio changes based on the saturation of haemoglobin with oxygen in arterial blood. Low SaO_2 means a higher amount of deoxygenated haemoglobin, which results

in higher red-light absorption (i.e. $AC_{660}/DC_{660} > AC_{940}/DC_{940}$) and a higher R value. For high SaO_2, infrared absorption predominates (i.e. $AC_{660}/DC_{660} < AC_{940}/DC_{940}$) and R is lower (Chan *et al.* 2013) (Figure 2.2). This relationship between arterial oxygen saturation and R is used to calculate an SpO_2 readout by comparing R to empirically derived oxygen saturation values obtained in healthy volunteers exposed to varying degrees of hypoxia (Hurford and Kratz 2004). SpO_2 can be calculated as shown, where K is an empirical device-specific constant:

$$SpO_2 = K \times R$$

Pulse oximeters provide accurate SpO_2 values only for their calibration range, usually for values between 70% and 100% saturation (Chan *et al.* 2013). Within this range, most manufacturers claim an accuracy of 2% between SpO_2 readouts and invasively measured SaO_2 values, however, inaccuracies > 4% are not uncommon (Milner and Mathews 2012). The accuracy of SpO_2 readings is limited by motion artefacts, low perfusion and the presence of dyshaemoglobins (Nitzan *et al.* 2014).

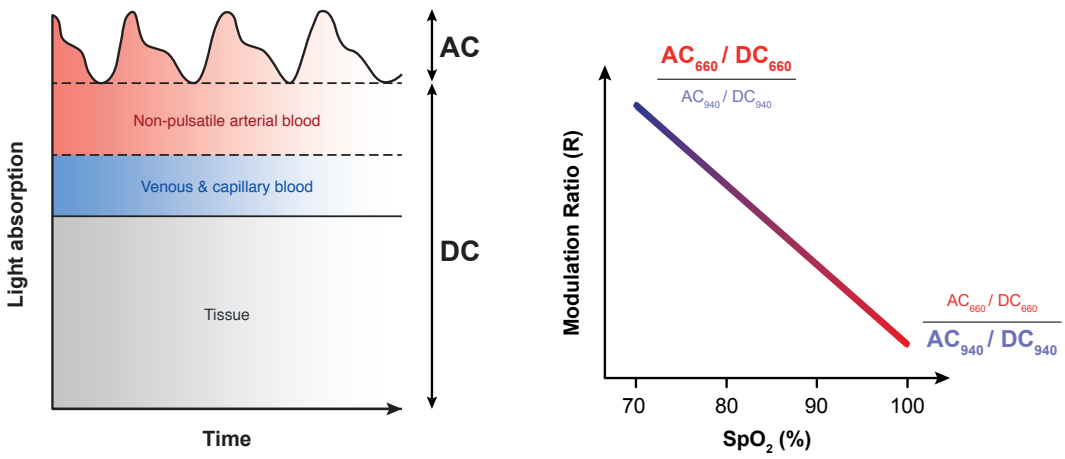


Figure 2.2 Overview of light absorbance by a pulse oximeter. The pulsatile change in arterial blood volume increases light absorption (*left*). By measuring the non-pulsatile "direct current (DC)" and the pulsatile "alternating current (AC)", the pulse oximeter can isolate the pulsatile component of the arterial blood. The double-ratio of the AC and DC components of red light to infrared light absorption changes based on the arterial oxygen saturation (*right*). This empirical correlation is used to derive the SpO_2 output. Based on figures from Bowes *et al.* (1989) and Chan *et al.* (2013).

2.2 Pulmonary function testing

Pulmonary function testing is routinely performed in the clinical setting to evaluate signs and symptoms of lung diseases, monitor disease progression and quantify treatment effectiveness (Al-Ashkar *et al.* 2003). Depending on the pulmonary function requiring assessment, tests can be divided into the following categories: spirometry, measurements of static lung volumes, and assessment of diffusion capacity. These can be performed individually or in combination.

2.2.1 Spirometry

Spirometry is the most basic pulmonary function test and assesses the ventilatory component of pulmonary function. Through various respiratory manoeuvres, different lung volumes and lung capacities (defined as the sum of two or more lung volumes) can be measured (Figure 2.3). Respiratory excursions beyond normal breathing (i.e. tidal volume) provide information about inspiratory and expiratory reserves as well as vital capacity (i.e. the maximum amount of air that can be expelled after maximal inspiration) (Al-Ashkar *et al.* 2003, Hall 2016).

Of particular clinical relevance are the forced vital capacity (FVC), the forced expiratory volume in one second (FEV_1) and their ratio (FEV_1/FVC) (Al-Ashkar *et al.* 2003). The FVC corresponds to the volume measured by forcefully exhaling as long as possible after deep inspiration. The volume forcefully expelled during the first second of that manoeuvre is referred to as FEV_1. These parameters allow the distinction of obstructive lung disease, where the passage of air is limited by airway narrowing, from restrictive lung disease, where lung capacity is reduced by intrapulmonary or extrapulmonary factors (Hughes 2008). Representative spirometry traces are shown in Figure 2.4.



Figure 2.3 Schematic of respiratory excursions, lung volumes, and lung capacities. Respiratory excursions during normal tidal volume (VT) breathing at rest and maximal inspiration and maximal expiration are shown. IRV: inspiratory reserve volume, ERV: expiratory reserve volume, RV: residual volume, VC: vital capacity, IC: inspiratory capacity, FRC: functional residual capacity, TLC: total lung capacity. Based on Hall (2016).

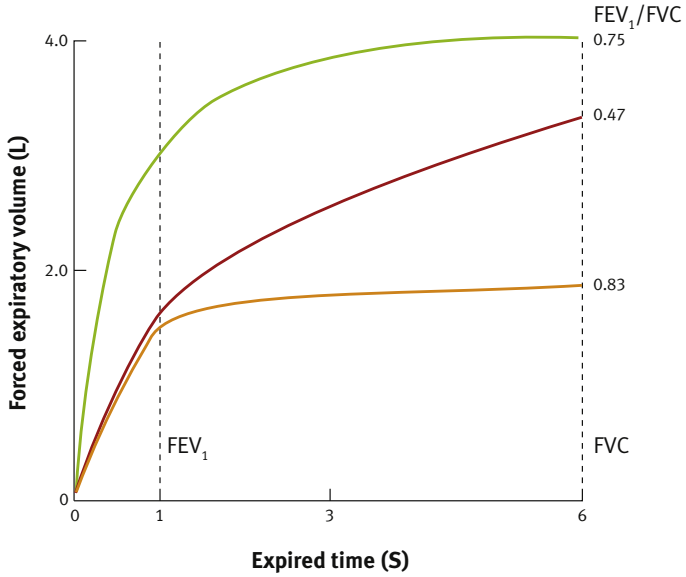


Figure 2.4 Typical forced spirometry traces. The expired volume from a forced expiratory manoeuvre is plotted against time. A healthy subject (*green trace*) is able to expel most of the vital capacity within the first second, resulting in a high FEV_1/FVC ratio. In obstructive (*magenta trace*) and restrictive (*orange trace*) lung disease these volumes are reduced. FEV_1: forced expiratory volume in one second, FVC: forced vital capacity. Reprinted from Hughes (2008) with permission of Elsevier. Copyright © 2007 Elsevier.

2.2.2 Static lung volumes

The volume of air remaining inside the lungs after maximal expiration – the residual volume (RV) – is not accessible to assessment by spirometry (West 2012). The functional residual capacity (FRC) is defined as the sum of the residual volume and the expiratory reserve volume (ERV), whereas the total lung capacity (TLC) is obtained by adding the residual volume to the vital capacity (VC) (Figure 2.3). These so-called static lung volumes provide additional clinical information, e.g. about air trapping and hyperinflation in obstructive disease, or the degree of restriction in restrictive disease (Al-Ashkar *et al.* 2003). The measurement of static lung volumes requires more complex equipment than spirometry: it involves either inhaling an inert gas like helium, the concentration of which is measured in the exhaled air after equilibration between breathing circuit and lung has occurred (multi-breath gas dilution), or measuring pressure changes caused by breathing inside an air-tight cubicle (body plethysmography) (Hughes 2008).

2.2.3 Diffusion capacity

In addition to ventilation, gas exchange can also be assessed as part of pulmonary function testing. Diffusion of gases between the alveoli and the blood depends on the surface area, the difference in partial pressures, the membrane thickness, and the solubility and molecular weight of the diffusing gas (Fick's law) (West 2012). Consequently, diffusion capacity can be reduced in a variety of conditions, such as diseases decreasing the surface area (e.g. emphysema) or conditions affecting membrane permeability (e.g. pulmonary fibrosis) (Al-Ashkar *et al.* 2003).

Diffusion capacity is measured by adding a small amount of carbon monoxide to the inhaled gas. Carbon monoxide is ideal for this purpose, since its transfer into blood is

solely limited by diffusion and not by the perfusion of the lung (West 2012). Measuring the concentration difference of carbon monoxide between the inhaled and exhaled air during a single breath held for 10 seconds allows for the calculation of the so-called transfer factor for carbon monoxide (TLCO). TLCO is the product of the rate of alveolar CO uptake during breath holding (expressed as rate constant KCO) and the alveolar volume (VA) during the measurement (the latter is calculated from the dilution of helium added to the inspired gas) (Hughes 2008).

2.3 Echocardiography

Transthoracic echocardiography is a widely performed investigation that allows a comprehensive assessment of cardiac structure and function in real time (Hillis and Bloomfield 2005). In the context of this thesis, cardiac ultrasound was primarily performed to assess pulmonary arterial pressure non-invasively by means of Doppler echocardiography. This approach has been used and validated extensively (Peacock *et al.* 1990, Allemann *et al.* 2000, Balanos *et al.* 2003, Talbot *et al.* 2005, Smith *et al.* 2008a, Smith *et al.* 2009).

The technique of measuring pulmonary arterial pressure is based on the Doppler principle, which allows the assessment of blood flow in echocardiography. A further important aspect allowing these measurements was the development of a successful method for quantifying the pressure gradient across valvular stenoses using a simplified Bernoulli equation (Gowda *et al.* 2004). In practical terms, an apical four chamber view is obtained and the tricuspid valve visualised (Figure 2.5). In a large percentage of healthy people, a small systolic regurgitant jet through the tricuspid valve is detectable by means of colour Doppler imaging (Yoshida *et al.* 1988, Jobic *et al.* 1993). Using continuous-wave spectral analysis, the velocity profile of this jet can be recorded (Figure 2.6). According to Bernoulli's principle, the velocity of a fluid increases as it passes through a narrow orifice (e.g. the closed tricuspid valve during systole). The driving force of this increased velocity is a pressure gradient across the obstruction, with higher pressures before and lower pressures after the obstruction. The maximum velocity of the tricuspid regurgitant (TR) jet can therefore be related to the maximum pressure gradient (ΔP_{max}) using Bernoulli's equation:

$$\Delta P_{max} = \frac{1}{2}\rho v^2$$

where ρ is the density of the blood and v the velocity of the jet. In clinical practice, this equation is often simplified by assuming the density of whole blood as constant at 1,060 kg/m^3 and converting pressure units to mmHg (1 mmHg = 133.3 Pa), which is more commonly used clinically to assess vascular pressures. Therefore:

$$\Delta P_{max} = \frac{1{,}060}{2 \times 133.3} v^2$$

This simplifies to:

$$\Delta P_{max} = 4v^2$$

For the purpose of echocardiography, this simplified Bernoulli equation is accurate enough and widely used to derive a pressure gradient from the peak velocity of the TR jet (Yock and Popp 1984, Skjaerpe and Hatle 1986). The pressure gradient, ΔP_{max}, represents the difference between right ventricular pressure (RVP) and right atrial pressure (RAP):

$$\Delta P_{max} = RVP - RAP$$

As RVP is equivalent to the pulmonary arterial systolic pressure (PASP), the latter can be derived:

$$PASP = \Delta P_{max} + RAP$$

RAP is usually low in healthy adults (approximately 3–5 mmHg) and therefore often assumed to be a fixed constant. Normal resting values for PASP are usually defined as peak pressure of less than approximately 35 mmHg (Rudski *et al.* 2010). While the described technique can be applied in most healthy individuals, lung diseases like COPD can complicate this approach, as pathological abnormalities (e.g. hyperinflation) can impede adequate echocardiographic imaging.

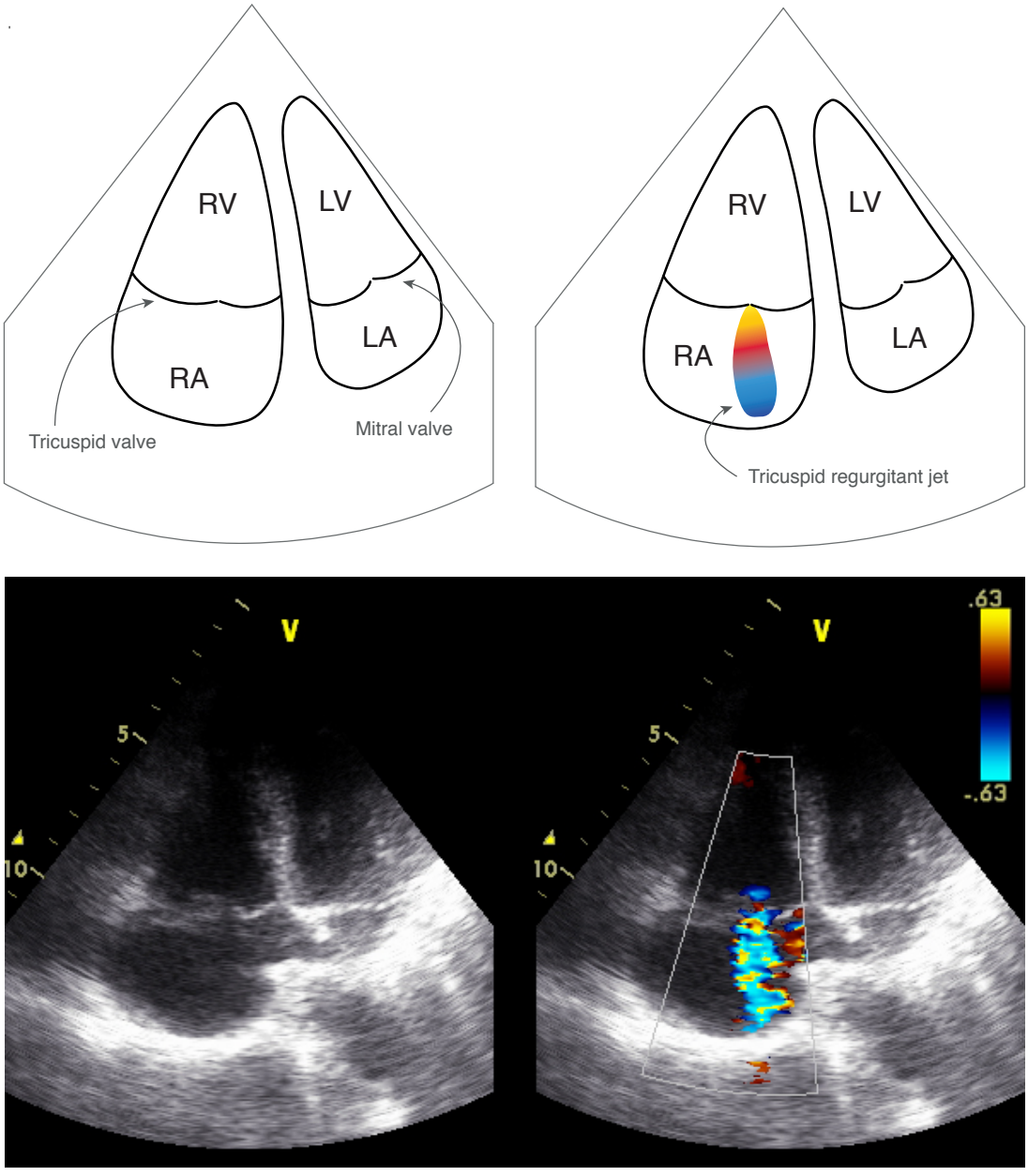


Figure 2.5 Visualisation of tricuspid regurgitation by Doppler echocardiography. In the apical four chamber view (*left*), the left and right atria (LA and RA) and ventricles (LV and RV) as well as the atrioventricular valves are visualised. Superimposing the colour Doppler signal (*right*) reveals a clear trans-tricuspid jet, which indicates tricuspid regurgitation. A schematic overview of the relevant features in the echocardiographic images is shown (*top*).

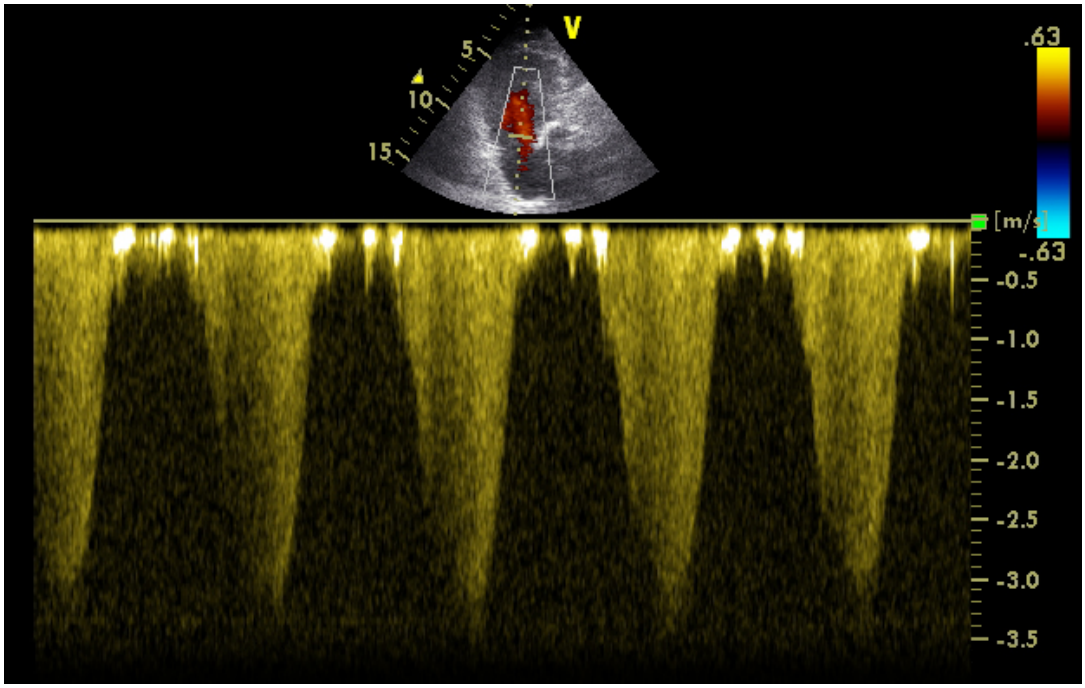


Figure 2.6 Measurement of ΔP_{max} by continuous-wave Doppler echocardiography. The apical four chamber view (*top*) was adjusted for the trans-tricuspid regurgitant jet (*red*) to align with the ultrasound probe (*dotted line*). The continuous-wave Doppler image (*bottom*) shows a maximum velocity of the TR jet of approximately 3.0 m/s, which corresponds to a ΔP_{max} of 36 mmHg, or a PASP of 41 mmHg assuming a right atrial pressure of 5 mmHg.

2.4 Assessments specific to chronic lung disease

This section highlights the theoretical background of assessments performed on patients with COPD (see study in Chapter 3). It is not meant to provide a comprehensive overview of assessment tools, but rather focuses on specific tests used in the context of this thesis.

2.4.1 Functional exercise capacity: Six-minute walk test

The assessment of exercise capacity provides important information about global and system responses of the body to increased metabolic requirements. Various modalities of evaluation are available, ranging from simple to highly complex tests. The gold standard in assessing exercise capacity remains cardiopulmonary exercise testing (CPET), which measures oxygen consumption at maximal exertion. While CPET provides very detailed and comprehensive information of all systems involved in exercise performance, it is also costly, time-consuming and often not feasible in elderly or chronically ill patients (Noonan and Dean 2000). Therefore, submaximal exercise tests are frequently used.

The 6-minute walk test (6MWT) is a simple test evaluating functional capacity on the basis of distance walked. This principle of correlating distance covered over a period of time with exercise capacity was originally established in the 1960s to test fitness in aviation (Balke 1963). Subsequently, a 12-minute walk test to assess the fitness of healthy individuals was developed (Cooper 1968). The 6MWT, which was introduced as an alternative for patients with respiratory disease unable to complete the longer version, performed just as well as the 12-minute version (Butland *et al.* 1982). In comparison to other available walk tests, the 6MWT was found to be “easy to

administer, better tolerated, and more reflective of activities of daily living than the other walk tests" (Solway *et al.* 2001).

In a 6MWT, the distance covered during six minutes of quick, self-paced walking on a flat surface is measured. Guidelines support a standardised and reproducible approach to the test (ATS Committee on Proficiency Standards for Clinical Pulmonary Function Laboratories 2002, Holland *et al.* 2014). In brief, test subjects are instructed to walk between two cones placed 30 metres apart on a flat and hard surface. Subjects can choose their own pace and are allowed to stop and rest during the test. Before and after the test, vital signs and the degree of breathlessness are recorded. Throughout the test, standardised phraseology should be used to avoid any impact of potential encouragement by the assessor.

While other submaximal exercise tests exist, the 6MWT has been chosen in the context of this thesis due to its widespread use in patients with COPD and its reflection of the ability to engage in activities of daily living.

2.4.2 Symptom, quality of life and prognostic indices

The scoring tools introduced in this section are commonly used in clinical and research settings to assess symptoms and quality of life among patients with COPD. This selection is not meant to provide a complete list of available indices, but rather to give an overview and relevant background of the tools specifically used in this thesis.

2.4.2.1 *BODE index*

The BODE index is a multidimensional scoring tool for the prediction of mortality in patients with COPD. It was developed based on the understanding that COPD is not a purely respiratory condition, but also has a systemic component (Celli *et al.* 2004). As

such, the BODE score integrates body mass index (B), airflow obstruction (O), dyspnoea (D), and exercise capacity (E). The score ranges from 0 to 10, with higher values indicating a higher risk of death (Table 2.1). By incorporating systemic markers of worse outcome, the BODE index performs better at predicting mortality in COPD than airflow obstruction alone (Celli *et al.* 2004).

Table 2.1 Components of the BODE index (Celli *et al.* 2004)

Variable	Points on BODE index			
	0	**1**	**2**	**3**
B - Body mass index (BMI), kg/m^2	> 21	≤ 21		
O - FEV$_1$, % predicted	≥ 65	50–64	36–49	≤ 35
D - Modified MRC dyspnoea score	0–1	2	3	4
E - Six-minute walk distance, m	≥ 350	250–349	150–249	≤ 149
Predicted 4-year survival: 0–2 points: 80%, 3–4 points: 67%, 5–6 points: 57%, 7–10 points: 18%.				

2.4.2.2 *Respiratory scores*

The *St. George Respiratory Questionnaire (SGRQ)* is a commonly used tool to measure quality of life in patients with obstructive airway disease. It covers 50 items with a total of 76 weighted responses divided into three components (Jones *et al.* 1991): the symptom score captures frequency and severity of respiratory symptoms, the activity score focuses on activities limited by dyspnoea, and the impact score encompasses social and psychological effects. Each score ranges from 0 to 100, with higher values indicating more severe limitation. In addition, a total score is calculated. The SGRQ showed good reproducibility and correlated well with other markers of disease activity such as 6-minute walk distance, dyspnoea and general health (Jones *et al.* 1992).

A downside of complex questionnaires such as the SGRQ is their lack of practicality in clinical settings. The *COPD Assessment Test™ (CAT)* has been developed and validated to solve this problem and to provide a short, simple, and patient-completed questionnaire to assess the impact of COPD on health-related quality of life (Jones *et al.* 2009). The test evaluates eight domains commonly affected in COPD (cough, sputum production, chest tightness, breathlessness, activity limitation, confidence leaving home, sleep, and energy). Each domain is graded on a 6-point differential scale from 0 (no symptoms) to 5 (most severe symptoms) points. The sum of points from each domain is the final score, which consequently ranges from 0 to 40. In the clinical assessment of COPD patients, the CAT score can provide additional information about a patient's risk of exacerbation, depression, deterioration of health status and mortality (Karloh *et al.* 2016).

Together with the modified MRC score (see below), the CAT is recommended for the assessment of symptoms in the latest consensus report of the Global Initiative for Chronic Obstructive Lung Disease (GOLD) (Vogelmeier *et al.* 2017).

2.4.2.3 *Dyspnoea indices*

Breathlessness, or dyspnoea, is a common symptom of cardiorespiratory disease. Several assessment scores have therefore been devised over time to quantify the degree of dyspnoea. One of the most commonly used scores is the *Medical Research Council (MRC) dyspnoea scale*. The simple five-point scale was first used in the 1950s as part of a longer respiratory questionnaire (Fletcher 1952, Fletcher *et al.* 1959), before adoption by the MRC in 1960 (Fletcher 1960). The score ranges from grade 1 (no incapacitation) to grade 5 (severe limitation by breathlessness) and assesses the level of activity necessary to induce breathlessness or the limitation of activity resulting from it (Stenton

2008). Over time, the original MRC scale has undergone minor changes and adaptations (Brooks *et al.* 1982, Mahler and Wells 1988). This *modified MRC dyspnoea scale* is widely used nowadays. It is very similar to the original scale with only minor alterations in phraseology and a point range from 0 to 4 (Table 2.2). This modified scale is integrated in the BODE index (see Chapter 2.4.2.1) and part of the GOLD recommendation for assessment of COPD symptoms (Vogelmeier *et al.* 2017).

Table 2.2 Modified MRC dyspnoea scale (Mahler and Wells 1988)

Grade	Description of breathlessness
0	Not troubled with breathlessness except with strenuous exercise.
1	Troubled by shortness of breath when hurrying on the level or walking up a slight hill.
2	Walks slower than people of the same age on the level because of breathlessness or has to stop for breath when walking at own pace on the level.
3	Stops for breath after walking about 100 yards or after a few minutes on the level.
4	Too breathless to leave the house or breathless when dressing or undressing.

The *Dyspnoea-12* is another score developed to assess the severity of breathlessness (Yorke *et al.* 2010). It consists of twelve questions about physical and affective consequences of dyspnoea, the answer to each of which can be “none”, “mild”, “moderate” or “severe”. As opposed to many other dyspnoea scores, it is not based on the activity level, but on the subjective experience of dyspnoea. The language of the questions reflects this by incorporating phraseology commonly used by patients (e.g. “My breathing is uncomfortable” or “I cannot get enough air”). The Dyspnoea-12 score correlates well with other assessment tools such as 6-minute walk distance and MRC grade (Yorke *et al.* 2010).

2.4.2.4 *Non-respiratory scores*

COPD is a respiratory disease with systemic, extrapulmonary manifestations. It is therefore important to consider non-respiratory symptoms and quality of life indices such as fatigue, anxiety and depression.

The *Fatigue Severity Scale* can be used to quantify fatigue. Participants are asked to indicate their level of agreement with nine statements about fatigue on a scale from 1 (strong disagreement) to 7 (strong agreement). The score was initially developed for use in multiple sclerosis and systemic lupus erythematosus (Krupp *et al.* 1989), but is also commonly used in other settings including COPD (Antoniu and Ungureanu 2015). In COPD, the fatigue severity score was found to correlate with multidimensional disease severity indices like BODE (Inal-Ince *et al.* 2010).

The psychological component of respiratory disease can be assessed with the *Hospital Anxiety and Depression Score (HADS)*. The scale was devised as a screening tool to be used in a general medical outpatient population (Zigmond and Snaith 1983). Two scores, each ranging from 0 to 21, are calculated from the responses to 14 questions (seven for each of the two components anxiety and depression). The cut-off score for an abnormal test is 11, with scores from 8 to 10 classified as borderline.

2.5 Enzyme-linked immunosorbent assay

The enzyme-linked immunosorbent assay (ELISA) is an analytical test commonly used to quantify the amount of a target protein in a liquid sample. The main principles underlying this technique are explained by antigen-antibody interactions and enzymatic reactions resulting in measurable changes of light absorption (O'Kennedy *et al.* 1990, Aydin 2015).

A "*direct ELISA"* constitutes the most basic form of this assay: the protein to be measured is immobilised on a solid surface before adding an enzyme-labelled detection antibody, which forms a complex with the antigen. In an *"indirect ELISA"*, a primary detection antibody binds to the target protein and is then recognised by a secondary detection antibody, which contains the enzymatic label. Addition of a clear substrate solution initiates a reaction catalysed by the antibody-bound enzyme and produces a visible colour change, which can subsequently be quantified by measuring the optical density. The initial concentration of the target protein is directly proportional to the colour change and can be empirically determined by using standard curves generated from samples of a known concentration.

In a *"sandwich ELISA"*, the vial or plate used to perform the test is coated with a capture antibody, which is specific for the protein of interest. Upon addition of the sample, only that protein will bind and become immobilised while other proteins in the sample will be removed in a washing step. This substantially increases the sensitivity and specificity of the assay. The subsequent steps are identical to the ones described before and involve primary and enzyme-labelled secondary detection antibodies.

Lastly, a *"competitive ELISA"* is based on the principle of competitive binding to the capture antibody. The sample containing the target protein is mixed with a reagent containing an enzyme-conjugated target protein. After addition to the test plate, the

target protein and the enzyme-conjugated protein compete for the antibody binding sites. Based on the concentration in the original sample, more or less of the target protein will be captured. If the concentration in the sample is high, most capture antibodies will form complexes with the target protein instead of the enzyme-conjugated protein. The opposite applies to samples with low concentrations of protein. After chemically stopping this reaction and adding a colourless enzyme substrate, the colour change of the substrate solution can be detected as described before. In contrast to the previously mentioned ELISA types, the intensity of the colour change is inversely related to the concentration of the target protein in the original sample.

The assays performed as part this thesis were either sandwich (e.g. erythropoietin, sTfR) or competitive ELISAs (e.g. hepcidin).

2.6 Intravenous iron preparations

Intravenous iron preparations have several advantages over oral iron formulations: higher doses can be administered, which permits iron repletion in fewer infusions or even a single dose, and common gastrointestinal side effects of oral iron administration can be avoided. Intravenous iron is furthermore also efficacious in situations of high hepcidin expression, which prevents oral iron from being absorbed. A significant downside of many of the earlier parenteral iron formulations (e.g. iron dextran) was the high risk of anaphylactic reactions. Newer intravenous iron preparations (e.g. ferric carboxymaltose) are less prone to hypersensitivity reactions and also bind iron more avidly, therefore limiting the release of toxic non-transferrin bound iron into the bloodstream after injection (Larson and Coyne 2014).

2.6.1 Ferric carboxymaltose

Ferric carboxymaltose (FCM) is a widely used and well-established high-dose parenteral iron formulation that allows rapid replenishment of the body's iron stores while maintaining a favourable safety profile (Scott 2018).

2.6.1.1 *Chemical and pharmacological properties*

The chemical structure of FCM consists of a macromolecular iron-hydroxide core of polynuclear iron(III)-hydroxide surrounded by a carbohydrate (carboxymaltose) shell (Figure 2.7) (Geisser 2009). The complex has a molecular weight of approximately 150,000 Daltons. The iron release from the FCM complex occurs gradually, which results in a much wider therapeutic window compared to other formulations. The stability of FCM – explained by the tight carbohydrate "wrapping" – prevents the quick release of iron from the complex after injection and the accumulation of toxic NTBI.

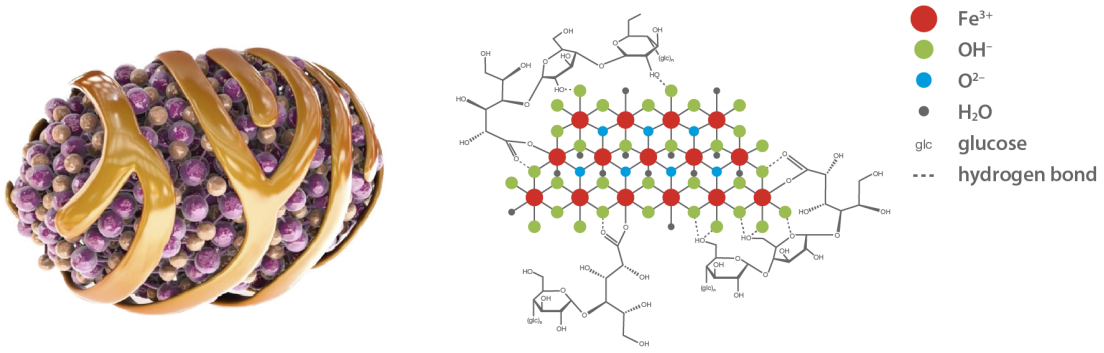


Figure 2.7 Schematic representation of the structure of ferric carboxymaltose. Ferric carboxymaltose (FCM) consists of a polynuclear iron(III)-hydroxide core stabilised within a carbohydrate shell. Provided by and reprinted with permission of Vifor Pharma. Copyright © 2017 Vifor Pharma UK Ltd.

Instead, the whole complex is quickly cleared from the bloodstream and taken up by macrophages of the reticuloendothelial system (Toblli and Angerosa 2014). After infusion, the carbohydrate shell is partially degraded by α-amylase in the blood. The complete degradation of the shell occurs in the endolysosome of the macrophages after the complex has been taken up through endocytosis. This leads to the release of ferric iron, which is then reduced to ferrous iron by STEAP3 and exported into the cytosol by DMT1. From there, it can then be exported by FPN, as described before.

2.6.1.2 *Safety profile*

FCM has a favourable safety profile due to its wide therapeutic range and the lower probability of anaphylactic reactions compared to older parenteral iron preparations. This allows the administration of relatively high single-bolus doses. Pharmacological safety studies further confirmed the absence of cardiovascular, respiratory, central nervous, renal, reproductive, or developmental toxicity of FCM (Funk *et al.* 2010).

According to the Summary of Product Characteristics, the most common side effect of FCM is nausea, occurring in 2.9% of cases (Vifor Pharma UK Limited 2017). Other common side effects (i.e. occurring in more than 1% of subjects) include headaches, dizziness, hypertension, hypophosphataemia and infusion site reactions. Uncommonly (i.e. in less than 1% but more than 1 in 1,000 subjects), FCM can cause tachycardia, hypotension, gastrointestinal complications (e.g. constipation, diarrhoea, vomiting), pruritus or rash, myalgia or arthralgia, as well as changes in laboratory parameters (e.g. increased liver function parameters). The Summary of Product Characteristics of FCM further lists the following as potential rare side effects (i.e. less than 1 in 1,000 subjects): anaphylactoid reactions, loss of consciousness, anxiety, bronchospasm, angioedema, and influenza-like symptoms.

CHAPTER 3:

Effects of intravenous iron in chronic obstructive pulmonary disease

3.1 Introduction

The study presented in this chapter forms the centrepiece of this thesis. Chronic obstructive pulmonary disease (COPD) is an excellent example of dysregulated iron and oxygen physiology in a clinical setting. Hypoxia is a crucial hallmark of the disease. Recently, iron mishandling has become a focus of COPD research as well. This chapter explores the potential benefits of intervening pharmacologically in these processes.

3.1.1 Definition

Chronic obstructive pulmonary disease is a slowly progressing airway disorder of global relevance. In an attempt to increase awareness, guide management, and improve prevention, the Global Initiative for Chronic Obstructive Lung Disease (GOLD) was formed in 1998. Shortly thereafter, a consensus report summarising existing evidence and deriving recommendations for diagnosis and treatment was published (Pauwels *et al.* 2001). Since then, the "Global Strategy for the Diagnosis, Management, and Prevention of COPD" has been regularly updated. In its latest executive summary, COPD is defined as follows (Vogelmeier *et al.* 2017):

> *Chronic obstructive pulmonary disease (COPD) is a common, preventable, and treatable disease characteri[s]ed by persistent respiratory symptoms and airflow limitation that is due to airway and/or alveolar abnormalities, usually caused by significant exposure to noxious particles or gases.*

This functional definition highlights the central feature of obstructive airflow in COPD. In contrast to other obstructive lung diseases such as asthma, the symptoms and airflow limitation in COPD are only partially reversible. This leads to a progressive lung

function decline and disabling symptoms such as dyspnoea, chronic cough and sputum production.

3.1.2 Epidemiology and socioeconomic impact

It is estimated that 4–10% of the world's population are affected by COPD (Halbert *et al.* 2003). Underdiagnosing contributes to substantial uncertainty about the true prevalence; the disease is often misdiagnosed or not diagnosed at all (Tinkelman *et al.* 2006, Shahab *et al.* 2006). A recent meta-analysis found an overall high prevalence of 14.3% in men and 7.6% in women (Adeloye *et al.* 2015).

Morbidity and mortality are equally alarming: in 2015, the disease caused 3.2 million deaths worldwide (GBD 2015 Chronic Respiratory Disease Collaborators 2017). COPD became the third most common cause of death globally in 2010 (Lozano *et al.* 2012), a rank previously only projected for 2020 (Murray and Lopez 1997). While mortality rates for other chronic conditions such as cancer, heart disease, or stroke have decreased over the past decades, mortality rates for COPD have increased since the 1970s (Jemal *et al.* 2005, Ma *et al.* 2015). However, some studies have also shown declines in mortality in more recent years. The Global Burden of Disease Study 2010 found decreases in the absolute number of COPD deaths from 1990 to 2010, even though the rank as a leading cause of death increased (Lozano *et al.* 2012). This apparent discrepancy can be explained by changes in age distribution and all-cause mortality (Burney *et al.* 2015). Similarly, the trend in overall mortality from COPD decreased in the United States from 1999 to 2011 (Ford 2015). Nevertheless, COPD death rates as well as burden of disease are projected to continue increasing globally in the decade leading up to 2030 (Mathers and Loncar 2006).

COPD is associated with significantly impaired quality of life, even in early disease stages (Carrasco Garrido *et al.* 2006). Exacerbations – sporadic episodes of acute worsening of symptoms requiring increased treatment and usually caused by bacterial or viral infections – can further substantially reduce health-related quality of life for COPD patients (Schmier *et al.* 2005, Qureshi *et al.* 2014).

Finally, COPD also represents a substantial economic burden on healthcare systems. In the United Kingdom, indirect costs associated with COPD were estimated at £982 million per year (Britton 2003). Costs associated with exacerbations contributed to a substantial portion of total COPD-related costs (Qureshi *et al.* 2014).

3.1.3 Aetiology and pathogenesis

COPD is a highly complex and heterogenous disorder. The GOLD report succinctly highlights key aspects of the pathogenesis of COPD (Vogelmeier *et al.* 2017):

> *The chronic airflow limitation that characteri[s]es COPD is caused by a mixture of small airway disease (e.g., obstructive bronchiolitis) and parenchymal destruction (emphysema), the relative contributions of which vary from person to person. Chronic inflammation causes structural changes, small airway narrowing, and destruction of lung parenchyma. A loss of small airways may contribute to airflow limitation and mucociliary dysfunction, a characteristic feature of the disease.*

The disease is caused by inhaled noxious particles or gases which initiate an inflammatory response ultimately leading to pathological changes. Tobacco smoking represents the most common exposure resulting in COPD. Other environmental and occupational exposures such as air pollutants, second-hand smoking, dusts, and fumes from biomass fuels play a role in the development of the disease as well (Eisner *et al.* 2010).

As mentioned, inflammatory processes represent a crucial link between the inhaled noxious stimuli and the structural damage in the lung (Barnes 2008). An overview of the immune cells and chemokines involved in the inflammatory response and associated pathological changes in COPD is depicted in Figure 3.1.

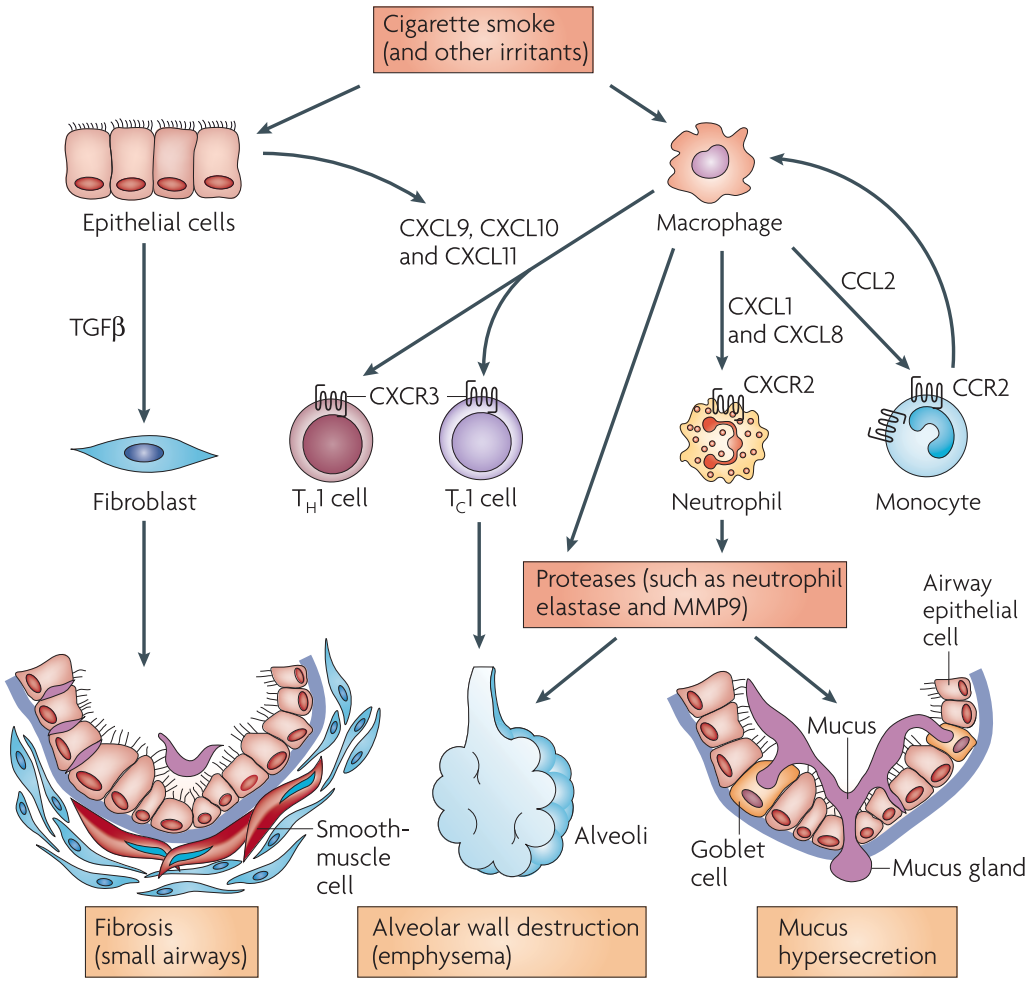


Figure 3.1 Immune cells involved in the inflammatory response in COPD. Alveolar epithelial cells and macrophages release chemotactic factors in response to inhaled particles resulting in the recruitment of immune cells such as monocytes, neutrophils and T cells. These cells release proteases, causing elastin degradation and emphysema, and neutrophil elastase, resulting in mucus hypersecretion. Transforming growth factor-β (TGF-β), released from epithelial cells and macrophages, stimulates fibroblast proliferation and leads to small airway fibrosis. CCL2: CC-chemokine ligand 2, CCR2: CC-chemokine receptor 2, CXCL: CXC-chemokine ligand, CXCR: CXC-chemokine receptor, MMP9: matrix metalloproteinase 9, T_H1: type 1 helper T cell, T_C1: type 1 cytotoxic T cell. Reprinted from Barnes (2008) with permission of Springer Nature. Copyright © 2008 Nature Publishing Group.

The response to noxious inhaled stimuli can be seen as a continuous process involving phases of initiation, progression, and consolidation of damage with inflammation, oxidative stress, extracellular matrix proteolysis, apoptosis, and autophagy interacting in a complex manner and contributing to the pathogenesis of COPD (Tuder and Petrache 2012).

In addition to exposure to risk factors, host factors such as genetic abnormalities may contribute to the development of COPD (Vogelmeier *et al.* 2017). In this context, deficiency of the alpha-1 antitrypsin protein is of interest as an inherited cause of COPD. The liver-derived protein protects the lung from proteolytic damage under physiological conditions. Mutations in the *SERPINA1* gene (also referred to as protease inhibitor (Pi) locus) result in misfolded alpha-1 antitrypsin proteins, systemic deficiency, and subsequent damage to alveolar membranes through increased neutrophil elastase activity. Individuals carrying the disease-associated S or Z alleles are at an increased risk for COPD, with ZZ homozygosity exhibiting particularly low alpha-1 antitrypsin levels (Greene *et al.* 2016).

COPD – although of pulmonary origin – is not purely limited to the lungs, but is associated with concomitant systemic manifestations further increasing morbidity and mortality (Vogelmeier *et al.* 2017). These systemic manifestations of COPD include skeletal muscle dysfunction, nutritional abnormalities, cachexia, cardiovascular and neurologic comorbidities, diabetes, osteoporosis, and depression (Agusti *et al.* 2003, Barnes and Celli 2009, Decramer *et al.* 2008). While some of the extrapulmonary comorbidities may arise on the basis of shared risk factors, a common underlying pathophysiology involving systemic inflammation and oxidative stress is likely to be a major contributor as well (MacNee 2005, Sevenoaks and Stockley 2006, Agusti 2007).

3.1.3.1 *Hypoxia, pulmonary hypertension and the role of iron*

The roles of hypoxia and pulmonary hypertension will be discussed in more detail, as they are particularly relevant in the context of imbalances in iron homeostasis.

Alveolar hypoxia and consequent hypoxaemia are central features in the pathogenesis of COPD and its aforementioned extrapulmonary manifestations. While many physiological responses to hypoxia are beneficial in ensuring tissue oxygen delivery, some are maladaptive in the setting of COPD and contribute to the development and progression of the disease (Kent *et al.* 2011). Hypoxaemia in COPD is primarily caused by a ventilation-perfusion ($\dot{V}/\dot{Q}$) mismatch as a result of limitations in airflow and obliteration of pulmonary capillaries due to emphysema (Wagner *et al.* 1977, Kent *et al.* 2011). Predominantly emphysematous disease can lead to high $\dot{V}/\dot{Q}$ ratios with increased ventilation of areas with poor perfusion (i.e. increased dead space), while low $\dot{V}/\dot{Q}$ ratios are observed in situations of poor ventilation due to airflow limitation (i.e. shunt). Inequalities in $\dot{V}/\dot{Q}$ occur early in the course of the disease and are detectable in patients with mild COPD already (Barbera *et al.* 1990).

Hypoxic pulmonary vasoconstriction – a physiological response aimed at reducing perfusion of poorly ventilated lung units and preventing $\dot{V}/\dot{Q}$ inequalities – is an example of a maladaptive process in COPD, which contributes to the development of pulmonary hypertension (Kent *et al.* 2011). Pulmonary arterial hypertension, which is defined as mean pulmonary arterial pressure ≥ 25 mmHg, frequently occurs in patients with COPD and is a strong predictor of decreased survival (Cuttica *et al.* 2010, Andersen *et al.* 2012). The role of hypoxia in the pathogenesis of pulmonary hypertension is further supported by the beneficial effects of supplemental oxygen administration, which attenuates the progression of pulmonary hypertension (Chaouat *et al.* 2008). While hypoxic vasoconstriction is an important contributor in the pathology

of pulmonary hypertension in COPD, there are several other contributing factors including inflammation, endothelial dysfunction, oxidative stress, emphysematous destruction of the vascular bed, and genetic predisposition. Combined, these factors result in structural changes in the form of pulmonary vascular remodelling (Chaouat *et al.* 2008, Minai *et al.* 2010).

As mentioned before, the physiological response to hypoxia depends on the availability of iron due to its central role in the hypoxia signalling cascade. This interaction has been extensively studied, as discussed in Chapter 1.3. Briefly, low levels of iron can mimic pulmonary responses similar to those seen in hypoxia, such as elevated pulmonary arterial pressure and altered pulmonary vascular responses, whereas iron administration can attenuate these effects (Smith *et al.* 2008a, Smith *et al.* 2009, Bart *et al.* 2016, Frise *et al.* 2016). Iron deficiency might therefore be particularly detrimental in conditions such as COPD, which are characterised by chronic hypoxia, as it can further augment hypoxia-driven pulmonary vasoconstriction and aggravate pulmonary hypertension. A recent study has indeed confirmed increased pulmonary arterial pressure in iron-deficient patients with COPD. In this study, the prevalence of elevated PASP was twice as high in iron-deficient patients with COPD compared to non-iron-deficient patients (Plesner *et al.* 2017).

3.1.4 Diagnosis and treatment

The diagnosis of COPD is primarily driven by the presence of respiratory symptoms or risk factors. This is highlighted in the latest GOLD consensus report, which lists the following key points for the diagnosis of COPD (Vogelmeier *et al.* 2017):

- *COPD should be considered in any patient with dyspn[o]ea, chronic cough or sputum production, and/or a history of exposure to risk factors.*
- *Spirometry is required to make the diagnosis; a post-bronchodilator FEV_1/FVC less than 0.70 confirms the presence of persistent airflow limitation.*
- *The goals of COPD assessment are to determine the level of airflow limitation, the impact of disease on the patient's health status, and the risk of future events (such as exacerbations, hospital admissions, or death) to guide therapy.*
- *Concomitant chronic diseases occur frequently in patients with COPD and should be treated because they can independently affect mortality and hospitali[s]ations.*

If respiratory symptoms and exposure to risk factors are present, spirometry following the application of an inhaled bronchodilator is required to establish the diagnosis. However, it should not be used independently of the clinical presentation, as screening asymptomatic patients for obstructive spirometry patterns does not provide benefits in terms of disease progression or patient outcomes (Siu *et al.* 2016). Once a diagnosis of COPD has been confirmed, spirometric measures of FEV_1 are further used to stage the disease based on the severity of airflow obstruction (Table 3.1). These stages correlate well with pathological findings of disease severity (Hogg *et al.* 2004) and mortality risk (Mannino *et al.* 2006). Additional investigations, such as chest radiography, computed

tomography, or blood testing, are not required to establish a diagnosis of COPD, but may be helpful in ruling out other pathologies or comorbidities.

Table 3.1 GOLD classification of airflow limitation in COPD (Vestbo *et al.* 2013)

FEV_1/FVC	FEV_1 (% predicted)	GOLD Stage	Severity of airflow limitation
< 0.7	≥ 80	**GOLD I**	Mild
	50–79	**GOLD II**	Moderate
	30–49	**GOLD III**	Severe
	< 30	**GOLD IV**	Very severe

Classification is based on post-bronchodilator FEV_1. FEV_1: forced expiratory volume in 1 second; FVC: forced vital capacity.

It is not the purpose of this introduction to provide comprehensive details on the treatment and management of COPD as this would exceed the limits and objectives of this thesis. The author rather wishes to give a brief overview of treatment options and concepts based on the latest GOLD recommendations (Vogelmeier *et al.* 2017). A key element in the treatment of COPD is smoking cessation. Even after years of tobacco exposure, smoking cessation can still alter the course of lung function decline and restore it to normal levels (Fletcher and Peto 1977). Efforts to stop smoking can be supported psychologically or pharmacologically to increase success rates. Pharmacotherapy, while highly complex, only represents a small piece in the management of COPD and is among the least cost-effective treatment options (British Thoracic Society 2012). It must be personalised to each patient's disease severity, risk of exacerbations, side effects, and comorbidities. Pharmacologic therapy usually includes inhaled bronchodilators such as β_2-agonists or muscarinic antagonists, either

individually or in combination. In more severe COPD, and in the case of frequent exacerbations, inhaled corticosteroids may be added for their anti-inflammatory properties. Proper technique of inhaler use must be emphasised and monitored regularly. Some patients may further benefit from mucolytic agents or prophylactic antibiotics. Vaccinations against seasonal influenza and pneumococci can reduce the risk of respiratory tract infections and should be considered in the management of COPD. Another important option in the management of COPD is pulmonary rehabilitation, an individualised intervention comprising exercise therapy, education, and support in self-management. Pulmonary rehabilitation can have significant benefits such as reduced symptoms as well as improved exercise capacity and overall health (Spruit *et al.* 2013). In very severe cases, further interventions are available. Patients with severe resting hypoxaemia can benefit from long-term oxygen therapy (LTOT), which has been shown to improve survival in this subgroup of patients (Cranston *et al.* 2005). If severe hypercapnia persists, long-term non-invasive ventilation can be considered. For patients with advanced, predominantly emphysematous disease, surgical interventions in the form of bullectomies or valve insertion can be an option. Finally, it must not be forgotten that palliative treatment options can improve quality of life for patients in terminal conditions.

3.1.5 Iron homeostasis in COPD

The topic of iron metabolism in COPD is complex and dysregulations of iron homeostasis occur frequently. Both systemic iron deficiency as well as local iron overload within the lungs have been reported and reviewed recently (Cloonan *et al.* 2017).

3.1.5.1 *Pulmonary iron homeostasis*

Before discussing common dysregulations of iron metabolism in COPD, it is worth reviewing normal iron handling within the lungs and how it contrasts from the systemic and cellular iron homeostasis described in Chapter 1.1. Key differences exist in terms of iron source and purpose of pulmonary iron handling. The lungs are exposed to exogenous inhaled iron from the air and – unlike the gastrointestinal system – do not serve a purpose in nutritional iron uptake. The overview given here is summarised from published reviews (Turi *et al.* 2004, Ghio 2009, Cloonan *et al.* 2017).

Iron represents one of the most abundant metals present in inhaled air. The daily amount of inhaled iron particles the upper respiratory tract is exposed to is approximately 10–25 µg. A main goal of iron handling in the lung is the sequestration of iron to prevent damaging effects from iron-induced oxidative stress. This process involves chemical reduction of iron, uptake into cells, storage in the form of ferritin, and eventually export in less reactive forms. The same proteins responsible for mediating these actions in the intestine are also present in the lung; their regulation is, however, targeted for the purpose of iron sequestration rather than nutritional uptake. Consequently, iron overload increases expression of proteins like Dcytb, DMT1, and FPN to facilitate the process of detoxification, while a contrary response to iron overload is needed in the intestine to prevent further uptake of iron. Proteins involved in iron transport within the lung are depicted in Figure 3.2. In addition to alveolar epithelial cells, alveolar macrophages and neutrophils (in conditions of iron excess and oxidative stress) are involved in pulmonary iron sequestration. The concept of detoxification by iron sequestration is highlighted by the fact that most pulmonary iron is located inside lung tissue (Heilig *et al.* 2006) and that ferritin concentrations in the lung are high (Ghio *et al.* 1998). Ferroportin also plays an important role in the detoxification process and is primarily located on the apical

membrane (Yang *et al.* 2005). In contrast to the basolateral location in enterocytes, this allows release of iron into the alveoli rather than the systemic circulation.

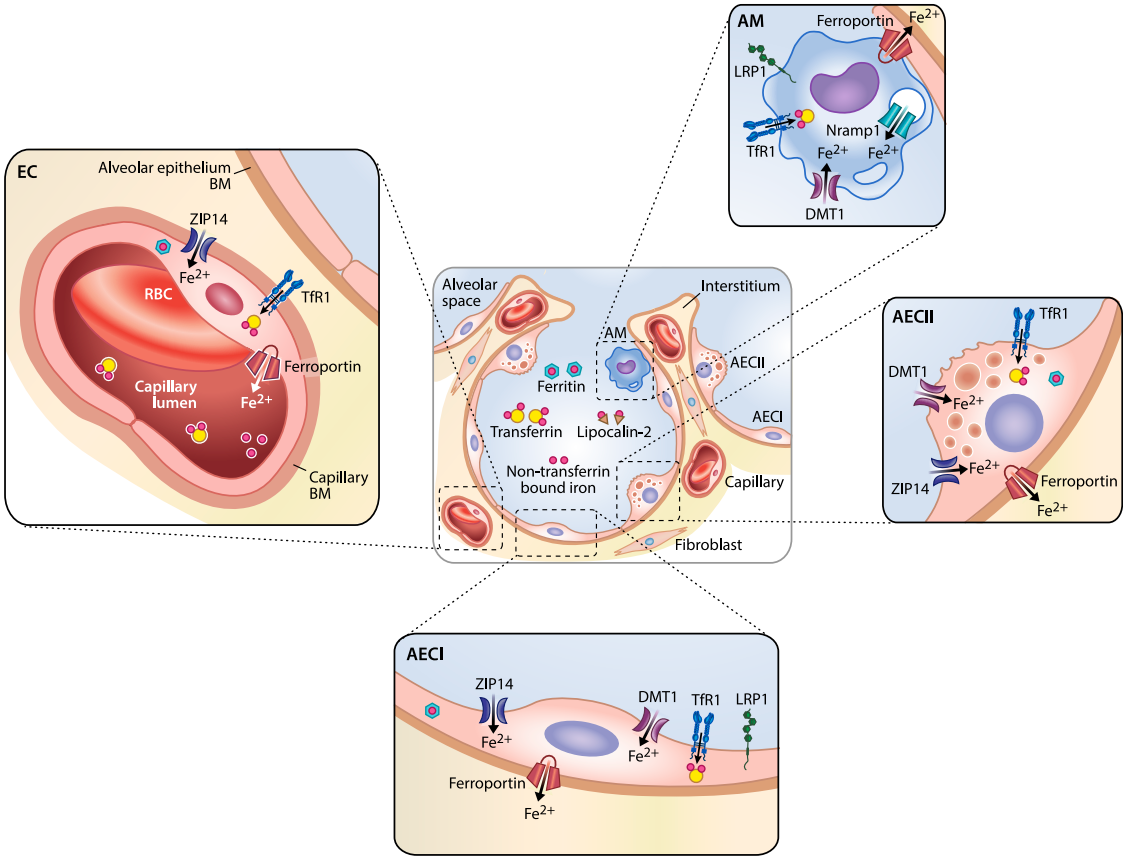


Figure 3.2 Proteins involved in pulmonary iron import and export. Iron sequestration within alveolar epithelial cells occurs through transferrin receptor 1 (TfR1), divalent metal transporter 1 (DMT1), the zinc transporter ZIP14 and the lactoferrin receptor low-density lipoprotein receptor-related protein 1 (LRP1). Alveolar macrophages can furthermore import iron through the natural resistance-associated macrophage protein 1 (NRAMP1). Iron export occurs through ferroportin, which (unlike in this figure) is primarily expressed apically. AECI/AECII: alveolar epithelial cell type I/II, AM: alveolar macrophage, EC: endothelial cell, RBC: red blood cell, BM: basal membrane. Reprinted from Cloonan *et al.* (2017) with permission of the American Thoracic Society. Copyright © 2017 American Thoracic Society.

3.1.5.2 *Dysregulation of iron homeostasis in COPD*

Non-anaemic iron deficiency frequently occurs among patients with COPD. In a retrospective cohort study, 18% of COPD patients were iron-deficient compared to only 5% in a matched control group (Nickol *et al.* 2015). In this study, iron deficiency was defined as any one of ferritin < 12 µg/L, transferrin saturation < 16% or soluble transferrin receptor > 28.1 nmol/L. Including patients with a transferrin saturation between 16% and 20% – an "*indeterminate*" group – iron deficiency was observed in 28% of COPD patients and 11% of healthy control subjects. Iron deficiency in COPD patients was furthermore associated with worse hypoxaemia, higher inflammatory markers and more exacerbations compared to iron-replete patients with COPD. The study also highlighted and discussed the difficulty of defining iron deficiency in COPD, a problem arising from the underlying chronic inflammation, which impacts several markers commonly used to assess iron status and might mask true iron deficiency. The authors therefore chose a fairly restrictive definition, as outlined. While the effects of iron deficiency on exercise tolerance were not clear in the cohort studied by Nickol *et al.* (2015), another trial could link non-anaemic iron deficiency in COPD to lower aerobic capacity before endurance training and an impaired response to pulmonary rehabilitation (Barberan-Garcia *et al.* 2015). Prevalence of iron deficiency was high in these study participants as well (48%). It must be noted, however, that a broader and more commonly applied definition of iron deficiency was chosen in this study (ferritin < 100 µg/L, or ferritin ≥ 100 but < 300 µg/L combined with a transferrin saturation < 20%). A similar rate of non-anaemic iron deficiency was reported in a recent study investigating the effects of iron deficiency on pulmonary arterial pressure in COPD (Plesner *et al.* 2017). In this study, 41% of participants met the criterion of ferritin < 100 µg/L, the definition of iron deficiency applied by the authors.

Traditionally, iron deficiency has been discussed and investigated in the setting of anaemia, which is unavoidable once low iron availability limits erythropoiesis. Anaemia is a common comorbidity in COPD that occurs in 10–30% of patients and is associated with worse outcomes (Vasquez and Logomarsino 2016). In COPD patients with anaemia, iron deficiency has a high prevalence as well (Silverberg *et al.* 2014). While it certainly is a contributing factor to the development of anaemia, iron deficiency is by far not the only cause (Boutou *et al.* 2015a). It therefore seems appropriate to consider anaemia and iron deficiency separately in COPD (Boutou *et al.* 2015b). The high prevalence of iron deficiency in patients without anaemia – as outlined above – further supports this.

In contrast to the described systemic iron deficiency, local iron accumulation within the lungs plays an important role in COPD as well. In addition to the continuous exposure to inhaled iron in ambient air, cigarette smoke significantly augments the iron load in the lung (Thompson *et al.* 1991). Smoking one pack of cigarettes can increase the daily exposure to inhaled iron by 5–14 µg (Cloonan *et al.* 2017). The inhaled particles alter iron homeostasis and lead to increased intracellular iron, which results in oxidative stress and tissue damage (Ghio *et al.* 2008). Alveolar macrophages have been identified as the predominant site of iron accumulation in the lungs of COPD patients, presumably representing a protective mechanism from iron-induced oxidative stress (Philippot *et al.* 2014). These authors furthermore described increased expression of iron transport and storage proteins in the lung as well as a correlation of pulmonary iron accumulation with disease severity.

A potentially important consequence of pulmonary iron accumulation is an increased risk of airway infections, given that most pathogens require iron to proliferate (Weinberg 2009). Exposure to inhaled particles in cigarette smoke can lead to an

elevated risk, in part through the increased availability of iron in the airway (Ghio 2014). Reduced inflammatory responses within the lung might furthermore promote infections. Preliminary research in mice with pulmonary iron overload has recently been presented and showed reduced upregulation of pro-inflammatory cytokines and attenuated recruitment of immune cells in response to lipopolysaccharide-induced inflammation (Neves *et al.* 2018). While the potential for an increased risk of infections exists, no clinical trial has linked intravenous iron administration to more infections in COPD so far.

Finally, genetic alterations associated with COPD susceptibility have been identified in recent years. In the context of altered iron metabolism, single nucleotide polymorphisms in the *IREB2* gene, which encodes the iron-regulatory protein 2 (IRP2), are of particular interest and have been associated with COPD susceptibility (DeMeo *et al.* 2009). This association has been confirmed in several other studies (Hardin *et al.* 2012, Ding *et al.* 2015b, Du *et al.* 2016) and was shown to be independent of lung function (Chappell *et al.* 2011) or tobacco exposure (Siedlinski *et al.* 2013). A potential association of *IREB2* polymorphisms with lung function in severe alpha-1 antitrypsin deficiency has also been reported (Kim *et al.* 2012). Alpha-1 antitrypsin deficiency is furthermore associated with disrupted iron homeostasis, as indicated by increased iron levels systemically and within the airway (Ghio *et al.* 2013, Fischer *et al.* 2009).

Animal models of COPD revealed that IRP2 regulates mitochondrial function in the lung and increases iron-loading in the mitochondria leading to experimental COPD in mice (Cloonan *et al.* 2016). IRP2 deficiency or treatment with a mitochondrial iron chelator protected these mice from the development of cigarette smoke-induced experimental COPD.

Of interest in the context of this thesis is the observation that a polymorphism in the *EGLN2* gene encoding the PHD1 enzyme, which is crucially involved in the hypoxia signalling cascade, is also associated with an increased risk of COPD (Ding *et al.* 2015a, Sakornsakolpat *et al.* 2018).

3.1.6 Aims and rationale

In light of the global impact of COPD and the fact that current treatments can be cumbersome and impact patients' daily life significantly (e.g. long-term oxygen therapy), new therapeutic options are needed. This randomised, controlled clinical trial was undertaken with the aims of establishing the feasibility and safety of iron administration in patients with COPD, understanding the effects on physiological and functional outcomes, and potentially providing a scientific foundation for further multi-centre research in the long-term setting. Given the high prevalence of iron deficiency in COPD as well as the importance of iron in the pathophysiology underlying the disease, it was hypothesised that the administration of intravenous iron to this population of patients might be beneficial and improve oxygenation and functional outcomes. Until now, this novel therapeutic approach has not been studied systematically in COPD. It is encouraging that intravenously administered iron significantly improved symptoms, quality of life, and exercise capacity in chronic heart failure (Okonko *et al.* 2008, Anker *et al.* 2009, Ponikowski *et al.* 2015), a condition sharing pathophysiological similarities, such as limited oxygen uptake and tissue hypoxia, with COPD. Patients with COPD might therefore derive similarly beneficial effects from intravenous iron.

3.2 Methods

3.2.1 Trial overview

This two-arm, parallel, randomised, controlled, double-blind, phase IV trial investigated the effects of intravenous iron administration in patients with COPD over an eight-week follow-up period. This study was reviewed by and received approval from the NHS South Central Berkshire Research Ethics Committee (Reference: 12/SC/0539), the Medicines & Healthcare Products Regulatory Agency (MHRA) and the Oxford University Hospitals R&D Department. The study was sponsored by the University of Oxford and funded by the NIHR Oxford Biomedical Research Centre. The trial was prospectively registered on the ISRCTN clinical trial registry (ISRCTN 09143837). All research was conducted in accordance with the approved study protocol, the Declaration of Helsinki, and Good Clinical Practice guidelines.

3.2.2 Participants

Participant recruitment for the study was initially limited to a previously established cohort of COPD patients at the Oxford Centre for Respiratory Medicine, who had participated in previous research studies and had given consent to be contacted for further research. Throughout the study period, recruitment efforts were expanded to include respiratory outpatient clinics and hospital wards, the newly established Respiratory Research Registry held at the Oxford Centre for Respiratory Medicine, primary care practices acting as Patient Identification Centres (PIC), as well as public outreach initiatives involving posters and advertisements.

Interested participants had to meet all of the following inclusion criteria before being enrolled in the study:

- Confirmed diagnosis of COPD with at least moderate airflow obstruction (GOLD stages II–IV, i.e. predicted FEV_1 < 80% and FEV_1/FVC < 70%)
- Significant smoking history (i.e. > 15 pack years, where a pack year was defined as the product of the average number of cigarettes smoked per day and the number of years smoked for divided by 20) or other definite cause of COPD
- Stable COPD for at least four weeks at study initiation, indicated by absence of exacerbations and no changes in prescribed respiratory medication
- Ability and willingness to comply with all study requirements, as judged by the investigator
- Ability and willingness to give informed consent for participation in the study
- Male or female adult aged 18 years or older

Participants not satisfying these criteria or who met any of the following were excluded:

- Females who were pregnant, lactating, planning pregnancy during the course of the study, or of child-bearing potential, unless using effective contraception for the duration of the study
- Patients taking iron supplements (in the six weeks preceding study initiation) or who have had a blood transfusion (in the six months preceding study initiation)
- Iron overload, defined as ferritin > 300 µg/L
- Hypersensitivity to previous iron infusion
- Evidence of bacteraemia or respiratory infection (participants with ongoing infections were given the opportunity to delay their participation to a point in time when they had sufficiently recovered)
- Significant renal or liver disease (as judged by the investigator)

3.2.3 Interventions

Study participants were randomly assigned to receive a single-dose infusion of intravenous iron or placebo in a 1:1 ratio. Details on the randomisation process are outlined later in this chapter. Participants allocated to the iron group were administered ferric carboxymaltose (Ferinject®) at a dose of 15 mg/kg bodyweight diluted in 250 mL of physiological saline (0.9% sodium chloride) solution, up to a maximum dose of 1 g. Individuals allocated to the placebo group received 250 mL of physiological saline solution. Infusions were administrated over a period of 15 minutes using an automated infusion pump. Investigators and participants were blinded to the treatment allocation as detailed in Chapter 3.2.6.

3.2.4 Study visits and assessments

This study comprised a total of four visits. All visits took place at the Oxford Respiratory Trials Unit at the Churchill Hospital in Oxford, United Kingdom. An overview of the study protocol is given in Figure 3.3.

3.2.4.1 *Screening visit*

Interested participants were invited to attend a screening visit at which informed written consent was obtained. As part of the eligibility assessment, each participant's medical history and medications were reviewed. In addition, a venous blood sample was obtained (see Chapter 3.2.9 for details). A full lung function test, including spirometry, plethysmography, and diffusion capacity measurement, was performed, if no spirometry results were available within one year prior to the visit. Post-bronchodilator spirometry – usually a requirement for the diagnosis of COPD – was not performed routinely, as most patients' diagnosis of COPD was previously confirmed by a respiratory physician.

Only if no documented prior consultation of a respiratory specialist was available, spirometry after application of an inhaled bronchodilator was performed to secure the diagnosis of COPD by a respiratory medicine consultant. All pulmonary function tests were performed by qualified respiratory physiologists at the Churchill Hospital.

Participants were instructed on how to measure peak expiratory flow rate and peripheral resting oxygen saturation at home and were asked to record these parameters as well as any changes in respiratory symptoms or medications on daily diary cards for the duration of the study. These records were used to determine the occurrence of exacerbations. For the purposes of this study, an exacerbation was defined as the presence of two or more new respiratory symptoms, one of which had to be a major symptom (i.e. breathlessness, increased sputum amount or sputum colour), for two or more consecutive days (Seemungal *et al.* 1998, Donaldson *et al.* 2002).

Participants meeting the eligibility criteria outlined in Chapter 3.2.2 were enrolled in the study. A minimum waiting period of four weeks between the screening and baseline visits was enforced to ensure documented absence of respiratory infections. If eligible participants exacerbated during this period, the baseline visit was delayed until a four-week exacerbation-free period was achieved.

3.2.4.2 *Baseline visit*

At the baseline visit, diary cards were reviewed to confirm the absence of exacerbations in the four weeks prior to the visit. Any changes in a participant's medical history since the screening visit were reviewed and documented. After obtaining anthropometric measurements, including height, weight, and body mass index, the participant was asked to lie down to ensure a well-rested state for subsequent measurements. Symptoms, burden of disease, and quality of life were assessed based on a variety of

questionnaires and scoring scales completed by the participant. These validated measures included the COPD Assessment Test™ (CAT), St George's Respiratory Questionnaire (SGRQ), Dyspnoea-12, Fatigue Severity Score, and Hospital Anxiety and Depression Scores (HADS) (see Chapter 2.4.2). Participants reporting restless leg symptoms also completed the restless leg syndrome rating questionnaire (Walters *et al.* 2003). In addition, participants reported the severity of their dyspnoea in the preceding days on the modified MRC dyspnoea scale. Breathlessness at the moment of the visit was graded by using a seven-point Likert scale (although not a validated tool to assess dyspnoea in COPD, this scale was used to confirm and document that participants were well-rested and not excessively breathless before further assessments). Symptoms in the week prior to each visit were further quantified by using visual analogue scales for dyspnoea, cough, sputum production, and sputum purulence (Brightling *et al.* 2001).

After a minimum resting period of 20 minutes in a supine position, participants' vital signs were obtained. In addition to blood pressure and respiratory rate, peripheral oxygen saturation and heart rate were measured every 30 seconds and averaged over a two-minute period. To minimise measurement inaccuracies, a standardised approach was used involving the same pulse oximeter (Pulsox-2, Konica Minolta, Osaka, Japan), measurement location and conditions at each visit. Prior to attending the baseline visit, participants also recorded their peripheral oxygen saturation continuously for one night by wearing a pulse oximeter with built-in memory (Pulsox-3i or Pulsox-300i, Konica Minolta) during the night before the visit. The overnight recording was downloaded on the day of the visit using Visi-Download software (Stowood Scientific Instruments Ltd, Oxford, United Kingdom) and used to analyse oxygen saturation and heart rate patterns.

A capillary blood sample was obtained from the earlobe for blood gas analysis. This was analysed instantly using a Radiometer ABL700 series or ABL90 FLEX blood gas

analyser (Radiometer Ltd, Crawley, United Kingdom). If a capillary blood sample could not be obtained, the study protocol allowed for an arterial sample to be drawn from the radial artery. Additionally, a venous blood sample was obtained from an antecubital vein or a vein on the dorsal aspect of the hand using standard phlebotomy techniques. Venous blood sample processing and analysis are described in detail in Chapter 3.2.9.

Transthoracic echocardiography was performed to attempt non-invasive measurement of pulmonary arterial pressures using a GE Vivid q ultrasound machine with a M4S-RS sector probe (GE Healthcare, Little Chalfont, United Kingdom). While this approach is well established in healthy individuals (see Chapter 2.3), it is complicated in COPD patients by a variety of pathological factors such as tachycardia or hyperinflation. In addition to obtaining measurements of pulmonary arterial pressure, a rationale of the present study was to obtain a better estimate of the proportion of patients with COPD in whom this technique can be employed successfully.

A standard 12-lead electrocardiogram was recorded for safety reasons. Bedside spirometry was performed in every participant using a Carefusion MicroLab Mk8 portable spirometer following standardised procedures (Miller *et al.* 2005). Participants were asked to sit comfortably and perform a minimum of three normal exhalations from maximum inhalation to determine vital capacity followed by a minimum of three forced expirations from maximum inspiration to measure forced volumes and capacities.

Lastly, participants completed a 6-minute walk test (6MWT) following published guidelines (ATS Committee on Proficiency Standards for Clinical Pulmonary Function Laboratories 2002). From a resting state, a participant's walking distance covered during a period of six minutes was recorded. As outlined in more detail in Chapter 2.4.1, walking pace was self-controlled by the participant and stops throughout the test were allowed in the event of breathlessness, desaturations, chest tightness or similar

symptoms. Heart rate, oxygen saturation and severity of dyspnoea were recorded before and after the test as well as at each stop during the test. Participants were allowed to use walking aids they normally use in their daily life, but participants on ambulatory oxygen treatment were asked to perform the test without the additional oxygen supply.

Some of the first enrolled participants were asked to provide a spontaneous sputum sample at each study visit, if possible. Due to the inconsistent yield of samples and the associated costs of processing, this was discontinued at a later stage.

Once all assessments were completed, venous cannulation was performed in preparation for the study infusion. A 20G or 22G cannula was inserted into a vein on the dorsal aspect of the hand, the forearm or the antecubital fossa. Next, the participant met with a non-blinded member of the research team who carried out the randomisation, and subsequently prepared and administered an infusion of the assigned study treatment. Pre- and post-infusion vital signs were recorded and participants remained under observation for 30 minutes after completion of the infusion to ensure no immediate adverse reactions occurred.

3.2.4.3 *Follow-up visits*

Participants were invited to attend two follow-up visits, the first one 7 ± 2 days after the baseline visit (week 1 visit), the second one after 56 ± 5 days (week 8 visit). A range of dates was permitted to allow some flexibility with scheduling. At each visit, the assessments performed at the baseline visit were repeated. If the attempt to measure the pulmonary arterial pressure by means of echocardiography was not successful at the baseline visit, no further attempts were made at the follow-up visits. Participants did not receive any infusion on either of the two follow-up visits. The completion of the eighth week follow-up visit marked the end of a participant's study participation.

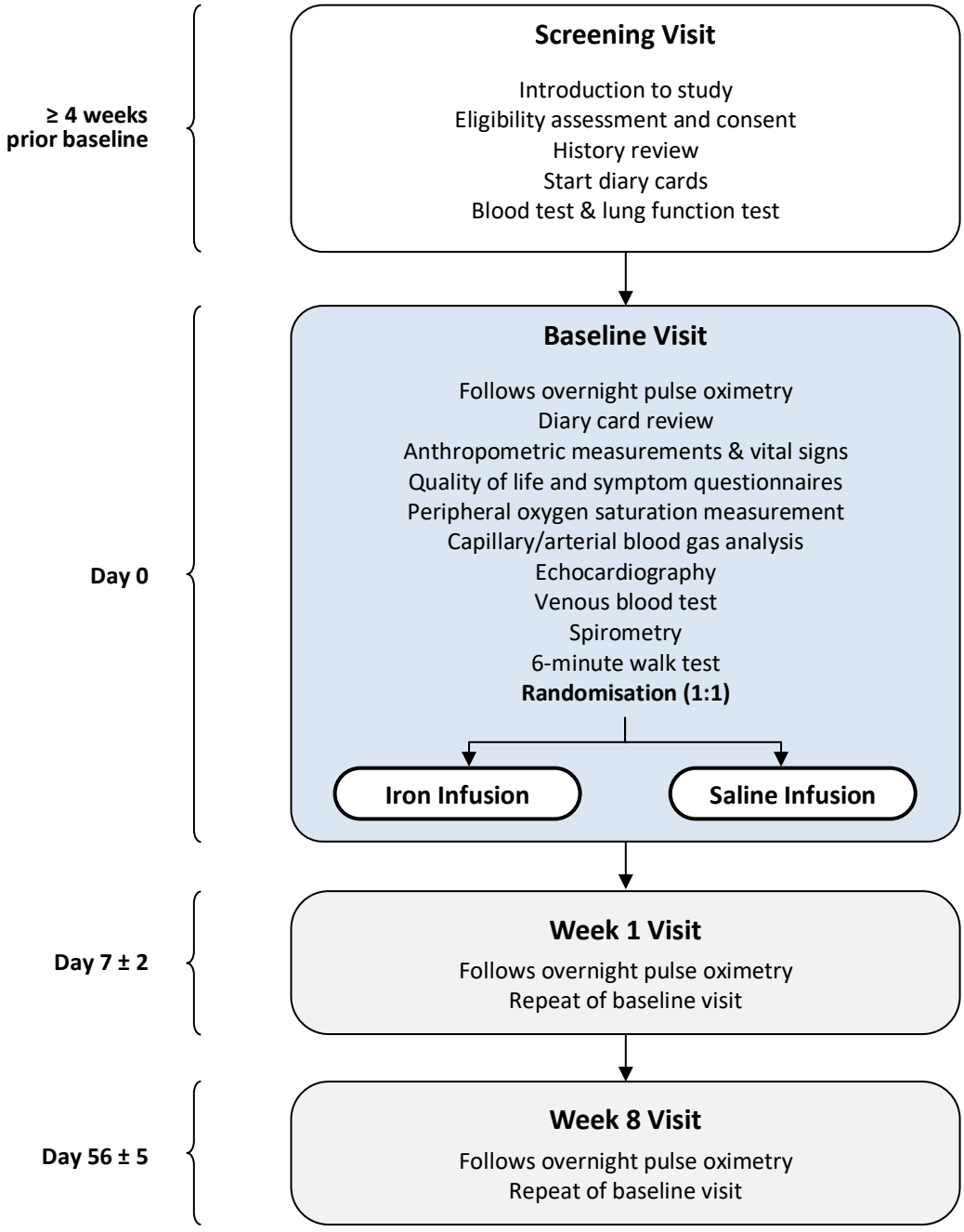


Figure 3.3 Overview of study protocol. A total of 48 participants were enrolled in the study and randomly allocated to an infusion of either iron or placebo at a ratio of 1:1 after an exacerbation-free period of at least four weeks. Treatment allocation was stratified based on oxygenation and iron status. Follow-up visits occurred one and eight weeks after randomisation and infusion.

3.2.5 Outcomes

The primary objective of this study was to determine whether intravenous iron improved oxygenation in patients with COPD one week following infusion. The primary outcome measure was therefore defined as the change in peripheral oxygen saturation one week after randomisation in patients receiving an iron infusion compared to placebo.

Secondary objectives of the study encompassed effects of intravenous iron on oxygenation, exercise capacity, pulmonary arterial pressure, blood parameters, exacerbations and quality of life. Secondary outcome measures included changes in the following parameters from baseline to weeks 1 and 8 in the iron-treated group compared to the placebo group:

- Peripheral oxygen saturation at rest at eight weeks using pulse oximetry
- Daily self-monitored peripheral oxygen saturation at home
- Overnight peripheral oxygen saturation with continuous pulse oximetry
- Oxygen saturation at rest in capillary or arterial blood gas analysis
- Distance walked during the 6-minute walk test (6MWT)
- Peripheral oxygen saturation at the beginning and end of exercise (6MWT)
- Quality of life and symptom indices
- Dyspnoea indices
- Echocardiography measures (pulmonary arterial systolic pressure, if measurable)
- Laboratory tests (haematinics, hepcidin, erythropoietin, haemoglobin, and inflammatory markers such as C-reactive protein and interleukin-6)
- Forced expiratory volume in one second (FEV_1)
- Time (in days) to first exacerbation (assessed from daily diary cards)

3.2.6 Randomisation and blinding

Participants were randomised on the day of the baseline visit by a non-blinded member of the research team not involved in any of the trial procedures and assessments. Randomisation was performed using a validated software randomisation system (Sealed Envelope) with an allocation ratio of 1:1 to either intravenous iron or placebo. This system used the method of randomisation by minimisation with a 30% chance of simple random allocation. Participants were stratified based on their oxygenation (resting baseline SpO_2 > 94% versus ≤ 94%) and iron status (transferrin saturation at screening < 20% versus ≥ 20%).

The double-blinded design of this study meant that both study participants and research staff involved in any of the study assessments were unaware of the treatment allocation. In order to minimise the risk of bias, treatment allocation as well as preparation and administration of the study drugs were carried out by non-blinded research staff not involved in any other study-related procedures. Due to the obvious colour differences of the infusions – iron has a brown colour whereas saline is colourless – infusion equipment, giving sets and injection sites were covered with opaque drapes to ensure adequate concealment of the treatment allocation from the participant. Additionally, participants were asked to wear a sleeping mask if they were comfortable with that option. Blinded members of the research team were asked to leave the room during preparation and administration of the infusion. Since post-infusion blood results clearly revealed the allocated treatment, review and data entry of those parameters was also performed by non-blinded members of the research team.

3.2.7 Sample size calculation

Previous research indicated that iron-deficient patients with COPD were significantly more hypoxaemic than iron-replete patients with COPD (Nickol *et al.* 2015). The median peripheral oxygen saturation was 2.5% lower in iron-deficient COPD patients. Based on these findings, the present study was designed to detect a change in peripheral oxygen saturation of 2% following the administration of intravenous iron compared to placebo one week after the infusion using an unpaired two-tailed Student t-test to compare the change in oxygen saturation between groups. Based on a standard deviation of the within-subject oxygen saturation change in COPD patients of 2.5%, a total of 24 participants was needed in each treatment group to detect a 2% increase in the primary outcome measure in the iron-treated group with a statistical power of 80% and a significance level of 5%. This resulted in a total sample size of 48 participants.

3.2.8 Safety reporting

Adverse events were recorded for a period of up to one week after administering the infusion. The ICH Guideline for Good Clinical Practice (GCP) (International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use 1996) defines an adverse event as:

> *[a]ny untoward medical occurrence in a patient or clinical investigation subject administered a pharmaceutical product and which does not necessarily have a causal relationship with this treatment.*

This includes any new diseases, symptoms, signs, or abnormal laboratory findings with a temporal link to the use of the study medication, whether related or unrelated to the study drug. Any adverse event considered to be causally related to the administration of the study drug was considered an adverse reaction. Adverse events were coded using to

the Common Terminology Criteria for Adverse Events (CTCAE) (National Cancer Institute 2009).

Serious adverse events were recorded from the time of the infusion to the end of the study follow-up period. According to ICH GCP guidelines (International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use 1996) a serious adverse event is:

> *[a]ny untoward medical occurrence that at any dose results in death, is life-threatening, requires inpatient hospitali[s]ation or prolongation of existing hospitali[s]ation, results in persistent or significant disability/incapacity, or is a congenital anomaly/birth defect.*

Adverse events that were both serious and potentially related to the study treatment were considered serious adverse reactions. In the present study, a number of adverse events were expected due to the underlying condition. These included exacerbations of COPD with infective or non-infective aetiology and complications associated with intermittent or continuous steroid use. Certain adverse events, such as nausea, headaches, or hypophosphataemia, were expected after an infusion of FCM as outlined in the Summary of Product Characteristics (Vifor Pharma UK Limited 2017).

All serious adverse events were reported to the study sponsor within 24 hours of the research team becoming aware that an event had occurred. Any serious adverse event that in its nature or severity deviated from the published product characteristics was additionally reported to the relevant regulatory authorities (MHRA, REC and NHS Trust) as a suspected unexpected serious adverse reaction (SUSAR). A Development Safety Update Report (DSUR) including serious adverse events and newly available safety information was submitted to the regulatory authorities on an annual basis to meet legal requirements.

3.2.9 Venous blood sample analysis

At each study visit, venous blood samples were obtained from the participants. During the baseline visit, the blood sample was taken before administration of the infusion. Blood samples were immediately sent to the haematology and clinical biochemistry laboratories at the Churchill or John Radcliffe hospital sites of the Oxford University Hospitals NHS Foundation Trust. Analysis included haematological parameters such as a full blood count, serum biochemistry (urea and electrolytes, liver function parameters, glucose, phosphate, C-reactive protein) as well as iron parameters (serum iron, ferritin, transferrin, and transferrin saturation). Phosphate levels were not routinely measured during the early study phase. After identifying reports of occasionally profound FCM-induced hypophosphataemia as part of the annual safety report in January 2016 (Vandemergel and Vandergheynst 2014, Hardy and Vandemergel 2015), routine phosphate measurements were implemented. In cases of a suspected hereditary cause of COPD due to alpha-1 antitrypsin deficiency, a sample was also sent to the clinical immunology laboratory of the Oxford University Hospitals NHS Foundation Trust for qualitative and quantitative analysis of alpha-1 antitrypsin, if no previous analyses were available.

At the baseline and the two follow-up visits, additional plasma and serum samples were obtained and stored for further analysis. For that purpose, blood was collected in EDTA-coated and serum separator tubes, respectively. Serum separator tubes were allowed to sit at room temperature for 30 minutes in order for clot formation to occur. Samples were then centrifuged for 15 minutes at 18°C and 3,000 rotations per minute. Serum and plasma supernatants were transferred to microcentrifuge tubes and duplicate aliquots stored at −80°C for further analysis. Upon study completion, stored serum and

plasma samples were analysed collectively by enzyme-linked immunosorbent assay (Chapter 2.5). Duplicate plasma samples were used to measure hepcidin (Hepcidin 25 bioactive HS ELISA, EIA-5782, DRG, Marburg, Germany), erythropoietin (Quantikine® IVD® human EPO ELISA, DEP00, R&D Systems, Abingdon, United Kingdom), and interleukin-6 (Quantikine® human IL-6 ELISA, D6050, R&D Systems) according to the manufacturer's instructions. Soluble transferrin receptor (Quantikine® IVD® human sTfR ELISA, DTFR1, R&D Systems) was measured in duplicate from stored serum samples.

All stored serum samples were retrospectively analysed for phosphate levels at the clinical biochemistry laboratory at the John Radcliffe Hospital of the Oxford University Hospitals NHS Foundation Trust.

3.2.10 Statistical analysis

Study analysis was by intention to treat, i.e. every randomised participant was included in the final analysis. Statistical analysis was conducted using IBM SPSS Statistics (version 24.0 for Macintosh, IBM Corporation, Armonk, NY, USA). Comparisons of means between two groups at a single time point were made by unpaired two-tailed Student t-test for normally distributed continuous data or Mann-Whitney U test for non-normal data. Categorical variables were analysed by means of Pearson's Chi-square test. If categorical data were arranged in a 2x2 table with expected cell counts of less than 5 in any cell, Fisher's Exact test was applied instead.

Longitudinal analysis of outcomes over time was performed by linear mixed effects modelling. Each model included time (visit week), allocated group, and status post iron infusion as fixed effects. Time was treated as continuous covariate to account for the unequal lengths of time between the visits. Allocated group and post-infusion status

were entered into the model as categorical factors. If exploratory data analysis suggested a possible curvilinear change over time, a quadratic time variable was introduced as fixed effect and kept in the final model if a significant main effect for that term was observed. All models included intercept as random effect. Within-subject correlation resulting from repeated measures was included in the model. Participants with partially missing data at single visits were included in the analysis, as linear mixed effects models are robust to missing data. Partially missing data points were, however, excluded from descriptive summaries in tables and graphs to avoid misleading interpretations. A p value of < 0.05 was considered statistically significant.

3.3 Results

3.3.1 Recruitment and participant flow

Participants for this study were recruited from COPD and respiratory research registries, outpatient clinics, primary care practices, as well as through direct contact with the research team in response to posters and advertisements. Between March 2015 and July 2017, a total of 244 potential participants were identified or contacted the research team, of whom 71 attended a screening visit. Out of 55 eligible participants, 48 were interested in proceeding with the study. All of the enrolled participants completed the follow-up period. The trial ended in July 2017, when the last enrolled participant completed the final follow-up visit. All participants were included in the final analysis. Detailed recruitment numbers are depicted in Figure 3.4.

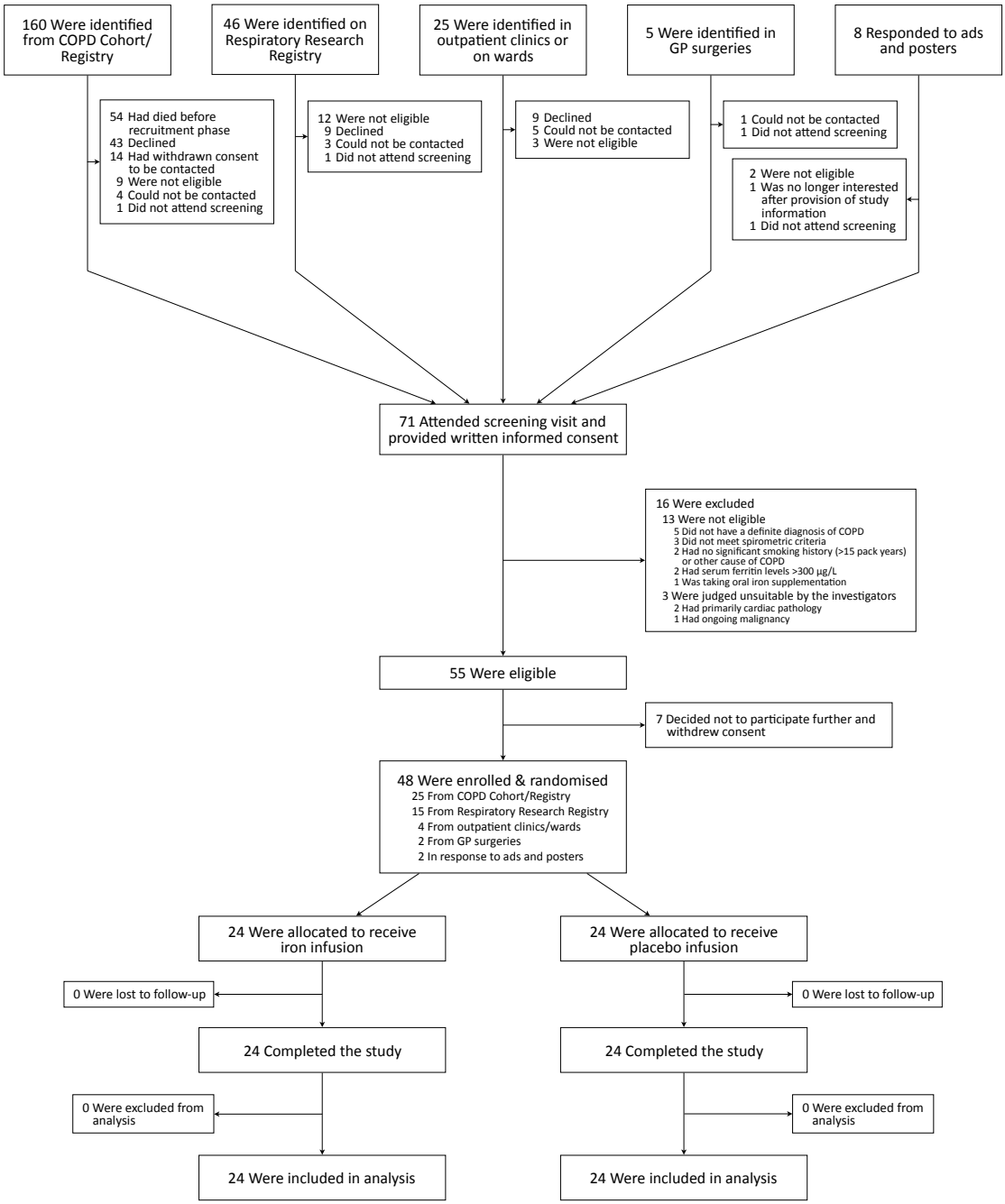


Figure 3.4 Participant flow diagram. Participants were recruited from a variety of sources including research registries, primary care practices, and public outreach initiatives. All participants enrolled in the study were randomised and completed the trial. No participants were lost to follow-up or excluded from the analysis.

3.3.2 Participant characteristics

All study participants had a diagnosis of COPD, which was confirmed by a respiratory physician. Groups were well-matched at baseline with regard to demographics, tobacco exposure, and disease onset. Participant demographics and baseline characteristics are shown in Table 3.2. In line with the chronic and slowly progressive nature of COPD, the predominantly male study population was, on average, of an advanced age with multiple decades of tobacco exposure. All but one participant fulfilled the eligibility criteria of a significant smoking history, i.e. a tobacco exposure of more than 15 pack years. One participant in the placebo group was a non-smoker but had a confirmed alpha-1 antitrypsin deficiency (alpha-1 antitrypsin level: 102 mg/dL (normal range: 107–209 mg/dL), PiMZ genotype) as underlying cause of COPD. Comorbidities were primarily cardiovascular and included hypertension, atherosclerosis, myocardial infarctions, arrhythmias, cerebrovascular events, and aneurysms. Other commonly observed comorbidities included gastro-oesophageal reflux, depression, peripheral oedemas, and less frequently, diabetes and osteoporosis. The majority of participants were on a COPD treatment regime including a short-acting beta-adrenoceptor agonist, an anticholinergic drug, and a combination inhaler containing a long-acting beta-adrenoceptor agonist and an inhaled corticosteroid. Participants had no changes to their respiratory medications in the four weeks preceding the baseline visit. Additionally, participants continued taking their prescribed medications as appropriate for their comorbidities.

Participants showed varying degrees of airflow obstruction, with most (81.3%) having moderate to severe obstruction (GOLD stages II and III), as confirmed by spirometry at the baseline visit (Table 3.3). Groups were well-matched regarding the severity of airflow obstruction. A full list of pulmonary function parameters obtained within one year prior to the screening visit is shown in Table 3.4. Overall, the two groups were

well-matched with similar baseline lung function results for most parameters. The iron group showed slightly worse baseline results, as indicated by significantly lower vital capacity, forced vital capacity, alveolar volume, and a higher RV/TLC ratio.

Table 3.2 Baseline participant demographics & characteristics

Characteristic, unit	**Iron group** (n = 24)	**Placebo group** (n = 24)	***p* value**
Age, years	69.2 ± 8.4	68.0 ± 7.0	0.603
Sex, n (%)			
Male	15 (62.5)	19 (79.2)	0.204
Female	9 (37.5)	5 (20.8)	
Weight, kg	71.0 ± 17.4	73.8 ± 11.2	0.497
Body mass index (BMI), kg/m^2	25.7 ± 6.1	25.4 ± 4.1	0.827
Blood pressure, mmHg			
Systolic	138 ± 20	140 ± 15	0.734
Diastolic	85 ± 11	86 ± 12	0.911
Smoking status, n (%)			
Former	18 (75.0)	16 (66.7)	0.550
Current	6 (25.0)	7 (29.2)	
Never	0	1 (4.2)	
Pack years	43 (31–67)	39 (28–67) [a]	0.932
Age at COPD onset, years	57.2 ± 11.3 [a]	58.2 ± 5.3 [b]	0.715
Age at smoking initiation, years	14.8 ± 2.9	14.6 ± 3.0 [a]	0.758
Age at smoking cessation, years	59.0 ± 6.5 [c]	57.5 ± 8.7 [d]	0.571
Exacerbations in previous year	1 (0–3) [a]	2 (1–3) [b]	0.392

Data are reported as mean ± SD, if normally distributed, or median (IQR), if not normally distributed. Statistical analysis was performed by unpaired Student t-test (normally distributed data), Mann-Whitney U test (non-normally distributed data), or Pearson's Chi-square test (categorical data). Missing data: [a] n = 23, [b] n = 22, [c] n = 18, [d] n = 16.

Table 3.3 Severity of airflow obstruction at baseline

FEV_1/FVC	FEV_1 predicted	GOLD Stage	Total (n = 48)	Iron group (n = 24)	Placebo group (n = 24)	*p* value
< 0.7	≥ 80%[1]	Mild (I)[1]	—	—	—	0.921
	50–79%	Moderate (II)	19 (39.6)	9 (37.5)	10 (41.7)	
	30–49%	Severe (III)	20 (41.7)	10 (41.7)	10 (41.7)	
	< 30%	Very severe (IV)	9 (18.8)	5 (20.8)	4 (16.7)	

[1] Stage I was not eligible for study participation.

All data are reported as n (%). Statistical analysis was performed by Pearson's Chi-square test.

Blood parameters measured at study enrolment were well-matched between groups for most parameters (Table 3.5). The placebo group exhibited slightly higher urea levels, which were still within normal limits. No significant differences in the baseline iron status were observed between the two groups. Iron deficiency was less common in this study population compared to a previously studied cohort of COPD patients (Nickol *et al.* 2015). Only 10.4% of participants fulfilled the criteria of iron deficiency (Table 3.6), as previously defined, compared to 17.7% in the cohort studied by Nickol *et al.* (2015).

Alpha-1 antitrypsin levels were not routinely measured, but available from previous research projects in 25 participants. Apart from the aforementioned patient, who was enrolled on the basis of the pre-existing alpha-1 antitrypsin deficiency, one other participant was deficient with levels of alpha-1 antitrypsin of 78.2 mg/dL (genotype not documented).

The median (IQR) dose of ferric carboxymaltose administered in the iron group at the baseline visit was 1,000 (786–1,000) mg.

Table 3.4 Pulmonary function test results

Parameter, unit		**Iron group** (n = 24)	**Placebo group** (n = 24)	***p* value**
Peak expiratory flow rate (PEFR)	*Measured, L min^{-1}*	246.6 ± 92.5	277.6 ± 74.1	0.205
	% predicted	60.3 ± 19.0	63.9 ± 18.8	0.518
Forced expiratory volume in one second (FEV_1)	*Measured, L*	1.16 ± 0.50	1.35 ± 0.38	0.162
	% predicted	48.0 ± 17.6	49.8 ± 16.9	0.714
Forced vital capacity (FVC)	*Measured, L*	2.56 ± 0.79	3.37 ± 0.84	**0.001**
	% predicted	83.0 ± 21.3	95.2 ± 19.9	**0.044**
Vital capacity (VC)	*Measured, L*	2.74 ± 0.84	3.48 ± 0.83 [a]	**0.004**
	% predicted	88.1 ± 22.5	96.8 ± 17.4 [a]	0.145
FEV_1/VC ratio	*Measured, %*	43.2 ± 9.9	39.1 ± 10.8 [a]	0.177
FEV_1/FVC ratio	*Measured, %*	44.8 ± 9.0	40.4 ± 10.2	0.116
Functional residual capacity (FRC)	*Measured, L*	5.11 ± 1.54 [b]	5.01 ± 1.50 [c]	0.838
	% predicted	156.5 (133.2–174.7) [b]	147.8 (123.7–175.2) [c]	0.616
Residual volume (RV)	*Measured, L*	4.59 ± 1.51 [b]	3.93 ± 1.27 [c]	0.169
	% predicted	191.0 (163.2–219.0) [b]	168.4 (120.0–198.5) [c]	0.107

Table 3.4 Pulmonary function test results (continued)

Parameter, unit		**Iron group** (n = 24)	**Placebo group** (n = 24)	***p* value**
Total lung capacity (TLC)	*Measured, L*	7.43 ± 1.50 [b]	7.59 ± 1.88 [c]	0.773
	% predicted	123.5 (118.7–133.2) [b]	124.8 (107.4–133.7) [c]	0.639
RV/TLC ratio	*Measured, %*	61.1 ± 10.8 [b]	51.1 ± 6.9 [c]	**0.002**
Airway resistance (R_{aw})	*Measured, kPa L^{-1} s^{-1}*	0.42 (0.32–0.56) [b]	0.43 (0.32–0.56) [c]	0.802
Specific airway conductance (sG_{aw})	*Measured, kPa^{-1} s^{-1}*	0.50 (0.27–0.61) [b]	0.45 (0.39–0.55) [d]	0.545
	% predicted	52.0 (24.4–82.3) [d]	50.5 (39.7–56.7) [e]	0.886
Alveolar volume (VA)	*Measured, L*	4.24 ± 1.18 [a]	5.06 ± 1.22 [a]	**0.025**
	% predicted	74.8 ± 14.0 [a]	81.8 ± 13.0 [a]	0.086
Transfer factor for carbon monoxide (T_{LCO})	*Measured, mmol min^{-1} kPa^{-1}*	3.80 ± 1.13 [a]	3.80 ± 1.28 [a]	0.995
	% predicted	50.1 ± 14.2 [a]	46.9 ± 15.3 [a]	0.467
Transfer coefficient for carbon monoxide (K_{CO})	*Measured, mmol min^{-1} kPa^{-1} L^{-1}*	0.92 ± 0.22 [a]	0.78 ± 0.27 [a]	0.057
	% predicted	67.6 ± 18.8 [a]	57.5 ± 15.2 [a]	0.051

Data are reported as mean ± SD, if normally distributed, or median (IQR), if not normally distributed. Statistical analysis was performed by unpaired Student t-test (normally distributed data) or Mann-Whitney U test (non-normally distributed data). Missing data: [a] n = 23, [b] n = 19, [c] n = 17, [d] n = 16, [e] n = 14.

Table 3.5 Screening blood results

Parameter, unit (normal range)	**Iron group** (n = 24)	**Placebo group** (n = 24)	***p* value**
Haematology			
Haemoglobin, g/dL (12–17)	14.7 ± 1.1 [a]	14.4 ± 1.3 [a]	0.379
Mean cell volume, fL (83–105)	91.2 ± 5.2 [a]	91.9 ± 3.5 [a]	0.581
Mean cell haemoglobin, pg (27–32)	30.5 ± 2.2 [a]	30.5 ± 1.3 [a]	0.904
Haematocrit, % (30–50)	43.9 ± 3.0 [a]	43.4 ± 3.7 [a]	0.599
White cell count, × 10^9/L (4–11)	7.10 (6.33–8.33) [a]	7.86 (7.06–8.84) [a]	0.191
Neutrophil count, × 10^9/L (2–7)	4.60 (3.94–5.66) [a]	5.34 (4.17–6.67) [a]	0.292
Eosinophil count, × 10^9/L (0–0.5)	0.19 ± 0.10 [a]	0.20 ± 0.13 [a]	0.802
Platelet count, × 10^9/L (150–400)	233 (218–287) [a]	287 (244–351) [a]	0.087
Serum biochemistry			
Sodium, mmol/L (135–145)	139.0 ± 2.9	138.5 ± 2.8	0.544
Potassium, mmol/L (3.5–5.0)	4.1 ± 0.5	4.1 ± 0.4	0.921
Urea, mmol/L (3.0–9.2)	4.8 ± 1.8	6.1 ± 1.9	**0.018**
Creatinine, µmol/L (49–104)	69.0 ± 14.3	78.6 ± 18.9	0.054
Glucose, mmol/L (3.8–6.6)	5.1 (4.7–5.7) [a]	5.4 (4.9–6.0) [b]	0.199
Bilirubin, µmol/L (0–21)	8.0 (6.0–10.8)	9.0 (6.0–14.0) [a]	0.486
Alanine aminotransferase, IU/L (10–45)	16.5 (14.0–21.3)	19.0 (15.0–26.0) [a]	0.376
Alkaline Phosphatase, IU/L (30–130)	74.1 ± 13.8	75.3 ± 20.4 [a]	0.817
Albumin, g/L (32–50)	37.1 ± 2.3	36.7 ± 2.4	0.497
Calcium, mmol/L (2.2–2.6)	2.35 ± 0.09	2.37 ± 0.08	0.546
Inflammatory markers			
C-reactive protein, mg/L (0–5)	3.2 (1.1–6.0)	2.3 (1.3–6.0)	0.967
Iron parameters			
Iron, µmol/L (11–31)	14.1 (12.3–18.9)	16.0 (13.0–19.2)	0.332
Ferritin, µg/L (10–300)	87.7 (49.9–127.6)	64.0 (40.9–158.8)	0.695
Transferrin, g/L (1.8–3.6)	2.52 ± 0.32	2.56 ± 0.33	0.698
Transferrin saturation, % (16–50)	26.5 (20.3–35.8)	27.5 (20.5–35.0)	0.672

Data are reported as mean ± SD, if normally distributed, or median (IQR), if not normally distributed. Statistical analysis was performed by unpaired Student t-test (normally distributed data) or Mann-Whitney U test (non-normally distributed data). Missing data: [a] n = 23, [b] n = 22.

Table 3.6 Iron status at baseline visit

Iron status	Total (n = 48)	Iron group (n = 24)	Placebo group (n = 24)	*p* value
Iron-deficient, defined as any one of:	5 (10.4)	3 (12.5)	2 (8.3)	0.893
Ferritin < 12 µg/L	1 (2.1)	0	1 (4.2)	
TSat < 16%	2 (4.2)	2 (8.3)	0	
sTfR > 28.1 nmol/L	2 (4.2)	1 (4.2)	1 (4.2)	
Indeterminate (16% ≤ TSat < 20%)	4 (8.3)	2 (8.3)	2 (8.3)	
Iron-replete (not iron-deficient & TSat ≥ 20%)	39 (81.3)	19 (79.2)	20 (83.3)	

TSat: transferrin saturation, sTfR: soluble transferrin receptor.

All data are reported as n (%). Statistical analysis was performed by Pearson's Chi-square test (comparing iron-deficient, indeterminate and iron-replete groups).

3.3.3 Treatment effect on iron status

The efficacy of the administered iron infusion was assessed by analysing parameters of iron status and their changes between groups over time. Both treatment groups were well-matched at baseline (Table 3.7 and Figure 3.5). As expected, body iron stores increased by week 1 in response to FCM administration, with significantly raised serum iron ($p = 0.003$), ferritin, transferrin saturation, and plasma hepcidin (all $p < 0.001$). On average, serum iron increased 1.7-fold, ferritin 11.9-fold, transferrin saturation 2.1-fold, and hepcidin 4.9-fold from baseline levels. Serum transferrin and soluble transferrin receptor significantly dropped, by 18.6% and 19.6% from baseline, respectively (both $p < 0.001$). By week 8, levels of serum iron and soluble transferrin receptor were no longer significantly different between groups, whereas differences in ferritin, transferrin, transferrin saturation, and hepcidin persisted.

Table 3.7 Temporal changes in iron parameters

Parameter, unit	**Iron group** (n = 24)			**Placebo group** (n = 24)			***p* value**
	Baseline	Week 1	Week 8	Baseline	Week 1	Week 8	
Iron, µmol/L	16.5 ± 1.5	28.8 ± 1.9	17.3 ± 1.1	16.8 ± 1.2	16.5 ± 1.4	15.3 ± 1.1	0.003
Ferritin, µg/L	96.4 ± 12.8	1,149.5 ± 57.6	395.2 ± 32.9	97.7 ± 15.9	91.4 ± 14.4	95.8 ± 16.2	< 0.001
Transferrin, g/L	2.53 ± 0.07	2.06 ± 0.05	2.08 ± 0.05	2.53 ± 0.06	2.48 ± 0.07	2.49 ± 0.07	< 0.001
Transferrin saturation, %	31.3 ± 3.1	64.9 ± 5.0	38.2 ± 2.5	31.4 ± 3.2	31.3 ± 3.5	29.0 ± 2.8	< 0.001
Soluble transferrin receptor, nmol/L	16.2 ± 0.9	13.0 ± 0.7	14.3 ± 0.7	17.1 ± 1.0	16.8 ± 1.1	16.8 ± 1.2	< 0.001
Hepcidin, ng/mL	21.8 ± 2.5	106.8 ± 6.9	58.5 ± 4.6	21.8 ± 3.9	18.7 ± 3.5	26.7 ± 5.2	< 0.001

All data are reported as mean ± SEM. Statistical analysis was performed by linear mixed effects modelling; p values are reported for the fixed effect of "status post iron infusion".

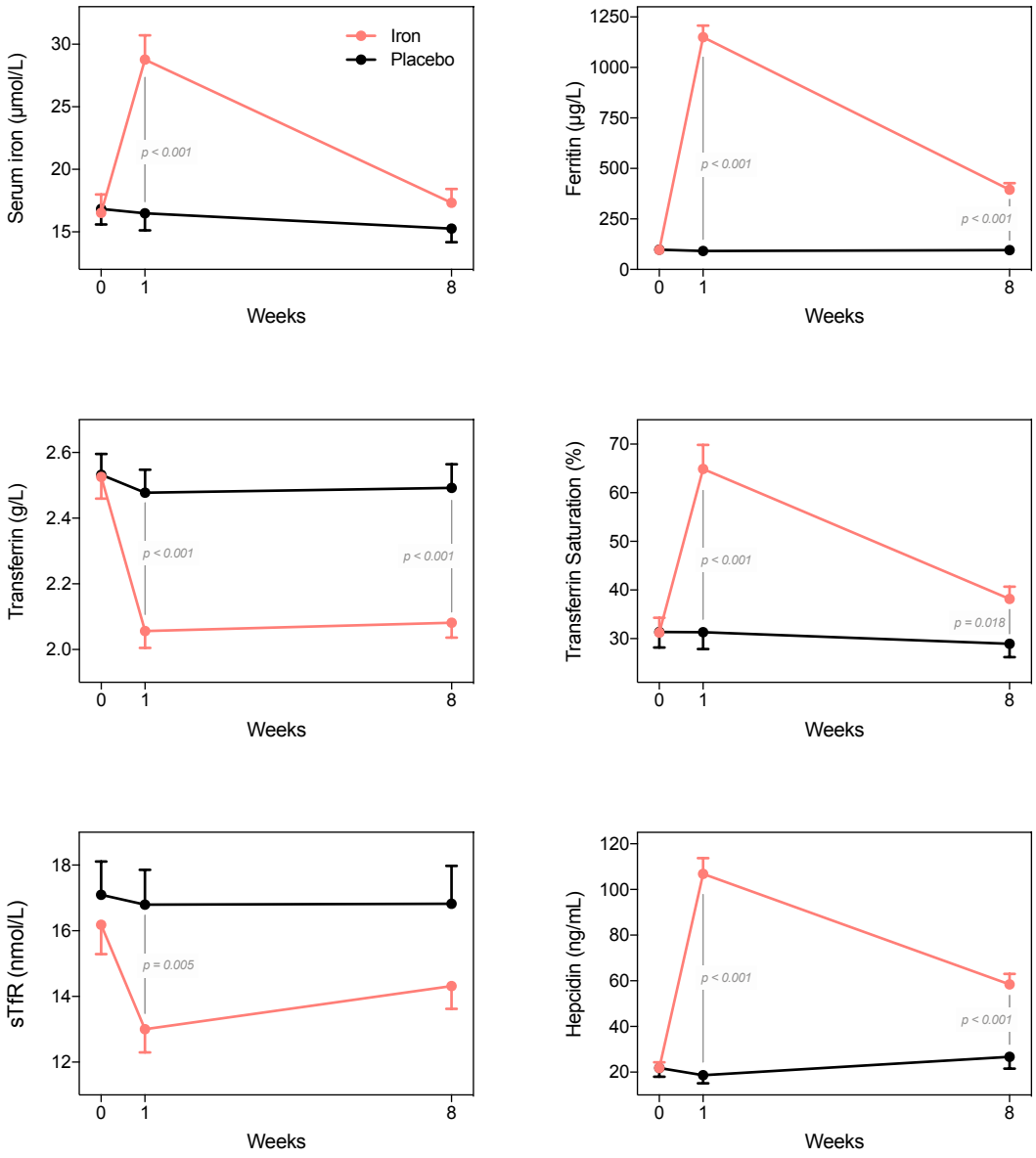


Figure 3.5 Temporal changes in iron parameters. Administration of ferric carboxymaltose resulted in significant changes of the iron indices. The concentrations of iron, ferritin, hepcidin, and transferrin saturation significantly increased, while transferrin saturation and soluble transferrin receptor dropped significantly. Statistical comparisons were performed by linear mixed effects modelling and unpaired two-tailed Student t-test; *p* values are reported for comparisons between groups at a single time point. All data are expressed as mean ± SEM.

3.3.4 Oxygen saturation measurements

The study's primary outcome – an improvement in resting peripheral oxygen saturation from baseline to week 1 following an infusion of FCM – was not reached. Oxygen saturation (mean ± SEM) changed by 0.39% ± 0.32% in the iron group and −0.39% ± 0.36% in the placebo group after one week, resulting in a mean difference of 0.77% (95% confidence interval: −0.20% to 1.74%) between the two groups (Figure 3.6 and Table 3.8). This difference did not reach statistical significance ($p = 0.116$) using an unpaired two-tailed Student t-test, the primary outcome analysis pre-specified in the study protocol. Excluding the single outlier in the placebo group – a patient with an ongoing exacerbation at the time of the first follow-up visit – diminished the change in the placebo group to −0.13% ± 0.26% and reduced the treatment effect to 0.56% (95% confidence interval: −0.33% to 1.36%, $p = 0.223$). By week 8, resting oxygen saturations returned to values similar to baseline (Table 3.8). Longitudinal analysis of resting oxygen saturation values revealed no significant differences between groups over the duration of the study.

Additional measures of oxygenation were analysed as secondary outcomes, including metrics derived from overnight pulse oximetry before each visit (Table 3.8) and participants' self-measured daily oxygen saturation values (Figure 3.7). No significant differences or longitudinal changes were observed between groups in any of these parameters. Oxygen saturation changes during exercise and oxygenation parameters from blood gas analysis are reported elsewhere in this chapter.

Collectively, these results suggest no significant effects of FCM administration on oxygenation parameters in this population of COPD patients.

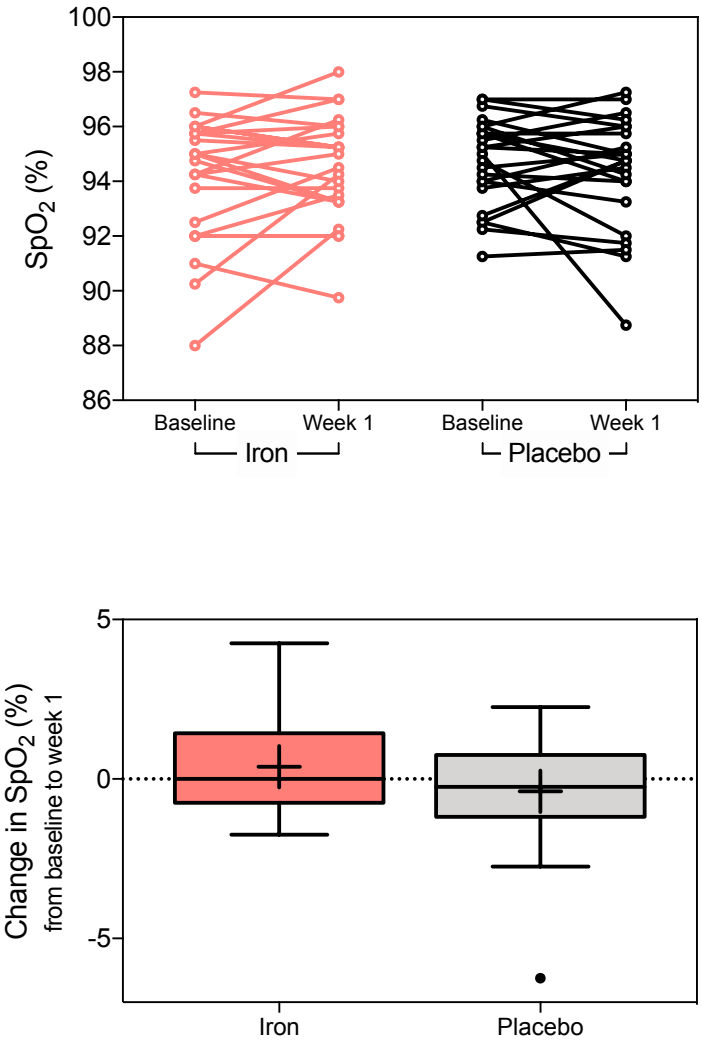


Figure 3.6 Primary study outcome: Peripheral oxygen saturation changes. Individual changes in peripheral oxygen saturation from baseline to week 1 for each participant in the iron and placebo group are shown (*top*). The box-and-whisker plot (*bottom*) shows the relative changes at week 1 by treatment allocation. Administration of ferric carboxymaltose did not significantly increase oxygen saturations (see text). The outlier (circle) in the placebo group (−6.25% change) was caused by an exacerbation at the follow-up visit. Solid lines indicate the median, boxes represent the interquartile range, whiskers extend to 1.5 times the interquartile range, and plus signs show the means. Statistical comparison was performed by unpaired two-tailed Student t-test.

Table 3.8 Overview of peripheral oxygen saturation measurements

Parameter, unit	**Iron group** (n = 24)			**Placebo group** (n = 24)			***p* value**
	Baseline	Week 1	Week 8	Baseline	Week 1	Week 8	
Resting SpO_2, %	94.1 ± 0.5	94.5 ± 0.4	93.7 ± 0.5	94.7 ± 0.3	94.4 ± 0.4	94.9 ± 0.3	0.931
Mean nocturnal SpO_2, %	91.8 ± 0.5 [a]	92.3 ± 0.4 [a]	92.0 ± 0.4 [a]	92.0 ± 0.5 [b]	92.3 ± 0.4 [b]	92.2 ± 0.5 [b]	0.317
Median nocturnal SpO_2, %	92.0 ± 0.5 [a]	92.6 ± 0.4 [a]	92.1 ± 0.5 [a]	92.3 ± 0.4 [b]	92.6 ± 0.5 [b]	92.4 ± 0.6 [b]	0.157
Minimum nocturnal SpO_2, %	80.7 ± 2.3 [a]	81.8 ± 1.3 [a]	81.1 ± 1.3 [a]	82.5 ± 1.1 [b]	81.3 ± 1.0 [b]	80.5 ± 1.7 [b]	0.431
Nocturnal SpO2 < 90%, % of time	14.7 ± 5.1 [a]	14.6 ± 3.9 [a]	13.8 ± 4.4 [a]	16.4 ± 5.5 [b]	15.5 ± 5.1 [b]	15.8 ± 5.3 [b]	0.945
Oxygen desaturation index (ODI)[1], h^{-1}	8.0 ± 1.9 [a]	7.3 ± 2.2 [a]	7.8 ± 2.7 [a]	6.4 ± 1.2 [b]	6.7 ± 1.2 [b]	7.5 ± 1.1 [b]	0.556

[1] Oxygen desaturation index (ODI) is defined as the number of SpO_2 desaturations ≥ 4% per hour measured by nocturnal pulse oximetry.

[a, b] Results are reported only for participants with a valid data point at each visit ([a] n = 23, [b] n = 22); cases with partially missing data were excluded from this table, but not from the statistical model.

All data are reported as mean ± SEM. Statistical analysis was performed by linear mixed effects modelling; p values are reported for the fixed effect of "status post iron infusion".

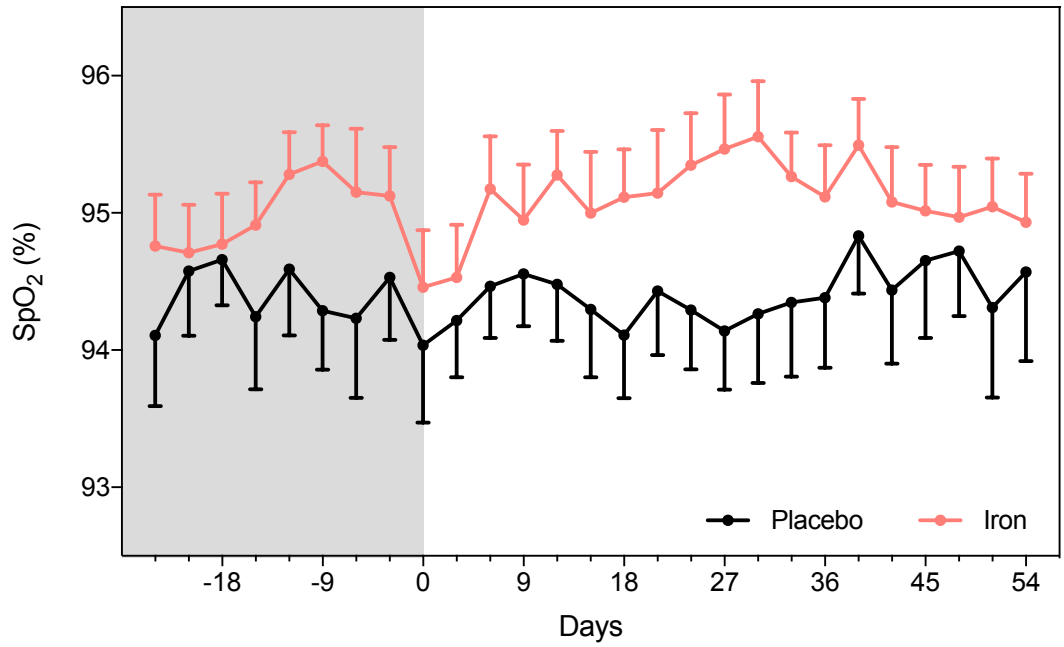


Figure 3.7 Daily self-measured oxygen saturations. Participants were asked to record their peripheral oxygen saturations on daily diary cards, starting at the screening visit. Self-measured oxygen saturations did not differ significantly between the two groups over time (p = 0.170 for the effect of iron administration). The perceived discrepancies between the groups after the baseline visit (day 0) can be attributed to normal daily variations in SpO_2 and measurement inaccuracies. Each data point (mean ± SEM) represents the average of the indicated day and the two preceding days. Statistical comparison was performed by linear mixed effects modelling; pre-baseline data (shaded area) were not included in the analysis.

3.3.5 Effects on exercise capacity

The ability of a participant to engage in physical activity was assessed by means of the 6-minute walk test. Results were well-matched at baseline with both groups covering similar distances (Figure 3.8 and Table 3.9). The iron group showed a marked increase in the distance covered during six minutes one week after the infusion. By the end of the study, the iron group had increased the average walking distance (mean ± SEM) by 24.0 ± 5.7 m compared to an increase of 10.2 ± 6.1 m in the placebo group (n = 22 in the placebo group due to missing walk tests for two participants at week 8). This change between the two groups was statistically significant (p = 0.025 for the effect of FCM). Oxygen saturation, heart rate, and reported dyspnoea were recorded before the start and at the end of each walk test. None of the pre-to-post-test changes differed significantly between groups over time (Table 3.9).

The number of participants achieving a minimum improvement in walking distance of 40 metres between baseline and follow-up visits is shown in Table 3.10. The proportion of participants improving their walking distance by at least 40 metres – a reasonable clinically important threshold (see discussion) – was significantly higher in the iron group one week after the infusion (29.2% vs. 0%, p = 0.009). By week 8, 45.8% of participants in the iron group reached or surpassed this threshold compared to 27.3% in the placebo group (p = 0.193). A few iron-treated individuals improved their walking distance by as much as 60 metres or more, while no participant in the placebo group achieved such an improvement (Figure 3.9).

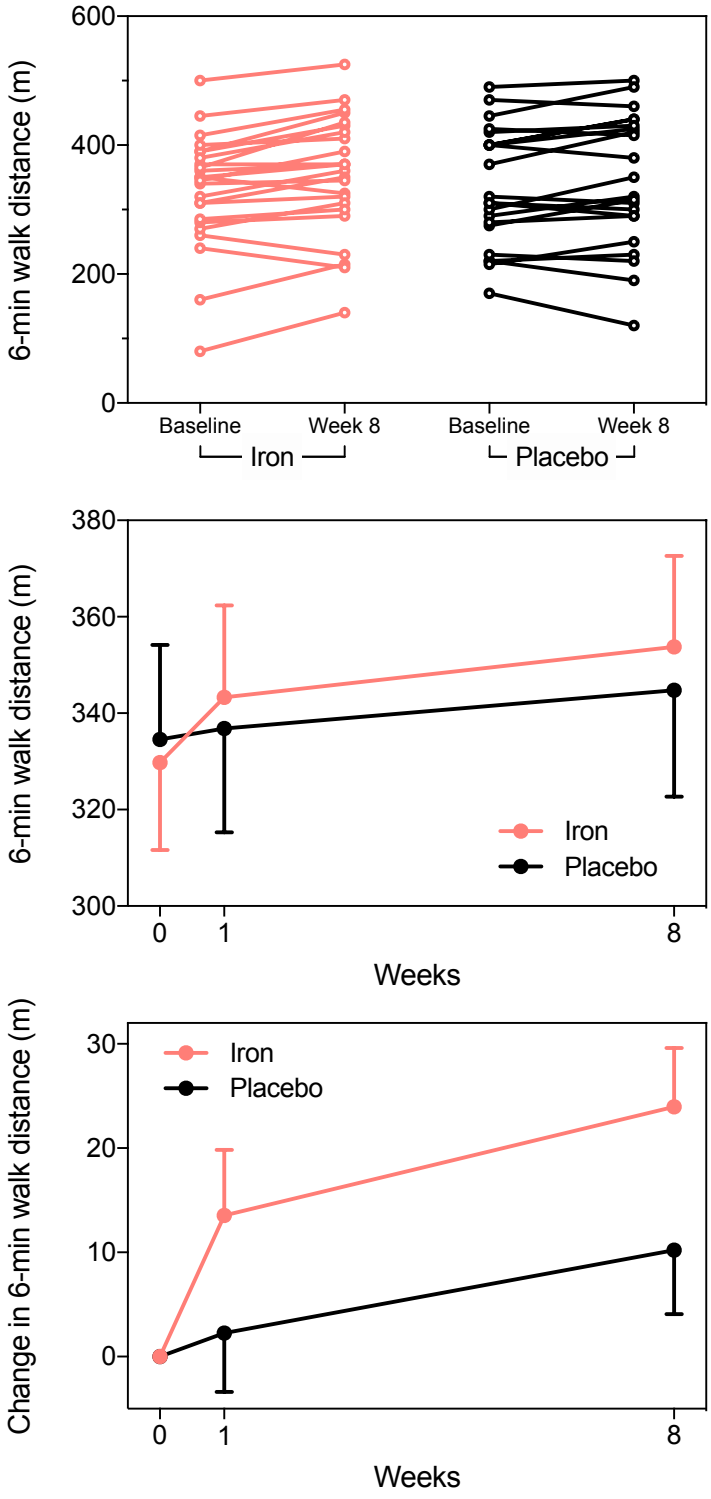


Figure 3.8 Temporal changes in 6-minute walk distance. Individual changes from baseline to week 8 (*top*), absolute mean values (*middle*), and relative changes in 6-minute walk distance (*bottom*) are shown. In the iron group, walking distance significantly improved over time compared to the placebo group. Statistical comparison was performed by linear mixed effects modelling. All data are expressed as mean ± SEM (n = 22 in the placebo group due to missing walk test results at the week 8 visit).

Table 3.9 Six-minute walk test results

Parameter, unit	**Iron group** (n = 24)			**Placebo group** (n = 24)			***p* value**
	Baseline	Week 1	Week 8	Baseline	Week 1	Week 8	
Distance, m	330 ± 18	343 ± 19	354 ± 19	335 ± 20 [a]	337 ± 22 [a]	345 ± 22 [a]	**0.025**
SpO_2 change[1], %	−4.1 ± 0.8	−4.1 ± 0.8	−4.3 ± 1.0	−5.6 ± 1.4 [a]	−5.0 ± 1.2 [a]	−5.2 ± 1.4 [a]	0.910
Heart rate change[1], min^{-1}	18.4 ± 3.4	14.0 ± 3.2	19.3 ± 3.6	17.7 ± 3.0 [a]	18.1 ± 3.5 [a]	17.5 ± 3.8 [a]	0.503
Dyspnoea score change[1]	2.3 ± 0.3	2.1 ± 0.4	2.1 ± 0.4	2.0 ± 0.2 [a]	2.0 ± 0.2 [a]	1.9 ± 0.2 [a]	0.673

[1] Changes in SpO_2, heart rate, and dyspnoea score reflect the differences from start to finish of the test. Dyspnoea was assessed using the Borg scale (range 0–10) as per guidelines (ATS Committee on Proficiency Standards for Clinical Pulmonary Function Laboratories 2002).

[a] Results are reported only for participants with a valid data point at each visit (n = 22); cases with partially missing data were excluded from this table, but not from the statistical model.

All data are reported as mean ± SEM. Statistical analysis was performed by linear mixed effects modelling; p values are reported for the fixed effect of "status post iron infusion".

Table 3.10 Proportion of participants with improvements in 6MWD of ≥ 40 m

Change in 6MWD of ≥ 40 m	**Iron group** (n = 24)	**Placebo group** (n = 24)	***p* value**
Baseline to Week 1	7 (29.2)	0	**0.009**
Baseline to Week 8	11 (45.8)	6 (27.3) [a]	0.193

All data are reported as n (%). Statistical analysis was performed by Pearson's Chi-square test or Fisher's exact test. Missing data: [a] n = 22.

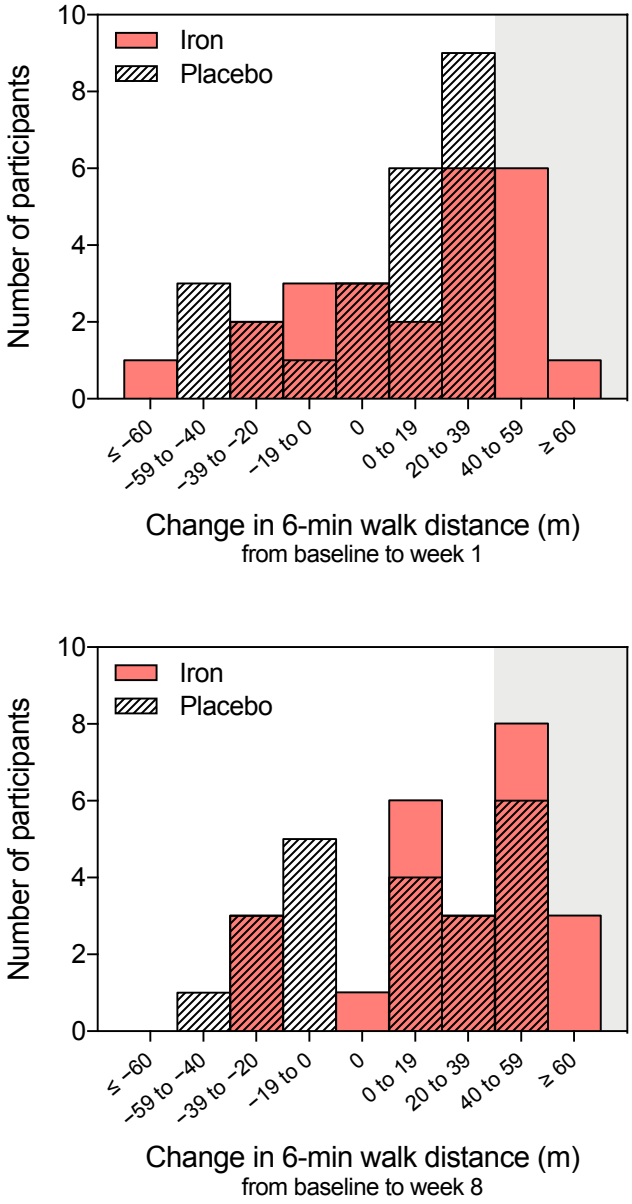


Figure 3.9 Distribution of changes in 6-minute walk distance. The number of participants reaching various thresholds of changes in 6-minute walk distance from baseline to week 1 (*top*) and from baseline to week 8 (*bottom*) are shown. An improvement of 40 metres or more was considered clinically important (grey-shaded areas). All data are expressed as absolute numbers of participants (n = 22 in the placebo group at week 8 due to missing walk test results).

3.3.6 Echocardiographic findings

The non-invasive assessment of PASP by transthoracic echocardiography has been well established in healthy individuals (see Chapter 2.3). In COPD patients, however, this approach is complicated by pathological abnormalities impeding or complicating the necessary measurement of a trans-tricuspid regurgitant (TR) jet. This was reflected in the present study, with only 21 participants (43.8%) having had a detectable TR jet at the baseline visit. Only in 15 (31.3%) of those participants could a quantitative measurement be obtained. Sample sizes were evenly distributed between the iron and placebo groups with n = 8 and n = 7, respectively. The average tricuspid pressure gradient (mean ± SD) among all participants with measurable TR jet at baseline was 31.9 ± 6.2 mmHg, which is in line with the increased pulmonary arterial pressure typically seen in COPD. Measurements were not well-matched between groups, however. The average baseline pressure was lower in the iron group at 30.0 ± 4.8 mmHg compared to 34.2 ± 7.1 mmHg in the placebo group. The mean trans-tricuspid pressure did not change significantly over time ($p = 0.087$ for the effect of FCM, Figure 3.10).

Isolated pairwise comparison between single time points revealed a within-group reduction in pressure gradient of 2.22 mmHg (95% confidence interval: 0.13 to 4.32 mmHg) in the iron group from baseline to week 1 ($p = 0.041$, paired Student t-test). Between-group difference in the pressure change from baseline to week 1, however, was not significant at 2.83 mmHg (95% confidence interval: −0.01 to 5.67 mmHg; $p = 0.05$, unpaired Student t-test) and in line with the results obtained from longitudinal data analysis by linear mixed effects modelling.

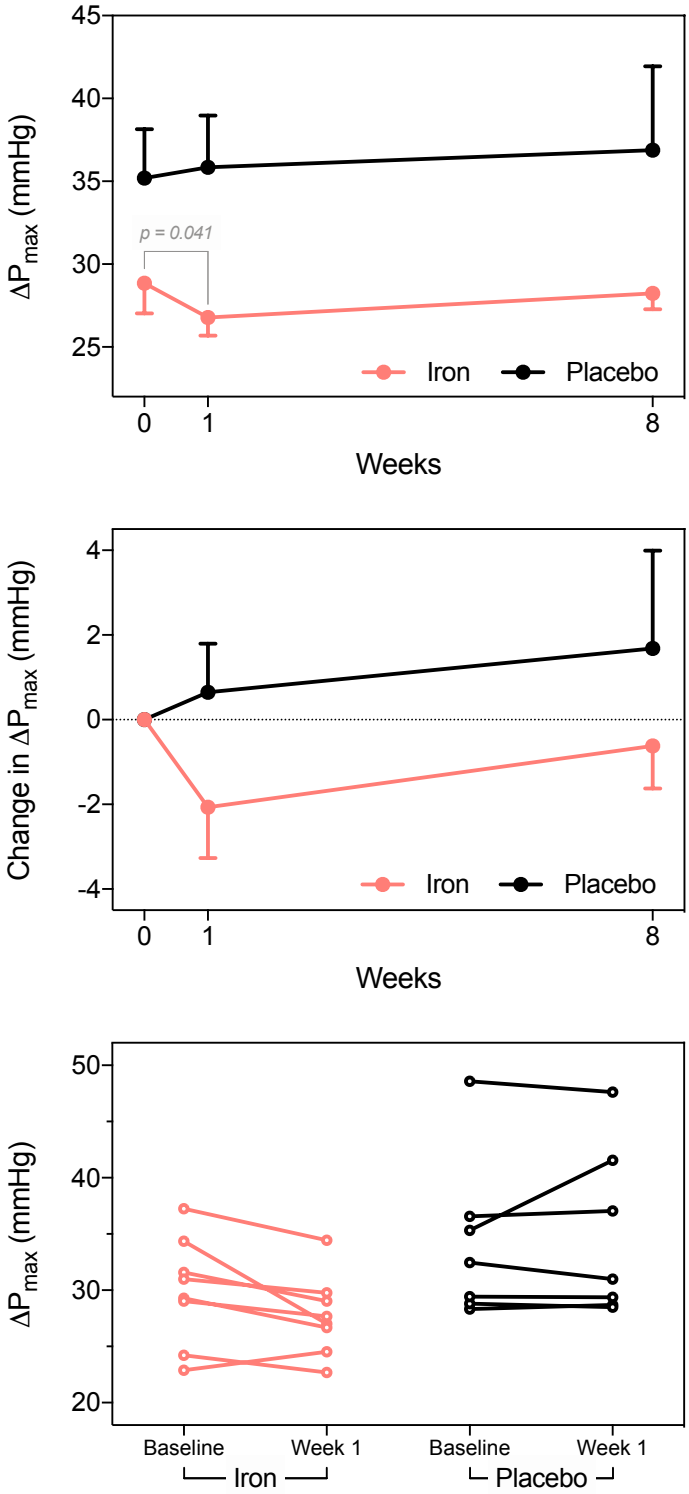


Figure 3.10 Temporal changes in maximum trans-tricuspid pressure gradient (ΔP_{max}). Absolute (*top*) and relative (*middle*) changes of ΔP_{max} (mean ± SEM) are shown for participants with a measurement at each visit (n = 6 in both groups due to missing data at week 8). Longitudinal data analysis showed no significant differences, although a significant within-group reduction in ΔP_{max} was evident at week 1 in the iron group (see text). Individual changes from baseline to week 1 by treatment group are also shown (*bottom*).

3.3.7 Quality of life and symptom questionnaires

A variety of scores and questionnaires were used to assess participants' symptoms, disease impact, and quality of life, as outlined in Chapter 3.2.4. In addition to the scores directly obtained at each study visit, the BODE index was calculated as a multidimensional scoring system for the prediction of long-term outcomes. A comprehensive overview of all symptom and quality of life scores obtained in the study is given in Table 3.11, with relative changes over time depicted in Figure 3.11.

The major finding was a marked difference in the distribution of reported MRC dyspnoea grades between groups. The proportions of the various severity grades were comparable between groups at baseline (Figure 3.12). In both groups, 9 participants (37.5%) had a dyspnoea grade of 1 or below, which corresponds to no dyspnoea or only some degree of breathlessness during physical activity (see Chapter 2.4.2.3). The number of subjects in this category increased to 16 (66.7%) by week 1 in the iron group, but remained relatively unchanged in the placebo group, where only 8 subjects (33.3%) had an MRC grade of 1 or below (p = 0.021). At week 8, 13 participants (54.2%) reported grade 1 or lower in the iron group compared to 7 participants (29.2%) in the placebo group (p = 0.079). The average MRC dyspnoea grade reflected this shift in proportions (Table 3.11 and Figure 3.11). By week 1, the average score (mean ± SEM) had dropped by 0.4 ± 0.1 points in the iron group, but remained relatively unchanged in the placebo group (p = 0.008 for the effect of FCM).

Other symptom and quality of life scores did not differ significantly between groups over time (Table 3.11). Figure 3.13 shows the distributions of quartiles of the BODE score, which are associated with survival in COPD (see Chapter 2.4.2.1). Proportions of quartiles were not significantly different at any point during the study.

Table 3.11 Overview of symptom and quality of life scores

Score	Iron group (n = 24)			Placebo group (n = 24)			*p* value
	Baseline	Week 1	Week 8	Baseline	Week 1	Week 8	
BODE score	3.5 ± 0.4 [a]	3.3 ± 0.4 [a]	3.4 ± 0.4 [a]	3.5 ± 0.4 [b]	3.5 ± 0.4 [b]	3.5 ± 0.4 [b]	0.222
COPD Assessment Test™ (CAT)	14.5 ± 1.3	14.8 ± 1.6	16.6 ± 1.5	16.5 ± 1.5	15.5 ± 1.6	18.3 ± 1.5	0.971
St George Respiratory Questionnaire (SGRQ)							
Total score	41.8 ± 3.2	39.8 ± 3.2	41.5 ± 3.1	46.1 ± 3.5	44.9 ± 3.7	47.9 ± 3.7	0.063
Impact score	27.4 ± 3.4	26.1 ± 3.1	27.4 ± 3.4	32.2 ± 3.8	32.6 ± 4.0	35.5 ± 3.9	0.271
Activity score	64.2 ± 4.0	59.7 ± 4.0	63.3 ± 3.6	69.4 ± 4.4	66.7 ± 4.6	67.0 ± 4.8	0.093
Symptom score	46.8 ± 3.9	47.5 ± 4.5	47.0 ± 4.9	48.0 ± 4.1	44.2 ± 4.2	52.7 ± 3.9	0.691
MRC dyspnoea scale	1.9 ± 0.2	1.5 ± 0.2	1.8 ± 0.2	2.0 ± 0.2	2.0 ± 0.2	2.0 ± 0.2	**0.008**
Dyspnoea-12 score	9.0 ± 1.4	9.5 ± 1.6	10.9 ± 1.6	10.1 ± 1.5	10.5 ± 1.7	11.1 ± 1.6	0.435
Likert scale: Dyspnoea at visit	1.9 ± 0.2	2.3 ± 0.2	2.1 ± 0.3	2.2 ± 0.3	2.0 ± 0.3	2.6 ± 0.3	0.307
Fatigue Severity Score	33.0 ± 3.2	31.0 ± 2.8	32.0 ± 2.6	39.8 ± 3.3	37.6 ± 3.4	41.1 ± 3.1	0.126
Hospital Anxiety and Depression Score (HADS)							
Anxiety score	4.3 ± 0.8	4.7 ± 0.9	4.5 ± 0.9	5.4 ± 0.8	5.3 ± 0.8	5.1 ± 0.8	0.313
Depression score	4.2 ± 0.5	4.2 ± 0.6	3.8 ± 0.5	5.9 ± 0.8	5.8 ± 0.7	5.7 ± 0.7	0.886

Table 3.11 Overview of symptom and quality of life scores (continued)

Score	Iron group (n = 24)			Placebo group (n = 24)			*p* value
	Baseline	Week 1	Week 8	Baseline	Week 1	Week 8	
Restless leg symptoms							
Symptoms reported, n (%)	7 (29.2)	7 (29.2)	5 (20.8)	7 (29.2)	8 (33.3)	9 (37.5)	
Restless leg syndrome rating scale [1]	22.3 ± 1.7 [c]	19.8 ± 3.4 [c]	22.5 ± 3.5 [c]	17.3 ± 2.4 [d]	17.0 ± 2.4 [d]	19.6 ± 2.7 [d]	0.462
Visual analogue scales (VAS)							
Dyspnoea	28.3 ± 4.1	32.1 ± 5.1	27.6 ± 4.4	32.4 ± 4.9	39.7 ± 5.2	33.8 ± 4.9	0.558
Cough	28.6 ± 5.2	34.4 ± 5.4	34.6 ± 5.6	31.3 ± 5.7	30.6 ± 5.3	29.9 ± 4.6	0.250
Sputum production	24.9 ± 5.3	21.4 ± 4.1	27.5 ± 4.9	21.2 ± 4.7	24.9 ± 4.8	25.6 ± 4.1	0.483
Sputum purulence	18.9 ± 4.5 [b]	17.1 ± 4.2 [b]	17.7 ± 4.7 [b]	24.4 ± 5.9 [b]	19.6 ± 4.2 [b]	26.0 ± 4.5 [b]	0.425

Scale ranges: BODE 0–10, COPD Assessment Test™ 0–40, St George Respiratory Questionnaire 0–100, MRC scale 0–4, Dyspnoea-12 score 0–36, Likert scale 1–7, Hospital Anxiety and Depression Score 0–21, Fatigue Severity Score 9–63, Restless leg syndrome rating scale 0–40, visual analogue scales 0–100. For all scores a lower value is better.

[a–d] Results are reported only for participants with a valid data point at each visit ([a] n = 23, [b] n = 21, [c] n = 4, [d] n = 7); cases with partially missing data were excluded from this table, but not from the statistical model.

[1] Restless leg syndrome rating scale was only performed if symptoms were reported.

All data are reported as mean ± SEM, unless otherwise stated. Statistical analysis was performed by linear mixed effects modelling; p values are reported for the fixed effect of "status post iron infusion".

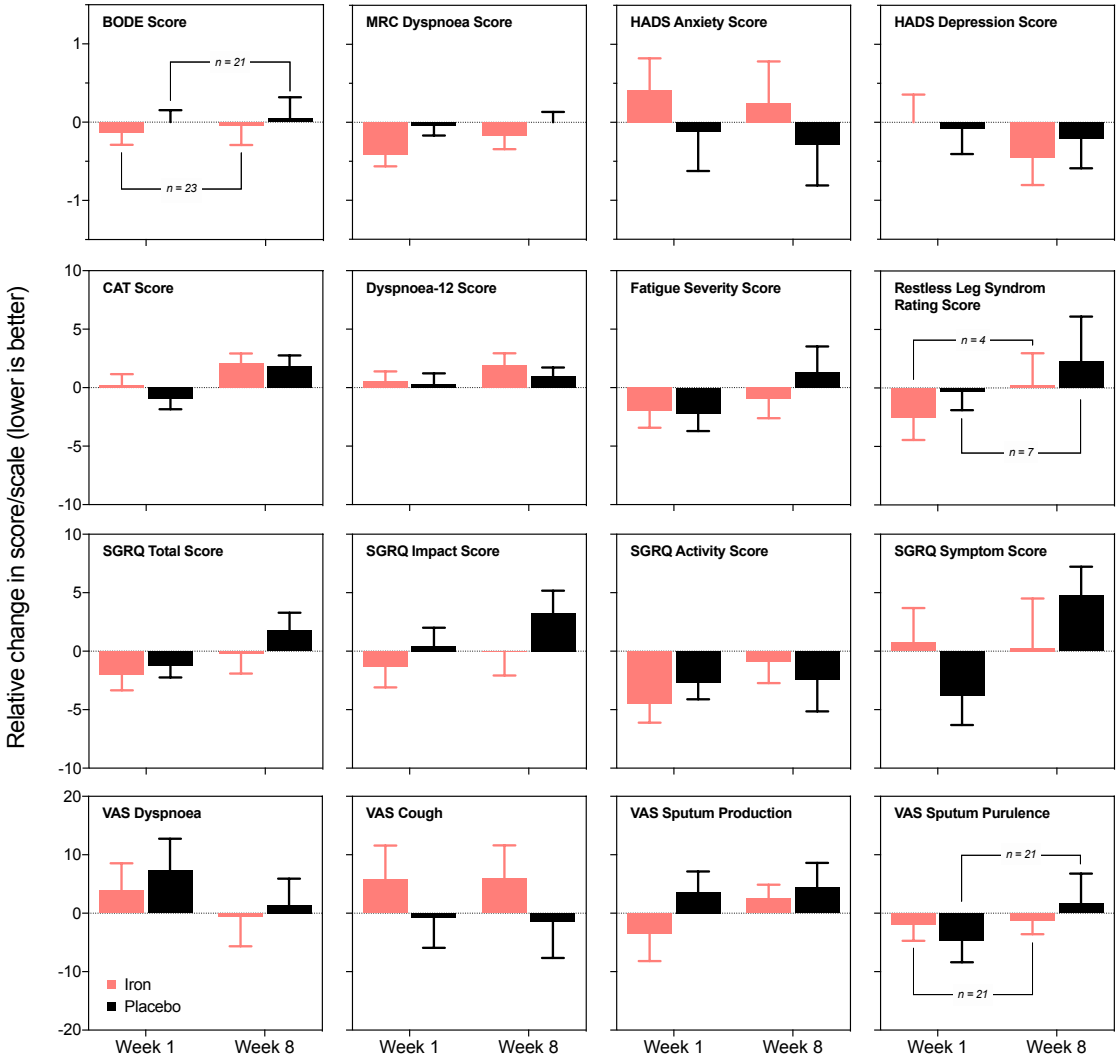


Figure 3.11 Relative changes in symptom and quality of life scores. Changes from baseline to follow-up visits are depicted. With the exception of MRC dyspnoea score (*top row, second panel*), no significant changes were observed following administration of intravenous FCM. Statistical comparisons were performed by linear mixed effects modelling (see **Table 3.11** for details). Only participants with valid data at each visit were included in the graphs (n = 24, unless otherwise indicated). All data are expressed as mean ± SEM.

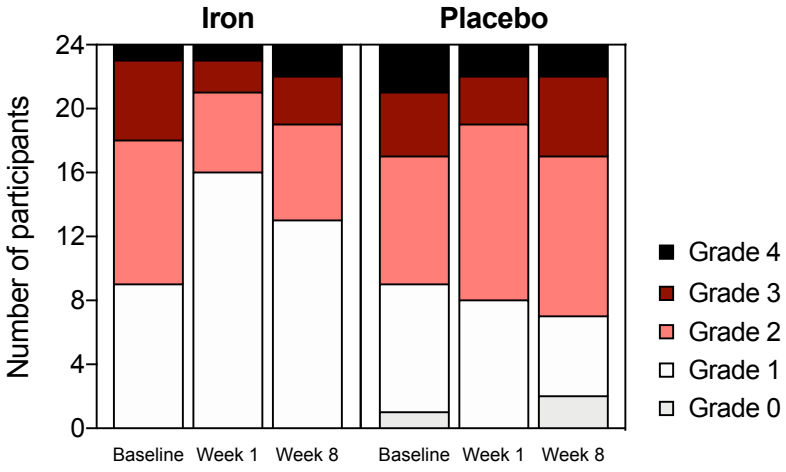


Figure 3.12 Proportions of MRC dyspnoea grades at each study visit. The fraction of subjects with an MRC grade of 1 or less was significantly increased one week after an infusion of iron compared to placebo (p = 0.021). Statistical comparisons were performed by Pearson's Chi-square test comparing the proportions of grade 1 or less to grade 2 or more at each time point.

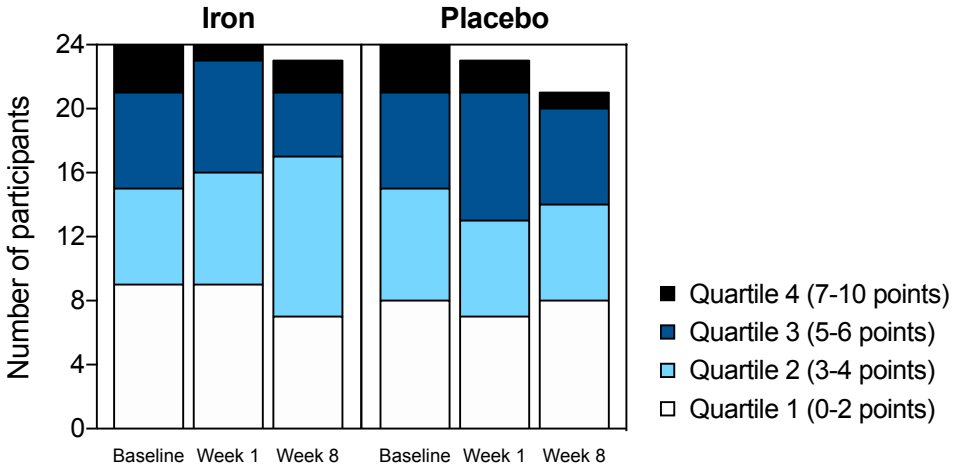


Figure 3.13 Proportions of BODE score quartiles. The quartiles of the BODE score serve as a predictor of outcome: higher scores are associated with decreased survival (see text). Proportions of the quartiles were well-matched at baseline (p = 0.987). No significant differences were observed at weeks 1 and 8 (p = 0.872 and p = 0.634, respectively). Statistical comparisons were performed by Pearson's Chi-square test comparing the proportions of quartiles 1–4 at each time point. Missing data were due to missing spirometry, walk distance, or BMI measurements (n = 23 for iron week 8 and placebo week 1, n = 21 for placebo week 8).

3.3.8 Haematology and blood biochemistry parameters

Blood samples were taken at each study visit and analysed for various haematological and serological parameters. Iron and inflammatory markers were also measured and are reported in Chapters 3.3.3 and 3.3.9, respectively. A comprehensive overview of haematological and serological parameters measured during the study is given in Table 3.12 and Table 3.13, respectively.

Both mean cell volume and haemoglobin steadily increased in the iron group over the 8-week study period ($p = 0.003$ and $p < 0.001$, respectively), which further confirmed treatment efficacy of FCM (Figure 3.14). Haemoglobin levels showed a noticeable dip one week after randomisation in both groups (Figure 3.14): levels decreased by 0.4 g/dL (95% confidence interval: 0.1 to 0.7 g/dL, $p = 0.007$) in the FCM group and by 0.4 g/dL (95% confidence interval: 0.2 to 0.6 g/dL, $p = 0.003$) in the placebo group. This effect on haemoglobin occurred independently of FCM administration, given that both groups experienced similar reductions. Other haematological indices were not significantly different between the two groups (Table 3.12).

Significant effects of FCM on blood biochemistry markers were observed for phosphate, creatinine, and the liver function parameters alanine aminotransferase (ALT) and alkaline phosphatase (Table 3.13). The latter two were mildly but significantly elevated – an expected adverse reaction of FCM infusion – but did not increase beyond normal limits (Figure 3.14). Creatinine levels, which were poorly matched at baseline, dropped by 2.5 ± 0.7 µmol/L (mean ± SEM) from baseline to week 1 in the iron group before returning to baseline levels at week 8 (Figure 3.14). Creatinine did not drop below the lower limit of normality either. While the effect of FCM on creatinine was statistically significant ($p = 0.005$), this reduction was too small to be of clinical importance.

Table 3.12 Haematology parameters

Parameter, unit	**Iron group** (n = 24)			**Placebo group** (n = 24)			***p* value**
	Baseline	Week 1	Week 8	Baseline	Week 1	Week 8	
Haemoglobin, g/dL	14.5 ± 0.3 [a]	14.1 ± 0.2 [a]	14.4 ± 0.3 [a]	14.4 ± 0.3 [a]	14.0 ± 0.3 [a]	14.1 ± 0.3 [a]	0.817
Mean cell volume, fL	90.6 ± 1.1 [a]	91.3 ± 1.1 [a]	92.8 ± 1.1 [a]	92.3 ± 0.7 [a]	92.6 ± 0.7 [a]	92.2 ± 0.8 [a]	**0.003**
Mean cell haemoglobin, pg	30.2 ± 0.5 [a]	30.4 ± 0.4 [a]	30.7 ± 0.4 [a]	30.7 ± 0.3 [a]	30.4 ± 0.2 [a]	30.3 ± 0.2 [a]	**< 0.001**
Haematocrit, %	43.6 ± 0.6 [a]	42.5 ± 0.7 [a]	43.6 ± 0.7 [a]	43.3 ± 0.8 [a]	42.7 ± 0.9 [a]	43.0 ± 0.9 [a]	0.478
Erythropoietin, mIU/mL	8.1 ± 0.7	7.8 ± 0.6	7.6 ± 0.8	9.9 ± 0.9	9.8 ± 1.3	9.8 ± 1.2	0.540
White cell count, × 10^9/L	7.7 ± 0.6 [a]	7.2 ± 0.6 [a]	7.6 ± 0.5 [a]	9.0 ± 0.6 [a]	8.4 ± 0.6 [a]	9.0 ± 0.4 [a]	0.152
Neutrophil count, × 10^9/L	5.2 ± 0.6 [a]	4.8 ± 0.5 [a]	5.0 ± 0.5 [a]	6.3 ± 0.5 [a]	5.7 ± 0.5 [a]	6.3 ± 0.4 [a]	0.167
Eosinophil count, × 10^9/L	0.18 ± 0.02 [a]	0.18 ± 0.02 [a]	0.19 ± 0.02 [a]	0.19 ± 0.03 [a]	0.19 ± 0.02 [a]	0.17 ± 0.03 [a]	0.689
Platelet count, × 10^9/L	261 ± 11 [a]	254 ± 14 [a]	270 ± 17 [a]	304 ± 30 [a]	296 ± 27 [a]	290 ± 24 [a]	0.294

[a] Results are reported only for participants with a valid data point at each visit (n = 23); cases with partially missing data were excluded from this table, but not from the statistical model.

All data are reported as mean ± SEM. Statistical analysis was performed by linear mixed effects modelling; p values are reported for the fixed effect of "status post iron infusion".

Table 3.13 Serum biochemistry parameters

Parameter, unit	**Iron group** (n = 24)			**Placebo group** (n = 24)			***p* value**
	Baseline	Week 1	Week 8	Baseline	Week 1	Week 8	
Sodium, mmol/L	138.4 ± 0.4	138.3 ± 0.5	138.5 ± 0.4	138.6 ± 0.8	138.4 ± 0.5	138.1 ± 0.5	0.870
Potassium, mmol/L	4.1 ± 0.1	4.0 ± 0.1	3.9 ± 0.1	4.2 ± 0.1	4.1 ± 0.1	4.2 ± 0.1	0.229
Phosphate, mmol/L	0.98 ± 0.03	0.52 ± 0.03	0.83 ± 0.05	0.97 ± 0.03	0.97 ± 0.03	0.97 ± 0.03	**< 0.001**
Urea, mmol/L	5.1 ± 0.4	4.8 ± 0.3	5.1 ± 0.4	6.2 ± 0.4	6.1 ± 0.4	6.3 ± 0.4	0.084
Creatinine, µmol/L	67.5 ± 3.1	65.0 ± 3.2	67.3 ± 2.9	79.3 ± 3.7	78.8 ± 3.4	80.0 ± 3.9	**0.005**
Glucose, mmol/L	5.5 ± 0.2 [a]	5.8 ± 0.4 [a]	5.6 ± 0.4 [a]	5.6 ± 0.3 [a]	5.4 ± 0.3 [a]	5.4 ± 0.2 [a]	0.238
Bilirubin, µmol/L	9.1 ± 0.6	8.1 ± 0.5	9.0 ± 0.7	10.5 ± 1.3	10.2 ± 1.2	10.0 ± 1.1	0.064
Alanine aminotransferase, IU/L	18.4 ± 1.3	23.6 ± 1.9	19.3 ± 1.4	19.5 ± 1.7	18.5 ± 1.7	20.1 ± 1.7	**0.001**
Alkaline Phosphatase, IU/L	72.4 ± 2.7	74.6 ± 2.8	81.4 ± 3.0	77.7 ± 5.2	75.0 ± 5.3	76.7 ± 5.4	**0.005**
Albumin, g/L	36.7 ± 0.6	35.6 ± 0.5	36.0 ± 0.6	36.4 ± 0.5	35.3 ± 0.6	35.3 ± 0.6	0.880

[a] Results are reported only for participants with a valid data point at each visit (n = 22); cases with partially missing data were excluded from this table, but not from the statistical model.

All data are reported as mean ± SEM. Statistical analysis was performed by linear mixed effects modelling; p values are reported for the fixed effect of "status post iron infusion".

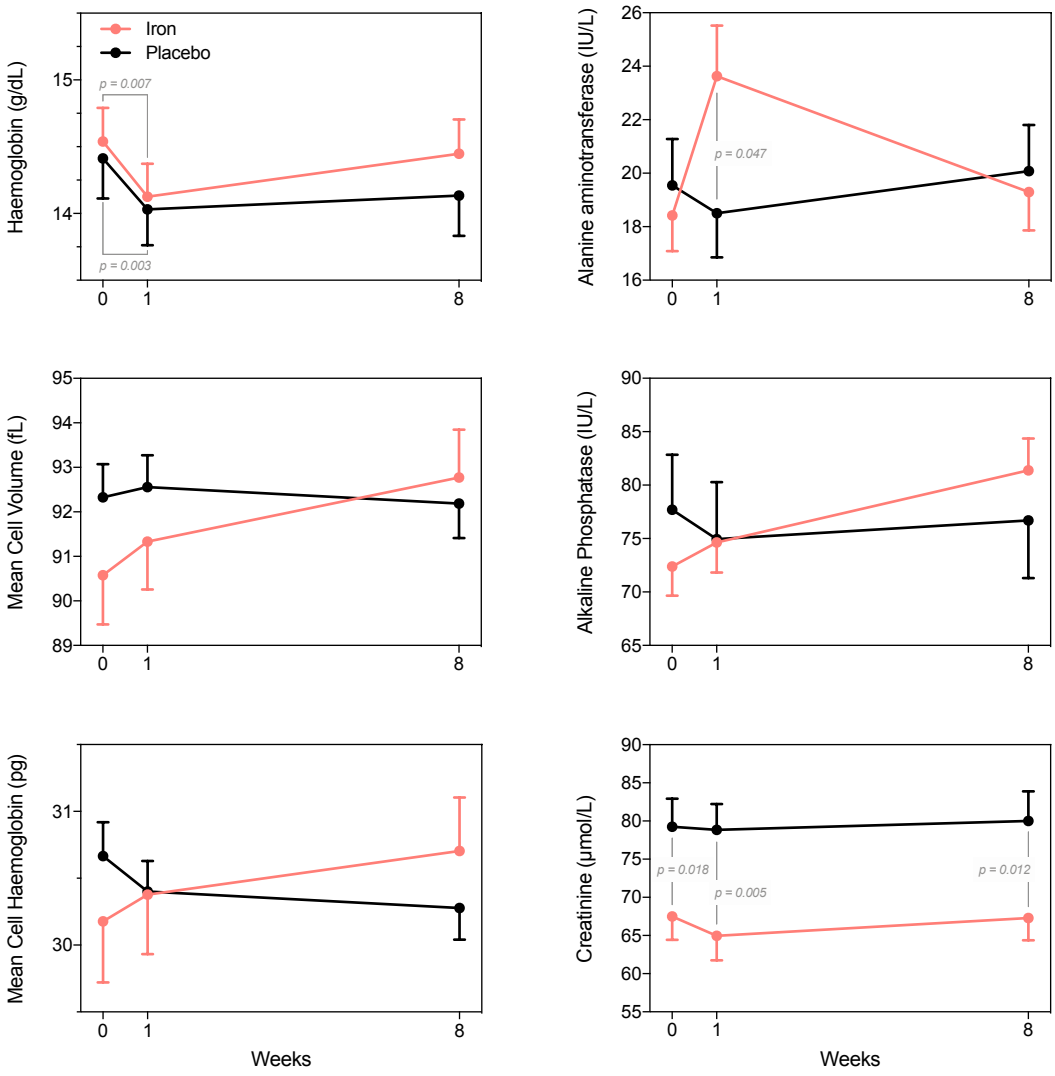


Figure 3.14 Main haematological and serological findings. Haematological parameters (*left*) indicated treatment efficacy of FCM with increasing levels of mean cell volume (MCV) and mean cell haemoglobin. Haemoglobin levels were not significantly different between groups, but showed a noticeable dip at week 1. Serological markers (*right*) significantly affected by FCM administration included alanine aminotransferase (ALT), alkaline phosphatase, and creatinine. Statistical comparisons were performed by linear mixed effects modelling (see **Table 3.12** and **Table 3.13**) and paired (haemoglobin) or unpaired (ALT, creatinine) two-tailed Student t-test; *p* values are reported for comparisons within groups between two timepoints (haemoglobin) or between groups at a single time point (ALT, creatinine). All data are expressed as mean ± SEM (n = 23 per group for haematological parameters, n = 24 per group for serological parameters).

Phosphate levels were significantly affected by FCM administration. Of the 29 participants, who were enrolled after January 2016 and had phosphate levels measured at each visit (see Chapter 3.2.9), 14 were randomised to receive FCM. All of those exhibited phosphate levels below the lower limit of normality (i.e. < 0.8 mmol/L) at week 1. Of those, five subjects received oral phosphate substitution due to levels dropping below 0.3 mmol/L. One participant did not respond to oral phosphate and was consequently administered an intravenous single-bolus replacement therapy. Stored serum samples were retrospectively analysed to establish the incidence of hypophosphataemia in the study cohort. Significantly more participants showed phosphate levels below 0.8 mmol/L in the iron group (91.7%) compared to placebo (12.5%) after one week ($p < 0.001$). All subjects receiving an iron infusion showed reduced levels, even though two (8.3%) did not drop below the lower limit of normality (Figure 3.15). Mean phosphate levels reflected this and were significantly lower ($p < 0.001$) after FCM infusion (Table 3.13 and Figure 3.15).

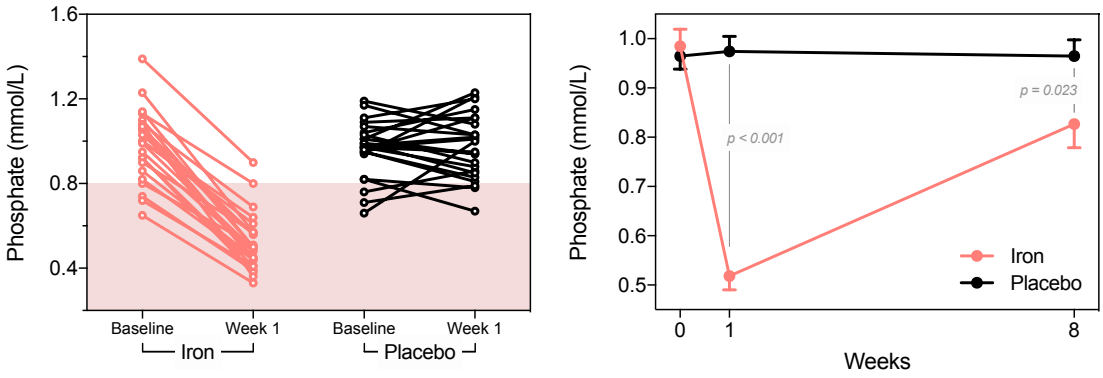


Figure 3.15 Changes in serum phosphate levels after infusion. Individual changes from baseline to week 1 (*left*) show reduced phosphate levels after FCM administration in all participants (area of values below normal limits is highlighted in red). This is reflected in significantly lower average (mean ± SEM) phosphate levels at week 1 (*right*). Statistical comparisons were performed by linear mixed effects modelling and unpaired two-tailed Student t-test; *p* values are reported for comparisons between groups at a single time point.

3.3.9 Inflammatory markers

In addition to the blood parameters described in the previous chapter, the inflammatory markers C-reactive protein (CRP) and interleukin-6 (IL-6) were analysed. Longitudinal analyses found no significant effect of FCM administration on either CRP or IL-6 (Table 3.14 and Figure 3.16), even though IL-6 levels were lower in the FCM group in isolated comparison between groups at week 8 (mean difference: 2.9 pg/mL, 95% confidence interval: 0.0 to 5.7 pg/mL, $p = 0.047$). The proportions of undetectable IL-6 values were not different between groups at any time point.

Table 3.14 Inflammatory markers

Parameter, unit	**Iron group** (n = 24)			**Placebo group** (n = 24)			***p* value**
	Baseline	Week 1	Week 8	Baseline	Week 1	Week 8	
C-reactive protein, mg/L	6.6 ± 3.0	8.0 ± 2.1	5.5 ± 1.3	6.5 ± 1.7	6.0 ± 1.8	5.8 ± 1.4	0.865
Interleukin-6[1], pg/mL	7.8 ± 2.7 (13)	4.8 ± 0.5 (11)	4.6 ± 0.4 (11)	8.2 ± 1.7 (8)	7.5 ± 1.3 (14)	7.5 ± 1.3 (6)	0.110

[1] *Values in parentheses indicate the number of data points with values below the assay's detection threshold of 3.13 pg/mL. These were not included in the descriptive statistics or the statistical model.*

All data are reported as mean ± SEM. Statistical analysis was performed by linear mixed effects modelling; p values are reported for the fixed effect of "status post iron infusion".

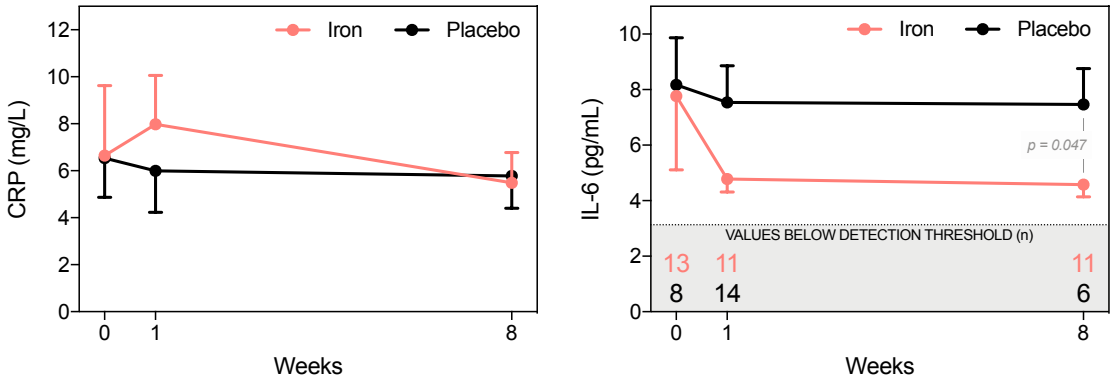


Figure 3.16 Temporal changes in inflammatory markers. C-reactive protein (CRP, *left*) and interleukin-6 (IL-6, *right*) did not differ significantly between groups over time. Statistical comparisons were performed by linear mixed effects modelling and unpaired two-tailed Student t-test; *p* value is reported for the comparison between groups at week 8. Proportions of undetectable IL-6 values were compared by Pearson's Chi-square test (Baseline: $p = 0.146$, Week 1: $p = 0.386$, Week 8: $p = 0.131$).

3.3.10 Blood gas analysis

Capillary blood samples were obtained at each visit to assess participants' oxygen and acid-base status. Results are shown in Table 3.15. Blood gas analysis did not reveal any significant changes in pH, partial pressures of oxygen and carbon dioxide, capillary oxygen saturation, base excess, or bicarbonate levels between the two groups. Capillary blood sampling was successful in all participants; no arterial samples had to be drawn.

Table 3.15 Capillary blood gas analysis

Parameter, unit	**Iron group** [a] (n = 24)			**Placebo group** [b] (n = 24)			***p* value**
	Baseline	Week 1	Week 8	Baseline	Week 1	Week 8	
pH	7.45 ± 0.01	7.45 ± 0.00	7.45 ± 0.00	7.45 ± 0.01	7.45 ± 0.01	7.44 ± 0.01	0.527
Partial pressure of CO_2 (PCO_2), kPa	4.85 ± 0.10	4.95 ± 0.11	4.99 ± 0.08	4.76 ± 0.09	4.81 ± 0.10	5.00 ± 0.13	0.968
Partial pressure of O_2 (PO_2), kPa	9.35 ± 0.24	9.14 ± 0.26	9.19 ± 0.23	9.37 ± 0.25	9.27 ± 0.23	9.04 ± 0.17	0.519
Oxygen saturation (SO_2), %	94.6 ± 0.4	94.4 ± 0.4	94.4 ± 0.4	94.6 ± 0.4	94.4 ± 0.3	94.1 ± 0.3	0.640
Base Excess, mmol/L	1.31 ± 0.43	1.62 ± 0.33	2.11 ± 0.36	0.92 ± 0.43	1.00 ± 0.44	1.40 ± 0.49	0.378
Bicarbonate (HCO_3), mmol/L	25.5 ± 0.4	25.8 ± 0.3	26.2 ± 0.3	25.2 ± 0.4	25.2 ± 0.4	25.6 ± 0.4	0.441

[a, b] Results are reported only for participants with a valid data point at each visit ([a] n = 22, [b] n = 23); cases with partially missing data were excluded from this table, but not from the statistical model.

All data are reported as mean ± SEM. Statistical analysis was performed by linear mixed effects modelling; p values are reported for the fixed effect of "status post iron infusion".

3.3.11 Spirometry

Spirometric measurements were obtained at each study visit to assess lung function and airway obstruction (Table 3.16). Predicted forced expiratory volume in the first second (FEV_1) and its ratio to forced vital capacity (FEV_1/FVC) – the two main spirometric variables used to assess airflow obstruction in COPD – showed no significant differences over time between the iron and placebo groups; thus, FCM administration had no effect on either.

Table 3.16 Spirometry results

Parameter, unit	**Iron group** (n = 24)			**Placebo group** (n = 24)			***p* value**
	Baseline	Week 1	Week 8	Baseline	Week 1	Week 8	
FEV_1, L	1.08 ± 0.10 [a]	1.04 ± 0.09 [a]	1.07 ± 0.09 [a]	1.25 ± 0.07	1.27 ± 0.08	1.27 ± 0.08	0.120
FEV_1, % of predicted	43.6 ± 3.3 [a]	42.3 ± 3.2 [a]	43.1 ± 3.0 [a]	46.0 ± 3.1	46.3 ± 3.3	46.5 ± 3.4	0.135
FVC, L	2.67 ± 0.17 [a]	2.53 ± 0.16 [a]	2.57 ± 0.15 [a]	3.24 ± 0.15	3.29 ± 0.17	3.29 ± 0.17	**0.017**
FVC, % of predicted	85.6 ± 5.4 [a]	81.3 ± 5.1 [a]	82.7 ± 4.4 [a]	91.3 ± 4.0	91.9 ± 3.7	92.0 ± 4.1	**0.015**
VC, L	2.87 ± 0.16	2.74 ± 0.16	2.79 ± 0.15	3.41 ± 0.17	3.40 ± 0.18	3.45 ± 0.18	**0.012**
VC, % of predicted	90.8 ± 4.7	87.0 ± 4.9	88.5 ± 3.9	92.8 ± 3.9	92.5 ± 4.0	94.0 ± 3.7	**0.016**
FEV_1/FVC, %	40.4 ± 2.2 [a]	41.3 ± 2.2 [a]	41.0 ± 2.1 [a]	39.8 ± 2.2	39.2 ± 2.3	39.3 ± 2.1	0.242
FEV_1/VC, %	37.3 ± 2.4 [a]	37.5 ± 2.1 [a]	37.8 ± 2.1 [a]	38.1 ± 2.4	38.0 ± 2.3	37.4 ± 2.3	0.694

[a] Results are reported only for participants with a valid data point at each visit (n = 23); cases with partially missing data were excluded from this table, but not from the statistical model.

All data are reported as mean ± SEM. Statistical analysis was performed by linear mixed effects modelling; p values are reported for the fixed effect of "status post iron infusion".

FCM administration, but not placebo, significantly affected vital capacity (VC) and forced vital capacity (FVC), both of which were lower during follow-up visits. Changes were small and must be seen in light of the poor baseline matching of these parameters, which was also seen in the pulmonary function tests at study enrolment (Chapter 3.3.2). The iron group had lower baseline values for all spirometric variables, indicating slightly worse lung function at the beginning of the study.

Participants recorded their peak expiratory flow rate on a daily basis (Figure 3.17). Measurements were not significantly different between groups at baseline (mean difference: 29 L/min, 95% confidence interval: −12 to 71 L/min, $p = 0.161$). Longitudinal analysis showed no significant effect of FCM administration on this parameter over time.

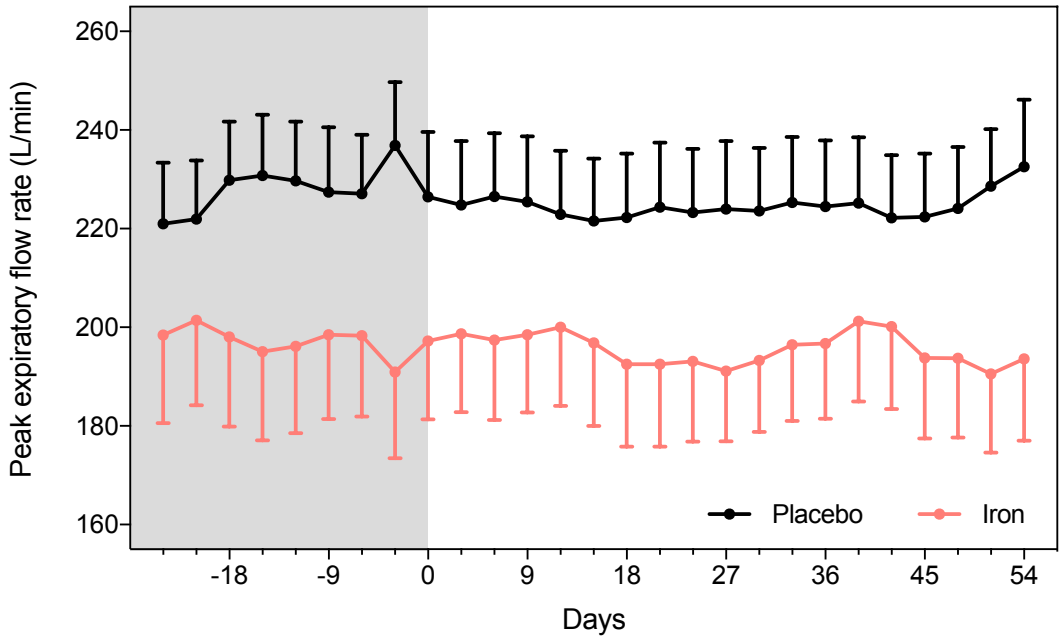


Figure 3.17 Daily self-measured peak expiratory flow rates (PEFR). Participants were asked to record peak expiratory flow rates (best out of 3 attempts) on daily diary cards, starting at the screening visit. Self-measured peak expiratory flow rates did not differ significantly between the two groups over time (p = 0.765 for the effect of iron administration). Day 0 marks the baseline visit. Each data point (mean ± SEM) represents the average of the indicated day and the two preceding days. Statistical comparison was performed by linear mixed effects modelling; pre-baseline data (shaded area) were not included in the analysis.

3.3.12 Exacerbations

The occurrence of exacerbations throughout the study period was documented based on self-reported symptoms recorded by each participant on daily diary cards. In each treatment group, 7 (29.2%) participants developed exacerbations of their COPD during the follow-up period (Table 3.17). Participants in the placebo group had repeated exacerbations (on average 1.7 events per participant), whereas only one exacerbation per participant was recorded in the iron group. The average cumulative duration spent with ongoing exacerbations as well as the average number of days to the occurrence of the first event did not differ between the two groups. No significant differences were seen in the use of antibiotics, steroids, or hospital facilities between the two groups.

In conclusion, exacerbations did not last longer, occur more frequently or sooner after administration of FCM compared to placebo.

Table 3.17 Exacerbations during the study period

	Iron group (n = 24)		**Placebo group** (n = 24)		***p* value**
	No. of patients (%)	No. of events	No. of patients (%)	No. of events	
Exacerbations	7 (29.2)	7	7 (29.2)	12	1.00
Use of antibiotics due to exacerbation	6 (25.0)	6	5 (20.8)	8	0.731
Use of steroids due to exacerbation	5 (20.8)	5	5 (20.8)	6	1.00
Hospitalisation due to exacerbation	0	0	1 (4.2)	1	1.00
	Mean ± SD		Mean ± SD		
Cumulative duration, days	13.0 ± 8.4		10.1 ± 6.4		0.490
Time to first event, days after infusion	22.9 ± 12.4		18.1 ± 20.6		0.613

Statistical analysis was performed by Pearson's Chi-square test or Fisher's exact test for differences in numbers of patients with a given event. Cumulative duration and time to first event (n = 7 per group) were analysed by unpaired Student t-test.

3.3.13 Adverse events

Administration of intravenous infusions of FCM or placebo was generally well-tolerated by participants. Adverse events occurring during the first week after the infusion were recorded and are shown in Table 3.18.

No significant differences in the overall occurrence of adverse events were observed. The most common adverse event was hypophosphataemia, which occurred more frequently in the iron group (91.7% vs. 8.3%, $p < 0.001$) as described in detail in Chapter 3.3.8. Abnormal laboratory parameters were identified retrospectively after study completion and were only considered as adverse events if they were not pre-existent at any point before randomisation or worsened from the pre-baseline state. None of the other observed laboratory abnormalities (Table 3.18) had clinical implications as they were all mild and represented only minimal deviations from normal limits.

Other commonly observed adverse events included post-infusion hypertension, dyspnoea, injection site haematomas, and headaches, of which none were significantly different between groups.

Adverse events observed in the iron group were expected as consequence of the underlying condition or outlined in the Summary of Product Characteristics of FCM as either common or uncommon adverse drug reactions (Vifor Pharma UK Limited 2017). No unexpected adverse reactions occurred.

Two adverse events in the placebo group met the criteria for reporting as serious adverse events due to the participants requiring inpatient hospitalisation as consequence of a fall and an infective exacerbation. No suspected unexpected serious adverse reactions (SUSAR) occurred.

Table 3.18 Adverse events

	Iron group (n = 24)		Placebo group (n = 24)		*p* value
	No. of patients (%)	No. of events	No. of patients (%)	No. of events	
Adverse events (all)	22 (91.7)	48	17 (70.8)	28	0.137
Hypophosphataemia[1]	22 (91.7)	22	2 (8.3)	2	**< 0.001**
Other abnormal laboratory tests[2]	8 (33.3)	10	7 (29.2)	8	0.755
Hypertension[3]	5 (20.8)	5	4 (16.7)	4	1.00
Dyspnoea	3 (12.5)	3	0	0	0.234
Headache	2 (8.3)	2	4 (16.7)	4	0.666
Haematoma	2 (8.3)	2	2 (8.3)	2	1.00
Fever	1 (4.2)	1	0	0	1.00
Rash	1 (4.2)	1	0	0	1.00
Upper respiratory infection	1 (4.2)	1	0	0	1.00
Cough	1 (4.2)	1	0	0	1.00
Nasal congestion	0	0	1 (4.2)	1	1.00
Lung infection	0	0	1 (4.2)	2	1.00
Vomiting	0	0	1 (4.2)	1	1.00
Diarrhoea	0	0	1 (4.2)	1	1.00
Dyspepsia	0	0	1 (4.2)	1	1.00
Dizziness	0	0	1 (4.2)	1	1.00
Fall	0	0	1 (4.2)	1	1.00
Serious adverse events (all)	0	0	2 (8.3)	2	0.489
Lung infection, requiring hospitalisation	0	0	1 (4.2)	1	1.00
Fall, requiring hospitalisation	0	0	1 (4.2)	1	1.00

Adverse events were coded using the Common Terminology Criteria for Adverse Events (CTCAE). Pre-existing events were not considered adverse events unless they changed in severity (increase by one grade or more) or frequency. Statistical analysis was performed by Pearson's Chi-square test or Fisher's exact test for differences in numbers of patients with a given event.

[1] Hypophosphataemia was defined as phosphate < 0.8 mmol/L. Numbers reported here are based on the retrospective analysis of stored serum samples.

[2] These included in order of frequency: elevated C-reactive protein, hypoalbuminaemia, thrombocytosis, hypokalaemia, anaemia, hyperkalaemia, elevated white cell and neutrophil counts (no significant between-group differences for any of those values).

[3] Hypertension was considered an adverse event if the elevated blood pressure was not pre-existent (i.e. the grade of hypertension as defined by CTCAE observed immediately post-infusion or at week 1 was not present on any occasion prior to infusion), or was pre-existent but worsened (i.e. an increase by at least one grade as defined by CTCAE immediately post-infusion or at week 1).

3.4 Discussion

3.4.1 Main study findings

The clinical trial presented in this chapter constitutes the first systematic study of the effects of intravenous iron administration in patients with COPD. While the infusion of ferric carboxymaltose did not affect oxygenation parameters, it significantly improved two relevant functional outcome measures: exercise capacity and dyspnoea. Furthermore, ferric carboxymaltose appeared to be safe and was well-tolerated in this patient population.

3.4.1.1 *Primary outcome: Oxygenation*

The study was designed to detect an improvement in peripheral oxygen saturation one week after infusing ferric carboxymaltose to patients with COPD. This primary outcome measure, as well as other secondary oxygenation variables, was not significantly different between iron and placebo groups. Several limitations could account for the absence of significant changes in oxygenation. Most study participants were well-oxygenated at baseline, as reflected by mean resting oxygen saturation and capillary partial pressure of oxygen values in the normal range in both groups. Any potential benefit of iron administration was therefore necessarily limited. Furthermore, fewer participants in this study met the criteria of iron deficiency compared to a previous cohort (Nickol *et al.* 2015), which may have reduced any beneficial effect of ferric carboxymaltose.

The rationale for choosing an improvement in oxygen saturation as primary outcome warrants discussion. An early version of the study protocol included changes in pulmonary arterial pressure measured by echocardiography as a primary endpoint. This technique has been successfully used in healthy volunteers to establish a link between

iron administration and pulmonary vascular responses (Smith *et al.* 2008a, Smith *et al.* 2009, Bart *et al.* 2016, Frise *et al.* 2016). However, the non-invasive assessment of pulmonary arterial pressure could not be successfully used here due to the difficulties in obtaining echocardiograms in COPD patients described elsewhere in this thesis. Based on the observation of lower oxygen saturation levels in iron-deficient patients with COPD (Nickol *et al.* 2015), peripheral oxygenation was chosen as an easily and universally measurable primary endpoint. The study by Nickol *et al.* (2015) was, however, a retrospective observational analysis and could consequently not establish a causal link between iron deficiency and more severe hypoxaemia. It cannot be ruled out that iron deficiency and hypoxaemia are two distinct and unrelated entities in COPD. Iron substitution or supplementation might therefore not necessarily affect oxygenation.

3.4.1.2 *Improvement in functional outcomes: Exercise capacity and dyspnoea*

Two secondary outcome measures, exercise capacity and MRC dyspnoea score, significantly improved after iron treatment compared with placebo. These findings are potentially highly relevant for the individual patient, who might subjectively benefit from an increased walk distance and reduced breathlessness. This was highlighted by qualitative statements from individual participants, who reported feeling overall better and being able to walk further after receiving an infusion of ferric carboxymaltose.

In this study, participants randomised to the iron treatment arm significantly increased their 6-minute walk distance over eight weeks compared to placebo-treated individuals. The 6-minute walk distance is a commonly used test to assess functional exercise capacity in COPD, which correlates with the ability of a patient to perform activities of daily living (ATS Committee on Proficiency Standards for Clinical Pulmonary Function Laboratories 2002). Lower walk distances are a predictor of higher mortality in COPD

(Celli *et al.* 2004). In the present study, the walk distance increased by a mean (± SEM) of 24 ± 6 metres in the iron group by week 8 compared to 10 ± 6 metres in the placebo group (mean difference: 14 ± 8 metres). This result is comparable to the within-group improvement observed in a study of pulmonary rehabilitation in patients with COPD, in whom walk distance (mean ± SD) increased by 25 ± 52 metres immediately post-rehabilitation (i.e. 6–12 weeks after baseline) (Laviolette *et al.* 2008). The minimal clinically important difference (MCID) in 6-minute walk distance used in that study was 54 metres, which was achieved by 27% of participants. In the study presented in this chapter, only 17% of iron-treated participants improved their walk distance by ≥ 50 metres, but 46% achieved an improvement of ≥ 40 metres (compared to 27% of placebo-treated participants).

There is no clear consensus in the scientific literature on what change in 6-minute walk distance constitutes a minimal clinically important improvement: A wide range of MCID thresholds have been reported, from as low as 14 metres (Bohannon and Crouch 2017) up to 80 metres (Wise and Brown 2005). In the present study, a significantly higher number of participants improved their walking distance by 40 metres or more after iron administration compared with placebo. This increase in walk distance is supported by a body of literature stating 30–40 metres as MCID threshold for this test in COPD (Puhan *et al.* 2008, Hernandes *et al.* 2011, Polkey *et al.* 2013, Holland and Nici 2013).

When assessing repeated measurements of walk distance, the potential of a learning effect should be considered. The learning effect of repeated walk tests can be substantial, but is primarily reported in the days immediately following the first test (Hernandes *et al.* 2011, Sciurba *et al.* 2003, Knox *et al.* 1988). As an example, Knox *et al.* (1988) described a 33% improvement in repeated tests administered during the first

three days after the initial test. The same authors found a smaller increase of 8.5% in walk distance over a one-month period. It was also shown that improved walking distance due to learning from repeated walks is maintained over a period of two months (Wu *et al.* 2003). The fairly linear improvements seen in the placebo group of the present study could reflect a learning effect due to repeated tests. Participants in the iron group increased their walk distance at a similar rate as the placebo group between the two follow-up visits, as reflected by parallel temporal changes in Figure 3.8. This might also indicate a learning effect and would suggest that the major effect of intravenous iron occurred within the first week after the infusion, when walk distance changed at different rates in the two groups. The potential learning effect does not alter the conclusions drawn in the present study, since the randomised controlled trial design accounts for this potential confounder.

While it is difficult to draw mechanistic conclusions from the data presented here, the lack of effect on oxygen saturation in the presence of significant changes in functional exercise capacity could suggest a peripheral effect on skeletal muscle and oxygen utilisation rather than an effect on the cardiopulmonary system.

The significant results of reduced breathlessness, measured by the MRC dyspnoea score, are intriguing. In the group treated with ferric carboxymaltose, almost twice as many participants reported a lower MRC score after one week than at baseline, whereas no change occurred in the control group. This finding is particularly noteworthy considering the insensitivity of this assessment tool to detect change over short timescales (Stenton 2008). The MRC score does not measure dyspnoea directly, but rather quantifies the amount of physical activity necessary to induce breathlessness (Williams 2017). As such, the improvement in the score following iron administration might reflect the previously described increases in functional exercise capacity. The

lack of significant changes in other, more nuanced and direct dyspnoea assessment tools is in agreement with this hypothesis. Irrespective of whether this finding is directly linked to or independent from the improvement in walk distance, it may have important implications, as lower MRC dyspnoea scores are associated with increased survival (Nishimura *et al.* 2002).

3.4.1.3 *Safety of ferric carboxymaltose in COPD*

Among intravenous iron preparations, ferric carboxymaltose has a favourable safety profile with a low risk of hypersensitivity reactions (Geisser 2009, Funk *et al.* 2010, Larson and Coyne 2014). This was confirmed in the present study. With the exception of hypophosphataemia, adverse events occurred at similar rates in both groups. The infusion was well-tolerated by participants and side effects were mild in nature.

Hypophosphataemia is a well-documented – usually transient and asymptomatic – side effect of ferric carboxymaltose (Hardy and Vandemergel 2015, Scott 2018). The mechanism underlying iron-induced hypophosphataemia is not completely understood, but fibroblast growth factor 23 (FGF23) is thought to play a major role. Ferric carboxymaltose causes increased levels of FGF23 by interfering with the cleavage of the protein, which ultimately leads to decreased tubular reabsorption of phosphate (Zoller *et al.* 2017). Hypophosphataemia occurred significantly more often in the treatment arm than in the placebo group. As described in Chapter 3.3.8, five participants received oral or intravenous phosphate substitution therapy due to phosphate levels falling below 0.3 mmol/L at the first follow-up visit. However, none of those participants showed symptoms of hypophosphataemia (such as weakness, myalgia, bone pain, or altered mental state). The phosphate substitution in those participants was deemed medically necessary by an unblinded physician in order to restore normal serum

levels. Whether this second pharmacological intervention, which occurred in addition to the main study drug, had an influence on outcome variables needs further discussion. Outcomes measured before and at the week 1 visit remained unaffected, as the phosphate substitution was administered after blood results from this first follow-up visit became available. The only outcome with significant changes after week 1 was the 6-minute walk distance, which showed the largest increase at week 8. To rule out any major contribution of phosphate administration on this variable, data points from participants receiving phosphate were excluded for the week 8 time point and the analysis was repeated with the remaining participants. The relative improvement in the iron group was slightly lower at week 8 (21.8 ± 6.9 m, mean ± SEM), but the effect of FCM remained significant in the longitudinal analysis ($p = 0.030$).

As discussed before, many pathogens rely on iron as a nutrient to proliferate. Therefore, another safety concern of administering intravenous iron to patients with COPD was the potential risk of promoting bacterial growth and increasing the rate of infective exacerbations (Maynor and Brophy 2007), which are associated with increased hospitalisations and mortality in patients with COPD (Qureshi *et al.* 2014). In the present study, the short-term occurrence of exacerbations was not different between groups. However, the 8-week follow-up period in this study was too brief to draw any conclusions about possible long-term effects on infective exacerbations.

No clear evidence exists suggesting an increased rate of infections after intravenous iron administration in COPD so far. For patients with cystic fibrosis and an ongoing exacerbation, individual cases of worsening symptoms and rising inflammatory markers after intravenous iron administration were reported (Hoo and Wildman 2012). Another study showed no adverse effects of iron supplementation in cystic fibrosis (Gifford *et al.* 2014). It is encouraging that no increased risk of infections or bacteraemia was

observed in a study of intravenous iron administration to anaemic intensive care patients, which – like COPD patients – are also prone to infective events (The IRONMAN Investigators *et al.* 2016). Furthermore, a recent study showed an increased risk of bloodstream infections in the setting of iron deficiency, not iron overload (Mohus *et al.* 2018).

3.4.1.4 *Other outcome variables*

A number of additional study outcomes deserve a more detailed discussion. As highlighted before, the echocardiographic assessment of pulmonary arterial pressure is particularly challenging in patients with COPD due to pathological features such as hyperinflation, which impede the optimal transthoracic visualisation of the heart. Detection rates of TR signals of sufficient quality to allow measurements of PASP range from 24% to 66% (Laaban *et al.* 1989, Torbicki *et al.* 1989, Tramarin *et al.* 1991, Arcasoy *et al.* 2003). This was reflected in the present study, where an adequate signal could only be obtained in a third of participants. Due to the small sample size in each group and the poor matching of baseline echocardiographic measurements, no definite conclusions could be drawn from these recordings. The observed decrease in the iron group was, however, consistent with previously reported effects of intravenous iron on pulmonary arterial pressure (Smith *et al.* 2008a, Smith *et al.* 2009, Frise *et al.* 2016, Bart *et al.* 2016).

Changes in blood parameters were mostly unremarkable and expected. An interesting observation was the temporal trend of interleukin-6 in the FCM group. While IL-6 did not statistically differ between the two groups over time, values appeared to be slightly lower in the iron group after administration of the infusion. This drop in IL-6 is physiologically interesting. Under normal conditions, IL-6 stimulates the production of

hepcidin and thereby mediates the functional iron deficiency associated with inflammation (Nemeth *et al.* 2004a). In the presence of abundant hepcidin levels, IL-6 might be downregulated as a consequence. Such an anti-inflammatory negative feedback loop has indeed been described *in vitro* (De Domenico *et al.* 2010, Maliken *et al.* 2011). Briefly, hepcidin binds to ferroportin, which in turn leads to JAK2-mediated activation of STAT3, increased levels of SOCS3, and suppressed expression of inflammatory cytokines IL-6 and TNF-α.

3.4.2 Study limitations

This study had several important limitations. As mentioned, it was designed on the basis of an observational study of iron deficiency in COPD (Nickol *et al.* 2015). In the study presented here, fewer participants were iron-deficient and effects on the primary outcome measure were limited by already high baseline oxygenation levels. This raises the question whether a more selective approach – limiting recruitment to iron-deficient and more hypoxic patients – would have resulted in a significant finding. Eligibility criteria in the present study were, however, deliberately broad in order to establish feasibility of iron administration in a diverse population of COPD patients.

The lower number of iron-deficient participants in the present study might be attributable to the relatively high percentage of deceased patients in the original COPD cohort, which was studied by Nickol *et al.* (2015) and used for recruitment in the present trial. As shown in Figure 3.4, 54 out of 160 participants (33.8%) in the cohort had died before this study started recruiting. The participants who had died might have been more severely ill with concomitant worse iron deficiency.

The choice of the primary outcome on the basis of a retrospective cohort study could be seen as limiting. Nickol *et al.* (2015) could only show an association between iron

deficiency and lower oxygen saturations, but no causal relationship between iron status and oxygen saturation levels.

The effect size of FCM on peripheral oxygen saturation might have been smaller than the anticipated 2% improvement, and therefore the sample size not sufficient to detect a significant difference. It is furthermore debatable whether an improvement of 2% in oxygen saturation holds any clinical relevance, particularly in participants with oxygen saturations within the normal range.

Finally, technical aspects of assessing oxygen saturation were another limiting factor. The measurement technique is susceptible to external influences (e.g. cold fingers, nail polish, poor circulation) and inherent inaccuracies of pulse oximetry devices, which are usually stated by manufacturers to be in the range of ± 2–3% (Milner and Mathews 2012).

3.4.3 The problem of defining iron deficiency in COPD

A significant finding underlying the research presented in this chapter was the frequent occurrence of non-anaemic iron deficiency among patients with COPD (Nickol *et al.* 2015). The diagnosis of iron deficiency in COPD is not trivial and complicated by the fact that several iron parameters, including ferritin, hepcidin, and transferrin, are affected by chronic inflammation (Cloonan *et al.* 2017). Various criteria for iron deficiency have been applied before, from broad definitions to more restrictive ones. Commonly used cut-off values defining iron deficiency are ferritin < 100 µg/L or ferritin < 300 µg/L with transferrin saturation < 20% (Anker *et al.* 2009). Applying those criteria to the present study, 69% of participants would have been labelled iron-deficient at baseline. In contrast, only 10% were iron-deficient using the more restrictive definition used by Nickol *et al.* (2015) and outlined in Chapter 3.1.5.2.

The aforementioned broader definition has recently been assessed in heart failure patients using bone marrow aspiration with iron staining as the gold standard to detect iron deficiency (Grote Beverborg *et al.* 2018). The authors found a sensitivity for these iron deficiency cut-off values of 82% and a specificity of 72%. A definition based on only transferrin saturation or serum iron performed even better and lead the authors to question the diagnostic value of ferritin. While these findings confirm the diagnostic accuracy of a broader definition in heart failure, similar validation studies have not been performed in the setting of COPD.

The more restrictive approach described by Nickol *et al.* (2015) and used to classify patients in the present study is based on criteria for absolute iron deficiency. A ferritin level < 12 µg/L is a very strong indicator of absolute lack of iron (Cavill 1999, Cook and Skikne 1982, Thomas *et al.* 2013). Similarly, transferrin saturation < 16% was associated with inadequate iron supply to the bone marrow (Bainton and Finch 1964). The inclusion of above-normal soluble transferrin receptor levels – an indication of unmet iron requirements – in this definition is particularly beneficial in a setting like COPD, as the marker is not significantly affected by chronic inflammation (Allen *et al.* 1998).

In the absence of a validated definition of iron deficiency in COPD, the true prevalence is hard to determine. Physiologically, it may be more appropriate to consider a spectrum of iron availability that can be modified by iron supplementation, rather than defining absolute cut-off values for iron deficiency. In line with this concept, the present study did not restrict recruitment to iron-deficient individuals only. Instead, iron administration might induce biological effects independent of the presence of iron deficiency and could therefore be beneficial in all patients with COPD.

3.4.4 Iron supplementation in other chronic disease conditions

Intravenous iron supplementation has had beneficial effects in chronic disease conditions that have pathophysiological similarities with COPD.

In their landmark FAIR-HF trial, Anker *et al.* (2009) described significant improvements in symptoms, quality of life, walk distance, and functional class among iron-deficient patients with heart failure who received repeated doses of FCM over 24 weeks. Interestingly, the effects were independent of the presence of anaemia, highlighting the role of iron deficiency as an independent risk factor in heart failure. The subsequent CONFIRM-HF trial showed similar results with significant improvements in 6-minute walk distance after FCM administration as well as improved symptoms and quality of life (Ponikowski *et al.* 2015). Treatment with FCM in heart failure was also reported to reduce recurrent hospitalisations and mortality in iron-deficient heart failure patients (Anker *et al.* 2018). The beneficial effects in heart failure were not specific to FCM: improvements in the same outcome variables in heart failure patients were also shown for iron sucrose (Okonko *et al.* 2008).

In iron-deficient patients with pulmonary arterial hypertension, intravenous FCM significantly improved exercise capacity and quality of life in two small clinical studies (Viethen *et al.* 2014, Ruiter *et al.* 2015). A larger clinical trial of FCM supplementation in idiopathic pulmonary arterial hypertension is currently being conducted (Howard *et al.* 2013).

It is worth pointing out that all of the mentioned studies described effects of parenteral iron on outcome variables similar or identical to the ones used in the present study.

3.4.5 Conclusions

In conclusion, the study presented in this chapter constitutes the first evidence of the effects of intravenous iron administration in COPD. The data suggest that this approach is safe and well-tolerated and beneficially affects functional outcomes including exercise capacity and breathlessness, despite a lack of evidence of improved hypoxaemia.

These findings are encouraging and thought-provoking: COPD is associated with significant morbidity and mortality worldwide, but therapeutic options to improve symptoms are limited. Intravenous iron therapy is a simple and widely available treatment and improved symptoms and walk distance can have substantial benefits for patients. Larger studies are needed and future work might focus on functional capacity as a primary endpoint, assess responses in the presence of more severe hypoxaemia and iron deficiency, and explore the administration of multiple doses to maintain or potentiate the observed effects. Such research will help to elucidate the mechanisms underlying these findings and to clarify the complex implications of COPD on iron homeostasis. Hopefully, this would further establish the feasibility of intravenous iron as a treatment option. Our data support the concept of iron supplementation, rather than replacement, in patients with COPD.

CHAPTER 4:
Iron parameters in critical illness

4.1 Introduction

In this chapter, an exploratory analysis of baseline iron status in critically ill patients is presented. The results are based on the retrospective analysis of data obtained in another trial. Stored samples and selected data were shared with the author for further analysis. Results presented here only reflect aspects of the project in which the author was personally involved. The author's specific contributions consisted in the exploratory analysis of provided deidentified data (demographic data, outcome variables, and laboratory parameters) and the analysis of stored serum samples for the quantification of hepcidin. The author was not involved in study design, sample collection, data acquisition, or any analysis pertaining to the main project.

4.1.1 Inflammation in critical illness

Critical illness can be defined as any life-threatening condition requiring support of vital organ functions (Bagchi and Zapol 2018). Patients on intensive care units often have certain pathophysiological manifestations in common, despite a wide spectrum of underlying diseases. This is easily explained by the local and systemic responses of several major organ systems triggered by acute injuries of various aetiologies (Bagchi and Zapol 2018). Many of the pathophysiological features of critical illness, such as fever, capillary leak, and inadequate perfusion, are often mediated by the host response rather than the initial noxious event (Muszynski *et al.* 2016).

The acute response to an injurious stimulus, such as trauma, infection, or surgery, is often dominated by an early proinflammatory phase, which leads to the systemic activation of the immune system referred to as *systemic inflammatory response syndrome (SIRS*) (Muszynski et al. 2016). The initial response is usually mediated by the innate immune system. Neutrophils, monocytes, and dendritic cells are activated

through binding of exogenous or endogenous molecules, such as pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs). The former are components of microorganisms (e.g. lipopolysaccharide on the cell membrane of bacteria), whereas the latter are released endogenously in response to stimuli such as hypoxia or mechanical trauma (e.g. heatshock proteins). Activated immune cells subsequently release proinflammatory cytokines, such as tumour necrosis factor alpha (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6), as well as chemokines like interleukin-8 (IL-8), reactive oxygen species (ROS), and other acute phase proteins. These mediators cause fever, increased vascular permeability, and vasodilation (Muszynski *et al.* 2016, Bagchi and Zapol 2018).

More recently, the occurrence of an anti-inflammatory response in parallel to the systemic activation of the immune system has become apparent (Muszynski *et al.* 2016, Marshall *et al.* 2008). Anti-inflammatory cytokines, like interleukin-10 (IL-10), are also expressed early on. This highlights the complexity underlying the body's responses in critical illness.

Clinically, SIRS manifests in abnormal body temperature, elevated heart rate, increased respiratory rate or signs of hyperventilation, and abnormal white blood cell count. Two or more of these signs are needed for a diagnosis of SIRS (Bone *et al.* 1992). A more severe stage of SIRS is sepsis, which essentially is a systemic inflammatory response mediated by infection. SIRS occurs very frequently in hospitalised patients: one in three of all in-hospital patients meet the diagnostic criteria for SIRS. The prevalence of SIRS is even higher among critically ill patients, with more than 50% of ICU patients and more than 80% of surgical ICU patients affected (Brun-Buisson 2000).

4.1.2 Iron homeostasis during inflammation

The inflammatory response and the homeostasis of iron metabolism are closely linked to each other. Functional iron deficiency due to iron sequestration is commonly seen in inflammatory conditions and serves a protective role in the defence against microbial invasion by limiting the iron supply required for bacteria to grow (Wessling-Resnick 2010). This reduction in iron availability is mediated by hepcidin. As described in Chapter 1.1.2.1, the expression of hepcidin is upregulated during inflammation by IL-6 (Nemeth *et al.* 2004a). Elevated hepcidin levels decrease iron export from enterocytes, hepatocytes, and macrophages. This iron sequestration is responsible for the functional iron deficiency and the associated iron-restricted erythropoiesis seen during inflammation (Heming *et al.* 2011).

Ferritin synthesis is upregulated through interleukin-1 (IL-1) during inflammation. This occurs independently of body iron stores, which makes ferritin an important acute phase reactant (Heming *et al.* 2011). The exact role of hyperferritinaemia in inflammation is not clearly defined: it ranges from the simple role as a biomarker to a more complex involvement in both protective and proinflammatory mechanisms (Kernan and Carcillo 2017). Increases in ferritin contribute to the development of functional iron deficiency due to overall increased iron-binding capacity (Darveau *et al.* 2004).

Other proteins involved in iron metabolism are also affected by inflammation. Ferroportin expression, for example, can be directly regulated in response to an inflammatory stimulus independently of hepcidin and therefore further promote iron retention (Wessling-Resnick 2010). On the other hand, transferrin shows reduced expression in inflammatory settings and is therefore regarded as a negative acute phase protein (Ritchie *et al.* 1999).

The perturbations of iron metabolism during inflammation are particularly troublesome in critical illness, which is essentially defined by the presence of inflammation. The assessment of iron status is therefore complicated substantially in those circumstances, as several proteins involved in iron metabolism respond as acute phase reactants and are consequently elevated or depressed, independent of iron status.

4.1.3 Anaemia of inflammation

The anaemia of inflammation or chronic disease is an obvious example of the link between disturbed iron homeostasis and inflammation.

Anaemia – defined as haemoglobin levels < 12 g/dL in women and < 13 g/dL in men – is very common in critically ill patients. On admission, two out of three patients are anaemic (Vincent *et al.* 2002, Heming *et al.* 2011). After 48 hours, almost 70% were found to have haemoglobin levels less than 12 g/dL (Corwin *et al.* 2004); by day 3, almost 95% have levels below the normal range. Anaemia in these patients persists: a vast majority (87%) were anaemic at discharge (Walsh *et al.* 2006) and about half of the patients had low haemoglobin levels six months after discharge (Bateman *et al.* 2009).

Several factors are involved in the aetiology of anaemia in critical illness. In addition to erythrocyte losses due to haemolysis, frequent phlebotomy, and surgical site bleeding, reduced red blood cell production plays a central role in causing anaemia in critically ill patients (Hayden *et al.* 2012, Prakash 2012). The mechanisms resulting in decreased erythrocyte production include dysregulated iron homeostasis, inhibited erythropoietin release, suppressed erythroid proliferation, and augmented haemophagocytosis (Weiss and Goodnough 2005, Zarychanski and Houston 2008, Hayden *et al.* 2012). Limited iron availability due to hepcidin-mediated iron sequestration leads to iron-restricted erythropoiesis, as described. Additionally, proinflammatory cytokines released during

inflammation directly inhibit erythropoietin transcription and interact with erythroid progenitor cells, which leads to reduced erythropoietic activity and impaired proliferation. Lastly, pharmacological agents commonly used in intensive care treatment (e.g. ACE inhibiters, beta blockers, calcium channel blockers) may also affect erythropoiesis by suppressing the physiological EPO release in the kidney (Hayden *et al.* 2012).

The presence of anaemia in critically ill patients is associated with worse outcomes (Hayden *et al.* 2012). The resulting administration of blood transfusions predicted increased hospital length of stay and higher mortality rates (Vincent *et al.* 2002, Corwin *et al.* 2004).

4.1.4 Aims and rationale

As highlighted in this introduction, iron homeostasis plays a central role in the responses to critical illness. Dysregulations of iron metabolism are frequent in the setting of inflammation and sequels of these perturbations – like anaemia – are associated with worse outcomes in critically ill patients. The systemic inflammatory response further complicates assessment of iron status, as many iron parameters can be misleadingly high (or low) due to their role in the acute phase response.

The retrospective and purely exploratory analysis presented in this chapter focused on iron parameters in a cohort of ICU patients. The goal was to identify changes in iron parameters caused by inflammation and to potentially derive information about the suitability of these markers for the assessment of iron status in critical illness. It was further investigated how these parameters changed over time and whether iron parameters on admission differed based on survival outcomes.

4.2 Study design

4.2.1 Overview of original study protocol

Data and serum samples discussed here were obtained from critically ill patients enrolled in the PERMIT (Pilot Effectiveness of Randomised Mandatory Insulin Therapy) trial. This randomised, clinical trial compared two blood glucose management strategies in a general, non-cardiac ICU setting: mandatory insulin infusion compared to usual care (Watkinson *et al.* 2018). The goal was to establish safety and feasibility of mandatory insulin therapy. Non-diabetic patients deemed to require at least 48 hours of intensive care treatment were eligible for inclusion. Exclusion criteria included pancreatitis, prior hepato-biliary or pancreatic transplant surgery, insulin-producing or pituitary gland tumours, primary diagnosis of head injury, stroke or intracranial haemorrhage as well as absence of a central line access. Data from the entire ICU stay and hospital discharge metrics were collected. Blood samples were obtained from all randomised patients for the duration of their stay in the ICU, specifically on admission and on days 3, 5, 8, and 15. Blood samples were analysed at a central laboratory and serum aliquots stored for further analysis. Additional details about the original study protocol have recently been published by Watkinson *et al.* (2018).

4.2.2 Source data

The author was provided with data and serum samples from 100 patients admitted to the ICU and enrolled in the PERMIT trial. Of those, 80 patients remained in the ICU for at least three days, 61 for at least five days, 44 for at least eight days, and 18 for 15 days or more. Provided laboratory results included haematological indices (haemoglobin, haematocrit, mean cell volume, mean cell haemoglobin, white cell count, platelet count), inflammatory markers (C-reactive protein, interleukin-6), and iron parameters

(serum iron, ferritin, transferrin, transferrin saturation, soluble transferrin receptor). Hepcidin results were not provided. Additional variables in the dataset included patient demographics, APACHE II scores, reasons for admission, hospital lengths of stay, ICU lengths of stay, and mortality data.

The APACHE II (Acute Physiology and Chronic Health Evaluation II) scoring system was used by the PERMIT investigators to classify severity of disease. The score is calculated on admission and integrates 12 routine physiological measurements (e.g. temperature, mean arterial pressure, serum electrolyte levels) and information about previous health status to calculate a score ranging from 0 to 71. Higher APACHE II scores are associated with increased mortality (Knaus *et al.* 1985).

4.2.3 Sample analysis

Frozen serum samples were provided and quantitatively analysed for hepcidin by enzyme-linked immunosorbent assay (see Chapter 2.5). Duplicate samples were analysed using a Quantikine® human hepcidin ELISA kit (DHP250, R&D Systems, Abingdon, United Kingdom) according to the manufacturer's instructions. Due to the expected high hepcidin values, samples were initially diluted by a factor of 1:200 and re-analysed with higher or lower dilution factors if the recorded values fell outside the assay's detection range.

4.2.4 Statistical analysis

Data were analysed using IBM SPSS Statistics (version 24.0 for Macintosh, IBM Corporation, Armonk, NY, USA) and GraphPad Prism (version 7.0a for Macintosh, GraphPad Software, La Jolla, CA, USA).

Comparisons of means between two groups at baseline were made by unpaired two-tailed Student t-test for normally distributed continuous data or Mann-Whitney U test for non-normal data. Comparisons of laboratory parameters between different days were performed using a paired two-tailed Student t-test, if data were normally distributed, or a Wilcoxon signed rank test for non-normally distributed data.

Pearson's correlation was used to analyse linear correlation between two variables. Variables were logarithmically transformed before calculating correlation coefficients if the data were considerably skewed or the correlation of the non-transformed data was not linear. A p value of < 0.05 was considered statistically significant.

4.3 Results

4.3.1 Participant characteristics

Characteristics of the 100 participants, from whom stored samples were available for analysis, are summarised in Table 4.1.

Table 4.1 Characteristics of participating patients

Characteristic, unit	**All participants** (n = 100)
Age, years	69.1 (56.6–78.9)
Sex, n (%)	
Male	68 (68.0)
Female	32 (32.0)
APACHE II Score[1]	22.4 ± 6.7
Reason for ICU admission, n (%)	
Pulmonary pathology	37 (37.0)
Surgical emergency	22 (22.0)
Cardiovascular pathology	17 (17.0)
Planned postoperative admission	13 (13.0)
Trauma	9 (9.0)
Intracranial pathology	1 (1.0)
Other	1 (1.0)
Length of stay, days	
in ICU	6.1 (3.3–12.3)
in hospital	21 (12–43)
Mortality, %	
Mortality in ICU	21
30 days after ICU admission	30
In-hospital mortality	37

[1] *APACHE II scores range from 0–71, higher scores correspond to more severe disease.*

Data are reported as mean ± SD, if normally distributed, or median (IQR), if not normally distributed.

4.3.2 Laboratory parameters on admission

On admission to the intensive care unit, venous blood samples were drawn. Haematological, inflammatory, and iron markers measured in those samples are summarised in Table 4.2. The frequency distributions of relevant admission parameters are described in more detail below.

Table 4.2 Blood parameters on ICU admission

Parameter, unit (normal range)	**All participants** (n = 100)
Haematology	
Haemoglobin, g/dL (12–17)	10.0 ± 1.8 [a]
Mean cell volume, fL (83–105)	91.0 ± 7.0 [a]
Mean cell haemoglobin, pg (27–32)	30.1 ± 2.4 [a]
Haematocrit, % (30–50)	30.2 ± 5.6 [a]
White cell count, × 10^9/L (4–11)	9.00 (5.75–14.19) [a]
Platelet count, × 10^9/L (150–400)	172 (98–251) [a]
Inflammatory markers	
C-reactive protein, mg/L (0–5)	108 (47–160) [b]
Interleukin-6, pg/mL	280.6 (96.3–1,520.9)
Iron parameters	
Iron, µmol/L (11–31)	2.0 (1.1–4.1)
Ferritin, µg/L (10–300)	293.8 (180.3–617.0) [a]
Transferrin, g/L (1.8–3.6)	1.15 ± 0.50
Transferrin saturation, % (16–50)	8.6 (5.0–18.0) [c]
Soluble transferrin receptor, nmol/L	11.3 ± 5.8
Hepcidin, ng/mL	126.5 (68.1–188.5) [c]

Data are reported as mean ± SD, if normally distributed, or median (IQR), if not normally distributed. Missing data: [a] n = 98, [b] n = 92, [c] n = 99.

4.3.2.1 *Haematological parameters*

A large number of participants were anaemic on admission to the intensive care unit, as highlighted in Figure 4.1. Haemoglobin and haematocrit levels were below the lower limits of normality (i.e. 12.5 g/dL for haemoglobin and 35% for haematocrit) in 91.8% and 83.7% of participants, respectively. Mean cell volume and haemoglobin content were within the normal range in the majority of cases – 87.8% and 79.6% respectively – suggesting mostly normocytic and normochromic anaemia.

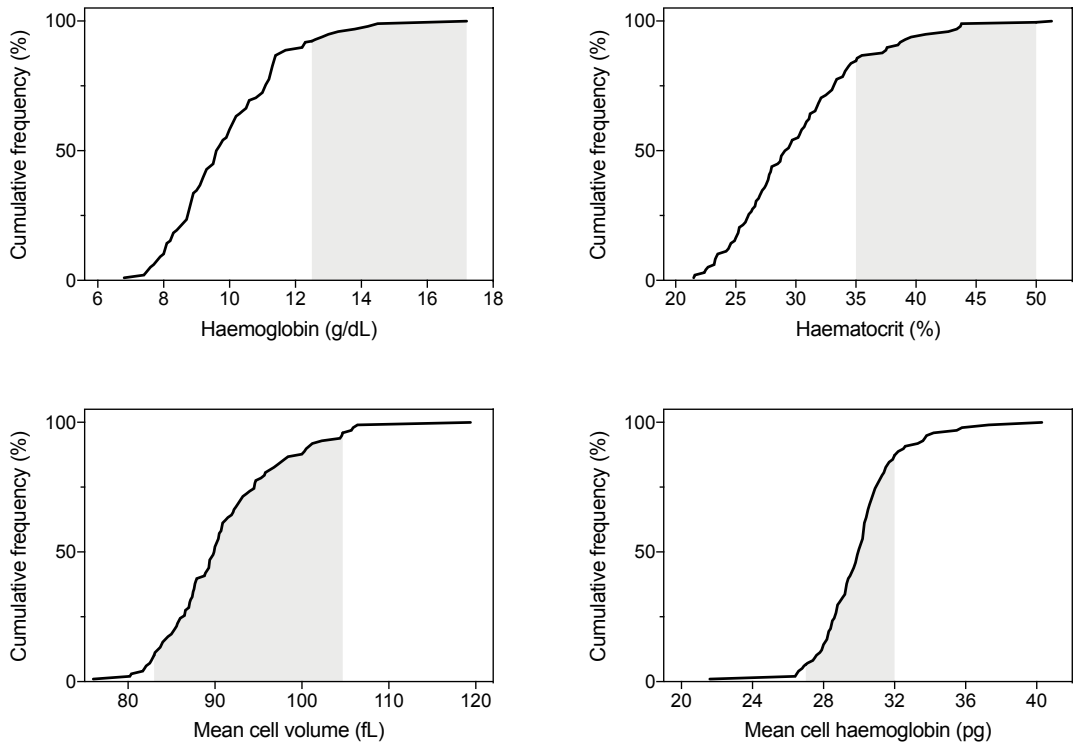


Figure 4.1 Distribution of haematological parameters on admission. Cumulative frequency distributions are plotted for haemoglobin (*top left*), haematocrit (*top right*), mean cell volume (*bottom left*), and mean cell haemoglobin (*bottom right*). The grey-shaded areas indicate the normal range of values for each parameter.

4.3.2.2 *Inflammatory parameters*

Cumulative frequency distributions of main inflammatory markers measured on admission to the intensive care unit are shown in Figure 4.2. White blood cell count – despite being a haematological parameter – is included here due to its role as a marker of infection and inflammation. All measured inflammatory parameters confirmed the presence of a pro-inflammatory state on admission. C-reactive protein levels were outside normal limits (i.e. > 5 mg/L) in 98.9% of participants and white cell count was elevated beyond normal limits (i.e. > 11 × 10^9/L) in 41.8%. All participants presented with interleukin-6 levels above 5.0 pg/mL. C-reactive protein and interleukin-6 levels were excessively raised in some participants and reached the upper detection limit of the respective assay.

4.3.2.3 *Iron parameters*

Disturbances in iron homeostasis were commonly observed in the studied cohort on admission to the intensive care unit, as shown by the cumulative frequency distributions of typical iron markers in Figure 4.3. Ferritin and hepcidin levels were increased beyond normal limits (i.e. > 300 µg/L for ferritin and > 55 ng/mL for hepcidin) in 49.0% and 80.8% of participants, respectively. Ferritin levels were raised by more than an order of magnitude in some participants. Serum iron, transferrin, and transferrin saturation were low in the majority of participants: 93.0% had iron levels below 11 µmol/L, 91.0% showed transferrin levels lower than 1.8 g/L, and 67.7% presented with a transferrin saturation of less than 16%. Soluble transferrin receptor measurements were within the normal reference limits of the assay (i.e. 8.7–28.1 nmol/L) in 60% of participants; only 2% had values higher than 28.1 nmol/L.

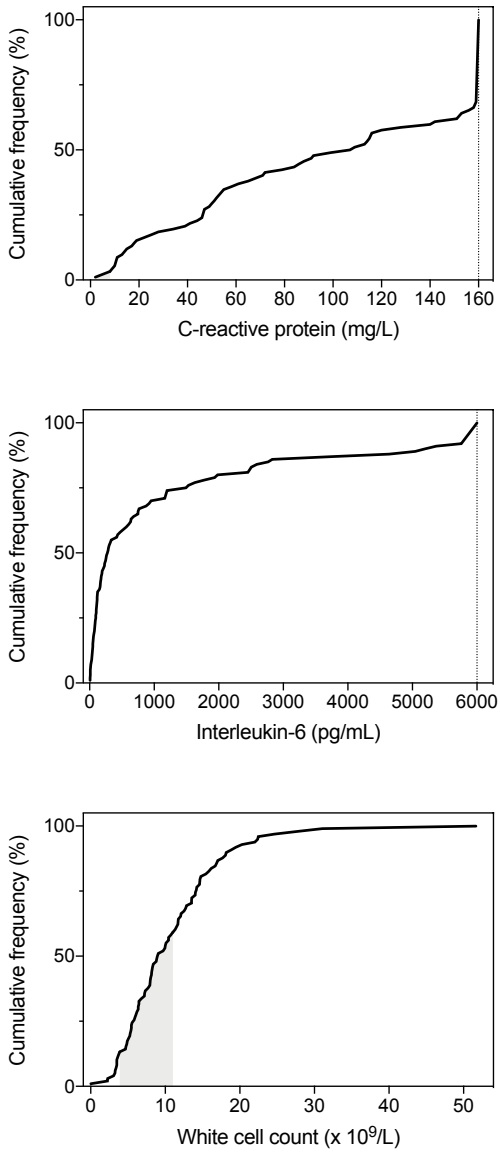


Figure 4.2 Distribution of inflammatory parameters on admission. Cumulative frequency distributions are plotted for C-reactive protein (*top*), interleukin-6 (*middle*), and white cell count (*bottom*). The grey-shaded areas correspond to the normal range of values for each parameter. Vertical dotted lines indicate the upper detection limit of the assay used.

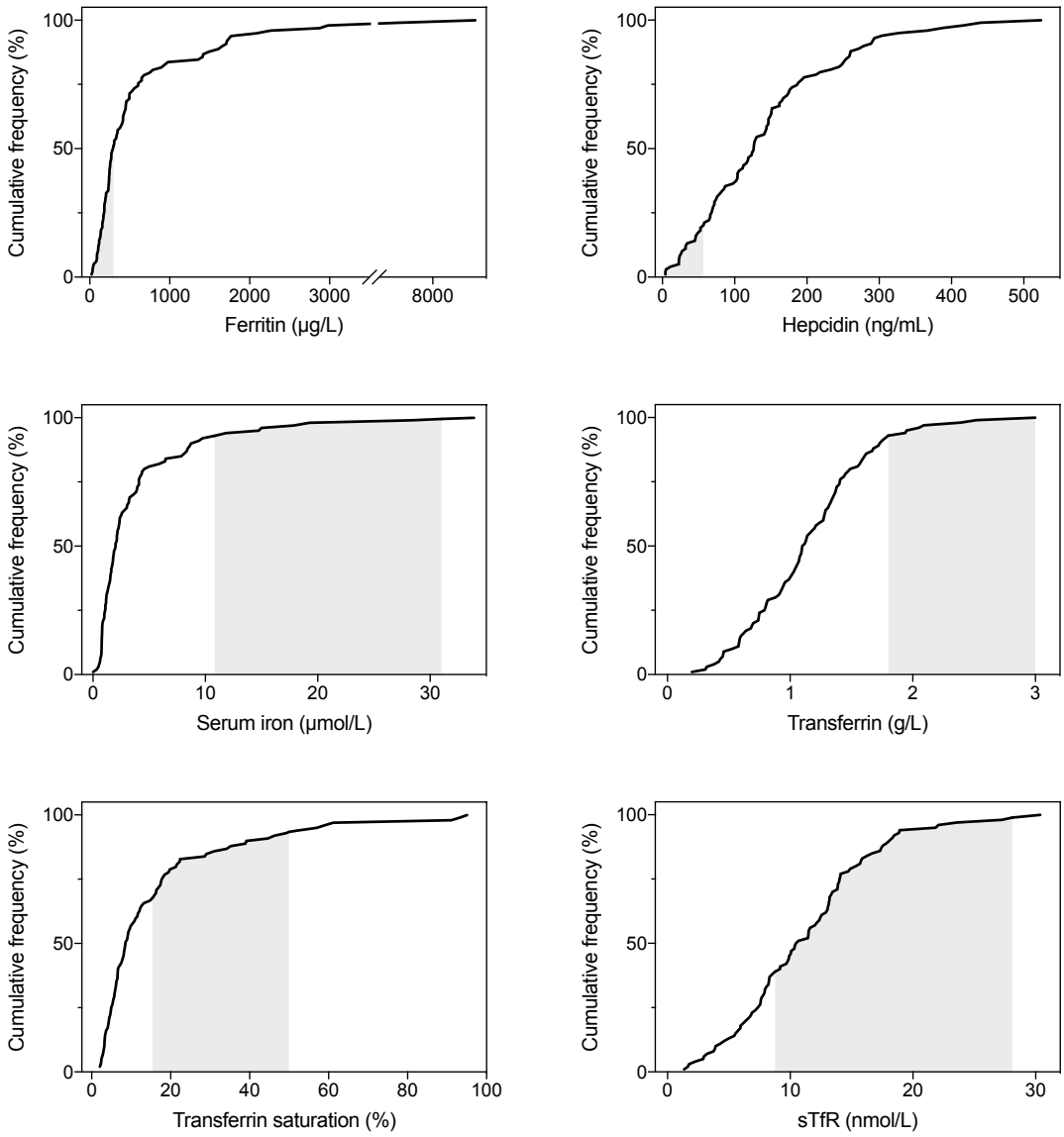


Figure 4.3 Distribution of iron parameters on admission. Cumulative frequency distributions are plotted for ferritin (*top left*), hepcidin (*top right*), serum iron (*middle left*), transferrin (*middle right*), transferrin saturation (*bottom left*), and soluble transferrin receptor (*bottom right*). The grey-shaded areas indicate the normal range of values for each parameter.

4.3.3 Correlation of inflammation and iron status

The correlations of iron parameters measured on admission with inflammatory markers are shown in Figure 4.4 for C-reactive protein and Figure 4.5 for interleukin-6. Several markers of iron status were significantly correlated with inflammation.

Elevated levels of C-reactive protein were associated with increases in ferritin ($r = 0.327$, $p = 0.002$) and hepcidin ($r = 0.448$, $p < 0.001$) as well as decreases in serum iron ($r = -0.515$, $p < 0.001$), transferrin ($r = -0.376$, $p < 0.001$), and transferrin saturation ($r = -0.341$, $p < 0.001$). Soluble transferrin receptor was not affected by changes in C-reactive protein ($r = -0.014$, $p = 0.895$).

For interleukin-6, similar results were found for hepcidin, serum iron, and transferrin. Hepcidin increased with rising IL-6 levels ($r = 0.463$, $p < 0.001$), while serum iron and transferrin decreased ($r = -0.407$ and $r = -0.442$, respectively, both $p < 0.001$). In contrast to CRP, ferritin and transferrin showed no correlation with IL-6 levels ($r = 0.149$, $p = 0.143$ and $r = -0.192$, $p = 0.057$, respectively). Interestingly, soluble transferrin receptor levels were lower in the presence of higher IL-6 concentrations ($r = -0.503$, $p < 0.001$).

These findings highlight a complex relationship between inflammatory markers and iron parameters. While hepcidin, transferrin, and serum iron were correlated with increasing CRP and IL-6 levels, ferritin and transferrin saturation were only significantly correlated with CRP changes. Soluble TfR on the other hand remained unaffected by CRP increases, but showed a strong negative correlation with IL-6.

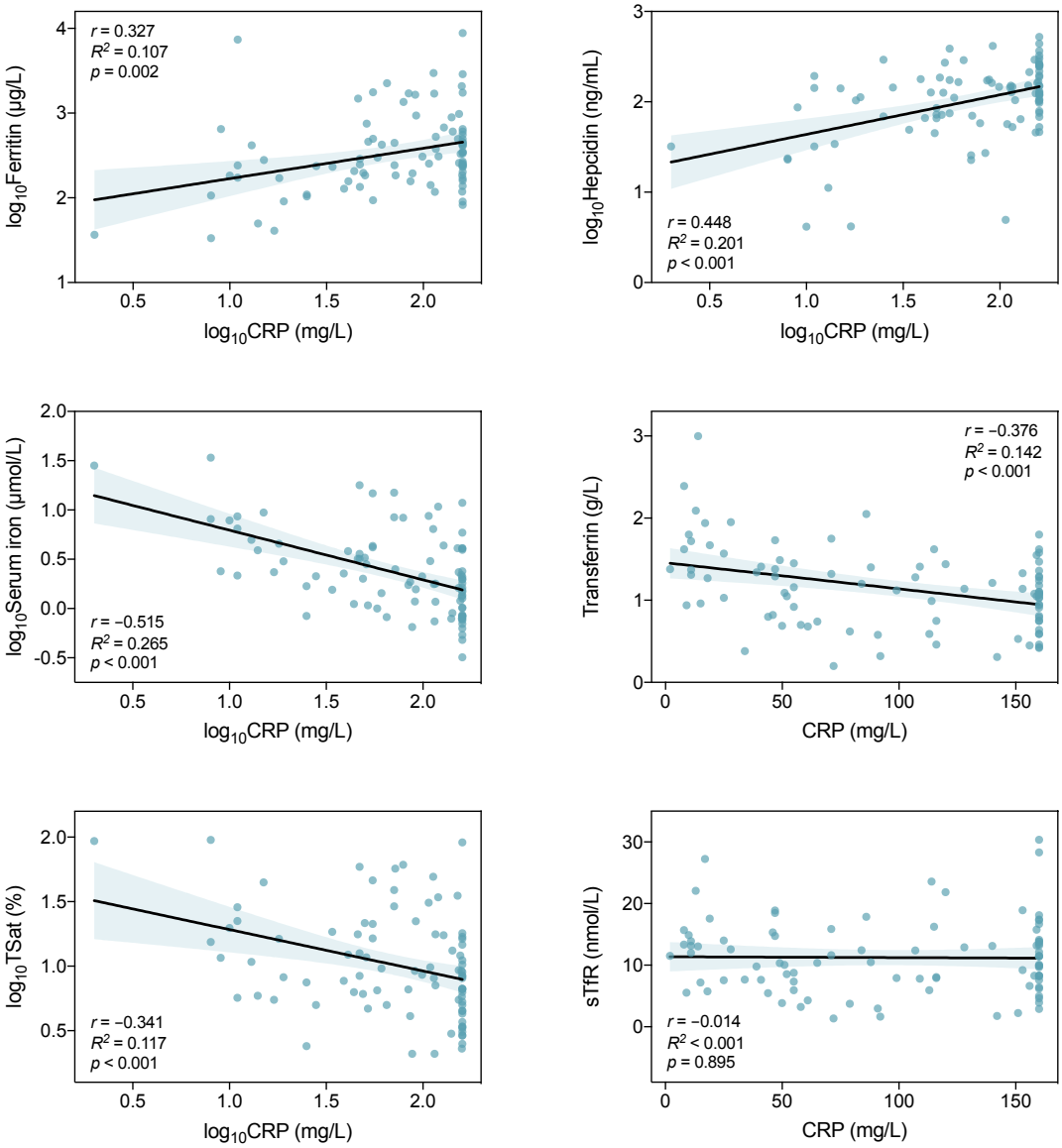


Figure 4.4 Correlation of C-reactive protein with iron status markers. The scatterplots show levels of C-reactive protein plotted against various markers of iron status measured on admission. CRP was significantly correlated with ferritin, hepcidin, iron, transferrin, and transferrin saturation, but not with soluble transferrin receptor. Coefficients of correlation (r) and determination (R^2), p values, as well as linear regression lines with 95% confidence bands (shaded areas) are shown for each graph.

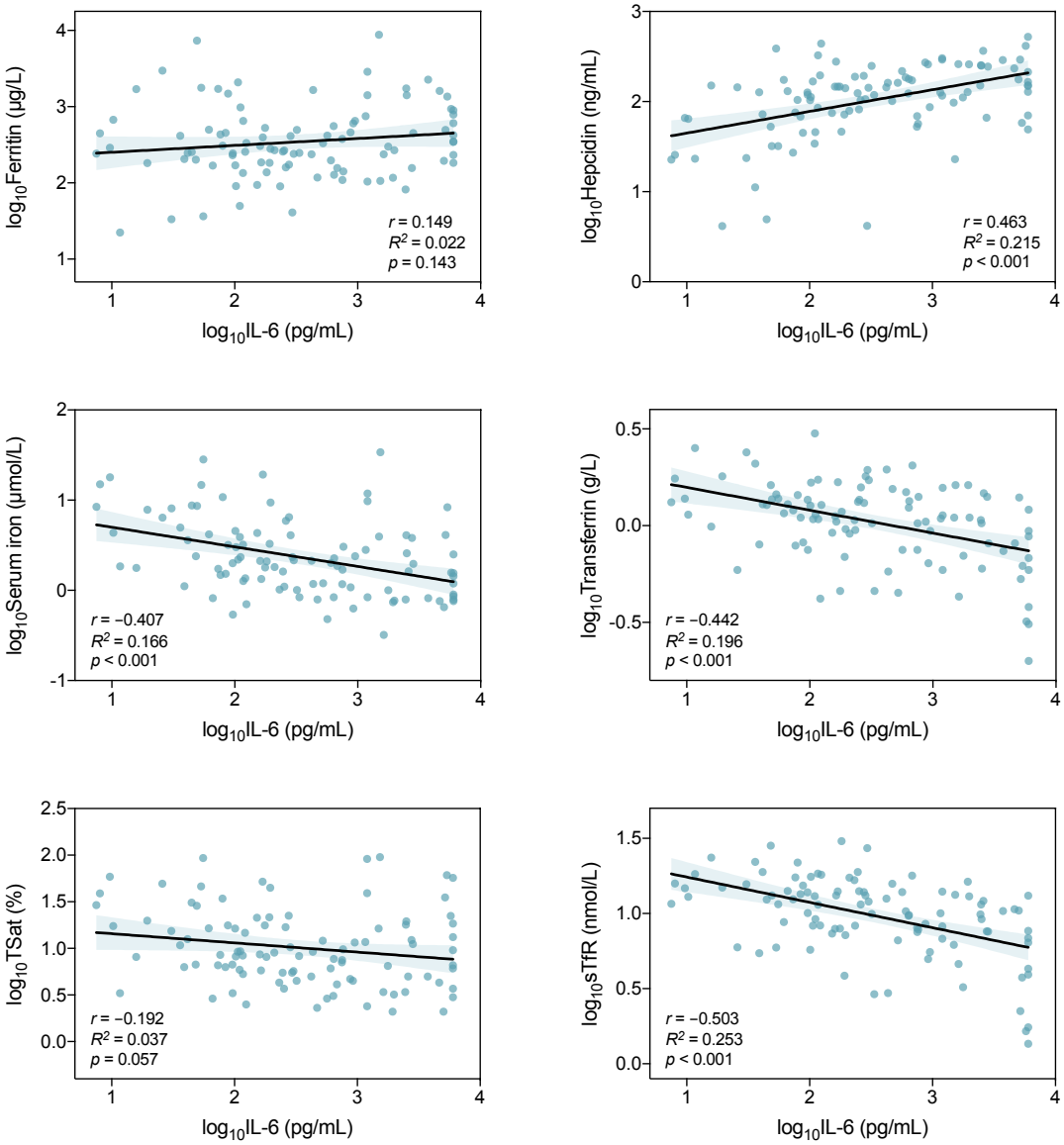


Figure 4.5 Correlation of interleukin-6 with iron status markers. The scatterplots show levels of interleukin-6 plotted against various markers of iron status measured on admission. IL-6 was significantly correlated with hepcidin, iron, transferrin, and soluble transferrin receptor, but not ferritin and transferrin saturation. Coefficients of correlation (*r*) and determination (R^2), *p* values, as well as linear regression lines with 95% confidence bands (shaded areas) are shown for each graph.

4.3.4 Survival and iron parameters

In Figure 4.6, iron parameters on admission are shown grouped by survival outcomes at hospital discharge. Overall, the mortality rate before discharge was 37%.

Of the six iron parameters, baseline transferrin was significantly different between the two outcome groups. Transferrin levels on admission were 0.21 g/L (95% confidence interval: 0.01–0.41 g/L, $p = 0.043$) lower in patients who eventually died before discharge compared to patients surviving their hospital stay. In both groups, however, the average baseline transferrin was already below the normal range (1.8–3.6 g/L): survivors had an admission transferrin level (mean ± SD) of 1.23 ± 0.50 g/L, compared to 1.02 ± 0.47 g/L in non-survivors. Furthermore, lower transferrin levels on admission were correlated with increased length of hospital stay ($r = -0.273$, $p = 0.006$).

Other iron parameters did not differ significantly on admission between the two outcome groups (ferritin: $p = 0.411$, hepcidin: $p = 0.070$, serum iron: $p = 0.043$, TSat: $p = 0.349$, sTfR: $p = 0.640$).

As expected, non-survivors had more severe disease. They were significantly older (mean difference: 14.2 years, 95% confidence interval: 8.1–20.4 years, $p < 0.001$), had higher APACHE II scores (mean difference: 4.0, 95% confidence interval: 1.3–6.7, $p = 0.004$) and higher IL-6 levels (median difference: 490.8 pg/mL, $p = 0.032$).

Comparisons between the two outcome groups were performed by Student t-test (transferrin, sTfR, age, and APACHE II) or Mann-Whitney U test (ferritin, hepcidin, iron, TSat, and IL-6).

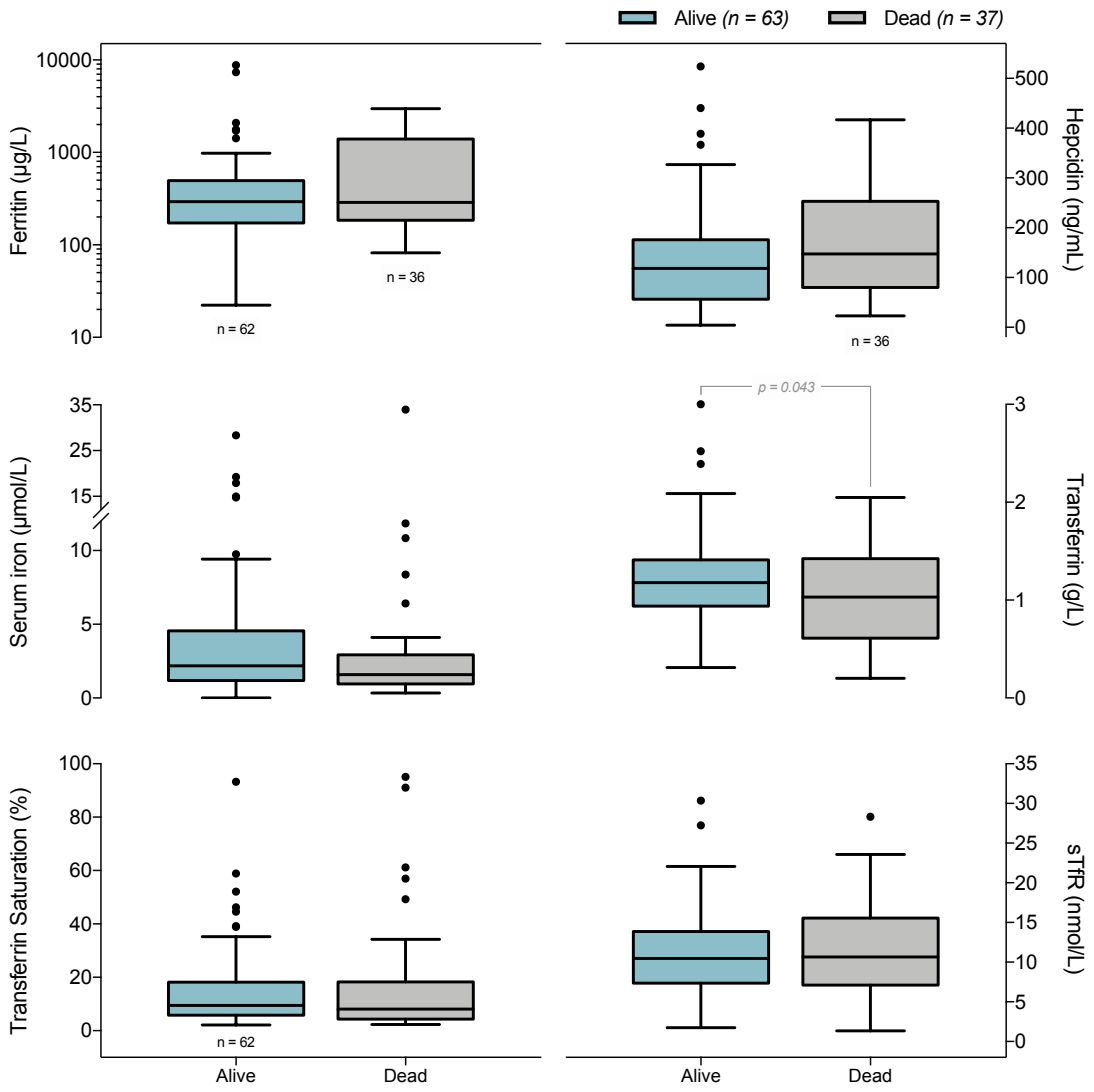


Figure 4.6 Hospital survival and markers of iron status. The boxplots show iron parameters measured on admission grouped by survival to hospital discharge. Transferrin was significantly lower in patients who did not survive the hospital stay. Other iron parameters were not different. Solid lines indicate the median, boxes represent the interquartile range, whiskers extend to 1.5 times the interquartile range, and circles show outliers. Statistical comparisons were performed by Student t-test (transferrin and sTfR) or Mann-Whitney U test (ferritin, hepcidin, iron, and transferrin saturation).

4.3.5 Temporal trends in laboratory parameters

Changes in haematological, inflammatory, and iron parameters over the course of the ICU stay are shown cumulatively in Figure 4.7 and Figure 4.8. Differences indicated below refer to the change from admission day to day 15.

On average, haemoglobin decreased from admission to subsequent days (mean difference: −1.1 g/dL, 95% confidence interval: −1.9 to −0.3 g/dL, $p = 0.014$), while white blood cells increased slightly during the first few days (median difference: 2.3×10^9/L, $p = 0.193$).

In agreement with its delayed acute phase response, CRP peaked on day 3, but did not significantly decrease from admission to day 15 (median difference: −45.5 mg/L, $p = 0.074$). IL-6 levels were highest on admission and dropped subsequently (median difference: −244.1 pg/mL, $p < 0.001$).

Ferritin levels remained mostly unchanged over time (median difference: 204.8 µg/L, $p = 0.071$), but hepcidin decreased significantly (median difference: −45.0 ng/mL, $p = 0.035$). Serum iron (median difference: 0.7 µmol/L, $p = 0.071$) and transferrin saturation (median difference: 1.3%, $p = 0.744$) showed no clear tendencies either. Transferrin (mean difference: 0.4 g/L, 95% confidence interval: 0.3–0.6 g/L, $p < 0.001$) and soluble transferrin receptor (mean difference: 7.2 ng/mL, 95% confidence interval: 4.1–10.3 ng/mL, $p < 0.001$) on the other hand both increased after admission.

In summary, anaemia worsened slightly after admission, while IL-6 levels improved. Changes in iron parameters included a decrease in hepcidin and increases in transferrin and soluble transferrin receptor levels.

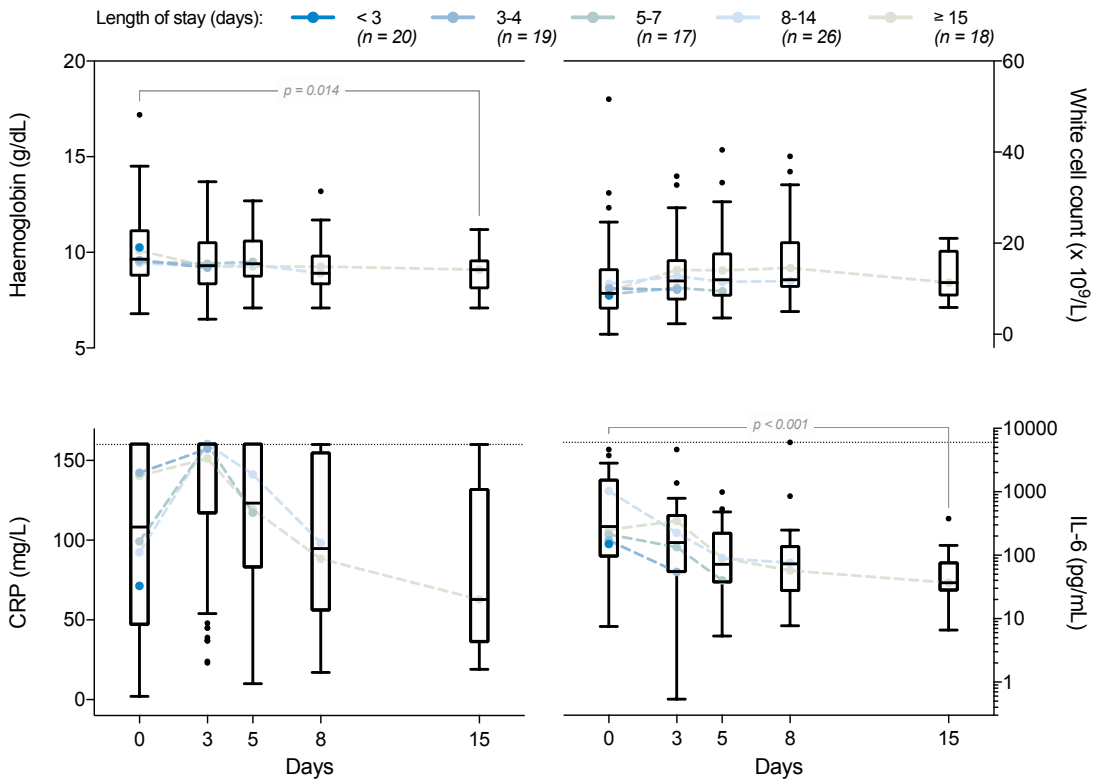


Figure 4.7 Temporal changes in haematological and inflammatory parameters. The boxplots show levels of haemoglobin, white cell count, CRP, and IL-6 cumulatively for all participants with measurements performed on a given day (day 0: n = 100, day 3: n = 80, day 5: n = 61, day 8: n = 44, day 15: n = 18). Dashed lines indicate temporal changes in medians (coloured circles) for subgroups of different lengths of stay. For boxplots: solid lines indicate the median, boxes represent the interquartile range, whiskers extend to 1.5 times the interquartile range, and black circles show outliers. Statistical comparisons between days 0 and 15 were performed by paired Student t-test (haemoglobin) or Wilcoxon signed rank test (white cell count, CRP, IL-6).

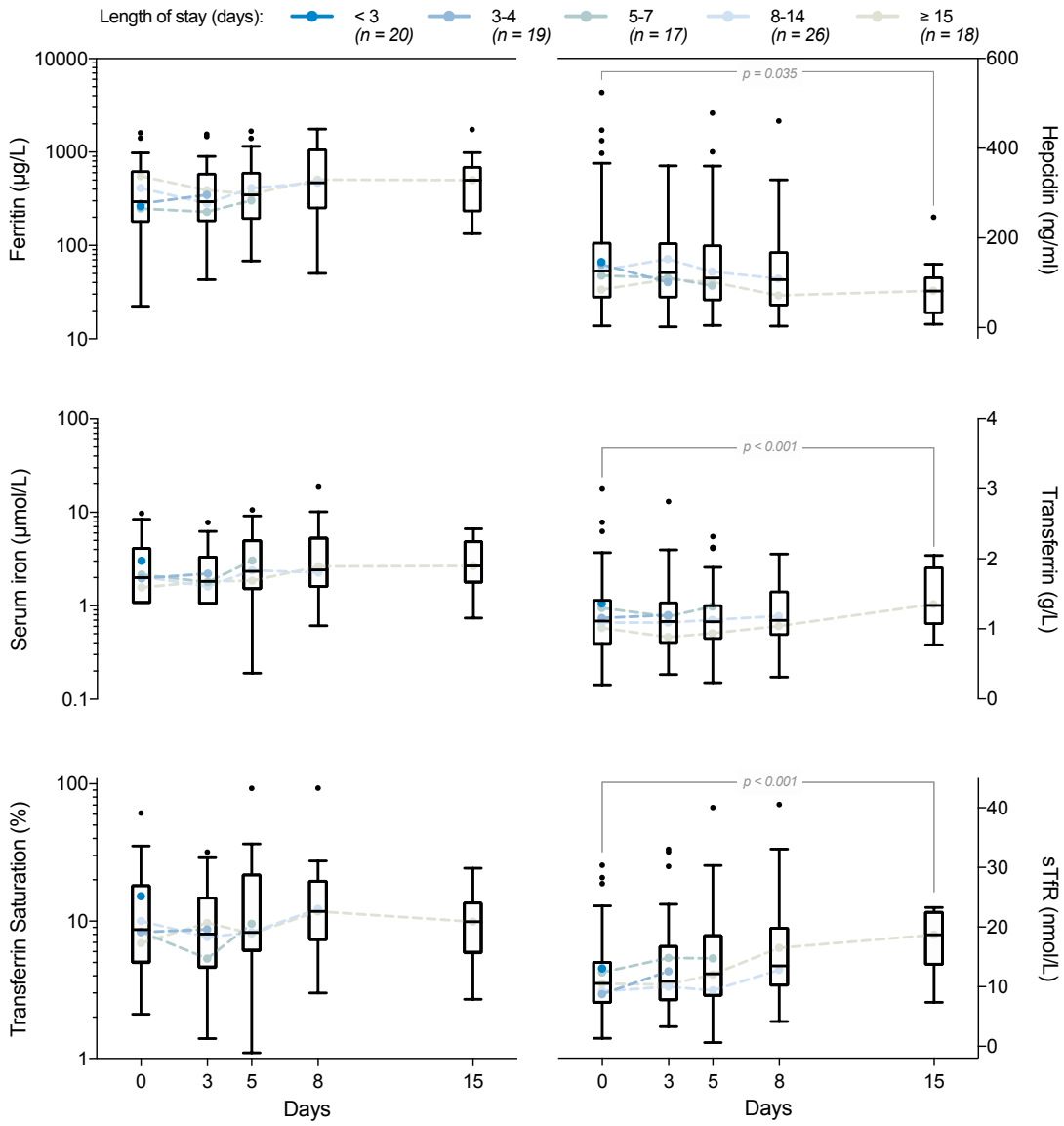


Figure 4.8 Temporal changes in iron parameters. The boxplots show levels of various iron parameters cumulatively for all participants with measurements performed on a given day (day 0: n = 100, day 3: n = 80, day 5: n = 61, day 8: n = 44, day 15: n = 18). Dashed lines indicate temporal changes in medians (coloured circles) for subgroups of different lengths of stay. For boxplots: solid lines indicate the median, boxes represent the interquartile range, whiskers extend to 1.5 times the interquartile range, and black circles show outliers. Statistical comparisons between days 0 and 15 were performed by paired Student t-test (transferrin and sTfR) or Wilcoxon signed rank test (ferritin, hepcidin, iron, and transferrin saturation).

4.4 Discussion

4.4.1 Main findings

The findings presented in this chapter provide insight into the complex physiological interplay of iron homeostasis and inflammation in critically ill patients. As was expected from the underlying pathophysiology and the literature, the cohort studied here had a high prevalence of anaemia, inflammation, and dysregulated iron parameters.

Consistent with previous reports, the observed prevalence of anaemia in the study cohort was high on admission, and haemoglobin levels declined further over time. With nine out of ten patients having abnormally low haemoglobin levels on admission, the presented cohort was even more anaemic than expected (Heming *et al.* 2011). Similarly, patients were in a highly pro-inflammatory state, with elevated levels of CRP and IL-6 in almost all cases. Abnormalities in iron parameters were equally common, with hepcidin and ferritin elevated and iron, transferrin, and transferrin saturation low in most patients. These observations are in agreement with the underlying physiology and have been reported in critically ill patients (Bobbio-Pallavicini *et al.* 1989). Soluble transferrin receptor, which is generally thought to be insensitive to inflammatory stimulation, was normal in most patients, in agreement with research on critically ill patient populations (Kolias *et al.* 2001, Munoz *et al.* 2005).

Correlations between inflammatory parameters and markers of iron homeostasis were analysed. Hepcidin, transferrin, and serum iron were significantly correlated with both CRP and IL-6 levels. Hepcidin's role in inflammation-mediated perturbations of iron homeostasis is well-established (Nemeth and Ganz 2009), as is its transcriptional upregulation by IL-6 (Nemeth *et al.* 2004a). Serum iron levels are consequently reduced due to hepcidin-mediated iron sequestration. Reduction in transferrin reflects its role as negative acute phase protein (Ritchie *et al.* 1999).

Correlation of inflammatory markers with ferritin and soluble transferrin levels revealed some ambiguity. Ferritin increased with increasing CRP concentrations, but was not correlated with IL-6. Elevated ferritin is an established acute phase reactant and therefore – unsurprisingly – elevated during inflammation. The lack of a significant correlation with IL-6 could be explained by the fact that ferritin expression in response to inflammatory stimulation is primarily mediated by IL-1 and TNF-α rather than IL-6 (Kernan and Carcillo 2017). This was also shown *in vitro*, where translational induction of ferritin was considerably greater in response to IL-1β than IL-6 (Rogers 1996). Comparisons between bacterial and viral infections led Slaats *et al.* (2016) to propose that distinct inflammatory pathways and different cytokines initiate the acute phase response of either CRP or ferritin.

Interestingly, the soluble transferrin receptor levels were not affected by CRP, but showed significant negative correlation with IL-6. The lack of correlation with CRP was expected, since sTfR is generally thought to be insensitive to inflammatory stimulation (Allen *et al.* 1998, Beguin 2003). The correlation with IL-6 is therefore intriguing and unexpected. A reduction of plasma transferrin receptor in response to inflammation has been described in rodents (Beguin *et al.* 1988). Transferrin receptor was also shown to be downregulated by the inflammatory cytokine interferon-γ (IFN-γ) on human monocytes (Byrd and Horwitz 1989, Byrd and Horwitz 1993). While these findings suggest a possible role for inflammation in modulating transferrin receptor – and therefore also its truncated fragment sTfR – the observed correlation with IL-6 lacks a clear mechanistic explanation.

Comparison of admission iron parameters grouped by survival to hospital discharge indicated that survivors had significantly higher transferrin levels when admitted. While this observation does not imply any causative effect due to the study design, it is in line

with previous research, which described similar differences and found a beneficial effect of higher transferrin values on short and long-term survival (Tacke *et al.* 2016).

The observed temporal changes of laboratory parameters resemble findings published recently. In a prospective study, Cherry-Bukowiec *et al.* (2018) examined time-dependent changes in iron, inflammatory, and haematological parameters in 100 surgical ICU patients. The authors found a significant decrease in hepcidin levels over time, which was associated with a reduction in inflammatory markers (CRP and IL-6), but no amelioration of anaemia. As shown in Chapter 4.3.5, similar changes were seen in the present cohort. The observed increases in transferrin and sTfR may reflect overall recovery from the hyperinflammatory baseline state.

4.4.2 Assessment of iron status in critical illness

Iron status assessment is complicated considerably in the presence of inflammation and anaemia of inflammation as several iron parameters are directly or indirectly affected by inflammation. Two commonly used markers in the diagnosis of iron deficiency are ferritin and transferrin saturation. Both are difficult to interpret in critically ill patients. As mentioned, inflammatory cytokines induce ferritin independently of the body's iron store. While this response is associated with a worse clinical status of the critically ill patient (Bobbio-Pallavicini *et al.* 1989), it limits the usefulness of ferritin in the diagnosis of iron deficiency. Bobbio-Pallavicini *et al.* (1989) correctly concluded, that ferritin "appears to be useful in predicting the patient's outcome, but is not reliable in evaluating iron stores in stressed patients". Thus, iron deficiency may exist in critically ill patients in the presence of normal or high ferritin levels (Heming *et al.* 2011). Transferrin saturation – the percentage of transferrin binding sites occupied by iron – is indirectly affected by alterations in iron and transferrin levels. Transferrin is reduced by

inflammation (negative acute phase protein), and so is serum iron due to sequestration mediated by hepcidin. Consequently, the interpretation of TSat in the setting of inflammation becomes difficult and its use for the diagnosis of iron deficiency limited.

Soluble transferrin receptor has been proposed as a promising marker. The protein remains relatively unaffected by inflammation, but is increased in iron deficiency and represents a marker for unmet iron need. It is therefore useful in the differentiation of absolute iron deficiency from functional iron deficiency in inflammatory conditions (Weiss and Goodnough 2005). Soluble TfR is a sensitive marker for identifying iron-deficient erythropoiesis, but it needs to be assessed in conjunction with other iron parameters (Chang *et al.* 2007).

The so-called ferritin index, defined as sTfR/$\log_{10}$Ferritin ratio, has been proposed as an even better, more sensitive marker to differentiate between anaemia of inflammation due to functional iron deficiency and absolute iron deficiency in the presence of inflammation (Punnonen *et al.* 1997). A low ratio suggests anaemia of inflammation, while a higher ratio indicates concomitant iron deficiency. The ferritin index has been extensively studied and found to be a valuable tool in the assessment of iron deficiency and anaemia in inflammation (Skikne *et al.* 2011).

Hepcidin might also be useful in the assessment of iron status. Hepcidin levels can be suppressed by iron deficiency even in the presence of inflammation and may therefore be useful in the diagnosis of iron deficiency in critical illness (Lasocki *et al.* 2010).

Other markers have been suggested for the diagnosis of iron deficiency in inflammation. These include the fraction of hypochromic red blood cells (increased in iron-restricted erythropoiesis), the reticulocyte haemoglobin content (decreased in iron-restricted erythropoiesis) as well as erythrocyte zinc protoporphyrin (increased in iron deficiency) (Heming *et al.* 2011).

4.4.3 Iron status and outcomes in critical illness

Much research concerned with outcomes in the context of iron metabolism and critical illness has focused on anaemia of inflammation as a consequence of functional iron deficiency. Overall, anaemia has been associated with worse outcomes, such as increased mortality, in critically ill patients (Prakash 2012, Hayden *et al.* 2012).

Increased levels of ferritin correlated with disease severity and outcomes in critically ill patients. In a small study of 51 post-operative ICU patients, Bobbio-Pallavicini *et al.* (1989) found a positive correlation between initial ferritin levels and severity of disease as measured by the Simplified Acute Physiology Score (SAPS, an admission screening score comparable to APACHE II). Ferritin also showed good prognostic value for patient outcomes: higher levels were associated with more severe complications.

The presence of functional iron deficiency was also shown to influence outcomes in critical illness. In a cohort of 51 patients, functional iron deficiency was present in 35% on admission (Patteril *et al.* 2001). These patients remained in the ICU longer and met SIRS criteria for prolonged periods of time compared with non-iron deficient patients. Length of stay was also correlated with the severity of functional iron deficiency. Mortality and APACHE II scores were not affected by functional iron deficiency though (Patteril *et al.* 2001).

In a more recent prospective study of 112 septic and 43 non-septic patients in intensive care units, the prognostic value of various iron parameters measured on admission was analysed (Tacke *et al.* 2016). Low serum iron, low transferrin saturation, and high transferrin were found to correlate with short and long-term survival. TSat in particular was a strong predictor of outcomes. However, the relevance of these findings was critiqued by others (Jiang *et al.* 2016, Talbot *et al.* 2016). Particularly the utility of TSat as a measure of iron availability in critically ill patients was questioned. As pointed out

before, interpretation of TSat in a setting of reduced transferrin and reduced iron levels may not be appropriate.

4.4.4 Limitations

Several limitations of this study need to be considered. It must not be forgotten that the study cohort used for the present analyses was preselected on the basis of the original study protocol (Watkinson *et al.* 2018). Selection bias was therefore inevitable. Eligibility criteria of the original PERMIT study excluded a number of clinical diagnoses, such as pre-existent diabetes, neurological disorders, and patients without central venous access. The latter might not be highly relevant, as most patients on intensive care units will have a central line placed as part of routine care. On the other hand, exclusion of diabetic patients – while necessary for the purposes of the original trial – may have skewed the presented results, as iron metabolism may influence glucose homeostasis and vice versa in a variety of tissues (Fernandez-Real *et al.* 2015). Due to varying lengths of ICU stay, sample size decreased over time, which may have biased the analysis of temporal changes. It is not unlikely that patient characteristics and disease severity shifted substantially over time. If the proportion of more severely ill patients increased over time, this would have influenced any observations about temporal trends.

The study design represents a further limitation: The analysis was purely exploratory and not hypothesis-driven. As such it was focused on reproducing important physiological concepts rather than deriving conclusions or predictions about outcomes. Adequately powered studies with prespecified hypotheses would be require to achieve the latter goal.

4.4.5 Conclusions

In conclusion, the present analysis confirms the complex relationship between iron status and inflammatory responses in critically ill patients. A high prevalence of inflammation, anaemia, and iron metabolism perturbation was observed in the studied cohort. These findings reflect the underlying physiology of iron homeostasis in inflammation and are mostly in agreement with the current knowledge base. However, the results also pose interesting questions (e.g. regarding the correlation of sTfR with IL-6). As highlighted, the assessment of iron status in critical care settings remains a challenge and will require further research.

CHAPTER 5:
Prolonged hypoxic exposure and markers of iron and oxygen homeostasis

5.1 Introduction

This chapter is concerned with a small human physiology study of mild exposure to hypoxia. The author was a collaborator on this project and only involved with certain aspects of conducting this study. Only results and analyses from investigations in which the author was personally involved are presented here. The specific contributions consisted in supervision of study participants and data collection during one of four exposure weeks as well as the retrospective analysis of stored plasma samples. Raw data about demographics and baseline parameters were provided. The author was neither involved in study design and recruitment, nor in data collection and analysis concerning the primary study endpoint.

5.1.1 Oxygen, hypoxia and HIF

Oxygen is the second largest component of ambient air, making up approximately 21% of the volume. Physiologically important is the partial pressure of oxygen, which determines the actual amount of oxygen molecules per volume unit of air. The partial pressure is defined as the product of atmospheric pressure and the percentage of oxygen in the air. Hypoxia can therefore result from a reduction in either one of those factors. Reduction in atmospheric pressure results in *hypobaric hypoxia* and is most commonly experienced at altitude or in commercial aviation. In contrast, reduction in oxygen percentage is referred to as *normobaric hypoxia*, which is normally induced artificially in controlled laboratory settings by manipulating the composition of inspired gases (Howard *et al.* 1995).

The physiology underlying oxygen sensing and responses to hypoxia has been extensively discussed in Chapter 1.2. To summarise, hypoxia triggers a wide range of physiological responses aimed at restoring and improving oxygen uptake, transport, and

utilisation. In particular the HIF signalling pathway plays a central role in mediating these responses at the systemic and cellular level (Smith *et al.* 2008b, Semenza 2009). As described before, HIF regulates the expression of a large variety of genes, including those involved in erythropoiesis (e.g. EPO) and iron homeostasis (e.g. hepcidin).

5.1.2 Effects of mild hypoxia

While severe oxygen deprivation and anoxia can have disastrous consequences, mild hypoxia is generally well-tolerated and encountered frequently at high altitudes, such as onboard commercial airplanes. As detailed in this section, systemic responses and activation of HIF can occur even at very mild levels of hypoxia.

The pressurised cabin onboard airliners provides an ideal setting to study these responses, as the partial pressure of oxygen at cruising altitude is reduced to a sea level equivalent of approximately 15%. Studies in healthy individuals have shown that EPO levels increased significantly within hours from take-off (Gunga *et al.* 1996). In addition to cellular adaptation to hypoxia, systemic responses were also shown. A short and mild hypoxic stimulus, such as long-haul air travel, can induce significant ventilatory acclimatisation (Fatemian *et al.* 2001). Furthermore, augmented hypoxic pulmonary vascular responses (as described in Chapter 1.2.3.3) were noted. Pulmonary arterial systolic pressure significantly increased in healthy volunteers during commercial air travel, which raised the question about potentially harmful effects of pulmonary hypertension in susceptible individuals travelling by air (Smith *et al.* 2013, Smith *et al.* 2012).

Exercise capacity is also improved upon exposure to hypoxia. Altitude acclimatisation resulted in improved aerobic performance and increased erythropoiesis in endurance athletes living at high altitude but training at lower elevations (Levine and Stray-

Gundersen 1997, Wehrlin *et al.* 2006, Brugniaux *et al.* 2006). While increased production of red blood cells seems to be an obvious explanation for improved performance, other non-haematological mechanisms, such as improved mitochondrial efficiency, play a role as well (Gore *et al.* 2007). Intermittent hypoxia also improved exercise performance and tolerance in healthy elderly men (Shatilo *et al.* 2008), as well as in patients with mild COPD (Burtscher *et al.* 2009) and coronary artery disease (Burtscher *et al.* 2004).

Long-term exposure to mild hypoxia can further affect survival. In the late 1970s, Mortimer *et al.* (1977) described an inverse relationship between mortality from atherosclerotic heart disease and altitude in groups of men residing at five different elevations. Similarly, Faeh *et al.* (2009) found living at higher altitude to be protective from cardiovascular and stroke mortality.

5.1.3 Implications for perioperative medicine

The observations described in the previous section support the idea that mild hypoxia – sufficient to provoke physiological responses and activate gene expression – can improve oxygen metabolism.

During and after major surgery, oxygen requirements can be increased significantly. The physiological stress response induced by the surgical trauma leads to a systemic inflammatory insult, which can persist for days (Minto and Biccard 2014). The release of cytokines and catecholamines causes endocrine and metabolic alterations resulting in a catabolic state with an associated increased demand for oxygen (Burton *et al.* 2004, Moor *et al.* 2017). Under normal circumstances, the elevated oxygen consumption is met by an increase in cardiac output and tissue oxygen extraction. However, not all patients have the necessary physiological reserve to increase their oxygen delivery

(DO_2) appropriately. These patients are particularly at risk of major post-operative complications and might derive benefits from the preoperative DO_2 optimisation (Davies and Wilson 2004). This requires the adequate preoperative identification of patients at risk. A method commonly used for that purpose is cardiopulmonary exercise testing, which objectively quantifies an individual's ability to meet increased oxygen requirements (Minto and Biccard 2014) and also predicts long-term survival (Colson *et al.* 2012).

Identifying and potentially optimising high-risk patients preoperatively is important considering the relatively high rates of morbidity and mortality following major surgery. A large cohort study of over 45,000 non-cardiac surgical patients across nearly 500 European hospitals identified an overall mortality rate of 4% following surgery (Pearse *et al.* 2012). High-risk surgical patients accounted for most perioperative deaths (Pearse *et al.* 2011).

5.1.4 Aims and rationale

Mild hypoxia has beneficial physiological effects and even reduces mortality. It might therefore be a beneficial intervention when oxygen demands are increased, such as in the perioperative period. Exposure to mild hypoxia could potentially be a risk modifier in those scenarios.

This small human physiology study aimed to evaluate the feasibility of prolonged mild hypoxic exposure in a group of 50–70-year-old volunteers. While the principal aim of the study was to determine whether such an exposure could improve cardiopulmonary functional capacity, the work in this chapter primarily focuses on the physiological secondary outcomes: effects of mild hypoxia on erythropoietin and hepcidin, as markers of oxygen and iron metabolism respectively.

5.2 Study design

5.2.1 Study overview

This randomised, controlled, single-blind, crossover study determined the feasibility of exposing sedentary 50–70-year-old volunteers to a week of mild hypoxia. The effects of this exposure on fitness (not presented in this thesis) as well as markers of iron and oxygen homeostasis were investigated. This study was a collaborative project between the Universities of Oxford and Limerick (Ireland). The study protocol was reviewed and approved by the research ethics committee of the Faculty of Education and Health Sciences at the University of Limerick (Reference: 2014/02/33/EHS). All research was conducted in accordance with the approved study protocol, the Declaration of Helsinki, and Good Clinical Practice guidelines.

5.2.2 Residential hypoxia facility

This research study was conducted at the National Altitude Training Centre of the University of Limerick in Ireland. This facility consists of a residential building, which was converted to allow entire rooms to be exposed to hypoxic air, and an outbuilding, which houses the air compressor and hypoxic filtration system required to generate the low-oxygen air supply (Figure 5.1). The "altitude house" is fully furnished and equipped for prolonged stays. It has a total living area of 122.4 m^2 and is composed of 7 bedrooms (each 13.2 m^2) and a communal living and cooking area (30 m^2). Oxygen levels can be controlled for each room independently by preselecting the desired target value on a control unit, which receives input from oxygen sensors in each room and regulates the supply of hypoxic air (i.e. nitrogen-enriched air) to maintain the pre-set target with an accuracy of 0.1%. Oxygen levels can be lowered to a minimum of 11%.



Figure 5.1 Residential hypoxia facility. The facility (*top*) consists of a converted residential building and an outbuilding housing air compressor and hypoxic air generator. The interior is equipped to allow comfortable stays for prolonged periods of time (*middle*). Oxygen levels can be controlled precisely through a touch screen display (*bottom left*), which controls valves regulating the distribution of hypoxic air for each room separately (*bottom right*).

Hypoxic air is generated using a Hypoxico K2-3000 hypoxic air generator (Hypoxico Inc., New York, NY, USA), which separates compressed air into a nitrogen-enriched (i.e. oxygen-deprived) and an oxygen-enriched air stream. For that purpose, pressurised air is passed through semipermeable hollow fibre membranes (Figure 5.2). Separation is achieved due to the different diffusion rates of the various gas components in the air: oxygen and water vapour have high diffusion rates and pass through the membrane rapidly, whereas nitrogen diffuses at a much slower rate. The nitrogen fraction increases as a consequence. Pressurised nitrogen-enriched air is released at the outlet of the membranes and used to "hypoxicate" the rooms of the facility, while the oxygen-enriched air is discarded. Compared to hypobaric hypoxia, where the partial pressure of oxygen is reduced due to an overall lower barometric pressure, hypoxia inside the house is normobaric and achieved through reducing the fraction of oxygen in the air.

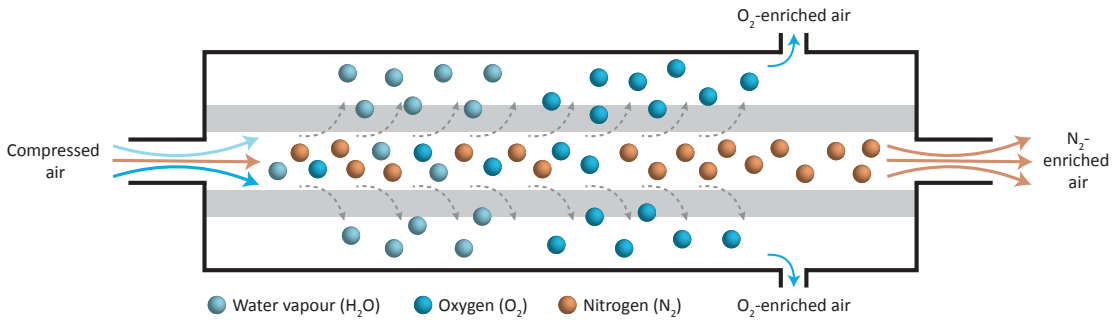


Figure 5.2 Schematic overview of hypoxic air generation by membrane separation. Compressed air is led through hollow fibre membranes (grey-shaded). Oxygen and water vapour diffuse more quickly across the membranes than nitrogen, which results in an increase in the fraction of nitrogen. Oxygen-deprived, nitrogen-enriched air is released at the outlet and introduced into individual rooms to produce a hypoxic environment. Based on Kotliar (1998).

5.2.3 Participants

Recruitment involved contacting healthy individuals who had taken part in previous research studies at the University of Limerick as well as distribution of recruitment emails among staff of the University. After difficulties in recruiting the desired number of 12 participants (this was later amended to a more practical and easily achievable number of 8 participants), recruitment efforts were extended to target bridge clubs in the Limerick area. It was the hope of the investigators that members of these clubs were more inclined to participate in this study and more likely to come forward in a group of four (the number of participants that can comfortably be accommodated within the facility at a time). This strategy resulted in successful recruitment of two groups of four eligible participants.

Interested participant had to meet the following inclusion criteria:

- Male or female adults aged 50–70 years
- Good health with sedentary lifestyle (i.e. not physically active)
- No contraindications identified in the participant's clinical examination, history, and pre-altitude screening by the research clinician
- Availability and suitability to complete the required residential period within the residential hypoxia facility at the University of Limerick

Exclusion criteria were as follows:

- Known intolerance to altitude identified in the participant's clinical examination, history, or pre-altitude screening by the research clinician
- Non-availability and/or suitability to complete the required residential period within the residential hypoxia facility at the University of Limerick

5.2.4 Study procedures and interventions

Interested participants who met eligibility criteria were invited to attend a screening visit, at which informed written consent was obtained. All participants underwent a clinical examination and medical history review by a general practitioner licensed to practice in Ireland in order to establish suitability for mild hypoxic exposure.

Eligible participants were randomly divided into two groups of four people each: group A was exposed to a week of hypoxia initially, followed by group B, which started the study with a week-long exposure to normoxia. After a two-week washout period, in which participants returned to their normal living environments, each group underwent the alternate exposure (crossover design). Participants were unaware of the sequence of exposures assigned to their group (single-blinded study design). A flow diagram and timeline of study visits is shown in Figure 5.3 and a detailed study schedule is depicted in Table 5.1.

Participants were slowly acclimated to the hypoxic environment with stepwise decreases in oxygen concentrations and intermittent periods of leaving the house (as required by University of Limerick regulations for the use of the facility) over the first two days. To ensure participant blinding, the same timeline was followed in normoxic weeks, but oxygen concentrations in the house were not altered. From day 3 onward, participants were exposed to a constant oxygen concentration (15% for hypoxic weeks and 21% for normoxic weeks) and did not leave the house until the end of the study week. To make the stay as comfortable as possible, participants were provided with daily newspapers, tablet computers, as well as catered meals three times a day.

During the stay in the house, daily measurements of vital signs (peripheral oxygen saturation, heart rate, respiratory rate, and blood pressure) were performed. Participants also completed the Lake Louise Scoring questionnaire every day to assess and

document symptoms associated with hypoxia. The Lake Louise Score was developed to evaluate symptoms of acute mountain sickness (AMS) resulting from hypoxia by assessing the occurrence of headaches, gastrointestinal symptoms, fatigue and weakness, dizziness, light-headedness, and sleeping difficulties (Roach *et al.* 1993). Each symptom category can be rated from 0 (no symptoms) to 3 (severe symptoms), resulting in a total score ranging from 0 to 15. A score of 3–5, combined with matching clinical symptoms and exposure history, suggests mild AMS whereas a score greater than 6 indicates severe AMS.

At baseline and on days 4 and 8, venous blood samples were drawn and processed as described in Chapter 5.2.6. At the beginning and end of each week, participants also underwent cardiopulmonary exercise testing (since this aspect of the study is not covered in this thesis, no further details are provided here).

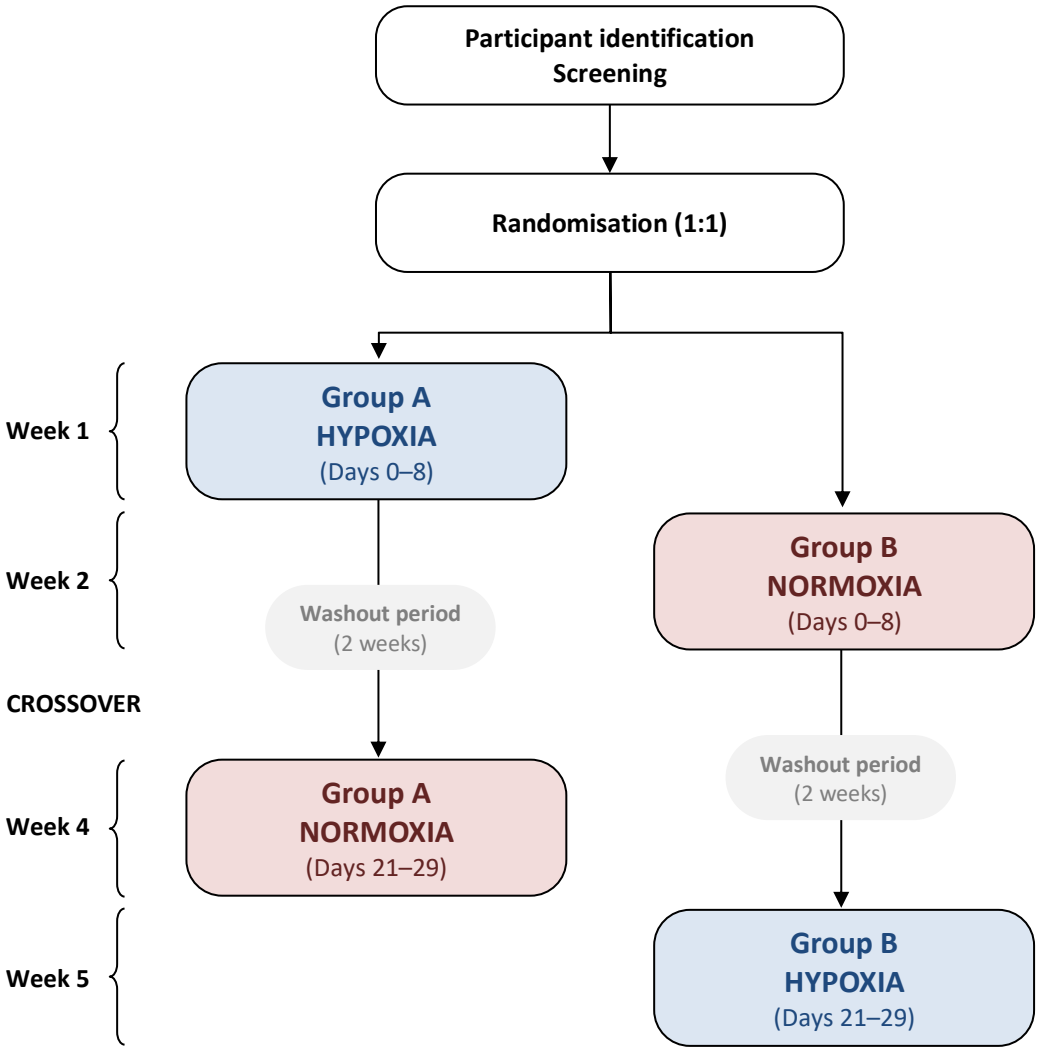


Figure 5.3 Overview of study protocol. A total of eight participants were enrolled into the study and randomly allocated to group A (hypoxia followed by normoxia) or group B (normoxia followed by hypoxia) at a ratio of 1:1. A washout period of two weeks was included between study weeks.

Table 5.1 Study schedule

Day	FiO_2, %		Comments	Assessments and procedures			
	Group A	Group B		Blood sample	Vital signs	LLS	CPET
Day 0			Baseline assessments	●	●		●
Day 1	17	21	Enter at 12:00 – 5 h acclimatisation 1 h leave – 14 h overnight exposure			●	
Day 2	16	21	Exit at 8:00 – 10 h leave 14 h overnight exposure		●	●	
Day 3	15	21	Exit at 8:00 – 1 h leave Permanent stay from 9:00		●	●	
Day 4	15	21	Constant exposure	●	●	●	
Day 5	15	21	Constant exposure		●	●	
Day 6	15	21	Constant exposure		●	●	
Day 7	15	21	Constant exposure		●	●	
Day 8			Exit at 8:00	●	●	●	●
			Washout period				
Day 21			Baseline assessments	●	●		●
Day 22	21	17	Enter at 12:00 – 5 h acclimatisation 1 h leave – 14 h overnight exposure			●	
Day 23	21	16	Exit at 8:00 – 10 h leave 14 h overnight exposure		●	●	
Day 24	21	15	Exit at 8:00 – 1 h leave Permanent stay from 9:00		●	●	
Day 25	21	15	Constant exposure	●	●	●	
Day 26	21	15	Constant exposure		●	●	
Day 27	21	15	Constant exposure		●	●	
Day 28	21	15	Constant exposure		●	●	
Day 29			Exit at 8:00	●	●	●	●

FiO_2: fraction of inspired oxygen, LLS: Lake Louise Score, CPET: cardiopulmonary exercise test.

5.2.5 Outcomes

The study's primary objective was to determine whether a one-week exposure to mild hypoxia improves exercise capacity measured by cardiopulmonary exercise testing in 50–70-year-old sedentary individuals. These measurements and findings are not reported as part of this thesis, since assessment and analysis of these data were performed without the author's involvement.

Secondary objectives included effects of prolonged hypoxic exposure on physiological parameters and biomarkers of oxygen and iron homeostasis. Secondary outcome measures included changes in the following parameters between hypoxic and normoxic exposure:

- Peripheral oxygen saturation
- Heart rate, respiratory rate, and blood pressure
- Symptoms of hypoxic exposure (Lake Louise Score)
- Haematological parameters (haemoglobin and haematocrit)
- Erythropoietin as a marker of hypoxic response
- Hepcidin as a marker of iron homeostasis

5.2.6 Sample analysis

Venous blood samples were obtained the day before each study week (baseline) as well as on days 4 and 8 of each week. Blood samples were immediately analysed using an Abbot i-STAT 1 handheld blood analyser (Abbot Point of Care Inc., Princeton, NJ, USA) with EG6+ cartridges to obtain measurements of haemoglobin and haematocrit. At baseline, serum samples were additionally sent to the Limerick regional hospital for analysis of iron parameters (serum iron, ferritin, transferrin, and transferrin saturation).

Lastly, plasma samples were obtained and stored for further analysis on each occasion. Blood was collected in EDTA-coated tubes and processed as described before (Chapter 3.2.9). Duplicate aliquots were stored at −80°C until further analysis. After the end of the study, duplicate samples were collectively analysed by enzyme-linked immunosorbent assay to measure erythropoietin (Quantikine® IVD® human EPO ELISA, DEP00, R&D Systems, Abingdon, United Kingdom) and hepcidin (Quantikine® human hepcidin ELISA, DHP250, R&D Systems).

5.2.7 Statistical analysis

This study was designed as a pilot study to establish feasibility of the proposed approach, so no data were available for a statistical sample size calculation. Hence, the sample size of 8 participants was chosen arbitrarily.

Statistical analysis was performed using IBM SPSS Statistics (version 24.0 for Macintosh, IBM Corporation, Armonk, NY, USA) and GraphPad Prism (version 7.0a for Macintosh, GraphPad Software, La Jolla, CA, USA). Comparisons of means between two groups at a single time point were made by unpaired or paired two-tailed Student t-test for normally distributed continuous data. Longitudinal analysis of the effects of different exposures on outcomes was performed by linear mixed effects modelling. Each model included the factors exposure and day as well as their interaction term as fixed effects. All models included intercept as random effect. Where *p* values from linear mixed effects models are reported they refer to the interaction term of exposure and day. Linear correlation between two variables was analysed by means of Pearson's correlation. Variables were logarithmically transformed before calculating correlation coefficients if the data were skewed or the correlation of non-transformed data was not linear. A p value of < 0.05 was considered statistically significant.

5.3 Results

5.3.1 Participant characteristics

All eight enrolled volunteers completed the study; all participants were included in the analysis (Figure 5.4). Baseline participant characteristics, spirometry results, and blood parameters are shown in Table 5.2. All participants were in good general health at the time of the study, although some presented with comorbidities. Individual participants had histories of hypertension, COPD, asthma, hypothyroidism, as well as one case of successfully treated prostate cancer. One participant presented with well-controlled myasthenia gravis. Six participants had a history of previous tobacco exposure.

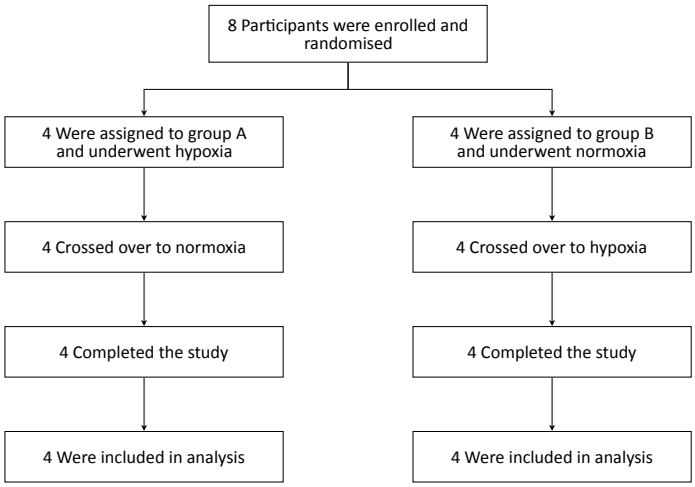


Figure 5.4 Participant flow diagram. A total of eight participants were enrolled in the study. All participants underwent hypoxic and normoxic exposures and completed the trial. No data were excluded from analysis.

Table 5.2 Baseline characteristics of study participants

Characteristic, unit (normal range)	**All participants** (n = 8)
Age, years	67.7 (60.8–69.0)
Sex, n (%)	
Male	3 (37.5)
Female	5 (62.5)
Body mass index (BMI), kg/m^2	27.8 ± 3.9
Blood pressure, mmHg	
Systolic	127 ± 16
Diastolic	71 ± 8
Resting SpO_2, %	96.5 ± 2.0
Heart rate, min^{-1}	80 ± 13
Respiratory rate, min^{-1}	16 (16–23)
FEV_1, L	2.11 ± 0.83
FEV_1, % of predicted	75.5 ± 25.5
FVC, L	2.96 ± 0.94
FVC, % of predicted	79.5 ± 17.3
FEV_1/FVC, %	72.3 ± 19.0
Haemoglobin, g/dL (12–17)	11.0 ± 1.5
Haematocrit, % (30–50)	32.3 ± 4.3
Iron, µmol/L (11–31)	15.5 ± 4.2
Ferritin, µg/L (10–300)	95.0 (59.5–200.8)
Transferrin, g/L (1.8–3.6)	2.55 (2.40–2.70)
Transferrin saturation, % (16–50)	24.1 ± 7.6

Data are reported as mean ± SD, if normally distributed, or median (IQR), if not normally distributed.

5.3.2 Environmental monitoring data

Oxygen levels throughout the hypoxia facility were continuously measured and recorded. As is shown in Figure 5.5, levels were maintained accurately during the entire study period. Oxygen levels were slightly above the target level of 15% during the first two days of hypoxic weeks due to the stepwise reduction in ambient oxygen levels. The oxygen levels (mean ± SD) throughout the house (i.e. common room and bedrooms used by participants) averaged 20.4 ± 0.2% in normoxic weeks and 15.4 ± 0.6% in hypoxic weeks ($p < 0.001$). Other environmental parameters were similar throughout the study period with an average temperature of 22.0 ± 1.1°C, average humidity of 48.8 ± 5.8%, and average barometric pressure of 765 ± 6 mmHg.

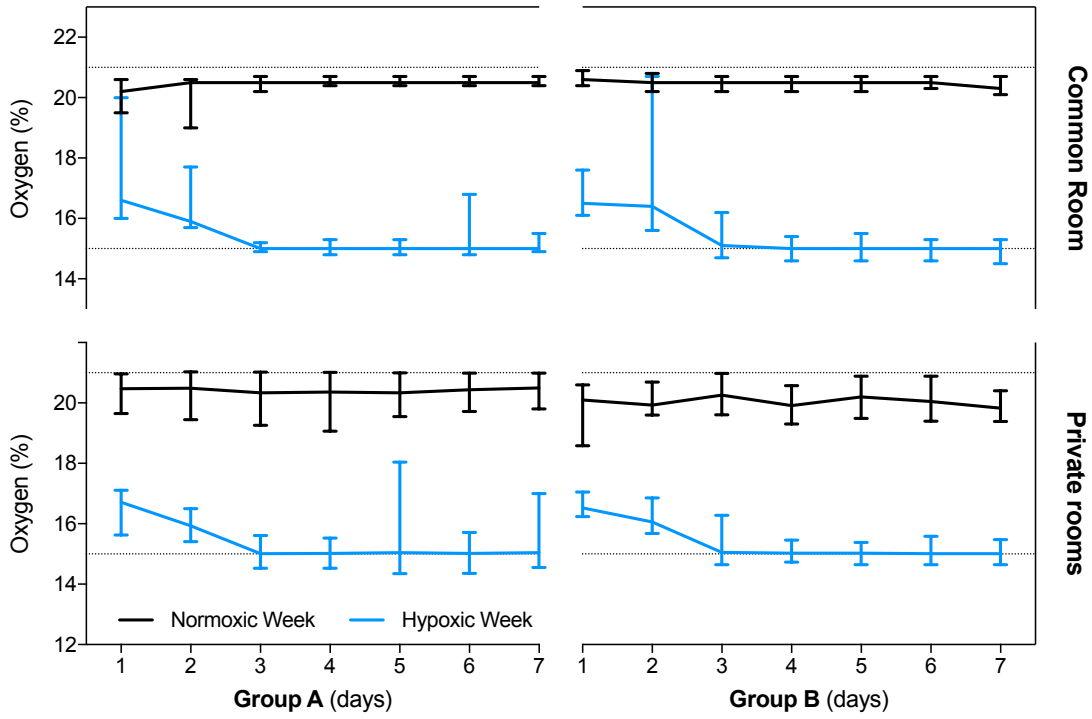


Figure 5.5 Oxygen levels in the residential hypoxia facility during study weeks. Average daily (mean ± range) oxygen levels measured in the common room (*top*) and bedrooms (*bottom*, average of all bedrooms in use by study participants) are shown for each group and week. Dotted lines mark the pre-set target levels of 21% (for normoxic weeks) and 15% (for hypoxic weeks).

5.3.3 Oxygenation and other physiological measurements

Peripheral oxygen saturations were measured on a daily basis to assess the efficacy of the hypoxic exposure and to monitor the degree of hypoxaemia in each participant (Figure 5.6). Longitudinal analysis of temporal changes in oxygen saturation revealed significantly lower values in hypoxic weeks (p = 0.029). Oxygen saturations (mean ± SEM) averaged 93.0 ± 0.3% during hypoxia and 96.4 ± 0.2% during normoxia, resulting in a difference of 3.4% (95% confidence interval: 2.7% to 4.2%, $p < 0.001$).

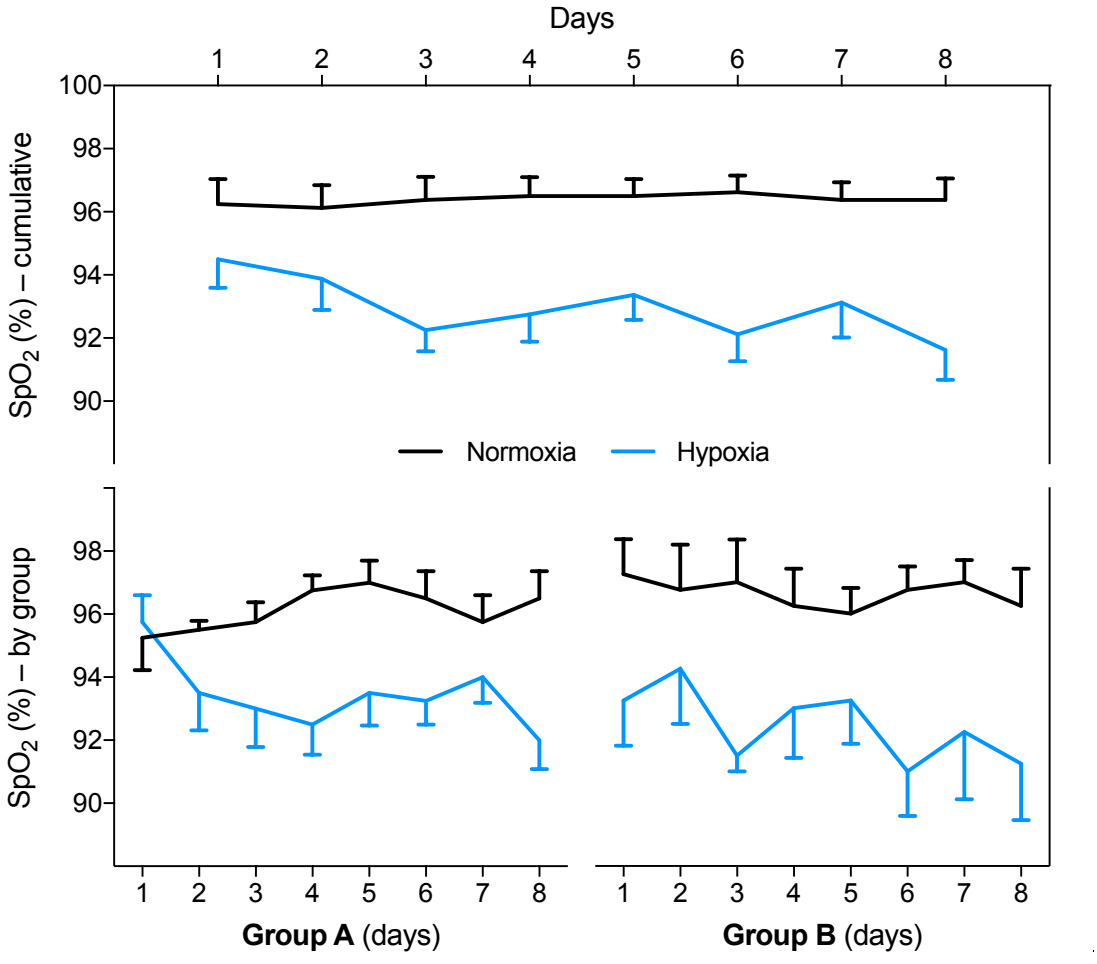


Figure 5.6 Effects of hypoxic exposure on peripheral oxygen saturation. Oxygen saturations during hypoxia and normoxia were measured daily and are shown cumulatively for all participants (*top*) and separately for each group (*bottom*). Values were significantly lower during hypoxic exposure (see text). All data are expressed as mean ± SEM.

Other physiological measurements are summarised in Figure 5.7. Heart rate and respiratory rate significantly increased over time in response to hypoxia ($p = 0.001$ and $p < 0.001$, respectively), whereas blood pressure remained unaffected ($p = 0.093$ for systolic and $p = 0.211$ for diastolic pressure). Exposure to hypoxia was generally well-tolerated with only a few participants reporting symptoms on the Lake Louise Scoring Scale; no participant reached a score higher than 3. Longitudinal analysis of Lake Louise Scores revealed no significant differences between hypoxic and normoxic exposure either ($p = 0.120$).

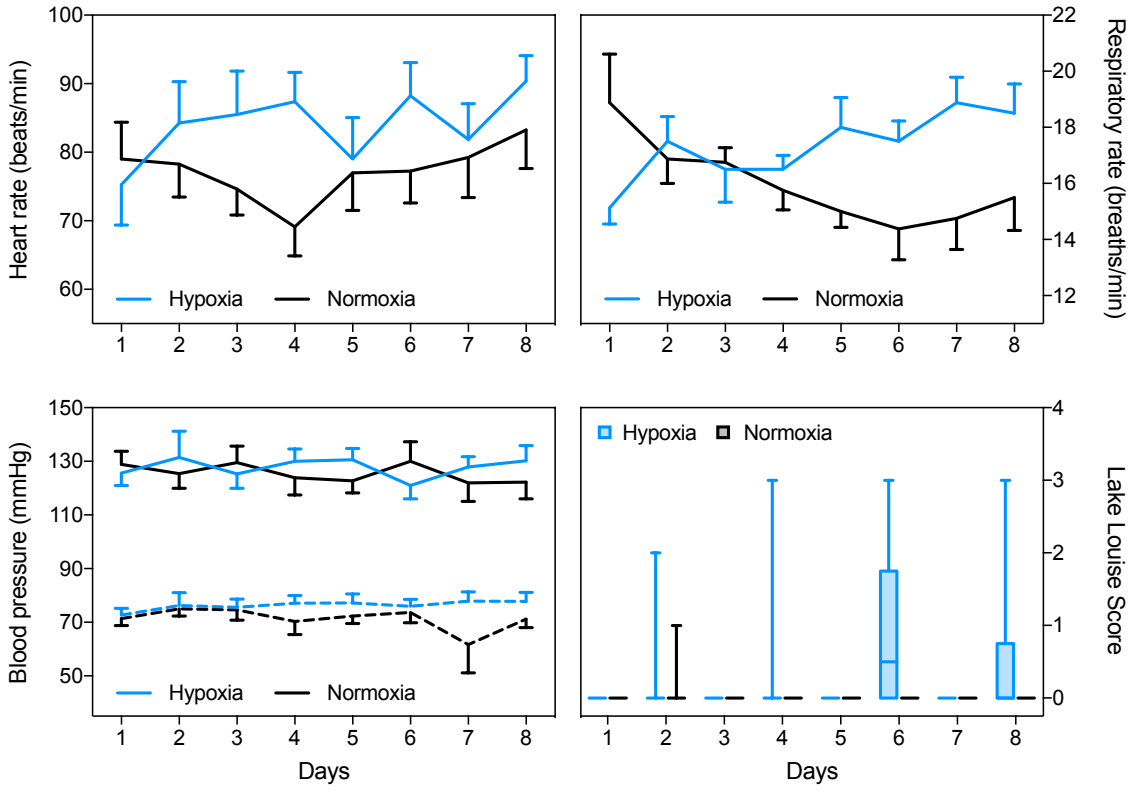


Figure 5.7 Effects of hypoxic exposure on vital signs and Lake Louise Score. Heart rate (*top left*) and respiratory rate (*top right*) both increased during hypoxic exposure. Blood pressure (*bottom left*, solid lines: systolic pressure, dashed lines: diastolic pressure) and Lake Louise Scores (*bottom right*) did not change. All data are expressed as mean ± SEM, except Lake Louise Scores, which are shown as box-and-whisker plot with solid lines indicating the median, boxes representing the interquartile range, and whiskers extending to the maximum values.

5.3.4 Haematology parameters

Blood samples were obtained at baseline and on days 4 and 8 during normoxic and hypoxic exposure. Changes in haemoglobin and haematocrit during the course of the study are shown in Figure 5.8. Both parameters were well-matched at baseline and day 4 but diverged by the end of the study period with both haemoglobin and haematocrit exhibiting higher levels after hypoxic exposure. The effect of hypoxia on both markers was statistically significant ($p = 0.028$ for haemoglobin and $p = 0.022$ for haematocrit).

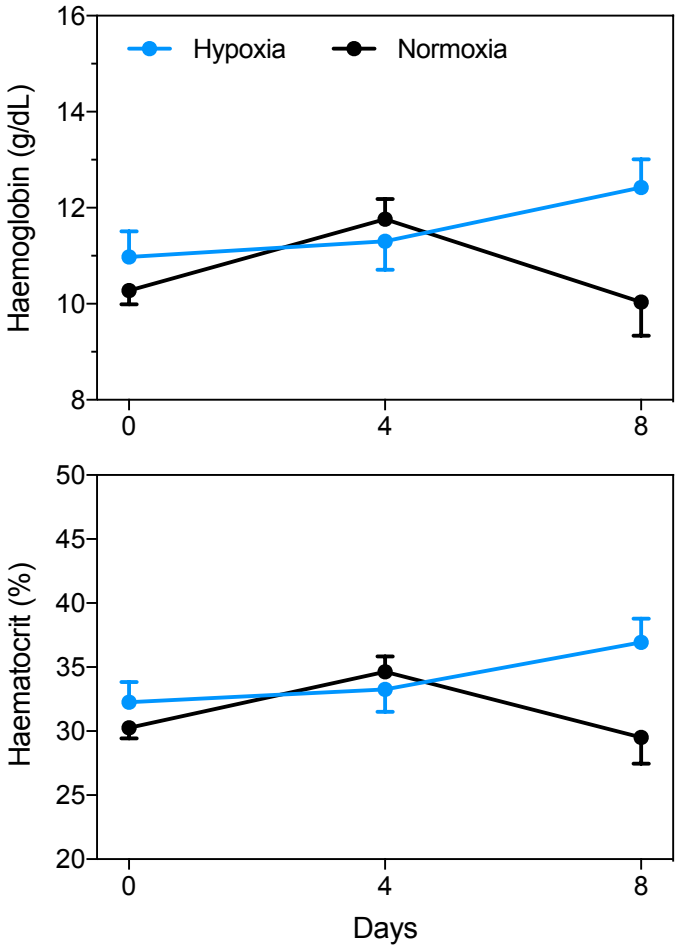


Figure 5.8 Temporal changes in haemoglobin and haematocrit. Haemoglobin (*top*) and haematocrit (*bottom*) were measured at baseline (day 0) and on days 4 and 8. Both parameters were significantly affected by hypoxic exposure. All data are expressed as mean ± SEM.

5.3.5 Effects on erythropoietin

Erythropoietin was analysed as a biomarker typically upregulated in response to hypoxic exposure. Baseline levels measured before exposure to hypoxia were well-matched between normoxic and hypoxic groups (Figure 5.9). During the hypoxic week, erythropoietin increased 2.1-fold by day 4 before dropping to levels similar to the control group by the end of the exposure period. This temporal trend was highly statistically significant (p = 0.004). Erythropoietin levels and relative changes for each group of participants are shown in Figure 5.10.

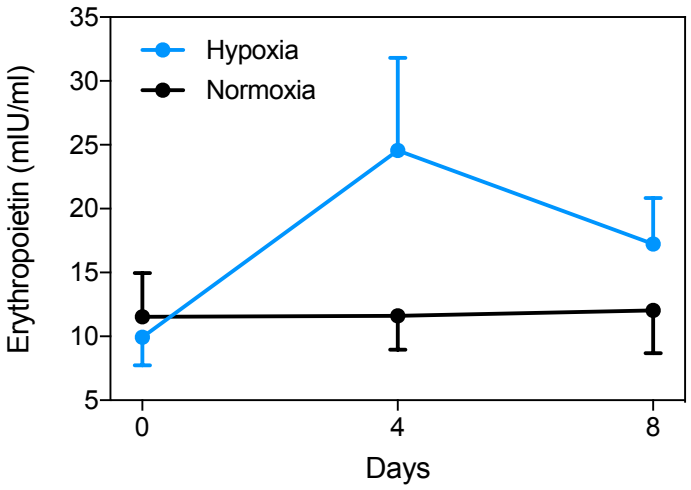


Figure 5.9 Temporal changes in erythropoietin levels. Erythropoietin levels were measured by ELISA at baseline and on days 4 and 8 during the exposure to hypoxia and normoxia. EPO levels significantly increased in response to hypoxia by day 4 before returning to baseline levels by the end of the study. All data are expressed as mean ± SEM.

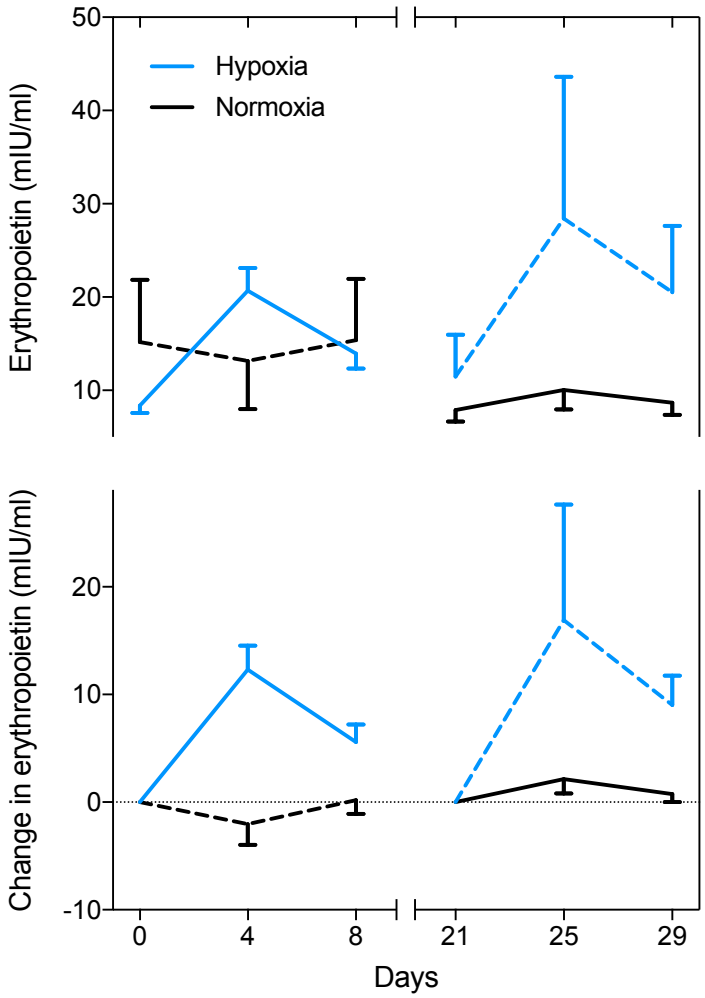


Figure 5.10 Changes in erythropoietin levels by group. Absolute (*top*) and relative (*bottom*) changes in erythropoietin levels during hypoxic and normoxic weeks are shown separately for group A (solid line) and group B (dashed line). EPO increased in both groups in response to hypoxia. All data are expressed as mean ± SEM.

5.3.6 Effects on hepcidin

The effects of prolonged hypoxic exposure on hepcidin as the key hormone in the regulation of iron homeostasis were analysed. Baseline values at the start of the hypoxic and normoxic weeks were similar (Figure 5.11). Hepcidin levels dropped after exposure to hypoxia ($p = 0.026$ for within-group comparison of baseline and day 8), but the effect of hypoxia was not significantly different from normoxia over time ($p = 0.881$).

Group-wise analysis allowed for a more detailed interpretation (Figure 5.12). Both groups A and B showed reductions in mean hepcidin levels throughout the respective hypoxic week: hepcidin dropped by 53.0% in group A and 46.9% in group B ($p = 0.038$ and $p = 0.118$, respectively). In group B, hepcidin levels did not change during the normoxic control week (days 0–8). In contrast, group A showed significantly higher hepcidin levels at the start of their normoxic week (days 21–29), which followed the hypoxic exposure in that group. No causes for this increase could be identified.

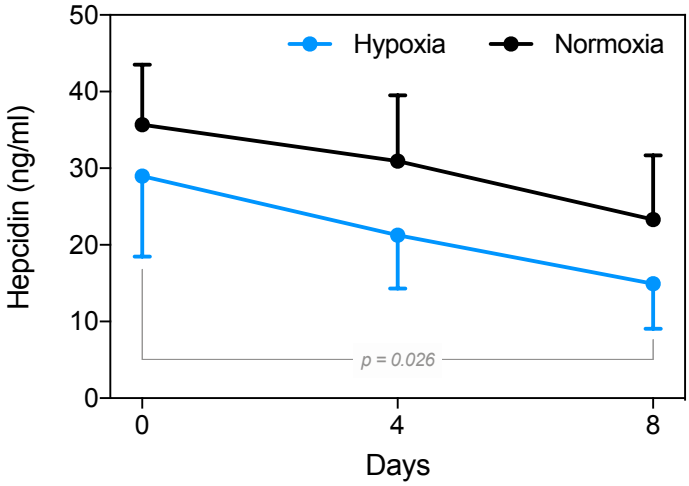


Figure 5.11 Temporal changes in hepcidin levels. Hepcidin was analysed by ELISA at baseline and on days 4 and 8 during the exposure to hypoxia and normoxia. Mean hepcidin levels decreased in both groups over time. Comparison of baseline levels with measurements at day 8 in the hypoxic group revealed a significant within-group difference ($p = 0.026$, paired t-test); the effect of hypoxia, however, was not significantly different from normoxic exposure. All data are expressed as mean ± SEM.

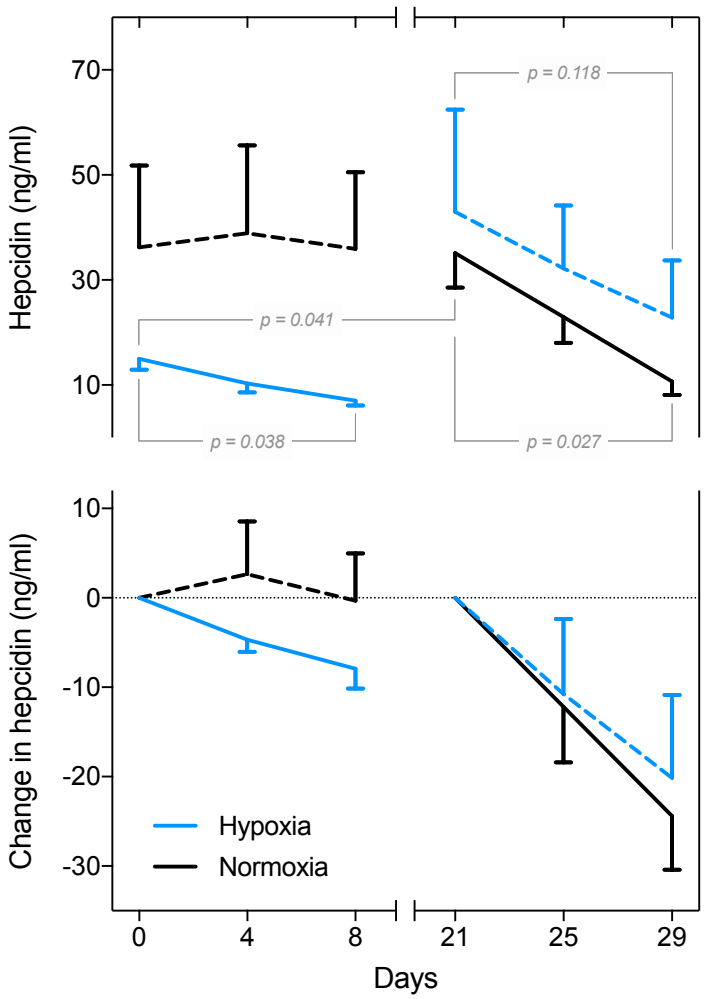


Figure 5.12 Changes in hepcidin levels by group. Absolute (*top*) and relative (*bottom*) changes in hepcidin levels during hypoxic and normoxic weeks are shown separately for group A (solid line) and group B (dashed line). Hepcidin decreased significantly in response to hypoxia in group A, but not group B. In group A, hepcidin levels were significantly higher at the start of the normoxic week (day 21) and dropped back to baseline levels during the course of the week. *p* values are given for pairwise comparisons between two single timepoints by paired samples t-test. All data are expressed as mean ± SEM.

5.3.7 Correlations between oxygenation and iron homeostasis

To gain additional insight into the physiology of oxygen and iron homeostasis in response to hypoxia, correlations between various measured variables were analysed.

As described in Chapters 5.3.5 and 5.3.6, both erythropoietin and hepcidin responded to hypoxia with increased and decreased levels, respectively. In Figure 5.13, values for erythropoietin and hepcidin are plotted against the corresponding peripheral oxygen saturations measured at the same time point. In contrast to results described before, the correlation coefficients for erythropoietin ($r = -0.238$) and hepcidin ($r = 0.116$) did not suggest changes in erythropoietin or hepcidin levels with lower oxygen saturations ($p = 0.103$ and $p = 0.433$, respectively).

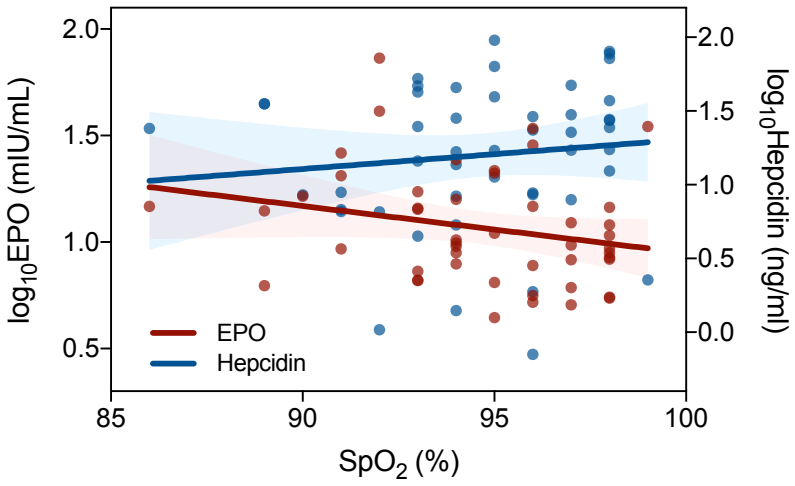


Figure 5.13 Correlation of SpO_2 with erythropoietin and hepcidin. The scatterplot shows individual log-transformed erythropoietin (left y-axis) and hepcidin (right y-axis) values plotted against the peripheral oxygen saturation measured at the same time point. Correlation coefficients for erythropoietin ($r = -0.238$) and hepcidin ($r = 0.116$) were not statistically significant. Regression lines with 95% confidence bands (shaded areas) are also shown.

The correlation between erythropoietin and hepcidin was also analysed (Figure 5.14). A significant negative correlation existed between the two parameters (r = −0.566, p < 0.001) with hepcidin levels decreasing in the presence of increased erythropoietin.

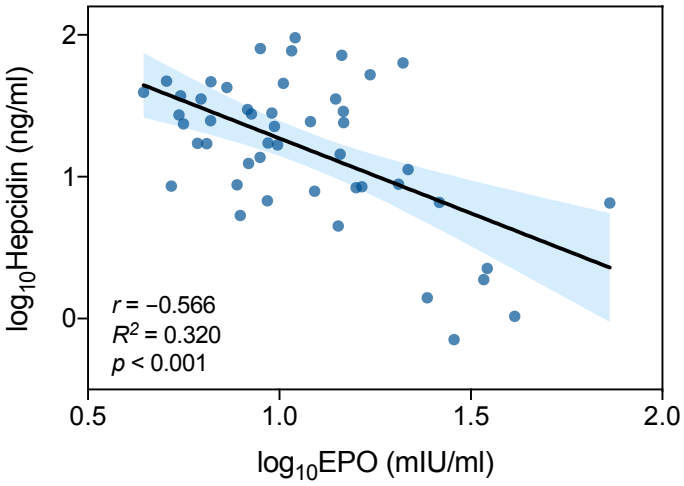


Figure 5.14 Correlation of erythropoietin with hepcidin. The scatterplot shows individual log-transformed hepcidin values plotted against log-transformed erythropoietin levels measured at the same time point. A significant negative correlation (r = −0.566) existed between the two parameters, indicating decreases in hepcidin in response to increased erythropoietin. The linear regression line with a 95% confidence band (shaded area) is also shown.

5.4 Discussion

5.4.1 Main findings

In this small study, mild hypoxia induced a variety of physiological adaptations during a one-week exposure period. Hypoxia levels were well-maintained in the facility, which translated into a reduced saturation of haemoglobin with oxygen, as shown in the significantly lower SpO_2 values during hypoxic weeks. The increases in heart and respiratory rates in response to hypoxia reflected the acute cardiopulmonary adaptations described in Chapter 1.2.3.

This study found prolonged mild hypoxic exposure to be safe and well-tolerated. While the potential to induce hypoxia-related symptoms was a theoretical risk of the studied exposure, Lake Louise Scores were consistently low, indicating the absence of any major symptoms. This was expected considering that levels of hypoxia similar to those used in the present study are commonly encountered onboard airplanes or at higher altitudes without major complications. Furthermore, mild hypoxia was shown to be safe and well-tolerated in elderly individuals, even with pre-existing cardiovascular or pulmonary disease (Roach *et al.* 1995, Hobkirk *et al.* 2013).

Changes in markers of oxygen and iron physiology were the main focus of this secondary outcome analysis. Erythropoietin is the prototypical marker of activated HIF signalling (see Chapter 1.2.3.4). The observed time course of EPO production, with the peak after four days and a subsequent decline, aligns with previous reports. Increased EPO levels in serum can be detected very early after the onset of hypoxia (Eckardt *et al.* 1989). Afterwards, levels continue to increase and peak after several days before decreasing back to baseline values (Abbrecht and Littell 1972, Milledge and Cotes 1985). As a response to the increased EPO production, haemoglobin and haematocrit were raised in the present study after one week of hypoxia.

Hepcidin, as the main regulator of iron homeostasis, was the second marker assessed in this study. Hepcidin expression is negatively regulated by hypoxia (Rishi *et al.* 2015), but the underlying mechanism is not entirely clear (see Chapter 1.3). The observed findings in this study are ambiguous. While cumulative analysis of both groups suggested a within-group reduction in the hypoxic week, a similar decrease was observed during normoxia. Analysis by group revealed that hepcidin levels decreased in group B during the week of hypoxia, but were markedly increased after the two-week wash-out period before falling back to baseline values by the end of that week. This could suggest a possible carry-over effect with compensatory increase after hypoxia, but this explanation remains hypothetical. Measurement errors or technical mistakes in performing the ELISA quantification of hepcidin were also considered. However, the strong correlation between laboratory-measured ferritin levels and corresponding hepcidin values makes this theory unlikely (Figure 5.15). Considering the small sample size, a conclusive explanation cannot be provided. Nevertheless, the observed decreases in hepcidin during hypoxic weeks agree with the expected physiological response.

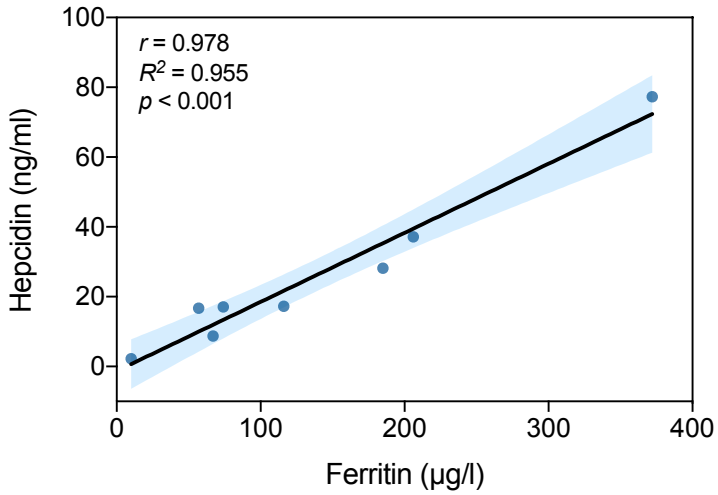


Figure 5.15 Correlation of ferritin with hepcidin. The scatterplot shows ferritin values measured at baseline plotted against the corresponding hepcidin levels. The two parameters were strongly correlated (r = 0.978). The linear regression line with a 95% confidence band (shaded area) is also shown.

Lastly, a significant inverse relationship between EPO and hepcidin levels was observed. Considering that both markers are affected by hypoxia, this finding was not surprising. As mentioned before, the mechanism of hypoxia-induced hepcidin regulation remains unclear (Rishi *et al.* 2015). Both direct HIF-mediated suppression, as well as indirect regulation through erythropoiesis, would explain the observed correlation.

5.4.2 Limitations

The major limitation of the presented study is the small sample size. Originally designed to recruit a larger number of participants, it had to be downsized in light of recruitment difficulties. As a consequence of the small study cohort, comparisons between groups and conclusions are inevitably preliminary in nature. Nevertheless, even very few participants can be sufficient to show a physiological principle, if the effect size is large enough (e.g. EPO in this study).

While the levels of hypoxia during the study were tightly regulated and monitored, other factors were not. Diet and caloric intake, for example, were not considered.

Finally, the practical applicability of the approach needs to be considered. While the unique hypoxia facility in Limerick made a continuous exposure to hypoxia for one week feasible and adequately comfortable for participants, this method is most likely not transferrable into other setting, which are more restrained and space-limited.

5.4.3 Context and conclusion

The primary goal of the original study was to establish a link between mild hypoxia and cardiopulmonary function, with the long-term goal to explore hypoxic conditioning in clinical research. Even though the data presented in this chapter did not cover the

cardiopulmonary component of this study, it is still worthwhile to briefly discuss the findings presented here in the context of the primary study outcome and goal.

The findings presented in this chapter confirmed that mild degrees of hypoxia are sufficient to induce physiological response mechanisms. This did, however, not translate into improved cardiopulmonary exercise capacity. This observation seems to be reflective of research concerned with hypoxic conditioning in general. Verges *et al.* (2015) concisely summarised, that "[a]lthough the bench-based foundation for ischemic and hypoxic conditioning is strong, the translation of conditioning strategies into effective, validated therapies and clinical practice is so far mostly missing". While a full discussion of this topic is beyond the scope of this chapter, the idea of improving perioperative outcomes with hypoxic exposure should not be dismissed. Future research is required to investigate whether longer exposure periods or more profound hypoxic episodes have any effect. Other modalities of hypoxia (e.g. intermittent exposure) might deserve consideration for further studies as well. Investigations of clinical outcomes, rather than surrogate markers of such outcomes, might also be an interesting strategy to pursue.

In conclusion, the approach of prolonged mild hypoxic exposure in 50–70-year-old volunteers is feasible and safe. The results presented here confirm the effectiveness of this approach in activating distinct physiological response pathways, but further research is necessary. Until such evidence becomes available, the clinical significance of the presented approach remains uncertain.

CHAPTER 6:
Conclusions

6.1 Final summary

Iron and oxygen are both indispensable for human life. Many physiological processes would not be possible in the absence of either one or both. This thesis has summarised the complex interplay between iron and oxygen homeostasis. The three presented studies focused on three different clinical areas in which iron and oxygen physiology are important: respiratory medicine, critical care, and anaesthesia. In three distinctly different studies, common principles of iron and oxygen homeostasis became evident.

Chapter 3 introduced a clinical trial of intravenous iron administration in COPD. Abnormal oxygen homeostasis and chronic hypoxia have long been established as a crucial feature of the condition's pathogenesis. As highlighted in detail, dysregulated iron metabolism has recently become a focus of clinical physiology research due to its importance in pulmonary and systemic processes contributing to COPD. Modification of the body's iron stores in affected patients resulted in significant improvements in symptomatology and functional endpoints, such as exercise capacity.

In critically ill patients, oxygen physiology is equally important, as systemic responses often involve disrupted oxygen delivery and malperfusion. As shown in *Chapter 4*, these systemic responses crucially affect iron homeostasis, which has important diagnostic and prognostic implications. Iron status is difficult to assess under these circumstances, and the altered iron homeostasis leads to complications, such as anaemia of inflammation.

Lastly, the small human physiology study presented in *Chapter 5* originated conceptually from the field of perioperative medicine. In anaesthesia, oxygen is commonly administered and hypoxia is usually regarded as an adverse event that clinicians aim to avoid. Mild hypoxia, however, might be beneficial under certain circumstances. The presented study explored this idea with the goal of inducing

potentially beneficial changes in oxygen metabolism in the future. Although not related, it is noteworthy that iron metabolism as well has become important in the field of anaesthesia in recent years. Advances in so-called "patient blood management" include guidelines on assessment and optimisation of perioperative iron status.

6.2 Future directions

The need for future research has been discussed in each study chapter. In particular, the clinical trial on intravenous iron in patients with COPD warrants further follow-up studies due to the potential impact for the affected patients. Such studies should focus more specifically on functional endpoints and include larger numbers of patients and recruitment sites. The underlying physiology and the results from the research presented here support this approach.

The two small studies presented in Chapters 4 and 5 left open questions that could justify further research. In contrast to the COPD trial, these should aim at addressing important physiological questions about iron and oxygen metabolism in pathophysiological conditions, such as inflammation and during surgical stress.

6.3 Concluding remarks

In conclusion, the work presented in this thesis highlights the complex and close relationship between iron and oxygen in the human body. It further shows how intricate and important the involvement of these two elements and their regulatory pathways are in health and disease. Further research is necessary to develop safe and effective clinical applications that improve quality of life among patients affected by oxygen- and iron-related diseases.

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APPENDICES

Appendix A: Validation of hepcidin measurements

Hepcidin values presented in this thesis were measured by using different ELISA kits. In Chapter 3, a competitive assay (Hepcidin 25 bioactive HS ELISA, EIA-5782, DRG, Marburg, Germany) was used, which has been validated against mass spectrometry (van der Vorm *et al.* 2016). The assay used in Chapters 4 and 5 (Quantikine® human hepcidin ELISA, DHP250, R&D Systems, Abingdon, United Kingdom) was recently introduced, is based on the "sandwich" method, and has not been performed in the author's research group before. For this reason, a small internal validation study was performed to compare results from this new assay with values previously obtained using another well-established competitive assay (Hepcidin-25 EIA kit, S-1337, Bachem, Peninsula Laboratories, San Carlos, CA, USA), which also showed good correlation with reference mass spectrometry results (van der Vorm *et al.* 2016).

As shown in Figure A1, hepcidin results from the new R&D assay correlated strongly with the previously obtained measurements ($r = 0.978$, $R^2 = 0.957$, $p < 0.0001$). For the purpose of this thesis, results from this new assay were therefore considered equivalent to hepcidin values obtained with other kits, even though formal validation with a reference method has not been published yet.

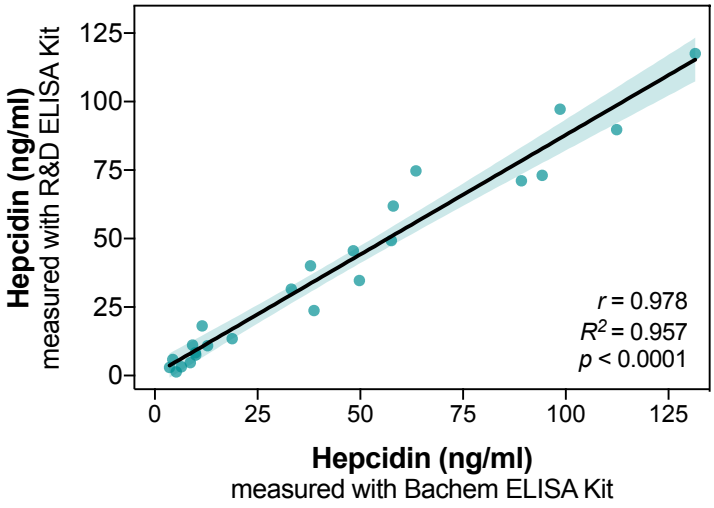


Figure A1 Correlation of hepcidin measurements obtained with different assays. Values obtained with the new R&D assay showed a strong and highly significant correlation with measurements previously obtained using the Bachem kit. The regression line had a slope of 0.87 (95% confidence interval: 0.79–0.96) and an intercept of 0.57 (95% confidence interval: −4.08–5.21).

Appendix B: Contributions

Clinical and human subjects research can hardly ever be achieved by a single individual. Particularly clinical trials require a collaborative effort. Certain aspects, such as randomisation and blinding, would not be possible without adequate support. Listed below are those people who made contributions leading to the successful completion of each project. For the clinical trial of intravenous iron in COPD (Chapter 3), the author was involved in every aspect of the study. For the two smaller trials presented in Chapters 4 and 5, the author's contributions have been disclosed at the beginning of each chapter. Data presented in this thesis is only concerned with the aspects of those studies in which the author was personally involved. All data analysis and interpretation reported in this thesis was undertaken entirely by the author.

Chapter 3: Study on iron in COPD

Dr Annabel Nickol	Chief investigator, conception and design of study
Anne McGahey	Research nurse, assistance with patient assessments
Dr Matthew Frise	Administration of study drugs, design of study
Dr Nayia Petousi	Administration of study drugs, design of study
Dr Nick Talbot	Administration of study drugs
Dr Chris Turnbull	Review of blinded laboratory results for safety purposes
Prof Mona Bafadhel	Interim chief investigator
Dr Richard Baskerville	Assistance with patient recruitment
ORTU team	Administrative and logistical assistance, randomisation of participants, administration of study drugs

Chapter 4: Study on iron parameters in critically ill patients

Dr Nick Talbot	Provision of samples and dataset used for analysis

Chapter 5: Study on prolonged hypoxic exposure

Dr Tom Smith	Study conception and design
Dr Lisa Brown	Recruitment, supervision of study, and data collection (for 3 out of 4 study weeks)
Dr John Griffiths	Blinded CPET analysis
Prof Phil Jakemann	Principal investigator (University of Limerick), study conception and design

Appendix C: Scientific communications associated with this thesis

Poster presentations

P Santer, A McGahey, M Frise, N Petousi, N Talbot, M Bafadhel, R Baskerville, A Nickol and P Robbins (2018). The effects of intravenous iron in COPD: Results from a randomized clinical trial. *European Iron Club 2018 Annual Meeting; Zürich, Switzerland; February 2018*

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