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Targeting proinflammatory mechanisms in atherosclerotic stroke

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Luka Živković

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Erstes Gutachten:	Prof. Dr. Martin Dichgans
Zweites Gutachten:	Prof. Dr. Konstantin Stark
Drittes Gutachten:	Prof. Dr. Anna-Sophia Wahl-Ommer

Dekan: Prof. Dr. med. Thomas Gudermann

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München, den 16. Oktober 2025 Luka Živković
Munich, October 16, 2025

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List of abbreviations

AIM2:	Absent in melanoma 2
ApoE:	Apolipoprotein E
BTK:	Bruton's tyrosine kinase
CCL2:	C-C chemokine ligand 2
CCR2:	C-C chemokine receptor 2
CXCL10:	C-X-C chemokine ligand 10
CXCR3:	C-X-C chemokine receptor 3
Casp-1:	Caspase-1
HDAC9:	Histone deacetylase 9
hsCRP:	High-sensitivity CRP
IKKβ:	IκB kinase beta
IL-1β:	Interleukin-1 beta
IL-6:	Interleukin-6
IL-18:	Interleukin 18
JNK1:	C-Jun-N-terminal kinase 1
LDL:	Low-density lipoprotein
MACE:	Major adverse cardiovascular event(s)
NEK7:	NIMA related kinase 7
NF-κB:	Nuclear factor kappa B
NLRP3:	NOD like receptor family pyrin domain containing 3
PRR:	Pattern recognition receptor
TLR:	Toll-like receptor
TNFα:	Tumor necrosis factor alpha

List of publications

1. **Živković L**, Asare Y, Bernhagen J, Dichgans M, Georgakis MK. THERAPEUTIC TARGETING OF THE CCL2/CCR2 AXIS ALLEVIATES ATHEROSCLEROSIS IN MICE: A META-ANALYSIS OF PRECLINICAL STUDIES. *European Stroke Journal* 2021;6:1 suppl. p.61. doi:10.1177/23969873211034932 *(Conference Abstract*)

2. **Živković L**, Asare Y, Bernhagen J, Dichgans M, Georgakis MK. Pharmacological Targeting of the CCL2/CCR2 Axis for Atheroprotection: A Meta-Analysis of Preclinical Studies. *Arteriosclerosis, thrombosis, and vascular biology.* 2022;42:e131-e144. doi: doi:10.1161/ATVBAHA.122.317492

3. Asare Y, Shnipova M, **Živković L**, Schlegl C, Tosato F, Aronova A, Brandhofer M, Strohm L, Beaufort N, Malik R, et al. IKKβ binds NLRP3 providing a shortcut to inflammasome activation for rapid immune responses. *Signal Transduction and Targeted Therapy*. 2022;7:355. doi: 10.1038/s41392-022-01189-3

4. Prapiadou S, **Živković L**, Thorand B, George MJ, Laan SWvd, Malik R, Herder C, Koenig W, Ueland T, Kleveland O, et al. Proteogenomic Data Integration Reveals CXCL10 as a Potentially Downstream Causal Mediator for IL-6 Signaling on Atherosclerosis. *Circulation.* 2024;149:669-683. doi: doi:10.1161/CIRCULATIONAHA.123.064974

Underlined are publications included in this dissertation.

Summary

Stroke is one of the leading causes of death and disability worldwide. Ischemic stroke, the most common subtype, occurs when a brain-supplying artery is suddenly occluded, limiting the supply of oxygen and nutrients to the affected central nervous tissue. Around 20% of all ischemic strokes are attributed to large artery atherosclerosis and require the management of cardiovascular risk factors for both primary and secondary prevention.

Atherosclerosis is initiated by hemodynamic damage to the endothelial lining, which results in the accumulation of lipids in the arterial intima layer and elicits an innate immune response that gradually derails due to the insufficient clearing of the accumulating lipids and the resulting inflammation. Over time, atherosclerotic plaques begin to convey a risk of erosion and rupture, which, in turn, cause thrombosis and occlusion of distal arteries. Proneness to rupture is, among other factors, determined by the activity of pro-inflammatory signaling pathways through modulating plaque composition; high intraplaque inflammation is commonly associated with vulnerable plaque phenotypes.

The C-C chemokine ligand 2/C-C chemokine receptor 2 (CCL2/CCR2) axis is a mainstay of atherogenic inflammation that facilitates the invasion of circulating monocytes into atherosclerotic plaques. Epidemiologic and genetic studies have pinpointed CCL2 concentrations and activity as conveying risk of atherosclerotic disease, while animal models of CCL2/CCR2 inactivation revealed decreased plaque burden, suggesting the CCL2/CCR2 axis as a potential anti-inflammatory drug target.

The Nuclear factor-kappa B (NF-κB) pathway, a key regulator of innate immunity, drives CCL2 expression along with a large amount of other pro-inflammatory genes. Its signaling relies on the activity of the IKK kinases. NF-κB is also responsible for the expression of NLRP3, a pattern recognition receptor that, once activated, causes the production and release of Interleukin-1 beta (IL-1β). IL-1β is a potent cytokine that upregulates the local and systemic immune response through further activation of NF-κB and is thus essential for atherogenic inflammation.

IL-1β also licenses the expression of IL-6, a cytokine with wide-reaching systemic effects that governs the acute phase reaction in inflammation. Like CCL2 and IL-1β, IL-6 has been causally implicated in atherosclerotic disease by converging preclinical, epidemiological and genetic evidence. The utility of IL-6 and the acute phase C-reactive protein (CRP) as circulating biomarkers for risk stratification in atherosclerotic diseases has been demonstrated in large cohort studies.

Drugs targeting pro-inflammatory mechanisms in coronary artery disease and stroke have entered clinical trials and, in the case of Colchicine, received regulatory approval

for the prevention of cardiovascular events in high-risk patients. We identify three obstacles remaining in the way of further clinical translation, namely (1) the lack of systematic overviews of studies testing anti-inflammatory agents in animal models for scrutinizing and prioritizing drug targets, (2) the need for exploration of interactions between different molecular actors of inflammation in atherosclerosis for the discovery of druggable mechanisms, and (3) the need for characterizing the downstream effects of interleukin signaling inhibition for the identification of suitable athero-specific targets.

In Publication I, we systematically searched the available literature for studies investigating direct pharmacological inhibition of CCL2 or CCR2 in animal models of atherosclerosis. Pooling 14 mouse studies of 11 different agents in random-effects meta-analyses, we observed a significant reduction in plaque burden across multiple vascular frames and an increase in composition features conveying plaque stability. We also noted that the reduction of plaque size correlated with reduction of plaque macrophage content, confirming the role of CCL2/CCR2 for macrophage accumulation, and no differences in efficacy were observed when comparing ligand and receptor inhibition. In Publication II, we investigated the role of the NF-κB kinase IKKβ on NLRP3 inflammasome priming and activation. We detected direct mechanistic binding of IKKβ to NLRP3 in both overexpressed and endogenous cell cultures that was further confirmed using in-vitro binding assays, colocalization imaging and proteomic profiling. In murine macrophages, inhibition of IKKβ using a small-molecule agent and short interfering RNA (siRNA) suppressed NLRP3 activation and abrogated IL-1β maturation. To uncouple the putative effect of IKKβ for NLRP3 activation from its NF-κB-mediated effects on NLRP3 priming, we inhibited IKKβ in rapid priming and post-priming inhibition experiments, maintaining the observed effect.

In Publication III, we examined the proteomic signature of genetically proxied IL-6 receptor (IL-6R) signaling inhibition, revealing 70 differentially expressed circulating proteins. After examining the association of genetic instruments proxying circulating levels of these proteins with cardiovascular outcomes, we found C-X-C motif chemokine ligand 10 (CXCL10) to be associated with risk of coronary artery disease (CAD), large-artery atherosclerotic stroke and peripheral artery disease. We then looked at associations between circulating levels of CXCL10 and major adverse cardiovascular events (MACE) in a population-based cohort, noting that individuals with higher CXCL10 levels exhibited higher risk of MACE. Lastly, we observed increased lipid core size and a pro-inflammatory transcriptomic profile in carotid artery plaques with increased CXCL10 expression, suggesting a causal role in atherosclerosis.

Taken together, our findings further solidify the role of pro-inflammatory mechanisms in atherosclerotic stroke while aiming to inform the design of future clinical trials.

Zusammenfassung

Der Schlaganfall ist eine der weltweit führenden Ursachen für Tod und Behinderung. Sein häufigster Subtyp, der ischämische Schlaganfall, ist definiert durch einen plötzlichen Verschluss hirnversorgender Arterien und die darauffolgende Unterbrechung der Sauerstoff- und Nährstoffzufuhr des betroffenen Nervengewebes. Etwa 20% aller ischämischen Schlaganfälle sind makroangiopathischer, genauer atherosklerotischer Genese und erfordern daher die Reduktion kardiovaskulärer Risikofaktoren als primär- und sekundärpräventive Maßnahmen.

Atherosklerose wird als Antwort auf hämodynamische Beanspruchung des arteriellen Endothels gesehen, welche zur Einlagerung von Lipiden in die Gefäßwand und einer zunehmend entgleisenden Entzündungsreaktion führt. Atherosklerotische Plaques ziehen mit der Zeit das Risiko der Ruptur und Erosion nach sich, welche Thrombosen und schlussendlich Ischämien nach sich verursachen. Maßgeblich beeinflusst wird die Plaquestabilität durch die Aktivität pro-inflammatorischer Signalwege und ihren Einfluss auf Morphologie und Zusammensetzung; dabei ist ein hohes Entzündungsniveau innerhalb der Plaque klassischerweise mit Vulnerabilitätsmerkmalen assoziiert.

Die CC-Chemokin-Ligand-2/CC-Chemokin-Rezeptor-2 (CCL2/CCR2)-Achse spielt eine tragende Rolle in der Invasion von Monozyten, den Vorläuferzellen von Makrophagen, aus der Blutbahn in die Gefäßwand. Epidemiologische und genetische Studien haben CCL2-Spiegel und -Aktivität als kausalen Risikofaktor für atherosklerotische Krankheitsbilder nahegelegt, während Tierstudien Reduktionen in Größe und Anzahl von Plaques nach Ausschaltung der CCL2/CCR2-Achse erbringen konnten. Diese Belege sprechen für eine Rolle der CCL2/CCR2-Achse als therapeutischen Angriffspunkt in Atherosklerose.

Der NF-kappaB (NF-κB)-Signalweg, ein Schlüsselspieler des angeborenen Immunsystems, reguliert die Genexpression von CCL2 gemeinsam mit zahlreichen anderen pro-inflammatorischen Genen. Innerhalb des NF-κB-Signalwegs sind insbesondere die IKK-Kinasen maßgeblich für die Aufrechterhaltung der Signalkaskade. NF-κB ist außerdem verantwortlich für die Expression von NLRP3, einem Pattern-Regocnition-Rezeptor, welcher in seinem aktivierten Zustand Interleukin 1β (IL-1β) produziert und freisetzt. IL-1β ist ein potentes Zytokin, welches NF-κB weiter aktiviert und damit sowohl die lokale als auch die systemische Entzündungsantwort hochreguliert; IL-1β ist daher essenziell für Entzündung in Atherosklerose.

IL-1β reguliert hierdurch auch die Expression von IL-6, einem Zytokin, welchem durch die Kontrolle der Akute-Phase-Reaktion eine weitreichende Bedeutung in der systemischen Immunantwort eingeräumt wird. Wie CCL2 und IL-1β konnte auch IL-6 durch vorklinische, epidemiologische und genetische Studien kausal in der Pathophysiologie der Atherosklerose verankert werden. Der Nutzen von IL-6- und CRP-Spiegeln in der Risikostratifizierung für atherosklerotische Erkrankungen wurde in großen Kohortenstudien vielfach dargelegt.

Die Entwicklung und Testung anti-inflammatorischer Pharmaka gegen die Koronare Herzkrankheit (KHK) und den Schlaganfall hat klinische Studienphasen und im Fall Colchicin sogar die Zulassung in der Sekundärprävention des akuten Myokardinfarkts erreicht. Nichtsdestotrotz verbleibt die klinische Translation an einigen Punkten auf der Stelle. Zu den Ursachen hierfür zählen wir einen Mangel an systematischen Übersichtsarbeiten präklinischer Studien mit anti-inflammatorischen Therapieansätzen als Thema, fehlende Erkenntnisse über Interaktionen zwischen den verschiedenen molekularen Akteuren in Atherosklerose sowie die weitgehend ausgebliebene Erforschung nachgeschalteter Effekte unter Interleukin-Inhibition.

In Publikation I durchsuchten wir etablierte Literaturdatenbanken nach Studien, welche über experimentelle Daten zur Inhibition der CCL2/CCR2-Achse in Atherosklerose-Tiermodellen verfügen. Nach der Auswertung von 14 Mausstudien von 11 Inhibitoren in Zufallseffekt-Metaanalysen stellten wir eine signifikante Abnahme der Plaquebelastung und eine signifikante Zunahme histologischer Stabilitätsmerkmale unter CCL2/CCR2-Inhibition fest. Wir beobachteten außerdem eine Korrelation der Reduktion von Plaquegröße und Makrophagengehalt, einhergehend mit der Rolle der CCL2/CCR2-Achse in der Akkumulation von Monozyten. Ob CCL2 oder CCR2 pharmakologisch inhibiert wurden, ergab unseren Ergebnissen zufolge keinen Unterschied mit Blick auf Atherosklerose-hemmende Effekte.

In Publikation II untersuchten wir die Rolle der NF-κB-Kinase IKKβ in der Bahnung und Aktivierung von NLRP3. Wir beobachteten eine direkte, mechanistische Interaktion zwischen IKKβ und NLRP3 sowohl in Überexpressions- als auch in endogenen Zellkulturmodellen, welche durch in-vitro-Bindungsassays, Kolokalisierungsversuche und proteomische Untersuchungen weiter bestätigt werden konnte. Die pharmakologische Ausschaltung von IKKβ in murinen Makrophagen führte zur Aussetzung der NLRP3-Aktivierung und Aufhebung der IL-1β-Produktion. Diese Effekte waren auch zu beobachten, als wir durch Modifizierung des Inhibitionsansatzes den direkten, mechanistischen Effekt von IKKβ auf NLRP3 von dem durchaus zu erwartenden, NF-κB-vermittelten Bahnungseffekt entkoppelten.

In Publikation III analysierten wir unter Zuhilfenahme eines Mendelschen Randomisierungsansatzes proteomische Merkmale reduzierter IL-6-Rezeptor (IL6-R) -Aktivität. Hierbei beobachteten wir unterschiedliche Expressionsprofile für 70 zirkulierende Proteine. Daraufhin konstruierten wir genetische Instrumente stellvertretend für die Blutspiegel dieser Proteine und untersuchten ihre Assoziation mit kardiovaskulären Erkrankungen. Dabei beobachteten wir für den CX-Chemokin-Liganden 10 (CXCL10) eine Assoziation mit erhöhtem Risiko für KHK und makroangiopathischem Schlaganfall. Als nächstes betrachteten wir CXCL10-Spiegel in einer bevölkerungsbezogenen Studie, wobei diese abermals mit kardiovaskulären Ereignissen assoziierten. Schließlich legten wir in einer Auswertung von Plaqueproben aus humanen Carotiden dar, dass Plaques mit höherer Expression von CXCL10 einem höheren Risiko für große Lipidkerne und einem pro-inflammatorischen transkriptomischen Profil ausgesetzt sind, was auf eine kausale Rolle in Atherosklerose deutet.

Abschließend betrachtet festigen unsere Ergebnisse die Bedeutung proinflammatorischer Mechanismen für die Entstehung des atherosklerotischen Schlaganfalls und liefern Erkenntnisse für die Planung künftiger klinischer Studien.

1. Introduction

1.1 Epidemiology, pathophysiology, treatment and prevention of stroke

Stroke is caused by the sudden restriction of blood supply to central nervous tissue, leading to temporary or permanent disability and death. The Global Burden of Disease Study estimated that in 2021 7.3 million deaths worldwide were caused by stroke, making it the third most common cause of death.[1] Approximately one in 100 humans alive in 2021 has survived a stroke in their lifetime and is at risk of recurrence. Stroke also continues to be one of the leading global causes of disability-adjusted life years (DALYs). Macroeconomic implications of stroke were estimated to be about $2.1 trillion in 2019.[2]

Strokes are either ischemic or hemorrhagic in nature depending on the pathophysiological mechanism. Ischemic stroke results from the obstruction of a brain-supplying artery. Hemorrhagic stroke arises from the rupture of an artery in the subarachnoid space (then termed subarachnoid hemorrhage, SAH) or inside the brain parenchyma as an intracerebral hemorrhage (ICH).[3]

Ischemic stroke comprises the majority of stroke cases.[1] According to the TOAST classification, ischemic strokes are further categorized according to cause of infarction as either large-artery atherosclerosis, cardioembolism, small vessel occlusion, other or undetermined (cryptogenic) etiology.[4] Large artery atherosclerosis, requiring a grade of luminal stenosis above 50%, is determined to be causal in about one in five ischemic strokes; however, imaging studies of cryptogenic stroke patients suggested non-stenosing atherosclerotic plaques in carotid arteries as potential culprits for a substantial proportion of cases.[5-7]

The neuronal tissue of the central nervous system is particularly vulnerable to ischemia. Once arterial blood flow is suddenly restricted, the resulting hypoxia causes rapid depletion of cellular energy reserves, which are required to maintain a polarized membrane. Irreversible neuronal damage begins within minutes and progresses throughout the entire ischemic vascular territory if reperfusion is not established. The infarct core, which comprises unsalvageable brain tissue, undergoes necrosis and becomes highly susceptible to hemorrhage due to the resulting blood-brain barrier disruption, placing initially unaffected tissue at risk.

Thus, the acute management of ischemic stroke focuses on the timely reperfusion of the affected vascular territory, aiming to rescue as much affected neuronal tissue as possible

from cell death. Established treatment algorithms offer either systemic thrombolysis using recombinant tissue plasminogen activators (rtPA), or endovascular intervention (typically in the form of mechanical thrombectomy), or a combination of both. The advent of these treatment options in the 1990s, along with the treatment of stroke patients at specialized, interdisciplinary wards and public health education efforts, more than halved stroke mortality rates in Germany from 1998 to 2015.[8-10] Despite this, the incidence of stroke continues to be on the rise globally and incident stroke increases the risk of recurrent events even under optimal treatment, placing special importance on preventative measures.[11]

Ischemic stroke is a multi-factorial disease. Age, race, sex, and genetic risk factors are unmodifiable, whereas obesity, tobacco consumption, arterial hypertension, lipid derangement, heart disease and diabetes are addressed through a combination of lifestyle recommendations and medical treatment.[12] To inform clinical decision making on long-term oral anticoagulation, the CHA_2DS_2-VASc score can be used to estimate one-year stroke risk.[13,14] Patients with underlying cardiovascular risk factors or evidence of atherosclerosis are at increased risk of large-artery atherosclerotic stroke and should thus receive antihypertensive, lipid-lowering and antiplatelet therapies, in addition to dietary and smoking cessation counseling.[15] In patients with carotid artery stenosis, surgical or catheter-guided revascularization is recommended both in primary and secondary prevention of stroke based on symptom status, degree of stenosis and perceived stroke risk.[16,17]

1.2 Pro-inflammatory signaling in atherosclerosis

1.2.1 Atherosclerosis epidemiology and the history of its research

Atherosclerosis is the most common pathology underlying large-vessel ischemic stroke, ischemic heart disease, aortic aneurysm, mesenteric ischemia and peripheral artery disease. Taken together, these atherosclerotic disease entities constitute the leading global causes of death.[1] While ischemic events rarely occur in young humans, subclinical atherosclerosis has been observed to already manifest in childhood and young adulthood, with increasing risk in the presence of cardiovascular risk factors.[18,19]

Imaging studies of mummies from multiple ancient populations reinforce the notion that the presence of atherosclerotic plaques can be traced back throughout modern human history.[20] In 1858, Virchow studied lipid-rich deposits located inside the arterial wall of deceased individuals, implicating vascular inflammation in their emergence.[21] In 1912, Anitschkow and colleagues published their experimental work on cholesterol-fed rabbits,

observing extensive atherosclerotic lesions across the aorta.[22] The second half of the 20th century saw a marked increase in the incidence of atherosclerotic cardiovascular disease, giving substantial momentum to atherosclerosis research and resulting in the discovery of the statin by Endo in 1976.[23,24] In 1992, the creation of a mouse line lacking both alleles of the murine *Apoe* gene was reported, enabling the previously unseen development of atherosclerotic plaques in mice and giving rise to one of the most-widely used biological systems in atherosclerosis research.[25,26]

1.2.2 Pathophysiology of atherosclerosis

These advances have furthered the understanding of atherosclerotic pathophysiology, which led to its characterization as a lipid-driven inflammatory disease. Atherogenesis typically begins in the vicinity of arterial branches, as these sites are subjected to disturbances in laminar blood flow exacerbated by hypertension. The vascular endothelial barrier becomes permeable for low-density-lipoprotein (LDL) cholesterol, which accumulates in the intima layer of the artery.[27] Resident macrophages then initiate the uptake of the accumulating lipids, resulting in LDL-engorged foam cells, which are characteristic of early atherogenesis.[28] Macrophages, in conjunction with damage-sensing endothelial cells, subsequently release chemokines which attract circulating monocytes and other leukocyte subsets to the site of vascular injury.[29] These monocytes, in turn, transmigrate from circulation into the intimal layer with the help of adhesion molecules and endothelial glycoproteins, clearing apoptotic cells overladen with LDL and thus perpetuating the cycle of lipid uptake and pro-inflammatory cytokine release. Meanwhile, smooth-muscle cells cross the subintimal space, where a subset de-differentiates and assume a macrophage-like phenotype.[30]

Early atherosclerotic lesions over time progress into atheromata of variable morphology and composition, typically consisting of lipid cores filled with cholesterol crystals, cellular debris and calcium deposits. Advanced plaques exhibit fibrous caps laying atop the lipid core and the surrounding, collagen-rich extracellular matrix. While the arterial lumen is initially unchanged as the plaque grows eccentrically, in later stages it is characterized by expansive and constrictive growth.[31] Originating from the vasa vasorum of large arteries, plaque neovascularization can be observed, posing a risk for intraplaque hemorrhage.[32,33]

Atherosclerotic plaques typically do not cause ischemia due to a slow occlusion of the arterial lumen over time; rather, it is the rupture of the fibrous cap that exposes the highly thrombogenic matrix of plaques to circulating blood, causing the formation of a thrombus that either occludes the artery at the site of rupture or is carried further distally. Both in

research and in clinical routine, plaques are characterized as either stable or unstable depending on their proneness towards rupture and erosion.[34-36] Unstable or vulnerable plaques typically exhibit expansive lipid cores, thin fibrous caps as well as high macrophage content and observable intraplaque hemorrhage. While a number of different pathomechanisms act in conjunction to convey plaque vulnerability, recent proteomic and spatial transcriptomic characterizations of human and murine plaques confirmed the essential role of pro-inflammatory signaling and its molecular effectors for the vulnerable plaque phenotype.[37,38] Signaling pathways communicating local and systemic inflammation have therefore been in the spotlight of the field for decades.[39-41]

1.2.3 The CCL2/CCR2 axis

The chemokine system involves at least 50 different ligand proteins and approximately 20 G-protein-coupled receptors, known as chemokine ligands and receptors.[42] Chemokines are known for inducing targeted migration of cells through specific ligand-receptor binding.[42] The C-C chemokine ligand 2 (CCL2, also referred to as "Monocyte chemoattractant protein-1" or MCP-1) directs monocytes to areas of ongoing immune response.[43] CCL2, through binding to C-C chemokine receptor 2 (CCR2), elicits the mobilization of leukocytes from the bone marrow into circulation.[44,45] In atherogenic inflammation, endothelial and vascular smooth-muscle cells as well as resident macrophages release CCL2 to attract monocytes to vascular injury sites, before facilitating their adhesion to the endothelial lining and migration into the intimal layer.[29,46]

The CCL2/CCR2 axis has been investigated extensively in preclinical atherosclerosis research. Atherogenic mouse models of CCL2 and CCR2 deficiency demonstrated alleviation of plaque burden and drastically reduced plaque macrophage content.[47-49] This was later corroborated by pharmacological inhibition studies reporting the same outcomes.[50-52]

Converging evidence using human studies corroborated the proatherogenic role of the CCL2/CCR2 axis. Mendelian randomization studies investigating genetically proxied increase in circulating CCL2 levels demonstrated an association with increased risk of atherosclerotic stroke and coronary artery disease, augmenting meta-analyses of clinical studies associating circulating CCL2 levels with increased risk of ischemic stroke and cardiovascular mortality.[53-55] Studies utilizing human carotid artery plaques found intraplaque CCL2 levels to be associated with vulnerability features.[56]

Taken together, these findings suggest that inhibition of the CCL2/CCR2 axis may constitute a viable therapeutic strategy in atherosclerosis.[57]

1.2.4 The NF-κB pathway

The expression of chemokines like CCL2 is licensed by nuclear factor κB (NF-κB) pathway activity, termed a "master regulator" of innate immunity, owing to its pleiotropic effects.[58,59] It is considered to be among the oldest immune responses, having been observed in a large majority of multi-cellular organisms.[60] NF-κB typically presents as a homo- or heterodimer of five transcription factors that translocate into the nucleus to mediate the transcription of several hundred target genes.[61] In their inactive state, NF-κB family proteins are coupled to a class of inhibitory kinases (IκB, most commonly IκBα) which prevent entry into the nucleus.[59]

The NF-κB pathway is activated by a multitude of membrane-based sensors, such as the tumor necrosis factor (TNF) and toll-like receptors (TLRs).[62] Their downstream signaling pathways converge in the activation of the IκB kinase (IKK), which consists of the subunits α, β and γ. IKK then phosphorylates IκB at multiple sites, enabling proteasomal degradation and allowing for the release of NF-κB.[63]

The NF-κB pathway is essential to atherogenic inflammation.[64-66] In macrophages, NF-κB signaling governs the expression of genes commonly associated with the pro-inflammatory M1 phenotype.[67] NF-κB signaling has been implicated in vascular smooth-muscle cells through its modulation of proliferation rate and pro-apoptotic, pro-inflammatory secretion profile.[68,69] In endothelial cells, the surface expression of cellular adhesion molecules and chemokines experiences marked upregulation after activation of the NF-κB pathway.[70,71] Previous research has also identified mechanistic regulators of NF-κB activity that directly influence atheroprogression. For instance, histone deacetylase 9 (Hdac9) binding to IKK α and β kinases has been shown to license NF-κB activity and driving atherosclerosis in mice. Previously, a variant within the HDAC9 gene robustly associated with large-vessel stroke had been identified in a large, meta-genome-wide association study.[72,73]

1.2.5 NLRP3 and IL-1β

One of the key molecular actors of innate immunity whose expression is driven by the NF-κB pathway is the NOD-like receptor with pyrin domain 3 (NLRP3) a cytosolic damage sensor that, once activated, swiftly induces interleukin production and release. NLRP3 is an extensively studied member of the NOD-like-receptor family, a class of tripartite proteins that act as pattern recognition receptors (PRRs) and mount pro-inflammatory responses. NLRP3 activity is dependent upon its transcriptional upregulation via NF-κB, called "priming", and its activation induced by pathogen- or damage- associated

molecular patterns (PAMPs and DAMPs, respectively).[74-76] PAMPs and DAMPs communicate the presence of microbial organisms and cellular stress; NLRP3 is commonly activated by the bacterial proteins Nigericin and α-Hemolysin, as well as extracellular Adenosine triphosphate (ATP), silica and urate crystals. These molecules typically induce NLRP3 activation via potassium efflux. Once activated, NLRP3 oligomerizes into inflammasomes, wheel-shaped structures with Apoptosis-associated speck-like proteins (ASC) to cleave Pro-caspase 1 into Caspase 1 (Casp-1).[77] Casp-1 then begins cleaving pro-interleukins 1β and 18 (IL-1β/IL-18) as well as Gasdermin D (GSDMD), a pore-forming protein that facilitates the release of the now cleaved and active interleukins during pyroptosis.

NLRP3 and ASC rely on a number of post-translational modifiers and mechanistic interaction partners to assemble into the inflammasome.[76,78] For instance, kinases JNK1 and BTK phosphorylate NLRP3, thus enabling its oligomerization.[79,80] During the activation step, potassium-efflux triggers binding of NEK7 to NLRP3, providing a scaffold for inflammasome assembly.[81,82] Recent work has uncovered that Hdac9 deacetylates NLRP3, thereby increasing IL-1β release and aggravating atherosclerosis in mouse models.[83]

The cytokine IL-1β is a potentiator of inflammation with both local and systemic effects.[84] Like NLRP3, IL-1β's precursor form is not constitutively expressed in most cell-types and thus requires NF-κB-dependent priming. Monocytes and macrophages, by contrast, also exhibit notable expression of NLRP3 and pro-IL-1β in an unprimed state, thus allowing for accelerated inflammasome assembly termed "rapid priming".[85,86] Once released, IL-1β initiates a multitude of proatherogenic programs like the expression of leukocyte adhesion molecules, chemokines and collagen-degrading matrix metalloproteinases.[87].

Crucially, IL-1β has been shown to bind to TLR4, inducing NF-κB activation and thus begetting its own expression.[88] Recently, it has been shown that in mouse models of atherosclerosis and clonal hematopoiesis of indeterminate potential (CHIP) IL-1β may also suppress the expansion of fibroblast-like cells implicated in plaque stability.[89]

1.2.6 IL-6

IL-1β induces the expression of Interleukin-6 (IL-6), a cytokine that amplifies both the acute immune response and chronic low-grade inflammation.[90] Like IL-1β, IL-6 is secreted primarily by immune cells and governs pro-inflammatory phenotype shifts, chemokine release, leukocyte adhesion and proliferation.[91] Critically, IL-6 regulates the he-

patic secretion of C-reactive protein (CRP), as well as other acute phase proteins. Downstream signaling from IL-6/IL-6 receptor (IL-6R) interaction relies on the JAK/STAT pathway.[92]

An extensive body of literature has cemented the pivotal role of IL-6 in atherogenic inflammation. Both pharmacological and genetic inhibition of IL-6 and its receptor alleviated plaque burden in animal models of atherosclerosis while increasing susceptibility to infection and hampering hepatic regeneration.[93] In humans, large cohort studies demonstrated that circulating IL-6 and high-sensitivity CRP (hsCRP) levels associate with the risk of recurrent myocardial infarction, incident ischemic stroke and cardiovascular death. Circulating IL-6 was found to be associated with increased plaque burden, vulnerability and progression in 4334 participants of the Cardiovascular Health Study.[94] Another, larger cohort study found that third tercile baseline levels of circulating IL-6 were associated with increased risks of all-cause mortality and heart failure, with consistent outcomes across multiple ethnic backgrounds.[95]

These findings are flanked by Mendelian randomization studies leveraging large consortium datasets underpinning the causal role of IL-6 in atherosclerotic disease entities. The *IL6R* variant rs9182284 was associated with a reduction in coronary heart disease risk.[96] Genetically proxied IL-6 signaling downregulation was associated with lower risks of ischemic stroke and coronary artery disease in Mendelian randomization and phenome-wide association studies.[97-100]

1.3 Previous anti-inflammatory drug trials in coronary heart disease and ischemic stroke

As outlined previously, multiple lines of evidence support the causal role of the CCL2/CCR2 axis and the IL-1β/IL-6 signaling pathways in atherosclerotic disease. The development of anti-inflammatory therapies for the prevention of coronary heart disease and large-vessel stroke has consequently gained momentum.

Among the first explorations of CCL2/CCR2 inhibition in humans was a phase II trial of rheumatoid arthritis, where intravenous gavage of the monoclonal CCR2 antibody MLN1202 led to a significant reduction in hsCRP in the treatment group compared to placebo.[101] Another randomized trial involving 112 individuals with two or more cardiovascular risk factors demonstrated significant reductions in circulating hsCRP levels in the MLN1202-treated group.[102] A phase 2 trial of the small molecule CCL2/7/8 inhibitor bindarit reported reduced rates of in-stent late loss following coronary intervention in the

treatment group.[103] Since then, further trials investigating cardiovascular or cerebrovascular outcomes have not been started to date.

Pharmacological targeting of IL-1β was explored in the CANTOS trial, where the monoclonal IL-1β antibody Canakinumab previously used in rheumatoid arthritis was administered to patients with a history of myocardial infarction at baseline and myocardial infarction, stroke and cardiovascular deaths as primary endpoints. While Canakinumab successfully reduced the rate of recurrent myocardial infarctions, it failed to demonstrate a reduction for risks of stroke and cardiovascular mortality while reporting significantly increased risk of fatal infections in the treatment groups.[104] Secondary analyses of trial data reported that patients achieving larger hsCRP and IL-6 reductions under treatment also achieved greater reductions in major adverse cardiovascular events (MACE), indicating that treatment efficacy scaled with the reduction of hsCRP/IL-6 concentrations.[105,106] These findings are in line with large patient-level meta-analyses demonstrating linear associations between circulating hsCRP and IL-6 levels and MACE.[93,107,108]

A clinical trial investigating IL-6 inhibition and cardiovascular outcomes is currently underway in the form of the ZEUS trial, which is including patients with chronic kidney disease and atherosclerotic cardiovascular disease (NCT05021835).[109] Previous phase 2 trials demonstrated significant reductions in hsCRP in similar cohorts under treatment with the IL-6 antibody ziltivekimab.[110,111]

Other trials have focused on the anti-inflammatory properties of colchicine, a small-molecule compound found in the autumn crocus that has previously been in use for the treatment of gout. Colchicine's effect on the immune response in atherogenic inflammation is incompletely understood. Available evidence points towards several capabilities which include NLRP3 inflammasome inhibition, microtubular toxicity in monocytes, reduced lipoprotein oxidation and the inhibition of plaque-destabilizing matrix metalloproteinases.[112] The COLCOT and LoDoCo2 trials showed significant reductions in MACE in patients with myocardial infarction or stable coronary artery disease at baseline following administration of low-dose colchicine, leading to its approval for the secondary prevention of myocardial infarctions in the United States.[113,114] Drawing from these results, further phase 3 RCTs were initiated that tested low-dose colchicine in patients diagnosed with ischemic stroke at baseline, both of which showed no reduction in the risk of either ischemic stroke recurrence or MACE.[115,116] Recently, the CLEAR cross-over trial reported no effect of low-dose colchicine on MACE or stroke risk in patients with myocardial infarction and coronary intervention at baseline.[117] Further trials using low-dose colchicine in various atherosclerotic disease entities are currently underway.

Taken together, to date, no anti-inflammatory therapy targeting either the CCL2/CCR2 or the NLRP3/IL-1β/IL-6 axes has gained regulatory approval for the prevention of large-vessel atherosclerotic stroke.

1.4 Challenges to successful clinical translation

Examining the available preclinical, epidemiological, and genetic evidence as well as previous clinical trials, several obstacles remain in the way of bench-to-bedside translation for anti-inflammatory treatment strategies in atherosclerosis:

First, decades of preclinical research testing anti-inflammatory treatment strategies in animal models of atherosclerosis have produced an unwieldy body of literature, employing an innumerable amount of inhibition strategies, with varying treatment paradigms, measurements and outcomes.[118] This heterogeneity in preclinical study reporting impedes on clinical translation and can cause duplication of efforts.[119,120] Despite this, newly approved drugs continue to build on preclinical research data using animal models of disease.

Second, the mechanistic properties of atherogenic inflammation remain incompletely understood with respect to inter-pathway crosstalk. The NF-κB, IL-1β and IL-6 signaling pathways form an interconnected system of mutual upregulation, adding to the complexity in the search for druggable molecular targets that balance atheroprotective effects with the pitfalls of immune suppression. Elucidating novel mechanisms that link pro-inflammatory signaling pathways could provide insights into the biology of atherosclerosis, thereby refining anti-inflammatory treatment strategies.

Third, the downstream impact of interleukin signaling inhibition is unclear. The increase in risk of fatal infections in the CANTOS trial indicated a need for careful consideration between drug efficacy and adverse reactions in the form of impaired host defense or potential compensatory mechanisms.[104,121] Therefore, there is a need for examining proteomic signatures of interleukin inhibition to anticipate potential undesired effects and explore downstream proteins with causal basis in atherogenic inflammation that could serve as alternative drug targets.

1.5 Aims of the thesis

To address these challenges, we have set out the following aims:

1. To summarize available preclinical evidence by meta-analyzing studies testing pharmacological inhibition of the CCL2/CCR2 axis in mouse models of atherosclerosis;[118]

2. To investigate molecular junctions linking NF-κB signaling and IL-1β maturation through mechanistic exploration of a novel binding between IKKβ and NLRP3;[122]

3. To reveal the downstream proteomic footprint of genetically-proxied IL-6 signaling inhibition and explore its associations with cardiovascular outcomes and atherosclerotic plaque phenotype.[121]

2. Contributions to publications

2.1 Contribution to publication I[118]

I executed a systematic literature screening in the PubMed and Embase repositories using a search algorithm co-developed with M.K.G, as described in the Methods section of the publication. After applying the inclusion criteria, I identified eligible studies, screened their reference lists for other potentially eligible studies unrevealed by the initial search algorithm and extracted descriptive statistics (mean, standard deviation or standard error of the mean, number of observations) from experiments relevant to the prespecified outcomes. I performed random-effects meta-analyses of standardized mean differences (expressed as *Hedges' g*) for plaque size as main outcome, plaque composition features (macrophage/smooth-muscle-cell/collagen content) as secondary outcomes, and several other outcomes outlined in the supplementary material. After reviewing the heterogeneity statistics, I performed subgroup and meta-regression analyses of plaque size as the main outcome/dependent variable to explore sources of between-study heterogeneity, as well as created funnel and Egger plots to assess publication bias. Next, I applied a risk of bias assessment tool to the study pool. I wrote all sections of the manuscript with corrections and feedback from M.K.G. and created all figures and tables both in the main manuscript and in the supplementary material. I performed all submissions of the manuscript and handled revisions along with reviewer correspondence under M.K.G.'s supervision.

2.2 Contribution to publication II[122]

I performed the initial co-immunoprecipitation (Co-IP) experiments suggesting mechanistic interaction between IKKβ and NLRP3. Then, I performed further experiments to solidify the specificity of this interaction by demonstrating absent Co-IP of Absent in melanoma 2 (AIM2), another inflammasome-forming protein, when co-overexpressed with IKKβ and investigated binding of IKKβ to the different domains of NLRP3. Next, I explored the effect of pharmacological inhibition of IKKβ using TPCA-1 in bone-marrow-derived macrophages (BMDMs) from *Apoe*$^{-/-}$ mice. To uncouple the suppression of NLRP3 priming that IKKβ inhibition would inevitably cause from the inhibition of inflammasome assembly, I modified the experimental set-up to first prime the cells, then inhibit IKKβ, then provide inflammasome activation triggers. This "post-priming" approach [123](Fig. S4) provided further evidence for a direct mechanistic effect of IKKβ on NLRP3 function. I then performed several experiments that aimed to explore whether the ob-

served mechanistic effect was a phosphorylation of NLRP3 directly by IKKβ that indicated no post-translational modifications on part of IKKβ. I also investigated the effects of IKKβ inhibition on IL-1β release employing various known triggers of NLRP3 activation, like monosodium urate (MSU) crystals, ATP, silicium dioxide (SiO_2), and double-stranded DNA (dsDNA). I reviewed the initial manuscript prior to submission and assisted in performing review experiments. In the final manuscript, I provided data and representative images for Figs. S4 and S5 a-e. Experiments represented in Fig 1a-d initially performed by me were repeated and extended upon by M. S.

2.3 Contribution to publication III[121]

Using a search algorithm based on previous work[118], I systematically screened PubMed for studies on pharmacological or genetic inhibition of CXCL10 or its receptor CXCR3 in animal models of atherosclerosis. Screening 2103 titles and abstracts from beginning of indexing until September 29, 2023, I identified 34 potentially eligible entries. After reviewing full texts, 18 entries were removed for not fulfilling any of the previously outlined inclusion criteria, eight entries were classified as “Pharmacological or genetic inhibition of CXCL10/CXCR3 in other cardiovascular disease models”, two entries fulfilled all inclusion criteria but did not contain plaque size as outcomes. Among the remaining six publications, three utilized genetic knock-out models of either CXCL10 or CXCR3[123-125] and three administered pharmacological inhibitors.[52,126,127] Because a meta-analysis of these six studies would have been underpowered, I summarized the findings from these studies and included them in the Discussion section of the final manuscript, along with a subsection detailing the current understanding of the CXCL10/CXCR3 axis’ role in vascular physiology. Finally, I reviewed the final submission and provided comments and edit suggestions.

3. Publication I

Title: Pharmacological Targeting of the CCL2/CCR2 Axis for Atheroprotection: A Meta-Analysis of Preclinical Studies
Authors: **Luka Živković**, Yaw Asare, Jürgen Bernhagen, Martin Dichgans, Marios K. Georgakis
Year: 2022
Journal: Arteriosclerosis, Thrombosis, and Vascular Biology, Vol. 42 (5) pp. e131-e144
DOI: 10.1161/ATVBAHA.122.317492

The dataset underlying the meta-analysis can be accessed under the provided DOI.

Pharmacological targeting of the CCL2/CCR2 axis for atheroprotection: a meta-analysis of preclinical studies

Running title: CCL2/CCR2 Inhibition Mitigates Atherosclerosis

Luka Živković[1], Yaw Asare, PhD[1], Jürgen Bernhagen, PhD[1,2,3], Martin Dichgans, MD[1,2,4*], Marios K. Georgakis, MD, PhD[1*]

[1] Institute for Stroke and Dementia Research (ISD), University Hospital, LMU Munich, Munich, Germany

[2] Munich Cluster for Systems Neurology (SyNergy), Munich, Germany

[3] Munich Heart Alliance, German Center for Cardiovascular Diseases (DZHK), 80802 Munich, Germany

[4] German Centre for Neurodegenerative Diseases (DZNE), Munich, Germany

* These authors jointly supervised this work.

Correspondence to:

Marios K. Georgakis, MD, PhD
Neurology resident & Postdoctoral researcher
Institute for Stroke and Dementia Research, University Hospital LMU
Ludwig-Maximilians-University (LMU), Munich, Germany
Feodor-Lynen-Str. 17, 81377 Munich, Germany
Telephone: +49-89-4400-46063; Fax: +49-89-4400-46040
e-mail: marios.georgakis@med.uni-muenchen.de

Martin Dichgans, MD
Director
Institute for Stroke and Dementia Research, University Hospital LMU
Ludwig-Maximilians-University (LMU), Munich, Germany
Feodor-Lynen-Str. 17, 81377 Munich, Germany
Telephone: +49-89-4400-46018; Fax: +49-89-4400-46040
e-mail: martin.dichgans@med.uni-muenchen.de

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Abstract

Background: The CC-chemokine ligand-2 (CCL2)/ CC-chemokine receptor-2 (CCR2) axis governs monocyte recruitment to atherosclerotic lesions. Genetic and epidemiological studies show strong associations of CCL2 levels with atherosclerotic disease. Still, experimental studies testing pharmacological inhibition of CCL2 or CCR2 in atheroprone mice apply widely different approaches and report variable results, thus halting clinical translation.

Methods: We systematically searched the literature for studies employing pharmacological CCL2/CCR2 blockade in atheroprone mice and meta-analyzed their effects on lesion size and morphology.

Results: In a meta-analysis of 14 studies testing 11 different agents, CCL2/CCR2 blockade attenuated atherosclerotic lesion size in the aortic root or arch (g=-0.75 [-1.17 to -0.32], $p=6\times10^{-4}$; N=171/171 mice in experimental/control group), the carotid (g=-2.39 [-4.23 to -0.55], p=0.01; N=24/25), and the femoral artery (g=-2.38 [-3.50 to -1.26], $p=3\times10^{-5}$; N=10/10). Furthermore, CCL2/CCR2 inhibition reduced intralesional macrophage accumulation and increased smooth muscle cell content and collagen deposition. The effects of CCL2/CCR2 inhibition on lesion size correlated with reductions in plaque macrophage accumulation, in accord with a prominent role of CCL2/CCR2 signaling in monocyte recruitment. Subgroup analyses showed comparable efficacy of different CCL2- and CCR2-inhibitors in reducing lesion size and intralesional macrophages. The quality assessment revealed high risk of detection bias due to lack of blinding during outcome assessment, as well as evidence of attrition and reporting bias.

Conclusions: Preclinical evidence suggests that pharmacological targeting of CCL2 or CCR2 might lower atherosclerotic lesion burden, but the majority of existing studies suffer major quality issues that highlight the need for additional high-quality research.

Non-standard abbreviations and Acronyms

CCL2 – CC-chemokine ligand 2
CCR2 – CC-chemokine receptor 2
WTD – Western-type diet

Introduction

Stroke and coronary artery disease remain the leading causes of long-term disability and mortality.[1] Multiple lines of experimental and clinical evidence implicate inflammatory mechanisms in atherosclerosis, the predominant pathology underlying cardiovascular disease. Recent clinical trials have provided proof-of-concept for the role of inflammation in atherosclerosis by demonstrating the potential of anti-inflammatory therapies to lower cardiovascular risk.[2, 3] Specifically, canakinumab[2] and colchicine[3, 4] were found to lower the risk of recurrent cardiovascular events in patients with a history of coronary artery disease. While interventional studies in humans have so far mostly focused on the inflammasome-interleukin-1β (IL-1β)-interleukin-6 (IL-6) axis,[5] recent experimental and epidemiological studies place emphasis on other mediators of inflammation, as has specifically been shown for the chemokine system.[6, 7] Targeting alternative inflammatory pathways with a more specific role in atherosclerosis could increase efficacy and improve the safety profile of anti-inflammatory approaches, thus moving them closer to clinical translation.

CC-motif chemokine ligand 2 (CCL2), is one of the first CC family chemokine described and implicated in atherosclerosis.[8, 9] CCL2 primarily acts by binding to CC-chemokine receptor 2 (CCR2) on the surface of classical monocytes, thus mobilizing them from the bone marrow to the circulation and attracting them to sites of inflammation[10] including the arterial subendothelium.[8, 11] CCL2/CCR2 signaling governs rolling and adhesion of monocytes on the endothelial lining of atherosclerotic lesions.[12] Hyperlipidemic atheroprone mice deficient for either *Ccl2*[13] or *Ccr2*[9] exhibit substantial reductions in the number and size of atherosclerotic lesions, as well as reductions in lipid deposition and macrophage accumulation in the arterial walls, thus supporting a causal role of the CCL2/CCR2 pathway in atherogenesis.

The potential importance of these findings for the development of therapeutic strategies is illustrated by recent studies demonstrating a causal role of CCL2 in human atherosclerosis. First, using a Mendelian randomization approach, we recently found higher genetically proxied circulating levels of CCL2 to be associated with a higher risk of ischemic stroke, in particular large artery stroke, and a higher risk of coronary artery disease and myocardial infarction.[14] Second, higher measured levels of circulating CCL2 were associated with a higher risk of ischemic stroke, coronary artery disease, and cardiovascular mortality in population-based cohorts of individuals free of cardiovascular disease at baseline.[15, 16] Third, CCL2 levels quantified in atherosclerotic plaques from individuals undergoing carotid endarterectomy showed significant associations with histopathological, clinical, and molecular features of plaque vulnerability.[17]

While these studies identify the CCL2/CCR2 axis as a promising pharmacological target for the treatment of atherosclerosis, there are only limited data from randomized trials specifically targeting this pathway in the context of human atherosclerosis. In a study on 108 patients with cardiovascular risk factors and high circulating levels of high-sensitivity C-reactive protein (hsCRP), those treated with a single intravenous infusion of MLN1202, a humanized monoclonal antibody against CCR2, exhibited significant reductions in hsCRP levels after four weeks and continuing through 12 weeks after dosing.[18] However, this phase II trial was not designed to investigate clinical endpoints.

Preclinical studies have explored various pharmacological approaches targeting the CCL2/CCR2 axis in models of atherosclerosis. Still, these studies reported largely variable and partly inconsistent results, possibly reflecting differences in the properties of the individual pharmacological agents, the selected drug targets (CCL2 or CCR2), the molecular sites in their structures targeted by the agents, the animal models under study, lesion stages at initiation of the intervention, duration of treatment, and the vascular beds under examination. Against this background, we aimed to analyze the available evidence from preclinical studies testing pharmacological inhibition of the CCL2/CCR2 pathway in atherosclerosis-prone mice and quantify the effects of the interventions on lesion size and cellular and extracellular plaque components (macrophage accumulation, smooth muscle cell content, and collagen

deposition). We further aimed to detect potential sources of heterogeneity of their efficacy including those related to pharmacological properties of the inhibitor, vascular bed, animal model, duration of treatment, and stage of atherosclerosis. We therefore performed a systematic review and meta-analysis of preclinical studies in an effort to inform the design of future clinical trials in humans.

Methods

This meta-analysis follows the PRISMA guidelines on systematic reviews (**Online Table I**). The dataset underlying this work is available online as an online dataset.

Search strategy

The SyRF 9-step outline and SYRCLE's protocol template for conception of animal meta-analyses[19] were used to design the search strategy, study and outcome selection, and statistical processing of the extracted data for this systematic review and meta-analysis. The Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA)[20] were used as reporting guidelines of this meta-analysis (**Online Table I**). To identify eligible articles, we screened MEDLINE and Embase from their inception to January 20th, 2022 without restrictions in language or publication year, using the following predefined search strategy: ("CC chemokine ligand 2" OR "C-C chemokine ligand 2" OR "C-C motif chemokine ligand 2" OR "C-C motif chemokine receptor type 2" OR "C-C chemokine receptor type 2" OR "CC chemokine receptor type 2" OR "monocyte chemoattractant protein" OR "monocyte chemotactic protein" OR CCL2 OR MCP-1 OR MCP1 OR CCR2) AND (atherogenesis OR atherosclerosis OR atheroprogression OR atherosclerotic OR plaque OR stroke OR ((cardiovascular OR ischemic OR cerebrovascular OR coronary) AND disease) OR (myocardial AND infarction)). A published search filter was employed to limit displayed entries to those referring to animal experiments.[21] Additionally, the reference lists of all eligible studies were screened. Eligible articles were evaluated for potential overlap of data. One reviewer (L.Z.) performed the initial screening and all potentially eligible articles were further independently screened by an additional reviewer (M.G.); differences were resolved in consensus.

Eligibility criteria

The eligibility criteria applied for our study selection strategy were pre-defined before the start of the literature search.

Population

Articles were deemed eligible if they described experimental inhibition of CCL2 or CCR2 in an *in vivo* mouse model of atherosclerosis. Specifically, eligible studies were required to use atherosclerosis-prone mouse models, such as *Apoe*$^{-/-}$, *Ldlr*$^{-/-}$, or ApoE3Leiden mice that were fed a normal laboratory diet or high-fat "Western-type" diet (WTD). Models of accelerated atherosclerosis following arterial injury in atherosclerosis-prone mice were also considered eligible.[22, 23] Studies referring to other animals beyond mice were not included in this review.

Intervention

Eligible studies had to explore the effects of a pharmacological intervention directly interfering with and inhibiting CCL2 or CCR2, such as orthosteric or allosteric receptor antagonists, competitive inhibitors of chemokine-receptor interaction, or antibodies. Studies that made use of gene therapy by means of transfecting plasmids, such as 7ND,[24-27] were also considered eligible, provided that the encoded protein was a direct inhibitor of CCL2 or CCR2. Studies that examined pharmacological agents or nutritional compounds that indirectly downregulate the CCL2/CCR2 axis by interfering with upstream agents or downregulated CCL2 or CCR2 expression were deemed ineligible. Eligible studies also required a control group of animals which were injected with a vehicle or were fed an inhibitor-free diet.

Outcomes

Eligible studies needed to provide a quantified measurement of atherosclerotic plaque burden as an outcome. Histopathological quantification of lesion size/area, plaque size/area,

neointimal area, or lipid-staining area following hematoxylin-eosin, Oil Red O, trichrome or pentachrome staining of vessel cross-sections were required for inclusion in the meta-analysis. Studies providing measurements of intima/media ratio were excluded, because these readouts fail to distinguish atherosclerotic plaque burden from intimal hyperplasia and vascular thickening. Apart from the well-established lesion quantification in the aortic root or arch, studies measuring carotid or femoral artery lesions were also included in the meta-analysis.

Additional predefined outcomes entailed plaque cellular and extracellular components including macrophage accumulation (expressed as Mac2/3-positive or Moma-2/3-positive content), smooth muscle cell content (expressed as smooth-muscle-actin-positive content), and collagen deposition. Plaque feature outcomes were only included if they were normalized to plaque size. We further explored the following as secondary outcomes: effects of the intervention on additional measurements that had not been predetermined, when explored by at least three individual studies. These included body weight, plasma cholesterol and triglyceride levels, circulating monocyte count, plasma CCL2 levels, and aortic expression of CCR2, IL-6, and TNF-α.

Study quality assessment

We examined potential sources of bias with the SYRCLE risk of bias tool that was specifically designed for preclinical studies.[28] The tool evaluates studies for selection bias (3 items), performance bias (2 items), detection bias (2 items), attrition bias (1 item), reporting bias (1 item), and other sources of bias (1 item). Full texts, figure legends and supplementary materials were considered in the risk of bias assessment. The specific criteria used in risk of bias assessment are shown in **Online Table II**. Furthermore, we explored whether the included studies followed the ARRIVE guidelines for reporting animal research for sample size, randomization, and blinding.[29]

Data abstraction

Absolute values, number of specimens, and either standard error or standard deviation in both intervention and control groups were extracted for each outcome (**Online Table III**). Where numerical data was not available, values were extracted from figures. Additionally, information pertaining to experimental setup such as inhibitor used and its target (CCL2 or CCR2), blood vessel under examination, mouse background and genetic model, sex, type of diet and (where applicable) start and duration of WTD feeding, start and duration of inhibitor administration, and additional pharmacological interventions were recorded for each experimental group. Where abstractable data were not presented in the published article or the supplementary materials, the corresponding author was contacted for providing the required information. One reviewer (L.Z.) performed the data abstraction and all data were further checked by a second reviewer (M.G.).

Meta-analysis

For all studies and outcomes, we calculated standardized mean differences (Hedges' *g*) between the experimental and the control groups using the Hedges approach to account for the small sample sizes. We then pooled the individual study estimates using DerSimonian-Laird random effects models to account for the expected heterogeneity between studies. For the main outcomes (lesion size and plaque characteristics), we performed the analysis separately for the different examined vessels (aortic root or arch, carotid artery, femoral artery). We calculated between-study heterogeneity with the I^2 and the Cochran *Q* statistic. I^2 exceeding 50% or 75% was considered as moderate and high heterogeneity, respectively.[30] Finally, we performed Egger regression to explore potential small-study effects in our main analysis that would indicate presence of publication bias.[31] Funnel plots were also created and visually inspected for asymmetry due to small-study effects.

To account for potential sources of heterogeneity, we performed a series of subgroup and meta-regression analyses. Specifically, we explored if the stage of lesion progression at the time of intervention start influenced the intervention effects. Lesion stage was classified as early, intermediate or advanced on the basis of mouse model, diet used, and age at intervention: *Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$ mice fed a normal laboratory diet were assumed to exhibit early lesions until they were 15 weeks old, intermediate lesions between 15-20 weeks, and advanced lesions after 20 weeks of age. The respective intervals in WTD-fed mice were until 10 weeks (early lesions); 10-15 weeks (intermediate lesions), and after 15 weeks (advanced lesions).[32, 33] We further performed subgroup analyses by target of intervention (CCL2 vs. CCR2), animal model (*Apoe*$^{-/-}$ vs. *Ldlr*$^{-/-}$), type of diet (normal laboratory vs. WTD) and sex. Finally, we carried out meta-regression analyses, exploring whether the effects of the interventions on plaque characteristics or other secondary outcomes, could explain heterogeneity in the effects on lesion size.

All analyses were performed using Stata 16.1 (College Station, United States).

Data availability

The dataset generated from the data abstraction of the included studies is available as a separate Online Supplementary File. The Stata code used for our analyses is available on Dataverse: https://doi.org/10.7910/DVN/KMKD0J.

Results

The results of the search strategy are summarized in **Fig. 1**. The MEDLINE and Embase searches returned 4,551 entries after excluding duplicates, out of which 288 articles were assessed for eligibility through inspection of their full texts. Of them, 15 articles met our eligibility criteria. Two of them presented data already available in another publication[34, 35] . One additional article[36] was identified through screening the reference lists of the eligible studies. A total of 14 articles[11, 24-27, 36-44] were eventually deemed eligible for inclusion in our systematic review and meta-analysis.

Characteristics of eligible studies

Table 1 summarizes key characteristics of the included studies, which were published between 2001 and 2018. All 14 eligible studies used hyperlipidemic mouse models of atherosclerosis. Specifically, 12 studies used *Apoe*$^{-/-}$ mice, one *Ldlr*$^{-/-}$ mice, and one ApoE3Leiden mice. Nine studies relied on WTD feeding in their experimental setup, whereas in four studies mice were fed normal laboratory diet, with one study not specifying any type of diet. Most of the studies that used a WTD initiated high-fat feeding before the age of ten weeks. Timing of intervention and duration of treatment differed widely in the eligible studies ranging from four to 30 weeks (age at initiation of treatment) and three to 12 weeks (treatment duration), respectively.

Twelve studies targeted CCR2 with an inhibitory compound. Three studies used commercially available inhibitors.[36, 43, 44] Specifically, Yamashita et al.[44] tested Propagermanium, an organometallic which has been used in the treatment of chronic hepatitis B,[45] whereas Van Wanrooij et al. used TAK-779, a small molecule inhibitor of CCR2, CCR5, and CXCR3[38] that is under examination as an HIV entry inhibitor.[36] Winter et al.[43] tested RS102895, a CCR2 small molecule antagonist in a chronopharmacological study. Four studies examined proprietary inhibitors that were developed in-house[37, 38, 41, 42] and four studies utilized a plasmid encoding for an N-terminal deletion mutant of CCL2 (termed 7ND) as a therapeutic compound,[24-27] which inhibits CCL2 signaling by functioning as a dominant-negative inhibitor of CCL2.[46, 47] Finally, Liehn et al.[40] opted for a similar approach using a recombinant N-terminal truncate of CCL2. Only two studies used approaches directly targeting CCL2: Lutgens et al.[11] employed a monoclonal anti-CCL2 antibody, whereas Cynis et al.[39] used a small molecule blocking essential posttranslational modifications on the N-terminus of CCL2. Regarding study outcomes, all but two studies examined plaque burden either in the aortic root or arch, whereas three studies explored lesions in the carotid artery. Cynis et al.[39] opted for a femoral artery wire injury model in ApoE3Leiden mice, where lesion development was artificially induced and accelerated. Similarly, Liehn et al.[40] conducted wire injury in the carotid artery of *Apoe*$^{-/-}$ mice.

Inhibiting CCL2 or CCR2 reduces lesion size and skews plaques towards a stable phenotype

Twenty-two treatment arms from all 14 studies were included in the meta-analysis for lesion size, as displayed in **Fig. 2**. Blockade of CCL2 or CCR2 resulted in a significant decrease in atherosclerotic lesion size in the aortic root or arch (g=-0.75 [-1.17 to -0.32], $p=6\times10^{-4}$), as derived after pooling 18 study arms (171 animals in experimental group, 171 controls). Significant decreases were also found in both the carotid (g=-2.39 [-4.23 to -0.55], p=0.01, k= 3 study arms, 24 animals in experimental group, 25 controls) and femoral arteries (g=-2.38 [-3.50 to -1.26], $p=3\times10^{-5}$, k= 1 study arm, 10 animals in experimental group, 10 controls). There was a significant difference in the effects of CCL2/CCR2 inhibition across the three vascular beds (p=0.01) with larger effects seen in the carotid and femoral arteries, as compared to the aortic root and arch.

CCL2/CCR2 inhibition further reduced the intralesional macrophage accumulation in the aortic arch and root (g=-0.76 [-1.11 to -0.41], $p=2\times10^{-5}$, k= 12 study arms, 112 animals in experimental group, 111 controls) (**Fig. 3**), while leading to an increase in collagen deposition (g=0.70 [0.16 to 1.24], p=0.011, k= 6 study arms, 60 animals in experimental group, 60

controls) and smooth-muscle cell content (g=0.95 [0.24 to 1.66], p=0.009, k= 6 study arms, 61 animals in experimental group, 61 controls), consistent with a more stable plaque phenotype.[48]

Associations between CCL2/CCR2 inhibition and secondary outcomes are presented in **Online Figure I** and **Online Table III**. The experimental groups did not undergo changes in body weight, plasma triglycerides or blood monocytes. However, there was a significant increase in CCL2 plasma levels across studies inhibiting CCR2 and a significant decrease in IL-6 expression levels within plaques. There was a borderline association between CCL2/CCR2 inhibition and lower plasma total cholesterol levels.

Subgroup analyses reveal no differences by lesion stage or intervention target

There was at least moderate heterogeneity (I^2>50%) for all main outcomes except for macrophage accumulation (I^2=42%). To explore whether other study variables could explain the between-study differences we performed a series of subgroup analyses (**Fig. 4** and **Online Table IV**). There were no significant differences between subgroups of different stages of atherosclerosis progression (early, intermediate, advanced) at the time of onset of intervention, although there was a tendency for smaller effect sizes in mice with more advanced lesions. Similarly, we observed no difference in the effects of intervention on lesion size in the aortic arch or root between targets of intervention, with both CCL2 and CCR2 inhibition showing significant reductions. Lesion size reduction differed significantly between WTD-fed mice and mice fed normal laboratory diet (p=0.048) with the latter showing no significant reduction in lesion size. All but one study examining aortic lesions used *Apoe*$^{-/-}$ models of atherosclerosis, but the single study using *Ldlr*$^{-/-}$ mice also showed a significant reduction in lesion size. No significant differences in effects were detected between male- and female-specific analyses. None of the subgroup analyses resolved the heterogeneity between studies. A meta-regression analysis revealed an association between longer intervention duration and larger atheroprotective effects on lesion size (β=-0.153 [-0.285 to -0.021], p=0.023; **Online Figure IIA**), but failed to account for study heterogeneity (residual I^2=67%).

Effects of CCL2/CCR2 inhibition on intralesional macrophage accumulation predict the reductions in lesion size

To further explore sources of the derived heterogeneity, we performed meta-regression analyses exploring the associations between the effects of CCL2/CCR2 inhibition on plaque features and their effect on lesion size. We found a significant association between the effects of different interventions on macrophage accumulation within plaques and the effects on the overall aortic lesion size (β=0.789 [0.263 to 1.314], p=0.003; **Fig. 5**). Residual heterogeneity (I^2) after meta-regression was 27% compared to the initial 73%, thus invoking the notion that differences across the interventions in their effects on macrophage accumulation within plaques could explain 62% of the differences in overall effect on lesion size. There was no significant association between the effects of CCL2/CCR2 inhibition on plasma CCL2 levels and its effect on lesion size (**Online Figure IIB**).

Publication bias and risk of bias assessment

Applying the Egger's test, we detected a significant small-study effect (β=-7.95 [-12.08 to -3.82] p=0.0002) in the main analysis exploring the effects of CCL2/CCR2 inhibition on aortic lesion size, thus indicating presence of potential publication bias. After visual inspection of the respective funnel plot (**Online Figure III**), we explored whether a single outlier study[44] could account for the observed effect. Following exclusion of this study, the observed small-study effect was attenuated (β=-5.79, [-11.77 to 0.19], p=0.058), while the overall effect of CCL2/CCR2 inhibition on aortic lesion size remained stable (g=-0.55, [0.93 to -0.17], p=0.005).

Finally, all eligible studies underwent a thorough quality assessment with the SYRCLE risk of bias tool, which was used to define the assessment criteria we lay out in **Online Table II**.[28] The detailed results are presented in **Online Figure IV**. Importantly, there was evidence of

high risk of detection bias caused by a lack of assessor blinding during outcome assessment in eleven eligible studies. Furthermore, we detected high risk of attrition bias in eight studies, which failed to report exact sample sizes for every experiment or reasons for differing sample sizes across experiments of the same cohorts. All eligible studies were also assigned a high risk of reporting bias, because of the lack of a published pre-defined study protocol. The tool items referring to selection or performance bias, as well as another aspect of detection bias represented by randomness of outcome assessment order, could not be assessed for most eligible studies due to insufficient information provided within the respective publications. The adherence of the included studies to the ARRIVE guidelines for sample size, randomization, and blinding is summarized in **Online Figure V**. While all of the studies reported on samples sizes across tested subgroups, no study reported *a priori* sample size calculations. Furthermore, only 3 of the 14 studies clearly reported their randomization and blinding protocols.

Discussion

Pooling data from 14 preclinical studies of experimental atherosclerosis, we found that pharmacological blockade of CCL2 or CCR2 in mice leads to a significant reduction in atherosclerotic lesions in the aorta, the carotid, and the femoral artery. Furthermore, pharmacological inhibition of CCL2 or CCR2 is associated with reductions in intralesional macrophage accumulation and increases in plaque smooth muscle cell content and collagen deposition. These effects were similar when targeting either CCL2 or CCR2 but were stronger for lesions in the carotid and femoral arteries than in the aorta. While there was substantial heterogeneity in the extent of CCL2/CCR2 inhibition on atherosclerotic lesion size, these effects were highly correlated with the effects of the interventions on macrophage accumulation within plaques, thus supporting the notion that intralesional macrophage reduction can serve as a surrogate marker of efficacy of CCL2/CCR2 inhibition. Still, despite the promising results, our quality assessment detected sources of detection, attrition, and reporting bias in the majority of existing studies.

The consistently large effects of pharmacological CCL2/CCR2 inhibition on atherosclerotic lesions across studies with different designs and across different vascular beds, when seen in conjunction with previous findings in *Ccl2*$^{-/-}$[13] or *Ccr2*$^{-/-}$[9] mice testing the genetic deletion of the ligand or the receptor, provide strong preclinical support for the candidacy of CCL2/CCR2 signaling as a promising target in atherosclerosis. While data from clinical trials remain limited,[18] recent results from genetic[14] and epidemiological[15, 16] studies emphasize a causal role of the CCL2/CCR2 axis in human atherosclerosis. Hence, there is coherent evidence from preclinical, genetic, epidemiological, and early-phase clinical trials that targeting the CCL2/CCR2 pathway may be a viable strategy to mitigate the risk of atherosclerotic disease.

Interestingly, we found stronger attenuating effects of CCL2/CCR2 inhibition on atherosclerotic lesions in the carotid artery, as compared to lesions in the aortic arch and root. While these findings cannot be directly translated to humans, they are consistent with the stronger associations we previously found between both measured and genetically determined CCL2 circulating levels and risk of ischemic stroke, as compared to coronary artery disease and myocardial infarction.[14-16] Despite the common mechanisms underlying atherogenesis and atheroprogression across different vascular territories, differences in the effects of established risk factors, such as smoking and hypertension, on atherosclerotic manifestations from different vascular beds are well-known.[49] Whether pharmacological targeting of the CCL2/CCR2 axis and inflammation in humans differentially affects atherosclerotic lesion formation in different vessels would need to be further explored in future studies. Still, this could have implications for the selection of the right population for future clinical trials.

Aside from its influence on lesions size, CCL2/CCR2 inhibition further exerted an effect on plaque composition. Specifically, mice in the intervention arms exhibited a lower macrophage accumulation and a higher smooth muscle cell and collagen content, consistent with a smaller core and a thicker fibrous cap, both characteristics of a plaque less vulnerable to rupture and subsequent complications like acute ischemic stroke or coronary syndromes.[48] Our results are consistent with those of a recent cross-sectional study of plaque samples from patients undergoing carotid endarterectomy, which showed associations between CCL2 levels within plaques and histopathological features of plaque vulnerability.[17] Thus, these data support a role of CCL2/CCR2 beyond the early stages of atherogenesis and highlight the potential benefits of targeting this axis even in patients with established atherosclerotic disease in future trials.

In a meta-regression analysis, we found the heterogeneity of the effects of CCL2/CCR2 inhibition on aortic lesion size to be to a large extent explained by the effects on plaque macrophage accumulation. This observation agrees with the key role of the CCL2/CCR2 axis in attracting monocytes to the atherosclerotic lesion, but also indicates that the effects of pharmacological approaches targeting the CCL2/CCR2 axis on intralesional macrophage

accumulation could be used as a surrogate marker of the overall efficacy of CCL2/CCR2 inhibition. The latter could have implications for the design of future early-phase clinical trials and the identification of a proper readout for drug response and efficacy beyond clinical endpoints that would require very large sample sizes.

Another important finding of the current meta-analysis is the lack of heterogeneity in efficacy between studies targeting either CCL2 or CCR2. The consistency in the effects of molecules targeting either the ligand CCL2 or its receptor CCR2 for either decreasing lesion size or improving the plaque composition indicate no superiority of one approach over the other in animal models of atherosclerosis. This is important given the different structural and targeting properties of the pharmaceutical agents employed. Features such as surface coverage, binding affinity, or bioavailability differ substantially between antibodies, orthosteric small molecule inhibitors, or decoy ligands. Also, it should be noted that there are fewer agents targeting chemokine ligands, as compared to chemokine receptors,[50] which is also reflected in the low number of studies inhibiting CCL2 in our meta-analysis. Moving towards clinical trials in humans, it would be important to consider both approaches as potentially promising.

Our study has limitations. First, there was considerable between-study heterogeneity in almost all analyzed outcomes, which could bias the derived effect estimates. It is possible that differences in experimental design as well as in the efficacy of the tested interventions underly this heterogeneity. More specifically, there were differences in the background strains of mice, models that were examined, the stages of lesion progression at the time of intervention, the duration of treatment, the pharmacological properties of the different CCL2/CCR2 inhibitors, the mode of administration of the different agents, the dosage, the vessels that were examined, and even the methods for quantifying lesion formation. While we aimed to account for these differences in subgroup and meta-regression analyses, this was not possible for all of these factors, whereas several study subgroups were too small for meaningful analyses. For instance, the finding that there were no significant differences in lesion size reduction between early, intermediate, and advanced lesions may have been driven by the small number of studies reporting effects on already established atherosclerotic plaques. While our subgroup analyses based on the time of intervention was rather underpowered, individual studies support a larger impact when treatment is started at early stages before lesions have been formed, corroborating the important role of the CCL2/CCR2 axis in atherosclerotic lesion initiation.[37, 51] There were also substantial differences in fat source, percentage, and cholesterol content of the atherogenic Western-type diets that were used by the included studies, which may explain part of the observed heterogeneity. The small number of studies with detailed information on the exact composition of the Western-type diet prohibited a meaningful analysis of the impact of diet composition on our results. Second, there was evidence of small-study effects indicating publication bias. While publication bias could indeed influence the effect estimates, we found the small-study effect to be primarily driven by a single outlier study. Reassuringly, the effects were only slightly attenuated after exclusion of this study from the meta-analysis. Third, some of our analyses, such as the analyses of lesions in the carotid and femoral arteries, the analyses for CCL2 inhibition, the subgroup analyses per stage of atherosclerotic lesions, and some meta-regression analyses were based on a rather small number of study arms and are thus limited by low statistical power. The disproportional number of studies exploring lesions in the aortic root or arch is to be expected because of the higher prevalence of atherosclerotic lesions in the aorta of genetically atheroprone mice, as compared to other vessels, which makes it the vascular bed of choice for the majority of studies exploring mouse models of atherosclerosis.[52] Fourth, the lesion staging used in the subgroup analysis relied on the age of mice, feeding, and treatment durations rather than histopathological lesion assessment due to paucity of data. Fifth, the exclusion of studies not meeting our eligibility criteria might have biased our results. However, because our selection strategy was pre-defined before the start of the literature search and our criteria were relatively broad, as reflected in the considerable between-study differences in experimental design, we believe that there is no systematic bias towards a selective inclusion of published studies with specific findings. Lastly, agents used in some studies, like 7ND, appear impractical for therapeutic approaches in humans compared to small molecule inhibitors.

An important element of our review that should be highlighted is the high risk of several forms of bias in the included studies, as detected in our quality assessment. We found the majority of the included studies to fulfill few of the quality criteria and to be vulnerable to detection, attrition, and reporting bias. For example, none of the included studies was performed according to a pre-published protocol, whereas the eligible studies provided no information on random animal housing, caretaker blinding, and random outcome assessment. This necessitates cautious interpretation of the findings, as sources of bias in preclinical studies can contribute to lack of translation of promising preclinical experiments into successful clinical trials.[53]

In view of these limitations, our findings offer key insights into the impact of CCL2/CCR2 inhibition on atheroprotection beyond the results of individual studies entering the meta-analysis. Specifically, these novel findings may have important implications for the transition of strategies targeting the CCL2/CCR2 axis for atheroprotection towards clinical testing. First, the lack of a difference in the efficiency of agents targeting either CCL2 or CCR2 highlights the importance of further investment in the development of agents targeting either molecules and implies that efficient agents against either target could be promoted for clinical testing. Second, our finding that the efficiency of different agents for reducing plaque size is proportional to their effects on macrophage accumulation opens a new pathway for development of biomarkers capturing plaque macrophage content, which could be used in trials testing such agents. Imaging methods currently under development, such as PET-based assessment of plaque inflammation could prove useful in this regard.[54] Third, pooling studies in a meta-analysis offered sufficient power to detect highly consistent effects for an effect of CCL2/CCR2 inhibition on plaque features beyond plaque size, which emphasizes the potential utility of this approach for patients with established atherosclerotic lesions. Fourth, our finding of a stronger effect on carotid and femoral lesions has implications for the selection of target populations that could benefited from such approaches. Beyond patients with coronary artery disease that have been the primary target population of previous anti-inflammatory trials for atheroprotection,[2-4, 55] our results suggest that clinical testing could likewise be expanded to other populations, such as patients with carotid stenosis, large artery atherosclerotic stroke, and established peripheral artery disease.

In conclusion, preclinical evidence supports a potential atheroprotective effect of the pharmacological targeting of the CCL2/CCR2 axis in mouse models of atherosclerosis. However, our systematic review detected high risk of bias in the published studies, thus highlighting the need for additional research based on robust methodology in this translationally relevant field. While still weak, this preclinical evidence adds to the recent data from human studies that imply a translational potential of targeting CCL2/CCR2 signaling in atherosclerotic disease and provides insights for informing the design of future clinical trials.

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Disclosures

None.

Supplemental Materials

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Highlights

- Pharmacological inhibition of either CCL2 or CCR2 is associated with reduced lesion sizes in hyperlipidemic mouse models of atherosclerosis.
- CCL2/CCR2 inhibition further changes plaque features like macrophage accumulation, collagen deposition, and smooth-muscle cell content.
- Effects on CCL2/CCR2 inhibition on macrophage accumulation are significantly associated with effects on lesion size.
- There was no sufficient power to detect differences in the effects of CCL2 or CCR2 inhibition by stage of atherosclerosis at the time of treatment onset.
- The majority of existing studies suffer major quality issues that highlight the need for additional high-quality research.

Table 1. **Descriptive characteristics of eligible studies included in the meta-analysis.** N/A = Information not provided in the study; m = male, f = female; WTD = Western-type diet; d = day; p.o. = oral gavage, i.p. = peritoneal injection, i.m. = intramuscular injection, s.c. = subcutaneous injection.

First author, year	Mouse strain & model	Sex	Diet (manufacturer & for WTD fat composition, where available)	Start of diet/ weeks	Intervention	Dosage, route, interval	Start of intervention/weeks (duration/ weeks)	Study groups (n)	Lesion site(s)
AIELLO, 2010[37]	C57BL/6, *Apoe*$^{-/-}$	m	normal laboratory diet (Purina Prolab® RMH 3000)		INCB-3344 (small molecule CCR2 antagonist)	50mg kg^{-1} d^{-1}, p.o.	7 (4) 10 (6) 10 (10) 20 (6)	4-week treatment (8) & control (8) 10-week-old, 6-week treatment (7) & control (11) 20-week-old, 6-week treatment (8) & control (10) 10-week treatment (7) & control (8)	Aortic root, brachiocephalic artery (20-week-old mice, 6-week treatment)
BOT, 2017[38]	N/A *Apoe*$^{-/-}$	m	WTD (SDS, 15% cocoa butter, 0.25% cholesterol)	10-12	15a (small molecule CCR2 antagonist)	5mg kg^{-1}, i.p. 1x daily	10-12 (4)	Treatment (9) & control (10)	Aortic root, Carotid artery (cuff placement)
CYNIS, 2011[39]	C57BL/6J, ApoE3 Leiden	m	WTD (manufacturer N/A, 15% cocoa butter, 1% corn oil, 1% cholesterol, 2% choline, 0.05% cholate)	12	PQ50 (small molecule glutaminyl cyclase/iso-glutaminyl cyclase inhibitor)	2,4mg mL^{-1} in drinking water, p.o. for 7 days, then 1,2mg mL^{-1}	12 (5)	Treatment (10) & control (10)	Femoral artery (cuff placement)
INOUE, 2002[27]	C57BL/6, *Apoe*$^{-/-}$	N/A	normal laboratory diet (Oriental Yeast)		7ND (plasmid encoding an N-terminal CCL2 deletion variant)	100µg, i.m. 1x biweekly with electroporation	20 (8)	Treatment (10) & control (10)	Aortic arch
LIEHN, 2010[40]	N/A, *Apoe*$^{-/-}$	N/A	WTD (manufacturer and composition not specified)		PA508 (recombinant CCL2 variant (CCL2-based "decoy" chemokine incapable of CCR2 activation)	10µg, i.p. 1x daily	8 (3)	Treatment (5) & control (5)	Carotid artery (wire injury)
LUTGENS, 2005[11]	C57BL/6 *Apoe*$^{-/-}$	m	N/A		11K2 (monoclonal CCL2 antibody)	100µg, i.p. 2x/week	5 (12) 17 (12)	5-week-old, early treatment & control 17-week-old, delayed treatment (all 15 per group)	Aortic arch

Ni, 2001[24]	C57BL/6J, *Apoe*$^{-/-}$	N/A	WTD (Oriental Yeast, 20% fat, 0.15% cholesterol)	7-8	7ND (plasmid encoding an N-terminal CCL2 deletion mutant; encapsulated in 7HVJ liposome)	5µg, i.m. 1x every 3 weeks	7-8 (6)	Treatment (8) & control (8)	Aortic root
Ni, 2004[25]	C57BL/6 *Apoe*$^{-/-}$	m	normal laboratory diet		7ND (plasmid encoding an N-terminal CCL2 deletion mutant)	100µg, i.m. 1x biweekly with electroporation	30 (4)	Saline infusion, treatment & control Angiotensin-II infusion, treatment & control (all 10)	Aortic root
Okamoto, 2012[41]	C57BLKS/J, *Apoe*$^{-/-}$	m	WTD (Oriental Yeast, 15% fat, 1.25% cholesterol)	4	TLK19705 (small molecule CCR2 antagonist)	10mg kg^{-1} d^{-1}, p.o.	4 (8)	Treatment (10) & control (8)	Aortic root
Olzinski, 2010[42]	N/A, *Apoe*$^{-/-}$, human CCR2 knock-in	N/A	WTD (21% milk fat, 0.2% cholesterol)	22-24	GSK1344386B (small molecule CCR2 antagonist)	10mg kg^{-1} d^{-1}, p.o.	22-24 (5)	Treatment (20) & control (20), both Angiotensin-II-treated	Aortic root
de Waard, 2010[26]	N/A, *Apoe*$^{-/-}$	m	WTD (SDS, composition N/A)	8-10	7ND (plasmid encoding an N-terminal CCL2 deletion mutant)	unknown, i.m. once	12-14 (4-5)	Treatment & control, both Angiotensin-II-treated (26 total)	Aortic root & arch
van Wanrooij, 2005[36]	N/A, *Ldlr*$^{-/-}$	f	WTD (manufacturer N/A 15% cocoa butter, 0.25% cholesterol)	15	TAK-779 (small molecule CCR2/CCR5/ CXCR3 antagonist)	100µg, s.c. every 2 days	17 (6) 15 (6)	15-week-old treatment & control, 17-week-old, collar-implanted treatment & control (all 10)	Aortic root, carotid artery (cuff placement)
Winter, 2018[43]	C57BL/6J, *Apoe*$^{-/-}$	m&f	WTD (ssniff cat. # E15721, 21% fat, 0.15% cholesterol)	8	RS102895 (small molecule CCR2 antagonist)	5mg kg^{-1}, i.p. 1x daily	8 (4)	Zeitgeber time 5 & 17 both treatment & control each (all 8)	Aortic root
Yamashita, 2002[44]	C57BL/6, *Apoe*$^{-/-}$	N/A	WTD (Oriental Yeast, normal laboratory diet plus 7.5% cocoa butter, 1.25% cholesterol, 0.5% cholate)	4	Propagermanium (small molecule CCR2 antagonist)	0,005% of diet, p.o.	4 (8) 4 (12)	8-week & 12-week both treatment & control each (all 8)	Aortic root

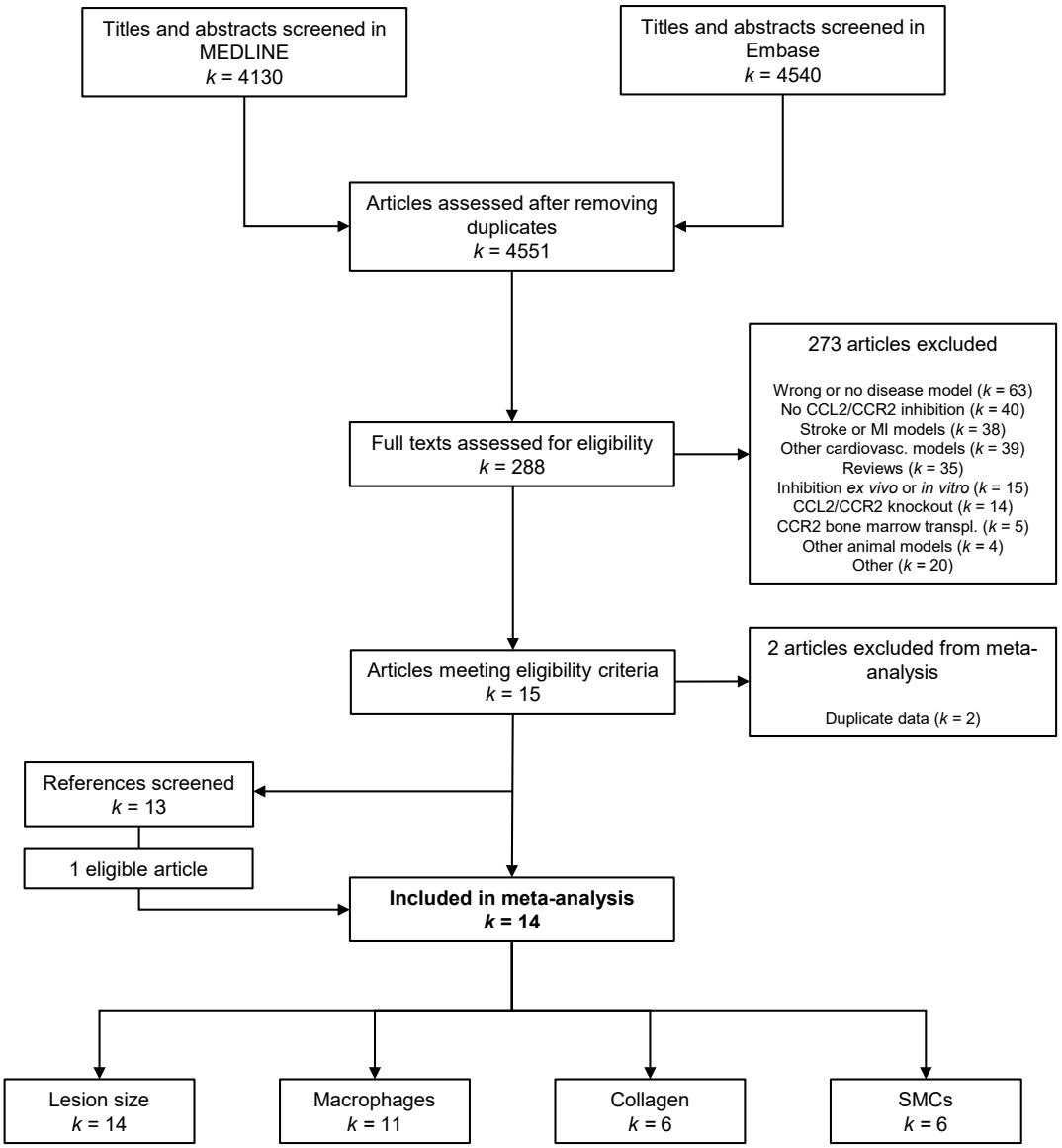


Figure 1. Flowchart of the study selection process. The search was performed in MEDLINE through the PubMed engine as well as Embase. Articles were evaluated for eligibility on the basis of their titles, abstracts, and full texts. k = number of articles. SMCs = smooth-muscle cells.

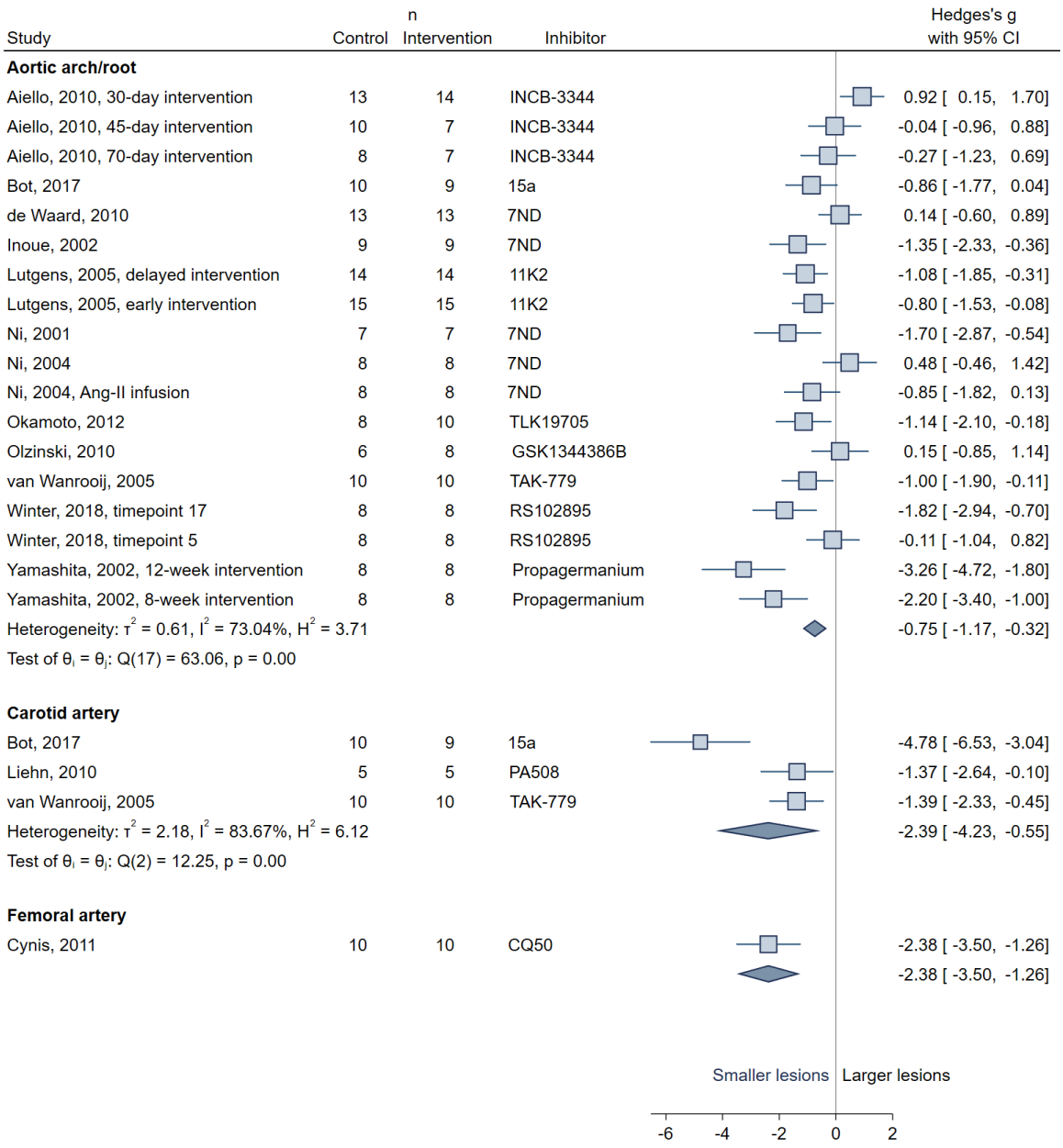


Figure 2. Forest plot of the effects of CCL2/CCR2 inhibition versus control (Hedges' g) on atherosclerotic lesion size in the aortic arch and root, the carotid, and the femoral artery. Shown are the standardized mean differences, calculated as Hedges' *g*, with their respective 95% confidence intervals per study. Plot squares are weighted for study size and correspond to individual effects, whereas plot whiskers correspond to the 95% confidence intervals. Diamonds indicate the pooled effects for each vascular bed. τ^2, I^2 and H^2 as indicators of group heterogeneity as well as test of group homogeneity $\theta_i - \theta_j$ are displayed for the groups containing more than one study.

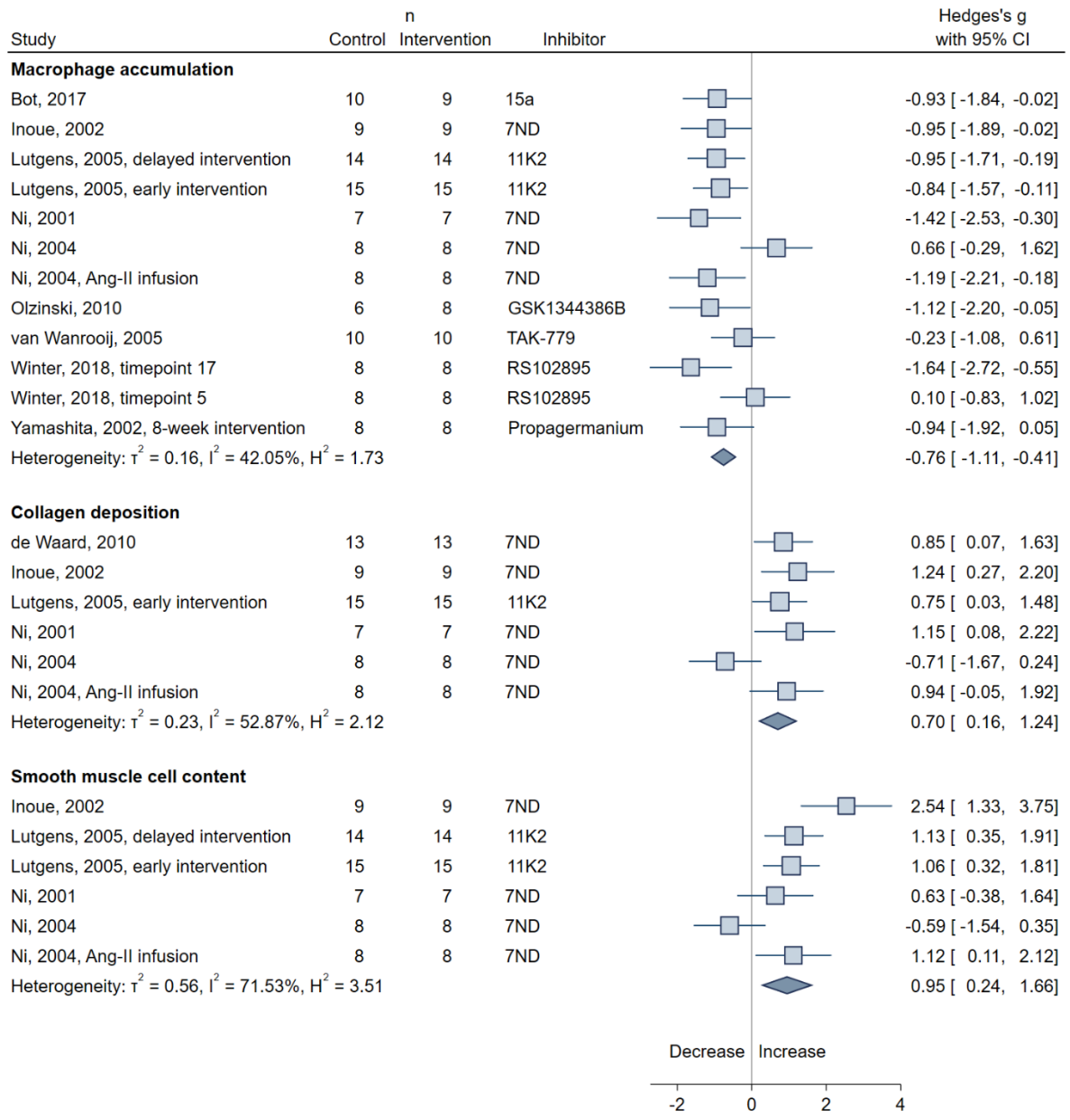


Figure 3. Forest plot of the effects of CCL2/CCR2 inhibition versus control (Hedges' g) on macrophage accumulation, collagen deposition, and smooth muscle content in plaques of the aortic arch or root. Shown are the standardized mean differences, calculated as Hedges' *g*, with their respective 95% confidence intervals per study. Plot squares are weighted for study size and correspond to individual effects, whereas plot whiskers correspond to the 95% confidence intervals. Diamonds indicate the pooled effects for each outcome. τ^2, I^2 and H^2 as indicators of group heterogeneity are displayed for the groups containing more than one study.

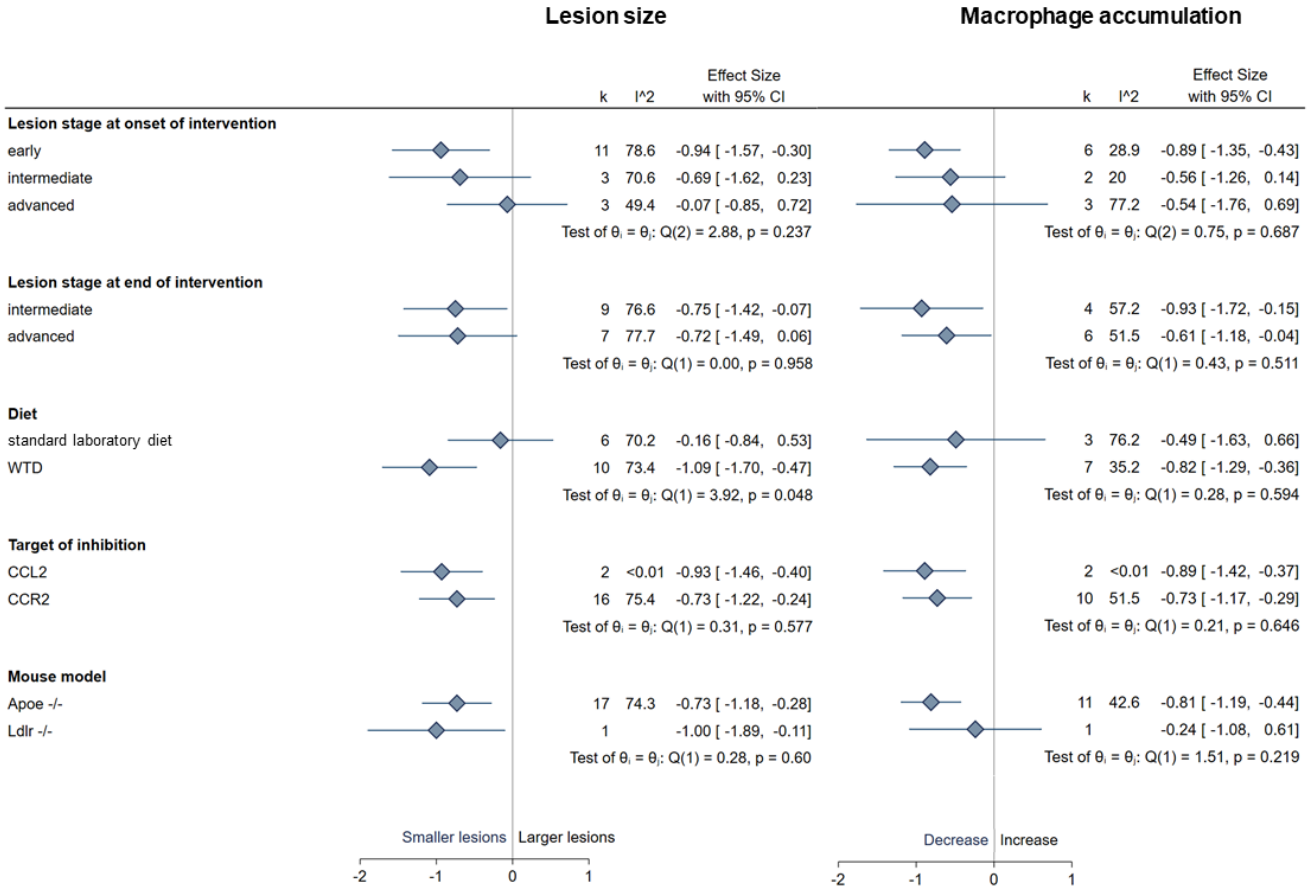


Figure 4. Subgroup analyses regarding the effects of CCL2/CCR2 inhibition versus control (Hedges' g) on aortic plaque burden and macrophage accumulation by various study characteristics. Shown are the pooled standardized mean differences, calculated as Hedges' *g*, with their respective 95% confidence intervals for each subgroup. Diamonds correspond to pooled effects per subgroup, whereas whiskers correspond to the 95% confidence intervals. Number of study arms (*k*) and heterogeneity measures (I^2) per subgroup are displayed. The Cochran's Q test and its p-value are provided as measures of between-subgroup differences. WTD = western-type diet.

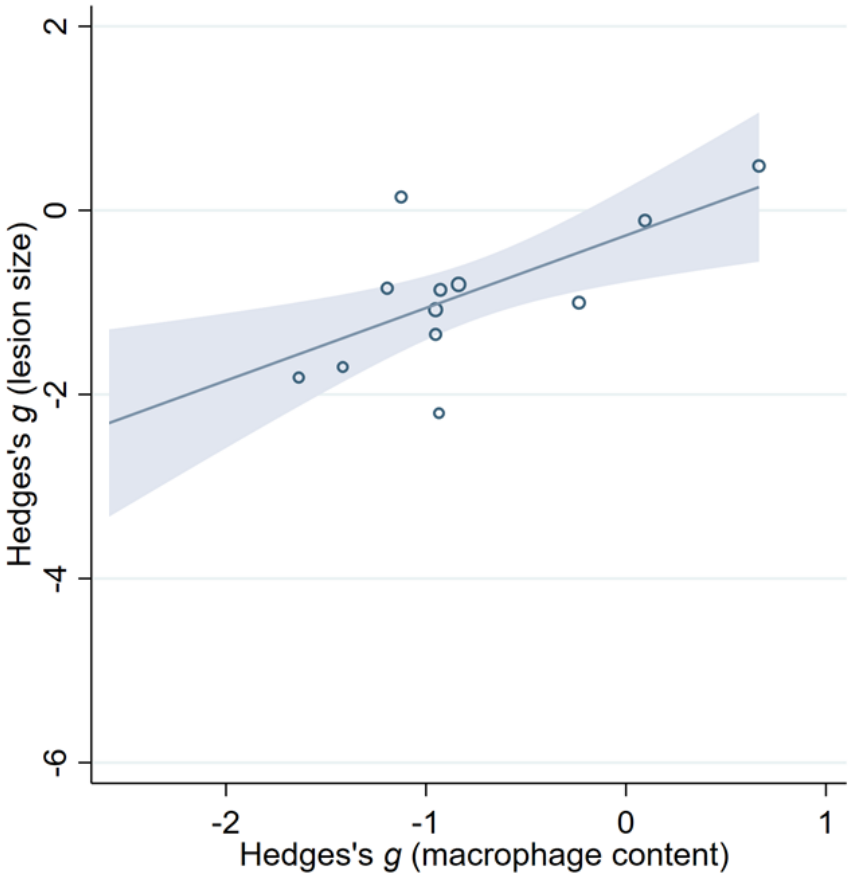


Figure 5. Meta-regression analysis of the effects of CCL2/CCR2 inhibition versus control (Hedges' g) on macrophage accumulation on the effects of the intervention on atherosclerotic lesion size in the aortic arch and root. Data points indicate individual studies around the regression line with its 95% confidence interval (shaded area).

Pharmacological targeting of the CCL2/CCR2 axis for atheroprotection: a meta-analysis of preclinical studies

SUPPLEMENTAL MATERIAL

Luka Živković[1], Yaw Asare, PhD[1], Jürgen Bernhagen, PhD[1,2,3], Martin Dichgans, MD[1,2,4]*, Marios K. Georgakis, MD, PhD[1]*

[1] Institute for Stroke and Dementia Research (ISD), University Hospital, LMU Munich, Munich, Germany

[2] Munich Cluster for Systems Neurology (SyNergy), Munich, Germany

[3] Munich Heart Alliance, German Center for Cardiovascular Diseases (DZHK), 80802 Munich, Germany

[4] German Centre for Neurodegenerative Diseases (DZNE), Munich, Germany

* These authors jointly supervised this work.

Correspondence to:

Marios K. Georgakis, MD, PhD
Neurology resident & Postdoctoral researcher
Institute for Stroke and Dementia Research, University Hospital LMU
Ludwig-Maximilians-University (LMU), Munich, Germany
Feodor-Lynen-Str. 17, 81377 Munich, Germany
Telephone: +49-89-4400-46063; Fax: +49-89-4400-46040
e-mail: marios.georgakis@med.uni-muenchen.de

Martin Dichgans, MD
Director
Institute for Stroke and Dementia Research, University Hospital LMU
Ludwig-Maximilians-University (LMU), Munich, Germany
Feodor-Lynen-Str. 17, 81377 Munich, Germany
Telephone: +49-89-4400-46018; Fax: +49-89-4400-46040
e-mail: martin.dichgans@med.uni-muenchen.de

Online Table I. PRISMA checklist for the evaluation of systematic reviews and meta-analyses[20]. N/A = item not applicable or not reported on.

Section and Topic	Item #	Checklist item	Location where item is reported
TITLE			
Title	1	Identify the report as a systematic review.	Title (p.1)
ABSTRACT			
Abstract	2	See the PRISMA 2020 for Abstracts checklist.	Abstract (p.2)
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of existing knowledge.	Introduction (pp.4f.)
Objectives	4	Provide an explicit statement of the objective(s) or question(s) the review addresses.	
METHODS			
Eligibility criteria	5	Specify the inclusion and exclusion criteria for the review and how studies were grouped for the syntheses.	Eligibility criteria (pp. 6f..)
Information sources	6	Specify all databases, registers, websites, organisations, reference lists and other sources searched or consulted to identify studies. Specify the date when each source was last searched or consulted.	Search strategy (p.6)
Search strategy	7	Present the full search strategies for all databases, registers and websites, including any filters and limits used.	Search strategy (p.6)
Selection process	8	Specify the methods used to decide whether a study met the inclusion criteria of the review, including how many reviewers screened each record and each report retrieved, whether they worked independently, and if applicable, details of automation tools used in the process.	Search strategy (p.6)
Data collection process	9	Specify the methods used to collect data from reports, including how many reviewers collected data from each report, whether they worked independently, any processes for obtaining or confirming data from study investigators, and if applicable, details of automation tools used in the process.	Data abstraction (p.7)
Data items	10a	List and define all outcomes for which data were sought. Specify whether all results that were compatible with each outcome domain in each study were sought (e.g. for all measures, time points, analyses), and if not, the methods used to decide which results to collect.	Outcomes (pp.6f.)
	10b	List and define all other variables for which data were sought (e.g. participant and intervention characteristics, funding sources). Describe any assumptions made about any missing or unclear information.	Data abstraction (p.7) Meta-analysis (pp.7f.)
Study risk of bias assessment	11	Specify the methods used to assess risk of bias in the included studies, including details of the tool(s) used, how many reviewers assessed each study and whether they worked independently, and if applicable, details of automation tools used in the process.	Study quality assessment (p.7), Online Table II (suppl. p.4.)
Effect measures	12	Specify for each outcome the effect measure(s) (e.g. risk ratio, mean difference) used in the synthesis or presentation of results.	Meta-analysis (pp.7f.)
Synthesis methods	13a	Describe the processes used to decide which studies were eligible for each synthesis (e.g. tabulating the study intervention characteristics and comparing against the planned groups for each synthesis (item #5)).	Meta-analysis (pp.7f.)
	13b	Describe any methods required to prepare the data for presentation or synthesis, such as handling of missing summary statistics, or data conversions.	Meta-analysis (pp.7f.)
	13c	Describe any methods used to tabulate or visually display results of individual studies and syntheses.	Meta-analysis (pp.7f.)
	13d	Describe any methods used to synthesize results and provide a rationale for the choice(s). If meta-analysis was performed, describe the model(s), method(s) to identify the presence and extent of statistical heterogeneity, and software package(s) used.	Meta-analysis (pp.7f.)
	13e	Describe any methods used to explore possible causes of heterogeneity among study results (e.g. subgroup analysis, meta-regression).	Meta-analysis (pp.7f.)
	13f	Describe any sensitivity analyses conducted to assess robustness	Meta-analysis (pp.7f.)

		of the synthesized results.	
Reporting bias assessment	14	Describe any methods used to assess risk of bias due to missing results in a synthesis (arising from reporting biases).	Study quality assessment (p.7)
Certainty assessment	15	Describe any methods used to assess certainty (or confidence) in the body of evidence for an outcome.	Meta-analysis (pp.7f.)
RESULTS			
Study selection	16a	Describe the results of the search and selection process, from the number of records identified in the search to the number of studies included in the review, ideally using a flow diagram.	Characteristics of eligible studies (p.9), Fig.1
	16b	Cite studies that might appear to meet the inclusion criteria, but which were excluded, and explain why they were excluded.	Characteristics of eligible studies (p.9)
Study characteristics	17	Cite each included study and present its characteristics.	Characteristics of eligible studies (p.9), Table 1 (pp.21f.)
Risk of bias in studies	18	Present assessments of risk of bias for each included study.	Publication bias and risk of bias assessment (pp.10f.), Online Figure IV (suppl. p.10)
Results of individual studies	19	For all outcomes, present, for each study: (a) summary statistics for each group (where appropriate) and (b) an effect estimate and its precision (e.g. confidence/credible interval), ideally using structured tables or plots.	Fig.2, Fig.3, Online Table III (suppl. p.5), Online Table IV (suppl. p.6), Online Figure I (suppl. p.7)
Results of syntheses	20a	For each synthesis, briefly summarise the characteristics and risk of bias among contributing studies.	Results (pp.9ff.)
	20b	Present results of all statistical syntheses conducted. If meta-analysis was done, present for each the summary estimate and its precision (e.g. confidence/credible interval) and measures of statistical heterogeneity. If comparing groups, describe the direction of the effect.	Results (pp.9ff.), Fig.2, Fig.3, Fig.4, Online Table III (suppl. p.5), Online Table IV (suppl. p.5), Online Figure I (suppl. p.7)
	20c	Present results of all investigations of possible causes of heterogeneity among study results.	Results (pp.9ff.), Fig.4, Fig.5
	20d	Present results of all sensitivity analyses conducted to assess the robustness of the synthesized results.	Results (pp.9ff)
Reporting biases	21	Present assessments of risk of bias due to missing results (arising from reporting biases) for each synthesis assessed.	Publication bias and risk of bias assessment (p.10f.), Online Figure IV (suppl. p.10)
Certainty of evidence	22	Present assessments of certainty (or confidence) in the body of evidence for each outcome assessed.	Confidence intervals throughout the results and in Figs. 2-5
DISCUSSION			
Discussion	23a	Provide a general interpretation of the results in the context of other evidence.	Discussion (pp.12ff.)
	23b	Discuss any limitations of the evidence included in the review.	
	23c	Discuss any limitations of the review processes used.	
	23d	Discuss implications of the results for practice, policy, and future research.	
OTHER INFORMATION			
Registration and protocol	24a	Provide registration information for the review, including register name and registration number, or state that the review was not registered.	N/A
	24b	Indicate where the review protocol can be accessed, or state that a protocol was not prepared.	
	24c	Describe and explain any amendments to information provided at registration or in the protocol.	
Support	25	Describe sources of financial or non-financial support for the review, and the role of the funders or sponsors in the review.	Sources of funding (p.14)
Competing interests	26	Declare any competing interests of review authors.	Disclosures (p.14)
Availability of data, code and other materials	27	Report which of the following are publicly available and where they can be found: template data collection forms; data extracted from included studies; data used for all analyses; analytic code; any other materials used in the review.	Data availability (p.8), Major Resources Table (suppl. p.12)

Online Table II. Risk of bias evaluation criteria. The column to the left contains the individual items of the SYRCLE risk of bias tool, along with the respective type of bias.[28] The following columns contain predefined statements that, if applicable to the respective study, indicated a specific type of bias ("low", "high", "unclear"). The results of the risk of bias assessment are displayed in **Online Figure IV**.

Item	**Bias type**	**Indicators**		
		Low RoB	High RoB	Unclear RoB
Sequence generation	Selection bias	The authors describe random assignment of mice to treatment or control group.	The assignment of mice was based on physiological parameters (e.g. body weight) or other non-random procedure.	The procedure of assigning mice to either treatment or control group is not described.
Baseline characteristics	Selection bias	Treatment and control group mice were similar or identical in all baseline characteristics (sex, age, weight).	Treatment and control group differed in sex of mice, baseline weight, or age at intervention in weeks.	The authors do not specify all baseline characteristics, e.g. only report sex of the mice.
Allocation concealment	Selection bias	The investigators did not have a way to predict whether a mouse was assigned to control or treatment group.	The assignment of mice to treatment or control group was predictable or foreseeable to the investigators.	Details on allocation concealment are not provided in the study, neither are there apparent ways of predicting allocation.
Random housing	Performance bias	Mice were randomly assigned to cages that were randomly placed within the animal facility.	Mice of the same group were housed in the same cage while cages were placed non-randomly.	The authors do not provide details on housing of the animals.
Caregiver blinding	Performance bias	Caregivers or investigators administering treatment or control were appropriately blinded.	Caregivers or investigators had a way of knowing whether a mouse was to receive either treatment or control.	No details on caregiver blinding are being provided.
Random outcome assessment	Detection bias	The order of outcome assessment in mice (e.g. sacrificing, histological processing) was randomly generated.	The order of outcome assessment in mice was predetermined or not based on random components.	The authors do not provide details on the order in which outcomes have been assessed.
Assessor blinding	Detection bias	The investigator assessing any outcome was blinded with regards to whether a mouse had been given treatment or control.	The investigator assessing any outcome was aware or had a way of knowing whether he was assessing a treatment or control group mouse.	N/A
Incomplete outcome data	Attrition bias	Study outcomes were balanced with regards to number of mice in both treatment and control group.	Survival rates in treatment and control groups were different, or outcomes had different numbers of animals in one group compared to the other.	N/A
Selective outcome reporting	Reporting bias	All experiments were performed based on a pre-published protocol.	There is no available pre-published protocol for this study. **Exception**: Studies reporting lesion size as well as two measurements of plaque characteristics were marked with an * in **Online Figure IV**.	N/A
Other sources of bias		No other drugs or agents were administered to the mice in addition to treatment or control.	Other drugs or agents (e.g. Angiotensin II) were administered.	N/A

Online Table III. Summary effects of CCL2/CCR2 inhibition on secondary outcomes. Shown are overall Hedges' g, along with 95% confidence interval and p-value, as derived from random-effect meta-analyses. Heterogeneity is expressed as I^2, and the number of study arms included in meta-analysis with *k*. * Significant at $p < 0.05$.

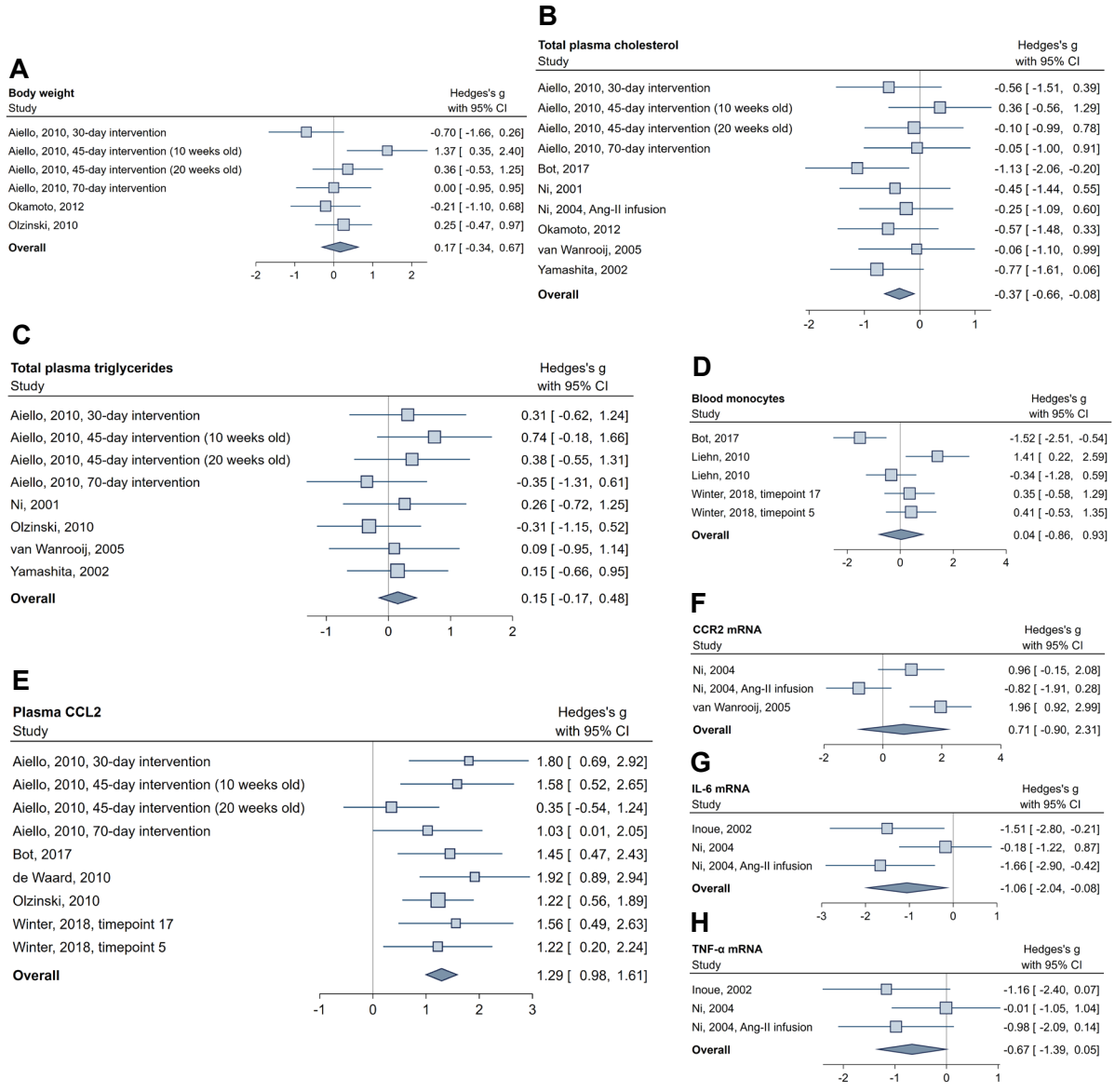
	Body weight	**Total plasma cholesterol**	**Total triglycerides**	**Blood monocytes**
g	0.17	-0.37	0.15	0.04
[95% CI], p	[-0.34; 0.67], 0.522	[-0.66; -0.08], 0.013*	[-0.17; 0.48], 0.363	[-0.86; 0.93]; 0.938
I^2	47.4%	<0.01%	<0.01%	75.5%
k	6	10	8	5

	Plasma CCL2	**CCR2 mRNA**	**IL-6 mRNA**	**TNF-α mRNA**
g	1.29	0.71	-1.06	-0.67
[95%-CI], p	[0.98; 1.61], $< 1\times10^{-5}$*	[-0.90; 2.31], 0.384	[-2.04; -0.08], 0.035*	[-1.39; 0.05], 0.069
I^2	<0.01%	84.9%	50.8%	18.4%
k	9	3	3	3

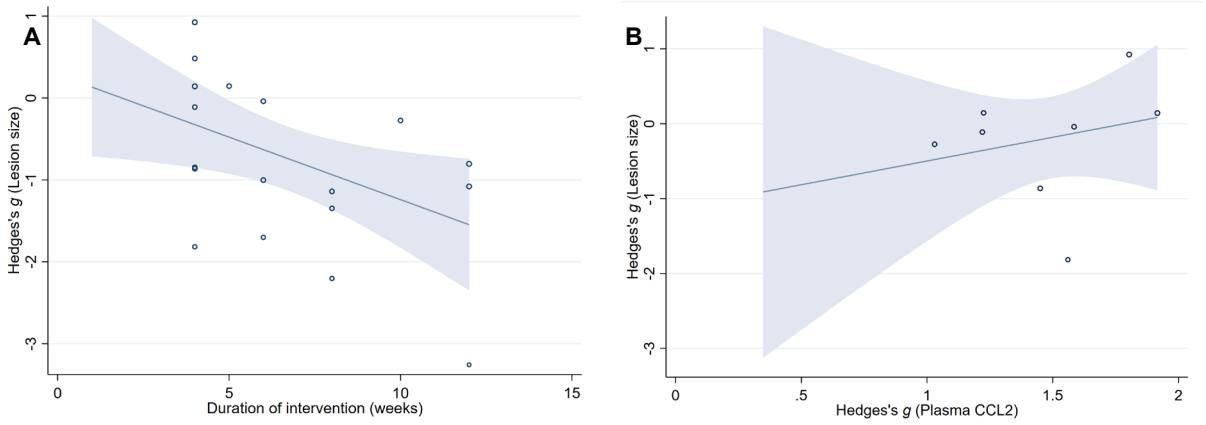
Online Table IV. Subgroup analyses regarding the effects of CCL2/CCR2 inhibition versus control (Hedges' g) on collagen and smooth muscle cell (SMC) content in aortic lesions. Shown are the pooled standardized mean differences, calculated as Hedges' *g*, with their respective 95% confidence intervals for each subgroup. Number of study arms (*k*) and heterogeneity measures (I^2) per subgroup are displayed. The Cochran's Q test and its p-value are provided as measures of between-subgroup differences. * Significant at p<0.05.

		Collagen content			SMC content		
	Subgroups	*g*	[95% CI], p	I^2 (%) *k*	*g*	[95% CI], p	I^2 (%) *k*
Lesion stage at intervention	early	0.89	[0.28; 1.48], 0.003*	<0.01 2	0.91	[0.31; 1.51], 0.003*	<0.01 2
	intermediate	1.00	[0.40; 1.60], 0.001*	<0.01 2	2.54	[1.33; 3.75], 0.0004*	(-) 1
	delayed	0.11	[-1.51; 1.73], 0.897	82.0 2	0.25	[-1.43; 1.93], 0.764	83.0 2
	Q_b; *p*		1.03; 0.597			6.79; 0.030*	
Diet	Normal laboratory diet	0.48	[-0.71; 1.68], 0.430	78.1 3	0.99	[-0.76; 2.74], 0.267	88.0 3
	WTD	0.96	[0.33; 1.59], 0.003*	<0.01 2	0.63	[-0.38; 1.64], 0.219	(-) 1
	Q_b; *p*		0.47; 0.494			0.12; 0.728	
Target	CCL2	0.75	[0.03; 1.48], 0.041*	(-) 1	1.10	[0.56; 1.64], <0.0001*	<0.01 2
	CCR2	0.69	[0; 1.38], 0.050	(-) 5	0.89	[-0.34; 2.11], 0.155	82.03 4
	Q_b; *p*		0.02; 0.897			0.09; 0.76	
Model	*Apoe*$^{-/-}$	0.68	[0.14; 1.21], 0.013*	52.8 6	0.95	[0.24; 1.66], 0.009	71.5 6
	Ldlr$^{-/-}$		(-)			(-)	
	Q_b; *p*		(-)			(-)	

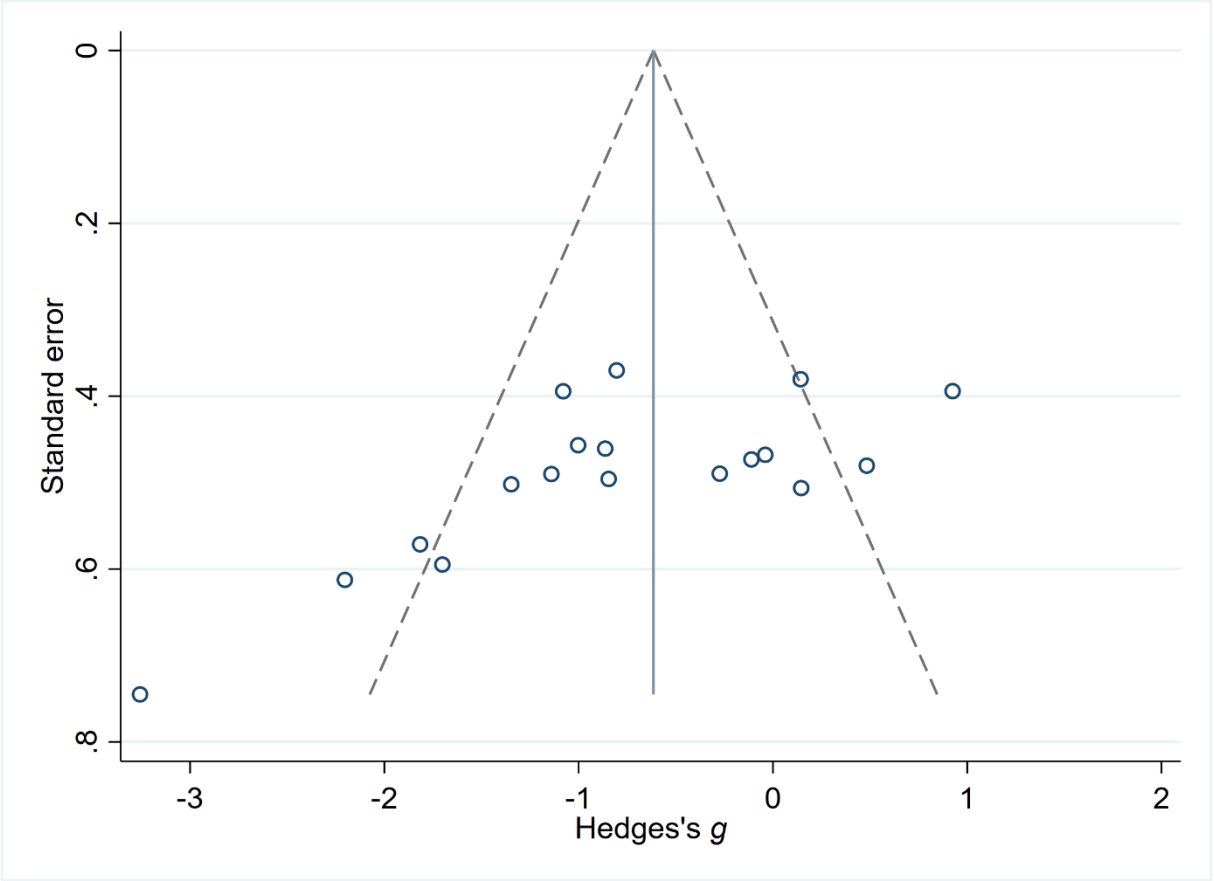
Online Figure I. Forest plots of the effects of CCL2/CCR2 inhibition versus control (Hedges' *g*) on secondary outcomes. Shown are the results for **(A)** mouse body weight, **(B)** plasma cholesterol and **(C)** triglycerides, **(D)** circulating blood monocytes, **(E)** circulating CCL2, as well as tissue mRNA expression of **(F)** CCR2, **(G)** IL-6, and **(H)** TNF-α. Individual study arms' Hedges' *g* is provided alongside 95% confidence interval (95% CI) in brackets. Weighted plot squares indicate Hedges' *g*, whiskers depict the confidence interval.



Online Figure II. Meta-regression analysis of (A) duration of intervention and (B) the effects of the intervention on plasma CCL2 levels on the effects of the intervention on atherosclerotic lesion size in the aortic arch and root. Predicted regression lines with 95% confidence interval are depicted for lesion size in the aortic arch/root against duration of intervention in weeks (**A**; (β=-0.153, 95%CI=[-0.285; -0.021], p=0.023, residual I^2=67.4%), and plasma CCL2 levels at end of intervention (**B**; β=0.634, 95%CI=[-1.248; 2.517], p=0.509, residual I^2=65.1%). Data points indicate individual studies around the regression line with its 95% confidence interval (blue shaded area).



Online Figure III. Funnel plot of the effects of CCL2/CCR2 inhibition versus control (Hedges' g) in the individual studies on atherosclerotic lesion size in the aortic arch or root. Circles indicate individual studies. A standard-error-adjusted pseudo-95% confidence range is indicated with dotted lines. The vertical blue line corresponds to the pooled Hedges' *g* for lesion size in the random-effects meta-analysis.



Online Figure IV. Assessment of risk of bias of included studies with the SYRCLE tool.

* No study protocol was available, but key outcomes were all reported on. Key outcomes were pre-specified as lesion size plus two out of the following outcomes: Macrophage accumulation, collagen deposition, smooth muscle cell content.

	Selection bias (sequence generation)	Selection bias (baseline characteristics)	Selection bias (allocation concealment)	Performance bias (random housing)	Performance bias (blinding)	Detection bias (rdm. outcome assessment)	Detection bias (blinding)	Attrition bias	Reporting bias	Other sources of bias
Aiello, 2010	unclear	unclear	unclear	unclear	unclear	unclear	high	high	high	high
Bot, 2017	unclear	low	unclear	unclear	unclear	unclear	high	low	high*	low
Cynis, 2011	high	low	high	unclear	unclear	unclear	high	low	high	high
Inoue, 2002	low	unclear	unclear	unclear	unclear	unclear	low	high	high*	low
Liehn, 2010	unclear	low	unclear	unclear	unclear	unclear	high	low	high*	high
Lutgens, 2005	unclear	unclear	unclear	unclear	unclear	unclear	high	low	high*	low
Ni, 2001	low	unclear	unclear	unclear	unclear	unclear	low	high	high	low
Ni, 2004	low	low	unclear	unclear	unclear	unclear	low	high	high*	high
Okamoto, 2012	unclear	unclear	unclear	unclear	unclear	unclear	high	high	high	low
Olzinski, 2010	unclear	unclear	unclear	unclear	unclear	unclear	high	high	high	high
de Waard, 2010	unclear	low	unclear	unclear	unclear	unclear	high	high	high	high
van Wanrooij, 2005	unclear	low	unclear	unclear	unclear	unclear	high	low	high*	low
Winter, 2018	unclear	low	unclear	unclear	unclear	unclear	high	low	high	low
Yamashita 2002	unclear	unclear	unclear	unclear	unclear	unclear	high	high	high	low

Online Figure V. Assessment of compliance with the ARRIVE guidelines on sample size, randomization, and blinding[29]. The top row specifies the respective guideline under examination. Studies were assessed based on whether they complied with the guideline ("yes") or not ("no"), or whether studies provided insufficient details to ascertain compliance with the guidelines ("not described"). Whenever parts of the guideline were sufficiently covered by the study, numbers indicate the according part.

	2a. *„Specify the exact number of experimental units allocated to each group* **(1)**, *and the total number in each experiment* **(2)**. *Also indicate the total number of animals used* **(3).***"*	**2b.** *"Explain how the sample size was decided. Provide details of any a priori sample size calculation, if done."*	**4a.** *"State whether randomization was used to allocate experimental units to control and treatment groups. If done, provide the method used to generate the randomization sequence."*	**4b.** *"Describe the strategy used to minimize potential confounders such as the order of treatments and measurements, or animal/cage location. If confounders were not controlled, state this explicitly".*	**5.** *"Describe who was aware of the group allocation at the different stages of the experiment (during the allocation* **(1)**, *the conduct of the experiment* **(2)**, *the outcome assessment* **(3)**, *and the data analysis* **(4)** *)."*
Aiello, 2010	**yes[1,2]**	**not described**	**not described**	**not described**	**not described**
Bot, 2017	**yes[1,2]**	**not described**	**not described**	**not described**	**not described**
Cynis, 2011	**yes[1,2,3]**	**not described**	**no**	**not described**	**not described**
Inoue, 2002	**yes[2]**	**not described**	**yes**	**not described**	**yes[3]**
Liehn, 2010	**yes[2]**	**not described**	**not described**	**not described**	**not described**
Lutgens, 2005	**yes[1,2]**	**not described**	**not described**	**not described**	**not described**
Ni, 2001	**yes[2]**	**not described**	**yes**	**not described**	**yes[3]**
Ni, 2004	**yes[2]**	**not described**	**yes**	**not described**	**yes[3]**
Okamoto, 2012	**yes[1,2]**	**not described**	**not described**	**yes**	**not described**
Olzinski, 2010	**yes[1,2]**	**not described**	**not described**	**yes**	**not described**
de Waard, 2010	**yes[1,2,3]**	**not described**	**not described**	**not described**	**not described**
van Wanrooij, 2005	**yes[1,2]**	**not described**	**not described**	**not described**	**not described**
Winter, 2018	**yes[1]**	**not described**	**not described**	**not described**	**not described**
Yamashita 2002	**yes[1,2,3]**	**not described**	**not described**	**not described**	**not described**

Major Resources Table

In order to allow validation and replication of experiments, all essential research materials listed in the Methods should be included in the Major Resources Table below. Authors are encouraged to use public repositories for protocols, data, code, and other materials and provide persistent identifiers and/or links to repositories when available. Authors may add or delete rows as needed.

Data & Code Availability

Description	Source / Repository	Persistent ID / URL
Meta-analysis dataset	Online Supplemental Material	
Meta-analysis software code	*The Dataverse Network*	https://doi.org/10.7910/DVN/KMKD0J

4. Publication II

Title: IKKβ binds NLRP3 providing a shortcut to inflammasome activation for rapid immune responses
Authors: Yaw Asare, Margarita Shnipova, **Luka Živković**, Christina Schlegl, Federica Tosato, Arailym Aronova, Markus Brandhofer, Laura Strohm, Nathalie Beaufort, Rainer Malik, Christian Weber, Jürgen Bernhagen, Martin Dichgans
Year: 2022
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LETTER OPEN

IKKβ binds NLRP3 providing a shortcut to inflammasome activation for rapid immune responses

Signal Transduction and Targeted Therapy (2022)7:355 ; https://doi.org/10.1038/s41392-022-01189-3

Dear Editor,

A rapid immune response to signals released from pathogens and injuries is critical for maintaining tissue integrity and restoring homeostasis. This response is largely mediated by the concerted action of pattern recognition receptors (PRRs). Such cooperativity has been described for Toll-like receptors (TLRs) and NACHT, LRR, and pyrin domain-containing protein 3 (NLRP3), but the underlying molecular mechanisms remain incompletely understood. Inflammasomes are multi-protein complexes defined by a cytosolic innate immune sensor, usually a PRR, which recruits the adaptor molecule apoptosis-associated speck-like protein containing a caspase-recruitment domain (ASC) to activate the effector caspase-1 leading to the release of matured IL-1β and IL-18. Active caspase-1 further cleaves gasdermin D (GSDMD) allowing the N-terminal domain of GSDMD (GSDMD-N) to form pores in the plasma membrane, thus facilitating the release of matured IL-1β and IL-18. Pore-forming GSDMD-N further induces pyroptosis, an inflammatory form of cell death.[1] NLRP3 inflammasome activation typically entails NF-κB-driven transcriptional priming, which in turn licenses the cell for inflammasome assembly and activation.[1] A previously discussed paradigm of rapid inflammasome assembly without the requirement for NF-κB-driven transcriptional priming involves simultaneous engagement of TLRs and NLRP3.[2,3] While increasingly recognized, the molecular mechanisms and ensuing biological effects remain largely undefined. Recent work has demonstrated a role of IKKβ in activation of the NLRP3 inflammasome by recruiting NLRP3 to the dispersed trans-Golgi network.[4] Given the activating effect of TLR signaling on IKK and the central role of NF-κB in inflammasome signaling,[1,4] we scrutinized whether IKK could directly activate the inflammasome on top of its effects on priming.

To systematically study the role of IKKβ in inflammasome activation beyond transcriptional priming, we assessed possible interactions between IKK and the inflammasome. Co-immunoprecipitation experiments in transfected HEK293 cells revealed binding of IKKβ to NLRP3 but not NLRC4, NLRP1, and AIM2 (Fig. 1a, b). To determine the specific domains of NLRP3 interacting with IKKβ, we co-transfected HA-IKKβ with Flag-tagged full-length or mutant NLRP3 and subjected the lysate to immunoprecipitation. We found that IKKβ binds to full-length as well as mutant NLRP3 lacking the amino-terminal pyrin domain, the singly expressed NACHT and LRR domains, but not the PYD (Fig. 1c, d). Next, we examined whether the enzymatic activity of IKKβ is required for this interaction and found that the wild-type, constitutively active, and catalytically inactive IKKβ all bind to NLRP3 (Supplementary Fig. 1a). An appreciable proportion of NLRP3 was found in IKKβ precipitates and vice versa (Supplementary Fig. 1b, c). As demonstrated by confocal microscopy, IKKβ and NLRP3 colocalized in bone marrow-derived macrophages (BMDMs) transfected with HA-IKKβ and stimulated with LPS and nigericin (Fig. 1e). Importantly, IKKβ promoted the oligomerization of NLRP3 (Fig. 1f), possibly in part through effects on the recruitment of NLRP3 to the dispersed TGN.[4,5] To further analyze this interaction at endogenous levels and determine the effect of inflammasome activation on this interaction, we treated human THP-1 macrophages with LPS and NLRP3 activators (nigericin or ATP) and subjected the lysate to immunoprecipitation. Consistent with the results in HEK293 cells, we found that IKKβ binds to NLRP3 at endogenous levels in THP-1 macrophages stimulated with LPS and NLRP3 activators or left unstimulated, indicating the constitutive nature of this interaction (Fig. 1g). We found no binding of p38 MAP Kinase to NLRP3 (Supplementary Fig. 1d), further supporting the specificity of the identified interactions. As a last step to corroborate this interaction, we performed microscale thermophoresis (MST) analysis by titrating MST-Red-NLRP3 against increasing concentrations of IKKβ. The obtained curve revealed a direct interaction between IKKβ and NLRP3 and a K_D value of 1.43 ± 0.35 μM was derived for the binding affinity (Fig. 1h). Collectively, these findings identify IKKβ as an interactor of NLRP3 promoting its oligomerization.

To detail the consequence of this interaction, we pharmacologically inhibited IKKβ with TPCA-1 in BMDMs. The dose of TPCA-1 used was chosen after titration (Supplementary Fig. 2a–e). Quantitative proteomic profiling of IKKβ-inhibited BMDMs revealed a perturbation of proteins related to signaling in the immune system and pyroptosis including Gsdmdc1, Dhx9, Cycs, and Casp8 (Supplementary Fig. 3a–d). Given this evidence and our data indicating that IKKβ binds NLRP3, we employed two treatment and stimulation paradigms to assess the effects of IKKβ inhibition on NLRP3 inflammasome activation. The first paradigm involved inhibition of IKKβ before LPS priming and nigericin stimulation (pre-priming inhibition), while the second involved LPS priming followed by IKKβ inhibition and subsequent nigericin activation (post-priming inhibition). The latter served as an attempt to examine the direct effect of IKKβ on inflammasome activation rather than priming. Post-priming inhibition of IKKβ in BMDMs resulted in reduced cleavage of caspase-1 and GSDMD (Supplementary Fig. 4a–e), which went along with reduced maturation and secretion of IL-1β and IL-18 (Supplementary Fig. 4f, g) and limited pyroptosis in macrophages as revealed by reduced LDH release (Supplementary Fig. 4h). Similar observations were made for pre-priming inhibition, when cells were treated with either TPCA-1 or siRNA-mediated knockdown of IKKβ (Supplementary Fig. 2f and 5a–i).

Next, we investigated the involvement of IKKβ in rapid assembly of the NLRP3 inflammasome by challenging cells simultaneously with LPS and nigericin for 60 min (Fig. 1i). Inhibition of IKKβ limited rapid assembly of the inflammasome as demonstrated by reduced NLRP3 oligomerization (Fig. 1j). Immunostaining with anti-caspase-1 and anti-ASC antibodies revealed caspase-1 and ASC in a high molecular-mass NLRP3

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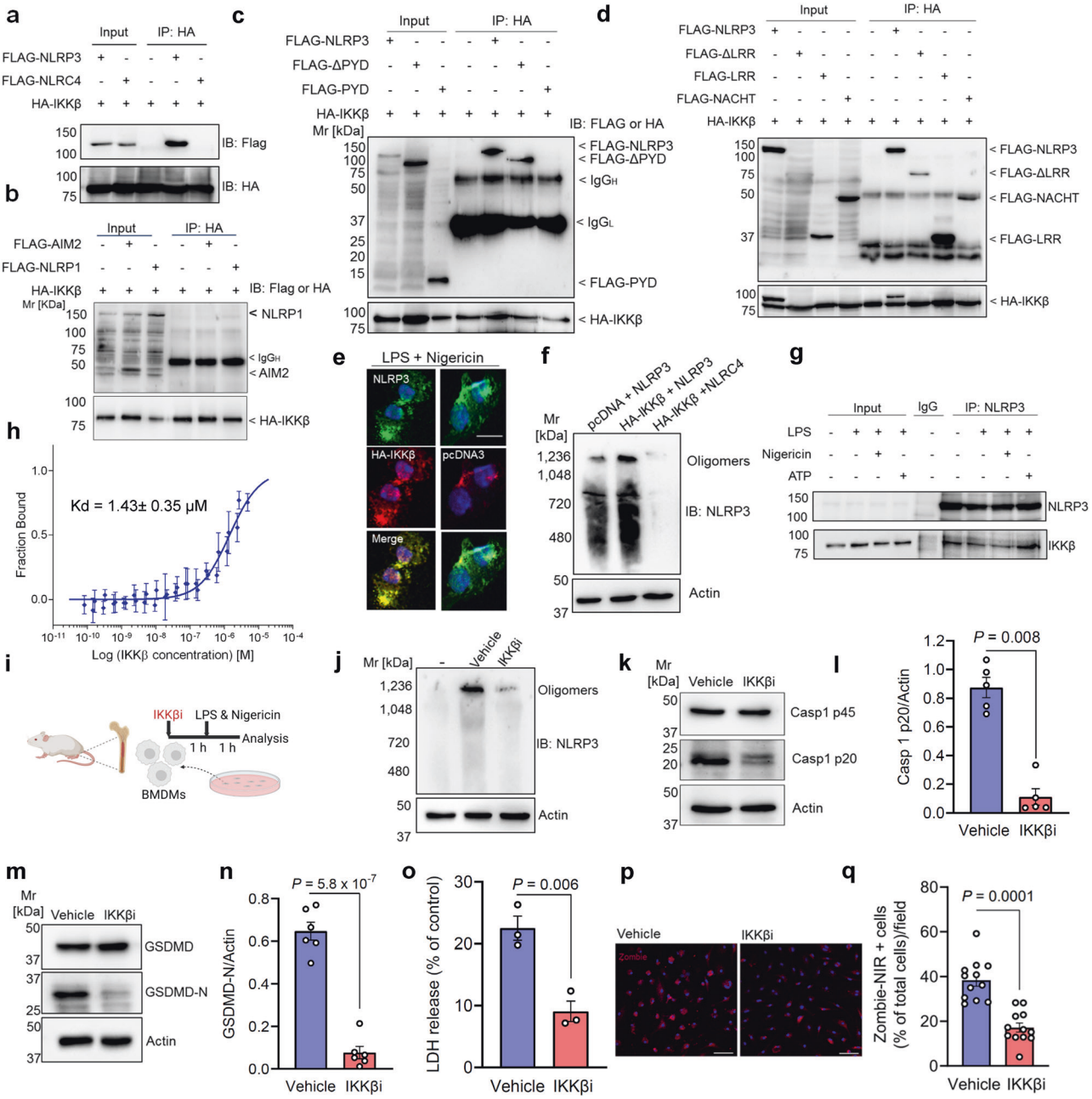

Fig. 1 IKKβ binds NLRP3 to induce rapid inflammasome assembly and pyroptotic cell death. **a**–**d** HEK293 cells were transiently cotransfected with HA-tagged full-length IKKβ, Flag-tagged full-length or mutant NLRP3, and Flag-tagged full-length NLRC4, AIM2, or NLRP1. Shown are representative immunoblots depicting the binding of IKKβ to NLRP3, but not NLRC4, AIM2, and NLRP1 (**a**, **b**), and specific domains of NLRP3 (**c**, **d**). $n = 3$ to 4 independent experiments. **e** Determination of colocalization between IKKβ and NLRP3 in BMDMs by confocal microscopy. Shown are representative immunostainings of three independent experiments. Scale bar = 20 μm. **f** Representative immunoblot showing the assessment of NLRP3 oligomerization in transfected HEK293 cells stimulated with nigericin and analyzed by blue native PAGE. $n = 3$ independent experiments. **g** THP-1 macrophages were either stimulated with LPS (200 ng/mL) for 4 h and nigericin (5 μM) for 60 min or ATP (5 mM) for 30 min or left unstimulated. Shown is a representative immunoblot depicting the endogenous binding of IKKβ to NLRP3. $n = 3$ independent experiments. **h** Protein–protein interactions between IKKβ and NLRP3 were analyzed in solution applying microscale thermophoresis (MST). 50 nM MST-Red-NLRP3 was titrated against increasing concentrations of IKKβ. Plotted is the fraction of bound MST-Red-NLRP3 (fraction bound) over the indicated concentrations of IKKβ (log scale). Data are represented as mean ± SD, with 3–4 data points for each concentration of IKKβ. Two independent titrations measured in two separate sets of capillaries. **i**–**q** Simultaneous engagement of TLRs and NLRP3. BMDMs were treated with TPCA-1 (500 nM) for 1 h and were simultaneously stimulated with LPS (200 ng/mL) and nigericin (5 μM) for 1 h. **i** Experimental outline. **j** Representative immunoblot of NLRP3 Oligomerization. $n = 4$ independent experiments. **k** Representative immunoblot depicting caspase-1 cleavage. **l** Quantification of cleaved caspase-1 normalized to actin. $n = 5$ independent experiments. **m** Representative immunoblots of GSDMD cleavage. **n** Quantification of GSDMD cleavage normalized to actin. $n = 6$ independent experiments. **o** Measurement of LDH release. $n = 3$ independent experiments. **p** Determination of Zombie uptake. Shown are representative immunostainings of three independent experiments. Scale bar = 50 μm. **q** Quantification of Zombie uptake. Data are represented as mean ± SEM.Two-sided unpaired t test was used in the statistical analyses after testing for normality with Shapiro–Wilk-Test

complex. Pharmacological inhibition of IKKβ limited the formation of this large oligomeric complex (Supplementary Fig. 6a, b). IKKβ inhibition further decreased caspase-1 activation (Fig. 1k, l), reduced IL-18 release (Supplementary Fig. 5j, k), and GSDMD cleavage (Fig. 1m, n). This restricted the formation of pore-forming GSDMD-N to induce pyroptosis in macrophages as demonstrated by reduced LDH release (Fig. 1o) and decreased uptake of Zombie NIR, an independent readout of pyroptosis (Fig. 1p, q). Hence, aside from effects on transcriptional priming, IKKβ further bridges TLR and NLR signaling providing a "shortcut" to inflammasome activation for prompt immune response.

Taken together, the results presented here show that (i) IKKβ directly interacts with NLRP3; (ii) IKKβ promotes the oligomerization of NLRP3; and (iii) pharmacological inhibition of IKKβ reduces rapid caspase-1 activation and limits GSDMD cleavage to restrict macrophage pyroptosis. Our findings thus provide a mechanistic explanation for rapid immune responses induced by the TLR-NLR signaling axis and implicate IKKβ as an essential regulator of pyroptotic cell death. This should be accounted for when designing therapeutic strategies that block TLR-NLR signaling in order to avoid increased rates of infection as recently seen in therapeutic neutralization of IL-1β.[6]

DATA AVAILABILITY

All data and materials are presented in the main manuscript or supplementary materials and are available on request. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD036118.

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AUTHOR CONTRIBUTIONS

Y.A. and M.D. designed research; Y.A., M.S., L.Z., C.S., F.T., A.A., L.S., and M.B. performed research; Y.A., N.B., M.B., R.M., C.W., J.B., and M.D. analyzed data; Y.A. and M.D. wrote the paper.

ADDITIONAL INFORMATION

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41392-022-01189-3.

Competing interests: The authors declare no competing interests.

Ethics: Isolation of bone marrow-derived macrophages from mice were approved by the Institutional Animal Care Committee of Regierung von Oberbayern (ROB-55.2-2532.Vet_02-14-187).

Yaw Asare[1]✉, Margarita Shnipova[1], Luka Živković[1], Christina Schlegl[1], Federica Tosato[1], Arailym Aronova[1], Markus Brandhofer[1], Laura Strohm[2], Nathalie Beaufort[1], Rainer Malik[1], Christian Weber[2,3], Jürgen Bernhagen[1,2] and Martin Dichgans[1,2]✉

[1]Institute for Stroke and Dementia Research (ISD), University Hospital, Ludwig-Maximilians-University (LMU), Munich, Germany; [2]Munich Cluster for Systems Neurology (SyNergy), Munich, Germany and [3]Institute for Cardiovascular Prevention (IPEK), LMU, Munich, Germany

Correspondence: Yaw Asare (yaw.asare@med.uni-muenchen.de) or Martin Dichgans (martin.dichgans@med.uni-muenchen.de)

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Supplementary Materials

IKKβ binds NLRP3 providing a shortcut to inflammasome activation for rapid immune responses

Yaw Asare[1]*, Margarita Shnipova[1], Luka Živković[1], Christina Schlegl[1], Federica Tosato[1], Arailym Aronova[1], Markus Brandhofer[1], Laura Strohm[2], Nathalie Beaufort[1], Rainer Malik[1], Christian Weber[2,3], Jürgen Bernhagen[1,2], Martin Dichgans[1, 2]*

[1]Institute for Stroke and Dementia Research (ISD), University Hospital, Ludwig-Maximilians-University (LMU), Munich, Germany; [2]Munich Cluster for Systems Neurology (SyNergy), Munich, Germany; [3] Institute for Cardiovascular Prevention (IPEK), LMU

* Correspondence to: martin.dichgans@med.uni-muenchen.de & yaw.asare@med.uni-muenchen.de

This PDF file includes:

Materials and Methods

Supplementary Figures. S1 to S6

Materials and Methods

Reagents

All NLRP3 constructs were a kind gift from Dr. Florian Schmidt and NLRC4 construct was received from Dr. Veit Hornung. Human IKKβ-HA (pUNO1-hIKBKB-HA3x) was purchased from *in vivo* gene. FLAG-IKKbeta (S177E, S181E)-pcw107 was a gift from David Sabatini & Kris Wood (Addgene plasmid # 64609)[1] while IKKβ K44M was a gift from Anjana Rao (Addgene plasmid # 11104)[2]. HA-Tag (C29F4) #3724, GSDMD (E9S1X) #39754, and IKKβ (D30C6) #8943 antibodies were purchased from Cell Signalling. NLRP3 (Cryo-2) and anti-Caspase 1 (p20) were from Adipogen. Anti-Flag M2 antibody (F1804) was from Sigma. ON-TARGETplus Mouse Ikbkb siRNA and ON-TARGETplus Non-targeting Control Pool were purchased from Horizon.

Generation of bone marrow-derived macrophages and THP1 macrophages

Mouse bone marrow-derived macrophages (BMDMs) were generated as established [3]. In brief, bone marrow cells were flushed from the femur and tibiae with PBS, filtered through 40-µm cell strainer, and cultured in RPMI medium supplemented with 15% L929-conditioned medium. Stimulations with LPS and nigericin were performed in serum-free medium. For generation of THP1 macrophages, THP1 monocytes were cultured with 100 nM PMA for 72 h before stimulating the cells with LPS, nigericin, or ATP for the endogenous interactions.

Cell lysis, co-immunoprecipitation and Western blot analysis

For total cell lysates, cells were washed with cold PBS and lysed either directly with 1x NuPAGE-LDS-sample buffer (Invitrogen) containing 1 mmol/l DTT (Sigma Aldrich) or with 1x cell lysis buffer (#9803, Cell signaling) containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na_3VO_4, 1 µg/mL leupeptin. Protease and phosphatase inhibitors (Roche) were added to all buffers. For immunoprecipitation experiments, HEK293 cells were transiently co-transfected with HA-tagged full-length or mutants of IKKβ and Flag-tagged full-length or mutants of NLRP3 (ΔPYD, LRR, NACHT, PYD, and ΔLRR domains). Further co-

transfections involved the use of Flag-NLRC4, Flag-AIM2, HA-IKKα, and HA-IKKγ. Endogenous interactions were performed in THP1 macrophages stimulated with LPS (200 ng/ml) for 4 h and nigericin (5 μm) for 1 h or ATP (5 mM) for 30 min or left unstimulated. Cell lysates were precleared with Protein A or G beads. Following incubation of lysates with primary antibodies (anti-Flag, anti-HA, or anti-NLRP3) overnight at 4°C, the ensuing protein complexes were incubated with protein A or G beads for 2 h. Antibody-antigen complexes were eluted from the beads after washing three times with 1x cell lysis buffer containing 150 mM NaCl and a final stringent wash with 250 mM NaCl for two times. Total cell lysates or coimmunoprecipitated proteins were separated by SDS-PAGE, transferred to a PVDF membrane (Bio-Rad), and detected with the appropriate antibodies. Primary antibodies were incubated overnight at 4ºC. HRP-conjugated anti-mouse or anti-rabbit antibodies were used as secondary antibodies and blots were developed with Immobilon Western HRP Substrate (Merck Millipore). Protein bands were visualized with a Fusion Fx7 and quantified using Image J 1.47v software (Wayne Rasband).

Microscale thermophoresis (MST)

Direct protein-protein interactions between IKKβ and fluorescently labeled NLRP3 were analyzed by microscale thermophoresis on a Monolith NT.115 instrument equipped with green/red filters (NanoTemper Technologies GmbH, Munich, Germany). For labeling of recombinant human NLRP3 (OriGene Technologies, Inc, Rockville, USA), the Monolith Protein Labeling Kit RED-NHS 2nd Generation from NanoTemper (Munich, Germany) was used, following the manufacturer's instructions. Experiments were performed essentially following a previously described protocol [4,5]. Measurements were performed in standard NanoTemper Monolith capillaries at 25°C with 80% MST power and 95% LED excitation power to obtain an initial fluorescence count in the range of 180 to 240. MST traces were recorded for 40 s (-5 s to +35 s), at default settings, with the sample being heated between 0 s and 30 s. All measurements were performed in MST assay buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 mM $MgCl_2$, 0.05% Tween-20). MST-Red-NLRP3 was used at a fixed concentration of 50 nM and mixed with equal volumes of serial dilutions of recombinant human IKKβ (ActiveMotif, Waterloo, Belgium), resulting in a final NLPR3 concentration of 25 nM. Prior to loading capillaries for measurements, the prepared samples were incubated for at least 40 min on ice. Three to four MST traces per IKKβ concentration, prepared in two individual serial dilutions each, were analyzed according to the K_D model using the default settings, analyzing the temperature related intensity change (TRIC) of the fluorescent label ("cold region" from -1 to 0 s, "hot region" from 0.5 to 1.5 s) and using the MO.AffinityAnalysis V2.3 software (NanoTemper Technologies). Curve fitting for data representation was performed by GraphPad Prism Version 6.07 ('one site – total binding') based on data exported from MO.AffinityAnalysis software.

ELISA

For maturation and release of Il-1β and Il-18, BMDMs were stimulated with LPS and nigericin. Levels of Il-1β and Il-18 were measured in supernatants using commercially available ELISA kits (R&D systems).

LDH release assay

LDH release was determined in supernatants of BMDMs stimulated with LPS and nigericin. The assay was performed using the Pierce LDH cytotoxicity assay kit following manufacturer's instructions (Thermo Fisher Scientific). LDH release was calculated as LDH release [%] = (LDH activity in stimulated cells – spontaneous LDH activity in unstimulated cells) / (Maximum LDH activity after complete lysis – spontaneous LDH activity) × 100.

Zombie uptake assay

Uptake of Zombie dye, a second independent methodology to assay membrane integrity, was assessed in BMDMs stimulated with LPS and nigericin following manufacturer's instructions (Biolegend). Cells were washed with 1× PBS and fixed with 4% paraformaldehyde-PBS solution for 10 minutes. Nuclei was counterstained with 4,6-diamidino-2-phenylindol and images acquired with 40× oil objective.

Confocal Microscopy

BMDMs were transfected with HA-IKKβ using electroporation. Cells were allowed to recuperate and either stimulated with LPS and nigericin or left unstimulated. Following washing with 1× PBS, cells were fixed with 4% paraformaldehyde-PBS solution for 10 minutes, and permeabilized using 0.1% Triton X. Cells were then blocked for 1 hour with 0.2% FCS, 0.2% BSA, and 0.002% fish skin gelatin in 1× PBS. Primary antibodies against NLRP3 and HA were incubated overnight at 4°C. 4,6-diamidino-2-phenylindol as well as Cy3 and Alexa Fluor 488–labeled secondary antibodies were incubated for 1 hour at room temperature. Cells were washed and sealed with a coverslip coated in fluoromount mounting medium (Sigma). Imaging was performed with the confocal microscope (LSM 880, Zeiss) using the 40× oil objective and analyzed with the ZEN software (Zeiss).

Blue native PAGE

Blue native gel electrophoresis was performed using the Bis-Tris Native PAGE system as previously described [6,7]. 2.5×10^6 HEK293 cells were plated in 60 mm dishes and transfected with 2 µg each of the indicated plasmids. Following transfections, the cells were stimulated with 5 µM nigericin and washed once with cold PBS and lysed in ice-cold native lysis buffer (20 mM Bis-tris, 500mM ε-aminocaproic acid, 20 mM NaCl, 10% (w/v) glycerol, 0.5% digitonin, 0.5 mM Na_3VO_4, 1mM PMSF, 0.5mM NaF, 1× EDTA-free Roche protease inhibitor cocktail, pH 7.0) for 15 min on ice. Cell lysates were clarified by centrifugation at 18.000g for 30 min at 4°C and analysed without further purification steps. Total proteins were quantified using the Pierce BCA protein assay (#23225, ThermoFisher).

For experiments with primary macrophages, 4.5×10^6 BMDMs were seeded in 10 cm dishes. On the following day, cells were serum-starved for 1 h and then treated with 500 nM TPCA-1 or DMSO for 1 h. Subsequently, the cells were stimulated with 5 µM nigericin and 200 ng/ml LPS for 1 h. Cell lysis was performed as above. Samples were separated on 3-12% blue native PAGE (#BN1001BOX, Invitrogen). Native gels were incubated in 10% SDS solution for 5 min before transfer to PVDF membranes (Millipore), followed by conventional western blotting.

Proteomics analysis

BMDMs treated with TPCA-1, were stimulated with TNF-α (50 ng/mL) for 24 h. TNF-α, implicated in priming of the inflammasome[8], was used as an endogenous pro-inflammatory stimulus and an inducer of sterile inflammation. Thereafter cells were collected and lysed in urea buffer (9 M Urea, 50 mM Tris pH 8, 150 mM NaCl, 1x Roche protease inhibitor cocktail) followed by short sonification. Samples were cleared by centrifugation and protein amounts were adapted. Protein reduction was performed with dithiothreitol (DTT; 5 mM final) for 25 min at 56°C and protein alkylation by the addition of iodoacetamide (14 mM final) for 30 min at room temperature. Protein mixtures were quenched with DTT and diluted 1:5 with 1 M Tris-Hcl, pH 8.2. For increased peptide recovery, proteins were digested at room temperature for 3 h with LysC (FUJIFILM, 2 µl/100 µg protein) before overnight tryptic digest at 37°C (0.5 µg/100µg protein). The following day, digestion was stopped with 10% TFA. To increase analysis depth, samples were pre-fractioned by a C18-SCX custom-made stage tip[9]. Fractions

were eluted stepwise with increasing NH4AcO concentrations (20 mM to 500 mM) and desalted on a separate C18 stage tip. Desalted peptides were loaded on a custom-made 75 mm x 15 cm fused silicia capillary filled with C18-AQ resin (Reprosil Pur 120, 1.9 µm, Dr. Maisch HPLC) using an Easy-nLC1200 liquid chromatography. Samples were separated for 75 min with a 5-95% ACN gradient in 0.5% acetic acid using a Q Exactive HF mass spectrometer (Thermo Scientific). MS raw data were processed with MaxQuant (version 1.6.0.1). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE[10] partner repository with the dataset identifier PXD036118. Intensities were log2-transformed and normalized using variance stabilizing normalization (vsn). Relative quantification and statistical analysis were performed for all proteins identified by at least 2 peptides in at least two samples per group. Pyroptosis-related proteins were identified using Uniprot keywords (https://www.uniprot.org). Pathway enrichment analyses were performed using DAVID (https://david.ncifcrf.gov).

Statistical analysis

Statistical analyses were performed with Graphpad Prism 9 using two-tailed unpaired T-test or Mann-Whitney test as appropriate after normality testing with Shapiro-Wilk-Test. One-way Anova with Holm-Sidak's or Sidak's multiple comparisons were used as appropriate. Sample sizes were chosen based on experience from previous studies[3,11].

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Supplementary Figures

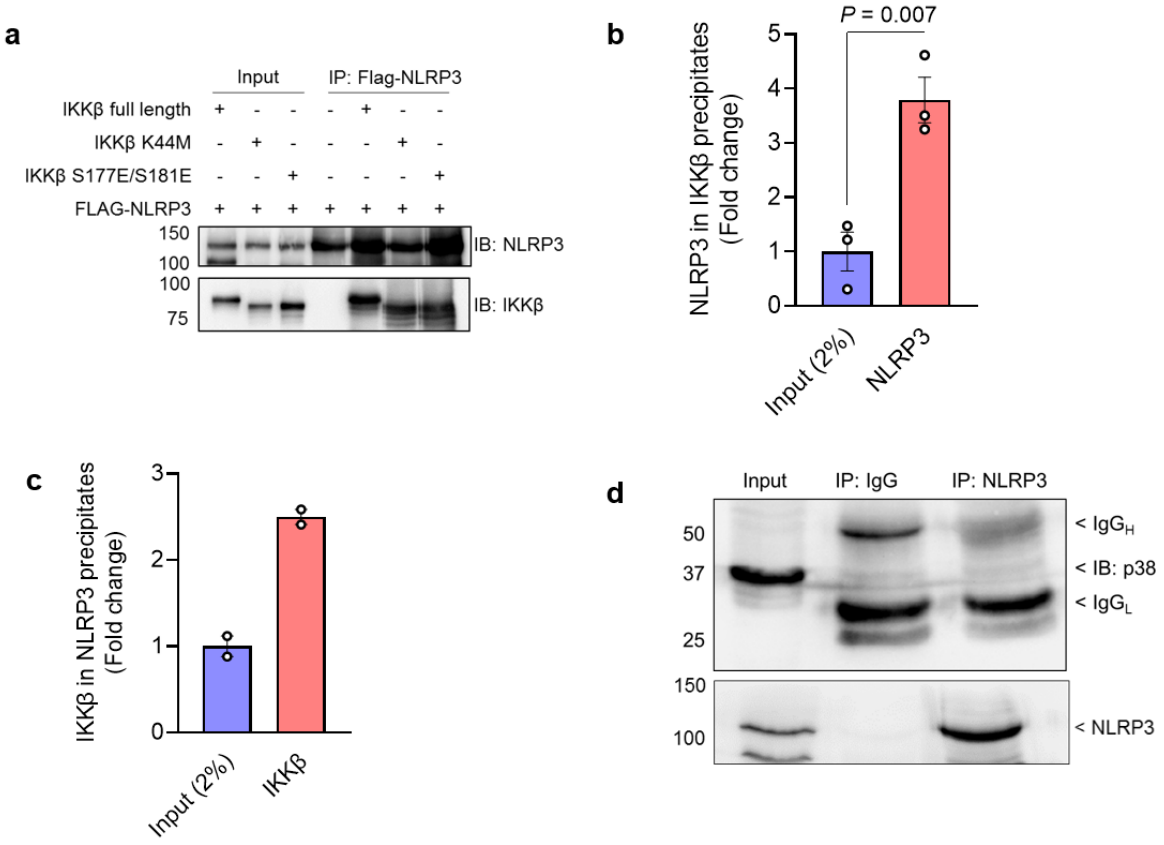

Figure. S1. **Binding of IKKβ to NLRP3 and effects on oligomeric complex formation**. (**a-c**) HEK293 cells were transiently cotransfected with full-length or mutant IKKβ and Flag-tagged full-length NLRP3. **a**) Shown are representative immunoblots depicting interaction between full-length or mutant IKKβ and NLRP3 upon immunoprecipitation of Flag-NLRP3. n=2 independent experiments. (**b**) Quantification of the proportion of NLRP3 in IKKβ precipitates. n=3 independent experiments. (**c**) Quantification of the proportion of IKKβ in NLRP3 precipitates. n=2 independent experiments. (**d**) Representative immunoblot of p38 and NLRP3 upon immunoprecipitation of NLRP3 in THP-1 macrophages. n=3 independent experiments.

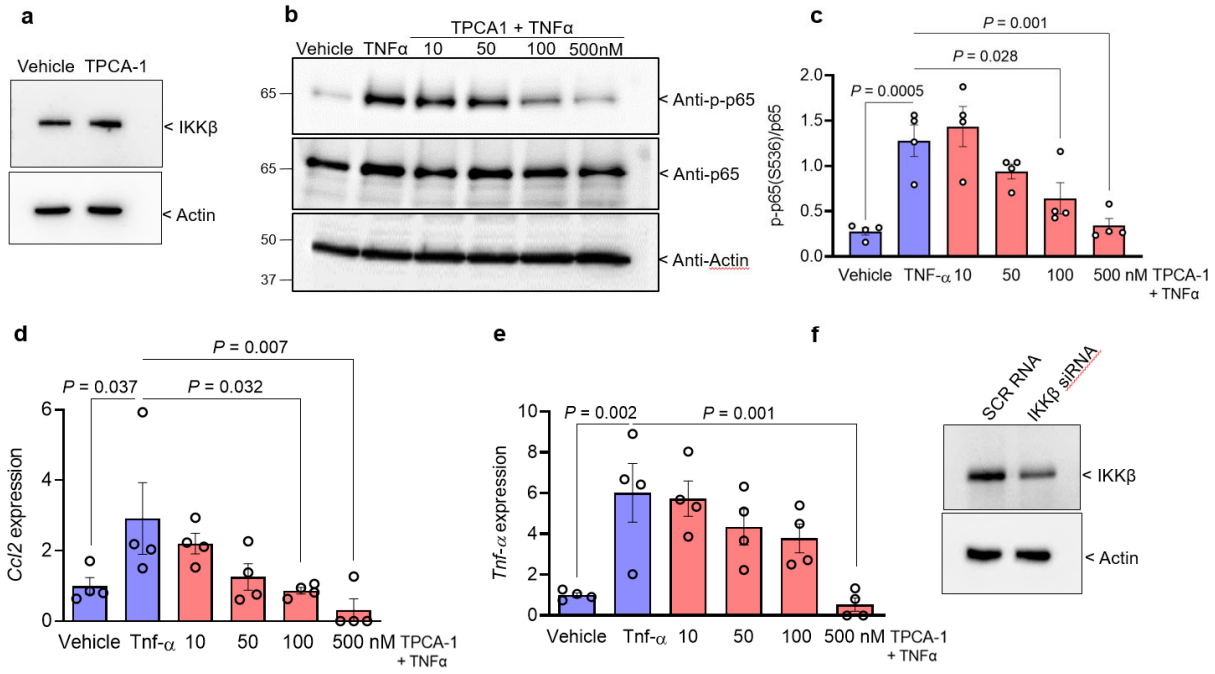

Figure. S2. Pharmacological inhibition and siRNA-mediated knockdown of IKKβ in Mϕ. (**a-e**) BMDMs were pre-treated with different concentrations of TPCA-1 or vehicle for 1 h and were stimulated with TNF-α. (**a**) Representative immunoblots of IKKβ and actin in non-stimulated cells pre-treated with 500 nM TPCA-1. n=3 independent experiments. (**b**) Representative immunoblots of P-p65, p65, and actin. (**c**) Quantification of immunoblots in B. n=4 independent experiments. (**d, e**) Gene expression of *Ccl2* and *Tnf-α*. n=4 independent experiments. (**f**) Representative immunoblot of siRNA-mediated IKKβ depletion in BMDMs. n=3 independent experiments. One-way Anova with Holm-Sidak's or Sidak's multiple comparisons were used in the statistical analyses.

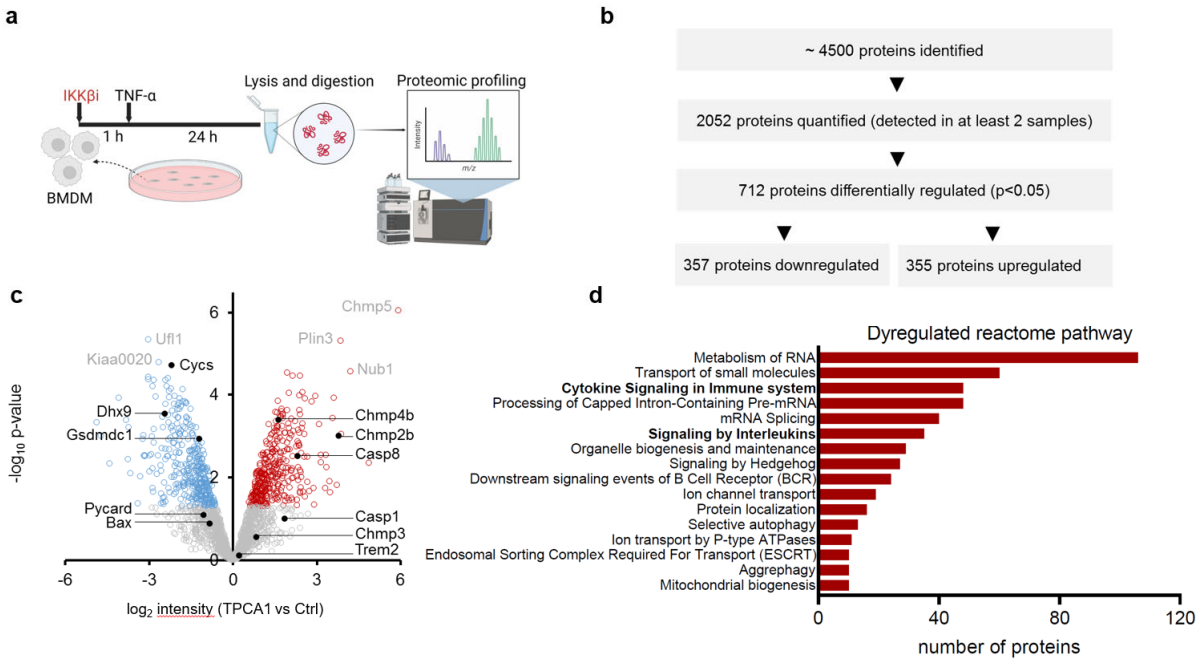

Figure. S3. Proteomic profiling of IKKβ-inhibited BMDMs reveals a signature for perturbation of proteins involved in immune system signaling and pyroptosis. BMDMs were treated with TPCA-1 (500 nM; designated IKKβi) or vehicle for 1 h and stimulated with TNF-α (50 ng/mL) for 24 h. Following lysis and digestion, the cellular proteome was resolved by LC-MS/MS and label-free quantification (LFQ). (**a**) Experimental outline. (**b**) Flowchart of proteins identified, quantified and differentially regulated. (**c**) Volcano plot of $\log_2$ intensity ratio (TPCA1 vs. control) and $-\log_{10}$ p values of all quantified proteins (n = 4 mice per condition). Red and blue circles represent proteins with a higher and a lower abundance respectively (p-value < 0.05). Proteins involved in pyroptosis are highlighted in black. Proteins are labelled by gene name. (**d**) Reactome pathway showing the signature of differentially regulated proteins (p value < 0.05**).**

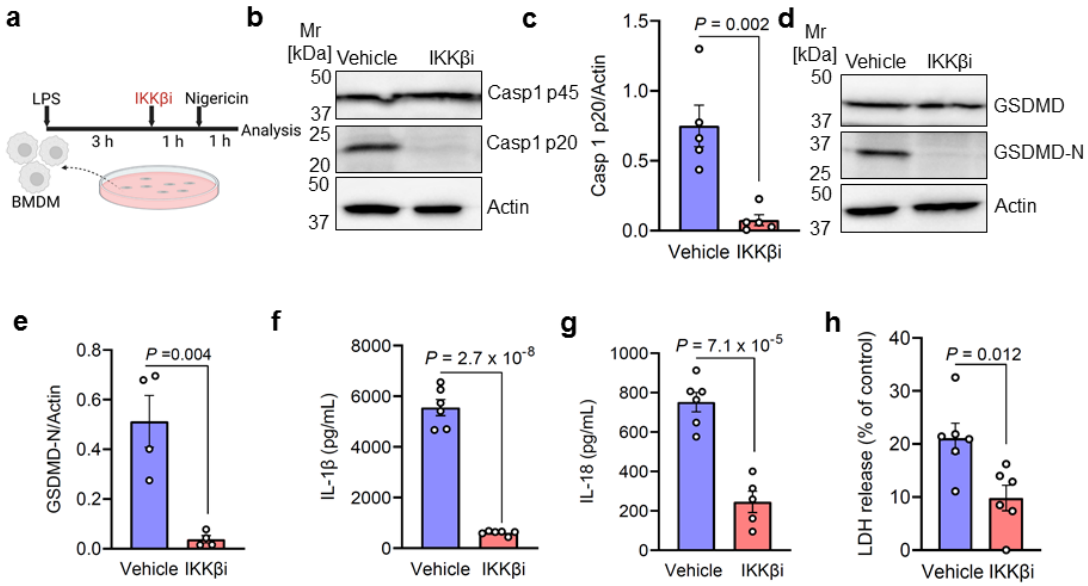

Figure. S4. Post-priming inhibition of IKKβ reduces inflammasome activation to limit pyroptosis in macrophages. (**a-h**) Post-priming inhibition. BMDMs were stimulated with LPS (100 ng/mL) for 3 h and treated with TPCA-1 (500 nM, designated IKKβi) for 1 h prior to nigericin (5 μM) stimulation for 1 h. (**a**) Experimental outline. (**b**) Representative immunoblots of caspase-1 cleavage. (**c**) Quantification of cleaved caspase-1 normalized to actin. n=5 independent experiments. (**d**) Representative immunoblots of gasdermin D cleavage. (**e**) Quantification of gasdermin D normalized to actin. n=4 independent experiments. (**f**) Quantification of IL-1β and (**g**) IL-18 release. n = 5-6 independent experiments. (**h**) Measurement of LDH release. n = 6 independent experiments.

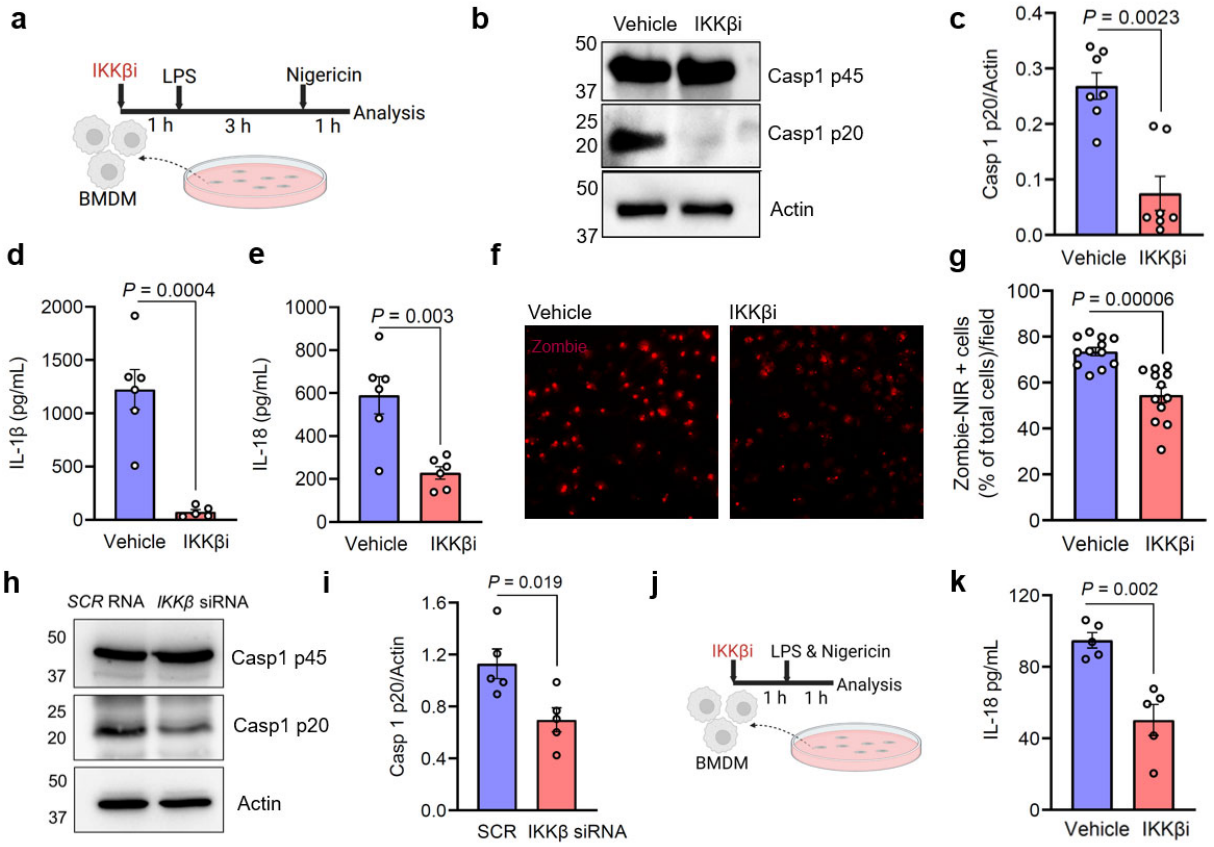

Figure. S5. Effects of Pre-priming inhibition of IKKβ on inflammasome activation. (**a-g**) Pre-priming inhibition. BMDMs were treated with TPCA-1 (500 nM; designated IKKβi) for 1 h and then stimulated with LPS (100 ng/mL) for 3 h and nigericin (5 µM) for 1 h. (**a**) Experimental outline. (**b**) Representative immunoblots of caspase-1 cleavage. (**c**) Quantification of cleaved caspase-1 normalized to actin. n=7 independent experiments. (**d**) Quantification of IL-1β and (**e**) IL-18 release. n = 5-6 independent experiments. (**f**) Determination of Zombie uptake. Shown are representative immunostainings of 3 independent experiments. (**g**) Quantification of Zombie uptake as a readout for pyroptosis. (**h**, **i**) siRNA-mediated knockdown of IKKβ in BMDMs. (**h**) Representative immunoblot of caspase-1 cleavage. (**i**) Quantification of cleaved caspase-1. n=5 independent experiments. (**j, k**) Simultaneous engagement of TLRs and NLRP3. BMDMs were treated with TPCA-1 (500 nM) for 1 h and were simultaneously stimulated with LPS (200 ng/mL) and nigericin (5 µM) for 1 h. (**j**) Experimental outline. (**k**) Quantification of IL-18 release. n=5 independent experiments. Two-sided unpaired t test was used in all after testing for normality with Shapiro-Wilk-Test except in C where Mann-Whitney test was used.

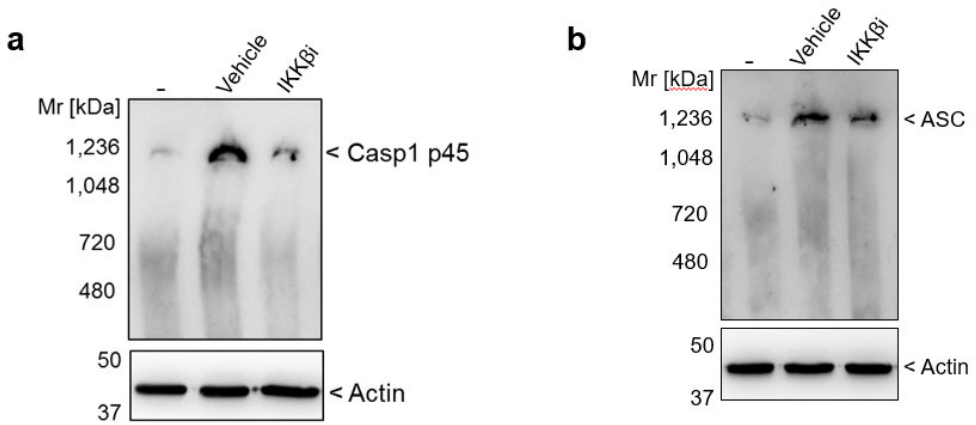

Figure. S6. Presence of caspase-1 and ASC in high molecular-mass NLRP3 complex (**a**, **b**) Simultaneous engagement of TLRs and NLRP3. BMDMs were treated with TPCA-1 (500 nM; designated IKKβi) for 1 h and were simultaneously stimulated with LPS (200 ng/mL) and nigericin (5 μM) for 1 h. Representative immunoblots of caspase-1 (**a**) and ASC detection (**b**) in high molecular-mass NLRP3 complex. n=3 independent experiments.

5. Publication III

Title: Proteogenomic Data Integration Reveals CXCL10 as a Potentially Downstream Causal Mediator for IL-6 Signaling on Atherosclerosis
Authors: Savvina Prapiadou, **Luka Živković**, Barbara Thorand, Marc J. George, Sander W. van der Laan, Rainer Malik, Christian Herder, Wolfgang Koenig, Thor Ueland, Ola Kleveland, Pål Aukrust, Lars Gullestad, Jürgen Bernhagen, Gerard Pasterkamp, Annette Peters, Aroon D. Hingorani, Jonathan Rosand, Martin Dichgans, Christopher D. Anderson, Marios K. Georgakis
Year: 2024
Journal: Circulation, Vol. 149 (9) pp. 669-683
DOI: 10.1161/CIRCULATIONAHA.123.064974

Supplementary tables containing the datasets used in this study can be accessed under the provided DOI.

Circulation

ORIGINAL RESEARCH ARTICLE

Proteogenomic Data Integration Reveals CXCL10 as a Potentially Downstream Causal Mediator for IL-6 Signaling on Atherosclerosis

Savvina Prapiadou, MD; Luka Živković; Barbara Thorand, PhD; Marc J. George, MB, ChB, PhD; Sander W. van der Laan, PhD; Rainer Malik, PhD; Christian Herder, PhD; Wolfgang Koenig, MD; Thor Ueland, PhD; Ola Kleveland, MD, PhD; Pål Aukrust, MD, PhD; Lars Gullestad, MD, PhD; Jürgen Bernhagen, PhD; Gerard Pasterkamp, MD; Annette Peters, PhD; Aroon D. Hingorani, MB, BS, PhD; Jonathan Rosand, MD; Martin Dichgans, MD; Christopher D. Anderson, MD, MMSc; Marios K. Georgakis, MD, PhD

BACKGROUND: Genetic and experimental studies support a causal involvement of IL-6 (interleukin-6) signaling in atheroprogression. Although trials targeting IL-6 signaling are underway, any benefits must be balanced against an impaired host immune response. Dissecting the mechanisms that mediate the effects of IL-6 signaling on atherosclerosis could offer insights about novel drug targets with more specific effects.

METHODS: Leveraging data from 522 681 individuals, we constructed a genetic instrument of 26 variants in the gene encoding the IL-6R (IL-6 receptor) that proxied for pharmacological IL-6R inhibition. Using Mendelian randomization, we assessed its effects on 3281 plasma proteins quantified with an aptamer-based assay in the INTERVAL cohort (n=3301). Using mediation Mendelian randomization, we explored proteomic mediators of the effects of genetically proxied IL-6 signaling on coronary artery disease, large artery atherosclerotic stroke, and peripheral artery disease. For significant mediators, we tested associations of their circulating levels with incident cardiovascular events in a population-based study (n=1704) and explored the histological, transcriptomic, and cellular phenotypes correlated with their expression levels in samples from human atherosclerotic lesions.

RESULTS: We found significant effects of genetically proxied IL-6 signaling on 70 circulating proteins involved in cytokine production/regulation and immune cell recruitment/differentiation, which correlated with the proteomic effects of pharmacological IL-6R inhibition in a clinical trial. Among the 70 significant proteins, genetically proxied circulating levels of CXCL10 (C-X-C motif chemokine ligand 10) were associated with risk of coronary artery disease, large artery atherosclerotic stroke, and peripheral artery disease, with up to 67% of the effects of genetically downregulated IL-6 signaling on these end points mediated by decreases in CXCL10. Higher midlife circulating CXCL10 levels were associated with a larger number of cardiovascular events over 20 years, whereas higher *CXCL10* expression in human atherosclerotic lesions correlated with a larger lipid core and a transcriptomic profile reflecting immune cell infiltration, adaptive immune system activation, and cytokine signaling.

CONCLUSIONS: Integrating multiomics data, we found a proteomic signature of IL-6 signaling activation and mediators of its effects on cardiovascular disease. Our analyses suggest the interferon-γ–inducible chemokine CXCL10 to be a potentially causal mediator for atherosclerosis in 3 vascular compartments and, as such, could serve as a promising drug target for atheroprotection.

Key Words: atherosclerosis ■ interleukin-6 ■ Mendelian randomization analysis

Correspondence to: Marios K. Georgakis, MD, PhD, Institute for Stroke and Dementia Research, Ludwig-Maximilians-University Hospital, Feodor-Lynen-Str. 17, 81377 Munich, Germany. Email marios.georgakis@med.uni-muenchen.de or mgeorgak@broadinstitute.org

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For Sources of Funding and Disclosures, see page 681.

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Clinical Perspective

What Is New?

- Integrating genomic and proteomic data, we found genetic downregulation of IL-6R (interleukin-6 receptor)–mediated signaling to be associated with changes in circulating levels of 70 proteins involved in cytokine production regulation and immune cell trafficking, which were consistent with the proteomic effects of the pharmacological inhibition of IL-6R.
- Among the 70 proteins, genetically proxied levels of the chemokine CXCL10 (C-X-C motif chemokine ligand 10) were associated with risk of coronary artery disease, large-artery atherosclerotic stroke, and peripheral artery disease, and changes in CXCL10 levels mediated a significant proportion of the effects of IL-6 (interleukin-6) signaling on these end points.
- Higher midlife circulating CXCL10 levels were associated with higher cardiovascular risk over 20 years.

What Are the Clinical Implications?

- Our findings provide evidence for a downstream proteomic signature of genetic IL-6 signaling downregulation that could be used as potential biomarkers of response to drugs targeting the IL-6 signaling cascade.
- CXCL10 might be a potentially causal mediator of the effects of IL-6 signaling on atherosclerosis and could serve as a promising drug target for atheroprotection that should be further explored in clinical trials.

Nonstandard Abbreviations and Acronyms

CAD	coronary artery disease
CANTOS	Canakinumab Anti-Inflammatory Thrombosis Outcomes Study
CBS	Cystathionine Beta-Synthase
CRP	C-reactive protein
CXCL10	C-X-C motif chemokine ligand 10
CXCR3	C-X-C motif chemokine receptor 3
IL-1	interleukin-1
IL-6	interleukin-6
IL-6R	interleukin-6 receptor alpha chain
IP-10	interferon-γ-induced protein 10
IVW	inverse variance weighted
LAAS	large artery atherosclerotic stroke
MR	Mendelian randomization
NSTEMI	non–ST-segment–elevation myocardial infarction
PAD	peripheral artery disease
sIL-6R	soluble IL-6R
STARNET	Stockholm-Tartu atherosclerosis reverse networks engineering task
TNF-α	tumor necrosis factor-α

Anti-inflammatory treatments have emerged as a promising approach for lowering the risk of atherosclerotic cardiovascular disease.[1] The IL-6 (interleukin-6) pathway has attracted major attention because of converging evidence supporting its relevance in atherosclerosis.[2] First, pharmacological inhibition of IL-6 or IL-6R (IL-6 receptor) leads to reductions in atherosclerotic lesion formation in mouse models of atherosclerosis.[3,4] Second, prospective cohort studies have consistently found circulating levels of IL-6 to be associated with the manifestations of atherosclerotic disease, including coronary artery disease[5] and ischemic stroke.[6] Third, human genetic studies have shown that polymorphisms in the gene encoding the IL-6R resulting in downregulated IL-6 signaling activity are associated with a lower lifetime risk of coronary artery disease,[7] atherosclerotic ischemic stroke,[8] peripheral artery disease,[9] and abdominal aortic aneurysm,[10] as well as a favorable cardiometabolic profile.[11]

In line with these results, the CANTOS trial (Canakinumab Anti-Inflammatory Thrombosis Outcomes Study), which tested a monoclonal antibody targeting IL-1β (interleukin-1β), an upstream regulator of IL-6 signaling, showed considerable reductions in major adverse cardiovascular events among patients with a recent history of myocardial infarction.[12] Post hoc analyses of CANTOS showed that canakinumab was particularly protective among patients achieving substantial decreases in IL-6 levels, thus providing indirect evidence that interfering with IL-6 signaling could reduce cardiovascular risk.[13] On the basis of these findings, a recent phase 2 trial (RESCUE [Trial to Evaluate Reduction in Inflammation in Patients With Advanced Chronic Renal Disease Utilizing Antibody Mediated IL-6 Inhibition]) tested ziltivekimab, a monoclonal antibody that is directed against IL-6, and found that it effectively and safely reduces biomarkers of inflammation and thrombosis among patients with chronic kidney disease and evidence of inflammation (high-sensitivity C-reactive protein [CRP] levels ≥2 mg/L).[14] The cardiovascular benefit of this approach remains unknown, as an ongoing large-scale phase 3 cardiovascular outcomes trial testing ziltivekimab will not be completed before 2025.[15]

However, any benefits from anti–IL-6 treatments would need to be balanced against an impaired host response. Canakinumab has been associated with a higher risk of fatal infections,[12] and genetic analyses support that downregulation of IL-6 signaling is associated with a higher risk of bacterial infections.[11] To this end, IL-6 is known as a key component of the host

innate immune defense system.[16] As such, although trials directly targeting IL-6 signaling are ongoing, it would be useful to identify alternative drug targets in the same signaling pathway that might mediate the atheroprotective effects of IL-6 inhibition. Such molecules might be more specific and therefore better anti-inflammatory treatment targets for atherosclerotic cardiovascular disease.

We previously leveraged genetic data from large-scale population-based studies and detected 26 variants in the gene encoding IL-6R that mimic the effects of pharmacologically inhibiting this protein.[17,18] Here, we expand these analyses to blood proteomic data derived from the INTERVAL study (n=3301) to explore: (1) the causal circulating signature of interfering with IL-6 signaling that could inform the discovery of biomarker signatures of drug response to IL-6 inhibitors, and (2) potential mediators of the favorable cardiovascular effects of IL-6 inhibition that could serve as alternative and perhaps more selective drug targets. Following up on our findings, we compared our results with those of clinical trials applying pharmacological inhibition of IL-6 signaling. Triangulating the evidence, we explore in observational studies associations between circulating levels of potential protein drug targets and incident cardiovascular disease, as well as the histological, transcriptomic, and cellular profile of atherosclerotic lesions associated with these targets.

METHODS

Data Availability and Ethical Approval

Details for access to summary statistics used for the current analyses are provided in the Extended Methods of the Supplemental Material. All included studies received ethical approval from the respective institutional review boards, and all participants provided informed consent, as detailed in the Extended Methods of the Supplemental Material.

This study adheres to the MR-STROBE (Strengthening the Reporting of Observational Studies in Epidemiology using Mendelian Randomization) guidelines.[19] The checklist is provided in the Extended Methods of the Supplemental Material.

Genetic Instrument Selection

To construct an instrument for IL-6R–mediated signaling,[20] we used variants within the *IL6R* gene or a region 300 kB upstream or downstream of it. In accordance with an approach we described previously,[17,18] we selected variants associated in a genome-wide association study meta-analysis of 522 681 individuals with circulating levels of CRP, a downstream biomarker of IL-6 signaling[7] that was used as a functional readout for IL-6 signaling activity. The methodology and criteria used to obtain this instrument are outlined in the Extended Methods of the Supplemental Material and the genetic variants comprised in this study are listed in Table S1. As previously described, this genetic instrument is associated with circulating IL-6, CRP, and fibrinogen levels, consistent with results of clinical trials testing tocilizumab, a monoclonal antibody against IL-6R, which have been previously described.[17]

Proteome-Wide Mendelian Randomization Analyses

To detect the downstream proteomic effects of genetically proxied IL-6 signaling, we applied a proteome-wide 2-sample Mendelian randomization approach using plasma data from the INTERVAL study (n=3301 with SOMAscan aptamer-based proteomics; see Extended Methods of the Supplemental Material) Using these data, after excluding exposure instruments that were also used as instruments for IL-6 signaling, we applied 2-sample Mendelian randomization (MR) analysis, applying the inverse variance weighted (IVW) method as our primary approach. As a measure of pleiotropy, heterogeneity was quantified with the I^2, and significance was tested with the Cochran Q statistic ($P<0.05$ was considered significant).[21] To correct for multiple comparisons across the proteome, we corrected the *P* values derived from the IVW approach using the false discovery rate method.[22] *P* values <0.05 were considered statistically significant. To validate our results against bias arising from horizontal pleiotropy, for significant proteins, we also applied the weighted median approach[23] and MR-Egger regression,[24] which are more robust against the use of pleiotropic instruments. Furthermore, for significant proteins, we applied a colocalization analysis to ensure that the same genetic signal influencing IL-6 signaling also influences protein levels, and that the detected association was not the result of pleiotropic effects of variants in linkage disequilibrium with the selected instruments.[25] We applied a Bayesian framework for pairwise colocalization, within 300 kB of the *IL6R* gene, using the coloc package in R and tested competing models (H0: the genomic region does not include any variant influencing either the exposure or the outcome; H1: the genomic region includes a variant influencing only the exposure; H2: the genomic region includes a variant influencing only the outcome; H3: the genomic region includes separate variants influencing the exposure and the outcome; and H4: the genomic region includes a variant that influences both the exposure and the outcome).[26] The previous probability of any random variant being associated with both traits was set at 10^{-5}. A posterior probability of association of ≥0.8 for the last model (H4) was defined as providing evidence of colocalization. The effects of the selected genetic variants on proteins were extracted from the PhenoScanner database through the phenoscanner function in R,[27] and MR analyses were performed in R using the TwoSampleMR package.[28] Pooling the significant proteins, we explored enrichment in biological pathways using the Gene Ontology resource.[29]

Comparisons of the Proteomic Effects of Genetically Proxied IL-6 Signaling Downregulation With the Pharmacological Effects of IL-6R Inhibition

To test the validity of our approach for detecting the downstream proteomic signature of a signaling pathway with proteome-wide MR, we aimed to correlate the effects derived from our MR approach with the effects derived from pharmacological IL-6R inhibition. We used data from a follow-up study of the Norwegian tocilizumab NSTEMI trial (non–ST-segment–elevation myocardial infarction), which tested IL-6R blockade with tocilizumab in patients with NSTEMI. In the original randomized control trial, patients with NSTEMI received a

single intravenous dose of 280 mg of tocilizumab or placebo (n=117).[30] In a follow-up analysis, the proteomic profile of serum samples was quantified at baseline and 4 days after treatment among a subgroup of 48 patients from the tocilizumab arm using the SOMAscan aptamer-based proteomics assay.[31] After matching the proteins available in both data sets, we quantified the Pearson *r* correlation between the MR effects and the pharmacological effects across the proteome.

Associations With Clinical End Points in Mediation MR Analyses

After detecting proteins significantly associated with genetically proxied IL-6 signaling, we then explored associations between genetically proxied levels of these proteins and the 3 primary manifestations of atherosclerosis to identify which of these proteins could mediate the effects of IL-6 signaling on clinical end points. Clinical outcomes included coronary artery disease (CAD), peripheral artery disease (PAD), and large artery atherosclerotic stroke (LAAS). For CAD, we used data from 63 746 CAD cases and 130 681 controls of predominantly European ancestry (≈80%) from the CARDIoGRAMplusC4D Consortium (Coronary Artery Disease Genome wide Replication and Meta-analysis plus the Coronary Artery Disease [C4D] Genetics).[32] For PAD, we used summary statistics from the Million Veteran Program cohort, a longitudinal cohort study containing electronic health records and genetic data across 50 veterans facilities in the United States. This cohort includes 31 307 cases and 211 753 controls of European, African, and Hispanic ancestry.[33] For LAAS, we leveraged data from the MEGASTROKE consortium of primarily individuals of European ancestry, consisting of 6688 patients with stroke and 245 201 controls from 29 studies.[34]

Again, we applied 2-sample IVW MR analyses using genetic variants associated with the proteins that were significantly associated with genetically proxied IL-6 signaling in the previous step as instruments. To avoid overlap in the used data sets, for this step, we used data from the deCODE study (Diabetes Epidemiology: Collaborative analysis of Diagnostic criteria in Europe) in the Icelandic population.[35] This study explored the genetic architecture of 4907 circulating proteins quantified with the aptamer-based SOMAScan approach among 35 559 Icelanders.[35] For the proteins significantly associated with genetically proxied IL-6 signaling, we selected genetic variants throughout the genome associated with the levels of these proteins at a $P<5\times10^{-8}$ and clumped at an $r^2<0.01$. For all proteins, beyond the IVW approach, we applied the MR-Egger and the weighted median methods. Furthermore, for significant proteins, we applied *cis*-MR analyses, selecting genetic variants associated with the proteins within or 300 kB upstream and downstream of the genes encoding these proteins at $P<10^{-5}$ after clumping at $r^2<0.1$. Significant results were screened on the basis of a false discovery rate–corrected $P<0.05$.

For proteins showing significant associations with both genetically proxied IL-6 signaling and risk of cardiovascular end points, we then applied 2-step mediation MR analyses[36] to explore whether any of the effects of IL-6 signaling on the outcomes could be explained by changes in circulating protein levels. We first performed multivariable MR exploring associations of genetic proxies for significant proteins on the risk of the cardiovascular end points adjusting for the effects of the respective genetic instruments on sIL-6R (soluble IL-6R) concentration. Then, by multiplying effects of the genetic proxies for IL-6 signaling on protein levels with the multivariable MR association estimates between genetically proxied protein levels and cardiovascular outcomes, we obtained indirect effects of the genetic proxies for IL-6 signaling on the outcomes mediated through the tested protein.[36] We divided these estimates by the total effects of the genetic proxies of IL-6 signaling on risk of CAD, PAD, and LAAS and obtained proportions of the effects mediated through the tested proteins.

Phenome-Wide Association Study

To explore potential adverse effects associated with promising proteins, we tested associations with the full range of clinical phenotypes encoded in the UK Biobank. We used the Phecode Map 1.2 to map UK Biobank *International Classification of Diseases, 10th Revision* (*ICD-10*) codes to Phecodes[37] using all *ICD-10* codes (main position, secondary position, and death records) from the UK Biobank. Individuals were assigned a case status if more than one *ICD-10* code was mapped to the respective phecode. To approximate effect size in a logistic regression framework, we used minor allele carrier status as an independent variable and age at baseline, sex, and 5 ancestry principal components as covariates.

Replication in an Observational Population-Based Setting

CXCL10/interferon-γ–inducing protein 10 (IP-10) showed particularly promising associations as a potential mediator of genetically proxied IL-6 signaling on all tested atherosclerotic outcomes (as shown in Results). To replicate these associations in an observational setting, we used data from the population-based MONICA (Monitoring Trends and Determinants in Cardiovascular Disease)/KORA cohort (Cooperative Health Research in the Region Augsburg),[38] a prospective population-based study of inhabitants of Augsburg, Germany, who were 25 to 74 years of age at baseline (1984–1995) and followed them up until 2016. Further information on the study design, outcome assessment, characteristics of the selected study participants, and the CXCL10 measurement methodology can be found in the Extended Methods of the Supplemental Material.

To explore associations between baseline CXCL10 levels with risk of future cardiovascular events (stroke or CAD), we applied a Cox proportional hazards model adjusting for age, sex, baseline survey (recruitment period: 1984–1985, 1989–1990, and 1994–1995; model 1), and additionally for vascular risk factors (body mass index [1 kg/m^2 increment], smoking [current versus noncurrent], estimated glomerular filtration rate [1 mL·min^{-1}·1.73 m^{-2} increment], history of CAD, diabetes, total cholesterol, high-density lipoprotein cholesterol, and hypertension defined as blood pressure >140/90 mm Hg, or use of antihypertensive medications if participants were aware of having hypertension; model 2). Based on the assumption that CXCL10 is a downstream effector of IL-6 signaling on atherosclerosis, we explored correlations between baseline IL-6 and CXCL10 levels and, in sensitivity analyses, included IL-6, CXCL10, or both in our models to see how the effects of each cytokine on cardiovascular events changed after adjusting for the other.

Analyses in Human Atherosclerotic Samples

We used data from the Athero-EXPRESS study, a biobank of carotid endarterectomy samples in which the expression levels of CXCL10 have been quantified in transcriptomic analyses to assess associations with histological features of plaque vulnerability. For 700 samples, RNA was isolated and libraries were prepared for sequencing as previously described.[39] We tested associations between the normalized expression values of CXCL10 and standard histological features of plaque vulnerability (large lipid core, intraplaque hemorrhage, extensive collagen content, and plaque calcification), as determined in sections from the plaque segment with the highest atherosclerosis burden. Methods for the evaluation of the histological images have been described previously in detail.[40]

To explore the changes in atherosclerotic plaque associated with higher expression of CXCL10, we used data from STARNET (Stockholm-Tartu Atherosclerosis Reverse Networks Engineering Task). We used transcriptomics data from atherosclerotic aortic root samples obtained with informed consent during coronary artery bypass grafting from 514 individuals with CAD.[41] We explored genes coexpressed with CXCL10 by estimating Spearman correlations with the expression of 16214 genes in this tissue. Genes significantly coexpressed with CXCL10 were moved forward to pathway analyses in Reactome.[42] To explore the cell landscape of the transcriptomic profile associated with upregulated *CXCL10* expression, we then used publicly available single-nuclei transcriptomic data from aortic tissue and tested which of the identified cell groups were enriched for these genes.[43]

RESULTS

Effects of Genetically Proxied IL-6 Signaling on Circulating Proteins

The overall study design is presented in Figure 1. The 26 CRP-lowering genetic variants within or close to the gene encoding IL-6R that were used as proxies for IL-6 signaling downregulation are presented in Table S1. First, we explored associations between genetically proxied IL-6R–mediated signaling as captured by these variants, with proteomic changes across 3281 plasma proteins quantified with the aptamer-based SOMAScan assay among 3301 participants of the INTERVAL study, as illustrated in Figure 2A. A detailed list of the associations as derived from IVW MR analyses is provided in Table S2. After correction for multiple comparisons

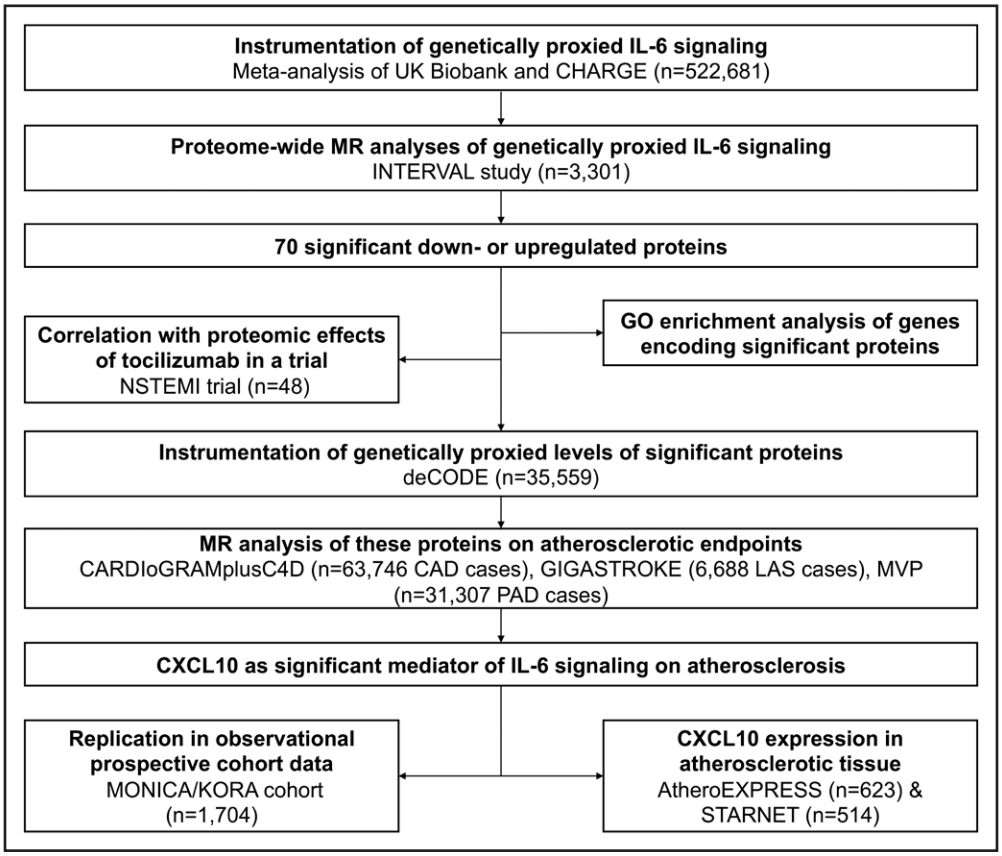

Figure 1. Study overview.
Overview of major analytical approaches and data sources used in the current study to identify proteins upregulated or downregulated by genetically proxied IL-6 (interleukin-6) signaling, as well as mediators of its effect on atherosclerotic disease. CAD indicates coronary artery disease; CHARGE, Cohorts for heart and aging research in genomic epidemiology; CXCL10, C-X-C motif chemokine ligand 10; GO, Gene Ontology; LAS, large artery atherosclerotic stroke; MR, Mendelian randomization; NSTEMI, non–ST-segment–elevation myocardial infarction; and PAD, peripheral artery disease.

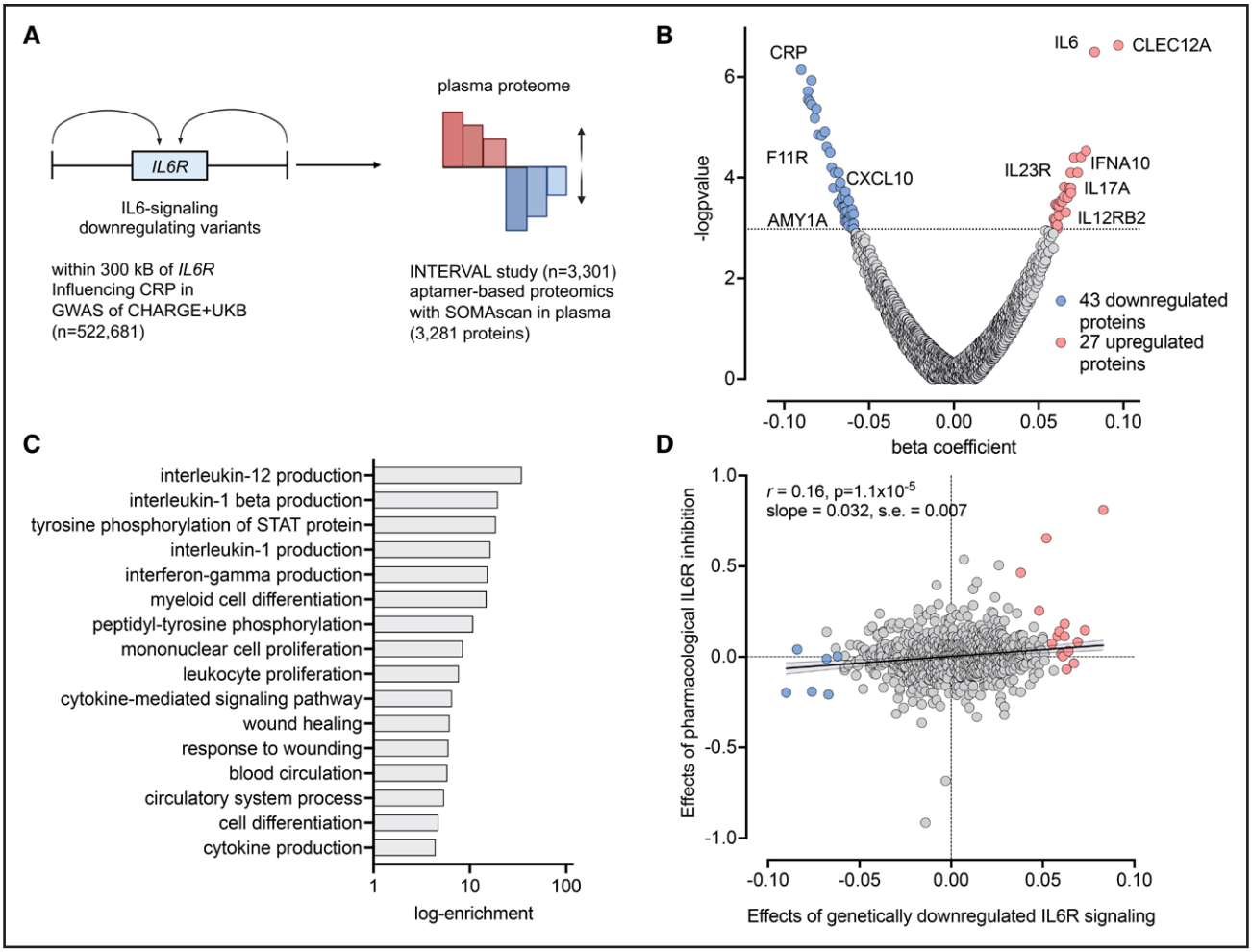

Figure 2. Plasma proteomic changes in association with genetically proxied IL-6 signaling.
A, Schematic representation of the study design and data sources. **B**, Volcano plot of the associations of genetically downregulated IL-6 signaling with plasma proteins in the INTERVAL study (n=3301). The results are derived from random-effects inverse-variance weighted Mendelian randomization analyses. Log *P* value on the *y* axis refers to the log base 10 logarithm. The dotted line corresponds to a false discovery rate–corrected $P<0.05$. **C**, Significant (false discovery rate–corrected $P<0.05$) Gene Ontology Pathway enrichment analysis for significant proteins. **D**, Correlation between Mendelian randomization estimates for proteins associated with genetically downregulated signaling and estimates from linear regression for pharmacological IL-6R inhibition among 24 individuals treated with tocilizumab vs 24 individuals treated with placebo in the Norwegian tocilizumab non–ST-segment–elevation myocardial infarction study (the blue and red dots correspond to proteins that were found to be downregulated and upregulated, respectively, in the genetic analysis of **B**). CRP indicates C-reactive protein; CXCL10, C-X-C motif chemokine ligand 10; GWAS, genome-wide association study; IL-6, interleukin-6; and IL-6R, interleukin-6 receptor.

(false discovery rate–adjusted *P* value<0.05), we found that genetically proxied downregulation of the IL-6 signaling pathway is associated with lower levels of 43 and higher levels of 27 circulating proteins, explaining between 1% and 15% of their variance (Figure 2B; Table S3). Of those proteins, 54 also showed evidence of colocalization, as determined by a posterior probability of association >0.80 for at least one common genetic variant in the examined genomic region influencing both sIL-6R and the respective protein levels. It should be noted that a low probability of association for the hypothesis of no common variant (probability of association 3) for specific proteins might indicate lack of power of colocalization to detect a common signal rather than lack of a common genetic variant. Among these proteins, 45 also showed significant and directionally consistent associations in the weighted median approach and no evidence of directional pleiotropy in MR-Egger regression ($P_{intercept}>0.05$). Of those, only one showed minimal evidence of heterogeneity (MYO6, I^2=27%; Table S3).

Among the 70 proteins, there were well-known components of the pathway, such as IL-6 itself or downstream mediators and effectors, such as CRP and the fibrinogen C-domain–containing protein-1 (Figure 2B; Table S3). A Gene Ontology pathway enrichment analysis with the significant proteins (Figure 2C; Table S4) showed enrichment in several cytokine production pathways, such as regulation of IL-12 and IL-1β production and regulation of the IFN-γ pathway, as well as in

immune cell-trafficking pathways, such as myeloid cell differentiation and mononuclear cell or leukocyte proliferation. Furthermore, there was enrichment in tyrosine phosphorylation of STAT in line with the known signaling transduction pathway of IL-6R activation.[44]

Correlation Between MR-Derived Proteomic and Pharmacological Effects

We compared the proteomic effects derived from a genetically proxied downregulation of IL-6 signaling in MR analyses with the effects of pharmacological IL-6 signaling inhibition on the same proteins. Leveraging data from 48 patients with NSTEMI from the Norwegian tocilizumab NSTEMI study (24 who underwent treatment with tocilizumab and 24 in the control arm),[31] we obtained differences between tocilizumab and placebo across 1074 proteins also assessed with a different version of the aptamer-based SOMAScan assay within 48 hours after randomization and treatment. Matching the available proteins with the proteins available in the INTERVAL study, we were able to explore correlations across 785 proteins between the MR and the trial effects (Table S5). We found a significant correlation across the proteome (Pearson r 0.16, $P=1.1\times10^{-5}$; Figure 2D). This correlation was even stronger when restricting the analysis to the 44 proteins reaching a $P<0.05$ in the trial analysis ($r=0.48$, $P=8\times10^{-4}$).

Effects of Significant Proteins on Cardiovascular End Points and Mediation Analysis

Given the well-established associations of genetically downregulated IL-6 signaling with CAD, PAD, and ischemic stroke,[8,9,11] we next explored to what extent the 70 proteins associated with genetically proxied IL-6 signaling are also associated with these cardiovascular end points. We proxied the levels of 58 of these proteins in the deCODE proteogenomic database from 35 559 Icelanders, in which 4907 circulating proteins were also quantified with the aptamer-based SOMAScan assay.[35] Across these 58 proteins, we found the genetically proxied circulating levels of 4 to be associated with the examined cardiovascular outcomes in IVW MR analyses after correction for multiple comparisons (false discovery rate–corrected $P<0.05$). Genetically proxied levels of CXCL10 were associated with all 3 atherosclerosis end points, whereas CBS (cystathionine β-synthase), CRP, and IL-23R (interleukin-23 receptor) showed significant associations only with CAD (Figure 3A; Table S6). The results for CXCL10 and IL-23R were consistent in sensitivity analyses, including the weighted median approach and MR-Egger regression.

To minimize the risk of pleiotropic effects, we next performed a sensitivity analysis based solely on *cis*-acting variants for these proteins. Using genetic variants in minimal linkage disequilibrium ($r^2<0.1$) associated with the circulating levels of these proteins at $P<10^{-5}$ and within 300 kB upstream or downstream of the encoding genes, we confirmed the association between genetically proxied CXCL10 levels and odds for PAD (Table S7). Figure 3B illustrates the colocalization of the genomic signals between circulating sIL-6R and CXCL10 around the same genetic variants at the *IL6R* locus. The posterior probability H4 ranged from 99.97% for a prior P_{12} of 10^{-3} to 19% for a prior P_{12} of 10^{-8}. Given the emergence of CXCL10 as the most promising mediator in the examined associations, we performed mediation MR analyses. There was no overlap in genetic instruments for IL-6R signaling and circulating CXCL10 levels (Tables S1, S8, and S9). We found changes in circulating CXCL10 levels to mediate 39%, 67%, and 25% of the effects of genetically proxied IL-6 signaling on PAD, LAAS, and CAD, respectively (all $P<0.05$; Figure 3C).

There was no evidence of a reverse effect of genetically proxied CXCL10 on IL-6 levels (β: 0.10 [95% CI, −0.04 to 0.24], $P=0.15$). Furthermore, we performed a sensitivity analysis in a population-based study of 876 participants from the Lothian Birth Cohort 1936, where CXCL10 was quantified with the Olink technology.[45] The IVW MR analyses showed an association similar in direction and magnitude to INTERVAL (β_{Olink}: −0.05 [95%CI, −0.11 to 0.01] versus $\beta_{SOMAScan}$: −0.06 [95% CI, −0.09 to −0.03]). An analysis of the larger UK Biobank data set, in which CXCL10 was also quantified with Olink, showed no association (β: −0.01 [95% CI, −0.02 to 0.01], $P=0.55$). In a PheWAS analysis, we found no evidence of significant associations between genetically proxied CXCL10 and infectious diseases (Table S10).

Circulating CXCL10 Levels and Risk of Cardiovascular Events in a Population-Based Prospective Cohort Study

Following up on the signal for CXCL10, we used data from the population-based MONICA/KORA study,[38] in which circulating levels of CXCL10 have been quantified with a Luminex cytokine multiplex assay at midlife in 1704 individuals (47% women; median age, 53 years [interquartile range: 44–61]) without overt cardiovascular disease at baseline. The baseline characteristics of the study participants are presented in Table S11. There was a significant positive correlation between serum IL-6 and CXCL10 levels among study participants ($r=0.19$, $P=1.2\times10^{-14}$; Figure 4A). Next, we tested associations of circulating CXCL10 levels with the risk of a composite end point of stroke and CAD over a median follow-up of 20.9 years (interquartile range: 12.9–26.1). After adjustments for age, sex, and baseline survey (model 1) in a Cox regression model, we found circulating CXCL10 levels to be associated with a higher risk of the

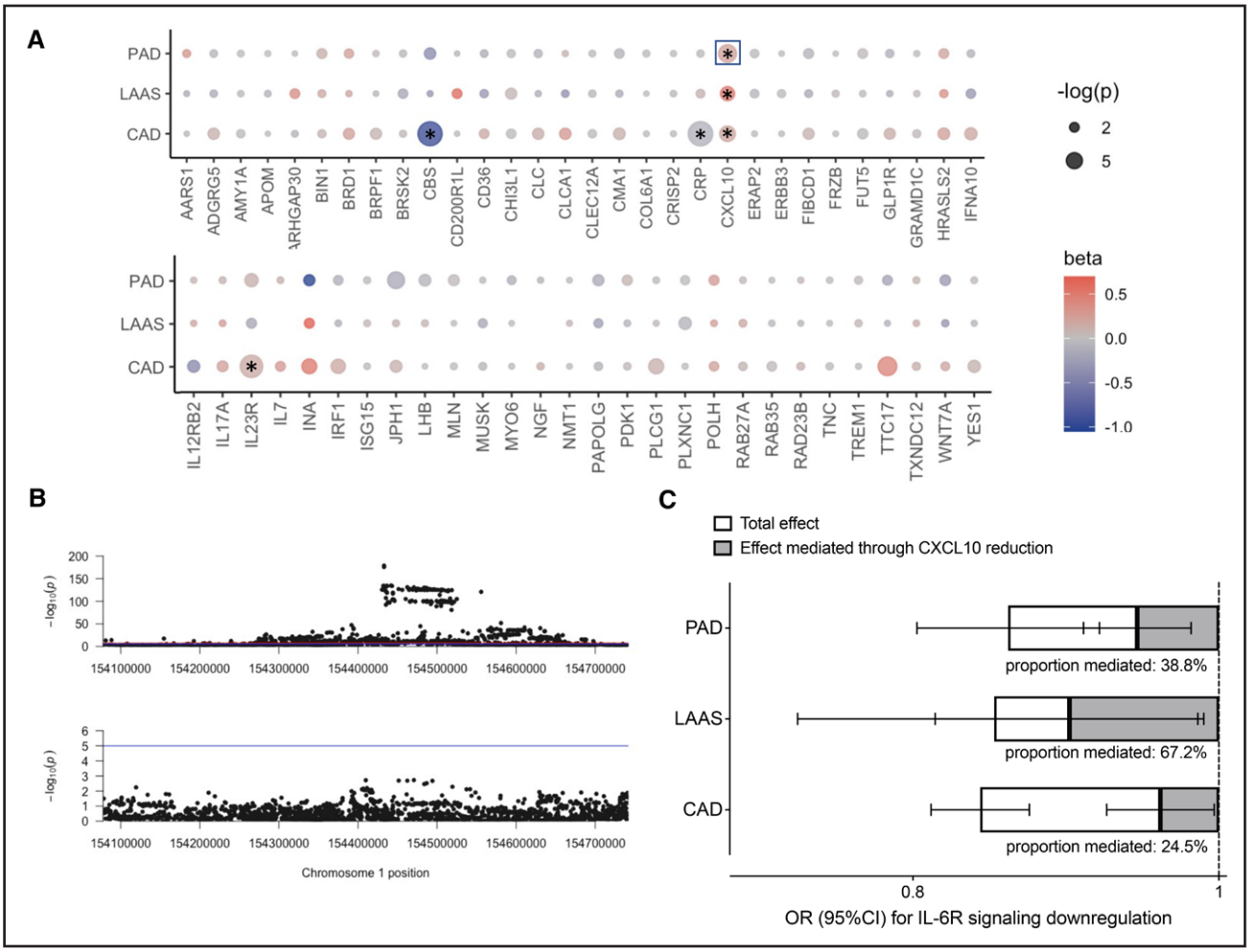

Figure 3. Genetically proxied levels of proteins associated with genetically downregulated IL-6 (interleukin-6) signaling and atherosclerotic cardiovascular disease.

A, Associations of genetically proxied levels of proteins associated with genetically downregulated IL-6 signaling with peripheral artery disease (PAD), large artery atherosclerotic stroke (LAAS), and coronary artery disease (CAD), as derived from inverse variance–weighted Mendelian randomization analyses. The stars indicate significant associations at a false discovery rate–corrected $P<0.05$, whereas the box around significant associations highlights associations that were also significant in *cis*–Mendelian randomization analyses. **B**, Regional association plots at the *IL-6R* locus for associations with soluble IL-6 receptor levels (**upper**) and CXCL10 (C-X-C motif chemokine ligand 10) levels (**lower**) demonstrating colocalization of the signal. **C**, Mediation Mendelian randomization analysis for the total effects of genetically downregulated IL-6 signaling on PAD, LAAS, and CAD, and the indirect effects mediated through changes in CXCL10 levels.

composite end point (hazard ratio per SD increment in log-transformed CXCL10 levels: 1.20 [95% CI, 1.05–1.36]; Figure 4B and 4C). These results remained significant in a model further adjusting for common vascular risk factors at baseline (model 2), and when including both circulating CXCL10 and IL-6 levels in the same model (model 3; Figure 4C; Table S12).

CXCL10 Expression in Atherosclerotic Tissue Associated With Plaque Vulnerability

To explore whether CXCL10 expression in human atherosclerotic lesions is associated with plaque vulnerability, we used data from 623 individuals from the Athero-EXPRESS Biobank in Utrecht, the Netherlands, who underwent carotid endarterectomy because of symptomatic or asymptomatic stenosis and for whom both transcriptomic and histological analyses were available (Table S13; Figure 5A). After adjustments for age and sex, we found that higher normalized CXCL10 expression in carotid atherosclerotic plaques was associated with a larger lipid core (odds ratio, 1.19 [95% CI, 1.02–1.40], $P=0.01$; Figure 5B). No associations were detected with intraplaque hemorrhage, extensive collagen content, or plaque calcification (Figure 5B).

To test in more depth the phenotypic plaque profile associated with elevated *CXCL10* expression, we used transcriptomic data from atherosclerotic aortic root tissue from 514 samples from patients with CAD in the STARNET network and explored the genes that were correlated with the expression of *CXCL10* in a coexpression analysis. A total of 98 genes were positively

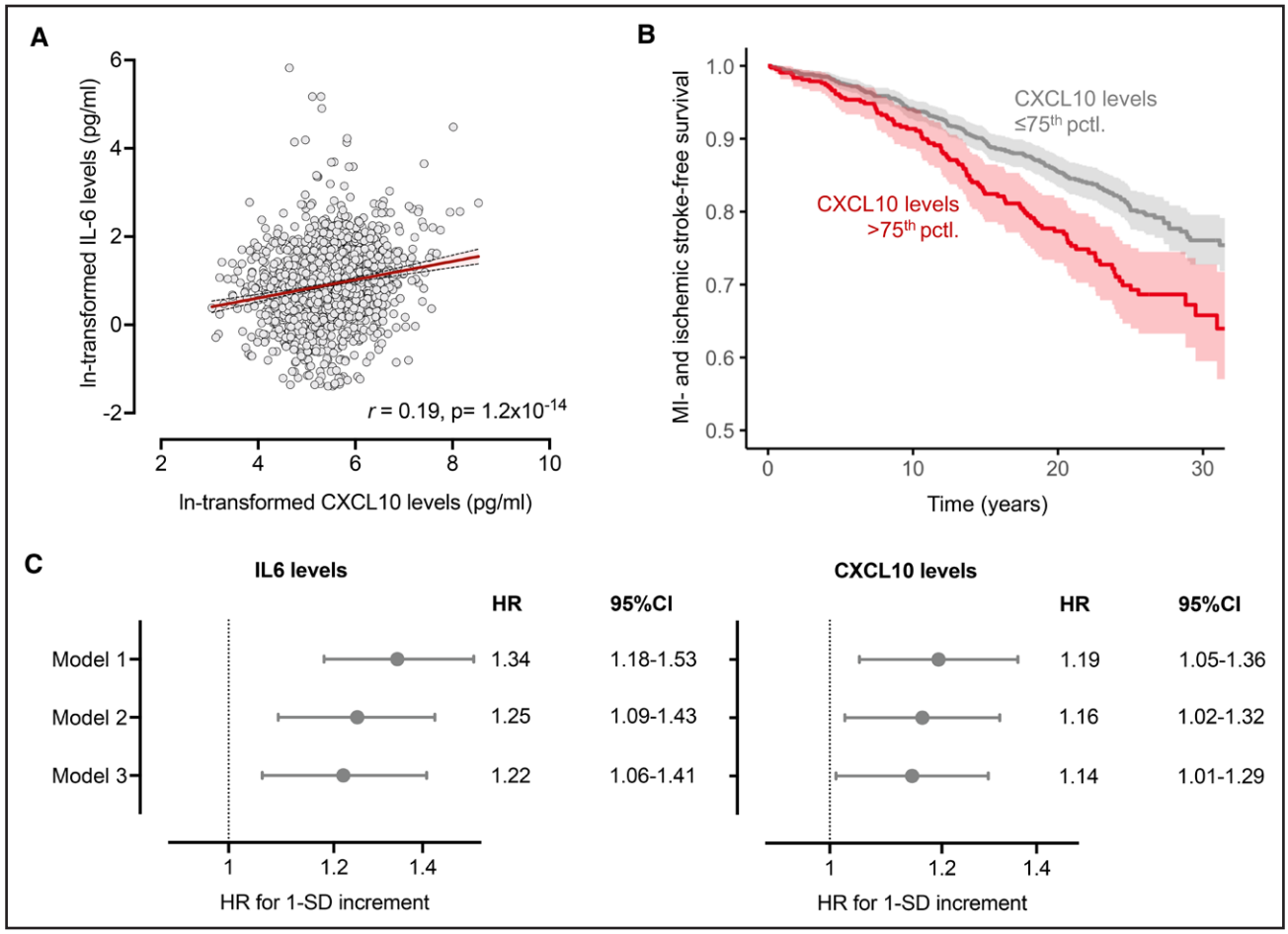

Figure 4. Circulating CXCL10 levels in association with circulating IL-6 and major adverse cardiovascular events in the population-based MONICA/KORA cohort.

A, Correlations between circulating IL-6 and CXCL10 levels among 1704 participants of the MONICA/KORA cohort. **B**, Kaplan-Meier curve of the associations between baseline circulating CXCL10 levels and risk of coronary artery disease or stroke over a follow-up period extending up to 30 years. **C**, Hazard ratios of the associations of circulating IL-6 and CXCL10 levels with risk of coronary artery disease or stroke in models adjusted for age, sex, and baseline survey (model 1), models adjusted for age, sex, baseline survey, and vascular risk factors (model 2), and a model adjusted for all variables of model 2 and both proteins simultaneously included (model 3). CXCL10 indicates C-X-C motif chemokine ligand 10; HR, hazard ratio; IL-6, interleukin-6; and MI, myocardial infarction.

correlated with *CXCL10* expression and 6 genes were negatively correlated with *CXCL10* expression (Figure 5C; Table S14). The positively correlated genes were enriched in immune system pathways, as captured by Reactome, and included CXCL9 and CXCL11, which are also ligands of the CXCR3 (C-X-C motif chemokine receptor 3), the main CXCL10 receptor.[46] These pathways included cytokine signaling pathways, and, in particular, the interferon and interleukin pathways, such as interferon-γ, IL-6, and IL-1 signaling, as well as pathways related to the adaptive immune system (Figure 5D; Table S15). As a final step, we explored whether specific cell types, as captured by single-nuclei RNA sequencing from aortic tissue,[43] are enriched for expressing the top genes positively coexpressed with *CXCL10* (r>0.3) to infer the cellular landscape associated with higher *CXCL10* expression. According to these data, endothelial cells, lymphocytes, and macrophages were the cell types primarily expressing these genes (*PARP14* and *STAT1* most strongly enriched in endothelial cells, *NLRC5* and *PARP14* most strongly enriched in lymphocytes, and *PARP14* and *TYMP* most strongly enriched in macrophages [Figure 5E; Table S16]).

DISCUSSION

Integrating data from large-scale genomic and proteomic studies, we explored the downstream effects of genetic downregulation of the IL-6R–mediated signaling on the blood proteome and their mediating role in lowering the risk of atherosclerotic cardiovascular disease. We found changes in circulating levels of 70 proteins involved in regulation of cytokine production and immune cell trafficking, which were consistent with the proteomic effects of the pharmacological inhibition of IL-6R with tocilizumab in a clinical trial.

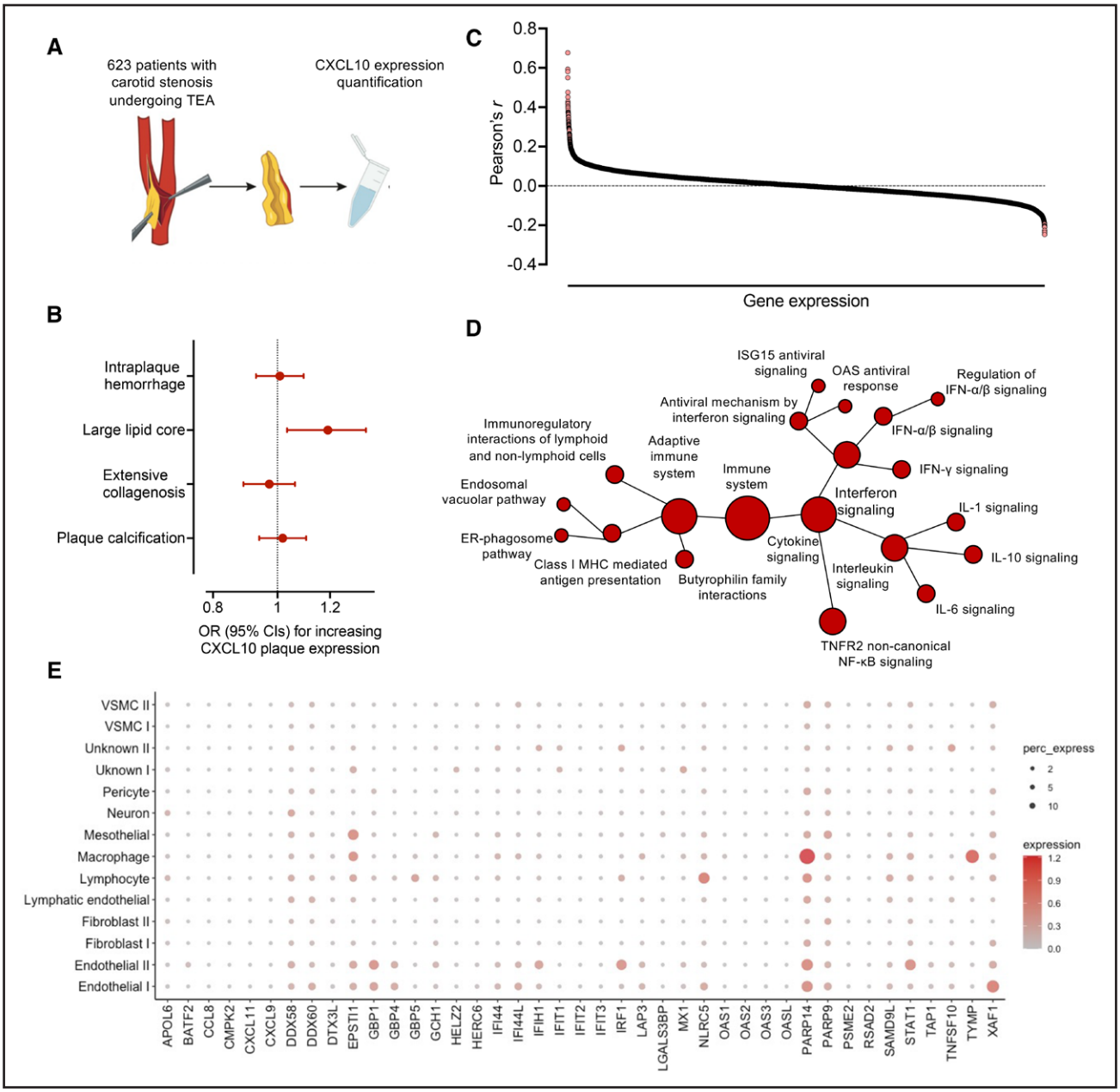

Figure 5. Expression of *CXCL10* within atherosclerotic lesions.
A, Schematic representation of CXCL10 mRNA quantification among 623 carotid atherosclerotic plaques from patients with carotid stenosis in the Athero-EXPRESS Biobank who underwent endarterectomy. **B**, Associations of plaque *CXCL10* expression with histological features of plaque vulnerability. **C**, Coexpression of *CXCL10* with 16214 other genes in atherosclerotic aortic root tissue from 514 participants in the STARNET network. **D**, Pathway enrichment analysis of genes coexpressed with *CXCL10*, as derived from Reactome. **E**, Cell-specific expression of top 42 genes coexpressed with CXCL10 at an r>0.3 in aortic tissue, as derived from single-nuclei RNA-sequencing analysis in human aorta samples. CXCL10 indicates C-X-C motif chemokine ligand 10; ER, endoplasmic reticulum; IFN, interferon; IL-6, interleukin-6; ISG15, interferon-stimulated gene 15; MHC, major histocompatibility complex; OAS, 2′,5′-oligoadenylate synthetase; OR, odds ratio; TEA, thromboendarterectomy; TNFR2, tumor necrosis factor receptor 2; and VSMC, vascular smooth muscle cell.

Across the 70 proteins affected by IL-6 signaling, genetically proxied levels of the chemokine CXCL10 were associated with risk of CAD, LAAS, and PAD, and changes in CXCL10 levels mediated a significant proportion of the effects of IL-6 signaling on these end points. In follow-up analyses, we found circulating CXCL10 levels at midlife in a population-based setting to be associated with the long-term risk of CAD or stroke. Higher expression of CXCL10 in human atherosclerotic plaques was correlated with a larger lipid core and a transcriptomic profile consistent with macrophage and lymphocyte infiltration, as well as activation of the adaptive immune system and cytokine signaling.

Our results linking CXCL10 levels with risk of atherosclerotic disease are consistent with experimental data

showing a key role for this chemokine in atherogenesis and atheroprogression. The administration of a monoclonal antibody against CXCL10 produced a histologically more stable atherosclerotic plaque phenotype with increased collagen and smooth muscle cell content and a smaller necrotic lipid core in the common carotid arteries of atheroprone *Apoe*$^{-/-}$ mice fed a high-fat diet.[47] This is in line with our results suggesting an association of higher intraplaque *CXCL10* expression with a larger lipid core, a hallmark of unstable plaques. In another model, both sexes of *Cxcl10*$^{-/-}$ *Apoe*$^{-/-}$ mice exhibited significantly smaller atherosclerotic lesion areas across the aortic arch, thoracic and abdominal aorta, and different durations of treatment.[48] The atheroprotective effect of CXCL10 blockade in translational experiments is further corroborated by evidence of decreased atherosclerotic burden in the aortic arches and carotid arteries of *Ldlr*$^{-/-}$ mice after pharmacological targeting of CXCR3, the receptor of CXCL10, with a specific small molecule antagonist,[49] and a CXCR3/CCR5 dual inhibitor.[50] Furthermore, experimental data also suggest that IL-6, and particularly IL-6 *trans*-signaling through STAT3, exerts an effect on T-cell immigration directly through regulation chemokine secretion, including CXCL10.[51] CXCL10/CXCR3 interaction induces T_H1 cell differentiation and migration into the atherosclerotic lesion where they release TNF-α (tumor necrosis factor-α) and interferon-γ, increasing the recruitment and activation of macrophages and contributing toward a proinflammatory lesion environment.[52] Within the plague the T cells directly interact with foam cells through the binding of the CD40 to the CD40 receptor and induce foam cells to produce inflammatory molecules and uptake more lipids, exacerbating the formation of the lipid core.[53] Current evidence collectively indicates that CXCL10 binding to CXCR3 drives atherosclerotic plaque progression through differentiation and homing of proinflammatory T-lymphocyte subsets; 2 isoforms (CXCR3-A and CXCR3-B) of this receptor have been identified more specifically in humans, which lead to different downstream effects. Through binding to the CXCR3-A receptor, CXCL10 has chemoattractant properties and induces the migration, proliferation, and survival of leukocytes. On the other hand, the CXCR3B isoform of the receptor has antiangiogenic properties and inhibits cell proliferation and migration while also stimulating apoptosis.[46] It should be noted that CXCR3 also binds the chemokines CXCL9 and CXCL11.[46]

Beyond detecting mediators of the effects of IL-6 signaling on atherosclerosis, our findings also provide evidence for a downstream proteomic signature of genetic IL-6 signaling downregulation that could be correlated with trial data from pharmacological IL-6R inhibition. This finding has important implications for a more widespread use of a proteome-wide MR analysis in the context of detecting the downstream proteomic consequences of perturbing drug targets. Proteomic signatures detected with this approach could be used as potential biomarkers of response to drugs targeting the upstream targets. Although such response biomarkers are well-established for drugs targeting the IL-6 signaling cascade, our approach supports use of the method for identifying response biomarkers for drugs under earlier development. Despite the significant correlation with trial data, there were also discrepancies between genetic downregulation and pharmacological inhibition of IL-6 signaling, including a lack of effect of tocilizumab on CXCL10 levels in the NSTEMI trial. There are several reasons that might explain this discrepancy. First, genetic downregulation reflects a lifelong effect, whereas the trial captured the effects of a single dose of tocilizumab 4 days after treatment. Second, the effects of genetic downregulation were studied in a general population setting, whereas the trial tested tocilizumab in a cohort of patients with NSTEMI shortly after the event, when an inflammatory response to the infarct might influence cytokine levels. Third, tocilizumab blocks both the classical and *trans*-IL-6 signaling.[54] Whether this is the case for most of the variants used as proxies for IL-6R signaling (selected based on their effects on CRP, primarily reflecting classical signaling) remains untested. Animal studies have shown decreases in CXCL10 levels and T-cell recruitment after targeted pharmacological inhibition of the *trans*-signaling cascade.[51] Alternative or even competing downstream effects of the classical and *trans*-signaling inhibition with tocilizumab versus genetic downregulation might explain part of the discrepancies. Fourth, the uncertainty in the effects of tocilizumab because of the small trial sample size (20 treated versus 20 placebo arm) should also be considered. Future studies should systematically explore how proteomic alterations detected in clinical trials correlate with those detected in MR analyses.

Among the proteins affected by genetic IL-6 signaling downregulation is the circulating soluble form of IL-6R itself (sIL-6R). The IL-6R variant with the largest effect size that largely drives the reported associations is rs2228145, which leads to the substitution of aspartic acid with alanine in the extracellular domain of IL-6R. This substitution is located within the cleavage site of the ADAM17 protease, which is responsible for ectodomain shedding of the membrane-bound IL-6R, resulting in its conversion from the membrane-bound to the soluble form of the receptor (sIL-6R).[55] The result of this substitution is an increased proteolytic cleavage of the membrane-bound IL-6R and a profound increase in circulating sIL-6R and IL-6. Although the levels of the sIL-6R increase, membrane-bound IL-6R expression (especially in CD4+ T cells and monocytes) decreases, which impairs IL-6 signaling.[55,56]

Using MR to integrate multiomics data is a method increasingly used for bridging data from different omics layers and detecting causal relationships between them.[57,58] Scaling up mediation analyses to be integrated

to this approach allows explorations of associations with clinically relevant outcomes, and as such the discovery of novel therapeutic targets. Furthermore, this approach enables the dissection of pathways leading from genomic alterations to cardiovascular disease, thus providing novel insights into pathophysiology. Expanding the focus of the approach to other omics layers beyond proteomics, to more drug targets and more outcomes could provide novel insights in future explorations.

Our study has limitations. First, despite leveraging the largest available samples, such integrating approaches are characterized by decreasing power when moving downstream across omics layers.[59] As such, several of our analyses, especially for the outcome of LAAS, might have been relatively underpowered, highlighting the need to repeat the analyses with increasing sample sizes. Second, although the proteomic analyses across all studies were done with the SOMAscan aptamer-based assay, there were considerable differences in proteins available in the INTERVAL study, deCODE study, and the Norwegian tocilizumab NSTEMI trial, which led to an incomplete overlap between the studies. Although the associations between genetically proxied IL-6 signaling and circulating CXCL10 were directionally consistent in 2 data sets performing protein quantifications with the proximity extension assay-based Olink assay, they did not reach statistical significance and should thus be interpreted with caution. Considerable differences have been previously reported for levels of the same proteins when quantified with Somascan or Olink.[60] Third, directional pleiotropy might confound the analyses exploring associations between genetically proxied circulating proteins and cardiovascular end points, especially instruments based on variants from throughout the genome may be particularly prone to directional pleiotropy and, as such, might lead to false-positive findings. Although analyses based on *cis*-variants are less prone to this kind of bias, such variants are rare and not directly available for proxying all proteins. Furthermore, selecting such variants might lead to weak instrument bias because of more lenient thresholds and, as a result, estimates biased to the null that might lead to false-negative findings. It is important to note that when restricting our analysis to *cis*-instruments we were unable to find significant associations of genetically proxied CXCL10 with CAD or LAAS and thus those results should be interpreted with caution. Fourth, the majority of individuals explored in current analyses were of European ancestry and as such our results may not be generalizable to individuals of other ancestries. Fifth, data from deCODE were derived from an Icelandic population that has substantial differences from European populations. Although integration of data from deCODE with data from European data sets has been extensively used in the past, providing important insights into disease pathogenesis, we cannot exclude the introduction of population stratification that could bias our MR analyses in the second step (Figure 2) of our approach. Sixth, an additional caveat to our study is that we used proxies of IL-6R inhibition as genetic instruments, meaning that proteins found upstream would be upregulated as a result of positive feedback, whereas downstream molecules would be downregulated. This could create confusion as to the directionality of the levels of these proteins in real-life clinical trials. Seventh, to proxy IL-6 signaling we used CRP levels, which increase in response to the activation of the IL-6 cascade. However, IL-6 signaling is a complex cascade with a classical component, exerted through the membrane-bound IL-6R, and a *trans*-signaling component exerted through the soluble form of IL-6R. The 2 subpathways exert unique actions,[16,61] but disentangling them with genetic instruments probably goes beyond the limits of MR. Eighth, by design, MR analyses assess the effects of lifetime downregulated IL-6 signaling, which might differ from a shorter pharmacological inhibition with IL-6R blockade. Ninth, the estimates of the direct and indirect effects of genetically proxied IL-6 signaling on atherosclerotic outcomes in the mediation analyses might be biased because of noncollapsibility of odds ratios when using a binary outcome. Tenth, colocalization analyses are based on an assumption of a single causal variant per trait in the examined regions, which is probably not realistic in most scenarios.

In conclusion, integrating genomic and proteomic data, we found a proteomic signature of IL-6 signaling activation and mediators of its effect on cardiovascular disease. Our analyses suggest that CXCL10 is a potentially causal mediator for atherosclerotic end points in 3 different vascular beds and, as such, might serve as a promising drug target for atheroprotection that should be further explored in clinical trials.

ARTICLE INFORMATION

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Affiliations

University of Patras School of Medicine, Greece (S.P.). Center for Genomic Medicine, Massachusetts General Hospital, Boston (S.P., J.R., C.D.A.). Broad Institute of MIT and Harvard, Cambridge, MA (S.P., J.R., C.D.A., M.K.G.). Institute for Stroke and Dementia Research, Ludwig-Maximilians-University of Munich, Germany (L.Z., R.M., J.B., M.D., M.K.G.). Institute of Epidemiology, Helmholtz Zentrum München, German Research Center for Environmental Health (GmbH), Neuherberg, Germany (B.T., A.P.). Department of Clinical Pharmacology, Division of Medicine, University College London, United Kingdom (M.J.G., A.D.H.). Central Diagnostics Laboratory, Division Laboratories, Pharmacy, and Biomedical Genetics, University Medical Center Utrecht, The Netherlands (S.W.v.d.L., G.P.). Institute for Clinical Diabetology, German Diabetes Center, Leibniz Center for Diabetes Research at Heinrich Heine University, Düsseldorf, Germany (C.H.). Department of Endocrinology and Diabetology, Medical Faculty and University Hospital, Heinrich Heine University, Düsseldorf, Germany (C.H.). German Center for Diabetes Research, Partner Düsseldorf, Neuherberg, Germany (C.H.). German Centre for Cardiovascular Research (DZHK), Partner Site Munich Heart Alliance, Germany (W.K., J.B., M.D.). Institute of Epidemiology and Medical Biometry, University of Ulm, Germany (W.K.). German Heart Center Munich, Technical University of Munich, Germany (W.K.). Thrombosis Research Center (TREC), Division of internal medicine, University hospital of North Norway, Tromsø (T.U.). Research Institute of Internal Medicine, Oslo University Hospital Rikshospitalet, Norway (T.U., P.A.). Institute of Clinical Medicine, University of Oslo, Norway (T.U., P.A.). Clinic of Cardiology, St Olavs Hospital, Trondheim, Norway (O.K.). Department of Circulation and Medical Imaging, Norwegian University of Science and Technology, Trondheim (O.K.). Section of Clinical Immunology and Infectious Diseases, Oslo University Hospital

Rikshospitalet, Norway (P.A., M.D.). Department of Cardiology Oslo University Hospital Rikshospitalet, Norway (L.G.). Munich Cluster for Systems Neurology (SyNergy), Germany (J.B.). Chair of Epidemiology, Institute for Medical Information Processing, Biometry, and Epidemiology, Ludwig-Maximilians-University Munich, Germany (A.P.). Munich Heart Alliance, German Center for Cardiovascular Health (DZHK e.V., partner-site Munich), Germany (A.P.). Centre for Translational Genomics, Institute of Cardiovascular Science, University College London, United Kingdom (A.D.H.). Henry and Allison McCance Center for Brain Health, Massachusetts General Hospital, Boston (J.R.). German Center for Neurodegenerative Diseases (DZNE, Munich), Germany (M.D.). Department of Neurology, Brigham and Women's Hospital, Boston, MA (C.D.A.).

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Supplemental Material

Expanded Methods

Tables S1–S16

References 62–66

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SUPPLEMENTAL MATERIAL

Prapiadou *et al*. **Proteogenomic data integration reveals CXCL10 as a potentially downstream causal mediator for IL-6 signaling on atherosclerosis**

1. Extended Methods

1.1. Genetic Instrument Selection

As previously described, we meta-analyzed a GWAS for CRP levels of 204,402 individuals of European ancestry from the Cohorts for Heart and Aging Research in Genomic Epidemiology (CHARGE) Consortium[62] with data from a GWAS for CRP levels for 318,279 White British individuals in the UK Biobank dataset.[63] We selected as instruments genetic variants associated with CRP levels at a $p<5x10^{-8}$ after clumping for linkage disequilibrium at $r^2<0.1$ with the 1000G European reference panel. We weighed the variants according to their effects on circulating levels of soluble IL-6R (sIL6R). The values of sIL6R in INTERVAL were rank-inverse normalized, thus all Mendelian randomization analyses for genetically proxied IL6R signaling downregulation should be interpreted as the effect of 1 standard deviation increment in the inverse rank-transformed sIL6R values. We selected this approach over weighing the variants according to their effects on CRP in order to minimize the distance between genetic variation and its biological downstream effects. We obtained the effects of the variants on sIL6R levels from the INTERVAL cohort of 3,301 individuals of European ancestry.[64]

1.2. INTERVAL Study

The INTERVAL study is comprised of 50,000 blood donors from 25 centers across England recruited into a randomized trial of blood donation frequency. Focusing on 3,301 randomly selected trial participants of European ancestry, 3,281 proteins were quantified in plasma samples with an aptamer-based multiplex protein assay (SOMAscan®) and their genetic architecture was explored.[64]

1.3. MONICA/KORA Study

CXCL10 was measured in serum obtained at the baseline visit with the Luminex® multiplex technology using a Luminex 100 analyzer (Luminex Corporation, Austin, TX, recombinant proteins and antibodies purchased from R&D systems) in non-fasting samples stored at -80°C in a case-cohort study on incident coronary artery disease[38] and type 2 diabetes[65]. For the present analysis, CXCL10 measurements within the random sub cohort of the case-cohort study were used and the follow-up period was extended up to 2016. Medical, demographic, and biochemical data were collected at baseline visits in 1984/85, 1989/90 and 1994/95. Vital status was ascertained through the population registries inside and outside the study area. Death certificates were obtained from the local health authorities. Follow-up questionnaires were sent to all participants with consent and current address information who were still alive in 1997-1998, in 2001-2002, in 2008-2009 and in 2016 to obtain information on the occurrence of chronic diseases and risk factors.

The present analysis used a combined endpoint of incident coronary artery disease and incident stroke. All coronary artery disease events occurring until the age of 74 years (or the age of 84 years from 2009 onwards) were ascertained through the local MI registry. Coronary artery disease events occurring in participants > 74 years (or > 84 years from 2009 onwards) or in participants residing outside the study area, as well as non-fatal strokes were assessed by postal follow-up

questionnaires sent out to the participants at four time points during the follow-up period as described above. All self-reported incident strokes (ischemic and hemorrhagic) as well as fatal strokes identified by death certificates and self-reported MIs and death certificate-identified fatal coronary artery disease events occurring outside the study area or in persons > 74 years (or >84 years from 2009 onwards) as well as the date of diagnosis were validated using information obtained from participants' hospital records or from their attending physicians[66].

1.4. Data Availability

Summary statistics of the genetic analyses from the INTERVAL and deCODE studies are publicly available online at https://ega-archive.org/datasets/EGAD00001004080/files and https://www.decode.com/summarydata/. The genetic instruments used for the main analyses are provided in Supplementary Table S1 and are available from previous publications.[17,18] GWAS summary statistics for coronary artery disease and large artery stroke from the CardioGRAMPLUSC4D Consortium and the MEGASTROKE Consortium are available at http://www.cardiogramplusc4d.org/data-downloads/ and https://www.megastroke.org/download.html, respectively. GWAS summary statistics for PAD have been accessed through dbGAP (pha005161.1) with an approved application by JR and CDA for the Veterans Administration (VA) Million Veteran Program (MVP) Summary Results from Omics Studies. Summary statistics from the NSTEMI trial were accessed through contact of the corresponding authors of the study and are available to interested authors through an application and a short research proposal submitted to the study. Data from the MONICA/KORA study are not publicly available because the data are subject to national data protection laws and restrictions were imposed by the ethics committee of the Bavarian Chamber of Physicians to ensure data privacy of the study participants. However, data are available on request to researchers through a project agreement from KORA (https://helmholtz-muenchen.managed-otrs.com/external/). Requests should be sent to kora.passt@helmholtz-munich.de and are subject to approval by the KORA board. The datasets from Athero-Express analyzed for the current study are available upon reasonable request and application to Athero-Express Biobank Study through a Data Transfer Agreement due to consent restrictions and local regulations. The gene expression dataset from STARNET was accessed through dbGAP with an approved application by JR and CDA (phs001203.v1.p1). Data for the single-nuclei transcriptomic analyses were accessed through the Single Cell Portal (https://singlecell.broadinstitute.org/single_cell). The YFS is a population-based prospective follow-up study on cardiovascular risk factors in Finland. It has been carried out in all five Finnish university cities with medical schools and their rural surroundings. The first cross-sectional study was conducted in 1980. Altogether 4,320 children and adolescents aged 3, 6, 9, 12, 15 and 18 years were randomly recruited from the population register of these areas to produce a representative subsample of Finnish children. Of these individuals 3,596 (83%) participated in year 1980. Since then, regular follow-up visits have been performed in 1980, 1983, 1986, 2001, 2007, 2011 and 2018/2020. Follow-up using registry data (diagnoses, medications and mortality) have been extended by 2018. The YFS has been approved by the Joint Commission on Ethics of the Turku University and the Turku University Central Hospital and has been conducted according to the guidelines of the Declaration of Helsinki, and informed consent has been obtained from all participants or their parents.

1.5. Ethical Considerations

All studies have received ethical approvals from the respective ethical authorities. All participants who donated samples to deCODE gave informed consent, and the National Bioethics Committee of Iceland approved the study, which was conducted in agreement with conditions issued by the Data Protection Authority of Iceland (VSN_14-015). Personal identities for the participant's data and biological samples were encrypted by a third-party system (Identity Protection System), approved and monitored by the Data Protection Authority. The study protocol of Athero-EXPRESS conforms to the Declaration of Helsinki and was approved by the ethics committee on research on humans of the University Medical Center Utrecht. All participants provided written informed consent. For all KORA studies as well as the MONICA/KORA case-cohort study approval has been obtained from the Ethics Committee of the Bavarian Medical Association (Bayerische Landesärztekammer). All KORA studies have been approved by the Bavarian commissioner for data protection and privacy (Bayerischer Datenschutzbeauftragter). All study participants have provided written consent after being informed about the study. All subjects have the option to restrict their consent to specific procedures, e. g. by denying storage of biosamples. All participants in INTERVAL also gave informed consent before joining the study and the study received approval from the National Research Ethics Service approved this study (11/EE/0538). Finally, the Norwegian tocilizumab NSTEMI trial has received approval from the Regional Committee for Medical and Health Research Ethics of South-Eastern Norway and the Norwegian Medicines Agency, and conducted according to the Declaration of Helsinki. All participants provided written informed consent. The STARNET protocol has been approved from an institutional review committee (Ethics Review Committee on Human Research of the University of Tartu).

2. Supplementary Tables S1-S16

- **Table S1**. Genetic variants used as instruments for IL-6 receptor/mediated signaling.
- **Table S2**. Inverse-variance weighted Mendelian randomization results of genetically proxied IL-6R-mediated signaling downregulation and proteomic changes.
- **Table S3.** Sensitivity analyses for proteins significantly associated with gentically downregulated IL-6 singaling (heterogeneity statistics, weighted median Mendelian randomization, colocalization).
- **Table S4.** Gene ontology (GO) enrichment analysis for the genes encoding the 70 proteins that were significantly associated with genetically downregulated IL-6 signaling.
- **Table S5.** Comparisons of the effects of tocilizumab and genetically proxies IL-6 signaling with Mendelian randomization on human proteome.
- **Table S6.** Inverse-variance weighted, MR- Egger and Weighted Medican Mendelian randomization associations of genetically proxied protein levels significantly associated with IL-6 signaling with atherosclerotic cardiovascular endpoints.
- **Table S7.** Sensitivity cis-acting Mendelian randomization analyses for protein-outcome pairs showing significant associations in trans-acting Mendelian randomization.
- **Table S8.** Genetic variants used as instruments for circulating CXCL10 levels.

- **Table S9.** Genetic variants used as instruments for multivariable MR analysis to obtain the direct effect of genetically proxied CXCL10 on atherosclerotic outcomes after adjusting for the effects of the instruments on IL6R.

- **Table S10.** Phenome-Wide Association study of geneticaly proxied CXCL10 levels.

- **Table S11.** Baseline characteristics of 1,704 MONICA/KORA participants included in presented analysis.

- **Table S12.** Associations of baseline predictors with incidence of coronary artery disease or stroke in MONICA/KORA, as derived from Cox proportional hazard models.

- **Table S13.** Baseline characteristics of the 623 patients included in the AtheroEXPRESS study.

- **Table S14.** Genes, whose expression in atherosclerotic aortic root tissue was significantly (FDR-corrected p-value<0.05) associated with the expression of CXCL10 among 514 STARNET participants.

- **Table S15.** Reactome pathway analysis for genes significantly co-expressed with CXCL10 in atherosclerotic tissue.

- **Table S16.** Single-cell expression in human aortic tissues, as derived from single-nuclei RNA sequencing analysis of 41 genes significantly positively co-expressed with CXCL10 in atherosclerotic tissue.

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Erklärung zur Übereinstimmung der gebundenen Ausgabe der Dissertation mit der elektronischen Fassung

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Hiermit erkläre ich, dass die elektronische Version der eingereichten Dissertation mit dem Titel:

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