ISBN 978-82-326-3704-1 (printed version)
ISBN 978-82-326-3705-8 (electronic version)
ISSN 1503-8181

Doctoral theses at NTNU, 2019:50

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Results from a randomized clinical trial

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Thesis for the degree of Philosophiae Doctor

Trondheim, February 2019

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Printed by Skipnes Kommunikasjon as

Norsk sammenfatning:

Inhibering av interleukin-6 ved hjerteinfarkt uten ST-segment elevasjon i EKG. Resultater fra en randomisert klinisk studie.

Dette arbeidet inneholder resultater fra en klinisk, randomisert, dobbel-blindet, placebo-kontrollert studie, som ble designet for å belyse effekten av å hemme aktiviteten til det pro-inflammatoriske cytokinet interleukin-6 (IL-6) ved akutt hjerteinfarkt uten ST-segment elevasjon i EKG (NSTEMI). På tross av dagens behandling som inkluderer blodplatehemmende og plakkstabiliserende medisinsk behandling, samt revaskularisering med perkutan koronar intervensjon (PCI) eller koronar bypass kirurgi, så er fortsatt akutt hjerteinfarkt en diagnose som er belastet med høy morbiditet og mortalitet. Økt betennelsesaktivitet i forbindelse med hjerteinfarktet, og særlig økte sirkulerende nivåer av IL-6, er en robust markør for dårligere prognose hos disse pasientene. I denne studien ble aktiviteten til IL-6 hemmet ved intravenøs administrering av én enkelt dose av IL-6 reseptor antagonisten tocilizumab i akuttforløpet av NSTEMI, umiddelbart i forkant av prognostisk utredning med koronar angiografi. I denne studien ble enten tocilizumab eller placebo gitt i tillegg til standard behandling av hjerteinfarkt i henhold til gjeldende norske og europeiske retningslinjer. Effekten av behandlingen ble vurdert med repeterte blodprøver under innleggelsen og ved langtidskontroller etter 3 og 6 måneder. Det ble også utført ultralyd av hjertet under innleggelsen og etter 6 måneder. Dette var en to-senter studie som representerer et samarbeid mellom NTNU, Universitetet i Oslo, St. Olavs hospital og Oslo universitetssykehus Rikshospitalet. 117 pasienter med NSTEMI ble inkludert i studien. Pasientene ble fortløpende inkludert ved Klinikk for hjertemedisin, St. Olavs hospital, og ved Kardiologisk avdeling, Oslo universitetssykehus Rikshospitalet, i perioden august 2011 til og

med november 2013. Hver pasient ble fulgt i 6 måneder, og siste kontroll ble gjennomført i april 2014.

Artikkel 1:

Inflammasjon reflektert ved stigning av C-reaktivt protein (CRP) i blod ved hjerteinfarkt, er positivt korrelert med infarktstørrelse og assosiert med utvikling av hjertesvikt og dårligere prognose. Ved betennelse er det i hovedsak IL-6 som stimulerer leveren til å produsere CRP, og i likhet med CRP er også IL-6 assosiert med infarktstørrelse og dårligere prognose ved akutt hjerteinfarkt. I denne studien så vi derfor på effekten av tocilizumab på CRP-nivåer, og sekundært om medikamentet kunne påvirke hjerteskade bedømt ved nivåer av den spesifikke hjerteskademarkøren troponin T i blod. Pasienter som fikk tocilizumab fikk betennelsesaktiviteten signifikant redusert med 50 % bedømt ved repeterte målinger av CRP under innleggelsen. Videre reduserte også tocilizumab troponin T verdiene, justert for nivåer målt før medikamentet ble gitt (baseline). Effekten på troponin T ble kun sett hos pasienter som ble behandlet med PCI, det vil si ballongutblokking av trange kransårer inn til hjertemuskelen med samtidig levering av stent. Denne artikkelen viser at tocilizumab reduserer betennelse ved hjerteinfarkt, og at medikamentet kan redusere hjerteskaden som kan oppstå som konsekvens av PCI-behandling. Funnene åpner for at medikamentet kan være prognostisk gunstig for pasienter med hjerteinfarkt.

Artikkel 2:

I denne studien evaluerte vi hvorvidt tocilizumab påvirket koronar- og systemisk endotelfunksjon. Pasienter med akutt hjerteinfarkt karakteriseres både av økt betennelse og dårligere endotelfunksjon. Betennelse er delvis årsaken til redusert endotelfunksjon i denne settingen, og både betennelse og redusert endotelfunksjon er assosiert med dårligere prognose.

Den totale karfunksjonen er i hovedsak betinget av funksjonen til glatt muskulatur i karveggen, samt funksjonen til endotelcellene som dekker innsiden av blodårene. I denne artikkelen undersøkte vi karfunksjonen i koronarkarene ved å måle koronar blodstrømsreserve (CFR) med ultralyd under stimulering med medikamentet adenosin. CFR ble undersøkt én gang under innleggelsen etter at medikamentet var gitt, og etter 6 måneder. Videre målte vi markører for endotelfunksjon i blod (VCAM-1, ICAM-1 og vWF). Vi fant ingen effekt av tocilizumab på CFR under innleggelsen eller etter 6 måneder. VCAM-1 var signifikant høyere i tocilizumab-gruppen, men i motsetning til pasienter som fikk placebo, fant vi ingen invers korrelasjon mellom VCAM-1 verdier og koronar karfunksjon bedømt ved CFR hos disse pasientene. Denne artikkelen viser at tocilizumab ikke påvirker koronar karfunksjon i akuttforløpet, samt at tocilizumab øker nivået av VCAM-1, men uten at dette er et uttrykk for forverret koronar karfunksjon. Tocilizumab ser dermed ikke ut til å ha akutte effekter på kar- og endotelfunksjon som eventuell forklaring på de gunstige funnene på troponin T observert i artikkel 1.

Artikkel 3:

I denne studien så vi på effekten av IL-6 hemming på cytokinnettverket ved NSTEMI. Betennelsesresponsen utløst av hjerteinfarkt inkluderer oppregulering av en rekke cytokiner som potensielt kan ha både beskyttende og skadelige effekter i forhold til infarktskade og reparasjonsprosessen av hjertet i etterkant. Vi analyserte plasmaverdiene av 27 forskjellige cytokiner på samtlige tidspunkt. Vi fant at tocilizumab førte til en betydelig stigning av kjemokinene interferon gamma-induserbart protein (IP-10) og makrofag inflammatorisk protein-1β (MIP-1β) som vedvarte under innleggelsen. I tocilizumabgruppen var MIP-1β inverst korrelert til nøytrofile granulocytter og troponin T. Nært samtlige cytokiner analysert i denne studien var oppregulert under innleggelsen sammenliknet med senkontroller, men kun

IP-10 og MIP-1β ble påvirket av behandlingen. Denne studien viser at tocilizumab har en begrenset og selektiv effekt på det aktiverte cytokinnettverket ved NSTEMI, med oppregulering av IP-10 og MIP-1β i akuttfasen. Betydningen av disse funnene er usikker, men antyder at MIP-1β kan ha en gunstig effekt i forhold til infarktstørrelse. Tocilizumabs begrensede effekt på andre cytokiner ved hjerteinfarkt, kan også indikere at de mulig positive effektene av IL-6 hemming ved hjerteinfarkt er direkte forårsaket av inhibering av IL-6, og ikke via sekundære effekter på andre cytokiner.

Navn kandidat: Ola Kleveland

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Hovedveileder: Professor Rune Wiseth, Institutt for sirkulasjon og bildediagnostikk, Fakultet for Medisin og Helsevitenskap, NTNU

Biveileder: Professor Jan Kristian Damås, Institutt for klinisk og molekylær medisin, Fakultet for Medisin og Helsevitenskap, NTNU

Finansieringskilder: Samarbeidsorganet mellom Helse Midt-Norge og NTNU. Helse Sør-Øst.

Ovennevnte avhandling er funnet verdig til å forsvares offentlig

for graden PhD i Klinisk medisin.

Disputas finner sted i auditorium LA21, Laboratoriesenteret, St. Olavs hospital

Torsdag 21.02.19 kl 10.15 og 12.15

Contents

ACKNOWLEDGEMENTS

I am grateful to *the Liaison committee for Education, Research and Innovation in Central Norway and NTNU* for funding my position as a PhD-graduate at the Department of Circulation and Medical Imaging, Faculty of Medicine and Health Sciences, NTNU, from 2011 through 2017. I also wish to thank *the South-Eastern Norway Regional Health Authorities* who provided further funding for the conduction of the project on which this thesis is based. I also express my gratitude to the Clinic of Cardiology, St. Olavs hospital, and the Department of Cardiology, Oslo university hospital Rikshospitalet, for their contributions economically and not at least for the patience, enthusiasm and assistance of the clinical staff at both institutions, which were essential for realizing this project.

My deepest respect and gratitude goes to all patients who participated in this project. I thank them for their motivation and persistency through follow-up, and not at least for their admirable tolerance towards numerous phlebotomies, adenosine-infusions and MRI imaging. Many of them travelled long distances to attend follow-up visits, making it possible for us to answer our research questions.

During these years I have had the privilege of having professor Rune Wiseth both as my supervisor on this project, and also as chief of cardiology with regard to my clinical work. I thank him for having faith in me, motivating me and giving me the possibility to do research. I thank him for facilitating an environment and a research organization within the clinic which made the inclusion and follow-up of patients in this project friction-free, and making the Clinic of Cardiology an ideal arena for further clinical research projects. Through these years Rune's door has always been open and I have never ever heard him say "no" when I have asked for his help and guidance, despite his very busy schedule. Rune's unique clinical

and scientifical expertise has been of great value for my progression these years both clinically and as a researcher, and for the quality of the work in this thesis.

Many, many thanks to my co-supervisor professor Jan Kristian Damås. I am deeply grateful and feel fortunate that he spotted me as a candidate for this project during our short spell as colleagues at the Department of Infectious Diseases back in 2009. Professor Damås was the driver of the initiation of this project, and I also thank him for introducing me to the inflammatory cardiac research group at Oslo university hospital Rikshospitalet, with whom we have enjoyed an outstanding cooperation during this project. I thank him for his limitless availability, wisdom and valuable contributions to the interpretation of the results in our project. However, how a man of such intellectual clarity can support Liverpool FC, still remains an unanswered conundrum after all these years of research.

I also want to express my immense gratitude to all co-authors and research staff involved in this project at Oslo university hospital Rikshospitalet for a brilliant collaboration. Especially principal investigator professor Lars Gullestad, who steered us through this project and its' review by the Norwegian Medicines Agency with a steady hand. I am indebted to Marte Bratlie and Gabor Kunszt, who included the patients at Rikshospitalet. I also thank Marte for her substantial and essential contributions in finalizing the scientific and formal preparations of this project, and Gabor for admirably stepping in on short notice so that the patient recruitment could be finalized. I owe a sincere and special thank you to professor Thor Ueland for biochemical analyses, his brilliant statistical insight, ideas and guidance during manuscript preparations. I am especially indebted and very thankful to professor Pål Aukrust who has been a pivotal driver in this project and has been an invaluable mentor during the work-up of manuscripts. I am grateful and immensely impressed by his continuous availability, his work capacity, his remarkable "boomerang-style" ability to respond to e-mails, his ideas, insight, enthusiasm, efficiency and endurance during this project. I have

learnt so much. I am indebted to professor Tom Eirik Mollnes for his insight and contributions to the interpretation of cytokine network analyses. A special thanks also to Kaspar Broch for his efforts during patient inclusion at Rikshospitalet, and for his important insight and contributions throughout manuscript preparations. Thank you to Wenche Stueflotten and Bjørn Bendz for welcoming me and being very helpful during my research visits at Rikshospitalet these years.

A special mention and thank you to my friend, colleague and co-lead-author of Paper II, Espen Holte, who was the principal echocardiographist at St. Olavs hospital in this study and performed all analyses of coronary flow reserve. Writing Paper II with Espen was a joyful experience both scientifically and socially. We should do it again sometime. Thanks to Brage Amundsen for assisting with echocardiographies in Espen's absence, and for being available to perform MRI-scans during many of his lunch breaks. I am very grateful to research staff at the Clinic of Cardiology, St. Olavs hospital; Tove Vindsetmo and Ann Mari Myraunet for their unlimited availability to perform randomization and drug preparation; Eli Skogan Granviken, Eva Kjøl Slind and Ann-Elise Antonsen for excellently organizing follow-up visits and assistance during echocardiography and CFR-measurements. Thank you to senior engineer Liv Ryan for performing the multiplex cytokine analyses for Paper III, and to colleague Øystein Sørdal for including patients while I was on paternal leave. I am also grateful for the great collaboration offered by Erik Madssen, Vegard Malmo and Erlend Prytz, with whom I shared my clinical responsibilities at the Clinic of Cardiology during these years.

Finally, I want to thank my whole family for support and love during these years. Especially I want to thank the most important persons in my life; my lovely wife Marte who deserves special thanks for her continuous support and patience through these years, and our two amazing and energetic sons Aksel (6) and Sigurd (3) who fill our lives with love and joy every day.

LIST OF PAPERS

I. Kleveland O, Kunszt G, Bratlie M, Ueland T, Broch K, Holte E, Michelsen AE, Bendz B, Amundsen BH, Espevik T, Aakhus S, Damås JK, Aukrust P, Wiseth R, Gullestad L. Effect of a single dose of the interleukin-6 receptor antagonist tocilizumab on inflammation and troponin T release in patients with non-ST-elevation myocardial infarction: a double-blind, randomized, placebo-controlled phase II trial. Eur Heart J 2016;37:2406-2413.

II. Holte E, Kleveland O, Ueland T, Kunszt G, Bratlie M, Broch K, Michelsen AE, Bendz B, Amundsen BH, Aakhus S, Damås JK, Gullestad L, Aukrust P, Wiseth R. Effect of interleukin-6 inhibition on coronary microvascular and endothelial function in myocardial infarction. Heart 2017;103:1521-1527.

III. Kleveland O, Ueland T, Kunszt G, Bratlie M, Yndestad A, Broch K, Holte E, Ryan L, Amundsen BH, Bendz B, Aakhus S, Espevik T, Halvorsen B, Mollnes TE, Wiseth R, Gullestad L, Aukrust P, Damås JK. Interleukin-6 receptor inhibition with tocilizumab induces a selective and substantial increase in plasma IP-10 and MIP-1β in non-ST-elevation myocardial infarction. Int J Cardiol 2018;271:1-7.

SELECTED ABBREVIATIONS

ACS = acute coronary syndrome

AUC = area under the curve

CAD = coronary artery disease

CFR = coronary flow reserve

CRP = C-reactive protein

DAPT = dual antiplatelet therapy

ECG = electrocardiogram

EF = ejection fraction

hs = high-sensitivity

IL = interleukin

IR = ischaemia-reperfusion

iv = intravenous

ICAM-1 = intercellular adhesion molecule-1

LAD = left anterior descending artery

LV = left ventricle / left ventricular

NSTE-ACS = non-ST-segment elevation acute coronary syndrome

NSTEMI = non-ST-segment elevation myocardial infarction

NT-proBNP = N-terminal pro-brain natriuretic peptide

PCI = percutaneous coronary intervention

PMI = periprocedural myocardial injury

RA = rheumatoid arthritis

ROS = reactive oxygen species

sc = subcutaneous

STEMI = ST-segment elevation myocardial infarction

TTE = transthoracic echocardiography

VCAM-1 = vascular cell adhesion molecule-1

vWF = von Willebrand factor

1 INTRODUCTION

1.1 General background

Over the last decades, remarkable progress has been made in the prevention, diagnosis and treatment of atherosclerotic coronary artery disease (CAD) and acute coronary syndromes (ACS). Mortality from CAD has declined due to increased awareness and management of risk factors, beneficial public health policies and legislation, advances in pharmacotherapy, and the ubiquitous availability of coronary revascularization therapy.[1] However, despite the significant advances made, CAD remains the major cause of death in Western societies,[1, 2] and accounts for 20 % of all deaths in Europe.[1] Thus, current state-of-the-art management of CAD only prevents a fraction of coronary ischaemic events and related complications, and patients with established CAD are still exposed to an increased risk for future ACS and death.[3, 4] This underscores the need for further research to improve care and prognosis for these patients.

1.2 Acute coronary syndromes. Current treatment.

ACS is a serious complication of CAD that is associated with substantial morbidity and mortality.[1, 2] ACS is triggered by destabilization and ultimately disruption (i.e. endothelial erosion or plaque rupture) of a coronary atherosclerotic plaque, with subsequent superimposed thrombus formation (Figure 1).[5] The coronary thrombus obstructs blood flow to the myocardium, resulting in prolonged ischaemia due to a persistent mismatch between myocardial oxygen supply and demand, and ultimately myocardial necrosis.[6] Patients presenting with ACS usually develop symptoms of myocardial ischaemia at rest, typically various combinations of chest, upper extremity, mandible or epigastrial pain lasting > 20 minutes.[7] Clinically, ACS is divided into two categories based on acute changes in the

electrocardiogram (ECG): ST-segment elevation myocardial infarction (STEMI) and non-ST-segment elevation ACS (NSTE-ACS), the latter comprising patients with non-STEMI (NSTEMI) and unstable angina.[7] While patients with myocardial infarction (MI) (STEMI and NSTEMI) show evidence of myocardial necrosis (reflected in an acute rise-and-fall of the cardiac biomarker troponin), the ischaemia in patients with unstable angina has not resulted in myocardial necrosis.[7] The main treatment objectives in all patients with ACS is to achieve symptom relief, stabilize the atherothrombotic disease process, restore normal coronary blood flow, and minimize infarct size by the use of anti-ischaemic and anti-thrombotic therapy, lipid-lowering therapy with statins, and coronary revascularization therapy predominantly through percutaneous coronary intervention (PCI).[8, 9] We use ST-segment changes in the ECG to stratify patients to different initial treatment strategies in ACS.[7-9] Patients with STEMI usually have a complete thrombotic occlusion of an epicardial coronary artery and therefore benefit from immediate pharmacological (intravenous (iv) thrombolysis) or mechanical revascularization (PCI) therapy to minimize infarct size.[8] In most patients presenting with NSTE-ACS, the thrombus does not cause total occlusion of the coronary artery, and the final infarct size is usually modest. In contrast to STEMI patients, most patients with NSTE-ACS do not benefit from immediate revascularization. The initial treatment strategy in the majority of these patients is medical stabilization with anti-ischaemic and anti-thrombotic therapy in addition to statins, followed by early coronary angiography and revascularization therapy as appropriate.[9] To minimize the risk of future atherothrombotic events, patients with ACS are treated with dual antiplatelet therapy (DAPT) with aspirin and a P2Y12-inhibitor (clopidogrel, prasugrel or ticagrelor) for at least 12 months, followed by indefinite monotherapy with aspirin. Intensive lipid-lowering therapy with statins is recommended.[8, 9] In the reperfusion-era of ACS-treatment, the benefit of prolonged use of betablockers in patients subjected to complete coronary revascularization (i.e. no residual coronary substrates for myocardial

ischemia) and with no or modest reduction of left ventricular (LV) systolic function is uncertain.[8, 9] However, in patients with larger infarct sizes and reduced LV systolic function (i.e. LV ejection fraction (EF) < 40 %) after ACS, life-long treatment with beta-blockers as well as angiotensin-converting enzyme (ACE) inhibitors are the mainstay of treatment to prevent unfavorable LV remodeling, the development of heart failure, and to improve prognosis.[10]

While current therapy has significantly reduced morbidity and mortality, patients with established CAD remain at increased risk of future coronary events.[3, 4] Furthermore, in patients with ACS infarct size is a strong predictor of the development of adverse LV remodeling, heart failure and premature death.[11, 12] Currently, the most effective way to limit infarct size, especially in patients with acute STEMI, is to re-establish perfusion through PCI.[8] However, whereas the abrupt restoration of blood flow undoubtedly has a net beneficial effect, it may also have detrimental effects through ischaemia-reperfusion (IR) injury. IR injury represents a clinically significant and unresolved issue in these patients, and may account for as much as 50 % of the myocardial damage during MI. This injury contributes to the development of heart failure and increased risk of death.[13] Furthermore, whereas PCI improves the prognosis in patients with NSTE-ACS,[9] significant iatrogenic periprocedural myocardial injury (PMI) can occur with negative impact on prognosis in these patients as well.[14] Thus, major objectives for future research on CAD and ACS should be to develop adjunctive therapy to (i) further reduce the risk of new ischaemic events in the long term, and (ii) reduce infarct size by addressing the IR and PCI-related PMI in these patients.

1.3 The role of inflammation in coronary artery disease and acute coronary syndromes

Interestingly, as early as in the mid-19th century, there was considerable interest in a possible link between inflammation and cardiovascular disease. Similar to what we have

witnessed in recent times, there was an ongoing debate in the scientific community whether inflammation was the driver of atherosclerosis or only a secondary phenomenon with no impact on the disease process.[15] However, when the link between cholesterol and atherosclerosis was established in the early 1900's followed by the metabolic hypothesis, the scientific community's interest in the role of inflammation in cardiovascular disease vanished for the most of the 20th century.[15] With the identification of risk factors such as hypercholesterolemia, hypertension, diabetes mellitus and smoking, our understanding and our ability to prevent and treat cardiovascular disease has increased substantially. However, cardiovascular disease, and CAD and ACS in particular, remain significant health threats with a remaining need for novel treatment strategies to improve outcome.

On this background, we have witnessed a renewed and considerable interest in the role of inflammation in CAD and ACS during the last three decades.[15] Today CAD is widely regarded as an inflammatory disease.[5] Our understanding of the mechanisms underlying atherosclerosis and CAD has evolved beyond the view that the disease process reflects a progressive and passive accumulation of lipids and cellular debris in the vascular wall only. It is now established that inflammation plays a pivotal role in all stages of CAD, from endothelial dysfunction and plaque formation to plaque destabilization and disruption with superimposed thrombosis (Figure 1),[5] and the inflammatory response triggered by acute MI is essential for cardiac repair and healing.[16] However, an imbalanced inflammatory response in acute MI may have detrimental effects by expanding IR injury[13] and PMI,[14] and can also contribute to adverse LV remodeling and the development of heart failure.[11] Accordingly, the intensity of the inflammatory response in MI, as reflected by circulating levels of the systemic inflammatory marker C-reactive protein (CRP), is associated with infarct size,[17] LV remodeling, heart failure and death.[18-21] Thus, different inflammatory mediators, including cytokines, have been proposed as potential therapeutic targets in CAD and ACS.[4, 22, 23]

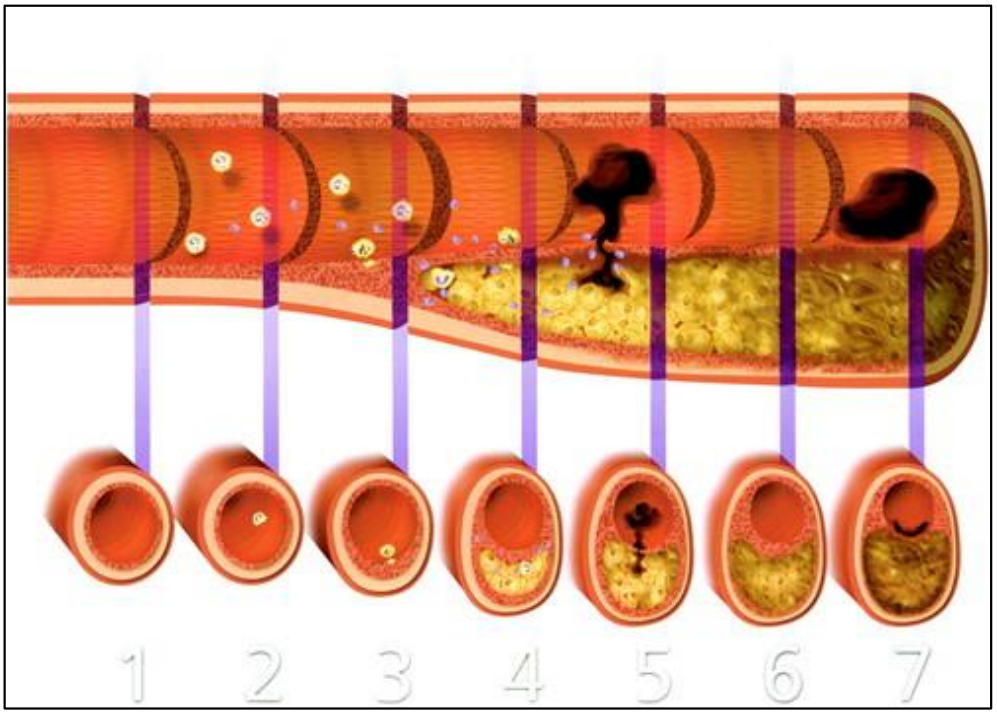

Figure 1 Initiation, progression and complication of human coronary atherosclerotic plaque. Top, Longitudinal section of artery depicting "timeline" of human atherogenesis from normal artery (1) to atheroma that caused clinical manifestations by thrombosis or stenosis (5, 6, 7). Bottom, Cross sections of artery during various stages of atheroma evolution. 1, Normal artery. Note that in human arteries, the intimal layer is much better developed than in most other species. The intima of human arteries contains resident smooth muscle cells often as early as first year of life. 2, Lesion initiation occurs when endothelial cells, activated by risk factors such as hyperlipoproteinemia, express adhesion and chemoattractant molecules that recruit inflammatory leukocytes such as monocytes and T lymphocytes. Extracellular lipid begins to accumulate in intima at this stage. 3, Evolution to fibrofatty stage. Monocytes recruited to artery wall become macrophages and express scavenger receptors that bind modified lipoproteins. Macrophages become lipid-laden foam cells by engulfing modified lipoproteins. Leukocytes and resident vascular wall cells can secrete inflammatory proteins and growth factors that amplify leukocyte recruitment and cause smooth muscle cell migration and proliferation. 4, As lesion progresses, inflammatory mediators cause expression of tissue factor, a potent procoagulant, and of matrix-degrading proteinases that weaken fibrous cap of plaque. 5, If fibrous cap ruptures at point of weakening, coagulation factors in blood can gain access to thrombogenic, tissue factor-containing lipid core, causing thrombosis on nonocclusive atherosclerotic plaque. If balance between prothrombotic and fibrinolytic mechanisms prevailing at that particular region and at that particular time is unfavorable, occlusive thrombus causing acute coronary syndromes may result. 6, When thrombus resorbs, products associated with thrombosis such as thrombin and mediators released from degranulating platelets, including platelet-derived growth factor and transforming growth factor-β, can cause healing response, leading to increased collagen accumulation and smooth muscle cell growth. In this manner, the fibrofatty lesion can evolve into advanced fibrous and often calcified plaque, one that may cause significant stenosis, and produce symptoms of stable angina pectoris. 7, In some cases, occlusive thrombi arise not from fracture of fibrous cap but from superficial erosion of endothelial layer. Resulting mural thrombus, again dependent on local prothrombotic and fibrinolytic balance, can cause acute myocardial infarction. Superficial erosions often complicate advanced and stenotic lesions, as shown here. However, superficial erosions do not necessarily occur after fibrous cap rupture, as depicted in this idealized diagram. *With permission from Wolters Kluwer Health, Inc. Libby P. Current concepts of the pathogenesis of the acute coronary syndromes. Circulation 2001;104(3):365-72.*

1.3.1 Inflammation in coronary atherosclerosis and the the inflammatory hypothesis in coronary artery disease

During the last decades, the concept of an important interleukin (IL)-1 → IL-6 → CRP axis in coronary vascular inflammation has been established.[24] CRP is a downstream, acute-phase protein acting as a marker of the overall systemic inflammatory activation. Several studies have identified CRP as a reliable marker of future coronary events both in healthy individuals[25, 26] and in patients with established CAD.[27, 28] However, CRP itself seems not to contribute to the disease process, as Mendelian randomization analyses have failed to identify a causal role for CRP in CAD.[29] However, IL-6 is the major upstream inducer of CRP during the acute phase response,[30] and also IL-6 levels are shown to predict future coronary events both in healthy subjects[26, 31] and patients with CAD.[32] Interestingly, and in contrast as for CRP, recent Mendelian randomization analyses have identified IL-6 to have a causal role in the development CAD and subsequent ACS.[33] Thus, the ability of CRP to predict future coronary events seems at least partly to be explained by its' ability to reflect upstream IL-6 mediated coronary inflammation. Moving further upstream in the inflammatory cascade, the apical inflammatory cytokine IL-1β is a major inducer of the IL-6 pathway[34, 35] and also has a strong association to CAD.[24] In fact, evidence has recently emerged showing that cholesterol crystals present in atherosclerotic plaques, probably represent a mechanistic link between cholesterol deposition and inflammation.[36] Cholesterol crystals have been shown to induce the production of IL-1β via activation of the NOD-like receptor family pyrin domain containing 3 (NLRP3) inflammasome,[36] which in turn results in activation of the IL-6 signalling pathway causally associated with progression of CAD. Interestingly, previous studies have shown that among patients treated with statins and aspirin, both of which possess anti-inflammatory properties, those with elevated CRP levels benefit the most in terms of risk reduction with regard to future coronary events.[25, 37] However, while these studies suggested that lowering

inflammation is beneficial in these patients, these studies could not confirm that reducing inflammation per se reduces coronary risk, due to the drugs' anti-thrombotic (aspirin)[25] and lipid-lowering (statins)[37] properties. However, the inflammation theory and the concept of an important IL-1 → IL-6 → CRP axis in coronary atherosclerosis was finally confirmed in the Antiinflammatory Therapy with Canakinumab for Atherosclerotic Disease (CANTOS) trial.[4] The CANTOS trial showed that in patients with previous MI and a CRP level > 2 mg/ml, treatment with the IL-1β antibody canakinumab reduced the rate of recurrent cardiovascular events compared to placebo, and, importantly, independent of lipid-level lowering. These results could have imminent impact on clinical practice, as they indicate that patients with established CAD and residual inflammatory risk (CRP > 2 mg/ml) could be further protected from future coronary events with anti-inflammatory treatment, in addition to treatment with high-intensity lipid-lowering therapy.

1.3.2 The role of inflammation in myocardial infarction

Inflammation seems to be involved in all aspects of myocardial damage in the context of acute MI.[13, 14, 16] Acute MI and cardiomyocyte necrosis trigger a comprehensive inflammatory response,[16] and the intensity of this inflammatory response reflected by CRP levels is positively correlated to infarct size,[18] death[19, 21] and the development LV remodeling and heart failure.[20] While an adequate inflammatory response is essential for cardiac repair and healing after MI,[16] an exaggerated inflammatory response may contribute to detrimental effects such as IR injury[13] and PMI.[14] Thus, modulating the inflammatory response could be an attractive therapeutic target in acute MI.

1.3.2.1 The inflammatory response to acute myocardial infarction

There is an extensive body of evidence exploring the role of inflammation during MI. The complex inflammatory response to MI and cardiomyocyte necrosis is summarized and described in several review articles.[16, 22, 23, 38] The inflammatory response in acute MI is usually divided into three phases: (i) the alarm phase, (ii) the leukocyte mobilization phase, and the (iii) resolution phase (Figure 2).[22] During the initial alarm phase, necrotic cardiomyocytes release several signals to induce an innate immune response. These, among

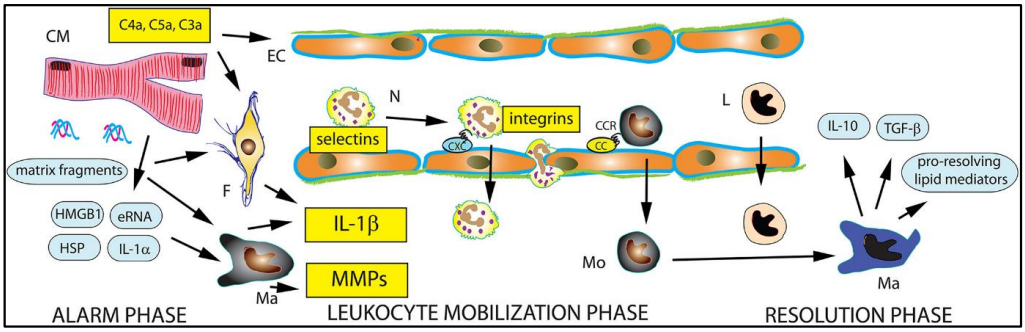

Figure 2 The inflammatory response following MI can be divided into three phases: the alarm phase, the leukocyte mobilization phase and the resolution phase. Necrotic cardiomyocytes (CM) release alarmins (heat shock proteins [HSP], high mobility group box 1 [HMGB1], extracellular RNA/eRNA, IL-1α and other danger signals) that activate innate immune signaling pathways. Extracellular matrix (ECM) fragments also trigger inflammatory signaling. Induction of pro-inflammatory cytokines, such as IL-1, and chemokines mediates recruitement of neutrophils (N) and pro-inflammatory monocytes (Mo) through interactions with endothelial cells (EC) that involve selectins and integrins. Clearance of dead cells and matrix debris from the infarct triggers transition to the resolution phase. Anti-inflammatory lymphocyte (L) and macrophage (Ma) subsets release mediators that suppress pro-inflammatory signaling, such as IL-1, TGF-β and pro-resolving lipid mediators. Experimental studies suggest that inhibition of the complement cascade, IL-1β antagonism, CCL2 inhibition, selectin and leukocyte integrin neutralization may be promising therapeutic strategies for patients with MI. F, fibroblast. *With permission from John Wiley and Sons. Huang S, Frangogiannis NG. Anti-inflammatory therapies in myocardial infarction: failures, hopes and challenges. Br J Pharmacol 2018;175(9):1377-1400.*

others, include reactive oxygen species (ROS) and so-called damage-associated molecular pattern proteins (DAMPs), such as extracellular RNA and DNA, mitochondrial DNA, heat-shock proteins and IL-1α. These signals activate the complement cascade, pattern recognition receptors like Toll-like-receptors and the NLRP3 inflammasome, which in concert contribute to up-regulation of the apical and pro-inflammatory cytokine IL-1β, which in turn activates downstream mediators including the IL-6 pathway and several other cytokines. Up-regulation

of cytokines then introduces the leukocyte mobilization phase of inflammation during MI. Predominantly by stimulating the endothelial cell expression of adhesion molecules (i.e. intercellular adhesion molecule-1 [ICAM-1] and vascular cell adhesion molecule-1 [VCAM-1]) and increased production of chemokines, pro-inflammatory cytokines direct leukocytes such as neutrophils, monocytes and macrophages, to the infarcted myocardium. During the first 3 days post-MI, these pro-inflammatory actors contribute to clear debris and necrotic cells from the infarct area. Thereafter a transition to a resolution phase occurs approximately from days 4-7, which facilitates wound healing, activation of myofibroblasts and scar formation. In this phase, suppression of pro-inflammatory mechanisms and increased presence of anti-inflammatory actors such as IL-10, IL-6, and a shift to an anti-inflammatory phenotype of leukocytes (e.g. shift from pro-inflammatory M1 macrophages to anti-inflammatory M2 macrophages) is observed.

1.3.2.2 Ischaemia reperfusion injury

While this complex inflammatory surge seems to be essential for infarct healing and cardiac repair, an unfavorable imbalance between pro-inflammatory and anti-inflammatory phases in this process can lead to an exaggerated and/or protracted inflammatory response with detrimental effects.[16] Especially in the setting of an acute STEMI, where the coronary artery is totally occluded by a thrombus, the inflammatory response to IR injury occurring after restoration of blood flow caused either by PCI or thrombolysis, is important in defining the final infarct size.[13] While revascularization undoubtedly contributes to net reduction of infarct size, the reperfusion injury which occurs after restoration of coronary blood flow, leads to additional death of cardiomyocytes that were viable immediately prior to reperfusion, and exceeds the injury caused by ischemia itself. This process usually occurs in the viable border-zone of the infarcted area, and represents a multifactorial phenomenon.[13] Major components

in reperfusion injury is the generation of ROS, reenergization of mitochondrias, intracellular calcium overload, rapid normalization of pH, as well as inflammation.[13] The major inflammatory contribution to reperfusion injury seems to be accumulation of neutrophils in the infarcted area and in the viable border-zone, due to upregulation of adhesion molecules such as ICAM-1.[13, 39] Neutrophils can contribute to accentuate myocardial injury by several means, including further generation of ROS, microvascular plugging and the release of degradative enzymes.[13] Targeting inflammatory mechanisms involved in reperfusion injury might therefore contribute to the reduction of infarct size and related complications.

1.3.2.3 Periprocedural myocardial injury following PCI

IR injury as described above, is predominantly observed in STEMI patients when a totally occluded coronary artery is reperfused. However, although most patients with NSTE-ACS or stable CAD do not suffer from completely obstructed coronary arteries, PCI of stenotic arteries in these patients can also lead to PMI, with negative prognostic impact.[14, 40] PMI can be divided in proximal type I PMI and distal type II PMI (Figure 3).[14] Type I PMI is predominantly an anatomical phenomenon caused by various degrees of side branch obstruction during PCI. If blood flow in the affected side branch is sufficiently compromised, myocardial injury results as a consequence of the PCI-procedure. While type I accounts for approximately 25 %, peripheral type II PMI causes up to 75 % of PMI during PCI-procedures.[14] Similar to the IR injury mostly observed in STEMI patients, type II PMI is a multifactorial phenomenon in which inflammation presumably plays a major part. Type II PMI can be caused by distal embolization of atherosclerotic debris and thrombus material. Furthermore, platelet activation occurs after PCI-induced plaque rupture. Content in the ruptured plaque, such as von Willebrand factor (vWF), tissue factor and collagen, is exposed to blood and activates platelets, contributing to further thrombus formation and microvascular

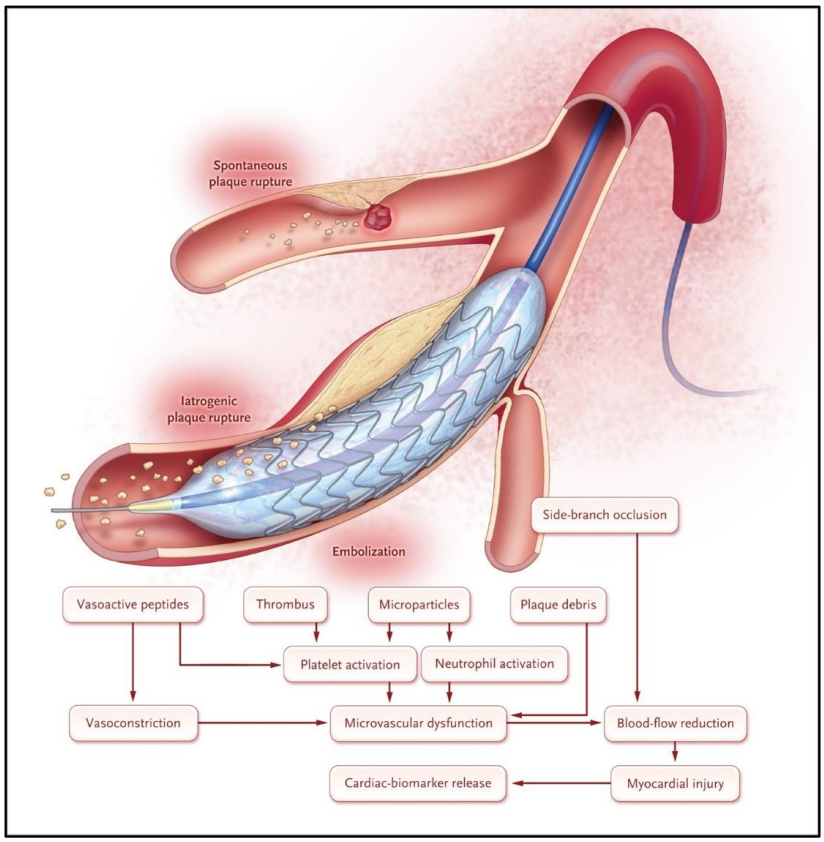

Figure 3 Mechanisms Underlying Periprocedural Myocardial Infarction. Cardiac biomarker-elevation before percutaneous coronary intervention (PCI) is primarily due to spontaneous plaque rupture of vulnerable plaques, epicardial thrombosis, and subsequent myocardial injury. In the absence of abrupt, PCI-related epicardial-artery closure, periprocedural myocardial infarction is related to either side-branch occlusion or iatrogenic plaque rupture by balloons and stents, which promotes microvascular injury owing to distal embolization, the release of vasoactive peptides, or both. *Reproduced with permission from Prasad A, Herrman J. Myocardial infarction due to percutaneous coronary intervention. N Engl J Med 2011;364(5):453-64. Copyright Massachusetts Medical Society.*

plugging of neutrophils. Other contributors could be vascular dysfunction, oxidative stress and inflammation.[14] With regard to platelet activation in this setting, state-of-the-art antiplatelet treatment with preloading with DAPT reduces the the occurrence of PMI.[41] Interestingly, increase of inflammatory markers such as CRP and IL-6 are observed after PCI in patients with both NSTE-ACS[42] and stable CAD,[43] and these inflammatory markers seems to be higher in patients with significant troponin release after the PCI-procedure.[43] Moreover, statins have been shown to reduce PMI.[44] However, whether this can be attributed to their plaque-stabilizing effect, anti-inflammatory properties, or both, is not clearly established.

Nonetheless, the positive association between inflammation and PMI suggest that anti-inflammatory therapy could offer cardioprotection in this context.

1.3.2.4 Previous research on modulating inflammation in acute myocardial infarction

Extensive experimental research has been conducted targeting different components in the inflammatory response using both reperfused and non-reperfused animal models of MI, often with promising results.[23] However, so far, testing these treatment strategies in humans with acute MI have yielded disappointing and neutral results. Experimental studies using acute MI models in animals evaluating neutrophil inhibition,[45, 46] different modalities of complement inhibition,[47, 48] and inhibition of IL-1,[49] have all shown reduction of infarct size. However, inhibitors of neutrophil adhesion (rhuMAB and Hu23F2G) showed no beneficial effects on coronary bloodflow, infarct size or clinical endpoints when administrated prior to iv thrombolysis or acute PCI in patients with acute STEMI.[50, 51] Furthermore, even though small clinical studies in humans on complement inhibition using iv infusions of C1 inhibitor prior to revascularization in STEMI have shown effect on markers of myocardial injury (CK-MB or troponins),[52-54] larger randomized clinical trials on complement inhibition with iv administration of the anti-C5 antibody pexelizumab prior to primary PCI, did not show any effect on either infarct size or clinical endpoints such as cardiogenic shock, heart failure or death.[55, 56] Moreover, while animal studies have shown reduced infarct size and improved cardiac function in mice treated with IL-1 inhibition, clinical studies in humans have not yet provided evidence that IL-1 inhibition affects infarct size. A randomized trial (MRC-ILA-heart) assessing the effect of subcutaneous (sc) administration of anakinra (an IL-1 receptor antagonist) for 14 days in patients with NSTEMI showed that IL-1 inhibition reduced inflammation as reflected by decreasing levels of CRP and IL-6 in the acute phase of NSTEMI. However, no between-group difference was observed regarding troponin T as a

marker of myocardial injury. In fact, in the anakinra-arm, a concerning significant increase in major adverse cardiovascular events (MI, death, stroke) was observed at 1 year follow-up.[57] The same treatment with anakinra for 14 days has been further tested in two small randomized trials (VCU-ART 1 and 2) in patients with acute STEMI.[58-60] These trials did not show any effect on infarct size, but suggest that IL-1 inhibition have beneficial effects on LV remodeling and development of clinical heart failure. The lack of effect on infarct size in the IL-1 inhibition studies could potentially be explained by study designs, as patients in the NSTEMI study could be included for up to 48 hours after symptom onset when the primary myocardial injury had already occurred. In the VCU-ART studies anakinra was given several hours after acute reperfusion, at a timepoint when most of the myocardial necrosis had been established.

The aforementioned studies serve as examples of the challenges faced when assessing inflammatory mediated injury in acute MI: So far, very promising results from animal studies usually do not translate into significant beneficial effects in humans. Several factors could explain this like differences between species. Animals used in these studies are mostly young and healthy with possibly a different inflammatory response during acute MI than more elderly human subjects. In acute MI models in animals, coronary occlusion and reperfusion is achieved by permanent or transient ligation of normal and healthy coronary arteries, while in acute MI in humans a completely different process involving rupture of often a necrotic atherosclerotic plaque with superimposed thrombus is the cause of injury. Components in the thrombus and in the ruptured plaque, which these animal models lack, may affect final infarct size in humans via e.g. distal embolization. Furthermore, humans suffering an acute MI often have relevant co-morbidities and concomitant pharmacological treatment which also may have an effect on infarct size and outcome. An additional challenge is that several actors in the inflammatory response during MI, could have opposing functions (pro- vs anti-

inflammatory) in the different phases of infarct related inflammation. Thus, permanently inhibiting one factor during MI can be beneficial during e.g. the initial pro-inflammatory phase, but could have unfavorable effects in the resolution phase of inflammation. Thus, to improve the translation of encouraging results from the animal lab to clinical benefit in humans with acute MI, there is a need to establish animal models of MI which in every way possible resemble the pathophysiological and clinical setting in humans. Furthermore, the right timing and duration of anti-inflammatory therapy in humans with acute MI might be of crucial importance to achieve clinical benefit.

1.4 Interleukin-6

1.4.1 The role of interleukin-6 in inflammation and diseases

IL-6, first discovered in 1986 as a B-cell differentiation factor,[61] is a multifunctional and often pro-inflammatory cytokine produced by a spectrum of cells, including cells in the cardiovascular system.[62] IL-6 is produced and released in response to tissue injury and infections, and contributes to inflammation and host defense.[63] IL-6 exerts a wide range of biological activities, including regulation of acute phase-reactions, inflammation, immune responses and haemopoiesis. However, overexpression of IL-6 seems to be of importance in the pathogenesis of several diseases, including cancer and autoimmune diseases such as rheumatoid arthritis (RA), systemic juvenile idiopathic RA and Castleman's disease.[62]

In inflammation, IL-6 serves as a secondary downstream mediator of apical cytokines such as IL-1.[34, 35] IL-6 mediates its' biological effects by binding to both the membrane-bound (m) and the soluble (s) IL-6 receptor (IL-6R). To activate intracellular signaling pathways and ultimately target gene transcription, the IL-6 – IL-6R complex is dependent on interaction with the receptor subunit glycoprotein 130 (gp130). Binding of IL-6 to IL-6R induces

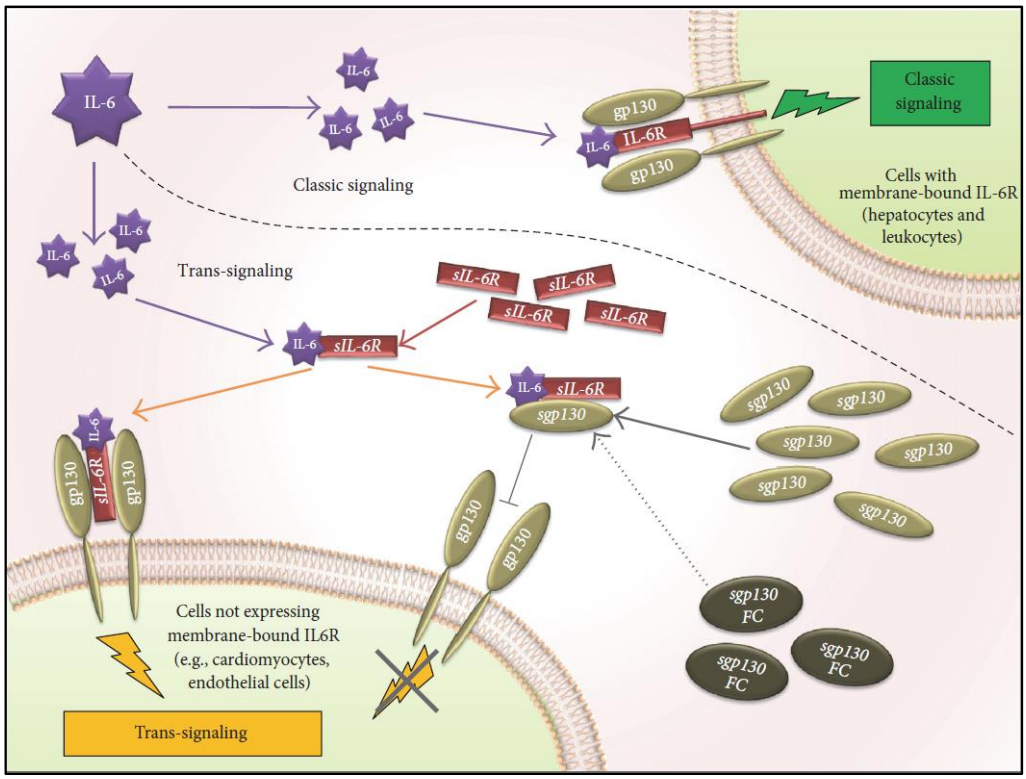

Figure 4 IL-6 signal transduction via classic and trans-signaling. The upper part of the figure depicts IL-6 signaling in cells expressing the membrane-bound receptor for IL-6 (IL-6R). In these cells (e.g. hepatocytes and several white blood cells), circulating IL-6 binds directly to IL-6R that forms a signaling complex with the membrane-bound glycoprotein 130 (gp130); this pathway is known as classic signaling. The bottom part depicts the IL-6 signaling in those cells that do not express the membrane-bound IL-6R. In these cells, membrane-bound gp130 (ubiquitously expressed) is activated by the circulating IL-6/sIL-6R complex (composed of IL-6 and the circulating soluble portion of IL-6R, sIL-6R). This pathway, known as trans-signaling, could be inhibited by the circulating soluble portion of gp130 (sgp130), which, by means of binding the circulating IL-6/sIL-6R complex, blocks the activation of the membrane-bound gp130. sgp130fc is a recombinant fusion protein of soluble gp130 and human IgG1 Fc that blocks IL-6 trans-signaling mimicking sgp130 functions. *From Morieri ML, Passaro A, Zuliani G. Interleukin-6 "trans-signaling" and ischemic vascular disease: the important role of soluble gp130. Mediators of Inflammation, vol. 2017, Article ID 1396398, 6 pages, 2017. https://doi.org /10.1155/2017/1396398.*

homodimerization of gp130, forming a high affinity complex of IL-6, IL-6R and gp130 (Figure 4). This results in the activation of primarily two intracellular pathways (the janus kinase/signal transducer and activator of transcription [JAK/STAT] and the Src homology domain-containing protein tyrosine phosphatase-2/extracellular-signal-regulated kinase/mitogen-activated protein kinase [SHP-2/ERK MAPK]), which in turn activates target gene transcription to exert IL-6 mediated biological actions. While mIL-6R is expressed only in a few cell types (e.g. hepatocytes and haemopoietic cells), gp130 seems to be ubiquitously

expressed in all cell types. Thus, IL-6 can potentially exert its' effects on all cell types through the interaction between circulating sIL-6R – IL-6 complexes and cellular gp130, called IL-6 transsignalling. While membrane-bound gp130 is essential for intracellular IL-6 signalling, circulating sgp130 works as a decoy receptor for the IL-6 – sIL-6R complex, thus inhibiting and being a natural moderator of IL-6 activity.[62]

1.4.2 Interleukin-6 as a potential therapeutic target in coronary artery disease and acute coronary syndromes

Inhibiting IL-6 signalling appears an attractive therapeutic target in CAD and ACS because of its central role in the progression of atherosclerosis, as well as its' strong association with poor outcome in ACS.

IL-6 has been linked to vascular inflammation and seems to be a major player in the inflammatory arm of atherosclerotic CAD.[33, 64, 65] IL-6 signalling contributes to both atherosclerotic plaque development and destabilization through various mechanisms, including the release of other inflammatory cytokines, oxidation of lipoproteins by phospholipases, and stimulation of acute-phase proteins such as CRP with subsequent complement activation. It causes the release of pro-thrombotic mediators and activation of matrix metalloproteinases. This results in weakening of the atherosclerotic fibrous cap which becomes vulnerable to rupture and the development of ACS.[64, 65] Accordingly, high levels of circulating IL-6 and CRP, the latter predominantly induced by IL-6, is associated with increased risk of future coronary events both in healthy individuals and patients with established CAD.[25, 26, 31, 32] Moreover, Mendelian randomization studies have recently provided strong evidence of a causal role of IL-6 signalling in the development of CAD.[33]

IL-6 and CRP are also substantially up-regulated in ACS. In this context, it is likely that CRP to a significant extent reflects upstream IL-6 activity, as it is strongly correlated to

IL-6[66] and IL-6 is the main inducer of hepatic production of CRP.[30] In acute MI, IL-6 has been shown to be elevated on admission, peaking 1-2 days after symptom onset, and staying above normal levels for up to three months post-MI.[66] While IL-6 and CRP seem to reflect inflammation at the coronary plaque level in stable patients, in acute MI levels of these inflammatory markers rather seem to reflect the intensity of the inflammatory response to myocardial injury.[19, 21] In ACS, IL-6 seems to be upregulated both systemically[19] and at the site of the culprit coronary lesion.[67] IL-6 levels are strongly associated with poor outcome in ACS.[19, 68] While circulating levels of IL-6 and CRP during acute MI are associated with infarct size,[17] it is not clearly established whether IL-6 mediates or merely reflects myocardial injury in MI. However, evidence suggest that IL-6 could mediate detrimental effects in IR injury, possibly by inducing neutrophil mediated myocardial injury[39, 69-71] and by contributing to the no-reflow phenomenon post-PCI.[67] Furthermore, IL-6 is also associated with PMI in PCI-treated patients.[43] Moreover, IL-6 is associated with adverse LV remodeling and heart failure post-MI,[66, 72] and experimental studies in mice show that inhibiting IL-6 might attenuate this process.[73] IL-6 is also associated with vascular and endothelial dysfunction during ACS, which may contribute to PMI[14] and also increased risk of future ischaemic events.[74]

On this background, inhibiting IL-6 signalling in patients with ACS could potentially contribute to coronary plaque stabilization and to reduce infarct size by attenuating the inflammatory response and potentially by specifically preventing IL-6 mediated injury during IR and PMI.

1.4.3 Tocilizumab

Tocilizumab (RoActemra®) is a recombinant humanized monoclonal antibody of the immunoglobulin G1 subclass directed against sIL-6R and mIL-6R. Tocilizumab prevents

dimerisation of gp130 molecules on the cell membrane and thus blocks IL-6 signal transmission.[75] RoActemra® is approved by the Norwegian Medicines Agency for the treatment of RA and systemic juvenile idiopathic RA. Tocilizumab confers a low risk of antibody production compared with murine or chimeric antibodies because it is humanized.[75]

Iv tocilizumab has been effective and well tolerated in several clinical studies in patients with RA.[75] Pooled data from several large clinical trials have shown that long-term treatment with tocilizumab is associated with increased incidence of respiratory tract infections, headache and increase in liver enzymes. In fact, treatment with tocilizumab causes reversible, moderate increases in serum levels of total cholesterol, low- and high-density lipoprotein cholesterol and triglycerides.[75] However, this does not seem to increase the risk of cardiovascular events in the long term in patients treated with tocilizumab.[76]

Temporary IL-6 inhibition with tocilizumab holds the potential to promote plaque-stabilisation, attenuate IR injury, PMI and reduce infarct size through reduced myocardial inflammation. On the other hand, tocilizumab could theoretically inhibit repair processes within the infarcted area, in particular if administered for a prolonged period. Keeping this in mind, no studies have been conducted regarding the effects of IL-6 inhibition in human atherosclerotic disease, ACS or revascularization of these patients. Therefore, to take into account potentially harmful effects on infarct healing, we chose to include patients with acute NSTEMI with presumably small infarct sizes in this first study in humans. Furthermore, to avoid detrimental effects on the healing process as well as unfavorable pro-atherogenic effects caused by prolonged tocilizumab treatment, we chose to explore the effect of a single iv infusion of a moderate and fixed dose of 280 mg tocilizumab. This regimen provides a temporary, complete IL-6 inhibition in patients with acute NSTEMI for approximately two to three weeks.

2 AIMS OF THE THESIS

2.1 General aims

The general aims of this thesis were to evaluate the effects of short-time IL-6 inhibition with a single iv dose of tocilizumab vs placebo on inflammation, myocardial injury and systemic and coronary vascular function in patients with acute NSTEMI scheduled for coronary angiography. Our hypothesis was that tocilizumab would have attenuating and beneficial effects on inflammation, and secondarily beneficial effects on troponin T release.

2.2 Specific aims

1. To evaluate the ability of a single administration of tocilizumab to modulate CRP during the acute phase of NSTEMI by assessing the area under the curve (AUC) calculated from serum levels measured at seven different timepoints during the first 3 days after inclusion. Similarly, the effect of tocilizumab on troponin T release, a sensitive and specific marker of myocardial injury, was also assessed by AUC during the first 3 days after inclusion (Paper I).

2. To evaluate effects of tocilizumab during the acute phase of NSTEMI on systemic and coronary vascular function as assessed by AUCs for levels of circulating markers of systemic endothelial cell activation (VCAM-1, ICAM-1 and vWF), and coronary flow reserve (CFR) measured during echocardiography (day 2 or 3) (Paper II).

3. To examine the effects of tocilizumab on the cytokine network during the acute phase of NSTEMI, by measuring 27 different cytokines at 7 different timepoints from day 1 to day 3 using a multiplex cytokine assay (Paper III).

3 MATERIAL AND METHODS

3.1 Study design and patients

This study was a two-centre, randomized, double-blind, placebo-controlled trial designed to evaluate the effects of a single dose of the anti-IL-6R antibody tocilizumab in patients with NSTEMI scheduled for coronary angiography. The three papers included in this thesis present results from the same patient cohort.

The study was performed at Oslo university hospital Rikshospitalet, Oslo, and St. Olavs hospital, Trondheim, Norway. The study was approved by the Regional Committee for Medical and Health Research Ethics of South-Eastern Norway and the Norwegian Medicines Agency, and conducted according to the Helsinki Declaration. All participants provided written, informed consent.

Patients admitted to hospital with acute NSTEMI were included on the day of scheduled coronary angiography. Patients between 18 and 80 years of age with NSTEMI presumed to be due to CAD were eligible for inclusion. Major exclusion criteria were clinically significant cardiac disease other than CAD, clinical instability, diseases or medication affecting inflammation, contraindications to tocilizumab, and any condition that could interfere with protocol adherence. A complete list of inclusion- and exclusion criteria is provided in Table 1.

Table 1. Inclusion and exclusion criteria for patients assessed for eligibility for enrollment in a randomized, clinical trial evaluating the effect of interleukin-6 receptor inhibition in patients with acute NSTEMI.

Inclusion criteria
• Clinical type I NSTEMI according to the European Society of Cardiology guidelines.[77]
• Troponin T level at admission ≥ 30 ng/L.
• Scheduled for coronary angiography during hospitalization.
• The patient's age is 18-80 years.
• Patient consent to participation.
Exclusion criteria
• STEMI (ST segment elevation in ECG).
• Previous chronic cardiac disease other than coronary heart disease (i.e., known cardiomyopathy, severe valvular heart disease, chronic heart failure).
• Clinical instability with symptoms/signs of cardiogenic shock.
• Have cardiac arrest.
• Have ventricular fibrillation.
• Have significant concomitant disease known to affect CRP such as infection, malignancies or autoimmune disease.
• Recent (<2 weeks) treatment with immunosuppressive drugs other than non-steroid anti-inflammatory drugs (NSAIDS)
• Severe renal failure with estimated glomerular filtration rate < 30 ml/minutes.
• Pregnancy.
• Other contraindications to study medication (including adenosine).
• Contraindications to MRI (pacemaker, CRT, ICD, certain ferro-magnetic implants, severe claustrophobia, allergy to contrast medium).
• Any condition which interferes with the patient's ability to comply with protocol.

NSTEMI, non-ST-elevation myocardial infarction. STEMI, ST-elevation myocardial infarction. ECG, electrocardiogram. CRP, C-reactive protein. NSAIDs, non-steroidal anti-inflammatory drugs. MRI, magnetic resonance imaging. CRT, chronic resyncronization therapy. ICD, implantable cardioverter defibrillator.

Baseline blood samples were obtained at inclusion. The patients were then randomized to receive a single iv infusion of tocilizumab 280 mg or matching placebo prior to coronary angiography. Patients remained in the study ward for 50 ± 1 hours for post-treatment blood sampling (Day 1: evening; Day 2: morning, afternoon, evening; Day 3; morning, afternoon), transthoracic echocardiography (TTE) and safety assessment. Follow-up with blood sampling, safety assessment and TTE (only 6 months) was performed at three and six months (Table 2). Measurements of CFR during TTE in the acute phase and at 6 months were only performed in patients included at St. Olavs hospital, which represented half of the study population.

Table 2: Baseline and follow-up investigations

Time schedule:	Day 1				Day 2			Day 3		Follow up Months	
	Baseline[1]			10 pm	8 am	3 pm	10 pm	8 am	3 pm	3	6
Informed consent	x										
Clinical status[2]	x	Study drug infusion[7]	Coronary angiography		x			x		x	x
ECG	x				x			x		x	x
Blood samples 1[3]	x			x	x	x	x	x	x	x	x
Blood samples 2[4]	x			x	x			x		x	x
Blood samples 3[5]	x				x			x		x	x
Echocardiography					<---------- x ---------->						x
CFR[6]					<---------- x ---------->						x
Tonometry					<---------- x ---------->						x
MRI					<---------- x ---------->						x

ECG, electrocardiogram. CFR, coronary flow reserve. MRI, magnetic resonance imaging.
1: Baseline investigations performed at a median of 2 days after symptom onset and immediately prior to study drug infusjon
2: Adverse and clinical events
3: high-sensitivity C-reactive protein, high-sensivitity troponin T, markers of endothelial cell activation, cytokines
4: Safety blood samples / other secondary endpoints
5: Tempus blood RNA samples
6: CFR only performed in patients included at St. Olavs hospital, Trondheim
7: Intravenous infusion of tocilizumab 280 mg or placebo (100 ml NaCl 0.9%), duration 1 hour

3.2 Randomization, masking and treatment allocation

A randomization list was generated using a computerized procedure by Unit for Applied Clinical Research (UACR), Faculty of Medicine and Health Sciences, Norwegian University of Science and Technology, Trondheim, Norway. Personnel from UACR were not involved in the rest of the trial. Block randomization with two blocks was used. Before the first patient was included, a complete set of sealed opaque envelopes for all patients were prepared by a person not involved in patient enrolment, treatment or follow-up. The envelopes, marked with patient study number, contained information regarding treatment allocation (tocilizumab or placebo) and study drug preparations. Patients were enrolled by trial personnel who also were involved in patient follow-up. To ensure double-blindness to treatment allocation for patients, clinicians and trial personnel, personnel from a separate ward not involved in patient inclusion, treatment or follow-up opened the sealed opaque envelope with treatment allocation and prepared either the tocilizumab or placebo infusion bag. The tocilizumab and placebo solutions had similar appearances. The study drug infusion

bag was labelled with time, date, patient initials and the inscription “this bag contains either tocilizumab 280 mg or NaCl 0.9 %”. The nurse in charge at the patient ward, blinded to treatment allocation, administered the study drug. Both patients and trial personnel remained blinded throughout the whole trial period beyond follow-up of the last included patient. A second sealed opaque envelope with the treatment allocation was kept available in the Case Report Form in case breaking the randomization code was necessary for serious and/or unexpected complications.

3.3 Drug dose, administration and pharmacological profile of tocilizumab

During the inclusion period, tocilizumab (RoActemra®) was purchased consecutively from the hospitals’ pharmacies and stored at 2-8 °C in a locked refrigerator on site. When a patient was included, a standardized dose of 280 mg tocilizumab was prepared by replacing 14 ml 0.9% NaCl from a 100 ml infusion bag with 14 ml tocilizumab (20 mg/ml), to achieve the recommended dilution of 280 mg/100 ml. The placebo infusion consisted of 100 ml 0.9% NaCl. The infusions were administered intravenously over 1 hour, as recommended by the drug manufacturer (1.67 ml/min).

3.3.1 Pharmacological profile of a single iv dose of 280 mg tocilizumab

The tocilizumab dose was 280 mg irrespective of weight, which resulted in a mean dose of 3.2 (0.5) mg/kg in the treatment arm (range 1.9-4.9 mg/kg). Halflife ($t_{1/2}$) of tocilizumab is dependent on mg/kg dose. Based on previous reports of pharmacokinetics and pharmacodynamics after a first dose of tocilizumab, the mg/kg doses used in this study give a $t_{1/2}$ of ± 86 hrs (3.6 days)[78] and provide complete IL-6 blockade of ± 2 weeks,[78, 79] depending on body weight.

3.4 Endpoints

3.4.1 Paper I

The primary endpoint in paper I was the between-group difference in the AUC for high-sensitivity (hs) CRP during hospitalization (days 1-3). AUC for hsCRP was also the pre-defined overall main endpoint for the trial, and the total number of patients included in this trial was therefore based on power calculations regarding this endpoint. The between-group difference in the AUC for hs troponin T during hospitalization was defined as the most important secondary endpoint. Within-group differences in absolute values, and between-group differences in changes from baseline at separate timepoints during hospitalization were also analyzed. Other secondary endpoints were IL-6 related parameters, N-terminal pro-brain natriuretic peptide (NT-proBNP), routine clinical biochemistry (safety analyses), echocardiographic LV EF and dimensions, and serious adverse events. We also evaluated the impact of tocilizumab treatment on the primary and secondary endpoints at 3 and 6 months follow-up.

3.4.2 Paper II

The primary endpoint in paper II was the between-group difference for CFR during hospitalization, as a measure of coronary microvascular function, which in part also reflects coronary endothelial function. Secondary endpoints were: (1) the between-group difference in the change in CFR from hospitalization to 6 months; (2) the between-group differences in the AUCs for circulating levels of markers of endothelial cell activation (ICAM-1, VCAM-1 and vWF) during hospitalization. We also evaluated the impact of tocilizumab treatment on markers of endothelial cell activation at 3 and 6 months follow-up.

3.4.3 Paper III

The main endpoint in paper III was the temporal development in the cytokine concentrations in the two treatment groups, looking for significant tocilizumab * time interactions. The effects of tocilizumab treatment on 27 different components of the cytokine network during hospitalization were analyzed. We also evaluated the impact of tocilizumab treatment on the cytokine network at 3 and 6 months follow-up.

3.5 Statistics

3.5.1 Power calculation

The power calculation for this trial is based on estimations regarding the trial's overall main endpoint, which was the between-group difference in AUC for hsCRP during hospitalization. Our estimations are based on data from a similar population where CRP was collected less frequently than in the present study, but over a longer period.[80] To observe a 50 % reduction in the AUC for hsCRP on active treatment when compared with placebo, with an α of 5 % and a power of 80 %, we needed a total of 98 patients (49 per group). To allow for drop-outs and analyses of secondary endpoints, we aimed to enrol 120 patients.

3.5.2 Statistical analyses

In all papers, statistical analyses were performed with a per-protocol approach.

AUCs during hospitalization (baseline to day 3) for hsCRP and hs troponin T (Paper I), markers of endothelial cell activation (Paper II) and cytokines (Paper III) were calculated using the trapezoidal rule.[81] For analyses of between-group differences in AUCs during hospitalization (representing main endpoints in Paper I and II), log AUC was compared between the treatment groups using linear regression adjusting for baseline values (i.e. log CRP or log troponin T). A different statistical approach was chosen to evaluate the main

endpoint (i.e. between-group differences for various cytokines during hospitalization analyzed by a multiplex cytokine assay) in Paper III. Several different plates were used for these analyses, and we assumed that while the within-plate precision was good for these biochemical analyses, the between-plate precision was less reliable, and therefore AUC was deemed less suitable to analyze these results. We therefore opted to evaluate between-group differences for the different cytokines during hospitalization with tocilizumab * time interactions using a mixed between-within subjects analysis of variance (ANOVA) test, adjusted for baseline values. For these ANOVA analyses, log transformed values were used to achieve near normalisation.

Changes from baseline were calculated for each timepoint during hospitalization (i.e. timepoint – baseline). Normally distributed variables are expressed as mean ± standard deviation, while most non-normally distributed data were log transformed prior to analyses and are expressed as geometric mean and 95 % confidence intervals. Some skewed data were not normalised with log transformation and are expressed as median and interquartile ranges. Most data were compared with parametric statistics throughout, but skewed data were compared using non-parametric statistics. For differences between two groups at the same timepoint (e.g. "differences in changes-from-baseline in the evening day 2"), either unpaired t-tests or Mann-Whitney U tests were used. For within-group longitudinal data analyses either a one-way ANOVA or a Friedman test was used *a* priori when > 2 timepoints were compared, and if significant followed by a paired t-test or Wilcoxon signed-rank test, respectively, comparing specific timepoints with baseline within each treatment group. For categorical data either the Chi-square test for independence or the Fisher's exact test (preferred if the expected count in at least one of the cells in a 2 x 2 table was < 5) was used. Correlations between variables were evaluated with either the Pearson or the Spearman Rho correlation coefficient depending on distribution. All statistical analyses were performed as two-sided tests, with a p

< 0.05 regarded as significant. All statistical analyses were performed using IBM SPSS Statistics versions 21.0, 22.0, 23.0 and 24.0 (IBM Corp., Armonk, NY, USA).

3.6 Blood sampling protocol

Timepoints for repeated blood sampling in this trial (Papers I-III) is outlined in Table 2. Peripheral venous blood was drawn into endotoxin free blood collection tubes with EDTA as anticoagulants (plasma) and with no additives (serum). The EDTA tubes were immediately placed on melting ice and centrifuged within 30 minutes at > 2500 × *g* for 20 minutes to obtain platelet-poor plasma. Serum was centrifuged at > 2100 × *g* for 15 minutes after full coagulation (~ 45 minutes) in room temperature. Immediately following centrifugation, both plasma and serum aliquots were stored at -80 °C until analyses in multiple aliquots.

Whole blood (3 ml) was collected in Tempus Blood RNA tubes (ThermoFischer, Paisley, UK) and stored at -80 °C.

3.7 Biochemical analyses

3.7.1 Paper I

Serum levels of hsCRP was analyzed on a MODULAR platform (Roche Diagnositics, Basel, Switzerland). Serum levels of hs troponin T was measured by a electrochemiluminescence immunoassay (ELICA; Elecsys 2010 analyzer, Roche Diagnostics).

Serum levels of IL-6, sIL-6R and sgp130 were analyzed using enzyme immunosassays (EIA) (R&D Systems, Minneapolis, MN). Assay sensitivities were 0.4 pg/ml (IL-6), 3.5 pg/ml (sIL-6R) and 40 ng/ml (sgp130).

Safety analyses (haemoglobin, platelets, leucocytes, neutrophils, total cholesterol, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, triglycerides, aspartate

aminotransferase, alanine aminotransferase, creatinine) and NT-proBNP were analyzed consecutively during inclusion using routine methods at the hospitals' clinical biochemistry laboratories.

3.7.2 Paper II

Serum levels of ICAM-1 and VCAM-1 were measured using enzyme immunoassays (R&D Systems, Minneapolis, MN). Serum levels of vWF was measured by an enzyme immunoassay with antibodies from Dako Cytomation (Glostrup, Denmark). The intra- and inter-assay co-efficient of variation was less than 10 % for all assays.

Results from analyses on hsCRP and hs troponin T performed for Paper I were also used in Paper II.

3.7.3 Paper III

Components of the cytokine network were analyzed in plasma according to the manufacturer's protocol. We used a multiplex cytokine assay (Bio-Plex Pro™ Human Cytokine Plex-27 Assay, Bio-Rad Laboratories, Hercules, CA) that quantifies IL-1β, IL-1 receptor antagonist (IL-1ra), IL-2, IL-4, IL-5, IL-7, IL-8/CXCL-8, IL-9, IL-10, IL-12 (p70), IL-13, IL-15, IL-17, basic fibroblast growth factor (bFGF), granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon-gamma (IFN-ɣ), eotaxin/CCL11, IFN-ɣ-inducible protein (IP-10)/CXCL10, macrophage chemoattractant protein (MCP-1)/CCL2, macrophage inflammatory protein-1α (MIP-1α/CCL3), MIP-1β/CCL4, regulated on activation, normal T-cell expressed and secreted (RANTES)/CCL5, tumor necrosis factor (TNF), platelet-derived growth factor (PDGF), and vascular endothelial growth factor (VEGF). Inter-assay and intra-assay co-efficient of variations were < 15 and < 11 for all analytes, respectively.

Additional analyses in plasma of IFN-α2a, IFN-β, and IL-29/IFN-λ were performed with a U-plex Interferon Combo kit (human), Cat. No K15094 K-1, obtained from Meso Scale Diagnostics, LLC, 1601 Research Blvd, Rockville, MD 20850 USA. The chemiluminescence was quantified using MESO® QuickPlex SQ 120 (Meso Scale Diagnostics).

Total RNA was isolated from whole blood at Aaros Applied Biotechnology, Aarhus, Denmark, and stored at -80 °C. Complementary DNA (cDNA) was synthesised using High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). Quantification of gene expression of IP-10 and MIP-1β was performed in duplicate using the ABI Prism 7900HT (Applied Biosystems), Perfecta SYBR Green Fastmix ROX (Quanta Bioscience), and sequence-specific PCR primers designed using the Primer Express software, version 3.0 (Applied Biosystems). Gene expression of the housekeeping gene ribosomal protein large p0 (RPLP0) was used for normalisation.

Results from analyses on hsCRP, hs troponin T and neutrophils performed for Paper I were also used in Paper III.

3.8 Echocardiography and coronary flow reserve

TTE was performed in all patients using a M5S-probe on a Vivid E9 XDclear (GE Vingmed Ultrasound, Horten, Norway) ultrasound system. All images were analyzed offline by an observer blinded to treatment group and baseline/follow-up status. EF was measured by the biplane Simpson method.

3.8.1 Transthoracic coronary flow measurements (Paper II)

CFR with adenosine reflects both endothelial-dependent and endothelial-independent vasodilation.[82] The coronary endothelial and microvascular dysfunction affects the LV globally, with the left anterior descending artery (LAD) being the most important coronary

vessel determining the LV function.[83] Also, the feasibility of CFR measurements in the LAD is superior to other coronary arteries.[84] Thus, we evaluated CFR only in the mid to distal LAD by TTE using a Vivid E9 XDclear (GE Vingmed Ultrasound, Horten, Norway) ultrasound system connected to a M5Sc-D transducer, using spectral Doppler signals with the use of iv adenosine (0.14 mg/kg/min, within 2 min) to induce hyperaemia.[84] CFR expresses the ratio of hyperaemic (measured during adenosine) to basal peak (measured prior to adenosine) diastolic coronary flow velocities. CFR was measured at least twice in each patient per visit, recording at least three consecutive cardiac cycles to average flow velocities both before and during during adenosine infusion. The best series was used for the CFR value. Coronary microvascular dysfunction was defined as CFR < 2.5.

4 SUMMARY OF RESULTS

A total of 121 patients with NSTEMI were enrolled in this trial between August 2011 and November 2013. Of these, 117 patients (placebo n=59, tocilizumab n=58) were included in the final analyses. An overview of the screening and inclusion process is provided in Figure 5. Follow-up ended in April 2014. Details regarding study drop-outs (placebo n=3, tocilizumab n=3) are provided in Table 3. Among these, one patient in the tocilizumab group suffered prolonged cardiac arrest during coronary angiography on the first day of inclusion, and subsequently severe clinical instability and eventually in-hospital death. This led to the randomization code being broken at day 2 after inclusion for safety reasons. This patient suffered from critical coronary 3-vessel disease. The safety committee opted to temporarily halt the study while reviewing this event. However, the safety monitoring board concluded that the patient's death was as a result of the critical CAD, and could not be viewed as a likely complication of tocilizumab treatment. The two patients who withdrew from the study during follow-up, were included in statistical analyses for as long as they were followed (during hospitalization and 3 months follow-up, respectively).

The majority of patients (75 %) were initially admitted to their local hospital and subsequently transferred to the trial centres (OUS Rikshospitalet or St. Olavs hospital) for scheduled coronary angiography within 72 hours. The remaining patients were directly admitted to either OUS Rikshospitalet or St. Olavs hospital. Patients were included in the study at the trial centres on the day of scheduled coronary angiography. Thus, the study

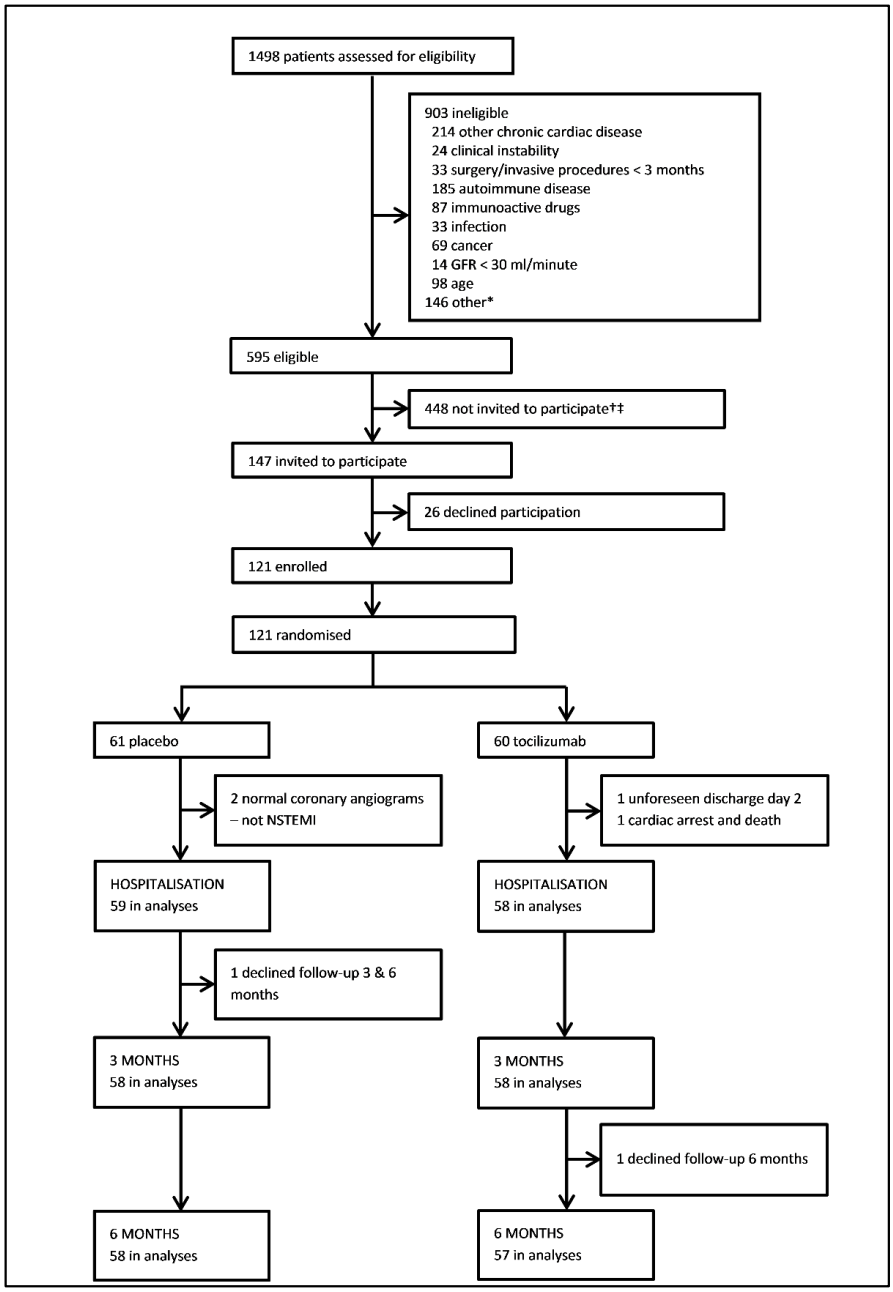

Figure 5 Flowchart for the screening, inclusion process and analyses of NSTEMI patients admitted to Oslo university hospital Rikshospitalet and St. Olavs hospital between August 11, 2011 and November 13, 2013, and follow-up until April 28, 2014. *Absolute/relative contraindications to tocilizumab and/or magnetic resonance imaging; deemed unable to comply with protocol (e.g. alcohol/drug abuse, dementia, significant language barrier); recent significant trauma; already included in current trial; included in other trials. †Too long travel distance to attend follow-up visits. ‡Not able to include all eligible patients on same day or week. NSTEMI, non-ST-elevation myocardial infarction. GFR, glomerular filtration rate. *Kleveland O, Kunszt G, Bratlie M, Ueland T, Broch K, Holte E, Michelsen AE, Bendz B, Amundsen BH, Espevik T, Aakhus S, Damås JK, Aukrust P, Wiseth R, Gullestad L. Effect of a single dose of the interleukin-6 receptor antagonist tocilizumab on inflammation and troponin T release in patients with non-ST-elevation myocardial infarction: a double-blind, randomized, placebo-controlled phase 2 trial. Eur Heart J 2016;37(30):2406-13. By permission of Oxford University Press.*

Table 3. Details on patient drop-outs / exclusions.

Patient	Group	Drop-out phase	Reason for drop-out / exclusion
1	Placebo	Hospitalization	Normal CA and deemed not to suffer from NSTEMI.
2	Placebo	Hospitalization	Normal CA and deemed not to suffer from NSTEMI.
3	Tocilizumab	Hospitalization	Patient unintentionally transferred to local hospital on day 2, initiated by personnel not aware of the patient's trial participation. Not available for further follow-up.
4	Tocilizumab	Hospitalization	Prolonged cardiac arrest during CA. Subsequent instability and death. Randomization code was broken on day 2 for safety reasons. The safety monitoring board concluded that death was due to critical 3-vessel coronary heart disease and not related to tocilizumab treatment.
5	Placebo	3 months	Withdrew from trial due to personal reasons.
6	Tocilizumab	6 months	Withdrew from trial due to personal reasons.

CA, coronary angiography. NSTEMI, non-ST-elevation myocardial infarction. *Kleveland O, Kunszt G, Bratlie M, Ueland T, Broch K, Holte E, Michelsen AE, Bendz B, Amundsen BH, Espevik T, Aakhus S, Damås JK, Aukrust P, Wiseth R, Gullestad L. Effect of a single dose of the interleukin-6 receptor antagonist tocilizumab on inflammation and troponin T release in patients with non-ST-elevation myocardial infarction: a double-blind, randomized, placebo-controlled phase 2 trial. Eur Heart J 2016;37(30):2406-13. By permission of Oxford University Press.*

design allowed for a wide distribution of time from symptom onset to inclusion, and patients were included at a median of 2 days after symptom onset in both treatment groups (Table 4). Patients were also well matched with regard to other baseline characteristics (Table 4). Myocardial damage prior to study inclusion was modest in both treatment groups. Pharmacological treatment was provided in adherence to prevailing guidelines.[77] We performed coronary angiography in all patients. PCI was performed in 47 patients (80 %) allocated to placebo and 41 patients (71 %) allocated to tocilizumab. In no patients coronary artery bypass grafting was performed during the first 3 days following inclusion, meaning that patients in need of surgery had this performed subsequent to the repeated blood sampling during hospitalization from baseline to day 3. While TTE was performed in all patients (placebo n=59, tocilizumab n=58), additional CFR-measurements, presented in Paper II, were only performed in patients included at St. Olavs hospital (placebo n=22, tocilizumab n=20).

Table 4. Baseline Characteristics according to Treatment Group

	Placebo (n=59)	Tocilizumab (n=58)	*P*-Value
Age, y, mean (SD)	60.1 (9.9)	59.8 (7.7)	0.859
Female, n (%)	5 (8.5)	9 (15.5)	0.364
Body mass index, kg/m^2, mean (SD)	27.4 (4.4)	28.8 (3.3)	0.055
Blood pressure, systolic, mm Hg, mean (SD)	136.8 (18.0)	139.7 (18.1)	0.389
Blood pressure, diastolic, mmHg, mean (SD)	80.5 (12.1)	82.9 (12.0)	0.273
Hypertension, n (%)	17 (28.8)	26 (44.8)	0.109
Diabetes Mellitus, n (%)	10 (16.9)	11 (19.0)	0.966
Current Smoking, n (%)	17 (28.8)	15 (26.3)	0.926
Previous Myocardial Infarction, n (%)	7 (11.9)	9 (15.5)	0.760
GRACE score, mean (SD)	90.3 (20.2)	86.3 (19.1)	0.272
Symptom onset to inclusion, days, median (25th, 75th percentiles)	2 (1, 3)	2 (1, 3.5)	0.197
Maximum TnT before baseline, ng/L, n=85, geometric mean (CI)	310 (214-449)	334 (217-514)	0.789
Maximum TnI before baseline, ng/L, n=31, geometric mean (CI)	1571 (517-4773)	2221 (826-5976)	0.626
Aspirin, n (%)	59 (100)	57 (98.3)	0.496
Clopidogrel, n (%)	32 (54.2)	32 (55.2)	1.0
Ticagrelor, n (%)	27 (45.8)	26 (44.8)	1.0
Low molecular weight heparin, n (%)	54 (91.5)	51 (89.5)	0.952
Statin, n (%)	53 (89.8)	53 (91.4)	1.0
Betablocker, n (%)	45 (76.3)	45 (77.6)	1.0
PCI, n (%)	47 (79.7)	41 (70.7)	0.367
Stents per PCI-treated patient, mean (SD)	1.85 (1.28)	1.85 (1.15)	0.982
CABG, n (%)	7 (11.9)	6 (10.3)	1.0
Medical treatment, n (%)	5 (8.5)	11 (19.0)	0.167
SYNTAX score, median (25th, 75th percentiles)	8.0 (5, 14)	8.0 (4, 18)	0.629

TnT, troponin T. TnI, troponin I. PCI, percutaneous coronary intervention. CABG, Coronary Artery Bypass Grafting. *Kleveland O, Kunszt G, Bratlie M, Ueland T, Broch K, Holte E, Michelsen AE, Bendz B, Amundsen BH, Espevik T, Aakhus S, Damås JK, Aukrust P, Wiseth R, Gullestad L. Effect of a single dose of the interleukin-6 receptor antagonist tocilizumab on inflammation and troponin T release in patients with non-ST-elevation myocardial infarction: a double-blind, randomized, placebo-controlled phase 2 trial. Eur Heart J 2016;37(30):2406-13. By permission of Oxford University Press.*

4.1 Paper I

In Paper I, the main objectives were to evaluate the effects of a single dose of tocilizumab 280 mg on inflammation and myocardial injury in patients hospitalized with acute NSTEMI, as reflected by serum levels of hsCRP (main endpoint) and hs troponin T (most important secondary endpoint), respectively. Other secondary endpoints were IL-6 related parameters, leucocytes with subpopulations, NT-proBNP, echocardiographic LV dimensions and EF, safety measures (lipid profile, liver function, general biochemistry), and clinical events.

Effects on CRP and troponin T release

The AUC for hsCRP during hospitalization (baseline to day 3) was 2.1 times higher in the placebo than in the tocilizumab group (4.2 vs 2.0 mg/L/hr, p<0.001). While CRP levels increased significantly in the placebo group, we observed a substantial decrease in tocilizumab treated patients during hospitalization (Figure 6).

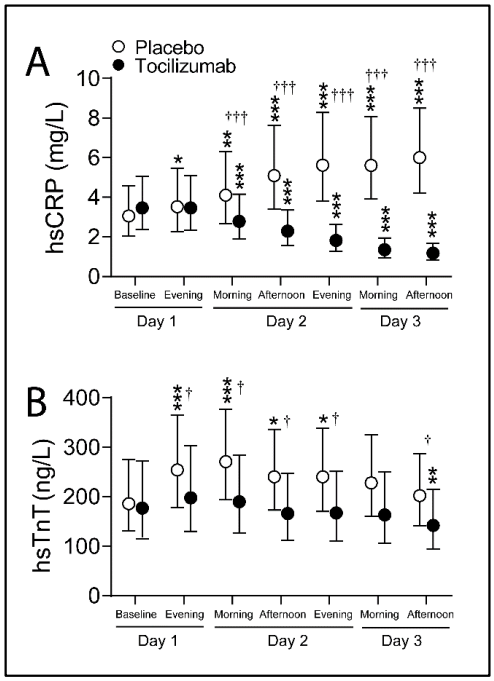

Figure 6 Serum levels of high-sensitivity C-reactive protein (hsCRP) (A) and high-sensitivity troponin T (hsTnT) (B) in patients with non-ST-elevation myocardial infarction receiving placebo (n=59) or tocilizumab (n=58) during hospitalization. Circles and bars represent geometric means and 95 % confidence intervals, respectively. *p < 0.05, **p < 0.01, ***p < 0.001 vs baseline. †p < 0.05, ††p < 0.01, ††† p < 0.001 comparing differences in changes from baseline between placebo and tocilizumab. *Kleveland O, Kunszt G, Bratlie M, Ueland T, Broch K, Holte E, Michelsen AE, Bendz B, Amundsen BH, Espevik T, Aakhus S, Damås JK, Aukrust P, Wiseth R, Gullestad L. Effect of a single dose of the interleukin-6 receptor antagonist tocilizumab on inflammation and troponin T release in patients with non-ST-elevation myocardial infarction: a double-blind, randomized, placebo-controlled phase 2 trial. Eur Heart J 2016;37(30):2406-13. By permission of Oxford University Press.*

The AUC for hs troponin T was 1.5 times higher in the placebo compared to the tocilizumab group (234 vs 159 ng/l/hr, p=0.007) during hospitalization. While we observed a significant rise in hs troponin T levels in the placebo group from baseline through days 1 and 2, no significant increase of hs troponin T was observed in the tocilizumab group (Figure 6).

Further analyses showed that even though tocilizumab attenuated CRP levels significantly during hospitalization regardless of revascularization therapy, the inflammatory response was clearly more intense, and the between-group differences in CRP levels were more pronounced in patients treated with PCI. Furthermore, the significant reduction in hs troponin T release was observed in PCI-treated patients only. Moreover, only patients included early with regard to symptom onset (<2 days) experienced a significant reduction of hs troponin T release on tocilizumab treatment. However, also in the early included patients, PCI seemed to be the main driver behind tocilizumab's attenuating effect on troponin T release. In contrast, the effect of tocilizumab in terms of CRP reduction remained unaffected by time from symptom onset.

Levels of hsCRP and hs troponin T returned to normal, and no between-group differences were observed for these biomarkers at 3 and 6 months follow-up.

IL-6 related parameters and leucocyte counts

While tocilizumab treatment had no impact on levels of IL-1β, a cytokine which is a major upstream inducer of IL-6 in the inflammatory cascade,[34, 35, 57] there were significant between-group differences for IL-6 and sIL-6R during hospitalization. While IL-6 increased moderately in the placebo group, we observed a rapid and substantial increase in the tocilizumab group through hospitalization. sIL-6R remained stable in the placebo group, but as for IL-6, sIL-6R increased substantially in the tocilizumab group during hospitalization. IL-6 and sIL-6R were lower compared to baseline and similar in both treatment groups at 3 and 6 months follow-up.

Compared to placebo, tocilizumab treatment also led to a significant fall in leucocyte counts, which predominantly reflected a substantial fall in neutrophils which remained stable throughout hospitalization. Leucocyte and neutrophil counts became normalised in the tocilizumab group and there were no between-group differences at 3 and 6 months.

Correlations between key biomarkers

During hospitalization we observed significant positive correlations between changes from baseline for hsCRP and hs troponin T, while we observed an inverse correlation between changes from baseline for hsCRP and IL-6 in the tocilizumab group.

NT-proBNP and cardiac function

NT-proBNP levels were similar and modestly elevated in both treatment groups at baseline, and no between-group differences were observed during hospitalization or follow-up. Accordingly, echocardiographic LV EF and dimensions were normal and similar in both treatment groups during hospitalization and follow-up.

Safety and clinical events

During hospitalization we observed a significant between-group difference in changes from baseline for alanine aminotransferase (ALT), reflecting a modest increase in the tocilizumab group. However, there were no between-group differences for ALT at 3 and 6 months, and levels for aspartate aminotransferase and lipid parameters were the same in both treatment groups during hospitalization and follow-up.

There were no between-group differences in clinical events from baseline through follow-up at 6 months. There were no significant differences in the occurrences of major adverse cardiovascular events (placebo n=6, tocilizumab n=3, p=0.490) or serious infections (placebo n=4, tocilizumab n=3, p=0.717).

Key findings: A single iv dose of 280 mg tocilizumab attenuated the inflammatory response as reflected by CRP in patients hospitalized with acute NSTEMI. Tocilizumab also attenuated

PCI-related troponin T release in these patients. No apparent safety issues were detected in the tocilizumab group during follow-up.

4.2 Paper II

In this pre-defined sub-study, we sought to examine the effects of tocilizumab on coronary microvascular dysfunction by CFR measured by TTE and circulating markers of endothelial cell activation during hospitalization and follow-up for NSTEMI.

Markers of endothelial cell activation were analyzed in a total of 117 patients (placebo n=59, tocilizumab n=58) and CFR was measured in 42 of these patients (placebo n=20, tocilizumab n=22). The infarct related artery was LAD in 12 out of 42 patients, with no significant between-group differences in distribution.

There were no between-group differences in CFR during hospitalization or 6 months. CFR increased significantly in both treatment groups after 6 months follow-up with no between-group difference in changes in CFR from hospitalization. Coronary microvascular dysfunction defined as CFR < 2.5 was present in 10 of 42 patients (24 %) during hospitalization. Whereas CFR improved significantly at 6 months in patients without initial coronary microvascular dysfunction, this was not observed in patients with coronary microvascular dysfunction during hospitalization.

Adjusting for baseline VCAM-1, the AUC for VCAM-1 during hospitalization was significantly higher in the tocilizumab group than in the placebo group (median 622 vs 609 ng/ml/hr, tocilizumab and placebo respectively). The patterns of markers of endothelial cell activation in the subset of patients evaluated for CFR (placebo n=20, tocilizumab n=22) were similar to the patterns observed for the total population (placebo n=59, tocilizumab n=58). In the placebo group, there was a strong and inverse correlation between VCAM-1 and CFR

during hospitalization. In contrast, there were no significant correlations between markers of endothelial cell activation and CFR in the tocilizumab group during hospitalization.

There also was a strong inverse correlation between VCAM-1 and CFR in the placebo-treated patients at 6 months, and at follow-up we also observed a strong inverse correlation between vWF and CFR in the placebo group. In the tocilizumab group, however, no significant correlations between CFR and markers of endothelial cell activation were found at follow-up. In patients evaluated for CFR, there were positive and similar correlations between hsCRP and hs troponin T in both treatment groups during hospitalization, but not at follow-up. There were no relevant correlations between hsCRP or hs troponin T and CFR or markers of endothelial cell activation during hospitalization or at 6 months follow-up.

When we evaluated CFR in patients without and with LAD as the infarct related artery separately, there was still no significant between-group differences in CFR during hospitalization or in the change from hospitalization to 6 months. However, in the placebo group, the associations between the different markers of endothelial cell activation and the association between these markers and CFR were enhanced when we excluded patients with LAD as the culprit artery from the analyses. Notably, this was not observed among tocilizumab treated patients.

Key findings: In the acute phase of NSTEMI 24 % of the patients had coronary microvascular dysfunction defined as CFR < 2.5. Tocilizumab did not affect CFR during hospitalization or at 6 months. Moreover, VCAM-1 was significantly increased in the tocilizumab group during the acute phase of NSTEMI. In the placebo group, but not in the tocilizumab group, there was an inverse correlation between VCAM-1 and CFR.

4.3 Paper III

In paper III, we explored the effects of tocilizumab treatment on the cytokine network in the acute phase of NSTEMI and during follow-up.

27 different cytokines were analyzed with a multiplex cytokine assay. However, IL-2, IL-15 and PDGF were excluded from further statistical analyses as these cytokines had > 10 % of measured concentrations out-of-lower-range (OOR). To avoid loss of low level data, values OOR were set to 1.0 pg/ml for all other cytokines, except for IL-1β for which values OOR were set to 0.5 pg/ml. Even though IL-8 had 15 % of concentrations OOR, IL-8 was still included in statistical analyses as all values OOR stemmed from a single malfunctional plate, and no values from any of the other plates were OOR.

While most cytokines remained unaffected by tocilizumab treatment, we observed significant between-group differences for IP-10 and MIP-1β during the acute phase (baseline through day 3), representing a rapid and substantial increase in tocilizumab treated patients (Figure 7). Levels for IP-10 and MIP-1β remained stable compared to baseline in the placebo group. A significant, but more subtle, between-group difference during hospitalization was also observed for IL-8, reflecting a significant decrease in the placebo group. Also, the finding of a substantial increase for IL-6 in the tocilizumab group in analyses performed for paper I, was replicated by the multiplex cytokine assay. The effects of tocilizumab on IP-10 and MIP-1β levels during hospitalization were not affected by revascularization therapy or time from symptom onset.

At 3 and 6 months follow-up, levels of most cytokines were significantly lower compared to baseline. However, different patterns were observed for IP-10 and MIP-1β. Irrespective of treatment allocation, IP-10 increased at 3 months compared to baseline, but returned to baseline levels at 6 months. At 3 and 6 months, levels of MIP-1β were similar to baseline in both the tocilizumab and the placebo group.

During hospitalization, we observed strong and positive correlations between AUCs for most cytokines in both treatment groups. However, while IL-6 was positively correlated to all cytokines in the placebo group except for IP-10 and MIP-1β, all correlations to other cytokines were lost in the tocilizumab group. Furthermore, IP-10 and MIP-1β were positively correlated to only a moderate (IP-10) or few (MIP-1β) number of other cytokines in the

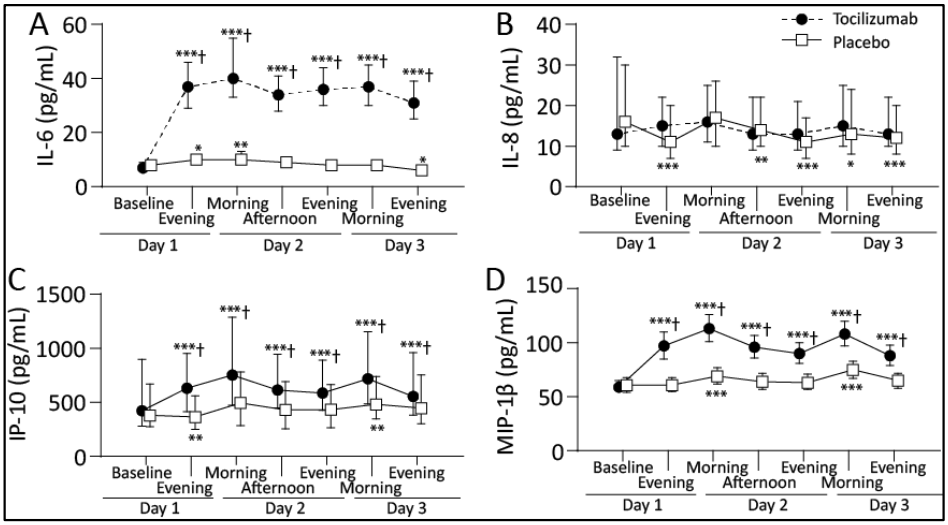

Figure 7 Interleukin-6 (IL-6), interferon-gamma inducible protein/CXCL10 (IP-10), macrophage inflammatory protein-1β (MIP-1β) (all placebo n=59, tocilizumab n=58), and IL-8 (placebo n=49, tocilizumab n=46) during hospitalization in patients with non-ST-elevation myocardial infarction. Circles and squares represent geometric means (IL-6 and MIP-1β) and median values (IL-8 and IP-10), bars represent 95 % confidence intervals (IL-6 and MIP-1β) and 25th and 75th percentiles (IL-8 and IP-10). *p < 0.05, **p < 0.01, ***p < 0.001 vs baseline within group. †p < 0.001 for between-group differences in changes from baseline at separate timepoints. *Kleveland O, Ueland T, Kunszt G, Bratlie M, Yndestad A, Broch K, Holte E, Ryan L, Amundsen BH, Bendz B, Aakhus S, Espevik T, Halvorsen B, Mollnes TE, Wiseth R, Gullestad L, Aukrust P, Damås JK. Interleukin-6 receptor inhibition with tocilizumab induces a selective and substantial increase in plasma IP-10 and MIP-1β in non-ST-elevation myocardial infarction. Int J Cardiol 2018.*

placebo group, and even fewer (IP-10) or no other than IP-10 (MIP-1β) in the tocilizumab group.

We also checked associations between AUCs for IP-10 and MIP-1β, and hsCRP, hs troponin T and neutrophils in these patients during the acute phase. While IP-10 was significantly and positively correlated with hsCRP in the placebo group, we found significant inverse correlations between MIP-1β and hs troponin T and neutrophils in the tocilizumab group.

To explore potential mechanisms behind the rise in IP-10 and MIP-1β during hospitalization in the tocilizumab group, further analyses were performed on type I and III interferons, as well as mRNA levels in peripheral blood for IP-10 and MIP-1β. Levels of IFN-α, IFN-β and IL-29 were not affected by tocilizumab treatment, and except for a weak and inverse correlation between MIP-1β and IFN-α, there were no correlations between AUCs for these interferons and IP-10 or MIP-1β in the tocilizumab group. mRNA levels in peripheral blood were significantly elevated for IP-10 in the tocilizumab compared to the placebo group during hospitalization, while no between-group differences were observed for MIP-1β.

Key findings: Except for IP-10 and MIP-1β, most cytokines were upregulated during the acute phase of NSTEMI compared to follow-up. However, tocilizumab induced a selective and substantial increase for IP-10 and MIP-1β during the acute phase in patients with NSTEMI. Increased peripheral production seems to contribute to the increased levels observed for IP-10 in tocilizumab treated patients. In contrast to the majority of cytokines, IP-10 and MIP-1β were poorly correlated to other components in the cytokine network during acute NSTEMI. However, MIP-1β was inversely correlated to neutrophils and hsTnT in the tocilizumab group.

5 DISCUSSION

The work in this thesis is based on a randomized, double-blind, placebo controlled phase II trial evaluating the effects of IL-6 inhibition with a single iv dose of the IL-6R antagonist tocilizumab 280 mg during hospitalization and follow-up of patients with acute NSTEMI. The main findings during this work were the following:

1. Tocilizumab attenuated the inflammatory response as reflected by CRP levels, and reduced PCI-related troponin T release (Paper I).

2. Tocilizumab did not affect coronary microvascular and endothelial function as reflected by CFR during hospitalization or follow-up. Tocilizumab induced a modest increase in circulating levels of VCAM-1, a marker of endothelial cell activation, during hospitalization, while circulating ICAM-1 and vWF remained unaffected by tocilizumab (Paper II).

3. Tocilizumab exerted a modest and selective impact on the cytokine network during the acute phase of NSTEMI, with a rapid, substantial and stable increase in plasma levels of chemokines IP-10 and MIP-1β during hospitalization (Paper III).

To summarize, a single dose of tocilizumab exerted potentially beneficial effects in the acute phase of NSTEMI by attenuating inflammation and PCI-related troponin T release. However, these beneficial effects do not seem to be mediated through acute effects on vascular or endothelial function. The modest impact on the cytokine network during the acute phase, and the uncertain relevance of the increase in IP-10 and MIP-1β, suggest that the potentially beneficial effects of tocilizumab in NSTEMI may be directly attributed to IL-6 inhibition, and not to secondary effects on other cytokines.

5.1 Drug dose and route of administration

In approved clinical use for the treatment of autoimmune diseases such as RA, the iv dosage of tocilizumab is weight-based.[75] Furthermore, the most efficient dose in terms of CRP-reduction and clinical response in patients with RA is 8 mg/kg.[79] In contrast, in this trial we used a fixed and moderate iv dose of 280 mg tocilizumab (i.e. 4 mg/kg in a patient weighing 70 kg). We chose this fixed and moderate dose for three reasons: (i) for simplicity during the inclusion; (ii) to examine if the effects of tocilizumab were dependent on dose per kg body weight; (iii) for safety reasons, as this was the first trial in humans regarding the effects of tocilizumab in acute MI, we had to take into account potentially unwanted effects of IL-6 blockade on infarct healing and LV remodeling as a result of prolonged IL-6R inhibition.

A weight-based dose of tocilizumab could be the more logical choice, as the effect of tocilizumab, at least in the long term, is dependent on the patients' weight.[79] However, in the current trial we found no correlation between the effects of tocilizumab on CRP or troponin T, as the two most important endpoints, and body weight. Furthermore, as the primary aim of this study was to provide and evaluate short-term (acute phase, days 1-3) IL-6 blockade with a single dose of tocilizumab in NSTEMI, an exact dose of tocilizumab in terms of mg/kg becomes less important. This is because the dose range according to the patients' weight and the fixed dose of 280 mg in this study (1.9 – 4.9 mg/kg) will provide > 95 % IL-6 blockade in all patients regardless of weight during the period of primary interest for the study (the acute phase, days 1-3). This is supported by a previous study which showed that as long as tocilizumab is detectable in serum (> 1 μg/ml), > 95 % of membrane-bound as well as soluble IL-6 receptors will be blocked by tocilizumab. Furthermore, the same study showed that after a first (i.e. single) dose of tocilizumab 2 mg/kg, tocilizumab will remain detectable in serum for up to two weeks.[78] Accordingly, in the current trial, where we showed that tocilizumab caused a firm suppression of CRP, we found no correlation between the percentage change in

CRP and weight in tocilizumab treated patients during the acute phase, suggesting that the patients' weight and differences in mg/kg doses did not have any significant impact on the degree of IL-6 blockade in these patients during the acute phase. Thus, even though doses in terms of mg/kg varied between patients, they all seemed to have similar effect in terms of CRP reduction during the acute phase, as expected and in accordance with the pharmacological findings in the aforementioned study.[78]

While all patients seemed to achieve total IL-6 blockade regardless of weight with the iv 280 mg dose during the acute phase, weight clearly determines the duration of IL-6 blockade.[75, 78] This could be important in patients with MI. It is reasonable to assume that the patients with the lowest weight in this study were subjected to a significantly longer period of IL-6 blockade compared to others. The design, size and population of this trial, unfortunately makes it incapable of clarifying the optimal length of IL-6 blockade in the aftermath of acute MI. It has to be emphasized that the patients included had small MIs with normal LV function on echocardiography. It is possible that IL-6 exerts different pro- vs anti-inflammatory effects in different phases of the inflammatory response and repair/healing process after acute MI.[38] Given the observed reduction of PCI-related troponin T-release, the current trial suggests that inhibiting IL-6 immediately before myocardial injury is beneficial. However, IL-6 might execute beneficial anti-inflammatory effects in the subsequent resolution phase of MI-related inflammation,[38] and IL-6 inhibition in this phase could potentially have adverse effects with regard to e.g. scar formation. Thus, it is possible that a more patient-tailored dose of tocilizumab in mg/kg, with a more predictable duration of action compared to a fixed dose, could be the right strategy to provide the "optimal surgical cut" with regard to IL-6 activity during the inflammatory response in acute MI. These temporal aspects need to be considered in future studies of tocilizumab and all other anti-inflammatory interventions during MI. Keeping this in mind, a single iv dose as low as 1 mg/kg to provide a fixed and shorter

duration of IL-6 inhibition could potentially be the optimal tocilizumab treatment during acute MI, in contrast to the higher doses needed to achieve significant effects in chronic autoimmune diseases.[75, 79] Future studies need to address this.

In order to achieve desired immediate effects on inflammatory mediated IR injury or PMI in acute MI, choice of drug administration route needs consideration. By using iv administration of tocilizumab (the only option available at the time), we observed immediate effects (< 12 hours from baseline) on both CRP and troponin T levels in the current trial (Figure 2). Interestingly, the short-term application of tocilizumab during myocardial infarction (STAT-MI) trial recently evaluated the effects of a single sc administration of 162 mg tocilizumab < 24 hours after symptom onset in patients with acute STEMI or NSTEMI.[85] A major limitation of the STAT-MI trial is its' premature termination resulting in only 28 included patients (placebo n=16, tocilizumab n=12). Nevertheless, an interesting and rather surprising observation from the STAT-MI trial, is that CRP levels in fact were elevated compared to baseline in the tocilizumab group 36 hours after drug administration. This is the opposite of what was observed in the current trial. This elevation of CRP after 36 hours in the tocilizumab group, could reflect that significant IL-6 inhibition had not been achieved at that timepoint in the STAT-MI trial. Thus, sc administration of tocilizumab, compared to iv administration, does not seem to provide the necessary rapid IL-6 blockade to affect inflammation and potentially related myocardial injury within the desired timeframe. While this could be caused by the smaller dose used in the STAT-MI trial, it is more likely due to differences in the pharmacological profiles of iv vs sc administration.[86] Taken together, the current and the STAT-MI trial suggest that iv administration of tocilizumab should be the route of choice when aiming to achieve immediate effects on inflammation and myocardial injury in acute MI or PMI.

5.2 Effects of tocilizumab on troponin T release

In this trial, attenuated troponin T release during the acute phase of NSTEMI was demonstrated. Interestingly, in both treatment groups, there were positive associations between both absolute values and changes from baseline values for CRP and troponin T during the acute phase. These findings suggest a link between the degree of inflammation and the degree of myocardial injury in these patients.

Importantly, the effect of tocilizumab on troponin T release only occurred in patients treated with PCI during hospitalization. Thus, these results suggest that IL-6 inhibition has beneficial effects on PMI in patients with NSTEMI. However, the attenuation of troponin T release in tocilizumab treated patients was modest, and the clinical significance of this effect is uncertain and needs further clarification. Even though this trial only showed effect on troponin T release in PCI-treated patients, it is important to emphasize that the design of the study was not suited to exclude that IL-6 inhibition also can have an effect on the primary myocardial injury in NSTEMI, caused by spontaneous plaque rupture and thrombosis. The reason for this, is that patients were included on the day of scheduled coronary angiography at a median of 2 days after symptom onset, a timepoint when the primary myocardial injury from the acute NSTEMI already could have occurred. Thus, with regard to troponin T release, one could view this trial as an investigation of the effects of tocilizumab on PCI-related troponin T release in the *context* of acute NSTEMI, rather than the effects on troponin T release caused by the NSTEMI itself. To be able to explore tocilizumab's effect on the primary myocardial injury in MI, future studies should exclusively include patients with a short time (e.g. < 6 hours) from symptom onset to drug infusion.

5.3 Effects of tocilizumab on inflammation

While the effects on troponin T release was modest, the CRP reduction in the tocilizumab group was substantial during the acute phase. As IL-6 is the major inducer of CRP-production,[30] this finding was to some extent expected. Importantly, this study also showed that tocilizumab had significant acute effects on other inflammatory parameters beyond CRP during the acute phase of NSTEMI, which might be of further relevance to the finding of reduced PMI in these patients. Tocilizumab caused a substantial increase in serum IL-6 and sIL-6R levels, a profound fall in neutrophil counts, and a substantial and stable increase in chemokines IP-10 and MIP-1β.

5.3.1 Interleukin-6, CRP and neutrophils

The significant rise in IL-6 and sIL-6R levels that we observed after tocilizumab administration have been demonstrated in previous studies of tocilizumab treatment in different autoimmune disorders. Under usual circumstances, IL-6 has a short dwell time in the circulation. However, the increase in IL-6 during tocilizumab treatment seems to reflect attenuated elimination of IL-6 from the circulation due to decreased IL-6R-mediated clearance of this cytokine caused by tocilizumab-mediated IL-6R blockade.[87] Importantly, this rise in free IL-6 has been shown to be accompanied by decreased IL-6 bioactivity *in vivo* as assessed by decreased CRP levels as also shown in the present study. In fact, we found that changes in CRP were inversely correlated with changes in IL-6 only in the tocilizumab group, underscoring that the rise in IL-6 could be a marker of tocilizumab efficiency *in vivo* rather than the opposite. Similarly, the increased levels of sIL-6R are probably due to delayed elimination due to the formation of sIL-6R – tocilizumab immunecomplexes.[87]

The findings in this thesis suggest that the potentially beneficial effects of tocilizumab on inflammation and PMI in the acute phase of NSTEMI, could be directly attributed to the

inhibiton of IL-6 itself, and not predominantly due to secondary effects on e.g. vascular/endothelial function or other cytokines. It is known that cholesterol crystals are important prerequisites for coronary plaque inflammation, which through the NLRP3 inflammasome activates the important IL-1β → IL-6 → CRP axis in the progression and destabilization of coronary atherosclerosis.[4, 24, 36] Interestingly, while it is established that cholesterol crystals play an important role in the progression of CAD, they may also play an important role in mediating inflammatory injury in acute MI. Cholesterol crystals are part of the atherosclerotic debris that can cause peripheral embolization and vascular obstruction after plaque rupture and/or PCI. Furthermore, cholesterol crystals are known to cause end-organ injuries in various conditions such as livedo reticularis,[88, 89] amaurosis fugax[90] and stroke.[91] A recent study by Abela et al showed that in patients hospitalized with acute MI, larger cholesterol crystal clusters in culprit coronary aspirates were associated with increased inflammation as reflected by IL-1β levels, increased arterial narrowing, and diminished reflow as assessed by TIMI-flow and myocardial blush grade.[92] On this background, the authors hypothesize that large cholesterol crystal clusters can contribute to myocardial injury both by directly obstructing the myocardial micovasculature, but also by increased myocardial inflammation due to the association between cluster size and IL-1β. It is known that while the size of PMI after PCI is associated with increasing CRP and IL-6 levels, pretreatment with statins attenuate both PMI and CRP in these patients.[14, 93] In the study by Abela et al,[92] statin use was more frequent among patients hospitalized with a recurrent acute MI, as compared with those hospitalized for first-time MI. Interestingly, patients with recurrent MI had smaller cholesterol crystal clusters and lower troponin and IL-1β levels. Moreover, the *current* trial suggests that IL-6 inhibition beneficially affects PMI. Thus, taken together, it is tempting to hypothesize that while cholesterol crystal composure is a provoker of myocardial inflammation and injury, statins seem to be protective by possibly modifying the structure of

these crystals to a more benign form, maybe less able to directly cause vascular obstruction and provoke inflammation. Furthermore, as IL-1β mediates its' inflammatory actions primarily through the IL-6 pathway, the results from the *current* trial could suggest that the beneficial effects of tocilizumab, at least partly, is caused by its' ability to prevent an acute deleterious inflammatory response in the myocardium to pro-inflammatory stimuli like embolization of cholesterol crystals, among others. Thus, as for the progression of coronary atherosclerosis, the cholesterol crystal → IL-1β → IL-6 → CRP axis might also be of importance for the evolution of complications of CAD, such as MI and PMI.

While IL-6 could be a crucial mediator of inflammatory myocardial injury in these patients, the exact mechanisms are uncertain. IL-6 is the major upstream inducer of CRP production in the liver,[30] and experimental animal studies suggest that CRP via complement activation could expand myocardial injury in MI.[94, 95] In contrast to these findings, complement inhibition prior to acute PCI in patients with STEMI did not have any effects on infarct size and clinical endpoints in humans.[56] However, a substudy from this patient cohort[56] showed that this treatment strategy in fact failed to inhibit activation of the terminal complement complex in acute STEMI, probably because pexelizumab was administered to late.[96] Thus, it is still somewhat unclear whether targeting CRP and complement activation could diminish infarct size or not. However, the aforementioned studies[56, 96] indicate that complement inhibition must be administered very early after, or maybe even prior to, the onset of MI to be able to inhibit activation of the terminal complement complex and have the desired cardioprotective effect. In the real-world clinical setting of an acute MI, this could be extremely challenging to achieve. Keeping that in mind, in the current trial we were able to administrate IL-6 inhibition *prior to* the onset of myocardial injury due to PMI.

In the current trial, tocilizumab treatment caused a rapid (at the first timepoint after baseline: evening day 1, results not shown) and highly significant decrease in neutrophil

counts. AUCs for troponin T and neutrophils during hospitalization were positively and significantly correlated in both groups (*not published post-hoc analysis:* Spearman Rho r = 0.448 and r = 0.354, respectively, $p < 0.01$ for both). Changes from baseline for troponin T and neutrophils did not show any correlation at various timepoints during hospitalization (results not shown). However, some relation between circulating neutrophil levels and troponin T seems to exist in both treatment groups in the current trial. Neutrophils have been suggested as important mediators of inflammatory injury in acute MI and IR injury.[13, 39, 69-71] Following MI, circulating neutrophils rapidly increase due to mobilization from the bone marrow,[38] and possibly also due to an IL-6 induced shift from the marginated to the circulating pool of neutrophils.[97] Neutrophils are the first inflammatory cells arriving in the infarcted myocardium, and their presence increase after reperfusion.[38] While neutrophils are important for clearing the infarct area of necrotic cells and debris,[22] they might aggravate myocardial injury by microvascular plugging contributing further to ischemia, the production of ROS and degradative enzymes.[13] IL-6 released from hypoxic cardiomyocytes in the viable border-zone of the infarcted area could facilitate neutrophil migration, adhesion and neutrophil mediated myocyte injury by inducing ICAM-1 in cardiomyocytes.[39, 69] However, despite promising results showing reduced infarct size by attenuating neutrophil adhesion in experimental models of MI and IR injury,[45, 46] neutral results were observed in reperfused acute MIs in humans.[50, 51] Moreover, the results from the current trial do not give signals that tocilizumab attenuates the ability of neutrophils to adhere to e.g. endothelial cells or hypoxic myocytes, as circulating levels of the endothelial cell marker ICAM-1 remained unaffected by tocilizumab treatment, and VCAM-1 was in fact modestly increased in tocilizumab treated patients. Furthermore, a recent trial showed that tocilizumab does not attenuate neutrophil function, meaning that neutrophil respiratory burst and phagocytic activity remains intact during tocilizumab treatment.[97] Circulating neutrophils during tocilizumab treatment thus

appears “healthy” and able to respond adequately to pathogens, and potentially also exert cardiotoxic effects in MI. Furthermore, tocilizumab does not seem to reduce the half-life of neutrophils. Thus, neutropenia seems mainly to be caused by a tocilizumab-induced shift from the circulating to the marginated pool, predominantly by increased neutrophil retention in the liver and the spleen.[97] Taken together, and given the positive association between troponin T and neutrophils in the tocilizumab group in the current trial, a neutrophil-related contribution to the cardioprotective effects of tocilizumab may predominantly relate to significantly fewer circulating neutrophils only, rather than attenuation of neutrophil adhesion and function. However, these issues are far from clear, and needs to be further explored in future studies.

Finally, IL-1 is an apical cytokine in the inflammatory cascade and the major inducer of IL-6 signalling pathways in inflammation,[34, 35] and probably also during MI.[98] The effects of anakinra, an IL-1 receptor antagonist, have been evaluated in patients with acute NSTEMI (MRC-ILA-heart study)[57] and STEMI (VCU-ART 1 and 2).[58-60] In the NSTEMI study, in which patients were given the first sc administration of anakinra prior to coronary angiography, similar to iv tocilizumab in the current trial, IL-1ra suppressed downstream IL-6 and CRP. However, anakinra did not attenuate troponin T levels during the acute phase.[57] At first glance, these results contradict the findings of attenuated troponin T release in the current trial. However, in the MRC-ILA-heart study, troponin T was measured less frequently than in the current trial, reducing the power to detect a treatment effect on troponin T release. Furthermore, the effect of anakinra on troponin T release in PCI treated patients only, was not evaluated in that study. In the studies evaluating the effects IL-1 receptor antagonism in acute STEMI, anakinra did not reduce infarct size.[58-60] However, in these studies, anakinra was administrated often several hours after acute PCI, limiting its ability to potentially affect myocardial necrosis and IR injury. In summary, it has to be concluded that the designs of the

studies on IL-1 receptor antagonism in acute MI have not been optimal to clarify if IL-1 inhibition have an impact on infarct size.

5.3.2 The cytokine network

While tocilizumab caused a profound decrease in both CRP and neutrophil counts, tocilizumab only exerted moderate and selective effects on the overall upregulated cytokine network during the acute phase of NSTEMI. While tocilizumab had a modest effect on IL-8 and substantially upregulated plasma levels of IP-10 and MIP-1β, all other components in the cytokine network remained unaffected by treatment during the acute phase.

MIP-1β is a chemokine which seems to be upregulated in MI and to be involved in leukocyte recruitment to the infarcted myocardium.[99] MIP-1β was inversely correlated to both troponin T and neutrophils in the tocilizumab group during the acute phase in the current trial. This suggests that the increase in MIP-1β somehow could be related to the potentially cardioprotective role of reduced neutrophil counts. On the other hand, increased levels of MIP-1β together with reduced neutrophil counts, could be a signal of increased recruitment of neutrophils to the myocardium, which in turn could be potentially detrimental with regard to myocardial injury. However, as MIP-1β was inversely correlated to troponin T, it seems more likely that reduced neutrophil counts primarily reflects increased liver- and spleen retention,[97] rather than increased recruitment to the myocardium. Previous evidence have so far failed to clearly identify this chemokine as a mediator of either beneficial or harmful effects in MI. One study has shown that MIP-1β levels correlate positively with infarct size in patients with reperfused MI,[100] possibly suggesting an adverse effect of this chemokine in the reperfused myocardium. In contrast, in an animal MI model, MIP-1β seemed to exert beneficial effects in terms of reduced post-MI inflammation and also attenuated post-MI LV remodeling.[99] Our results seem to be in conflict with the former study,[100] as AUCs for MIP-1β were inversely

correlated with AUCs for troponin T during the acute phase in tocilizumab treated patients. Thus, to date, current evidence, including the results from the present study, is not sufficient to clearly determine whether MIP-1β has a beneficial or harmful role in MI with or without revascularization.

IP-10 is a chemokine which is also known to be upregulated during MI.[100, 101] A role of IP-10 in IR injury in MI has been suggested, possibly related to effects on leukocyte subpopulations.[100] However, previous studies have so far reported conflicting results regarding IP-10's association with infarct size in patients with reperfused MI.[100, 101] Furthermore, there were no associations between IP-10 and troponin T levels during hospitalization in either treatment group in the present study. Thus, the current body of evidence does not provide compelling evidence that IP-10 holds a central position in the inflammatory arm of myocardial injury, and the potentially cardioprotective effect of IL-6 inhibition in patients with NSTEMI in the current trial does not appear to be mediated via this chemokine. IP-10 also exhibits angiostatic and anti-fibrotic properties.[102] Accordingly, it has been suggested that the timely suppression of IP-10 which has been observed in the course of MI,[100-102] might be necessary to provide neovascularization in order to achieve an optimal healing process, and to facilitate formation of scar tissue in the infarcted myocardium.[103] However, in an experimental study on mice undergoing myocardial IR protocols, IP-10 deficient mice experienced accentuated LV remodeling compared to controls, which seem contradictory to the notion that suppression of IP-10 is essential to counteract LV remodeling following MI.[102] In addition, infarct size was not affected by IP-10 deficiency in these animals.[102] Thus, neither previous data nor the present study provide convincing evidence that high levels of IP-10 are either beneficial or clearly harmful during MI. On the other hand, low or absent IP-10 could potentially be harmful for these patients.[102]

The mechanisms behind the increase of plasma levels IP-10 and MIP-1β in the tocilizumab group remain uncertain. mRNA levels in peripheral blood was significantly increased in the tocilizumab group for IP-10, but not for MIP-1β during hospitalization. Thus, an increase in peripheral production may at least partly explain the increase in IP-10 levels, but not for MIP-1β. However, these issues are far from clear, and we did not perform measurements from different cardiac compartments to further explore if e.g. the increase in MIP-1β was caused by increased cardiac production. Moreover, especially type I interferons have been shown to induce both IL-6, IP-10 and MIP-1β in certain clinical settings.[104, 105] However, tocilizumab did not have any impact on type I or III interferons, and they do not seem to be crucial in the upregulation of IP-10 and MIP-1β during tocilizumab treatment in acute NSTEMI. The mechanisms behind tocilizumab's effect on IP-10 and MIP-1β needs to be further explored in future research.

In the current trial, the vast majority of cytokines in the cytokine network were upregulated during hospitalization compared to follow-up visits. However, beyond the substantial increase of IP-10 and MIP-1β, tocilizumab had very modest (IL-8) or no effects on this broad inflammatory surge during the acute phase. The limited impact of tocilizumab on the cytokine network was somewhat surprising, as tocilizumab has been shown to downregulate several inflammatory cytokines in the treatment of RA.[106] The conspicuously absent effect of IL-6 inhibition on the majority of components in the inflammatory network, might be due to the wide distribution in time from symptom onset to inclusion in this study, as patients could have been included at a time when the primary myocardial injury already had occurred. Thus, we may have missed an initial rise-and-fall for some of these cytokines, reducing the power to detect a treatment effect. Furthermore, the inflammatory surge is modest in NSTEMI compared to STEMI patients, which also could reduce the power to detect a treatment effect on biomarkers analyzed in this study. Moreover, we evaluated the effects of

tocilizumab during a short period (days 1-3) immediately after drug administration. While tocilizumab clearly have immediate effects on IL-6 related parameters, CRP, neutrophils, IP-10 and MIP-1β during the first three days after a first dose of tocilizumab, maybe other cytokines need longer time to respond to IL-6 inhibition, as suggested by the aforementioned study in which downregulation of other cytokines were reported as late as 24 weeks.[106] The design of the current trial unfortunately did not allow for evaluating effects on the cytokine network during the period from day 4 to 3 months, during which it is possible that one could have detected some transient late-effects on other cytokines. Nonetheless, the impact of tocilizumab on only a few inflammatory mediators during the acute phase, suggest that the potentially beneficial effects of tocilizumab on e.g. PMI can be attributed directly to IL-6 effects, maybe via neutrophils or CRP, and not to secondary effects via other cytokines, which at least in the acute phase showed an indifferent response to tocilizumab. This could indirectly signal a direct role of IL-6 in inflammatory mediated myocardial injury.

5.4 Effects on coronary microvascular and endothelial function

Patients with ACS are characterized by widespread coronary inflammation[107] associated with systemic[74] and coronary endothelial dysfunction.[108] In ACS, inflammation and endothelial dysfunction interact, and both are related to adverse outcomes.[19, 74] Furthermore, patients with ACS are characterized by reduced CFR, which partially reflects coronary endothelial function.[82, 84] In the current trial, irrespective of treatment group, we found that CFR was reduced during the acute phase of NSTEMI, and was normalized at follow-up visits. This is in agreement with previous studies.[108, 109] While previous studies suggest that IL-6 is associated with endothelial dysfunction in ACS,[74] tocilizumab had no significant impact on CFR during the acute phase or follow-up in the current trial. Furthermore, with regard to circulating markers of endothelial dysfunction, tocilizumab in

fact induced a modest increase in VCAM-1, while ICAM-1 and vWF remained unaffected by tocilizumab. Taken together, tocilizumab does not seem to have any beneficial effects on coronary microvascular or endothelial function during the acute phase of NSTEMI. Thus, the presumably positive effects of tocilizumab in acute NSTEMI does not seem to be mediated via effects on microvascular or endothelial function. Of note, while VCAM-1 showed a strong and inverse correlation with CFR in the placebo group, there were no significant correlations between CFR and VCAM-1 in the tocilizumab group during hospitalization or follow-up. Thus, the modest increase in VCAM-1 in the tocilizumab group does not necessarily reflect an unfavorable effect on coronary endothelial function of tocilizumab, and may suggest that tocilizumab exerts unidentified effects on microvascular/endothelial function which supersede the role of VCAM-1. Nonetheless, the increase in VCAM-1 in the tocilizumab group was modest, and its clinical significance may be questioned. Interestingly, the lack of association between circulating markers of endothelial dysfunction and CFR extended to 6 months follow-up in the tocilizumab group. While the dissociation between CFR and VCAM-1 during hospitalization could be explained by unidentified effects of active tocilizumab treatment, the mechanisms explaining dissociation at 6 months remain elusive. This may indicate that tocilizumab has a persistent effect on vascular inflammation and/or function exceeding the drug's presence in the body. On the other hand, this observation may merely be related to the relatively few number and possibly a skewed between-group distribution of patients examined with CFR.

The lack of an observed effect of tocilizumab on coronary vascular function in NSTEMI could be due to the timing of measurements. In the current trial, circulating markers of endothelial dysfunction were measured as early as days 1-3, and CFR 1-2 days after drug administration. In patients with RA treated with tocilizumab, endothelial function improved gradually over 6 months.[110] This may indicate that a tocilizumab-induced reduction of

inflammation needs a longer period to translate into beneficial effects on endothelial and vascular function. Furthermore, while the current trial had sufficient power to detect a large increase in CFR, it did not have sufficient power to detect smaller effects.

To summarize, the effect of tocilizumab on coronary microvascular function was indifferent, and coronary microvascular function does not appear to play an important role in mediating tocilizumab's presumably beneficial effects during the acute phase of NSTEMI.

5.5 Effects on cardiac function and heart failure

IL-6 is associated with adverse LV remodeling and the development of heart failure after an acute MI.[66, 72] However, experimental studies show conflicting results regarding the effect of IL-6 inhibition on LV remodeling.[73, 111] In humans, IL-1 receptor antagonism with a duration of 2 weeks initiated shortly after reperfusion in acute STEMI, mitigated inflammation, LV remodeling and the onset of clinical heart failure.[58-60] Furthermore, IL-1 receptor antagonism attenuates IL-6 levels in acute MI.[57] However, it remains unclear if the potentially beneficial effects of IL-1 receptor antagonism on LV remodeling and heart failure after MI is mediated through suppression of IL-6. In fact, experimental studies rather suggest that IL-1's unfavorable effects on LV function is mediated through IL-18, and not IL-6.[112]

In the current trial, known heart failure and clinical instability were exclusion criteria, and the myocardial damage from NSTEMI was modest in both treatment groups. NT-proBNP levels were only modestly elevated and there were no between-group difference during hospitalization. Accordingly, LV systolic function was similar and normal during hospitalization, and remained normal and similar between groups at 6-months follow-up. While there were no between-group differences for NT-proBNP and LV systolic function, it is important to emphasize that the current trial cannot serve any conclusions regarding the effects of IL-6R inhitibion on LV remodeling and heart failure. This is because patients had

normal heart function at inclusion, and the acute NSTEMI resulted in very small infarct sizes judged by troponin T release and echocardiography. Thus, most patients lacked necessary prerequisites to develop adverse LV remodeling. The effects of IL-6R inhibition on LV remodeling and heart failure needs to be addressed in future studies, preferably in patients with acute STEMI with more substantial myocardial injury and thereby risk of developing adverse LV remodeling and heart failure.

5.6 Adverse events and safety

There were no between-group differences in clinical adverse events during 6 months follow-up. However, due to the moderate number of patients in each treatment group, this trial was not sufficiently powered to evaluate the effects of tocilizumab on clinical events after NSTEMI. The clinical efficacy and safety of tocilizumab in these patients must be addressed in larger and adequately powered studies. However, no apparent safety issues were detected. We did not observe any increase in serious infections. With regard to infection risk, one concern was the observation of the substantial fall in neutrophil counts during hospitalization. However, this phenomenon has not been associated with increased risk for infections in patients with RA.[75] This could be explained by the fact that neutrophil function does not seem to be attenuated by tocilizumab.[97] There were modest between-group differences in changes from baseline for ALT and total cholesterol due to higher levels in the tocilizumab group during hospitalization. However, no between-group differences for these parameters were observed at follow-up. Thus, a single dose of tocilizumab in acute NSTEMI does not seem to have concerning short- or long-term effects on liver function or lipid-profile.

5.7 Limitations

This trial has some limitations. The study did not have power to evaluate the clinical efficacy and safety of tocilizumab in NSTEMI. While the power estimation of this study was based on the expected between-group difference for AUC for CRP during hospitalization, power estimations with regard to secondary endpoints were not performed, and results should be interpreted accordingly. A major limitation of this trial is the wide distribution of time from symptom onset to inclusion. Thus, most patients were included at a time when the primary myocardial injury from NSTEMI already had occurred. More strict inclusion criteria with shorter time from symptom onset to drug infusion is required to fully evaluate the effect of tocilizumab on the primary myocardial injury in MI. On the other hand, a strength of the study design, with tocilizumab being administrated shortly before coronary angiography and PCI in all patients, is that it allowed for a precise investigation of the effects of tocilizumab on PCI-related inflammation and troponin T release (PMI). Normal LV function and small MIs in all patients left the trial with little ability to address the effects of IL-6 inhibition on LV remodeling and heart failure, which is important issues that need clarification in this context. We did not evaluate endpoints between day 4 and 3 months, thereby potentially missing some relevant effects of 2 weeks of total IL-6 blockade. E.g., by evaluating CFR very early after symptom onset, maybe a transient effect occurring at a later timepoint was missed. The choice of a fixed dose of tocilizumab can be questioned, as drug half-life is significantly dependent on patient weight. As discussed above, the differences in mg/kg doses between patients does not affect the degree of IL-6 blockade during the first three days after hospitalization, but could be of importance later on with regard to the inflammatory arm of cardiac healing and the development of adverse LV remodeling. The most ideal dose and duration of tocilizumab treatment in acute MI needs further clarification in larger clinical trials, preferably including patients with larger infarct sizes (STEMI). In this study, only plasma levels of various

cytokines were evaluated (Paper III). However, plasma levels do not necessarily reflect cytokine levels and acitivity at the site of inflammation, or clarify the origin of cytokine release. Unfortunately, we did not perform measurements of intracardiac gradients, which could have given further insight in the effects of tocilizumab on the cytokine network in NSTEMI.

6 MAIN CONCLUSIONS

1. This trial provides encouraging data concerning short-time inhibition of IL-6 with tocilizumab in patients with NSTEMI. We observed an attenuated inflammatory response, a reduction in PCI-related troponin T release, and a favorable safety profile. We need further studies that also give priority to early inclusion after symptom onset, to assess the potential effects of IL-6 inhibition in clinical outcomes in ACS.

2. The present study provides new insight into coronary endothelial function as reflected by transthoracic CFR, how it relates to markers of endothelial cell activation as well as the effect of tocilizumab on these parameters in the course of NSTEMI. Our findings suggest that the promising effects of tocilizumab on inflammation and troponin T release during the acute phase of NSTEMI do not involve inhibition of endothelial cell activation. However, the link between VCAM-1 as a marker of endothelial cell activation and coronary endothelial function seems to be "uncoupled" by tocilizumab treatment, thus suggesting an effect of anti-IL-6-therapy on vascular inflammation in this setting of uncertain mechanism and relevance. Forthcoming studies should examine further the potential effects of tocilizumab during MI and evaluate the clinical impact and the mechanisms of action of the enhancing effect of tocilizumab on VCAM-1 levels in these patients.

3. In this trial we show that except for a selective increase in IP-10 and MIP-1β, tocilizumab has only minor effects on the cytokine network during hospitalization in NSTEMI patients. Further studies are needed to clarify the role of IP-10 and MIP-1β in NSTEMI. However, the limited impact of IL-6

inhibition on the cytokine network suggests that attenuation of troponin T release may be caused directly by IL-6, or through CRP, possibly underscoring the direct role of this cytokine in post-MI inflammation.

7 CLINICAL PERSPECTIVE AND FUTURE DIRECTIONS FOR RESEARCH

This trial has provided encouraging results regarding the effect of IL-6 inhibition in NSTEMI, by attenuating inflammation and reducing PCI-related troponin T release, without any apparent safety issues. While these results clearly motivate further research on the use of tocilizumab in the management of ACS, several important questions need to be answered before tocilizumab can be applied in clinical use. Obviously, the clinical efficacy and safety of tocilizumab needs to be evaluated in sufficiently powered randomized trials. While we show that a fixed dose of 280 mg iv tocilizumab rapidly attenuated CRP and troponin T during the acute phase of NSTEMI, the optimal dose and duration of tocilizumab treatment needs further clarification. However, given the uncertainty in IL-6's role with regard to cardiac healing and adverse LV remodeling, it is not clear whether a reduced dose (shorter duration of IL-6 blockade) or an increased dose (longer duration) is the optimal strategy. Also, due to the different phases and the possible different and opposing roles of IL-6 during the acute inflammatory response to MI, maybe a dose in mg/kg is the most optimal strategy, as it yields a more precise and similar duration of action in all patients, targeting the right phase of IL-6 activity.

Some of the questions raised above, could be answered in the ongoing Assessing the effect of anti-IL-6-treatment in myocardial infarction (ASSAIL-MI) trial (ClinicalTrials.gov, NCT03004703). In the ASSAIL-MI trial, the aim is to evaluate the effect on myocardial damage of 280 mg iv tocilizumab vs placebo administered immediately prior to acute PCI in patients with a first-time acute STEMI < 6 hours after symptom onset. The primary endpoint in the ASSAIL-MI trial is the between-group difference in the myocardial salvage index as

measured by MRI during hospitalization. Secondary endpoints include parameters assessing LV remodeling, infarct size, clinical efficacy and safety parameters. While the current trial showed that tocilizumab could have an impact on PMI in patients with NSTEMI, the ASSAIL-MI trial will investigate whether tocilizumab is able to reduce IR injury in STEMI, which is somewhat different to PMI e.g. due to a more extensive impact by the generation of ROS.[14] The STEMI population and the endpoints in the ASSAIL-MI trial are also better suited to assess the impact of approximately 2 weeks of total IL-6 blockade on adverse LV remodeling. Furthermore, this study could potentially clarify if a shorter or longer duration of IL-6 blockade is the best strategy with regard to LV remodeling.

We showed that tocilizumab attenuated PMI in NSTEMI. Importantly, in the current trial we were able to administrate tocilizumab *before* the impact of myocardial injury and the new inflammatory surge following PCI. While e.g. the ASSAIL-MI trial has strict inclusion criteria with regard to symptom onset and tocilizumab will be administrated prior to reperfusion and potential IR injury, tocilizumab will still be given potentially several hours after the onset of the inflammatory response to MI, at a timepoint which could be too late to gain significant impact on the detrimental inflammation. The large randomized trial evaluating the effects of complement inhibition with pexelizumab before acute PCI in STEMI, had similar inclusion criteria as the ASSAIL-MI trial. Disappointingly, pexelizumab had no impact on infarct size or clinical endpoints in STEMI.[56] Interestingly, even though pexelizumab was administered < 6 hours from symptom onset, the drug did not succeed in inhibiting activation of the terminal complement complex.[96] This could suggest that, despite the strict inclusion criteria, pexelizumab was possibly administered too late in STEMI to achieve its' therapeutic potential. Keeping this in mind, and while we await the results from the ASSAIL-MI trial, it is tempting to suggest that future studies on various anti-inflammatory strategies on myocardial injury in acute MI, should be moved from the cardiac

catheterization laboratory to the prehospital arena. Results from the pexelizumab study, especially regarding the apparently very early activation of the terminal complement complex in acute MI, could suggest that, as for iv thrombolytic therapy, anti-inflammatory therapy may have its' "golden hour" at the very inception of MI.

The current trial showed encouraging results regarding CRP and troponin T in the context of MI. However, it is important to recognize that, so far, the history of studies on anti-inflammatory therapies in MI tells a disappointing tale of translating encouraging results from surrogate endpoints in humans, as well as positive results in animal MI-models, to clinical benefit. As discussed above, there is a need to improve animal models of MI if they are to be useful tools to plan meaningful clinical investigations in humans in the future. The CANTOS trial[24] is a success story which partly builds on the evidence gained by Mendelian randomization analyses, which could be a more efficient alternative compared to biomarker studies to target relevant novel treatment strategies for different diseases, including CAD and acute MI.[33]

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PAPER III

International Journal of Cardiology 271 (2018) 1–7

Contents lists available at ScienceDirect

International Journal of Cardiology

journal homepage: www.elsevier.com/locate/ijcard

Interleukin-6 receptor inhibition with tocilizumab induces a selective and substantial increase in plasma IP-10 and MIP-1β in non-ST-elevation myocardial infarction

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ARTICLE INFO

Article history:
Received 5 September 2017
Received in revised form 8 April 2018
Accepted 30 April 2018
Available online 29 June 2018

Keywords:
Tocilizumab
Interleukin-6
Inflammation
Myocardial infarction
IP-10
MIP-1β

ABSTRACT

Aim: To evaluate the effect of interleukin-6 inhibition with tocilizumab on the cytokine network in patients with acute non-ST-elevation myocardial infarction (NSTEMI).
Methods: 117 patients with acute NSTEMI were randomised to an intravenous infusion of 280 mg tocilizumab or placebo prior to coronary angiography. Blood samples were obtained at baseline, at 6 consecutive points in time during hospitalisation, and at follow-up after 3 and 6 months. Cytokines ($n = 27$) were analysed with a multiplex cytokine assay.
Results: Using a mixed between-within subjects analysis of variance, we observed a significant ($p < 0.001$) between-group difference in changes for interferon gamma-inducible protein (IP-10) and macrophage inflammatory protein-1β (MIP-1β), due to significant increases in the tocilizumab group during hospitalisation (i.e., IP-10 median change from baseline during hospitalisation (m_Δ), placebo: 3 (−60, 68) pg/ml vs tocilizumab: 209 (69, 335) pg/ml; MIP-1β m_Δ, placebo: 5 (−2, 12) pg/ml vs tocilizumab: 39 (24, 63) pg/ml). MIP-1β was inversely correlated to troponin T ($r = -0.28$, $p < 0.05$) and neutrophils ($r = -0.32$, $p < 0.05$) in the tocilizumab group. In contrast, tocilizumab had only modest or no effects on the other examined cytokines.
Conclusions: Tocilizumab led to a selective and substantial increase in IP-10 and MIP-1β during the acute phase of NSTEMI, with no or only minor effects on the other measured cytokines. ClinicalTrials.gov, NCT01491074.

1. Introduction

Inflammation plays a crucial role in chronic atherosclerotic disease as well as during plaque rupture [1]. When a coronary plaque ruptures, the resulting myocardial infarction (MI) triggers a comprehensive inflammatory response characterised by homing of leukocytes to the infarcted myocardium and up-regulation of several cytokines and chemokines [2]. While this complex inflammatory surge seems to be essential for infarct healing and cardiac repair, an exaggerated or protracted inflammatory response may contribute to increased infarct size [2]. The ischaemia-reperfusion (I/R) injury that occurs with restoration of blood flow may add to the deleterious inflammation [3]. Thus, therapies targeting the inflammatory overshoot could

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https://doi.org/10.1016/j.ijcard.2018.04.136
0167-5273/

have beneficial effects on plaque rupture as well as on I/R injury and infarct size.

The recently published Canakinumab Antiinflammatory Thrombosis Outcome Study (CANTOS) showed that inhibition of interleukin (IL)-1β reduces inflammation and prevents cardiovascular events in patients with coronary atherosclerosis [4]. IL-1β is an upstream activator of IL-6, a multifunctional and pro-inflammatory cytokine which appears to hold a central position in the inflammatory response during plaque destabilisation [5] and MI [6]. In MI, elevated levels of IL-6 are associated with larger infarct sizes and worse prognosis [6]. Furthermore, IL-6 seems to be involved in myocardial I/R injury [7,8], and elevated levels of IL-6 also predict adverse left ventricular remodeling after acute MI [9]. In acute MI, IL-6 acts as an intermediate cytokine which is induced by apical cytokines such as IL-1 and tumor necrosis factor (TNF) [10], and is the major inducer of downstream acute phase responses, including hepatic production of C-reactive protein (CRP) [11].

We have recently shown that IL-6 inhibition by a single intravenous administration of 280 mg of the IL-6 receptor (R) antagonist tocilizumab attenuated inflammation and percutaneous coronary intervention (PCI)-related troponin T (TnT) release in patients with non-ST-elevation MI (NSTEMI) [8]. These results might reflect a beneficial effect of IL-6 inhibition on inflammatory mediated I/R injury in these patients. However, cytokines operate in complex networks, and whether tocilizumab affects other inflammatory and anti-inflammatory mediators in NSTEMI is not known. In this pre-defined sub-study of the same patient cohort [8], we sought to evaluate the effects of IL-6 inhibition on the cytokine network in patients with acute NSTEMI. A dose of 280 mg of tocilizumab provides total IL-6 blockade for 2–3 weeks [12], and it is important to explore to which extent IL-6 inhibition affects different actors in the cytokine network not only in the acute phase (i.e., during hospitalisation), but also during follow-up (i.e., 3 and 6 months post-MI).

2. Methods

2.1. Study population and design

This pre-defined sub-study was part of a two-center, randomised, double-blind, placebo-controlled trial investigating the effect of a single dose of the anti-IL-6R antagonist tocilizumab in patients with NSTEMI scheduled for coronary angiography (ClinicalTrials.gov, NCT01491074). The study was performed at Oslo university hospital Rikshospitalet, Oslo, and St. Olavs hospital, Trondheim, Norway. The study was approved by the Regional Committee for Medical and Health Research Ethics of South-Eastern Norway and the Norwegian Medicines Agency, and conducted according to the Helsinki Declaration. All participants provided written, informed consent.

The study design has been reported previously [8]. In short, patients with acute NSTEMI presumed to be caused by coronary artery disease were included on the day of scheduled coronary angiography. All patients received pharmacological treatment in adherence to prevailing guidelines [13]. Major exclusion criteria were other clinically significant heart disease, clinical instability, medication or diseases affecting inflammation, and any condition that could interfere with protocol adherence. After baseline blood sampling, patients received a 1 h infusion containing either 280 mg tocilizumab or matching placebo (100 ml 0.9% NaCl) intravenously, prior to coronary angiography. After coronary angiography and PCI when appropriate, further blood samples were obtained at six further points in time during hospitalisation (day 1: evening, day 2: morning, afternoon, evening, day 3: morning, afternoon). Blood samples were also obtained at 3 and 6 months follow-up. We chose a fixed dose of tocilizumab for simplicity during the inclusion and to examine if the effects of tocilizumab were dependent on dosage per kg body weight [8].

Details on study drug pharmacology are given in the Supplementary Material online. Details on randomisation, treatment allocation and study drug administration are reported elsewhere [8].

2.2. Blood sampling protocol

Peripheral venous blood was drawn into endotoxin-free blood collection tubes with EDTA as anticoagulant (plasma) and with no additives (serum). The EDTA tubes were immediately placed on melting ice and centrifuged within 30 min at >2500 ×*g* for 20 min to obtain platelet-poor plasma. Serum was centrifuged at >2100 ×g for 15 min after full coagulation (~45 min) in room temperature. Immediately following centrifugation, the aliquots were frozen and stored at −80 °C until analyses in multiple aliquots. Samples were thawed only once.

Whole blood (3 ml) was collected in Tempus Blood RNA tubes (ThermoFischer, Paisley, UK) and stored at −80 °C.

2.3. Biochemical analyses

Components of the cytokine network were analysed in plasma according to the manufacturer's protocol. We used a multiplex cytokine assay (Bio-Plex Pro™ Human Cytokine Plex-27 Assay, Bio-Rad Laboratories, Hercules, CA) that quantifies IL-1β, IL-1 receptor antagonist (IL-1ra), IL-2, IL-4, IL-5, IL-6, IL-7, IL-8/CXCL-8, IL-9, IL-10, IL-12 (p70), IL-13, IL-15, IL-17, basic fibroblast growth factor (bFGF), granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon-gamma (IFN-γ), eotaxin/CCL11, IFN-γ-inducible protein (IP-10)/CXCL10, macrophage chemoattractant protein (MCP-1)/CCL2, macrophage inflammatory protein-1α (MIP-1α/CCL3), MIP-1β/CCL4, regulated on activation, normal T-cell expressed and secreted (RANTES)/CCL5, tumor necrosis factor (TNF), platelet-derived growth factor (PDGF), and vascular endothelial growth factor (VEGF). Inter-assay and intra-assay co-efficient of variations were <15 and <11 for all analytes, respectively.

IFN-α2a, IFN-β and IL-29/IFN-λ were measured in plasma with a U-plex Interferon Combo kit (human), Cat. No K15094 K-1, obtained from Meso Scale Diagnostics, LLC, 1601 Research Blvd., Rockville, MD 20850 USA. The chemiluminescence was quantified using MESO® QuickPlex SQ 120 (Meso Scale Diagnostics).

High-sensitivity C-reactive protein (CRP) was analysed on a MODULAR platform (Roche Diagnostics, Basel, Switzerland), and high-sensitivity troponin T (TnT) was measured by an electrochemiluminescence immunoassay (ELICA; Elecsys 2010 analyzer, Roche Diagnostics). Leukocytes with subgroups were counted consecutively using routine methods at the hospitals' clinical biochemistry laboratories.

Total RNA was isolated from whole blood at Aaros Applied Biotechnology, Aarhus, Denmark, and stored at −80 °C. Complementary DNA (cDNA) was synthesised using High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). Quantification of gene expression of IP-10 and MIP-1β was performed in duplicate using the ABI Prism 7900HT (Applied Biosystems), Perfecta SYBR Green Fastmix ROX (Quanta Bioscience), and sequence-specific PCR primers designed using the Primer Express software, version 3.0 (Applied Biosystems). Gene expression of the housekeeping gene ribosomal protein large p0 (RPLP0) was used for normalisation. Primer sequences can be provided upon request.

2.4. Endpoints

The main objective of this pre-defined sub-study was to evaluate the effects of treatment with tocilizumab on 27 different components of the cytokine network in patients with acute NSTEMI during hospitalisation. To assess the effect of treatment, we analysed the temporal development in the cytokine concentrations in the two treatment groups, looking for significant tocilizumab * time interactions. Secondary objectives were to determine the effects of tocilizumab on components of the cytokine network at 3 and 6 months' follow-up, and to analyse within-group changes in levels at follow-up visits vs. baseline. We also evaluated associations between cytokines significantly affected by tocilizumab treatment and CRP, TnT, IL-6 and neutrophils.

2.5. Statistics

The statistical analyses were performed with a per-protocol approach. IL-2, IL-15 and PDGF had in total (of all values in 117 patients over all timepoints) >10% values out-of-lower-range (OOR). These cytokines were therefore left out of further statistical analyses. 15% of the samples for IL-8 had to be excluded due to technical problems with one of the plates, and analyses could unfortunately not be repeated. However, IL-8 was still included in the final analyses, as all missing values for this cytokine stemmed from this malfunctional plate only. To avoid loss of low level data, values OOR and values <1.0 pg/ml were set to 1.0 pg/ml for all cytokines included in further statistical analyses, except for IL1-β for which values OOR and <0.5 pg/ml were set to 0.5 pg/ml. Further details regarding power estimation and statistical analyses are described in the Supplementary Material online.

3. Results

3.1. Baseline characteristics

We enrolled 121 patients with NSTEMI between August 2011 and November 2013. Follow-up ended in April 2014. 117 patients were included in the final analyses. There were no between-group differences in baseline characteristics (Table 1). Details regarding the screening and inclusion process, and patient dropout (placebo $n = 3$, tocilizumab n = 3) have been described previously [8].

3.2. Cytokine network during hospitalisation

We have previously reported that we observed a significant increase in circulating levels of IL-6 in the tocilizumab arm during hospitalisation

[8]. This finding was replicated with the IL-6 multiplex assay (Fig. 1, Table 2). In the current study, we also observed rapid and substantial increases in the plasma concentrations of IP-10 and MIP-1β in the tocilizumab arm (Fig. 1, Table 2). In patients allocated to placebo, the levels of these cytokines were stable during hospitalisation. A more subtle, but significant effect of tocilizumab was observed for IL-8, where we observed a fall in the placebo group and no increase in the tocilizumab group (Fig. 1, Table 2).

In contrast, treatment with tocilizumab did not significantly affect plasma concentrations of the other cytokines that were evaluated in this study (Table 2).

3.3. Cytokine network at 3 and 6 months follow-up

For the majority of cytokines, circulating levels were significantly lower at 3 and 6 months than at baseline (Table 3). Irrespective of treatment allocation, levels of IP-10 increased significantly from baseline to 3 months, but returned to baseline levels after 6 months. Levels of MIP-1β did not change between baseline and 3 and 6 months' follow-up. Except for a modest between-group difference for bFGF, treatment with tocilizumab did not affect cytokine levels at 3 and 6 months' follow-up (Table 3).

3.4. Impact of revascularisation therapy on cytokine levels during hospitalisation

As previously reported for IL-6 [8], there was no interaction between revascularization (PCI vs no PCI) and the effects of tocilizumab on IP-10 and MIP-1β (Supplementary Tables S1 and S2). However, while there was no between-group difference during hospitalisation for IL-8 in patients treated with PCI, a significant tocilizumab * time interaction was observed for IL-8 in patients not treated with PCI, reflecting a decrease in IL-8 in the placebo group (Supplementary Table S2).

Table 1
Baseline Characteristics according to Treatment Group.

	Placebo ($n = 59$)	Tocilizumab ($n = 58$)	*p*-Value
Age, y, mean (SD)	60.1 (9.9)	59.8 (7.7)	0.859
Female, n (%)	5 (8.5)	9 (15.5)	0.364
Body mass index, kg/m^2, mean (SD)	27.4 (4.4)	28.8 (3.3)	0.055
Blood pressure, systolic, mm Hg, mean (SD)	136.8 (18.0)	139.7 (18.1)	0.389
Blood pressure, diastolic, mmHg, mean (SD)	80.5 (12.1)	82.9 (12.0)	0.273
Hypertension, n (%)	17 (28.8)	26 (44.8)	0.109
Diabetes Mellitus, n (%)	10 (16.9)	11 (19.0)	0.966
Current Smoking, n (%)	17 (28.8)	15 (26.3)	0.926
Previous Myocardial Infarction, n (%)	7 (11.9)	9 (15.5)	0.760
GRACE score, mean (SD)	90.3 (20.2)	86.3 (19.1)	0.272
Symptom onset to inclusion, days, median (25th, 75th percentiles)	2 (1, 3)	2 (1, 3.5)	0.197
Maximum TnT before baseline, ng/L, $n = 85$, geometric mean (CI)	310 (214–449)	334 (217–514)	0.789
Maximum TnI before baseline, ng/L, $n = 31$, geometric mean (CI)	1571 (517–4773)	2221 (826–5976)	0.626
Aspirin, n (%)	59 (100)	57 (98.3)	0.496
Clopidogrel, n (%)	32 (54.2)	32 (55.2)	1.0
Ticagrelor, n (%)	27 (45.8)	26 (44.8)	1.0
Low molecular weight heparin, n (%)	54 (91.5)	51 (89.5)	0.952
Statin, n (%)	53 (89.8)	53 (91.4)	1.0
Betablocker, n (%)	45 (76.3)	45 (77.6)	1.0
PCI, n (%)	47 (79.7)	41 (70.7)	0.367
Stents per PCI-treated patient, mean (SD)	1.85 (1.28)	1.85 (1.15)	0.982
CABG, n (%)	7 (11.9)	6 (10.3)	1.0
Medical treatment, n (%)	5 (8.5)	11 (19.0)	0.167
SYNTAX score, median (25th, 75th percentiles)	8.0 (5, 14)	8.0 (4, 18)	0.629

TnT, troponin T; TnI, troponin I; PCI, percutaneous coronary intervention; CABG, Coronary Artery Bypass Grafting.

3.5. Impact of time from symptom onset on cytokine levels during hospitalisation

For patients included ≤2 days (early) from symptom onset, the same between-group differences as for all patients were observed for IP-10 and MIP-1β as well as for IL-6 [8], but not for IL-8 (Supplementary Table S3). However, in patients included >2 days (late) from symptom onset, equivalent significant between-group differences were observed for MIP-1β only, in addition to IL-6, but not for IL-8 or IP-10 (Supplementary Table S4).

3.6. Correlations between all cytokines during hospitalisation

Correlations between area under the curve (AUC) for all cytokines during hospitalisation are presented as a heatmap (Supplementary Fig. S1). Except for IP-10 and MIP-1β, the vast majority of cytokines were significantly and positively correlated to each other during hospitalisation in both treatment groups. Whereas IP-10 and MIP-1β were positively correlated in both treatment groups, IP-10 and MIP-1β were positively correlated to only a moderate (IP-10) or few (MIP-1β) number of other cytokines in the placebo group, and even fewer (IP-10) or no other than IP-10 (MIP-1β) in the tocilizumab group. IL-6 was positively correlated to all other cytokines in the placebo group, while all these correlations were lost in the tocilizumab group.

3.7. Correlations between IP-10, MIP-1β and IL-6, hsCRP, neutrophils and hsTnT during hospitalisation

We have previously shown that tocilizumab treatment caused a significant attenuation of CRP levels and PCI-related TnT release, as well as a substantial rise in IL-6 levels (reflecting attenuated elimination, but decreased IL-6 bioactivity), and a profound decrease in neutrophils during hospitalisation in these patients [8]. There were no associations between the AUCs for IL-6 and AUCs for IP-10 or MIP-1β in either treatment group during hospitalisation (Table 4). However, there was a significant positive association between AUCs for IP-10 and CRP in the placebo group, and significant inverse associations between AUCs for MIP-1β and AUCs for neutrophils and TnT in the tocilizumab group (Table 4).

3.8. Whole blood gene expression of IP-10 and MIP-1β during hospitalisation and follow-up

In order to evaluate if the increase in IP-10 and MIP-1β in the tocilizumab group reflected increased peripheral production, we measured peripheral blood mRNA-levels of these chemokines during hospitalisation (baseline, morning day 1, morning day 2). The levels of IP-10 mRNA were significantly higher in the tocilizumab group than in the placebo group, but there was no between-group difference for MIP-1β (Supplementary Fig. S2). No between-group differences were observed for either IP-10 or MIP-1β at 6 months follow-up (Supplementary Fig. S2).

3.9. Type I and III interferon levels and associations with IP-10, MIP-1β and IL-6 during hospitalisation

To further explore potential mechanisms behind the observed increases in IP-10 and MIP-1β in the tocilizumab group during hospitalisation, we examined plasma levels of the type I interferons IFN-α and IFN-β, as well as for the type III interferon IL-29. Tocilizumab did not affect levels of these interferons during hospitalisation (Supplementary Table S5). Except for a weak inverse correlation between MIP-1β and IFN-α, there were no significant correlations between type I and III interferons and IP-10 or MIP-1β in the tocilizumab group (Supplementary Table S6).

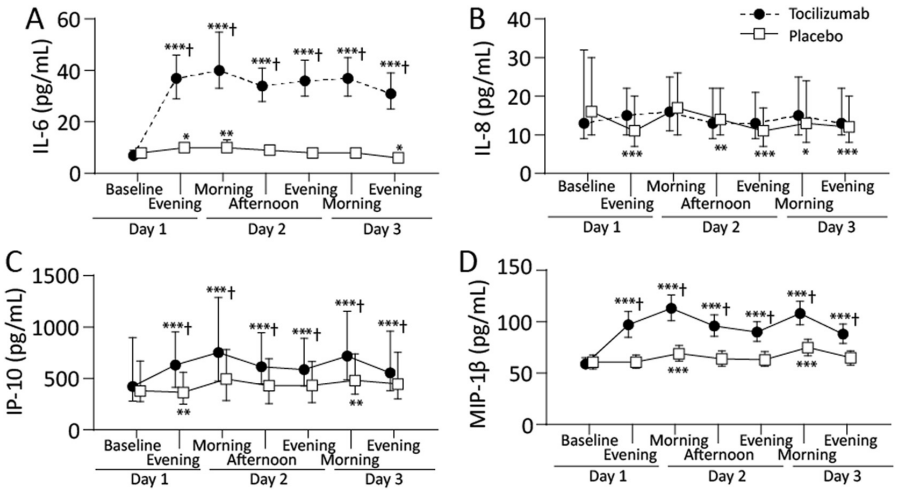

Fig. 1. Interleukin-6 (IL-6), interferon gamma-inducible protein/CXCL10 (IP-10), macrophage inflammatory protein-1β (MIP-1β) (all placebo $n = 59$, tocilizumab $n = 58$), and IL-8 (placebo $n = 49$, tocilizumab $n = 46$) during hospitalisation in patients with non-ST-elevation myocardial infarction. Circles and squares represents geometric means (IL-6 and MIP-1β) and median values (IL-8 and IP-10), bars represent 95% confidence intervals (IL-6 and MIP-1β) and 25th and 75th percentiles (IL-8 and IP-10). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs baseline within group. $^{\dagger}p < 0.001$ for between-group differences in changes from baseline at separate timepoints.

4. Discussion

We have previously shown that IL-6 inhibition with tocilizumab attenuated inflammation and PCI-related TnT-release in patients with NSTEMI [8]. In this pre-defined sub-study, we explored the effects of tocilizumab on the cytokine network in these patients. Our main findings were; (i) Most cytokines remained unaffected by tocilizumab treatment. (ii) However, tocilizumab led to a profound and selective increase in IP-10 and MIP-1β, whereas the effect on IL-8 was more complex. Thus, tocilizumab seemed to exert modest and selective effects on the inflammatory surge during the acute phase of NSTEMI, suggesting that the effects of tocilizumab on CRP and TnT may be directly attributed to IL-6 inhibition and not secondary to its effects on other inflammatory mediators.

Somewhat surprisingly, treatment with tocilizumab did not affect the levels of most mediators, except for IP-10, MIP-1β and IL-8. There are some in vitro studies showing that tocilizumab inhibits TNF [14] and other inflammatory cytokines [15], and tocilizumab has been reported to down-regulate IL-21 in humans [16]. There is also one relatively small study ($n = 42$) showing that tocilizumab down-regulates several inflammatory cytokines in patients with rheumatoid arthritis [17], but this study had no placebo group. The present study is, to the best of our knowledge, the first to assess the effect of tocilizumab on the cytokine network in a placebo-controlled randomised trial in humans. It has been claimed that IL-6 functions primarily as a secondary signaling cytokine, with a rather narrow effect on the cytokine network, and our results partly support such a notion. However, tocilizumab markedly down-regulated CRP in our study [8], underscoring the role of IL-6 as a major inductor of CRP production.

While the increase of IL-6 in these patients seems to be explained by delayed receptor-mediated clearance from the circulation as tocilizumab inhibits circulating IL-6's interaction with its receptors [18], the mechanisms behind the selective increase in IP-10 and MIP-1β in response to tocilizumab treatment remain elusive. The mRNA levels in peripheral blood suggest that the increased levels of IP-10, but not MIP-1β, were to some extent caused by increased peripheral production. However, these issues are far from clear and more accurate analyses from different cardiac compartments (i.e. intracardiac gradients) are needed to make firm conclusions. Interestingly, type I interferons are known to induce production of both IP-10 and MIP-1β [19,20]. However, we found no impact of treatment with tocilizumab on either type I or type III interferons. Thus, the exact mechanisms behind the rise of these cytokines after treatment with tocilizumab remain uncertain. Nonetheless, IP-10 and MIP-1β were standing out from the concerted pattern of cytokine upregulation during NSTEMI, as illustrated by our heatmap presentation, suggesting that their production, as well as the substantial upregulation in tocilizumab treated patients, is specifically regulated and share a common, yet unidentified, stimulus.

The potential consequences of the effects of tocilizumab on IP-10 and MIP-1β are unclear, but some possibilities exist. IP-10 is a chemokine which is known to be up-regulated during MI [21,22], potentially playing a role in I/R-injury through effects on leukocyte subpopulations [21]. However, previous studies have so far reported conflicting results regarding IP-10's association with infarct size in patients with reperfused MI. One study showed that IP-10 measured before revascularization in acute MI correlated inversely with infarct size, which might suggest a cardioprotective role of IP-10 [22]. In contrast, another study showed a positive correlation between IP-10 levels 2 days after acute PCI and final infarct size [21]. In our patients there were no associations between IP-10 and TnT levels during hospitalisation in either treatment arm. Thus, the role of IP-10 during NSTEMI remains unclear, and the potentially cardioprotective effect of IL-6 inhibition in our patients [8] does not appear to be mediated via this chemokine.

MIP-1β seems to be up-regulated in MI and to be involved in leukocyte recruitment to the infarcted myocardium [23]. Levels of MIP-1β have been shown to correlate positively to infarct size in patients with reperfused MI, which in turn could suggest an unfavourable role in I/R injury [21]. On the other hand, in a rat myocardial I/R-model, MIP-1β signaling through one of its receptors, CCR5, seemed to attenuate postinfarction inflammation and adverse left ventricular remodeling through increased myocardial recruitment of T regulatory cells [23]. In our patients, MIP-1β increased substantially during hospitalisation in patients treated with tocilizumab. We have previously reported that tocilizumab caused a profound decrease in neutrophil counts during hospitalisation [8]. This phenomenon may be involved in the putative cardioprotective effect of IL-6 inhibition reflected by the attenuated TnT release in these patients [8]. Interestingly, MIP-1β was inversely correlated to both TnT and neutrophils in the tocilizumab group, suggesting that MIP-1β may be involved in tocilizumab's suppressive and potentially

Table 2
Multiplex cytokine assay during hospitalisation in patients with non-ST-elevation myocardial infarction receiving placebo (n = 59) and tocilizumab (n = 58).

	Group	Baseline	Evening day 1	Morning day 2	Afternoon day2	Evening day 2	Morning day 3	Afternoon day 3	Repeated measures, P†
IL-1β (pg/ml)	Placebo	5.8 (2.9,9.7)	3.0 (1.3,5.1)	5.9 (2.8,8.1)	4.3 (2.0,7.2)	3.5 (1.3,5.7)	3.9 (2.2,7.5)	3.5 (2.1,5.7)	0.151
	Tocilizumab	4.8 (2.0,10.2)	4.2 (1.6,6.1)	4.3 (2.2,8.0)	4.1 (2.1,6.8)	4.1 (1.6,7.0)	4.8 (2.1,8.2)	3.3 (1.8,6.7)	
IL-1ra (pg/ml)	Placebo	113 (73–176)	76 (52–111)	115 (86–155)	99 (71–139)	80 (55–116)	82 (57–118)	85 (60–122)	0.657
	Tocilizumab	147 (103—210)	98 (71–134)	112 (85–148)	109 (83–143)	104 (75–144)	94 (69–129)	98 (73–130)	
IL-4 (pg/ml)	Placebo	4.4 (2.4,6.2)	2.8 (1.0,4.7)	4.1 (2.2,5.3)	3.4 (2.2,5.1)	3.0 (1.2,4.7)	3.1 (1.9,4.9)	3.2 (1.7,4.6)	0.353
	Tocilizumab	4.1 (2.1,6.6)	3.3 (1.7,4.7)	3.3 (2.0,5.5)	3.6 (2.0,4.9)	3.3 (1.8,4.8)	2.8 (2.1,5.1)	3.1 (1.7,4.7)	
IL-5 (pg/ml)	Placebo	21 (12–29)	14 (7–21)	19 (12–25)	17 (10—23)	14 (8–21)	14 (8–22)	13 (10—22)	0.588
	Tocilizumab	19 (11–29)	14 (8–22)	16 (9–23)	16 (9–22)	15 (7–24)	16 (9–20)	13 (7–20)	
IL-6 (pg/ml)	Placebo	8 (6–10)	10 (8–12)*	10 (9–13)**	9 (8–11)	8 (6–9)	8 (6–9)	6 (5–8)*	***<0.001***
	Tocilizumab	7 (6–9)	37 (29–46)***C	40 (33–50)***C	34 (28–41)***C	36 (30–44)***C	37 (30–45)***C	31 (25–39)***C	
IL-7 (pg/ml)	Placebo	26 (21—33)	17 (14–21)	24 (20—30)	22 (17–27)	16 (13—20)	22 (18–28)	19 (15–23)	0.530
	Tocilizumab	27 (20–34)	19 (15–24)	27 (22—32)	20 (15–26)	18 (15–23)	23 (18–30)	22 (17–27)	
IL-8 (pg/ml)‡	Placebo	16 (10,30)	11 (7,20)***	17 (10,26)	14 (10,22)**	11 (7,17)***	13 (8,24)*	12 (8,20)***	***0.019***
	Tocilizumab	13 (9,32)	15 (10,22)	16 (11,25)	13 (9,22)	13 (9,21)	15 (10,25)	13 (10,22)	
IL-9 (pg/ml)	Placebo	16 (12—22)	10 (8–13)	16 (13—21)	14 (11–18)	10 (7–13)	14 (10–19)	11 (8–15)	0.303
	Tocilizumab	18 (13–25)	12 (9–17)	18 (14–23)	14 (10–19)	13 (10–17)	15 (11—21)	15 (11—20)	
IL-10 (pg/ml)	Placebo	21 (15–30)	11 (8–16)	20 (15–27)	16 (12—23)	12 (8–16)	16 (12–23)	13 (9–19)	0.278
	Tocilizumab	22 (15–33)	13 (9–18)	17 (12–23)	13 (9–19)	11 (8–15)	15 (11–21)	14 (10—20)	
IL-12 (pg/ml)	Placebo	33 (24–46)	18 (13–24)	31 (24–41)	25 (19–34)	18 (13–25)	26 (18–37)	21 (15–28)	0.343
	Tocilizumab	32 (23–45)	19 (14–26)	27 (21–35)	21 (16–29)	18 (13–24)	24 (18–33)	23 (17–32)	
IL-13 (pg/ml)	Placebo	22 (16–30)	10 (7–15)	17 (13—23)	14 (10–20)	10 (7–14)	14 (10–19)	12 (9–17)	0.407
	Tocilizumab	22 (16–30)	12 (9–17)	16 (13–21)	15 (11–19)	12 (9–15)	14 (11–19)	15 (12—20)	
IL-17 (pg/ml)	Placebo	39 (21,68)	24 (8,44)	38 (20,61)	29 (14,57)	27 (11,50)	33 (12,58)	28 (13,46)	0.204
	Tocilizumab	42 (18,80)	31 (18,57)	39 (19,65)	39 (20,60)	35 (19,52)	37 (18,64)	34 (17,58)	
Eotaxin (pg/ml)	Placebo	91 (66,127)	78 (56,104)	86 (68,120)	95 (64,115)	77 (59,100)	83 (57,109)	75 (58,111)	0.299
	Tocilizumab	100 (59,157)	84 (56,135)	83 (62,169)	87 (59,168)	84 (59,151)	87 (59,137)	90 (61,147)	
Bas FGF (pg/ml)	Placebo	64 (41,87)	45 (24,62)	64 (37,82)	54 (32,74)	49 (28,73)	51 (32,76)	48 (32,70)	0.684
	Tocilizumab	63 (36,93)	48 (31,66)	54 (32,80)	57 (29,77)	51 (29,69)	50 (30,78)	52 (29,78)	
G-csf (pg/ml)	Placebo	58 (45–73)	39 (31–49)	55 (45–66)	43 (32–57)	36 (27–49)	42 (32–55)	46 (37–56)	0.322
	Tocilizumab	61 (50–74)	46 (37–57)	54 (45–46)	50 (42–61)	48 (40–59)	49 (40–61)	51 (42–63)	
GM-csf (pg/ml)	Placebo	57 (25,67)	39 (18,54)	49 (28,68)	45 (24,63)	39 (18,59)	35 (23,63)	40 (20,59)	0.403
	Tocilizumab	51 (20,82)	40 (19,65)	41 (22,66)	43 (22,63)	40 (18,65)	42 (18,67)	41 (18,58)	
IFN-γ (pg/ml)	Placebo	162 (83,232)	100 (33,168)	161 (75,210)	119 (77,199)	111 (36,184)	116 (57,190)	116 (64,170)	0.857
	Tocilizumab	138 (69,261)	114 (66,182)	133 (60,211)	124 (68,198)	121 (54,176)	112 (64,216)	117 (59,197)	
IP-10 (pg/ml)	Placebo	381 (275,672)	366 (252,561)**	495 (286,783)	431 (257,694)	433 (266,664)	481 (349,741)**	447 (305,757)	***<0.001***
	Tocilizumab	421 (282,899)	630 (418,954)***C	754 (477,1289)***C	613 (450,947)***C	587 (429,892)***C	718 (487,1155)***C	556 (384,961)***C	
MCP-1 (pg/ml)	Placebo	40 (25,73)	30 (17,47)	41 (24,65)	37 (22,59)	28 (17,53)	33 (20,60)	29 (18,51)	0.265
	Tocilizumab	40 (20,83)	35 (20,60)	37 (21,65)	35 (20,64)	36 (17,53)	36 (23,65)	31 (17,61)	
MIP-1α (pg/ml)	Placebo	85 (47,131)	57 (29,85)	86 (50,114)	70 (40,104)	58 (29,98)	74 (43,114)	55 (37,98)	0.491
	Tocilizumab	77 (47,135)	63 (39,100)	72 (44,110)	70 (37,92)	58 (39,84)	74 (40,113)	64 (41,101)	
MIP-1β (pg/ml)	Placebo	61 (54–68)	61 (55–68)	69 (62–77)***	64 (57–72)	63 (57–71)	75 (67–83)***	65 (58–72)	***<0.001***
	Tocilizumab	59 (54–65)	97 (85–110)***C	113 (101–126)***C	96 (86–107)***C	90 (81–100)***C	108 (97–120)***C	88 (79–98)***C	
RANTES	Placebo	451 (213,1231)	551 (195,1385)	570 (182,1300)	677 (197,1143)	406 (216,1697)	393 (164,1008)	610 (285,1272)	0.900
	Tocilizumab	418 (249,1161)	411 (196,896)	366 (211,881)	470 (181,1203)	437 (148,2201)	352 (162,818)	386 (198,1298)	
TNF (pg/ml)	Placebo	60 (21,78)	27 (8,51)	49 (16,65)	37 (15,61)	29 (8,60)	29 (15,67)	31 (12,57)	0.220
	Tocilizumab	46 (16,95)	29 (15,59)	31 (15,70)	31 (15,65)	33 (13,60)	32 (14,69)	29 (13,59)	
VEGF (pg/ml)	Placebo	23 (16–34)	14 (10–19)	23 (17–31)	19 (14–26)	14 (10–20)	18 (13–26)	15 (11–21)	0.452
	Tocilizumab	24 (17–35)	15 (10—21)	19 (14–26)	17 (12–24)	15 (11–21)	18 (13–25)	18 (13–25)	

Data are geometric mean and (95% confidence interval) and median (25th, 75th percentiles). *p < 0.05, **p < 0.01, ***p < 0.001 vs baseline. ^{C}p < 0.001 changes from baseline vs placebo same timepoint. †interaction tocilizumab * time in mixed between-within subjects analysis of variance during hospitalisation. IL-1β, interleukin-1β. IL-1ra, interleukin-1 receptor antagonist. IL-4, interleukin-4. IL-5, interleukin-5. IL-6, interleukin-6. IL-7, interleukin-7. IL-8, interleukin-8. IL-9, interleukin-9. IL-10, interleukin-10. IL-12, interleukin-12. IL-13, interleukin-13, IL-17, interleukin-17. Bas FGF, basic fibroblast growth factor. G-csf, granulocyte colony stimulating factor. GM-csf, granulocyte macrophage colony stimulating factor. IFN-γ, interferon γ. IP-10, interferon gamma-inducible protein/CXCL10. MCP-1, monocyte chemoattractant protein-1. MIP-1α, macrophage inflammatory protein-1α. MIP-1β, macrophage inflammatory protein-1β. RANTES, regulated on activation, normal T cell and secreted. TNF, tumor necrosis factor. VEGF, vascular endothelial growth factor. ‡placebo $n = 50$, tocilizumab $n = 49$.

beneficial effects on neutrophil counts. Based on the important role of neutrophils in the induction of superficial intimal erosion [24] this may be of particular relevance in the NSTEMI population. On the other hand, it is possible that the inverse correlation between MIP-1β and neutrophils reflects that this chemokine is directing the leukocytes into the myocardium, away from the circulation, thereby mediating harmful effects. However, the causes and consequences of neutropenia associated with tocilizumab treatment have not been established [25].

4.1. Limitations

This study has some limitations. The moderate number of patients does not provide sufficient power to evaluate effects on clinical outcomes. The conspicuously absent effect of IL-6 inhibition on the majority of cytokines, might be due to the variable delay from symptom onset to inclusion. It is possible that the peak levels of some of the cytokines had already passed at the time of inclusion. Furthermore, plasma levels do not necessarily reflect cytokine levels at the site of inflammation. It is possible that tocilizumab exerts a stronger effect within the myocardium and the coronary plaque. We did not measure intracardiac gradients, which could be a more sensitive method to assess this and also clarify the origin of the cytokine release. Finally, associations do not prove causal relationship. Mechanistic studies are needed to elucidate the effects of IL-6 inhibition in NSTEMI.

4.2. Conclusions

Inflammation plays a crucial role in the pathogenesis of atherosclerosis, plaque rupture and MI, and neutrophils also seem to be involved in plaque erosion [24]. The CANTOS trial recently demonstrated the efficacy

Table 3
Multiplex cytokine assay in patients with non-ST-elevation myocardial infarction at baseline and during follow-up receiving placebo (n = 59) and tocilizumab (n = 58).

	Group	Baseline	3 months	6 months	Repeated measures, *P*†
IL-1β (pg/ml)	Placebo	5.8 (2.6,9.7)	2.9 (1.3,4.3)***	2.8 (0.7,4.7)***	0.617
	Tocilizumab	4.8 (1.9,10.3)	3.0 (1.2,5.0)***	3.7 (1.1,4.5)***	
IL-1ra (pg/ml)	Placebo	119 (77–186)	72 (48–108)	78 (47–130)	0.763
	Tocilizumab	146 (103–205)	72 (46–114)***	95 (61–148)	
IL-4 (pg/ml)	Placebo	4.5 (2.4,6.3)	2.7 (1.0,4.0)***	2.3 (1.0,4.2)***	0.829
	Tocilizumab	4.0 (2.1,6.5)	2.7 (1.0,4.0)***	2.6 (1.0,4.1)***	
IL-5 (pg/ml)	Placebo	21 (12,29)	14 (6,22)***	11 (4,23)**	0.614
	Tocilizumab	20 (11,30)	13 (4,23)***	12 (4,24)***	
IL-6 (pg/ml)	Placebo	7.7 (6.9,9.8)	3.2 (2.6–3.9)***	4.3 (3.4–5.4)***	0.878
	Tocilizumab	7.5 (5.9–9.5)	3.4 (2.7–4.2)***	4.5 (3.6–5.6)**	
IL-7 (pg/ml)	Placebo	26 (21−32)	11 (9–14)***	10 (8–13)***	0.744
	Tocilizumab	26 (20−33)	13 (10–16)***	11 (8–14)***	
IL-8 (pg/ml)‡	Placebo	15 (10,30)	9 (6,13)***	9 (6,11)***	0.763
	Tocilizumab	13 (9,30)	8 (6,12)***	8 (6,10)***	
IL-9 (pg/ml)	Placebo	16 (12–22)	7 (6–10)***	6 (5–9)	0.969
	Tocilizumab	19 (22–26)	8 (6–12)***	7 (5–10)***	
IL-10 (pg/ml)	Placebo	20 (14–29)	9 (6–12)***	7 (5–9)***	0.784
	Tocilizumab	21 (15–31)	8 (6–12)***	7 (5–10)	
IL-12 (pg/ml)	Placebo	32 (22–45)	15 (11–20)***	12 (9–15)***	0.580
	Tocilizumab	31 (22–43)	13 (9–17)***	11 (9–15)***	
IL-13 (pg/ml)	Placebo	22 (16–30)	8 (6–10)***	6 (4–8)***	0.625
	Tocilizumab	21 (16–29)	6 (5–9)***	6 (5–8)***	
IL-17 (pg/ml)	Placebo	38 (21,69)	25 (9,37)***	22 (7.36)***	0.434
	Tocilizumab	42 (18,76)	24 (11,41)***	26 (8,39)***	
Eotaxin (pg/ml)	Placebo	91 (65,127)	69 (57,104)***	72 (59,129)*	0.884
	Tocilizumab	96 (59,152)	71 (57,124)***	78 (59,137)	
Bas FGF (pg/ml)	Placebo	64 (38,87)	46 (27,59)***	38 (20,55)***	***0.034***
	Tocilizumab	63 (36,88)	44 (22,66)**	42 (42,61)***	
G-csf (pg/ml)	Placebo	57 (45–73)	34 (26–44)**	32 (25–42)***	0.501
	Tocilizumab	60 (49–73)	31 (22–43)***	36 (28–48)***	
GM-csf (pg/ml)	Placebo	57 (25,67)	33 (13,59)***	33 (13,57)***	0.607
	Tocilizumab	51 (21,82)	40 (14,64)**	39 (19,60)***	
IFN-γ (pg/ml)	Placebo	162 (80,232)	95 (36,142)***	107 (34,163)***	0.402
	Tocilizumab	138 (69,261)	104 (25,149)***	116 (28,162)***	
IP-10 (pg/ml)	Placebo	381 (273,672)	464 (318,872)***	408 (314,1044)	0.522
	Tocilizumab	420 (276,783)	456 (351,772)**	429 (291,857)	
MCP-1 (pg/ml)	Placebo	40 (24,73)	23 (13,37)***	24 (10,36)***	0.496
	Tocilizumab	40 (21,80)	26 (10,44)***	26 (13,43)**	
MIP-1α (pg/ml)	Placebo	83 (42,131)	44 (33,64)***	48 (23,58)***	0.317
	Tocilizumab	75 (46,132)	45 (31,73)***	42 (29,61)***	
MIP-1β (pg/ml)	Placebo	61 (54–68)	58 (52–64)	59 (53–66)	0.138
	Tocilizumab	59 (55–65)	60 (56–65)	56 (52–61)	
RANTES (pg/ml)	Placebo	460 (262,1231)	342 (190,2137)	491 (166,2324)	0.533
	Tocilizumab	460 (261,1288)	422 (168,1916)	629 (196,2722)	
TNF (pg/ml)	Placebo	60 (21,78)	23 (8,55)***	31 (12,65)*	0.765
	Tocilizumab	46 (16,95)	25 (7,61)**	36 (10,69)**	
VEGF (pg/ml)	Placebo	23 (16–33)	11 (8–15)***	10 (7–14)***	0.862
	Tocilizumab	23 (16–33)	12 (9–16)***	11 (8–14)***	

Data are geometric mean and (95% confidence interval), median and (25th, 75th percentiles) and mean (standard deviation). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs baseline. [A]$p < 0.05$, [B]$p < 0.01$, [C]$p < 0.001$ changes from baseline vs placebo same timepoint. †interaction tocilizumab*time in mixed between-within subjects analysis of variance during follow-up. IL-1β, interleukin-1β. IL-1ra, interleukin-1 receptor antagonist. IL-4, interleukin-4. IL-5, interleukin-5. IL-6, interleukin-6. IL-7, interleukin-7. IL-8, interleukin-8. IL-9, interleukin-9. IL-10, interleukin-10. IL-12, interleukin-12. IL-13, interleukin-13, IL-17, interleukin-17. Bas FGF, basic fibroblast growth factor. G-csf, granulocyte colony stimulating factor. GM-csf, granulocyte macrophage colony stimulating factor. IFN-γ, interferon γ. IP-10, interferon gamma-inducible protein/CXCL10. MCP-1, monocyte chemoattractant protein-1. MIP-1α, macrophage inflammatory protein-1α. MIP-1β, macrophage inflammatory protein-1β. RANTES, regulated on activation, normal T cell and secreted. TNF, tumor necrosis factor. VEGF, vascular endothelial growth factor. ‡placebo n = 50, tocilizumab n = 49.

Table 4
Pearson r correlations between area under the curve during hospitalisation for IP-10, MIP-1β and IL-6, CRP, neutrophils and troponin T in patients with non-ST-elevation myocardial infarction. Placebo n = 59, Tocilizumab n = 58.

	AUC IP-10		AUC MIP-1β	
	Placebo	Tocilizumab	Placebo	Tocilizumab
AUC IL-6	0.168	−0.129	0.205	−0.111
AUC CRP	**0.346****	−0.079	0.061	−0.158
AUC Neutr	0.208	−0.155	−0.078	**−0.321***
AUC TnT	0.109	−0.077	−0.055	**−0.279***

AUC, area under the curve. IP-10, interferon gamma-inducible protein/CXCL10. MIP-1β, macrophage inflammatory protein-1β. IL-6, interleukin-6. CRP, C-reactive protein. Neutr, neutrophils. TnT, troponin T.
* $p < 0.05$.
** $p < 0.01$.

of immunosuppression via IL-1β in patients with coronary artery disease [4], and our results suggest that inhibition of IL-6 can reduce TnT release in patients with NSTEMI [8]. Herein we show that except for a selective increase in IP-10 and MIP-1β, tocilizumab has only minor effects on the cytokine network during hospitalisation in NSTEMI patients. Further studies are needed to clarify the role of IP-10 and MIP-1β in NSTEMI. However, the limited impact of IL-6 inhibition on the cytokine network suggests that attenuation of TnT release may be caused directly by IL-6, or through CRP, possibly underscoring the direct role of this cytokine in post-MI inflammation.

Funding

This work was supported by the South-Eastern Norway Regional Health Authorities, Oslo, Norway (grant number 2011124).

Conflicts of interest

Lars Gullestad has participated in an expert meeting sponsored by F. Hoffman-La Roche AG in 2014. The other authors declare no conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijcard.2018.04.136.

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