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Inflammatory biomarkers in ischemic heart disease

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Chapter 1

GENERAL INTRODUCTION

Scope of the problem

Cardiovascular disease (CVD) is a major cause of death in the western world and accounts for 17.8 million deaths per year, representing 31% of all global deaths[1]. Around 50% of these cardiovascular deaths are due to coronary artery disease (CAD), leading to ischemic heart disease (IHD), myocardial infarction and eventually to death. Although the age-standardized mortality rates are decreasing (compared to 1980 a decrease of 70% in men and 63% in women), the burden of the disease remains high (with healthcare costs of 10.2 billion euro in 2017, which is 11.7% of the total health care costs) and novel therapeutic strategies are still needed to improve clinical outcome [1–3].

The cardiovascular disease continuum

CVD has been depicted as a continuum of events, rather than a single disease process by Dzau *et al.*[4]. This continuum of complex events is initiated by a vast number of risk factors and progresses through numerous pathophysiological pathways to the development of end-stage heart disease (**Figure 1**). Intervention anywhere along the continuum of events can disrupt the pathophysiological process. However, even within these specific processes, factors like timing seem crucial while certain factors may be harmful at one time but may be considered protective in other circumstances.

FIGURE 1. Adapted from Dzau *et al.* (Circulation (2006)) [4]. The cardiovascular disease continuum.

Myocardial infarction

Myocardial infarction (MI) is defined as the presence of acute myocardial injury detected by abnormal cardiac biomarkers in the setting of evidence of acute myocardial ischaemia [5,6]. This might be due to a rupture or fissuring of an atherosclerotic plaque, which exposes the blood to thrombogenic lipids and leads to activation of platelets and the coagulation cascade. Thrombus formation takes place next to the atherosclerotic lesion and can result in a (sub) total occlusion of the coronary artery and myocardial ischemia. This is the so-called type 1 MI[6]. The presence of prolonged ischemia causes irreversible injury and includes death of cardiac myocytes, which already starts after 15-30 minutes. Depending on the electrocardiogram (ECG) features two different forms of MI can be distinguished, i.e. ST-elevation MI (STEMI) and non-ST elevation MI (NSTEMI) [5]. STEMI is in the majority of cases caused by a complete blockage of coronary artery requiring fast reperfusion with primary percutaneous coronary intervention (PCI) or thrombolysis.

Myocardial infarction and inflammation

Besides timely reperfusion, the treatment of STEMI patients has improved tremendously over the past decades [7]. After successful introduction of antiplatelet inhibitors, beta-blockers, statins and renin-angiotensin-aldosterone inhibitors, more recently there is increasing interest to target inflammation, which is known to play an important role along the different stages of the cardiovascular disease continuum [4,8–16]. Epidemiological studies suggest that circulating concentrations of inflammatory markers, such as C-reactive protein (CRP), are associated with subsequent risk of atherosclerosis formation, coronary heart disease (CHD) and cardiac remodelling [17]. Zougari and colleagues observed in mice extensive interaction between the immune cells B lymphocytes and monocytes after acute MI. This interaction occurred via the circulating cytokines CCL7 and BAFF (i.e. small proteins that are important in cell signalling) and was associated with decreased heart function [16]. They also showed that high concentrations of CCL7 and BAFF in MI patients predicts increased risk of death or recurrent MI. This supports the hypothesis that cell-cell interaction, via cytokines, is of importance along the cardiovascular disease continuum and might be a potential therapeutic target in the treatment of MI and its cardiac remodelling. Another recent example is the CANTOS trial. In 2017, the CANTOS trial showed that anti-inflammatory therapy targeting the interleukin-1 β (IL-1 β) immunity pathway with the monoclonal antibody canakinumab leads to a significantly lower rate of recurrent cardiovascular events in patients with previous myocardial infarction (MI)[18]. As IL-1 β drives the IL-6 signalling pathway, IL-6 could be a potential therapeutic target in the treatment of MI as

well [19]. However, IL-6 has a context-dependent pro- and anti-inflammatory properties and the signalling cassette that controls the activity of IL-6 is complicated[20]. Therefore, it is relevant to investigate the role of IL-6 after MI more thoroughly.

Interleukin 6

IL-6 is a pleiotropic cytokine, produced by many cells of the body (leukocytes, ischemic myocytes), and regulates the acute-phase response[20]. IL-6 signalling is activated through two different ways (**Figure 2**): 1. IL-6 binds to the membrane-bound IL-6 receptor (IL-6R), which is only expressed on hepatocytes and certain subpopulations of leukocytes, and then associates with the signal transducing receptor glycoprotein 130 (gp130)[21]. This association initiates activation of intracellular pathways and is known as classic signalling. 2. IL-6 binds to a soluble form of IL-6R (sIL-6R), and this complex binds to gp130 on cells (trans-signalling). Soluble gp130 (sgp130) could neutralize the IL-6/sIL-6R complex[9,10].

FIGURE 2. Adapted from Gottschalk *et al.* (Front. Immunol. (2015)) [22]. IL-6 signaling pathway.

The Interleukin-6 Receptor Mendelian Randomisation Analysis Consortium suggests that IL-6R signalling could have a causal role in development of CHD[23]. In a collaborative meta-analysis of 82 studies, including the Groningen based PREVEND study, the IL6R Genetics Consortium and Emerging Risk Factors Collaboration have compared the Asp358Ala variant frequency in 51,441 CHD patients and 136,226 controls. For every copy of 358Ala inherited, risk of CHD was reduced by 3.4% (1.8 – 5.0). Furthermore, previous research showed that IL-6 is involved in ischemia-reperfusion myocardial injury, and is

associated with myocardial damage and mortality in patients presenting with myocardial infarction[24,25]. In addition, the effect of the humanized anti-IL-6R antibody Tocilizumab on inflammation has been studied in patients with NSTEMI[26]. Tocilizumab binds to both membrane-bound and soluble (s)IL-6R, preventing the IL-6 signaling, and is effective and generally well tolerated in patients with autoimmune disorders. Administration of Tocilizumab in NSTEMI patients attenuated the inflammatory response and primarily PCI-related troponin T (TnT) release [26]. Currently, the 'ASSessing the effect of Anti-IL-6 treatment in MI' (ASSAIL-MI) trial examines whether a single administration of Tocilizumab can increase myocardial salvage in STEMI patients [27]. This trial is still ongoing and it is expected to have the first results at the end of this year.

Single-cell RNA sequencing

Despite our increasing knowledge and gains in therapeutics (i.e. CANTOS trial [18]), there is still room for improvement in the prevention and treatment of MI and its consequences, i.e. cardiac remodeling, since the complex human immune system has not been fully elucidated yet[15,28,29]. The method of single-cell RNA sequencing (scRNA-seq) is of value as it can improve our understanding of the mechanisms underlying MI by comprehensively mapping cell types, showing cell-type specific gene expression profiles, and creating gene regulatory networks[30,31]. So far, this has been successfully applied in 45 healthy participants from the Lifelines DEEP cohort. Furthermore, scRNA-seq has been used in endothelial cells to study the mechanisms of neovascularization in the adult mouse heart following MI[32]. Nevertheless, scRNA-seq has not been used in MI patients so far and has been considered promising in providing new insights into the pathophysiology of cardiovascular diseases and potential therapeutic targets.

Microbiome

Recently, accumulating evidence has suggested that the human gut microbiome may play an important role in the pathophysiology of MI through the modulation of the immune response related to the event [33,34]. The first studies on the human gut microbiome in cardiovascular disease analyzed the non-human metabolite trimethylamine N-oxide[35]. This molecule, generated via gut microbial metabolism, was shown to be associated with an increased risk of atherosclerosis [35]. Furthermore, in a study of the 1135 participants of the Groningen LifeLines-DEEP cohort, a lower abundance of *Eubacterium eligens* was observed in individuals who had developed a MI in the past [36]. However, the study included ten cases for which stool was not collected in the acute setting of MI and who already used cardiovascular medications, which was also associated with variation in the gut microbiome[36]. Recent studies in that same LifeLines DEEP cohort have observed functional links between the gut

microbiome and immune system [37,38]. Finally, specific microbe-associated molecular patterns have been detected that induce immune activation via cognate pattern-recognition receptors on circulating host immune cells [39]. Since pattern recognition receptors on immune cells already have been linked to IHD, gene expression profiles of circulating immune cells might be an indicator of immune activation and could result into an increased risk of MI and/or cardiac remodeling[40]. Nevertheless, a detailed characterization of microbe-host association in MI is still lacking.

Aims and outline of the thesis

In the past decades, inflammation has been recognized as an important factor throughout all stages of atherogenesis and the development of IHD. The increase in understanding the inflammatory mechanisms has uncovered an intriguing diversity of potentially targetable pathways. However, the exact role of inflammation still remains to be further elucidated.

The main aims of this thesis are:

1. To improve our understanding of the involvement of the immune system across the CVD continuum and the potential therapeutic targets within the inflammatory field.
2. To gain insights into the association of inflammatory markers and cardiac function in acute STEMI.
3. To further unravel the role of the immune system underlying the pathophysiological development of IHD and to explore potentially new pathways.

OUTLINE OF THIS THESIS

Part I. Inflammation in ischemic heart disease

In **part I**, a general overview of the immune system across the CVD continuum and an update are provided on the development of drugs targeting inflammation. It is generally appreciated that inflammation plays a pivotal role in the pathophysiology of atherosclerosis and CVD [41,42]. Nevertheless, these supporting data contrast with neutral clinical trials studying the effect on anti-inflammatory agents on (a composite of) major cardiovascular events, emphasizing the complexity of the human immune system and the need for more studies unravelling this complex mechanism [15]. In **chapter 2** leukocyte profiles are studied in different stadia of the CVD continuum in

individuals participating in the UK Biobank. In **chapter 3** an translational overview is provided on cytokine inhibiting therapies tested in experimental models and clinical studies in the setting of acute MI or chronic heart failure (HF).

Part II. Inflammation and cardiac function in myocardial infarction

In **chapters 4,5** and **6**, the time course of hs-CRP, IL-6, sIL-6R, and sgp130 is studied in patients presenting with acute STEMI. Furthermore, the association between the previously mentioned inflammatory markers and myocardial reperfusion and cardiac function is studied. In **chapter 4**, the time course of hs-CRP and the association with reperfusion are studied in STEMI patients participating in de GIPS-III trial. This trial was designed to evaluate the effect of metformin treatment on preservation of left ventricular function in STEMI patients without diabetes. In **chapter 5**, focus is shifted to IL-6. In 70 STEMI patients the association between sIL-6R and reperfusion success is studied [43]. Coronary angiograms post-PCI were analysed for myocardial blush grade (MBG) as indicator of myocardial reperfusion. Serum IL-6 and sIL-6R were measured using IL-6 and sIL-6R enzyme-linked immunosorbent assays (ELISA). In **chapter 6**, levels of IL-6, sIL-6R, and sgp130 are studied in 380 STEMI patients. Furthermore, the association between the IL-6 cascade and cardiac function 4 months after MI is studied. Infarct size and left ventricular ejection fraction (LVEF) were assessed by magnetic resonance imaging. Diastolic function (E/e') was determined by echocardiography.

Part III. Newest insights in ischemic heart disease – new therapeutic targets?

In **chapter 7**, associations are studied between genetic variants, known to affect the gut microbiome, and its relation to food intake, previous and new-onset disease, and health in 422,417 participants of the UK Biobank. Finally, in **chapter 8** the new method of scRNA-seq is used to study gene expression levels of peripheral blood mononuclear cells (PBMCs) in 24 STEMI patients at three different time points (hospital admission, 24 hours post-PCI, and 6-8 weeks post-PCI). In **chapter 9** the main findings of this thesis are summarized and future directions regarding inflammation in IHD are described.

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Chapter 2

LEUKOCYTE PROFILES ACROSS THE CARDIOVASCULAR DISEASE CONTINUUM: A POPULATION- BASED COHORT STUDY

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ABSTRACT

Introduction: Inflammation plays a pivotal role across all stadia of the cardiovascular disease (CVD) continuum, i.e. non-obstructive coronary artery disease (CAD), myocardial infarction (MI), and ischemic heart failure (iHF). However, inflammation across CVD continuum has not been studied yet within one population. Therefore, we mapped leukocyte profiles across the continuum within the UK Biobank.

Methods: The UK Biobank cohort study includes more than 500,000 participants aged 40 to 70 years who were recruited from 22 assessment centers across the United Kingdom from 2006 to 2010. A total of 333,218 individuals with available laboratory measurements at baseline were included in this study. These consisted of controls and individuals who had progression of CVD during follow-up (i.e. who developed CAD, MI, or iHF during follow-up). We investigated whether leukocytes and subtypes of leukocytes at baseline differed among the CVD continuum. Furthermore, we studied the possible interactions between sex and CVD on leukocytes.

Results: Of 333,218 individuals, 325,054 (97.5%) individuals were categorized as controls, and 8,164 (2.5%) individuals had progression of CVD during follow-up. Of those 8,164 individuals, 4,552 (1.4%) developed CAD during follow-up, 2,839 (0.9%) MI, and 773 (0.2%) in iHF. Compared to controls, mean leukocyte levels at baseline increased across the CVD continuum from $6.8 \cdot 10^9$ cells/L (SD $1.7 \cdot 10^9$ cells/L) to $7.7 \cdot 10^9$ cells/L (SD $1.9 \cdot 10^9$ cells/L) ($P_{\text{trend}} = 2.19 \cdot 10^{-132}$) in individuals who developed iHF. This increase mainly depended on an increase in neutrophils. Furthermore, controls with leukocyte levels in the highest quartile at baseline had a 1.44 higher chance of being diagnosed with CAD during follow-up compared with individuals with leukocyte levels in lower quartiles (OR 1.44, 95% CI 1.34 – 1.56 $P = 9.63 \cdot 10^{-21}$). A similar increased chance was observed for neutrophils, lymphocytes, monocytes, and eosinophils. There was a significant interaction between sex and CVD continuum on lymphocytes ($P = 8.49 \cdot 10^{-5}$).

Conclusion: Overall leukocyte count increased across the CVD continuum, which mainly depended on the increase in neutrophil count. High leukocytes in individuals not having CAD at baseline were predictive for the development of CAD during follow-up. Women had a greater increase of lymphocytes across the CVD continuum compared to men. Understanding which cells are key players in which stadium, could serve as a starting point for the identification of new potential therapeutic targets in CVD.

INTRODUCTION

It is generally appreciated that inflammation plays a pivotal role in the pathophysiology of atherosclerosis and cardiovascular disease (CVD). This has been shown on genetic, biologic, epidemiologic and clinical trial level[1,2]. Nevertheless, these supporting data contrast with neutral clinical trials studying the effect on anti-inflammatory agents on (a composite of) major cardiovascular events, emphasizing the complexity of the human immune system and the need for more studies unravelling this complex mechanism[3]. Recently, the CANTOS trial showed improved CVD outcomes using the anti-interleukin-1 β antibody canakinumab, although CVD mortality did not improve and there was an increase in the rate of infections. Therefore, optimization of immunotherapy for CVD is required (i.e. identification of novel drug targets that block inflammatory pathways, but do not exhibit immune-suppressive side effects). In order to achieve this optimization it is of value to first obtain an overview of the immune response in CVD patients. This has been done in the European Prospective Investigation into Cancer in Norfolk (EPIC-Norfolk) study, which studied differential white blood cell count in approximately sixteen thousand apparently healthy individuals [4,5]. They observed associations between increased leukocytes and the risk for CVD. However, leukocyte profiles in different conditions of CVD (i.e. coronary artery disease (CAD), myocardial infarction (MI), and ischemic heart failure (iHF)), also known as the cardiovascular disease continuum (CVD continuum)[6], within one population have not been studied yet. In combination with an increased sample size, information on the different components of granulocytes (i.e. neutrophils, eosinophils, and basophils), and further exploration of potential sex differences, this might be of additive value to the existing data from the EPIC-Norfolk study. Understanding which immune cells are present in particular stadia of the continuum may aid in increasing our knowledge of the underlying pathophysiologic process and expanding our efforts of optimising therapies. Therefore, we aimed to study leukocyte profiles in different stadia of the CVD continuum and to provide a broader overview of associations between leukocytes and CVD in individuals participating in the UK Biobank.

METHODS

UK Biobank participants

The UK Biobank study design and population have been described in detail elsewhere [7]. In brief, UK Biobank is a large community-based prospective study in the United Kingdom that recruited >500,000 participants aged 40 to 69 years old with the aim of improving prevention, diagnosis, and treatment of a plethora of illnesses including

cancer, diabetes mellitus, stroke, and heart diseases. All participants gave informed consent for the study. UK Biobank has approval from the relevant institutional review boards, namely, the North West Multi-centre Research Ethics Committee for the UK, from the National Information Governance Board for Health & Social Care for England and Wales, and from the Community Health Index Advisory Group for Scotland [8]. The present study was conducted under application number 12006 of the UK Biobank resource. The data from the UK Biobank resource are available for other researchers following an approved research proposal [9]. We used self-reported diagnoses and medication, and Hospital Episode Statistics data, as previously described [10]. We excluded individuals with a non-Caucasian ethnicity ($n = 29,750$), missing laboratory values ($n = 21,960$), and disease and/or therapy at baseline present which could affect cell counts ($n = 117, 631$). This included: C-reactive protein ≥ 10 mg/L ($n = 37,908$), HIV ($n = 15$), malignant cancer ($n = 64,351$), immunosuppressants/chemotherapy ($n = 3,373$), rheumatoid arthritis ($n = 3,650$), rheumatic heart disease (2,347), and gout ($n = 5,987$). We created a set of 333,218 individuals. **Figure 1** shows a flowchart of the study sample selection.

Definition of new-onset cardiovascular diseases

For our analyses we included individuals who had progression of CVD during follow-up. We used the following categories: controls, individuals who developed CAD during follow-up, individuals who developed MI during follow-up, and individuals who developed iHF during follow-up. Explanation of CVD continuum categories can be found in **Supplementary File 1**. Individuals were only placed in one category. When individuals initially could belong to more categories, they were placed in the 'worst' category.

Follow-up for disease outcomes was censored on 31-03-2015 for participants from England, 31-08-2014 for participants from Scotland, and on 28-02-2015 for participants from Wales.

Vital signs and blood count

At the baseline visit, vital signs and biological samples (i.e. prior to the progression of CVD during follow-up) were collected, together with data of self-completed questionnaires, interviews, and physical measurements. Blood pressure was measured twice, automated or manually, and average values were used. Automated measurements were corrected as proposed by Stang *et al* [11].

FIGURE 1. Flowchart for the selection of the analysed study sample from the UK Biobank study.

Statistical analysis

Continuous variables were summarized as mean±standard deviation if normally distributed or median and interquartile range if skewed. Discrete variables were presented as frequencies and percentages. We performed linear regression analysis to study the association between the different stages of the CVD continuum and cells. Age, sex, and CVD continuum stage were included in multivariate regression analysis.

Afterwards, we performed post-hoc analyses to study trends across the CVD continuum and to study sex interactions with the CVD continuum on leukocytes. To validate our findings, we repeated the linear regression analyses with a smaller set of controls, based on propensity score matching (age and sex) to the MI individuals, since the MI individuals had the average age and male percentage of the CAD, MI, and iHF group combined. Logistic regression analysis was performed to assess the chance of being diagnosed with CAD, MI or iHF during follow-up compared to a stage less worse at baseline. We adjusted for age and sex. We used Mann-Whitney to test for differences in leukocyte profiles between iHF and non-ischemic HF. To maximize the likelihood of reporting true findings, we set the α at .005 instead of .05 and used Bonferroni correction to adjust for multiple testing[12]. We considered 2-sided P values less than $1.39 \cdot 10^{-4}$ (P value of less than .005 divided by the number of independent tests, calculated using the Nyholt method, ie, $.005/36$) statistically significant for all analyses[13,14]. P values less than $1.39 \cdot 10^{-3}$ (ie, $.05/36$) were considered of suggestive significance. All analyses were performed using Stata version 15 (StataCorp).

RESULTS

Population characteristics

From the 502,559 individuals, we included a total of 333,218 individuals for the present analyses (**Figure 1**). Overall, 153,043 (46%) were male. Mean age was 57 years (SD 8 years) and median follow-up of 6 years (interquartile range [IQR] 5 – 7). Baseline characteristics, including cardiovascular risk factors and medical history of coronary heart diseases are provided in **Table 1**.

Leukocytes increase across the CVD continuum

Of 333,218 individuals, 325,054 (97.5%) individuals were categorized as controls and 8,164 (2.5%) individuals had progression of CVD during follow-up. Of those 8,164 individuals, 4,552 (1.4%) developed CAD, 2,839 (0.9%) MI, and 773 (0.2%) in iHF. The absolute and relative number of all leukocytes is shown in **Table 2**. Compared to controls, mean leukocyte levels at baseline increased across the CVD continuum from $6.8 \cdot 10^9$ cells/L (SD $1.7 \cdot 10^9$ cells/L) to $7.7 \cdot 10^9$ cells/L (SD $1.9 \cdot 10^9$ cells/L) ($P_{\text{trend}} = 2.19 \cdot 10^{-132}$) in individuals who developed iHF during follow-up. Baseline neutrophil count increased across the continuum with a range from $4.2 \cdot 10^9$ cells/L (SD $1.3 \cdot 10^9$ cells/L) in controls to $4.9 \cdot 10^9$ cells/L (SD $1.5 \cdot 10^9$ cells/L) in individuals who developed iHF during follow-up ($P_{\text{trend}} = 4.65 \cdot 10^{-112}$). **Figure 2** shows the association between CVD continuum categories and leukocyte and neutrophil counts. After adjustment for age and sex, being in a 'worse' CVD continuum category during follow-up remained significantly associated

with an increase in total leukocytes and neutrophils compared to controls. A similar phenomenon was observed for absolute cell counts of lymphocytes, monocytes, eosinophils, and basophils (**Supplementary Table 2**), and also in the analyses using the control group ($n = 2,837$) based on propensity score matching (**Supplementary Table 3**).

TABLE 1. Baseline characteristics

Characteristic	Controls	CAD	MI	iHF
Total, No.	325,054	4,552	2,839	773
Male	147,427 (45.4%)	2,949 (64.8%)	2,060 (72.6%)	607 (78.5%)
Age, mean (SD), y	56.4 (8.1)	61.0 (6.5)	60.1 (6.8)	62.4 (6.1)
Blood pressure, mean (SD), mmHg				
Systolic	132.6 (17.8)	140.6 (17.8)	142.0 (18.2)	140.2 (19.2)
Diastolic	81.9 (8.5)	83.7 (8.6)	84.9 (8.9)	81.8 (9.5)
Heart rate, beats per minute	68.9 (11.0)	69.0 (11.7)	69.6 (12.2)	67.9 (13.4)
Smoking				
Never or <100 cigarettes	183,337 (57.8%)	2,084 (46.9%)	1,173 (42.2%)	252 (33.4%)
Stopped >12 months	100,243 (31.6%)	1,823 (41.0%)	1,008 (36.2%)	318 (42.2%)
Stopped ≤12 months	1,427 (0.4%)	22 (0.5%)	22 (0.8%)	13 (1.7%)
Active occasionally	8,579 (2.7%)	95 (2.1%)	95 (3.4%)	32 (4.2%)
Active daily	23,642 (7.5%)	424 (9.5%)	484 (17.4%)	139 (18.4%)
Body mass index, kg/m ²	27.1 (4.5)	28.6 (4.7)	28.3 (4.4)	29.6 (5.2)
Hypertension	85,950 (26.4%)	2,231 (49.0%)	1,262 (44.5%)	551 (71.3%)
Hyperlipidemia	54,354 (16.7%)	1,656 (36.4%)	879 (31.0%)	510 (66.0%)
Type 2 Diabetes	8,261 (2.5%)	436 (9.6%)	230 (8.1%)	174 (22.5%)

Data is expressed as number (%) and as mean (standard deviation (SD)) for a normal distribution. CAD = coronary artery disease; MI = myocardial infarction; iHF = ischemic heart failure.

Prognostic value of leukocytes for progression CVD

Furthermore, we investigated the predictive value of high leukocytes levels at baseline in the progression of CVD during follow-up (**Figure 3, Table 3**). We defined this progression as a change in category between baseline and follow-up (i.e. no CAD to CAD, CAD to MI, MI to iHF). Individuals without CAD with leukocyte levels in the highest quartile at baseline had a 1.44 higher chance of being diagnosed with CAD during follow-up compared with individuals with leukocyte levels in lower quartiles (OR 1.44, 95% CI 1.34 – 1.56 $P = 9.63 \cdot 10^{-21}$). A similar increased change was observed for neutrophils,

TABLE 2. Leukocyte profiles across the cardiovascular disease continuum.

	Controls	CAD	MI	iHF	P value for trend
Number	325,054	4,552	2,839	773	
Leukocyte count, 10 ⁹ cells/L	6.8 (1.7)	7.1 (1.8)	7.3 (1.9)	7.7 (1.9)	2.19·10 ⁻¹³²
Neutrophil count, 10 ⁹ cells/L	4.2 (1.3)	4.4 (1.4)	4.6 (1.5)	4.9 (1.5)	4.65·10 ⁻¹¹²
Percentage of neutrophils, %	60.8 (8.1)	61.0 (8.1)	61.6 (8.1)	62.8 (8.5)	6.91·10 ⁻¹⁵
Lymphocyte count, 10 ⁹ cells/L	0.9 (0.4)	0.9 (0.5)	0.9 (0.5)	0.9 (0.5)	1.24·10 ⁻²⁸
Percentage of lymphocytes, %	29.0 (7.1)	28.4 (7.1)	27.8 (7.0)	26.3 (7.2)	6.45·10 ⁻²²
Monocyte count, 10 ⁹ cells/L	0.5 (0.2)	0.5 (0.2)	0.5 (0.2)	0.6 (0.2)	1.59·10 ⁻⁴³
Percentage of monocytes, %	7.1 (2.6)	7.3 (2.5)	7.3 (2.5)	7.6 (3.3)	0.164
Eosinophil count, 10 ⁹ cells/L	0.13 (0.10 – 0.20)	0.16 (0.10 – 0.23)	0.18 (0.10 – 0.25)	0.19 (0.10 – 0.25)	3.64·10 ⁻³³
Percentage of eosinophils, %	2.11 (1.38 – 3.27)	2.27 (1.50 – 3.40)	2.31 (1.52 – 3.47)	2.24 (1.15 – 3.29)	1.00·10 ⁻⁴
Basophil count, 10 ⁹ cells/L	0.02 (0.00 – 0.04)	0.02 (0.00 – 0.04)	0.02 (0.00 – 0.05)	0.03 (0.00 – 0.06)	1.56·10 ⁻³⁶
Percentage of basophils, %	0.43 (0.30 – 0.67)	0.43 (0.30 – 0.66)	0.41 (0.30 – 0.65)	0.47 (0.30 – 0.70)	0.060

Data is expressed as mean with standard deviation (SD) or median with interquartile range (IQR). CAD = coronary artery disease; MI = myocardial infarction; iHF = ischemic heart failure. P value for trend was derived from the post-hoc linear regression analysis.

lymphocytes, monocytes, and eosinophils. This increased chance was not observed for individuals with progression of their CVD in a later stadium (CAD to MI, or MI to iHF) (Table 3).

FIGURE 2. Associations between CVD continuum and leukocytes, neutrophils, and monocytes, corrected for age and sex. CAD = coronary artery disease; MI = myocardial infarction; iHF = ischemic heart failure.

FIGURE 3. Chance of getting diagnosed with CAD during follow-up, when having no CAD at baseline. Highest quartile of leukocytes (Q4) vs. lower quartiles (Q1 – Q3). CAD = coronary artery disease.

To see whether the increase in leukocyte levels was really related to the inflammatory and ischemic component of the CVD continuum, we studied the differences in levels between iHF and non-ischemic HF individuals. Level of leukocytes, lymphocytes, and monocytes were significantly higher in individuals with iHF compared with individuals with non-ischemic HF (Supplementary Table 4).

TABLE 3. The chance of being diagnosed with progression across the CVD continuum during follow-up: individuals with leukocyte levels in the highest quartile (Q4) compared with individuals with leukocyte levels in the lower quartiles (Q1 – Q3).

	No CAD at baseline to CAD	P value	CAD at baseline to MI	P value	MI at baseline to iHF	P value
Leukocyte count (Q4 vs. Q1-3)	1.44 (1.34 – 1.56)	$9.63 \cdot 10^{-21}$	1.43 (1.14 – 1.80)	0.002	1.26 (1.02 – 1.55)	0.032
Neutrophil count (Q4 vs. Q1-3)	1.39 (1.29 – 1.50)	$1.58 \cdot 10^{-16}$	1.49 (1.19 – 1.87)	0.001	1.43 (1.16 – 1.76)	0.001
Percentage of neutrophils (Q4 vs. Q1-3)	1.04 (0.96 – 1.13)	0.330	1.21 (0.95 – 1.54)	0.115	1.33 (1.07 – 1.66)	0.010
Lymphocyte count (Q4 vs. Q1-3)	1.25 (1.16 – 1.36)	$3.20 \cdot 10^{-8}$	1.07 (0.83 – 1.39)	0.591	0.94 (0.75 – 1.19)	0.620
Percentage of lymphocytes (Q4 vs. Q1-3)	0.93 (0.85 – 1.01)	0.089	0.79 (0.58 – 1.08)	0.133	0.86 (0.48 – 0.89)	0.008
Monocyte count (Q4 vs. Q1-3)	1.31 (1.21 – 1.41)	$2.39 \cdot 10^{-11}$	1.24 (0.99 – 1.55)	0.066	1.41 (1.14 – 1.74)	0.001
Percentage of monocytes (Q4 vs. Q1-3)	1.00 (0.92 – 1.08)	0.984	1.19 (0.94 – 1.50)	0.152	1.15 (0.93 – 1.43)	0.187
Eosinophil count (Q4 vs. Q1-3)	1.21 (1.11 – 1.31)	$5.12 \cdot 10^{-6}$	1.09 (0.86 – 1.39)	0.454	1.10 (0.89 – 1.37)	0.373
Percentage of eosinophils (Q4 vs. Q1-3)	1.05 (0.97 – 1.14)	0.205	0.98 (0.77 – 1.26)	0.855	0.94 (0.75 – 1.18)	0.606
Basophil count (Q4 vs. Q1-3)	1.16 (1.05 – 1.28)	0.003	1.40 (1.06 – 1.86)	0.018	1.52 (1.18 – 1.95)	0.001
Percentage of basophils (Q4 vs. Q1-3)	1.06 (0.97 – 1.15)	0.177	1.12 (0.86 – 0.45)	0.406	1.36 (1.08 – 1.72)	0.010

Data is expressed as odds ratio (OR) and 95% Confidence Interval (CI). CAD = coronary artery disease; MI = Myocardial infarction; iHF = ischemic heart failure. Logistic regression was performed, adjusted for age and sex.

To explore whether the increase in leukocyte levels across the CVD continuum would reflect a difference in functionality of the immune system, we investigated whether there was a difference in pneumonia incidence during follow-up between controls and individuals with iHF. In the iHF group, the incidence of pneumonia during follow-up was significantly higher compared to controls (13.8% vs. 1.0%, $P < 5.00 \cdot 10^{-324}$) (**Figure 4**). This difference remained in the analysis using the control group (n = 2,837) based on propensity score matching.

FIGURE 4. Incidence of pneumonia during follow-up in controls and individuals with iHF. iHF = ischemic heart failure.

Sex differences in leukocyte profiles

Overall, women had higher leukocyte counts compared to men ($P = 8.04 \cdot 10^{-5}$) (**Supplementary Table 5**). In addition, men had relatively higher levels of neutrophils, monocytes, and eosinophils, whereas women had relatively higher levels of lymphocytes and basophils. Furthermore, we investigated whether there were any interaction between sex and leukocytes. A significant overall interaction between CVD continuum and sex was observed for lymphocytes, where these increased more in women than in men ($P = 8.49 \cdot 10^{-5}$).

DISCUSSION

In this large community-based population of more than 330,000 individuals, we mapped leukocyte profiles across the different stadia of the CVD continuum (CAD, MI, and iHF). First, overall leukocyte count increased across the continuum, which mainly depended on the increase in neutrophil count. Second, high levels of leukocytes at baseline were

predictive for the progression of CVD during follow-up. Third, women showed overall higher leukocyte levels and there was an interaction between CVD continuum and sex on lymphocytes, suggesting a sex specific component in leukocyte profiles across the CVD continuum.

Leukocyte profiles across the CVD continuum

Across the CVD continuum, we observed an increase in leukocyte count, mainly depending on the increase in neutrophils. This might indicate that neutrophils alone may account for more vascular damage than any other single cell type, as they are often the first cells which are recruited into the damage site [15,16]. We observed the largest increase in neutrophil count between controls and individuals who developed CAD during follow-up, but this increase continues in individuals who developed MI and iHF during follow-up, supporting that the immune system plays a role across all stages of the CVD continuum[1]. The phenomenon of the increasing neutrophils across the different stages of the CVD continuum is also in line with previous research that showed that an increased ratio between neutrophils and lymphocytes is an independent predictor of cardiac mortality in stable CAD patients[17]. The possibility to show this increase in neutrophils across the different CVD continuum stages is of additive value, since it enables to obtain the bigger picture. Furthermore, our results support earlier research that postulated that neutrophil extracellular traps (NETs) are not only present in plaques and thrombi, but also may play a causative role in triggering atherosclerotic plaque formation and arterial thrombosis[18,19]. Of interest is the predictive value of increased leukocyte levels at baseline on the progression of CAD during a six years follow-up. This association did not remain in individuals with either CAD or MI at baseline, and getting diagnosed with MI or iHF during follow-up. Although this finding is solely observational and suggestively significant, our study is of additional value to earlier results of the European Prospective Investigation into Cancer in Norfolk (EPIC-Norfolk) study, that also showed the association between increased leukocytes and a higher risk for coronary heart disease and CVD, and might implicate that early intervention in the inflammation pathway is of importance to gain as much effect as possible[3,4].

Furthermore, we observed higher leukocyte levels in iHF individuals compared with non-ischemic HF individuals. Levels in both groups were higher compared to controls. Additionally, the incidence of pneumonia was fourteen-fold higher in the iHF group compared to controls, which may also point to a functional change of the leukocytes. Although data on the functionality of leukocytes was not available and there might be other explanations for this difference (i.e. presence of pulmonary oedema), this finding supports the need for prophylactic vaccination in iHF individuals.

Sex differences

It is generally appreciated that sex differences exist in the innate and adaptive immune response in adults[20]. In this study, sex differences were also observed. Women showed higher levels of total leukocytes, and specifically lymphocytes and basophils. The opposite was observed for neutrophils, monocytes, and eosinophils, which were higher in men compared with women. Furthermore, of interest is the interactions between sex and CVD continuum on lymphocytes. T-lymphocytes are known to stimulate macrophages expressing collagen-degrading enzymes and thereby increasing the risk of plaque rupture[21]. Estrogen has been shown to be a cardioprotective agent in pre-menopausal females, partially by reducing atherosclerosis via the estrogen receptor α [22]. Although highly speculative, it might be hypothesized that the increase in lymphocytes in women compared to men might reflect that women are less prone to inflammation (due to the protective function of estrogen), before they develop MI[23]. Estimating lymphocytes in pre- and post-menopausal women in the UK Biobank did, however, not provide additional support. On the other hand, in a study of 6,050 patients that underwent coronary computed tomography angiography, women had a higher relative risk for high-risk plaque features compared to men, which might correspond with higher lymphocyte levels[24]. Nevertheless, the difference in lymphocyte trends between women and men across the CVD continuum invites to further investigate potential mechanisms underlying. Furthermore, the difference in leukocytes between men and women may also cohere with the a difference in gene expression between sexes. Previous research showed a greater upregulation of the gene *FAM5C* in women [23]. *FAM5C* is associated with increased monocyte adhesion, suggesting that women may need less monocytes to experience the similar amount of monocyte adhesion compared to men[23]. The increased association in both men and women between the CVD continuum and monocytes is intriguing, since this observation is contrary to previous research in the EPIC-Norfolk study[5]. They found an inverse relation between monocytes and the risk of developing HF, whereas we observed an increased association between CVD continuum and monocytes. This could be due to another definition of HF, where they based HF on drug treatment only and we tried to focus on ischemic HF solely. Nevertheless this finding warrants further exploration of the underlying mechanism.

Strengths and limitations

In this study, we were able to provide an elegant overview of the different leukocyte profiles across the CVD continuum within one population. The major strengths of this study were the large sample size, variety of information, and prospective design of

the UK Biobank study. Furthermore, in addition to the EPIC-Norfolk study, we did have information on the components of granulocytes (neutrophils, eosinophils, basophils) to further study their specific effect[4].

However, this study possesses some limitations. We only had information on the quantity of the cells. It would be of value to also possess information on the functionality and activity of cells, and cytokine levels, since this could provide new insights into the cellular cross-talk between immune cells and their related pathways[25]. Nevertheless, this study could serve as a starting point, providing new insights where to focus concerning new possible therapeutic targets

Future perspectives

Single-cell RNA sequencing data of immune cells in different stages of the CVD continuum might be a potential way to gain more insights into the inflammatory pathophysiological mechanisms underlying CVD, as this provides additional information on the cell-cell interactions in the heart. Until now, the cellular interactions and their dynamics during the different stages of the CVD continuum remain poorly characterized[26]. This method has already been performed in animals and in healthy humans, but has not yet been applied to cardiovascular patients [26,27]. Furthermore, we provided evidence for sex differences in leukocyte profiles across the CVD continuum. Further research on these differences is worthwhile for the development of more patient-tailored therapies.

Conclusions

Our study provided an overview of the different leukocyte profiles across different stadia of the CVD continuum. Overall leukocyte count increased across the continuum, which mainly depended on the increase in neutrophil count. Furthermore, high levels of leukocytes at baseline were predictive for the progression of CVD during follow-up. Last, women showed overall higher leukocyte levels and there was an interaction between CVD continuum and sex on lymphocytes, suggesting a sex specific component in leukocyte profiles across the CVD continuum. Understanding which cells are key players in various stadia, could serve as a starting point for the identification of new potential therapeutic targets in CVD.

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Disclosure of potential conflicts of interest

All authors have completed and submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest and none were reported.

Explanation of the role of funder/sponsor

The funding agencies had no role in the study design, analysis, or interpretation of data; the writing of the manuscript; or in the decision to submit the article for publication.

Data statement

The data, analytical methods, and study materials will be made available to other researchers for purposes of reproducing the results or replicating the procedure (contact the corresponding author).

Supplementary File 1 – Definition of CVD continuum categories

We defined the CVD continuum categories as follows;

Control: Absence of CAD, MI or iHF at baseline and during follow-up.

Coronary artery disease: No CAD at baseline, but development of CAD during follow-up; described as follows: ICD10 code I70 (Atherosclerosis), I24 (Other acute ischemic heart diseases), I25 (Chronic ischemic heart diseases), ICD9 code 440 (Atherosclerosis), 414 (Other forms of chronic ischemic heart disease), operational code K75 but absence of non-cardiac vascular disease, MI or iHF.

Myocardial infarction: No MI at baseline, but development of MI during follow-up; described as follows: ICD10 code I21 (Acute myocardial infarction), I22 (Subsequent myocardial infarction), I23 (Certain current complications following acute myocardial infarction), I25.2 (Old myocardial infarction), ICD9 code 410 (Acute myocardial infarction), 412 (Old myocardial infarction), touch screen UKBB n_6150 = 1, self-reported non-cancer illness code 1075 (heart attack/myocardial infarction), but absence of iHF.

Ischemic heart failure: No iHF at baseline, but development of iHF during follow-up; described as follows: ICD 10 code I50 (Heart failure), ICD 9 code 428 (Heart failure), self-reported non-cancer illness code 1076 (heart failure/pulmonary oedema), and presence of MI.

Non-ischemic heart failure: No non-ischemic HF at baseline, but development of non-ischemic HF during follow-up; described as: ICD 10 code I50 (Heart failure), ICD 9 code 428 (Heart failure), self-reported non-cancer illness code 1076 (heart failure/pulmonary oedema), but absence of CAD, MI, myocarditis, hypertrophic cardiomyopathy

SUPPLEMENTARY TABLE 2. Association between CVD continuum and leukocytes (linear regression analysis), crude model and model adjusted for age and sex.

	Absolute cell counts (crude model)				Adjusted for age and sex		
	Beta	95% CI	P value		Beta	95% CI	P value
Total leukocytes							
CVD continuum				CVD continuum			
Control (ref.)				Control (ref.)			
CAD	0,315	0.266 – 0.365	1,42·10 ⁻³⁵	CAD	0,302	0.252 – 0.352	1,53·10 ⁻³²
MI	0.551	0.488 – 0.614	2,01·10 ⁻⁶⁶	MI	0.537	0.475 – 0.600	5,08·10 ⁻⁶³
iHF	0.893	0.774 – 1.013	2,51·10 ⁻⁴⁸	iHF	0,874	0.754 – 0.994	3,01·10 ⁻⁴⁶
Neutrophils							
CVD continuum				CVD continuum			
Control (ref.)				Control (ref.)			
CAD	0,203	0.164 – 0.242	3,02·10 ⁻²⁴	CAD	0,198	0.159 – 0.237	4,63·10 ⁻²³
MI	0,389	0.340 – 0.439	1,07·10 ⁻⁵³	MI	0,379	0.330 – 0.429	5,64·10 ⁻⁵¹
iHF	0,703	0.608 – 0.797	3,37·10 ⁻⁴⁸	iHF	0,692	0.598 – 0.787	9,52·10 ⁻⁴⁷
Lymphocytes							
CVD continuum				CVD continuum			
Control (ref.)				Control (ref.)			
CAD	0,034	0.021 – 0.047	2,61·10 ⁻⁰⁷	CAD	0,048	0.036 – 0.061	1,71·10 ⁻¹³
MI	0,047	0.030 – 0.063	2,41·10 ⁻⁰⁸	MI	0,069	0.053 – 0.085	1,04·10 ⁻¹⁶
iHF	0,03	-0.001 - 0.061	0,059	iHF	0,056	0.025 – 0.087	3,71·10 ⁻⁰⁴
Monocytes							
CVD continuum				CVD continuum			
Control (ref.)				Control (ref.)			
CAD	0,041	0.035 – 0.047	5,98·10 ⁻⁴⁵	CAD	0,019	0.014 – 0.025	1,48·10 ⁻¹¹
MI	0,056	0.049 – 0.063	2,00·10 ⁻⁵²	MI	0,03	0.023 – 0.037	6,30·10 ⁻¹⁷
iHF	0,099	0.086 – 0.113	1,45·10 ⁻⁴⁵	iHF	0,066	0.052 – 0.079	1,74·10 ⁻²¹
Eosinophils							
CVD continuum				CVD continuum			
Control (ref.)				Control (ref.)			
CAD	0,136	0.109 – 0.164	2,05·10 ⁻²²	CAD	0,010	0.072 – 0.127	1,16·10 ⁻¹²
MI	0,21	0.176 – 0.245	1,53·10 ⁻³²	MI	0,159	0.125 – 0.194	1,89·10 ⁻¹⁹
iHF	0,232	0.166 – 0.298	6,00·10 ⁻¹²	iHF	0,169	0.103 – 0.235	4,66·10 ⁻⁰⁷
Basophils							
CVD continuum				CVD continuum			
Control (ref.)				Control (ref.)			
CAD	0,084	0.046 – 0.121	1,56·10 ⁻⁰⁵	CAD	0,113	0.075 – 0.151	5,23·10 ⁻⁰⁹

SUPPLEMENTARY TABLE 2. Continued.

Absolute cell counts (crude model)				Adjusted for age and sex			
	Beta	95% CI	P value		Beta	95% CI	P value
MI	0.171	0.121 – 0.220	1.12·10 ⁻¹¹	MI	0.205	0.155 – 0.254	4.31·10 ⁻¹⁶
iHF	0.356	0.263 – 0.448	4.72·10 ⁻¹⁴	iHF	0.402	0.309 – 0.494	1.60·10 ⁻¹⁷
Percentages (crude model)				Adjusted for age and sex			
Neutrophils							
CVD continuum				CVD continuum			
Control (ref.)				Control (ref.)			
CAD	0.218	0.020 – 0.456	0.073	CAD	0.219	-0.02 – 0.458	0.072
MI	0.759	0.458 – 1.060	7.42·10 ⁻⁰⁷	MI	0.701	0.400 – 1.00	5.12·10 ⁻⁰⁶
iHF	2.029	1.454 – 2.604	4.52·10 ⁻¹²	iHF	1.988	1.413 – 2.562	1.20·10 ⁻¹¹
Lymphocytes							
CVD continuum				CVD continuum			
Control (ref.)				Control (ref.)			
CAD	-0.604	-0.81 – -0.397	1.07·10 ⁻⁰⁸	CAD	-0.268	-0.474 – -0.062	0.011
MI	-1.191	-1.452 – -0.93	3.91·10 ⁻¹⁹	MI	-0.708	-0.968 – -0.449	8.80·10 ⁻⁰⁸
iHF	-2.67	-3.169 – -2.172	9.69·10 ⁻²⁶	iHF	-2.087	-2.582 – -1.591	1.46·10 ⁻¹⁶
Monocytes							
CVD continuum				CVD continuum			
Control (ref.)				Control (ref.)			
CAD	0.265	0.190 – 0.341	5.59·10 ⁻¹²	CAD	-0.03	-0.104 – 0.044	0.424
MI	0.265	0.170 – 0.361	4.76·10 ⁻⁰⁸	MI	-0.094	-0.187 – -0.001	0.049
iHF	0.537	0.355 – 0.719	7.48·10 ⁻⁰⁹	iHF	0.07	-0.108 – 0.248	0.439
Eosinophils							
CVD continuum				CVD continuum			
Control (ref.)				Control (ref.)			
CAD	0.087	0.058 – 0.116	3.03·10 ⁻⁰⁹	CAD	0.046	0.017 – 0.075	0.002
MI	0.115	0.079 – 0.152	4.67·10 ⁻¹⁰	MI	0.06	0.024 – 0.096	0.001
iHF	0.057	-0.012 – 0.127	0.104	iHF	-0.011	-0.08 – 0.058	0.749
Basophils							
CVD continuum				CVD continuum			
Control (ref.)				Control (ref.)			
CAD	-0.003	-0.031 – 0.025	0.822	CAD	0.018	-0.01 – 0.046	0.197
MI	-0.031	-0.066 – 0.005	0.088	MI	-0.005	-0.04 – 0.03	0.789
iHF	0.048	-0.019 – 0.115	0.159	iHF	0.082	0.015 – 0.149	0.017

CI = confidence interval; CVD = cardiovascular disease; CAD = coronary artery disease; MI = myocardial infarction; iHF = ischemic heart failure; ref = reference

SUPPLEMENTARY TABLE 3. Association between CVD continuum and leukocyte counts (linear regression analysis), crude model and model adjusted for age and sex, using the control group (n = 2,837) based on propensity score matching (age and sex) to the MI individuals.

	Absolute cell counts (crude model)				Adjusted for age and sex		
	Beta	95% CI	P value		Beta	95% CI	P value
Total leukocytes							
CVD continuum				CVD continuum			
Control (ref.)				Control (ref.)			
CAD	0.293	0.210 – 0.376	5.11·10 ⁻¹²	CAD	0.290	0.206 – 0.373	6.81·10 ⁻¹¹
MI	0.528	0.436 – 0.620	4.18·10 ⁻²⁹	MI	0.528	0.436 – 0.621	3.74·10 ⁻²⁹
iHF	0.871	0.730 – 1.011	1.53·10 ⁻³³	iHF	0.889	0.748 – 1.030	1.06·10 ⁻³⁴
Neutrophils							
CVD continuum				CVD continuum			
Control (ref.)				Control (ref.)			
CAD	0.196	0.131 – 0.262	1.03·10 ⁻¹¹	CAD	0.199	0.133 – 0.264	2.76·10 ⁻⁹
MI	0.383	0.310 – 0.455	3.74·10 ⁻²⁹	MI	0.383	0.310 – 0.455	5.45·10 ⁻²⁵
iHF	0.696	0.585 – 0.807	1.06·10 ⁻³⁴	iHF	0.698	0.587 – 0.809	1.35·10 ⁻³⁴
Lymphocytes							
CVD continuum				CVD continuum			
Control (ref.)				Control (ref.)			
CAD	0.057	0.035 – 0.078	3.28·10 ⁻⁰⁷	CAD	0.049	0.027 – 0.070	8.67·10 ⁻⁶
MI	0.069	0.045 – 0.093	1.96·10 ⁻⁰⁸	MI	0.069	0.045 – 0.093	1.30·10 ⁻⁸
iHF	0.053	0.016 – 0.089	0.005	iHF	0.071	0.035 – 0.108	1.29·10 ⁻⁰⁴
Monocytes							
CVD continuum				CVD continuum			
Control (ref.)				Control (ref.)			
CAD	0.014	0.004 – 0.023	0.004	CAD	0.018	0.009 – 0.027	1.34·10 ⁻⁴
MI	0.029	0.018 – 0.039	5.15·10 ⁻⁸	MI	0.029	0.019 – 0.039	2.92·10 ⁻⁸
iHF	0.072	0.056 – 0.088	2.88·10 ⁻¹⁹	iHF	0.063	0.048 – 0.079	1.16·10 ⁻¹⁵
Eosinophils							
CVD continuum				CVD continuum			
Control (ref.)				Control (ref.)			
CAD	0.079	0.035 – 0.122	3.72·10 ⁻⁴	CAD	0.091	0.047 – 0.134	4.35·10 ⁻⁵
MI	0.153	0.105 – 0.201	5.16·10 ⁻¹⁰	MI	0.152	0.104 – 0.200	5.49·10 ⁻¹⁰
iHF	0.175	0.101 – 0.248	3.20·10 ⁻⁶	iHF	0.171	0.098 – 0.245	4.93·10 ⁻⁰⁶
Basophils							
CVD continuum				CVD continuum			
Control (ref.)				Control (ref.)			

SUPPLEMENTARY TABLE 3. Continued.

	Absolute cell counts (crude model)				Adjusted for age and sex		
	Beta	95% CI	P value		Beta	95% CI	P value
CAD	0.094	0.034 – 0.155	0.002	CAD	0.090	0.030 – 0.151	0.003
MI	0.181	0.113 – 0.250	1.84·10 ⁻⁷	MI	0.181	0.113 – 0.249	1.84·10 ⁻⁷
iHF	0.366	0.263 – 0.470	5.24·10 ⁻¹²	iHF	0.387	0.283 – 0.491	3.68·10 ⁻¹³

CI = confidence interval; CVD = cardiovascular disease; CAD = coronary artery disease; MI = myocardial infarction; iHF = ischemic heart failure; ref = reference

SUPPLEMENTARY TABLE 4. Leukocytes in iHF vs. non-ischemic HF individuals. Data of controls is shown as a reference.

	Controls	Non-ischemic HF	iHF	P value (noniHF vs. iHF)
Number	325,054	674	773	
Leukocyte count, 10 ⁹ cells/L	6.8 (1.7)	7.2 (1.9)	7.7 (1.9)	1.53·10 ⁻⁶
Neutrophil count, 10 ⁹ cells/L	4.2 (1.3)	4.6 (1.6)	4.9 (1.5)	2.59·10 ⁻⁴
Percentage of neutrophils, %	60.8 (8.1)	63.4 (7.9)	62.8 (8.5)	0.210
Lymphocyte count, 10 ⁹ cells/L	0.9 (0.4)	0.8 (0.5)	0.9 (0.5)	1.20·10 ⁻⁵
Percentage of lymphocytes, %	29.0 (7.1)	26.4 (6.8)	26.3 (7.2)	0.870
Monocyte count, 10 ⁹ cells/L	0.5 (0.2)	0.5 (0.2)	0.6 (0.2)	4.09·10 ⁻⁹
Percentage of monocytes, %	7.1 (2.6)	7.2 (2.4)	7.6 (3.3)	0.005
Eosinophil count, 10 ⁹ cells/L	0.13 (0.10 – 0.20)	0.15 (0.10 – 0.22)	0.19 (0.10 – 0.25)	5.00·10 ⁻⁴
Percentage of eosinophils, %	2.11 (1.38 – 3.27)	2.10 (1.32 – 3.28)	2.24 (1.15 – 3.29)	0.088
Basophil count, 10 ⁹ cells/L	0.02 (0.00 – 0.04)	0.02 (0.00 – 0.05)	0.03 (0.00 – 0.06)	0.012
Percentage of basophils, %	0.43 (0.30 – 0.67)	0.40 (0.30 – 0.64)	0.47 (0.30 – 0.70)	0.041

Data is expressed as number (%) and as mean with standard deviation (SD) or median with interquartile range (IQR) for continuous variables with a skew distribution. iHF = ischemic heart failure ; HF = heart failure.

SUPPLEMENTARY TABLE 5. Leukocyte profiles across the CVD continuum for men and women.

	Controls	CAD	MI	IHF	P value for sex difference	P value for sex interaction
Men	147,427 (45.4%)	2,949 (64.8%)	2,060 (72.6%)	607 (78.5%)		
Leukocyte count, 10 ⁹ cells/L					8.04·10 ⁻⁵	3.20·10 ⁻³
Men	6.8 (1.7)	7.1 (1.7)	7.3 (1.8)	7.6 (1.8)		
Women	6.8 (1.7)	7.2 (1.8)	7.5 (2.0)	7.8 (1.9)		
Neutrophil count, 10 ⁹ cells/L					6.93·10 ⁻³⁹	0.287
Men	4.2 (1.4)	4.4 (1.4)	4.5 (1.4)	4.9 (1.5)		
Women	4.1 (1.3)	4.4 (1.4)	4.6 (1.5)	4.9 (1.5)		
Percentage of neutrophils, %					6.48·10 ⁻⁷²	0.159
Men	61.1 (8.2)	61.3 (8.1)	61.9 (8.1)	63.0 (8.6)		
Women	60.6 (8.1)	60.4 (8.1)	60.8 (8.1)	62.3 (8.4)		
Lymphocyte count, 10 ⁹ cells/L					5.00·10 ⁻³²⁴	8.49·10 ⁻⁵
Men	0.8 (0.4)	0.9 (0.4)	0.9 (0.5)	0.9 (0.5)		
Women	0.9 (0.4)	1.0 (0.5)	1.0 (0.5)	1.0 (0.5)		
Percentage of lymphocytes, %					5.00·10 ⁻³²⁴	0.071
Men	28.0 (7.0)	27.6 (7.0)	27.1 (6.9)	25.9 (7.0)		
Women	29.8 (7.0)	29.8 (7.1)	29.6 (7.1)	27.9 (7.5)		
Monocyte count, 10 ⁹ cells/L					5.00·10 ⁻³²⁴	0.576
Men	0.5 (0.2)	0.5 (0.2)	0.5 (0.2)	0.6 (0.3)		
Women	0.4 (0.2)	0.5 (0.2)	0.5 (0.2)	0.5 (0.2)		
Percentage of monocytes, %					5.00·10 ⁻³²⁴	0.257

SUPPLEMENTARY TABLE 5. Continued.

	Controls	CAD	MI	iHF	P value for sex difference	P value for sex interaction
Men	7.6 (2.6)	7.7 (2.4)	7.6 (2.6)	7.9 (3.5)		
Women	6.6 (2.5)	6.6 (2.4)	6.5 (2.1)	6.5 (2.3)		
Eosinophil count, 10 ⁹ cells/L					5.0010 ⁻³²⁴	0.017
Men	0.15 (0.10 – 0.23)	0.17 (0.10 – 0.25)	0.19 (0.10 – 0.26)	0.20 (0.10 – 0.26)		
Women	0.12 (0.10 – 0.20)	0.15 (0.10 – 0.20)	0.16 (0.10 – 0.22)	0.16 (0.10 – 0.20)		
Percentage of eosinophils, %					5.0010 ⁻³²⁴	0.173
Men	2.30 (1.49 – 3.52)	2.38 (1.50 – 3.60)	2.40 (1.60 – 3.60)	2.30 (1.50 – 3.30)		
Women	2.00 (1.30 – 3.01)	2.10 (1.40 – 3.10)	2.20 (1.40 – 3.13)	2.05 (1.30 – 2.96)		
Basophil count, 10 ⁹ cells/L					2.4210 ⁻⁸⁹	0.549
Men	0.02 (0.00 – 0.04)	0.02 (0.00 – 0.04)	0.02 (0.00 – 0.05)	0.03 (0.00 – 0.06)		
Women	0.02 (0.00 – 0.04)	0.02 (0.00 – 0.05)	0.02 (0.00 – 0.05)	0.03 (0.00 – 0.07)		
Percentage of basophils, %					6.9410 ⁻¹⁰⁹	0.512
Men	0.41 (0.30 – 0.62)	0.42 (0.30 – 0.66)	0.40 (0.30 – 0.64)	0.46 (0.30 – 0.70)		
Women	0.44 (0.30 – 0.70)	0.44 (0.30 – 0.68)	0.44 (0.30 – 0.70)	0.50 (0.30 – 0.74)		

Data is expressed as number (%) and as mean with standard deviation (SD) or median with interquartile range (IQR). CAD = coronary artery disease; MI = Myocardial infarction; iHF = ischemic heart failure. P value for sex differences is derived from the regression analyses. P value for sex interaction is also shown, derived from the post-hoc regression analyses.

Chapter 3

TRANSLATIONAL OVERVIEW OF CYTOKINE INHIBITION IN ACUTE MYOCARDIAL INFARCTION AND CHRONIC HEART FAILURE

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ABSTRACT

Many cytokines are currently under investigation as potential target to improve cardiac function and outcome in the setting of acute myocardial infarction (MI) or chronic heart failure (HF). Here we aim to provide a translational overview of cytokine inhibiting therapies tested in experimental models and clinical studies. In various experimental studies, inhibition of interleukin-1 (IL-1), -6 (IL-6), -8 (IL-8), monocyte chemoattractant protein-1 (MCP-1), CC- and CXC chemokines, and tumor necrosis factor- α (TNF- α) had beneficial effects on cardiac function and outcome. On the other hand, neutral or even detrimental results have been reported for some (IL-1, IL-6, IL-8, and MCP-1). Ambivalence of cytokine function, differences in study designs, treatment regimens and chosen endpoints hamper the translation of experimental research into clinical practice. Human studies are currently limited to IL-1 β inhibition, IL-1 receptor antagonists (IL-1RA), IL-6 receptor antagonists (IL-6RA) or TNF inhibition. Despite favorable effects on cardiovascular events observed in retrospective cohort studies of rheumatoid arthritis patients treated with TNF inhibition or IL-1RA, most prospective studies reported disappointing and inconsistent results. Smaller studies ($n < 100$) generally reported favorable results of anticytokine therapy on cardiac function, but only one of the larger studies ($n > 100$) evaluating IL-1 β inhibition presented positive results on outcome. In conclusion, of the 10 anticytokine therapies tested in animals models beneficial effects have been reported in at least one setting. In larger clinical studies, findings were unsatisfactory in all but one. Many anticytokine therapies with promising animal experimental data continue to require further evaluation in humans.

INTRODUCTION

Acute myocardial infarction (MI) and chronic heart failure (HF) are associated with decreased quality of life and unfavorable long-term outcome [1], [2], [3] and novel therapeutic strategies are still needed to improve clinical outcome. After successful introduction of antiplatelet inhibitors, beta-blockers, statins, and renin-angiotensin-aldosterone inhibitors, more recently there is increasing interest to target inflammation more specifically by immunomodulation or specific anticytokine treatment.

Cardiac remodeling is one of the major contributors to progression of MI to HF and considered to be importantly mediated by inflammation [4]. Epidemiological studies suggest that circulating concentrations of inflammatory markers, such as C-reactive protein (CRP), are associated with subsequent risk of atherosclerosis formation, coronary heart disease (CHD) and cardiac remodeling [5]. In the setting of acute MI, elevated CRP levels are associated with impaired myocardial reperfusion [6]. In principle, the inflammatory response is a protective mechanism short-term but may lead to chronic overcompensatory failure. It is a complex conjunction between innate (quick and non-specific) and adaptive (slow and specific) immune systems [4], [7]. Upon tissue damage or endothelial cell stress, cardiomyocytes, leukocytes and platelets can release various inflammatory cytokines attracting antigen presenting cells. Antigen presenting cells such as dendritic cells, monocytes and macrophages from the innate immune system will recognize released self-antigens or danger signals and start to interact with B and T cells from the adaptive immune system [8], [9]. This interaction may be caused by the formation of receptor complexes and via cytokine production further activates and amplifies the instigated inflammatory response. Cytokines, such as interleukin-1 (IL-1), monocyte chemoattractant protein-1 (MCP-1), tumor necrosis factor- α (TNF- α) were previously found to be elevated in MI and HF [10], [11]. They are known to promote cell death of cardiomyocytes and cell hypertrophy by induction of intracellular signaling cascades such as NF- κ B, JAK/STAT and PI3K pathways in leukocytes. Some cytokines may even function as biomarker(s) while the extent of elevation has been associated with outcome and degree of cardiac injury [12], [13]. Anticytokine therapy targeting inflammation is widely used and successful in rheumatoid arthritis [14] and is currently an active field of investigation for treatment of MI and HF. Since their fluctuations during the process of cardiovascular remodeling and observed associations with clinical outcome, cytokines have attracted attention as potential therapeutic targets. The aim of this review is to provide a contemporary and translational overview of potential effects of cytokine inhibition on cardiac function and outcome in the setting of acute MI and chronic HF.

OUTLINE OF THIS REVIEW

For the selection of clinical studies, a total of 923 articles were screened (**Supplementary Methods**). Irrelevant articles based on article type, study design, patient population, and drug therapy were excluded. In total, 25 articles including 14 randomized clinical trials (RCTs) were reviewed thoroughly. For selection of animal experimental studies, we included search results and references of the clinical search and initially reviewed 56 articles of which 50 articles were considered relevant and are discussed. Since there are many pro-inflammatory cytokines being studied in the experimental field, we mainly focused on those that are currently under investigation in the clinical setting.

ANTICYTOKINE THERAPY IN EXPERIMENTAL MI MODELS

IL-1 inhibition in small animals

A variety of experimental MI models evaluated the effect of IL-1 inhibition. Pre-treatment with IL-1 receptor antagonist (IL-1RA) showed positive effects on left ventricular ejection fraction (LVEF) and infarct size in a murine ischemia reperfusion model (**Figure 1**) [15]. IL-1 receptor 1 knockout mice (IL-1R1, one of the receptors of the IL-1R superfamily) undergoing permanent coronary artery ligation, had larger infarct size compared to controls [16]. This aligns well with the observation that genetically engineered rat overexpressing IL-1RA in an ischemia reperfusion model had reduced infarct size and apoptosis [17]. IL-1RA overexpression in mice undergoing permanent coronary artery ligation had an equivalent effect on cardiac function and in the infarct-remote zone collagen expression was reduced, suggesting involvement of IL-1 in cardiac fibrosis [18]. Both pre- and post-treatment with IL-1RA have been reported to exert beneficial effects on cardiac function, dimensions and infarct size after permanent coronary artery ligation and ischemia reperfusion in mice and rats. Anakinra (recombinant human IL-1RA inhibitor) treatment initiated in the first weeks after permanent coronary artery ligation also resulted in improved left ventricular (LV) dimensions and fractional shortening (FS) [19]. These findings have been replicated in a comparable study with immediate and delayed treatment of anakinra causing a reduction in infarct size [20]. IL-1 inhibition with IL-1 trap, also known as rilonacept, a long-acting IL-1 inhibiting agent, was likewise successful in a chronic MI model. Using different dosages, less apoptosis and smaller infarct size was observed and LV dimensions and FS were attenuated [21]. Moreover, specific IL-1 β inhibition after permanent coronary artery ligation led to less LV dilatation and increased FS [22]. Interestingly, in a larger chronic MI study, detrimental effects have been reported with mice having larger infarct size, lower collagen gene expression

and more ventricular ruptures after treatment with a similar dose of IL-1 β antibody [23]. Taken together, there appears to be evidence for both beneficial as well as detrimental effects of IL-1 inhibition on cardiac function and infarct size in experimental MI.

FIGURE 1. Effects of cytokine suppletion, overexpression and inhibition in MI.

IL-6 inhibition in mice and rats

Few studies investigated the effect of activation and inhibition of interleukin-6 (IL-6) and its receptor. One study evaluated IL-6 receptor antagonist (MR16-1) or placebo treatment after permanent coronary artery ligation in mice [24]. FS increased, left ventricular end-diastolic diameter (LVEDD) was smaller and the survival rate was higher than controls. In an opposed model using gp130 knockout mice, the IL-6 binding common receptor, increased IL-6 and STAT3 expression, LV dilatation, LV rupture and mortality was seen compared to the wild-type [25]. The effect was attenuated with an additional genetic reduction of STAT3, suggesting the destructive mechanism behind gp130 impaired signaling is STAT3 dependent. Contradictory with previous studies, infarct size increased and LVEF decreased in a different study with mice treated with IL-6 monoclonal antibody prior to permanent coronary artery ligation. Neutrophil infiltration was reduced in the treatment group suggesting the inflammatory response initiated by IL-6 also has functional and cardioprotective properties [26]. In addition, treatment with IL-6/soluble IL-6 receptor (sIL-6R) complex has reduced cardiomyocyte apoptosis and lowered the infarct area vs. area at risk percentage in an ischemia reperfusion model [27]. In conclusion, with contradictory findings on its inhibition illustrated by impaired cardiac function observed after pre-treatment and opposite effects after post-treatment, IL-6 appears a difficult target for therapy.

TNF- α inhibition in small and large mammals

In various ischemia reperfusion models with TNF- α inhibitor post-treatment reduced MI size [28], [29], [30]. In an ex vivo study, treatment with monoclonal TNF- α antibodies after a period of ischemia in isolated rat hearts showed positive effects on LV pressure, coronary flow and oxygen consumption [31]. However, TNF- α blockade had no effect when tested in adiponectin knockout mice, whereas adiponectin supplementation did, suggesting the effect of TNF- α inhibition is adiponectin dependent [29]. Adiponectin itself has been shown to be cardioprotective in ischemia reperfusion and adiponectin blocks the pro-inflammatory effects of TNF- α , while elevated TNF- α can inhibit adiponectin production [29]. To the contrary, administration of low-dose TNF prior to ischemia reperfusion in isolated murine hearts resulted in reduced infarct size, suggestive of a potential preconditioning effect [32]. One chronic MI rat model receiving TNF- α inhibition post-treatment showed better LV pressures and diastolic function compared to controls [33]. In addition, less leukocyte infiltration and increased thickness of the LV free wall in the infarct area were seen. The effects of TNF- α antagonists have also been evaluated in larger mammals, including rabbits, swine, and dogs. In one rabbit study, two groups received anti-murine TNF- α sheep antibodies pre-treatment and in one group this was combined with short periods of coronary artery occlusion (ischemic

preconditioning) before the main coronary artery procedure [34]. Infarct size was reduced in all treatment groups compared to controls. The concentration of circulating TNF- α correlated with infarct size. The authors suggested that ischemic preconditioning was as effective as anti-TNF- α administration in reducing infarct size. Anti-TNF- α treatment was again tested before permanent coronary artery ligation in another rabbit study and led to less necrosis, less circulating endothelial cells and neutralized levels of TNF- α compared to controls [35]. In a permanent coronary artery balloon occlusion model in swine inducing ventricular fibrillation, treatment with TNF- α inhibition resulted in survival rates almost twice as high [36]. In a similar study in swine, Infliximab treatment after induction of ventricular fibrillation and concomitant resuscitation was associated with higher mean arterial pressure and stroke work [37]. In dog ischemia reperfusion models, TNF- α inhibition after coronary artery balloon occlusion, or prior to coronary artery ligation, was associated with smaller infarct size [38], [39]. Pre- and post-treatment with TNF- α antagonists in smaller and larger mammals reduced infarct size in both ischemia reperfusion and permanent myocardial ischemia models. Only two studies reported data on cardiac function after treatment with TNF- α antagonists and showed improvement of LV pressures.

CC and CXC chemokine inhibition in mice

Evasin-3 is a chemokine binding protein discovered in tick saliva. Evasin-3 binds CXCL1, CXCL8, and macrophage inflammatory protein-2 and inhibits neutrophil cell recruitment and has been tested in experimental MI models [40]. In a model of in vivo and ex vivo coronary artery ligation lasting 30 minutes, Evasin-3 post-treatment reduced infarct size and reactive oxygen species levels [41]. The beneficial effects of post-treatment in a mouse ischemia reperfusion model were attributed to the prevention of neutrophil infiltration, which is induced by CXC chemokines. In a subsequent study, Evasin-3 and Evasin-4, chemokine binding proteins inhibiting CC chemokines (including CCL5 and CCL11), administration after inducing permanent coronary artery ligation in mice was associated with lower levels of CXCL1 and CCL2, less leukocyte infiltration and smaller infarct size [42]. The effect of Evasin-4 on circulating chemokine levels was accelerated and survival after infarction improved compared to Evasin-3. Cardiac function did not differ between Evasin-3 and -4 groups and controls. Interestingly, direct inhibition of CXCL1 and receptor CXCR2 in several regimens was not successful in mice [43]. Only anti-CXCR2 antibody improved LVEF and decreased infarct size when administered for a longer period up to 3 weeks. Inhibition of the CXCR2 and CXCR4 binding cytokine, macrophage migration inhibitory factor (MIF), was further tested in a genetic ischemia reperfusion model with chimeric mice lacking CXCR2 and wildtype mice [44]. Pre-treatment with anti-MIF resulted in larger infarct size and impaired LVEF in wildtype

mice and in the mice generated with CXCR2 lacking bone marrow derived inflammatory cells. In contrast, chimeric mice with CXCR2 lacking cardiomyocytes receiving anti-MIF treatment showed an improved LVEF compared to chimeric mice with control antibody treatment. Blocking MIF has detrimental effects presumably via CXCR2 cardiomyocytes as opposed to cardioprotective effects in CXCR2-deficient cardiomyocytes. Miller et al. [45] investigated effects of genetic deletion of MIF (MIF^{-/-}) in an ischemia reperfusion mouse model and observed larger infarct size compared to wildtype mice. In conclusion, Evasin-3 and -4 post-treatment, targeting CC and CXC chemokines, were associated with smaller infarct size. CXCR2 inhibition during a longer period attenuated cardiac function in experimental MI whereas contradictory findings are reported regarding inhibition of its ligand MIF.

MCP-1 (CCL2) inhibition in mice

Anti-MCP-1 treatment in mice, administered before and after permanent coronary artery ligation, resulted in improved survival and reduced LVEDD and improved FS [46]. Extracellular matrix metalloproteinase 9 concentration, involved in collagen degradation and thereby remodeling, was lower in the anti-MCP-1 treated group. In contrast, in transgenic MCP-1 overexpressing mice infarct size was reduced in ischemia reperfusion experiments but not in permanent coronary artery ligation [47]. Likewise, in an ischemia reperfusion model with isolated hearts of MCP-1 overexpressing transgenic mice, improved LV pressures were observed [48]. Taken altogether, the effects of MCP-1 inhibition and overexpression in experimental MI are ambiguous.

CCL5 (RANTES) inhibition in mice

Several pharmacological and genetic knock-out studies have been undertaken to determine the effect of CCL5 inhibition. Mice treated with anti-CCL5 after permanent coronary artery ligation or ligation for 30 minutes resulted in improved LVEF and smaller infarct size [49]. A decline in infarct size was also observed in another ischemia reperfusion model in which mice received CCL5 antagonist prior to reperfusion [50]. On the other hand, blocking CCR5, a receptor binding CCL5 and others, in a knockout model had detrimental effects [51]. Though, CCL5 was not induced, which corresponds with the previous study suggesting CCL5 inhibition may exert its effects via CCR1 and not via de CCR5 receptor [50]. The preliminary effects of CCL5 inhibition are promising but the mechanism or receptor of action needs to be elucidated.

IL-8 inhibition in rabbits and rats

Both genetic overexpression and inhibition of interleukin-8 (IL-8) have been studied. In an ischemia reperfusion model, rabbits received a monoclonal antibody against IL-8 prior

to coronary artery ligation that was associated with reduced infarct size [52]. In a chronic MI model with rats, treatment with endothelial cell transfection overexpressing IL-8 receptors at several hours after permanent coronary artery ligation decreased infarct size, inflammatory cells and improved LVEF was observed [53]. Thus far, experimental MI studies are contradictory on the effect of IL-8 inhibition and overexpression.

ANTICYTOKINE THERAPY IN MI IN HUMANS

IL-1 inhibition

The recombinant human IL-1RA (anakinra) is currently registered for the treatment of rheumatoid arthritis. Two pilot studies and a phase 2 study with anakinra have been performed in ST-segment elevation myocardial infarction (STEMI) patients (**Table 1**). In the pilot study VCU-ART, 10 patients received anakinra 100 mg/day and showed a decrease in LV end-diastolic and end-systolic volume indices, compared to placebo [54]. Details on study design and results can be found in **Table 1**. In a consecutive RCT, 30 STEMI patients undergoing percutaneous coronary intervention (PCI) were treated with anakinra 100 mg/day or placebo during the first 2 weeks and the primary end point, left ventricular end-systolic volume index, did not differ [55]. A meta-analysis combining the data with the previous pilot study, VCU-ART, showed a lower incidence of HF symptoms in the anakinra treated group [54]. In a further analysis including extended follow-up data of these patients, treatment was associated with decreased incidence of new-onset HF diagnoses and death [56]. In the MRC ILA Heart Study, 182 acute non-ST-segment elevation myocardial infarction (NSTEMI) patients were randomized to anakinra 100 mg/day or placebo for a period of 2 weeks [57]. No differences were found in levels of high sensitive C-reactive protein (hsCRP), Troponin or von Willebrandfactor 1 week after MI. In a later publication of the same author, it was stated that the primary endpoint, hsCRP area under the curve over first 7 days, was significantly lower [58]. Unfortunately, more major adverse cardiac events (MACE) also occurred in the IL-1RA treatment group during 1-year follow-up. In contrast, the recent CANTOS RCT including 10,061 patients with previous MI and hsCRP levels ≥ 2 mg/L showed promising results. Treatment with canakinumab, an IL-1 β targeting monoclonal antibody, resulted in a lower incidence of cardiovascular events [59]. As can be expected, fatal infection or sepsis was more prevalent in the treatment groups. Nonetheless, fatal cancer, with almost double the incidence rate, was significantly lower in the treatment groups. Although future studies are needed to evaluate the safety profile and to reaffirm these study results, canakinumab is the most promising anticytokine therapy studied in MI. The double-blind RCT VCU-ART3 in STEMI patients is currently ongoing, evaluating IL-1RA treatment on CRP [60].

TABLE 1. Cytokine inhibition in MI—randomized clinical trials.

Targeted cytokine	Main findings	N, age	Treatment	Follow-up period	Author, year
Double blind RCT on IL-1RA after STEMI	+ stable LV function, lower incidence of heart failure	30. Mean age 58.7	Anakinra 100 mg/day SC during 14 days compared to placebo treatment	14 weeks	Abbate et al., 2013 [55] VCU-ART2
Double blind RCT on IL-1RA in NSTEMI	– no difference in levels of hsCRP, Troponin and vWF, IL-6 levels lower in placebo	182. Mean age 61	Anakinra 100 mg/day SC during 14 days compared to placebo treatment	1 year	Morton and Foley, 2011 [57]
Double blind RCT on IL-1RA after STEMI	+ safe and favorably affects LV remodeling (higher LVESVI)	10. Mean age 47.8	Anakinra 100 mg/day SC during 14 days compared to placebo treatment	14 weeks	Abbate et al., 2010 [54] VCU-ART
Double blind RCT on IL-1β after MI	++ safe and lower incidence of cardiovascular events	10,061 Mean age 61.1	Canacinumab 50 mg, 150 mg and 300 mg SC every 3 months	Median follow-up 3.7 years	Ridker et al., 2017 [59] CANTOS
Double blind RCT on IL-6RA in NSTEMI	+/- lowers hsCRP and Troponin levels, no effects on cardiac function	117. Mean age 60	Single injection of tocilizumab 280 mg compared to placebo treatment	6 months	Kleveland et al., 2016 [62]

Abbreviations: hsCRP, high-sensitive C-reactive protein; IL-1, interleukin-1; LV, left ventricular; LVESVI, left ventricular end-systolic volume index; mg, milligram; N, number; NSTEMI, non-ST-segment elevation myocardial infarction; RA, receptor antagonist; RCT, randomized clinical trial; SC, subcutaneous; STEMI, ST-segment elevation myocardial infarction; vWF, von Willebrand factor.

TNF- α inhibition

TNF- α antagonists (infliximab, etanercept, and adalimumab) are commonly used anti-inflammatory agents and inhibit TNF- α signaling by binding to its soluble receptors sTNFR1 and sTNFR2. Only one double-blind RCT evaluated the effect of etanercept 10 mg or placebo treatment in 26 acute NSTEMI patients [61]. Etanercept reduced neutrophil and IL-6 levels, although an increase in platelet-monocyte aggregation was seen. Cardiac function and infarct size were not assessed in this study, prohibiting a hard conclusion on the effects of etanercept.

IL-6 inhibition

Promising results have recently been reported on the effect of a single dose of tocilizumab, an IL-6 receptor antagonist, on the primary endpoint hsCRP levels in NSTEMI patients (Table 1) [62]. In this double-blind trial, Area under the curves of hsCRP and Troponin T were higher in the placebo group, suggesting the inflammatory response can be attenuated by tocilizumab. Echocardiography at 6 months follow-up showed no difference in cardiac function between the groups, although the trial was not primarily powered for this endpoint.

ANTICYTOKINE THERAPY IN EXPERIMENTAL HF MODELS

IL-1 inhibition in mice

Only limited data are available on IL-1 inhibition in HF. One experimental model induced HF by injecting IL-1 β (3 μ g/kg) causing a significant reduction in FS. When IL-1RA anakinra was administered prior to this injection, LVEF and stroke volume improved in anakinra treated mice [63].

TNF- α inhibition in rats and dogs

TNF- α inhibition has been evaluated in experimental HF induced by Isoproterenol or chronic pacing or related to an animal constitution with hypertension or diabetes. In one of these experimental HF models, spontaneously hypertensive and healthy rats underwent treatment with etanercept (TNF- α inhibitor) or placebo during 12 weeks [64]. Spontaneously hypertensive rats were suggested to display an early stage of HF with increased relative wall thickness and heart weight. After 12 weeks, FS did not differ, although relative wall thickness decreased and cardiac reserve increased compared to controls. Furthermore, TNF- α expression was not affected and blood pressure was increased only in the etanercept treatment group. In healthy rats, etanercept resulted

in increased levels of β -1-adrenergic receptor mRNA expression, suggesting a positive inotropic effect. These findings indicate that anti-TNF- α treatment is ineffective and may even aggravate HF. In a different HF model with diabetic rats associated with enlarged thinned left ventricles with impaired LV function, no long-term beneficial effects within the context of cardiac function and remodeling were seen with etanercept [65]. Etanercept treatment was studied yet in another HF model with isoproterenol [66]. A single injection of isoproterenol, a systemic β -adrenergic receptor agonist, is associated with myocardial damage and numerous other characteristics resembling HF [67]. FS and LV dilatation was indeed ameliorated in rats receiving etanercept. Noteworthy, not TNF- α levels, but IL- β levels in the left ventricle were lower in the etanercept group. In a distinct HF model, dogs were paced chronically for 4 weeks and received placebo or etanercept treatment twice a week [68]. The chronic pacing resulted in reduced LVEF and LV dilatation. LV dilatation was less severe and LVEF was partially preserved with etanercept treatment. Also, mitochondrial respiratory chain enzyme complexes II and V in the etanercept group were completely or partially restored. DNA fragments, Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)-positive cells and Aldehyde levels were normalized in the etanercept group whereby an increase was seen in the placebo group. This suggested that apoptosis and mitochondrial dysfunction in this HF model was attenuated by etanercept. Due to the variety of HF models used to test the effect of anti-TNF- α , a general conclusion is hard to determine.

ANTICYTOKINE THERAPY IN HF IN HUMANS

IL-1 inhibition in human

Few RCTs, observational and cohort studies have evaluated the effect of TNF and IL-1 inhibition in relation to HF (**Table 2, Table 3**). In one study, 14-day treatment with IL-1 receptor antagonist anakinra in 7 HF patients improved median peak oxygen consumption [63]. A similar treatment protocol was followed in the D-HART study, including 12 HF patients with preserved ejection fraction (HFpEF). Anakinra treatment led to improvement in peak oxygen consumption and decrease in CRP levels [69]. The subsequent RED-HART trial including 60 HF patients treated with anakinra for 12 weeks and a different study of the same author including 30 acute decompensated HF patients treated for 2 weeks showed similar results with regards to peak oxygen consumption and reduction of CRP levels [70], [71]. Furthermore, the investigators of the RED-HART trial observed an improvement in LVEF with 2 week anakinra treatment, but total incidence of death or readmission for HF did not differ among the treatment groups. In a double-blind cross-over trial, 46 rheumatoid arthritis patients received 150 mg anakinra daily treatment for 30 days [72]. The echocardiographic index of LV

TABLE 2. Cytokine inhibition in HF—(randomized) clinical trials.

Targeted cytokine	Main findings	N, age	Treatment	Follow-up period	Author, year
RCT on IL-1RA in HF patients	–/+ improved median peak oxygen consumption in 12 weeks treatment group	60, Median age 57 and 55 (IL-1RA groups)	Injection of anakinra 100 mg daily for 2 weeks or 12 weeks or placebo for 12 weeks	24 weeks	Van Tassel et al., 2017 [70] RED-HART
Double blind RCT on IL-1RA in ADHF patients	+ reduction in CRP	30, Median age 60 (IL1RA group)	Injection of anakinra 100 mg twice daily during 3 days and once daily during following 11 days compared to placebo	14 days	Van Tassel et al., 2016 [71]
Clinical trial on IL-1RA in HF patients	–/+ improved median peak oxygen consumption	7, Mean age 48	Anakinra 100 mg/day SC during 14 days	2 weeks	Van Tassel et al., 2012 [63]
Double blind crossover RCT on IL-1RA in HFpEF patients	+ improved peak oxygen consumption, reduction in CRP	12, Median age 62	Anakinra 100 mg/day SC during 14 days or placebo treatment followed by alternative treatment	2 weeks	Van Tassel et al., 2014 [69]
Double blind (R)CT on IL-1RA in RA patients	+ increase in LV performance (E/Em, LongS and LongSRS)	46, Mean age 56	Single injection (randomized) and 30 days (non-randomized) anakinra 150 mg SC treatment compared to prednisolone treatment	1 month	Ikonomidis et al., 2011 [72]
Double blind crossover IL-1RA RCT in CAD patients with RA	+ increase in LVEF, improved LV myocardial deformation and twisting	60, Mean age 59.5	Single injection of anakinra 100 mg SC or placebo followed by the alternative treatment after 48 hours	3 hours	Ikonomidis et al., 2014 [74]
Double blind RCT on TNFi treatment in HF patients	– no benefit of etanercept on death/HF hospitalization	2,048, Mean age 63.5	Etanercept 25 mg SC once, twice or three times weekly, compared to placebo treatment	24 weeks	Mann et al., 2004 [89] RENEWAL
Double blind RCT on TNFi treatment in HF patients	– no improvement, possible CHF worsening, LVEF improved with 5 mg/kg	150, Mean age 61.4	Infliximab 5 mg/kg or 10 mg/kg IV at baseline, week 2 and week 6 compared to placebo treatment	28 weeks	Chung et al., 2003 [88] ATTACH

TABLE 2. Continued.

Targeted cytokine	Main findings	N, age	Treatment	Follow-up period	Author, year
Double blind RCT on TNFi treatment in HF patients	+ significant dose-dependent improvement in LV structure and function	47. Mean age 55.0	Etanercept 5 mg/m ² , 12 mg/m ² SC twice weekly for 3 months compared to placebo treatment	3 months	Bozkurt et al., 2001 [85]
Controlled clinical trial on TNFi in CHF patients	+ improved systemic endothelial vasodilator capacity	18. Mean age 53.6	Single dose of etanercept 25 mg SC compared to controls without treatment	1 week	Fichtlscher-er et al., 2001 [87]
Double blind RCT on TNFi treatment in HF patients	+ significant improvement of LVEF in 4 or 10 mg/m ² group	18. Mean age 63.3	Single infusion of etanercept 1, 4 or 10 mg/m ² IV or placebo treatment	2 weeks	Deswal et al., 1999 [86]

Abbreviations: ADHF, acute decompensated heart failure; CAD, coronary artery disease; CHF, congestive heart failure; CRP, C-reactive protein; E, early transmitral flow velocity; Em, early diastolic mitral annulus velocity; HF, heart failure; IV, intravenously; kg, kilogram; LongS, longitudinal end-systolic strain; LongSPS, longitudinal peak systolic strain rate; LV, left ventricular; LVEF, left ventricular ejection fraction; mg, milligram; m², square meters; N, number; RA, rheumatoid arthritis; RCT, randomized clinical trial; SC, subcutaneous; TNFi, TNF inhibitors.

TABLE 3. Cytokine inhibition and CVD—cohort and retrospective studies.

Targeted cytokine	Main findings	N, age	Treatment	Follow-up period	Author, year
Cohort study on TNFi in RA patients	++ modest increase in LVEF and reduced biochemical HF markers	23. Mean age 51.3	Infliximab 3 mg/kg body weight per infusion every 8 weeks	1 year	Kotyla et al., 2012 [76]
Cohort study on TNFi in RA patients and CV events	++ reduced risk of non-fatal and fatal cardiovascular events	10,156 Mean age 59	TNFi treatment in person years of exposure	Mean 22.9 months	Greenberg and Kremer, 2011 [82]
Cohort study on TNFi in RA patients and HF incidence	+ no increased risk of (worsening) HF	2,757. Mean age 53.7	Infliximab, etanercept, Adalimumab treatment compared to conventional DMARD treatment (<i>n</i> = 1491)	3 years	Listing et al., 2008 [79]
Cohort study on TNFi in elderly RA patients and HF incidence	– increased risk HF hospitalization and exacerbation, TNFi treated patients had more severe RA	1,002. Mean age 73/77 in HF	At least one prescription of etanercept, infliximab and adalimumab compared to MTX using controls (<i>n</i> = 5593)	Mean 1.6/1.7 years in HF	Setoguchi et al., 2008 [80]
Cohort study on TNFi and MI incidence in RA patients	~/* no difference in MI incidence, but reduced incidence of MI in responders	10,829. Mean age 56.8	TNFi treatment of minimal 6 months (<i>n</i> = 8659) compared to DMARD treatment (<i>n</i> = 2170)	Mean 18 months	Dixon et al., 2007 [106]
Retrospective study TNFi and exacerbation of CHF	– no difference between CHF, but mortality rates non-significantly different	103. Mean age 58.7	At least one dose of infliximab, adalimumab or etanercept compared to RA and non-RA controls (<i>n</i> = 200)	max. 4 years	Cole et al., 2007 [77]
Retrospective study on TNFi and prevalence of HF in RA/CD patients	~/* no significant increase in HF incidence	4,018. Mean age RA 40/ 38	At least three prescriptions for etanercept or infliximab compared to controls	15/21 months	Curtis et al., 2007 [78]
Retrospective study on TNFi in RA patients and CVD risk	+ risk of CVD lower in TNF blocker treated RA patients	983. Mean age 58	TNFi treatment (<i>n</i> = 531) compared to no TNFi treatment patients (<i>n</i> = 543)	max. 2 years	Jacobsson et al., 2005 [84]
Cohort study on TNFi in RA patients and HF incidence	+ HF less common in TNFi treated RA patients	13,171. Mean age 61	Infliximab or etanercept treatment compared to no TNFi treated controls	2 years	Wolfe and Michaud, 2004 [83]

Abbreviations: CD, Crohn's disease; CHF, congestive heart failure; CV, cardiovascular disease; DMARD, disease-modifying antirheumatic drugs; HF, heart failure; kg, kilogram; LVEF, left ventricular ejection fraction; MI, myocardial infarction; MTX, Methotrexate; mg, milligram; N, number; RA, rheumatoid arthritis; RCT, randomized clinical trial; TNFi, TNF inhibitors.

diastolic filling pressure, longitudinal strain measurements and LVEF improved after treatment with anakinra. However, these patients were not primarily diagnosed with HF; LVEF at baseline was within normal range according to current guidelines [73] and for the remaining echocardiographic parameters normal values are not yet available. One study reported a substantial improvement of cardiac function measured a few hours after single anakinra injection in 80 coronary artery disease patients with on average decreased LVEF at baseline [74]. In conclusion, IL-1 inhibition thus far seems a successful therapy in HF. A substudy of the CANTOS trial is currently evaluating improvement of peak oxygen consumption at 3 months after canakinumab in a selected group of patients with prior MI, elevated hsCRP, reduced LVEF and symptomatic HF [75].

TNF- α inhibition in human

Few small and larger cohort studies have focused on the potential beneficial effect of TNF inhibition on cardiac function and cardiovascular endpoints. In one of these cohort studies, 23 female rheumatoid arthritis patients without overt or latent history of HF underwent infliximab treatment for 1 year and LVEF increased significantly [76]. As mean LVEF was still within the normal range, it may be premature to extrapolate these results to clinical overt HF. In an observational study of 303 rheumatoid patients and controls, no difference was found in mortality and the incidence or worsening of HF [77]. Results from a larger observational study in 4018 rheumatoid arthritis and Crohn's disease patients were also neutral. The risk for HF was non-significantly increased in patients receiving TNF inhibition compared to non-biologicals [78]. In a similar neutral study, 2757 rheumatoid arthritis patients receiving either infliximab, etanercept or adalimumab were compared to 1491 patients receiving non-biological disease-modifying drugs (DMARDs) regarding the incidence or worsening of HF [79]. When corrected for cardiovascular disease (CVD) risk factors, increased incidence of HF was mainly related to disease activity scores. There was no significant risk related to TNF inhibition. The authors suggested that anti-TNF- α treatment is more beneficial than harmful in the context of risk of HF. One study reported negative effects of TNF inhibition on HF hospitalizations in 1002 rheumatoid arthritis elderly patients compared to 5593 methotrexate users [80]. Baseline characteristics of TNF inhibition vs. methotrexate users showed that patients taking TNF inhibitory drugs had more severe rheumatoid arthritis, indicated by higher CRP levels, more co-medication or injections and more comorbidities, though statistical significance was not reported. In addition, the study was not randomized and selection bias in TNF inhibitory drug prescription reserved to patients with more severe disease could have influenced the results. Methotrexate may also be a suboptimal control treatment as it is reported to reduce incidence of cardiovascular events [81]. In a cohort study of Dixon et al., the incidence of MI was reduced in responders to TNF inhibitory

treatment. In addition, in one of the larger cohort studies in 10,156 rheumatoid arthritis patients, the risk of non-fatal (MI) and fatal cardiovascular events was lower in the TNF inhibition group compared to patients taking DMARDs [82]. Positive effects were also observed in a study including 13,171 rheumatoid arthritis and 2568 osteoarthritis patients and in a smaller cohort showing a lower HF prevalence in TNF inhibitory drug treated patients [83], [84]. When pre-existing CVD was absent, there was a low risk of HF unrelated to TNF inhibitory therapy. However, age, sex and comorbidity differed significantly between the TNF inhibitory drug and non-TNF inhibitory drug-treated groups so it remains to be determined if TNF inhibition lowers the risk for HF in these patients.

A RCT including 47 HF patients for safety and tolerability of etanercept showed LVEF improvement in a dose-dependent manner [85]. In a similar double-blind RCT including 18 HF patients, a single intravenous injection of etanercept was associated with improvement of LVEF [86] and in a different set of 18 congestive HF patients, etanercept improved endothelial vasodilator capacity [87]. In another RCT, 150 patients with HF NYHA class III-IV and LVEF $\leq$ 35% received placebo or infliximab treatment 5 mg/kg or 10 mg/kg at baseline, week 2 and week 6 [88]. LVEF increased in the 5 mg/kg infliximab group, but the primary endpoint, clinical functional status after 2 weeks, did not improve. Conversely, 10 mg/kg infliximab treatment was associated with increased risk for HF hospitalization or death of any cause at 28 weeks.

To evaluate the effect of etanercept on HF hospitalization and death, data were combined of two RCTs; RECOVER and RENAISSANCE, including 1366 HF patients receiving placebo or etanercept 25 mg once, twice or three times a week [89]. Both studies were prematurely stopped because of a lack of benefit. Etanercept treatment did not reduce mortality or HF hospitalizations. The results did not change after subgroup analyses for NYHA class or LVEF.

In smaller RCTs, TNF- α antagonists ameliorated cardiac function. However, this positive effect is absent in previously mentioned large RCTs. In high dose, TNF- α antagonists seem to have adverse effects on clinical outcome. To summarize, TNF- α inhibition in HF patients did improve cardiac function in several smaller studies and is more likely to have a beneficial effect on cardiovascular events in patients with rheumatoid arthritis, but has failed to have such an effect in large RCTs. There are currently no large studies ongoing that evaluate TNF- α inhibition treatment in HF.

DISCUSSION

In a wide range of experimental animal models of MI and in various HF models, cytokine inhibition has shown promising results. Few clinical studies investigated the effects of anticytokine therapy in MI and HF patients. Larger clinical trials to date have failed to show an improvement on cardiac function and outcome, except the CANTOS trial, with less cardiovascular events in MI patients treated with canakinumab. Several explanations are possible to explain this failure of translation from animal studies to human trials. First of all, the association of cytokines with MI and HF is not completely elucidated. The time course of cytokine activation and elevation may appear obvious at first glance; however it is not clear whether, for instance, the duration of ischemia and the degree of successful reperfusion (or co-medication) has an impact on the inter- and intra-individual biochemical curves of cytokine levels. The complex pathways underlying cytokine activation and interactions are still only partly understood. The presence of potential negative feedback loops correcting hormone imbalances, such as SOCS3 inhibiting IL-6 [90], are not yet clarified for most cytokines involved in MI and HF and its presence and function are not readily translatable from animals to humans [91]. Furthermore, inhibition of some cytokines may lead to increased expression of other cytokines [64]. When looking at effects of anticytokine therapy, levels of related cytokines should therefore also be taken into account.

Another complexity is that many cytokines appear to have an ambivalent role. The function and effects of cytokine activation could be time-dependent, as is contemplated for instance for IL-6 [92]. Prolonged activation and excessive cytokine production may be detrimental. Pro-inflammatory effects are counterbalanced by anti-inflammatory downstream signaling, as in the case of TNF- α [93], [94], and selective inhibition of pro-inflammatory pathways is therefore challenging. In addition, other agents not discussed in this review, such as rapamycin, an immunosuppressive agent inhibiting mammalian target of rapamycin (mTOR), could have a key position in the inflammatory chain reaction. Rapamycin is supposed to have anti-atherosclerotic effects and inhibits different cytokines, including MCP-1 [95], [96]. Further studies are needed to evaluate the potential therapeutic value of rapamycin and other agents involved in the inflammatory cascade in patients with MI and HF.

At times, effects of anticytokine therapy and cytokine overexpression were in disagreement. Both anti-MCP-1 treatment [46] and MCP-1 overexpression [47] showed positive effects on cardiac function. Ischemia reperfusion and permanent coronary artery ligation models might have different effects on cytokine signaling. Lower cytokine levels have been reported in mice undergoing ischemia reperfusion vs. permanent

coronary artery ligation [97]. Hypothetically, when reperfusion is achieved, the cytokine burst is interrupted together with the initial inflammatory response that is associated with beneficial effects on myocardial healing [98]. Therefore, cytokine overexpression might have a place in ischemia reperfusion, whereas anticytokine treatment is more sensible in chronic MI models. Pre-treatment could also downregulate cytokine receptors and after a second stimulation caused by infarction cardiomyocytes may be less prone to cytokine activation. Yet, in the later phase after MI, the CANTOS trial showed that a selected group of MI patients with increased inflammatory response did benefit from the IL-1 β antibody canakinumab. In a previous study in diabetes patients, canakinumab reduced inflammatory markers IL-6 and hsCRP, without an effect on LDL-cholesterol [59]. These studies together endorse the hypothesis that inhibition of inflammation prevents atherothrombosis, without a major influence on other factors involved in this process, such as cholesterol. In contrast to other studies, the CANTOS trial only included patients with elevated levels of the inflammatory marker hsCRP. It makes sense that in order to be effective the target inflammatory cytokine(s) of a particular drug should be elevated in the first place. To which extent the >20% current smokers in the CANTOS trial might explain the persistent pro-inflammatory response and if canakinumab has additional positive effects on top of quitting smoking in these patients remains unknown.

In some of the studies described, inhibition of cytokines or its receptors had contradictory consequences and interacting pathways involved in inhibition of each cytokine are as yet not cleared up. This prohibits making a general conclusion on the responsible targets and potential clinical use of anticytokine therapy. Different treatment regimens are practiced. In experimental MI mode, timing and duration of treatment vary widely. This is illustrated by a model where mice received anti-MCP-1 gene therapy 3 days before and 14 days after coronary artery ligation [46]. Effective plasma concentrations of the MCP-1 receptor binding protein are reached for 14 days after the injection, explaining the choice for this treatment regimen. Instead of long-lasting treatment, others focused on pre-treatment [15], or short-term post-treatment [28]. After initial inflammatory response in MI, a second cytokine burst has been observed after 8 days [98]. Hence, the optimal timepoint to interfere with pro-inflammatory effects of cytokines might also be after the first week, which is barely studied in the discussed experimental MI models.

Other explanations for observed discrepancies in MI models may be the different design, namely ischemia reperfusion vs. permanent coronary artery ligation. Ischemia reperfusion is believed to trigger a more pronounced inflammatory response [99]. For instance, contradictive findings have been reported for IL-6 and IL-8 inhibition. In addition, anti-inflammatory properties have been ascribed to IL-8 [100]. Hypothetically,

anti-inflammatory actions by IL-6R inhibition and IL-8 overexpression may have positive effects on cardiac function during chronic MI while the opposite is true during ischemia reperfusion. Again, in experimental HF, a wide variety of models was used. In one study, HF was induced by injecting IL-1 β and at the same time IL-1RA was administered [63]. As HF is a complicated disease with many underlying factors, it may be too simplistic to imitate and evaluate treatment in a model with addition and inhibition of a single cytokine. The dog model with chronic overpacing [68] might be a good model for HF caused by atrium fibrillation, but may not account for other etiologies linked to HF. Recently, guidelines have been proposed to enhance similarity of experimental animal studies and human HF and might help in providing a structured approach for translation to humans [101].

Chosen endpoints in experimental MI and HF were also disparate. In experimental models, applied methods and timing of the evaluation of the inflammatory response and cardiac remodeling were very different. To illustrate, in an ischemia reperfusion model, the inflammatory response was studied and TNF- α , IL-1 β , and IL-6 were markedly upregulated 6 hours after MI [102]. The authors of this study endorsed the assessment of inflammatory mediators to be performed during the first 3 days. They also recommended that assessment of dilatative remodeling should take place at least 4 weeks after MI. LV dilatation increased significantly between 1 and 4 weeks after MI reflecting progressive LV remodeling. In many of the previous experimental models, these criteria for measuring LV dilatation at a later time point are not met.

Again, chosen endpoints in clinical MI and HF studies differ from each other and from experimental endpoints. This makes it even harder to judge if experimental findings can be readily translated to humans. Also, publication bias in experimental studies could play a part in the neutral results found in humans. In the European Society of Cardiology guidelines, LVEF is stated as an important prognostic parameter after MI [103]. However, surrogate endpoints, including LVEF, might not be good representatives for long-term outcome. In the reviewed experimental animal studies regarding anticytokine therapy in MI, endpoints vary widely. Apart from enzymatic and functional infarct size and LVEF, other parameters, such as LV dilatation, LV pressures, LV mass and stroke work, are used to evaluate treatment effects. In the two smaller clinical MI studies, positive effects of IL-1RA were seen on NYHA class, LV dimensions, incidence of HF and death. In one larger study, they found no effects on Troponin levels 1 week after NSTEMI and the incidence of MACE after 1-year follow-up did not differ. In HF, reduced LVEF is generally associated with worse outcome [104]. In HFrEF, important predictors of HF hospitalization and cardiovascular death were LV hypertrophy, increased pulmonary artery and LV filling pressures [105]. The reviewed experimental HF model evaluated

effects of IL-1RA and TNF inhibition on LVEF, FS, LV dilatation and stroke work. Human studies on anti-TNF- α inhibition mainly focused on LVEF, NYHA class, HF hospitalizations and death.

Conclusion and future perspectives

In this review we summarize the rapidly developing field of anticytokine therapy in cardiovascular disease and highlighted the contradictory findings in experimental MI and HF compared to the neutral results in clinical studies. In various experimental studies, inhibition of IL-1, -6, -8, MCP-1, CC chemokines, CXC chemokines and TNF- α had profound beneficial effects on cardiac function and outcome. On the other hand, neutral or even detrimental results have been reported for some (IL-1, IL-6, IL-8, and MCP-1) of these cytokines. Ambivalence of cytokine function, differences in study designs, species, treatment regimens, and chosen endpoints appear to hamper the successful translation of experimental research into clinical practice. In the clinical setting, only TNF- α inhibition, IL-1RA, IL-1 β inhibition and IL-6RA have been tested so far. Promising results were seen in smaller studies, but until now only one large RCT showed positive results on outcome. Many other anticytokine therapies with encouraging animal experimental data require further evaluation in humans, but the first clinical studies suggest this translation can be troublesome. We recommend investigators of future clinical MI and HF studies to carefully consider the intensity of treatment as well as the treatment period and monitor a set of related cytokine levels throughout and after the treatment period. Furthermore, inclusion of patients should be based on a pre-specified number of primary end-point events, such as reinfarction, incidence of HF, readmission for HF, and cardiovascular death. Widely accepted surrogate endpoints that have been shown to be strongly related to outcome and are also frequently used in experimental studies, e.g., LVEF and infarct size, may be more suited in smaller clinical trials.

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Supplementary Methods - Literature search

Clinical studies: a systematic search through the journal databases of PubMed, EMBASE and Cochrane Library was performed. Several (if available MeSH) terms were used: 'cytokines', 'antagonists and inhibitors', 'cardiovascular diseases', 'antibodies, monoclonal, humanized', 'biological therapy', 'biologicals', 'B-DMARDs', 'anticytokine therapy', 'cytokines/therapeutic use', 'interleukins', 'cardiovascular diseases', 'coronary artery disease', 'myocardial infarction', 'ventricular remodeling', 'heart failure'. In addition, we included European and American Food and Drug Administration registered monoclonal antibodies and monoclonal antibodies from the PubChem Substance database with the MeSH keyword monoclonal antibody aimed at inhibiting or blocking pro-inflammatory cytokines into the search (adalimumab; afelimomab; anti-IGF-1R antibody A12; briakinumab; brodalumab; canakinumab; certolizumabpegol; dupilumab; elsilimomab; fontolizumab; gevokizumab; golimumab; infliximab; lebrikizumab; LY2439821; mepolizumab; nerelimomab; olokizumab; pascolizumab; reslizumab; rontalizumab; sarilumab; secukinumab; sifalimumab; siltuximab; sirukumab; tildrakizumab; tocilizumab; tralokinumab; ustekinumab). Related articles denoted while screening the PubMed articles and references were also studied and included if relevant as determined by patient group, disease and chosen therapy. The population was restricted to subjects aged 18 years or older. The intervention is the use of cytokine inhibitors in the specified population. The principal outcomes were changes in cardiac function, new onset or worsening of CVD and mortality after intervention with the specified inhibitors. Study design includes (randomized) clinical trials, retrospective and cohort studies.

Experimental animal studies: a general search was performed with combinations of the following terms: 'cytokines', 'antagonists and inhibitors', 'cardiovascular diseases', 'antibodies, monoclonal, humanized', 'biological therapy', 'biologicals', 'B-DMARDs', 'anticytokine therapy', 'cytokines/therapeutic use', 'interleukin', 'cardiovascular diseases', 'coronary artery disease', 'myocardial infarction', 'ventricular remodeling', 'heart failure'. Relevant articles referred to in the clinical studies and experimental hits in the clinical search were reviewed as well. Timeline for selection was through January 1989 and March 2015 (extended to December 2017) for studies in the form of English written full-length articles, both published or scheduled for publication.

Chapter 4

HIGH-SENSITIVITY C-REACTIVE PROTEIN AND LONG TERM REPERFUSION SUCCESS OF PRIMARY PERCUTANEOUS INTERVENTION IN ST- ELEVATION MYOCARDIAL INFARCTION

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ABSTRACT

Aims: In STEMI patients, success of reperfusion of primary PCI predicts cardiac remodeling and clinical outcome. This success may depend on inflammation. We aimed to investigate the association between inflammation and reperfusion success, left ventricular function and long-term mortality in STEMI patients.

Methods: In 376 consecutive STEMI patients of the GIPS-III trial hs-CRP levels were measured at baseline, 2weeks, 7weeks and 4months post-PCI. Myocardial blush grade was used to determine success of myocardial reperfusion. In multivariate models sex, age, hs-CRP levels at baseline, NT-proBNP levels at baseline, ischemia time, heart rate, TIMI flow, and CK, CKMB and troponin AUC were included. Follow-up was complete until 4months.

Results: Baseline hs-CRP levels were 2.1mg/L (IQR 0.5-4.2mg/L). hs-CRP levels were associated with impaired reperfusion (OR 1.239, 95% CI 1.006-1.527) and remained higher compared to patients with normal reperfusion up to 2months after PCI (hs-CRP 1.9mg/L (IQR 0.9-3.7mg/L) versus 1.5mg/L (IQR 0.7-2.7mg/L), $p=0.041$). In multivariate analysis baseline hs-CRP levels remained independently associated with impaired reperfusion. In patients with impaired reperfusion, hs-CRP and NT-proBNP levels remained higher during 4months of follow-up. No correlation was observed between hs-CRP at baseline and left ventricular function at 4months. The number of events was small and we observed no differences in mortality.

Conclusion: Increased hs-CRP levels at presentation are associated with impaired microvascular reperfusion after PCI in STEMI patients and remain higher until 2 months follow-up.

INTRODUCTION

In patients with acute myocardial infarction (AMI) morbidity and mortality remains high despite advances in treatment like timely reperfusion of coronary stenosis by primary percutaneous coronary intervention (PCI). After AMI left ventricular function is often impaired due to impaired left ventricular remodeling and results in heart failure (HF) [1–3]. Inflammation is considered to play a substantial role in the pathophysiological process of cardiac remodeling [4,5]. AMI triggers a systemic acute-phase response, in which neutrophils and monocytes/ macrophages track to the infarcted region of the heart [2]. Epidemiological studies suggest that circulating concentrations of inflammatory markers, such as C-reactive protein (CRP) are associated with subsequent risk of atherosclerosis formation, coronary heart disease (CHD) and cardiac remodeling. In the setting of an AMI elevated CRP levels on admission are associated with impaired myocardial reperfusion as depicted by lower Thrombolysis in Myocardial Infarction (TIMI) flow grades [6–9]. A clear difference in CRP patterns between different forms of acute coronary syndrome (ACS) was previously described in the early days after admission [10]. Currently, sparse data is available about time course of CRP levels and its association with reperfusion success after AMI. The aim of this study was to investigate the association between inflammation, as determined by high sensitivity C-reactive protein (hs-CRP), and reperfusion success, left ventricular function and long-term mortality in patients with ST elevation myocardial infarction (STEMI).

METHODS

Study population and design

We included all patients participating in the GIPS-III trial. This trial was designed to evaluate the effect of metformin treatment on preservation of left ventricular function in STEMI patients without diabetes. Details on the design of the GIPS-III trial have been reported previously [11]. In brief, all patients admitted to the University Medical Center Groningen between January 1st, 2011, and May 26th 2013, via the STEMI protocol were considered eligible for the trial. Inclusion criteria were age older than 18 years, the presence of STEMI, and primary PCI with implantation of at least 1 stent with a diameter of at least 3 mm resulting in TIMI flow grade 2 or 3 post PCI. Major exclusion criteria were previous myocardial infarction, known diabetes, the need for coronary artery bypass graft surgery, severe renal dysfunction, and standard contraindications for magnetic resonance imaging (MRI) [12]. The study protocol of the GIPS-III trial was in accordance

with the Declaration of Helsinki and was approved by the local ethics committee (Groningen, the Netherlands) and national regulatory authorities. Informed consent was obtained for inclusion of the patients.

Data collection

On admission, standard laboratory assessment was performed and standard physical examination parameters were measured according protocol. Patients were seen in the outpatient clinic 2 weeks, 7 weeks, 4 months, and one year after discharge. During hospitalization, blood was sampled at baseline (initial admission) and at 3, 6, 9, 12, and 24 h after PCI to monitor values of cardiac enzymes and high sensitive troponin. Less frequently during hospitalization and at every visit to the outpatient clinic hemoglobin, leucocytes (including neutrophils, lymphocytes), platelets, glucose, hs-CRP and N-terminal pro B-type natriuretic peptide (NT-proBNP) were determined. Furthermore, during PCI, 24 h after PCI, and at every visit to the outpatient clinic, blood samples for additional analyses were collected [11]. The incidence of major adverse cardiac events (MACE; the combined end point of death, reinfarction, or target-lesion revascularization) was recorded until 4 months. hs-CRP was assayed by nephelometry with a lower limit of 0.175 mg/l (BNII N; Dade Behring, Marburg Germany). A hs-CRP level below 1 mg/l was considered as normal [13]. Leucocytes were determined on the XE2100 – system (Sysmex, Japan). As the neutrophil/ lymphocyte ratio is associated with cardiovascular outcomes, we calculated this ratio as well [14]. NT-proBNP levels were examined (Roche (Modular, Germany)), as NTproBNP is known as a biomarker for the development of HF and inflammation is thought to play a role in the development of HF [15]. Furthermore, we calculated the ratio between hs-CRP and NT-pro BNP levels evaluating whether these ratios would be different between reperfusion groups.

Myocardial blush grade

Myocardial blush grade (MBG) represents an angiographic measurement of myocardial perfusion [16]. It reflects myocardial response to ischemic injury and reperfusion. MBG was categorized as follows: 0 = no myocardial blush, or contrast density; 1 = minimal myocardial blush; 2 = moderate myocardial blush but less than that obtained during angiography of a contralateral or ipsilateral non-infarct-related coronary artery; 3 = normal myocardial blush comparable to that obtained during angiography of a contralateral or ipsilateral non-infarct-related coronary artery [17]. The patients were categorized as having normal (MBG 3) versus impaired (MBG 0–2) reperfusion. Coronary angiograms were analysed by two physicians blinded to clinical data.

Statistical analysis

Continuous variables were summarized as mean $\pm$ standard deviation if normally distributed or median and interquartile range if skewed. Discrete variables were presented as frequencies and percentages. To compare groups, we used Student's t-test for normally distributed continuous variables, Mann–Whitney U test for skewed continuous variables, Chi-square and Fisher's exact test for categorical variables. In order to be able to compare hs-CRP levels within reperfusion groups (between consecutive time points) and between reperfusion groups on the same time points, we used mixed model analysis. We modeled hs-CRP using a random intercepts regression model in terms of MBG and time points. Area under the curve (AUC) and peak values were calculated over the first 72 h post-PCI using the trapezoid method as previously described. No curve was calculated for patients having 2 measurements or only measurements with a timespan of 10 h. In multivariate models (logistic regression) for impaired reperfusion (impaired vs. normal), sex, age, hs-CRP levels at baseline, NT-proBNP levels at baseline, ischemia time, heart rate, TIMI flow (pre- and postintervention), and CK, CKMB and troponin AUC were included. Statistical significance was considered at a 2-tailed p-value of ≤ 0.05 . Statistical analyses were performed with Stata version 13.0 (StataCorp).

RESULTS

Study population

From 1 January 2011–26 May 2013, 376 out of 1473 patients admitted to the hospital via the STEMI protocol were included in the analysis [12]. Baseline characteristics are presented in Table 1. Clinical characteristics did not significantly differ between patients with impaired and normal reperfusion. The average age was 59 ± 12 years and 25% were female. Time between first complaints and catheterization, i.e. ischemia time, was longer in patients with impaired reperfusion than in patients with normal reperfusion. Levels of CK, CKMB, and NT-pro BNP at admission were higher in patients with impaired reperfusion than in patients with normal reperfusion. Furthermore levels of leucocytes and neutrophils, and the neutrophil/lymphocyte ratio were higher in patients with impaired reperfusion (**Table 1**).

TABLE 1. Baseline characteristics of patients with impaired reperfusion and normal reperfusion

Characteristics	Impaired reperfusion (MBG 0-2) (n=113)	Normal reperfusion (MBG 3) (n=263)	P-value
Age, mean (SD), years	60.2 ± 12.4	58.1 ± 11.3	0.056
Female sex – No. (%)	27 (23.5)	67 (25.6)	0.665
BMI, mean (SD), kg/m ²	27.0 ± 4.0	27.0 ± 3.7	0.450
Cardiovascular related history – No. (%)			
Hypertension	31 (27.0)	80 (30.5)	0.483
Dyslipidemia	69 (60.0)	169 (64.5)	0.404
Current smoking	59 (51.3)	148 (56.5)	0.554
Stroke	1 (0.9)	2 (0.8)	0.915
Peripheral artery disease	0	0	-
Previous PCI	2 (1.7)	2 (0.8)	0.395
Blood pressure, mean (SD) mmHg			
Systolic	135 ± 23	134 ± 24	0.456
Diastolic	84 ± 14	85 ± 15	0.719
Heart rate, mean (SD), beats/min	78 ± 18	75 ± 15	0.056
Ischemia time, median (IQR), min	190 (125 – 326)	145 (102 – 229)	0.001
Single vessel disease – No. (%)	79 (69)	177 (68)	0.827
Infarct-related artery TIMI flow – No. (%)			0.005
Preintervention grade			0.011
0	72 (63)	135 (52)	
1	11 (10)	16 (6)	
2	20 (17)	46 (18)	
3	12 (10)	65 (25)	
Post intervention grade			0.000
2	31 (27)	2 (1)	
3	84 (73)	260 (99)	
Laboratory values at admission			
CK, median (IQR), U/L	138 (93 – 196)	110 (80 – 178)	0.015
Myocardial band of CK, median (IQR), U/L	17 (14 – 25)	15 (12 – 22)	0.001
Troponin, median (IQR), ng/L	76 (30 – 230)	43 (22 – 99)	0.001
Creatinine, median (IQR), umol/L	73 (61 – 80)	71 (63 – 81)	0.911
NT-proBNP, median (IQR), ng/L	82 (42 – 274)	78 (37 – 156)	0.023
Glucose (median (IQR), mmol/L	7.7 (6.9 – 9.4)	8.5 (7.2 – 10.1)	0.327

TABLE 1. Continued.

Characteristics	Impaired reperfusion (MBG 0-2) (n=113)	Normal reperfusion (MBG 3) (n=263)	P-value
HbA1c, median (IQR), %	6 (5.7 – 6.2)	5.9 (5.7 – 6.1)	0.086
Peak values			
Peak CK, median (IQR), U/L	1755 (811 – 3610)	1123 (440 – 2882)	0.002
Peak myocardial band of CK, median (IQR), U/L	216 (103 – 419)	142 (57 – 300)	0.002
Peak troponin, median (IQR), ng/L	3808 (1832 – 7118)	2330 (792 – 5865)	0.003
AUC			
AUC CK, median (IQR), U/L sec ^{-10⁻⁸}	1.43 (0.58 – 2.60)	0.83 (0.35 – 1.83)	0.000
AUC myocardial band of CK, median (IQR), U/L sec ^{-10⁻⁸}	0.13 (0.07 – 0.26)	0.09 (0.04 – 0.17)	0.000
AUC troponin, median (IQR), ng/L sec ^{-10⁻⁸}	3.00 (1.15 – 6.64)	1.62 (0.56 – 3.91)	0.000
Blood count and biochemistry			
Leucocytes (10 ⁹ /l)	12 (9 – 14)	11 (8 – 13)	0.012
Thrombocytes (10 ⁹ /l)	240 (203 – 276)	232 (204 – 267)	0.315
Neutrophils (10 ⁹ /l)	9 (7 – 11)	7 (5 – 9)	0.000
Lymphocytes (10 ⁹ /l)	2 (1 – 2)	2 (2 – 3)	0.061
N/L ratio	5.19 (2.92 – 7.34)	3.32 (2.19 – 5.36)	0.001

Data are expressed as mean ± standard deviation (SD), median (interquartile range (IQR)), or as number (%). AUC = Area under the curve; BMI = Body Mass Index; TIMI = Thrombolysis in Myocardial Infarction; CK = creatine kinase; NT-proBNP = N-terminal pro brain natriuretic peptide; HbA1c = glycated hemoglobin; N/L = neutrophil/lymphocyte.

Temporal course of hs-CRP

hs-CRP levels are presented in **Figure 1A and B**. Levels of hs-CRP decreased over time in both groups. Patients with normal reperfusion had lower hs-CRP levels at baseline, 2 weeks, and 7 weeks compared to patients with impaired reperfusion. At 4 months, hs-CRP levels were not significantly different.

hs-CRP and associations

hs-CRP levels at baseline were positively correlated with leucocyte levels at baseline, NT-proBNP levels at baseline, and ischemia time ($r = 0.19$, $p = 0.001$; $r = 0.21$, $p < 0.001$; $r = 0.22$, $p < 0.001$, respectively) and negatively correlated with myocardial reperfusion ($r = -0.12$, $p = 0.035$) (**Table 2**). After multivariate adjustment, higher hs-CRP levels at baseline remained positively associated with impaired reperfusion. Higher infarct-related artery TIMI flow (postintervention) had a negative association with impaired reperfusion (**Table 3**).

FIGURE 1. Hs-CRP levels and NT-proBNP levels over time in 376 patients with impaired reperfusion and normal reperfusion. Mixed model analysis was used to compare hs-CRP and NT-proBNP levels within and between reperfusion groups. Data are presented as mean and standard error of the mean (SEM). NS = not significant. Figure 1A depicts the proportion of patients with impaired reperfusion between the lowest (baseline 0–1 mg/L, 2w 0–0.9 mg/L, 7w 0–0.8 mg/L, 4m 0–0.7 mg/L) and highest quartile (baseline >4.3 mg/L, 2w >5.0 mg/L, 7w >3.3 mg/L, 4m >2.5 mg/L) of hs-CRP levels. Figure 1B and 1C depict hs-CRP and NT-proBNP levels between the reperfusion groups on the same time points. Figure 1D shows the decrease within reperfusion groups between consecutive time points. Hs-CRP remained significantly higher in patients with impaired reperfusion for 7 weeks (Figure A). NT-proBNP levels were also higher in these patients (Figure B). Figure C depicts a combination of figure A and figure B in order to show the parallelism of the biomarkers in both reperfusion groups. Furthermore, hs-CRP levels decreased significantly in both reperfusion groups between 2 and 7 weeks, whereas NT-pro BNP levels differed between all consecutive time points (Figure C).

Temporal course of hs-CRP, NT-proBNP, and CRP/NT-proBNP ratio

Levels of NT-proBNP were higher in patients with impaired reperfusion in time **(Figure 1C)**. NT-proBNP increased in both groups between baseline and two weeks, these levels decreased thereafter. At 4 months NT-proBNP levels remained higher in patients with impaired reperfusion. Furthermore, ratio between hs-CRP and NTproBNP levels over time did not significantly differ between groups **(Supplemental Figure 1)**.

Outcomes

Clinical outcomes at 4 months did not significantly differ between patients with impaired and normal reperfusion (data not provided). However, the number of clinical events in overall population was low, mortality rate at 1 year was 0%, reinfarction rate at 1 year was 1.8%.

TABLE 2. Correlation between hs-CRP at baseline and other variable

Characteristic	Hs-CRP at baseline	P-value
Leucocytes at baseline	0.19	0.001
NT-proBNP at baseline	0.21	0.000
Ischemic time	0.22	0.000
MBG (0,1,2,3) at baseline	-0.12	0.035
MBG (reduced vs. normal) at baseline	-0.13	0.029
Infarct size (%) at 4 months	-0.04	0.533
LVEF (%) at 4 months	-0.06	0.353

hs-CRP = high sensitivity C-reactive protein; NT-proBNP = N terminal probrain natriuretic peptide; MBG = Myocardial blush grade; LVEF = Left ventricular ejection fraction

TABLE 3. Univariate and multivariate predictors for impaired reperfusion.

Variable	Univariate analysis			Multivariate analysis		
	OR	95% CI	P value	OR	95% CI	P value
Sex (female)	0.96	0.58 - 1.60	0.887	0.76	0.39 - 1.50	0.431
Age (per year)	1.02	1.00 - 1.04	0.060	1.01	0.99 - 1.04	0.351
hs-CRP level at baseline (per doubling)	1.27	1.08 - 1.50	0.004	1.24	1.00 - 1.53	0.042
NT-pro BNP levels at baseline (per doubling)	1.19	1.06 - 1.34	0.004	1.10	0.92 - 1.32	0.304
Time between symptom onset and catheterization (per hour)	1.18	1.08 - 1.28	0.000	1.05	0.93 - 1.20	0.429
Heart rate (bpm)	1.01	1.00 - 1.03	0.064	1.01	0.99 - 1.03	0.240
Infarct-related artery TIMI flow, preintervention grade	0.75	0.62 - 0.91	0.003	0.79	0.60 - 1.04	0.100
Infarct-related artery TIMI flow, postintervention grade	0.02	0.01 - 0.09	0.000	0.04	0.01 - 0.19	0.000
CK AUC (per doubling)	1.28	1.11 - 1.48	0.001	0.99	0.48 - 2.04	0.975
Myocardial band of CK AUC (per doubling)	1.36	1.16 - 1.59	0.000	0.90	0.45 - 1.82	0.773
Troponin AUC (per doubling)	1.26	1.11 - 1.42	0.000	1.15	0.74 - 1.79	0.536

AUC = area under the curve; Bpm = beats per minute; CI = confidence interval; CK = creatine kinase; hs-CRP = high sensitivity C-reactive protein; NT-pro BNP = N-terminal probrain natriuretic peptide; OR = odds ratio; TIMI = Thrombolysis in Myocardial Infarction

DISCUSSION

This study investigated the temporal course of hs-CRP levels in patients who presented with a first STEMI and treated with primary PCI. The principle findings were as follows. First, higher hs-CRP levels at presentation are associated with decreased reperfusion success and these levels remained higher in patients with impaired reperfusion for up to two months after PCI. Second, hs-CRP at baseline is positively correlated with ischemia time, although the correlation is not strong, and together with TIMI flow an independent predictor for myocardial reperfusion. Third, NT-proBNP levels follow the same trend as hs-CRP in time with slightly higher levels in patients with impaired reperfusion. Last, LV systolic function was preserved in these STEMI patients and we did not observe an association between hs-CRP levels and impaired LV function and/or long term outcomes.

Temporal course of hs-CRP

Earlier studies suggested that CRP levels in STEMI patients reached their peak values between 36 and 42 h after admission [10]. The degree of inflammation marked by CRP was associated with the degree of myocardial necrosis. We indeed observed an association with ischemia time and impaired reperfusion resulting in increased myocardial necrosis. The ischemic time might, in part, also explain the higher hs-CRP levels in these patients at baseline. We also measured hs-CRP at 2 weeks, 7 weeks and 4 months after admission, and in addition to Patti et al. we report on the temporal course of hs-CRP in STEMI patients [8,9,18, 19]. In addition, we used more sensitive assay to determine the hsCRP levels, allowing detection of CRP below 5 mg/L and facilitating the detection of subtle differences between patients with normal and impaired reperfusion. Currently, it remains to be elucidated why hs-CRP levels continue to remain increased for a substantial long period in patients with impaired reperfusion. It may be a consequence of chronic damage of the microvasculature of the heart and the inability to recover quickly due to impaired reperfusion, which causes the liver to continue producing hs-CRP. Furthermore, inflammation plays a key role in wound healing and scar formation after myocardial infarction [20]. Perhaps this reparative mechanism and the remodeling of the heart takes longer in patients with impaired reperfusion, which causes the liver to produce hs-CRP for a longer period. In addition to increased hs-CRP levels in patients with impaired reperfusion, the increased levels of leucocytes, neutrophils, and their ratio in these patients suggest that these patients have a higher inflammatory state. As the significant difference in hs-CRP levels between the impaired and normal reperfusion persists until 7 weeks after admission, and reperfusion success is negatively correlated with infarct size and positively correlated with left ventricular ejection fraction, it could

be important to continue monitoring hs-CRP levels until at least 7 weeks [21]. Although this study does not show a direct association between hs-CRP levels and left ventricular function, monitoring these levels could still be of importance, since increased hs-CRP levels in STEMI patients with mildly impaired left ventricular function are associated with adverse cardiovascular events [22]. However, it remains important to critically consider the possible function of hs-CRP [23]. Furthermore, in this study they seem to correspond with the kinetics of NT-proBNP, which might still implicate a potential association between inflammation and HF. The ability to evaluate hs-CRP levels and to detect subtle differences over time in such a number of patients is of additional value to previous studies [10].

Hs-CRP and ischemia time as predictors for reperfusion

hs-CRP at baseline is an independent predictor for reperfusion success. This result is consistent with recent studies showing the association of elevated CRP levels at baseline and impaired reperfusion in STEMI patients [8,9]. However, we studied whether subtle differences in hs-CRP, measured with a high-sensitive array allow differentiating between patients with normal and impaired reperfusion. A doubling in hs-CRP level remains an independent predictor for impaired reperfusion after multivariate adjustment, together with impaired TIMI flow (postintervention). It might be plausible that these findings (hs-CRP and impaired TIMI flow) are linked because of a higher risk of periprocedural myocardial infarction in patients with higher CRP levels at baseline [24]. Previous data suggested that a higher level of hs-CRP (≥ 3 mg/l) is an independent predictor of long-term clinical outcomes in late-presenting STEMI patients (i.e. ischemia time ≥ 6 h) [18]. We investigated whether ischemia time, hsCRP and reperfusion were associated. Ischemia time was positively correlated with hs-CRP levels, hs-CRP was negatively correlated with reperfusion, and ischemia time was negatively correlated with reperfusion. Although most of our patients reported a shorter ischemia time than 6 h, patients with impaired reperfusion reported longer ischemia time than patients with normal reperfusion. However, ischemia time did not remain an independent predictor for poor reperfusion after multivariate adjustment. It is reasonable to assume that ischemia time influences hs-CRP levels and thereby indirectly influences the extent of reperfusion after PCI, and that hs-CRP and TIMI flow are stronger predictors for reperfusion than ischemia time.

hs-CRP as surrogate biomarker for the development of HF?

In addition to the measurements of hs-CRP levels at different time points, we evaluated NT-proBNP levels measured at same time points. Interestingly, similar to the differences in hs-CRP levels between the reperfusion groups, NT-proBNP levels were also higher

over time in patients with impaired reperfusion. Currently, many studies report on the role of inflammation in the development of HF [15,25,26]. Because of the potential association between inflammation and HF, we considered the similarity of the patterns of hs-CRP and NT-proBNP in both reperfusion groups as an interesting phenomenon. It seemed interesting to investigate in which way the hs-CRP levels and NT-proBNP levels correspond to each other, not only at one moment, but also for a longer time period, and whether the association between these levels would differ between reperfusion groups. One way to do this is to calculate the ratio between these levels. The hs-CRP/NT-proBNP ratio at baseline in the normal reperfusion group does not differ from the ratio in the impaired reperfusion group. This is in accordance with the separate hs-CRP and NT-proBNP levels, because levels of both biomarkers are higher in the impaired reperfusion group, and implies that a similar process is going on in these patient groups. As it seems that the kinetics of hs-CRP corresponds with the kinetics of NT-proBNP, hs-CRP could be used as an appropriate biomarker to monitor STEMI patients for the development of HF since it is likely to represent the same pathophysiological process as NT-proBNP. This finding, in addition to the increased leucocyte levels and neutrophil levels, provides a little more insight in the possible link between inflammation and HF development.

Limitations

Some limitations should be taken into consideration. The GIPS III study consisted of non-diabetic patients presenting with a first STEMI and as a consequence of rapid primary PCI the myocardial infarct size was limited and their systolic LV function well preserved. Possibly, small infarct size and preserved LV-function resulting in limited variation might explain why we did not observe an association between hs-CRP and systolic LV-function. Besides, this was a single-center study in a specific STEMI population with a high proportion of TIMI-3 flow before intervention and our observations should be carefully extrapolated to other populations. Furthermore, our study was not designed to translate hs-CRP levels to clinical decision making and therefore, our results are not readily applicable to the clinical arena in terms of relevance for patient management. However, the observed subtle differences in hs-CRP between reperfusion groups and the phenomenon of resembling the kinetics of NT-proBNP does provide data that might be of mechanistic insight. Although the mechanism between inflammation and reperfusion is not fully elucidated yet, we hope that we gained a little more insight in this mechanism with our findings and that further research could continue with revealing this mechanism. In addition, due to the nature of the condition studied, hs-CRP was not measured before presentation. These data could have added more information about the involvement of inflammation in plaque rupture and the time course of hs-CRP.

Future perspectives

As hs-CRP is just one marker in the inflammation network, it is necessary to investigate more participants in the inflammatory cascade. It is interesting to dig further into the mechanisms of other inflammatory markers and to evaluate their course after intervention for STEMI. Currently, we see a difference in biomarker levels between patients with impaired and normal reperfusion for a longer time period. As the current follow-up is not very long, we could just speculate on longer term outcomes. In a few years, we have more information about these patients and are hopefully able to state whether differences in biomarker level influences long term outcomes in patients with impaired and normal reperfusion.

Conclusion

Higher hs-CRP levels at presentation are associated with lower reperfusion success and higher NT-proBNP levels. Impaired reperfusion is also associated with long-term higher hs-CRP levels compared to optimal reperfusion. Advancing our understanding of the temporal course of inflammatory markers in STEMI provide new insights required to further develop novel strategies of treatment.

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Supplement

SUPPLEMENTAL FIGURE 1. The ratio between hs-CRP and NT-proBNP over time in 376 patients with impaired reperfusion and normal reperfusion. Mixed model analysis was used to compare between reperfusion groups. Data are presented as mean and standard error of the mean (SEM). NS = not significant. The ratio did not differ between reperfusion groups.

Chapter 5

SOLUBLE INTERLEUKIN 6 RECEPTOR LEVELS ARE ASSOCIATED WITH REDUCED MYOCARDIAL REPERFUSION AFTER PERCUTANEOUS CORONARY INTERVENTION FOR ACUTE MYOCARDIAL INFARCTION

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ABSTRACT

Aims: Interleukin-6 receptor (IL-6R) signalling has been suggested to play a causal role in the development and outcome of coronary heart disease (CHD). The aim of this study was to investigate the association of sIL-6R levels with myocardial reperfusion after percutaneous coronary intervention (PCI) for acute ST-elevated myocardial infarction (STEMI).

Methods: Blood was sampled from 70 patients presenting with STEMI at 6 different time-points (baseline, post-PCI, t=1h, t=6h, t=24h, t=2w). Coronary angiograms post-PCI were analysed for myocardial blush grade (MBG) as indicator of myocardial reperfusion. Serum IL-6 and sIL-6R were measured using IL-6 and sIL-6R enzyme-linked immunosorbent assays (ELISA).

Results: sIL-6R levels fluctuated biphasic during the two weeks after STEMI. Reduced MBG was associated with a larger change in sIL-6R levels between baseline and post-PCI compared to optimal MBG (-13.40; SEM 2.78ng/ml vs -1.99; SEM 2.35ng/ml, respectively; $p < 0.001$). Patients with reduced MBG also showed a larger increase in sIL-6R levels after PCI and 1h after myocardial infarction (MI) compared to optimal MBG (respectively 11.56; SEM 2.68ng/ml vs 3.02; SEM 2.39ng/ml; $p = 0.018$). IL-6/sIL-6R ratio was also more increased in patients with reduced MBG at 24h after myocardial infarction (0.23; SEM 0.08-0.51 vs 0.10; SEM 0.05-0.21; $p = 0.024$). An optimal MBG was associated with a 10ng increase in sIL-6R level between baseline and post-PCI (OR 1.687, CI 1.095-2.598; $p = 0.018$).

Conclusions: sIL-6R levels fluctuate biphasic during the two weeks after MI with larger changes and increased IL-6/sIL-6R ratio in patients with reduced MBG. Further research is needed to increase our understanding of the possible causality of these associations.

INTRODUCTION

Inflammation is recognized as an important factor throughout all stages of atherosclerosis and the development of coronary heart diseases (CHD) [1,2]. The continuous increase of our understanding on the involved inflammatory mechanisms has uncovered an intriguing diversity of potentially targetable mechanisms [1–6]. Data from observational epidemiological studies suggest that circulating concentrations of liver-derived inflammation biomarkers, such as C-reactive protein (CRP), fibrinogen and interleukin-6 (IL-6), are associated with subsequent risk of CHD [7–9]. Pro-inflammatory cytokines, like IL-6, are thought to be involved in repair processes and scar tissue formation after myocardial infarction [10]. The IL-6 signalling is activated through two different ways: 1. IL-6 binds to the membrane-bound IL-6 receptor (IL-6R), which is only expressed in some cell populations, and then associates with the signal transducing receptor glycoprotein 130 (gp130). This association initiates activation of intracellular pathways. 2. IL-6 binds to a soluble form of IL-6R (sIL-6R), and this complex binds to gp130 on cells. In this transsignalling pathway, cells do not have to express IL-6R, as gp130 is widely expressed in most cell types. In the past years, evidence has been provided to suggest that sIL-6R functions as pro-inflammatory molecule [11]. Nevertheless, as the pathway of IL-6, sIL-6R, and gp130 has not been completely elucidated yet and knowledge about additional properties of sIL-6R will enlarge the biologic understanding, it is of importance to further investigate these molecules and their mechanisms [12]. Several lines of evidence have suggested that IL-6R signalling is involved in the development of CHD. Circulating levels of IL-6 are correlated with impaired left ventricular systolic and diastolic function in patients with myocardial infarction (MI) [13]. Furthermore, IL-6R signalling plays an important role in the modulation of the inflammatory response after MI and it is associated with adverse left ventricular remodelling in animal models [14–16]. Finally, genetic evidence in humans derived from Mendelian randomization analyses have elucidated that IL-6R signalling seems to have a causal role in development of CHD [17,18]. Previously, Ueda et al. described fluctuations of sIL-6R in ST-segment Elevation MI (STEMI) patients. We here expand on these findings by evaluating these fluctuations in STEMI patients with reduced versus optimal reperfusion. We hypothesize that an increased extent of myocardial damage is associated with greater fluctuations in sIL-6R levels. The aim of the present study was to emphasize possible associations between sIL6R levels and temporal changes that occur during myocardial reperfusion, after percutaneous coronary intervention for acute STEMI.

METHODS

Study design and population

All patients participating in the Biobank of the Comparison of Intracoronary versus Intravenous Abciximab Administration During Emergency Reperfusion of ST-Segment Elevation Myocardial Infarction (CICERO) trial were included in this study. Details on the CICERO trial have been reported previously [19].

Regarding the use of abciximab in the CICERO trial, consecutive STEMI patients undergoing primary PCI were randomly assigned to either an intracoronary or an intravenous bolus of abciximab (0.25 mg/kg body weight; ReoPro 2 mg/mL; Centocor BV, Leiden, the Netherlands). After randomization, a bolus of abciximab was administered through the guiding catheter proximal to the lesion in the infarct-related artery over a period of 1 min in patients assigned to intracoronary administration directly after first restoration of antegrade flow. In patients assigned to intravenous administration, abciximab was administered during PCI, but the exact timing of administration was not specified by protocol [19].

From the 534 patients, 75 patients were enrolled in a substudy of the CICERO trial. A total of 5 patients were excluded because of absence of serum samples (**Figure 1**). Serum was sampled at baseline, directly after PCI, one hour after PCI, six hours after PCI, 24 h after PCI, and two weeks after PCI. Samples were stored at -80 °C until analysed.

Clinical follow-up was obtained from the central personal records database and hospital records. We used serum samples from 23 healthy participants of the Telosophy study as controls. The Telosophy study was an observational, prospective case control study [20]. Details on the Telosophy study have been published previously [20]. Samples were stored at -80 °C until analysed. MBG was categorized as follows [21]: 0 = no myocardial blush, or contrast density; 1 = minimal myocardial blush; 2 = moderate myocardial blush but less than that obtained during angiography of a contralateral or ipsilateral non-infarct-related coronary artery; 3 = normal myocardial blush comparable to that obtained during angiography of a contralateral or ipsilateral non-infarct-related coronary artery. The primary analysis was MBG (reduced vs normal, 0, 1, 2 vs 3). Coronary angiograms were analysed by a physician blinded to clinical data (AvdH).

IL-6 & sIL-6R

To determine the IL-6 levels, we performed enzyme-linked immunosorbent assay (ELISA) in serum, using human IL-6 HS Quantikine ELISA kit (R&D, cat. No. HS600B).

To determine the sIL-6R levels, we performed ELISA in serum, using R&D Systems components. These components were MoAb anti-H IL-6R (R&D, cat. No. MAB227), r-hIL-6R (R&D, cat. No. 227-SR-025), and PoAb anti-H IL-6R biotine (R&D, cat. No. BAF 227). Samples were randomly analysed. In our laboratory, the inter-assay coefficient of variation (CV) was 8.8%.

FIGURE 1. Flowchart of patient enrolment.

Statistical analysis

Skewed variables were natural log transformed to acquire a near normal distribution. Continuous variables were summarized as mean $\pm$ standard deviation if normally distributed or median and interquartile range if skewed distributed. Discrete variables were presented as frequencies and percentages. To compare groups, we used Student's t-test for normally distributed continuous variables, Mann-Whitney U-test for skewed continuous variables, Chi-square and Fisher's exact test for categorical variables. Univariate and multivariate logistic regression analyses were applied to study the association between sIL-6R levels and myocardial blush grade. We reported crude and adjusted odds ratios together with corresponding 95% confidence intervals. Statistical significance was considered at a 2-tailed p-value of <0.05 . Statistical analyses were performed with the Statistical Package for the Social Sciences version 20.0 (SPSS Inc, Chicago, IL).

RESULTS

Baseline characteristics of the study population are presented in **Table 1**. Clinical characteristics did not significantly differ between patients with reduced and patients with normal MBG. The average age was 62 ± 13 years and 70% were male. 54% of the patients had hypertension, 58% a positive family history of CHD, and 41% were current smokers. Concerning PCI related characteristics, culprit artery and the amount of anterior infarctions did not significantly differ between groups. In patients with reduced reperfusion, Thrombolysis in Myocardial Infarction (TIMI) flow pre-PCI was more scored as 0, compared to patients with normal reperfusion (67% vs 40%; $p = 0.027$). The average ischemic time was 192 min (IQR 128–242 min) and did not differ significantly between groups. Regarding the substudy of the CICERO trial, we investigated whether there was a significant interaction between the way of abciximab administration and sIL-6R levels. There was no significant interaction between those variables ($p = 0.110$ for interaction).

Temporal course of sIL-6R

sIL-6R levels fluctuated during two weeks (**Figure 2A and C**). Changes in sIL-6R levels between two consecutive time points were compared. sIL-6R level at baseline was 80.92 ng/ml (IQR 57.17–100.45 ng/ml), directly post-PCI sIL-6R level decreased with 6.85 ng/ml (SEM 1.91 ng/ml) and between post-PCI and 1 h, sIL-6R level increased with 6.95 ng/ml (SEM 1.85 ng/ml; $p < 0.01$). After 6 h, it increased with 1.16 ng/ml (SEM 3.50 ng/ml; $p = 0.13$). After 24 h, sIL-6R levels decreased with 8.34 ng/ml (SEM 3.00 ng/ml; $p = 0.06$). Two weeks after baseline, the average increase was 13.13 ng/ml (SEM 3.56 ng/ml; $p < 0.01$).

sIL-6R levels differed significantly between STEMI patients and healthy controls at $T = \text{post-pci}$, and $t = 1$ h. Levels at baseline, $t = 6$ h, and $t = 24$ h were not significantly lower than sIL-6R level at 2 weeks (**Table 2**). The median of the sIL-6R level at two weeks after baseline in STEMI patients was the same as sIL-6R level in healthy controls (93.48 ng/ml (IQR 69.31–117.98 ng/ml) vs 94.90 ng/ml (IQR 73.02–117.56 ng/ml); $p = 0.703$) (**Figure 2B**). The coefficient of variation of sIL-6R in healthy persons is $5.6 \pm 1.6\%$ (SEM) [8].

TABLE 1. Baseline characteristics reduced and normal MBG

Characteristics	Reduced MBG (n=30)	Normal MBG (n=40)	P-value
Male sex (%)	22 (73)	27 (68)	0.598
Age (years), mean $\pm$ SD	64 $\pm$ 12	61 $\pm$ 13	0.210
Body Mass Index (kg/m ²), mean $\pm$ SD	27 $\pm$ 3	27 $\pm$ 4	0.530
Blood pressure (mmHg), mean $\pm$ SD			
Systolic	131 $\pm$ 27	132 $\pm$ 29	0.872
Diastolic	78 $\pm$ 12	76 $\pm$ 14	0.524
Heart rate (beats/min), mean $\pm$ SD	79 $\pm$ 13	80 $\pm$ 20	0.834
Medical history (n(%))			
Systemic hypertension	17 (57)	21 (53)	0.729
Diabetes	4 (13)	3 (8)	0.690
Smoking	11 (37)	18 (45)	0.484
Family history	16 (55)	24 (60)	0.688
Hypercholesterolemia	8 (27)	14 (36)	0.415
Previous MI	2 (7)	2 (5)	1.000
CVA	0 (0)	2 (5)	0.503
Previous CABG	0 (0)	0 (0)	-
Previous PCI	2 (7)	3 (8)	1.000
Angina	8 (27)	15 (38)	0.340
Medication (n(%))			
Aspirin	30 (100)	39 (98)	1.000
Heparin	29 (97)	39 (98)	1.000
LMWH	1 (3)	0 (0)	0.435
Beta blocker	8 (27)	7 (18)	0.391
ACE inhibitor	4 (13)	9 (23)	0.371
AT II antagonist	2 (7)	5 (13)	0.368
Diuretics	7 (23)	6 (15)	0.375
Coumarin derivatives	0 (0)	2 (5)	0.503
Platelet aggregation inhibitor	4 (13)	8 (20)	0.464
Calcium antagonist	4 (13)	5 (13)	1.000
Statin	5 (17)	11 (28)	0.285
Antiarrhythmics	0 (0)	1 (3)	1.000
Insulin	0 (0)	1 (3)	1.000

TABLE 1. Continued.

Characteristics	Reduced MBG (n=30)	Normal MBG (n=40)	P-value
Oral antidiabetics	3 (10)	2 (5)	0.645
Culprit artery			
Left main artery	0 (0)	1 (3)	1.000
LAD	15 (50)	16 (40)	0.470
Cx	1 (3)	4 (10)	0.383
RCA	14 (47)	19 (48)	0.945
Anterior infarction	15 (50)	17 (43)	0.533
Pre TIMI			
0	20 (67)	16 (40)	0.027
1	0 (0)	4 (10)	0.130
2	7 (23)	9 (23)	1.000
3	3 (10)	11 (28)	0.070
Post TIMI			
0	0 (0)	1 (3)	1.000
1	1 (3)	1 (3)	1.000
2	5 (17)	8 (20)	0.723
3	24 (80)	30 (75)	0.776
Ischemic time (minutes)	196 (154 – 233)	171 (128 – 277)	0.618

Data are expressed as mean $\pm$ standard deviation, median (interquartile range), or as number (%). STEMI = ST-elevated myocardial infarction; MI = myocardial infarction; CVA = cerebrovascular accident; CABG = coronary artery bypass graft; PCI = percutaneous coronary intervention; LMWH = low molecular weight heparin; ACE = angiotensin-converting enzyme; AT II = angiotensin II; LAD = left anterior descending; Cx = circumflex; RCA = right coronary artery; TIMI = Thrombolysis in Myocardial Infarction grade

sIL-6R levels and myocardial blush grade (MBG)

Comparing the changes in sIL-6R levels among the different time points, we observed remarkable differences between patients with reduced MBG (MBG 0, 1, 2) compared to normal MBG (MBG 3). sIL-6R level at baseline was 89.21 ng/ml (IQR 59.56–108.69 ng/ml) in patients with reduced MBG, and 71.14 ng/ml (IQR 55.54–95.66 ng/ml) in patients with normal MBG ($p = 0.126$). The change between baseline and post-PCI sIL-6R levels, in patients with reduced MBG was more substantial compared to patients with an optimal MBG (–13.40 ng/ml vs –1.99 ng/ml; $p < 0.001$; Fig. 3). Also a larger change in sIL-6R levels was observed between post-PCI and T = 1 h in patients with a reduced MBG compared to those with an optimal MBG (11.56 ng/ml vs 3.02 ng/ml; $p = 0.018$; **Figure 3**).

FIGURE 2. sIL-6R levels in STEMI patients at different time points (baseline $n = 70$, Post-PCI $n = 68$, T = 1 h $n = 65$, T = 6 h $n = 64$, T = 24 h $n = 48$, T = 2w $n = 42$). Data are presented as median and interquartile range (2A). sIL-6R levels in patients two weeks post-MI compared to sIL-6R levels in controls ($n = 23$). Data are presented as median and interquartile range (2B). Change in sIL-6R levels in STEMI patients between time points. Data are presented as mean and SEM (2C).

TABLE 2. sIL-6R levels in STEMI patients (different time points) and healthy controls (one time point).

Time	sIL-6R (ng/ml)		p-value
	STEMI	Control	
Baseline	80.92 (57.17 – 100.45)	94.90 (73.02 – 117.56)	0.052
Post-PCI	73.91 (56.60 – 92.29)	94.90 (73.02 – 117.56)	0.006
T=1h	80.39 (57.60 – 98.23)	94.90 (73.02 – 117.56)	0.038
T=6h	81.90 (65.30 – 98.97)	94.90 (73.02 – 117.56)	0.098
T=24h	78.56 (59.78 – 102.06)	94.90 (73.02 – 117.56)	0.058
T=2w	93.48 (69.31 – 117.98)	94.90 (73.02 – 117.56)	0.703

Data are expressed as median (interquartile range). STEMI = ST-elevation myocardial infarction

FIGURE 3. Change in sIL-6R levels between different time points (MBG reduced vs MBG normal). Data are presented as mean and SEM.

To further investigate the possible association of MBG and Δ sIL-6R (baseline – post-PCI), we applied logistic regression. The unadjusted odds ratio per 10 ng. Δ sIL-6R was 1.790 (CI 1.169–2.742; $p = 0.007$) for optimal MBG. After adjustment for age, sex, hypertension, and diabetes the odds ratio was only slightly reduced 1.687 (CI 1.095–2.598; $p = 0.018$) (**Table 3**).

TABLE 3. Multivariate regression on MBG

Variable	OR	95% CI	P-value
Δ sIL-6R baseline – post-PCI (per 10 ng/ml)	1.687	1.095 – 2.598	0.018
Sex (female)	1.479	0.421 – 5.190	0.541
Age (per year)	0.980	0.933 – 1.029	0.417
Hypertension	0.818	0.283 – 2.367	0.712
Diabetes	0.584	0.283 – 2.367	0.526

CI = confidence interval; OR = odds ratio; PCI = percutaneous coronary intervention.

IL-6/sIL-6R ratio

In both groups, sIL-6 level increased over time. This increase was more pronounced in patients with reduced reperfusion compared to patients with optimal reperfusion (16.55 pg/ml vs 7.35 pg/ml at T = 24 h; $p = 0.008$) (**Table 4A**). sIL-6R level was not significantly lower in patients with reduced reperfusion. In patients with reduced reperfusion, the

sIL-6R level decreased during the first 24 h, in contrast to sIL-6R level in patients with optimal reperfusion (**Table 4B**). IL-6/sIL-6R ratio at T = 24 h was higher in patients with reduced reperfusion (0.23 vs 0.10; $p = 0.024$) (**Table 4C**).

TABLE 4A. IL-6 levels reduced and normal MBG

Time	IL-6 (pg/ml)		p-value
	MBG reduced	MBG normal	
Baseline	2.79 (1.65 – 6.28)	2.85 (1.91 – 4.50)	0.76
T=1h	5.49 (2.71 – 10.61)	2.97 (2.21 – 6.31)	0.047
T=24h	16.55 (7.05 – 51.53)	7.35 (4.42 – 15.43)	0.008

Data are expressed as median (interquartile range)

MBG = myocardial blush grade

TABLE 4B. sIL-6R levels reduced and normal MBG

Time	sIL-6R (ng/ml)		p-value
	MBG reduced	MBG normal	
Baseline	89.21 (59.56 – 108.69)	71.14 (55.54 – 95.66)	0.126
T=1h	82.53 (60.05 – 104.02)	74.09 (54.50 – 97.46)	0.350
T=24h	82.09 (67.07 – 102.66)	77.82 (58.74 – 98.48)	0.580

Data are expressed as median (interquartile range)

MBG = myocardial blush grade

TABLE 4C. IL-6/sIL-6R ratio reduced and normal MBG

Time	IL-6/sIL-6R		p-value
	MBG reduced	MBG normal	
Baseline	0.03 (0.02 – 0.08)	0.04 (0.02 – 0.06)	0.965
T=1h	0.06 (0.03 – 0.14)	0.05 (0.03 – 0.08)	0.211
T=24h	0.23 (0.08 – 0.51)	0.10 (0.05 – 0.21)	0.024

Data are expressed as median (interquartile range)

MBG = myocardial blush grade

Platelet count

Platelet count at baseline was not significantly higher in patients with reduced reperfusion (**Table 5**).

TABLE 5. Platelet count in reduced and normal MBG

Time point	Reduced MBG (n=30)	Normal MBG (n=40)	P-value
<i>Platelets (10⁹/l)</i>			
Baseline	272 (196 – 292)	227 (178 – 277)	0.124

Data are expressed as median (interquartile range). MBG = myocardial blush grade

Leukocytes and hs-CRP

Leukocyte count was not significantly higher in patients with reduced reperfusion compared to patients with normal reperfusion, at baseline as well as after 24 h. Same phenomenon was visible in high sensitive (hs) CRP at baseline (**Table 6**).

TABLE 6. Leucocytes and hs-CRP in reduced and normal MBG

Time point	Reduced MBG (n=30)	Normal MBG (n=40)	P-value
<i>Leukocytes (10⁹/l)</i>			
Baseline	11.70 (7.35 – 16.50)	11.65 (10.00 – 14.23)	0.802
T=24h	10.05 (8.98 – 14.20)	9.80 (8.65 – 11.45)	0.375
<i>hs-CRP (mg/l)</i>			
Baseline	3.50 (1.48 – 6.80)	1.80 (0.70 – 5.20)	0.197

Data are expressed as median (interquartile range). MBG = myocardial blush grade; hs-CRP = high sensitive C-reactive protein

DISCUSSION

In the current study we observed an association of sIL-6R with myocardial reperfusion after percutaneous coronary intervention for acute STEMI; the biphasic fluctuations of sIL-6R were more pronounced in patients with reduced MBG compared to normal MBG. IL-6/sIL-6 ratio increased more over time in patients with reduced reperfusion. The difference in fluctuation of sIL-6R levels and the difference in IL-6/sIL-6R ratio between reduced and normal MBG could be explained by the fact that the reduced

MBG is caused by a greater extent of inflammation. Thus, greater extent of inflammation leads to more elevation in inflammatory markers, but it takes also more effort to return to a stable state of inflammatory markers. This effort could be seen as the extent of fluctuation in sIL-6R.

Our data are in line with earlier observations that sIL-6R levels fluctuate after myocardial infarction [22,23]. Although the time frame and the amount of time points differed between our study and the previously reported, the observation that sIL-6R levels fluctuated was consistent.

MBG is considered a measure of reperfusion and reperfusion injury and reflects the microvascular damage [21]. Previous studies showed that the presence of persistent microvascular obstruction increases the risk for the development of cardiac remodelling [24–26]. In addition, it has been shown that improvement in microvascular function in the infarcted territory is significantly associated with improved left ventricular ejection fraction [27]. Finally, MBG is a strong predictor of mortality in STEMI patients [21,28].

Ueda et al. also noticed that a larger decrease of sIL-6R was associated with a larger extent of myocardial inflammation and injury as determined by CRP, white blood cell count, erythrocyte sedimentation rate, and creatine kinase and lactic dehydrogenase. We did not find significant higher levels of leukocyte count and hs-CRP in patients with reduced reperfusion compared to patients with optimal reperfusion, neither did Blancke et al. [29]. The reason for this difference in findings compared to Ueda et al. might be the difference in sample size.

We now provide further data that fluctuation in inflammation markers might also be associated with the amount of myocardial reperfusion and myocardial injury. This is consistent with the earlier finding that the difference in left ventricular ejection fraction between 7 days and 6 months after primary PCI in STEMI patients was negatively correlated with the extent of change in serum IL-6 levels [13].

The extent of cell death in the late phase after reperfusion may relate to the fluctuations in these inflammatory cytokine levels. In experimental animal studies of cardiac ischemia reperfusion, the process of apoptotic cell death continues to increase in the late phase of reperfusion, between 6 and 24 h and concomitant necrosis peaked at 24 h [30].

Nian et al. also reported that cytokine gene expression may re-increase as a second wave of cytokine activation when there are other ongoing myocardial stress factors,

or when the infarct size is large [15]. Although it is not clear yet which exact factors are causally involved, our finding that sIL-6R levels fluctuate more in patients with worse reperfusion after myocardial infarction lend supports to this finding of Nian et al.

Modifying IL-6R signalling might be a target of therapy. In Mendelian randomization analyses, the causal role of IL-6R signalling in the development of CHD has been investigated, and it seemed that IL6R blockade could provide a novel therapeutic approach to prevention of CHD [17,18]. Furthermore, in previous mice experiments, the role of anti-IL-6R antibodies in the process of myocardial inflammation after myocardial infarction has been studied [14]. Balb/c male mice were subjected to MI by ligating the left anterior descending coronary artery. These mice were then treated with an intraperitoneal injection of anti-IL-6R antibody (MR16-1; 500 µg/body) or control IgG. This resulted in that administration of the selective IL-6R antagonist MR16-1 reduced leukocyte and macrophage infiltration and attenuated matrix metalloproteinase activation, leading to a marked improvement in LV dilatation, contractile dysfunction, and lifespan [14]. Blocking the sIL-6R in patients with myocardial infarction, could be a target of therapy in order to prevent these patients from developing left ventricular remodelling. Currently an ongoing study is testing the hypothesis that administration of the IL-6R antagonist Tocilizumab, in patients with NSTEMI, may interrupt the self-perpetuating inflammatory loops which could improve plaque stability, with potential secondary beneficial effects on myocardial damage (NCT01491074). This randomized, double blind, placebo controlled study will include 120 patients presenting with non ST-elevation myocardial infarction and the primary outcome will be change in high-sensitive CRP area under the curve.

Some limitations should be considered. First, using MBG to assess the degree of reperfusion is subjective [31]. However, the physician evaluating MBG in our study was blinded to all other clinical data of the patients and MBG was determined apart from measuring sIL-6R levels. Second, this was a retrospective study with a relative small sample size. Third, unfortunately, we did not investigate levels of gp130. Data of gp130 levels could have strengthened our hypothesis. However, this is the first study reporting associations of fluctuation in sIL-6R levels and myocardial reperfusion.

Conclusions

In conclusion, our study shows an association of sIL-6R with MBG after percutaneous coronary intervention for acute STEMI. sIL-6R levels fluctuate biphasic during two weeks after STEMI with larger fluctuations observed in patients with reduced MBG.

An increased IL-6/sIL-6R ratio was observed in patients with reduced MBG. Further research is required to determine the possible causality of these associations and the efficacy of intervening in sIL-6R signalling on development of cardiac dysfunction.

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Chapter 6

PLASMA INTERLEUKIN 6 LEVELS ARE ASSOCIATED WITH CARDIAC FUNCTION AFTER ST-ELEVATION MYOCARDIAL INFARCTION

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ABSTRACT

Background and aims: Myocardial infarction triggers an inflammatory response involved in cardiac repair. We studied the association of the interleukin 6 (IL-6) cascade with infarct size and cardiac function after ST-elevation myocardial infarction (STEMI).

Methods: In 369 STEMI patients IL-6, soluble IL-6 receptor (sIL-6R), and soluble glycoprotein (sgp) 130 were measured at baseline (hospital admission), 24 hours, 2 weeks, 7 weeks, 4 months, and 1 year post-PCI and sIL-6R/IL-6 ratio was calculated. At 4 months, infarct size and left ventricular ejection fraction (LVEF) were assessed by magnetic resonance imaging. Diastolic function (E/e') was determined by echocardiography.

Results: Hospital admission levels for IL-6, sIL-6R, sgp 130 were 3.7 pg/ml (IQR 2.1 – 6.7 pg/ml), 51.6 ng/ml (IQR 37.3 – 69.0 ng/ml), and 332 ng/ml (IQR 280 – 399 ng/ml) respectively. 24 hours after admission, IL-6 had increased threefold compared to baseline ($p < 0.001$) and returned below baseline ($p < 0.001$) 2 weeks after STEMI. sIL-6R and sgp130 levels at 24 hours remained similar to baseline but were increased at 2 weeks ($p < 0.001$; $p < 0.001$, respectively). IL-6 and sIL-6R/IL-6 ratio at 24 hours were independently associated with infarct size (β 5.4 (95% CI 3.3 – 7.5); $p < 0.001$, β -4.0 (95% CI -6.1 – -1.9); $p < 0.001$, respectively). Higher levels of IL-6 at 24 hours were associated with lower LVEF (β -4.2 (95% CI -6.7 – -1.8); $p = 0.001$).

Conclusions: Higher IL-6 and lower sIL-6R/IL-6 ratio early after presentation with STEMI are indicative for larger infarct size and decreased cardiac function at 4 months.

INTRODUCTION

The past decades, a substantial amount of research has been dedicated to enhancing our understanding of the role of inflammation throughout the cardiovascular disease continuum. In myocardial infarction (MI) an intense inflammatory response that is essential for cardiac repair is observed, but which is also implicated in the pathogenesis of post-infarction remodeling and heart failure [5]. An intriguing diversity of potentially targetable mechanisms in the inflammatory cascade has been discovered [2,3,8,14,15,25,29,32]. Recently, the Canakinumab Anti-inflammatory Thrombosis Outcome Study (CANTOS) demonstrated that anti-inflammatory therapy targeting the interleukin-1 β (IL-1 β) pathway with the monoclonal antibody canakinumab leads to a significantly lower rate of recurrent cardiovascular events in patients with previous MI [21].

IL-1 β is known to drive the IL-6 signaling pathway and therefore this pathway could be another potential therapeutic target in the treatment of coronary artery disease [20]. IL-6 itself is conserved to be a central hub in cardiometabolic signaling and can be produced by many different cells regulating the acute-phase response [16,20]. IL-6 can bind to the membrane bound IL-6 receptor (IL-6R) and the soluble form of IL-6R (sIL-6R). It contributes to atherosclerotic plaque development and destabilization [30]. Furthermore, mendelian randomization studies of the IL-6R provided evidence for a causal mechanism of IL-6 signaling in the development of coronary artery disease [9,10]. Downstream signaling of the IL-6R also is thought to play a role in the regulation of cardiomyocyte loss, cardiac hypertrophy, and loss of cardiac function [4]. Studies in patients presenting with an acute coronary syndrome also suggested that IL-6 is associated with ischemia-reperfusion myocardial injury and mortality [24,31].

The humanized anti-IL-6R antibody tocilizumab showed to block IL-6 binding on the receptor, and to be effective and generally well tolerated in patients with autoimmune disorders. In patients with non-ST-elevation MI treated with percutaneous coronary intervention (PCI) tocilizumab treatment was associated with a reduced inflammatory response and lower troponin T levels [12].

Whether the IL-6 pathway can be linked to MI size and cardiac function is unknown. We hypothesized that levels of the IL-6 cascade components during admission for MI are associated with infarct size and cardiac function.

METHODS

Study population and design

We included all patients participating in the GIPS-III trial. This trial was designed to evaluate the effect of metformin treatment on preservation of left ventricular function in STEMI patients without diabetes and to establish a biobank. Details on the design of the GIPS-III trial have been reported previously [13]. In brief, all patients admitted to the University Medical Center Groningen between January 1st, 2011, and May 26th 2013, via the STEMI protocol were considered eligible for the trial. Inclusion criteria were age older than 18 years, the presence of STEMI, and primary PCI with implantation of at least 1 stent with a diameter of at least 3 mm resulting in TIMI flow grade 2 or 3 post PCI. Major exclusion criteria were previous myocardial infarction, known diabetes, the need for coronary artery bypass graft surgery, severe renal dysfunction, and standard contraindications for magnetic resonance imaging (MRI)[14]. The study protocol of the GIPS-III trial was in accordance with the Declaration of Helsinki and was approved by the local ethics committee (Groningen, the Netherlands) and national regulatory authorities. Informed consent was obtained for inclusion of the patients.

Data Collection

On admission, standard laboratory assessment was performed and standard physical examination parameters were measured according to protocol. Patients were seen in the outpatient clinic 2 weeks, 7 weeks, 4 months, and one year after discharge.

During hospitalization, blood was sampled at baseline (initial admission) and at 3, 6, 9, 12, and 24 h after PCI to monitor values of cardiac enzymes and high sensitive troponin. Less frequently during hospitalization and at every visit to the outpatient clinic hemoglobin, leucocytes (including neutrophils, lymphocytes), platelets, glucose, hs-CRP and N-terminal pro B-type natriuretic peptide (NT-proBNP) were determined. Furthermore, during PCI, 24 h after PCI, and at every visit to the outpatient clinic, blood samples for additional analyses were collected [13]. LVEF was measured by MRI 4 months after infarction. Imaging was performed on a 3.0 Tesla whole-body MRI scanner (Achieva; Philips) using a phased array cardiac receiver coil. Electrocardiogram-gated cine steady state, free precession magnetic resonance images were acquired during repeated breath holds in contiguous short-axis slices of 1 cm covering the entire left ventricle. The endocardial borders were outlined in end-systolic and end-diastolic images. Left ventricular end-systolic volumes and left ventricular end-diastolic volumes were calculated using the summation of slice method multiplied by slice thickness. An independent core laboratory (Image Analysis Center, VU University

Medical Center, Amsterdam, the Netherlands) evaluated the MRI scans and assessed the primary efficacy measure, blinded for treatment allocation and clinical patient data. According to the guidelines, LVEF $\geq 50\%$ was considered as normal, LVEF $< 40\%$ was considered as reduced, LVEF between 40 – 49% was considered as 'mid-range' [18]. Additionally, transthoracic echocardiograms were performed in left lateral decubitus position using a Vivid 7 echo system (General Electric, Horton, Norway) at 4 months. The echocardiographic data from these echocardiograms were digitally stored in DICOM format and analyzed off-line by an independent core lab (Groningen Imaging Core Laboratory, Groningen, the Netherlands) that was blinded to treatment allocation and clinical information. E/e' was used as a measure of diastolic function. Mitral valve early filling flow (E) and early diastolic tissue velocities (e') from both the septal and lateral wall were measured in accordance with current guidelines [17]. Mean e' was calculated as (e' septal + e' lateral)/2. E/e' was determined as E/mean e' and was deemed abnormal if ≥ 13 . The incidence of major adverse cardiac events (MACE; the combined endpoint of death, reinfarction, or target-lesion revascularization) was recorded until 4 months [13].

IL-6, sIL-6R, and sgp130 measurements

To determine the IL-6 levels, we performed enzyme-linked immunosorbent assay (ELISA) in serum, using human IL-6 DuoSet ELISA (R&D, cat. no. DY206). To determine the sIL-6R levels, we used human IL-6R DuoSet ELISA (R&D, cat. no. DY227), and for determining sgp130, we used human sgp130 DuoSet ELISA (R&D, cat. no. DY228). Samples were randomly analyzed. In our laboratory, the inter-assay coefficients of variation (CV) for IL-6, sIL-6R, and sgp130 were respectively 6.3%, 11.6%, and 5.9%. The cytokines were measured in duplicate and the detectable limits for IL-6, sIL-6R, and sgp130 were respectively 0.4 pg/ml, 1.4 ng/ml, 0.1 ng/ml. Since IL-6 binds to IL-6R we also calculated the IL-6R/IL-6 ratio.

Myocardial blush grade

Myocardial blush grade (MBG) represents an angiographic measurement of myocardial perfusion [7]. It reflects a myocardial response to ischemic injury and reperfusion. MBG was categorized as follows: 0 = no myocardial blush, or contrast density; 1 = minimal myocardial blush; 2 = moderate myocardial blush but less than that obtained during angiography of a contralateral or ipsilateral non-infarct-related coronary artery; 3 = normal myocardial blush comparable to that obtained during angiography of a contralateral or ipsilateral non-infarct-related coronary artery [28]. The patients were categorized as having normal (MBG 3) versus impaired (MBG 0-2) reperfusion. Coronary angiograms were analyzed by two physicians blinded to clinical data.

Statistical analysis

Continuous variables were summarized as mean $\pm$ standard deviation if normally distributed or median and interquartile range if skewed. Discrete variables were presented as frequencies and percentages. To compare groups, we used Student's t-test for normally distributed continuous variables, Mann-Whitney U-test for skewed continuous variables, Chi-square and Fisher's exact test for categorical variables. We used mixed model analysis to analyze biomarker levels between patient groups over time. We modeled IL-6, sIL-6R, sIL-6R/IL-6 ratio, and sgp130 using a random intercepts regression model in terms of MBG, LVEF, and E/e', and time points. Regarding IL-6, sIL-6R, sIL-6R/IL-6 ratio, and sgp130, we created quartiles in order to use them in regression analysis (using the first quartile as reference). Relevant variables (Table 1) were assessed as potential confounders. Variables with a $p < 0.2$ in univariate analysis were included in the multivariate linear regression model. In accordance with Benjamin *et al.*, a 2-tailed p -value of <0.005 was considered significant. P -value between 0.05 and 0.005 was considered suggestive [1]. Statistical analyses were performed with Stata version 14.0 (StataCorp). Figures were created with GraphPad Prism version 7.02 for Windows.

RESULTS

Baseline characteristics

Baseline characteristics are presented in **Table 1** and in **Supplementary Tables 1, 2 and 3** according to quartiles of IL-6, sIL-6R and sgp130. The average age at presentation was 59 years (± 12 year) and 25% of the population was female. One third had hypertension, more than half smoked, and two third had dyslipidemia. The median ischemic time was 161 minutes (IQR 109 – 250 minutes). Of the total 379 patients participating in the GIPS-III trial, we had plasma samples available of 369 patients. The exact number of samples which was available for the measurements of IL-6, sIL-6R and sgp130 at the different time points is presented in **Supplementary Table 4**. The range varied between 369 samples at baseline and 252 samples at 1 year follow-up.

TABLE 1. Baseline characteristics

Characteristics	Total (n = 369)
Age, mean (SD), years	58.8 ± 11.6
Female sex – No. (%)	95 (25)
BMI, mean (SD), kg/m ²	27.0 ± 3.8
Cardiovascular related history – No. (%)	
Hypertension	112 (30)
Dyslipidemia	239 (63)
Current smoking	209 (55)
Stroke	3 (0.8)
Peripheral artery disease	0
Previous PCI	4 (1.1)
Blood pressure, mean (SD) mmHg	
Systolic	134 ± 23
Diastolic	84 ± 15
Heart rate, mean (SD), beats/min	76 ± 16
Ischemia time, median (IQR), min	161 (109 – 250)
Single vessel disease – No. (%)	258 (68)
Infarct-related artery TIMI flow – No. (%)	
Preintervention grade	
0	208 (55)
1	27 (7.1)
2	66 (17)
3	78 (21)
Post-intervention grade	
2	34 (9.0)
3	345 (91)
Myocardial blush grade	
0	10 (2.6)
1	29 (7.7)
2	74 (20)
3	263 (69)
Laboratory values at admission	
CK, median (IQR), U/L	129 (83 – 210)
Myocardial band of CK, median (IQR), U/L	16 (13 – 25)

TABLE 1. Continued.

Characteristics	Total (n = 369)
Troponin, median (IQR), ng/L	50 (23 – 136)
Creatinine, median (IQR), $\mu\text{mol/L}$	72 (62 – 82)
NT-proBNP, median (IQR), ng/L	81 (40 – 200)
Glucose (median (IQR), mmol/L	8.2 (7.0 – 9.6)
HbA1c, median (IQR), %	5.8 (5.6 – 6.0)
Blood count and biochemistry	
Leucocytes ($10^9/\text{L}$)	11 (9 – 14)
Thrombocytes ($10^9/\text{L}$)	234 (204 – 269)
Neutrophils ($10^9/\text{L}$)	8 (5 – 10)
Lymphocytes ($10^9/\text{L}$)	2 (1 – 3)
N/L ratio	3.63 (2.39 – 6.07)
Hs-CRP (mg/L)	2.1 (1 – 4.2)

Data are expressed as mean $\pm$ standard deviation (SD), median (interquartile range (IQR)), or as number (%). BMI = Body Mass Index; TIMI = Thrombolysis in Myocardial Infarction; CK = creatine kinase; NT-proBNP = N-terminal pro brain natriuretic peptide; HbA1c = glycated hemoglobin; N/L = neutrophil/lymphocyte.

Interleukin 6, soluble interleukin 6 receptor, and soluble glycoprotein 130

Median baseline IL-6 level was 3.7 pg/ml (IQR 2.1 – 6.7 pg/ml) and after 24 hours it was increased a threefold to 10.3 pg/ml (IQR 5.8 – 19.8 pg/ml) ($p < 0.001$) and subsequently decreased to 1.8 pg/ml (IQR 1.1 – 2.9; $p < 0.001$) at 2 weeks to remain stable (**Figure 1A**). The median sIL-6R at baseline was 51.6 ng/ml (IQR 37.3 – 69.0 ng/ml), did not change at 24 hours, but increased somewhat after 2 weeks to 62.9 ng/ml (IQR 47.8 – 81.6 ng/ml; $p < 0.001$) and remained stable thereafter (**Figure 1B**). Baseline sgp130 was 332 ng/ml (IQR 280 – 399 ng/ml) and showed a comparable trend as sIL-6R levels (**Figure 1C**).

Baseline ratio between sIL-6R and IL-6 was 13,651 (IQR 6780 – 26102), decreased a 2.8-fold to 4856 (IQR 2,350 – 8,382; $p < 0.001$) after 24 hours and increased 8.7-fold to 36,843 (IQR 20,078 – 62,352; $p < 0.001$) at 2 weeks (**Supplementary Figure 1A**). The sIL-6R/IL-6 ratio at 24 hours was ~10 times smaller compared to the sIL-6R/IL-6 ratio at 1 year (**Supplementary Figure 1B**). Hs-CRP levels decreased significantly between 2 and 7 weeks post-PCI ($p < 0.001$) (**Figure 1**).

Figure 1. Log IL-6 and log hs-CRP (a), log sIL-6R (b), and log sgp130 levels (c). Levels are depicted in mean and SEM. IL-6 levels at baseline significantly differed from all

consecutive time points ($p < 0.001$). hs-CRP levels at baseline significantly differed from hs-CRP at 7 weeks and 4 months ($p < 0.001$). sIL-6R and sgp130 levels at baseline significantly differed from sIL-6R at 2 weeks, 7 weeks, 4 months, and 1 year ($p < 0.001$).

Interleukin 6 and reperfusion

Next, we studied IL-6 levels in relation to reperfusion grade. IL-6 levels were suggestively higher in a patient with impaired (MBG 0-2) compared to normal (MBG 3) reperfusion grade ($p = 0.019$; **Supplementary Figure 2A**). sIL-6R levels did not differ between groups (**Supplementary Figure 2B**) but the sIL-6R/IL-6 ratio was lower in patients with impaired reperfusion ($p = 0.003$; **Supplementary Figure 2C**). Sgp130 levels were also comparable between groups (data not shown).

FIGURE 2. Log IL-6 (a), log sIL-6R/IL-6 (b) levels in STEMI patients with LVEF $\geq 50\%$, LVEF 40-49%, LVEF $< 40\%$. Levels are depicted in mean and SEM. P values for trend are shown. IL-6 and sIL-6R/IL-6 ratio levels at baseline significantly differed from all consecutive time points ($p < 0.001$).

Differences in levels of the IL-6 cascade between normal and reduced cardiac function

We compared IL-6 levels in patients categorized for their LVEF at 4 months. IL-6 levels were significantly higher in patients with reduced LVEF compared to the other categories ($p=0.006$; **Figure 2A**). Also the sIL-6R/IL-6 ratio was significantly lower in patients with reduced LVEF ($p=0.003$; **Figure 2B**). sIL-6R, sgp 130, and hs-CRP levels did not differ between categories (data not shown).

When studying the IL-6 cascade markers in relation to diastolic function, indicated by E/e', IL-6 levels were suggestively higher in patients with abnormal E/e' ($p=0.013$) (**Supplementary Figure 3A**). sIL-6R/IL-6 ratio was significantly lower in patients with abnormal E/e' ($p=0.005$) (**Supplementary Figure 3B**). Similar to the biomarkers and LVEF, sIL-6R and sgp 130 levels did not differ between groups (data not shown).

Associations between covariates and infarct size, cardiac function, and cardiac markers

Univariate associations between covariates and infarct size, LVEF or E/e' are given in **Table 2**. After adjustments for relevant covariates, Q4 of IL-6 measured at baseline, compared to the lowest quartile (Q1), was still suggestively associated with infarct size with a coefficient of 2.87 (95% CI 0.74 – 5.00; $p=0.008$; adj. R^2 0.19) (**Supplementary Figure 4**) and Q4 of sgp130 was suggestively associated with lower E/e' (β -0.82 (95% CI -1.54 – -0.11); $p=0.024$; adj. R^2 0.19).

When analyzing for the association between biomarker levels at 24 hours and infarct size, LVEF or E/e', the Q4s of IL-6 and sIL-6R/IL-6 ratio versus the lowest quartile were significantly associated with infarct size, also after adjusting for covariates (β 5.41 (95% CI 3.33 – 7.48); $p<0.001$; adj. R^2 0.23, β -4.00 (95% CI -6.11 – -1.89); $p<0.001$; adj. R^2 0.20, respectively). The Q4 of sIL-6R at 24 was suggestively associated with smaller infarct size (β -2.45 (95% CI -4.56 – -0.35); $p=0.023$; adj. R^2 0.17).

Looking at LVEF, the Q4 of IL-6 at 24 hours remained significantly associated after adjustment (β -4.24 (95% CI -6.67 – -1.80); $p=0.001$; adj. R^2 0.08). The Q4 of sIL-6R/IL-6 ratio at 24 hours was suggestively associated with higher LVEF (β 2.63 (95% CI 0.18 – 5.08); $p=0.035$; adj. R^2 0.06).

The Q4 of IL-6 at 24 hours was suggestively associated with diastolic function as indicated by the E/e' (β 0.92 (95% CI 0.23 – 1.62; $p=0.010$; adj. R^2 0.20) (**Figure 3**). All associations persisted after correction for hs-CRP.

TABLE 2. Univariate associations between covariates and infarct size, LVEF, and E/e' ratio measured at 4 months

	Infarct size		Left ventricular ejection fraction		E/e' ratio	
	Coefficient (95% CI)	p value	Coefficient (95% CI)	p value	Coefficient (95% CI)	p value
Sex, female	-0.35 (-2.7 - 2.0)	0.77	1.6 (-0.91 - 4.0)	0.22	2.0 (1.3 - 2.6)	<0.001
Age, years	0.03 (-0.06 - 0.11)	0.54	-0.01 (-0.10 - 0.74)	0.76	0.06 (0.04 - 0.09)	<0.001
BMI, kg/m ²	-0.24 (-0.53 - 0.04)	0.097	0.16 (-0.13 - 0.45)	0.27	0.08 (-0.00 - 0.16)	0.059
Heart rate, bpm	0.01 (-0.05 - 0.08)	0.66	-0.02 (-0.09 - 0.04)	0.45	0.01 (-0.00 - 0.03)	0.20
Hypertension	-0.73 (-2.9 - 1.5)	0.51	0.42 (-1.8 - 2.7)	0.71	1.7 (1.0 - 2.4)	<0.001
Hypercholesterolemia	1.4 (-0.67 - 3.4)	0.19	-1.6 (-3.7 - 0.45)	0.13	0.14 (-0.49 - 0.78)	0.66
Cerebrovascular accident	-5.6 (-21.3 - 10.0)	0.48	7.4 (-9.3 - 24.1)	0.38	0.67 (-2.6 - 3.9)	0.68
PTCA in medical history	3.4 (-5.7 - 12.4)	0.46	-1.7 (-10.2 - 6.7)	0.69	-1.2 (-4.4 - 2.0)	0.47
Smoking	-1.1 (-3.1 - 0.86)	0.27	0.21 (-1.8 - 2.2)	0.84	-0.75 (-1.4 - -0.13)	0.018
TIMI (pre-intervention)	-2.6 (-3.3 - -1.8)	<0.001	1.5 (0.68 - 2.3)	<0.001	-0.79 (-0.33 - 0.17)	0.54
TIMI (post-intervention)	-3.8 (-7.8 - -0.18)	0.06	3.3 (-0.85 - 7.5)	0.12	-0.26 (-1.4 - 0.88)	0.66
MBG	-1.9 (-3.3 - -0.49)	0.008	2.1 (0.67 - 3.5)	0.004	-0.14 (-0.57 - 0.30)	0.54
Ischemic time, min	0.00 (-0.00 - 0.01)	0.51	-0.01 (-0.01 - 0.00)	0.15	0.00 (0.00 - 0.00)	0.60
IL-6 levels at baseline (Q4 vs Q1)	3.3 (1.0 - 5.6)	0.005	-2.1 (-4.5 - 0.23)	0.077	0.53 (-0.23 - 1.3)	0.47
IL-6 levels at 24 hours (Q4 vs Q1)	6.0 (3.7 - 8.2)	<0.001	-4.6 (-7.0 - -2.1)	<0.001	1.2 (0.46 - 2.0)	0.002
sIL-6R levels at baseline (Q4 vs Q1)	-1.5 (-3.8 - 0.79)	0.20	0.07 (-2.3 - 2.5)	0.96	-0.10 (-0.83 - 0.64)	0.79
sIL-6R levels at 24 hours (Q4 vs Q1)	-3.13 (-5.36 - -0.89)	<0.001	1.5 (-0.96 - 3.9)	0.23	-0.28 (-1.1 - 0.49)	0.47
Ratio sIL-6R/IL-6 at baseline (Q4 vs Q1)	-2.8 (-5.1 - -0.46)	0.019	2.0 (-0.43 - 4.4)	0.11	-0.77 (-1.5 - -0.48)	0.037
Ratio sIL-6R/IL-6 at 24 hours (Q4 vs Q1)	-4.5 (-6.8 - -2.3)	<0.001	3.3 (0.84 - 5.7)	0.008	-0.90 (-1.7 - -0.14)	0.021
Sgp130 at baseline (Q4 vs Q1)	-0.65 (-3.2 - 1.9)	0.61	0.91 (-1.7 - 3.5)	0.49	-0.93 (-1.7 - -0.15)	0.020
Sgp130 at 24 hours (Q4 vs Q1)	-2.6 (-5.2 - -0.08)	0.043	3.1 (0.34 - 5.8)	0.028	-0.35 (-1.2 - 0.47)	0.40

95% CI = 95% Confidence Interval; BMI = Body Mass Index; PTCA = Percutaneous Transluminal Coronary Angioplasty; TIMI = Thrombolysis in Myocardial Infarction; MBG = Myocardial Blush Grade; IL-6 = interleukin 6; sIL-6R = soluble interleukin 6 receptor; sgp130 = soluble glycoprotein 130; Q1 = lowest quartile; Q4 = highest quartile.

FIGURE 3. Associations between members of the interleukin-6 signaling cascade measured in STEMI patients at 24 h and infarct size, LVEF, and E/e' measured at 4 months, depicted as β and 95% CIs obtained from linear regression models. 95% CI 95% Confidence Interval, IL-6 interleukin 6, sIL-6R soluble interleukin 6 receptor, sgp130 soluble glycoprotein 130, Q1 lowest quartile, Q4 highest quartile, STEMI ST-elevation myocardial infarction, LVEF left ventricular ejection fraction. Multivariate analysis on infarct size: adjusted for age, sex, BMI, hypercholesterolemia, TIMI (pre- and post-intervention), MBG. Multivariate analysis on LVEF: adjusted for age, sex, hypercholesterolemia, TIMI (pre- and post-intervention), ischemic time. Multivariate analysis on E/e': adjusted for age, sex BMI, heart rate, hypertension, smoking.

There were no associations of members of the IL-6 cascade and diastolic function within the different LVEF groups (data not shown). Furthermore, IL-6 at baseline was significantly associated with troponin T, CK, CK-MB and NT-proBNP (0.19, $p < 0.001$; 0.19, $p < 0.001$; 0.22, $p < 0.001$; 0.22, $p < 0.001$ respectively) (**Supplementary Table 5**).

Outcomes

The number of clinical events in the overall population was low, mortality rate at 2 years was 1.1%, reinfarction rate at 2 years was 3.4%. There were no associations between the components of the IL-6 system and reinfarction or mortality (data not provided).

DISCUSSION

In patients presenting with a first STEMI treated with primary PCI, the temporal course of the IL-6 cascade components is associated with infarct size and cardiac function. IL-6 levels are increased during the first two weeks and reach a steady state afterward. Furthermore, sIL-6R and sgp130 levels are decreased during the first two weeks, after which they reach a stable level, similar to IL-6. Most importantly IL-6 and sIL-6R/IL-6 ratio at 24 hours are associated with infarct size and cardiac function measured at 4 months. sIL-6R and gp130 alone are not associated with these outcomes, although higher levels of sgp130 are suggestively associated with lower E/e'.

Inflammation after myocardial infarction

Several pre-clinical studies show the benefits of intervening in the inflammatory cascade during/after MI [4]. So far, beneficial effects of intervening in the IL-6 cascade have not been observed in clinical studies, probably because of the pleiotropic characteristics of IL-6.

Targeting the IL-1 β pathway with canakinumab led to a lower rate of recurrent cardiovascular events than placebo [21]. Whether anti-inflammatory therapy is also protective in STEMI patients, is not elucidated yet. In contrast to chronic inflammation, which is considered to be harmful, the first acute inflammatory response in STEMI patients might even be cardioprotective facilitating repair of the infarction [4]. However, if this process continues and becomes chronic, this could cause excessive damage and fibrosis development eventually leading to loss of cardiac function [4].

Temporal course of IL-6, sIL-6R, and sgp130

We studied the temporal course of IL-6, sIL-6R, and sgp130 during a long time span and their association with infarct size, LVEF and E/e' [12,22,23,31]. IL-6, sIL-6R as well

as sgp130 reach a stable state around approximately 2 weeks after MI. Our findings are expanding the time horizon of these biomarker trajectories in comparison to earlier studies only evaluating the acute phase. We also present a broader picture of the IL-6 pathway by also measuring sIL-6R and sgp130, and calculating the ratio between sIL-6R and IL-6 [11,19,27]. Finally, we provide the first link of IL-6 pathway to normal/impaired reperfusion, systolic function and diastolic function, adding to the increasing level of evidence linking interleukin pathways in MI and the future development of decreased cardiac function and the risk of heart failure [6,26].

IL-6, infarct size, and cardiac function

In agreement with previous research, we observed associations between the IL-6, sIL-6R, sgp130 and infarct size and cardiac function [23]. We did not observe any associations between sgp130 and LVEF. This difference might be explained by differences in method and time course of LVEF measurements. Furthermore, the median time interval from symptoms to blood sampling was shorter compared to our study [23]. This is a small difference, although these hours could make a difference in case of the acute and sub-acute phase of the inflammatory response. In agreement with previous research we did not observe a significant association between sIL-6R and LVEF either [23]. We could not confirm previous associations between the IL-6 cascade and long-term outcomes [22, 33]. This could be explained by a difference in sample size, since 989 and 525 STEMI patients were included. Since both studies observed an association between IL-6 signaling and outcome in both the general population and STEMI patients, this could be a promising marker of cardiovascular risk and even be used to select patients for anti-inflammatory therapy.

Limitations

Some limitations should be taken into consideration. First, the GIPS III study recruited non-diabetic patients presenting with a first STEMI and as a consequence of rapid primary PCI the myocardial infarct size was limited and their systolic LV function well preserved. Second, we report associations and cannot draw conclusions about causality. Finally, our study was not powered to translate IL-6, sIL-6R, and sgp130 levels to clinical decision-making and therefore, our results are not directly applicable to the clinical arena in terms of relevance for patient management but should be considered in the light of other available data.

Future perspectives

Our study supports further research into targeting the IL-6 cascade in the treatment of acute myocardial infarction. One earlier study observed attenuation of the inflammatory

response and troponin T release by tocilizumab. The associations we observed between IL-6, infarct size and cardiac function are additive to these results [12]. Furthermore, they suggested investigating the time of administration of tocilizumab regarding the inflammatory response.

Conclusion

Members of the IL-6 cascade measured at 24 hours after myocardial infarction are indicative for larger infarct size and decreased cardiac function measured at 4 months. These results support the concept of early intervention in the inflammatory cascade in order to prevent the heart from myocardial damage.

Conflict of Interest

The authors declare that they have no conflict of interest.

Ethical standards

The study protocol of the GIPS-III trial was in accordance with the Declaration of Helsinki and was approved by the local ethics committee (Groningen, the Netherlands) and national regulatory authorities. Informed consent was obtained for inclusion of the patients.

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SUPPLEMENTARY TABLE 1. Baseline characteristics per IL-6 quartile

Characteristics	Q1 (n = 93)	Q2 (n = 92)	Q3 (n = 92)	Q4 (n = 92)	P value
IL-6 levels (pg/ml)	1.5 (1.1 - 1.8)	2.8 (2.4 - 3.2)	4.9 (4.1 - 5.6)	10.3 (7.9 - 17.1)	<0.001
Age, mean (SD), years	57.4 ± 10.6	58.4 ± 11.4	58.6 ± 12.2	60.9 ± 11.9	0.22
Female sex – No. (%)	24 (26)	19 (21)	26 (28)	24 (26)	0.68
BMI, mean (SD), kg/m ²	27.0 ± 3.9	27.0 ± 3.7	27.0 ± 4.4	26.7 ± 3.3	0.69
Cardiovascular related history – No. (%)					
Hypertension	25 (27)	24 (26)	24 (26)	35 (38)	0.21
Dyslipidemia	62 (67)	54 (59)	51 (55)	63 (68)	0.20
Current smoking	58 (62)	47 (51)	50 (54)	50 (54)	0.46
Stroke	1 (1)	1 (1)	1 (1)	0 (0)	0.80
Peripheral artery disease	0 (0)	0 (0)	0 (0)	0 (0)	1.00
Previous PCI	1 (1.1)	1 (1.1)	1 (1.1)	1 (1.1)	1.00
Blood pressure, mean (SD) mmHg					
Systolic	133 ± 22	137 ± 24	134 ± 24	133 ± 24	0.57
Diastolic	82 ± 14	85 ± 15	84 ± 15	85 ± 15	0.36
Heart rate, mean (SD), beats/min	74 ± 15	75 ± 14	74 ± 16	80 ± 20	0.08
Ischemia time, median (IQR), min	164 (118 - 216)	140 (96 - 244)	173 (110 - 273)	158 (111 - 288)	0.24
Single vessel disease – No. (%)	65 (70)	63 (69)	63 (68)	64 (70)	0.99
Culprit vessel – No (%)					0.07
LAD	29 (31%)	38 (41%)	39 (42%)	39 (42%)	
LCX	15 (16%)	18 (20%)	8 (9%)	20 (22%)	
RCA	49 (53%)	36 (39%)	45 (49%)	33 (36%)	
Infarct-related artery TIMI flow – No. (%)					
Pre-intervention grade					0.26
0	43 (46)	54 (59)	59 (64)	50 (54)	
1	7 (8)	4 (4)	7 (8)	7 (8)	
2	21 (23)	14 (15)	9 (10)	20 (22)	
3	22 (24)	20 (22)	17 (18)	15 (16)	
Post-intervention grade					0.05
2	5 (5)	6 (7)	15 (16)	8 (9)	

SUPPLEMENTARY TABLE 1. Continued.

Characteristics	Q1 (n = 93)	Q2 (n = 92)	Q3 (n = 92)	Q4 (n = 92)	P value
3	88 (95)	86 (93)	77 (84)	84 (91)	
Myocardial blush grade					0.58
0	1 (1)	3 (3)	3 (3)	3 (3)	
1	4 (4)	5 (5)	10 (11)	10 (11)	
2	16 (17)	21 (23)	19 (21)	18 (20)	
3	71 (77)	63 (68)	58 (64)	61 (66)	
Laboratory values at admission					
CK, median (IQR), U/L	122 (77 - 177)	126 (86 - 171)	136 (93 - 255)	143 (89 - 343)	0.038
Myocardial band of CK, median (IQR), U/L	14 (12 - 22)	16 (13 - 21)	16 (13 - 23)	20 (13 - 55)	<0.001
Troponin, median (IQR), ng/L	37 (20 - 86)	44 (22 - 89)	52 (27 - 165)	68 (27 - 315)	0.007
Creatinine, median (IQR), $\mu\text{mol/L}$	71 (61 - 80)	71 (64 - 82)	71 (60 - 81)	76 (64 - 85)	0.44
NT-proBNP, median (IQR), ng/L	66 (35 - 138)	80 (32 - 166)	86 (42 - 176)	112 (52 - 487)	0.004
Glucose (median (IQR), mmol/L	7.7 (6.7 - 8.5)	8.6 (7.3 - 9.7)	8.7 (7.2 - 9.7)	8.8 (7.5 - 10.4)	<0.001
HbA1c, median (IQR), %	5.8 (5.6 - 6)	5.8 (5.6 - 6)	5.8 (5.6 - 6.1)	5.8 (5.6 - 6.1)	0.95
Blood count and biochemistry					
Leucocytes ($10^9/\text{L}$)	11 (8.3 - 13.6)	10.5 (8.4 - 13)	11.4 (9.0 - 13.8)	11.4 (9.5 - 13.9)	0.14
Thrombocytes ($10^9/\text{L}$)	234 (206 - 267)	224 (195 - 260)	243 (210 - 282)	245 (210 - 273)	0.099
Neutrophils ($10^9/\text{L}$)	8.2 (5.5 - 9.9)	7.18 (4.96 - 9.04)	8.5 (5.7 - 9.8)	7.9 (5.9 - 10.4)	0.37
Lymphocytes ($10^9/\text{L}$)	1.8 (1.3 - 2.4)	2.1 (1.5 - 2.6)	1.8 (1.4 - 2.5)	2.1 (1.5 - 2.9)	0.16
N/L ratio	3.9 (2.9 - 6.6)	3.3 (1.9 - 5.4)	4.2 (2.8 - 6.3)	3.4 (2.1 - 6.4)	0.26
hsCRP (mg/L)	1.6 (0.6 - 3)	1.9 (0.9 - 3.1)	2.3 (1.1 - 4.2)	3.8 (1.6 - 7.6)	<0.001

Data are expressed as mean $\pm$ standard deviation (SD), median (interquartile range (IQR)), or as number (%). BMI = Body Mass Index; TIMI = Thrombolysis in Myocardial Infarction; CK = creatine kinase; NT-proBNP = N-terminal pro brain natriuretic peptide; HbA1c = glycated hemoglobin; N/L = neutrophil/lymphocyte.

SUPPLEMENTARY TABLE 2. Baseline characteristics per sIL-6R quartile

Characteristics	Q1 (n = 92)	Q2 (n = 91)	Q3 (n = 92)	Q4 (n = 91)	P-value
sIL-6R levels (ng/ml)	52180 (37164 - 71582)	50730 (36787 - 69125)	51170 (37175 - 69009)	52241 (37920 - 68547)	<0.001
Age, mean (SD), years	58.3 ± 10.6	60.4 ± 11.1	58.3 ± 11.7	58.2 ± 12.9	0.52
Female sex - No. (%)	23 (25)	23 (25)	25 (27)	23 (21)	0.94
BMI, mean (SD), kg/m ²	26.9 ± 5.0	26.3 ± 3.1	27.1 ± 3.7	27.6 ± 3.4	0.17
Hypertension	30 (33)	27 (30)	26 (28)	25 (27)	0.88
Dyslipidemia	59 (64)	55 (60)	60 (65)	54 (59)	0.82
Current smoking	58 (63)	45 (49)	56 (61)	45 (49)	0.12
Stroke	1 (1)	0 (0)	0 (0)	2 (2)	0.29
Peripheral artery disease	0 (0)	0 (0)	0 (0)	0 (0)	1.00
Previous PCI	1 (1)	1 (1)	1 (1)	1 (1)	1.00
Blood pressure, mean (SD) mmHg					
Systolic	134 ± 22	137 ± 27	131 ± 25	134 ± 20	0.29
Diastolic	85 ± 14	86 ± 16	82 ± 16	84 ± 12	0.20
Heart rate, mean (SD), beats/min	79 ± 13	76 ± 20	75 ± 17	72 ± 14	0.06
Ischemia time, median (IQR), min	175.5 (107.5 - 260.5)	161 (108 - 266)	148 (109.5 - 217)	161 (105 - 254)	0.78
Single vessel disease - No. (%)	64 (70)	69 (76)	65 (71)	56 (62)	0.22
Culprit vessel - No. (%)					0.22
LAD	45 (49)	39 (43)	28 (30)	32 (35)	
LCX	12 (13)	13 (14)	19 (21)	17 (19)	
RCA	35 (38)	39 (43)	45 (49)	42 (46)	
Infarct-related artery TIMI flow - No. (%)					
Pre-intervention grade					0.48
1	56 (61)	56 (62)	49 (53)	44 (48)	
2	7 (8)	8 (9)	4 (4)	6 (7)	
3	12 (13)	13 (14)	20 (22)	18 (20)	
4	17 (18)	14 (15)	19 (21)	23 (25)	
Post-intervention grade					0.88
1	7 (8)	10 (11)	9 (10)	8 (9)	

SUPPLEMENTARY TABLE 2. Continued.

Characteristics	Q1 (n = 92)	Q2 (n = 91)	Q3 (n = 92)	Q4 (n = 91)	P-value
Myocardial blush grade	85 (92)	81 (89)	83 (90)	83 (91)	
	2 (2)	3 (3)	3 (3)	2 (2)	0.99
	7 (8)	9 (10)	5 (6)	8 (9)	
	21 (23)	17 (19)	18 (20)	17 (19)	
	62 (67)	62 (68)	63 (71)	64 (70)	
Laboratory values at admission					
CK, median (IQR), U/L	139 (83 - 255)	129 (86 - 192)	122 (85 - 208)	126 (85 - 198)	0.89
Myocardial band of CK, median (IQR), U/L	18 (13 - 42.5)	16 (13 - 25)	15 (12 - 22.5)	16 (12 - 23)	0.09
Troponin, median (IQR), ng/L	42 (21 - 174)	60 (29 - 133)	40 (16 - 135)	50 (29 - 115)	0.34
Creatinine, median (IQR), umol/L	70 (59 - 80.5)	72 (60 - 82)	76 (65.5 - 85)	71 (64 - 82)	0.19
NT-proBNP, median (IQR), ng/L	116 (41 - 277)	91 (54 - 233)	65.5 (30 - 167)	67 (35 - 170)	0.03
Glucose (median (IQR), mmol/L	8.2 (6.5 - 9.7)	8.4 (7.2 - 9.8)	8.25 (7.05 - 9.2)	8.2 (7 - 9.4)	0.82
HbA1c, median (IQR), %	5.8 (5.6 - 6)	5.8 (5.6 - 6.1)	5.7 (5.6 - 6)	5.9 (5.5 - 6.1)	0.47
Blood count and biochemistry					
Leucocytes (10e9/l)	11.4 (8.5 - 14.05)	11.4 (8.9 - 13.7)	11 (9.65 - 13.6)	10.2 (8.4 - 12.9)	0.17
Thrombocytes (10e9/l)	233 (206 - 267)	239 (200 - 274)	243 (215 - 278)	220 (188 - 266)	0.06
Neutrophils (10e9/l)	7.9 (5.5 - 11.2)	7.6 (5.3 - 10.6)	8.3 (6.0 - 9.8)	6.9 (5.2 - 9.0)	0.13
Lymphocytes (10e9/l)	1.97 (1.5 - 2.4)	2.0 (1.5 - 2.7)	2.0 (1.4 - 2.7)	2.0 (1.4 - 2.6)	0.98
N/L ratio	3.7 (2.6 - 6.7)	3.8 (2.3 - 6.1)	4.0 (2.9 - 6.9)	3.3 (2.2 - 5.6)	0.48
hsCRP (mg/l)	2.55 (1.4 - 5)	2.4 (1.1 - 3.7)	1.8 (.8 - 3.5)	1.7 (.9 - 4.1)	0.28

Data are expressed as mean $\pm$ standard deviation (SD), median (interquartile range (IQR)), or as number (%). BMI = Body Mass Index; TIMI = Thrombolysis in Myocardial Infarction; CK = creatine kinase; NT-proBNP = N-terminal pro brain natriuretic peptide; HbA1c = glycated hemoglobin; N/L = neutrophil/lymphocyte.

SUPPLEMENTARY TABLE 3. Baseline characteristics per sgp130 quartile

Characteristics	Q1 (n = 88)	Q2 (n = 85)	Q3 (n = 87)	Q4 (n = 86)	P-value
sgp130 levels (ng/ml)	235 (195 - 259)	311 (292 - 320)	364 (345 - 381)	453.5 (425 - 513)	<0.001
Age, mean (SD), years	59.0 ± 11.4	60.6 ± 11.2	57.9 ± 12.7	59.0 ± 11.0	0.50
Female sex - No. (%)	28 (32)	28 (33)	19 (22)	14 (16)	0.03
BMI, mean (SD), kg/m ²	26.8 ± 3.8	26.8 ± 4.2	26.8 ± 4.0	27.4 ± 3.6	0.74
Cardiovascular related history - No. (%)					
Hypertension	24 (27)	26 (31)	27 (31)	27 (31)	0.93
Dyslipidemia	57 (65)	50 (59)	51 (59)	56 (65)	0.70
Current smoking	46 (52)	46 (54)	51 (59)	47 (55)	0.86
Stroke	0 (0)	0 (0)	1 (1)	2 (2)	0.29
Peripheral artery disease	0 (0)	0 (0)	0 (0)	0 (0)	1.00
Previous PCI	1 (1)	0 (0)	2 (2)	1 (1)	0.56
Blood pressure, mean (SD) mmHg					
Systolic	133 ± 22	131 ± 24	135 ± 23	138 ± 25	0.21
Diastolic	84 ± 13	81 ± 14	86 ± 15	87 ± 16	0.02
Heart rate, mean (SD), beats/min	74 ± 13	77 ± 18	77 ± 17	77 ± 16	0.51
Ischemia time, median (IQR), min	163 (108 - 241)	153 (109 - 236)	161 (96 - 304)	160 (120 - 229)	0.99
Single vessel disease - No. (%)	57 (65)	62 (73)	61 (70)	58 (67)	0.69
Culprit vessel - No (%)					
LAD	33 (38)	38 (45)	32 (37)	36 (42)	0.74
LCX	11 (13)	12 (14)	15 (17)	16 (19)	
RCA	44 (50)	35 (41)	40 (46)	34 (40)	
Infarct-related artery TIMI flow - No. (%)					
Pre-intervention grade					
0	54 (61)	47 (55)	48 (55)	48 (56)	0.15
1	4 (5)	3 (4)	11 (13)	4 (5)	
2	14 (16)	18 (21)	17 (20)	12 (14)	
3	16 (18)	17 (20)	11 (13)	22 (26)	
Post-intervention grade					
2	6 (7)	11 (13)	10 (11)	6 (7)	0.40
3	82 (93)	74 (87)	77 (89)	80 (93)	

SUPPLEMENTARY TABLE 3. Continued.

Characteristics	Q1 (n = 88)	Q2 (n = 85)	Q3 (n = 87)	Q4 (n = 86)	P-value
Myocardial blush grade					
0	1 (1)	5 (6)	3 (4)	1 (1)	0.12
1	10 (11)	8 (9)	8 (10)	3 (3)	
2	12 (14)	22 (26)	20 (24)	18 (21)	
3	65 (74)	50 (59)	53 (63)	64 (74)	
Laboratory values at admission					
CK, median (IQR), U/l	131 (86 - 241)	108 (81 - 176)	143 (100 - 213)	140 (89 - 260)	0.12
Myocardial band of CK, median (IQR), U/l	15 (12 - 24)	17 (13 - 25)	18 (13 - 31)	16 (14 - 23)	0.76
Troponin, median (IQR), ng/l	42 (28 - 137)	60 (26 - 130)	49 (20 - 158)	48 (25 - 134)	0.97
Creatinine, median (IQR), umol/l	70 (60 - 79)	74 (60 - 81)	70 (62 - 81)	77 (65 - 85)	0.12
NT-proBNP, median (IQR), ng/l	88 (37 - 230)	80 (37 - 200)	64 (41 - 143)	105 (51 - 266)	0.41
Glucose (median (IQR), mmol/L	8.2 (7.3 - 9.9)	9 (7.6 - 10.5)	8.1 (6.9 - 9)	8.2 (7.1 - 9.3)	0.02
HbA1c, median (IQR), %	5.7 (5.6 - 6)	5.8 (5.6 - 6)	5.8 (5.6 - 6.1)	5.8 (5.6 - 6.1)	0.70
Blood count and biochemistry					
Leucocytes (10e9/l)	11.1 (8.9 - 14.0)	11.6 (8.5 - 14.1)	11.1 (8.9 - 13.4)	10.7 (8.6 - 13.8)	0.88
Thrombocytes (10e9/l)	245 (212 - 276)	242 (209 - 276)	227 (187 - 270)	231 (204 - 262)	0.15
Neutrophils (10e9/l)	7.5 (5.6 - 9.8)	8.2 (5.0 - 9.8)	7.6 (5.7 - 10.1)	8.1 (5.3 - 10.8)	0.82
Lymphocytes (10e9/l)	1.9 (1.4 - 2.5)	2.1 (1.5 - 2.9)	2.0 (1.6 - 2.6)	1.9 (1.3 - 2.4)	0.12
N/L ratio	3.6 (2.8 - 5.8)	3.3 (2.1 - 5.1)	3.4 (2.4 - 6.4)	4.7 (2.4 - 7.4)	0.11
hsCRP (mg/l)	2 (1.8 - 3.8)	2.7 (1.4 - 5.1)	2.6 (1.3 - 5.1)	1.7 (1.8 - 3.9)	0.09

Data are expressed as mean $\pm$ standard deviation (SD), median (interquartile range (IQR)), or as number (%). BMI = Body Mass Index; TIMI = Thrombolysis in Myocardial Infarction; CK = creatine kinase; NT-proBNP = N-terminal pro brain natriuretic peptide; HbA1c = glycated hemoglobin; N/L = neutrophil/lymphocyte.

SUPPLEMENTARY TABLE 4. Number of samples available for measurements of IL-6, sIL-6R and sgp130 at the different time points

	IL-6	sIL-6R	sgp130
Baseline	369	366	346
24 hours	341	342	316
2 weeks	325	329	309
7 weeks	316	319	297
4 months	313	314	293
1 year	262	268	252

IL-6 = interleukin 6; sIL-6R = soluble interleukin 6 receptor; sgp130 = soluble glycoprotein 130

SUPPLEMENTARY TABLE 5. Associations between IL-6, sIL-6R and sgp130 and cardiac markers at baseline. * = $p < 0.005$, # = $p < 0.05$.

	Troponin T	CK	CK-MB	NT-proBNP
IL-6	0.19*	0.19*	0.22*	0.22*
sIL-6R	-0.02	-0.02	-0.08	-0.12#
sgp130	0	0	0.03	0.03

SUPPLEMENTARY FIGURE 1. Log sIL-6R/IL-6 ratio (mean and SD) (A). We divided log sIL-6R levels by IL-6 levels in order to be able to show a clear and understandable figure. Reduction factor of log sIL-6R/IL-6 ratio using log sIL-6R/IL-6 ratio at 1 year as a reference (B).

SUPPLEMENTARY FIGURE 2. Log IL-6 (A), log sIL-6R (B), and log sIL-6R/IL-6 ratio (C) levels in STEMI patients with normal (MBG 3) and impaired reperfusion (MBG 0-2). Levels are depicted in mean and SEM (IL-6 and sIL-6R) or mean and SD (sIL-6R/IL-6 ratio). p values for trend are shown.

SUPPLEMENTARY FIGURE 3. Log IL-6 (A), log sIL-6R/IL-6 (B) levels in STEMI patients with normal E/e' (<13) and elevated E/e' (≥ 13). Levels are depicted in mean and SEM. p values for trend are shown.

SUPPLEMENTARY FIGURE 4. Associations between members of the interleukin-6 signaling cascade measured in STEMI patients at baseline and infarct size, LVEF, and E/e' ratio measured at 4 months, depicted as β and 95% CIs obtained from linear regression models. 95% CI = 95% Confidence Interval; IL-6 = interleukin 6; sIL-6R = soluble interleukin 6 receptor; sgp130 = soluble glycoprotein 130; Q1 = lowest quartile; Q4 = highest quartile; STEMI = ST-elevation myocardial infarction; LVEF = left ventricular ejection fraction.

Multivariate analysis on infarct size: adjusted for age, sex, BMI, hypercholesterolemia, TIMI (pre- and post-intervention), MBG.

Multivariate analysis on LVEF: adjusted for age, sex, hypercholesterolemia, TIMI (pre- and post-intervention), ischemic time.

Multivariate analysis on E/e': adjusted for age, sex, BMI, heart rate, hypertension, smoking.

Chapter 7

HUMAN GENETIC DETERMINANTS OF THE GUT MICROBIOME AND THEIR ASSOCIATIONS WITH HEALTH AND DISEASE: A PHENOME- WIDE ASSOCIATION STUDY

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ABSTRACT

Background: Small-scale studies have suggested a link between the human gut microbiome and highly prevalent diseases. However, the extent to which the human gut microbiome can be considered a determinant of disease and healthy aging remains unknown. We aimed to determine the spectrum of diseases that are linked to the human gut microbiome through the utilization of its genetic determinants as a proxy for its composition.

Methods: 180 single nucleotide polymorphisms (SNPs) known to influence the human gut microbiome were used to assess the association with health and disease outcomes in 422,417 UK Biobank participants. Potential causal estimates were obtained using a Mendelian randomization (MR) approach.

Results and conclusion: From the total sample analysed (mean age was 57 ± 8 years), 194,567 (46%) subjects were male. Median exposure was 66-person years (interquartile range 59 to 72). Eleven SNPs were significantly associated with 28 outcomes (Bonferroni corrected P value $< 4.63 \cdot 10^{-6}$) including food intake, hypertension, atopy, COPD, BMI, and lipids. Multiple SNP MR pointed to a possible causal link between *Ruminococcus flavefaciens* and hypertension, and *Clostridium* and platelet count. Microbiota and their metabolites might be of importance in the interplay between overlapping pathophysiological processes, although challenges remain in establishing causal relationships.

INTRODUCTION

It has been suggested that different diseases may share more pathophysiological mechanisms than initially assumed (1–6). An enhanced understanding of these complex shared disease causing mechanisms is of paramount importance to further improve our strategies to study, prevent and treat diseases.

One of the possible shared systems is the human gut microbiome, which has been suggested as a key inter-player in a variety of individual disease entities (7). For example, the gut microbiome has been linked to immune system activation, inflammatory processes and metabolic phenotypes. Moreover, it has been associated with thrombosis and the development of cardiovascular disease (CVD) (8–17), Alzheimer's disease, and cancer (18–20). Human genetic variants have been linked to the microbiome composition (21–23) and with this knowledge, rather than actual measurements of the microbiome itself, human genetic variants can be used as a proxy to inform about human gut microbiome composition. This is of interest because large cohorts in which genetic information is available can be studied even if the microbiome itself has not been measured. In addition, the use of genetic determinants can be useful to study the potential long-term health effects of having increased or decreased levels of specific gut microbiota.

Although several associations between the human gut microbiome and individual diseases have been reported, a broad characterization of a large spectrum of health and disease states is lacking but is highly desired as it may help identify potentially common mechanistic pathways (17,24,25). Hence, this study aimed to explore the associations between known genetic determinants of the human gut microbiome and the presence of health and its determinants (absence of disease, vital and blood biomarkers and food intake) and disease states in a very large and comprehensive population cohort and investigate possible causal mechanisms using a Mendelian randomization (MR) approach.

RESULTS

We studied 422,417 unrelated individuals with a mean age of 57±8 years, of which 194,567 (46%) were male (**Table 1**). **Figure 1** presents a flowchart of the study sample selection. Median follow-up was 6 years (interquartile range (IQR) 5–7) and the median total exposure was 66-person years (interquartile range (IQR) 59–72).

TABLE 1. Baseline characteristics.

Characteristic	No.
Total, no.	422,417
Male	194,567 (46.1%)
Age, mean (SD), y	57 (8)
Years of exposure, median (IQR), y	66 (59 – 72)
Body mass index (kg/m ²)	27.4 (4.8)
Systolic blood pressure (mmHg)	133 (20)
Diastolic blood pressure (mmHg)	82 (9)
Smoking behaviour	
Active daily	32,829 (8.0%)
Active occasionally	11,545 (2.8%)
Stopped <= 12 months	1,961 (0.5%)
Stopped > 12 months	131,800 (32.0%)
Never or <100 cigarettes	233,186 (56.7%)
Medical history	
Hypertension	125,243 (29.6%)
Diabetes Mellitus type 2	16,815 (4.0%)
Hyperlipidemia	80,712 (19.1%)

SD = standard deviation; IQR = interquartile range

Phenome-wide scan on outcomes

A total of eleven microbiome SNPs was associated with 28 outcomes after a stringent Bonferroni correction (P value < $4.63 \cdot 10^{-6}$) (**Figure 2**). The strongest associations with binary traits were observed with diseases related to the following general ICD-10 categories: circulatory system, respiratory system, and respiratory/skin. Complementary information on associations between the SNPs, bacteria, and clinical outcomes is provided in **Supplementary Table 1**.

An even large number of significant associations with linear traits was discovered; especially health-related measurement including food intake, hemodynamic parameters, anthropometric and laboratory measurements were highlighted (**Figure 2**). Extended information on the associations between the SNPs, bacteria, and continuous outcomes is provided in **Supplementary Table 2**. We identified no significant interactions between sex and genetically determined changes in microbiome.

FIGURE 1. Flowchart for the selection of the analysed study sample from the UK Biobank Study.

Mendelian randomization analyses

Single SNP

Single SNP Mendelian randomization (MR) analyses using the Wald estimate were performed for all 28 outcomes. All Wald estimates of all 28 exposure-outcomes were significant (highest P value $4.4 \cdot 10^{-6}$). Results are shown in **Figure 3A**, are schematically depicted in **Supplementary Figure 1** and full results can be found in **Supplementary Table 3**. F-statistics were all higher than 10, indicating little chance of weak-instrument bias based on the summary statistics. MR-steiger filtering indicated reversed causation to be likely in the association between *Clostridium cellulolyticum* and platelet count ($P_{steiger} < 5.6 \cdot 10^{-119}$).

FIGURE 2. Manhattanplot for associations between microbiome SNPs and health and disease outcomes and continuous outcomes. On the x-axis, the health and disease outcomes (according to the ICD 10 code), and continuous are shown. On the y-axis, the P-value is shown. The red line indicates the significance threshold using Bonferroni correction.

Under the ideal scenario that all MR assumptions are met, one could cautiously conclude the following based on these results. Genetically determined higher levels of *Clostridium* cluster IV were associated with a decreased risk for COPD (β -0.34, SE 0.05, $P = 1.96 \cdot 10^{-10}$) and a decreased risk for atopy (β -0.54, SE 0.04, $P = 1.45 \cdot 10^{-37}$). Genetically determined higher levels of the species *Ruminococcus flavefaciens* were associated with an increased risk for hypertension (β 0.05, SE 0.01, $P = 4.97 \cdot 10^{-7}$).

Genetically determined higher levels of the genera *Slackia* and *Pseudobutyrvibrio* were associated with an increase in eosinophil count. Higher levels of the species *Clostridium xylanovorans* were associated with a decrease in resting heart rate and body mass index (BMI). An increase of the genus *Desulfovibrio* was also associated with a decrease in resting heart rate. Genetically determined higher levels of Methanobacteria on family level were associated with an increase in alcohol intake frequency. Conversely, it was associated with a decrease in mean arterial blood pressure, systolic blood pressure,

diastolic blood pressure, BMI, waist hip ratio, reticulocyte count , and erythrocyte count. Interestingly, genetically determined higher levels of the genus *Bifidobacterium* were associated with increased levels over cholesterol, apolipoprotein A1, HDL, and grip strength. This might seem paradoxical to previous findings in animals and human studies in which a favourable effect of specific *Bifidobacterium* strains on obesity and cholesterol levels was observed (26–28).

A genetically increase in the genus *Shigella* was associated with an increase in reticulocyte count. Higher levels of *Clostridium* cluster IV were associated with a decrease in eosinophil count. Genetically determined higher levels of the species *Ruminococcus flavefaciens* were associated with an increase in systolic blood pressure and mean arterial pressure. Lastly, genetically determined higher levels of the species *Lactivibrio alcoholicus* were associated with a decrease in lymphocyte count.

FIGURE 3A. Heatmap showing the Wald β 's of the associations between microbiome SNPs and health and disease outcomes, and continuous outcomes. Only significant associations (P value < 4.63·10⁻⁶) are shown. BMI = body mass index; COPD = Chronic Obstructive Pulmonary Disease; HDL = high density lipoprotein; MAF = minor allele frequency.

Combined SNPs

To investigate whether the MR assumptions were truly met, we increased the amount of SNPs to assess the exposure-outcome association (**Supplementary Table 3**).

We were able to pool SNPs for a total of 26 exposure-outcome associations (**Supplementary Table 4**). Heterogeneity was indicated using Cochran's Q statistic for all exposure-outcome associations ($P < 0.05$, **Supplementary Table 5**), except for the Clostridiaceae family and *Clostridium* genus with platelet count and the genus *Ruminococcus* with hypertension. This indicates at least balanced pleiotropy to be affecting the results. Given that assumptions of inverse variance weighted (IVW) effects model are violated under this scenario, we took forward the IVW random effects model as most liberal model (**Figure 3B, Supplementary Table 4**). Compared to 27 causal estimates provided by the Wald ratio (one excluded by MR-Steiger filtering), now only 3 exposure-outcome associations remained suggestively significant (i.e. $P < 0.05$, but not significant for multiple testing ($0.05/28$)). These were the causal estimates of the Clostridiaceae family and *Clostridium* genus with platelet count (β 0.43, SE 0.16, $P = 7.28 \cdot 10^{-3}$ and β 0.43, SE 0.19, $P = 1.99 \cdot 10^{-2}$; respectively) and the genus *Ruminococcus* with hypertension (β 0.45, SE 0.18, $P = 1.11 \cdot 10^{-2}$).

Intriguingly, the association between Clostridiaceae/*Clostridium* and platelet count remained significant considering the main driver (rs58368959, associated with *Clostridium cellulosyticum*) was removed as a result of MR-steiger filtering. The results were also robust to several sensitivity analyses, including MR-PRESSO and MR-Lasso, which are more outlier-robust, and the "unweighted" MR, which corrects for potential differences in effect size units as effect sizes depict relative abundance (**Supplementary Table 4**). The Rucker model showed no differences between the heterogeneity estimates of Cochran's Q and Rucker's Q, indicating at the absence of unbalanced horizontal pleiotropy and thus an inverse variance weighted model to be preferred (**Supplementary Table 5**). The results were not robust to MR-Egger, weighted median and weighted mode analyses (**Supplementary Table 4**). A forest and scatter plot of the MR between Clostridiaceae/*Clostridium* and platelet count can be found in **Supplementary Figure 2-5**.

The association between the genus *Ruminococcus* with hypertension was also robust to the "unweighted" MR, as expected considering the 2 SNPs included in the MR were obtained from the same study. Further sensitivity analyses could not be performed considering the amount of SNPs. One taxonomic level higher, 11 SNPs were pooled for the Ruminococcaceae family and hence more sensitivity analyses could be performed. The IVW random effect estimate was already insignificant ($\beta = 6.94 \cdot 10^{-3}$, SE = $6.63 \cdot 10^{-3}$).

³, $P = 0.29$). This suggests that, if the association between genus *Ruminococcus* and hypertension is true, it is specific for that genus within the Ruminococcaceae family. A forest and scatter plot of the MR between Ruminococcaceae/*Ruminococcus* and hypertension can be found in **Supplementary Figure 6 and 7**.

FIGURE 3B. Heatmap showing the inverse variance weighted random effects β 's of the associations between the genetically determined increase of bacteria (family, genus and species level if possible) and health and disease outcomes, and continuous outcomes. Please note that the inverse variance weighted random effects β 's are in some cases liberal, considering the Rucker framework indicated unbalanced horizontal pleiotropy in the estimates as indicated by a significant ($P < 0.05$) Q-Q' (and thus the MR-Egger estimate to be a better fit, please see Supplementary Table 5 for these results). BMI = body mass index; COPD = Chronic Obstructive Pulmonary Disease. # indicates suggestively significant.

To investigate whether estimated effects of the Methano- and Bifidobacterium on associated outcomes were consistent when considering a community effect of the combined pool of gut bacteria, additional sensitivity analyses were performed (**Supplementary Table 6**). The results remained similar to the initial single SNP estimates.

Lastly, we performed a look-up in MR-Base, a platform for Mendelian randomization analysis, to explore whether the eleven SNPs were associated with other traits (P -value significance threshold of $5.00 \cdot 10^{-8}$) in other studies than UK Biobank. The variant rs4548017 (Methanobacteria) was associated with ulcerative colitis, inflammatory bowel disease, and psoriasis(29,30). The variant rs1446585 (*Bifidobacterium*) was associated with height (31). We additionally performed a look-up in GeneCards for extra information about the genes related to the eleven significantly associated SNPs (**Supplementary Table 7**).

DISCUSSION

In the present study, we used a data-driven approach to identify human health and diseases parameters that are associated with genetic variants thought to influence the microbiome and identified 28 associations with 11 genetic variants. We found relevant associations with food intake, hypertension, BMI, lipids, atopy, and COPD. The possibility of a causal nature of these associations was tested using a MR approach. Although 27 out of 28 associations were indicated to be causal using a single SNP approach, only three of these associations were consistent across some, but not all sensitivity analyses.

Our findings highlight diseases that have been investigated in previous observational studies (32–41). However, contrary to earlier observational research on the human gut microbiome and CVD, associations with CAD and MI did not reach statistical significance in our phenome-wide scan of microbiome associated SNPs(8,34). While this might be due to a lack of study power or a small effect size of the evaluated genetic variants, associations with pathways via which the human gut microbiome may influence the development of CAD or MI (i.e. inflammation, arterial blood pressure, and circulating lipoproteins) were documented. As these factors are strongly related to CVD development, it is highly interesting to further unravel their, by microbiome influenced, effect in the long-term.

The association between a genetically determined increase of *Bifidobacterium* and elevated lipids is notable, since *Bifidobacterium* is considered to exert beneficial effects in human health(42). Small sample sizes ($n = 19 - 32$ humans), differences in microbiome behaviour between obese and lean individuals, and the hypothesis that probiotic supplements may disturb the original composition of the microbiome could be possible explanations for this discrepancy(43–45). In fact, a previous MR between

genetically determined Bifidobacterium and HDL and LDL also found a positive causal estimate(46). It is likely that total composition of the gut microbiome and bacteria ratios may eventually tell us more than single particular increases or decreases.

Strengths and limitations

This study is the first to investigate the association between previously established microbiome SNPs and a wide variety of health and disease states simultaneously. The major strengths are the considerable sample size, the variety of explored outcomes without an a priori hypothesis, the prospective design of the UK Biobank study. This study could lead other researchers in certain directions to further explore the role of the gut microbiome in health and disease. In addition, we performed a large amount of MR sensitivity analyses (when enough SNPs were available). Another strength is that the MR was performed at different taxonomic levels. This allowed for the investigation of specific strains (species level) while also being able to increase the number of instruments when investigating at genus or family level. The potential importance was illustrated by the consistent estimates between Clostridiaceae family and *Clostridium* genus with platelet count, and the inconsistent estimates between the family Ruminococcaceae and genus Ruminococcus with hypertension. We believe this might provide insights how to perform and interpret MR estimates within the microbiome research.

We first address limitations most pertinent to a MR approach within microbiome research. It is of importance to note that the human gut microbiome is shaped for a large part by environmental factors (47). However, the importance of a genetic component has been established as well(23,48). In addition, the genetic approach allowed us to identify new pathways that are interesting to further unravel in the large cohort of the UK Biobank without real stool samples. A second limitation includes the lack of reliable SNPs associated with the gut microbiome. Although we adapted a fairly stringent P value threshold ($5.00 \cdot 10^{-8}$) for inclusion criteria and all F statistics were larger than 10, our results could still be influenced by weak-instrument bias. "Winner's curse", i.e. overestimation of genetic associations within the dataset in which the SNPs were first identified, is likely considering the failure of replication of previously established SNPs(21,48–51) in newer MWAS(47,52). Since a two-sample approach was used without sample overlap, any bias due to weak instruments is directed to the null and therefore does not lead to false positive findings(53). Another limitation is that the biological function through which the SNPs influence the gut microbiome is unsure and complex, especially considering the possible bilateral nature of the association within the GWAS exposure (i.e. the human microbiome influencing health status and health status influencing the human microbiome). We therefore performed an array of sensitivity

analyses to assess whether the results of the single SNP estimates were robust to pleiotropic effects; this was not the case for most exposure-outcome associations. However, whether associations in multiple SNP MR analyses due to pleiotropic effects or weak instrument bias through the "winner's curse" cannot be differentiated with the current set of analyses. In addition, we applied MR-Steiger filtering to evaluate potential reversed causation. We were unable to validate that the traits investigated share the same causal variant at a particular locus for both the exposure and outcome using colocalization methods, as we did not have the full Linkage Disequilibrium structure of the SNPs investigated. We did use MR-Base to check whether the SNPs were already known to be associated with other traits.

Considering the drawbacks of the current approach, we are very cautious in labelling the associations as "causal". The current study should be considered as a broad hypothesis free scan in which causality of some associations is strengthened by contextualisation with previously described possible mechanisms (for example, the known risk factor hypertension in the development of CVD). In addition, it highlights the need for strict MR analyses in the microbiome context, as more extensively discussed previously(54).

We believe that current study is of additive value to the multi-omics approach required to further dissect the role of the human microbiome in human health and disease (55).

Future perspectives

Our study could constitute a useful tool to identify bacteria of interest in order to thoroughly investigate the mechanisms between these bacteria and clinical outcomes; not only in the field of cardiology, but also in other fields. Second, the human gut microbiome might be used as a biomarker in disease risk stratification. Third, the observed associations can point towards new possibilities for therapeutic treatment. Furthermore, the abundance of particular bacteria or their metabolites could influence therapeutic treatment efficacy, as recently demonstrated in the treatment of Parkinson's disease (56).

Conclusions

Human genetic determinants of the gut microbiome are associated with 28 specific health and disease outcomes including hypertension, atopy, COPD, lipids, and BMI. Multiple SNP MR pointed to a possible causal link between *Ruminococcus flavefaciens* and hypertension, and *Clostridium* and platelet count. Microbiota and their metabolites might be of importance in the interplay between overlapping pathophysiological

processes, and could serve as potential therapeutic targets for the maintenance of health and prevention and treatment of diseases. However, many challenges remain in establishing causal relationships using current genetic data and approaches.

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METHODS

Study population

Data from the UK Biobank was accessed and analysed under an approved research proposal (application ID: 12006) (57). The UK Biobank is a large community-based prospective study in the United Kingdom that recruited approximately 500,000 participants aged 40 to 69 years old with the general objective to improve the prevention, diagnosis, and treatment of diseases. All methods were performed in accordance with the relevant guidelines and regulations. The study design and population have been extensively described elsewhere (58). All participants provided informed consent to participate (59). The UK Biobank obtained approval from the relevant institutional review boards, that is, the North West Multi-centre Research Ethics Committee for the UK, the National Information Governance Board for Health and Social Care for England and Wales, and the Community Health Index Advisory Group for Scotland (60).

Identification of single nucleotide polymorphisms

Single nucleotide polymorphisms associated with the gut microbiome were collected from 6 previous GWASes (21,22,50–52,61), using a genome-wide significance threshold of $P < 5 \times 10^{-8}$ for their inclusion in the current study. If the same SNPs and effect sizes were found across different taxonomic level within a single study, we took forward the association with the most differentiated taxonomic level to reduce multiple testing burden. SNPs were clumped within study per bacterium (on the lowest taxonomic level available) using the PLINK (version 1.9(62)) clumping procedure to prune genetic

variants at a stringent linkage disequilibrium (LD) of $R^2 < 0.005$ within a five megabase window. We did not clump the 25 SNPs from Goodrich *et al.* due to the character of the study (twin study instead of GWAS). In the end, this resulted in a total of 180 SNPs associated with the gut microbiome. For more information on the SNP selection, please see **Table 2**.

Genotyping and imputation

The genotyping process and arrays used in the UK Biobank study have been previously described (60). Briefly, participants were genotyped using the custom UK Biobank Lung Exome Variant Evaluation Axiom (Affymetrix; $n = 49,949$), which includes 807,411 SNPs, or the UK Biobank Axiom array (Affymetrix; $n = 452,713$), which includes 820,967 SNPs (60,63). The arrays share over 95% of insertion and deletion markers (60,63). Imputed genotype data were provided by the UK Biobank, based on merged UK10K and 1000 Genomes phase 3 panels (64). Participants were excluded in case of missing genotype or sex mismatch ($n = 378$). Participants with high missingness or excess heterozygosity were also excluded ($n = 963$). Furthermore, 64,535 participants were excluded because of familial relatedness and being of non-Caucasian British descent. Lastly, participants without information available on the previously reported SNPs associated with the composition of the gut microbiome were also excluded ($n = 14,243$) (21,22). **Figure 1** presents a flowchart of the study sample selection.

Definition of (cardiovascular) health, prevalent disease and new-onset disease

Two "health state" variables were generated. Cardiovascular health was defined as the absence of type 2 diabetes mellitus (DM2), stroke, and myocardial infarction (MI) (65). Total health was based on the WHO top 10 causes of deaths in high-income countries. It was defined as the absence of coronary artery disease (CAD), stroke, Alzheimer's disease, lung cancer, chronic obstructive pulmonary disorder (COPD), pneumonia, colon cancer, rectum cancer, DM, kidney diseases, and breast cancer at baseline and during follow-up (66). The diseases was defined using self-reported diagnoses and medication (Hospital Episode Statistics data) as well as ICD-9 and ICD-10 codes as described previously (67). Definitions of prevalent (established) and incident (new-onset) disease (outcomes) are shown in **Supplementary Table 8**.

Vital signs, blood count, and food intake

Vital signs and biological samples were collected during the baseline visit together with data of self-completed questionnaires (including food questionnaires), interviews,

TABLE 2. Studies used for SNP extraction

Author (year)	Table	Cohort (N)	Age range	Method	Unit	N SNPs (before clumping)	Clumped (lowest taxa)	N SNPs (after clumping)	Note
Davenport <i>et al.</i> (2015)	S7	Hutterites (n = 184)	18 – 75	16S rRNA	Abundance	1	No	-	
Bonder <i>et al.</i> (2016)	S3	LifeLines Deep (n = 984)	18 – 84	Metagenomics sequencing	Abundance	58	Yes	10	Allele frequencies obtained from HaploReg v3
Goodrich <i>et al.</i> (2016)	S5	TwinsUK (n = 2,139)	18 – 89	16S rRNA	Relative abundance	25	No	NB	
Wang <i>et al.</i> (2016)	T2	PopGen (n = 914), FoCus (n = 1,115)	25 – 83, 18 – 83	16S rRNA	Relative abundance	43	Yes	-	
Turpin <i>et al.</i> (2016)	S6	GEM Project (n = 1,098)	6 – 35	16S rRNA	Relative abundance	58	Yes	56	Effect sizes obtained from log-normal model
Scepanovic <i>et al.</i> (2018)	S13	Milieu Intérieur (n = 858)	20 – 69	16S rRNA	Relative abundance	188	Yes	48	

N SNPs denotes number of SNPs, S denotes Supplementary Table, T denotes Table. Please note that the studies from Rothschild *et al.* Was not taken forward in the current analyses since effect sizes for discovered SNPs were not provided in the manuscript.

and physical measurements. Blood pressure was measured twice for consistency and the average value was used. Automated measurements were corrected according to previously described methodology (68).

Phenome-wide scan on outcomes

Outcomes with a prevalence of $\leq 1\%$ were excluded from the analyses. Logistic regression modelling was performed to assess the effect of microbiome SNPs on combined prevalent and new-onset diseases, and health outcomes in term of the resulting odds ratios (OR) and corresponding 95% confidence intervals (CIs). Genotypes were coded according to a dose-response model (0,1,2), where the allele coded with a 2 corresponded with an increased abundance of a certain bacterium. Linear regression analyses were conducted to assess the effect of microbiome SNPs on continuous outcome measures (anthropometric, hemodynamic and laboratory variables as well as longevity and food intake at baseline) in terms of the resulting β coefficients and associated standard errors (SE). Since sex differences exist regarding the human gut microbiome, we tested for sex-interactions in the association between the SNPs and all outcomes. All regression analyses were adjusted for age (at the moment of the last follow-up or age at baseline visit), sex (except when sex-interactions were tested), genotyping chip, and the first 30 principal components (to adjust for population structure) provided by the UK Biobank. A stringent Bonferroni correction was used to correct for multiple testing (180 SNPs, 60 outcomes; $0.05/10,800 = P \text{ value} < 4.63 \cdot 10^{-6}$). Outcomes reaching statistical significance after Bonferroni correction were considered of interest for further analyses (see **Mendelian randomization** section below). Analyses described above are performed using Stata version 15 (StataCorp, Texas, United States)

Mendelian randomization analyses

In order to explore the possibility of a causal relationship between the exposure (the gut microbiome) and the outcomes reaching statistical significance after Bonferroni correction, we first performed a single SNP Mendelian randomization (MR) using the Wald-estimate(69). Weak instrument bias was assessed per SNP using the F-statistic, calculated with the following formula: $F = R^2(n-2)/(1-R^2)$. In this formula, n is the sample size of the exposure and R^2 is the amount of variance of the exposure explained by the SNP(70). R^2 was calculated using a previously established formula(71). Allele frequencies and SNP position were obtained for the SNPs obtained from the study of Wang *et al*(72). In order to explore the possibility of reversed causation to be a driver of the current results, we applied MR-Steiger filtering. MR-Steiger filtering calculates the R^2 for the exposure and outcome and removes variants if the R^2 of the exposure is significantly lower ($P \text{ value} < 0.05$) than the R^2 of the outcome(73). R^2 for linear traits

was calculated as mentioned above(71). R^2 for binary outcomes was calculated on the liability scale according to previously established methods(74). These results can be found in **Supplementary Table 3**.

Pleiotropy within MR analyses refers to a genetic variant having multiple effects and distorts MR estimates when genetic variants affect the outcome independently(75). Given the uncertainty of the biological mechanisms underlying the SNP-exposure association, and hence uncertainty on whether the genetic variants affect the outcome independently, we aimed to gain additional insight in pleiotropy by increasing the amount of SNPs to assess the exposure-outcome association. SNPs associated with the same exposure and their effect estimates on the outcome (possibly not reaching Bonferroni significance) were obtained from the pool of all 180 SNPs. This resulted in an additional 153 exposure-outcome associations, for which single SNP MR estimates, F-statistics(70) and MR-Steiger(73) results can be found in **Supplementary Table 3** as well. By definition, effect sizes used to obtain pooled estimates should reflect the same unit. We therefore only took forward SNPs with effect sizes reflecting relative abundance, given the fact that a) only one SNP (rs4548017) reached statistical significance on the tested outcomes after Bonferroni correction, b) 169 out of 180 SNPs reflected relative abundance and c) the relative effects better reflect the biology of the *milieu intérieur*. Due to difference in in-depth classification of the taxonomic rank in the SNP-exposure association between and within study, SNPs were pooled on species, genus and family level. Again, SNPs were clumped (between-studies) for every bacterium per level. Detailed information on the amount of SNPs per bacterium per taxonomic level and the outcomes tested can be found in **Supplementary Table 4**. Three methods were applied based on the amount of genetic variants available: a) if after pooling only one SNP remained available, only Wald ratio, MR-Steiger filtering(73) and F-statistics (70) were calculated as explained above (28 exposure-outcome associations, see **Supplementary Table 3**), b) if two SNPs were available, inverse variance weighted fixed effects and random effects were calculated; I^2 -index(76) and Cochran's Q(77) were used to assess heterogeneity and differentiate between models (6 exposure-outcome associations) (**Supplementary Table 4 and 5**) and c) if more than two SNPs were available, a more extensive pallet of analyses was performed (22 exposure-outcome associations) (**Supplementary Table 4 and 5**). First of all, this extended pallet included the Rucker framework. Heterogeneity was and thus potential pleiotropy was first assessed in the IVW effect estimate using Cochran's Q statistic. A P value of <0.05 was considered as an indication of at least balanced horizontal pleiotropy, indicating the IVW random effects model to be preferred over a fixed effects model. heterogeneity and, as a consequence, of pleiotropy. Next, the MR-Egger test was performed(78). The MR-Egger relies on different assumptions than the IVW method, as it does not assume all instruments to be valid by allowing for a non-

zero intercept. The intercept therefore represents unbalanced horizontal pleiotropy. The MR-Egger intercept, the standard error and p-value were therefore calculated. A MR-Egger intercept P value of <0.05 was considered to be an indicator of unbalanced horizontal pleiotropy. Heterogeneity was assessed within the MR-Egger by calculating Rucker's Q. A significant difference between these two heterogeneity statistics (Q-Q', $P < 0.05$) was considered indicative of unbalanced horizontal pleiotropy as well and thus the MR-Egger test to be the preferred method over the IVW. I^2_{GX} was calculated to assess weak-instrument bias within MR-Egger estimates(79). An I^2_{GX} of $>95\%$ was considered to indicate a low risk of weak-instrument bias within the MR-Egger test. In addition, MR-Lasso(80) and MR-PRESSO(81)(the latter when 4 or more SNPs were available) were performed, two tests that are robust to outliers due horizontal pleiotropy when the SNPs used are valid instrumental variables. Lastly, weighted median(82) and weighed mode(83) effect estimates were calculated. These methods allow for estimation of causal effect estimates when the majority ($>50\%$) or the plurality (no larger subset of invalid instruments estimating the same causal parameter) of SNPs are valid instruments.

Under scenario b) and c), we additionally performed an "unweighted" MR by calculated an inverse variance weighted fixed and random effects estimate after equally weighting all genetic instruments (i.e. negative and positive beta's were set to -1 and 1 respectively) and were assumed to be estimated with infinite precision (i.e. the stand error was set to 0). This was performed considering that relative abundance might still reflect different biological implications between studies/populations. We recalculated Cochran's Q using these fixed weights to differentiate between the fixed and random effects model.

MR analyses were performed using R (version 3.6.3), the TwoSampleMR package 0.5.3(84), MR-PRESSO(81) (version 1.0), and the source code for MR-Lasso(80) in a Two-Sample MR setting as proposed in the article of Rees *et al.*

Conditional analyses on the Methano- and Bifidobacterium

To investigate whether estimated effects of the Methano- and Bifidobacterium on associated outcomes were consistent when considering a community effect of the combined pool of gut bacteria, we performed additional sensitivity analyses. First, we took forward SNPs containing a) taxonomic information up to family level and b) effect sizes depicting relative abundance, totalling to 136 SNP. After clumping on family level, 32 unweighted GRS for 32 different bacterial families were constructed. These families included the Acidaminococcaceae, Bacteroidaceae, Barnesiellaceae, Bifidobacteriaceae, Clostridiaceae, Coriobacteriaceae, Desulfovibrionaceae, Enterobacteriaceae, Erysipelotrichaceae, Eubacteriaceae, Firmicutes, Lachnospiraceae, Lactobacillaceae,

Leuconostocaceae, Marinilabiliaceae, Micrococcaceae, Mogibacteriaceae, Moraxellaceae, Odoribacteraceae, Paenibacillaceae, Pasteurellaceae, Peptococcaceae, Peptoniphilaceae, Porphyromonadaceae, Rhodospirillaceae, Rikenellaceae, Ruminococcaceae, Streptococcaceae, Synergistaceae, Veillonellaceae, Victivallaceae and the Bifidobacteriaceae/Methanobacteriaceae family (for the association of the Methanobacteriaceae/Bifidobacteriaceae respectively). We opted for an unweighted GRS based on the same rationale as the choice for performing an “unweighted” MR as sensitivity analysis. These were subsequently used as covariates in the SNP (rs4548017 and rs1446585 for the Methano- and Bifidobacteriaceae, respectively)-outcome association in the UK Biobank.

Look-ups

We performed a look-up in MR-Base, a platform for Mendelian randomization analysis, to explore whether the SNPs would be associated with other traits in order to gain insights in potential pleiotropic effects (84). Furthermore, we consulted GeneCards for extra information about the genes related to the analysed SNPs (85).

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All authors have completed and submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest and none were reported. Author NV is an employee of Genomics plc.

Availability of data and material (data transparency)

Data is available on request.

Authors' contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Hilde E Groot and Yordi J van de Vegte. The first draft of the manuscript was written by Hilde E Groot and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY TABLES

<https://bit.ly/3lihMgU>

Chapter 8

MAPPING THE IMMUNE RESPONSE AFTER MYOCARDIAL INFARCTION USING SINGLE-CELL RNA SEQUENCING

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ABSTRACT

The immune response after myocardial infarction (MI) plays pivotal role in both the acute and chronic phase to initiate cardiac repair and is nowadays considered as a therapeutic target. It is known that a delicate, time-dependent balance exists between the beneficial and harmful effects of the immune system on outcome post-MI. However, to better understand the complexity of the immune system in MI, more detailed longitudinal knowledge is required. To gain this in-depth insight, the role of individual immune cells and their interactions need to be dissected. Single-cell RNA-sequencing (scRNA-seq) provides this resolution by unbiasedly profiling the gene expression pattern of millions of individual immune cells. Here, we generated scRNA-seq data of ~60,000 peripheral blood mononuclear cells from 24 MI patients, collected at three time points (hospital admission, 24 hours after MI, 6-8 weeks after MI). This data was used to study how cell type composition and gene expression levels in 6 major immune cell types (CD4⁺ T cells, CD8⁺ T cells, monocytes, natural killer cells, dendritic cells, and B cells) were changed during and after the course of a MI. We observed suggestive longitudinal quantitative changes in monocytes and CD8⁺ T cells. Next, we observed 74 genes to be differentially expressed over time after a MI, of which the majority was detected in dendritic cells. *S100A8* and *S100A9*, genes involved in neutrophil chemotaxis and aggregation, were shortly upregulated (the first 24 hours post-MI). Other inflammatory related genes remained upregulated in the chronic phase after MI compared to time of hospital admission, where *IL1B* appeared to be centrally placed. This study is a proof of concept to study more extensively the immunological pathways during and after MI, eventually leading to new therapeutic targets.

INTRODUCTION

Myocardial infarction (MI) represents one of the most important causes of mortality and morbidity globally. Our knowledge of the immune system in MI has increased over the past decade, which has led to new therapeutics (i.e. CANTOS trial using the anti-interleukin-1 β antibody canakinumab on cardiovascular disease outcomes[1]). In addition, it is known that an adequate inflammatory response is required for the healing process after MI, but a prolonged response could exert adverse remodelling [2–6]. However, this delicate balance between beneficial and adverse effects has not been elucidated sufficiently yet [6]. Until recently, it was difficult to provide an unbiased comprehensive overview/full picture of the immune system and to identify specific immune cell subpopulations that may more closely identify specific inflammatory pathways underlying MI [7,8]. The state-of-the art technology single-cell RNA sequencing (scRNA-seq) enables to study large sets of cells simultaneously on single-cell level from an unbiased approach; by comprehensively mapping cell types, showing cell-type specific gene expression profiles, and creating gene regulatory networks[7,9]. So, sc-RNA seq may therefore pose an extremely valuable tool for broadening our knowledge of the mechanisms underlying MI. Several studies have shown that scRNA-seq can be applied to dissect the pathogenesis of cardiovascular disease with increased resolution[9,10]. For example, scRNA-seq has been applied to study the leukocyte diversity in atherosclerotic mice aortas, where the cellular repertoire was more diverse in the atherosclerotic aortas compared to healthy aortas [11]. Despite these early efforts, no studies have been conducted on MI in human subjects. To fill this gap, and understand how previous mouse data can be translated to the human situation, we applied scRNA-seq in peripheral blood mononuclear cells (PBMCs) of 24 MI patients, collected during the acute and chronic phase of MI. By comparing the cell type composition and cell type-specific gene expression changes over time, this study increased the resolution by which the involvement of the immune system in MI has been dissected.

METHODS

Study population

All patients admitted to the University Medical Center Groningen (UMCG) between January 1st and December 31st 2018 via the ST-elevation MI (STEMI) protocol were considered eligible for the study. Inclusion criteria were adults that had a STEMI, a primary percutaneous intervention (PCI) with implantation of at least one stent with a diameter of at least 3 mm resulting in TIMI flow grade 2 or 3 post PCI, and which showed

less than 6 h symptoms before undergoing PCI. Major exclusion criteria were previous MI, medical history of diabetes, inflammatory disease or malignancies, medication affecting inflammation and clemastine or desloratadine use during intervention. Initially, all patients presenting with a STEMI at hospital admission were included in the study if they met the inclusion criteria, giving a total of 57 patients. Due to missing samples 24 hours and/or 6-8 weeks after MI (because of early transmission to other hospitals), 30 patients were excluded from the study. One patient was further excluded due to statin use which was not initially known upon presentation at hospital admission. Furthermore, two patients were excluded due to malignancy and/or diabetes. Finally, a total of 24 patients was eligible for the study. Informed consent was obtained for inclusion of all patients. **Figure 1** presents a flowchart of the study sample selection. The study was part of CardioLines, a single-center, observational biobank aimed to study potential factors related to success or failure of diagnosis and treatment both from a patient as well as from a medical perspective, and was approved by the ethics committee of the UMCG, document number METC UMCG 2012/296.

FIGURE 1. Flowchart for the selection of the analysed study sample from CardioLines.

Data collection

Upon admission, standard laboratory assessment was performed and standard physical examination parameters were measured according to protocol. During hospitalization, blood samples were collected at three distinct time intervals: at hospital admission before PCI (baseline), at 24 hours and 6-8 weeks after PCI. PBMCs were isolated from the blood samples for scRNA-seq and EDTA plasma was stored at -80°C for future Olink expression.

Isolation and preparation of PBMCs

PBMCs were collected and stored as previously reported [7]. For each patient, whole blood was collected in two 10 ml EDTA-vacutainer (BD) tubes prior to PCI. Within 2h, PBMCs were isolated using Cell Preparation Tubes with sodium heparin (BD). PBMCs were kept in RPMI1640 supplemented with 50 µg/mL gentamicin, 2 mM L-glutamine, and 1mM pyruvate. Isolated PBMCs were cryopreserved in RPMI1640 supplemented with 40% FCS and 10% DMSO and were stored in liquid nitrogen afterwards. Within one year, PBMCs were further processed for scRNA-seq. Cells were thawed in a 37 °C water bath until almost completely thawed, after which the cells were washed. After washing, cells were resuspended in medium and incubated for 1h in a 5° slant rack at 37 °C in a 5% CO₂ incubator. After resting, cells were washed twice in medium supplemented with 0.04% bovine serum albumin. Cells were counted using a hemocytometer and cell viability was assessed by Trypan Blue assay.

Single-cell library preparation and sequencing

9 time point-balanced pools of 8 patients each were prepared, aiming for a targeted recovery of 1,000 cells per patient. Single cells were captured with the 10xChromium controller (10xGenomics) according to the manufacturer's instructions (document CG00026) and as described earlier[12]. Each sample pool was loaded into a different lane of a 10xchip (Single Cell A Chip Kit, 120236). cDNA libraries were generated with the Single Cell 3' Library & Gel Bead kit version 2 (120237) and i7 Multiplex kit (120262) according to the company's guidelines. These libraries were sequenced using a custom program (27-9-150) on an Illumina NovoSeq6000 using a 150-bp paired-end kit, per BGI (Hong Kong) sequencing guidelines. In total, 66,521 cells were captured and sequenced to an average read depth of 46,122 per cell (ranging from 37,282 to 67,029 reads per cell).

QC and preprocessing of sequencing data

Cell Ranger v3.0.2 software with default settings was used to generate FASTQ files, align the sequencing reads to the hg19 reference genome, filter cell and UMI (unique

molecular identifier) barcodes, and count gene expression per cell. Genotyping was performed successfully for 24 samples, using the Illumina Infinium Global Screening Array-24 (hg19). Imputation was performed on Michigan Imputation Server (MIS) using Minimac3 pipeline. Pre-imputation QC-steps were performed on MIS pipeline, excluding SNPs containing alleles other than A, C, G or T, duplicate sites, indels, monomorphic sites, alleles with mismatches between reference panel (1000G phase 3-v5, hg19) and study data and SNPs with call rate < 90%. A total of 380,850 genotyped SNPs were included for imputation. SNPs were first phased using Eagle2 v2.3[13] and then imputed using Minimac v3 and 1000G phase 3-v5 (hg19) reference panel on MIS. Imputations yielded 47,109,600 SNPs unfiltered. Applying a Minor Allele Frequency (MAF) filter of $MAF > 0.01$ and an imputation quality filter of $R^2 > 0.3$, resulted in a remaining set of 9,399,490 variants.

Version 3.0 of the R package Seurat was used to read the UMI counts output from Cell Ranger [14]. First, all genes that were not detected in ≥ 3 cells were removed. The following cells were discarded: cells in which $\geq 10\%$ of the UMIs mapped to the mitochondrially encoded genes were discarded [15], cells expressing ≤ 200 genes, cells expressing ≥ 10 UMIs of the HBB gene, and all cells that were marked as doublets or inconclusive by the SoupCell method [16] (**Supplementary Table 1**). Clusters output by SoupCell were correlated to the genotypes collected for the patients and the cells in the clusters were assigned to the best matching patient. The combination of the 10X lane and the participant assignment then allowed cells to be assigned their condition. After QC filtering, a total of 60,029 cells remained for analysis.

Cell type classification

The gene expression count data was normalized using SCTransform method[17]. Principal Component analysis was run on the normalized gene expression data and the first 30 principal components were used for cell clustering using Seurat's FindNeighbors (dims = 1:30) and FindCluster function (default parameters, resolution 1.0). A UMAP plot was used to visualize the clustering in 2D space. Based on known marker genes and differentially expressed genes per cluster (found using Seurat's FindAllMarkers function), we could assign 15 cell types to the clusters, including some smaller cell subtypes (**Table 1** (<https://bit.ly/3gpwj6z>), **Figure 2**).

FIGURE 2. UMAP of cell type clusters.

Statistical analysis

Discrete variables were represented as frequencies and percentages. Continuous variables with a normal distribution were summarized as mean $\pm$ standard deviation and if skewed were represented as median with inter-quartile range. The Friedman test was used to study the difference in cell composition over time. For differential expression analysis, log normalization was performed on the UMI-collapsed gene expression for each barcode, after which the library was scaled to 10,000 reads per cell. MAST was used to identify differential expression changes over time for each major cell type [18]. We considered an adjusted P value <0.05 statistically significant. To correct for multiple

testing we applied Bonferroni correction. Statistical analysis was performed using Stata version 15.0 (StataCorp) or R. Figures were created using GraphPad Prism version 8 and R. We used the STRING database for several network and pathway analyses (including Reactome and Gene Ontology) [19]. STRING is a database of known and predicted protein-protein interactions and include direct (physical) and indirect (functional) interactions.

RESULTS

Population characteristics

Baseline characteristics of the patients are presented in **Table 2**. The average age was 60 ± 10 years and one fifth of the participants (21%) was female. The median BMI was 26.6 (IQR 24.3-28.7) kg/m^2 . Patients most frequently presented with one stenosed vessel, and the degree of stenosis was 100% in 13 out of 24 patients (54%).

Cell type composition in myocardial infarction

After clustering, we could assign 6 major cell types, that could be further subdivided into 15 finer cell types (**Figure 2**). To perform a robust cell type composition analysis, we only focussed on the major cell types (that consisted of at least 1.0% of the total PBMC population in each of the time points: CD4⁺ T cells, CD8⁺ T cells, monocytes, natural killer (NK) cells, dendritic cells (DCs), and B cells. The cells mostly present at baseline were CD4⁺ T cells (32.4%), monocytes (28.9%), and CD8⁺ T cells (19.4%). Studying the cell composition over time, we observed an increase in percentage of CD8⁺ T cells over time ($P = 0.021$). Monocytes increased at 24 hours after STEMI and decreased at 8 weeks after STEMI ($P = 0.014$) (**Figure 3**). After Bonferroni correction (0.05/6), the differences over time did not remain significant. The absolute and relative numbers of cells per patient are provided in **Table 1**.

Gene expression profiles – Time course MI

Using differential expression analysis in MAST, we detected 22 unique genes in bulk-like analysis, compared to 74 unique genes studying gene expression over time per cell type in five out six PBMC clusters, i.e. all except CD4⁺ T cells (Bonferroni corrected P value $[0.05/18] = 2.78 \cdot 10^{-3}$) (**Figure 4**). The nine genes that were overlapping included *LST1*, *FOS*, *S100A4*, *S100A12*, *FTH1*, *CYP1B1*, *S100A9*, *S100A8*, and *LYZ*. Network analysis of these genes showed a significant protein-protein interaction (PPI) enrichment (P value $3.57 \cdot 10^{-6}$) (**Supplementary Figure 1A**). Significantly associated Reactome pathways were amongst others 'Toll-like receptor cascades', 'Neutrophil degranulation', and 'Innate Immune System'.

TABLE 2. Baseline characteristics

Characteristics	Total (n=24)
Age, mean (SD), years	60 (10)
Female sex – no. (%)	5 (21%)
Height, median (IQR), cm	175 (172, 180)
Weight, median (IQR), kg	82 (72, 91)
Blood pressure, mmHg	
Systolic, mean (SD)	127 (17)
Diastolic, mean (SD)	81 (14)
Heart rate, mean (SD), bpm	78 (17)
Hypertension, no. (%)	1 (4%)
Hyperlipidemia, no. (%)	2 (8%)
BMI, median (IQR), kg/m ²	26.6 (24.3, 28.7)
Location of STEMI, no. (%)	
Anterior	11 (46)
Lateral	8 (33)
Inferior	11 (46)
Posterior	6 (25)
No of stenosed vessels, no. (%)	
1	14 (58)
2	6 (25)
3	4 (17)
Degree of stenosis, no. (%)	
70-89%	2 (8)
90-99%	9 (38)
100%	13 (54)
Total ischemic time, median (IQR), min	128 (110, 175)
Infarct-related artery TIMI flow – no. (%)	
Pre-intervention grade	
0	12 (52)
1	1 (4)
2	2 (8)
3	8 (35)
Post-intervention grade	
0	1 (4)

TABLE 2. Continued.

Characteristics	Total (n=24)
2	1 (4)
3	22 (92)
Laboratory values at admission	
CK, median (IQR), U/L	128 (110, 195)
Myocardial band of CK, median (IQR), U/L	17 (14, 34)
Troponin T, median (IQR), ng/L	73 (25, 179)
Creatinine, mean (SD), $\mu\text{mol/L}$	77 (15)
NT-proBNP, median (IQR), ng/L	108 (32, 467)
Glucose, median (IQR), mmol/L	8 (7, 9)
HbA1c, mean (SD), %	9 (1)
MCV, mean (SD), fL	88 (4)
C-reactive protein, median (IQR), mg/L	2 (1, 3)
Blood count at admission	
Leukocytes, median (IQR), 10^9 cells/L	9 (7, 14)
Neutrophils, median (IQR), 10^9 cells/L	6 (5, 10)
Lymphocytes, median (IQR), 10^9 cells/L	2 (1, 3)
Monocytes, median (IQR), 10^9 cells/L	0.6 (0.5, 0.8)
Eosinophils, median (IQR), 10^9 cells/L	0.1 (0.1, 0.2)
Basophils, median (IQR), 10^9 cells/L	0.05 (0, 0.1)
Undif. Granulocytes, median (IQR), 10^9 cells/L	0.05 (0.03, 0.11)
Thrombocytes, mean (SD), 10^9 cells/L	252 (67)

Data is expressed as number (%), as mean $\pm$ standard deviation (SD) for continuous variables with normal distributions and as median with inter-quartile range (IQR) for continuous variables with a skew distribution. BMI: Body Mass Index, STEMI: ST-Elevation Myocardial Infarction, TIMI: Thrombosis In Myocardial Infarction, CK: Creatine Kinase, NT-proBNP: N-terminal pro brain natriuretic peptide, HbA1c: Glycated hemoglobin, MCV: Mean corpuscular volume.

We observed 125 significant differences in cell type-specific gene expression (in 74 unique genes); 2 in CD8⁺ T, 11 in NK cells, 13 in B cells, 48 in monocytes, and 51 in DC. Some genes were differentially expressed in more than one cell type (*ACTB* and *S100A4* (DC, NK), *CCL4* and *MT2A* (monocyte, NK), *FOS* (B cell, DC, monocyte, NK), *FTH1* (CD8⁺ T, NK), *HLA-DRB1* (B cell, DC), and *IL1B* (DC, monocyte)).

FIGURE 3. PBMC composition over time.

Network analysis of all genes, that were upregulated at 24 h after MI compared to baseline, resulted in a network with a PPI enrichment P value of $4.77 \cdot 10^{-12}$ (**Supplementary Figure 1B**) [19]. The most significantly associated Gene Ontology (GO) biological processes were '(Positive regulation of) immune system process', and 'Immune response-regulating signalling pathway'. Network analysis of all genes, that were upregulated at 8 weeks after MI compared to baseline, showed a PPI enrichment of $P < 1.00 \cdot 10^{-16}$ (**Supplementary Figure 1C**). Significantly associated Reactome pathways were 'Interleukin-10 signaling', 'Signaling by interleukins', and 'Cytokine signaling in immune system'. Network analysis of all genes, that were upregulated at 8 weeks after MI compared to 24 hours after MI, showed also PPI enrichment of $P < 1.00 \cdot 10^{-16}$ (**Supplementary Figure 1D**). Interestingly, the gene *IL1B* seemed to have a central role in the upregulated gene networks after MI (**Supplementary Figure 1B-D**). Extensive information on the genes included in the network analyses can be found in **Supplementary Table 2**. Network analyses

of all genes, that were downregulated at 24 hours or 8 weeks after MI compared to baseline, did not result in an enriched PPI network (both PPI enrichment P value = 0.04). Interestingly, the genes *S100A9*, *S100A12*, *S100A8*, and *CYP1B1* were downregulated at 8 weeks compared to 24 hours (PPI enrichment P value = $1.99 \cdot 10^{-5}$). This longitudinal difference stresses the importance to take a specific time window into account and supports previous research where short-term blockade of *S100A9* post-MI appeared to be beneficial for cardiac function (**Figure 1E**)[20]. Most significantly associated Gene Ontology (GO) biological processes in this case were 'Neutrophil aggregation' and 'Neutrophil chemotaxis'. **Supplementary Table 3** provides an overview on the cell type-specific and bulk differential expression.

FIGURE 4. Venn diagram of identified genes using bulk and cell type specific analysis with MAST.

DISCUSSION

In this proof of concept study, we used an unbiased approach to study gene expression profiles of circulating immune cells at single-cell level during the acute and chronic phase of MI. First, we identified 15 different cell types. These 15 cell types included six main cell types: B cells, CD4⁺ T cells, CD8⁺ T cells, DCs, monocytes, and NK cells. The other cells were subsets of these main types, megakaryocytes, hematopoietic stem cells, or Th17 cells. Studying cell type composition over time we observed suggestive changes in monocytes and CD8⁺ T cells, although this did not remain after correction for multiple testing. Second inflammatory related genes remain upregulated in the chronic phase after MI compared to time of hospital admission (baseline). Third, a difference in upregulation of specific genes might exist in the time span between baseline and

24 hours after MI compared to the time span between 24 hours and 8 weeks after MI. Although preliminary, this study might aid to gain new insights into the immunological pathways during and after MI and to provide new clues for future therapeutic targets.

The complexity of the immunological pathways underlying myocardial infarction and the translational failures from bench to bedside until now have stimulated us to further study these processes as precisely as possible[5]. scRNA-seq has opened a wide range of opportunities to gain more knowledge in the cardiovascular field and numerous groups are currently using this technique[21]. For example, scRNA-seq has been applied in the setting of a murine nonischemic, pressure-overload heart failure model[22]. Here, the authors were able to show immune activation occurred across a wide range of immune cell types. Of interest, oncostatin M was upregulated in proinflammatory macrophages and PD-1 in regulatory T cells. Similar to the murine model, we also observed cell-type specific upregulation of particular genes. For example, *IL1B*, known as target in the CANTOS trial[1,23], was upregulated in DCs and monocytes at 24 hours after MI compared to hospital admission and remained upregulated at 8 weeks after MI. Furthermore, in our network analyses, *IL1B* appeared to be centrally placed in the protein-protein network, supporting previous clinical research and suggesting that this pathway is of value to further elucidate[1]. One could hypothesize that cell-type specific gene expression responses upon MI could aid in developing more precise therapies instead of a 'one size fits all'-approach and thereby decreasing the risk of potential side effects.

Intriguingly, most of the inflammatory related genes remained upregulated during the chronic phase after MI. This supports the hypothesis that the chronic inflammatory state after MI eventually contributes to the progression towards ischemic heart failure[5,24,25]. The genes *S100A8*, *S100A9*, *S100A12* were one of the few genes that were downregulated at 8 weeks compared to 24 hours after MI. These genes are known to be involved in neutrophil aggregation and chemotaxis, and it is known that neutrophils are attracted to the infarcted region of the heart in the first hours after MI[26,27]. Furthermore, the proteins *S100A8/S100A9* are involved in *IL1 β* pathway activation. Although speculative, our observation of upregulation in the acute phase could indicate that therapies focusing on *S100A8/S100A9* might only be successful when applied in the first 24 hours after MI[20,28].

Strengths and limitations

Current study provides valuable information concerning the gene expression profiles of circulating immune cells in MI patients. We were the first to use clinical samples of humans instead of animal models. The availability of longitudinal data enabled us to

gain valuable insights into both the acute and chronic immune response after a MI. This could be of importance, since we know a delicate balance exists between the beneficial and detrimental effects of the immune system during and after MI[5]. However, this study also had limitations. This study was limited by the lack of data from healthy controls. In the future we will study differences in gene expression between matched MI patients and healthy controls, using data from the LifeLines Deep cohort [7]. Furthermore, we will add data of circulating proteins (as products of the gene expression profiles). This could add more translatable properties to this study, since circulating proteins are often used as biomarkers in the clinic. Last, our sample size of 24 patients did not allow us to draw robust conclusions concerning rare cell types. Expanding our sample size would overcome this limitation in the future.

Future perspectives

The findings of this study can be used to specifically identify immunological pathways underlying MI and determine what targets could be considered as potential therapeutics in the future. Gene expression could be linked to clinical parameters, such as infarct size, left ventricular ejection fraction, but also re-infarction. This is relevant since it currently remains challenging to develop anti-inflammatory therapies for MI and ischemic heart failure. Additional data of circulating proteins are of value to proceed gaining more insights.

Conclusions

Longitudinal scRNA-seq data of MI patients aid in further unravelling the complex immune system. This enables to identify potential therapeutic targets for the treatment of MI.

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SUPPLEMENTARY MATERIALS

SUPPLEMENTARY FIGURE 1A. Network of genes that were overlapping in bulk and cell-type specific analysis. Edges between nodes represent associations. Light blue = from curated databases, pink = experimentally determined, light green = gene neighborhood, red = gene fusions, yellow = text mining, black = co-expression.

SUPPLEMENTARY FIGURE 1B. Network of genes that were significantly upregulated 24 hours vs. baseline. Edges between nodes represent associations. Light blue = from curated databases, pink = experimentally determined, light green = gene neighborhood, red = gene fusions, yellow = text mining, black = co-expression.

SUPPLEMENTARY FIGURE 1C. Network of genes that were significantly upregulated 8 weeks vs. baseline. Edges between nodes represent associations. Light blue = from curated databases, pink = experimentally determined, light green = gene neighborhood, red = gene fusions, yellow = text mining, black = co-expression.

SUPPLEMENTARY FIGURE 1D. Network of genes that were significantly upregulated 8 weeks vs. 24 hours. Edges between nodes represent associations. Light blue = from curated databases, pink = experimentally determined, light green = gene neighborhood, red = gene fusions, yellow = text mining, black = co-expression.

SUPPLEMENTARY FIGURE 1E. Network of genes that were significantly downregulated 8 weeks vs. 24 hours. Edges between nodes represent associations. Light blue = from curated databases, pink = experimentally determined, light green = gene neighborhood, red = gene fusions, yellow = text mining, black = co-expression.

SUPPLEMENTARY TABLE 1. Overview of quality check steps prior to cell type classification. Eventually, 60,029 cells were used for sc-RNA seq analysis.

	N of cells	% of total
Start	66,521	100
Mitochondrial leakage	2,328	3.5
≤ 200 genes expressed	399	0.6
≥ 10 UMIs of HBB gene	81	0.1
Doublets or inconclusive	3,684	5.5
Total	60,029	90.2

UMI = unique molecular identifier

Supplementary Table 2. Differentially expressed genes.

<https://bit.ly/3lg3e1g>

Supplementary Table 3. Cell type specific differential expression.

<https://bit.ly/2QpJLwK>

Chapter 9

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

GENERAL DISCUSSION

In this thesis, we investigated the role of inflammation in patients suffering from ischemic heart disease (IHD).

Inflammation in IHD:

- is involved in various phases of the disease
- can be further unraveled using single cell RNA sequencing (sc-RNA seq)
- might be influenced by the gut microbiome

In the first part of this thesis, we obtained a general overview of leukocyte profiles across the cardiovascular disease (CVD) continuum and provided an update on the therapeutic opportunities of cytokine inhibition in acute myocardial infarction and chronic heart failure. In the second part, we studied the association between the inflammatory markers hs-CRP and IL-6 with cardiac function directly after PCI and during 4 months follow up. In the third part, we explored the human gut microbiome and genetic profiles of circulating immune cells as potential therapeutic targets in the treatment of IHD.

Part I. Inflammation in ischemic heart disease

In **Chapter 2** of this thesis, we studied leukocyte profiles across different stadia of the CVD continuum, including (non-obstructive) coronary artery disease (CAD), myocardial infarction (MI), and ischemic heart failure (iHF). We first investigated whether leukocyte profiles changed during the CVD continuum, and found that the overall leukocyte count increased across the continuum. In addition, we found higher leukocyte counts at baseline were predictive for CVD progression during follow-up. The increase in leukocyte counts mainly depended on the increase in neutrophil count. This may indicate that neutrophils alone are accountable for more vascular damage compared to other inflammatory cells (1,2). We also investigated potential sex differences in leukocyte profiles and found women had overall higher leukocyte levels. There was also an interaction between the CVD continuum and sex on lymphocytes. Lymphocytes increased more in women than in men across the CVD continuum, suggesting a sex specific component in leukocyte profiles across the CVD continuum. Although we characterized the leukocyte profiles in different CVD stages, the investigation of cell-specific up- or downregulation of genes involved in the inflammatory response during and after MI may provide additional insights into the role of the immune system in the CVD continuum. This remains to be elucidated in future research.

We observed an association between increased leukocytes and development of CAD in individuals who were healthy at baseline and developed CAD during follow-up. Our observational study is complementary to earlier results of the European Prospective Investigation into Cancer in Norfolk (EPIC-Norfolk) study, which showed associations between increased leukocyte counts and higher risks of CAD and overall CVD. Together, these studies provide further support for the notion that early intervention in the inflammatory pathway is of importance to gain as much effect as possible in the prevention/treatment of CVD(3,4). Although speculative, leukocyte levels might be considered as an additional biomarker on top of classical risk stratification scores to aid decision making on when to start with preventive therapy for CAD (i.e. statins or future anti-inflammatory therapy) in individuals who would not yet receive this according to the current guidelines. The differences in leukocyte profiles between men and women remain to be further elucidated. Understanding which immune cells are key players in various stadia of the CVD continuum in men and women could serve as a starting point for the identification of new potential therapeutic targets in CVD.

Chapter 3 provides a contemporary and translational overview of the potential effects of cytokine inhibition on cardiac function and outcome in the setting of acute MI and chronic HF in experimental models and clinical studies. In a wide range of experimental animal models of MI and in various HF models, cytokine inhibition has shown promising results. Unfortunately, larger clinical trials have failed to show an improvement on cardiac function and outcome, except the Canakinumab Anti-Inflammatory Thrombosis Outcomes Study (CANTOS), which reported fewer cardiovascular events in MI patients treated with the monoclonal antibody, targeting interleukin-1 β , canakinumab (5). Ambivalence of cytokine function, differences in study designs, species, treatment regimens, and chosen endpoints appear to hamper the successful translation of experimental research into clinical practice. For example, treatment intensity and treatment periods should be carefully chosen, while monitoring cytokines before, during and after treatment periods. Due to the complexity of the immune system it remains challenging to gain a complete overview of the consequences that follow upon an intervention in the majority of studies. Nevertheless, the inflammatory field remains promising in terms of finding new therapeutic targets for CVD.

Part II. Inflammation and cardiac function in myocardial infarction

It is generally appreciated that inflammation plays a substantial role in the pathophysiological process or cardiac remodeling after MI(6). However, sparse data are available about the time course of inflammatory markers after MI and their association with reperfusion success, infarct size, and cardiac function. In part II, hs-CRP and

interleukin 6 are studied in patients of the GIPS III cohort, since genetic and clinical evidence suggests causality for the IL-6R signaling pathway on both MI and cardiac remodeling (5,7,8).

Chapter 4 reports the temporal course of hs-CRP levels in patients who presented with a first ST-elevation MI (STEMI) and were treated with primary percutaneous coronary intervention (PCI). We found higher hs-CRP levels at presentation to be associated with decreased reperfusion success. These levels remained higher in patients with impaired reperfusion for up to two months after PCI. Furthermore, hs-CRP at baseline was positively correlated with ischemia time and together with TIMI flow an independent predictor for myocardial reperfusion. Last, there was no association observed between hs-CRP levels and impaired LV function and/or long-term outcomes. It is unknown why hs-CRP levels continue to remain increased for a substantially longer period in patients with impaired reperfusion compared to normal reperfusion. This may be a consequence of permanent damage of the heart microvasculature and the inability to recover quickly due to impaired reperfusion, which causes the liver to continue producing hs-CRP for a longer period. In addition to increased hs-CRP levels in patients with impaired reperfusion, we observed increased levels of leucocytes, neutrophils, and their ratio in these patients, which suggest an overall higher inflammatory state amongst patients with impaired reperfusion after STEMI.

As hs-CRP is just one marker in the complex inflammation network, it is necessary to investigate participating biomarkers in the inflammatory cascade to gain a more complete understanding of the role and characteristics of inflammation in the course of an acute STEMI. Therefore, we studied the role of cytokine interleukin 6 (IL-6) in MI. First, we studied the association of the soluble IL-6 receptor (sIL-6R) with myocardial reperfusion after PCI for acute STEMI in **chapter 5**. We observed biphasic fluctuations of sIL-6R levels during two weeks after STEMI, which were more pronounced in patients with impaired reperfusion compared to patients with normal reperfusion. Earlier research reported that cytokine gene expression, after an initial drop, may re-increase in the case of ongoing myocardial stress factors or with large infarctions, causing a second wave of cytokine activation (9). Although we did not establish which factors are causally involved, our finding that sIL-6R levels fluctuate more in patients with worse reperfusion after myocardial infarction lend support to this finding. **Chapter 6** showed that IL-6 levels were associated with cardiac function after STEMI. IL-6 levels were increased during the first two weeks, after which they reached a steady state. Furthermore, sIL-6R and sgp130 levels were decreased during the first two weeks, after which they also reached a stable level, similar to IL-6. We found IL-6 and the sIL-6R/IL-6 ratio at 24 hours after STEMI were associated with infarct size and cardiac function measured

at four months. sIL-6R and gp130 alone were not associated with these outcomes. Although it remains challenging to distinguish the cause and consequence in these associations, these results provide support for the concept of early intervention in the inflammatory cascade to reduce the myocardial damage as much as possible. Some insight into whether such early interventions may be beneficial may be provided by the "ASSessing the effect of Anti-IL-6 treatment in MI (ASSAIL-MI) trial" (NCT03004703), which examines whether IL-6 inhibition by tocilizumab can increase myocardial salvage in STEMI patients (10). This trial is currently ongoing, although the results are expected to become available this year.

Part III. Newest insights into ischemic heart disease – new therapeutic targets?

Part I and **II** of this thesis support the evidence for the involvement of the immune system, and in particular IL-6, in IHD. Nevertheless, the complexity of the immune system makes it challenging to identify new therapeutic targets (11,12). **Part III** of this thesis focuses on the newest insights into IHD and inflammation. Accumulating evidence suggest that the human gut microbiome plays an important role in the pathophysiology of MI through the modulation of the immune response related to the event (13,14). The gut microbiome can be considered as a virtual endocrine organ that communicates with distal organs. This occurs through the interaction of signaling molecules (lipopolysaccharides) on gut bacteria, with pattern recognition receptors on host mucosal surface cells, causing numerous downstream effects(15). Previous research demonstrated that a core microbiome exists, but that deviations from this core are associated with different physiological states (obese vs. lean) (16). However, deviations of the core microbiome have not been directly assessed in patients presenting with MI. It has been shown that gut metabolites such as trimethylamine N-oxide (TMAO) can act as danger-associated molecular patterns (DAMPs), which can activate inflammatory pathways that eventually lead to atherosclerosis (17). In addition, TMAO production has been associated with increased platelet reactivity and thrombosis potential [19,20]. Furthermore, gut microbiota might trigger plaque rupture by causing a harmful inflammatory response. However, to obtain an optimal understanding of the associations between the gut microbiome and the immune response in patients with MI, a systems based approach including multiple levels of data measured during the course of MI is necessary. (11,20).

In **chapter 7**, we explored associations between known genetic determinants (single nucleotide polymorphisms [SNPs]) of the human gut microbiome and the presence or development of health (absence of disease, vital and blood biomarkers and food intake) and disease states in the UK Biobank. Eleven SNPs were significantly associated with

28 outcomes including food intake, hypertension, atopy, chronic obstructive pulmonary disease, body mass index, and lipids. Mendelian randomization analyses using a multiple instrument approach pointed to a possible causal link between genetically determined higher levels of *Ruminococcus flavefaciens* and hypertension, but also between higher levels of *Clostridium* and platelet count. Although 27 out of 28 associations were indicated to be causal using a single SNP approach, only three of these associations were consistent in effect across some, but not all, sensitivity analyses. Our findings highlight diseases that have been investigated in previous observational studies(21–23). However, contrary to earlier observational research on the human gut microbiome and CVD, we found no associations with CAD or MI in our phenome-wide scan using the microbiome associated SNPs(13). While this might be due to a lack of study power or a small effect size of the evaluated genetic variants, associations with pathways via which the human gut microbiome may influence the development of CAD or MI (i.e. inflammation, arterial blood pressure, and circulating lipoproteins) were observed in our study. As these factors are strongly related to CVD development, it is interesting to further unravel their effect, which is influenced by the gut microbiome, in the long-term. In conclusion, gut microbiota and their metabolites may be of importance in the interplay between overlapping pathophysiological processes, and could serve as potential therapeutic targets for the prevention and treatment of diseases. Further research using more or stronger genetic variants associated with gut microbiota are needed to investigate the evidence for causal links with diseases.

In **chapter 8**, we studied gene expression levels of circulating immune cells from STEMI patients at an individual level. We generated scRNA-seq data of ~60,000 peripheral blood mononuclear cells from 24 MI patients, collected at three time points (hospital admission, 24 hours after MI, 6-8 weeks after MI). We used this data study how cell type composition and gene expression levels in 6 major immune cell types (CD4⁺ T cells, CD8⁺ T cells, monocytes, natural killer cells, dendritic cells, and B cells) were changed during and after the course of a MI. We observed suggestive longitudinal quantitative changes in monocytes and CD8⁺ T cells. In total, 74 genes were differentially expressed over time after a MI, of which the majority was detected in dendritic cells. Intriguingly, most of the inflammatory related genes remained upregulated during the chronic phase after MI. This supports the hypothesis that the chronic inflammatory state after MI eventually contributes to the progression towards ischemic heart failure (24–26). The genes S100A8, S100A9, S100A12 were one of the few genes that were downregulated at 8 weeks compared to 24 hours after MI. These genes are known to be involved in neutrophil aggregation and chemotaxis, and it is known that neutrophils are attracted to the infarcted region of the heart in the first hours after MI (27,28). Furthermore, the proteins S100A8/S100A9 are involved in IL1 β pathway activation. Although speculative,

our observation of upregulation in the acute phase could indicate that therapies focusing on S100A8/S100A9 might only be successful when applied in the first 24 hours after MI (29,30). In conclusion, this study can be considered as a proof of concept to study more extensively the immunological pathways during and after MI. Longitudinal scRNA-seq data of MI patients aid in further unraveling the complex immune system. This enables to identify novel potential therapeutic targets for the treatment of MI.

Future perspectives

This thesis supports the hypothesis that inflammation plays an important role across all stages of the CVD continuum. It is associated with a decrease in cardiac function after MI, but adequate anti-inflammatory therapies that may aid in reducing this decline remain to be developed or to be proven to be effective (10). It is therefore essential to further unravel the complex pathophysiological inflammatory mechanisms underlying MI. New techniques such as sc-RNA-seq, can help us to further study these complex mechanisms. However, because sc-RNA-seq is a relatively costly technique, collaboration on the generation of sc-RNA-seq data is necessary. This will be possible through, for example, the introduction of the single-cell eQTLGen consortium, which is a large-scale, international collaborative effort that has been set up to identify the upstream interactors and downstream consequences of disease-related genetic variants in individual immune cell types(31). Similar initiatives have been launched in the human gut microbiome field with the MiBioGen consortium (32). Although the interplay of the immune system and the human gut microbiome in MI is a relatively young field of research, a better understanding of these two may aid in the improvement of risk stratification, therapeutic treatment efficacy, and prognosis of MI. For example, the gut microbiome could be used as a biomarker in risk stratification for MI, eventually aiding in the decision when to start with preventive treatment in high risk individuals. The LifeLines Deep cohort has gathered gut microbiome data at baseline and has 10 years of follow-up data at the moment of writing, allowing it to be used as a validation cohort to test whether individuals with a specific change in microbiome suffer from MI more often compared to others. Furthermore, studies have already shown that incorporating microbiome data may aid in modifying cardiometabolic risks. Segal and colleagues demonstrated in 800 volunteers that using personal and microbiome features in a machine-learning algorithm enabled accurate prediction of postprandial glycemic response (33). A blinded randomized controlled dietary intervention based on this algorithm subsequently resulted in significantly lower postprandial responses, suggesting that personalized diets, based on microbiome data, may successfully modify elevated postprandial blood glucose and its metabolic cardiovascular consequences. Identification of new specific targets will enable us to develop targeted therapies,

modifying the composition of the gut microbiome (i.e. antibiotics, fecal transplantation). Over 50 drugs have already been shown to be metabolized by the gut microbiome. Gut microbiota can directly and indirectly influence drug response either by interfering with drug pharmacokinetics or pharmacodynamics (34). As Tuteja and colleagues stated: *"Comprehensive deep phenotyping studies are required to understand the directionality and complex relationship between the host, the microbiome, and medications. A deeper understanding of the molecular mechanisms by which the gut microbiome contributes to CVD risk and drug response will enable us to improve outcomes for patients with CVD and move toward microbiome-informed precision medicine."* It is clear that we are just at the start of a new era with the integration of multiple layers of information, including genomics, transcriptomics, and microbiomics, to gain a better understanding of the pathophysiology of MI. This thesis modestly contributes to this new era of data by supporting the need for data integration and starting the collection of data in multiple layers in IHD patients for future research.

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Appendices

DUTCH SUMMARY
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DUTCH SUMMARY | NEDERLANDSE SAMENVATTING

Inleiding

Hart- en vaatziekten zijn momenteel één van de grootste doodsoorzaken wereldwijd en zijn verantwoordelijk voor 31% van de sterfgevallen per jaar. Dit gaat om 17,8 miljoen mensen^(1,2). De helft van deze groep overlijdt ten gevolge van een ischemische hartziekte, waarbij de kransvaten rond het hart door aderverkalking vernauwen, hierna ook kunnen verstoppert en zorgen voor zuurstoftekort voor de hartspier (ischemie). Dit kan leiden tot een hartinfarct en uiteindelijk ook tot overlijden. Hoewel de mortaliteitscijfers sinds 1980 gedaald zijn, blijft de ziektelast ten gevolge van ischemische hartziekten hoog. Dit gaat tevens gepaard met hoge zorgkosten. Naast de huidige therapieën is het dan ook van belang te blijven zoeken naar nieuwe behandelstrategieën om klinische uitkomsten na een hartinfarct te verbeteren.

Hart- en vaatziekten worden gezien als een 'keten van opeenvolgende ziektebeelden', waarbij deze keten begint met de traditionele risicofactoren, zoals hypertensie, diabetes, roken, een positieve familiegeschiedenis⁽³⁾. Hierop volgend kan men aderverkalking ontwikkelen, hetgeen kan leiden tot pijn op de borst (stabiel coronairlijden) bij bijvoorbeeld inspanning, kou of stress. Wanneer deze aderverkalking zodanig ernstig wordt dat de kransvaten rond het hart geheel verstoppert, is er sprake van zuurstoftekort voor de hartspier, met een hartinfarct als gevolg. Een deel van de hartspier sterft tijdens een hartinfarct af en dit dode spierweefsel wordt vervangen door littekenweefsel. De pompfunctie van het hart kan hierdoor verminderen. Dit leidt uiteindelijk tot hartfalen. Deze opeenvolging van (ischemische) cardiovasculaire ziektebeelden wordt ook wel het cardiovasculaire ziektecontinuüm genoemd⁽³⁾.

Het is bekend dat inflammatie een belangrijke rol speelt gedurende dit gehele cardiovasculaire ziektecontinuüm. Het exacte effect is echter nog onbekend; zo kan inflammatie zowel beschermende als ook schadelijke effecten hebben in het continuüm. Ook is lang niet altijd bekend of inflammatie een oorzakelijk verband heeft met het continuüm of dat het juist een uiting is van de schade die al is geleden. Geleidelijk komt de wetenschap steeds meer te weten over de rol van inflammatie in het cardiovasculaire ziektecontinuüm. Zo heeft de CANTOS (Canakinumab ANti-inflammatory Thrombosis Outcome Study) studie het medicijn canakinumab onderzocht in patiënten met stabiel coronairlijden⁽⁴⁾. Canakinumab blokkeert het inflammatoire pad dat wordt gedreven door het ontstekingseiwit interleukine 1 β en zorgde in deze studie voor een lager aantal cardiovasculaire events. Ook zijn er aanwijzingen dat het ontstekingseiwit interleukine 6 (IL-6) een belangrijke rol speelt in de ontwikkeling van hart- en vaatziekten. Desondanks

is met de huidige onderzoekstechnieken, zoals flow cytometrie, de complexiteit van het immuunsysteem nog lang niet ontrafeld. Om daadwerkelijk nieuwe therapieën te kunnen ontwikkelen op het gebied van inflammatie is daarom extra onderzoek (met nieuwe technieken) van belang(5). Recentelijk is gebleken dat darmbacteriën inflammatoire reacties kunnen veroorzaken en zo mogelijk kunnen bijdragen aan het ontwikkelen van hart- en vaatziekten(6). Momenteel is op dit gebied veel onderzoek gaande. Concluderend speelt inflammatie een belangrijke rol bij de ontwikkeling van hart- en vaatziekten, maar is het van belang dit complexe systeem nog beter te begrijpen en daarmee ook het samenspel tussen darmbacteriën en inflammatie bij hart- en vaatziekten.

Doel van dit proefschrift

Het eerste doel van dit proefschrift is om inzichten te verkrijgen in de rol van het immuunsysteem in het cardiovasculaire ziektecontinuüm en de therapeutische mogelijkheden met betrekking tot inflammatie. Het tweede doel is om de associatie te bestuderen tussen inflammatoire markers en hartfunctie in patiënten met een acuut hartinfarct. Hierbij worden de ontstekingseiwitten C-reactief proteïne (CRP) en IL-6 bestudeerd. Tot slot wordt gekeken naar nieuwe potentiële aangrijpingspunten, zoals het microbiom. Tevens is het doel om de functie van het immuunsysteem bij hart- en vaatziekten verder te ontrafelen door middel van de techniek single cell RNA sequencing (scRNA-seq).

Deel I. Inflammatie bij ischemische hartziekten

In **deel I** is beschreven hoe de samenstelling van leukocyten, ook wel leukocytenprofielen genoemd, zich verhoudt tot de verschillende ziektebeelden van het cardiovasculaire ziektecontinuüm. Tevens is in de vorm van een review een overzicht gegeven van alle therapeutische mogelijkheden op inflammatoir gebied die recentelijk zijn onderzocht in dier- en mensstudies met betrekking tot een hartinfarct dan wel chronisch hartfalen. In **hoofdstuk 2** zijn de leukocytenprofielen van meer dan 300.000 deelnemers van het Engelse UK Biobank cohort bestudeerd. In dit hoofdstuk wordt beschreven dat het aantal leukocyten stijgt gedurende het cardiovasculaire ziektecontinuüm. Dit is vooral te zien bij de neutrofiele granulocyten. Tevens bleek dat het hebben van hoge leukocytenlevels in mensen zonder coronairlijden voorspellend was voor het ontwikkelen van coronairlijden gedurende de follow-up tijd van zes jaar. De verhoudingsgewijs grote toename in neutrofiele granulocyten zou erop kunnen wijzen dat voornamelijk deze cellen voor schade zorgen, gezien zij in het inflammatoire proces vaak als eerste aanwezig zijn op de plaats van schade(7,8). Echter, kwantiteit zegt niet alles, en het is dan ook van belang om in toekomstig onderzoek informatie te verkrijgen over de functie van de

cellen, door bijvoorbeeld individuele genexpressie te bestuderen. Het gegeven dat hoge leukocytenlevels in mensen zonder coronairlijden voorspellend zijn voor het ontwikkelen van coronairlijden de jaren erop, suggereert dat leukocytenlevels mogelijk als extra biomarker kunnen fungeren om bijvoorbeeld te bepalen wanneer te starten met cardiovasculaire primaire preventie. Men kan zich voorstellen dat mensen met een nog normaal cholesterol, maar al wel hoge inflammatoire parameters, baat hebben bij het eerder starten van primaire preventie dan mensen bij wie de inflammatoire parameters nog laag zijn(9,10). Voordat dit kan worden toegepast is hier natuurlijk verder onderzoek voor nodig, maar dit zou zeker iets kunnen zijn dat eenvoudig in de praktijk kan worden toegepast. In **hoofdstuk 3** wordt middels een review een overzicht gegeven van alle dier- en mensstudies (hartinfarct danwel chronisch hartfalen) die op het gebied van inflammatoire therapieën zijn gedaan de afgelopen jaren. Wat voornamelijk blijkt is dat dierstudies vaak veelbelovende resultaten laten zien, maar dat de translatie naar de mens zeer moeizaam gaat. De al eerder genoemde CANTOS studie is hier een uitzondering op(4). Het feit dat deze translatie zo lastig gaat, komt door meerdere aspecten. Allereerst is het immuunsysteem zeer complex en wordt vaak maar een klein deel van dit complexe systeem onderzocht. Hierdoor is lastig te bepalen wat up- of downstream effecten zijn van bepaalde therapieën. Tevens lijkt het tijdswindow waarin medicatie wordt getest, van belang. Inflammatie kan namelijk zowel beschermende als schadelijke effecten hebben, afhankelijk van het tijdstip (acuut vs. chronisch). Verder lijken eindpunten vaak te verschillen tussen studies, waardoor vergelijkingen moeilijk te maken zijn. Eenduidigheid zou hierbij kunnen helpen. Desalniettemin, lijkt het wel zinvol om inflammatie binnen het cardiovasculair ziektecontinuüm te blijven onderzoeken.

Deel II. Inflammatie en hartfunctie bij patiënten met een hartinfarct

In **deel II** wordt de associatie tussen de inflammatoire markers CRP en IL-6 en hartfunctie in patiënten met een hartinfarct beschreven. Het is bekend dat inflammatie betrokken is bij zowel het ontwikkelen van een hartinfarct als ook bij de verlittekening na een hartinfarct (met hartfalen als gevolg)(11). Er is echter nog weinig bekend over het beloop van deze markers na een hartinfarct en hun associatie met hartfunctie. In **hoofdstuk 4** is de associatie tussen het ontstekings eiwit CRP en hartfunctie na een hartinfarct onderzocht. Hierbij zagen we dat patiënten met een verminderde doorbloeding van het hart na het dotteren, hogere CRP-waardes in het bloed hielden. Dit bleef het geval tot twee maanden na het hartinfarct. Hogere CRP-waardes bij opname waren niet geassocieerd met een grotere infarctgrootte dan wel slechtere linkerventrikelfunctie gemeten met MRI op vier maanden. Een hypothese is dat deze wondgenezing langer duurt bij patiënten met een verminderde doorbloeding en dat ze daardoor hogere waarden houden. Het feit dat CRP niet geassocieerd was met infarctgrootte en

hartfunctie kan komen doordat CRP een vrij aspecifieke marker is. Gezien IL-6 hogerop in de inflammatoire cascade zit, is in **hoofdstukken 5 en 6** IL-6 bestudeerd. Hierbij is zowel IL-6 als ook de oplosbare IL-6 receptor en het eiwit sgp130 onderzocht. Sgp130 is namelijk in staat het IL-6/sIL-6R complex te neutraliseren, waardoor geen signalen van de ene op de andere cel worden doorgegeven. In **hoofdstuk 5** zagen we voornamelijk dat grotere fluctuaties in sIL-6R waarden geassocieerd waren met slechtere doorbloeding van het hart na infarct. Dit reflecteert mogelijk dat deze patiënten wellicht meer moeite hebben met het opnieuw vinden van een stabiel inflammatoir evenwicht, wat meer schade aan het hart zou kunnen weerspiegelen(12). In **hoofdstuk 6** bleek dat IL-6 levels een piek vertoonden op 24 uur na het hartinfarct en vanaf twee weken stabiele waarden hadden. sIL-6R en sgp130 waren juist verlaagd tijdens de eerste twee weken. Tevens waren hoge IL-6 levels op 24 uur na het hartinfarct geassocieerd met een groter infarct en slechtere hartfunctie gemeten op 4 maanden. Het blijft lastig om oorzaak en gevolg te onderscheiden. Wellicht dat de klinische trial "ASSessing the effect of Anti-IL-6 treatment in MI (ASSAIL-MI)" (NCT03004703) ons meer inzichten zal verschaffen. Deze studie onderzoekt of IL-6 blokkade middels het medicijn tocilizumab post-infarct schade kan beperken in STEMI patiënten(13).

Deel III. Nieuwste inzichten bij ischemische hartziekten – nieuwe therapeutische aangrijpingspunten?

Concluderend uit **deel I en deel II** zijn er velerlei aanwijzingen voor de betrokkenheid van het immuunsysteem bij ischemische hartziekten, zeker voor IL-6. Toch blijft het ingewikkeld om te verklaren waarom het immuunsysteem van verschillende mensen dan weer zo van elkaar kan verschillen en nieuwe therapiën te identificeren(14,15). Daarom ligt in **deel III** de focus op de nieuwste inzichten omtrent inflammatie en ischemische hartziekten. De laatste tijd zijn er steeds meer aanwijzingen dat er een relatie is tussen darmbacteriën en het risico op het krijgen van een hartinfarct(16,17). Men denkt dat dit als volgt werkt: bacteriën in de darmwand kunnen door 'signaalstoffen' uit te scheiden in het bloed cellen van het immuunsysteem activeren(18,19). Het immuunsysteem speelt op zijn beurt een belangrijke rol in het proces van slagaderverkalking, welke op termijn tot een hartinfarct leidt. Uit data van het Groninger cohort LifeLines DEEP (n = 1135) is gebleken dat de bacterie *Eubacterium* eligens verminderd aanwezig was in de darmen van mensen die een hartinfarct hadden doorgemaakt(20). Naar aanleiding hiervan hebben wij gekeken naar genetische varianten die reeds geassocieerd waren met een verandering in de samenstelling van darmbacteriën en hun associaties met ziekte. In **hoofdstuk 7** observeerden wij in het Engelse cohort UK Biobank, met 500.000 deelnemers, 11 genetische varianten die geassocieerd waren met 28 uitkomsten. De genetische variant, die geassocieerd was met een toename in de bacterie *Ruminococcus*

flavefaciens, was bijvoorbeeld geassocieerd met een verhoogde kans op hypertensie. Daarnaast was de variant, geassocieerd met een toename in de *Clostridium* bacterie, geassocieerd met een toename in bloedplaatjes. Hoewel associatief, lijken er wel aanwijzingen te zijn voor de betrokkenheid van darmbacteriën bij de ontwikkeling van verscheidene ziektebeelden die het risico op hart- en vaatziekten vergroten. Er is echter nog veel onderzoek nodig om bijvoorbeeld causaliteit aan te tonen.

Het is tot nu toe lastig gebleken de complexiteit van het immuunsysteem te ontrafelen. Dit komt onder andere doordat met bulkanalyse het vaak niet mogelijk is om subtypes binnen bepaalde cellen van elkaar te onderscheiden. Middels de techniek scRNA-seq is dit wel mogelijk(5,21). Daarom is deze nieuwe techniek toegepast in **hoofdstuk 8**, waarbij individuele genexpressieprofielen van circulerende immuuncellen in infarctpatiënten zijn bestudeerd. Middels deze data hebben we gekeken naar veranderingen binnen de samenstelling en genexpressieprofielen van zes typen immuuncellen (CD4⁺ T-cellen, CD8⁺ T-cellen, monocyt, natural killer cellen, dendritische cellen en B-cellen) tijdens en na een hartinfarct. Wat betreft celtype samenstelling viel op dat er longitudinale kwantitatieve verschillen waarneembaar waren bij de monocyt en CD8⁺ T-cellen. In totaal kwamen er 74 genen differentieel tot expressie, waarvan de meeste in dendritische cellen. Inflammatoire genen bleken opgereguleerd zowel in de acute fase als ook in de chronische fase na het hartinfarct; het gen *IL1β* leek in de 'pathway' analyses telkens centraal te worden geplaatst. De genen *S100A8*, *S100A9* en *S100A12* waren een van de weinige genen die alleen opgereguleerd waren tijdens de acute fase van het hartinfarct. Deze genen zijn betrokken bij aggregatie van neutrofiele granulocyten en het is bekend dat neutrofiele granulocyten worden aangetrokken tot de infarctlocatie in de eerste fase van het hartinfarct(22–26). De korte opregulatie van deze genen zou kunnen betekenen dat alleen vroeg ingrijpen op deze genen een effectieve therapie is na een hartinfarct. Kortom, deze studie kan worden beschouwd als een 'proof of concept' om immunologische cascades bij een hartinfarct nog gedetailleerder te kunnen onderzoeken, hetgeen de wetenschap helpt om uiteindelijk nieuwe therapieën te ontwikkelen.

Conclusie

In dit proefschrift wordt beschreven dat inflammatie bij ischemische hartziekten van belang is bij het gehele cardiovasculaire ziekte continuüm. Middels nieuwe technieken zoals scRNA-seq en nieuwe inzichten, zoals de betrokkenheid van darmbacteriën, biedt het inflammatoire vlak mogelijkheden voor de ontwikkeling van nieuwe therapieën om zo winst te behalen in de behandeling van ischemische hartziekten.

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ABOUT THE AUTHOR

Hilde Emmy Groot was born in Bloemendaal, the Netherlands, on the 19th of April 1991. She attended primary school in Bloemendaal, after which she moved with her parents and two brothers to Santpoort-Zuid. After finishing high school (Gymnasium diploma Natuur & Gezondheid, *cum laude*) at Het Stedelijk Gymnasium Haarlem, she moved to the University of Groningen to study Medicine. In 2009 she started her medical school and during her first years as a medical student she actively participated in the M.F.V. Panacea. In 2011 she was a board member of the M.F.V. Panacea. During her bachelor of Medicine, she also obtained her Bachelor Honours degree.

In 2013 Hilde started her Master in Medicine with her research internship at the department of Cardiology under supervision of professor P. van der Harst. Starting 2014 she performed her first year of medical rotations at the UMCG, Groningen. In 2015 she officially started her MD/PhD trajectory on the role of inflammatory biomarkers in ischemic heart disease. During that year, Hilde also attended the board of the Dutch National Student Orchestra (NSO). She continued her medical rotations in 2016 at Medisch Spectrum Twente, Enschede. Thereafter, Hilde visited the African Population and Health Research Center, Nairobi, Kenya, for a 2-month internship on cardiovascular disease in developing countries. This internship was under supervision of Catherine Kyobutungi (MBChB, MSc, PhD), Tilahun Haregu (PhD), and Caroline Wainaina (BSc, MPH). In May 2017, Hilde returned to Groningen, continued with her research and started her final clinical internship at the department of Cardiology at the UMCG under supervision of dr. P.P. van Geel. She obtained her MD-degree in December 2017 and continued with her research at the department of Cardiology. Her research in the field of inflammation and ischemic heart disease involved the initiation of the 'single-cell sequencing pilot', where she started a collaboration with the Genetics department (Prof. L. Franke and dr. M.P.G. van der Wijst) and the department of Gastroenterology (Prof. R. Weersma). This pilot is currently ongoing and will be proceeded by MD/PhD students I.V. van Blokland and F.M. Prins. Hilde presented her research at various national and international congresses including the Dutch Society for Cardiology congress (Papendal, 2017), the Dutch Vascular Forum congress (Utrecht, 2020), the European Society of Cardiology congress (Barcelona, 2014; Rome, 2016; Amsterdam, 2020) and the European Society of Cardiology Heart Failure congress (Vienna, 2018).

The completion of the MD/PhD-programme eventually resulted in this thesis entitled "Inflammatory biomarkers in ischemic heart disease", which she plans to defend on February 3rd 2021. In November 2020 Hilde started her clinical cardiology training. She is currently working at the department of Internal Medicine at the Martini Hospital Groningen, as part of her cardiology training.

LIST OF PUBLICATIONS

1. Almesned, M.A.M., Prins, F.M., Lipsic, E., Dullaart, R.P., **Groot, H.E.**, van der Harst, P. (2021). Trimethylamine N-oxide and its post-PCI trends in STEMI patients. Submitted.
2. Prins, F.M., Almesned, M.A.M., Lipsic, E., Dullaart, R.P., **Groot, H.E.**, van der Harst, P. (2021). Microbial metabolites in human plasma and their association with inflammation in patients with ST-elevated myocardial infarction. Submitted.
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4. Van Blokland, I.V., **Groot, H.E.**, Franke, L.H., van der Wijst, M.G.P., & van der Harst, P. (2020). Translational insights from single-cell RNA sequencing across the cardiovascular disease continuum. Submitted.
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