Acute ST-elevation Myocardial Infarction and Interleukin-6 Inhibition Cardioprotection and Novel Therapeutic Strategies

Thesis for the degree of Philosophiae Doctor (PhD)

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“Awaken people´s curiosity. It is enough to open minds; do not overload them. Put there just a spark. If there is some good inflammable stuff, it will catch fire.”
Anatole France

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Selected Abbreviations

ACS	Acute coronary syndrome
CMR	Cardiac magnetic resonance
CRP	C-reactive protein
ECG	Electrocardiogram
IL-6	Interleukin 6
MSI	Myocardial salvage index
NSTEMI	Non-ST elevation myocardial infarction
NT-proBNP	N-terminal pro-B-type natriuretic peptide
PCI	Percutaneous coronary intervention
SPM	Specialized pro-resolving lipid mediators
STEMI	ST-elevation myocardial infarction
TnT	Troponin T

Thesis summary

Acute myocardial infarction is a serious condition where time to proper treatment is important for outcome. Acute myocardial infarction can be life-threating, and it might cause considerable morbidity in the form of heart failure. Infarct size is the main determinant of death and complications in myocardial infarction.

Today, percutaneous coronary intervention (PCI) is the mainstay of treatment for patients presenting with ST-elevation myocardial infarction (STEMI). Prompt revascularization of the occluded coronary artery is essential to limit infarct size and improve outcome. Nevertheless, the ischemia-reperfusion injury following PCI can substantially contribute to the myocardial injury. Therefore, adjuvant therapeutic strategies targeting inflammation are of interest. However, modulation of the inflammatory response is a therapeutic challenge because of the dual effect of inflammation in myocardial infraction. While a certain inflammatory response is necessary to remove debris and initiate repair, too much inflammation is presumed to be harmful in this setting. Inflammation is beneficial as well as harmful to the myocardium. Mediators of the body´s active system to resolve inflammation are other targets of interest. However, the main focus of this thesis is to examine the effect of anti-inflammatory treatment by blocking the interleukin-6 (IL-6) receptor in patients with STEMI presenting for acute PCI.

In this project, my co-authors and I have designed, initiated, run and completed the ASSessing the effect of Anti-IL-6 treatment in Myocardial Infarction (ASSAIL-MI) trial. In this clinical trial, the concept of anti-inflammatory treatment in STEMI patients to limit infarct size was tested. This “proof-of-principle” trial was designed to evaluate the effect of tocilizumab, an interleukin-6 receptor inhibitor, on the primary outcome measure myocardial salvage index (MSI). The trial was randomized, placebo-controlled, phase 2 trial. The study was approved by the Regional Ethics Committee and the Norwegian Medicines Agency. It was conducted in accordance with Good Clinical Practice. A trial steering committee and an independent data monitoring and safety committee were formed prior to initiation. The study was investigator-initiated with limited financial support and supply of study drugs from the pharmaceutical industry.

The trial was conducted at three high-volume PCI-centre in Norway during 2017-2020. A total of 199 patients presenting with first-time STEMI for acute PCI participated. There were two treatment arms, and the allocation was 1:1. Half of the study population received intravenous tocilizumab (n=101), and the other half received placebo (n=98), an intravenous infusion of sodium chloride. Patients and study personnel were blinded to treatment allocation. The study drug was administered at the catheterization lab, immediately prior to PCI in the acute setting. All patients gave consent to participate prior to randomization.

The primary endpoint, the adjusted myocardial salvage index (MSI) measured 3-7 days after the infusion, was higher in the tocilizumab arm than in the placebo arm (69 ± 19% vs. 64 ± 21%, p = 0.04). MSI is an index calculated from late-enhancement cardiac magnetic resonance imaging (CMR) images based on the amount of reversible and irreversible myocardial injury. The implication of this result was that IL-6 inhibition resulted in less myocardial damage after PCI. However, at 6 months follow up, this beneficial effect was not detectable with regard to secondary endpoints, such as final infarct size, left ventricular end-diastolic volumes, and the biomarker N-terminal pro-B-type natriuretic peptide (NT-proBNP). There was attenuated inflammation as reflected by C-reactive protein (CRP) levels in the active treatment group during acute phase, but just a positive trend regarding a reduction in Troponin T (TnT).

The aim of this thesis is mainly related to the ASSAIL-MI trial: to describe the rationale and design of the ASSAIL-MI trial, to investigate the effect and safety of IL-6 inhibition in a phase 2 study of first time STEMI patients, and to elucidate immune cell profile in the two intervention arms with regard to treatment effects on leucocytes and their subsets. Finally, we examined moderators of resolution beyond IL-6 inhibition in a small STEMI population in search for therapeutic targets in the active resolution process after acute myocardial infarction.

Overall, we have explored the anti-inflammatory hypothesis in STEMI patients and gained novel insights regarding inflammation, its resolution, and IL-6 inhibition in an exciting research field straddling cardiology and immunology. The future will show if IL-6 inhibition might have a place in the treatment of acute myocardial infarction. Larger clinical trials are

needed to explore the effect of anti-inflammatory treatment observed in the phase 2 ASSAIL-MI trial for this strategy to reach clinical use.

Norsk sammendrag

Akutt hjerteinfarkt er en alvorlig tilstand og rask behandling er viktig for godt utkomme. I noen tilfeller er den dødelig, mens i andre tilfeller fører den til økt sykelighet på bakgrunn av hjertesvikt. Størrelsen på hjertemuskelskaden er den viktigste avgjørende faktoren for død og sykelig.

I dag er utblokkende behandling med PCI den viktigste behandlingen som tilbys pasienter med akutt hjerteinfarkt hvor man mistenker en tilstoppet kransåre som årsak. Ved å blokke ut den tilstoppende kransåren, gjenopprettes blodstrømmen til hjertemuskulaturen og hjerteskaden blir mindre. Imidlertid kan den plutselige gjenopprettingen av blodtømmen bidra i vesentlig grad til den endelig hjertemuskelskaden, ofte kalt iskemi-reperfusjonsskade. Nye behandlingsstrategier som påvirker denne betennelsesprosessen er interessante med hensyn på å redusere potensiell hjertemuskelskade. Å harmonisere betennelsesprosessen på en gunstig måte kan være utfordrende. Andre mulige angrepspunkter kan være regulatorer av aktive prosesser som avslutter inflammasjon.

Denne avhandlingen omfatter i hovedsak The ASSAIL-MI studien. Fokuset har vært på studiens design, på inklusjon og oppfølging av studiepasienter og analyser av effektmål og utforskning av hovedfunn på immuncellenivå. Denne kliniske intervensjonsstudien er gjennomført med henblikk på IL6 som et mulig angrepspunkt i den påfølgende betennelsesprosessen hos STEMI pasienter til akutt PCI for å redusere infarktstørrelse. Studien ble designet for å teste effekt av IL-6 reseptor blokking på det primære endepunktet MSI i en randomizert, placebo-kontrollert studie med mulighet for å vise årsakssammenheng i en fase II studie. Studien var godkjent av regional etisk komite og norsk legemiddelverk og ble utført i samsvar med god klinisk praksis. En styringsgruppe og en uavhengig komité som overvåket sikkerhetsdata ble etablert i forkant av studiestart. Forskningsmiljøet selv hadde regi på ide, studieforløp, analyse av data og videre implementering av data. Legemiddelfirma bidro med legemidler og tilskudd.

Studien ble gjennomført ved tre PCI sentra i Norge i tidsperioden 2017-2020. Totalt 199 pasienter med førstegangs STEMI til akutt PCI deltok. Det var to behandlingsarmer, og tildelingen var 1:1. Halvparten av studiepasientene mottok intravenøst tocilicumab (n=101) og den andre halvparten mottok placebo (n=98) som var intravenøs natriumklorid.

Studiedesignet var dobbelt-blindet slik at både pasient og studiepersonell var blindet med hensyn på tildeling av behandlingsarm. Studiemedikamentet ble gitt rett før PCI på kateteriserings labben i en akuttsituasjon, og alle studiedeltagere samtykket til deltagelse før randomiseringen.

Vi fant behandlingseffekt i favør tocilizumab sammenliknet med placebo på det primære endepunktet justert MSI (69 ± 19% vs. 64 ± 21%) målt 3-7 dager etter infusjon av studiemedisin. MSI er en indeks kalkulert fra mål estimert på magnetisk resonans bilder utført med late-enhancement teknikk som skiller reversibel og irreversibel skade av hjertemuskulatur. Sekundære endepunkter som endelig infarkt størrelse, venstre ventrikkel endediastolisk volumer og biomarkør for hjertesvikt ved 6 måneder viste ingen sikker forskjell mellom behandlingsgruppene. Vi påvist dempet CRP som markør på inflammasjon 3-7 dager etter infusjon, og positiv trend på markør for hjerteskade som TnT.

Formålet med denne avhandlingen var i hovedtrekk å beskrive bakgrunnen for IL-6 hemming i STEMI for akutt PCI og dernest valg av design og endepunkter for å undersøke effekt og sikkerhet av denne behandlingen i en fase 2 studie. Avslutningsvis utforsket vi forskjeller i hvite blodcellers profil i de to behandlingsarmene for å bedre forstå innvirkningen på betennelsesprosessen. I tillegg til å dempe betennelse, er andre regulatorer også interessante angrepspunkt i aktive prosesser for å terminere inflammasjon til rett tid.

Mine medforfattere og jeg har undersøkt betennelsesdempende behandling i STEMI og fått ny innsikt i dette spennende område som forener hjertemedisin med immunologi. Fremtiden vil vise om IL-6 hemming vil få en plass i behandlingen av akutt hjerteinfarkt for å redusere sykelighet og dødelighet. Større kliniske studier er nødvendig for å følge opp resultatene av denne fase 2 studien for data på harde kliniske endepunkter.

Articles in the thesis

I. Anstensrud AK, Woxholt S, Sharma K, Broch K, Bendz B, Aakhus S, Ueland T, Amundsen B, Damås JK, Hopp E, Kleveland O, Stensæth KH, Opdahl A, Kløw NE, Seljeflot I, Andersen GØ, Wiseth R, Aukrust P, Gullestad L.
Rationale for the ASSAIL-MI-trial: a randomised controlled trial designed to assess the effect of tocilizumab on myocardial salvage in patients with acute ST-elevation myocardial infarction (STEMI). Open Heart. 2019. 2019 Aug;46:264-273.6. https://doi.org/10.1136/openhrt-2019-001108

II. Broch K*, Anstensrud AK*, Woxholt S, Sharma K, Tøllefsen IM, Bendz B, Aakhus S, Ueland T, Amundsen BH, Damås JK, Berg, ES, Bjørkelund E, Bendz C, Hopp E, Kleveland O, Stensæth KH, Opdahl A, Kløw NE, Seljeflot I, Andersen GØ, Wiseth R, Aukrust P', Gullestad L'.
Randomized Trial of Interleukin-6 Receptor Inhibition in Patients With Acute ST-Segment Elevation Myocardial Infarction. J Am Coll Cardiol. 2021 Apr 20;77(15) 1845-1855. https://doi.org/10.1016/j.jacc.2021.02.049

III. Huse C*, Anstensrud AK*, Michelsen AE, Ueland T, Broch K, Woxholt S, Yang K, Sharma K, Tøllefsen IM, Bendz B, Amundsen BH, Damås JK, Berg ES, Bjørkelund E, Quiles-Jiménez A, Bjerkeli V, Bendz C, Kleveland O, Stensaeth KH, Opdahl A, Kløw NE, Andersen GØ, Wiseth R, Halvorsen B, Gullestad L, Seljeflot I, Aukrust P, Osnes L, Dahl TB.
Interleukin-6 inhibition in ST-elevation myocardial infarction: Immune cell profile in the randomised ASSAIL-MI trial. EBioMedicine 2022 Jun;80:104013. https://doi.org/10.1016/J.ebiom.2022.104013

IV. Fosshaug LE, Colas RA*, Anstensrud AK*, Gregersen I, Nymo S, Sagen EL, Michelsen A, Vinge LE, Øie E, Gullestad L, Halvorsen B, Hansen TV, Aukrust P, Dalli J, Yndestad A.
Early increase of specialised pro-resolving lipid mediators in patients with ST-elevation myocardial infarction. EBioMedicine 2019 Aug; 46:264-273. https://doi.org/10.1016/j.ebiom.2019.07.024

1. Introduction

All four papers in this thesis concern patients with STEMI admitted to hospital for mainstay treatment with PCI to reperfuse an assumedly occluded coronary artery. The subsequent activation of the inflammatory cascade may to a large extent contribute to the final infarct size (1). In papers I through III, we assessed the effect of anti-inflammatory treatment with an Interleukin-(IL-) 6 inhibitor on myocardial salvage. As we show in paper no. IV, termination of inflammation through active resolution processes is another potential therapeutic strategy in STEMI.

1.1 Acute coronary syndrome

Acute coronary syndromes (ACS) are characterized by a sudden decrease in blood supply to the heart. Atherosclerosis is the dominant underlying pathology of coronary artery disease and is the primary cause of ACS. The clinical presentation in acute coronary syndrome varies. Acute myocardial infarction but also unstable angina pectoris, are encompassed by this definition. Most often, acute coronary syndrome is due to plaque rupture and thrombus formation. When this occurs, the disease evolves from a stabile situation to an acute setting. Atherosclerosis is a chronic disease of low-grade inflammation (2). Several inflammatory actors may drive the atheroprogression from atherosclerotic cardiovascular disease to acute coronary syndrome. These actors may play a role in all stages from the initiation, progression and rupture of the coronary plaque, but also in the ischemia-reperfusion injury and in the remodeling, following acute myocardial infarction (3).

1.1.1 ST-elevation myocardial infarction (STEMI)

The third universal definition of myocardial infarction is used in this thesis. According to this definition, evidence of acute myocardial injury must be present with at least one value of cardiac troponin above the 99th percentile upper reference limit in the setting of ischemia. (4)

STEMI accounts for about 30% of ACS (5)

1.1.1.1 Clinical presentation, diagnosis and management

Acute myocardial infarction is categorized into two main groups, STEMI and non-ST-elevation myocardial infarction (NSTEMI), respectively (6). The 12-lead electrocardiogram

(ECG) is used to evaluate changes to the ST-segment in patients with ischemic symptoms as a tool to guide further management. New ST-elevations in at least two contiguous leads are required for the diagnosis of STEMI. Prompt diagnosis and care ensure urgent reperfusion of the myocardium at risk for myocardial injury to limit infarct size. Abrupt cessation of blood flow to the myocardium puts the patient at risk for sudden death according to arrhythmias. However, in the long term, dysfunctional remodeling and heart failure contributes to morbidity and mortality (7).

1.1.1.2 Epidemiology

Over the past three decades, the mortality in ischemic heart disease has declined in Europe (8). However, over the last decade the improvements in mortality rates have decelerated. (9). Worldwide ischemic heart disease remains the most common cause of death, and in Europe it accounts for 20% of all deaths (10). Furthermore, the incidence rate of STEMI in European countries varies from 43 to 144 per 100 000 per year (11). The relative incidence of STEMI is decreasing. On the other hand, the incidence of NSTEMI is increasing. These two entities are unlike in regard to pathology and clinical presentation, as well as to prognosis and therapeutic strategy. Relative to NSTEMI, STEMI is more common in younger people compared to older, and more frequent in men (12).

1.1.1.3 Pathophysiology

STEMI is the most often caused by coronary thrombosis after plaque rupture, classified as type 1 myocardial infarction. Some atherosclerotic plaques have a stable fibrous cap. But others are considered as "vulnerable" plaques and have thin caps. This is the most common kind of plaque in ACS. A vulnerable plaque contains a large lipid core enriched with macrophage foam cells and debris. With plaque rupture or plaque erosion, the thin fibrous cap rich in extracellular matrix macromolecules, is disrupted or ulcerated. This exposes the blood flow to tissue factors, activates the clotting cascade and leads to intravascular thrombosis (13). Plaque rupture and thrombosis frequently occurs at plaques that cause only modest coronary stenosis (<50% luminal narrowing) (14, 15).

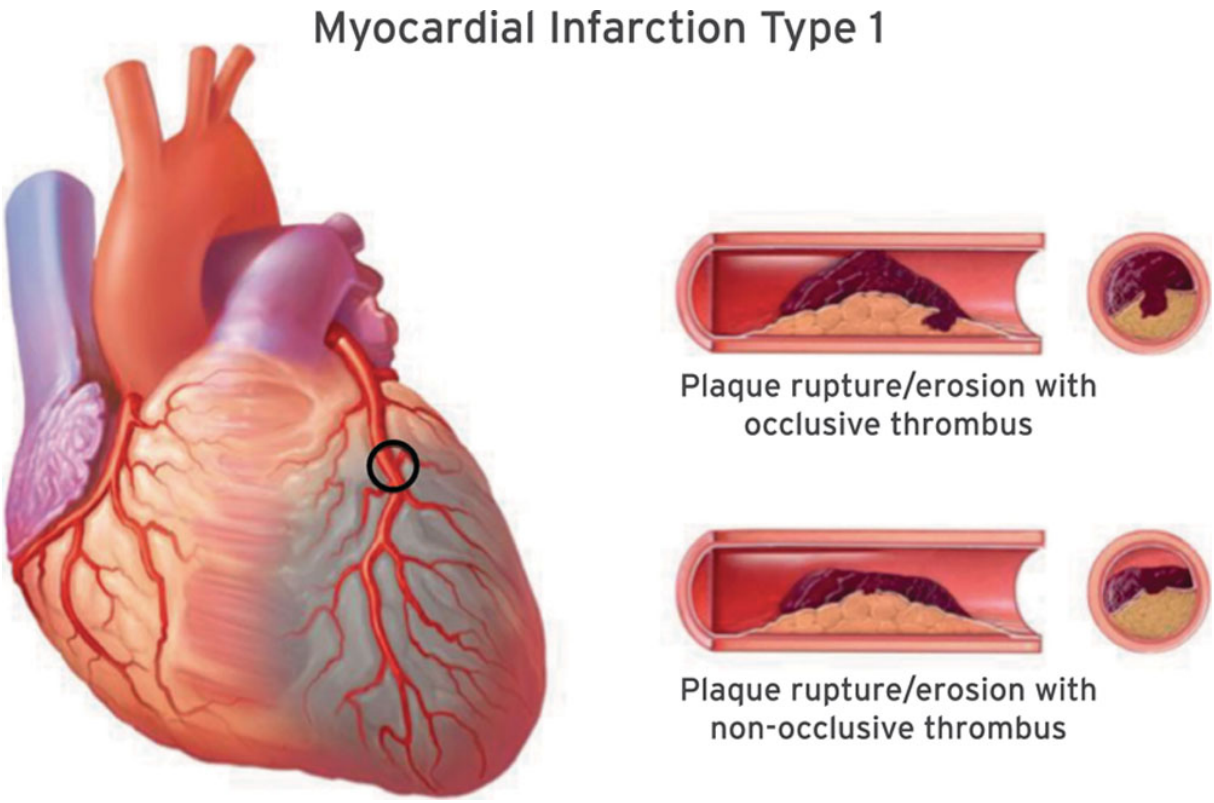

Figure 1. Myocardial infarction type 1

Kristian Thygesen. Circulation. Fourth Universal Definition of Myocardial Infarction (2018), Volume: 138, Issue: 20, Pages: e618-e651, DOI: (10.1161/CIR.0000000000000617). ©2018 The European Society of Cardiology, American College of cardiology Foundation, American Heart Association, Inc, and the World Heart Federation.

1.1.1.4 Prognosis

Infarct size and microvascular obstruction are major independent predictors of clinical outcomes in STEMI (16, 17). The time from symptom onset to reperfusion is a key factor to limit reperfusion injury and subsequent infarct size. It was not until 1977 that the first revascularization procedure, at the time termed percutaneous transluminal coronary angioplasty, was performed in man to open a stenosis in the left anterior descending coronary artery (18). Today, myocardial revascularization holds an IA recommendation in international guidelines (6), underscoring the importance of timely reperfusion therapy in STEMI. In a prospective cohort of The EURObservational Research Programme STEMI Registry, encompassing 29 countries, 72 % of hospitalized STEMI patients with symptom onset < 24 hours underwent PCI, 19 % fibrinolysis, and 9 % no reperfusion therapy. The corresponding in-hospital mortality rates from any cause were 3.1%, 4.4%, and 14.1%, respectively (19).

Percutaneous coronary intervention (PCI) is held to be one of the most important technological and therapeutic advances made in medicine of the twentieth century, defining a new era within the field of cardiology.

1.1.1.5 Future perspective in the management of STEMI patients

According to Eugene Braunwald, often referred to as the father of modern cardiology, treatment strategies in the management of STEMI may be classified as belonging to one of 4 phases: phase 1 (1912-1961) bed rest and "expectant treatment", phase 2 (1961-1974) the coronary care unit, and phase 3 (1975-present) myocardial reperfusion. Lastly, phase 4 comprises the effort to reduce myocardial perfusion injury as well as regenerative medicine (20).

The main focus of this thesis is on phase 4. By attacking interleukin-6 signaling, we may counteract plaque destabilization and thrombus formation in coronary arteries, impair the ischemia-reperfusion injury and inhibit maladaptive remodeling without attenuating the repair process. The ASSAIL-MI trial was designed to elucidate the hypothesis of beneficial effect of anti-inflammatory treatment with IL-6 inhibition in STEMI. In this thesis, 3 of 4 papers are based on the ASSAIL-MI trial. Paper 4 expands our focus to search for novel treatment strategies, examining modulation of active resolution to finally "break" the inflammation following PCI in STEMI patients.

2.2 Ischemia and ischemia-reperfusion injury

2.2.1 STEMI, ischemia-reperfusion injury, and infarct size

In acute myocardial infarction, a blocked or narrowed lumen of the infarct-related artery interrupts the oxygen supply to the myocardium. This may give rise to large infarcts, as is often the case in STEMI patients. The full thickness of the heart muscle may be involved, and the transmural ischemia has the potential to permanently injury a large part of the myocardium if prompt revascularization is not achieved (21). Several factors are known to affect final infarct size such as i) the initial area at risk, ii) collateral blood flow and microvascular dysfunction, iii) the time from symptom onset to revascularization, the duration of ischemia, and iv) additional injury as part of the reperfusion process (21). Of note, as much

as 50% of the final infarct size might be due to the ischemia-reperfusion injury (22). Myocardial stunning, ventricular arrhythmias and microvascular dysfunction are some of the detrimental consequences of this process (7). Thus, attacking the following inflammatory cascade after reperfusion is of interest to limit infarct size.

2.2.2. Inflammation, ischemia-reperfusion injury and microvascular obstruction

The ischemia-reperfusion injury leads to both metabolic and structural changes. The depletion of cell energy is a main factor during ischemia. In reperfusion, other key factors are oxidative and microvascular stress, inflammation and apoptosis (23). In this thesis, the inflammatory cascade after reperfusion, and in particular the role of IL-6 signaling, will be further discussed. A too long-lasting or a too strong inflammatory process after acute myocardial infarction may both be harmful to the myocardium. However, immune cells and signaling molecules of the inflammatory cascade, also play a role in myocardial repair by removing dead cells and pave the way for reparative processes (24).

1.3 Interleukin-6

1.3.1 Interleukin-6 – a pleiotropic cytokine

IL-6 is a small glycoprotein produced by several cell types such as mononuclear macrophages, T helper 2 cells, B cells, vascular endothelial cells, smooth muscle cells, and fibroblasts in response to inflammation and infection (25-27). It regulates a wide spectrum of biological processes and is involved in regulation of the acute phase response, inflammation, immune responses, and hemopoiesis (28, 29) .

1.3.2 Interleukin-6 in ACS

In cardiovascular diseases, IL-6 may have several effects/important roles. It is involved in the initiation of the atherosclerotic plaque, plaque growth and rupture, but also in reperfusion injury, myocardial tissue repair, remodeling, and the development of heart failure, see Figure 2. The causal role of IL-6 signaling in the development of cardiovascular disease has been proven in Mendelian randomization studies (30). IL-6 signaling promotes the production of acute phase proteins such as CRP and subsequently the activation of the complement cascade. The following release of pro-thrombotic mediators and activation of matrix metalloproteinases may weaken the atherosclerotic fibrous cap to the point of rupture and

cause ACS. However, the rise in IL-6 levels seen in acute myocardial infarction probably reflects the myocardial injury, rather than inflammation due to plaque rupture.

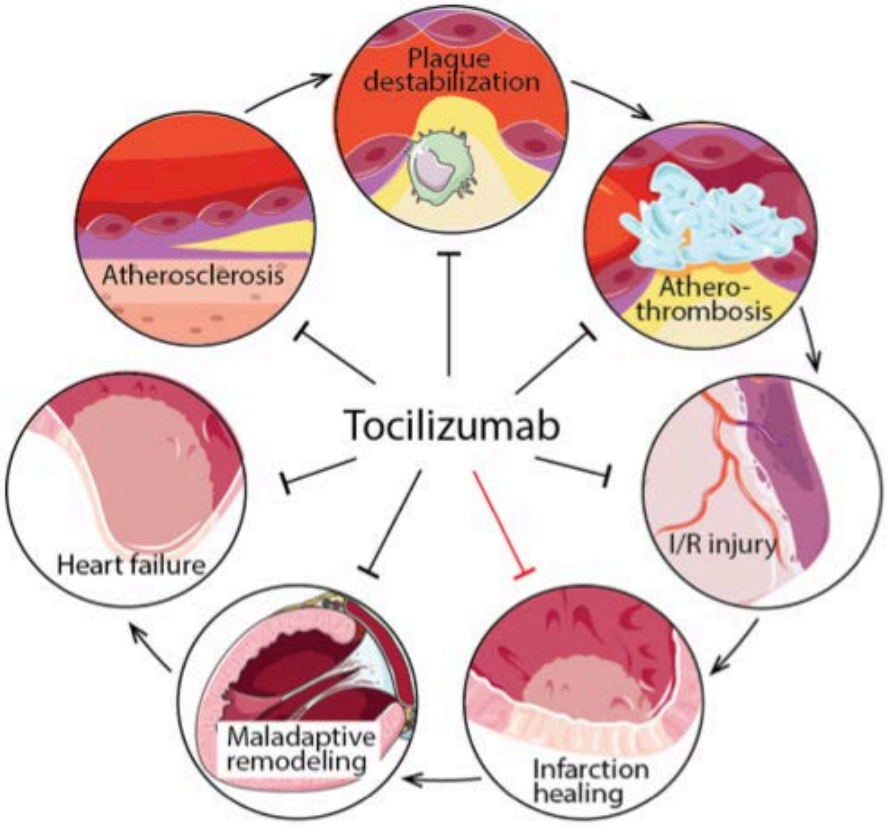

Figure 2. The figure shows possible actions for IL-6R blocking by tocilizumab in CVD.
Figure made by Thor Ueland for the ASSAIL-MI trial and printed with his permission

Studies have shown that elevated IL-6 levels in coronary artery disease and in ACS put the patient at increased risk for future cardiovascular events. In the landmark Canakinumab Antinflammatory Thrombosis Outcome Study (CANTOS) trial, targeting IL-1β, an upstream regulator of IL-6, significantly reduced cardiovascular events in patients with previous myocardial infarction and residual inflammatory risk (C-reactive protein >2 mg/l). Importantly, this benefit was not mediated through effects on lipid levels or blood pressure (31). The CANTOS trial provided proof to the inflammatory theory of atherothrombosis. Of note, the magnitude of risk reduction observed in this trial was related directly to the magnitude of IL-6 reduction observed in the individual trial participant (32).

1.3.3 IL-6 signaling

Intercellular IL-6 signaling is in the following described according to classical and trans-signaling pathway, respectively. However, recently a third intercellular IL-6 signaling has been described (33). In classical signaling, IL-6 binds to its membrane-bound receptor (IL-6R). Of note, the IL-6/IL-6R complex has no signaling capacity until forming a high affinity complex of IL-6, IL-6R, and glycoprotein (gp) 130 (34). Glycoprotein 130 is a membrane-bound protein that interacts with the complex to activate intracellular signaling and ultimately affect gene transcription. Notably, only a few cell lines express IL-6R, such as macrophages, neutrophils, cluster of differentiation (CD) 4+ T cells, podocytes, hepatocytes and endothelial cells. In trans-signaling, IL-6 binds to soluble IL-6R and the sIL6/IL6 complex can activate intracellular signaling in cells that not express IL-6R on their cell membrane. Again, this is only possible due to gp130, which is present on all cells and can activate intracellular signaling. This latter signaling pathway is in a way a natural moderator of IL-6 activity (35). These IL-6 signaling pathways, along with the third one known as trans-presentation, involving dendritic cells and a receiver T cell, underscore the role of IL-6 as a pleiotropic cytokine.

Studies on myocardial infarction have shown that IL-6-signaling is protective to the myocardium in the short term, but pathogenic in the long term (29). IL-6 inhibits both pro- and anti-inflammatory properties. In the short term, its pro-inflammatory properties may be beneficial in myocardial repair processes, but in the long term it is harmful. The classical signaling IL-6 pathway is most often regarded as anti-inflammatory while the trans-signaling IL-6 signaling pathway is regarded as mainly pro-inflammatory (36).

1.3.4 Tocilizumab

Tocilizumab is a recombinant, humanized monoclonal antibody. It is an antagonist directed towards the IL-6R but blocks all three kinds of IL-6 signaling by inhibition of soluble gp130 activity against IL-6 signaling. In rheumatology, tocilizumab is approved for the treatment of rheumatoid arthritis and systemic juvenile idiopathic arthritis. In these rheumatic diseases along with Castleman disease and Crohns disease, elevated serum-IL6 has been found to correlate with disease activity (37-39). Furthermore, inhibition of IL-6 signaling by tocilizumab ameliorates inflammation and is therapeutically effective in these diseases (39,

40). However, IL-6 inhibition in human atherosclerotic disease and ACS has not been evaluated until recently. Given the potentially beneficial effects of reducing the adverse effects of IL-6 in cardiovascular diseases, IL-6 signaling is an interesting target for novel treatment strategies in STEMI patients.

The balance between beneficial and harmful effects of modulating cytokine signaling is not straightforward (41). Both too much and too little may be harmful. Myocardial hypertrophy may be a consequence of increased IL-6 activity. In the opposite case, when IL-6 is prohibited from binding to its receptor, severe cardiac dilation is induced (42). In the ASSAIL-MI trial, we used a modest dosage of tozilizumab, similar to the dose used in the NSTEMI trial performed by our research group in advance of this trial (43). There were no safety issues regarding this fixed dosage. Probably, it provided a transient, complete IL-6 inhibition for 2-3 weeks (44). Long-term inhibition of IL-6 signaling may be more detrimental to the myocardium due to inhibited repair process within the infarct area.

1.3.5 IL-6 inhibition in ACS

In a placebo-controlled randomized trial, Kleveland and other members of our research group showed that interleukin-6 inhibition with tocilizumab attenuated inflammation and reduced troponin T release in acute NSTEMI, especially in patients treated with PCI (43). IL-6 may be involved in ischemia-reperfusion injury and myocardial remodeling, which are of special interest in this thesis. IL-6 levels are elevated in acute myocardial infarction and are associated with poor outcomes in ACS (45-47). Increased levels of circulating IL-6 are associated with procedure-related myocardial infarction and with adverse left-ventricle remodeling (47-49). Of note, it is associated with vascular and endothelial dysfunction during ACS (50). Experimental studies suggest that IL-6 inhibition can limit infarct size through anti-inflammatory mechanisms (51). Potential effects on infarct size and microvascular obstruction are of major interest in the search for attractive targets to improve outcomes in ACS. The ASSAIL-MI trial was designed prove the effect of IL-6 inhibition on myocardial salvage in reperfused STEMIs.

1.4 The immune system

The immune system is composed of two intertwining systems, the innate immune system and the adaptive immune system. The innate immune system is characterized by an immediate response to foreign invaders while the adaptive immune response is slow to develop on first exposure to a new pathogen (52). All immune cells are developed in the bone marrow.

The IL-6 cytokine is a player in the innate as well as in the adaptive immune system (53). It is a pivotal cytokine in the innate immune system. However, it was first discovered as a factor that induced B cells to produce immunoglobulins (54). Given its pleiotropic properties, it bridges the innate and the adaptive immune system, see Figure 3. In this thesis, we have elucidated the effect of tocilizumab on immune cells representing the innate and adaptive immune system, neutrophils and lymphocytes, respectively.

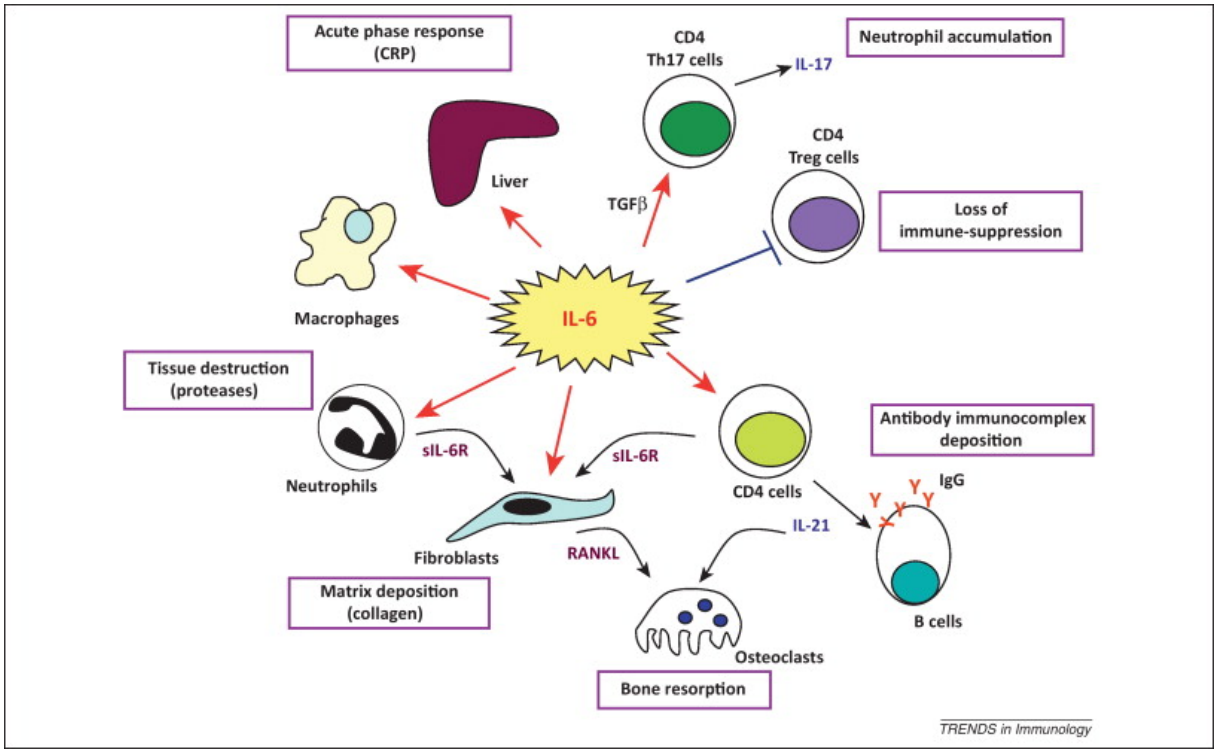

Figure 3. Interleukin-6 is a pleiotropic cytokine and regulates many pathways involved in inflammation. Picture used under licence from Trends of Immunology (26)

1.4.1 The innate immune system

When the integrity of tissues is under threat, the innate immune response with its neutrophils, macrophages, monocytes, dendritic cells, complement system, and cytokines, acts as the first line of defense. After myocardial infarction, the innate immune system coordinates the functions of its immune cells to remove dying cells and promote tissue repair. A balanced inflammatory cascade controls the overall outcome to promote cardiac tissue regeneration versus repair and scar formation. Neutrophils, monocytes/macrophages, and other immune cells of the innate immune system play central roles in infarct healing. However, neutrophils of the innate immune system are a topic of this thesis, regarding IL-6 inhibition and the resolution of inflammation in STEMI.

Neutrophils

Neutrophils infiltrate the ischemic myocardium in large numbers shortly after acute myocardial infarction and act as first responders to initiate the inflammatory response (55, 56). This abundant leucocyte is short-lived (57). It usually survives for only one day, or maximally two or three days in tissues. To maintain their numbers, they are continuously produced by the bone marrow, but also in the spleen to a very lesser extent. Release of damage-associated molecular patterns (DAMPs) due to cardiac cell damage leads to the recruitment of neutrophils on demand from resident macrophages and the endothelium. Even though neutrophils are beneficial because they clear away dead cell debris, their detrimental effects in myocardial infarction are thought to be excessive.

Neutrophils promote myocardial injury in several ways through the release of i) reactive oxygen species (ROS), ii) granular components such as myeloperoxidase (MPO), serine proteases, and matrix metalloproteinases (MMPs), and iii) pro-inflammatory mediators such as cytokines (tumor necrosis factor [TNF]-alfa, IL-1beta, and IL-8) and chemokines (C-X-C motif ligand 1, 2,3, and 8)(55, 58). They also form extracellular traps. Their detrimental effect is of clinical relevance, surmised from the fact that high peripheral neutrophil counts are associated with adverse outcomes and high mortality in patients with coronary syndromes (59, 60). Clinical endpoints like infarct size, death, and heart failure positively correlate with circulating neutrophil counts (61, 62). The association between neutrophils and microvascular plugging and no-reflow after ischemia/reperfusion is also of interest. However, growing

evidence suggests that neutrophils facilitate resolution of inflammation and cardiac repair by mediating the polarization of machrophages to a reparative phenotype (63).

The process of resolution of inflammation takes place when apoptotic neutrophils are removed by macrophages by efferocytosis. Macrophages remove tissue neutrophils when they have fulfilled their roles. This process stimulates anti-inflammatory and pro-resolving mediators such as specialized pro-resolving mediators (SPMs) to break the inflammation and promote cardiac repair (64). In this thesis, we measured levels of SPMs in acute STEMI to demonstrate their dynamic changes after PCI. Studies have shown that delayed neutrophil apoptosis occurs in acute coronary syndromes. Early initiation of resolution mechanisms is of interest since SPMs such as lipoxin A4, resolvin E1, and AnxA1 may induce neutrophil apoptosis and promote their removal by efferocytosis. Persistence of neutrophils can cause tissue damage and chronic inflammation. Our study provides new data regarding SPMs in the acute phase of STEMI.

1.4.3 The adaptive immune system

The adaptive immune system involves antigen-specific responses. Specialized B- and T-cells are the main players. In contrary to the innate immune system, much remains unknown regarding the contribution of lymphocytes to the inflammatory cascade in myocardial infarction. Previously, the role of ischemia/reperfusion injury was uncertain: Their inherent property is to act as a secondary defence army. However, increasing data has emerged on their roles as mediators of the inflammatory response. Neutrophil accumulation occurs via tumor growth factor beta stimulation of CD4 Th17 cells, loss of immune-suppression is caused by antibody immunocomplex deposition is by CD4+ regulatory T-cells. IL-6 affects several different cells in the inflammatory cascade following myocardial infarction, both regarding the removal of dead cells and in the repair process that follows. IL-6 plays may promoting differentiation or proliferation of B and T cells (65) Therefore, it is of interest to look into the effect of tocilizumab on lymphocyte subpopulations in the ASSAIL-MI trial given the pleiotropic effects of IL-6 on lymphocyte subpopulations.

Lymphocytes

Hematopoietic progenitors of both B- and T cells reside in bone marrow but mature in secondary lymphoid organs such as the spleen and lymph nodes (B-cells), and in the thymus (T-cells). Antigen presenting cells such as monocytes, machrophages, dendritic cells, and B-cells, activate naive T cells. This occurs when T cell receptors bind antigens in the presence of co-stimulatory molecules on the surface of antigen presenting cells to induce effector cells. Helper T cells (CD4+), cytotoxic T cells (CD8+), and regulatory T cells are all subsets of matured T cells from the thymus. CD4+ cells are involved in the regulation of leucocyte activity through the release of various cytokines. Regulatory T cells suppress the immune system, but also prevent self-reactivity. Lastly, CD8+ are killer cells. Taken together, these cells play diverse roles such as; i) various antigen recognition, ii) homeostasis maintenance, iii) tolerance, and iv) immunological memory. Studies have shown a skewed T-cell differentiation in ACS towards aggressive effector phenotypes and defective regulatory T, resulting in less ability to suppress the excessive immune response (66).

Limited evidence exists regarding the role of lymphocytes in myocardial ischemia-reperfusion injury. Yang et al have found a critical role for interferon-gamma-producing CD4+ T cells in experimental studies of myocardial ischemia-reperfusion injury in a mouse model (67). However, more clinical research is needed to elucidate its role. In a retrospective analysis of 1377 STEMI patients admitted to a single tertiary center in United Kingdom, lymphopenia after PCI for STEMI was associated with poor outcome (68). Furthermore, there was an association between effector T cells and microvascular obstruction, suggesting a role for effector T cells.

1.4.4 Neutrophil/lymphocyte ratio

The neutrophil-lymphocyte ratio is well established as an independent predictor for mortality and major adverse cardiovascular events in patients treated with PCI for STEMI (69-71). IL-6 affects cells in both the innate and adaptive immune system and the neutrophil-lymphocyte ratio is therefore an interesting parameter in the evaluation of the effects of IL-6 treatment on immune cells. Both the recruitment and function of the leucocyte subsets of interest in this thesis, have been examined due counts in peripheral blood and gene expression, respectively.

1.4.5 Resolution of inflammation

The regulation of mediators involved in termination of inflammation is of interest to establish novel therapeutic opportunities for treating reperfusion injury. Until recently, the process of terminating inflammation has been thought to be passive, but lipid mediators derived from specialized SPMs play an active role in resolving inflammation (72). Limiting myocardial damage through a balanced inflammatory process is undoubtedly favorable for patients presenting with acute MI to restore homeostasis and limit inflammation.

Sterile inflammation as seen in acute myocardial infarction is both beneficial to the myocardium, but also may be harmful. This fine-tuned balance is of major interest in research. By combining research in cardiology and immunology, we can gain new insight and new treatment strategies may evolve.

The duration and strength of the inflammatory response is determined by both pro-inflammatory mediators, but also by key effectors of resolution of inflammation (73-75). The core of this is to harmonize these processes in favor of tissue integrity and function. A prolonged and unresolved inflammation can result in adverse cardiac remodeling and heart failure with implication for morbidity and mortality. The role of SPMs in cardiac reperfusion-injury and in optimal healing after MI is unknown. In this thesis, we have explored plasma levels of SPMs during the first week in PCI-treated patients with STEMI.

Taken together, in this thesis we elucidate not only anti-inflammatory intervention by IL-6 inhibition, but also explore the potential to target mediators involved in resolving inflammation and tissue repair after STEMI.

2. Thesis aims

The overall aim of this thesis was to assess the effect of the anti-inflammatory strategy of IL-6 inhibition as an adjuvant therapy to improve myocardial salvage and limit irreversible myocardial injury in patient presenting with acute STEMI. Targeting inflammation in acute myocardial infarction has the potential to change clinical practice and improve morbidity and mortality in these patients.

The specific aims were as follow:

1. To describe the rationale and design for interleukin-6 inhibition by tocilizumab in patients presenting for PCI for acute STEMI.

2. To assess the effect of interleukin-6 inhibition on myocardial salvage in patients with acute STEMI presenting for PCI

3. To examine the effect of tocilizumab on a broad spectrum of leukocyte subpopulations and their potential relationships to outcomes

4. To investigate the role of resolution in acute myocardial infarction by assessing SPMs

3. Patients and methods

This thesis encompasses four papers. Paper I-III regard the ASSAIL-MI trial and IL-6 inhibition in acute STEMI patients presented for urgent revascularization with primary PCI. Paper IV is about resolution of inflammation in patients undergoing PCI for acute STEMI without interventions beyond usual care. All four papers have different study designs. The first is a design study, the second reports on the results of a randomized placebo-controlled trial, the third describes an exploratory study design, and the fourth details an observational study. Study patients were enrolled prospectively during my PhD period, beginning in 2017.

The ASSAIL-MI trial enrolled patients at three high-volume PCI centers in Norway; i) the Department of Cardiology, OUS Rikshospitalet, ii) the Clinic of Cardiology, St. Olavs Hospital, and iii) the Department of Cardiology, OUS Ullevål. The patients presented in Paper IV were enrolled at the Department of Cardiology, OUS Rikshospitalet only.

All enrolled STEMI patients had the same predefined inclusion and exclusion criteria with one main exception. In paper IV, patients with previous myocardial infarctions were allowed to participate also.

3.1 Design and study populations

3.1.1 Paper I-II

Paper I describes the design of the ASSAIL-MI trial, a randomized placebo-controlled trial. It illustrates the rationale for the stated hypothesis and gives an overview of the research field at the time this trial was initiated in 2017. The proof-of-concept ASSAIL-MI study was a phase II clinical trial designed to provide data regarding treatment effect and safety. The randomized control trial design is the gold standard method to prove treatment effect of an intervention and can provide strong evidence for causality.

In this prospective study, patients were screened successively for eligibility upon admittance for acute STEMI at either of the three participating sites. In Table 1, all inclusion criteria are listed for the ASSAIL-MI trial, an in the following Table 2 all the exclusion criteria are listed consecutively. Of note, all patients were screened in the acute setting within a short time limit

en route to the catheterization lab just after admittance to hospital with intent to perform PCI. Therefore, oral consent was obtained prior to study drug administration and was confirmed within 24 hours in writing.

Table 1: Inclusion criteria of the ASSAIL-MI trial

Inclusion criteria
• New ST elevation at the J-point in two contiguous leads (cut-points: 0.2mV in men and >0.15 mV in women in leads V2-V3 and/or >0.1 mV in other leads) in combination with symptoms consistent with acute MI
• Presentation within 6 hours of chest pain
• Indication for urgent coronary angiography with intent to reperfuse presumed occluded vessel
• Age between 18 and 80 years
• Informed consent obtained and documented according to ICH/GCP, and national/local

ICH/GCP, International Conference on Harmonization/Good Clinical practice

Table 2: Exclusion criteria for the ASSAIL-MI trial

Exclusion criteria
• NSTEMI (non-ST segment elevation in ECG)
• Left bundle branch block in ECG
• History of previous MI
• Cardiogenic shock
• Fibrinolytic therapy within 72 hours prior to admission
• Cardiac arrest / ventricular fibrillation
• History of severe renal failure with estimated glomerular filtration rat < 30 ml/minutes
• Known, current liver disease
• History of concurrent inflammatory, biliary obstructive or malignant disease
• A history of chronic or concurrent infectious disease, including a history of HIV, tuberculosis, or hepatitis B or C

• Known, uncontrolled lower gastrointestinal (GI) disease such as diverticulitis, Crohn´s disease, ulcerative colitis, or other symptomatic lower GI conditions that could predispose to GI perforations
• Major surgery within 8 weeks prior or after baseline
• History of central nervous system demyelinating or seizure disorders
• History of primary or secondary immunodeficiency
• Treatment with immunosuppressants other than lower dose corticosteroids (equivalent to 5 mg of prednisolone or less) at the time of randomization,
• Immunization with a live/attenuated vaccine within 4 weeks prior to baseline
• History of severe allergic or anaphylactic reactions to humanized or murine monoclonal antibodies or to tocilizumab
• Other contraindications to study medication
• Pregnancy, possible pregnancy or breast-feeding - women of child-bearing potential or breastfeeding mothers cannot participate. A woman is considered of childbearing potential following menarche and until becoming post-menopausal unless permanently sterile. Permanent sterilization methods include hysterectomy, bilateral salpingectomy and bilateral oophorectomy. A postmenopausal state is defined as no menses for 12 months without an alternative medical cause
• Contraindications to CMR (pacemaker, CRT, ICD, certain ferromagnetic implants, severe claustrophobia, allergy to contrast medium
• Any condition/circumstances believed to interfere with the ability to comply with protocol
• Any reason why, in the opinion of the investigator, the patient should not participate
• Failure to obtain written, informed consent by patient or next of kin, for instance in case of patient death after consent has been provided in oral.

MI, myocardial infarction. ICH/GCP, International Conference on Harmonization/Good Clinical practice. NSTEMI, non ST-segment elevation myocardial infarction. ECG, electrocardiogram. CMR, Cardiac Magnetic Resonance. CRT, cardiac resynchronization therapy. ICD, implantable cardioverter defibrillator.

The numbers of patients in Paper I and II, are not the same. Paper I is a design study, and presents demographic data of the first 100 patients enrolled in the ASSAIL-MI trial. Paper II

comprises all the 199 patients who consented to participate in the main trial. In a randomized controlled trial, it is important to calculate the minimum sample size needed for the trial to show a difference between the treatment arms based on the expected effect size.

3.1.2 Paper III

Paper III is an exploratory sub-study of the ASSAIL-MI trial. It was predefined and conducted to gain insight to immune cells and their subsets. The sub-study was designed to evaluate white blood cell counts and cell function from admittance to hospital just prior to PCI and throughout the acute phase and follow-up. We correlated leucocyte subpopulations and their subsets with the myocardial salvage index and circulating levels of troponin T to shed light on the mechanisms through which tocilizumab might exert its effects. Little is known about IL-6 inhibition by tocilizumab in acute myocardial infarction and the following reperfusion injury. New insight may pave the way for further investigations to evolve this knowledge base and the role of IL-6 inhibition in the future.

There are several patient populations presented in Paper III. All of them were enrolled prospectively.

- First, we assessed the study population of the ASSAIL-MI trial, comprising 199 patients allocated 1:1 to a single dose of tocilizumab intravenous or placebo, respectively.

- Second, a subgroup of 69 of the patients recruited at Oslo University Hospital Rikshospitalet in the ASSAIL-MI trial underwent extended flow cytometry analyses. Of these, 37 patients received tocilizumab. The laboratory analyses were conducted at the Department of Immunology, also at Oslo University Hospital.

- Third, in 20 patients allocated to each treatment arm in the ASSAIL-MI trial, we performed gene expression analysis at the Research Institute of Internal Medicine at Oslo University Hospital Rikshospitalet. The two groups were matched regarding age, gender, HbA1c and cholesterols levels.

- Last, we collected blood samples of 20 patients with stabile angina admitted to Oslo University Hospital Rikshospitalet for elective coronary angiography. The blood samples were drawn after we obtained written consent and in accordance with specified inclusion and exclusion criteria. Previous cardiac events, such as myocardial infarction, were allowed, and the patient were not required to have ST-elevation myocardial infarction, but otherwise we used the same criteria as in the ASSAL-MI trial.

3.1.3 Paper IV

The study described in Paper IV was designed to provide observational data on SPMs derived from polyunsaturated fatty acids. These SPMs act as markers of resolution in acute STEMI. Blood sampling was performed in three distinct populations: Patients with acute STEMI, patients with chronic coronary artery disease, and healthy control subjects.

The three study populations were enrolled prospectively. The study population of interest was patients with STEMI with presumed plaque-rupture and no underlying comorbidities known to modulate inflammation. Thus, patients with autoimmune diseases, infections, and malignancies, and patients using immunomodulating drugs, such as steroids and non-steroidal anti-inflammatory drugs were not allowed to participate. However, previous myocardial infarction was not an exclusion criteria. The other two study populations were control groups.

- Study population no 1 encompassed 15 patients with acute STEMI. They were enrolled successively at the catheterization lab just after admittance to hospital for urgent PCI. The inclusion criteria were as follows, i) STEMI according to ESC guidelines, ii) significant coronary artery disease with at least 50% coronary artery stenosis by angiography at patient presentation at the catheterization lab, and iii) increase in cardiac troponin T. We obtained blood samples i) at admittance to hospital just prior to PCI, ii) on day one post MI, and iii) at day 8 post MI
- Study population no 2 comprized healthy individuals (n=10) without known coronary artery disease or medication use. We collected blood samples once from these individuals.

- Study population no 3 constituted patients with chronic coronary artery disease (n=10). In these patients, we drew blood samples once before elective coronary angiography.

Study population no 2 and 3 were matched with the STEMI patients with regard to age- and sex. Notably, patients with chronic coronary artery disease are often older and have more comorbidities that patients with STEMI.

3.2 Study procedures

3.2.1 Paper I, II and III

Informed consent was obtained from all participants of the ASSAIL-MI trial prior to drug infusion, first orally and then in writing within 24 hours. The main reason for this strategy, which was approved by the Regional Ethical Committee, was to avoid delayed PCI. Clinical examination, blood pressure measurement, and an electrocardiogram (ECG) was done at admittance to hospital and at all successive follow-up visits. At admittance, the pre-hospital ECG was used to confirm diagnostic ST-segment elevations in at least two contiguous leads.

3.2.1.1 Randomization and blinding

In the ASSAIL-MI trial reported in Paper II patients were randomized in a 1:1 fashion at the catheterization lab just prior to PCI to either a single dose of tocilizumab or placebo. Study patients and study personnel were blinded with regard to treatment allocation. Identical-looking infusion bottles were pre-prepared by un-blinded personnel and given unique randomization numbers, stratified by short (<3hours) or long (3 to 6 hours) time from symptom onset. At the time of randomization, study personnel just used the next-in-line bottle according to whether the patient was admitted less than 3 hours after symptom onset or 3-6 hours after symptom onset. The Research Support Unit at Oslo University Hospital generated the balanced, permuted block randomization list with varying block sizes. The randomization was stratified according to site.

3.2.1.2 Study drug

The active drug in the ASSAIL-MI trial was a single i.v. dose of tocilizumab (RoActemra®), 20 mg/ml; 14 ml (280mg) dissolved in 100 ml NaCl 0.9%. Placebo was 100 ml of NaCl 0.9%,

i.v. The participants were given either active drug or placebo according to randomization. The infusion was given in a peripheral vein at the catheterization lab, just prior to PCI.

Table 1: Study drug

Drug Name	Dosage Form (e.g.vial)	Strength	Quantities per kit
Roactemra® (tocilizumab)	Vial	20mg/ml; 10 ml	1
Matching placebo	Vial	10 ml	1

mg, miligrams. ml, mililiter

Figure 4. Study drug. Identical looking vials for active drugs and placebo produced by the pharmaceutical company.

3.2.1.3 Study tests at baseline and follow up

Table 2 shows study procedures of the ASSAIL-MI trial. The participants underwent physical examination, ECG-test, and blood sampling at all clinical visits from inclusion to last clinical follow-up visit; thus i) inclusion /admission, ii) 14-33 hours post MI (24h), iii) in the acute phase 3-7 days post infusion, iv) at 3 months post infusion, and v) 6 months post infusion.

Table 2: Flow chart study procedures

Time schedule	Baseline[1]	Hours post infusion	Months

				6 - 8	12 - 16	14 - 33	72 - 168	3	6
Informed consent	x	Study drug infusion	PCI						
Clinical examination	x					x		x	x
ECG	x							x	x
Biobank samples[2]	x					x	x	x	x
Safety samples[3]	x			x	x	x	x	x	x
Randomization	x								
Echocardiography							x		x
Quality of life									x
CMR							x		x
Adverse events				←x→					

[1]Immediately prior to infusion of study drug and coronary angiography. [2]Secondary endpoints (i.e, NT-proBNP, parameters of platelet activation, inflammatory and anti-inflammatory mediators, markers of ECM remodeling, lipid parameters). [3]Hematological parameters, renal function, serum electrolytes, tests of liver damage, TnT (also efficacy parameter), CK-MB, CRP.

CMR and echocardiography were performed 3-7 days after infusion and at 6 months' follow-up. The patients answered a quality-of-life questionnaire approximately 24 hours after infusion and at 6 months' follow-up. Safety data was registered at all visits as; i) no events, ii) adverse events, iii) severe adverse events, or iv) suspected unexpected serious reactions.

3.2.1.4 Cardiac myocardial resonance - protocol and measurements

Cardiac magnetic resonance imaging was performed twice in the ASSAIL-MI trial (Paper I-III). We used the same imaging protocol in the acute phase (3-7 days after study drug administration) and at 6 months follow-up. In all cases, 1.5 T systems (Siemens Avanto, Philips Ingenia) were used. According to the study protocol, patients were informed about the CMR scan at arrival, and we assessed possible contraindications and creatinine levels. Adequate vector-ECG was established up front, and standard localizing sequences were conducted before a Gadolinium contrast agent was administered (0.15 mmol/kg gadobutrol or 0.22 mmol/kg Gd-DOTA).

After 5 minutes, we measured i) size and function of the left ventricle, and ii) oedema of the left ventricular myocardium. Variables such as end-diastolic volume, end-systolic volume, and ejection fraction and left ventricular mass were quantified by using retrospective ECG-gated, segmented, balanced steady-state free precession cine sequence with minimum echo and repetition times to acquire left ventricular long- and short-axis images. The protocol specified slice thickness of 8 mm with no interslice gap and spatial- and temporal resolution of 1.5 x 1.5 mm and 30-35 ms respectively. Left and right ventricular volumes and ejection fraction were calculated by summation of short-axis slices. Enhancement in contrast indicated area-at-risk due to oedema of the left ventricle myocardium.

After 15 minutes, late enhancement inversion recovery fast low angle shot (IR FLASH) sequences were recorded in the same image positions as the balanced steady-state free precession cine sequence after a Look-Locker sequence was used in advance. We combined the early and late images to quantify the myocardial salvage index. The area at risk and the final infarct size were quantified semi-automatically in off-line analyses. Microvascular obstruction was defined as dark areas surrounded by bright areas. All CMR images were analyzed by the core lab at St. Olavs Hospital in Trondheim, using Segment (Medviso, Lund, Sweden), se Figure 5.

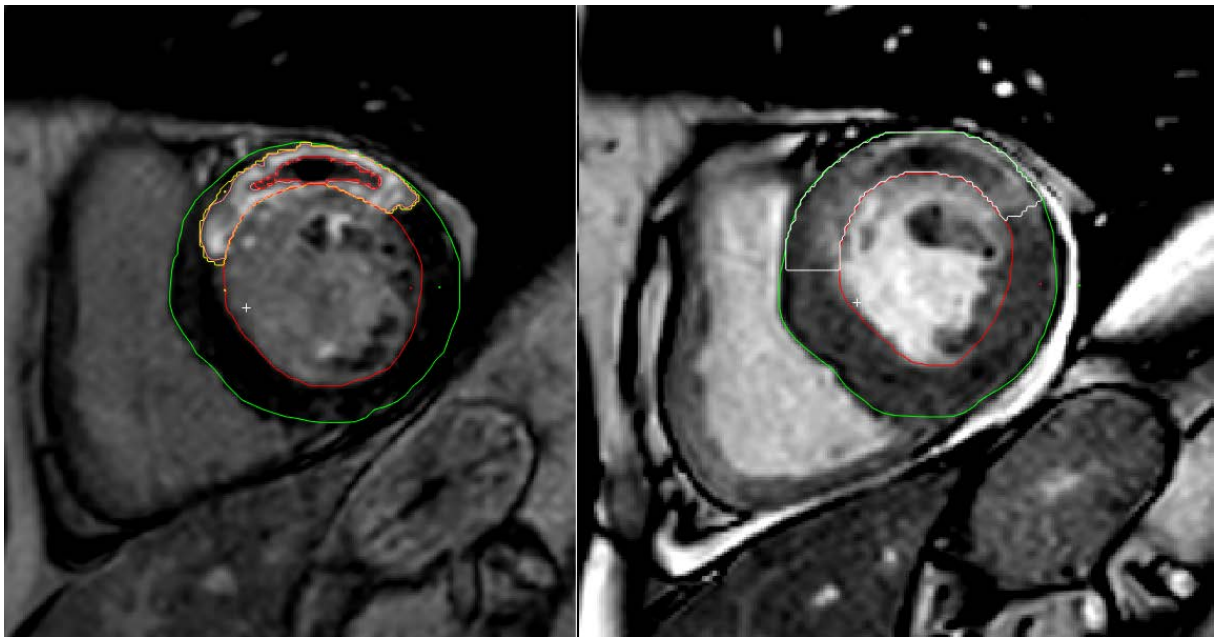

Figure 5. Measurements necessary for calculating the myocardial salvage index. Short-axis images of the left ventricle in a patient with ST-elevation myocardial infarction due to LAD occlusion. Left panel: late gadolinium-enhanced image, with delineation of hyperintense transmural scar in the

anterior wall. The dark area surrounded by hyperintense myocardium respresents microvascular obstruction. Right panel: Early gadolinium-enhanced image with the area-at-risk outlined by the increased signal intensity in the anterior wall. Microvascular obstruction is observed. *Representative image of one of the trial participants. Courtesy of Sindre Woxholt, St. Olav´s Hospital, Trondheim, Norway.*

3.2.1.5 Blood sampling, laboratory analysis and biobank

Arterial blood samples were collected directly from the right radial artery, at the catheterization lab just prior to heparin administration and acute PCI. Thereafter, venous blood samples were collected at 6-8 h and 12-16h, and at all clinical follow-up visits thereafter at 14-33 h (24h), at 3-7 days, at 3 months, and at 6 months, as seen in Table 2.

The hospital laboratories analyzed conventional biomarkers and safety samples per routine. NT-proBNP was assayed on a MODULAR platform (Roche Diagnostics, Basel, Switzerland), CRP was determined by a high-sensitivity particle-enhanced immunoturbidimetric assay (Tina-quant CRP (Latex) HS, Roche Diagnostic, Basel, Switzerland), and TnT was assessed using a high-sensitive immunoassay (Roche hs-TnT). Leucocytes and differential counts were analyzed on Sysmex XN-10 (Sysmex, Kobe, Japan). Safety samples included hematology, renal function, serum electrolytes, tests of liver damage, but also TnT, creatin kinase (CK) MB and CRP. Safety samples were performed at all clinical follow-up visits.

Biobank samples were collected at all visits (Table 2). Tubes containing ethylenediaminetetraacetic acid (EDTA) and tubes with citrate as the anticoagulant were put on ice immediately after sampling and centrifuged according to protocol 20 minutes. The tubes without additives were left at room temperature for 1-2 hours prior to centrifugation for 15 minutes, see Figure 6. The resulting plasma and serum were stored in multiple aliquots and labelled with the unique trial subject number and the letter A to E signifying the visit. Biobank samples of serum and plasma were collected and stored at -80ºC.

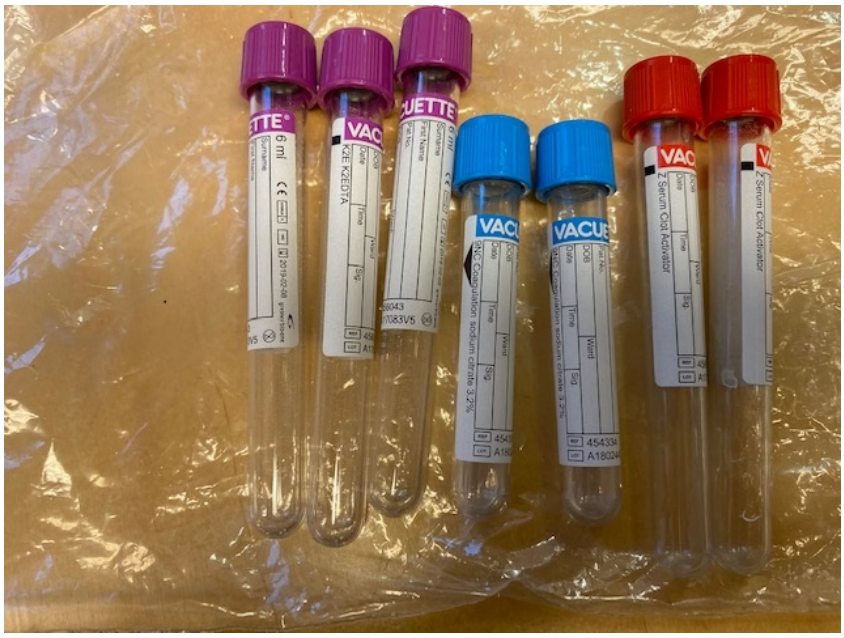

Figure 6. Biobank sampled at all 5 clinical visits. i) Tubes containing ethylenediaminetetraacetic 6 ml x 3 (purple lid), ii) tubes containing coagulant sodium citrate 3.2 %, 3 ml x 2 for plasma (blue lid), and iii) tubes without additives 6 ml x 2 (red lid). *Private photo*.

PAXgene^{TM} Blood ribonucleic acid (RNA) tubes (BD, Frankline Lakes, NJ) were collected at admission, at 3-7 days post infusion, and lastly at 6 months follow up. They were stored at -80 degrees Celsius. Later, they were used to RNA isolation in whole blood (Paper III).

3.2.1.6 Additional procedures

Echocardiography

Echocardiography was performed with a General Electrics healthcare Vivid E9 Doppler ultrasound scanner or a similar. Echocardiograms were obtained at the 3-7 days visit and at 6 months follow-up. Patients were examined in the lateral recumbent position. Transthoracic echocardiograms were obtained according to guidelines recommended by the European society of echocardiography, using standard ultrasonic techniques and imaging planes (76). We obtained grey-scale two-dimensional cine loops from parasternal long/short axis, apical 4 chamber/5chamber, apical 2 chamber, and apical long axis views, and Color Doppler and Tissue Doppler images from the apical views. Three heartbeats were recorded in each position. Data were digitally stored to be analyzed at the core lab later.

3.2.3 Paper III

Additional blood sampling was performed at Oslo University Hospital Rikshospitalet for Paper III. Flow-cytometry analyses were performed on EDTA-blood. Lastly, blood samples were collected in sodium heparin tubes for isolation of pheripheral blood mononuclear cells.

3.2.3.1 Flow cytometry

The Department of Immunology at Oslo University Hospital holds an ISO (International Standard Organization) certification. The flow cytometry analyses in Paper III were performed there in accordance with standard operating procedure.

3.2.1.9 Immune cell isolation – RNA and protein isolation analyses

The Research Institute of Internal Medicine at Oslo University Hospital performed isolation of peripheral blood mononuclear cells. A method based on density gradient was used to differentiate cells. Lymphocytes, monocytes and platelets were separated from granulocytes and red blood cells, and the medium itself. A band consisting of mononuclear cells were finally pipetted and washed. Cell subsets of T cells were isolated by using magnetic bead isolation.

RNA and protein isolation analyses were also performed at the Research Institute of Internal Medicine at Oslo University Hospital by experienced personnel in accordance with established procedures.

3.2.4 Paper IV

Several biomarkers reflecting inflammation and its resolution were measured per routine. Plasma levels of polyunsaturated fatty acids were determined by established criteria. Liquid chromatography with tandem mass spectrometry (LC-MS/MS) was performed to identify each lipid mediator. Furthermore, cytokine analyses were performed by enzyme immunoassay with the Meso Scale (Rockville, MD) V-Plex Human proinflammatory kit. Interleukin-6, Interleukin-8 and TNF were estimated.

3.3 Statistics

Statistical Package for the Social Sciences (SPSS) verison 25 (IBM Corp, Armok, New York) and GraphPad Prism 8.3.0 (GraphPad Softeware, La Jolla CA) were the programs used for statistical analyses.

Paper I

Categorical data were reported as numbers and percentages. Continuous data were summarized by the mean ± SD. In some instances, we also reported the range from lowest to highest values.

Paper II

Analyses were directed by a pre-specified statistical analysis plan. End point analyses were performed according to the intention-to-treat principle. Thus, randomized participants were analyzed according to treatment assignment, and noncompliance after randomization was ignored in the final analyses. Continuous data were summarized by the mean ± SD due to normal distribution. If distributions were skewed, continuous data were summarized by median and interquartile-range. Categorical data were reported as numbers and percentages. Analysis of covariance (ANCOVA) was used to compare treatment groups regarding MSI, the primary endpoint measure adjusted for time from symptom onset. ANCOVA test was used to compare baseline-adjusted between-group difference in left ventricular end-diastolic volume as a secondary outcome measure. AUC was calculated for CRP and TnT using the quadratic method. To compare groups where distributions were skewed, a Mann-Whitney *U* test was used, as for CRP, TnT, microvascular obstruction and final infarct size.

Paper III

Within the ASSAIL-MI trial population, we analyzed endpoints pertaining to inflammation and cell populations according to treatment allocation tocilizumab/placebo and time from symptom onset (<3 h /3-6 h). We also compared the results to those of a control group of patients with stabile angina pectoris. A one-way ANOVA with Dunnett´s multiple comparison test was performed to compare the participants of the ASSAIL-MI trial to those of the control group. In addition, we performed mixed effect analyses with Bonferroni´s multiple comparison test or unpaired two-tailed t tests where appropriate. Spearman´s

correlation coefficient was calculated in search for correlation between treatment allocation and outcome measures such as MSI and TnT, but also between time from symptom onset and the two outcome measures. In the analyses of gene expression, false discovery rate adjustment was performed and adjusted p-values reported.

Paper IV

We used Student´s t-tests (paired and unpaired) and Chi-square tests or Fisher exact tests (for observation <5) when comparing continuous data and categorical variables, respectively. ANOVA was used when comparison of more than two groups. We performed repeated measures when one-way analysis of variance was significant. Lastly, we used non-parametric statistics o search for associations between SPMs and parameters reflecting cardiac function and inflammation.

4 Summary of results

Our main finding in this thesis was a favorable effect of IL-6 inhibition in acute STEMI presenting for PCI. Beyond the effect on the primary endpoint, the ASSAIL-MI trial was designed to show treatment effect on multiple secondary outcome measures, as well as safety. Tocilizumab, as an IL-6 receptor inhibitor, affected neutrophils, but to a substantially lesser degree lymphocytes. Changes in neutrophil levels were associated with measures of infarct size. However, causality may only be inferred from RCT-studies, not from correlation studies.

4.1 Paper I

Rationale for the ASSAIL-MI-trial: a randomized controlled trial designed to assess the effect of tocilizumab on myocardial salvage in patients with acute ST-elevation myocardial infarction (STEMI)

- In Paper I, we described the rationale for attacking IL-6 signaling in PCI treated STEMI patients to attenuate inflammation due to reperfusion injury. Possible mechanisms of actions for tocilizumab was discussed in relation to the atherothrombotic process in cardiovascular disease. The ASSAIL-MI trial was a proof-of-concept-study designed to demonstrate treatment effect and safety by using the RCT- study design, the gold standard in clinical intervention studies, see Figure 7.

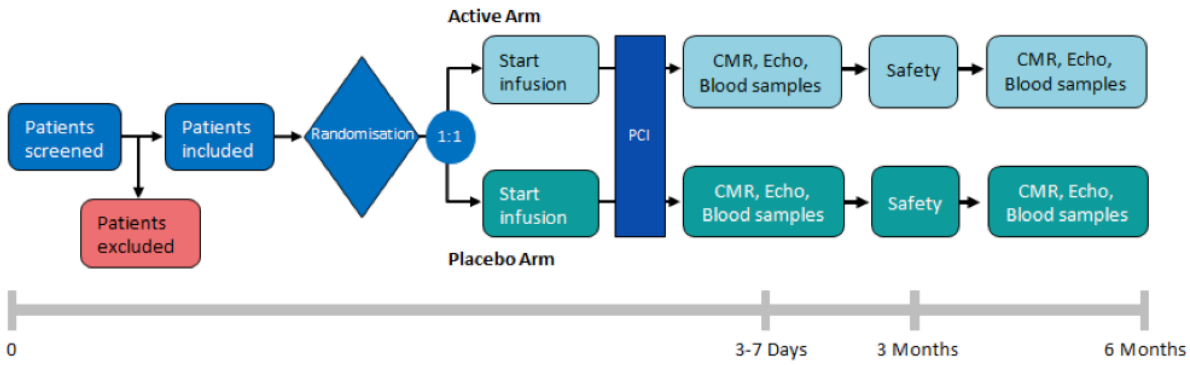

Figure 7. Overall study design of the ASSAIL-MI trial

- We also reported preliminary data from the ongoing ASSAIL-MI trial. As of 2019, we had enrolled 135 patients (67.5%) from the beginning in March 2017. Table 3

highlights to the main demographic and clinical data seen in the first 100 patients enrolled. The age, gender and cardiovascular risk profile is as would be expect in a general STEMI population without a previous history of myocardial infarction.

Table 3: Selected baseline characteristics of the first 100 patients in the ASSAIL-MI trial

Demography and clinical data	
Age, years (range)	61.0±8.7 (38-79)
Male, gender, n (%)	80 (80.0)
Cardiovascular risk factors, n (%)	
Current smoker/former smoker	40 (40.0)/26 (26.0)/34 (34.0)
Hypertension	33 (33.0)
Diabetes mellitus	7 (7.0)
Peripheral vascular disease	2 (2.0)
Body mass index, kg/m^2 (range)	27.0 (15.7-39.2)±4.3

Data are given as number (percent) or mean±SD (range)

4.2 Paper II

Randomized Trial of Interleukin-6 Receptor Inhibition in Patients With Acute ST-Segment Elevation Myocardial Infarction

The ASSAIL-MI trial enrolled patients in the time period from March 2017 to February 2020. 4735 patients were admitted with STEMI during the recruitment period, and of these, 200 patients were enrolled and randomized. One patient withdrew his/her consent to participate, leaving 199 patients for the final analysis in the ASSAIL-MI trial. 101 patients were randomized to the tocilizumab arm, and 98 patients to the placebo arm. Few study patients were lost to follow-up with no significant between-group differences.

- Figure 8, sums up the ASSAIL-MI trial. The adjusted myocardial salvage index as measured in the acute phase was higher in the tocilizumab arm than in the placebo arm (69 ± 19% vs. 64 ± 21%) with a between-group difference of 5.6 percentage points (95% confidence interval: 0.2 to 11.3; p = 0.04).

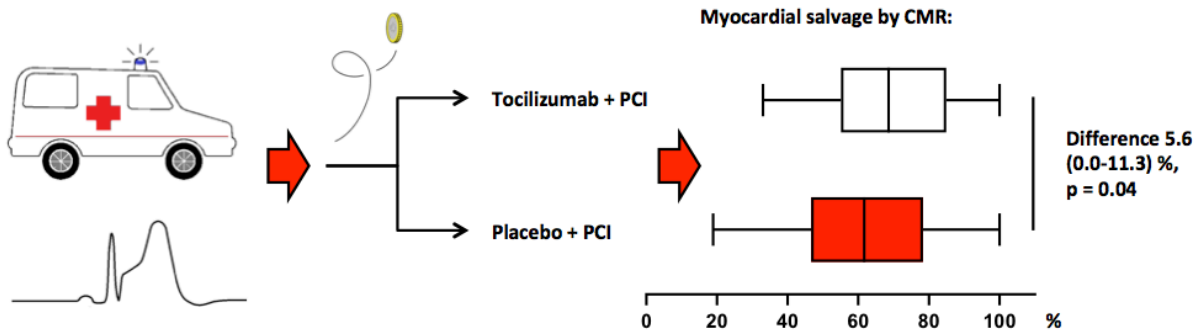

Figure 8. Central illustration: Design and primary result of the ASSAIL-MI trial. Illustration made by K. Broch. Printed with permission.

- Secondary outcome measures are presented in Table 4. As seen, i) the extent of microvascular obstruction during acute phase was significantly less in the tocilizumab arm than in the placebo arm (p = 0.03), ii) the area under the curve of CRP and TnT during this time period was lower in the tocilizumab group than in the placebo group, but only statistically significant for CRP (p < 0.001) and just numerical for TnT (p = 0.13), iii) after 6 months, the median final infarct size was 21% lower in the tocilizumab arm, but this difference was not statistically significant (p = 0.08), iv) measures of cardiac remodeling and heart failure at 6 months follow-up did not differ between treatment arms, and v) there were no safety-issues.

Table 4. Secondary Outcomes

	Tocilizumab	n	Placebo	n	p
Final infarct size (g)	12.6 (6.9-23.6)	97	15.0 (6.8-31.1)	93	0.08
Extent of microvascular obstruction (% LV volume)	0 (0-14)	99	4 (0-18)	96	0.03
Troponin T AUC (ng/l/h)	1614 (860-3515)	101	2357 (97-4127)	98	0.13
C-reactive protein AUC (mg/l/h)	1.9 (0.9-4.9)	101	8.6 (5.0-17.9)	98	<0.001
NT-proBNP at 6 months (ng/l)	79 (50-187)	98	63 (50-148)	97	0.25

LVEDV at 6 months (ml)	157 (151-166)	97	160 (153-166)	93	0.54
Any serious event	19	99	15	96	0.57
Infections requiring hospitalization at 6 months	3	99	2	96	0.10

Values are mean±SD, n, or median (interquartile range), unless otherwise indicated. *We did not adjust for multiple testing, and app p values are nominal only. AUC = area under the curve; CI = confidence interval; LVEDV = left ventricular and-diastolic volume; NT-proBNP = N-terminal pro-B-type natriuretic peptide

- Heterogeneity of treatment effects was seen in only one of six pre-specified subgroups, see Figure 9. Patients presenting >3 hours after symptom onset seemed to benefit more from tocilizumab treatment. Sex may be of importance, but the interaction between treatment and sex did not reach nominal statistical significance in the exploratory subgroup analysis.

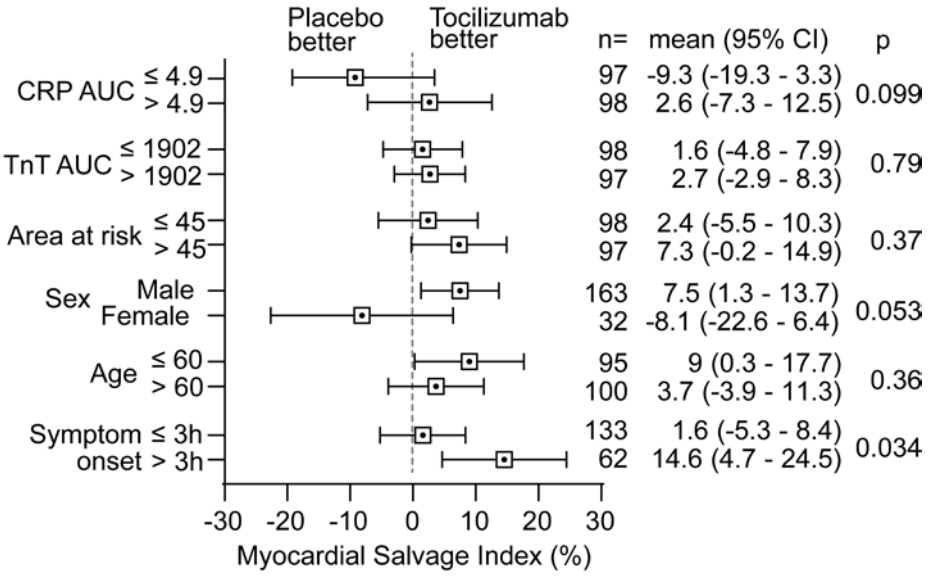

Figure 9: Primary Outcome in Pre-Specified Subgroups

4.3 Paper III

Interleukin-6 inhibition in ST-elevation myocardial infarction: Immune cell profile in the randomized ASSAIL-MI trial

This exploratory sub-study was based on results from the ASSAIL-MI trial. Levels of leukocyte subsets were measured repeatedly after PCI. We reported leukocyte counts according to treatment allocation and stratified by time from symptom onset. Statistical analyses was done to search for correlations between immune cells and outcome measures such as MSI and TnT. We compared the results with those of patients with chronic coronary artery disease scheduled for coronary angiography at Oslo University Hospital. The main findings were:

- In the patients with acute STEMI, neutrophil levels were elevated at admission and declined gradually after PCI (Figure 10A). However, in the tocilizumab arm, this decline in neutrophil levels was more rapid and pronounced (Figure 10B). The reductions in neutrophil counts after 24 h differed significantly between the treatment groups, 56% decline in the tocilizumab arm versus 8% mean decline in the placebo arm.

- The neutrophil-lymphocyte ratio was higher in the STEMI patients at admission and 24 hours than in the control subjects with chronic coronary artery disease (Figure 10D). This result was mainly driven by the elevated levels of neutrophils. The lymphocyte levels changed little over the first 24 hours (Figure 10C).

- Correlation analyses showed that the larger the change in the number of neutrophils from admission to 24 hours the higher the myocardial salvage (tocilizumab: r=0.206, p=0.045; placebo: r= 0.049, p=0.65) and the lower the peak TnT concentration (tocilizumab: r=0.227, p=0.03; placebo: r=0.015, p=0.89).

- Patients presenting > 3 hours from symptom onset to PCI, had a more pronounced effect of tocilizumab on myocardial salvage. However, in the tocilizumab group, the decrease in neutrophil counts and neutrophil-lymphocyte ratio was independent of

time stratification (short < 3 hours, or late 3 to 6 hours). Only, a low number of $CD8^+$ T cells was associated with increased myocardial salvage in patients presenting late.

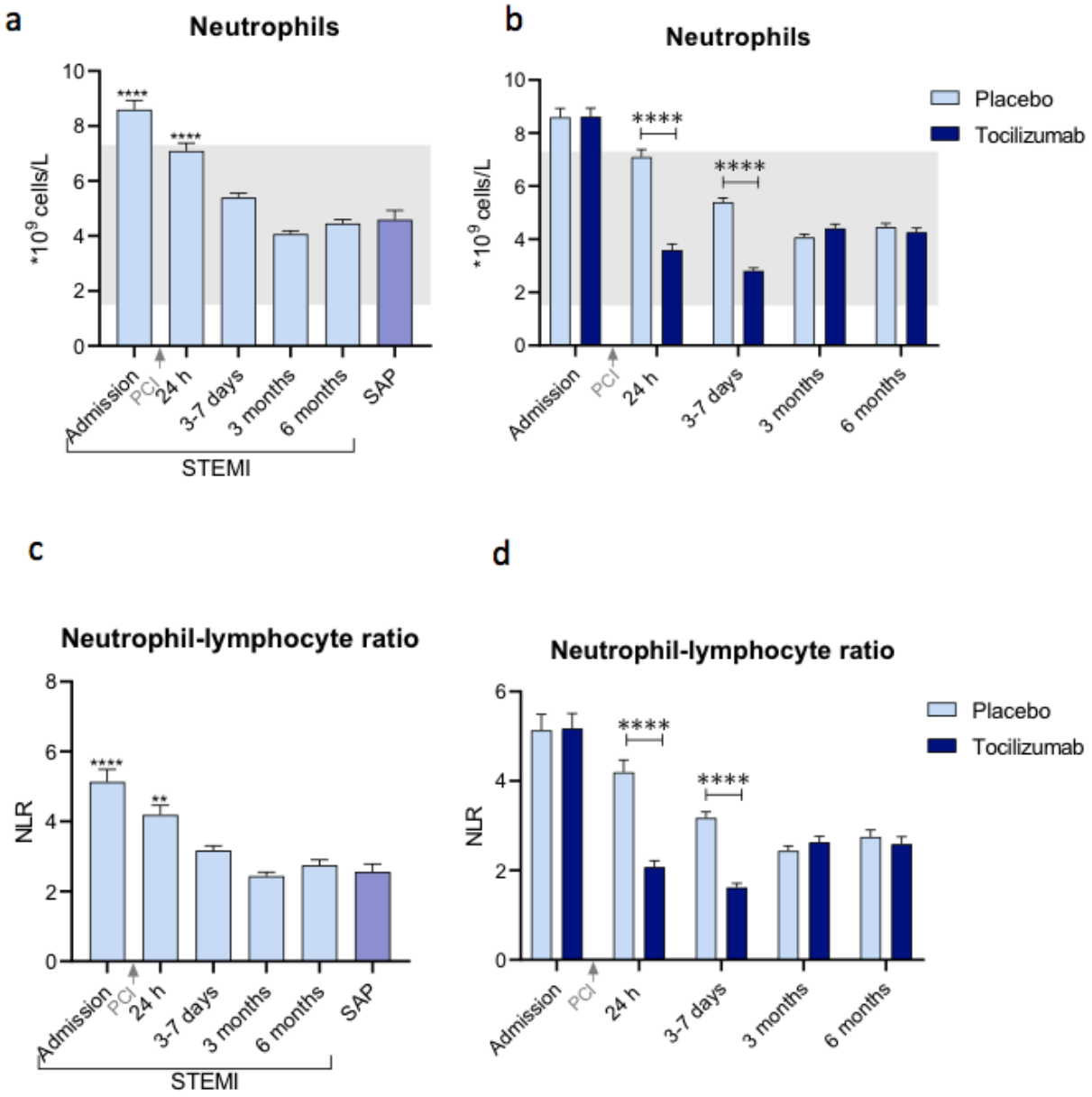

Figure 10. A: Levels of circulating neutrophils in the placebo group of the ASSAIL-MI trial and in patients with chronic coronary artery disease. B: Levels of circulating neutrophils according to treatment allocation in the trial participants. C: Neutrophil-lymphocyte ratio in the placebo arm of the ASSAIL-MI trial and in patients with chronic coronary artery disease. D: Neutrophil-lymphocyte ratio by treatment allocation in the ASSAIL-MI trial. ASSAIL-MI = ASSessing the effect of Anti-IL-6 treatment in Myocardial Infarction, SAP = stabile angina pectoris, STEMI = ST-segment elevation myocardial infarction.

- Forty-six genes were expressed differently between the treatment groups. Most of the genes that were expressed differently, were down-regulated in the tocilizumab arm. At 6 months follow-up, there were only minor changes in gene expression. Highlighted altered pathways relevant for neutrophil function were "neutrophil degranulation" and the "MAPK" cascade.

4.4 Paper IV

The study described in Paper IV demonstrates an early increase of specialized pro-resolving lipid mediators in patients with ST-elevation myocardial infarction

- Plasma levels of biomarkers for myocardial injury and inflammation are presented in Figure 11. As seen in the figure, neutrophil counts peaked markedly in patients with STEMI presenting for PCI, and neutrophil counts peaked before TnT and CRP.

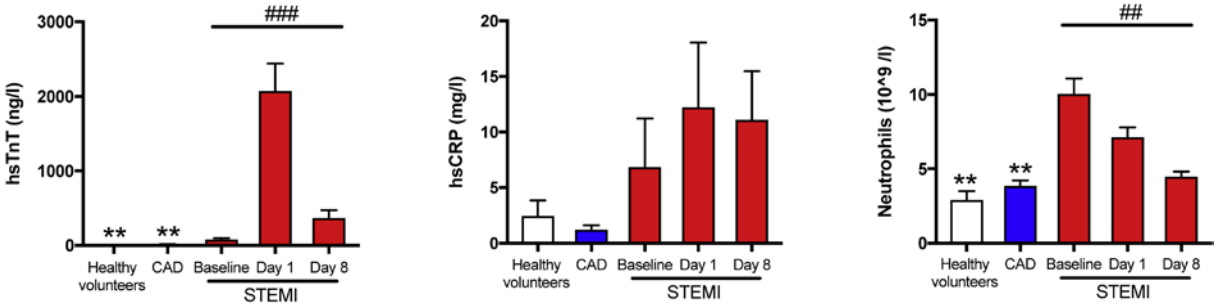

Figure 11. Plasma levels of high sensitive troponin T (hsTnT), high sensitive C-reactive protein (hsCRP), and neutrophils in three different patient populations i) healthy volunteers (*n* = 10), ii) patients with chronic coronary artery disease (*n* = 10), and iii) patients with ST-segment elevation myocardial infarction (STEMI) (*n* = 15).

- Specialized pro-resolving lipid mediators were markedly increased upon first presentation in patients with STEMI, and rose prior to peak levels of TnT.

- The rapid increase in neutrophil counts correlated with several individual protectins as products of SPMs, but not to CRP levels.

- Docosapentaenoic acid- and docosahexaenoic acid-derived protectin families were the main drivers of early SPM response in STEMI.

- Pro-inflammatory lipid mediator levels in STEMI and chronic CAD may be lowered due to aspirin use.

5. Discussion of main findings

In this thesis, we show that IL-6 inhibition improves myocardial salvage in acute STEMI. Final infarct size at 6 months was not statistically reduced. However, the median final infarct size was 21% lower in the tocilizumab arm compared to placebo. We did show that tocilizumab attenuated the inflammatory cascade following PCI. CRP levels were statistically lower in the acute phase and neutrophil counts were markedly reduced. The relative reduction in neutrophils from admission to 24 hours was associated with MSI, suggesting that the effect of tocilizumab could manifest through changes in neutrophil counts. In the tocilizumab group, several genes related to neutrophil function and signal transduction were downregulated. No safety issues were apparent even though we interfered with the fine-tuned balance of pro- and anti-inflammatory processes taking place after myocardial infarction. Taken together, our results suggest a net beneficial effect of IL-6 inhibition in STEMI. Other targets involved in the termination of inflammation are of interest in the quest for new treatment strategies in STEMI. Resolvins of inflammation increase rapidly after STEMI. These compounds may limit the damage to the myocardium and could represent targets for therapy in myocardial infarction.

5.1 Why perform a proof-of-concept study on IL-6 inhibition in STEMI?

Anti-cytokine and anti-inflammatory therapies in coronary artery disease have emerged as methods for treatment and prevention of atherosclerotic diseases (77). Recently, IL-6, a central signaling cytokine of the innate immunity, has been given renewed attention as a promising target in this field. Circulating levels of IL-6 are associated with vascular events in primary and secondary prevention (78, 79). High levels of IL-6 are associated with final infarct size and adverse clinical events in patients with STEMI (80). Recently, we assessed the effect of IL-6 inhibition in patients with NSTEMI (43). In this thesis, we have examined the efficacy and safety of IL-6 inhibition in STEMI.

Between the initiation of the ASSAIL-MI trial in 2017 and the submission of this thesis in 2023, a tremendous development has taken place in this research field. Targeted inhibition of

inflammation through the NOD-, LRR- and pyrin domain-containing protein 3 (NLRP3 inflammasome), IL-1, and IL-6 has attracted major interest in cardiovascular disease (81). Two milestone clinical trials, the CANTOS and low-dose colchicine (LoDoCo) 2 trials with randomized controlled designs and sample sizes ranging from approximately 5000 to 10 000 participants, have been game changers in this regard (31, 82). These phase III clinical trials, in addition to several phase II trials like the Colchicine Cardiovascular Outcomes Trial (COLCOT) and the LoDoCo trial, have provided support for anti-inflammatory therapy in patients with coronary disease (83, 84). However, the Cardiovascular Inflammation Reduction Trial (CIRT) demonstrated the importance of targeted inhibition: not all anti-inflammatory treatments seem to result in better cardiovascular outcomes. In CIRT, low-dose methotrexate did not reduce cardiovascular events compared to placebo in patients with known coronary artery disease and/or diabetes/metabolic syndrome, and it did not affect the IL-1β-IL-6-CRP cascade (85). Unlike CANTOS, this trial did not mandate residual inflammation among enrolled patients. Interleukin-6 has a pivotal place in a central signaling pathway in cardiovascular inflammation. It sits downstream of IL-1 and upstream of CRP, and is today possibly the most interesting target when aiming to translate immune-modulating therapies into clinical cardiovascular medicine. The data obtained through our work can help guide our research concerning acute coronary syndromes, atherothrombosis and IL-6 inhibition.

In the ASSAIL-MI trial, our main objective was to assess the effect of IL-6 inhibition in STEMI. Detailed knowledge of IL-6 biology in terms of its pleiotropic effect on numerous cell types is important to understand the potential fallout of tampering with its signaling. For anti-inflammatory treatment of IL-6 inhibition to be beneficial, it would have to attenuate the pro-inflammatory activity of IL-6. The IL-6 trans-signaling pathway acts on numerous cell types to accumulate neutrophils and differentiate T- and B cells. High-levels of soluble-IL-6 receptors in the acute phase of STEMI are associated with subsequent major cardiac adverse events (86). On the other hand, classical signaling is important for regenerative and protective function. In classical signaling, IL-6 binds to cell surface receptor only present at a few cell types and act through the signaling complex with gp130 signaling. Hepatocytes synthesize and release acute phase proteins when IL-6 binds to its cell surface receptor and act through the signaling complex with gp130 signaling. Both these signaling pathways are blocked by tocilizumab. Thus, a well-balanced blockage - not too long or too strong - is of importance when searching for a dose to harmonize efficiency and safety.

5.2 IL-6 inhibition in STEMI and efficiency

Tocilizumab had a statistically significant effect on the primary outcome but not on final infarct size. In the protocol of the ASSAIL-MI trial, we stated that a 20% reduction in infarct size with tocilizumab would be of clinical significance. However, the effect of tocilizumab on myocardial necrosis assessed at 6 months follow-up was smaller than anticipated. Several interesting points are to be made in the further discussion.

But first, a short review of our main outcome measures. Of note, our outcome measures in the ASSAIL-MI trial comprised several markers of myocardial injury in the acute phase. Myocardial salvage, microvascular obstruction, and TnT are all surrogate markers of infarct size However, the MSI innately adjusts for the area at risk, which is a major confounder when assessing the effect of a therapy that is thought to ameliorate ischemia/reperfusion injury. We therefore knew that given a certain sample size, the chances of achieving statistical significance was larger with the MSI than with the other markers of infarct size. The trial was therefore powered to show a between-group difference in the MSI; whereas, we knew up-front that the trial was underpowered to show differences in the other parameters. Despite the relatively large relative reduction in TnT and final infarct size in the tocilizumab arm, these results were not statistically significant. This should not be surprising, given the broad confidence intervals of these variables. On the other hand, the fact that all of the measures of myocardial damage were nominally smaller in the tocilizumab arm, contribute to the credibility of the results: These did not seem to reflect statistical flukes where one parameter increased and another decreased.

At 6 months follow-up, final infarct size and myocardial remodeling were assessed by CMR, TnT and NT-proBNP. At this point, there were no statistically significant between-group differences. However, given the small absolute difference in infarct size between the arms, differences in left ventricular remodeling were not to be expected. In fact, the average NT-proBNP-level and left ventricular diameter in the placebo arm was within the normal range, suggesting that no meaningful remodeling had occurred. In other words, there was no adverse remodeling to mitigate.

The small average infarct size in the placebo arm may be the reason why final infarct size and TnT did not differ between the treatment groups. Several factors may explain the small

average infarct size: i) patient enrolment criteria did not include hemodynamic unstable patients, cardiogenic shock, or patients with cardiac arrest prior to admission, ii) informed consent had to be obtained in the acute setting not to delay standard care of treatment with PCI, iii) all patients received well-established prehospital treatment with double antiplatelet treatment and anticoagulants, iv) time from symptom onset was short, v) we included STEMIs caused by the occlusion of any coronary artery, not only the larger anterior wall infarctions. Differences in time from symptom onset, infarct related arteries, and sex differences may have confounded the results. We aimed for larger infarcts than those that we actually enrolled. This has implication for the assumption made in term of sample size in the first place. So why did we include a patient population with these characteristics, and how generalizable are our results to the general STEMI population?

As stated in the introduction, several factors affect final infarct size. Our patient population was highly selected and did not include the patients with the potentially largest infarctions. Patients with hemodynamic instability might present to a larger degree with occluded, rather than severely stenotic or spontaneously reopened vessels. In the ASSAIL-MI trial, participants were included based on ECG changes at presentation, and not according to verified occlusions on the angiogram. In this way, our study population was heterogenic with regard to the patency of the infarct-related artery. However, the mix of patent and occluded infarct related artery may have influenced our primary outcome measure in term of smaller infarcts than expected. Studies have shown that patients with STEMI presenting for PCI at the catheterization lab with patent infarct-related artery are more likely to have lower infarct size and improved prognosis (87, 88).

The myocardium at risk, defined by the region supplied by the occluded coronary artery, is a major independent predictor for infarct size. The area at risk depends upon proximal versus distal localization within the coronary artery, but also upon which of the three coronary arteries that is affected. The left anterior coronary artery serves a larger area, and an occlusion of this vessel may potentially produce larger infarcts. However, we did not limit participation to anterior wall STEMIs only. All participants received antiplatelet therapies and anticoagulation prior to admission to a tertiary hospital for PCI. Real-life data shows benefits on mortality when PCI-procedures are performed at high-volume centers and by experienced

operators. Door – to – ballon time was short in the ASSAIL-MI trial (23 ± 10 vs 23 ± 11 minutes), and is a quality parameter in the management of STEMI (89, 90).

Thrombus aspiration was not performed routinely in the ASSAIL-MI trial. Two large RCTs reported no benefit on clinical outcome to recommend against routine (91-94). In contrast, small-scale studies had previously suggested a potential benefit in term of reduced microvascular obstruction (95). Microvascular obstruction was significantly different between treatment groups in the ASSAIL-MI trial, and is further discussed in chapter 6, which examines methods. Almost none of the participants in the ASSAIL-MI trial underwent thrombus aspiration.

According to sub-group analyses of the six pre-specified groups (Figure 8), time from symptom onset was the only parameter that significantly interacted with the treatment effect. This will be further discussed in the context of CMR measures and microvascular obstruction in Chapter 6. There may be a true difference in the treatment effect between the sexes. In our trial, the effect of tocilizumab nominally favored men. However, more data is needed. As most clinical cardiovascular studies, the ASSAIL-MI trial enrolled more men than women.

Outcome measures pertaining to left ventricular remodeling and heart failure development did not vary between treatment groups, and the result might be linked to the small average infarct size. However, we managed to show treatment effect on myocardial salvage, and this finding may therefore be further evaluated in larger clinical trials.

5.3 IL-6 inhibition in STEMI and safety

There were no safety issues in the ASSAIL-MI trial. This is in line with previous research in a NSTEMI population. A modest dose was used to limit the risk for severe events, and this dose had previously not been associated with safety issues in a NSTEMI population.

Tocilizumab can induce sustained neutropenia, increases in hepatic enzymes, and increased circulating levels of triglycerides (96). In the ASSAIL-MI trial, there were few hospitalization

for serious infections. Increased hepatic enzymes and altered cholesterol-levels were seen in the treatment group, but there was no significance difference between the treatment groups.

A novel IL-6 ligand inhibitor, ziltivekimab, has recently shown promising effect in patients with high atherosclerotic risk, but without a systemic disorder (81). It is now tested in an ongoing phase III trial (97). Ziltivekimab binds to the IL-6 ligand to inhibit IL-6 signaling. However, results of the phase II trial was promising in term of effect and safety. These trials demonstrate the continued interest regarding IL-inhibition in cardiovascular disease and data regarding safety profile.

The “lipid paradox” affecting rheumatic arthritis patients deserves mentioning in relation to our discussion on IL-6 inhibition and side effects. A worsened lipid profile in term of increased total cholesterol, low-density lipoprotein cholesterol, and high-density lipoprotein cholesterol are found to be inversely associated with the presence of rheumatic arthritis, which is a chronic inflammatory state with increased cardiovascular disease risk, indicating a benefit of immune-modulating therapies. However, the cholesterol elevation that is observed in these patients during the first weeks of tocilizumab treatment does not translate to a worsening of the atherogenic index, which is the most accurate predictor of cardiovascular risk in rheumatic arthritis patients (36).

5.4 IL-6 inhibition in STEMI and the immune system

IL -6 is secreted by immune cells (neutrophil and macrophages), fibroblasts, and endothelial cells among others, as response to inflammation. As a pivotal cytokine of the innate immune system, it also link to the adaptive immune system through promotion of CD4+ T cells (98). It induces acute phase proteins such as CRP, but also fibrinogen and plasminogen activator inhibitor involved in thrombosis formation, to precipitate ACS (99). Studies have shown that IL-6 levels are elevated in acute myocardial infarction from admission to discharge with a peak at 1-2 days after symptom onset, and also in patients not undergoing PCI (47). Immune cells may predict short-and long term mortality in patients with acute coronary syndrome in term of the neutrophil-lymphocyte ratio (71). The neutrophil-lymphocyte ratio links the innate

and the adaptive immune system and is a well-established marker of immune response. It is also easy to obtain.

In Paper III, we investigated levels of neutrophils and lymphocytes from baseline, during the acute phase and until 6 months follow-up in both treatment arms and stratified by time from symptom onset. Our results suggest that tocilizumab markedly reduced the number of circulating neutrophils during the first 24h, in contrast to just minor changes in lymphocytes levels. Subgroup analysis of the ASSAIL-MI trial suggested a beneficial effect in patients presenting late for PCI. The assumed larger area at risk in these patients may benefit more from anti-inflammatory treatment than the smaller injuries in patients with short ischemic time prior to reperfusion. However, significant differences in neutrophil and lymphocyte levels between treatment arms were not found in term of time stratification.

In our exploratory study, Paper III, the changes in the neutrophil-lymphocyte ratio were mainly driven by the rapid drop in neutrophils during the first 24h and until day 3-7 after myocardial infarction. The drop in neutrophils at 24h correlated with increased MSI and lower circulating levels of troponin T, indicating a beneficial association with myocardial salvage and necrosis. Our findings are in line with a previous study that reported correlation between levels of neutrophils and infarct size in ACS (59). Downregulation of genes in the tocilizumab arm suggests attenuated neutrophil function in the active treatment arm. The "neutrophil degranulation" pathway was the pathway that was the strongest affected in our trial. Taken together, we observed not only a marked drop of circulating neutrophils short time after reperfusion, but also altered inflammatory potential of the circulating neutrophils in term of dampened function, to be associated with myocardial damage.

It is known from previous studies in rheumatic diseases that tocilizumab may give side effects like neutropenia. Our result in the STEMI study was in line with the rapid decrease in neutrophil count seen in patients with NSTEMI allocated to tocilizumab. However, in contrast to the NSTEMI study, changes from baseline did not correlate between troponin T and neutrophils in the ASSAIL-MI trial, whereas the AUC for troponin T and neutrophils during the acute phase were positively and significantly correlated in NSTEMI.

The loss of circulating neutrophils may be due to increased migration of neutrophils from the circulation to the injured tissue as first responders of the innate immunity. The main role of neutrophils in this setting is to clear up dead cell debris and then undergo apoptosis. Increased degree of apoptosis may also give lower neutrophil count. However, we were only able to assess neutrophils in peripheral blood and not in tissue. On the other hand, delayed removal of neutrophils may be harmful to the myocardium and stimulate the pro-inflammatory state to persist (55). Neutrophils are mobilized from the bone marrow and spleen upon IL-6 stimulation (100). However, tocilizumab blocks IL-6 receptors and may inhibit neutrophil release.

Pro-inflammatory mechanisms and endogenous pathways involved in the resolution of inflammation are countervailing mechanisms that modulate inflammation in ACS. In this thesis, novel therapeutic strategies in cardio protection is the overall frame. Our main focus has been on anti-inflammatory treatment and attacking IL-6, a central cytokine in the pro-inflammatory cascade following reperfusion (Paper I-III). However, in Paper IV, we have looked at other contributors to the fine-tuned balance of inflammation and repair. Specialized pro-resolving lipid mediators are key mediators in the active resolution processes to avoid chronic inflammation and to restore homeostasis.

Neutrophils have dual roles in myocardial infarction. Their phenotype changes over time from pro-inflammatory properties to a switch in phenotype to promote resolution processes (101). The first responders are involved in phagocytosis and degranulation. The second phenotype may upon activation trigger pro-resolution mediators to abrogate neutrophil recruitment and induce neutrophil apoptosis. This again promotes an environment of local mediators to enhance resolution, also by changing phenotypes in macrophages.

Anti-inflammatory effects and pro-resolving effects differ with respect to their action: inhibitory/blocking action versus apoptosis or efferocytosis, respectively (102). Paper I-III describes anti-inflammatory treatment with tocilizumab to block IL-6 signaling and its specific pathways. In contrast, a hypothetical pro-resolving therapeutic drug to attack reperfusion injury in the future might act on a plethora of mechanisms to ensure that resolution takes place.

Paper III and Paper IV showed a rapid increase in neutrophil counts in patients with STEMI undergoing PCI compared to patients with chronic coronary disease. Tocilizumab markedly reduced neutrophils within 24 hours. In Paper IV, we observed markedly increased levels of SPMs compared to control groups immediately after the onset of myocardial infarction. Several individual protectins were correlated with the rapid increase in neutrophil counts, but not with CRP. The recruitment of inflammatory cells and activation of inflammatory pathways ensure tissue repair if not prolonged into a more chronic inflammatory state (64, 73).

Little is known about IL-6 inhibition in STEMI with regard to the adaptive immune system. Our result did not show any major differences between treatment groups. However, IL-6 is involved in immune responses, such as the differentiation of T helper cells in the regulation to balance between interleukin-17 producing T helper 17 cells and regulatory T- cells. IL-6 is also involved in the process to differentiate B cells into anti-body-producing T cells. More research is needed with respect to the clinical significance of these functions.

5.6 Targeting immunology in cardiovascular medicine

A major breakthrough in this research era was the acknowledgement of atherosclerosis as a chronic inflammatory disease with interaction between lipids and inflammation as major characteristic. The seminal CANTOS trial, published in 2017, changed the landscape to establish evidence in support of the inflammatory hypothesis in cardiovascular disease. Recently, Colchicine was included in the new recommendation for secondary cardiovascular prevention of the European Society of Cardiology (103). Colchicine is an orally administrated and potent anti-inflammatory medication with its main mechanism to interact on the NLRP3 inflammasome, a component of the innate immune system.

In this thesis, we have mainly focused our attention on anti-inflammatory treatment by blocking cytokine signaling of IL-6. The IL1β – IL6 – CRP cascade is linked to the NLRP3 inflammasome (104). Pro-inflammatory pathways may be modulated at different levels in cascades: Colchicine acts on NLRP3, Canakinumab on IL-1β, and down-stream of IL-1β,

tocilizumab blocks IL-6 signaling. Depending on the level of blockade, effects, but also side effects, may differ.

Modulating inflammatory activity to obtain cardioprotection in patients presenting with STEMI, but also in ACS, might guide us towards precision medicine in future work. Elucidating the complex interactions that occur between the cells and signal molecules of the immune system, may give rise to hypotheses that can be tested stepwise to bring knowledge from - bench to clinic - in our search for novel clinical treatment options for patients with cardiovascular disease.

Crea and colleagues have argued for addressing inflammation with regard to three different mechanisms that contribute to the inflammatory response in ACS. They group patients according to i) coronary instability, ii) myocardial necrosis, and iii) distal embolization to guide treatment in subpopulation with similar characteristics (105). These treatment strategies build on mechanistic insight of the diverse underlying causes of ACS and point towards precision medicine (106). Interaction with inflammatory cascades at different levels may benefit patients with some characteristics more than others, and such selectivity may serve to limit undesirable side effects. In this picture, anti-inflammatory treatment with tocilizumab show promise in reperfusion injury. In other cases, adaptive immunity may be targeted to treat plaque fissure (105).

Active resolution processes involve derivates of the omega-3 fatty acids eicosapentanoic acid and docosahexanoic acid, such as lipoxin, resolvins and protectins (77). In Paper IV, we found an immediately increase in specialized pro-resolving mediators that were mainly driven by the omega-3 fatty acid-derivate protectins. Individual protectins correlated with neutrophil counts and not CRP. Our data suggest an early activation of resolution processes. The study had several limitation, such as sample size, the different use of aspirin across groups, and the lack of registration of diet. Bearing in mind to guide future work in this field: cellular targets involving resolution process may also promote anti-inflammatory agent and pro-resolving properties at the same time to reach homeostasis (102)

6. Methodological considerations

6.1 Study design and choice of main endpoint, ASSAIL-MI-trial

Hypothesis testing requires a research question to be tested. In the ASSAIL-MI trial our research question was specific and formulated before commencement of the trial. Our statistical null hypothesis was that there would be no effect of the intervention in the study population. We conducted a randomized controlled trial to minimize potential errors in term of bias, confounding factors, and errors that may happen by chance. The methodology was defined in the pre-specified protocol. This crucial step in trial design allows for evaluation of the hypothesis being tested and has impact on the clinical interpretation of the test results.

Randomized controlled trials are the current gold-standard study design for the determination of the efficacy and safety of medical interventions. In a clinical intervention trial, such as the ASSAIL-MI trial, two major tasks must be taken into consideration in the design process. First, to isolate the intervention and the outcomes from extraneous influences. Second, to generalize the results from sample to population. Test results must always be interpreted with caution in term of clinical relevance, population selections, and protocol adherence. However, randomized controlled trials are the most stringent way to provide evidence for a cause-effect relationship between the intervention and the outcome (107).

Clinical intervention and clinical endpoints

In clinical intervention trials such as the ASSAIL-MI trial, assessment of benefit and risk is the main goal. Well-defined clinical endpoints, such as survival and myocardial infarction, are among the most reliable way to assess clinical impact of a therapeutic intervention (108). However, these trials are often time consuming and requires a large number of patients for the clinical endpoints to be achieved.

Proof-of-concept study, primary outcome measure and infarct size

The ASSAIL-MI trial was designed to show treatment effect on myocardial salvage. The main objective of undergoing reperfusion therapy in patients with acute myocardial infarction is to obtain myocardial salvage (109). The degree of improvement in myocardial salvage is an independent predictor of outcome (110). CMR with gadolinium late enhancement images

were used to calculate myocardial salvage index. This estimate took into account measures of area at risk and irreversible injury as a ratio of area of risk for permanent injury.

Myocardial salvage index = $\frac{Area\ at\ risk - infarct\ size}{area\ at\ risk}$

The primary outcome measure was performed 3-7 days post infusion. Final infarct size, a secondary outcome, was measured at 6 months' follow-up. Infarct size is a major predictor of clinical endpoints like mortality and morbidity (16, 17). The ASSAIL-MI trial was a phase 2 clinical intervention study designed to provide the strongest evidence for causality between the intervention and MSI (111), but also focused on efficacy and safety (112). Surrogate endpoints for clinical outcome measures allow for smaller sample sizes and reduce costs. Potentially favorable effect of our intervention must be further evaluated in larger clinical trials with outcome measure as mortality and morbidity before tocilizumab is adapted for clinical use in myocardial infarction.

6.2 Myocardial salvage index (MSI) and sample size

The ASSAIL-MI trial was designed to assess the effect of treatment with tocilizumab on myocardial salvage in patients with STEMI. The sample size analysis was based on *post hoc* analyses from similar STEMI trials (CHILL-MI, MITOCARE) as reported by Engblom et al (113-115). The sample size analysis was based on the primary endpoint: the myocardial salvage index (MSI), and not infarct size. We considered a treatment effect of a 20% relative reduction to be clinically relevant. Based on the reported analyses (using Monte Carlo simulation) from previous trials, we would need 82 patients in each group to achieve a power of 90%. This number was based on the assumption that we would obtain a similar MSI with similar standard deviation in the placebo group in the ASSAIL-MI trial as in the CHILL-MI and MITOCARE trials. So, we did not perform sample size calculation by ourselves, but based our assumption on previous trials as reported by Engblom et al (115).

According to these previous trials the mean ± standard deviation for the myocardial salvage index was 54.0 ± 19%.4% and the mean ± infarct size 17.4 ± 10.5% of left ventricular mass. Our trial thus had the power to detect an underlying treatment effect on the myocardial salvage index of 9.2 percentage points (SD of 20% and 200 patients). In patients with first-

time, acute anterior wall myocardial infarction treated with PCI within 12 hours of the onset of symptoms, final infarct size is typically 20 % of total myocardial volume (116). Studies have shown that an absolute reduction in infarct size of approximately 3-5 % of myocardial volume, i.e. a relative reduction of 14 – 25 %, given an infarct size of 20 %, is associated with improved survival and a reduction in clinical events (117). We therefore considered a decrease in (1- the myocardial salvage index) of 20 % to be clinically relevant. However, we ended up with enrolling patients with smaller infarcts than expected. We detected a 5.6 percentage point difference in MSI between treatment groups, indicating that our trial was underpowered.

6.3 Cardiac magnetic resonance (CMR) and outcome measures

Cardiac magnetic resonance imaging (CMR) is a well-established technique used to assess myocardial viability and function. In a single examination, different complementary CMR techniques are used to assess the ischemic cascade in acute myocardial infarction in terms of myocardial edema, infarcted tissue, and microvascular obstruction (118). In the ASSAIL-MI trial, these measures were assessed as area at risk for permanent injury, irreversible injury, and microvascular obstruction, respectively. Late gadolinium enhancement technique is valuable in predicting recovery of function, to depict myocardial scar vs myocardial viability (119). CMR also provide information on myocardial perfusion and volume of myocardial injury (120). CMR is the preferred methodology performed in experimental and clinical trial to identify potential benefits of novel cardio- protective strategies. However, universal standardization is needed in term of i) time of post-MI scan, ii) acquisition protocols, and iii) selection of endpoints. In 2019 an expert panel came forward with recommendation for CMR endpoints in experimental and clinical trials and is presented in Figure 12 (121).

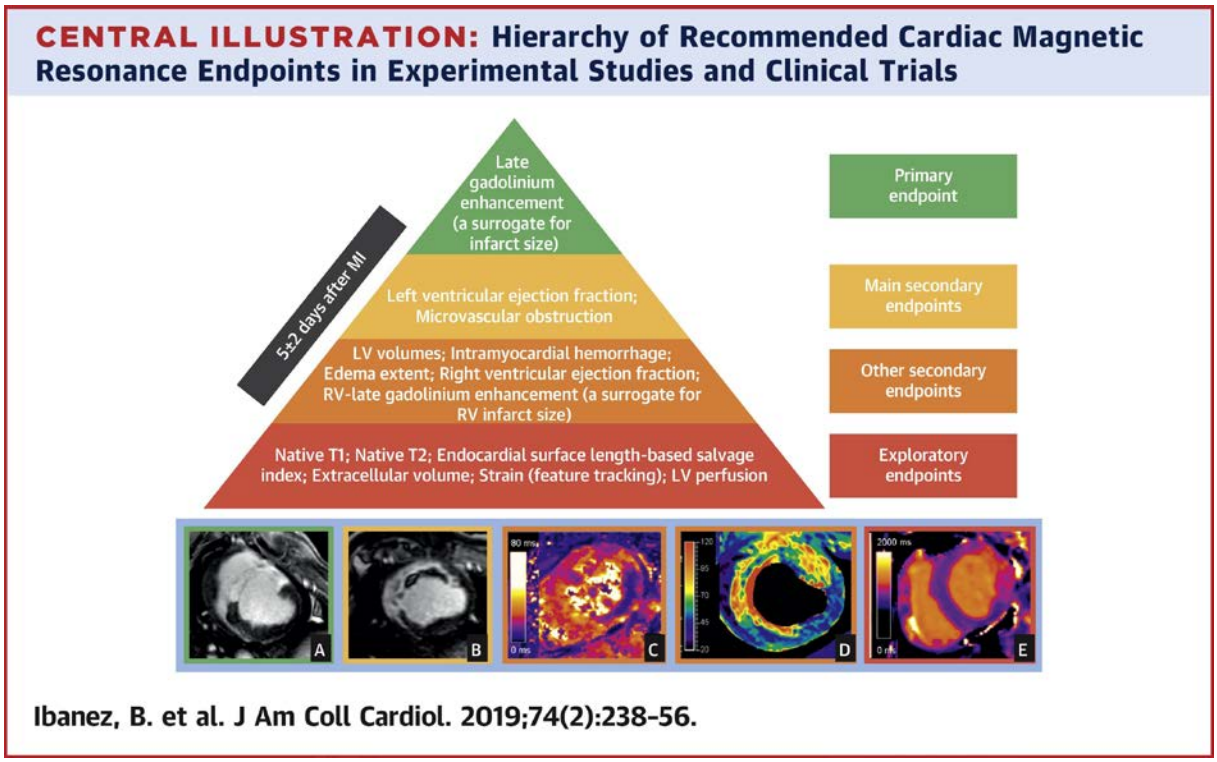

Figure 12. Hierarchy of recommended cardiac magnetic resonance endpoints in experimental studies and clinical trials A: infarct size on late gadolinium enhancement, B: extensive microvascular obstruction within late gadolinium enhancement area, C: Intramyocardial hemorrhage (IMH) in the lateral wall on T2* mapping, D: intense emdema in anteroseptal wall on T2 mapping; E: T1 mapping abnormalities in lateral wall. A, B, and D corresponds to pig ischemia-reperfusion experiments (images courtesy of Borja Ibanez and Rodrigro Fernandez-Jimenez, CNIC), whereas C and E corresponds to human post-STEMI cases (clinical images courtesy of Colin Berry and Peter McCartney, University of Glasgow) (121).

Myocardial salvage index– primary outcome measure

The primary outcome measure in the ASSAIL-MI trial was the MSI assessed 3-7 days after the index event. Our predefined outcome measure was in line with previous clinical studies and novel recommendations according to time window for the assessment (113, 114, 121, 122). However, new CMR recommendations for standardization of cardio-protection trials was set forward after the initiation of the ASSAIL-MI trial. Time of post-MI scan was according to recommendations, 5.0 ± 1.3 days after PCI in the tocilizumab arm, and 5.0 ± 1.3 days in the placebo group, receptively. A standardized protocol must take into account the bimodal pattern of edema post-MI (123).

Acquisition protocol was well implemented, and we used *E*xpectation *M*aximization, weighted intensity, *a* priori information (EWA-method) as was well validated (124). The MSI is based on edema-sensitive methodologies to estimate the area at risk. However, therapy may effect edema formation. Thus, infarct size assessed by late gadolinium enhancement is now recommended as the first choice because it is not edema-sensitive. Taken together, MSI is still a well-established measure in cardioprotection trials with prognostic value. In cardioprotection trials to come, the use of edema-sensitive methodologies, such as MSI, may be reduced in favor of infarct size as the preferred outcome measure.

Secondary outcome measures on CMR

Microvascular obstruction evolves over time and is stabilized 48 to 72 hours after myocardial infarction in experimental studies (125). Ischemic time is associated with larger areas of microvascular obstruction (121). Capillary permeability increases as the ischemia progress, and upon reperfusion it may be overwhelmed and result in microvascular damage (17).

In the ASSAIL-MI trial, most patients in the tocilizumab group had no detectable microvascular obstruction. In the majority of patients who had microvascular obstruction, the area of microvascular obstruction was very small. These results may be in line with the fact that we enrolled relative small infarcts with limited ischemic time. However, we did find a difference between treatment groups in favor of tocilizumab. Several factors may contribute to microvascular obstruction after reperfusion, and capillary leucocyte aggregation followed by increased load of reactive oxygen species, may play a partial role (1).

Inter- and intra-observer reliability

All CMR images were evaluated at a core lab located at St. Olav's Hospital in Trondheim. One PhD-candidate did most of theCMR analyses. However, it was checked for both inter- and intra-observer reliability with a senior doctor and of the PhD-candidate himself to determine consistency.

6.4 Biomarkers of inflammation, myocardial necrosis and ventricular remodeling

It is common to use biomarkers in diagnosing, staging, monitoring disease, as well as in determining response to therapy (108). In clinical intervention trials, the measurement of a biomarker may be useful in the assessment of efficacy, as well as safety. In the ASSAIL-MI trial several biomarkers were measured regarding efficacy and safety.

Inflammation

CRP is a product of the acute phase response, and is classified as a positive acute phase reactants with upregulated serum concentrations during inflammation (98). IL-6 is the primary cytokine responsible for inducing the production in the liver (126). CRP may also be induced by other mediators of inflammation as interleukin-1, TNF-alpha, and interferon-gamma. Importantly, CRP is not a direct mediator of inflammation.

However, it is a convenient and reliable biomarker of overall inflammatory status. The main functions of CRP is in the innate immune system and to promote phagocytosis due to pathogens. In the ASSAIL-MI trial, CRP act as a biomarker of overall inflammatory status as the surrogate endpoint of interest regarding attenuated inflammatory cascade by IL6 inhibition by tocilizumab. In most patients with ACS, CRP is elevated at presentation.
One of our main findings in the ASSAIL-MI trial was that inflammation was attenuated to a large extent in the tocilizumab group compared to placebo. This is in line with previous research and was in a way expected. However, our treatment response seen on myocardial salvage is interesting in the context of attenuated inflammation, and CRP serve as a biomarker of inflammation.

Myocardial necrosis and heart failure

Biomarkers, such as TnT and NT-proBNP did not differ significantly between treatment groups. TnT was numerical lower in the tocilizumab group. A rationale for the intervention was to prevent the development of heart failure. However, the myocardial salvage index was higher than expected in the placebo arm. Few patients developed heart failure. We enrolled patients with smaller infarcts than first expected.

6.5 ASSAIL-MI – internal and external validity

The validity of a trial is the extent to which the interpretation of the trial results can be trusted and depends on the study methods and the representativeness of the study sample. Internal validity refers to the appropriateness of the study design and conduct, and is about the degree to which the observed differences between treatment groups can be attributed to the actual intervention. External validity concerns whether the results of the trial can be generalized to the broader population (127). Internal validity is shortly discussed with regard to i) randomization, ii) allocation, iii) blinding, and iv) intention to treat analysis. External validity depends on internal validity and the representativeness of the trial population.

Internal validity

Correct randomization eliminates selection bias and minimizes the effect of confounding variables. The randomization procedure was performed according to the pre-specified protocol, and is described in detail in chapter 3, the methods section. Permuted block randomization was used to randomly allocate participants to treatment groups to maintain a balance across treatment groups. The patient characteristics were well-balanced between treatment groups in the ASSAIL-MI trial.

The investigators and the participants were blinded to study drug allocation throughout the study period. The double-blind design of the ASSAIL-MI trial was an additional safeguard to ensure unbiased follow-up.

Intention-to-treat analyses were used in final analysis, and not analyses per protocol. The intention-to-treat analyses serve to preserve benefits of the randomization and strengthen generalizability. Overall, there were few drop-outs in the ASSAIL-MI trial. However, drop-outs are of interest because patients who do not follow protocol often have a different prognosis than those who remain in the trial. ITT analysis is the most conservative estimate of treatment effect. With the randomized controlled, double blind design, robust procedures to maintain blinding, blinded end-point analyses by dedicated personnel, and the low drop-out rate, we consider the ASSAIL-MI trial to have high internal validity.

External validity

The ASSAIL-MI trial design gives highly internal validity for generalization to the real-life patient. However, judging the external validity of a RCT is not the same as looking at score systems for the judgement of RCT quality (128). There are several issues that potentially affect external validity; i) Setting of the trial, ii) selection of patients, iii) characteristics of randomized patients, iv) differences between the trial protocol and routine practice, v) outcome measures and follow-up, vi) adverse effects of treatment.

The ASSAIL-MI trial enrolled a highly selected patient population. First, we used strict selection criteria, excluding hemodynamically unstable patients, patients with long time from symptom onset, patients with significant co-morbidities, and patients with prior myocardial infarction. As a result, we probably ended up with "healthier" patients than the average patient with STEMI. As discussed above, the selection criteria may also have resulted in smaller infarct sizes than we had powered the trial for. The main purpose of this trial was to give proof to a concept. We therefore aimed for a "clean" patient population with few confounding issues. It was a phase II trial, and its results have to be tested in larger clinical trials to show clinical relevance in routine clinical practice in a less selected patient population, such as a phase III trials. However, our results may be interpreted with respect to the possible treatment effect and the favorable safety results. Clinical interpretation must always be made with caution, and has to be conservative.

Study designs of Paper III and IV

The study designs of Paper III and Paper IV were not designed to provide evidence of causality, only associations. The trial designs were explorative and observational. Both trials enrolled patients prospectively.

6.6 Statistics

The ASSAIL-MI trial was designed to test for treatment effect of tocilizumab compared to placebo in patients with STEMI with regard to the primary endpoint, MSI. The continuous efficacy variable MSI was analyzed using ANCOVA and time from symptom as a covariate. The between-group difference of the adjusted MSI was statistically significant in favor of the tocilizumab arm (69 ± 19% vs. 64 ± 21%). However, the between-group difference was small, only 5.6 percentage points (95% confidence interval: 0.2 to 11.3; p = 0.04) at the two-sided

5% level of significance. In advance, a detection of 20% between-group difference was defined to be clinically relevant. Notably, the sample size assumptions were made with regard to the clinical effect size determined in advance.

The between-group difference of the adjusted primary outcome measure was big enough statistically (5.6 percentage points) to probably not happen just by chance at the 5% confidence level. The null hypothesis (no difference between means of the two study population regarding the adjusted MSI) was therefore rejected. Thus, the alternative hypothesis was approved, indicating a difference in treatment effect on the adjusted primary endpoint. However, several remarks should be made regarding the interpretation of this result and some statistical considerations are made.

Statistical significance and clinical significance are not equivalent. The results of clinical trials must be put into clinical. Also, introducing new treatment strategies into clinic is subject to strict regulations. Small principle-of-concept studies on treatment effect must be followed by larger clinical trials with clinical outcome measures like morbidity and mortality. How big the between-group difference needs to be to count as a clinically significant difference is debatable and calls for clinical judgement. The probability of achieving a statistically significant result will increase as the sample size increases, even if the effect size remains the same.

The bigger the difference, the more confidently we can reject the null hypothesis that there is no real difference between the means of the study populations. These data must be predefined before initiating the clinical trial. Awareness of type I and type II errors is crucial in this regard, and so is the awareness of their inversely related relationship. A type II error is to fail to recognize a difference as being real, even when it is. To reduce the risk for type II error, we may increase the risk of type I error, which means running the risk of accepting a result as “true” when it is, in fact, not. The emphasis in clinical research is on avoiding type I errors.

The ASSAIL-MI study was designed to show causality given the RCT-design. In the exploratory study in Paper III, we looked into correlations between leucocyte subpopulations and outcome measures such as CRP and TnT. Associations do not prove causal relationships. However, the results might be hypotheses generating. Furthermore, in Paper III and IV,

several groups were compared including a control group and not only two groups as in the ASSAIL-MI trial. Thus, the t-test did not apply any longer, but comparison of within-samples variance and the between-samples variance of the groups were made in these cases.

In paper IV we used non-parametric methods. Non-parametric methods are used when assumption of normal distribution does not hold, as for categorical data. Of note, non-parametric tests usually require larger effect sizes than parametric tests for the results to achieve statistical significance. This is due to the loss of statistical information when measured data are reduced to a few categories or rank-position only. This means we have more difficulty in detecting differences that are significant. Non-parametric tests increase the risk that we will accept the null hypothesis when it is false (type II error)

Sample size

A critical step in designing a RCT to prove treatment efficacy is the estimation of the trial´s sample size. Optimal sample size is critical in several regards, but mainly to ensure adequate power to detect statistical significance. These trials are often time consuming and costly. Thus, if underpowered it will be statistically inconclusive. In the opposite case, too many participants may be costly and expose an unnecessary large number of patients to any risk associated with the intervention. In addition, recruiting more participants than needed to show an effect, might have ethical considerations. In the ASSAIL-MI trial the sample size was not based on own calculation, but on previous research estimating sample size in myocardial infarction.

6.7 Limitations

From study population to overall population – generalizability of the ASSAIL-MI

Randomized controlled trials represent the current gold-standard study design for the determination of the efficacy and safety of medical interventions. The populations studied in randomized trials are often highly selected and have a lower risk profile than real-world populations because of i) strict adherence to structured protocol, ii) the use of restrictive inclusion and exclusion criteria, and iii) patient randomization. Frequently, elderly patients and patients with co-morbidities are excluded from trial participation.

Patients enrolled in the ASSAIL-MI trial were highly selected according to the pre-defined inclusion and exclusion criteria. Only patients with first-time myocardial infarction (STEMI) admitted to hospital for acute coronary angiography within 6 hours from symptom onset and aged 18-80 years were eligible for randomization. No exclusion criteria such as previous myocardial infarction, inflammation/infection, serious comorbitities or hemodynamic instability should be present. Thus, the patients in the ASSAIL-MI trial were not representative of the overall STEMI population. The aim of this phase 2 trial was to elucidate efficacy and safety and to minimize the possibility of bias regarding the effect of an intervention. The implications for external validity of trial results must be further evaluated in larger trials.

7. Ethical considerations

The ASSAIL-MI trial (Paper I-III) was approved by the Regional Committee for Medical and Health Research Ethics (REK), South-East Norway. It was also approved by the Norwegian Medicines Agency as warranted when medicinal product are used in an intervention study. The study was initiated before 2018. Thus, it did not need additional approval from the local Department of Privacy and Data Security. The trials was conducted in compliance with the Declaration of Helsinki. Furthermore, it was conducted according to Good Clinical Practice guidelines. Before the initiation of the ASSAIL-MI trial, it was registered with clinicaltrials.gov, ID number: NCT03004703, URL: https://www.clinicaltrials.gov/ct2/show/NCT03004703

7.1 Informed consent

All patients confirmed to participate in advance of study specific procedures and study drug administration. According to the approval from REK, participants were allowed to commit orally in the acute setting and in writing within 24 hours in the ASSAIL-MI trial. All patients enrolled in clinical trials must be given sufficient and individually customized information. In this thesis enrolment of STEMI patients took place at the catheterization lab in an acute setting. Therefore, the study personnel were extra aware of the vulnerable position the patient was in when asked to participate. In cases where the patient was unable to take this information into consideration, no further questions about study participation was asked. All participants were given written information in addition to oral information by study personnel within 24 hours and were given the opportunity to ask questions. One patient withdrew consent on the day he/she was asked to confirm consent in writing. The patient was given the opportunity to attend follow-ups without registration of data in the study database, but declined. All patients were informed that they could withdraw their consent at any time without reason.

7.2 Patient-physician relationship

The participants were enrolled and followed-up by a limited number of study personnel throughout the whole trial period. Only one patients withdrew consent, and few patients were lost to follow-up. One reason might be the organization of the study and the trust achieved by not regularly exposing the patients to new study personnel. According to study design, study patients and investigators were both blinded, also in the follow-up situations. This allowed for neutral registration of clinical signs and reports of side effects.

7.3 Risks and benefits

In advance of initiating a trial such as the ASSAIL-MI trial, potential benefits and risks for the participants are closely evaluated. Approvals by REK and the Norwegian Medicines Agency are obligatory to secure patient safety. After initiation, an independent data monitoring and safety committee was responsible for the regular monitoring of trial data. Throughout the study, they evaluated the accumulated safety data, and could terminate the trial according to safety issues. Participation in clinical trials may benefit patients by way of frequent visits and close contact with health personnel. However, some patients must travel far to attend study visits and the time they are willing to spend on the research project must be appreciated.

8. Conclusions and implications

In this thesis my co-authors and I elucidated a new concept of anti-inflammatory treatment in STEMI. We showed a beneficial effect of IL-6 inhibition on myocardial salvage in the acute phase of STEMI. Moreover, we provided valuable cellular and mechanistic insight to our results. We showed that tocilizumab attenuated the innate immune response and dampened signal transduction. Novel therapeutic strategies attacking inflammation have potential to limit myocardial damage following reperfusion in PCI-treated patients with STEMI.

In the randomized-placebo-controlled double-blinded ASSAIL-MI trial, we show that STEMI patients receiving IL-6 inhibition just prior to PCI had a larger MSI. We also found that a marked and rapid decrease in neutrophil counts at 24 hours post PCI was associated with improved MSI and decreased peak TnT. Our results suggest that the beneficial effect of tocilizumab seen in these patients may be related to attenuation of the innate immune response, involving neutrophil function and signal transduction. In an observational study with a prospective design, we found that ‘pro-resolving mediators are biosynthesized from polyunsaturated fatty acids soon after STEMI. Our results suggest a prompt activation of resolution processes that might begin in parallel with inflammation.

9. Future perspective

The ASSAIL-MI trial was a proof-of-concept-trial that showed promising results regarding IL-6 inhibition by tocilizumab in STEMI patients undergoing acute PCI. As described in the introduction of this thesis, attacking the reperfusion injury may be the next step in evolving new treatment strategies to improve outcomes in these patients. Several targets in the inflammatory cascade have been of interest in this context. However, the complexity involved in inflammation and resolution is challenging. Likely, future research will shed light on pathways and targets worth further investigation. The next step will be to demonstrate their potential therapeutic benefits on clinical endpoints like morbidity and mortality in patients with myocardial infarction.

Interleukin-6 is a central hub in cardiometabolic signaling. Recently, it has received increasing interest as a target for intervention. The concept of selective and targeted inhibition of the IL-1β- IL-6- CRP pathway in acute ischemia and chronic atherosclerosis is promising. However, more data is required from large clinical trials beyond the CANTOS (31), CIRT (85), and COLCOT (83) trials. These trials have set the stage in recent years and act as guides for future work. Our research group has contributed with data on IL-6 inhibition in NSTEMI and STEMI patients in small proof-of-concept trials. However, the beneficial results we have observed must be tested in larger clinical trials powered to provide results regarding morbidity and mortality. First then may IL-6 inhibition come into clinical use if still found to be beneficial and safe. Data on the clinical effect of IL-6 inhibition in cardiovascular disease are pending. The RESCUE trial (81) showed markedly reduced biomarkers of inflammation and thrombosis relevant to atherosclerosis. A large-scale cardiovascular outcome trial based on this trial might add valuable insight on IL-6 inhibition in cardiovascular disease (97). Interleukin-6 has emerged as a pivotal factor in atherothrombosis, and IL-6 inhibition may have several possible actions in the atherothrombotic process.

In this thesis my focus has been on patients with STEMI and anti-inflammation in the reperfusion setting following PCI. The optimal goal is to save as much myocardium as possible from permanent damage and in this way prohibit dysfunctional cardiac remodeling and heart failure development. Future research may dig deeper into anti-inflammatory therapy in this setting, but also investigate interventions enhancing the body's pro-resolving capacity to limit myocardial damage and dysfunctional myocardial remodeling. Finally, this may also

pave the way for more personalized precision medicine in inflammatory cardiovascular disease.

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Rationale for the ASSAIL-MI-trial: a randomised controlled trial designed to assess the effect of tocilizumab on myocardial salvage in patients with acute ST-elevation myocardial infarction (STEMI)

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ABSTRACT

Introduction Interleukin-6 (IL-6) may be involved in ischaemia-reperfusion injury and myocardial remodelling after myocardial infarction (MI). We have recently shown that IL-6 inhibition by tocilizumab attenuates systemic inflammation and troponin T-release in patients with acute non-ST elevation MI (NSTEMI). Experimental studies suggest that IL-6 inhibition can limit infarct size through anti-inflammatory mechanisms, but this has not been tested in clinical studies. With the *ASS*essing the effect of *A*nti-*IL*-6 treatment in *MI* (ASSAIL-MI) trial, we aim to examine whether a single administration of the IL-6 receptor antagonist tocilizumab can increase myocardial salvage in patients with acute ST-elevation MI (STEMI).

Methods and analysis The ASSAIL-MI trial is a randomised, double blind, placebo-controlled trial, conducted at three high-volume percutaneous coronary intervention (PCI) centres in Norway. 200 patients with first-time STEMI presenting within 6 hours of the onset of chest pain will be randomised to receive tocilizumab or matching placebo prior to PCI. The patients are followed-up for 6 months. The primary endpoint is the myocardial salvage index measured by cardiac MRI (CMR) 3–7 days after the intervention. Secondary endpoints include final infarct size measured by CMR and plasma markers of myocardial necrosis. Efficacy and safety assessments during follow-up include blood sampling, echocardiography and CMR.

Ethics and dissemination Based on previous experience the study is considered feasible and safe. If tocilizumab increases myocardial salvage, further endpoint-driven multicentre trials may be initiated. The ASSAIL-MI trial has the potential to change clinical practice in patients with STEMI.

Registration Clinicaltrials.gov, identifier NCT03004703.

Key questions

What is already known about this subject?

- Interleukin-6 (IL-6) may be involved in ischaemia-reperfusion injury and myocardial remodelling after myocardial infarction. Experimental studies suggest that IL-6 inhibition can limit infarct size through anti-inflammatory mechanisms.

What does this study add?

- The trial is designed to test the hypothesis that treatment with tocilizumab, an IL-6 receptor antagonist, before and during PCI will increase myocardial salvage in patients presenting with acute ST-elevation myocardial infarction (STEMI).

How might this impact on clinical practice?

- If tocilizumab increases myocardial salvage, IL-6 inhibition might be an adjuvant therapeutic target for patients with STEMI. Larger endpoint-driven multicentre trials may be initiated.

INTRODUCTION

Myocardial infarction (MI) is a major cause of morbidity and mortality in the Western world.[1 2] Infarct size is the main determinant of death and complications after MI. Limiting the extent of the myocardial necrosis has therefore been an important objective. In patients presenting with acute ST segment elevation MI (STEMI), urgent myocardial reperfusion with percutaneous coronary intervention (PCI) is the most effective treatment to this end. However, the morbidity and mortality in patients with STEMI remain substantial.[1 2] Other adjuvant strategies are therefore required to reduce infarct size

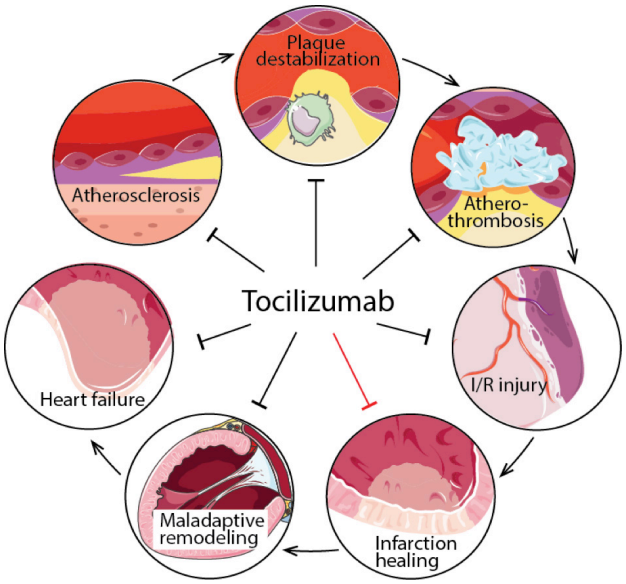

Figure 1 The figure shows possible actions for interleukin-6 (IL-6) and targets for the anti-IL-6 antagonist, tocilizumab in the atherothrombotic process. Tocilizumab could (1) reduce initiation and progression of the atherosclerotic process, (2) stabilise atherosclerotic plaques, (3) inhibit initiation and propagation of the thrombus, (4) reduce ischaemia/reperfusion (I/R) injury, (5) inhibit the maladaptive left ventricular remodelling process and (6) prevent development of symptomatic heart failure. However, tocilizumab could potentially also attenuate infarct healing.

and improve outcomes. The role of inflammation in plaque destabilisation and post-MI myocardial remodelling has been thoroughly investigated. Several studies have also examined the role of IL-6 in these processes. However, at present, there are no data on the effect of IL-6 inhibition in patients with STEMI.

We recently conducted a double blind, placebo controlled trial in 117 patients with non-ST segment elevation MI (NSTEMI) who presented at a median of 2 days after the onset of chest pain. In this study, a single, intravenous dose of the IL-6 receptor antagonist tocilizumab reduced C-reactive protein (CRP) levels by more than 50% during hospitalisation. Importantly, tocilizumab also reduced PCI-related troponin T (TnT) levels, suggesting that tocilizumab reduced periprocedural myocardial injury.[3] The favourable effects of tocilizumab were limited to patients who underwent PCI and patients who were randomised within 2 days after symptom debut. However, as expected in patients with non-STEMI (NSTEMI), the peak TnT plasma concentrations were relatively low (approximately 300 ng/L before baseline), and the study was not designed to examine the effect on myocardial remodelling or infarct size. The beneficial effect of tocilizumab in the NSTEMI population may reflect an effect on periprocedural myocardial injury (PMI) following PCI, and a stabilising effect on the affected coronary plaque, rather than an effect on infarct size and myocardial remodelling. Thus, while our recent study indicates that IL-6 inhibition has largely favourable effects in NSTEMI, it is unknown whether similar, beneficial effects can be obtained in patients with STEMI. The pathophysiologies in these patients have similarities, but also differences.[4] On this background, we wanted to investigate the effect of a single fixed dose of tocilizumab (280 mg) in patients with acute STEMI. We postulate that a single dose of tocilizumab (RoActemra) will have favourable effects on myocardial salvage, as assessed by cardiac MRI (CMR) and markers of myocardial necrosis, without negative consequences for the myocardial repair process in these patients.

The *ASS*essing the effect of *A*nti-*IL*-6 treatment in *M*yocardial *I*nfarction (ASSAIL-MI) trial is a randomised, controlled, double blind, parallel arm trial designed to test the hypothesis that treatment with tocilizumab before and during PCI will increase myocardial salvage in patients presenting with acute STEMI. Our ambition is to improve the prognosis of patients with STEMI, and if we fulfil this ambition, our research has the potential to change clinical practice.

Acute coronary syndromes (ACS), ischaemia/reperfusion (I/R) injury and inflammation

Whereas the abrupt restoration of blood flow by PCI undoubtedly has a net beneficial effect, it may also have detrimental consequences, including myocardial stunning, ventricular arrhythmias and microvascular dysfunction.[5] The I/R injury may account for as much as 50% of the myocardial damage during MI.[6] While the myocardial response to the reperfusion injury aims to restore homeostasis within the heart through repair and adaptive remodelling, a dysregulated process can induce maladaptive remodelling and subsequent myocardial failure. An ideal therapeutic approach to MI should (1) counteract plaque destabilisation and thrombus formation, (2) impair the I/R injury, (3) minimise infarct size and (4) inhibit maladaptive remodelling without attenuating the repair process. At present, we lack the necessary tools to fully achieve these goals.

Inflammation is involved in several aspects of coronary artery disease (CAD) and ACS. Plaque destabilisation (ie, endothelial erosions and fibrous cap rupture) with subsequent thrombus formation and vascular obstruction is the principal cause of acute coronary events. Inflammation is involved in all these features of ACS.[7 8] In addition, different cytokines as well as reactive oxygen species (ROS), intracellular calcium overload and myocardial infiltration of leucocytes may contribute to the reperfusion injury occurring after the opening of occluded coronary arteries. Enhanced oxidative stress activates inflammatory pathways within the myocardium, which again enhances ROS production, in a vicious cycle after PCI.[6] Myocardial inflammation is also a major contributor to infarct size through the activation and release of matrix degrading enzymes and induction of cardiomyocyte and fibroblast apoptosis and necrosis. However, while the injurious role of inflammation in MI is well established, how to modulate the activation of the inflammatory responses remains unclear. While massive or sustained

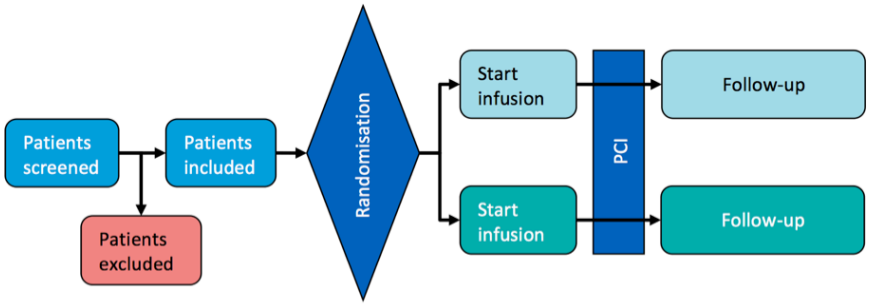

Figure 2 Overall study design.

inflammatory responses are harmful and may contribute to myocardial damage and increased infarct size, inflammation is also prerequisite for tissue repair. This dual role of inflammation following MI represents a therapeutic challenge.

Therapeutic intervention during MI

Recently, a great breakthrough on therapeutic approaches targeting inflammation in atherosclerotic disorders was achieved with the Canakinumab Anti-inflammatory Thrombosis Outcomes Study trial.[9] In this trial, anti-inflammatory therapy with the interleukin-1 beta (IL-1β) antibody canakinumab led to a significantly lower rate of recurrent cardiovascular events in patients with previous MI. However, few studies have explored anti-inflammatory strategies in MI. A randomised trial evaluating IL-1 receptor (IL-1R) antagonism in NSTEMI showed a significantly lower level of CRP in the intervention arm, but not during the first 2 days after drug administration, and without effects on TnT.[10] A larger multicentre study on the effect of the IL-1R antagonist anakinra in patients with STEMI is ongoing.[11] In patients with STEMI, pexelizumab (n=960), which inhibits the C5 complement protein[12] mitigates inflammation. Moreover, whereas pexelizumab had no measurable effect on infarct size, it showed a significant reduction in mortality. Subsequent studies, however, have failed to demonstrate effects on clinical outcomes.[13] In fact, used adjunctively with fibrinolytic agents, pexelizumab did not reduce infarct size, as assessed by creatine kinase-myocardial band (MB), or the risk of adverse clinical events.[14] Recently, Defteros and colleagues showed that in patients with STEMI (n=151), the antirheumatic drug colchicine could reduce creatine kinase-MB, and, in a subgroup of patients, infarct size as assessed by CMR.[15] Moreover, inclacumab targeting P-selectin, an adhesion molecule that facilitates the interaction between platelets, endothelial cells and leucocytes, was found to reduce TnI after PCI in patients with NSTEMI (n=322).[16] More recently, the p38 mitogen-activated protein kinase inhibitor losmapimod, known to attenuate a range of inflammatory pathways, failed to improve outcomes in a relatively large randomised trial in patients with NSTEMI (n=3503).[17]

To date, there are no studies on IL-6 inhibition in patients with STEMI. Several drugs (eg, glycoprotein IIb/IIIa antagonists) attenuate the levels of circulating IL-6 after ACS and PCI.[18] However, there are studies suggesting that IL-6 is insufficiently suppressed with contemporary therapies. In the Myocardial Ischemia Reduction with Acute Cholesterol Lowering study, treatment with atorvastatin reduced the level of circulating CRP, but failed to reduce IL-6 levels.[19] Moreover, numerous studies have shown that, despite 'optimal' therapy, IL-6 levels are higher in patients with ACS than in patients with stable coronary disease and healthy control subjects. In summary, there seems to be an unfulfilled need for treatment targeting IL-6. This unfulfilled need forms the scientific background for the ASSAIL-MI trial.

The role of IL-6

IL-6 is a multifunctional cytokine that is produced by a spectrum of cell types in the cardiovascular and immune systems and can influence the cardiovascular system in several ways (figure 1). It has been linked to vascular inflammation and myocardial remodelling. IL-6 and its signalling through the IL-6-receptor complex contribute to atherosclerotic plaque development and plaque destabilisation[20] and has also been related to the progression of myocardial failure.[21] IL-6 is a pleiotropic cytokine with a wide range of effect on immune cells and other cells with relevance for cardiovascular disease.[22] IL-6 is the main promoter of acute-phase proteins, such as CRP, and the subsequent complement activation. It causes the release of prothrombotic mediators and activation of matrix metalloproteinases. This result in matrix degradation and weakening of the atherosclerotic fibrous cap and may also have harmful effects on the repair process after MI.

IL-6 plays a significant role in myocardial remodelling predisposing to the development of overt heart failure. Experimental mice models have shown that enhanced IL-6 activation promote myocardial hypertrophy. However, deficiency of its receptor induced severe cardiac dilation.[23] These findings illustrate the balance between beneficial and harmful effects of this cytokine where too much and too little can both be harmful. Interestingly, in an experimental mice model, Kobara *et al* found that therapy targeting the IL-6 receptor downregulated inflammation and attenuated LV remodelling after MI,[24] illustrating the potential for similar interventions

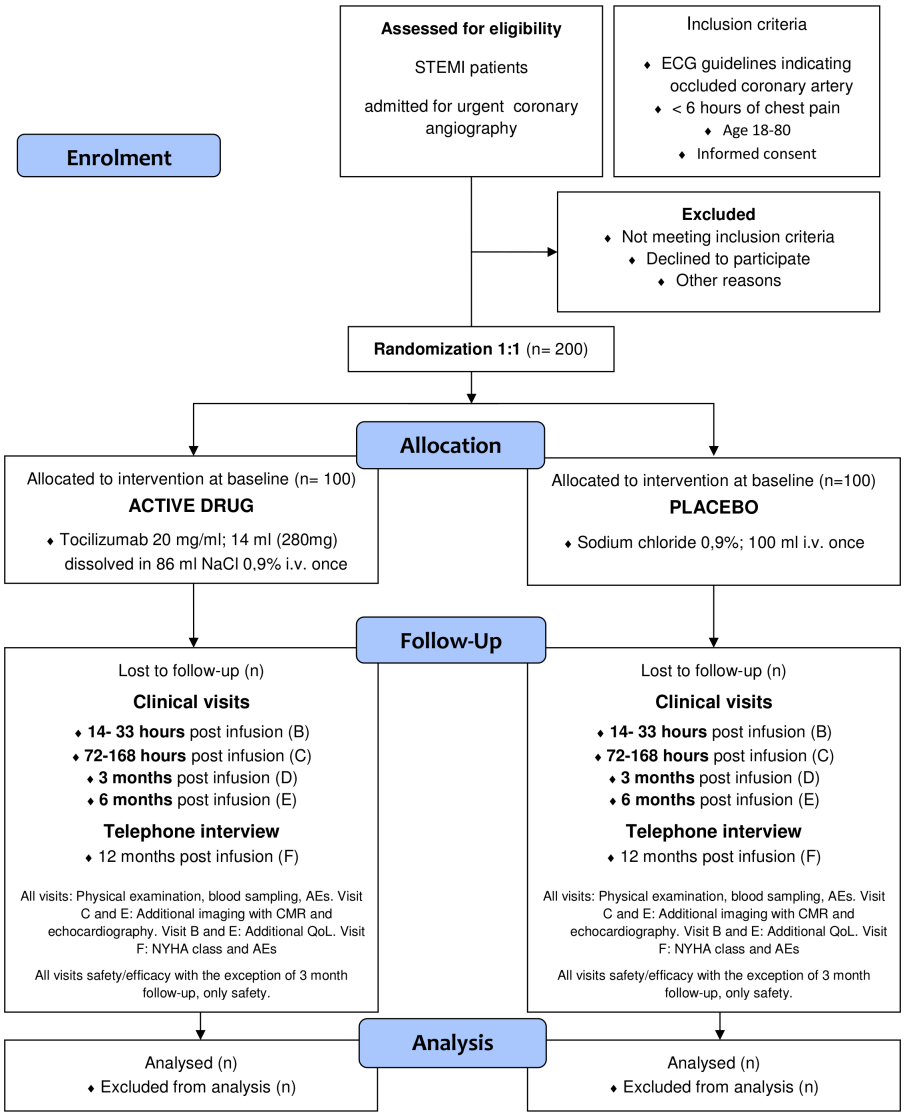

Figure 3 Study flow chart.

in humans. We have shown that high levels of soluble IL-6 receptors (ie, IL-6R and gp130) in the acute phase of STEMI are associated with subsequent major cardiac adverse events, underscoring the potential importance of the IL-6 signalling system in patients with MI.[25] To conclude, IL-6-related pathways seem to be involved in several pathogenic processes during MI, from plaque destabilisation and thrombus formation to I/R injury and maladaptive myocardial remodelling and our hypothesis is that IL-6 inhibition has several beneficial effects in STEMI (figure 1).

Known and potential benefits and risks of targeting IL-6 therapy in STEMI

IL-6 inhibition with a single dose of tocilizumab may have several merits. First, in contrast to long-term anti-inflammatory therapy in various autoimmune disorders, we hypothesise that one dose of tocilizumab administered on admission may be sufficient to induce beneficial effects on infarct size and myocardial remodelling. Accordingly, the patients will not be exposed to long-term immunosuppression with its potential harmful effects. Second, in contrast to IL-1β, which is an upstream inflammatory cytokine, IL-6 is a more downstream mediator. Although the effect of IL-1β inhibition seems at least partly to be mediated through reduction of levels of IL-6,[26] we found only minor alterations in the cytokine network during tocilizumab intervention in patients with NSTEMI, suggesting a more narrow effect of IL-6 inhibition.[27] We therefore expect that the targeted IL-6 therapy provided in the ASSAIL-MI trial will to a lesser degree interfere with other inflammatory pathways, some of which may be of importance for infarct healing. Third, to balance the beneficial and harmful effect of anti-inflammatory therapy, we have chosen a moderate

and fixed dose of tocilizumab (280 mg) that will provide complete IL-6 blockade of approximately 2 weeks. Notably, in the NSTEMI study, we found no association between body mass index and the effect of tocilizumab on CRP, suggesting that its effect is influenced by body weight to a minor degree only.[3] There are several potential challenges with anti-IL6 inhibition during STEMI. We know that tocilizumab is associated with an increased risk of infections. However, this risk is probably low after a single dose only. In the NSTEMI study, we recorded no severe infectious complications in patients receiving tocilizumab.[3] Tocilizumab induced a significant reduction in blood levels of neutrophils,[3] but based on the role of these cells during MI, this could be a beneficial rather than a harmful effect.[28] As discussed above, there is also increased risk of impaired healing after MI during IL-6 inhibition, potentially increasing the risk of myocardial rupture and cardiac dilation. However, we consider this risk to be small in patients treated within 6 hours of symptom onset. Finally, there have been some concern about the hyperlipidaemic effects of tocilizumab, but hyperlipidaemia was not observed in the NSTEMI trial. This may reflect differences in the interaction between inflammation and lipid metabolism between patients with CAD and patients with autoimmune disorders like rheumatoid arthritis, potentially related to the fact that nearly all patients with CAD receive statins.[29]

METHODS AND ANALYSIS

Design

The ASSAIL-MI trial is a phase II, double blind, randomised, placebo-controlled trial conducted at three experienced, high volume PCI centres in Norway. Participants are randomised in a 1:1 fashion to receive a single intravenous fixed dose of tocilizumab (280 mg) tocilizumab or matching placebo prior to PCI. The study is designed to show superiority with regard to the primary endpoint in patients assigned to active treatment vs patients allocated to the placebo arm. The overall study design is presented in figure 2 and study procedures in figure 3.

Eligibility

The inclusion and exclusion criteria are presented in box 1.

Patients with first time STEMI scheduled for acute PCI within 6 hours from the onset of chest pain are screened for eligibility on admittance at either participating site. Informed consent is obtained for all enrolled patients aged between 18 and 80, fulfilling ECG criteria for STEMI, and accepted for urgent coronary angiography with the intention to reperfuse a presumed occluded vessel within the given time limit. Haemodynamically unstable patients and patients with previous MI or severe comorbidities are not included in this trial. Exclusion criteria include concomitant diseases that we assume affect inflammation.

Box 1 The main inclusion and exclusion criteria

Inclusion criteria

- New ST elevation at the J-point in two contiguous leads (cut-points: 0.2 mV in men and >0.15 mV in women in leads V2–V3 and/or >0.1 mV in other leads) in combination with symptoms consistent with acute myocardial infarction (MI).
- Presentation within 6 hours of chest pain.
- Indication for urgent coronary angiography with intent to reperfuse presumed occluded vessel.
- Age between 18 and 80 years.
- Informed consent obtained and documented according to International Council for Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH)/Good Clinical Practice and national/local regulations.

Exclusion criteria

- Non-ST elevation MI (NSTEMI) (non-ST segment elevation in ECG).
- Left bundle branch block in ECG.
- History of previous MI.
- Cardiogenic shock.
- Fibrinolytic therapy within 72 hours prior to admission.
- Cardiac arrest/ventricular fibrillation.
- History of severe renal failure with estimated glomerular filtration rate <30 mL/min.
- Known, current liver disease.
- History of concurrent inflammatory, biliary obstructive or malignant disease.
- Ongoing infection.
- A history of chronic or concurrent infectious disease, including a history of HIV, tuberculosis or hepatitis B or C.
- Known, uncontrolled lower gastrointestinal (GI) disease such as diverticulitis, Crohn's disease, ulcerative colitis or other symptomatic lower GI conditions that could predispose to GI perforations.
- Major surgery within 8 weeks prior to or after baseline.
- History of central nervous system demyelinating or seizure disorders.
- History of primary or secondary immunodeficiency.
- Treatment with immunosuppressants other than low dose corticosteroids (equivalent to 5 mg of prednisone or less) at the time of randomisation.
- Immunisation with a live/attenuated vaccine within 4 weeks prior to baseline.
- History of severe allergic or anaphylactic reactions to humanised or murine monoclonal antibodies or to tocilizumab.
- Pregnancy, possible pregnancy or breast feeding—women of childbearing potential or breastfeeding mothers cannot participate. A woman is considered of childbearing potential following menarche and until becoming postmenopausal unless permanently sterile. Permanent sterilisation methods include hysterectomy, bilateral salpingectomy and bilateral oophorectomy. A postmenopausal state is defined as no menses for 12 months without an alternative medical cause.
- Contraindications to cardiac MRI (pacemaker, cardiac resynchronisation therapy, implantable cardioverter defibrillator, certain ferromagnetic implants, severe claustrophobia, allergy to contrast medium).
- Any condition/circumstances believed to interfere with the ability to comply with protocol.
- Any reason why, in the opinion of the investigator, the patient should not participate.

Continued

Box 1 Continued

- Failure to obtain written, informed consent by patient or next of kin, for instance in case of patient death after consent has been provided in oral.

Objectives

Primary objective

The primary objective of the ASSAIL-MI trial is to examine the effect of tocilizumab on myocardial salvage index as measured by CMR with gadolinium late enhancement 3–7 days after study drug infusion.

Secondary objectives

Secondary objectives are to assess the impact of treatment on: (1) troponin release, (2) final infarct size, (3) other biomarkers of myocardial damage (ie, aspartate aminotransferase and creatine kinase-MB), (4) CRP levels during hospitalisation, (5) markers of left ventricular (LV) remodelling and (6) safety and tolerability.

Explorative endpoints

Endpoints that could explain the mechanisms of action of tocilizumab in STEMI: (1) additional markers of inflammation, (2) markers of extracellular matrix remodelling, (3) lipid parameters, (4) markers of platelet activation and additional prothrombotic and antithrombotic parameters. Endpoints are detailed in box 2.

Rationale for study endpoint

Infarct size is a major determinant of prognosis after MI and can be accurately measured by gadolinium late enhancement CMR.[30] CMR is also the method of choice for the evaluation of LV function in this setting. Infarct size is mainly determined by two factors: the size of the myocardial territory supplied by the occluded vessel and the efficacy of the therapeutic actions taken to restore normal blood flow and minimise myocardial damage. In the present study, we will study the effect of tocilizumab on myocardial damage during reperfusion. This effect can be evaluated by the myocardial salvage index, which is calculated by indexing the area of the salvaged myocardium to the area at risk of irreversible injury, thus relating the size of the damage to the area at risk. The myocardial salvage index has recently been used in several large clinical cardioprotection trials (CHILL-MI,[31] MITOCARE,[32] METOCARD-CNIC,[33] LIPSIA CONDITIONING).[34] The area at risk can be quantified with early gadolinium enhancement images, applying a standard CMR sequence.

In addition to infarct size and the salvage index, CMR can assess microvascular obstruction, which is considered a marker of severe myocardial damage and poor prognosis.[35] Soluble markers of myocardial damage, in particular TnT which is the most established marker for assessment of myocardial damage during STEMI, will be an important secondary endpoint. Explorative endpoints will elucidate possible mechanisms of action of tocilizumab in STEMI.

Box 2 Prespecified endpoints in the *ASS*essing the effect of *A*nti-*IL*-6 treatment in *M*yocardial *I*nfarction trial

Primary endpoint

- The between-group difference in myocardial salvage index as measured in the acute phase by cardiac MRI (CMR) with late gadolinium enhancement (LGE).

Secondary endpoints

- The between-group differences in the area under the curve (AUC) for troponin T during index hospitalisation (baseline adjusted).
- Final infarct size as measured by CMR 6 months after randomisation.
- The between-group difference in the AUC for aspartate aminotransferase and creatine kinase-myocardial band during index hospitalisation (baseline adjusted).
- The between-group differences in the AUC of C-reactive protein during index hospitalisation (baseline adjusted).
- The extent of microvascular obstruction as measured by CMR after 3–7 days.
- Left ventricular size as assessed by CMR six6 months after randomisation.
- Markers of myocardial remodelling as assessed by echocardiography.
- Baseline-adjusted N-terminal prohormone of brain natriuretic peptide at 6 months.
- Safety and tolerability as assessed by clinical assessment, blood samples and imaging.

Explorative endpoints

- Additional inflammatory and anti-inflammatory mediators.
- Markers of extracellular matrix remodelling (eg, matrix metalloproteinases and their endogenous inhibitors).
- Lipid parameters (eg, low-density lipoprotein and high-density lipoprotein cholesterol and triglycerides).
- Markers of platelet activation (eg, platelet-derived inflammatory markers) and additional prothrombotic and antithrombotic parameters (eg, tissue factor and plasminogen activator inhibitor).

Statistical considerations

This trial is designed to assess the effect of treatment with tocilizumab on myocardial salvage in patients with STEMI. In patients with first time, acute anterior wall MI treated with PCI within 12 hours of the onset of symptoms, final infarct size is typically 20% of total myocardial volume.[36] Studies have shown that an absolute reduction in infarct size of approximately 3%–5% of myocardial volume, that is, a relative reduction of 14%–25%, given an infarct size of 20%, is associated with improved survival and a reduction in clinical events.[37] We therefore consider a decrease in (1–the myocardial salvage index) of 20% (equalling 20% reduction in infarct size) to be clinically relevant. With a two-sided α of 0.05 and a power of 90 %, detection of a 20% between-group difference in (1–myocardial salvage index) requires 86 patients in each arm.[38]

In a recent trial on the effect of IL-6 inhibition in patients with NSTEMI, we showed that treatment with tocilizumab was associated with reduced PCI-released release of TnT.[3] In the ASSAIL-MI trial, the baseline-adjusted between-group difference in AUC for TnT will be an important secondary endpoint. In order to reduce the AUC for TnT by 25%, with an α of 0.05 and a power of

Table 1 Baseline characteristic of the first 100 patients in the *ASS*essing the effect of *Anti-IL*-6 study

Demography	
Age, years (range)	61.0±8.7 (38–79)
Male gender, n (%)	80 (80.0)
Race/ethnicity, n (%)	
White/Black/Asian	96 (96.0)/0 (0.0)/4 (4.0)
Relationship status, n (%)	
Married/single/widow(er)	73 (73.0)/22 (22.0)/5 (5.0)
Work status, n (%)	
Working/sick leave/pensioner	61 (61.0)/4 (4.0)/34 (34.0)
Cardiovascular risk factors, n (%)	
Current smoker/former smoker	40 (40.0)/26 (26.0)/34 (34.0)
Hypertension	33 (33.0)
Diabetes mellitus	7 (7.0)
Peripheral vascular disease	2 (2.0)
Chest pain to hospital arrival time, hours (range)	2.4±1.2 (0.42–6.0)
Concomitant medication, n (%)	
ACE-inhibitor/angiotensin receptor blocker	26 (26)
Aldosterine antagonist	1 (1)
Anticoagulants	4 (4)
Antiplatelets	10 (10)
Beta blocker	7 (7)
Calcium antagonist	14 (14)
Diuretics	10 (10)
Statin	10 (10)
Other	25 (25)
Clinical characteristics	
Blood pressure, mm Hg (range)	
Systolic	132.3±21.5 (80.0–180.0)
Diastolic	82.7±16.2 (50.0–121.0)
Heart rate, beats per minute (range)	70.8±16.1 (40.0–125.0)
Body mass index, kg/m^2 (range)	27.0 (15.7–39.2)±4.3
Killip classification, n (%)	
I/II/III/IV	95 (95)/4 (4)/0 (0)/0 (0)
New York Heart Association functional status, n (%)	
I/II/III/IV	62 (62)/27 (27)/3 (3)/7 (7)

Data are given as number (percent) or mean±SD (range)

80%, we need approximately 75 patients in each arm.[38] To allow for dropout and to improve the chances of obtaining significance for secondary endpoints, we therefore aim to include all together 200 patients.

All statistical tests will be performed using a two-sided 5% level of significance. Continuous efficacy variables will be analysed using independent t-tests for comparisons between the treatment arms and baseline-adjusted analysis of covariance (ANCOVA) for difference in changes. If necessary, values will be log-transformed to meet the assumptions of the tests. All analyses will be analysed according to the intention-to-treat principle. Between-group differences in ordinal categorical variables, such as New York Heart Association class, will be analysed using ordinal logistic regression, whereas the count variables will be assessed by Poisson regression. Demographic, efficacy and safety data will be summarised by treatment group using means, minimums, medians, maximums, IQRs and SD for continuous variables and frequency counts and percentages for categorical variables. Per-protocol analyses will be performed using the same methods as for the intention-to-treat analyses.

The between-group difference in the myocardial salvage index as measured by CMR 3–7 days after study drug infusion will be assessed by an independent Student's t-test according to the intention-to-treat principle, the statistical null hypothesis being that the myocardial salvage index does not differ between the two treatment arms. Secondary analyses will be made according to the per-protocol principle. For TnT, the baseline-adjusted between-group difference in AUC for TnT will be calculated by ANCOVA, if necessarily after log transformation. The statistical null hypothesis is that the TnT release does not differ between patients allocated to tocilizumab and patients allocated to placebo.

Safety analyses will include tabulation of the type and frequency of adverse events. Any serious adverse events will be reported with comprehensive narratives. Any value of safety laboratory parameters outside normal ranges will be identified.

Study management

A trial steering committee oversees the progress of the trial. An independent data monitoring and safety committee is responsible for the regular monitoring of trial data, and it will give advice on whether the accumulated safety data, together with the results from other relevant research, necessitate premature termination of the trial.

ETHICS AND DISSEMINATION

The ASSAIL-MI trial is designed to assess the effect of a therapeutic intervention with the aim to improve outcome in a population with considerable known mortality and morbidity. The trial is conducted according to Good Clinical Practice guidelines. Based on previous trials and drug pharmacodynamics, we do not expect a substantial number of drug-related severe adverse events. The trial complies with the Declaration of Helsinki. All patients provide written informed consent before enrolment and randomisation.

This study was initiated in February 2017 and is currently recruiting patients at all three participating sites. As of April 2019, 135 patients have been enrolled. Baseline characteristics of the first 100 of these patients are shown in table 1.

We expect patient recruitment to be complete by the end of 2019. We expect the trial to be completed and results available in 2020.

If positive, this study may be followed by larger multicentre studies with clinical endpoints like mortality and hospitalisation as primary endpoints. The study has the potential to change clinical practice in the management of patients with ACS.

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Competing interests None declared.

Patient consent for publication Not required.

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Provenance and peer review Not commissioned; internally peer reviewed.

Data availability statement Data are available in a public, open access repository.

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ORIGINAL INVESTIGATIONS

Randomized Trial of Interleukin-6 Receptor Inhibition in Patients With Acute ST-Segment Elevation Myocardial Infarction

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ABSTRACT

BACKGROUND Prompt myocardial revascularization with percutaneous coronary intervention (PCI) reduces infarct size and improves outcomes in patients with ST-segment elevation myocardial infarction (STEMI). However, as much as 50% of the loss of viable myocardium may be attributed to the reperfusion injury and the associated inflammatory response.

OBJECTIVES This study sought to evaluate the effect of the interleukin-6 receptor inhibitor tocilizumab on myocardial salvage in acute STEMI.

METHODS The ASSAIL-MI trial was a randomized, double-blind, placebo-controlled trial conducted at 3 high-volume PCI centers in Norway. Patients admitted with STEMI within 6 h of symptom onset were eligible. Consenting patients were randomized in a 1:1 fashion to promptly receive a single infusion of 280 mg tocilizumab or placebo. The primary endpoint was the myocardial salvage index as measured by magnetic resonance imaging after 3 to 7 days.

RESULTS We randomized 101 patients to tocilizumab and 98 patients to placebo. The myocardial salvage index was larger in the tocilizumab group than in the placebo group (adjusted between-group difference 5.6 [95% confidence interval: 0.2 to 11.3] percentage points, p = 0.04). Microvascular obstruction was less extensive in the tocilizumab arm, but there was no significant difference in the final infarct size between the tocilizumab arm and the placebo arm (7.2% vs. 9.1% of myocardial volume, p = 0.08). Adverse events were evenly distributed across the treatment groups.

CONCLUSIONS Tocilizumab increased myocardial salvage in patients with acute STEMI. (ASSessing the effect of Anti-IL-6 treatment in Myocardial Infarction [ASSAIL-MI]; NCT03004703) (J Am Coll Cardiol 2021;77:1845–55) © 2021 by the American College of Cardiology Foundation.

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ABBREVIATIONS AND ACRONYMS

CMR = cardiac magnetic resonance imaging

CRP = C-reactive protein

IL = interleukin

I/R = ischemia/reperfusion

MI = myocardial infarction

NT-proBNP = N-terminal pro-B-type natriuretic peptide

PCI = percutaneous coronary intervention

STEMI = ST-segment elevation myocardial infarction

TnT = cardiac troponin T

The mortality and morbidity associated with ST-segment elevation myocardial infarction (STEMI) have fallen in the era of primary percutaneous coronary intervention (PCI) (1); however, the residual morbidity is substantial. A large proportion of patients subsequently develop heart failure, which is associated with an increased risk of death (2). The area at risk, that is, the volume of the myocardium that is rendered ischemic by the coronary occlusion, is the most important determinant of the final infarct size (3), which in turn influences outcomes (4). Another important factor is myocardial salvage; the extent to which the ischemic myocardium recovers after reperfusion (5).

Paradoxically, the restoration of blood flow to the ischemic area may result in further myocardial injury. The pathophysiological mechanisms causing this ischemia/reperfusion (I/R) injury are not fully elucidated, but may involve the generation of reactive oxygen species, intracellular calcium overload, and acidosis (6). The I/R injury may account for as much as 50% of the myocardial damage in myocardial infarction (MI), and inflammatory mechanisms seem to contribute to the I/R injury (6). A dysregulated inflammatory process can increase the final infarct size, induce maladaptive remodeling within the myocardium, and lead to heart failure (7). Targeted therapy against inflammatory pathways that are activated during reperfusion could be a target for reducing the final infarct size and improve prognosis after STEMI. Cardiac magnetic resonance imaging (CMR) can be used to quantify the extent of myocardial ischemia and necrosis and thus to estimate the effect of such intervention.

The inflammatory cytokine interleukin (IL)-6 is an important mediator of the inflammatory process in coronary artery disease, and may also contribute to the I/R injury in MI (8,9). Levels of IL-6 increase substantially after MI and are associated with poor short-term outcomes (10). Tocilizumab is a recombinant humanized monoclonal antibody that binds to the IL-6 receptor to block its signal transmission. Tocilizumab is approved for the treatment of rheumatoid arthritis, juvenile idiopathic arthritis, and giant cell arteritis and protects against cardiovascular events induced by chimeric antigen receptor T-cell therapy (11).

We recently conducted a double-blind, placebo-controlled trial in 117 patients with non-STEMI who presented within 72 h of the onset of chest pain. In this study, a single, intravenous dose of tocilizumab reduced levels of C-reactive protein (CRP), a downstream marker of IL-6, by >50% in the days after the intervention (12). Importantly, tocilizumab also reduced levels of troponin T (TnT) after revascularization, suggesting that tocilizumab reduced the magnitude of the I/R injury. On the other hand, the potential for myocardial salvage is larger in transmural infarctions. We therefore designed the ASSAIL-MI (ASSessing the effect of Anti-IL-6 treatment in Myocardial Infarction) trial to test the hypothesis that prompt administration of tocilizumab would increase the myocardial salvage index in patients presenting with acute STEMI (13).

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METHODS

TRIAL DESIGN AND PARTICIPANTS. This phase II, parallel arm, double-blind, randomized, placebo-controlled trial was conducted at 3 high-volume PCI centers in Norway (Oslo University Hospital Rikshospitalet, Oslo University Hospital Ullevål, and St. Olav's Hospital, Trondheim). Patients aged between 18 and 80 years were eligible for participation if presenting with chest pain within 6 h of symptom onset and ST-segment elevation in 2 contiguous electrocardiogram leads consistent with acute transmural MI (14). Key exclusion criteria

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The authors attest they are in compliance with human studies committees and animal welfare regulations of the authors' institutions and Food and Drug Administration guidelines, including patient consent where appropriate. For more information, visit the Author Center.

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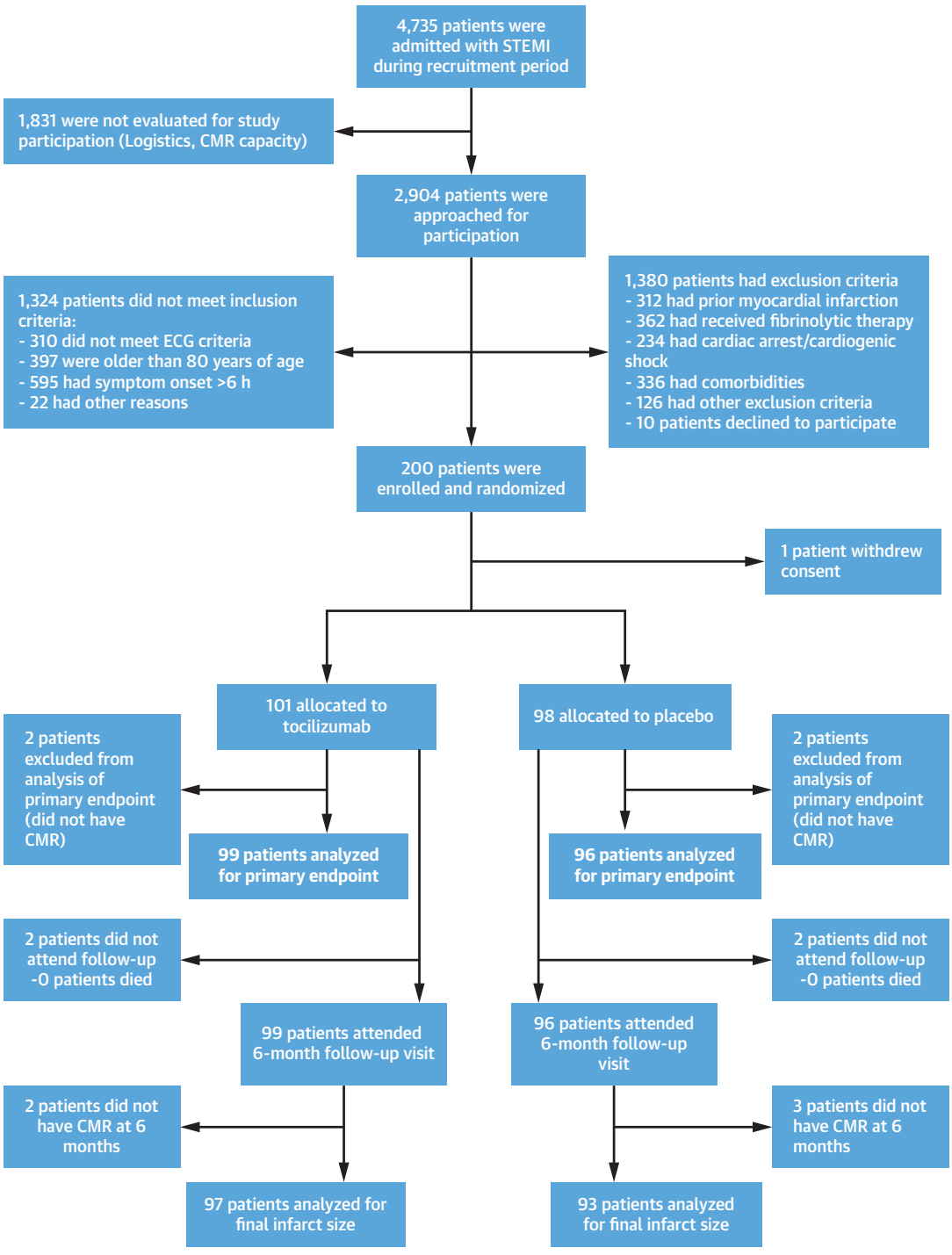
FIGURE 1 **Screening, Randomization, and Follow-Up**



Flow chart illustrating patient selection, randomization, and follow-up. CMR = cardiac magnetic resonance imaging; ECG = electrocardiogram; STEMI = ST-segment elevation myocardial infarction.

were previous MI; left bundle branch block; cardiogenic shock; resuscitated cardiac arrest; fibrinolytic therapy within the last 72 h; a history of severe renal failure, liver failure, malignant disease, chronic infection, or chronic autoimmune or inflammatory disease; uncontrolled bowel disease; ongoing infectious or immunologic disease; major surgery within the past 8 weeks; or treatment with

TABLE 1 Baseline Characteristics

	Tocilizumab (n = 101)	Placebo (n = 98)
Demographics		
Age, yrs	62 ± 10	60 ± 9
Men	80 (79)	87 (89)
Body mass index, kg/m^2	27.1 ± 4.5	27.5 ± 4.3
White	99 (98)	94 (96)
Smoking status		
Never smokers	38 (38)	36 (37)
Previous smokers	33 (33)	24 (24)
Current smokers	30 (30)	38 (39)
Prior conditions		
Angina pectoris	1 (1)	1 (1)
Cerebrovascular disease	4 (4)	2 (2)
Other vascular disease	1 (1)	3 (3)
Diabetes mellitus	8 (8)	6 (6)
Hypertension	33 (33)	30 (31)
Treatment		
ACE inhibitor or ARB	22 (22)	25 (26)
Aldosterone antagonist	1 (1)	0 (0)
Oral anticoagulants	5 (5)	2 (2)
Platelet inhibitor	12 (12)	5 (5)
Beta-blocker	8 (8)	3 (3)
Calcium antagonist	13 (13)	10 (10)
Diuretic	8 (8)	8 (8)
Statin	19 (19)	9 (9)
Up-front DAPT	101 (100)	98 (100)
Clinical characteristics		
Blood pressure at admission, mm Hg		
Systolic	131 ± 23	132 ± 22
Diastolic	81 ± 17	84 ± 16
Heart rate at admission, beats/min	71 ± 15	73 ± 18
Time from symptom onset to arrival at PCI center, min	151 ± 71	149 ±72
Door-to-balloon time, min	23 ± 10	23 ± 11
Killip class		
I	96 (95)	95 (97)
II	4 (4)	3 (3)
III	0 (0)	0 (0)
IV	1 (1)	0 (0)
GRACE risk score	140 ± 25	135 ± 21

Continued on the next page

immunosuppressants other than low-dose steroids (equivalent to a systemic exposure to 5 mg prednisone per day). Detailed inclusion and exclusion criteria are provided in Supplemental Table 1.

ETHICAL CONSIDERATIONS. The trial protocol was approved by the regional ethics committee (REK Sør-Øst 2016/1223), and all participants provided written informed consent. An independent Data and Safety Monitoring Board oversaw the safety of the trial. The trial was conducted in compliance with the declaration of Helsinki and with the rules outlined in the guidelines for Good Clinical Practice. Before commencing enrollment, we registered the trial with ClinicalTrials.gov, number NCT03004703.

STUDY SETTING AND INTERVENTION. Patient eligibility was assessed after admission, *en route* to the catheterization laboratory. The study procedures were designed not to delay revascularization. A brief physical examination was performed on the operating table as per usual routine. Oral consent was obtained before study drug administration, and confirmed in writing the next day.

The participants were randomized in a 1:1 fashion to receive a single intravenous dose of tocilizumab or matching placebo during PCI. Tocilizumab was administered at a fixed dose of 280 mg dissolved in 100 ml NaCl 0.9%. The intravenous infusion was administered over 1 h, as recommended by the drug manufacturer (1.67 ml/min). Patients allocated to placebo received an identical-looking intravenous infusion of 100 ml NaCl 0.9%.

RANDOMIZATION AND MASKING. The Research Support Unit at Oslo University Hospital generated a balanced, permuted block randomization list with varying block sizes. The randomization was stratified by center and by whether the time from symptom onset was shorter or longer than 3 h. Patients, study personnel, and caretakers were blinded to treatment allocation. Unblinded personnel pre-prepared identical-looking infusion bottles containing the active study drug or placebo. For treatment allocation, the blinded study personnel selected the next-in-sequence infusion container, according to whether the time from symptom onset was <3 h or 3 h or more, and registered the randomization number. This method was selected for expedient study drug allocation in the emergency care setting.

OUTCOMES. The primary endpoint was the myocardial salvage index (%) defined as:

$$\left(\frac{area\ at\ risk - infarct\ size}{area\ at\ risk}\right) \times 100$$

measured by CMR 3 to 7 days after the intervention. The area at risk is the myocardial volume that is rendered ischemic by the coronary occlusion, whereas the infarct size is the volume of necrotic myocardium. Pre-specified secondary endpoints included: 1) final infarct size (in % of left ventricular mass) as measured by CMR 6 months after the intervention; 2) microvascular obstruction; 3) the area under the curve for TnT; 4) CRP during index hospitalization; 5) N-terminal pro-B-type natriuretic peptide (NT-proBNP); 6) baseline-adjusted left ventricular end-diastolic volume at 6 months; and 7) safety and tolerability. For details, see Supplemental Table 2.

SAMPLE SIZE. We did not perform a sample size analysis based on assumptions about the data that we expected to obtain in the ASSAIL-MI trial, but relied on sample size calculations from the CHILL-MI (Efficacy of Endovascular Cooling Combined With Cold Saline for the Treatment of Acute Myocardial Infarction) and MITOCARE (Treatment of reperfusion injury using a mitochondrial targeted approach: towards a better understanding of the disease) trials as described by Engblom et al. (15). We assumed that our patients would not differ substantially from the patients enrolled in these trials, in whom the mean ± SD for the myocardial salvage index was 54.0% ± 19.4%, and the mean ± SD of the infarct size was 17.4% ± 10.5% of left ventricular mass.

With a SD of 20% and 2 × 100 patients, our trial has 90% power to statistically detect an underlying treatment effect on the myocardial savage index of 9.2 percentage points. Studies have shown that a treatment effect of this magnitude is associated with improved survival and a reduction in clinical events (16).

FOLLOW-UP. The patients were hospitalized for a minimum of 3 days after PCI. Blood samples for assessments of efficacy and safety were drawn before administration of the investigational medicinal product, and again after approximately 8, 16, 24, and 72 to 168 h after admission, as well as after 3 and 6 months. CMR was performed at 3 to 7 days, and at 6 months.

ASSESSMENTS. CMR was performed on 1.5-T systems (Siemens Avanto, Philips Ingenia). A gadolinium contrast agent was administered (0.15 mmol/kg gadobutrol or 0.22 mmol/kg Gd-DOTA), and after 5 min, we acquired a stack of short-axis images of the left ventricle using a retrospectively electrocardiogram-gated, steady-state free precession cine sequence with minimum echo and repetition times. The slice thickness was 8 mm, there were no interslice gaps, the spatial resolution was approximately 1.5 × 1.5 mm, and the temporal resolution 30 to 35 ms. After 15 min, corresponding late enhancement images were acquired in the same image positions (inversion recovery snapshot fast low-angle shot [FLASH]). The same protocol was used at the 6-month examination.

All CMR images were analyzed by a core laboratory at the Department of Circulation and Medical Imaging, Norwegian University of Science and Technology, Trondheim, Norway, using the Segment software (Medviso, Lund, Sweden) (17). Left ventricular mass, volumes, and ejection fraction were analyzed according to recommendations. The area at risk was quantified using the short-axis early contrast-enhanced images as previously described (17), with end-diastolic and end-systolic values averaged. Infarct size was quantified using the expectation maximization, weighted intensity, a priori information method with manual correction. This method has been experimentally and clinically validated, and agrees well with expert delineation (18).

High-sensitivity CRP and NT-proBNP were analyzed on a MODULAR platform (Roche Diagnostics, Basel, Switzerland), and high-sensitivity TnT was measured by electrochemiluminescence immunoassay (Elecsys 2010 analyzer, Roche Diagnostics). Safety samples were analyzed consecutively using routine laboratory methods. In addition, safety was assessed through comprehensive patient interviews, review of patient records, physical examination, and blood samples for safety, as well as echocardiography and CMR.

STATISTICS. Analyses were performed on an intention-to-treat basis. Continuous data are summarized by the mean ± SD or median (interquartile range) if distributions were skewed. Categorical data

TABLE 1 Continued

	Tocilizumab (n = 101)	Placebo (n = 98)
Infarct location		
Left anterior descending branch	38 (38)	36 (37)
Circumflex or marginal	11 (11)	13 (13)
Right coronary artery	47 (47)	46 (49)
Other	5 (5)	3 (3)
Laboratory values		
Hemoglobin, g/l	143 ± 13	144 ± 12
Platelet count, 10^9/l	253 ± 59	260 ± 62
Total white blood cell count, 10^9/l	11.6 ± 3.4	11.6 ± 3.4
Aspartate transaminase, U/l	28 (22-37)	30 (24-37)
Troponin T, ng/l	44 (22-163)	49 (28-95)
CK-MB, μg/l	5.0 (2.6-14.0)	5.3 (3.0-10.0)
NT-proBNP, ng/l	79 (50-178)	63 (50-146)
Creatinine, mmol/l	74 ± 17	78 ± 20
Glucose, mmol/l	9 ± 3	9 ± 3
HbA1c, mmol/mol	37 (34-41)	37 (34-40)
Total cholesterol, mmol/l	5.3 ± 1.2	5.2 ± 1.0
HDL cholesterol, mmol/l	1.2 (0.9-1.3)	1.1 (0.9-1.3)
LDL cholesterol, mmol/l	3.7 ± 1.1	3.7 ± 0.9
C-reactive protein, mg/l	2.4 (0.9-5.0)	2.9 (1.4-5.0)
Albumin, g/l	42 ± 3	42 ± 3

Values are mean ± SD, n (%), or median (interquartile range). Baseline characteristics stratified by treatment allocation.

ACE = angiotensin-converting enzyme; ARB = angiotensin receptor blocker; CK-MB = creatine kinase myocardial band; DAPT = dual anti-platelet therapy; HDL = high-density lipoprotein; LDL = low-density lipoprotein; PCI = percutaneous coronary intervention; NT-proBNP = N-terminal pro-B-type natriuretic peptide.

are reported as numbers and percentages. Normally distributed endpoints, including the primary endpoint, were analyzed using parametric methods. The analysis of the primary endpoint was adjusted for the time from symptom onset, the pre-determined stratification variable. The baseline-adjusted between-group difference in left ventricular end-diastolic volume was calculated by analysis of covariance with treatment as a fixed effect and the baseline volume as a covariate. The areas under the curve of TnT and CRP were calculated by the quadratic method. The between-group differences in TnT, CRP, microvascular obstruction, and final infarct size were assessed by Mann-Whitney *U* tests due to skewed distributions. There were no imputations for missing data.

We did not perform interim outcome analyses. We assessed the consistency of the treatment effect on the primary endpoint among 6 pre-specified subgroups that were analyzed individually and then in a multivariable model. The following a priori subgroup analyses were planned: age younger than versus older than 60 years, duration from symptom onset to study drug infusion less than versus at least 3 h, female versus male sex, area at risk above versus below median, and the areas under the curve for TnT and CRP above versus below median. Safety analyses included tabulation of type and frequency of all adverse events and severe adverse events. All statistical analyses were performed in SPSS version 25 (IBM Corp., Armonk, New York). Two-sided probability values were considered significant at $p < 0.05$. The p values and 95% confidence intervals presented in this report have not been adjusted for multiplicity, and therefore inferences drawn from these statistics may not be reproducible.

RESULTS

Between March 16, 2017, and February 13, 2020, we enrolled 200 patients. One patient gave oral consent, but later refused to participate in the trial and would not allow the use of his or her data. In 4 patients, the CMR at 3 to 7 days was not performed. Therefore, 195 patients had data available for the analysis of the primary endpoint: 96 patients in the placebo group and 99 in the tocilizumab group (**Figure 1**). Baseline data were well balanced between the study arms (**Table 1**).

Coronary angiography was performed in all patients. The door-to-balloon time was 23 ± 10 min. All patients underwent primary PCI except for 8 patients who were deemed not to have MI, 5 patients in the tocilizumab arm and 3 allocated to placebo. Optimal medical therapy was provided according to prevailing guidelines. No patients received urgent coronary artery bypass grafting. The primary endpoint CMR was performed 5.0 ± 1.3 days after inclusion in the tocilizumab arm and 5.0 ± 1.3 days after inclusion in the placebo arm ($p = 1.00$). A total of 195 patients attended the 6-month follow-up-visit (99 in the tocilizumab arm and 96 in the placebo arm). Vital status was known for all participants. No patients died during 6 months of follow-up.

Table 2 shows the results for the primary and key secondary endpoints. The adjusted myocardial salvage index was higher in the tocilizumab arm than in the placebo arm (69 ± 19% vs. 64 ± 21%). The between-group difference was 5.6 percentage points (95% confidence interval: 0.2 to 11.3; $p = 0.04$) (**Figure 2**). The median final infarct size measured 6 months after the intervention was 21% lower in the tocilizumab arm, but this difference was not statistically significant ($p = 0.08$). The area under the curve of TnT during hospitalization was numerically lower in patients allocated to tocilizumab, but once again, the between-group difference was not statistically significant ($p = 0.13$). On the other hand, the extent of microvascular obstruction was significantly less in the tocilizumab arm than in the placebo arm ($p = 0.03$). The area under the curve of CRP during hospitalization was substantially lower in the tocilizumab group than in the placebo group ($p < 0.001$). Finally, there were no between-group differences in the baseline-adjusted left ventricular volume ($p = 0.54$) or the plasma concentration of NT-proBNP at 6 months ($p = 0.25$). Comprehensive results of the CMR examinations are provided in **Table 3** and the laboratory analyses in Supplemental Table 3.

The primary outcome in the 6 pre-specified subgroups is shown in **Figure 3**. There was heterogeneity of the treatment effect regarding the time from symptom onset. Notably, the positive effect of tocilizumab on the primary endpoint seemed to be limited to patients presenting >3 h after symptom onset. Men appeared to benefit more than women from treatment with tocilizumab, but the interaction between sex and treatment was of borderline statistical significance ($p = 0.053$).

We observed 77 minor adverse events in the tocilizumab group and 85 events in the placebo arm during 6 months' follow-up. Most of the events were mild and deemed not to be associated with the study drug. Serious adverse events are tabulated in **Table 4**. There were 19 serious adverse events in patients allocated to tocilizumab and 15 serious adverse events in patients allocated to placebo ($p = 0.57$). Notably, there were no myocardial ruptures. There

TABLE 2 Primary and Secondary Outcomes

	Tocilizumab		Placebo		Between-Group Difference (95% CI)	p Value*
Myocardial salvage index, %	69.3 ± 19.3	99	63.6 ± 20.8	96	5.6 (0.2 to 11.3)	0.04
Final infarct size at 6 months (% of left ventricular mass)	7.2 (2.6 to 11.8)	97	9.1 (2.9 to 16.3)	93	-	0.08
Extent of microvascular obstruction (% of left ventricular volume)	0 (0 to 14)	99	4 (0 to 18)	96	-	0.03
Troponin T AUC, ng/l/h	1,614 (860 to 3,515)	101	2,357 (97 to 4,127)	98	-	0.13
C-reactive protein AUC, mg/l/h	1.9 (0.9 to 4.9)	101	8.6 (5.0 to 17.9)	98	-	<0.001
Baseline-adjusted LVEDV at 6 months, ml	157 (151 to 166)	97	160 (153 to 166)	93	3 (−6 to 11)	0.54
NT-proBNP at 6 months, ng/l*	79 (50 to 187)	98	63 (50 to 148)	97	32 (−84 to 149)	0.25

Values are mean ± SD, n, or median (interquartile range), unless otherwise indicated. Primary and secondary endpoints. *We did not adjust for multiple testing, and all p values are nominal only.

AUC = area under the curve; CI = confidence interval; LVEDV = left ventricular end-diastolic volume; NT-proBNP = N-terminal pro-B-type natriuretic peptide.

were 3 infections requiring prolongation of the index hospitalization or renewed hospitalization in the tocilizumab arm and 2 such infections in the placebo arm. No patients died or developed heart failure during follow-up.

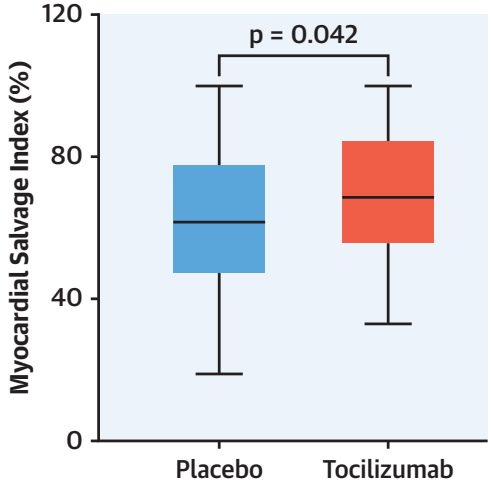
There were minor differences in biochemical variables between the 2 treatment groups that could potentially reflect side effects of tocilizumab (Supplemental Table 3). First, there was an early decrease in neutrophils and monocytes in the tocilizumab arm. Second, low-density lipoprotein and triglycerides increased in the tocilizumab arm compared with the placebo arm. Finally, we observed a very modest increase in liver enzymes in the tocilizumab group. Importantly, at 3 and 6 months, there were no between-group differences in these parameters.

DISCUSSION

This randomized trial showed that prompt, intravenous treatment with the IL-6 inhibitor tocilizumab may improve myocardial salvage in patients presenting with acute STEMI (**Central Illustration**). This effect seemed to be limited to patients with symptom onset >3 h before PCI. The extent of microvascular obstruction was less in the tocilizumab arm than in the placebo arm.

Inflammation seems to be involved in all stages of atherosclerotic disease, from the development of the initial lesion to plaque progression, rupture, and erosion, and appears to contribute to the I/R injury after revascularization. The Canakinumab Antiinflammatory Thrombosis Outcomes Study (19), the Colchicine Cardiovascular Outcomes Trial (20), and the Low-dose colchicine for secondary prevention of cardiovascular disease trial (21) showed that anti-inflammatory treatment can reduce cardiovascular events in patients with coronary artery disease. However, the effect of anti-inflammatory treatment on the I/R injury has been explored to a limited degree only (22). In a small randomized trial, methotrexate did not reduce infarct size and seemed to impair left ventricular function after STEMI (23). Treatment with the IL-1 receptor antagonist anakinra was recently shown to attenuate inflammation in the wake of STEMI. Although there was no difference in left ventricular volume or ejection fraction between the anakinra arm and the placebo arm, the incidence of death or new-onset heart failure or of death and

FIGURE 2 Myocardial Salvage



Bar chart showing myocardial salvage in patients treated with tocilizumab and patients treated with placebo.

TABLE 3 Cardiac Magnetic Resonance Imaging Data

	3-7 Days After Randomization			6 Months After Randomization		
	Tocilizumab	Placebo	p Value for Difference	Tocilizumab	Placebo	p Value for Difference*
LVEDV, ml	149 ± 39	157 ± 40	0.17	153 ± 45	163 ± 47	0.12
LVESV, ml	80 ± 22	81 ± 18	0.88	85 ± 222	88 ± 19	0.46
LVEF, %	55 ± 10	53 ± 10	0.18	56 ± 11	55 ± 10	0.53
Area at risk, g	46 ± 24	51 ± 30	0.16	N/A	N/A	
LV mass, g	131 ± 29	135 ± 34	0.46	118 ± 33	124 ± 32	0.22
Infarct size, g	12.6 (6.9–23.6)	15.0 (6.8–31.2)	0.14	7.9 (2.7–15.5)	11.7 (3.3–21.9)	0.09
MVO	35 (35)	49 (51)	0.02	10 (10)	11 (12)	0.46

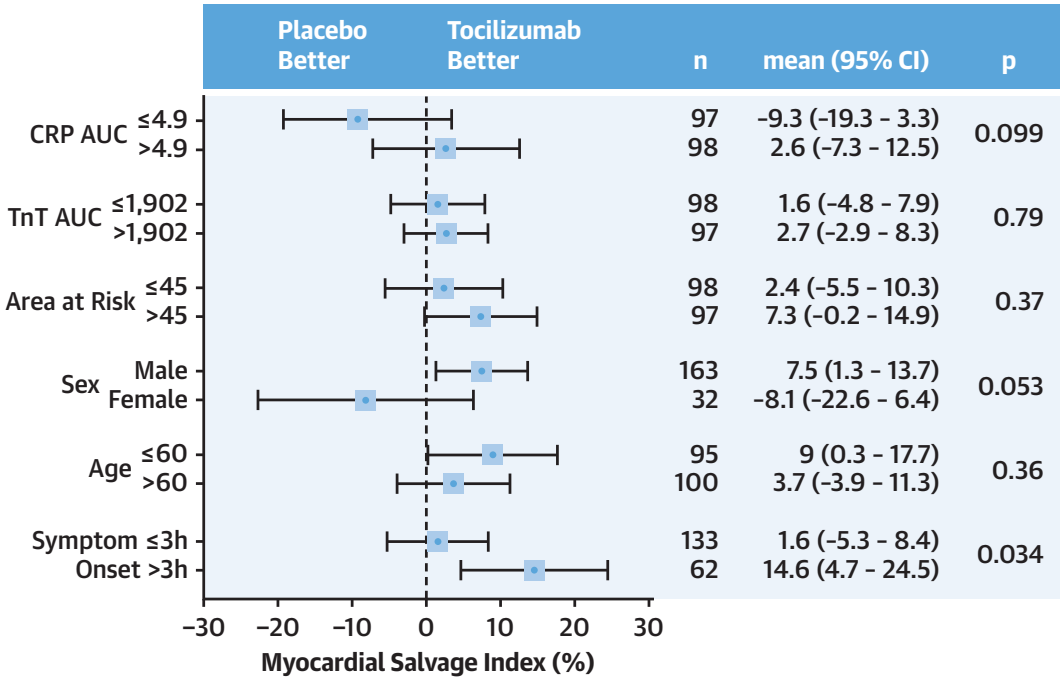
Values are mean ± SD or median (interquartile range) depending on distribution, or n (%). *p values are nominal.
LV = left ventricle; LVEDV = left ventricular end-diastolic volume; LVEF = left ventricular ejection fraction; LVESV = left ventricular end-systolic volume; MVO = microvascular obstruction.

hospitalization for heart failure was lower in patients treated with anakinra (24). Similar results were observed in a pooled analysis of the pilot trials (25). On the other hand, we recently showed that tocilizumab tempered the inflammatory response after non-STEMI and diminished the release of TnT, in particular in patients who underwent PCI, suggesting that tocilizumab could mitigate the I/R injury (12).

The ASSAIL-MI trial was a first-in-human, proof-of-concept trial. It was designed to test whether IL-6 receptor inhibition could attenuate the inflammatory overshoot that occurs during MI and reperfusion in patients with acute STEMI and thereby reduce the harmful effects of inflammation. We assumed that a reduction in the I/R injury would be reflected in a larger degree of myocardial salvage, a surrogate endpoint that is associated with clinical outcomes (5). The myocardial salvage index reports myocardial salvage as a fraction of the area at risk, which reduces the otherwise large variability in measures of infarct size and allows for a smaller sample size (15). We showed that tocilizumab improved myocardial salvage and reduced the extent of microvascular obstruction, suggesting that there is a potential for targeted therapy against the inflammatory cytokine IL-6 in these patients.

We assessed the area at risk with early gadolinium enhancement steady-state free precession images. Our data show that the area at risk was numerically smaller in the tocilizumab arm. A recent meta-analysis showed that a reduction in the area at risk was mainly observed in studies in which the intervention reduced the final infarct size (26). Final

FIGURE 3 Primary Outcome in Pre-Specified Subgroups



Primary outcome in pre-specified subgroups (age younger than 60 years vs. at least 60 years, duration from symptom onset to study drug infusion <3 h vs. at least 3 h, female sex vs. male sex, area at risk above median vs. below median, and the areas under the curve [AUC] for troponin T [TnT] and C-reactive protein [CRP] above vs. below median).

TABLE 4 Patients With Serious Adverse Events and Events of Special Interest (6 Months' Follow-Up)

Event	Tocilizumab	Placebo
Any serious adverse event	19	15
Infections requiring hospitalization	3	2
New malignancy	2	0
Cardiovascular events	9	10
Myocardial infarction	0	4
CABG	1	0
Chest pain	5	4
Resuscitated VF	1	1
VT	1	0
Ischemic stroke	0	1
SAH	1	0
Worsening renal function*	0	0
Liver-associated events†	0	0

*Defined as a doubling in serum creatinine from baseline, a >50% fall in the estimated glomerular filtration rate, or the need for renal replacement therapy. †Defined as an elevation of aspartate transaminase to above 3 times the upper limit of normal beyond the acute phase or Child Pugh stage II or II liver failure.

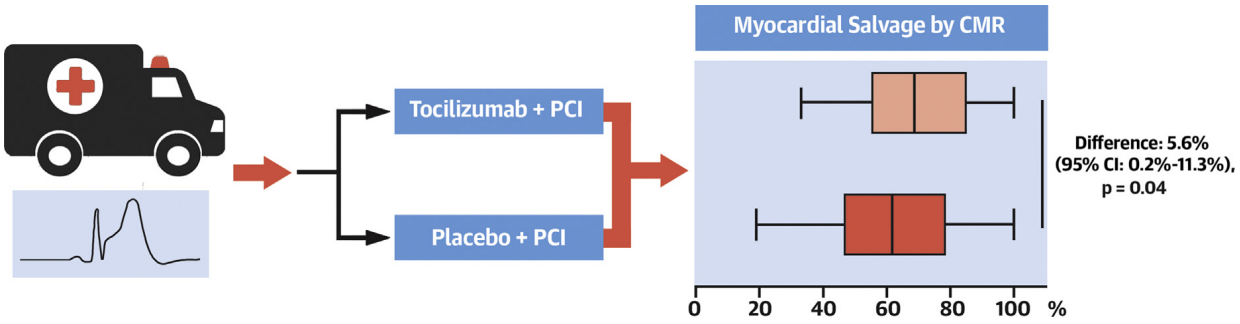
CABG = coronary artery bypass grafting; SAH = subarachnoid hemorrhage; VF = ventricular fibrillation; VT = ventricular tachycardia.

infarct size was also numerically smaller in the group receiving tocilizumab. Whether the modest gain in myocardial salvage can translate into a clinical benefit in patients with STEMI should be confirmed in larger trials with clinical endpoints.

The absolute effect of tocilizumab on myocardial necrosis was smaller than we assumed when we designed the trial. This may explain why there was no significant reduction in infarct size as measured by CMR or the release of TnT and CK-MB. Although the relative reduction in the median infarct size was 21%, the median infarct size in the placebo arm was limited. We included nonanterior MIs, we randomized patients before evaluating target vessel coronary blood flow, and we excluded patients with <6 h of ischemia, all of which may have contributed to the smaller than expected infarct sizes. The small extent of myocardial necrosis may also explain why at 6 months there were no signs of material left ventricular remodeling in either treatment group, and why, despite the improved myocardial salvage in the tocilizumab arm, there was no between-group difference in NT-proBNP.

Because IL-6 inhibition was untested in STEMI, we selected a modest dose of tocilizumab designed to provide short-lived full suppression of IL-6 signaling. The dose was selected to minimize the potential negative effect on myocardial healing but may have been too small to achieve maximal anti-inflammatory effect. Reassuringly, there were no major safety issues and specifically no myocardial ruptures. On the other hand, we observed a robust reduction in CRP in the tocilizumab arm, suggesting that we achieved powerful inhibition of the IL-6 pathway. However, not all relevant effects of IL-6 are reflected in circulating levels of CRP, and this important issue should be explored in forthcoming studies.

CENTRAL ILLUSTRATION Design and Primary Result of the ASSAIL-MI Trial



Broch, K. et al. J Am Coll Cardiol. 2021;77(15):1845–55.

In the ASSAIL-MI trial, we randomized 199 patients with acute ST-segment elevation myocardial infarction (illustrated by ambulance and ST-segment elevation in electrocardiogram) to prompt treatment with the interleukin-6 receptor inhibitor tocilizumab or placebo during percutaneous coronary intervention (PCI). As illustrated in the bar chart, the primary endpoint, the myocardial salvage index as measured by cardiac magnetic resonance imaging (CMR), was higher in patients allocated to tocilizumab (between-group difference 5.6% [95% confidence interval: 0.2% to 11.3%] of left ventricular volume; p = 0.04). ASSAIL-MI = ASSessing the effect of Anti-IL-6 treatment in Myocardial Infarction.

Bearing in mind that the subgroup analyses were exploratory only, the effect on the primary endpoint seemed to be stronger in patients presenting >3 h after symptom onset. It is conceivable that the inflammatory response, and therefore the potential effect of the anti-inflammatory intervention, is smaller in short-lasting ischemia. Prompt revascularization may minimize the area amenable to salvage in patients with a short history of chest pain. For safety reasons, we excluded patients with a time from symptom onset of >6 h, as well as patients with cardiogenic shock or resuscitated cardiac arrest.

STUDY LIMITATIONS. The ASSAIL-MI trial was designed to show the effect of tocilizumab on myocardial salvage in patients presenting with acute STEMI. Investigators have recently questioned the validity of the myocardial salvage index (27); however, the salvage index was a favored endpoint in clinical trials aiming for cardioprotection when the trial was designed. Simulations based on multisite, multivendor data showed that the sample size could be substantially reduced if the effect of the intervention was evaluated by the myocardial salvage index instead of infarct size (15). The number of patients was limited, but the trial was designed to detect a clinically meaningful increase in myocardial salvage. However, the myocardial salvage index was higher than expected in the placebo group, limiting the statistical power of the trial. Immediate and powerful inhibition of inflammation is a novel treatment concept in STEMI, and safety was therefore emphasized. The strict inclusion and exclusion criteria may have limited the effect of the intervention. The narrow inclusion criteria also limit the generalizability of the results.

CONCLUSIONS

Early treatment with tocilizumab augmented myocardial salvage in patients presenting with acute STEMI within 6 h of symptom onset. There was a trend toward less myocardial necrosis and smaller final infarct sizes in the tocilizumab arm. In exploratory subgroup analyses, the effect of tocilizumab seemed to be limited to patients who were randomized >3 h after the onset of symptoms. The drug was well tolerated and there were no major safety concerns. The clinical significance of the observed increase in myocardial salvage is uncertain. Larger studies should explore the effect of tocilizumab on clinical endpoints, optimize the dose of tocilizumab, and perhaps select patients who present several hours after symptom onset.

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PERSPECTIVES

COMPETENCY IN MEDICAL KNOWLEDGE: In a proof-of-concept study, administration of the interleukin-6 receptor inhibitor tocilizumab in patients with acute STEMI was associated with myocardial salvage, as assessed by magnetic resonance imaging.

TRANSLATIONAL OUTLOOK: Larger trials are necessary to confirm whether inhibition of inflammation ameliorates post-ischemic myocardial reperfusion injury and improves clinical outcomes in patients with acute STEMI.

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KEY WORDS inflammation, infarct size, myocardial salvage, randomized controlled trial, reperfusion injury, ST-segment elevation myocardial infarction

APPENDIX For supplemental tables, please see the online version of this paper.

Supplementary material, Interleukin-6 receptor inhibition in acute ST-segment elevation myocardial infarction: A randomized placebo-controlled trial (ASSAIL-MI) by Broch, Anstensrud et al.

Table 1 Exclusion/inclusion criteria

Inclusion criteria	• New ST elevation at the J-point in two contiguous ECG[‡] leads (cut-points: 0.2mV in men and > 0.15 mV in women in leads V2-V3 and/or > 0.1 mV in other leads) in combination with symptoms consistent with acute myocardial infarction. • Presentation within six hours of chest pain. • Indication for urgent coronary angiography with intent to reperfuse presumed occluded vessel. • Age between 18 and 80 years. • Informed consent obtained and documented according to ICH/GCP[#], and national/local regulations.
Exclusion criteria	• NSTEMI[**] (non-ST segment elevation in ECG[‡]). • Left bundle branch block in ECG[‡] • History of previous MI[††] • Cardiogenic shock. • Fibrinolytic therapy within 72 hours prior to admission. • Cardiac arrest / ventricular fibrillation. • History of severe renal failure with estimated glomerular filtration rate < 30 ml/minutes. • Known, current liver disease • History of concurrent inflammatory, biliary obstructive or malignant disease • Ongoing infection

	• A history of chronic or concurrent infectious disease, including a history of HIV[§], tuberculosis, or hepatitis B or C. • Known, uncontrolled lower gastrointestinal disease such as diverticulitis, Crohn's disease, ulcerative colitis, or other symptomatic lower gastrointestinal conditions that could predispose to gastrointestinal perforations • Major surgery within eight weeks prior to or after baseline • History of central nervous system demyelinating or seizure disorders • History of primary or secondary immunodeficiency • Treatment with immunosuppressants other than low dose corticosteroids (equivalent to 5 mg of prednisone or less) at the time of randomisation • Immunization with a live/attenuated vaccine within four weeks prior to baseline • History of severe allergic or anaphylactic reactions to humanized or murine monoclonal antibodies or to tocilizumab • Pregnancy, possible pregnancy or breast-feeding – women of child-bearing potential or breastfeeding mothers cannot participate. A woman is considered of childbearing potential following menarche and until becoming post-menopausal unless permanently sterile. Permanent sterilisation methods include hysterectomy, bilateral salpingectomy and bilateral oophorectomy. A postmenopausal state is defined as no menses for 12 months without an alternative medical cause.

	• Contraindications to CMR* (pacemaker, CRT†, ICD?, certain ferromagnetic implants, severe claustrophobia, allergy to contrast medium). • Any condition/circumstances believed to interfere with the ability to comply with protocol. • Any reason why, in the opinion of the investigator, the patient should not participate. • Failure to obtain written, informed consent by patient or next of kin, for instance in case of patient death after consent has been provided in oral.

*CMR = Cardiac magnetic resonance imaging; †CRT = cardiac resynchronization; ‡ECG = electrocardiogram; §HIV = human immunodeficiency virus; ?ICD = implantable cardioverter defibrillator; #ICH/GCP = International Conference on Harmonization/Good Clinical Practice: **NSTEMI = non-ST-segment elevation myocardial infarction; ††MI = myocardial infarction

Supplementary Table 2 Endpoints

Primary endpoint	The myocardial salvage index as measured in the acute phase, three to seven days after randomization, by CMR[§] with late gadolinium enhancement.
Secondary endpoints	• Final infarct size as measured by CMR[§] 6 months after randomization • Microvascular obstruction as measured by CMR[§] after three to seven days • Left ventricular end diastolic volume as assessed by CMR[§] six months after randomization • The AUC[†] for troponin T during index hospitalization • The AUC[†] of C-reactive protein during index hospitalization • N-terminal pro-B-type natriuretic peptide at six months • Safety and tolerability as assessed by clinical assessment, blood samples and imaging
Exploratory endpoints	• Additional inflammatory and anti-inflammatory mediators • Markers of left ventricular myocardial remodeling as assessed by echocardiography • Markers of platelet activation (e.g., platelet-derived inflammatory markers) and additional pro- and anti-thrombotic parameters (e.g., tissue factor and plasminogen activator inhibitor) • The baseline-adjusted AUC[†] for AST[*] and CK-MB[‡] during index hospitalization

[*]AST = Aspartate transaminase; [†]AUC = Area under the curve; [‡]CK-MB = creatine kinase myocardial band; [§]CMR = cardiac magnetic resonance imaging

Supplementary Table 3 Biochemistry

	Treatment	Baseline	24 hours	168 hours	3 months	6 months
NT-proBNP[§] – ng/l	Placebo	63 (50-146)	988 (584-1743)	462 (212-915)	199 (81-463)	133 (58-316)
	Tocilizumab	79 (50-178)	1009 (418-2001)	476 (172-866)	175 (78-509)	133 (56-357)
C-reactive protein – mg/l	Placebo	2.0 (1.0-4.2)	7.5 (4.5-14.8)	8.4 (3.1-21.2)	1.1 (0.7-2.1)	1.0 (0.6-2.0)
	Tocilizumab	1.7 (0.8-3.4)	2.1 (1.1-3.8)[c]	0.6 (0.6-0.7)[c]	1.1 (0.6-2.6)	0.9 (0.6-2.2)
Troponin T – ng/l	Placebo	49 (28-95)	2807 (1071-4853)	1446 (294-2952)	11 (9-16)	10 (7-13)
	Tocilizumab	44 (22-163)	1884 (1076-3994)	1121 (499-2209)	12 (8-18)	10 (8-15)
CK-MB[‡] – µg/l	Placebo	5 (3-10)	79 (40-152)	3 (2-4)	2 (2-3)	2 (2-3)
	Tocilizumab	5 (3-14)	73 (41-122)	4 (3-7)[c]	3 (2-4)[a]	3 (2-4)
Hemoglobin – g/l	Placebo	144±12	141±13	144±14	146±11	147±11
	Tocilizumab	143±13	142±14	150±14b	145±12	145±12
Platelets – *109/l	Placebo	260±62	239±59	266±67	246±57	245±54
	Tocilizumab	253±59	235±47	248±53[a]	250±58	244±55
White blood cell count – *109/l	Placebo	11.6±3.4	10.1±3	8.3±2	6.8±1.7	7.1±1.7
	Tocilizumab	11.6±3.4	6.3±2.5[c]	5.6±1.6[c]	7.2±1.9	7±1.9
Neutrophils – *109/l	Placebo	8.6±3.3	7.1±2.8	5.4±1.6	4.1±1.1	4.5±1.4
	Tocilizumab	8.6±3.3	3.6±2.3[c]	2.8±1.2[c]	4.4±1.6	4.3±1.5
Monocytes – *109/l	Placebo	0.72±0.22	0.88±0.26	0.78±0.24	0.66±0.23	0.66±0.26
	Tocilizumab	0.7±0.27	0.63±0.25[c]	0.66±0.25[c]	0.66±0.22	0.66±0.22
Cholesterol – mmol/l	Placebo	5.2±1	4.8±1	4.1±1	3.4±0.7	3.4±0.8
	Tocilizumab	5.3±1.2	5.2±1.2[a]	4.6±1.1[c]	3.4±0.6	3.6±0.7
Low density cholesterol – mmol/l	Placebo	3.7±0.9	3.3±0.9	2.6±0.9	1.9±0.5	1.9±0.6
	Tocilizumab	3.7±1.1	3.5±1	2.9±0.9[a]	1.9±0.6	2±0.6
High density cholesterol – mmol/l	Placebo	1.19±0.41	1.11±0.28	1.03±0.26	1.23±0.37	1.24±0.32
	Tocilizumab	1.18±0.36	1.14±0.34	1.07±0.27	1.22±0.29	1.29±0.35
Triglycerides – mmol/l	Placebo	1.22±0.95	1.52±0.69	1.36±0.41	1.13±0.55	1.08±0.38
	Tocilizumab	1.5±1.59	2.04±2.43[a]	2.07±1.72[c]	1.32±0.99	1.22±0.68
AST[†] – U/l	Placebo	35±19	232±180	47±27	33±32	29±16
	Tocilizumab	37±29	191±133	57±31[a]	29±10	30±12
ALT[*] – U/l	Placebo	30±14	56±29	40±16	47±72	40±33
	Tocilizumab	29±16	50±27	55±33c	37±23	37±25
Creatinine – µg/l	Placebo	78±20	79±18	85±19	83±20	83±22
	Tocilizumab	74±17	75±14a	85±16	79±16	79±14

Blood values stratified by treatment. Data are presented as mean ± standard deviation or median (interquartile range) depending on distribution. [a]Between-group difference significant at $p < 0.05$. [b]Between-group difference significant at $p < 0.01$. [c]Between-group difference significant at $p < 0.001$. [*]ALT = Alanine transaminase; [†]AST = Aspartate transaminase; [‡]CK-MB: creatine kinase myocardial band; [§]NT-proBNP: N-terminal pro-B-type natriuretic peptide.

III

Interleukin-6 inhibition in ST-elevation myocardial infarction: Immune cell profile in the randomised ASSAIL-MI trial

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Summary

Background We recently showed that interleukin (IL)-6 inhibition by tocilizumab improves myocardial salvage in ST-elevation myocardial infarction (STEMI). However, the mechanisms for this effect are not clear.

Methods In this exploratory sub-study of the ASSAIL-MI trial, we examined leukocyte differential counts and their relation to myocardial salvage and peak troponin T (TnT) in STEMI patients randomised to tocilizumab (n = 101) or placebo (n = 98). We performed RNA-sequencing on whole blood (n = 40) and T cells (n = 20). B and T cell subpopulations were examined by flow cytometry (n = 69).

Findings (i) STEMI patients had higher neutrophil counts at hospitalisation compared with stable angina patients. (ii) After percutaneous coronary intervention there was a gradual decline in neutrophils, which was significantly more pronounced in the tocilizumab group. (iii) The decrease in neutrophils in the tocilizumab group was associated with improved myocardial salvage and lower peak TnT. (iv) RNA-sequencing suggested that neutrophil function was also attenuated by tocilizumab. (v) B and T cell sub-populations changed only minimally after STEMI with minor effects of tocilizumab, supported as well by RNA-sequencing analyses of T cells. (vi) However, a low $CD8^+$ count was associated with improved myocardial salvage in patients admitted to the hospital > 3 h after symptom onset.

Interpretation Tocilizumab induced a rapid reduction in neutrophils and seemed to attenuate neutrophil function in STEMI patients potentially related to the beneficial effects of tocilizumab on myocardial salvage.

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Keywords: ST-elevation myocardial infarction; Neutrophils; Lymphocytes; Tocilizumab; Interleukin-6; Inflammation

Research in context

Evidence before this study

One study has shown that interleukin-6 inhibition with tocilizumab reduces C-reactive protein and troponin T in patients with NSTEMI. Recently, we showed that a single dose of tocilizumab administered before percutaneous coronary intervention was associated with improved myocardial salvage and reduced levels of circulating neutrophils in patients with STEMI. The effect on myocardial salvage was more pronounced in the patients who received treatment >3 h after symptom onset. However, (i) the kinetics of the effects of tocilizumab in STEMI patients, (ii) whether tocilizumab also modulates other leukocyte subpopulations, and most importantly, (iii) if these effects on leukocytes are related to the beneficial effects of tocilizumab in STEMI are not known.

Added value of this study

We characterize leukocyte levels and subpopulations in STEMI patients treated with tocilizumab in this exploratory sub-study of the clinical ASSAIL-MI trial. Repeated assessments were made from hospital admission to six months into remission. Our major findings were: (i) High neutrophil levels were observed at hospitalisation. Following percutaneous coronary intervention (PCI) there was a gradual decline in neutrophils, which was more pronounced in the tocilizumab group. (ii) A similar decline was also seen for the neutrophil-lymphocyte ratio. (iii) The decrease in neutrophils in the tocilizumab group was associated with improved myocardial salvage and lower peak troponin. (iv) RNA-sequencing and Reactome analysis of whole blood revealed that tocilizumab attenuated the innate immune response and signal transduction. (v) Cell type deconvolution and gene expression imputation analysis revealed several altered pathways relevant for neutrophil function. (vi) B and T cell sub-populations changed only minimally after STEMI and were only slightly affected by tocilizumab. Only minor changes were seen in RNA-sequencing analyses of T cells. (vii) However, the lack of rising in $CD8^+$ T cells in the tocilizumab arm in patients who were admitted > 3 h after symptom onset, previously reported to have the best effect of tocilizumab, was associated with improved myocardial salvage.

Implications of all the available evidence

Our findings provide evidence that tocilizumab markedly affects neutrophil levels and function in patients treated with PCI for acute STEMI. We suggest that the beneficial effects of tocilizumab on myocardial salvage in STEMI patients may, at least partly, be related to the observed effect on neutrophils, emphasising the major importance of these cells during STEMI.

Introduction

Inflammation plays a crucial role in atherosclerotic disease and might be a therapeutic target in acute coronary syndrome (ACS).[1] Both local and systemic inflammation have been documented in patients with myocardial infarction (MI), and can potentially contribute to plaque destabilization and myocardial damage.[1] However, following MI, inflammation also plays an important role in infarct healing, in which both too much and too little inflammation could potentially be harmful.[2] Several inflammatory cytokines are upregulated in coronary artery disease and in particular during acute MI[2,3] and, from a therapeutic point of view, much focus has been put on interleukin (IL)-1.

In the landmark "Canakinumab Antiinflammatory Thrombosis Outcome Study" (CANTOS), canakinumab, a monoclonal antibody against IL-1β, significantly reduced the hazard of cardiovascular events in patients with previous MI.[4] Interestingly, the beneficial effect of canakinumab in the CANTOS study was particularly strong in those who obtained a reduction in IL-6.[4] IL-6 is an inflammatory cytokine that is upregulated during MI and affects both plaque destabilization and myocardial remodeling.[4] We have previously shown that a single dose of tocilizumab, a humanized monoclonal antibody that blocks IL-6 signalling by binding to the soluble and membrane-bound IL-6 receptor (IL-6R), significantly reduced C-reactive protein (CRP) and troponin T (TnT) in patients with non-ST segment elevation MI (NSTEMI).[5] In our recently published randomised trial "Assessing the effect of Anti-IL-6 treatment in MI" (ASSAIL-MI), patients with ST-elevation MI (STEMI) had an improved myocardial salvage and reduced CRP (area under the curve during

hospitalisation) when receiving a single intravenous dose of tocilizumab (280 mg) just prior to percutaneous coronary intervention (PCI).[6]

Tocilizumab reduced the absolute neutrophil count in STEMI and NSTEMI.[5,6] However, the kinetic and the molecular consequence of this effect and its clinical relevance is not clear. IL-6 is known to have pleiotropic effects on other leukocyte subpopulations, in particular lymphocyte subpopulations. These effects might have relevance for the development of myocardial injury after acute MI.[7] However, whether tocilizumab affects these cells in patients with STEMI is still not known. In this predefined exploratory sub-study of the ASSAIL-MI trial we examined the effect of tocilizumab on a broad spectrum of leukocyte subpopulations.

Methods

Ethics

The trial protocol was approved by the regional ethics committee (REK South-East 2016/1223) and all participants provided written informed consent. An independent Data and Safety Monitoring Board oversaw the safety of the trial. The trial was approved by The Norwegian Medicines Agency and was conducted in compliance with the Declaration of Helsinki and the rules outlined in the guidelines for Good Clinical Practice.

Patients and study design

In the phase 2 ASSAIL-MI trial (Clinicaltrials.gov: NCT03004703), we investigated the hypothesis that a single dose of intravenous tocilizumab would be superior to placebo in improving myocardial salvage in patients admitted with acute STEMI. The study design and participants have been described previously and the groups were found to be well balanced after inclusion.[6,8] The trial was conducted at three high-volume PCI centres in Norway (Oslo University Hospital Rikshospitalet, Oslo University Hospital Ullevål, and St. Olav's Hospital, Trondheim). Briefly, 200 patients were randomised in the ASSAIL-MI trial and stratified according to the three PCI centres. One patient withdrew consent, leaving 199 patients for analyses in this investigation. The trial was double-blinded, placebo-controlled, and the patients were allocated in a 1:1 fashion in the period from March 2017 until February 2020. The key inclusion criteria were STEMI and symptom onset less than 6 h before PCI. Patients with previous MI; chronic infection, or chronic autoimmune or inflammatory disease; uncontrolled inflammatory bowel disease; ongoing infectious or immunologic disease; major surgery within the past eight weeks; or treatment with immunosuppressants other than low-dose steroids (equivalent to a systemic exposure to 5 mg prednisone per day) were excluded. The full inclusion and exclusion criteria, as well as the study design, have been published elsewhere.[8] Baseline characteristics of the study population are described in Table 1.

The primary endpoint of the ASSAIL-MI trial was the myocardial salvage index (MSI; %). MSI is defined as: [(area at risk- infarct size): area at risk] x 100, assessed by magnetic resonance imaging (MRI) 3–7 days after intervention.

For this predefined explorative sub-study, blood samples were also obtained from 20 patients admitted to Oslo University Hospital Rikshospitalet for elective coronary angiography due to stable angina pectoris. Except for previous MI, these patients had the same exclusion criteria as in the main study population. These patients were recruited in the autumn of 2020 providing written consent. Baseline characteristics for these patients are described in Supplemental Table S3.

Blood sampling protocol

The trial participants received double antiplatelet therapy and unfractionated heparin (5000–7500 IE) intravenous before PCI. Most of the patients (76%) had also received unfractionated heparin (5000 IE) in the ambulance before arrival at the hospital. Arterial blood samples were collected at admission, just before PCI, before intra-arterial unfractionated heparin and intravenous study medication were administrated at the catheterisation laboratory. Thereafter, venous blood samples were collected at 14–33 h (24 h), at 3–7 days, at 3 months, and at 6 months. For RNA isolation of whole blood, BD PAXgene™ Blood RNA tubes (BD, Franklin Lakes, NJ) were collected at admission before PCI,at 3–7 days and at 6 months. These tubes were used for RNA isolation in whole blood. Leukocytes and differential counts were analysed on Sysmex XN-10 (Sysmex, Kobe, Japan) per routine. The neutrophil-lymphocyte ratio (NLR), an independent risk factor for mortality after cardiac events, was calculated by dividing the absolute neutrophil count by the absolute lymphocyte count. High-sensitivity TnT was measured by electrochemiluminescence immunoassay (Elecsys 2010 analyzer, Roche Diagnostics, Basel Switzerland). In a subgroup of patients, EDTA-blood was sampled for flow cytometry at hospital admission and at three time-points post-treatment; 14–33 h, at 3–7 days and at 6 months. Venous blood samples from patients with stable angina pectoris were collected on the day before PCI. These patients had received platelet inhibitors but not heparin.

Isolation of T cells

Peripheral blood mononuclear cells (PBMCs) were obtained from sodium-heparin-anticoagulated blood by Isopaque-Ficoll (Lymphoprep; Axis-Shield, Oslo, Norway) gradient centrifugation. PBMCs were then

	Tocilizumab (*n* = 101)	Placebo (*n* = 98)
Demographics		
Age, years	62 ± 10	60 ± 9
Men	80 (79)	87 (89)
Body mass index, kg/m^2	27.1 ± 4.5	27.5 ± 4.3
Caucasian	99 (98)	94 (96)
Smoking status		
Never smokers	38 (38)	36 (37)
Previous smokers	33 (33)	24 (24)
Current smokers	30 (30)	38 (39)
Prior conditions		
Other vascular disease	6 (6)	6 (6)
Aortic disease	0	2
Angina pectoris	1	1
Cerebrovascular disease	4	2
Peripheral vascular disease	1	1
Diabetes mellitus	8 (8)	6 (6)
Hypertension	33 (33)	30 (31)
Treatment		
ACE inhibitor or ARB	22 (22)	25 (26)
Aldosterone antagonist	1 (1)	0 (0)
Oral anticoagulants	5 (5)	2 (2)
Platelet inhibitor	12 (12)	5 (5)
Beta-blocker	8 (8)	3 (3)
Calcium antagonist	13 (13)	10 (10)
Diuretic	8 (8)	8 (8)
Statin	19 (19)	9 (9)
Up-front DAPT	101 (100)	98 (100)
Time from symptom onset to arrival at PCI centre, min	151 ± 71	149 ± 72
Door-to-balloon time, min	23 ± 10	23 ± 11
Laboratory values		
Haemoglobin, g/l	143 ± 13	144 ± 12
Platelet count, 10^9/l	253 ± 59	260 ± 62
Total white blood cell count, 10^9/l	11.6 ± 3.4	11.6 ± 3.4
Aspartate transaminase, U/l	28 (22–37)	30 (24–37)
Troponin T, ng/l	44 (22–163)	49 (28–95)
CK-MB, μg/l	5.0 (2.6–14.0)	5.3 (3.0–10.0)
NT-proBNP, ng/l	79 (50–178)	63 (50–146)
Creatinine, mmol/l	74 ± 17	78 ± 20
Glucose, mmol/l	9 ± 3	9 ± 3
HbA1c, mmol/mol	37 (34–41)	37 (34–40)
Total cholesterol, mmol/l	5.3 ± 1.2	5.2 ± 1.0
HDL cholesterol, mmol/l	1.2 (0.9–1.3)	1.1 (0.9–1.3)
LDL cholesterol, mmol/l	3.7 ± 1.1	3.7 ± 0.9
C-reactive protein, mg/l	2.4 (0.9–5.0)	2.9 (1.4–5.0)
Albumin, g/l	42 ± 3	42 ± 3

***Table 1*: Baseline characteristics for the STEMI population before treatment and study drug administration.**
Values are mean ± SD, *n* (%), or median (interquartile range). Note, all laboratory values including total white blood cell counts and CRP reflect values before the administration of tocilizumab.
ACE = angiotensin-converting enzyme; ARB = angiotensin receptor blocker; CK-MB = creatine kinase myocardial band; DAPT = dual antiplatelet therapy; HDL = high-density lipoprotein; LDL = low-density lipoprotein; PCI = percutaneous coronary intervention; NT-proBNP = N-terminal pro-B-type natriuretic peptide.

resuspended in autoMACS® rinsing buffer (Miltenyi Biotec, Bergisch Gladbach, Germany) for negative selection of pan-T cells with Pan-T cell Isolation Kit (Miltenyi Biotec) following manufacturer's instructions. Pelleted T cells were stored at -80 °C before RNA isolation.

RNA isolation and RNA-sequencing

Total RNA was isolated from BD PAXgene™ Blood RNA tubes using MagMAX™ for Stabilized Blood Tubes RNA Isolation Kit (Invitrogen™, Waltham, MA) following the manufacturer's instructions. Total RNA from T cells was isolated under RNase-free conditions using Allprep DNA/RNA/Protein Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The isolated RNA samples were sent to Novogene (UK) Company Limited. rRNA depletion library preparation was used for the RNA isolated from whole blood, while mRNA enrichment method was used for T cell RNA. The fastp (v0.23.0) was used to remove contaminated adapters and low-quality reads with phred score below 30 in the pair-end mode.[9] Filtered reads were mapped to the human transcriptome (Gencode Human Release H37), and transcripts were quantified with 200 bootstrap iterations by Salmon (v1.5.2).[10,11] The Salmon outputs were summarised to gene-level and imported into DESeq2 (v1.34.0) via tximeta (v.1.12.3).[12,13] For better accuracy, haemoglobin mRNAs were removed from the neutrophils before the analysis of differentially expressed genes (DEGs).[14] Raw counts were uploaded to the Reactome Pathway Knowledgebase[15] for pathway analyses. For neutrophil deconvolution and gene expression imputation, the CIBERSORTx high-resolution mode and its build-in LM22 reference matrix were used.[16] Differentially regulated imputed genes with more than 50 counts and a *p*-value < 0.01 were uploaded to Metascape[17] for gene annotation analyses. Cytoscape[18] was used for network plot visualization. For gene set enrichment analysis (GSEA), all imputed gene were imported into GSEA software (version 4.2.2) and analysed against the gene sets "Neutrophil degranulation" Reactome pathway and "MAPK cascade" GO term, respectively (MSigDB version 7.5.1).[19,20]

Flow cytometry

In a subgroup of 69 patients treated with tocilizumab (*n* = 37) or placebo (*n* = 32) recruited at Oslo University Hospital Rikshospitalet, we performed an extended flow cytometry analysis of lymphocyte subpopulations at the Department of Immunology. Patient characteristics are provided in Supplemental Table S3 showing no significant differences in baseline characteristics between the two treatment arms. Routine analyses of absolute counts for B-, T-, and NK-cells were analysed in Trucount tubes (BD) on a FacsCanot II instrument and

analysed in BD FACSCanto™ Clinical Software according to the manufacturer's instructions. Instrument settings were standardised as recommended and daily quality run with CS&T-Beads (BD) and 7-color Setup Beads (BD) ensuring high reproducibility. The laboratory follows standard operation procedure and also have ISO (International Standard Organization) certification. Further sub-classification of B- and T-cells was performed on a Gallios Flow cytometer (Beckman Coulter, San Diego, CA). For B-cell analysis, the blood samples were washed twice before incubation with antibodies. T-cell analysis was performed in unwashed blood samples. Briefly, EDTA-blood was incubated with optimally titrated antibodies for 15 min at room temperature, followed by erythrocyte lysis (BD FACSLysing Solution, Beckman Dickinson, CA). Data acquisition was performed using Kaluza Software (Beckman Coulter). For T-cells, 1×10^5 cells was acquired; for B-cells, 1×10^6 cells if possible. The antibodies that were used and their RRID tags are provided in Supplemental Table 1. B-cell were gated as $CD19^+$ and further sub-classified as naive (IgD^+, IgM^+, $CD27^-$), IgM memory ($CD27^+$, IgD^+, IgM^+), class switched ($CD27^+$, IgM^-, IgD^-), plasmablasts ($CD19^+$ dim, $CD27^{++}$, $CD38^{++}$), transitional (IgM^+ $^+$, $CD38^{++}$, $CD24^+$) and CD21 low B cells (CD38 low, CD21 low). T-cells were gated as $CD3^+$ and further as naive $CD4^+$ ($CD4^+$, $CD45RA^+$), recent thymic emigrants ($CD4^+$, $CD45RA^+$, $CD31^+$), $CD4^+$ memory ($CD4^+$, $CD45RO^+$), follicular like $CD4^+$ ($CD4^+$, $CD45RO^+$, $CCR5^+$), regulatory T-cells ($CD4^+$, $CD25^{++}$, $CD127^-$), naive $CD8^+$ ($CD8^+$, $CD27^+$, $CD28^+$), $CD8^+$ early effector memory ($CD8^+$, $CD27^+$, $CD28^-$), $CD8^+$ late effector memory ($CD8^+$, $CD27^-$, $CD28^-$). Gating strategy is provided in Supplemental Figure 1. Reference values are 5–95 percentile for 65 normal controls (blood donors).

Statistics

Continuous data are presented as mean (standard deviation or standard error of the mean) or median (interquartile range) if distributions were skewed. Categorical data are reported as numbers and percentages. One-way ANOVA with Dunnett's multiple comparison test was performed to investigate significant differences between the stable angina pectoris group compared with the STEMI-group. To investigate differences between the intervention groups for counts or imputed gene expression, or between patients admitted ≤ 3 or > 3 h after the symptom onset, we either used mixed effect analysis with Bonferroni's multiple comparison test or unpaired two-tailed t tests. Correlations were calculated using Spearman's correlation coefficient. The percent change from admission to 24 h, that takes into account the differences in admission levels, was calculated as: [absolute neutrophil count (24 h) − absolute neutrophil count (admission)]: absolute neutrophil count (admission). *P*-values less than 0.05 were considered statistically significant. For RNAseq we performed FDR adjustment and report adjusted *p*-values. Patient number (given as *n*) might vary over time for both counts and RNA analyses due to missing samples or quality issues. The amount of missing data was evenly distributed between the treatment groups and the missing values are assumed to be missing at random. Statistical analyses were performed in SPSS version 25 (IBM Corp., Armonk, New York) and in GraphPad Prism 8.3.0 (GraphPad Software, La Jolla, CA).

Restrictions to availability of material and data

Ethical restrictions from the Regional Committee for Medical and Research Ethics in South–East Norway prohibits data from individual patients to be made available on a publicly available repository. However, an institutional data transfer agreement can be established and data can be shared if the aims of data use are covered by ethical approval and patient consent. The procedure will involve an update to the ethical approval as well as a review by legal departments at both institutions, with the process typically taking 2 to 4 months from the first contact.

Role of funders

The funders of the study had no role in the study design, data collection, analysis, interpretation, or writing of the report.

Results

Neutrophil and neutrophil-lymphocyte ratio after STEMI: downregulatory effects of tocilizumab

Patients in the placebo arm of the ASSAIL-MI trial had higher neutrophil counts at admission and after 24 h than patients with stable angina pectoris ($p < 0.0001$ and $p < 0.0001$, respectively) (Figure 1a, showing the placebo arm only). Following hospital admission and PCI, there was a gradual decline in the level of circulating neutrophils. However, while there was no difference in neutrophils between the two treatment arms at admission, the decrease in the number of neutrophils during hospitalisation was more pronounced in the tocilizumab arm ($p < 0.0001$ at 24 h, $p < 0.0001$ at 3–7 days) (Figure 1b). The STEMI patients also had higher neutrophil-lymphocyte ratios (NLR) at admission ($p < 0.0001$) and after 24 h ($p = 0.0068$) than patients with stable angina pectoris (Figure 1c, showing the placebo arm only). Tocilizumab significantly reduced the NLR at 24 h ($p < 0.0001$) and day 3-7 ($p < 0.0001$) after admission compared with placebo (Figure 1d). The decrease in NLR was driven mainly by the decrease of neutrophils. Meanwhile, the lymphocyte counts did not change significantly over time or differ between the intervention groups (Supplemental Figure S2). The

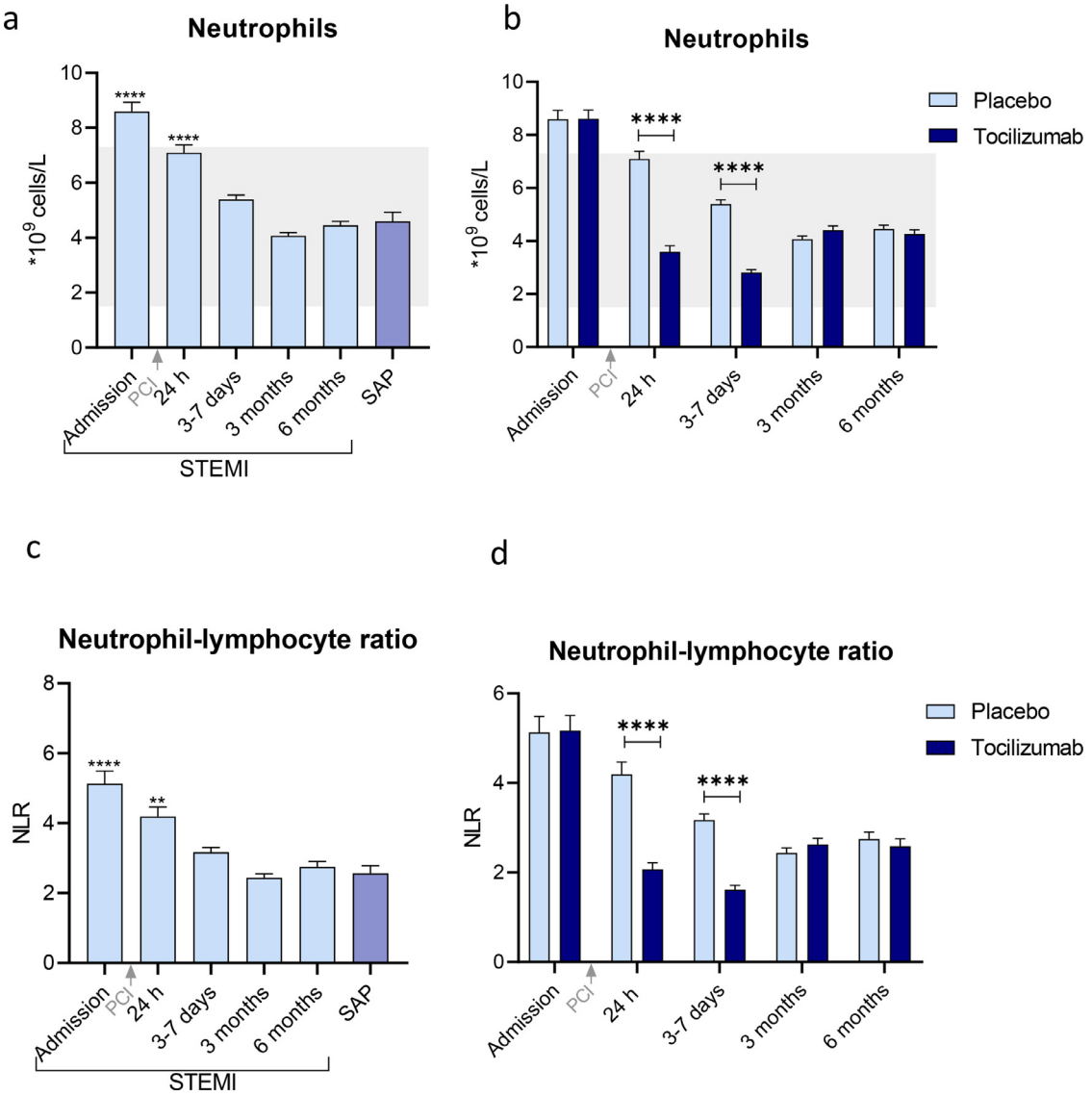

Figure 1. Downregulatory effects by tocilizumab treatment on neutrophils and neutrophil-lymphocyte ratio (NLR) following STEMI. *Note, Panel A and C show only the placebo group to compare the pattern in STEMI patients with levels in stable angina pectoris.* Panel a shows the level of circulating neutrophils in STEMI patients treated with PCI (*only placebo*) over time compared with patients with stable angina pectoris (SAP) (n = 20) as mean with SEM. Grey-shaded area shows normal range. ****$p < 0.0001$ versus SAP (One-way ANOVA with Dunnett's multiple comparisons test). Panel b shows a comparison of the effect of tocilizumab treatment at all time-points between the neutrophil counts in the tocilizumab arm and in the placebo arm as mean with SEM. ****$p < 0.0001$ comparing the two treatment groups (Mixed effect analyses with Bonferroni's multiple comparisons test). Panel c shows NLR in STEMI patients treated with PCI (*only placebo*) over time compared with patients with stable angina pectoris (SAP) (n = 20) as mean with SEM. ****$p < 0.0001$ and **$p < 0.01$ versus SAP (Dunnett's multiple comparisons test). Panel d shows a comparison of the effect of tocilizumab treatment at all time-points between the tocilizumab arm and the placebo arm as mean with SEM. ****$p < 0.0001$ comparing the two treatment groups (Mixed effect analyses with Bonferroni's multiple comparisons test). Data are given as mean and SEM. Hospital admission was within 6 h after symptom onset. Placebo (n = 98): at hospitalisation (n = 93), 24 h (n = 98), 3-7 days (n = 96), 3 months (n = 94) and 6 months (n = 96). Tocilizumab (n = 101): at hospitalisation (n = 98), 24 h (n = 99), 3-7 days (n = 100), 3 months (n = 99) and 6 months (n = 99).

effect of tocilizumab on the MSI was more pronounced in patients undergoing PCI more than 3 h after symptom onset. The decrease in neutrophil counts and NLR in the tocilizumab group was, however, not dependent on the time since symptom onset (Supplemental Figure S3).

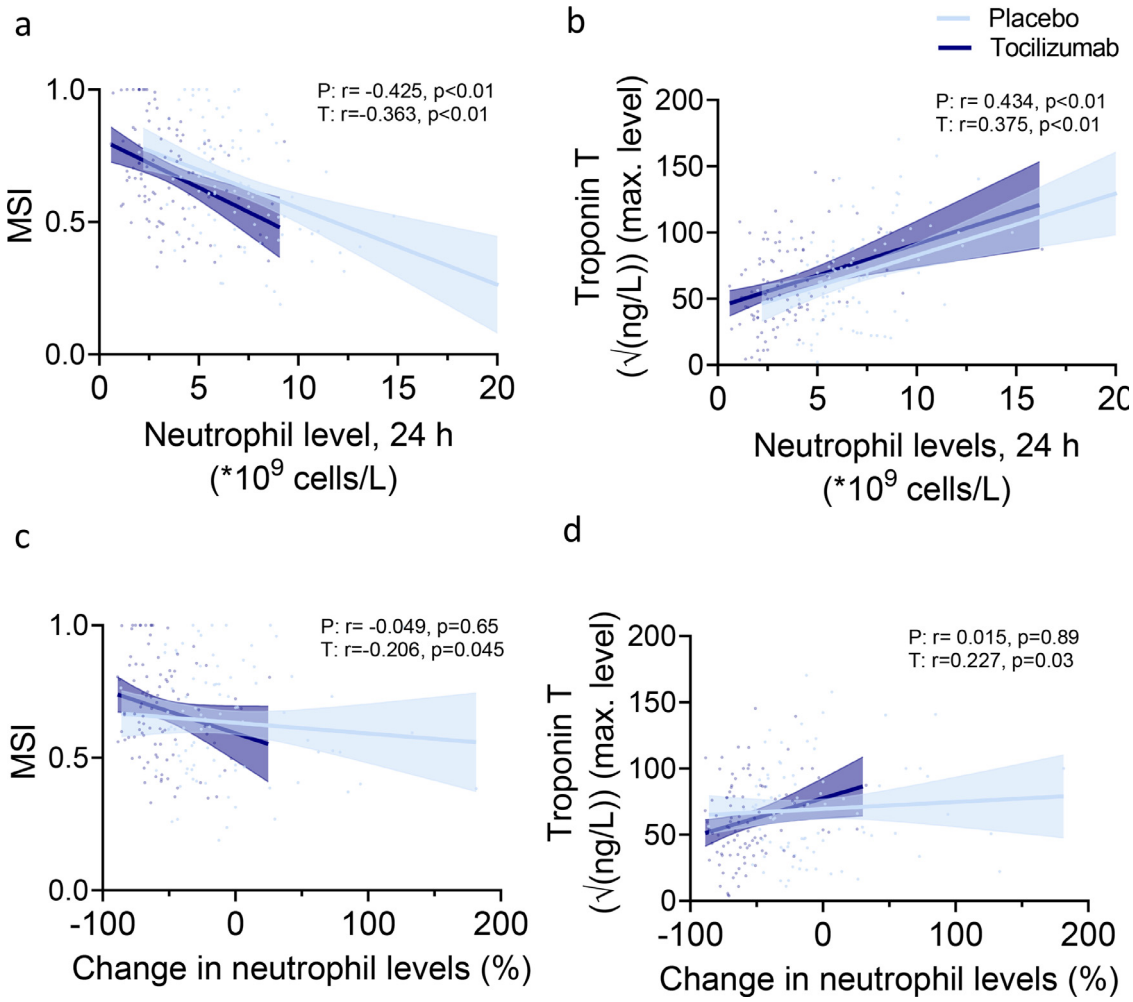

Figure 2. Correlation between neutrophil counts *and* myocardial salvage index (MSI) and maximum troponin T (TnT) levels in STEMI patients. Panel a and b shows correlation between absolute neutrophil counts at 24 h *and* MSI (a) and maximum TnT levels (b) in the tocilizumab (T) and the placebo (P) group. Panel c and d show correlation between percent neutrophil change from admission to 24 h *and* MSI (c) and maximum TnT levels (d) in the tocilizumab (T) and the placebo (P) group. Trend line is indicated for easier visualization. Correlations were calculated using Spearman's correlation coefficient (r). Placebo (n = 98): at hospitalisation (n = 93), 24 h (n = 98). Tocilizumab (n = 101): at hospitalisation (n = 98), 24 h (n = 99).

The decrease in neutrophils after tocilizumab treatment: potential effects on MSI and TnT release

The absolute neutrophil counts at 24 h correlated inversely with MSI (placebo: r = -0.425, p < 0.01; tocilizumab: r = -0.363, p < 0.01) and positively with maximum TnT levels (placebo: r = 0.434, p < 0.01; tocilizumab: r = 0.375, p < 0.01). This relationship was independent of treatment allocation (Figure 2a, b). When examining the percentage changes in neutrophil counts after 24 h, we found that the marked and rapid decrease in neutrophil counts in the tocilizumab group (mean 56% decline) was significantly associated with increased MSI (r = -0.206, p = 0.045) and decreased peak TnT (r = 0.227, p = 0.03) (Figure 2c, d). However, such correlations were not seen in the placebo-treated patients, potentially reflecting that the percentage decrease in this group was modest (MSI: r = -0.049, p = 0.65; TnT: r = 0.015, p = 0.89) (mean 8% decline). Thus, it seems that the decrease in neutrophils in the tocilizumab arm compared with the placebo arm may be associated with a beneficial effect on MSI and TnT release.

Gene expression in whole blood: attenuated innate immunity response after treatment with tocilizumab

We extracted and sequenced RNA from whole blood drawn from 20 patients who were allocated to placebo in the ASSAIL-MI trial and from 20 patients allocated to tocilizumab. Patients were selected to obtain equal distribution of age and gender as well as levels of HbA1C, LDL and HDL cholesterol, and triglycerides between the

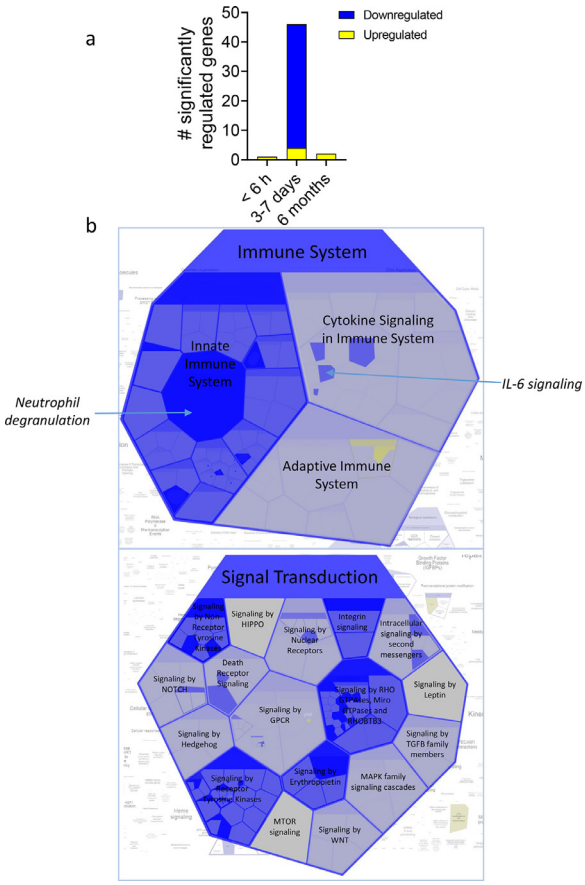

Figure 3. Comparison of gene expression in whole blood after 3-7 days between placebo and tocilizumab treated STEMI patients. Panel a shows the number of significantly differentially regulated genes (adjusted *p*-value < 0.05) at hospitalisation (placebo [p]: *n* = 18, tocilizumab [t]: *n* = 19), 3-7 days after hospitalisation (p: *n* = 19, t: *n* = 20) and after 6 months (p: *n* = 18, t: *n* = 20) between the two treatment arms. Panel b shows the pattern of differential regulation between placebo and tocilizumab treated patients of the two Reactome pathways "Immune system" (R-HSA-168256) and "Signal transduction" (R-HSA-162582), when including all genes with a *p*-value < 0.01. Dark blue is significantly downregulated in the tocilizumab-treated compared with the placebo-treated patients, while bright yellow is significantly upregulated in the same groups.

treatment groups. Few differences were observed at hospitalisation and after 6 months between the treatment groups. However, after 3-7 days, a total of 46 genes were significantly differentially expressed between treatment groups. Importantly, 42 of these genes were expressed at lower levels in the tocilizumab arm than in the placebo arm (Figure 3a). Reactome analysis of all genes revealed an attenuated expression of the pathway "Immune system" (R-HSA-168256). It is worth noticing that the "Innate immune system" (R-HSA-168249) pathway and the underlying "Neutrophil degranulation" (R-HSA-6798695) pathway were downregulated in the tocilizumab arm compared with the placebo arm (Figure 3b). In addition, the "Signal transduction" (R-HSA-162582) pathway along with several relevant sub-pathways (e.g., "Signalling by Receptor Tyrosine Kinases" (R-HSA-9006934) and "RHO GTPase cycle" (R-HSA-9012999)) were reduced in patients treated with tocilizumab compared with patients allocated to placebo (Figure 3B). The "IL-6 signalling" pathway was also downregulated in the tocilizumab arm, in accordance with tocilizumab treatment.

Downregulation of genes related to neutrophil function and signal transduction after tocilizumab

To estimate neutrophil gene expression, we used CIBERSORTx for deconvolution of whole blood RNA-sequencing data and to impute the gene expression profile of neutrophils. The enrichment analysis of significantly differentially expressed genes revealed that "Neutrophil degranulation" (R-HSA-6798695) was the most significantly regulated pathway between the treatment groups 3-7 days after hospitalisation (Figure 4a). "MAPK cascade" (GO:0000165), "signalling by Receptor Tyrosine Kinases" (R-HSA-9006934), "RHO GTPase cycle" (R-HSA-9012999), and the "immune response-regulating signalling pathway" (GO:0002764) were also regulated differently between the treatment groups. All these pathways are important for signal transduction in immune cells. In addition, gene ontology (GO) terms related to neutrophil function and cell development, like "positive regulation of cell migration" (GO:0030335), "organelle localization" (GO:0051640) and "regulation of cell development" (GO:0060284) were significantly altered (Figure 4A). We plotted the fold change of genes associated with these pathways. Most of them were downregulated, suggesting a dampened function of these GO terms (Figure 4b, c). To determine the association between the patient groups and genes involved in the two most pronounced GO terms "Neutrophil degranulation" and Reactome pathways "MAPK cascade" we ran a gene set enrichment analysis (GSEA). The results from this clearly showed that the genes in these two terms are most highly expressed in the placebo group, indicating a downregulation in the tocilizumab arm (Figure 4d, e).

After performing corrections for multiple testing, 86 genes were significantly altered (adjusted *p*-value < 0.05). Of these, 4 were higher and 82 were lower in the tocilizumab arm compared with the placebo arm (Supplemental Table S2). Several of these genes may be of importance for cell migration, exosome function and inflammation (Supplemental Table S2).

Minor changes in lymphocyte subpopulation composition following STEMI: no effect of tocilizumab

In a subgroup of 69 patients from the ASSAIL-MI trial and in 20 patients with stable angina, we performed a

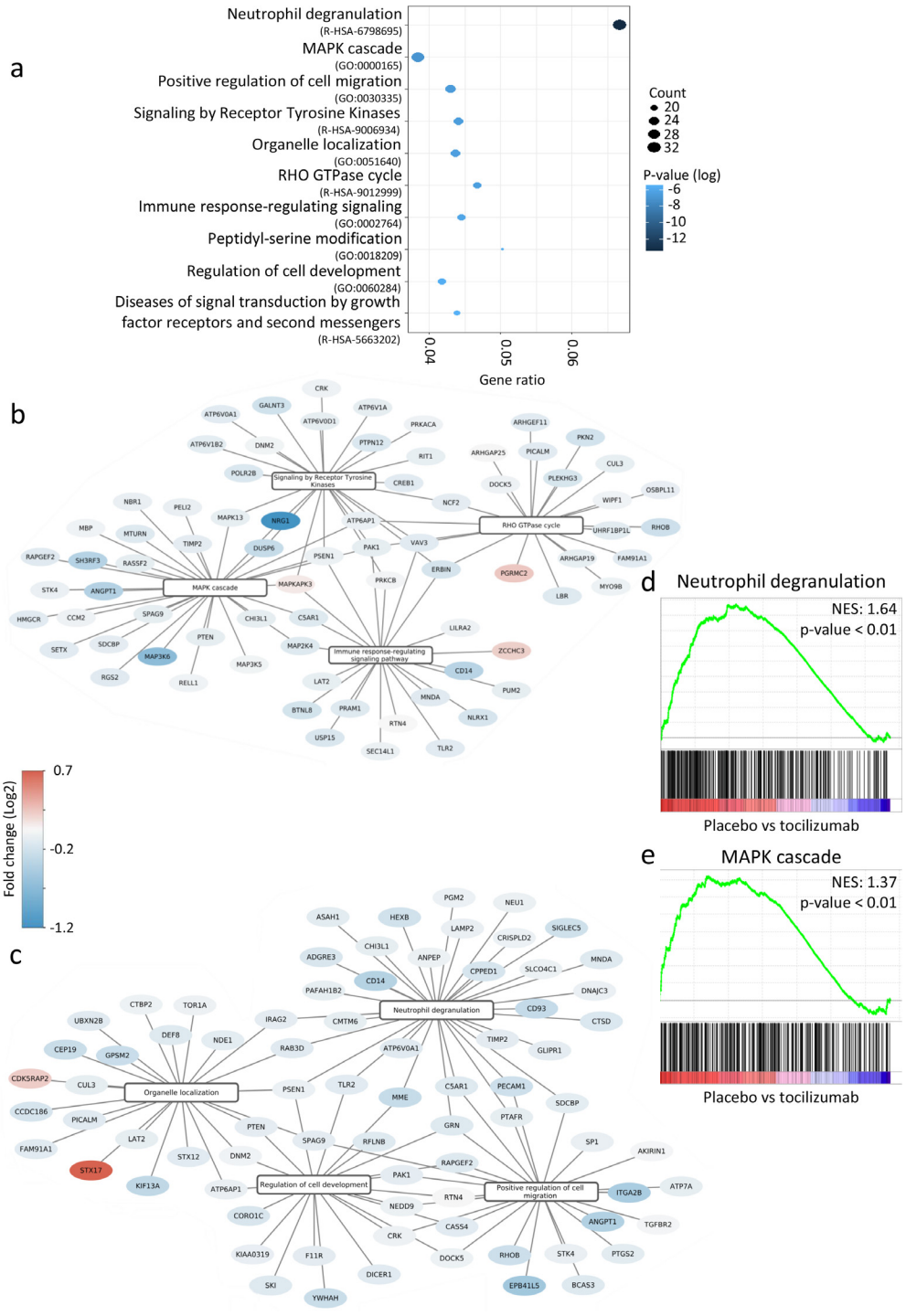

Figure 4. Gene expression imputation in neutrophils derived from whole blood analyses. Panel a shows the 10 most enriched pathways (Reactome) and Gene Ontology (GO) terms from analysis with the gene expression level of the 423 genes belonging to neutrophils that had more than 50 average counts and a p-value < 0.01 (unpaired t test) after the deconvolution analysis performed on RNA-sequencing results from whole blood by CIBERSORTx. Panel b shows a network plot for the four most relevant Reactome pathways and GO terms for signal transduction, and Panel c shows pathways relevant for neutrophil function and cell development. Blue shows lower gene level (log2 fold change), while red shows higher gene level in the tocilizumab group compared with placebo treated patients. Panel d shows GSEA analysis for genes related to the Reactome pathway "Neutrophil degranulation", and Panel e shows the GSEA analysis for the GO term "MAPK cascade".

more thorough analysis of lymphocyte populations by flow cytometry. Patient characteristics are presented in Supplemental Table S3. The level of NK cells was higher immediately after hospital admission than at all later assessments, but the level only slightly exceeded the normal range (grey area), and the difference did not reach statistical significance (p = 0.079) (Figure 5a). There were only minor and mostly non-significant changes in the other lymphocyte subpopulations ($CD19^+$ B-cells, $CD4^+$ T-cells and $CD8^+$ T-cells) following STEMI (Figure 5B–D). B and T cell subpopulations barely differed from normal values at time of hospitalisation and changed little during follow-up after STEMI. In the patients with STEMI, plasmablasts peaked at 3-7 days (p = 0.04) compared with stable angina patients (Supplemental Table S4). Importantly, for all these subpopulation analyses there were no differences between the tocilizumab arm and the placebo arm (Supplemental Table S4). A RNA-sequencing on isolated T cells revealed few differences between the treatment groups when looking at differentially expressed genes (adjusted p-value < 0.05, average gene expression > 50), with 4 regulated genes at time of hospitalisation, 6 at 24 h, none after 3-7 days and 1 after 6 months (Figure 5e). The fold change of the regulated genes was in general modest and the net effects of these changes are uncertain (Supplemental Table S5).

The effect of tocilizumab on $CD8^+$ T cells is dependent on the time from symptom onset

We found significantly lower levels of $CD4^+$ T cells (p = 0.02) and $CD8^+$ T cells (p = 0.02), but no NK cells (p = 0.2) or B cells (p = 0.06), in patients admitted > 3 h after symptom onset than in patients admitted ≤ 3 h after symptom onset (Figure 6A). Notably, in the tocilizumab arm, the levels of $CD8^+$ T cells, but not the $CD4^+$ T cells (Supplemental Table S6), remained low in the patients admitted to the hospital > 3 h after symptom onset compared with patients receiving tocilizumab ≤ 3 h after symptom onset. The placebo-treated patients arriving > 3 h after symptom onset had an increase in $CD8^+$ T cells after hospital admission (p = 0.02), not found in the tocilizumab group (Figure 6B). Moreover, tocilizumab significantly reduced the absolute number of late effector/memory $CD8^+$ T cells (p = 0.01 at 24 h), but not the numbers of naive $CD8^+$ T cells (p = 0.2 at 24 h) and early effector/memory $CD8^+$ T cells (p = 0.08 at 24 h) in patients arriving to the hospital > 3 h after symptom onset (Supplemental Figure S4). In the patient group as a whole, we found an inverse correlation between $CD8^+$ T cell counts and MSI in those hospitalezed later (> 3 h after symptom onset) (24 h: r = -0.6, p = 0.002) (Table 2). This correlation was only seen in the placebo group (24 h: r = -0.64, p = 0.02) and not in the tocilizumab group (24 h: r = -0.49, p = 0.13) (Table 2), either for TnT (24 h, whole population: r = 0.366, p = 0.07) (Supplemental Table S7).

Discussion

We have previously shown that tocilizumab improved myocardial salvage in STEMI, and that tocilizumab reduced TnT levels in NSTEMI.[5,6] In both studies, we observed lower neutrophil counts during hospitalisation in the tocilizumab group than in the placebo group. In this exploratory and predefined sub-study, we present novel data on associations between neutrophils and MSI and TnT in STEMI patients. Our results may suggest that the beneficial effects of tocilizumab in these patients could at least partly be mediated through an attenuation of neutrophil functions. Our data show that tocilizumab not only reduces the number of circulating neutrophils but also mitigates the inflammatory potential of the remaining circulating neutrophils. However, these associations do not prove any causal relationship and it could be claimed that the decrease in neutrophils in the tocilizumab group could be caused by a lower degree of myocardial damage and not *vice versa*.

Acute STEMI and the subsequent ischaemia/reperfusion (I/R) injury promotes an inflammatory cascade that contributes to the replacement of damaged tissue and tissue repair. However, this inflammatory reaction also has detrimental effects if, in particular, the inflammatory responses are too strong or persist for too long. Experimental and clinical studies have suggested that neutrophils contribute to tissue damage, including cardiomyocyte apoptosis, following MI.[21] During I/R, neutrophils are major contributors to myocardial damage, at least partly through oxidative stress.[21] Moreover, clinical studies have shown that high neutrophil counts predict both acute and chronic cardiovascular disease (CVD), and a high neutrophil count is proposed as a clinical predictor of poor outcomes after coronary events.[22] Interestingly, an elevated NLR, reflecting the balance between the innate and the adaptive immune responses, has been associated not only with the presence of CVD but also with short-term adverse outcomes, including mortality, in patients with CVD.[23] In accordance with previous reports,[24,25] we found higher levels of neutrophils and NLR in STEMI patients at admission compared with patients with stable angina. However, herein we showed that there was a significant decrease in neutrophil counts and NLR, mainly driven by a reduction in neutrophil counts, during hospitalisation in the tocilizumab arm compared with placebo. A reduction in neutrophils has been suggested to be a side effect that predisposes patients treated with IL-6 inhibitors to infectious complications.[26] However, we found that a high number of neutrophils in STEMI patients 24 h after hospital admission correlated inversely with MSI and positively with maximum levels of TnT. Moreover, we found that the rapid decrease in neutrophils

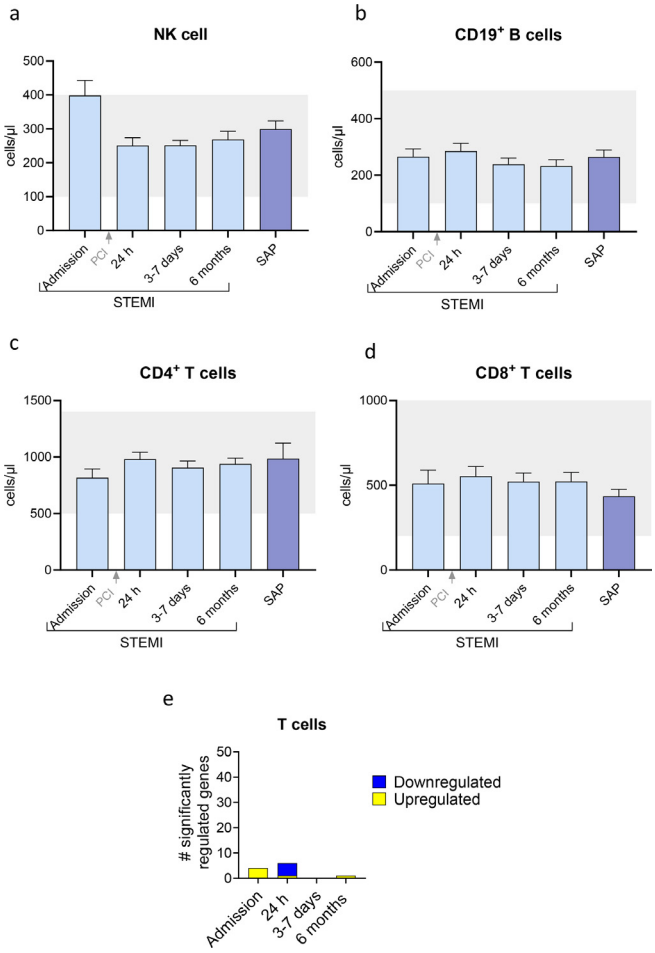

Figure 5. B cells, T cells and NK cells following STEMI. Levels of (a) NK cells, (b) B cells (CD19), (c) $CD4^+$ T cells and (d) $CD8^+$ T cells in STEMI patients (*only placebo*) at hospitalisation ($n = 30$), 24 h ($n = 31$), 3-7 days ($n = 29$) and 6 months ($n = 30$) compared with stabile angina pectoris (SAP) patients ($n = 20$). Data are presented as mean with SEM. Grey-shaded area: normal range. *$p < 0.05$ versus SAP (One-way ANOVA with Dunnett's multiple comparisons test). Data are given as mean and SEM. Hospital admission was within 6 h after symptom onset. Panel e shows significantly DEGs (adjusted p-value < 0.05, average gene expression > 50) in T cells between the treatment groups at hospitalisation (placebo [p]: $n = 10$, tocilizumab [t]: $n = 6$), 24 h after hospitalisation (p: $n = 8$, t: $n = 8$), after 3-7 days (p: $n = 8$, t: $n = 8$) and after 6 months (p: $n = 10$, t: $n = 9$).

from admission to 24 h in the tocilizumab treated patients was associated with increased MSI and decreased peak TnT. These data suggest that the beneficial effect of tocilizumab in STEMI may involve an attenuated neutrophil level.

Our data suggests that a sustained high number of neutrophils may be harmful during STEMI and may contribute to myocardial damage. However, we do not know whether the reduced neutrophil counts in the tocilizumab group are due to increased migration to the infarct site, increased neutrophil apoptosis or, more likely, due to a reduced influx to the circulation from the bone marrow or spleen, or due to a combination thereof. IL-6 stimulation is suggested to mobilize neutrophils from the bone marrow into the circulation, possibly through the CX_3CR1 receptor (fractalkine receptor).[27,28] It could be hypothesized that the reduced neutrophil level in the tocilizumab treated patients is due to a direct inhibition of the IL-6 receptor of the cells in the bone marrow. In the RNA-sequencing of whole blood 3–7 days after tocilizumab treatment, we found no difference in the CX_3CR1 receptor gene expression between the two treatment arms. Further data on this subject is needed. Notably, it has also been suggested that the decrease in lymphocytes during MI could reflect enhanced myocardial infiltration of these cells at least partly involving increased expressing of CX_3CR1 on these lymphocytes.[29,30] In addition, neutrophils have been found to change their phenotype over time to regulate not only tissue damage but also the resolution of the inflammation following MI.[31] To elucidate these important issues, forthcoming studies should clarify

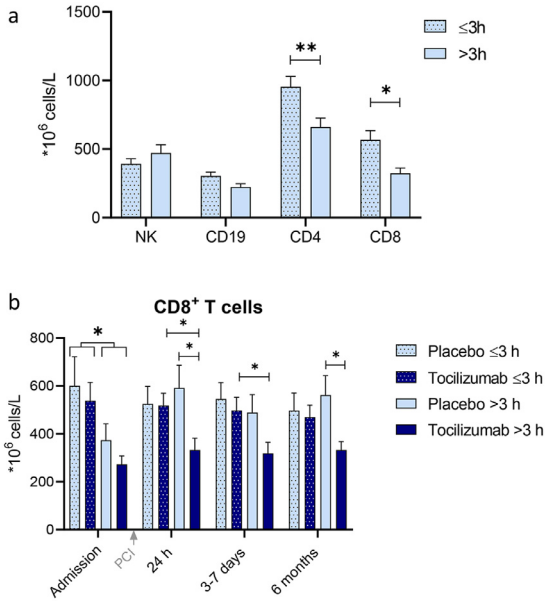

Figure 6. Effect of tocilizumab on numbers of NK cells, B (CD19) cells, $CD4^+$ T cells and $CD8^+$ T cells according to time since symptom onset. Panel a shows numbers of NK cells, B (CD19) cells, $CD4^+$ T cells, and $CD8^+$ T cells at admission (before drug administration and PCI) according to time since symptom onset ($\leq$ 3 h, n = 40 and $>$ 3 h n = 24) as mean with SEM. Panel b shows $CD8^+$ T cells counts for STEMI patients in the placebo and tocilizumab group based on time since symptom onset as mean with SEM. *$p < 0.05$ (Unpaired t test between relevant groups). Data are given as mean and SEM. Placebo $\leq$ 3 h (admission: n = 18, 24 h: n = 18, 3-7 days: n = 17, 6 months: n = 18); placebo $>$ 3 h (admission: n = 12, 24 h: n = 13, 3-7 days: n = 12, 6 months: n = 12); tocilizumab $\leq$ 3 h (admission: n = 22, 24 h: n = 21, 3-7 days: n = 25, 6 months: n = 23); tocilizumab $>$ 3 h (admission: n = 12, 24 h: n = 12, 3-7 days: n = 12, 6 months: n = 12). Hospital admission was within 6 h after symptom onset.

which neutrophil subtypes are decreased by tocilizumab.

Attenuation of neutrophil responses due to tocilizumab treatment was reflected at the RNA level. The innate immunity response was dampened and signal transduction was reduced 3–7 days after treatment with tocilizumab. Further, pathways related to neutrophil activity, like neutrophil degranulation and several of the signalling pathways, were downregulated in the tocilizumab arm compared with the placebo arm. The same tendency was also observed at the estimated gene expression level in neutrophils, where the downregulated "Neutrophil degranulation" pathway and the "MAPK cascade" GO term potentially promote a dampened inflammatory phenotype in the tocilizumab-treated patients. Genes associated with the RHO GTPase cycle, the MAPK cascade and signalling by Receptor Tyrosine Kinases were also downregulated. Signal transduction through these three pathways have roles in neutrophil recruitment, including polarization, trans-endothelial migration and chemotaxis, as well as neutrophil activation.[32–34] Thus, a downregulation of these signalling pathways might contribute to an attenuated neutrophil function. Moreover, one of the few upregulated genes by tocilizumab was *Syntaxin 17*. Syntaxin 17 is involved in the initiation of autophagy, a process of importance for maintaining cardiac homeostasis and a survival mechanism that is upregulated during myocardial stress.[35] Interestingly, a proteomic sub-study of the LoDoCo2 study, using low dose colchicine-treatment to reduce the risk of cardiovascular events after MI also revealed an attenuation of neutrophil degranulation-related proteins thought to be beneficial in reducing atherogenesis.[36,37] Finally, we have recently shown that tocilizumab may induce the formation of neutrophil extracellular traps (NETs) in NSTEMI.[38] NETS may have a harmful effect during plaque destabilization and their potential role in STEMI needs clarification.

In the present study, we analysed a wide range of lymphocyte subpopulations in the ASSAIL-MI study. However, except for a peak at 3-7 days for plasmablasts, these cell populations remained surprisingly stable and did not differ across treatment groups. Thus, whereas IL-6 is known to have pronounced effects on B cells,[39] we found no substantial differences in B cell subpopulations between the two treatment groups. It has previously been reported that an immediate reduction in T cells within the first 24 h after PCI is associated with poor outcomes for STEMI patients,[29,40] but we do not have data on the immediate T cell levels after PCI in our intervention trial. Moreover, in contrast to neutrophils, we observed no pronounced differences in gene expression of T cells in the tocilizumab arm compared with the placebo treated patients, indicating that tocilizumab treatment has few effects on both T cell subpopulations and T cell function. Cormark *et al.* showed that a single dose of ciclosporin induced a significant and incidental decrease in T cell counts.[41] Importantly, nevertheless, the mechanisms of action differ widely between ciclosporin and tocilizumab affecting mainly IL-2 and IL-6, respectively. In patients who were admitted $>$ 3 h after symptom onset, however, $CD8^+$ T cell counts did not increase during hospitalisation in the tocilizumab arm, whereas a pronounced increase in the placebo arm was observed. Inhibition of IL-6-mediated differentiation of $CD8^+$ T cells,[42] which affects the late effector/memory $CD8^+$ T cells in particular, could be operating in the tocilizumab arm in this subgroup of patients. In patients admitted to the hospital $>$ 3 h after symptom onset, there was a negative correlation between $CD8^+$ T cell counts and the MSI in the placebo group, suggesting harmful effects of $CD8^+$ T cells in this subgroup. However, although $CD8^+$ T cells have been suggested to contribute to myocardial damage following MI, their roles in this context are far from clear. Further studies

		Total population					
		≤ 3 h			> 3 h		
		r	***P* value**	***n***	***r***	***P* value**	***n***
Absolute count of circulating $CD8^+$ T cells	24 h	0.09	0.57	39	-0.60	0.002	24
	3-7 days	0.13	0.41	42	-0.60	0.002	23
		Placebo					
		≤ 3 h			> 3 h		
		r	*P* value	*n*	*r*	*P* value	*n*
Absolute count of circulating $CD8^+$ T cells	24 h	0.09	0.73	18	-0.64	0.02	13
	3-7 days	0.07	0.79	17	-0.76	0.004	12
		Tocilizumab					
		≤ 3 h			> 3 h		
		r	*P* value	*n*	*r*	*P* value	*n*
Absolute count of circulating $CD8^+$ T cells	24 h	0.15	0.51	21	-0.49	0.13	11
	3-7 days	0.17	0.41	25	-0.39	0.24	11

***Table 2*: Correlations between $CD8^+$ T cell counts and MSI according to time of hospitalisation after symptom onset.**
Significant values are marked in bold. *r* is the Spearman's correlation coefficient.

are needed to clarify if the effect of tocilizumab on $CD8^+$ T cells is beneficial in STEMI.

Our study has some limitations. Associations do not necessarily imply causal relationships, and association between the circulating numbers of neutrophils and the myocardial salvage does not prove that a reduced number of neutrophils *causes* improved salvage. Further, due to the strict inclusion criteria, the study participants are presumably not representative of the whole STEMI population. However, this was a proof-of-concept trial where we knowingly "purified" the population, and any between-group difference must be regarded as a treatment effect due to the randomization. Forthcoming studies should evaluate tocilizumab treatment in an unselected study population. Moreover, the number of patients who underwent extended flow cytometry was limited and the data derived from these analyses should be interpreted with caution. This is particularly important in the interpretation of the analyses looking at time between symptom onset and hospitalisation, where the groups are small. The cells we investigated were from peripheral blood. Our findings may therefore not necessarily reflect the processes that occur within the infarcted myocardium and we do not know if the reduced number of circulating neutrophils in the tocilizumab arm might reflect less mobilization from the bone marrow or a less likely migration to the myocardium. Single-cell mRNA-sequencing, as well as proteomic analyses and measurements of neutrophil-derived mediators in plasma, would have strengthened the results. Studies in animal models and *in vitro* studies would have also strengthened our conclusion and given more mechanistic insight into the molecular mechanisms of action of tocilizumab in STEMI patients and its effect on the myocardium. Forthcoming studies should include such experiments.

In conclusion, tocilizumab treatment reduces the number of circulating neutrophils and seemingly also dampens neutrophil function when administered prior to PCI in patients with STEMI. Finally, our data suggest that these effects could be related to the beneficial effects of tocilizumab on myocardial salvage in these patients.

Contributors

Study conception and design: PA, BH, LO, CH, AKA, TBD, LG, KB, RW, GÄA, IS, JKD, TU, BB

Recruitment of patients, laboratory analysis and collection of data. CH, AKA, TBD, AM, TU, KY, LO, RW, AQJ, VB, GÄA, SW, KB, KS, IMT, BB, BHA, ESB, EB, CB, OK, KHS, AO, NEK

Contributed to analysis and interpretation of data. TBD, CH, PA, AKA, AM, TU, LO

Verification of all data; Clinical data verification. CH, AKA, TU. RNA-sequencing data verification. CH, KY, TBD. Flow cytometry data verification. CH, LO, TBD

Contributed to the original drafting of the article. CH, AKA, KY, TBD, PA, TU

All authors contributed to review and editing of the article and has given final approval of the version to be submitted.

Declaration of interests

Kaspar Broch has received lecture fees from Pharmacosmos, AstraZeneca, Boehringer Ingelheim, Pfizer, Orion Pharma, and Vifor Pharma, and has been on advisory boards for Pfizer and AstraZeneca. Lars L. Gullestad has received lecture fees from AstraZeneca, Boehringer Ingelheim, Novartis, and Amgen. He has also been a member of the local advisory board in AstraZeneca and Boehringer Ingelheim. Annika E. Michelsen is a stock holder in Pfizer. Bente E. Halvorsen has a patent on fish derived fatty acids, is a SAB member in CircM, Linkøping, Sweden, and is a leader of the scientific evaluation committee, Svensk Hjerte Lung Fonden.

Data sharing statement

Because of ethical restrictions from the Regional Committee for Medical and Research Ethics in South−East Norway, the data from the individual patients will unfortunately not be made available to other researchers for purposes of reproducing the results or replicating the procedure. However, researchers and others can contact the corresponding authors for more information. Regarding RNA-sequencing data, an institutional data transfer agreement can be established, and data can be shared if the aims of data use are covered by ethical approval and patient consent. The procedure will involve an update to the ethical approval as well as review by the legal departments at both institutions, and the process will typically take 2−4 months from the firts contact.

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Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.ebiom.2022.104013.

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Supplemental Appendix

for

Interleukin-6 inhibition in ST-elevation myocardial infarction: Immune cell profile in the randomised ASSAIL-MI trial

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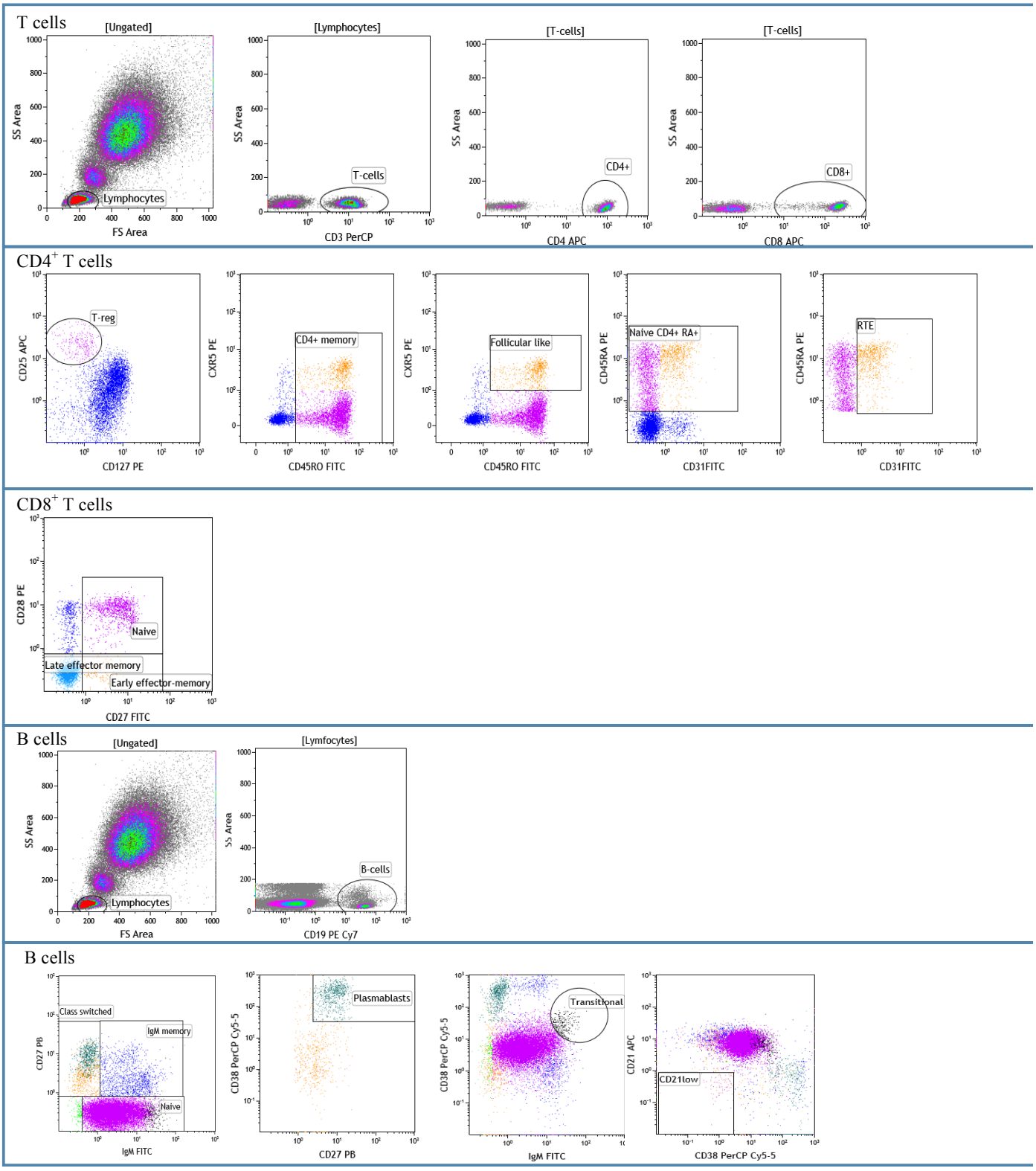
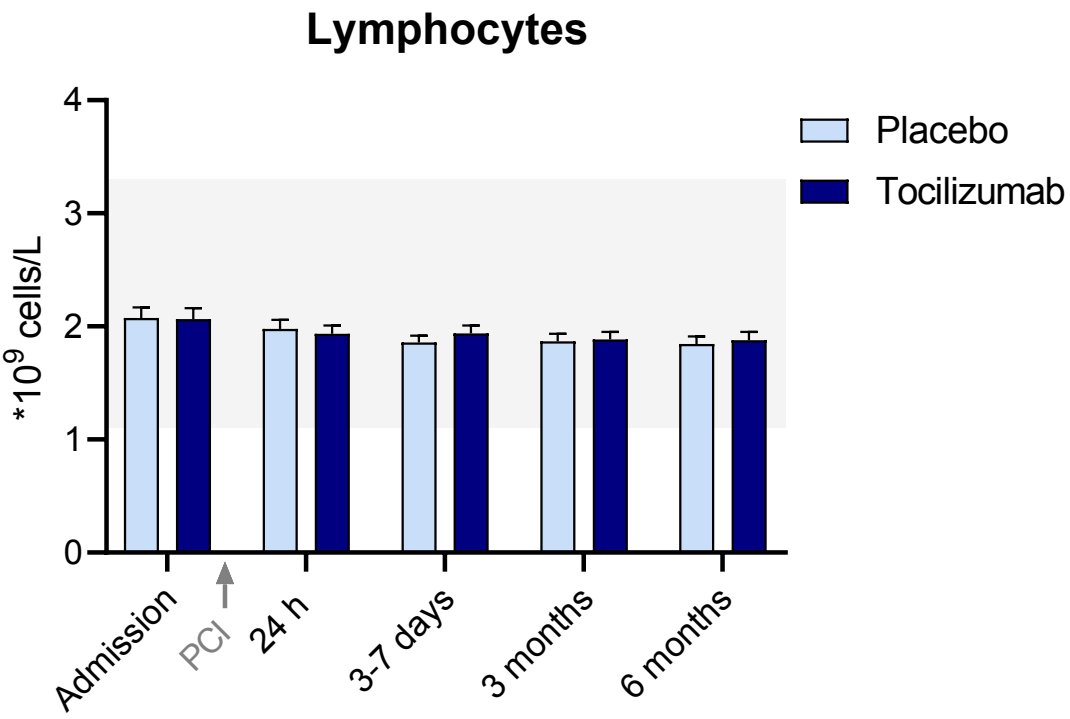


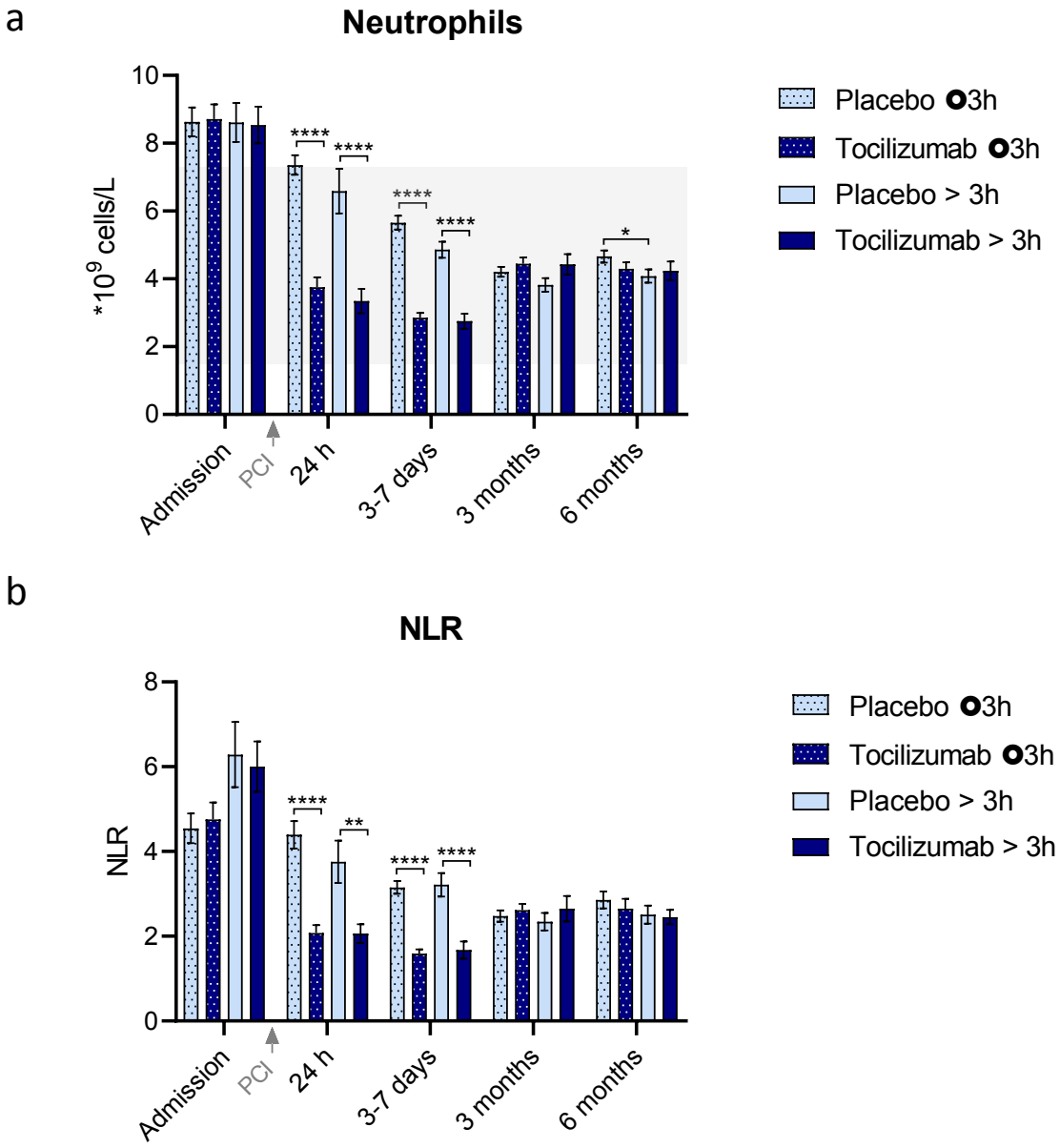
Supplemental Figure S2: Lymphocyte levels in STEMI patients

Lymphocyte levels for patients with STEMI treated with PCI over time. Dark blue is patients receiving a single injection of tocilizumab before PCI treatment. Shaded area: normal range.



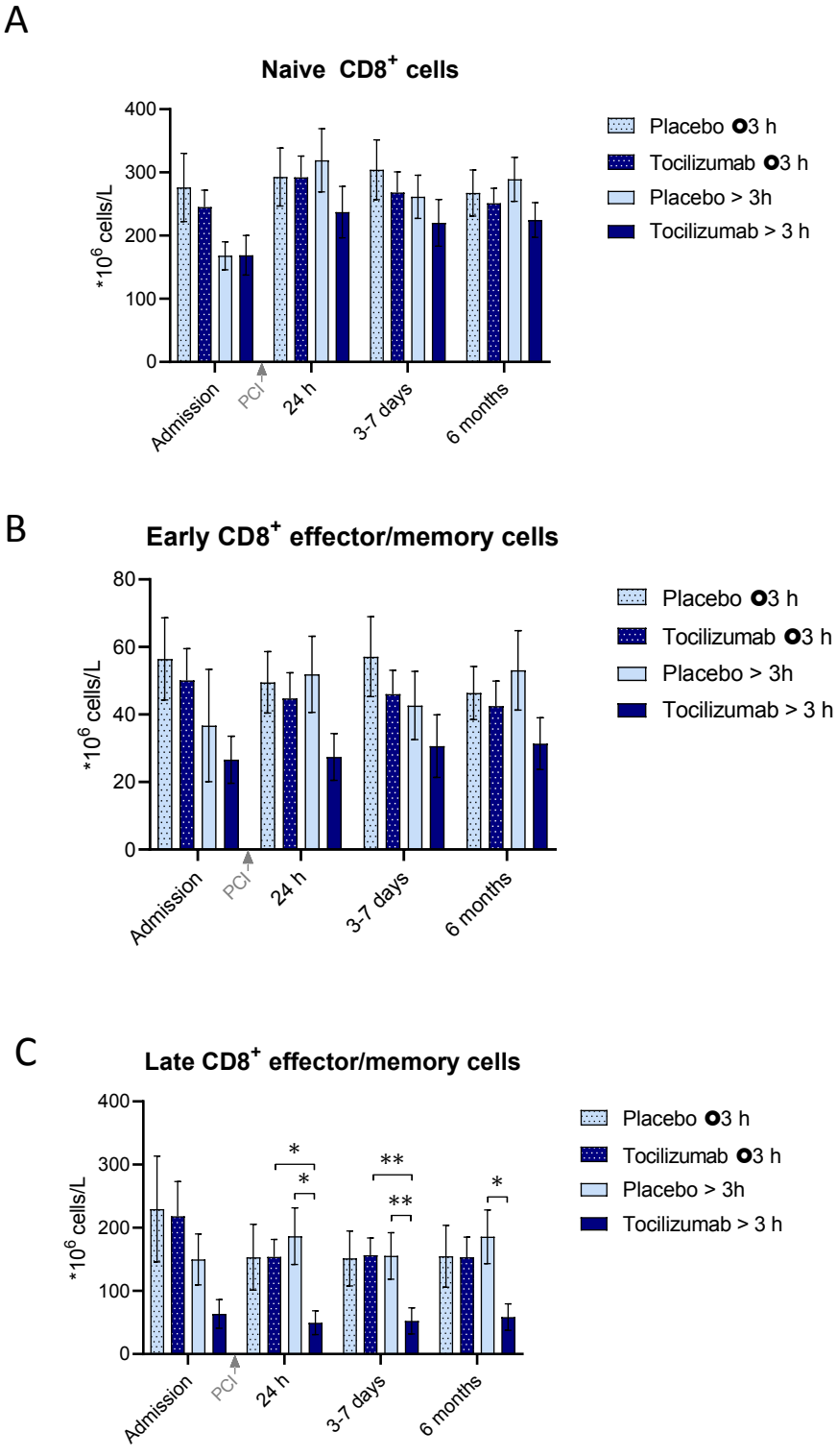
Supplemental Figure S3: Neutrophil levels and neutrophil lymphocyte ratio (NLR) in STEMI patients dependent on time of hospitalisation and treatment.

Panel **a** shows neutrophil counts from STEMI patients in the placebo and tocilizumab group based on time since symptom onset. Panel **b** shows neutrophil-lymphocyte ratio (NLR). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 (unpaired t test between relevant groups). Placebo ≤ 3 hours (admission: n= 61, 24 h: n=67, 3-7 days: n=66, 3 months: n=64, 6 months: n=66); placebo > 3 hours (admission: n= 31, 24 h: n=31, 3-7 days: n=30, 3 months: n=30, 6 months: n=30); tocilizumab ≤ 3 hours (admission: n= 65, 24 h: n=67, 3-7 days: n=67, 3 months: n=67, 6 months: n=67); tocilizumab > 3 hours (admission: n= 33, 24 h: n=32, 3-7 days: n=33, 3 months: n=32, 6 months: n=32). Hospital admission was within 6 hours after symptom onset.



Supplemental Figure S4: CD8$^+$ T cell subsets in STEMI patients dependent on time of hospitalisation and treatment.

Panel **a** shows naive CD8$^+$ T cell levels from STEMI patients in the placebo and tocilizumab group based on time since symptom onset. Panel **b** shows early CD8$^+$ effector/memory cells, and panel **C** shows late CD8$^+$ effector/memory cells. Placebo ≤ 3 hours (admission: n= 18, 24 h: n=18, 3-7 days: n=17, 6 months: n=18); placebo > 3 hours (admission: n= 12, 24 h: n=13, 3-7 days: n=12, 6 months: n=12); tocilizumab ≤ 3 hours (admission: n= 22, 24 h: n=21, 3-7 days: n=25, 6 months: n=23); tocilizumab > 3 hours (admission: n= 12, 24 h: n=12, 3-7 days: n=12, 6 months: n=12). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 (unpaired t test between relevant groups).



Supplemental Table S1: Antibodies used for flow cytometry analyses of lymphocyte subsets.

Monoclonal Antibody	Fluorochrome	Clone	Vendor	RRID
CD4	FITC	RPA-T4	BD Pharmingen	AB_395751
CD8	FITC	SK1	BD Biosciences	AB_2868800
IgM	FITC	G20-127	BD Pharmingen	AB_396117
CD24	PE	ML5	BD Pharmingen	AB_395822
CD3	PerCP	SK7	BD Biosciences	AB _2783791
CD38	PerCPCy5.5	HIT2	BD Pharmingen	AB_394184
CD4	APC	SK3	BD Biosciences	AB_2868799
CD8	APC	SK1	BD Biosciences	AB_2868803
CD25	APC	2A3	BD Biosciences	AB_2819021
IgD	APCH7	IA6-2	BD Pharmingen	AB_10645792
CD27	FITC	M-T271	Dako, Agilent	AB_579572
CD31	FITC	5.6E	Beckman Coulter	AB_13101
CD45RO	FITC	UCHL1	Beckman Coulter	*
CD28	PE	CD28.2	Beckman Coulter	AB_2833011
CD45RA	PE	ALB11	Beckman Coulter	**
CD127	PE	R34.34	Beckman Coulter	AB_131301
CD19	PC7	J3-119	Beckman Coulter	AB_10638575
CD27	PB	1A4CD27	Beckman Coulter	***
TCR αβ	PE	IP26	eBioscience	AB_10597891
CD21	APC	HBS	eBioscience	AB_1582217
CXCR5	PE	J252D4	R&D	AB_2089666
CD45	PO	HI30	Invitrogen	AB_1475776

*Schmidt, R.E., Non lineage / natural killer section report: new and previously defined clusters, 1989, Leucocyte Typing IV, White Cell Differentiation Antigens. Knapp, W., et al., Eds., Oxford University Press, 517-542

** Schlossman, S.F., et al., Differentiation Antigens, Eds., Oxford University Press, p 391-393

***Ritz,J., Trinchieri, G., Lanier, L.L., NK cell antigens: section report. 1995 in Leucocyte Typing V, White Cell Differentiation Antigens, Schlossman, S.F., et al., Eds., Oxford Univ. Press, p.1367-1372

BD,Becton Dickinson (Franklin Lakes, New Jersey, USA), Beckman Coulter (San Diego, CA) R&D Systems (Minneapolis, MN), eBioscience (San Diego, CA), Invitrogen (Waltham, MA, Dako Agilent (Santa Clara, CA), Biolegend (San Diego, CA).

Supplemental Table S2: Significantly differentially expressed imputed genes between placebo and tocilizumab treated patients in neutrophils

After two-tailed multiple t-tests, these genes were significantly differentially regulated between tocilizumab- and placebo-treated patients 3-7 days after hospitalisation. The gene-level was predicted using CIBERSORTx, and the threshold for significant regulation was set to $p < 0.01$.

Gene name	Log2Fold change	Gene name	Log2Fold change	Gene name	Log2Fold change
DNPEP	0.900242	CASS4	-0.13571	HOMER3	-0.39533
STX17	0.710718	CYP4F3	-0.14041	DRAIC	-0.41336
VAC14	0.440306	FAM91A1	-0.14086	CYP27A1	-0.42304
MIA2	0.028954	RSBN1L	-0.14287	KIF27	-0.44393
ARHGAP25	-0.00714	WLS	-0.14831	RMDN2	-0.44431
TGFBR2	-0.01322	RGS2	-0.15508	AC007342.5	-0.47599
WIPF1	-0.04457	SKI	-0.1576	LETM2	-0.55245
RELL1	-0.04535	RAPGEF2	-0.15769	AL118508.3	-0.61038
PIGX	-0.04675	ZNF398	-0.15983	GRAMD1C	-0.70411
ITGA5	-0.04865	MAP2K4	-0.16139	MAP3K6	-0.71493
CACUL1	-0.04948	BCAS3	-0.16299	CACNB4	-0.83341
DOCK5	-0.05792	RESF1	-0.17839	NRG1	-1.21772
PRKACA	-0.06065	CDK19	-0.18433		
FBXL20	-0.06289	ADGRE3	-0.19472		
LILRA2	-0.06356	JARID2	-0.1952		
MYO9B	-0.06645	FEZ2	-0.20228		
FBXL13	-0.06743	AHCTF1	-0.21281		
IKBIP	-0.07596	NLRX1	-0.21646		
LUCAT1	-0.08555	ARRDC3	-0.21784		
CD302	-0.08575	ERBIN	-0.22487		
LAMP2	-0.08794	AC026401.3	-0.2277		
VNN3	-0.09028	PKN2	-0.2402		
STK4	-0.09104	AC009716.2	-0.24266		
AOC3	-0.09156	PPIF	-0.24789		
STXBP5	-0.09884	VPS8	-0.2605		
MAPK13	-0.10301	GALNT3	-0.26118		
AFTPH	-0.10487	RHOB	-0.28137		
UBR5.AS1	-0.1051	PTP4A3	-0.28663		
CHI3L1	-0.10671	GPSM2	-0.30076		
PTEN	-0.1114	PLOD1	-0.30265		
IRAG2	-0.11167	MPZL1	-0.31611		
PUM2	-0.11465	MME	-0.31678		
SLC43A2	-0.11904	ZNF117	-0.32545		
NDE1	-0.12042	CD93	-0.32797		
CUL4B	-0.12072	TUFT1	-0.37162		
ATP6V1B2	-0.12784	GTF2H2C	-0.37648		
OGA	-0.1304	ASF1B	-0.37718		

Supplemental Table S3: Baseline characteristics of the populations included in flow cytometry analysis before treatment

Variable	Tocilizumab (n=37)	Placebo (n=32)	SAP (n=20)
Demography			
Age – years	61 ± 10	59 ± 9	68 ± 6*
Men – no (%)	30 (81)	30 (94)	15 (75)
Body mass index – kg/m^2	27.0 ± 4.6	27.5 ± 3.2	27.0 ± 3.5
Caucasian race – no (%)	37 (100)	31 (97)	20 (100)
Smoking status – no (%)			
Never smokers	16 (43)	17 (53)	5 (25)
Previous smokers	13 (35)	5 (16)	10 (50)
Current smokers	8 (22)	10 (31)	5 (25)
Prior conditions – no (%)			
Angina Pectoris	0 (0)	1 (3)	9 (45)***
Cerebrovascular disease	1 (3)	0 (0)	1 (5)
Diabetes mellitus	3 (8)	0 (0)	2 (10)
Hypertension	9 (24)	6 (19)	14 (70)***
Previous myocardial infarction	0 (0)	0 (0)	6 (30)***
Prior CABG	0 (0)	0 (0)	4 (20)
Prior PCI	0 (0)	1 (3)	8 (40)
Treatment – no (%)			
ACE* inhibitor or ARB†	6 (16)	6 (19)	10 (50)**
Aldosterone antagonist	0 (0)	0 (0)	0 (0)
Oral anticoagulants	2 (5)	0 (0)	2 (10)
Platelet inhibitor	3 (8)	1 (3)	18 (90)***
Beta blocker	4 (11)	0 (0)	9 (45)***
Calcium antagonist	4 (11)	2 (6)	7 (35)***
Diuretic	3 (8)	3 (9)	3 (15)
Statin	5 (14)	4 (13)	20 (20)***
Up-front DAPT‡	37 (100)	32 (100)	n/a
Time from symptom onset to arrival at PCI centre – min	156 ± 78	162 ±76	n/a
Door-to-balloon time – min	20 ± 9	18 ± 5	n/a
Infarct location			
Left anterior descending branch	15 (41)	10 (31)	n/a
Circumflex or marginal	4 (11)	6 (19)	n/a
Right coronary artery	16 (43)	15 (47)	n/a
Other	2 (5)	1 (3)	n/a
Laboratory values			
Haemoglobin – g/l	144 ± 11	145 ± 8	141 ± 11***
Platelet count – 10^9/l	252 ± 57	269 ± 71	249 (217-284)
Total white blood cell count – 10^9/l	11.7 ± 3.0	11.7 ± 3.1	7.5 ± 2.0***
Aspartate transaminase – U/l	30 (21-43)	33 (25-43)	26.6 ± 7.3
Troponin T – ng/l	45 (22-170)	52 (24-125)	10.5 (6.5-13.0)***
CK-MB§ – µg/l (placebo minus 1)	5.0 (2.7-16.0)	5.9 (3.2-13.3)	2.0 (2.0-3.8)***
NT-proBNP‖ – ng/l	67 (50-460)	62 (50-159)	118 (50-201)
Creatinine – mmol/l	71 ± 13	79 ± 16	87 ± 23*
Glucose – mmol/l	8 (7-10)	8 (7-10)	5.3 (5.1-5.8)**
HbA1c – mmol/mol	36 (34-41)	37 (35-39)	40 (39-43)
Total cholesterol – mmol/l	5.5 ± 1.4	5.2 ± 1.0	3.8 ± 0.6***
HDL¶ cholesterol – mmol/l	1.2 (1.1-1.4)	1.2 (0.9-1.4)	1.4 ± 0.4
LDL# cholesterol – mmol/l	3.8 ± 1.0	3.8 ± 0.9	2.0 ± 0.5***
C-reactive protein – mg/l	1.9 (0.8-4.1)	2.5 (1.2-5.8)	1.3 (0.6-3.1)
Albumin – g/l	42 ± 3	42 ± 3	n/a

Baseline characteristics stratified by treatment allocation. Values are presented as mean ± SD, median (interquartile range) or number (%) as appropriate. *ACE = angiotensin-converting enzyme; †ARB = angiotensin receptor blocker; ‡DAPT = dual anti-platelet therapy; §CK-MB = creatine kinase myocardial band; || NT-proBNP = N-terminal pro-B-type natriuretic peptide; ¶HDL = high-density lipoprotein; #LDL = low-density lipoprotein. For continuous variables, we used a one-way ANOVA or a Kruskal-Wallis test depending on the distribution. Categorical variables were tested using Chi-square. SAP vs STEMI populations showed as expected several differences in baseline characteristics: *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001. There were no differences between the two treatment arms of STEMI patients.

Supplemental Table S4: Lymphocyte subgroups over time in STEMI patients

Cell type (Mean+SEM)	Treatment	Admission	24h	3-7 days	6 months	SAP
NK cells	**Placebo**	398 ± 44.4	251 ± 23.0	251± 14.8	268 ± 24.6	299 ± 24.4
(*10^6 cells/L)	**TOC**	441.32±48.98	210.88±22.35	224.46±19.38	269.46±21.01	
CD19 (B cells)	**Placebo**	265 ± 27.9	285 ± 27.9	239 ± 21.6	233 ± 21.8	264 ± 24.9
(*10^6 cells/L)	**TOC**	281.27±29.41	274.24±33.12	262.38±31.19	230.77±24.21	
Transitional B cells	**Placebo**	2.55 ± 0.35	3.21 ± 0.53	2.50 ± 0.27	2.60 ± 0.30	3.15 ±0.42
(% of $CD19^+$ cells)	**TOC**	2.24±0.29	2.83±0.3	2.35±0.28	2.47±0.27	
Naïve B cells	**Placebo**	71.3 ± 2.58	69.6 ± 2.57	69.3 ± 2.49	71.6 ± 1.88	67.5±3.26
(% of $CD19^+$ cells)	**TOC**	73.19±2.35	72.77±2.21	70.51±2.41	70.63±2.12	
Plasmablasts	**Placebo**	1.03 ± 0.29	1.48 ± 0.65	**2.75 ± 0.71***	0.67 ± 0.13	0.88 ±0.27
(% of $CD19^+$ cells)	**TOC**	0.88±0.19	1.2±0.33	2.92±0.55	0.98±0.19	
CD21 low B	**Placebo**	3.07 ± 0.49	3.16 ± 0.72	2.53 ± 0.58	3.37 ± 0.72	4.91 ±0.98
(% of $CD19^+$ cells)	**TOC**	2.89±0.55	2.88±0.61	2.34±0.45	3.3±0.67	
Class-switched B cells	**Placebo**	10.4 ± 0.88	11.1 ± 0.94	12.6 ± 1.19	10.4 ± 0.83	13.3 ±1.74
(% of $CD19^+$ cells)	**TOC**	10.95±1.36	10.18±1.01	13.46±1.27	13.04±1.49	
IgM memory B cells	**Placebo**	13.2 ± 1.50	14.3 ± 1.37	14.1 ± 1.47	13.3 ± 1.22	13.1 ±1.99
(% of $CD19^+$ cells)	**TOC**	12.02±1.53	11.96±1.54	11.82±1.4	11.61±1.25	
CD3 (T cells)	**Placebo**	1357 ± 132	1553 ± 102	1449± 85.2	1485 ± 83.1	1412 ±151
(*10^6 cells/L)	**TOC**	1316.32±119.67	1541.97±103.8	1418.68±86.43	1370.51±72.24	
CD4 (T helper cells)	**Placebo**	817 ± 77.3	981 ± 61.4	905 ± 58.8	940 ± 50.5	985 ± 138
(*10^6 cells/L)	**TOC**	868.77±79.39	1091.27±82.29	972.84±61.43	944.63±58.64	
$CD4^+$ naïve T cells	**Placebo**	51.5 ± 2.12	47.5 ± 2.07	49.6 ± 2.12	51.5 ± 2.35	50.2 ±3.93
(% of $CD4^+$ T cells)	**TOC**	49.74±2.91	47.45±2.78	48.63±2.66	50.75±2.93	
Follicular $CD4^+$ T cells	**Placebo**	10.5 ± 0.84	10.3 ± 0.65	10.7 ± 0.60	10.6 ± 0.58	11.6 ±0.90
(% of $CD4^+$ T cells)	**TOC**	10.6±0.76	10.71±0.74	10.82±0.67	11.18±0.7	
$CD4^+$ memory cells	**Placebo**	63.4 ± 2.51	67.7 ± 2.10	66.6 ± 2.21	64.0 ± 2.38	66.4 ±3.26
(% of $CD4^+$ T cells)	**TOC**	67.11±2.79	67.45±2.8	67.41±2.58	65.74±2.56	
CD8 (T cytotoxic cells)	**Placebo**	510 ± 79.3	553 ± 57.1	522 ± 50.1	523 ± 53.6	436 ± 40.5
(*10^6 cells/L)	**TOC**	444.5±54.97	450.42±39.74	439.92±41.62	422.71±36.4	
$CD8^+$ naïve T cells	**Placebo**	54.6 ± 4.09	60.2 ± 3.76	60 ± 3.77	60.3 ± 3.71	62 ± 4.35
(% of $CD8^+$ T cells)	**TOC**	54.87±3.56	62.15±3.11	59.9±2.92	60.99±2.89	
Early $CD8^+$ effector/memory T cells	**Placebo**	9.87 ± 1.50	9.83 ± 1.28	10.3 ± 1.66	10.1 ± 1.34	8.53 ± 1.6
(% of $CD8^+$ T cells)	**TOC**	10.05±1.27	8.79±1	9.7±1.07	9.43±1.01	
Late $CD8^+$ effector/memory T cells	**Placebo**	29.9 ± 3.82	24.7 ± 3.36	24.5 ± 3.26	24.4 ± 3.31	23.3 ±3.84
(% of $CD8^+$ T cells)	**TOC**	30.06±3.98	23.7±3.23	24.98±3.05	24.31±3.21	
Double-negative T cells	**Placebo**	0.51 ± 0.044	0.50 ± 0.047	0.46 ± 0.044	0.46 ± 0.046	0.53 ±0.07
(% of T cells)	**TOC**	0.59±0.06	0.51±0.04	0.56±0.05	0.57±0.06	
Regulatory T cells	**Placebo**	7.35 ± 0.34	7.32 ± 0.38	6.57 ± 0.36	6.63 ± 0.28	7.76 ±0.58
(% of $CD4^+$ T cells)	**TOC**	7.43±0.38	6.77±0.25	6.27±0.24	6.96±0.39	
Recent thymic emigrants	**Placebo**	32.4 ± 2.06	31.1 ± 2.02	31.2 ± 2.09	32.6 ± 2.18	29.7 ±3.42
(% of $CD4^+CD45RA^+$ T cells)	**TOC**	32.7±2.08	31.86±2.12	32.86±1.85	31.22±2.12	

Significant values between the placebo group and SAP are marked in bold. No significant changes were found at any time point between placebo and tocilizumab.

Supplemental Table S5: Significantly differentially expressed imputed genes between placebo and tocilizumab treated patients in T cells

After multiple testing corrections, these genes were significantly differentially regulated (adjusted p-value < 0.05, average gene expression > 50) between tocilizumab- and placebo-treated patients 24 hours after hospitalisation.

Gene name	Log2Fold change
SOCS3	-2.39766
TRGC1	-1.5322
VCAN	-2.2150
IRF1	0.61399
SIGLEC17P	-1.50452
PON2	-0.78162

Supplementary Table S6: NK, $CD19^+$ and $CD4^+$ cells dependent on the time of hospitalisation and treatment.

NK cells ($*10^6$ cells/L)					
Time after symptom onset		**Placebo <3h**	**Placebo >3h**	**Tocilizumab <3h**	**Tocilizumab >3h**
Admission	Mean±SEM	369.94±45.19	441.08±89.32	409.23±59.18	500.17±87.44
24 h	Mean±SEM	212.39±21.01	304.31±43.23	208.29±19.9	215.42±52.27
3-7 days	Mean±SEM	253.35±20.47	248.58±21.8	213.6±17.67	247.08±47.1
6 months	Mean±SEM	254.61±28.98	289.17±44.35	248.87±19.11	308.92±48.62
$CD19^+$ B cells ($*10^6$ cells/L)					
		Placebo <3h	**Placebo >3h**	**Tocilizumab <3h**	**Tocilizumab >3h**
Admission	Mean±SEM	304±39.78	207.33±30.9	303.95±40.69	239.67±36.23
24 h	Mean±SEM	282.83±37.21	288.15±43.75	273.71±45.18	275.17±47.77
3-7 days	Mean±SEM	233.65±28.98	245.42±33.63	271.92±43.12	242.5±36.20
6 months	Mean±SEM	226.33±29.64	242.58±32.82	240.09±33.55	212.92±30.45
$CD4^+$ T cells ($*10^6$ cells/L)					
		Placebo <3h	**Placebo >3h**	**Tocilizumab <3h**	**Tocilizumab >3h**
Admission	Mean±SEM	940.11±107.5	632.25±86.02	966.91±105.55	688.83±110.15
24 h	Mean±SEM	945.67±88.06	1029.69±92.58	1140.48±100.22	1005.17±145.43
3-7 days	Mean±SEM	892.24±72.9	924.08±101.25	999.16±78.25	918.0±9.23
6 months	Mean±SEM	903.17±63.73	994.25±83.47	944.04±73.89	945.75±100.34

Supplementary Table S7: Correlations between CD8+ T cell counts and troponin T according to time of hospitalisation after symptom onset

Correlations between neutrophil absolute counts or change from admission for the total population, the placebo or the tocilizumab treated patients towards troponin T based on time of hospitalisation.

		Total population					
		≤ 3 h			> 3 h		
		r	P value	n	r	P value	n
Absolute count	CD8 (24 h)	-0.043	0.795	39	0.366	0.072	25
	CD8 (3-7 days)	-0.033	0.836	42	**0.423**	**0.040**	24
Change from baseline (%)	CD8 (24 h)	-0.303	0.061	39	-0.013	0.952	24
	CD8 (3-7 days)	-0.256	0.116	39	0.055	0.802	23

		Placebo					
		≤ 3 h			> 3 h		
		r	P value	n	r	P value	n
Absolute count	CD8 (24 h)	0.086	0.735	18	0.379	0.201	13
	CD8 (3-7 days)	0.064	0.808	17	**0.608**	**0.036**	12
Change from baseline (%)	CD8 (24 h)	-0.255	0.307	18	0.021	0.948	12
	CD8 (3-7 days)	-0.265	0.305	17	0.027	0.937	11

		Tocilizumab					
		≤ 3 h			> 3 h		
		r	P value	n	r	P value	n
Absolute count	CD8 (24 h)	-0.355	0.115	21	0.280	0.379	12
	CD8 (3-7 days)	-0.262	0.206	25	0.280	0.379	12
Change from baseline (%)	CD8 (24 h)	-0.286	0.209	21	-0.056	0.863	12
	CD8 (3-7 days)	-0.234	0.294	22	0.182	0.572	12

Significant values are marked in bold. r is the Spearman's correlation coefficient.

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Research paper

Early increase of specialized pro-resolving lipid mediators in patients with ST-elevation myocardial infarction

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ABSTRACT

Background: Termination of acute inflammation is an active process orchestrated by lipid mediators (LM) derived from polyunsaturated fatty acids, referred to as specialized pro-resolving mediators (SPM). These mediators also provide novel therapeutic opportunities for treating inflammatory disease. However, the regulation of these molecules following acute myocardial infarction (MI) remains of interest.
Methods: In this prospective observational study we aimed to profile plasma levels of SPMs in ST-elevation MI (STEMI) patients during the first week following MI. Plasma LM concentrations were measured in patients with STEMI ($n = 15$) at three time points and compared with stable coronary artery disease (CAD; $n = 10$) and healthy controls ($n = 10$).
Findings: Our main findings were: (i) Immediately after onset of MI and before peak troponin T levels, STEMI patients had markedly increased levels of SPMs as compared with healthy controls and stable CAD patients, with levels of these mediators declining during follow-up. (ii) The increase in SPMs primarily reflected an increase in docosapentaenoic acid- and docosahexaenoic acid-derived protectins. (iii) Several individual protectins were correlated with the rapid increase in neutrophil counts, but not with CRP. (iv) A shift in 5-LOX activity from the leukotriene B_4 pathway to the pro-resolving RvTs was observed.

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Interpretation: The temporal regulation of SPMs indicates that resolution mechanisms are activated early during STEMI as part of an endogenous mechanism to initiate repair. Thus strategies to boost the activity and/or efficacy of these endogenous mechanisms may represent novel therapeutic opportunities for treatment of patients with MI.
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Research in context

Evidence before this study

MI leads to a sterile inflammatory reaction, a response vital for tissue repair. However, if the inflammation is prolonged and not resolved, it could aggravate tissue damage with unfavorable effects on the myocardium. Indeed, resolution of inflammation is a prerequisite for restoration of tissue integrity and function following MI. Specialized pro-resolving lipid mediators (SPMs) are key effectors of resolution of inflammation and are endogenously formed from n-3 polyunsaturated fatty acids (PUFAs), the balance of pro-inflammatory mediators and SPMs regulates the duration and strength of the inflammatory response. To this end, however, the role of SPMs and resolution in acute MI is unexplored in humans. We hypothesized that timely induction and resolution of inflammation is required for optimal MI healing and aimed to profile plasma levels of SPMs in STEMI patients during the first week following MI. The sources searched were all PubMed publications available and the search terms were myocardial infarction and resolution, with emphasis on ST-elevation myocardial infarctions.

Added value of this study

This is the first report on the regulation of SPMs during acute MI in humans. Our findings show that pro-resolving mechanisms are increased early during STEMI, indicating that the "inflammation breaks" are activated immediately after MI onset. Our novel findings, along with the existing evidence on resolution mechanisms in inflammatory disease, could represent the start of a new era in relation to targeting inflammation during MI, focusing not only of anti-inflammatory intervention, but also on enhancing the pro-resolving capacity.

Implications of all the available evidence

Our data may lead to new targets for therapy in STEMI, not only focusing on how to down-regulate harmful inflammation, but also on how to enhance resolving and repair mechanisms. Future research should try to identify the most important pathways in these processes and their cellular targets, and if successful, these studies could lead to a new paradigm in the management of these patients.

1. Introduction

Cardiovascular disease (CVD) is the leading cause of death worldwide of which atherosclerotic disorders are the most important [1]. Atherosclerosis is now considered a chronic inflammatory disease, with an interaction between lipids and inflammation as the major characteristic. In clinical studies, increased levels of inflammatory markers are associated with accelerated disease and worsened prognosis following atherosclerotic complications like myocardial infarction (MI) [2,3]. MI leads to a sterile inflammatory reaction involving recruitment of inflammatory cells and activation of inflammatory pathways within the myocardium, responses that are vital for tissue repair [4]. However, if sterile inflammation is prolonged and not resolved, such inflammatory responses could aggravate tissue damage with unfavorable effects on the myocardium. Indeed, resolution of inflammation is a prerequisite for restoration of tissue integrity and function following MI [4,5].

Specialized pro-resolving lipid mediators (SPM) are key effectors of resolution of inflammation and are endogenously formed from n-3 (omega-3) polyunsaturated fatty acids (PUFA) [6]. SPMs are classified according to their n-3 PUFA precursor and further divided into subsets of functional families, such as resolvins (Rv), maresins, and protectins. The balance of pro-inflammatory mediators and SPMs during acute inflammation, like in MI, regulates the duration and strength of the inflammatory response [5,7]. Commonly used medications in CVD may influence the levels of these lipid mediators (LM). Thus, while aspirin irreversibly inhibits the ability of cyclooxygenase (COX)-1 to form pro-inflammatory LM such as prostaglandins (PG), aspirin also switches the activity of COX-2 leading to a shift in the LM profile from the inflammation-initiating PG to epimeric forms of the protectins, resolvins, and lipoxins primarily mediating anti-inflammatory and pro-resolving effects [8,9].

An exaggerated and prolonged inflammatory response after MI has been proposed to be detrimental for cardiac function, both in the short and longer term [4,10–12]. We recently found that resolution mechanisms are altered in stable atherosclerotic disorders [13]. However, the role of resolution in acute MI is unexplored in humans. In this prospective and observational study, we hypothesize that timely induction and resolution of inflammation is required for optimal MI healing and we therefore aimed to profile plasma levels of SPMs in ST-elevation MI (STEMI) during the first week following MI.

2. Materials and methods

2.1. Study population

Patients presenting with STEMI, subjected to primary percutaneous coronary intervention (PCI) who met all the following inclusion criteria: (i) significant ST-segment elevation on ECG, ii) elevated high-sensitivity troponin T (hsTnT) levels, and iii) an occluded or stenotic coronary artery (>50%) presumed to be the culprit lesion on angiography were consecutively included in the study. STEMI patients were admitted within 5 h (median 2·6 h) after onset of symptoms (Table 1). Coronary angiography and PCI were performed in all patients and pharmacological treatment (prehospital, procedural, and secondary preventive treatment) was provided in adherence to prevailing guidelines. The STEMI patients were compared with patients with stable coronary artery disease (CAD) ($n = 10$) defined as episodes of reversible ischemic chest pain with atherosclerotic plaques demonstrated by coronary angiography. To keep confounding factors at a minimum, patients with

developing signs of heart failure within the observation period, other known inflammatory comorbidities (e.g. autoimmune disease, infections, and malignancies), and patients using immune modulating drugs (e.g. steroids) and COX-inhibitors were excluded. Blood samples were also obtained from ten age- and sex-matched apparently healthy controls. The healthy volunteers were all apparently healthy based on clinical examination and history, mean age 61 years, and none used any medications. They all had high-sensitivity C-reactive protein (hsCRP) <2·5 mg/L and their clinical and biochemical characteristics are presented in Tables 1 and 2. One patient in each group used a daily n-3 PUFA supplement, in the STEMI group 240 mg n-3 PUFA daily, in the stable CAD group 2000 mg n-3 PUFA daily, and among healthy volunteers 1200 mg n-3 PUFA daily.

2.2. Blood sampling

Blood samples were collected from patients and controls at the time of inclusion (in STEMI patients, mean time from symptoms debut 2·5 h; Table 1). In blood samples from STEMI patients were also collected at day one and eight. In STEMI and stable CAD patients, peripheral arterial blood was drawn prior to procedural heparinization and catheterization on inclusion into endotoxin-free blood collection tubes with ethylenediaminetetraacetic acid (EDTA) as anticoagulant. In healthy volunteers and STEMI patients at day one and eight, peripheral venous blood collected into EDTA tubes was used. The EDTA tubes were immediately placed on melting ice and centrifuged within 30 min at 2000 ×*g* for 20 min to obtain platelet-poor plasma. Immediately following centrifugation, the vials were stored in several aliquots at -80 °C for less than six months and thawed only once prior to analyses.

2.3. Targeted lipid mediator profiling

Plasma samples for liquid chromatography-tandem mass spectrometry (LC-MS/MS)-based LM profiling were extracted using solid-phase (C-18) extraction columns as previously described [14]. Prior to sample extraction, deuterated internal standards, representing each region in the chromatographic analysis (500 pg each) were added to facilitate quantification in 4 vol of cold methanol. Samples were kept at −20 °C for a minimum of 45 min to allow protein precipitation. Supernatants were subjected to solid phase extraction, methyl formate fraction were collected, brought to dryness and suspended in phase (methanol/water, 1:1, vol/vol) for injection on a Shimadzu LC-20 AD HPLC and a Shimadzu SIL-20 AC autoinjector, paired with a QTrap 6500 plus (Sciex). An Agilent Poroshell 120 EC-C18 column (100 mm × 4·6 mm × 2·7 μm) was kept at 50 °C and mediators eluted using a mobile phase consisting of methanol-water-acetic acid of 20:80:0·01 (vol/vol/vol) that was ramped to 50:50:0·01 (vol/vol/vol) over 0·5 min and then to 80:20:0·01 (vol/vol/vol) from 2 to 11 min, maintained till 14·5 min and then rapidly ramped to 98:2:0·01 (vol/vol/vol) for the next 0·1 min. This was subsequently maintained at 98:2:0·01 (vol/vol/vol) for 5·4 min, and the flow rate was maintained at 0·5 ml/min. QTrap 6500 plus was operated using a multiple reaction monitoring method. Each LM was identified using established criteria including matching retention time to synthetic and authentic materials and at least 6 diagnostic ions. Calibration curves were obtained for each using synthetic compound mixtures at 0·78, 1·56, 3·12, 6·25, 12·5, 25, 50, 100, and 200 pg that gave linear calibration curves with a r^2 values of 0·98–0·99 and co-efficient of variation of 1–4% [14,15].

2.4. Cytokine analyses

Interleukin (IL)-6, IL-8, and tumour necrosis factor (TNF) were analysed by an enzyme immunoassay (EIA) with the Meso Scale (Rockville, MD) V-Plex Human proinflammatory kit. Intra- and inter-assay coefficients of variation were < 10%.

Table 1
Clinical and hemodynamic characteristics of STEMI patients, stable CAD, and healthy volunteers.

	Healthy volunteers ($n = 10$)	Stable CAD (n = 10)	STEMI ($n = 15$)
Age, years, mean (SEM)	61 (3·4)	67 (2·2)*	59 (2·8)
Female, *n* (%)	2 (20)	4 (60)	2 (13)
Body mass index, kg/m^2, mean (SEM)	24·5 (0·6)	31·0 (1·7)**	26·2 (0·7)
Blood pressure, systolic, mmHg, mean (SEM)	125 (5·4)	154 (4·0)*	131 (8·2)
Blood pressure, diastolic, mmHg, mean (SEM)	80 (3·4)	80 (2·8)	79 (3·6)
Symptom onset to inclusion, hours, mean (min, max)	–	–	2·6 (0·9, 4·7)
Maximum hsTnT, ng/L, mean (min, max)	–	–	2681 (1096, 9557)
GRACE score, mean (SEM)	–	–	105 (6·0)
Multi vessel disease, n (%)	0	4 (40)	7 (47)
History			
Diabetes mellitus, n (%)	0	2 (20)	0
Current smoking, n (%)	0	0	8 (53)
Previous myocardial infarction, n (%)	0	1 (10)	1 (7)
Medication on admittance			
Aspirin, n (%)	0	10 (100)	15 (100)
Clopidogrel, n (%)	0	1 (6·3)	15 (100)
Ticagrelor, n (%)	0	0	0
Prasugrel, n (%)	0	0	0
Low molecular weight heparin, n (%)	0	0	15 (100)
Statin, n (%)	0	9 (90)	3 (20)

Data given as mean (SEM) or number of subjects (%). hsTnT, high-sensitivity Troponin T.
*$p < 0·05$, **$p < 0·01$ vs. STEMI baseline

2.5. Biochemical analyses

Blood samples were analysed consecutively using routine methods in Medical Biochemistry laboratories at Oslo University Hospital Rikshospitalet including analyses of hsCRP on a MODULAR platform (Roche Diagnostics, Basel, Switzerland) and hsTnT by electrochemiluminescence immunoassay (ELICA; Elecsys 2010 analyzer, Roche Diagnostics).

2.6. Statistical analyses

Multivariate analysis (PLS-DA) was performed using SIMCA 14·1 with mean centring and unit variance scaling. All other statistical calculations were performed with Prism 7 for Mac OS X (GraphPad Software, SD, California). All continuous variables were compared with paired or unpaired Student's *t*-tests and categorical variables with the Chi-square test or Fisher exact test for observations <5. For comparison of more than two groups, ANOVA and repeated measures ANOVA were used and subsequent analyses were performed only if the one-way analysis of variance was significant. Association of SPMs to cardiac and inflammatory parameters was performed only on significantly elevated SPMs after MI onset with non-parametric statistics (Spearman-rho correlation). A value of $p \leq 0·05$ was considered statistically significant, but should be interpreted with caution.

Ethics statement

The study was approved by the Regional Committee for Medical and Health Research Ethics of South-Eastern Norway and conducted according to the Helsinki Declaration. All participants provided written, informed consent.

Table 2
Clinical biochemistry at MI onset and during follow-up of STEMI patients, stable CAD, and healthy volunteers.

	Healthy	Stable	STEMI ($n = 15$)		
	Volunteers ($n = 10$)	CAD (n = 10)	Baseline	Day 1	Day 8
hsTnT baseline, ng/L, mean (SEM)	8 (0·9)	15 (5·4)	79 (22·1)	2073 (449)	369 (103)
NT-proBNP, ng/L, mean (SEM)	64 (6·6)	295 (58·2)	251 (106·3)	1196 (258)	406 (95)
hsCRP, mg/L, mean (SEM)	2·5 (1·4)	1·2 (0·4)	6·9 (5·3)	13·4 (7·7)	11·1 (5·6)
Hb, g/Dl, mean (SEM)	14·6 (0·3)	14·1 (0·3)	14·6 (0·3)	13·9 (0·3)	14·3 (4·5)
Leucocytes, 10^9/L, mean (SEM)	6·6 (0·6)	6·4 (0·5)	12·9 (1·4)	10·2 (0·9)	7·4 (0·5)
Neutrophils, 10^9/L, mean (SEM)	2·9 (0·6)	3·9 (0·4)	10·0 (1·3)	7·2 (0·8)	4·5 (0·4)
Monocyte, 10^9/L, mean (SEM)	0·7 (0·1)	0·6 (0·1)	0·8 (0·1)	1·0 (0·1)	0·8 (0·1)
Lymphocyte, 10^9/L, mean (SEM)	2·6 (0·5)	1·8 (0·2)	1·9 (0·3)	1·9 (0·2)	2·0 (0·2)
Platelets, 10^9/L, mean (SEM)	260 (19·6)	234 (23·7)	260 (15·5)	244 (19·3)	295 (30·4)
Creatinine, μmol/L, mean (SEM)	88·9 (4·6)	81·1 (5·8)	81·3 (4·6)	76·6 (2·8)	84·4 (2·8)
Total cholesterol, mmol/L, mean (SEM)	5·4 (0·3)	4·2 (0·3)	4·2 (0·3)	4·9 (0·3)	3·9 (0·2)
Triglycerides, mmol/L, mean (SEM)	1·5 (0·2)	1·4 (0·3)	1·3 (0·4)	1·6 (0·3)	1·2 (0·3)
LDL cholesterol, mmol/L, mean (SEM)	3·3 (0·3)	2·4 (0·3)	3·5 (0·3)	3·2 (0·2)	2·3 (0·2)
HDL cholesterol, mmol/L, mean (SEM)	1·7 (0·2)	1·6 (0·2)	1·3 (0·3)	1·2 (0·1)	1·0 (0·1)
AST, U/L, mean (SEM)	26 (0·8)	26 (1·1)	33 (4·7)	149·6 (22·1)	27 (2·6)
ALT, U/L, mean (SEM)	25 (2·1)	34 (4·3)	28 (2·4)	40 (4·6)	34 (3·2)
HbA1c, %, mean (SEM)	5·4 (0·1)	6·0 (0·5)	5·6 (0·1)	5·7 (0·1)	5·8 (0·1)

Data are given as mean (SEM). hsTnT, high-sensitivity Troponin T; BNP, brain natriuretic protein; hsCRP, high-sensitivity C-reactive protein; Hb, haemoglobin; LDL, low density lipoprotein; HDL, high-density lipoprotein; AST aspartate aminotransferase; ALT, alanine aminotransferase.

3. Results

3.1. Study population

Baseline clinical characteristics were comparable in STEMI patients and stable CAD patients. However, the stable CAD patients were slightly older, had higher body mass index (BMI), and hypertension was more prevalent (Table 1). Further, all patients with stable CAD and STEMI had received aspirin (300 mg) prior to baseline sampling. Healthy controls were matched for age, gender and actual blood pressure with the two patient groups and for BMI with the STEMI group (Table 1). As shown in Fig. 1, STEMI patients had hsTnT and hsCRP peak levels on day 1. In contrast to this pattern, neutrophil counts peaked and were tripled already at MI onset compared with stable CAD and healthy volunteers, demonstrating an early inflammatory response during MI, with decreasing levels throughout the observation period. All recorded biochemical parameters are listed in Table 2.

3.2. Patients with STEMI have a distinct and early increase in peripheral SPM concentrations

SPMs and eicosanoids from the eicosapentaenoic acid (EPA), n-3 docosapentaenoic acid (n-3 DPA), docosahexaenoic acid (DHA), and arachidonic acid (AA) bioactive metabolomes were identified and quantified in accordance with published criteria, including matching retention times on for liquid chromatography (LC) and tandem mass spectrometry (MS/MS) fragmentation spectra [15]. Supplementary Fig. S1a-b depict representative multiple reaction monitoring chromatograms of selected ion pairs for protectin (PD)1 and $PD2_{n\text{-}3\ DPA}$ along with representative MS/MS spectra and diagnostic ions employed for their identification. To assess if STEMI was linked to changes in SPM profiles, we first performed a Partial least squares discriminant analysis (PLS-DA) (Fig. 2a top) with results obtained from LC-MS/MS profiling. The PLS-DA plot shows the systematic clusters among observations (closer plots presenting higher similarity in the data matrix) [16] and demonstrates a separation between the healthy controls, stable CAD, and STEMI clusters (actual levels with SEM for all the SPMs in each patient group are shown in Supplementary Table S1). The corresponding loading plot (Fig. 2a below), that describes the magnitude and manner the SPMs contribute to the cluster separation in the score plot [16], demonstrated that plasma from STEMI patients was characterized by higher levels of several individual LMs such as PD1 and $PD2_{n\text{-}3\ DPA}$ (VIP score ≥ 1·0).

Combining (cumulative values) all individual SPMs (DHA-, DPA-, EPA-derived and lipoxins) and pro-inflammatory LM (AA-derived leukotrienes, prostaglandins, and thromboxane), respectively, STEMI patients had an almost doubling of SPM levels (Fig. 2b top) only hours after onset of MI symptoms and before the observed peak in hsTnT, compared with both healthy controls and stable CAD, with declining levels throughout the observation period. Of note, the levels of SPMs in plasma were identified within physiologically relevant concentrations: 1 pM-10 nM [17,18]. In line with the administration of aspirin, patients with stable CAD ($p < 0·001$) had decreased plasma eicosanoid concentrations (Fig. 2b, below) and in STEMI patients this was seen at onset of MI ($p = 0·003$) and during follow-up. Our findings so far show that STEMI patients have increased levels of SPMs immediately after MI onset and this response is most likely part of the endogenous damage limitation pathway to minimise secondary damage post MI.

3.3. STEMI patients have elevated levels of DHA- and n-3 DPA-derived protectins

Having found distinct SPM profiles in STEMI patients when compared with both CAD patients and healthy volunteers, we next examined the contribution of each of the LM families to the overall profiles. The definitions of which individual SPMs that are included in each SPM family are defined in Supplementary Table S1. In STEMI patients we observed an increase of DHA-derived SPMs ($p = 0·03$ and $p = 0·02$ vs CAD and controls, respectively) when compared with stable CAD and healthy volunteers (Fig. 3a). Of note, we also found increases in both n-3 DPA and DHA-derived protectins during the early hours post MI that decreased during follow-up to levels comparable with those measured in both stable CAD patients and healthy volunteers (3- and 4-fold increase, respectively; Fig. 3b-c).

Assessment of individual mediator concentrations with the protectin metabolomes demonstrated an increase in the DHA-derived SPM PD1 and its double deoxygenation isomer 10S,17S-diHDHA (also referred to as PDX), that also carries biological actions including anti-platelet actions [19,20], after MI onset in STEMI as compared with stable CAD and controls, with a decrease during follow-up (Fig. 4a, right). The regulation of the n-3 DPA derived protectins followed similar dynamics as those observed for the DHA-derived congeners, whereby $PD2_{n\text{-}3\ DPA}$ and the protectin pathway marker 10S,17S-diHDPA, increased by three-fold and ten-fold, respectively, in STEMI patients at onset of MI compared with stable CAD and controls with normalization during follow up (Fig. 4b).

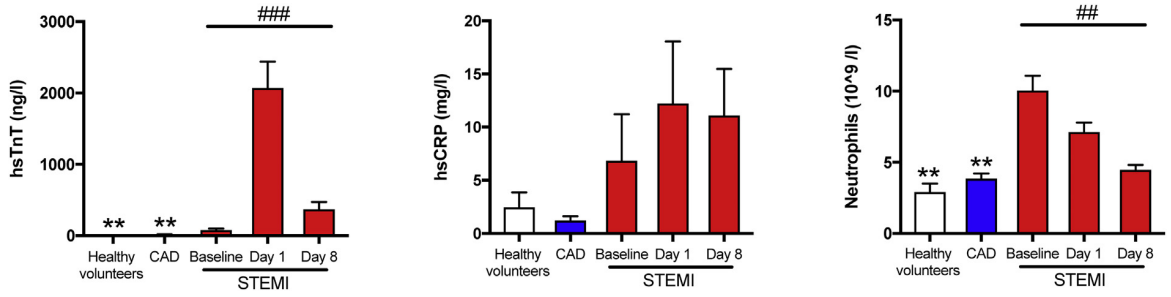

Fig. 1. STEMI patients present with elevated troponin T and inflammatory markers. Plasma from healthy volunteers ($n = 10$) and patients with stable CAD ($n = 10$) and STEMI ($n = 15$) were collected after MI onset. Repeated samples were drawn from STEMI patients. The figure show plasma levels of hsTnT, hsCRP, and neutrophils. All results are expressed as mean ± SEM. $^{*}p < 0{\cdot}05$, $^{**}p < 0{\cdot}01$, $^{***}p < 0{\cdot}001$ vs. STEMI baseline. $^{\#}p < 0{\cdot}05$, $^{\#\#}p < 0{\cdot}01$, $^{\#\#\#}p < 0{\cdot}001$ for repeated measures ANOVA for STEMI-baseline, day 1, and 8.

In contrast with the protectin pathways, the production of $RvD5_{n\text{-}3\ DPA}$, which we recently found to exert vasculo-protective actions [21], was reduced in all STEMI patients at all three intervals tested and in stable CAD patients when compared with healthy volunteers.

Taken together, these findings strongly indicate a modulated biosynthesis of several SPMs with a temporal up regulation of DHA- and n-3 DPA-derived protectins immediately after vascular injury suggesting that these mediators may play a role in limiting further tissue damage. In contrast, the potential vasculo-protective $RvD5_{n\text{-}3\ DPA}$ was reduced.

3.4. Association of SPMs with markers of inflammation and hsTnT in the STEMI population

We next assessed whether there was a correlation between individual significantly regulated SPMs and markers of inflammation. Whereas we found no associations between significantly regulated individual SPMs and hsCRP, we found significant correlations between PD1 ($r = 0{\cdot}26$, $p < 0{\cdot}04$), 10*S*,17*S*-diHDA (PDX) ($r = 0{\cdot}28$, $p < 0{\cdot}03$), and $PD2_{n\text{-}3\ DPA}$ ($r = 0{\cdot}32$, $p < 0{\cdot}01$) with neutrophil counts. Furthermore, data on cytokine levels (i.e. IL-6, IL-8 and TNF) are shown in Supplementary Fig. S2. While IL-8 and TNF were not correlated with the individual protectins, IL-6 levels was correlated with PDX ($r = 0.28$, $p = .02$). Finally, 10*S*,17*S*-diHDA (PDX) ($r = 0{\cdot}35$, $p = 0{\cdot}005$) was also associated with increased levels of hsTnT.

3.5. Low pro-inflammatory LM levels in STEMI and stable CAD primarily reflect effects of aspirin

Aspirin influences biosynthesis of certain LM and the aspirin triggered pathway for AA is shown in Fig. 5a. As expected and shown by others [22], in accordance with the observed decrease in pro-

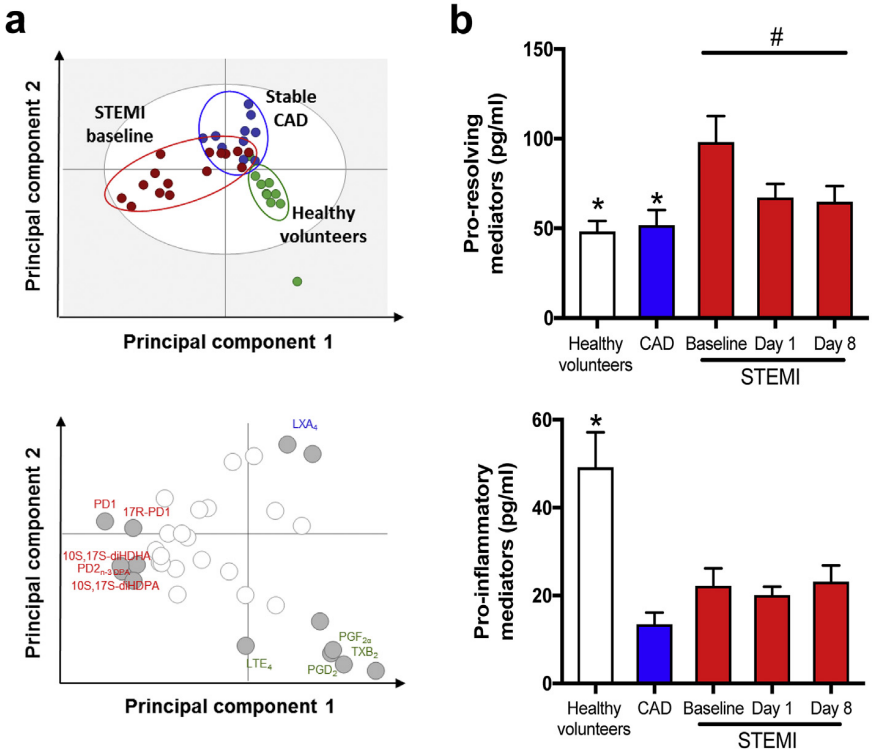

Fig. 2. Patients with STEMI have a distinct and early increase in SPM biosynthesis. Plasma from healthy volunteers ($n = 10$) and patients with stable CAD (n = 10) and STEMI ($n = 15$) were collected after MI onset. Repeated samples were drawn from STEMI patients. LM profiles were obtained using LC-MS/MS. (a) Partial least squares discriminant analysis of the LM-profiles. Top panel, two-dimensional score plot; lower panel, two-dimensional loading plot. Gray ellipse in the score plot denotes 95% CI regions. (b) Cumulative values of SPMs and pro-inflammatory LMs (prostaglandins, leukotrienes, and thromboxane B_2) after MI onset and during study follow up. All results are expressed as mean ± SEM. $^{*}p < 0{\cdot}05$, $^{**}p < 0{\cdot}01$, $^{***}p < 0{\cdot}001$ vs. STEMI baseline. $^{\#}p < 0{\cdot}05$, $^{\#\#}p < 0{\cdot}01$, $^{\#\#\#}p < 0{\cdot}001$ for repeated measures ANOVA for STEMI-baseline, day 1, and day 8.

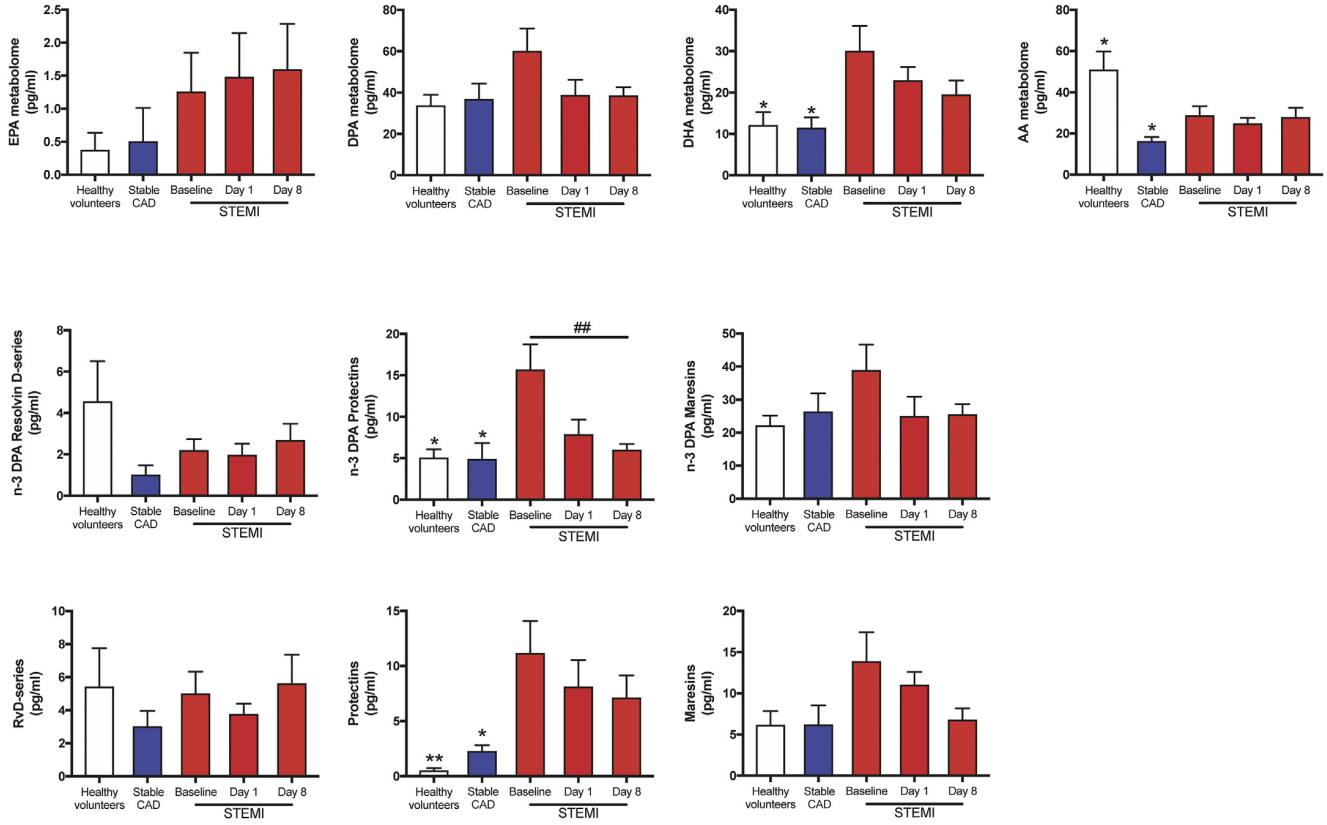

Fig. 3. STEMI patients have higher levels of n-3 DPA and DHA derived protectins. Plasma from healthy volunteers ($n = 10$) and patients with stable CAD ($n = 10$) and STEMI ($n = 15$) were collected after MI onset. Repeated samples were drawn from STEMI patients. LM were quantified using LC-MS/MS. (a) DHA, n-3 DPA, EPA, and AA metabolomes after MI onset and during study follow up. (b) n-3 DPA derived resolvin D-series, protectins, and maresins. (c) DHA-derived resolvin D-series, protectins, and maresins. EPA derived series is not included as there is only one series embedded in the metabolome. All results are expressed as mean ± SEM. $^{*}p < 0{\cdot}05$, $^{**}p < 0{\cdot}01$, $^{***}p < 0{\cdot}001$ vs. STEMI baseline. $^{\#}p < 0{\cdot}05$, $^{\#\#}p < 0{\cdot}01$, $^{\#\#\#}p < 0{\cdot}001$ for repeated measures ANOVA for STEMI-baseline, day 1, and day 8.

inflammatory LM (Fig. 2d), STEMI and CAD patients had lower plasma levels of the n-6 PUFA AA-derived PG family (i.e., PGD_2, PGE_2 and $PGF_{2\alpha}$) and thromboxane $(Tx)B_2$ compared with healthy volunteers (Fig. 5b). In fact, as all patients in the stable CAD and STEMI group were given 300 mg aspirin prior to the first blood sampling the observed pattern is in line with the mechanism of action of aspirin [23]. In addition to a decrease in AA-derived inflammatory LM, aspirin may trigger the biosynthesis of the pro-resolving aspirin triggered (AT)-SPM and notably, patients with STEMI had raised levels of AT-PD1 and AT-LXA_4 (Fig. 5c-d; Supplementary Table S1). There was a decline in AT-LXA_4 levels at day 1 following STEMI, but the differences were not significant, and forthcoming studies should clarify if these changes are real and not by chance.

3.6. Modulation of 5-LOX dependent mediators

As both pro-inflammatory leukotrienes (LT) and several SPM families, including Rv of the *E*-series, D-series, and T-series, are 5-LOX dependent pathways [7], we lastly looked into the balance and timing of their biosynthesis in STEMI. In terms of AA-derived 5-LOX products, LT levels as a whole were comparable in healthy controls and STEMI patients, but for 12-epi-6-*trans*-LTB_4, a biosynthetic pathway marker of the potent leukocyte chemoattractant LTB_4, the increase was significant (Fig. 6). Interestingly, SPMs in all the major 5-LOX dependent families were detected in circulation during STEMI and especially, RvT4 was the most abundant with peaking levels on day 8 ($p = 0{\cdot}03$ vs healthy volunteers). Of note we observed that the ratio of RvT4 to LTB_4 was higher after one week compared with MI onset, potentially reflecting a shift in the product profile of the 5-LOX enzyme from LTB_4 to the pro-resolving RvT4.

Having observed changes in both classic eicosanoid pathways as well as omega-3 derived SPM we next assessed whether there were changes in circulating of unesterified essential fatty acids that are substrates in the formation of the lipid mediators. In STEMI patients at baseline we found a ~ 5-fold increase in plasma n-3 DPA concentrations and ~2-fold increase in the concentrations of AA, EPA and DHA when compared with concentrations measured in healthy volunteers. Of note, levels of these fatty acids returned to those measured in healthy volunteers after 1 day. Thus, these findings highlight an acute increase in the plasma concentrations of lipid mediator substrates following STEMI.

3.7. SPM levels in relation to clinical characteristics

One control and one patient in each group (CAD and STEMI) used n-3 supplements prior to inclusion, but importantly, these individuals did not differ from the other individuals in their respective groups in relation to the actual SPM parameters (data not shown). Moreover, we cannot exclude that dietary differences (e.g., intake of fish) between the different patient groups could have influenced our findings. However, Supplementary Fig. S3 on circulating free fatty acid levels between the different patient groups show that the levels of all four fatty acids are elevated at baseline in STEMI patient, levels that rapidly return to concentration levels measured in both healthy volunteers and CAD patients. These data suggest that the increases in SPM concentrations are via an upregulation of SPM biosynthetic pathways and not merely reflecting difference in FA availability. Finally, the fact that only the CAD group included diabetic patients (20%) and only the STEMI group included

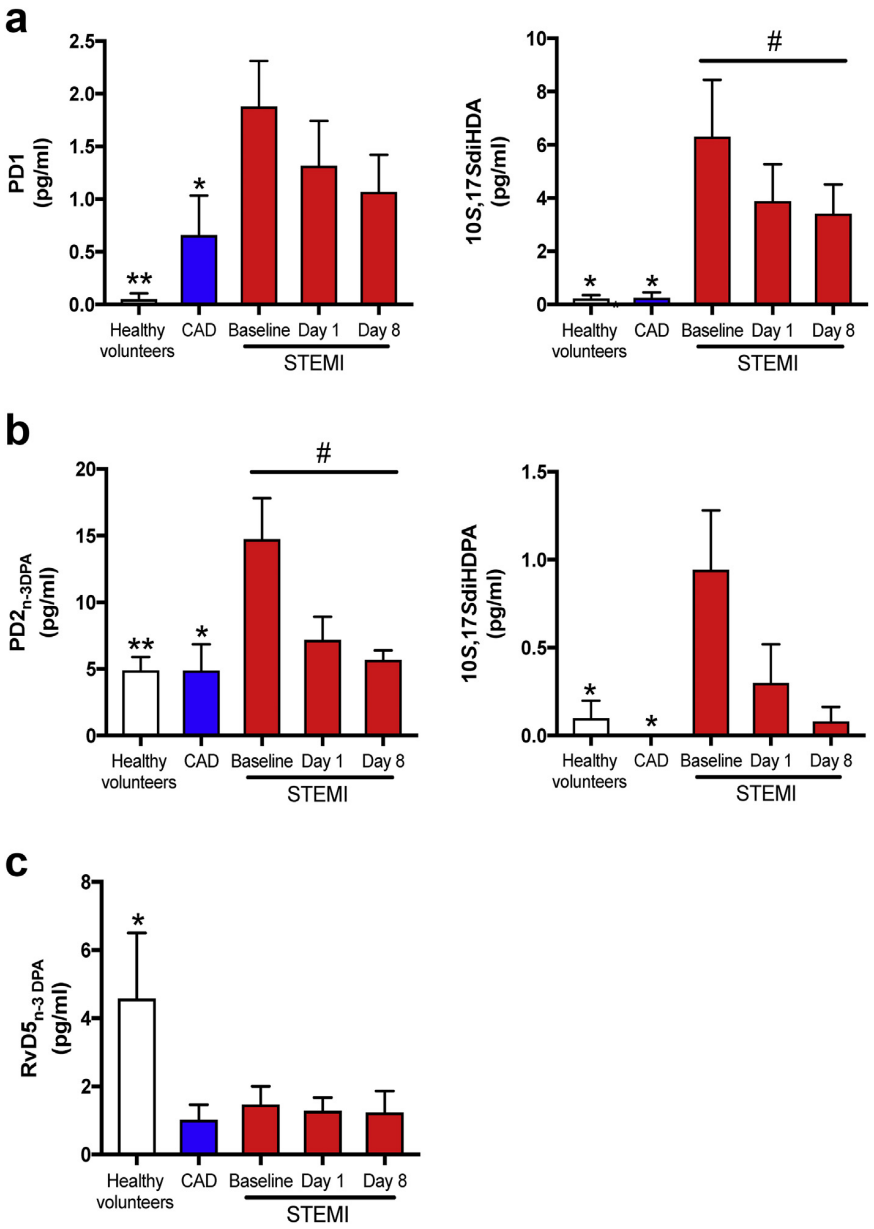

Fig. 4. Significantly modulated individual SPMs during STEMI. Plasma from healthy volunteers ($n = 10$) and patients with stable CAD ($n = 10$) and STEMI ($n = 15$) were collected after MI onset. Repeated samples were drawn from STEMI patients. LM were obtained using LC-MS/MS. (a) Quantification of PD1, 10*S*,17*S*-diHDA, (b) $PD2_{n\text{-}3\ DPA}$, 10*S*,17*S*diHDPA, and (c) $RvD5_{n\text{-}3\ DPA}$. Results are expressed as pg/ml and mean ± SEM $^{*}p < 0{\cdot}05$, $^{**}p < 0{\cdot}01$, $^{***}p < 0{\cdot}001$ vs. STEMI baseline. $^{\#}p < 0{\cdot}05$, $^{\#\#}p < 0{\cdot}01$, $^{\#\#\#}p < 0{\cdot}001$ for repeated measures ANOVA for STEMI-baseline, day 1, and day 8. LM, lipid mediators.

smokers (53%) is clearly a limitation of study, but importantly, patients in these subgroups (diabetic and smokers) did not differ from the other individuals in their respective groups in relation to the individual significantly regulated SPMs (Supplementary table S2).

4. Discussion

While data on the regulation of inflammatory pathways during MI is abundant, this is, to the best of our knowledge, the first report on the regulation of SPMs during acute MI. We show that circulating SPMs are markedly modulated at the onset of STEMI, indicating an early activation of resolution processes during MI. We found that circulating SPM levels peak within hours after onset of MI symptoms, even before the observed maximum release of hsTnT, corresponding with the early neutrophil response in circulation. Moreover, in contrast to this rise in pro-resolving mediators, there was a marked decline in pro-inflammatory PG and TxB_2 throughout the observation period, reflecting the use of aspirin as previously shown by others [22]. Finally, in contrast to the rapid increase in overall SPM biosynthesis, there seemed to be a delayed shift from pro-inflammatory to pro-resolving LM in the LOX-dependent pathway during STEMI. Our findings show that pro-resolving mechanisms are activated early during STEMI, underscoring that resolution of inflammation is an active and regulated process.

Our observations suggest that post-MI inflammation and wound healing programs are activated in parallel and indicate that the "inflammation breaks" are activated immediately after MI onset. Whereas this is the first report on resolution mediators during the acute phase of STEMI in humans, SPMs have been shown to modulate post-MI wound healing in experimental animal models [24–26]. Diminished resolution of inflammation worsened prognosis after experimental MI,

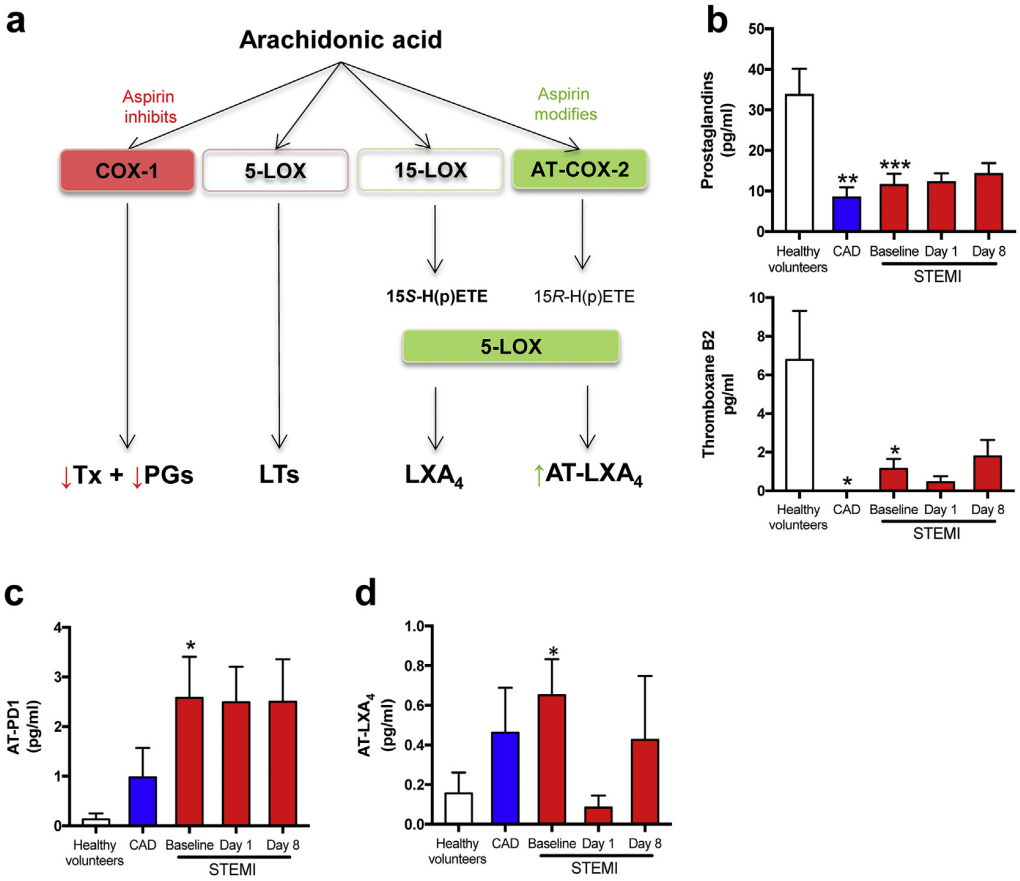

Fig. 5. Aspirin triggered SPM biosynthesis during STEMI. Plasma from healthy volunteers ($n = 10$) and patients with stable CAD ($n = 10$) and STEMI ($n = 15$) were collected after MI onset. Repeated samples were drawn from STEMI patients. LM were obtained using LC-MS/MS. (a) Biosynthetic pathways involved in AT-SPMs. (b) Quantification of prostaglandins, TxB_2, (c) AT-PD1 (d) AT-LXA_4. Results are expressed as pg/ml and as mean ± SEM. $^{*}p < 0{\cdot}05$, $^{**}p < 0{\cdot}01$, $^{***}p < 0{\cdot}001$ vs. STEMI baseline. $^{\#}p < 0{\cdot}05$, $^{\#\#}p < 0{\cdot}01$, $^{\#\#\#}p < 0{\cdot}001$ for repeated measures ANOVA for STEMI-baseline, day 1, and day 8. Refer to Table 1 for patient demographics.

as well as promoted plaque instability [5,24]. We have reported data in patients with stable CAD showing diminished levels of several SPMs [27] and demonstrated the diurnal regulation and lower levels of $RvD_{n\text{-}3\ DPA}$ in CAD patients admitted for PCI [13]. Herein we extend these findings by showing a much more marked regulation of SPMs in STEMI patients. Also, and most importantly, in contrast to the previously reported decreased RvD1 concentrations in stable atherosclerotic disorders, we found marked up-regulation of several SPMs immediately after onset of symptoms in STEMI patients, with a gradual decline during the first week. In contrast, however, the potential vasculo-protective $RvD5_{n\text{-}3\ DPA}$ was reduced during MI, and therapy that counteract this decrease could be of potential interest in STEMI patients.

The early SPM response in STEMI was mainly driven by the DHA- and n-3 DPA-derived protectin families, and especially PD1, 10*S*,17*S*-diHDA (PDX), $PD2_{n\text{-}3\ DPA}$, and 10*S*,17*S*-diHDPA were higher in STEMI patients shortly after MI onset. DHA derived protectins are formed via the stereoselective conversion of DHA by 15-LOX, with both PD1 and 10S,17S-diHDA (PDX) carrying anti-inflammatory properties [19]. Moreover, 10*S*,17*S*-diHDA (PDX) have been shown to inhibit TxA_2-induced platelet aggregation [28] and during mouse ischemia-reperfusion injury, PD1 administration before ischemia resulted in a reduction in functional and morphological kidney injury [29]. The n-3 DPA-derived protectins have been found to enhance the resolving capacity of macrophages, partly through induction of efferocytosis and a reprogramming of the macrophage phenotype [30]. Of note, $PD2_{n\text{-}3\ DPA}$ reduces neutrophil recruitment during sterile inflammation [14] and interestingly, in contrast to maresins that are mostly produced by pro-resolving (M2) macrophages, protectins may also be produced by neutrophils [6]. Indeed, herein $PD2_{n\text{-}3\ DPA}$, as well as PD1, were positively correlated with neutrophil counts shortly after MI onset, and it is tempting to hypothesize that the increase in the protectin levels may represent a counteracting mechanism to dampen the harmful effect of the initial rise in neutrophils following STEMI.

Aspirin irreversibly inhibits COX-1 with a subsequent reduced biosynthesis of pro-inflammatory LM. All our patients (STEMI and CAD) received aspirin prior to baseline sampling, and as expected we observed suppressed biosynthesis of COX-1 dependent pro-inflammatory LM in these patients (PGs and TxB_2). Furthermore, aspirin modifies the enzymatic activity of COX-2, where it promotes a shift in COX-2 activity to 15-LOX-like activity, with the consequential biosynthesis of AT-SPMs contributing to additional anti-inflammatory effects of aspirin [31]. Hence, the effects of low-dose aspirin beyond inhibition of PGs and thromboxane are becoming increasingly apparent. AT-PD1 exhibits significant higher metabolic stability than PD1 [19] and in our study AT-PD1 was clearly elevated throughout the observation period. Chiang et al. [22] showed that aspirin administered in doses ranging from 80 to 650 mg for 8 weeks to healthy volunteers both inhibited PG biosynthesis and increased AT-LXA_4 in circulation, the latter a pattern that was also observed when assessing plasma concentrations of STEMI patients when compared to healthy volunteers (Supplementary Table S1). Of note we also found that 1 day after aspirin administration the concentrations of this mediator were markedly reduced. Given that STEMI patients received a single dose of aspirin, these observations are in line with the transient effects that aspirin exerts on changing the activity of leukocyte COX-2. Indeed, at variance to the biological actions of aspirin on platelet COX-1 which last up to 2 weeks since platelet COX-1

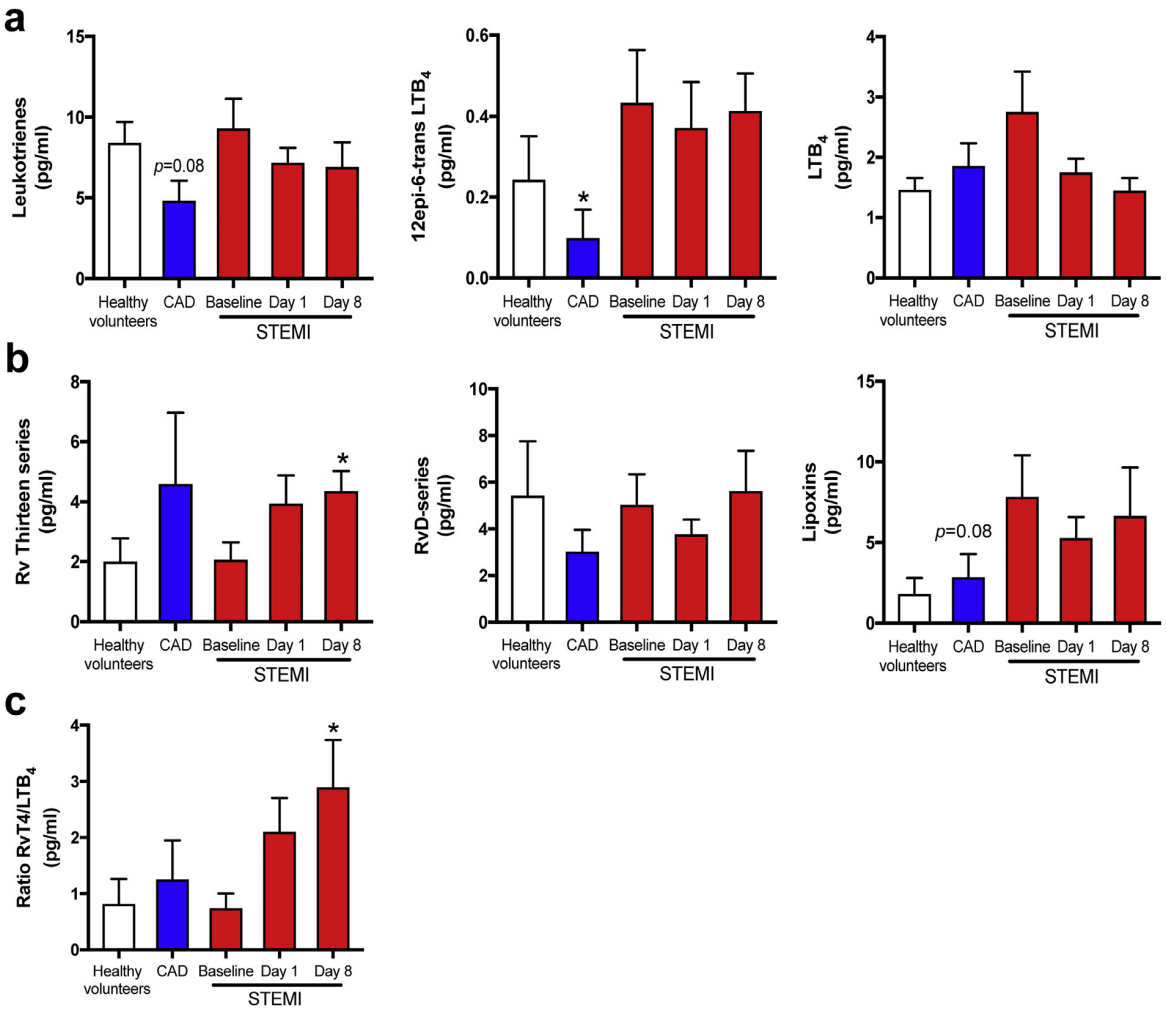

Fig. 6. Modulation of 5-LOX dependent mediators. Plasma from healthy volunteers ($n = 10$) and patients with stable CAD ($n = 10$) and STEMI ($n = 15$) were collected after MI onset. Repeated samples were drawn from STEMI patients. LM were obtained using LC-MS/MS. (a) AA-derived 5-LOX products. (b) n-3 PUFA-derived 5-LOX products (c) Ratio of RvT4 to LTB_4. Results are expressed as pg/ml and as mean ± SEM. $^{*}p < 0{\cdot}05$, $^{**}p < 0{\cdot}01$, $^{***}p < 0{\cdot}001$ vs. STEMI baseline. $^{\#}p < 0{\cdot}05$, $^{\#\#}p < 0{\cdot}01$, $^{\#\#\#}p < 0{\cdot}001$ for repeated measures ANOVA for STEMI-baseline, day 1, and day 8.

is not turned over in these cells, leukocyte COX-2, like many other leukocyte proteins, is turned over and therefore the acetylated protein is readily degraded.

In contrast to the early increase in 15-LOX products, we found a delayed increase in the biosynthesis of the 5-LOX SPMs. While 5-LOX activity may also be increased during the initial stages, this activity produces LTs. Later in the timeline, possibly regulated by protectins, 5-LOX remains in the cytosol where it produces RvTs. Moreover, Fredman et al. [7] also showed decreased levels of 5-LOX SPMs in unstable atherosclerotic lesion in both human samples and murine models of atherosclerosis and one might speculate if the late (1 week after MI) rise in 5-LOX SPMs could inhibit further plaque destabilization. Earlier reports have shown that 5-LOX deletion impairs wound healing and promotes cardiac rupture after MI, indicating a crucial role in post-MI inflammation [32]. If peak level was reached within the study observation period, or if the 5-LOX SPMs will continue to rise even further, is at present not clear and the importance of this "second hit" of SPMs needs to be further clarified. Of note, this increase in plasma SPM concentrations in the early phases post MI was linked with an increase in circulating free fatty acid concentrations, however the levels of these fatty acids returned to levels found in both healthy volunteers and CAD patients at a later stage (Supplementary Fig. S3). Thus, suggesting that underscoring a role selective regulation of these pathways in the latter stages post STEMI. Moreover, whereas the early changes in some of the SPM could be related to a marked increase in neutrophils, the cellular or tissue origin of the late increase in 5-LOX is at present not clear and should be explored in forthcoming studies.

4.1. Study limitations

This study has some important limitations. First of all there is a low number of individuals included that limit the impact of our findings. Moreover, the use of aspirin in all patients but not in any controls may clearly have influenced comparative analyses between patients and controls. Furthermore, diet may influence levels of various SPM, and the lack of detailed dietary information of the study population limit the interpretation of the data. Finally, correlation analyses do not necessarily document any causal relationship, and forthcoming studies should include more mechanistic studies.

5. Conclusion

This is the first report on the regulation of SPMs during acute MI in humans. Our findings show that pro-resolving mechanisms are increased early during STEMI, indicating that the "inflammation breaks" are activated immediately after MI onset. These findings could

contribute to the start of a new era in relation to targeting inflammation during MI, focusing not only on anti-inflammatory intervention, but also on enhancing the pro-resolving capacity.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ebiom.2019.07.024.

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Declaration of Competing of Interests

None declared.

Author contributions

Fosshaug, Linn E performed and planned study, did lab work, and wrote article.

Colas, Romain A performed lab work and statistical analyses.

Anstensrud, Anne K recruited patients and sampled data.

Gregersen, Ida recruited patients, sampled data, and did lab work.

Nymo, Ståle, performed and advised on statistical analyses.

Sagen, Ellen L performed lab work.

Michelsen, Annika performed lab work.

Vinge, Leif Erik planned study, clinical advice, and wrote article.

Øie, Erik planned study, clinical advice, and wrote article.

Gullestad, Lars sampling of data, biobanking, and conceptual work.

Halvorsen, Bente sampling of data, biobanking, and conceptual work.

Hansen, Trond V performed labwork, did conceptual work, and wrote article.

Aukrust, Pål wrote article, gave scientific insight, statistical analyses, and interpretation of data.

Dalli, Jess planned study, facilitated and performed lipid analyses, and wrote article.

Yndestad, Arne planned study, facilitated data biobanking and analyses, labwork, and wrote article.

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Supplementary material

Early increase of specialized pro-resolving lipid mediators in patients with ST-elevation myocardial infarction

Fosshaug LE et al.

Supplementary figure legends

Supplementary figure S1. Patients with STEMI have a distinct and early increase in SPM levels

Plasma from healthy volunteers (n=10) and patients with stable CAD (n=10) and STEMI (n=15) were collected after MI onset. Repeated samples were drawn from STEMI patients. LM profiles were obtained using LC-MS/MS. (a) Representative multiple reaction monitoring (MRM) chromatograms of the LM identified in healthy controls and patients with stable CAD and STEMI. Peak heights represent the relative levels of each LM. (b) Accompanying MS/MS spectra used for identification of PD1 and $PD2_{n\text{-}3\ DPA}$.

Supplementary figure S2. STEMI patients present with elevated inflammatory markers

Plasma from healthy volunteers (*n*=10) and patients with stable CAD (*n*=10) and STEMI (*n*=15) were collected after MI onset. Repeated samples were drawn from STEMI patients. The figure show plasma levels of interleukin (IL)6, IL8, and tumour necrosis factor (TNF). All results are expressed as mean±SEM. $^{**}p<0{\cdot}01$, $^{***}p<0{\cdot}001$ vs. STEMI baseline. $^{\#\#}p<0{\cdot}01$ for repeated measures ANOVA for STEMI-baseline, day 1, and 8.

Supplementary Figure S3. Increase in circulating free fatty acids in STEMI patients. Plasma from healthy volunteers (*n*=10) and patients with stable CAD (*n*=10), and STEMI (*n*=15) were collected after MI onset and circulating unesterrified concentrations of (a) DHA, (b) n-3 DPA (c) EPA and (d) AA were quantified using lipid mediator profiling. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001 using ANOVA followed by Mann Withney test for multiple comparisons.

Supplementary Tables

Supplementary Table S1. MI onset and temporal lipid mediators identified in STEMI, stable CAD, and healthy volunteers

	MRM transition		Lipid mediator levels (plasma; pg/ml)				
			Healthy	Stable	STEMI		
	Q1	Q3	volunteers	CAD	Baseline	Day 1	Day 8
DHA Bioactive Metabolome							
Resolvin D-series							
RvD1	***375***	***215***	2·3 ± 1·5	0·0 ± 0·0	1·0 ± 0·8	0·2 ± 0·1	0·1 ± 0·1
RvD2	***375***	***141***	0·0 ± 0·0	0·0 ± 0·0	0·0 ± 0·0	0·0 ± 0·0	0·1 ± 0·1
RvD3	***375***	***147***	0·0 ± 0·0	0·0 ± 0·0	0·0 ± 0·0	0·0 ± 0·0	0·0 ± 0·0
RvD4	***375***	***101***	1·8 ± 1·0	2·1 ± 1·1	1·3 ± 0·7	1·3 ± 0·6	3·1 ± 1·8
RvD5	***359***	***199***	0·1 ± 0·1	0·1 ± 0·1	1·3 ± 0·7	0·3 ± 0·2	0·8 ± 0·4
RvD6	***359***	***159***	1·3 ± 0·3	0·8 ± 0·5	1·4 ± 0·6	2·0 ± 0·6	1·7 ± 0·7
AT-RvD1	***375***	***233***	0·0 ± 0·0	0·0 ± 0·0	0·0 ± 0·0	0·0 ± 0·0	0·0 ± 0·0
AT-RvD3	***375***	***147***	0·0 ± 0·0	0·0 ± 0·0	0·0 ± 0·0	0·0 ± 0·0	0·0 ± 0·0
Protectins							
PD1	***359***	***153***	0·1 ± 0·1**	0·7 ± 0·4*	1·9 ± 0·4	1·3 ± 0·4	1·1 ± 0·4
AT-PD1	***359***	***153***	0·1 ± 0·1*	1·0 ± 0·6	2·6 ± 0·8	2·5 ± 0·7	2·6 ± 0·9
10*S*,17*S*diHDHA	***359***	***153***	0·2 ± 0·1*	0·3 ± 0·2*	6·3 ± 2·2	3·9 ± 1·4	3·5 ± 1·2
22-OH-PD1	***375***	***153***	0·1 ± 0·1	0·4 ± 0·3	0·4 ± 0·2	0·4 ± 0·2	0·1 ± 0·1
PCTR1	***650***	***231***	0·0 ± 0·0	0·0 ± 0·0	0·0 ± 0·0	0·0 ± 0·0	0·0 ± 0·0
PCTR2	***521***	***231***	0·0 ± 0·0	0·0 ± 0·0	0·0 ± 0·0	0·0 ± 0·0	0·0 ± 0·0
PCTR3	***446***	***231***	0·0 ± 0·0	0·0 ± 0·0	0·0 ± 0·0	0·0 ± 0·0	0·0 ± 0·0
Maresins							
MaR1	***359***	***221***	3·1 ± 1·5	2·7 ± 1·3	5·2 ± 2·1	7·0 ± 1·5	3·0 ± 1·0
MaR2	***359***	***191***	0·0 ± 0·0	0·0 ± 0·0	0·2 ± 0·2	0·0 ± 0·0	0·1 ± 0·1
22-OH-MaR1	***375***	***221***	0·0 ± 0·0	0·0 ± 0·0	0·0 ± 0·0	0·0 ± 0·0	0·0 ± 0·0
14-oxo-MaR1	***357***	***248***	0·0 ± 0·0*	0·0 ± 0*	0·8 ± 0·3	0·3 ± 0·2	0·8 ± 0·3
7*S*,14*S*diHDHA	***359***	***221***	2·3 ± 1·1	1·6 ± 1·1	5·5 ± 2·1	2·3 ± 1·3	1·7 ± 1·0
4*S*,14*S*diHDHA	***359***	***101***	0·8 ± 0·4	1·9 ± 1·1	2·2 ± 0·9	1·4 ± 0·5	1·3 ± 0·5
MCTR1	***650***	***191***	0·0 ± 0·0	0·0 ± 0·0	0·0 ± 0·0	0·0 ± 0·0	0·0 ± 0·0
MCTR2	***521***	***191***	0·0 ± 0·0	0·0 ± 0·0	0·0 ± 0·0	0·0 ± 0·0	0·0 ± 0·0
MCTR3	***446***	***191***	0·0 ± 0·0	0·0 ± 0·0	0·0 ± 0·0	0·0 ± 0·0	0·0 ± 0·0
n-3 DPA Bioactive Metabolome							

Resolvin thirteen series							
RvT1	***377***	***211***	0·0 ± 0·0	0·0 ± 0·0	0·0 ± 0·0	0·0 ± 0·0	0·0 ± 0·0
RvT2	***377***	***255***	0·8 ± 0·5	0·7 ± 0·6	0·2 ± 0·2	0·4 ± 0·2	0·7 ± 0·5
RvT3	***377***	***197***	0·0 ± 0·0	0·0 ± 0·0	0·1 ± 0·1	0·1 ± 0·1	0·0 ± 0·0
RvT4	***361***	***193***	1·2 ± 0·7	3·9 ± 2·1	1·8 ± 0·6	3·4 ± 1·0	3·6 ± 0·8
Resolvin D-series							
$RvD1_{n3\ DPA}$	***377***	***143***	0·0 ± 0·0	0·0 ± 0·0	0·0 ± 0·0	0·0 ± 0·0	0·0 ± 0·0
$RvD2_{n3\ DPA}$	***377***	***261***	0·0 ± 0·0	0·0 ± 0·0	0·7 ± 0·4	0·7 ± 0·4	1·4 ± 0·8
$RvD5_{n3\ DPA}$	***361***	***199***	4·6 ± 2·0*	1·0 ± 0·5	1·5 ± 0·5	1·3 ± 0·4	1·3 ± 0·7
Protectins							
$PD1_{n3\ DPA}$	***361***	***183***	0·1 ± 0·1	0·0 ± 0·0	0·0 ± 0·0	0·4 ± 0·2	0·2 ± 0·2
$PD2_{n3\ DPA}$	***361***	***263***	4·9 ± 1·0**	4·9 ± 2·1*	14·7 ± 3·2	7·2 ± 1·8	5·7 ± 0·8
10I,17*S*diHDPA	***361***	***183***	0·1 ± 0·1*	0·0 ± 0·0*	0·9 ± 0·3	0·3 ± 0·2	0·1 ± 0·1
Maresins							
$MaR1_{n3\ DPA}$	***361***	***205***	0·1 ± 0·1	0·6 ± 0·3	0·3 ± 0·2	0·4 ± 0·4	0·8 ± 0·4
$MaR2_{n3\ DPA}$	***361***	***193***	21·8 ± 3·1*	25·0 ± 5·6	39·9 ± 8·3	24·0 ± 5·8	24·5 ± 3·2
7*S*,14*S*diHDPA	***361***	***205***	0·3 ± 0·2*	0·8 ± 0·4*	0·0 ± 0·0	0·6 ± 0·3	0·4 ± 0·2
EPA Bioactive Metabolome							
Resolvin E-series							
RvE1	***349***	***161***	0·0 ± 0·0	0·0 ± 0·0	0·0 ± 0·0	0·2 ± 0·2	0·6 ± 0·5
RvE2	***333***	***199***	0·0 ± 0·0	0·0 ± 0·0	0·0 ± 0·0	0·2 ± 0·2	0·0 ± 0·0
RvE3	***333***	***201***	0·4 ± 0·3	0·5 ± 0·5	1·3 ± 0·6	0·9 ± 0·4	1·1 ± 0·5
AA Bioactive Metabolome							
Lipoxins							
LXA_4	***351***	***115***	0·1 ± 0·0	0·2 ± 0·1*	0·0 ± 0·0	0·1 ± 0·1	0·1 ± 0·0
LXB_4	***351***	***115***	0·0 ± 0·0	0·5 ± 0·5	0·4 ± 0·5	1·2 ± 0·6	0·0 ± 0·0
5·15-diHETE	***335***	***235***	1·4 ± 1·1	1·7 ± 1·2	6·6 ± 2·7	3·8 ± 1·2	6·4 ± 3·3
AT-LXA_4	***351***	***115***	0·2 ± 0·1*	0·5 ± 0·2	0·7 ± 0·2	0·1 ± 0·1	0·5 ± 0·4
AT-LXB_4	***351***	***221***	0·0 ± 0·0	0·0 ± 0·0	0·0 ± 0·0	0·0 ± 0·0	0·0 ± 0·0
13·14-dehydro-15-oxo-LXA_4	***351***	***217***	0·2 ± 0·1	0·0 ± 0·0	0·1 ± 0·1	0·0 ± 0·0	0·1 ± 0·1
15-oxo-LXA_4	***349***	***115***	0·0 ± 0·0	0·0 ± 0·0	0·0 ± 0·0	0·0 ± 0·0	0·0 ± 0·0
Leukotrienes							
LTB_4	***335***	***195***	1·5 ± 0·2	1·9 ± 0·4	2·8 ± 0·7	1·8 ± 0·2	1·5 ± 0·2
5*S*,12*S*diHETE	***335***	***195***	0·0 ± 0·0	0·1 ± 0·1	0·3 ± 0·2	0·0 ± 0·0	0·1 ± 0·1

6-trans-LTB_4	***335***	***195***	0·0 ± 0·0	0·0 ± 0·0	0·1 ± 0·1	0·1 ± 0·1	0·0 ± 0·0
12-epi-6-trans-LTB_4	***335***	***195***	0·2 ± 0·1	0·1 ± 0·1*	0·4 ± 0·1	0·4 ± 0·1	0·4 ± 0·1
20-OH-LTB_4	***351***	***195***	0·1 ± 0·1	0·0 ± 0·0	0·1 ± 0·1	0·1 ± 0·1	0·0 ± 0·0
20-COOH-LTB_4	***365***	***195***	0·0 ± 0·0	0·1 ± 0·1	0·0 ± 0·0	0·0 ± 0·0	0·2 ± 0·1
LTC_4	***626***	***189***	0·0 ± 0·0	0·0 ± 0·0	0·0 ± 0·0	0·0 ± 0·0	0·0 ± 0·0
LTD_4	***497***	***189***	0·0 ± 0·0	0·0 ± 0·0	0·0 ± 0·0	0·0 ± 0·0	0·0 ± 0·0
LTE_4	***440***	***189***	6·6 ± 1·2	2·7 ± 1·4	5·7 ± 1·3	4·8 ± 0·8	5·0 ± 1·6
Prostaglandins							
PGD_2	***351***	***189***	7·4 ± 2·0*	2·4 ± 0·7	3·3 ± 0·7	1·7 ± 0·4	2·3 ± 0·7
PGE_2	***351***	***189***	9·6 ± 3·1**	0·3 ± 0·3	0·6 ± 0·4	0·4 ± 0·2	1·3 ± 0·5
$PGF_{2\alpha}$	***353***	***193***	16·9 ± 3·2*	5·9 ± 2·0	7·7 ± 2·3	10·3 ± 1·9	10·9 ± 2·7
TXB_2	***369***	***169***	6·8 ± 2·6**	0·0 ± 0·0	1·2 ± 0·5	0·5 ± 0·3	1·9 ± 0·9

Quantification and values obtained in plasma of STEMI patients, stable CAD patients, and healthy volunteers. Specific bioactive lipid mediator and precursor/pathway markers where: Q1: M-H (parent ion) and Q3 (daughter ion): diagnostic ion in the MS-MS along with mean ± SEM values for each of the mediators identified. Results are expressed as pg/ml and the detection limit was 0·1 pg/ml. All results are expressed as mean ± SEM. *p<0·05. **p<0·01. ***p<0·001 vs. STEMI baseline. $^{\#}p$<0·05 $^{\#\#}p$<0·01. $^{\#\#\#}p$<0·001 for repeated measures ANOVA for STEMI-baseline, day 1, and day 8. LM, lipid mediators. DHA, docosahexaenoic acid; Rv, resolvin; AT, aspirin triggered; PD, Protectin; MaR, maresin; EPA, ecosapentaenoic acid; AA, arachidonic acid; LX, lipoxins; LT, leukotriene; PG, prostaglandin; TX, thromboxane.

Supplementary table S2. Coronary artery disease and smoking did not impact the levels of significantly regulated SPMs

	STEMI		CAD	
	Non smokers (*n*=7)	**Smokers (*n*=8)**	**No diabetes (*n*=8)**	**Diabetes (*n*=2)**
PD1	1.7±0.6	2.0±0.7	0.7±0.5	0.5±0.5
10*S*,17*S*diHDHA	4.5±2.8	7.9±3.2	0.2±0.2	0.4±0.4
$PD2_{n3\ DPA}$	16.8±6.1	13.0±2.5	5.7±2.4	1.8±1.8
10I,17*S*diHDPA	1.2±0.6	0.7±0.3	0±0	0±0
$RvD5_{n3\ DPA}$	1.2±0.9	1.7±0.7	1.3±0.5	0±0

Values for each of the significantly regulated mediators identified. Results are expressed as pg/ml and the detection limit was 0·1 pg/ml. All results are expressed as mean±SEM. *p<0·05. STEMI,