Defining atherothrombotic risk in peripheral artery disease

DEFINING ATHEROTHROMBOTIC RISK IN PERIPHERAL ARTERY DISEASE

Bram Kremers

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CHAPTER 1

GENERAL INTRODUCTION

PERIPHERAL ARTERY DISEASE

Cardiovascular disease (CVD) is a universal term for disorders of the heart and blood vessels. Most CVDs are caused by atherosclerosis, including coronary artery disease (CAD), cerebrovascular disease (CeVD), and peripheral artery disease (PAD), and are taking almost 18 million lives each year [1]. PAD is a progressive disorder characterized by atherosclerotic narrowing of large and medium-sized arteries, other than coronary arteries or arteries supplying the brain. PAD is, in most cases, located in the lower extremities, although upper extremities can also be affected. Symptoms of lower extremity PAD may be defined using the Rutherford classification (R0-6) or the Fontaine classification (FI-IV). PAD is diagnosed using the ankle-brachial index (ABI) where the systolic blood pressure at the ankle is divided by the systolic blood pressure in the arm [2]. An overview of the Rutherford/Fontaine classification in relation to symptomatology is shown in *Table 1*, and ABI values in relation to diagnosis in *Table 2.* In the case of a non-compressible artery, the toe-brachial index is alternatively used where a value below 0.7 is abnormal [3].

Rutherford stage	Fontaine stage	Symptoms
0	I	Asymptomatic
1	IIa	Mild claudication
2	IIa/IIb	Moderate claudication
3	IIb	Severe claudication
4	III	Rest pain
5	IV	Minor tissue loss
6	IV	Major tissue loss

Table 1: Overview of the Rutherford/Fontaine classification with respective symptoms.

ABI	Interpretation
>1.40	Non-compressible
1.00-1.40	Normal
0.91-0.99	Equivocal or borderline PAD
0.41-0.90	Mild to moderate PAD
<0.40	Severe PAD

Table 2: Ankle-brachial index (ABI) with appropriate interpretation according to the AHA/ACC Guideline on the Management of Patients with Lower Extremity Peripheral Artery Disease.

Prevention of cardiovascular events in patients with PAD is based on reducing the cardiovascular risk using medical treatment strategies, combined with lifestyle changes such as structured exercise, dietary changes, and smoking cessation. Medical treatment strategies include the use of antiplatelet agents (aspirin or clopidogrel) for all PAD patients as well as the use of lipid-lowering drugs to lower serum cholesterol levels [3].

ATHEROSCLEROSIS

Underlying PAD is the process of atherosclerosis, characterized by a chronic inflammatory process where plaques build up in arteries resulting in a gradual narrowing of the lumen *(Figure 1)*. The vascular wall homeostasis is mainly regulated by the endothelium, with endothelial cells maintaining a normal vascular tone and low levels of oxidative stress. Vascular permeability, platelet and leukocyte adhesion, and aggregation are also actively regulated by the endothelium [4]. The process of atherosclerosis is initiated when the endothelium becomes dysfunctional. An important mechanism is the disruption of the nitric oxide balance by free radicals, inducing increased permeability and expression of adhesion molecules, synthesis of pro-inflammatory markers, and oxidative stress [5]. Cardiovascular risk factors including hyperlipidemia, smoking, diabetes, and hypertension are associated with such an imbalance in the nitric oxide homeostasis [4,6]. Following endothelial dysfunction, fatty streaks are formed via the trapping of lipoproteins in the lesion site. Low-density lipoproteins (LDL) pass the endothelium through transcytosis and increased concentrations of LDL within the intimal layer lead to cell oxidation [7]. LDL particles further induce endothelial dysfunction and the expression of more cell adhesion molecules, leading to the adhesion of monocytes and lymphocytes. Infiltrated monocytes mature into macrophages and absorb lipids to become foam cells that characterize an early fatty streak lesion [8]. Foam cell formation is followed by migration of vascular smooth muscle cells from the medial layer towards the intima, leading to a collagen-enriched fibrous cap. The cap thickness is important in defining plaque vulnerability to rupture, along with the necrotic core formation where dead macrophages accumulate. Although the fibrous cap prevents the necrotic core from getting in contact with the circulating blood, the cap can rupture, and the ensuing interaction between the procoagulant plaque matter and the blood results in thrombus formation. Thrombi can, however, not only form after plaque rupture but also as a result of plaque erosion, which is characterized by the local absence of endothelium, minimal inflammation, and large amounts of vascular smooth muscle cells in the intimal layer [9].

HEMOSTASIS

Primary and secondary hemostasis are responsible for pathological thrombus formation as a result of plaque rupture or erosion. Hemostasis is a physiological process to stop bleeding and prevent infection at sites of injury while maintaining normal blood flow elsewhere in the circulation. Upon damage to the endothelial layer, the subendothelial matrix is exposed to the blood, activating the coagulation system to initiate clot formation [10].

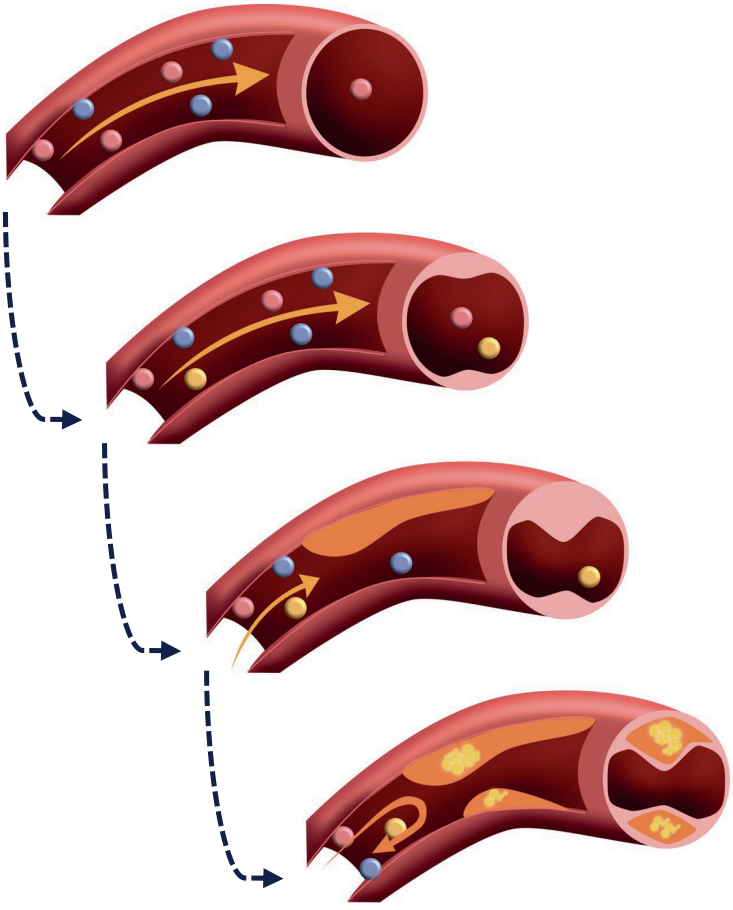

Figure 1: Atherosclerotic plaque formation leading to gradual narrowing of the lumen.

Primary hemostasis

In primary hemostasis, blood platelets become activated, aggregate, and form a platelet plug adhering to the site of injury. Multiple receptors on platelet surfaces are responsible for these actions, including receptor glycoprotein (GP) Ib which binds to von Willebrand factor [11]. This binding facilitates the contact between the GPVI receptor and collagen, inducing the release of secondary platelet agonists thromboxane A2 and adenosine diphosphate (ADP). These agonists further contribute to platelet activation together with locally produced thrombin. Together with other agonists, their downstream signaling pathways synergize to form a platelet plug. To prevent unwanted platelet activation and platelet plug formation, antiplatelet agents are used in patients with PAD. Specifically, aspirin is used to inhibit thromboxane A2 production [12], while clopidogrel inhibits the effects of ADP by irreversibly blocking the P2Y12 receptor [13].

Secondary hemostasis

In secondary hemostasis, a cascade of serine proteases results in the conversion of fibrinogen to fibrin monomers. Fibrin formation following thrombin cleavage occurs simultaneously with platelet aggregation. As in primary hemostasis, vascular wall injury causes the blood to be exposed to extravascular tissue which is rich in tissue factor (TF). TF forms a complex with factor VIIa which activates factors X and IX. This extrinsic pathway then proceeds with the activation of thrombin leading to not only fibrin formation but also a positive feedback loop to factor XI to propel the coagulation as shown in *Figure 2* [10]. Currently, direct oral anticoagulants (DOACs) are most widely used to treat venous thromboembolism and patients with atrial fibrillation, amongst others [14,15]. These DOACs downregulate the coagulation cascade by inhibiting factor Xa or thrombin itself. In the past, there was no place for anticoagulant therapy in patients with PAD, apart from patients undergoing a below-the-knee bypass [16]. Recently, however, it was shown that the addition of a low dose of an anticoagulant, rivaroxaban, combined with aspirin improves cardiovascular risk prevention in PAD patients [17]. As anticoagulant therapy appears beneficial in treating arterial and venous thrombosis, common pathophysiological pathways may be involved in these two different diseases. Based on patient characteristics, various risk factors of arterial thrombosis such as obesity, diabetes, hypertension, and hyperlipidemia, also promote venous thrombosis [18]. Evidence of common pathophysiological pathways is however scarce, although endothelial dysfunction may play a role as the presence of microalbuminuria, a marker for dysfunction, increases the risk of both arterial and venous thrombosis [19,20].

CLINICAL PROBLEM

In contrast to coronary and cerebrovascular disease, PAD remains an underappreciated condition despite being extremely prevalent as 236 million people worldwide are affected by the disease [21], especially populations aged 80 years and older [22]. Patients diagnosed with PAD not only suffer from daily symptoms as described by the Rutherford criteria, but they are also at an increased risk of cardiovascular events and death. PAD patients have a 70% increased risk of cardiovascular events and 80% increased risk of death as compared to people without PAD [23]. The addition of anticoagulant drugs or anti-inflammatory agents [24,25] to the regular medication strategies reduces the cardiovascular risk in PAD patients. The underlying pathophysiological pathways driving the increased risk, however, remain to be elucidated. Due to limited knowledge of these pathways, clinicians are hindered in improving their management strategies to prevent cardiovascular events and mortality.

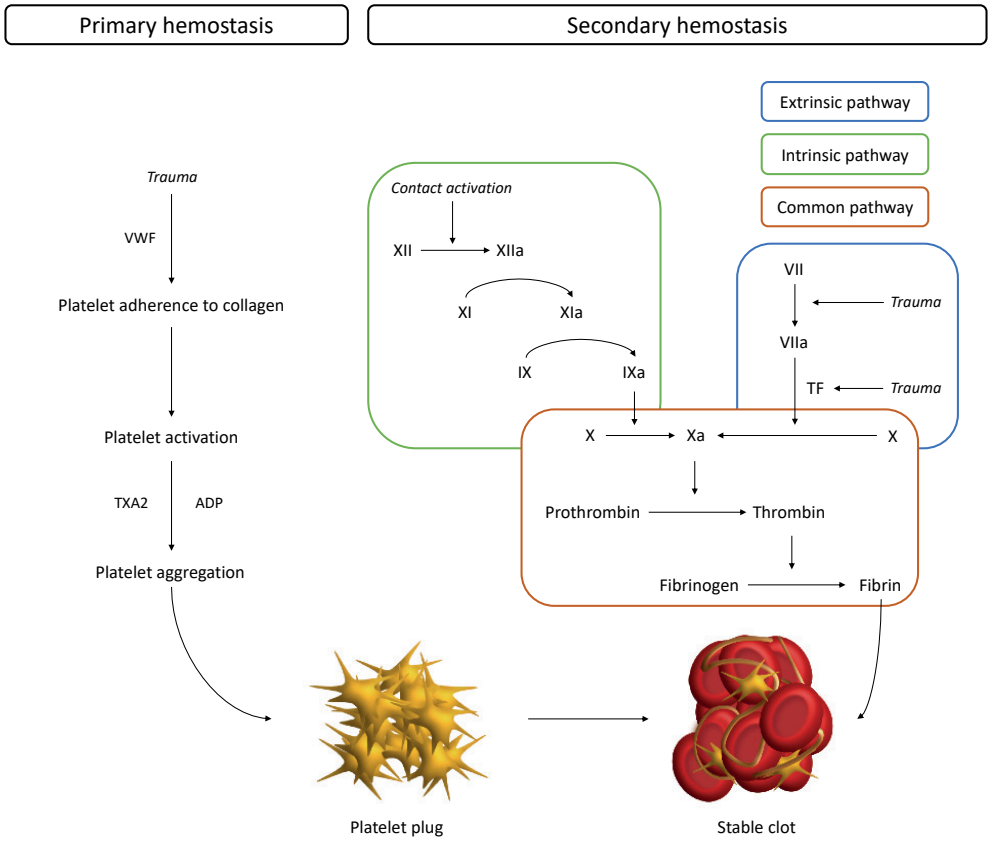

Figure 2: Primary and secondary hemostasis with the formation of a stable clot. VWF = von Willebrand factor, TXA2 = thromboxane A2, ADP = adenosine diphosphate, TF = tissue factor.

AIMS AND OUTLINE OF THE THESIS

This thesis aims to better understand why some PAD patients suffer from cardiovascular events despite current medical treatment strategies, while others do not. The unraveling of this increased cardiovascular risk is explored in this thesis through the characterization of high-risk patients combined with the investigation of important pathophysiological pathways driving cardiovascular events and mortality in PAD. Biomarkers related to these pathways can be used to identify patients at increased risk of such events.

In **chapter two**, we provide an overview of the current evidence on known pathophysiological pathways in atherosclerosis and atherothrombosis underlying PAD. The interplay between inflammation and coagulation is discussed, with a specific focus on protease-activated receptors to elaborate on the role of platelets in hypercoagulability. Additionally, potential pharmacological treatment targets to promote vascular protection are considered.

In **chapter three**, neutrophil and platelet activation are investigated as possible drivers of cardiovascular events in PAD patients, based on the insights provided in chapter two. Considering that these patients share clinical risk factors and treatment strategies with patients that suffer from venous thromboembolism, the existence of a pathophysiological link is explored.

In **chapter four** an overview of all investigated plasmatic biomarkers in association with cardiovascular outcome in PAD is given, to create a comprehensive overview of state-of-the-art biomarkers testing. As biomarkers are useful in determining the cardiovascular risk in PAD patients, this overview gives an insight into what is already known and what is yet to be explored.

Chapter five describes the patient population of the PAD Clinical Study. In this observational prospective cohort study, patient characteristics are investigated in relation to the occurrence of cardiovascular events and mortality. Moreover, the medical treatment with antiplatelet agents and lipid-lowering drugs is also evaluated to find targets for improved management.

Chapter six presents a subgroup of the PAD Clinical Study in which target finding is performed and 184 plasmatic biomarkers are measured using the Olink cardiovascular panels. In addition to the plasmatic biomarkers as described in chapter three, new predictive biomarkers are presented.

As chapter five mostly discusses the role of inflammatory pathways of PAD, **chapter seven** focuses on pathways leading to a hypercoagulable state in PAD patients. In this chapter, thrombin generation testing and coagulation complex measurements are performed to explore these pathways in PAD and their role in the occurrence of atherothrombotic events.

REFERENCES

1. Wang, H. *et al.* Global, regional, and national life expectancy, all-cause mortality, and cause-specific mortality for 249 causes of death, 1980–2015: a systematic analysis for the Global Burden of Disease Study 2015. *Lancet* **388**, 1459–1544 (2016).
2. Al-Qaisi, M., Nott, D. M., King, D. H. & Kaddoura, S. Ankle brachial pressure index (ABPI): An update for practitioners. *Vasc. Health Risk Manag.* **5**, 833–841 (2009).
3. Gerhard-Herman, M. D. *et al.* 2016 AHA/ACC guideline on the management of patients with lower extremity peripheral artery disease: Executive summary: A report of the American college of cardiology/American heart association task force on clinical practice guidelines. *J. Am. Coll. Cardiol.* **69**, 1465–1508 (2017).
4. Sitia, S. *et al.* From endothelial dysfunction to atherosclerosis. *Autoimmun. Rev.* **9**, 830–834 (2010).
5. Cyr, A. R., Huckaby, L. V., Shiva, S. S. & Zuckerbraun, B. S. Nitric oxide and endothelial dysfunction. *Crit. Care Clin.* **36**, 307–321 (2020).
6. Rajendran, P. *et al.* The vascular endothelium and human diseases. *Int. J. Biol. Sci.* **9**, 1057–1069 (2013).
7. Zhang, X., Sessa, W. C. & Fernández-Hernando, C. Endothelial transcytosis of lipoproteins in atherosclerosis. *Front. Cardiovasc. Med.* **5**, 130 (2018).
8. Galkina, E. & Ley, K. Vascular adhesion molecules in atherosclerosis. *Arterioscler. Thromb. Vasc. Biol.* **27**, 2292–2301 (2007).
9. Bentzon, J. F., Otsuka, F., Virmani, R. & Falk, E. Mechanisms of plaque formation and rupture. *Circ. Res.* **114**, 1852–1866 (2014).
10. Gale, A. J. Continuing education course #2: current understanding of hemostasis. *Toxicol. Pathol.* **39**, 273–280 (2011).
11. Savage, B., Almus-Jacobs, F. & Ruggeri, Z. M. Specific synergy of multiple substrate-receptor interactions in platelet thrombus formation under flow. *Cell* **94**, 657–666 (1998).
12. Warner, T. D., Nylander, S. & Whatling, C. Anti-platelet therapy: cyclo-oxygenase inhibition and the use of aspirin with particular regard to dual anti-platelet therapy. *Br. J. Clin. Pharmacol.* **72**, 619–633 (2011).
13. Secco, G. G. *et al.* P2Y12 inhibitors: pharmacologic mechanism and clinical relevance. *Cardiovasc. Hematol. Agents Med. Chem.* **11**, 101–105 (2013).
14. Ortel, T. L. *et al.* American Society of Hematology 2020 guidelines for management of venous thromboembolism: treatment of deep vein thrombosis and pulmonary embolism. *Blood Adv.* **4**, 4693–4738 (2020).
15. Hindricks, G. *et al.* 2020 ESC Guidelines for the diagnosis and management of atrial fibrillation developed in collaboration with the European Association for Cardio-Thoracic Surgery (EACTS): The Task Force for the diagnosis and management of atrial fibrillation of the European Society of Cardiology (ESC) Developed with the special contribution of the European Heart Rhythm Association (EHRA) of the ESC. *Eur. Heart J.* **42**, 373–498 (2021).
16. Dick, F. *et al.* Chapter VI: Follow-up after revascularisation. *Eur. J. Vasc. Endovasc. Surg.* **42 Suppl 2**, S75-90 (2011).
17. Anand, S. S. *et al.* Rivaroxaban with or without aspirin in patients with stable peripheral or carotid artery disease: an international, randomised, double-blind, placebo-controlled trial. *Lancet* **391**, 219–229 (2018).

18. Prandoni, P. Is there a link between venous and arterial thrombosis? A reappraisal. *Intern. Emerg. Med.* **15**, 33–36 (2020).
19. Migliacci, R. *et al.* Endothelial dysfunction in patients with spontaneous venous thromboembolism. *Haematologica* **92**, 812–818 (2007).
20. Mahmoodi, B. K. *et al.* Microalbuminuria and risk of venous thromboembolism. *JAMA* **301**, 1790–1797 (2009).
21. Song, P. *et al.* Global, regional, and national prevalence and risk factors for peripheral artery disease in 2015: an updated systematic review and analysis. *Lancet Glob. Health* **7**, e1020–e1030 (2019).
22. Norman, P. E., Eikelboom, J. W. & Hankey, G. J. Peripheral arterial disease: prognostic significance and prevention of atherothrombotic complications. *Med. J. Aust.* **181**, 150–154 (2004).
23. Grenon, S. M. *et al.* Peripheral artery disease and risk of cardiovascular events in patients with coronary artery disease: insights from the Heart and Soul Study. *Vasc. Med.* **18**, 176–184 (2013).
24. Kaplovitch, E. *et al.* Rivaroxaban and aspirin in patients with symptomatic lower extremity peripheral artery disease: A subanalysis of the COMPASS randomized clinical trial. *JAMA Cardiol.* **6**, 21–29 (2021).
25. Ridker, P. M. *et al.* Antiinflammatory therapy with canakinumab for atherosclerotic disease. *N. Engl. J. Med.* **377**, 1119–1131 (2017).

CHAPTER 2

PLEIOTROPIC EFFECTS OF THE HEMOSTATIC SYSTEM

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ABSTRACT

Atherothrombosis is characterized by the inflammatory process of atherosclerosis combined with a hypercoagulable state leading to superimposed thrombus formation. In atherosclerotic plaques, cell signaling can occur via protease-activated receptors (PARs), four of which have been identified so far (PAR1-PAR4). Proteases that are able to activate PARs can be produced systemically, but also at the site of lesions, and they include thrombin and activated factor X. After PAR activation, downstream signaling can lead to both proinflammatory effects and a hypercoagulable state. Which specific effect occurs, depends on the type of protease and activated PAR, and the site of activation. Hypercoagulable effects are mainly exerted through PAR1 and PAR4, whereas proinflammatory responses are mostly seen after PAR1 and PAR2 activation. PAR signaling pathways contribute to atherothrombosis, suggesting that inhibition of these pathways possibly prevents cardiovascular events based on this pathophysiological mechanism. In this review, we highlight the pathways by which PAR activation leads to proinflammatory responses and a hypercoagulable state. Furthermore, an overview is given of potential pharmacological treatment targets that promote vascular protection.

INTRODUCTION

A common definition of hemostasis is the process that arrests bleeding, or, in other words, the biological mechanisms that keep blood within a damaged blood vessel. The processes involved have been described elsewhere [1], and include platelet adhesion, activation, and aggregation, as well as coagulation activation and propagation. The final product of hemostasis is the formation of a platelet plug stabilized by crosslinked fibrin fibers. According to the definition of hemostasis, pleiotropic effects of hemostasis are the actions of components or key players of this mechanism beyond the activity of platelets and the formation of fibrin. For the structure of this article, we followed this reasoning and sectioned the text into actions of platelets and coagulation proteases not causing a platelet/fibrin clot, with a focus on cardiovascular disease and atherosclerosis.

PLATELETS

The role of platelets in cardiovascular disease, and more specifically in atherosclerosis, is diverse. Although atherosclerosis is a long-term process, platelets are known to exert effects throughout all stages of the inflammatory disease. Platelets contribute to early atherosclerosis by adhering to the activated endothelial monolayer, in a process mediated by platelet P-selectin [2]. By adhering to the endothelium, platelets are activated, leading to the release of proinflammatory cytokines such as IL-1β [3]. The interaction between endothelial cells and platelets also induces the expression of adhesion molecules and thereby promotes leukocyte adhesion and monocyte recruitment [4,5]. After an activated platelet transmigrates into the plaque, it secretes even more IL-1β, amongst other proinflammatory cytokines. Furthermore, and perhaps even more importantly, platelets interact with dendritic cells, which are classical antigen-presenting cells. This interaction initiates and promotes lesion growth via the recruitment of lipids into atherosclerotic plaques [6]. In the late stages of atherosclerosis, platelets are well known to play a key role in thrombus formation on eroded plaques or after plaque rupture [7].

COAGULATION PROTEASES

Protease-activated receptors

Although it was already known for many years that thrombin can activate platelets, it was not until the last decade of the 20th century that the so-called protease-activated receptors (PARs) were discovered. To date, a total of four PARs (PAR1, 2, 3, and 4) have been identified, and their expression has been demonstrated on platelets, vascular smooth muscle cells, endothelial cells, and leukocytes among other cell types. The PARs belong to the family of G-protein-coupled receptors, and activation of the receptor takes place by proteolysis of the N-terminal domain, generating a new so-called tethered ligand to be exposed to the receptor.

The presence of PARs on platelets has been demonstrated by *Vu et al.* [8], and so far only PAR1 and PAR4 have been identified on human platelets [9]. In contrast, mouse platelets express PAR3 and PAR4 [10,11]. For human platelets, PAR1 plays a key role in activation, requiring only very low thrombin concentrations, whereas PAR4 needs high concentrations of thrombin to induce an effect on platelets [12]. Upon activation of PAR1, heterotrimeric G-proteins have, through intracellular signaling, several effects, including thromboxane A_2 production, ADP release, activation of P-selectin, and platelet aggregation. The hemostatic effect of PAR activation on platelets is further facilitated by the formation of a procoagulant phospholipid membrane through stimulation of phosphatidylserine exposure [13–15]. The role of PAR4 in platelets is less evident; however, it is thought that PAR4 acts as a support mechanism for thrombin-mediated PAR1 signaling.

Activators of PARs can be divided into coagulation proteases and non-coagulation proteases. All four PARs can be activated by one or more coagulation proteases. PAR1, PAR3, and PAR4 are activated by thrombin, whereas PAR2 is activated by activated factor X (FXa) and activated factor VII (FVIIa) [1,16]. Recent studies, however, have also shown activation of PAR2 by high concentrations of thrombin, and activation of PAR1 and PAR3 by FXa [17–19]. PAR1 can also be activated by the anticoagulant activated protein C (APC) at a site downstream of thrombin activation, resulting in a different amino acid composition of the N-terminal tethered ligand [20,21]. Other proteases that can activate PAR1 include neutrophil-derived cathepsin G [22], neutrophil elastase [23], and several metalloproteinases such as ADAM17 [24,25] and the matrix metalloproteinases MMP-1, -9, and -13. Cathepsin S, chitinase, and matriptase/MT-SP1 have been identified as agonists of PAR2 [26]. Regarding PAR4 activation, cathepsin G, kallikrein 1 (KLK-1), and plasmin are known for their proatherogenic properties, inducing cell-specific events such as proliferation and adhesion [27,28].

In addition to undergoing monomeric activation, PARs can form homodimers, heterodimers, and even oligomers, thereby diversifying their effects on biology and pathology. After the formation of a PAR1-PAR2 complex, β-arrestin-mediated extracellular signal-regulated kinase (ERK) 1/2 signaling is greatly enhanced. It is tempting to speculate that PAR1-PAR2 complex formation is pro-atherogenic, given the fact that ERK1/2 activation is highly proatherogenic. [29,30].

Activation of PARs induces site-specific effects, and the effects may differ between receptors. Functions of PAR activation become visible early on when PAR1 is required for normal vascular development in the embryo. PARs also play an important role in vascular tone regulation, with PAR1 and PAR2 being able to induce vasorelaxation through an endothelial-dependent mechanism. During vascular injury, PAR1 increases endothelial permeability and the extravasation of proteins. Furthermore, PAR1 recruits platelets and leukocytes to injured surfaces via increased

expression of cytokines and adhesion molecules. Vascular remodeling is promoted by PARs via stimulation of endothelial and smooth muscle cell proliferation [31].

Mechanisms of PAR activation in atherosclerosis and atherothrombosis

Early studies investigating mouse vascular tissue demonstrated that PARs might play a pathophysiological role in the development of experimental atherosclerosis *(Figure 1)* [32,33]. In these early years, many effects of PARs were identified, including modulation of the vascular tone by a nitric oxide mechanism [34], induction of vascular smooth muscle cell growth [35], and inflammatory-driven atherogenesis [36].

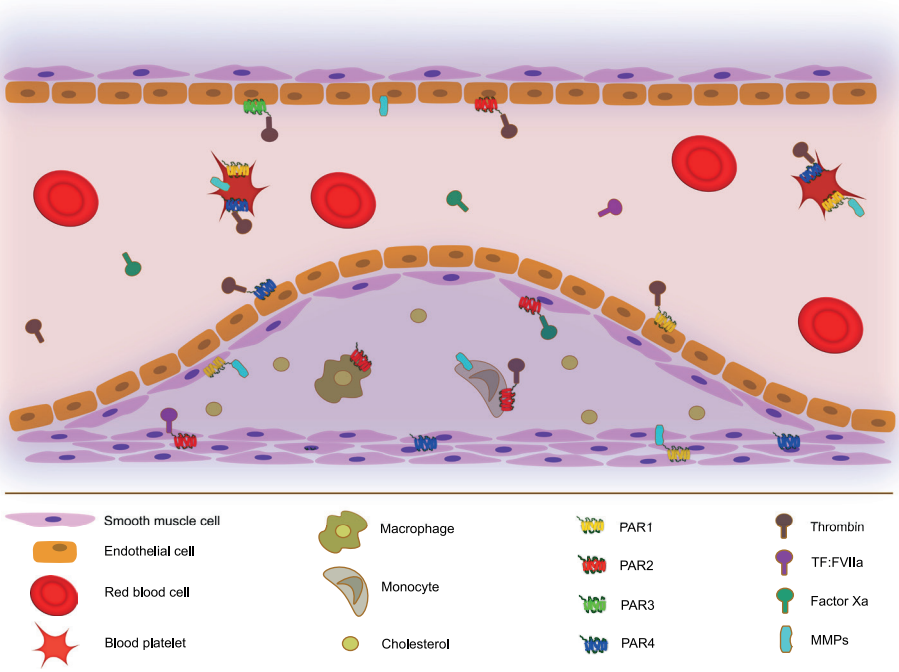

Figure 1: Visualization of proteases activating protease-activated receptors (PARs) on different cell types in an atherosclerotic plaque. FVIIa, activated factor VII; FXa, activated factor X; MMP, matrix metalloproteinase; TF, tissue factor.

Evidence for a role of PARs in the development and progression of atherosclerosis stems from experiments with several animal models with deficiencies of the receptors on an atherogenic background. Deficiency of PAR2 has been shown to attenuate atherosclerosis [37], whereas, in contrast, PAR4 deficiency on an ApoE$^{-/-}$ background did not alter the extent of atherosclerosis as compared to wild-type animals [38]. Although the exact mechanisms of PARs in atherosclerosis have yet to be established, several potential pathways have been studied. In the early stages of

atherosclerosis, proliferation and migration of smooth muscle cells take place, together with apoptosis of endothelial cells. The proliferation of smooth muscle cells is partially induced by PAR2 activation, facilitated by factors such as tumor necrosis factor (TNF)-α and IL-1β. Activation of PAR2 can take place on macrophages vascular smooth muscle cells, among other cells, in atherosclerotic plaques. Consequently, a complex of MT-SP1/matriptase stimulates the synthesis of proinflammatory cytokines IL-8 and IL-6, enhancing inflammatory processes in the endothelial layer of the arterial vessel wall and thereby inducing the progression of atherosclerosis *(Figure 2)*. MT-SP1/matriptase does not seem to be a key regulator in the pro-inflammatory response, but can rather be seen as an amplifier of inflammation [26]. Furthermore, activation of PAR2 leads to nuclear factor-κB signaling in coronary smooth muscle cells accompanied by increased production of cyclooxygenase, both of which are associated with increased inflammation and progression of atherosclerosis [39]. PAR2 activation also directly increases the adhesion of inflammatory cells to the vascular endothelium, as leukocyte adhesion is significantly decreased in PAR2$^{-/-}$ mice [40]. Finally, as PAR2 can modulate the vascular tone, it is considered to be an important physiological regulator of blood pressure; upregulation of PAR2 can lower the blood pressure, whereas a downregulation increases the blood pressure [41].

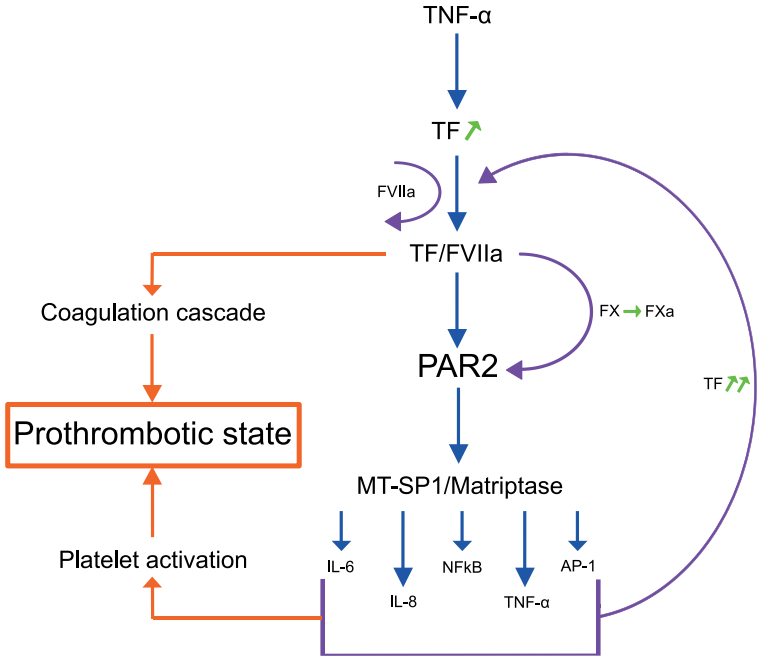

Figure 2: Indirect formation of a prothrombotic state via protease-activated receptor 2 (PAR2) activation. FVIIa, activated factor VII; FXa, activated factor X; IL, interleukin; NF-κB, nuclear factor-κB; TF, tissue factor; TNF, tumor necrosis factor.

MMP-9 mediated PAR1 activation contributes to endothelial dysfunction, which is one of the initial stages of atherogenesis and is potentially involved in thrombosis and restenosis [42]. MMP-9 is a member of the zinc-dependent endopeptidases family, which can be divided into collagenases, gelatinases, stromelysins, and metalloelastases. Almost all MMPs are secreted as zymogens and are then activated after their inhibitory domain is cleaved off. Along with MMP-9, four other metalloproteinases (MMP-1, -2, -13, -14) are expressed in the vascular wall. The expression of MMP-1 by macrophages, smooth muscle cells, and endothelial cells, is increased in atherosclerosis and is mostly seen in vulnerable regions of atherosclerotic plaques. The role of MMP-2 and PAR1 activation remains unclear, but it has been suggested that MMP-2 is involved in monocyte migration or macrophage proliferation [43]. It has been demonstrated that atherosclerotic plaques are significantly reduced in MMP-2$^{-/-}$/ApoE$^{-/-}$ mice [44]. Summarizing the above-described MMP effects, it is possible that, by antagonizing the MMP-PAR1 activation, the processes of atherosclerosis and atherothrombosis could be counteracted. The best antagonist that could be used in such a situation would be a PAR1 antagonist [45]. However, administration of a PAR1 antagonist also counteracts several APC-mediated protective mechanisms of PAR1 activation. One of the most important mechanisms is induced by PAR1 activation on endothelial cells by APC, leading to the upregulation of monocyte chemotactic protein 1 (MCP-1), which promotes both anti-inflammatory effects through immune-modulatory chemokine networks [46] and pro-inflammatory effects, by influencing the growth of different cell types [47]. It would be ideal to inhibit PAR1 activation through blockage of a specific agonist such as an MMP. Unfortunately, there is no clinical evidence available yet regarding selectively blocking PAR agonists, although research is progressing. The use of pepducins, which are peptides acting as intracellular inhibitors/agonists of signaling from receptor to G-protein, is currently under investigation [48]. A newly developed PAR2 pepducin antagonist, for example, caused suppression of leukocyte infiltration *in vivo* [49].

Role of specific coagulation factors in atherosclerosis

Tissue factor (TF) is an important cytokine receptor that is a key player in the initiation of atherothrombosis and is the high-affinity receptor for FVIIa [50]. It is mostly expressed by extravascular cells [51], but high concentrations have been identified in atherosclerotic plaques as well [52]. In plaques, TF mainly originates from macrophages, but vascular smooth muscle cells and endothelial cells are also able to express TF [53,54]. Even though TF:FVIIa might be an important direct (PAR2) or indirect (coagulation activation) regulator of atherosclerotic progression, the main clinical effect of this protein can be seen when it initiates atherothrombosis. TF appears to be the most abundant protein in thrombogenicity during plaque rupture. The rupture itself depends on the composition of the plaque, but the size of the thrombus that is formed upon rupturing is dependent on the concentration of TF present [55]. Furthermore, it has been suggested that TF plays an important role in the progression of atherosclerotic plaques by increasing macrophage apoptosis and necrosis in the lesion [52,56]. Studies with genetically modified mice on

an atherosclerotic background that express no wild-type TF but express a low level of human TF have failed to show a contribution of TF in atherogenesis [57]. However, the TF:FVIIa complex has been shown to activate PAR2 [58], and is known to upregulate cytokines (e.g. IL-6, IL-8) and chemokines that induce leukocyte recruitment at the site of atherosclerosis, leading to further progression of the lesion. Furthermore, TF:FVIIa-mediated PAR2-signaling increases the migration of vascular smooth muscle cells, which express receptors that are needed for lipid accumulation inside the plaque [56].

Factor X is expressed by macrophages and vascular smooth muscle cells in atherosclerotic lesions. The active protease, FXa, can activate both PAR1 and PAR2 on cells within the (atherosclerotic) vessel wall, including endothelial cells, leukocytes, smooth muscle cells, and macrophages. Responses mediated by FXa signaling include tissue remodeling and fibrosis, through the induction of chemokine and cytokine expression [59,60]. Inhibition of FXa, e.g. by administration of rivaroxaban, reduces the expression of pro-inflammatory mediators (e.g. IL-6, MCP-1) in ApoE$^{-/-}$ mice. FXa inhibition also increases the fibrotic cap thickness and reduces medial erosion, both of which lead to greater plaque stability [61]. Furthermore, following the administration of an FXa inhibitor, leukocyte adhesion is attenuated in mice with metabolic syndrome [62].

Thrombin can be locally generated within the atherosclerotic lesion because FVII and FX are, along with TF, functionally expressed by both macrophages and smooth muscle cells within atherosclerotic plaques [63,64]. In the early stages of atherosclerosis, thrombin stimulates reactive oxygen species (ROS) production, leading to apoptosis and lipid peroxidation. Furthermore, thrombin induces the recruitment of monocytes into the vessel wall through the synthesis of MCP-1 and increases the expression of interleukins and TNF-α. Thrombin also contributes to atherogenesis by upregulating the expression of vascular adhesion molecule (VCAM) and intercellular adhesion molecule (ICAM), both of which aid leukocyte transmigration through the endothelium [65,66]. In a study by *Borissoff et al.*, ApoE$^{-/-}$ mice with a genetically-imposed 50% reduction of prothrombin were investigated. During atherogenesis, neutrophil mobilization was enhanced by a hypercoagulable state imposed in mice that had a point mutation in the thrombomodulin gene, creating a hypercoagulable state with diminished cytoprotective activation of APC. In these animals, there were increases in oxidative stress and apoptosis, both of which promote atherosclerotic plaque progression and eventually atherothrombosis [67]. In contrast, another study with comparable mice genotypes demonstrated that, during *de novo* atherogenesis, thrombin-induced signaling via PAR1 signaling impairs the migration of monocytes, resulting in a reduction in macrophage accumulation in atherosclerotic plaques, thereby having a protective effect [68]. Although a clear explanation for the contradictory data obtained with comparable genotypes is still lacking, the effects of different conditions, including the age of animals and administered diet, cannot be excluded.

Other coagulation factors have been studied in less detail. Studies performed with ApoE$^{-/-}$ mice showed a roughly 30% reduction in atherosclerotic lesion volume in factor XI (FXI) deficient animals [69]. Even though the direct link between FXI and inflammation is still unknown, decreased levels of TNF-α and IL-6 levels have been observed in septic mice with inhibition of FXI activation [70], thereby providing a potential link to reduced atherosclerosis in the absence of FXI. Factor XII (FXII) deficiency attenuated atherosclerosis in mice on an atherogenic background and reduced levels of inflammatory markers such as serum IL-1β and IL-12 levels suggest an anti-inflammatory mechanism [71]. FVIII deficiency is also associated with sustained protection against atherosclerotic progression in ApoE$^{-/-}$ mice, but not in LDL-R$^{-/-}$ mice, suggesting an underlying lipid-dependent mechanism in LDL-R$^{-/-}$ mice. [72]. FIX deficiency does not seem to protect against atherosclerosis in an experimental model [73].

ANTICOAGULANTS AND PROTECTION AGAINST ATHEROSCLEROSIS

Given the pivotal role of coagulation proteases in the development and progression of atherosclerosis, it seems straightforward to hypothesize that anticoagulant therapy might be beneficial for the reduction of atherosclerosis. However, the picture is two-faced as the outcome of coagulation inhibition on the extent of atherosclerosis seems to be dependent on the class of anticoagulant therapy used. Although the vitamin K antagonists (VKAs) such as warfarin and coumarin derivatives decrease the incidence of cardiovascular events [74,75], it has been demonstrated that long-term administration of VKAs leads to an increase in vascular calcification [76] as a result of incomplete γ-glutamyl carboxylation of matrix Gla protein [77,78]. In contrast, direct inhibition of coagulation enzymes may have antiatherosclerotic properties. Regarding FXa anticoagulation, several experimental animal studies demonstrated reduced atherosclerosis following treatment with a direct FXa inhibitor. A study by *Hara et al.* demonstrated that the development of atherosclerosis in mice is reduced after administration of the FXa inhibitor rivaroxaban. The diminished progression of atherosclerosis was accomplished by stabilization of already developed atherosclerotic plaques through inhibition of lipid deposition, but also by reducing collagen loss and accumulation of macrophages [79]. In a different study, the reduction of atherosclerotic volume upon treatment with rivaroxaban was less evident, although a small but significant increase in minimum thickness of the fibrous cap was noticed, suggesting a more stable plaque phenotype [61]. The inhibition of FXa was accompanied by reduced expression of the pro-inflammatory markers IL-6, TNF-α, and MCP-1, which are known to promote atherosclerotic progression.

The second group of DOACs consists of direct thrombin inhibitors (DTIs), which exert their effect on thrombin itself rather than on its amplification pathway. One of the first experimental studies demonstrating reduced atherosclerosis upon treatment with a thrombin inhibitor was

performed with melagatran [80]. Atherosclerosis in ApoE$^{-/-}$ mice receiving a diet containing melagatran was reduced in terms of lesion area and thickness, as well as by increased plaque stability through reduced activation of proinflammatory transcription factors and reduced synthesis of MMP-9. These observations were confirmed by using dabigatran etexilate as DTI. After demonstrating the vascular protective effects of melagatran in mice, *Kadoglou et al.* found that, after administration of dabigatran etexilate, plaques became smaller and more stable, owing to an increase in collagen and elastin. The fibrous cap also became thicker, with an increase in mechanical strength via upregulation of smooth muscle cells [81]. Several years later, *Lee et al.* demonstrated that treatment of ApoE$^{-/-}$ mice with dabigatran etexilate attenuated atherosclerosis by reducing oxidative stress, leading to smaller-sized atherosclerotic lesions along with an improvement in endothelial function. Furthermore, the recruitment of inflammatory cells and platelet aggregation were inhibited [82]. *Borissoff et al.* demonstrated diminished lesion formation and increased plaque stability in FII$^{-/-}$/ApoE$^{-/-}$ mice after administration of dabigatran etexilate [67]. It has been suggested that DTIs have the most effect on early atherosclerotic lesions, owing to a more stimulated coagulation system in the early stages of atherosclerosis. In these early stages, thrombin may be a particularly important driver of macrophage recruitment by inducing the transcription of proinflammatory genes. High levels of macrophages are present in atherosclerotic lesions, induced by thrombin itself. Administration of a DTI might therefore be most profitable in the early stage of atherosclerosis [83].

ATHEROTHROMBOTIC PROTECTION IN CLINICAL STUDIES

DOACs have been clinically introduced mainly on the basis of studies on the prevention and treatment of venous thromboembolism and prevention of stroke in subjects with atrial fibrillation [84]. Over the past few years, clinical research has been expanded to involve the prevention of atherothrombosis in both acute and stable manifestations of atherosclerotic vascular disease *(Figure 3)*. Patients with acute coronary syndrome treated with rivaroxaban in conjunction with platelet inhibitors have a reduced risk for a secondary event or cardiovascular death as compared to solely platelet inhibitors [85,86]. The price for combining rivaroxaban and platelet inhibitors was an increase in major bleeding, without an increased risk of fatal intracerebral bleeding. The APPRAISE-2 trial investigated the combination of apixaban with aspirin or aspirin/clopidogrel in patients with acute coronary syndrome and showed no clear benefit in terms of reduction of recurrent arterial events or death, and at a price of increased risk of major, including fatal, bleeding [87]. From these studies, the concept of a potential benefit of a combination of a relatively low dose of a DOAC (rivaroxaban) with antiplatelet therapy, emerged; however, practical implementation appears to be limited by the associated bleeding risk.

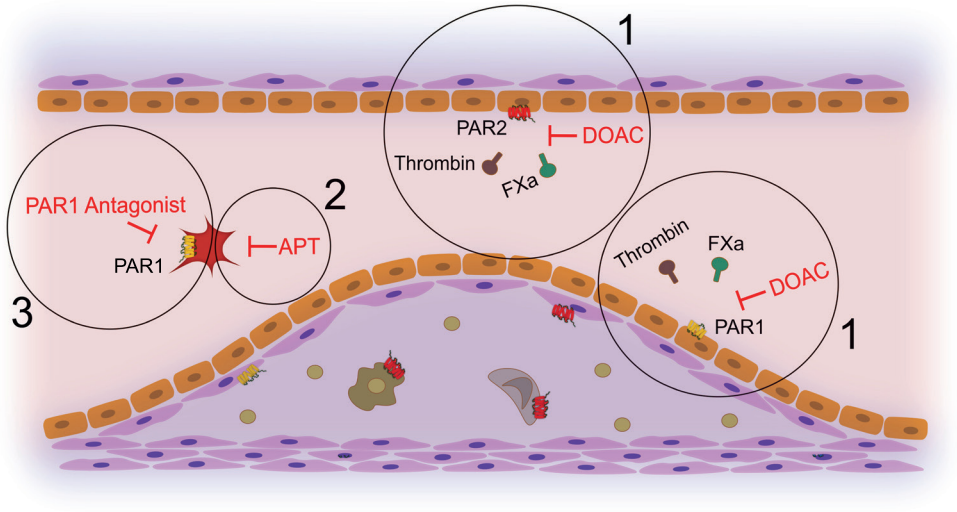

Figure 3: Therapeutic vascular protection options. 1, Direct oral anticoagulants (DOACs); 2, Antiplatelet therapy (APT); 3, Protease-activated receptor (PAR) 1 antagonists.

There are no studies on the management of DOACs in patients with acute ischemic stroke; here, the use of single or short-lasting double antiplatelet therapy remains standard [88]. Large studies have addressed the prevention of acute ischemic stroke (and other vascular events, including mortality) in patients with atrial fibrillation [89,90]. None of the studies addressed effects on atherosclerosis, except for the RE-LY study, which showed an increased risk of myocardial infarction in those treated with dabigatran as compared to those treated with warfarin [91–93]. The origin of this effect remains unclear, and there are no indications that effects on plaque stability, as suggested by preclinical studies with dabigatran (see above), are involved, but this has also not been addressed specifically. In the management of patients with stable (i.e. non-acute) atherosclerotic vascular disease, the use of platelet inhibitors has been a cornerstone of antithrombotic management. In general, this involves the use of one antiplatelet agent, i.e. aspirin or clopidogrel, and rarely dual antiplatelet therapy (beyond the guideline-supported interval use after a coronary intervention), because of the associated increased risk of bleeding complications as compared to single-agent therapy [94].

The recent COMPASS trial triggered interest in the use of combined anticoagulant and antiplatelet therapy in patients with relatively high-risk atherosclerotic disease. In particular, in patients with peripheral artery disease, combined rivaroxaban (2.5 mg) and aspirin were more effective than either agent alone in reducing major adverse cardiovascular events and major adverse limb events, although at the cost of more bleeding events [95]. The potential advantage of this combined antithrombotic treatment may be profound, at least from a conceptual point of view. On the

one hand, the use of platelet inhibitors and anticoagulants makes sense, as atherothrombosis is dependent not only on platelets but also on fibrin formation. On the other hand, pre-clinical data from mouse studies suggest that (long-term) administration of anticoagulants (at least DOACs) may also have beneficial effects on atherosclerosis. The challenge will be to address the underlying mechanisms of such combined therapy that may extend beyond simply lowering thrombosis risk (also for these low doses of rivaroxaban supported by marked attenuation of thrombin generation levels [96]). This requires further preclinical studies, also with other DOAC alone and in combination with antiplatelet therapy. In addition, we argue for more clinical studies addressing the potential vascular effects of (long-term) oral anticoagulant therapy, to carefully address the existence and magnitude of such effects in patients with defined vascular disease; such studies will require sensitive imaging tools and biomarkers, rather than clinical endpoints.

CONCLUSION

As cardiovascular disease is the most important cause of death worldwide, optimization of secondary prevention remains a huge health care issue. Although secondary cardiovascular prevention is based on different treatment strategies, anticoagulant therapy is a key player. Preclinical evidence suggests that DOACs in particular may have vascular effects beyond the prevention of thrombosis. The clinical efficacy of relatively low doses of a DOAC in combination with an antiplatelet therapy suggests, although at the cost of bleeding, that efficacy regarding cardiovascular events can still be optimized. More clinical studies on DOACs in secondary cardiovascular prevention are necessary to investigate efficacy and safety, especially in subgroups such as patients with peripheral artery disease.

REFERENCES

1. Versteeg, H. H., Heemskerk, J. W. M., Levi, M. & Reitsma, P. H. New fundamentals in hemostasis. *Physiol. Rev.* **93**, 327–358 (2013).
2. Massberg, S. *et al.* A critical role of platelet adhesion in the initiation of atherosclerotic lesion formation. *J. Exp. Med.* **196**, 887–896 (2002).
3. Lindemann, S. *et al.* Activated platelets mediate inflammatory signaling by regulated interleukin 1beta synthesis. *J. Cell Biol.* **154**, 485–490 (2001).
4. Gawaz, M. *et al.* Activated platelets induce monocyte chemotactic protein-1 secretion and surface expression of intercellular adhesion molecule-1 on endothelial cells. *Circulation* **98**, 1164–1171 (1998).
5. Siegel-Axel, D. I. & Gawaz, M. Platelets and endothelial cells. *Semin. Thromb. Hemost.* **33**, 128–135 (2007).
6. Manthey, H. D. & Zernecke, A. Dendritic cells in atherosclerosis: functions in immune regulation and beyond. *Thromb. Haemost.* **106**, 772–778 (2011).
7. Badimon, L. & Vilahur, G. Thrombosis formation on atherosclerotic lesions and plaque rupture. *J. Intern. Med.* **276**, 618–632 (2014).
8. Vu, T. K., Hung, D. T., Wheaton, V. I. & Coughlin, S. R. Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. *Cell* **64**, 1057–1068 (1991).
9. Kahn, M. L., Nakanishi-Matsui, M., Shapiro, M. J., Ishihara, H. & Coughlin, S. R. Protease-activated receptors 1 and 4 mediate activation of human platelets by thrombin. *J. Clin. Invest.* **103**, 879–887 (1999).
10. Ishihara, H., Zeng, D., Connolly, A. J., Tam, C. & Coughlin, S. R. Antibodies to protease-activated receptor 3 inhibit activation of mouse platelets by thrombin. *Blood* **91**, 4152–4157 (1998).
11. Kahn, M. L. *et al.* A dual thrombin receptor system for platelet activation. *Nature* **394**, 690–694 (1998).
12. Nieman, M. T. & Schmaier, A. H. Interaction of thrombin with PAR1 and PAR4 at the thrombin cleavage site. *Biochemistry* **46**, 8603–8610 (2007).
13. Harper, M. T. *et al.* Transient receptor potential channels function as a coincidence signal detector mediating phosphatidylserine exposure. *Sci. Signal.* **6**, ra50 (2013).
14. Keuren, J. F. W. *et al.* Synergistic effect of thrombin on collagen-induced platelet procoagulant activity is mediated through protease-activated receptor-1. *Arterioscler. Thromb. Vasc. Biol.* **25**, 1499–1505 (2005).
15. Posma, J. J. N., Posthuma, J. J. & Spronk, H. M. H. Coagulation and non-coagulation effects of thrombin. *J. Thromb. Haemost.* **14**, 1908–1916 (2016).
16. Nieman, M. T. Protease-activated receptors in hemostasis. *Blood* **128**, 169–177 (2016).
17. Mihara, K. *et al.* Thrombin-mediated direct activation of proteinase-activated receptor-2: Another target for thrombin signaling. *Mol. Pharmacol.* **89**, 606–614 (2016).
18. Bukowska, A. *et al.* Coagulation factor Xa induces an inflammatory signalling by activation of protease-activated receptors in human atrial tissue. *Eur. J. Pharmacol.* **718**, 114–123 (2013).
19. Stavenuiter, F. & Mosnier, L. O. Noncanonical PAR3 activation by factor Xa identifies a novel pathway for Tie2 activation and stabilization of vascular integrity. *Blood* **124**, 3480–3489 (2014).
20. Ludeman, M. J. *et al.* PAR1 cleavage and signaling in response to activated protein C and thrombin. *J. Biol. Chem.* **280**, 13122–13128 (2005).

21. Griffin, J. H., Zlokovic, B. V. & Mosnier, L. O. Activated protein C: biased for translation. *Blood* **125**, 2898–2907 (2015).
22. Sambrano, G. R. *et al.* Cathepsin G activates protease-activated receptor-4 in human platelets. *J. Biol. Chem.* **275**, 6819–6823 (2000).
23. Mihara, K., Ramachandran, R., Renaux, B., Saifeddine, M. & Hollenberg, M. D. Neutrophil elastase and proteinase-3 trigger G protein-biased signaling through proteinase-activated receptor-1 (PAR1). *J. Biol. Chem.* **288**, 32979–32990 (2013).
24. Andrews, R. K., Karunakaran, D., Gardiner, E. E. & Berndt, M. C. Platelet receptor proteolysis: a mechanism for downregulating platelet reactivity. *Arterioscler. Thromb. Vasc. Biol.* **27**, 1511–1520 (2007).
25. Trivedi, V. *et al.* Platelet matrix metalloprotease-1 mediates thrombogenesis by activating PAR1 at a cryptic ligand site. *Cell* **137**, 332–343 (2009).
26. Seitz, I. *et al.* Membrane-type serine protease-1/matriptase induces interleukin-6 and -8 in endothelial cells by activation of protease-activated receptor-2: potential implications in atherosclerosis. *Arterioscler. Thromb. Vasc. Biol.* **27**, 769–775 (2007).
27. Schurgers, L. J. & Spronk, H. M. H. Differential cellular effects of old and new oral anticoagulants: consequences to the genesis and progression of atherosclerosis. *Thromb. Haemost.* **112**, 909–917 (2014).
28. Quinton, T. M., Kim, S., Derian, C. K., Jin, J. & Kunapuli, S. P. Plasmin-mediated activation of platelets occurs by cleavage of protease-activated receptor 4. *J. Biol. Chem.* **279**, 18434–18439 (2004).
29. Lin, H. & Trejo, J. Transactivation of the PAR1-PAR2 heterodimer by thrombin elicits beta-arrestin-mediated endosomal signaling. *The Journal of biological chemistry* **288**, 11203–15 (2013).
30. Hu, Y., Dietrich, H., Metzler, B., Wick, G. & Xu, Q. Hyperexpression and activation of extracellular signal-regulated kinases (ERK1/2) in atherosclerotic lesions of cholesterol-fed rabbits. *Arterioscler. Thromb. Vasc. Biol.* **20**, 18–26 (2000).
31. Steinberg, S. F. The cardiovascular actions of protease-activated receptors. *Mol. Pharmacol.* **67**, 2–11 (2005).
32. al-Ani, B., Saifeddine, M. & Hollenberg, M. D. Detection of functional receptors for the proteinase-activated-receptor-2-activating polypeptide, SLIGRL-NH2, in rat vascular and gastric smooth muscle. *Can. J. Physiol. Pharmacol.* **73**, 1203–1207 (1995).
33. Hollenberg, M. D., Saifeddine, M., Al-Ani, B. & Gui, Y. Proteinase-activated receptor 4 (PAR4): action of PAR4-activating peptides in vascular and gastric tissue and lack of cross-reactivity with PAR1 and PAR2. *Can. J. Physiol. Pharmacol.* **77**, 458–464 (1999).
34. Emilsson, K. *et al.* Vascular effects of proteinase-activated receptor 2 agonist peptide. *J. Vasc. Res.* **34**, 267–272 (1997).
35. Bono, F., Lamarche, I. & Herbert, J. M. Induction of vascular smooth muscle cell growth by selective activation of the proteinase activated receptor-2 (PAR-2). *Biochem. Biophys. Res. Commun.* **241**, 762–764 (1997).
36. Sandberg, W. J. *et al.* Inflammatory interaction between LIGHT and proteinase-activated receptor-2 in endothelial cells: potential role in atherogenesis. *Circ. Res.* **104**, 60–68 (2009).
37. Zuo, P. *et al.* Protease-activated receptor-2 deficiency attenuates atherosclerotic lesion progression and instability in apolipoprotein E-deficient mice. *Front. Pharmacol.* **8**, 647 (2017).

38. Hamilton, J. R., Cornelissen, I., Mountford, J. K. & Coughlin, S. R. Atherosclerosis proceeds independently of thrombin-induced platelet activation in ApoE-/- mice. *Atherosclerosis* **205**, 427–432 (2009).
39. Indrakusuma, I., Romacho, T. & Eckel, J. Protease-activated receptor 2 promotes pro-atherogenic effects through transactivation of the VEGF receptor 2 in human vascular smooth muscle cells. *Front. Pharmacol.* **7**, 497 (2016).
40. Tennant, G. M., Wadsworth, R. M. & Kennedy, S. PAR-2 mediates increased inflammatory cell adhesion and neointima formation following vascular injury in the mouse. *Atherosclerosis* **198**, 57–64 (2008).
41. Cicala, C. *et al.* Protease-activated receptor-2 involvement in hypotension in normal and endotoxemic rats in vivo. *Circulation* **99**, 2590–2597 (1999).
42. Florence, J. M., Krupa, A., Booshehri, L. M., Allen, T. C. & Kurdowska, A. K. Metalloproteinase-9 contributes to endothelial dysfunction in atherosclerosis via protease activated receptor-1. *PLoS One* **12**, e0171427 (2017).
43. Austin, K. M., Covic, L. & Kuliopulos, A. Matrix metalloproteases and PAR1 activation. *Blood* **121**, 431–439 (2013).
44. Kuzuya, M. *et al.* Effect of MMP-2 deficiency on atherosclerotic lesion formation in apoE-deficient mice. *Arterioscler. Thromb. Vasc. Biol.* **26**, 1120–1125 (2006).
45. Ahn, H.-S., Chackalamannil, S., Boykow, G., Graziano, M. P. & Foster, C. Development of proteinase-activated receptor 1 antagonists as therapeutic agents for thrombosis, restenosis and inflammatory diseases. *Curr. Pharm. Des.* **9**, 2349–2365 (2003).
46. Riewald, M. & Ruf, W. Proteinase-activated receptor activation by coagulation proteinases. *Drug Dev. Res.* **59**, 400–407 (2003).
47. Lin, J., Kakkar, V. & Lu, X. Impact of MCP-1 in atherosclerosis. *Curr. Pharm. Des.* **20**, 4580–4588 (2014).
48. Kuliopulos, A. & Covic, L. Blocking receptors on the inside: pepducin-based intervention of PAR signaling and thrombosis. *Life Sci.* **74**, 255–262 (2003).
49. Sevigny, L. M. *et al.* Interdicting protease-activated receptor-2-driven inflammation with cell-penetrating pepducins. *Proc. Natl. Acad. Sci. U. S. A.* **108**, 8491–8496 (2011).
50. Saha, D., S, S., Sergeeva, E. G., Ionova, Z. I. & Gorbach, A. V. Tissue factor and atherothrombosis. *Curr. Pharm. Des.* **21**, 1152–1157 (2015).
51. Drake, T. A., Morrissey, J. H. & Edgington, T. S. Selective cellular expression of tissue factor in human tissues. Implications for disorders of hemostasis and thrombosis. *Am. J. Pathol.* **134**, 1087–1097 (1989).
52. Wilcox, J. N., Smith, K. M., Schwartz, S. M. & Gordon, D. Localization of tissue factor in the normal vessel wall and in the atherosclerotic plaque. *Proc. Natl. Acad. Sci. U. S. A.* **86**, 2839–2843 (1989).
53. Thiruvikraman, S. V. *et al.* In situ localization of tissue factor in human atherosclerotic plaques by binding of digoxigenin-labeled factors VIIa and X. *Lab. Invest.* **75**, 451–461 (1996).
54. Tatsumi, K. & Mackman, N. Tissue factor and atherothrombosis. *J. Atheroscler. Thromb.* **22**, 543–549 (2015).
55. Toschi, V. *et al.* Tissue factor modulates the thrombogenicity of human atherosclerotic plaques. *Circulation* **95**, 594–599 (1997).
56. Owens, A. P., 3rd & Mackman, N. Role of tissue factor in atherothrombosis. *Curr. Atheroscler. Rep.* **14**, 394–401 (2012).
57. Tilley, R. E. *et al.* Atherosclerosis in mice is not affected by a reduction in tissue factor expression. *Arterioscler. Thromb. Vasc. Biol.* **26**, 555–562 (2006).

58. Camerer, E., Huang, W. & Coughlin, S. R. Tissue factor- and factor X-dependent activation of protease-activated receptor 2 by factor VIIa. *Proc. Natl. Acad. Sci. U. S. A.* **97**, 5255–5260 (2000).
59. Spronk, H. M. H. *et al.* Pleiotropic effects of factor Xa and thrombin: what to expect from novel anticoagulants. *Cardiovasc. Res.* **101**, 344–351 (2014).
60. Borensztajn, K., Peppelenbosch, M. P. & Spek, C. A. Factor Xa: at the crossroads between coagulation and signaling in physiology and disease. *Trends Mol. Med.* **14**, 429–440 (2008).
61. Zhou, Q. *et al.* Evaluation of plaque stability of advanced atherosclerotic lesions in apo E-deficient mice after treatment with the oral factor Xa inhibitor rivaroxaban. *Mediators Inflamm.* **2011**, 432080 (2011).
62. Iba, T. *et al.* Factor Xa inhibitor attenuates leukocyte adhesion and thrombus formation in an experimental mouse model of the metabolic syndrome. *Cardiovasc. Ther.* **31**, 280–284 (2013).
63. Wilcox, J. N., Noguchi, S., Casanova, J. R. & Rasmussen, M. E. Extrahepatic synthesis of FVII in human atheroma and smooth muscle cells in vitro. *Ann. N. Y. Acad. Sci.* **947**, 433–438 (2001).
64. Borissoff, J. I. *et al.* Early atherosclerosis exhibits an enhanced procoagulant state. *Circulation* **122**, 821–830 (2010).
65. Borissoff, J. I., Spronk, H. M. H., Heeneman, S. & ten Cate, H. Is thrombin a key player in the "coagulation-atherogenesis" maze? *Cardiovasc. Res.* **82**, 392–403 (2009).
66. Kalz, J., ten Cate, H. & Spronk, H. M. H. Thrombin generation and atherosclerosis. *J. Thromb. Thrombolysis* **37**, 45–55 (2014).
67. Borissoff, J. I. *et al.* Genetic and pharmacological modifications of thrombin formation in apolipoprotein e-deficient mice determine atherosclerosis severity and atherothrombosis onset in a neutrophil-dependent manner. *PLoS One* **8**, e55784 (2013).
68. Seehaus, S. *et al.* Hypercoagulability inhibits monocyte transendothelial migration through protease-activated receptor-1-, phospholipase-Cbeta-, phosphoinositide 3-kinase-, and nitric oxide-dependent signaling in monocytes and promotes plaque stability. *Circulation* **120**, 774–784 (2009).
69. Shnerb Ganor, R. *et al.* Factor XI deficiency protects against atherogenesis in apolipoprotein E/factor XI double knockout mice. *Arterioscler. Thromb. Vasc. Biol.* **36**, 475–481 (2016).
70. Tucker, E. I. *et al.* Inhibition of factor XI activation attenuates inflammation and coagulopathy while improving the survival of mouse polymicrobial sepsis. *Blood* **119**, 4762–4768 (2012).
71. Vorlova, S. *et al.* Coagulation factor XII induces pro-inflammatory cytokine responses in macrophages and promotes atherosclerosis in mice. *Thromb. Haemost.* **117**, 176–187 (2017).
72. Fabri, D. R., de Paula, E. V., Costa, D. S. P., Annichino-Bizzacchi, J. M. & Arruda, V. R. Novel insights into the development of atherosclerosis in hemophilia A mouse models. *J. Thromb. Haemost.* **9**, 1556–1561 (2011).
73. de Paula, L. Í. S., Urban Paffaro, A., Costa, D. S. P., de Paula, E. V. & Annichino - Bizzacchi, J. M. The role of clotting factor IX in the development of atherosclerosis. *Thromb. Res.* **157**, 79–81 (2017).
74. Hylek, E. M., Go, A. S. & Chang, Y. Effect of intensity of oral anticoagulation on stroke severity and mortality in atrial fibrillation. *ACC Curr. J. Rev.* **13**, 13–14 (2004).
75. Brouwer, M. A. *et al.* Aspirin plus coumarin versus aspirin alone in the prevention of reocclusion after fibrinolysis for acute myocardial infarction: results of the Antithrombotics in the Prevention of Reocclusion In Coronary Thrombolysis (APRICOT)-2 Trial. *Circulation* **106**, 659–665 (2002).

76. Schurgers, L. J. *et al.* Vitamin K-antagonists accelerate atherosclerotic calcification and induce a vulnerable plaque phenotype. *PLoS One* **7**, e43229 (2012).
77. Schurgers, L. J., Aebert, H., Vermeer, C., Bültmann, B. & Janzen, J. Oral anticoagulant treatment: friend or foe in cardiovascular disease? *Blood* **104**, 3231–3232 (2004).
78. Schurgers, L. J. *et al.* Post-translational modifications regulate matrix Gla protein function: importance for inhibition of vascular smooth muscle cell calcification. *J. Thromb. Haemost.* **5**, 2503–2511 (2007).
79. Hara, T. *et al.* Rivaroxaban, a novel oral anticoagulant, attenuates atherosclerotic plaque progression and destabilization in ApoE-deficient mice. *Atherosclerosis* **242**, 639–646 (2015).
80. Bea, F. *et al.* Melagatran Reduces Advanced Atherosclerotic Lesion Size and May Promote Plaque Stability in Apolipoprotein E– Deficient Mice. *Arteriosclerosis, Thrombosis, and Vascular Biology* **26**, 2787–92 (2006).
81. Kadoglou, N. P. E. *et al.* The beneficial effects of a direct thrombin inhibitor, dabigatran etexilate, on the development and stability of atherosclerotic lesions in apolipoprotein E-deficient mice : dabigatran etexilate and atherosclerosis. *Cardiovasc. Drugs Ther.* **26**, 367–374 (2012).
82. Lee, I.-O., Kratz, M. T., Schirmer, S. H., Baumhäkel, M. & Böhm, M. The effects of direct thrombin inhibition with dabigatran on plaque formation and endothelial function in apolipoprotein E-deficient mice. *J. Pharmacol. Exp. Ther.* **343**, 253–257 (2012).
83. Preusch, M. R. *et al.* Dabigatran etexilate retards the initiation and progression of atherosclerotic lesions and inhibits the expression of oncostatin M in apolipoprotein E-deficient mice. *Drug Des. Devel. Ther.* **9**, 5203–5211 (2015).
84. Weitz, J. I. *et al.* Rivaroxaban or aspirin for extended treatment of venous thromboembolism. *J. Vasc. Surg. Venous Lymphat. Disord.* **5**, 767 (2017).
85. Mega, J. L. *et al.* Rivaroxaban in patients with a recent acute coronary syndrome. *N. Engl. J. Med.* **366**, 9–19 (2012).
86. Oldgren, J. *et al.* New oral anticoagulants in addition to single or dual antiplatelet therapy after an acute coronary syndrome: a systematic review and meta-analysis. *Eur. Heart J.* **34**, 1670–1680 (2013).
87. Alexander, J. H. *et al.* Apixaban with antiplatelet therapy after acute coronary syndrome. *N. Engl. J. Med.* **365**, 699–708 (2011).
88. Kernan, W. N. *et al.* Guidelines for the prevention of stroke in patients with stroke and transient ischemic attack: a guideline for healthcare professionals from the American Heart Association/American Stroke Association. *Stroke* **45**, 2160–2236 (2014).
89. Connolly, S. J. *et al.* Apixaban in patients with atrial fibrillation. *N. Engl. J. Med.* **364**, 806–817 (2011).
90. Patel, M. R. *et al.* Rivaroxaban versus warfarin in nonvalvular atrial fibrillation. *N. Engl. J. Med.* **365**, 883–891 (2011).
91. Connolly, S. J. *et al.* Dabigatran versus warfarin in patients with atrial fibrillation. *N. Engl. J. Med.* **361**, 1139–1151 (2009).
92. Hohnloser, S. H. *et al.* Myocardial ischemic events in patients with atrial fibrillation treated with dabigatran or warfarin in the RE-LY (Randomized Evaluation of Long-Term Anticoagulation Therapy) trial. *Circulation* **125**, 669–676 (2012).

93. Uchino, K. & Hernandez, A. V. Dabigatran association with higher risk of acute coronary events: meta-analysis of noninferiority randomized controlled trials. *Arch. Intern. Med.* **172**, 397–402 (2012).
94. Investigators, S. P. S. *et al.* Effects of clopidogrel added to aspirin in patients with recent lacunar stroke. *N Engl J Med* **367**, 817–25 (2012).
95. Eikelboom, J. W. *et al.* Rivaroxaban with or without aspirin in stable cardiovascular disease. *N. Engl. J. Med.* **377**, 1319–1330 (2017).
96. Borst, O. *et al.* Inhibitory mechanisms of very low-dose rivaroxaban in non-ST-elevation myocardial infarction. *Blood Adv.* **2**, 715–730 (2018).

CHAPTER 3

SEARCHING FOR A COMMON THROMBO-INFLAMMATORY BASIS IN PATIENTS WITH DEEP VEIN THROMBOSIS OR PERIPHERAL ARTERY DISEASE

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ABSTRACT

Background

Inflammation and hypercoagulability play a pivotal role in venous thromboembolism and atherothrombosis. Since venous thrombosis increases the risk of atherothrombotic events and vice versa, common mechanisms may be involved.

Objective

To elucidate the role of neutrophils and coagulation in the occurrence of atherothrombotic events in patients with a history of deep vein thrombosis (DVT or peripheral artery disease (PAD).

Methods

We studied 115 patients from two cohorts (75 DVT, 40 PAD). Of those with PAD, 20 patients had progressive disease; from those with DVT, 25 patients had a recurrent DVT, and 25 suffered from post-thrombotic syndrome (PTS); patients were age and sex-matched to DVT and PAD patients without events. Markers of neutrophil recruitment (p-selectin) and activation [nucleosomes, human neutrophil elastase- α1anti-trypsin (HNE-AT)], an anti-inflammatory marker (Lipoxin A4), and a clotting activity marker (d-dimer), were measured with ELISA. Coagulation potential was analyzed by thrombin generation (CAT method).

Results

Higher nucleosome levels were found in DVT patients [11.3 U/mL (7.4–17.7)] compared to PAD patients [7.1 U/mL (5.1–13.8)], lower HNE-AT levels were found in DVT patients [33.4 ng/mL (23.5–40.5)] in comparison to PAD patients [158 ng/mL (88.1–283)]. No difference in nucleosome levels was found between DVT patients with cardiovascular (CV) events [12.6 U/mL (8.2–16.1)], and PAD patients with CV events [6.9 U/mL (4.9–11.2)]. Lipoxin A4 levels appeared to be significantly lower in DVT [2.4 ng/mL (1.7–4.8)] vs. PAD [35.6 ng/mL (16.6–80.1)], with similar results in DVT patients with CV events vs. PAD patients with CV events. Thrombin generation showed higher ETP [160.4% (141.1–215.4)], and peak height [292.1% (177.9–330)] values in DVT patients. D-dimer levels were significantly lower in the DVT cohort [330 ng/mL (220–550)] compared to the PAD cohort [550 ng/mL (369–959)].

Conclusion

In DVT patients, neutrophil activity does not appear to be an important driver of CV events. Although neutrophil activity is more pronounced in PAD, its effect is partly dampened by Lipoxin A4. Moreover, no associations were found between NET products and coagulation activity, suggesting that neutrophil activation does not play a pivotal role in the risk of thrombosis in either DVT or PAD.

INTRODUCTION

There is an increased risk for atherothrombosis following deep venous thrombosis (DVT) [1–3]. *Prandoni et al.* showed that the prevalence of symptomatic atherosclerotic plaques is significantly higher in patients with a history of DVT, suggesting there might be an overlap in the pathogenesis of both diseases [4]. Moreover, epidemiological studies have highlighted the association between venous and arterial thrombosis, mainly focusing on the presence of common risk factors including obesity, diabetes, and hypertriglyceridemia [5]. Although the increased risk of cardiovascular events following venous thrombosis has consistently been reported [6,7], the opposite association is much less defined. Some authors report an increased risk of venous thromboembolism (VTE) in the first 3 to 6 months after an acute arterial cardiovascular event, but after that period, the increased risk disappears [8,9]. Common pathophysiological mechanisms linking arterial and venous thrombosis are most likely related to inflammation and hypercoagulability. Both arterial thrombosis, and to a lesser extent venous thrombosis, have been associated with increased markers of inflammation [10,11]. Additionally, increased thrombin generation *in vivo* [12] and *ex vivo* [13] has been associated with coronary artery disease as well as peripheral artery disease (PAD) and is also related to increased VTE occurrence [14]. One mechanism linking inflammation and coagulation is the activation of neutrophils and the ability to adhere to platelets and endothelial surfaces. These cellular interactions are likely to contribute to a "prethrombotic state" or overt hypercoagulability. P-selectin is a cell adhesion molecule expressed by activated endothelium and activated platelets; its role in inflammation lies in the recruitment of neutrophils. Upregulation of p-selectin expression was shown to be associated with the progression of atherosclerotic lesions [15] while inhibition resulted in accelerated thrombolysis and restoration of blood flow in thrombosed veins in animal studies [16,17]. Activation of neutrophils induces the formation of neutrophil extracellular traps (NETs), an important mechanism in the interplay between inflammation and coagulation. NETs are webs of de-condensed chromatin in the extracellular space with citrullinated histones and proteases, produced by activated neutrophils. NETs have thus far been described in relation to their antimicrobial activity [18], a scaffold function for red blood cells and platelets [19], and activation of platelets [20], facilitating the onset of thrombosis with increased thrombin generation (TG) [21–24]. Investigating the inflammatory and hypercoagulable role of NETs can be challenging because several markers are non-specific for NET formation. Extracellular nucleosomes, for example, are not only released during NET formation, but levels can also be elevated due to necrosis and apoptosis [25]. Unlike nucleosomes, human neutrophil elastase α1anti-trypsin (HNE-AT) complexes are more specific for the formation of NETs [26]. HNE is known to cleave tissue factor pathway inhibitor (TFPI) and stimulate fibrin formation [16]. Lipoxin, a metabolite of arachidonic acid, is a product of the leukocyte-platelet interaction with an important role in dampening the inflammatory response. The role of lipoxins in ischemia-reperfusion is well-known [27], so one could speculate that lipoxin could also be protective in PAD and DVT. Our aim with this explorative study is to assess platelet activation and neutrophil recruitment as well as anti-inflammatory markers and markers of coagulation in patients with

non-acute DVT and patients with peripheral artery disease (PAD), in order to elucidate the role of neutrophils and substantiate arguments for a common biochemical background for the occurrence of atherothrombotic events in these patient populations with chronic vascular disease.

METHODS

Patients and controls

Plasma was collected from patients from two previously established cohorts (PAD and DVT) at the Maastricht University Medical Center+ (MUMC+) [28,29]. From the cohort of patients with PAD, 40 patients were selected; 20 patients who experienced a cardiovascular (CV) event during follow-up and 20 patients who did not experience such an event, matched for age and sex to patients with CV events. Cardiovascular events were defined as myocardial infarction or angina pectoris (n = 1), stroke (n = 1), acute limb ischemia or revascularization procedure (n = 17), or the need for interventional treatment (n = 1). Patients from the PAD cohort were newly diagnosed by ankle-brachial index (ABI) measurements, an ABI < 0.9 was considered to indicate PAD [30]. Most of these patients were classified as stage IIa or IIb in the Fontaine classification [31]. Patients were excluded based on the use of medication affecting coagulation (except for platelet aggregation inhibitors), documented congenital coagulation disorders, documented chronic inflammatory diseases, active malignancy, pregnancy, and age <18. Patients with DVT were selected from a cohort of patients that experienced at least one DVT. From this cohort, we selected a total of 75 patients; 25 patients who had experienced a recurrent event during follow-up, 25 age and sex-matched patients that did not experience a recurrent thrombotic event, and 25 patients from the same cohort that developed post-thrombotic syndrome (PTS). Patients with documented chronic inflammatory diseases or with known venous insufficiency were excluded. The Medical Ethics Committee (METC) of the Maastricht University Medical Center (MUMC+) approved both the PAD study (NL19929.068.07) and the DVT study (METC 15-4-256).

Blood collection and storage

Venous blood was drawn from subjects in resting condition and collected by antecubital venipuncture with 21-gauge needles and 3.2% (w/v) citrated Vacutainer glass tubes. The tubes were processed using the standard platelet-poor plasma (PPP) centrifugation protocol used at our laboratory (2,000 × g for 5 min, 10,000 × g for 10 min). Samples were frozen and stored at –80°. Analysis was performed at one point in time, avoiding repeated freeze-thaw cycles.

Measurements

Plasma levels of extracellular nucleosomes and HNE-AT were assayed using in-house ELISAs as described previously [32]. To detect extracellular nucleosomes, monoclonal antibody CLB-ANA/60 (Sanquin, Amsterdam, the Netherlands) which recognizes histone 3 was coated. Biotinylated F(ab)2 fragments of monoclonal antibody CLB-ANA/58 (Sanquin, Amsterdam, the Netherlands), that recognizes the histone 2A/2B complex when bound to DNA was used for detection. As a standard, culture supernatant of apoptotic Jurkat cells (1×10^6 cells/mL) was used. One unit is the number of nucleosomes released by ≈100 Jurkat cells. The lower detection limit of the assay was 2.5 U/mL, the coefficients of variation were 8.5% (inter-assay) and 4.3% (intra-assay).

HNE-AT complexes were detected using plates coated with a polyclonal rabbit anti-human neutrophil elastase antibody (Sanquin, Amsterdam, the Netherlands). Bound complexes were detected by incubation with biotinylated monoclonal anti–α1-antitrypsin antibody followed by poly-horseradish peroxidase-labeled streptavidin. Results are expressed in ng/mL by reference to a standard curve of normal human citrated plasma in which HNE-AT complexes were generated by incubating with purified elastase for 15 min at room temperature. The lower detection of the assay was 2 ng/mL. The coefficients of variation were 9.5% (inter-assay) and 5.7% (intra-assay).

Soluble p-selectin levels were assessed in plasma samples using commercially available ELISA (R&D MyBioSource, San Diego, California, USA). Lipoxin plasma levels were evaluated using a commercially available ELISA kit (Human lipoxin A4 (LXA4), Bio-Connect Services, Huissen, the Netherlands). All commercial ELISA's were performed according to the manufacturer's instructions. The coagulation potential in plasma was assessed using the calibrated automated thrombin generation (CAT) assay (Thrombinoscope BV). This method employs a low-affinity fluorogenic substrate for thrombin, and thereby enables continuous monitoring of thrombin activity in clotting plasma. For each measurement, 80 µL of human PPP was added to 20 µL of fluorogenic substrate, 20 µL of trigger reagent, and calcium chloride, as previously reported [33]. D-dimer fragments, a marker for fibrin formation and cleavage were determined as part of routine patient management using a latex-enhanced immunoassay (Innovance assay, Siemens Healthcare, Marburg, Germany).

Statistical analysis

Baseline characteristics were collected and differences between cohorts were analyzed using Chi-square testing. Differences in plasma levels of all markers between the DVT and PAD cohort were analyzed using the two samples *t*-test (parametric) or Mann-Whitney test (non-parametric). *T*-test results are shown as the mean and standard deviation, and Mann-Whitney results are shown as the median and 25th and 75th percentile. Subgroup analysis was performed with the one-way ANOVA (parametric) or the Kruskal-Wallis test (non-parametric). ANOVA

results are shown as the mean and standard deviation, and Kruskal-Wallis results are shown as the median and 25th and 75th percentile. Correlations between d-dimer levels and markers of neutrophil activation were analyzed using linear regression. $P < 0.05$ was considered statistically significant. Identification and exclusion of outliers were performed using the ROUT method. Testing for normality was carried out with the D'Agostino Pearson test. All analyses were performed using GraphPad Prism version 7 for MAC OS X, GraphPad Software, La Jolla California USA, www.graphpad.com.

RESULTS

Baseline characteristics

Plasma from a total of 115 patients, 75 DVT patients and 40 PAD patients, was analyzed. Patient characteristics for these patients are shown in *Table 1*. Within the selected population of 75 patients from the DVT cohort we identified 10 patients with cardiovascular (CV) events, 2 of whom also had CV events prior to the DVT. These 10 patients were evenly distributed over the subgroups, with, respectively 3 patients in the DVT group without recurrence, 3 patients in the DVT group with recurrence, and 4 patients in the DVT group with PTS, leaving 65 DVT patients without CV events *(Figure 1)*. Patients in the DVT cohort who experienced a CV event were older compared to patients in the other groups. PAD patients with CV events were more likely to be female. As expected, the use of oral anticoagulants was higher in DVT patients (63%) compared to PAD patients (0%), while the use of antiplatelet agents was much lower in DVT (5%) than in PAD (95%).

	DVT	**DVT**	**PAD**	**PAD**
	CV event	**No CV event**	**CV event**	**No CV event**
	n = 10	n = 65	n = 20	n = 20
Mean age (SD)	**76 (7.5)***	69 (13.1)	68.7 (8.3)	68.3 (6)
Male gender (%)	7 (70)	42 (76.4)	9 (45)	**15 (75)***
Anticoagulants (VKA, DOAC) (%)	7 (70)	40 (72.7)	0 (0)	0 (0)
Antiplatelet medication (%)	**3 (30)***	1 (1.8)	20 (100)	18 (90)
Statin (%)	**3 (30)***	5 (9.1)	14 (70)	15 (75)

Table 1: Baseline characteristics, significant differences ($p < 0.05$) within cohorts depicted with *. VKA, vitamin K antagonist; DOAC, direct oral anticoagulant.

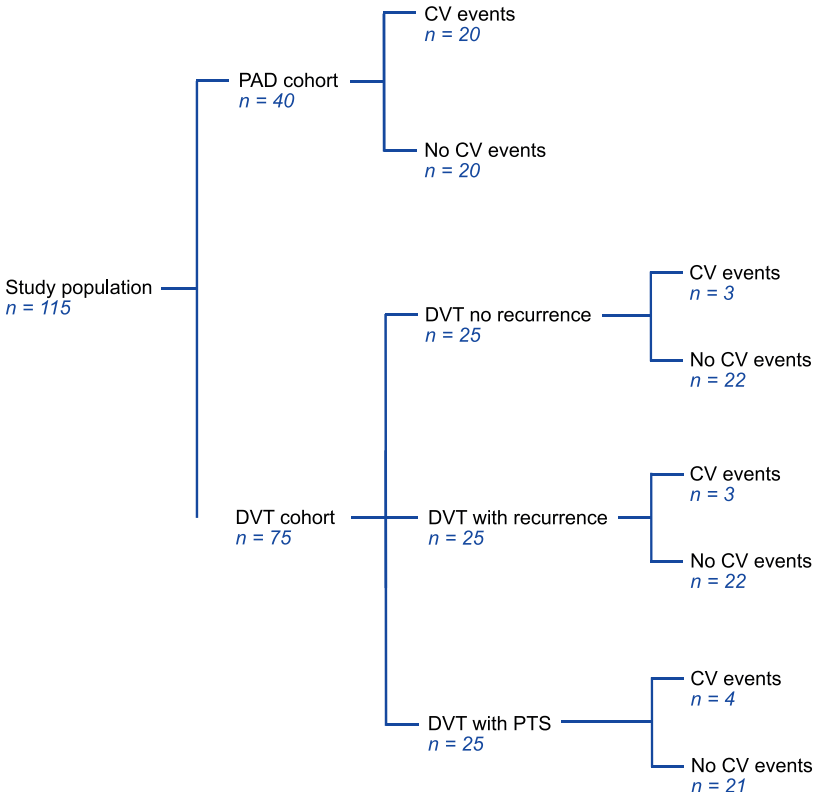

Figure 1: Cohort distribution of 40 patients with PAD and 75 patients with DVT.

Markers of inflammation

Plasma levels of p-selectin were assessed to gather information on the activation of blood platelets and endothelial cells, with potential impact on the recruitment of neutrophils. No difference in p-selectin levels in plasma from DVT and PAD patients was found (DVT 35.6 ng/mL (27.3–40.9), PAD 37.9 ng/mL (30.4–46.2), $p = 0.08$) *(Figure 2A)*. P-selectin levels did not differ in DVT patients with CV events compared to PAD patients with events *(Table 2, Figure 2B)*. P-selectin levels neither differed between the DVT subgroups *(Table 3)*.

Neutrophil activation was assessed by measuring plasma nucleosome levels and levels of HNE-AT. Nucleosome plasma levels were significantly higher in the DVT cohort compared to the PAD cohort (11.3 U/mL (7.4–17.7) vs. 7.1 U/mL (5.1–13.8), $p < 0.01$) *(Figure 2C)*. Plasma nucleosome levels in DVT patients with CV events were similar to nucleosomes levels in PAD patients with CV events [12.6 U/mL (8.2–16.1) vs. 6.9 U/mL (4.9–11.2), $p = 0.32$] *(Figure 2D)*. Analysis of the DVT subgroups showed significantly higher nucleosome levels in patients with PTS *(Table 3)*.

Plasma levels of HNE-AT were substantially lower in DVT patients compared to PAD patients (33.4 ng/mL (23.5–40.5) vs. 158 ng/mL (88.1–283), $p < 0.001$) *(Figure 2E)*. DVT patients with CV events also had significantly lower HNE-AT levels compared to PAD patients with CV events *(Table 2, Figure 2F)*. DVT subgroup analysis showed higher HNE-AT plasma levels in PTS patients *(Table 3)*.

Anti-inflammatory marker lipoxin A4 was found to be significantly lower in DVT patients compared to PAD patients [2.4 ng/mL (1.7–4.8) vs. 35.6 ng/mL (16.6–80.1), $p < 0.001$] *(Figure 2G)*. DVT patients with CV events had significantly lower lipoxin A4 levels compared to PAD patients with CV events *(Table 2, Figure 2H)*. No differences were observed between DVT subgroups *(Table 3)*.

	All DVT	All PAD	P-value	DVT + CV event	PAD + CV event	P-value
P-selectin (ng/mL)	35.6 (27.3-40.9)	37.9 (30.4-46.2)	NS	35.5 (25.3-40.4)	42.9 (32.3-47.8)	NS
Nucleosomes (U/mL)	11.3 (7.4-17.7)	7.1 (5.1-13.8)	< 0.01	12.6 (8.2-16.1)	6.9 (4.9-11.2)	NS
HNE-AT (ng/mL)	33.4 (23.5-40.5)	158 (88.1-283)	< 0.001	33.4 (23-39.4)	33.2 (22.9-38.1)	NS
Lipoxin A4 (ng/mL)	2.4 (1.7-4.8)	35.6 (16.6-80.1)	< 0.001	2 (1.5-3)	51.2 (18.7-88.7)	< 0.001

Table 2: Inflammatory markers in all DVT and PAD patients, and specifically in DVT and PAD patients with CV events. P-values ≥ 0.05 are shown as non-significant (NS).

	DVT SUBGROUPS				
	No recurrence	Recurrence	P-value	PTS	P-value
P-selectin (ng/mL)	30.4 (23-39.2)	36.4 (29.5-45.9)	NS	37 (27.8-42.9)	NS
Nucleosomes (U/mL)	8.7 (5.5-11.3)	11.3 (7.3-19.3)	NS	17 (15.3-24.9)	< 0.001
HNE-AT (ng/mL)	31.3 (20.6-35.9)	35.1 (24.5-38)	NS	40.4 (31.3-44.6)	0.046
Lipoxin A4 (ng/mL)	2.1 (1.6-4.3)	2.6 (1.8-4.2)	NS	3.4 (1.7-10.5)	NS

Table 3: Inflammatory markers in the DVT subgroups a) no recurrence, b) recurrence, and c) PTS. P-values ≥ 0.05 are shown as non-significant (NS).

Markers of coagulation

The CAT thrombin generation assay yields data on lag time, endogenous thrombin potential (ETP), and peak height. Both the ETP and peak height are shown as "normalized" values. Thrombin generation and d-dimer levels were only assessed in patients not on anticoagulant treatment *(Figure 3)*. The lag time was equal in DVT patients and PAD patients, with, respectively 5.5 min (4.8–6.7) and 5.5 min (4.7–6.2), $p = 0.83$. No difference in lag time was found between DVT patients with CV events and PAD patients with CV events *(Table 4)*. Subgroup analysis of the DVT cohort showed no differences either *(Table 5)*. We observed a higher ETP in DVT patients [160.4% (141.1–215.4)] compared to PAD patients [75.7% (59.3–93.1)], $p < 0.001$. A non-significant difference was found between DVT patients with CV events [144.7% (98.1–192.3)] and PAD patients with CV events [70.4 % (57.6–92.6)], $p = 0.11$. Subgroup analysis of the DVT cohort showed no differences in ETP *(Table 5)*. Peak height was significantly higher in DVT patients compared to PAD patients [292.1% (177.9–330) vs. 82.2% (53.8–103.7), $p < 0.001$]. Higher peak height levels were also observed in DVT patients with CV events compared to PAD patients with CV events *(Table 4)*. No differences in peak height were found between the DVT subgroups *(Table 5)*.

D-dimer levels were significantly lower in the DVT cohort compared to the PAD cohort, respectively 330 ng/mL (220–550) and 550 ng/mL (369–959), $p = 0.003$. No differences in d-dimer levels were observed between DVT patients with CV events and PAD patients with CV events [380 ng/mL (199–500) vs. 524 ng/mL (362.3–1024), $p = 0.99$]. DVT subgroup analysis showed significantly lower d-dimer levels in DVT patients without recurrence compared to patients with recurrence and patients with PTS *(Table 5)*. D-dimer levels in DVT patients did not correlate with nucleosome levels ($p = 0.42$) or HNE-AT levels ($p = 0.14$). There neither was a correlation with nucleosome levels ($p = 0.49$) nor HNE-AT levels ($p = 0.38$) in PAD patients.

	All DVT	All PAD	P-value	DVT + CV event	PAD + CV event	P-value
Lag time (min)	5.5 (4.7-6.2)	5.5 (4.8-6.7)	NS	5.8 (4.9-6.9)	5.5 (4.3-6.6)	NS
ETP (%)	160.4 (141.1-215.4)	75.5 (59.3-93.1)	< 0.001	144.7 (98.1-192.3)	70.4 (57.6-92.6)	NS
Peak height (%)	292.1 (177.9-330)	82.2 (53.8-103.7)	< 0.001	238 (169.3-319)	74.4 (51.4-100.9)	< 0.001
D-dimer (ng/mL)	330 (220-550)	550 (369-959)	0.003	380 (199-500)	524 (362.3-1024)	NS

Table 4: Coagulation markers in all DVT and PAD patients, and specifically in DVT and PAD patients with CV events. P-values ≥ 0.05 are shown as non-significant (NS).

	DVT SUBGROUPS				
	No recurrence	**Recurrence**	**P-value**	**PTS**	**P-value**
Lag time (min)	5.7 (4-6.7)	5.2 (4.8-6.7)	NS	6.5 (4.3-7.6)	NS
ETP (%)	151 (63.4-198.6)	183.2 (150.5-223.5)	NS	160.4 (111.5-203.6)	NS
Peak height (%)	236.2 (103.2-329.5)	295.1 (214.3-412.7)	NS	290.3 (173.6-330)	NS
D-dimer (ng/mL)	225 (199-297)	580 (330-1950)	0.008	380 (257-500)	0.001

Table 5: Coagulation markers in the DVT subgroups a) no recurrence, b) recurrence, and c) PTS. P-values ≥ 0.05 are shown as non-significant (NS).

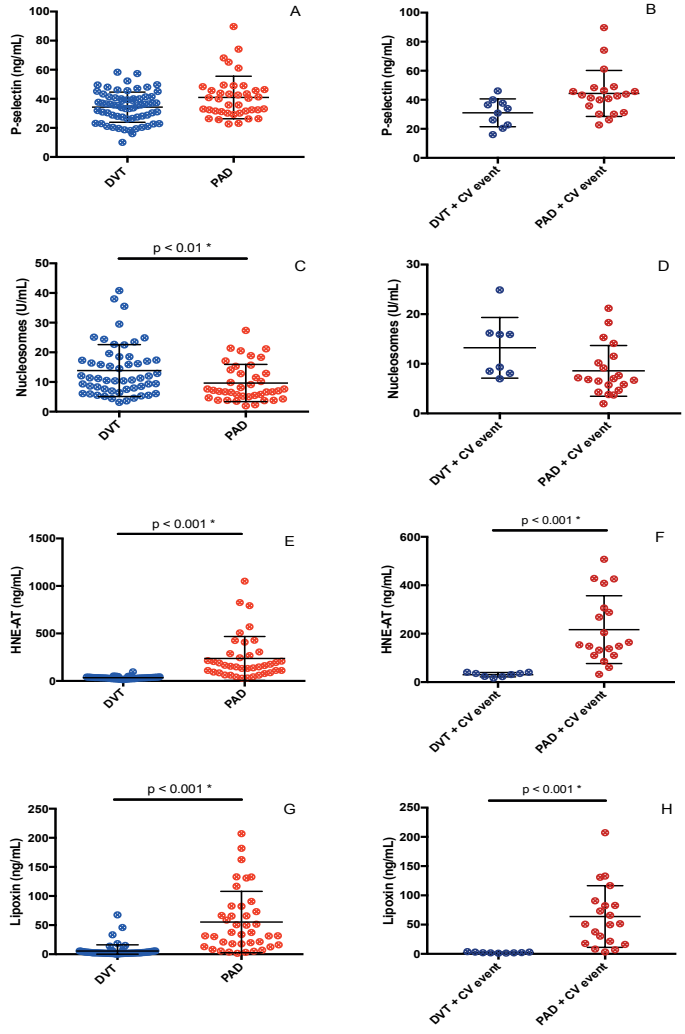

Figure 2: Inflammatory (A-F) and anti-inflammatory (G, H) markers in DVT patients and PAD patients.

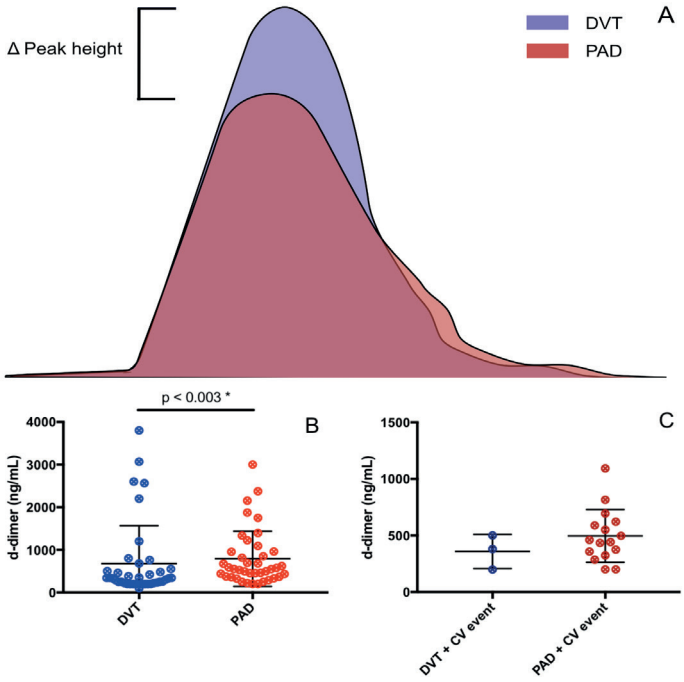

Figure 3: Coagulation markers in DVT patients and PAD patients. Figure A resembles thrombin generation curves in PAD and DVT patients, with an increased peak height seen in DVT patients.

DISCUSSION

In this explorative study, we assessed the role of inflammation and coagulation in patients with non-acute DVT and patients with PAD in order to demonstrate a possible common biochemical background for the occurrence of atherothrombotic events in these patient populations. We used highly selected markers reflecting the interplay between platelet and endothelial activation (soluble p-selectin), neutrophil activation (nucleosomes and HNE-AT) as well as an inflammation inhibiting pathway component (lipoxin A4); Finally, we explored the activity of the coagulation system by probing the potential to generate thrombin and by assessing a sensitive marker of coagulation activity, utilizing d-dimer *(Figure 4)*.

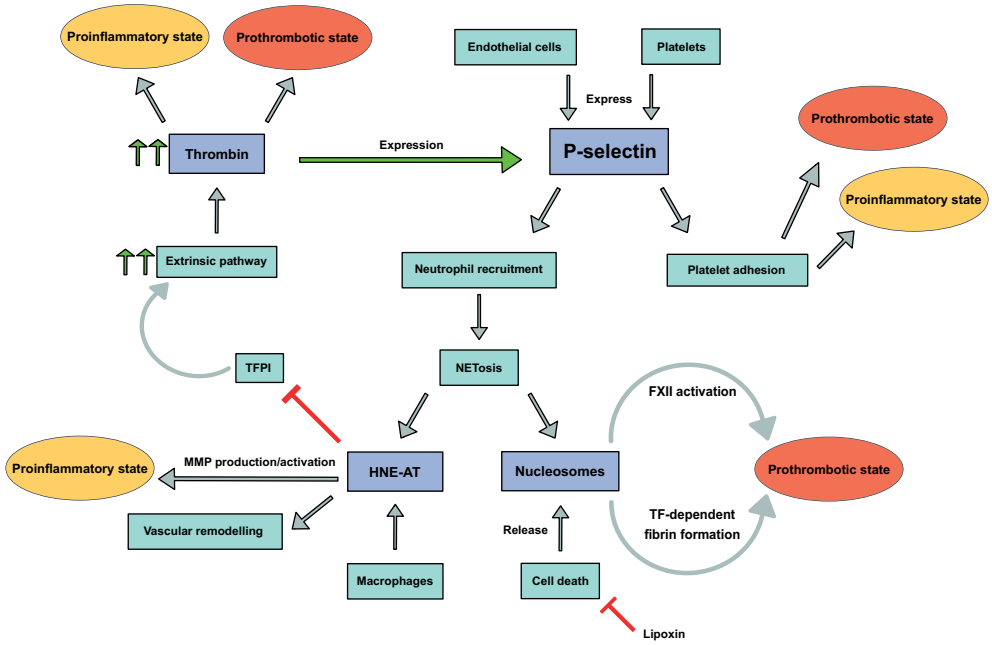

Figure 4: P-selectin expression with downstream pathways leading to a prothrombotic and/or proinflammatory state. FXII, Factor XII; TF, tissue factor; TFPI, tissue factor pathway inhibitor; MMP, matrix metalloproteinase.

We postulated that p-selectin would play a role in the interactions among platelets, leukocyte populations, and endothelial cells, together with CD40 and several chemokines derived from platelets [34,35]. We demonstrate non-significantly higher levels of p-selectin in PAD patients compared to DVT patients, indicating that at least in the non-acute phase of vascular disease, there is no major difference in the degree of platelet activation and to a lesser extent endothelial activation. This may be in part related to the fact that the clear majority of PAD patients were on antiplatelet therapy attenuating platelet reactivity to some extent [36].

In contrast to the lack of difference in p-selectin levels between patients with venous or arterial vascular disease, substantial differences in plasma levels of neutrophil products were observed. We demonstrate higher nucleosome levels in non-acute DVT patients, whereas higher HNE-AT levels were observed in PAD patients: the differences in plasma levels of both biomarkers were unexpected and may in part be explained by their specificity for NETosis. Activated neutrophils secrete nucleosomes, but apoptosis in any cell type will also give rise to increased nucleosome levels due to cell destruction [37]. One could speculate that cell death, for instance, due to venous congestion secondary to venous thrombosis, is a more prominent phenomenon in patients with a prior DVT than in those with arterial vascular disease. Subgroup analysis demonstrated different nucleosome levels within the DVT cohort, as DVT patients with PTS had higher

nucleosome levels compared to DVT patients without PTS or recurrence. These results suggest more pronounced apoptosis with the release of nucleosomes in relation to post-thrombotic vascular remodeling. In contrast, HNE-AT is a more specific marker for NETosis by neutrophils, although macrophages may also secrete HNE. Both neutrophils and macrophages are key players in atherosclerosis and atherothrombosis. Dollery et al. demonstrated the presence of HNE in macrophage-rich shoulders of atherosclerotic plaques but not in normal arteries. Moreover, these macrophages were shown to contain HNE mRNA. One may indeed expect that systemic HNE levels are also higher in PAD patients, reflecting the burden of atherosclerotic disease. It remains uncertain how substantial the contribution of neutrophil activation is toward the measured HNE-AT levels. While the association between nucleosomes and vascular remodeling has yet to be investigated, neutrophil elastase (measured as HNE-AT) is known to contribute to vascular remodeling and promote plaque rupture [38]. We did, however, not find an increase in CV events in patients with higher HNE-AT levels. Moreover, plasma levels of nucleosomes and HNE-AT in DVT patients with CV events differed significantly from plasma levels in PAD patients with these events, making it highly unlikely that neutrophil activation is a common mechanism of comparable significance in the occurrence of CV events in both cohorts.

A wide variety of mechanisms that counteract inflammatory processes have been identified in the past, and we aimed to assess anti-inflammatory capabilities by measuring lipoxin A4 levels. We demonstrate significantly higher lipoxin A4 levels in PAD patients in comparison to DVT patients. Lipoxin levels were not associated with the occurrence of CV events in both DVT and PAD patients. The relatively high levels of lipoxin A4 may at least in part be attributed to the actions of low-dose aspirin, known to stimulate the levels of this anti-inflammatory mediator *in vivo* [39]. Another factor may be the stronger activity of macrophages in patients with atherosclerosis, as compared to the DVT cohort, which may also modulate the production of lipoxin A4. Via this mechanism, a macrophage can limit its apoptosis pathway, as previous studies have shown inhibition of apoptosis by lipoxin A4 [40].

Neutrophils and hypercoagulability

We assessed hypercoagulability by measuring thrombin generation and d-dimer levels. Thrombin generation appeared to be increased in DVT patients, whereas d-dimer levels were higher in PAD patients. We found no difference in lag time, indicating that the onset of thrombin generation is essentially the same. ETP and peak height were, on the other hand, higher in DVT patients, implicating that a prethrombotic state is more pronounced in DVT patients. Due to low numbers, we were not able to show significantly higher ETP values in DVT patients with CV events in comparison to PAD patients with CV events. These thrombin generation results are consistent with the concept that venous thrombosis is stronger dependent on blood coagulation reactivity, as compared to atherothrombotic events in atherosclerosis. A recent study pointed out that PAD patients have a “normal” thrombin generation profile compared to healthy

controls, which is in line with our results. *Kleinegris et al.* however also demonstrated an increased ability to form stable clots in comparison to healthy controls, most likely due to an increase in fibrinogen in atherosclerosis [28]. These conclusions support our finding that d-dimer levels are increased in PAD patients. As more fibrin is formed, more fibrinolysis will likely occur, and thus more d-dimer will be formed.

Activated neutrophils can support coagulability via several mechanisms. In DVT, where nucleosomes are more pronounced, FXII can be auto-activated by negatively charged DNA backbones [41]. Moreover, nucleosomes inhibit TFPI and thereby also promote coagulation [42]. Human neutrophil elastase, increased in PAD, stimulates matrix metalloproteinase (MMP) production and activation. Furthermore, through inhibition of TFPI by elastase, thrombin and subsequent fibrin production as well as vascular remodeling may be promoted [16]. Together, these data suggest different neutrophil (and probably macrophage) mediated mechanisms to be operational in venous and arterial vascular disease.

Strengths and limitations

Thrombo-inflammation is involved in both arterial and venous thrombosis, and common pathophysiological pathways have yet to be elucidated. This study gives insights into the possible involvement of neutrophil activation in the occurrence of thrombotic events. Our results are hypothesis-generating regarding pathogenic mechanisms in DVT and PAD.

The number of patients in the DVT and PAD cohort was too small to reliably analyze subgroups. Here, we also had to exclude DVT patients on anticoagulants to prevent interference with the outcome of thrombin generation testing and d-dimer levels. In general, the selected biomarkers are insufficient to document the contribution of platelets, neutrophils, and other relevant cells and microvesicles in these complex pathologies. In addition, even statistically significant differences for markers like nucleosomes should be considered with caution, given the lack of knowledge on the biological significance. For this matter, our data and interpretation of differences between populations must be regarded as hypothesis-generating.

Conclusion

Our findings suggest that in patients with previous DVT neutrophil activity is not an important driver of CV events. In subjects with PAD, neutrophil activity is more pronounced and in part dampened by increased lipoxin A4. We did not detect any associations between neutrophil and nucleosome levels and markers of coagulation activity, suggesting that neutrophil activation is not a common driver of thrombosis risk in DVT and PAD.

REFERENCES

1. Prandoni, P. *et al.* Venous thromboembolism and the risk of subsequent symptomatic atherosclerosis. *J. Thromb. Haemost.* 4, 1891–1896 (2006).
2. Schulman, S. *et al.* Post-thrombotic syndrome, recurrence, and death 10 years after the first episode of venous thromboembolism treated with warfarin for 6 weeks or 6 months. *J. Thromb. Haemost.* **4**, 734–742 (2006).
3. Andrei, M.-C. & Andercou, A. Is there a link between atherothrombosis and deep venous thrombosis? *Maedica (Buchar)* **9**, 94–97 (2014).
4. Prandoni, P. *et al.* An association between atherosclerosis and venous thrombosis. *N. Engl. J. Med.* **348**, 1435–1441 (2003).
5. Lowe, G. D. O. Common risk factors for both arterial and venous thrombosis. *Br. J. Haematol.* **140**, 488–495 (2008).
6. Sørensen, H. T., Horvath-Puho, E., Pedersen, L., Baron, J. A. & Prandoni, P. Venous thromboembolism and subsequent hospitalisation due to acute arterial cardiovascular events: a 20-year cohort study. *Lancet* **370**, 1773–1779 (2007).
7. Lind, C. *et al.* Family history of myocardial infarction and cause-specific risk of myocardial infarction and venous thromboembolism: the Tromsø Study. *Circ. Cardiovasc. Genet.* **7**, 684–691 (2014).
8. Sørensen, H. T. *et al.* Arterial cardiovascular events, statins, low-dose aspirin and subsequent risk of venous thromboembolism: a population-based case-control study. *J. Thromb. Haemost.* **7**, 521–528 (2009).
9. Rinde, L. B. *et al.* Impact of incident myocardial infarction on the risk of venous thromboembolism: the Tromsø Study. *J. Thromb. Haemost.* **14**, 1183–1191 (2016).
10. Horvei, L. D. *et al.* C-reactive protein, obesity, and the risk of arterial and venous thrombosis. *J. Thromb. Haemost.* **14**, 1561–1571 (2016).
11. Zacho, J., Tybjaerg-Hansen, A. & Nordestgaard, B. G. C-reactive protein and risk of venous thromboembolism in the general population. *Arterioscler. Thromb. Vasc. Biol.* **30**, 1672–1678 (2010).
12. Merlini, P. A. *et al.* In vivo thrombin generation and activity during and after intravenous infusion of heparin or recombinant hirudin in patients with unstable angina pectoris. *Arterioscler. Thromb. Vasc. Biol.* **20**, 2162–2166 (2000).
13. Smid, M. *et al.* Thrombin generation in the Glasgow Myocardial Infarction Study. *PLoS One* **8**, e66977 (2013).
14. Korte, W., Clarke, S. & Lefkowitz, J. B. Short activated partial thromboplastin times are related to increased thrombin generation and an increased risk for thromboembolism. *Am. J. Clin. Pathol.* **113**, 123–127 (2000).
15. Zhang, N., Liu, Z., Yao, L., Mehta-D'souza, P. & McEver, R. P. P-selectin expressed by a human SELP transgene is atherogenic in apolipoprotein E-deficient mice. *Arterioscler. Thromb. Vasc. Biol.* **36**, 1114–1121 (2016).
16. Fuchs, T. A., Brill, A. & Wagner, D. D. Neutrophil extracellular trap (NET) impact on deep vein thrombosis. *Arterioscler. Thromb. Vasc. Biol.* **32**, 1777–1783 (2012).
17. Myers, D. D., Jr *et al.* P-selectin inhibition enhances thrombus resolution and decreases vein wall fibrosis in a rat model. *J. Vasc. Surg.* **36**, 928–938 (2002).
18. Brinkmann, V. *et al.* Neutrophil extracellular traps kill bacteria. *Science* **303**, 1532–1535 (2004).
19. Fuchs, T. A. *et al.* Extracellular DNA traps promote thrombosis. *Proc. Natl. Acad. Sci. U. S. A.* **107**, 15880–15885 (2010).

20. Semeraro, F. *et al.* Extracellular histones promote thrombin generation through platelet-dependent mechanisms: involvement of platelet TLR2 and TLR4. *Blood* **118**, 1952–1961 (2011).
21. Wang, Y. *et al.* Neutrophil extracellular trap-microparticle complexes enhance thrombin generation via the intrinsic pathway of coagulation in mice. *Sci. Rep.* **8**, (2018).
22. McDonald, B. *et al.* Platelets and neutrophil extracellular traps collaborate to promote intravascular coagulation during sepsis in mice. *Blood* **129**, 1357–1367 (2017).
23. Gould, T. J. *et al.* Neutrophil extracellular traps promote thrombin generation through platelet-dependent and platelet-independent mechanisms. *Arterioscler. Thromb. Vasc. Biol.* **34**, 1977–1984 (2014).
24. Borissoff, J. I. *et al.* Elevated levels of circulating DNA and chromatin are independently associated with severe coronary atherosclerosis and a prothrombotic state. *Arterioscler. Thromb. Vasc. Biol.* **33**, 2032–2040 (2013).
25. Radic, M., Marion, T. & Monestier, M. Nucleosomes are exposed at the cell surface in apoptosis. *J. Immunol.* **172**, 6692–6700 (2004).
26. Kaufman, T. *et al.* Nucleosomes and neutrophil extracellular traps in septic and burn patients. *Clin. Immunol.* **183**, 254–262 (2017).
27. Zhao, Q. *et al.* Lipoxin a4 preconditioning and postconditioning protect myocardial ischemia/reperfusion injury in rats. *Mediators Inflamm.* **2013**, 231351 (2013).
28. Kleinegris, M.-C. F. *et al.* Increased clot formation in the absence of increased thrombin generation in patients with peripheral arterial disease: A case-control study. *Front. Cardiovasc. Med.* **4**, 23 (2017).
29. Nagler, M., ten Cate, H., Prins, M. H. & ten Cate-Hoek, A. J. Risk factors for recurrence in deep vein thrombosis patients following a tailored anticoagulant treatment incorporating residual vein obstruction. *Res. Pract. Thromb. Haemost.* **2**, 299–309 (2018).
30. Mota, A. P. L. *et al.* Hypercoagulability markers in patients with peripheral arterial disease: association to ankle-brachial index. *Angiology* **60**, 529–535 (2009).
31. Sartori, M. *et al.* Thrombophilic risk factors and peripheral arterial disease severity. *Thromb. Haemost.* **104**, 71–77 (2010).
32. van Montfoort, M. L. *et al.* Circulating nucleosomes and neutrophil activation as risk factors for deep vein thrombosis. *Arterioscler. Thromb. Vasc. Biol.* **33**, 147–151 (2013).
33. Spronk, H. M. H. *et al.* Assessment of thrombin generation II: Validation of the Calibrated Automated Thrombogram in platelet-poor plasma in a clinical laboratory. *Thromb. Haemost.* **100**, 362–364 (2008).
34. Thomas, M. R. & Storey, R. F. The role of platelets in inflammation. *Thromb. Haemost.* **114**, 449–458 (2015).
35. Koedam, J. A. *et al.* P-selectin, a granule membrane protein of platelets and endothelial cells, follows the regulated secretory pathway in AtT-20 cells. *J. Cell Biol.* **116**, 617–625 (1992).
36. van Geffen, J. P. *et al.* Normal platelet activation profile in patients with peripheral arterial disease on aspirin. *Thromb. Res.* **135**, 513–520 (2015).
37. Wu, D. *et al.* Apoptotic release of histones from nucleosomes. *J. Biol. Chem.* **277**, 12001–12008 (2002).
38. Dollery, C. M. *et al.* Neutrophil elastase in human atherosclerotic plaques: production by macrophages. *Circulation* **107**, 2829–2836 (2003).

39. Chiang, N., Bermudez, E. A., Ridker, P. M., Hurwitz, S. & Serhan, C. N. Aspirin triggers antiinflammatory 15-epi-lipoxin A4 and inhibits thromboxane in a randomized human trial. *Proc. Natl. Acad. Sci. U. S. A.* **101**, 15178–15183 (2004).
40. Chandrasekharan, J. A. & Sharma-Walia, N. Lipoxins: nature's way to resolve inflammation. *J. Inflamm. Res.* **8**, 181–192 (2015).
41. Schulz, C., Engelmann, B. & Massberg, S. Crossroads of coagulation and innate immunity: the case of deep vein thrombosis. *J. Thromb. Haemost.* **11 Suppl 1**, 233–241 (2013).
42. Broze, G. J., Jr & Girard, T. J. Tissue factor pathway inhibitor: structure-function. *Front. Biosci. (Landmark Ed.)* **17**, 262–280 (2012).

CHAPTER 4

PLASMA BIOMARKERS TO PREDICT CARDIOVASCULAR OUTCOME IN PATIENTS WITH PERIPHERAL ARTERY DISEASE: A SYSTEMATIC REVIEW AND META-ANALYSIS

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ABSTRACT

Background and objective

Patients with lower extremity peripheral artery disease (PAD) are at increased risk of major adverse cardiovascular events (MACE). Numerous plasma biomarkers have been investigated in lower extremity PAD, but none are used for clinical risk assessment. We aimed to provide a comprehensive overview of biomarker testing in PAD as a first step to improving risk stratification.

Methods and results

A systematic literature review in MEDLINE/PubMed, Cochrane, and Embase was performed, identifying all studies investigating plasma biomarkers in association with cardiovascular events and mortality in lower extremity PAD. Forty-seven studies comprising 21473 PAD patients met our criteria and were included. Effect estimates were provided by the studies based on a minimum follow-up of one year. Meta-analyses were performed by pooling studies per biomarker for each endpoint. Patients with increased hs-CRP levels had a RR of 1.86 (1.48-2.33) for MACE and a RR of 3.49 (2.35-5.19) for mortality. Increased fibrinogen and d-dimer levels were associated with an increased RR of mortality of 2.08 (1.46-2.97) and 2.22 (1.24-3.98), respectively. Additionally, patients with increased NT-proBNP and hs-cTnT levels were at an even higher risk of mortality with RRs of 4.50 (2.98-6.81) and 3.33 (2.70-4.10), respectively.

Conclusion

This systematic review identifies promising biomarkers representing different pathophysiological processes implicated in lower extremity PAD, including hs-CRP, NLR, fibrinogen, d-dimer, NT-proBNP, and hs-cTnT. Clinical implementation should be preceded by a management study to test the utility of a combination of these markers for individual risk stratification. Ultimately, this may contribute to tailored treatment and increased effectiveness of current treatment strategies in PAD.

INTRODUCTION

Peripheral arterial occlusive disease is a manifestation of systemic atherosclerosis resulting in progressive blood flow restriction in peripheral arteries, ultimately leading to atherothrombosis. Although PAOD can occur in multiple arterial beds, the focus of this systematic review will be on lower extremity peripheral artery disease (PAD). Like any atherosclerotic disease, the prevalence of lower extremity PAD continues to increase worldwide, now affecting 5% of the population aged 45-49 years up to 18% at the age of 85-89 years in high-income countries [1]. Risk factors contributing to this high prevalence include hypertension, obesity, hyperlipidemia, diabetes, and smoking [2]. Lower extremity PAD patients have increased mortality rates compared to non-PAD populations, mainly due to higher incidences of myocardial infarction and stroke [3–6]. These patients typically have more extensive coronary artery disease as well as increased progression of atherosclerosis [7].

While on average the risk of cardiovascular complications including death is increased, there is marked heterogeneity among patients. Individual risk estimation in PAD patients is based on the Fontaine or Rutherford classification in combination with the ankle-brachial index which is the current gold standard for vascular severity classification. These classifications divide lower extremity PAD into two major groups, namely patients with claudication (Fontaine I and II) and patients with chronic limb-threatening ischemia (Fontaine III and IV). Patients with claudication tend to be the mild PAD group, whereas patients with chronic limb-threatening ischemia are the more severe PAD patients. To improve the risk estimation in lower extremity PAD as a whole, many plasma biomarkers have been investigated. Since systemic atherosclerosis is characterized by chronic inflammation, most of the investigated biomarkers are inflammation-related. A recent systematic review specifically focused on C-reactive protein (CRP) and showed an association with major cardiovascular events in lower extremity PAD populations [8].

Coagulation markers, such as d-dimer and fibrinogen have also been extensively studied. A systematic review conducted in 2013, found a two-fold increased risk of arterial thrombotic events and cardiovascular mortality in subjects with lower extremity PAD and increased levels of d-dimer. Short-term prediction of cardiovascular events was found to be more successful compared to long-term prediction (> 4 years) [9]. The third group of biomarkers contains cardiac markers including N-Terminal pro-Brain Natriuretic Peptide (NT-proBNP) [10,11] and high-sensitive cardiac Troponin T (hs-cTnT) [10], but these biomarkers have not been reviewed systematically in lower extremity PAD thus far. Apart from these three major groups of plasma biomarkers, there are more than thirty proteins that were found to be associated with cardiovascular risk and increased mortality.

In this systematic review and meta-analysis, we provide a comprehensive overview of state of the art of biomarker testing in PAD, as a first step to improved risk assessment.

METHODS

We conducted and reported this systematic review in accordance with the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) statement [12]. The protocol was published on PROSPERO (submitted November 4, 2019; awaiting number) (https://www.crd.york.ac.uk).

Literature search

This systematic review used MEDLINE/PubMed, Cochrane, and Embase to identify all cohort studies and case-control studies on plasma biomarkers in populations with peripheral artery disease. All databases were searched systematically up to April 2019 by two independent researchers using identical search terms. Used terms were "peripheral artery disease", "peripheral vascular disease", or "intermittent claudication", or "critical limb ischemia", or "critical limb-threatening ischemia"; and "plasma biomarker"; and "cardiovascular outcome" or "cardiovascular mortality", or "mortality", or "cardiovascular event", or "myocardial infarction", or "stroke", or "limb loss, or "amputation". When available, we also included the attached MeSH-term to the search and also applied the following filters: publication date from January 2000 and later, studies conducted on humans and the English language. To prevent missing studies which did not have "plasma biomarker" as a keyword, we added the specific names of biomarkers to the search and also performed hand searching. Although the focus of this review is on lower extremity PAD, we used the search term PAD as this term was more commonly used in the past. Both researchers performed an eligibility assessment. Eligible studies were screened on title and abstract first using the in-and exclusion criteria.

Study eligibility

In this systematic review, case-control studies and cohort studies, both prospective and retrospective analyzing patients with lower extremity PAD, were included. Lower extremity PAD had to be confirmed with an ankle-brachial index below 0.90 or by the use of medical records showing that patients had undergone an intervention for lower extremity PAD. Furthermore, only studies that investigated a plasma biomarker and had defined a cardiovascular endpoint after at least one year of follow-up, were included. Biomarkers that were reported in multiple studies (>2) were included in this review. Investigation of a non-plasmatic or calculation-based biomarker, studies on a population other than a PAD population, or the absence of a cardiovascular outcome were reasons for exclusion.

Data extraction

Two authors independently performed, the search, selection of studies, data extraction, and assessment of quality (BK, LW). Disagreements were resolved in a consensus discussion. A third author (AtC) checked for accuracy and made the final decision. Data was systematically extracted from the full-text articles and was categorized per biomarker. Duplicate publications of the same study were checked for additional data. Categorized data consisted of the author's name and publication year, period and location of investigation, study design, and study population (total number of patients and distribution of intermittent claudication and chronic limb-threatening ischemia), setting, and follow-up duration, outcome, (adjusted) results and conclusion.

Quality assessment

Risk of bias in the included studies through assessment of the methodological quality was carried out using the Newcastle Ottawa Assessment Scale [13]. Cohort studies were scored on the following topics: representativeness of the exposed cohort, selection of the non-exposed cohort, ascertainment of exposure, demonstration that outcome of interest was not present at the start of the study, comparability of cohorts on the basis of the design or analysis, assessment of outcome, follow-up length and adequacy of follow-up. Case-control studies were scored on other topics: adequacy of case definition, representativeness of the cases, selection of controls, definition of controls, comparability of bases and controls on the basis of the design or analysis, ascertainment of exposure, method of ascertainment between cases and controls and non-response rate. Based on these topics, studies were allocated stars, ranging from 0 (worst possible) and 9 (best possible).

Analysis

The total number of events and the total population within a study were collected and risk ratios were calculated across different thresholds. Dichotomous outcomes were expressed as relative risks (RR) with their 95% confidence intervals (CI). All results in the meta-analyses were unadjusted and may therefore differ from the adjusted hazard risks (HRs) as presented in some of the underlying studies. If a study did only report time to event curves without exact numbers of events within a group, the event rate was estimated by using the reported Kaplan-Meier curves. Meta-analyses were performed by pooling studies per biomarker for each outcome using the Mantel-Haenszel method in a random effects meta-analysis model. Heterogeneity among included studies was explored qualitatively and quantitatively by using the chi-square test of heterogeneity and I^2 statistics. In cases with more than 50% heterogeneity, sensitivity analyses were carried out. All meta-analyses were carried out using Review Manager (RevMan) [Computer program]. Version 5.3. Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration, 2014. P-values < 0.05 are significant.

RESULTS

Study identification

The full search was performed in three databases and resulted in 1688 hits, of which 1608 were excluded after title and abstract inspection. The remaining 80 articles were screened by full-text inspection, leading to exclusion of another 15 articles. The remaining 65 articles were then clustered by specific type of biomarker. Of the 65 remaining articles, 18 were on biomarkers that were only studied once; these specific biomarkers did not occur in any other studies. We, therefore, chose to just refer to these studies and not to include them in our qualitative or quantitative assessments *(Figure 1)*.

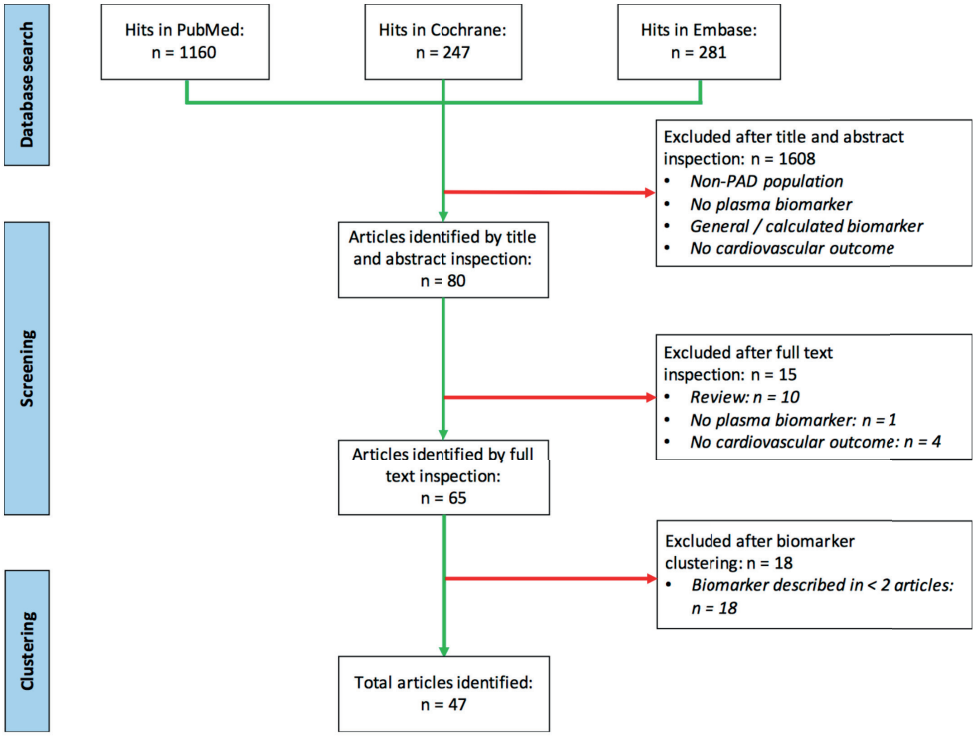

Figure 1: Search and study selection.

Study characteristics

The 47 studies selected, were performed all over the world, but were mostly conducted in western countries. The period in which these studies were carried out ranged from 1990 to 2015. Most studies (n = 41) had a cohort study design, of which 10 were retrospectively analyzed. The remaining 6 studies had a case-control study design. The study setting could be divided into 4 categories; random (outpatients and inpatients), inpatients only, pre-intervention and post-intervention. The mean/median follow-up was at least 12 months *(Supplemental table 1-4)*.

The 47 included studies were sorted per biomarker; several studies appeared in different groups as they investigated multiple biomarkers. The following biomarkers were studied: 13 studies investigated high-sensitive CRP (hs-CRP) [11,14–26], 2 reported on growth differentiation factor 15 (GDF-15) [14,27], 2 on Myeloperoxidase (MPO) [24,28], 6 on the neutrophil-Lymphocyte ratio (NLR) [29–34], 2 assessed serum amyloid A (SAA) [22,35], 6 reported on fibrinogen [19,35–39], another 7 assessed d-dimer [22,25,38,40–43], 7 investigated NT-proBNP [11,15,17,44–47], 5 high-sensitive cardiac Troponin T (hs-cTnT) [10,44,45,48,49], 4 dimethylarginine [50–53], 4 assessed adiponectin [54–57] and 3 homocysteine [18,22,58]. Investigated outcomes were heterogeneous across studies but mainly included all-cause mortality, cardiovascular mortality, cardiovascular events, major adverse cardiovascular events (MACE), major adverse limb events (MALE), coronary events, amputation-free survival, amputation, reintervention for lower extremity PAD, and graft patency. Studies investigating hs-CRP, fibrinogen, d-dimer, NT-proBNP, hs-cTnT, and adiponectin provided sufficient data to perform meta-analyses. The remaining biomarkers did not have sufficient data to create forest plots and are therefore not visualized.

The 18 articles that were not selected studied α-defensin [21], matrix metalloproteinase 10 [59], galectin-3 [60], soluble tumor necrosis-like weak inducer of apoptosis [61], ferritin [62], activated protein C-protein C inhibitor complex [63], angiopoietin-related growth factor [64], fatty acid-binding protein 4 [65], alkyl-phosphatidylcholine and alkenylphosphatidylcholine lipids [66], high-density lipoprotein cholesterol [66], malondialdehyde-modified low-density lipoprotein [67], lipoprotein-associated phospholipase A2 [68], cardiac Troponin I [69], phosphate [70], carboxy-terminal telopeptide of type I collagen [15], cholinesterase [71], eicosapentaenoic acid to arachidonic acid ratio [72] or endothelin-1 [73].

Patient characteristics

A total number of 21473 lower extremity PAD patients were investigated in the studies, with an average age between 56 and 75 years. 8378 Patients were classified as claudicants, 5313 patients had chronic limb-threatening ischemia and for another 7782 lower extremity PAD severity was not documented. The classifications for each biomarker are shown in *Table 1.*

Biomarker	# Studies	# Patients	# IC	# CLTI	# Unknown	Age (years)
hs-CRP	13	3866	1873	879	1114	64-75
GDF-15	2	632	260	362	10	67-70
MPO	2	562	451	90	21	67
NLR	6	3108	798	1754	556	64-74
SAA	2	488	41	50	397	63-69
Fibrinogen	6	2879	1592	412	875	62-72
D-dimer	7	2493	114	9	2370	56-72
NT-proBNP	7	1312	969	182	161	64-75
hs-cTnT	5	1676	1019	657	0	58-72
ADMA	4	2119	132	106	1881	69-74
Adiponectin	4	1229	736	493	0	66-71
Homocysteine	3	1109	393	319	397	69-76

Table 1: PAD severity and average age of the patient populations for each biomarker. IC = intermittent claudication, CLTI = chronic limb-threatening ischemia.

Quality assessment

The results of the quality assessment are shown in *Table 2a* for the cohort studies and in *Table 2b* for the case-control studies. In general, the cohort studies scored higher compared to the case-control studies due to the lack of representativeness of the patients. In most cases, the exposed cohort was representative for the general lower extremity PAD population. Studies that did not score a star on this topic had either a population of only male patients or had a very specific patient population such as patients undergoing a lower extremity bypass intervention [25,26,33,37,45,48,49,54,57]. All studies performed well in the selection of the non-exposed cohort, ascertainment of exposure, and demonstration of the absence of outcome at the start of the study. Also, only studies with a follow-up longer than one year were selected and loss to follow-up was minimal in all studies. Only one study did not correct for risk factors [24] and ten studies did not elaborate on the assessment of outcome (mortality) [20,23,26,27,29,33,47,54,57,58]. For the case-control studies, almost all studies provided a clear description of the cases and controls. The selection of controls was imperfect as some of these populations were considered hospital controls instead of community controls. Furthermore, the ascertainment of exposure was not noted in two studies [14,51], and neither was the method of ascertainment for cases and controls. Lastly, the non-response rate was not shared in three studies [10,14,18].

	1. Representativeness of the exposed cohort	2. Selection of the non-exposed cohort	3. Ascertainment of exposure	4. Demonstration that outcome of interest was absent at start	5. Comparability of cohorts on the basis of the design/analysis	6. Assessment of outcome	7. Was follow-up long enough for outcomes to occur	8. Adequacy of follow-up of cohorts	9. Total number of stars
Otaki 2017 [15]									9
Vrsalovic 2015 [16]									9
Stone 2014 [17]									9
Bleda 2013 [19]									9
Owens 2012 [20]									8
Urbonaviciene '12 [21]									9
Criqui 2010 [22]									9
Vainas 2005 [26]									7
Brevetti 2008 [24]									7
Mueller 2009 [46]									9
Shadman 2007 [47]									9
Clemens 2019 [44]									8
Bosevski 2006 [38]									7
Komarov 2002 [43]									9
Vidula 2008 [42]									9
Vidula 2010 [41]									9
Schlager 2009 [23]									8
Musicant 2006 [25]									8
De Haan 2017 [27]									8
Owens 2007 [35]									9
Amrock 2016 [30]									9
Erturk 2014 [32]									9
Pourafkari 2018 [29]									8
Luo 2015 [31]									9
Chan 2014 [33]									8
Gonzalez 2014 [34]									9
Haslacher 2012 [28]									9
Altes 2018 [36]									9
Bartlett 2009 [37]									8
Doweik 2003 [39]									9
Heneghan 2008 [58]									8
Owens 2010 [54]									7
Urbonaviciene '10 [55]									9
Dieplinger 2009 [56]									9
Komai 2009 [57]									7
Falkensammer'15 [45]									8
Szczeklik 2017 [48]									8
Linnemann 2014 [49]									8
Staniszewska '15 [50]									9
Wilson 2010 [52]									9
Mittermayer 2006 [53]									9

Table 2a: Quality assessment of cohort studies by the use of the Newcastle Ottawa Assessment Scale; green = present, yellow = partially present, red = absent

	1. Is the case definition adequate	2. Representativeness of the cases	3. Selection of controls	4. Definition of controls	5. Comparability of cases and controls on the basis of this design	6. Ascertainment of exposure	7. Same method of ascertainment for cases and controls	8. Non-response rate	9. Total number of stars
Hsu 2017 [14]									2
Mueller 2014 [18]									7
Skoglund 2014 [11]									8
McDermott 2015 [40]									8
Pohlhammer 2014 [10]									8
Boger 2010 [51]									4

Table 2b: Quality assessment of case-control studies by the use of the Newcastle Ottawa Assessment Scale; green = present, yellow = partially present, red = absent

Inflammatory markers and cardiovascular outcomes in lower extremity PAD patients

An overview of all outcomes for the biomarkers is shown in *Table 3*.

	All-cause mortality		Cardiovascular mortality		MACE/ MALE/AFS	
hs-CRP	+++	RR 3.49	-		+++	RR 1.86
GDF-15	+		0		+	HR 1.57-1.70
MPO	**0**		0		+	HR 1.68-6.80
NLR	+++	HR 1.10-1.97	+	HR 2.04	+++	HR 1.09-2.33
SAA	-		-		-	
Fibrinogen	+++	RR 2.08	+	HR 2.68	+	
D-dimer	++	HR 1.17	+	RR 2.15	-	
NT-proBNP	+++	RR 4.60	-		++	HR 1.55-1.60
hs-cTnT	+++	RR 3.14	0		+++	HR 2.20-3.71
ADMA	++	HR 1.31-2.23	0		++	HR 1.70-5.20
Adiponectin	+++	RR 1.99	0		-	
Homocysteine	-		0		+	OR 3.4

Table 3: Associations between plasma biomarkers and different outcomes. +++ = association found in three or more studies, ++ = association found in two studies, + = association found in one study, 0 = association not investigated, - = no association found in any study.

hs-CRP: a total of thirteen studies investigated hs-CRP levels and cardiovascular outcome in patients with lower extremity PAD. Twelve studies were of good methodological quality and only one was of poor quality. Hazard ratios or relative risks were provided in most studies and, when possible, a multivariate analysis was performed to control for bias. The overall population in which hs-CRP was measured, consisted of mostly claudicants (1873 patients) and but also more severe PAD (879 patients). Both newly diagnosed PAD patients and patients who already underwent revascularization of the lower limbs were included. All-cause mortality was most widely used as outcome, the results of which are shown in a forest plot *(Figure 2A)*. Overall, the risk ratio for all-cause mortality in patients with elevated hs-CRP levels was 3.49 (2.35-5.19) without heterogeneity between studies. Cardiovascular mortality specifically was reported in three studies [21,22,25] but did not show a significant increase in patients with higher hs-CRP levels. As shown in *Figure 2B*, Patients with high hs-CRP levels also had a higher risk ratio for MACE compared to patients with low hs-CRP levels (RR 1.86 (1.48-2.33)).

GDF-15: the stress-responsive cytokine GDF-15 is produced among others by macrophages, vascular smooth muscle cells, and adipocytes [74]. Only two studies investigated GDF-15 as a biomarker to predict outcome in lower extremity PAD populations [14,27]. Both studies were qualitatively assessed as good and had a combined population of 632 patients (260 claudication patients, 362 chronic limb-threatening ischemia patients, 10 patients missing). Included patients either were undergoing an iliofemoral endarterectomy or were not eligible for conventional revascularization. One study showed a decreased risk of amputation-free survival in patients with elevated GDF-15 levels, with HRs ranging from 1.57 (1.02-2.41) to 1.78 (1.18-2.69) [27]. The second study used all-cause mortality as outcome and showed higher levels of GDF-15 in patients who died, compared to patients who survived during the follow-up period (5749.6 pg/mL vs 2849.4 pg/mL, p 0.028) [14].

MPO: this white blood cell-derived inflammatory enzyme is classified as belonging to the peroxidases. MPO generates reactive oxidants and radical species that initiate oxidative degradation of lipids. MPO is mostly expressed in neutrophils [75], but can also be found in monocytes and macrophages [76]. Levels of MPO in regard to cardiovascular outcome were reported in two studies, one of which was conducted as a prospective cohort study while the other was a retrospective cohort study. Both studies mainly investigated patients with chronic limb-threatening ischemia, both newly diagnosed patients and patients undergoing endovascular therapy. The first study reports an HR of 6.80 (1.20-38.69, p 0.031) for cardiovascular events [24], while the other study reports an HR of 1.68 (1.09-2.60, $p < 0.05$) for MACE [28].

NLR: the ratio of number of neutrophils to the number of lymphocytes [77] was reported in six studies. All of these were cohort studies; only one of which was carried out prospectively. Nonetheless, all studies were assessed as qualitatively good studies with at least seven out of nine

stars. Chronic limb-threatening ischemia was present in most patients (n= 1754), while patients suffering from claudication were underrepresented (n = 798). All-cause mortality was used most often as outcome and showed an association with increased NLR (HR 1.20, p 0.012 (30); HR 1.10, p < 0.001 (29); HR 1.97, p 0.03) [33]. Comparable results were seen for cardiovascular mortality (HR 2.04, p 0.004) [32], MALE (HR 1.09, p < 0.001) [29], amputation (HR 1.14, p < 0.001) [31] and amputation-free survival (HR 2.38, p 0.000) [34].

SAA: this group of apolipoproteins is upregulated during the acute phase of inflammation. Two studies reported results for SAA, both of which were cohort studies of good quality. Although the follow-up period differed substantially between the studies, their results show comparable outcomes. None of both showed significant associations with cardiovascular outcome. One study reported no association between SAA and MACE (HR unknown) [35], while the other study found no association between SAA and all-cause mortality (HR unknown, p 0.12) and cardiovascular mortality (HR unknown, p 0.19) [22].

Coagulation-inflammation crosstalk markers and cardiovascular outcome in lower extremity PAD patients

Fibrinogen: levels of fibrinogen were measured in six studies, all prospective cohort studies which all scored as qualitatively good (7-9 stars). Patient samples were randomly taken, including outpatients and inpatients with PAD. In three out of five studies, all-cause mortality was significantly increased (OR 1.44 (1.02-1.94) [37], HR 1.90 (1.11-3.41, p 0.02) [39], with higher fibrinogen levels (446.35 mg/L) in patients who died versus 349.8 mg/L in patients who survived, p 0.013 [19] *(Figure 2C)*. Cardiovascular mortality was increased in patients with higher fibrinogen levels (HR 2.68 (1.39-5.16, p 0.003)) [39]. MACE, however, did not show an association with levels of fibrinogen [35].

D-dimer: seven studies investigated d-dimer levels in relation to cardiovascular outcome in PAD patients. The distribution of intermittent claudication and chronic limb-threatening ischemia patients was only indicated in one study. Nonetheless, all studies were qualitatively assessed as good. The severity of the lower extremity PAD was not described in most studies, but overall, most patients were newly diagnosed with lower extremity PAD. All-cause mortality was significantly increased in patients with high d-dimer levels in two studies (HR 2.55 and HR 1.17 (1.04-1.32, p 0.007)) [38,42]. Cardiovascular mortality was increased in one (RR 1.97 (1.06-3.65)) [41], with an overall RR for all studies of 2.15 (1.19-3.88) *(Figure 2D)*. Specifically, coronary events were more abundant in patients with elevated d-dimer levels (p 0.028) [40].

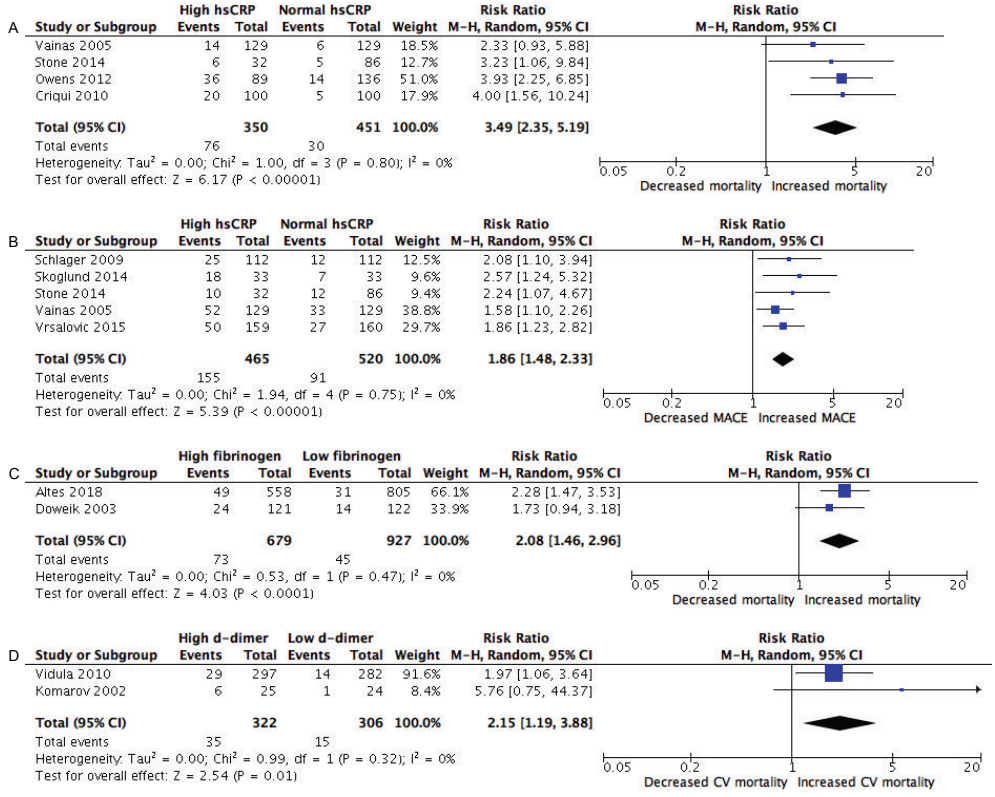

Figure 2: Forest plots for inflammatory and coagulation markers. (A) shows hs-CRP levels and the risk of mortality, (B) shows hs-CRP levels and the risk of MACE, (C) shows fibrinogen levels and the risk of mortality, (D) shows d-dimer levels and the risk of mortality (all-cause and CV mortality). The diamond and its width indicate the pooled risk ratio (RR) and the corresponding 95% confidence interval (CI). M-H = Mantel – Haenszel.

Cardiac markers and cardiovascular outcome in lower extremity PAD patients

NT-proBNP: seven studies reported NT-proBNP as biomarker. All seven studies were of good quality and HRs were present for all studies. Overall, most included patients were diagnosed with intermittent claudication (n = 969) and only a small group had chronic limb-threatening ischemia (n = 182). All-cause mortality was the most investigated outcome and increased HRs were seen in patients with elevated NT-proBNP levels. Three studies provided data for meta-analysis and are presented in a forest plot *(Figures 3A and 3B).* It is shown that patients with increased NT-proBNP levels are at increased risk of mortality compared to PAD patients with normal NT-proBNP levels with a risk ratio of 4.60 (2.09-10.10). MACE only appeared to be associated with higher levels of NT-proBNP in one out of three studies, with an HR of 1.60 (1.16-2.22, $p < 0.01$) [15].

Hs-cTnT: this cardiac marker was investigated in five studies, four of which were cohort studies and one was a case-control study. All studies were of good quality (7-9 stars) and contained both inpatient and outpatient PAD populations. Within the total investigated population of 1676 patients, 1019 had intermittent claudication and 657 had chronic threatening limb ischemia. All-cause mortality was increased in patients with elevated hs-cTnT levels with an RR of 3.14 (1.56-6.34), and 2.28 (1.78-2.93), after performing the sensitivity analysis *(Figures 3C and 3D)*. MACE was also increased in two out of three studies, with HRs of 2.89 (p 0.004) [48], 3.25 (p 0.01) [10] and 1.04 (p 0.562) [45] respectively.

Biomarkers of arterial vessel wall damage and cardiovascular outcome in lower extremity PAD patients

ADMA: asymmetric dimethylarginine is a known risk marker in vascular disease, inhibiting the NO synthase and causing endothelial dysfunction, vasoconstriction, elevation of blood pressure, and aggravation of atherosclerosis. ADMA was investigated in four studies, all of methodological good quality. A total of 2119 patients were investigated comprising both inpatients and outpatients with lower extremity PAD. All-cause mortality was increased with high ADMA levels (HR 2.23, p 0.024 [50] and HR 1.31, p 0.037) [51]. MACE was also increased in patients with higher levels of ADMA (HR 5.2, $p < 0.001$ [52] and HR 1.70, p 0.043) [53].

Adiponectin: the adipocyte-specific adiponectin was investigated in four prospective cohort studies which were all published in 2009 or 2010. These studies yielded a total of 1129 patients which were mostly claudicants (n = 736). All-cause mortality was increased in lower extremity PAD patients with higher adiponectin levels (RR 1.99 (1.29-3.07)) *(Figure 3E)* in contrast to studies investigating specifically cardiovascular events, which showed fewer cardiovascular events in patients with higher levels of adiponectin (HR 0.73 (0.54-0.98)) [55].

Homocysteine: in three studies, levels of homocysteine were investigated, two cohort studies and one case-control study. The PAD population was a mixed population of claudicants and patients with chronic limb-threatening ischemia; most of them were included during hospital admission. All-cause mortality was not associated with homocysteine levels (RR 1.17, p 0.444) [18], but graft occlusion was more abundant in patients with higher homocysteine levels (OR 7.97, $p < 0.0001$) [58].

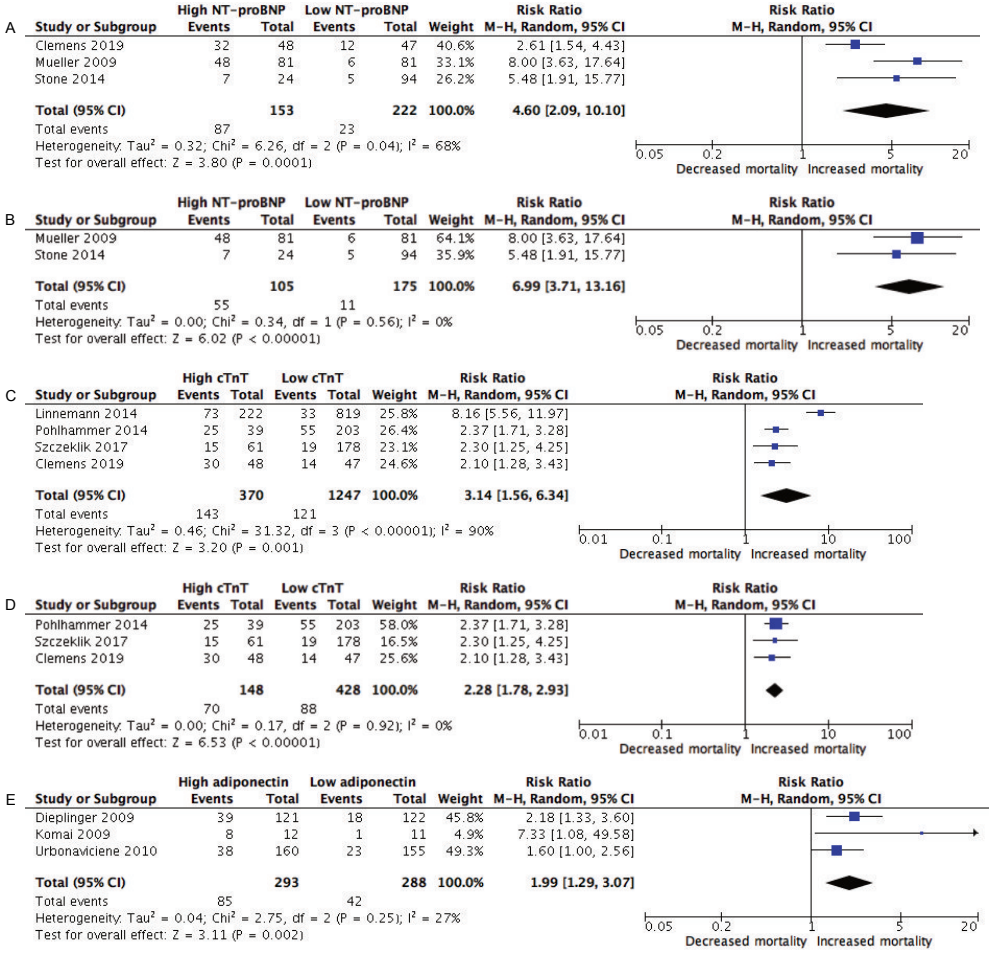

Figure 3: Forest plots for cardiac markers and markers for arterial vessel wall damage. (A) shows NT-proBNP levels and the risk of mortality, (B) shows the sensitivity analysis of A, (C) shows hs-cTnT levels and the risk of mortality, (D) shows the sensitivity analysis of C, (E) shows adiponectin and the risk of mortality. The diamond and its width indicate the pooled risk ratio (RR) and the corresponding 95% confidence interval (CI). M-H = Mantel – Haenszel.

DISCUSSION

This systematic review aimed to provide a comprehensive overview of biomarker testing in lower extremity PAD as a first step to improving risk stratification. We categorized the biomarkers studied based on the underlying pathophysiological processes, into markers of inflammation, coagulation, cardiac damage, or vessel wall damage *(Figure 4)*. Several biomarkers which could potentially be used for risk stratification in PAD patients were identified. The inflammatory markers hs-CRP and NLR were found to be associated with a two to threefold increased risk of all-cause mortality and MACE. Coagulation markers d-dimer and fibrinogen were associated with a more than twofold increase in both all-cause and cardiovascular mortality. The cardiac markers NT-proBNP and hs-cTnT were associated with a two to fourfold risk of all-cause mortality and MACE, while the markers of vessel wall damage: ADMA and adiponectin were only weakly associated with all-cause mortality. Despite the identification of these potentially useful plasma biomarkers, surprisingly so far none are used for clinical risk stratification in lower extremity PAD.

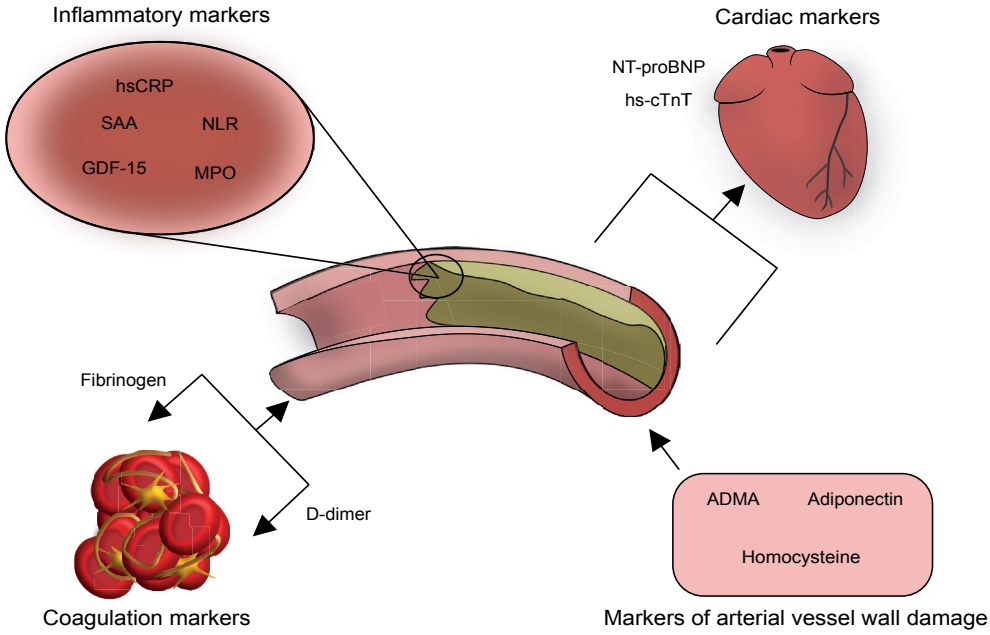

Figure 4: Plasma biomarkers included in this review. hs-CRP indicates a state of chronic inflammation with increased numbers of neutrophils (NLR) expressing pro-atherogenic markers such as MPO. Fibrinogen plays an important role in early atherogenesis, apart from its role in coagulation, whereas d-dimer marks a hypercoagulable state. Cytokines such as GDF-15 and acute phase proteins like SAA are implicated in further progression of atherosclerotic lesions. Within these lesions, adiponectin, ADMA, and homocysteine cause further damage and disruption of the arterial vessel wall. Progression of atherosclerosis leads to ischemia in organs, including the heart. Cardiac markers NT-proBNP and hs-cTnT reflect myocardial ischemia, which can be primarily due to coronary artery disease but can also be a sign of systemic microvascular disease and inflammation.

Recently, new treatment strategies to prevent atherothrombotic complications were developed and introduced into clinical practice. These new treatment strategies target coagulation [78], inflammation [79,] and lipid metabolism [80], and are all associated with comparable and substantial reductions in overall mortality varying from 14% to 32%.

The COMPASS-trial [78] showed that in a population with stable chronic arterial disease, including lower extremity PAD, dual pathway inhibition based on a low-dose anticoagulant combined with an antiplatelet drug further improved cardiovascular outcomes and reduced mortality when compared to treatment with antiplatelet therapy alone. This indicates that hypercoagulability presents a cardiovascular risk in lower extremity PAD patients. A recent cost-effectiveness analysis identified subgroups of patients with varying benefits of dual pathway inhibition, indicating that there could be added value in better selection of patients [81]. It could be expected that markers of coagulation such as d-dimer, and possibly fibrinogen, might be useful to identify hypercoagulable patients that could benefit most from this intensified antithrombotic therapy. Fibrinogen is, apart from its role in coagulation, also an inflammatory marker associated with atherosclerotic plaque formation [82]. It is thought that fibrinogen is important in early atherogenesis, preceding or facilitating low-density lipoprotein accumulation [83], indicating that fibrinogen could be a high potential marker to predict long-term all-cause and cardiovascular mortality in lower extremity PAD. Specific anti-inflammatory treatment strategies targeting IL-1β, the key mediator of the inflammatory response, are effective in reducing cardiovascular events [79]. In the CANTOS trial patients treated with an IL-1β lowering drug showed a dose-dependent reduction in plasma CRP levels, and administration of the drug in higher doses showed a significant reduction in cardiovascular events and death [84]. Thus, the use of inflammatory markers could potentially improve patient stratification and management in that respect. NLR, an intensively investigated inflammatory marker and an indicator of relative inflammatory cellular activities, is easy to measure and interpret. Although several studies show an association between NLR and all-cause mortality, this association is weaker compared to hs-CRP. NLR could however be a potential biomarker to predict short-term (< 2 years) cardiovascular mortality in lower extremity PAD, but to date, only one study has observed this particular association. NT-proBNP and hs-cTnT are already embedded in the ABC-score for the prediction of stroke in patients with atrial fibrillation [85]. Both are cardiac markers, NT-proBNP is clinically used as a marker for heart failure, and troponins are used to diagnose cardiac ischemia [86,87]. High levels of NT-proBNP are associated with vulnerable plaque components [88] and have been shown to predict outcome in patients with coronary heart disease and patients who suffered an ischemic stroke [89,90]. In PAD patients, similar results have been published with a strong association between all-cause mortality and increased NT-proBNP levels. The association with MACE and NT-proBNP is less frequently studied but also showed a moderately increased risk. The contribution to risk stratification in patients with PAD could be in identifying patients that also have evidence of heart failure (with preserved ejection fraction), a combination of entities with a particularly poor outcome [91,92]. Hs-

cTnT also appears to be a good predictor for all-cause mortality and MACE in lower extremity PAD patients. In comparison to NT-proBNP, the studies investigating hs-cTnT had more patients with chronic limb-threatening ischemia and were, therefore, more prone to MACE. The increased hs-cTnT levels in high-risk PAD patients may reflect microvascular organ damage of the heart as more than one in two PAD patients suffer from concomitant coronary artery disease [2,49]. However, hs-cTnT does not only reflect myocardial ischemia due to coronary artery disease, but it can also indicate systemic vascular (including microvascular) disease, associated with heart and kidney failure [49,93,94]. Comparing the risk ratios of both markers, NT-proBNP does seem to be a stronger predictor for all-cause mortality and especially for long-term mortality. Finally, markers of arterial vessel wall damage are weak predictors of cardiovascular outcome in lower extremity PAD. Adiponectin has been thoroughly investigated in patients with coronary heart disease, with ambiguous outcomes being positively or negatively associated with mortality and cardiovascular risk [95,96]. Similar results were found in this review, as high adiponectin levels were associated with increased all-cause mortality but not with amputation-free survival [54,56,57]. Although studies investigating ADMA show promising results, more studies need to be performed to confirm the predictive potential of this biomarker. Homocysteine is not recommended to be used as a predictive marker for cardiovascular outcome.

How can these data be translated to practice? One way forward may be to design management studies addressing the value of a panel of biomarkers as discussed, including hs-CRP, NLR, fibrinogen, d-dimer, NT-proBNP, and hs-cTnT. Tailoring based on biomarker results as compared to standard care without such biomarkers could reveal the utility of such an approach for early identification of specific contributing risks, including inflammation, hypercoagulability, and heart failure. Now that more potent pharmacological interventions are becoming available to target specific mechanisms, a biomarker-supported strategy may help identify those patients with lower extremity PAD, that may benefit most from intensified treatment. The financial consequences of using biomarkers may be very limited as one may assume that most of these markers may be tested on one or only few occasions per patient. In fact, we previously explored the cost-effectiveness of d-dimer and the societal value (headroom) of a hypothetical perfect biomarker for risk assessment and subsequent tailored treatment allocation in lower extremity PAD patients. We concluded that further risk assessment and treatment stratification based on the use of d-dimer could be a cost-effective health intervention. Identification of high-risk patients and prescription of intensified antithrombotic therapy could potentially save substantial costs and improve chances of survival [97]. It can be expected that this will also be the case for other biomarkers in lower extremity PAD with similar risk associations.

By investigating which of the biomarkers are increased in an individual patient, the treatment strategy may be adapted to provide optimal personalized vascular protection. For instance, in a patient with increased levels of inflammatory markers but normal levels of coagulation markers, intensifying anti-inflammatory treatment would better suit the preventive strategy than escalating anticoagulant therapy. By individualizing treatment to the needs of each specific patient, the risk of adverse cardiovascular events, bleeding, or other complications can be expected to be limited. This study has several limitations, mainly due to the heterogeneity of the underlying evidence. Although a total number of fifty studies were included in this systematic review, the number of studies per biomarker was limited. We were only able to include studies that provided sufficient data for meta-analysis. For each biomarker, the primary endpoint differed between studies, and therefore only studies with the same endpoint could be included in the meta-analyses. Within each meta-analysis, we included studies with a different, but minimal follow-up of one year. Lastly, we used unadjusted results in the meta-analyses, which could differ from reported adjusted results in each study. Several studies did not report baseline data on PAD severity, such as the Fontaine classification, which makes it difficult to interpret results for subgroups within PAD. Notwithstanding these limitations, this systematic review was able to provide a comprehensive overview of biomarker testing in lower extremity PAD which can be used as a first step to improve risk stratification.

Conclusion

The clinical application of biomarkers to stratify patients at increased risk for adverse cardiovascular events in lower extremity PAD is urgently needed. This systematic review identifies promising candidate biomarkers representing different pathophysiological processes implicated in lower extremity PAD, including hs-CRP, NLR, fibrinogen, d-dimer, NT-proBNP, and hs-cTnT. Combining these markers for individual risk stratification might result in improved treatment choices and increased effectiveness of current treatment strategies in lower extremity PAD patients and is expected to be societally cost-effective. This strategy needs testing in management studies.

REFERENCES

1. Fowkes, F. G. R. *et al.* Comparison of global estimates of prevalence and risk factors for peripheral artery disease in 2000 and 2010: a systematic review and analysis. *Lancet* **382**, 1329–1340 (2013).
2. Bhatt, D. L. *et al.* International prevalence, recognition, and treatment of cardiovascular risk factors in outpatients with atherothrombosis. *JAMA* **295**, 180–189 (2006).
3. Meves, S. H. *et al.* Peripheral arterial disease as an independent predictor for excess stroke morbidity and mortality in primary-care patients: 5-year results of the getABI study. *Cerebrovasc. Dis.* **29**, 546–554 (2010).
4. Cea Soriano, L., Fowkes, F. G. R., Johansson, S., Allum, A. M. & García Rodriguez, L. A. Cardiovascular outcomes for patients with symptomatic peripheral artery disease: A cohort study in The Health Improvement Network (THIN) in the UK. *Eur. J. Prev. Cardiol.* **24**, 1927–1937 (2017).
5. Inglis, S. C. *et al.* Peripheral artery disease and outcomes after myocardial infarction: an individual-patient meta-analysis of 28,771 patients in CAPRICORN, EPEHESUS, OPTIMAAL and VALIANT. *Int. J. Cardiol.* **168**, 1094–1101 (2013).
6. Spencer, F. A. *et al.* Treatment practices and outcomes of patients with established peripheral arterial disease hospitalized with acute myocardial infarction in a community setting. *Am. Heart J.* **153**, 140–146 (2007).
7. Moussa, I. D. *et al.* Prevalence and prediction of previously unrecognized peripheral arterial disease in patients with coronary artery disease: the Peripheral Arterial Disease in Interventional Patients Study. *Catheter. Cardiovasc. Interv.* **73**, 719–724 (2009).
8. Singh, T. P., Morris, D. R., Smith, S., Moxon, J. V. & Golledge, J. Systematic review and meta-analysis of the association between C-reactive protein and major cardiovascular events in patients with peripheral artery disease. *Eur. J. Vasc. Endovasc. Surg.* **54**, 220–233 (2017).
9. Kleinegris, M.-C. F., ten Cate, H. & ten Cate-Hoek, A. J. D-dimer as a marker for cardiovascular and arterial thrombotic events in patients with peripheral arterial disease. A systematic review. *Thromb. Haemost.* **110**, 233–243 (2013).
10. Pohlhammer, J. *et al.* High-sensitivity cardiac troponin T in patients with intermittent claudication and its relation with cardiovascular events and all-cause mortality--the CAVASIC Study. *Atherosclerosis* **237**, 711–717 (2014).
11. Skoglund, P. H., Arpegård, J., Ostergren, J. & Svensson, P. Amino-terminal pro-B-type natriuretic peptide and high-sensitivity C-reactive protein but not cystatin C predict cardiovascular events in male patients with peripheral artery disease independently of ambulatory pulse pressure. *Am. J. Hypertens.* **27**, 363–371 (2014).
12. Moher, D., Liberati, A., Tetzlaff, J., Altman, D. G. & PRISMA Group. Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. *Int. J. Surg.* **8**, 336–341 (2010).
13. Stang, A. Critical evaluation of the Newcastle-Ottawa scale for the assessment of the quality of nonrandomized studies in meta-analyses. *Eur. J. Epidemiol.* **25**, 603–605 (2010).
14. Hsu, L.-A. *et al.* Growth differentiation factor 15 may predict mortality of peripheral and coronary artery diseases and correlate with their risk factors. *Mediators Inflamm.* **2017**, 1–13 (2017).
15. Otaki, Y. *et al.* Serum carboxy-terminal telopeptide of type I collagen (I-CTP) is predictive of clinical outcome in peripheral artery disease patients following endovascular therapy. *Heart Vessels* **32**, 149–156 (2017).

16. Vrsalović, M., Vučur, K., Car, B., Krčmar, T. & Vrsalović Presečki, A. C-reactive protein, renal function, and cardiovascular outcome in patients with symptomatic peripheral artery disease and preserved left ventricular systolic function. *Croat. Med. J.* **56**, 351–356 (2015).
17. Stone, P. A. *et al.* C-reactive protein and brain natriuretic peptide as predictors of adverse events after lower extremity endovascular revascularization. *J. Vasc. Surg.* **60**, 652–660 (2014).
18. Mueller, T. *et al.* Mortality rates and mortality predictors in patients with symptomatic peripheral artery disease stratified according to age and diabetes. *J. Vasc. Surg.* **59**, 1291–1299 (2014).
19. Bleda, S. *et al.* Inflammatory burden predicts long-term outcomes in endovascular therapy in peripheral arterial disease. *Ann. Vasc. Surg.* **27**, 459–466 (2013).
20. Owens, C. D. *et al.* An integrated biochemical prediction model of all-cause mortality in patients undergoing lower extremity bypass surgery for advanced peripheral artery disease. *J. Vasc. Surg.* **56**, 686–695 (2012).
21. Urbonaviciene, G. *et al.* Markers of inflammation in relation to long-term cardiovascular mortality in patients with lower-extremity peripheral arterial disease. *Int. J. Cardiol.* **160**, 89–94 (2012).
22. Criqui, M. H. *et al.* Biomarkers in peripheral arterial disease patients and near- and longer-term mortality. *J. Vasc. Surg.* **52**, 85–90 (2010).
23. Schlager, O. *et al.* Inflammation and adverse cardiovascular outcome in patients with renal artery stenosis and peripheral artery disease. *Atherosclerosis* **205**, 314–318 (2009).
24. Brevetti, G. *et al.* Myeloperoxidase, but not C-reactive protein, predicts cardiovascular risk in peripheral arterial disease. *Eur. Heart J.* **29**, 224–230 (2008).
25. Musicant, S. E. *et al.* Prospective evaluation of the relationship between C-reactive protein, D-dimer and progression of peripheral arterial disease. *J. Vasc. Surg.* **43**, 772–80; discussion 780 (2006).
26. Vainas, T. *et al.* C-reactive protein in peripheral arterial disease: relation to severity of the disease and to future cardiovascular events. *J. Vasc. Surg.* **42**, 243–251 (2005).
27. De Haan, J. J. *et al.* Growth differentiation factor 15 is associated with major amputation and mortality in patients with peripheral artery disease. *J. Am. Heart Assoc.* **6**, (2017).
28. Haslacher, H. *et al.* Plasma myeloperoxidase level and peripheral arterial disease. *Eur. J. Clin. Invest.* **42**, 463–469 (2012).
29. Pourafkari, L. *et al.* Neutrophil-lymphocyte ratio is a marker of survival and cardiac complications rather than patency following revascularization of lower extremities. *Vasc. Med.* **23**, 437–444 (2018).
30. Amrock, S. M. & Weitzman, M. Multiple biomarkers for mortality prediction in peripheral arterial disease. *J. Vasc. Surg.* **63**, 1661–1662 (2016).
31. Luo, H. *et al.* Post-treatment neutrophil-lymphocyte ratio independently predicts amputation in critical limb ischemia without operation. *Clinics (Sao Paulo)* **70**, 273–277 (2015).
32. Erturk, M. *et al.* Predictive value of elevated neutrophil to lymphocyte ratio for long-term cardiovascular mortality in peripheral arterial occlusive disease. *J. Cardiol.* **64**, 371–376 (2014).
33. Chan, C., Puckridge, P., Ullah, S., Delaney, C. & Spark, J. I. Neutrophil-lymphocyte ratio as a prognostic marker of outcome in infrapopliteal percutaneous interventions for critical limb ischemia. *J. Vasc. Surg.* **60**, 661–668 (2014).
34. González-Fajardo, J. A. *et al.* Prognostic significance of an elevated neutrophil-lymphocyte ratio in the amputation-free survival of patients with chronic critical limb ischemia. *Ann. Vasc. Surg.* **28**, 999–1004 (2014).

35. Owens, C. D. *et al.* Elevated C-reactive protein levels are associated with postoperative events in patients undergoing lower extremity vein bypass surgery. *J. Vasc. Surg.* **45**, 2–9; discussion 9 (2007).
36. Altes, P. *et al.* Raised fibrinogen levels and outcome in outpatients with peripheral artery disease. *Angiology* **69**, 507–512 (2018).
37. Bartlett, J. W., De Stavola, B. L. & Meade, T. W. Assessing the contribution of fibrinogen in predicting risk of death in men with peripheral arterial disease. *J. Thromb. Haemost.* **7**, 270–276 (2009).
38. Bosevski, M., Kostoska, S., Tosev, S. & Borozanov, V. Usefulness of D-Dimers and fibrinogen plasma determination in patients with polyvascular disease. *Angiol. Sosud. Khir.* **12**, 9–15 (2006).
39. Doweik, L. *et al.* Fibrinogen predicts mortality in high risk patients with peripheral artery disease. *Eur. J. Vasc. Endovasc. Surg.* **26**, 381–386 (2003).
40. McDermott, M. M. *et al.* Changes in D-dimer and inflammatory biomarkers before ischemic events in patients with peripheral artery disease: The BRAVO Study. *Vasc. Med.* **21**, 12–20 (2016).
41. Vidula, H. *et al.* Comparison of effects of statin use on mortality in patients with peripheral arterial disease with versus without elevated C-reactive protein and D-dimer levels. *Am. J. Cardiol.* **105**, 1348–1352 (2010).
42. Vidula, H. *et al.* Biomarkers of inflammation and thrombosis as predictors of near-term mortality in patients with peripheral arterial disease: a cohort study. *Ann. Intern. Med.* **148**, 85–93 (2008).
43. Komarov, A. *et al.* D-dimer and platelet aggregability are related to thrombotic events in patients with peripheral arterial occlusive disease. *Eur. Heart J.* **23**, 1309–1316 (2002).
44. Clemens, R. K. *et al.* Cardiac biomarkers but not measures of vascular atherosclerosis predict mortality in patients with peripheral artery disease. *Clin. Chim. Acta* **495**, 215–220 (2019).
45. Falkensammer, J. *et al.* Prognostic relevance of ischemia-modified albumin and NT-proBNP in patients with peripheral arterial occlusive disease. *Clin. Chim. Acta* **438**, 255–260 (2015).
46. Mueller, T. *et al.* Amino-terminal pro-B-type natriuretic peptide as predictor of mortality in patients with symptomatic peripheral arterial disease: 5-year follow-up data from the Linz Peripheral Arterial Disease Study. *Clin. Chem.* **55**, 68–77 (2009).
47. Shadman, R., Allison, M. A. & Criqui, M. H. Glomerular filtration rate and N-terminal pro-brain natriuretic peptide as predictors of cardiovascular mortality in vascular patients. *J. Am. Coll. Cardiol.* **49**, 2172–2181 (2007).
48. Szczeklik, W. *et al.* Myocardial injury after endovascular revascularization in critical limb ischemia predicts 1-year mortality: a prospective observational cohort study. *Clin. Res. Cardiol.* **107**, 319–328 (2018).
49. Linnemann, B. *et al.* Elevated cardiac troponin T is associated with higher mortality and amputation rates in patients with peripheral arterial disease. *J. Am. Coll. Cardiol.* **63**, 1529–1538 (2014).
50. Staniszewska, A., Rajagopalan, S., Al-Shaheen, A., Thies, F. & Brittenden, J. Increased levels of symmetric dimethylarginine are associated with all-cause mortality in patients with symptomatic peripheral arterial disease. *J. Vasc. Surg.* **61**, 1292–1298 (2015).
51. Böger, R. H. *et al.* Asymmetric dimethylarginine as an independent risk marker for mortality in ambulatory patients with peripheral arterial disease. *J. Intern. Med.* **269**, 349–361 (2011).
52. Wilson, A. M. *et al.* Asymmetric dimethylarginine correlates with measures of disease severity, major adverse cardiovascular events and all-cause mortality in patients with peripheral arterial disease. *Vasc. Med.* **15**, 267–274 (2010).

53. Mittermayer, F. *et al.* Asymmetric dimethylarginine predicts major adverse cardiovascular events in patients with advanced peripheral artery disease. *Arterioscler. Thromb. Vasc. Biol.* **26**, 2536–2540 (2006).
54. Owens, C. D. *et al.* Novel adipokines, high molecular weight adiponectin and resistin, are associated with outcomes following lower extremity revascularization with autogenous vein. *J. Vasc. Surg.* **51**, 1152–1159 (2010).
55. Urbonaviciene, G., Frystyk, J., Flyvbjerg, A., Henneberg, E. W. & Lindholt, J. S. Association of serum adiponectin with risk for cardiovascular events in patients with peripheral arterial disease. *Atherosclerosis* **210**, 619–624 (2010).
56. Dieplinger, B., Haltmayer, M., Poelz, W. & Mueller, T. Value of adiponectin as predictor of 5-year all-cause mortality in patients with symptomatic peripheral arterial disease: results from the Linz Peripheral Arterial Disease (LIPAD) study. *Clin. Chim. Acta* **408**, 87–91 (2009).
57. Komai, H., Shigematsu, H. & Juri, M. PP47. Impact of reduced endogenous anti-coagulation protein activity on vascular events of peripheral arterial disease. *J. Vasc. Surg.* **49**, S28 (2009).
58. Heneghan, H. M. & Sultan, S. Homocysteine, the cholesterol of the 21st century. Impact of hyperhomocysteinemia on patency and amputation-free survival after intervention for critical limb ischemia. *J. Endovasc. Ther.* **15**, 399–407 (2008).
59. Martinez-Aguilar, E. *et al.* Matrix metalloproteinase 10 is associated with disease severity and mortality in patients with peripheral arterial disease. *J. Vasc. Surg.* **61**, 428–435 (2015).
60. Madrigal-Matute, J. *et al.* Galectin-3, a biomarker linking oxidative stress and inflammation with the clinical outcomes of patients with atherothrombosis. *J. Am. Heart Assoc.* **3**, (2014).
61. Urbonaviciene, G. *et al.* Impact of soluble TWEAK and CD163/TWEAK ratio on long-term cardiovascular mortality in patients with peripheral arterial disease. *Atherosclerosis* **219**, 892–899 (2011).
62. Depalma, R. G. *et al.* Ferritin levels, inflammatory biomarkers, and mortality in peripheral arterial disease: a substudy of the Iron (Fe) and Atherosclerosis Study (FeAST) Trial. *J. Vasc. Surg.* **51**, 1498–1503 (2010).
63. Blomstrand, D., Kölbel, T., Lindblad, B. & Gottsäter, A. Activated protein C-protein C inhibitor complex in peripheral arterial disease. *Ann. Vasc. Surg.* **24**, 588–595 (2010).
64. Nie, H. *et al.* Angiopoietin-related growth factor is independently associated with lower extremity peripheral arterial disease. *J. Diabetes Complications* **31**, 433–438 (2017).
65. Höbaus, C. *et al.* FABP4 and cardiovascular events in peripheral arterial disease. *Angiology* **69**, 424–430 (2018).
66. Moxon, J. V. *et al.* Baseline serum phosphatidylcholine plasmalogen concentrations are inversely associated with incident myocardial infarction in patients with mixed peripheral artery disease presentations. *Atherosclerosis* **263**, 301–308 (2017).
67. Takamura, T.-A. *et al.* Circulating malondialdehyde-modified low-density lipoprotein (MDA-LDL) as a novel predictor of clinical outcome after endovascular therapy in patients with peripheral artery disease (PAD). *Atherosclerosis* **263**, 192–197 (2017).
68. Fatemi, S. *et al.* Lp-PLA2 activity and mass and CRP are associated with incident symptomatic peripheral arterial disease. *Sci. Rep.* **9**, 5609 (2019).
69. Eisen, A. *et al.* High-sensitivity troponin I in stable patients with atherosclerotic disease in the TRA 2°P - TIMI 50 trial. *Clin. Chem.* **63**, 307–315 (2017).
70. Zettervall, S. L. *et al.* Elevated serum phosphate levels are associated with decreased amputation-free survival after interventions for critical limb ischemia. *J. Vasc. Surg.* **65**, 431–437 (2017).

71. Gremmel, T. *et al.* Serum cholinesterase levels are associated with 2-year ischemic outcomes after angioplasty and stenting for peripheral artery disease. *J. Endovasc. Ther.* **23**, 738–743 (2016).
72. Hishikari, K. *et al.* The prognostic value of the serum eicosapentaenoic acid to arachidonic acid ratio in relation to clinical outcomes after endovascular therapy in patients with peripheral artery disease caused by femoropopliteal artery lesions. *Atherosclerosis* **239**, 583–588 (2015).
73. Chen, N., Chen, L., Jiang, S., Wang, Z. & Liu, T. Predictive value of P-selectin and endothelin-1 for vascular restenosis after interventional procedures for peripheral artery disease. *Exp. Ther. Med.* **17**, 3907–3912 (2019).
74. Milks, M. W. & Nambi, V. Cardiac injury, maladaptation, and heart failure incidence. in *Biomarkers in Cardiovascular Disease* 81–96 (Elsevier, 2019).
75. Schultz, J. & Kaminker, K. Myeloperoxidase of the leucocyte of normal human blood. I. Content and localization. *Arch. Biochem. Biophys.* **96**, 465–467 (1962).
76. Bos, A., Wever, R. & Roos, D. Characterization and quantification of the peroxidase in human monocytes. *Biochimica et Biophysica Acta (BBA) - Enzymology* **525**, 37–44 (1978).
77. Aykan, A. C. *et al.* Neutrophil-to-lymphocyte ratio may be a marker of peripheral artery disease complexity. *Anatol. J. Cardiol.* (2015) doi:10.5152/anatoljcardiol.2015.6240.
78. Eikelboom, J. W. *et al.* Rivaroxaban with or without aspirin in stable cardiovascular disease. *N. Engl. J. Med.* **377**, 1319–1330 (2017).
79. Ridker, P. M. *et al.* Antiinflammatory therapy with canakinumab for atherosclerotic disease. *N. Engl. J. Med.* **377**, 1119–1131 (2017).
80. Karatasakis, A. *et al.* Effect of PCSK9 inhibitors on clinical outcomes in patients with hypercholesterolemia: A meta-analysis of 35 randomized controlled trials. *J. Am. Heart Assoc.* **6**, (2017).
81. Petersohn, S., Pouwels, X., Ramaekers, B., Ten Cate-Hoek, A. & Joore, M. Rivaroxaban plus aspirin for the prevention of ischaemic events in patients with cardiovascular disease: a cost-effectiveness study. *Eur. J. Prev. Cardiol.* **27**, 1354–1365 (2020).
82. Lowe, G. D. & Rumley, A. Fibrinogen and its degradation products as thrombotic risk factors. *Ann. N. Y. Acad. Sci.* **936**, 560–565 (2001).
83. Spronk, H. M., van der Voort, D. & Ten Cate, H. Blood coagulation and the risk of atherothrombosis: a complex relationship. *Thromb. J.* **2**, 12 (2004).
84. Ridker, P. M. *et al.* Relationship of C-reactive protein reduction to cardiovascular event reduction following treatment with canakinumab: a secondary analysis from the CANTOS randomised controlled trial. *Lancet* **391**, 319–328 (2018).
85. Berg, D. D. *et al.* Performance of the ABC scores for assessing the risk of stroke or systemic embolism and bleeding in patients with Atrial Fibrillation in ENGAGE AF-TIMI 48. *Circulation* **139**, 760–771 (2019).
86. Iwanaga, Y. *et al.* B-type natriuretic peptide strongly reflects diastolic wall stress in patients with chronic heart failure: comparison between systolic and diastolic heart failure. *J. Am. Coll. Cardiol.* **47**, 742–748 (2006).
87. Shah, M. R. *et al.* Rapid assay brain natriuretic peptide and troponin I in patients hospitalized with decompensated heart failure (from the Evaluation Study of Congestive Heart Failure and Pulmonary Artery Catheterization Effectiveness Trial). *Am. J. Cardiol.* **100**, 1427–1433 (2007).

88. Hong, Y. J. *et al.* Relation between N-terminal pro-B-type natriuretic peptide and coronary plaque components in patients with acute coronary syndrome: virtual histology-intravascular ultrasound analysis. *Coron. Artery Dis.* **20**, 518–524 (2009).
89. Yang, J. *et al.* Association between increased N-terminal pro-brain natriuretic peptide level and poor clinical outcomes after acute ischemic stroke. *J. Neurol. Sci.* **383**, 5–10 (2017).
90. Mishra, R. K. *et al.* The association of five-year changes in the levels of N-terminal fragment of the prohormone brain-type natriuretic peptide (NT-proBNP) with subsequent heart failure and death in patients with stable coronary artery disease: The heart and Soul Study. *Cardiology* **137**, 201–206 (2017).
91. Sandesara, P. B., Hammadah, M., Samman-Tahhan, A., Kelli, H. M. & O'Neal, W. T. Peripheral artery disease and risk of adverse outcomes in heart failure with preserved ejection fraction. *Clin. Cardiol.* **40**, 692–696 (2017).
92. Smith, S. L., Matthews, E. O., Moxon, J. V. & Golledge, J. A systematic review and meta-analysis of risk factors for and incidence of 30-day readmission after revascularization for peripheral artery disease. *J. Vasc. Surg.* **70**, 996-1006.e7 (2019).
93. Matsushita, K. *et al.* High-sensitivity cardiac troponin and natriuretic peptide with risk of lower-extremity peripheral artery disease: the Atherosclerosis Risk in Communities (ARIC) Study. *Eur. Heart J.* **39**, 2412–2419 (2018).
94. Tanindi, A. & Cemri. Troponin elevation in conditions other than acute coronary syndromes. *Vasc. Health Risk Manag.* 597 (2011).
95. Zhang, H. *et al.* Adiponectin levels and risk of coronary heart disease: a meta-analysis of prospective studies. *Am. J. Med. Sci.* **345**, 455–461 (2013).
96. Sook Lee, E. *et al.* Association between adiponectin levels and coronary heart disease and mortality: a systematic review and meta-analysis. *Int. J. Epidemiol.* **42**, 1029–1039 (2013).
97. Vaidya, A., Joore, M. A., ten Cate-Hoek, A. J., ten Cate, H. & Severens, J. L. Cost-effectiveness of risk assessment and tailored treatment for peripheral arterial disease patients. *Biomark. Med.* **8**, 989–999 (2014).

CHAPTER 5

INADEQUATE RESPONSE TO ANTIPLATELET THERAPY IN HIGH-RISK PATIENTS WITH PERIPHERAL ARTERY DISEASE

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ABSTRACT

Background

Patients with peripheral artery disease (PAD) are treated with preventive strategies to improve the cardiovascular risk. The incidence of cardiovascular events and mortality however remains high in PAD populations. We therefore aimed to better characterize PAD patients suffering from cardiovascular events and mortality in order to tailor preventive treatment.

Methods

Between 2018 and 2020, 246 PAD outpatients (17 newly diagnosed, 229 with known PAD) were prospectively enrolled in this observational cohort study. Patient data and blood samples were collected after inclusion, and the primary composite endpoint (myocardial infarction, elective coronary revascularization, ischemic stroke, acute limb ischemia, mortality) was evaluated after one year. Secondary outcomes included platelet reactivity, measured using the VerifyNow assay, and medication adherence, assessed using the Morisky Medication Adherence Scale-8 (MMAS-8). Logistic regression models were used to identify associations between characteristics and the occurrence of events.

Results

The cohort comprised 207 patients with claudication and 39 with chronic limb threatening ischemia. Twenty-six (10.6%) patients suffered from an event during follow-up. Prior myocardial infarction (OR 3.3 [1.4-7.7]), prior ischemic stroke (OR 4.5 [1.8-10.9]), higher levels of creatinine (OR 5.2 [2.2-12.6]), lower levels of high-density lipoprotein (OR 4.2 [1.5-10.6]) and lower haemoglobin levels (OR 3.1 [1.3-7.1]) were associated with events. Patients with events had more often high on-treatment platelet reactivity (HTPR) on aspirin (OR 5.9 [1.4-25.1]) or clopidogrel (OR 4.3 [1-19.3]). High adherence to medication was associated with the occurrence of events (OR 4.1 [1-18]).

Conclusions

Patients suffering from cardiovascular events and mortality were characterized by prior cardiovascular events as compared to patients who did not experience any events. Antiplatelet therapy was not optimally protective despite high medication adherence, and HTPR was independently associated with the occurrence of events. More research is needed on alternative treatment strategies such as dual antiplatelet therapy or combinations with anticoagulant drugs.

BACKGROUND

Peripheral artery disease (PAD) is a vascular disease characterized by atherosclerosis-driven narrowing of peripheral arteries. The prevalence of PAD worldwide in individuals aged twenty-five years and older was estimated at 236 million in 2015 [1]. Despite its high prevalence, PAD remains underdiagnosed as many patients are asymptomatic and thus not aware of the disease [2]. However, both asymptomatic and symptomatic PAD patients are at risk of atherothrombotic events such as myocardial infarction and ischemic stroke with incidences of 15% over a period of three years [3,4]. Within the symptomatic patient population, intermittent claudication, a mild manifestation of PAD, can be distinguished from the more severe chronic limb threatening ischemia. Intermittent claudication is classified as Fontaine II with typical symptoms of muscle pain during walking. Chronic limb threatening ischemia is classified as Fontaine III with rest pain and Fontaine IV with ischemic ulcer formation [5,6]. PAD patients with chronic limb threatening ischemia are at a higher risk of adverse cardiovascular events with high mortality rates as compared to patients with intermittent claudication [4]. Current preventive strategies for cardiovascular events and mortality in PAD patients are based on risk management in which lipid-lowering drugs, antihypertensive drugs and antiplatelet drugs are the main treatment modalities. Statins are most widely used to improve the lipid profile targeted at a low-density lipoprotein (LDL) value of 1.8 mmol/L for PAD patients of 70 years or younger and a value of 2.5 mmol/L for PAD patients above 70 years [7]. By effectively lowering LDL levels, the incidence of cardiovascular events can be reduced significantly [8]. Addition of antihypertensive drugs to overcome hypertension as well as the use of antiplatelet drugs to effectively inhibit platelet activation reduces the incidence of cardiovascular events even further. Aspirin and clopidogrel are the antiplatelet drugs most often used as first-line treatment depending on national guidelines [5]. Although the CAPRIE-study demonstrated that clopidogrel was more effective than aspirin in reducing the combined risk of ischemic stroke, myocardial infarction, and cardiovascular death, this is no preferential treatment strategy [9]. Despite the established efficacy of antiplatelet regimes with regard to the reduction of cardiovascular events, high on-treatment platelet reactivity (HTPR) for both aspirin and clopidogrel may still occur and interfere with atheroprotective effects. HTPR is referred to as the failure of the antiplatelet agent to inhibit the target of its action [10,11]. Aspirin HTPR prevalence is estimated at 17-26% in PAD populations [12–14] while clopidogrel HTPR appears to be more common with a prevalence up to 54% [12,14–16]. The incidence of cardiovascular events in PAD populations remains high despite current treatment strategies [3]. Therefore, the aim of this observational cohort study was to better characterize PAD patients at risk of cardiovascular events and mortality in order to find targets for improved management.

METHODS

Study design

Between May 2018 and May 2020, patients visiting the outpatient clinic of the department of Vascular Surgery of the Maastricht University Medical Center (MUMC+) were screened for PAD. Patients were eligible to participate in the study when the PAD was objectively diagnosed with an ankle-brachial index (ABI) of 0.9 or below. Fontaine II (intermittent claudication) and Fontaine III (chronic limb threatening ischemia) patients were selected and patients with Fontaine IV were excluded because of expected increased inflammatory parameters associated with ulcer formation. Further exclusion criteria were active malignancy, chronic inflammatory disease, coagulation disorders, pregnancy, age below 18, and the use of anticoagulant therapy. All eligible patients that were willing to participate were included after written informed consent was obtained. The Medical Ethics Committee (METC) of the MUMC+ approved the study (NL63235.068.17) and the study was registered in the Netherlands Trial Register (NTR7250; https://www.trialregister.nl/trial/7045).

Blood collection and sample storage

Venous blood was drawn from the patients immediately after informed consent was signed. Blood drawing took place in a resting state and blood was collected by antecubital venipuncture with 21-gauge needles and 3.2% (w/v) citrated Vacutainer tubes, EDTA Vacutainer tubes and VACUETTE 9NC Coagulation 3.2% (w/v) Sodium Nitrate tubes. After blood drawing, the EDTA tubes and the citrate tubes were directly processed using the standard platelet-poor plasma centrifugation protocol used at our laboratory (4000 x g for 5 minutes followed by 11000 x g for 10 minutes). Thereafter samples were, within two hours after blood drawing, frozen and stored at -80° Celsius for further analysis. The VACUETTE 9NC tubes were immediately used to perform the VerifyNow assays for aspirin and clopidogrel.

Data collection and measurements

Age, sex and date of PAD diagnosis of each patient were registered upon inclusion. The medical history of each patient including prior cardiovascular events such as myocardial infarction, ischemic stroke and PAD revascularization was collected from patient records. Each patient provided an updated medication list from which the use of lipid-lowering drugs, antihypertensive drugs and antiplatelet drugs were collected. The intensity of lipid-lowering strategies was categorized as high, medium and low intensity according to the ACC/AHA guideline [17]. Current smoking status, diabetes mellitus type 2 (DM2) and body mass index (BMI) were recorded. Patients were classified based on their symptoms upon inclusion using the Fontaine classification, and were then grouped as having intermittent claudication (Fontaine II) or chronic limb threatening ischemia (Fontaine III). The ABI at the time of diagnosis was measured and grouped by ratio as greater than 1.3 (incompressible), between 0.91 and 1.3, between 0.7 and 0.9, between 0.4 and 0.69 and below 0.4.

A complete blood cell count was performed at baseline and included levels of haemoglobin, haematocrit, thrombocytes and leukocytes with respective subpopulations. Platelet reactivity was assessed using the VerifyNow Aspi assay for Aspirin and VerifyNow P2Y12 assay for Clopidogrel (Accumetrics, San Diego, CA, USA). Blood collected in the VACUETTE 9NC tube was used in the optical detection system using a specific cartridge. The cut-off value for aspirin and clopidogrel HTPR was based on the most recent consensus document on the definition of on-treatment platelet reactivity, and was set at Aspirin Reaction Units (ARU) >550 for aspirin [18] and P2Y12 Reaction Units (PRU) >208 for clopidogrel [11]. Laboratory results that were collected from recent blood drawing included kidney function (creatinine, estimated glomerular filtration rate-Chronic Kidney Disease Epidemiology Collaboration (eGFR (CKD-EPI))), lipid profile (cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL), triglycerides) and haemoglobin A1c levels (HbA1c). The KDIGO guideline was used to classify the kidney function, and the stage of chronic kidney disease in each patient when appropriate [19].

Medication adherence was assessed by the licensed Morisky Medication Adherence Scale-8 (MMAS-8), which was developed by Morisky et al. The MMAS-8 is a validated assessment tool verified by numerous studies, consisting of eight questions to assess medication adherence [20–22]. Patients that were completely adherent scored a maximum score of 8, whereas the lowest possible adherence was scored 0. Each point decrease marked lower adherence to the medical treatment. According to MMAS-8 user guidelines the adherence was categorized as high (8 points), medium (7 or 6 points) and low (5 points or below).

Outcome

The primary outcome consisted of a composite endpoint comprising myocardial infarction, ischemic stroke, acute limb ischemia, elective percutaneous intervention (PCI) or coronary artery bypass grafting (CABG) and all-cause mortality during the one-year follow-up. The outcome was assessed at 3, 6 and 12 months and was verified by telephone calls to the patient combined with hospital records. Patients who reached the composite endpoint were grouped as the "PAD event group" while patients who did not reach the composite endpoint were grouped as the "PAD no event group". The secondary outcomes were platelet reactivity, HTPR and medication adherence.

Statistical analysis

Baseline characteristics were collected for all patients and presented for patients with and without events during follow-up. Differences between both groups were analyzed using the chi-square test for dichotomous and categorical variables. For continuous variables, differences were analyzed using the parametric two-samples t-test or the non-parametric Mann-Whitney U test, as appropriate. Youden's index was used, when appropriate, to determine optimal cut-off values for continuous variables. Univariable logistic regression models were used to test the associations

of characteristics with the occurrence of events, reported as odds ratios with respective 95% confidence intervals (OR [95% CI]). Characteristics with an association with the occurrence of events ($p < 0.05$) in the univariable analysis were then used in multivariable models with backward stepwise logistic regression analysis for the occurrence of events, reported as odds ratios with respective 95% confidence intervals. Statistical significance was reached when $p < 0.05$. All analyses were performed using SPSS (IBM SPSS Statistics for Macintosh, Version 27.0. Armonk, NY: IBM Corp). All figures were created using GraphPad Prism (GraphPad Prism version 9 for Mac OS X, GraphPad Software, San Diego, California USA, www.graphpad.com).

RESULTS

Patient characteristics

The cohort comprised 246 patients and baseline characteristics of the entire cohort as well as the distribution of patients with and without events are shown in *Table 1*. All patients had been diagnosed with PAD at a median of 33 (8-101) months prior to inclusion, while in 17 (6.9%) patients the diagnosis was established at the time of inclusion. Upon inclusion, most patients had intermittent claudication (207 (84.1%)) and 39 (15.9%) had chronic limb threatening ischemia. The cohort consisted of 141 (57.3%) male patients and the mean age was 68.7±9.2 years. Most patients (109 (44.3%)) had an ABI between 0.7 and 0.9, while 99 (40.2%) and 21 (8.5%) patients had an ABI between 0.4 and 0.69 or below 0.4, respectively. The remaining 17 (6.9%) patients had incompressible arteries. Patient history revealed that 151 (61.4%) patients had previously undergone a peripheral revascularization procedure. Moreover, 72 (29.3%) patients had a prior myocardial infarction and 37 (15%) had a prior ischemic stroke. DM2 was present in 67 (27.2%) patients and the mean BMI was 26.4±4.41 kg/m^2. Of all patients, 229 (93.1%) had a history of smoking and 94 (38.2%) were current smokers with a median of 29 (15-40) pack years upon inclusion. A normal kidney function (G1) was observed in 41 (16.7%) patients, a mildly decreased kidney function (G2) in 138 (56.1%) patients, a mildly to moderately decreased kidney function (G3a) in 41 (16.7%) patients, a moderately to severe decreased kidney function (G3b) in 19 (7.7%) patients, a severely decreased kidney function (G4) in 6 (2.3%) patients and kidney failure (G5) in 1 (0.4%) patient.

	Total cohort (n = 246)	Event group (n = 26)	No event group (n =220)	P-value
	Mean±SD / Median (IQR) / n (%)	Mean±SD / Median (IQR) / n (%)	Mean±SD / Median (IQR) / n (%)	
Age (years)	68.7±9.2	71.5±7.9	68.3±9.3	0.093
Male gender	141 (57.3)	17 (65.4)	124 (56.4)	0.379
Newly diagnosed PAD patient upon inclusion	17 (6.9)	2 (7.7)	15 (6.8)	0.868
Chronic PAD patient upon inclusion	229 (93.1)	24 (92.3)	205 (93.2)	0.868
Time between diagnosis and inclusion (months)	33 (8-101)	81 (19-121)	26 (7-89)	**0.028***
Intermittent claudication	207 (84.1)	22 (84.6)	185 (84.1)	0.945
Chronic limb ischemia	39 (15.9)	4 (15.4)	35 (15.9)	0.945
History of myocardial infarction	72 (29.3)	14 (53.8)	58 (26.8)	**0.004***
History of stroke	37 (15)	10 (38.5)	27 (12.3)	**0.001***
Current smoking	94 (38.2)	11 (42.3)	83 (37.7)	0.649
Pack years	29 (15-40)	33 (18-50)	29 (15-40)	0.175
BMI (kg/m^2)	26.4±4.41	27.4±5.9	26.3±4.2	0.390
DM2	67 (27.2)	10 (38.5)	57 (25.9)	0.174
HbA1c (mmol/mol)	43 (37-51)	43 (37-53)	43 (37-50)	0.752
Creatinine (mmol/L)	85 (72-104)	101 (77-145)	83 (71-101)	**0.006***
eGFR (ml/min/1.73 m^2)	71 (58-83)	59 (42-80)	72 (59-84)	**0.022***
Haemoglobin (mmol/L)	8.66±0.97	8.3±1.1	8.7±0.95	**0.026***
Haematocrit (L/L)	0.42±0.04	0.4±0.04	0.42±0.04	**0.027***
Thrombocytes (x 10^3 /mm^3)	272±87	277±82	271±88	0.714
Leukocytes (x 10^9/L)	7.89±2.2	8.44±3.1	7.83±2	0.342
Neutrophils (%)	62.2±8.2	64±7.5	62±8.2	0.228
Lymphocytes (%)	25.8±6.8	24.1±6.5	26±6.9	0.168
Eosinophils (%)	2 (1-3)	2 (2-3)	2 (1-3)	0.254
Basophils (%)	1 (1-1)	1 (1-1)	1 (1-1)	0.544
Monocytes (%)	8.83±2.4	8.8±3.2	8.8±2.3	0.901
NLR	2.4 (1.9-3.1)	2.5 (2-3.4)	2.4 (1.9-3.1)	0.288
ABI at diagnosis				0.156
>1.30 (incompressible)	17 (6.9)	0 (0)	17 (7.7)	
0.91-1.30	0 (0)	0 (0)	0 (0)	
0.70-0.90	109 (44.3)	9 (34.6)	100 (45.5)	
0.40-0.69	99 (40.2)	16 (61.5)	83 (37.7)	
<0.40	21 (8.5)	1 (3.8)	20 (9.1)	

Table 1: Baseline characteristics for the whole cohort and distribution between patients with and without cardiovascular events and mortality during follow-up. Significance was reached when P <0.05 (*), significant values are in bold. PAD = peripheral artery disease, BMI = body mass index, DM2 = diabetes mellitus type 2, HbA1c = haemoglobin A1c, eGFR = estimated glomerular filtration rate, NLR = neutrophil-lymphocyte ratio, ABI = ankle-brachial index, SD = standard deviation, IQR = interquartile range.

Composite endpoint

All patients were followed for one year in which 26 (10.6%) patients reached the composite endpoint. Ten (38.5%) myocardial infarctions, four (15.4%) elective coronary revascularizations, five (19.2%) ischemic strokes and seven (26.9%) deaths were recorded. No differences were observed between patients with and without events regarding smoking status, DM2 and BMI. Both prior myocardial infarction (OR 3.3 [1.4-7.7]) and prior ischemic stroke (OR 4.5 [1.8-10.9]) were associated with the occurrence of events *(Figure 1)*. Also, decreased kidney function (plasma creatinine level > 111 μmol/L, OR 5.2 [2.2-12.6]) and plasma haemoglobin levels < 8.1 mmol/L (OR 3.1 [1.3-7.1]) were associated with the occurrence of events. Leukocyte and thrombocyte count were not associated.

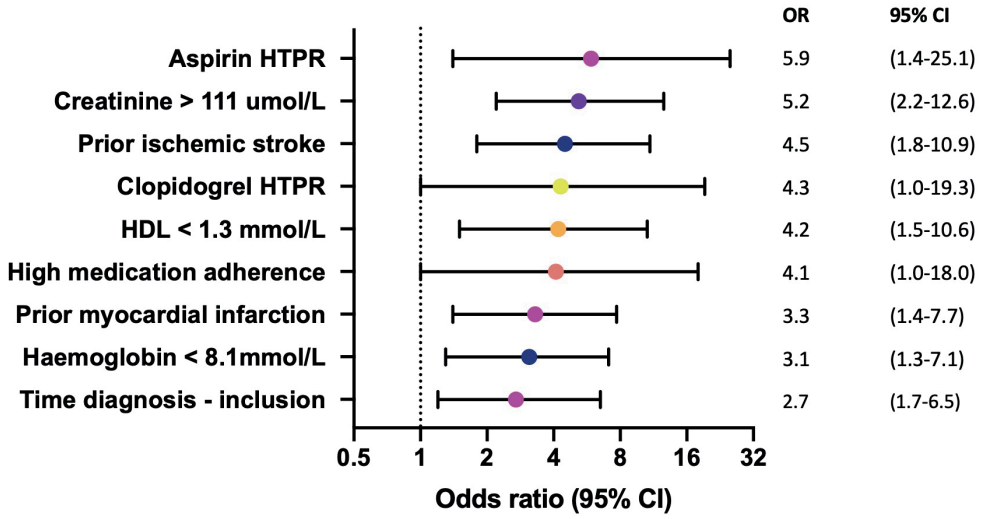

Figure 1: Univariable logistic regression analysis of characteristics associated with the occurrence of cardiovascular events and mortality, with corresponding odds ratios and 95% confidence intervals. HTPR = high on-treatment platelet reactivity, HDL = high-density lipoprotein, SD = standard deviation, IQR = interquartile range.

Evaluation of medication strategies

All patients were treated according to current guidelines [5] which included the use of antihypertensive drugs, lipid-lowering drugs and antiplatelet drugs. The prescription of antihypertensive drugs (73.1% vs 72.7%, p = 0.834), lipid-lowering drugs (88.5% vs 90%, p = 0.396) and antiplatelet drugs (100% vs 100%, p = 1.000) did not differ between patients with and without events. Lipid-lowering strategies were prescribed in different intensities. A total of 71 (32.6%) patients were on high intensity lipid-lowering therapy without differences between patients with and without events (31.8% vs 32.7%, p = 0.937). Moderate and low intensity therapy had been applied in 138 (63.3%) and 9 (4.1%) patients, but also in these groups no differences were observed between patients with and without events (63.6% vs 63.3%, p = 0.973

and 4.5% vs 4.1%, p = 0.917 respectively). The effectiveness of the lipid-lowering therapies was assessed using the cholesterol profile, showing similar mean LDL levels of 2.44±1.07 mmol/L between patients with events and those without (2.31±1.14 mmol/L vs 2.45±1.06 mmol/L, p = 0.542). In 58.5% of patients above 70 years old the LDL target level of 2.5 mmol/L was reached (2.49±1.18 mmol/L), while the target level of 1.8 mmol/L was not reached in 95 (72%) patients 70 years or younger (2.39±0.97 mmol/L). HDL levels were significantly lower in patients who experienced an event during follow-up (OR 4.2 [1.5-10.6]). The use of antiplatelet agents was evenly distributed in the cohort with 130 (52.8%) patients on aspirin, 127 (51.6%) on clopidogrel. Additionally, 11 (4.5%) patients were on dual antiplatelet therapy. During the conduct of this study, there was a transitioning of aspirin to clopidogrel as first choice antiplatelet agent in the hospital where patients were recruited. Therefore, some patients were using aspirin upon inclusion, while others were using clopidogrel. The median ARU on aspirin was 435 (402-482) and 12 (8.5%) patients had HTPR. The ARU in patients with events during follow-up was significantly higher compared to those without events (521 (452-554) vs 428 (401-478), p = 0.011) and HTPR was associated with the occurrence of events (OR 5.9 [1.4-25.1]). PRU in patients on clopidogrel were 100 (46-155) for the whole cohort and significantly higher in patients with events (144 (102-190) vs 96 (43-144), p = 0.019) *(Figure 2)*. HTPR on clopidogrel was observed in 8 (5.8%) patients and was associated with events (OR 4.3 [1-19.3]). In the multivariable analysis the adjusted OR for antiplatelet therapy was 5.2 [1.5-18.5] (*Figure 3)*.

High medication adherence was observed in 188 (76.4%) patients and was positively associated with the occurrence of events (OR 4.1 [1-18]). Medium adherence was observed in 46 (18.7%) patients and low adherence in 12 (4.9%) patients, both were not associated with the occurrence of events.

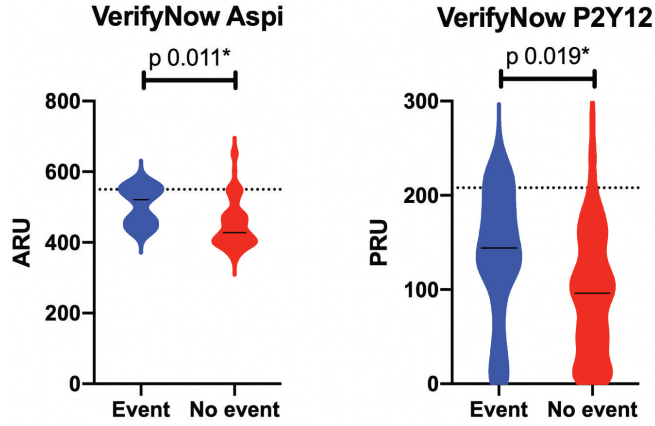

Figure 2: Platelet reactivity measured by the use of the VerifyNow assay in Aspirin Reactions Units (ARU) for aspirin users and P2Y12 Reaction Units (PRU) for clopidogrel users. Dotted lines represent HTPR which is an ARU > 550 for aspirin and a PRU > 208 for clopidogrel.

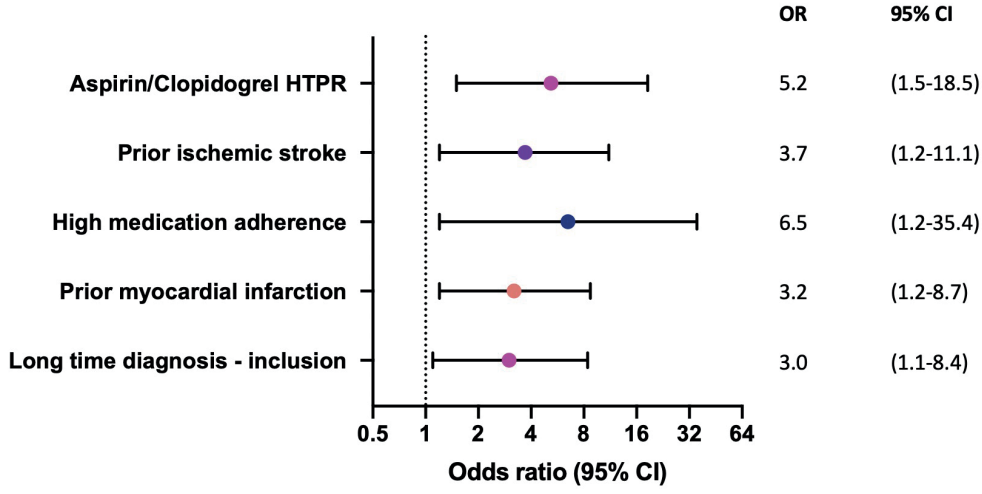

Figure 3: Multivariable logistic regression of characteristics associated with the occurrence of cardiovascular events and mortality, with corresponding odds ratios and 95% confidence intervals. HTPR = high on-treatment platelet reactivity, HDL = high-density lipoprotein, SD = standard deviation, IQR = interquartile range.

DISCUSSION

In this prospective observational cohort study, we characterized PAD patients with enhanced risk for cardiovascular events and mortality with the aim to find targets for improved management. Most patients in our cohort had prevalent PAD with a chronic state of atherosclerosis with years of plaque build-up and involvement of multiple vascular beds with associated decrease in renal function in conjunction with lower haemoglobin levels. Patients with such polyvascular disease

are at increased risk for cardiovascular events with worsening prognosis when more vascular beds are affected [23]. In almost half of the patients in the cohort this polyvascular diseased state was present.

All patients in our cohort were treated with lipid-lowering agents, antiplatelet therapy and antihypertensive drugs. Interestingly, the medication prescribed did not appear to be sufficiently protective for PAD patients experiencing cardiovascular events and mortality, despite adequate adherence to the medication. Suboptimal target LDL levels were observed. The intensity of the lipid-lowering strategies prescribed was medium to high according to the ACC/AHA guidelines [7]. The LDL target level of 2.5 mmol/L was indeed reached in more than half of all patients older than 70 years. On the other hand, average LDL levels in patients of 70 years or younger were similar to the older patient group, while in these patients LDL levels of 1.8 mmol/L or lower are recommended. Especially in these younger patients the lipid-lowering regimen should ideally be intensified, which will likely result in a reduced incidence of cardiovascular events as the association between increased LDL levels and cardiovascular risk is well established [24]. Potentially, LDL target values of 1.8 and 2.5 mmol/L could even be lowered further as a recent study showed that lower concentrations of LDL may even better prevent cardiovascular events [25]. Also lower HDL levels were seen in patients that suffered from an event during follow-up, which indirectly supports the known atheroprotective effects of HDL including counteracting inflammation [26] and oxidative stress [27]. Several studies have found an association between lower HDL levels and cardiovascular risk in patients with coronary artery disease [28,29] and low concentrations of HDL as one of the strongest lipoprotein risk factors for PAD [30,31].

The VerifyNow assay was used to measure platelet reactivity while on aspirin or clopidogrel (or both). The residual platelet reactivity in patients on aspirin or on clopidogrel was significantly higher in patients experiencing events, indicating that platelets are less efficiently inhibited. Lack of medication adherence could have caused residual platelet reactivity. However, this did not seem to be the case as the results of the adherence score revealed that highly adherent patients were in the majority in the event group, which could be the result of increased awareness in this patient group as these patients more often experienced prior myocardial infarctions and ischemic strokes. Therefore, assuming that the adherence assessment is reliable, the current antithrombotic regime appears to be insufficient for adequate cardiovascular protection in these high-risk patients. Published studies show conflicting results regarding the association of HTPR with cardiovascular outcome. Two studies investigating clopidogrel HTPR found a significant association with cardiovascular events while two other studies did not [12,14–16]. These studies used the same cut-off values for HTPR and follow-up duration was also similar. The contradicting results may however be explained by the lack of power. One study found a non-significant trend between clopidogrel HTPR and cardiovascular events [12], while the other study found a non-significantly increased hazard ratio in patients with HTPR [14]. In all four studies the prevalence of

clopidogrel HTPR was higher as compared to the HTPR prevalence in our study, which could be explained by differences in medication adherence. We were not able to confirm this as other studies did not report on adherence. The strength of the risk association of HTPR that we found for both aspirin and clopidogrel suggests that optimization of antiplatelet therapy is an important management target for improvement. For aspirin, there is no known mechanism for biochemical resistance, but high platelet turnover could be a reason for residual platelet hyperreactivity [32]. In patients taking clopidogrel the HTPR could be explained by genetic polymorphisms in platelet receptor P2Y12 [33,34] or polymorphisms of the CYP2C9 and CYP2C19 genes [35,36]. Recent studies in patients with coronary artery disease investigated pharmacogenomics based on CYP2C19 gene variations to optimize therapy [37,38]. Moreover, a meta-analysis concluded that the use of ticagrelor or prasugrel appeared more effective than clopidogrel in reducing the cardiovascular risk in patients with CYP2C19 gene variants [39]. Similar studies have yet to be performed in patients with PAD. The association between P2Y12 polymorphisms and the risk for cerebrovascular events in PAD patients has been established in the past [40]. Indeed, recent studies suggest that a twice-daily dosing of aspirin could improve its pharmacological efficacy. In patients with essential thrombocythemia a once-daily dose of aspirin as antithrombotic regime appeared inadequate in reducing platelet activation, while a dosing interval of 12 hours increased the antiplatelet response to aspirin [41,42]. For clopidogrel, studies with increased dosing to compensate for the low inhibitory efficacy have been performed in the past [43], assuming "resistance" to be in part explained by too low concentrations of active clopidogrel at the platelet surface [44,45]. However, apart from the use of loading doses in patients undergoing percutaneous coronary interventions, such regimens were never introduced in clinical practice in patients with PAD [46]. Dual antiplatelet therapy (DAPT) has been studied in the large CHARISMA trial [47]. Except for exceptionally thrombogenic conditions DAPT has not been introduced for long-term treatment of patients with PAD because of increased bleeding risk as compared to single antiplatelet therapy. Guidelines only recommend DAPT for a short period of time following percutaneous interventions and stenting in PAD. Several studies demonstrated that fibrinogen [48,49] and d-dimer [50] levels were increased in high-risk PAD patients indicating an underlying hypercoagulable state. Anticoagulant treatment may counteract this prothrombotic state in PAD patients which is characterized by increased clot formation [51]. The COMPASS-trial has shown that dual pathway inhibition with aspirin and a low dose rivaroxaban reduced the incidence of cardiovascular events in high-risk PAD patients [52], suggesting that a reasonably low level of anticoagulation on top of antiplatelet therapy provides additional benefit. In spite of its demonstrated cost-effectiveness in at least a subset of PAD patients [53], the use of dual pathway inhibition in practice is still hindered by low uptake due to concerns about the number of pills per day in combination with an increased risk of major bleeding, even though fatal or critical organ bleeding events remained limited [52].

Limitations

The use of the VerifyNow assay to identify HTPR may be perceived as a possible limitation of this study. Several studies have however shown that this assay correlates well with the "gold standard" of light transmission aggregometry for both aspirin [54] and clopidogrel [55]. The recorded rates of HTPR within our study population were lower than rates reported by most other studies [12,14–16], this can however be explained by the overall high medication adherence rate that we recorded. The positive association that was found between high medication adherence and higher risk for cardiovascular events in the multivariable analysis may be confounded as this risk is likely to be primarily attributed to the higher rate of comorbidities and prior cardiovascular events leading to the increased motivation to be adherent to medication in these patients. Finally, due to sample size limitations, there is a lack of precision surrounding the estimates which demonstrates that there is still uncertainty about the actual effect size and that further information is needed.

Conclusion

In our single-center cohort of PAD patients, current treatment strategies appeared to be insufficient for the reduction of cardiovascular risk. Lipid-lowering strategies should be intensified to further reduce LDL levels and improve the lipid profile. Antiplatelet agents were found to be inadequate despite high medication adherence, as platelet reactivity was insufficiently decreased in patients experiencing cardiovascular events. More research is needed on alternative treatment strategies such as dual antiplatelet therapy or combinations with anticoagulant drugs.

REFERENCES

1. Song, P. *et al.* Global, regional, and national prevalence and risk factors for peripheral artery disease in 2015: an updated systematic review and analysis. *Lancet Glob. Health* **7**, e1020–e1030 (2019).
2. Fowkes, F. G. *et al.* Edinburgh Artery Study: prevalence of asymptomatic and symptomatic peripheral arterial disease in the general population. *Int. J. Epidemiol.* **20**, 384–392 (1991).
3. Alberts, M. J. *et al.* Three-year follow-up and event rates in the international REduction of Atherothrombosis for Continued Health Registry. *Eur. Heart J.* **30**, 2318–2326 (2009).
4. Sartipy, F., Sigvant, B., Lundin, F. & Wahlberg, E. Ten year mortality in different peripheral arterial disease stages: A population based observational study on outcome. *Eur. J. Vasc. Endovasc. Surg.* **55**, 529–536 (2018).
5. Aboyans, V. *et al.* 2017 ESC Guidelines on the Diagnosis and Treatment of Peripheral Arterial Diseases, in collaboration with the European Society for Vascular Surgery (ESVS): Document covering atherosclerotic disease of extracranial carotid and vertebral, mesenteric, renal, upper and lower extremity arteriesEndorsed by: the European Stroke Organization (ESO)The Task Force for the Diagnosis and Treatment of Peripheral Arterial Diseases of the European Society of Cardiology (ESC) and of the European Society for Vascular Surgery (ESVS). *Eur. Heart J.* **39**, 763–816 (2018).
6. Fontaine, R., Kim, M. & Kieny, R. Surgical treatment of peripheral circulation disorders. *Helv. Chir. Acta* **21**, 499–533 (1954).
7. Stone, N. J. *et al.* Treatment of blood cholesterol to reduce atherosclerotic cardiovascular disease risk in adults: synopsis of the 2013 American College of Cardiology/American Heart Association cholesterol guideline. *Ann. Intern. Med.* **160**, 339–343 (2014).
8. Collins, R. *et al.* Interpretation of the evidence for the efficacy and safety of statin therapy. *Lancet* **388**, 2532–2561 (2016).
9. CAPRIE Steering Committee. A randomised, blinded, trial of clopidogrel versus aspirin in patients at risk of ischaemic events (CAPRIE). *Lancet.* **348**, 1329-1339 (1996).
10. Bonello, L. *et al.* Consensus and future directions on the definition of high on-treatment platelet reactivity to adenosine diphosphate. *J. Am. Coll. Cardiol.* **56**, 919–933 (2010).
11. Tantry, U. S. *et al.* Consensus and update on the definition of on-treatment platelet reactivity to adenosine diphosphate associated with ischemia and bleeding. *J. Am. Coll. Cardiol.* **62**, 2261–2273 (2013).
12. Yeo, K.-K. *et al.* Aspirin and clopidogrel high on-treatment platelet reactivity and genetic predictors in peripheral arterial disease. *Catheter. Cardiovasc. Interv.* **91**, 1308–1317 (2018).
13. Pasala, T. *et al.* Aspirin resistance predicts adverse cardiovascular events in patients with symptomatic peripheral artery disease. *Tex. Heart Inst. J.* **43**, 482–487 (2016).
14. Armstrong, E. J. *et al.* Association of dual-antiplatelet therapy with reduced major adverse cardiovascular events in patients with symptomatic peripheral arterial disease. *J. Vasc. Surg.* **62**, 157-165.e1 (2015).
15. Pastromas, G. *et al.* Clopidogrel responsiveness in patients undergoing peripheral angioplasty. *Cardiovasc. Radiol.* **36**, 1493–1499 (2013).
16. Spiliopoulos, S., Kassimis, G., Hatzidakis, A. & Krokidis, M. High on-treatment platelet reactivity in peripheral endovascular procedures. *Cardiovasc. Radiol.* **37**, 559–571 (2014).

17. Stone, N. J. *et al.* 2013 ACC/AHA guideline on the treatment of blood cholesterol to reduce atherosclerotic cardiovascular risk in adults: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines. *J. Am. Coll. Cardiol.* **63**, 2889–2934 (2014).
18. Lee, P.-Y. *et al.* Low-dose aspirin increases aspirin resistance in patients with coronary artery disease. *Am. J. Med.* **118**, 723–727 (2005).
19. Kidney Disease: Improving Global Outcomes (KDIGO) Blood Pressure Work Group. KDIGO 2021 clinical practice guideline for the management of blood pressure in chronic kidney disease. *Kidney Int.* **99**, S1–S87 (2021).
20. Morisky, D. E., Ang, A., Krousel-Wood, M. & Ward, H. J. Predictive validity of a medication adherence measure in an outpatient setting. *J. Clin. Hypertens. (Greenwich)* **10**, 348–354 (2008).
21. Krousel-Wood, M. *et al.* New medication adherence scale versus pharmacy fill rates in seniors with hypertension. *Am. J. Manag. Care* **15**, 59–66 (2009).
22. Morisky, D. E. & DiMatteo, MR. Improving the measurement of self-reported medication nonadherence: response to authors. *Journal of clinical epidemiology* vol. 64 255–7; discussion 258-63 (2011).
23. Subherwal, S. *et al.* Polyvascular disease and long-term cardiovascular outcomes in older patients with non-ST-segment-elevation myocardial infarction. *Circ. Cardiovasc. Qual. Outcomes* **5**, 541–549 (2012).
24. Ference, B. A. *et al.* Low-density lipoproteins cause atherosclerotic cardiovascular disease. 1. Evidence from genetic, epidemiologic, and clinical studies. A consensus statement from the European Atherosclerosis Society Consensus Panel. *Eur. Heart J.* **38**, 2459–2472 (2017).
25. Sabatine, M. S., Wiviott, S. D., Im, K., Murphy, S. A. & Giugliano, R. P. Efficacy and safety of further lowering of low-density lipoprotein cholesterol in patients starting with very low levels. *JAMA Cardiol.* (2018) doi:10.1001/jamacardio.2018.2258.
26. Barter, P. J. *et al.* Antiinflammatory properties of HDL. *Circ. Res.* **95**, 764–772 (2004).
27. Kontush, A. & Chapman, M. J. Antiatherogenic function of HDL particle subpopulations: focus on antioxidative activities. *Curr. Opin. Lipidol.* **21**, 312–318 (2010).
28. Giugliano, R. P. *et al.* Clinical efficacy and safety of achieving very low LDL-cholesterol concentrations with the PCSK9 inhibitor evolocumab: a prespecified secondary analysis of the FOURIER trial. *Lancet* **390**, 1962–1971 (2017).
29. Wilson, P. W., Abbott, R. D. & Castelli, W. P. High density lipoprotein cholesterol and mortality. The Framingham Heart Study. *Arteriosclerosis* **8**, 737–741 (1988).
30. Murabito, J. M. *et al.* Prevalence and clinical correlates of peripheral arterial disease in the Framingham Offspring Study. *Am. Heart J.* **143**, 961–965 (2002).
31. Newman, A. B. *et al.* Ankle-arm index as a marker of atherosclerosis in the Cardiovascular Health Study. Cardiovascular Heart Study (CHS) Collaborative Research Group. *Circulation* **88**, 837–845 (1993).
32. Cesari, F. *et al.* Relationship between high platelet turnover and platelet function in high-risk patients with coronary artery disease on dual antiplatelet therapy. *Thromb. Haemost.* **99**, 930–935 (2008).
33. Fontana, P. *et al.* Adenosine diphosphate-induced platelet aggregation is associated with P2Y12 gene sequence variations in healthy subjects. *Circulation* **108**, 989–995 (2003).
34. Lee, S.-J. *et al.* Identification of P2Y12 single-nucleotide polymorphisms and their influences on the variation in ADP-induced platelet aggregation. *Thromb. Res.* **127**, 220–227 (2011).

35. Brandt, J. T. *et al.* Common polymorphisms of CYP2C19 and CYP2C9 affect the pharmacokinetic and pharmacodynamic response to clopidogrel but not prasugrel. *J. Thromb. Haemost.* **5**, 2429–2436 (2007).
36. Hulot, J.-S. *et al.* Cytochrome P450 2C19 loss-of-function polymorphism is a major determinant of clopidogrel responsiveness in healthy subjects. *Blood* **108**, 2244–2247 (2006).
37. Claassens, D. M. F. *et al.* A genotype-guided strategy for oral P2Y12 inhibitors in primary PCI. *N. Engl. J. Med.* **381**, 1621–1631 (2019).
38. Pereira, N. L. *et al.* Effect of genotype-guided oral P2Y12 inhibitor selection vs conventional clopidogrel therapy on ischemic outcomes after percutaneous coronary intervention: The TAILOR-PCI randomized clinical trial. *JAMA* **324**, 761–771 (2020).
39. Pereira, N. L. *et al.* Effect of CYP2C19 genotype on ischemic outcomes during oral p2y12 inhibitor therapy: a meta-analysis. *JACC Cardiovasc Interv.* **14**, 739-750 (2021).
40. Ziegler, S. *et al.* Association of a functional polymorphism in the clopidogrel target receptor gene, P2Y12, and the risk for ischemic cerebrovascular events in patients with peripheral artery disease. *Stroke* **36**, 1394–1399 (2005).
41. Rocca, B. *et al.* A randomized double-blind trial of 3 aspirin regimens to optimize antiplatelet therapy in essential thrombocythemia. *Blood* **136**, 171–182 (2020).
42. Larsen, M. L. *et al.* Once- versus twice-daily aspirin treatment in patients with essential thrombocytosis. *Platelets* **30**, 322–328 (2019).
43. Gladding, P. *et al.* The antiplatelet effect of higher loading and maintenance dose regimens of clopidogrel: the PRINC (Plavix Response in Coronary Intervention) trial. *JACC Cardiovasc. Interv.* **1**, 612–619 (2008).
44. Louca, J. I., Mina, G. S., Habib, B. W. & Sadek, S. E. The effect of doubling the dose of clopidogrel on platelet aggregation in patients with clopidogrel resistance. *Egypt. Heart J.* **66**, 259–262 (2014).
45. Aleil, B. *et al.* Clopidogrel 150 mg/day to overcome low responsiveness in patients undergoing elective percutaneous coronary intervention: results from the VASP-02 (Vasodilator-Stimulated Phosphoprotein-02) randomized study. *JACC Cardiovasc. Interv.* **1**, 631–638 (2008).
46. Siller-Matula, J. M. *et al.* Impact of clopidogrel loading dose on clinical outcome in patients undergoing percutaneous coronary intervention: a systematic review and meta-analysis. *Heart* **97**, 98–105 (2011).
47. Bhatt, D. L., Topol, E. J. & Clopidogrel for High Atherothrombotic Risk and Ischemic Stabilization, Management, and Avoidance Executive Committee. Clopidogrel added to aspirin versus aspirin alone in secondary prevention and high-risk primary prevention: rationale and design of the Clopidogrel for High Atherothrombotic Risk and Ischemic Stabilization, Management, and Avoidance (CHARISMA) trial. *Am. Heart J.* **148**, 263–268 (2004).
48. Altes, P. *et al.* Raised fibrinogen levels and outcome in outpatients with peripheral artery disease. *Angiology* **69**, 507–512 (2018).
49. Doweik, L. *et al.* Fibrinogen predicts mortality in high risk patients with peripheral artery disease. *Eur. J. Vasc. Endovasc. Surg.* **26**, 381–386 (2003).
50. Kleinegris, M.-C. F., ten Cate, H. & ten Cate-Hoek, A. J. D-dimer as a marker for cardiovascular and arterial thrombotic events in patients with peripheral arterial disease. A systematic review. *Thromb. Haemost.* **110**, 233–243 (2013).
51. Kleinegris, M.-C. F. *et al.* Increased clot formation in the absence of increased thrombin generation in patients with peripheral arterial disease: A case-control study. *Front. Cardiovasc. Med.* **4**, 23 (2017).

52. Anand, S. S. *et al.* Rivaroxaban with or without aspirin in patients with stable peripheral or carotid artery disease: an international, randomised, double-blind, placebo-controlled trial. *Lancet* **391**, 219–229 (2018).
53. Petersohn, S., Pouwels, X., Ramaekers, B., Ten Cate-Hoek, A. & Joore, M. Rivaroxaban plus aspirin for the prevention of ischaemic events in patients with cardiovascular disease: a cost-effectiveness study. *Eur. J. Prev. Cardiol.* **27**, 1354–1365 (2020).
54. Paniccia, R. *et al.* Comparison of different methods to evaluate the effect of aspirin on platelet function in high-risk patients with ischemic heart disease receiving dual antiplatelet treatment. *Am. J. Clin. Pathol.* **128**, 143–149 (2007).
55. Paniccia, R. *et al.* Different methodologies for evaluating the effect of clopidogrel on platelet function in high-risk coronary artery disease patients. *J. Thromb. Haemost.* **5**, 1839–1847 (2007).

CHAPTER 6

DISCOVERY OF FOUR PLASMATIC BIOMARKERS POTENTIALLY PREDICTING CARDIOVASCULAR OUTCOME IN PERIPHERAL ARTERY DISEASE

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ABSTRACT

Peripheral artery disease (PAD) patients have an increased cardiovascular risk despite pharmacological treatment strategies. Biomarker research improving risk stratification only focused on known atherothrombotic pathways, but unexplored pathways might play more important roles. To explore the association between a broad cardiovascular biomarker set and cardiovascular risk in PAD. 120 PAD outpatients were enrolled in this observational cohort study. Patients were followed for one year in which the composite endpoint (myocardial infarction, coronary revascularization, stroke, acute limb ischemia and mortality) was assessed. Patient data and blood samples were collected upon inclusion, and citrated platelet-poor plasma was used to analyze 184 biomarkers in Olink Cardiovascular panel II and III using a proximity extension assay. Fifteen patients reached the composite endpoint. These patients had more prior strokes and higher serum creatinine levels. Multivariate analysis revealed increased plasma levels of protease-activated receptor 1 (PAR1), galectin-9 (Gal-9), tumor necrosis factor receptor superfamily member 11A (TNFRSF11A) and interleukin 6 (IL-6) to be most predictive for cardiovascular events and mortality. Positive regulation of acute inflammatory responses and leukocyte chemotaxis were identified as involved biological processes. This study identified IL-6, PAR1, Gal-9, TNFRSF11A as potent predictors for cardiovascular events and mortality in PAD, and potential drug development targets.

INTRODUCTION

Peripheral artery disease (PAD) involves atherosclerotic plaque formation in peripheral vascular beds leading to progressive blood flow restriction in large and medium-sized arteries. PAD patients are at increased risk of atherothrombotic events such as myocardial infarction, ischemic stroke or cardiovascular death, with an incidence of 5% to 14% each year [1]. This high incidence of cardiovascular events within PAD populations is partly caused by concomitantly affected vascular beds, such as the coronary arteries, in more than 60% of all PAD patients [2,3]. The rate of complications illustrates the need to better identify patients at highest risk that would benefit from more intensive cardiovascular risk management. Although several biomarkers have been identified as predictors of cardiovascular events and mortality in previous studies, as summarized in our recent systematic review [4], surprisingly none have been implemented yet in clinical management. One reason is the perception that current biomarkers including high-sensitivity c-reactive protein (hs-CRP), neutrophil-lymphocyte ratio (NLR), fibrinogen, d-dimer, N-terminal pro brain natriuretic peptide (NT-proBNP) and high-sensitivity cardiac troponin T (hs-cTnT) still lack power to tailor individual patient management. In other vascular diseases, like atrial fibrillation, the ABC-score comprising two biomarkers NT-proBNP and hs-cTnT, can be used to estimate stroke risk [5]. Pursuing a similar strategy in PAD patients should start with searching candidate biomarkers with the potential to identify patients with an increased cardiovascular risk. We therefore decided to explore a broad set of cardiovascular biomarkers from different biological processes that were not known to be associated with risk stratification in PAD.

METHODS

Study design

Outpatients of the department of Vascular Surgery of the Maastricht University Medical Center (MUMC+) were screened for PAD between 2018 and 2020. Eligibility for study participation was based on the ankle-brachial index, which had to be 0.9 or below. Within the selection of patients with an abnormal ankle-brachial index, we selected patients with Rutherford 1-2-3 / Fontaine IIa-IIb (intermittent claudication) or Rutherford 4 / Fontaine III (chronic limb threatening ischemia). Patients with Rutherford 5-6 / Fontaine IV were not eligible due to increased inflammatory parameters rising from ulcer formation or tissue loss. Active malignancy, chronic inflammatory disease, coagulation disorders or anticoagulant therapy, pregnancy and age below eighteen were other exclusion criteria. All eligible patients willing to participate were included after written informed consent was obtained. Upon inclusion, patient characteristics were collected and blood was drawn from the patient. All patients were followed for one year in which the primary outcome was assessed. The Medical Ethics Committee of the MUMC+ approved the study (NL63235.068.17) and the study was registered in the Netherlands Trial

Register (NTR7250; https://www.trialregister.nl/trial/7045). All experiments were performed in accordance with the relevant guidelines and regulations.

Blood collection and sample storage

Venous blood was drawn upon inclusion by antecubital venipuncture with 21-gauge needles and 3.2% (w/v) citrated Vacutainer tubes. The blood collection tubes were immediately processed using the standard platelet-poor plasma centrifugation (4000 x g for 5 minutes and 11000 x g for 10 minutes). After centrifugation, the plasma aliquots were frozen and stored at -80°C.

Data collection and outcome

Patient characteristics were recorded at baseline including gender and age of each patient as well as a history of myocardial infarction, ischemic stroke and PAD revascularization. Each patient provided an updated medication list from which the use of lipid-lowering drugs, antihypertensive drugs and antiplatelet drugs were collected. Information on the presence of traditional risk factors smoking, renal insufficiency, diabetes mellitus type 2 (DM2) and body mass index (BMI) was also obtained. Kidney function was evaluated by measuring plasma creatinine levels. The outcome of the study comprised a composite endpoint of myocardial infarction, stroke, acute limb ischemia, elective percutaneous coronary intervention or coronary artery bypass grafting and all-cause mortality during one year of follow-up. Outcome verification took place by a combination of telephone calls to the patient and analysis of hospital records.

Biomarker analysis

Citrated platelet-poor plasma was used to measure protein concentrations using the ProSeek Cardiovascular II and III panels (Olink Biosciences, Uppsala, Sweden). These panels are based on proximity extension assay (PEA) technology allowing simultaneous measurements of 92 protein biomarkers per panel. In total, 184 different proteins were measured in each patient. Pairs of oligonucleotide-labeled antibodies bind pairwise to target proteins present in 1μL of plasma, leading to the formation of a new polymerase chain reaction (PCR) target sequence formed by a proximity-dependent DNA polymerization event. The resulting sequence is subsequently detected and quantified by standard real-time PCR. Measurements are specified as Normalized Protein Expression (NPX), generated from the PCR quantification cycles. NPX data are then used to establish protein signatures where high NPX values equal high proteins concentrations and low NPX values equal low proteins concentrations.

Statistical analysis

Statistical analysis was performed on the whole cohort and a comparison was made between patients who reached the composite endpoint (event group) and patients who did not (no event group). Differences in baseline characteristics for continuous variables were presented as mean with standard deviation or median with interquartile range, as appropriate. Dichotomous and categorical variables were defined as frequencies with percentages and compared using the Fisher's Exact test or chi-square testing, while continuous variables were compared using the parametric two-samples t-test or the non-parametric Mann-Whitney U test. Protein expression levels following non-normal distributions were transformed into normal distributions using logarithmic transformation. Missing values were imputed to prevent a loss of statistical precision and to reduce the likelihood of selection bias, using random forest imputation, implemented in the R package 'missForest' [40]. The relation of the standardized biomarker levels (mean=0 and SD=1) with the cardiovascular outcome was assessed using individual Cox Hazard proportional regression models adjusted for age, gender, prior myocardial infarction, prior stroke and plasma creatinine levels. Then, to identify a subset of best predictive biomarkers for cardiovascular events and mortality during follow-up, LASSO regression analysis was performed with a 10-fold cross validation to increase generalizability of the models (glmnet package [41]). The selected biomarkers were shown as hazard ratios and 95% confidence intervals from previous individual Cox regression models. Due to the explorative nature of this study, a nominal p-value < 0.05 was used to reach statistical significance. All analyses were performed using R (R Core Team (2013) version 3.5.3. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

Baseline characteristics for the whole cohort are shown in *Table 1*. All 120 patients completed the one-year follow-up, although 4 patients were unable to give blood and could therefore not be included in the biomarker analysis. The cohort comprised 70 (58.3%) male patients with an average age of 67.7 (±9.6) years. Most patients had symptoms of intermittent claudication (88 (73.3%)) while the remaining 32 (26.7%) had chronic limb threatening ischemia. Many patients had a prior PAD revascularization 84 (70%) while 39 (32.5%) had a prior myocardial infarction and another 14 (11.7%) suffered from a prior stroke. The average BMI was 26.5 (±4.5) kg/m^2 and 53 (44.2%) patients were current smokers upon inclusion. DM2 was diagnosed in 31 (25.8%) patients and the median plasma creatinine level was 90 (74-105 μmol/L).

Within the whole cohort 15 patients suffered from an event during follow-up with recordings of 9 myocardial infarctions, 3 elective coronary interventions, 2 ischemic strokes and 1 death. Age and gender did not differ between patients with and without events, however more prior strokes were observed in patients with events (5 (33.3%) vs 9 (8.6%), p=0.016). Although not

significant, more prior myocardial infarctions were observed in patient with events (8 (53.3% vs 31 (29.5%), p 0.064). Kidney function was significantly worse in patients with events (plasma creatinine 97 (81-154) μmol/L vs 86 (73-104) μmol/L, p=0.036) while other cardiovascular risk factors were similar between the groups.

	Total cohort	**Event group**	**No event group**	**P-value**
	Mean±SD / Median (IQR) / n (%)	Mean±SD / Median (IQR) / n (%)	Mean±SD / Median (IQR) / n (%)	
Age (years)	67.7±9.6	72.1±7.7	67.1±9.7	0.056
Male gender	70 (58.3)	8 (53.3)	62 (59)	0.440
Prior myocardial infarction	39 (32.5)	8 (53.3)	31 (29.5)	0.064
Prior ischemic stroke	14 (11.7)	5 (33.3)	9 (8.6)	**0.016***
Prior PAD revascularization	84 (70)	12 (80)	72 (68.9)	0.281
Current smoking	53 (44.2)	8 (53.3)	45 (42.9)	0.312
Body Mass Index (kg/m²)	26.5±4.5	27.1±5.2	26.4±4.5	0.557
Diabetes Mellitus type 2	31 (25.8)	5 (33.3)	26 (24.8)	0.335
Creatinine (μmol/l)	90 (74-105)	97 (81-154)	86 (73-104)	**0.036***
Antiplatelet drugs	120 (100)	15 (100)	105 (100)	1.000
Lipid-lowering drugs	115 (95.8)	13 (86.7)	102 (97.1)	0.058
Antihypertensive drugs	90 (75)	11 (73.3)	79 (75.2)	0.908
Rutherford classification				0.090
Rutherford 1	9 (7.5)	1 (6.7)	8 (7.6)	
Rutherford 2	34 (28.3)	2 (13.3)	32 (30.5)	
Rutherford 3	45 (37.5)	4 (26.7)	41 (39)	
Rutherford 4	32 (26.7)	8 (53.3)	24 (22.9)	

Table 1: Baseline characteristics for the whole cohort and distribution between patients with and without cardiovascular events during follow-up. Significance was reached when $p < 0.05$ (*).

All biomarkers were added to the multivariate cox regression analysis with correction for age, gender, prior myocardial infarction or stroke and creatinine levels, revealing 13 proteins to be positively predictive for cardiovascular events and mortality. Placenta growth factor (PGF) appeared to have the highest hazard ratio (HR [95% Confidence interval (CI)]) (HR 4.03 [1.48-10.95]) followed by heat shock protein 27 (HSP; HR 3.18 [1.37-7.35]), protease-activated receptor 1 (PAR1; HR 3.15 [1.40-7.07]), adrenomedullin (ADM; 3.10 [1.16-8.29]), galectin-9 (Gal-9; HR 3.03 [1.45-6.32]), tumor necrosis factor superfamily member 11A (TNFRSF11A; HR 2.46 [1.20-5.03]), interleukin-6 (IL-6; HR 2.02 [1.35-3.02]), brain natriuretic peptide

(BNP; HR 2.02 [1.20-339]), N-terminal pro brain natriuretic peptide (NT-proBNP; HR 2.01 [1.01-4.00]), interleukin-4 receptor subunit alpha (IL4ra; HR 1.99 [1.22-3.26]), Dickkopf-related protein 1 (Dkk1; HR 1.93 [1.01-3.68]), matrix metalloproteinase-12 (MMP12; HR 1.89 [1.06-3.39]) and chitinase-3-like protein 1 (CHI3L1; HR 1.83 [1.03-3.27]). Another 2 proteins were negatively predictive for cardiovascular events and mortality, being p-selectin glycoprotein ligand 1 (PSGL1; HR 0.57 [0.34-0.98]) and plasminogen activator inhibitor 1 (PAI-1; HR 0.45 [0.23-0.88]) *(Figure 1).*

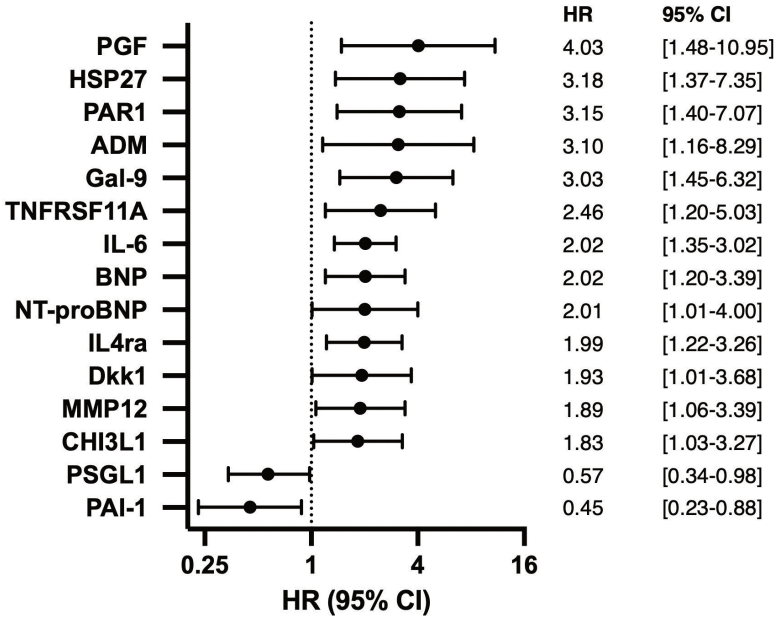

Figure 1: Biomarkers with a significant positive or negative predictive value for cardiovascular events and mortality, identified by multivariate cox regression analysis.

To identify the most predictive biomarkers across all proteins, LASSO regression analysis was performed (C-index 0.84) that revealed protease-activated receptor 1 (PAR1), galectin-9 (Gal-9), tumor necrosis factor receptor superfamily member 11A (TNFRSF11A) and interleukin 6 (IL-6) as most predictive biomarkers for cardiovascular events and mortality *(Figure 2)*. PAR1 showed the highest predictive value with a hazard ratio of 3.15 [1.40-7.07] followed by Gal-9 (HR 3.03 [1.45-6.32]), TNFRSF11A (HR 2.46 [1.20-5.03] and IL-6 (2.02 [1.35-3.02]).

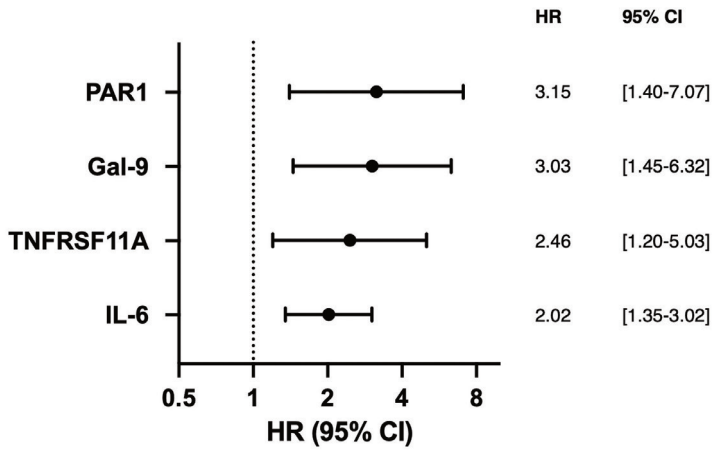

Figure 2: Biomarkers with significantly increased plasma levels in patients with events, identified by multivariate cox regression analysis with LASSO.

DISCUSSION

Patients with PAD are at increased risk of (recurrent) cardiovascular events and death despite current treatment strategies. Biomarkers may potentially help to identify patients with different risk profiles. The main finding of our study is that four proteins (IL-6, PAR1, TNFRSF11A and Gal-9) are linked to cardiovascular outcomes in PAD patients and therefore are novel candidate biomarkers for risk assessment in PAD.

The pro-inflammatory cytokine IL-6 induces the synthesis and release of several acute phase proteins such as C-reactive protein (CRP) and fibrinogen [6]. IL-6 has a variety of functions, making it a key player in the inflammatory response, also in different stages of atherosclerosis. In early stages, IL-6 coordinates influx of inflammatory cells into atherosclerotic lesions. IL-6 increases expression of intercellular adhesion molecule 1 (ICAM-1) and thereby enables leukocytes to be recruited and transmigrated into the vessel wall [7]. IL-6 has also been shown to increase the surface expression of tissue factor on cultured monocytes, thereby initiating the coagulation cascade [8]. In later stages of atherosclerosis, growth and progression of atherosclerotic lesions are promoted by IL-6 through induction of platelet-derived growth factor (PDGF) which causes growth of vascular smooth muscle cells [9]. In the final stages of atherosclerosis, including atherothrombosis, IL-6 induces aggregation and activation of platelets and thereby accelerates thrombus formation. Aggregation of platelets is stimulated through the production of fibrinogen [10] while activation is enhanced through increased expression of P-selectin [11]. Combining these functions, IL-6 appears to play an important pro-atherogenic role throughout all stages of atherosclerosis, including thrombus formation and arterial occlusion. This important role is also seen in clinical studies, where IL-6 is a significant predictor of PAD progression independently

of traditional cardiovascular risk factors. Moreover, IL-6 is predictive of changes in ankle-brachial index, thereby illustrating a change in the degree of atherosclerosis [12]. Surprisingly, IL-6 has not been investigated regarding occurrence of cardiovascular events in PAD populations specifically. Studies in other populations, such as patients with prior stroke and polyvascular disease, show an increased risk of recurrent stroke in patients with elevated IL-6 levels [13].

PAR1 is a membrane-bound protein mostly found on endothelial cells, platelets and vascular smooth muscle cells. As PAR1 is a transmembrane receptor, measured plasma concentrations of soluble PAR1 do not represent functional receptor. However, PAR1 is known to be internalized by multivesicular bodies (MVB). Cargo from MVBs can be degraded by lysosomes or secreted as exosomes. This suggests that PAR1 concentrations in plasma might reflect exosome PAR1 concentrations as a marker of cellular receptor processing. Furthermore, PAR1 plasma levels can also reflect cell death [14]. Thrombin is the main activator of PAR1 [15] and given the increased thrombin production in subjects with atherosclerosis, this may also result in further upregulation of PAR1 receptor expression [16]. PAR1, like IL-6, plays an important role throughout various stages of atherosclerosis. In early stages, matrix metalloproteinase-9 (MMP-9)-mediated PAR1 activation induces endothelial dysfunction leading to a loss of vascular integrity [17]. PAR1 on endothelial cells can also be activated by activated protein C (APC) upregulating monocyte chemotactic protein 1 (MCP-1). MCP1 promotes not only pro-inflammatory effects, but also anti-inflammatory effects, indicating anti-atherogenic effects of PAR1 activation [18,19]. In later stages, pro-atherothrombotic effects become more visible as activation of PAR1 on platelets leads to platelet activation through thromboxane A2 production and aggregation through P-selectin upregulation [20,21]. Overall, activation of PAR1 induces mostly pro-atherosclerotic effects and thereby increases the cardiovascular risk, making it a potent predictive plasma biomarker. PAR1 has not been investigated thoroughly as a potential biomarker, but is well-known for its selective inhibition by vorapaxar. The TRA2P-TIMI 50 trial demonstrated a significant benefit of vorapaxar in reducing cardiovascular death and ischemic events in patients with a history of acute myocardial infarction, ischemic stroke or peripheral artery disease [22].

TNFRSF11A is commonly known as receptor activator of nuclear factor kappa-B (RANK) and is part of the RANK/RANKL/OPG signaling pathway which regulates osteoclast differentiation and activation [23]. Activation of NF-kB is usually mediated by RANK ligand, however overexpression of RANK itself is sufficient to activate this pathway [24]. Via NF-kB signaling, endothelial cells become activated and express various chemokines (e.g. MCP1) and adhesion molecules (e.g. ICAM-1) responsible for chemotaxis and transmigration of leukocytes into atherosclerotic plaques [25]. Progression and evolvement of plaques is further stimulated through NF-kB by accumulation and proliferation of VSMCs [26]. In late stages of atherosclerosis, NF-kB plays an important role in regulating activation and aggregation of platelets however underlying

mechanisms remain to be elucidated [27]. TNFRSF11A has not been investigated as biomarker in association with progression of atherosclerosis in PAD or the occurrence of cardiovascular events.

Gal-9 is an important immune regulator which is abundantly present in several chronic inflammatory diseases such as inflammatory bowel disease [28] and systemic lupus erythematosus [29]. Expressed by many different cell types such as endothelial cells, macrophages, and T-lymphocytes [30], Gal-9 is thought to be anti-inflammatory via TIM-3 signaling. Important effects of this signaling include apoptosis of pro-inflammatory Th1 and Th17 cells [30,31] and stimulation of regulatory T cell activity [32], both to dampen atherosclerotic progression. Serum levels of Gal-9 were found to be decreased in patients with coronary artery disease, specifically those with acute coronary syndrome. However, other studies reported higher serum Gal-9 levels in patients with DM2 and chronic kidney disease, two morbidities that were abundantly present in our cohort [33]. Gal-9 seems to be anti-inflammatory and thus atherosclerosis-dampening, however certain factors such as kidney function and the presence of DM2 may alter levels of Gal-9 [33]. Therefore, Gal-9 should be used with caution and the presence of co-morbidities should be considered. As with TNFRSF11A, Gal-9 has not been investigated as biomarker in association with progression of atherosclerosis in PAD or the occurrence of cardiovascular events.

PSGL1 and PAI-1 were negatively predictive for cardiovascular events and mortality, indicating that increased levels of these biomarkers are associated with fewer events. PSGL1 plays an important role as inflammatory marker in atherogenesis with involvement in leukocyte recruitment and activation. Studies investigating the role of PSGL1 in atherosclerosis mostly found accelerating effects of PSGL1 on the atherosclerotic process. The absence of PSGL1 in $PSGL^{-/-}$ transgenic mice reduced atherosclerotic plaque surface area, inflammatory cell infiltration and hyperplasia [34]. Clinical studies investigating PSGL1 are limited, but *Ozaki et al.* reported that expression levels of PSGL1 on monocytes were high in acute coronary syndrome patients [35]. The increased levels of PSGL1 in association with fewer events in our study may be explained by the function of PSGL1 as it activates intracellular protein kinases [36]. Hereby atherosclerosis may be accelerated, but not necessarily lead to the formation of unstable plaques. A similar mechanism may explain the increased levels of PAI-1, which appeared to be protective of events in our cohort. While a recent systematic review found an association between increased PAI-1 levels and the occurrence of cardiovascular events, lower PAI-1 levels were associated with increased restenosis [37]. Although both PSGL1 and PAI-1 appeared protective in our study, other studies have found positive associations between these biomarkers and the occurrence of events, which in part can be explained by variations in pro-inflammatory status and other cardiovascular risk factors [38,39]. It remains to be elucidated what the exact roles of PSGL1 and PAI-1 are in the process of atherosclerosis.

This study has several limitations. First, all biomarkers were measured in Olink panels using the Proximity Extension Assay (PEA) technology followed by PCR. This semiquantitative technology provides relative concentrations of a biomarker in plasma rather than absolute concentrations. Therefore, these results must be validated in a quantitative assay to measure quantitative concentrations. Second, our sample size was limited to 120 patients, yielding higher confidence intervals in several biomarker hazard ratio calculations. Lastly, patients were only followed for 12 months in total, limiting the total event rate.

In conclusion, risk stratification models in PAD patients are necessary to predict future cardiovascular events and death. This study identified IL-6, PAR1, TNFRSF11A and Gal-9 as promising biomarkers to aid in risk stratification. These proteins are involved in prominent atherosclerotic biological processes including activation of endothelial cells, positive regulation of acute inflammatory responses, leukocyte chemotaxis and platelet activation. This semi-quantitative biomarker discovery is a first step to improve risk stratification in PAD. The next step would be to perform quantitative assays to confirm the association with cardiovascular outcome, preferably in a separate PAD population.

REFERENCES

1. Alberts, M. J. *et al.* Three-year follow-up and event rates in the international REduction of Atherothrombosis for Continued Health Registry. *Eur. Heart J.* **30**, 2318–2326 (2009).
2. Bhatt, D. L. *et al.* International prevalence, recognition, and treatment of cardiovascular risk factors in outpatients with atherothrombosis. *JAMA* **295**, 180–189 (2006).
3. Aday, A. W. & Matsushita, K. Epidemiology of peripheral artery disease and polyvascular disease. *Circ. Res.* **128**, 1818–1832 (2021).
4. Kremers, B. *et al.* Plasma biomarkers to predict cardiovascular outcome in patients with peripheral artery disease: A systematic review and meta-analysis. *Arterioscler. Thromb. Vasc. Biol.* **40**, 2018–2032 (2020).
5. Berg, D. D. *et al.* Performance of the ABC scores for assessing the risk of stroke or systemic embolism and bleeding in patients with Atrial Fibrillation in ENGAGE AF-TIMI 48. *Circulation* **139**, 760–771 (2019).
6. Heinrich, P. C., Castell, J. V. & Andus, T. Interleukin-6 and the acute phase response. *Biochem. J.* **265**, 621–636 (1990).
7. Romano, M. *et al.* Role of IL-6 and its soluble receptor in induction of chemokines and leukocyte recruitment. *Immunity* **6**, 315–325 (1997).
8. Neumann, F. J. *et al.* Effect of human recombinant interleukin-6 and interleukin-8 on monocyte procoagulant activity. *Arterioscler. Thromb. Vasc. Biol.* **17**, 3399–3405 (1997).
9. Ikeda, U. *et al.* Interleukin 6 stimulates growth of vascular smooth muscle cells in a PDGF-dependent manner. *Am. J. Physiol.* **260**, H1713-7 (1991).
10. Tsakadze, N. L., Zhao, Z. & D'Souza, S. E. Interactions of intercellular adhesion molecule-1 with fibrinogen. *Trends Cardiovasc. Med.* **12**, 101–108 (2002).
11. Oleksowicz, L., Mrowiec, Z., Isaacs, R., Dutcher, J. P. & Puszkin, E. Morphologic and ultrastructural evidence for interleukin-6 induced platelet activation. *Am. J. Hematol.* **48**, 92–99 (1995).
12. Tzoulaki, I. *et al.* C-reactive protein, interleukin-6, and soluble adhesion molecules as predictors of progressive peripheral atherosclerosis in the general population: Edinburgh Artery Study. *Circulation* **112**, 976–983 (2005).
13. Tian, Y. *et al.* Association of polyvascular disease and elevated interleukin-6 with outcomes in acute ischemic stroke or transient ischemic attack. *Front. Neurol.* **12**, 661779 (2021).
14. McGough, I. J. & Vincent, J.-P. Exosomes in developmental signalling. *Development* **143**, 2482–2493 (2016).
15. Nieman, M. T. & Schmaier, A. H. Interaction of thrombin with PAR1 and PAR4 at the thrombin cleavage site. *Biochemistry* **46**, 8603–8610 (2007).
16. Chen, B., Siderovski, D. P., Neubig, R. R., Lawson, M. A. & Trejo, J. Regulation of protease-activated receptor 1 signaling by the adaptor protein complex 2 and R4 subfamily of regulator of G protein signaling proteins. *J. Biol. Chem.* **289**, 1580–1591 (2014).
17. Florence, J. M., Krupa, A., Booshehri, L. M., Allen, T. C. & Kurdowska, A. K. Metalloproteinase-9 contributes to endothelial dysfunction in atherosclerosis via protease activated receptor-1. *PLoS One* **12**, e0171427 (2017).
18. Lin, J., Kakkar, V. & Lu, X. Impact of MCP-1 in atherosclerosis. *Curr. Pharm. Des.* **20**, 4580–4588 (2014).
19. Posma, J. J. *et al.* Roles of coagulation proteases and PARs (protease-activated receptors) in mouse models of inflammatory diseases. *Arterioscler. Thromb. Vasc. Biol.* **39**, 13–24 (2019).

20. Keuren, J. F. W. *et al.* Synergistic effect of thrombin on collagen-induced platelet procoagulant activity is mediated through protease-activated receptor-1. *Arterioscler. Thromb. Vasc. Biol.* **25**, 1499–1505 (2005).
21. Kremers, B. M. M., Ten Cate, H. & Spronk, H. M. H. Pleiotropic effects of the hemostatic system. *J. Thromb. Haemost.* **8**, 1464-1473 (2018).
22. Morrow, D. A. *et al.* Vorapaxar in the secondary prevention of atherothrombotic events. *N. Engl. J. Med.* **366**, 1404–1413 (2012).
23. Walsh, M. C. & Choi, Y. Biology of the RANKL-RANK-OPG system in immunity, bone, and beyond. *Front. Immunol.* **5**, 511 (2014).
24. Anderson, D. M. *et al.* A homologue of the TNF receptor and its ligand enhance T-cell growth and dendritic-cell function. *Nature* **390**, 175–179 (1997).
25. Monaco, C. *et al.* Canonical pathway of nuclear factor kappa B activation selectively regulates proinflammatory and prothrombotic responses in human atherosclerosis. *Proc. Natl. Acad. Sci. U. S. A.* **101**, 5634–5639 (2004).
26. Bourcier, T., Sukhova, G. & Libby, P. The nuclear factor kappa-B signaling pathway participates in dysregulation of vascular smooth muscle cells in vitro and in human atherosclerosis. *J. Biol. Chem.* **272**, 15817–15824 (1997).
27. Kojok, K., El-Kadiry, A. E.-H. & Merhi, Y. Role of NF-κB in platelet function. *Int. J. Mol. Sci.* **20**, 4185 (2019).
28. Chen, H.-Y. *et al.* Intracellular galectin-9 enhances proximal TCR signaling and potentiates autoimmune diseases. *J. Immunol.* **204**, 1158–1172 (2020).
29. Matsuoka, N. *et al.* Galectin-9 as a biomarker for disease activity in systemic lupus erythematosus. *PLoS One* **15**, e0227069 (2020).
30. Zhu, C. *et al.* The Tim-3 ligand galectin-9 negatively regulates T helper type 1 immunity. *Nat. Immunol.* **6**, 1245–1252 (2005).
31. Oomizu, S. *et al.* Galectin-9 suppresses Th17 cell development in an IL-2-dependent but Tim-3-independent manner. *Clin. Immunol.* **143**, 51–58 (2012).
32. Seki, M. *et al.* Galectin-9 suppresses the generation of Th17, promotes the induction of regulatory T cells, and regulates experimental autoimmune arthritis. *Clin. Immunol.* **127**, 78–88 (2008).
33. Kurose, Y. *et al.* Serum galectin-9 levels are elevated in the patients with type 2 diabetes and chronic kidney disease. *BMC Nephrol.* **14**, 23 (2013).
34. Li, B. *et al.* The metabonomics study of P-selectin glycoprotein ligand-1 (PSGL-1) deficiency inhibiting the progression of atherosclerosis in LDLR-/- mice. *Int. J. Biol. Sci.* **14**, 36–46 (2018).
35. Ozaki, Y. *et al.* Association between P-selectin glycoprotein ligand-1 and pathogenesis in acute coronary syndrome assessed by optical coherence tomography. *Atherosclerosis* **233**, 697–703 (2014).
36. Ye, Z. *et al.* The P-selectin and PSGL-1 axis accelerates atherosclerosis via activation of dendritic cells by the TLR4 signaling pathway. *Cell Death Dis.* **10**, 507 (2019).
37. Jung, R. G. *et al.* Association between plasminogen activator inhibitor-1 and cardiovascular events: a systematic review and meta-analysis. *Thromb. J.* **16**, (2018).
38. Srikanthan, K., Feyh, A., Visweshwar, H., Shapiro, J. I. & Sodhi, K. Systematic review of metabolic syndrome biomarkers: A panel for early detection, management, and risk stratification in the west Virginian population. *Int. J. Med. Sci.* **13**, 25–38 (2016).

39. Alessi, M.-C. & Juhan-Vague, I. PAI-1 and the metabolic syndrome: links, causes, and consequences. *Arterioscler. Thromb. Vasc. Biol.* **26**, 2200–2207 (2006).

40. Stekhoven, D. J. & Bühlmann, P. Missforest-Non-parametric missing value imputation for mixed-type data. *Bioinformatics*.

41. Friedman, J., Hastie, T. & Tibshirani, R. Regularization paths for generalized linear models via coordinate descent. *J. Stat. Softw.* **33**, 1–22 (2010).

CHAPTER 7

CONTRIBUTION OF THE INTRINSIC PATHWAY TO OBSERVED HYPERCOAGULABILITY IN PATIENTS WITH CHRONIC PERIPHERAL ARTERY DISEASE: A PROSPECTIVE COHORT STUDY

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In preparation

CHAPTER 8

GENERAL DISCUSSION

SUMMARY

IMPACT PARAGRAPH

CURRICULUM VITAE

LIST OF PUBLICATIONS AND PRESENTATIONS

ACKNOWLEDGEMENTS

GENERAL DISCUSSION

Defining the atherothrombotic risk in patients with peripheral artery disease (PAD) has been a challenge for years. Known factors contributing to this risk include obesity, smoking, diabetes, hyperlipidemia, and hypertension [1]. Current treatment strategies therefore aim to counteract these factors through lifestyle changes and the use of antidiabetic drugs, lipid-lowering drugs, and antihypertensive drugs. To prevent thrombus formation at sites of atherosclerotic plaques, antiplatelet agents, in the form of aspirin or clopidogrel, are added to the treatment [2]. Despite this broad set of treatment options, the incidence of adverse cardiovascular events and death remains high in patients with PAD [3,4]. The pivotal question that rises from this observation is why these patients still suffer from events despite seemingly adequate treatment. One reason for this could be that current medical treatment strategies are not sufficiently decreasing cardiovascular risk. We showed that suboptimal levels of low-density lipoprotein (LDL) were reached, while the prescribed medication intensity was medium to high according to the ACC/AHA guidelines [5] and patients were adherent to their medication. While at the time of the study the LDL target values were 2.5 mmol/L (patients > 70 years) and 1.8 mmol/L (patients >70 years), current guidelines recommend that very high-risk patients should achieve an even lower LDL target value of 1.4 mmol/L [6]. This recommendation is supported by various studies showing that further lowering LDL values can reduce the risk of cardiovascular events [7]. Although adapting the lipid-lowering strategy based on this knowledge could reduce the risk, the role of antiplatelet agents should also not be overlooked. Depending on national guidelines, aspirin and clopidogrel are the antiplatelet agents most often used as first-line treatment [2]. The efficacy of these antiplatelet regimes is well established in the light of the reduction of cardiovascular events [8]. Although these drugs are prescribed in the same dose to all PAD patients, this therapy does not appear to be a one-size-fits-all therapy. Higher-than-expected platelet reactivity in certain patients, the so-called high on-treatment platelet reactivity (HTPR), is common worldwide [9–11]. Patients with genetic polymorphisms in the P2Y12 platelet receptor reveal this increased reactivity and, as shown in this thesis and other studies, are at increased risk of cardiovascular events [10,12]. The ongoing GENPAD study investigates a genotype guided antithrombotic treatment in regard to the occurrence of cardiovascular events in patients who are carriers of loss-of-function alleles. Recently, a meta-analysis showed that in these patients the use of ticagrelor or prasugrel appeared more effective in reducing cardiovascular events than clopidogrel [13]. Another ongoing study, the CLEAR-PATH study, investigates a combination of antiplatelet agents, aspirin and clopidogrel, rather than the use of ticagrelor or prasugrel. Apart from switching to a different antiplatelet agent, the addition of an anticoagulant drug has also been investigated. The COMPASS trial showed that this so-called dual pathway inhibition with aspirin and a low dose of rivaroxaban reduced the incidence of cardiovascular events in high-risk PAD patients [14]. The reasoning behind this is the inhibition of both platelets and the coagulation system and thereby the prevention of thrombus formation at sites of atherosclerosis, including coronary and cerebral arteries. The pathophysiology behind the hypercoagulable state in these patients has yet to be unraveled.

In this thesis it is shown that increased fibrinogen levels and d-dimer levels are predictive of cardiovascular events and mortality, supporting the presence of this hypercoagulable state. We postulated that in acute settings of inflammation or ischemia, contact activation becomes a relevant amplifier of coagulation. This would explain why the addition of an anticoagulant drug could further reduce the risk of cardiovascular events and mortality. Alternative to the use of anticoagulant drugs, the CANTOS trial demonstrated that the use of the anti-inflammatory interleukin-1β inhibitor canakinumab led to a reduction in cardiovascular events and death [15]. Moreover, the use of canakinumab reduced plasma c-reactive protein (CRP), a plasmatic biomarker of which increased levels are associated with cardiovascular events and mortality in patients with PAD [16]. These new treatment strategies targeting coagulation [17] and inflammation [15] substantially reduce mortality varying from 14% to 32%, but do come at a cost. Patients receiving antiplatelet therapy combined with a low dose of rivaroxaban in the COMPASS trial had more often a major bleeding event, although no significant differences were found in regard to fatal bleedings [17]. The CANTOS trial showed that patients receiving canakinumab had more often a fatal infection and sepsis as compared to patients receiving a placebo [15]. These results indicate that the new treatment strategies can definitely be of benefit in certain patients, but should perhaps not be used in all patients with PAD. The use of these treatment strategies could be based on the cardiovascular risk of each patient suffering from a cardiovascular event. Risk stratification in PAD patients is, however, challenging, but studies in other patient populations may pave the road to successful risk prediction. An example is the use of the ABC-score for the prediction of stroke in patients with atrial fibrillation [18]. Here, patient characteristics including age and prior stroke are combined with plasmatic biomarkers high-sensitivity Troponin T (hsTnT) and N-Terminal pro-Brain Natriuretic Peptide (NT-proBNP). A similar strategy could be pursued to create a risk stratification model for patients with PAD. Although various studies have investigated the association between plasmatic biomarker levels and the occurrence of cardiovascular events or death [16], none have been implemented as a risk predictor. Therefore, in this thesis, we identified four biomarkers associated with the occurrence of cardiovascular events and mortality that could potentially help to identify patients with different risk profiles. The question remains how to proceed with these biomarkers and their associations with cardiovascular risk, as research seems to be stuck at this step. One way forward may be to design management studies addressing the value of a panel of biomarkers as discussed in this thesis, i.e. combining an inflammatory biomarker with a coagulation biomarker and a cardiac biomarker. The philosophy of a combined panel of biomarkers resides in the complex pathophysiology of the process of atherosclerosis, where inhibition of multiple pathways might be necessary to prevent a cardiovascular event. A potent biomarker panel could then be added to a risk stratification model including the patient characteristics associated with cardiovascular events and mortality as presented in this thesis (i.e., prior stroke, prior myocardial infarction, kidney function). The use of risk stratification models enables physicians to tailor treatment strategies, where a patient with an increased risk and an elevated inflammatory biomarker could be treated with an anti-inflammatory agent. By

individualizing treatment to the needs of each patient, the risk of cardiovascular events can be expected to be limited, while not all patients with PAD are exposed to side effects of additional treatment strategies such as major bleeding and fatal infections.

REFERENCES

1. Bhatt, D. L. *et al.* International prevalence, recognition, and treatment of cardiovascular risk factors in outpatients with atherothrombosis. *JAMA* **295**, 180–189 (2006).
2. Aboyans, V. *et al.* 2017 ESC Guidelines on the Diagnosis and Treatment of Peripheral Arterial Diseases, in collaboration with the European Society for Vascular Surgery (ESVS): Document covering atherosclerotic disease of extracranial carotid and vertebral, mesenteric, renal, upper and lower extremity arteriesEndorsed by: the European Stroke Organization (ESO)The Task Force for the Diagnosis and Treatment of Peripheral Arterial Diseases of the European Society of Cardiology (ESC) and of the European Society for Vascular Surgery (ESVS). *Eur. Heart J.* **39**, 763–816 (2018).
3. Alberts, M. J. *et al.* Three-year follow-up and event rates in the international REduction of Atherothrombosis for Continued Health Registry. *Eur. Heart J.* **30**, 2318–2326 (2009).
4. Sartipy, F., Sigvant, B., Lundin, F. & Wahlberg, E. Ten year mortality in different peripheral arterial disease stages: A population based observational study on outcome. *Eur. J. Vasc. Endovasc. Surg.* **55**, 529–536 (2018).
5. Stone, N. J. *et al.* 2013 ACC/AHA guideline on the treatment of blood cholesterol to reduce atherosclerotic cardiovascular risk in adults: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines. *J. Am. Coll. Cardiol.* **63**, 2889–2934 (2014).
6. Mach, F. *et al.* 2019 ESC/EAS Guidelines for the management of dyslipidaemias: lipid modification to reduce cardiovascular risk. *Eur. Heart J.* **41**, 111–188 (2020).
7. Sabatine, M. S., Wiviott, S. D., Im, K., Murphy, S. A. & Giugliano, R. P. Efficacy and safety of further lowering of low-density lipoprotein cholesterol in patients starting with very low levels. *JAMA Cardiol.* **3**, 823-828 (2018).
8. Jimenez, J. C. & Wilson, S. E. A randomised, blinded, trial of clopidogrel versus aspirin in patients at risk of ischaemic events (CAPRIE). in *50 Landmark Papers Every Vascular and Endovascular Surgeon Should Know* 199–203 (CRC Press, 2020).
9. Yeo, K.-K. *et al.* Aspirin and clopidogrel high on-treatment platelet reactivity and genetic predictors in peripheral arterial disease. *Catheter. Cardiovasc. Interv.* **91**, 1308–1317 (2018).
10. Pastromas, G. *et al.* Clopidogrel responsiveness in patients undergoing peripheral angioplasty. *Cardiovasc. Radiol.* **36**, 1493–1499 (2013).
11. Spiliopoulos, S., Kassimis, G., Hatzidakis, A. & Krokidis, M. High on-treatment platelet reactivity in peripheral endovascular procedures. *Cardiovasc. Radiol.* **37**, 559–571 (2014).
12. Pasala, T. *et al.* Aspirin resistance predicts adverse cardiovascular events in patients with symptomatic peripheral artery disease. *Tex. Heart Inst. J.* **43**, 482–487 (2016).
13. Pereira, N. L. *et al.* Effect of genotype-guided oral P2Y12 inhibitor selection vs conventional clopidogrel therapy on ischemic outcomes after percutaneous coronary intervention: The TAILOR-PCI randomized clinical trial. *JAMA* **324**, 761–771 (2020).
14. Anand, S. S. *et al.* Rivaroxaban with or without aspirin in patients with stable peripheral or carotid artery disease: an international, randomised, double-blind, placebo-controlled trial. *Lancet* **391**, 219–229 (2018).

15. Ridker, P. M. *et al.* Antiinflammatory therapy with canakinumab for atherosclerotic disease. *N. Engl. J. Med.* **377**, 1119–1131 (2017).
16. Kremers, B. *et al.* Plasma Biomarkers to Predict Cardiovascular Outcome in Patients With Peripheral Artery Disease: A Systematic Review and Meta-Analysis. *Arterioscler. Thromb. Vasc. Biol.* **40**, 2018–2032 (2020).
17. Eikelboom, J. W. *et al.* Rivaroxaban with or without aspirin in stable cardiovascular disease. *N. Engl. J. Med.* **377**, 1319–1330 (2017).
18. Berg, D. D. *et al.* Performance of the ABC Scores for Assessing the Risk of Stroke or Systemic Embolism and Bleeding in Patients With Atrial Fibrillation in ENGAGE AF-TIMI 48. *Circulation* **139**, 760–771 (2019).

SUMMARY

This thesis aimed to better understand why some patients with peripheral artery disease (PAD) suffer from cardiovascular events despite current medical treatment strategies, while others do not. In this thesis the risk of cardiovascular events and mortality was explored through the characterization of high-risk patients, combined with the unraveling of important pathophysiological pathways driving cardiovascular events and mortality in PAD. Biomarkers related to these pathways could be used to identify patients at increased risk of such events, enabling clinicians to improve their management strategies to prevent cardiovascular events and mortality.

Chapter two provides an overview of the pleiotropic effects of coagulation enzymes and current evidence on pathophysiological pathways in atherosclerosis and atherothrombosis. Although platelet activation and formation of fibrin are crucial in thrombosis, inflammatory pathways and pleiotropic effects of the coagulation system substantially drive the process of atherosclerosis. Cell signaling in these pathways predominantly occurs via protease-activated receptors (PARs), which can be activated by a wide range of proteins, including coagulation factors IIa (thrombin) and Xa. Upon activation, downstream signaling leads to pro-inflammatory effects and a hypercoagulable state, thereby accelerating atherosclerotic plaque formation. Hypercoagulable effects are mainly exerted through platelet PAR1 and PAR4, while pro-inflammatory responses are mostly seen after vascular PAR1 and PAR2 activation. Inhibition of PAR signaling pathways, for example by the use of PAR1 antagonist vorapaxar, could be explored as a way to lower cardiovascular events and mortality. In this chapter, therefore, we also discussed how to improve vascular protection beyond the prevention of thrombosis. Clinical efficacy to be gained from the search to further decrease cardiovascular events might lie in the use of a low dose of a direct oral anticoagulant (DOAC) combined with antiplatelet therapy. More clinical studies on DOACs in secondary cardiovascular prevention are however necessary to investigate efficacy and safety, especially in subgroups such as patients with peripheral artery disease.

Provided by the insights of chapter two and the knowledge that the use of DOACs may lower the cardiovascular event and mortality risk in PAD, we explored a possible common biochemical background in patients with PAD and patients with non-acute deep vein thrombosis (DVT), as the latter patient group greatly benefits from treatment with a DOAC. In **chapter three**, we assessed these possible common pathways by using biomarkers reflecting the interplay between platelet and endothelial activation, neutrophil activation, and inhibition of inflammation. Neutrophil activity was an important driver of cardiovascular events, although more activity was recorded in PAD patients as compared to DVT patients, even with dampening by lipoxin A4. We found no differences in platelet activation, keeping in mind that PAD patients use antiplatelet therapy. A hypercoagulable state was observed in PAD patients with higher d-dimer levels, but venous thrombosis appeared to be more strongly dependent on blood coagulation activity.

The investigated pathways, as discussed in chapter three, did not appear to play pivotal roles in the cardiovascular event and mortality risk in PAD patients. We therefore decided to create an overview in **chapter four** of all investigated plasmatic biomarkers in association with cardiovascular outcome in PAD. During the last decades, various biomarkers have been investigated concerning cardiovascular outcome in PAD. This systematic review identified promising candidate biomarkers representing different pathophysiological processes in PAD, with biomarkers playing important roles in inflammatory signaling (hsCRP, NLR), the coagulation cascade (fibrinogen, d-dimer), and cardiac (patho)physiology (NT-proBNP, hs-cTnT). These biomarkers should not be used solitary but added to risk stratification models with multiple biomarkers from different pathophysiological pathways, as atherosclerosis is a multifactorial disease. Now that more potent pharmacological interventions are becoming available to target specific mechanisms such as inflammation and hypercoagulability, biomarker-supported risk stratification may help identify PAD patients that may benefit most from intensified treatment. To facilitate this, management studies should address the value of biomarker panels combined with traditional risk factors and patient characteristics.

Following the prior chapters, we conducted a clinical study to further dissect the cardiovascular event and mortality risk in PAD patients. **Chapter five** describes the patient population of the prospective cohort study that we performed as a continuation of the already gathered biomarker evidence. In this observational study, we aimed to find patient characteristics associated with cardiovascular risk in order to find targets for improved management. The patient population had prevalent PAD with a chronic state of atherosclerosis, defined by multiple affected vascular beds, decreased renal function, and lower hemoglobin levels. All patients were treated according to current guidelines with lipid-lowering agents, antiplatelet therapy, and antihypertensive drugs. We observed suboptimal low-density lipoprotein (LDL) levels, indicating that lipid-lowering strategies should be intensified to improve the lipid profile further. Antiplatelet agents were found to be inadequate despite high medication adherence, as platelet reactivity was insufficiently decreased in patients experiencing cardiovascular events. The addition of anticoagulant treatment in the form of a DOAC may counteract the remaining prothrombotic state in PAD patients. This so-called dual-pathway inhibition is, however, still hindered due to concerns about the number of pills per day and the increased risk of major bleeding. Nevertheless, more research is needed on alternative treatment strategies such as dual antiplatelet therapy or combinations with anticoagulant drugs.

Although various biomarkers have been identified as predictors of cardiovascular outcome in PAD, as summarized in chapter four, none have been implemented yet in clinical management. One explanation may be that unexplored biomarkers may play more pivotal roles in cardiovascular risk prediction. Therefore, in **chapter six**, a subgroup of the cohort study was investigated to discover new predictive biomarkers in addition to the already known biomarkers, as presented in chapter

four. Using proximity extension assay technology, we explored the association between a broad set of cardiovascular biomarkers and cardiovascular risk and identified interleukin-6, PAR1, tumor necrosis factor receptor superfamily receptor 11A, and galectin 9 as promising biomarkers to aid in risk stratification. These proteins are involved in prominent atherosclerotic biological processes including activation of endothelial cells, positive regulation of acute inflammatory responses, leukocyte chemotaxis, and platelet activation. This semi-quantitative biomarker discovery can be seen as a first step to improving risk stratification in PAD with new protein biomarkers. Quantitative assays are required to confirm the association with cardiovascular outcome. Potent biomarkers can then be implemented in risk stratification models.

As the association between hypercoagulability and cardiovascular is established, it remains to be elucidated which mechanisms are responsible for this prothrombotic state. To elucidate potential mechanisms, we focused on pathways leading to this state in PAD in **chapter seven**. Activation of the intrinsic pathway, assessed by coagulation enzyme:inhibitor complexes and thrombin generation, was not different in PAD patients at increased risk for cardiovascular events and mortality. This observation may be the result of a profound coagulation activity with systemic atherosclerosis as primary driver of coagulation. It could be that platelets, rather than the hypercoagulability, are responsible for the actual occurrence of cardiovascular events.

IMPACT PARAGRAPH

Peripheral artery disease (PAD) is a common manifestation of atherosclerosis with a global prevalence of 5.6% and an even higher prevalence in high-income countries (7.4%). These numbers are estimates, as the disease remains underdiagnosed due to lack of awareness of clinical manifestations. Awareness was improved after the introduction of the ankle brachial index as diagnostic tool, but still millions of people with PAD are not being treated with the current medical treatment strategies as they have yet to be diagnosed with the disease. The patients that are diagnosed with PAD do receive the current optimal treatment strategies. They are however still at increased risk for cardiovascular events, with cardiovascular mortality being increased three-fold in PAD patients as compared to non-PAD patients. People who are diagnosed with the disease also have, apart from the increased adverse event risk, two times higher odds of having impaired physical function, with PAD being consistently associated with a reduction of the physical component of quality of life. Moreover, PAD considerably increases medical expenditure, especially in PAD requiring major amputation where the expenditure is twelve times higher as compared to people without PAD.

To decrease the number of adverse events and medical expenditure, as well as to improve quality of life, it is important to understand why certain PAD patients are at increased for vascular complications and why the current medical treatment strategies are insufficient in preventing adverse events. This thesis revealed that the lipid-lowering strategy can be intensified to further improve lipid profiles. Moreover, antiplatelet agents appear to inadequate in preventing adverse events with platelet reactivity being insufficiently decreased in patients who suffer from such events. Alternative medical strategies have been proposed, where the addition of a low dose of a direct anticoagulant drug to the standard antiplatelet therapy reduces the risk of cardiovascular events by 24%. This specific treatment strategy not only improves health outcomes, it also appeared to be cost-effective as compared to the standard antiplatelet therapy, especially in comorbid patients.

An important question however remains; who would benefit from such alternative treatment strategy? The combination of a low dose anticoagulant drug comes with a 70% increase in major bleeding events, and it is important not to expose patients to this increased bleeding risk when they are successfully treated with antiplatelet drugs only. Risk stratification could aid in creating tailored treatment strategies for PAD patients. In this thesis, biomarkers were identified, both newly found and as identified in the systematic review, that could be used for risk stratification. Along with these biomarkers, other variables such as prior ischemic events and decreased kidney function could be used in risk stratification models. Being able to stratify cardiovascular risk in PAD patients, medication strategies can be tailored and thereby the number of cardiovascular events can be reduced and quality of life can be preserved.

CURRICULUM VITAE

Bram Kremers was born on February 17, 1992, in Roosteren, the Netherlands. He attended high school education (Atheneum) in Maaseik, Belgium. After that, his aim was to study Medicine, but the study was based on acceptance by random selection and he was not selected. He therefore studied Biomedical Sciences at Maastricht University and obtained his first year's degree. He was then selected to study Medicine and obtained his bachelor's degree in 2017 and master's degree in 2017. During the last years of the study, he became increasingly interested in cardiovascular diseases, and followed an internship at the Department of Internal Medicine of the Maastricht University Medical Center. During his scientific internship under the supervision of dr. A.J. ten Cate-Hoek, he investigated predictive markers for arterial cardiovascular events and recurrent venous thrombotic events in patients with deep vein thrombosis. As the interest in cardiovascular disease had only grown further, he took the opportunity to become a PhD-candidate at the Department of Biochemistry under the supervision of dr. H.M.H. Spronk, and co-supervisors dr. A.J. ten Cate-Hoek and dr. B.M.E. Mees. During his years as a PhD-candidate he searched for common thrombo-inflammatory pathways in patients with arterial thrombosis and venous thrombosis, and later focused on unravelling the atherothrombotic risk in patients with peripheral artery disease. During these years, he also worked as a medical doctor for the Department of Internal Medicine and for the Thrombosis Service Maastricht. The highlights of his work are presented in this thesis.

LIST OF PUBLICATIONS

Iding AFJ, ***Kremers BMM****, Pallares Robles A, Ten Cate H, Ten Cate-Hoek AJ.* Residual venous obstruction as an indicator of clinical outcomes following deep vein thrombosis: A management study. Thromb Haemost. **2023** Apr 12.

Kremers BMM*, Daemen JHC, Ten Cate H, Spronk HMH, Mees BME, Ten Cate-Hoek AJ.* Inadequate response to antiplatelet therapy in patients with peripheral artery disease: a prospective cohort study. Thromb J. **2023** Jan 10;21(1):5.

Iding AFJ, ***Kremers BMM****, Nagy M, Pallares Robles A, Ten Cate H, Spronk HMH, ten Cate-Hoek AJ.* Translational insights into mechanisms underlying residual venous obstruction and the role of factor XI, P-selectin and GPVI in recurrent venous thromboembolism. Thromb Res. **2023** Jan;221:58–64.

Kremers BMM*, Posma JN, Heitmeier S, Glunz J, Ten Cate H, Pallares Robles A, Daemen JHC, ten Cate-Hoek AJ, Mees BME, Spronk HMH.* Discovery of four plasmatic biomarkers potentially predicting cardiovascular outcome in peripheral artery disease. Sci Rep. **2022** Nov 1;12(1):18388.

van der Bruggen MM, ***Kremers BMM****, van Oerle R, van Oostenbrugge RJ, Ten Cate H.* Potential value of the calibrated automated thrombogram in patients after a cerebral venous sinus thrombosis; an exploratory study. Thromb J. **2021** Nov 4;19(1):81.

Wübbeke LF, ***Kremers BMM****, Daemen J-WHC, Snoeijs MGJ, Jacobs MJ, Mees BME.* Mortality in octogenarians with chronic limb threatening ischaemia after revascularisation or conservative therapy alone. Eur J Vasc Endovasc Surg. **2021** Feb;61(2):350–1.

Kremers BMM*, Wübbeke L, Mees B, Ten Cate H, Spronk H, Ten Cate-Hoek A.* Plasma Biomarkers to Predict Cardiovascular Outcome in Patients With Peripheral Artery Disease: A Systematic Review and Meta-Analysis. Arterioscler Thromb Vasc Biol. **2020** Sep;40(9):2018–32.

Ordieres-Ortega L, Demelo-Rodríguez P, Galeano-Valle F, ***Kremers BMM****, Ten Cate-Hoek AJ, Ten Cate H.* Predictive value of D-dimer testing for the diagnosis of venous thrombosis in unusual locations: A systematic review. Thromb Res. **2020** May;189:5–12.

d'Alessandro E, Becker C, Bergmeier W, Bode C, ..., ***Kremers BMM****, …, et al.* Thrombo-inflammation in cardiovascular disease: An expert consensus document from the third Maastricht Consensus Conference on thrombosis. Thromb Haemost. **2020** Apr;120(4):538–64.

***Kremers BMM**, Birocchi S, van Oerle R, Zeerleder S, Spronk HMH, Mees BME, Luken BM, ten Cate H, ten Cate-Hoek AJ*. Searching for a common thrombo-inflammatory basis in patients with deep vein thrombosis or peripheral artery disease. Front Cardiovasc Med. **2019** Apr;6:33.

***Kremers BMM**, Ten Cate H, Spronk HMH.* Pleiotropic effects of the hemostatic system. J Thromb Haemost. **2018** May; 39(8):1464-1473

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