Atherosclerotic cardiovascular disease in rheumatoid arthritis: aspects of pathogenesis and risk

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A man with a fork in a world of soup
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Abstract

Patients with rheumatoid arthritis (RA) have an increased prevalence and severity of atherosclerosis, and a corresponding increased risk of cardiovascular disease. The mechanisms causing this are not well elucidated, but both traditional cardiovascular risk factors and RA-associated factors have been associated with atherosclerosis and increased risk of cardiovascular events in patients with RA. Cardiovascular risk estimation based on traditional cardiovascular risk factors, often underestimates the risk in patients with RA. The aims of this thesis were to examine factors and biomarkers associated with atherosclerosis in patients with RA, and to evaluate an algorithm for cardiovascular risk estimation in patients with RA.

Methods Patients with early RA in the four northernmost counties of Sweden have since 1995 been included in a prospective study of both the progress of RA and comorbidities. Besides clinical data, radiographs, genetic markers and autoantibodies are registered. Paper I includes 665 patients aged 40-80 years from that cohort, in whom the 10-year risk of a first cardiovascular event was estimated with both Expanded Cardiovascular Risk Prediction Score in Rheumatoid Arthritis (ERS-RA), and the general population based ACC/AHA algorithm. The estimations were then compared to the actual outcomes. Paper II examines factors associated with coronary artery calcification (CAC) in 22 patients with long-standing RA. Papers III and IV use data from a cohort of patients <60 years of age at diagnosis of RA (n=79), in whom development of atherosclerosis has been prospectively followed since diagnosis of RA. This is a subset of patients from the larger cohort in paper I. Controls matched for age and sex (n=44) are examined as well. In paper III, phenotypes of T-cells and IgG-antibodies against cytomegalovirus (CMV) are analysed in relation to carotid intima-media thickness (IMT). In paper IV, bone mineral density and markers and regulators of bone metabolism are analysed in relation to IMT.

Results Cardiovascular risk estimation with the RA-specific algorithm ERS-RA is not superior to estimation with the ACC/AHA algorithm. Both algorithms underestimate the risk in patients with a high grade of inflammation and in patients with an estimated moderate risk. In patients with long-standing RA, presence of CAC is associated with inflammatory activity, both at time of examination and in earlier stages of RA. Presence of anti-CMV IgG antibodies and altered T-cells (both CD4+ and CD8+) lacking the co-stimulatory molecule CD28 (CD28null) are associated with a higher IMT, and patients IgG-positive for CMV have a rapid increase in IMT after onset of RA. Regulators of bone metabolism (sclerostin, osteoprotegerin and osteocalcin) are associated with a higher IMT in patients with RA.
Conclusion Cardiovascular risk estimation in patients with RA still needs to be improved. The fact that CMV-positivity, altered populations of T-cells and IMT all are associated, and that also regulators of bone metabolism reflect IMT, suggests that the pathogenesis of atherosclerosis in patients with RA is multifactorial. This thesis provides knowledge of the accelerated development of atherosclerosis in RA and could possibly be relevant also in other chronic inflammatory diseases, where markers of accelerated atherosclerosis and increased cardiovascular risk are lacking.
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ACC/AHA</td>
<td>The American College of Cardiology/American Heart Association risk score</td>
</tr>
<tr>
<td>ACPA</td>
<td>Antibodies against citrullinated peptides</td>
</tr>
<tr>
<td>Anti-CCP</td>
<td>Antibodies against cyclic citrullinated peptides</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under the curve</td>
</tr>
<tr>
<td>BMD</td>
<td>Bone mineral density</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>BP</td>
<td>Blood pressure</td>
</tr>
<tr>
<td>CAC</td>
<td>Coronary artery calcification</td>
</tr>
<tr>
<td>CDAI</td>
<td>Clinical disease activity</td>
</tr>
<tr>
<td>CIC</td>
<td>Circulating immune complexes</td>
</tr>
<tr>
<td>CMV</td>
<td>Human cytomegalovirus</td>
</tr>
<tr>
<td>CT</td>
<td>Computed tomography</td>
</tr>
<tr>
<td>CTX</td>
<td>C-terminal cross-linked telopeptide</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>DAS28</td>
<td>Disease activity score of 28 joints</td>
</tr>
<tr>
<td>DMARD</td>
<td>Disease modifying anti-rheumatic drug</td>
</tr>
<tr>
<td>DXA</td>
<td>Dual-energy x-ray absorptiometry</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immune-assay</td>
</tr>
<tr>
<td>ERS-RA</td>
<td>Expanded Cardiovascular Risk Prediction Score for Rheumatoid Arthritis</td>
</tr>
<tr>
<td>ESR</td>
<td>Erythrocyte sedimentation rate</td>
</tr>
<tr>
<td>FMD</td>
<td>Flow-mediated dilation</td>
</tr>
<tr>
<td>HAQ</td>
<td>Health assessment questionnaire</td>
</tr>
<tr>
<td>HDL</td>
<td>High-density lipoproteins</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IL2sR</td>
<td>Soluble receptor of interleukin-2</td>
</tr>
<tr>
<td>IMT</td>
<td>Intima-media thickness</td>
</tr>
<tr>
<td>LDL</td>
<td>Low-density lipoproteins</td>
</tr>
<tr>
<td>MDA-LDL</td>
<td>Malondialdehyde-modified LDL</td>
</tr>
<tr>
<td>NETS</td>
<td>Neutrophil extracellular traps</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>OCN</td>
<td>Osteocalcin</td>
</tr>
<tr>
<td>OPG</td>
<td>Osteoprotegerin</td>
</tr>
<tr>
<td>OPLS</td>
<td>Orthogonal projection of latent structures</td>
</tr>
<tr>
<td>ox-LDL</td>
<td>Oxidized LDL</td>
</tr>
<tr>
<td>PAI</td>
<td>Plasminogen activator inhibitor-1 mass</td>
</tr>
<tr>
<td>PET</td>
<td>Positron-emission tomography</td>
</tr>
<tr>
<td>P1NP</td>
<td>Procollagen type I N-terminal propeptide</td>
</tr>
<tr>
<td>PTPN22</td>
<td>Protein tyrosine phosphatase, non-receptor type 22</td>
</tr>
<tr>
<td>PWV</td>
<td>Pulse-wave velocity</td>
</tr>
<tr>
<td>RA</td>
<td>Rheumatoid arthritis</td>
</tr>
<tr>
<td>RANKL</td>
<td>Receptor activator of nuclear factor κB</td>
</tr>
<tr>
<td>RF</td>
<td>Rheumatoid factor</td>
</tr>
<tr>
<td>ROC</td>
<td>Receiver operating characteristic</td>
</tr>
<tr>
<td>SE</td>
<td>HLA-DRB1 shared epitope</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>SCORE</td>
<td>Systematic Coronary Risk Evaluation algorithm</td>
</tr>
<tr>
<td>T0</td>
<td>Baseline examination</td>
</tr>
<tr>
<td>T1.5</td>
<td>Examination 1.5 years from baseline</td>
</tr>
<tr>
<td>T11</td>
<td>Examination eleven years from baseline</td>
</tr>
<tr>
<td>TG</td>
<td>Triglycerides</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>tPA</td>
<td>Tissue plasminogen activator antigen</td>
</tr>
<tr>
<td>VAS</td>
<td>Visual analogue scale</td>
</tr>
<tr>
<td>VIP</td>
<td>Variable importance in projection</td>
</tr>
</tbody>
</table>
List of papers


*Equal contributors

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Enkel sammanfattning på svenska

Reumatoid artrit (ledgångsreumatism; RA) är en kronisk inflammatorisk ledsjukdom, som drabbar ungefär 0,7 % av Sveriges befolkning. Varför en individ insjuknar i RA är inte klarlagt, men man har identifierat såväl bakomliggande ärtliga faktorer som miljöfaktorer, i första hand rökning.

Det dominerande symptomet hos patienter med RA är inflammation i lederna, vilket leder till värk och stelhet och ofta även permanenta skador i lederna. Förutom att RA leder till lidande, har patienter med RA en ökad risk för hjärt-kärlsjukdom, i första hand hjärtinfarkt och stroke, vilket leder till en förkortad livslängd. Denna riskökning beror på ökad förekomst av ateroskleros, som är den bakomliggande förklaringen till huvudparten av hjärt-kärlsjukdom. Orsaken till den ökade förekomsten av ateroskleros hos patienter med RA är inte helt klarlagd, och inte heller finns något etablerat verktyg för att bedöma risken för framtida hjärt-kärlsjukdom hos patienter med RA.

Syftet med denna avhandling var att studera faktorer som skulle kunna vara relaterade till den ökade förekomsten av ateroskleros hos patienter med RA, och att utvärdera ett riskvärderingsverktyg för hjärt-kärlsjukdom hos patienter med RA.

I delarbete I jämfördes träffsäkerheten med det för patienter med RA utvecklade riskberäkningsverktyget ERS-RA med träffsäkerheten för ett riskberäkningsverktyg för vanlig befolkning och med den faktiska förekomsten av hjärt-kärlsjukdom. Inget av dessa verktyg var helt träffsäkert, utan båda hade svagheter i att bedöma risken för framtida hjärt-kärlsjukdom, särskilt när beräkningen baserades på patientens och den undersökande läkarens uppgifter om höga blodfetter och högt blodtryck.

Delarbete II är en uppföljning av en tidigare studie av ateroskleros hos patienter som haft RA i över 30 år, där det visades att det finns ett samband mellan inflammation under hela sjukdomstiden och graden av ateroskleros, mätt som förkalkning i hjärtatets kranskärl.

I delarbete III och IV studerades RA-patienter som följts med avseende på aterosklerosutveckling sedan de insjuknade i RA elva år tidigare. I delarbete III konstaterades att infektion med cytomegalovirus (CMV), ett virus som de flesta människor är bärare av, var associerat med en snabb utveckling av ateroskleros efter insjuknande i RA. Patienter som var infekterade med CMV hade också ökad förekomst av en typ av avvikande T-lymfocyter, CD28null-T-celler, som även de var associerade med ateroskleros. I delarbete IV studerades markörer för
benomsättning i relation till ateroskleros, eftersom patienter med benskörhet har ökad risk för hjärt-kärlsjukdom. I denna studie visades att osteoprotegerin, en av många molekyler som reglerar omsättningen av ben, var associerad till ateroskleros. Även osteocalcin, ett hormonliknande protein som frisätts från ben, hade samband med ateroskleros, medan det inte fanns något samband mellan markörer för själva benomsättningen och ateroskleros.

Sammanfattningsvis visar denna avhandling att det behöver utvecklas nya verktyg för beräkning av risken för hjärt-kärlsjukdom hos patienter med RA, och att aktiv mätning av blodfetter och blodtryck är en förutsättning för att kunna göra en korrekt riskbedömning. Vidare visar jag att det finns ett samband mellan inflammation och förkalkning i hjärtats kransfärd. Man kan också dra slutsatsen att CMV-infection, CD28null-T-cell och ateroskleros är associerade, och att de benrelaterade molekylerna osteoprotegerin och osteocalcin har ett samband med ateroskleros, trots att benomsättningen i sig inte har något samband med ateroskleros hos patienter med RA.
Background

Rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic autoimmune disease. At onset, inflammation of the intra-articular synovium is the predominant symptom, causing pain and stiffness. The inflamed synovium becomes hyperplastic and can erode the cartilage and bone in the affected joints, causing permanent disability. Furthermore, malaise and fatigue are common in RA, due to systemic inflammation (Smolen et al. 2018).

Many patients with RA have inflammation not only in the synovium, but also in other organs (extraarticular RA). The most recognized extra-articular manifestation is rheumatic nodules, usually located in the subcutis, but sometimes in other organs. More severe manifestations (e.g., pleuritis, pericarditis, Felty’s syndrome, and vasculitis) are seen in about 10% of patients with RA (Turesson et al. 2003, Myasoedova et al. 2011). Patients suffering from RA have a reduced life expectancy, not only due to extra-articular RA, but mainly due to cardiovascular disease (Mutru et al. 1985, Wallberg-Jonsson et al. 1997).

Epidemiology of rheumatoid arthritis

The prevalence of RA varies globally between regions and populations. The global prevalence of RA has been estimated to 0.24%, whereas in Western Europe, the prevalence is 0.6% (Cross et al. 2014). This is in line with studies of the Swedish population, where the prevalence of RA is estimated to 0.5% (Simonsson et al. 1999) or 0.7% (Neovius et al. 2011). Globally, the highest prevalences are seen in indigenous peoples of North America, where prevalences ranging from 0.6 to 6.8% have been reported (McDougall et al. 2017).

In both the global population and the Swedish population, females have a higher risk of developing RA, making the prevalence of RA about twice as high in females as it is in males (Neovius et al. 2011, Cross et al. 2014). The incidence of RA varies with age, and the highest incidence of RA in Sweden is seen in individuals 70-79 years of age (Eriksson et al. 2013). Mean age at onset of RA was in recent Swedish studies 55-60 years (Innala et al. 2011, Eriksson et al. 2013).

Aetiology of rheumatoid arthritis

Rheumatoid arthritis is an autoimmune disease, involving both the innate and the adaptive immune system (Smolen et al. 2018). Despite extensive research, it is still unknown what initiates the autoimmune response causing RA, but several risk factors have been identified. The risk factors identified are of several kinds -
environmental, genetic, as well as hormonal - and there are also indications of interaction between them, making the aetiology of RA hard to elucidate and hitherto insufficiently known.

**Genetic risk factors**
A hereditary component in the aetiology of RA was identified several decades ago, when studies showed a higher frequency of RA in first degree relatives to patients with RA, than in the common population (Lawrence 1970). The heritability of RA has been estimated to 60% (MacGregor et al. 2000). Today more than 100 loci have been associated with risk of RA (Okada et al. 2014). The most important hereditary factor for development of RA, and the first one to be identified, was the HLA DRB1 alleles called shared epitope (SE) (Gregersen et al. 1987). The association between RA and SE was later specified to patients with the rheumatoid factor (RF) antibodies (Padyukov et al. 2004) and antibodies against citrullinated peptides (ACPA) (van der Woude et al. 2009).

The second most important risk loci in RA is the protein-tyrosine-phosphatase non-receptor type 22 (PTPN22) (Begovich et al. 2004). As for SE, PTPN22 is primarily associated with ACPA-positive RA (Kallberg et al. 2007).

**Environmental risk factors**
Smoking increases the risk of RA (Vessey et al. 1987), in a dose-dependent manner (Sugiyama et al. 2010). The association between smoking and RA is predominantly seen for RF-positive RA (Symmons et al. 1997, Sugiyama et al. 2010) and ACPA-positive RA (Klareskog et al. 2006). Inhalation of dust is another risk factor for RA. This has been shown for exposure to silica (Khuder et al. 2002), but also for exposure to textile dust (Too et al. 2016). The increased risk in textile dust exposed individuals is not limited to developing ACPA-positive RA, but also ACPA-negative RA.

**Hormonal risk factors**
Rheumatoid arthritis is more common in females, with a female to male ratio of 4:1 in individuals less than 50 years of age and 2:1 in individuals over 60 years of age (Alpízar-Rodríguez et al. 2017). This suggests that hormonal factors are involved in the pathogenesis of RA, but the role and mechanisms of hormonal factors remain controversial. The incidence of RA is lower during pregnancy, but regarding for example breast feeding, hormonal replacement therapy and oral contraceptives, the results are conflicting (Alpízar-Rodríguez et al. 2017).

**Microbiota**
Periodontal disease is associated with an increased incidence of RA (de Pablo et al. 2008) and also to disease activity in patients with RA (Rodríguez-Lozano et al. 2019). It has been suggested that the increased risk at least in part is mediated by
the oral microbiota, in particular Porphyromonas gingivalis and Aggregatibacter actinomycetemcomitans, bacteria that are able to citrullinate peptides. The gut microbiota is different in patients with RA compared with the general population, but the microbiota changes with time of disease and treatment of RA (Guerreiro et al. 2018).

*Interactions between risk factors*

There is a clear interaction between genetic and environmental factors on development of RA. Smoking interacts with shared epitope (SE), as well as PTPN22, and the combination of all three of these risk factors increase the risk 20-fold for development of ACPA-positive RA (Kallberg et al. 2007). There is also an association between textile dust and SE, where dust exposed individuals carrying the SE had a 40-fold increased risk of anti ACPA-positive RA. Regarding interaction between environmental risk factors, individuals exposed to both smoking and silica dust have a higher risk of development of RA, than individuals exposed to either smoking or silica dust (Stolt et al. 2010).

*Antibodies*

Rheumatoid arthritis is a disease, where extensive studies of antibodies have been undertaken. Numerous antibodies have been detected, although there are patients in whom no RA-specific antibodies can be detected. The first antibody to be discovered was RF (Waaler 1940, Rose and Ragan 1948). Although RF is present in about 75% of patients with RA, it is not restricted to patients with RA, but common in many other inflammatory conditions and in healthy individuals, where the prevalence of RF increases with age (van Boekel et al. 2002). The presence of RF is associated with a more aggressive disease and a higher risk of joint erosions (van der Heijde et al. 1992). The terms seropositive and seronegative RA are still used to denote RA with or without RF, although the possible presence of other antibodies makes the terms somewhat inappropriate.

Of other antibodies in patients with RA, ACPAs are highly relevant. Although they have been known for decades, their clinical importance has arisen in the last 15 years, when studies showed them to be highly specific of RA (Nishimura et al. 2007), prevalent before disease onset (Rantapää-Dahlqvist et al. 2003) and associated with a more aggressive disease phenotype (Lindqvist et al. 2005, Berglin et al. 2006). The ACPA test used in most clinical laboratories is the second generation anti-cyclic citrullinated peptides (anti-CCP).
Pathogenesis

The pathogenesis of RA often begins years before onset of clinical symptoms. This has been shown in studies where antibodies have been detected before disease onset (Rantapää-Dahlqvist et al. 2003, Brink et al. 2013) and even up-regulation of pro-inflammatory cytokines have been detected before onset of disease (Kokkonen et al. 2010). What initiates the disease and what initiates the onset of symptoms is insufficiently known, although some environmental and hereditary risk factors are known, as described in previous pages.

At disease onset, inflammation of joints usually is the dominant symptom. This inflammation is located in the synovium, and involves several mechanisms. As described by others (McInnes and Schett 2011, Smolen et al. 2018), the synovitis is caused by infiltration of adaptive immune cells into the synovial lining. A large part of the inflammatory cells in the inflamed synovium consists of CD4+ T-cells interacting with B-cells in what has been proposed as an ongoing maturation of the immune response in the synovium. Cells representing the innate immune system, in particular macrophages, are also abundant in the synovium in patients

Table 1. The 1987 American College of Rheumatology classification criteria for rheumatoid arthritis (Arnett et al. 1988).

<table>
<thead>
<tr>
<th>Criteria</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Morning stiffness</td>
<td>Stiffness in and around joints, lasting at least 1 hour before maximal improvement.</td>
</tr>
<tr>
<td>2. Arthritis of 3 or more joints</td>
<td>At least 3 joint areas have had soft tissue swelling observed by a physician. The 14 possible areas are right or left proximal interphalangeal (PIP) joints, metacarpophalangeal (MCP) joints, wrist, elbow, knee, ankle, and metatarsophalangeal (MTP) joints.</td>
</tr>
<tr>
<td>3. Arthritis of hand joints</td>
<td>Swelling (arthritis) of the PIP, MCP, or wrist joints.</td>
</tr>
<tr>
<td>4. Symmetric arthritis</td>
<td>Simultaneous involvement of the same joint areas (as defined in 2.) on both sides of the body.</td>
</tr>
<tr>
<td>5. Rheumatoid nodules</td>
<td>Subcutaneous nodules, over bone prominences, or extensor surfaces, or in juxtaarticular regions observed by a physician.</td>
</tr>
<tr>
<td>6. Serum rheumatoid factor</td>
<td>Demonstration of abnormal amounts of serum rheumatoid factor by any method for which the result has been positive in ≤5% of normal control subjects.</td>
</tr>
<tr>
<td>7. Radiographic changes</td>
<td>Radiographic changes typical for RA on posteroanterior hand and wrist radiographs, which must include erosions or unequivocal bony decalcification in or most marked adjacent to the involved joints (osteoarthritis changes alone do not qualify).</td>
</tr>
</tbody>
</table>

Criteria 1-4 must have been present for at least 6 weeks. Rheumatoid arthritis is defined by the presence of 4 or more criteria.
with RA. Besides the infiltration of inflammatory cells, the synoviocytes are active in the inflammation. The synovial lining becomes hypertrophic, and fibroblast-like synoviocytes produce cytokines and chemokines, as well as matrix metalloproteinases. The synoviocytes thereby cause destruction to the joint both directly, and by promoting the inflammation. Production of receptor activator of nuclear factor κB (RANKL) and pro-inflammatory cytokines activate osteoclasts, that degrade bone matrix and causes bone erosions.

**Diagnosis**

Diagnosing RA is a clinical task where joint symptoms, clinical findings, assessment of acute phase reactants, analysis of RF and anti-CCP and also x-ray are considered. No diagnostic criteria exist, but classification criteria have been used since 1956. In 1987, a set of criteria was developed by the American college of rheumatology (Arnett et al. 1988), that was in use until 2010, when they were

**Table 2.** The 2010 American College of Rheumatology/European League Against Rheumatism classification criteria for RA (Aletaha et al. 2010).

<table>
<thead>
<tr>
<th>Joint involvement</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 large joint</td>
<td>0</td>
</tr>
<tr>
<td>2-10 large joints</td>
<td>1</td>
</tr>
<tr>
<td>1-3 small joints</td>
<td>2</td>
</tr>
<tr>
<td>4-10 small joints</td>
<td>3</td>
</tr>
<tr>
<td>&gt;10 joints (at least 1 small joint)</td>
<td>5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Serology</th>
<th>Score</th>
</tr>
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<tbody>
<tr>
<td>Negative RF and negative ACPA</td>
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</tr>
<tr>
<td>Low-positive RF or low-positive ACPA</td>
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</tr>
<tr>
<td>High-positive RF or high-positive ACPA</td>
<td>3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Acute-phase reactants</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal CRP and normal ESR</td>
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</tr>
<tr>
<td>Abnormal CRP or abnormal ESR</td>
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</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Duration of symptoms</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;6 weeks</td>
<td>0</td>
</tr>
<tr>
<td>≥6 weeks</td>
<td>1</td>
</tr>
</tbody>
</table>

Joint involvement: tender or swollen joints on examination. Distal interphalangeal joints, first carpometacarpal joints, and first metatarsophalangeal joints are excluded from assessment.

Large joints: shoulders, elbows, hips, knees, and ankles.
Small joints: metacarpophalangeal joints, proximal interphalangeal joints, second through fifth metatarsophalangeal joints, thumb interphalangeal joints, and wrists.
Low-positive: above upper limit of normal (ULN), but ≤3 times the ULN.
High-positive: >3 times ULN

Patients with scores ≥6 are classified as having definite RA.
replaced by new criteria from a collaboration between the American college of rheumatology (ACR) and the European league against rheumatism (EULAR) (Aletaha et al. 2010). The two sets of criteria are presented in Tables 1 and 2.

**Pharmacological treatment of RA**

Pharmacological treatment of RA aims to reduce or eliminate the inflammation, in order to improve physical function and reduce joint erosion, as well as the risk of extra-articular disease and comorbidities. In order to achieve this, several drugs are used. Disease modifying anti-rheumatic drugs (DMARD) is a term to designate drugs that not only relieve the symptoms, but also have an impact on inflammation. Although corticosteroids have a disease modifying effect, they are not included in the term DMARD. The cornerstone of DMARDs is methotrexate, that has been extensively used since the effects on clinical measures of disease activity (Weinblatt and Maier 1990) and radiological progression (Rau et al. 1991) were identified. Other commonly used DMARDs are sulfasalazine, hydroxychloroquine, leflunomide, cyclosporine and gold salts. These agents are all small molecules, developed without exact knowledge of mode of action, and are called synthetic DMARDs, since they are produced by chemical methods. The word synthetic is to separate them from the biologic DMARDs, that are large molecules produced in biological processes. The first biological DMARDs were the tumor necrosis factor inhibitors, that were introduced in the 1990´s and since then have been extensively used to treat RA. Other biological DMARDs used to treat RA are the B-cell depletion antibody rituximab, the interleukin 6 inhibitor tocilizumab, and the T-cell activation inhibitor abatacept. In the last years targeted synthetic DMARDs, small molecules with a specific target for mode of action, have been introduced. Inhibitors of janus kinases are the first agents in this class of DMARDs. Beside the DMARDs described above, glucocorticoids are anti-inflammatory and disease modifying in patients with RA, but have side effects that limit their use. Both EULAR (Smolen et al. 2017) and the Swedish association of rheumatology (Swedish society of rheumatology 2018) have guidelines for treatment of RA.

**Measurement of disease activity and severity**

Measurement of disease activity in RA relies on several different assessments, that are combined in scores that represent general disease activity. Clinical examination, where swollen and tender joints are registered, is fundamental in all scores, but also visual analogue scales (VAS) for patient´s assessment of global health and pain are used, as well as physician´s assessment of global health. Analysis of erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) are routinely used as laboratory measures of inflammation. Two composite scores that have widespread use are the Disease Activity Score of 28 joints (DAS28) and Clinical Disease Activity Index (CDAI). DAS28 is calculated in a somewhat
intricate way, using VAS for patient’s global health and pain, swollen and tender joints, and ESR (Prevoo et al. 1995). CDAI is calculated as the sum of swollen joints, tender joints, patient’s assessment of global health and physician’s assessment of global health (Aletaha et al. 2005). In clinical trials where improvement of disease activity is the primary outcome, the American College of Rheumatology definition of improvement often is used. This measurement is based on relative improvement in swollen and tender joints, and improvement in patient’s and physician’s global assessments, pain, disability, and acute-phase reactants (Felson et al. 1995).

Another measure of disease activity and severity is the Health Assessment Questionnaire (HAQ), a patient reported scale that yields a numeric measure of function in daily life (Fries et al. 1982). It is worth noting, that reduced function possibly can have different causes in new-onset RA and in long-term RA: in the new-onset patient, reduced function and elevated HAQ likely reflects ongoing inflammation, whereas in long-term disease, joint erosions can contribute to impairment of physical function, thus making HAQ a measure of not only present inflammation.

Severity of RA can be assessed by conventional x-ray, where joint erosions and joint space narrowing are detected. Scoring systems often used in research and clinical trials are the modified Larsen score (Larsen 1995) and Sharp score modified by van der Heijde (Van Der Heijde et al. 1989).

**Rheumatoid arthritis and atherosclerosis**

In the 1950s and 1960s, reports of increased mortality in patients with RA were published (Cobb et al. 1953, Duthie et al. 1964). The most important cause of increased mortality was later shown to be cardiovascular disease (Mutru et al. 1985), suggesting that patients with RA could have an increased prevalence of atherosclerosis. The first study confirming this was published in 2001 (Wållberg-Jonsson et al. 2001), an observation that has been corroborated in several studies (Ambrosino et al. 2015).

**Atherosclerosis**

Cardiovascular disease is the leading cause of death in the world (Global Burden of Disease 2018). It is a heterogenous class of diseases, that includes both thrombo-embolic diseases and clinical manifestations of atherosclerosis, as well
as for example congenital heart disease, cardiomyopathy and diseases of the heart valves. Of these different types of cardiovascular disease, atherosclerotic cardiovascular diseases are by far the most common and cause 85% of deaths from cardiovascular disease (Global Burden of Disease 2018).

Although the onset of symptoms of disease often is sudden, the underlying atherosclerosis is a slowly progressive disease. The phenomenon where plaques are developed in the arteries is referred to as atherogenesis (Figure 1). As reviewed by others (Hansson and Hermansson 2011, Libby et al. 2011), it starts with endothelial activation, where the endothelial cells lining the inner arterial surface interact with inflammatory cells via expression of adhesion molecules, as a response to irritation from for example hypertension, dyslipidaemia or inflammation. Parallel to the interaction with inflammatory cells, the endothelial permeability is increased, promoting entry of low-density lipoproteins (LDL) into the arterial wall. When LDL is modified to oxidized LDL, it is subject of endocytosis by macrophages derived from monocytes, that have migrated through the endothelium. As the intracellular cholesterol increases, the macrophages become cholesterol filled foam cells. In this stage of the atherogenesis, fatty streaks in the vessel wall can be detected. The fatty streaks can disappear, or progress into more

![Figure 1. Progression of atherosclerosis.](https://commons.wikimedia.org/wiki/File:Endo_dysfunction_Athero.PNG)
advanced atheromas and subsequent plaques. Formation of plaques involves synthesis of extracellular matrix and migration of smooth muscle cells from the medial layer of the arterial wall, that forms a fibrous cap surrounding a lipid core containing cholesterol crystals and often necrotic macrophages. In addition to monocytes, T-cells migrate through the intima into the plaque, where they are supposed to have immunoregulatory functions, and also B-cells, neutrophils, dendritic cells and mast cells enter the plaque. Inflammation in the plaque makes the fibrous cap weaker and more prone to rupture, exposing the pro-coagulant material from the core to the coagulation system and thrombocytes, thus causing a thrombus that obstructs the blood flow. A plaque with a thick fibrous cap, containing extracellular matrixproteins and calcium, is less prone to rupture (“stable”), whereas a plaque with a thin fibrous cap and a high grade of inflammation is more prone to rupture (“unstable”) (Muller et al. 1989).

**Cardiovascular risk factors**

Several risk factors for development of cardiovascular disease have been identified. Age, male sex and a family history of premature cardiovascular disease are fixed factors that are not modifiable for an individual, but in the large, international INTERHEART study, the modifiable risk factors abnormal lipids, smoking, hypertension, diabetes, abdominal obesity, psychosocial factors, consumption of fruits, vegetables, and alcohol, and regular physical activity accounted for over 90% of the risk of myocardial infarction (Yusuf et al. 2004). A role of infections in the process of atherogenesis has been hypothesized. Antibodies against human cytomegalovirus (CMV) have been associated with atherosclerosis and cardiovascular disease in several studies (Sorlie et al. 2000, Dahal et al. 2017). Another infectious agent suggestedly associated with atherosclerosis and cardiovascular disease is Chlamydia pneumoniae (Liu et al. 2005, Dahal et al. 2017).

**Cardiovascular risk in patients with RA**

As stated above, patients with RA have an increased risk of cardiovascular disease. Compared with the general population, the morbidity in cardiovascular disease is increased by 50% and the mortality by 60% (Meune et al. 2009, Avina-Zubieta et al. 2012). The exact mechanisms have been hard to identify, but both traditional cardiovascular risk factors and inflammation contribute to the increased risk (Wållberg-Jonsson et al. 1999, Innala et al. 2011, Nurmohamed et al. 2015). In a recent multi-national study using several cohorts of patients with RA, 30% of the cardiovascular events were attributable to RA-characteristics, whereas 49% were attributable to traditional risk factors (Crowson et al. 2018).
When RA-characteristics and traditional risk factors were combined, 70% of the cardiovascular events were explained, leaving 30% of the events unaccounted for. Figure 2 presents results from a meta-analysis of cardiovascular risk in patients with RA.

**Traditional risk factors in patients with RA**
The risk of cardiovascular disease is in both patients with RA and the general population to a great extent explained by the traditional risk factors. In the above mentioned study of patients with RA, smoking and hypertension were the traditional risk factors that had the largest impact on the cardiovascular risk (Crowson et al. 2018). Some of the traditional risk factors are more prevalent in patients with RA, compared with the general population.

*Smoking* is a shared risk factor for both cardiovascular disease and RA, and smoking is more prevalent prior to onset of RA (Kokkonen et al. 2017) and seropositive inflammatory arthritis (Goodson et al. 2004).

*Dyslipidaemia* is more common in patients with RA and ongoing inflammation. Levels of high-density lipoproteins, low-density lipoproteins and total cholesterol are reduced in patients with active RA (Choy and Sattar 2009), but to a great extent normalized when the disease activity is lowered (Steiner and Urowsitz 2009). There are also qualitative changes in the lipoproteins in patients with RA and inflammatory activity (Rantapää-Dahlqvist et al. 1991).

*Glucose tolerance* is impaired in patients with RA (Svenson et al. 1988, Hoes et al. 2011) and the prevalence of diabetes has been reported to be higher (Solomon et al. 2010).

*Hypertension* has been studied in patients with RA, with conflicting results regarding the prevalence, as reviewed by Panoulas (Panoulas et al. 2008). However, hypertension has been reported to be underdiagnosed and undertreated in patients with RA (Stoep et al. 2016).

*Obesity* is a field of conflicting results in patients with RA, but an altered body composition with sarcopenia and increased trunc fat is reported (Book et al. 2009, Dessein et al. 2016, Turk et al. 2018). This condition is often referred to as rheumatoid cachexia.
**RA-associated cardiovascular risk factors**

Patients with RA have cardiovascular risk factors that are associated with the disease. Some of the RA-associated risk factors are mentioned below.

*Inflammation* is associated with an increased risk of cardiovascular disease in patients with RA (Wållberg-Jonsson et al. 1999, Innala et al. 2011). Plausible mechanisms of this finding are inflammation-induced endothelial activation (Libby et al. 2011) and increased instability of plaques due to inflammation (Aubry et al. 2007, Karpouzas et al. 2014).

*Genetic susceptibility* of RA and cardiovascular disease is common for some genetic traits. Studies have mainly focused on the HLA DRB1 shared epitope, that is a risk factor for anti-CCP-positive RA, but also a risk factor for myocardial infarction in both patients with RA (López-Mejías et al. 2016) and the general population (Björkbacka et al. 2010, Paakkanen et al. 2012). On the other hand, PTPN22 is not associated with cardiovascular events nor atherosclerosis in patients with RA (Palomino-Morales et al. 2010). A population-based Swedish study identified an increased risk of acute coronary syndrome in siblings of patients with RA (Westerlind et al. 2019).

*Antibodies against citrullinated peptides* have been suggested to be associated with cardiovascular death in patients with RA (Ajeganova et al. 2016).

Table 3. Previous studies of T-cell markers and atherosclerosis in patients with RA.

<table>
<thead>
<tr>
<th>Reference</th>
<th>T-cell markers</th>
<th>Measures of atherosclerosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gerli et al. 2004</td>
<td>CD4 CD28</td>
<td>IMT</td>
</tr>
<tr>
<td>Pingiotti et al. 2007</td>
<td>CD4 CD28 CX3CR1</td>
<td>IMT, FMD</td>
</tr>
<tr>
<td>Winchester et al. 2016</td>
<td>CD4 CD8 CD28 CD56 CD57 HLA-DR</td>
<td>CAC</td>
</tr>
</tbody>
</table>

IMT: intima-media thickness. FMD: Flow-mediated dilation. CAC: coronary artery calcification
Neutrophil extracellular traps (NETS) are chromatin and proteins released by neutrophils to control infections (Brinkmann and Zychlinsky 2012). NETS are dysregulated in patients with rheumatoid arthritis (Apel et al. 2018), but also in atherogenesis (Qi et al. 2017).

CD28null T-cells are expanded in patients with RA (Schmidt et al. 1996). Increased numbers of CD28null T-cells are associated with atherosclerosis and an increased risk of cardiovascular disease in the general population (Dumitriu et al. 2009). No studies have examined the relation between CD28null T-cells and cardiovascular disease in patients with RA, but CD28null T-cells are associated with atherosclerosis in patients with RA (Gerli et al. 2004). Table 3 summarizes previous studies of T-cells and atherosclerosis in patients with RA.

Osteoporosis is associated with increased risk of cardiovascular disease and atherosclerosis (den Uyl et al. 2011). Osteoporosis is more common in patients with RA compared with the general population (Haugeberg et al. 2000). Patients with RA and a fragility fracture have almost a doubled risk of a cardiovascular event, compared with patients without fractures (Ni Mhuircheartaigh et al. 2017). Table 4 summarizes previous studies of atherosclerosis and aspects of bone turnover in patients with RA.

Table 4. Previous studies of bone markers and atherosclerosis in patients with RA.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Bone marker</th>
<th>Measures of atherosclerosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tanaka et al. 2006</td>
<td>Bone mineral density</td>
<td>Pulse-wave velocity</td>
</tr>
<tr>
<td>Asanuma et al. 2007</td>
<td>Osteoprotegerin</td>
<td>CAC</td>
</tr>
<tr>
<td>Asanuma et al. 2013</td>
<td>Osteoprotegerin</td>
<td>Carotid plaque</td>
</tr>
<tr>
<td>Dessein et al. 2014</td>
<td>Osteoprotegerin</td>
<td>Intima-media thickness, Markers of endothelial activation</td>
</tr>
<tr>
<td>Beyazal et al, 2015</td>
<td>Osteoprotegerin</td>
<td>Pulse-wave velocity, Intima-media thickness</td>
</tr>
<tr>
<td>Provan et al. 2017</td>
<td>Bone mineral density</td>
<td>Cardiovascular death</td>
</tr>
<tr>
<td></td>
<td>Osteoporosis</td>
<td></td>
</tr>
<tr>
<td>Ni Mhuircheartaigh et al. 2017</td>
<td>Fragility fracture</td>
<td>Cardiovascular event</td>
</tr>
</tbody>
</table>

CAC: coronary artery calcification
Impact of risk factors in patients with RA

The impact of cardiovascular risk factors in patients with RA is somewhat different from what we know from the healthy population. According to a large study of heterogenous international cohorts (Crowson et al. 2018) and a meta-analysis (Baghdadi et al. 2015), hypertension, smoking, diabetes and hypercholesterolaemia are associated with an increased cardiovascular risk, whereas BMI and physical inactivity are not. When RA-associated factors are assessed, the impact of high inflammatory activity and positivity for RF or anti-CCP were comparable to the impact of smoking and hypertension, and higher than the impact of blood lipids and diabetes, as presented in figure 3 (Crowson et al. 2018).

There are also several studies of patients with RA, where treatment with tumor necrosis factor (TNF) inhibitors and methotrexate have been associated with a lower cardiovascular risk (Choi et al. 2002, Barbhaiya and Solomon 2013), but whether this is due to a cardioprotective effect of the drugs, reduction of inflammation or selection of patients to treatment is not sufficiently known. However, the risk of myocardial infarction in patients with RA is associated with increased ESR and CRP (Meissner et al. 2016), and a Swedish study showed that patients with a good response to treatment with TNF inhibitors have a cardiovascular risk comparable to the risk in the general population (Ljung et al. 2016). On the other hand, endothelial function is improved independent of inflammatory response, when treatment with methotrexate and TNF inhibitors is initiated in patients with RA (Deyab et al. 2017), indicating that the effect possibly is explained not only by reduced inflammation.

Assessment of atherosclerosis

Atherosclerosis is in most cases subclinical and causes no symptoms, until the affected individual suffers from an atherosclerotic cardiovascular event. Therefore, methods have been developed to study subclinical atherosclerosis.

Flow-mediated dilation

Flow-mediated dilation (FMD) uses ultrasound to measure the diameter of the brachial artery before and after occlusion (Celermajer et al. 1992). The endothelium produces vasodilating nitric oxide (NO) when the artery is occluded, but dysfunctional endothelium, an early phenomenon in atherogenesis, has reduced capacity of NO production, thus making FMD reduced. FMD is a method that requires a standardized procedure, but performed adherent to guidelines,
FMD is reproducible and reliable (Greyling et al. 2016). Decreased FMD is a predictor of future atherosclerotic cardiovascular disease (Perticone et al. 2001, Matsuzawa et al. 2015).

**Pulse wave velocity**
Arterial stiffness is a sign of atherosclerosis, that can be measured as increased pulse wave velocity (PWV). The method is based on the fact that the velocity of the pulse wave increases with reduced elasticity in the arterial wall. Several methods for assessing PWV and arterial stiffness have been developed (Laurent et al. 2006). Increased PWV is a predictor of future atherosclerotic cardiovascular disease (Blacher et al. 1999, Laurent et al. 2006, Simon et al. 2007).

**Intima-media thickness**
Once the atherogenesis is started, the thickness of the innermost part of the arterial wall (intima and media layers) is increased. This can be measured using B-mode ultrasound, where the intima-media thickness (IMT) is measured, usually in the far wall of the common carotid artery proximal to the bulb (Pignoli et al. 1986). Increased IMT is a predictor of future atherosclerotic cardiovascular disease (Chambless et al. 1997, Simon et al. 2007).

**Assessment of coronary artery calcium by computed tomography**
X-rays are attenuated as they pass through calcified matter. This holds true also for calcified plaques, that can be detected using x-ray techniques. The most commonly used method for assessment of calcified plaques is computed tomography (CT) of the coronary arteries and subsequent quantification of coronary artery calcium (CAC) according to Agatson (Agatston et al. 1990). This method detects both fully calcified plaques, that are considered stable, and plaques with heterogenous calcification, that are more prone to rupture. Nevertheless, a higher CAC is a predictor of future atherosclerotic cardiovascular disease (Detrano et al. 1996, Simon et al. 2007).

**Positron-emission tomography**
Positron-emission tomography (PET) is a nuclear medicine functional imaging method that uses radioactive tracers to observe metabolic processes. When PET is combined with computed tomography (PET-CT), the anatomical structure corresponding with the tracer uptake can be located. $^{18}$F-fluorodeoxyglucose ($^{18}$F-FDG), an analogue to glucose, is a tracer that is concentrated in tissues with increased metabolism, e.g. sites of inflammation, and has been used to identify inflammation in plaques, whereas $^{18}$F-sodium fluoride ($^{18}$F-NaF) is a marker of bone mineralization and vascular calcification (McKenney-Drake et al. 2018). Long-term studies of the prognostic value of PET-CT are scarce, but arterial
uptake of $^{18}$F-FDG in aorta has been associated with future cardiovascular events in one study (Iwatsuka et al. 2018). Traditional cardiovascular risk factors are stronger associated with arterial wall uptake of $^{18}$F-NaF, than with uptake of $^{18}$F-FDG (McKenney-Drake et al. 2018).

**Management of cardiovascular risk**

It has been estimated that 90% of cardiovascular disease in the general population could be prevented (McGill Henry C. et al. 2008). Since atherosclerotic cardiovascular disease is the leading cause of increased mortality in patients with RA, prevention of disease is an urgent task in this population. Very recently an interventional study was published, where intense treatment of traditional cardiovascular risk decreased the risk of atherosclerotic cardiovascular events and reduced the rate of progress of IMT in patients with RA (Burggraaf et al. 2019). This implies that the cardiovascular risk in patients with RA actually can be reduced by successful treatment of both RA and traditional cardiovascular risk factors, but in order to maximize the impact of primary prevention, patients with a high risk must be identified.

**Estimation of cardiovascular risk**

Several tools have been developed to calculate the risk of a cardiovascular event or cardiovascular death for an individual, based on the traditional risk factors. When used in patients with RA, these risk estimation tools do not perform well, mostly due to underestimation of the risk (Crowson et al. 2012, 2017a, Arts et al. 2015a). A modification of the European Systematic Coronary Risk Evaluation algorithm (SCORE) that included RA-characteristics was developed, but did not perform much better than the original algorithm (Arts et al. 2015b). Neither did the RA-specific calculator created by the Trans-Atlantic Cardiovascular Risk Consortium for Rheumatoid Arthritis perform better than calculators for the general population (Crowson et al. 2017b). Another RA-specific risk calculator, the Expanded Risk Score for cardiovascular disease in RA (ERS-RA) was developed using the North American patient cohort CORRONA (Solomon et al. 2015), but tested in a multi-national cohort of patients with RA, it did not outperform risk calculators for the general population (Crowson et al. 2017a), although ERS-RA performed well when externally validated in Swedish patients with RA (Ljung et al. 2018). Table 5 presents previous studies of cardiovascular risk scores in patients with RA. The EULAR recommendations for management of cardiovascular risk, state that the estimated risk from general population risk scores shall be multiplied by 1.5 for patients with RA (Agea et al. 2017).
Table 5. Previous studies of cardiovascular risk estimation scores in patients with RA.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Risk scores studied</th>
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<tbody>
<tr>
<td>Crowson et al, 2012</td>
<td>Framingham</td>
</tr>
<tr>
<td></td>
<td>Reynolds</td>
</tr>
<tr>
<td>Arts et al, 2015a</td>
<td>SCORE</td>
</tr>
<tr>
<td></td>
<td>Framingham</td>
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<tr>
<td></td>
<td>Reynolds</td>
</tr>
<tr>
<td></td>
<td>QRiskII</td>
</tr>
<tr>
<td>Arts et al, 2015b</td>
<td>SCORE</td>
</tr>
<tr>
<td></td>
<td>Adapted SCORE</td>
</tr>
<tr>
<td>Crowson et al, 2017a</td>
<td>ERS-RA</td>
</tr>
<tr>
<td></td>
<td>Framingham</td>
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<tr>
<td></td>
<td>Reynolds</td>
</tr>
<tr>
<td></td>
<td>QRiskII</td>
</tr>
<tr>
<td></td>
<td>ACC/AHA</td>
</tr>
<tr>
<td>Crowson et al, 2017b</td>
<td>SCORE</td>
</tr>
<tr>
<td></td>
<td>Framingham</td>
</tr>
<tr>
<td></td>
<td>QRiskII</td>
</tr>
<tr>
<td></td>
<td>ACC/AHA</td>
</tr>
<tr>
<td></td>
<td>New developed score</td>
</tr>
<tr>
<td>Ljung et al, 2018</td>
<td>ERS-RA</td>
</tr>
</tbody>
</table>

AIMS

Although there is overwhelming evidence that patients with RA have an increased prevalence of atherosclerosis and atherosclerotic cardiovascular disease, the underlying pathogenic mechanisms are not sufficiently known. Neither is there enough knowledge of how the risk of cardiovascular disease in patients with RA should be estimated. The aims of this thesis were to address these tasks, with an emphasis on the following:

- To validate the performance of a cardiovascular risk score specific for patients with RA.

- To investigate factors associated with coronary artery calcification in patients with long-term RA.

- To investigate the impact of cytomegalovirus and subsets of T-cells on development of atherosclerosis after onset of RA.

- To investigate the associations between atherosclerosis, bone mineral density, and regulators and markers of bone turnover after onset of RA.
Study populations

The studies included in this thesis are undertaken in cohorts of patients with RA in Northern Sweden. Papers III and IV also include matched controls. Figure 4 presents the cohorts included in papers I, III, and IV.

Paper I

Paper I uses data from a cohort of patients with early RA in the four northernmost counties of Sweden (Jämtland, Västernorrland, Västerbotten and Norrbotten), that was initiated in 1995. All patients fulfilling the 1987 criteria for RA (Arnett et al. 1988) and with symptoms of RA for less than one year, are invited to the ongoing study. Paper I uses data from patients included until October 2009. The number of patients was initially 950, but was reduced to 810 after exclusion of patients with a previous cardiovascular disease, or without any data on inflammatory activity at inclusion. ERS-RA estimates 10-year risk of a cardiovascular event for patients 20-80 years of age. However, the American College of Cardiology/American Heart Association risk score (ACC/AHA), that was used as comparator in the study, does not calculate 10-year risk for patients less than 40 years of age. Those patients were consequently excluded, leaving 665 patients (458 female, 207 male) 40-80 years old for risk estimation with ERS-RA. The upper limit of age for risk estimation with ACC/AHA is 79 years, making the number of patients 662 (455 female, 207 male) in risk estimation with ACC/AHA.

Paper II

Paper II is in part a follow-up of an earlier study (Wållberg-Jonsson et al. 2001), where all eligible patients diagnosed with RA at the Department of Rheumatology at Umeå university hospital 1974-1978 and less than 66 years old at the time of examination, were invited. All patients fulfilled the ACR 1987 criteria for RA (Arnett et al. 1988). That previous study included 39 patients, but at the time of follow-up, seven patients were deceased and one emigrated. Since seven patients declined inclusion in the follow-up, most of them due to severe disability, and two patients did not complete the CT examination, 22 patients (18 female, 4 male) were included.

Paper III and IV

Papers III and IV report data from an ongoing longitudinal study of atherosclerosis in patients with RA. The cohort is a subpopulation of the cohort in paper I. In the years 2000-2004, all patients with early RA, not more than 60 years old and resident in Jämtland, Västerbotten or Norrbotten were consecutively invited to participate in the study. Of the 87 patients invited, six declined participation
and two were excluded (one due to pregnancy, one due to advanced malignancy), leaving 79 patients that were included in the baseline examinations (To). Follow-up was undertaken after eleven years (n=62), hereafter denominated T11. Of the 62 patients examined at T11, eight patients did not have data on IMT due to a hardware crash and four were not assessed by flow-cytometry analysis, making the number of patients 50 in paper III and 54 in paper IV. At baseline, 44 controls matched for age and sex were included, of whom 31 were re-examined at T11. A subset of 26 unselected patients and their 27 matched controls were subjects to an additional examination 18 months after inclusion (T1.5).

Figure 4. Illustration of the cohorts in papers I, III and IV. All patients were intially included in the regional cohort of patients with new-onset RA.
Methods

The studies included in this thesis include data from several different measures of clinical, laboratory and radiological examinations, that are described in the following sections.

Measures of disease activity

In papers I, III and IV, data on disease activity was read from the Swedish register of early RA and completed by thorough reading of patients´ records, whereas in paper II the data on disease activity was registered at the study examination. In all papers, the data on disease activity includes number of swollen and tender joints, VAS scales for pain and global assessment, HAQ, DAS28, and the laboratory measures ESR and CRP. Calculation of CDAI at baseline was undertaken in paper I. In paper II, haptoglobin, soluble receptor of interleukin 2 (IL2sR), and interleukin 6 were analysed as measures of inflammation, and accumulated disease activity (Baecklund et al. 1998) was calculated at the baseline examination 1997-1998.

Cardiovascular risk factors

In order to assess the presence of cardiovascular risk factors at the time of diagnosis of RA, all patients´ records until the time of diagnosis of RA were thoroughly read and cardiovascular risk factors registered according to a protocol for every patient in paper I, III, and IV. In papers III and IV, patients´ records were also read during the study. A survey including questions on cardiovascular risk factors was sent to the patients in paper I when they were included in the study. The patients in papers II, III and IV filled in surveys on cardiovascular risk factors at the study examinations.

In the patients in papers I, II and IV, cholesterol, high-density lipoproteins (HDL), low-density lipoproteins (LDL) and triglycerides were analysed at the time of diagnosis of RA or soon thereafter, completed by repeated analysis at the subsequent study examinations for the patients in papers III and IV. Analyses were done by routine methods at the local laboratories of clinical chemistry at the present hospital, where the patient was examined. In paper III, fasting blood lipids were analysed at the study examination.

Risk estimations and outcomes

The risk of a cardiovascular event for the patients in paper I was estimated by both the ERS-RA and the ACC/AHA algorithm. Risk estimations using both risk scores were for every individual patient performed according to the algorithms,
based on collected data, completed by multiple imputation for missing values. The risk was calculated from ERS-RA in two settings: the first estimation used patients’ and physicians’ reported data on hypertension and hyperlipidaemia, whereas the second estimation used measured values of blood pressure and blood lipids. Hypertension was defined as blood pressure >140/90 and hyperlipidaemia defined according to the Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) Final Report (Wilkins 2002). The estimations from ACC/AHA was analysed both as crude risk estimation, and multiplied by 1.5 according to the EULAR recommendations (Agca et al. 2017).

Data on the cardiovascular outcomes for the patients in paper I was retrieved from the Cause-of-Death-register and National Inpatient register at the National Board of Health and Welfare (Socialstyrelsen 2017). Events until December 31 2013 were included.

**Radiographs**

In patients included in paper IV, conventional radiographs of hands, wrists and feet from baseline were analysed by trained rheumatologists and evaluated according to Larsen score (Larsen 1995).

**Bone mineral density**

Bone mineral density (BMD) was measured in patients and controls resident in Västerbotten, using dual-energy x-ray absorptiometry (DXA) (Lunar DPX-L, software version 1:3, Lunar, Madison, WI, USA). This technique is based on an X-ray generator using two energies (“dual energy X-ray”; DXA). Using DXA, bone can be separated from surrounding tissues, due to the different attenuations of the x-rays (Pietrobelli et al. 1996). BMD for the spine was derived from the whole body composition scan using region of interest for the spine.

**Biomarkers**

In paper II, several previously analysed biomarkers were included in the analysis. These biomarkers included presence of antibodies with affinity for oxidized LDL of immunoglobulin class A (IgA) (ox-LDL IgA), immunoglobulin class G (IgG) (ox-LDL IgG) and immunoglobulin class M (IgM) (ox-LDL IgM), as well as antibodies directed against malondialdehyde-modified LDL of classes IgA (MDA-LDL IgA), IgG (MDA-LDL IgG) and IgM (MDA-LDL IgM). Endothelial activation and adhesion molecules were analysed, including soluble intercellular adhesion molecule-1 (ICAM-1), E-selectin, and circulating immune complexes (CIC).
Hemostatic factors analysed were von Willebrand factor, plasminogen activator inhibitor-1 mass (PAI), tissue plasminogen activator antigen (tPA), D-dimer, fibrinogen and anti-cardiolipin antibodies of class IgA, IgG and IgM. Leptin, lipoprotein(a) and homocystein levels were also analysed. The procedures of analyses have previously been described in detail (Wällberg-Jonsson et al. 2001, 2002).

**Markers of bone turnover**

Markers of bone turnover were analysed in frozen samples collected at baseline and T11, presented in table 6. The samples were drawn at the study examinations at baseline and T11, and then stored at -80° Celsius.

Serum concentrations of dickkopf-1, osteopontin, osteoprotegerin (OPG), sclerostin, osteocalcin (OCN), and parathyroid hormone (PTH) were determined using a multiplex assay (HBNMAG-51K-07, Millipore Corporation, Billerica, MA) according to manufacturer’s protocol. Serum concentration of receptor activator of nuclear factor κB ligand (RANKL) was analysed using an enzyme linked immune-assay (ELISA; Human RANKL ELISA (BioVendor, Karasek, Czech Republic)) according to the manufacturer’s protocol. All analyses were done in duplicates. Procollagen type I N-terminal propeptide (P1NP), C-terminal cross-linked telopeptide (CTX), thyroid stimulating hormone (TSH), thyroxin, 25-OH vitamin D, phosphate, and calcium were analysed at the laboratory of clinical

<table>
<thead>
<tr>
<th>Bone marker</th>
<th>Baseline</th>
<th>T11</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dickkopf-1</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Osteopontin</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Osteoprotegerin</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Sclerostin</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Osteocalcin</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Parathyroid hormone</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Fibroblast growth factor 23</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>RANKL</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>P1NP</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>CTX</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>TSH</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Thyroxin</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>25-OH vitamin D</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Phosphate</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Calcium</td>
<td>X</td>
<td></td>
</tr>
</tbody>
</table>

**Table 6.** Bone markers studied in paper IV.

RANKL: receptor activator of nuclear factor κB ligand. P1NP: procollagen type 1 N-terminal propeptide. CTX: C-terminal cross-linked telopeptide. TSH: thyroid stimulating hormone.
chemistry at Karolinska University Hospital, using routine methods. Table 6 presents the bone markers.

**Antibodies**

Analysis of RF was performed by Waaler-Rose hemagglutination test at the local hospitals using local routine methods. ACPA was determined using an ELISA for anti-CCP2 antibodies (Euro-Diagnostica, Malmö, Sweden) at the local hospitals.

**CMV serologies**

In paper III, analysis of anti-CMV IgG-antibodies was done from frozen plasma samples collected at T0 and T11, stored at -80°C. An in-house ELISA was used at Karolinska institutet (Sundqvist and Wahren 1982, Rahbar et al. 2004). Paired samples from patients and matched controls were assessed on the same plate.

**Flow cytometry**

In paper III, flow-cytometry analysis of T-cells subsets was undertaken at T11, using a LSRII flow cytometer (Becton-Dickinson, San Jose, CA). The cell surface markers analysed were CD45 (common leukocyte antigen), CD3 (T-cell marker), CD4, CD8, CD28, CD56, and CX3CR1. Obtained data was analysed using the BDDiva software (BD Biosciences, San Jose, CA, USA).

**Carotid ultrasound**

Ultrasound examinations of the right common carotid artery were performed with the patient in supine position and the head turned to the left, using a Sequoia 512 and an 8L5 linear transducer (both from Siemens, Acuson, Upplands Väsby, Sweden) at 8MHz. Three longitudinal measurements of IMT in the far wall in an approximately 10 mm long segment proximal to the bulb was undertaken. The average of the three measurements was calculated and used in further analysis.

**Coronary artery CT**

In paper II, measurement of CAC was performed by multidetector computed tomography (LightSpeed VCT, GE Healthcare, Milwaukee, WI, US). The images were analysed on a workstation (Advantage 4.4, GE Medical systems, Milwaukee, WI, USA) and CAC was detected by a dedicated software (SmartScore version 4.0, GE Medical systems, Milwaukee, WI, USA). The CAC score was quantified according to the Agatston method (Agatston et al. 1990). Patients with CAC 0-10 were classified as having low CAC, whereas patients with CAC >10 were classified as having high CAC. This classification was based on a large study (Budoff et al. 2007), where CAC >10 was a strong independent predictor of mortality, but
individuals with CAC 1-10 had only a small increase in mortality compared to individuals without CAC.

**Genetic analyses**
A polymerase chain reaction kit with sequence specific primers (Dynal, Oslo, Norway) was performed for genotyping of the HLA-DRB1 shared epitope alleles *0101, *0401, *0404, *0405, and *0408. The PTPN22 1858T/C polymorphism was identified using a 5´nuclease assay. Detection of the genotypes was made using ABI PRISM 7900HT Sequence Detector System and the data was processed using the SDS 2.1 software (both Applied Biosystems, Foster City, CA, USA).

**Statistical analyses**
For comparison of mean or median values between groups, Mann-Whitney U test or student´s t-test was used, depending on distribution. Kruskal-Wallis test was used when more than two groups were compared, Bonferroni corrected in pairwise comparisons. Linear and logistic regression models were used for analysis of associations between variables. Variables with skewed distribution were log-transformed before entering linear regression models. Linear regression models initially included several independent variables chosen on clinical assumptions and results from univariable regression, but variables that lowered $R^2$ were subsequently excluded. Goodness of fit of multivariable logistic regression models was tested with Hosmer-Lemeshow test. Collinearity of independent variables was tested in multiple linear regression models, and a variable inflation factor of more than four was regarded intolerable. P-values <0.05 were regarded statistically significant.

In paper I, data from onset of RA was used for calculation of every patient´s individual 10-year risk for a cardiovascular event. When the duration of follow-up was less than 10 years, the risk was adjusted proportionally to the time of follow-up. Multiple imputation with five repetitions was performed when there was a missing value of a risk factor. The accuracy of estimations was assessed in terms of calibration (comparing observed and estimated events) and discrimination (the ability to correctly rank individuals from low to high risk). Calibration was assessed using deciles of estimated risk, comparing observed events and estimated risk. Discrimination was assessed by calculation of the area under the curve (AUC) of receiver operating characteristic (ROC) curves.

In paper II, multivariate discriminant analysis was performed using the orthogonal projection to latent structures discriminant analysis (OPLS) algorithm (Trygg and Wold 2002). The concept of OPLS is transition from a large number of descriptive variables, to a small number of vectors, representing latent
variables. The first latent variable is the latent variable that best explains the variation in the response variable, whereas the subsequent latent variables are orthogonal to vector 1, meaning that they represent information that varies in a non-random pattern, but is independent of the response variable. The models were modified by repeated exclusion of variables without importance in the model (variable importance in projection (VIP) <0.5). ROC-curves were made for each final model, to evaluate sensitivity and specificity in predicting high or low CAC.

Statistical analyses were performed using SPSS for Windows version 21-25 (IBM SPSS INC, Chicago, Illinois, USA) or, for OPLS, Simca version 13.0 (Umetrics, Umeå, Sweden).
Results

Paper I

In paper I, the risk estimations from the RA-specific risk score ERS-RA in 665 patients (458 female, 207 male) patients with new-onset RA were validated. The estimations were compared with the actual outcomes, and with the risk estimations from the general population ACC/AHA risk score. The risk score ERS-RA was used in two settings: one based on patients´ and physicians´ reports on hyperlipidaemia (“ERS-RA reported”), and one based on measured values of blood pressure and blood lipids (“ERS-RA measured”). The ACC/AHA risk score was analysed in two settings as well: crude data (ACC/AHA), and multiplied by 1.5 as recommended by EULAR (ACC/AHAx1.5) (Agca et al. 2017).

The estimated median 10-year risk was 6.3% for ERS-RA (reported), 8.3% for ERS-RA (measured), 6.3% for ACC/AHA and 9.2% for ACC/AHAx1.5. During the follow-up, 73 patients (11.0%) developed a cardiovascular event (9 cases of death due to cardiovascular disease, 25 patients suffered a myocardial infarction, 39 patients suffered a stroke). Of the female patients, 38 (8.3%) had an event, compared with 35 (16.9%) of the male patients. One event occurred in a patient 80 years old at inclusion, making the number of observed events 72 when ACC/AHA data was analysed. Figure 5 illustrates the percentage of observed and estimated events in deciles of estimated risk. When the deciles of observed and estimated events for each of the four risk estimation scores were compared with the Hosmer-Lemeshow test, ERS-RA (reported) had the lowest agreement and the two models of ACC/AHA the highest agreement between estimated and predicted events. Discrimination (ranking the patients according to risk) was good for all four risk estimation scores with AUC of 0.76-0.77.

All risk scores were less accurate in terms of discrimination in patients with 5-15% estimated risk. ERS-RA (reported) underestimated remarkably, with lower AUC than the other risk scores (0.52 vs 0.59-0.60) and also the highest grade of underestimation in that subset of patients.

In high-inflammatory patients (defined as ESR ≥ 40), the calibration of all risk scores was poor. The observed proportion of events in this subset of patients was 12.1%, whereas the estimated proportion was 7.2%, 9.8%, 7.6% and 11.4% for ERS-RA (reported), ERS-RA (measured), ACC/AHA, and ACC/AHAx1.5 respectively. Thus, ACC/AHA adjusted according to EULAR recommendations (ACC/AHAx1.5), produced higher risk estimations than both variants of ERS-RA, although ERS-RA includes variables on disease activity.
The distribution of events in patients classified to a risk <7.5% or ≥7.5% was investigated for the risk scores. The positive and negative predictive values of an estimated risk <7.5% or ≥7.5% did not differ much, but both the highest positive and negative predictive values were seen for ERS-RA (measured). The risk scores were compared to each other in terms of reclassification of patients with future events from the low-risk (<7.5%) to the high-risk group (≥7.5%). Using ERS-RA (reported) as comparator, ERS-RA (measured) and both variants of ACC/AHA correctly reclassified patients with future events to the high-risk group (≥7.5%).

**Figure 5.** Observed and predicted proportion of events in deciles of estimated risk from ERS-RA (reported) (upper left), ERS-RA (measured) (upper right), ACC/AHA (lower left), and ACC/AHAx1.5 (lower right). Black bars are observed proportion of events, grey bars estimated proportion of events.
Paper II

Paper II reports the results from a long-term follow-up of the first study that identified a higher prevalence of atherosclerosis in patients with RA. The first study (“baseline”) was undertaken in 1997-1998 and included both IMT and several biomarkers (Wållberg-Jonsson et al. 2001, 2002). In the present follow-up, the presence of CAC was examined, and factors associated with presence of CAC were investigated.

The study included 22 patients (4 male/18 female, mean age 65 years, RA-duration 30-36 years) from the baseline (n=39) cohort of patients with seropositive RA. The amount of CAC was quantified according to Agatston (Agatston et al. 1990). Patients with CAC 0-10 were classified as having low CAC (n=10), whereas patients with CAC >10 (range 18-1700) were classified as having high CAC (n=12), a classification based on previous studies (Budoff et al. 2007). Patients with high CAC had significantly higher ESR (24.3 vs 9.9 mm/h), swollen joint count (2 vs 0) and DAS 28 (3.61 vs 2.31) than patients with low CAC.

When patients with high or low CAC were analysed in multivariable OPLS models, the information in the independent variables from baseline and follow-up to a great extent separated the two groups of patients from each other. In the first OPLS model (“model 1”), independent variables both from baseline and follow-up were included. Figure 6 illustrates how patients with high CAC and low CAC are separated by the score vectors in the model. Score vector 1 is the latent variable that best separates patients with CAC 0-10 from patients with CAC >10, whereas score vector 2 contains information that does not separate the two groups of patients from each other, but still varies in a non-random manner. Figure 7 presents how the variables in the model are related to each other and how they contribute to the discrimination between patients with high CAC and low CAC. The more to the right a variable is plotted, the stronger is the association with high CAC. R² for this model was 0.87. Subsequent ROC analysis displayed a sensitivity of 89% and a specificity of 85% in discriminating between high or
When the baseline ultrasound variables (IMT and plaque) were omitted from the model, \( R^2 \) was 0.86, sensitivity was 80% and specificity 83%.

The next OPLS model to be investigated included baseline variables, but not the follow-up variables, nor delta values. This model was tested to investigate if the baseline variables could predict CAC status 13 years later. \( R^2 \) for this model was 0.67, sensitivity 73% and specificity 82% in separating patients with high CAC from patients with low CAC. Variable importance in projection is presented in Figure 8. All variables except HDL and leptin were positively related to high CAC.
The last OPLS model (not shown) included initially all baseline variables but IMT and plaque, yielding an $R^2$ of 0.58. Sensitivity was 67% and specificity 80% in predicting high or low CAC at follow-up 13 years later.

**Papers III and IV**

Papers III and IV comprise the same cohort of patients with early RA, in whom atherosclerosis has been prospectively studied with ultrasound measurement of carotid IMT. A cohort of matched controls is examined as well. Paper III reports the associations between IMT measured at baseline (T0), after 1.5 years (T1.5) and after 11 years (T11), infection with CMV at T0 and CD28null T-cells assessed at T11. Paper IV reports the associations between IMT, bone mineral density at T11, and markers of bone turnover measured both at T0 and T11. This paper includes data from 50 patients with RA and 29 controls. In both patients and
controls, 66% of the individuals were IgG-positive for CMV at T0. Neither did the concentration of antibodies differ between patients and controls.

Patients with RA who were CMV IgG-positive had a rapid increase in IMT after onset of RA, compared with controls and CMV IgG-negative patients, as presented in Figure 9. However, the number of individuals in each category was small, contributing to p-values 0.01-0.08 in pairwise comparisons of IMT at T1.5.

Furthermore, CMV-positive patients had a higher percentage of CD28null T-cells of both CD4+ (median 7% vs 0.8%) and CD8+ types (mean 52% vs 27%). Regression models revealed significant associations between presence of CMV IgG and CD28null T-cells of both CD4+ and CD8+ types (Table 7). Since the median percentage of CD4+CD28null T-cells have been determined to 1.6-1.9% in healthy individuals of the general population (Schmidt et al. 1996, Goronzzy et al. 2001), a level of CD4+CD28null ≥2% was regarded elevated in further analyses of the relation between CD4+CD28null T-cells and other variables.

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**Figure 9.** Median IMT in patients with early RA during 11 years of follow-up. Results from carotid ultrasound in 20 patients with RA and 17 controls, grouped by CMV-status. Pairwise comparisons of change in IMT from To to T1.5 (CMV-positive patients as reference): CMV-positive controls p=0.06; CMV-negative controls p=0.01; CMV-negative patients p=0.08. CMV-positive patients n=10; CMV-positive controls n=12; CMV-negative controls n=5; CMV-negative patients n=10.
When relations between subsets of T-cells and IMT at T11 were analysed in linear regression models, a elevated percentage of CD4+CD28null T-cells (≥2%) was associated with a higher IMT at T11, adjusted for systolic blood pressure (Table 8). Furthermore, there was a positive association between the percentage of CD8+CD28null T-cells and IMT, adjusted for blood pressure (Table 8). In regression models including age, neither CD28null T-cells nor blood pressure were significantly associated with IMT at T11. CX3CR1 was abundant in CD28null T-cells of both CD4+ and CD8+ type, but CX3CR1 per se was not associated with IMT (data not shown).

Paper IV presents data from examinations of 54 patients and 31 controls, in whom several biomarkers associated with bone turnover were measured both at T0 and T11. Moreover, BMD was measured at T11 in patients resident in Västerbotten. When patients and controls were compared, there were no significant differences in BMD or concentrations of bone biomarkers, except for OPG (higher in controls at T0 and higher in patients at T11) and PTH (higher in controls at T0).

<table>
<thead>
<tr>
<th>Variable</th>
<th>CD4+CD28null &gt;2%, yes (logistic regression)</th>
<th>CD8+CD28null, % (linear regression)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, year</td>
<td>1.0 (0.95; 1.15)</td>
<td>0.75 (0.1; 1.4)*</td>
</tr>
<tr>
<td>Anti-CCP, yes</td>
<td>0.41 (0.07; 2.3)</td>
<td>-2.1 (-13.3; 9.2)</td>
</tr>
<tr>
<td>Positive CMV IgG at T0, yes</td>
<td>20.3 (3.2; 130)**</td>
<td>16.2 (3.1; 29.3)*</td>
</tr>
</tbody>
</table>

R² 0.55                              R² 0.32

T11: Follow-up eleven years from baseline. *: p<0.05. **: p<0.01.

Table 7. Multivariable regression models with CD4+CD28null and CD8+CD28null at T11 as dependent variables.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Model 1 B (95% CI)</th>
<th>Model 2 B (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD8+CD28null, %</td>
<td>0.02 (0.006; 0.04)*</td>
<td></td>
</tr>
<tr>
<td>CD4+CD28null ≥2%, yes</td>
<td>0.93 (0.12; 1.7)*</td>
<td></td>
</tr>
<tr>
<td>Systolic blood pressure</td>
<td>0.05 (0.02; 0.07)**</td>
<td>0.05 (0.02; 0.07)**</td>
</tr>
<tr>
<td>T11, mmHg</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

R² 0.23                              R² 0.25

T11: Follow-up eleven years from baseline. *: p<0.05. **: p<0.01.

Table 8. Multivariable linear regression models with IMT at T11 (1/10 mm) as dependent variable.
In patients with RA, OPG and OCN at T11 were significantly associated with IMT at T11, adjusted for age and systolic blood pressure (Table 9). Change in IMT from T0 to T11 was associated with OPG at T0 (B 2.0, p=0.07) and OCN at T0 (B -0.07, p=0.02). However, none of the collagen derived markers of pure bone turnover P1NP and CTX, nor BMD measured at T11, was significantly associated with IMT at T11, in linear regression models including traditional cardiovascular risk factors.

The relation between IMT, OPG and OCN was more pronounced in patients with erosions at baseline, where a linear regression model with IMT at T11 as dependent variable and independent variables OPG (B 9.6, p=0.005) and OCN (B -0.2, p=0.04) yielded an adjusted R² of 0.45. Furthermore, RANKL at T11 showed a strong association with IMT at T11 in patients with erosions at onset of RA (standardized β=0.98, p=3x10⁻⁵).
Discussion

As shown in paper I, cardiovascular risk estimation in patients with RA still needs to be improved. The RA-specific risk score ERS-RA did not make more accurate risk estimations than the ACC/AHA risk score designed for the general population, although ERS-RA comprises not only traditional cardiovascular risk factors, but also variables reflecting inflammatory activity in RA, corticosteroid use and duration of RA. Inflammation is well known to be associated with a higher risk of cardiovascular disease in patients with RA (Wållberg-Jonsson et al. 1999, Maradit-Kremers et al. 2005, Innala et al. 2011), so inclusion of this parameter in a risk score is expected to make it perform better than risk scores for the general population. ERS-RA has recently been externally validated in large Swedish cohorts and found to perform well (Ljung et al. 2018), but it was not compared to other risk scores. When compared to risk scores for the general population, no RA-specific risk score has outperformed the general population risk scores (Arts et al. 2015a, Crowson et al. 2017a). Neither did an RA-adapted version of SCORE (Arts et al. 2015b), nor the RA-specific risk score developed by the ATACC-RA consortium perform (Crowson et al. 2017b) better than risk scores for the general population. This raises questions: are assessments of the traditional risk factors reliable in patients with RA? Are the variables reflecting disease activity and severity in RA reliable in the context of risk estimation?

The first question could be answered no. We know that blood lipids in patients with ongoing inflammation in general are lower (Choy and Sattar 2009) and that there are qualitative changes in the blood lipids as well (Rantapää-Dahlqvist et al. 1991). Furthermore, hypertension and hyperlipidaemia are underdiagnosed and undertreated in patients with RA (Burggraaf et al. 2019). This points to the need of activity in risk estimation – when risk factors are not assessed, they cannot be included in risk estimation, and neither can they be treated. In ERS-RA, not only smoking and diabetes, but also hypertension and hyperlipidaemia are dichotomous variables. If blood lipids and blood pressure are not measured, they will not be included in the risk estimation. This is illustrated in paper I, where ERS-RA did not perform very well when risk estimation was done using patient’s or physician’s diagnosis of hypertension and hyperlipidaemia, whereas the estimations were more accurate when measured levels of blood pressure and blood lipids were used.

When it comes to the question of variables reflecting RA in risk estimation, one has to keep in mind what the commonly used variables represent. The HAQ is a patient reported disability index, where a high score probably can represent different pathologies in patients with early disease, and in patients with a long duration of RA, respectively. In early RA, an elevated HAQ most probably is
caused by ongoing inflammation, whereas it in patients with long-standing disease can be caused by erosions and other long-term effects of RA. Thus, an elevated HAQ is not a reliable marker of inflammation in patients with long-term RA. Furthermore, disease activity measured by DAS28 is much affected by patient’s reported components pain, tender joints and general health. According to a recent meta-analysis (Duffield et al. 2018), about 20% of patients with RA have concomitant fibromyalgia, elevating DAS28 with 1.24 points compared to patients with RA alone. This difference is not caused by differences in the objective inflammatory components ESR or swollen joints, but comes from patient reported components tender joints and pain, making DAS28 and other scores including these components unreliable in patients with RA and fibromyalgia. Of course this causes problems in all aspects of management of RA, not only risk estimation.

The process of development of cardiovascular risk scores is another potential confounder in evaluation of RA-specific risk scores. Cardiovascular risk scores are developed from cohorts of patients, in whom potential predictors of cardiovascular events are registered. After a period of follow-up, the relation between possible predictors of events and actual events during follow-up is analysed, and an algorithm based on the predictors values at baseline is constructed. Thus, a new risk score is a representation of risk factors from the past, so if the population changes over time, the risk score can become obsolete. When it comes to RA, the treatment strategies have obviously changed over time. Recommendations today comprise intense treatment and ambitious targets of treatment, in addition to the new drugs available (Smolen et al. 2017), making modern populations of patients with RA different from earlier populations. It is also plausible that genetic variation and geographical differences in treatment strategies limit the generalizability of populations with RA. This points out the need of developing and evaluating RA-specific risk scores in sub-populations of patients with RA, where patients are classified according to duration of disease, age at disease onset, time of disease onset, geography, genetic background, disease activity, and treatment.

One aspect of risk estimation, is whether it is useful to identify patients with an elevated risk. If the risk cannot be decreased, identification of high-risk individuals is useless. A recent study examined the effect of cardiovascular prevention in patients with RA, and found that intense treatment of cardiovascular risk factors decreased the progress of IMT and reduced the risk of cardiovascular events, although the number of events was small (Burggraaf et al. 2019). This tells us that cardiovascular risk prevention by treatment of traditional cardiovascular risk factors in patients with RA is possible. Considering the observations of a relationship between inflammation and risk of myocardial infarction and acute coronary syndrome (Ljung et al. 2016, Meissner et al. 2016),
it is reasonable that the risk of atherosclerotic cardiovascular disease to a great extent is modifyable. The relation between inflammation and atherosclerosis has been studied in the general population, where several trials of prevention of cardiovascular events using anti-inflammatory agents have been performed, but mostly with negative results (Vaidya et al. 2019). A recent study with negative results, was the Cardiovascular Inflammation Reduction Trial (CIRT), where methotrexate did not reduce the risk of cardiovascular disease in patients with diabetes and a previous myocardial infarction (Ridker et al. 2018), an observation that contradicts the observations in patients with RA. Unlike other substances investigated in this area, canakinumab (Ridker et al. 2017) and colchicine (Nidorf et al. 2013) both have been effective in cardiovascular prevention in the general population. Interestingly, these two substances have a common effect: canakinumab is a potent inhibitor of interleukin 1β, while colchicine by inhibiting the NLRP3-inflammasome decreases the production of interleukin 1β. This implies that reduction of inflammation is not enough to have effect on atherosclerosis: the mechanism is crucial. If this holds true in RA is yet to be explored, but the reduced risk of cardiovascular events in patients with RA treated with methotrexate (Choi et al. 2002), suggests that the mechanisms linking inflammation and atherosclerosis are different in patients with and without RA.

As shown by Crowson et al (Crowson et al. 2018), traditional cardiovascular risk factors and RA-associated risk factors together explain 70% of the cardiovascular risk in patients with RA. This suggests that more risk factors are yet to be identified. My study presented in Paper IV, identifies OPG, and possibly OCN, as potential markers of atherosclerosis in patients with RA. Previous studies of OPG in patients with RA have been consistent with this: OPG has been associated with CAC (Asanuma et al. 2007), carotid plaque (Asanuma et al. 2013), endothelial activation (Dessein et al. 2014), IMT (Dessein et al. 2014, Beyazal et al. 2016), and pulse wave velocity (Beyazal et al. 2016). These measures represent stages of atherosclerosis from the early endothelial activation, to calcified plaques, implying that the relation between OPG and atherosclerosis is consistent, further corroborated by a study linking increased levels of OPG in patients with RA to established cardiovascular disease (López-Mejias et al. 2015). The question is, whether OPG is a risk factor or a marker of atherosclerosis. In prospective cohort studies, higher concentrations of OPG have been independently associated with future atherosclerotic cardiovascular disease, adjusted for traditional risk factors (Vik et al. 2011, Mogelvang et al. 2013, Tschiderer et al. 2017), although OPG is associated with several traditional risk factors (Mogelvang et al. 2012). In patients with RA, no prospective studies have been published.

A potential confounder in studies of OPG, is the fact that it is produced not only by osteoblasts, but also in several other tissues and cell types, including vascular...
smooth muscle cells and endothelial cells (Rochette et al. 2018), from the latter secreted as a response to inflammation (Zannettino et al. 2005). Despite this, the relation between OPG and inflammation is inconsistent in patients with RA. A meta-analysis did not show a significant relation with DAS28, the only measure of inflammation reported (Wang et al. 2017). In Paper IV, OPG was associated with ESR, but not with other measures of inflammation, as in previous studies of atherosclerosis, where a relation with measures of inflammation was reported in one in one study (Asanuma et al. 2007), but not in others (Dessein et al. 2014, López-Mejias et al. 2015, Beyazal et al. 2016). Also in general population cohorts, the relation between OPG and inflammation measured as high-sensitive CRP has been contradictory (Vik et al. 2011, Mogelvang et al. 2012). This suggests that OPG is not the final link between atherosclerosis and inflammation in patients with RA, but still is a marker of atherosclerosis. However, more longitudinal studies are required, since my study is the first in patients with RA, where a cohort of patients is followed over time.

Paper II reports results from a long-term follow-up of the very first study that reported increased atherosclerosis in patients with RA (Wållberg-Jonsson et al. 2001). In the baseline study IMT was measured, but in this follow-up CAC was measured, and found to be associated with inflammation in all stages of RA. A large amount of biomarkers was measured at baseline, but no biomarkers reflecting or regulating bone turnover. Otherwise that study might have shed some light on the relations between atherosclerosis, inflammation and bone biomarkers in patients with RA in patients with long-standing disease.

The mechanisms linking OPG and other markers of bone turnover and atherosclerosis are not sufficiently elucidated, but bearing in mind that the process of plaque calcification is an active process (Rochette et al. 2018), it is reasonable to think of it as independent from the regulation of bone turnover. My study supports this idea, since levels of the collagen degradation product CTX, released from osteoclasts during bone resorption, was not associated with atherosclerosis. Neither was P1NP, a product from bone formation, associated with atherosclerosis, indicating that neither resorption nor formation of bone was related to IMT, albeit OPG was. On the other hand, OCN, a protein only secreted by osteoblasts (Moser and van der Eerden 2018), was negatively associated with atherosclerosis, providing a link between low osteoblast activity and atherosclerosis. However, OCN has been found to be less a regulator of calcification, than a hormone regulating such disparate things as brain development, response to exercise and glucose metabolism (Moser and van der Eerden 2018). In accordance with this finding, results from studies of atherosclerosis and OCN in the general population have been disparate, with the exception of histological studies of vascular calcification, where OCN or OCN-
positive cells consequently have been associated with calcification (Millar et al. 2017).

The hypothesis of infection causing atherosclerosis has been studied (Dahal et al. 2017), and several serological studies have linked infection with CMV to atherosclerosis and cardiovascular disease (Sorlie et al. 2000, Dahal et al. 2017). This hypothesis was tested in paper III, where IMT increased rapidly after onset of RA in CMV IgG-positive patients (Figure 9). This suggests an additional effect of RA and CMV, propagating the process of atherosclerosis when a CMV-positive individual develops RA. Furthermore, the presence of CD28null T-cells was higher in CMV IgG-positive patients, as in previous studies in patients with RA (Hooper et al. 1999, Broadley et al. 2017), and the general population (Gratama et al. 1987, Derhovanessian et al. 2011). CD28null T-cells are terminally differentiated, with properties different from the normal CD28+ T-cells: CD4+CD28null T-cells are no longer T-helper cells, but are cytotoxic, whereas CD8+CD28null T-cells demonstrate both increase and decrease in cytotoxic properties, pointing to the fact that the population of CD28null T-cells is heterogenous (Mou et al. 2014). Nevertheless, CD28null T-cells were associated with atherosclerosis in paper III, as well as in previous studies in patients with RA (Gerli et al. 2004, Pingiotti et al. 2007, Winchester et al. 2016). It has been suggested that CMV-infection through induction of CD4+CD28null T-cells is the cause of increased cardiovascular mortality in patitents with RA (Broadley et al. 2017), and our results do not contradict this idea, although more evedence definitely is needed. Thinking of CMV and atherosclerosis, the hypothesis of an immediate effect of CMV on atherosclerosis cannot be rejected: the endothelial cells are in fact infected by CMV and become activated from infection with CMV, and although the immediate response is downregulated, the endothelium is chronically infected (Jeffery et al. 2013). Thus, it is reasonable to think of CMV in itself as a potential cause of atherosclerosis.
Conclusions

From this study of atherosclerosis and risk of cardiovascular disease in patients with RA, the following conclusions can be made:

- Risk estimation scores for patients with RA need to be improved.
- Blood pressure and blood lipids must be measured in patients with RA, to enable accurate risk estimation.
- The amount of coronary artery calcification is related to both present and previous inflammation in patients with RA.
- CD28null T-cells are associated with increased atherosclerosis in patients with RA.
- CMV IgG-positive patients with RA have increased atherosclerosis.
- Osteoprotegerin, and possibly osteocalcin, could be used as indicators of atherosclerosis in patients with RA.
- Bone turnover per se is not associated with atherosclerosis in patients with RA.
Future perspectives

As stated in previous parts of this thesis, there are still many areas where more knowledge is needed. The first of them, is how to improve the cardiovascular risk estimation in patients with RA. There is a need for an RA-specific risk score that performs better than risk scores for the general population. This probably will not be one uniform risk score applicable to all patients with RA, but rather a risk score where the patients are categorized according to disease characteristics, and perhaps genetic and geographic background. The ACC/AHA risk score includes interaction between the included variables, since the impact of a variable depends on other variables. This probably holds true even more for patients with RA, where there are more potential interactions to include in the risk estimation, e.g. the well-known interaction between inflammation and blood lipids. The first step would be to recalibrate the ACC/AHA algorithm using patients with RA, and investigate if this improves the performance. Secondly, characteristics of RA, genetics, and geography could be included in the recalibrated and the original ACC/AHA risk score. The characteristics of RA would preferably include not only measures of present inflammation, RF- or ACPA-status and HAQ, but also duration of disease, age at disease onset, time of disease onset, geography, genetic background, and treatment. As a last step, inclusion of new variables could be included, e.g. OPG, OCN and CMV IgG-positivity.

Another question to be answered, is if antiviral treatment for CMV could be efficient in reducing atherosclerosis and atherosclerotic cardiovascular disease in patients with RA. A randomized controlled trial using antiviral treatment could provide not only an opportunity to treat cardiovascular risk, but also give useful information whether CMV per se is involved in atherogenesis. If analysis of the T-cell repertoire is performed along, some light could be shed on the interaction between CMV, T-cells and atherosclerosis in patients with RA.

Numerous studies of biomarkers in terms of atherosclerosis have been performed in patients with RA, but the histological studies are scarce. Patients with RA are subject to vascular surgery, and studies of specimen from these patients would give an opportunity to examine cell types and biomarkers in situ, in contrast to soluble biomarkers and circulating cells. As a matter of fact, the disease is located to the arterial wall, not what circulates in the lumen.
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