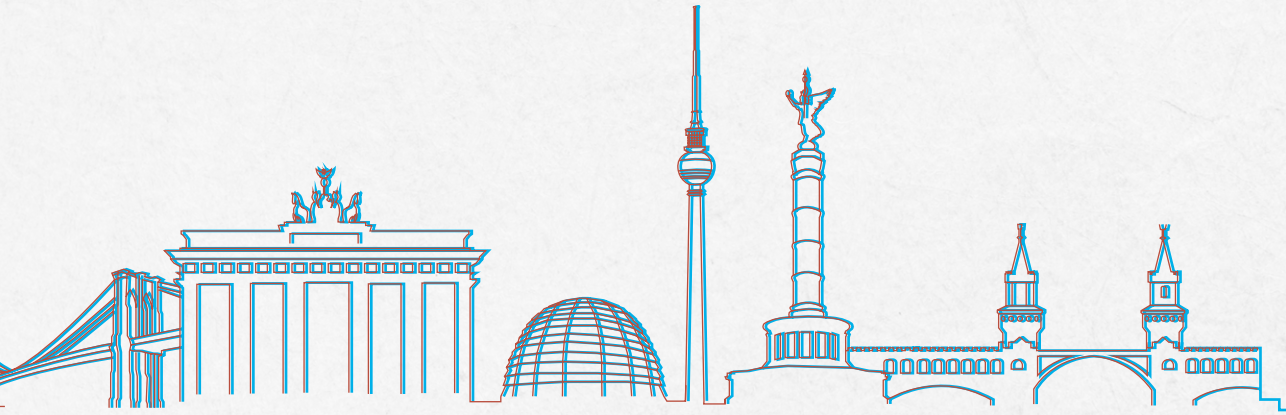
The impact of antithrombotic therapy on endothelial function and clinical outcomes in peripheral arterial disease



Loes H. Willems

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The impact of antithrombotic therapy on endothelial function and clinical outcomes in peripheral arterial disease

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Part IV

Chapter 1

General introduction, aim and outline of the thesis

Peripheral arterial disease (PAD) is a chronic condition rooted in atherosclerosis; a pathological process recognized since ancient times. Calcifications observed in mummified remains from ancient Egypt suggest early evidence of arterial disease. However, it was not until the late 19th and early 20th centuries that atherosclerosis and its clinical consequences, including PAD, were systematically studied and described. The modern understanding of PAD as a distinct clinical syndrome emerged in the mid-20th century, as advances in vascular pathology, surgery, endovascular therapies and non-invasive diagnostics refined its characterization. Initially termed "arteriosclerosis obliterans", PAD often leads to mobility impairment and significantly reduced quality of life for affected individuals and is increasingly recognized as a manifestation of systemic atherosclerosis with substantial global health implications.

Today, PAD affects approximately 202 million individuals worldwide, with a disproportionate burden on low- and middle-income countries (141 million cases) compared to high-income countries (61 million cases).[1] This global distribution underscores both the widespread nature of the disease and the significant health disparities that exist in its prevention and management.

The understanding of PAD has advanced alongside recognition of its major risk factors, including aging, smoking, hypertension, dyslipidemia, and diabetes mellitus.[2] These factors drive the progressive arterial narrowing that underlies PAD, necessitating continued efforts to mitigate their impact through both public health initiatives and individualized care.

Clinical symptoms and staging

The clinical presentation of PAD is variable, depending on the extent of arterial narrowing and the availability of collateral circulation.[3-4] Two major classification systems are commonly used to stage PAD and guide clinical decision-making: the Rutherford[5] and Fontaine[6] classifications.

The **Rutherford classification**[5] divides PAD into six stages:

- **Stage 0:** Asymptomatic arterial insufficiency, characterized by restricted blood flow without clinical complaints.
- **Stages 1-3:** Intermittent claudication, presenting as exercise-induced ischaemic muscle pain (typically in the calf, but sometimes in the upper leg or buttock) that is relieved by rest.
- **Stage 4:** Rest pain, a hallmark of chronic limb threatening ischemia.

- **Stages 5-6:** Tissue loss, including ulceration (stage 5) and gangrene (stage 6), reflecting severe ischemic damage.4-Weitz, 5-Rutherford]

The **Fontaine classification**[6], developed earlier in the 1950s, provides a complementary system for staging PAD:

- **Stage I:** Asymptomatic disease.
- **Stage II:** Intermittent claudication, with subcategories:
  - **IIa:** Mild claudication, not limiting lifestyle.
  - **IIb:** Severe claudication, limiting daily activities.
- **Stage III:** Ischemic pain at rest.
- **Stage IV:** Ischemic ulcers or gangrene.

Both classifications highlight the spectrum of PAD severity, from asymptomatic disease to critical limb-threatening ischemia. Together, they provide clinicians with robust frameworks for assessing disease progression and tailoring interventions.

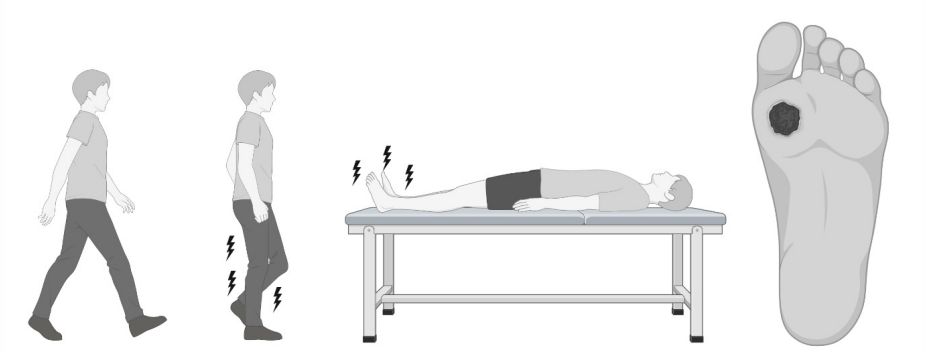

***Figure 1**: Clinical presentation of Peripheral arterial disease with asymptomatic disease, intermittent claudication, ischemic pain at rest and Ischemic ulcers, respectively. [Created with BioRender.com]*

Pathophysiology atherosclerosis

Peripheral arterial disease (PAD) is a manifestation of systemic atherosclerosis, a chronic inflammatory disease of the arterial wall characterized by the progressive accumulation of lipids, inflammatory cells, and fibrous elements. Advances in our understanding of atherosclerosis highlight its complex pathophysiology, which begins with dysfunction of the arterial endothelium, a critical regulator of vascular homeostasis. This endothelial dysfunction is particularly likely to occur at arterial bifurcations, where disturbed blood flow leads to low wall shear stress, disrupting endothelial integrity and promoting the initiation of atherosclerotic lesions.[7]

The endothelium, the innermost layer of the arterial wall, acts as a dynamic barrier and plays a pivotal role in maintaining vascular tone by interacting with vascular smooth muscle cells (VSMCs) and bioactive agents such as nitric oxide (NO), endothelin, and prostacyclin.[8] In the early stages of atherosclerosis, the endothelium is exposed to oxidized low-density lipoproteins (ox-LDL), which induces endothelial dysfunction. This dysfunction disrupts the regulation of vascular permeability and tone, while simultaneously enhancing the expression of pro-inflammatory cytokines and adhesion molecules. These changes facilitate the recruitment and adhesion of monocytes to the endothelium, which subsequently migrate into the intimal layer and differentiate into macrophages.

Macrophages absorb ox-LDL, transforming into foam cells that aggregate to form fatty streaks, the earliest visible lesion in atherosclerosis.[8] This stage reflects the shift from a reversible inflammatory response to a more sustained and progressive process.

As the disease advances, the ongoing expression of cytokines and adhesion molecules promotes the migration, accumulation, and proliferation of VSMCs. Foam cells release growth factors and cytokines, further amplifying these processes and contributing to the development of fibrotic plaques. These plaques consist of a lipid-rich necrotic core covered by a fibrous cap, providing structural integrity to the plaque.

The disruption of a fibrous plaque represents a critical event in atherosclerosis. Plaque rupture or erosion exposes thrombogenic substances, triggering activation of the coagulation cascade and leading to the formation of a thrombus. This process, known as atherothrombosis, can precipitate acute clinical events such as myocardial infarction, stroke, or acute limb ischemia, depending on the affected vascular territory.[9-10]

In addition to causing acute events, atherothrombosis also contributes to the progression of chronic lesions and symptoms. The repetitive formation and resolution of small thrombi can lead to chronic plaque growth, further narrowing the arterial lumen (stenosis). This progressive stenosis reduces blood flow to downstream tissues, exacerbating ischemia and contributing to the gradual worsening of chronic symptoms, such as intermittent claudication and, in severe cases, chronic critical limb ischemia. Moreover, chronic low-grade thrombotic activity can promote persistent inflammation within the arterial wall, further accelerating plaque progression and increasing the risk of both chronic and acute complications.

Thus, atherothrombosis not only plays a pivotal role in acute ischemic events but also perpetuates the chronic ischemic burden of PAD, underlining the importance of effective therapeutic strategies targeting both thrombosis and atherosclerosis to mitigate disease progression and symptomatology.

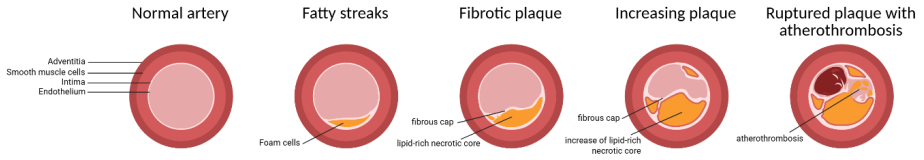

***Figure 2**: Sequential development of atherosclerotic lesions [Created with BioRender.com]*

Endothelial dysfunction

As previously described, endothelial dysfunction represents an early and critical event in the pathogenesis of atherosclerosis. It not only initiates the process of atherosclerosis, but also contributes to its progression and the subsequent formation of atherosclerotic plaques, which eventually may narrow the lumen of conduit arteries and impair blood flow.[9-10]

The endothelium, under normal conditions, maintains vascular homeostasis by regulating vascular tone, permeability, and the interaction between circulating blood elements and the vessel wall. In atherosclerosis, however, exposure to factors such as oxidized low-density lipoprotein (ox-LDL), smoking, and hyperglycemia disrupts these functions, resulting in endothelial dysfunction. This dysfunction is characterized by reduced bioavailability of nitric oxide (NO), increased oxidative stress, and upregulated expression of pro-inflammatory cytokines and adhesion molecules.

Emerging evidence has highlighted the role of crosstalk between coagulation and inflammatory pathways in exacerbating endothelial dysfunction. This interaction is mediated through protease-activated receptor (PAR) signaling, which links the coagulation cascade to vascular inflammation. PAR activation amplifies the recruitment and migration of leukocytes to the arterial wall, perpetuating inflammation and contributing to atherogenesis.[7,9-10]

Through this intricate interplay of dysfunctional endothelial signaling, inflammation, and coagulation, endothelial dysfunction becomes both a driver and a perpetuator of the atherosclerotic process, underscoring its central role in the pathophysiology of PAD.

Endothelial dysfunction in PAD

Endothelial dysfunction has been identified in association with nearly all major risk factors for PAD, including aging[11], hypertension[12], hyperlipidemia[13], obesity[14] and diabetes mellitus[15]. Interventions aimed at reducing atherosclerosis risk, such as physical activity and lipid-lowering therapies, have been shown to improve endothelial function.[16-18]

Notably, endothelial dysfunction is strongly linked to the occurrence of major adverse cardiovascular and limb events in PAD patients, regardless of the underlying disease state or other risk factors.[19-20] Previous studies underscore the prognostic value of endothelial dysfunction assessment, highlighting its role as an independent predictor of adverse outcomes across different vascular conditions. Impaired endothelial function has been consistently linked to an increased risk of major cardiovascular and limb events in patients with PAD, acute coronary syndrome, and ischemic stroke.[21-24]

Methods for testing endothelial dysfunction

Several methods have been developed to evaluate endothelial function. Traditionally, endothelium-dependent vasodilation of coronary arteries was assessed using angiography following the infusion of acetylcholine, which stimulates the release of endothelium-derived nitric oxide[25], or by administering medications that increase blood flow.[26] To study a more accessible vascular bed, researchers investigated the vasodilator response in the forearm using venous occlusion plethysmography in response to similar invasive stimuli.[27]

Given the risks associated with invasive techniques, non-invasive methods have gained prominence. Among these, flow-mediated dilation (FMD) measured via vascular ultrasound has attracted significant interest. FMD evaluates changes in the diameter of the brachial artery using ultrasound following a period of forearm ischemia induced by inflating a blood pressure cuff above systolic levels.[28-29] Compared to invasive techniques, FMD is safer, more feasible, and provides valuable prognostic insights into future cardiovascular events. However, the method suffers from low specificity and lacks standardized protocols, limiting its widespread utility.[30]

The development of alternative, standardized methods for assessing endothelial dysfunction could significantly enhance preventive strategies for PAD.

Carotid artery reactivity

In recent years, assessing carotid artery diameter changes in response to a cold pressor test (sympathetic stimulation) has proven useful for evaluating endothelial

dysfunction.[31-34] This method, known as the Carotid Artery Reactivity (CAR) test, is a straightforward and non-invasive procedure.

During the CAR test, the carotid artery is visualized in a longitudinal plane using B-mode ultrasound while the subject lies in a supine position. The arterial diameter is measured at baseline (for 30 seconds) and during a 3-minute immersion of the hand in ice water (≤4.0 °C).[31,34-35] In individuals with normal endothelial function, the carotid artery exhibits a dilatory response, while diseased endothelium demonstrates an attenuated or even reversed (constrictive) response.

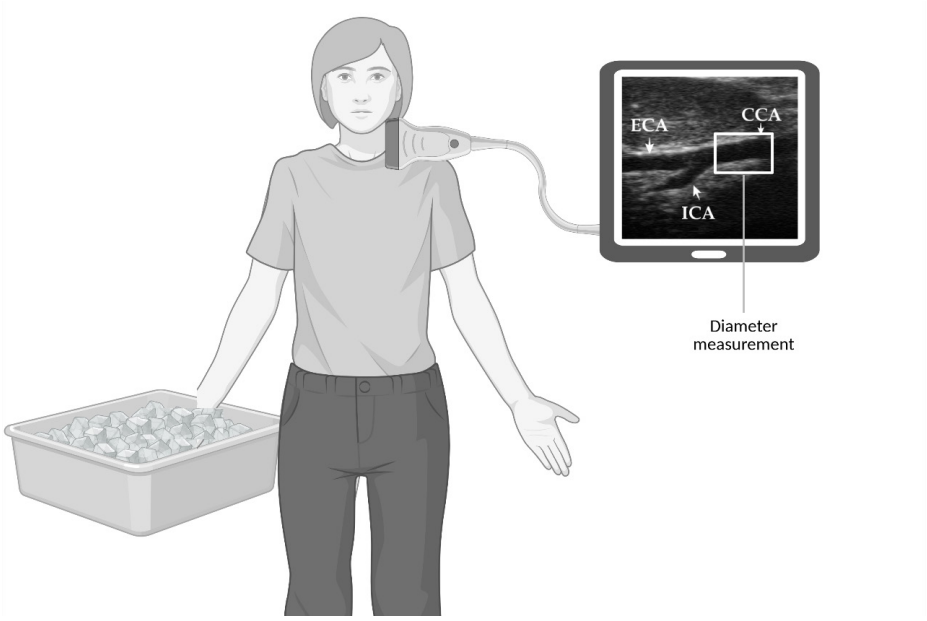

***Figure 3**: Assessing carotid artery diameter changes with handheld ultrasound, in response to a cold pressor test (hand in ice water) initiating a sympathetic stimulation [Created with BioRender.com]*

The CAR response is quantified as CAR%, representing the maximum relative change in carotid artery diameter from baseline. This response has been shown to independently predict the occurrence of major cardiovascular events within one year.[34]

Applicability of arterial diameter measurements during CAR testing has been limited by the use of expensive and static high-end ultrasound machines. Over the past decades, an increasing number of clinicians have started using handheld US devices.[36-37] Important advantages of handheld US devices include their lower costs in comparison with high-end US devices and their simplicity of use, which

makes handheld US applicable in outpatient clinics and general practices. The use of handheld ultrasound for endothelial dysfunction testing could facilitate the implementation of the assessment of endothelial dysfunction in every care facility across the world, creating the opportunity to identify patients with high cardiovascular risk in an early stage.

Secondary cardiovascular risk prevention in PAD

Peripheral artery disease (PAD) is associated with a substantial risk of arterial thrombotic events, including myocardial infarction, ischemic stroke, lower-extremity amputation, and cardiovascular death.[4,38-41] Additionally, PAD significantly impairs physical function, reduces quality of life, and leads to increased healthcare costs over the course of a person's lifetime.[42-43]

Secondary prevention is therefore essential in PAD and involves both non-pharmacological and pharmacological interventions. General cardiovascular risk management includes smoking cessation, adherence to a healthy diet, weight loss, and regular physical activity.[44-45] Pharmacological strategies also play a crucial role. Recommendations include lowering LDL cholesterol levels to below 1.8 mmol/L or achieving a reduction of more than 50%.[45] Additionally, blood pressure management with a target of <140/90 mmHg is advised.[46]

Antithrombotic drugs

In patients with peripheral artery disease (PAD), antithrombotic therapy is indicated for those who are symptomatic or have undergone revascularization. Current guidelines recommend single antiplatelet therapy (SAPT) as the first-line treatment for symptomatic PAD.[3,47] Acetylsalicylic acid (ASA) monotherapy has been extensively studied for this purpose.[48] The CAPRIE trial demonstrated that clopidogrel, a P2Y12 inhibitor, was more effective than ASA in reducing arterial thrombotic events while maintaining a similar safety profile, making clopidogrel a preferred option for symptomatic PAD.[3,49]

Alternative P2Y12 inhibitors, such as ticagrelor and prasugrel, are not approved by major international authorities (e.g., EMA and FDA) and are not currently recommended for secondary prevention in PAD. For patients who have undergone peripheral revascularization with endovascular stenting or infra-inguinal prosthetic bypass grafting, dual antiplatelet therapy (DAPT) with ASA and clopidogrel for at least one month is advised.[3,47] However, this recommendation is based on limited high-quality evidence, and the optimal duration of DAPT remains uncertain.[50] Following venous bypass surgery, the Dutch BOA trial found fewer graft occlusions

with oral anticoagulation, but this benefit came at the cost of a twofold increase in major bleeding risk.[51] Moreover, a subsequent trial – the WAVE study – was unable to confirm these benefits, as it showed no significant reduction in cardiovascular events with the addition of oral anticoagulation, while again observing an increased risk of major bleeding.[52]

Dual-pathway inhibition

DPI, combining ASA (80–100 mg once daily) with low-dose rivaroxaban (2.5 mg twice daily), has shown superior efficacy compared to ASA monotherapy in two large international trials. The COMPASS trial demonstrated that DPI significantly reduces major adverse cardiovascular and limb events in patients with symptomatic PAD[53] and in high-risk patients with coronary artery disease.[54] Although an increased risk of major bleeding was observed, the net clinical benefit of DPI was clear. The VOYAGER-PAD trial reinforced these findings by showing similar benefits in PAD patients following peripheral revascularization.[55]

Personalized antithrombotic drugs

Despite these advancements, questions remain regarding the comparative effectiveness of DPI versus clopidogrel monotherapy, as no randomized controlled trials have directly compared these approaches. Clopidogrel's efficacy depends on its activation by the CYP2C19 enzyme, and polymorphisms in CYP2C19 can influence treatment outcomes.[56] This suggests that clopidogrel's effectiveness could be enhanced through CYP2C19 genotype-guided therapy, potentially improving its potency.

In summary, while significant progress has been made, selecting the optimal antithrombotic therapy for PAD patients continues to involve uncertainties, highlighting the need for further research and personalized approaches.

Endothelial dysfunction in COVID-19

Although the primary focus of this thesis is on endothelial dysfunction in the context of PAD, a section on COVID-19 is included given the unique timing of this research during the COVID-19 pandemic. The global health crisis brought renewed attention to the endothelium, as COVID-19 has been closely linked to endothelial dysfunction and increased cardiovascular risk. Autopsy studies have revealed significant endothelial cell damage in individuals who succumbed to COVID-19.[57] Furthermore, markers of endothelial cell activation are markedly elevated in severe cases of COVID-19.[58] Severe COVID-19 is also strongly associated with a high incidence of thromboembolic complications.[59-61]

The risk of severe COVID-19 is notably higher in individuals with pre-existing cardiovascular disease or established cardiovascular risk factors such as advanced age, smoking, hypertension, dyslipidemia, and diabetes.[61-62] Interestingly, all of these factors—cardiovascular disease, older age, obesity, hypertension, and diabetes—not only correlate with worse COVID-19 outcomes but also predispose individuals to endothelial dysfunction.[10-12,19-20]

Goal thesis

Peripheral artery disease (PAD) is a highly prevalent manifestation of atherosclerosis, associated with significant morbidity, mortality, and healthcare costs. Endothelial dysfunction is one of the earliest indicators of atherosclerosis, often present years before clinical symptoms emerge. Antithrombotic drugs play a critical role in the secondary prevention of PAD following the onset of symptoms. However, the impact of antithrombotic therapies on endothelial function and their relationship with the development of clinical symptoms or complications remains poorly understood.

The aim of this thesis is therefore threefold:

1. **To explore the use of carotid artery reactivity (CAR) testing** as a simple, non-invasive method to assess endothelial function.
2. **To evaluate the effect of dual-pathway inhibition (DPI)** as a promising antithrombotic regimen on endothelial function.
3. **To provide an evidence-based overview of antithrombotic therapies** in PAD to guide the selection of optimal treatments for secondary prevention in clinical practice.

Outline thesis

The **first part** of this thesis investigates endothelial dysfunction and related biomarkers in patients recovered from acute COVID-19. Specifically, this section explores carotid artery reactivity (CAR) testing and circulating biomarkers of 1) endothelial function, 2) coagulation, and 3) vascular inflammation. *Chapter 2* examines the construct validity of handheld ultrasound devices for measuring carotid artery diameter. *Chapters 3 and 4* focus on CAR testing using handheld ultrasound in patients who have recovered from COVID-19, providing insights into endothelial function, coagulation activation, and vascular inflammation during the acute phase and at mid- (3 months) and long-term (18 months) follow-up. *Chapter 5* studies changes in circulating biomarkers of endothelial function, coagulation, and vascular inflammation following ChAdOx1 vaccination.

The **second part** of this thesis evaluates the effects of dual-pathway inhibition (DPI) on endothelial function and systemic inflammation in patients with PAD and coronary artery disease (CAD). *Chapters 6 and 7* assess the impact of DPI on microvascular and macrovascular endothelial function in symptomatic PAD patients. *Chapter 8* investigates the effects of DPI on systemic inflammation and immune cell responsiveness in PAD and high-risk CAD patients.

The **third part** of this thesis provides an overview of current and emerging evidence on antithrombotic therapy across different atherosclerotic conditions, including PAD, CAD, and stroke. *Chapter 9* uses network meta-analysis to compare the effectiveness and safety of antithrombotic regimens for secondary prevention in PAD. *Chapter 10* reviews the impact of CYP2C19 metabolizer status on cardiovascular outcomes in patients with PAD, CAD, or stroke. *Chapter 11* describes the study protocol for an ongoing randomized controlled trial (RCT) comparing a CYP2C19 genotype-guided antithrombotic treatment strategy to standard clopidogrel therapy in patients with PAD.

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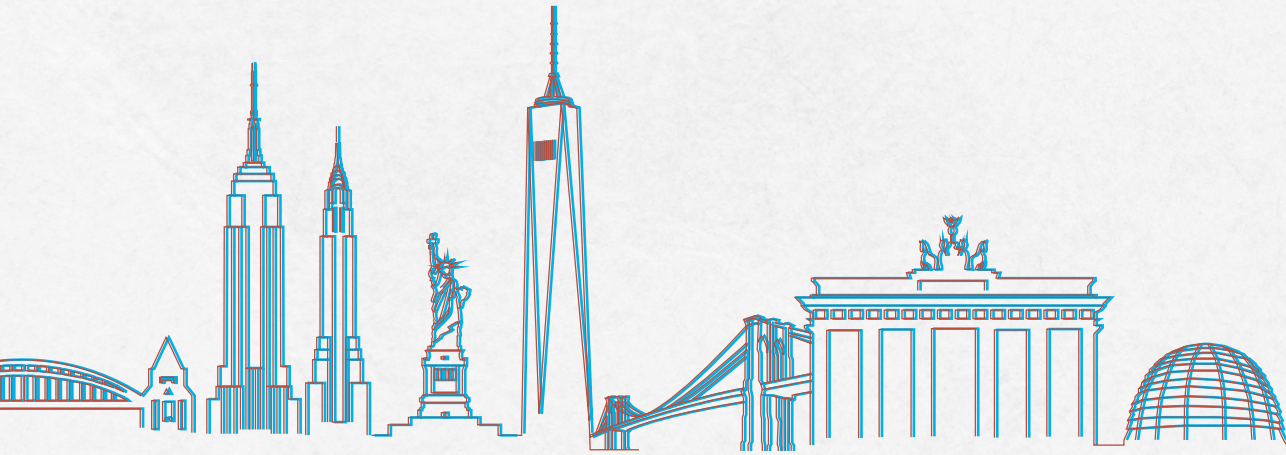
Part I

Effect of COVID-19 on endothelial dysfunction and the cardiovascular system



Chapter 2

Construct validity and reproducibility of handheld ultrasound devices in carotid artery diameter measurement

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Abstract

The construct validity and reproducibility of three commonly used handheld ultrasound (US) devices in measuring carotid arterial diameter was evaluated: Telemed MicrUs EXT-1H (Telemed, Vilnius, Lithuania), Butterfly iQ (Butterfly Network, Inc., Guilford, CT, USA) and Philips Lumify (Philips Healthcare, Best, The Netherlands). An *In vitro* setup was built to evaluate construct validity, compared with high-end US, and intra-observer variability of handheld US devices. Handheld devices showed a mean difference of 0.023 § 0.030 cm, 0.012 § 0.037 cm and 0.009 § 0.046 cm for, respectively, Telemed, Butterfly and Lumify in comparison with high-end US devices. Intraclass agreement with the high-end system as well as intra-observer variability for handheld US devices was classified as excellent, with all values greater than 0.95. Subsequently, inter-observer variability of handheld US devices was investigated in an *in vivo* setup with 20 healthy volunteers. Inter-observer variability was classified as excellent for Telemed (0.901), good for Lumify (0.827) and moderate for Butterfly (0.684) with a difference of, respectively, 0.005 § 0.031 cm, 0.020 § 0.050 cm and 0.003 § 0.033 cm. In conclusion, handheld US devices demonstrated an excellent construct validity and intra-observer variability. Additionally, excellent-to-good inter-observer variability for Telemed and Lumify was observed, and Butterfly demonstrated a moderate inter-observer agreement. These results indicate that handheld US devices are effective for measuring carotid arterial diameter.

Introduction

Endothelial dysfunction is one of the first signs of systemic atherosclerosis and contributes to its progression by promoting coagulation, vasoconstriction, and deficient vascular repair, ultimately leading to thickening of the arterial wall with narrowing of conduit arteries as result.[1,2] Measuring arterial diameter changes in response to physiological stimuli, such as shear stress (e.g., flow-mediated dilation) and sympathetic stimulation (e.g., carotid artery reactivity), using ultrasound (US) has emerged useful to assess endothelial dysfunction.[3-7]

Arterial diameter measurements during endothelial function testing currently depends on high-end US machines. High costs and the static nature of these machines prevent the applicability of these measurements at first- and second-line clinical centers. Over the past decades, an increasing number of clinicians have started using handheld US devices.[8,9] Important advantages of handheld US devices include their lower costs in comparison with high-end US devices and their simplicity of use, which makes handheld US applicable in outpatient clinics and general practices. Moreover, handheld US may facilitate the implementation of the assessment of artery diameters and diameter responses to physiological responses. To date, little is known about the validity and reproducibility of contemporary handheld US to examine arterial diameter.

The purpose of this study was to evaluate the construct validity and reproducibility of three commonly used handheld US devices (Telemed MicrUs EXT-1H (Telemed, Vilnius, Lithuania), Butterfly iQ (Butterfly Network, Inc., Guilford, CT, USA) and Philips Lumify (Philips Healthcare, Best, The Netherlands)) in measuring carotid arterial diameter. For this purpose, first, *In vitro* evaluation of handheld US devices in a phantom setup was performed to evaluate the construct validity of handheld US devices in comparison to a high-end US device. Subsequently, experiments were performed, comparing intra- and interobserver variability of the handheld US devices within respectively an *In vitro* and *in vivo* setup.

Materials and methods

Design

In the first part of this study, the construct validity of handheld US devices was evaluated using an *In vitro* setting to create a controlled environment with fixed parameters like acoustic (speed of sound, acoustic impedance and attenuation,

backscattering)[10] and mechanical (tissue elasticity and viscosity)[11] tissue properties for diameter detection of the US devices. In total, 28 measurements were performed per US device, which were compared against a contemporary high-end US machine. Measurements were repeated on a second day to evaluate intraobserver variability. In the second part of this study, repeated measurements of the carotid artery diameter were performed within twenty healthy individuals. The carotid artery was chosen for diameter assessment because the carotid artery is easily accessible by US and commonly used for the evaluation of atherosclerosis development.[12]

Handheld US devices

The following three commonly used handheld US devices were used to evaluate construct validity and intra- and interobserver reproducibility: 1) Telemed MicrUs EXT-1H (Telemed, Vilnius, Lithuania) with a linear array probe with a frequency range of 5-12 MHz, 2) Butterfly iQ (Butterfly Network, Inc., Guilford, Connecticut, United States) with a single probe emulating a linear and phased array probe by means of microsensors with a frequency range 1-10 MHz, and 3) Philips Lumify (Philips Healthcare, Best, The Netherlands) with a linear array probe with a frequency range of 4-12 MHz. To evaluate construct validity using the *In vitro* setting, handheld US machines were compared against a high-end US system with a linear array probe with a frequency range of 5-14 MHz (Terason 3300, Terason Ultrasound, Burlington, MA, USA).

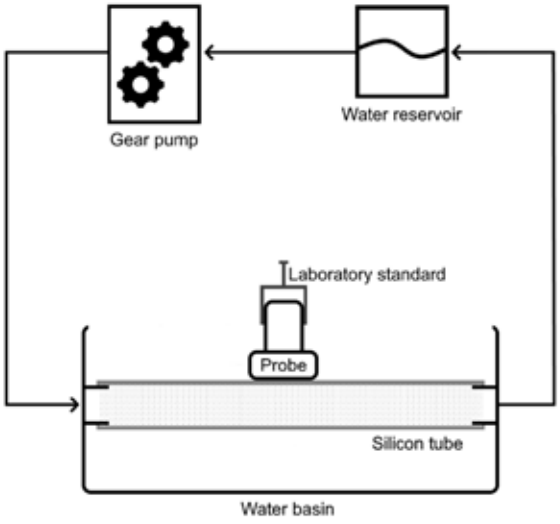

***Figure 1**: Schematic overview of experimental setup of the in vitro experiment, where water from the water reservoir was pumped around by the gear pump through the silicon tube, which was placed in a water basin. The probe of each ultrasound device was mounted in the laboratory standard and positioned above the silicon tube such that a longitudinal plane was visualized.*

In vitro: construct validity and intra-observer variability

Experimental setup

An experimental setup was built to perform US measurements on a custom-made flexible polyvinyl alcohol phantom mimicking an artery; *figure 1* shows a schematic overview. The phantom artery was positioned in an US compatible box (water basin) and connected to an in-house built circulatory system with physiological flow and pressure conditions.[13] Different flow volumes were applied to simulate different phantom diameters.

Measurement protocol

The gear pump, connected to the phantom artery circulation, was set at a continuous flow of 0.3 L/min. The US transducer was longitudinally aligned with the phantom artery and this position was maintained by use of a laboratory standard. Basic carotid ultrasonography presets were used. Gain and depth were adapted when considered necessary. Consensus of the optimal position and settings was reached by two skilled sonographers (JV, LW) and was kept the same for each device. The phantom artery was recorded during a 10 second interval. Thereafter, the flow was increased with 0.1 L/min, corresponding with an approximately 1 millimeter increase in diameter per minute, and the phantom artery was recorded again. These steps were repeated to a flow of 0.9 L/min. Subsequently, the pressure regulator was set on a pulsatile flow of 0.3 – 0.9 L/min, with 60 pulses/minute, with the phantom artery being recorded for 10 seconds periods. These procedures were repeated for all devices.

Measurements were repeated on a second day, which was performed within 30 days, to determine the intraobserver variability. We ensured that all procedures were kept similar, including the order of testing.

In vivo: interobserver variability

Participants

A total of 20 volunteers were recruited. Inclusion criteria were age between 18 and 65 years and a body mass index of 18 - 30 kg/m2. No participants with previously diagnosed carotid artery occlusive disease were included. Written informed consent was obtained prior to participation from all volunteers. Approval of the local Medical Ethical Committee (study number: CMO 2020-6700) and the local Institutional Review Board was obtained. This study was conducted in accordance with the latest revision of the Helsinki Declaration of 1964.

Procedures

Data on sex, age, height, weight, smoking behavior, medical history, and the familial occurrence of cardiovascular diseases were collected. Participants visited the hospital once. During the visit, US measurements of the common carotid artery were performed. Participants were in supine position with the neck extended and had rested at least 5 minutes before the start of US measurements. Room temperature was kept constant and only one type of US gel was used. The left common carotid artery was longitudinally visualized using the three handheld US devices and one high-end US device, which were applied in randomized order. After image optimalization by the examiner (JV, LW), the carotid artery diameter was recorded for 10 seconds. Subsequently, the probe was removed from the participant and handed over to the second experienced examiner without adjusting US settings. This was followed by repositioning the transducer at the artery. Subsequently followed the recording of the carotid artery diameter for another 10 seconds. The order of the two examiners was also randomized.

Diameter analysis

Dependent on US device, data were saved as or converted to an Audio Video Interleave (AVI) file. US videos of the Butterfly device were converted using Movavi Video Converter 20 (Movavi Software, Wildwood, MO, USA) using the original size (resolution 1696 x 1080) and MPEG-4 codec. Additionally, US videos of the Lumify device were converted using MatLab R2018b (The Mathworks, Natick, MA, USA) using the VideoWriter function with quality index 90. This resulted in a video resolution varying from 512 x 296 to 512 x 444 depending on the depth setting during the measurement. For the Terason ultrasound videos, Camtasia (Camtasia Softonic, Barcelona, Spain) was used to record the screen containing ultrasound images. This was saved as an AVI file with a resolution of 1024 x 768. The Telemed ultrasound video was directly saved as AVI file with a resolution of 1556 x 868. This corresponds to an axial resolution of 28, 68, 39 and 35 micron for the Butterfly, Lumify, Terason and Telemed device, respectively.

Diameter analysis of the recorded US videos of the phantom and carotid arteries was performed by a single-blinded investigator using BloodFlow Software (Version 4.0; National Instruments LabVIEW, Austin, TX, USA) with a semiautomated edge-detection and wall-tracking algorithm. This software enables the identification of a region of interest (ROI) in the longitudinal plane of an artery. ROIs were identified for each US video. Within the ROI, the lumen-arterial wall interface was detected (*figure 2*). The diameter was determined multiple times per frame depending on the size of the ROI. Subsequently, a median diameter per frame was determined

and eventually a median diameter of all frames was determined for the resulting diameter per measurement. For the resulting diameter, full cardiac cycles were included to minimize bias of the average diameter. More details on this technique have been described previously.[14] The software is largely independent of investigator bias.[15]

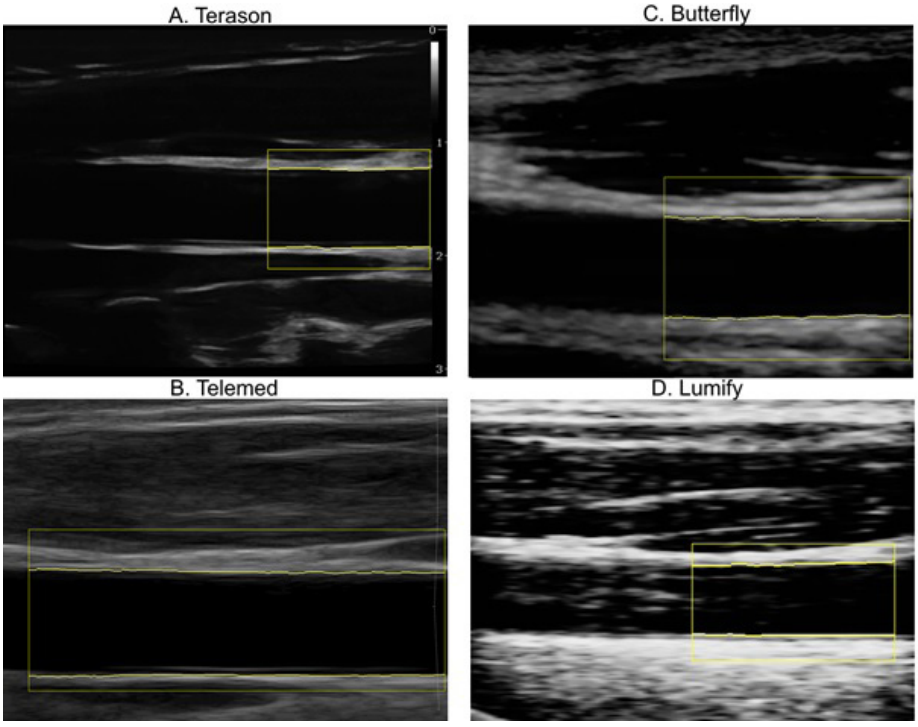

***Figure 2**: The detected borders of the lumen-arterial wall interface in participants for A) Terason, B) Telemed, C) Butterfly and D) Lumify device, where the yellow square represents the drawn region of interests and the yellow lines represent the detected border.*

Statistical analysis

Phantom and carotid artery diameters were reported as the mean ± standard deviation (SD) for each measurement. Baseline characteristics of the participants were reported as the median with interquartile range [Q1, Q3], and categorical variables are presented as percentages. Bland-Altman plots were created to determine the agreement of measured diameters between the handheld devices and the high-end US device and to determine the intra- and interobserver variability of the three handheld US devices for *In vitro* and *in vivo* measurements. Differences were plotted against the mean per comparison. Bland-Altman plots are visualized with one solid black line representing the mean and two dotted lines representing

the limits of agreement (1.96 * standard deviation).[16] Variability of measurements was assessed using intra- and interobserver variability by determining the intraclass correlation coefficient (ICC), which is presented for the between-day comparison for the *In vitro* setup and between-observers comparison for the *in vivo* setup, respectively. ICC were reported according to the guideline of Koo and Li (2016)17, in which a coefficient <0.50, between 0.50 and 0.75, between 0.75 and 0.90 and >0.90 represents respectively poor, moderate, good and excellent agreement. Additionally, coefficients of variation were calculated per participant, per device and between observers by using the ratio of the standard deviation and the mean absolute differences between observers. After Bonferroni correction, p-values <0.01 were considered significant. Statistical analysis was performed using SPSS Statistics version 25 (IBM Corporation, Armonk, NY, USA).

Results

In vitro: construct validity and intra-observer variability

The Bland-Altman plots for variability in *In vitro* measurements between handheld devices and the high-end US device are shown in *figure 3*. Compared with the high-end US device, the Telemed demonstrated a significantly larger diameter (0.023 ± 0.030 cm, $p < 0.001$, *able 1*), while no such difference was reported for the Butterfly (0.012 ± 0.037 cm) or Lumify (0.009 ± 0.046 cm). Visually inspecting the Bland-Altman plots, we found comparable limits of agreement across a large range of diameters between the three handheld US devices. The ICC comparing the handheld US and high-end US was 0.996, 0.994 and 0.990 for Telemed, Butterfly and Lumify, respectively.

No significant difference was found between measurement days for the Telemed (0.013 ± 0.059cm) and Butterfly (-0.012 ± 0.048cm), while a small but significant difference was found for the Lumify (0.008 ± 0.009cm, $p = 0.008$, *table 1*). Bland-Altman plots (*figure 4*) reveal comparable limits of agreement across the three handheld US devices. The ICC comparing both measurements per handheld US device was 0.986, 0.990 and 1.000 for Telemed, Butterfly and Lumify, respectively.

In vivo: interobserver variability

The median age of the participants was 21.0 years [20.0, 22.0] and 40.0% were male. Additionally, the median body mass index was 21.7 [20.4, 23.6], 10% were current smokers and 45% had a family history of cardiovascular disease. Bland-Altman plots for *in vivo* measurements comparing the interobserver variability of the handheld

US devices are shown in *figure 5*. No significant difference in carotid artery diameter was found between operators for the Telemed (0.005 ± 0.031 cm), Butterfly (0.020 ± 0.050 cm) or Lumify (-0.003 ± 0.033 cm, *table 1, figure 5*). Limits of agreement were smallest for the Lumify, with similar patterns and limits observed for the Telemed and Butterfly. The ICC for carotid artery diameter between the operators per device was classified as excellent for the Telemed (0.901), good for Lumify (0.827), and moderate for the Butterfly (0.684). Average coefficients of variation per participant, per device between observers were 2.4% ± 2.5%, 2.2% ± 2.0% and 5.2% ± 2.9% for Telemed, Lumify and Butterfly, respectively.

Discussion

This study has demonstrated that the three studied handheld devices show a good construct validity and strong ICC compared with high-end US and excellent between-day intraobserver variability using an *In vitro* setting for measuring arterial diameters. Between-observer reproducibility of the handheld US devices within the *in vivo* setting revealed an excellent-to-good interobserver variability for the Telemed and Lumify, but a moderate variability for the Butterfly.

Good consistency and excellent reliability were observed between handheld and high-end US devices in an *In vitro* setting, as all ICCs were well above 0.95. Nonetheless, a significant difference between Telemed and the high-end US device was found, which may suggest limited validity of the Telemed. One possible reason for this difference is (not) taking the intima-media thickness into account when analyzing the diameter. Such consistent difference in determining the diameter may result in structural difference between US devices. An example of this can be seen in *figure 2*, where the Lumify analysis detects the intima and the other devices detect the outer wall. Furthermore, it is important to realize that Telemed demonstrated the smallest SD. Taken this together, all three handheld US devices showed excellent construct validity.

Although *In vitro* setups are commonly used to determine validity of US devices, few studies have focused on understanding (construct) validity using an *In vitro* setup for handheld US devices. Two studies were found comparing US devices. One study investigated carotid strain assessment applying US speckle tracking using a clinical and high-end US device[18], where the other study investigated optic nerve sheath diameters using a pocket-sized US unit compared to a previously validated portable unit.[19] The study of Larsson et al.[18] showed an ICC of, respectively, 0.73 for the clinical

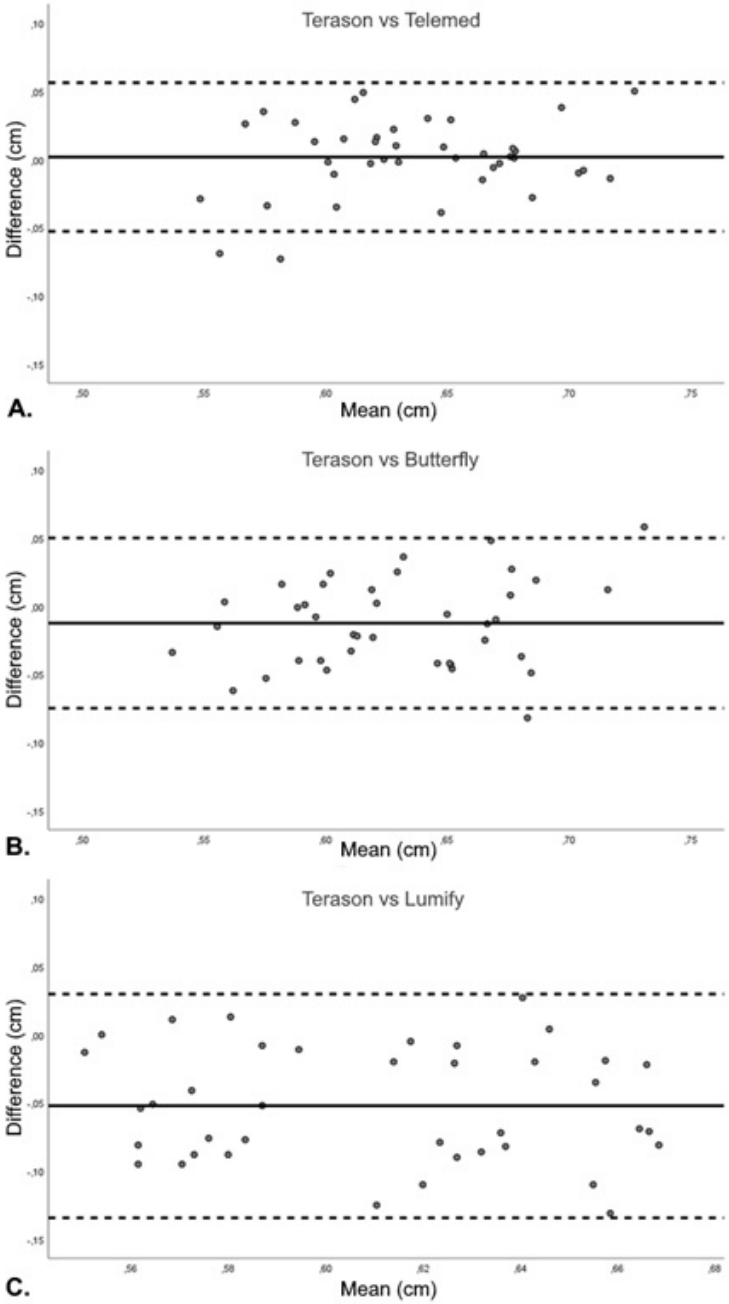

***Figure 3**: Bland-Altman plots that compare assessment of the phantom diameters of the A) Telemed, B) Butterfly and C) Lumify handheld ultrasound (US) devices against the high-end US device (Terason), where the solid black line represents the mean difference and the dotted black lines represent the limits of agreement per comparison.*

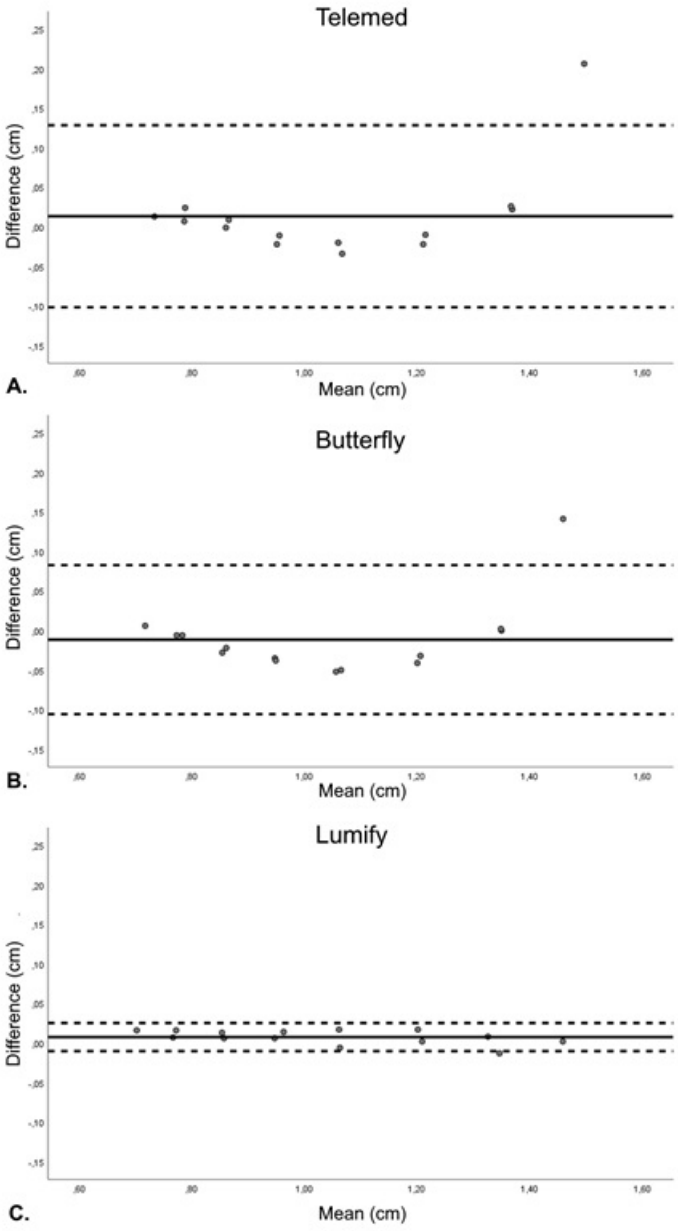

***Figure 4**: Comparison of the between-day variation of the in vitro measurement of diameter for the A) Telemed, B) Butterfly and C) Lumify devices, where the solid black line represents the mean difference and the dotted black lines represent the limits of agreement per comparison.*

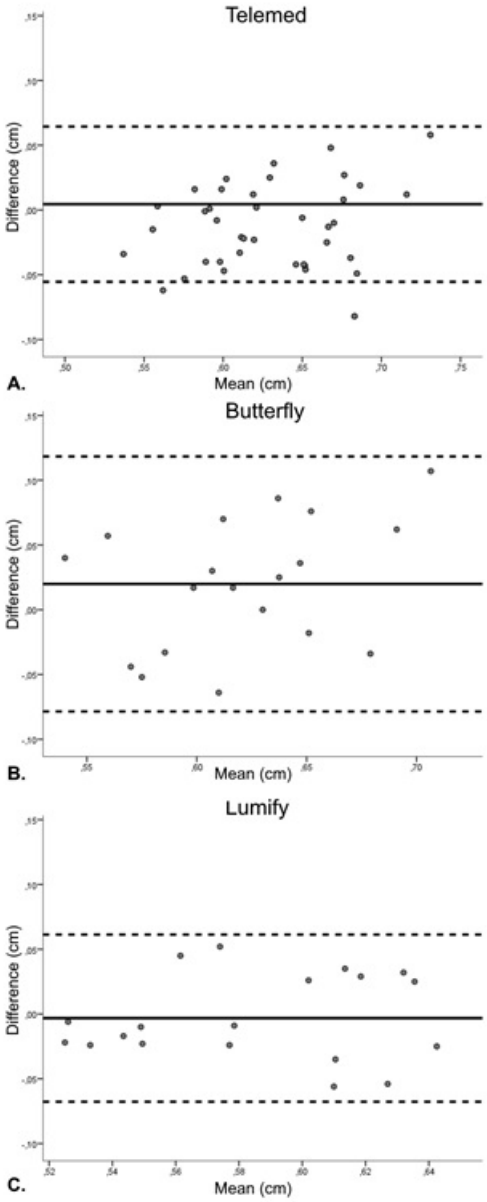

***Figure 5**: Bland-Altman plots of in vivo measurements of the carotid diameter comparing both operators using the A) Telemed, B) Butterfly and C) Lumify, where the solid black line represents the mean difference and the dotted black lines represent the limits of agreement per comparison.*

US device and 0.90 for the high-frequency US device. Johnson et al.[19] demonstrated an ICC of 0.75 for between-device comparison (pocket size versus previously validated portable unit) and 0.83 for interobserver variability of the pocket-sized US device. These values seem slightly lower than the results presented in our study. Importantly, these previous studies focused on other outcome measures. Other studies that evaluated the validity of handheld US directly compared handheld US devices with each other[8,20-21] or adopted other imaging modalities[22] using patients. A strength of our study is therefore that the handheld US devices were both tested in an *In vitro* setup and afterwards evaluated *in vivo* in volunteers.

In line with our results, other studies reporting on vascular US have positively addressed the use of handheld US devices (e.g. Acuson P10[23], Vscan[24] and Butterfly[25]). Importantly, US devices were tested in relation to varying pathological screening areas (e.g. size of liver, spleen and kidneys[23], carotid artery plaques[24] and abdominal aorta[25]). At the least, these studies provide further support that handheld US devices are feasible and reliable, with an ICC of 0.8 with high-end systems.[23-25] However, the validity and reproducibility must be considered within its specific use, which was related to the carotid artery diameter in our study.

In contrast to the interobserver variability of the Telemed and Lumify, we found a moderate variability for the Butterfly device. This latter observation may, at least in part, be explained by the US transducer specifications of the Butterfly. While Telemed and Lumify utilize a classic linear array probe, the butterfly probe is shaped differently and emulates a linear array probe by means of microsensors. The relatively small probe head of the Butterfly device allows for more variability in probe positioning, possibly resulting in some inter-operator variability. Evaluation of arterial diameter is influenced by probe positioning (more proximal or distal), but also artery shape, blood pressure variation, and tissue properties.[26-28] Therefore, inter-operator variability *in vivo* can be multifactorial and does not necessarily indicate lack of quality of the US device. Accordingly, it is important to highlight that the Butterfly device has already proven to have a good interobserver variability in assessing carotid artery plaque assessment.[25] This highlights the importance of (construct) validity studies for the large range of handheld US devices, as device specifications may importantly determine the potential (clinical) application of a specific US device.

A limitation of this study relates to analyzing standard B-mode images instead of using raw radiofrequency data. The latter has a higher spatial resolution and might be preferred as the golden standard. Previous studies, however, have shown

standard B-mode images to be robust for measuring arterial characteristics with good precision and accuracy.[29,30] Using standard B-mode based analysis made it possible to make the analysis comparable and consistent between the three handheld US devices. However, to optimize the US videos for analysis, ultrasound settings were adjusted between devices or participants, but kept constant between operators. Nevertheless, this could have had an impact on the final results.

B-mode images obtained from the various US machines had differences in format and quality. Some US videos had to be converted to AVI files, which may have caused loss of quality of the US videos (specifically affecting Lumify and Butterfly). The use of a reliable converter software and converting packages effectively minimized loss of quality, which was further supported by visual inspection of the US videos after conversion. Our software has proven to be reliable and largely independent of investigator bias.[15] Woodman et al.[15] described the method of analysis as well as some coefficients of variations for different determined parameters with the software, with the largest coefficient of variation being 6.7%. However, we cannot fully exclude a bias caused by different types of videos obtained with the different US machines. Nevertheless, because the quality of US devices has also improved over the past two decades just as converting software has, the impact of this quality (e.g. image resolution and video compression) on analysis with this software is expected to be minimized. Importantly, despite this possible bias, all devices showed excellent construct validity compared to the high-end US device and excellent between-day reproducibility. Another limitation could be the small sample size for Bland-Altman analysis.[31] Due to the explorative character of the *in vivo* part of this study, no sample size calculation was performed.

Conclusion

All handheld devices showed an excellent construct validity and intraobserver variability *In vitro* and are therefore suitable to analyze carotid artery diameter. Interobserver variability *in vivo* of the handheld devices was excellent-to-good for Telemed and Lumify, and Butterfly showed a moderate variability. Although analysis software has proven to be reliable, Butterfly and Lumify did not provide compatible US video, which could have caused minor variation between the handheld devices. Nevertheless, this study demonstrated that handheld US devices, especially Telemed and Lumify, are effective in measuring carotid arterial diameter.

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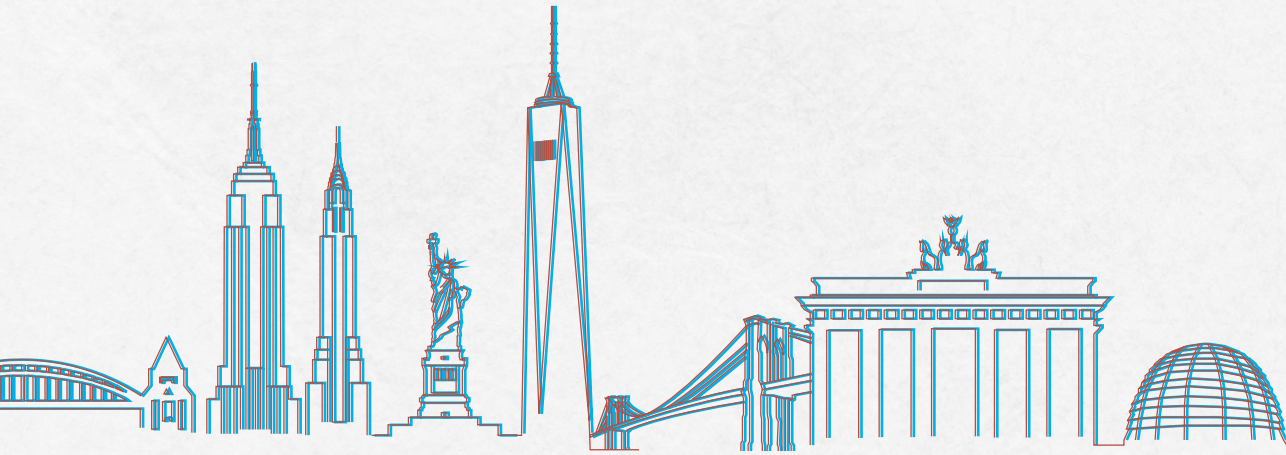

Chapter 3

Sustained inflammation, coagulation activation and elevated endothelin-1 levels without macrovascular dysfunction at 3 months after COVID-19

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Abstract

Introduction: Endothelial damage and thrombosis caused by COVID-19 may imperil cardiovascular health. More than a year since the WHO declared COVID-19 pandemic, information on its effects beyond the acute phase is lacking. We investigate endothelial dysfunction, coagulation and inflammation, 3 months post-COVID-19.

Materials and Methods: A cohort study was conducted including 203 patients with prior COVID-19. Macrovascular dysfunction was assessed by measuring the carotid artery diameter in response to hand immersion in ice-water. A historic cohort of 312 subjects served as controls. Propensity score matching corrected for baseline differences. Plasma concentrations of endothelin-1 were measured in patients post-COVID-19, during the acute phase, and in matched controls. Coagulation enzyme:inhibitor complexes and inflammatory cytokines were studied.

Results and conclusions: The prevalence of macrovascular dysfunction did not differ between the COVID-19 (18.6%) and the historic cohort (22.5%, RD -4%, 95%CI: -15–7, p=0.49). Endothelin-1 levels were significantly higher in acute COVID-19 (1.67±0.64 pg/mL) as compared to controls (1.24±0.37, p<0.001), and further elevated 3 months post-COVID-19 (2.74±1.81, p<0.001). Thrombin:antithrombin(AT) was high in 48.3%. Markers of contact activation were increased in 16-30%. FVIIa:AT (35%) and Von Willebrand Factor:antigen (80.8%) were elevated. Inflammatory cytokine levels were high in a majority: interleukin(IL)-18 (73.9%), IL-6 (47.7%), and IL-1ra (48.9%). At 3 months after acute COVID-19 there was no indication of macrovascular dysfunction; there was evidence, however, of sustained endothelial cell involvement, coagulation activity and inflammation. Our data highlight the importance of further studies on SARS-CoV-2 related vascular inflammation and thrombosis, as well as longer follow-up in recovered patients.

Introduction

Coronavirus disease 2019 (COVID-19) results from infection with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), preferentially affecting the upper airways and pulmonary system.[1] Depending on the severity of infection, dissemination towards multiple other organs occurs and systemic COVID-19 is associated with a high incidence of thromboembolic complications and risk of multi-organ failure.[2-4] Endothelial vascular injury and thrombo-inflammation are emerging key factors in COVID-19 pathophysiology.[5-8] Pronounced endothelial cell damage was found in COVID-19 autopsy studies[5], and markers of endothelial cell activation are significantly increased in severe COVID-19[9] and associated with organ damage, including liver, heart and brain[10]. This endothelial cell activation and dysfunction may be aggravated by the interplay of thrombo-inflammatory mediators and cells, including neutrophils that trigger contact and complement activation, perturbing the barrier function and contributing to thrombosis and cardiovascular disease.[11]

Severe COVID-19 occurs more often in subjects with established cardiovascular disease or in those with cardiovascular risk factors.[4,12] Notably, all factors associated with worse disease outcome, such as cardiovascular disease, higher age, obesity, hypertension and diabetes, also predispose to endothelial dysfunction.[13-17] In different cohorts of cardiovascular patients without COVID-19, the presence of endothelial dysfunction is strongly related to the occurrence of cardiovascular events, including stroke, myocardial infarction and limb events.[18-20]

Although it has been more than a year since the WHO declared COVID-19 a global pandemic, there is a paucity of information related to the effects of COVID-19 on the cardiovascular system beyond the acute phase.[21] Moreover, the SARS-Cov-2 pandemic represents a tremendous health problem including the frequent occurrence of often unexplained prolonged complaints and morbidity long after the acute infection (so-called "long COVID"[22]). We hypothesize that sustained inflammation, coagulation activation and endothelial cell activation may ultimately lead to macrovascular dysfunction with an increased risk for developing cardiovascular complications later on.[11]

Therefore, we firstly investigated whether macrovascular dysfunction could be observed 3 months after recovery from acute COVID-19. Furthermore, we determined whether signs of endothelial cell activation[9,23-25], coagulation system activation[26], and circulating inflammatory cytokines[9,27-28], as observed during the acute phase, are present 3 months after recovery from acute COVID-19 symptoms.

Materials and Methods

Study design

COVAS was a cross-sectional observational cohort study, initiated at Radboudumc (Nijmegen, the Netherlands) and conducted at Bernhoven hospital (Uden, the Netherlands). The study was approved by the regional ethics committee Arnhem-Nijmegen (reference number NL74101.091.20), and local approval has been obtained of the local directory boards. This study was conducted in accordance with the latest revision of the Declaration of Helsinki. Data available on request from the authors.

Participants

Patients who had experienced SARS-CoV-2 infection, confirmed by polymerase chain reaction on nasopharyngeal swab, sputum or bronchoalveolar lavage, were recruited. Patients had to be aged 16 years or older and recovered from acute COVID-19 symptoms for at least 6 and no more than 20 weeks. Exclusion criteria were recent (<3 months) episode of angina pectoris, myocardial infarction, stroke or heart failure, and abnormalities of the upper extremities restricting cold pressor testing. Abnormalities of the upper extremities included severe bilateral Raynaud syndrome, scleroderma, complex regional pain syndrome, or presence of arteriovenous fistula or open wounds. Written informed consent was obtained from all participants.

Procedures

Data about cardiovascular risk factors, comorbidities, medication use, and severity of acute COVID-19 infection were retrieved from electronic patient files. Case report forms were used to obtain information about lifestyle and COVID-19 symptoms.

Measurement of macrovascular dysfunction: carotid artery reactivity test

Eligible patients visited the hospital once. During the visit, the carotid artery reactivity (CAR) test[29-31] was performed. The CAR test is a simple, non-invasive procedure to examine macrovascular function by measuring the carotid artery diameter in reaction to a cold pressor test (sympathetic stimulus).[29-31] Participants rest in the supine position with the neck extended. The left carotid artery is visualized using L12-4 MHz linear array probe of Philips Lumify, ultrasound device. The carotid artery diameter is measured with custom-designed edge-detection and wall-tracking software during baseline (30 seconds) and during hand-immersion in icy water of 4°C (sympathetic stimulus) for 3 minutes.

A historic control cohort was created from all studies initiated by Radboudumc that completed data collection before November 2019, i.e. before the pandemic

and therefore not affected by COVID-19. In these studies, the CAR test had been used to determine macrovascular function and data on risk factors for endothelial dysfunction were collected. Prespecified risk factors for endothelial dysfunction were age[13], sex[32], body mass index (BMI)[14], smoking status[33], atherosclerotic cardiovascular disease[15], hypertension[16], hyperlipidaemia[34] and diabetes mellitus[17]. Subjects were either healthy volunteers, patients with cardiovascular risk factors or patients with symptomatic cardiovascular disease. Incomplete case files were excluded from final analyses. Propensity score matching was used to select individuals, based on baseline characteristics and cardiovascular risk profile, to match those with COVID-19.

Measurement of endothelin-1, coagulation and inflammatory cytokines:

Whole blood of recovered COVID-19 patients was collected by venipuncture in Lithium-Heparin (Vacuette) tubes. Platelet poor plasma was prepared by centrifuging whole blood at 2500g for 10 min followed by a second centrifugation step at 2500g for 20 min, both at room temperature. Subsequently, platelet poor plasma was snap frozen and stored at -80°C until use. Plasma concentrations of endothelin-1 (ET-1), interleukin (IL)-18, IL-6, and IL-1ra were quantified using commercial ELISA kits (R&D, Minneapolis, Minnesota. Catalogue numbers DET100, DL180, D6050 and DRA00B, respectively). Activated coagulation factors in complex with their natural inhibitors, including thrombin:antithrombin (TAT), factor(F)IXa:AT, FVIIa:AT, FXIa:AT, FXIa:alpha-1-antitrypsin (α1AT), FXIa:C1-esterase-inhibitor (C1inh) and kallikrein:C1inh (PKa:C1Inh), as well as von Willebrand factor antigen (VWF:Ag) levels, were quantified by in-house developed enzyme-linked immunosorbent assay (ELISA) methods as described previously[35]. The use of lithium-heparin plasma for the mentioned biomarkers was validated by comparing citrate with lithium-heparin plasma from 38 healthy volunteers. ET-1 was also measured in samples of patients who consented with blood plasma preservation during the acute phase of COVID-19 (subjects of the ongoing BioMarCo-19 study, (M de Groot, unpublished data, 2021, https://www.clinicaltrialsregister.eu/ctr-search/trial/2020-001325-31/NL) and in a matched control group of patients without a history of COVID-19 infection. Plasma concentrations below the lower detection limit were set on the lowest detectable value.

Endpoints

The primary endpoint was the prevalence of macrovascular dysfunction, defined as a constrictive response to the CAR test in the COVAS cohort compared to the historic control cohort.[29] The CAR response was either a dilatory or a constrictive reaction, dichotomized by an area under the curve of at least zero or below zero,

respectively. The secondary endpoint was the plasma concentration of ET-1 in the COVAS cohort compared to the acute phase and matched controls.

Statistical analysis

This study was designed to detect a 2.5-fold excess risk of macrovascular dysfunction in the COVAS cohort compared to the matched historic control cohort. With a power of 0.8 and an alpha of 5%, 97 patients with COVID-19 infection were required. Assuming an acceptable match rate of 50%, the final sample size was set on 200 participants.

For the CAR test, propensity score matching (PSM) was applied to correct for systematic differences in baseline characteristics that could influence endothelial function (i.e. age, sex, BMI, smoking status, atherosclerotic cardiovascular disease, hypertension, hyperlipidemia and diabetes) between post-COVID-19 patients and historic controls. The propensity score is defined as the chance to be in the COVAS cohort, based on baseline characteristics. Propensity score was estimated using logistic regression analysis with the group (COVAS or historic control) as dependent variable in relation to baseline characteristics. The balance in covariates was evaluated using standardized mean differences (SMD), where a SMD of 0.1 indicates a negligible correlation.[36] After PSM, univariable logistic regression analysis was performed to estimate the difference in CAR response and value between the historic control cohort and the COVAS cohort.

ET-1 levels of the COVAS cohort were compared to matched controls using the Mann-Whitney U test. Sub-analyses were performed on patients with preserved blood plasma during the acute phase of infection to compare ET-1 concentrations of the acute phase with ET-1 concentrations post-COVID-19.

Blood plasma markers of coagulation activation and inflammatory cytokines were presented as mean ± standard deviation (SD) and the proportion of patients with concentrations above normal range. Normal ranges of in-house developed ELISA methods were defined as above normal mean + 1 SD (based on previous validation studies[35]). The Mann-Whitney U test was used to compare post-COVID-19 values of patients that stayed home during the acute phase of infection to patients that needed hospitalization.

Correlations between endothelial dysfunction, markers of coagulation and inflammatory cytokines were explored using Spearman's rank correlation coefficient. Analyses were performed using R 4.0.3 and IBM SPSS Statistics 25. P values below 0.05 were considered significant.

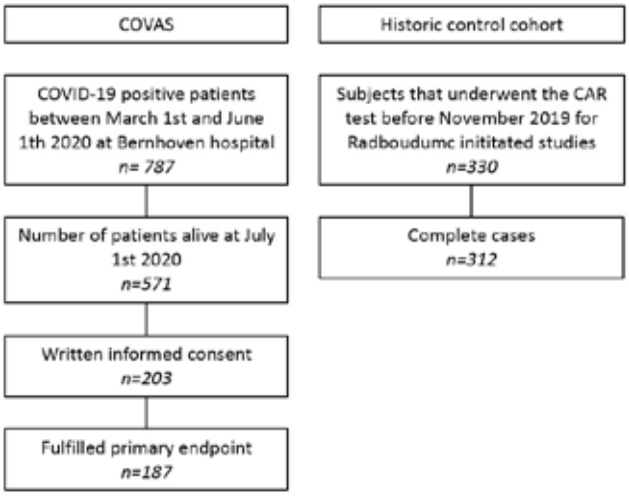

***Figure 1**: Flow-chart showing the composition of the post-COVID-19 cohort and the historic control cohort*

Results

Post-COVID-19 patients: In total, 787 patients were diagnosed with COVID-19 between February 1st and June 1st 2020 within the Bernhoven hospital, of which 571 patients survived (72.5%). Ultimately, 203 eligible patients gave written informed consent.

Historic controls: For the control cohort, 330 participants of 5 different studies underwent the CAR test before November 2019 and were previously assessed for the prespecified risk factors for endothelial dysfunction. Complete case files were obtained of 312 participants (*figure 1*).

The mean age at COVID-19 diagnosis was 62.7 years and 63.5% of the patients were male (*table 1*). The most common comorbidities were hypertension (43.3%), hyperlipidemia (26.6%) and coronary artery disease (17.7%). Thrombocytopenia (<150*10^9/l) was present in 16.6% of the patients. Four patients had asymptomatic disease and were diagnosed with COVID-19 during a visit to the emergency ward for other medical reasons. For patients with symptomatic COVID-19, the median duration of COVID-19 related symptoms was 18 days (range 1-80 days). Fatigue, dyspnea and fever above 39 degrees were the most frequently experienced symptoms, 94.1%, 77.3% and 70.9%, respectively. Hospitalization was needed for 130 (64.0%) patients and 28 (21.5%) of these patients were transferred to the intensive care unit.

Table 1: *Baseline characteristics and COVID-19 infection course of patients included in the COVAS cohort and of all patients that were diagnosed with COVID-19 between February 1st and June 1st 2020 within the Bernhoven hospital*

		COVAS selection *(n=203)*	**COVID-19 cohort** *(n=787)*		
			All *(n=787)*	**Non-survivors** *(n=217)*	**Survivors** *(n=570)*
Patient characteristics	Age, mean (SD)	62.7 (12.4)	70.0 (14.2)	78.4 (8.8)	66.8 (14.5)
	Male sex, No (%)	129 (63.5)	470 (59.7)	148 (68.2)	322 (56.5)
	BMI, mean (SD)	28.3 (4.3)	28.52 (5.2)	28.35 (5.0)	28.58 (5.2)
	History of smoking, No (%)	137 (67.5)	371 (56.3)	86 (53.8)	285 (57.1)
			Missing 128	Missing 57	Missing 71
Comorbidities	Missing		12 (1.5)	7 (3.2)	5 (0.9)
	Hypertension, No (%)	88 (43.3)	377 (48.6)	121 (57.6)	256 (45.3)
	Hyperlipidemia, No (%)	54 (26.6)	149 (19.2)	44 (21.0)	105 (18.6)
	Coronary artery disease, No (%)	36 (17.7)	147 (19.0)	55 (26.2)	92 (16.3)
	Stroke/TIA, No (%)	23 (11.3)	111 (14.3)	43 (20.5)	68 (12.0)
	Peripheral arterial disease, No (%)	6 (3.0)	70 (9.0)	33 (15.7)	37 (6.5)
	Diabetes Mellitus, No (%)	32 (15.8)	183 (23.6)	58 (27.6)	125 (22.1)
Disease severity	Days of illness, median [range]	18 [1-80]			
	Hospital care *(n=121)*	130 (64.0)	516 (65.6)	176 (81.1)	340 (59.6)
	Days, median [range]	7 [1-61]	11 (0-103)	8 (0-60)	12 (0-103)
	Intensive care *(n=27)*	28 (13.8)	117 (14.9)	41 (18.9)	76 (13.4)
	Days, median [range]	16 [1-42]	19 (0-82)	15 (0-56)	21 (1-82)
	Days recovered, median [range]	114 [50-157]			

*SD = standard deviation, TIA = transient ischemic attack.*Patient characteristics and infection course

Recovery from COVID-19 infection does not result in macrovascular dysfunction

The prevalence of the prespecified risk factors for endothelial dysfunction in the historic control cohort and the COVAS cohort are shown in *figure 2*. Besides age, history of smoking and history of stroke, all risk factors substantially differed between the historic control cohort and the COVAS cohort, with the most noticeable differences seen in the rate of male participants (42.9% vs 62.6%), the prevalence of hyperlipidemia (49.0% vs 27.3%) and the prevalence of peripheral arterial disease (58.7% vs 2.7%). Macrovascular dysfunction, as measured by carotid artery reactivity

testing, was more prevalent in the historic control cohort compared to the COVAS cohort, 32.7% vs 16.6% (RD -16.1%, 95%CI: -23.4 to -8.5, p<0.001). PSM was used to account for differences between the historic control cohort and the COVAS cohort and resulted in a mean SMD below 0.1 for most variables and below 0.15 for all variables. After PSM, the prevalence of macrovascular dysfunction was not different between the cohorts, 22.5% vs 18.6% (RD -4%, 95%CI: -15 to 7, p=0.49), respectively (*figure 2*).

	Raw data			Propensity score matching		
	Historic *(n=312)*	COVAS *(n=187)*	SMD	Historic *(n=102)*	COVAS *(n=102)*	SMD
Balance in covariates between the historic control cohort and the COVAS cohort						
Age, mean (SD)	61.1 (15.9)	62.4 (12.6)	0.090	58.1 (14.0)	59.0 (12.5)	0.064
Male sex, n (%)	134 (42.9)	117 (62.6)	0.401	58 (56.9)	58 (56.9)	0.000
BMI, mean (SD)	27.4 (5.2)	28.3 (4.3)	0.197	29.2 (5.1)	28.7 (4.4)	0.104
HSM, n (%)	205 (65.7)	124 (66.3)	0.013	46 (45.1)	53 (52.0)	0.145
HT, n (%)	169 (54.2)	78 (41.7)	0.251	28 (27.5)	29 (28.4)	0.020
HL, n (%)	153 (49.0)	51 (27.3)	0.460	14 (13.7)	9 (8.8)	0.104
CAD, n (%)	82 (26.3)	32 (17.1)	0.224	5 (4.9)	3 (2.9)	0.050
Stroke/TIA, n (%)	39 (12.5)	21 (11.2)	0.039	3 (2.9)	6 (5.9)	0.091
PAD, n (%)	183 (58.7)	5 (2.7)	1.528	5 (4.9)	5 (4.9)	0.000
PAD Mild, n (%)*	147 (47.1)	5 (2.7)	1.198	5 (4.9)	5 (4.9)	0.000
PAD Severe, n (%)†	36 (11.5)	0 (0.0)	0.511	0 (0.0)	0 (0.0)	0.000
DM, n (%)	63 (20.2)	29 (15.5)	0.123	14 (13.7)	11 (10.8)	0.077
Carotid artery reactivity response, before and after propensity score matching						
	Historic *(n=312)*	COVAS *(n=187)*	RD [95%CI] p-value	Historic *(n=102)*	COVAS *(n=102)*	RD [95%CI] p-value
Constriction, n (%)	102 (32.7)	31 (16.6)	-16.1% [-23.4 – -8.5] p < 0.001	23 (22.5)	19 (18.6)	-3.9% [-15.0 – 7.2] p = 0.49

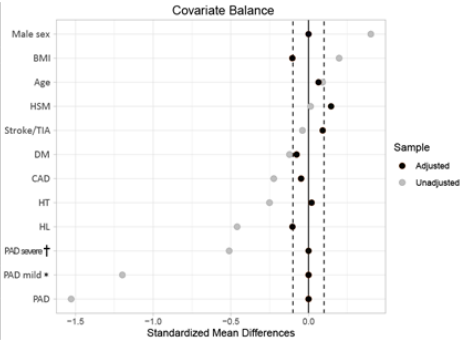

Figure 2: *Carotid artery reactivity response, based on propensity score matching of covariates in the historic control cohort and the COVAS cohort.SMD = standard mean difference, SD = standard deviation, HSM = history of smoking, HT = hypertension, HL = hyperlipidemia, CAD = coronary artery disease, TIA = transient ischaemic attack, DM = diabetes mellitus, RD = risk difference, 95%CI = 95% confidence interval.*

** Rutherford stage I-III. † Rutherford stage IV-VI.*

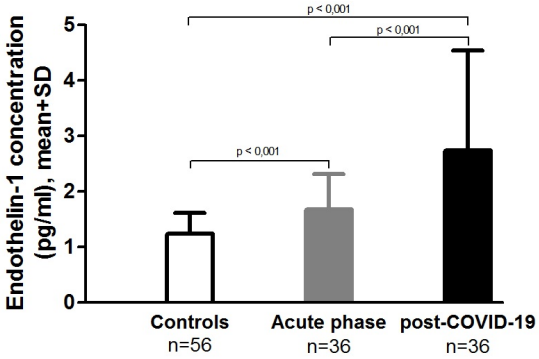

Figure 3: *Elevated levels of endothelin-1 during the acute phase of COVID-19 and further elevated in recovered patients (n = 36) when compared to controls (n = 56); Mann-Whitney U test.*

Elevated levels of endothelin-1 during the acute phase of COVID-19 and even higher levels in recovered patients

Concentrations of ET-1 were significantly higher in the COVAS cohort as compared to matched controls (2.52 ± 1.50 vs 1.24 ± 0.37 pg/mL, p<0.001. A selection of 36 patients had consented with blood plasma preservation during the acute phase of COVID-19. Levels of ET-1 of those 36 patients were significantly higher during acute phase COVID-19 (1.67 ± 0.64 pg/mL) as compared to controls (1.24 ± 0.37 pg/mL, p<0.001), and were further elevated 3 months post-COVID-19 (2.74±1.81 pg/mL, p<0.001, *figure 3*).

Markers of coagulation and inflammatory cytokines are elevated in recovered COVID-19 patients

In vivo coagulation activity was measured in the post-COVID-19 cohort and increased coagulation activity was observed in patients 6-20 weeks after recovery of acute COVID-19 (*table 2*). TAT and FIXa:AT complexes, reflecting a prothrombotic state, were elevated in 48.3% and 29.6% of the patients, respectively. FVIIa:AT, a marker of the extrinsic pathway, was increased in a third of the patients (35%), while markers of contact activation were elevated in a minority of the patients (PKa:C1inh (16.3%), FXIa:AT (16.3%), FXIa:α1AT (20.7%), and FXIa:C1inh (17.7%)). VWF:Ag was predominantly elevated in 80.8% of the post-COVID-19 patients. Inflammatory cytokine levels were determined. IL-18 levels were high in a majority of patients (73.9%), but IL-6 and IL-1ra were also frequently elevated (47.7% vs 48.9%, respectively).

Table 2: *Sustained inflammation, coagulation activation and elevated endothelin-1 levels*

	Normal range	All (n=203)		Home (n=73)		Hospital (n=130)		Home vs hospital
		Mean±SD	High, %	Mean±SD	High, %	Mean±SD	High, %	MWU
Markers of endothelial dysfunction								
CAR (n=187)	≥0%	3.5 ± 4.8	16.6	3.8 ± 5.2	19.4	3.4 ± 5.3	15.0	p=0.334
ET-1 (n=203)	0.87-1.61 pg/mL*	2.52 ± 1.50	64.5	2.25 ± 1.17	61.6	2.67 ± 1.64	66.2	p=0.171
Coagulation factor:inhibitor complexes (n=203)								
TAT	≤ 4.0 ug/L	4.8 ± 4.0	48.3	6.0 ± 6.2	63.0	4.2 ± 1.5	40.0	p=0.002
FXIa:AT	7.0-12.5 pg/mL*	25.0 ± 85.5	16.3	20.4 ± 57.3	16.4	27.6 ± 98.0	16.2	p=0.983
FXIa:α1AT	78.6-120.1 pg/mL*	206.6 ± 698.0	20.7	210.8 ±855.6	19.2	204.3 ± 595.3	21.5	p=0.768
FXIa:C1inh	176.7-396.7 pg/mL*	633.0 ± 2102.5	17.7	516.1 ± 1664.1	20.5	698.7 ± 2316.1	16.2	p=0.773
FIXa:AT	187.3-265.9 pg/mL*	259.1 ± 87.9	29.6	270.8 ± 126.3	34.2	252.5 ± 55.4	26.9	p=0.745
FVIIa:AT	237.7-374.6 pg/mL*	463.7 ± 743.1	35.0	519.2 ± 1157.6	37.0	432.6 ± 337.8	33.8	p=0.999
Pka:C1inh	1.2-2.2 ng/mL*	17.7 ± 93.1	16.3	19.7 ± 98.7	17.8	16.6 ± 90.2	15.4	p=0.994
VWF:Ag	≤160%	267 ± 133	80.8	289 ± 151	78.1	254 ± 120	82.3	p=0.097
Inflammatory cytokines (n=176)								
IL-18	37-215 pg/mL	306.5 ± 128.2	73.9	281.7 ± 124.4	66.1	319.9 ± 128.7	78.1	p=0.026
IL-6	≤ 1.8 pg/mL	3.1 ± 7.7	47.7	3.7 ± 12.6	41.9	2.8 ± 2.7	50.9	p=0.033
IL-1ra	100-400 pg/mL	491.3 ± 364.3	48.9	489.8 ± 475.5	48.8	492.1 ± 288.9	49.1	p=0.194

*SD = standard deviation, MWU = Mann-Whitney U test. * normal mean±SD*

Poor correlations between coagulation and inflammation markers in patients recovered from COVID-19 infection

A heatmap of correlations between markers of endothelial dysfunction, markers of coagulation and inflammatory cytokines is presented in *figure 4*. Macrovascular dysfunction as represented by a negative CAR, was correlated with lower levels of VWF:Ag (correlation coefficient (r) = 0.172, 95%CI 0.034 – 0.303, p=0.019), and higher levels of IL-1ra (r = -0.150, 95%CI -0.283 – -0.012, p=0.047). An association exists between ET-1 and contact activation markers FXIa:AT (r = 0.155, 95%CI 0.017 – 0.287, p=0.027) and FXIa:α1AT (r = 0.163, 95%CI 0.025 – 0.295, p=0.020).

Strong correlations exist between the coagulation enzyme:inhibitor complexes themselves, especially the complexes related to the contact pathway (FXIa:AT, FXIa:α1AT, FXIa:C1inh, PKa:C1inh). Markers of a prothrombotic state (TAT and FIXa:AT), as well as FVIIa:AT, show moderate correlations between themselves and with the complexes related to the contact pathway. Moderate correlations were established between the inflammatory cytokines. There was a negative correlation between T:AT and Il-18 (correlation coefficient (r) = -0.204, 95%CI -0.334 – -0.067, p=0.006), but not between any of the other coagulation markers and inflammatory cytokines.

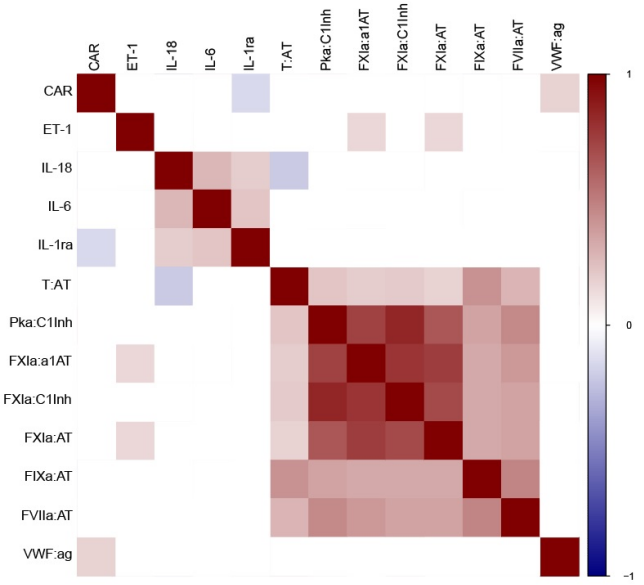

***Figure 4**: Correlation heatmap showing rho correlations between factors involved with endothelial dysfunction, inflammation and coagulation. Cell colors indicate Spearman's rank correlations from blue (negative) to red (positive), where only p values lower than 0.05 are colored. Elevated levels of endothelin-1 during the acute phase of COVID-19 and even higher levels in recovered patients*Discussion

This study is, to our best knowledge, the first to investigate macrovascular dysfunction, coagulation activation and vascular inflammation in patients that have recovered from previous COVID-19 infection. Our data demonstrate that, 3 months after COVID-19 infection, patients manifest with sustained inflammation, coagulation activation and elevated endothelin-1 levels. These changes, however, do not coincide with macrovascular dysfunction. Whilst previous studies on the effects of COVID-19 on the cardiovascular system mainly focused on the immediate (i.e. days following infection) cardiovascular complications[3,37-38], our data are amongst the first to reveal potentially detrimental lasting effects beyond the acute phase. In line with our findings, Fien et al reported sustained prothrombotic changes based on enhanced thrombin-generating capacity and decreased plasma fibrinolytic potential in 52 patients with a resolved COVID-19 infection, 4 months after hospital discharge.[39] However few groups have endeavored to study sustained elevations in inflammation following recovery from acute COVID infection.

Acute COVID-19 is associated with a remarkably high incidence of thrombotic complications, which can be explained by the unique and complex interplay between SARS-CoV-2, pneumocytes, endothelial cells, the local and systemic inflammatory response, and the coagulation system. Although clinical recovery is paralleled by normalization of C-reactive protein (CRP) and D-dimer levels, we demonstrate that 3 months after acute COVID-19, low grade activation of coagulation, inflammation and signs of endothelial dysfunction are still present. COVID-19 infection is strongly associated with thrombus formation, both in venous and arterial vasculature[3,37-38], and coagulation factors in COVID-19-associated coagulopathy show a strong correlation with disease severity[40]. Different mechanisms have been proposed, including neutrophil and complement activation, vascular damage, and tissue factor expression. Recently, our group demonstrated that neutrophils and contact activation of coagulation are potential drivers of COVID-19.[23] Both the intrinsic and extrinsic pathways of coagulation, do not only amplify fibrin generation, but also link to inflammation. Protease Activated Receptor (PAR) 1 is a high-affinity thrombin receptor which is highly expressed on platelets as well as on endothelial cells.[41] Thrombin-induced endothelial PAR1 activation, leads to regulation of vascular tone, permeability, and signals for endothelial adhesion molecules (vascular cell adhesion molecule-1, intercellular adhesion molecule-1, and E-selectin).[42-43] FXa is an important agonist for PAR2 receptors, which is present on monocytes, macrophages and Kupffer cells.[44] Through PAR2 activation, FXa contributes to the production of inflammatory cytokines.[45] Therefore, activation of the coagulation system could lead to inflammation mediated through PAR-receptor activation on endothelial and immune cells.

The COVAS cohort showed indicators of a prothrombotic state, as demonstrated by elevated TAT, in half of the patients, and signs of continued inflammation in the majority of the patients, approximately 3 months after resolution of acute COVID-19 symptoms. Acute COVID-19 infection is associated with an exaggerated inflammatory response, where cases of deadly cytokine storm are common in severe infections, often leading to multiorgan failure and death. In the acute state, levels of IL-1 family of cytokines and IL-6 have been reported to be elevated.[9,24-25] Interestingly, we found that IL-18 levels remained elevated in the vast majority of recovered patients, with IL-6 and IL-1ra also remaining elevated in half of the individuals. Heightened IL-18 and IL-6 levels are seen in states of vascular inflammation and have been associated with worse outcome in cardiovascular disease.[46-47] Our data, therefore, suggest that there may be a state of chronic low-grade inflammation in the arteries of acute COVID-19 survivors. The activation and secretion of the IL-1 family of cytokines is mediated through the formation and activity of the Nod-like receptor family pyrin domain containing 3 (NLRP3) inflammasome, which is similarly enhanced in states of chronic inflammation.[45,48] Elevated TAT is also associated with worse outcome for coronary artery disease[48], suggesting a link with vascular inflammation as well.

Widespread endothelial involvement in acute COVID-19 and in COVID-19-associated coagulopathy has been extensively suggested.[11,49] Varga et al[27] and Rovas et al[28] provided evidence that COVID-19 could directly infect the endothelial cell and cause severe alterations of the microcirculation, accumulation of inflammatory cells and diffuse endothelial inflammation in patients suffering from COVID-19. Decreased endothelium-dependent vasodilator responses and increased serum cytokines and chemokines involved in the regulation of vascular function, indicating endothelial dysfunction, were found in hospitalized patients with COVID-19.[50] We add the novel insight that, also 3 months following COVID-19 infection, markers of endothelial activation remained elevated in the greater share of recovered patients. This persisting endothelial activation could reflect disruption of vascular integrity which may lead to the sustained exposure of the thrombogenic basement membrane and the activation of the intrinsic coagulation pathway. This contact pathway could contribute to PAR2 activation[44], and lead to the secretion of IL-1 family cytokines by monocytes[45], and eventually to vascular inflammation with macrovascular dysfunction, subsequently contributing to accelerating cardiovascular disease progression[46-47].

The proposed causes of the global health care problem termed 'long COVID', are thought to derive from virus-specific pathophysiological changes, inflammatory damage, and sequelae of critical illnesses (i.e. microvascular damage, immobility and metabolic

alterations).[22] Our data suggest not only an indication of chronic inflammation, but also potential microvascular damage, reflected by persistent endothelial activation, in the majority of acute COVID-19 survivors (*table 2*). Remarkably, endothelial activation was present to a similar degree in patients that experienced critical illness and patients that remained at home during the acute phase of infection. An important question remains whether the observed sustained inflammation and endothelial activation relate to long COVID complaints and morbidity.

Macrovascular dysfunction, represented by carotid artery vasoconstriction upon sympathetic stimulation, was successfully determined in 187 patients of the COVAS cohort and in 330 historic controls. To correct for systematic differences in known risk factors for macrovascular dysfunction, propensity score matching was used. Although a perfect balance could not be achieved for three of the variables (BMI, history of smoking and hyperlipidemia), most SMDs could be reduced to below 0.1 and all SMDs were below 0.15, indicating minimal difference in baseline characteristics after correction. After PSM, the COVAS study found no signs of COVID-19-induced macrovascular dysfunction 6-20 weeks after recovery of acute COVID-19. Our study, therefore, suggests that macrovascular dysfunction is not yet present 3 months following COVID-19. As changes of the larger arteries may not occur within 3 months, a longer follow-up may be required to detect COVID-19-induced macrovascular dysfunction. It has been demonstrated previously that an elevated risk of cardiovascular disease persists during 10 years following hospitalization for pneumonia.[51]

Despite explicit elevations in both coagulation enzyme:inhibitor complexes and inflammatory cytokines, no correlation was established between the two, suggesting a dissociation between thrombotic and inflammatory states in COVID-19, or at some point during recovery. An association exists between different contact activation markers and ET-1, supporting the hypothesis of endothelial cell activation, likely partially mediated through PAR1 stimulation. ET-1, as an indicator of microvascular dysfunction[52], was not related to macrovascular dysfunction. Since matched controls clearly confirmed a connection between high levels of ET-1 and past COVID-19, while macrovascular dysfunction was not present 3 months post-COVID-19, this is as expected. A moderate positive correlation exists between macrovascular dysfunction and IL-1ra, which could be explained by the role of IL-1 family cytokines in arterial/vascular inflammation (*figure 5*).

This study investigated endothelial dysfunction, coagulation activation and vascular inflammation in patients recovered from COVID-19 infection. The availability of a large historic control cohort with known risk factors for macrovascular dysfunction

enabled an appropriate comparison between patients recovered from acute COVID-19 and patients that had unquestionably not experienced COVID-19. CAR measurements may vary over time and may depend on the observer, which could unfortunately not be controlled for in the present study. Semi-automated edge-detection and wall-tracking software was used to reduce observer variation.

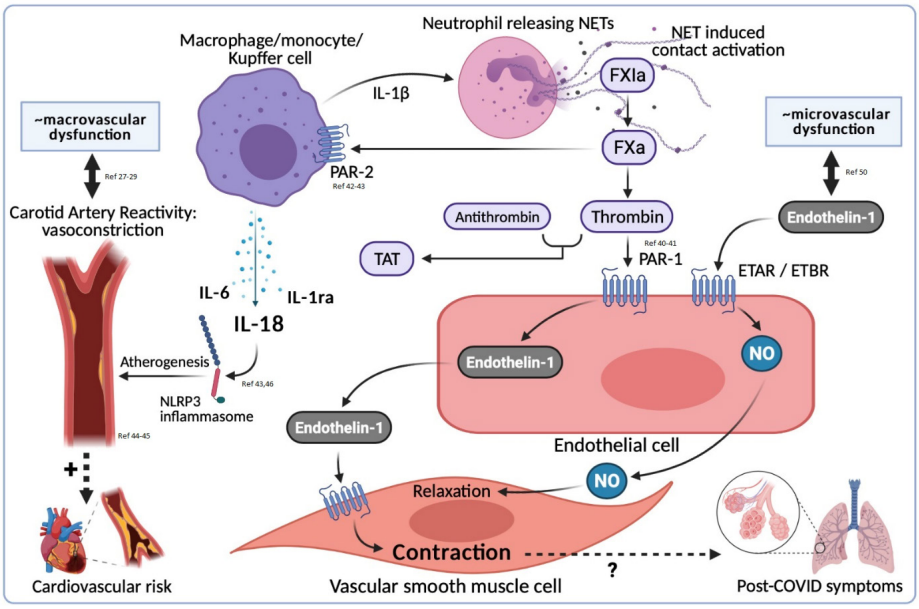

***Figure 5**: Reflection on elevated endothelin-1 levels, coagulation activation and sustained inflammation, in the medium to long term post-COVID-19.*

NET = Neutrophil Extracellular Traps, ETAR = Endothelin Type A Receptor, ETBR = Endothelin Type B Receptor, NO = nitric oxide

Conclusions

Based on this unique design, we found evidence of sustained endothelial cell activation, coagulation activation and inflammation. However, these changes did not coincide with macrovascular dysfunction, 3 months after acute COVID-19. Our data highlight the importance of further studies on SARS-CoV-2 related coagulation activation and vascular inflammation, with longer follow-up periods in recovered patients, with the newly raised hypothesis of IL-1 family cytokines, IL-18 in particular, induced arterial inflammation. Sustained endothelial activation as reflected by high ET-1 levels could be maintained by contact pathway activation, possibly induced through PAR1 stimulation. Future studies should pursue if high ET-1 levels as a marker of microvascular dysfunction play a role in long-term COVID complications.

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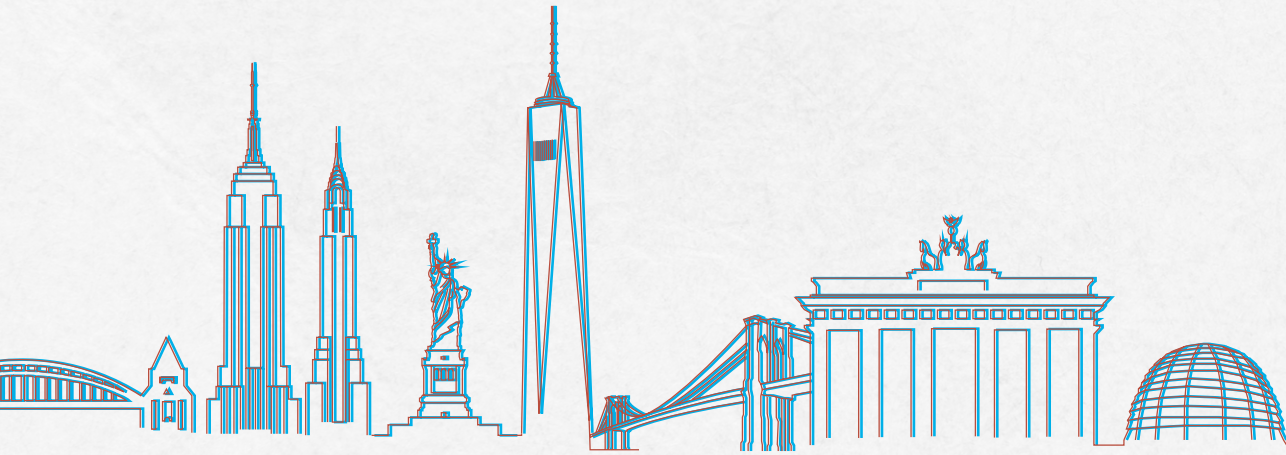

Chapter 4

Vascular function, systemic inflammation, and coagulation activation 18 months after COVID-19 infection: an observational cohort study

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Abstract

Introduction: Among its effect on virtually all other organs, COVID-19 affects the cardiovascular system, potentially jeopardizing the cardiovascular health of millions. Previous research showed no indication of macrovascular dysfunction as reflected by carotid artery reactivity, but sustained microvascular dysfunction, systemic inflammation and coagulation activation at 3 months after acute COVID-19. The long-term effects of COVID-19 on vascular function remain unknown.

Materials and Methods: This cohort study involved 167 patients who participated in the COVAS trial. At 3 months and 18 months after acute COVID-19, macrovascular dysfunction was evaluated by measuring the carotid artery diameter in response to cold pressor testing. Additionally, plasma endothelin-1, von Willebrand Factor, Interleukin(IL)-1ra, IL-6, IL-18, and coagulation factor complexes were measured by ELISA techniques.

Results: The prevalence of macrovascular dysfunction did not differ between 3 months (14.5%) and 18 months (11.7%) after COVID-19 infection ($p = 0.585$). However, there was a significant decrease in absolute carotid artery diameter change, 3.5% ± 4.7 vs 2.7% ± 2.5, p – 0.001, respectively. Also, levels of vWF:Ag were persistently high in 80% of COVID-19 survivors, reflecting endothelial cell damage and possibly attenuated endothelial function. Furthermore, while levels of the inflammatory cytokines interleukin(IL)-1RA and IL-18 were normalized and evidence of contact pathway activation was no longer present, the concentrations of IL-6 and thrombin:antithrombin complexes were further increased at 18 months versus 3 months (2.5 pg/mL ± 2.6 vs 4.0 pg/mL ± 4.6, p=.006 and 4.9 µg/L ± 4.4 vs 18.2 µg/L ± 11.4, p<.001, respectively).

Discussion: This study shows that 18 months after COVID-19 infection, the incidence of macrovascular dysfunction as defined by a constrictive response during carotid artery reactivity testing is not increased. Nonetheless, plasma biomarkers indicate sustained endothelial cell activation (vWF), systemic inflammation (IL-6) and extrinsic/common pathway coagulation activation (FVII:AT, TAT) 18 months after COVID-19 infection.

Introduction

A substantial amount of research has been performed to uncover the mechanism of action and the consequences of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). COVID-19 mostly affects the upper airways and pulmonary system[1]. However, virtually all organs can be affected. Among others, COVID-19 induces inflammation, platelet activation, hypercoagulability and endothelial cell (EC) dysfunction, which contribute to the occurrence of complications.[2-3] As a result, COVID-19 may jeopardize the cardiovascular health of millions.[4]

Previous results from our group revealed that at 3 months after acute COVID-19, sustained EC involvement, inflammation, and coagulation activity were present.[5] A heightened innate immune response state, and a prothrombotic state promote macrovascular EC dysfunction and damage, which impair important functions of the endothelium[6-8], resulting in an increased risk of developing major arterial thrombotic events such as myocardial infarction or stroke.[9-10] The literature already revealed a higher incidence of large-vessel stroke[11-12] and myocardial infarction[13] in acute COVID-19 patients.

Our research group found no indication of macrovascular dysfunction at 3 months after acute COVID-19; however, macrovascular dysfunction might take a longer time to develop.[5] The effect of COVID-19 infection on ECs has been studied elaborately, but the long-term effects of the disease on ECs and blood vasculature remain to be determined.[7,14-15] Therefore, this study aimed to determine the long-term effects (18 months) of acute COVID-19 infection on macrovascular endothelial function. Additionally, this study reveals whether EC involvement, elevated circulating inflammatory cytokines and coagulation system activation remain present in patients 18 months after recovery.

Materials and Methods

Study design and participants

The COVAS study was an observational cohort study, initiated at Radboudumc (Nijmegen, the Netherlands) and conducted at Bernhoven hospital (Uden, the Netherlands). Detailed description of the design, in- and exclusion criteria, methods and cross-sectional data at 3 months post-COVID were published previously.[5] In brief, patients aged 16 years or older who had experienced SARS-CoV-2 infection were recruited. Patients with a recent (<3 months) episode of angina pectoris,

myocardial infarction, stroke or heart failure, or upper-extremity conditions contra-indicating cold pressor testing were excluded. The study was approved by the regional ethics committee Oost-Nederland (reference number NL74101.091.20), and local approval was obtained of the local directory boards. This study was conducted in accordance with the latest revision of the Declaration of Helsinki. Written informed consent was obtained from all participants before the start of any study-related procedures. Data are available on request from the authors.

Procedures and endpoints

Participants of the COVAS study were followed for a period of 18 months after recovery from acute COVID-19 symptoms. The first visit was planned at least 6 and no more than 20 weeks after recovery from acute COVID-19. The second study visit was planned at 18 months after COVID-19 infection. During both visits, macrovascular endothelial function was assessed using the carotid artery reactivity (CAR) test and whole blood was collected for determining markers of microvascular EC dysfunction, inflammation and coagulation system activation.

Measurement of macrovascular dysfunction by the carotid artery reactivity (CAR) test

The CAR test is a non-invasive procedure, measuring the response of the carotid artery diameter to cold pressor testing (CPT), a 3-minute immersion of the hand in ice water (≤4.0°C).[5,16-18] Carotid artery reactivity was classified as dilatation (normal endothelial function) or constriction (macrovascular endothelial dysfunction) and the relative carotid artery diameter change from baseline (CAR%) was determined to quantify this response. Recorded videos of the carotid artery diameter were analyzed with edge-detection and wall-tracking software, custom-designed by Philips Healthcare (Best, The Netherlands). Details on how this test was performed can be found elsewhere.[5] In short, the left common carotid artery was visualized using L12-4 MHz linear array probe of Philips Lumify ultrasound device with the participant laying in supine position. Participants were asked to adhere to several guidelines prior to CAR testing: (1) No food or drinks other than water within 6 hours prior to the study visit; (2) no coffee, tea, soft drinks, alcohol, vitamin C rich products or chocolate within 18 hours prior to the study visit; and (3) no intensive physical exercise within 24 hours prior to the study visit.

Determining markers of microvascular EC dysfunction, inflammation and coagulation system activation

Platelet poor plasma was obtained from whole blood samples after centrifuging for 30 minutes in two steps (first 10 minutes, second 20 minutes), both at 2500g at room temperature. The plasma was snap-frozen at -80 °C until further assay. Concentrations

of endothelin-1 (ET-1), interleukin-1 receptor antagonist (IL-1RA), IL-6 and IL-18 were measured using manufacturer's protocol by commercial quantikine ELISA kits (R&D Systems, Minneapolis, Minnesota). Concentrations of von Willebrand factor antigen (VWF:Ag) and activated coagulation factors in complex with their natural inhibitors were measured using in-house developed ELISA methods.[5,19] Measured coagulation factor inhibitor complexes included thrombin:antithrombin (TAT), factor(F)VIIa:AT; FIXa:AT, FXIa:AT, FXIa:alpha-1-antitrypsin (α1AT), FXIa:C1-esterase-inhibitor (C1inh) and kallikrein:C1inh (PKa:C1inh).

Adverse cardiovascular events

During the second study visit at 18 months after COVID-19 infection, participants were asked if they experienced any adverse events after their first study visit. Specifically, participants were asked if they had experienced myocardial infarction, stroke, acute limb ischemia, revascularization procedures, or above ankle amputation of a lower extremity. Additionally, patient files of all participants were checked for the before mentioned events and death.

Statistical analysis

The change in the proportion of participants with vasoconstriction during the CAR test at 3 months versus 18 months was tested using McNemar's test. Changes in CAR%, markers of microvascular EC dysfunction, markers of inflammation and markers of coagulation system activation at 3 months versus 18 months were compared using the Wilcoxon signed rank test. The proportion of participants with concentrations of previous mentioned markers above normal range was reported. Normal ranges of in-house developed ELISA methods were defined as above normal mean ± 1 SD, based on previous validation studies.[5,19] Analyses were performed using IBM SPSS Statistics 27. P-values below 0.05 were considered statistically significant.

Results

Study population

During the first study visit, 203 patients provided written informed consent. All participants were invited for a follow-up visit 18 months after acute COVID-19 and 167 participants accepted the invitation and visited Bernhoven hospital between November 1st, 2021, and February 16th, 2022. Thirty-six participants refused the invitation and did not complete the study due to loss to follow-up (4), withdrawal of consent (16), researcher's decision (2), death (4), or other reasons (10). *Figure 1* demonstrates the study flowchart. Baseline patient characteristics are shown in *table 1*.

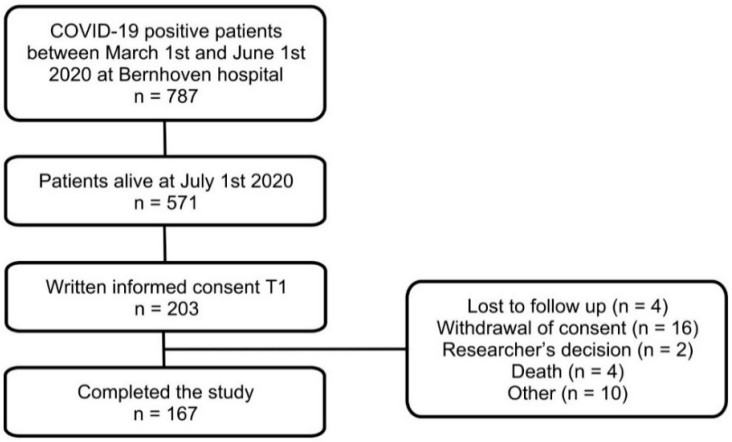

Figure 1: *Flow chart of the study population.*
T1: 3 months after acute COVID-19.

Table 1: *Baseline characteristics of all participants who completed the study.*

		n = 167
Patient characteristics	Age, mean ± SD	62.3 ± 12.1
	Male sex, n (%)	112 (76.1)
	BMI, mean ± SD	27.8 ± 4.1
	History of smoking, n (%)	112 (67.1)
Comorbidities	Hypertension, n (%)	71 (42.5)
	Hyperlipidemia, n (%)	45 (26.9)
	Cardiovascular disease, n (%)	68 (40.7)
	Diabetes mellitus, n (%)	20 (12.0)
Disease severity	Days of illness, median [range]	19 [1-80]
	Hospital care, n (%)	109 (65.3)
	Days, median [range]	6 [1-61]
	Intensive care, n (%)	24 (14.4)
	Days, median [range]	16 [1-42]

*SD = standard deviation, BMI = body mass index*No indication of delayed onset macrovascular dysfunction, but reduced CAR% at 18 months after COVID-19

No differences in macrovascular dysfunction as assessed by CAR testing were found when comparing the incidence at 3 months to 18 months after acute COVID-19 (*table 2*). At 3 months after acute COVID-19, vasoconstriction occurred in 14.5% of participants and at 18 months after acute COVID-19 in 11.7% of participants,

p=.584. (*table 2*) There was a significant decrease, however, in CAR% between 3 months and 18 months after infection, 3.5 ± 4.7% versus 2.7 ± 2.5% respectively.

Table 2: *total participants with a vasodilatation and vasoconstriction response at 3 months and 18 months after acute COVID-19*

		CAR response at T2, 18 months after acute COVID-19		
		Dilatation	**Constriction**	**Total**
CAR response at T1, 3 months after acute COVID-19	Dilatation	111	13	124
	Constriction	17	4	21
	Total	128	17	145
Constriction, n (%)				
3 months after acute COVID-19		21 (14.5)		p=.584†
18 months after acute COVID-19		17 (11.7)		
CAR%, mean ± SD				
3 months after acute COVID-19		3.5 ± 4.7		p = 0.001‡
18 months after acute COVID-19		2.7 ± 2.5		

CAR = carotid artery reactivity, SD = standard deviation

†The change in the proportion of participants with vasoconstriction during the CAR test was tested using McNemar's test.

‡ Changes in CAR% were compared using the Wilcoxon signed rank test.

Partial normalization of microvascular EC dysfunction, inflammation and contact pathway activation, but further increase in IL-6 and extrinsic pathway activation at 18 months versus 3 months after COVID-19 recovery

ET-1 as marker of microvascular EC dysfunction showed significant decrease of plasma concentrations with mean levels being in normal range, while vWF:AG levels, representing endothelial damage, remained elevated in the majority of patients at 18 months after acute COVID-19. IL-1RA and IL-18 which were both highly activated at 3 months after COVID-19, were significantly decreased and restored to normal means at 18 months after COVID-19. However, IL-6 appeared to be further elevated at 18 months versus 3 months after acute COVID-19, 4.0 ± 4.6 pg/mL vs 2.5 ± 2.6 pg/mL (p=.006), with 19.4% of participants having IL-6 levels above normal range at 18 months after COVID-19. Predominantly normalization of markers of contact activation was demonstrated with a decrease in mean concentrations of FIXa:AT, FXIa:α1AT and FXIa:C1inh below the upper limit of normal and decrease, but not normalization, of FXIa:AT. On the other hand, TAT, reflecting ongoing thrombin generation, as well as FVIIa:AT, an extrinsic pathway activity marker, were further elevated 4.9 ± 4.4 vs 18.2 ± 11.4 (p<.001) and 411.9 ± 317.0 vs 426.8 ± 356.0 (p=.005), respectively. (*table 3*).

Table 3: *Endothelial cell activation, circulating inflammatory cytokines and markers of coagulation system activation at 3 months versus 18 months after recovery of acute COVID-19*

	Normal range	T1 (+3 months)		T2 (+18 months)		*p*
		mean ± SD	High, %	mean ± SD	High, %	
ET-1	0.87-1.61 pg/mL	2.56 ± 1.42	68.3	1.44 ± 0.53	30.5	**<.001**
VWF:Ag	≤160%	273.2 ± 138.3	81.0	244.2 ± 89.6	80.0	.090
IL-1RA	100-400 pg/mL	494.8 ± 383.0	47.9	363.5 ± 541.4	22.5	**<.001**
IL-6	4.6-5.7 pg/mL[20]	2.5 ± 2.6	3.7	4.0 ± 4.6	19.4	**.006**
IL-18	37-215 pg/mL	307.0 ± 126.9	75.4	207.8 ± 126.5	34.5	**<.001**
TAT	≤ 4.0 µg/L	4.9 ± 4.4	47.2	18.2 ± 11.4	95.7	**<.001**
FVIIa:AT	237.7-374.6 pg/mL	411.9 ± 317.0	34.4	426.8 ± 356.0	35.0	**.005**
FIXa:AT	187.3-265.9 pg/mL	255.2 ± 66.0	27.6	234.6 ± 62.5	25.2	**<.001**
FXIa:AT,	7.0-12.5 pg/mL	22.7 ± 85.2	16.0	15.3 ± 28.8	4.9	**<.001**
FXIa:α1AT	78.6-120.1 pg/mL	163.4 ± 487.5	19.0	70.6 ± 6.8	0.6	**<.001**
FXIa:C1inh	176.7-396.7 pg/mL	500.2 ± 1604.3	17.8	348.7 ± 814.0	11.7	**<.001**

ET-1 = endothelin-1; VWF:Ag = von Willebrand factor antigen; IL-1RA = interleukin 1 receptor antagonist; TAT = thrombin:antithrombin; F = factor; α1AT = alpha-1-antitrypsin; C1inh = C1-esterase-inhibitor; PKa = kallikrein

Major adverse cardiovascular events

Some participants experienced adverse cardiovascular events during the time between the study visits. Myocardial infarction occurred in one participant and one participant had a stroke. Two participants underwent revascularization. There was no significant difference between participants that did or did not experience a major adverse cardiovascular event for any of the variables.

Discussion

This study revealed that COVID-19 does not cause delayed onset macrovascular endothelial dysfunction as measured by carotid artery reactivity testing. Nonetheless, there is still evidence of sustained endothelial cell damage with largely restored levels of ET-1, but persistent high levels of vWF:Ag. Sustained inflammation and coagulation activation as previously demonstrated at 3 months after acute COVID-19, were partly restored in the long-term (18 months). However, further increase of IL-6 levels and TAT were present.

CAR testing revealed no increased macrovascular dysfunction reflected by constriction of the carotid artery as a reaction to CPT. However, the CAR% was

significantly reduced at 18 months compared to 3 months after acute COVID-19. As the vascular endothelium is critical for maintaining vascular tone and homeostasis, endothelial dysfunction switches the vascular equilibrium to vasoconstriction.[14-15] The decrease in CAR% could therefore represent COVID-19 induced reduced vascular wall relaxation capacity. The clinical importance of such changes in CAR%, without influencing the prevalence of macrovascular dysfunction, may not be immediately evident. Also, other factors might have influenced the CAR%. Previous research described decreased vasomotor response with advancing age.[17,21] In the current study, the age difference between follow-up moments was 15 months, which might slightly influence the CAR%. Another explanation might be the difference in adherence to the guidelines prior to CAR testing. At 3 months, 75% of participants adhered to all guidelines, while at the second study visit only 57% of participants adhered to all guidelines. The guideline that was adhered to the least was not drinking coffee, tea, or soft drinks 18 hours prior to the CAR test, with 17% and 32% of participants not following these instructions at 3 months and at 18 months after COVID-19 infection, respectively. Coffee exerts an acute unfavorable effect on endothelial function[22] that might have resulted in a decrease in CAR%.

Mean blood plasma levels of inflammatory cytokines IL-1RA and IL-18 were lower and no longer elevated at 18 months versus 3 months after COVID-19 infection. This suggests at least a partial restoration of the chronic low-grade inflammation state that was present 3 months after COVID-19 infection.[5] Circulating IL-18 is moderately and independently associated with cardiovascular disease (CVD) and also higher levels of IL-1RA are positively associated with incident CVD.[23-24] Therefore, a reduction in plasma concentrations of IL-18 and IL-1RA may be beneficial for the cardiovascular health of COVID-19 survivors.

Most notably, however, data from this study also indicate a significant increase in blood plasma levels of the inflammatory cytokine IL-6 between 3 months and 18 months after COVID-19 recovery. The amount of participants with IL-6 levels above normal range compared to literature increases from 3.7% to 19.4%. Compared to their own levels, the plasma concentrations increase with 60% in the 15 months following first study visit, 2.5 ± 2.6 pg/mL versus 4.0 ± 4.6 pg/mL, p=0.006. COVID-19 infection can cause endothelial dysfunction and thrombosis by two proposed mechanisms: acting directly on the endothelium and impairing its anti-thrombogenic and barrier properties, or acting indirectly through a local cytokine storm and systemic inflammation resulting in endothelial injury.[7] Cytokines are believed to play a major role during control and resolution of COVID-19 infection.[25] Most infected patients develop mild to moderate symptoms, but in

some COVID-19 patients, exacerbated pro-inflammatory cytokine release occurs known as cytokine release syndrome (CRS). CRS results in systemic inflammation and can in turn lead to multiple organ failure.[25-27] IL-6 plays an important role in CRS and is positively correlated with COVID-19 severity.[26,28] Persisting and even further elevation of IL-6 levels in the long-term post-covid might be an alarming signal of persistent COVID-19 induced inflammatory state. An important sidenote is that the COVID-19 booster vaccination campaign was initiated during the course of this study. Vaccination against COVID-19 has been shown to increase IL-6 levels with an early small peak 1 day after vaccination.[29-30] Recent research demonstrates that vaccination induced IL-6 level increase is no longer present at 4 weeks after vaccination.[31] The exact number of patients that has been vaccinated in the days or weeks preceding the second study visit is unknown. The influence of IL-6 levels in study participants is expected to be limited since the peak is described to be short and early. Still, COVID-19 vaccination may have resulting in higher levels of IL-6 at 18 months versus 3 months post COVID-19 in some participants. No changes in IL-18 levels have been observed after COVID-19 vaccination.[29] Another important issue is that ageing is associated with a chronic progressive increase in the proinflammatory status, called inflammaging.[32] There is strong evidence that serum concentrations of the pro-inflammatory cytokine IL-6 increase with age.[33] However, since the age difference between follow-up moments was only 15 months, and the increase was not seen in other inflammatory cytokines, the effect of inflammaging is expected to be limited in our cohort.

Ongoing contact activation has been confirmed in patients with active COVID-19 disease with a clear link to disease severity, possibly explaining the high incidence of thrombotic complications in severe COVID-19.[34] After 3 months, only a minority of participants show elevated markers of contact activation, where long-term follow-up demonstrates further normalization. On the other hand, TAT, a marker of thrombin generation, is further increased at 18 months compared to 3 months after acute COVID-19. Other studies have demonstrated evidence of increased thrombin generation up to 1 year after acute COVID-19 infection[35,36], proposedly caused by the underlying mechanisms of persistent endothelial damage. Indeed, in the current study, we found evidence of sustained elevation of vWF:Ag levels, with 80% of participants still having vWF:Ag levels above normal range. Additionally, we found increased levels of IL-6 which is a driver of tissue factor expression. Tissue factor is an initiator of the extrinsic coagulation pathway, ultimately leading to thrombin generation.[37] We found increased levels of markers of both extrinsic pathway activity (FVII:AT) and thrombin generation (TAT).

Since previous research on COVID vaccination demonstrates no effect on TAT, the booster vaccination campaign initiated during this study, is an unlikely explanation for the ongoing elevation of TAT.

This study has some limitations. First, adherence to guidelines prior to CAR testing at the second study visit was somewhat modest compared to the first study visit. Participants were asked to adhere to several guidelines prior to CAR testing to minimize the influence of oral intake and behavior on endothelial function. At 3 months, 75% of participants adhered to all guidelines, compared to 57% at 18 months. The difference was mostly explained by the intake of coffee, tea, or (possibly caffein containing) soft drinks in the 18 hours preceding CAR testing. This might have led to a slight deviation from the true effect. However, this deviation would be in the disadvantage of the second follow-up visit, where a lower prevalence of macrovascular dysfunction was observed. Therefore, we are convinced that there is no indication of late-onset macrovascular dysfunction at 18 months after COVID-19 infection. Second, information about factors which could influence IL-6 levels in the blood, such as recent vaccinations or re-infection with COVID-19, were not collected from the participants. COVID-19 vaccination leads to an increase in IL-6 for at least 1 day, but no longer than 4 weeks.[29-31] The effect of re-infection on IL-6 concentrations is not well described in literature, but in primary infection, rise in cytokines appear to play a major role.[25] Since the booster campaign was initiated during the study period, and the pandemic continued, we cannot be sure whether or not the observed increase in IL-6 concentrations were caused by COVID-19 vaccination or re-infection.

In conclusion, this study showed that COVID-19 does not increase the incidence of macrovascular dysfunction as assessed by carotid artery reactivity testing until 18 months after infection. Nevertheless, our results indicate that, 18 months after COVID-19, there is evidence of: 1) sustained EC damage with persistent high levels of vWF:Ag in almost all survivors and a slight increase in CAR% which might indicate attenuated endothelial function; 2) sustained and further rise of circulating IL-6 levels compared to 3 months after covid-19, possibly indicating a chronic low-grade inflammatory state; and 3) no longer evidence of contact pathway activation but further rise in the marker of thrombin generation TAT and the extrinsic pathway marker FVII:AT, which indicates persistent prothrombotic state, possibly triggered by increased tissue factor expression through IL-6 . Therefore, based on the findings of the current study, the long-term cardiovascular risk after acute COVID-19 infection could be increased. Future research should focus on the clinical consequences of chronic inflammatory and prothrombotic state, such as cardiovascular complications.

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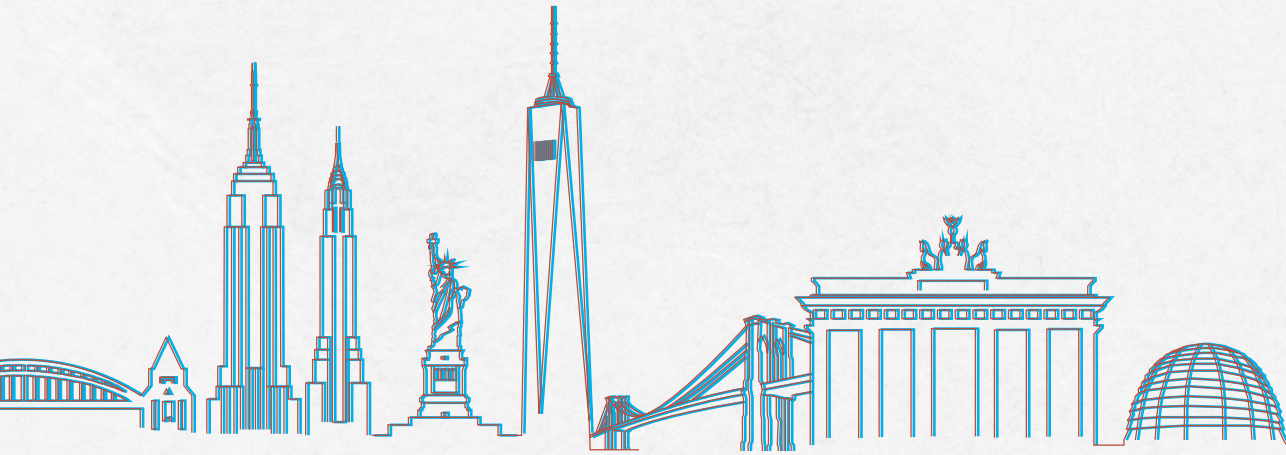

Chapter 5

ChAdOx1 vaccination, blood coagulation, and inflammation: No effect on coagulation but increased interleukin-6

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Abstract

Background: Vaccination is the leading approach in combatting the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic. ChAdOx1 nCoV-19 vaccination (ChAdOx1) has been linked to a higher frequency of rare thrombosis and thromboembolism. This study aimed to explore markers related to the blood coagulation system activation and inflammation, before and after ChAdOx1 vaccination.

Patients and Methods: An observational cohort study including forty health care workers. Whole blood samples were collected prior to, and either 1 or 2 days after vaccination. Activated coagulation factors in complex with their natural inhibitors were determined by custom ELISAs, including thrombin:antithrombin (T:AT), kallikrein:C1-esterase-inhibitor (PKa:C1Inh), factor(F)IXa:AT, FXa:AT, FXIaAT, FXIa:alpha-1-antitrypsin (α1AT), FXIa:C1inh, and FVIIa:AT. Plasma concentrations of interleukin (IL)-6 and IL-18 were quantified via ELISA. Analyses were performed using Wilcoxon Signed Ranks Test.

Results: Levels of FVIIa:AT decreased with a median (IQR) of 707 (549–1028) pg/mL versus 598 (471–996) pg/mL, p=0.005; and levels of IL-6 increased, 4.0 (1.9–6.8) pg/mL versus 6.9 (3.6–12.2) pg/mL, p=0.023, after vaccination. No changes were observed in T:AT, PKa:C1Inh, FIXa:AT, FXa:AT, FXIaAT, FXIa:α1AT, FXIa:C1inh and IL18.

Conclusion: ChAdOx1 leads to an inflammatory response with increased levels of IL-6. We did not observe activation of the blood coagulation system 1-2 days following vaccination.

Background

Thrombosis is a frequent complication in patients during the acute and recovery phase of the coronavirus disease 2019 (COVID-19).[1-4] Reflecting this increased thrombotic risk, complexes of activated coagulation factors and their inhibitors were elevated in plasma of individuals with COVID-19. These factors similarly vary with disease severity.[5] Unpublished data from our group show that, ~3 months after COVID-19 infection, the same markers of blood coagulation system activation remain increased in 40-50% of all patients, alongside elevated inflammatory cytokines [submitted for publication]. As novel variants of the severe acute respiratory syndrome coronavirus 2 (Sars-Cov-2) continue to infect individuals worldwide, vaccination is the leading approach in mitigating this pandemic. By August 2021, four different COVID-19 vaccines have been approved by the European Medicines Agency. Among these the ChAdOx1 nCoV-19 vaccine (ChAdOx1) of AstraZeneca, which has been shown to be safe and effective in randomized controlled trials.[6] Despite this evidence in favor of the use of ChAdOx1, the vaccination program was discontinued in several European countries due to observations of extensive thrombosis in atypical sites with associated thrombocytopenia occurring at a low frequency.[7] This manifestation, now known as Vaccine-induced Immune Thrombotic Thrombocytopenia (VITT), is associated with anti-platelet 4 antibodies activating platelets.[8-9] Besides the rare occurrence of VITT, researchers have described a higher frequency of general thrombosis and thromboembolism in individuals recently vaccinated with ChAdOx1, when compared to individuals vaccinated with other COVID-19 vaccines[10], and compared to the general population.[11] This raises the question whether ChAdOx1 could induce activation of the blood coagulation system, comparable to the activation of the blood coagulation system by SARS-CoV-2 itself. This study aimed to explore changes in circulating biomarkers of the activated blood coagulation system and proinflammatory cytokines before and after ChAdOx1 vaccination.

Methods

An observational cohort study was performed including forty health care workers of the Radboud university medical center (Radboudumc, Nijmegen, the Netherlands), scheduled for the first dose of ChAdOx1 vaccination, recruited by open invitation between April 13th and May 6th 2021. This study was approved by the regional ethics committee and the directory board of the Radboudumc. Individuals meeting any of the following criteria were excluded from participation: 1) individuals with a

bleeding disorder; 2) individuals on vitamin K antagonists, low-molecular weight heparin, or direct oral anticoagulants; or 3) individuals on immunosuppressant and/or anti-inflammatory therapy, including glucocorticoids, cytostatic agents, antibodies, immunophilins, interferons, TNF-binding proteins, mycophenolate and interleukin antagonists. Written informed consent was obtained from all participants. Baseline demographics and clinical data regarding medical history and medication use were requested. Whole blood samples were collected prior to vaccination. A second whole blood sample collection was scheduled for either one or two days post-vaccination, endeavoring to include equal numbers of participants in both groups and considering participants preferences for the day of second collection. Whole blood samples were collected and processed to platelet-poor plasma by standard procedures.[12] Participants were contacted by telephone four weeks after vaccination to inform about any health complaints experienced since vaccination, including thrombosis and thromboembolism. Activated coagulation factors in complex with their natural inhibitors, including thrombin:antithrombin (T:AT), kallikrein:C1-esterase-inhibitor (PKa:C1Inh), factor(F) IXa:AT, FXa:AT, FXIaAT, FXIa:alpha-1-antitrypsin (α1AT), FXIa:C1inh, and FVIIa:AT were quantified by in-house developed enzyme-linked immunosorbent assays (ELISA).[13] Plasma concentrations of interleukin (IL)-6 and IL-18 were quantified using commercial ELISA kits (R&D, Minneapolis, Minnesota). Data were analyzed by performed Wilcoxon Signed Ranks Test to detect changes in levels of activated coagulation factor:inhibitor complexes and proinflammatory cytokines from baseline to post-vaccination. Subanalyses were performed for the groups with the second blood sample collection one day post vaccination, and two days post-vaccination, separately. Analyses were performed using IBM SPSS Statistics 25 and p-values below 0.05 were considered significant.

Results and discussion

Forty health care workers, scheduled for the first dose of ChAdOx1 vaccination, were recruited by open invitation. One subject did not meet the in- and exclusion criteria and was replaced. Subjects were assessed for changes in circulating biomarkers of blood coagulation activation and proinflammatory cytokines, before and one or two days after exposure to ChAdOx1 vaccination. All participants were aged between 60 and 65 years, the mean body mass index was 26 kg/m^2 and 20% of the subjects were male. Comorbidities, most frequently hypertension (22.5%) and cardiovascular disease (22.5%), were present in 65% of individuals. Nineteen subjects had their second blood sample collection one day post-vaccination, and

twenty-one subjects had their second blood sample collection two days post-vaccination. No significant differences were established in baseline characteristics or medical history between the two groups (*table 1*).

In vivo blood coagulation activity was measured and compared before and after ChAdOx1 vaccination. No changes in T:AT, PKa:C1Inh, FIXa:AT, Fxa:AT, FXIaAT, FXIa:α1AT and FXIa:C1inh were observed after exposure to ChAdOx1. FVIIa:AT was significantly decreased after vaccination, median (IQR) 707 (549–1028) pg/mL versus 598 (471–996) pg/mL, p=0.005. Levels of IL-6 increased after ChAdOx1 vaccination (4.0 (1.9–6.8) pg/mL versus 6.9 (3.6–12.2) pg/mL, p=0.023), while IL-18 levels were unchanged. Sub-analyses were performed separately on the one day and two day post-vaccination groups. Results of both groups were similar to the overall population with decreasing tendencies in FVIIa:AT levels and increasing tendencies in IL-6 levels. (*table 2*)

***Table 1**: Baseline characteristics and medical history*

	All, n=40	Second visit at +1 day, n=19	Second visit at +2 days, n=21	P-value
Baseline characteristics				
Male, n (%)	8 (20)	5 (26.3)	3 (14.3)	0.342
Age, y, mean±SD	61.2±1.3	61.2±1.4	61.2±1.2	0.961
Body mass index, mean±SD	26.0±4.6	26.7±3.7	25.5±5.3	0.396
Smoking behavior, n (%) Never Former Current	13 (32.5) 23 (57.5) 4 (10)	7 (36.8) 11 (57.9) 1 (5.3)	6 (28.6) 12 (57.1) 3 (14.3)	0.600
Race, n (%) Caucasian	40 (100)	19 (100)	21 (100)	NA
Medical history				
Hypertension, n (%)	9 (22.5)	6 (31.6)	3 (14.3)	0.191
Hyperlipidemia, n (%)	3 (7.5)	2 (10.5)	1 (4.8)	0.489
Cardiovascular disease, n (%)	9 (22.5)	5 (26.3)	4 (19.0)	0.583
Diabetes mellitus, n (%)	1 (2.5)	0 (0.0)	1 (4.8)	0.335
Past COVID-19 infection, n (%)	3 (7.5)	2 (10.5)	1 (4.8)	0.489
Antiplatelet therapy, n (%)	6 (15)	1 (10.5)	4 (19.0)	0.451

SD = standard deviation, COVID-19 = Corona Virus Disease 2019, NA = not applicable

Table 2: *Circulating concentrations of coagulation markers and inflammatory cytokines*

	All, n=40			Second visit at +1 day, n=19			Second visit at +2 days, n=21		
	Before, n=40, median (IQR)	**After, n=40, median (IQR)**	**P**	**Before, n=19, median (IQR)**	**After, n=19, median (IQR)**	**P**	**Before, n=21, median (IQR)**	**After, n=21, median (IQR)**	**P**
Coagulation enzyme : inhibitor complexes									
T:AT, µg/L	1.2 (1.2–1.9)	1.2 (1.2–1.5)	0.150	1.2 (1.2–2.2)	1.2 (1.2–1.5)	0.261	1.2 (1.2–1.7)	1.2 (1.2–1.4)	0.332
Pka:C1Inh, ng/mL	2.6 (2.2–4.1)	2.5 (2.2–4.5)	0.534	3.2 (2.2–4.9)	3.1 (2.2–6.7)	0.686	2.5 (2.3–3.7)	2.5 (2.2–3.5)	0.587
FIXa:AT, pg/mL	195 (195–212)	195 (195–197)	0.152	195 (195–260)	195 (195–247)	0.310	195 (195–199)	195 (195–195)	0.345
FXa:AT, pg/mL	200 (185–221)	200 (182–215)	0.548	209 (188–222)	203 (182–218)	0.809	191 (184–220)	195 (181–215)	0.455
FXIa:AT, pg/mL	22.4 (17.2–35.5)	22.3 (15.0–32.8)	1.000	25.1 (14.8–51.0)	28.5 (15.6–52.2)	0.979	21.1 (18.0–26.6)	20.7 (14.7–28.3)	0.958
FXIa:α1AT, pg/mL	50 (50–151)	50 (50–128)	0.501	50 (50–227)	50 (50–181)	0.260	50 (50–55)	50 (50–63)	0.866
FXIa:C1Inh, pg/mL	76 (76–308)	76 (76–322)	0.427	76 (76–748)	76 (76–722)	0.086	76 (76–112)	76 (76–146)	0.173
FVIIa:AT, pg/mL	707 (549–1028)	598 (471–996)	0.005	717 (563–1275)	620 (478–1083)	0.020	705 (525–874)	577 (454–936)	0.099
Inflammatory cytokines									
IL-6, pg/mL	4.0 (1.9–6.8)	6.9 (3.6–12.2)	0.023	5.0 (2.2–7.0)	9.1 (4.6–13.9)	0.053	3.9 (1.3–5.9)	4.2 (3.0–9.9)	0.289
IL-18, pg/mL	56 (5–107)	71 (0–155)	0.447	73 (15–158)	98 (0–170)	0.796	38 (2–96)	61 (0–145)	0.500

T:AT = thrombin:antithrombin, Pka = kallikrein, C1Inh = C1-esterase-inhibitor, F = factor, α1AT = alpha-1-antitrypsin, IL = interleukin, IQR = interquartile range

Increased systemic levels of IL-6 have been previously observed following various types of vaccination, such as foot and mouth disease vaccination[14], Bacillus Calmette-Guérin[15], diphtheria toxoid vaccination[16] and influenza vaccination[17]. An explanation has been proposed by Farsakoglu et al. who demonstrated elevations in IL-6 secretion by CD11b+ dendritic cells following influenza vaccination, this response was initiated by interferon-γ production from natural killer cells.[17] A natural killer cell response has been previously established for single-dose ChAdOx1[18] and potentially justifies the elevated levels of IL-6 after vaccination in the current study.

IL-6 is known to induce the expression of tissue factor (TF), which plays a key role in the regulation of hemostasis.[19] TF forms complexes with activated FVII where TF:FVIIa complexes activate FIX and FX, which subsequently results in thrombin generation. Binding of FVII to TF could have reduced the availability of FVIIa to bind to AT, its natural inhibitor, thus resulting in the observed decrease in levels of FVIIa:AT. However, a type I error cannot be excluded. We found no further evidence of extrinsic pathway activation as reflected by comparable levels of FIXa:AT, Fxa:AT and T:AT, before and after ChAdOx1 vaccination.

All participants were followed for health complaints until four weeks after vaccination. Thirty-two (80%) of the subjects reported health complaints, including injection site tenderness, myalgia, headache, malaise, fever, chills, nausea or diarrhea. All health complaints resolved within four days following vaccination with ChAdOx1. No complications related to thrombosis or thromboembolism were reported.

This study had some limitations. All participants were aged 60-65 years, and the sample size was small, which limits the generalizability of the study results. Changes in markers related to blood coagulation system activation and inflammation were measured 24-48 hours after vaccination. Our results, therefore, represent the immediate response of the blood coagulation and inflammatory system. In previous literature, the immediate inflammatory response following vaccination is measurable at 24 hours after the trigger.[17] Coagulation system activation is related to inflammation and, more specific, to IL-6 release.[21] The onset of thrombosis and thromboembolism following ChAdOx1 vaccination usually occurs after the first 24 hours. By measuring changes in markers related to blood coagulation system activation and inflammation at 24 and 48 hours after vaccination, both the immediate inflammatory response and an eventually increasing trend in coagulation system activation, should be noticed. The occurrence of VITT, which is

generally diagnosed four or more days after vaccination, was not studied. Also, not all possibilities for immediate blood coagulation activation were assessed, such as platelet aggregation, which was not altered in previous literature.[20]

In conclusion, the current study found no evidence of immediate activation of the blood coagulation system 1-2 days following ChAdOx1 vaccination. ChAdOx1 leads to an inflammatory response with increased levels of IL-6, as seen previously with other types of vaccinations. The increase in IL-6, however, does not coincide with extrinsic pathway activation.

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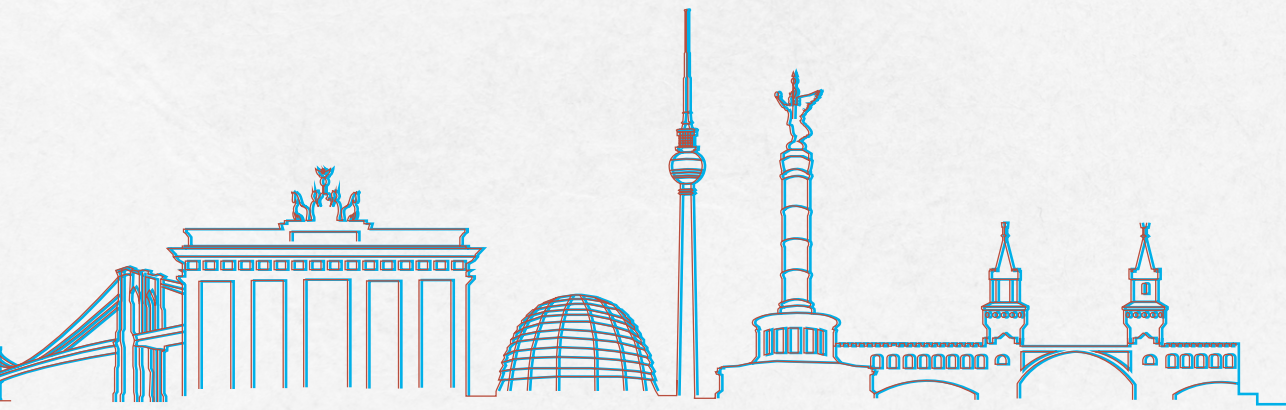
Part II

Dual-pathway inhibition



Chapter 6

A protocol for DUAL pathway inhibition (low-dose rivaroxaban and aspirin) as compared to aspirin only to improve endothelial function in peripheral artery disease

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Abstract

Background: Peripheral artery disease (PAD) patients are at high risk of major adverse cardiovascular events and major adverse limb events. Recent trials demonstrate that rivaroxaban, a factor Xa inhibitor, in addition to single antiplatelet therapy results in lower mortality. A potential explanation is that Factor Xa improves endothelial function through crosstalk between coagulation and inflammatory pathways, subsequently attenuating the occurrence of major adverse cardiovascular and major adverse limb events. In this study, we hypothesize that combined treatment of low-dose rivaroxaban and acetylsalicylic acid improves endothelial function in PAD patients.

Methods: DUAL-PAD is a multicenter, two-arm, phase IV clinical trial. Two cohorts of patients with symptomatic lower extremity PAD are enrolled: a:) moderate PAD (intermittent claudication), b) severe PAD (critical limb ischemia). Participants are treated with acetylsalicylic acid for a 1-month run-in period, followed by 3-months of dual pathway inhibition with acetylsalicylic acid and low-dose rivaroxaban. The primary outcome is the change in proportion of patients with endothelial dysfunction, measured as carotid artery vasoconstriction upon sympathetic stimulation. The secondary endpoint is the change in level of endothelial dysfunction, as reflected by plasma endothelin-1 levels.

Discussion: the aim of the study is to examine if dual pathway inhibition improves endothelial function in patients with moderate or severe PAD.

Introduction

Peripheral artery disease (PAD) patients are at high risk of major adverse cardiovascular events (MACE) and major adverse limb events (MALE), including major limb amputation.[1-4] Recent literature demonstrates that PAD patients benefit from treatment with acetylsalicylic acid (ASA) and low dose rivaroxaban, an oral factor Xa inhibitor.[5] However, the precise mechanism underlying the improved clinical outcomes in PAD patients is currently unknown.

According to the international guidelines, single antiplatelet therapy is indicated if patients have symptomatic PAD, to prevent MACE, MALE and PAD progression.[6] The added value of different anticoagulant strategies have been studied. Oral vitamin K antagonists have not been shown to be superior in PAD patients and have increased rates of major bleedings.[7] Relatively new in the treatment of PAD are direct oral anticoagulants, specifically, rivaroxaban. Rivaroxaban is an oral factor Xa inhibitor and has shown to be effective in treating venous thromboembolic events.[8] In acute coronary syndromes, it has been shown that low-dose rivaroxaban (2.5 and 5.0 mg twice a day), used in addition to dual antiplatelet therapy, reduced the incidence of MACE. However, both doses increased the rates of major and fatal bleedings.[9] Dual pathway inhibition (DPI) by combining low-dose rivaroxaban (2.5 mg twice daily) with single antiplatelet therapy (ASA 100 mg once daily) was studied in PAD patients (COMPASS trial).[10] The COMPASS trial showed that a combination of ASA and low-dose rivaroxaban reduced morbidity and mortality from MACE and MALE in patients with stable PAD, compared to ASA alone. Although a higher rate of major bleedings was identified, the incidence of fatal or critical organ bleedings was not increased.[5]

PAD is a manifestation of systemic atherosclerosis. One of the first signs of atherosclerosis is endothelial dysfunction, which is already present years before clinical symptoms appear.[11] Endothelial dysfunction has been reported in relation with most, if not all, risk factors for atherosclerosis, such as hypertension[12], diabetes[12], hyperlipidemia[14], and ageing[15], and contributes to its progression by promoting coagulation, vasoconstriction, and deficient vascular repair.[16] Presence of endothelial dysfunction, independent from disease state and other risk factors, is strongly related to occurrence of MACE.[17-19] and MALE[17-18].

Accumulating evidence suggests that factor Xa is an important modulator of cellular signaling mechanisms through activation of protease-activated receptor (PAR) mediated signaling. PAR is located on endothelial cells. It has been postulated

that the cross-talk between coagulation and inflammatory pathways contributes to various mechanisms, such as inflammation, leukocyte migration, and endothelial dysfunction, resulting in the initiation of atherosclerosis.[20-22] To prevent PAD progression, reducing vascular inflammation and thereby improving endothelial function may provide an important explanation for the reduced incidence of MACE and MALE as observed in the COMPASS trial. In this study we hypothesize that combined treatment of low-dose rivaroxaban and ASA improves endothelial function in PAD patients with moderate (intermittent claudication) and severe (critical limb ischemia) symptoms. This mechanism may ultimately contribute to the improved clinical outcomes of DPI in PAD patients.

Methods

Objectives

Primary Objective

To study the effectiveness of low-dose rivaroxaban + ASA in improving endothelial function, as measured by the carotid artery reactivity (CAR) test, in patients with (a) intermittent claudication (Rutherford stages I-III) and (b) critical limb ischemia (Rutherford stages IV-VI).[23]

Secondary Objective

To study the effectiveness of low-dose rivaroxaban with ASA in decreasing plasma endothelin-1 levels, as a biomarker of endothelial function, in patients with (a) intermittent claudication (Rutherford stages I-III) and (b) critical limb ischemia (Rutherford stages IV-VI).[23]

Design

DUAL-PAD is an interventional, non-randomized, parallel trial of two single clinical cohorts involving patients with PAD, investigating the effect of combined treatment of low-dose rivaroxaban and ASA on endothelial function. The study is conducted at the Radboud University Medical Center Nijmegen and the Rijnstate Hospital Arnhem, the Netherlands. The protocol has been approved by the regional ethics committee (2019-6036), and local approval has been obtained of each participating site. This study is conducted in accordance with the latest revision of the Declaration of Helsinki and Good Clinical Practice regulations and is registered at ClinicalTrials.gov on January 6th 2020 (URL https://clinicaltrials.gov/ct2/show/NCT04218656?term=NCT04218656&draw=2&rank=1).

Participants

All patients with lower extremity PAD visiting the outpatient clinic are screened. Patients are found eligible if they are aged 16 years or older, have an indication for single antiplatelet therapy according to the international guidelines[6], and have one of the following: (1) intermittent claudication, (2) lower limb pain at rest, (3) lower limb ulcers, (4) previous percutaneous transluminal angioplasty or intentional extraluminal revascularization of the lower limb, (5) previous aorta-iliac, aorta-femoral or lower limb bypass surgery for arterial occlusive disease, (6) previous above-foot amputation. The highest Rutherford classification[23] (now or in the past) per participant is leading for classification into one of the cohorts: (a) intermittent claudication (Rutherford I-III) or (b) critical limb ischemia (Rutherford IV-VI). Detailed exclusion criteria are found in Supplementary Material S1. Key exclusion criteria included: increased bleeding risk, prosthetic valve, severe renal impairment, systemic treatment with CYP3A4 inhibitors or inducers, concomitant treatment with other anticoagulants, and known hypersensitivity to ASA or rivaroxaban.

Procedures

The clinician determines eligibility of patients for inclusion in the study. Eligible patients are informed about the study. Written informed consent is obtained before proceeding with any of the trial procedures. Eligible patients interrupt their daily single anti-platelet therapy and start ASA 100 mg once a day orally (T0) during a 30-day run-in period, except for those who already use ASA 80-100 mg once a day orally. Patients that already use ASA 80-100 mg once daily orally continue their single anti-platelet therapy during the run-in period. After the run-in period (T1), medication adherence is evaluated by interview. When medication adherence is less than 80%, the participant is excluded and replaced with a maximum of 10 participants per group. When medication adherence is at least 80%, endothelial function is determined by the CAR test and plasma endothelin-1 levels (measured by enzyme-linked immunosorbent assay, ELISA). Subsequently, participants receive DPI with low-dose rivaroxaban 2.5 mg twice a day orally and ASA 100 mg once a day orally. After 3 months (T2), the CAR test and venous blood sampling for plasma endothelin-1 measurement, are repeated. Hereafter, DPI is discontinued and replaced by single antiplatelet therapy. Continuation of DPI can be discussed with the clinician. Participants can leave the study at any time for any reason if they wish to do so without any consequences. The investigator can decide to withdraw a subject from the study for urgent medical reasons. *Table 1* represents an overview of the time schedule and study procedures.

Table 1: *study schedule*

Activity	T0 *(study start)*	T1 *(T0 + 1 month)*	T2 *(T0 + 4 months)*
Informed consent	X		
Stop regular single antiplatelet therapy	X		
Start acetylsalicylic acid 100 mg once daily	X		
Start rivaroxaban 2.5 mg twice daily		X	
Carotid artery reactivity test		X	X
Venous blood sampling for plasma endothelin-1 ELISA		X	X
Stop study medication			X
Restart regular single antiplatelet therapy			X

CAR test

The CAR test assesses endothelial function as reflected by carotid artery diameter response to sympathetic stimulus. In agreement with coronary arteries[24], the carotid artery dilates in response to sympathetic stimulation in healthy individuals, whilst vasoconstriction is present in those with cardiovascular risk factors or disease[25-26]. Participants are in the supine position with the neck extended for assessment of the carotid artery. Left carotid artery diameter is recorded continuously during baseline (30 seconds) and during immersion of the right hand up to the wrist in icy water (4°C) for 3 minutes with a L12-4 MHz linear array probe of Philips Lumify (Philips Healthcare, Best, The Netherlands), app-based ultrasound solution, linked to a compatible smart device. Custom-designed edge-detection and wall-tracking software is used. Baseline diameter is calculated as the mean across 30 seconds preceding cold water immersion (control interval). Area under the curve (AUC) relative to baseline is calculated for the subsequent 3 minutes. A net negative AUC is defined as CAR constriction and a net positive AUC is defined as CAR dilatation. The absolute CAR value (CAR%) is the peak change percentage constriction or dilatation computed as the mean diameter over a 10-second interval – excluding control interval – most deviating from baseline diameter, divided by baseline diameter, multiplied by 100%.[24-26]

Venous blood sampling

Blood samples will be taken at T1 and T2. Poor platelet plasma will be separated from whole blood samples and stored at -80°C. At the end of the study, endothelin-1 levels – a marker of atherosclerosis grade – will be determined by ELISA according to manufacturer's instructions.

Adverse events

Participants are requested to report any adverse events experienced during study participation. Adverse events are defined as any undesirable experience occurring to a subject during the study, whether or not considered related to the investigational product/study procedure. All adverse events reported by the subject or observed by the research team are recorded.

Outcomes

The primary outcome is the change in endothelial dysfunction expressed as the change in the proportion of participants with CAR constriction from baseline (ASA alone) to 3 months after adding low-dose rivaroxaban.

The secondary outcome is the change in mean endothelin-1 level from baseline (ASA alone) to 3 months after adding low-dose rivaroxaban.

Exploratory outcomes are MACE, MALE, (major) bleeding complications and medication adherence. MACE include non-fatal myocardial infarction, non-fatal ischemic stroke, and cardiovascular death. MALE include acute limb ischemia, deterioration of Rutherford classification[23], need for peripheral revascularization, and amputation. Major bleeding is defined as the composite of bleeding that was fatal, symptomatic bleeding into a critical organ, surgical site bleeding requiring reoperation, or bleeding requiring hospitalization (including presentation to an acute care facility without an overnight stay).

Sample size

The COMPASS trial[5] demonstrated in a cohort of PAD patients that 9% of participants treated with acetylsalicylic acid and 6% of participants receiving DPI developed adverse cardiovascular events. Based on previous literature[25], 40% of the patients with intermittent claudication (Rutherford I-III) and 60% of the patients with critical limb ischemia (Rutherford IV-VI) will have a CAR constriction at baseline and the hazard ratio for development of adverse cardiovascular events in this population is 4, with CAR dilatation used as a reference. These data suggest a relative reduction in the proportion of patients with CAR constriction after addition of rivaroxaban twice a day 2.5 mg of approximately 40%. Based on the correlation between the two CAR response measurements of the first 30 participants, power has been calculated for 35% discordant pairs using McNemar's Z-test. With an alpha of 5% and a power of 80%, 105 patients with intermittent claudication (Rutherford I-III) and 46 patients with critical limb ischemia (Rutherford VI-VI) are required to detect a 40% relative reduction in the proportion of participants with CAR constriction from baseline (T1) to 3 months

of DPI (T2), resulting in a final sample size of 151. To allow for a dropout rate of 5%, the final sample size was 159 participants (111 Rutherford I-III and 48 Rutherford IV-VI).

Data collection and management

Study subjects are identified only by a unique subject code, consisting of two parts. The first part refers to the study site where the participant is under treatment, and the second part is a unique personally assigned identification code. Baseline values, such as gender, age, height, weight, smoking, comorbidities (hyperlipidemia, hypertension, and diabetes), and medication use are collected from the medical records and stored on the secured Castor (Castor Electronic Data Capture, Amsterdam, the Netherlands) servers. Case report forms are used to obtain information about current height, weight, smoking behavior, drinking behavior and past infection with the corona virus disease 2019 (COVID-19). During the participants' visits, ultrasound videos are recorded of the CAR test and stored for subsequent analysis. All patient data and ultrasound videos will be stored at the Digital Research Environment of Radboudumc for 15 years. Collected poor platelet plasma will be stored at the surgical laboratory of the Radboudumc for 5 years.

Analysis

All patients are evaluated, except those who withdraw and those with incomplete follow-up regarding the primary endpoint. Change in proportion of participants with CAR constriction from baseline to 3 months after addition of rivaroxaban per group is analyzed using McNemar's Z-test, 2-sided equality. CAR% is reported as mean with minimum and maximum value and analyzed using the paired sample t-test. Differences in plasma endothelin-1 levels are analyzed using the paired sample t-test. Other secondary endpoints (MACE, MALE, major bleeding, and medication adherence) are dichotomized and reported as percentage. Categorical and continuous baseline variables are recorded as percentage and mean, respectively. Analyses are performed using IBM SPSS statistics 25. P values below 0.05 are considered significant.

Monitoring

An independent, accredited monitor is appointed to verify that participants' well-being is protected, reported trial data are accurate, and the trial is conducted in compliance with the currently approved protocol. Periodic monitoring visits are conducted with adequate frequency. Site initiation visits are performed when the site requirements for study participation are met. The local research team is informed about most recent version of study protocol, standard operating procedures, case report forms, and management of the online system for data collection.

Discussion

The DUAL-PAD trial is an investigator-initiated multicenter phase IV clinical trial to evaluate the effect of combined treatment of low-dose rivaroxaban and ASA on endothelial function in patients with PAD. The benefit of ASA monotherapy in symptomatic PAD has been established in a meta-analysis of 42 randomized controlled trials. In 9200 patients with symptomatic PAD, ASA monotherapy significantly reduced MACE.[27] The COMPASS trial demonstrated an additional reduction in both MACE and MALE by adding low-dose rivaroxaban to standard ASA treatment in patients with stable PAD.[5] Very recently, the VOYAGER-PAD trial established comparable benefits of low-dose rivaroxaban in patients who had undergone lower-extremity revascularization.[28] No studies, however, have investigated the mechanism underlying the benefit of rivaroxaban in PAD. Understanding this mechanism is relevant to further optimize the treatment of PAD patients. Indeed, if the positive outcomes of the COMPASS trial are closely related to specific improvements in endothelial function, future work should specifically aim at enhancing endothelial function. Moreover, studies can use endothelial function as an early marker to explore effectiveness of (non)pharmacological strategies in PAD.

Endothelial dysfunction is a strong predictor of MACE and MALE[17-19] and occurs in relation with cardiovascular risk factors.[11-15,29] The CAR test is validated to assess endothelial function by measuring the carotid artery diameter in response to sympathetic stimulation. The CAR-response appears strongly related to cardiovascular risk, is closely related to coronary artery endothelial function, and independently predicts 1-year occurrence of MACE in patients with lower extremity PAD.[24-25] An alternative source of information on endothelial function and PAD development is blood. A marker with potential prognostic value is the plasma endothelin-1 level. Plasma endothelin-1 levels correlate with atherosclerosis grade.[30] Other information, obtained from blood samples, relates to peripheral gene expression. Literature suggests that many risk factors for PAD (i.e. age, smoking, diabetes) promote inheritable changes in gene expression.[31] The effect of DPI on endothelin-1 levels, and peripheral gene expression, but also the prognostic value of gene expression in PAD, are an unexplored field and might evolve valuable.

The DUAL-PAD trial is a multicenter trial, conducted at a secondary and tertiary health care centrum. No patients from primary care will be included. Therefore, the applicability of our results will be limited to the patients with more advanced PAD. Since MACE and MALE are most common in advanced PAD, this group may have the most potential for clinical benefit. Few studies suggest good reproducibility of the

CAR test in asymptomatic, healthy volunteers.[24] However, no literature is available regarding the intraindividual variability of the CAR test in patient with symptomatic cardiovascular disease. To prevent insufficient power, the sample size has been calculated based on the prevalence of discordant pairs of the first 30 participants.

The results of the DUAL-PAD trial will improve knowledge about the mechanism of action of rivaroxaban in PAD by investigating its effect on improving endothelial function. Additionally, the trial will examine the benefit for patients with intermittent claudication and critical limb ischemia separately. This knowledge is required for implementation of rivaroxaban in current practice and for identifying patients who will benefit most from DPI.

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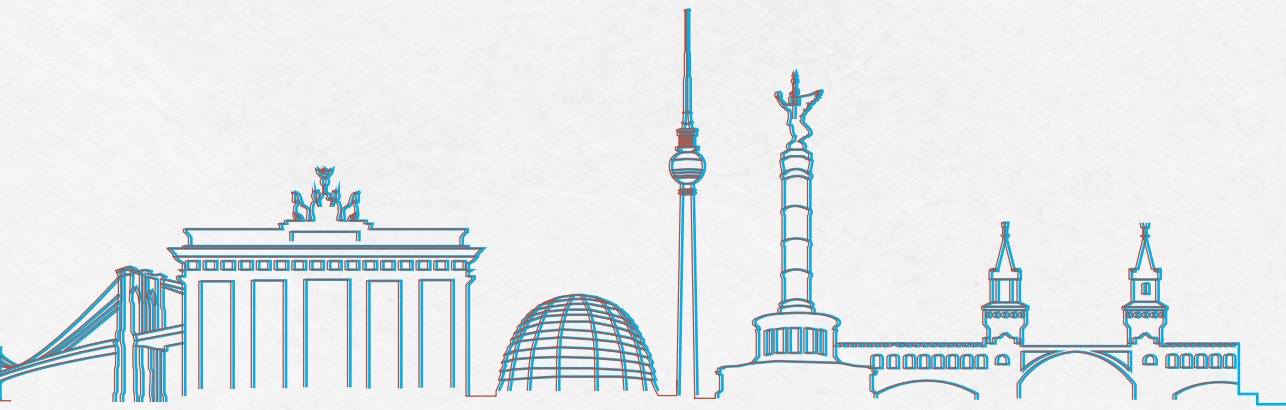
Supplemental material 1 (S1): exclusion criteria

A potential subject who meets any of the following criteria will be excluded from participation in this study:

- Patients having or at risk of major bleeding:
  - Gastrointestinal ulceration
  - Current malignant neoplasms
  - Brain or spinal injury
  - Brain, spinal or ophthalmic surgery
  - Intracranial hemorrhage
  - Known or suspected esophageal varices
  - Arteriovenous malformations
  - Major intraspinal or intracerebral vascular abnormalities
  - Hepatic disease associated with coagulopathy and clinically relevant bleeding risk, including cirrhotic patients with Child Pugh B and C
  - Use of selective serotonin reuptake inhibitors or serotonin-norepinephrine reuptake inhibitors
- Patients with prosthetic valves
- Patients with a history of asthma attacks caused by salicylates
- Severe renal impairment (creatinine clearance <30 ml/min)
- Systemic treatment with moderate and strong CYP3A4inhibitors:
  - Protease inhibitors: ritonavir, indinavir, nelfinavir, saquinavir
  - Some antibiotics: clarithromycin, telithromycin, erythromycin, roxithromycin, chloramphenicol, ciprofloxacin
  - Azole antifungals: ketoconazole, itraconazole, posaconazole, voriconazole, fluconazole, miconazole
  - Some antidepressants: nefazodone, fluvoxamine
  - Some antiarrhytmic medication: amiodarone, donedarone
  - Some calcium channel blockers: verapamil, diltiazem
  - Other medication: cobicistat, aprepitant, cimetidine, cyclosporin, imatinib
- Systemic treatment with CYP3A4 inducers
  - Some androgen antagonists: enzalutamide, apalutamide
  - Some antiepileptic drugs: carbamazepine, phenytoin, oxcarbazepine, topiramate, phenoparbital
  - Some antibiotics: rifampicin, rifabutin
  - Some antiretroviral drugs: efavirenz, nevirapine
  - Other medication: modafinil

- Concomitant treatment with other anticoagulants
- Concomitant treatment with methotrexate at a weekly dosage of >15 mg
- Pregnant or lactating
- Known hypersensitivity to acetylsalicylic acid or rivaroxaban



Chapter 7

Dual pathway inhibition as compared to acetylsalicylic acid monotherapy in relation to endothelial function in peripheral artery disease, a phase IV clinical trial

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Abstract

Objective: Dual pathway inhibition (DPI) by combining acetylsalicylic acid (ASA) with low-dose rivaroxaban has been shown to reduce cardiovascular events in patients with peripheral arterial disease (PAD) when compared to ASA monotherapy. A potential explanation is that inhibition of factor Xa improves endothelial function through crosstalk between coagulation and inflammatory pathways, subsequently attenuating the occurrence of cardiovascular events. We hypothesize that the addition of rivaroxaban to ASA in PAD patients leads to improved endothelial function.

Design: An investigator-initiated, multicenter trial investigating the effect of DPI on endothelial function.

Methods: Patients, diagnosed with PAD, were enrolled in two cohorts: cohort A (Rutherford I-III) and cohort B (Rutherford IV-VI). Participants received ASA monotherapy for a 4-weeks run-in period, followed by 12 weeks of DPI. Macro- and microvascular endothelial dysfunction were studied by measuring carotid artery reactivity upon sympathetic stimulus and by measuring plasma endothelin-1 concentrations, respectively. All measurements were performed during the use of ASA (baseline) and after 12 weeks of DPI.

Results: 159 PAD patients (111 cohort A, 48 cohort B) were enrolled. Twenty patients discontinued study drugs early. Carotid artery constriction upon sympathetic stimulation at baseline (ASA) and after 12 weeks of DPI was similar in the total group, 22.0% versus 22.7% (p=1.000), and in the subgroups (Cohort A 22.6% versus 23.7%, p=1.000; cohort B 20.5% versus 20.5%, p=1.000), respectively. The mean concentration of plasma endothelin-1 at baseline and after 12 weeks of DPI did not differ, 1.70±0.57 pmol/L versus 1.66±0.64 pmol/L (p=.440) in the total group, 1.69±0.59 pmol/L versus 1.62±0.55 pmol/L in cohort A (p=.202), and 1.73±0.53 pmol/L versus 1.77±0.82 pmol/L in cohort B (p=.682), respectively.

Conclusion: Macro- and microvascular endothelial dysfunction, as reflected by carotid artery reactivity and plasma endothelin-1 concentrations, are not influenced in PAD patients by addition of low-dose rivaroxaban to ASA monotherapy for 12 weeks.

Introduction

Patients with peripheral arterial disease (PAD) are at high risk of developing cardiovascular events[1-2], for which single antiplatelet therapy (SAPT) is indicated.[3-4] Recently, the COMPASS trial demonstrated that dual pathway inhibition (DPI), where acetylsalicylic acid (ASA) is combined with low-dose rivaroxaban (2.5 mg twice daily), reduces the rate of major adverse cardiovascular (MACE) and limb (MALE) events, compared to ASA monotherapy.[5] The VOYAGER-PAD trial confirmed these findings in PAD patients, who underwent peripheral revascularization.[6] The precise mechanism underlying the benefit of rivaroxaban in PAD patients is currently unclear.

Rivaroxaban is an oral inhibitor of factor Xa, the first enzyme in the 'common pathway' of the coagulation cascade. Besides this prominent role in the coagulation cascade, previous literature demonstrates that factor Xa can activate protease-activated receptors (PAR) on the surface of endothelial cells in the inner lining of the arterial wall. By binding to PAR, factor Xa is capable of modulating inflammatory pathways, contributing to vascular inflammation, leukocyte migration and endothelial dysfunction.[7-9]

Endothelial dysfunction contributes to the development and progression of atherosclerosis[10] and is generally present years before the patient develops symptomatic disease[11]. Other risk factors for developing atherosclerotic disease have also been related to endothelial dysfunction, including hypertension[12], hyperlipidaemia[13], diabetes mellitus[14], and ageing[15]. Presence of endothelial dysfunction, independent from disease state and other risk factors, is strongly related to the occurrence of MACE[16-18] and MALE[16-17]. Risk-reducing interventions for atherosclerosis, such as physical activity and lipid-lowering drug therapy, have been shown to improve endothelial dysfunction.[19-21] Rivaroxaban may therefore potentially improve endothelial function, thus contributing to the previously demonstrated reduction in MACE in PAD patients.

A simple and non-invasive test has been developed and validated to assess macrovascular endothelial function by measuring carotid artery reactivity (CAR) in response to a sympathetic stimulus. The CAR response was proven to be strongly related to cardiovascular risk, closely related to coronary artery endothelial function, and independently predictive for the 1-year occurrence of MACE in PAD patients.[22-23] Microvascular endothelial dysfunction, on the other hand, has been strongly related to plasma concentrations of endothelin-1 (ET-1).[24] ET-1 is a potent

vasoconstrictor peptide[25] and has been suggested to be a potential target for treating microvascular endothelial dysfunction in atherosclerosis[26].

In this study, we hypothesize that DPI, by combining ASA with low-dose rivaroxaban for 12 weeks, reduces macro- and microvascular endothelial dysfunction in symptomatic PAD patients.

Materials and Methods

This is an investigator-initiated, non-randomized, multicenter parallel trial of two clinical cohorts investigating the effect of DPI (ASA with low-dose rivaroxaban) on endothelial function in PAD patients. The study was approved by the regional ethics committee and the local directory boards. The study was conducted in accordance with the latest revision of the Declaration of Helsinki and Good Clinical Practice regulations and is registered at ClinicalTrials.gov on January 6th, 2020 (NCT04218656) and at the Dutch Trial Register on September 22nd, 2020 (NL8908). Written informed consent was obtained from all participants. The protocol has been published previously.[27]

Participants

Patients with lower extremity PAD and an indication for SAPT according to the current guidelines[3-4] were recruited. Based on the severity of PAD, patients were divided into cohort A (intermittent claudication, Rutherford I-III) and cohort B (chronic limb-threatening ischemia (CLTI), Rutherford IV-VI), where the highest Rutherford classification recorded for a patient (either now or in the past) was used to allocate a patient into one of the two cohorts.[28] Patients with an increased bleeding risk, severe renal impairment, systemic treatment with CYP3A4 inhibitors/inducers, concomitant treatment with other anticoagulants, and known hypersensitivity to ASA/rivaroxaban were excluded. A detailed description of the in- and exclusion criteria can be found in our previously published protocol.[27]

Procedures

Eligible patients received low-dose (80-100 mg once daily) ASA monotherapy during a 4-weeks run-in period. After 4 weeks, medication adherence was evaluated by interview (T1). Participants with an adherence below 80% were excluded and replaced. Patients who dropped out before T1 and did not fulfil the run-in period were considered non-adherent and were replaced. Subsequently, at T1, participants were prescribed DPI by ASA (100 mg once daily) plus low-dose rivaroxaban (2.5 mg

twice daily) for 12 weeks. After these 12 weeks (T2), DPI was discontinued, and participants could resume SAPT. During study participation, the occurrence of severe adverse events (SAE) was recorded, including myocardial infarction, stroke, acute limb ischemia (ALI), deterioration of PAD as classified by Rutherford[28], need for peripheral revascularization, lower extremity amputation, major bleeding and death.

Outcomes

The primary outcome was a change in macrovascular endothelial dysfunction represented by a change in the proportion of participants with a constrictive CAR response upon sympathetic stimulus from T1 (ASA monotherapy) to T2 (after 12 weeks of DPI). The secondary outcome is a change in microvascular endothelial dysfunction represented by a change in mean plasma ET-1 level from T1 to T2.

We explored changes in the activation of the common pathway by measuring markers of thrombin generation, including thrombin:antithrombin (TAT) complexes and prothrombin fragment 1+2 (F1.2). Additionally, activated coagulation factor XI in complex with its natural inhibitor antithrombin (FXIa:AT) was measured, in order to assess for changes in activation of the intrinsic pathway (positive feedback loop mechanism).

The carotid artery reactivity test

The CAR test assesses endothelial function by measuring the change in diameter of the common carotid artery in response to a sympathetic stimulus (cold pressor test). The common carotid artery was visualized using Philips Lumify ultrasound device (Philips Healthcare, Best, The Netherlands) with a L12-4 MHz linear array probe, during a 30 second baseline, and during a subsequent 3 minutes of sympathetic stimulation by hand in ice water immersion. The common carotid artery diameter was measured with semi-automatic custom-designed edge-detection and wall-tracking software by an investigator, blinded for the test moment (T1 or T2). The area under the curve (AUC) relative to baseline was calculated. A net positive AUC represents a dilatory response, and a net negative AUC represents a constrictive response. Additionally, the peak change percentage dilatation or constriction (CAR%) was computed as the mean diameter over a 10-second interval – excluding control interval – most deviating from baseline diameter, relative to baseline.

To limit the influence of external factors on the CAR test, participants were instructed 1) not to eat or drink anything except for water in the 6 hours preceding their appointment, 2) not to have beverages with caffeine, alcohol, or any products that are high in vitamin C in the 18 hours preceding their appointment, and 3) not

to do heavy exercise training in the 24 hours preceding their appointment. Each patient underwent endothelial testing twice, at T1 and at T2. Time of appointment was generally between 9 and 12 AM and was equal for both T1 and T2.

Blood sampling

Venous blood samples were collected by venipuncture in 10 ml Lithium-Heparin (Vacuette) tubes. Platelet poor plasma was prepared by centrifuging whole blood at 2500g for 10 minutes followed by a second centrifugation step at 2500g for 20 minutes, both at room temperature. The platelet poor plasma was stored at -80°C until further analysis. Plasma concentrations of ET-1 were quantified using commercial ELISA kits (R&D, Minneapolis (Minnesota), United States of America). Plasma concentrations of TAT and FXIa:AT were quantified by in-house developed ELISA methods as described previously[29] and F1.2 was quantified using commercially available assays (Enzygnost™ F1+2, Siemens Healthineers, The Hague, the Netherlands).

Statistical analyses

This study was powered to detect a relative reduction in the proportion of patients with CAR constriction when switching from ASA to DPI of approximately 40%. Based on previous literature, the expected prevalence of CAR constriction at baseline was 40% in cohort A (intermittent claudication) and 60% in cohort B (CLTI).[22] With an alpha of 5%, a power of 80%, and allowing for a drop-out rate of 5%, the final sample size was 159 participants (111 in cohort A and 48 in cohort B).

Change in proportion of participants with CAR constriction from T1 to T2 was compared using McNemar's Z-test, 2-sided equality. Differences in CAR% and plasma ET-1 levels from T1 to T2 was analyzed using the paired sample t-test. Differences in plasma concentrations of the coagulation markers TAT, F1.2 and FXIa:AT were explored using the Wilcoxon signed ranks test.

Categorical and continuous baseline variables are recorded as percentage and mean, respectively. SAEs were reported as numbers. Analyses are performed using IBM SPSS statistics 25. P values below .05 were considered significant.

Results

Enrolment and baseline characteristics

Between June 2020 and August 2021, 159 patients with symptomatic PAD were enrolled, with an additional 12 patients being enrolled to replace participants with

an adherence below 80% at T1 (*figure 1*). In total, 111 patients with intermittent claudication (cohort A) and 48 patients with CLTI (cohort B) completed the run-in period and were started on DPI. Of these, 20 discontinued the trial. The most common reasons for not continuing the trial were side effects associated with the study medication (*figure 1*). Baseline characteristics are shown in *table 1*. The mean age of the participants was 67 years and 66% were male. Most patients used ASA as SAPT (64.8%) before study participations, while 35.2% used clopidogrel.

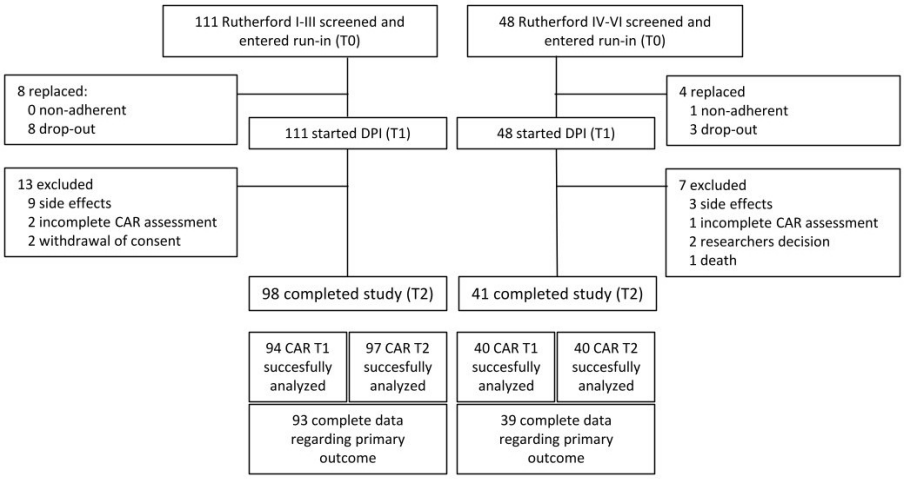

Figure 1: *Flow diagram of enrolment and outcomes*

Endothelial function

The CAR response was successfully assessed in 134/139 patients after a 4-weeks run-in period of ASA monotherapy (T1) and in 137/139 patients after 12 weeks of DPI (T2). Complete paired assessment was obtained of 132 participants, 93 in Cohort A and 39 in cohort B (figure 1). The numbers of patients with a CAR dilatory response and a CAR constrictive response at T1 and T2, respectively, are presented in *table 2*. The proportion of patients with a constrictive CAR response at T1 and T2 was similar in the total group, 22.0% versus 22.7% (p=1.000), respectively. Subsequent analysis on cohort A and cohort B revealed comparable trends in constrictive response rates, 22.6% versus 23.7% (p=1.000) for cohort A and 20.5% versus 20.5% (p=1.000) for cohort B, respectively. Consistent with this, the peak change in percentage of carotid artery diameter revealed no differences between T1 and T2 with a mean CAR% of 1.92 ± 2.88 versus 1.69 ± 3.09 (p=.510) in the total group, 2.03 ± 2.98 versus 1.71 ± 2.96 in cohort A (p=.442), and 1.66 ± 2.64 versus 1.63 ± 3.42 in cohort B (p=.969), respectively (*table 3*).

The mean plasma ET-1 levels at T1 and T2 did not differ significantly and were 1.70 ± 0.57 pg/mL versus 1.66 ± 0.64 pg/mL (p=.440) for the total group, 1.69 ± 0.59 pg/mL versus 1.62 ± 0.55 pg/mL (p=.202) for cohort A, and 1.73 ± 0.53 pg/mL versus 1.77 ± 0.82 pg/mL (p=.682) for cohort B (*table 3*).

Table 1: *baseline characteristics of the patients that successfully completed the run-in period*

	All, n=159	**Cohort A, n=111**	**Cohort B, n=48**
Age (mean ± SD)	67 ± 8	67 ± 8	68 ± 9
Male (n, %)	105 (66.0)	77 (69.4)	28 (58.3)
BMI (mean ± SD)	26.8 ± 4.6	27.1 ± 4.7	26.1 ± 4.2
Tobacco use (n, %)			
Current	52 (32.7)	31 (27.9)	21 (43.8)
Former	98 (61.6)	74 (66.7)	24 (50.0)
Never	9 (5.7)	6 (5.4)	3 (6.3)
Alcohol use (n, %)			
Never	43 (27.0)	29 (26.1)	14 (29.2)
Rarely	17 (10.7)	11 (9.9)	6 (12.5)
Monthly	14 (8.8)	13 (11.7)	1 (2.1)
Weekly	46 (28.9)	33 (29.7)	13 (27.1)
Daily	39 (24.5)	25 (22.5)	14 (29.2)
Previous intervention for PAD (n, %)	105 (66.0)	64 (57.7)	41 (85.4)
Endovascular revascularization	100 (62.9)	64 (57.7)	36 (75.0)
Thrombendarteriectomy	12 (7.5)	5 (4.5)	7 (14.6)
Bypass surgery	18 (11.3)	6 (5.4)	12 (25.0)
Lower extremity amputation	5 (3.1)	0 (0.0)	5 (10.4)
Amputation of toe(s)	3 (1.9)	0 (0.0)	3 (6.3)
Thrombolysis	2 (1.3)	0 (0.0)	2 (4.2)
Embolectomy	1 (0.6)	0 (0.0)	1 (2.1)
Comorbidity (n, %)			
Hypertension	114 (71.7)	79 (71.2)	35 (72.9)
Hyperlipidemia	71 (44.7)	51 (45.9)	20 (41.7)
Ischaemic heart disease	50 (31.4)	33 (29.7)	17 (35.4)
CVA/TIA	20 (12.6)	12 (10.8)	8 (16.7)
Diabetes mellitus	52 (32.7)	36 (32.4)	16 (33.3)
Asthma/COPD	42 (26.4)	30 (27.0)	12 (25.0)
Medication before study participation (n, %)			
Acetylsalicylic acid	103 (64.8)	75 (67.7)	28 (58.3)
Clopidogrel	56 (35.2)	36 (32.4)	20 (41.7)
Lipid lowering drugs	146 (91.8)	102 (91.9)	44 (91.7)
Antihypertensive drugs	117 (73.6)	82 (73.9)	35 (72.9)

Cohort A: intermittent claudication, Rutherford I-III. Cohort B: chronic limb-threatening ischemia, Rutherford IV-VI.

BMI = body mass index, PAD = peripheral arterial disease, CVA = cerebrovascular accident, TIA = transient ischaemic attack, COPD = chronic obstructive pulmonary disease.

Table 2: *total patients with a CAR dilatory response and a constrictive response at the test moments T1 and T2*

All participants, N=132		**CAR response at T2, after 12 weeks of dual pathway inhibition**		
		Dilatation	**Constriction**	**Total**
CAR response at T1, after 4 weeks of ASA monotherapy	Dilatation	80	23	103
	Constriction	22	7	29
	Total	102	30	132
Cohort A, n=93		CAR response at T2, after 12 weeks of dual pathway inhibition		
		Dilatation	Constriction	Total
CAR response at T1, after 4 weeks of ASA monotherapy	Dilatation	55	17	72
	Constriction	16	5	21
	Total	71	22	93
Cohort B, n=39		CAR response at T2, after 12 weeks of dual pathway inhibition		
		Dilatation	Constriction	Total
CAR response at T1, after 4 weeks of ASA monotherapy	Dilatation	25	6	31
	Constriction	6	2	8
	Total	31	8	39

Cohort A: intermittent claudication, Rutherford I-III. Cohort B: chronic limb-threatening ischemia, Rutherford IV-VI.
CAR = carotid artery reactivity, ASA = acetylsalicylic acid

Coagulation activity

In vivo coagulation activity of the common and intrinsic pathway was measured at T1 and T2 (*table 4*). The mean plasma concentration of TAT and F1.2 significantly decreased with DPI compared to ASA monotherapy, 1.13±3.37 µg/L versus 0.99±3.82 µg/L (p=.013) and 386.43±204.41 pmol/L versus 258.24±153.79 pmol/L (p<.001), at T1 and T2 respectively. There was no significant difference in FXIa:AT levels between T1 and T2, 17.68±25.69 pM versus 16.90±21.59 pM (p=.949).

Table 3: *the effect of DPI on endothelial dysfunction as represented by the CAR response, CAR%, and plasma endothelin-1 levels*

	T1 (ASA)	T2 (DPI)	*p*
CAR response, constriction, n (%)			*McNemar's test*
All	29 (22.0)	30 (22.7)	1.000
Cohort A	21 (22.6)	22 (23.7)	1.000
Cohort B	8 (20.5)	8 (20.5)	1.000
CAR%, mean ± SD			*Paired sample t-test.*
All	1.92 ± 2.88	1.69 ± 3.09	510
Cohort A	2.03 ± 2.98	1.71 ± 2.96	.442
Cohort B	1.66 ± 2.64	1.63 ± 3.42	.969
Plasma endothelin-1, pg/mL, mean ± SD			*Paired sample t-test.*
All	1.70 ± 0.57	1.66 ± 0.64	440
Cohort A	1.69 ± 0.59	1.62 ± 0.55	.202
Cohort B	1.73 ± 0.53	1.77 ± 0.82	.682

Cohort A: intermittent claudication, Rutherford I-III. Cohort B: chronic limb-threatening ischemia, Rutherford IV-VI.
CAR = carotid artery reactivity, SD = standard deviation, ASA = acetylsalicylic acid, DPI = dual pathway inhibition

Table 4: *the effect of DPI on activation of the common and intrinsic pathway of coagulation*

	T1 (ASA)	T2 (DPI)	p
TAT, µg/L, mean ± SD	1.13±3.37	0.99±3.82	.013
F1,2, pmol/L, mean ± SD	386.43±204.41	258.24±153.79	<.001
FXIa:AT, pM, mean ± SD	17.68±25.69	16.90±21.59	.949

*TAT = thrombin:antithrombin, F1,2 = fragment 1+2, FXI:aAT = factorXI:antithrombin, SD = standard deviation, ASA = acetylsalicylic acid, DPI = dual pathway inhibition*Adverse events

In total, 18 SAEs were experienced by 16 patients (*supplementary table 1*). The two patients who experienced ALI had a second severe adverse event: one underwent a peripheral transluminal angioplasty of the iliac arteries, while the other decided for a palliative policy and died. Both patients used ASA before study participation and suffered from ALI during the run-in phase. The other 14 SAEs occurred during DPI treatment (*supplementary table 1*).

Possible side effects were reported by 31 participants, 4 of whom reported two possible side effects. The onset of the possible side effects was during DPI treatment for 28 of 31 participants. The most common side effects were minor bleeding problems (n=11), skin rash (n=6) and gastro-intestinal complaints (n=5). Thirteen participants withdrew from study participation because of possible side effects of study medication (ASA 1, rivaroxaban 12).

Discussion

In this study we investigated the potential impact of switching ASA monotherapy to DPI, by combining ASA with low-dose rivaroxaban, on macro- and microvascular endothelial dysfunction in patients with PAD and observed no differences. While the addition of rivaroxaban resulted in suppression of markers of thrombin formation, demonstrating an overall anticoagulant effect, the carotid artery response upon sympathetic stimulation and plasma ET-1 concentrations were similar after 4 weeks of ASA monotherapy and after 12 weeks of DPI.

Classically, macrovascular endothelial dysfunction was angiographically determined by detecting endothelium-dependent vasodilatation of the coronary arteries as response to an increase of endothelium derived nitric oxide by infusion of acetylcholine[30] or blood flow increasing medication[31]. Due to risks related to the invasive character of these methods, non-invasive detection of endothelial dysfunction by vascular ultrasound gained interest. In this study, the CAR in response to cold pressor testing (sympathetic stimulus) was used. The CAR test is a simple, non-invasive test using an easily accessible vascular bed to assess macrovascular endothelial function. The CAR test closely relates to coronary artery endothelial function as tested by the classical invasive methods and has shown to be strongly related to cardiovascular risk in the population of peripheral arterial disease.[22] Endothelial functions such as modulation of vascular tone, thrombogenicity and inflammation, are regulated by molecules, amongst which ET-1.[32] Plasma concentrations of ET-1 strongly relate to microvascular endothelial function[24] and are therefore an easy target to evaluate changes in its function. Previous research has even suggested ET-1 as a potential for treating microvascular endothelial dysfunction in atherosclerosis.[26]

To our best knowledge, this study is the first to investigate changes in endothelial function by antithrombotic drugs. Previous literature has addressed the effect of lipid-lowering drugs on endothelial dysfunction. The impaired endothelial-dependent responses (i.e., nitric-oxide mediated vasodilatation), present in patients with hypercholesterolemia, can be reversed by lowering cholesterol levels using lipid-lowering therapies. This effect can already be observed after one month and persists with continued therapy.[20,33-34] Furthermore, other cardiovascular risk reducing interventions have been shown to improve endothelial function. Exercise training augments endothelial dependent vasodilatation, provoked by acetylcholine infusion, in both coronary vessels and resistance vessels in atherosclerotic patients.[19] The same effect can be observed by non-invasive

methods of measuring endothelial dysfunction. Buckley et al demonstrated significant improvements in vascular health after a 12-week physical activity program in patients with cardiovascular risk factors and in patients with manifest cardiovascular disease, with reversed carotid artery constriction in response to sympathetic stimulus and increased brachial artery flow-mediated dilatation.[21,35]

Improved endothelial dysfunction thus correlates with a reduction in cardiovascular risk factors and might subsequently reduce the occurrence of MACE. This is in line with the results of van Mil et al, who demonstrated that patients with a constrictive CAR response have a 4-fold increased risk of developing MACE and a 2-fold increased risk for clinical deterioration, in patients with manifest PAD. Low-dose rivaroxaban in addition to ASA has been shown to reduce the occurrence of myocardial infarction, stroke, ALI and cardiovascular death, in patients with PAD.[5-6] The current study, however, could not confirm an improvement in endothelial function underlying this benefit of rivaroxaban in PAD patients.

Rivaroxaban is an oral inhibitor of factor Xa, which plays a crucial role in the coagulation cascade by cleaving prothrombin, yielding the active thrombin. During this process, a fragment of prothrombin called F1,2 is released next to thrombin itself. Thrombin activates the intrinsic pathway through a positive feedback loop mechanism converting FXI into its active metabolite FXIa.[36] Both thrombin and FXIa will be rapidly bound by antithrombin in circulation, generating TAT and FXI:AT, respectively. Treatment with rivaroxaban thus leads to a decrease in generation of active thrombin, coinciding with a decrease of TAT, FXIa, FXI:AT and F1,2. Since active thrombin is only present in circulation for a very short time, TAT, FXI:AT and F1,2 are acknowledge as more useful measures for thrombin level in the blood. A significant decrease in markers of thrombin generation is observed in participants at T2 compared to T1. This is consistent with the addition of rivaroxaban to ASA monotherapy and provides a strong indication that the negative findings regarding endothelial function in our study were not caused by non-adherence to medication. Since other studies on anticoagulants failed to show a clinical benefit on MACE in both the short- and long-term follow-up, it is highly unlike that the clinical benefit of rivaroxaban is fully explained by its effect on coagulation.[37]

In addition to its role in the coagulation cascade, factor Xa has been identified as a direct agonist of PAR-1 leading to thrombin independent platelet activation and thrombus formation. By inhibiting factor Xa, rivaroxaban can attenuate platelet aggregation, in addition to its antithrombotic effect.[7-8,38-39] In mice, administration of rivaroxaban for 20 weeks reduced thrombus formation and atherosclerotic plaque

destabilization.[38,40] In humans, platelet aggregation and thrombus formation under arterial flow conditions are attenuated in the presence of rivaroxaban, but thrombus regression has not been investigated.[38]

Morphological improvement of atherosclerotic lesions has also been shown with dietary treatment in monkeys, and this improvement coincides with restoration of endothelial function.[41] Since morphological improvement of atherosclerotic lesions has only been observed with long-term (20 weeks) treatment with rivaroxaban, the interventional period of 12-weeks DPI in the current study, might be too short to establish improved endothelial function. However, the clinical benefit of rivaroxaban as observed in the COMPASS and VOYAGER-PAD trial, is not delayed, but visible from study onset. Therefore, if improving endothelial dysfunction underlies the clinical benefit of rivaroxaban in PAD patients, one would expect to see some signs of improvement within a 12-week period of DPI.

Future research should address other possible long-term PAR-related benefits of rivaroxaban in humans, such as the capability of rivaroxaban to regress atherosclerotic plaques[42], and whether this relates to other (easily measurable) elements of PAR inhibition, such as improved endothelial function (after >12 weeks of rivaroxaban) or reduced vascular inflammation.

The strengths of this study are mainly related to its straightforward approach. By establishing a run-in period of ASA monotherapy, followed by 12 weeks of DPI, we could compare both antithrombotic strategies using paired assessments. Furthermore, the study protocol has been pre-published, facilitating replicability. There are also limitations that should be addressed. The prevalence of endothelial dysfunction, represented by CAR constriction, was lower than anticipated. While we predicted that 40% of the participants with claudication, and 60% of the participants with CLTI would show a constrictive response, in our study, the prevalence of CAR constriction varied between 20-25% in both cohorts. The expected high prevalence of CAR constriction was based on the CAVIPAD study, in which the CAR response was evaluated in 172 patients with PAD.[22] A lower proportion of constrictive CAR in our study can be explained by our relatively strict in- and exclusion criteria. By selecting patients solely on SAPT, we implicitly may have excluded most patients with more severe (i.e. acute coronary syndrome in the past year, multivessel disease) concomitant coronary artery disease, and patients with a recent vascular intervention. Also, patients with a current malignancy and patients with a glomerular filtration rate below 30 were excluded. This might have led to the selection of a relatively "healthy" cohort of PAD patients. In addition,

we classified patients into cohort A or B, based on their highest Rutherford classification ever, rather than on current classification. Therefore, patients in cohort B, might not have been as severely diseased as the patients with CLTI in the CAVIPAD study. A prevalence of 20-25% CAR constriction is in line with other studies that determined the CAR response in patients with atherosclerosis. The COVAS study, and the study by Buckley et al, both found a prevalence of 24% CAR constriction in respectively 50 patients with various expressions of atherosclerosis and 95 patients with coronary artery disease.[35,43] Another limitation is the relatively high drop-out rate. By replacing all participants that dropped out before starting DPI, we endeavored to obtain as much paired assessments of the primary and secondary outcomes as possible. Another twenty patients, however, dropped out during 12 weeks of DPI. Noteworthy, is the high number of side effects reported to the study team, which mainly underlies the high drop-out rate. Possible side effects of low-dose rivaroxaban were reported by 28 (17.6%) participants, and for 12 (7.5%) participants, these side effects were such that a stop in study medication was requested. The high occurrence of side effects should be considered when prescribing DPI in PAD patients. Last, some patients experienced peripheral revascularization during their DPI treatment. An eventual improvement of mobility with subsequent improvement in endothelial function, was not corrected for. As the number of patients undergoing revascularization was relatively small, we believe that this was not a relevant source of bias, although a certain influence cannot be ruled out

In conclusion, macro- and microvascular endothelial dysfunction, as determined by determining the CAR response and measuring plasma ET-1 concentrations, is not influenced by addition of low-dose rivaroxaban to ASA monotherapy during 12 weeks in PAD patients.

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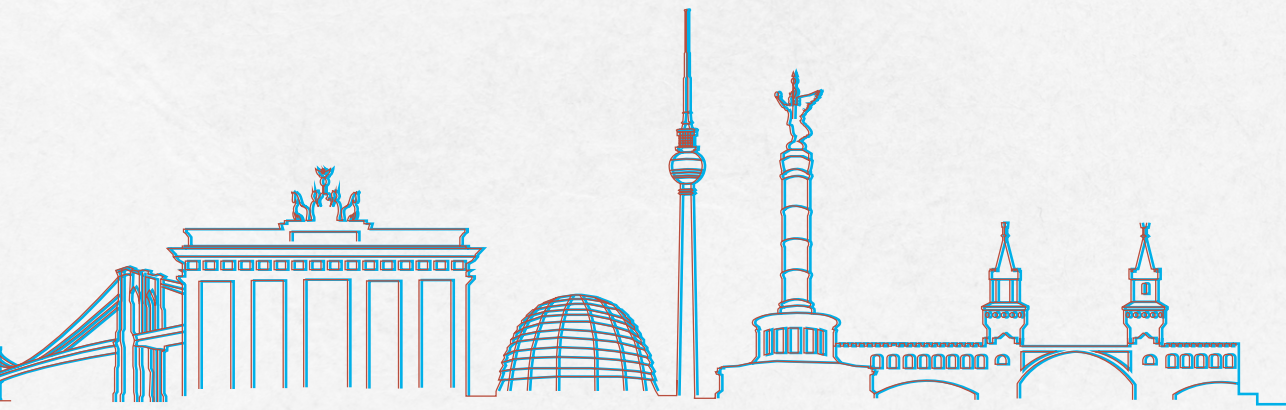
Supplementary table 1: *severe adverse events and possible side effects of study drugs.*

Severe adverse events	**Total number**	**Onset during run-in**	**Onset during DPI**
Acute limb ischemia	2	2	
Peripheral revascularization	13	1	12
Cardiovascular death	1	1	
Non-cardiovascular death	1		1
Gastro-intestinal hemorrhage	1		1
Possible side effects	**Total number**	**Onset during ASA**	**Onset during DPI**
Bleeding problems	11		11
Dysmenorrhea	2		2
Anemia	1		1
Bleeding hemorrhoids	1		1
Gastro-intestinal hemorrhage	1		1
Easy bruising	3		3
Hematuria	1		1
Epistaxis	2		2
Skin rash	6	1	5
Gastro-intestinal	5		5
Stomach complaints	4	1	3
Obstipation	1		1
Palpitations	1		1
Arm, leg and/or back pain	5		5
Dizziness	2		2
Headache	2		2
Shortness of breath	1		1
Decreased kidney function	1	1	
Fatigue	1		1



Chapter 8

Dual-pathway inhibition with rivaroxaban and low-dose aspirin does not alter immune cell responsiveness and distribution in patients with coronary artery disease

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Abstract

Introduction: Cardiovascular diseases (CVD) are the leading cause of death globally. Inflammation is an important driver of CVD where tissue damage may lead to the formation of deadly thrombi. Therefore, anti-thrombotic drugs, such as platelet inhibitors, are crucial for secondary risk prevention in coronary (CAD) and peripheral artery disease (PAD). For severe forms of the disease, dual-pathway inhibition (DPI) where low-dose aspirin is combined with rivaroxaban has shown improved efficacy in reducing cardiovascular mortality.

Methods: Given this greater improvement in mortality, and the importance of inflammation in driving atherosclerosis, the potential for off-target inflammation-lowering effects of these drugs were evaluated by looking at the change in immune cell distribution and responsiveness to *ex vivo* lipopolysaccharide (LPS) stimulation after 3 months of DPI in patients with CAD.

Results: We observed no changes in whole blood or peripheral blood mononuclear cells (PBMC) immune cell responsiveness to LPS after 3 months of DPI. Additionally, we did not observe any changes in the distribution of total white blood cells, monocytes, neutrophils, lymphocytes, or platelets during the study course. Signs of systemic inflammation were studied using Olink proteomics in 33 patients with PAD after 3 months of DPI. No changes were observed in any of the inflammatory proteins measured after the treatment period, suggesting that the state of chronic inflammation was not altered in these subjects.

Conclusion: In conclusion, 3 months of DPI does not result in any meaningful change in immune cell responsiveness and distribution in patients with CAD or PAD.

Background

The leading cause of mortality globally is cardiovascular disease (CVD).[1] Although there are many underlying causes for CVD, coronary artery disease (CAD) and peripheral artery disease (PAD) are primarily caused by atherosclerotic plaques within arterial walls. Tissue injury associated with atherosclerosis, particularly plaque rupture, can activate platelets and proteins of the coagulation cascade, resulting in the formation of a thrombi restricting blood flow through the artery, potentially leading to ischemic tissue damage. Therefore, patients are generally prescribed anti-thrombotic medication to reduce complications associated with thrombi formation.

Amongst the most widely prescribed anti-thrombotic drugs is aspirin, which reduces hemostasis by inhibiting thromboxane production in platelets reducing their activation and aggregation. Although this course of treatment is effective, many patients will still experience recurrent events every year.[2] The COMPASS trial demonstrated that for more complex cases of recurrent cardiovascular events, aspirin combined with low-dose rivaroxaban improved the event-free survival when compared to mono-therapy of either drug.[3] Rivaroxaban works by directly inhibiting factor X, an important rate-limiting enzyme in the coagulation cascade. Therefore, combined aspirin and rivaroxaban serves as a method of dual-pathway inhibition (DPI) with regards to thrombus formation.

Much of the benefit of DPI are likely the result of reducing the formation of blood-clots within the arteries of patients. However, apart from thrombus formation, a state of low-grade chronic inflammation is known to play an important role in CVD onset and progression.[4] Aspirin has well studied modulatory effects on inflammation and immune cell function.[5-6] Furthermore there is cross-talk between proteins of the coagulation cascade and inflammatory cells, most notably thrombin and PAR2 receptors on monocytes/macrophages.[7-8] Thus opening the possibility for indirect effects of rivaroxaban on immune cell modulation. Therefore, we hypothesize that part of the additive benefits of DPI is via off-target by modulation of immune cell driven inflammation. This study aimed to investigate whether the beneficial effects of DPI were a result of reducing systemic inflammation, and if these changes were accompanied by alterations in immune cell responsiveness and circulating distribution.

Materials and Methods

Study design

An explorative interventional trial investigating the effect of DPI on monocyte-driven inflammation was initiated by and conducted in the Radboud university medical center (Nijmegen, the Netherlands). Approval of the Medical Research Ethics Committee Oost-Nederland (file number: 2021-13291) and the local institutional review board was obtained. This study was conducted in accordance with the latest revision of the Helsinki Declaration of 1964 and Good Clinical Practice regulations and is registered at ClinicalTrials.gov on January 27th, 2022 (registration number NCT05210725). Written informed consent was obtained from all participants.

Participants

Patients with stable CAD and an indication for single antiplatelet therapy according to the leading international guidelines[9] were recruited at our outpatient clinic. Patients were considered eligible when they were 1) currently treated with Aspirin (80-100mg once daily) monotherapy, 2) at high risk of developing recurrent vascular events based on a SMART Risk Score ≥ 20%, 3) at least 1 year after myocardial infarction or suffering from multi-vessel CAD, and 4) aged ≥ 16 years. Exclusion criteria were concomitant use of immunosuppressant/anti-inflammatory therapies and known contra-indications to rivaroxaban. Contra-indications to rivaroxaban include hypersensitivity, at significant risk for major bleeding, severe hepatic disease, severe kidney failure (estimated glomerular filtration rate < 15 ml/min or requiring dialysis), severe heart failure (ejection fraction < 30% or New York Heart Association class III or IV symptoms), concomitant treatment with medication with a strong pharmacokinetic interaction with rivaroxaban.

Procedures

Eligible patients visited the hospital three times: once at baseline and twice during follow-up (after 4 and 12 weeks of DPI treatment, respectively).

Baseline

At baseline (T0), written informed consent was obtained. Data regarding demographics, lifestyle, medical history, and medication use (including recent vaccinations, ≤ 1 month before screening) were recorded using standardized case report forms and electronic patient files. Measurement of blood pressure, height, weight, and hip-waist circumference took place. Venous blood samples were collected. Finally, participants were prescribed rivaroxaban 2.5mg twice daily for a 12-week period and follow-up visits were scheduled

Follow-up

Patients were scheduled for follow-up after 4 (T1) and 12 (T2) weeks of DPI treatment. During follow-up visits, adverse events were reported, and medication adherence was evaluated by interview. If medication adherence was below 80%, rivaroxaban was discontinued, and participants were excluded from further study participation. Venous blood samples were collected.

Whole blood stimulation

Per well of a round-bottom 96 wells-plate 100 µl whole blood was added along with 400 µl RPMI 1640 Medium ("Dutch modification" containing 11 mM glucose; Thermo-Fischer, Waltham, MA, USA) supplemented with 10 µg/mL gentamicin, 2 mM GlutaMAX and 1 mM pyruvate, or 400 µl culture medium supplemented with 12.5 ng/ml for a final concentration of 10ng/ml of *Escherichia coli* lipopolysaccharide (LPS) (serotype 055:B5 Sigma-Aldrich, St. Louis, MO). Blood was then incubated at 37°C 5% CO_2 for 24 h, supernatants were then collected and stored at -80°C until cytokine assessment.

Peripheral blood mononuclear cell isolation and stimulation

Peripheral blood mononuclear cells (PBMCs) were isolated from blood of study participants by dilution in phosphate-buffered saline (PBS) and density-gradient centrifugation using Ficoll-Paque (GE healthcare, Chicago, IL, USA). PBMCs were washed three times with cold PBS and resuspended in RPMI 1640 Medium. For stimulations experiments, 5×10^5 PBMCs were seeded per well in a round-bottom 96 wells-plate (Corning, NY, USA) and stimulated for 24 h with either culture medium or culture medium supplemented with 10ng/ml of *Escherichia coli* lipopolysaccharide (LPS) (serotype 055:B5 Sigma-Aldrich, St. Louis, MO, USA) at 37°C 5% CO_2. After 24 h incubation, supernatants were stored after plate centrifugation at –80°C until cytokine assessment.

Cytokine measurements

Levels of TNFα and IL-6 were measured in supernatants using the IL-6 and TNF-alpha DuoSet ELISA kits (R&D Systems, Minneapolis, MN, USA).

Immune cell measurements via Sysmex

Complete blood counts were performed on EDTA whole blood and PBMC fractions after Ficoll isolation, on the Sysmex XN-450 hematology analyzer (Sysmex AmericaInc, Lincolnshire, IL, USA).

Olink analysis in patients with PAD

An additional cohort, a subset of the DUALPAD study [NCT04218656][10], of 33 patients with PAD were included and assessed for circulating plasma protein expression. Circulating plasma protein expression both at baseline and after 3 months of DPI was assessed using the commercially available multiplex proximity extension assay from Olink® Proteomics AB (Uppsala, Sweden). The Target 96 Inflammation Panel was run where 96 inflammatory proteins were measured.

Statistical analysis

Since this is exploratory research, detailed sample size calculation is not appropriate. We aimed to include 15 to 20 patients based on the average LPS induced TNF alpha production in patients with symptomatic atherosclerosis[11], and an expected 20% increase in cytokine production capacity from baseline (Aspirin) to 3 months of DPI (Aspirin with rivaroxaban). Statistical testing was performed by using the Wilcoxon matched pairs signed rank test. For volcano plots, data were analyzed by Mann-Whitney test, the Benjamini-Hochberg procedure was employed to correct multiple testing errors. False discovery rate (FDR)-adjusted p-values smaller than 0.05 were considered statistically significant. Statistical analysis and data visualization were performed with Graphpad Prism v9.3.1 (GraphPad software, La Jolla, CA) or R/Bioconductor (https://www.R-project.org/).

Results

Patient characteristics

Between March 2022 and April 2022, 16 patients with CAD were enrolled. Medication adherence was above 80% for all participants. Three participants dropped out during the study course. Reasons for drop-out were: 1) side effects of rivaroxaban, 2) development of atrial fibrillation with need for more intensive antithrombotic treatment, and 3) active infection with the coronavirus disease 2019 during last episode of study, with high risk of affecting study outcomes. Patient characteristics are shown in *table 1*. The median age of the participants was 72 years and 31% were female. The median age of the additional cohort of 33 patients with PAD[10] was 67 and 33% were female.

Table 1: *patient characteristics*

		DUALCAD *(n=16)*	**DUALPAD** *(n=33)*
Demographics	Age (median, range)	72 [52-82]	67 [49-85]
	Female sex (n,%)	5 (31.3)	11 (33.3)
	Ethnicity (n,%)		
	Caucasian	16 (100.0)	32 (97.0)
	Other		1 (3.0)
Lifestyle	Smoking behavior (n,%)		
	Current	6 (37.5)	6 (18.2)
	Former	9 (56.3)	25 (75.8)
	Never	1 (6.3)	2 (6.1)
Vascular state	Coronary episode leading to inclusion (n,%)		NA
	Myocardial infarction	11 (68.8)	
	Episode of unstable angina pectoris	4 (25.0)	
	Stable angina pectoris	1 (6.3)	
	PAD severity* leading to inclusion (n,%)	NA	
	Intermittent claudication		27 (81.8)
	CLTI		6 (18.2)
	Previous intervention for PAD (n,%)	NA	24 (72.7)
Comorbidity	Hypertension (n,%)	8 (50.0)	22 (66.7)
	Hyperlipidemia (n,%)	7 (43.8)	14 (42.4)
	Ischemic heart disease (n,%)	16 (100.0)	11 (33.3)
	CVA/TIA (n,%)	3 (18.8)	4 (12.1)
	PAD (n,%)	5 (31.3)	33 (100.0)
	Diabetes mellitus (n,%)	8 (50.0)	12 (36.4)
Drugs	Lipid lowering drugs (n,%)	14 (87.5)	33 (100.0)
	Antihypertensive medication (n,%)	15 (93.8)	21 (63.6)
Physical examination	BMI (median, range)	27.3 [21.1-38.6]	27.1 [21.0-38.2]
	Blood pressure, mmHg (median, range)		
	Systolic	130 [105-178]	148 [100-214]
	Diastolic	76 [60-88]	76 [57-96]

Data are for 16 participants of the DUAL-CAD study who were evaluated for complete blood cell counts, whole blood stimulation, peripheral blood mononuclear cells (PBMC) isolation and stimulation, and cytokine levels of TNFa and IL-6; and patient characteristics of 33 participants of the DUAL-PAD study with inflammatory profiles determined using Olink proteomics

PAD = peripheral arterial disease, CLTI = chronic limb threatening ischemia, CVA = cerebrovascular accident, TIA = transient ischemic attack, BMI = body mass index, NA = not applicable

**Most severe presentation of PAD, now or in the past*

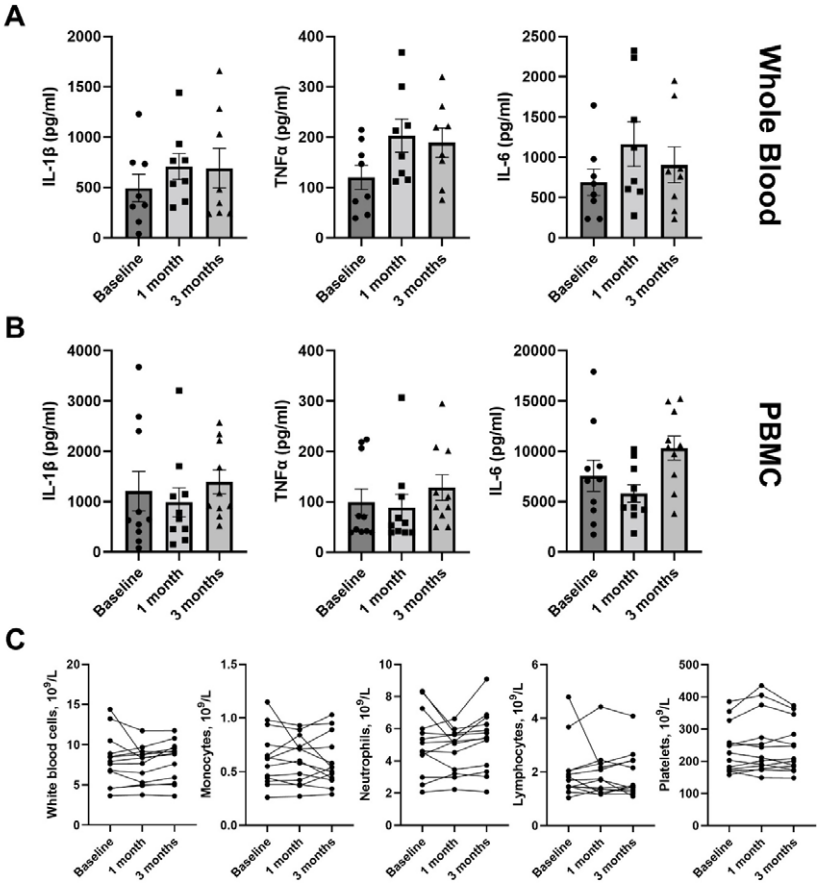

Figure 1: *Three months of dual-pathway inhibition (DPI) does not alter ex vivo immune cell responsiveness to lipopolysaccharide (LPS) stimulation nor circulating immune cell distribution. Levels of TNFa, IL-6, and IL1b in ex vivo stimulated A whole blood (n = 8) and B peripheral blood mononuclear cells (PBMC) from study participants (n = 10). C Sysmex results showing the peripheral cell counts of white blood cells, monocytes, neutrophils, lymphocytes and platelets (n = 13). Data are represented as mean ± standard error of the mean (SEM), Wilcoxon signed-rank test*

DPI does not change *ex vivo* immune cell responsiveness and distribution

When comparing *ex vivo* responsiveness of whole blood to stimulation with LPS following 1 months and 3 months of DPI, we found no significant alterations in TNFα, IL-6 or IL-1β production [*figure 1A*, n = 8]. Similarly, we found that PBMC's isolated from these volunteers did not demonstrate any changes in immune responsiveness following *ex vivo* LPS stimulation [*figure 1B*, n = 10]. In line with the cytokine levels, we did not measure any changes in specific immune cell populations or

distributions. White blood cells, neutrophils, monocytes, and lymphocytes showed no meaningful changes in abundance across the different measurements [*figure 1C*, n = 13]. Similarly, platelet counts also did not appear to be altered. This is in line with literature that shows low-dose aspirin does not affect platelet count in circulation but only affects their activation characteristics.[14-15]

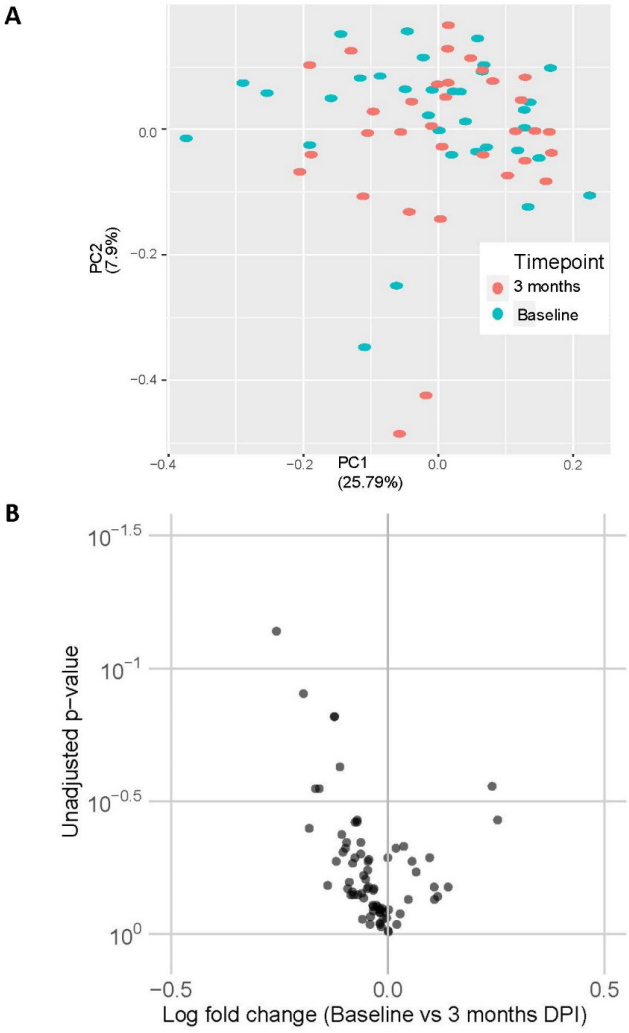

Figure 2: *Circulating markers of inflammation are not altered by 3 months of dual-pathway inhibition (DPI) in patients with peripheral artery disease (PAD). A Principal component analysis (PCA) comparing plasma levels of inflammatory proteins at baseline and after 3 months of DPI (n = 33, data dimensionality is visualized by principal component 1 (PC1) and PC2). B Volcano plot depicting a comparison of plasma levels of inflammatory proteins at baseline to 3 months of DPI in patients with PAD (n = 33)*

DPI does not change markers of systemic inflammation in patients with PAD

CVD is driven by a state of chronic-systemic inflammation, therefore we sought to determine whether DPI has any effects on markers of systemic inflammation. We evaluated plasma samples from 33 patients with PAD who were enrolled in the DUAL-PAD study.[10] Mirroring the DUAL-CAD study, patients received 4 weeks aspirin run-in followed by 3 months of DPI of low-dose aspirin and rivaroxaban. Circulating levels of inflammatory cytokines were than assessed for relative expression by Olink proteomics (Inflammation Target 96 panel). Principal Component Analysis (PCA) showed that baseline samples clustered closely with samples collected after 3 months of DPI [*figure 2A*]. Similarly, paired statistical analysis comparing baseline with 3 months of DPI verified that there were no changes in systemic inflammation in these patients following combined treatment with low-dose aspirin and rivaroxaban [*figure 2B*].

Discussion

Recent landmark studies such as the CANTOS[12] trial have convincingly demonstrated that inflammation plays a pivotal role in the pathophysiology of atherosclerotic diseases. However, due to some practical restraints such as costs, these anti-inflammatory treatment avenues remain under-utilized within the clinic. In the COMPASS trial, patients with severe forms of CAD and PAD benefited from DPI where low-dose aspirin is co-administered alongside the factor X inhibitor rivaroxaban.[3] Given that there is considerable cross talk between platelets and the coagulation with the immune system[13], we aimed to determine whether any of the beneficial effects conveyed by DPI were due to dampening of inflammation.

Previously it was demonstrated that patients with active CAD not only have elevated levels of circulating cytokines but also elevated immune cell responsiveness to *ex vivo* LPS stimulation.[11] A strength of the current study design is that each participant serves as their own control, allowing for a comparison of DPI treatment to a background of aspirin monotherapy. This negates the need for an aspirin-only monotherapy control group which would require greater participant recruitment as well as appropriate randomization to control for variables. We did not observe any meaningful lowering of whole blood and/or PBMC *ex vivo* cytokine production following LPS stimulation from subjects with CAD. No changes in the distribution of immune cell populations were observed either. By accessing a larger cohort of 33 subjects with PAD and measuring relative levels of 96 inflammatory proteins

in serum both at baseline and after 3 months of DPI, we did not measure a single significantly altered protein. Given that this proteomic analysis includes both pro-inflammatory as well as anti-inflammatory, it is apparently clear that inflammation is neither changed by lowering of pro-inflammatory cytokines, nor by elevations in anti-inflammatory cytokines.

A limitation of this current study is the relatively short treatment period which may not be long enough to capture changes in systemic and cellular inflammation. However, in the COMPASS trial, the beneficial effects of DPI could be seen immediately upon switching medication. Therefore, the lack of change in immune cell responsiveness, as well as changes in markers of systemic inflammation following 3 months of DPI, we do not believe that inflammation is playing a driving role in the beneficial mechanism of action of rivaroxaban combined with aspirin.

Given that DPI also failed to show meaningful changes in markers of macro- and microvascular endothelial function in the recently published DUAL-PAD study, we therefore hypothesize that the main mechanism by which DPI lowers the risk of severe outcomes is due to its primary role in reducing coagulation and thrombosis.

A further limitation of the current study is the relatively limited number of CAD patients included. Despite this limitation, we do not observe any trends in *ex vivo* cytokine production or immune cell population shifts in the 13 subjects with CAD. Additionally, we feel our conclusions are supported by the adequately sized proteomic analysis similarly showing no alterations in circulating inflammatory proteins in subjects with PAD after 3 months of DPI.

Conclusion

To conclude, 3 months of low-dose aspirin combined with rivaroxaban treatment does not alter the immunophenotype of patients with CVD. DPI treatment did not alter relative levels of inflammatory proteins in the blood of patients with following 3 months of DPI. Additionally, 3 months of DPI did not alter immune cell responsiveness to *ex vivo* stimulation with LPS, nor the circulating distribution of different immune cell types. Therefore, our results indicate that the clinical benefit of DPI over aspirin alone[3] is more likely related to the intensified level of anti-coagulation, and not to the inhibition of inflammatory pathways.

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Part III

Optimal antithrombotic treatment for peripheral arterial disease: trial evidence

Chapter 9

Antithrombotic therapy for symptomatic peripheral arterial disease: a systematic review and network meta-analysis

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Abstract

Background: High-quality evidence from trials directly comparing single antiplatelet therapies in symptomatic peripheral arterial disease (PAD), to dual antiplatelet therapies or acetylsalicylic acid (ASA) plus low-dose rivaroxaban is lacking. Therefore, we conducted a network meta-analysis on the effectiveness of all antithrombotic regimens studied in PAD.

Methods: A systematic search was conducted to identify randomized controlled trials. The primary endpoints were major adverse cardiovascular events (MACE) and major bleedings. Secondary endpoints were major adverse limb events (MALE) and acute limb ischemia (ALI). For each outcome, a frequentist network meta-analysis was used to compare relative risks between medication and ASA. ASA was the universal comparator since a majority of studies used ASA as reference group.

Results: Twenty-four randomized controlled trials were identified including 48,759 patients. With regard to reducing MACE, clopidogrel [RR 0.78, 95%CI 0.66-0.93], ticagrelor [RR 0.79, 95%CI 0.65-0.97], ASA plus ticagrelor [RR 0.79, 95%CI 0.64-0.97], and ASA plus low-dose rivaroxaban [RR 0.84, 95%CI 0.76-0.93] were more effective than ASA, and equally effective to one another. As compared to ASA, major bleedings occurred more frequently with vitamin K antagonists, rivaroxaban, ASA plus vitamin K antagonists, and ASA plus low-dose rivaroxaban. All regimens were similar to ASA concerning MALE, while ASA plus low-dose rivaroxaban was more effective in preventing acute limb ischemia [RR 0.67, 95%-CI 0.55-0.80]. Subgroup analysis in patients undergoing peripheral revascularization revealed that ≥3 months after intervention, evidence of benefit regarding clopidogrel, ticagrelor and ASA plus ticagrelor was lacking, while ASA plus low-dose rivaroxaban was more effective in preventing MACE [RR 0.87, 95%CI 0.78-0.97] and MALE [RR 0.89, 95%CI 0.81-0.97], compared to ASA. ASA plus clopidogrel was not superior to ASA in preventing MACE ≥3 months after revascularization. Evidence regarding antithrombotic treatment strategies within 3 months after a peripheral intervention was lacking.

Conclusion: Clopidogrel, ticagrelor, ASA plus ticagrelor, and ASA plus low-dose rivaroxaban are superior to ASA monotherapy and equally effective to one another in preventing MACE in PAD. Of these four therapies, only ASA plus low-dose rivaroxaban provides a higher risk of major bleedings. More than 3 months after peripheral vascular intervention, ASA plus low-dose rivaroxaban is superior in preventing MACE and MALE compared to ASA but again at the cost of a higher risk of bleeding, while other treatment regimens show non-superiority. Based on

the current evidence, clopidogrel may be considered the antithrombotic therapy of choice for most PAD patients, while in patients who underwent a peripheral vascular intervention, ASA plus low-dose rivaroxaban could be considered for the long term (>3 months) prevention of MACE and MALE.

Introduction

Peripheral arterial disease (PAD) is a manifestation of atherosclerosis in the major arteries of the lower extremities, leading to various clinical symptoms such as intermittent claudication, ischaemic rest pain and gangrene.[1-2] Globally, approximately 202 million patients suffer from PAD, of whom 141 live in low-income or middle-income countries and 61 million live in high-income countries.[3] PAD is associated with a significant risk of arterial thrombotic events, such as myocardial infarction, ischaemic stroke, lower-extremity amputation and cardiovascular death[2,4-8]. The relative risk of cardiovascular death over a period of 10 years in patients with PAD is six times increased, compared to non-PAD individuals.[8] Furthermore, during lifetime, PAD is associated with impaired physical function, reduced quality of life and increased health care costs.[9-10] Within the framework of secondary prevention, antithrombotic therapy is recommended. According to the current guidelines, the first choice of antithrombotic therapy in symptomatic PAD is single antiplatelet therapy (SAPT).[1,11] The benefit of ASA monotherapy in symptomatic PAD has been extensively studied.[12] Clopidogrel, a P2Y12 inhibitor, was more effective in reducing arterial thrombotic events with a similar safety profile compared to ASA in the CAPRIE study.[13] Therefore, clopidogrel may be preferred over ASA as antithrombotic therapy in patients with symptomatic PAD.[1] The use of alternative P2Y12 inhibitors, such as ticagrelor and prasugrel, is not approved by international authorities (i.e. the European Medicines Agency and the United States Food and Drug Administration) and has therefore no place in the secondary prevention of PAD patients. After peripheral revascularization by endovascular stenting or infra-inguinal prosthetic bypass grafting, dual antiplatelet therapy (DAPT) with ASA plus clopidogrel for at least one month is recommended.[1,11] However, this recommendation is not supported by high-quality trial evidence and the optimal duration of DAPT after an intervention is unknown.[14] After venous bypass surgery, fewer graft occlusions have been demonstrated with oral anticoagulation in the Dutch BOA trial, unfortunately at the expense of a 2-fold increased risk of major bleeding.[15] Recently, two large randomized controlled trials, respectively including patients with symptomatic PAD or patients undergoing a peripheral vascular intervention (endovascular or surgical), demonstrated superiority of ASA plus low-dose rivaroxaban over ASA monotherapy.[16-17] Whether the benefits of ASA plus low-dose rivaroxaban also apply when compared to clopidogrel monotherapy, is unclear. Choosing the best antithrombotic therapy for PAD patients entails significant uncertainties, since only few randomized controlled trials (RCTs) directly compared the latest treatment options. By use of network meta-analysis, treatment options can be indirectly compared using a universal comparator.[18] This study aimed to evaluate the effectiveness and safety of antithrombotic regimens for secondary prevention in PAD patients.

Methods

Data sources and searches:

This systematic review and network meta-analysis was performed in accordance with the PRISMA Extension Statement for Reporting of Systematic Reviews Incorporating Network Meta-analyses of Health Care Interventions (PRISMA-NMA). The review protocol is registered with Open Science Framework, number 4nz9t. A systematic search was performed using the electronic databases PubMed, MEDLINE and EMBASE for English language RCTs published from January 1st, 1995 up to December 31st, 2021. Earlier performed RCTs were excluded to minimize the risk of suboptimal secondary prevention biasing the protective effect of antithrombotic treatment.[19] The search combined terms for PAD with terms for antithrombotic treatment. The electronic database search was supplemented with a manual search for RCTs in the reference list of the selected articles. ClinicalTrials.gov and the Cochrane Central Register of Controlled Trials were searched to identify further studies and unpublished RCTs with results. Details of the search strategies are described in Appendix A.

Study selection:

Two authors (LW and DM) independently screened titles and abstracts of the articles collected through the searches. Of all eligible articles, the full text was independently assessed by two authors (LW and DM). Disagreement was resolved by discussion. In case no agreement was obtained, arbitration of a third author was requested (MW). In case different articles contained duplicate data, the article with the largest sample size or the most complete information was selected.

Studies on patients with symptomatic lower extremity PAD, based on a clinical presentation of intermittent claudication or chronic limb-threatening ischemia that either was related to an ankle brachial index below 0.9, and/or resulted in the need for peripheral revascularization were considered eligible if: 1) two or more antithrombotic treatment strategies were compared and 2) patients were followed for clinical outcome measurements. Clinical outcome measurements included death, myocardial infarction, stroke, acute limb ischemia (ALI), need for revascularization, major amputation, and bleeding events. Study reports meeting one or more of the following criteria were excluded: 1) studies on patients using anticoagulant therapy for venous thromboembolic disease, 2) studies on patients using anticoagulant therapy for the prevention of systemic embolism in atrial fibrillation, 3) studies on patients with known congenital bleeding or thrombotic disorders, 4) studies with intravenous or intra-arterial antithrombotic treatment as

intervention, 5) studies in which the clinical outcomes were described for a follow-up period that was twice or more the duration of the intervention period, 6) animal studies, and 7) in vitro studies.

Data extraction

Data were extracted from the selected articles by two independent reviewers (LW and DM) using a standardized form. Discrepancies were resolved by discussion. Extracted data included: study acronym, last name of the first author, full title, publication year, study setting (primary, secondary, tertiary care), population, inclusion criteria, exclusion criteria, clinical severity of PAD, intervention (drug and dose), comparison (drug and dose), sample size, baseline characteristics of the participants, compliance, outcome measurements and duration of follow-up.

The primary cardiovascular effectiveness endpoint was the occurrence of major adverse cardiovascular events (MACE). The primary safety endpoint was major bleeding. Secondary endpoints were major adverse limb events (MALE) and ALI. Variations in definitions were allowed as long as they assessed cardiovascular complications of atherosclerotic cardiovascular disease, but not end points related to other pathophysiological mechanisms such as venous thromboembolism. End points were assessed at the longest follow-up available.

Quality assessment:

Risk of bias was assessed by two independent reviewers (LW and DM) using Cochrane Collaboration's risk of bias tool for RCTs. In brief, a quality judgment was performed on the randomization process, allocation concealment, missing outcome data, measurement of the outcome, and selection of the reported results.[20] Studies with a low risk of bias in all domains or studies that raised concerns in a maximum of one domain, are considered to have a low risk of bias. Studies that raised concerns in two domains are considered to have a medium risk of bias. Studies with a high risk of bias in one or more domains, and studies that raised concerns in more than two domains, are considered to have a high risk of bias. Discrepancies between reviewers were resolved through discussion.

Certainty of evidence

We assessed the certainty of evidence contributing to network estimates of the main outcomes with the Grading of Recommendations Assessment, Development and Evaluation (GRADE) framework.[21] Indirect estimates were rated, starting with the lower of the ratings from the direct estimates forming the dominant first order loop. If intransitivity was present, the rating was further downgraded. The estimate,

direct or indirect, that contributed most was the basis for the certainty of evidence. In case of similar amounts of contribution, the higher of certainty judgments was chosen. If evidence of imprecision between direct and indirect estimates, the certainty was downgraded. If the relative risk estimate was ≥ 1 and the lower limit of the confidence interval is below 0.75, or if the relative risk estimate was ≤ 1 and the upper limit of the confidence interval was above 1.25, the certainty was downgraded.[22]

Statistical methods

We conducted a frequentist network meta-analysis with random-effects models to estimate the aggregate effects in MACE, major bleeding, MALE, and ALI for each type of antithrombotic medication compared with ASA and with each other. Pooled estimates were expressed as relative risks (RRs) with their corresponding 95%CIs.

Network plots were produced for each outcome to visualize network geometry and node connectivity. We estimated the ranking probabilities of the different antithrombotic treatments based on their P-scores. The P-score is measured on a scale from 0 (worst) to 1 (best), with a higher score indicating better overall performance of the competing treatment. The numerical P-score values are nearly identical to the surface under the cumulative ranking curve.[23] It is also important to consider the relative risk and corresponding 95%CI for each comparison when interpreting the ranking results.[24]

Network heterogeneity across treatment contrasts was assessed using τ^2 and I^2 statistics. We applied the Q statistic to test for global inconsistency using a design-by-treatment interaction random effects model.[25] Local inconsistency was evaluated through a node split method by splitting the network estimates into direct and indirect evidence using a back-calculation method.[26-28] P-values were 2-sided, and P-values less than .05 were considered statistically significant.

Results were graphically displayed using forest plots.

We used the number needed to treat (NNT) as an absolute measure of effect used to communicate the effectiveness or safety of an intervention. The NNT provides insight into the clinical relevance of an effect size because it is defined as the average number of patients who need to be treated to prevent one extra person from having a bad outcome compared with another treatment. For positive outcomes, the NNT can be equivalently defined as the number of people that need to be treated to have one person with a good outcome. Similarly, the

number needed to harm (NNH) indicates how many people need to be treated in order for one patient to have a particular adverse effect. To avoid the unfavorable NNH term[29], we used the terms NNTB ('number needed to treat for an additional beneficial outcome') and NNTH ('number needed to treat for an additional harmful outcome') for positive and negative outcomes, respectively.

The NNTB and NNTH with their 95%CIs were calculated by taking the inverse of the risk difference (RD) as estimated from the network meta-analysis.[30] In NNT, values between -1 and 1 are impossible, and the domain of NNT uses two regions: 1) the NNTB region, including the union of 1 (where is the largest possible beneficial treatment effect) to ∞ (no treatment effect), and 2) the NNTH region, ∞ (no treatment effect) to 1 (where is the largest possible harmful treatment effect). For example, a non-statistically significant NNT 5 with CI -40 and 2 is a combination of the two regions (∞,40] and [2,∞). The suggested presentation of such non-statistically significant NNT is NNTB 5 (NNTH 40; ∞; NNTB 2).[29-30] We used this presentation for the 95%CIs of the NNT. Furthermore, the direct evidence proportion, mean path length, and aggregated minimal parallelism were quantified. The direct evidence proportion is the proportion of direct evidence contained in each network estimate. Minimal parallelism reflects the minimum number of independent paths contributing to the effect estimate on an aggregated level. Large values of parallelism can be interpreted as supporting the robustness of the estimate. Mean path length characterizes the degree of indirectness of an estimate. Higher mean path lengths indicate less reliable estimates, given that more similarity assumptions have to be made when serially combining direct comparisons. Comparisons with mean path lengths greater than two should be interpreted with caution.[31]

Population-based subgroup analysis was performed for studies with patients that were selected for undergoing a peripheral vascular intervention.

Sensitivity analyses were performed to assess the robustness of the model by excluding trials with a high risk of bias. We explored the potential for publication bias by visual inspection of the comparison-adjusted funnel plots.[32-33]

Analyses were performed using R (version 4.1.2; R Foundation for Statistical Computing, Vienna, Austria) with package 'netmeta'.[34]

Results

The study flowchart is presented in *figure 1*. In total, 5,862 records were identified by electronic database and additional records searching, of which 24 RCTs with 48,759 (range 20-13,885) patients were included in this network meta-analysis.[13,15-17,35-55] One of the included studies was retrieved by the search for unpublished literature in the trial registers. A network diagram of all the research scenarios of the primary outcomes is shown in *figure 2*. Study characteristics are shown in *table 1*. Five studies had broader inclusion criteria than symptomatic lower extremity PAD but were included since a great majority (>75%) of participants fulfilled the inclusion criteria of our network meta-analysis: (1) the CREDO study[43] which included 61 patients (22.4% of study population) with cerebrovascular disease, (2) the WAVE study[55] which included 394 patients (18.2% of study population) with PAD of the subclavian or carotid arteries, and 3-5) the CHARISMA[39], CLIPS[40] and PEGASUS TIMI 54[50] trials which included respectively 258 (8.3%), 82 (22.4%) and 217 (19.0%) patients with asymptomatic peripheral arterial disease, diagnosed by an ankle brachial index below 0.9. Two studies compared three antithrombotic regimens[16,50]. Drug dose variations were collectively analyzed, including ASA 75-325 mg daily, ticagrelor 60-90mg twice daily and ticlopidine 200-250mg twice daily. Target International Normalized Ratio's (INR) in studies combining SAPT with a vitamin K antagonist, ranged from INR 1.4-3.0. The one study[16] that investigated vitamin K antagonist monotherapy strived for a target INR of 3.0-4.5. Other drug doses were consistent among the included studies. Definitions of MACE, major bleeding and MALE differed between the studies. MACE was mostly defined as the composite of (cardiovascular) death, myocardial infarction and stroke. Major bleeding was defined according to TIMI, ISTH or GUSTO criteria[56], or to self-defined criteria of the individual articles. MALE was commonly defined as the composite of any peripheral vascular intervention for chronic or acute limb ischemia and major amputation, but occasionally includes elective peripheral revascularization for non-ischaemic reasons, vascular occlusion without intervention, or death. *Table 2* summarizes the variable definitions used in the individual RCTs.

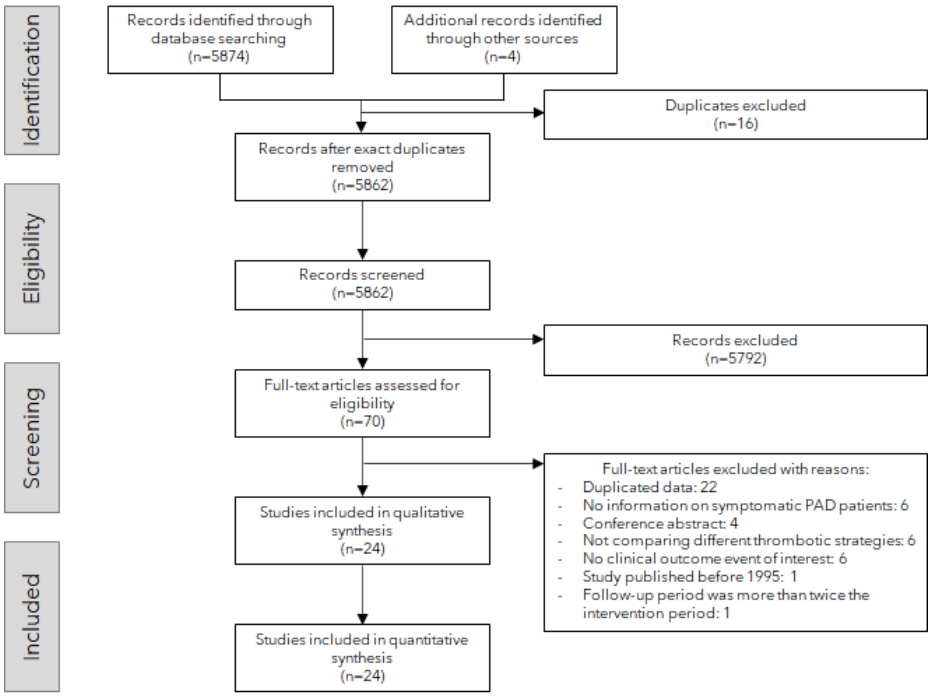


Figure 1: *Flow chart of study screening and selection.*

Risk of bias assessment

Some concerns on the risk of bias were noted for multiple studies in the following domains: randomization process (n=1), deviation from intended interventions (n=11), missing outcome data (n=3), and selection of the reported result (n=3). High risk of bias was found once, in the domain: selection of the reported results. Eventually, of the 24 included studies, most were classified as low risk for bias (n=20, 83.3%), two raised some concerns (8.3%), and two were classified as high risk of bias (8.3%). The risk of bias is presented in *table 3*.

Visual inspection of the comparison-adjusted funnel plots indicated no clear indication of publication bias, however, to draw definite conclusions the number of studies appears relatively low.

Figure 2A

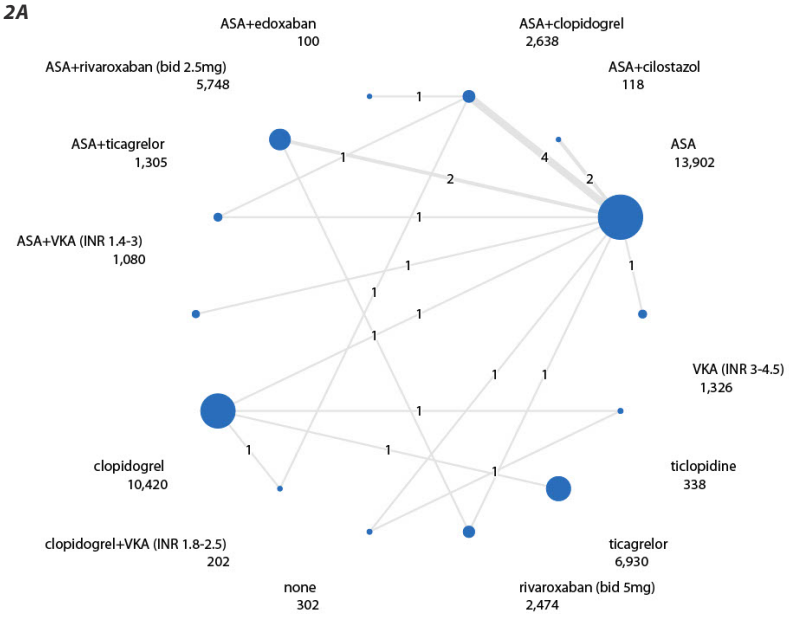


Figure 2B

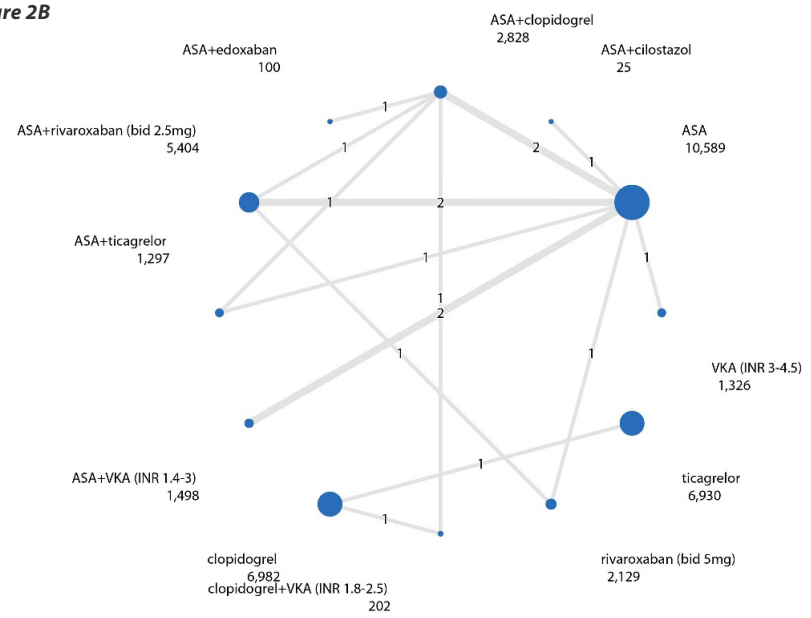


Figure 2: *Network diagram of antithrombotic regimens. The line width is proportional to the sample size of each direct comparison. The number in the middle of the line represents the number of direct comparisons. The number below the antithrombotic regimen corresponds with the total number of participants on that specific antithrombotic therapy. A: network diagram of MACE. B: network diagram of major bleedings.*

*ASA = acetylsalicylic acid, bid = bi-daily, VKA = vitamin K antagonist, INR = international normalized ratio. * patients that used no antithrombotic treatment, did receive placebo tablets*

Table 1: *study characteristics*

Ref	Study	Year	Sample size	Antithrombotic regimen	Population
36	Becquemin	1997	243	P vs TP2	PVI
15	BOA	2000	2690	A vs VKA2	PVI
37	CABBAGE	2017	50	A vs A+CI	PVI
13	CAPRIE	1996	6452	A vs C	PAD
38	CASPAR	2010	851	A vs A+C	PVI
39	CHARISMA	2009	3096	A vs A+C	PAD
40	CLIPS	2007	366	A vs P	PAD
16+41	COMPASS	2018	7470	A vs A+R1 vs R2	PAD
42	COOPER	2012	431	C vs TP1	PAD
33	CREDO	2006	272	A vs A+C	CAD
44	ePAD	2018	203	A+C vs A+E	PVI
35	EUCLID	2017	13885	C vs TG2	PAD
45	Gresele	2000	159	A vs A+CC	PAD
46	Johnson	2002	831	A vs A+VKA1	PVI
47	Li	2013	50	C vs C+VKA1	PVI
48	MIRROR	2011	80	A vs A+C	PVI
49	Monaco	2012	318	A+C vs C+VKA1	PVI
50	PEGASUS TIMI 54	2016	1143	A vs A+TG1 vs A+TG2	CAD
51	PLATO	2015	1144	A+C vs A+TG2	CAD
52	RIVAL-PAD	2020	20	A+C vs A+R1	PVI
53	Soga	2009	80	A+TP1 vs A+TP1+CI	PVI
54	STOP-IC	2013	200	A vs A+CI	PVI
17	VOYAGER-PAD	2020	6564	A vs A+R1	PVI
55	WAVE	2007	2161	A vs A+ VKA1	PAD

Antithrombotic regimen: A = Acetylsalicylic acid 75-325 mg daily; C = Clopidogrel 75 mg once daily, CC = Cloricromene 100 mg twice daily; CI = cilostazol 200 mg once daily; E = Edoxaban 60 mg once daily; P = Placebo only; R1 = Rivaroxaban 2.5 mg twice daily; R2 = Rivaroxaban 5 mg twice daily; S = Sarpogrelate 100 mg trice daily TG1 = Ticagrelor 60 mg twice daily; TG2 = Ticagrelor 90 mg twice daily; TP1 = Ticlopidine 200 mg twice daily; TP2 = Ticlopidine 250 mg twice daily; VKA1 = Vitamin K antagonist with target INR between 1.4 and 3; VKA2 = Vitamin K antagonist with target INR between 3 and 4.5

Population: PAD = Studies on patients who are solely selected for peripheral arterial disease, PVI = Studies on patients who underwent a peripheral vascular intervention for peripheral arterial disease, CAD = Studies on patients with coronary artery disease who coincide with PAD

HT = hypertension; HL = hyperlipidaemia; CSM = current smoker; HSM = history of smoking; CAD = coronary artery disease; CVD = cerebrovascular disease; DM = diabetes mellitus

Average follow-up (months)	Age (mean)	Male (%)	HT (%)	HL (%)	CSM (%)	HSM (%)	CAD (%)	CVD (%)	DM (%)
24	67	77	51	25	22		23		24
21	69	64							
3	73	74	88	50	14	40	54	26	74
23	64	72	51	45	38	90	54	14	21
12	66	76	70	50	38		35		37
26	60	70	72	70	32	85	25	16	36
21	66	77	62		26	80			76
21	68	72	79		28	75	65	7	45
3	71	88	74	56	25	88	13	18	33
12	67	66	76	74	30		100		32
2,7	67	29	83		35	86			40
30	66	72	78	76	31	78	29	12	39
6	66	86	45	64	84				25
38	64		88				25	17	36
12	74	66	70	24	51		59	20	42
6	70	53	78	63	40		33	19	38
77	67	70	81		24		61		48
36	66	78	85	81	30		100	3	42
9	66	75	79	66	38	74	100	15	38
3	67	60					0	0	
24	71	83	49	33	39		54	9	36
12	73	59	81	47		461	39		56
28	67	74	81	60	35		32		40
35	64	74	58		29	39	47	16	27

Table 2: *definitions of outcome measurements*

Ref.	Study	MACE	MB	MALE
36	Becquemin	1,3,4	-	-
15	BOA	2,3,4,6	1,2,3	-
37	CABBAGE	1,3,4	1,2,3,4	1,2,3
13	CAPRIE	2,3,4	-	-
38	CASPAR	-	GUSTO	1,3,4
39	CHARISMA	1,3,4	GUSTO	-
40	CLIPS	1,3,4	-	-
16+41	COMPASS	2,3,4	Mod. ISTH	1,3,5
42	COOPER	2,3,4	-	-
43	CREDO	1,3,4	-	-
44	ePAD	2,3,4	TIMI	-
35	EUCLID	2,3,5	TIMI	1,5
45	Gresele	2,3,4	-	-
46	Johnson	-	1,2,3,5,6	-
47	Li	1,3,4	1,2,5,6,7,8	-
48	MIRROR	n.s.	-	-
49	Monaco	2,3,4,7	1,2,3,6	-
50	PEGASUS TIMI 54	2,3,4	TIMI	1,5
51	PLATO	2,3,4	TIMI	-
52	RIVAL-PAD	-	TIMI	-
53	Soga	1,3,4	5,6,9	-
54	STOP-IC	1,3,4	-	3,4,5,6
17	VOYAGER-PAD	1,3,4,6,8	ISTH	1,3,5
55	WAVE	2,3,4	1,2,5,6,10	-

MACE = major adverse cardiovascular event; MB = major bleeding; n.s. = not specified; MALE = major adverse limb event

MACE: 1 = death; 2 = cardiovascular death; 3 = myocardial infarction; 4 = stroke; 5 = ischaemic stroke; 6 = amputation; 7 = urgent revascularization; 8 = ALI

MB: 1 = fatal bleeding; 2 = intracranial haemorrhage; 3 = bleeding requiring hospitalization; 4 = gastro-intestinal haemorrhage; 5 = bleeding requiring intervention; 6 = bleeding requiring blood product transfusion; 7 = hematoma with diameter >5 cm; 8 = haemoglobin reduction of >4 g/dL; 9 = hypotension requiring inotropic support; 10 = intraocular haemorrhage

GUSTO major bleeding defined as intracranial haemorrhage and/or haemodynamic compromise

ISTH major bleeding including 1) fatal bleeding, 2) symptomatic bleeding into a critical organ, or 3) bleeding causing a fall in haemoglobin level of 2 g/dL (1.24 mmol/L) or more, or leading to transfusion of two or more units of whole blood or red cells.

Table 2: *continued*

Modified ISTH major bleeding including 1) symptomatic bleeding into a critical organ, 2) surgical site bleeding requiring reoperation, or 3) any bleeding requiring hospitalisation (including presentation to an acute care facility without an overnight stay).
TIMI major bleeding including 1) any intracranial bleeding, 2) clinically overt signs of haemorrhage associated with a drop in haemoglobin of ≥5 g/dL or a ≥15% absolute decrease in haematocrit, and 3) fatal bleeding
MALE: 1 = peripheral revascularization; 2 = any revascularization; 3 = major amputation; 4 =re-occlusion/ revascularization of target lesion after intervention; 5 = ALI; 6 = death

Certainty of evidence assessment

Certainty of evidence contributing to network estimates as assessed by the GRADE framework was rated for each separate comparison of the four outcome measurements. Rates varied between high quality of evidence, moderate quality of evidence, low quality of evidence, and very low quality of evidence. The results of all certainty of evidence assessment are displayed in *supplementary table 1* to 4. The certainty of evidence of the main comparisons are discussed per outcome.

Clinical outcome

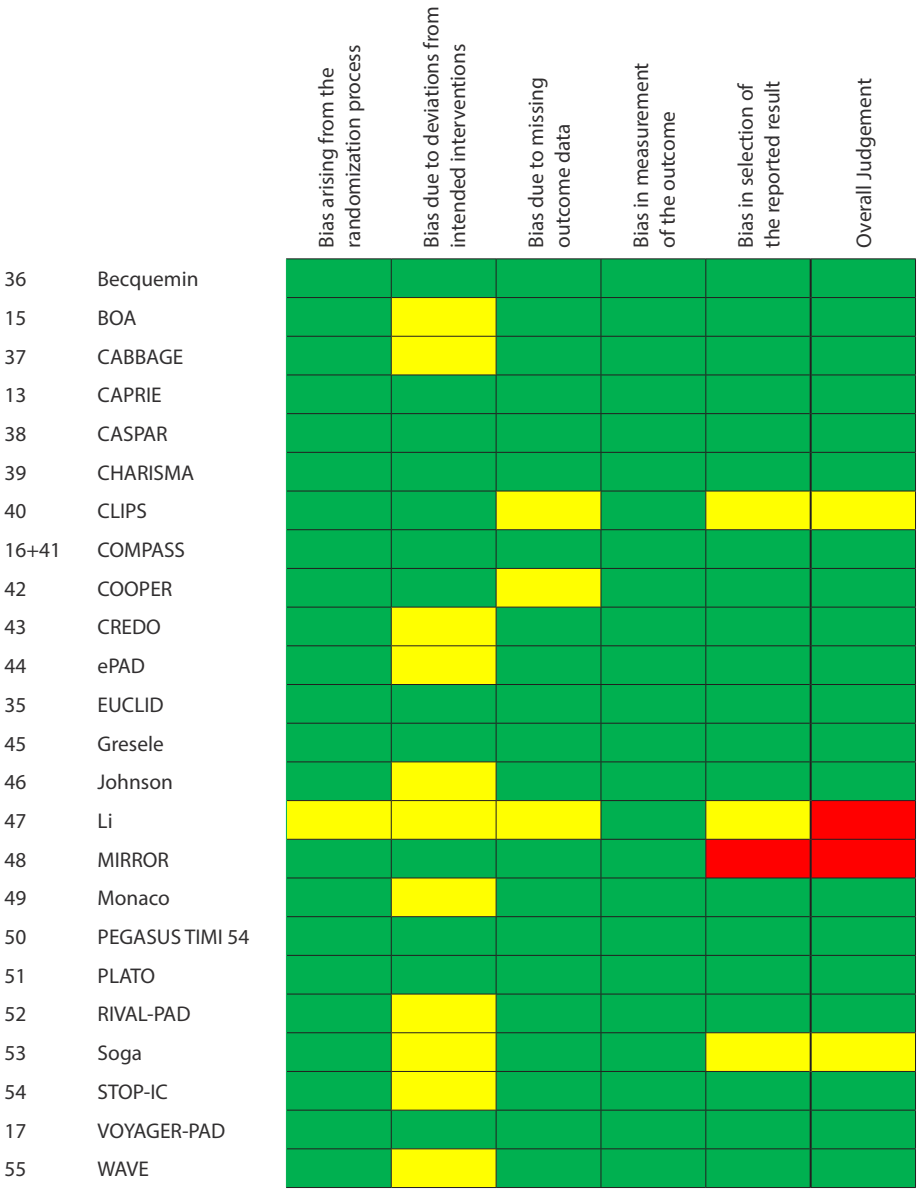
The number of events of the primary cardiovascular effectiveness outcome, the primary safety outcome, and secondary outcomes per study, are presented in *table 4*. *Figure 3A* shows the results of the network meta-analysis.

Major adverse cardiovascular events

Twenty-one studies with 46,961 patients reported MACE. One study[53] with 78 patients is not part of the network graph, since its treatment regimens did not connect to other studies. The certainty of evidence was incorporated in *supplementary table 1*. Compared to ASA, clopidogrel (RR 0.78, 95%CI 0.66-0.93; p-score 0.82), ticagrelor (RR 0.79, 95%CI 0.65-0.97; p-score 0.77), ASA plus ticagrelor (RR 0.79, 95%CI 0.64-0.97; p-score 0.79), and ASA plus low-dose rivaroxaban (RR 0.84, 95%CI 0.76-0.93; p-score 0.67) were more effective in reducing MACE, with according to GRADE, moderate certainty of evidence for the comparison ticagrelor monotherapy and high certainty of evidence for the other three regimens. None of these four antithrombotic regimens were superior to one-another (*supplementary table 1*). Only placebo significantly increased the risk of developing MACE (RR 2.25, 95%CI 1.07-4.73; P-score 0.09) (*figure 3A*).

In the network meta-analysis, no evidence of heterogeneity was found ($\tau^2 = 0$ and $I^2 = 0\%$; 95% CI, 0%-64.8%). There was no measurable global inconsistency based on a random effects design- by-treatment model ($\chi^2_4 = 1.43$; $P = .84$) or local inconsistency within the network.

Table 3: *quality assessment*



Major bleeding

Sixteen studies with 39,388 patients reported major bleeding. One study[53] with 78 patients was not part of the network graph. The certainty of evidence was incorporated in *supplementary table 2*. High-intensity VKA (RR 1.93, 95%CI 1.41-2.64; p-score 0.22), rivaroxaban 5mg twice daily (RR 1.47, 95%CI 1.06-2.05; p-score 0.39), ASA plus low-intensity VKA (RR 2.77, 95%CI 1.93-3.97; p-score 0.08), and ASA plus low-dose rivaroxaban (RR 1.46, 95%CI 1.18-1.80; p-score 0.40), all significantly increased the risk of major bleeding, compared to ASA monotherapy, with high certainty of evidence according to GRADE. No antithrombotic regimen or placebo reduced the risk of major bleeding (*figure 3A*).

No heterogeneity was observed ($\tau^2 = 0$ and $I^2 = 0\%$; 95%CI, 0%-74.6%), and there was no measurable global inconsistency based on a random effects design-by-treatment model ($\chi^2_3 = 0.60$; $p = .90$) or local inconsistency within the network.

Major adverse limb events

Seven studies with 29,015 patients reported MALE. A network graph could be built of six studies[16-17,37-38,50,54] with 15,130 patients. The EUCLID trial[35] was left out since its treatment options did not connect to other studies. The certainty of evidence was incorporated in *supplementary table 3*. ASA plus clopidogrel (RR 0.99, 95%CI 0.57-1.73, moderate certainty of evidence), ASA plus ticagrelor (RR 0.82, 95%CI 0.40-1.67, low certainty of evidence), rivaroxaban 5mg twice daily (RR 0.81, 95%CI 0.43-1.50, moderate certainty of evidence), ASA plus low-dose rivaroxaban (RR 0.75, 95%CI 0.49-1.14, high certainty of evidence) and ASA plus cilostazol (RR 0.69, 95%CI 0.34-1.39, moderate certainty of evidence) were compared to ASA, but none was superior in preventing MALE (*figure 3A*).

Some heterogeneity was observed ($\tau^2 = 0.07$ and $I^2 = 60\%$; 95%CI, 0%-88.7%). There was measurable inconsistency based on a random effects design-by-treatment model ($\chi^2_1 = 4.04$; $p = .04$).

Acute limb ischemia

Six studies with 31,406 patients reported ALI. A network graph could be built of four studies[16-17,50,55] with 17,278 patients. The certainty of evidence was incorporated in *supplementary table 4*. ASA plus low-dose rivaroxaban significantly reduced the occurrence of ALI, compared to ASA monotherapy (RR 0.67, 95%CI 0.55-0.80), with high certainty of evidence according to GRADE. No benefit was established for ASA plus ticagrelor, rivaroxaban 5mg twice daily or ASA plus low-intensity VKA (*figure 3A*).

Table 4: *number of events per study, sorted by study population*

Ref.	Study	Antithrombotic regimen			Sample Size		
		T1	T2	T3	N1	N2	N3
Studies on patients who are solely selected for peripheral arterial disease							
13	CAPRIE	A	C		3229	3223	
39	CHARISMA	A	A+C		1551	1545	
40	CLIPS	A	P		185	181	
16+41	COMPASS	A	A+R1	R2	2504	2492	2474
42	COOPER	C	TP1		215	216	
35	EUCLID	C	T2		6955	6930	
45	Gresele	A	A+CC		73	74	
55	WAVE	A	A+ VKA1		1081	1080	
Cumulative incidence of events with universal comparator A, n (%)							
Studies on patients who underwent a peripheral vascular intervention for peripheral arterial disease							
36	Becquemin	P	T2		121	122	
15	BOA	A	VKA2		1324	1326	
37	CABBAGE	A	A+CI		25	25	
38	CASPAR	A	A+C		426	425	
44	ePAD	A+C	A+E		101	100	
46	Johnson	A	A+VKA1		413	418	
47	Li	C	C+VKA1		25	25	
48	MIRROR	A	A+C		40	40	
49	Monaco	A+C	C+VKA1		157	161	
52	RIVAL-PAD	A+C	A+R1		11	9	
53	Soga	A+TP1	A+TP1+CI		39	39	
54	STOP-IC	A	A+CI		98	93	
17	VOYAGER-PAD	A	A+R1		3278	3286	
Cumulative incidence of events with universal comparator A, n (%)							
Studies on patients with coronary artery disease who coincide with PAD							
43	CREDO	A	A+C		140	132	
50	PEGASUS TIMI 54	A	A+TG1	A+TG2	404	368	371
51	PLATO	A+C	A+T2		578	566	
Cumulative incidence of events with universal comparator A, n (%)							

A = Acetylsalicylic acid 75-325 mg daily; C = Clopidogrel 75 mg once daily, CC = Cloricromene 100 mg twice daily; CI = cilostazol 200 mg once daily; E = Edoxaban 60 mg once daily; P = Placebo only; R1 = Rivaroxaban 2.5 mg twice daily; R2 = Rivaroxaban 5 mg twice daily; S = Sarpogrelate 100 mg trice daily TG1 = Ticagrelor 60 mg twice daily; TG2 = Ticagrelor 90 mg twice daily; TP1 = Ticlopidine 200 mg twice

MACE			MB			MALE			ALI		
N1	N2	N3	N1	N2	N3	N1	N2	N3	N1	N2	N3
277	215										
138	117		27	26							
9	19										
174	126	149	42	68	66	56	32	40	34	19	19
0	2										
740	751		109	113					115	117	
0	0										
144	132		24	74					44	42	
742/8623 (8.6%)			93/5136 (1.8%)			56/2504 (1.6%)			78/3583 (2.2%)		
31	28								17	16	
275	248		56	108							
2	1		0	1		2	3				
			5	9		151	149				
1	3		2	0							
			15	35							
2	1		0	1							
15	12										
5	7		13	15							
			0	0							
3	1		0	0							
9	11					28	16				
588	514		100	140		770	687		227	155	
889/4765 (18.7%)			676/5466 (12.4%)			951/3827 (24.8%)			277/3287 (8.4%)		
24	12										
71	47	54	4	4	5	26	23	17	4	2	3
112	93		46	58							
95/544 (17.5%)			4/404 (0.9%)			26/404 (6.4%)			4/404 (0.9%)		

daily; TP2 = Ticlopidine 250 mg twice daily; VKA1 = Vitamin K antagonist with target INR between 1.4 and 3; VKA2 = Vitamin K antagonist with target INR between 3 and 4.5
MACE = major adverse cardiovascular event; ACM = all-cause mortality; MALE = major adverse limb event; ALI = acute limb event; MB = major bleeding; AB = any bleeding

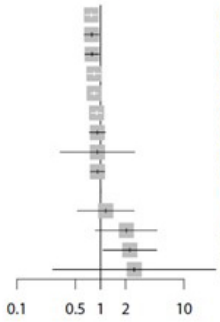
No heterogeneity was observed ($\tau^2 = 0$ and $I^2 = 0\%$; 95%CI not estimable). There was no measurable global inconsistency based on a random effects design-by-treatment model ($\chi^2_1 = 0.41$; $p = .52$) or local inconsistency within the network.

Sensitivity analysis

Exclusion of the high risk of bias studies[47,48] showed our results to be robust, with comparable relative risks and confidence intervals for MACE, MALE and ALI. For major bleeding, besides a high risk of bias study, an additional study[35] was excluded from the network graph because of loss of connection to other studies. Therefore, clopidogrel monotherapy and ticagrelor monotherapy could no longer be compared to ASA for major bleeding. However, all other antithrombotic regimens demonstrated comparable relative risks and confidence intervals as in the primary analysis.

Figure 3A

Treatment	MACE	RR	95%CI	NNT	95%CI	Direct comparisons	Proportion direct evidence	Mean path length	Minimal parallelism
clopidogrel		0.78	[0.66; 0.93]	NNTB 55	[32; 191]	1	0.99	1.02	1.00
ASA+ticagrelor		0.79	[0.64; 0.97]	NNTB 32	[NNTB 16; ∞; NNTH 556]	1	0.58	1.42	1.00
ticagrelor		0.79	[0.65; 0.97]	NNTB 62	[NNTB 31; ∞; NNTH2749]	0	0.00	2.02	1.00
ASA+clopidogrel		0.84	[0.70; 1.01]	NNTB 85	[NNTB 38; ∞; NNTH 345]	4	0.75	1.25	1.00
ASA+rivaroxaban (bid 2.5mg)		0.84	[0.76; 0.93]	NNTB 49	[32; 103]	2	1.00	1.00	1.00
VKA (INR 3-4.5)		0.90	[0.77; 1.05]	NNTB 48	[NNTB 20; ∞; NNTH 104]	1	1.00	1.00	1.00
ASA+VKA (INR 1.4-3)		0.92	[0.74; 1.14]	NNTB 91	[NNTB 26; ∞; NNTH 58]	1	1.00	1.00	1.00
clopidogrel+VKA (INR 1.8-2.5)		0.92	[0.33; 2.57]	NNTB 151	[NNTB 22; ∞; NNTH 30]	0	0.00	2.20	1.00
rivaroxaban (bid 5mg)		0.92	[0.76; 1.12]	NNTB 99	[NNTB 43; ∞; NNTH 351]	1	0.85	1.34	1.00
low-dose ASA (≤365mg/day)		1.00							
ASA+cilostazol		1.16	[0.53; 2.54]	NNTH 163	[NNTB 15; ∞; NNTH 13]	2	1.00	1.00	1.00
ticlopidine		2.04	[0.87; 4.78]	NNTB 123	[NNTB 36; ∞; NNTH 85]	0	0.00	2.00	1.00
none		2.25	[1.07; 4.73]	NNTH 21	[NNTB 22775; ∞; NNTH 10]	1	0.94	1.12	1.00
ASA+edoxaban		2.54	[0.27; 24.22]	NNTH 121	[NNTB 30; ∞; NNTH 20]	0	0.00	2.25	1.00



Treatment	Major bleeding	RR	95%CI	NNT	95%CI	Direct comparisons	Proportion direct evidence	Mean path length	Minimal parallelism
ASA+edoxaban		0.23	[0.01; 4.90]	NNTB 58	[NNTB 20; ∞; NNTH 60]	0	0.00	2.14	1.00
clopidogrel		0.46	[0.02; 12.00]	NNTB 44	[NNTB 8; ∞; NNTH 12]	0	0.00	3.14	1.00
ticagrelor		0.48	[0.02; 12.61]	NNTB 46	[NNTB 8; ∞; NNTH 11]	0	0.00	4.14	1.00
ASA+ticagrelor		0.95	[0.56; 1.62]	NNTB 13878	[NNTB 84; ∞; NNTH 85]	1	0.21	1.80	1.00
low-dose ASA (≤365mg/day)		1.00							
ASA+clopidogrel		1.14	[0.73; 1.78]	NNTH 380	[NNTB 191; ∞; NNTH 95]	2	0.86	1.14	1.00
clopidogrel+VKA (INR 1.8-2.5)		1.28	[0.55; 2.96]	NNTH 84	[NNTB 21; ∞; NNTH 14]	0	0.00	2.14	1.00
ASA+rivaroxaban (bid 2.5mg)		1.46	[1.18; 1.80]	NNTH 83	[54; 182]	2	1.00	1.00	1.00
rivaroxaban (bid 5mg)		1.47	[1.06; 2.05]	NNTH 89	[49; 458]	1	0.73	1.43	1.00
VKA (INR 3-4.5)		1.93	[1.41; 2.64]	NNTH 26	[17; 48]	1	1.00	1.00	1.00
ASA+VKA (INR 1.4-3)		2.77	[1.93; 3.97]	NNTH 21	[16; 32]	2	1.00	1.00	1.00
ASA+cilostazol		2.88	[0.12; 67.41]	NNTH 25	[NNTB 15; ∞; NNTH 7]	1	1.00	1.00	1.00

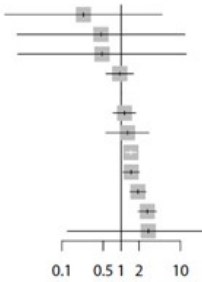
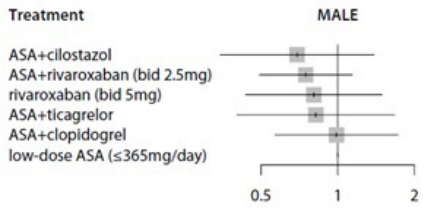
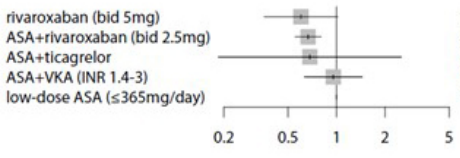


Figure 3A *continued*

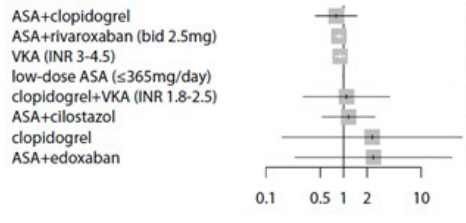


Treatment	RR	95%CI	NNT	95%CI	Direct comparisons	Proportion direct evidence	Mean path length	Minimal parallelism
ASA+cilostazol	0.69	[0.34; 1.39]	NNTB 16	[NNTB 6; ∞; NNTH 28]	2	1.00	1.00	1.00
ASA+rivaroxaban (bid 2.5mg)	0.75	[0.49; 1.14]	NNTB 59	[NNTB 29; ∞; NNTH896]	2	1.00	1.00	1.00
rivaroxaban (bid 5mg)	0.81	[0.43; 1.50]	NNTB 96	[NNTB 31; ∞; NNTH 88]	1	0.87	1.27	1.00
ASA+ticagrelor	0.82	[0.40; 1.67]	NNTB 86	[NNTB 21; ∞; NNTH 41]	1	1.00	1.00	1.00
ASA+clopidogrel	0.99	[0.57; 1.73]	NNTB 258	[NNTB 14; ∞; NNTH 16]	1	1.00	1.00	1.00
low-dose ASA (≤365mg/day)	1.00							



Treatment	RR	95%CI	NNT	95%CI	Direct comparisons	Proportion direct evidence	Mean path length	Minimal parallelism
rivaroxaban (bid 5mg)	0.60	[0.36; 1.02]	NNTB 104	[NNTB 33; ∞; NNTH 95]	1	0.89	1.32	1.13
ASA+rivaroxaban (bid 2.5mg)	0.67	[0.55; 0.80]	NNTB 75	[NNTB 34; ∞; NNTH386]	2	1.00	1.00	1.13
ASA+ticagrelor	0.68	[0.18; 2.53]	NNTB 319	[NNTB 37; ∞; NNTH 49]	1	1.00	1.00	1.13
ASA+VKA (INR 1.4-3)	0.96	[0.63; 1.45]	NNTB 551	[NNTB 35; ∞; NNTH 40]	1	1.00	1.00	1.13
low-dose ASA (≤365mg/day)	1.00							

Figure 3B



Treatment	RR	95%CI	NNT	95%CI	Direct comparisons	Proportion direct evidence	Mean path length	Minimal parallelism
ASA+clopidogrel	0.80	[0.43; 1.49]	NNTB 13	[NNTB 4; ∞; NNTH 8]	1	1.00	1.00	1.00
ASA+rivaroxaban (bid 2.5mg)	0.87	[0.78; 0.97]	NNTB 43	[24; 202]	1	1.00	1.00	1.00
VKA (INR 3-4.5)	0.90	[0.77; 1.05]	NNTB 48	[NNTB 20; ∞; NNTH104]	1	1.00	1.00	1.00
low-dose ASA (≤365mg/day)	1.00							
clopidogrel+VKA (INR 1.8-2.5)	1.09	[0.30; 3.94]	NNTB 16	[NNTB 4; ∞; NNTH 7]	0	0.00	2.00	1.00
ASA+cilostazol	1.16	[0.53; 2.54]	NNTH 163	[NNTB 15; ∞; NNTH 13]	2	1.00	1.00	1.00
clopidogrel	2.34	[0.16; 33.83]	NNTB 40	[NNTB 4; ∞; NNTH 5]	0	0.00	3.00	1.00
ASA+edoxaban	2.42	[0.24; 24.92]	NNTB 18	[NNTB 4; ∞; NNTH 6]	0	0.00	2.00	1.00

Figure 3B *continued*

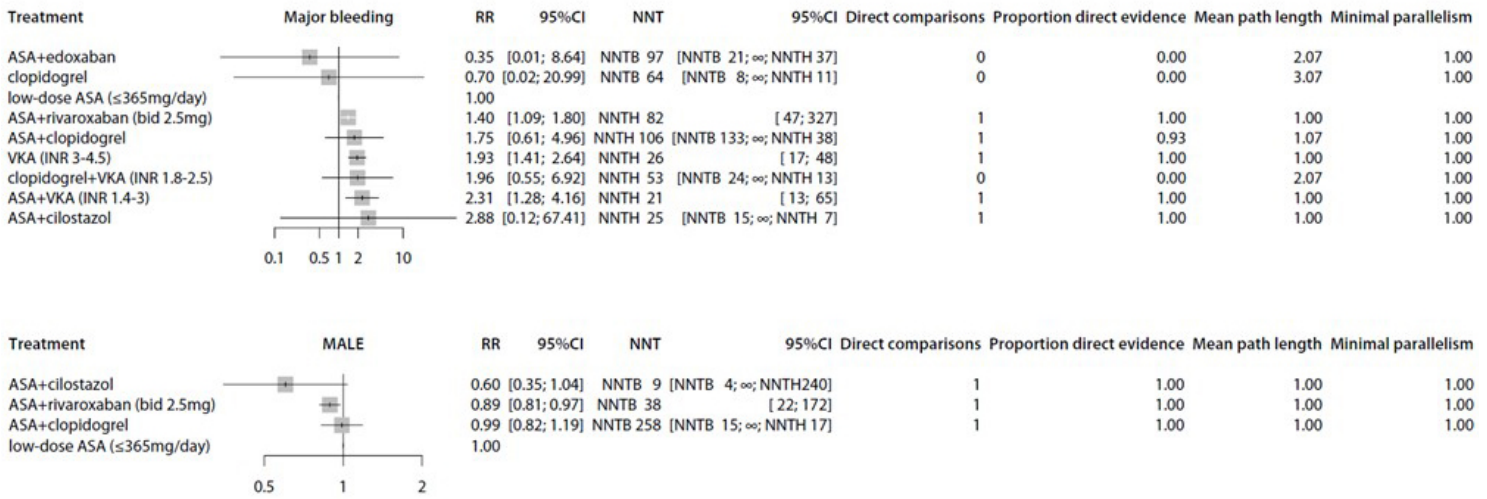


Figure 3: *Forest plots presenting estimated relative risks and corresponding 95% confidence intervals. Results are presented for different antithrombotic strategies compared to low-dose acetylsalicylic acid. NNT, number needed to treat; NNTB, number needed to treat for an additional beneficial outcome; NNTH, number needed to treat for an additional harmful outcome; ∞ , need to treat an infinite number of people to cause or avoid an event (i.e. no effect). Direct comparisons are the number of studies that directly compared the treatment option to the universal comparator. Hereafter the proportion of direct evidence is shown. The mean pathlength characterizes the degree of indirectness of an estimate. Minimal parallelism presents the minimum number of independent paths contributing to the effect estimate.*[22] *A: all patients. B:patients who underwent a peripheral vascular intervention for peripheral arterial disease. For the patients who underwent a peripheral vascular intervention, no network meta-analysis could be performed for acute limb events, since studies had no overlapping antithrombotic regimens.*

Subgroup analysis

There were 13 RCTs in which patients were selected for undergoing a peripheral vascular intervention (endovascular or surgical). *Table 4* provides an overview of the number of events, sorted per population, including the cumulative incidence of events of all patients taking the universal comparator ASA. MACE, major bleeding, MALE and ALI were all more common in patients who underwent a peripheral intervention for PAD, compared to patients who were solely selected for PAD, 18.7% vs 8.6%, 12.4% vs 1.8%, 24.8% vs 1.6% and 8.4% vs 2.2%, respectively. *Figure 3B* demonstrates the results of the network meta-analysis of patients who underwent a peripheral vascular intervention for PAD. The duration of antithrombotic treatment and follow-up of all studies was at least 3 months, starting at the day of intervention. Evidence regarding antithrombotic treatment strategies within 3 months after peripheral intervention was lacking.

In patients, ≥3 months after a peripheral vascular intervention, MACE was reported for ASA, clopidogrel, ASA plus clopidogrel, high-intensity VKA, ASA plus low-dose rivaroxaban, ASA plus edoxaban, ASA plus cilostazol and clopidogrel plus low-intensity VKA in eight studies with 10,073 patients. Only ASA plus low-dose rivaroxaban significantly reduced the risk of MACE (RR 0.87, 95%CI 0.78-0.97), compared to ASA. Major bleeding was reported for ASA, clopidogrel, ASA plus clopidogrel, high-intensity VKA, ASA plus low-intensity VKA, ASA plus low-dose rivaroxaban, ASA plus edoxaban, ASA plus cilostazol, and clopidogrel plus low-intensity VKA in nine studies with 11,503 patients. High-intensity VKA (RR 1.93, 95%CI 1.41-2.64), ASA plus low-intensity VKA (RR 2.31, 95%CI 1.28-4.16), and ASA plus low-dose rivaroxaban (RR 1.4, 95%CI 1.09-1.80) increased the risk of major bleeding. MALE was reported for ASA, ASA plus clopidogrel, ASA plus low-dose rivaroxaban, and ASA plus cilostazol in four studies with 7,596 patients. ASA plus low-dose rivaroxaban significantly reduced MALE (RR 0.89, 95%CI 0.81-0.97). No network meta-analysis could be performed for ALI, since studies describing ASA had no overlapping antithrombotic regimens.

Discussion

In this systematic review and network meta-analysis, we evaluated the effectiveness and safety of different antithrombotic regimens compared to ASA in symptomatic PAD patients. In the overall network meta-analysis, clopidogrel, ticagrelor, ASA plus ticagrelor, and ASA plus low-dose rivaroxaban, were all more effective than ASA monotherapy, and equally effective to one-other, in preventing MACE. ASA plus

low-dose rivaroxaban also reduced the risk of ALI, but increased the risk of major bleeding. Regarding the efficacy of clopidogrel in reducing MALE and ALI, evidence is lacking, while limited evidence indicates similar safety regarding bleeding complications of clopidogrel compared to ASA.

According to international guidelines, clopidogrel monotherapy is advised in symptomatic PAD, and ASA is an alternative.[1,11] The slight preference of clopidogrel over ASA, as mentioned by the European guidelines[1], is based on the results from the CAPRIE study.[13] CAPRIE was a RCT assessing the relative efficacy and safety of clopidogrel versus ASA in (stratified) subgroups of patients with atherosclerotic vascular disease (i.e. ischaemic stroke, recent myocardial infarction, or symptomatic PAD). The primary outcome (i.e. a composite of ischaemic stroke, myocardial infarction, and cardiovascular death) was significantly reduced with clopidogrel compared to ASA. The CAPRIE study reported no major differences in bleeding risk, however, safety profiles were not separately analyzed for the different subgroups, while we could not retrieve bleeding data of PAD patients from the investigators. In our network meta-analysis, the comparison between ASA and clopidogrel regarding bleedings is based on indirect evidence with a relatively high uncertainty, but demonstrated no increased bleeding risk. Altogether, it is plausible to assume that clopidogrel does not increase the risk of bleeding as compared to ASA.

The use of alternative P2Y12 inhibitors, such as ticagrelor, in PAD patients is not approved by international authorities (i.e. the European Medicines Agency and the United States Food and Drug Administration) and has therefore currently no place in the treatment of PAD. The network meta-analysis demonstrated superiority of ticagrelor over ASA, with similar effectiveness as compared to clopidogrel. This is in line with results from the EUCLID trial.[33] However, since clopidogrel is a prodrug which needs to be converted by the CYP2C19 enzyme, its effectiveness is related to CYP2C19 polymorphisms. The EUCLID trial did not compensate for the presence of CYP2C19 loss-of-function alleles. At least in theory, the effectiveness of clopidogrel could be further improved by CYP2C19 polymorphism guided prescription, suggesting that clopidogrel might be the more potent antithrombotic regimen. Results of the ongoing GENPAD study [https://clinicaltrials.gov/ct2/show/NCT04619927] will clarify the role of CYP2C19 polymorphism in symptomatic PAD patients treated with clopidogrel monotherapy.

The dual antiplatelet therapies ASA plus clopidogrel and ASA plus ticagrelor were also studied in this network meta-analysis. The combination of ASA plus clopidogrel has been studied in several RCTs concerning PAD patients, of which

the CHARISMA trial[39] was the largest. In line with the CHARISMA trial, our network meta-analysis found no significant improvement in MACE, and no increased rates of major bleeding, compared to ASA. In contrast, ASA plus ticagrelor, did result in fewer MACE compared to ASA monotherapy. However, this combination has only been studied in populations of PAD patients with manifest concomitant coronary artery disease[50-51], which might influence the extrapolation of these results to the overall PAD population.

The use of vitamin K antagonist monotherapy has only been studied in patients that underwent infrainguinal bypass grafting[15], but not in the overall population of symptomatic PAD. Antiplatelet therapy plus vitamin K antagonist, however, was studied in the overall population of symptomatic PAD. Similar to our results of the network meta-analysis, the WAVE trial[55] reported no reduction in MACE while the bleeding risk increased with ASA plus vitamin K antagonist compared to ASA monotherapy.

The recent COMPASS[16] and VOYAGER-PAD[17] studies demonstrated that the use of ASA plus low-dose rivaroxaban was associated with a reduction of MACE and MALE, but an increased risk of major bleeding, compared to ASA monotherapy. Altogether, a net-clinical benefit of ASA plus low-dose rivaroxaban was established over ASA monotherapy[16,57]. Trials directly comparing ASA plus low-dose rivaroxaban to clopidogrel monotherapy have not been performed. By use of network meta-analysis, we could indirectly compare clopidogrel to ASA plus low-dose rivaroxaban in their effectiveness to reduce MACE [RR 0.93 95%CI 0.76-1.13] (*supplementary table 1*), and found no significant difference. This is in line with a recent concise network meta-analysis.[58]

In the subgroup analysis of patients who underwent a peripheral vascular intervention, only ASA plus low-dose rivaroxaban was superior to ASA monotherapy for the reduction of MACE and MALE. This benefit, however, coincided with an increased risk of major bleeding. Quality trial evidence on clopidogrel or ticagrelor monotherapy was lacking. Patients undergoing a peripheral vascular intervention display a remarkably high risk of MACE and MALE, compared to the overall group of symptomatic PAD. In this subgroup of patients, a higher bleeding risk would be justified to reduce arterial thrombotic events, resulting in a net-clinical benefit. Based on the current evidence and the strongly increased thrombotic risk, the use of ASA plus low-dose rivaroxaban could be considered in patients who underwent a peripheral vascular intervention. This is supported by a commentary of Mukherjee who argues that adding rivaroxaban to ASA could be considered in PAD patients

who have had lower extremity revascularization, with reassessment of the patient-specific risks-benefit ratio beyond 1 year.[59] Furthermore, this is in line with the ESVS Global Vascular Guidelines on the Management of Chronic Limb-Threatening Ischemia which advises to consider low-dose rivaroxaban to reduce adverse cardiovascular events and lower extremity ischemic events in patients with chronic limb threatening ischaemia.[60]

Remarkably, no benefits were found for DAPT after peripheral revascularization. The use of DAPT after revascularization procedures has been widely studied in the field of cardiology. The combination of ASA with a P2Y12 inhibitor is recommended for 6 to 12 months after myocardial revascularization to reduce the risk of stent thrombosis.[61-62] In PAD, DAPT with ASA plus clopidogrel for at least one month after peripheral revascularization procedures (i.e. endovascular stent implantation, below-the-knee bypass with a prosthetic graft) is currently recommended in guidelines[1,11]. In our network meta-analysis, evidence regarding antithrombotic treatment strategies within 3 months after peripheral intervention was lacking. Therefore, we have no information on whether DAPT might improve outcomes in the first months after peripheral revascularization. For the long-term secondary prevention, we identified two RCTs comparing the combination of ASA and clopidogrel to ASA monotherapy. The CASPAR trial[38], which solely studied below-the-knee bypass grafting, indicated a benefit of ASA plus clopidogrel over ASA monotherapy in patients receiving prosthetic grafts. The MIRROR trial demonstrated a reduction in target lesion revascularization with ASA plus clopidogrel compared to ASA, in patients following endovascular femoropopliteal revascularization. These trials were not powered for MACE. A high quality RCT comparing DAPT to clopidogrel monotherapy for secondary prevention in patients undergoing peripheral vascular interventions, is needed to address these important gaps in evidence.

The strengths of our network meta-analysis are mainly related to its comprehensive approach by including all randomized controlled trials since 1995 comparing different antithrombotic treatments in symptomatic PAD patients. Also, no evidence of statistical heterogeneity and no measurable global or local inconsistency was found for the primary outcomes (MACE and major bleedings). However, this network meta-analysis also has some limitations that should be addressed. First, the definitions of MACE, MALE and major bleeding differed between the studies. MACE was generally defined as the composite of cardiovascular death, myocardial infarction and stroke, and MALE as the composite of chronic or acute limb ischemia and major amputation. Broader definitions were accepted if they concerned outcomes of interest. For example, MACE including amputation was

acceptable, but MACE including pulmonary embolism was not. In some studies, in which multiple composite outcomes were reported, we selected the composite outcome that was closest to the most common definitions of MACE and MALE. We expect that the differences in definitions of MACE, MALE and major bleeding did not have much impact on the relative risks calculated in our network meta-analyses, as the definitions were similar between the control and intervention arms of the respective studies. Second, the follow-up time varied between the studies. Since re-occlusion is more common in the first year after revascularization[63], this could have resulted in a relatively high risk of events in studies on patients who underwent a peripheral vascular intervention. The increased risk, however, applied to both control and intervention group and therefore not necessarily affected relative risks. Third, ASA was the universal comparator in the network meta-analyses, while current guidelines state that clopidogrel may be preferred over ASA as antithrombotic treatment in symptomatic PAD patients. Since the majority of studies used ASA as the comparator, choosing another antithrombotic regimen as universal comparator, would have increased the indirectness of the evidence resulting in higher uncertainties. Fourth, there is lack of individual patient data. Individual patient data network meta-analysis would generate more precise estimates, however individual patient data were not available for most important trials. Fifth, the subgroup analysis of patients after a peripheral vascular intervention, included both endovascular and surgical procedures. With the outcomes of interest mainly focusing on secondary prevention, we choose to select a broad group with relatively severe PAD. However, it is possible that the optimal long-term antithrombotic therapy for a patient after endovascular revascularization is not similar to the optimal therapy for a surgically treated patient.

In conclusion, clopidogrel, ticagrelor, ASA plus ticagrelor, and ASA plus low-dose rivaroxaban are superior to ASA monotherapy and equally effective to one another in preventing MACE in PAD patients. Of these four therapies, ticagrelor is not approved by international authorities, and ASA plus low-dose rivaroxaban provides a higher risk of major bleedings. Therefore, clopidogrel may be considered the first choice in symptomatic PAD patients. In PAD patients undergoing a vascular intervention, ASA plus low-dose rivaroxaban could be considered for the long term (>3 months) prevention of MACE and MALE, but a higher bleeding risk should be taken into account.

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Supplementary table 1: *estimated relative risk of the primary cardiovascular effectiveness outcome with 95%- confidence intervals*

	A+CI	A+C	A+E	A+R1	A+T	A+VKA1	C
A+CI	NA	1.38 (0.62 - 3.09)[4]	0.45 (0.04 - 4.95)[3]	1.37 (0.62 - 3.03)[3]	1.47 (0.65 - 3.32)[3]	1.26 (0.56 - 2.85)[3]	1.48 (0.66 - 3.31)[3]
A+C	0.73 (0.32 - 1.62)[4]	NA	0.33 (0.03 - 3.12)[2]	1 (0.81 - 1.22)[2]	1.07 (0.87 - 1.3)[1]	0.91 (0.69 - 1.22)[2]	1.08 (0.84 - 1.38)[2]
A+E	2.2 (0.2 - 23.9)[3]	3.03 (0.32 - 28.64)[2]	NA	3.02 (0.32 - 28.81)[3]	3.23 (0.34 - 30.83)[3]	2.77 (0.29 - 26.68)[3]	3.26 (0.34 - 31.25)[3]
A+R1	0.73 (0.33 - 1.61)[3]	1 (0.82 - 1.23)[2]	0.33 (0.03 - 3.16)[3]	NA	1.07 (0.85 - 1.35)[2]	0.92 (0.72 - 1.17)[2]	1.08 (0.89 - 1.31)[2]
A+T	0.68 (0.3 - 1.53)[3]	0.94 (0.77 - 1.14)[1]	0.31 (0.03 - 2.95)[3]	0.93 (0.74 - 1.17)[2]	NA	0.86 (0.63 - 1.16)[2]	1.01 (0.77 - 1.32)[2]
A+VKA1	0.79 (0.35 - 1.79)[3]	1.09 (0.82 - 1.45)[2]	0.36 (0.04 - 3.47)[3]	1.09 (0.86 - 1.39)[2]	1.17 (0.86 - 1.58)[2]	NA	1.18 (0.89 - 1.55)[2]
C	0.67 (0.3 - 1.51)[3]	0.93 (0.72 - 1.19)[2]	0.31 (0.03 - 2.94)[3]	0.93 (0.76 - 1.13)[2]	0.99 (0.76 - 1.3)[2]	0.85 (0.64 - 1.12)[2]	NA
C+VKA1	0.79 (0.22 - 2.9)[3]	1.1 (0.4 - 3.03)[2]	0.36 (0.03 - 4.26)[3]	1.09 (0.39 - 3.06)[3]	1.17 (0.42 - 3.29)[3]	1 (0.35 - 2.87)[3]	1.18 (0.42 - 3.32)[4]
A	0.86 (0.39 - 1.9)[2]	1.19 (0.99 - 1.43)[1]	0.39 (0.04 - 3.74)[4]	1.19 (1.08 - 1.31)[1]	1.27 (1.03 - 1.57)[1]	1.09 (0.87 - 1.36)[1]	1.28 (1.08 - 1.52)[1]
P	1.94 (0.66 - 5.73)[4]	2.68 (1.25 - 5.76)[3]	0.88 (0.08 - 9.48)[4]	2.67 (1.26 - 5.65)[3]	2.86 (1.32 - 6.18)[3]	2.45 (1.13 - 5.32)[3]	2.88 (1.35 - 6.17)[3]
R2	0.8 (0.35 - 1.79)[3]	1.1 (0.84 - 1.43)[2]	0.36 (0.04 - 3.48)[3]	1.09 (0.9 - 1.33)[1]	1.17 (0.88 - 1.56)[2]	1 (0.75 - 1.35)[2]	1.18 (0.91 - 1.53)[2]
T	0.69 (0.31 - 1.54)[3]	0.95 (0.73 - 1.23)[2]	0.31 (0.03 - 3)[3]	0.94 (0.76 - 1.17)[2]	1.01 (0.76 - 1.34)[2]	0.87 (0.64 - 1.16)[2]	1.02 (0.93 - 1.12)[1]
TP	1.76 (0.55 - 5.62)[3]	2.43 (1.02 - 5.8)[2]	0.8 (0.07 - 8.93)[3]	2.42 (1.03 - 5.71)[2]	2.6 (1.08 - 6.23)[2]	2.23 (0.92 - 5.36)[2]	2.62 (1.1 - 6.22)[1]
VKA2	0.78 (0.35 - 1.73)[3]	1.07 (0.85 - 1.36)[2]	0.35 (0.04 - 3.39)[3]	1.07 (0.89 - 1.28)[2]	1.14 (0.88 - 1.48)[2]	0.98 (0.75 - 1.28)[3]	1.15 (0.92 - 1.45)[2]

A = Acetylsalicylic acid 75-325 mg daily; CI = cilostazol 200 mg once daily; C = Clopidogrel 75 mg once daily; E = Edoxaban 60 mg once daily; R1 = Rivaroxaban 2.5 mg twice daily; T = Ticagrelor 60-90 mg twice daily; VKA1 = Vitamin K antagonist with target INR between 1.4 and 3; P = Placebo only; R2 = Rivaroxaban 5 mg twice daily; TP = Ticlopidine 200-250 mg twice daily; VKA2 = Vitamin K antagonist with target INR between 3 and 4.5. The certainty of the evidence (according to GRADE) was incorporated in this figure. [1]High quality of evidence, [2]Moderate quality of evidence, [3]Low quality of evidence, [4]Very low quality of evidence

C+VKA1	A	P	R2	T	TP	VKA2
1.26 (0.35 - 4.58)[3]	1.16 (0.53 - 2.54)[2]	0.51 (0.17 - 1.52)[4]	1.26 (0.56 - 2.82)[3]	1.46 (0.65 - 3.27)[3]	0.57 (0.18 - 1.8)[3]	1.29 (0.58 - 2.86)[3]
0.91 (0.33 - 2.52)[2]	0.84 (0.7 - 1.01)[1]	0.37 (0.17 - 0.8)[3]	0.91 (0.7 - 1.19)[2]	1.06 (0.81 - 1.38)[2]	0.41 (0.17 - 0.98)[2]	0.93 (0.74 - 1.18)[2]
2.76 (0.23 - 32.55)[3]	2.54 (0.27 - 24.22)[4]	1.13 (0.11 - 12.14)[4]	2.76 (0.29 - 26.51)[3]	3.2 (0.33 - 30.74)[3]	1.25 (0.11 - 13.85)[3]	2.82 (0.3 - 27.04)[3]
0.92 (0.33 - 2.57)[3]	0.84 (0.76 - 0.93)[1]	0.37 (0.18 - 0.79)[3]	0.91 (0.75 - 1.12)[1]	1.06 (0.85 - 1.32)[2]	0.41 (0.18 - 0.97)[2]	0.94 (0.78 - 1.12)[2]
0.86 (0.3 - 2.4)[3]	0.79 (0.64 - 0.97)[1]	0.35 (0.16 - 0.76)[3]	0.85 (0.64 - 1.14)[2]	0.99 (0.74 - 1.32)[2]	0.39 (0.16 - 0.92)[2]	0.87 (0.67 - 1.13)[2]
1 (0.35 - 2.85)[3]	0.92 (0.74 - 1.14)[1]	0.41 (0.19 - 0.89)[3]	1 (0.74 - 1.34)[2]	1.16 (0.86 - 1.55)[2]	0.45 (0.19 - 1.08)[2]	1.02 (0.78 - 1.33)[3]
0.85 (0.3 - 2.39)[4]	0.78 (0.66 - 0.93)[1]	0.35 (0.16 - 0.74)[3]	0.85 (0.65 - 1.1)[2]	0.98 (0.89 - 1.08)[1]	0.38 (0.16 - 0.91)[1]	0.87 (0.69 - 1.09)[2]
NA	0.92 (0.33 - 2.57)[3]	0.41 (0.12 - 1.45)[4]	1 (0.35 - 2.84)[3]	1.16 (0.41 - 3.28)[3]	0.45 (0.12 - 1.71)[3]	1.02 (0.36 - 2.89)[3]
1.09 (0.39 - 3.04)[3]	NA	0.44 (0.21 - 0.94)[2]	1.09 (0.89 - 1.32)[1]	1.26 (1.04 - 1.53)[2]	0.49 (0.21 - 1.15)[2]	1.11 (0.95 - 1.3)[1]
2.44 (0.69 - 8.68)[4]	2.25 (1.07 - 4.73)[2]	NA	2.44 (1.13 - 5.26)[3]	2.83 (1.32 - 6.09)[3]	1.1 (0.71 - 1.71)[4]	2.5 (1.17 - 5.33)[3]
1 (0.35 - 2.85)[3]	0.92 (0.76 - 1.12)[1]	0.41 (0.19 - 0.88)[3]	NA	1.16 (0.88 - 1.53)[2]	0.45 (0.19 - 1.08)[2]	1.02 (0.8 - 1.31)[2]
0.86 (0.31 - 2.44)[3]	0.79 (0.65 - 0.97)[2]	0.35 (0.16 - 0.76)[3]	0.86 (0.65 - 1.14)[2]	NA	0.39 (0.16 - 0.93)[2]	0.88 (0.69 - 1.13)[2]
2.22 (0.59 - 8.42)[3]	2.04 (0.87 - 4.78)[2]	0.91 (0.58 - 1.41)[4]	2.22 (0.93 - 5.3)[2]	2.57 (1.08 - 6.14)[2]	NA	2.27 (0.96 - 5.38)[2]
0.98 (0.35 - 2.77)[3]	0.9 (0.77 - 1.05)[1]	0.4 (0.19 - 0.86)[3]	0.98 (0.76 - 1.25)[2]	1.13 (0.88 - 1.45)[2]	0.44 (0.19 - 1.05)[2]	NA

Supplementary table 2: *estimated relative risk of the primary safety outcome with 95%- confidence intervals*

MB	A+CI	A+C	A+E	A+R1	A+T	A+VKA1
A+CI	NA	2.53 (0.1 - 60.94)[3]	12.51 (0.16 - 1009.02)[3]	1.98 (0.08 - 46.58)[3]	3.04 (0.12 - 74.38)[3]	1.04 (0.04 - 24.84)[3]
A+C	0.4 (0.02 - 9.55)[3]	NA	4.95 (0.24 - 101.83)[2]	0.78 (0.48 - 1.28)[2]	1.2 (0.84 - 1.72)[1]	0.41 (0.23 - 0.73)[2]
A+E	0.08 (0 - 6.45)[3]	0.2 (0.01 - 4.15)[2]	NA	0.16 (0.01 - 3.38)[3]	0.24 (0.01 - 5.1)[3]	0.08 (0 - 1.81)[3]
A+R1	0.51 (0.02 - 11.91)[3]	1.28 (0.78 - 2.08)[2]	6.32 (0.3 - 135.3)[3]	NA	1.54 (0.87 - 2.73)[2]	0.53 (0.35 - 0.8)[2]
A+T	0.33 (0.01 - 8.05)[3]	0.83 (0.58 - 1.18)[1]	4.11 (0.2 - 86.39)[3]	0.65 (0.37 - 1.15)[2]	NA	0.34 (0.18 - 0.65)[2]
A+VKA1	0.96 (0.04 - 22.94)[3]	2.43 (1.37 - 4.29)[2]	12.02 (0.55 - 260.74)[3]	1.9 (1.25 - 2.88)[2]	2.92 (1.53 - 5.57)[2]	NA
C	0.16 (0 - 14.86)[4]	0.4 (0.02 - 10.2)[4]	1.98 (0.02 - 166.47)[4]	0.31 (0.01 - 8.29)[4]	0.48 (0.02 - 12.52)[4]	0.17 (0.01 - 4.42)[4]
C+VKA1	0.44 (0.02 - 11.58)[3]	1.12 (0.55 - 2.28)[2]	5.55 (0.25 - 123.93)[3]	0.88 (0.37 - 2.08)[3]	1.35 (0.61 - 2.99)[3]	0.46 (0.19 - 1.15)[2]
A	0.35 (0.01 - 8.11)[2]	0.88 (0.56 - 1.37)[1]	4.34 (0.2 - 92.18)[3]	0.69 (0.56 - 0.85)[1]	1.05 (0.62 - 1.8)[3]	0.36 (0.25 - 0.52)[1]
R2	0.51 (0.02 - 12.17)[3]	1.29 (0.75 - 2.24)[2]	6.4 (0.3 - 138.34)[3]	1.01 (0.74 - 1.38)[2]	1.56 (0.83 - 2.91)[2]	0.53 (0.33 - 0.87)[2]
T	0.17 (0 - 15.58)[4]	0.42 (0.02 - 10.73)[3]	2.06 (0.02 - 174.53)[4]	0.33 (0.01 - 8.71)[4]	0.5 (0.02 - 13.16)[4]	0.17 (0.01 - 4.64)[4]
VKA2	0.67 (0.03 - 15.87)[3]	1.69 (0.98 - 2.9)[2]	8.36 (0.39 - 180.38)[3]	1.32 (0.91 - 1.93)[2]	2.03 (1.09 - 3.78)[2]	0.7 (0.43 - 1.12)[2]

A = Acetylsalicylic acid 75-325 mg daily; CI = cilostazol 200 mg once daily; C = Clopidogrel 75 mg once daily; E = Edoxaban 60 mg once daily; R1 = Rivaroxaban 2.5 mg twice daily; T = Ticagrelor 60-90 mg twice daily; VKA1 = Vitamin K antagonist with target INR between 1.4 and 3; R2 = Rivaroxaban 5 mg twice daily; VKA2 = Vitamin K antagonist with target INR between 3 and 4.5. The certainty of the evidence (according to GRADE) was incorporated in this figure. [1]High quality of evidence, [2]Moderate quality of evidence, [3]Low quality of evidence, [4]Very low quality of evidence

C	C+VKA1	A	R2	T	VKA2
6.31 (0.07 - 590.84)[4]	2.25 (0.09 - 58.84)[3]	2.88 (0.12 - 67.41)[2]	1.95 (0.08 - 46.49)[3]	6.06 (0.06 - 572.15)[4]	1.5 (0.06 - 35.56)[3]
2.5 (0.1 - 63.56)[4]	0.89 (0.44 - 1.82)[2]	1.14 (0.73 - 1.78)[1]	0.77 (0.45 - 1.34)[2]	2.4 (0.09 - 61.74)[3]	0.59 (0.34 - 1.02)[2]
0.5 (0.01 - 42.31)[4]	0.18 (0.01 - 4.03)[3]	0.23 (0.01 - 4.9)[3]	0.16 (0.01 - 3.38)[3]	0.48 (0.01 - 40.98)[4]	0.12 (0.01 - 2.58)[3]
3.19 (0.12 - 84.23)[4]	1.14 (0.48 - 2.7)[3]	1.46 (1.18 - 1.8)[1]	0.99 (0.72 - 1.35)[2]	3.06 (0.11 - 81.8)[4]	0.76 (0.52 - 1.1)[2]
2.07 (0.08 - 53.85)[4]	0.74 (0.33 - 1.64)[3]	0.95 (0.56 - 1.62)[3]	0.64 (0.34 - 1.2)[2]	1.99 (0.08 - 52.3)[4]	0.49 (0.26 - 0.92)[2]
6.06 (0.23 - 162.18)[4]	2.17 (0.87 - 5.39)[2]	2.77 (1.93 - 3.97)[1]	1.88 (1.16 - 3.05)[2]	5.82 (0.22 - 157.5)[4]	1.44 (0.89 - 2.32)[2]
NA	0.36 (0.02 - 8.41)[4]	0.46 (0.02 - 12)[4]	0.31 (0.01 - 8.27)[4]	0.96 (0.74 - 1.25)[1]	0.24 (0.01 - 6.33)[4]
2.8 (0.12 - 65.8)[4]	NA	1.28 (0.55 - 2.96)[3]	0.87 (0.35 - 2.13)[3]	2.69 (0.11 - 63.92)[4]	0.66 (0.27 - 1.63)[3]
2.19 (0.08 - 57.42)[4]	0.78 (0.34 - 1.81)[3]	NA	0.68 (0.49 - 0.94)[1]	2.1 (0.08 - 55.76)[4]	0.52 (0.38 - 0.71)[1]
3.23 (0.12 - 86.07)[4]	1.15 (0.47 - 2.84)[3]	1.47 (1.06 - 2.05)[1]	NA	3.1 (0.12 - 83.58)[4]	0.77 (0.49 - 1.21)[2]
1.04 (0.8 - 1.35)[1]	0.37 (0.02 - 8.85)[4]	0.48 (0.02 - 12.61)[4]	0.32 (0.01 - 8.69)[4]	NA	0.25 (0.01 - 6.65)[4]
4.21 (0.16 - 112.24)[4]	1.51 (0.62 - 3.69)[3]	1.93 (1.41 - 2.64)[1]	1.31 (0.83 - 2.05)[2]	4.05 (0.15 - 109)[4]	NA

Supplementary table 3: *estimated relative risk of the secondary outcome major adverse limb events with 95%- confidence intervals*

MALE	A+CI	A+C	A+R1	A+T	A	R2
A+CI	NA	0.7 (0.29 - 1.71)[3]	0.92 (0.41 - 2.08)[3]	0.84 (0.31 - 2.29)[3]	0.69 (0.34 - 1.39)[2]	0.86 (0.34 - 2.18)[3]
A+C	1.43 (0.59 - 3.49)[3]	NA	1.32 (0.66 - 2.65)[3]	1.21 (0.49 - 2.98)[3]	0.99 (0.57 - 1.73)[2]	1.23 (0.53 - 2.82)[3]
A+R1	1.08 (0.48 - 2.44)[3]	0.76 (0.38 - 1.52)[3]	NA	0.91 (0.4 - 2.09)[1]	0.75 (0.49 - 1.14)[1]	0.93 (0.49 - 1.75)[2]
A+T	1.18 (0.44 - 3.21)[3]	0.83 (0.34 - 2.05)[3]	1.09 (0.48 - 2.5)[1]	NA	0.82 (0.4 - 1.67)[3]	1.02 (0.4 - 2.61)[3]
A	1.44 (0.72 - 2.9)[2]	1.01 (0.58 - 1.76)[2]	1.33 (0.88 - 2.03)[1]	1.22 (0.6 - 2.49)[3]	NA	1.24 (0.67 - 2.3)[2]
R2	1.16 (0.46 - 2.95)[3]	0.82 (0.35 - 1.87)[3]	1.08 (0.57 - 2.03)[2]	0.98 (0.38 - 2.53)[3]	0.81 (0.43 - 1.5)[2]	NA

A = Acetylsalicylic acid 75-325 mg daily; C = Clopidogrel 75 mg once daily; R1 = Rivaroxaban 2.5 mg twice daily; T = Ticagrelor 60-90 mg twice daily; R2 = Rivaroxaban 5 mg twice daily. The certainty of the evidence (according to GRADE) was incorporated in this figure. [1]High quality of evidence, [2]Moderate quality of evidence, [3]Low quality of evidence

Supplementary table 4: *estimated relative risk of the secondary outcome acute limb ischemia with 95%-confidence intervals*

ALI	A+R1	A+T	A+VKA1	A	R2
A+R1	NA	0.98 (0.26 - 3.66)[3]	0.7 (0.44 - 1.1)[2]	0.67 (0.55 - 0.8)[1]	1.11 (0.65 - 1.89)[2]
A+T	1.03 (0.27 - 3.85)[3]	NA	0.72 (0.18 - 2.82)[2]	0.68 (0.18 - 2.53)[2]	1.14 (0.28 - 4.66)[3]
A+VKA1	1.43 (0.91 - 2.26)[2]	1.4 (0.35 - 5.52)[2]	NA	0.96 (0.63 - 1.45)[2]	1.59 (0.81 - 3.1)[2]
A	1.5 (1.24 - 1.81)[1]	1.46 (0.4 - 5.42)[2]	1.05 (0.69 - 1.58)[2]	NA	1.66 (0.98 - 2.81)[1]
R2	0.9 (0.53 - 1.54)[2]	0.88 (0.21 - 3.61)[3]	0.63 (0.32 - 1.23)[2]	0.6 (0.36 - 1.02)[1]	NA

A = Acetylsalicylic acid 75-325 mg daily; R1 = Rivaroxaban 2.5 mg twice daily; T = Ticagrelor 60-90 mg twice daily; VKA1 = Vitamin K antagonist with target INR between 1.4 and 3; R2 = Rivaroxaban 5 mg twice daily. The certainty of the evidence (according to GRADE) was incorporated in this figure. 1High quality of evidence, 2Moderate quality of evidence, 3Low quality of evidence

Chapter 10

Impact of CYP2C19 genotype status on clinical outcomes in patients with symptomatic coronary artery disease, stroke, and peripheral arterial disease, a systematic review

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Abstract

Background: Clopidogrel is widely used for the secondary prevention of atherothrombotic events in patients with coronary artery disease (CAD), ischemic stroke, and peripheral arterial disease (PAD). CYP2C19 plays a pivotal role in the conversion of clopidogrel to its active metabolite. Clopidogrel-treated carriers of a CYP2C19 loss-of-function allele (LOF) may have a higher risk of new atherothrombotic events. Previous studies on genotype-guided treatment were mainly performed in CAD and showed mixed results.

Purpose: To simultaneously investigate the impact of CYP2C19 genotype status on the rate of atherothrombotic events in the most common types of atherosclerotic disease (CAD, stroke, PAD).

Methods: A comprehensive search in Pubmed, EMBASE, and MEDLINE from their inception to July 23rd 2023 was performed. Randomized controlled trials (RCTs) comparing genotype-guided and standard antithrombotic treatment, and cohort studies and post hoc analyses of RCTs concerning the association between CYP2C19 genotype status and clinical outcomes in clopidogrel-treated patients were included. The primary efficacy endpoint was major adverse cardiovascular events (MACE) and the safety end point major bleeding. Secondary endpoints were myocardial infarction, stent thrombosis, and ischemic stroke.

Results: Forty-four studies were identified: 11 studies on CAD, 29 studies on stroke, and 4 studies on PAD. In CAD, genotype-guided therapy significantly reduced the risk of MACE [risk ratio (RR) 0.60, 95% confidence interval (CI) 0.43–0.83], myocardial infarction (RR 0.53, 95% CI 0.42–0.68), and stent thrombosis (RR 0.64, 95% CI 0.43–0.94), compared with standard antithrombotic treatment. The rate of major bleeding did not differ significantly (RR 0.93, 95% CI 0.70–1.23). Most RCTs were performed in patients after percutaneous coronary intervention (9/11). In stroke, LOF carriers had a significantly higher risk of MACE (RR 1.61, 95% CI 1.25–2.08) and recurrent ischemic stroke (RR 1.89, 95% CI 1.48–2.40) compared with non-carriers. No significant differences were found in major bleeding (RR 0.90, 95% CI 0.43–1.89). In the 6955 patients with symptomatic PAD treated with clopidogrel in the EUCLID trial, no differences in MACE or major bleeding were found between LOF carriers and non-carriers. In three smaller studies on patients with PAD treated with clopidogrel after endovascular therapy, CYP2C19 genotype status was significantly associated with atherothrombotic events.

Conclusions: Genotype-guided treatment significantly decreased the rate of atherothrombotic events in patients with CAD, especially after PCI. In patients with history of stroke, LOF carriers treated with clopidogrel had a higher risk of MACE and recurrent stroke. The available evidence in PAD with regard to major adverse limb events is too limited to draw meaningful conclusions.

Introduction

Coronary artery disease (CAD), cerebrovascular disease, and peripheral arterial disease (PAD) are all clinical manifestations of systemic atherosclerosis. In patients with these atherosclerotic diseases, the platelet aggregation inhibitor clopidogrel is widely used for the secondary prevention of thrombotic events. Nevertheless, in clinical practice, many patients receiving clopidogrel show a high residual platelet reactivity. Consequently, these patients are still at risk of adverse cardiovascular atherothrombotic events.

A part of this "clopidogrel resistance" may be explained by genetic variations. Clopidogrel is a thienopyridine prodrug that needs biotransformation by hepatic cytochrome P450 (CYP) enzymes to generate its clinically active metabolite. CYP2C19 plays a pivotal role in this activation process.[1] The CYP2C19 gene is, however, highly polymorphic, with CYP2C19*2 and CYP2C19*3 being the most common polymorphisms. These loss-of-function variants (LOF) can be used to classify patients as normal or extensive metabolizers (EM; CYP2C19 *1/*1), intermediate metabolizers (IM; CYP2C19 *1/*2 and *1/*3), or poor metabolizers (PM; CYP2C19 *2/*2, *2/*3 and *3/*3).

There is substantial evidence that CYP2C19 IMs and PMs have higher on-treatment platelet reactivity than EMs.[2,3] Most clinical studies on the rate of cardiovascular events in CYP2C19 IMs and PMs were performed in patients with acute coronary syndrome. In patients who underwent percutaneous coronary intervention (PCI), a higher risk of cardiovascular events was observed in CYP2C19 LOF carriers compared with non-carriers.[4,5] Moreover, the beneficial effects of a CYP2C19 genotype-guided treatment strategy on the reduction of cardiovascular events in this patient group was demonstrated in several meta-analyses.[6-9] In patients with ischemic stroke treated with clopidogrel, a meta-analysis showed that CYP2C19 LOF carriers had a 1.92 increased risk of recurrent stroke compared with non-carriers.[10] Less clinical evidence is available for patients with peripheral arterial disease.[11]

The aim of this systematic review and meta-analysis is to integrate the available research data in patients with acute coronary syndrome, stroke and peripheral arterial disease and, thus, to provide one large overview of the impact of CYP2C19 metabolizer status on the risk of cardiovascular events in patients with the most common manifestations of atherosclerotic disease.

Methods

The study protocol was registered with PROSPERO, number CRD42020220284, and Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines were followed.

Search Strategy

A comprehensive search in the electronic databases Pubmed, EMBASE, and MEDLINE from their inception to 23 July 2023 was performed. Search terms referring to CYP2C19 genotype, peripheral arterial disease, coronary artery disease, stroke, and treatment with platelet aggregation inhibitors were used in various configurations. Furthermore, the reference lists of the selected articles were searched manually to identify additional studies. Lastly, ClinicalTrials.gov was screened for completed but yet unpublished studies. Details of our electronic search strategy in Pubmed, EMBASE, and MEDLINE are provided in the appendix.

Study Selection

After removal of duplicates, two authors (D.M. and L.W.) independently screened the title and abstract of each record. The same authors independently assessed the full text of all eligible articles. Any disagreements between the authors were resolved via consensus or, in case of persistent discrepancies, by discussion with a third author (M.W.).

Studies on patients with atherosclerotic cardiovascular disease who had an indication for platelet aggregation inhibition therapy were considered eligible for inclusion if: (1) CYP2C19 genotype status was available and (2) clinical outcome measurements were reported. Clinical outcome measurements comprised major adverse cardiovascular events (MACE), myocardial infarction, (ischemic) stroke, cardiovascular death, stent thrombosis, and (major) bleeding. Animal studies, in vitro studies, reviews, editorials, and case reports were excluded. When different articles contained duplicate data, the article with the largest sample size or most complete information was included.

Two different types of studies were available: (1) randomized controlled trials (RCTs) comparing a genotype-guided antithrombotic treatment with standard treatment and (2) cohort studies or post hoc analyses of RCTs describing the association between CYP2C19 genotype status and clinical outcomes. Cohort studies were only included if clopidogrel was used as platelet aggregation inhibitor. For the post hoc analyses of RCTs, we included only the clopidogrel-treated arm in our analysis.

Our study population was divided in three subgroups based on the type of atherosclerotic cardiovascular disease: (1) patients with coronary artery disease (stable disease or acute coronary syndrome), (2) patients with stroke or transient ischemic attack (TIA), and (3) patients with peripheral arterial disease. For each subgroup, the available data were analyzed separately. For patients with coronary artery disease, only data from RCTs comparing genotype-guided and standard antithrombotic treatment were included, as this study type is generally considered to have the highest level of evidence. Because RCTs were not available in patients with peripheral arterial disease and scarcely available in patients with stroke or TIA (n = 3), only cohort studies or post hoc analyses of RCTs describing the association between CYP2C19 genotype status and clinical outcomes were included in these patient groups. The three RCTs in patients with stroke or TIA were described separately.

Data Extraction

Two reviewers (D.M. and L.W.) independently extracted data from the selected articles using a standardized form. Any disagreements were resolved by consensus. The following data were extracted: study acronym (if any), last name of the first author, full title, year of publication, study population, inclusion criteria, sample size, treatment groups (drug, dose, and duration of treatment), CYP2C19 genotype status, baseline patient characteristics, outcome measurements, and duration of follow-up. The primary efficacy endpoint was the occurrence of MACE. MACE was defined as a composite of cardiovascular death, myocardial infarction and stroke. However, broader definitions including other relevant complications of atherosclerotic cardiovascular disease were also allowed.

The primary safety end point was (major) bleeding. Secondary end points were cardiovascular death, myocardial infarction, stent thrombosis, and (ischemic) stroke.

Quality Assessment

The quality of all included studies was assessed by two independent reviewers (D.M. and L.W.). Any discrepancies were resolved by discussion. For the RCTs comparing genotype-guided and standard antithrombotic treatment, risk of bias was assessed with the Cochrane collaboration's risk of bias tool for randomized trials.[12] In brief, this tool is based on the judgement of the risk of bias in five distinct domains: the randomization process, deviations from the intended interventions, missing outcome data, measurement of the outcome, and selection of the reported results. An overall low risk of bias was assigned to studies with a low risk of bias for all domains or studies that raised concerns in a maximum of one domain. An overall

medium risk of bias was assigned to studies that raised concerns in two domains, and an overall high risk of bias to studies that raised concerns in more than two domains or studies with a high risk of bias in at least one domain. The quality of the cohort studies and post hoc analyses of RCTs was evaluated using the Newcastle–Ottawa Scale (NOS).[13] According to this tool, the quality of a study is judged in three different domains: selection of cohorts, comparability of cohorts, and assessment of outcome. An overall quality score (0–9 stars) was assigned for each included study.

CYP2C19 Alleles Measured in Cohort Studies and Post Hoc Analyses of RCTs

To determine CYPC219 genotype status, the loss-of-function (LOF) alleles *2 and/or *3 were measured in most studies. Few studies also described other LOF alleles: *4, *5, *6, *7, and *8. Furthermore, some studies also provided data on the gain-of-function allele *17. The included cohort studies and post hoc analyses of RCTs used different ways to classify patients according to CYP2C19 genotype status. Some studies compared clinical outcomes between LOF allele carriers (LOF carriers) and patients without a LOF allele (non-carriers), while other studies divided their study population in different types of metabolizers. In these last studies, extensive metabolizers had no LOF alleles (*1/*1), intermediate metabolizers had one copy of a LOF allele (*1/*2-*8), and poor metabolizers had two copies of a LOF allele (*2-*8/*2-*8). Some studies also provided data for ultrarapid metabolizers (patients carrying one or two copies of *17: *1/*17 or *17/*17) and/or unknown metabolizers (patients carrying one copy of a LOF allele and one copy of *17: *2-*8/*17).

To increase the uniformity of data in our analysis, we divided the participants into two groups: LOF carriers and non-carriers. Extensive metabolizers were classified as non-carrier and intermediate and poor metabolizers as LOF carriers. Ultrarapid and unknown metabolizers were, if possible, excluded from our analysis as the *17 allele was not consistently determined in all included studies and the presence of a *17 allele may influence the clinical outcome measurements.

Statistical Analysis

Statistical analyses were performed using Cochrane Review Manager software, version 5.3 (RevMan 5.3). For all clinical outcomes, pooled effect estimates were calculated using a random-effects model based on the Mantel–Haenszel method and expressed as risk ratios (RR) with corresponding 95% confidence intervals (95% CI). Results were graphically displayed using forest plots. The I 2 statistic was calculated to assess heterogeneity between studies. Moderate heterogeneity was assumed when I 2 was higher than 30% and substantial heterogeneity when I 2 was higher than 50%.

Results

A total of 9407 records were identified by the search in the electronic databases and other sources (Fig. 1). After removal of exact duplicates and screening of title and abstract, 161 full-text articles were assessed for eligibility. Eventually, 44 articles were included in our analysis: 11 articles on patients with coronary disease, 29 articles on patients with stroke or TIA, and 4 articles on patients with peripheral arterial disease. Moreover, three studies (two RCTs and one post hoc analysis of an RCT) in patients with stroke or TIA involving only CYP2C19 LOF carriers were identified. The main results of these three studies were described separately.

Coronary Disease

Eleven RCTs comparing genotype-guided and standard antithrombotic treatment were included (*table 1*).[14-24] These 11 RCTs contained a total of 11,740 patients (range 60–5276). Of these 11,740 patients, 5958 were treated with genotype-guided treatment and 5782 with standard antithrombotic treatment.

In three RCTs, the study population consisted of patients with acute coronary syndrome or stable coronary artery disease.[17,18,22] Of the remaining eight RCTs, seven studies included only patients with acute coronary syndrome[14-16,19,20,23,24], and one study included only patients with stable coronary artery disease.[21] Furthermore, performance of percutaneous coronary intervention (PCI) was part of the inclusion criteria in nine RCTs.[14,15,17-19,21-24] In the other two RCTs, not all patients underwent a revascularization procedure: in the PHARMCLO study, 62% underwent a PCI and 11% coronary artery bypass grafting[16], and in the study of Tam et al., 77% underwent a PCI and 4% coronary artery bypass grafting.[20]

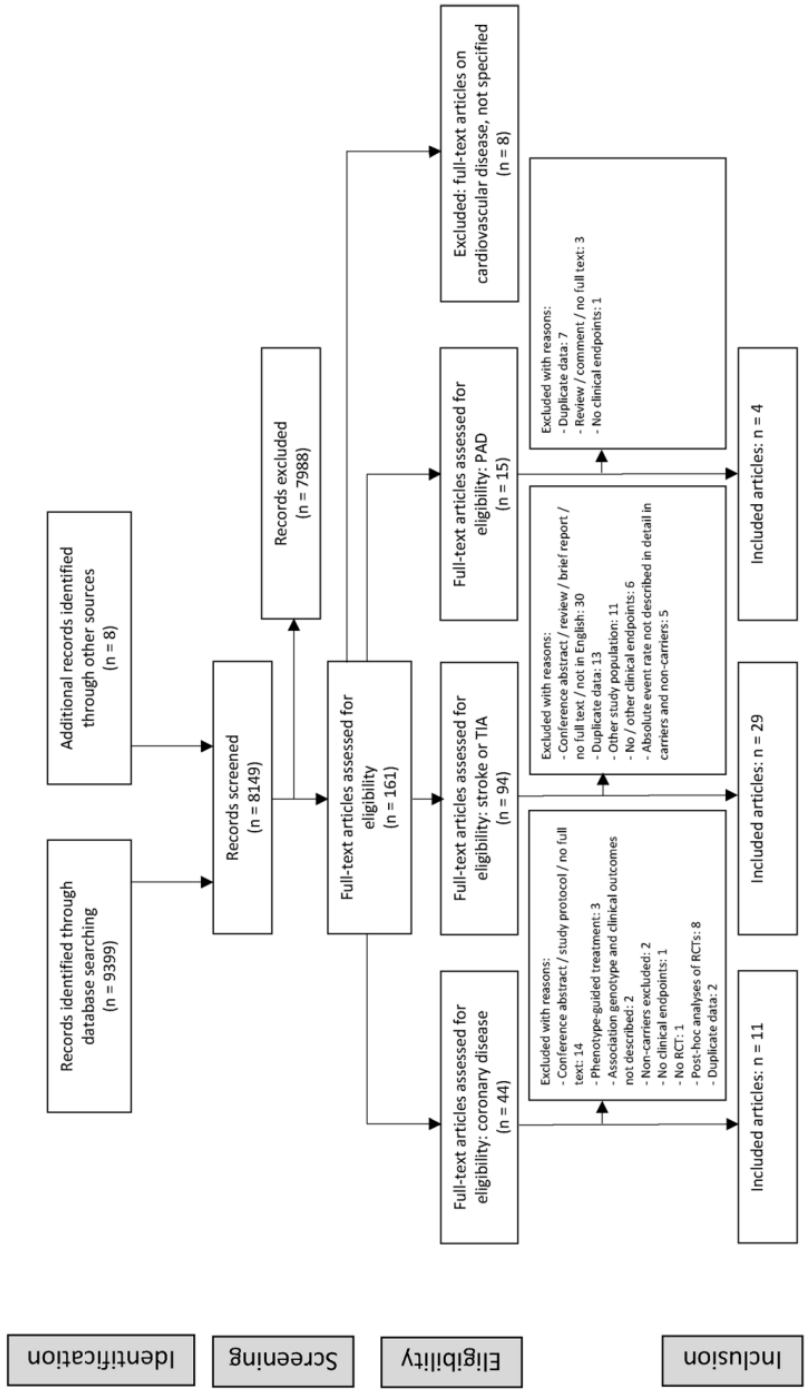

***Figure 1**: Flow chart*

Table 1. *Characteristics of studies on coronary artery* ***disease***

Study	Region	Study period	Patients	Sample size	Age, mean (y)	Male (%)
Al-Rubaish, 2021 (14)	Saudi Arabia	2013 - 2020	STEMI + PCI	687 G: 375 S: 312	56.2	80.8
Claassens, 2019 (15) POPular Genetics	Europe	2011 – 2018	STEMI + PCI	2488 G: 1242 S: 1246	61.7	74.8
Notarangelo, 2018 (16) PHARMCLO	Europe	2013 - 2015	ACS	888 G: 448 S: 440	70.9	68
Pereira, 2020 (17) TAILOR-PCI	US, Canada, South Korea, Mexico	2013 - 2018	ACS or stable CAD + PCI	5276 G: 2641 S: 2635	62 (median)	75.5
Roberts, 2012 (18)	Canada	2010 - 2011	NSTEMI or stable CAD + PCI	187 G: 91 S: 96	60.2	78.1
Shi, 2021 (19)	China	2019	STEMI, NSTEMI or unstable angina + PCI	301 G: 201 S: 100	59.7	75.1
Tam, 2017 (20)	China	2013 - 2015	STEMI, NSTEMI or unstable angina	132 G: 65 S: 67	60.9	80.3

Intervention		Method of genotyping + CYP2C19 alleles	Duration of follow-up
Genotype-guided	**Standard**		
LOF carriers: ticagrelor Non-carriers: clopidogrel All patients: DAPT for at least 12 months after STEMI	Clopidogrel All patients: DAPT for at least 12 months after STEMI	Spartan RX *2	12 months
LOF carriers: ticagrelor or prasugrel Non-carriers: clopidogrel	Ticagrelor or prasugrel	Spartan RX or TaqMan StepOnePlus *2, *3	12 months
Clopidogrel, prasugrel or ticagrelor based on an algorithm including genotyping and clinical characteristics	Clopidogrel, prasugrel or ticagrelor based on clinical characteristics	ST Q3 *2, *17	12 months
LOF carriers: ticagrelor (or prasugrel in case of ticagrelor intolerance) Non-carriers or inconclusive results: clopidogrel All patients: aspirin	Clopidogrel All patients: aspirin	TaqMan *2, *3	12 months
LOF carriers: prasugrel Non-carriers: clopidogrel	Clopidogrel	Spartan RX *2	30 days
Initial selection of P2Y12 receptor inhibitors based on clinical characteristics (ticagrelor or clopidogrel). After genetic results: reconsidered by cardiologists. LOF carriers: ticagrelor recommended. All patients: aspirin	Selection of P2Y12 receptor inhibitors based on clinical characteristics (ticagrelor or clopidogrel). All patients: aspirin	TL988A *2, *3	12 months
Clopidogrel LD 600 mg for STEMI + PCI, LD 300 mg for STEMI without PCI, NSTEMI or unstable angina. LOF carriers: additional LD of ticagrelor 180 mg, MD ticagrelor 2 dd 90 mg Non-carriers: clopidogrel MD 75 mg/d	Clopidogrel LD 600 mg for STEMI + PCI, LD 300 mg for STEMI without PCI, NSTEMI or unstable angina. Clopidogrel MD 75 mg/d	Verigene or Roche LightCycler 480 *2, *3	1 month

Study	Region	Study period	Patients	Sample size	Age, mean (y)	Male (%)
Tomaniak, 2017 (21) ONSIDE TEST	Poland	2012 - 2015	Stable CAD + PCI	60 G: 34 S: 26	62.1	77.3
Tuteja, 2020 (22) ADAPT	United States	2014 - 2016	ACS or stable CAD + PCI	504 G: 249 S: 255	63	73.5
Xie, 2013 (23)	China	2011	ACS or unstable angina + PCI	600 G: 301 S: 299	57.9	78
Zhang, 2020 (24)	China	2014 - 2017	ACS + PCI	617 G: 311 S: 306	64.1	70.3

Y: years. STEMI: ST-elevation myocardial infarction. PCI: percutaneous coronary intervention. G: genotype-guided treatment. S: standard treatment. LOF: loss-of-function allele. ACS: acute coronary syndrome. CAD: coronary artery disease. LD: loading dose. MD: maintenance dose. EM: extensive metabolizer. IM: intermediate metabolizer. PM: poor metabolizer.

	Intervention		Method of genotyping + CYP2C19 alleles	Duration of follow-up
	LOF carriers: aspirin + prasugrel LD 60 mg before PCI, MD 10 mg/d for 1 week, followed by therapy de-escalation to clopidogrel + aspirin Non-carriers: clopidogrel + aspirin	Clopidogrel + aspirin	Spartan RX *2	12 months
	EM: clopidogrel IM: prasugrel or ticagrelor PM: prasugrel or ticagrelor Rapid or ultrarapid metabolizer: clopidogrel	Choice of antiplatelet therapy: usual care decided by treating physician	Spartan RX or Infinium Global Screening Array *2, *3, *17	16.4 months (mean)
	EM: clopidogrel LD 300 mg, MD 75 mg/d IM: clopidogrel LD 600 mg, MD 150 mg/d PM: clopidogrel LD 600 mg, MD 150 mg + cilostazol LD 200 mg, MD 2 dd 100 mg	Clopidogrel LD 300 mg, MD 75 mg/d	Commercially available kits from Shanghai Baiao Technology Co *2, *3	180 days
	EM: clopidogrel 75 mg/d IM: clopidogrel 2 dd 75 mg PM: ticagrelor 2 dd 90 mg All patients: aspirin	Clopidogrel 75 mg/d All patients: aspirin	Sinochips Bioscience Co *2, *3	12 months

The drug regimens used as genotype-guided and standard treatment differed between the studies. In most RCTs, LOF carriers in the genotype-guided arm were treated with ticagrelor or prasugrel and non-carriers with clopidogrel. However, Xie et al. prescribed a double dose of clopidogrel in intermediate metabolizers and a combination of double-dose clopidogrel and cilostazol in poor metabolizers[23], Zhang et al. prescribed a double-dose of clopidogrel in intermediate metabolizers[24], and Tomaniak et al. used a treatment regimen with prasugrel for 1 week followed by therapy de-escalation to clopidogrel in LOF carriers.[21] In the standard treatment arm, most patients were treated with clopidogrel ± aspirin, except in the POPular genetics study, which used ticagrelor or prasugrel[15], and the PHARMCLO study[16], the ADAPT PCI study[22], and the study from Shi et al.[19], in which the choice of antiplatelet therapy was decided by the treating physician. Definition of MACE and major bleeding differed among the included studies (*table 2*).

Risk of Bias Assessment

Some concerns on the risk of bias due to deviations from intended interventions were noted in all studies and some concerns on the risk of bias in selection of the reported result in two studies (Fig. 2). In none of the studies, a high risk of bias was found. Overall, nine studies were classified as low risk for bias and two studies raised some concerns.

Clinical Outcomes

Compared with standard antithrombotic treatment, genotype-guided therapy significantly reduced the risk of MACE (RR 0.60, 95% CI 0.43–0.83, I 2 71%), myocardial infarction (RR 0.53, 95% CI 0.42–0.68, I 2 0%), and stent thrombosis (RR 0.64, 95% CI 0.43–0.94, I 2 0%) (Fig. 3). No significant differences between treatment groups were observed in the rate of cardiovascular death (RR 0.69, 95% CI 0.40–1.20, I 2 57%), stroke (RR 0.65, 95% CI 0.42–1.01, I 2 0%), and major bleeding (RR 0.93, 95% CI 0.70–1.23, I 2 0%).

Comparable results were found for all thrombotic and bleeding outcomes in a subgroup analysis excluding the two RCTs that raised some concerns (Supplementary Fig. 1), in a subgroup analysis on the nine RCTs that included only patients in whom a percutaneous coronary intervention was performed (Supplemental Fig. 2), and in a subgroup analysis that excluded the only RCT using a genotype-guided de-escalation strategy (POPular Genetics) (data not shown). In a subgroup analysis based on studies with a follow-up duration of 12 months or more and in a subgroup analysis on studies including only patients with acute coronary syndrome, the risk of stent thrombosis did not differ significantly between

genotype-guided and standard antithrombotic treatment (RR 0.68, 95% CI 0.46–1.02, I 2 0%, and RR 0.63, 95% CI 0.39–1.01, I 2 0%, respectively) (Supplementary Figs. 3d and 4d). A significantly decreased risk of stroke was found in the subgroup analysis on studies including only patients with acute coronary syndrome (RR 0.56, 95% CI 0.33–0.97, I 2 0%) (Supplementary Fig. 4e).

Table 2: *Definitions of outcome measurements in studies on coronary artery disease*

Study	MACE	Major bleeding
Al-Rubaish, 2021	1, 2, 3, 4	PLATO
Claassens, 2019	1, 2, 3, 5	PLATO
Notarangelo, 2018	1, 2, 3	BARC type 3 - 5
Pereira, 2020	1, 2, 3, 5, 6	TIMI
Roberts, 2012	1, 2, 5, 7	TIMI
Shi, 2021	2, 3, 5, 8, 9	BARC ≥ type 2
Tam, 2017	-	Not further specified
Tomaniak, 2017	1, 2, 5, 10	BARC type 3 + 5
Tuteja, 2020	1, 2, 3, 5, 9	BARC type 3 + 5
Xie, 2013	2, 3, 8	-
Zhang, 2020	2, 5, 8	BARC type 2, 3, 5

MACE: major adverse cardiovascular events.
1 = cardiovascular death; 2 = myocardial infarction; 3 = stroke; 4 = major bleeding; 5 = stent thrombosis; 6 = severe recurrent ischemia; 7 = readmission to hospital; 8 = all-cause death; 9 = urgent revascularization; 10 = revascularization at 1 year

Figure 2: *Risk of bias assessment in studies on coronary artery disease. The risk of bias was assessed with the Cochrane collaboration's risk of bias tool for randomized trials*

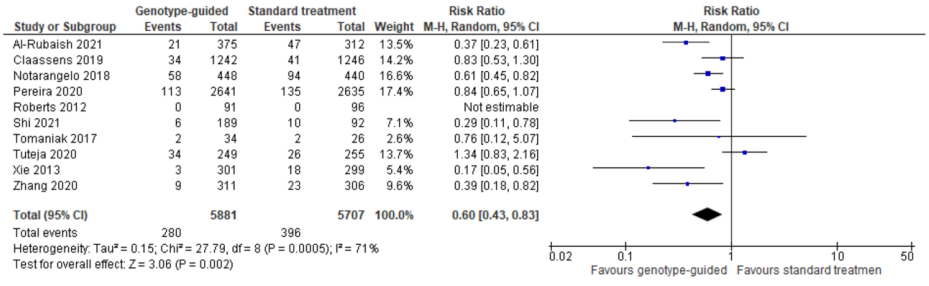

***Figure 3**: Forest plots for the ischemic and bleeding outcomes in studies on coronary artery disease*

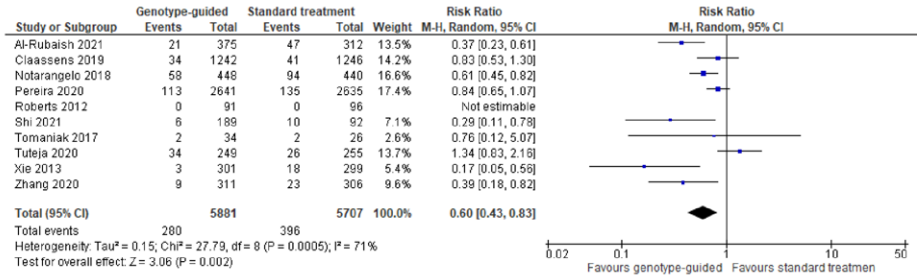

***Figure 3A**: MACE*

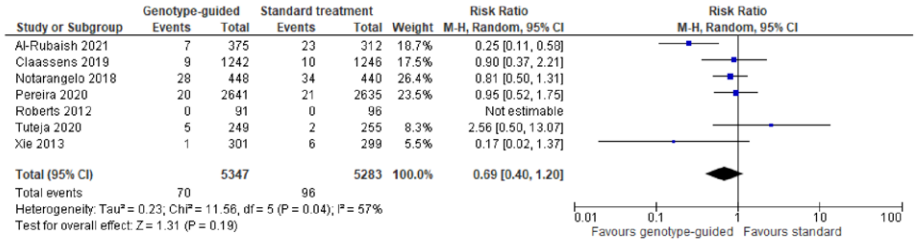

***Figure 3B:** Cardiovascular death*

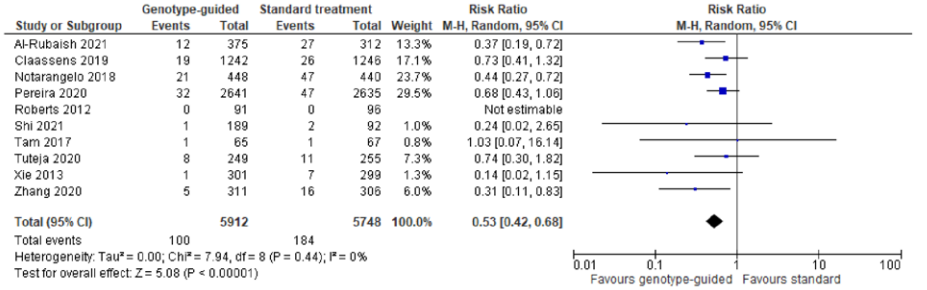

***Figure 3C:** Myocardial infarction*

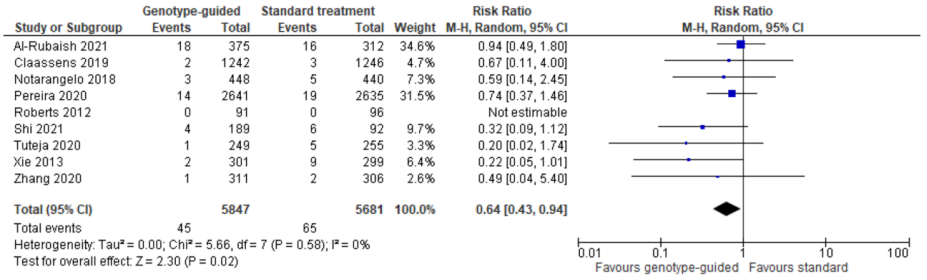

***Figure 3D:** Stent thrombosis*

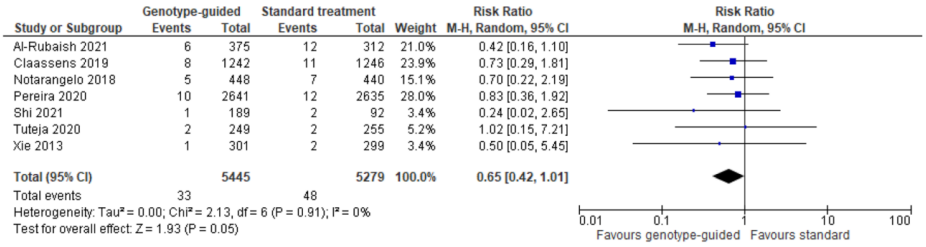

Figure 3E: *Stroke*

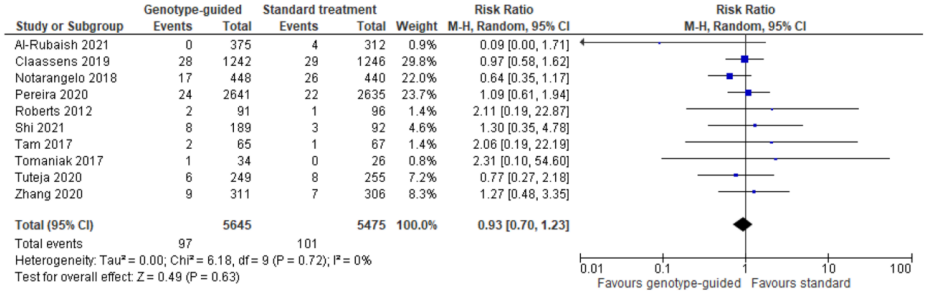

Figure 3F: *Major bleeding*

Stroke

A total of 29 studies describing the association between CYP2C19 genotype status, and clinical outcomes were eligible for inclusion in our analysis.[25-51] Of these 29 studies, 22 were cohort studies and 7 were post hoc analyses of RCTs. Characteristics of these studies are described in *table 3*.

The 22 cohort studies contained a total of 5182 patients (range 42–743), and the seven post hoc analyses of RCTs contained 3356 patients (range 112–1463). In seven studies, both ischemic stroke and TIA patients were included.[27,28,35,36,41,46,47] One of these studies specified ischemic stroke as symptomatic small subcortical stroke.[35] Two studies only included patients who received a cerebrovascular stent because of atherosclerotic stenosis.[30,50] In the study of Patel et al., only patients with asymptomatic extracranial carotid artery stenosis were included, and these patients were divided in a medical therapy cohort and a procedural cohort.[38] In the remaining 19 studies, only patients with history of ischemic stroke were included.[25,26,29,31-34,37,39,40,42-45,48,49,51] Definitions of MACE and bleeding differed among the included studies (*table 4*).

Table 3: *Characteristics of studies on stroke or TIA*

Study	Study type	Region	Study period	Patients	Sample size	Age, mean (y)
Al-Rubaish, 2022 (25)	Cohort	Saudi Arabia	2018 - 2019	Acute IS	42	-
Fu, 2020 (26)	Cohort	Chinese-Han		Acute IS	131	61.4
Fukuma, 2022 (27)	Cohort	Japan	2013 - 2015	Acute IS or TIA with large-artery atherosclerosis	194	-
Hoh, 2015 (28)	Cohort	USA		Stroke or TIA due to ICAD	188	67
Jardaq, 2022 (73)	Cohort	Kurdistan	2021	IS	60	-
Kitazono, 2023 (74)	Post-hoc analysis RCT (PRASTRO-III)	Japan	2018 – 2020	IS (large-artery atherosclerosis or small-vessel occlusion) + age ≥ 50 years + cardiovascular risk factors	112	70
Li, 2017 (29)	Cohort	China	2012 – 2016	Acute IS	196	62.9
Li, 2021 (30)	Cohort	China	2016 - 2019	Cerebrovascular stent due to atherosclerotic stenosis or occlusion of the internal carotid artery, subclavian artery, vertebral artery or intracranial arteries	154	61.2
Lin, 2018 * (31)	Cohort	China	2014 - 2015	Acute IS	375	69
Lin, 2021 (32)	Cohort	China	2016 - 2017	IS	89	65.1

Male (%)	Medication	Follow-up	Method of genotyping + CYP2C19 alleles	Mode of reporting results in original article
-	Clopidogrel 75 mg/d or clopidogrel 75 mg/d + aspirin 50 – 325 mg/d	6 months	Spartan RX, confirmed by TaqMan *2	LOF – no LOF
79	Clopidogrel 75 mg/d	6 months	PCR-RFLP *2, *3	LOF – no LOF
-	Clopidogrel 75 mg/d ± other antiplatelet agents (aspirin 200 mg/d, cilostazol 200 mg/d), anticoagulant agents (including argatroban injection)	90 days	TaqMan *2, *3, *17	EM – IM – PM (patients with *17 excluded from analysis)
63.3	Clopidogrel + aspirin	12 months	Sequenom, TaqMan or pyrosequencing (Qiagen) *2, *3, *8, *17	LOF – no LOF
57	Clopidogrel 75 mg/d	NR	ABI PRISM 3700 genetic analyzer *2, *3	EM – IM - PM
70.5	Clopidogrel 75 mg/d	24 – 48 weeks treatment, FU 2 weeks after completion or discontinuation of treatment	Independent laboratory (SRL Mediserch Inc) NR	EM – IM - PM
82.1	Clopidogrel 75 mg/d	6 months	ABI 3730 *2, *3	*2 LOF – no LOF
81.8	Clopidogrel 75 mg/d + aspirin 100 mg/d. In case of aspirin intolerance: cilostazol 200 mg/d	6 months	PCR-RFLP *2, *3	EM – IM – PM
63.8	Clopidogrel 75 mg/d Minor IS or symptomatic carotid or intracranial artery stenosis: clopidogrel 75 mg/d + aspirin 200 mg/d for 2 weeks, followed by clopidogrel 75 mg/d	10 days	MALDI-TOF MS *2, *3	*2 LOF – no LOF
57.3	Clopidogrel ± aspirin	12 months	Unknown *2, *3	LOF – no LOF

Table 3 Continued

Study	Study type	Region	Study period	Patients	Sample size	Age, mean (y)
Liu, 2020 (33)	Cohort	China	2015 - 2018	Acute IS	289	66.6
Lv, 2021 (34)	Cohort	China	2012 - 2013	Acute IS	311	67.9
McDonough, 2015 (35)	Post-hoc analysis RCT (SPS3)	North America, Latin America, Spain		Symptomatic small subcortical stroke or TIA	493	62.5
Meschia, 2020 (36)	Post-hoc analysis RCT (POINT)	North America, Europe		Minor IS or high-risk TIA	282	62.5 (median)
Ni, 2017 (37)	Cohort	China	2012 - 2014	Acute IS	191	61.5
Patel, 2022 (38)	Cohort	USA		Carotid artery stenosis without cerebral infarction: 1. Medical therapy cohort 2. Procedural cohort (endarterectomy or stenting)	Medical therapy cohort: 743 Procedural cohort: 60	Medical therapy cohort: 67.9 (median) Procedural cohort: 67.2 (median)
Qiu, 2015 (39)	Cohort	China	2012 – 2013	Acute IS	198	67.1
Sen, 2014 (40)	Cohort	Turkey		Acute ischemic cerebrovascular disease	51	66.4
Spokoyny, 2014 (41)	Cohort	USA	2010 - 2012	Stroke or TIA	43	69.6
Sun, 2015 (42)	Cohort	China	2008 – 2010	First-ever IS	625	61.6
Tomek, 2018 (43)	Cohort	Czech Republic	2010 - 2015	Acute IS	72	64.5

Male (%)	Medication	Follow-up	Method of genotyping + CYP2C19 alleles	Mode of reporting results in original article
58.1	Clopidogrel 75 mg/d	6 months (mean)	CYP2C19 genotyping kit (Shanghai, China) (DNA Microarray) *2, *3	EM – IM - PM
68.5	Clopidogrel 75 mg/d	54 months	Sequenom MassARRAY iPLEX platform *2, *3, *4, *5, *7, *8, *17	EM – IM – PM (ultrarapid excluded, unknown included)
62	Clopidogrel 75 mg/d + aspirin 325 mg/d	3.4 years (mean)	TaqMan *2, *17	UM-EM vs IM-PM
56.4	Clopidogrel 75 mg/d + aspirin 50 – 325 mg/d	90 days	TaqMan *2, *3, *17	EM – IM – PM (ultrarapid and unknown excluded)
67	Clopidogrel	9.5 months (mean)	iMIDR *2, *3	EM – IM - PM
Medical therapy cohort: 63.5 Procedural cohort: 68.3	Clopidogrel + aspirin	2.8 years (median)	Unknown *2, *3, *4, *5, *6, *7, *8, *17	EM – IM – PM (ultrarapid excluded, unknown included)
55.6	Clopidogrel 75 mg/d	6 months	PCR-RELP *2, *3	LOF – no LOF
41.2	Clopidogrel 75 mg/d	12 months	LightCycler 2.0 *2, *3	EM – IM - PM
53.4	Clopidogrel	NR	Unknown NR	EM – IM – PM Indeterminate and mixed ultrarapid/ poor excluded
74.4	Clopidogrel 75 mg/d	12.7 months (mean)	iMLDR *2, *3, *17	LOF – no LOF
60	Clopidogrel	14.9 months (mean)	LightScanner *2, *17	EM – IM Ultrarapid and unknown excluded. PM in article excluded.

Table 3 Continued

Study	Study type	Region	Study period	Patients	Sample size	Age, mean (y)
Tornio, 2017 (44)	Cohort	Scotland	1997 - 2007	Hospitalization for IS	94	74
Wang, 2016 (45)	Cohort	China	2009 - 2011	IS	321	62
Wang, 2016 (CHANCE) (47)	Post-hoc analysis RCT	China	2010 - 2012	Acute minor IS or TIA	1463	62.7 (median)
Wang, 2019 (46)	Post-hoc analysis RCT (PRINCE)	China	2015 - 2017	Acute minor IS or moderate to high risk TIA	329	60.4
Won Han, 2017 (51)	Post-hoc analysis RCT (MAESTRO)	South Korea	2010 - 2014	First time non-cardiogenic IS	393	61
Yi 2016 * (49)	Cohort	China	2014 - 2015	Acute IS	514	68.6
Yi, 2018 (48)	Post-hoc analysis RCT	China	2009 - 2011	Acute large-artery atherosclerosis IS	284	69.2
Zhu, 2016 (50)	Cohort	China	2012 - 2014	IS and carotid artery stenting	241	64.3

*Y: years. IS: ischemic stroke. USA: United States of America. TIA: transient ischemic attack. ICAD: intracranial atherosclerotic disease. RCT: randomized controlled trial. NR: not reported. * Lin 2018 and Yi 2016 included*

Male (%)	Medication	Follow-up	Method of genotyping + CYP2C19 alleles	Mode of reporting results in original article
62	Clopidogrel	24 months	Unknown *2	EM – IM - PM
75.5	Clopidogrel	12 months	iMLDR *2, *3, *4, *5, *6, *7, *8	LOF – no LOF
66.9	Clopidogrel 75 mg/d (3 months) + aspirin 75 mg/d (21 days)	90 days	Sequenom MassARRAY iPLEX platform *2, *3, *17	MACE + all strokes + all bleeding: EM – IM – PM (ultrarapid and unknown excluded) Other EP's: LOF – no LOF
73.3	Aspirin 100 mg/d (until day 21) + clopidogrel 75 mg/d (until day 90)	1 year	Sequenom MassARRAY iPLEX platform If results inconclusive: ABI 3500 *2, *3, *17	MACE + all strokes: EM – IM – PM (ultrarapid and unknown excluded) Other EP's: LOF – no LOF
67	Clopidogrel 75 mg/d	2.7 years (median)	Seeplex CYP2C19 ACE genotyping system or Real-Q CYP2C19 genotyping kit *2, *3, *17	IS + all strokes: EM – IM – PM Other EP's: UM-EM vs IM-PM-unknown
65.2	Clopidogrel 75 mg/d Minor IS or symptomatic carotid or intracranial artery stenosis: clopidogrel 75 mg/d + aspirin 200 mg/d for 2 weeks, followed by clopidogrel 75 mg/d	6 months	MALDI-TOF MS *2, *3	*2 LOF – no LOF
54.9	Aspirin 200 mg/d + clopidogrel 75 mg/d for 30 days, followed by clopidogrel 75 mg/d	5 years	MALDI-TOF MS *2	LOF – no LOF
90	3 – 5 days before intervention until 3 months after intervention (at least 1 month): clopidogrel 75 mg/d + aspirin 100 mg/d Long-term: clopidogrel 75 mg/d	1 year	Commercially available kit (BaiO Technology Co) *2, *3	EM – IM - PM

patients from the same study population but described other study endpoints: Lin 2018 ischemic stroke and all bleeding, Yi 2016 MACE.

Risk of Bias Assessment

The mean NOS score of included studies was 7.2 (*table 5*). A total of 22 studies were scored as high quality studies (NOS score ≥ 7); 4 of these 22 studies were rated with the highest NOS score of 9. Seven studies had a NOS score < 7.

Clinical Outcomes

Carriers of a LOF allele had a significantly higher risk of MACE compared with non-carriers (RR 1.61, 95% CI 1.25–2.08, I 2 62%) (Fig. 4). Moreover, the risk of ischemic stroke and all strokes was significantly higher in LOF carriers than in non-carriers (RR 1.89, 95% CI 1.48–2.40, I 2 16% and RR 1.43, 95% CI 1.08–1.89, I 2 0%, respectively).

No significant differences were found in the risk of myocardial infarction (RR 1.09, 95% CI 0.37–3.20, I 2 0%), major bleeding (RR 0.90, 95% CI 0.43–1.89, I 2 0%), and all bleeding (RR 0.96, 95% CI 0.70–1.32, I 2 0%). Subanalyses including only high-quality studies with a NOS score ≥ 7, studies with a duration of follow-up longer than 12 months, and only the post hoc analyses of RCTs demonstrated robust results (Supplementary Figs. 5, 6, and 7).

Table 4: *Definitions of outcome measurements in studies on stroke or TIA*

Study	MACE	All bleeding	Major bleeding
Fu, 2020	1, 2, 3, 4	-	-
Fukuma, 2022	-	-	1, 2, 3
Hoh, 2015	1, 2, 4, 5	-	-
Kitazono, 2023	1, 3, 9	-	-
Li, 2017	3, 6, 7	-	-
Li, 2021	2, 3, 4, 8	-	4, 5
Lin, 2018	-	1, 2, 3	-
Lin, 2021	-	Not further specified	-
Lv, 2021	1, 3, 4, 9	-	-
McDonough, 2015	-	-	6
Meschia, 2020	1, 3, 9	-	1, 7, 8, 9, 10
Ni, 2017	1, 3, 9	-	-
Qiu, 2015	1, 3, 9	-	-
Sun, 2015	1, 3, 9	4	-
Tomek, 2018	1, 3, 4, 9	5	11
Tornio, 2017	2, 10	-	-
Wang, 2016	1, 3, 9	-	-
Wang, 2016 (CHANCE)	1, 5, 9	4	12

Table 4 Continued

Study	MACE	All bleeding	Major bleeding
Wang, 2019	1, 4, 5, 9	6	13
Won Han, 2017	1, 5, 9	Not further specified	-
Yi, 2016	1, 3, 9	-	-
Yi, 2018	1, 2, 3, 4	-	-
Zhu, 2016	2, 8, 11, 12, 13	-	-

MACE: major adverse cardiovascular events

1 = myocardial infarction; 2 = death; 3 = (recurrence of) ischemic stroke; 4 = TIA; 5 = stroke; 6 = progressive ischemic stroke (an increase of NIHSS score ≥2) during admission; 7 = other ischemic diseases; 8 = stent thrombosis; 9 = (cardio)vascular death; 10 = hospitalization for an arterial thrombo-occlusive event (myocardial infarction, ischemic stroke, peripheral arterial disease); 11 = previous ischemic symptom recurrence; 12 = previous cerebrovascular transient ischemic attack; 13 = new cerebral infarction caused by previous cerebrovascular

All bleeding

1 = symptomatic or asymptomatic hemorrhagic transformation; 2 = symptomatic or asymptomatic intracerebral hemorrhage; 3 = extracranial hemorrhages; 4 = GUSTO; 5 = ISTH; 6 = PLATO (major, minor or minimal bleeding)

Major bleeding

1 = (symptomatic) intracranial hemorrhage; 2 = symptomatic bleeding (such as intraspinal, intraocular, retroperitoneal, intra-articular, pericardial or intramuscular bleeding with compartment syndrome); 3 = episodes that caused ≥ 2 g/dL decline in hemoglobin level or required at least 280 ml of red cell transfusion; 4 = gastro-intestinal hemorrhage; 5 = hemorrhagic stroke; 6 = major extracranial hemorrhage defined as serious or life-threatening bleeding requiring transfusion of red cells or surgery, or resulting in permanent functional sequelae or death; 7 = intraocular hemorrhage causing vision loss; 8 = transfusion of ≥ 2 units of red blood cells or an equivalent of whole blood; 9 = hospitalization or prolongation of an existing hospitalization; 10 = death due to hemorrhage; 11 = ISTH; 12 = GUSTO severe bleeding; 13 = PLATO

Table 5: *Quality assessment (Newcastle–Ottawa Scale score) of studies on stroke or TIA*

Study	Selection				Comparability	Outcome			Total score
	Representativeness of the exposed cohort	Selection of the nonexposed cohort	Ascertainment of exposure	Demonstration that outcome of interest was not present at start of study	Comparability of cohorts on the basis of the design or analysis	Assessment of outcome	Was follow-up long enough for outcomes to occur	Adequacy of follow-up of cohorts	
Al-Rubaish, 2022	★	★	★	★		★			5
Fu, 2020	★	★	★	★	★ ★	★			7
Fukuma, 2022	★	★	★	★	★ ★	★		★	8
Hoh, 2015		★	★	★	★ ★		★		6
Jardaq, 2022	★	★	★	★		★		★	6
Kitazono, 2023	★	★	★	★		★			5
Li, 2017	★	★	★	★	★ ★	★		★	8
Li, 2021	★	★	★	★	★ ★	★		★	8
Lin, 2018	★	★	★	★	★ ★	★			7
Lin, 2021	★	★	★	★	★ ★	★	★	★	9
Liu, 2020	★	★	★	★	★	★			7
Lv, 2021	★	★	★	★	★ ★	★	★		8
McDonough, 2015	★	★	★	★	★ ★	★	★	★	9
Meschia, 2020	★	★	★	★	★ ★	★			7
Ni, 2017	★	★	★	★	★ ★	★	★		8
Patel, 2022	★	★	★	★	★ ★	★	★		8
Qiu, 2015	★	★	★	★	★ ★	★		★	8
Sen, 2014		★	★				★	★	4

Table 5 Continued

Study	Selection				Comparability	Outcome			Total score
	Representativeness of the exposed cohort	Selection of the nonexposed cohort	Ascertainment of exposure	Demonstration that outcome of interest was not present at start of study	Comparability of cohorts on the basis of the design or analysis	Assessment of outcome	Was follow-up long enough for outcomes to occur	Adequacy of follow-up of cohorts	
Spokoyny, 2014		★	★	★					3
Sun, 2015	★	★	★	★	★ ★	★	★		8
Tomek, 2018	★	★	★	★	★	★	★	★	8
Tornio, 2017	★	★	★	★	★ ★		★		7
Wang, 2016	★	★	★	★	★ ★	★	★	★	9
Wang, 2016 (CHANCE)	★	★	★	★	★ ★	★		★	8
Wang, 2019	★	★	★	★	★	★		★	7
Won Han, 2017	★	★	★	★		★	★		6
Yi, 2016	★	★	★	★	★ ★	★		★	8
Yi, 2018	★	★	★	★	★ ★	★	★	★	9
Zhu, 2016		★	★	★	★ ★	★	★		7

***Figure 4**: Forest plots for the ischemic and bleeding outcomes in studies on stroke or TIA*

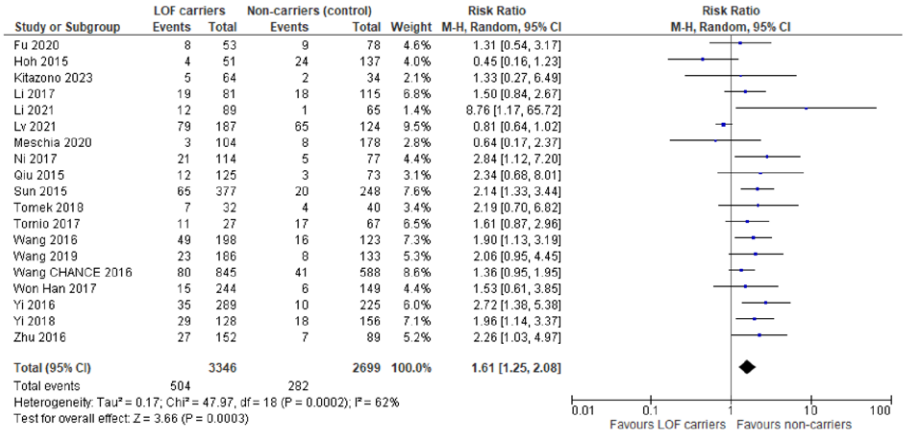

***Figure 4A:** MACE*

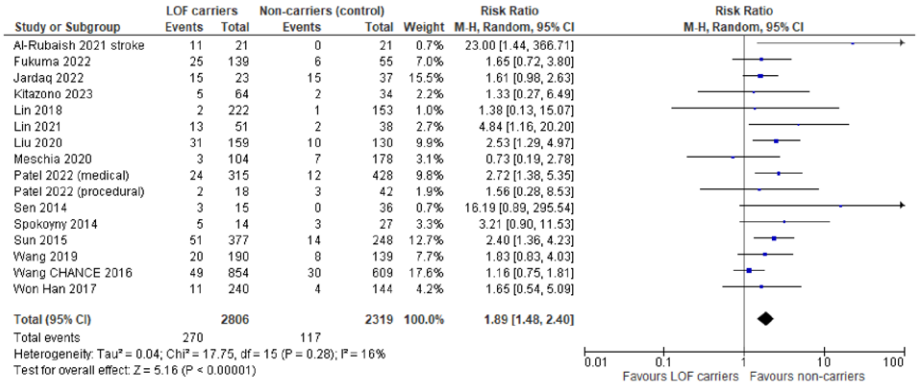

***Figure 4B:** Ischemic stroke*

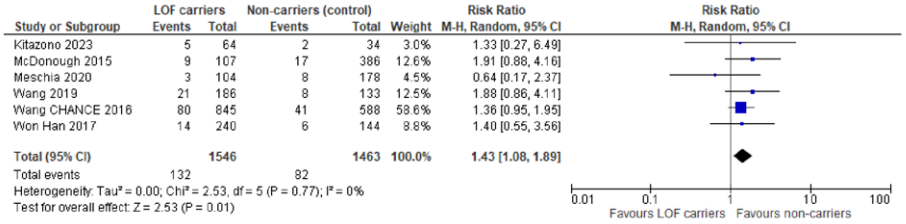

***Figure 4C:** All strokes*

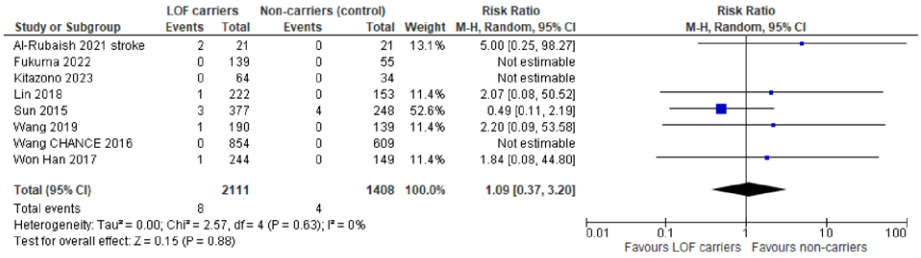

Figure 4D: *Myocardial infarction*

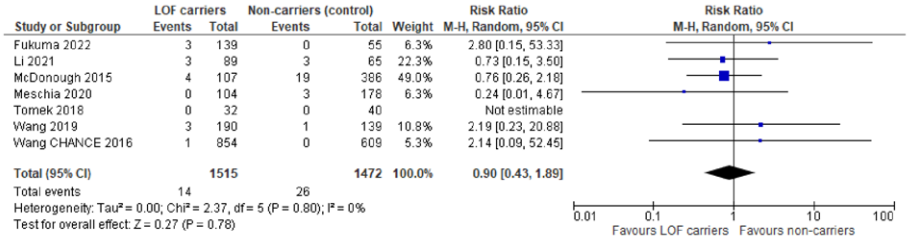

Figure 4E: *Major bleeding*

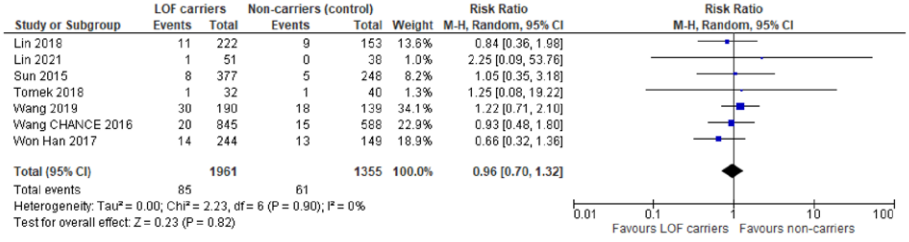

Figure 4F: *all bleeding*

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RCTs Including Only LOF Carriers: Description of Main Results

In our full-text screening, two RCTs involving only CYP2C19 LOF carriers were identified. The CHANCE-2 trial was a double-blind, placebo-controlled RCT among mainly Han Chinese patients (98%) with an acute minor ischemic stroke (NIHSS score ≤ 3) or high-risk TIA (ABCD2 score ≥ 4).[52] Patients were randomized to receive ticagrelor or clopidogrel through day 90. All patients received aspirin for 21 days. A total of 3205 patients were assigned to the ticagrelor group and 3207 to the clopidogrel group. Patients who were treated with ticagrelor-aspirin had a significantly lower risk of a new ischemic or hemorrhagic stroke within 90 days compared with patients with clopidogrel–aspirin [6.0% versus 7.6% respectively; hazard ratio (HR) 0.77, 95% CI 0.64–0.94]. Ticagrelor increased the risk of any bleeding compared with clopidogrel (5.3% versus 2.5% respectively, HR 2.18, 95%

CI 1.66–2.85). A post hoc analysis showed that these bleedings were generally mild and occurred mostly in the first 21 days after randomization.[53] No significant differences were found in the risk of severe or moderate bleeding (both 0.3%, HR 0.82, 95% CI 0.34–1.98).[52]

Another RCT included only single CYP2C19 LOF carriers (*1/*2, *1/*3) with an acute minor ischemic stroke (NIHSS score ≤ 5) and moderate-to-severe cerebral artery stenosis (> 50%). Patients were randomly assigned to receive a combination of high dose clopidogrel (150 mg per day) and aspirin (100 mg per day) or a combination of normal dose clopidogrel (75 mg per day) and aspirin (100 mg per day). After the first 21 days, clopidogrel was stopped and monotherapy with aspirin was continued during the 90-day observation period. In total, 62 patients with high dose clopidogrel and 69 patients with normal dose clopidogrel were analyzed. No significant differences in the vascular event rate were found between these two treatment groups.[54]

RCT Comparing GenotypeGuided with Standard Treatment

Our full-text screening identified one RCT that compared a personalized genotype-guided treatment strategy with standard treatment. In this RCT, 650 adult patients with a mild-to-moderate acute noncardioembolic ischemic stroke (NIHSS ≤ 5) or a moderate-to-high risk TIA (ABCD2 score ≥ 4) were included from 2019 to 2021 in China.[55] Patients were randomized in a pharmacogenetic or standard treatment group. In the pharmacogenetic group, all patients were treated with aspirin (until day 90). Ultrarapid (*17/*17), rapid (*1/*17), and extensive (*1/*1) metabolizers were also treated with clopidogrel 75 mg/day, intermediate (*1/*2, *1/*3, *17/*2, *17/*3) metabolizers with clopidogrel 150 mg/day, and poor (*2/*2, *2/*3, *3/*3) metabolizers with ticagrelor 90 mg twice daily (until day 21). The standard group was treated with aspirin (until day 90) and clopidogrel 75 mg/day (until day 21). After a 90-day follow-up, patients in the pharmacogenetic group had a significantly lower risk of new stroke (RR 0.27, 95% CI 0.08–0.97) and composite vascular events (RR 0.38, 95% CI 0.16–0.92). No differences in major bleeding were found between treatment groups (RR 1.50, 95% CI 0.25–8.95).[55]

Peripheral Arterial Disease

After full-text screening, four studies were eligible for inclusion: a retrospective study[56], a prospective study[57], a conference abstract[58], and a research letter describing the results from an RCT substudy.[59] Due to the low number of studies and the heterogeneity in study design, the main results of each study are described separately.

Lee et al. retrospectively analyzed the association between CYP2C19 genetic profiles and clinical outcomes (major amputation free survival and all-cause mortality) in a cohort of Taiwanese patients with critical limb ischemia treated with clopidogrel after endovascular treatment.[56] All patients had a Rutherford classification of V or VI. A total of 278 patients were included: 153 EMs, 79 IMs, and 46 PMs. Significant differences in the estimated amputation free 12-month survival rates (EM 82.1%, IM 66.1%, PM 56.6%) and overall one year survival rate (EM 83.7%, IM 72.2%, PM 71.3%) were found between the different types of metabolizers. Moreover, the gene polymorphism number had a significant negative association with the clinical outcomes in a multivariable analysis.

In the prospective study from Guo et al, 50 patients with peripheral arterial disease in the superficial femoral artery (TASC II A–C) in whom successful recanalization was achieved by endovascular therapy were included.[57] Of these 50 patients, 26 were LOF carriers. The risk of in-stent restenosis or occlusion was significantly higher in LOF carriers compared with non-carriers. The primary patency rate at 12 months was 73.1% in non-carriers compared with 34.6% in LOF carriers. Also, after adjustment for confounding factors, CYP2C19 genotypic classification was an independent risk factor for the occurrence of in-stent restenosis or occlusion. In a study among 72 patients with peripheral arterial disease treated with clopidogrel after percutaneous transluminal angioplasty, a significant association between carriage of CYP2C19 LOF alleles and the occurrence of atherothrombotic ischemic events was found in the univariate analysis [odds ratio (OR) 4.49, 95% CI 1.45–13.84, p = 0.009] and multivariate analysis (OR 4.89, 95% CI 1.32–12.83, p = 0.018).[58] Lastly, the EUCLID trial was an RCT involving 13.885 patients with symptomatic PAD who were assigned to receive ticagrelor or clopidogrel.[59] Of the 6955 patients treated with clopidogrel, 2873 were non-carriers, 1596 had one LOF allele, 10 had two LOF alleles, 1676 had one gain-of-function allele, 342 had two gain-of-function alleles, and 458 had a combination of one gain-of-function and one LOF allele. No differences in the rate of the primary composite endpoint (cardiovascular death, myocardial infarction or ischemic stroke) or TIMI major bleeding were found between these CYP2C19 polymorphism subgroups.[59]

Discussion

This systematic review and meta-analysis investigates the impact of CYP2C19 genotype status on the rate of adverse arterial thrombotic events in the most common types of atherosclerotic cardiovascular disease. In patients with coronary

artery disease, mostly undergoing PCI, genotype-guided treatment significantly reduced the risk of MACE, myocardial infarction and stent thrombosis, while the rate of major bleeding remained unchanged. In patients with history of stroke treated with clopidogrel, LOF allele carriage was associated with a higher risk of MACE and stroke compared with noncarriage. For PAD, it remains unclear whether LOF allele carriage is associated with the risk of thrombotic limb events in patients treated with clopidogrel.

Coronary artery disease, carotid artery disease, and peripheral arterial disease are all clinical manifestations of atherosclerosis and thus share the same underlying pathophysiological mechanisms.[60] In these patients, antiplatelet therapy is widely prescribed for the secondary prevention of atherothrombotic events. The specific regimen of antiplatelet therapy used in these different atherosclerotic diseases slightly differs but commonly exists of a P2Y12 inhibitor ± acetylsalicylic acid. In symptomatic peripheral arterial disease, international guidelines recommend the use of monotherapy with clopidogrel over acetylsalicylic acid.[61-63] For patients with a stroke or TIA, the current guidelines advise acetylsalicylic acid or clopidogrel monotherapy or the combination of acetylsalicylic acid and dipyridamole.[64] Guidelines on acute coronary syndrome advise the use of dual antiplatelet therapy with acetylsalicylic acid and prasugrel or ticagrelor over clopidogrel.[65] However, clopidogrel is globally still frequently prescribed in these patients. In the guideline and updated expert consensus statement (2019) of the European Society of Cardiology, the routine use of CYP2C19 genotyping in clinical practice is not recommended because of the lack of robust scientific evidence.[66] In selected patients, however, genotyping can be used in combination with clinical risk factors and platelet function tests to aid in the decision whether treatment should be escalated or de-escalated.[66] The last guideline for the management of acute coronary syndromes from the European Society of Cardiology (2023) includes the use of genetic testing to guide de-escalation of P2Y12 receptor inhibitor treatment after PCI as one of the research gaps that should be addressed in the following years.[67] In a recent real-world study in 2751 elderly patients (> 65 years) carrying a CYP2C19 LOF variant who underwent PCI after ACS, a similar rate of ischemic events and a higher bleeding rate was observed in patients who were treated with ticagrelor compared with clopidogrel. These results emphasize that more research data are needed to answer the question how to integrate genotyping, platelet function tests and clinical risk factors in clinical decision making.[68] The guidelines on carotid artery disease and peripheral arterial disease do not contain recommendations about the use of CYP2C19 genotyping.[69]

The results of our meta-analysis demonstrate that the CYP2C19 genotype status can be used to reduce the rate of adverse arterial thrombotic events in selected patient groups. Most evidence exists for patients with coronary artery disease, specifically for patients who underwent PCI in whom the risk of MACE, myocardial infarction and stent thrombosis were all reduced by the use of genotype-guided treatment. In patients with stroke or TIA, only few studies included patients who received a carotid stent or underwent a carotid endarterectomy. In three of four included studies on peripheral arterial disease, the study population was treated with a revascularization procedure. In all these studies, CYP2C19 LOF allele carriage was a risk factor for the occurrence of atherothrombotic events. In the EUCLID trial, no differences in ischemic or bleeding endpoints were found between the different CYP2C19 polymorphism subgroups. In the clopidogrel-treated arm of this study, however, only 57% underwent a revascularization procedure. The remaining patients had an ankle–brachial index of 0.80 or lower at screening. From these results in patients with coronary artery disease or peripheral arterial disease, it can be hypothesized that CYP2C19 genotyping is especially of additional value in patients who underwent a revascularization procedure for their atherosclerotic disease.

Currently, there is much debate whether CYP2C19 genotype status should be determined in all "new" patients with atherosclerotic disease. However, in the future, an enlarging number of patients will already know their CYP2C19 genotype status before they present to the emergency department or outpatient clinic with atherosclerotic disease as genetic testing is becoming more easily available. Irrespective of the discussion whether CYP2C19 genotyping should be performed, clinical guidelines on the management of patients with a known CYP2C19 genotype should be available. Recently, the Clinical Pharmacogenetics Implementation Consortium (CPIC) updated their guideline including recommendations on the management of the different CYP2C19 metabolizer types with coronary artery disease, cerebrovascular disease and peripheral arterial disease.[1]

The impact of CYP2C19 genotyping can be different according to the type of atherosclerotic cardiovascular disease. The latest guidelines on acute coronary syndrome recommend the use of ticagrelor or prasugrel over clopidogrel.[67] Therefore, in these patients, CYP2C19 genotyping could be used as de-escalation strategy with the use of clopidogrel in non-carriers and prasugrel or ticagrelor (if no contraindications) for all other patients. For patients with stroke and PAD, the "standard therapy" is clopidogrel. In these patients, CYP2C19 genotyping can be used to escalate antithrombotic treatment. For patients with stroke, alternative P2Y12 inhibitors are ticagrelor and ticlopidine. Patients with a history of stroke or

TIA have a contraindication for prasugrel.[1] For patients with PAD, genotype-guided escalation strategies are currently under investigation. The ongoing GENPAD trial does not use ticagrelor or prasugrel but explores the beneficial effects of double dose clopidogrel for intermediate metabolizers and acetylsalicylic acid plus rivaroxaban for poor metabolizers.[11]

Only 5–12% of the variation in clopidogrel response is explained by the CYP2C19*2 genotype [70–72]. Clinical characteristics (such as age, BMI, adherence, and comorbidity), use of comedication, and additional polymorphisms in other genes might also affect the response to clopidogrel. Most of the variability in clopidogrel response is, however, still unexplained. Future research should focus on the identification of other relevant factors that influence the response to clopidogrel. However, at this moment carriage of the loss-of-function CYP2C19*2 variant is the most important known determinant of the residual platelet aggregation on clopidogrel.

A great strength of this meta-analysis is that it provides an overview of all (recent) studies on the relationship between CYP2C19 polymorphisms and clinical outcomes in patients with one of the three most common types of atherosclerotic disease simultaneously. The meta-analysis was performed in accordance with PRISMA guidelines, and it was preregistered in PROSPERO. For each included study the same definitions were used for LOF carriers and non-carriers. However, our study also has some limitations. First, substantial heterogeneity was found for MACE in studies on coronary artery disease and cerebrovascular disease and for cardiovascular death in studies on coronary artery disease. Second, different definitions of MACE and major bleeding were used in the included studies. In general, MACE was used as a composite outcome including cardiovascular death, myocardial infarction, and/or stroke. Broader definitions including relevant endpoints (such as stent thrombosis, severe recurrent ischemia, revascularization, all-cause death, major bleeding, and readmission to hospital) were also accepted. Because we selected the definitions that most closely resembled the general definition of MACE and we included only the relevant endpoints for this meta-analysis, we do not expect that the differences in definitions influenced our study outcomes. For major bleeding, different definitions according to the most frequently used and globally accepted classification schemes were used (PLATO, BARC, TIMI). Third, the classification of patients according to CYP2C19 genotype status differed among studies. In some studies, the patient cohort was divided in extensive, intermediate and poor metabolizers, while in other studies information on the number of CYP2C19 LOF alleles was lacking as patients were only classified as LOF carrier or non-carrier.

Therefore, we unfortunately were not able to explore a potential association between clinical outcomes and the number of CYP2C19 LOF alleles. Fourth, the dosage and type of drugs used in the genotype-guided and standard treatment groups differed (slightly) between studies. Fifth, the length of follow-up differed between studies. Compared with the overall analysis, separate subgroup analyses based on studies with a follow-up duration of 12 months or more in patients with coronary artery disease and cerebrovascular disease demonstrated robust results for our primary outcomes. Sixth, in the majority of stroke studies, the type of ischemic stroke was not further specified or clinical outcomes were not reported separately for each stroke subtype. Finally, RCTs comparing a genotype-guided antithrombotic treatment with standard treatment were only available in patients with coronary artery disease. These RCTs are not performed in patients with peripheral arterial disease and are scarce in patients with cerebrovascular disease. For these patient categories, we used the best evidence that is currently available: cohort studies and post hoc analyses of RCTs describing the association between CYP2C19 genotype status and clinical outcome measurements. The pooled results of all RCTs comparing genotype-guided and standard antithrombotic treatment (i.e., all RCTs in CAD and one RCT in stroke) were comparable to the analyses described in individual indications (Supplementary Fig. 8A–E).

For patients with cerebrovascular disease, this is the first meta-analysis in recent years on the relationship between CYP2C19 genotype status and clopidogrel response. The last meta-analysis on this subject was published in 2017 by Pan et al.[10], but numerous new studies have been published since. The least evidence was available for peripheral arterial disease. However, our study is the first that included the results of a post hoc analysis of the EUCLID study. It is important to note that this post-hoc analysis did not include major adverse limb events.

In the future, (more) large RCTs comparing genotype-guided antithrombotic treatment with standard treatment are also required in patients with conservatively treated coronary artery disease and cerebrovascular disease. In recent years, one RCT comparing genotype-guided with standard treatment was conducted among patients with cerebrovascular disease, showing promising results.[55] The efficacy of a CYP2C19 genotype-guided treatment strategy in comparison to conventional clopidogrel treatment in patients with peripheral arterial disease is currently being investigated in the ongoing GENPAD trial.[11]

In conclusion, genotype-guided treatment significantly decreased the rate of adverse atherothrombotic events in patients with coronary artery disease, mostly

undergoing PCI. In patients with history of stroke, LOF carriers who were treated with clopidogrel had a higher risk of MACE and stroke. The evidence in peripheral arterial disease is limited but CYP2C19 LOF carriage seemed to be a risk factor for adverse events in patients who underwent a revascularization procedure. In the future, large RCT's comparing genotype-guided treatment with standard treatment are required, particularly in stroke and peripheral arterial disease, to give accurate management recommendations and to optimize care for these patients.

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Appendix

Pubmed

1. genotyp* OR polymorphism OR "Genetic Testing"[Mesh] OR "genotype"[Mesh] OR "polymorphism, genetic"[Mesh]

   891.494 results

2. CYP2C19 OR Cytochrome p-450 CYP2C19 OR CYPIIC19 OR P450 2C19 OR P-450 2C19 OR P450 CYP2C19 OR "Cytochrome P-450 CYP2C19"[Mesh]

   7.046 results

3. peripheral arterial diseases OR peripheral artery diseases OR peripheral vascular disease OR "peripheral arterial disease"[Mesh] OR "peripheral vascular diseases"[Mesh] OR acute coronary syndromes OR percutaneous coronary interventions OR coronary artery diseases OR coronary diseases OR ischemic heart diseases OR heart attacks OR "myocardial infarct*" OR myocardial ischemia OR "Cardiovascular Diseases"[Mesh:noexp] OR "cardiovascular disease*" OR stent* OR "acute coronary syndrome"[Mesh] OR "percutaneous coronary intervention"[Mesh] OR "myocardial ischemia"[Mesh] OR stroke OR transient ischemic attacks OR brain infarct* OR cerebral infarct* OR cerebrovascular accidents OR brain ischemia OR transient cerebral ischemia OR "cerebrovascular disorders"[Mesh]

   1.678.818 results

4. clopidogrel OR plavix OR iscover OR "clopidogrel"[Mesh] OR ticagrelor OR brilique OR "ticagrelor"[Mesh] OR prasugrel OR efient OR "prasugrel hydrochloride"[Mesh] OR P2Y12 inhibitor* OR P2Y12 antagonist* OR purinergic P2Y receptor antagonist* OR P2Y purinoceptor antagonist* OR platelet aggregation inhibitor* OR "purinergic P2Y receptor antagonists"[Mesh] OR "Platelet Aggregation Inhibitors"[Pharmacological Action] OR "Platelet Aggregation Inhibitors"[Mesh] OR "Purinergic P2Y Receptor Antagonists"[Pharmacological Action]

   168.904 results

1 OR 2	894.603 results
3 AND 4	51.613 results
(1 OR 2) AND (3 AND 4)	1.864 results

EMBASE

1. exp genetic screening/
2. exp genotype/
3. exp genetic polymorphism/
4. genotyp*.mp
5. polymorphism*.mp
6. pharmacogenetic.mp
7. pharmacogenomic.mp
8. genetic testing.mp
9. 1 or 2 or 3 or 4 or 5 or 6 or 7 or 8 — 1.139.027 results

10. CYP2C19.mp
11. P-450 CYP2C19.mp
12. P-450 2C19.mp
13. P450 2C19.mp
14. P450 CYP2C19.mp
15. CYPIIC19.mp
16. exp cytochrome P450 2C19/
17. 10 or 11 or 12 or 13 or 14 or 15 or 16 — 13.467 results

18. peripheral arterial disease*.mp
19. peripheral artery disease*.mp
20. peripheral occlusive artery disease*.mp
21. peripheral occlusive arterial disease*.mp
22. peripheral vascular disease*.mp
23. exp peripheral occlusive artery disease/
24. exp vascular stent/
25. peripheral vascular disease/
26. acute coronary syndrome*.mp
27. percutaneous coronary intervention*.mp
28. coronary artery disease*.mp
29. coronary disease*.mp
30. myocardial infarct*.mp
31. myocardial isch?emia*.mp
32. cardiovascular disease*.mp
33. stent*.mp
34. heart attacks.mp
35. ischemic heart diseases.mp
36. exp coronary artery disease/

37. exp percutaneous coronary intervention/
38. exp coronary stent/
39. exp ischemic heart disease/
40. carotid artery disease*.mp
41. stroke*.mp
42. transient ischemic attack*.mp
43. brain infarct*.mp
44. cerebral infarct*.mp
45. cerebrovascular accidents.mp
46. brain ischemias.mp
47. transient cerebral ischemias.mp
48. exp carotid artery disease/
49. exp cerebrovascular accident/
50. cerebrovascular disease/
51. OR/18-50 2.160.457 results

52. clopidogrel.mp
53. plavix.mp
54. iscover.mp
55. ticagrelor.mp
56. brilique.mp
57. prasugrel.mp
58. efient.mp
59. P2Y12 inhibitor*.mp
60. P2Y12 antagonist*.mp
61. purinergic P2Y receptor antagonist*.mp
62. P2Y purinoceptor antagonist*.mp
63. platelet aggregation inhibitor*.mp
64. exp purinergic P2Y receptor antagonist/
65. exp antithrombocytic agent/
66. OR/52-66 407.462 results

9 OR 17	1.144.832 results
51 AND 66	175.692 results
(9 OR 17) AND (51 AND 66)	5.701 results

MEDLINE

1. exp genetic testing/
2. exp genotype/
3. exp polymorphism, genetic/
4. genotyp*.mp
5. polymorphism*.mp
6. pharmacogenetic.mp
7. pharmacogenomic.mp
8. genetic testing.mp
9. 1 or 2 or 3 or 4 or 5 or 6 or 7 or 8 — 882.525 results

10. exp cytochrome P-450 CYP2C19/
11. CYP2C19.mp
12. P-450 CYP2C19.mp
13. P-450 2C19.mp
14. P450 2C19.mp
15. P450 CYP2C19.mp
16. CYPIIC19.mp
17. 10 or 11 or 12 or 13 or 14 or 15 or 16 — 6.356 results

18. peripheral arterial disease*.mp
19. peripheral artery disease*.mp
20. peripheral occlusive artery disease*.mp
21. peripheral occlusive arterial disease*.mp
22. peripheral vascular disease*.mp
23. exp peripheral arterial disease/
24. exp peripheral vascular diseases/
25. acute coronary syndrome*.mp
26. percutaneous coronary intervention*.mp
27. coronary artery disease*.mp
28. coronary disease*.mp
29. myocardial infarct*.mp
30. myocardial isch?emia*.mp
31. cardiovascular disease*.mp
32. stent*.mp
33. heart attacks.mp
34. ischemic heart diseases.mp
35. cardiovascular diseases/
36. exp coronary disease/

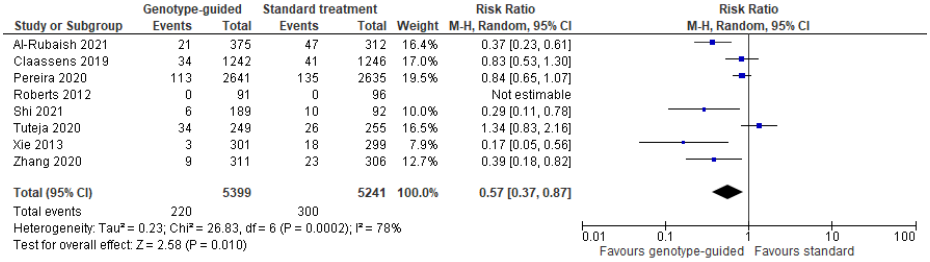
37. exp myocardial ischemia/
38. exp percutaneous coronary intervention/
39. exp coronary artery disease/
40. carotid artery disease*.mp
41. stroke*.mp
42. transient ischemic attack*.mp
43. brain infarct*.mp
44. cerebral infarct*.mp
45. cerebrovascular accidents.mp
46. brain ischemias.mp
47. transient cerebral ischemias.mp
48. exp carotid artery diseases/
49. exp stroke/
50. exp ischemic attack, transient/
51. exp cerebrovascular disorders/
52. OR/18-51 1.549.344 results

53. clopidogrel.mp
54. plavix.mp
55. iscover.mp
56. ticagrelor.mp
57. brilique.mp
58. prasugrel.mp
59. efient.mp
60. P2Y12 inhibitor*.mp
61. P2Y12 antagonist*.mp
62. purinergic P2Y receptor antagonist*.mp
63. P2Y purinoceptor antagonist*.mp
64. platelet aggregation inhibitor*.mp
65. exp purinergic P2Y receptor antagonists/
66. exp platelet aggregation inhibitors/
67. OR/53-66 141.585 results

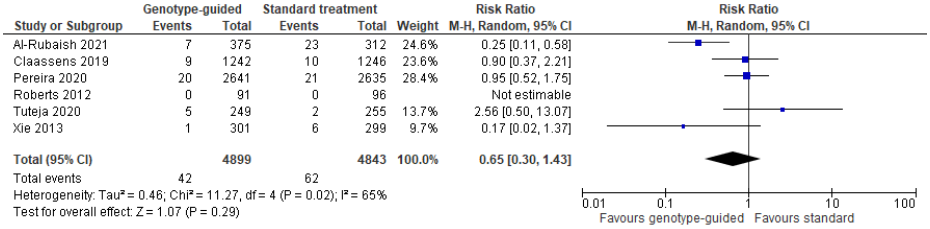
9 or 17	885.019 results
52 and 67	47.099 results
(9 or 17) and (52 and 67)	1.834 results

Supplementary Information

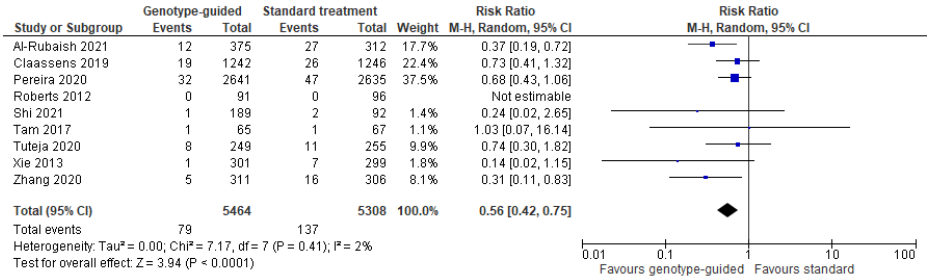
Supplemental figure 1: *Sensitivity analysis: forest plots for the ischemic and bleeding outcomes in studies on coronary artery disease with a low risk of bias*



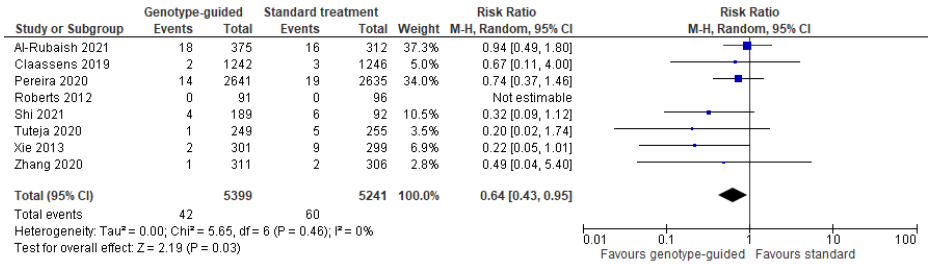
Supplemental figure 1A: *Major adverse cardiovascular events*



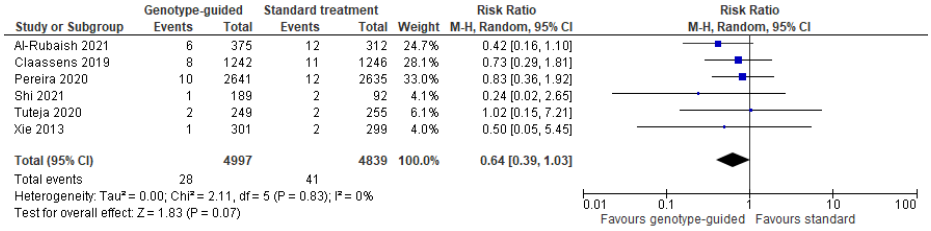
Supplemental figure 1B: *Cardiovascular death*



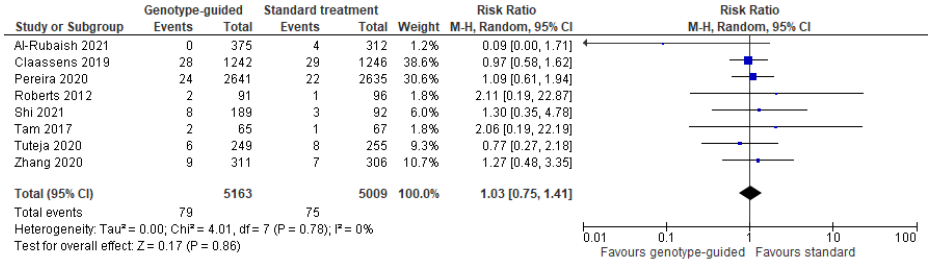
Supplemental figure 1C: *Myocardial infarction*



Supplemental figure 1D: *Stent thrombosis*

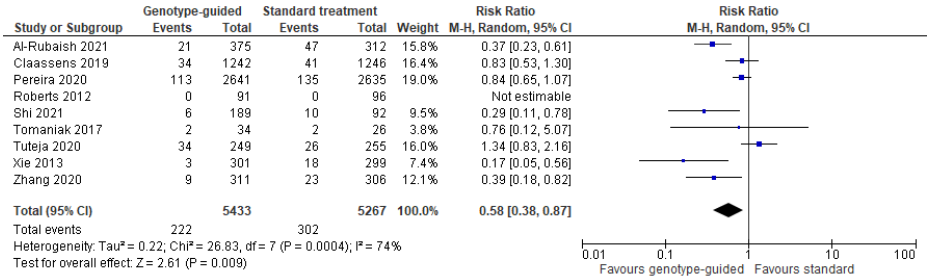


Supplemental figure 1E: *Stroke*

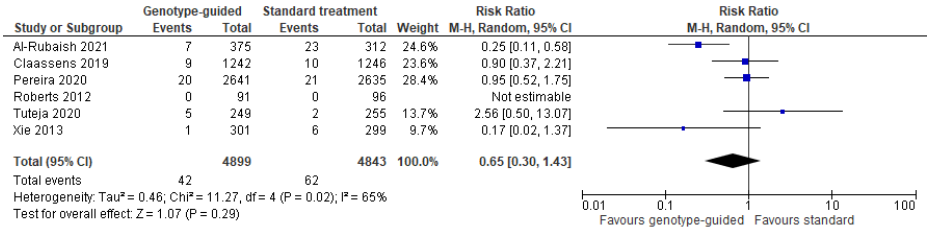


Supplemental figure 1F: *Major bleeding*

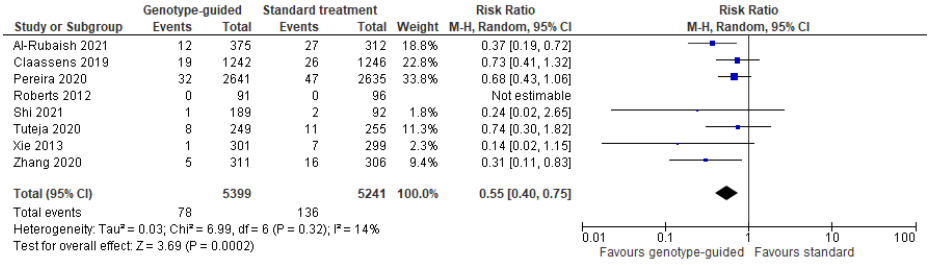
Supplemental figure 2: *Subanalysis: forest plots for the ischemic and bleeding outcomes in studies on coronary artery disease including only patients who underwent percutaneous coronary intervention*



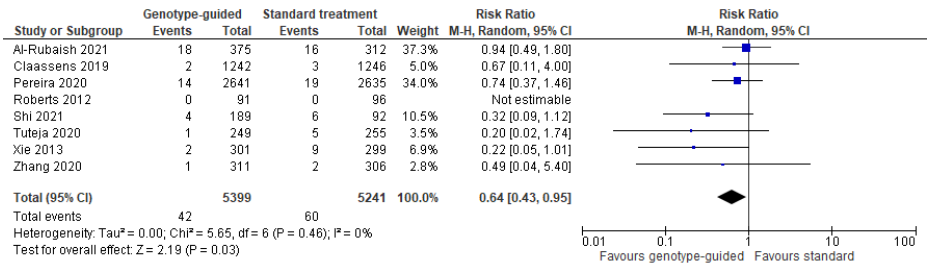
Supplemental figure 2A: *Major adverse cardiovascular events*



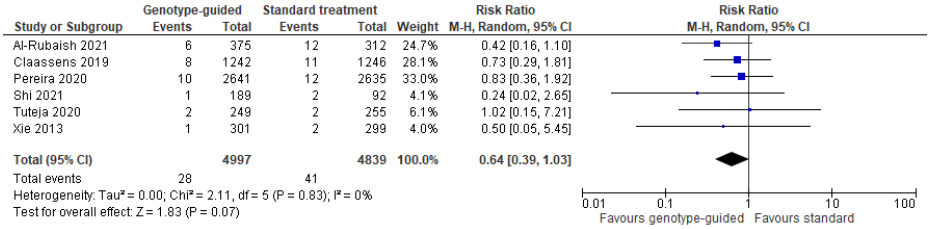
Supplemental figure 2B: *Cardiovascular death*



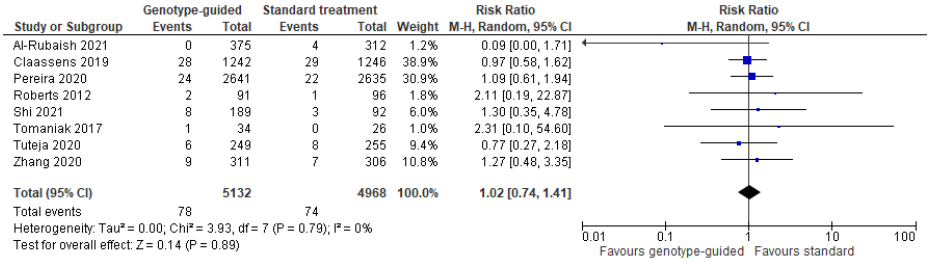
Supplemental figure 2C: *Myocardial infarction*



Supplemental figure 2D: *Stent thrombosis*

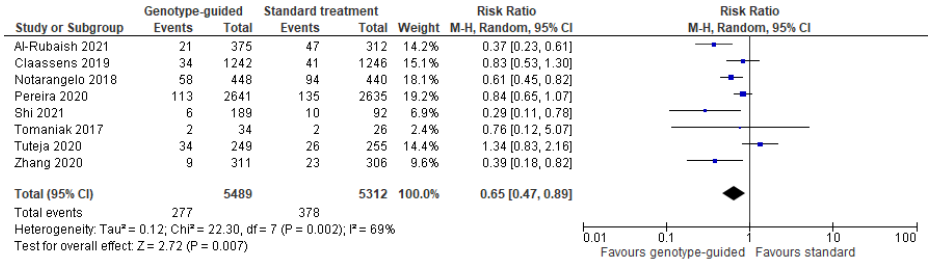


Supplemental figure 2E: *Stroke*

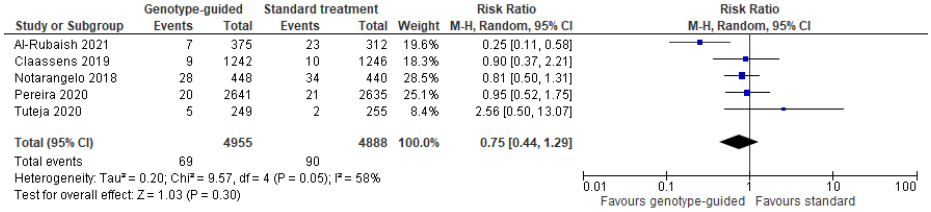


Supplemental figure 3F: *Major bleeding*

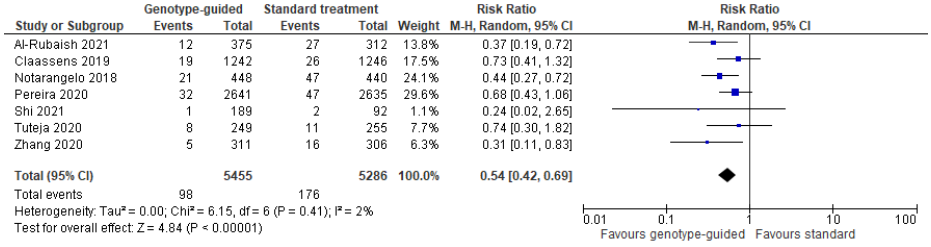
Supplemental figure 3: *Subanalysis: forest plots for the ischemic and bleeding outcomes in studies on coronary artery disease with a duration of follow-up ≥12 months*



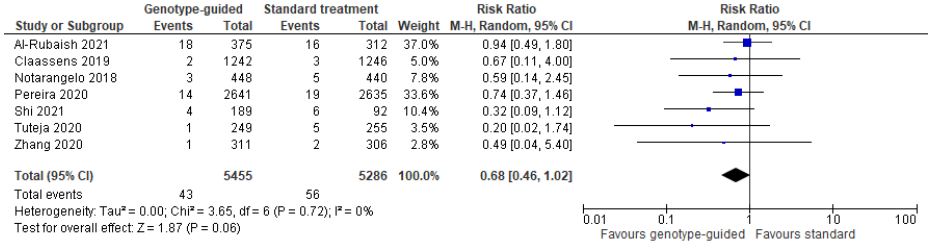
Supplemental figure 3A: *Major adverse cardiovascular events*



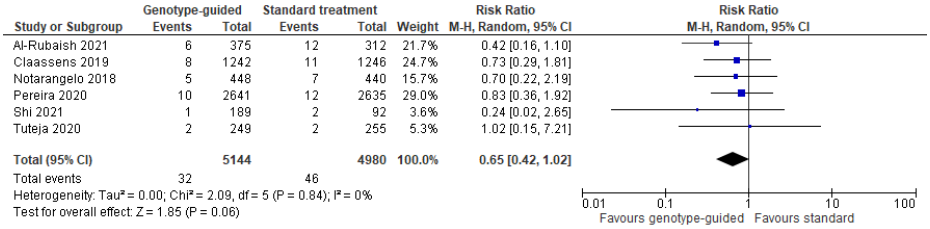
Supplemental figure 3B: *Cardiovascular death*



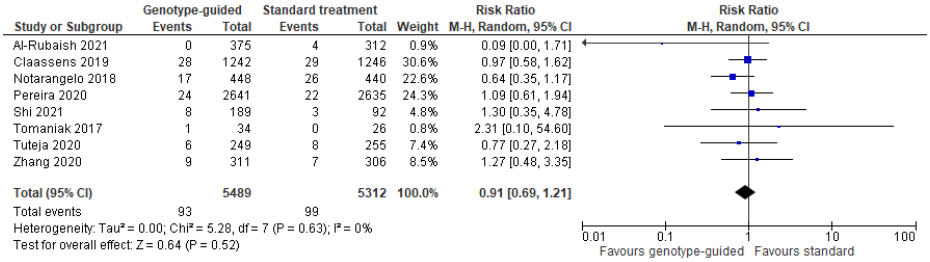
Supplemental figure 3C: *Myocardial infarction*



Supplemental figure 3D: *Stent thrombosis*

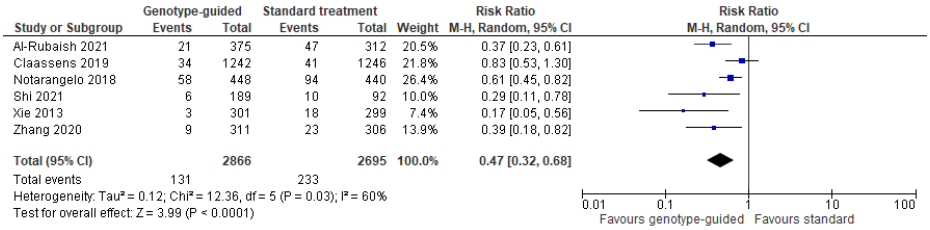


Supplemental figure 3E: *Stroke*

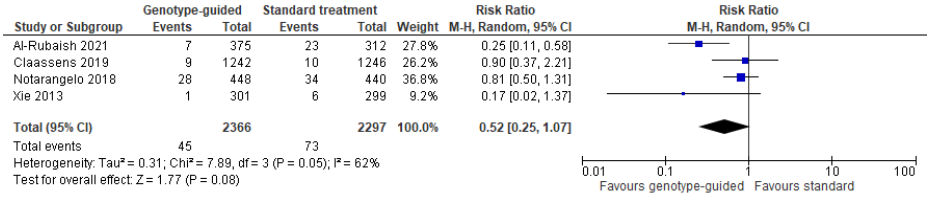


Supplemental figure 3F: *Major bleeding*

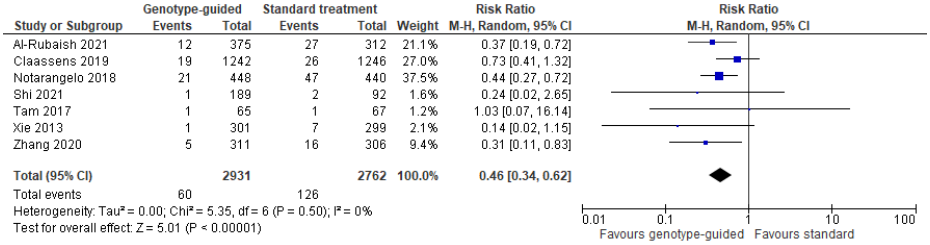
Supplemental figure 4: *Subanalysis: forest plots for the ischemic and bleeding outcomes in studies on coronary artery disease including only patients with acute coronary syndrome*



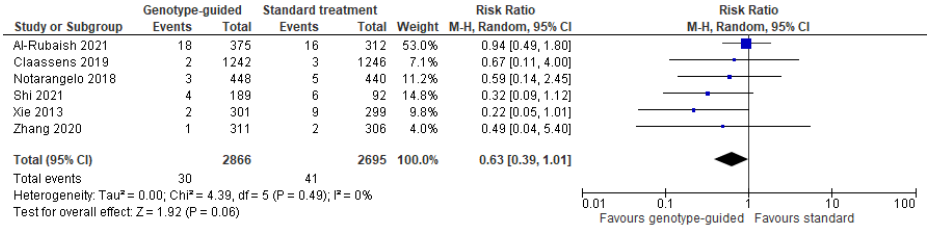
Supplemental figure 4A: *Major adverse cardiovascular events*



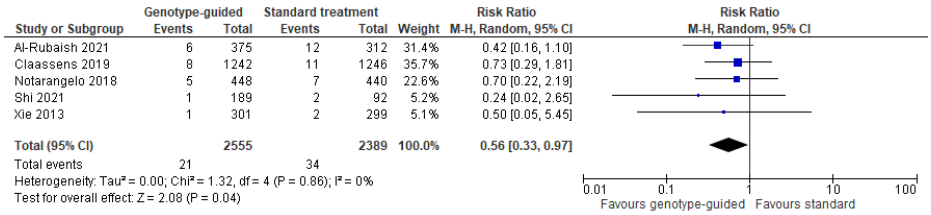
Supplemental figure 4B: *Cardiovascular death*



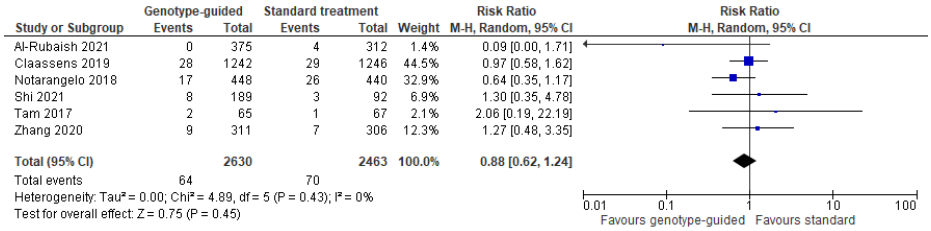
Supplemental figure 4C: *Myocardial infarction*



Supplemental figure 4D: *Stent thrombosis*

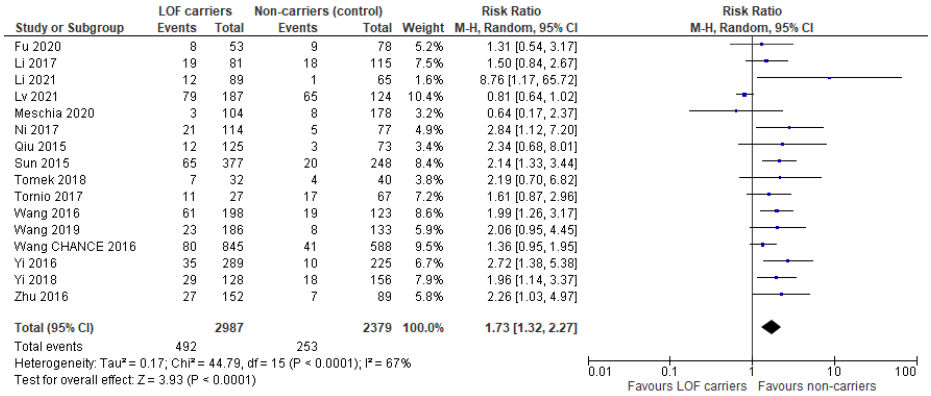


Supplemental figure 4E: *Stroke*

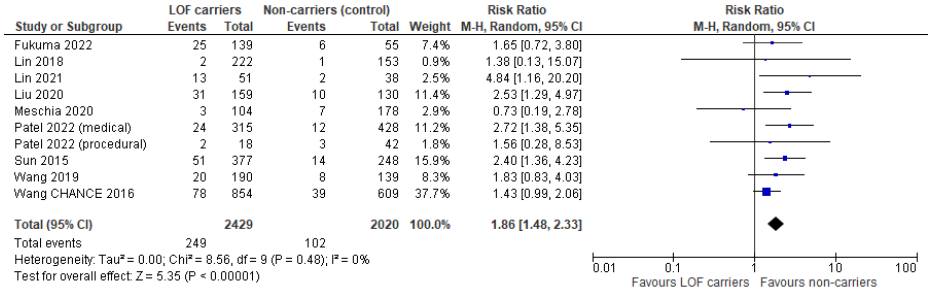


Supplemental figure 4F: *Major bleeding*

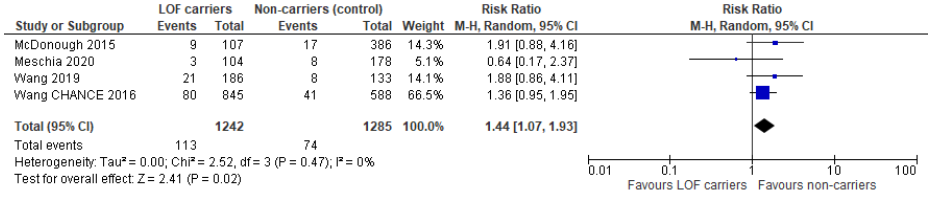
Supplemental figure 5: *Sensitivity analysis: forest plots for the ischemic and bleeding outcomes in high-quality studies on stroke or TIA (only studies with Newcastle-Ottawa Scale score ≥7)*



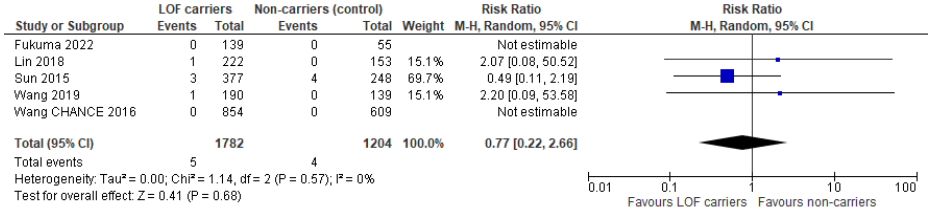
Supplemental figure 5A: *Major adverse cardiovascular events*



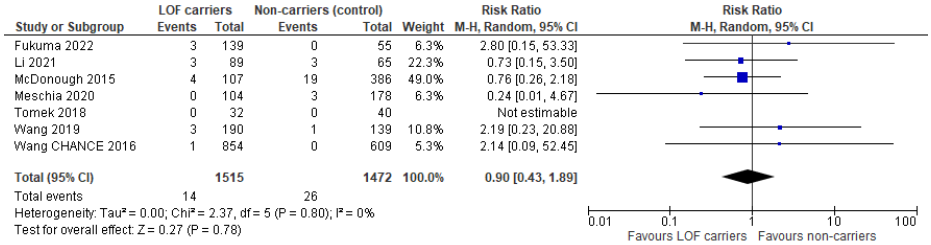
Supplemental figure 5B: *Ischemic stroke*



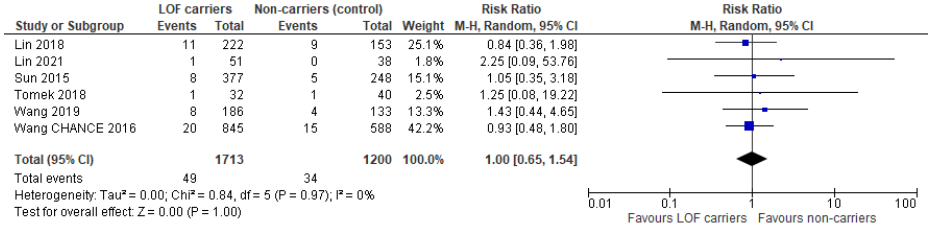
Supplemental figure 5C: *All stroke*



Supplemental figure 5D: *Myocardial infarction*

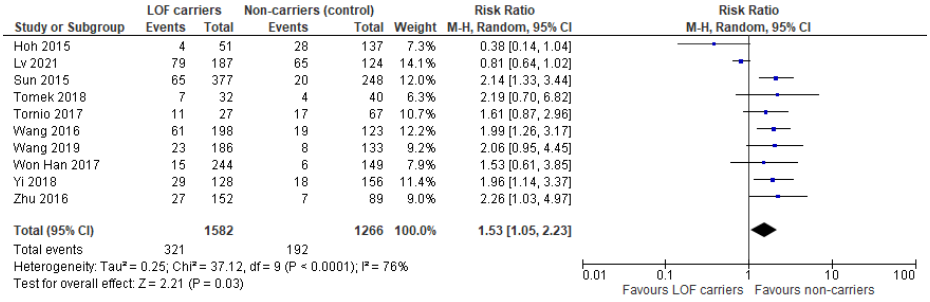


Supplemental figure 5E: *Major bleeding*

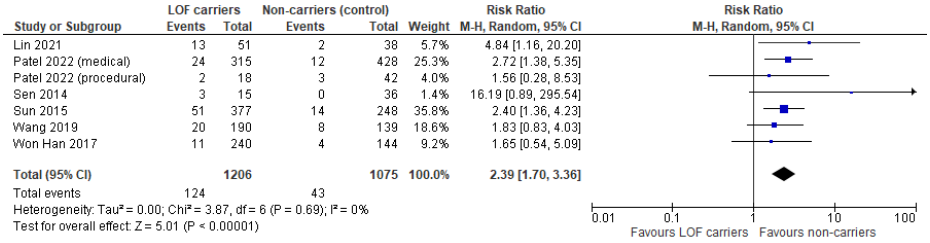


Supplemental figure 5F: All bleeding

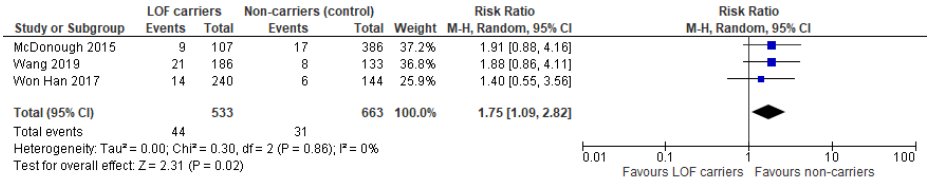
Supplemental figure 6: *Subanalysis: forest plots for the ischemic and bleeding outcomes in studies on stroke or TIA with a duration of follow-up ≥12*



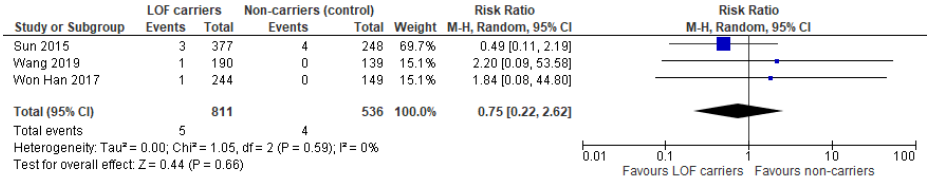
Supplemental figure 6A: *Major adverse cardiovascular events*



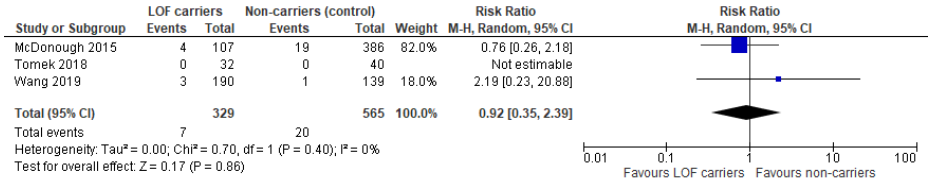
Supplemental figure 6B: *Ischemic stroke*



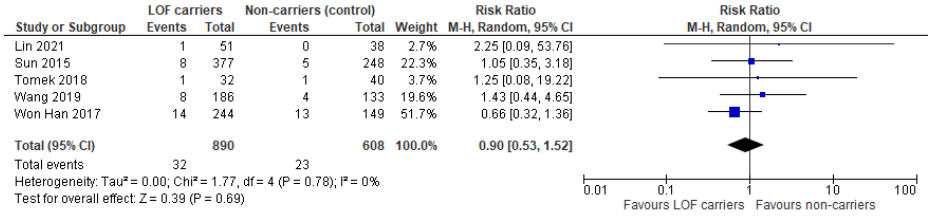
Supplemental figure 6C: *All stroke*



Supplemental figure 6D: *Myocardial infarction*

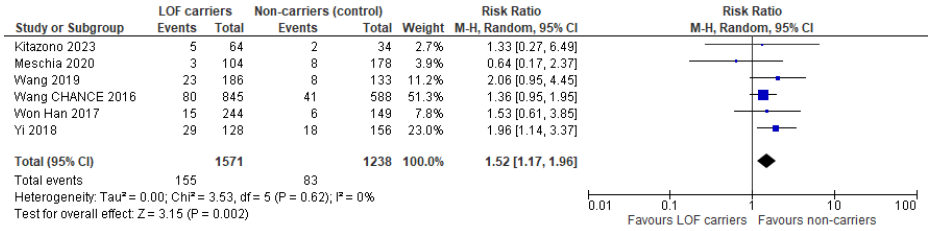


Supplemental figure 6E: *Major bleeding*

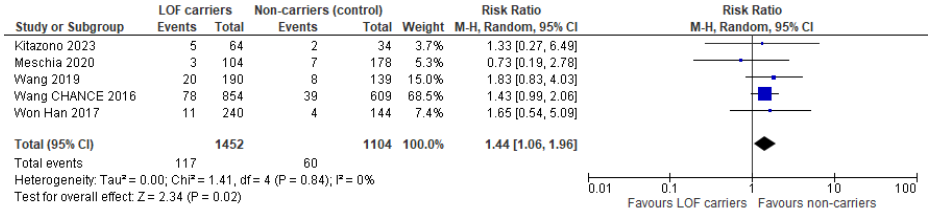


Supplemental figure 6F: *All bleeding*

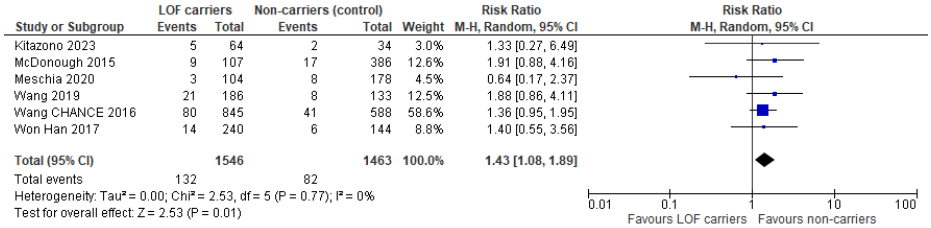
Supplemental figure 7: *Subanalysis: forest plots for the ischemic and bleeding outcomes in studies on stroke or TIA, only including post-hoc analyses of randomized controlled trials*



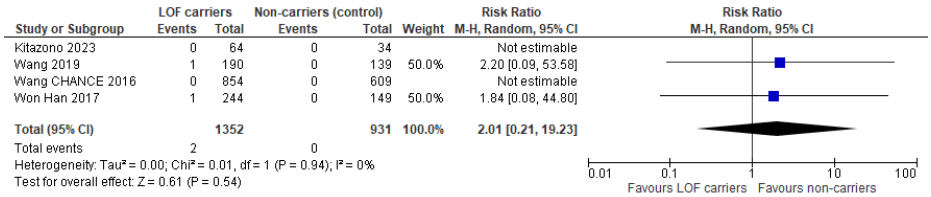
Supplemental figure 7A: *Major adverse cardiovascular events*



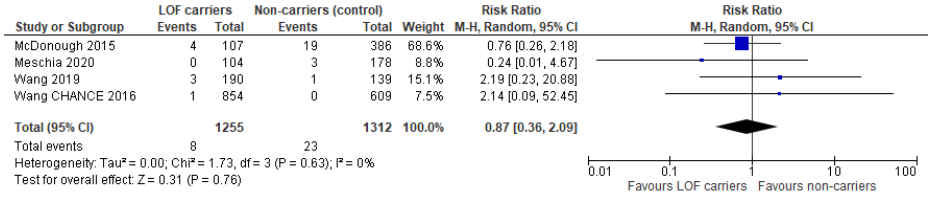
Supplemental figure 7B: *Ischemic stroke*



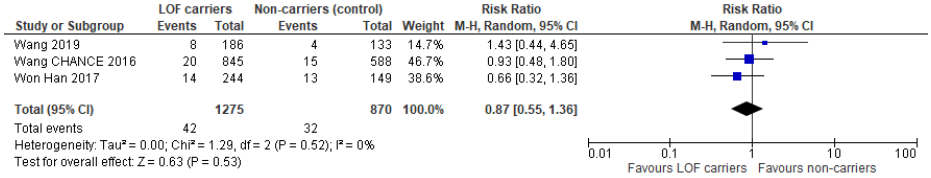
Supplemental figure 7C: *All stroke*



Supplemental figure 7D: *Myocardial infarction*

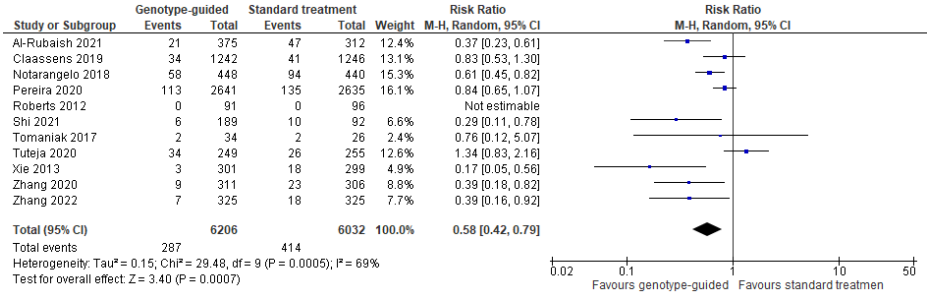


Supplemental figure 7E: *Major bleeding*

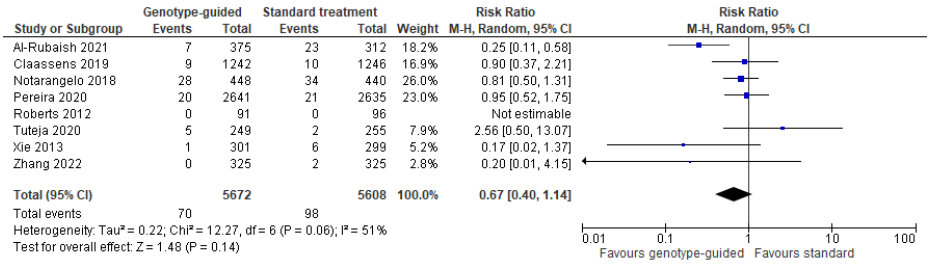


Supplemental figure 7F: *All bleeding*

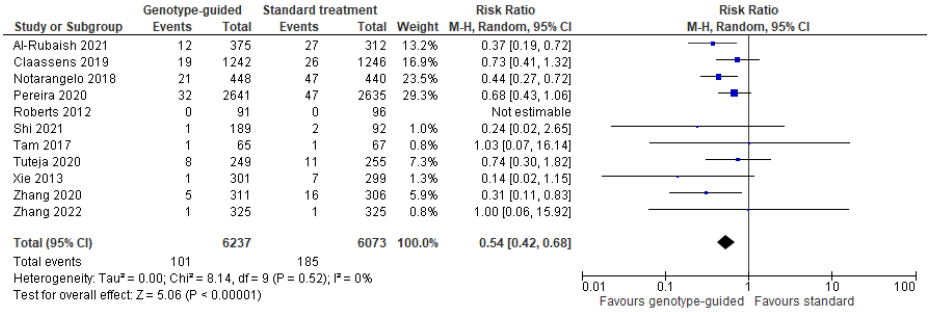
Supplemental Figure 8: *Forest plots for ischemic and bleeding outcomes in randomized controlled trials (RCTs) comparing genotype-guided and standard antithrombotic treatment in coronary artery disease patients and stroke patients (one RCT, Zhang 2022)*



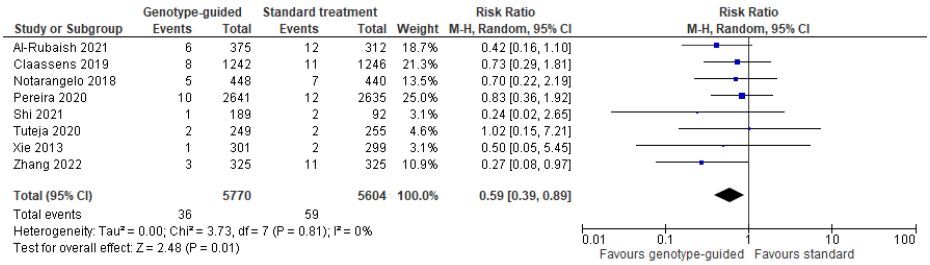
Supplemental figure 8A: *Major adverse cardiovascular events*



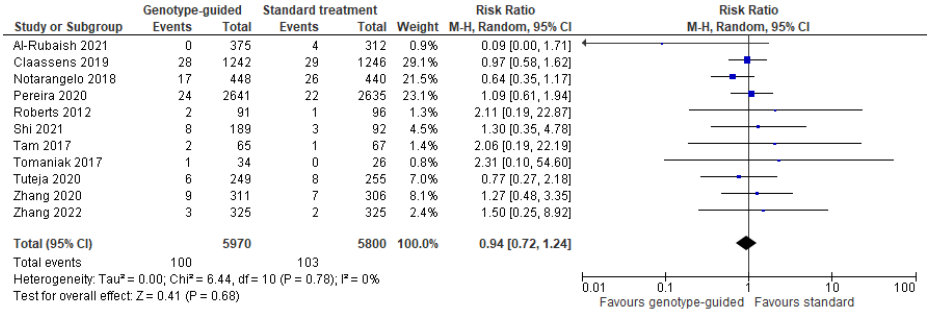
Supplemental figure 8B: *Cardiovascular death*



Supplemental figure 8C: *Myocardial infarction*



Supplemental figure 8D: *Stroke*



Supplemental figure 8E: *Major bleeding*

Chapter 11

CYP2C19 genotype-guided antithrombotic treatment versus conventional clopidogrel therapy in peripheral arterial disease: study design of a randomized controlled trial

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Am Heart J. 2022 Dec:254:141-148.

Abstract

Background: Clopidogrel is recommended in international guidelines to prevent arterial thrombotic events in patients with peripheral arterial disease (PAD). Clopidogrel itself is inactive and metabolism is dependent on the CYP2C19 enzyme. About 30% of Caucasian PAD patients receiving clopidogrel carry 1 or 2 CYP2C19 loss-of-function allele(s) and do not or to a limited extent convert the prodrug into its active metabolite. As a result, platelet inhibition may be inadequate which could lead to an increased risk of adverse clinical events related to arterial thrombosis. A CYP2C19 genotype-guided antithrombotic treatment might be beneficial for PAD patients.

Methods: GENPAD is a multicenter randomized controlled trial involving 2,276 PAD patients with an indication for clopidogrel monotherapy. Patients with a separate indication for dual antiplatelet therapy or stronger antithrombotic therapy are not eligible for study participation. Patients randomized to the control group will receive clopidogrel 75 mg once daily without pharmacogenetic guidance. Patients randomized to the intervention group will be tested for carriage of CYP2C19 ∗2 and ∗3 loss-of-function alleles, followed by a genotype-guided antithrombotic treatment with either clopidogrel 75 mg once daily for normal metabolizers, clopidogrel 150 mg once daily for intermediate metabolizers, or acetylsalicylic acid 80 mg once daily plus rivaroxaban 2.5 mg twice daily for poor metabolizers. The primary outcome is a composite of myocardial infarction, ischemic stroke, cardiovascular death, acute or chronic limb ischemia, peripheral vascular interventions, or death. The secondary outcomes are the individual elements of the primary composite outcome and clinically relevant bleeding complications.

Conclusion: The aim of the GENPAD study is to evaluate the efficacy, safety, and cost-effectiveness of a genotypeguided antithrombotic treatment strategy compared to conventional clopidogrel treatment in PAD patients. (Am Heart J 2022;254:141–148.)

Background

It is estimated that globally over 200 million people are affected by peripheral arterial disease (PAD).[1] Symptoms vary and may include intermittent claudication, pain at rest, or gangrene, typically categorized according to the Rutherford classification.[2] Despite adequate secondary prevention measures, cardiovascular morbidity and mortality remain high amongst PAD patients. According to the international PAD guidelines, clopidogrel 75 mg once daily is recommended to reduce the risk of arterial thrombosis.[3,4] These guidelines do not mention the influence of CYP2C19 genetic variants on the pharmacokinetics and the platelet inhibiting effect of clopidogrel.

Clopidogrel is a prodrug that needs to be metabolized into its active metabolite by the CYP2C19 enzyme. About 30% of the Caucasian population carries at least one loss-of-function (LOF) allele, such as the *2 or *3 alleles. This results in a limited ability to convert the prodrug clopidogrel into its active metabolite. Studies showed that carriers of CYP2C19 LOF alleles have lower plasma concentrations of the active metabolite of clopidogrel and increased platelet aggregation as compared to noncarriers.[5,6]

Previous studies in the fields of neurology and cardiology demonstrated the clinical relevance of CYP2C19 LOF alleles. A meta-analyses of Pan et al regarding stroke patients indicate that carriers of CYP2C19 LOF alleles receiving clopidogrel had a circa 2-fold increased risk of recurrent stroke as compared to noncarriers.[7] The largest body of evidence, including several meta-analyses, exists for patients with coronary artery disease (CAD). Carriers of CYP2C19 LOF alleles who are treated with clopidogrel and undergoing percutaneous coronary intervention (PCI) have a higher risk of adverse cardiovascular events compared to noncarriers. This is more pronounced in an Asian population (RR = 1.91), where LOF-alleles are more common, compared to a Caucasian population (RR = 1.20).[8,9] Since atherosclerosis is a systemic condition, extrapolation of results from previous studies in CAD and stroke patients is possible. However, CAD patients undergoing PCI with stenting are usually treated with dual antiplatelet therapy (DAPT) instead of monotherapy clopidogrel.

The association between CYP2C19 LOF alleles and increased risk of adverse cardiovascular events in patients using clopidogrel suggests that a CYP2C19 genotype-guided strategy could lead to improved clinical outcomes in patients with clinical manifestations of atherosclerosis. In CYP2C19 LOF-allele carriers with cerebrovascular disease, the use of ticagrelor has proven to be superior to clopidogrel in terms of reducing risk of recurrent stroke after 90 days.[10] In CAD

patients, multiple prospective trials have been performed investigating the use of a CYP2C19 genotype-guided antiplatelet therapy.[11-14] Several large meta-analyses showed that CYP2C19 genotype-guided strategies could reduce ischemic events in CAD patients, especially in patients undergoing PCI, compared to conventional therapy.[15-18] Pereira et al. showed that the superiority of the more potent P2Y12 inhibitors, ticagrelor, and prasugrel, in CAD patients was based primarily on CYP2C19 LOF-alleles since these treatment strategies significantly reduced ischemic events in CYP2C19 LOF-allele carriers, but not in non-carriers.[17]

So far, no large-scale prospective research has been performed to investigate the clinical relevance of CYP2C19 LOF-alleles in PAD patients treated with clopidogrel, nor is there any trial evidence regarding the added value of CYP2C19 genotype-guided treatment. We hypothesize that a CYP2C19 genotype-guided antithrombotic treatment strategy is superior in terms of reducing the rate of adverse clinical events related to arterial thrombosis in PAD patients, and that this approach is cost-effective compared to standard clopidogrel treatment.

Methods

Objectives

The primary aim of the GENPAD study is to determine whether a CYP2C19 genotype-guided antithrombotic treatment is superior in reducing adverse clinical events related to arterial thrombosis in comparison to standard clopidogrel treatment in PAD patients. The secondary objective is to evaluate the efficacy of CYP2C19 genotype-guided antithrombotic treatment in reducing the separate elements of the primary composite outcome end point and to compare the occurrence of clinically relevant bleeding complications of both treatment strategies. Furthermore, we will evaluate the cost-effectiveness of a CYP2C19 genotype-guided approach.

Design

The GENPAD study is a randomized, open label, multicenter, clinical trial. Patients will be randomized to either a CYP2C19 genotype-guided antithrombotic treatment strategy or standard treatment with clopidogrel. Patients and health care providers are not blinded for treatment allocation.

The study is initiated by the Radboud university medical center and conducted at the Radboud university medical center, university medical center Groningen, Maastricht university medical center, Amsterdam university medical centers,

Canisius Wilhelmina Ziekenhuis Nijmegen, Rijnstate Arnhem, Bernhoven Uden, Gelderse Vallei Ede, Gelre Ziekenhuizen, Máxima Medisch Centrum Veldhoven, Medisch Spectrum Twente, Ommelander Ziekenhuis Groningen, and Groene Hart Ziekenhuis Gouda. The recruitment schedule is designed to have a mean follow-up of 2 years for the entire study population. The follow-up time will range from 6 months to 3 years. The study has been approved by the regional medical ethics committee Oost-Nederland (reference number: 2020-7057), and local approval has been obtained for each participating site. This study is conducted in accordance with the latest revision of the Declaration of Helsinki and Good Clinical Practice regulations and is registered at ClinicalTrials.gov on November 6 2020 as NCT04619927 (https://www.clinicaltrials.gov/ ct2/show/NCT04619927) and at the Dutch Trial Register on November 2 as NL9027 (https://www.trialregister.nl/trial/9027). The GENPAD trial is supported by a grant from ZonMw, a Dutch organization funded by the government promoting health care research, and implementation of study results in daily practice. The authors are solely responsible for the design and conduct of this study, all study analyses, the drafting and editing of the paper and its final contents.

Participants and recruitment

All patients with lower extremity PAD visiting the outpatient clinic or vascular laboratory are screened. Patients already on clopidogrel monotherapy as well as newly diagnosed patients with an indication for clopidogrel monotherapy are considered eligible. Patients with an indication for dual antiplatelet therapy or stronger anticoagulant therapy (including NOACs, vitamin K antagonists or coumarins) are not eligible for participation. Inclusion criteria for this study are: 1) previous or current ankle-brachial index < 0.9 and/or toe brachial index < 0.5, 2) previous or current symptoms due to insufficient vascularization of 1 or 2 lower extremities including intermittent claudication, pain at rest, and/or gangrene (Rutherford classification 1-6), 3) consultation of the vascular surgery department for diagnosis, treatment and/or follow-up of PAD, 4) an indication for monotherapy clopidogrel 75 mg once daily, and 5) age 16 years or older. Detailed exclusion criteria are found in appendix A. Key exclusion criteria include: 1) known CYP2C19 genotype or metabolizer state, 2) (concomitant) treatment with other anticoagulants, 3) contraindications for clopidogrel, ASA, and/or rivaroxaban, 4) pregnant or breastfeeding women, and 5) patients who are unable to provide written informed consent.

Local computer programs select eligible patients by a pseudo-anonymous screening of the outpatient clinic and vascular laboratory of the vascular surgery

departments. In case a participating hospital cannot facilitate computer-controlled screening, a physician involved in the treatment of patients with PAD will manually perform screening. Eligible patients are informed about the study and written informed consent is obtained before proceeding with any of the trial procedures. Where possible, research procedures at baseline will be combined with the participants' planned visit to the vascular laboratory or the outpatient clinic.

Study procedures

Castor EDC's (Castor Electronic Data Capture, Amsterdam, the Netherlands) validated variable block randomization model randomly assigns participants in a one-to-one ratio to either CYP2C19 genotype-guided antithrombotic treatment or standard clopidogrel treatment, stratified by participating center. After randomization, data on baseline patient characteristics, vascular state, medical history, and medication use is collected. Height, weight, hip/waist circumference, and blood pressure is measured and blood samples are collected to perform CYP2C19 genotyping. Patient questionnaires are obtained at baseline and at 6, 12, 24, and 36 months, respectively. These questionnaires include the EQ-5D-5L, the WHOQol-Bref, and an adapted combined version of the Institute for Medical Technology Assessment (IMTA) medical cost questionnaire (iMCQ) and the IMTA productivity cost questionnaires (iPCQ) in Dutch language versions. Additionally, a GENPAD specific questionnaire is used during follow-up, including questions about medication adherence and occurrence of adverse events. An overview of the study procedures is shown in *table 1*.

Table 1: *Study schedule*

Study procedure	T0	T1	T2	T3	T4
Informed consent	X				
Randomization	X				
Patient characteristics	X				
Vascular state	X				
Medical history	X				
Medication use	X				
Blood sample withdrawal	X				
CYP2C19 genotyping (intervention grup)	X				
Questionnaires *	X	X	X	X	X
Review of medical record	X	X	X	X	X

T0 = study start, T1 = 6 months, T2 = 12 months, T3 = 24 months, T4 = 36 months
**Questionnaires include the EQ-5D-5L, the WHOQol-Bref, an adapted combined version of the IMTA medical cost questionnaire (iMCQ) and the IMTA productivity cost questionnaire (iPCQ), and the GENPAD specific questionnaire.*

CYP2C19 genotyping and genotype-guided prescription

For CYP2C19 genotyping, a 6 mL blood sample is collected in an EDTA tube of each participant at baseline. Samples of participants in the intervention group are directly analyzed for the presence of CYP2C19 LOF alleles. CYP2C19 genotyping will take place at the department of Human Genetics of the Radboud university medical center using Taqman assays (CYP2C19*2: C__25986767_70, CYP2C19*3: C__27861809_10) according to the protocols of the manufacturer (ThermoFisher Scientific, Bleiswijk, The Netherlands).

After CYP2C19 genotyping, participants in the intervention group will receive genotype-guided antithrombotic treatment. Patients without a *2 or *3 CYP2C19 LOF allele are considered extensive or normal metabolizers and will be treated with clopidogrel 75 mg once daily. Patients with 1 LOF allele, either a *2 or *3 CYP2C19 allele, are classified as intermediate metabolizers and will be treated with double-dose clopidogrel (150 mg) once daily. Finally, patients with 2 of these LOF alleles are classified as poor metabolizers and will be treated with ASA 80 mg once daily plus rivaroxaban 2.5mg twice daily. Blood samples of participants in the control group are stored at -80°C and will be analyzed for CYP2C19 LOF alleles at the end of the study. Participants in the control group are treated with clopidogrel 75 mg once daily.

Delivery and prescription of medication

GENPAD is a pragmatic trial thus all medication will be provided via usual care and expenses are covered by the national health insurance. All eligible patients have been diagnosed with PAD and are therefore already using clopidogrel or acetylsalicylic acid. New patients are prescribed clopidogrel standard treatment upon diagnosis after which they are considered for study participation. Patients in the control group using acetylsalicylic acid are switched to clopidogrel directly after inclusion. Patients in the intervention group continue their current platelet aggregation inhibitor, clopidogrel or acetylsalicylic acid, until genotyping results are available. Results of the CYP2C19 genotyping are available a maximum of 3 weeks after study inclusion. Requested medication modifications are directly communicated to the health care provider and monitored by the study team. Pharmacy delivery details will be checked regularly to ensure participants are treated according to protocol.

Outcomes

The primary composite outcome is the occurrence of myocardial infarction, ischemic stroke, cardiovascular death, acute or chronic limb ischemia, peripheral

vascular interventions, or death. The composite of myocardial infarction, ischemic stroke or cardiovascular death is referred to as major adverse cardiovascular events (MACE). The composite of acute limb ischemia, chronic limb ischemia, and peripheral vascular interventions is referred to as major adverse limb events (MALE). Acute limb ischemia is defined as limb-threatening ischemia that is confirmed by using limb hemodynamic parameters or imaging and leading to an acute vascular intervention within thirty days of onset of symptoms. Chronic limb threatening ischemia is defined as (1) continuing ischemic limb, foot, or digit pain leading to hospitalization and intervention and not meeting the definition of acute limb ischemia, or (2) participants with Rutherford classification 4 to 6 at baseline who had a peripheral vascular intervention over the course of the trial. Peripheral vascular interventions include pharmacological interventions (e.g., thrombolysis), plain balloon angioplasty with or without stenting, peripheral artery surgery/ reconstruction, and major or minor lower limb amputations. Major amputation is defined as an amputation above the forefoot due to a vascular event while minor amputations are defined as more distal amputations.

Secondary outcomes are the occurrence of the separate elements of the primary composite outcome, and the occurrence of major and clinically relevant bleeding complications. Bleeding complications are defined according to the International Society on Thrombosis and Haemostasis criteria.19 Major bleeding complications are defined as (1) fatal bleeding, (2) symptomatic bleeding into a critical organ, (3) bleeding causing a fall in hemoglobin level of 20 g L−1 (1.24 mmol L−1) or more or leading to transfusion of 2 or more units of whole blood or red blood cells, or (4) surgical site bleeding requiring reoperation.[19] Clinically relevant minor bleeding complications are bleedings leading to (1) hospitalization (including presentation to an acute care facility without an overnight stay), (2) a physician-guided medical or surgical treatment for bleeding, or (3) a change in antithrombotic treatment. An independent, blinded clinical end point committee will determine and grade all adverse clinical events. Other outcomes of interest include patient-reported health-related quality of life (WHOQolBref), patient-reported health state (EQ-5D-5L), amount of medical costs (iMCQ), and costs due to productivity loss (iPCQ).

Data collection and management

To ensure privacy, all study participants are identified by a unique subject number used in all correspondence and in the study database. Baseline values such as gender, age, comorbidities, vascular state, and medication use are collected from the medical records and stored on the secured Castor (Castor Electronic Data Capture, Amsterdam, the Netherlands) servers. Case report forms are used to obtain

relevant information about ethnicity, smoking behavior, alcohol consumption, and family history of cardiovascular disease. Current height, weight, hip/waist ratio, and blood pressure are measured at baseline. Study-related correspondence, patient records, signed informed consent forms, and source documents with an exception for the questionnaires, will be preserved at the participating site for 15 years. All questionnaires will be sent to and preserved at Radboud University medical center. Source data will be entered in the online database Castor and exported for statistical analyses afterward.

Power calculation

Based on previous literature we assume a risk ratio of 1.8 between carriers of CYP2C19 *2 and/or *3 allele(s) and noncarriers.[8,9,18,20-26] It is known that 30% percent of the Caucasian population is carriers of 1 or 2 of these LOF alleles.[27-29] Van Mil et al showed that 32% of PAD patients consulting a vascular surgeon at Radboud University Medical Center will develop an adverse clinical event within 1 year.[21] For the power calculation we used a more conservative percentage of 25% over 2 years follow-up to avoid insufficient power due to a lack of events. The 2-year risk for noncarriers to develop an adverse clinical event is 20%. We hypothesize that the risk of an adverse clinical event related to arterial thrombosis in patients with a relevant CYP2C19 variant that receive genotype-guided antithrombotic therapy will be reduced to the risk of patients without a genetic variant. With an alpha of 5% and a power of 80% a total of 1089 patients need to be included per group. To account for a drop-out rate of 5% we will include 2,276 patients in total.

Statistical analysis

Baseline characteristics for continuous data are described as mean and standard deviation while categorical data will be described as number and percentage. Data is analyzed according to intention-to-treat principle. Primary and secondary outcomes in the intervention and control groups are compared. Additionally, subgroup analyses are performed for intermediate and poor metabolizers.

For both the primary composite outcome as well as the secondary outcomes, Cox proportional-hazards model is used to analyze the primary and secondary time-to-event. Kaplan-Meier estimates of the cumulative proportion of patients with events are performed. Explorative subgroup analyses are performed to assess differences in efficacy between gender, age groups, and ethnic groups. The economic evaluation is embedded in the design of the study and will be undertaken as cost-effectiveness analysis (CEA) with the costs per adverse event avoided over a 2-year period. Additionally, an empirical cost-utility analysis (CUA) will be performed with

the costs per quality-adjusted life-year (QALY) as outcome over a 2-year time period. A long-term scenario will be explored by decision analytical modeling according the International Society for Pharmacoeconomics and Outcomes Research and the Netherlands Health Care Institute guidelines for economic evaluations.[30] The CEA closely relates to the results concerning the primary composite outcome, the CUA is performed to enable priority setting during health care policy making across patient groups, interventions, and health care settings. Both analyses will be performed from a societal perspective (as base-case) and the time for empirical data collection is set at 24 months, after which extrapolation to long term will be applied. With the 24 months, horizon discounting of costs and effects are unnecessary. In case of confounding the net monetary benefit (NMB) approach will be applied to incorporate the confounders in the regression model with NMB as dependent variable. In case of QALY as efficiency outcome results will be displayed graphically by means of cost-effectiveness planes and acceptability curves according to the Dutch guideline for economic evaluations.[30] For all statistical analyses, P-values of .05 or less are considered significant.

Present status

The first patient was enrolled on March 16, 2021. Currently, 10 participating study centers are actively enrolling patients and a total number of 749 patients have been included so far. Patient recruitment is expected to be completed in 2023.

Discussion

The GENPAD study is, to the best of our knowledge, the first randomized clinical trial to evaluate the efficacy of a CYP2C19 genotype-guided antithrombotic treatment strategy in PAD patients.

We hypothesize that PAD patients with CYP2C19 LOF alleles who are treated with clopidogrel are at increased risk of ischemic events. However, large-scale research on PAD patients is lacking, and the existing evidence regarding this association in PAD patients is conflicting. The EUCLID study was a large randomized trial investigating the effects of ticagrelor vs clopidogrel in 13,885 PAD patients.[31] The main conclusion of this trial was that ticagrelor did not reduce ischemic events in patients with PAD. During the EUCLID trial 6,955 patients were randomized to clopidogrel treatment, and 30% of these patients were found to have one CYP2C19 LOF-allele. All poor metabolizers were excluded, and major adverse limb events were not taken into account. In a secondary subgroup analyses of the EUCLID

trial, published as a research letter, carriage of one CYP2C19 LOF allele was not associated with an increased risk of the primary efficacy end point of cardiovascular death, MI, or ischemic stroke.[32] However, the studies of Guo et al and Lee et al in PAD patients do suggest a higher risk of adverse cardiovascular and limb events in CYP2C19 LOF-allele carriers treated with clopidogrel[33,34], which is in line with results from previous studies in the fields of cardiology and neurology that showed an increased risk of recurrent ischemic events in carriers of CYP2C19 LOF allele(s) with coronary or cerebral artery disease that were treated with clopidogrel.

Optimizing clopidogrel treatment is fundamental since clopidogrel is the mainly used antithrombotic drug in the treatment of symptomatic PAD patients. The CAPRIE trial showed that PAD patients receiving clopidogrel monotherapy had a lower risk of cardiovascular events than those using ASA.[35] The previously mentioned EUCLID trial compared the use of ticagrelor vs clopidogrel in symptomatic PAD, and ticagrelor was not shown to be superior to clopidogrel in reducing adverse cardiovascular events.[31] Both ticagrelor and prasugrel are not registered for PAD patients, so currently treatment option with an alternative P2Y12 inhibitor is lacking. More recent, the COMPASS trial, although increasing bleeding complications, showed superiority of dual pathway inhibition using low-dose rivaroxaban plus ASA compared to ASA alone in reducing adverse cardiovascular events in PAD patients.[36] Direct comparisons between clopidogrel and low-dose rivaroxaban plus ASA have not been made yet. Therefore, clopidogrel remains a pillar in the treatment of symptomatic PAD patients, which is why optimizing clopidogrel treatment is of great importance.

During the GENPAD trial, alternative treatment strategies are used for intermediate and poor metabolizers in the intervention group. Double-dose clopidogrel will be prescribed for intermediate metabolizers since previous research found that clopidogrel 150 mg once daily leads to better platelet inhibition in patients with low clopidogrel responsiveness.[13,14,37-48] Double dose clopidogrel is therefore recommended by the Dutch Pharmacogenetics Working Group (DPWG) of the Dutch Royal Pharmacist Association for patients with an intermediate metabolizer phenotype after PCI or after experiencing a stroke or TIA when use of alternative treatment options are lacking.[49] However, trial evidence for the effectiveness of this approach in patients with PAD is currently lacking which is a potential limitation of our study. Another potential limitation is that an interim analysis is not included in the protocol, so if our power calculation proves inaccurate our study might be underpowered.

The GENPAD trial aims to evaluate the efficacy, safety, and cost-effectiveness of a genotype-guided antithrombotic treatment strategy compared to conventional clopidogrel treatment in PAD patients. We expect to see a higher rate of ischemic events in patients with LOF-allele(s) who are treated with standard clopidogrel dose as compared to patients with LOF-allele(s) treated according to a CYP2C19 genotype-guided approach. Furthermore, we expect the genotype-guided strategy to be cost-effective. If so, pharmacogenetic testing should be implemented in daily practice and treatment strategies used in the GENPAD trial may be used to optimize treatment of CYP2C19 LOF-carriers. This knowledge is required to enhance treatment of PAD patients, possibly leading to improved outcomes and reduced cardiovascular morbidity and mortality while reducing health care costs.

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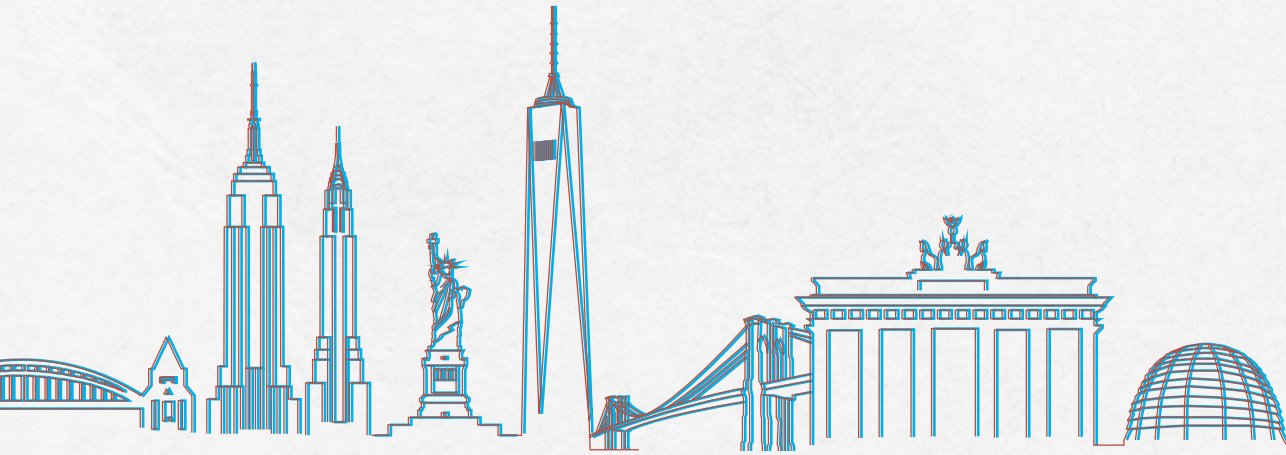
Appendix A: Exclusion Criteria

A potential subject who meets any of the following criteria will be excluded from participation in this study:

- Patients with a known CYP2C19 genotype or metabolizer state
- Patients treated with coumarins, Non-vitamin K Oral Anti-Coagulants (NOACs), unfractionated heparin (UFH), low molecular weight heparins (LMWH) or double antiplatelet therapy (DAPT) with ASA and a P2Y12 inhibitor for other indications
- Patients having one of the following contraindications for clopidogrel, ASA and/or rivaroxaban
    - Hypersensitivity to clopidogrel, ASA or rivaroxaban
    - History of asthma attacks, caused by salicylates or nonsteroidal anti-inflammatory drugs (NSAIDs)
    - Patients at significant risk for major bleeding:
        - Current gastrointestinal ulceration
        - Presence of malignant neoplasms, with the exception of non-melanoma skin cancer
        - Recent (<2 months) brain or spinal injury
        - Recent (<3 months) brain or spinal surgery
        - Recent (<3 months) intracranial, gastrointestinal or pulmonary hemorrhage
        - Presence of arteriovenous malformations
        - Major intraspinal or intracerebral vascular abnormalities
        - Congenital or acquired bleeding disorders
        - Uncontrolled severe arterial hypertension (180 mmHg or more systolic, or 110 mmHg or more diastolic
    - Patients with severe hepatic disease: Child-Pugh classification B or C
    - Patients with severe kidney failure: Patients with an estimated glomerular filtration rate < 15 mL/min or requiring dialysis
    - Patients with severe heart failure: Patients with a known ejection fraction of < 30% or New York Heart Association class III or IV symptoms
    - Patients using methotrexate at a weekly dose of 15 mg or more
    - Concomitant treatment with medication with a strong pharmacokinetic interaction with rivaroxaban, leading to contra-indication according to the 'Regionale richtlijn DOAC'[50]
- Patients who are pregnant or breastfeeding
- Patients who are unable to give informed consent, including not being able to understand the Dutch language

Part IV



Chapter 12

General discussion and future perspectives

Discussion and future perspectives

Peripheral arterial disease (PAD) is a common manifestation of atherosclerosis and contributes significantly to morbidity, mortality, and healthcare burden. A key feature of PAD is endothelial dysfunction, which can precede clinical symptoms by years. This highlights the importance of early detection and secondary cardiovascular prevention, including the use of antithrombotic therapy.

This thesis addressed three main objectives:

1. To explore the use of carotid artery reactivity (CAR) testing as a simple, non-invasive method to assess endothelial function.
2. To evaluate the effect of dual-pathway inhibition (DPI)—a combination of low-dose rivaroxaban and aspirin—on endothelial function.
3. To provide an overview of current antithrombotic therapies in PAD and support clinical decision-making for secondary cardiovascular prevention.

The studies included in this thesis focused on vascular health, specifically examining endothelial function, coagulation activity, and inflammation across different patient groups and treatment contexts. Due to the rapidly evolving circumstances and clinical urgency during the COVID-19 pandemic, the original first objective of this thesis was adjusted. As vascular health assessments such as endothelial function measures became immediately relevant, the initial part of this work was redirected to investigate vascular health in individuals recovering from SARS-CoV-2 infection or after ChAdOx1 nCoV-19 vaccine (ChAdOx1) CoV-19 vaccination. The second part evaluated the effects of DPI on vascular function and inflammation in patients with PAD. The third part reviewed current evidence on antithrombotic strategies for PAD, including genotype-guided therapy. The final section reflects on the implications of these findings and outlines directions for further research.

PART I assessment of endothelial function, coagulation activation and inflammation after COVID-19

The emerge of the coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus (SARS-CoV) 2, was officially declared a pandemic by the World Health Organization on March 11th 2020.[1] COVID-19 appeared first in Wuhan, China, in December 2019 and rapidly spread across the world.[1-2]

Thrombotic complications in COVID-19

The interaction between COVID-19 and the cardiovascular system has been demonstrated on multiple levels. First, coagulation activation is commonly observed in COVID-19 patients in the acute phase. Elevated D-dimer levels, often indicative of more severe disease[1,3-4] and acute respiratory distress syndrome[5], are commonly detected. Notably, increased D-dimer levels are present in almost all COVID-19-related fatalities, indicating widespread coagulation activation.[6-8]

Second, elevated cardiac troponin (cTn) levels are frequently observed in COVID-19 patients, indicating myocardial damage. Patients with elevated cTn levels are at a heightened risk of requiring intensive care unit (ICU) admission and mortality.[6,9]

Third, acute COVID-19 is associated with a high incidence of thrombotic complications, affecting both venous and arterial vasculature. Klok et al. documented 27% prevalence of venous thromboembolism among 184 ICU admitted COVID-19 patients in the Netherlands.[10] Similar rates were observed in a Chinese and a French cohort.[11-12] Additionally, Klok et al. reported a 4% incidence of arterial thromboembolism.[10] Furthermore, increased incidences of large-vessel stroke and myocardial infarction in acute COVID-19 patients have been described.[13-15] Proposed mechanisms for these complications include neutrophil and contact activation, vascular damage, and tissue factor expression.[16] While these thrombotic events suggest the involvement of the vasculature at a macrovascular level during acute COVID-19, the virus may also induce more subtle and persistent effects on the microvascular endothelium.

Indicators of micro- but not macrovascular endothelial dysfunction after COVID-19

Widespread endothelial cell involvement in the acute phase of COVID-19and in COVID-19-associated coagulopathy, particularly in the early waves of the pandemic (primarily associated with the original Wuhan strain and Alpha variant), has been extensively described.[17-18] Studies by Varga et al. and Rovas et al., conducted during

the first pandemic wave in 2020, have demonstrated evidence of direct infection of endothelial cells by SARS-CoV-2, leading to diffuse endothelial inflammation.[19-20] Moreover, hospitalized COVID-19 patients have exhibited reduced endothelium-dependent vasodilator responses and increased levels of serum cytokines and chemokines involved in vascular function regulation, indicating endothelial dysfunction.[21] This thesis provides the novel insight of persistent endothelial cell involvement beyond the acute phase, specifically persisting into the mid- (3 months) and long-term (18 months) after COVID-19 infection (*Chapter 3* and *Chapter 4*). The study population of *Chapter 3* and *Chapter 4* was infected between early and mid-2020, covering periods dominated by the original Wuhan strain and early Alpha variants. Although clinical recovery coincided with normalization of C-reactive protein and D-dimer levels, endothelin-1 (ET-1) levels, indicating endothelial cell activation, were significantly elevated during acute COVID-19 compared to controls, with a further increase observed 3 months post-COVID-19. Remarkably, endothelial activation persisted to a similar extent in patients with critical illness and managed at home during the acute infection phase, suggesting that disease severity alone does not fully explain persistent endothelial activation (*Chapter 3*).

Additionally, levels of von Willebrand factor antigen, reflecting endothelial damage, were not only elevated in approximately 80% of patients at 3 months post-COVID-19 (*Chapter 3*), but also remained above normal range in the majority of COVID-19 survivors at 18 months after acute infection (*Chapter 4*). These findings indicate long-term endothelial involvement. This persistent endothelial cell involvement may potentially lead to delayed onset macrovascular endothelial dysfunction and thus contribute to the long-tern development and progression of cardiovascular disease[22-23], as will be discussed later in this chapter (Paragraph 'Interplay between endothelial cells, coagulation and inflammation post-COVID-19').

However, macrovascular dysfunction, reflected by carotid artery constriction in response to sympathetic stimulation (cold pressor test), was not observed in this thesis (*Chapter 3* and *Chapter 4*). Nonetheless, the maximum relative change in carotid artery diameter in response to sympathetic stimulation (CAR%) was significantly reduced at 18 months compared to 3 months after acute COVID-19 (*Chapter 4*). This decrease in CAR% may indicate reduced vascular wall relaxation capacity, and thus microvascular endothelial dysfunction, induced by COVID-19. The clinical significance of such changes in CAR%, without affecting the prevalence of macrovascular dysfunction, may not be immediately apparent. Given that changes in the larger arteries may not manifest within 18 months, a longer follow-up may be required to detect COVID-19-induced macrovascular dysfunction. Previous research

has shown that the risk of cardiovascular disease remains elevated up to 10 years following hospitalization for pneumonia.[24]

Persistent prothrombotic alterations following COVID-19

The high incidence of thrombotic complications in the acute phase of severe COVID-19, as previously described, could be explained by excessive activation of the contact coagulation pathway. Busch et al. confirmed ongoing contact pathway activation during acute COVID-19 with a clear correlation to disease severity.[16] This thesis shows that elevated markers of contact activation persist in a subset of patients even 3 months after acute COVID-19 (*Chapter 3*), with further normalization observed after 18 months (*Chapter 4*).

Interestingly, thrombin-antithrombin (TAT) complex, indicative of thrombin generation and reflective of a prothrombotic state, increased further at 18 months compared to 3 months after acute COVID-19, which may be the result of persistent endothelial injury, as described earlier. Evidence of thrombin generation up to 1 year after COVID-19, likely due to enduring endothelial damage, has also been demonstrated by other studies.[25-26]

Additionally, Factor VII:AT, a marker of extrinsic pathway activation, was elevated in one-third of patients 3 months after acute COVID-19 (*Chapter 3*), and remained high at 18 months after COVID-19 (*Chapter 4*). These findings align with those of Meijenfeldt et al. reported sustained prothrombotic changes characterized by enhanced thrombin-generating capacity and reduced plasma fibrinolytic potential in 52 patients who had recovered from COVID-19, 4 months after hospital discharge.[25]

Beyond the infection itself, concerns have also been raised about prothrombotic risks following COVID-19 vaccination. As novel variants of SARS-CoV-2 continued to infect individuals worldwide, various vaccines have been developed and authorized for use, including ChAdOx1. While ChAdOx1 has demonstrated safety and efficacy in randomized controlled trials[27], its administration was halted in several European countries due to reports of rare thromboses at atypical sites accompanied by thrombocytopenia.[28] This phenomenon, now recognized as vaccine-induced immune thrombotic thrombocytopenia (VITT), is linked to the activation of platelets by anti-platelet factor 4 antibodies.[29-30]

In addition to the rare occurrence of VITT, studies have indicated a higher incidence of thrombosis and thromboembolism in individuals recently vaccinated with ChAdOx1 compared to other COVID-19 vaccines[31] and compared to the general

population.[32] This raised the question of whether ChAdOx1 could trigger activation of the blood coagulation system, comparable to the coagulation activation induced by SARS-CoV-2 itself.

To address this, this thesis measured coagulation activation 24 and 48 hours after ChAdOx1 vaccination, compared to pre-vaccination levels. No increases in markers of coagulation activation were observed (*Chapter 5*). The findings revealed a significant decrease in levels of Factor VIIa:AT following vaccination, possibly triggered by interleukin (IL)-6 induced tissue factor (TF) expression[33] leading to the binding of Factor VII to TF. However, no further evidence of extrinsic pathway activation was demonstrated.

Possible chronic low grade inflammation and COVID-19

Several key aspects regarding the persistent impact of COVID-19 on inflammation, as well as the effects of ChAdOx1 vaccination on inflammatory markers are addressed in this thesis. Acute COVID-19 induces an intense inflammatory response, with severe cases often progressing in cytokine storms and multiorgan failure or death. During the acute phase, elevated levels of IL-1 family cytokines and IL-6.[19-20,34]

Even after recovery, this thesis found evidence of a lingering low-grade systemic inflammation among acute COVID-19 survivors, characterized by elevated IL-18 levels in the majority and heightened IL-6 and IL-1RA levels in a significant portion, three months post-infection (*Chapter 3*). This sustained inflammation may be due to the direct endothelial infection by COVID-19 and consequential microcirculatory disturbances as described before. Elevated IL-18, IL-6 and IL-1RA levels are associated with vascular inflammation and adverse cardiovascular outcomes, implying a potential chronic low-grade arterial inflammation in acute COVID-19 survivors.[22-23,35] Activation of IL-1 family cytokines is mediated by the Nod-like receptor family pyrin domain containing 3 inflammasome, which is known to be upregulated in chronic inflammatory states.[36-37] Mean plasma levels of the inflammatory cytokines IL-1RA and IL-18 decreased and returned to normal at 18 months compared to 3 months post-COVID-19, suggesting at least partial resolution of the chronic low-grade inflammatory state (*Chapter 4*), which may confer cardiovascular benefits.[23,34]

Following ChAdOx1 vaccination, IL-6 levels increased, while IL-18 levels remained unchanged. Elevated IL-6 levels after non-COVID-19 vaccination have previously been reported.[38-41] This post-vaccination IL-6 increase, possibly triggered by interferon-γ production by natural killer cells[41], aligns with previous observations

following single-dose ChAdOx1 administration and may be related to IL-6's induction of tissue factor expression, which regulates hemostasis (*Chapter 5*).[33]

Interplay between endothelial cells, coagulation and inflammation post-COVID-19

The interaction between endothelial cells, coagulation, and systemic inflammation following COVID-19 is a focal point of this thesis. At 3 months post-recovery, patients demonstrate endothelial cell injury, coagulation activation, and low-grade systemic inflammation (*Chapter 3*). Despite explicit elevations in both coagulation activation and inflammatory cytokines, *Chapter 3* showed no correlation between the two, suggesting a dissociation between thrombotic and inflammatory states in COVID-19, or at some point during recovery. Follow-up at 18 months shows continued endothelial cell involvement, with only partial normalization of coagulation and inflammatory markers (*Chapter 4*). These findings suggest that vascular changes may extend beyond the acute phase.

Despite the normalization of acute-phase markers like CRP and D-dimer, signs of endothelial dysfunction and continued low-grade activation of inflammation and coagulation are present, potentially predisposing individuals to thrombotic complications. This state may increase the risk of thrombotic complications after recovery. Neutrophils and contact activation of coagulation are proposed as contributing mechanisms of COVID-19 related vascular pathology, emphasizing the interconnectedness of coagulation and inflammation.[16]

Activation of both intrinsic and extrinsic coagulation pathways promotes fibrin formation and contributes to inflammation through PAR-receptor activation on endothelial cells. Continued PAR-receptor activation may sustain vascular inflammation, and support atherogenesis, as reflected on in *figure 1*.[36,42]

Inflammatory markers change over time. Plasma levels of IL-6, a key inflammatory cytokine, increase from three months to 18 months post-COVID-19 recovery. While markers of contact pathway activation show partial normalization in the long term, thrombin generation, reflected in TAT levels, continues to increase, suggesting a persistent prothrombotic state. IL-6 may be involved in this process, driving tissue factor expression and stimulating thrombin generation via the extrinsic coagulation pathway.[43]

Macrovascular endothelial dysfunction is not observed at the three-month mark, nor does extended follow-up suggest delayed onset (*Chapter 3 and 4*). Von Willebrand factor

antigen levels remained elevated in nearly all survivors, indicating ongoing endothelial cell involvement. In addition, a small decline in CAR% at 18 months post-COVID-19 may reflect early functional impairment (*Chapter 4*). The hypothesis of microvascular endothelial dysfunction representing the first steps in the evolvement of apparent macrovascular dysfunction and potential long-term cardiovascular consequences is supported by observations in other chronic diseases, such as hypertension[44], hyperlipidemia[45] and diabetes mellitus[46], where microvascular pathology often precedes and predicts macrovascular complications. In the context of COVID-19, direct evidence for this progression remains lacking, but the long-lasting elevation of endothelial biomarkers and early changes in vascular responsiveness highlights the need for continued monitoring to assess long-term vascular risk in COVID-19 survivors.

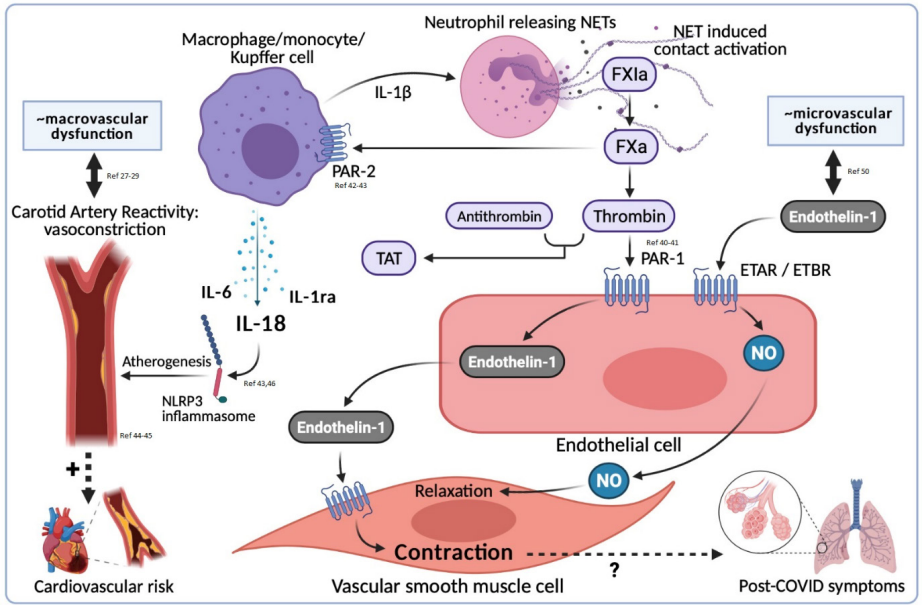


***Figure 1**: Reflection on elevated endothelin-1 levels, coagulation activation and sustained inflammation, in the long term post-COVID-19. NET = Neutrophil Extracellular Traps, ETAR = Endothelin Type A Receptor, ETBR = Endothelin Type B Receptor, NO = Nitric Oxid (Chapter 3)*

In light of the evolving SARS-CoV-2 variants, it is also important to consider that later variants, such as Delta and Omicron, have been associated with a more severe and milder clinical presentations, respectively, and potentially accordingly a more and less pronounced endothelial response. This underscores the need for variant-specific studies to determine whether the persistence and severity of endothelial dysfunction differ by viral strain.

PART II vascular health in relation to DPI in PAD patients

PAD significantly affects vascular health leading to debilitating symptoms and increased cardiovascular risk. In recent years, DPI—combining antiplatelet and anticoagulant therapy—has emerged, as a potential treatment strategy in PAD. DPI aims to target both platelet activation and the prothrombotic state associated with PAD, thereby reducing ischemic events and disease progression, and has shown significant success in achieving these outcomes.[47-49] Randomized controlled trials have demonstrated that DPI significantly lowers the risk of myocardial infarction, stroke, acute limb ischemia, and cardiovascular death. The increased risk of major bleeding remains a consideration, necessitating careful patient selection and monitoring. While the clinical benefits of DPI are well established, the underlying mechanisms by which anticoagulant therapy enhances vascular outcomes in PAD are not yet fully understood.

DPI and endothelial function in PAD patients

As previously discussed, endothelial function is vital for maintaining vascular health, and impaired endothelial function is a predictor of cardiovascular events and the progression of atherosclerosis in PAD patients. This thesis used CAR testing to assess macrovascular endothelial function in PAD patients. The CAR test is a simple, non-invasive method that uses an accessible artery and correlated well with the coronary artery endothelial function assessed by classical invasive methods and has been strongly linked to cardiovascular risk in PAD patients.[50-51] Compared to other non-invasive techniques such as flow-mediated dilation (FMD), the CAR test is relatively easy to perform due to the accessibility of the carotid artery for ultrasound imaging and the ability to stabilize the participant's head, as the sympathetic stimulus is applied to the arm. This likely reduces the risk of investigator bias and makes it a practical choice in both clinical and research settings.

Endothelial functions, such as modulation of vascular tone, thrombogenicity, and inflammation, are regulated by molecules, including ET-1.[52] In addition to the CAR test, this thesis measured plasma ET-1 concentrations. Plasma ET-1 levels are strongly related to microvascular endothelial function and can be used to monitor changes in this domain.[53] Previous research has even suggested ET-1 as a potential target for treating microvascular endothelial dysfunction in atherosclerosis since elevated serum levels of ET-1 were independently related to angiographically measured coronary microvascular dysfunction.[54]

In this thesis, the addition of low-dose rivaroxaban to Acetylsalicylic acid (ASA) monotherapy for 12 weeks in PAD patients, did not affect either macrovascular

or microvascular endothelial function (*Chapter 7*). To date, no other studies have reported on the impact of DPI or other antithrombotic drugs on endothelial function. However, other therapies have demonstrated clear effects on endothelial function. A 12-week physical activity has been shown to reverse carotid artery constriction and improve brachial artery flow-mediated dilation, suggesting improved endothelial function.[55-57] Additionally, lipid-lowering therapies improve macrovascular endothelial function, with effects observable within a month.[58-60]

Results from van Mil et al. showed that PAD patients with a constrictive CAR response have a fourfold increased risk of developing major adverse cardiovascular events and a twofold increased risk of clinical deterioration.[50] While DPI has been shown to reduce the occurrence of major adverse cardiovascular events and death in PAD patients, this thesis could not confirm that the improvement in these outcomes is due to enhanced endothelial function. It is possible that the 12-week intervention period was insufficient to induce measurable vascular improvements. This, however, would stand in contrast to the above mentioned findings from physical activity and lipid-lowering interventions, which have demonstrated significant improvements in endothelial function within similar or even shorter timeframes. Additionally, it is possible that changes in vascular tone, such as shifts from constrictive to dilatative responses or vice versa, did occur but were too subtle to be reliably detected with the current methodology.

DPI and systemic inflammation in atherosclerotic disease

Recent landmark studies, including the CANTOS trial, have convincingly established that inflammation is a key factor in the development and progression of atherosclerotic disease.[61] The accumulation of lipids and inflammatory cells in the arterial wall leads to the formation of atherosclerotic plaques, while chronic inflammation can destabilize these plaques, increasing the risk of rupture and subsequent cardiovascular events.[62]

ASA, although primarily an antiplatelet agent, has anti-inflammatory properties as it inhibits cyclo-oxygenase enzymes, reducing the production of pro-inflammatory prostaglandins and thromboxane.[63-64] Rivaroxaban, a direct inhibitor of Factor Xa in the coagulation cascade, may also exert anti-inflammatory effects. Factor Xa can activate protease activated receptor 1, leading to thrombin independent platelet activation and thrombus formation.[65-66] Previous research has shown that individuals with active coronary artery atherosclerosis, exhibit both higher levels of circulating cytokines and an increased immune cell response when exposed to ex vivo lipopolysaccharide stimulation.[67]

This thesis investigated whether DPI could offer additional benefits beyond thrombosis prevention, potentially by reducing inflammation, in both PAD and coronary artery disease patients. *(Chapter 8)* No significant differences in immune cell responsiveness – when exposed to ex vivo lipopolysaccharide stimulation – after three months of DPI were observed. Specifically, cytokine production – measured through levels of TNF-α, IL-6, and IL-1β – remained unchanged. Additionally, counts of immune cell populations (such as neutrophils, monocytes, lymphocytes, and platelets) showed no meaningful shifts, suggesting no alteration in overall immune cell distribution. Finally, the analysis of 96 inflammatory proteins by use of a proteomic approach confirmed that DPI did not impact systemic inflammation.

Despite the previous reported anti-inflammatory properties of both ASA and rivaroxaban, the primary mechanism of the clinical benefits demonstrated with DPI in reducing cardiovascular events, appears to be intensified coagulation prevention rather than immune modulation

Proposed mechanism of benefit of DPI in PAD

DPI with ASA and low-dose rivaroxaban benefits PAD patients by targeting both platelet activation and the prothrombotic state associated with atherosclerosis. ASA reduces platelet aggregation, while rivaroxaban inhibits factor Xa in the coagulation cascade. Together, these agents lower the risk of cardiovascular events.

Although DPI has shown clear benefits in reducing ischemic complications, the results presented in this thesis indicate that the observed benefits are unlikely to be mediated by improvements in vascular function or systemic inflammation. After 12 weeks of treatment, no changes were observed in macrovascular endothelial function (assessed by CAR), microvascular endothelial function (based on ET-1 levels), immune cell responsiveness (including cytokine production after ex vivo lipopolysaccharide stimulation), immune cell population counts, or systemic inflammation (based on inflammatory protein profiles) (*Chapter 7 and 8*). These findings suggest that the mechanism of benefit of DPI is most likely attributable to more enhanced anticoagulation. It is, however, possible that the duration of DPI treatment in these studies was insufficient to capture more subtle or delayed modulation of endothelial function or inflammatory pathways. Altogether, while the clinical efficacy of DPI is supported[47,49], its mechanistic pathways appear to be primarily anticoagulant in nature. Further research is warranted to explore whether longer-term treatment, more sensitive vascular assessments, or targeting specific patient subgroups could reveal additional vascular or immunomodulatory effects.

PART III the impact of antithrombotic therapy for secondary cardiovascular prevention in PAD patients

PAD is associated with a high risk of cardiovascular events, necessitating effective secondary cardiovascular prevention strategies. Antithrombotic therapy is central in reducing thrombotic complications in this population. Different classes of antithrombotic agents have been evaluated each with its own efficacy and safety profiles. This section provides an integrated overview of these therapies and highlights the latest evidence including insights from this thesis.

Antiplatelet therapy

Antiplatelet agents, such as ASA and clopidogrel, reduce thrombotic risk by inhibiting platelet aggregation, thereby reducing the risk of thrombus formation. This mechanism is central to preventing cardiovascular events in patients with atherosclerotic conditions, including PAD. Consequently, monotherapy with antiplatelet agents, is recommended for secondary cardiovascular prevention in symptomatic PAD.[68-69]

ASA monotherapy, at doses of 75–150 mg daily, has been widely studied in symptomatic PAD, and consistently shown to provide clinical benefit.[70] Clopidogrel, a P2Y12 inhibitor, at a dose of 75 mg daily, may be preferred due to its association with a lower incidence of major adverse cardiovascular events without a significant increase in bleeding risk, as demonstrated in the CAPRIE study.[71] This thesis supports a slight preference for clopidogrel in secondary cardiovascular prevention, but also identifies a gap in evidence regarding its bleeding risk relative to ASA monotherapy (*Chapter 9*).

As clopidogrel is a prodrug, activated by the Cytochrom P450 2C19 (CYP2C19) enzyme, its effectiveness may be influenced by genetic polymorphisms in CYP2C19. Theoretically, the effectiveness of clopidogrel could be further improved by CYP2C19 polymorphism-guided prescription (*Chapter 10*). Other P2Y12 inhibitors, such as Ticagrelor at a dose of 60-90 mg daily, did not demonstrate superiority over clopidogrel in trials and are therefore not recommended for routine secondary cardiovascular prevention in PAD.[72-74]

A combination of ASA plus clopidogrel in stable PAD patients has been evaluated across several studies, most notably the CHARISMA trial.[75] Dual antiplatelet therapy (DAPT) with ASA and clopidogrel did not yield a significant reduction in cardiovascular events. However, DAPT for at least one month may be considered

following certain peripheral revascularization procedures – i.e. endovascular stent implantation and below-the-knee bypass with a prosthetic graft – to further reduce the risk of cardiovascular and limb events, especially in high-risk patients.[68-69] This recommendation, however, is based on limited trial data and lacks statistical power to demonstrate differences in major cardiovascular outcomes.[76-77] This thesis supports clopidogrel monotherapy as a preferred antiplatelet agent and identifies opportunities for CYP2C19-guided therapy. It also confirms that routine DAPT in stable PAD is not warranted but may have a limited role in post-procedural settings.

Vitamin K antagonists

Vitamin K antagonists, such as acenocoumarol and fenprocoumon, inhibit the vitamin K-dependent synthesis of specific clotting factors within the liver (Factors II, VII, IX, and X) interrupting the coagulation cascade and lowering clot formation potential. Vitamin K antagonists are generally not recommended for long-term use in PAD due to an increased bleeding risk without substantial improvement in ischemic outcomes.[68-69,78-79] Although they have demonstrated some benefit in enhancing graft patency post-bypass surgery, particularly with autologous vein grafts[78], they require regular INR monitoring and carry a higher bleeding risk compared to single antiplatelet therapy. This thesis confirms that vitamin K antagonists increase bleeding risk relative to antiplatelet agents, without a corresponding reduction in major adverse cardiovascular events (*Chapter 9*) and reinforces their limited role in PAD management. VKAs may be used selectively in vascular surgery contexts but are otherwise obsolete in secondary cardiovascular prevention in PAD.

Direct oral anticoagulants

Direct oral anticoagulants (DOACs), including rivaroxaban, directly inhibit key enzymes in the coagulation cascade, such as Factor Xa or thrombin. Unlike vitamin K antagonists, direct oral anticoagulants have more predictable pharmacokinetics and pharmacodynamics, reducing the need for routine monitoring. Their efficacy in lowering thrombotic event rates in various cardiovascular conditions has supported their expanded use in PAD. In particular, low-dose rivaroxaban combined with ASA, referred to as DPI, is emerging as a promising option for secondary cardiovascular prevention in PAD patients, particularly post-revascularization.[68-69] Clinical trials such as COMPASS and VOYAGER PAD have demonstrated the efficacy of this regimen, though DPI carries a moderate bleeding risk, warranting caution.[47,49] This thesis provides an indirect comparison of this DPI regimen with currently recommended therapies (ASA and clopidogrel monotherapies) and finds that DPI shows high potential for secondary cardiovascular prevention following

revascularization. However, its benefit in stable PAD remains uncertain (*Chapter 9*). This finding aligns with a recent concise network meta-analysis.[80]

General advice regarding antithrombotic treatment in PAD

PAD patients represent a diverse group with varying degrees of ischemic burden, comorbid conditions, previous interventions, and bleeding risks. As a result, antithrombotic should be carefully tailored to individual patient profiles. For most patients with PAD, single antiplatelet therapy (preferably with clopidogrel over ASA) is sufficient for secondary cardiovascular prevention. For patients on clopidogrel, CYP2C19 genotyping may offer insights into treatment optimization, especially post-revascularization. This personalized approach may improve efficacy while addressing variability in drug response due to genetic factors. In specific high-risk patients following interventions, short-term DAPT may be considered. However, stronger evidence supports the long-term use of low-dose rivaroxaban with ASA in reducing cardiovascular and limb events after revascularization. DPI is especially indicated in high-risk patients, though balancing benefits with bleeding risk remains crucial. VKAs are generally discouraged for long-term use due to bleeding risks and minimal ischemic benefit but may retain a limited role in maintaining graft patency after bypass surgery.

New insights from this thesis include (*Chapter 9 and 10*):

- Confirmation that clopidogrel is more effective than ASA in the secondary cardiovascular prevention of stable PAD, and that DPI presents a promising new strategy with also greater potential than ASA monotherapy.
- Indirect evidence that clopidogrel offers similar safety to ASA and may be further optimized in terms of efficacy by genotype-guided use.
- Recognition that DAPT provides no consistent benefit in stable PAD or for long-term prevention after revascularization.
- Highlighting the absence of benefit of VKAs in secondary cardiovascular prevention, coupled with an elevated bleeding risk.
- Identification of DPI as a superior strategy after peripheral revascularization, though associated with a higher bleeding risk.

PART IV future perspectives

Building on the findings of this thesis, the following section outlines future directions for improving vascular health in COVID-19 survivors and refining antithrombotic therapy within the framework of secondary cardiovascular prevention in PAD patients.

The first part of this thesis focused on vascular health in the post-COVID-19 context. Although observational evidence suggests a prolonged inflammatory and prothrombotic state following COVID-19 infection, the clinical consequences, including the hypothesized heightened cardiovascular risk, remain largely unknown. This highlights the need for ongoing cardiovascular monitoring in COVID-19 survivors, ideally using standardized assessments of vascular health such as endothelial function testing (i.e. CAR), measurement of biomarkers of endothelial activation (i.e. ET-1, vWF) and general secondary prevention parameters It is advisable to integrate these evaluations into existing cardiovascular risk management programs, including routine blood pressure monitoring, lipid profiling, and other relevant risk factor assessments.

Additionally, better characterization of the relationship between inflammation, endothelial dysfunction, and long COVID symptoms remains an important research priority. The CORona Follow Up (CORFU) study, a prospective, multi-cohort investigation, is expected to provide further insight into these unresolved questions.[81]

Subsequent parts of this thesis addressed the impact and application of antithrombotic drugs in PAD patients. One objective was to evaluate vascular health, in terms of endothelial function and inflammation, in relation DPI. As DPI did not alter macrovascular or microvascular endothelial function, nor affect systemic inflammation after three months, it is hypothesized that its clinical benefit is primarily mediated through inhibition of coagulation and thrombosis. Nonetheless, the possibility of more subtle or delayed modulation of endothelial function or inflammatory pathways has not been excluded. A deeper understanding of the longer-term effects of DPI on endothelial function and inflammation would contribute to a more complete understanding of its benefits in preventing ischemic events and could be obtained by repeated endothelial function and inflammation measurements over a six to twelve month period.

This thesis also reviewed available evidence on antithrombotic therapy in PAD, providing a comparative analysis of available antithrombotic regimens for secondary cardiovascular prevention. Nevertheless, certain antithrombotic regimens lack

adequate trial evidence. It remains unclear whether the observed benefits of the emerging combination of low-dose rivaroxaban with ASA relative to ASA monotherapy, also apply when compared to the preferred clopidogrel monotherapy in stable PAD. A direct comparison in a randomized controlled trial, powered for major adverse cardiovascular events, is required to address this question.

Further, there are high levels of uncertainty regarding treatment recommendations for patient's post-peripheral revascularization, who may benefit from DAPT, although evidence is limited. A hypothesis is that DPI may offer superior protection against graft occlusion and limb ischemia compared to DAPT. This hypothesis is supported by indirect evidence in this thesis, but this requires confirmation including safety analyses addressing bleeding risks. High-quality trials with sufficient power to detect differences in major cardiovascular outcomes and major bleedings, comparing DAPT with clopidogrel monotherapy for secondary cardiovascular prevention following peripheral vascular interventions, as well as head-to-head comparisons of DAPT and DPI, are warranted. The ongoing CLEAR-PATH trial, a randomized, placebo-controlled, double-blind study comparing clopidogrel to DAPT (clopidogrel/ASA), may provide valuable insights into the former comparison.[82] The latter, however, remains unexamined at present. This thesis supports that antiplatelet monotherapy should be sufficient for most PAD patients in long-term secondary cardiovascular prevention, with clopidogrel being preferable to ASA. However, it is essential to investigate the potential of a personalized approach guided by CYP2C19 genotyping. The ongoing GENPAD trial is expected to provide valuable evidence on the efficacy, safety, and cost-effectiveness of a genotype-guided approach versus standard clopidogrel in PAD patients (*Chapter 11*).

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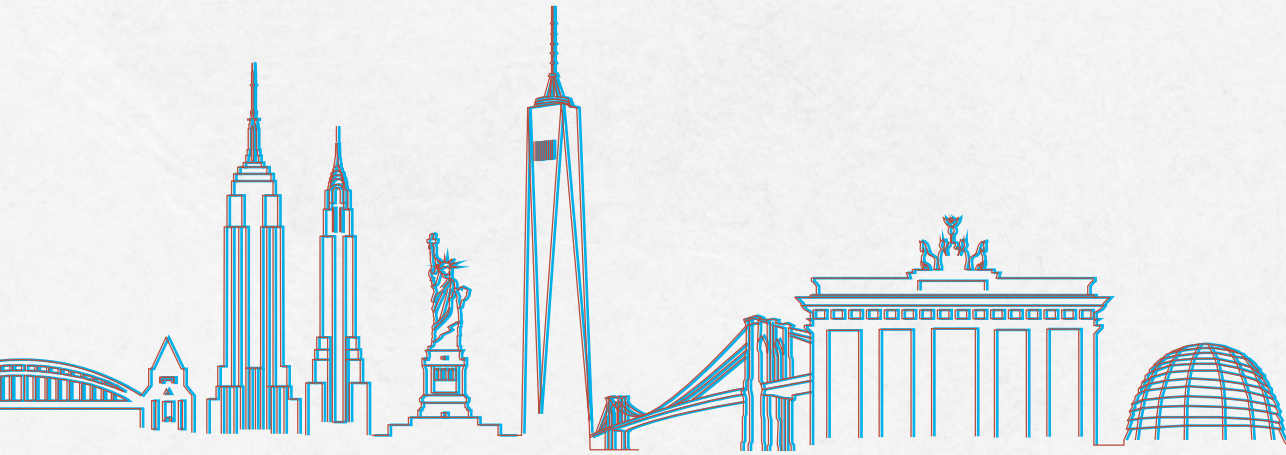

Chapter 13

Nederlandse samenvatting

Summary

Research data management

List of publications

PhD portfolio

Curriculum Vitae

Nederlandse samenvatting

Het algemene doel van dit proefschrift was 1) het verkennen van het gebruik van carotid artery reactivity (CAR) testing als een eenvoudige, niet-invasieve methode om de endotheelfunctie te beoordelen, 2) het evalueren van het effect van dual-pathway inhibitie (DPI), als een veelbelovende antitrombotische behandeling, op endotheelfunctie, en 3) het bieden van een evidence-based overzicht van antitrombotische therapieën bij perifeer arterieel vaatlijden (PAD) om de selectie van optimale behandelingen voor secundaire preventie in de klinische praktijk te ondersteunen. In Hoofdstuk 1 werd een algemene inleiding gegeven over de achtergrond en het doel van dit proefschrift. We beschreven de klinische presentatie, pathofysiologie en secundaire preventiemaatregelen van PAD en de rol van endotheeldysfunctie. Daarnaast werden meetinstrumenten voor vasculaire gezondheid beschreven.

In **Hoofdstuk 2**, onderzochten we de constructvaliditeit en reproduceerbaarheid van drie draagbare echografieapparaten voor het meten van de diameter van de halsslagader. *In vitro* experimenten toonden een uitstekende overeenkomst met een high-end echografieapparaat. De apparaten hadden minimale intra-observervariabiliteit. *In vivo* vertoonden de apparaten uitstekende tot goede inter-observer reproduceerbaarheid. Ondanks kleine video-compatibiliteitsproblemen blijkt dat draagbare echografieapparaten betrouwbaar zijn voor klinische metingen van de halsslagaderdiameter.

In **Hoofdstuk 3**, onderzochten we de effecten van COVID-19 op endotheelfunctie, stolling en inflammatie, drie maanden na herstel. Hoewel geen macrovasculaire dysfunctie werd waargenomen, bleven markers van endotheelactivatie, stolling, en inflammatie verhoogd bij veel patiënten. Deze bevindingen wijzen op aanhoudende endotheel- en inflammatieactivatie, wat kan bijdragen aan microvasculaire schade en chronische cardiovasculaire risico's. De studie benadrukt de noodzaak van verder onderzoek naar de vasculaire implicaties van post-COVID-19.

In **Hoofdstuk 4**, onderzochten we de lange-termijneffecten van COVID-19 op de vasculaire gezondheid, 18 maanden na infectie. Er werd geen toename van macrovasculaire dysfunctie vastgesteld, maar de procentuele CAR-reactiviteit nam af, wat wijst op verminderde vasculaire relaxatie. Markers van microvasculaire dysfunctie zoals endothelin-1 normaliseerden, maar de niveaus van von Willebrand-factor, die endotheelbeschadiging aangeven, bleven hoog. Ontstekingsmarkers IL-1RA en IL-18 keerden terug naar normale waarden, terwijl IL-6 toenam, wat duidt

op chronische laaggradige inflammatie. Bovendien namen trombinevorming en markers van de extrinsieke stollingscascade toe, wat wijst op een aanhoudende prothrombotische staat. Deze bevindingen suggereren mogelijke cardiovasculaire risico's op lange termijn bij COVID-19-overlevers

In **Hoofdstuk 5**, onderzochten we de effecten van het ChAdOx1 COVID-19-vaccin op bloedstolling en ontsteking. Bij 40 zorgmedewerkers werden markers gemeten vóór en 1–2 dagen na vaccinatie. De resultaten lieten geen activatie van het bloedstollingssysteem zien, wat duidt op geen direct risico op bloedstolsels. Wel stegen de niveaus van interleukine-6 (IL-6), een ontstekingsmarker, aanzienlijk, wat wijst op een inflammatiereactie. Hoewel sommige deelnemers milde bijwerkingen rapporteerden, werden er geen gevallen van trombose of embolieën waargenomen. Dit hoofdstuk suggereert dat het ChAdOx1-vaccin kortdurende ontsteking veroorzaakt zonder invloed op de bloedstolling.

In **Hoofdstuk 6**, beschrijven we een onderzoeksprotocol, naar het verbeteren van de endotheelfunctie bij PAD patiënten door de combinatie van lage doses rivaroxaban en aspirine. De studie hypothetiseert dat DPI vasculaire ontsteking vermindert en de endotheelfunctie verbetert, mogelijk een verklaring biedend voor de lagere sterfte en cardiovasculaire event incidentie die in eerdere studies zijn waargenomen. De studie omvat twee groepen – matige en ernstige PAD-patiënten – die een 3-maandse DPI-behandeling ondergaan na een maand aspirinegebruik. Primaire uitkomsten zijn verbeterde endotheelfunctie, gemeten via CAR en endotheline-1-niveaus.

In **Hoofdstuk 7**, worden de resultaten beschreven van de in **Hoofdstuk 6** behandelde studie. Na 12 weken behandeling werden geen significante verbeteringen waargenomen in de endotheelfunctie, gemeten via CAR en plasma-endotheline-1-niveaus. DPI verlaagde echter wel markers van stollingsactiviteit, wat de anticoagulerende effecten bevestigde. Ondanks deze bevindingen verklaarde de endotheelfunctie niet de klinische voordelen van DPI die in eerdere onderzoeken zijn waargenomen, wat suggereert dat andere mechanismen kunnen bijdragen aan de effectiviteit ervan.

In **Hoofdstuk 8**, onderzochten we of DPI met lage doses aspirine en rivaroxaban invloed heeft op immuunreacties bij patiënten met coronaire hartziekte (CAD) of PAD. DPI vermindert cardiovasculaire sterfte door bloedstolsels te voorkomen, maar de effecten op ontstekingen waren onduidelijk. De resultaten toonden geen significante veranderingen in immuuncelverdeling, responsiviteit of ontstekingsniveaus

gedurende drie maanden. De bevindingen suggereren dat de klinische voordelen van DPI voortkomen uit verbeterde antistolling en niet uit ontstekingsremmende effecten.

In **Hoofdstuk 9**, beschrijven we een systematische review en netwerk meta-analyse van antitrombotische therapieën bij patiënten met symptomatisch PAD. We onderzoeken de effectiviteit van behandelingen bij het voorkomen van grote cardiovasculaire en been events. Analyse van 24 gerandomiseerde gecontroleerde studies met 48.759 patiënten toont aan dat clopidogrel, ticagrelor, aspirine plus ticagrelor, en aspirine plus lage dosis rivaroxaban effectiever zijn dan aspirine alleen voor het verminderen van MACE. Aspirine met rivaroxaban verhoogt echter ook het bloedingsrisico. Clopidogrel wordt aanbevolen als eerstelijnstherapie voor de meeste PAD-patiënten, terwijl aspirine met rivaroxaban geschikt kan zijn voor langdurige preventie na vasculaire interventies, rekening houdend met bloedingsrisico's.

In **Hoofdstuk 10**, beschrijven we een systematische review en meta-analyse welke de invloed van het CYP2C19-genotype op klinische uitkomsten bij patiënten met CAD, cerebrovasculair accident (CVA) en PAD onderzoekt. Clopidogrel, veelgebruikt ter preventie van trombotische events, wordt geactiveerd via CYP2C19. Dragers van CYP2C19-loss-of-function (LOF) allelen kunnen verminderde effectiviteit ervaren. Genotype-geleide therapie verlaagt significant het risico op cardiovasculaire events bij CAD-patiënten, vooral na percutane intraluminale interventies (PCI). Bij CVA-patiënten met LOF-allelen is het risico op cardiovasculaire events en recidief CVA hoger. Bewijs voor PAD is echter beperkt. De studie benadrukt de voordelen van gepersonaliseerde behandelingen op basis van genetische profilering.

In **Hoofdstuk 11**, wordt een protocol voor een randomized controlled trial beschreven die de effectiviteit en kosteneffectiviteit onderzoekt van CYP2C19-genotype-gestuurde antitrombotische therapie vergeleken met standaard clopidogrelbehandeling bij patiënten met PAD. Deze gerandomiseerde studie omvat 2.276 PAD-patiënten, verdeeld in standaard clopidogrel- en genotype-gestuurde behandelingsgroepen. Bij de laatste wordt de therapie aangepast op basis van genetische tests: normal metabolizers blijven clopidogrel gebruiken, intermediate metabolizers krijgen een dubbele dosering, en poor metabolizers worden overgezet op alternatieve medicatie. De primaire uitkomst meet cardiovasculaire en been events, terwijl secundaire uitkomsten zich richten op bloedingscomplicaties en kosteneffectiviteit. De resultaten kunnen farmacogenetische testen ondersteunen als onderdeel van de routinezorg voor PAD.

Summary

The general aim of this thesis was 1) to explore the use of carotid artery reactivity (CAR) testing as a simple, non-invasive method to assess endothelial function, 2) to evaluate the effect of dual-pathway inhibition (DPI) as a promising antithrombotic regimen on endothelial function, and 3) to provide an evidence-based overview of antithrombotic therapies in peripheral arterial disease (PAD) to guide the selection of optimal treatments for secondary prevention in clinical practice. In *Chapter 1*, a general introduction on the background and rationale of this thesis was provided. We described the clinical presentation, pathophysiology and secondary prevention measures of PAD and the role of endothelial dysfunction. In addition, vascular health measures were described.

In *Chapter 2*, the construct validity and reproducibility of three handheld ultrasound devices for measuring carotid artery diameter were assessed. *In vitro* experiments demonstrated excellent agreement with a high-end ultrasound system. The devices showed minimal intra-observer variability. *In vivo*, devices displayed excellent-to-good inter-observer reproducibility. Despite minor video compatibility challenges, the findings suggest that handheld ultrasound devices are reliable for clinical carotid artery diameter measurements.

In *Chapter 3*, the effects of COVID-19 on endothelial function, coagulation, and inflammation were investigated, three months after recovery. Despite no observed macrovascular dysfunction, markers of endothelial activation, coagulation activity, and inflammation, remained elevated in many patients. These findings suggest persistent endothelial and inflammatory activation, potentially contributing to microvascular damage and chronic cardiovascular risks. The study emphasizes the need for further research into the vascular implications of post-COVID-19 conditions.

In *Chapter 4*, the long-term vascular effects of COVID-19, 18 months post-infection, were analyzed. Results revealed no increased incidence of macrovascular dysfunction, but CAR percentage decreased, indicating reduced vascular relaxation. Microvascular endothelial dysfunction markers like endothelin-1 normalized, while von Willebrand factor levels, indicating endothelial damage, remained elevated. Inflammatory markers IL-1RA and IL-18 returned to normal, but IL-6 levels increased, suggesting chronic low-grade inflammation. Additionally, thrombin generation and extrinsic coagulation pathway markers increased, pointing to a persistent prothrombotic state. These findings suggest potential long-term cardiovascular risks in COVID-19 survivors.

In *Chapter 5*, we investigated the effects of the ChAdOx1 COVID-19 vaccine on blood coagulation and inflammation. Conducted on 40 healthcare workers, it measured markers before and 1–2 days after vaccination. Results showed no activation of the blood coagulation system, suggesting no immediate risk of clotting. However, levels of interleukin-6 (IL-6), a marker of inflammation, increased significantly, indicating an inflammatory response. While some participants reported mild side effects, no cases of thrombosis or thromboembolism occurred. The chapter highlights that the ChAdOx1 vaccine may trigger short-term inflammation without affecting blood clotting mechanisms.

In *Chapter 6*, a research protocol is outlined, investigating whether combining low-dose rivaroxaban and aspirin improves endothelial function in patients with PAD. The study hypothesizes that DPI reduces vascular inflammation and improves endothelial function, potentially explaining lower mortality and event rates observed in previous studies. The trial involves two groups—moderate and severe PAD patients—who undergo a 3-month treatment with DPI after a 1-month aspirin run-in period. Primary outcomes include improved endothelial function, measured through CAR and endothelin-1 levels.

In *Chapter 7*, the results of the in *Chapter 6* described studies are described. After 12 weeks of treatment, no significant improvements were observed in endothelial function, measured through CAR and plasma endothelin-1 levels. However, DPI did reduce markers of coagulation activity, confirming its anticoagulant effects. Despite these findings, endothelial function did not explain the clinical benefits of DPI observed in previous trials, indicating other mechanisms may contribute to its efficacy.

In *Chapter 8*, we investigated whether DPI using low-dose aspirin and rivaroxaban impacts immune responses in patients with coronary artery disease (CAD) or PAD. DPI is known to reduce cardiovascular mortality by preventing blood clots, but its effects on inflammation were unclear. Results showed no significant changes in immune cell distribution, responsiveness, or inflammation levels over three months. The findings suggest that the clinical benefits of DPI are due to enhanced anticoagulation rather than anti-inflammatory effects.

In *Chapter 9*, a systematic review and network meta-analysis is presented comparing antithrombotic therapies for patients with symptomatic PAD. We evaluate the effectiveness of different treatments in preventing major cardiovascular and limb events. The analysis of 24 randomized controlled trials with 48,759 patients

shows that clopidogrel, ticagrelor, aspirin plus ticagrelor, and aspirin plus low-dose rivaroxaban are more effective than aspirin alone for reducing MACE. However, aspirin combined with low-dose rivaroxaban also increases bleeding risks. Clopidogrel is recommended as first-line therapy for most PAD patients, while aspirin with rivaroxaban may be suitable for long-term prevention after vascular interventions, considering bleeding risks.

In *Chapter 10*, a systematic review and meta-analysis is presented examining the impact of the CYP2C19 genotype on clinical outcomes in patients with CAD, stroke, and PAD. Clopidogrel, widely used for preventing thrombotic events, relies on CYP2C19 activation. Carriers of CYP2C19 loss-of-function (LOF) alleles may experience reduced efficacy. Results indicate that genotype-guided therapy significantly lowers major cardiovascular events in CAD patients, particularly post-PCI. Stroke patients with LOF alleles face higher risks of cardiovascular events and recurrent strokes, while evidence in PAD is insufficient. The chapter highlights the potential benefits of personalized treatment strategies based on genetic profiling.

In *Chapter 11*, a randomized controlled trial protocol is outlined investigating the efficacy and cost-effectiveness of CYP2C19 genotype-guided antithrombotic therapy compared to standard clopidogrel treatment in patients with PAD. This randomized trial includes 2,276 PAD patients, dividing them into standard clopidogrel or genotype-guided treatment groups. The latter adjusts therapy based on genetic testing: normal metabolizers continue clopidogrel, intermediate metabolizers receive double dosage, and poor metabolizers are switched to alternative medication. The primary outcome assesses cardiovascular and limb events, while secondary outcomes focus on bleeding complications and cost-effectiveness. Results could support the integration of pharmacogenetic testing into routine PAD care.

Research data management

This thesis is based on the results of human studies, which were conducted in accordance with relevant national and international legislation and regulations, guidelines, codes of conduct and Radboudumc policy. The recognized Medical Ethics Review Committee 'METC Oost-Nederland' has given approval to conduct these studies (file numbers: NL74101.091.20, NL77323.091.21, NL72452.091.19, NL 79727.091.21, NL75567.091.20). The institutional ethical review committee CMO Radboudumc, Nijmegen, the Netherlands has given approval to conduct one study (CMO Radboudumc dossier number: 2020-6700).

All projects are stored on the Radboudumc department server.

The studies in chapter 3-5 and 11 were co-funded by the Netherlands Organisation for Health Research and Development. The study in chapter 6-7 was co-funded by Bayer B.V.

In the studies in chapter 2-5 and 7-8 written informed consent was obtained from all participants to collect and process their data for this research project. The original of the informed consent form was stored in the department archive of the participating center. The participant was provided with a copy or a second original. In chapter 2-5 and 7 paper case report forms were collected. The paper data were stored in the department archive of the participating center. The department archives of the participating centers were: for chapter 2 Radboudumc, Nijmegen the Netherlands, department of physiology; for chapter 3-4 Bernhoven, Uden, the Netherlands, secretariat of the department of surgery; for chapter 5 Radboudumc, Nijmegen the Netherlands, surgical research department; for chapter 7 Radboudumc, Nijmegen the Netherlands, surgical research department and Rijnstate, Arnhem, the Netherlands, research department of vascular surgery. In chapter 8 electronic case report forms were collected by use of a standardized note in the electronic patient file EPIC of the Radboudumc. All case report forms were entered into the computer by use of Castor EDC. Data management and monitoring were also performed within Castor EDC. An audit trail was incorporated to provide evidence of the activities that has altered the original data.

The privacy of the participants in this study is warranted by use of encrypted and unique individual subject codes. This code correspondents with the code on the patient- and physicians' booklets. The code was stored separately from the

study data. Data were converged from Castor EDC to SPSS (SPSS Inc., Chicago, Illinois, USA).

The patient data for the analyses of the studies as presented in chapter 2-8 is stored on the department server in SPSS format: chapter 2 project USCA, chapter 3-4 project COVAS, chapter 5 project COCOS, chapter 6-7 project DUAL-PAD, chapter 8 project DUALCAD.

The data will be saved for 15 years after termination of the study (chapter 2, December 24th 2021; chapter 3-4, April 1st 2022; Chapter 5, May 31st 2021; Chapter 6-7, December 31st 2021; Chapter 8, July 1st 2022). Using these patient data in future research is only possible after a renewed permission by the patient as recorded in the informed consent.

The data underlying chapter 2-8 are not suitable for reuse because consent was only obtained for the corresponding studies and direct follow-up studies and will be archived with closed access for 15 years in DACs of the Radboud Data Repository after termination of the study (DOIs: 10.34973/pw48-cb80, 10.34973/41b6-qj83, 10.34973/m86t-yr24, 10.34973/px2a-c245, 10.34973/2pjr-5d62).

List of publications

Dominique PMSM Maas, Loes H Willems, Josephine Kranendonk, Cornelis Kramers, Michiel C Warlé. Impact of CYP2C19 genotype status on clinical outcomes in patients with symptomatic coronary artery disease, stroke, and peripheral arterial disease. Drugs. 2024 Oct;84(10):1275-1297. doi: 10.1007/s40265-024-02076-7.

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PhD portfolio of Loes Willems

Department: Surgery
PhD period: 01/01/2020 – 23/06/2025
PhD Supervisors: Ass. Prof. Dr MC Warlé, Prof. Dr. DHJ Thijssen, Prof. Dr. MMPJ Reijnen

Training activities	Hours
Courses	
• RIHS - Introduction course for PhD candidates (2020)	15.00
• RIHS PhD introduction course (2020)	21.00
• RU - Scientific Writing for PhD candidates (2020)	84.00
• At-home training of Laparoscopic Suturing (2020)	18.00
• Basic Life Support (2021)	7.00
• RU - Statistics for PhD's by using SPSS (2021)	56.00
• Radboudumc - Scientific integrity (2022)	20.00
• Vervolgcursus Duplexsonografie, perifere vaten (2024)	42.00
• Basiscursus Duplexsonografie (2025)	56.00
• Basiscursus regelgeving en organisatie voor klinisch onderzoekers (2020)	42.00
Seminars	7.00
• Vascular Rounds Nijmegen (2021)	7.00
• Kennissessie Wetenschap voor de Praktijk (2021)	7.00
• Vascular Rounds Nijmegen (2022) – oral presentation	100.00
• Radboud Research Rounds vascular damage (2020-2022) – 3x oral presentation	6.00
• AMC vascular rounds (2022) – oral presentation	6.00
• Vascular Rounds Nijmegen (2023)	6.00
• Research round UMCU (2023) – oral presentation	
Conferences	21.00
• Division of Vascular and Endovascular Surgery Conference (2021) – poster presentation	84.00
• ESC congress 2021 (2021) – oral presentation	42.00
• ARTERY conference (2021) – oral presentation	56.00
• CX symposium 2022 (2022) – oral presentation	14.00
• Division of Vascular and Endovascular Surgery Conference (2022) – oral presentation	56.00
• SCVS symposium (2023) – oral presentation	7.00
• Vaatsymposium Helios Gefäßzentrums Berlin-Buch (2024)	14.00
• Vaatsymposium Helios Gefäßzentrums Berlin-Buch (2025) – oral presentation	7.00
• Vaatsymposium Helios Gefäßzentrums Berlin-Brandenburg (2025)	
Teaching activities	
Lecturing	7.00
• Lecture clinical research for honors program students (2021)	7.00
• Lecture research at the department of vascular surgery VECTOR (2022)	7.00
• Lecture clinical research for honors program students (2022)	
Supervision of internships / other	84.00
• Supervision of medical research internship of 3 students (2020)	56.00
• Supervision of 1 biomedical master research internship (2022)	28.00
• Supervision of 1 medical research internship (2022)	
Total	**990.00**

Curriculum Vitae

Loes Henrieke Willems werd geboren op 19 mei 1993 te Mook, Nederland. In 2011 behaalde zij haar Gymnasium-diploma cum laude aan het Stedelijk Gymnasium te Nijmegen. Aansluitend begon zij aan de studie Geneeskunde aan de Radboud Universiteit Nijmegen. Na het afronden van haar bachelor in 2014 vervolgde Loes haar opleiding met de master Geneeskunde. In mei 2018 rondde Loes haar master succesvol af en behaalde zij haar artsdiploma.

Na haar afstuderen werkte Loes als arts-assistent chirurgie in het Radboudumc te Nijmegen. Haar interesse in wetenschappelijk onderzoek bracht haar vervolgens naar de afdeling Vaatchirurgie, waar zij van januari 2020 tot juli 2022 als PhD-kandidaat werkte onder begeleiding van Dr. M.C. Warlé, Prof. Dr. D.H.J. Thijssen en Prof. Dr. M.M.P.J. Reijnen. Tijdens haar promotieonderzoek heeft Loes zes klinische trials opgezet en gecoördineerd, waaronder een grote gerandomiseerde gecontroleerde multicenter trial waarbij ziekenhuizen uit heel Nederland betrokken waren. Daarnaast heeft zij als co-applicant een subsidie verkregen bij ZonMw binnen het COVID-19 programma "Science for Professional Practice". Hiermee heeft zij bijgedragen aan de versterking van zowel het wetenschappelijk onderzoek als de klinische samenwerking tussen verschillende instellingen. In het kader van haar promotietraject bracht Loes enkele maanden door aan New York University Langone Health (april 2022 – juli 2022), waar zij onder supervisie van Prof. Dr. T. Maldonado haar onderzoek verder verdiepte en internationale ervaring opdeed binnen de vaatchirurgie. Haar resultaten presenteerde zij op zowel nationale als internationale congressen.

Na deze onderzoeksjaren keerde Loes in augustus 2022 terug naar het klinisch werkveld als arts-assistent chirurgie en spoedeisende hulp in Ziekenhuis Gelderse Vallei te Ede waar zij tot augustus 2023 werkzaam was. Na afloop van deze functie koos zij ervoor om gedurende enkele maanden als organisatie-, advies- en projectmedewerker werkzaam te zijn bij Platform Talent voor Technologie, waar zij zich richtte op het in kaart brengen en verbeteren van het technologieonderwijs in Nederland, evenals op het analyseren van de aansluiting tussen opleidingen en de arbeidsmarkt binnen het vakgebied cybersecurity. Deze tijdelijke stap bood haar de mogelijkheid om zich voor te bereiden op haar geplande emigratie naar Duitsland.

In maart 2024 verhuisde Loes naar Berlijn, waar zij sinds april 2024 werkzaam is als arts in opleiding tot vaatchirurg in Helios Klinikum Berlin-Buch.

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