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ABSTRACT

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Clinical and molecular manifestations of lower limb peripheral artery disease

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Annales Universitatis Turkuensis, Turku, Finland 2017

Lower limb peripheral artery disease (PAD) is poorly known by the general public and even health care professionals. The manifestations of PAD are diverse depending on the prevailing cardiovascular risk factors leading to the disease. Also the outcome of patients is very different depending on the different manifestations of the disease. The end-stage of PAD, known as critical limb ischemia (CLI), is associated with very poor survival. In order to understand the heterogenic nature of PAD in depth, different molecular mechanisms of the disease in association with major cardiovascular risk factors and disease severity were investigated. The findings indicate that different cardiovascular risk factors are associated with diverse molecular pathology. This thesis work also shows that the presence of crural atherosclerosis and CLI are tightly associated with poor patient survival, and that especially CLI is associated with elevated levels of multiple circulating cytokines, which resembles a systemic inflammatory condition. The major future implications of the current findings are that CLI can no longer be referred to as a plain condition of the lower limb and effort should be put into the attenuation of the systemic inflammatory condition. Most importantly, the current findings imply that future effort should be put into studying the risk factor specific molecular mechanism leading to the disease in order to develop new specific medical therapies according to the prevailing inflammatory pathology.

Keywords: peripheral artery disease, critical limb ischemia, atherosclerosis, purinergic signalling, cytokines, cardiovascular risk factors

TIIVISTELMÄ

Juho Jalkanen
Perifeerisen valtimokovettumataudin kliinisiä ja molekylaarisia manifestaatioita

Turun yliopisto, Lääketieteellinen tiedekunta, kirurgia. Molekyylilääketieteen tohtoriohjelma. Turun yliopistollinen keskussairaala, verisuonikirurgian osasto

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Perifeerisellä valtimonkovettumataudilla tarkoitetaan alaraajojen ateroskleroosia eli ASO-tautia, joka tulee alkuperäisestä latinankielisestä nimityksestä atherosclerosis obliterans (ASO). Kansan parhaiten tuntema valtimonkovettumataudin ilmenemismuoto on sepelvaltimotauti. Alaraajojen valtimonkovettumatauti on yleisesti huonosti tunnettu. Sen äärimäiseen muotoon, alaraajojen kriittiseen iskemiaan eli jalkojen hapen puutteeseen, liittyy hyvin suuri riski toimintakyvyn heikkenemiseen ja jopa kuolemaan. Tässä tutkimuksessa perehdyttiin alaraajojen valtimonkovettumataudin kliinisiin ja molekylaarisiin ilmentymiin sekä taudin ennusteeseen.

Perifeerisen valtimokovettumataudin ilmentymät ovat hyvin heterogeenisiä. Tähän ilmiöön vaikuttaa tautiin johtava sydän- ja verisuonisairauksien riskitekijä. Riskitekijästä ja taudin ilmentymästä riippuen on potilaiden ennuste hyvin erilainen. Tämän tutkimuksen tarkoituksena on selvittää valtimokovettumataudin ilmentymien solu- ja molekylaarisia eroja sekä taudin sijainnin vaikutusta eliniän ennusteeseen.

Tutkimuksen tulokset osoittavat, että taudin riskitekijöillä on erilaisia molekyylipatologisia ilmentymiä. Taudin sijainnista riippuen on potilaiden ennuste myös hyvin erilainen. Erityisesti säärisuonien valtimonkovettumatauti ja alaraajojen kriittinen hapenpuute assosioituvat erittäin huonoon eliniän ennusteeseen. Tulokset myös osoittavat, että alaraajan kriittinen hapenpuute ei ole pelkkä paikallinen ongelma, vaan tautitilaan liittyy elimistön yleistynyt pahanlaatuinen tulehdustila, joka voi osaltaan selittää tautiin liittyvän huonon ennusteen. Kriittistä iskemiaa ei voida ajatella vain jalan ongelmana vaan pitäisi löytää uusia hoitomuotoja yleistyneen tulehdustilan hoidoksi. Nyt tehdyt löydökset luovat merkittävän tutkimuspohjan kohti yksilöllisempää taudin ymmärtämistä ja uusien yksilöllisten hoitomuotojen kehittämistä.

Avainsanat: perifeerinen valtimonkovettumatauti, kriittinen iskemia, ateroskleroosi, puriiniaineenvaihdunta, sytokiinit, sydän- ja verisuonisairauksien riskitekijät

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ABBREVIATIONS

ABI	ankle brachial index
ADP	adenosine diphosphate
AMP	adenosine monophosphate
ATP	adenosine triphosphate
CAD	coronary artery disease
CBVD	cerebrovascular disease
CKD	chronic kidney disease
CLI	critical limb ischemia
COPD	chronic obstructive pulmonary disease
CVD	cardiovascular disease
EC	endothelial cell
ESRD	end-stage renal disease
h	hour
IC	intermittent claudication
ICD-10	tenth international classification of diseases
mmHg	millimetres of mercury
mL	millilitres
NTPDase1	nucleoside triphosphate diphosphohydrolase-1
nmol	nano mole
PAD	peripheral artery disease
pg	pico grams
SMC	smooth muscle cell
SLE	systemic lupus erythematosus
TASC	trans-atlantic inter-society consensus (for the management of peripheral arterial disease)
TBI	toe brachial index
Th1	T helper 1 cell (lymphocyte)
Th2	T helper 2 cell (lymphocyte)
Treg	T regulatory cell (lymphocyte)
5’NT	5’(ecto)nucleotidase

LIST OF ORIGINAL PUBLICATIONS

1. Jalkanen J, Yegutkin GG, Hollmén M, Aalto K, Kiviniemi T, Salomaa V, Jalkanen S, Hakovirta H. Aberrant circulating levels of purinergic signaling markers are associated with several key aspects of peripheral atherosclerosis and thrombosis. *Circulation Research* 2015;116:1206–1215.

2. Jalkanen J, Wickström JE, Venermo M, Hakovirta HH. The extent of atherosclerotic lesions in crural arteries predicts survival of patients with lower limb peripheral artery disease: A new classification of crural atherosclerosis. *Atherosclerosis*. 2016 Aug;251:328-33.

3. Jalkanen J, Maksimow M, Hollmén M, Jalkanen S, Hakovirta H. Compared to Intermittant Claudication Critical Limb Ischemia Is Associated with Elevated Levels of Cytokines. *PLoS One*. 2016 Sep 9;11(9):e0162353.

4. Jalkanen J, Maksimow M, Hollmén M, Salomaa V, Jalkanen S, Hakovirta H. Immunological Pathology Differs Between Major Cardiovascular Risk Factors in Patients with Symptomatic Atherosclerosis. (Submitted)

1 INTRODUCTION

Patients with lower limb peripheral artery disease (PAD) are generally divided into three categories according to disease severity: the asymptomatic, patients with intermittent claudication (IC) and patients with critical limb ischemia (CLI) (Dormandy & Rutherford 2000). CLI is a condition in which atherosclerotic lesions occlude lower limb arteries and oxygenated arterial blood flow is insufficient to maintain viable tissue perfusion (Anon 1992; Michaels 1993). The milder state of a symptomatic disease is referred to as IC. It is characterized by insufficient arterial blood flow during exercise, e.g. walking, causing ischemic pain in the muscles under strain. These symptoms reside at rest (McDermott 2015). Opposite to IC, rest pain, non-healing ulcers, and gangrene characterize CLI (Thompson et al. 1993; Michaels 1993). Where as patients with IC can be managed conservatively by cessation of smoking, statins, antiplatelet therapy and exercise, patients with CLI require revascularization in the form of endovascular angioplasty and stenting or open surgical procedures, e.g. endarterectomy or bypass surgery to avoid amputation (Dormandy & Rutherford 2000; Hirsch et al. 2006; Rooke et al. 2011)

Atherosclerosis is a result of multiple cardiovascular risk factors and genetic susceptibility (P. W. Wilson et al. 1998; D'Agostino et al. 2001). Major risk factors include: age, hypertension, diabetes, dyslipidaemia, chronic kidney disease (CKD), rheumatic disorders, smoking and male gender (DAWBER et al. 1951; DAWBER et al. 1957; Mahmood et al. 2014). PAD is one of the three main forms of atherosclerosis (DeBakey et al. 1985; DeBakey & Glaeser 2000). The other two are coronary artery disease (CAD) and cerebrovascular disease (CBVD) (Aboyans et al. 2006). Compared to CAD or CBVD, lower limb PAD encompasses a vast range of vasculature from the aorta to the toes (DeBakey et al. 1985). None the less, the anatomical variation of these arteries is very different (Haimovici & Maier 1961; Haimovici 1991). Therefore, lower limb atherosclerosis is often categorized into three distinct anatomical regions: the aorto-iliac arteries, the femoro-popliteal arteries, and the crural arteries (Norgren, Hiatt, Dormandy, Nehler, Harris & Fowkes 2007; Norgren, Hiatt, Dormandy, Nehler, Harris, FowkesTASC II Working Group 2007).

From prior studies it is well documented that different cardiovascular risk factors are associated with different anatomical distribution of lower limb atherosclerosis (Smith et al. 1996; N. Diehm et al. 2006), and that different risk factors are associated with IC when compared to CLI (Wyss et al. 2015). Compared to IC, CLI is also associated with significantly worse over all survival (Golomb et al. 2006; Reinecke et al. 2015). Unfortunately, PAD is the least studied and poorly known

of atherosclerotic diseases (Hirsch et al. 2001; Hirsch et al. 2007), despite its vast manifestations and poor outcome (Sampson et al. 2014; Fowkes et al. 2014). In order to understand the poor outcome associated with CLI better, this thesis explores the clinical and molecular manifestations of lower limb atherosclerosis.

2 REVIEW OF LITERATURE

2.1 Atherosclerosis as an inflammatory disease

Atherosclerosis was once thought to be a passive process of the accumulation of cholesterol into the artery wall. This impression of the disease has become obsolete (Ross & Glomset 1976a; Ross & Glomset 1976b). Currently atherosclerosis is considered an inflammatory disease of the arterial wall (Ross 1999; Weber & Noels 2011), in which a variety of inflammatory mediators contribute to the accumulation of lipids and immune cells from both inside the artery via luminal endothelium, or outside the artery from the vasa vasorum, or even surrounding tertiary lymphatic structures (Libby et al. 2015). The main form of circulating immune cells that enter the atherosclerotic lesion are neutrophils, lymphocytes and especially monocytes, which turn into macrophages when entering into the artery wall (Aqel et al. 1984; Jonasson et al. 1986). Thus, immunology has become a cornerstone in understanding atherosclerosis (Hansson & Hermansson 2011).

2.1.1 Development of an atherosclerotic lesion/plaque

An atherosclerotic lesion is often also referred to as an atherosclerotic plaque because of calcification brought forth by dying lipid digesting foam cells (Faggiotto & Ross 1984). Dying foam cells form a necrotic core and fibrosis in the atherosclerotic lesion, which then turns into a very hard solid calcified accumulation of the digested lipids (Faggiotto et al. 1984). Foam cells originate from monocytes circulating in the blood (Ghattas et al. 2013). They enter the artery wall and turn into lipid digesting macrophages, which then are named foam cells after they are filled with digested lipids. In this process lipid and leucocyte accumulation, digestion and cell death causes artery wall thickening and finally occlusion of the artery either by atherosclerotic plaque rupture and thrombus formation inside the arterial lumen (Ross 1993), or the artery occludes by plain thickening as surprisingly often seen in PAD (O'Neill et al. 2015). The thickening of the arterial wall happens gradually during years, but a plaque rupture and occlusion of an artery can be sudden and cause acute symptoms (van der Wal, Becker, van der Loos & Das 1994).

Figure 1 below present a schematic illustration of normal artery and atherosclerotic stenosis seen in PAD. There is no exact explanation to what triggers the development of atherosclerosis at a certain location, but it is generally accepted that it requires endothelial dysfunction and damage for the process to begin. Endothelial damage can occur from aging, shear stress caused by hypertension, hyperglycaemia caused by diabetes, cell death caused by smoking or other toxins, and also some infections can cause endothelial damage and provoke the development of atherosclerosis (Hansson 2005).

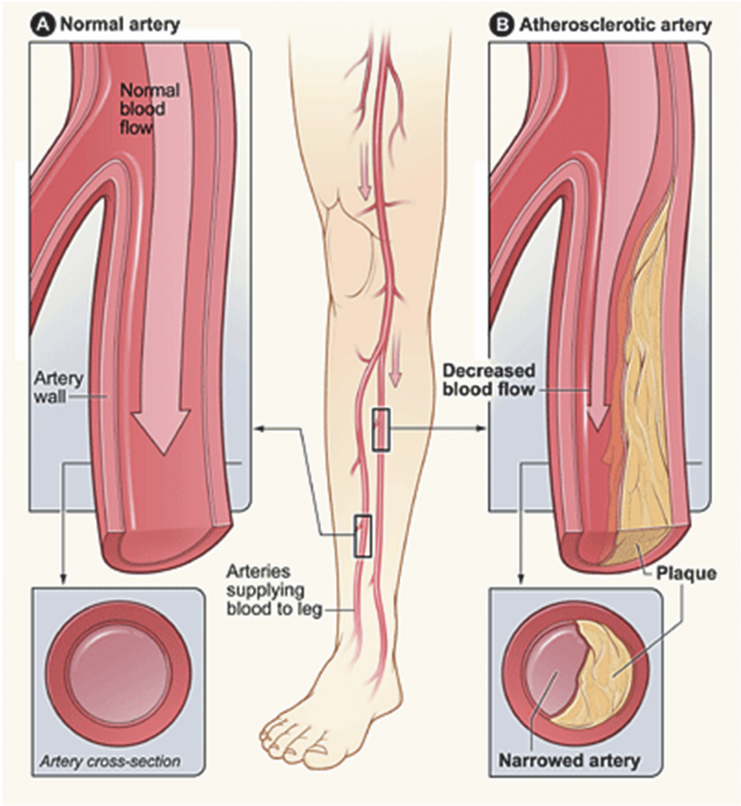

Figure 1. Schematic illustration of atherosclerotic lesion formation and stenosis of the crural artery in PAD. As presented by the American Heart Association under public domain free of restrictions under copyright law (http://www.nhlbi.nih.gov/health/health-topics/topics/pad/, accessed18.10.2016).

2.1.2 Cytokines, chemokines and growth factors in atherosclerosis

In addition to lipid accumulation, the infiltration and presence of macrophages and lymphocytes is essential in the development of atherosclerosis (Jonasson et al. 1986). Cytokines, chemokines and growth factors are an important part of the requiting of these inflammatory cells into the artery wall (Frostegård et al. 1999). These are molecules that are responsible for the cross-talk between cells in the developing plaque and recruitment of circulating immune cells (Larsen et al. 1990). Cytokines are a broad category of proteins involved in cell signalling (Oppenheim et al. 1991). Cytokines are produced by immune cells, endothelial cells, as well as fibroblasts and stromal cells, i.e. all cells that are involved in atherosclerosis (Galindo et al. 2001). Chemokines are a subclass of cytokines that have chemotactic properties and are also referred to as chemotactic cytokines (Luster 1998). Growth factors, on the other hand, are cytokines that stimulate cellular growth, proliferation and differentiation (Salcedo et al. 1999). Figure 2 below is a more de-

tailed presentation of the accumulation of lipids and immune cells into the atherosclerotic plaque using chemokines as the attractant of circulating cells (Bernhagen et al. 2007). Pro-inflammatory cytokines and growth factors from immune cells in the plaque promote smooth muscle cell proliferation and neovascularization (de Boer et al. 1997; Salcedo et al. 1999). The wall thickens causing also hypoxia and increased inflammation and neovascularization resulting in a vicious cycle (Galindo et al. 2001). Increasing local hypoxia and inflammation eventually causes foam cell apoptosis and also intra-plaque haemorrhage from fragile neovascular vessels. All this results in a lipid rich necrotic core with calcium deposits (Skeoch & Bruce 2015). Hence, the term calcification is also often used for describing an atherosclerotic plaque (van der Wal, Becker, van der Loos, Tigges, et al. 1994).

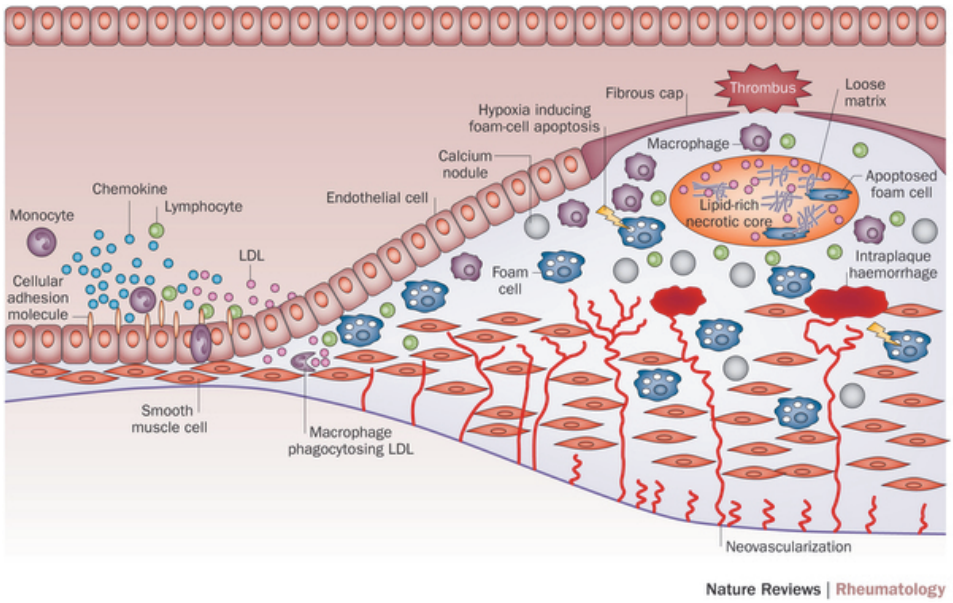

Figure 2. A closer look at plaque development. Endothelial dysfunction gives raise to inflammatory mediators (adhesion molecules, cytokines and chemokines). This increases vascular permeability and the infiltration of LDL-cholesterol particles and immune cells (mainly monocytes and lymphocytes) into the artery wall. Monocytes turn into LDL phagocytising macrophages, which then turn into foam cells. Macrophages and lymphocytes in the plaque produce pro-inflammatory cytokines and growth factor resulting smooth muscle cell proliferation, arterial wall thickening, hypoxia and neovascularization. This becomes a vicious cycle resulting in increasing thickening and occlusion of the artery (Skeoch & Bruce 2015). Reproduced with the permission of Nature Publishing Group.

In addition to the signals cytokines, chemokines and growth factor have within the plaque and its immediate surrounding the release of these molecular signals have systemic consequences (van der Wal et al. 1989). Cytokines in circulation, especially, growth factors enhance the proliferation of bone marrow and spleen derived myeloid progenitor cells, which turn into monocytes and then macrophages in the

process of atherosclerosis (Weber & Noels 2011). Thus, in many ways cytokines can enhance the development of atherosclerosis in association with cardiovascular risk factors that stress the body and vasculature. Most cytokines are pro-inflammatory, but there are also anti-inflammatory cytokines. Cytokines may also have pleiotropic effects. Thus, the field of cytokines and atherosclerosis is very broad and complex, and currently under vigorous research (Ait-Oufella et al. 2011; Zernecke & Weber 2014; Tousoulis et al. 2016). There are more than 100 cytokines known to date. To help classify them they are often clustered into the following groups: interleukins from 1 to 37 (IL-1 to IL-37), tumor necrosis factors (TNFs), interferons (IFNs), colony stimulating factos (CSFs), transforming growth factors (TGFs) and chemokines. Interleukins are then further divided into pro-inflammatory mediators of innate (IL-1, 2, 6, 8, 12, 16, 17, 18) and adaptive (IL-3, 4, 5, 7, 13) immunity, as well as anti-inflammatory interleukins (IL-1ra, IL-10, IL-33) (Ait-Oufella et al. 2011). Innate immunity mediators activate T helper 1 cells (Th1), neutrophils, natural killers and class 1 macrophages. Adaptive immunity mediators activate T helper 2 cells (Th2), eosinophils, B cells, mast cells, and class 2 macrophages. Anti-inflammatory mediators activate T regulatory cells (Tregs) (Hansson & Hermansson 2011). Figure 3 below is schematic illustration of cytokines involved in atherosclerosis and the cells that produce them.

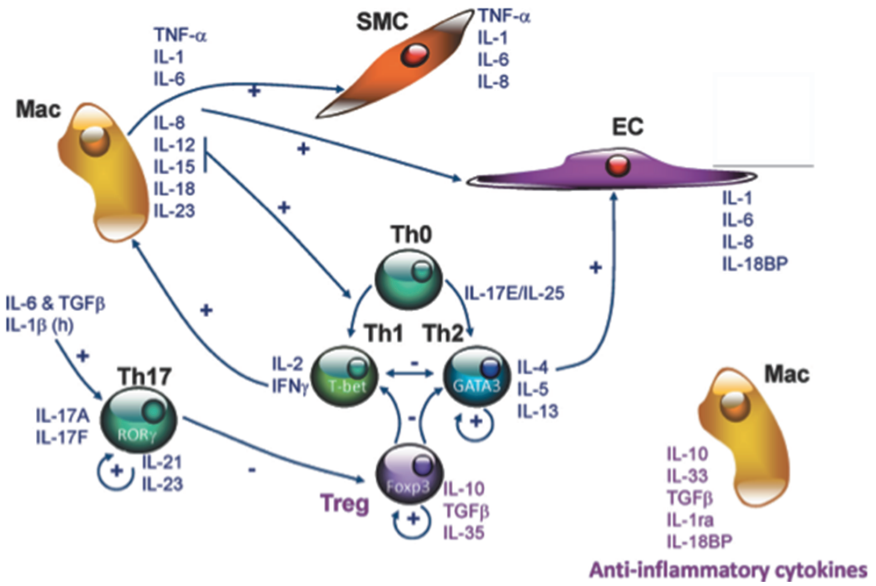

Figure 3. Cytokines involved in atherosclerosis, and the immune and vascular cells they activate. Cytokines are produced by several immune and vascular cells. IL-12 and IL-18, produced by macrophages (Mac), are potent inducers of IFN-γ and promote the differentiation of naïve T cells into pro-atherogenic Th1 cells. Macrophage-derived cytokines activate SMCs and ECs to produce pro-inflammatory cytokines. Anti-inflammatory cytokines IL-10 and TGFβ are also produced by macrophages, which then promotes anti-atherogenic Treg cell differentiation. Pro-atherogenic Th17 cell differentiation requires TGFβ, IL-1β, IL-6 and IL-17 (Ait-Oufella et al. 2011). Reproduced with the permission of Wolters Kluwer Health Inc.

The involvement of cytokines in atherosclerosis has been extensively studied in animal models, which has led to the understanding of their function. The presence of these cytokines has then been confirmed from analyses of human atherosclerotic lesions (Hansson 2005). As said, there are numerous cytokines involved in atherosclerosis. Following, the most important cytokines and their affect on immune cells and atherosclerosis are summarized in Table 1. Pro-inflammatory cytokines are in general divided into Th1 cytokines of innate immunity, Th2 cytokines of adaptive immunity, and Th17 cytokines.

Table 1. Cytokines involved in atherosclerosis grouped by primary cellular function and inflammatory activity. Adapted from (Tedgui & Mallat 2006; Zernecke & Weber 2014)

Group	Cytokine	Full name	Cellular source	Inflammatory activity
Th1	IL-1b	Interleukin-1 beta	Macrophages, ECs, SMCs	Stimulates EC and SMC inflammatory activity and leucocyte recruitment
	IL-2	Interleukin-2	Macrophages, T & B cells	T cell growth factor, stimulates natural killer (NK) activity
	IL-6	Interleukin-6	Macrophages, lymphocytes, ECs, SMCs	Induction of acute phase proteins, SMC proliferation
	IL-8	Interleukin-8	Neutrophils, T cells, monocytes	Pro-inflammatory, promotes leukocyte adhesion and arrest
	IL-12	Interleukin-12	T cells, macrophages	Promotes NK and cytotoxic lymphocyte activity and induces IFN-γ
	IL-15	Interleukin-15	ECs, T cells, macrophages	Enhances neutrophil chemokine production
	IL-16	Interleukin-16	Mast cells, T cells	T cell growth factor, enhances lymphocyte chemotaxis and adhesion molecules
	IL-18	Interleukin-18	Macrophages, T cells	Pro-inflammatory, induces Th1 cytokines and IFN-γ
Th2	IL-3	Interleukin-3	T cells, mast cells	Promotes proliferation and differentiation of mast cells
	IL-4	Interleukin-4	T & B cells, mast cells, macrophages	Proliferation and differentiation of B cells and Th2 cells
	IL-5	Interleukin-5	T cells, mast cells, Ecs	Stimulates growth and differentiation of B cells
	IL-7	Interleukin-7	Platelets	Pro-inflammatory towars monocytes, Th2 cells and B cells
	IL-13	Interleukin-13	Th2 cells	Promotes Ig (gammaglobulin) transcription in B cells
Anti-inflammatory	IL-10	Interleukin-10	Macrophages, mast cells, Tregs, B cells	Inhibits Th1 inflammatory responses, promotes proliferation and differentiation of regulatory T cells
Th17	IL-17	Interleukin-17	T regs, ECs, fibroblasts	Th 17 cell proliferation and differentiation, and secretion of IL-6, IL-8, G-CSF
TNFs	TNF-α	Tumor necrosis factor alpha	Many cells types	Pro-inflammatory, neutrophil activation and recruitment
	TRAIL	Tumor necrosis factor related apoptosis inducing ligand	Many cells types	Lymphocyte recruitment
IFNs	IFN-γ	Interferon gamma	Macrophages, Th1 lymphocytes, ECs, SMCs	Promotes Th1 cytokine expression and inhibits extracellular matrix synthesis by SMCs

Group	Cytokine	Full name	Cellular source	Inflammatory activity
Chemokines induced by IFN-γ	IP-10	Interferon gamma induced protein-10	Monocytes and lymphocytes	Recruitment of monocytes and lymphocytes
	CTACK	T cell attracting chemokine	SMCs and lymphocytes	Recruitment of lymphocytes
	MIG	Monocine induced by interferon gamma	Monocytes and lymphocytes	Recruitment of monocytes and lymphocytes
Monocyte recruiting chemokines	MCP-1	Monocyte chemotactic protein-1	Macrophages, SMCs	Monocyte mobilisation from bone marrow and neutrophil recruitment
	MIP-1a	Macrophage inflammatory protein-1 alpha	Macrophages, SMCs	Monocyte recruitment and arrest
	MIP-1b	Macrophage inflammatory protein-1 beta	Macrophages, SMCs	Monocyte recruitment and arrest
	MIF	Macrophage migration inhibitory factor	Macrophages, SMCs, lymphocytes	Monocyte and lymphocyte recruitment and accumulation
Growth factors	M-CSF	Macrophage colony stimulating factor	Macrophages, ECs, lymphocytes	Growth and differentiation of macrophages
	GM-CSF	Granulocyte-macrophage colony stimulating factor	Macrophages, ECs, lymphocytes	Growth and differentiation of macrophages
	G-CSF	Granulocyte colony stimulating factor	Macrophages, SMCs, lymphocytes	Growth and differentiation of hematopoietic stem cells
	PDGF	Platelet derived growth factor	Platelets	Platel growth and proliferation
	bFGF	Basic fibroblast growth factor	SMCs, granulocytes, fibroblasts	Growth and differentiation of fibroblasts
	HGF	Hepatocyte growth factor	SMCs, granulocytes, lymphocytes	Growth and differentiation of hematopoietic stem cells
	VEGF	Vascular endothelial growth factor	ECs, SMCs	Neovascularisation

2.1.3 Purinergic signaling in atherosclerosis

Purinergic signaling is the extracellular metabolism of circulating adenosine triphosphate (ATP) and adenosine diphosphate (ADP) (Eltzschig et al. 2012). ATP and ADP are released from dying or stressed cells and they act as signaling molecules. ATP is pro-inflammatory by nature and ADP enhances the adherence of platelets, i.e. is thrombotic (Birk et al. 2002). During this signaling pathway circulating ATP and ADP are first converted to adenosine monophosphate (AMP) by endothelial surface enzyme nucleoside triphosphate diphosphohydrolase-1 (NTPDase1), commonly known as CD39 (Enjyoji et al. 1999). After this AMP is converted to adenosine and phosphate by another endothelial cell surface enzyme named ecto-5'-nucleotidase (5'-NT), more commonly named CD73 (Figure 4). Adenosine then acts as a potent anti-inflammatory agent via different tissue specific adenosine receptors (Eltzschig et al. 2003). In summary, purinergic signaling or i.e. extra-cellular ATP metabolism is an anti-inflammatory response to a pro-inflammatory stimulus on healthy, normally functioning, endothelium.

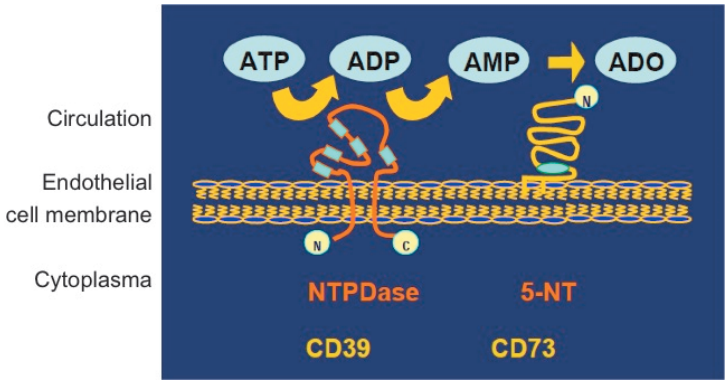

Figure 4. Purinergic signalling in a nutshell. Simplified, purinergic signalling is the conversion of circulating adenosine triphosphate (ATP) and adenosine diphosphate (ADP) into adenosine by endothelial bound ectonucleotidases CD39 and CD73.

Earlier pre-clinical studies have indicated that purinergic signaling as a key regulator of inflammation and vascular stability (Zernecke et al. 2006; Eltzschig et al. 2012), and impaired CD73 can lead to atherosclerosis (Buchheiser et al. 2011). Small observational findings in man have shown that impairment in purinergic signaling may lead to early severe PAD (Lecka et al. 2010; St Hilaire et al. 2011). For example, Hilaire et al. found that the cause of severe PAD at a relatively young age was associated with mutations in the gene encoding for CD73 {StHilaire:2011hi}. As atherosclerosis is an inflammatory disease and purinergic signaling is one of natures profound inflammatory signaling mechanisms it is bound to somehow be involved in atherosclerosis (Burnstock & Ralevic 2014), but little is still known of its actual role in the development atherosclerosis.

2.2 Peripheral artery disease amongst atherosclerotic diseases

Despite the scientific advances of modern medicine atherosclerosis remains a major cause of death in the Western world. Death from atherosclerosis has, however, shifted from a working age population to the more elderly (Jousilahti et al. 2016), and the burden of the disease prevails as the age structure of society has sifted significantly towards higher ages. Thus, we face the challenge of aging arteries. This is especially the case with PAD, which is very strongly associated with aging (Fowkes et al. 2013). An estimated 5% of people over 40 years of age (Menke et al. 2006), 16% of people from 55 – 74 years of age (Lee et al. 2004), and over 20% of people over 80 years of age suffer from the disease (Meijer et al. 1998; Hirsch et al. 2001). Altogether approximately 200 million people suffer from the disease world wide (Criqui & Aboyans 2015). The majority of people with PAD are unaware of the condition and do not express symptoms. Even an asymptomatic disease and the presence of unknown PAD is associated with sig-

nificant cardiovascular co-morbidity and poorer survival compared to the an age matched population (Lee et al. 2004; Menke et al. 2006).

In general, PAD is less known than CAD or CBVD by both the public (Hirsch et al. 2007) and even amongst healthcare professionals (Hirsch et al. 2001). This rises a concern since amongst the three forms of atherosclerosis, lower limb atherosclerosis is the least recognized, but associated with the highest cardiovascular mortality (Criqui et al. 1992; Allison et al. 2012). Experts claim that due to aging and the increasing prevalence of diabetes, PAD is an unrecognised upcoming health crisis (Sampson et al. 2014; Cooke & Chen 2015).

2.2.1 Risk factors of atherosclerosis and peripheral artery disease

The main risk factors for atherosclerosis and PAD are generally referred to as major cardiovascular disease risk factors, which traditionally include: age, male gender, smoking, diabetes, hypertension and dyslipidaemia in the form of high LDL-cholesterol and low HDL-cholesterol (Vartiainen et al. 2000). New comers amongst major cardiovascular risk factors are chronic kidney disease (CKD) (Manjunath et al. 2003; Go et al. 2004) and rheumatic disorders, mainly rheumatoid arthritis (Gabriel 2008) and systemic lupus erythematosus (SLE) (Erdozain et al. 2014). Compared to other atherosclerotic diseases the importance of dyslipidaemia is not that significant in PAD (Norgren, Hiatt, Dormandy, Nehler, Harris, Fowkes, TASC II Working Group, et al. 2007), but its importance cannot be neglected when talking about atherosclerosis. The most significant risk factors for PAD are smoking, age and diabetes (Norgren, Hiatt, Dormandy, Nehler, Harris, Fowkes, TASC II Working Group, et al. 2007). In addition, CKD has been established as significant risk factor for especially PAD and atherosclerosis in general (O'Hare et al. 2004; Lacroix et al. 2013). In similar fashion, tough clinically evident, the burden of rheumatic illnesses on cardiovascular morbidity has later on been established with the help of large cohorts studies and meta-analyses (Skeoch & Bruce 2015). Most patients with PAD also have hypertension. However, despite a strong association it is not completely clear weather hypertension is a result of arterial stiffening and the disease itself or is it a very significant risk factor leading to the disease (Norgren, Hiatt, Dormandy, Nehler, Harris, Fowkes, TASC II Working Group, et al. 2007; Korhonen et al. 2009).

2.2.2 The association of risk factors with anatomical distribution of lower limb atherosclerosis

For practitioners dealing with PAD it has become evident that the anatomical distribution of atherosclerotic lesions of the lower limbs has a lot to do with the

cardiovascular risk factor leading to the disease. In 2006 Diehm et al. very informatively captured this phenomenon into pictures (Figure 5). Summarized, proximal or large vessel PAD in above the knee arteries (i.e., the aorta, and iliac and femoral arteries) is mainly associated with smoking, dyslipidemia, and young age, whereas distal or small vessel PAD encompassing the crural arteries is associated with diabetes, CKD, and old age. This phenomenon has also been validated in other cohorts and discussed in reviews (Smith et al. 1996; N. Diehm et al. 2006; Aboyans et al. 2006; Aboyans et al. 2007). Since the original report by Diehm et al. also the impact of rheumatic disorder and use of cortisone has also been associated with distal PAD (Willenberg et al. 2010).

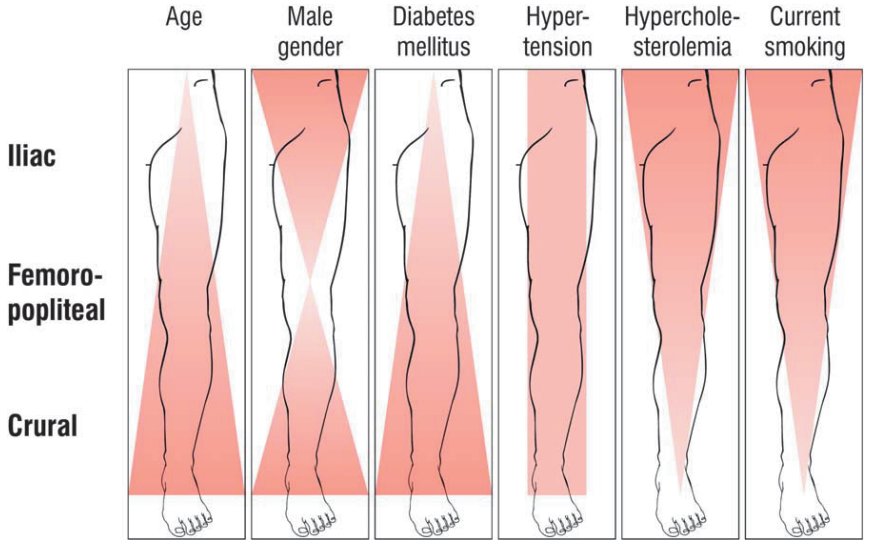

Figure 5. The association of major cardiovascular risk factors with the anatomical distribution of lower limb atherosclerosis (N. Diehm et al. 2006). Reproduced with the permission of Elsevier Ltd.

Already in 2006 Diehm et al. concluded that there must be molecular level differences behind these patterns. Unfortunately, little has been done to understand these patterns of PAD better. Using a similar approach it has now been established that certain risk factors are also associated with the severity of PAD (Wyss et al. 2015), and hence also outcome (Lüders et al. 2016; van Haelst et al. 2016).

2.3 Classifications of peripheral artery disease

2.3.1 Classification of intermittent claudication and critical limb ischemia

Now depending on how insufficient the arterial flow is following an occlusion or stenosis, and how well does collateral flow from different origin compensate, a sub-

ject suffers from symptoms, which delineate disease severity. Disease severity is crudely classified into the asymptomatic, subjects with intermittent claudication (IC), and subjects with critical limb ischemia (CLI). This categorization is important because subjects with CLI are in great need of surgical revascularization in order to prevent limb loss, while subjects with IC can be mainly managed conservatively (McGrath et al. 1983; Norgren, Hiatt, Dormandy, Nehler, Harris & Fowkes 2007).

IC is classified as limb pain distal to an arterial occlusion, which is brought forth during walking. It never starts at rest. Insufficient arterial circulation is confirmed using the ankle brachial index (ABI), which means systolic blood pressure is measured from the ankle and divided by the blood pressure measured from the brachial artery. Values ranging from 0.9 – 1.3 are considered normal (Hardman et al. 2014). In other words everything below 0.9 refers to insufficient blood flow of the lower limb. Everything above 1.3 is considered falsely high as result of arterial stiffening and incompressible tibial arteries at ankle level. This dilemma can be compensated by measuring blood pressure from the toe, because digital arteries of the toe do not generally suffer from incompressibility (Bhamidipaty et al. 2015; Forsythe & Hinchliffe 2016). When making blood pressure measurements from the toe, the absolute values in mmHg are considered more appropriate than the toe brachial index (TBI) (Holtman & Gahtan 2008). Table 2 below is a directional illustration of correlation of symptoms with levels of ankle and toe pressure measurements.

Table 2. The correlation of PAD symptoms with peripheral blood pressure measurements. Modified from the work of Holtman & Gahtan 2008.

Clinical status	ABI*	TBI** or toe systolic pressure
Incompressible arteries, all symptoms possible	> 1.3	All toe pressures possible and usually correlate with symptoms
Normal	0.9 – 1.3	0.8 – 0.9
Mild claudication	0.8 – 0.9	60 – 80 mmHg
Moderate-severe claudication	0.4 – 0.8	40 – 60 mmHg
Tissue loss	< 0.5	40 – 50 mmHg
Ischemic rest pain	< 0.4	index < 0.15 or pressure < 30mmHg
Threatened limb	< 0.15	0 mmHg

Given index and pressure values are non-definitive approximate values
*ABI = ankle brachial index
** TBI = toe brachial index

Compared to IC, CLI is characterized by rest pain and/or tissue loss. For rest pain to occur blood pressure at ankle level should be < 50 mmHg and < 30 mmHg at toe level. Otherwise other reasons for rest pain should be sought. Ulcers and tissue loss should not appear unless ABI is < 0.5 or absolute pressure at ankle level

is < 70 mmHg and < 50 mmHg at toe level (Norgren, Hiatt, Dormandy, Nehler, Harris, Fowkes, TASC II Working Group, et al. 2007; Holtman & Gahtan 2008).

2.3.2 The severity of the disease according to Fontaine and Rutherford classifications

There are two widely acknowledged classification systems for the severity of PAD according to the presented clinical symptoms: the Fontaine classification and Rutherford (Rutherford et al. 1986; Rutherford et al. 1997). Both classification systems are rather similar. They grade the severity of PAD to stage from I to IV according to the presented symptoms. The stages are not, however, identical. All forms of claudication fall under the stage I according to the Rutherford classification while stage I is an asymptomatic class according to the Fontaine classification and claudication is divided into staged IIa and IIb according to severity. On the contrary, end-stage PAD is III (ischemic rest pain) and IV (ulceration or gangrene) according to the Fontaine classification, while the Rutherford system classifies these stages more precisely from II to IV (Table 3). The Rutherford classification also gives numerical categories from 0 to 6 six for all symptom categories. This numerical category is often seen used in the current literature, because it the most precise. The Fontaine classification has also been widely used and could be referred as the classical one of these two.

Table 3. Fontaine and Rutherford classification for the severity of PAD. Modified from the work of Rutherford et al. 1997.

Fontaine classification		**Rutherford classification**		
Stage	Clinical symptom	Stage	Category	Clinical symptom
I	Asymptomatic	0	0	Asymptomatic
IIa	Mild claudication	I	1	Mild claudication
IIb	Moderate to severe claudication	I	2	Moderate claudication
		I	3	Severe claudication
III	Ischemic rest pain	II	4	Ischemic rest pain
IV	Ulceration or gangrene	III	5	Minor tissue loss
		IV	6	Major tissue loss

According to the Fontaine classification stages III and IV form the group of CLI. Correspondingly, CLI comprises of stages II, III and IV or categories 4, 5 and 6 for the Rutherford classification (Rutherford et al. 1997).

2.3.3 TASC classification by anatomical distribution

After major collaboration the The Trans-Atlantic Inter-Society Consensus Document on Management of Peripheral Arterial Disease (TASC) was published in 2000 (Dormandy & Rutherford 2000). In 2007 the second Inter-Society Consensus for the Management of Peripheral Arterial Disease (TASC II) was published (Norgren, Hiatt, Dormandy, Nehler, Harris, Fowkes, TASC II Working Group, et al. 2007). The TASC documents classify lower limb atherosclerotic lesions by length and severity in the anatomical regions of the aorto-iliac and femoropopliteal arteries, separately. The TASC classification scheme is a practical guide and research tool to asses treatment modalities for specific lesion types from A to D. Following is schematic illustration of these lesions types along with a rationale for their grading (Figures 6 & 7).

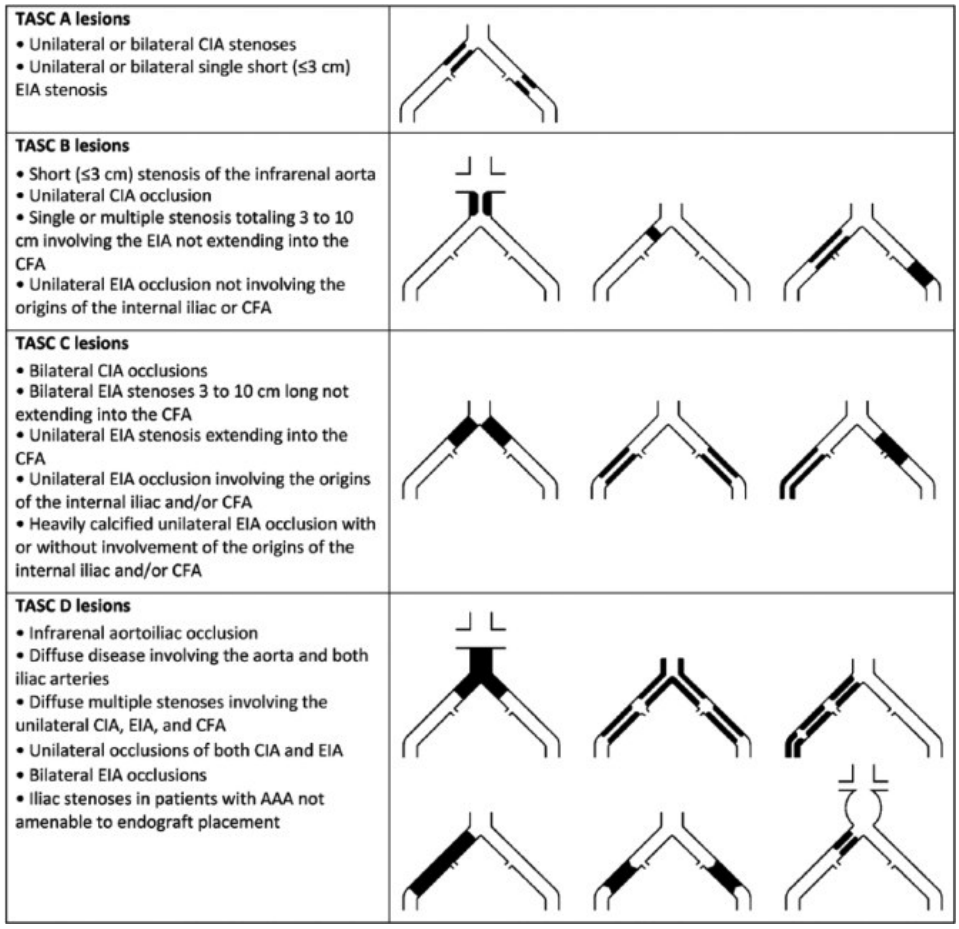

Figure 6. The TASC classification for the aorta and iliac arteries. CIA = common iliac artery, EIA = external iliac artery, CFA = common femoral artery, AAA = abdominal aortic aneurysm (Norgren, Hiatt, Dormandy, Nehler, Harris, Fowkes, TASC II Working Group, et al. 2007). Reproduced with the permission of Elsevier Ltd.

A and B type lesions being mild short lesions are handled solely by angioplasty (balloon dilatation and stenting, i.e. endovascular therapy), and D type lesions being severe long lesions usually require bypass surgery. C type lesions are borderline cases. They can often be treated by endovascular therapy. Even D type lesions can be treated by endovascular means the current literature shows that the more severe the lesion according to the TASC classification, the poorer is the long-term results of endovascular therapy. This especially applies to TASC D lesions and the femoro-popliteal arteries (Norgren, Hiatt, Dormandy, Nehler, Harris, Fowkes, TASC II Working Group, et al. 2007; Ihnat et al. 2008). The predictive value of the TASC classification on overall patient outcome is understudied.

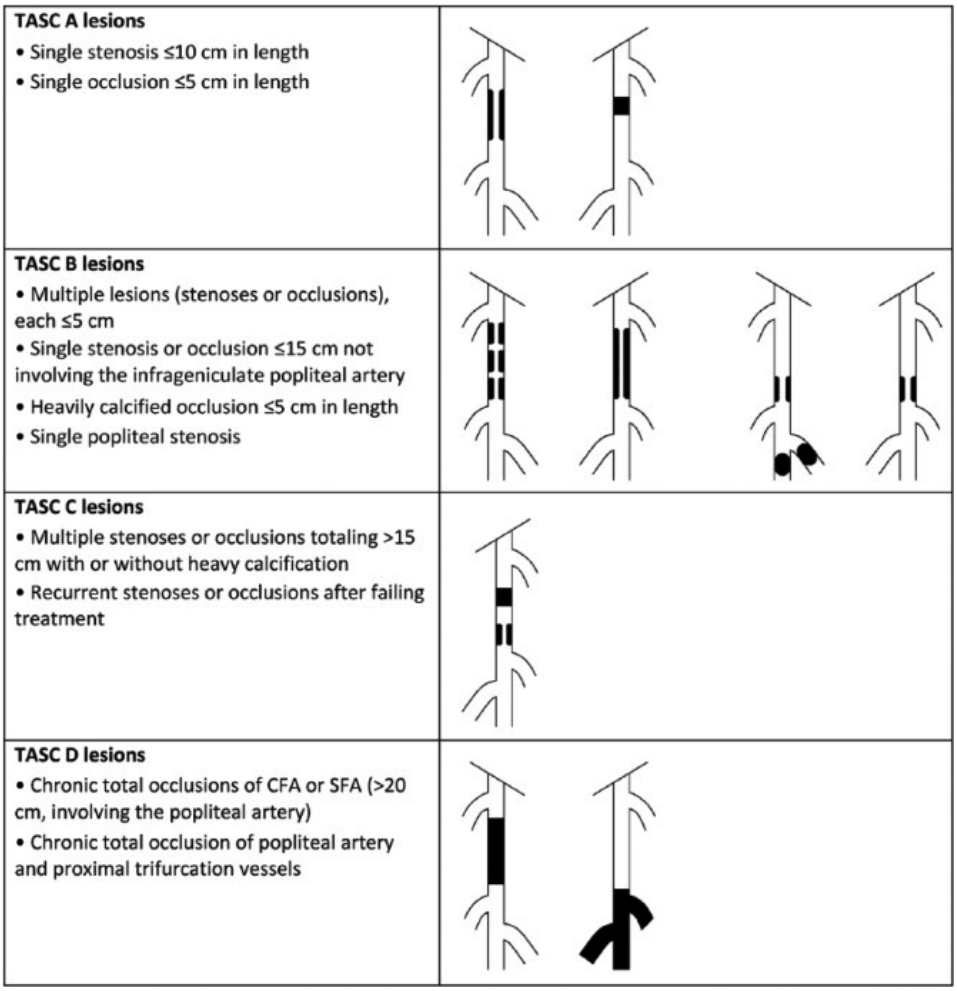

Figure 7. The TASC classification for the femoral and popliteal arteries. The diagonal line represents the inguinal ligament, from which there is a short segment of the common femoral artery (CFA) before it becomes the superficial femoral artery (SFA), and then into three crural arteries (Norgren, Hiatt, Dormandy, Nehler, Harris, Fowkes, TASC II Working Group, et al. 2007). Reproduced with the permission of Elsevier Ltd.

The original TASC classification poorly took into account the infra-popliteal arteries, which are also referred to as crural or tibial arteries. Thus, an update was published in 2015 to account for this (TASC Steering Committee et al. 2015). In similar fashion this update classifies atherosclerotic lesions of the crural arteries from A to D as presented below (Figure 8).

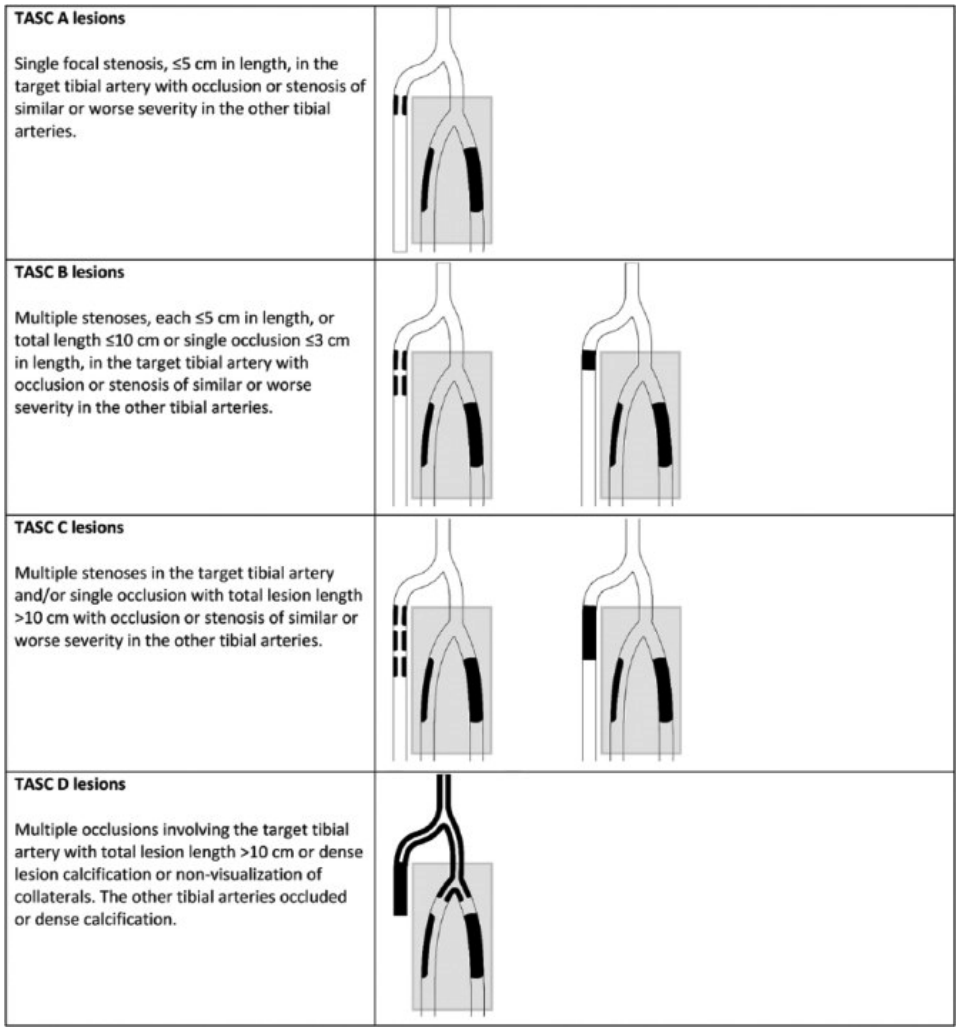

Figure 8. The updated TASC classification for infra-popliteal arteries. The un-shaded area represents the target lesion; area inside the shaded rectangle represents typical background disease (TASC Steering Committee et al. 2015). Reproduced with the permission of Sage Publications.

The three tibial arteries forming the trifurcation are the anterior tibial artery, which departs first (or highest) following by the posterior tibial artery and tibioperoneal artery, which depart simultaneously below the anterior tibial artery. The

TASC classification does not distinguish these three from each other, but concentrates on the target lesion under evaluation or treatment.

2.4 Outcome of peripheral artery disease

As previously mentioned PAD is associated with poor survival when compared to age matched controls (Criqui et al. 1992; Sampson et al. 2014). Patients with PAD mostly die of myocardial infarction (40 – 60%), ischemic stroke (10 – 20%), or other vascular events (10%) (Norgren, Hiatt, Dormandy, Nehler, Harris, Fowkes, TASC II Working Group, et al. 2007). This phenomenon is present in both symptomatic and asymptomatic subjects screened and determined to have PAD by ABI measurement (C. Diehm et al. 2009). However, the exact outcome of PAD is difficult to predict. For example, the severity of symptoms is not very prognostic. Thus far, the best prognostic factor of overall outcome of patients with PAD has been seen with low and very high ABI values (Heald et al. 2006; Fowkes et al. 2014). False high values are result of arterial stiffening and incompressible arteries during ABI measurement. This is primarily a result of diabetic mediasclerosis. Diabetes itself is a significant factor associated with poor survival (Norgren, Hiatt, Dormandy, Nehler, Harris, Fowkes, TASC II Working Group, et al. 2007).

Among subjects with PAD the outcome of subjects with a mild disease, i.e. an asymptomatic disease or claudication, is very different from subjects with CLI (Farber & Eberhardt 2016). It is estimated that within one year from the diagnosis of CLI 30 – 70% of patients are amputated or dead (Norgren, Hiatt, Dormandy, Nehler, Harris, Fowkes, TASC II Working Group, et al. 2007). The short and mid-term mortality rate of patients with CLI can be up to 4 times higher when compared to patients with IC (Golomb et al. 2006). This implies that even amongst patients with PAD the symptoms and peripheral hemodynamic measurements (ankle and toe systolic pressure) classified as CLI present an entirely different condition than the classification of IC.

3 AIMS OF THE STUDY

Lower extremity PAD is a diverse disease with many risk factors and clinical manifestations. This thesis aims to deepen knowledge on the clinical and molecular manifestations of the disease and especially manifestations associated with CLI and poor prognosis. The specific aims of the study were to investigate:

1. The role of purinergic signaling in patients with PAD
2. Do cardiovascular risk factors differ in molecular pathology in patients with PAD
3. The effect of disease severity and distribution in association with overall PAD patient survival
4. The circulating cytokine profile of patients with CLI compared to patients with IC

4 MATERIALS AND METHODS

4.1 Patient populations and healthy controls

The thesis work and original publication were performed using several patient and control populations. Two different PAD patient cohorts were used: the PURE ASO cohort to study inflammatory mechanisms of atherosclerosis, and the Crural Index cohort for the study of atherosclerotic lesion classification system associations with survival. In addition, healthy reference populations were sought for from the FINRISK cohorts of 1997 and 2002, and the same inflammatory pathways were also studied from the subjects with prevalent atherosclerotic cardiovascular disease (CVD) from the FINRISK 2002 cohort. Table 4 summarizes the use of different PAD patient cohorts and healthy controls obtained for comparison.

Table 4. Summary of PAD and CVD patient cohorts used in the original publications and healthy controls obtained for comparison.

Original publication	PAD/CVD cohort	Healthy controls	Study conduct
I	PURE ASO*	Healthy volunteers from university and age matched healthy controls from the FINRISK-97	Comparison of components of purinergic signaling between PAD patients and healthy controls
II	Crural Index**	N/A	Retrospective study of 887 consecutive DSAs and their association with 3 year patient outcome
III	PURE ASO*	N/A	Comparison of circulating cytokine expression between PAD patients with IC vs. CLI
IV	PURE ASO* + symptomatic CVD patients from the FINRISK-02 Study	Age and gender matched subjects from the FINRISK-02 Study free of CVD during 10-year follow-op	Multivariable modelling of circulating cytokine associations with cardiovascular risk factors in patient cohorts and comparison to healthy controls

* 226 consecutive patients that came for an elective non-emergency intervention to the Department of Vascular Surgery at Turku University Hospital, **887 consecutive (elective and emergency) patients which underwent DSA at the Department of Vascular Surgery at Turku University Hospital, DSA = digital substraction angiography, PAD = peripheral artery disease, CVD = cardiovascular disease, IC = intermittent claudication, CLI = critical limb ischemia, The FINRISK Study is large Finnish national epidemiologic study on the prevalence of cardiovascular risk factors and CVD outcome in population substraction of approximately 10 000 inhabitants conducted every 5 years (Borodulin et al. 2015).

4.1.1 The PURE ASO Study cohort and healthy controls from the same area and the FINRISK-97 Study

To study inflammatory mechanisms of PAD the prospective PURE ASO Study cohort was gathered during a one-year enrolment period from February 2012 to March 2013 from all consecutive elective patients coming into the Department of Vascular Surgery of the Turku University Hospital, Finland. The study was approved by the local Ethical Committee of the Hospital District of South-West Finland. Written informed consent was sought from all subjects prior entering the study. Both patients with claudication or CLI were included, but exclusion criteria consisted of severe tissue loss or infection or patients coming in from the emergency department and an acute condition. This exclusion was done in order to prevent acute and inflammatory conditions from interfering with laboratorial analyses.

For the PURE ASO cohort we sought for healthy controls from volunteering university personnel, for whom a duplex Doppler arterial ultrasound was performed to be sure that no underlying PAD existed. Twenty-three such controls were obtained. In addition, 41 young healthy volunteers from a prior study on purinergic signalling were used to establish reportedly normal reference values.

For more, and better age, matched controls we identified 89 subjects from that had participated in the FINRISK-97 cohort from the Hospital District of South-West Finland and were free of chronic heart diseases, PAD, stroke, diabetes mellitus, cancer, chronic obstructive pulmonary disease (COPD), and rheumatic illnesses.

4.1.2 The FINRISK-02 cohort and sub-populations with and without cardiovascular disease

The FINRISK-02 cohort has been described in detail elsewhere (Vartiainen et al. 2010; Borodulin et al. 2015). Local Hospital District Ethical Committees have approved the Study and all participants have given written informed consent before inclusion. In order to match the PURE ASO cohort we sought for subjects with prevalent PAD from a subset 2957 FINRISK-02 subjects aged 51 – 74 years. Only 26 subjects were found. Thus, we sought for all subjects with an atherosclerotic disease, i.e. prevalent CVD at baseline. Prevalent CVD was determined as a hospital diagnosis of PAD, a hospital diagnosis of myocardial infarction (MI) or unstable angina and coronary revascularization, or a hospital diagnosis of ischemic non-hemorrhagic stroke. From the initial sub-set of 2957 subjects of the FINRISK-02 cohort all subjects with prevalent malignancy at baseline or incident malignancy during 10 year follow were excluded. This exclusion was

done because underlying cancer could lead to the misinterpretation of the variety of measured inflammatory markers.

For healthy controls, from the subset of FINRISK-02 subjects without cancer, we identified 332 subjects that had completed 10 years of follow up and according to self reporting and National Registers were free of smoking and had not developed CVD or a hospital diagnosis of the following major cardiovascular risk factors or inflammatory diseases: hypertension, diabetes, dyslipidemia, rheumatoid arthritis, asthma, chronic obstructive pulmonary disease, inflammatory bowel disease, colitis ulcerosa, hepatitis or other significant liver disease, multiple sclerosis, heart failure, and just to be sure of self reporting, dementia. The majority of this healthy sub-population of 332 subjects were female (70%) and the mean age of the group was significantly younger than of the group of 165 FINRISK-02 subjects with prevalent CVD. Thus, 167 of the youngest females of the healthy controls were excluded, which resulted in 165 healthy controls that had an identical gender ratio with the group of subjects with prevalent CVD.

4.1.3 The Crural Index registry and outcome data

The Crural Index registry is a retrospective study of 887 consecutive subjects that had digital subtraction angiography (DSA) of the lower limbs performed because of PAD at the Department of Vascular Surgery of Turku University Hospital (Turku, Finland) from January 1st 2009 to July 30th 2011. All consecutive patients were included regardless of earlier PAD history. The DSA from the clinically worse or more severely affected lower limb was analysed. When both limbs met the criteria for critical limb ischemia, the limb with the lowest toe pressures was considered worse and entered into the study. If a patient had repeated DSAs during the study period, only the data from the first DSA was analysed. The study cohort consisted of both elective and urgent patients.

Deaths within the patient cohort were registered for the first 36 months, which was the cut-off point for follow up. The date and cause of death was provided by the Cause of Death Registry of Statistics Finland. Patient baseline characteristics were collected from the electronic hospital database of Turku University Hospital. Only ICD-10 coded diagnoses were registered. The following risk factors were recorded: coronary artery disease (CAD), carotid artery disease, hypertension, active smoking, diabetes, sleep apnoea, chronic obstructive pulmonary disease (COPD), end-stage renal disease (ESRD), and dyslipidaemia. The Rutherford classification was retrospectively determined from patient files to assess the clinical severity of ischemia.

4.2 Laboratory analyses of inflammatory mediators

4.2.1 Blood sampling

All blood samples were taken after fasting from the cubital vein. Blood sampling of the PURE ASO cohort was conducted by the accredited laboratory of Turku University Hospital, Tykslab (Turku, Finland). The serum sample tube was left to clot and cool to room temperature It was centrifuged at 2000g for 10 minutes after which serum was distracted for analysis. Both serum and plasma were stored at – 70°C until further analyses. Blood sampling of the FINRISK cohorts have been described previously and are stored at – 70°C (Vartiainen et al. 2010). Special analytics of cytokines and components of purinergic signaling were performed in the MediCity Research Laboratory for both PURE ASO and FINRISK cohorts, as well as healthy volunteers.

4.2.2 Multiplex analyses of circulating cytokines

The use of Bio-Plex Pro Human Cytokine 21- and 27-plex panels (Bio-Rad Laboratories) for cytokine analyses have been previously described (Nieminen et al. 2014; J. Jalkanen, Maksimow, S. Jalkanen, et al. 2016) and now summarized in Table 5 below These multiplex analyses were chosen because they have the most comprehensive representation of cytokines known to be involved in atherosclerosis. The multiplex cytokine analyses of the PURE ASO and FINRISK cohorts were done at different time points using different batches of reagents, which to our experience do not provide directly comparable values. Otherwise, all multiplex analyses were performed with the magnetic bead suspension array kit of Bio-Plex Pro Human Cytokine 21- and 27-plex panels according to the manufacturers instructions except that the amount of beads, detection antibodies and streptavidin-phycoerythrin conjugate were used at half of their recommended concentration.

Table 5. Summary of the analysed cytokines from human serum using the Bio-Rad Multiplex panels

Abbreviation	Full name
IL-1b	Interleukin-1 beta
IL-1ra	Interleukin-1 receptor antagonist
IL-2	Interleukin-2
IL-2Rα	Interleukin-2 receptor alpha
IL-3	Interleukin-3
IL-4	Interleukin-4
IL-5	Interleukin-5
IL-6	Interleukin-6
IL-7	Interleukin-7
IL-8	Interleukin-8
IL-9	Interleukin-9
IL-10	Interleukin-10
IL-12	Interleukin-12
IL-13	Interleukin-13
IL-15	Interleukin-15
IL-16	Interleukin-16
IL-17	Interleukin-17
IL-18	Interleukin-18
Eotaxin	Eotaxin
TNF-a	Tumor necrosis factor alpha
TNF-b	Tumor necrosis factor beta
IFN-g	Interferon gamma
IFN-a2	Interferon alpha 2
IP-10	Interferon gamma induced protein-10
CTACK	T cell attracting chemokine
MCP-1	Monocyte chemotactic protein-1
MCP-3	Monocyte chemotactic protein-3
LIF	Leukemia inhibitory factor
MIP-1a	Macrophage inflammatory protein-1 alpha
MIP-1b	Macrophage inflammatory protein-1 beta
MIF	Macrophage migration inhibitory factor
MIG	Monocine induced by interferon gamma
M-CSF	Macrophage colony stimulating factor
GM-CSF	Granulocyte-macrophage colony stimulating factor
G-CSF	Granulocyte colony stimulating factor
PDGF	Platelet derived growth factor
bFGF	Basic fibroblast growth factor
HGF	Hepatocyte growth factor
VEGF	Vascular endothelial growth factor
SCF	Stem cell factor
SCGF-b	Stem cell growth factor beta
SDF-1a	Stromal cell derived factor-1 alpha
b-NGF	Beta nerve growth factor
GROa	Growth regulated oncogene alpha
TRAIL	Tumor necrosis factor related apoptosis inducing ligand
RANTES	Regulated on activation normal T cell expressed and secreted

4.2.3 Components of purinergic signaling

Analyses of purinergic signaling biomarkers were done at different time points for the PURE ASO and FINRISK cohorts, but these analyses are very repeatable and directly comparable. Plasma ATP and ADP were determined by enzyme-coupled assay using ATPlite assay kit with a long-lived luminescent signal (Perkin Elmer, Groningen, The Netherlands) (Helenius et al. 2012). Soluble CD39 and CD73 activities were assayed radiochemically, as described earlier (Yegutkin et al. 2007). Enzymatic activities were expressed as nanomoles of ^{3}H-substrate metabolized per hour by 1 ml serum (Yegutkin 2015).

4.3 The formation of the Crural Index from angiographic images

Similar to the TASC classification of the aorto-iliac and femoro-popliteal regions we developed a four-grade classification system for tibial arteries and named it the Crural Index. In the same way as in the TASC classification lesions were scored by length as follows: No detectable occlusive disease: 0; occlusion less than 5 cm: 1; occlusion between 5 – 10 cm: 2; occlusion between 10 – 15 cm: 3; occlusion more than 15 cm: 4. All three tibial arteries were first analysed separately. The Crural Index was created by a sum of the three values obtained from each individual tibial artery. If the sum was 0 the Index was 0, if the sum was between 1 – 3 the Index was I, if the sum was 4 – 6 the Index was II, if the sum was 7 – 9 the Index was III, and if the sum was 10 – 12 the Index was IV.

4.4 Statistical analyses

Statistical analyses were done in collaboration with a professional biostatician from 4Pharma Ltd. (Turku, Finland). Basic descriptive analyses, correlation analyses, and differences between groups were done by the original researchers using IBM SPSS Statistics for Mac (version 22). Multivariable modeling was done by 4Pharma Ltd. using SAS Software for Windows (version 9.3).

4.4.1 Descriptive statistics

The prevalence of cardiovascular risk factors, medications, and other categorical baseline characteristics are presented as percentages, and differences between groups were examined using the Chi-square test or Fischer's exact test. Baseline characteristics of numeric values (age, blood pressure, cholesterol level etc.) and measured inflammatory biomarkers were analyzed for normality using the

Shapiro-Wilk test and log-transformed if necessary. For normally distributed data differences between groups were analyzed using the Student's T-test and the Mann-Whitney U-test for data that remained skewed after log-transformation. Pearson correlations were used to study the associations of log-transformed biomarkers with continuous baseline variables. Linear regression models were used to study the possible trends in the means of log-transformed marker values across PAD classes.

4.4.2 Multivariable modeling

For multivariable modeling association between baseline characteristics with each individual biomarker was explored using a linear regression model for log-transformed marker values. At first, all baseline characteristics encompassing cardiovascular risk factors and used medication were taken into account for the FINRISK cohort. For the prospective PURE ASO cohort also the presence of critical ischemia (CLI) vs. intermittent claudication (IC), and the localization of significant atherosclerotic lesions were taken into account. Variables to be included in the model were selected using nonparametric methods. Variables with a test P value <0.15 were entered into the model. Dichotomous variables were tested using the Wilcoxon rank-sum test, and variables in more than two categories were tested using the Kruskal–Wallis test. The full model for each marker was constructed by fitting all the variables selected using the *P* value criterion. In addition to the full model, a reduced model was constructed by removing variables that showed little or no association with the explored marker in the full model. This model was defined by removing the least significant variable from the model one by one until all remaining variables showed a *P* value <0.15. Thus, in the end the most decisive variables affecting each specific marker were left in the model. Model fit was inspected visually using studentized residuals showing a reasonable fit for all models.

4.4.3 Survival analyses and COX proportional hazard models

Survival analyses were performed by the original researchers using IBM SPSS Statistics for Mac (version 22). Kaplan-Meier survival curves representing cumulative survival were constructed for baseline characteristics using the entire study cohort of the Crural Index registry and statistical significance was tested using the log-rank test. A Cox proportional hazard model was performed to assess the final predictive value of factors affecting survival. Factors with *P* <0.2 in univariate analyses were selected as independent variables to the Cox proportional hazard model.

5 RESULTS

5.1 Baseline characteristics of study populations

5.1.1 The PURE ASO patient population

The PURE ASO patient cohort consisted of 226 participants of which 128 (56.6%) were male. All were of Caucasian origin. Overall mean age was 69.8 years (SD ± 11.44, 46–93 years) with a small tendency for males to be younger (males, 67.9 years [SD ± 11.3], and females, 72.3 years [SD ± 11.2]). Smoking, diabetes, and dyslipidemia had a tendency to be more prevalent in young age groups, while hypertension, renal failure, and rheumatic diseases tended to be more abundant in older age groups. In a similar fashion, smoking and dyslipidemia had a tendency to be more prevalent in proximal PAD, while renal failure, rheumatic diseases and diabetes tended to be more prevalent in distal PAD (Table 6).

Table 6. Characteristics of the PURE ASO cohort according to age, disease localization, and severity (n = 226). Modified from (J. Jalkanen et al. 2015).

	<60 years	**60–69 years**	**70–79 years**	**>80 years**
Age group	10.6%	32.7%	30.2%	26.5%
Male/female	63%/37%	67%/33%	59%/41%	35%/65%
History of smoking	75%	78%	64%	23%
Hypertension	50%	74%	78%	86%
Dyslipidemia	38%	40%	21%	33%
Diabetes	42%	40%	38%	22%
CKD	13%	14%	25%	37%
Rheumatic disease	8%	10%	24%	18%
	Aorta-iliac	**Femoro-popliteal**	**Crural**	**Pedal**
Localization	26.1%	43.8%	23.5%	6.2%
Male/female	56%/44%	58%/42%	(53%/47%)	50%/50%
Mean age (years)	66 (SD, 9.3)	71 (SD, 9.3)	78 (SD, 11.8)	74 (SD, 12.6)
History of smoking	86%	69%	26%	7%
Hypertension	63%	81%	83%	64%
Dyslipidemia	44%	34%	25%	7%
Diabetes	33%	43%	49%	50%
CKD	9%	16%	42%	64%
Rheumatic disease	7%	13%	25%	43%

CKD = chronic kidney disease

5.1.2 The Crural Index patient population

The Crural Index study cohort consisted of 887 consecutive retrospective patients with a male-to-female ratio of 57 % and 43 %, respectively. Mean age of the patient cohort was 72.4 years ranging from 40 to 98 years. There was no significant difference in the mean age between the sexes. Baseline characteristics are summarized according to disease localization in Table 7.

Table 7. Baseline characteristics of the Crural Index cohort according to disease localization. Modified from (J. M. Jalkanen, Wickström, Venermo & Hakovirta 2016b).

Most severe disease burden	Aorto-iliac	Femoro-popliteal	Crural
N	141	416	325
Male/female	65/35%	58/42%	51/49%
Age (years)	66.7 (SD, 10.1)	71.8 (SD, 10.1)	76.0 (SD, 10.7)
Hypertension	67 %	69 %	71 %
Diabetes	23 %	41 %	53 %
History of smoking	52 %	32 %	9 %
ESRD	6 %	8 %	15 %

ESRD = end-stage renal disease

5.2 The association of circulating cytokines, chemokines and growth factors with major cardiovascular risk factors in patients with PAD and CVD

5.2.1 Correlation of numeric baseline variables with cytokine values

Within the PURE ASO cohort age in years had a very significant positive correlation with Th1 and IFNγ-induced cytokines (Table 1): CTACK, MIG, IP-10, IL-2Rα and IL-16, but a negative correlation with PDGF and TRAIL. Measures of lipids (total cholesterol, LDL and triglycerides) had totally opposite correlations, e.g. positive correlation with PDGF and TRAIL, and negative correlation with IFNγ, CTACK, IL-2Rα and IL-6. Creatinine values, then again, had positive correlations with both Th1 cytokines and growth factors.

The PURE ASO cohort and FINRISK-02 cohort with prevalent CVD had rather similar correlations. In other words a strong correlation of age with Th1 and IFNγ-induced cytokines, a positive correlation of lipids with growth factors and a negative correlation with CTACK, and positive correlation of creatinine with both Th1 cytokines and growth factors. These correlations clearly differed from the correlations seen among the healthy controls. For example, subjects with CVD (both PURE ASO and FINRISK-02) had several significant positive corre-

lations with age and a number of cytokines, while healthy controls had mostly negative correlations with cytokines and age. The same applied with systolic blood pressure, total cholesterol and triglycerides.

5.2.2 Multivariable modeling of cytokine associations with cardiovascular risk factors

Following, the results of multivariable modeling of cytokine associations with major cardiovascular risk factors are presented for the PURE ASO cohort, i.e. patients with PAD, along with the results of the same set of analyses done with subjects with prevalent CVD of the FINRISK-02 cohort.

According to multivariable modeling elevated levels of IP-10, CTACK, MIG and SCF had a very strong independent association with increasing age (P <0.001 for all), while IL-18 had a steadily decreasing association with increasing age (P = 0.04). All of these chemokines are induced by IFN-γ, but no independent association of age with IFN-γ was found in the PURE ASO cohort but was seen in the among the FINRISK-02 subjects with prevalent CVD (Figure 9).

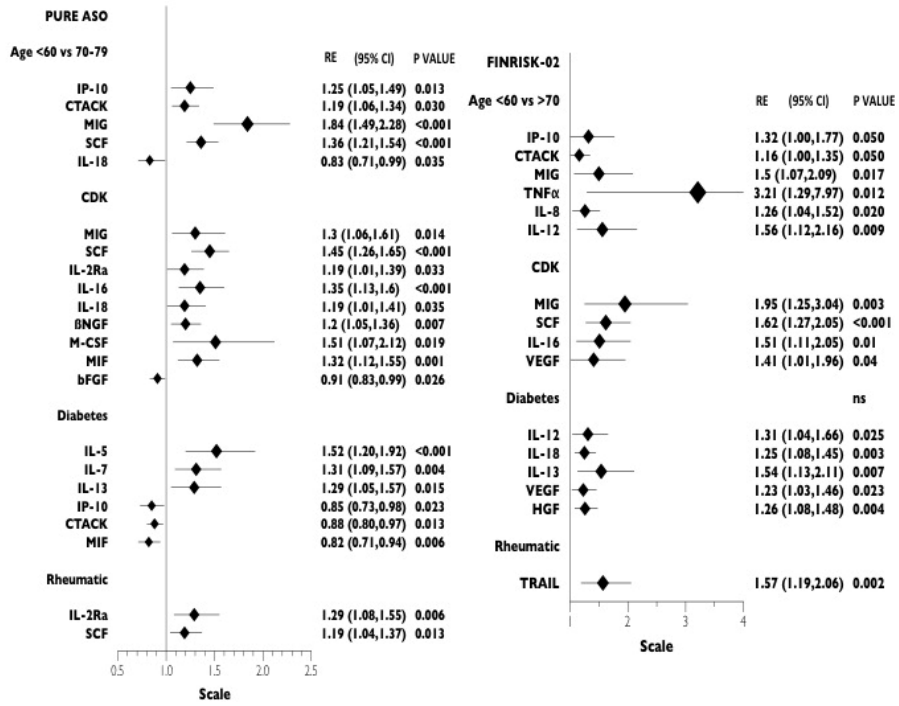

Figure 9. Forest plot summarizing the results of multivariable modelling for age, CKD, diabetes and rheumatic illnesses. RE = ratio estimates, e.g. the age group 70 – 79 years was independently associated with a 25% increase of IP-10 (95% confidence interval, 5 – 49%) when compared to the age group < 60 years olds in the PURE ASO cohort, and a 17% decrease in IL-18 (95% CI, 1 – 29% decrease).

Due to a low number of subjects with type I diabetes in the FINRISK-02 cohort, diabetic subjects were pooled into one category (diabetes vs. no diabetes). According to multivariable analyses of the PURE ASO cohort, opposite to aging, diabetes was associated with lower levels of MIF, CTACK and IP-10, and increased levels of Th2 related mechanisms: IL-5, IL-7, IL-13.

CKD, which is known to have devastating effects on the vasculature, was associated with elevated levels of a number of cytokines: IL-2Rα, IL-16, IL-18, MIF, MIG, M-CSF, SCF and □-NGF, but lower levels of bFGF. Similar to CKD, rheumatic illnesses were associated with elevated of IL-2Rα and SCF (Figure 9).

Active smoking was independently associated with elevated levels of IL-17 and MIF when compared to non-smokers. A predominant feature of smoking was an association with elevated levels of several growth factors: GM-CSF, PDGF, bFGF and HGF. Hypertension had somewhat similar associations, as did smoking. The presence of hypertension as a diagnosis in medical charts was independently associated with elevated levels of IL-17, MIP-1α and bFGF. Rather similar to smoking and hypertension, dyslipidemia as a diagnosis in medical charts was linked with elevated levels MIP-1β, PDGF and TRAIL (Figure 10).

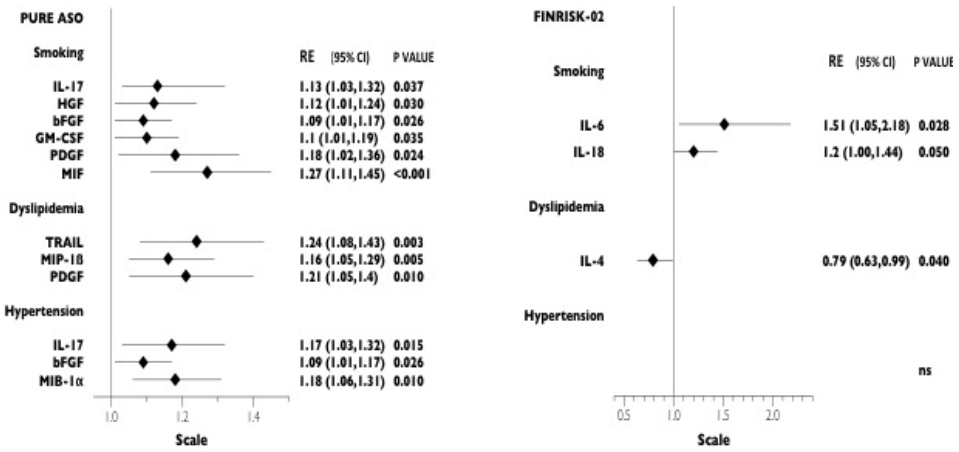

Figure 10. Forest plot summarizing the results of multivariable modelling for smoking, dyslipidemia and hypertension. RE = ratio estimates, e.g. smoking is independently associated with a 13% increase of IL-17 (95% confidence interval, 3 – 32%) among subjects of the PURE ASO cohort, and dyslipidemia is independently associated with a 21% decrease in IL-4 (95% CI, 1 – 37% decrease).

5.3 Purinergic signaling in patients with PAD – The PURE ASO Study

In patients with PAD purinergic signaling was highly disturbed. ATP, ADP and CD73 levels were significantly higher in the patient cohort when compared to controls ($P < 0.001$). This was also evident in the comparison of each age group specifically. In general CD39 was elevated in patients with PAD when compared to healthy controls, because young healthy controls tended to have very high CD39 activity (Figure 11)

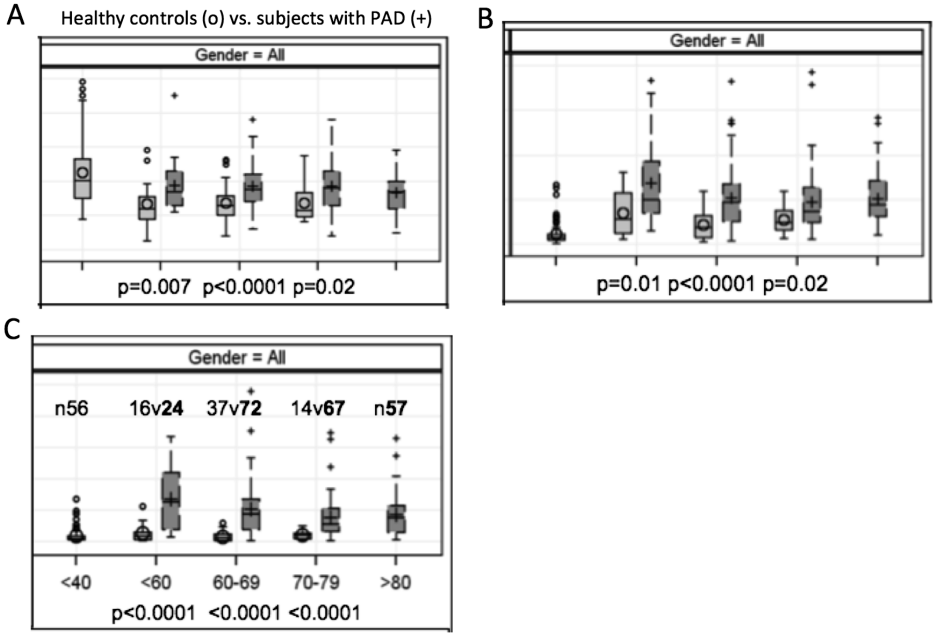

Figure 11. CD39 (A), ATP (B) and ADP (C) values of patients with PAD and healthy controls according to age group. *P* values represent age group specific comparison of healthy controls against PAD patients according to the Mann-Whitney U-test. High values of CD39, and low values of ATP and ADP are seen in the youngest healthy controls. According to linear regression CD39 significantly declined along with age ($P < 0.001$) among healthy controls (J. Jalkanen et al. 2015). Reproduced with the permission of American Heart Association.

5.3.1 Smoking increases circulating ATP and ADP, but also CD39

Especially, ATP and ADP levels were significantly higher in the patient cohort than among healthy controls. The observed extremely high values came from samples of young patients with proximal PAD. Clinically this relates to smoking. However, a solid direct association to smoking could not be detected in multivar-

iable analyses, but in the inspection of patients with only one major cardiovascular risk factor, smoking associated with the highest values of ADP (Table 8).

Table 8. ADP values in PAD patients with only one distinct risk factor

Risk factor	N	Mean	SE	*P*-value*
Smoking	17	6686 nmol/L	1220 nmol/L	NA
Hypertension	12	5190 nmol/L	1249 nmol/L	0.076
Dyslipidemia	9	4869 nmol/L	1553 nmol/L	0.050
Diabetes	8	4144 nmol/L	971 nmol/L	0.007

*P-value = comparison between patient subgroup vs. patient subgroups with only smoking as a risk factor with the Mann-Whitney U-test. Modified from (J. Jalkanen et al. 2015).

5.3.2 Low CD39 is associated with disease severity

The level of smoking (never smoked, quit smoking, active smoker) also associated with increased CD39, which cleanses ATP and ADP. Active smokers had significantly higher CD39 activity than those who had smoked but quit (P = 0.041) or those who had never smoked (P = 0.001), using log-transformation and the Student's t-test. Lower CD39 on the other hand significantly associated with the degree of disease progression according to the Rutherford classification according to linear regression (Figure 12).

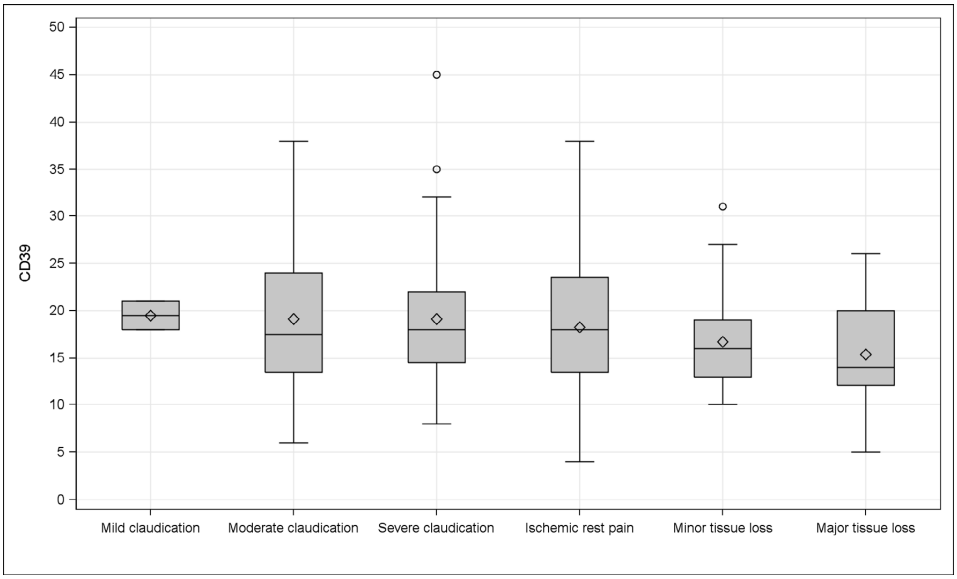

Figure 12. Lower CD39 is associated with disease progression according to the Rutherford classification. Linear regression R = -1.51, P = 0.025.

5.4 Survival of patients with PAD

5.4.1 The effect of clinical status and presence of major cardiovascular risk factors

During the 36-month follow-up of 887 consecutive patients of the Crural Index 295 (33 %) deaths were registered. Over 70% of these were due to cardiovascular reasons. The corresponding death rates for patients with IC or CLI at baseline were 23% vs. 43%, respectively at 36 months ($P < 0.001$, Fischer exact test between groups). According to our most recent unpublished data of these 887 patients at 80 months 44% of patients with IC at baseline are dead and 80% of patients with CLI are dead ($P < 0.001$, Fischer exact test between groups).

Patients with CAD had significantly poorer survival at 36 months (mean 27.8 months; SE, 0.69) than patients without CAD (mean 30.2 months; SE, 0.54, $P < 0.001$). Hypertension was also associated with significantly shorter survival (mean 28.5 months; SE, 0.53) when compared to patients without a hypertension diagnosis (mean 30.8 months; SE, 0.71), $P < 0.05$. As shown in several other survival studies diabetes and end-stage renal disease (ESDR) were associated with significantly shorter survival. The mean survival of PAD patients with diabetes was 27.6 months (SE, 0.70) compared to 30.4 months (SE, 0.53) with patients without diabetes, $P < 0.001$. For PAD patients with ESDR mean survival at 36 months was 25.2 months (SE, 1.5) and 29.6 months for patients without ESDR, $P < 0.001$.

5.4.2 TASC classifications and survival

The previously presented four-grade classification systems of aorto-iliac, femoro-popliteal and crural lesions were utilised for survival analyses. For aorto-iliac disease the survival analyses according to the TASC classification gave a statistically significant, but irrational result (Log-rank test, $P = 0.01$). Subjects with no aorto-iliac lesions or grade III (TASC C) lesions had poorer survival than TASC A, B or D lesions, which had similar survival (Figure 13 A). No significant difference in survival was detected between different grades femoro-popliteal disease classified by the TASC II classification (Log-rank test, $P = 0.34$) (Figure 13 B).

Crural Index grade IV had a very poor survival (mean estimated survival 23.2 months, SE 1.1) and significantly differed from other group wise comparison against other Crural Indexes *(*Log-rank test, $P < 0.001$). The survival curves of Crural Index grades 0-IV are illustrated in Figure 13 C.

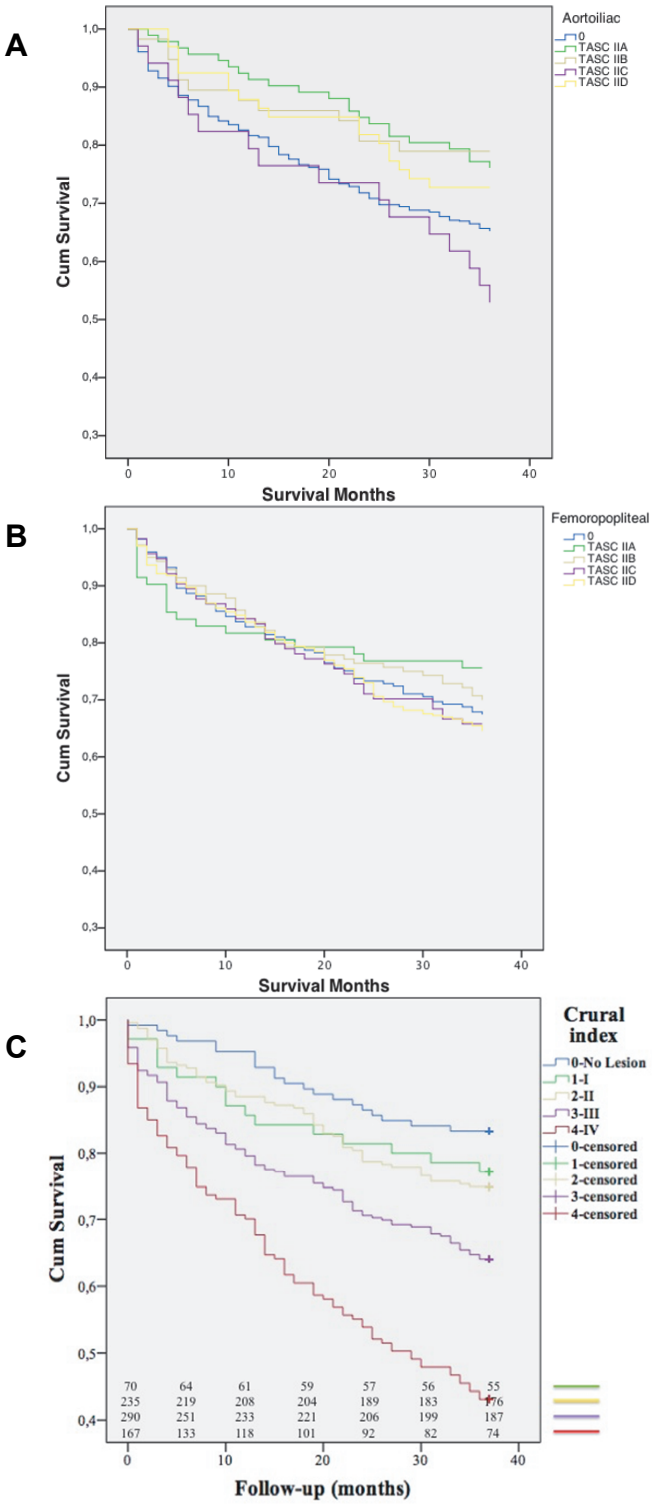

Figure 13. Kaplan-Meier analyses of cumulative survival up to 36-month follow up classified by A) the aorto-iliac TASC II classification (Log-rank test, $P = 0.01$), **B)** the femoro-popliteal TASC II classification (Log-rank test, P = 0.34), **C)** the Crural Index (Log-rank test, $P < 0.001$). Numbers at risk marked at defined time-point at the bottom line. Modified from (J. M. Jalkanen, Wickström, Venermo & Hakovirta 2016b) and (J. M. Jalkanen, Wickström, Venermo & Hakovirta 2016a).

5.4.3 The Crural Index and survival

A Cox proportional hazard model was run to assess the predictive value of cardiovascular risk factors and the Crural Index on survival at 36 months. Diabetes and end-stage renal disease associated with a significantly higher risk of death. However, the strongest predictor of death was the Crural Index IV (HR 2.20; 95% CI, 1.3 – 3.7; P = 0.003) (Table 9).

Table 9. Cox proportional hazard analyses assessing the predictive value of risk factors and severe atherosclerosis of crural vessels affecting survival during 36-months follow-up. Modified from (J. M. Jalkanen, Wickström, Venermo & Hakovirta 2016b).

	Hazard Ratio	95,0% CI	P Value
Crural Index 0	Reference	value	N.A.
Crural Index I	1.04	0.5 – 2.1	0.905
Crural Index II	1.05	0.63 – 1.8	0.846
Crural Index III	1.36	0.82 – 2.2	0.237
Crural Index IV	2.20	1.3 – 3.7	0.003
ABI	1.62	1.3 – 2.1	<0.001
Age	1.04	1.0 – 1.1	<0.001
CBVD	0.521	0.34 – 0.79	0.002
DM	1.42	1.1 – 1.8	0.015
CKD	2.09	1.4 – 3.0	<0.001
Active smoking	0.638	0.43 – 0.95	0.028

5.5 The cytokine profile of PAD patients with IC compared to patients with CLI

Crural atherosclerosis and CLI were previously shown to associate with very poor patient survival in PAD. Thus, the differences of patients with IC and CLI were examined more closely using the PURE ASO cohort with precise patient condition documentation. Patients with CLI were slightly older (74y vs. 68y; $P < 0.01$) than patients with IC. Patients with CLI had more CKD, rheumatic illnesses, and diabetes, which are all associated with crural atherosclerosis. Patients with IC had more dyslipidemia and a history of smoking.

Significant baseline differences existed between IC and CLI groups, thus the previously presented multivariable model taking into account the presence of all significant major cardiovascular risk factors was applied. According to the multivariable mode CLI was independently associated with a very large number of elevated cytokines (Figure 14).

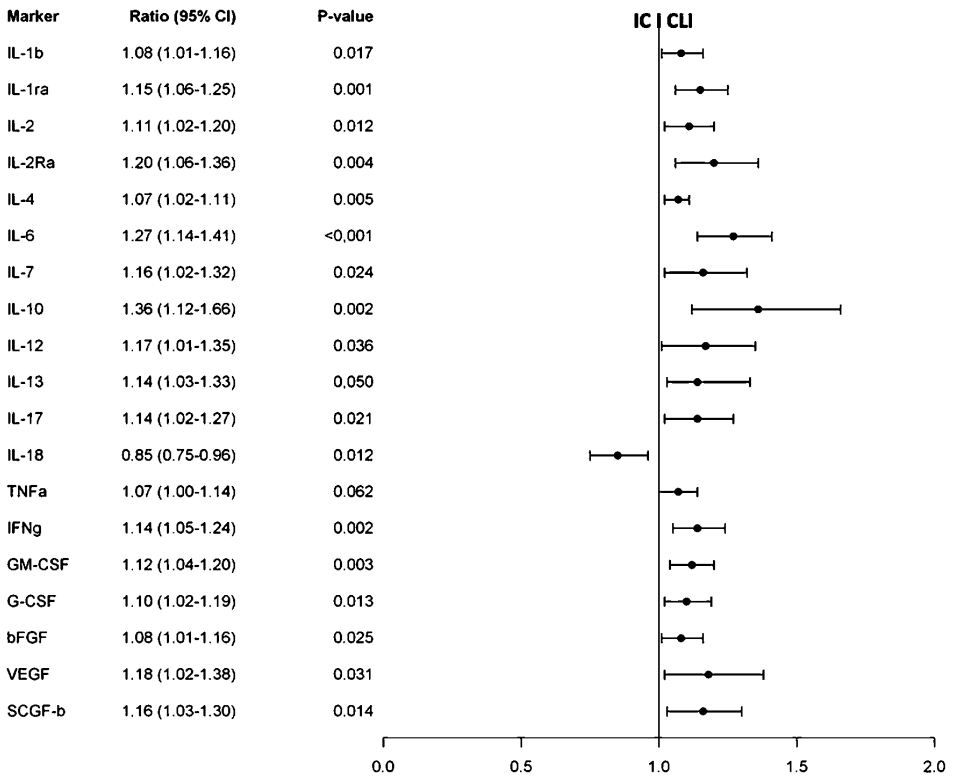

Figure 14. Cytokines associated with CLI when compared to IC. Forest plot illustrating the findings of multivariate modelling. The ratio value indicates that there is an independent 8% (95% CI, 1 – 16%) increase in the level of IL-1β associated with the presence of critical ischemia, an independent 15% (95% CI, 6 – 25%) increase in the level of IL-1ra in the presence of CLI, an independent 11% (95% CI, 2 – 20%) increase the level of IL-2 associated with the presence of critical ischemia etc. (J. Jalkanen, Maksimow, Hollmén, et al. 2016). Open access, personal copyright.

6 DISCUSSION

6.1 Atherosclerosis and PAD as an inflammatory disease

Atherosclerosis is an inflammatory disease, for which there is a strong body of pre-clinical evidence highlighting the involvement of different cytokines and chemokines in this process (Ait-Oufella et al. 2011; Zernecke & Weber 2014). Of the three major atherosclerotic diseases (CAD, CBVD and PAD) PAD remains the least studied (Cooke & Chen 2015) and least known (Hirsch et al. 2007) disease. The current literature on role of different cytokines in association with disease specific mechanism of PAD is non-existent. The knowledge of current biomarkers for PAD were mainly constricted to ABI, C-reactive protein, IL-6, certain growth factors (VEGF, HGF and FGF), lipids, adhesion molecules (VCAM and ICAM), and thrombosis related measures such as homocysteine, fibrinogen, and von Willebrand factor (Cooke & A. M. Wilson 2010). Prior to the current studies the role of purinergic signalling in a wider population of patients with atherosclerosis was un-charted despite extensive pre-clinical knowledge (Burnstock & Ralevic 2014).

6.1.1 Purinergic signaling and the importance of smoking cessation, statins and ADP inhibiting anti-platelet therapy

The results of the PURE ASO Study show that purinergic signaling is significantly altered in patients with PAD. Especially, smoking increases circulating ADP levels of PAD patients but not the ADP levels of presumably healthy individuals. This may explain why smoking cardiovascular patients benefit more from ADP antagonism when compared to non-smoking cardiovascular patients (Gagne et al. 2013). Individual CD39 activity may also be protective of disease progression and a future target of drug development. For the time being the results of the PURE ASO Study imply that PAD patients unable to quit smoking will most likely benefit from permanent ADP/$P2Y_{12}$ receptor anti-platelet therapy, especially if they have a vascular bypass. Smoking, independently, is the leading cause of vascular by pass thrombosis (Singh et al. 2008). Likewise smoking PAD patients undergoing any form of revascularization or treated conservatively will most likely benefit from maximal statin therapy as it is accustomed in the treatment of all patients suffering from CBVD and CAD. In this respect the use of statins and anti-platelet therapy has traditionally been poor in patients with PAD alone, not accompanied by CBVD or CAD (Subherwal et al. 2012). This needs to change. Recent large-scale meta-analyses now show that the use of statins and dual anti-platelet therapy have a significant risk reduction in prevent-

ing major amputation after revascularization (Katsanos et al. 2015). According to these recent findings purinergic signaling seems to be the key mechanism behind this phenomenon. The benefit of statins and dual antiplatelet therapy should be even more dramatic in a meta-analysis of smoking vs. non-smoking patients with PAD. This remains an interesting aspect of further investigations.

6.1.2 Major cardiovascular risk factors are associate with different immunological pathology

According to multivariable modeling of cardiovascular risk factors and circulating cytokines, aging is independently associated with MIG, CTACK and IP-10. All these paracrine factors are up-regulated by IFN-γ, a key driver of Th1 immune responses (Zernecke & Weber 2014). Chronic kidney disease also associated with Th1 mediated pathology and several growth factors. Smoking was associated with acute phase inflammation and growth factors known to induce myeloid progenitor cell proliferation, but not with IFN-γ induction. Similar to smoking, dyslipidemia associated with myeloproliferative factors. Diabetes had a negative association with IFN-γ induced mechanisms, but otherwise a positive association with Th1 and especially Th2 mediated inflammation.

The present findings are in line with the heterogenic appearance of patients with PAD in relation to specific cardiovascular risk factors (N. Diehm et al. 2006; Aboyans et al. 2007). This phenomenon and its molecular background is summarized in Figure 15. Small vessel or distal lower limb atherosclerosis is primarily associated with Th1 cytokines and especially IFN-γ, while risk factors associated with proximal or large vessel atherosclerosis are primarily associated with myeloid progenitor cell proliferation. No prior study has tackled this issue as such, but these findings are reinforced by similar more narrow findings primarily done in animals. For example, dyslipidaemia has been associated with increased expression of G-CSF and myeloid progenitor cell proliferation in a rabbits (Hu et al. 2013). Similar to dyslipidaemia, smoking shows affinity to factors inducing myeloid progenitor cell proliferation (Messner & Bernhard 2014; Butcher et al. 2012). In man, using somewhat similar methodology IP-10, and thus IFN-γ, have been associated with increasing age in subjects with CVD (Herder et al. 2006). The findings associated with diabetes were surprising, because the onset of diabetic vasculopathy has been thought to be switch from atheroprotective Th2 surrounding present in adipose tissue to a Th1 milieu. This concept has been questioned (Kraakman et al. 2014; Casella et al. 2015), and prior work has shown that a resident Th2 surrounding can the driving source of inflammation (Zeyda et al. 2007; Jenkins et al. 2011). According to the current findings Th2 related cytokines prevailed in the circulation of patients with PAD.

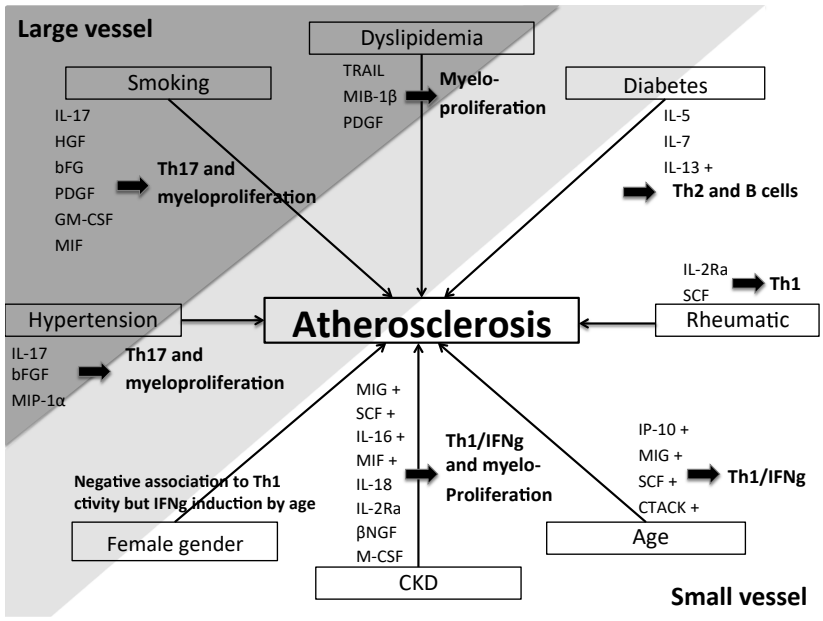

Figure 15. The association of major cardiovascular risk factors with circulating cytokines and their signature pathway leading to atherosclerosis. These are cytokine associations from the PURE ASO Study. + = also present in the FINRISK-02 Study.

The importance of each individual cytokine can be questioned. These findings need further validation, and the aim is not make definitive assumptions of the role of each individual cytokine. On the contrary, these finding wish to indicate the presence of distinct immunological pathology, i.e. myeloproliferation, Th1, Th2, or Th17 mediated inflammation in association with specific cardiovascular risk factors. Somewhat similar findings were also seen among subjects with prevalent CVD in the FINRISK-02 cohort. Although cytokine associations were not identical, they were substantiating and did not present conflicting cytokine profiles concerning immunological pathways.

6.2 Outcome in PAD and the malignant nature of CLI

The present findings very well highlight the poor prognosis of patients with PAD, and especially CLI. According to the recent follow up data of the Crural Index registry after 5 years only 20% patients diagnosed with CLI are alive. These devastating results are in line with data from other cohorts (Golomb et al. 2006; Reinecke et al. 2015).

The burden of death associated with PAD has constantly risen. For octogenarians the death rate per year has risen to a remarkable 28.7/100,000 (Sampson et al. 2014). In comparison, only aggressive types of cancers, such as pancreatic cancer and lung cancer, have as poor short/mid-term survival rates as does CLI. Opposite to the public awareness of for example lung cancer, CLI has remained in the shadows, although in light of prevalence and mortality it is a major cause of death, and has features of a malignant disease. In comparison to stroke or acute myocardial infarction and sudden death, lower limb atherosclerosis has unfortunately remained the most unappreciated of atherosclerotic diseases. However, in terms of disability and wearing mortality it presents both inhuman individual suffering and significant socio-economical cost (Farber & Eberhardt 2016).

From prior studies and the current findings it is clear that patients with CLI run an entirely different course than do patients with IC. This means that the conditions must in some way significantly differ from each other. Compared to IC, CLI is a systemic inflammatory condition associated with a variety of elevated cytokines. This difference is seen even in subtle CLI patients captured by elective non-emergency PURE ASO patients. Similar findings have also been seen in other cohorts (Findley et al. 2008; Teraa et al. 2013).

6.3 Limitations and strengths

The present studies are a compilation of a) the clinical insight of practicing vascular surgeons on the heterogeneity of patients with PAD, b) world class laboratory work on novel molecular analytics, and c) professional statistical multivariable modelling. The strength of these studies are in the combination these three groups of professionals. The validity of the PURE ASO cohort is that it presents the same traits as do very large cohorts, i.e. the association of diabetes and CKD to CLI (Wyss et al. 2015; J. Jalkanen, Maksimow, Hollmén, et al. 2016), and the association of different cardiovascular risk factors to different disease localization (N. Diehm et al. 2006; Aboyans et al. 2006).

These findings still should be validated in large prospective cohorts, and most importantly the same molecular mechanism leading to disease development and poorer prognosis should be studied at the vascular wall. The major limitation of the large number of performed tests and analyses is that these are reflections of vasculature conditions measured from circulation and definitive conclusions can not be made about the corresponding conditions at the vascular wall.

6.4 The Crural Index, novel biomarkers, and future studies

The TASC II document and classifications of PAD according to lesion morphology are thus far some of the best tools to work with when communicating research findings and interventional outcomes. Unfortunately, the TASC classifications had no predictive value of overall patient survival according to the current findings. However, the newly developed Crural Index was the most predictive clinical measure seen to date, surpassing both ESRD and diabetes, which are known for their bad reputation in relation to patient survival. At the time the Crural Index was developed the update for crural arteries of the TASC classification was not yet published. It too should be tested for predictive value. Simultaneously with the development of the Crural Index came a new classification of disease severity, the WIFi score, which stands for Wound, Ischemia and Foot infection (Mills et al. 2014). Predictive data on its utility is still preliminary, but in the future it may prove useful. Thus, the knowledge and analytical tools are expanding in the interpretation of the manifestations of PAD.

Building on the present findings of this thesis work, there too remains a lot of work ahead. First of all it would be essential to further understand the role of the studied molecular mechanisms at the actual arterial wall at different stages of plaque development. This will require new sampling. From the current cohorts the predictive value of the studied molecules on patient outcome remains a future study. In addition, the predictive role of the studied mechanism prior to a prevalent disease is a very interesting aspect. An important future study would be to investigate the inflammatory condition related to CLI after successful revascularization, and to see does it calm down or not, and if not, can it be attenuated with a novel anti-inflammatory medical therapy.

Most importantly, if different molecular mechanisms prevail in atherosclerotic diseases depending on the affected part of vasculature and the risk factor leading to the disease. There is a tremendous amount of work is ahead to develop individual therapy, which is dictated by the prevailing molecular background.

7 CONCLUSIONS

The findings of the presented studies lead to the following conclusions:

1. Purinergic signaling is significantly distorted in patients with PAD, especially in younger patients and in the form of elevated ATP and ADP signaling stress and pro-thrombotic conditions.

2. Different cardiovascular risk factors are associated with different immunological pathways in patients with PAD.

3. CLI and the degree of crural atherosclerosis is associated with very poor patient survival.

4. CLI is a systemic inflammatory condition, which needs attention. It cannot be considered as just a symptom of the leg.

ACKNOWLEDGEMENTS

This study was conducted at the Department of Vascular Surgery of Turku University Hospital and MediCity Research Laboratory, Department of Microbiology and Immunology, of Turku University during 2011 - 2017.

In 2010, I had a dream to understand peripheral artery disease better. Opposite to coronary artery disease, the peripheral disease form of atherosclerosis was not a mainstream interest and my study plans constantly fell short of funding. In addition, I was to study a molecular mechanism that was un-heard of to many. Two exceptional surgeons who shared my passion for molecules saved this thesis, because it was about to end before it even started. My deepest gratitude for the birth of this thesis goes to the Professor of Surgery at Turku University Matti Laato, who granted the initial funds to gather the study samples of PAD patients seen at the Department of Vascular Surgery, and Docent Chief of Vascular Surgery Harri Hakovirta, my Supervisor, a scientific surgeon like no other. Without the practical support and visionary scientific intellect of my Supervisor this thesis would not have been possible simultaneously with full time clinical practice. I am privileged to have had spend the last seven years under the supervision of Docent Hakovirta, my mentor in both surgery and science.

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The meaning of sisterhood I have learned from Katriina and Maija, my blood sisters. My non-family brothers have always envied our sisterhood, which runs deep. Thanks to you there has always been a more sensitive, slightly feminine, meaning to my life, which has shaped me into better man. Your meaning in my life goes beyond words.

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The path of my life has led to you Anna. You and our children Heikki, Enna, Aaro and Esko are my life. Everything else is nothing. Thank you.

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