Clonal Haematopoiesis and Inflammation in Patients with Heart Failure

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Abstract

Background

The prevalence of heart failure (HF) continues to grow. Its incidence rises substantially with age and the mean age of patients at HF diagnosis is almost 77 years. HF with preserved ejection fraction (HFpEF) now accounts for over half of cases and has an even closer relationship to ageing than does HF with reduced ejection fraction (HFrEF). Unlike HFrEF, limited evidence-based therapies currently exist for the treatment of patients with HFpEF which is more commonly associated with multi-morbidity, myocardial stiffening and coronary macro- and micro-vascular endothelial dysfunction.

Both HFrEF and HFpEF are associated with systemic inflammation. However, inflammation appears to be more important in the pathophysiology of HFpEF than HFrEF. High circulating levels of inflammatory markers have been consistently associated with worse outcomes in patients with both types of HF.

Clonal haematopoiesis of indeterminate potential (CHIP) reflects the accumulation of specific somatic genetic abnormalities with a variant allele frequency (VAF) ≥2% in haematopoietic stem cells with age. The consequence of this is the accumulation of mutant leukocytes that confer an increased risk of subsequent haematologic malignancy. Most carriers will never develop leukaemia and the progression rate is approximately 0.5% per year. Despite the low risk of progression to haematologically important diagnoses, all-comers with CHIP have a 40% higher mortality than those without CHIP. This excess is a reflection of cardiovascular (CV) events, including fatal, and non-fatal MI, percutaneous coronary intervention and fatal stroke (ischaemic and haemorrhagic). Importantly, however, the presence of CHIP also confers a substantially increased risk for CV disease independent of traditional risk factors and recently the presence of mutations with a VAF<2% has been associated with worse CV outcomes.

CHIP was initially found to be associated with an increased risk of coronary artery disease (CAD). Subsequently it has been associated with a wide range of CV disease including HF and cardiac arrhythmias. The presence of CHIP has also been associated with an increased risk of adverse CV outcomes including death and subsequent hospitalisation. The commonly detected CHIP mutations play a central role in the regulation of inflammation, and the

inflammatory and pro-atherogenic effects of CHIP have become a major recent focus for research.

To date, studies have mostly retrospectively examined the prevalence of CHIP in patients with HF and there is limited information on the presence of CHIP and its association with circulating cardiac and inflammatory biomarkers. There is also limited information on the roles and associations of specific circulating inflammatory biomarkers on adverse CV outcomes. Furthermore, there is also little information on whether standard HF therapies reduce levels of inflammation.

Aim

The main aim of this study was to investigate the prevalence of CHIP in an unselected cohort of patients with HF and to understand the clinical and HF characteristics of patients with CHIP while obtaining mechanistic data to inform therapeutic strategies for the treatment of HF, in particular, examining its association with inflammation.

To further obtain mechanistic data to inform future trials of anti-inflammatory therapy in both stable and decompensated HF, the role of inflammation was comprehensively investigated in different HF cohorts. In particular, interleukin-6 (IL-6) and relative lymphocyte count (RLC) were examined. In addition, I examined whether treatment with sacubitril/valsartan reduced levels of inflammation in stable patients with HFpEF and HFrEF.

Methods

I performed a prospective observational study of 96 patient (48 patients with HFpEF and 48 patients with HFrEF) admitted to NHS Greater Glasgow and Clyde Hospitals with decompensated HF over a one-year period. All patients recruited had their deoxyribonucleic acid (DNA) analysed for the presence of CHIP driver mutations and serum/plasma was analysed to examine levels of circulating cardiac and inflammatory biomarkers at the time of HF decompensation. Participants recruited to the study had detailed demographic and clinical data collected and they had an echocardiogram (echo) and electrocardiogram (ECG) performed at baseline.

In addition, to performing the prospective observational study, I also examined three large datasets of outpatients with decompensated HF. Two were large clinical trial datasets (PARADIGM-HF and PARAGON HF) and one was a large observational HF study (Microvolt T wave alternans). I specifically investigated the role of circulating levels of IL-6 and RLC in patients with HFrEF and HFpEF, and their association with adverse CV outcomes including death and subsequent HF hospitalisation. I also examined whether treatment with sacubitril/valsartan reduced circulating levels of inflammation over time.

Results

Of the 96 patients recruited to the prospective observational study, the average age of patients was 72 years. As expected, patients with HFpEF were older (74 years) than those with HFrEF (70 years). CHIP driver mutations with a VAF≥2% were detected in 5 patients with HFrEF (10%) and 8 patients with HFpEF (17%). CHIP driver mutations with VAF≥1% were detected in 25 patients with HFrEF (52%) and 21 patients with HFpEF (44%). There was an age-dependent increase in the prevalence of CHIP, and the total number of mutations was higher in older patients in both HF groups. The most common mutation identified in both HFpEF and HFrEF was in deoxyribonucleic acid methyltransferase 3 alpha (DNMT3A). Patients with CHIP were older than patients without CHIP. Baseline haematological parameters (WBC, neutrophiles and lymphocytes), CV disease and CV therapies were similar between the groups. Furthermore, there was no difference in circulating levels of relative lymphocyte count (RLC) or neutrophil lymphocyte ratio (NLR) according to CHIP status. The presence of CHIP was not associated with levels of N-terminal prohormone of brain natriuretic peptide (NTproBNP). The presence of CHIP was associated with higher levels of novel biomarkers of inflammation. Specifically, concentrations of interleukin-1 beta (IL-1β), interleukin-18 (IL-18) and transforming growth factor-beta 2 (TGF-β2) were higher in those with, compared to those without, CHIP. In HFpEF, the presence of CHIP was associated with elevated levels of IL-1β and upstream and downstream makers of nucleotide-binding oligomerization domain, leucine rich repeat and pyrin domain containing 3 (NLRP3) inflammasome activity. In HFrEF, the presence of CHIP was associated with elevated levels of IL-18 and TGF-β2. Surprisingly, the presence of CHIP was not associated with higher levels of circulating IL-6, another downstream marker of NLRP3 inflammasome activity.

IL-6 while not elevated in my CHIP cohort, is an important pro-inflammatory cytokine which appears to play an important role in CV disease. Therefore, I went on to examine levels of IL-6 in the observational study (Microvolt T wave alternans) dataset. Levels of IL-6 were high in patients with both decompensated HFpEF and HFrEF. Higher levels of IL-6 were associated with increased age in both groups. Higher levels of IL-6 were associated with an increased risk of all-cause mortality and CV death in HFrEF. Whereas higher levels of IL-6 were associated with an increased risk of all-cause mortality, CV death and 1st HF hospitalisation in patients with HFpEF. In HFrEF and HFpEF groups, IL-6 remained an independent predictor of events even after adjustment for established independent predictors or risk including BNP. This potentially suggests in my CHIP cohort that we saw a false negative due to small size and this warrants further investigation.

Levels of IL-6 are not routinely measured in clinical practice, and standard clinical haematological parameters such as RLC and NLR have found to be important in CV disease. In PARADIGM-HF and PARAGON-HF the distribution of RLC was similar in ambulatory patients with chronic HFrEF and HFpEF. Lower RLC was due to a higher total leukocyte (and neutrophil) count, as well as lower lymphocyte count. In each of HFrEF and HFpEF, lower RLC was associated with a similar higher risk of HF hospitalization and death (CV and all-cause). A 10% decrease in RLC was associated with a 17% higher risk of the primary composite end point in both the HFpEF and HFrEF. Importantly, treatment with sacubitril/valsartan appeared to attenuated reduction in RLC suggesting that HF therapies have an impact on circulating levels of inflammation over time.

Conclusion

CHIP is common in patients with both HFpEF and HFrEF. CHIP appears to be associated with elevated levels of inflammatory biomarkers associated with the NLRP3 inflammasome. In patients with recently decompensated HFrEF and HFpEF, circulating levels of IL-6 were particularly high and correlated with worse CV events even after adjustment for established independent predictors of risk including BNP. Considering the findings of my thesis, further research into CHIP and its role as a marker and mediator of inflammation in patients with CV disease is warranted. Further research is also necessary to determine if novel anti-inflammatory therapies might be beneficial for patients with HF and whether these may be personalised based on CHIP status and inflammatory biomarker profiles.

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Author's Declaration

The work presented in this thesis was performed during my employment as a Clinical Research Fellow in the Institute of Cardiovascular and Medical Sciences at the BHF Glasgow Cardiovascular Research Centre, University of Glasgow. I was supervised by Professor Ninian Lang and Professor Mark Petrie.

I performed the screening and recruitment, including obtaining informed consent, of all patients who participated in the CHIP study. I completed all the electronic case report forms for all the study visits, and I constructed the electronic database for the study. I took the required blood samples from each patient recruited to my study and performed all the statistical analysis with supervision from my supervisors. From the blood samples, I also separated the peripheral blood mononuclear cells from serum, which subsequently allowed the detection of CHIP driver mutations. CHIP-driver mutations were analysed by Dr Kristina Kirschner, Dr Tamir Chandra, Dr Neil Robertson, and Dr Maria Terradas. Alongside John Butler I analysed the levels of circulating inflammatory biomarkers from patients' serum and plasma using Meso Scale Discovery technology. To date, work from this thesis has been presented at the American Heart Association Conference 2022 and published in other high impact journals (see below).

I performed a retrospective analysis of IL-6 levels in a previous observational study, Microvolt T wave alternans, recruitment to this study and the study procedures were performed by Dr Colette Jackson at the University of Glasgow in 2009.

I confirm that this thesis has been composed by me solely and that it has not been submitted for any other degree at the University of Glasgow or any other institution. The writing of this thesis is entirely my own work, and all sources of information within this thesis have been specifically acknowledged.

Leanne Mooney

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COVID-19 Statement

This PhD was completed during the COVID-19 pandemic, and unfortunately due to all funding going towards the pandemic my application to recruit 500 patients was unsuccessful. However, this was the first study in NHS Greater Glasgow and Clyde which started recruitment during the pandemic. This study provided key evidence that recruitment to studies was safe for both esearchers and patients after a thorough risk assesment. It also provided evidence that even in during a pandemic patients were keen to be involved in important research. COVID-19 meant that I had to learn a range of new skills in the laboratory which has been invaluable for my learning.

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(PMCID:PMC6755854)

Presentations to Learned Societies

American Heart Association Scientific Sessions 2022: Prevalence and Clinical Characteristics of Patients With Decompensated Heart Failure and Clonal Haematopoiesis of Indeterminate Potential (Poster Presentation) November 2022.

European Society of Cardiology Congress 2021: Interleukin-6 and outcomes in patients recently hospitalised with heart failure and preserved ejection fraction (Poster Presentation). August 2021.

European Society of Cardiology Heart Failure Congress: Neutrophil/lymphocyte ratio and outcomes in patients with heart failure and preserved ejection fraction: An analysis of PARAGON-HF. (Oral Presentation) May 2020.

American Heart Association Scientific Session 2020: Impact of Chronic Obstructive Pulmonary Disease on Outcomes in Heart Failure With Preserved Ejection Fraction: An Analysis of PARAGON-HF (Poster Presentation) November 2020.

Abbreviations

5hmC- 5-hydroxymethylcystosine

5mc 5- methylcytosine

ASXL1- Additional sex comb-like 1

ARCH- Age related clonal haematopoiesis

AHA- American Heart Association

ACEi- Angiotensin converting enzyme inhibitor

ARB- Angiotensin receptor blocker

CANTOS- Anti-inflamamtory therapy with Canakinumab for Atherosclerotic Disease

ARIC- Atherosclerosis Risk in Communities Study

AF- Atrial fibrillation

BP- Blood pressure

BMI- Body mass index

BHF- British Heart Foundation

CRP- C-reactive protein

CO- Cardiac output

CV- Cardiovascular

CXR- Chest X-ray

CKD- Chronic kidney disease

CHIP- Clonal haematopoiesis of indeterminate potential

CABG- Coronary artery bypass graft

CAD- Coronary artery disease

COVID19- Coronavirus-19

DAMPS- Damage-associated molecular patterns

DNA- Deoxyribonucleic acid

DNMT3A- Deoxyribonucleic acid methyltransferase 3 alpha

Echo- Echocardiogram

ECG- Electrocardiogram

EC- Endothelial cell

eGFR- Estimation of the glomerular filtration rate

ESC- European Society of Cardiology

FBC- Full blood count

GP- General Practitioner

GRI- Glasgow Royal Infirmary

HBA1c- Glycosylated haemoglobin

GDF-15- Growth Differentiating Factor-15

HSCs- Haematopoietic stem cells

HF- Heart Failure

HFpEF- Heart Failure with Preserved ejection fraction

HFrEF- Heart Failure with Reduced Ejection Fraction

hsCRP- High sensitivity CRP

hstropT- High sensivity troponin T

HTN- Hypertension

IL1R1- Interleukin 1 receptor like 1

IL-1β- interleukin-1 beta

IL-18- Interleukin-18

IL-2- Interleukin-2

IL-6 Interleukin-6

JHS- Jackson Heart Study

Jak2- Janus kinase 2

KIM-1- Kidney Injury Molecule 1

LAD- Left anterior descending artery

LA- Left atrial

LVEF- Left Ventricular Ejection Fraction

LPS- Lipopolysaccharide

LFTS- Liver function tests

MMP-9- Matrix mettaloproteinase 9

MDRD- Modification of Diet in Renal Disease

MCP-1- Monocyte chemoattractant protein one

MI- Myocardial infaction

NTproBNP- N-terminal-pro hormone BNP

NLR- Neutrophil/Lymphocyte Ratio

NYHA- New York Heart Association

NO- Nitric oxide

NLRs- NOD-like receptors

NLRP3- Nucleotide-binding oligomerization domain, leucine rich repeat and pyrin domain containing 3

PAMPS- Pathogen-associated molecular patterns

PRRs- Pattern recognition receptors

PBMC- Peripheral blood mononuclear cells

PARADIGM-HF- Prospective Comparison of Angiotensin receptor-neprilysin inhibitor with angiotensin converting enzyme inhibitor to Determine Impact on Global Mortality and Morbidity in Heart Failure

PARAGON-HF- The Prospective Comparison of Angiotensin receptor–neprilysin inhibitor with Angiotensin-receptor blockers Global Outcomes in HF with Preserved Ejection Fraction

PPMD1- Protein phosphatase, magnesium/manganese-dependant 1D

QoL- Quality of life

QUEH- Queen Elizabeth University Hospital

ROS- Reactive oxygen species

Treg- Regulatory T cells

RLC- Relative lymphocyte count

RNA- Ribonucleic acid

SHFR- Seattle Heart Failure Risk

SRSF2- Serine/Arginine rich splicing Factor 2

SGLT-2- Sodium-glucose co-transporter 2

SF3B1- Splicing factor 3B, subunit 1

SD- Standard deviation

SBP- Systolic blood pressure

Th17- T helper 17 cells

TET2- Ten-eleven-translocation-2

TFTs- Thyroid function tests

TLR- Toll-like receptors

TP53- Transformation-related protein 53

TGF-β2 -Transforming growth factor beta 2

TAC- Transverse aortic contriction

TNF- Tumour necrosis factor

TNF-α- Tumour necrosis factor alpha

UK BB- UK Biobank

UK- United Kingdom

USA- United States of America

U&Es- Urea and electrolytes

VAF- Variant allele frequency

WBC- White blood count

WHI- Women's Health Initiative

Chapter 1 INTRODUCTION

1.1 What is heart failure?

1.1.1 Definition and classification of heart failure

Heart failure (HF) is a clinical syndrome characterised by symptoms including breathlessness, ankle swelling, fatigue and signs such as elevated jugular venous pressure, pulmonary crackles and peripheral oedema. HF occurs when the heart is unable to maintain adequate cardiac output (CO) either at rest or exertion, due to structural or functional abnormalities. HF is classified according to the duration of symptoms, aetiology of HF and the left ventricular ejection fraction (LVEF) (1).

Acute HF encompasses two groups of patients those who present urgently to hospital with a "de-novo" admission or patients with chronic HF who have suffered a deterioration in symptoms known also as "acute decompensated HF". Chronic HF is the term used to describe ambulatory patients who have experienced classical symptoms and structural/functional abnormalities of the heart for at least one month.

The aetiology of HF is diverse and varies according to age, geographical location and social circumstances (Table 1-1) (1). Understanding the aetiology of HF has several important implications from deciding on investigations (e.g genetic testing) and treatment options.

Table 1-1: Aetiology of HF adapted from the European Society of Cardiology (ESC) HF diagnosis and treatment guidelines (1)**.**

Aetiology	**Examples**
CAD	Previous myocardial infarction (MI), angina and microvascular disease.
Hypertension (HTN)	Primary HTN whereby the cause is unknown, common in elderly patients. Secondary HTN for example: • Renal disease. • Endocrine disease e.g., phaeochromocytoma. • Coarctation of the aorta.
Valvular heart disease	Severe aortic stenosis or acute mitral regurgitation.
Cardiomyopathies	Idiopathic dilated cardiomyopathy, Familial cardiomyopathy, peripartum cardiomyopathy and alcohol related cardiomyopathies.
Congenital heart disease	Congenitally corrected/ repaired congenital heart disease e.g., transposition of the great vessels.
Infective	Viral myocarditis (e.g. Coronovirus-19), human immunodeficiency virus.
Drug-induced	Anabolic steroids, immune checkpoint inhibitors and anthracyclines.
Infiltrative	Amyloid, sarcoidosis, and malignancy.
Storage disorders	Haemochromatosis, Fabry and glycogen storage disorders.
Endomyocardial disease	Eosinophilic myocarditis, carcinoid.
Pericardial disease	Constrictive and restrictive pericarditis.
Metabolic	Thiamine deficiency, vitamin B1 deficiency.

Neuromuscular disease	Muscular dystrophy, Friedreich ataxia.

Classically, HF has been divided into phenotypes based on LVEF which is typically measured using simple and easily accessible transthoracic echocardiogram (echo). LVEF can also be measured with other cardiac imaging modalities including cardiac magnetic resonance imaging and nuclear imaging techniques.

Both the American Heart Association (AHA) and ESC guidelines definitions of HF are outlined in Table 1-2. LVEF may improve with medical therapies and interventions over time, and this is referred to as HF with improved ejection fraction. Both guidelines recognise HF with mildly reduced ejection fraction (HFmrEF), which represents those with an LVEF of 41-49%, which is often referred to as the "grey zone" (1,2). Currently, the trajectory of these patients is unclear with some demonstrating improvement in their ejection fraction to HF with preserved ejection fraction (HFpEF) with time and others deteriorating to HFrEF. HFpEF is defined in both guidelines as LVEF ≥50% and evidence of spontaneous or provokable increased LV filling pressures and consistent cardiac structural abnormalities.

The recent large clinical trials in HFpEF/HFmrEF have used varying LVEF cut offs, with some opting to define HFpEF as LVEF of ≥40% (3,4), or to include HFmrEF and HFpEF in the same trial (5), or lastly to use an LVEF cut off ≥45% (6,7).

While EF cut-offs are required for the purpose of clinical trials and evidenced based HF therapies, it is problematic as it does not fully consider the pathophysiology of HF and often patients EF will vary over time. Furthermore, EF cut offs are not solely predictive of response to HF therapies and other factors such as sex and levels of inflammation appear to be just as important. Indeed, in women with HFpEF treatment sacubitril/valsartan reduced the risk of HF hospitalisation more than men (8). Therefore, this thesis includes patients with both decompensated HF patients and chronic stable HF patients.

Table 1-2: HF phenotypes according to ESC/ AHA guidelines.

HFrEF	HFmrEF	HFpEF
Symptoms ± Signs LVEF ≤ 40%	Symptoms ± Signs LVEF 41-49%	Symptoms ± Signs LVEF ≥50% Evidence of cardiac structural/ functional abnormalities e.g., raised natriuretic peptides.

HFrEF, I with reduced ejection fraction; HFmrEF, heart with mid-range ejection fracIheart failure with preserved ejection fraction; LVEF, left ventricular ejection fraction.

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1.1.2 Epidemiology of HF

HF is common and it is estimated that more than 1 million people in the UK have HF (more than 780,000 people are on the General Practitioner (GP) register with HF) (9). The prevalence of HF continues to grow and its incidence rises substantially with age: from around 1% for those aged <55 years to >10% in those aged over 70 years or over (10–13). HFpEF now accounts for over half the cases and has an even closer relationship to ageing than HFrEF (14,15). Around 24% of all UK deaths are caused by heart and circulatory disease (9). In a large longitudinal follow up study of 39,982 patients, all three groups of HF (HFrEF, HFmrEF and HFpEF), had a similar five year mortality and the composite of mortality and rehospitalization (16).

1.1.3 New York Heart Association (NYHA) classification of HF

Patients with HF experience a range of symptoms and even patients with mild symptoms may still have a high-risk of hospitalisation and death (17). The most common terminology used to describe the severity of HF is the NYHA functional class. NYHA classification involves doctors subjective and patient reported limitation in function, and intra/interobserver variability has been reported (18,19). While NYHA class can provide some information on patient prognosis there are other better prognostic indicators in HF such as markers of cardiac stretch, N-terminal-pro hormone BNP (NT-proBNP). Furthermore, there has been growing amount of

evidence to suggest that a multi and serial biomarker measurement may more accurately predict HF risk (20). In particular, recently there has been an increased enthusiasm for the use of specific inflammatory levels to assess responsiveness to treatment of HF, e.g. IL-1β (21). Whether or not a multiple biomarker approach coupled with more invasive and less subjective cardio-pulmonary exercise testing will improve prognostic information for patients with HF is currently unknown.

Table 1-3: NYHA functional class

Class I	No limitation of physical activity. Ordinary physical activity does not cause undue breathlessness, fatigue, or palpitations.
Class II	Slight limitation of physical activities. Comfortable at rest, but ordinary physical activity results in undue breathlessness, fatigue or palpitations.
Class III	Marked limitation of physical activity. Comfortable at rest, but less than ordinary physical activity results undue breathlessness, fatigue or palpitations.
Class IV	Unable to carry out on any physical activity without discomfort. Symptoms at rest can be present.

1.1.4 Pathophysiology of HF

In HFrEF, an initial insult or injury (such as MI) results in a reduction in CO. Following this, numerous compensatory mechanisms are activated to maintain adequate CO. This remodelling process is complex and involves activation of the adrenergic nervous system with increased levels of noradrenaline, the renin-angiotensin aldosterone system, and the inflammatory system which will be addressed in more detail later in the thesis.

There is substantial heterogeneity in the aetiology and pathophysiology of HFpEF. Although structural and functional abnormalities are becoming better defined. Cardiac biopsies obtained from patients with HFpEF reveal structural alterations including cardiomyocyte hypertrophy (22,23) and interstitial fibrosis (22,24–26), while functional changes include impaired myocardial relaxation (27) and increased myocardial stiffness (22,24,25). Cardiac biopsies also reveal higher levels of myocardial inflammatory cells in patients with HFpEF (28). Post mortem findings from patients with HFpEF reveal more extensive CAD, a greater burden of myocardial fibrosis and reduced microvascular density compared with controls without HF (29). Large artery stiffening may, at least in part, contribute to the pathophysiology of HFpEF (30).

Non-cardiac comorbidities are common in HFpEF, particularly obesity, diabetes, chronic kidney disease (CKD) and HTN (31). A novel paradigm proposes that the systemic inflammatory state induced by these comorbidities induces coronary microvascular endothelial dysfunction (32). The production of inflammation-induced reactive oxygen species (ROS) limits the bioavailability of nitric oxide (NO) with consequent impairment of cardiomyocyte protein kinase G activity, microvascular ischaemia, fibrosis and left ventricular concentric remodelling (33).

Figure 1-1: Inflammatory Paradigm in HFpEF

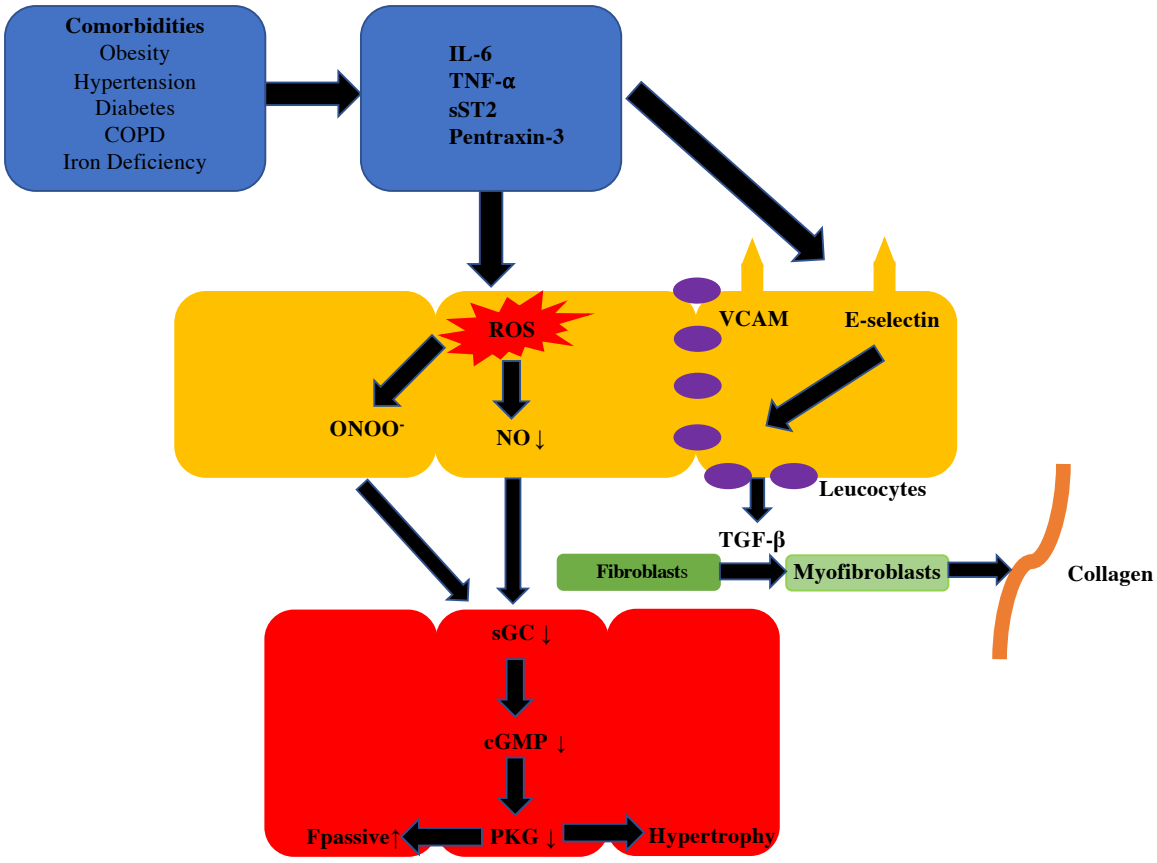


Figure adapted IParadigm for Heart Failure With Preserved Ejection Fraction: Comorbidities Drive Myocardial Dysfunction and Remodeling Through Coronary Microvascular Endothelial Inflammation. Paulus, WJ & Tschope, C. JACC Vol 62, Issue 4. Page 264. (32) *IL-6, Interleukin-6; TNF-α, Tumour necrosis factor-alpha; sST2, soluble suppression tumorigenesis-2; ROS, reactive oxygen species; NO, nitric oxide; ONOO⁻, peroxynitrite; VCAM, vascular cell adhesion protein; TGF-β, transforming growth factor beta; sGC, soluble guanylate cyclase; cGMP cyclic gyanosine monophosphate; PKG, protein kinase G.*

1.1.5 Prognosis of HF

The prognosis of HFrEF has improved considerably over the last decade, however patients still experience frequent hospital admissions and reduced quality of life (QoL). Observational studies have shown that patients with HFpEF and HFrEF have similar mortality rates and impairment of QoL (16,34–36). A large meta-analysis revealed that HFrEF diagnosed at a young age had a worse prognosis than HFpEF at a young age (37). It also revealed that systolic blood pressure (BP) had a stronger inverse association with mortality in patients with HFrEF (37). During the Coronavirus-19 (COVID-19) pandemic, admissions for acute CV conditions declined and patients who were admitted had shorter lengths of stay, but mortality during the pandemic were not different (38). Notably, the recruitment of patients for this thesis was during the COVID-19 pandemic and I discuss this in detail later in the thesis.

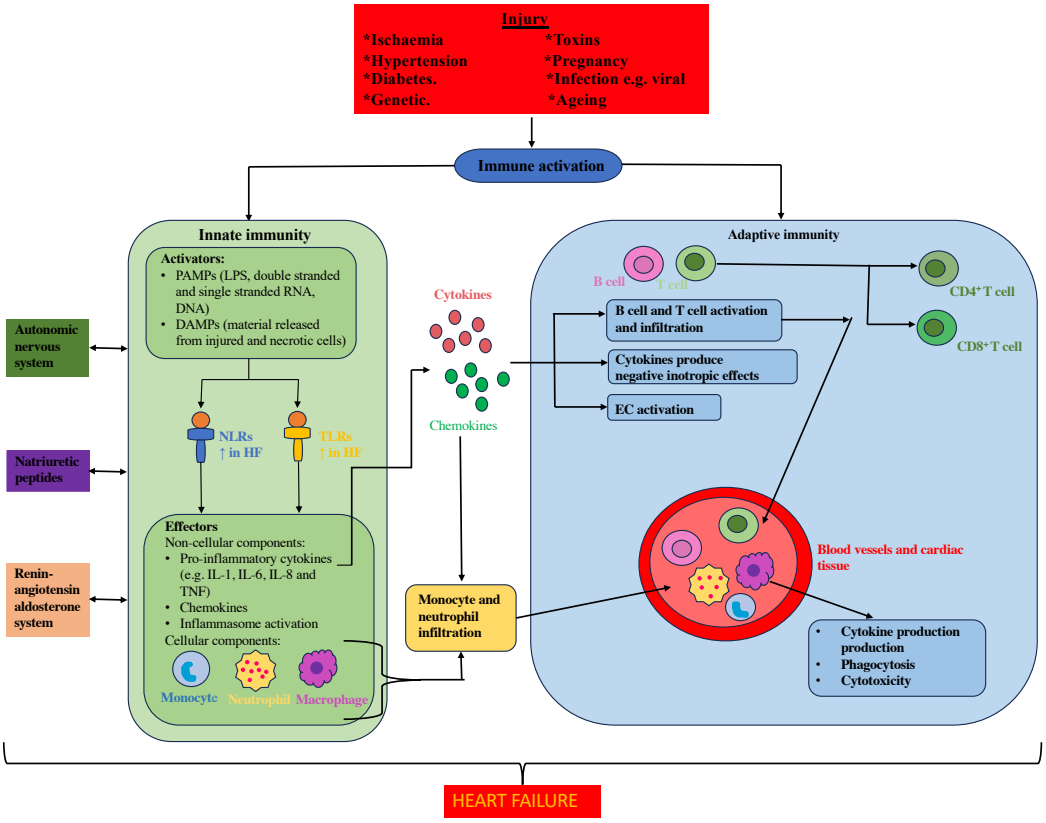
1.1.6 Summary

Both HFrEF and HFpEF affect a substantial proportion of the general population, and both types of HF are associated with similarly high levels of morbidity and mortality. HF places a considerable financial burden on our healthcare systems and leads to a significant reduction in patients' QoL. While there are good effective treatments available for HFrEF, only dapagliflozin and finerenone have been shown to be beneficial in patients with HFpEF. It has never been more important that new and alternative treatments for HF are explored, including anti-inflammatory therapy, to reduce the substantial morbidity and mortality experienced.

1.2 Inflammation and cardiovascular (CV) disease

The association between inflammation and CV disease has been recognised for over a decade. Inflammation plays an important role in the development, progression, and complications of several CV diseases. Despite this, initial clinical trials of anti-inflammatory therapy were largely unsuccessful or resulted in worse clinical outcomes (39,40). However, the recent Anti-inflammatory Therapy with Canakinumab for Atherosclerotic Disease (CANTOS) trial has re-invigorated the need to further understand and characterise inflammation in CV diseases. This randomized double-blind placebo-controlled trial demonstrated that therapy with canakunimab, an IL-1B inhibitor, led to a dose-dependent reduction in HF hospitalisation and the composite of HF hospitalisation or HF related mortality in a population of patients with prior MI and elevated levels of C-reactive protein (CRP). Furthermore, the relatively recent development of rapid multiplex assays has facilitated the measurement of multiple biomarkers from a very small volume of stored serum. This has made measuring multiple biomarkers of inflammation more accessible, quicker, and affordable for research purposes. In this thesis, I have explored the role of novel biomarkers and traditional simple biomarkers in both stable and decompensated HFpEF and HFrEF. The figure below provides an overview of the role of the immune system in the development of HF.

Figure 1-2: Immune mechanisms in HF pathophysiology



PAMPS, pathogen-associated molecular patterns; LPS, lipopolysaccharide; RNA, ribonucleic acid; DNA, deoxyribonucleic acid; DAMPS, damage-associated molecular patterns; IL, interleukin; TNF, tumour necrosis factor; EC, endothelial cell.

Initial cardiac injury activates the innate immune response in the heart through binding of pathogen-associated molecular patterns (PAMPS) or damage-associated molecular patterns (DAMPS) to pattern-recognition receptors (PRRs), such as toll-like receptors (TLRs) and NOD-like receptors (NLRS), which are present on cardiac myocytes and immune cells. This results in the release of pro-inflammatory cytokines and activation of the complement system, which leads to endothelial cell activation and the recruitment of monocytes and neutrophils. This then triggers the activation of the adaptive immune response through the recruitment of B cells and T cells.

1.2.1 Inflammation and incident HF

Several population and cohort studies have examined the association of inflammatory biomarkers with the development of HF in different population cohorts (Table 1-4). However, most of these studies have examined non-specific markers of inflammation including CRP and white blood cell (WBC) counts including its derivatives (NLR and RLC) have consistently shown that elevated levels of inflammation are associated with an increased risk of developing HF. The relatively recent discovery of CHIP and its role in HF, appears to suggest that specific inflammatory markers including IL-6 may be even more important in the development and progression of several CV disease.

Recently, the relationship of more specific inflammatory markers and different HF phenotypes has been examined. In particular, interleukin-6 (IL-6) has been shown to be predictive of incident HFpEF (HR 1.59, 95%CI [1.16-2.19], p=0.004), rather than HFrEF (HR 1.05, 95%CI [0.75-1.47], p=0.77)(41). The Framingham Heart Study found that the both growth differentiation factor-15 (GDF-15) (HFpEF; HR 1.67; 95%CI 1.32-2.12, P<0.001; HFrEF; HR 2.13 95% CI 1.52-1.99, P<0.001) and CRP (HFpEF; HR 1.46; 95%CI 1.17-1.83, p=0.001; HFrEF 1.46 95%CI 1.17-1.83, P<0.001) were associated with an increased risk of incident HFpEF and HFrEF, respectively but they did not differentiate between the two phenotypes (42). In other studies, tumour necrosis factor alpha (TNF-α), IL-6 and cystatin C were strongly associated with incident HFpEF, but less so with HFrEF (43–45). In the Multi-Ethnic Study of Atherosclerosis, interleukin-2 (IL-2) was associated with new onset HFpEF, but not HFrEF (46). Furthermore, the Atherosclerosis Risk in the Community Study (ARIC) recently studied ten proteins which contribute to neutrophil activity whose activity can be modified by colchicine (which included biomarkers such as matrix metalloproteinase 9 (MMP-9) (45). In unadjusted analysis, higher neutrophil activity was associated with a heightened risk of both incident HFpEF and incident HFrEF, but only incident HFpEF remained significant following further adjustment for age, sex, race and field centre (45). Importantly, higher neutrophil activity was associated with greater diastolic dysfunction (45). In this thesis, I have examined circulating levels of RLC & NLR in stable patients with HFrEF and HFpEF, and have also examined the more specific IL-6 cytokine in patients with decompensated HFrEF and HFpEF.

Table 1-4: Inflammatory markers and risk of incident HF

Inflammatory marker assessed	Cohort	Number of patients	Participant characteristics	Median follow up period	HRs 95%CIs	Ref
WBC	Atherosclerosis Risk in Communities (ARIC)	14485	US population without previous CVD.	15.5yrs (mean)	1.62 [1.34-1.96]	(47)
	EPIC-Norfolk study	17891	European men and women aged 39-79.	12.4yrs	1.09 [1.04-1.15]	(48)
ESR	ULSAM	2314	Community based study of men aged 50yrs old.	29.6yrs	1.31 [1.03-1.67]	(49)

CRP	Cardiovascular Health Study (CHS)	4017	Age≥65yrs without baseline CHF or diabetes.	12.2yrs	1.53 [1.34-1.75]	(50)
	Strong Heart Study Phase II	3098	American Indians without prevalent CVD.	11yrs (mean)	1.25 [0.97-1.32]	(51)
	British regional heart failure study	3569	Men without previous MI or HF.	16.3yrs (mean)	1.05 [0.95-1.17]	(52)
	ARIC	9978	US population without previous CVD.	15.5yrs (mean)	1.70 [1.14-2.53]	(47)
	Population based study of Finnish men	10106	Finnish men without HF at baseline.	8.8yrs	1.09 [1.04-1.15]	(53)

	Malmo diet and cancer study	4691	Swedish population cohort free from CVD at baseline.	13.2yrs (mean)	1.8 [1.4-2.2]	(54)
	MOGRAM project (20 cohorts from six countries)	52799	Individuals with/without diabetes and without HF at baseline.	14.1yrs	1.13 [1.03-1.24]	(55)
	Health ABC study	311	Community dwelling men and women age 70-79yrs without HF.	9.4yrs	1.60 [0.97-2.97]	(44)
	Multi-Ethnic Study of Atherosclerosis (MESA)	6814	Multicentre cohort study, aged 45-84yrs.	4.0yrs	1.38 [1.01-1.86]	(56)
	Rotterdam study	6437	Population based study, aged≥55 without HF.	6.5yrs	2.64 [2.04-3.43]	(57)

	Study of men born in 1943	747	Prospective study of men born in Sweden.	21yrs	2.61 [1.59-4.29]	(58)
IL-6	British regional heart failure study	3569	Men without previous MI or HF.	16.3yrs (mean)	1.09 [0.91-1.31]	(52)
	Prevention of Renal and Vascular End Stage disease	961	Case cohort study of people free from HF at baseline.	8.2yrs	1.45 [1.18-1.78]	(41)
	Health ABC study	311	Community dwelling men and women age 70-79yrs without HF.	9.4yrs	1.75 [1.20-2.67]	(44)
	Multi-Ethnic Study of Atherosclerosis (MESA)	6814	Multicentre cohort study, aged 45-84yrs.	4.0yrs	1.50 [1.10-2.03]	(56)

	Study of men born in 1943	747	Prospective study of men born in Sweden	21yrs	1.50 [0.94-2.39]	(58)
TNF-α family including TNF-αr1 & R2	Health ABC study	311	Community dwelling men and women age 70-79yrs without HF	9.4yrs	3.10 [2.4-3.96] (TNF-α)	(44)
	Health ABC study	1285	Community dwelling men and women age 70-79yrs without HF	11.4yrs	1.28 [1.02-1.61] (TNF-α)	(59)
					1.68 [1.15-2.46] (sTNF-R1)	
					1.15 [0.80-1.63] (sTNF-R2)	
	MESA	2869	Multicentre cohort study, aged 45-84yrs.	14.2yrs	1.43 [1.21-1.70]	(60)
IL-1 receptor antagonist	Population based study of Finnish men	10106	Finnish men without HF at baseline	8.8yrs	1.15 [1.05-1.26]	(53)

IL-2 receptor antagonist	MESA	2869	Multicentre cohort study, aged 45-84yrs.	14.2yrs	1.26 [1.04-1.53]	(60)
Galectin-3	Atherosclerosis Risk in Communities (ARIC)	8687	US population without previous CVD	20.5yrs	1.49 [1.18-1.88]	(61)

1.2.2 Inflammation in human HFrEF and HFpEF

HFpEF and HFrEF represent two distinct pathophysiological entities, however with time and CV therapies a patients EF can improve or decline. However, inflammation is common in both, and levels of inflammation appear to vary throughout the clinical course. Disease processes and comorbidities such as concurrent infection, diabetes, obesity and CKD are associated with higher levels of inflammation. To date, only a small number of studies have investigated the biomarker profiles in HFrEF, HFmrEF and HFpEF. The main findings of these studies have been summarised in (Table 1-5). Furthermore, the recent discovery of CHIP suggests that mutations in the HSC may drive inflammation predisposing to CV disease and worse prognosis. Therefore, it has never been more important to understand the role of circulating inflammatory biomarkers in stable and decompensated HF.

TIME-CHF was the first relatively large study to examine the role of a small number of biomarkers in HFrEF versus HFpEF (62). Patients with HFpEF had significantly higher levels of soluble interleukin 1 receptor-like-1 (IL1R1), high sensitivity CRP (hsCRP) and cystatin-C (62). However, the discriminative value for HFpEF versus HFrEF of each biomarker separately was non-significant in receiver operating characteristic curve analysis (62).

Subsequently, a study examined 33 biomarkers in 460 patients at discharge after hospitalisation for HFrEF or HFpEF (63). Patients with HFpEF had higher levels of hsCRP, while NTproBNP levels were higher in HFrEF (63). Linear regression followed by network analysis revealed prominent inflammation and angiogenesis-associated interactions in HFpEF, whereas HFrEF was mainly associated with cardiac stretch (63).

An even larger panel of 37 biomarkers was analysed twenty-four hours after admission to hospital in a cohort of 843 patients with acute decompensated HF. This was the first study to define the biomarker profile in HFmrEF, alongside HFrEF and HFpEF (64). Network analysis revealed similar results to previous studies with markers of cardiac stretch being mostly related to HFrEF, whereas in HFpEF biomarker interactions were mostly related to inflammation (64). This was the first study to demonstrate that in HFmrEF, biomarker interactions related to both inflammation and cardiac stretch (64).

More recently the largest biomarker profile to date (92 biomarkers) were studied of patients with chronic HFrEF, HFmrEF and HFmrEF. HFrEF was associated with upregulation of cellular growth and metabolism, whereas HFpEF was associated with inflammation and extracellular matrix reorganisation (65). Furthermore, there were specific unique biomarker correlations in HFrEF which included NTproBNP, GDF-15 and IL1R1, and in HFmREF IL-1B was specifically upregulated (65). However, in HFpEF there was a more general upregulation of biomarkers which may represent the heterogenous nature of HFpEF (65).

Overall, the above studies emphasize that markers of cardiac stretch are more important in HFrEF, and makers of inflammation appear more important in HFpEF. Therefore, targeting of specific inflammatory pathways may be of benefit for the tailored treatment of HF. To date, there is a significant lack of research demonstrating changes in inflammatory biomarkers over time and this warrants future research.

Table 1-5: Summary of studies examining biomarker profiles in patients with HFrEF, HFmrEF and HFpEF.

Cohort	Number of patients	Participant characteristics	Main findings	Ref
Time-CHF	458	CHF patients age ≥60 with NYHA class≥II	• HFpEF higher levels of IL1R1, hsCRP and cystatin C. • HFrEF higher levels of NTproBNP, hstrop-T and haemoglobin.	(62)
COACH trial	460	CHF patients with biomarkers measured at discharge after hospitalisation (HFrEF and HFpEF)	• HFrEF had significantly higher levels of cardiac stretch (NT-proBNP and proANP) when compared to HFpEF. • Levels of hsCRP were significantly higher in HFpEF when compared to HFrEF. • Network analysis revealed HFpEF was more associated with inflammation and remodelling, whereas HFrEF angiogenesis was a more prominent feature. • HFpEF mainly associated with IL-6 and pentraxin-3, whereas HFrEF showed exclusive interactions with NTproBNP.	(63)
PROTECT-trial	843	AHF at admission and 24hours (HFrEF, HFmrEF, HFpEF)	• Increasing LVEF showed a trend to increasing levels of CRP and KIM-1, and decreasing levels of troponin, BNP and GDF-15. • Change in troponin-I from admission to 24hours was more in patients with HFrEF than HFpEF or HFmrEF.	(64)

			• HFrEF- troponin I and BNP were central hubs at admission and BNP and endothelin-1 after 24hours. • HFmrEF- haemoglobin, endothelin-1, BNP and galectin-3 central hubs at admission. • HFpEF- angiogenin, haemoglobin, galectin-3 and D-dimer were hubs at admission and after 24hours were mainly associated with inflammation.	
BIOSTAT-CHF	1544	Scottish and European cohort of patients with CHF age≥18years	• HFrEF showed unique biomarker correlations for NT-proBNP, GDF-15 and IL1R1. • HFmrEF IL-1B was a central hub. • HFpEF associated with upregulation of inflammatory pathways. • HFpEF was a strong independent predictor of elevated IL-6 levels.	(65,66)

CHF; chronic heart failure, HFpEF; heart failure with preserved ejection fraction, HFrEF; heart failure with reduced ejection fraction, IL1R1, interleukin-1 receptor type 1, hsCRP: high sensitivity CRP, NTproBNP; N-terminal prohormone of brain natriuretic peptide, hstrop; high sensitivity troponin, proANP; pro atrial natriuretic peptide, KIM; kidney injury molecule, AHF; acute heart failure, LVEF; left ventricular ejection fraction, GDF-15; growth differentiating factor-15.

1.2.3 Inflammation and HF outcomes

Patients with HF have a high risk for CV events, including HF hospitalisation, CV death, stroke, MI and sudden cardiac death. Initial studies which examined the association between inflammation and CV events focused on non-specific markers of inflammation, including CRP and WBC differentials which are readily available in clinical practice. There are now multiple studies examining a range of inflammatory biomarkers in varying cohorts of patients with a range of CV diseases from HF to CAD. Despite this substantial evidence, until recently no specific anti-inflammatory therapy have clinical benefits. However, the recent CANTOS trial examined the effects of canakinumab, a monoclonal antibody directed against IL-1β, in patients with a history of prior MI and elevated CRP. Canakinumab reduced CRP and the incidence of atherosclerotic CV events by 15% versus placebo (21). Notably, canakinumab also reduced HF hospitalization and HF-related mortality by 23% in patients who achieved a CRP level of <2mg/L (67). Therefore, in this thesis I examined both non-specific RLC and NLR, and other more specific markers of inflammation including IL-6 and IL-1β.

1.2.4 Summary

Levels of inflammation not only predict the development of both HFrEF and HFpEF, but they are associated with worse HF outcomes. HFpEF appears to be more strongly associated with higher levels of inflammation than HFrEF. Evidence demonstrates that targeting inflammation with pharmacological therapies may be beneficial to reduce morbidity and mortality for both types of HF. This thesis explores the link between CHIP, inflammation and HF, while examining important inflammatory biomarkers and the change in levels of inflammation with treatment with sacubitril/valsartan in large datasets. Therefore, aiming to prove that personalised anti-inflamamtory therapy is the future of HF management.

1.3 Clonal Haematopoiesis of indeterminate potential (CHIP)

This section has been adapted from my published review article in Clinical Science which examined CHIP and its association with inflammation, vascular disease and heart failure (68).

1.3.1 Definition and Overview of CHIP

CHIP, also known as age related clonal haematopoiesis (ARCH), reflects the accumulation of potentially pre-leukaemic, somatic mutations in haematopoietic stem cells (HSCs) over time (69,70). However, whilst the risk of malignant transformation of CHIP is low, its presence confers a substantially greater risk of CV disease including HF and CAD (70–75). This increased risk appears to be driven by elevated levels of inflammation in particular IL-6 and IL-1β, rather than elevation of non-specific biomarkers including RLC and NLR.

Figure 1-3: Development of CHIP

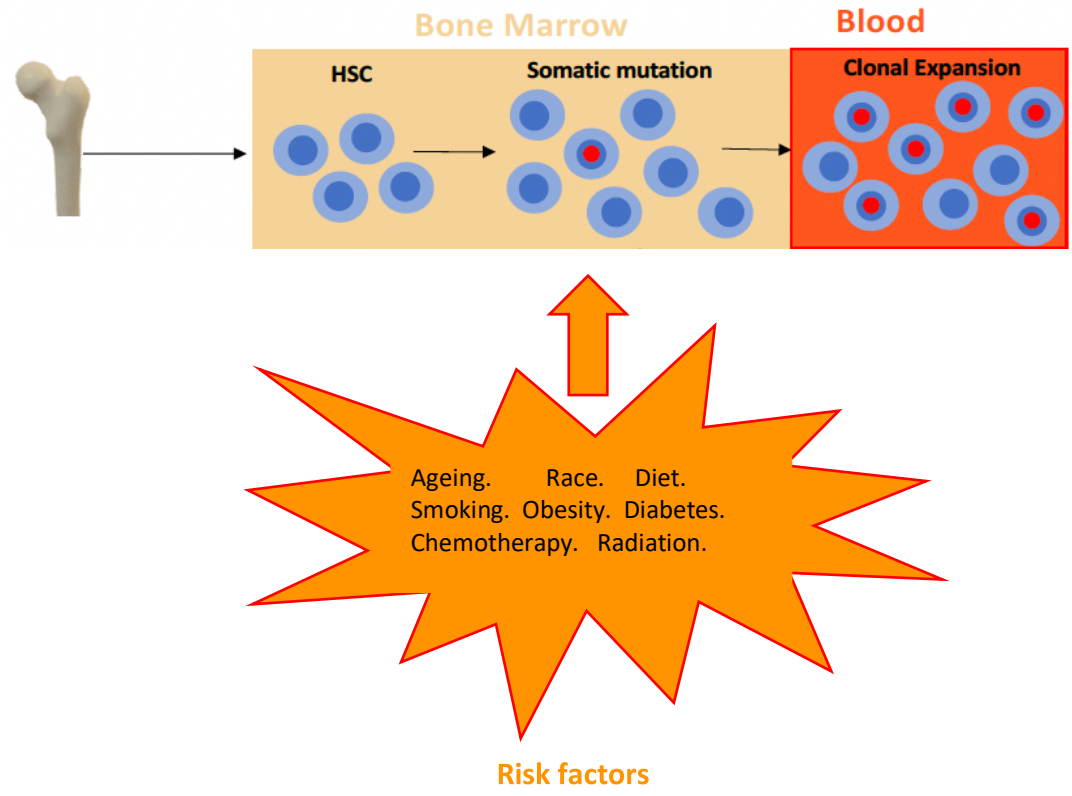


Table 1-5: Current diagnostic criteria for CHIP

1) the absence of overt haematological malignancy;
2) a normal peripheral blood count and;
3) mutant cells bearing relevant driver mutations in ≥2% of peripheral white blood cells (VAF ≥2%)

CHIP can be detected via DNA sequencing of peripheral blood, saliva and tumour samples and, initially the proposed 2% of VAF for the definition of CHIP was based on the lower limit of reliable detection of small somatic variants using whole exome sequencing and the clinical consequences of these smaller clonal population are largely unknown (76–79). The development of ultra-deep, error-corrected targeted sequences are now capable of detecting mutations with a VAF<0.5% (78).

To date, only one study has examined the effect of small clonal size (i.e. VAF<2%) on five year mortality in a cohort of 419 patients with chronic ischaemic heart disease. Those with a VAF of<2% had an increased risk of mortality (80). The exact VAF cut off was dependent of the type of mutation of mutation observed (80). Whether the same increased risk exists for the subsequent development of haematological cancer or CV events at lower VAF levels is currently unknown. However, malignant potential is probably more related to quantitative levels, features of the mutation (i.e. specific deletion) and the sequential acquisition of multiple mutations (70,75,78,81–83).

The most frequently encountered somatic mutations are within the driver genes ten-eleven-translocation-2 (TET2), DNA methyltransferase 3 (DNMT3), Janus kinase 2 (Jak2) and additional sex comb-like1 (ASXL1). CHIP-associated mutations are also found, albeit less frequently, in other driver genes outlined below (84).

Table 1-7: Most frequent somatic mutations in CHIP

Gene	Name	Description
TET2	Ten-eleven-translocation-2	A methylcytosine dioxygenase that catalyses the conversion of 5-methylcytosine to 5-hydroxymethylcytosine. An epigenetic regulator that can activate or repress transcription.
DNMT3A	DNA methyltransferase 3A	A *de novo* DNA methyltransferase.
Jak2	Janus kinase 2	Receptor tyrosine kinase involved in haematopoietic cytokine signalling and myelopoiesis.
ASXL1	Additional sex combs-like 1	Polycomb chromatin-binding protein that is involved in the transcriptional regulation of *Hox* genes.
PPMD1	Protein phosphatase, magnesium/manganese-dependent 1D	Protein phosphatase involved in dephosphorylation and inactivation of proteins in the DNA damage response pathway.
SF3B1	Splicing factor 3B, subunit 1	A component of the U2 small nuclear riboprotein that binds to the 3' branch site in pre-mRNA splicing and processing.
SRSF2	Serine/Arginine rich splicing Factor 2	Required for 5' and 3' spliceosome assembly, splice-site selection, U1 and U2 snRNP interactions with pre-mRNA, and alternative splicing.
TP53	Transformation-related protein 53	Tumour suppressor transcription factor that responds to cellular stress and DNA damage.

Adapted from Clonal Hematopoiesis: Crossroads of Aging, Cardiovascular Disease, and Cancer: JACC Review Topic of the Week. J Am Coll Cardiol(85);

DNA, deoxyribonucleic acid; mRNA, messenger ribonucleic acid; snRNP, small nuclear ribonucleioproteins.

1.3.2 Common CHIP mutations

1.3.2.1 DNMT3A

DNMT3A modulates gene transcription via the catalysis of DNA methylation and is the most frequently mutated gene in people with CHIP. DNMT3A mutations are thought to be loss of function mutations including missense mutations, nonsense mutations, insertions-deletions and splice site mutations (86). Although there are reports that some mutations may lead to gain of function, conferring increased HSCs self-renewal and subsequent clonal expansion (87,88).

DNMT3A also has multiple roles in the regulation of inflammation. In particular, it controls cytokine expression through the regulation of the scaffold protein IQ motif containing GTPase Activating Protein 2 in mast cells (89). In patients with osteoarthritis, IL-6 gene activity is associated with the expression of DNMT3A and significantly lower levels of IL-6 secretion are found in those with DNMT3A overexpression (90). In a small cohort of patients with HF, the presence of DNMT3A mutation was associated with higher transcription of IL-6, IL-1β, IL-8, NLRP3 inflammasome, and the macrophage inflammatory proteins CCL3 and CCL4 (91). Furthermore, in patients with severe aortic stenosis, the presence of DNMT3A mutations has been associated with significantly elevated T helper 17 cell(TH17): Regulatory T cells (Treg) ratio, representing pro-inflammatory T-cell polarization(74).

1.3.2.2 TET2

Mutation of TET2 was the first somatic genetic abnormality to be reported in blood cells from individuals with CHIP without overt haematological malignancy (92). TET2 is a member of a family of enzymes located on chromosome 4q23 and is an epigenetic regulator of DNA methylation. It catalyses the oxidation of 5-methylcytosine (5mC) to 5-hydroxymethylcystosine (5 hmC) as the first step in cytosine demethylation (93). This activity is critical for maintaining the normal development of HSCs. TET2 mutations are loss-of-function mutations associated with a decrease in 5hmC availability and consequently this has been proposed as a potential diagnostic and prognostic biomarker in haematological malignancy (94). Whether it holds the same potential utility in the prediction of CHIP/TET2 mutation-associated CV disease remains to be tested.

TET2 has an important role in restraining the expression of inflammatory genes in macrophages. TET2-deficient macrophages show increased inflammation, both spontaneous and in response to lipopolysaccharide, in particular higher expression of IL-1β and IL-6 (95). In an unselected cohort of patients without CV disease, the presence of TET2 mutation was associated with over two-fold higher circulating concentrations of IL-8 than in those without this mutation (71). Furthermore, analysis of the Trans-Omics for Precision Medicine (TOPMed) cohort revealed significantly increased serum IL-1β levels among TET2 carriers (96).

1.3.2.3 ASXL1

ASXL1 is the third most commonly mutated gene in CHIP and encodes an epigenetic regulator which binds to chromatin. It is one of the most frequently mutated genes in myeloid neoplasms and its presence is associated with poor prognosis (97–99). The majority of mutations are frameshift or nonsense mutations and frequently coexist with TET2, IDH1 and IDH2 mutations (99–102). However, whether these truncations of the protein lead to loss or gain of function remains controversial (103–105).

Observational studies detail a link between ASXL1 mutations with smoking and among patients with HIV (106,107). Mutations of ASXL1 are common in patients with atherosclerosis and chronic ischaemic HF, but the mechanisms by which ASXL1 enhances inflammation is poorly understood. To date, no study has investigated the downstream inflammatory effects of ASXL1 CHIP carriers.

1.3.2.4 Jak2^{V617F}

Of CHIP-associated genetic abnormalities, Jak2^{V617F} gain of function mutation has been linked most clearly to inflammatory processes. V617F somatic mutation of the Jak2 gene reflects substitution of phenylalanine for valine at position 617. Jak2^{V617F} mutations are commonly associated with myeloproliferative neoplasms including essential thrombocythaemia, polycythaemia vera and myelofibrosis (108). These conditions result in increased blood viscosity and a pro-coagulant state and are associated with an increased risk of stroke, MI and deep vein thrombosis. However, Jak2^{V617F} mutations are increasingly recognised in individuals with normal peripheral blood counts, and remain associated with increased CV mortality (109–112).

1.3.4 Epidemiology, triggers and risk factors of CHIP

The natural process of ageing results in an increased likelihood of retaining somatic mutations. Such mutations are rare in people under the age of 40 and by the age of 70 years, 10 to 20% of the otherwise healthy population will have CHIP (85,113,114). It has been postulated that all adults have some CHIP mutations at extremely low VAFs (78,115), indeed in a study of individuals ≥80years old 62% of participants had CHIP with a VAF≥1% (116). At the molecular level, DNA damage, telomere shortening and autophagy appear to be central mechanisms underlying age-related functional impairment and decline in the durability of HSCs (68,117,118). Chronic low-grade inflammation occurs with ageing (recently described as *inflammageing*) and may also be partly responsible (68,119). Indeed, exposure of mice to the pro-inflammatory mediator, TNF-α, promotes the expansion of TET2 mutant clones and exposure to inflammatory stress in myeloid cells results in the rapid increase in frequency and absolute number of TET2-mutated myeloid cells (68,120,121).

There is also a difference in the distribution of mutated CHIP genes with age, de novo DNA methyltransferase DNMT3A and JAK2 are observed in the third and fourth decade of life, whereas clones carrying mutations in spliceosome are generally not detected until after the fifth and sixth decade of life (69,96,122). The reason for this age-dependant difference in mutations is an area of ongoing investigation but is likely due to several contributing factors such as the HSCs ability to repair, regenerate and exposure to exogenous stress that occurs with ageing.

The prevalence of CHIP varies across several demographic features; with an increased prevalence observed in men and in comparison, to those of European ancestry CHIP occurs less frequently in individuals of Hispanic, East Asian and African origin (70,96,123).

Exogenous stressors that directly provoke inflammation, DNA damage, telomere shortening and production of ROS may lead to the premature exhaustion of HSCs and an increased likelihood of retaining somatic mutations at a younger age (124). Consistent with this hypothesis, prior chemotherapy and radiotherapy, in particular TP53 and PPM1D, are associated with an increased susceptibility to the retention of these somatic mutations in humans (125,126). Several environmental factors have also been shown to be associated with the development of CHIP including smoking, diet and diabetes (3,13,28). In particular, a study has showed that mutations in ASXL1 and genes coding for spliceosomes are strongly

associated with exposure to DNA-damaging agents due to substance abuse such as smoking (116), but not with DNMT3A and TET2 variants.

Table 1-8 Risk factors for CHIP

		Degree of risk	Ref
Non-modifiable risk factors	Age	↑	(70,75,109)
	Male sex	↑	(70)
	Race Hispanic ancestry	↓	(70)
	Asian ancestry	↓	(96)
	African ancestry	↓	(123)
Modifiable risk factors	Smoking	↑	(106,109)
	Diabetes	↑	(70)
	Unhealthy diet	↑	(127)
	Radiation exposure	↑	(125)
	Chemotherapy exposure (PPM1D mutations) Platinum agents (cisplatin, carboplatin and oxaliplatin)	↑	(125,128)
	Topoisomerase inhibitor (etoposide)	↑	

Ref, References

1.3.4 Risk of haematological malignancy and CV disease in patients with CHIP

CHIP belongs to a spectrum of haematological pre-malignant states and is associated with the development of various haematological malignancies including leukaemia, lymphoma and myeloma (3,26). However, most carriers will not develop malignancy and the progression rate is approximately 0.5%-1% per year (129). Malignant transformation or progression generally requires the acquisition of multiple mutations and directly correlates with the mean VAF

(70,130). There is variation in the rate and type of CHIP mutations seen in patients with different form of cancers. It is notable that patients found to have CHIP at the time of autologous stem cell transplantation are at an increased risk for the subsequent development of therapy-related myeloid neoplasm (myelodysplastic syndrome and acute myeloid leukaemia) (82). PPM1D mutations have also been shown to be present in patients with breast, ovarian and lung cancer and are significantly associated with prior exposure to chemotherapy, and there appears to be a dose response effect with doxorubicin (131–135). In a murine model PPM1D mediated therapy-related clonal haematopoiesis was associated with increased cardiac stress and elevated pro-inflammatory cytokines (136).

Despite the low risk of progression to haematologically important diagnoses, all-comers with CHIP have a 40% higher mortality than those without CHIP, and this striking excess is a reflection of CV events including fatal, and non-fatal MI, percutaneous coronary intervention and fatal stroke (ischaemic and haemorrhagic) (70). The presence of CHIP confers a substantially increased risk for CV disease independent of traditional risk factors including diabetes and hypercholesterolaemia (70,71).

1.3.5 CHIP and inflammation

The effects of specific CHIP-associated mutations are yet to be fully described, but a core feature appears to be the establishment of a pro-inflammatory state. Compared to those without CHIP, people with evidence of CHIP have higher circulating concentrations of pro-inflammatory markers including IL-6, TNF-α and monocyte chemoattractant protein one (MCP-1) (96,137). Driver-gene-specific analysis of a large cohort of individuals with CHIP highlighted the association of TET2 mutations with increased IL-1β whereas Jak2 and SF3B1 mutations were associated with higher circulating IL-18 (96). Other, potentially less sensitive markers of inflammation such as WBC, neutrophil count, CRP and erythrocyte sedimentation rate (ESR) are not normally elevated in people with CHIP (96,137). It has been proposed that the role of inflammation in CHIP is bidirectional, whereby inflammation initially predisposes to the development of CHIP, with consequent unregulated pro-inflammatory cytokine release via a feedback loop (138). To date, it is unknown whether specific mutations in the TET2, DNMT3A and ASXL1 genes have different clinical consequences. Several different mutations have been reported to occur in each gene and the pathophysiologic effects of these have not yet been individually characterised (70,130).

1.3.6 CHIP and vascular disease

Atherosclerotic CV disease remains a leading cause of vascular disease worldwide. Atherosclerosis is an inflammatory disease, predominantly of the microvasculature. Fatty and fibrous material accumulates in the intima of arteries. Over time the atherosclerotic plaques become more fibrous and accumulate calcium, which can impede blood flow and cause tissue ischaemia. Almost 60% of elderly patients with atherosclerosis have either no conventional risk factors (e.g. HTN or hypercholesterolaemia) or have only one risk factor, thus implying the presence of otherwise unidentified predisposing conditions (139). CHIP has been identified as a potential factor closely linked to the initiation and progression of atherosclerosis (71).

Microvascular disease affects vessels with a diameter less than 300μm, including arterioles, capillaries and venules. It involves a complex interplay between upstream atherosclerosis, inflammation and endothelial dysfunction. The presence of microvascular disease is associated with adverse CV outcomes (140).

Of the CHIP-related mutations, the role of TET2 has been most clearly defined in relation to vascular disease and normal TET2 function has been implicated in several important regulatory processes in both the macro- and microcirculation (141–145). These include suppression of vascular smooth muscle cell phenotypic transformation, protective effects upon endothelial cells as well as anti-inflammatory and anti-atherogenic effects(141–145).

1.3.6.1 CHIP and human atherosclerosis

Nested case-control analyses of prospective cohorts, that together enrolled 4,726 participants with CAD and 3,529 controls, reveal that carriers of CHIP (DNMT3A, TET2 and ASXL1 mutations) have a risk of CAD that is substantially greater than controls. (71) Indeed, patients with CHIP were twice as likely to have a history of MI or coronary revascularisation than people without CHIP (71). CHIP-associated DNMT3A mutation was associated with a hazard ratio of 1.7 for CAD while TET2 mutation conferred a hazard ratio of 1.9. Those with Jak^{V617F} mutation had the highest increased risk of CAD, which was twelve times greater than people with no mutation. In younger patients, the association between CHIP and atherosclerotic risk was even stronger than in older individuals (71). In the same study, people with CHIP without a prior diagnosis of CAD were three times more likely to have a computed tomography

coronary artery calcification score of at least 615 Agatston units (71), the empirical cut off for the identification of older patients at high risk of coronary events (146). This coronary artery score correlated positively with percentage VAF implying a 'dose effect' of the accumulation of mutated cells. Patients with large mutant clone populations (VAF >10%) without a prior diagnosis of CAD were twelve times more likely to have a coronary artery calcium score over 615 Agatston units (71). In a large genome wide association study, the presence of CHIP-associated Jak2 mutation was associated with increased risk of CAD despite lower levels of triglycerides and low density lipoprotein (LDL) cholesterol (147). In 485 patients with ST-elevation MI, DNMT3A or TET2 mutations (≥2%) were observed in 12.4% of patients. The presence of these mutations was associated with an increased risk of major adverse CV events (death, MI, stroke or HF hospitalisation) and significantly elevated plasma levels of IL-6 & IL-1β post-ST elevation MI (148).

Endothelial dysfunction is the earliest feature in the development of atherosclerosis. Patients with coronary endothelial dysfunction (assessed via vasomotor responses to intra-coronary acetylcholine infusion) have significantly higher prevalence of CHIP-associated mutations in comparison to people with normal coronary endothelial function (9.2% versus 1.5%, respectively) (149). Furthermore, somatic mutations in ASXL1, DNMT3A and TET2 were associated with higher levels of IL-6 and IL-8 in this group (149). The relationship between CHIP and CAD is most studied, there is recent evidence to suggest that it extends to the entire arterial system, indeed the presence of TP53 was associated with a 1.7 fold increase in incident peripheral arterial disease (150).

The potential association between CHIP, inflammation and CV disease was assessed in 35,416 people included in the UK Biobank (Table 1-9) (72). Participants did not have a history of CV disease at inclusion but those with DNMT3A or TET2 mutation had a 27% higher risk of CV disease over 6.9 years of follow-up when compared to those without these CHIP mutations (72). This risk was larger in those with larger clones denoted by VAF >10% (hazard ratio 1.59 [95% CI 1.21-2.09]) (72). Furthermore, to examine the potential interaction with inflammation, the effect of carrying a genetic proxy of IL-6 inhibition (IL6R p.Asp358Ala) and simultaneous CHIP was also assessed (72). In people with large CHIP clones (VAF>10%), the presence of this genetic proxy was associated with a 54% lower risk of CV disease events and was without effect upon CV disease event risk in individuals without CHIP (72). In those aged over 50 years with a history of prior MI and CHIP, each additional IL6R p.Asp358Ala allele attenuated the risk of CV disease events (72). Not only do these genetic data provide further mechanistic insight concerning interactions between CHIP, inflammation

and CV disease, they also give weight to the hypothesis that therapeutic inhibition of IL-6 signalling may prove to be beneficial in patients with large CHIP clones and CVD. Furthermore, in postmenopausal women from the UK Biobank aged 40-70 and from the Women's Health Initiative aged 50-79 years premature menopause was independently associated with CHIP (151). Among postmenopausal middle-aged women the presence of CHIP was independently associated with incident CAD (151,152).

Table 1-9: CHIP mutations and associated CV risk

Cohort	Mutation	Age	CV Association Examined	HR	Ref
US Population based	Any CHIP mutation	Median 58 years	Incident CAD	2.0 (1.2-3.5)	(70)
			Ischaemic Stroke	2.6 (1.3-4.8)	
PROMIS	Any CHIP mutation	< 50 years	Early onset MI (before the age of 50 years)	4.0 (2.4-6.7)	(71)
ATVB				5.4 (2.3-13.0)	
UK Biobank	Any CHIP mutation	Mean 61 years	MI, coronary artery revascularisation, stroke or death	1.27 (1.04-1.56)	(72)
UK Biobank & Women's Health Initiative (Premature menopause)	Any CHIP mutation	Mean 60 years (Biobank) Mean 68 years (WHI)	Incident CAD	1.36 (1.07-1.73)	(151)
Health & Anemia	Any CHIP mutation	Median 83 years	History of MI or coronary revascularisation	1.61 (1.28-3.21)	(153)
Chronic ischaemic HFrEF	TET2 or DNMT3A	Median 69 years	HF hospitalization or all-cause death	2.1 (1.1-4.0)	(73)
Post-ST-elevation MI	TET2 or DNMT3A	Median 67 years	Composite of death, MI, stroke or HF hospitalisation	1.83 (1.15-2.91)	(148)

Severe aortic stenosis undergoing transcatheter aortic valve replacement	TET2 or DNMT3A	Median 83 years	Risk of death following transcatheter aortic valve replacement	3.1 (1.17-8.08)	(74)
UK Biobank & MGB Biobank	All CHIP mutations	Mean 60 years	Incident peripheral arterial disease	1.67 (1.32-2.11)	(150)
6 cohort studies (ARIC, CHS, FHS, JHS, MESA & WHI) 2 electronic health records (UK Biobank & MGB Biobank)	All CHIP mutations	Mean 61 years	Risk of stroke (haemorrhagic or ischaemic)	1.14 (1.03-1.27)	(154)

1.3.6.2 TET2- Preclinical vascular models

The first murine model to implicate the role of CHIP in atherosclerosis used a competitive bone marrow transplantation strategy to generate atherosclerotic prone, low-density lipoprotein receptor deficient ($Ldlr^{-/-}$) chimeric mice with a small proportion of TET2-deficient HSC (10% $TET2^{-/-}$ bone marrow) (145). Importantly, when compared to control mice, there was no difference in body weight, plasma cholesterol levels, glucose and systemic insulin sensitivity (145). Following nine weeks of a high fat/high cholesterol diet, TET-2 deficient mice (10% knockout [KO]-BMT) developed aortic root plaques that were 60% larger than those of control animals (145). There was an increase in total macrophage content in the intima and these TET2-deficient macrophages exhibited markedly increased expression of pro-inflammatory cytokines (145). In particular, levels of IL-1β in macrophages of the aortic arch were doubled and treatment with the NLRP3 inflammasome inhibitor, MCC950, reduced atherosclerotic plaque burden and IL-1β secretion. Therefore, suggesting that TET2 deficiency affects NLRP3 mediated IL-1β secretion. These findings have been replicated in other murine models of TET2 deficiency, confirming the association of TET2 deficiency in accelerated atherosclerosis through induction of a pro-inflammatory state (71). There has been a suggestion from a small cohort of TET2 deficient atherosclerotic-prone mice (n=30) that the response to IL-1β inhibition may be sex dependent although this needs further exploration (155).

TET2 is highly expressed in human coronary artery smooth muscle cells, and following arterial injury, TET2 less of function exacerbates intimal hyperplasia (156). TET2 is an important regulator of vascular smooth muscle cell phenotypic transformation (156). Furthermore, TET2 is also an important regulator of autophagy, and abnormalities of autophagy have been implicated in endothelial cell dysfunction, development of atherosclerosis, microvascular dysfunction and HF. Following low shear stress, endothelial cell autophagy was reduced via the downregulation of TET2 (144). Furthermore, in the $ApoE^{-/-}$ murine model, autophagy is upregulated by TET2 overexpression and decreased by TET2 silencing (144).

1.3.6.3 Jak^{V617F} – Preclinical vascular models

The Jak^{V617F} mutation has also been examined in a mouse model of atherosclerosis. Irradiated Ldlr$^{-/-}$ mice were transplanted with bone marrow from either wild type or Jak2^{VF617} mutant mice and subsequently fed a high fat/ high cholesterol diet. Despite lower plasma cholesterol levels, the aortic root atherosclerotic lesion size was 1.6-fold higher in Jak2^{VF617F} mice in comparison to WT (157). Furthermore, Jak2^{VF617F} macrophages had greater expression of pro-inflammatory cytokines and chemokines including, IL-1β, IL-6, IL-18, TNF-α and MCP-1 following challenge with LPS (157). Even in the absence of LPS stimulation, Jak2^{V617F} mice had higher plasma levels of IL-18 compared to WT controls (157). However, these Jak2^{V617F} mice developed marked erythrocytosis, thrombocytosis and neutrophilia which is more consistent with a myeloproliferative neoplastic phenotype than CHIP and these confounding effects limit further interpretation. A subsequent mouse model used an Mx1-driven Cre recombinase to generate mice expressing Jak2^{VF}, importantly this model did not produce any changes in blood cell counts or cholesterol levels and is therefore more consistent with CHIP (158). These mice displayed a 2 fold increase in atherosclerotic lesion area and increased macrophage staining of IL-1β and the downstream inflammasome components caspase 1 and 11 when compared to control mice (158). There was also increased levels of AIM2 and NLRP3 inflammasome activation in human JAK2^{VF} macrophages derived from induced pluripotent stem cells when compared to isogenic controls (158). The respective roles of the NLRP3 and AIM2 inflammasomes23 in atherosclerosis were then assessed, *Jak2VFNLRP3*$^{-/-}$ or *Jak2VFAim2*$^{-/-}$ bone marrow were transplanted into *Ldlr*−/− mice and fed them a Western-type diet for 12 weeks (158). The *NLRP3* deficiency had no significant effect on the areas of lesions or necrotic cores, *Aim2* deficiency markedly reduced both (158).

Endothelial function was assessed in the common carotid artery of LDLr$^{-/-}$ mice transplanted with Jak2^{V617F} bone marrow cells following constrictive cuff placement across the artery (159). The carotid arteries of these Jak2^{V617F} mice displayed increased endothelial permeability, reduced endothelial continuity, increased intimal neutrophil extracellular trap accumulation with a subsequent increase in thrombus formation (159). Treatment with ruxolitinib, a Jak1/2 inhibitor, reduced endothelial cell apoptosis and improved endothelial continuity in Jak2^{V617F} mice (159).

1.3.6.4 Other CHIP preclinical models

The effect of DNMT3A inactivation on atherosclerosis development in murine models is unclear. However, in macrophages, DNMT3A haploinsufficiency leads to increased expression of pro-atherogenic genes including several CXCL family members, but it also increases production of IL13 in T cells which is protective against atherosclerosis development (160,161). Further research is required to understand the role of DNMT3A in atherosclerosis. However, a number of experimental studies have evaluated the role of p53, Ppm1d and other DNA damage response genes in atherosclerosis (162–166).

1.3.7 HF and CHIP

In 56,597 individuals from five cohorts (Atherosclerosis Risk in Communities Study [ARIC], Cardiovascular Health Study [CHS], Jackson Heart Study [JHS], UK Biobank [UKBB] & Women's Health Initiative [WHI]) the association of CHIP with incident HF was examined (Table 1-10) (167). CHIP was present in 3,406 (6%) individuals and 4,694 (8.4%) developed HF over a follow up period of 20 years (167). The presence of CHIP (any sequence of mutations) was associated with a 25% increased risk of HF in meta-analysis (167). Interestingly ASXL1, TET2 and JAK2 were associated with an increased risk of HF, whereas DNMT3A was not associated with increased risk and only ASXL1 was significantly associated with reduced ejection fraction (167). The increased risk of incident HF was slightly stronger in individuals with a higher VAF (167).

Table 1-10 Risk of incident HF in cohort studies (167)

	Study	No. of patients	Events	HR (95%CI)
	ARIC	9900	2171	1.25 (1.04-1.50)
	CHS	2400	942	1.16 (0.97-1.40)
	JHS	2423	188	0.93 (0.48-1.81)
	UKBB	36660	188	1.33 (1.05-1.69)
	WHI	5214	626	1.36 (1.10-1.68)
Total		56597	4694	1.25 (1.13-1.38)

No.; number, HR; hazard ratios, CI; confidence intervals.

Table adapted from Association of Clonal Hematopoiesis with Incident Heart Failure, Yu, B et al. JACC. Vol 78 2021 (167)

Table 1-11 Individual CHIP mutations and risk of incident HF; fixed effect analysis from five cohort studies (167)

CHIP Mutation	Risk of incident HF (HR 95% CIs)
TET2	1.59 (1.18-2.14)
JAK2	2.50 (1.35-4.64)
ASXL1	1.58 (1.20-2.08)

HR; hazard ratios, CI; confidence intervals.

Table adapted from Association of Clonal Hematopoiesis with Incident Heart Failure, Yu, B et al. JACC. Vol 78 2021 (167)

1.3.7.1 Human HFrEF and CHIP

Bone marrow derived mononuclear cells were obtained from 200 patients with chronic ischaemic HF enrolled in clinical trials of autologous stem cell therapy. In this relatively young cohort (median age 65 years) with a mean LVEF of 31%, CHIP was present in 18.5%. (73). DNMT3A mutations were observed in 30% of patients and 18% of patients had mutations in TET2 and these CHIP mutations were independently associated with HF hospitalisation and death (HR 2.1; 95% CI 1.1-4.0) (73). Notably, the majority of this mortality was attributable to progressive HF with only one death occurring as a result of subsequent MI. There was a significant association between clinical outcome and %VAF, implying a 'dose effect' of CHIP

(73,168). VAF cut-off values of ≥0.73% and ≥1.15% for TET2 and DNMT3A mutations, respectively, were predictive of poorer prognosis (80). To examine the importance of other CHIP mutations at low VAFs, this cohort of patients was expanded with a longer duration of follow-up (median 3.95yrs) to 399 patients with chronic ischaemic HF, 87% of patients carried at least one mutation with a VAF≥0.5% and importantly 82% of patients younger than 50 years old harboured at least one mutation (169). The prevalence of CHIP increased with age, and the number of mutations and VAF also increased with age (169). The presence of less common mutations CHIP mutations including KMT2A, CBL, CEBPA, SRSF2, U2AF1, SMC1A, EZH2, GNB1 and PHF6 was associated with an increased risk of death when compared to patients without CHIP (169). Importantly, this increased risk of death was not confounded by co-occurring CHIP-mutations in DNMT3A, TET2 or other CHIP-driver genes with a VAF≥2% (169). Another study went on to the examine the importance of TET2 and DNMT3A in ischaemic and non-ischaemic HFrEF. This showed that the presence of either mutations was associated with increased risk of adverse outcomes and progression of HF irrespective of HF aetiology (170).

1.3.7.2 Human HFpEF and CHIP

Both HFpEF and CHIP are considered to be diseases of the ageing population, and both are associated with a systemic pro-inflammatory state. The incidence and prevalence of HFpEF rises sharply with age (171–175), and the mean age of patients with HFpEF in recent cohorts is 72 years (4,35,43,172–174,176–193). In the context of findings describing the prevalence of CHIP in all-comers, it is reasonable to expect that CHIP is found in at least 10-20% of patients with HFpEF. However, this may be a substantial under-estimate. CHIP was found in 27% of patients with chronic ischaemic HFrEF aged between 70 and 79 years and in an elderly population (median age 83 years) with severe aortic stenosis undergoing transcatheter aortic valve implantation (TAVI), the prevalence of CHIP was 33% (73,74). In this cohort of patients with severe aortic stenosis, the presence of TET2 or DNMT3A was also associated with an elevated pro-inflammatory subset of circulating leucocytes and conferred a profound increased in mortality even after successful correction of the aortic valve stenosis (HR 3.1 [95%CI 1.17-8.08]) (74).

Alongside ageing, the prevalence of comorbidity rises in patients with chronic HF, and nearly half of patients with HFpEF have five or more comorbidities (194,195). Many of these comorbidities are associated with a pro-inflammatory state and, circulating markers of inflammation are predictive of incident HFpEF (44). Diabetes occurs in approximately 40% of male patients with HFpEF and 30% of female patients with HFpEF (195). Diabetes is associated with a two-fold increased risk of developing CHIP and individuals with both diabetes and CHIP have a higher burden of CV comorbidities than those with diabetes alone (72,196). It is unclear to what extent these pro-inflammatory comorbidities, considered to be central to the concept of *inflammageing*, are the cause or effect of CHIP, but it is likely that a positive feedback loop is established between them (138).

Younger patients with HFpEF are more likely to be male and have a history of obesity and diabetes (197–199), both of which are strongly associated with chronic low grade inflammation (197–199). The presence of CHIP may be of even greater relevance in these younger patients as an indicator of increased epigenetic age. Indeed, the presence of any CHIP mutation confers a 4-year increase in epigenetic age, while CHIP-related TET2 mutation confers a 6 year increase (200). Deviations from chronological age towards an increased epigenetic age are associated with increased risk of earlier mortality and age related morbidities (201,202).

In 5,214 postmenopausal women included in the WHI dataset, the presence of any of the top three CHIP-associated mutations (TET2, DNMT3A and ASXL1) was associated with incident HFpEF but not HFrEF (203).Women with premature menopause have increased risk of HF, stroke, coronary and peripheral arterial disease (204). Furthermore, systemic markers of inflammation, including CRP, are higher in post-menopausal women than they are in those who are pre-menopausal (205,206). It is of note that, in women included in the UKBB and WHI, the prevalence of CHIP was 60% higher in women with premature menopause compared to those without and the presence of CHIP was independently associated with incident CAD (207). Whether or not the presence of CHIP and early onset menopause is associated with the risk of developing HFpEF is unknown.

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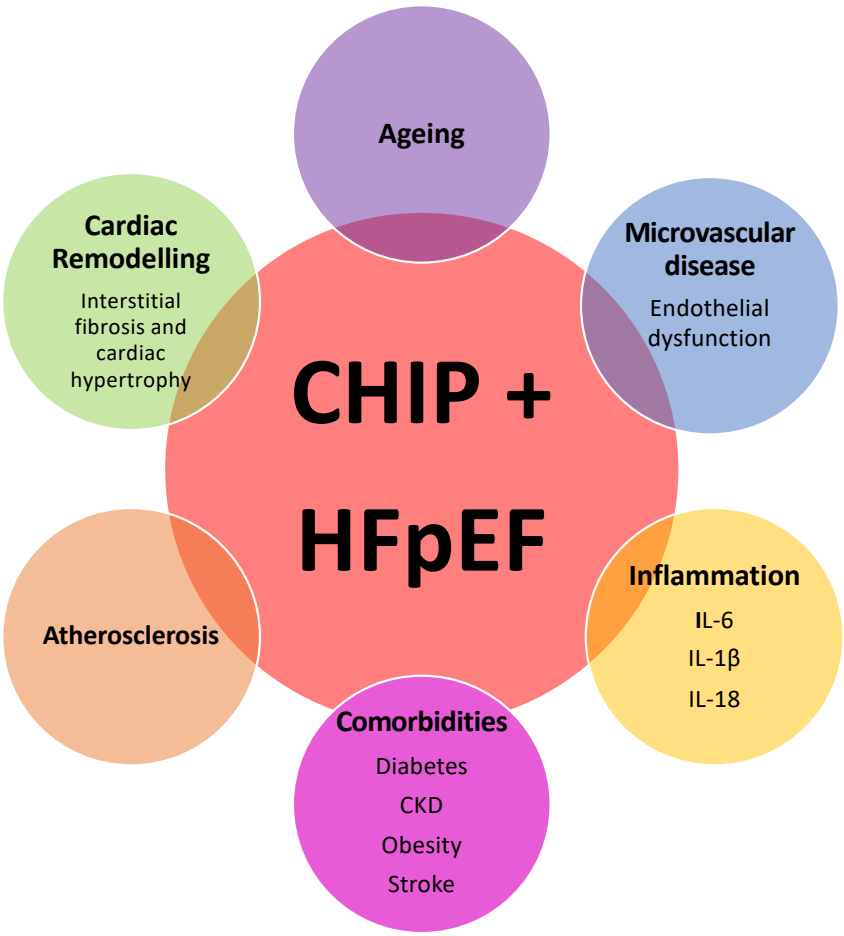
Table 1-12 Prevalence of HF and outcomes in patients with CHIP

Study	Total number of patients	VAF cut off	Prevalence of CHIP	Outcome	Median follow up	HR 95% CIs	Ref
Several RCTs including TOPCARE-CHD, Cellwave or REPEAT	200	VAF≥2%	18.5%	Death or HF hospitalisation	4.4yrs	2.1 [1.1-1.40]	(73)
Several RCTs including TOPCARE-CHD, Cellwave or REPEAT	399	VAF≥0.5%	87%	All cause death	3.95yrs	3.1 [1.8-5.4]	(169)
Prospective registries MUSIC REDINSCOR	62	VAF>2%	38.7%	HF hospitalisation or HF death	3.65yrs	2.02 [1.10-3.72]	(170)
Several RCTs including	419	TET2 VAF≥0.73%	23.6%	All-cause mortality	4yrs	1.77 [1.08-2.90)	(80)

TOPCARE-CHD, Cellwave or REPEAT		and/ or DNMT3A≥1.151					

Figure 1-4: Potential mechanistic links between CHIP and HFpEF



IL; interleukin, CKD; chronic kidney disease

1.3.7.3 HF and CHIP- preclinical models

HSC-specific TET2 mutation is associated with the accelerated development of HF in murine models of HF as a result of left ventricular pressure overload induced by transverse aortic constriction (TAC) and as a consequence of chronic ischaemia induced by ligation of the left anterior descending artery (LAD) (208). While TAC has been employed as a murine model of HFpEF, after 2-3weeks TAC results in a reduction in systolic function and progression to HFrEF (209–211). Following the permanent ligation of the LAD, 10% TET2 KO mice had significantly reduced ejection fraction and this was associated with increased transcription of pro-inflammatory mediators including IL-1β, IL-18, Chemokine (C-X-C motif) ligand 2 (Cxcl2), Chemokine (C-C motif) ligand 2 (Ccl2) and 5 (Ccl5) (208). Myeloid-specific TET2-deficient mice also had worse cardiac remodelling following LAD ligation with lower LVEF

and increased fibrotic area when compared to control mice. 10% TET2 KO mice subjected to TAC exhibit marked left ventricular hypertrophy with greater posterior wall thickness and cardiac fibrosis when compared to WT mice. These structural changes were also associated with higher concentrations of circulating IL-1β when compared to control mice (208). IL-1β cleavage is mediated by the NLRP3 inflammasome, a complex intracellular protein which upon activation, cleaves procaspase-1 protein to functional caspase-1. The primary function of caspase-1 is the conversion of the inactive pro-inflammatory cytokines pro-IL-1β and pro-IL-18 into their active, potently pro-inflammatory states. Over time, TET2 knockout mice subjected to TAC also developed systolic impairment. Importantly, administration of MCC950, an NLRP3 inflammasome inhibitor, was associated with significant protection from adverse cardiac remodelling in both models (208).

Bone marrow-specific deletion of TET2 or DNMT3A is associated with cardiac hypertrophy, fibrosis and impaired LV fractional shortening after infusion of angiotensin II in comparison to WT controls (212). TET2 deletion promoted the expression of IL-1β and IL-6, whereas DNMT3 deletion significantly increased the expression of IL-6 with a trend towards increased IL-1β (212). Importantly, DNMT3A has both direct and indirect roles in maintaining overall cardiomyocyte homeostasis and function (213). Specifically, DNMT3A$^{-/-}$ engineered human induced pluripotent stem cell-derived cardiomyocytes have upregulation of pathways involved in cardiac hypertrophy and cardiac proliferation pathways when compared to WT (213). DNMT3A knock-out also affected contraction kinetics, cell diameter was greater and intracellular lipid accumulation was greater in comparison to the WT (213).

Myeloid-specific Jak2^{V617F} mutation in mice is not associated with abnormalities of peripheral blood count, as would be expected in human CHIP. These animals also do not appear to have abnormalities of cardiac structure or function in the unstressed state (214). However, following LAD ligation or TAC these mice have greater myocardial macrophage infiltration and concentrations of IL-6 and IL-1β are greater than WT. It has been proposed that Jak2^{V617F} activates the interferon gamma receptor 1 Jak2 signalling transduction pathway (IFNGR1-Jak2-STAT1) resulting in the release of pro-inflammatory cytokines (214). In the myeloid-specific Jak2^{V617F} model, this mutation was associated with a more substantial deterioration in cardiac function, larger infarct size and increased cardiac fibrosis following TAC/LAD ligation (214). Furthermore, the adoptive transfer of Jak2^{V617F} bone marrow cells into mice exposed to chronic hypoxia was associated with increased right ventricular systolic pressure

and increased muscularization of pulmonary vessels when compared to control chronically hypoxic mice (215).

While these models have focused upon the investigation of the effects of an exogenous injury or stressor, a recent investigation has attempted to replicate the effects of CHIP in the otherwise 'unstressed' state. By transferring TET2-mutant bone marrow cells into mice without prior myeloablative irradiation preconditioning, an attempt was made to replicate the accumulation of somatically abnormal cells over time (216). In this model, TET2-deficient cardiac macrophages had an overrepresentation of immune response effectors, with specific elevation in IL-1β, Ccl17 levels and the IL1-receptor antagonist gene (216). Concentrations of brain natriuretic peptide (released in response to cardiac pressure overload) were significantly higher in TET2 mutant mice and these animals had greater posterior wall dimension, left ventricular end systolic volume, heart weight and cardiac fibrosis in comparison to control. While LVEF declined slightly, all mice had an LVEF of ≥40% providing evidence that CHIP may be important in the development of HFpEF (216).

1.3.8 Personalised CV management

Historical trials of anti-inflammatory therapy for the treatment of CV disease have mainly been disappointing. However, CANTOS has reinvigorated this area and highlights CHIP as a potential biomarker to inform personalised therapy. CANTOS examined the effects of canakinumab, a monoclonal antibody directed against IL-1β, in patients with a history of prior MI and elevated CRP. Canakinumab reduced CRP and the incidence of atherosclerotic CV events was decreased by 15% versus control (21). Notably, canakinumab also reduced HF hospitalization and HF-related mortality by 23% in patients who achieved a CRP level of <2mg/L (67). Given the association of CHIP with inflammation and, in particular, the secretion of IL-1β (the immediate upstream precursor to IL-6), CHIP has been proposed as a potential biomarker for personalized therapy with canakinumab and potentially other anti-inflammatory therapies. Indeed, in an exploratory analysis of CANTOS, canakinumab reduced the relative risk of MACE by 64% in those with TET2 mutations and by 15% in the treatment overall (217). Whether or not this impressive effect will also be seen in patients with HF is unknown.

Inzomelid, a novel small-molecule inhibitor of the NLRP3 inflammasome, is currently under clinical investigation for its safety and tolerability in humans (NCT04015076). Whether any

potential effect is amplified in patients with CHIP may be a logical future step in its assessment. Recent data revealed that the sodium-glucose co-transporter 2 (SGLT-2) inhibitor, dapagliflozin, reduces IL-1β via up-regulation of serum β-hydroxybutyrate (218). Again, the potential benefits of personalisation of SGLT-2 inhibitor therapy based on CHIP status is an intriguing but, as yet untested, hypothesis.

1.3.9 CHIP and other human disease processes

There is some evidence to suggest that CHIP may interact with other disease beyond cancer and CV disease, in particular diseases where inflammation is a key feature. To date these include:

- Chronic obstructive pulmonary disease (106,219).
- Progression of CKD (220).
- Adult onset haemophagocytosis (221).
- Acquired aplastic anaemia (222).
- Anti-neutrophil cytoplasmic-antibody associated vasculitis (223).
- Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (224).
- Human Immunodeficiency Virus (HIV) (223).

1.4 Study Aims

1.4.1 Hypotheses

CHIP is associated with both inflammation and ageing, and HFpEF is more commonly associated with ageing when compared to HFrEF. Therefore, I hypothesised that CHIP would be more prevalent in patients with HFpEF than those with HFrEF. A core feature of the common CHIP mutations appears to be the establishment of a pro-inflammatory state. Importantly, CHIP does not affect circulating levels of the commonly measured traditional inflammatory markers, including haemoglobin, WBC, lymphocytes and neutrophils. Therefore, I hypothesised that the presence of CHIP was associated with other novel inflammatory biomarkers, such as IL-6 and IL-1B (part of the NLRP3 inflammasome pathway), which are not measured in routine clinical practice. I also hypothesised that the inflammatory profile would be different for CHIP patients with HFpEF and those with HFrEF, with HFrEF being more associated with biomarkers associated with cardiac stretch. I

hypothesised that the identification of CHIP in patients with HF may identify those patients who would receive the greatest benefit from anti-inflammatory therapy.

While the simple and traditional markers of inflammation are not different in patients with and without CHIP, they are commonly measured in clinical practice and form part of the SEATTLE-HF risk score. I hypothesised that these were still important for determining CV outcomes in both types of stable HF. Therefore, alongside my clinical recruitment, I examined RLC and NLR in stable HFrEF and HFpEF using two large clinical trial datasets (PARAGON-HF and PARADIGM-HF). I hypothesised that RLC (low) and NLR (high) levels would be associated with worse CV outcomes in patients with stable HFpEF and HFrEF. I also hypothesised that these levels would be more important for patients with stable HFpEF when compared to those with HFrEF. To date, there has been relatively little information regarding changes in inflammation over time and with the treatment of HF. Therefore, I hypothesised that treatment with sacubitril/valsartan would only reduce levels of inflammation in patients with HFrEF and have no effect in patients with HFpEF, considering treatment with sacubitril/valsartan showed no overall benefit in HFpEF. This analysis further adds to the hypothesis that personalisation of therapy for HF may be beneficial and that further studies are required to understand changes in inflammation over time in patients with HF.

There is a lot of information regarding the role of simple and traditional markers of inflammation in the risk of developing HF and subsequent worse outcomes. However, these simple markers do not identify a specific therapeutic receptor target, such as the NLRP3 inflammasome. Recently, there has been a huge drive to develop novel anti-inflammatory therapy targeting specific pathways. Therefore, I hypothesised that levels of circulating IL-6, part of the NLRP3 inflammasome pathway, would be elevated in patients with decompensated HFrEF and HFpEF and that these elevated levels would be associated with worse HF outcomes. IL-6 has been demonstrated to be elevated in patients with CHIP without CV disease. Therefore, therapies targeting the NLRP3 inflammasome may be beneficial for patients with HF and CHIP, further strengthening the argument that personalised HF treatment based on circulating levels of inflammation may be the future in HF.

1.4.2 Aims

- To demonstrate that simple and novel markers of inflammation are elevated in patients with stable and decompensated HF.

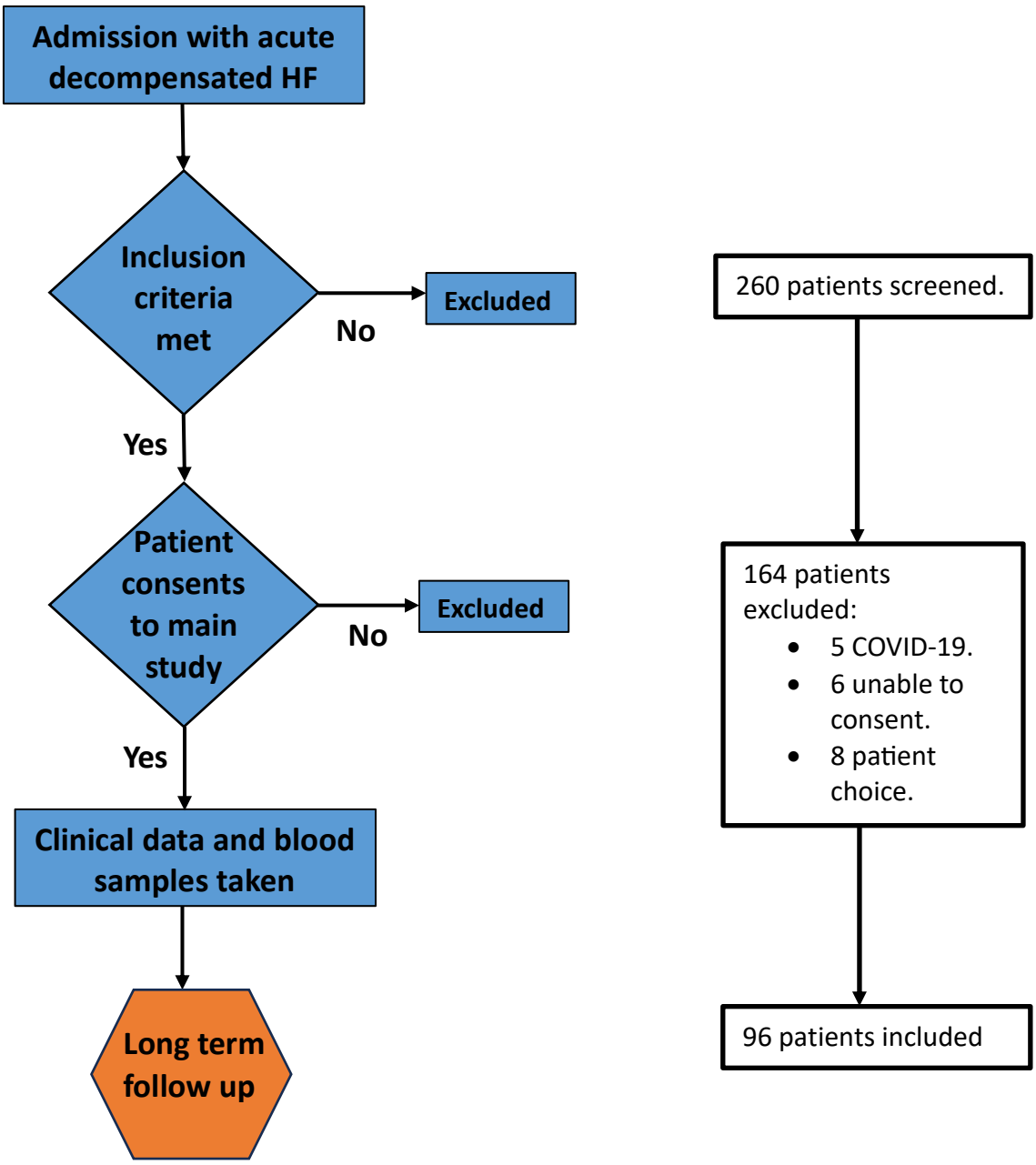
- To understand whether inflammation is important in both stable and decompensated HF.
- To understand the association between inflammation and CV outcomes in stable/decompensated patients with HFrEF and HFpEF.
- To characterise the clinical characteristics of patients with CHIP and decompensated HF.
- To investigate the association between CHIP and circulating inflammatory biomarkers in patients with recently decompensated HF including RLC and NLR.
- To understand whether treatment with sacubitril/valsartan reduced levels of inflammation in HFpEF and HFrEF.
- To obtain mechanistic data to inform therapeutic strategies for the treatment of HF, in particular whether novel anti-inflammatory therapy targeting the NLRP3 inflammasome pathway would be potentially beneficial for patients with HF.
- To provide further evidence that personalisation of therapies would be beneficial for patients with HF.

Chapter 2 METHODS

This chapter will outline the methods used in this thesis. I designed a prospective observational study to investigate the prevalence of CHIP and its association with the inflammatory phenotype in patients hospitalised with HF. The study was approved by the West of Scotland (REC 20/WS/0027) and the study was funded by the British Heart Foundation core fund (BHF Centre of Research Excellence Award [RE 18/6/34217]). A summary of the study is provided in Figure 2-1, and the results of this observational study are outlined in Chapter 3. In addition to designing a prospective observational study, I also examined the role of circulating inflammatory biomarkers in large HF datasets, and the results of these are outlined in Chapter 4,5 & 6.

2.1 CHIP in Patients with HF: A Prospective Cohort Study

Figure 2-1: CHIP Study Design and consort diagram



2.2 Study Population

2.2.1 Identification of patients

All patients were recruited from the West of Scotland, from two hospitals based in the City of Glasgow: Queen Elizabeth University Hospital (QUEH) or Glasgow Royal Infirmary (GRI). Patients recruited into this study were admitted to these hospitals by self-referral or by a clinician (e.g. GP). All patients recruited were admitted to either a Coronary Care Unit or a cardiology ward, while patients with decompensated HFpEF and HFrEF may have been admitted to other wards, such as medicine of the elderly, I only recruited from cardiology wards to limit the spread of COVID-19 and to ensure there were no other competing reasons for admission to hospital. Near consecutive admissions were screened daily at both hospitals between the 12th of October 2020 and the 25th of September 2021 by myself, and by limiting recruitment to the cardiology wards this meant I was unlikely to miss patients suitable for the study. The recruitment period was during the COVID-19 pandemic and each patient was required to have a negative COVID-19 test prior to recruitment. Patients who were exposed to COVID-19 while in hospital were not recruited.

All admissions to the two hospitals were screened for evidence of decompensated HF. This involved reviewing the case records for all new admissions for documentation of the following:

- Symptoms and clinical signs of HF.
- Radiological evidence suggestive of HF.
- BNP/NT-BNP levels at time of admission (within 24hours).

Symptoms of HF included shortness of breath and peripheral oedema. Clinical signs of HF included a raised jugular venous pressure, lung crepitations and pitting peripheral oedema. Radiological evidence of HF included cardiomegaly and signs of pulmonary oedema.

All patients without obvious contraindications to enrolment based on review of case notes were approached for study screening. Potential participants were then approached by their clinical care team, and those who were interested in taking part in the study were given a Patient Information Sheet (Appendix I)

Patients who agreed to participate in the study provided written informed consent (Appendix II). Copies of the consent form were given to the patient and filed in their medical case records. A letter and information sheet were issued to every participant's GP, which also provided contact information for the research team (Appendix III).

2.2.2 Inclusion Criteria

Patients were invited to participate in the study if they met all the following criteria:

- Age ≥18 years.
- HF (NYHA II-IV).
- Clinical evidence of decompensated HF.
- Elevated natriuretic peptide levels: B-type natriuretic peptide (BNP) ≥100 pg/ml or NT-proBNP≥300 pg/ml.

HFpEF group:

- LVEF≥40%; and evidence of relevant structural heart disease on echo (i.e. LV hypertrophy [maximal diastolic LV septal or posterior wall thickness ≥13 mm]; left atrial (LA) dilatation (indexed LA volume ≥34 ml/m2); and/or evidence of elevated LV filling pressures [E/e' >13]) in accordance with the ESC guidelines.

HFrEF group:

- LVEF <40%.

At the time of designing the study, the inclusion criteria were set based on what laboratory test were being used at the time. In GG&C, both BNP and NTproBNP were used initially, but at the time of recruitment all hospitals within GG&C had moved to measuring solely NTproBNP. NT-proBNP levels were not adjusted for age or AF, in view of the fact patients were required to have been admitted with decompensated HF and have clinical evidence of decompensation. For the HFpEF group we defined this as LVEF≥40% which does vary from the ESC and AHA guidelines, however, is in line with the recent clinical trials in HFpEF. This cut off was also used to reduce the number of groups for statistical analysis due to the small sample size (96 patients). Furthermore, patients EF is not static and may improve or deteriorate and 40-49% still represents a grey zone due to the scarce data available.

2.2.3 Exclusion Criteria

Patients were not eligible for participation if they met any of the following exclusion criteria:

- Patients who were unwilling or unable to provide consent.
- Pre-existing myeloproliferative disorder or haematological malignancy.
- Haemoglobin <100g/L, platelets <100x10^9/L or neutrophils <1.0 x10^9/L.
- Acute coronary syndrome, coronary revascularisation (percutaneous or surgical) or stroke within the last three months.
- Severe left-sided valvular disease (except functional mitral regurgitation).
- Known or suspected hypertrophic/infiltrative cardiomyopathy or constrictive pericarditis.
- Chronic treatment with immunomodulating therapy.
- Ongoing infection requiring treatment with antibiotics.
- Life expectancy of less than one year as a result of a non-cardiac condition.

2.3 Data Collection

Every patient recruited into the study had detailed demographic and clinical data collected. Data was obtained through history taking, clinical examination and review of the medical records. Each participant was allocated a unique and anonymous study identification number. Data were recorded on a secure online Good Clinical Practice-approved electronic case report form (eCRDF) (Castor EDC, Amsterdam, Netherlands).

Baseline data were recorded on the eCRF under the following headings:

- Baseline characteristics which included height, weight, past medical history.
- HF characteristics including time since diagnosis, NYHA class, total number of HF admissions and any cardiac device therapy.
- In hospital symptoms including orthopnoea, paroxysmal nocturnal dyspnoea, ankle swelling, abdominal distention, wheeze, palpitations and fatigue.
- Baseline medications prior to admission.
- In hospital treatment.
- COVID-19 testing results.
- Baseline blood tests including full haematological and biochemical profile.

- Previous CV imaging where applicable including findings from invasive or CT coronary angiography.
- Social history including family history of previous premature CV disease, alcohol and smoking history.
- Full clinical examination.
- Chest X-ray findings.
- Frailty and KCCQ baseline scores.

2.4 ECG

Each patient underwent a 12 lead ECG at baseline using the Mortara ECG machine, which is frequently tested and appropriately calibrated. ECGs are frequently abnormal in patients with HF, and often, the ECG can allude to the primary aetiology of the HF, for example previous MI. Therefore, specific ECG parameters were recorded including heart rate, rhythm, PR interval, QRS duration, and the QT interval which was corrected for heart rate. QTc was calculated using the Bazetts and Fridericia formula. The presence or absence of left bundle branch block (LBBB), right bundle branch block (RBBB) or pathological Q waves (defined as >40ms wide, 2mm deep and >25% of depth of QRS complex) was also recorded in the eCRF.

2.5 Chest X-ray (CXR)

Each patient had a baseline CXR performed on admission to the hospital. CXR often assists in the diagnosis of acute HF and allowed me to ensure there was no other primary cause for symptoms or HF decompensation, such as pneumonia. It is important to note that a normal CXR does not exclude the diagnosis of HF. The following signs were recorded:

- Cardiomegaly.
- Upper lobe venous diversion.
- Interstitial oedema.
- Perihilar oedema.
- Pleural effusion- unilateral or bilateral.

2.5 Blood sampling

2.5.1 Routine biochemical and haematological tests

The ESC recommends many routine laboratory investigations in the evaluation of patients with HF. These routine blood tests can provide information on the aetiology of HF, degree of congestion and response to treatment.

All patients had the following blood tests taken routinely on admission to hospital with HF decompensation: urea and electrolytes (U&E's), liver function tests (LFT) and full blood count (FBC), thyroid function (TFTs), glycosylated haemoglobin (HbA1c), CRP, ferritin glucose and full lipid profile. All routine biochemical and haematological tests were performed at the time of admission and analysed in the hospital biochemistry and haematology laboratories within four hours of venesection.

Renal function is not solely assessed by serum creatinine but also assessed by the estimation of the glomerular filtration rate (eGFR) using the Modification of Diet in Renal Disease (MDRD) equation. The MDRD has been validated in patients with severe chronic HF, and this was calculated for all patients recruited to the study. This formula calculated eGFR as follows

$$\text{eGFR (ml/min/1.73m}^2\text{)} = 32788 \times (\text{serum creatinine in } \mu\text{mol/l})^{-1.154} \times (\text{age})^{-0.203} \times [1.210 \text{ if black or } 0.742 \text{ if female}]$$

This then allows renal function to be classified using the National Kidney Foundation classification. This guideline classifies renal function as:

- Normal eGFR ≥90ml/min/1.73m^2.
- Mild impairment eGFR 60-89 ml/min/1.73m^2.
- Moderate and severe renal impairment eGFR 30-59 ml/min/1.73m^2.
- Severe <30ml/min/1.73m^2.

The FBC includes several parameters important useful for assessing inflammation and HF prognosis. These include haemoglobin, WBC, lymphocyte, neutrophil and eosinophil count all of which were recorded in the eCRF. If a patient had abnormal blood count on admission to hospital as per the exclusion criteria, they were not recruited. RLC was calculated by dividing the total number of lymphocytes by the total number of white blood cells and

multiplying by one hundred. NLR was calculated by dividing the total number of neutrophils by the total number of lymphocytes.

2.5.2 Cardiac biomarkers

Blood samples were collected at baseline to assess plasma levels of NT-proBNP. Some patients had levels of hs-TnI measured at baseline if the clinical team felt this was appropriate and this was done in the NHS GG&C hospital laboratory. Hs-TnI was measured using the Elecsys Troponin I assay (F.Hoffmann-La Roche, Basel, Switzerland), respectively on a cobas e402 analyser.

NT-proBNP was an essential test in this study, with patients requiring an elevated NT-proBNP to be included in the study. Blood samples for NT-proBNP were performed as close to hospital admission as possible, the majority before 72 hours. Blood samples were collected in a potassium ethylene diamine tetra acetic acid tube (EDTA) and sent to the department of biochemistry at either the QEUH or GRI. The blood samples for NT-proBNP were centrifuged for 15 minutes at 1,000g. Samples were analysed using the Elecsys proBNP II (F.Hoffmann-La Roche, Basel, Switzerland), respectively on a cobas e411 analyser.

2.5.3 Inflammatory biomarker analysis

Blood samples (15 mL) were collected for analysis of inflammatory biomarkers. I centrifuged these for 15 minutes at 1,000g. Serum and plasma were aliquoted, stored at -80°C and thawed immediately before the assays were performed. I used the Meso Scale Discovery Platform (Meso Scale Diagnostics (MSD), 1601 Research Blvd, Rockville, Maryland) to measure a range of inflammatory biomarkers using electrochemiluminescence technology. I chose this platform for several reasons: 1) it provided a rapid and convenient method for measuring multiple inflammatory markers from a small volume of blood; 2) it was a cost-effective way of measuring multiple biomarkers and; 3) it had been previously used successfully within our wider research team.

While designing my study, I performed a literature search on inflammation and HF. This allowed me to curate a specific list of relevant inflammatory markers of greatest relevance for assessment. In the introduction, I have provided a brief overview of the current most important research examining inflammatory biomarkers in HF. I curated a list of the important available

inflammatory biomarker panels I wished to examine. I decided that the following assays/platforms would be most relevant for assessment in my study: U-PLEX (IL-18, IL-33, TGF-β group MCP), S-PLEX (IL-1β and IL-6) and V-PLEX (CRP, VCAM and ICAM-1). I was subsequently trained in using the Meso-scale discovery (MSD) platform by Mr John Butler, a biochemist from MSD. He assisted me in the examination of these plasma and serum inflammatory biomarkers. All samples were examined according to manufacturer instructions. Briefly, the samples were inserted into a 96 well plate with standard controls. The MSD platform makes use of electrochemiluminescence technology. Ten antibody coated carbon electrons integrated in the 96 well plates provide the solid phase of the sandwich immunoassay. Following binding of the antigen, a ruthenium-conjugated secondary antibody, emitting light upon electrochemical stimulations, provides the means for quantification. The signal is then amplified by microscopy and images are captured and analysed using the MSD-specific software.

2.5.4 CHIP driver mutation calling

The methods used for detecting CHIP have been previously well-established. I worked with expert colleagues in CHIP who were based at the Beatson Institute for Cancer Research University of Glasgow (Dr Kristina Kirschner, (PhD), Dr Neil Robertson, (PhD), and Dr Maria Terradas, (PhD)) and MRC Human Genetics Unit University of Edinburgh (Dr Tamir Chandra, (PhD)).

Stage 1: Peripheral Blood Mononuclear cell isolation

The first stage of identifying CHIP mutations is to isolate peripheral blood mononuclear cells (PBMC) from fresh blood. Prior to the COVID-19 outbreak, the plan was for this to be performed by colleagues at the Beatson Institute for Cancer Research. However, limited access to the laboratory and working from home restrictions meant that this was no longer possible. Therefore, I learnt laboratory technique and isolated PBMCs myself in laboratory accommodation at the QUEH. I used the EasySep Direct Human PBMC Isolation Kit/Big EasySep Magnet (STEMCELL Technologies, 1618 Station Street, Vancover, BC, Canada) for PBMC isolation. The standard operating procedure has been provided in Appendix IV taken from the manufacturer protocol. The first step in isolating the PBMC is to add a small amount (2mls) of blood to a 14ml round bottom tube with the subsequent addition of 'isolation cocktail' to achieve a final concentration of 50μL/mL. This was then incubated for five

minutes and topped up with phosphate buffer solution (PBS) to double the original sample volume. While this was incubating, the EasySep rapid spheres were vortexed until they appeared evenly dispersed. After five minutes, the Rapid Spheres were added to the sample and mixed by pipetting. The tube was then placed into the EasySep Big Magnet and incubated for five minutes. After five minutes, the magnet was picked up and, in one continuous motion, was inverted, pouring the enriched cells into a new tube. The rapid spheres were then added to the new tube, and this step was repeated a further two times until the isolated cells were ready.

Step 2: DNA isolation

DNA was then isolated using the Qiagen blood DNA extraction kit using the manufacturer standard operating procedure (Appendix V). The samples were then stored at -80°C. The quality and amount of DNA isolated was checked prior to the calling of CHIP mutations. Table 2-1 provides evidence that the samples we collected had adequate DNA for CHIP mutation calling.

Table 2-1 Qubit DNA Quantification

Sample	Qubit concentration (ng/μl)	Total Yield (ng)
00/00/03	10.26	2052
00/00/04	11.46	2292
00/00/05	14.10	2820
00/00/06	17.02	3404
00/00/07	10.80	2160
00/00/08	14.18	2836

Step 3: CHIP driver mutation calling

The following protocol was then followed by collaborating colleagues (led by Dr Kristina Kirschner) at the Beatson Institute to detect and call the CHIP mutations. This protocol has previously been employed by that group in a prior study examining the longitudinal dynamics of CHIP (225). Libraries were prepared from 200 ng of each DNA sample using the Archer VariantPlex® 75 Myeloid gene panel and VariantPlex® Somatic Protocol for Illumina sequencing (Invitae, AB0108, and VariantPlex®-HGC Myeloid Kit for Illumina), including modifications for detecting low allele frequencies. This comprehensive panel allowed the

identification of the most commonly described CHIP mutations with high confidence. The method is based upon Anchored Multiplex PCR (AMP), a rapid and scalable method to generate target-enriched libraries for next-generational sequencing (NGS). The kit is designed for low nucleic acid input, this process delivers robust performance across a variety of sample types and has been validated on PBMCs. AMP utilizes unidirectional gene-specific primers (GSPs) that enrich for both known and unknown mutations. Adapters that contain both molecular barcodes and sample indices permit quantitative multiplex data analysis, read deduplication and accurate mutation calling.

Sequencing of each pool was performed using the NextSeq 500/550 High-Output version 2.5 (300 cycles) kit on the NextSeq 550 platform (Illumina). To ensure reproducibility, background model for error and batch correction, they sequenced two GIAB DNA samples in each batch of samples (DNA NA12878,Coriell Institute) (226). Reads were filtered for phred ≥30 and adapters removed using Trimmomatic(version 0.27) (227) before undergoing guided alignment to human genome assembly hg19 using bwa-mem (version 0.7.17) (228) and bowtie2 (version 2.2.1) (229). Unique molecular barcodes (ligated before PCR amplification) were used for read de-duplication to support quantitative multiplexed analysis and confident mutation detection. Within targeted regions, variants were called using three tools (Lofreq (version 2.1.0)(230), Freebayes (231) and Vision (ArcherDX version 6.2.7), building a consensus from the output of all callers.

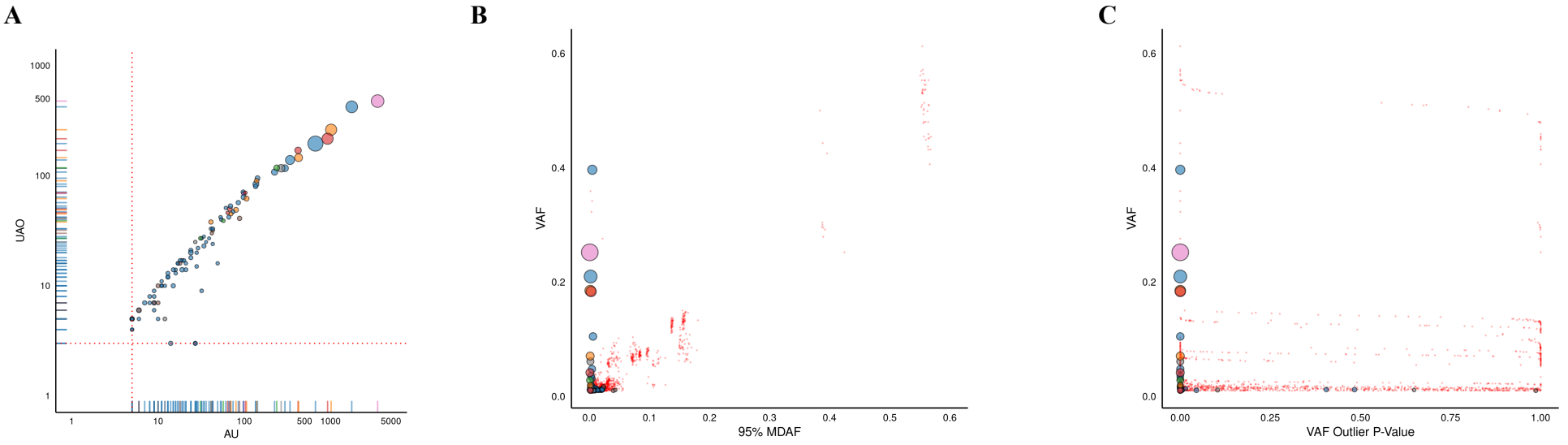
All filtered variants at 2% VAF met the following criteria:
1. The number of reads supporting the alternative allele surpasses the coverage criteria while exhibiting no directional biases (AO ≥ 5, UAO ≥ 3);
2. Variants are significantly underrepresented in the Genome Aggregation Database (gnomAD; P ≤ 0.05) (232);
(3) Variants are not obviously germline variants (stable VAF across all waves ~0.5 or ~1);
(4) Contain events that are overrepresented across the dataset—generally frameshift duplications and deletions—whose reads share some sequence homology to target regions yet are likely misaligned artifact from the capture method (Appens).

In addition, we manually curated this list, checking for variants that were previously reported, as per Jaiswal et al (70), in COSMIC (233) or in the published literature.
To further mitigate against the diverse sources of noise that can occur in any sequencing experiment, which can become especially problematic when attempting to detect variants at low VAFs, the ArcherDX variant-calling platform leverages the pan-dataset coverage levels

of each sample, and the GIAB controls to establish a position-specific noise profile and, thus, ascertain the limit of detection (LOD) for each variant discovered in our panel (Figure 2-1).
(1) the minimal detectable allele fraction (95% MDAF; Extended Data Fig. 1c), which describes the minimum VAF that a variant can be detected in the data, in essence describing the limit of detection for each event.
(2) the VAF outlier p value, which denotes the probability that any variant call could have been generated by sequencing noise given the position-specific noise distribution across our GIAB controls and the pan-dataset coverage levels of our samples, thus allowing us to discern overrepresented sequencing artefacts from real events.

This method allowed detection of VAF of ≥1%, which was important as studies have shown that low level VAFs (i.e. those less than <2%) are also associated with worse outcomes for both CV disease and cancer.

Figure 2-2: Quality Control Metrics



A. Plotted here are the AO (the number of sequenced reads supporting the alternative allele (mutation)) against the UAO (the number of sequenced reads with unique start sites that support the alternative allele- an additional measure of molecular complexity in our sequencing libraries). The red dotted lines denote the filter thresholds in both measurements (AO ≥ 5, UAO ≥ 3) and points are scaled by the VAF of the somatic mutation.

B. The 95% MDAF (Minimal Detectable Allele Fraction with 95% Confidence) versus the VAF for each event. This metric describes the minimum VAF that a variant can be detected in our data - in essence, describing the Limit of Detection (LOD) for each event. All variants used in our analysis above 2% VAF are scaled by their clone size and coloured by their functional consequence. Points in red are events that failed to pass our quality criteria and are removed from subsequent work.

C. The VAF Outlier P-Value versus VAF for each event. This parameter describes the probability that any observed variant could have been generated by sources of artefact given the position-specific noise distribution across our Genome in a Bottle controls and the pan-dataset coverage levels of our samples; thus, allowing us to discern recurrent sources of artefact from real events.

2.6 Statistical Handling

2.6.1 Data Handling

All participant data were recorded on a secure online GCP approved data management system (Castor EDC, Amsterdam, Netherlands). I manually entered all the data into the eCRF. No patient-identifying material was entered into the electronic database. Patients were anonymised and identified by their unique study identification number. Quality control of the data was ensured by a robust system whereby all data were checked manually and underwent pre-specified electronic data validation checks, while all queries were investigated, and dates appropriately amended in the eCRF.

2.6.2 Power Calculation

When the study was designed, there was no information relating to the prevalence of CHIP in patients with HFpEF, and the only limited data on the incidence of CHIP in patients with HFrEF was in a highly selected group of patients with ischaemic HF (73). Initially, I aimed to recruit 500 patients, but the COVID-19 pandemic coincided with funding applications, and my applications were unsuccessful. The power calculation for this funding application, assumed that in 500 patients, 18.5% would have CHIP giving the study 80% power at the 5% level of significance.

However, with my pre-existing funding (BHF Centre of Research Excellence Fund), I had enough funding to recruit and assess 96 patients. Previous studies found that CHIP was prevalent in 18.5% of patients with chronic ischaemic HF (73). Considering the limited evidence and the need to identify the prevalence of CHIP in a broader range of patients with HF, including patients with HFpEF, I believe that my sample size provided adequate hypotheses-generating data and provide preliminary evidence of feasibility for performing a larger cohort study.

2.6.3 Statistical Analysis

Categorical variables are expressed as the number and percentage of patients. Continuous variables following a normal distribution are expressed as means with standard deviation, while those not following a normal distribution are expressed as medians with interquartile range. Comparison of categorical variables between groups at baseline was performed using Chi-square or Fisher's exact test. Differences in continuous variables between groups were assessed with the t-test or Wilcoxon rank sum test. Univariate and multivariable regression models were used to assess for the association between baseline factors and the presence of CHIP. A p-value of >0.05 indicates the absence of a statistically significant effect. All analyses were performed using Stata version 16.0 (Stat Corp., College Station, TX, USA) or higher.

2.7 Role of inflammatory biomarkers in patients with HF

In addition to designing a prospective observational study, I also examined the role of circulating inflammatory biomarkers in large HF datasets. Data were examined from the following large clinical trials Angiotensin-Neprilysin Inhibition versus Enalapril in Heart Failure (PARADIGM-HF) and Angiotensin-Neprilysin Inhibition versus Enalapril in Heart Failure with Preserved Ejection Fraction (PARAGON-HF) which will be discussed in more detail below. In addition, I also examined inflammatory data from a large observational HF study (Microvolt T-wave alternans in chronic HF). At the outset of my PhD, I enquired about measuring levels of IL-6 and hsCRP in PARADIGM-HF and PARAGON-HF, however, unfortunately this was not possible due to funding and rights over the ownership of blood tests.

These analysis provided further insight into the role of important inflammatory markers in both stable and decompensated HFrEF and HFpEF, while also providing evidence that HF therapies can reduce circulating levels of inflammation. The table below outlines which inflammatory biomarker was examined in each dataset (Table 2-2).

Table 2-2: Inflammatory biomarkers examined in available HF datasets.

Principal inflammatory biomarker of interest	Number of patients (n)	Dataset
IL-6	286 HFpEF 301 HFrEF	Microvolt T-wave alternans
Neutrophil/lymphocyte ratio	7978 HFrEF 4795 HFpEF	PARADIGM-HF PARAGON-HF

2.7.1 Study Population

Eligibility criteria for the patients enrolled in these studies are detailed in Table 2-3. Patients in PARADIGM-HF (ejection fraction of 40% or less) were randomised to receive either sacubitril/valsartan (a combination of an angiotensin receptor-neprilysin inhibitor [sacubitril] plus angiotensin receptor blocker [valsartan]) or enalapril (angiotensin-converting enzyme inhibitor receptor (ACEi). In PARAGON-HF, patients with an ejection fraction of 45% or higher were randomised to receive either sacubitril/valsartan or valsartan (angiotensin receptor blocker (ARB)). In both trials, recruited patients were required to have 'stable' (not decomensated HF). Ongoing therapy with an ACE inhibitor or ARB was stopped and, patients entered a sequential run-in, first receiving ACE inhibitor or ARB inhibitor followed by sacubitril/valsartan. Patients tolerating both run-in periods were randomly assigned to double blind therapy in a 1:1 ratio.

In the Microvolt T-wave alternans observational study, patients were recruited if they were admitted to one of NHS Greater Glasgow and Clyde Hospitals with decompensated HF. The design of these two trials and one observational study have been published previously (234–236).

Table 2-3 Eligibility criteria of the study populations analysed

PARADIGM-HF (HFrEF)	**PARAGON-HF (HFpEF)**	**Microvolt T-wave alternans (Both HFpEF and HFrEF)**
Inclusion criteria		
Age≥18 years old, male or female. LVEF ≤40% (measurement done anytime within past 6months); changed to ≤35% by amendment. BNP≥150pg/ml (NT-proBNP≥400mg/ml) and unplanned hospitalisation with HF with 12 months prior to visit 1. ACEi or ARB at a stable dose of at least 10mg/day or equivalent for at least 4 weeks before screening. Beta-blockers for at least 4 weeks prior to screening if not contraindicated).	Age≥50 years old, male or female LVEF≥45% at screening or within 6months prior. Symptoms of HF requiring treatment with diuretic(s) for at least 30 days prior to screening visit. Current symptoms of HF (NYHA class II to IV) at screening visit. Structural heart disease evidenced by at least 1 of the following echo findings:	Age≥18 years old, male or female. Symptoms and signs of HF PLUS radiological evidence of HF or clinical response to intravenous diuretics. Elevated BNP (<100pg/mL).

	a) LA enlargement defined by at least 1 of the following LA width (diameter) ≥3.8cm or LA length≥5.0cm or LA≥20cm^2 or LA volume≥55ml or LA volume index≥29ml/m^2 b) LVH defined by septal thickness or posterior wall thickness ≥1.1cm At least 1 of the following: a) HF hospitalisation within 9 months prior to screening visit and NT-proBNP>200pg/ml for patients not in AF OR >600pg/ml for patient in AF on screening ECG, or b) NT-proBNP>300pg/ml for patient not in AF or >900pg/ml for patients in AF on the screening visit ECG.	
Exclusion criteria		
Hypersensitivity or allergy to any of the study drugs, drugs of similar chemical classes, ACEi's, ARB's or NEP inhibitors as well as know or suspected C/Is to the study drugs.	Any prior echo measurement of LVEF<40%. Acute coronary syndrome, cardiac surgery, other major CV surgery, or urgent PCI within	Primary presentation with MI. Prior sustained ventricular arrhythmic event.

Previous history of intolerance to recommended target dose of ACEi or ARBs. Current acute decompensated HF. Requirement of treatment with both ACEis and ARBs. Known history of angioedema. Systematic hypotension and/or SBP<100mmHg at screening and/or <95mmHg at visit 3 or randomisation (visit 5). eGFR<30 at screening, visit 3 or visit 5 (randomisation) or decrease in eGFR of >25% between screening and visit 3 or between visit 3 and randomisation.	3months prior to visit 1 or an elective PCI within 30 days prior to visit 1. History of hypersensitivity to any of the study drugs or to drugs of similar classes. Current acute decompensated HF. Requirement of treatment with both ACEis and ARBs, or a renin inhibitor. Known history of angioedema. Any clinical event within the 6months prior to visit 1 or elective PCI within 30 days prior to visit 1. Probable alternative diagnosis that could account for HF symptoms. Specifically, patients with the following: a) Severe pulmonary disease	Significant cognitive impairment. Concurrent systemic disease likely to result in reduced life expectancy. Geographical or social factors making study participation or follow-up impractical.

Screening potassium≥5.2 or ≥5.4 at visit 3 or randomisation. Acute coronary syndrome, stroke, transient ischaemic attack, carotid or major vascular surgery, percutaneous coronary intervention or carotid angioplasty within past 3 months prior to screening. Coronary or carotid artery disease likely to require surgical or PCI within the 6months after screening. History of severe pulmonary disease. Diagnosis of peripartum or chemotherapy induced cardiomyopathy within 12 months prior to screening. History of heart transplant or who are on the transplant list with an LVAD.	b) Haemoglobin <10g/dl c) BMI>40kg/m^2 Patients with any of the following: a) SBP≥180mmHg at visit 1, or b) SBP>150mmHg AND <180mmHg at visit 1 unless the patient is receiving 3 or more anti-hypertensive drugs. c) SBP<110mmHg at visit 1, or d) SBP<100mmHg or symptomatic hypotension at visit 103 or visit 199/201. Use of other investigational drugs. Patients with a history of any dilated cardiomyopathy. Evidence of right sided HF in the absence of left sided structural heart disease.	

Documented ventricular arrhythmia with syncopal episodes within past 3 months prior to screening that is untreated. Symptomatic bradycardia or second-degree heart block without a pacemaker. Implantation of a CRT device within the prior 3months to screening or intent to implant a CRT device. Presence of haemodynamically significant mitral and/ or aortic valve disease except mitral regurgitation secondary to left ventricular dilatation. Presence of other haemodynamically significant obstructive lesions of left ventricular outflow tract including aortic stenosis. Any surgical or medical condition which might significantly alter the absorption, distribution, metabolism or excretion of study drugs.	Known pericardial constriction, genetic hypertrophic cardiomyopathy or infiltrative cardiomyopathy. Clinically significant congenital heart disease. Presence of haemodynamically significant valvular heart disease. Stroke, transient ischaemic attack, or valvular heart disease likely to require surgical or percutaneous intervention. Life-threatening or uncontrolled dysrhythmia, including symptomatic or sustained VT and AF or AFl with a resting ventricular rate >110bpm. Patients with a CRT device.	

Presence of any disease with a life expectancy <5years.	Patients with prior major organ transplant or intent to transplant. Any surgical or medical condition that might significantly alter the absorption, distribution, metabolism, or excretion of study drugs. Evidence of hepatic disease Patients with one of the following a) eGFR<30ml/min/1.73m^2 at visit 1 b) eGFR<25ml/min/1.73m^2 at visit 103 or 199/201, or c) eGFR reduction >35% (compared with visit 1) at visit 103 or visit 199/201 Presence of significant bilateral renal artery stenosis. Patients with either of the following: a) Serum potassium>5.2mmol/l at visit 1	

	b) Serum potassium>5.4mmol/l at visit 103 or visit 199/201 Life expectancy <3years History of noncompliance or history/evidence of drug/alcohol abuse. History of malignancy of any organ system treated or untreated within the past 5 years. Pregnant or lactating women or women of childbearing potential.	

LVEF; left ventricular ejection fraction, BNP; brain natriuretic peptide, LA; left atrium, LVH; left ventricular hypertrophy, SBP; systolic blood pressure, CRT; cardiac resynchronisation therapy, ACEi; angiotensin converting enzyme inhibitor, ARB; angiotensin receptor blocker, NEP; neprilysin inhibitor, PCI; percutaneous coronary intervention, LVAD; left ventricular assist device, AF; atrial fibrillation, ALF; atrial flutter, eGFR; estimated glomerular filtration rate.

2.7.2 Outcomes of interest

The primary outcome in PARADIGM HF trial was a composite of CV death or a 1st hospitalisation for HF, with a median follow up time of 27 months (stopped early due to efficacy). Whereas in PARAGON HF the primary outcome was all HF hospitalisations (including first and repeat admissions) or CV death, the median follow-up in PARAGON-HF was 35 months (completed follow-up the target number of events). In this thesis, the primary outcome used in both trials was time to first occurrence of HF hospitalisation or CV death. All events in the two trials were adjudicated by the same endpoints committee.

In microvolt T-wave alternans the primary outcome was all cause mortality. All outcome data was obtained from linkage provided by ISD. Causes of death were classified according to the ICD-10 classification as documented on the death certificates. Causes of death were not adjudicated.

2.7.3 Statistical Analysis

Data were analysed in several different ways in keeping with the themes of each of the chapters in this thesis. All analysis were conducted using Stata (Collage station, TX, USA). Continuous variables were summarised as number of observed values, number of missing values, mean and standard deviation (SD). Non-parametric continuous variables were presented as medians with interquartile range. Categorical data was summarised number of observed values, number and percentage. Comparison of categorical variables between groups at baseline was performed using Chi-squared or Fisher's exact test. Differences in continuous variables between groups at baseline were assessed with the t-test or Wilcoxon rank sum test.

Time-to-event analyses were displayed using Kaplan-Meier curves according to tertiles. Inflammatory biomarkers were also modelled as a continuous variable. Restricted cubic spline was generated and displayed graphically using the xblc command in STATA. Fractional polynomial and entered into the model as an interaction term with treatment. The results of the interaction were displayed graphically using the mfpi command in STATA. Models were adjusted for important clinical confounders which are outlined in each chapter of the thesis. Pearson correlation coefficients were examined to evaluate the correlation between

inflammatory biomarkers and other clinically important variables. A p value<0.05 was considered statistically significant.

CHAPTER 3 The prevalence of CHIP and its association with inflammation in patients with HF

3.1 Introduction

In this chapter, I will report the prevalence of CHIP and associated clinical characteristics in patients admitted to hospital with HF. I will describe the mutations observed and the correlations with inflammatory and cardiac biomarkers.

Ageing is a major risk factor for the development of CV disease and cancer. CHIP is associated with HF and cancer. CHIP reflects the accumulation of potentially pre-leukaemic, somatic mutations in HSCs over time. CHIP is detected via DNA sequencing of peripheral blood, saliva and tumour samples (77–79). The diagnosis of CHIP requires the absence of overt haematological malignancy, a normal peripheral blood count and mutant cells bearing relevant driver mutations in ≥2% of peripheral white blood cells (VAF≥2%). The VAF represents the fraction of variant sequencing within a genetic locus. The VAF cut off to diagnose CHIP was initially set based on sequencing methods available at the time, however the development of next generation sequencing now allows for VAFs of less than <0.5% to be detected. Until recently, the clinical relevance of VAF's less than 0.5% was uncertain. A recent study identified that a VAF cut off of less than 0.5% was associated with an increased risk of cardiac and all cause death (237).

Mutations in DNMT3A, TET2, ASXL1 represent approximately 80% of all detected mutations, with DNMT3A responsible for nearly 60% of the total mutational burden (70,75). Other commonly detected mutations include JAK2, TP53, PPM1D, SF3B1 and SRSF2 (70). CHIP was initially found to be associated with an increased risk of CAD, however subsequently it has been associated with a wide range of CV diseases including HF and cardiac arrhythmias (73,238–241). The presence of CHIP has also been associated with an increased risk of adverse CV outcomes including death and subsequent hospitalisation.

The commonly detected CHIP mutations play a central role in the regulation of inflammation, particularly IL-6, IL-8, MCP-1 and TNFα (137). There appears to be specific cytokine

associations with DNMT3A being associated with elevated levels of TNFα, whereas TET2 has associations with IL-6 (137). The upregulation of these inflammatory pathways may provide the mechanistic link between CHIP and the development of CV disease. For decades, inflammation has been associated with the development of HF, progression and worse prognosis in both HFpEF and HFrEF. In comparison to HFrEF, the pathophysiology of HFpEF is still poorly understood, but elevated levels of systemic inflammation may be relatively more important in HFpEF than HFrEF. There are currently limited treatment options for HFpEF, and the initial clinical trials examining anti-inflammatory therapy in HF were disappointing, showing either negative results or increased risk. However, the recent CANTOS trial has re-invigorated interest in anti-inflammatory therapy for the treatment of HF. Canakinumab, a monoclonal antibody directed against IL-1β, in patients with a history of prior MI reduced the incidence of atherosclerotic CV events and HF hospitalisation (21,67).

To date, the prevalence of CHIP has been retrospectively assessed in outpatients with chronic ischaemic HF and outpatients with HFpEF (73,238). This is the first study to prospectively examine the presence of CHIP in patients admitted to hospital with decompensated HF both HFrEF and HFpEF. It is also the first study to examine the association of CHIP with circulating levels of inflammation in patients with decompensated HF. Understanding the association of CHIP, inflammation and CV disease is of vital importance, especially given the results of the exploratory analysis of the CANTOS trial. Canakinumab reduced the relative risk of major CV events by 64% in those with TET2 mutations, a prior MI and elevated levels of CRP compared to 15% in the overall population (217). The presence of CHIP may therefore allow for the personalisation of CV therapies, although this hypothesis has yet to be tested.

In this study, I describe the prevalence of CHIP and its association with inflammatory and cardiac biomarkers in a cohort of patients with decompensated HFpEF and HFrEF. I compare clinical and biomarker differences in patients with CHIP and HFpEF and those with CHIP and HFrEF.

3.2 Methods

I performed a prospective observational study to investigate the prevalence of CHIP and its association with the inflammatory in patients with hospitalised HFpEF and HFrEF. The study was approved by the West of Scotland (REC 20/WS/0027) and the study was funded by the British Heart Foundation core fund (BHF Centre of Research Excellence Award [RE 18/6/34217]). A summary of the study has been previously provided (Figure 2-1). Recruitment to this study took place during the COVID-19 pandemic and each patient was required to have a negative COVID-19 test prior to recruitment. I screened near consecutive admissions to cardiology wards between the 12th of October and the 25th 2020 and the 25th of September 2021, at the QUEH and the GRI hospitals.

3.2.1 Study design

Potential participants were approached by their clinical team and asked if they were interested in taking part in the study. I provided patients who were interested in participating with verbal and written information on the study. Those who agreed to participate had their clinical information recorded and blood was taken to analyse their DNA and inflammatory biomarkers.

3.2.2 Inclusion criteria

Patients were invited to participate in the study if they met all the following criteria:

- Age ≥18 years.
- HF (NYHA II-IV).
- Clinical evidence of decompensated HF.
- Elevated natriuretic peptide levels.
    - In-patients: B-type natriuretic peptide (BNP) ≥100 pg/ml or NT-proBNP≥300 pg/ml).
- ***HFpEF group-*** LVEF ≥40%; and evidence of relevant structural heart disease on echo (i.e. LV hypertrophy [maximal diastolic LV septal or posterior wall thickness ≥13 mm]; LA dilatation (indexed LA volume ≥34 ml/m2); and/or evidence of elevated LV filling pressures [E/e' >13])

- ***HFrEF group-*** LVEF <40%

3.2.3 Exclusion criteria

To ensure the study population represented a "real-world" HF population without any underlying haematological malignancy. The exclusion criteria were:

- Patients who are unwilling or unable to provide consent.
- Pre-existing myeloproliferative disorder or haematological malignancy.
- Haemoglobin <100g/L, platelets <100x10^9/L or neutrophils <1.0 x10^9/L
- Acute coronary syndrome, coronary revascularisation (percutaneous or surgical) or stroke within the last three months.
- Severe left-sided valvular disease (except functional mitral regurgitation).
- Known or suspected hypertrophic/infiltrative cardiomyopathy or constrictive pericarditis.
- Chronic treatment with immunomodulating therapy.
- Ongoing infection requiring treatment with antibiotics.
- Life expectancy of less than one year as a result of a non-cardiac condition.

3.3 Study procedures

3.3.1 Main visit

Once the patient consented to taking part in the main study the following investigations were performed and all results were recorded in a study-specific electronic case report form (eCRF) (Castor EDC, Amsterdam, Netherlands). I completed this for all the ninety-six patients enrolled in the study:

- Full physical examination- including measurement of height, weight, resting heart rate and BP.

- A record of the patients past medical history (including previous cancer and cancer treatment) and previous CV investigations including detail of previous percutaneous coronary intervention.
- Baseline biochemical and haematological tests.
- Baseline echo performed by an experienced sonographer.
- Chest X-ray results.
- 12-lead ECG.
- Spot urine collection.
- Venepuncture- 75mls of venous blood was withdrawn for next generation DNA sequencing, haematological, biochemistry and cardiac/inflammatory biomarkers.
- NYHA class.

3.3.2 Echo

The echo was performed on admission to hospital, and the imaging protocol used in each scan is described in Chapter 2. Briefly, this included assessment of LV systolic function, diastolic function, RV function, as well as valvular function. They were performed by a BSE accredited sonographer but were not performed by a single operator.

3.3.3 CXR

Each patient had a baseline CXR performed, and the following signs were recorded: cardiomegaly, upper lobe venous diversion, interstitial oedema, perihilar oedema and the presence of pleural effusions.

3.3.4 ECG

A 12-lead ECG was performed at baseline, I recorded the following parameters: heart rate, rhythm, PR interval, QRS duration and the QT interval corrected for heart rate (QTc). The ECG was also examined for evidence of bundle branch block and ischaemic ST and T wave

abnormalities. Additionally, evidence of left ventricular hypertrophy was measured as defined by the Sokolow and Lyon criteria (S V_1 + R V_5 or V_6 >35mm).

3.3.5 Laboratory analysis

3.3.5.1 Detection of CHIP mutations

The full method for sampling, processing, storage and analysis are outlined in Chapter 2. In brief, immediately following collection, samples were centrifuged for 15 minutes at 1,000g. Serum was then aliquoted and PBMC cells were separated using the PBMC Isolation Kit/Big EasySep Magnet (STEMCELL Technologies, 1618 Station Street, Vancover, BC, Canada). The DNA quality and quantity was then tested and documented. CHIP mutations were then called using archer reagent kits and corresponding target enrichment panels to produce high-complexity libraries for use with Illumina® next-generation sequencing instruments, the full method is outlined in Chapter 2.

3.3.5.2 Serum cardiac biomarkers analysis

Cardiac biomarkers were measured at the time of enrolment and were processed. NTproBNP and hstropT levels provide evidence of cardiac stress and high levels are associated with poorer functional class and worse CV outcomes. The methods for sampling processing, storage and analysis are outlined in Chapter 2. In brief, immediately following collection, samples were centrifuged for 15 minutes at 1,000g. Serum was then aliquoted and stored at -80°C. Samples were thawed immediately before the assays were performed using an ELISA technique.

3.3.5.3 Serum inflammatory biomarkers

Samples for these serum inflammatory biomarkers were collected at baseline. The methods for sample processing, storage and analysis are outlined in Chapter 2. In brief, immediately following collection, samples were centrifuged for 15minutes at 1,000g. Serum and plasma was then aliquoted and stored at -80°C. Samples were thawed immediately before the assays

were performed using the Mesoscale U-PLEX (IL-18, IL-33, TGF-β group, MCP), S-PLEX (IL-1β and IL-6) and V-PLEX (CRP, VCAM and ICAM-1) immunoassays.

3.3.6 Statistical analysis

Categorical variables are expressed as number and percentage of patients. Continuous variables following a normal distribution are expressed as means with standard deviation, while those not following a normal distribution are expressed as medians with interquartile range. Differences between groups were assessed using t-tests, Mann-Whitney tests, Wilcoxon signed rank test or Fisher tests where appropriate. A p-value of >0.05 indicates the absence of a statistically significant effect. All analyses were performed using Stata verion 16.0 (Stat Corp., College Station, TX, USA) or higher.

3.4 Results

3.4.1 Baseline characteristics of patients with decompensated HF

Baseline characteristics are reported in Table 3-1. A total of 96 patients agreed to participate in the study; 48 patients had HFrEF and 48 patients had HFpEF. Patients had an average age of 72.2years, and as expected patients with HFpEF were older (74.4 yrs) than those with HFrEF (70.1 yrs). Almost all patients were White (99%), apart from one. AF/ flutter was common (64%), and a past history of cancer was less common (14%). Rates of vascular disease were low in my cohort; MI (19%), stroke (9%), previous coronary artery bypass operation (CABG) (8%).

Table 3-1 Baseline characteristics of patients hospitalised with HF

	All HF (n=96)	**HFpEF (n=48)**	**HFrEF (n=48)**
Age, years	72.2±12.9	74.4±11.9	70.1±13.5
Female sex, no. (%)	44 (46)	27 (56)	17 (35)
Ethnicity, no. (%)			
White	95 (99)	48 (100)	47 (98)
Black	1 (1)	0 (0)	1 (2)
Weight (kg)	88.9±29.2	91.3±33.3	86.4±24.4
BMI (kg/m^2)	30.9±9.9	32.6±9.4	29.1±10.1
Smoking history, no. (%)			
Current smoker	18 (19)	6 (12)	12 (25)
Ex-smoker	30 (31)	13 (27)	17 (35)
Alcohol intake, no. (%)			
Alcohol excess	21 (22)	7 (15)	14 (30)
Medical History, no. (%)			
HTN	55 (58)	31 (66)	24 (50)
MI	18 (19)	8 (17)	10 (21)
Previous CABG	8 (8)	5 (10)	3 (6)
Stroke	9 (9)	6 (12)	3 (6)
AF/ Flutter	61 (64)	30 (62)	31 (65)
Diabetes	32 (33)	15 (31)	17 (35)
COPD	21 (22)	12 (25)	9 (19)
Previous cancer	13 (14)	7 (15)	6 (12)

BMI; body mass index, HTN; hypertension, MI; myocardial infarction, CABG: coronary artery bypass operation, COPD; chronic obstructive pulmonary disease.

3.4.2 HF characteristics of patients with decompensated HF

Table 3-2 outlines the HF characteristics of the patients recruited to the study. In my cohort, 44 patients (24 (50%) with HFpEF and 20 (42%) with HFrEF) had a diagnosis of HF prior to admission to hospital and 26 patients had experienced a previous HF hospitalisation (33% with HFpEF and 20% with HFrEF). As expected, in HFrEF group LVEF was an average of 23.6% and in HFpEF group LVEF was an average of 55.5%, in keeping with diagnostic criteria for the type of HF. The majority of patients were either NYHA class III or IV. Patients experienced the symptoms expected for those admitted to hospital with decompensated HF.

CV treatments were as expected at the time of this study. Forty-seven patients (49%) were receiving treatment with a loop diuretic prior to admission to hospital. In patients with HFrEF; 20 (42%) were receiving an ACEi/ARB, 6 (13%) were receiving sacubitril/valsartan and 22 (47%) were receiving a beta-blocker. At the time of recruitment, SGLT2 inhibitors were not licensed for the treatment of HFpEF or HFrEF. Fineronone was also not licensed for the treatment of HFpEF at the time of recruitment. Therefore, only one patient recruited to this study was receiving an SGLT2 inhibitor.

Table 3-2: HF characteristics of patients with decompensated HF

	All HF (n=96)	**HFpEF (n=48)**	**HFrEF (n=48)**
Previous HF diagnosis no. (%)	44 (46)	24 (50)	20 (42)
Previous HF hosp. no. (%)	26 (27)	16 (33)	10 (21)
Total number of HF hosp. no.	1.4±0.7	1.4±0.7	1.3±0.7
LVEF, %	40.0±19.5	55.5±12.4	23.6±9.7
NYHA class, no. (%)			
II	16 (17)	10 (21)	6 (12)
III	62 (65)	28 (60)	34 (71)
IV	17 (18)	9 (19)	8 (17)
HR, b.p.m	78.6±19.5	71.9±15.6	85.5±20.8
SBP (mmHg)			
Signs and symptoms, no. (%)			
Orthopnea	59 (61)	31 (65)	28 (58)
PND	46 (48)	20 (43)	26 (54)
Ankle swelling	72 (76)	41 (85)	31 (66)
Fatigue	45 (47)	26 (55)	19 (40)
ECG			
LBBB, no. (%)	20 (21)	5 (10)	15 (31)
QRS duration (ms)	108±28	99±23	117±30
CV Treatment, no. (%)			
Beta Blocker	46 (48)	24 (50)	22 (47)
ACEi or ARB	40 (42)	20 (42)	20 (42)
Sacubitril/valsartan	6 (6)	0 (0)	6 (13)
Aspirin	20 (21)	9 (19)	11 (23)
Other antiplatelet	8 (8)	7 (15)	1 (2)

Warfarin	9 (9)	4 (8)	5 (10)
Loop diuretic	47 (49)	29 (60)	18 (38)
Thiazide diuretic	7 (7)	5 (10)	2 (4)
MRA	9 (9)	2 (4)	7 (15)
SGLT2	1 (1)	0 (0)	1 (2)

LVEF; left ventricular ejection fraction, PND; paroxysmal nocturnal dyspnoea, LBBB; left bundle branch block, ACEi; angiotensin converting enzyme inhibitor, ARB; angiotensin receptor blocker, MRA; mineralocorticoid receptor antagonist.

3.4.3 The prevalence of CHIP in patients with decompensated HFpEF and HFrEF

The initial diagnostic criteria for CHIP was determined based on the sequencing methods available at the time, which were only able to accurately detect VAF of ≥2%. However, with the introduction of next generation sequencing, low level VAFs can now be detected accurately. Therefore, I decided to adopt a VAF ≥1% cut off, as most studies have shown that low level VAFs are also associated with worse outcomes for both CV disease and cancer.

CHIP driver mutations with a VAF≥2% were detected in 5 patients with HFrEF (10%) and 8 patients with HFpEF (17%). CHIP driver mutations with VAF≥1% were detected in 25 patients with HFrEF (52%) and 21 patients with HFpEF (44%) (Figure 3-1).

There was an age-dependent increase in the prevalence from 4% (≤50years old) to 44% (≥80years), and the total number mutations also increased with age in both HFpEF and HFrEF groups with a VAF≥1% (Figure 3-2).

Figure 3-1: Frequency of CHIP according to VAF and HF phenotype.

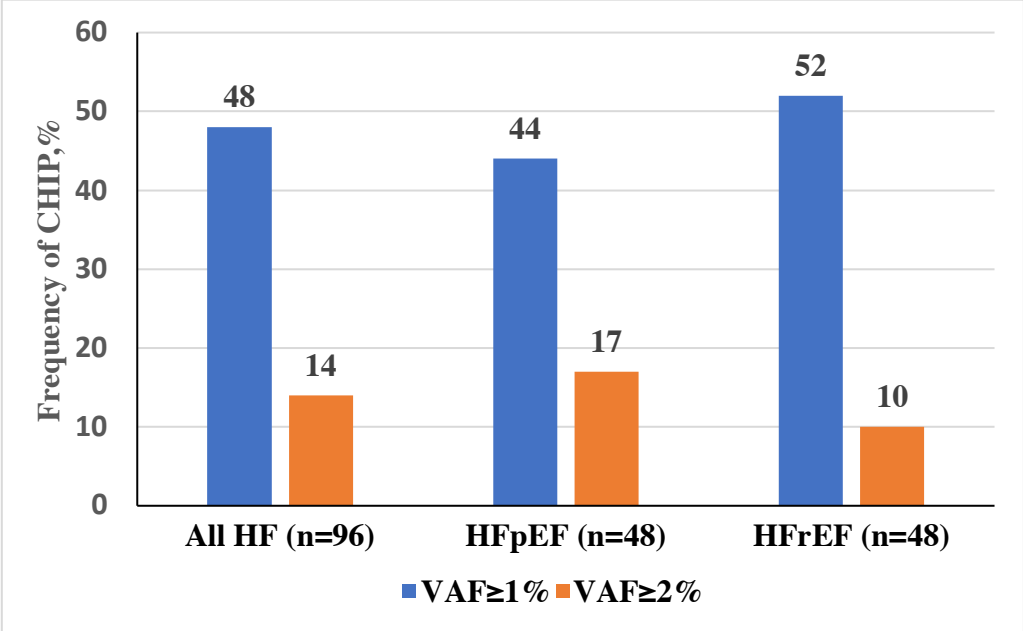
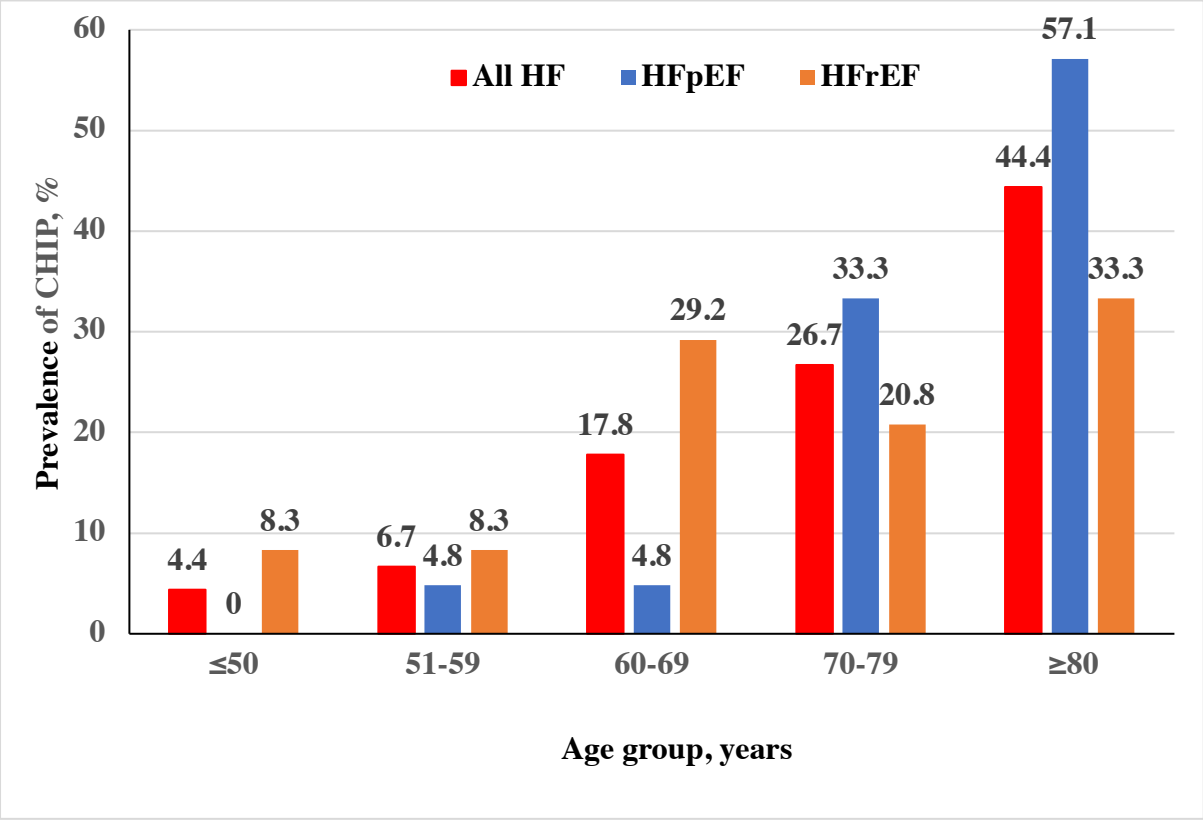


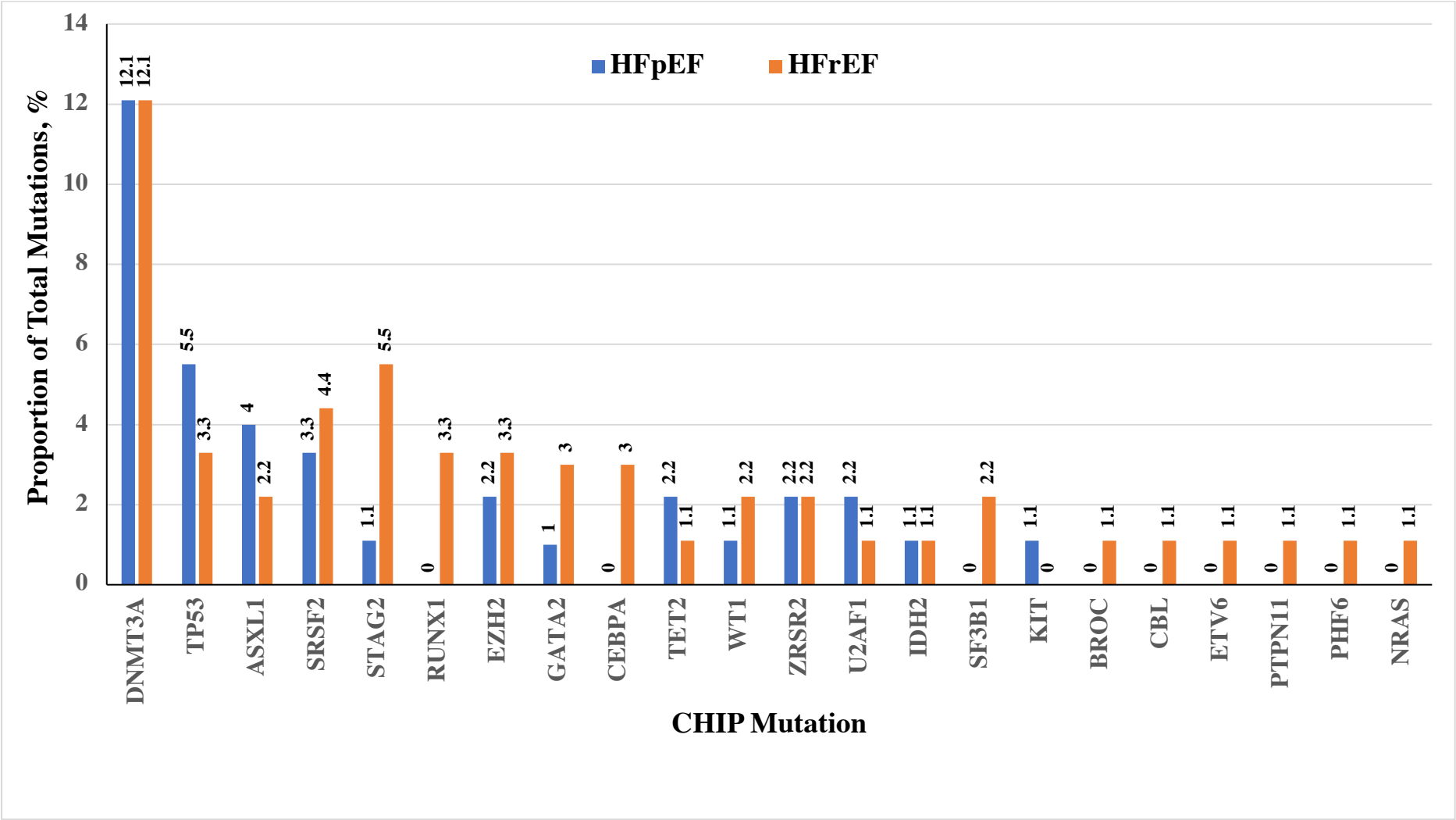
Figure 3-2: Prevalence of CHIP according to age group (VAF≥1%)



3.4.4 CHIP associated mutations

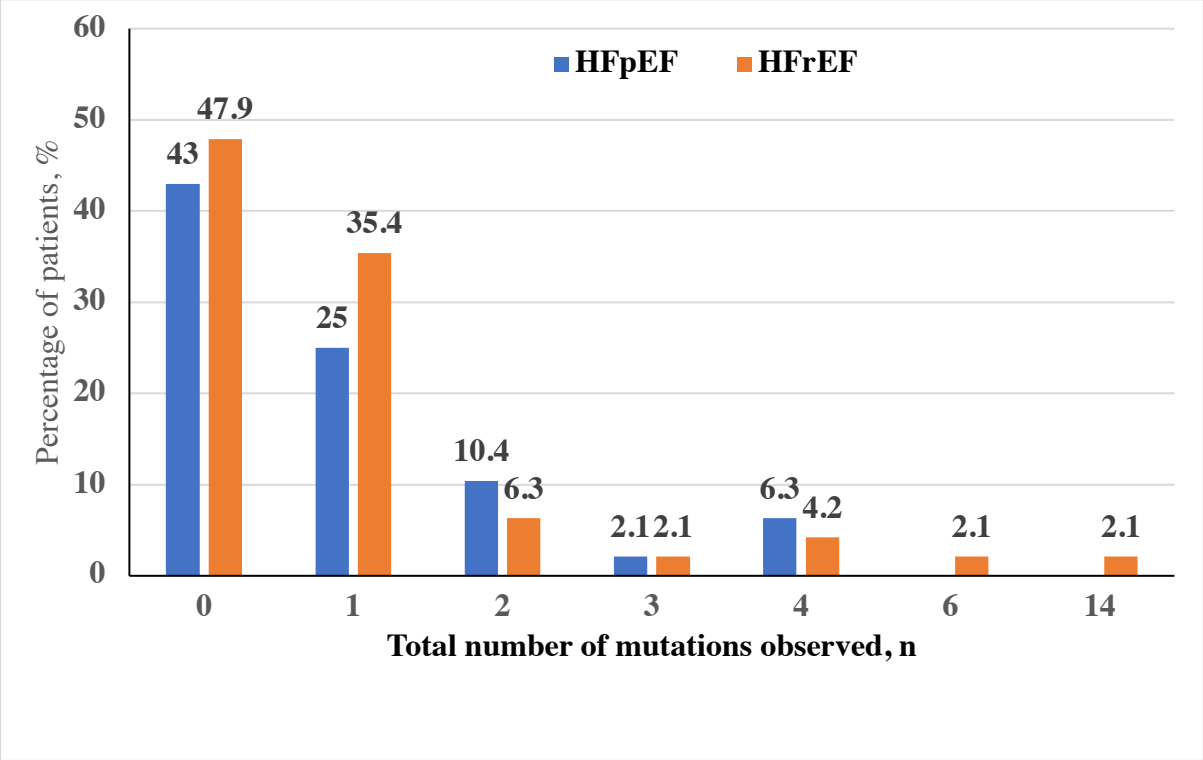
A total of 37 mutations were identified in those with HFpEF and 54 mutations were identified in those with HFpEF. In Appendix VI**,** I have provided a full list and combination of mutations observed in patients with both HFrEF and HFpEF. The most common mutation identified in both HFpEF and HFrEF was DNMT3A (Figure 3-3). Notably, only two patients with HFpEF and only one patient with HFrEF had a TET2 mutation (2.2% and 1.1% of total mutations, respectively). The second most common mutation was TP53 in patients with HFpEF and STAG2 in patients with HFrEF. No RUNX1 mutations were observed in patients with HFrEF, but RUNX1 mutation was observed in three patients with HFpEF.

Figure 3-3: Proportion of Total mutations



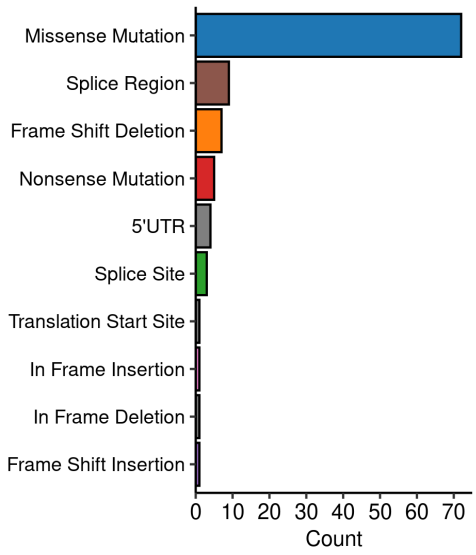
The majority of patients had only one mutation, but one patient with HFrEF had fourteen mutations in total (see appendix VI and figure 3-4).

Figure 3-4: Total number of mutations observed in patients with HF



The majority of mutations observed were missense mutations, with splice region mutations being the second most common mutation followed by frame shift deletions (Figure 3-5).

Figure 3-5: Type of mutations observed in patients with HF



3.4.5 VAF of associated mutations

Patients can harbour more than one CHIP associated mutation as outlined in Figure 3-4. The dominant mutation (i.e the one with the highest VAF) was selected for the purpose of calculation of the median VAF for patients with HFpEF and HFrEF. The range of VAF in patients with HFpEF was 1.0% to 39.7%, with a median of 1.5%. In HFrEF, the range of VAF was 1.0% to 18.5%, with a median of 1.2%. Although the median VAF was numerically higher in the HFpEF group when compared to the HFrEF group this was not statistically significant.

Cumulative VAF is defined as the sum of the VAFs of all mutations observed in a patient. The clinical significance of cumulative VAF is relatively unknown. Patients with HFpEF had a numerically higher cumulative VAF (median 2.6%) when compared to those with HFrEF (median 1.4%), but this was not statistically significant (Figure 3-7).

Figure 3-6: VAF of individual mutations

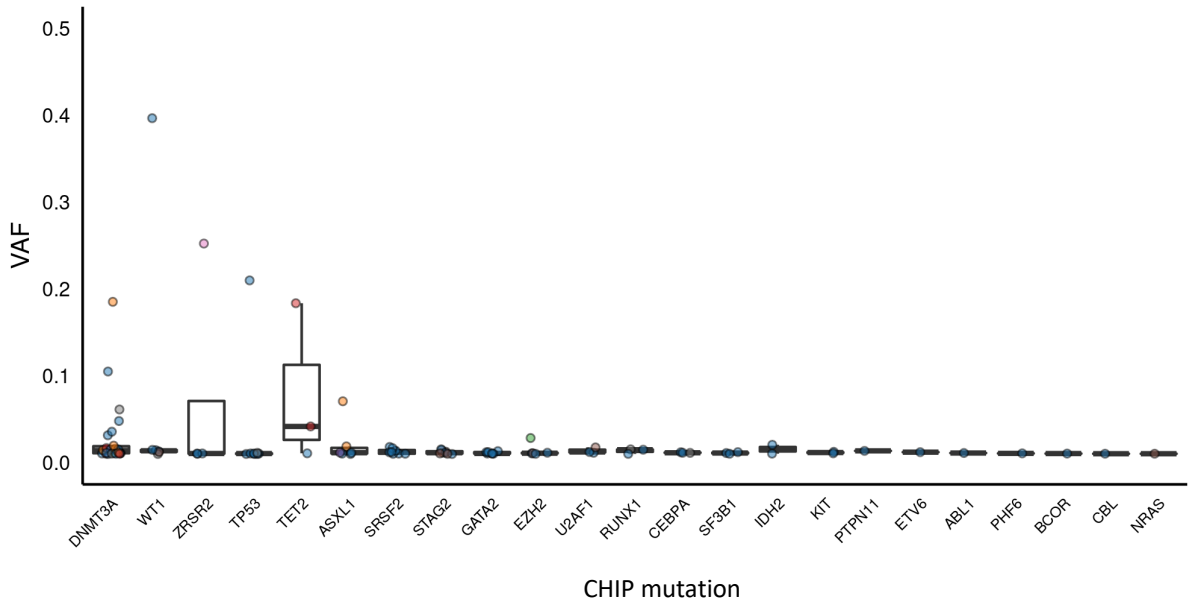
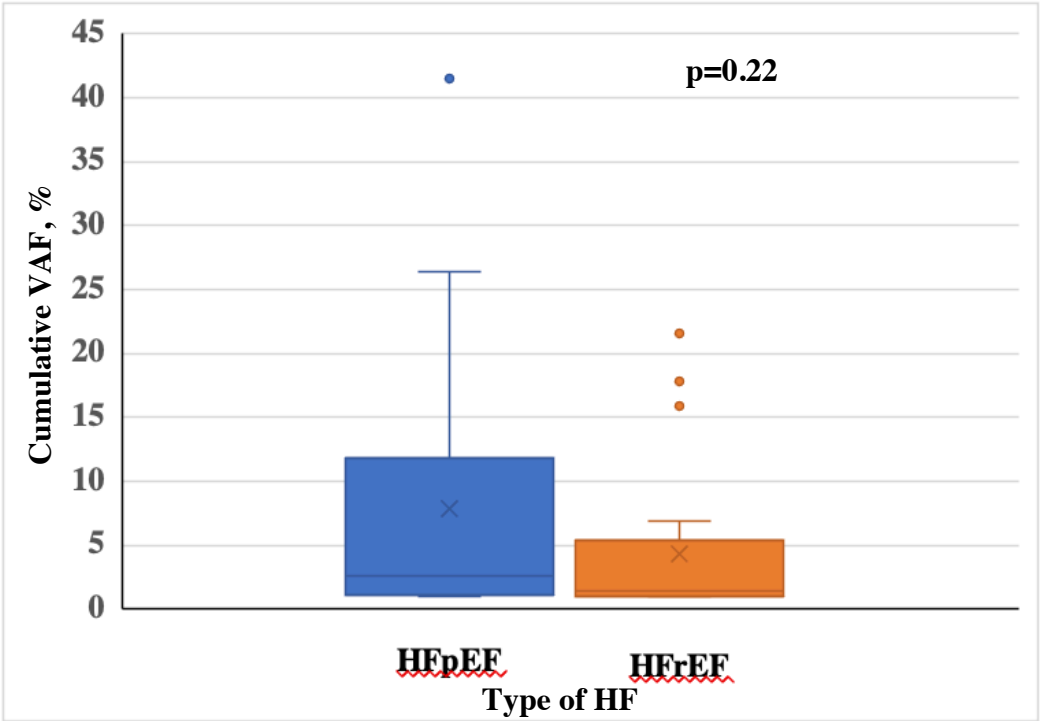


Figure 3-7: Cumulative VAF in patients with HFpEF and HFrEF



3.4.6 Clinical characteristics of patients with CHIP

The clinical characteristics of patients according to CHIP status are outlined in Table 3-3. Patients with CHIP were older than patients without CHIP (75.2yrs versus 69.5yrs, p=0.03). Patients with CHIP and HFpEF were older than those without CHIP and HFpEF (80.1yrs versus 70.0yrs, p=0.002). In the HFrEF group, there was no statistically significant difference in age in those with and without CHIP, although there was a trend to those with CHIP being older than those without CHIP. There was a no significant trend toward a lower BMI in those with CHIP when compared to those without CHIP across the groups (CHIP 28.9 kg/m^2 versus No CHIP 32.9 kg/m^2, p=0.06). In this cohort, the prevalence of previous vascular disease (including MI and stroke) was low overall, including in the HFrEF group. Rates of cancer were similar for patients with or without CHIP in both HF groups.

Table 3-3: Baseline characteristic according to CHIP status

	All HF		**p**	**HFpEF**		**p value**	**HFrEF**		**p**
	CHIP	**No CHIP**		**CHIP**	**No CHIP**		**CHIP**	**No CHIP**	
	n=46	**n=50**		**n=21**	**n=27**		**n=25**	**n=23**	
Age, years	75.2±12.8	69.5±12.5	**0.03**	80.1±8.8	70.0±12.3	0.002	71.1±14.2	68.9±13.0	0.58
Female sex, no. (%)	21 (46)	23 (46)	0.97	13 (62)	14 (52)	0.49	8 (32)	9 (39)	0.61
Ethnicity, no. (%)									
White	45 (98)	50(100)	0.29	21 (100)	27 (100)	-	24 (96)	23 (100)	0.33
Black	1 (2)	0 (0)		-	-		1 (4)	0 (0)	
Weight (kg)	83.9±26.5	93.4±31.2	0.12	84.5±28.8	96.4±36.0	0.23	83.5±25.0	89.6±23.8	0.41
BMI (kg/m^2)	28.9±9.7	32.9±9.8	0.06	30.5±8.0	34.3±10.2	0.17	27.6±10.8	31.0±9.1	0.27
Smoking history, no. (%)									
Current smoker	5 (11)	13 (26)	0.06	0 (0)	6 (22)	0.02	5 (20)	7 (30)	0.40
Ex-smoker	15 (33)	15 (30)	0.78	5 (24)	8 (30)	0.65	10 (40)	7 (30)	0.49
Medical History, no. (%)									
HTN	25 (56)	30 (60)	0.66	12 (60)	19 (70)	0.46	13 (52)	11 (48)	0.77
MI	9 (20)	9 (18)	0.88	4 (19)	4 (15)	0.74	5 (20)	5 (22)	0.88

Previous CABG	3 (7)	5 (10)	0.54	2 (10)	3 (11)	0.86	1 (4)	2 (9)	0.50
Stroke	4 (9)	5 (10)	0.83	2 (10)	4 (15)	0.58	2 (8)	1 (4)	0.60
AF/ Flutter	31 (67)	30 (60)	0.45	15 (71)	15 (56)	0.26	16 (64)	15 (65)	0.93
Diabetes	14 (30)	18 (36)	0.56	4 (19)	11 (41)	0.11	10 (40)	7 (30)	0.49
COPD	9 (20)	12 (24)	0.60	4 (19)	8 (30)	0.40	5 (20)	4 (17)	0.82
Previous cancer	6 (13)	7 (14)	0.89	3 (14)	4 (15)	0.96	3 (12)	3 (13)	0.91

BMI; body mass index, HTN; hypertension, MI; myocardial infarction, CABG; coronary artery bypass graft, AF; atrial fibrillation, COPD; chronic obstructive pulmonary disease

3.4.7 HF characteristic according to CHIP status

The HF characteristics of patients according to CHIP status are outlined in Table 3-4. There was no difference in rates of previous HF hospitalisations across the groups according to CHIP status. The mean LVEF was similar for patients with CHIP when compared to those without CHIP for both HFpEF and HFrEF groups (52.4% versus 58.9% and 23.2% versus 23.9%, respectively). SBP was not higher in all patients with HF and CHIP when compared to patients with HF without CHIP. NYHA class was similar across the groups, alongside the commonly encountered HF symptoms.

There was no statistically significant difference in CV treatments according to CHIP status.

Table 3-4: HF characteristics according to CHIP status

	All HF		**p**	**HFpEF**		**p value**	**HFrEF**		**p**
	CHIP **n=46**	**No CHIP** **n=50**		**CHIP** **n=21**	**No CHIP** **n=27**		**CHIP** **n=25**	**No CHIP** **n=23**	
Previous HF diagnosis, no. (%)	22 (48)	22 (44)	0.71	9 (43)	15 (56)	0.38	13 (52)	7 (30)	0.13
Previous HF hosp. no. (%)	14 (30)	12 (24)	0.52	7 (33)	9 (33)	1.00	7 (28)	3 (14)	0.23
Total number of HF hosp. no.	1.5±0.8	1.3±0.6	0.21	1.5±0.9	1.3±0.4	0.21	1.4±0.7	1.3±0.7	0.59
LVEF, %	37.4±18.3	43.6±21.2	0.38	52.4±14.1	58.9±9.9	0.30	23.9±8.2	23.2±12.6	0.89
NYHA class, no (%)									
II	6 (13)	10 (20)	0.67	3 (15)	7 (26)	0.66	3 (12)	3 (13)	0.98
III	31 (69)	31 (62)		13 (65)	15 (56)		18 (72)	16 (70)	
IV	8 (18)	9 (18)		4 (20)	5 (19)		4 (16)	4 (17)	
HR, b.p.m	79.5±17.2	77.8±21.6	0.67	75.3±12.6	69.4±17.4	0.20	82.9±19.7	88.6±22.2	0.36
SBP (mmHg)	122.9±22.9	126.0±27.3	0.55	130.7±21.7	137.2±27.5	0.39	116.4±22.1	113.9±21.6	0.70
Signs and symptoms, no. (%)									
Orthopnea	26 (57)	33 (66)	0.34	15 (71)	16 (59)	0.38	11 (44)	17 (74)	0.04
PND	14 (31)	32 (64)	0.001	7 (35)	13 (48)	0.37	7 (28)	19 (83)	<0.001

Ankle swelling	34 (76)	38 (76)	0.96	20 (95)	21 (78)	0.09	14 (58)	17 (74)	0.26
Fatigue	20 (44)	25 (50)	0.59	10 (50)	16 (59)	0.53	10 (40)	9 (39)	0.95
ECG									
LBBB, no. (%)	9 (20)	11 (22)	0.77	0 (0)	5 (19)	0.04	9 (36)	6 (26)	0.46
QRS duration (ms)	109.2±31.5	106.54±24.2	0.64	91.8±16.4	104.7±25.2	0.05	123.9±33.9	108.7±23.4	0.08
CV Treatment, no. (%)									
Beta Blocker	26 (57)	20 (41)	0.13	12 (57)	12 (44)	0.38	14 (56)	8 (36)	0.18
ACEi or ARB	14 (30)	26 (52)	0.03	5 (24)	15 (56)	0.27	9 (36)	11 (48)	0.41
Sac/val	2 (4)	4 (8)	0.44	0 (0)	0 (0)	-	2 (8)	4 (18)	0.30
Aspirin	9 (20)	11 (22)	0.73	4 (19)	5 (19)	0.96	5 (20)	6 (27)	0.56
Other antiplatelet	2 (4)	6 (12)	0.17	2 (10)	5 (19)	0.38	0 (0)	1 (5)	0.28
Warfarin	5 (11)	4 (8)	0.63	2 (10)	2 (7)	0.79	3 (12)	2 (9)	0.71
Loop diuretic	24 (52)	23 (47)	0.61	15 (71)	14 (52)	0.17	9 (36)	9 (41)	0.73
Thiazide diuretic	3 (7)	4 (8)	0.76	1 (5)	4 (15)	0.26	2 (8)	0 (0)	0.18
MRA	6 (13)	3 (6)	0.25	0 (0)	2 (7)	0.20	6 (24)	1 (5)	0.06
SGLT2 inhibitor	0 (0)	1 (2)	0.33	0 (0)	0 (0)	-	0 (0)	1 (5)	0.28

LVEF; left ventricular ejection fraction, HR; heart rate, SBP; systolic blood pressure, PND; paroxysmal nocturnal dysponea, LBBB; left bundle branch block, ACEi; angiotensin converting enzyme inhibitor; ARB, angiotensin receptor blocker, Sac/val; sacubitril/valsartan, MRA; mineralocorticoid receptor antagonist, SGLT2; sodium glucose co-transporter-2 inhibitor

3.4.8 Baseline haematological parameters according to CHIP status

The diagnosis of CHIP requires the presence of no peripheral cytopenia as outlined in the introduction. There was no difference in the haematological parameters, including RLC and NLR between the groups according to CHIP status.

Table 3-5: Haematological parameters according to CHIP status

	HF Type		**p**	**HFpEF**		**p**	**HFrEF**		**p**
	CHIP	**No CHIP**		**CHIP**	**No CHIP**		**CHIP**	**No CHIP**	
	n=46	**n=50**		**n=21**	**n=27**		**n=25**	**n=23**	
Hb, g/l	134.5 (117-149)	136 (117-152)	0.52	133 (119-141)	134 (116-153)	0.50	135 (117-151)	143 (128-152)	0.91
Haematocrit, l/l	41.9±6.4	42.3±5.9	0.76	40.3±5.4	42.7±6.3	0.32	43.3±7.0	42.6±6.7	0.72
MCV, fl	94.8 (89.3-97.7)	93.2 (89.5-96.7)	0.56	92.3 (89.4-96.3)	92.8 (88.6-94.7)	0.63	96.3 (87.7-98.9)	95.4 (89.6-100.8)	0.95
WBC, $x10^9$/l	7.5±1.9	8.0±1.8	0.27	7.3±1.8	7.9±1.8	0.22	7.7±2.0	8.0±1.8	0.68
Lymphocyte, $x10^9$/l	1.5 (1.1-2.0)	1.7 (1.2-2.1)	0.37	1.4 (1.2-1.5)	1.7 (0.9-2.1)	0.66	1.6 (1.1-2.1)	1.7 (1.2-2.1)	0.56
RLC, %	21.2 (14.7-27.5)	20.3 (15.0-24.5)	0.99	21.4 (14.7-27.5)	19.8 (13.6-27.8)	0.85	21.1 (15.4-26.6)	20.3 (17.7-24.1)	0.76
Neutrophil, $x10^9$/l	4.9±1.5	5.3±1.6	0.17	4.7±1.6	5.4±1.7	0.17	5.0±1.5	5.3±1.4	0.58
NLR	3.0 (2.1-4.6)	3.2 (2.5-4.9)	0.77	3.0 (2.0-4.8)	3.4 (2.2-5.3)	0.62	3.1 (2.2-4.3)	3.1 (2.7-4.1)	1.00
Monocyte, $x10^9$/l	0.8 (0.7-0.9)	0.7 (0.6-0.8)	0.10	0.8 (0.7-0.9)	0.7 (0.5-0.9)	0.23	0.8 (0.7-1.0)	0.7 (0.6-0.8)	0.23
Eisinophils, $x10^9$/l	0.2 (0.1-0.3)	0.2 (0.1-0.3)	0.84	0.2 (0.1-0.4)	0.16 (0.1-0.3)	0.61	0.2 (0.2-0.3)	0.2 (0.2-0.3)	0.36

Hb; haemoglobin, MCV; mean corpuscular volume, WBC; white blood count, RLC; relative lymphocyte count, NLR; neutrophil lymphocyte ratio.

3.4.9 Humoral biomarkers according to CHIP status

In this section I examine the association of CHIP with circulating biomarkers (Table 3-5). There was no statistically significant difference in renal or liver function measurements across the groups. NTproBNP and troponin levels were similar in patients with and without CHIP across the groups.

Table 3-6: Cardiac biomarkers and biochemistry parameters according to CHIP status

	All HF		p	HFpEF		p value	HFrEF		p
	CHIP n=46	No CHIP n=50		CHIP n=21	No CHIP n=27		CHIP n=25	No CHIP n=23	
Biochemistry									
Creatinine, µmol/L	94 (77-140)	98 (78-131)	0.87	93 (71-140)	108 (81-135)	0.68	95 (82-122)	92 (70-124)	0.73
E-GFR, ml/min	60 (38-60)	57.5 (42-60)	0.77	49 (37-60)	57 (39-60)	0.87	60 (49-60)	60 (42-60)	0.71
Cystatin-c	1.5 (1.2-2.0)	1.5 (1.1-1.7)	0.37	1.6 (1.3-2.2)	1.5 (1.2-2.0)	0.53	1.4 (1.1-1.7)	1.4 (1.2-1.6)	0.78
Bilirubin, µmol/L	16 (10-21)	14 (10-20)	0.22	16 (10-19)	11 (9-22)	0.53	17 (11-21)	15 (10-17)	0.26
ALT, U/L	24 (15-42)	21 (14-33)	0.31	18 (15-35)	22 (14-31)	0.94	29 (21-43)	20 (16-38)	0.17
Alk Phos, U/L	109.7±60.0	106.9±45.7	0.80	117.2±65.4	115.2±51.1	0.90	103±55.5	97.2±37.1	0.66
AST, U/L	22 (18-34)	23.5 (17-33)	0.78	21 (18-32)	23 (16-32)	0.95	26 (18-34)	24 (18-32)	0.61
Glucose, mmol/L	5.9 (5.4-7.9)	6.3 (5.4-8.2)	0.78	6.3 (5.3-7.9)	5.7 (5-7.2)	0.25	5.8 (5.4-8.9)	7 (5.9-8.8)	0.18
HBA1c, %	50 (42.5-61.5)	45 (38-55)	0.39	61 (44-62)	55 (38-64)	0.95	50 (41-60)	41.5 (38-47.5)	0.17
Cardiac Biomarkers									

NT-proBNP, pg/ml	3912 (1400-9226)	2943 (1446-6437)	0.26	2487 (1189-4246)	1837 (907-3334)	0.16	7059 (2147-11597)	6323 (3261-11367)	0.86
Troponin, ng/L	21 (11-50)	32 (11-58)	0.66	44 (11-50)	18 (4-54)	0.42	18.5 (11-58)	35 (22-64)	0.15
Total cholesterol, mmol/L	3.9 (3.4-5.45)	4.5 (3.6-5.6)	0.50	4.4 (2.9-5.3)	3.7 (3.2-5.0)	1.00	3.9 (3.6-5.6)	4.8 (4-5.8)	0.32
HDL, mmol/L	1.25 (0.9-1.8)	1.0 (0.8-1.3)	0.24	1.7 (1.1-1.9)	1.1 (0.9-1.3)	0.17	1.2 (0.9-1.3)	0.9 (0.8-1.3)	0.84
LDL, mmol/L	2.8 (1.6-3.4)	2.3 (1.7-2.9)	0.58	2.5 (1.7-3.1)	2.0 (1.6-2.2)	0.29	3.1 (1.5-3.4)	2.8 (1.8-3.1)	0.79
Triglyceride, mmol/L	1.3 (1.1-1.6)	1.4 (0.9-1.7)	0.97	1.3 (1.1-1.3)	1.7 (0.9-2.2)	0.33	1.4 (1.0-2.0)	1.2 (1.0-1.7)	0.46

e-GFR; estimated glomerular filtration rate, ALT; alanine transaminase, AST; aspartate aminotransferase, Alk Phos; alkaline phosphatase, HBA1c; glycosylated haemoglobin, NTproBNP; N-terminal pro-B-type natriuretic peptide, HDL; high density lipoprotein, LDL; low density lipoprotein.

3.4.10 Inflammatory biomarkers according to CHIP status

Table 3-6 outlines the inflammatory biomarkers findings according to CHIP status. Overall, in this cohort of patients, levels of inflammatory activation were high. In all patients with HF and CHIP, concentrations of IL-1β, IL-18 and TGF-β2 were higher when compared to those without CHIP. Levels of ICAM-1 were numerically higher in all patients with HF and CHIP when compared to those without, but narrowly missed statistical significance (p=0.07).

In patients with CHIP and HFpEF levels of IL-1β were higher than in patients without CHIP and HFpEF (202.5fg/mL versus 141.3fg/mL, p=0.03). Upstream and downstream markers of IL-1β activity, IL-18 and CRP, were numerically higher in patients with CHIP and HFpEF when compared to patients with HFpEF without CHIP, although this did not reach statistical significance. However, levels of IL-6 were not higher in patients with HFpEF with and without CHIP.

In patients with CHIP and HFrEF, there was no difference in levels of IL-1β, IL-6 or CRP when compared to HFrEF patients without CHIP.

Table 3-7 Inflammatory biomarkers according to CHIP status

	All HF		p	HFpEF		p	HFrEF		p
	CHIP n=46	No CHIP n=50		CHIP n=21	No CHIP n=27		CHIP n=25	No CHIP n=23	
CRP (ng/mL)	9845.0 (4672.8-30827.8)	10477.4 (4054.8-20015.7)	0.29	11779.0 (5176.4-22096.3)	8996.9 (3415.5-14526.2)	0.19	8046.9 (4672.8-36527.2)	13043.2 (4540.7-21125.6)	0.82
IL-6 (fg/mL)	3338.0 (1932.7-6478.0)	4255.3 (2610.1-6628.1)	0.25	4100.9 (2527.1-7411.5)	4650.6 (2773.1-6645.6)	0.51	2542.1 (1702.9-6141.7)	3760.0 (2384.2-6628.1)	0.40
IL-1β (fg/mL)	195.5±146.5	141.4±56.3	0.02	202.4±120.9	141.3±54.0	0.03	141.5±60.1	189.9±167.2	0.20
IL-18 (pg/mL)	1102.0±458.4	925.4±293.9	0.03	1082.4±424.9	927.9±300.7	0.15	1118.4±492.9	922.2±292.6	0.12
IL-33 (pg/mL)	0.5 (0.2-1.2)	0.3 (0.1-0.7)	0.58	0.3 (0.1-1.3)	0.2 (0.1-0.7)	0.49	0.5 (0.3-0.9)	0.5 (0.3-0.6)	0.85
TGF-β1 (pg/mL)	7696.3 (4665.6-10552.6)	6128.3 (4082.7-11550.3)	0.74	7296.2 (4112.4-10069.4)	6420.5 (4428.3-10791.8)	0.88	7783.3 (5244.3-10921.5)	6033.1 (3989.9-13027.0)	0.63
TGF-β2 (pg/mL)	76.5±51.1	57.6±34.2	0.05	66.4±40	56.6±36.5	0.40	85.1±58.6	58.8±31.7	0.09
TGF-β3 (pg/mL)	4.2 (2.4-6.4)	3.1 (2.0-5.2)	0.12	3.4 (2.3-5.5)	2.6 (2.0-4.9)	0.20	4.5 (3.2-6.4)	3.6 (2.9-5.6)	0.46
MCP-1 (pg/mL)	230.3 (198.2-281.2)	230.6 (197.9-282.6)	0.93	228.3 (178.9-261.6)	233.1 (195.8-270.7)	0.83	232.6 (201.0-281.2)	229.9 (201.5-282.6)	0.70
ICAM1	1059.3	923.4	0.07	1115.5	858.8	0.16	1036.0	1116.7	0.34

(ng/mL)	(836.2-1873.5)	(652.5-1281.6)		(745.7-1361.4)	(668.6-1154.5)		(883.1-1873.5)	(652.5-1345.7)	
VCAM1 (ng/mL)	828.3 (652.7-1108.9)	790.6 (628.0-924.9)	0.29	849.4 (660.9-983.5)	800.9 (674.7-938.4)	0.53	824.0 (580.0-1110.3)	766.6 (605.6-853.1)	0.42

CRP; C-reactive protein, IL-6; interleukin-6, IL-1β; interleukin-one beta, IL-18; interleukin eighteen, IL-33; interleukin 33, TGF-B; transforming growth factor beta, MCP-1; monocyte chemoattractant protein-1, ICAM1; intercellular adhesion molecule 1, VCAM1; vascular cell adhesion molecule

3.4.11 Logistic regression according to CHIP status

Univariable logistic regression analysis was used to determine if certain variables were associated with CHIP status (Table 3-7). Patients with CHIP were more likely to be older (p=0.03). Patients with CHIP appeared to have higher levels of IL-1β, although this narrowly missed statistical difference (p=0.06).

Patients with HFpEF and CHIP were also more likely to be older in age (p=0.007), although this was not the case for patients with HFrEF and CHIP (p=0.57). Patients with HFpEF and CHIP had significantly higher levels of IL-1β (p=0.05).

Levels of NTproBNP were not associated with CHIP status in all groups.

Table 3-8: Univariable logistic regression model according to CHIP status

	Odds ratio (95% CI)		
	All HF	**HFpEF**	**HFrEF**
Age	1.04 (1.00-1.07) p=0.03*	1.09 (1.03-1.17) p=0.007*	1.01 (0.97-1.06) p=0.57
Type of HF	1.39 (0.63-3.12) p=0.41	-	-
Female sex	0.99 (0.44-2.20) p=0.97	1.51 (0.47-4.81) p=0.49	0.73 (0.22-2.40) p=0.61
SBP	0.99 (0.98-1.01) p=0.55	0.99 (0.97-1.01) p=0.38	1.01 (0.98-1.03) p=0.69
NTproBNP	1.00 (1.00-1.00) p=0.22	1.00 (1.00-1.00) p=0.63	1.00 (1.00-1.00) p=0.41
Creatinine	1.00 (0.99-1.01) p=0.80	1.00 (0.99-1.01) p=0.81	1.00(0.99-1.02) p=0.51
Log(CRP)	1.25 (0.86-1.83) p=0.24	1.50 (0.83-2.71) p=0.18	1.07 (0.65-1.77) p=0.78
Log(IL-6)	0.70 (0.41-1.20) p=0.19	0.71 (0.31-1.65) p=0.43	0.71 (0.35-1.45) p=0.35
Log(IL-1β)	2.20 (0.96-5.08) p=0.06	4.06 (1.00-16.4) p=0.05*	1.53 (0.52-4.48) p=0.44
Log(IL-18)	3.07 (0.96-9.84) p=0.06	3.10 (0.58-16.6) p=0.19	3.03 (0.59-15.6) p=0.19

*SBP; systolic blood pressure, NTproBNP; N-terminal pro-B-type natriuretic peptide, CRP; C-reactive protein; IL-6; interleukin 6; IL-1*β; interleukin-one beta, IL-18; interleukin-18.

3.4.12 Previous cancer and cancer therapy according to HF type

Previous cancer history was similar across the groups. The most common previous cancer observed in my cohort was breast cancer followed by prostate cancer (Figure 3-8 & Figure 3-9). The majority of patients with previous cancer exhibited only one mutation (67%), while one patient exhibited six mutations (17%). DNMT3A was the most common mutation observed in patients with previous cancer. The numbers of patients with previous cancer were too small to analyse whether the type of mutation observed varied according to previous cancer, cancer treatment and HF type.

Figure 3-8: Type of previous cancer according to type of HF

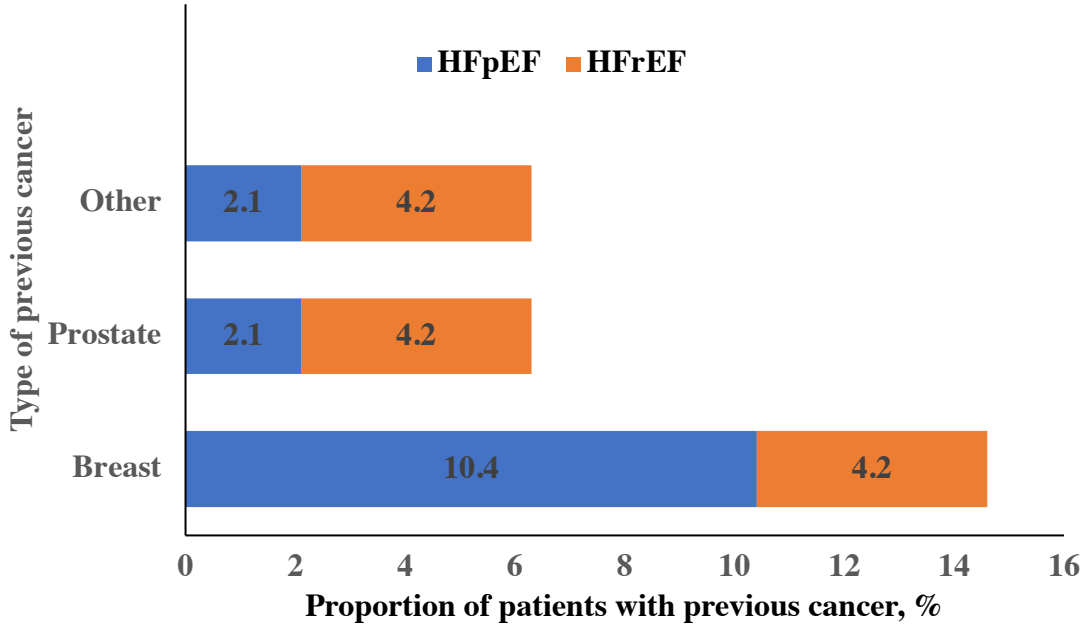
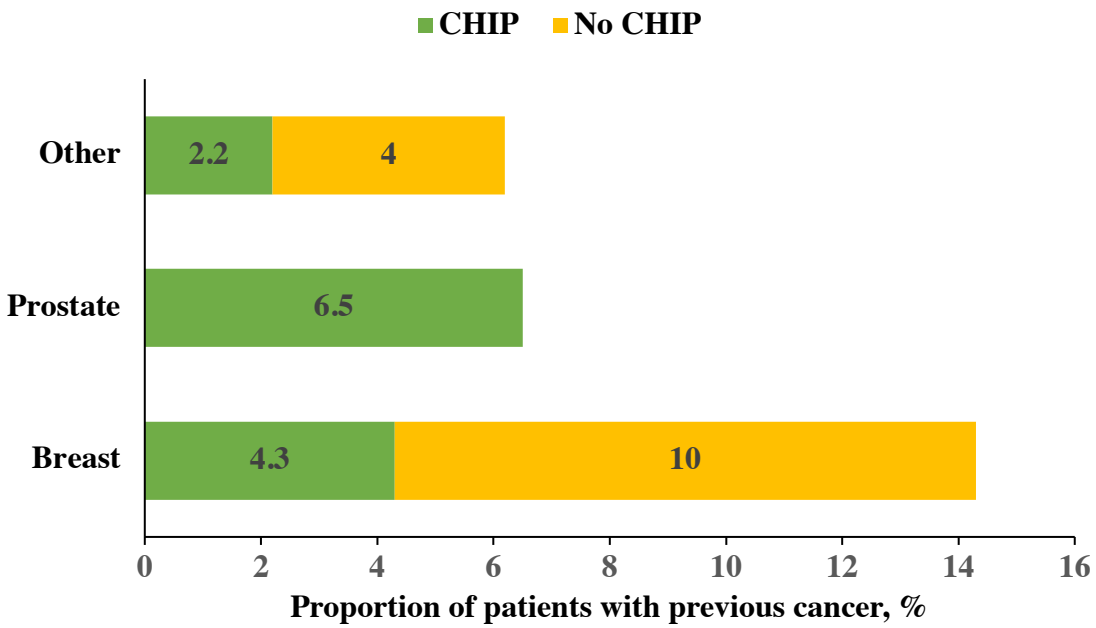


Figure 3-9: Type of previous cancer according to CHIP status in all patients with HF



3.5 Discussion

In this study, I observed:

- CHIP is common in patients with both types of HF, with the prevalence being slightly higher in patients with HFpEF when compared to HFrEF.
- While the prevalence of CHIP rises with age overall, the association between CHIP and HFrEF appeared to be independent of increasing age.
- The presence of CHIP was associated with higher circulating levels of inflammatory biomarkers in particular IL-1β, IL-18 and TGF-β2 in all patients.
- The presence of CHIP was not associated with a difference in standard clinical haematological parameters or their differentials.
- In patients with HFpEF, the presence of CHIP was associated with higher levels of IL-1β and its associated downstream markers.
- In patients with HFpEF and HFrEF, the presence of CHIP was surprisingly not associated with higher levels of IL-6.

3.5.1 Recruitment and baseline characteristics

The eligibility criteria for this study were designed to recruit a "real world" cohort of patients admitted to hospital with decompensated HF. I assessed the prevalence of CHIP in patients with both HFrEF and HFpEF. The exclusion criteria ensured that patients with pre-existing or undiagnosed myeloproliferative disorder and patients with specific causes for HF (e.g., severe valvular heart disease) were not recruited. To accurately assess the role of inflammation and CHIP in patients with decompensated HF, patients with ongoing infection or chronic immunomodulating therapy were excluded. Prior to undertaking this study, it was unclear what impact the COVID-19 pandemic would have on recruitment. Of 260 patients screened, only five (2%) were excluded due to COVID-19 infection or concerns regarding COVID-19 contacts. The remaining patients were excluded for a variety of reasons, but most commonly abnormal baseline haematological parameters, in particular low haemoglobin (9%), and active infection (5%). This study provided evidence that recruitment to studies was still possible during a pandemic, which was pivotal at the time as all other research had been suspended over concerns of the impact of COVID-19. It also provided proof that recruitment for a larger study would be feasible, if subsequent funding was available.

I believe that the cohort of patients recruited to this study has several qualities. Firstly, I screened near consecutive patients admitted to hospital with decompensated HF, therefore limiting selection bias. In view of reducing the spread of COVID-19, I only recruited patients from coronary care units and cardiology wards. This ensured that the patients recruited were solely admitted due to decompensated HF and not due to other common comorbidities such as chronic liver disease or renal impairment. Baseline demographics and comorbidities were in line with those observed in other HF studies. Additionally, the high rate of uptake and success of recruitment to this study also highlight the feasibility of this study. Although, this study largely represents a real-world population, one notable exception is regarding the ethnicity of enrolled patients. All patients apart from one were White, and although ethnic minority groups make up a small proportion of the Scottish population, they are under-represented in this study. This may reflect lower socioeconomic status, cultural and language barriers.

3.5.2 HF characteristics

The patients recruited to this study, were a mixture of patients with de novo HF and acute on chronic HF. It is possible that the patients with de novo HF were more inflamed than those with acute on chronic HF, as it is likely that HF therapies reduce levels of inflammation although sample size is too small to compare this theory. The majority of patients recruited were NYHA class III/IV and were symptomatic of decompensated HF, emphasizing that the correct patients were recruited. It is important to note that at the time of recruitment to this study, there were no licensed therapies for the treatment of HFpEF, and therefore very few patients with HFpEF were receiving SGLT2 inhibitors and no patients were receiving finerenone. Otherwise, most patients received treatment a loop diuretic indicating that our cohort of patients had decompensated HF. Surprisingly, treatment with sacubitril/valsartan was low in the HFrEF group a potential explanation for this may be that the COVID-19 pandemic impacted on optimisation of HF therapies. It would have been interesting to examine whether levels of inflammation were lower in patients who decompensated on optimal medical therapy when compared to those with de novo HF admission.

3.5.3 Prevalence of CHIP

The initial diagnostic criteria for CHIP was determined based on the sequencing methods available at the time, which were only able to accurately detect VAF of ≥2%. However, with the introduction of next generation sequencing, low level VAFs can now be detected accurately. Therefore, I decided to adopt a VAF ≥1% cut off, as studies have shown that low level VAFs are also associated with worse outcomes for CV disease (80,169).

This study demonstrates that CHIP is common in patients who are hospitalised with HF. CHIP driver mutations with a VAF≥2% were observed more commonly in patients with HFpEF (17%) when compared to HFrEF (10%). However, CHIP driver mutations with a VAF≥1% were more commonly observed in patients with HFrEF (52%) when compared to HFpEF (44%).

One of the aims of this study was to determine if CHIP is more common in patients with HFpEF. I hypothesized that CHIP would be more common in patients with HFpEF for several reasons. Indeed, patients with HFpEF tend to be older than patients with HFrEF, and HFpEF is thought to be more associated with inflammation and vascular stiffness. While I observed that CHIP driver mutations with a VAF≥2% were more common in patients with HFpEF, this was not the case at lower VAF cut offs in this study, although the robustness of this finding is potentially limited by sample size issues. This observation that CHIP (VAF≥2%) appears more common in HFpEF requires further confirmation in a larger cohort of HF patients.

3.5.4 CHIP mutations

In keeping with previous studies, I observed that DNMT3A was a common mutation found in both HFpEF and HFrEF and missense mutations were the most common type of mutation (70,74,169). However, unlike previous studies I only found three patients with TET2 mutations (2 HFpEF patients and 1 HFrEF patient) (70,74,169). A potential explanation could be that this cohort of patients had a low prevalence of vascular disease (MI and stroke) when compared to previous studies (19% versus 30%) (169). This raises the possibility that different HF phenotypes may be associated with different mutations, and that TET2 is more commonly associated with vascular disease including CAD.

The largest VAF size observed in this cohort was in the WT1 mutation, followed by the TET2 mutation. In this cohort, it was common for patients to have multiple mutations. There is no consensus on how best to deal with multiple mutations and the clinical significance of cumulative VAF is relatively unknown. VAF increases over time and studies to date have shown that increased mutation burden and clonal heterogeneity correlate with worse survival outcomes in patients with haematological malignancy (242–244). In patients with HF, both larger VAF (2-5% versus >5%) clones and cumulative VAF were both significantly associated with increased risk of HF related death and HF hospitalisation (170). In my study, I did not study VAF changes over time, but cumulative (total) VAF incorporating all CHIP-associated driver mutations was analysed and presented in Figure 3-3 for both HFpEF and HFrEF. Median and cumulative VAF was higher overall in patients with HFpEF and CHIP, when compared to HFrEF patients with CHIP. Whether this is explained by the process of ageing on the HSC is unknown, but it raises the possibility that CHIP may be more important overall in HFpEF, especially if mutation clone size is related to elevated levels of inflammation. Furthermore, it is currently unknown whether CV outcomes are more closely associated with specific individual mutations or with the presence of a combination of different CHIP-driver mutations.

3.5.5 Clinical characteristics of patient with CHIP

In line with previous research, I observed that patients with CHIP were older than those without (70,73) I observed that current smokers were less likely to have CHIP and this may be explained by the effect of statistical chance in my relatively small cohort of patients. However, analysis of UK BB revealed that smoking was not associated with the presence of CHIP, but that certain CHIP mutations may be more associated with smoking than others (e.g. ASXL1) (245). Overall, the comorbidities were similar in those with and without CHIP. Previous studies have raised the possibility that HTN and higher BMI are more common in patients with CHIP (73), although I did not observe this. Importantly, ischaemia was not more common in those with CHIP than those without in both HF groups.

A previous study showed that the presence of CHIP was associated with increased risk of HF hospitalisations (170). In this cohort of patients, patients with CHIP were not more likely to have had a previous HF hospitalisation. However, a substantial proportion of the patients recruited to this study had de novo HF decompensation.

LBBB is associated with poor HF outcomes in patients with HF and is associated with a higher risk of sudden cardiac death (246,247). Patients with CHIP had a longer QRS complex duration than patients without CHIP in both types of HF. This increased QRS duration was more marked in patients with HFrEF with CHIP when compared to the HFpEF cohort. While it is currently unknown the mechanisms by which CHIP would result in this prolonged QRS, previous examination of a murine model of TET2 has implicated altered calcium channel signalling as an arrhythmogenic mechanism (248). Therefore, there is a potential that patients with CHIP may be more predisposed to cardiac arrhythmias than those without, but this remains to be robustly assessed in large, dedicated studies. It is also possible that only certain CHIP mutations result in increased arrhythmic risk and this needs further investigation.

3.5.6 Biomarker analysis

NT-proBNP is an important biomarker associated with adverse HF outcomes (249). In my cohort, while CHIP status was associated with numerically higher levels of NT-proBNP in both types of HF this was not observed in univariable logistic regression analysis. It is currently unclear whether the addition of CHIP status to a robust HF model such as the MAGGIC HF risk stratification tool would improve its predictive ability.

CHIP has been associated with atherosclerosis, through synergy with high levels of LDL cholesterol in the general population (250). In my cohort, the presence of CHIP was associated with elevated levels of LDL cholesterol, but overall, these patients had a low frequency of vascular disease (previous MI and stroke).

3.5.7 Inflammation and CHIP

As described previously, a core feature of CHIP is the establishment of a pro-inflammatory state. Previous murine and human studies have demonstrated high circulating levels of pro-inflammatory marker (96,137). One of the aims of this study was to demonstrate whether the presence of CHIP was associated with inflammation. As expected in this HF cohort the presence of CHIP was not associated with any difference in routine clinical haematological

markers of inflammation, including WBC, RLC and NLR, therefore suggesting that more specific inflammatory pathways are important.

In line with other studies, I observed numerically elevated levels of pro-inflammatory cytokines involved in NLRP3 inflammasome activation according to CHIP status. In particular, higher levels of IL-1β, CRP and IL-18 were observed in patients with CHIP. HFpEF and HFrEF represent different pathophysiological processes and overall circulating levels of inflammatory markers appeared to be higher in the HFpEF cohort when compared to the HFrEF cohort. In both types of HF, the presence of CHIP was associated with higher circulating levels of IL-18. However, levels of IL-1β levels were not elevated in the HFrEF cohort. Markers of vascular stiffness including ICAM-1 and VCAM-1 were also numerically higher in patients with CHIP in both types of HF and vascular stiffness is a strong predictor of CV events (251). HFpEF appears to be more associated with vascular stiffness than HFrEF and in line with this I observed that markers of vascular stiffness were numerically higher in the HFpEF cohort. Due to the sample size of the cohort I was unable to identify whether specific CHIP mutations were associated with specific elevations in circulating inflammatory markers.

Surprisingly, I did not observe that levels of IL-6 were associated with CHIP status. One possible explanation is that this was a chance result in view of the small numbers in this cohort. However, another potential explanation is that when the initial IL-6 MSD assay was run there was an error and it had to be repeated. Whether the extra freezing and thawing impacted this result is unknown. Furthermore, the MSD platform for measuring IL-6 was relatively new at the time of this study which may have also contributed to this result. A possible option for future, would be to re-run the IL-6 assay using the standard well-validated ELISA.

As expected, CHIP was associated with ageing when assessed overall. However, there was not a robust association between ageing and CHIP in the HFrEF cohort. This may indicate that, in contrast to patients with HFpEF, the relative mechanistic (rather than age-associated) role of CHIP may be greater in patients with HFrEF. Interestingly, CHIP status had no association with levels of NT-proBNP. This therefore suggests that the main potential pathophysiological mechanism of CHIP in HF is via inflammation, acting independently of conventional neurohormonal pathways. Furthermore, this also raises the possibility that the use of anti-inflammatory drugs in patients with HF could provide effects that are additive to contemporary

treatments which primarily target neurohormonal activation rather than having direct anti-inflammatory properties. The presence of CHIP in patients with HFpEF was strongly associated with elevated levels of IL-1β. This raises the potential that IL-1β inhibition may be beneficial for patients with HFpEF and potentially indicating that CHIP status may allow personalisation of HF therapies in both HFrEF and HFpEF.

3.6 Strengths and Limitations

The main strength of this study was that it is the first prospective study examining the prevalence of CHIP in patients with both types of HF. Previous studies have mostly been retrospective analyses and have not examined HFrEF and HFpEF simultaneously. It also provides novel insights into the role of CHIP in inflammation in patients with decompensated HF as inflammatory markers were analysed when patients were most unwell. Another strength of this study was demonstrating that safe recruitment was possible during a worldwide pandemic.

The main limitation of this study is that the number of patients recruited was relatively small. These small numbers mean there is a potential to be unable to identify important results. Also, these small numbers mean there is a potential to miss important statistical results.

3.7 Conclusion

Overall, the findings indicated that CHIP is common in patients with HF and is associated with elevated levels of inflammatory biomarkers, but further research is required to further understand these relationships. The presence of CHIP does not appear to be associated with standard clinical haematological parameters of inflammation, but more specific inflammatory pathways which may represent therapeutic targets for the treatment of HF. Whether or not the presence of CHIP could be used to personalise therapies for patients with HF warrants further investigation. I observed that patients with HF often displayed multiple CHIP mutations and whether this impacts on prognosis or levels of inflammation needs future analysis in larger cohorts of patients.

The majority of research regarding CHIP to date has been in older patients, and my finding that ageing was not associated with CHIP status in HFrEF warrants further investigation into CHIP in younger patients with CV disease, in particular HFrEF.

3.8 Next steps

- To examine whether levels of circulating IL-6 are elevated and associated with worse CV outcomes in patients with decompensated HF, providing further evidence that personalised anti-inflammatory therapy may be beneficial.
- To examine whether stable HF is associated with elevated levels of routine haematological markers of inflammation and worse cardiovascular outcomes.
- To understand whether HF therapies, specifically sacubitril/ valsartan reduced levels of inflammation.

CHAPTER 4: The effects of interleukin-6 in patients recently hospitalised for HFpEF

4.1 Introduction

In this chapter, I will report the clinical and HF characteristics of patients recently hospitalised for HFpEF according to levels of IL-6. I also assess the relationship between levels of IL-6 and all-cause death, CV death and subsequent HF hospitalisation. The content of this chapter has been published previously in Circulation: Heart Failure for which I solely completed all statistical analysis and led the academic writing (252). My co-authors reviewed and approved the work submitted to *Circulation: Heart Failure* (252).

The importance of the IL-6 signalling pathway in CV disease is increasingly recognized. To date, there is limited information addressing the association between circulating levels of IL-6 and prognosis for patients with HFpEF are limited (66,253,254). IL-6 is principally responsible for the synthesis of acute phase proteins in the liver including CRP and fibrinogen. High circulating levels of IL-6 have been associated with the development of HFpEF, greater symptom severity and poorer exercise capacity (41,44,255–257). The addition of IL-6 levels to the MAGGIC risk score (Meta-Analysis in Chronic Heart Failure Risk Score) improved prediction of the composite end point of death or HF related hospitalisation in patients with symptomatic HFpEF defined as a LVEF of ≥45% from the TOPCAT clinical trial (254). A meta-analysis revealed that IL-6 levels are higher in HFpEF patients when compared to patients with HFrEF (258) and examination of IL-6 levels one year post HFpEF decompensation revealed that IL-6 levels remained similar (259). Furthermore, the administration of IL-6 to murine models induced the key characteristics of HFpEF including cardiac fibrosis, hypertrophy and diastolic dysfunction (260,261).

Unlike HFrEF, there are still limited licensed therapies for the treatment of HFpEF. Based on evidence from murine models and the encouraging results of the CANTOS trial IL-6 inhibition may improve the symptoms and outcomes for patients with HFpEF. The CANTOS trial reported that, in patients with a history of prior MI and elevated hs-CRP, treatment with the IL-1β inhibitor, canakinumab, reduced HF hospitalization and HF related mortality, although

examination of these effects specifically upon HFpEF was not addressed (21,67). Currently, ZEUS (Ziltivekimab CV Outcomes Study) is a large large Phase 3 trial examining the effect of IL-6 inhibition upon CV outcomes in patients with CKD and elevated hsCRP (262). This trial incorporates baseline and serial assessment of LVEF and includes HF events as an outcome measure (262).

Given the noteworthy association of HFpEF with micro- and macrovascular disease, ventricular hypertrophy and inflammation, and the current lack of therapeutic options for patients with HFpEF understanding the association with IL-6 and HFpEF is vital. Therefore, I examined the relationship between IL-6 and clinical outcomes in patients recently hospitalised with HFpEF.

4.2 Methods

4.2.1 Data source

Data was taken from a previous study examining Microvolt T-Wave Alternans in patients hospitalised with HF (236). This study enrolled near-consecutive patients admitted with decompensated HF (irrespective of LVEF) at three hospitals in the West of Scotland between the 1st December 2006 and 10th January 2009 (236). HF was defined according to the criteria outlined by the ESC (1). Eligible patients were required to be 18 years of age or older and to have an elevated BNP>100 pg/ml. The main exclusion criteria were primary presentation with MI or concurrent systemic disease likely to result in reduced life expectancy. Attendance for the study visit and measurement of inflammatory biomarkers took place one month after discharge from hospital. For this analysis I only included patients with LVEF>40%. This threshold was selected as it reflects the LVEF inclusion criterion used in recent major clinical trials in HFpEF and is consistent with cut-offs used in prior analyses of inflammatory markers in HFpEF (66,263,264).

Of 1,003 patients originally enrolled, 628 patients (65%) returned for the study visit. Failure to attend was due to death (n=115), deterioration in health (n=73), or withdrawal of consent (n=167). 317 patients had LVEF >40%. IL-6 data was unavailable for 31 patients. These 286 patients with HFpEF and IL-6 data comprise the current study population.

4.2.2 Study Funding and ethics

The study complied with the Declaration of Helsinki and was approved by the Local Ethics Committee. All patients provided written informed consent. All data provided are anonymized to respect the privacy of patients who have participated in line with applicable laws and regulations. This work was funded by The Scottish Executive Chief Scientist Office grant no. CZH/4/439 for the original study to which the patients were recruited.

4.2.3 Measurement of LVEF

LVEF was measured by 2-dimensional echo. Analysis was performed offline, using the biplane method of discs (modified Simpson's rule) by a single operator blinded to patient information.

4.2.4 Measurement of humoral biomarkers

Whole blood was drawn by venepuncture. Samples were processed immediately by centrifugation at 3,000 g for 15 minutes and serum and plasma fractions were aliquoted for storage at -80 °C until assay. Immunoassays were used to measure IL-6, KIM-1, TNF-α (Singulex, Singulex INC, California, USA) and hsCRP (Siemens BN II Nephelometer, Siemens Healthcare Diagnostics GmbH, Marburg, Germany). Plasma BNP was measured using the Abbott Architect assay (Abbott Diagnostics, Maidenhead, United Kingdom (UK)). hsTnI was measured using the Architect assay (Abbott Laboratories, Abbott Park, IL, United States of America (USA)). Galectin-3 was measured by ELISA (BG Medicine, Waltham, Massachusetts, USA). All other biochemical and haematological assays were performed in local National Health Service laboratories in Glasgow, UK, and these assays all performed adequately in the relevant national external quality assurance schemes.

4.2.5 Outcome measurements

Outcomes were captured using routinely collected data. Patients were 'flagged' using the Information Services Division of the Scottish Health Service data on hospital admissions as well as in-hospital and out-of-hospital deaths, held by the General Register Office for Scotland. First HF hospitalisation was defined as any hospitalisation with any of the following ICD codes: I110, I255, I420, I426, I427, I428, I429, I50, I500, I501, I509.

4.2.6 Statistical methods

Patients were divided into tertiles according to IL-6 levels. Baseline characteristics are presented as frequencies and percentages for categorical variables and means with standard deviations or medians with interquartile range (IQR) for continuous variables. A non-

parametric test for trend across groups, an extension of the Wilcoxon rank sum test, was used to examine for variation in baseline characteristics across IL-6 tertiles. All continuous variables were log transformed as appropriate to normalise their distribution. The primary outcome (All-cause mortality), CV death and first HF hospitalisation were analysed for each tertile using Cox regressions. Times to events are displayed using Kaplan Meier curves according to tertile. Models were adjusted for validated clinical risk factors which included age, sex, creatinine, systolic BP, ejection fraction, BMI, diabetes, previous MI, stroke, HF hospitalisation prior to the enrolment episode and BNP at the time of enrolment. IL-6 and other additional relevant biomarkers were examined as continuous variables for all outcomes in both univariable and multivariable Cox regression models. Pearson correlation coefficients were examined to evaluate the correlation between IL-6 and other clinically important variables. A restricted cubic spline of IL-6 was generated and displayed graphically using the xblc command in STATA. A p value of <0.05 was considered statistically significant. All statistical analysis was performed using STATA version 16.0 or later.

4.3 Results

4.3.1 Baseline characteristics according to IL-6 tertiles

Data from 286 participants with HFpEF were analysed. Tertile ranges of IL-6 were as follows: tertile 1 (T1): 0.7-4.16 pg/mL, tertile 2 (T2): 4.20-7.84 pg/mL and tertile 3 (T3): 7.85-236.32 pg/mL. 61.2% of patients had concentrations of IL-6 that were greater than the previously reported 95th centile of normal values (4.45 pg/mL) (265). Table 4-1 summarises the baseline characteristics (at time of admission to hospital) of the patients in each tertile. Patients in tertile 3 (highest IL-6), compared to tertile 1 (lowest IL-6) were less likely to be female (44.2%% versus 64.6%, p=0.005) and had higher serum creatinine (117.7±45.7 μmol/l versus 101.3±36.4 μmol/l, p=0.002). Haemoglobin levels were lower according to IL-6 tertile, although this did not reach statistical significance. CV treatment and the prevalence of the commonly encountered cardiac and non-cardiac comorbidities including stroke, MI, AF/flutter, diabetes and COPD were similar across the tertiles. Recruitment to this study was before the introduction of SGLT-2 inhibitors for the treatment of HF.

Table 4-1 Baseline characteristics according to IL-6 tertiles

	All patients	IL-6			
	n=286	Tertile 1 n=96	Tertile 2 n=95	Tertile 3 n=95	p
IL-6, median (range) pg/ml	5.71 (0.71-236.32)	3.10 (0.71-4.16)	5.73 (4.20-7.84)	13.11 (7.85-236.32)	
Age (years)	72.1±9.5	70.9±10.0	72.3±9.9	73.1±8.6	0.128
Female Sex	153 (53.5)	62 (64.6)	49 (51.6)	42 (44.2)	0.005
Race					
White	282 (98.6)	94 (97.9)	94 (98.9)	94 (98.9)	0.544
South Asian	4 (1.4)	2 (2.1)	1 (1.1)	1 (1.1)	
Physiological Measures					
Systolic BP (mmHg)	142.6±27.3	144.2±26.7	144.7±29.1	138.9±25.9	0.067
Heart rate (bpm)	76.3±14.2	77.1±12.9	75.0±15.0	76.7±14.7	0.810
BMI (kg/m^2)	29.1±6.7	27.8±5.4	30.8±7.3	28.9±6.9	0.386
Laboratory Investigations					
Haemoglobin (gd/L)	12.4±2.0	12.6±1.9	12.5±2.1	12.2±2.1	0.163
WBC ($x10^9/L$)	9.5±3.9	10.1±4.7	9.2±3.4	9.2±3.5	0.288
Creatinine (μmol/l)	113.3±47.4	101.3±36.4	121.1±56.2	117.7±45.7	0.002
Sodium (mmol/l)	137.8±4.4	137.9±4.7	138.5±3.9	137.3±4.6	0.163
Potassium (mmol/l)	4.1±0.6	4.1±0.6	4.0±0.5	4.2±0.6	0.350

Social History					
Current Smoker	49 (17.1)	18 (18.8)	14.0 (14.7)	17.0 (17.9)	0.874
Alcohol Excess	22 (7.7)	4 (4.2)	8 (8.4)	10 (10.5)	0.100
Medical History					
HTN	203 (71.0)	58 (60.4)	75 (78.9)	70 (73.7)	0.043
MI	100 (35.0)	30 (31.2)	40 (42.1)	30 (31.6)	0.958
Stroke	63 (22.0)	23 (24.0)	23 (24.2)	17 (17.9)	0.314
AF/ Flutter	167 (58.4)	55 (57.3)	48 (50.5)	64 (67.4)	0.160
Diabetes	81 (28.3)	22 (22.9)	32 (33.7)	27 (28.4)	0.397
COPD	83 (29.0)	27 (28.1)	26 (27.4)	30 (31.6)	0.601
CV treatments					
Beta Blocker	159 (55.6)	49 (51.0)	52 (54.7)	58 (61.1)	0.165
MRA	12 (4.2)	3 (3.1)	4 (4.2)	5 (5.3)	0.462
ACEi/ARB	147 (51.4)	49 (51.0)	47 (49.5)	51 (53.7)	0.716
Digoxin	50 (17.5)	13 (13.5)	15 (15.8)	10 (10.5)	0.081
Statin	201 (7030)	65 (67.7)	68 (71.6)	68 (71.6)	0.558

Values are mean ± SD, n (%), or median (interquartile range), for continuous measures and number (%) for categorical measurements. IL-6: interleukin-6; bpm: beats per minute: BP: blood pressure; mmHg: millimetre of mercury; BMI: body mass index (kg/m^2); WBC: white blood cell count; kilograms per metre squared; COPD: chronic obstructive pulmonary disease; ACEi: angiotensin-converting enzyme inhibitor; ARB: angiotensin receptor blocker; MRA: mineralocorticoid receptor antagonist.

4.3.2 HF characteristics according to IL-6 tertiles

LVEF did not vary across IL-6 tertiles and neither did the frequency of previous HF hospitalisation (Table 4-2). NYHA class did not vary with the levels of IL-6. Patients in tertile 3 had higher hsTnI concentrations when compared to patients in tertile 1 ($p<0.05$) and although there was a trend towards higher BNP levels, this did not reach statistical significance ($p=0.068$). Markers of inflammation (TNF-α, hsCRP) and fibrosis (galectin-3) were higher in tertile 3 when compared to tertile 1 ($p<0.05$). Markers of kidney injury (KIM-1) were also higher in tertile 3 when compared to tertile 1 ($p<0.001$). Patients in tertile 3 had more peripheral oedema when compared to patients in tertile 1.

Table 4-2 HF characteristics according to IL-6 tertiles

	All patients	IL-6			
	n=286	Tertile 1 n=96	Tertile 2 n=95	Tertile 3 n=95	p
Ejection Fraction (%)	50.3±7.0	50.9±6.8	49.7±7.0	50.2±7.1	0.358
Previous HF Hospitalisations	86 (30.1)	26 (27.1)	26 (27.4)	34 (35.8)	0.191
NYHA Class, n (%)					
II	89 (31.1)	34 (35.4)	28 (29.5)	27 (28.4)	0.421
III	154 (53.8)	47 (49.0)	54 (56.8)	53 (55.8)	
IV	43 (15.0)	15 (15.6)	13 (13.7)	15 (15.8)	
Etiology of HF, n (%)					
Ischaemic	134 (46.9)	42 (43.8)	50 (52.6)	42 (44.2)	0.946
Signs and symptoms, n (%)					
Ankle swelling	190 (66.4)	54 (56.2)	62 (65.3)	74 (77.9)	0.002
Orthopnea	210 (73.4)	74 (77.1)	70 (73.7)	66 (69.5)	0.235
PND	138 (48.3)	46 (47.9)	44 (46.3)	48 (50.5)	0.720
ECG					
LBBB	27 (9.4)	14 (14.6)	4 (4.2)	9 (9.5)	0.226

QRS duration	100.4±23.6	101.2±25.3	97.6±19.3	102.3±25.7	0.454
Biomarkers					
BNP (pg/ml)	528.5 (243.0-1045.0)	446.5 (241.0-934.0)	530.0 (233.0-1025.0)	566.0 (331.0-1209.0)	0.068
hsCRP (mg/l)	4.3 (2.0-9.8)	2.3 (1.1-4.2)	4.1 (2.1-8.2)	11.6 (4.9-26.6)	<0.001
hsTnI (ng/L)	5.4±12.6	5.1±8.7	4.6±2.2	6.6±20.0	0.042
KIM-1 (pg/ml)	322.0 (205.0-530.0)	255.5 (149.5-401.5)	343.0 (243.0-568.0)	355.0 (236.0-694.0)	<0.001
TNF-alpha (pg/ml)	5.6 (4.2-7.1)	4.3 (3.6-5.5)	6.1 (4.6-7.0)	6.8 (5.2-8.5)	<0.001
Galectin-3 (pg/ml)	20.0 (15.6-25.9)	16.9 (14.2-21.1)	19.9 (15.4-27.8)	22.3 (18.4-27.1)	<0.001

Values are mean±SD, n(%), or median (interquartile range), for continuous measures and number (%) for categorical measurements. IL-6: interleukin-6; NYHA: New York Heart Association class; PND: paroxysmal nocturnal dysponea; LBBB: left bundle branch block; BNP: brain natriuretic peptide; hsCRP: high sensitivity C-reactive protein; KIM-1: Kidney injury molecule-1; TNF-alpha: tumour necrosis factor alpha.

4.3.3 Correlation between log IL-6 with clinical variables and biomarkers

Levels of IL-6 correlated positively with circulating levels of hsCRP (r=0.533), TNF-α (r=0.347) and galectin-3 (r=0.315) but were only weakly correlated with age (r=0.134) and KIM-1 (r=0.203). IL-6 did not correlate with BNP (r=0.076) or systolic BP (r=0.287) (Table 4-3).

Table 4-3: Correlation between log IL-6 with clinical variables and biomarkers

	r	p
Age, years	0.121	0.041*
BMI (kg/m^2)	0.011	0.850
Ejection Fraction, %	-0.016	0.786
BNP (pg/ml)	0.076	0.198
Systolic BP (mmHg)	-0.063	0.287
Creatinine (μmol/l)	0.109	0.066
Haemoglobin (g/dL)	-0.111	0.0615
WBC (x10^9/L)	-0.039	0.515
Log (hsCRP)	0.533	<0.001*
Log (TNF-alpha)	0.347	<0.001*
Log (KIM-1)	0.203	<0.001*
Log (Galectin-3)	0.315	<0.001*

IL-6: Interleukin-6; BNP: brain natriuretic peptide; BP: blood pressure; WBC: white blood count; BMI: body mass index; hsCRP: high sensitivity C-reactive protein; KIM-1: kidney injury molecule-1; TNF-alpha: tumour necrosis factor-alpha.

4.3.4 Association between IL-6 and clinical outcomes

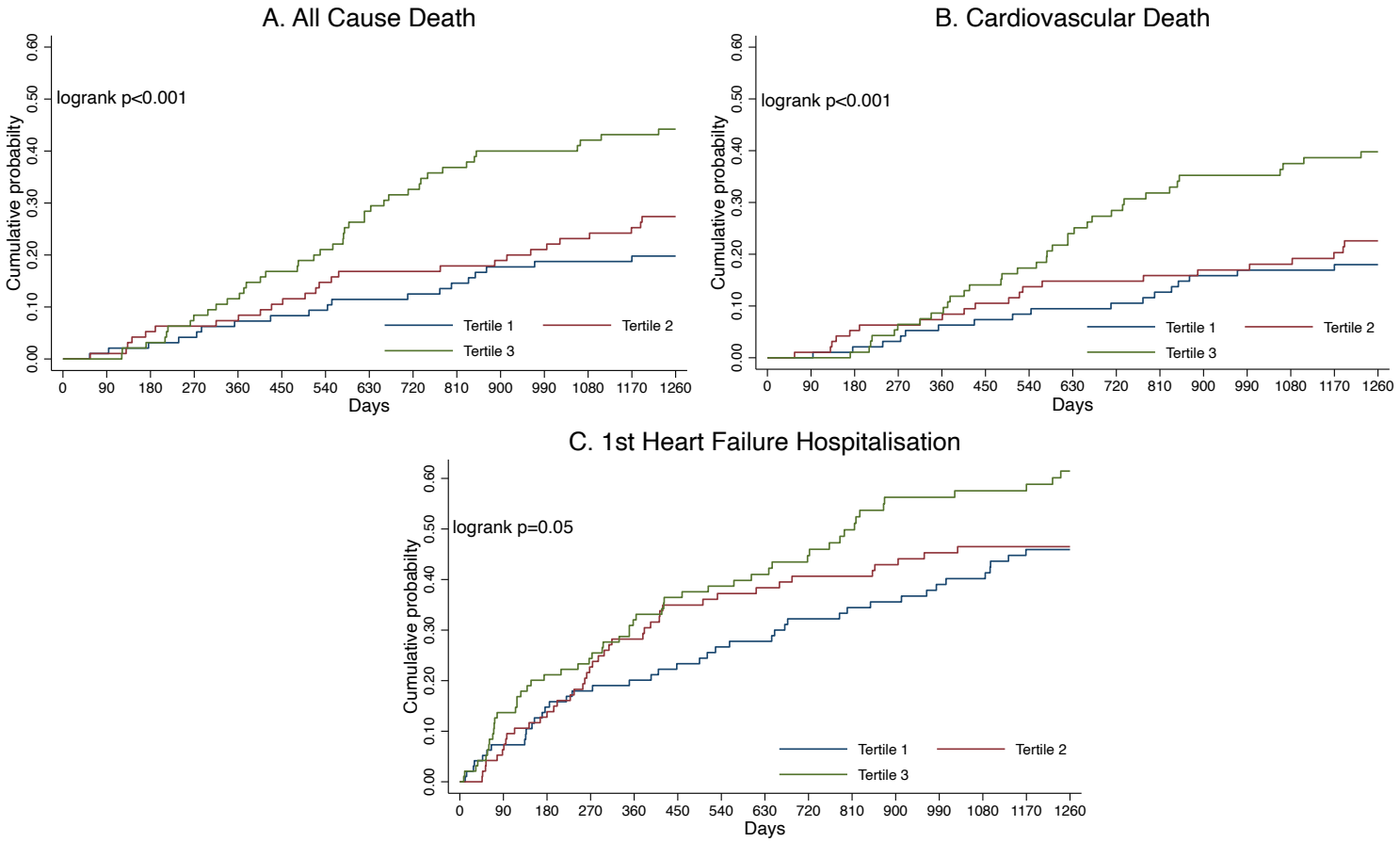
The mean follow-up was 3.2 ± 1.5 years, during which time 110 patients (38.5%) died. All-cause mortality occurred in 51 patients (18.2 per 100 patient-years) in tertile 3 and 27 patients (7.3 per 100 patient-years) in tertile 1 (Table 4-4 and Figure 4-1). The adjusted risk of all-cause mortality and CV death was higher in IL-6 tertile 3 when compared to tertile 1 (adjusted HR for all-cause mortality 2.47 [95% CI 1.49-4.11, p<0.001] and CV death 2.46 [95%CI 1.43-4.22, p<0.001]). Although the rate of first HF hospitalisation was numerically higher for tertile 3 versus tertile 1, there was no increased risk of HF hospitalisations after adjustment. When assessed as a continuous variable and after adjustment, one log unit increase in IL-6 was associated with a higher risk of all-cause mortality (HR 1.46 [1.17-1.81], p=0.001), CV death (HR 1.40 [1.10-1.77], p=0.005) and first HF hospitalisation (HR 1.24 [1.01-1.51], p=0.044), (Table 4-5 and Figure 4-2).

Table 4-4 Outcomes according to IL-6 tertiles

	Tertile 1 n=96	**Tertile 2 n=95**	**Tertile 3 n=95**
All-cause mortality			
Event number	27	32	51
Event rate per 100.pt years	7.33 (5.03-10.69)	9.72 (6.87-13.75)	18.19 (13.82-23.93)
Unadjusted HR	1.00 (ref)	1.36 (0.81-2.27)	**2.56 (1.60-4.10)*** **
Adjusted HR	1.00 (ref)	1.45 (0.83-2.52)	**2.47 (1.49-4.11)*** **
CV death			
Event number	24	27	45
Event rate per 100.pt years	6.52 (4.37-9.72)	8.20 (5.62-11.96)	16.05 (11.98-21.49)
Unadjusted HR	1.00 (ref)	1.30 (0.75-2.26)	**2.57 (1.56-4.24)*** **
Adjusted HR	1.00 (ref)	1.34 (0.74-2.42)	**2.46 (1.43-4.22)*** **
1st HFH			
Event number	49	43	58
Event rate per 100.pt years	18.35 (13.87-24.28)	18.34 (13.60-24.73)	29.22 (22.59-37.79)
Unadjusted HR	1.00 (ref)	0.97 (0.65-1.47)	**1.48 (1.01-2.17)***
Adjusted HR	1.00 (ref)	0.99 (0.62-1.56)	1.42 (0.94-2.14)

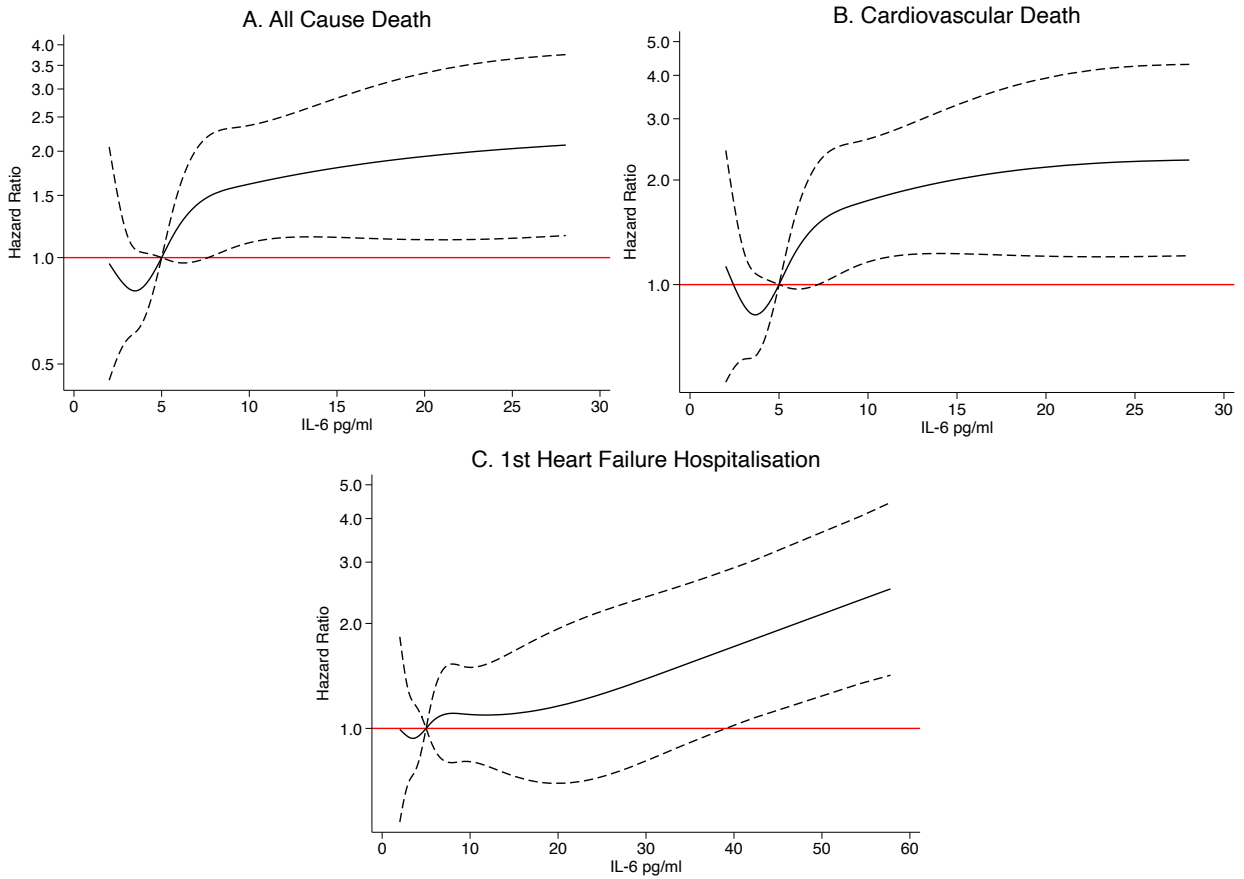
HR: hazard ratio; IL-6: interleukin-6; pt: patient; CV: cardiovascular death; HFH: heart failure hospitalisations. *Adjusted for age, sex, systolic BP, creatinine, body mass index, NYHA class, diabetes, MI, stroke, prior heart failure hospitalisation and log(BNP)* ***p<0.05 **p <0.01 ***p<0.001***

Figure 4-1 Clinical outcomes for patients with HFpEF according to IL-6 tertiles



A, Cumulative probability for all-cause death. **B,** Cumulative probability for cardiovascular death. **C.** Cumulative probability for 1st HF hospitalisation.

Figure 4-2 Association between IL-6 levels and risk of all-cause death, CV death and first HF hospitalisation (restricted cubic spline analysis)



Model adjusted for age, female sex, systolic BP, creatinine, body mass index, NYHA class, diabetes, MI, stroke, prior HF hospitalisation and log(BNP).

4.3.5 Association between other biomarkers and clinical outcomes

When modelled as a continuous variable, one log unit increase of TNF-α, KIM-1 and galectin-3 was associated with higher risk of all-cause mortality, CV death or 1st HF hospitalisation in univariable analysis (Table 4-5). After adjustment, high-sensitivity hsCRP and KIM-1 remained associated with a higher risk of all-cause mortality. hsCRP was associated with a higher risk of CV death after adjustment but this association was not seen for TNF-α, KIM-1 or galectin 3 after adjustment. Unlike IL-6, one log unit increase of hsCRP was not associated with first HF hospitalisation after adjustment. TNF-α was the only other biomarker associated with risk for first HF hospitalisation.

Table 4-5: Univariable and multivariable analysis of outcomes according to other biomarkers

One unit (log) increase	All-cause mortality	CV death	HFH
IL-6			
Univariable	**1.57 (1.28-1.93)*** **	**1.51 (1.21-1.88)*** **	**1.24 (1.03-1.50)***
Multivariate	**1.46 (1.17-1.81)*** **	**1.40 (1.10-1.77)***	**1.24 (1.01-1.51)***
hsCRP			
Univariable	**1.32 (1.12-1.55)*** **	**1.32 (1.11-1.56)****	1.14 (0.99-1.30)
Multivariate	**1.35 (1.15-1.59)*** **	**1.35 (1.13-1.61)*** **	1.14 (0.99-1.30)
TNF-alpha			
Univariable	**1.61 (1.14-2.28)****	**1.49 (1.00-2.22)***	**1.52 (1.11-2.09)****
Multivariate	1.11 (0.73-1.70)	1.12 (0.70-1.78)	**1.52 (1.04-2.22)***
KIM-1			
Univariable	**1.50 (1.20-1.88)*** **	**1.41 (1.09-1.82)****	**1.31 (1.06-1.61)***
Multivariate	**1.36 (1.02-1.82)***	1.25 (0.91-1.70)	1.17 (0.91-1.51)
Galectin-3			
Univariable	**1.99 (1.27-3.11)*** **	**1.72 (1.03-2.88)***	**1.81 (1.24-2.64)****
Multivariate	1.49 (0.86-2.58)	1.31 (0.72-2.38)	1.35 (0.83-2.18)

IL-6, interleukin-6; hsCRP; high sensitivity CRP; KIM-1. Kidney injury molecule-1; TNF-alpha, tumour necrosis factor alpha.

Adjusted for age, sex, systolic BP, creatinine, body mass index, NYHA class, diabetes, MI, stroke, prior HF hospitalisation and log(BNP)

p<0.05 **p <0.01 ***<0.001

4.4 Discussion

In this study, I observed:

- Levels of IL-6 were high in patients with recently decompensated HFpEF.
- Patients with higher levels of IL-6 were at an increased risk of all-cause mortality, CV death and 1st HF hospitalisation.
- IL-6 remained an independent predictor of events even after adjustment for established independent clinical risk factors including BNP.
- The association between IL-6 and clinical outcomes appears to be stronger than these associations with CRP, the downstream product of IL-6.

This is the first study to demonstrate the prognostic significance of circulating levels of IL-6 following hospital admission because of HFpEF. By virtue of the enrolment of near-consecutive patients admitted to hospital with HF the patients included in this analysis represent a typical cross-section of people admitted to hospital with HFpEF. Indeed, in keeping with a common HFpEF patient profile, the majority were elderly, over half were female and the prevalence of both HTN and AF was high. Echo measurements were performed by a single operator, therefore ensuring consistency and reliability in the acquisition of measurement of LVEF in these patients and reducing the risk of bias.

In this high-risk population, 61.2% had a concentration of IL-6 that was greater than the previously reported 95th centile of the normal range (4.45 pg/mL) and hsCRP was ≥2 mg/dL in 77%, reflecting substantial inflammatory activity persisting one month post hospital discharge (21,265). Even after adjustment for clinically important prognostic variables, including BNP, patients in the highest tertile of IL-6 concentration had over two-fold increased risk for all-cause mortality and were at 2.8 times higher risk for CV death during follow-up. Although the association between IL-6 tertile and first HF hospitalisation was not apparent after adjustment, when these outcomes were assessed in relation to IL-6 as a continuous variable, the association between higher IL-6 and HF hospitalisation remained, even after adjustment. Indeed, each one-unit log increase in IL-6 was associated with a 24% increased risk of first HF hospitalisation. IL-6 is therefore a marker of adverse outcomes in patients recently hospitalised with HFpEF.

IL-6 is an important pleotropic cytokine which regulates the release of CRP and other acute phase proteins. Therefore, it is not surprising that concentrations of IL-6 were correlated with its downstream product, hsCRP. However, in contrast to IL-6, hsCRP was not associated with risk for HF hospitalisation which may be due to hsCRP levels being influenced by other inflammatory pathways independent of the IL-6 pathway. Furthermore, point estimates suggest that the increase in risk for all-cause and CV mortality was relatively greater for each log unit increase in IL-6 than it was for an equivalent log unit increase in hsCRP. IL-6 may, therefore, be of particular relevance in the stratification of patients for potential enrolment in trials of anti-inflammatory therapies, of particular relevance in the context of anti-IL-6 drug development. While circulating concentrations of IL-6 also correlated with TNFα, after multivariable adjustment, TNFα was associated with first HF hospitalisation but not associated with all-cause or CV mortality. This finding may be of note in the context of previous neutral results from trials investigating anti-TNFα drugs, including infliximab and etanercept, in the treatment of HF (39,40).

Patients with the highest IL-6 concentrations had more frequent evidence of peripheral oedema. Those patients may also have intestinal oedema and increased permeability for gut endotoxins to enter the systemic circulation and evoke pro-inflammatory effects (266). Although levels of IL-6 were higher in those with higher serum creatinine, they were not associated with an increased prevalence of the common cardiac and non-cardiac comorbidities such as diabetes and stroke. Notably, higher levels of IL-6 were also not associated with prior MI. While IL-6 has been clearly associated with atherogenesis, in patients with HFpEF, IL-6 may exert relatively more important pathophysiological effects in the promotion of endothelial dysfunction, coronary microvascular disease and increased arterial stiffness (267,268). These processes are all relevant to the development and progression of HFpEF and the inflammatory hypothesis underlying the initiation and progression of HFpEF is increasingly well established (32). Although higher IL-6 levels were also associated with galectin-3, a biomarker related to the fibrotic effects of inflammation, in keeping with prior reports this marker was not associated with clinical outcomes after adjustment (269–271). In the cohort I examined, KIM-1 was associated with higher risk of all-cause death after adjustment. KIM-1 is released from the proximal tubule of the kidney in response metalloproteinase activity and has previously been associated with rehospitalisation for HF after adjustment in an acute HFrEF cohort. However, it has not previously been explored in relation to outcomes for patients with HFpEF (272–275).

The clinical significance of IL-6 in HF (HFrEF and HFpEF combined) was previously examined in the BIOlogy Study to TAilored Treatment in Chronic Heart Failure (BIOSTAT-CHF) cohort (66). This multi-centre observational study included patients from in-patient and out-patients settings, but only 10% of the cohort had HFpEF (66). Although poorer clinical outcomes were found in patients with IL-6 above the 95th centile, outcomes were reported for the cohort as a whole and associations were not specifically examined in patients with HFpEF in isolation (66). However, patients with HFpEF were 1.6 times more likely to have IL-6 concentrations above 95th centile than patients with HFrEF. In samples obtained from 379 participants with HFpEF in the Treatment of Preserved Cardiac Function Heart Failure with an Aldosterone Antagonist (TOPCAT) trial, a machine learning approach was used to examine multi-biomarker clusters to predict outcomes for patients with HFpEF (254). In this analysis, IL-6 was predictive of a composite of death and HF related hospital admission (254). Unlike my analysis of data from recently hospitalised patients, those in TOPCAT were recruited from both in-patient and out-patient settings and were less symptomatic than patients in our study (38% of patients had class III/IV symptoms at enrolment in comparison to 69% of our patients). The number of events in this TOPCAT cohort was accordingly low, with only 94 events observed in 379 subjects over 2.9 years, in comparison to the 260 events (all-cause death and HF hospitalisation) observed in our cohort (254). The biomarker assessment in the TOPCAT recruits a multiplex analysis of 49 biomarkers, and the authors acknowledge that whilst providing informative data, this method has assay-specific limits of detection that may not be equivalent to those of established quantitative assays (254).

4.5 Clinical Application

In this study, higher IL-6 levels were associated with adverse outcomes even after adjustment for BNP. Furthermore, IL-6 and BNP were not significantly correlated. These findings suggest the potential for additive, independent beneficial effects of anti-inflammatory therapies in the treatment of HF. This strategy would be complementary to current therapies with an emerging evidence basis for the treatment of HFpEF, including SGLT2 inhibiton and potentially angiotensin-neprilysin inhibition, whose primary therapeutic effects are not anti-inflammatory. Given the promising signal toward reduction in HF events seen in CANTOS and, particularly now with the large scale clinical assessment of IL-6 inhibitors upon CV events, including HF across the spectrum of ejection fractions, better understanding of the association between IL-6

and CV outcomes has never been more relevant (21,67). While elevated hsCRP has been used for trial entry, there may be a growing argument to use IL-6 thresholds (276). Personalisation of therapy on the basis of elevated IL-6 as evidence of residual inflammatory risk, particularly in the high-risk post hospitalisation period, remains an intriguing concept for exploration.

4.6 Strengths and limitations

The main strength of this study is that it is the largest study to date to examine the effects of IL-6 in patients with recently decompensated HFpEF. Another strength of this study is the fact patients were enrolled on near consecutive days and echo was performed by a single trained operator. The study also observed a large number of events which were reported using the standard accepted international classifications of diseases.

However, there are limitations. This analysis was retrospective and had not been prespecified. Only a single baseline measurement of IL-6 was available, and this was measured approximately one month post hospitalisation for HFpEF. However, while the trajectory of IL-6 and other biomarker concentrations between hospital discharge and follow-up may be interesting, the post-discharge assessments utilised here may be less susceptible to acute fluctuations in the setting of multiple concomitant conditions during and after hospitalisation. To date, only one study has evaluated changes in IL-6 levels over time in patients with HFpEF in which it was observed that patients with HFpEF did not demonstrate any statistically significant change in levels of IL-6 despite clinical stabilisation (259). By enrolling patients at the time of HF hospitalisation the patients included in my analysis were more symptomatic and at higher risk of adverse outcomes than the general HFpEF population. However, I believe that these higher risk patients are worthy of focused attention. Furthermore, recruitment of hospitalised patients allows greater confidence in the veracity of the HFpEF diagnosis which can be challenging, and particularly so in ambulatory patients whose condition may be relatively more confounded by non-cardiac comorbidities such as obesity, chronic lung disease and physical deconditioning. Almost all the patients in this study were white and the potentially important effect of race upon outcomes and biomarkers has not been addressed here.

4.7 Conclusions

Overall, the findings demonstrate that higher IL-6 levels are associated with an increased risk of all-cause death, CV death and 1st HF hospitalisation. Importantly, IL-6 remained an independent predictor of these events even after adjustment for established independent clinical factors including BNP. Therefore, this highlights that circulating IL-6 is not only important in the development of HFpEF (41), but also plays a pivotal role in clinical outcome. Further research is required to demonstrate whether IL-6 levels can be used for the personalisation of HF therapy.

4.8 Next steps

- To understand the importance of circulating levels of IL-6 in patients with recently decompensated HFrEF.
- To understand whether routine haematological parameters RLC and NLR are associated with worse CV outcomes in patients with stable HF.
- To understand whether treatment with sacubitril/valsartan reduces inflammation.

CHAPTER 5 The effects of IL-6 in patients recently hospitalised for HFrEF

5.1 Introduction

In this chapter, I will report the clinical and HF characteristics of patients recently hospitalised for HFrEF according to levels of IL-6. I will also assess the relationship between levels of IL-6 and all-cause death, CV death and 1st HF hospitalisation.

HFrEF affects around 1-3% of the general population and the incidence rises with age (277). Unlike HFpEF, there are several evidence-based therapies for the treatment of HFrEF. Despite this, morbidity and mortality remain high alongside healthcare costs (277). Chronic systemic inflammation has long been associated with the development and progression of HFrEF, with poorer functional status and worse clinical outcomes. However, anti-inflammatory therapy is not a licensed treatment for HFrEF as the initial large clinical trials targeting TNF-α were unsuccessful and provided evidence that larger doses were detrimental (39,40). Recently, however, treatment with an SGLT2 inhibitor significantly reducted levels of IL-6 and improved QoL in inpatients with HFrEF and diabetes mellitus (278). Furthermore, higher levels of IL-6 were associated with a higher risk of CV death or time to first HF hospitalisation independent of levels of NTproBNP in patients with HFrEF from the VICTORIA trial (Vericiguat Global Study in Subjects with Heart Failure with reduced ejection fraction) (279). Whether therapies directly targeting the IL-6 signalling pathway will improve outcomes and QoL of patients with HFrEF is currently unknown.

IL-6 is an important inflammatory mediator for CV disease including HF and CAD. However, the significance of IL-6 in CV disease has yet to be fully recognised. IL-6 levels have been shown to increase with age and a single nucleotide polymorphism of the IL-6 receptor has been shown to be protective against the development of CAD independent of lipid concentration (280,281). Levels of IL-6 are high in patients with HFrEF when compared to controls, however, there are conflicting reports when levels of IL-6 are directly compared in patients with HFpEF versus HFrEF (66,258,259,282,283). High levels have been associated with decreased function capacity, reduced LVEF and increased risk of subsequent hospitalisation for HF (284–287).

However, unlike HFpEF in the recent MESA (Multi-Ethnic Study of Atherosclerosis) study doubling of IL-6 levels was not associated with the development of HFrEF (45). Another meta-analysis revealed that a year following decompensation IL-6 levels significantly reduced with stability of HFrEF (259).

Understanding the role of IL-6 in patients with HFrEF is of pivotal importance and levels may be even more important in different patient groups for example following MI. Levels of IL-6 may also indicate patients who would receive the most benefit from anti-inflammatory therapy.

5.2 Methods

The methods used here replicate those in Chapter 4 and data has been taken from the previous study examining Microvolt T-Wave Alternans in patients hospitalised with HF (236). The details of which have been outlined above in Chapter 2 and Chapter 4.

5.2.1 Measurement of LVEF

LVEF was measured by 2-dimensional echo. Analysis was performed offline, using the biplane method of discs (modified Simpson's rule) by a single operator blinded to patient information. Of 1,003 patients originally enrolled, 628 patients (65%) returned for the study visit. Failure to attend was due to death (n=115), deterioration in health (n=73), or withdrawal of consent (n=167). Withdrawl of consent was likely driven by patients having to complete exercise testing on their follow-up study visit. IL-6 data was unavailable for 11 patients. These 301 patients with HFrEF and IL-6 data comprise the current study population.

5.2.3 Statistical analysis

Patients were divided into tertiles according to IL-6 levels. Baseline characteristics are presented as frequencies and percentages for categorical variables and means with standard deviations or medians with interquartile range (IQR) for continuous variables. A non-parametric test for trend across groups, an extension of the Wilcoxon rank sum test, was used to examine for variation in baseline characteristics across IL-6 tertiles. All continuous variables were log transformed as appropriate to normalise their distribution. The primary outcome (All-cause mortality), CV death and first HF hospitalisation were analysed for each tertile using Cox regressions. Times to events are displayed using Kaplan Meier curves according to tertile. Models were adjusted for validated clinical risk factors which included age, sex, creatinine, systolic BP, ejection fraction, creatinine, BMI, diabetes, previous MI, stroke, HF hospitalisation prior to the enrolment episode and BNP at the time of enrolment. IL-6 and other additional relevant biomarkers were examined as continuous variables for all outcomes in both univariable and multivariable Cox regression models. Pearson correlation coefficients were examined to evaluate the correlation between IL-6 and other clinically important variables. A restricted cubic spline of IL-6 was generated and displayed graphically using the xblc command

in STATA. A p value of <0.05 was considered statistically significant. All statistical analysis was performed using STATA version 16.0 or later.

5.3 Results

5.3.1 Baseline characteristics according to IL-6 tertile

Data from 301 participants with HFrEF were analysed. Tertile ranges of IL-6 were as follows: tertile 1 (T1): 0.47-4.73pg/mL, tertile 2 (T2): 4.77-10.13pg/mL and tertile 3 (T3): 10.22-660.0pg/mL. 70.1% of patients had concentrations of IL-6 that were greater than the previously reported 95th centile of normal values (4.45pg/mL) (265). Table 5-1 summaries the baseline characteristics of the patients in each tertile. Patients in tertile 3 (highest IL-6), compared to tertile 1 (lowest IL-6) were older (72.6 years versus 66.2 years, p=0.001), had a lower haemoglobin (12.0gd/L±2.0 versus 13.1±1.9gd/L, p<0.001) and a higher serum creatinine (132.8±63.2µmol/l versus 108.9±40.6µmol/l, p<0.001). Patients in tertile 3 when compared to tertile 1 had a higher prevalence of HTN (62% versus 45%, p= 0.012), diabetes (44% versus 15.8%, p=0.049) and COPD (30% versus 17.8%, p=0.049). CV treatment were similar across the tertiles. Recruitment to this study was before the introduction of SGLT-2 inhibitors for the treatment of HFrEF.

Table 5-1 Baseline characteristics according to IL-6 tertile

	All patients	**IL-6**			
	n=301	**Tertile 1 n= 101**	**Tertile 2 n= 100**	**Tertile 3 n= 100**	**p**
IL-6, median (range) pg/ml	6.90 (0.47-660.0)	3.43 (0.47-4.73)	6.93 (4.77-10.13)	16.65 (10.22-660.0)	
Age (years)	69.7±11.7	66.2±13.8	70.3±10.7	72.6±9.1	0.001
Female Sex	101 (33.6)	38 (37.6)	36 (36.0)	27 (27.0)	0.112
Race					
White	294 (97.7)	99 (98.0)	97 (97.0)	98 (98.0)	0.991
South Asian	5 (1.7)	2 (2.0)	1 (1.0)	2 (2.0)	
Black	2 (0.7)	0 (0.0)	2 (2.0)	0 (0.0)	
Physiological Measures					
Systolic BP (mmHg)	131.7±25.7	129.3±23,8	131.9±28.1	134.0±25.1	0.231
Heart rate (bpm)	77.6±16.4	73.5±14.7	81.2±17.3	78.2±16.3	0.044
BMI (kg/m^2)	27.7±6.2	27.2±5.4	27.9±6.6	27.9±6.6	0.705
Laboratory Investigations					

Haemoglobin (gd/L)	12.6±1.9	13.1±1.9	12.6±1.9	12.0±2.0	<0.001
Creatinine (µmol/l)	120.3±51.0	108.9±40.6	119.3±44.0	132.8±63.2	<0.001
Sodium (mmol/l)	138.6±4.2	138.6±3.9	138.8±3.8	138.3±4.7	0.875
Potassium (mmol/l)	4.2±0.5	4.2±0.5	4.1±0.6	4.2±0.6	0.037
Social History					
Current Smoker	83.0 (27.6)	23 (22.8)	32 (32.0)	28 (28.0)	0.406
Alcohol Excess	31 (10.3)	17 (16.8)	8 (8.0)	6 (6.0)	0.012
Medical History					
HTN	174 (57.8)	45 (44.6)	67 (67.0)	62 (62.0)	0.012
MI	153 (50.8)	44 (43.6)	57 (57.0)	52 (52.0)	0.231
Stroke	71 (23.6)	16 (15.8)	26.0 (26.0)	29 (29.0)	0.028
AF/ Flutter	144 (47.8)	43 (42.6)	46 (46.0)	55 (55.0)	0.079
Diabetes	94 (31.2)	16 (15.8)	34 (34.0)	44 (44.0)	<0.001
COPD	78 (25.9)	18 (17.8)	30 (30.0)	30 (30.0)	0.049
CV treatments					
Beta Blocker	143 (47.5)	43 (42.6)	48 (48.0)	52 (52.0)	0.182
MRA	23 (7.6)	9 (8.9)	10 (10.0)	4 (4.0)	0.192
ACEi/ARB	159 (52.8)	46 (45.5)	57 (57.0)	56 (56.0)	0.138
Digoxin	44 (14.6)	11 (10.9)	14.0 (14.0)	19.0 (19.0)	0.104

Statin	203 (67.4)	59 (58.4)	75.0 (75.0)	69.0 (69.0)	0.109

Values are mean ± SD, n (%), or median (interquartile range), for continuous measures and number (%) for categorical measurements. IL-6: interleukin-6; bpm: beats per minute: BP: blood pressure; mmHg: millimetre of mercury; BMI: body mass index; kg/m^2: kilograms per metre squared; COPD: chronic obstructive pulmonary disease; ACEi: angiotensin-converting enzyme inhibitor; ARB: angiotensin receptor blocker; MRA: mineralocorticoid receptor antagonist.

5.3.2 HF characteristics according to IL-6 tertiles

LVEF did not vary across IL-6 tertiles (Table 5-2). There was a trend towards a higher frequency of previous HF hospitalisations and higher levels of BNP in patients in tertile 3 when compared to patients in tertile 1, although this did not reach statistical significance (p=0.068 and p=0.097, respectfully). Patients in tertile 3 were more likely to be NYHA class IV than those in tertile 1 (24% versus 11.9%, p=0.017) and were more likely to have peripheral oedema (72% versus 46.5%, p<0.001). Markers of inflammation (TNF-α, hsCRP) and fibrosis (galectin-3, KIM-1) were higher in tertile 3 when compared to tertile 1 (p<0.001).

Table 5-2 HF characteristics according to IL-6 tertiles

	All patients	IL-6			
	n=301	Tertile 1 n= 101	Tertile 2 n= 100	Tertile 3 n= 100	p
Ejection Fraction (%)	30.6±7.1	30.9±7.1	30.0±7.7	31.0±6.3	0.829
Previous HF Hospitalisations	105 (34.9)	30.0 (29.7)	33.0 (33.0)	42.0 (42.0)	0.068
NYHA Class, n (%)					
II	55 (18.3)	24.0 (23.8)	16.0 (16.0)	15.0 (15.0)	0.017
III	184 (61.1)	65.0 (64.4)	58.0 (58.0)	61.0 (61.0)	
IV	62.0 (20.6)	12.0 (11.9)	26.0 (26.0)	24.0 (24.0)	
Etiology of HF, n (%)					
Ischaemic	183 (60.8)	49.0 (48.5)	69.0 (69.0)	65.0 (65.0)	0.017
Signs and symptoms, n (%)					
Ankle swelling	182 (60.5)	47.0 (46.5)	63.0 (63.0)	72.0 (72.0)	<0.001
Orthopnea	236 (78..4)	77 (76.2)	85.0 (85.0)	74.0 (74.0)	0.705
PND	141.0 (46.8)	44 (43.6)	50.0 (50.0)	47.0 (47.0)	0.625

ECG					
LBBB	85.0 (28.2)	34.0 (33.7)	23.0 (23.0)	28.0 (28.0)	0.371
QRS duration	118.4±28.1	117.9±26.5	120.3±30.8	117.2±26.9	0.802
Biomarkers					
BNP (pg/ml)	1170.0 (575.0-2322.0)	993.0 (420.0-2012.0)	1234.5 (694.0-2380.0)	1186.5 (666.0-2511.5)	0.097
hsCRP (mg/l)	4.9 (1.9-10.2)	2.0 (1.0-3.6)	4.8 (2.5-8.0)	10.5 (6.7-22.1)	<0.001
hsTnI (µg/l)	6.3±10.9	5.8±7.6	7.5±16.8	5.7±4.4	0.092
KIM-1 (pg/ml)	421.0 (259.0-629.5)	320.0 (198.0-501.0)	215.0 (155.0-386.0)	346.5 (220.0-585.0)	<0.001
TNF-alpha (pg/ml)	6.9 (4.8-8.8)	5.3 (4.2-7.5)	4.6 (3.6-5.7)	5.7 (4.5-6.9)	<0.001
Galectin-3 (pg/ml)	22.5 (15.7-31.9)	19.0 (15.1-25.1)	17.2 (13.6=20.0)	19.4 (15.8-25.4)	<0.001

Values are mean±SD, n(%), or median (interquartile range), for continuous measures and number (%) for categorical measurements. IL-6: interleukin-6; NYHA: New York Heart Association class; PND: paroxysmal nocturnal dysponea; LBBB: left bundle branch block; BNP: brain natriuretic peptide; hsCRP: high sensitivity C-reactive protein; KIM-1: Kidney injury molecule-1; TNF-alpha: tumour necrosis factor alpha.

5.3.3 Correlation between log IL-6 with clinical variables and biomarkers

Levels of IL-6 correlated positively with circulating levels of hsCRP (r=0.574) and TNF-α (r=0.371) but were only weakly correlated with age (r=0.251), KIM-1 (r=0.263) (Table 5-3), galectin-3 (r=0.232) and creatinine (r=0.140). IL-6 levels did not correlate with BNP or LVEF.

Table 5-3: Correlation between log IL-6 with clinical variables and biomarkers

	r	p
Age, years	0.251	<0.001
Ejection Fraction, %	0.073	0.198
BNP (pg/ml)	0.105	0.069
Systolic BP (mmHg)	0.035	0.541
Creatinine (μmol/l)	0.140	0.015
Haemoglobin (g/dL)	0.182	0.002
Log (hsCRP)	0.574	<0.001
Log (TNF-alpha)	0.371	<0.001
Log (KIM-1)	0.263	<0.001
Log (Galectin-3)	0.232	<0.001

IL-6: Interleukin-6; BNP: brain natriuretic peptide; hsCRP: high sensitivity CRP; KIM-1: kidney injury molecule-1; TNF-alpha: tumour necrosis factor-alpha.

5.3.4 Association between IL-6 and clinical outcomes

The mean follow up was 3.2 ± 1.5 years, during which time 159 patients (52.8%) died. All-cause mortality occurred in 63 patients (24.5 per 100 patient-years) in tertile 3 and 38 patients (10.7 per 100 patient-years) in tertile 1 (Table 5-4 and Figure 5-1). The adjusted risk of all-cause mortality and CV death was higher in IL-6 tertile 3 when compared to tertile 1 (1.91 [95% CI 1.23-2.97, p<0.01] and (1.99 [95%CI 1.23-3.19, p<0.01], respectively). There was no increased risk of 1st HF hospitalisations in tertile 3 when compared to tertile 1. When assessed as a continuous variable and after adjustment, one log unit increase in IL-6 was associated with a higher risk of all-cause mortality (HR 1.40 [1.18-1.67], p<0.001) and CV death (HR 1.46 [1.22-1.75]), but not HF hospitalisation (Table 5-5 & Figure 5-2).

Table 5-4 Outcomes according to IL-6 tertiles

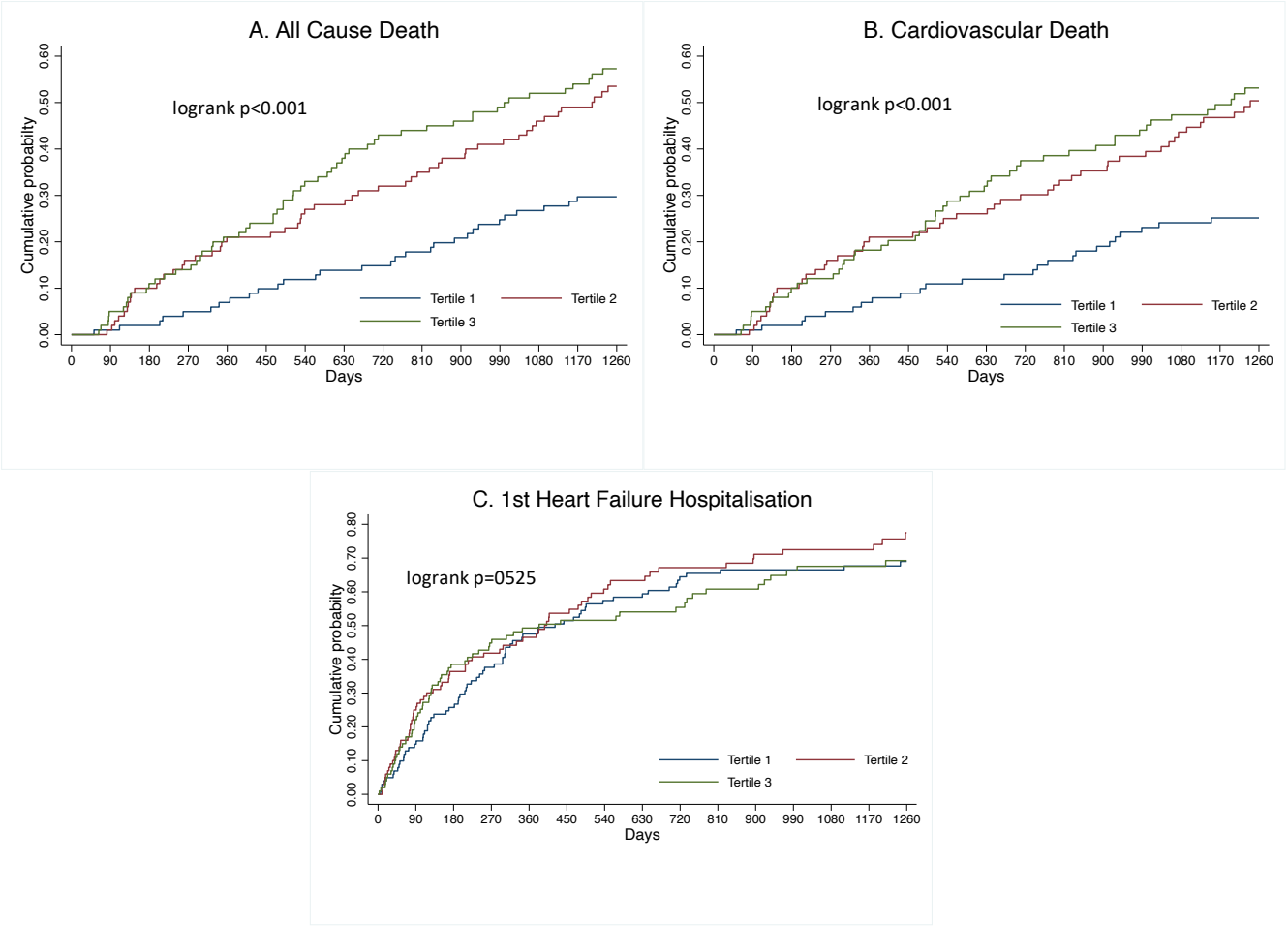
	Tertile 1 n= 101	**Tertile 2 n= 100**	**Tertile 3 n=100**
All-cause mortality			
Event number	38	58	63
Event rate per 100.pt years	10.71 (7.79-14.71)	20.60 (15.93-26.65)	24.47 (19.11-31.32)
Unadjusted HR	1.00 (ref)	**1.93 (1.28-2.90)****	**2.28 (1.52-3.41)*****
Adjusted HR	1.00 (ref)	**1.74 (1.14-2.67)***	**1.91 (1.23-2.97)****
CV death			
Event number	33	52	54
Event rate per 100.pt years	9.30 (6.61-13.08)	18.47 (14.08-24.24)	20.97 (16.06-27.38)
Unadjusted HR	1.00 (ref)	**1.98 (1.28-3.06)****	**2.23 (1.44-3.44)*****
Adjusted HR	1.00 (ref)	**1.83 (1.16-2.89)****	**1.99 (1.23-3.19)****
1st HFH			
Event number	72	70	65
Event rate per 100.pt years	39.11 (31.05-49.28)	52.29 (41.37-66.09)	42.37 (33.22-54.03)
Unadjusted HR	1.00 (ref)	1.20 (0.86-1.67)	1.03 (0.74-1.45)
Adjusted HR	1.00 (ref)	1.19 (0.83-1.68)	1.04 (0.72-1.50)

HR: hazard ratio; IL-6: interleukin-6; pt: patient; CV: cardiovascular death; HFH: heart failure hospitalisations.

Adjusted for age, sex, systolic BP, creatinine, body mass index, NYHA class, diabetes, MI, stroke, prior heart failure hospitalisation and log(BNP)

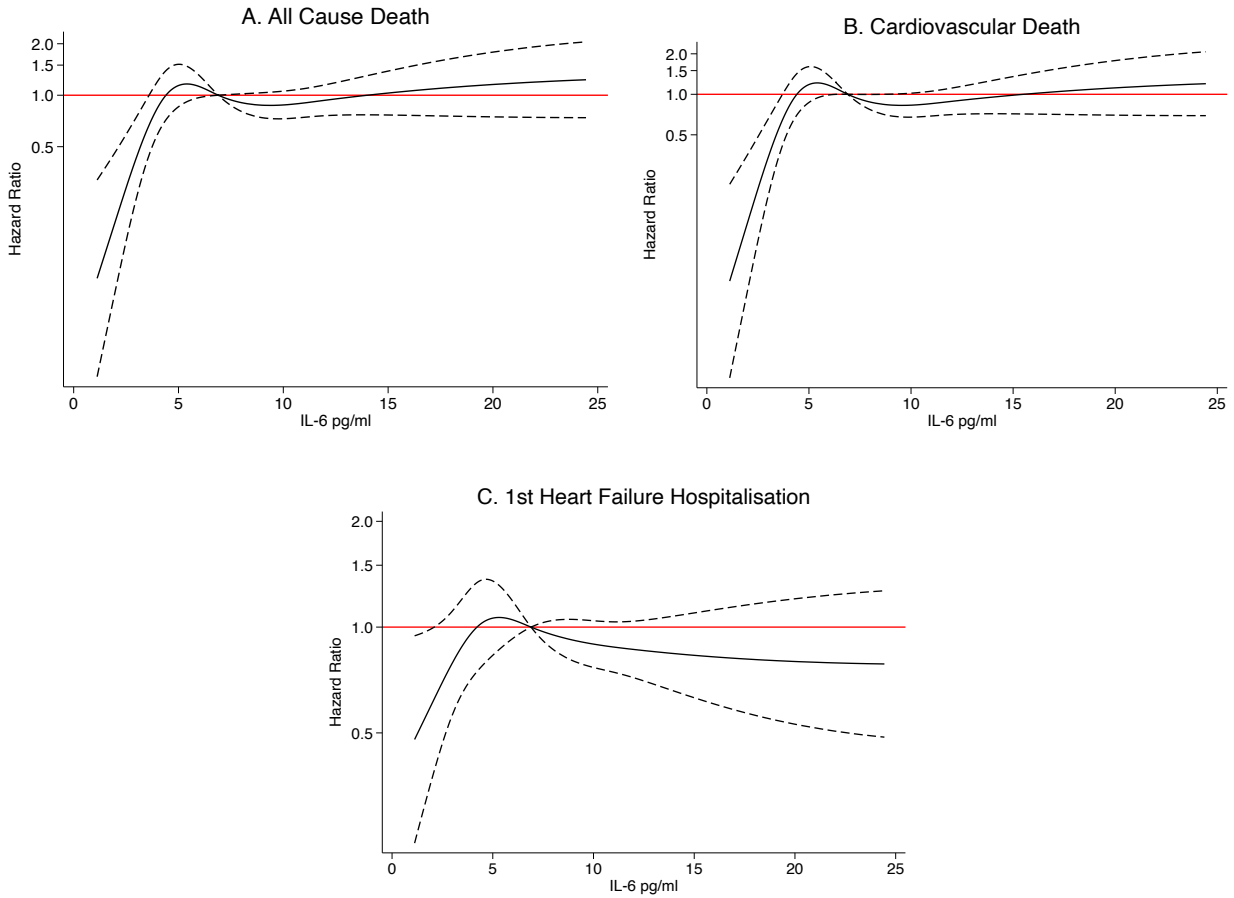
p<0.05 **p <0.01 ***p<0.001

Figure 5-1 Clinical outcomes for patients with HFrEF according to IL-6 tertiles



A, Cumulative probability for all-cause death. **B,** Cumulative probability for cardiovascular death. **C.** Cumulative probability for 1st heart failure hospitalisation

Figure 5-2 Association between IL-6 levels and the risk of all-cause death, CV death and HF hospitalisation (restricted cubic spline analysis). *Model adjusted for age, female sex, systolic BP, creatinine, body mass index, NYHA class, diabetes, MI, stroke, prior HF hospitalisation and log(BNP)*



5.3.5 Association between other biomarkers and clinical outcomes

When modelled as a continuous variable, one log unit increase of TNF-α, KIM-1 and galectin-3 was associated with a higher risk of all-cause mortality and CV death (Table 5-5). After adjustment, only hsCRP and TNF-α remained associated with a higher risk of all-cause mortality and CV death. None of the other biomarkers were associated with risk for first HF hospitalisation.

Table 5-5 Univariable and multivariable analysis of outcomes according to other biomarkers

One unit (log) increase	All-cause mortality	CV death	HFH
IL-6			
Univariable	**1.39 (1.21-1.59)*** **	**1.40 (1.21-1.62)*** **	1.04 (0.91-1.20)
Multivariate	**1.40 (1.18-1.67)*** **	**1.46 (1.22-1.75)*** **	1.05 (0.91-1.22)
hsCRP			
Univariable	**1.22 (1.08-1.39)*** **	**1.20 (1.05-1.36)*** **	1.06 (0.95-1.19)
Multivariate	**1.28 (1.12-1.46)*** **	**1.26 (1.10-1.45)*** **	1.08 (0.96-1.21)
TNF-alpha			
Univariable	**1.74 (1.21-2.30)*** **	**1.79 (1.34-2.40)*** **	0.97 (0.72-1.31)
Multivariate	**1.49 (1.07-2.08)***	**1.59 (1.13-2.25)** **	1.02 (0.72-1.45)
KIM-1			
Univariable	**1.42 (1.18-1.70)*** **	**1.39 (1.14-1.69)*** **	1.06 (0.89-1.28)
Multivariate	**1.27 (1.01-1.61)***	1.25 (0.97-1.61)	1.08 (0.87-1.33)
Galectin-3			
Univariable	**1.73 (1.23-1.45)** **	**1.76 (1.22-2.55)** **	1.25 (0.91-1.72)
Multivariate	1.35 (0.88-2.07)	1.39 (0.89-2.19)	1.31 (0.91-1.90)

IL-6, interleukin-6; hsCRP; high sensitivity CRP; KIM-1. Kidney injury molecule-1; TNF-alpha, tumour necrosis factor alpha. *Adjusted for age, sex, systolic BP, creatinine, body mass index, NYHA class, diabetes, MI, stroke, prior HF hospitalisation and log(BNP)* ***p<0.05 **p <0.01 ***<0.001***

5.4 Discussion

In this study, I observed:

- Levels of IL-6 were high in patients with recently decompensated HFrEF.
- Patients with higher levels of IL-6 were at an increased risk of all-cause mortality and CV death.
- IL-6 remained an independent predictor of all-cause mortality and CV death even after adjustment for established clinical risk factors including BNP.
- Levels of IL-6 were not associated with an increased risk of 1st HF hospitalisation.
- The association between IL-6 and all-cause mortality and CV death appears to be numerically stronger than these associations with CRP, the downstream product of IL-6.

This is the first largest retrospective study to examine clinical characteristics and outcomes in patients according to levels of IL-6 in patients with recently decompensated HFrEF in patients recently hospitalised with decompensated HFrEF, who are not enrolled in a clinical drug trial. Unlike the VICTORIA clinical trial, I believe that this retrospective study represents a more "real-life" population of patients with HFrEF. Firstly, in VICTORIA, HFrEF was defined as an LVEF of less than 45% which is not the accepted definition of HFrEF in either AHA or ESC guidelines. Secondly, the study exclusion criteria were not as strict as in VICTORIA (288). Indeed, in keeping with a common HFrEF profile in the cohort analysed here, patients were elderly, the majority were male and the prevalence of previous MI was high.

In this "real-life" population, 70.1% of patients had a concentration of IL-6 that was greater than the previously reported 95th centile of the normal range (4.45pg/mL), reflecting substantial inflammatory activity persisting one month post hospital discharge (265). In this study, patients with the highest IL-6 concentration were older, had a lower haemoglobin, higher serum creatinine and were more likely to have diabetes. These findings are in keeping with the recent BIOSTAT-CHF cohort which examined the clinical significance of IL-6 in HF (HFrEF and HFpEF combined) (66). However, approximately one third of those recruited in BIOSTAT were not hospitalised and patients with HFpEF were also included (66). In comparison to BIOSTAT and the VICTORIA trial, the upper limit of IL-6 in this cohort was substantially higher (660pg/mL versus 30.4pg/mL and 11.2pg/ml, respectively) (66,279). In keeping with

these elevated levels of IL-6, I also observed a substantially higher hsCRP, the downstream marker of IL-6, when compared to VICTORIA (upper limit 10.2 versus 9.5mg/L, respectively). This may be partially explained by the fact that in BIOSTAT and VICTORIA a multiplex analysis was used which may not be directly equivalent to those of established quantitative assays. However, it may also reflect the fact that stable patients may be expected to have less active inflammation. Indeed, 85% of patients in the cohort I examined were NYHA class III/IV at enrolment compared to 38.8% in BIOSTAT and 40% in VICTORIA (66,279). I also observed a much higher risk of all-cause mortality (40% versus 22% risk) and CV death (46% versus 16%) after adjustment for each one unit log increase of IL-6 when compared to BIOSTAT (66). Furthermore, in comparison to VICTORIA, I observed a much higher risk of CV death (46% versus 12%) after adjustment for each one-unit log increase of IL-6. Similar to findings from VICTORIA, one unit log increase in IL-6 was not associated with an increased risk of HF hospitalisation which I previously observed in patients with decompensated HFpEF cohort (252), this may be potentially due the lack of evidence based therapies for HFpEF.

Anaemia has long been associated with poor HF outcomes and the reduction in haemoglobin with rising levels of IL-6 suggest that IL-6 may be an important biological pathway leading to anaemia via its effects on the acute phase protein hepcidin (289). Patients with higher IL-6 levels in this cohort also had more evidence of peripheral oedema, and gut oedema may evoke further pro-inflammatory effects through increased gastrointestinal permeability (266). Both, hs-CRP and TNF-alpha correlated with circulating levels of IL-6 and were also found to be associated with a higher risk of all-cause mortality and CV death after adjustment. This increased risk of CV death with hs-CRP was also observed to a lesser effect in VICTORIA (10% versus 26% in the cohort I examined) (279). IL-6 upstream levels can directly influence circulating levels of TNF-alpha and hsCRP. Therefore it is not surprising that these pro-inflammatory cytokines were also associated with a higher mortality risk which has previously been documented (290–294). However, IL-6 appeared to be associated with the highest risk of mortality. Whether or not direct inhibition of IL-6 will be as beneficial as targeting the upstream IL-1 pathway (which mainly drives IL-6 signalling) is currently unknown.

In this cohort, I found that patients with higher IL-6 levels were more likely to have an ischaemic aetiology. IL-6 levels have previously been found to predict future vascular risk in apparently health populations and have been shown to correlate with endothelial dysfunction and arterial stiffness (267,268,295–297). Preliminary data from a single dose study of

tocilizumab (an IL-6 antagonist) post non-ST-elevation MI showed a significant reduction in levels of CRP and a trend towards reduction in troponin (298). Further research is required to determine whether treatment with tocilizumab reduces the risk of developing HF with reduced ejection fraction following MI (298).

5.5 Clinical application

Despite significant advancements in therapies for patients with HFrEF, this condition is still associated with considerable morbidity and mortality. In this study, I found that the majority of recently hospitalised patients with HFrEF had high circulating levels of IL-6 and high circulating levels of IL-6 are associated with adverse outcomes including all cause death and CV death even after adjustment of BNP. This suggests that anti-inflammatory therapy may complement currently available therapies, and it may be even more beneficial for certain patient groups potentially including those with ischaemic HFrEF and those recently hospitalised for decompensated HF. Further research is required to determine the safety and efficacy of IL-6 inhibition in patients with HFrEF.

5.6 Strengths and Limitations

The main strength of this study is that it is the first study to retrospectively examine the effects of IL-6 in "real-world" patients with recently decompensated HFrEF. Another strength of this study is the fact that patients were enrolled on near consecutive days. The study also observed a large number of events which were reported using the standard accepted international classification of disease.

However, there are limitations. The analysed described were retrospective and had not been prespecified. Only a single baseline measurement of IL-6 was available, and this was measured approximately one month post hospitalisation of HFrEF. By enrolling patients at the time of HF hospitalisation the study included a population that was more symptomatic and at higher risk of adverse outcomes than the general HFrEF population. Almost all the patients in this study were white and the potentially important effect of race upon outcomes and biomarkers has not been addressed here. Lastly, the recruitment of this study took place before the introduction of SGLT2 inhibitors for treatment of HFrEF. SGLT2 inhibitors have been found

to reduce circulating levels of IL-6 and whether or not the effect of IL-6 would have been so apparent if the patients had been on optimal medical therapy is unknown.

5.7 Conclusions

Overall, the findings demonstrate that IL-6 is an independent predictor of all-cause mortality and CV death in patients recently hospitalised with decompensated HFrEF, even after adjustment for established independent clinical factors including BNP. These findings highlight the adverse association between inflammation and outcomes and are particularly important in the context of personalisation of therapy.

5.8 Next steps

- To understand whether routine haematological parameters RLC and NLR are associated with worse CV outcomes in patients with stable HF.
- To understand whether treatment with sacubitril/valsartan reduces inflammation.

CHAPTER 6 Relative lymphocyte count and neutrophil lymphocyte ratio in patients with HF with reduced and preserved ejection fraction: An analysis of the PARADIGM-HF and PARAGON-HF trials

6.1 Introduction

Relative lymphocyte count (RLC) and neutrophil to lymphocyte ratio (NLR) are easily obtainable, routinely measured and cost-effective markers of inflammation. Little is known about the role of NLR and RLC in ambulant patients with HF and even less is known about these markers in patients with HFpEF than it is in HFrEF. Low RLC and high NLR (the inverse) have been associated with worse outcomes in CV and non-CV diseases (299–315).

In this chapter, I examined RLC and NLR in two large clinical trials. Firstly, I examined PARADIGM-HF, which enrolled a large cohort of ambulatory HFrEF patients with predominantly mild symptoms, who were receiving contemporary therapy, including a beta-blocker in >90% of participants (235). Subsequently, I examined PARAGON-HF which enrolled ambulant patients with HFpEF (LVEF ≥45%) (234). As well as describing RLC distribution, and the relationship between RLC and outcomes, in each HF phenotype, I also examined the effect of neprilysin inhibition according to baseline RLC and on RLC after randomization to see whether treatment with sacubitril/valsartan reduced circulating levels of inflammation. I also compared RLC with a related (but inverse) index, the neutrophil to lymphocyte ratio (NLR) the results of which are included in Appendix VI.

RLC is defined as the proportion of total peripheral white blood cells that are lymphocytes and NLR is the neutrophil to lymphocyte ratio. Several studies have identified a high prevalence of low RLC in patients hospitalized because of worsening HF and confirmed the association between low RLC and poor outcomes (7,9). Proposed explanations for the low RLC in patients hospitalized with HF include elevated levels of pro-inflammatory cytokines which may cause lymphocyte apoptosis, possibly related, in part to increased gut permeability as a result of

congestion, splanchnic sequestration and the effects of elevated corticosteroid and catecholamine levels (65,299,316–319).

Only a few reports have described the distribution of RLC in outpatients with HF, most of these have been from small, single-centre, studies, often in patients with severe HF (1,2,5,6). The one exception is a report describing the development and validation of the Seattle Heart Failure Model (SHFM) (320). However, in that study patients were not receiving contemporary treatment for HF and reduced ejection fraction (HFrEF). This is an important consideration given that beta-blockers may protect against adrenergic mediated reduction in RLC (319). Furthermore, in the SHFM, it is not clear whether RLC is an independent predictor of outcomes as this model was not adjusted for natriuretic peptide concentrations, the single most powerful prognostic variable in HF (321).

6.2 Methods

6.2.1 Data source

The results of PARADIGM-HF and PARAGON-HF have previously been published (234,235). Each was a randomized, double-blind, controlled trial which compared sacubitril/valsartan with a renin-angiotensin system blocker alone. Each trial enrolled adults (≥18 years in PARADIGM-HF and ≥50 years in PARAGON-HF) with symptomatic HF (defined as NYHA functional class II to IV). Patients also needed to have functional or structural cardiac disease and elevated natriuretic peptides. In PARADIGM-HF, patients were required to have a LVEF ≤40% and in PARAGON a LVEF ≥45% and left ventricular hypertrophy, left atrial enlargement or both. In PARADIGM-HF, BNP had to be ≥150pg/ml or NT-proBNP≥600pg/ml (or ≥100pg/ml or ≥400pg/ml, respectively, if hospitalized in the previous 12 months). In PARAGON-HF, NT-proBNP had to be >300pg/ml (>900pg/ml if in AF) or if hospitalized for HF within 9 months, NT-proBNP>200pg/ml (>600pg/ml for AF).

In each trial, patients entered an initial run-in period during which they received the comparator (enalapril or valsartan), followed by a second period of treatment with sacubitril/valsartan. Patients tolerating both run-in periods were randomized 1:1 to sacubitril/valsartan (target dose 97/103mg twice daily) and either enalapril (target dose10 mg twice daily) in PARADIGM-HF or valsartan (target dose 160mg twice daily) in PARAGON-HF. Each trial was approved by an Ethic Committee at investigative sites and all patients provided written informed consent. Each was event-driven and the median follow-up in PARADIGM-HF was 27 months (stopped early for efficacy) and in PARAGON-HF it was 35 months (completed follow-up to target number of events).

6.2.2 Leukocyte samples

A complete blood count, including total leukocyte, neutrophil and lymphocyte count, was analysed in a central laboratory at randomization and at twelve months after randomization in both trials. RLC was calculated as the ratio of lymphocytes to the total number of white cells and NLR was calculated as the ratio of neutrophils to lymphocytes.

6.2.3 Outcome analysis

In PARADIGM-HF, the primary endpoint was time to first occurrence of HF hospitalization or CV death whereas in PARAGON-HF the primary outcome was all HF hospitalizations (including first and repeat admissions) or CV death. In this analysis, the primary outcome used in both trials was time to first occurrence of HF hospitalization or CV death. All events in the two trials were adjudicated by the same endpoints committee.

6.2.4 Statistical Analysis

In the two trials, I divided patients into tertiles according to RLC. The primary composite outcome, its components and all-cause mortality were analysed for each tertile using Cox regression. Time-to-event analyses were displayed using Kaplan-Meier curves according to tertile. For each outcome, the effect of sacubitril-valsartan compared with enalapril was examined across each tertile in a Cox regression model. RLC was also modelled as a continuous variable. A restricted cubic spline of RLC was generated and displayed graphically using the xblc command in STATA. A fractional polynomial was constructed of RLC and entered into the model as an interaction term with treatment. The results of the interaction were displayed graphically using the mfpi command in STATA. Models were adjusted for treatment, age, sex, race, region, systolic BP, heart rate, body mass index, serum creatinine, clinical features of HF (ischaemic cardiomyopathy, LVEF, NT-proBNP (log)), NYHA class, HTN, diabetes, AF, hospitalization for HF, MI, stroke, duration of HF. All analyses were conducted using STATA version 16 (College Station, TX: StataCorp LLC). A p value of <0.05 was considered statistically significant.

6.3 Results

6.3.1 Haematological parameters in PARADIGM and PARAGON-HF

In PARADIGM-HF the median (Q1, Q3) white blood cell (leukocyte) count was 6.8 (5.6, 8.1) $x10^9$/L**;** mean (SD) was 6.98±1.97$x10^9$/L**.** Median neutrophil count was 4.18 (3.32, 5.22) $x10^9$/L**;** mean was 4.39±1.56$x10^9$/L**.** Median lymphocyte count was 1.77 (1.39, 2.22)$x10^9$/L; mean was 1.87±0.85$x10^9$/L.

In PARAGON-HF the median white blood cell (leukocyte) count was 6.4 (5.3, 7.6) $x10^9$/L; mean was 6.62±1.88 $x10^9$/L. Median neutrophil count was 4.1 (3.2, 13.4) $x10^9$/L; mean 2.89±1.55 $x10^9$/L. Median lymphocyte count was 1.6 (1.3, 3.6) $x10^9$/L; mean was 1.71±0.69 $x10^9$/L.

6.3.2 Baseline characteristics in PARADIGM-HF according to RLC tertile

RLC was not available for 421 patients at randomisation, leaving 7978 participants for analysis. Median RLC in PARADIGM-HF was 26.6 (21.3, 32.4)%; mean was 27.3±8.6%. The proportion of patients with a reduced RLC (<20%) was 19.7%.

The range of RLC in each tertile was: Tertile 1 30.4-87.3 (median 35.2)%, Tertile 2 23.3-30.3 (median 26.6)% and Tertile 3 1.8-23.2 (median 19.3)%. Table 6-1 summarises the baseline characteristics of the patients in each tertile.

Patients in Tertile 1 (highest RLC), compared to Tertile 3 (lowest RLC), were more likely to be women (27.5% versus 17.3%, P<0.001) and were younger (62.5±11.7years versus 65.3±11.0 years, P<0.001). Participants in Tertile 3 were more likely than those in Tertile 1 to have an ischemic etiology and had a higher serum creatinine (104.2±28.0 μmol/L versus 95.0±23.9 μmol/L, P<0.001) and body mass index (28.4±5.7 kg/m^2 versus 27.8±5.4 kg/m^2, P=0.01). They had also a higher prevalence of diabetes, HTN, AF and previous MI. LVEF did not vary across RLC tertiles. Patients in Tertile 3 had the highest median NT-proBNP, had more evidence of congestion (rales and oedema) and were more commonly prescribed diuretics (Table 6-1).

Table 6-1 Baseline characteristics in PARADIGM-HF according to RLC tertile

	Tertile 1 n=2648	**Tertile 2 n=2641**	**Tertile 3 n=2689**	**p**
Leukocytes, median (range)- x10^9/L	6.3 (1.7-33.5)	6.7 (2.6-15.7)	7.3 (2.0-22.2)	<0.001
Neutrophils, median (range)- x10^9/L	3.31 (0.25-8.57)	4.24 (1.33-10.87)	5.15 (0.55-16.81)	<0.001
Lymphocyte, median (range) x10^9/L	2.25 (0.57-29.2)	1.79 (0.67-4.1)	1.34 (0.12-3.31)	<0.001
RLC, median (range) – %	35.2 (30.4-87.3)	26.6 (23.3-30.3)	19.3 (1.8-23.2)	<0.001
Age – years	62.5±11.7	63.6±11.2	65.3±11.0	<0.001
Female sex – no. (%)	727 (27.5)	562 (21.3)	466 (17.3)	<0.001
Race– no. (%)				<0.001
White	1567 (59.2)	1704 (64.5)	1978 (73.6)	
Black	233 (8.8)	108 (4.1)	72 (2.7)	
Asian	521 (19.7)	530 (20.1)	416 (15.5)	
Other	327 (12.3)	299 (11.3)	223 (8.3)	
Region – no. (%)				0.002
North America	143 (5.4)	137 (5.2)	292 (10.9)	
Latin America	550 (20.8)	447 (16.9)	361 (13.4)	
Western Europe and other	577 (21.8)	639 (24.2)	724 (26.9)	
Central Europe	859 (32.4)	898 (34.0)	906 (33.7)	
Asia-Pacific	519 (19.6)	520 (19.7)	406 (15.1)	

Systolic BP – mmHg	121±15	121±15	122±15	0.001
Heart rate – beats/min	72±12	72±12	73±12	<0.001
Body mass index – kg/m^2	27.8±5.4	28.2±5.4	28.4±5.7	0.002
Serum creatinine – µmol/L	95.0±23.9	98.9±26.3	104.2±28.0	<0.001
Clinical features of HF				
Ischemic etiology – no. (%)	1456 (55.0)	1575 (59.6)	1729 (64.3)	<0.001
LVEF – %	29.4±6.2	29.5±6.2	29.4±6.3	0.93
NT-proBNP– pg/ml	1468 (815-2795)	1562 (889-3059)	1867 (1003-3954)	<0.001
NYHA class				<0.001
I	170 (6.4)	121 (4.6)	85 (3.2)	
II	1892 (71.5)	1906 (72.2)	1851 (69.0)	
III	563 (21.3)	591 (22.4)	731 (27.2)	
IV	20 (0.8)	21 (0.8)	16 (0.6)	
Signs of HF – no. (%)				
Jugular venous distention	241 (9.1)	255 (9.7)	278 (10.4)	0.13
Oedema	486 (18.4)	501 (19.0)	674 (25.1)	<0.001
Third heart sound	235 (8.9)	265 (10.0)	263 (9.8)	0.3
Rales	175 (6.6)	205 (7.8)	253 (9.4)	<0.001
Prior HF hospitalization no. (%)	1640 (61.9)	1638 (62.0)	1729 (64.3)	0.07

Medical history – no. (%)				
HTN	1786 (67.4)	1876 (71.0)	1984 (73.8)	<0.001
Diabetes	788 (29.8)	916 (34.7)	1052 (39.1)	<0.001
AF	916 (34.6)	905 (34.3)	1099 (40.9)	<0.001
MI	1003 (37.9)	1143 (43.3)	1270 (47.2)	<0.001
Stroke	199 (7.5)	232 (8.8)	253 (9.4)	0.014
Pre-trial use of ACE inhibitor	2028 (76.6)	2051 (77.7)	2123 (79.0)	0.04
Pre-trial use of ARB	630 (23.8)	596 (22.6)	574 (21.3)	0.03
Treatments at randomisation – no. (%)				
Diuretic	2055 (77.6)	2122 (80.3)	2220 (82.6)	<0.001
Digitalis	785 (29.6)	800 (30.3)	832 (30.9)	0.33
Beta-blocker	2483 (93.8)	2465 (93.3)	2479 (92.2)	0.02
Mineralocorticoid receptor antagonist	1501 (56.7)	1495 (56.6)	1450 (53.9)	0.04
Insulin-treated diabetes	180 (6.8)	211 (8.0)	298 (11.1)	<0.001
ICD	213 (8.0)	240 (9.1)	335 (12.5)	<0.001
CRT	133 (5.0)	166 (6.3)	244 (9.1)	<0.001

Values reported are either numbers (%), means (±standard deviations) or medians (range), where stated. *RLC- Relative lymphocyte count, NYHA- New York Heart Association , NT-proBNP- N terminal B-type natriuretic peptide, ACE- angiotensin-converting enzyme, ARB- angiotensin receptor blocker, ICD- Implantable Cardiac Defibrillation, ICD- Implantable Cardioverter Defibrillator, CRT- Cardiac Resynchronisation Therapy*

6.3.3 Baseline characteristics in PARAGON-HF according to RLC tertile

RLC was not available for 27 patients at randomisation, leaving 4,795 participants for analysis. Median RLC in PARAGON-HF was 26.0 (21.0, 31.0); mean was 26.45±8.15%. The proportion of patients with a reduced RLC (<20%) was 23.4%.

The range of RLC in each tertile was: Tertile 1 30.0-84.0 (median 34.0)%, Tertile 2 24.0-29.0 (median 26.0)% and Tertile 3 4.0-23.0 (median 19.0)%, Table 6-2 summarises the baseline characteristics of the patients in each tertile.

Patients in Tertile 1 (highest RLC), compared to Tertile 3 (lowest RLC), were more likely to be women (60.3% versus 45.9%, P<0.001) and were younger (72.3±8.6 versus 73.6±8.3 years). Participants in Tertile 3 were more likely than those in Tertile 1 to have a higher serum creatinine (100.9±28.4μmol/L versus 92.4±26.0μmol/L, P<0.001). In comparison to PARADIGM-HF, body mass index did not vary between the tertiles. Patients in Tertile 3 also had a higher prevalence of diabetes and AF. LVEF did not vary across RLC tertiles. Patients in Tertile 3 (lowest RLC) had the highest median NT-proBNP, had more evidence of congestion (oedema) and were more commonly prescribed diuretics.

Table 6-2 Baseline characteristics in PARAGON-HF according to RLC tertile

	Tertile 1 n=1550	**Tertile 2 n=1455**	**Tertile 3 n=1790**	**p**
Leukocytes, median (range)- x10^9/L	5.9 (1.7-21.4)	6.3 (2.5-14.1)	6.9 (2.4-19.7)	<0.001
Neutrophils, median (range)- x10^9/L	3.3 (0.4-9)	4.0 (1.5-9.2)	5.0 (0.2-15.0)	<0.001
Lymphocyte, median (range) x10^9/L	2.0 (0.7-17.9)	1.7 (0.6-3.8)	1.3 (0.4-3.4)	<0.001
RLC, median (range) – (%)	34.0 (30-84)	26.0 (24-29)	19.0 (4-23)	<0.001
Age – years	72.3±8.6	72.2±8.4	73.6±8.3	<0.001
Female sex – no. (%)	935 (60.3)	722 (49.6)	822 (45.9)	<0.001
Race– no. (%)				<0.001
White	1202 (77.5)	1184 (81.4)	1521 (85.0)	
Black	52 (3.4)	31 (2.1)	19 (1.1)	
Asian	221 (14.3)	186 (12.8)	199 (11.1)	
Other	75 (4.8)	54 (3,7)	51 (2.8)	
Region – no. (%)				<0.001
North America	131 (8.5)	130 (8.9)	298 (16.6)	
Latin America	143 (9.2)	118 (8.1)	109 (6.1)	
Western Europe and other	394 (25.4)	415 (28.5)	580 (32.4)	
Central Europe	613 (39.5)	556 (38.2)	546 (30.5)	

Asia-Pacific	269 (17.4)	236 (16.2)	257 (14.4)	
Systolic BP – mmHg	131±15	131±15	130±16	0.07
Heart rate – beats/min	70±12	71±12	71±12	0.03
Body mass index – kg/m^2	30.1±5	30.2±5	30.3±5.1	0.32
Serum creatinine – µmol/L	92.4±26.0	95.2±26.5	100.9±28.4	<0.001
Clinical features of HF				
Ischemic etiolgy – no. (%)	562 (36.3)	510 (35.1)	651 (36.4)	0.91
LVEF – %	57.5 (7.9)	57.6 (8.0)	57.5 (7.7)	0.88
NT-proBNP– pg/ml	761 (422-1448)	902 (466-1572)	1015 (522-1794)	<0.001
NYHA class				0.63
I	38 (2.5)	48 (3.3)	51 (2.8)	
II	1201 (77.5)	1139 (78.3)	1365 (76.3)	
III	305 (19.7)	263 (18.1)	364 (20.3)	
IV	5 (0.3)	4 (0.3)	10 (0.6)	
Signs of HF (%)				
Jugular venous distension	197 (12.8)	168 (11.6)	289 (16.3)	0.003
Oedema	546 (35.3)	538 (37.0)	741 (41.4)	<0.001
Third heart sound	42 (2.7)	25 (1.7)	43 (2.4)	0.62
Rales	114 (7.4)	102 (7.0)	129 (7.2)	0.87
Hospitalisation for HF	712 (45.9)	707 (48.6)	887 (49.6)	0.04

Medical history – no. (%)				
HTN	1477 (95.3)	1394 (95.8)	1712 (95.6)	0.64
Diabetes	574 (37)	608 (41.8)	879 (49.1)	<0.001
AF	439 (28.4)	478 (32.9)	635 (35.6)	<0.001
MI	342 (22.1)	321 (22.1)	420 (23.5)	0.32
Stroke	154 (9.9)	154 (10.6)	200 (11.2)	0.24
Pretrial use of ACE inhibitor	635 (41.0)	635 (43.6)	711 (39.7)	0.42
Pretrial use of ARB	731 (47.2)	678 (46.6)	840 (46.9)	0.90
Treatments at randomisation – no. (%)				
Diuretic	1464 (94.5)	1393 (96.1)	1722 (96.2)	0.02
Digitalis	116 (7.5)	144 (9.9)	190 (10.6)	0.002
Beta-blocker	1237 (79.8)	1175 (80.8)	1408 (78.7)	0.38
Mineralocorticoid receptor antagonist	358 (23.1)	392 (26.9)	489 (27.3)	0.006
Insulin-treated diabetes	161 (10.4)	182 (12.5)	314 (17.5)	<0.001
Implantable cardiac defibrillator	4 (0.3)	6 (0.4)	8 (0.4)	0.003

Values reported are either numbers (%), means (±standard deviations) or medians (range), where stated. *RLC- Relative lymphocyte count, NYHA- New York Heart Association , NT-proBNP- N terminal B-type natriuretic peptide, ACE- angiotensin-converting enzyme, ARB- angiotensin receptor blocker, ICD- Implantable Cardiac Defibrillation, ICD- Implantable Cardiac Defibrillator, CRT- Cardiac Resynchronisation Therapy.*

6.3.4 PARADIGM-HF outcomes according to RLC tertile

The primary outcome occurred in 519 (9.3 per 100 person-years) patients in Tertile 1 (highest RLC) and 781 patients (14.7 per 100 person-years) in Tertile 3 (lowest RLC). The cumulative probability of the composite primary outcome, death from CV causes, hospitalization for HF and death from any cause was lowest for patients in Tertile 1 and highest for patients in Tertile 3 (Figure 6-1). The adjusted risk of all outcomes was significantly higher in Tertile 3 compared with Tertile 1 (Table 6-4) - adjusted HR for the composite primary end point: 1.31 (95%CI 1.16-1.47), hospitalization for HF 1.41 (1.21-1.64), CV death 1.24 (1.07-1.44) and death from any cause 1.36 (1.19-1.55). Examination of RLC as a continuous variable showed that a 10% decrease in RLC was associated with a 17% higher risk of the primary composite end point (adjusted HR 1.42, 95% CI 1.08-1.21) and a higher risk of the other outcomes of interest (Table 6-4 and Figure 6-2- restricted cubic spline analysis).

Table 6-3 PARADIGM-HF outcomes according to tertile of RLC (referent to Tertile 1) and per 10% decrease in RLC

	Tertile 1 n=2648	**Tertile 2 n=2641**	**Tertile 3 n= 2689**	**Per 10% decrease in RLC n=7978**
Primary End Point				
Event number	519	622	781	
Event rate per 100 patient years (95% CI)	9.3 (8.56-10.16)	11.5 (10.64-12.45)	14.7 (13.71-15.78)	
Unadjusted HR	1.00 (ref)	1.24 (1.11-1.40)	1.57 (1.41-1.76)	1.26 (1.19-1.33)
Adjusted HR[a]	1.00 (ref)	1.16 (1.04-1.31)	1.31 (1.16-1.47)	1.17 (1.08-1.27)
Hospitalization for HF				
Event number	286	348	494	
Event rate per 100 patient years (95% CI)	5.14 (4.58-5.77)	6.4 (5.80-7.15)	9.3 (8.52-10.16)	
Unadjusted HR	1.00 (ref)	1.25 (1.07-1.46)	1.72 (1.49-2.00)	1.36 (1.26-1.46
Adjusted HR[a]	1.00 (ref)	1.16 (0.99-1.36)	1.41 (1.21-1.64)	1.21 (1.12-1.31)
Death from CV Causes				

Event number				
Event rate per 100	322	399	466	
patient years (95% CI)	5.5 (4.91-6.11)	6.9 (6.25-7.61)	8.0 (7.27-8.72)	
Unadjusted HR				
Adjusted HR[a]	1.00 (ref)	1.29 (1.11-1.49)	1.53 (1.33-1.77)	1.22 (1.13-1.31)
	1.00 (ref)	1.19 (1.02-1.38)	1.24 (1.07-1.44)	1.09 (1.01-1.17)
Death from Any Cause				
Event number	373	495	597	
Event rate per 100	6.3 (5.73-7.02)	8.56 (7.84-9.35)	10.2 (9.41-11.05)	
patient years (95% CI)				
Unadjusted HR	1.00 (ref)	1.38 (1.20-1.58)	1.67 (1.47-1.91)	1.27 (1.19-1.35)
Adjusted HR[a]	1.00 (ref)	1.27 (1.11-1.46)	1.36 (1.19-1.55)	1.13 (1.06-1.20)

[a]*Adjusted for: region, treatment, age, sex, race, systolic BP, heart rate, body mass index, serum creatinine, clinical features of HF(ischemic etiology, LVEF, NT-proBNP (log)), NYHA class, HTN, diabetes, AF, hospitalization for HF, MI, stroke, duration of HF.*

Figure 6-1 Kaplan-Meier curves for outcomes of interest, according to tertile of RLC in PARADIGM-HF

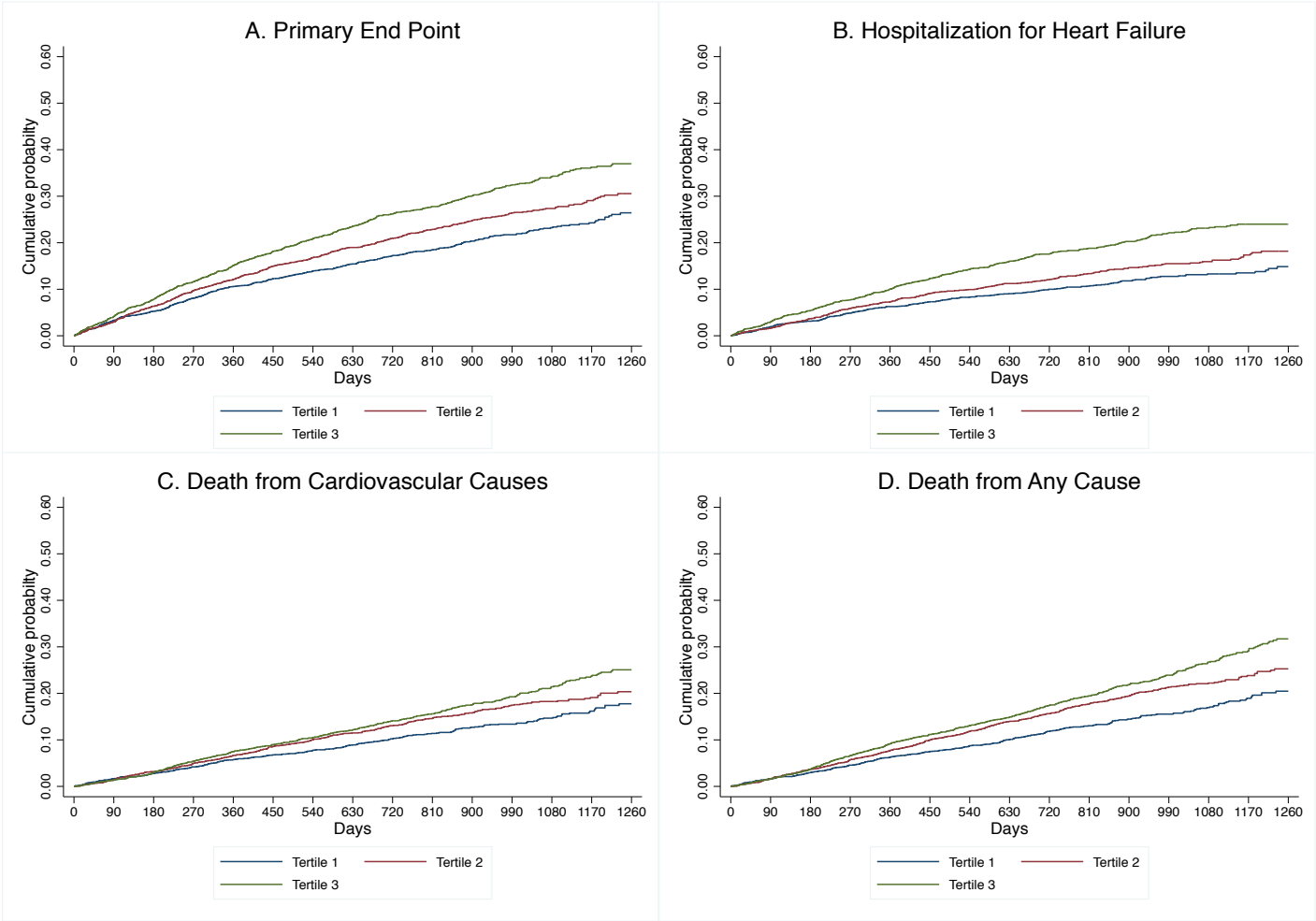
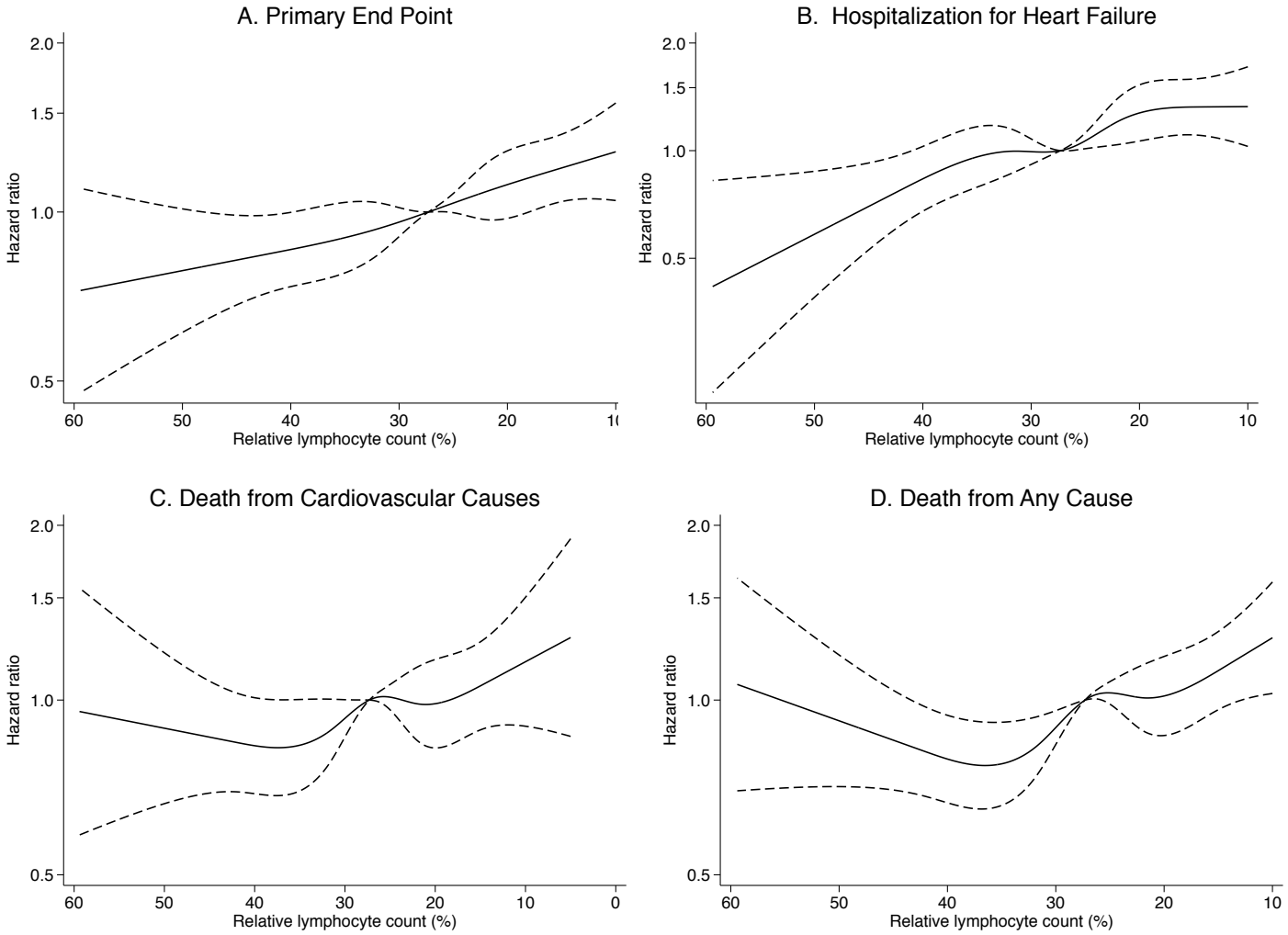


Figure 6-2: PARADIGM-HF adjusted splines for outcomes according to RLC relative to median RLC



6.3.5 PARAGON-HF outcomes according to RLC tertile

The primary outcome occurred in 292 (6.9 per 100 person-years) in Tertile 1 (highest RLC) compared to 482 patients (10.7 per 100 person-years) in Tertile 3 (lowest RLC) (Table 6-4). The cumulative probability of the composite primary outcome, death from CV cause, hospitalisation for HF and death from any cause was lowest for patients in Tertile 1 and highest for patients in Tertile 3 (Figure 6-3). The adjusted risk of all outcomes was significantly lower in Tertile 1 compared with Tertile 3-adjusted HR for the composite primary end point: 1.25 (95%CI 1.08-1.46), hospitalisation for HF 1.28 (95%CI 1.07-1.52), CV death 1.36 (95%CI 1.06-1.74) and death from any cause 1.46 (95% CI 1.20-1.77). Examination of RLC as a continuous variable showed that a 10% decrease in RLC was associated with a 17% higher risk of the primary composite end point (adjusted HR 1.17, 95%CI 1.08-1.27) and a higher risk of the other outcomes of interest (Figure 6-4- restricted cubic spline analysis).

Table 6-4 PARAGON-HF outcomes according to tertile of RLC (referent to Tertile 1) and per 10% decrease in RLC

	Tertile 1 n=1550	**Tertile 2 n=1455**	**Tertile 3 n=1790**	**Per 10% decrease in RLC n=4795**
Primary End Point				
Event number	292	308	482	
Event rate per 100 patient years (95% CI)	6.9 (6.14-7.73)	7.9 (7.11-8.89)	10.7 (9.75-11.65)	
Unadjusted HR	1.00 (ref)	1.15 (0.98-1.35)	1.45 (1.25-1.68)	1.27 (1.17-1.37)
Adjusted HR[a]	1.00 (ref)	1.12 (0.95-1.31)	1.25 (1.08-1.46)	1.17 (1.08-1.27)
Hospitalization for HF				
Event number	220	234	383	
Event rate per 100 patient years (95% CI)	5.2 (4.55-5.93)	6.0 (5.31-6.86)	8.5 (7.66-9.36)	
Unadjusted HR	1.00 (ref)	1.15 (0.96-1.39)	1.47 (1.25-1.74)	1.30 (1.19-1.42)
Adjusted HR[a]	1.00 (ref)	1.12 (0.92-1.35)	1.28 (1.07-1.52)	1.20 (1.10-1.32)
Death from CV Causes				
Event number				

Event rate per 100 patient years (95% CI)	105 2.3 (1.98-2.78)	120 2.8 (2.38-3.40)	191 3.8 (3.28-4.35)	
Unadjusted HR				
Adjusted HR[a]	1.00 (ref) 1.00 (ref)	1.26 (0.97-1.63) 1.22 (0.93-1.59)	1.67 (1.31-2.12) 1.36 (1.06-1.74)	1.30 (1.15-1.48) 1.15 (1.01-1.30)
Death from Any Cause				
Event number	168	196	326	
Event rate per 100 patient years (95% CI)	3.7 (3.16-4.27)	4.6 (4.03-5.33)	6.4 (5.78-7.18)	
Unadjusted HR	1.00 (ref)	1.28 (1.04-1.57)	1.80 (1.49-2.17)	1.37 (1.24-1.52)
Adjusted HR[a]	1.00 (ref)	1.23 (0.99-1.51)	1.46 (1.20-1.77)	1.20 (1.09-1.33)

[a] *Adjusted for: region, treatment, age, sex, race, systolic BP, heart rate, body mass index, serum creatinine, clinical features of HF (ischemic etiology, LVEF, NT-proBNP (log)), NYHA class, HTN, diabetes, AF, hospitalization for HF, MI, stroke, duration of HF.*

Figure 6-3: Kaplan-Meier curves for outcomes of interest, according to tertile of RLC in PARAGON-HF

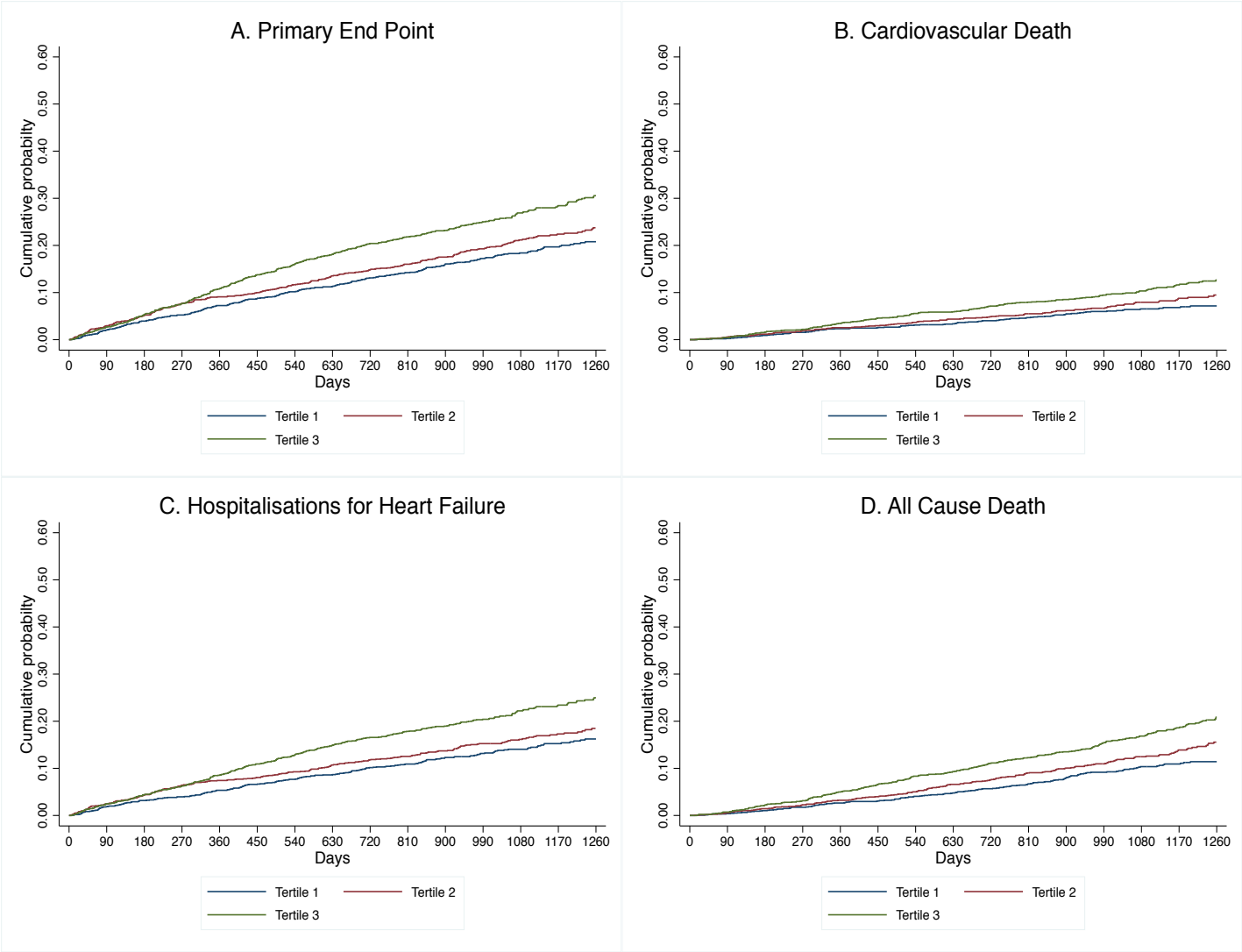
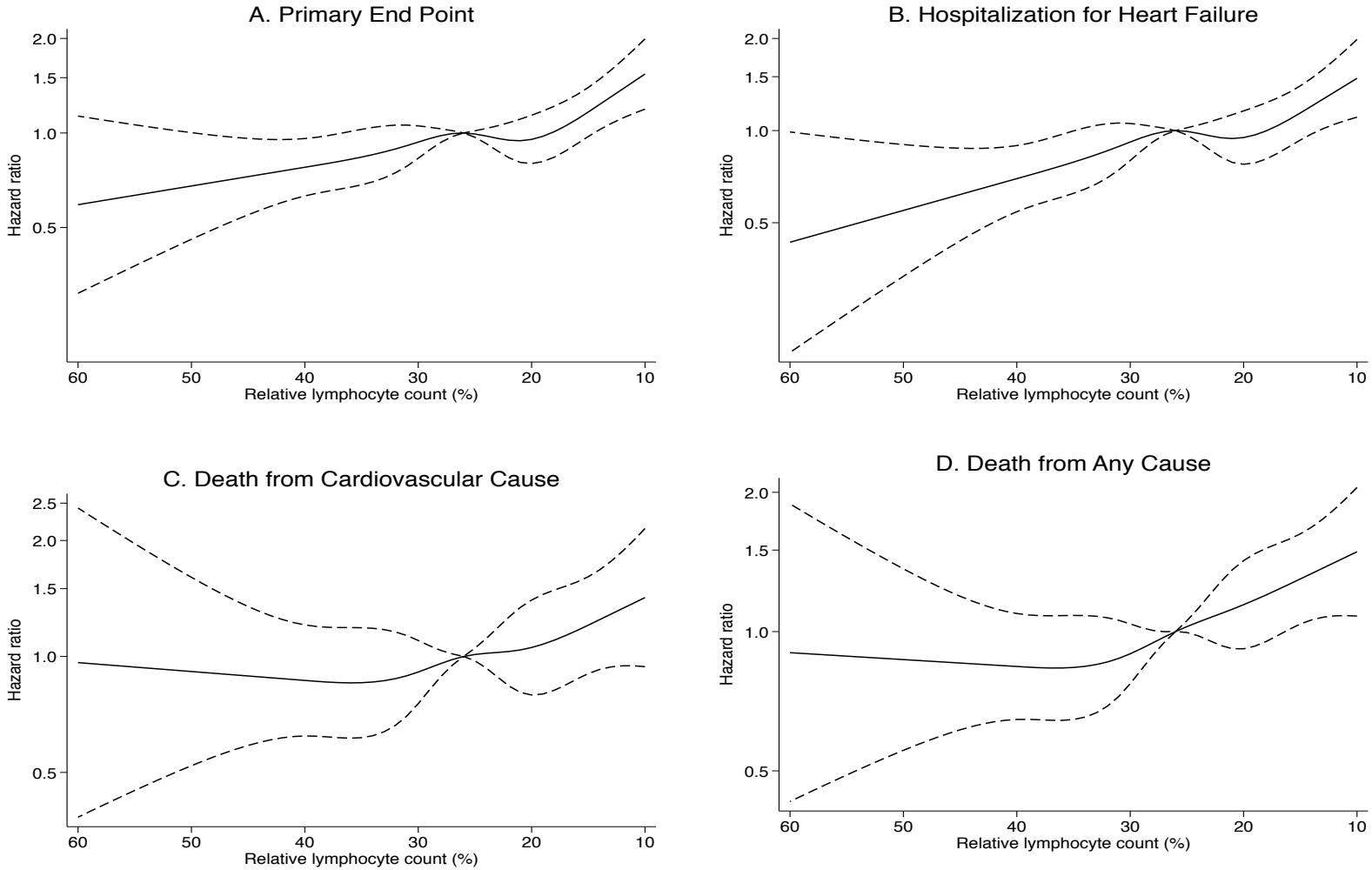


Figure 6-4: PARAGON-HF adjusted splines for outcomes according to RLC relative to median RLC



6.3.6 Change in RLC with sacubitril/valsartan treatment

In both trials RLC decreased over time but the decrease was less in the sacubitril/valsartan group than in the comparator group.

6.3.6.1 PARADIGM-HF

RLC decreased from baseline to twelve months in the enalapril group by 1.42%±7.32% and by 1.09%±7.0% in the sacubitril-valsartan group. The difference between groups was 0.33 (95%CI -.067-0.01)%, p=0.06.

6.3.6.2 PARAGON-HF

RLC decreased from baseline to twelve months by 1.37±6.48% in the valsartan group and by 0.90±6.71% in the sacubitril-valsartan group. The difference between groups was 0.47 (95%CI -0.87-0.08), p=0.02.

6.3.7 Effect of sacubitril/valsartan on clinical outcomes according to baseline RLC in PARADIGM-HF

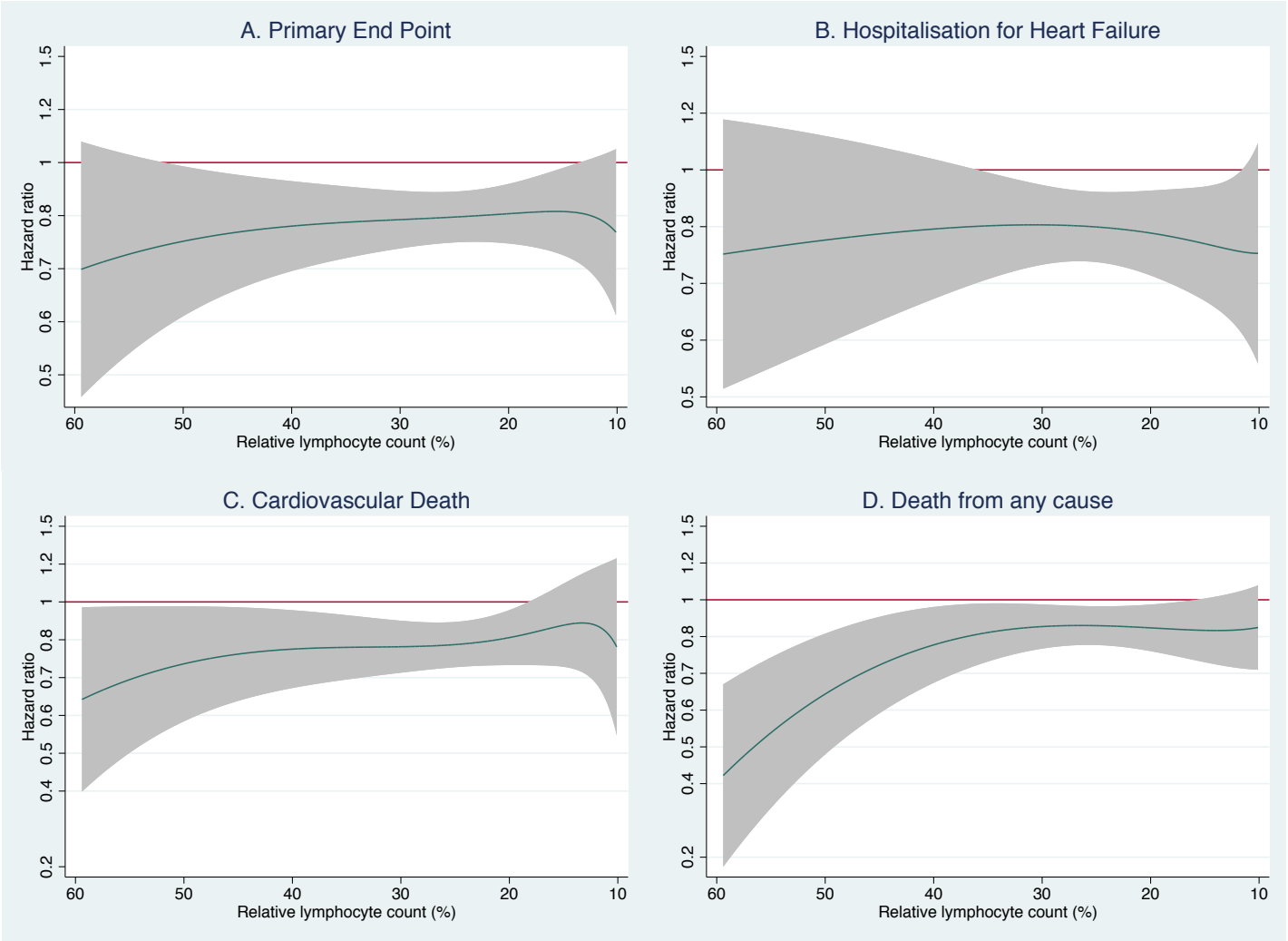
The rate (per 100 person-years) for each outcome was higher in the enalapril group than in the sacubitril-valsartan group, overall, and in each RLC tertile (Table 6-5). Compared with enalapril, treatment with sacubitril-valsartan reduced the risk of the composite primary endpoint (P for interaction with RLC tertile=0.84), death from CV causes (P for interaction=0.56), hospitalization for HF (p for interaction=0.75) and death from any cause (P for interaction=0.3), irrespective of RLC at baseline (Figure 6 – fractional polynomial analysis)

Table 6-5: PARADIGM-HF: Outcomes and treatment effect according to RLC

	Tertile 1		Tertile 2		Tertile 3		Interaction p
	Enalapril n=1331	**Sac-val n= 1317**	**Enalapril n=1292**	**Sac-val n=1349**	**Enalapril n=1379**	**Sac-val n=1310**	
Primary End Point							
No.	282	237	336	286	438	343	
Rate	10.1 (9.0-11.4)	8.5 (7.5-9.7)	13.1 (11.8-14.6)	10.1 (9.0-11.3)	16.3 (14.8-17.9)	13.1 (11.8-14.5)	0.78
Unadjusted HR	0.84 (0.71-1.00)		0.77 (0.66-0.91)		0.80 (0.70-0.92)		
Death from CV Causes							
No.	177	145	215	184	267	199	
Rate	6.0 (5.2-6.9)	4.9 (4.2-5.8)	7.8 (6.8-8.9)	6.1 (5.3-7.0)	8.9 (7.9-10.0)	7.0 (6.1-8.0)	0.94
Unadjusted HR	0.82 (0.66-1.02)		0.78 (0.64-0.95)		0.79 (0.66-0.95)		
Hospitalization for HF							
No.	154	132	191	157	275	219	
Rate.	5.5 (4.7-6.5)	4.8 (4.0-5.6)	7.4 (6.5-8.6)	5.5 (4.7-6.5)	10.2 (9.1-11.5)	8.4 (7.3-9.5)	0.70
Unadjusted HR	0.86 (0.68-1.09)		0.75 (0.61-0.93)		0.81 (0.68-0.96)		

Death from Any Cause No. Rate.	203 6.9 (6.0-7.9)	170 5.8 (5.0-6.7)	257 9.3 (8.2-10.5)	238 7.9 (6.9-9.0)	334 11.1 (10.0-12.4)	263 9.2 (8.2-10.4)	0.99
Unadjusted HR	0.84 (0.68-1.03)		0.84 (0.71-1.01)		0.84 (0.71-0.98)		

Figure 6-5: PARADIGM-HF: Treatment effect according to RLC



6.3.7 Effect of sacubitril/valsartan on clinical outcomes according to baseline RLC in PARAGON-HF

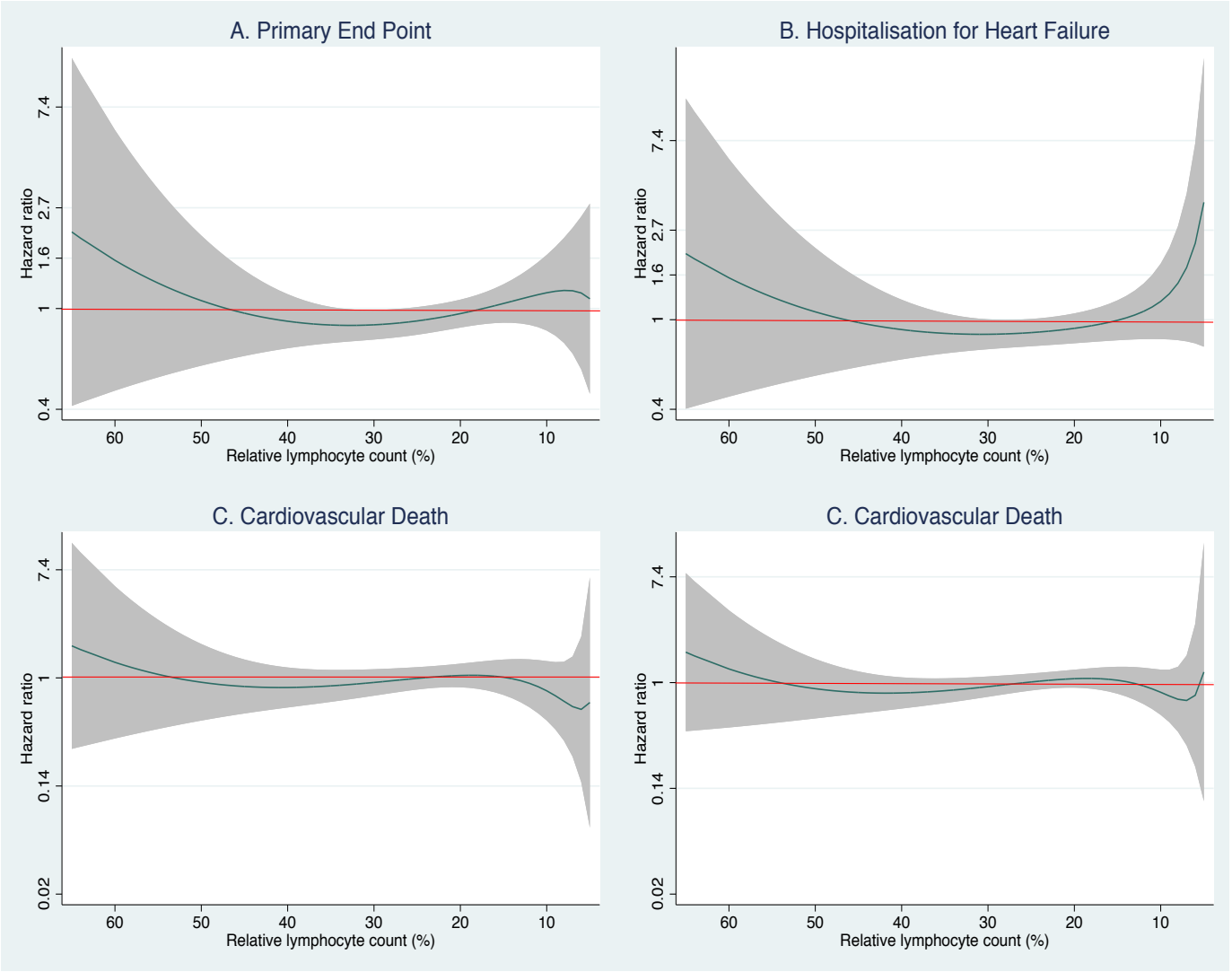
The rate (per 100 person-years) for each outcome was higher in the valsartan group than in the sacubitril-valsartan group for patients in RLC tertile 1. The effect of sacubitril/valsartan compared to valsartan is shown in Table 6-6 and Figure 6-6 (fractional polynomial analysis).

Table 6-6: PARAGON-HF: Outcomes and treatment effect according to RLC

	Tertile 1		Tertile 2		Tertile 3		Interaction p
	Valsartan n= 774	**Sac-val n= 776**	**Valsartan n= 714**	**Sac-val n= 741**	**Valsartan n= 900**	**Sac-val n= 890**	
Primary End Point							
No.	159	133	157	151	240	242	0.30
Rate	7.7 (6.5-8.9)	6.2 (5.2-7.3)	8.2 (7.0-9.6)	7.7 (6.6-9.0)	10.6 (9.4-12.0)	10.7 (9.4-12.1)	
Unadjusted HR	0.80 (0.63-1.00)		0.93 (0.75-1.17)		0.99 (0.83-1.18)		
Death from CV Causes							
No.	60	45	56	64	96	95	0.64
Rate	2.7 (2.1-3.4)	1.9 (1.5-2.6)	2.7 (2.1-3.5)	3.0 (2.4-3.8)	3.8 (3.1-4.6)	3.8 (3.1-4.6)	
Unadjusted HR	0.73 (0.50-1.08)		1.12 (0.78-1.61)		0.99 (0.75-1.32)		
Hospitalization for HF							
No.	119	101	121	113	192	191	0.39
Rate.	5.7 (4.8-6.9)	4.7 (3.9-5.7)	6.3 (5.3-7.6)	5.8 (4.8-6.9)	8.5 (7.4-9.8)	8.5 (7.3-9.7)	

Unadjusted HR	0.80 (0.61-1.04)		0.92 (0.71-1.18)		0.97 (0.79-1.18)		
Death from Any Cause No. Rate.	93 4.1 (3.4-5.0)	75 3.2 (2.6-4.1)	94 4.5 (3.7-5.5)	102 4.8 (3.9-5.8)	161 6.3 (5.4-7.4)	165 6.5 (5.6-7.6)	0.53
Unadjusted HR	0.79 (0.59-1.07)		1.07 (0.81-1.42)		1.03 (0.83-1.28)		

Figure 6-6 PARAGON-HF: Treatment effect according to RLC



6.4 Discussion

The main findings of Chapter 6 were as follows:

- RLC was associated with clinical outcomes in both HFrEF and HFpEF.
- Low RLC was an independent predictor of both death and hospitalization.
- Sacubitril/valsartan slightly attenuated the decline in RLC during follow-up and the benefit of sacubitril-valsartan over enalapril was consistent across the range of RLC found in the patients randomized in PARADIGM-HF.

RLC is a simple and widely accepted measure of systemic inflammatory state, with low RLC indicative of heightened inflammation. I found that the distribution of RLC was similar in ambulatory patients with chronic HFrEF and HFpEF, contrary to what I anticipated. In each of these two distinct HF phenotypes, lower RLC was accounted for by a higher total leukocyte (and neutrophil) count, as well as lower lymphocyte count. In each of HFrEF and HFpEF, lower RLC was associated with a similar higher risk of HF hospitalization and death (CV and all-cause).

In each HF phenotype, patients with the lowest RLC were older, more often male and had more comorbidities, including diabetes, AF and renal impairment. Individuals with a low RLC also had more oedema and higher natriuretic peptide levels. It was notable that the distribution of RLC, and the differences in patient characteristics according to RLC, were so similar in HFrEF and HFpEF. This was contrary to what I hypothesized and seemingly at variance with the recent hypothesis that inflammation plays a particularly key pathophysiological role in HFpEF (32,322). However, HFrEF is also associated with inflammation, with several studies showing similar elevations of inflammatory biomarkers to those reported in HFpEF (323–326). Moreover, the "inflammation hypothesis" in HFpEF is centred on the importance of comorbidities in driving inflammation and the overall burden of comorbidities does not differ greatly between HFrEF as HFpEF (327–329). It was also notable that in both HF phenotypes oedmea, and other evidence of congestion, was more prominent in patients in the lowest RLC tertile. Congestion in HF has been linked to increased intestinal permeability and entry of gut endotoxins into the circulation, leading to elevated systemic levels of pro-inflammatory cytokines (266,330). This may lead directly to lymphocyte apoptosis. Lymphocytes may also

be lost into the gut lumen or sequestrated in lymphoid tissue, possibly also aggravated by splanchnic congestion.

Importantly, the association between lower RLC and higher risk persisted after extensive adjustment for the comorbidities described above and other variables associated with worse outcomes, including NT-proBNP. This is consistent with what was found by the investigators who developed the SHFR Score, although none of the studies included in that report had a measurement of either BNP or NT-proBNP which are, individually, by far the strongest prognosticators in HF (18). Most patients enrolled in the studies included in the Seattle report were not treated with a beta-blocker either (17), which may be relevant as enhanced adrenergic activity may play a role in reducing RLC (16). Indeed, the mean RLC in these studies ranged from 22 to 26%, somewhat less than the mean of 27.3% in PARADIGM-HF.

The only other large investigation of RLC in HFrEF was in the Efficacy of Vasopressin Antagonism in Heart Failure Outcome Study with Tolvaptan trial (EVEREST) (313). The patients in EVEREST were quite different as they were enrolled during a hospital admission for decompensated HF, circumstances likely to lead to both increased adrenergic activity and greater inflammation. It is notable, therefore, that the median RLC in EVEREST was 21% compared with 26.6% in PARADIGM-HF. Moreover, in EVEREST, RLC increased after discharge, in contrast to the slight decrease over time in PARADIGM-HF after randomization. In EVEREST, RLC was independently associated with a short-term mortality and the composite of CV mortality or HF hospitalization in the first 100 days after discharge, but not in longer term outcomes.

In this analysis I have also shown that RLC was an independent predictor of outcomes in HFpEF and know of no other similar report. Consequently, RLC would appear to be a simple, routinely available, and inexpensive test which provides prognostic information for ambulatory patients with HF, irrespective of ejection fraction phenotype.

The decrease in RLC over time was attenuated significantly by sacubitril/valsartan in PARAGON-HF with a similar, but not statistically significant, trend in PARADIGM-HF. The reason for this small effect of sacubitril/valsartan on RLC is unclear. It could reflect either a direct anti-inflammatory action, for which there is some experimental evidence (303,331), or an indirect effect, for example in improving congestion or slowing the rate of decline in renal

function (332–334). This small effect on RLC is unlikely to have contributed to the benefit of sacubitril/valsartan over enalapril, which was consistent across baseline RLC tertile.

This study cannot determine whether inflammation, as identified by low RLC, is a mediator or marker of risk in HFrEF and HFpEF, a differentiation that could only be made by demonstration of benefit from a therapy specifically directed at inflammation. Although initial anti-inflammatory interventions in HF with anti-cytokine therapy were unsuccessful, these were not targeted to patients with elevated cytokine levels (39,40,335). Other data suggest possible benefit from anti-inflammatory therapy. Rosuvastatin appeared to reduce HF hospitalization in a prespecified subgroup of HFrEF patients with an elevated hsCRP level (and reduced hsCRP in these individuals) (336). The recent Canakinumab Anti-Inflammatory Thrombosis Outcome Study (CANTOS) showed that canakinumab, a monoclonal antibody directed against IL-1β, reduced the risk of developing HF in patients with a history of prior MI and elevated CRP (67).

I also examined another related, reciprocal, index of inflammation, the neutrophil to lymphocyte ratio (NLR) and found it to give essentially identical findings to RLC, although it had been suggested that NLR might be superior in predicting outcomes in patients hospitalized with acute HF (337) (Appendix VI).

6.5 Strengths and limitations

As with any study of this type, there are limitations. The analyses described were retrospective and had not been prespecified. The patients included in both trials were selected, with certain comorbidities explicitly excluded by protocol (e.g. severe lung and CKD, and severe anaemia). Because of this and the demands of participating in a trial, these patients were likely to be less frail and comorbid than in the "real world", which may mean I underestimated the potential prevalence of inflammation in HF. I did not have other measures of inflammatory status, such as hsCRP and IL-6 which would have been interesting to measure. However, the main strength of this analysis if that to date this is the largest study examining RLC levels in ambulatory patients with HFrEF and HFpEF. It also provides evidence that HF therapies may reduce levels of inflammation.

6.6 Conclusions

In summary, I found that RLC was associated with clinical outcomes in both HFrEF and HFpEF, with low RLC being an independent predictor of both death and hospitalization. Sacubitril/valsartan slightly attenuated the decline in RLC during follow-up and the benefit of sacubitril-valsartan over enalapril was consistent across the range of RLC found in the patients randomized in PARADIGM-HF. To date, there is limited information looking at levels of inflammation over time and whether certain CV treatments reduce inflammation. It is possible, that some patients who have higher levels of inflammation and CHIP may receive the greatest benefit from anti-inflammatory therapy. RLC and NLR do not appear to be difference according to CHIP status, therefore it may be that these routine markers of inflammation are not enough for personalisation of therapies and more specific markers of inflammation such as IL-6 and IL-1B are required.

6.7 Next steps

- To understand whether traditional haematological parameters can help predict those who will gain benefit from anti-inflammatory therapy, or whether more specific markers of inflammation such as those involved in the NLRP3 inflammasome pathway are more important.
- To understand whether standard HF therapies reduce levels of inflammation.
- To understand whether personalisation of HF therapies is beneficial for patients with high circulating levels of inflammation.

CHAPTER 7: Discussion

7.1 Summary of findings

The prevalence of HF continues to grow, and ageing is a major risk factor for the development of CV disease. HFpEF now accounts for over half the cases (14) and has an even closer relationship to ageing than does HFrEF (15). Unlike HFrEF, limited evidence-based therapies currently exist for the treatment of patients with HFpEF. Both HFrEF and HFpEF are associated with systemic inflammation, and elevated levels of inflammation are consistently shown to be associated with poor outcomes in HF as outlined in Chapter 1. Whilst the cumulative effect of exposure to conventional CV risk factors is important, recent evidence highlights clonal CHIP as a further key risk factor (70–75). CHIP reflects the accumulation of somatic, potentially pro-leukaemic gene mutations within HSCs over time (69,70). The most common mutations associated with CHIP and CV disease occur in genes that also play central roles in the regulation of inflammation.

As I outlined in Chapter 1, the incidence of CHIP in patients with HF is not well-defined and previous studies examining CHIP and HF have mainly been retrospective analysis and no study has simultaneously examined HFrEF and HFpEF patients. Additionally, no study has prospectively examined CHIP and circulating levels of inflammatory biomarkers in patients with decompensated HFpEF and HFrEF.

This thesis has provided a comprehensive evaluation of the role of inflammation in well-defined populations of patients with both stable and decompensated HF from a range of sources including large clinical trial datasets and a prospective observational studies. It also examines and outlines the prevalence, clinical characteristics and circulating levels of inflammation in patients with decompensated HF and CHIP. It also examines whether treatment with sacubitril/valsartan reduces circulating levels of inflammation in patients with stable HF. The results of this thesis provide evidence that personalised anti-inflammatory therapy may be beneficial for patients with HF and that further work is required to examine levels of circulating inflammatory levels over time.

7.2 Clonal Haematopoiesis and inflammation in patients with HF: A prospective cohort study

Prior to undertaking recruitment to this study, I applied for funding to the BHF to examine the prevalence of CHIP in 500 patients. However, my funding application was examined at the outset of the COVID-19 pandemic which meant that research into COVID-19 took priority and unfortunately my application was unsuccessful. However, due to the lack of research into CHIP and HF, I decided to examine the role of CHIP in 96 patients with decompensated HF with the funds I had available from my BHF Core Fund. At the time of recruitment, the only studies examining CHIP had been retrospective and focused solely on ischaemic HFrEF. Therefore, I felt that examining 96 patients would provide key insights into the prevalence of CHIP in both types of HF and its associated clinical and inflammatory biomarker characteristics. It has also provided evidence that a larger clinical prospective study would be feasible and cost-effective if subsequent funding became available.

7.2.1 Study recruitment and baseline characteristics

Just prior to starting recruitment, the COVID-19 pandemic hit. This meant that I had several other factors to consider in the planning of the study, and at the time all other HF studies were suspended within NHS GG&C. To prove that my study was both safe for myself and patients, I had to complete a thorough risk assessment which was submitted to both the University and NHS GG&C research governance team. My study recruited only inpatients with decompensated HF, this meant that all my patients would be tested for COVID-19 at baseline and if they tested positive, they were excluded from recruitment due to the presence of active infection. I was the only person involved in recruitment, making patient contact limited to two visits and reducing the likelihood of spread of the virus, furthermore I also limited recruitment to cardiology wards to prevent spread. Initially, I was unsure whether patients would be reluctant to take part in a study due to the pandemic, but I found that patient recruitment was as I initially expected (approximately 2 patients a week). Of the 260 patients I screened only five (2%) were excluded due to COVID-19 infection or concerns regarding recent COVID contacts. My safe recruitment of patients provided evidence for other studies that recruitment was possible if all factors were considered.

One factor I feel I did not consider enough prior to undertaking my study was patients' literacy ability. The Scottish government report that around 26% of people have issues with literacy (338) and I discovered that during recruitment that 6 patients were unable to take part due to literacy ability, despite them wanting to participate. For future studies, I would consider how to improve patients' ability to consent to research, for example having video information available.

Of the 260 patients with decompensated HF screened for inclusion in the study, 96 were recruited. The majority of the cohort was elderly, the mean age of patients who agreed to participate in this study was 72 years, and as expected patients with HFpEF were older (74 years) than those with HFrEF (70 years). Patients were very symptomatic of their decompensation with most being NYHA class III and IV and experienced expected symptoms of HF including orthopnea, PND, ankle swelling and fatigue. CV co-morbidities were highly prevalent at baseline, the commonest was AF (64%), followed by HTN (57%) and diabetes (33%). In our cohort, rates of vascular disease were lower than expected with previous MI only occurring in 19% of patients, previous CABG (18%) and previous stroke (19%). LVEF was as expected in both groups of HF (HFpEF 55.5±12.4 & HFrEF=23.6±9.7). CV medications were common at baseline and the majority of patients were receiving a diuretic at baseline indicating decompensation. At the time of recruitment SGLT2 inhibitors and fineronone were not licensed for the treatment of HFpEF therefore only one patient with HFpEF was receiving this treatment for diabetes.

7.2.2 Main findings

The main findings in chapter 3 were:

- CHIP is common in patients with both types of HF, with the prevalence being slightly higher in patients with HFpEF when compared to HFrEF.
- While the prevalence of CHIP rises with age overall, the association between CHIP and HFrEF appeared to be independent of increasing age.
- The presence of CHIP was not associated with a difference in traditional routine clinical haematological markers of inflammation, including its derivatives RLC and NLR.

- The presence of CHIP was associated with higher circulating levels of inflammatory biomarkers in particular IL-1β, IL-18 and TGF-β2 in all patients.
- In patients with HFpEF, the presence of CHIP was associated with higher levels of IL-1β and its associated downstream markers.
- In patients with HFpEF and HFrEF, the presence of CHIP was surprisingly not associated with higher levels of IL-6.

7.2.3 The prevalence of CHIP in patients with HF

As outlined in the introduction, the prevalence of CHIP in all comers with HF is not well defined. I found that CHIP driver mutations with a VAF≥2% were detected in 5 patients with HFrEF (10%) and 8 patients with HFpEF (17%). CHIP driver mutations with VAF≥1% were detected in 25 patients with HFrEF (52%) and 21 patients with HFpEF (44%). In line with other studies, I observed an age-dependent increase in the prevalence from 4% (≤50years old) to 44% (≥80years), and the total number mutations also increased with age in both HFpEF and HFrEF groups. However, there was not a robust association between ageing and CHIP in the HFrEF cohort. This raises the possibility that CHIP is more important in younger patients with HFpEF and warrants further investigation.

The most common mutation observed in both types of HF was DNMT3A. Unlike other studies, I observed lower than expected rates of TET2 mutation, occurring in only one patient with HFrEF and two patients with HFpEF (70,73,169). A possible hypothesis for this is that we observed low rates of vascular disease in our cohort than other studies examining CHIP (169). In line with other studies, we observed that the majority of mutations observed were missense mutations, followed by splice region mutations and then frame shift deletions (70,73,169).

7.2.4 Diagnostic criteria of CHIP

The initial diagnostic criteria of CHIP were set based on the sequencing methods available at the time. However, the development of next generation sequencing means that very low levels CHIP driver mutations can be accurately detected. To date, it is currently unknown what VAF cut off is important for patients, but different VAF cut-off levels have been reported for different mutations when patient outcomes are examined (80,169). There is growing evidence

that even very low levels of CHIP-driver mutations are important in the patient outcomes (80,169). This is why in this thesis I used a VAF cut off as greater or equal to 1% to diagnose CHIP. In addition, the importance of multiple mutations is currently unknown. It is plausible that different CHIP mutations are associated with different CV disease depending on the CHIP-driver mutation mechanism of action, for example TET2 appears to be more associated with vascular disease.

7.2.5 Previous cancer history

In line with the UK cancer statistics as reported by Cancer Research, I observed that the most common previous cancers in my cohort were breast and prostate cancer (339). Previous oncological therapies including chemotherapy and radiotherapy increase the risk of the development of CHIP in the future (125,128). Unfortunately, my cohort was too small to demonstrate whether previous cancer history or previous cancer therapies were associated with CHIP status.

7.2.6 Biomarker levels in patients with decompensated HF and CHIP

NTproBNP levels is an important biomarker for future adverse CV outcomes, in this study we observed that CHIP status was associated with numerically higher levels of NT-proBNP in both type of HF. However, higher levels of NT-proBNP were not associated with CHIP status. High levels of NTpro-BNP are important for patient risk stratification and it is currently unknown whether the addition of CHIP status to a robust HF model such a MAGGIC HF risk stratification tool would improve its prediction ability (37,340,341). Currently, the presence of CHIP and levels of VAF are used to risk stratify patients with underlying cancer (82,342).

7.2.7 Inflammatory biomarkers in patients with decompensated HF

In line with other studies, I observed numerically elevated levels of pro-inflammatory cytokines involved in NLRP3 inflammasome activation with CHIP status, in particular numerically higher levels of IL-1β, CRP and IL-18 were observed (96). HFpEF and HFrEF represent

different pathophysiological processes, and overall circulating levels of inflammation appeared to be higher in the HFpEF cohort when compared to the HFrEF cohort.

Logistic regression analysis revealed that as expected CHIP was associated with ageing, however the effect of ageing was numerically less in the HFrEF cohort. The presence of CHIP in patients with HFpEF was strongly associated with elevated levels of IL-1β. This raises the potential that IL-1β inhibition may be beneficial for patients with HFpEF and indicates that CHIP status may allow personalisation of HF therapies in both types of HF.

Surprisingly, I did not observe that levels of IL-6 were associated with CHIP status. One possible explanation is that this was a chance result in view of the small numbers in this cohort. However, another potential explanation is that when the initial IL-6 assay was run there was an error and it had to be repeated. Whether the extra freezing and thawing of the samples impacted this result is unknown. Furthermore, the MSD platform for measuring IL-6 was relatively new at the time of this study which may have also contributed to this result. A potential way to discover whether these results were true, would be to re-run the IL-6 assay using the well validated traditional IL-6 ELISA. While some studies have shown that repeated sample thawing does reduce levels of circulating biomarkers, this is largely unknown and would be worth further research.

7.2.8 The potential for anti-inflammatory therapies in patients with HF

In Chapter 1, I outlined how high circulating levels of inflammation are associated with development of HF and worse CV outcomes. Historical trials of anti-inflammatory therapy for the treatment of CV disease have mainly been disappointing. CANTOS examined the effects of canakinumab, a monoclonal antibody directed against IL-1β, in patients with a history of prior MI and elevated CRP. Canakinumab reduced CRP and the incidence of atherosclerotic CV events was decreased by 15% versus control (21). Notably, canakinumab also reduced HF hospitalization and HF-related mortality by 23% in patients who achieved a CRP level of <2mg/L (67). Given the association of CHIP with inflammation and, in particular, the secretion of IL-1β (the immediate upstream precursor to IL-6), CHIP has been proposed as a potential biomarker for personalized therapy with canakinumab and potentially other anti-inflammatory therapies. Indeed, in an exploratory analysis of CANTOS, canakinumab reduced the relative

risk of major adverse CV events by 64 % in those with TET2 mutations and by 15% in the treatment overall (217). Whether or not this impressive effect will also be seen in patients with HF is unknown.

Inzomelid, a novel small-molecule inhibitor of the NLRP3 inflammasome, is currently under clinical investigation for its safety and tolerability in humans (NCT04015076). Whether any potential effect is amplified in patients with CHIP may be a logical future step in its assessment. Recent data revealed that the SGLT-2 inhibitor, dapagliflozin, reduces IL-1β via up-regulation of serum β-hydroxybutyrate (218). Again, the potential benefits of personalisation of SGLT2 inhibitor therapy on the basis of CHIP status is an intriguing but, as yet, untested hypothesis.

7.3 The role of IL-6 in HFpEF and HFrEF

CHIP status does not affect standard clinical haematological parameters and surprisingly in my cohort CHIP status was not associated with a difference in circulating levels of IL-6. To date, the literature indicates that IL-6 is an important pro-inflammatory cytokine in CVD, and the recent CANTOS study demonstrated that treatment with canakinumab reduced circulating levels of IL-6 (343). I therefore went on to examine circulating levels of IL-6 in a different cohort of patients with recently decompensated HFrEF and HFpEF in chapters 4 &5.

The main findings of chapters 4 and 5 are as follows:

- Levels of IL-6 were high in patients with recently decompensated HFrEF and HFpEF.
- Higher levels of IL-6 were associated with an increased risk of all-cause mortality and CV death in both HFrEF and HFpEF.
- Higher levels of IL-6 were associated with an increased risk of 1st HF hospitalisation in HFpEF only.
- IL-6 remained an independent predictor of events even after adjustment for established independent clinical risk factors including BNP, in HFrEF this was only for all-cause mortality and CV death.

This study is the first to demonstrate the prognostic significance of circulating levels of IL-6 in patients with HFrEF and HFpEF. The study enrolled near consecutive patients admitted to hospital and represent a typical cross-section of people admitted to hospital with HFpEF and

HFrEF. The study also observed a large number of clinical events which were reported using the standard accepted international classifications of disease.

In these "real-life" populations, in the HFrEF cohort 70.1% of patients and in the HFpEF cohort 61.2% of patients had a concentration of IL-6 that was greater than the previously reported 95th centile of the normal range (4.45pg/mL).

In the HFpEF cohort, even after adjustment for clinically important prognostic variables, including BNP, patients in the highest tertile of IL-6 concentration had over two-fold increased risk for all-cause mortality and were at 2.8 times higher risk for CV death during follow-up. Although the association between IL-6 tertile and first HF hospitalisation was not apparent after adjustment, when these outcomes were assessed in relation to IL-6 as a continuous variable, the association between higher IL-6 and HF hospitalisation remained, even after adjustment. Indeed, each one unit log increase in IL-6 was associated with a 24% increased risk of first HF hospitalisation.

In the HFrEF cohort, even after adjustment for clinically important prognostic variables, including BNP, patients in the highest tertile of IL-6 concentration had an almost 2 times higher risk of all-cause mortality and CV death. IL-6 is therefore a marker of adverse outcomes in patients with decompensated HFrEF and HFpEF, and numerically appeared more important in HFpEF when compared to HFrEF.

Indeed, levels of IL-6 were only associated with an increased risk of first HF hospitalisation in the HFpEF cohort suggesting that anti-inflammatory therapy could be more beneficial for reducing hospitalisations in patients with HFpEF than in those with HFrEF. IL-6 may also help identify those who would gain the most benefit from IL-6 inhibition, especially as it is now relatively cheap, quick and simple to measure circulting levels of IL-6.

7.4 RLC and NLR in patients with HFpEF and HFrEF

The main findings of Chapter 6 were as follows:

- RLC was associated with clinical outcomes in both HFrEF and HFpEF.
- Low RLC is an independent predictor of both death and hospitalization.

- Sacubitril/valsartan slightly attenuated the decline in RLC during follow-up and the benefit of sacubitril-valsartan over enalapril was consistent across the range of RLC found in the patients randomized in PARADIGM-HF.

As outlined in Chapter 1, the initial studies examining the role of inflammation in HF used simple blood count derivatives. I found that the distribution of RLC was similar in ambulatory patients with chronic HFrEF and HFpEF, contrary to what I anticipated. In each of these two distinct HF phenotypes, lower RLC was accounted for by a higher total leukocyte (and neutrophil) count, as well as lower lymphocyte count. In each of HFrEF and HFpEF, lower RLC was associated with a similar higher risk of HF hospitalization and death (CV and all-cause).

In both HF phenotypes, the patient characteristics according to RLC were similar with patients with the lowest RLC were older, more often male and had more comorbidities. In both HF phenotypes evidence of congestion, was more prominent in patients in the lowest RLC tertile. Congestion in HF has been linked to increased intestinal permeability and entry of gut endotoxins into the circulation, leading to elevated systemic levels of pro-inflammatory cytokines (266,330). This may lead directly to lymphocyte apoptosis. Lymphocytes may also be lost into the gut lumen or sequestrated in lymphoid tissue, possibly also aggravated by splanchnic congestion.

In both HF phenotypes, lower RLC was associated with a higher risk of composite primary end point, hospitalisation for HF, CV death and death from any cause. Examination of RLC as a continuous variable showed that a 10% decrease in RLC was associated with a 17% higher risk of the primary composite end point in HFpEF cohort and the HFrEF cohort.

I also examined another related, reciprocal, index of inflammation, the neutrophil to lymphocyte ratio (NLR) and found it to give essentially identical findings to RLC, although it had been suggested that NLR might be superior in predicting outcomes in patients hospitalized with acute HF (337) (Appendix VI).

7.5 Strengths

To my knowledge, this is the first study examining the prevalence of CHIP and the clinical characteristics and cardiac biomarkers in patients admitted to hospital with both decompensated HFpEF and HFrEF. This is also the first study to prospectively examine a large number circulating inflammatory biomarkers in patients with decompensated HF according to CHIP status. The finding of this study provides important data for informing further studies in anti-inflammatory therapy in HF, which is important as there are limited therapies for the treatment of HFpEF. Finally, perhaps one of the biggest strengths of this study was that it was completed in the context of the challenges of the COVID-19 pandemic.

Another strength of this thesis is that the introduction provides a comprehensive overview of inflammatory biomarkers in HF. This is also the first study to examine the role of circulating levels of IL-6 in patients with recently decompensated HFpEF and HFrEF. While also examining simple readily available markers of inflammation (RLC and NLR) in patients with both types of HF. The results from these studies provide important information informing future research into the role of anti-inflammatory therapy in HF.

7.6 Limitations

The main limitation of the CHIP study is that the number of patients recruited was relatively small, which makes drawing conclusions from associations challenging. Also, these small numbers mean there is a potential to miss important statistical results. Furthermore, I am unable to assess the role of CHIP in the CV outcomes of patients recruited to this study due to the low number of events observed.

The analyses of inflammatory markers were retrospective and had not been prespecified. Almost all patients examined in this these were white and potentially the important effects of race upon outcomes and biomarkers has not been addressed here. Recruitment to the studies examined was prior to the introduction of SGLT-2 inhibitors for the treatment of HF. Lastly, all inflammatory biomarkers examined in this thesis were only examined at one time point.

7.7 Future directions

Many aspects of the findings in this study are hypothesis-generating and pose many questions around the role of CHIP in patients with HF. Firstly, CHIP is common in patients with HF but it is currently unknown whether the presence of CHIP is associated with worse outcomes in both types of HF and what VAF level for each mutation is important. It is also unknown which CHIP-driver mutation(s) are most important for patients with HF and whether identification of CHIP can lead to improved risk stratification and identify those patients likely to gain the most benefits from anti-inflammatory therapies. It is also currently unknown whether different CHIP driver mutations are associated with different CV disease from CAD to aortic valve stenosis. The pathophysiological mechanisms of each CHIP-driver mechanisms warrant further research. It is possible that CHIP status effects circulating levels of inflammation over time and may effect response to conventional HF therapies, in particular SGLT-2 inhibitors which appear to impact the NLRP3 inflammasome pathway. It may be benefical to study post-mortem HFpEF and HFrEF hearts levels of inflammation according to CHIP status. CHIP appears to be associated with increased risk of death, but it is currently unknown if CHIP is associated with an increased risk of arrhythmia and sudden cardiac death, in my cohort I observed that CHIP status was associated with an increase in QRS duration and this warrants further investigation.

Chapters 4 & 5 consistently show that elevated circulating levels of IL-6 are associated with worse outcomes for patients with decompensated HF. It is currently unknown whether therapeutic IL-6 inhibition would be beneficial in patients with decompensated HF. However, reduction in levels of IL-6 in the recent CANTOS trial suggest that targeting the NLRP3 inflammasome may be beneficial for patients with elevated levels of IL-6 and IL-1B. It is unknown whether IL-6 levels improve risk stratification in patients with decompensated HF over the classical lymphocyte levels which are included in the Seattle HF model. Furthermore, there is limited information on the trends of IL-6 in patients with HF and with HF therapies and this warrants further information, considering this thesis demonstrates that treatment with sacubitril/valsartan appeared to reduce levels of inflammation.

7.8 Conclusions

CHIP is common in patients with both types of HF and appears to be associated with elevated levels of inflammatory biomarkers associated with the NLRP3 inflammasome. Furthermore, in patients with decompensated HFrEF and HFpEF, circulating levels of IL-6 are particularly high and consistently correlated with worse CV outcomes even after adjustment for established independent clinical factors including BNP. These findings provide important information supporting the clinical relevance of inflammation as a marker for adverse CV outcomes and as a therapeutic target in patients with HF. The use of CHIP status as a component of personalisation of care remains valid for further research. With the development of novel HF therapies, including those acting against IL-6, it is highly conceivable that the next major advance for HF treatment will come from an anti-inflammatory drug.

APPENDICES

Appendix I: Patient Information Sheet

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PARTICIPANT INFORMATION SHEET

Age-related Clonal Haematopoiesis and Inflammation in Patients with Heart Disease.

You have been invited to take part in a clinical research study. Prior to agreeing to take part, it is important to understand why the research is being done, and what the study will involve for you. Please read through this information sheet, and we will be happy to answer any questions you may have (using the contact details above).

WHAT IS THE PURPOSE OF THE STUDY AND WHY HAVE I BEEN INVITED TO TAKE PART?

You have been invited to take part in the study because you have been diagnosed with coronary artery disease without heart failure.

In the United Kingdom, coronary artery disease effects a large proportion of people. Although we have made substantial progress in the treatment of heart disease, we wish to know more to help develop future therapies. Genes carry the information that determines your features or characteristics. Each cell in the human body contains about 25,000 to 35,000 genes. Genes that have been changed are called mutations. Although some mutations can be passed from parent to child, we are interested in mutations that develop over time as you get older rather than changes that can be passed to your offspring. Age Related Clonal Haematopoiesis (ARCH) is the development of mutations in the genes involved in blood cell production. These changes have also been found in people with heart failure and heart artery disease and seem to be associated with higher levels of inflammation (the bodies response to injury or infection). We would like to understand more about how ARCH is involved with inflammation and coronary artery disease. The strongest known link of these mutations is with heart artery disease, for which you are already receiving treatment. However, these mutation (changes) also carry a very slightly increased risk of developing disorders of the blood, including blood cancer (0.5% per year). Your blood count will be checked as part of the study to ensure you don't show any worrying changes just now. We will be assessing ARCH in a research laboratory for research purposes only and not to guide your treatment. Having features of ARCH would not mean that you require any further follow-up or treatment for this. This would be the case whether or not you were in a research study. As outlined above, the most important thing for people with ARCH is to have heart-related issues treated and you are already receiving the appropriate care for that. The risk of blood disorders is low and if you were found to have gene changes consistent with ARCH then no further investigation of the blood

or treatment would be suggested. For these reasons, we do not plan to tell you or your GP about the presence of changes in genes associated with ARCH. Your GP will continue to manage you according to standard routine clinical care guidelines should you develop any abnormalities in your blood count in the future.

We will also carry out blood tests to look for markers of inflammation in your bloodstream. We will also review your clinical notes for the results of your previous cardiovascular tests. This will include the results of your heart scan which looks at the pumping of your heart and any previous imaging looking at the vessels which supply the heart with blood (coronary arteries).

Some patients may be interested in attending a second study visit for our sub-study. This will involve returning to the Queen Elizabeth University Hospital in six to twelve weeks later, at a time convenient for you. We will use non-invasive methods to look at the structure and function of your blood vessels as well as taking more blood to look for signs of inflammation and to check your cardiac function. At this visit we will not repeat any ARCH analysis.

By improving our understanding of how many people with heart artery disease have ARCH and how this affects inflammation, we hope that this will guide the development of future heart disease treatments and might also identify a group of patients who could respond to new treatments more than others.

DO I HAVE TO TAKE PART?

No – it is entirely voluntary and your decision whether or not you choose to take part in this study. If you agree to take part, in addition to this information sheet, you will be asked to sign a consent form. You are free to withdraw consent at any stage and are not required to give a reason for doing so. Should you withdraw from the study at any stage, with your consent, we would retain your stored samples and data along with the other participants'.

WHAT WILL HAPPEN TO ME IF I AGREE TO TAKE PART?

If you agree to take part in the study, we will only see you at one study visit unless you decide to take part in the sub-study. If you agree to take part in the sub-study, you will require to attend the Queen Elizabeth Hospital Clinical Research Centre six to twelve weeks after your initial study visit. This will be arranged at a convenient time for yourself and transport can be arranged if required.

By agreeing to take part, we will require the following:

- **Blood pressure (BP) measurement –** your blood pressure will be measured as part of your routine at your appointment.

- **Electrocardiogram (ECG) -** this is a tracing of the electrical activity within your heart and will involve attaching electrode stickers to the chest and limbs. This will be taken as part of your routine clinical at your appointment, this result will be taken from your clinical notes.

- **Echocardiogram –** this is an ultrasound heart scan to assess the pumping function of the heart, which was performed as part of your routine clinical care. This result will be taken from your clinical notes.

- **Blood samples –** you will be asked to provide a blood sample; we will aim to do this alongside your routine blood samples where possible. The blood will be checked for the presence of ARCH and for markers of inflammation. With your sample, we will ***only*** test genes responsible for ARCH. These tests will not have any bearing on inheritance or other family members. Approximately 40mL (2

tablespoons) of blood will be taken in total, some of this blood will be stored anonymously for up to 10 years after the end of the study for analysis of any future relevant tests as they become available and for use in future ethically approved studies if appropriate.

- **Urine samples –** you will be asked to provide a urine sample. This will be used to check for the amount of protein in your urine.

- **Follow Up-** Patients who consent to the study will consent to their clinical notes to be accessed for ten years by members of the research team.

SUB-STUDY:

If you are interested in taking part in the optional 'sub-study', this will require a single visit to the Clinical Research Facility at the Queen Elizabeth Hospital, 6 to 12 weeks after your initial involvement in the study. We would perform several tests to assess the function and structure of your blood vessels in order to understand how ARCH and inflammation affect these in people like you. This will take up to two hours to complete, and you will be required to **abstain from tobacco, alcohol and caffeine for at least eight hours before**. The following tests would be performed:

- **Blood sample-** you will be asked to provide a further blood sample; this will be analysed for markers of inflammation. **Your DNA will not be analysed again**. This will allow us to see changes in the levels of inflammation in your blood. Approximately 20mls (1 tablespoon) of blood will be taken in total, some of this blood will be stored anonymously for up to 10 years after the end of the study for analysis of any future relevant tests as they become available and for use in future ethically approved studies if appropriate.

- **Brachial Aortic Arterial Pulse Wave Velocity-** this is a non-invasive technique which allows us to assess blood vessel function. A blood pressure cuff is applied to your arm, and then we will use a small probe applied to the skin over the artery in your neck and arm to assess the pulse. This should not be uncomfortable but required you to be still. This test allows us to understand how 'stiff' your blood vessels are.

- **Brachial Artery Flow Mediated Dilatation-** this is a quick, simple non-invasive technique which allows us to asses blood vessel function. It involves resting your arm on a table and placing a blood pressure cuff around your upper arm, very similar to having your blood pressure checked. The cuff is inflated and deflated. The cuff does inflate to quite a high pressure for five minutes. This can be slightly uncomfortable but usually well tolerated. If you find this uncomfortable you can ask to stop. The whole test takes around twenty-five minutes. We use an ultrasound probe to assess the response of your arteries to blood flow, providing a measure of blood vessel function.

- **Carotid Intima Media Thickness-** this is also a non-invasive technique which allows us to use an ultrasound machine to look at the structure of your blood vessel in your neck. It involves you lying flat with your head to the left-hand side. An ultrasound scan to looks at the inside of the main vessel in your neck and allows us to look at thickening in the artery walls.

- **Retinal Optical Coherence Topography-** the blood vessels in your eyes share similar characteristics to the vessels in your heart and we wish to understand more about this in people with coronary

artery disease. The scan is frequently performed as part of routine eye appointments. It involves taking a quick photo of the back of your eye with your eyes aligned in front of a camera. Because there is a brief flashing light with this test we would not perform if you have a history of epilepsy. We will also ask about a history of previous eye problems, whether or not you wear strong spectacles or have diabetes. If you do, we would not perform this part of the sub-study, but you would be able to participate in other parts.

WHAT ARE THE BENEFITS OF TAKING PART?

It is hoped that by taking part in this study, you will be providing valuable information to help us work out how ARCH and inflammation may contribute to coronary artery disease. Although taking part in this study will not affect your normal heart failure care, you will receive closer monitoring of your cardiovascular health than you might otherwise. We hope the results will help us to design a future study of a drug aimed at treating these cardiovascular problems, particularly for people who have high levels of inflammation or ARCH.

WHAT ARE THE POSSIBLE DISADVANTAGES OF TAKING PART?

It is possible that we will discover clinically relevant information or another medical condition during the study. If so, we will inform both you and the Cardiologist responsible for your care if necessary, and arrange further follow up, investigation or treatment as appropriate.

Blood tests- the collection of blood can occasionally lead to bruising although this usually resolves without any specific treatment.

Brachial Aortic Arterial Pulse Wave Velocity- occasionally some patients find inflation of the blood pressure cuff uncomfortable this is only inflated for a short period of time and the discomfort is only very mild. If it causes significant discomfort the test can be stopped at any time.

Brachial Arterial Flow Mediated Dilatation- occasionally some patients find inflation of the blood pressure cuff uncomfortable this is only inflated for a short period of time and the discomfort is only very mild. If it causes significant discomfort the test can be stopped at any time.

Carotid Intima Media Thickness- occasionally some patients can find the ultrasound scan of their neck uncomfortable, but the procedure is very quick and can be stopped immediately if necessary.

Retinal Optical Coherence Topography- occasionally some patients can find the flashing light in their eye uncomfortable. The light only flashes once for each image and we will aim to only take one photography. Occasionally the first image we take is not of good enough quality to interpret and therefore we need to repeat the image.

WHAT IF SOMETHING GOES WRONG?

We do not anticipate that anything will go wrong during the study, however in the event that something did go wrong, and you were harmed due to someone's negligence then you may have grounds for legal action against the University of Glasgow/NHS Greater Glasgow and Clyde, but you may have to pay the legal costs for this.

http://www.nhsggc.org.uk/get-in-touch-get-involved/complaints/

Phone us: 0141 201 4500

Email us: complaints@ggc.scot.nhs.uk

WILL MY TAKING PART IN THIS STUDY BE KEPT CONFIDENTIAL?

If you consent to take part in the study, the research doctor will view your medical records for purposes of analysing the results. Representatives of the Sponsor, NHS Greater Glasgow and Clyde, may also look at these in relation to checking the study has been carried out appropriately. All information collected about you during the study will be kept strictly confidential. All of the information relating to your participation in the study will be securely stored using a unique study number that only the study investigators have access to. NHS Greater Glasgow and Clyde is a GDPR compliant organisation and all information will be processed in compliance with these 2018 regulations.

NHS Greater Glasgow and Clyde is the sponsor for this study based in the United Kingdom. We will be using information from you and your medical records in order to undertake this study and will act as the data controller for this study. This means that we are responsible for looking after your information and using it properly. NHS GGC will keep identifiable information about you for 10 years after the study has finished.

Your rights to access, change or move your information are limited, as we need to manage your information in specific ways in order for the research to be reliable and accurate. If you withdraw from the study, we will keep the information about you that we have already obtained. To safeguard your rights, we will use the minimum personally-identifiable information possible.

You can find out more about how we use your information http://www.nhsggc.org.uk/patients-and-visitors/faqs/data-protection-privacy/

WILL MY GP BE INFORMED THAT I AM TAKING PART?

With your consent, we would like to inform your GP that you are participating in this study.

WHAT WILL HAPPEN TO THE RESULTS OF THE STUDY?

Once the study has been completed and the results analysed, the findings are likely to be published in medical journals, which will be available to the public. You will be contacted by post once the study is complete informing you of progress and the outcomes of investigations in the form of a newsletter. Following this, the results will be published online on our Institute of Cardiovascular Medical Science website, and we will provide you with the details on how to access this. Any reports or publications from the research will not contain any personal details.

WHO IS ORGANISING AND FUNDING THE RESEARCH?

The research is being carried out by doctors employed by the University of Glasgow and NHS Greater Glasgow and Clyde. The research is funded by the British Heart Foundation Centre of Excellence award and departmental funding.

WHO HAS REVIEWED THE STUDY?

The Study has been reviewed by an independent doctor, Dr. Pardeep Jhund (Clinical Senior Lecturer in Cardiology and Honorary Consultant Cardiologist), and has received a favourable ethical opinion from the South East Scotland Research Ethics Committee XX, which is an independent panel.

THANK YOU FOR TAKING THE TIME TO READ THIS INFORMATION SHEET.

If you would like more information about the study and wish to speak to someone **not** closely linked to the study, please contact **Dr Pardeep Jhund (0141 330 2000 or email pardeep.jhund@glasgow.ac.uk)** Alternatively, please don't hesitate to contact a member of the research team:

Study Doctor

Dr Leanne Mooney

Clinical Research Fellow

Telephone: 0141 330

Email: Leanne.mooney@glasgow.ac.uk

Supervisors

Dr Ninian Lang

Telephone: 0141 330 2000

Professor Mark Petrie

Telephone: 0141 330 2000

Independent Doctor

Dr Pardeep Jhund

Telephone: 0141 330 2000

Appendix II Patient Consent Form

BHF Glasgow Cardiovascular Research Centre
126 University Place
Glasgow, G12 8TA

Enquiries to: Dr Leanne Mooney
Telephone: 0141 330 2237
E-mail: Leanne.mooney@nhs.net

PARTICIPANT CONSENT FORM

ARCH-HF: Age related changes, inflammation and heart failure.

Please initial the box

Patient ID:_____

I confirm that I have read and understand the information sheet (heart failure/ coronary artery disease) dated _____ (version ____) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.	☐
I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected.	☐
I agree to have data and samples retained and stored should I withdraw from the study for any reason.	☐
I agree to undergo blood testing (including genetic testing for age-related clonal haematopoiesis) and urine tests as described in the Patient Information Sheet.	☐
I agree to have my blood and urine samples stored and used in future ethically approved Studies for ten years.	☐
I agree to allow the research team to examine my clinical notes to access previous cardiovascular scan results.	☐
I understand that sections of my medical notes may be looked at by the research team and by representatives of the Sponsor, NHS Greater Glasgow and Clyde, where it is relevant to my taking part in the research. I give permission for these people to have access to my records for ten years after I have taken part in the study.	☐

	Yes	No
I agree to take part in the optional 'sub-study' which requires a further study visit and to undergo the procedures described in the Patient Information Leaflet.	☐	☐
I agree to my GP being informed of my participation in the study.	☐	
I agree to take part in the above study.	☐	

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Name of participant	Date	Signature
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Name of researcher	Date	Signature

Version 1.3 18/2/2020

Appendix III GP Information Sheet

BHF Glasgow Cardiovascular Research Centre
126 University Place
Glasgow
G12 8TA

Enquiries to: Dr Leanne Mooney
Telephone: 0141 330 2237
E-mail: Leanne.mooney@nhs.net
Date:

GP INFORMATION LETTER

Age-related Clonal Haematopoiesis and Inflammation in Patients with Heart Disease

Dear Doctor,

Patient name:

CHI number:

Recruited to substudy: Yes/No

I am carrying out a research project involving patients with heart failure and in patients who have coronary artery disease. Your patient has kindly agreed to take part in the study.

This study is investigating the prevalence of Age-Related Clonal Haematopoiesis (ARCH) in these patients and its association with inflammation and vascular function. ARCH is the accumulation of somatic DNA mutations in the haematopoietic stem cell, which confers an increased risk of cardiovascular events in the general population. Patients with ARCH also have a slightly increased risk of haematological malignancy with a progression rate of 0.5% per year. By selecting patients without substantial cytopaenia at baseline we are including a group who should be at lower risk for haematological malignancy.

The relevant Participant Information Leaflet is attached here for your information. Your patient will be recruited from one of the Greater Glasgow and Clyde Hospitals with either a diagnosis of decompensated heart failure or coronary artery disease. Patients recruited to the main study will have demographic and routine clinical data recorded, blood and urine will be collected to assess inflammatory and cardiovascular biomarkers and for the presence of ARCH. Patients also recruited to the sub-study will have repeat blood tests 6 to 12 weeks after their initial enrolment and will also undergo the following non-invasive assessments: brachial arterial pulse wave velocity, flow mediated dilatation, carotid intima media thickness and retinal optical coherence topography.

The study is being conducted over a period of two years. The study does not involve any additional medications. We will notify the patient's Consultant Cardiologist should any clinically significant information arise from their participation in this study, who may arrange further investigation, treatment and inform you as required. The presence of one or more ARCH-related mutations is not a reflection of a 'disease' *per se* and there is no specific treatment, nor routinely mandated haematological follow-up for patients on the basis of ARCH status. Therefore, we do not intend to disclose ARCH assessment results to you or your patient and he/she has been advised of this.

If you have any questions or require any further information, please do not hesitate to contact me on the above telephone number or e-mail address.

Yours faithfully,

Dr Leanne Mooney
Clinical Research Fellow
University of Glasgow

Investigators – Dr Ninian Lang, Professor Mark Petrie, Professor John McMurray, Professor Carl Goodyear, Professor Mhairi Copland, Dr Kristina Kirschner and Dr Leanne Mooney.

Appendix IV SOP for PBMC separation

Table 2. EasySep™ Direct Human PBMC Isolation Kit Protocol for CORD BLOOD

		EASYSEP™ MAGNETS	
STEP	INSTRUCTIONS	EasySep™ (Catalog #18000)	"The Big Easy" (Catalog #18001)
1	Prepare sample within the volume range.	0.5 - 1.5 mL	1 - 6 mL
	Add sample to required tube.	5 mL (12 x 75 mm) polystyrene round-bottom tube (e.g. Catalog #38007)	14 mL (17 x 95 mm) polystyrene round-bottom tube (e.g. Catalog #38008)
2	Add EDTA to sample.	At a final concentration of 6 mM EDTA	At a final concentration of 6 mM EDTA
3	Add Isolation Cocktail to sample. NOTE: Do not vortex cocktail.	50 µL/mL of sample	50 µL/mL of sample
	Mix and incubate.	RT for 5 minutes	RT for 5 minutes
4	Add recommended medium to top up the sample to the indicated volume. Mix by gently pipetting up and down 2 - 3 times.	Top up to double the original sample volume	Top up to double the original sample volume
5	Vortex RapidSpheres™. NOTE: Particles should appear evenly dispersed.	30 seconds	30 seconds
6	Add RapidSpheres™ to sample and mix.	50 µL/mL of original sample volume NOTE: No incubation, IMMEDIATELY proceed to next step	50 µL/mL of original sample volume NOTE: No incubation, IMMEDIATELY proceed to next step
7	Place the tube (without lid) into the magnet and incubate.	RT for 5 minutes	RT for 5 minutes
8	Pick up the magnet, and in one continuous motion invert the magnet and tube, pouring the enriched cell suspension* into a new tube.	Use a new 5 mL tube	Use a new 14 mL tube
9	Add RapidSpheres™ to the new tube containing the enriched cells and mix.	Use same volume as in step 6 NOTE: No incubation, IMMEDIATELY proceed to next step	Use same volume as in step 6 NOTE: No incubation, IMMEDIATELY proceed to next step
10	Remove the tube from the magnet; place the tube from step 9 (without lid) into the magnet and incubate for a second separation.	RT for 5 minutes	RT for 5 minutes
11	Pick up the magnet, and in one continuous motion invert the magnet and tube,** pouring the enriched cell suspension into a new tube.	Use a new 5 mL tube	Use a new 14 mL tube
12	Remove the tube from the magnet; place the tube from step 11 (without lid) into the magnet and incubate for a third separation.	RT for 5 minutes	RT for 5 minutes
13	Pick up the magnet, and in one continuous motion invert the magnet and tube,** pouring the enriched cell suspension into a new tube.	Isolated cells are ready for use	Isolated cells are ready for use

RT - room temperature (15 - 25°C)
* Following the first magnetic separation, the collected cells may contain a significant amount of RBCs and may look similar to the original unprocessed human cord blood sample.
** To minimize RBC contamination in the isolated cells, pour off the sample along a clean area of the tube (i.e. the opposite side to where the sample was poured in).

Appendix V: Standard operating procedure DNA isolation (Qiagen)

Midi Spin

Protocol: Purification of DNA from Whole Blood using the QIAamp Blood Midi Kit (Spin Protocol)

This protocol is for purification of genomic DNA from up to 2 ml of whole blood.

Important points before starting

- Blue (marked with a ■) denotes values for 0.3–1 ml of whole blood; red (marked with a ▲) denotes values for 1–2 ml of whole blood.
- Do not use more than 2 x 10^7 white blood cells. For samples containing more than 2 x 10^7 white blood cells, use the QIAamp DNA Blood Maxi Kit.
- All centrifugation steps are carried out at room temperature (15–25°C). Do not use a fixed-angle rotor.
- For samples with low white blood cell counts (i.e., <20,000 genome equivalents), add carrier DNA at the start of the procedure. Since the carrier DNA will co-purify with the DNA from the blood, make sure that this will not interfere with any downstream analyses, such as PCR.

Things to do before starting

- Equilibrate samples to room temperature (15–25°C) before starting.
- Prepare a 70°C water bath for use in step 4 of the protocol.
- Ensure that Buffer AW1, Buffer AW2, and QIAGEN Protease have been prepared according to instructions (page 17).
- If a precipitate has formed in Buffer AL, redissolve by incubating at 56°C.

Procedure

1. **Pipet ■ 100 µl or ▲ 200 µl QIAGEN Protease into the bottom of a 15 ml centrifuge tube (not provided).**
2. **Add ■ 0.3–1 ml or ▲ 1–2 ml blood and mix briefly.**

   Bring the volume of the sample up to ■ 1 ml or ▲ 2 ml with PBS, if necessary, before adding to the centrifuge tube.

   Note: QIAGEN Protease (or proteinase K) can be added to samples that have already been dispensed into centrifuge tubes. In this case, it is important to ensure proper mixing after adding the enzyme.
3. **Add ■ 1.2 ml or ▲ 2.4 ml Buffer AL, and mix thoroughly by inverting the tube 15 times, followed by additional vigorous shaking for at least 1 min. Invert multiple tubes simultaneously by clamping them into a rack using another empty rack, grasping both racks, and inverting them together.**

   To ensure adequate lysis, the sample must be mixed thoroughly with Buffer AL to yield a homogenous solution.

   Note: Do not add QIAGEN Protease directly to Buffer AL.

4. **Incubate at 70°C for 10 min.**

   DNA yield reaches a maximum after lysis for 10 min at 70°C, but longer incubation times will not adversely affect yield.

5. **Add ■ 1 ml or ▲ 2 ml ethanol (96–100%) to the sample, and mix by inverting the tube 10 times, followed by additional vigorous shaking.**

   To ensure efficient binding, it is essential that the sample is mixed thoroughly after addition of ethanol to yield a homogeneous solution.

   Note: Only use 96–100% ethanol. Other alcohols may result in reduced yield and purity. Do not use denatured alcohol, which contains other substances such as methanol or methylethylketone.

6. **Carefully transfer ■ all or ▲ one half of the solution from step 5 onto the QIAamp Midi column placed in a 15 ml centrifuge tube (provided), taking care not to moisten the rim. Close the cap and centrifuge at 1850 x *g* (3000 rpm) for 3 min. Proceed with ■ step 8 (skip step 7) or ▲ step 7.**

   ■ If the initial sample volume was ≤1 ml, apply all of the lysate to the QIAamp Midi column in the step above. Skip step 7 and go directly to step 8.

   If more convenient, lysate can be loaded onto the QIAamp Midi column up to the level of the top screw thread on the 15 ml centrifuge tube.

   If the solution has not completely passed through the membrane, centrifuge again at a slightly higher speed.

   Do not overtighten caps. If caps are tightened until they snap they may loosen during centrifugation and damage the centrifuge.

   Note: Always hold the closed QIAamp Midi columns in an upright position as liquid may pass through the ventilation slots on the rims of the columns even if caps are closed.

7. **▲ Remove the QIAamp Midi column, discard the filtrate, and place the QIAamp Midi column back into the 15 ml centrifuge tube. Load the remainder of the solution from step 5 onto the QIAamp Midi column. Close the cap and centrifuge again at 1850 x *g* (3000 rpm) for 3 min.**

   ■ This step is not necessary when starting with ≤1 ml blood. Proceed directly with step 8.

   Note: Wipe off any spillage from the thread of the 15 ml centrifuge tube before re-inserting the QIAamp Midi column.

   Do not wet the rim of the QIAamp Midi column. Close each column to avoid cross-contamination during centrifugation. If the solution has not completely passed through the membrane, centrifuge again at a slightly higher speed.

8. **Remove the QIAamp Midi column, discard the filtrate, and place the QIAamp Midi column back into the 15 ml centrifuge tube.**

   Note: Wipe off any spillage from the thread of the 15 ml centrifuge tube before re-inserting the QIAamp Midi column.

   If the filtrate is not removed, the nozzle of the QIAamp Midi column will be submerged in the filtrate, and washing efficacy will be reduced.

9. **Carefully, without moistening the rim, add 2 ml Buffer AW1 to the QIAamp Midi column. Close the cap and centrifuge at 4500 x *g* (5000 rpm) for 1 min.**

   Note: Do not discard the flow-through at this stage. Continue directly with step 10.

10. **Carefully, without moistening the rim, add 2 ml Buffer AW2 to the QIAamp Midi column. Close the cap and centrifuge at 4500 x *g* (5000 rpm) for 15 min.**

    Note: The increased centrifugation time should remove all traces of Buffer AW2 from the QIAamp Midi column before elution. If the centrifugal force is below 4000 x *g*, incubating the QIAamp Midi column for 10 min at 70°C in an incubator to evaporate residual ethanol is recommended. Residual ethanol in the eluate may cause inhibition of PCR leading to false-negative results.

11. **Place the QIAamp Midi column in a clean 15 ml centrifuge tube (provided), and discard the collection tube containing the filtrate.**

    Note: Use a wet tissue paper to wipe any spillage off the QIAamp Midi column before insertion into the 15 ml centrifuge tube.

12. **Pipet ■ 200 µl or ▲ 300 µl Buffer AE or distilled water, equilibrated to room temperature (15–25°C), directly onto the membrane of the QIAamp Midi column and close the cap. Incubate at room temperature for 5 min, and centrifuge at 4500 x *g* (5000 rpm) for 2 min.**

    For long-term storage of DNA, eluting in Buffer AE and storing in aliquots at –30 to –15°C is recommended since DNA is subject to acid hydrolysis if dissolved in water.

13. **For highly concentrated DNA, continue with step 13a. For maximum DNA yield, continue with step 13b.**

    Note: Highly concentrated DNA may be desirable for applications such as restriction digestion and Southern blotting. For other applications, increasing the total DNA yield may be preferable. (For detailed information regarding elution efficiency, see Tables 2A and 2B on page 12.)

13a. **For maximum concentration: Reload the eluate (■ 200 µl or ▲ 300 µl) containing the DNA onto the membrane of the QIAamp Midi column. Close the cap and incubate at room temperature for 5 min. Centrifuge at 4500 x *g* (5000 rpm) for 2 min.**

    Note: Less than ■ 200 µl or ▲ 300 µl will be eluted from the column, but this has no effect on DNA yield.

Midi Spin

13b. **For maximum yield: Pipet ■ 200 µl or ▲ 300 µl fresh Buffer AE or distilled water, equilibrated to room temperature, onto the membrane of the QIAamp Midi column. Close the cap and incubate at room temperature for 5 min. Centrifuge at 4500 x *g* (5000 rpm) for 2 min.**

    Note: Less than ■ 200 µl or ▲ 300 µl will be eluted from the column, but this has no effect on DNA yield.

Appendix VI Complete list of CHIP mutations observed in patients with decompensated HF

Study ID	CHIP mutations observed in HFpEF			
00/00/03	ASXL1	WT1		
00/00/04	KIT	TP53		
00/00/08	TP53			
00/00/10	DNMT3A	U2AF1		
00/00/28	DNMT3A			
00/00/30	DNMT3A	GATA2	KIT	SRSF2
00/00/32	ASXL1	DNMT3A	EZG2	
00/00/33	DNMT3A			
00/00/38	STAG2			
00/00/44	IDH2	SRSF2	TET2	TP53
00/00/45	DNMT3A	TP53		
00/00/47	ZRSR2			
00/00/49	DNMT3A			
00/00/66	TET2			
00/00/71	TP53			
00/00/72	DNMT3A			
00/00/76	DNMT3A			
00/00/80	DNMT3A	ZRSR2		

00/00/82	EZH2			
00/00/88	ASXL1			
00/00/94	ASXL1	DNMT3A	SRSF2	UR2AF1

Study ID	**CHIP mutations observed in HFrEF**													
00/00/05	CEBPA													
00/00/11	TP53													
00/00/12	DNMT3A													
00/00/17	DNMT3A													
00/00/18	ZRSR2													
00/00/21	STAG2													
00/00/22	RUNX1	TP53												
00/00/23	DNMT3A	SRSF2												
00/00/24	ZRSR2													
00/00/26	DNMT3A													
00/00/27	WT1													
00/00/29	EZH2													
00/00/31	ASXL1	DNMT3A	EZH2	NRAS	STAG2	WT1								
00/00/34	GATA1	SRSF2	STAG2	WT1										
00/00/36	ASXL1	CEBPA	DNMT3A	ETV5	EZH2	GATA2	IDH2	PTPN11	RUNX1	SF3B1	SRSF2	STAG2	U2AF1	WT1
00/00/37	PHF6													

00/00/39	BCOR	CBL	DNMT3A	STAG2										
00/00/40	GATA2													
00/00/42	CEBP1	TP53												
00/00/43	SF3B1	SRSF2	TET2											
00/00/46	RUNX1													
00/00/48	DNMT3A													
00/00/51	DNMT3A													
00/00/59	DNMT3A													
00/00/95	DNMT3A													

Appendix VII- NLR in PARADIGM AND PARAGON-HF

Table 1 Baseline characteristics according to NLR tertile in PARADIGM-HF

	Tertile 1 n=2659	Tertile 2 n=2659	Tertile 3 n=2660	P value
Neutrophil, median (range)	5.22 (1.46-16.81)	4.23 (1.45-9.37)	3.25 (0.25-8.57)	<0.001
Lymphocyte, median (range)	1.35 (0.12-3.39)	1.79 (0.6-3.95)	2.23 (0.57-29.2)	<0.001
NLR, median (range)	3.67 (2.87-53.83)	2.37 (1.94-2.87)	1.53 (0.09-1.94)	<0.001
Age- years	65.2±11.0	63.6±11.3	62.6 ±11.7	<0.001
Female sex- no. (%)	487 (18.3)	570 (21.4)	698 (26.2)	<0.001
Race or Ethnic group- no (%)				<0.001
White	1935 (72.8)	1750 (65.8)	1564 (58.8)	
Black	75 (2.8)	106 (4.0)	232 (8.7)	
Asian	414 (15.6)	497 (18.7)	556 (20.9)	
Other	235 (8.8)	306 (11.5)	308 (11.6)	
Region- no. (%)				<0.001
North America	278 (10.5)	154 (5.8)	140 (5.3)	

Latin America	365 (13.7)	471 (17.7)	522 (19.6)	
Western Europe and other	706 (26.6)	645 (24.3)	589 (22.1)	
Central Europe	907 (34.1)	898 (33.8)	858 (32.3)	
Asia-Pacific	403 (15.2)	491 (18.5)	551 (20.7)	
Systolic Blood Pressure- mmHg	122±16	121±15	121±15	<0.001
Heart rate- beats/min	73±12	72±12	72±12	<0.001
Body Mass Index- kg/m^2	28.4±5.7	28.3±5.4	27.7±5.4	<0.001
Serum creatinine- μmol/L	103.6±27.9	99.2±26.7	95.4±23.9	<0.001
Clinical features of heart failure				
Ischemic etiology – no. (%)	1693 (63.7)	1595 (60)	1472 (55.3)	<0.001
Left ventricular ejection fraction – %	29.3±6.3	29.6±6.1	29.4±6.2	0.92
NT-pro-BNP- pg/ml	1854 (987-393s4)	1554 (891-3026)	1484 (821-2874)	<0.001
NYHA class- no. (%)				<0.001
I	90 (3.4)	118 (4.4)	168 (6.3)	
II	1820 (68.6)	1936 (72.9)	1893 (71.2)	
III	726 (27.4)	584 (22.0)	575 (21.6)	
IV	18 (0.7)	18 (0.7)	21 (0.8)	
Signs of heart failure- no. (%)				
Jugular venous distension	274 (10.3)	255 (9.6)	245 (9.2)	0.18

Edema	655 (24.7)	526 (19.8)	480 (18.1)	<0.001
Third heart sound	258 (9.7)	260 (9.8)	245 (9.2)	0.54
Rales	241 (9.1)	216 (8.1)	176 (6.6)	0.001
Prior hospitalization for heart failure- no (%)	1708 (64.2)	1651 (62.1)	1648 (62)	0.09
Medical history – no. (%)				
Hypertension	1981 (74.5)	1880 (70.7)	1785 (67.1)	<0.001
Diabetes	1065 (40.1)	916 (34.4)	785 (29.5)	<0.001
Atrial fibrillation	678 (25.9)	610 (23.3)	637 (24.3)	0.18
Myocardial infarction	1231 (46.3)	1181 (44.4)	1004 (37.7)	<0.001
Stroke	250 (9.4)	232 (8.7)	202 (7.6)	0.02
Pre-trial use of ACE inhibitor	2082 (78.3)	2075 (78)	2045 (76.9)	0.21
Pre-trial use of ARB	585 (22)	590 (22.2)	625 (23.5)	0.19
Treatments at randomisation – no. (%)				
Diuretic	2191 (82.4)	2130 (80.1)	2076 (78)	<0.001
Digitalis	827 (31.1)	797 (30)	788 (29.6)	0.24
Beta-blocker	2449 (92.1)	2485 (93.5)	2493 (93.7)	0.02
Mineralocorticoid receptor antagonist	1433 (53.9)	1511 (56.8)	1502 (56.5)	0.06
Insulin-treated diabetes	298 (11.2)	221 (8.3)	170 (6.4)	<0.001
ICD	320 (12)	252 (9.5)	216 (8.1)	<0.001
CRT	238 (9)	179 (6.7)	126 (4.7)	<0.001

Values reported are either numbers (%), means (±standard deviations) or medians (range), where stated. *RLC- Relative lymphocyte count, NYHA- New York Heart Association , NT-proBNP- N terminal B-type natriuretic peptide, ACE- angiotensin-converting enzyme, ARB- angiotensin receptor blocker, ICD- Implantable Cardiac Defibrillation, ICD- Implantable Cardioverter Defibrillator, CRT- Cardiac Resynchronisation Therapy.*

Table 2: Baseline characteristics according to NLR tertile in PARAGON-HF

	Tertile 1 **n=1550**	**Tertile 2** **n=1637**	**Tertile 3** **n=1608**	**P value**
Neutrophil, median (range)	5.1 (1.7-15)	4.1 (1.5-9.2)	3.2 (0.2-9)	<0.001
Lymphocyte, median (range)	1.3 (0.4-3.4)	1.6 (0.6-3.8)	2.0 (0.7-17.9)	<0.001
NLR, median (range)	3.88 (3.04-19.8)	2.5 (>2.08-3)	1.67 (0.13-<2.08)	<0.001
Age- years	73.6±8.3	72.4±8.4	72.3±8.6	<0.001
Female sex- no. (%)	719 (46.4)	820 (50.1)	940 (58.5)	<0.001
Race or Ethnic group- no (%)				<0.001
White	1312 (84.6)	1346 (82.2)	1249 (77.7)	
Black	18 (1.2)	25 (1.5)	59 (3.7)	
Asian	173 (11.2)	209 (12.8)	224 (13.9)	
Other	47 (3.0)	57 (3.5)	76 (4.7)	
Region- no. (%)				<0.001
North America	266 (17.2)	153 (9.3)	140 (8.7)	
Latin America	97 (6.3)	120 (7.3)	153 (9.5)	
Western Europe and other	512 (33)	460 (28.1)	417 (25.9)	
Central Europe	452 (29.2)	636 (38.9)	627 (39)	
Asia-Pacific	223 (14.4)	268 (16.4)	271 (16.9)	

Systolic Blood Pressure- mmHg	130±16	131±15	131±15	0.12
Heart rate- beats/min	71±13	71±12	70±12	0.01
Body Mass Index- kg/m^2	30.3±5.1	30.2±4.9	30.2±5.0	0.56
Serum creatinine- μmol/L	100.8±28.4	95.2±26.5	93.3±26.6	<0.001
Clinical features of heart failure				
Ischemic etiology – no. (%)	562 (36.3)	579 (35.4)	582 (36.2)	0.96
Left ventricular ejection fraction – %	57.6±7.7	57.5±8.0	57.5±7.9	0.68
NT-proBNP– pg/ml	1039 (527-1850)	889 (467-1540)	769 (425-1468)	<0.001
NYHA class- no. (%)				0.44
I	46 (3.0)	51 (3.1)	40 (2.5)	
II	1175 (75.8)	1279 (78.2)	1251 (77.8)	
III	319 (20.6)	302 (18.5)	311 (19.4)	
IV	10 (0.6)	4 (0.2)	5 (0.3)	
Signs of heart failure- no. (%)				
Jugular venous distension	256 (16.7)	199 (12.2)	199 (12.5)	0.001
Oedema	634 (41.5)	618 (37.8)	564 (35.1)	<0.001
Third heart sound	40 (2.6)	27 (1.7)	43 (2.7)	0.86
Rales	106 (6.8)	122 (7.5)	117 (7.3)	0.63
Prior heart failure hospitalization - no (%)	774 (49.9)	793 (48.4)	739 (46.0)	0.03
Medical history – no. (%)				

Hypertension	1480 (95.5)	1572 (96.0)	1531 (95.2)	0.7
Diabetes	769 (49.6)	702 (42.9)	590 (36.7)	<0.001
Atrial fibrillation	551 (35.7)	547 (33.5)	454 (28.3)	<0.001
Myocardial infarction	363 (23.4)	367 (22.4)	353 (22)	0.33
Stroke	175 (11.3)	169 (10.3)	164 (10.2)	0.32
Pre-trial use of ACE inhibitor	616 (39.7)	697 (42.6)	668 (41.5)	0.31
Pre-trial use of ARB	726 (46.8)	776 (47.4)	747 (46.5)	0.83
Treatments at randomisation – no. (%)				
Diuretic	1495 (96.5)	1569 (95.8)	1520 (94.5)	0.008
Digitalis	174 (11.2)	157 (9.6)	119 (7.4)	<0.001
Beta-blocker	1204 (77.7)	1341 (81.9)	1275 (79.3)	0.27
Mineralocorticoid receptor antagonist	433 (27.9)	433 (26.5)	373 (23.2)	0.002
Insulin-treated diabetes	279 (18.0)	209 (12.8)	169 (10.5)	<0.001
Implantable cardiac defibrillator	8.0 (0.5)	5.0 (0.3)	5.0 (0.3)	0.35

Values reported are either numbers (%), means (±standard deviations) or medians (range), where stated.*RLC- Relative lymphocyte count, NYHA- New York Heart Association , NT-proBNP- N terminal B-type natriuretic peptide, ACE- angiotensin-converting enzyme, ARB- angiotensin receptor blocker, ICD- Implantable Cardiac Defibrillation, ICD- Implantable Cardioverter Defibrillator, CRT- Cardiac Resynchronisation Therapy.*

Table 3 Outcomes according to tertile of neutrophil/lymphocyte ratio (referent to Tertile 3) and per 10% increase in NLR in PARADIGM-HF

	Tertile 1 n=2659	**Tertile 2 n=2659**	**Tertile 3 n=2660**	**Per 10% increase in NLR n=7918**
Primary End Point				
Event number	777	612	533	
Event rate per 100 patient years (95% CI)	14.9 (13.87-16.0)	11.1 (10.3-12.07)	9.6 (8.8-10.42)	
Unadjusted HR	1.00 (ref)	0.76 (0.68-0.85)	0.65 (0.58-0.72)	1.99 (1.67-2.36)
Adjusted HR[a]	1.00 (ref)	0.85 (0.77-0.95)	0.77 (0.69-0.86)	1.45 (1.18-1.79)
Hospitalisation for Heart Failure				
Event number	487	352	289	
Event rate per 100 patient years (95% CI)	9.3 (8.53-10.19)	6.4 (5.78-7.12)	5.2 (4.63-5.83)	
Unadjusted HR	1.00 (ref)	0.72 (0.63-0.83)	0.58 (0.5-0.68)	2.1 (1.72-2.55)
Adjusted HR[a]	1.00 (ref)	0.82 (0.71-0.94)	0.71 (0.61-0.83)	1.51 (1.18-1.93)

Death from Cardiovascular Causes				
Event number	461	390	336	
Event rate per 100 patient years (95% CI)	8.0 (7.3-8.77)	6.6 (6.01-7.33)	5.7 (5.13-6.35)	
Unadjusted HR	1.00 (ref)	0.81 (0.71-0.93)	0.68 (0.59-0.78)	1.91 (1.49-2.46)
Adjusted HR[a]	1.00 (ref)	0.91 (0.80-1.05)	0.83 (0.71-0.96)	1.31 (0.96-1.80)
Death from Any Cause				
Event number	586	483	396	
Event rate per 100 patient years (95% CI)	10.2 (9.38-11.03)	8.2 (7.52-8.99)	6.7 (6.1-7.42)	
Unadjusted HR	1.00 (ref)	0.79 (0.70-0.90)	0.64 (0.56-0.73)	2.16 (1.79-2.61)
Adjusted HR[a]	1.00 (ref)	0.90 (0.79-1.01)	0.77 (0.68-0.88)	1.6 (1.25-2.02)

[a] *Adjusted for: region, treatment, age, sex, race, systolic blood pressure, heart rate, body mass index, serum creatinine, clinical features of heart failure (ischemic etiology, LVEF, NT-proBNP (log)), NYHA class, hypertension, diabetes, atrial fibrillation, hospitalization for heart failure, myocardial infarction, stroke, duration of heart failure.*

Table 4 Outcomes according to tertile of NLR (referent to Tertile 3) and per 10% increase in NLR in PARAGON-HF

	Tertile 1 n=1550	**Tertile 2 n=1637**	**Tertile 3 n=1608**	**Per 10% increase in NLR n=4795**
Primary End Point				
Event number	418	356	308	
Event rate per 100 patient years (95% CI)	10.7 (9.72-11.77)	8.2 (7.39-9.09)	7.0 (6.28-7.86)	
Unadjusted HR	1.00 (ref)	0.82 (0.71-0.94)	0.70 (0.61-0.82)	2.92 (2.11-4.03)
Adjusted HR[a]	1.00 (ref)	0.92 (0.80-1.07)	0.82 (0.70-0.96)	2.23 (1.56-3.17)
Hospitalization for Heart Failure				
Event number	333	275	229	
Event rate per 100 patient years (95% CI)	8.5 (7.65-9.49)	6.3 (5.62-7.12)	5.2 (4.59-5.95)	

Unadjusted HR	1.00 (ref)	0.82 (0.70-0.96)	0.68 (0.58-0.81)	2.90 (2.01-4.17)
Adjusted HR[a]	1.00 (ref)	0.92 (0.78-1.09)	0.80 (0.67-0.95)	2.31 (1.55-3.45)
Death from Cardiovascular Causes				
Event number	166	135	115	
Event rate per 100 patient years (95% CI)	3.8 (3.26-4.41)	2.8 (2.40-3.37)	2.4 (2.02-2.92)	
Unadjusted HR	1.00 (ref)	0.75 (0.60-0.94)	0.63 (0.50-0.81)	3.06 (1.86-5.03)
Adjusted HR	1.00 (ref)	0.90 (0.71-1.13)	0.78 (0.61-1.0)	1.97 (1.14-3.41)
Death from Any Cause				
Event number	288	222	180	
Event rate per 100 patient years (95% CI)	6.6 (5.86-7.38)	4.7 (4.1-5.34)	3.8 (3.28-4.4)	
Unadjusted HR	1.00 (ref)	0.7 (0.59-0.84)	0.56 (0.47-0.68)	3.4 (2.34-4.95)
Adjusted HR[a]	1.00 (ref)	0.83 (0.69-1.0)	0.7 (0.58-0.85)	2.19 (1.44-3.33)

[a] Adjusted for: region, treatment, age, sex, race, systolic blood pressure, heart rate, body mass index, serum creatinine, clinical features of heart failure (ischemic etiology, LVEF, NT-proBNP (log)), NYHA class, hypertension, diabetes, atrial fibrillation, hospitalization for heart failure, myocardial infarction, stroke, duration of heart failure.

Figure 1 Kaplan-Meier curves for outcomes of interest, according to tertile of NLR in PARADIGM-HF

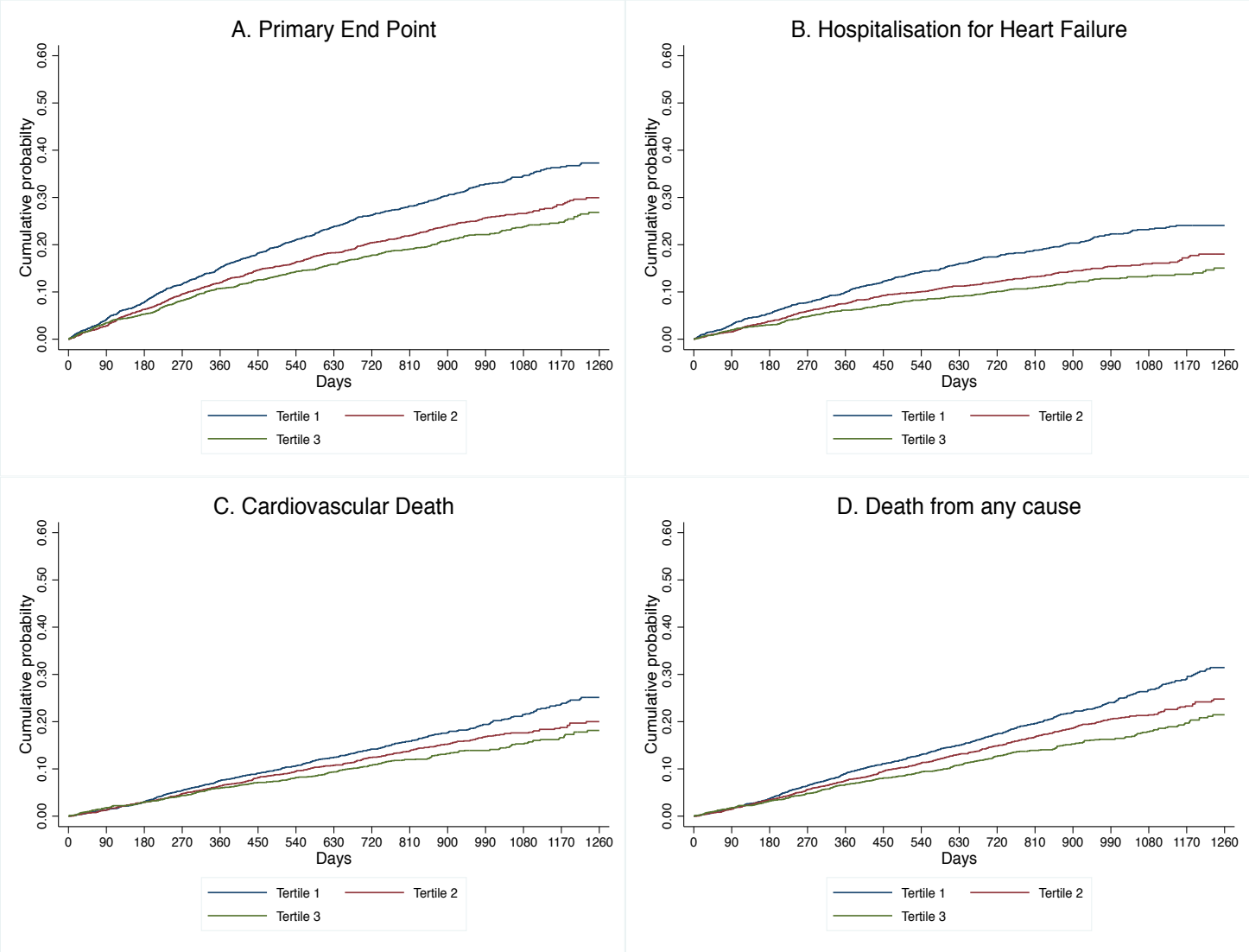

Figure 2 Kaplan-Meier curves for outcomes of interest, according to tertile of NLR in PARAGON-HF

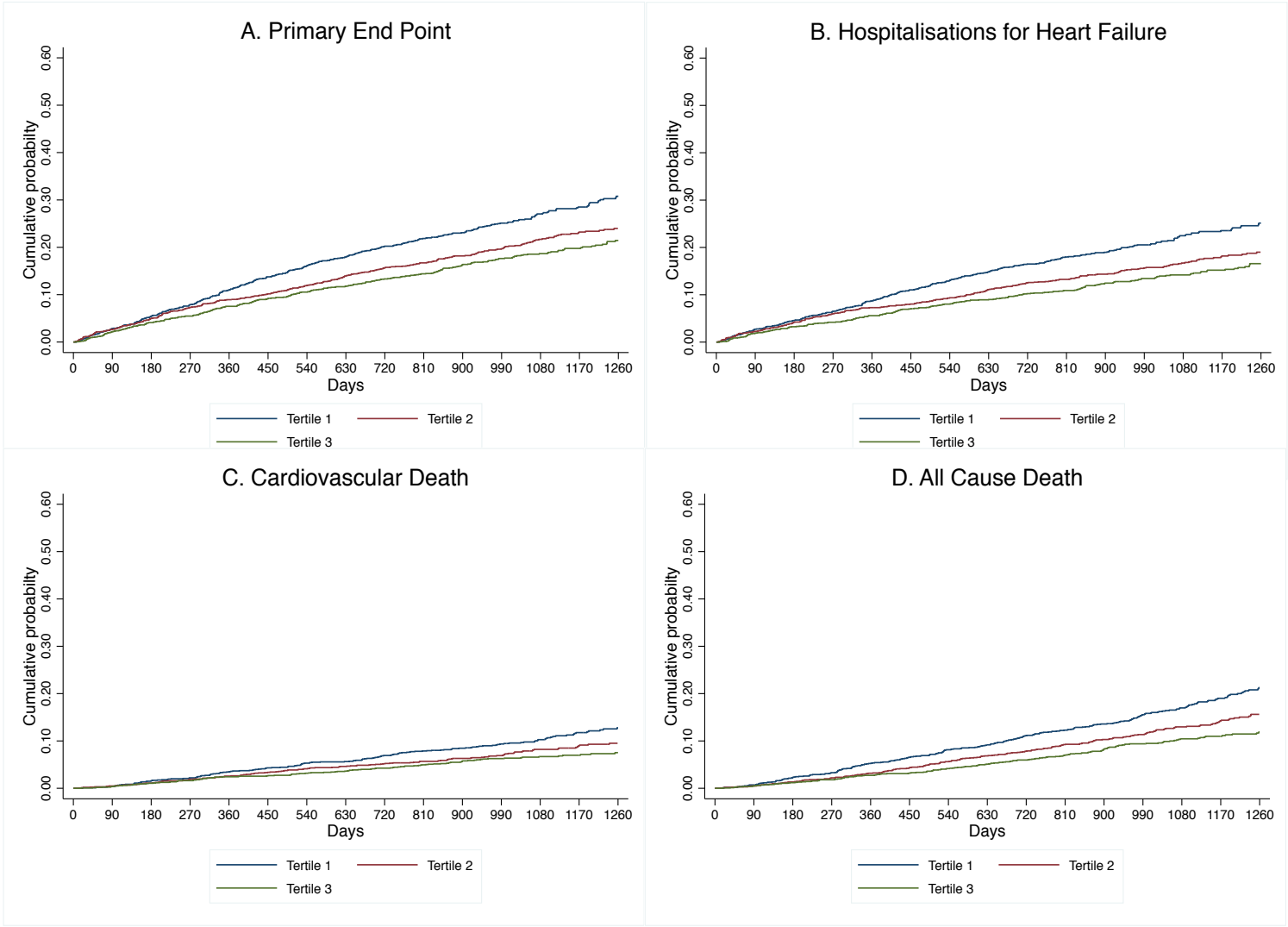

Figure 3: Adjusted splines for outcomes according to relative lymphocyte count relative to median RLC in PARADIGM-HF

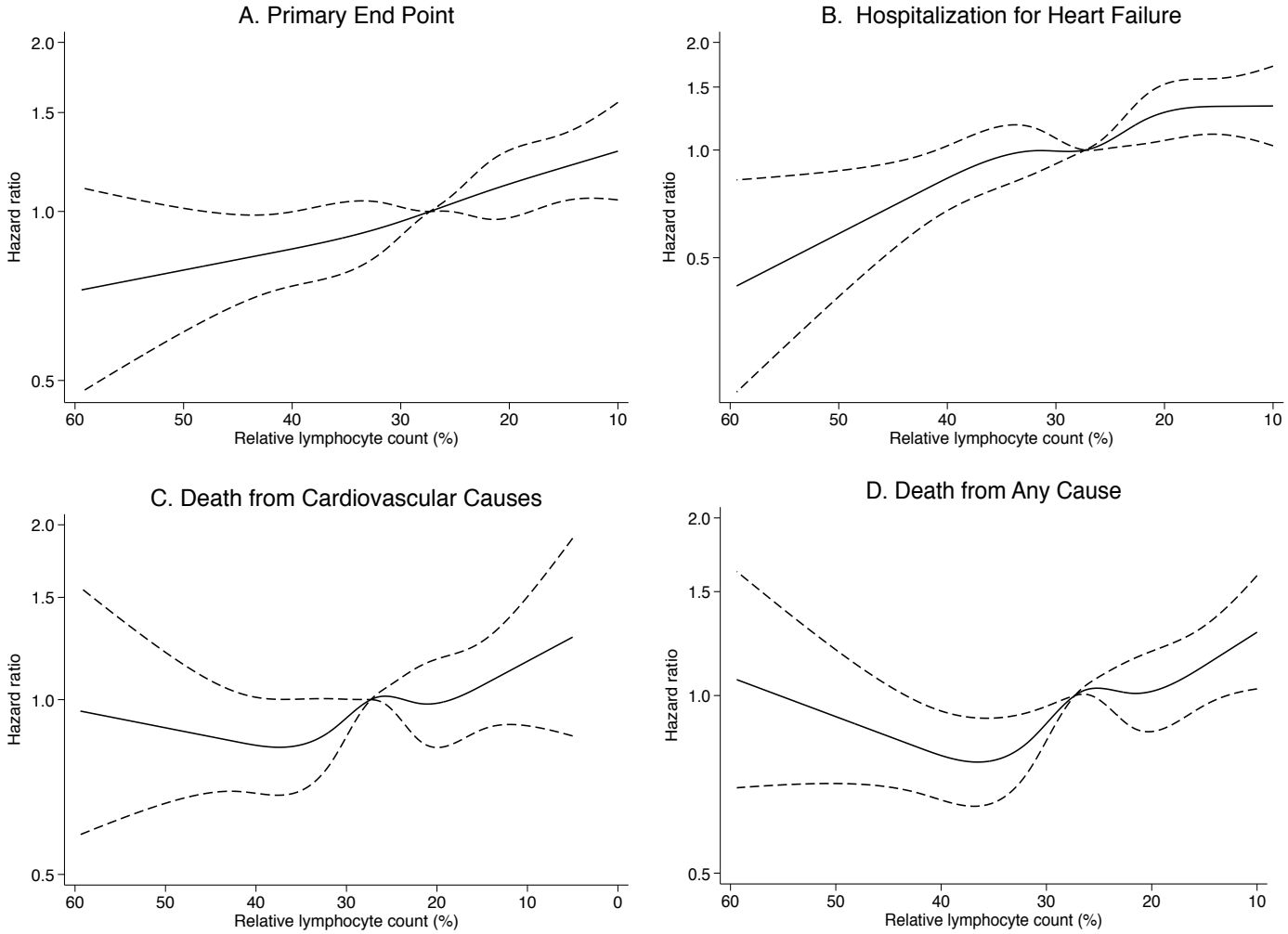

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Figure 4: Adjusted splines for outcomes according to relative lymphocyte count relative to median RLC in PARAGON-HF

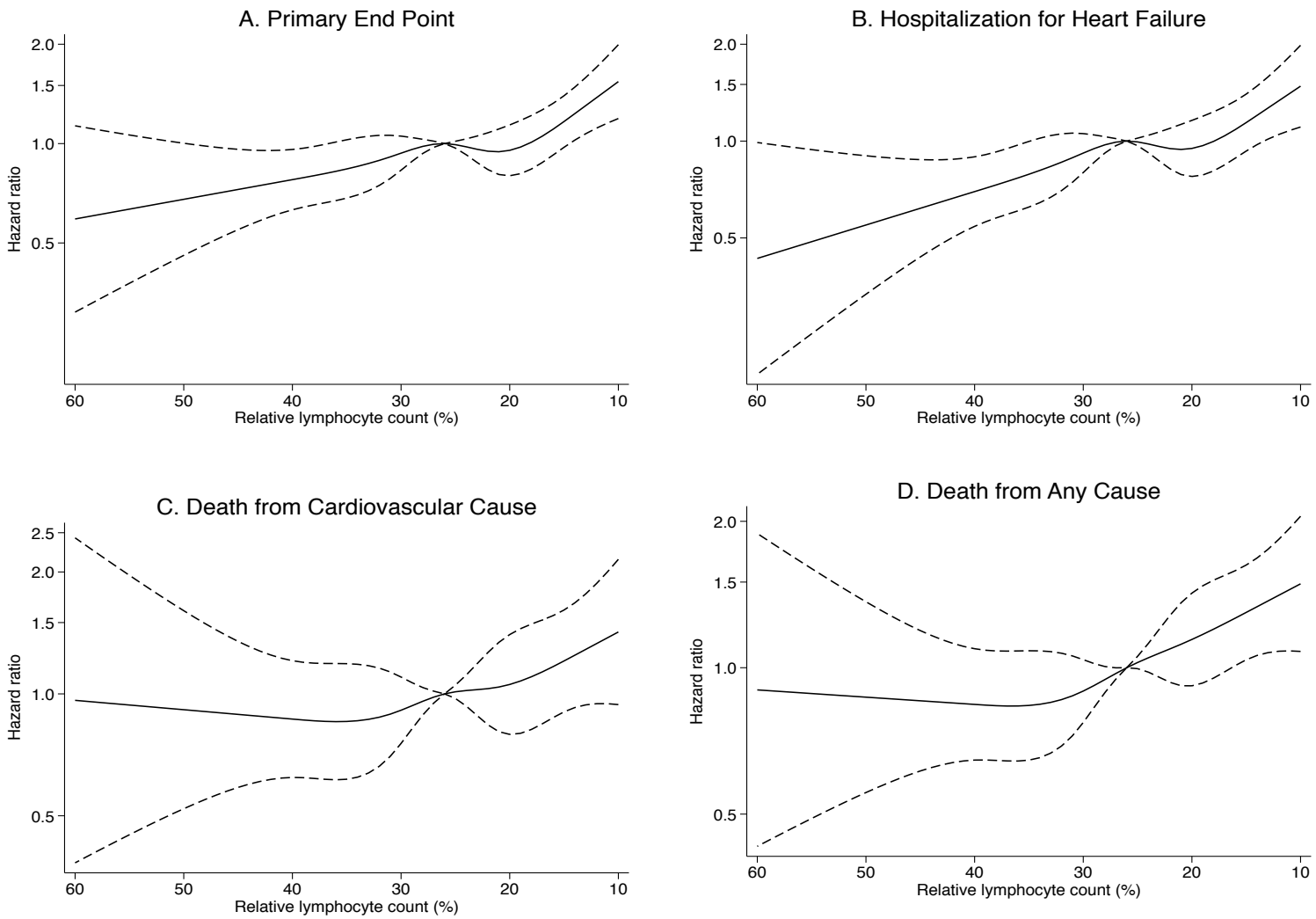


Figure 5: Treatment effect according to RLC in PARADIGM-HF

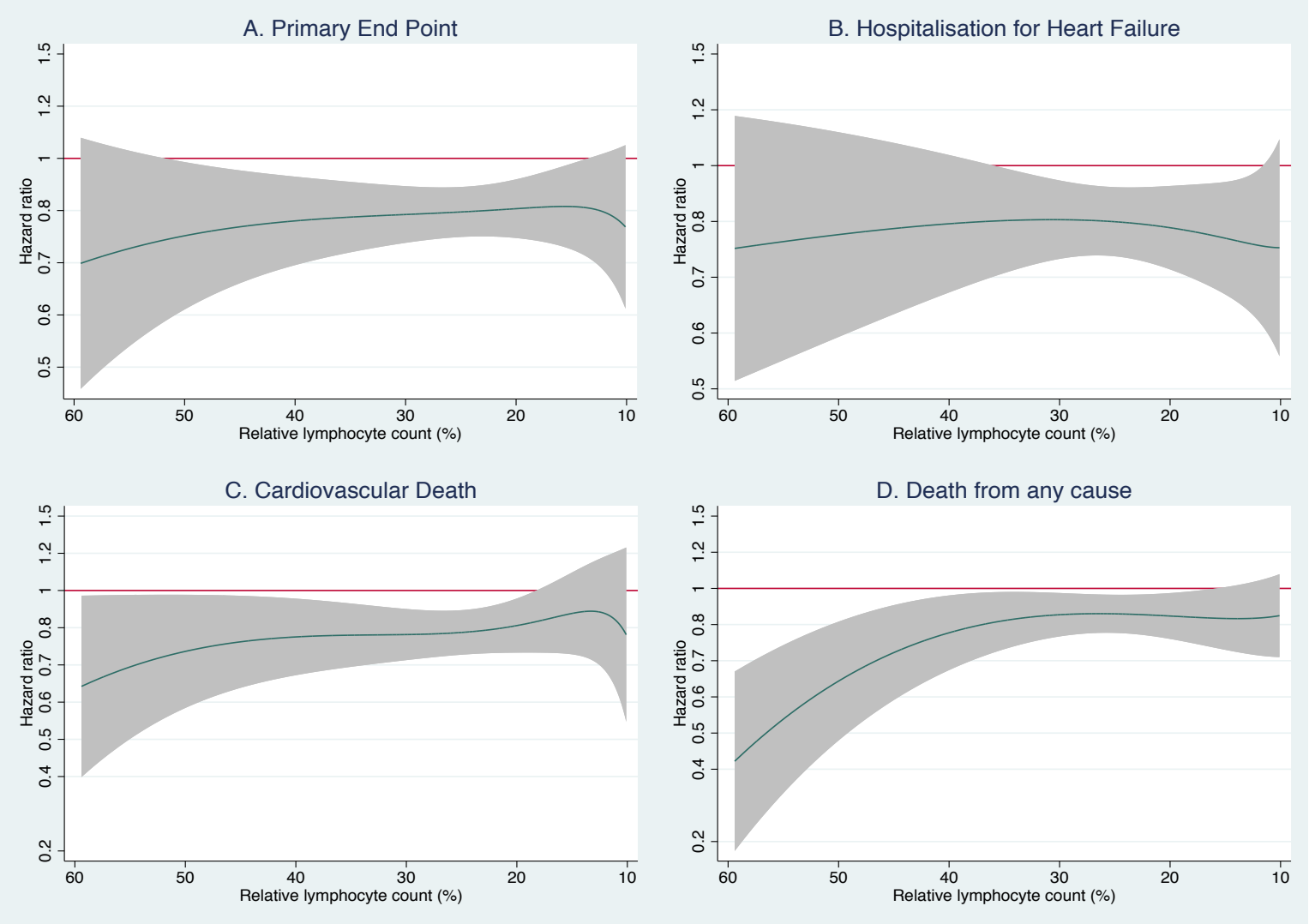
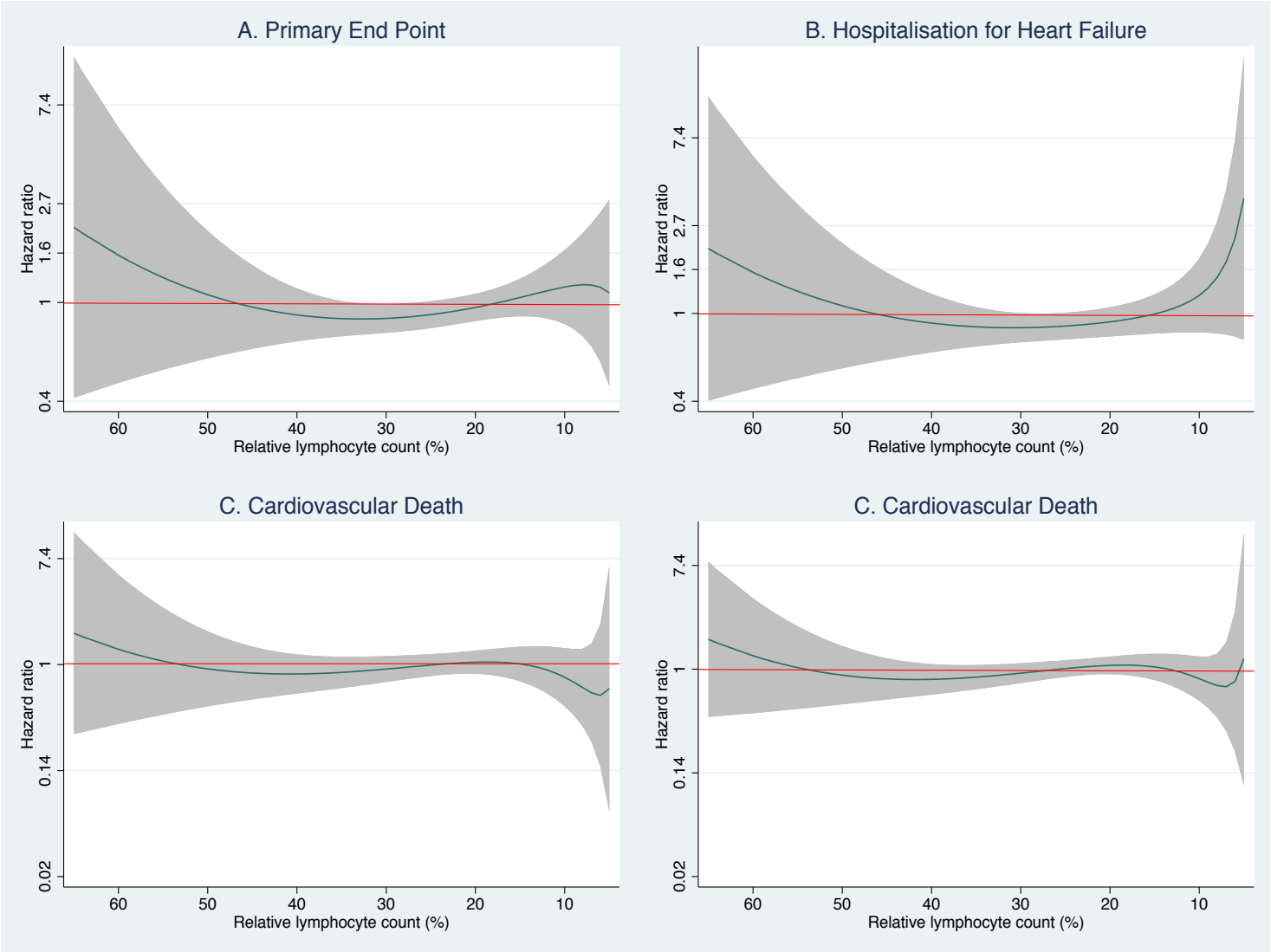


Figure 6: Treatment effect according to RLC in PARAGON-HF



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