Biological relevance and prognostic significance of radial artery intima-media thickness

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Cover illustration: Charlotte Eklund

You are never too old to set another goal or to dream a new dream.

C. S. Lewis

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ABSTRACT

Cardiovascular disease (CVD) is the major cause of death both in Sweden and globally. Atherosclerotic vascular changes are considered the major underlying pathology leading to clinical manifestations of CVD, e.g. myocardial infarction and stroke. Hyperlipidemia and chronic subclinical inflammation have been identified as major pathophysiological mechanisms driving atherosclerosis, which is a life-long vascular disease. Inflammatory diseases such as rheumatoid arthritis (RA) are known to be associated with increased risk of CVD. To be able to detect and follow atherosclerosis from its subclinical phase to its clinical stage, we are in great need of novel high-sensitive imaging tools to characterize functional and morphological vascular changes. Our group has successfully used very high frequency ultrasound to follow atherosclerosis in genetically modified mice models. In the current thesis, we adopted this technique to patients and evaluated biological relevance and prognostic significance of high-frequency ultrasound-measured radial artery intima-media thickness (rIMT) in patients with suspected myocardial ischemia. Further, rIMT and coronary flow reserve (CFR) in relationship to systemic inflammation were explored in a group of recent diagnosed RA patients.

Clinical correlates and prognostic values of rIMT were evaluated in 416 patients with suspected myocardial ischemia undergoing myocardial perfusion imaging. Relationship between rIMT, CFR and systemic inflammatory status was explored in patients with recent-onset RA, immediately following diagnosis and four months after standard anti-rheumatic treatment.

Radial artery IMT correlated with conventional cardiovascular (CV) risk factors. Presence of myocardial ischemia and significant coronary artery narrowing, as verified by myocardial perfusion scintigram respectively coronary angiogram, were associated with increased rIMT. Increased rIMT was seen in patients with CV events compared to those without events. Further, rIMT values above the median indicated an independent two-fold increased risk of CV events after multivariate adjustment. At RA onset, CFR correlated with rIMT, number of swollen joints and systemic inflammation. A reduced number of swollen joints after four months treatment were associated with improvement in CFR.

In conclusion, rIMT can be accurately measured with good feasibility and reproducibility. Radial artery IMT is related to multiple CV risk factors and confers prognostic information in patients with suspected myocardial ischemia. Potential use of this vascular surrogate marker to follow atherosclerotic vascular changes and response to treatment warrants further studies

POPULÄRVETENSKAPLIG SAMMANFATTNING

Hjärt- och kärlsjukdom till följd av åderförkalkning är en av de vanligaste dödsorsakerna i världen och förväntas bli ett växande hälsoproblem då det sedan några år tillbaka sker en kraftig ökning i låg- och medelinkomstländer. Även i Sverige är hjärt- och kärlsjukdom den främsta dödsorsaken hos både män och kvinnor. Åderförkalkning är en livslång sjukdom. Den börjar hos barn och tillväxer med små förändringar hela livet, med olika hastighet beroende på vilka riskfaktorer som finns. Inflammatoriska sjukdomar t.ex. ledgångsreumatism har visat sig ge en ökad tillväxtshastighet. För att upptäcka tidiga stadier av åderförkalkning samt följa förändring efter behandling, krävs en metod som ger betydligt högre bildkvalitet än dagens konventionellt använda metod. Hittills har ultraljud av halspulsådern använts, men metoden är inte tillräckligt känslig för att se små eller tidiga förändringar.

Denna avhandling utvärderar användandet av en ny högfrekvent ultraljudsmetod, vars bildkvalitet är betydligt förbättrad och tydligt kan urskilja strukturer så små som 20 µm. Metoden har använts för undersökning av kärl och åderförkalkning hos möss. Metoden fungerar utmärkt för att titta på ytliga kärl hos människor bl.a. på kärl i armen (radialisartären), där man ser kärlväggens struktur detaljrikt. Undersökningen är enkel och snabb att utföra. Tidigare studier med högfrekvent ultraljud har visat att man kan se början till åderförkalkning hos överviktiga barn. Hos vuxna har man sett samband mellan förekomst av åderförkalkning i radialisartären och åderförkalkning i andra kärl såsom benens artärer och halspulsådern. Detta tyder på att åderförkalkning förekommer i alla kroppens kärl, att det är en systemisk sjukdom.

Syftet med avhandlingen var dels att utvärdera om åderförkalkningsgraden i radialisartären, undersökt med högfrekvent ultraljud, kan förutsäga risk att drabbas av framtida hjärt- och kärlhändelser, och dels att undersöka om ledgångsreumatism har samband med åderförkalkning i radialisartären samt vilken effekt antireumatisk behandling har på kärlen.

I delarbete I och II ingick patienter remitterade till hjärtskintigrafi, för utvärdering av bröstsmärta med misstanke om kärlkramp. De undersöktes med ultraljud av radialisartären och halspulsådern. Blodprov togs för att mäta blodfetter och andra riskfaktorer. Patienterna följdes under tre år med avseende på hjärtinfarkt, stroke, död och behov av ballongvidgning av hjärtats kranskärl. I delarbete III ingick patienter med nydebuterad ledgångs-

reumatism, remitterade till reumatologiska kliniken på Sahlgrenska Universitetssjukhuset. De undersöktes med ultraljud av radialisartären, halspulsådern, samt hjärtat där vi mätte blodflödesreserven i kranskärlen. Patienterna kom på ett första besök före insatt behandling och ett andra besök efter fyra månaders antireumatisk behandling. Patienter med stabil och välbehandlad ledgångsreumatism ingick i en kontrollgrupp.

Resultatet visade att patienter med ökad grad av åderförkalkning i radialisartären oftare drabbades av hjärtinfarkt, stroke, död eller behov av kranskärlsvidgning. Åderförkalkning i radialisartären hade samband med förekomst av riskfaktorer för hjärt- och kärlsjukdom samt åderförkalkning i hjärtats kranskärl. Hos patienter med nydebuterad ledgångsreumatism såg vi samband mellan försämrad blodflödesreserv i kranskärlen, hög sjukdomsaktivitet och ökad grad av åderförkalkning i radialisartären. Efter fyra månaders antireumatisk behandling skedde en förbättring av blodflödesreserven i kranskärlen i samband med att patienterna förbättrades i sin ledgångsreumatism.

Sammanfattningsvis, åderförkalkning i radialisartären verkar spegla graden av åderförkalkning i hjärtats kranskärl. Patienter med ökad grad av åderförkalkning i radialisartären har ökad risk att drabbas av hjärt- och kärlhändelser. Vi tror att åderförkalkning i radialisartären, påvisad med högfrekvent ultraljud, har förutsättningar att kunna användas som surrogatmarkör för hjärt- och kärlsjukdom samt till att följa kärlförändringar efter eventuell behandling.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Eklund C, Friberg P, Gan LM. High-resolution radial artery intima-media thickness and cardiovascular risk factors in patients with suspected coronary artery disease – comparison with common carotid artery intima-media thickness.
    Atherosclerosis 2012; 221: 118-123

II. Eklund C, Omerovic E, Haraldsson I, Friberg P, Gan LM. Radial artery intima-media thickness predicts major cardiovascular events in patients with suspected coronary artery disease.
    Eur Heart J Cardiovasc Imaging. 2014 Jan 26. (Epub ahead of print). PMID: 24469155

III. Eklund C, Gjertsson I, Larsson K, Wåhlander K, Friberg P, Gan, LM. Cardiovascular status in patients with recent-onset rheumatoid arthritis.
    In manuscript

Paper I and II are reprinted with permission.

Papers not included in the thesis

Svedlund S, Eklund C, Robertsson P, Lomsky M, Gan LM. Carotid artery longitudinal displacement predicts 1-year cardiovascular outcome in patients with suspected coronary artery disease.
Arterioscler Thromb Vasc Biol. 2011;31(7):1668-74

Gan LM, Svedlund S, Eklund C, Gao S, Matejka G, Albertsson P, Lerman A, Incremental Additive Values of Transthoracic Doppler Echocardiography-Assessed Coronary Flow Reserve in Patients with Suspected Myocardial Ischemia Undergoing Myocardial Perfusion Scintigraphy.
Submitted for publication.

CONTENT

ABBREVIATIONS

BMI   Body Mass Index

CABG   Coronary Artery Bypass Grafting

CAD   Coronary Artery Disease

CBFV   Coronary Blood Flow Velocity

CI   Confidence Interval

cIMT   Carotid Artery Intima-Media Thickness

CFR   Coronary Flow Reserve

CRP   C-reactive protein

CVD   Cardiovascular Disease

CV   Cardiovascular

DAS28   Disease Activity Score including 28 tender and swollen joints

DMARD   Disease Modifying Anti-rheumatic Drugs

ECG   Electrocardiogram

ESR   Erythrocytes Sedimentation Rate

HDL   High-density Lipoprotein

HR   Hazard Ratio

IL   Interleukin

IMT   Intima-media Thickness

IVUS   Intravascular Ultrasound

LAD   Left Anterior Descending Coronary Artery

LDL   Low-density Lipoprotein

MACE   Major Adverse Cardiovascular Event

MI   Myocardial Infarction

MPS   Myocardial Perfusion Scintigram

RA   Rheumatoid Arthritis

RF   Rheumatoid Factor

rIMT   Radial Artery Intima-Media Thickness

TNF-α   Tumor Necrosis Factor alpha

1 INTRODUCTION

General introduction and overall aim of the thesis

Cardiovascular disease (CVD) can be regarded as an evolving global epidemic. According to the World Health Organization the death rate in CVD has begun to rise in low- and middle-income countries during the last years. A large proportion of these deaths occurred before the age of 60. In Sweden CVD is still the most common cause of death in both women and men, although a trend of decrease has been seen during the last 20 years *(NBH 2013)*. CVD is indeed a major health problem in the world and there is an urgent need to find cost-effective methods to detect and prevent the development of CVD.

The most common cause of CVD is atherosclerosis. Atherosclerosis is a life-long process; it begins already in childhood and progresses with small changes throughout life. To be able to follow subtle structural vascular changes associated with this chronic vascular disease, there is a need of using a very high-resolution imaging tool. This thesis evaluates the use of very high frequency ultrasound with resolution down to 20µm, which obtains a significant improvement of the imaging resolution compared to conventional imaging methods. Although the method has promising characteristics, there are hitherto limited studies performed in humans. Due to the short penetration depth of high frequency ultrasound, the method is only feasible on peripheral arteries e.g. the radial artery. The radial artery has been shown to have several similarities to the coronary arteries, atherosclerosis is also common in this vascular bed in spite of the rare occurrence of severe stenosis *(Ruengsakulrach, Sinclair et al. 1999; Barry, Touati et al. 2007)*. High frequency ultrasound has previously been validated with good accuracy, and the method has been used to image the vascular wall in the radial artery in healthy humans aged 10 to 90 years *(Osika, Dangardt et al. 2007)*. The method has further been used to detect atherosclerotic changes in children with obesity and in small groups of patients *(Dangardt, Osika et al. 2008; Mohler, Sibley et al. 2009; Johansson, Myredal et al. 2010; Myredal, Gan et al. 2010)*. The relevance of the method has not yet been evaluated in larger patient populations with atherosclerotic disease, and whether the method has a prognostic value for cardiovascular (CV) risk is still unknown.

Another point of interest is to evaluate the influence of systemic inflammation on vascular status, since inflammatory mechanisms has shown to play a major role in increasing the atherosclerotic development. Patients with systemic inflammatory diseases e.g. rheumatoid arthritis (RA) are

known to have an accelerated atherosclerosis progress with increased risk of myocardial infarction (MI) *(del Rincon, Williams et al. 2001)*. It seems relevant to evaluate the relationship between systemic inflammation and vascular structure and function, and further investigate the effects of RA treatment. The vascular function is most relevant to study in the coronary arteries. With non-invasively measurements of coronary flow reserve (CFR) assessed by transthoracic echocardiography, it is possible to obtain the vascular function of the coronary arteries. This method has shown to be predictive to CV outcome *(Cortigiani, Rigo et al. 2011)*. To evaluate short-time effects of standard clinical RA treatment, high frequency ultrasound of the radial artery may be an adequate method to use for detection of small changes in vascular structure.

The overall aim of this thesis is to evaluate the relevance of radial artery intima-media thickness (rIMT), assessed by high frequency ultrasound, as a relevant marker of CV risk. Further, we aim to evaluate the influence of systemic inflammation on peripheral vascular structure and coronary artery function in RA patients, as well as short-time effects of clinical treatment.

1.1 Atherosclerosis

Vascular wall in normal arteries

Elastic arteries (e.g. aorta, carotid artery) consist mostly of elastin, compared to muscular arteries (e.g. coronary artery, radial artery) which comprise mostly of smooth muscle cells. The arterial wall consists of three layers: intima, media and adventitia. In human muscular artery the normal intima layer contains of resident smooth muscle cells. The media layer contains of smooth muscle cells embedded in a complex extracellular matrix with a few elastic laminae. Further, the adventitia layer includes mast cells, microvessels and nerve fibers *(Barry, Foulon et al. 2003)* *(Libby, Ridker et al. 2011)*. The layers are separated by clearly defined laminaes composed of elastin and collagen *(Siegel, Chae et al. 1993)*.

Development of atherosclerotic lesions

The development of atherosclerosis is a complex process. The "response to retention" theory has gained increasing popularity during the past decades *(Williams and Tabas 1995; Tabas, Williams et al. 2007)*. This theory represents that hyperlipidemia leads to increased lipid accumulation in the sub-intimal space of the vascular wall and foam cell formation occurs originated from lipid-filled macrophages. Response to lipid retention will thus cause a local inflammatory process in the vascular wall and further recruitment of

inflammatory cells. Research during the last decades indicates that systemic risk factors may maintain and enhance the inflammatory reaction within the arterial wall, resulting in an accelerated atherogenesis as well as its final clinical manifestation, e.g. MI and stroke. *(Bui, Prempeh et al. 2009) (Libby 2002)*.

Early stages of atherosclerosis are seen in young children, and development of asymptomatic atherosclerotic plaques occurs during adolescence *(Velican and Velican 1980; Berenson, Wattigney et al. 1992)*. In the age group of 13-19 years, 17% had atherosclerotic lesions in the coronary arteries, detected by intravascular ultrasound (IVUS)*(Tuzcu, Kapadia et al. 2001)*. Indeed, fatty streaks, as considered an early step in atherogenesis, have been observed in new born infants *(Napoli, D'Armiento et al. 1997; Ikari, McManus et al. 1999)*. Although fatty streaks found in young may regress, these findings indicate that the atherosclerotic process is a life-long chronic process.

Atherosclerotic lesion progression

Early vascular changes are seen as endothelial dysfunction in the arterial wall caused by systemic risk factors such as increased blood pressure, pro-inflammatory mediators and increased levels of low-density lipoprotein (LDL). Those are stimuli activating the endothelium to express adhesion molecules, which facilitate monocyte adhesion and migration into the intima layer where they differentiate into macrophages *(Libby, Ridker et al. 2011)*.

An accumulation of foam cells forms fatty streaks within the intima layer *(Stary, Chandler et al. 1994)*. The lesion progresses with migration of smooth muscle cells from the media layer into the intima layer, leading to a thickening of the intima layer and a thinning of the media layer *(Gussenhoven, Frietman et al. 1991)*.

At later stages, atherosclerotic plaque development occurs. In advanced lesions lipids from dying cells accumulate in the center of the plaque forming a lipid core. Plaques that are considered to be vulnerable are characterized by an active inflammation. Inflammatory mechanisms weaken the fibrous cap that covers the plaque, leading to increased risk of rupture and e.g. MI *(Virmani, Burke et al. 2006; Libby, Ridker et al. 2011)*.

The development of atherosclerosis from normal artery to thrombosis or stenosis is illustrated in Figure 1.

Lumen-narrowing atherosclerosis in the coronary arteries is known to cause ischemia, but myocardial ischemia is also associated with residual CV risks among both symptomatic and asymptomatic coronary artery disease (CAD) *(Zellweger, Hachamovitch et al. 2009)*. Indeed, transient ischemia could also lead to sudden death due to lethal arrhythmia *(Reichenbach, Moss et al. 1977)*.

Atherosclerosis is a systemic disease, but the growth of atherosclerotic lesions is known to vary in different segments of the arterial tree. This may be due to different hemodynamics or due to the type of arteries *(Barry, Foulon et al. 2003; Barry, Touati et al. 2007; Majesky 2007)*. One interesting theory is that the lineage diversity of smooth muscle cells in the vascular wall plays an important role, and determines whether a segment is atherosclerosis-resistant or atherosclerosis-prone *(Majesky 2007)*.
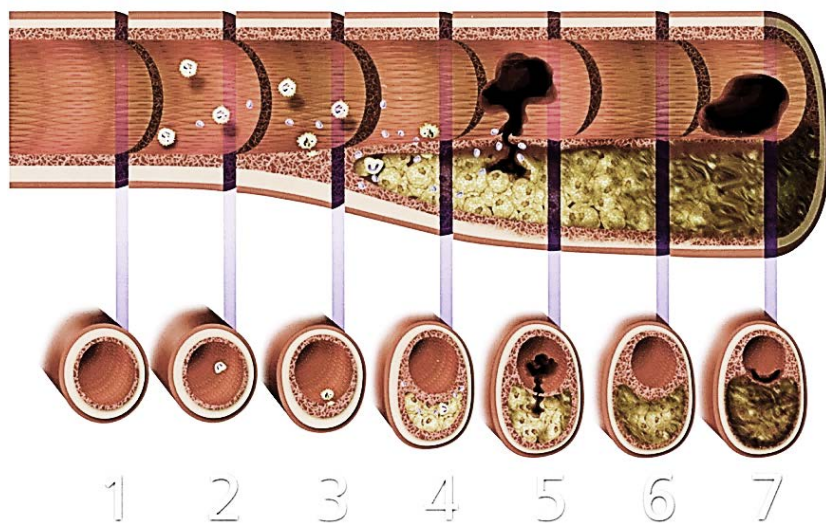
Figure 1. Initiation, progression, and complication of human atherosclerotic plaque. Top, Longitudinal section of artery depicting "timeline" of human atherogenesis. Bottom, Cross sections of artery during various stages of atheroma evolution. 1, Normal artery.2, endothelial dysfunction with accumulation of lipids in intima. 3, Fatty streaks develops.4, Lesion progression. 5, plaque rupture. 6-7, advanced fibrous and calcified plaque, stenosis and thrombosis. Adapted with permission from Lippincott Williams and Wilkins/Wolters Kluwer Health: Libby P., Circulation 2001;104(03) 365-372

1.2 Inflammation and cardiovascular disease

Systemic inflammatory response seems to be associated with CVD, although acute infections may have a transient increase of vascular events during a few days *(Smeeth, Thomas et al. 2004)*. Endothelial dysfunction seen in the brachial artery due to infection in childhood (cough, cold, fever, and sore throat) was recovered after one year in most of the children *(Charakida, Donald et al. 2005)*. Recent studies have shown that inflammation in peripheral respiratory airways caused by air pollution, acute viral respiratory infections and bacterial pneumonia are strongly related to development of atherosclerosis and an increased risk of CV mortality *(Van Eeden, Leipsic et al. 2012)*. The inflammatory response may be local in the airways at first, but a "spill over" of pro-inflammatory mediators into the blood stream contributes to the systemic effects. Also patients with autoimmune rheumatoid diseases and individuals infected with human immunodeficiency virus (HIV) are known to have an increased prevalence of CVD *(Lo and Plutzky 2012; Hollan, Meroni et al. 2013)*.

Inflammation contributes to an accelerated atherosclerotic process, since many mechanisms appear to be of similar origin as in the atherosclerotic pathogenesis. As a response to the systemic inflammation an increase of cytokines such as interleukin-6 (IL-6), interleukin-1 (IL-1) and tumor necrosis factor alpha (TNF-α) are known to activate vascular endothelium causing increased permeability and dysfunction and increased levels of adhesion molecules that stimulates more leukocytes to migrate into the intima *(Libby and Ridker 2004)*. Within the intima layer more foam cells are formed contributing to intima thickening, migration of smooth muscle cells and later plaque formation. Inflammatory cytokines are further involved in processes that weaken the fibrous cap, making the plaque more vulnerable. Recent studies have shown that increased levels of IL-6 are associated with increased risk of CV events *(Danesh, Kaptoge et al. 2008)*. IL-6 has also been shown to be related to several autoimmune disorders e.g. RA *(Nishimoto 2006)*.

Inflammation impairs vascular function

The healthy endothelium controls many functions including regulation of vascular tone, coagulation as well as inflammatory response. Vasodilatation is regulated by nitric oxide (NO) produced and released by the endothelium *(Behrendt and Ganz 2002)*. Systemic risk factors such as diabetes mellitus, hypertension, obesity, and smoking may all cause systemic low-grade inflammation leading to cytokine activation e.g. elevation of IL-6 and C-

reactive protein (CRP). The inflammatory process leads to increased levels of reactive oxygen species (ROS), which reduces NO bioavailability and causes endothelial dysfunction. In the coronary arteries, both macrovascular and microvascular endothelial function seems to be affected since they have shown to be independently predictive of CV events *(Halcox, Schenke et al. 2002)*. Endothelial dysfunction is a systemic process since peripheral arteries have shown to be affected as well as coronary arteries *(Anderson, Gerhard et al. 1995)*.

*Figure 2. Endothelial cell activation can be initiated by inflammation, which leads to an increase of adhesion molecules and migration of monocytes into the vessel wall. Within the intima, monocytes transform into macrophages, which bind oxidized LDL, thereby leading to the formation of foam cells. Accumulation of lipids and foam cells leads to the formation of fatty streaks. Foam cells produce ROS and proinflammatory cytokines (TNF, IL-1β, IL-6) that, in turn, activate the expression of adhesion molecules, facilitating further recruitment of T cells and macrophages. Reprinted by permission from Macmillan Publishers Ltd: Libby, P. Nature **420**, 868–874 (2002) , copyright 2014*

1.3 Rheumatoid arthritis and cardiovascular disease

There are interesting parallels between the pathogenesis of RA and atherosclerosis. RA is a chronic inflammatory disease, and a number of pro-inflammatory cytokines e.g. TNF-α, IL-1 and IL-6 and adhesion molecules that are involved in RA disease, overlap with the pathogeneses in atherosclerosis causing endothelial dysfunction and further increased inflammation in the vascular wall *(Libby 2008)* (*Fig 2*). Other mechanisms that

may promote accelerated atherogenesis have been ascribed to increased levels of the atherogenic small-dense LDL, despite normal or even slightly decreased level of total cholesterol *(Sattar, McCarey et al. 2003; Cavagna, Boffini et al. 2012)*. Moreover, as a consequence of the enhanced systemic inflammation in RA patients, decreased level of high-density lipoprotein (HDL) as well as impaired capacity of HDL to remove cholesterol from atherosclerotic lesions, i.e. reverse cholesterol transport, have been described *(Cavagna, Boffini et al. 2012)*. When the systemic inflammation is suppressed by anti-inflammatory treatment an improvement of the lipid profile has been shown *(Steiner and Urowitz 2009)*.

Patients with RA are known to have an increased prevalence of CVD, studies have shown a two to threefold increase in rates of MI compared to the general population *(del Rincon, Williams et al. 2001)*. The systemic inflammation promotes increased vascular inflammation and appearance of unstable atherosclerotic plaques with an increased risk of rupture. Unrecognized MI and sudden cardiac death are more common in RA patients compared with non-RA subjects *(Maradit-Kremers, Crowson et al. 2005)*. Development of CVD seems to occur early in the RA disease. Patients with less than twelve months RA disease duration present with increased intima-media thickness in the carotid artery (cIMT), and a more rapid rate of wall thickening with increasing age compared to healthy *(Hannawi, Haluska et al. 2007)*. The increase in cIMT seems to be more rapid during the first six years after RA onset compared to longer disease duration *(Giles, Post et al. 2011)*. Further, increased CV risk is seen in patients with positive rheumatoid factor (RF) compared to RF negative patients. During the first four years after RA onset the rate of CV death has been shown to be ten times higher in RF positive patients compared to RF negative patients *(Goodson, Wiles et al. 2002)*. This demonstrates the importance of early recognizing and prevention strategies of CV risk in RA patients.

Anti-rheumatic therapies have various effects on atherosclerosis and CV risk *(Cavagna, Boffini et al. 2012)*. Methotrexate (belonging to the group Disease Modifying Anti-rheumatic Drugs [DMARD]) is one of the most common anti-rheumatic drugs and seems to reduce CV risk although the underlying mechanisms are unknown *(Choi, Hernan et al. 2002; Westlake, Colebatch et al. 2010)*. Treatment with anti-TNF-α therapy, that reduces the systemic inflammation in RA and improves endothelial function, may also be associated with reduced atherosclerotic process and reduced CV risk *(Avouac and Allanore 2008)*.

Peripheral arteries are affected as well. Studies have described accelerated changes of vascular function in the brachial artery related to degree of inflammation in patients with early RA disease. An improvement of endothelial function in the brachial artery was seen after twelve weeks anti-rheumatic treatment with anti-TNF-α therapy, as well as after one year with a combination treatment including Methotrexate *(Hurlimann, Forster et al. 2002; Hannawi, Marwick et al. 2009).*

1.4 Non-invasive imaging of atherosclerosis

There are several imaging methods used today to detect atherosclerosis, but they are either invasive (IVUS, coronary angiography), associated with ionizing radiation (computed tomography, positron emission tomography) or associated with high costs and time-consuming (magnetic resonance imaging). The widely used method so far in large clinical trials is ultrasound of cIMT. Ultrasound has the benefit of being non-invasive and inexpensive.

Ultrasound of cIMT

Ultrasound of cIMT is an established method for evaluating patients risk for CVD. It is well known that cIMT relates to CV risk factors and, importantly, several studies have shown that cIMT is a predictor of CV events. *(Lorenz, Markus et al. 2007) (Heiss, Sharrett et al. 1991).* Lorenz et al summarized the data from outcome studies in a meta-analysis study and concluded that an increase of 0.1mm in cIMT was associated with 10-15% increased risk for MI *(Lorenz, Markus et al. 2007).*

Most of the cIMT studies performed during the years do not conform to a uniform protocol, which makes it difficult to compare studies. During the past years a consensus of recommendations has been developed to further standardize the ultrasound measurements of IMT and plaque in carotid arteries *(Touboul, Hennerici et al. 2012).* According to this, IMT is recommended to be measured in plaque-free regions, since IMT reflects the systemic disease with both early stages of atherosclerosis and remodeling in the vascular wall due to systemic risk factors, while plaque formation reflects later stages of atherosclerosis and local hemodynamic effects. Progresses of atherosclerotic changes are recommended to be monitored as IMT *(Touboul, Hennerici et al. 2012).*

It requires large cohorts to measure morphological changes in the vascular wall, since the changes in intima-media thickness (IMT) usually are minor. Studies have shown a cIMT progress of about 0.015 mm/year in healthy middle-aged men *(Weber 2009).* Changes due to intervention may be even

smaller, changes from baseline as small as -0.001mm/year and -0.014mm/14 months have been reported in intervention studies with rosuvastatin and niacin *(Crouse, Raichlen et al. 2007; Taylor, Villines et al. 2009)*. Since the image resolution is about 0.1-0.2 mm, often several hundred patients are needed to gain enough power. However, during recent years, studies using a standardized protocol, as mentioned previously, have gained improved reproducibility of cIMT measurements *(Taylor, Villines et al. 2009)*. Ultrasound imaging of the cIMT has been accepted as a reliable surrogate marker for atherosclerosis and used in a numerous clinical trials, although a higher resolution would have a benefit in assessing minor changes *(Bots 2006)*.

1.5 High frequency ultrasound

Conventional ultrasound used in clinical routine operates in frequencies up to 20 MHz. A new application in human studies is ultrasound with almost microscopic resolution. It is known as ultrasound biomicroscopy (UBM), micro-ultrasound, high frequency ultrasound or very high resolution ultrasound and operates at 30-100 MHz. The advantage of using ultrasound with higher frequency is the improved definition of structures, and possibility to detect smaller structures, since the resolution of ultrasound is depending on the frequency used. High frequency ultrasound is today a rapidly growing field *(Foster, Pavlin et al. 2000; Shung 2009; Foster, Hossack et al. 2011)*.

There are a dozen commercial high frequency ultrasound systems available. The first system was presented in the 1990´s, a system for imaging of human eye and skin using 50-100 MHz ultrasound *(Pavlin, Harasiewicz et al. 1991; Atta 1995)*. The first commercial high frequency ultrasound system for small animal research was released in 2000, provided with 40-55 MHz transducers as considered to be the minimum frequency needed to resolve anatomical structures in the mouse embryo *(Foster, Zhang et al. 2002)*. However, the accompanying transducers of these systems were mechanical with fixed focal depth. The image quality was considerably improved when linear-array transducers, provided with multiple focal depths and improved technique, was launched in 2008 *(Foster, Mehi et al. 2009)*.

Preclinical usage of high frequency ultrasound

- **Embryonic development**; imaging of cardiac structure and function in mouse embryos, published 1998 *(Srinivasan, Baldwin et al. 1998)*.
- **Cancer**; monitoring tumor growth and changes related to anti-cancer therapy, and visualization of microcirculation in tumors *(Foster, Hossack et al. 2011)*.
- **Cardiovascular diseases** i.e. atherosclerosis; follow progression of atherosclerotic lesion and intima-media thickness (IMT) in atherosclerotic mouse models *(Gan, Gronros et al. 2007)*. It is possible to visualize the morphology of coronary arteries in mice models, in spite of the small lumen diameter. Measurement of coronary flow velocity reserve has been evaluated as a non-invasive method to assess degree of coronary artery stenosis in mice models and coronary microvascular dysfunction in rat models *(Wikstrom, Gronros et al. 2005; Gronros, Wikstrom et al. 2006; Gronros, Jung et al. 2011)*.

Field of applications in humans

- **Ophtalmic imaging**; high frequency ultrasound in the range of 40-100 MHz is used for ocular imaging on the human eye. The system was developed 1990 and initial studies published 1991 *(Atta 1995)*. Clinically it may be used for diagnosing e.g. glaucoma and ocular tumors.
- **Dermatology**; the method was early used in skin imaging, contributing to assessment of skin tumors and evaluated with preoperative value, first published in 1993 *(Harland, Bamber et al. 1993; Turnbull, Starkoski et al. 1995; Lassau, Mercier et al. 1999)*.
- **Vascular research**; use of high frequency ultrasound (55 MHz) in human peripheral arteries was initially reported by our research group in 2007 *(Osika, Dangardt et al. 2007)*. The resolution is sufficient to distinguish the different layers in the vascular wall.
- **Imaging of neonatal and young children** are new areas to use the technology in. Since their vessels are smaller than 2 mm, the 50 MHz transducer has been helpful e.g. for visualization of peripheral veins and arteries in infants and children before cannulation of the vessels at surgery. *(Latham, Bosenberg et al. 2013; Latham, Veneracion et al. 2013)*.

1.5.1 High frequency ultrasound of radial artery

The amount of studies of rIMT is limited since the resolution of conventional ultrasound is not high enough to measure vessel wall structure in the radial artery. The highest resolution in the system used by our group is obtained with a 50-55 MHz transducer. High frequency gives a high resolution but a limited penetration depth, the 50 MHz transducer has a depth of 12 mm.

Our group has previously shown that when using high frequency ultrasound (55MHz) it is possible to image intima and media layer separately in both the radial and anterior tibial artery in humans aged 10 to 90 years *(Osika, Dangardt et al. 2007)*. Mohler et al further evaluated the feasibility of measuring vascular wall layers using this method *(Mohler, Sibley et al. 2009)*. The method has been used to detect early-stage atherosclerosis in the radial artery in children with obesity *(Dangardt, Osika et al. 2008)* and for evaluation of rIMT in small group of patients with end-stage renal disease, hypertension as well as peripheral artery disease *(Osika, Dangardt et al. 2007; Johansson, Myredal et al. 2010; Myredal, Gan et al. 2010)*.

Our research group has previously validated the high frequency ultrasound (55MHz) technique against silicon layers of thicknesses between 20µm to 120µm showing a correlation coefficient of 0.98 *(Fig 3)* and a Bland-Altman plot with mean difference and standard deviation of 3.5 ± 8.8µm. The high frequency ultrasound was further validated against histologic vascular specimens with good accuracy showing a correlation coefficient of 0.92 *(Osika, Dangardt et al. 2007)*.

Relevance of radial artery for atherosclerosis

The histological structure of the radial artery is similar to the coronary arteries, they are both muscular arteries, and they are also of a similar size *(Barry, Foulon et al. 2003; Khot, Friedman et al. 2004; Kobayashi 2009)*. Due to this similarity, the radial artery has in several studies been evaluated as a graft in coronary artery bypass surgery (CABG), however the results were not satisfying since the radial artery seemed to be as prone to atherosclerosis as the coronary arteries. In contrast, the elastic internal thoracic artery had a much lower incidence of atherosclerosis, suggesting that different type of arteries are different atherosclerotic prone *(Barry, Touati et al. 2007)*. Advanced atherosclerosis was seen in 5.3% of the radial arteries, and intima thickening in 94%, in patients undergoing CABG *(Ruengsakulrach, Sinclair et al. 1999)*. The atherosclerotic burden in the radial artery has been further evaluated by IVUS in a recently published study, where patients with CAD

had a greater percent atheroma volume in the radial artery compared to patients without CAD *(Moon, Kim et al. 2013)*.

Cardiovascular risk factors such as age, smoking, diabetes and peripheral vascular disease has been shown to correlate with intima thickening and atherosclerosis in the radial artery as evaluated in a histological study *(Ruengsakulrach, Sinclair et al. 1999)*.

The endothelial function in this vascular bed has also been shown to be associated with endothelial function in the coronary arteries *(Anderson, Uehata et al. 1995; Park, Youn et al. 2006)*.
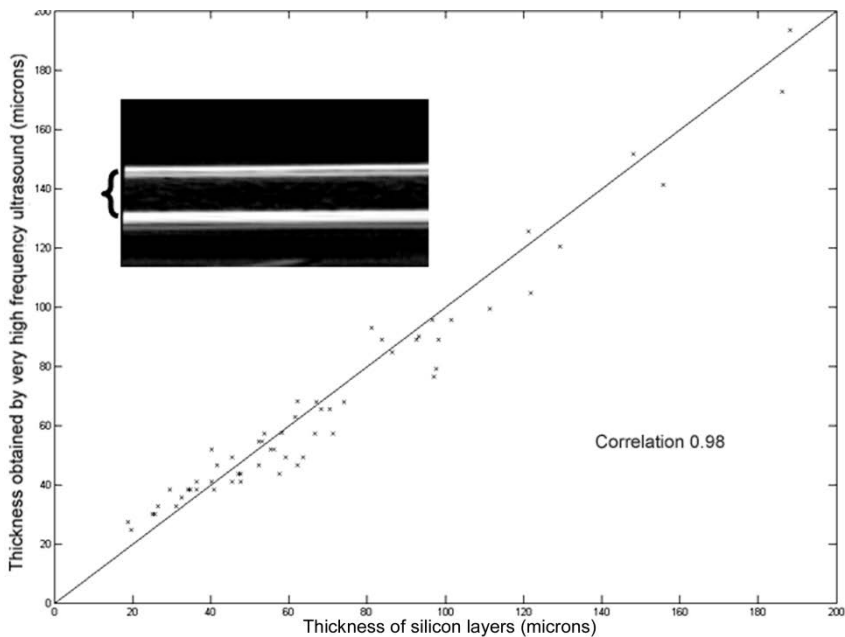
Figure 3. Validation of high frequency ultrasound (55 MHz); comparison with silicone layers of various thickness

1.6 Coronary flow reserve to assess vascular function

Vascular function in the coronary arteries may be assessed non-invasively by measuring CFR in left anterior descending coronary artery (LAD) during vasodilator stress echocardiography. CFR is an integrative measurement reflecting the entire coronary vascular function. Impaired CFR is caused by

significant coronary lesions as well as microvascular dysfunction, increased blood viscosity due to hyperlipidemia, and systemic inflammation in the myocardial capillaries *(Fig 4)*. Markers of low grade systemic inflammation such as CRP have been shown to be related to CFR. Interestingly, impaired CFR has been associated with impaired endothelial function in peripheral arteries, in patients with chest pain *(Park, Youn et al. 2006)*.
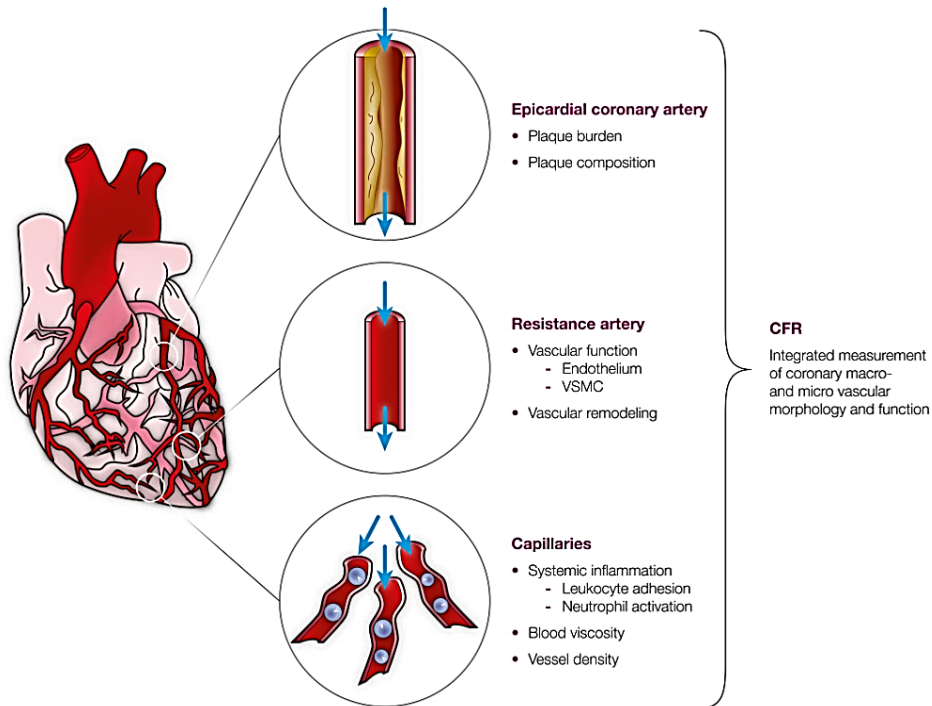

Figure 4. CFR provides an integrative assessment of coronary vascular health at all levels of the vascular tree. (Gan, Wikstrom et al. 2013) figure 1, copyright with kind permission from Springer Science and Business Media

CFR assessed with echocardiography is considered to be a feasible method with low inter- and intra-day variability *(Hozumi, Yoshida et al. 1998; Wittfeldt, Emanuelsson et al. 2013)*. Studies have shown that reduced CFR is a predictor of CV events *(Cortigiani, Rigo et al. 2011)*. Several studies with CV treatments have shown improvement of CFR both acute within a couple of hours and long-term improvements during 3-12 months *(Gan, Wikstrom et al. 2013)*.

1.7 Current status and unresolved issues

There is a need of inexpensive imaging methods with higher resolution than the ones that are used today, especially when investigating early pre-atherosclerotic changes or when following effect of clinical treatment, since the yearly changes in IMT are very small. High frequency ultrasound appears as a promising method with possibility to study changes in the vascular wall with greater detail due to the significant improvement of the imaging resolution compared to conventional imaging methods. Radial artery IMT, assessed by high frequency ultrasound, can be measured relatively simply and has shown good accuracy, but the method needs further validation.

Previous studies have been conducted in both children and adults, although the number of studies are limited. The value of high frequency ultrasound of rIMT has not yet been evaluated in larger clinical relevant patient populations, the studies performed so far have examined small groups of patients. Atherosclerosis is a systemic disease, affecting both peripheral and coronary arteries. But the demonstrated association between CV risk factors and development of intima thickening and atherosclerosis in the radial artery is derived mainly from autopsy studies. It is unknown whether the influence by conventional risk factors can be assessed by high frequency ultrasound of rIMT. An important issue to unravel is further the ability of rIMT to predict future clinical CV events. This has yet to be evaluated. The ability to measure short-term changes in rIMT is also unknown, and needs further evaluation.

Of further interest is the influence of systemic inflammation, since many mechanisms appear to be similar as in the atherosclerotic pathogenesis. An increased risk of CV disease is seen due to systemic inflammatory response caused by e.g. pneumonia or autoimmune rheumatoid diseases. The endothelial function of the coronaries and peripheral arteries has been shown to be affected, but whether rIMT is influenced by systemic inflammation is not known.

2 AIM

The general aim of this thesis is to evaluate whether rIMT can be used as a predictive tool to assess CV risk. Further, we aimed to study the relationship between rIMT, coronary microvascular function and systemic inflammation in recent-onset RA patients, and investigate the possibility to use the method in measuring short-time effects of anti-rheumatic treatment.

Specific aims

- Determine the relationship between rIMT and conventional cardiovascular risk factors

- Examine the relationship between presence of CAD and rIMT

- Evaluate the prognostic value of rIMT for future CV events

- Investigate the relationship between systemic inflammation, rIMT and coronary artery vascular function assessed by CFR, in patients with recent-onset RA

- Investigate whether short-term clinical anti-rheumatic treatment reduces rIMT

3 PATIENTS AND METHODS

3.1.1 Patient populations

Suspected myocardial ischemia

We selected a medium-high risk population to evaluate the relationship between rIMT and CV risk factors as well as the prognostic value for CV events (Paper I and II). We recruited 416 patients referred to myocardial perfusion scintigram (MPS) for evaluation of chest pain and suspected myocardial ischemia. The patients were consecutively offered participation in the study at the time of their MPS, at the Department of Clinical Physiology at Sahlgrenska University Hospital, between February 2006 and April 2008. This patient category was chosen since it is a clinical relevant population and MPS is a well-established method for the diagnosis of patients with suspected myocardial ischemia. The patients were examined according to the study protocol within four weeks after the MPS (mean two weeks). The results from MPS were blinded to operators and the patients. The exclusion criteria were atrial fibrillation or other cardiac arrhythmia, chronic obstructive pulmonary disease (COPD), other severe disease (e.g. cancer), treatment with dipyridamol (Persantin, Asasantin), not able to assimilate information about the study or unwillingness to participate. Patients with acute coronary syndromes were not included.

Rheumatoid arthritis

In Paper III we selected a population with systemic inflammation known to have an increased risk of atherosclerosis. Patients with recent-onset RA were recruited consecutively at time of diagnosis at the Department of Rheumatology at Sahlgrenska University Hospital, between February 2010 and January 2011. We enrolled a total of 23 consecutive patients with newly diagnosed RA according to American College of Rheumatology 1987 criteria. The patients made a first visit before the initiation of any immunosuppressant treatment. The second visit was accomplished in adjunct to their four months follow-up seeing a physician at the Department of Rheumatology. A total number of 20 patients completed both study visits. Equal examinations with the same investigators were performed at both visits. Exclusion criteria were unstable angina, known hypersensitivity to adenosine, treatment with statins or dipyridamol (Persantin, Asasantin), as well as contraindication associated with use of adenosine, e.g. atrial fibrillation, cardiac arrhythmia, atrioventricular (AV) block, COPD and asthma.

RA disease activity score (DAS28) for each patient was obtained by physicians at the Department of Rheumatology. The score was calculated as a number on a scale with range 0 to 10, indicating the current activity of the rheumatoid disease. DAS28 takes into consideration 28 tender and swollen joints (including shoulders, elbows, wrists, knees, metacarpophalangeal joints and proximal interphalangeal joints of the hands), erythrocytes sedimentation rate (ESR) and the general health of the patient measured on a visual analog scale.

A control group with patients that were well-treated and stable in their RA disease was also recruited at the Department of Rheumatology. The included patients were stable in their value of DAS28 and had obtained unchanged medical treatment during at least one year before inclusion (indicating unchanged disease status). We enrolled 26 patients, of which 23 completed both visits. Patients in the control group were examined with the same study protocol as in the recent-onset RA group. Patients in the control group received unchanged medication throughout the study.

Ethics (Paper I–III)
All studies were approved by the local ethics committee in Gothenburg (S462-03, Dnr 449-06 and 427-09). All study participants received oral and written information about the study, and written informed consent was obtained from each patient before enrollment in the studies.

3.1.2 Follow–up (Paper II)
The patient cohort with suspected myocardial ischemia was followed during a period of three years after their initial study visit and major adverse cardiovascular events (MACE) were recorded. The follow-up was accomplished by scripted telephone calls to the patients and confirmed through hospital records. Recorded events were as follows: MI, stroke, coronary revascularization (percutaneous coronary intervention or CABG); and death and cause of death. Five patients were excluded due to death from other causes than CV (four cancers and one accident)

3.1.3 Ultrasound methods

High frequency ultrasound of radial artery (Paper I–III)
We used the highest frequency available in an ultrasound system originally developed for small animal research (Visualsonics, Toronto, Canada). In Paper I and II we used 55MHz transducer RMV708, Vevo 770. In Paper III we used at the time the recently released linear-array transducer MS700,

50MHz, Vevo2100. Figure 5 shows the image quality of the same radial artery imaged with different ultrasound frequencies. Note the highly improved image quality obtained by higher frequency compared to conventional ultrasound.

The imaging procedure was performed using a standardized protocol. Both left and right arteries were examined in longitudinal view, with both near and far walls clearly visible. Digital cine-loops of four consecutive cardiac cycles were stored for offline analysis. To reduce variability between measurements we performed the imaging at the same segment of the artery. We placed the transducer in regio antebrachii anterior at the second skinfold proximal to palma manus, according to the earlier standardized protocol *(Osika, Dangardt et al. 2007)*.
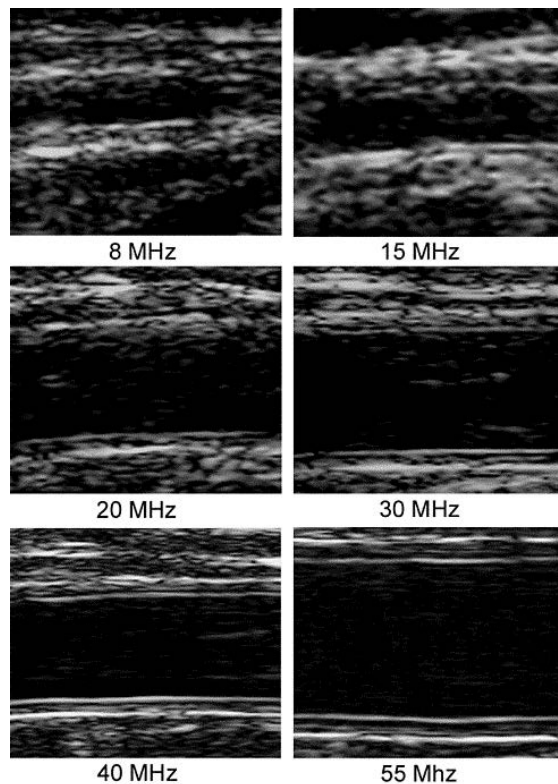
Figure 5. Radial artery in healthy, showing the improved image quality obtained with higher ultrasound frequency.

Ultrasound of carotid artery (Paper I–III)

Images of left and right carotid arteries, assessed with an 8 MHz linear transducer (Siemens, Acuson Sequoia 512, Mountainview), was acquired in longitudinal view with both near and far walls clearly visible according to standardized recommendations *(Touboul, Hennerici et al. 2007)*. Plaque screening was performed with and without color Doppler. Electrocardiogram-signals (ECG) were simultaneously recorded. Digital cine-loops of three consecutive cardiac cycles were stored for further analysis.

Transthoracic color Doppler Echocardiography of Coronary flow reserve (Paper III)

Image acquisition of CFR was performed according to a standardized method *(Wittfeldt, Emanuelsson et al. 2013)*. Imaging of LAD was performed with a 4V1C transducer and 3.5 MHz color Doppler (Siemens, Acuson Sequoia 512, Mountainview). Coronary blood flow velocity (CBFV) was acquired at rest, and during 5 minutes of pharmacological stimuli with adenosine infusion to obtain maximal flow reserve capacity. Doppler images were stored for offline analysis. To ensure that the same LAD segment was measured at the next visit, we documented the position and location of the transducer at the chest. A cine-loop of the LAD color Doppler image was also stored.

Offline measurements

All offline measurements were performed by a single reader who was blinded to the patients' clinical characteristics. Both rIMT and cIMT was measured in far wall, with IMT defined as the distance between the leading edges of the lumen-intima and media-adventitia interface. Definitions of IMT, carotid artery plaque, and their measurements, were according to recommendations *(Touboul, Hennerici et al. 2007)*.

Radial artery IMT: Measurement of rIMT has previously been standardized *(Osika, Dangardt et al. 2007)*. The measurements were performed in peak systole (defined as the frame in cardiac systole at which the artery had its largest diameter in a cine loop). IMT was measured in two different heart beats and averaged. The mean IMT value between left and right arteries was used for analysis. Workstation VisualSonics Vevo 770 (Version 3.0.0) was used in Paper I and II. In Paper III measurements of rIMT were performed using semi-automated edge-detecting software tracing 2 mm of the far vessel wall.

Carotid artery IMT and plaque area: Measurements of common carotid artery IMT was obtained 1 cm proximal to the bifurcation. Measurements were performed in a similar way to rIMT in paper I and II, in peak systole

and in two different heart beats. Mean values between left and right carotid artery were used in the statistical analysis. Plaque area was manually traced. Only plaques in the carotid bulb were included. The total sum of plaque area in left and right carotid bulb was used in the analysis. Workstation Image-Arena (version 2.9.1, Tomtec, Germany) was used in Paper I and II. In Paper III Workstation Syngo US (version 3.5.6.34, Siemens Medical Solutions) was used with automatic edge detection of mean IMT over a distance of 1 cm, at the R-wave in the ECG signal.

Coronary flow reserve (Paper III): CBFV values were obtained by manually tracing the diastolic flow velocity in the Doppler image. CFR was calculated as the ratio between maximal CBFV during hyperemia and CBFV during rest. Workstation Image Arena (2.9.1, TomTec Imaging Systems GmbH, Unterschleissheim, Germany) was used.

3.1.4 Coronary angiography

Some of the patients (n=133) in paper II underwent coronary angiography due to clinical indications during the follow-up period. To evaluate the extent of coronary atherosclerosis and its relevance with rIMT, we compared the results of coronary angiography in this subset of patients. The angiographic procedure was performed according to standard clinical protocol at the Department of Cardiology, Sahlgrenska University Hospital. The clinical indications were as follows: 69 with stable angina pectoris, 29 with unstable angina pectoris, 7 with ST elevation MI, 18 with atypical chest pain, 2 with heart failure/cardiomyopathy, 2 with silent myocardial ischemia, 2 with cardiac arrest and 4 with valvular heart disease.

The results of the diagnostic angiograms were classified into two categories: normal/atheromatosis or significant CAD (pathological). Presence of one or more lesions in one or more coronary arteries with >50% narrowing was defined as a pathological angiogram. An angiogram was classified as pathological if there was severe and diffuse lumen narrowing, even in the absence of focal lesions.

3.1.5 Radionuclide myocardial perfusion scintigram

MPS was a part of the clinical examination performed before the patients were included in study I and II. MPS was performed according to standard clinical protocol at the Department of Clinical Physiology, Sahlgrenska University Hospital. The examination included a 2-day stress/rest protocol where stress provocation was performed either by exercise test or with

pharmacological provocation using adenosine. Radionuclide Technetium (99mTc) sestamibi was used together with single-photon emission computed tomography (SPECT). Images were acquired with rotating dual-head SPECT cameras (Infinia or Hawkeye, General Electric, USA) equipped with low energy, high-resolution collimators. Images obtained shows perfusion and function of the left ventricle.

An experienced physician interpreted the images. The ischemia area was scored as small, medium, or large (extent of left ventricular perfusion defect: <10%, 10–19%, or >19%, scored as 1, 2, 3, respectively). Severity was scored as low, medium, or high (1, 2 and 3, respectively). Clinical ischemia score was calculated as the product of the ischemia severity and area scores. MPS-verified ischemia was defined as a clinical ischemia score >1.

3.1.6 Healthy volunteers

As a reference value to the performed study II we investigated the rIMT value in age-matched healthy subjects (Paper II). We recruited 20 healthy volunteers (10 males and 10 females, mean age 61 ± 3 years). Inclusion criteria were normal lipid levels (HDL, cholesterol, LDL, and triglycerides), no diabetes or hypertension, no lipid-lowering treatment or any other medication, no family history of MI and no previously known CAD. All subjects were non-smokers or had not smoked within the last 10 years. Further, to exclude CAD, all subjects underwent a standard exercise ECG test with normal ECG, heart rate, and blood pressure reactions.

3.1.7 Laboratory analyses (Paper I and III)

We performed blood sampling in all patients.

Triglycerides and total cholesterol in serum were measured using reagent systems from Roche (Triglycerides/GB kit No; 12146029216, Cholesterol kit no. 2016630, Roche Diagnostics GMBH, Mannheim Germany).

The Apolipoprotein A1 (ApoA1) concentration (Paper I) was measured with turbidimetric technique, using polyclonal rabbit anti-human antibodies (Q 0496 and Q 0497, Daco Cytomation, Glostrup, Denmark).

HDL in plasma was measured using an enzymatic colorimetric method (Direct HDL-Cholesterol, RANDOX cat no CH2652). The assay was performed on a Cobas Mira Analyser (Hoffman-La Roche & Co., Basel Switzerland).

The value of LDL was calculated with Friedewalds equation (only in patients with triglyceride <4 mmol/l): "LDL = Total Cholesterol – HDL - (0.45 × Triglycerides)". For all patients in Paper I, the ratio "Total cholesterol/HDL" was also calculated.

Analyses of leukocytes, neutrophils, lymphocytes, monocytes, CRP, and ESR were performed by the routine laboratory at Sahlgrenska University Hospital according to standard methods (Paper III).

Clinical immunological analyses were performed in clinic by the routine immunology laboratory at Sahlgrenska University Hospital according to standard methods (Paper III). The following auto-antibodies were analyzed: RF with ELISA method, anti-CCP (cyclic citrullinated peptide) with multiplex immunoassay (Bio-Plex), and antinuclear antibody (ANA) with both immunofluorescence and ELISA.

3.1.8 Method variability and statistical analysis

Variability

Intra- and inter-observer variability of offline repeated measurements of rIMT demonstrated a coefficient of variation of 5% and 6%, respectively (Paper I). This was assessed in 19 consecutive patients, with a single reader and between readers, respectively. The measurements were blinded to previous measurement and repeated with one week apart.

Total intra-sonographer variability, from image acquirement to analysis, had a coefficient of variation of 13%. This was evaluated in 27 consecutive patients who were scanned twice with one-hour interval by a single sonographer. Offline analysis was performed in a blinded way by a single reader.

Total inter-sonographer variability, with imaging performed by two different sonographers and analysis performed by a single reader, was evaluated in 13 consecutive patients and acquired with the new ultrasound system Vevo2100. Coefficient of variation was of likewise 13%. This similar result between intra- and inter-observer variability may be due to the improved imaging and easy acquisition in Vevo2100.

The semi-automatic edge-detection program (Paper III) demonstrated an intra-observer variability of 3% for offline measurements of rIMT, and a total intra-sonographer variability of 9%. Variability of offline measurements was evaluated by a single reader in 30 patients, with repeated measurements after

one week blinded to the previous measurements. Total intra-sonographer variability was evaluated in 18 consecutive patients who were scanned twice by a single sonographer, and images analyzed by a single reader.

Variability of both offline measurements and the total imaging procedure is presented in Table 1.

Bland-Altman plot of total intra-sonographer variability shows that the disagreement between rIMT in repeated imaging procedures was equally distributed throughout the range of rIMT *(Paper I)*. Intra-sonographer mean difference and standard deviation was 0.0±0.03mm, indicating the possibility to measure small structures or small changes in the vascular wall. Inter-sonographer mean difference was 0.01±0.04mm. To compare with inter-sonographer difference in cIMT described as between 0.06±0.09mm and 0.20±0.26mm in different populations *(Lorenz, Markus et al. 2007)*.

Total intra-observer variability of CFR was evaluated between two visits with the same sonographer, in 15 patients. Both variability of the method and inter-day variability of the measurement are included, giving a coefficient of variation of 20%. In well-controlled studies when minimizing environmental influences the variability of CFR is very low <5% *(Wittfeldt, Emanuelsson et al. 2013)*

Table 1. Variability of rIMT imaging and offline measurements presented as coefficient of variation.

	Intra-observer	Inter-observer
Offline measurements	5%[1]	6%[1]
Offline semi-automatic measurements	3%[2]	-
Total imaging	13%[1]	13%[2]
Total imaging and semi-automatic measurements	9%[2]	-

Data presented are percentage. 1) Imaging with Vevo770, 2) Imaging with Vevo2100

Statistical analyses (Paper I–III)

A more detailed presentation is found in respective paper.

Pearson correlation or **Fishers exact test** was used to explore relationship between different variables.

Student's *t*-test for normal distributed parameters and its non-parametric alternative **Wilcoxon rank-sum test** were used to compare groups. **Chi square test** was used to compare categorical variables.

Paired t-test and its non-parametric alternative **Wilcoxon rank-sum test** for variables with non-normal distribution was used to explore changes between repeated measurements at visit 1 and 2.

Multivariate logistic regression was used when exploring relationship to categorical variables in multivariate models.

The Kaplan-Meier survival plot was used to visualize cumulative CV events during the follow-up period categorized according to rIMT values divided into above/below median or tertiles.

The log-rank test was used to compare difference in outcome between two groups, while the log-rank test for trends was used for tertiles.

Cox proportional-hazards regression was used to assess the association between rIMT and events. Adjusted hazard ratios (HR) with corresponding 95% confidence intervals (CI) were estimated in both univariate and multivariate models. The following variables were included in the model: rIMT, cIMT, age, gender, body mass index (BMI), smoking habits, diabetes, hyperlipidemia, hypertension, and inducible ischemia on MPS; and with prior known CAD added as a separate model. A backward stepwise model was used and variables with p-values <0.05 and >0.1 were entered and removed from the model, respectively.

Coefficient of variation was calculated as SD($x-y$)/mean(x,y) × 100%.

P-values <0.05 (2-tailed) were considered statistically significant. Analyses were performed with Stata and SPSS software.

4 RESULTS AND DISCUSSION

4.1.1 Cardiovascular risk factors and rIMT

The relationship between rIMT and CV risk factors was evaluated in a relatively large patient population (n=416) with suspected myocardial ischemia *(Paper I)*. We compared our findings with cIMT that is a commonly used surrogate marker for atherosclerosis.

We found that rIMT was related to a number of traditional CV risk factors such as age, gender, BMI, blood pressure, cIMT, carotid plaque area and lipids in terms of total cholesterol, HDL and ApoA1. We found that both rIMT and cIMT was related to the same risk factors, except BMI that was only related with rIMT in this population. Increased BMI is previously known to be associated with increased cIMT, however the range of BMI was small in our study since obese patients were not specifically recruited. Due to this finding, rIMT seems to be equivalent to cIMT considering relevance to CV risk factors. This supports the theory that IMT reflects the systemic nature of atherosclerotic disease, even in peripheral arteries.

No relationship was found between rIMT and the subgroups of patients diagnosed with diabetes type II (n=47), peripheral disease (n=53), or with current smoking (n=51) in spite of their known relevance to atherosclerosis. Nor was there any relationship between these patient groups and cIMT in our study. However, the groups were relatively small, and the severity of the diseases and smoking habits are unknown. Osika et al. have previously reported a thickening of rIMT in patients with peripheral artery disease awaiting operation with femoral bypass, however this patient group may have been presented with a more severe disease compared to the one in our study *(Osika, Dangardt et al. 2007)*. Patients with diabetes or current smokers have been reported to have a thickening of the intima layer in radial artery, evaluated in a histological study *(Ruengsakulrach, Sinclair et al. 1999)*.

Dangard et al. studied radial artery wall thickness in obese children and found thicker intima layer in girls but no gender difference in rIMT *(Dangardt, Osika et al. 2008)*. In our study rIMT was thicker in men compared to females, which may reflect the difference of the vessel diameter, since rIMT is correlated with the diameter of the radial artery (r=0.43, p<0.001). Maximal diameter of the radial artery in men was 2.3 ± 0.4mm (range 1.2 - 3.4mm) and in female 2.0 ± 0.3mm (range 1.2 - 3.5mm). The use of rIMT/lumen ratio

or wall cross-sectional area (calculated as $\pi(Re^2\text{-}Ri^2)$, where Re is mean internal radius+IMT and Ri is mean internal radius) have been described *(MacKay, Hamilton et al. 2001; Osika, Dangardt et al. 2007)*. In comparison, to minimize the influence of diameter on cIMT in intervention studies it has been suggested to calculate the cross-sectional area of cIMT *(Simon, Gariepy et al. 2002)* estimated by IMT $\times$ π $\times$ (IMT + diameter at the site of IMT-measurement). However the absolute value of cIMT has become an accepted variable to predict CV risk, and in consideration to that, we will use rIMT and not the cross-sectional area or wall/lumen ratio in further analysis.

Radial artery IMT and myocardial ischemia *(Paper I)*

The relevance of rIMT to coronary atherosclerosis was investigated in the study population with suspected myocardial ischemic referred to MPS. We explored the relationship between MPS-verified myocardial ischemia and rIMT in the whole study population and in a subgroup of patients without prior known CAD. We found that MPS-verified ischemia in both analysis was associated with a thickening of rIMT. In patients without prior CAD, rIMT was 0.34 ± 0.06mm in patients with MPS verified ischemia versus 0.31 ± 0.06mm in patients without, p=0.002. Figure 6 shows high frequency ultrasound images of radial artery in healthy compared to patients with MPS-verified myocardial ischemia, demonstrating thickening of IMT and occurrence of plaque.

Patients in the study population with established CAD in terms of previously known MI or coronary revascularization (n=96) was analyzed in a subgroup. Apart from their MPS results (since some of them did not obtain myocardial ischemia on MPS), previously known CAD was associated with an increased rIMT *(Paper I)*. To further evaluate the relevance of rIMT to presence of coronary artery disease, we added patients with MPS-verified ischemia together with patients with known CAD in a subgroup (n=143). Presence of myocardial ischemia and/or prior known CAD had a significant relationship with thickening of rIMT (r=0.25, p<0.001), and the relationship remained in multivariate analysis (rIMT p=0.037) adjusting for age and gender.

Coronary angiography verified CAD *(Paper II)*

Whether rIMT is associated with established coronary atherosclerosis was further evaluated in a subgroup of patients referred to coronary angiography on clinical indications within the three-year follow-up period. If a patient underwent more than one angiography, the angiogram closest to the study date and before revascularization was chosen for analysis, suggesting that one to be the most relevant in reflecting the coronary atherosclerotic burden of the patient. Patients with significant CAD (>50% narrowing) on angiogram

had increased rIMT compared to the rest of the patients in this subgroup
(0.35 ± 0.06mm vs 0.32 ± 0.06mm, p=0.028). We observed no difference in
rIMT between patients with single vessel and multi vessel disease, which
may be due to the small cohort size and/or that the majority of those patients
were on lipid lowering treatment that may affect the progress of rIMT.

In consideration to these findings, IMT in the radial artery seems indeed
reflect atherosclerotic disease in the coronary arteries, as indicated with these
two different imaging methods (MPS and coronary angiography). In line with
our findings, a recently published study performed by IVUS reports a greater
percent atheroma volume in the radial artery in patients with CAD compared
to patients without CAD *(Moon, Kim et al. 2013)*. This study finally confirmed
that atherosclerosis indeed occurs in the radial artery and its burden is an
indicator of CAD disease burden. In light of these confirmatory data
generated by high-end invasive technique, our non-invasive highly feasible
approach indeed become a promising tool to serve as a vascular surrogate
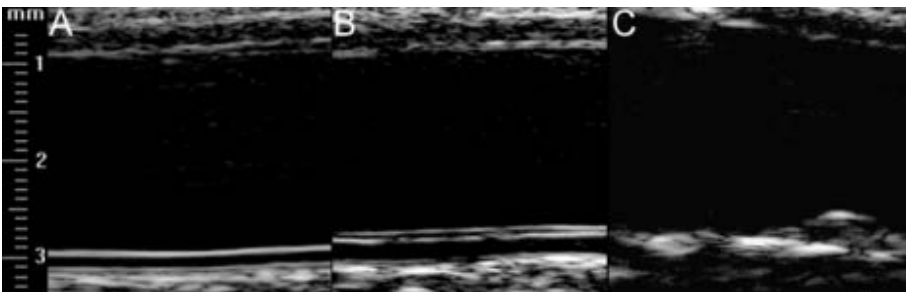marker of CAD.

*Figure 6. Radial artery in healthy (A,) with intimal thickening (B) and plaque
formation (C). Both patient B and C experienced MI during the follow up period.
Patient C was diagnosed with multi vessel disease on coronary angiography.*

4.1.2 Prognostic value of radial artery IMT

To evaluate the prognostic value of rIMT, outcome was assessed during a follow-up period of 3 years ± 1 month (Paper II). During this period 77 CV events were recorded in 66 patients. The event rate was 19% during 3 years. Composite MACE was defined as time to first MI, stroke, CV death or coronary revascularization. Hard MACE was defined as time to first MI, stroke or CV death.

First time composite MACE was 7 non-fatal MI, 4 non-fatal strokes, 2 CV deaths and 53 coronary revascularizations. To study the relationship between rIMT and CV events, the study population was divided into groups according to rIMT-values below or above the medium value. In Kaplan-Meier analysis, greater event-free survival from composite MACE was seen in patients with rIMT below median value *(Fig 7)*. Patients with above-median rIMT had a nearly three-fold greater risk for occurrence of composite MACE (HR 2.8, 95% CI 1.6-4.8), demonstrating rIMT as a valuable predictive marker of CV events *(Table 2)*.

First-time hard MACE revealed 8 non-fatal MI, 5 non-fatal strokes and 2 CV deaths. Similar as when analyzing composite MACE, patients with above-median rIMT values were more likely to experience hard MACE, compared to the rest *(Table 2)*. There was a relatively small number of hard MACE, in consideration to that we chose to use composite MACE in the survival analysis.

Table 2. Hazard Ratio and incidence of cardiovascular events in patients grouped according to rIMT below or above the median value

	rIMT<0.318mm	rIMT≥0.318mm	p-value	Hazard Ratio (95% CI)
Composite MACE(n=66)	8.9 % (18)	23.1 % (48)	<0.001	2.8 (1.6 - 4.8)
Hard MACE (n=15)	1.5% (3)	5.8% (12)	<0.05	4.0 (1.1-14.0)

Data presented are percentage (number). MACE, major adverse cardiovascular events; rIMT, radial artery intima-media thickness; CI, confidence interval

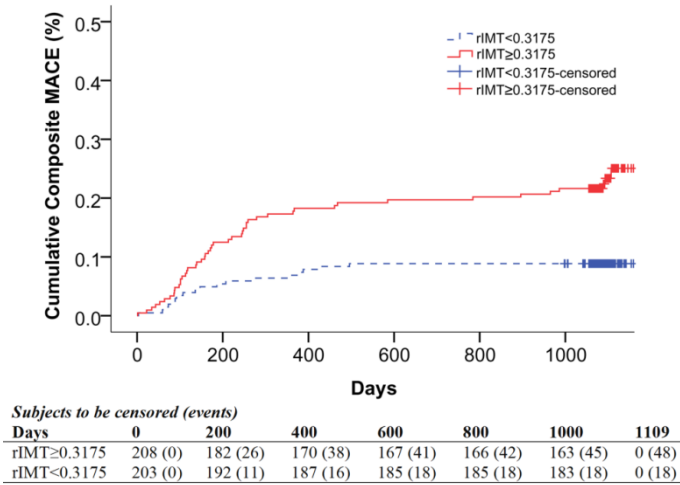| Days	0	200	400	600	800	1000	1109
rIMT≥0.3175	208 (0)	182 (26)	170 (38)	167 (41)	166 (42)	163 (45)	0 (48)
rIMT<0.3175	203 (0)	192 (11)	187 (16)	185 (18)	185 (18)	183 (18)	0 (18)

Figure 7. Kaplan-Meier survival graph showing cumulative composite MACE during 3 year follow-up, grouped according to rIMT values above or below the median (p<0.001, log rank test). The number of patients and composite MACE are shown. Reprinted with permission (paper II, Fig 1).

When the study population was divided into tertiles of rIMT, patients in the upper tertile (rIMT>0.35mm) had the highest risk of developing composite MACE (HR 3.2, 95% CI 1.6-6.4, upper tertile versus the rest) *(Fig 8)*.
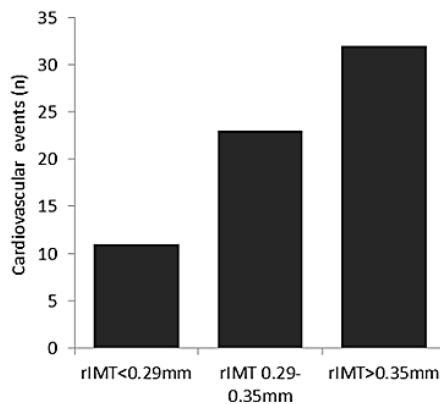
Figure 8. Distribution of composite MACE in tertiles of radial artery IMT. Patients in the upper tertile had the highest number of CV events.

Survival analysis including the whole study population

To explore the ability of rIMT to predict composite MACE, we used multivariable cox regression models generated with the following variables: age, gender, smoking, BMI, hypertension, hyperlipidemia, diabetes type II, MPS-verified ischemia. In additional analysis, prior known CAD was added as a variable in two separate models (model 3 and 4). Radial artery IMT and cIMT vas added to the models as continuous variables or as below/above median value respectively.

Model 1, (with rIMT and cIMT as continuous variables): rIMT (p=0.010, HR 1.6, 95% CI 1.1-2.4), diabetes mellitus type II (p=0.012), BMI (p=0.023), and MPS-verified ischemia (p<0.001) are independently statistically significant predictors for composite MACE, while age, gender, smoking, hypertension, hyperlipidemia, and cIMT are not *(paper II)*.

Model 2, (with rIMT>median and cIMT>median): In this model rIMT>median (p=0.006, HR 2.2, 95% CI 1.3-4.0) is a significant predictor for composite MACE, along with diabetes mellitus type II (p=0.019), BMI (p=0.019), MPS-verified ischemia (p<0.001), and cIMT>median (p=0.030, HR 1.9, 95% CI 1.1-3.5), independent of age, gender, smoking, hypertension, and hyperlipidemia.

Additional statistical analysis

When adding the variable "prior known CAD" in the models above, we obtain similar results *(Table 3)*.

Model 3, adjusted for prior known CAD (with rIMT and cIMT as continuous variables): Adding the variable "prior known CAD" in model 1 results in the following significant predictors of composite MACE; rIMT (p=0.045, HR 1.5 (1.0-2.2)), diabetes type II (p=0.018) and MPS-verified ischemia (p<0.001) *(Table 3)*.

Model 4, adjusted for prior known CAD (with rIMT>median and cIMT>median): When adding "prior known CAD" as a variable in model 2, predictors of composite MACE are rIMT>median (p=0.008 HR 2.2 (1.2-4.0)), cIMT>median (p=0.017), BMI (p=0.022), diabetes type II (p=0.011) and MPS-verified ischemia (p<0.001) *(Table 3)*.

In these models, patients with rIMT ≥ 0.318mm were associated with a two-fold increased risk of CV events. Adjusting for prior known CAD does not seem to change the prognostic value of rIMT. Carotid artery IMT was

obtained with a similar risk. Carotid artery IMT has been reported with a risk of 1.4 to 2.2 for MI in various studies and a risk of 2.3 for CVD, which is supportive to our findings *(Simon, Megnien et al. 2010)* . This further supports the theory that IMT in both radial and carotid artery reflects the systemic nature of atherosclerotic disease. Our finding indicates that rIMT may be equivalent to cIMT in its prognostic value, independently of age and gender.

Table 3. Hazard Ratios for predictors of composite MACE in multivariate survival analysis adjusted with prior known MI or coronary revascularization.

	Model 3 Hazard Ratio (95% CI)	p-value	Model 4 Hazard Ratio (95% CI)	p-value
Age		0.424		0.664
Male		0.235		0.392
Smoking		0.923		0.849
BMI		0.056	0.9 (0.8-1.0)	0.022
Hypertension		0.175		0.374
Hyperlipidemia		0.564		0.624
Diabetes type II	2.2 (1.1-4.2)	0.018	2.3 (1.2-4.3)	0.011
Prior known CAD		0.068		0.124
MPS-verified ischemia	5.5 (3.2-9.4)	<0.001	5.5 (3.2-9.5)	<0.001
rIMT	1.5 (1.0-2.2)	0.045	-	-
cIMT		0.516	-	-
rIMT>median	-	-	2.2 (1.2-4.0)	0.008
cIMT>median	-	-	2.1 (1.1-3.8)	0.017

Model 3 with rIMT and cIMT as continuous variables, model 4 with IMT grouped in below/above median values. CI, confidence interval; BMI, body mass index; CAD, coronary artery disease (in terms of MI or revascularization); MPS, myocardial perfusion scintigram; rIMT, radial artery intima-media thickness; cIMT, coronary artery intima-media thickness

Subgroup of patients with and without prior known CAD (additional statistical analysis)

In Paper II, a total of 95 patients had prior known MI or coronary revascularization. This is indicating a more severe atherosclerotic disease burden and the risk of CV events may be different in those patients compared to patients presenting only chest pain without any prior known CAD. To further evaluate the prognostic value of rIMT in respectively group, we divided the patients according to suspected or known CAD as previously done in Paper I. As found in Paper I, patients with previously known CAD had thicker rIMT compared to the rest.

We further divided these groups of patients according to occurrence of CV events. In patients with suspected CAD (without prior known CAD), a significantly thicker rIMT was obtained in patients that experienced CV events during follow up *(Table 4)*.

In patients with prior known CAD, no significant difference was seen in patients with events compared to event-free subjects. This may be due to that they already had diagnosed and established atherosclerotic disease and thereby were more likely to have lipid lowering or anti-hypertensive treatment compared to the rest which may reduce the atherosclerotic progress (lipid lowering treatment in 84% of the patients with known CAD versus 29% of the patients with suspected CAD, $p<0.001$, and anti-hypertensive treatment in 57% of the patients with known CAD versus 41% of the patients with suspected CAD, $p=0.007$).

Table 4. Radial and carotid artery IMT in patients with or without CV events, grouped according to known or suspected CAD.

	Suspected CAD (n=316)			Previously known CAD (n=95)		
	Event free (n=278)	Event (n=38)	*p*-value	Event free (n=67)	Event (n=28)	*p*-value
rIMT (mm)	0.31 ± 0.06	0.35 ± 0.06	$<0.001^{*}$	0.34 ± 0.07	0.35 ± 0.07	0.766
cIMT (mm)	0.61 ± 0.21	0.67 ± 0.15	0.072	0.66 ± 0.16	0.70 ± 0.13	0.350

Data presented as mean $\pm$SD. *$p<0.05$ when adjusting for age and gender

Survival analysis in subgroup of patients with and without known CAD

In patients with suspected CAD (without prior known CAD), patients with rIMT above the median of this subgroup (rIMT > 0.3125mm) had three-fold greater risk for MACE compared to patients with rIMT below the median in univariate analysis (HR 3.3, 95% CI 1.6 – 6.8), $p=0.001$)

Survival analysis with multivariate Cox regression was further evaluated for the two subgroups, using the same models as used in the whole study population.

Survival analysis in the group of patients without prior known CAD, using model 1 as described previously, showed that rIMT ($p=0.004$, HR 2.3 (95% CI 1.3-4.0)) was a predictor of composite MACE, alongside with diabetes type II ($p<0.001$), BMI ($p=0.004$) and MPS-verified ischemia ($p<0.001$), independently of age, gender, current smoking, hypertension, lipids or cIMT.

A similar result was obtained when using model 2 in survival analysis in the same group; rIMT>median (p=0.020, HR 2.5 (95% CI 1.2-5.3)) was predictive of composite MACE, alongside with diabetes type II (p=0.004), BMI (p=0.006) and MPS-ischemia (p<0.001), independently of age, gender, current smoking, hypertension, lipids or cIMT>median (p=0.092).

Survival analysis in patients with previously known CAD, using both model 1 and 2, showed that only MPS-verified ischemia was a predictor of composite MACE. None of the other variables were significant in this patient group.

In conclusion, increased rIMT seems to be a predictor of CV events in patients undergoing MPS due to suspected myocardial ischemia, even after adjustment for prior CAD history. Further, in the subgroup analysis including only patients without prior CAD, rIMT was stronger than cIMT to predict CV events. However, in the group of patients with known CAD, neither rIMT nor cIMT seem to be a predictor of new events. Whether this finding has a biological explanation or is only due to poor statistical power in this sub analysis will be further evaluated in a recently completed study (RECIPE, Dnr 494-09), where rIMT has been measured in patients with recent acute coronary syndrome.

Additive values to MPS?

We made an interesting finding among patients free from MPS verified ischemia (n=334). Patients with above-median rIMT values but no MPS-verified ischemia, had a higher risk of developing composite MACE (p=0.014, HR 2.5 (95% CI 1.2 – 5.1)), compared to patients with below-median rIMT values. However, the event rates are small in this group (33 composite MACE) and further studies are needed to evaluate the additive value. Since MPS measures flow limiting CAD, early atherosclerosis may be challenging to detect. In this setting, rIMT as a marker of systemic atherosclerosis burden seems to have additional prognostic value.

Distribution of rIMT

It is of clinical relevance to define a threshold value beyond which rIMT may be considered abnormally high. The distribution of rIMT has previously been evaluated within healthy populations from children to adults *(Osika, Dangardt et al. 2007)*, describing that normal rIMT values are increased with aging. We tried to evaluate the range of rIMT in our study population by distinguish a subgroup (n=72) that seemed to have a better CV health compared to the rest (Paper II). The criteria was no MPS-verified ischemia and no medication (no history of MI or coronary revascularization, no diabetes mellitus type II, and no diagnosed hypertension). In this subgroup mean rIMT was significantly thinner compared to the rest of the study population (rIMT in subgroup 0.30 ± 0.05mm compared to 0.33 ± 0.07mm in the rest of population, $p<0.001$). The difference in rIMT remained after adjusting for age. No difference between genders was seen. However, although these patients had a better CV health compared to the rest, they still had suspected myocardial ischemia since they were referred to MPS due to evaluation of their chest pain. One of four patients in this group had a family history of MI, and 11% (n=8) were current smokers. During the follow-up, 3 patients in this group had events (1 stroke, 2 revascularizations).

As a reference group, we further evaluated rIMT in healthy volunteers age-matched to the study population. Mean rIMT was 0.28 ± 0.03 mm in this healthy group.

A threshold value for normal rIMT needs to be further evaluated. According to the results, including previous studies in healthy together with our findings, the distribution of rIMT in healthy seems to be 0.21± 0.04mm to 0.28±0.03mm. While a rIMT value of 0.35±0.06mm or above indicates atherosclerotic diseases with significant CAD or events *(Table 5)*.

Table 5. Distribution of radial artery IMT in different populations

	rIMT (mm)	cIMT (mm)	Age (year)
Healthy children[1] (n=17)	0.21 ± 0.04	-	10-17
Healthy adults (n=20)	0.28 ± 0.03	-	61 ± 3
Subgroup better CV health (n=72)	0.30 ± 0.05	0.60 ± 0.29	58 ± 7
Suspected without prior CAD or event (n=278)	0.31 ± 0.06	0.61 ± 0.21	60 ± 9
Patients with CV event (n=66)	0.35 ± 0.06[2]	0.68 ± 0.14	65 ± 8
Significant CAD on angiogram (n=94)	0.35 ± 0.06[2]	0.68 ± 0.15	65 ± 8
Patients with angiographic verified stenos in lower extremities[1] (n=12)	0.36 ± 0.08[2]	-	73 ± 8

Data presented as mean ± SD. 1) rIMT in healthy children and patients with stenosis in lower extremities has been evaluated by Osika et al (*Osika, Dangardt et al. 2007*) ; 2) significant thicker rIMT compared to age-adjusted healthy controls

4.1.3 Systemic inflammation and vascular structure and function

In Paper III we showed that coronary microvascular function is associated with degree of systemic inflammation as well as with peripheral vascular structure in patients with recent-onset RA (n=23), before onset of any anti-rheumatic treatment. This study also investigated short time changes in rIMT and CFR following four months of standard of care treatment.

At time of RA diagnosis, before onset of treatment, we found a relationship between CFR and number of swollen joints and ESR level. Patients with a greater number of swollen joints or higher level of ESR showed reduced CFR. We also found that reduced CFR was associated with a thickening of rIMT. However, no correlation was seen between vascular structure (neither rIMT nor cIMT) and parameters of systemic inflammation or RA disease activity.

After four months of conventional anti-rheumatic treatment, patients with recent-onset RA improved in their disease activity and ESR level. A reduced number of swollen joints were associated with an improvement in CFR. There was no detectable change in rIMT, in spite of the improvement in coronary microvascular function.

It is reasonable to assume that vascular changes will occur at a functional level first. Given that CFR reflects both macro- and microvascular function, the acute proinflammatory status in recent-onset RA patients may lead to systemic microvascular dysfunction measured in the heart as impaired CFR. After four months of treatment an improvement in systemic inflammation was attained that may lead to improvement in endothelial function reflected as improved CFR. Our findings are supported by previous studies that shows an early improvement in endothelial function due to anti-rheumatic treatment (methotrexate or anakinra) within 6 hours, after 1 month and 6 weeks, respectively *(Ikonomidis, Lekakis et al. 2008; Hjeltnes, Hollan et al. 2012)*.

There are limited studies performed on vascular structural changes due to treatment in patients with recent-onset RA. Studies performed are disordered in consideration to treatment therapy and disease duration. A previous study on early RA patients with longer treatment period (18 months) with methotrexate, supports our findings since they detected an improvement in CFR but could neither detect any changes in IMT *(Turiel, Tomasoni et al. 2010)*. Another study in early RA patients with combination therapy of methotrexate and prednisolone noted a reduction in cIMT of 0.20mm after 1

year treatment, associated with changes in inflammatory markers *(Georgiadis, Voulgari et al. 2008)*, suggesting an increased treatment effect on IMT although the patients had higher level of ESR and CRP at baseline compared to our study. It has been suggested that combination therapy with prednisolone and methotrexate have an additional treatment effect *(Bakker, Jacobs et al. 2012)*. In our study group patients with recent-onset RA were mostly treated with DMARDs (methotrexate or sulfasalazine) in combination with either non-steroidal anti-inflammatory drugs (NSAID) (n=11) or low-dose prednisolone (n=7), however the groups were too small to be able to compare IMT changes due to different therapies.

Among the patients with recent-onset RA, the subgroup with RF positive patients (n=16) showed an improvement of CFR between the first and the second study visit. There was no corresponding change detected in rIMT or cIMT in this subgroup. RF positive patients have been described to have a greater impairment of endothelial function at time of diagnosis, and with a rapid improvement of the endothelial function ending on the same level as RF negative patients after only 6 weeks treatment *(Hjeltnes, Hollan et al. 2012)*.

Since RA patients, due to the systemic inflammation, are more prone to atherosclerosis, it will most likely lead to vascular wall thickening. However, it was not possible to detect any changes in IMT during four months treatment, which indicates that there was not sufficient structural change in IMT in spite of the very high resolution of the imaging system. However, in our study one limitation is treatment duration and another limitation is a rather small patient group. Longer treatment period, maybe with various treatment therapies and more patients, are needed to be able to address this question.

Our group of patients with recent-onset RA showed an average rIMT in line with healthy. Only 6 patients were presented with CV risk factors such as hyperlipidemia, hypertension, diabetes or prior MI. When analyzing the subgroup presented with CV risk factors (n=6), those patients showed a significantly thicker rIMT at time of diagnosis compared to the rest (rIMT in patients with CV risk factors 0.33±0.04mm versus 0.25±0.04mm in patients without, p=0.001). An indication of improvement was seen in their rIMT during four months treatment, (0.33±0.04mm at visit 1, compared to 0.27±0.02mm at visit 2, p=0.004), while no change in rIMT occurred in patients without CV risk factors (0.25±0.05mm at visit 1 compared to 0.27±0.07mm at visit 2, p=0.259). Further studies are warranted to verify these results, indicating that RA patients presented with CV risk factors have an increased atherosclerosis process at time of diagnosis and that RA patients

with thickened rIMT at diagnosis might benefit from anti-rheumatic treatment.

Sample size was calculated from a pilot study before onset of study III, by a consulted statistician. To be able to detect a change in rIMT of 0.03mm between two visits, with 80% power, we would need 13 patients *(Fig 9)*. The above described change in rIMT between two visits, in recent-onset RA patients with CV risk factors, was 0.06mm.
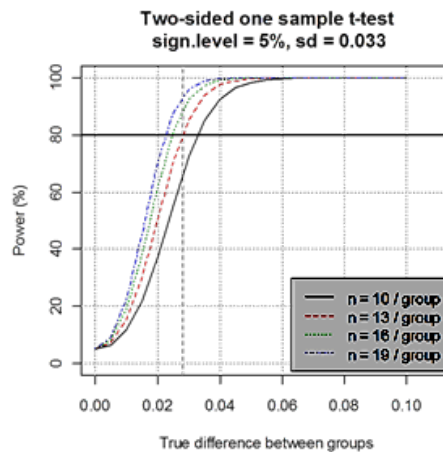

Figure 9. The graph shows the sample size needed to detect a difference of 0.03mm in rIMT between two visits, with 80% power.

4.2 Methodological considerations

Previous studies using high frequency ultrasound have measured thickness of the intima layer separately, in both children and adults. It is known that the different layers of the vascular wall are impacted under different disease conditions. Thickening of the media layer occurs due to aging or hypertension. Since thickening of the intima layer represents early stage atherosclerosis it may be more relevant to measure the intima thickness (IT) alone. Changes in IT occur before any changes in IMT may be detected. While a thickening occurs in intima layer in early atherosclerosis, the media layer seems to get thinner, which will get an IMT that may be less sensitive in detection of atherosclerosis in the early stage *(Gussenhoven, Frietman et al. 1991)*. However, an advantage of measuring IMT is the clearly defined edges that provide a more precise measurement compared to IT. The coefficient of variation has been reported to 10% for radial artery IT, compared to 5% for

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rIMT *(Mohler, Sibley et al. 2009)*. Further, IMT may be a "composite" marker of multiple risk factors of CV risk, since e.g. age and hypertension that affects media thickness may portend increased risk for events.

It has been questioned if it is possible to measure intima layer with the leading edge method. Measurement of IT in the radial artery has been defined as the distance from the leading edge of the lumen-intima to the leading edge of the intima-media interface *(Mohler, Sibley et al. 2009; Myredal, Gan et al. 2010)*. The media layer are echolucent in muscular arteries, but histological studies of muscular arteries show that the vascular wall layers are separated by clearly defined laminaes composed of elastin and collagen that are strong echo reflectors *(Siegel, Chae et al. 1993)*. The internal elastic laminae, placed between the intima and media layer, reflects a bright echo comparable with the external elastic laminae adjacent to the adventitia that are used as leading edge when measuring IMT. However, in patients with no disease, the intima layer and the internal elastic lamina seems to cause a single echogenic interface. It is first when intima thickening occurs that it is possible to detect the intima layer separately from the elastic lamina. The thickness of the radial artery intima layer in healthy adults was reported to be 58-68µm in the age 27-57 years and 74µm at age 64-83 years, assessed by 55MHz ultrasound. A significantly thicker intima was obtained in patients with peripheral artery disease compared to healthy *(Osika, Dangardt et al. 2007; Mohler, Sibley et al. 2009)*. Histopathology of radial artery in patients undergoing CABG described an intima thickness in the range of 50-1630 µm, in patients aged 42 to 83 years, almost all had intima thickening or atherosclerosis. In spite of possible overestimation of the intima measurements when using high frequency ultrasound, substantial differences in thickness was found between healthy adults and patients with peripheral artery disease in the studies presented by Mohler et al and Osika et al, indicating the value of the measurements. Whether IT will have an additive prognostic value to IMT needs further evaluation.

5 SUMMARY AND CONCLUSION

For the first time, rIMT has been measured in a relatively large patient cohort. We have demonstrated that rIMT was related to a number of CV risk factors, similar to cIMT, suggesting they both are markers for systemic atherosclerotic burden. Presence of coronary atherosclerosis seems to be reflected in rIMT, since myocardial ischemia and significant coronary artery narrowing were all associated with thickening of rIMT. Our finding is further confirmed by recent IVUS findings by an independent research group. The present studies are the first to show that it is possible to obtain these data non-invasively. Most importantly, our results indicate that rIMT has a prognostic value with the ability to predict future CV events in patients with suspected myocardial ischemia. Hence, both rIMT and cIMT are predictive of CV risk factors and outcome, which reflects the systemic nature of the atherosclerotic disease. These findings support the contention that rIMT may be a relevant surrogate marker for presence of coronary artery disease and CV risk.

In patients with recent-onset RA we found a relationship between coronary vascular function and the degree of RA disease activity and level of systemic inflammation. Interestingly, reduced coronary vascular function was associated with thickening of rIMT at time of RA diagnosis. After four months conventional anti-rheumatic treatment, an improvement in microvascular function of the coronary vascular bed was seen associated with the reduced RA disease activity. Although no structural changes in IMT could be detected in average in recent-onset RA patients after four months of RA treatment, an indication of regress in rIMT was seen in a subgroup of patients with CV risk factors, indicating that it may be possible to detect short-time effects of anti-rheumatic treatment in patients with thickening of rIMT at diagnosis. However, further studies are needed to verify this finding. Moreover, coronary microvascular function was improved in RF positive patients following standard RA treatment, which was not seen in RF negative patients, suggesting that RF positive patients may have had an increased disease activity at diagnosis or that patients may respond differently to various therapies depending on the phenotype of their disease.

High frequency ultrasound assessed rIMT seems to be a relevant surrogate marker for CV risk in patients with suspected myocardial ischemia. Its relevance in a general population and in high-risk patients, e.g. post acute coronary syndrome, needs to be further evaluated. Further, thanks to the significantly improved resolution and great reproducibility of the imaging

technique compared to other existing imaging approaches, it is highly likely that this novel technique could be a sensitive tool to follow vascular effects of future interventions. Future well-designed intervention studies are warranted to prove these interesting aspects of the technique.

Finally, rIMT will only serve as a structure surrogate marker for CV risk, combination with a proper functional risk marker, especially in the coronary vascular bed, e.g. coronary flow reserve, will most likely provide a more holistic and clinically relevant tool to risk-stratify patients and follow effects of interventions.

Known before the study
High frequency ultrasound (55MHz) has the ability to measure structures down to 20µm of size, and good accuracy is obtained in imaging of superficial arteries. IMT of the radial artery has successfully been measured in healthy children and adults, as well as in small group of patients. Histological studies have shown that the radial artery is atherosclerotic prone and CV risk factors are associated with intima thickening.

What this thesis adds
This thesis establishes that rIMT reflects systemic atherosclerosis. Further, rIMT confers prognostic information in patients with suspected myocardial ischemia. Relationship between rIMT, coronary atherosclerosis and coronary microvascular function has been shown, indicating that rIMT may be a relevant marker of CVD risk.

6 FUTURE PERSPECTIVES

Using high frequent ultrasound it may be possible to obtain an essentially improvement of the IMT measurements and detect smaller structural changes. Further technological improvements, of both the imaging system and the analysis package, towards the tools used in clinic today, will most likely make high frequency imaging even more powerful in the future.

Future potential applications of high frequency imaging of rIMT:

1. Detection of early onset of vascular changes i.e. in young adolescent, to prevent atherosclerosis development in the future, through e.g. life-style intervention etc.

2. High precision in measurements of wall structure will make it possible to use smaller cohorts in intervention studies.

3. It may even be possible to follow high-risk CAD patients on yearly basis as a risk-stratification tool and/or follow effects of various interventions.

4. It is possible to measure vascular function (flow-mediated dilatation - FMD) and structure in the same vascular bed for improved vascular characterization. FMD measured with high frequency ultrasound in the radial artery is a method under evaluation in our group.

5. In patients with recent-onset RA, the combination of functional and structural examinations in coronary and peripheral arteries, may be valuable as a non-invasive and inexpensive package in monitoring those patients´ CV risk, or to follow responses to novel treatment. The effects may be different in different RA-patients due to the clinical as well as molecular background of their disease.

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