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Karolinska Institutet, Stockholm, Sweden

EVALUATION OF BIOMARKERS IN CHRONIC INFLAMMATORY DISEASES

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Stockholm 2020

Evaluation of biomarkers in chronic inflammatory diseases

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To Behrouz and Nova

&

To those who do not surrender in difficulties

"I don't have any particular recipe . . . Doing research is challenging as well as attractive. It is like being lost in a jungle and trying to use all the knowledge that you can gather to come up with some new tricks, and with some luck you might find a way out."

Maryam Mirzakhani, The First Woman To Win Math's Most Prestigious Prize, Field's Medal 2014

ABSTRACT

The overall aim of this thesis was to assess the role of biomarkers in chronic inflammatory disease. The goal of the first study was to investigate the association of sIL-6R and sgp130 levels and risk of first myocardial infarction (MI), and to explore the potential interaction between these two biomarkers in association with risk of MI.

In the second study, the aim was to identify genetic loci associated with the circulation of sgp130, known as an inhibitor of the IL-6 trans-signaling, and also to test the association between the single nucleotide polymorphisms (SNPs) associated with sgp130 and carotid intima-media thickness (c-IMT) as a surrogate marker of atherosclerosis.

The main objective of the third study was to investigate whether environmental risk factors of rheumatoid arthritis (RA) affect the occurrence of different reactivity patterns to citrullinated peptides and the presence of rheumatoid factor (RF) isotypes.

Study I was carried out in the Stockholm Heart Epidemiology Program (SHEEP), a case-control study designed to determine new risk factors. sIL-6R and sgp130 levels were measured in serum of 682 and 664 MI cases and 1103 and 1062 controls, respectively. Increased concentrations of sIL6R were associated with a high occurrence of MI while very high levels of sgp130 had an inverse association with incidence of MI. The obtained results indicated presence of an interaction between the low level of sgp130 and the high level of sIL6R that might have a synergistic effect on increased risk of MI. Our results highlighted the necessity of focusing on molecular pathways instead of only one biomarker when estimating the risk of CVDs.

Study II was performed in IMPROVE, a European multicenter prospective study recruited subjects with high risk profile for cardiovascular diseases. c-IMT was measured at baseline and after 30 months follow-up. Genomic DNA of 3703 participants was genotyped using the CardioMetaboChip and ImmunoChip. A linear regression model was used to assess the association of 360,842 SNPs and sgp130 levels. Model was adjusted for gender, age and population structure. rs10935473 and rs1929666, located at chromosome 3 and 10 respectively, had an association with sgp130 circulation. Besides, 24 SNPs showed the suggestive association with sgp130 levels. A positive association between rs17688225 and serum level of sgp130 was observed while this SNP showed a negative association with c-IMT. Our results indicated that sgp130 levels is regulated by multiple genetic loci, which is likely these loci partly also regulate c-IMT measures.

In **study III**, analyses were based on data from two Swedish RA patients cohorts: Epidemiological Investigation of RA (EIRA, n=2859), and early RA from Umeå (eRA-Umeå, n=1011). Our results corroborate the wide-spread co-occurrence of different RA-specific antibodies, which is likely mediated by epitope spreading or cross-reactivity. When analyzing all antibodies jointly, we found that smoking is mainly associated with the presence of anti-Cit-Fibα_{36-50}, anti-CEP-1 as well as with IgA-RF. No conclusive associations were found between low alcohol consumption or high BMI with the presence of any specific autoantibody. Our study indicated smoking might play a part in pathogenesis of RA through specific anticitrulline immunity by increasing the exposure of these antigens in affected tissues like lungs.

SAMMANFATTNING PÅ SVENSKA

Det övergripande syftet med denna avhandling var att utvärdera biomarkörernas roll i kronisk inflammatorisk sjukdom. Målet med den första studien var att undersöka sambandet mellan sIL-6R- och sgp130-nivåerna och risken för en första episod av hjärtinfarkt, samt undersöka den potentiella interaktionen mellan dessa två biomarkörer i risk för hjärtinfarkt. I den andra studien syftade vi till att identifiera genetiska varianter kopplade till sgp130-serumnivåer, den naturliga antagonisten mot IL6-transignaleringen. Vi undersökte även sambandet mellan genotyperna förknippade med sgp130 och en markör för subklinisk åderförkalkning (carotis intima-media tjocklek, c-IMT). Målet med den tredje studien var att undersöka om miljöfaktorer för reumatoid artrit (RA) påverkar förekomsten av sjukdomsspecifika antikropparna mot citrullerade peptider och reumatoid faktor (RF) isotyper.

Studie I genomfördes i en stor befolkningsbaserad fallkontrollstudie, Stockholm Heart Epidemiology Program (SHEEP). Vi mätte nivåer av sIL6R och sgp130 i serumprover från 682 respektive 664 hjärtinfarktspatienter, och 1103 respektive 1062 kontroller. Förhöjda koncentrationer av sIL6R var förknippade med en ökad förekomst av hjärtinfarkt och mycket höga nivåer av sgp130 var associerade med en reducerad förekomst av hjärtinfarkt. Våra resultat indikerar även en interaktion mellan dessa två biomarkörer, som föreslår att låga sgp130-nivåer tillsammans med höga sIL6R-nivåer öka risken för hjärtinfarkt mer än förväntat.

Studie II utfördes i IMPROVE, en europeisk prospektiv multicenterstudie utformad för att undersöka sambandet mellan c-IMT och c-IMT-progression med risken för framtida kardiovaskulära sjukdomar. Genomiskt DNA genotypades med hjälp av CardioMetaboChip och ImmunoChip. rs10935473 (på kromosom 3) och rs1929666 (på kromosom 10) var signifikant förknippade med sgp130-nivåer och 24 genetiska varianter visade svagare association med sgp130-nivåer. En av de genetiska varianterna med svagare association, rs17688225 på kromosom 14, visade en negativ association med c-IMT. Våra resultat indikerar att sgp130-nivåerna regleras av flera genetiska varianter som till viss del överlappar dem som reglerar c-IMT.

Studie III använde data från två svenska kohorter av RA-patienter: Epidemiologisk undersökning av RA (EIRA, n = 2859) och tidig RA från Umeå (eRA-Umeå, n = 1011). Våra resultat bekräftar att patienter oftast utrycker flera olika antikroppar som binder flera olika auto-antigen samtidigt. Detta medieras troligen av epitopspridning eller korsreaktivitet. När vi analyserade alla antikroppar gemensamt fann vi att rökning huvudsakligen är förknippat med närvaron av anti-Cit-Fibα$_{36-50}$, anti-CEP-1 såväl som med IgA-RF. Vi hittade inga tydliga samband mellan låg alkoholkonsumtion eller hög BMI med närvaron av någon specifik autoantikropp.

خلاصه پایان نامه مقطع دکتری

اهداف

هدف اصلی این پایان نامه بررسی نقش شاخص های زیستی (Biomarkers) در بیماری های مزمن التهابی است.

اهداف مطالعه اول این پایان نامه از این قرار است:

۱. بررسی رابطه ی میزان شکل محلول گیرنده اینترلوکین شماره ۶ (SIL6R) و شکل محلول گلیکو پروتئین شماره ی ۱۳۰ (SGP130) با خطر وقوع اولین سکته قلبی.

۲. بررسی امکان برهمکنش احتمالی بین این دو شاخص زیستی در رابطه با خطر سکته قلبی.

در مطالعه دوم این پایان نامه ما برآنیم تا انواع رویه های ژنتیکی مرتبط با سطح گلیکو پروتئین شماره ۱۳۰ درخون انسان را بیابیم. همچنین کوشش کردیم تا ارتباط بین گوناگونی های ژنتیکی جمعیت انسانی از نوع چند ریختی تک نوکلئوتیدی ژن گلیکو پروتئین شماره ی ۱۳۰ (SGP130) را با ضخامت لایه ی اینتیمامدیای (داخلی ترین لایه) سرخرگ کاروتید به عنوان یک نشانگر زیستی زودهنگام برای بیماری تصلب شرائین را بررسی نمائیم.

هدف مطالعه سوم این پایان نامه بررسی تاثیر خطرات محیطی مرتبط با بیماری روماتیسم مفصلی بر روی فراوانی انواع مختلف پپتیدهای سیترولینه ی کنش گر و همچنین حضور ایزوتوپ های فاکتور روماتئید می باشد.

روش ها و نتایج:

مطالعه شماره ۱-

این مطالعه به صورت یک تحقیق/ مطالعه ی جمعیتی بزرگ از نوع مورد - شاهدی در سازمان همه گیر شناسی بیماری قلبی شهر استکهلم انجام گرفت.

در این تحقیق، ما غلظت دو پروتئین گیرنده محلول اینترلوکین شماره ۶ (SIL6R) و گلیکو پروتئین شماره ی ۱۳۰ (SGP130) را در بیش از هزار نمونه سرم بیماران مبتلا به سکته قلبی و کنترل هایشان اندازه گیری کردیم. غلظت بالای پروتئین گیرنده محلول اینترلوکین شماره ۶ با افزایش خطر سکته قلبی مرتبط است و از سوی دیگر غلظت بسیار بالای گلیکو پروتئین شماره ی۱۳۰(SGP130) با کاهش خطر سکته قلبی همراه است.

نتایج ما بیانگر وجود یک نوع رابطه ی هم افزایشی بین این دو نشانگر زیستی می باشد. به عبارتی، غلظت های پایین گلیکو پروتئین شماره ی ۱۳۰ (SGP130) و غلظت های بالای گیرنده ی محلول اینترلوکین شماره ۶، طی نوعی رابطه ی هم افزایشی منجر به افزایش خطر وقوع سکته قلبی می شوند.

مطالعه شماره ۲ -

مطالعه دوم در پروژه ی آینده نگر IMPROVE که همزمان چند کشور مختلف اروپایی در آن شرکت دارند، انجام شد.

هدف این تحقیق بررسی ارتباط بین شاخص ضخامت لایه ی اینتیما مدیای سرخرگ کاروتید و تغییرات آن با خطر بروز عوارض قلبی و عروقی در آینده می باشد.

در این تحقیق توالی ژنوم (genomic DNA) افراد مورد مطالعه با استفاده از دو روش Cardiometabech و Immune chip تعیین شد. بخش rs10935473 کروموزوم شماره ۳ و بخشrs1929666 از کروموزوم شماره ۱۰با غلظت گلیکو پروتئین شماره ی ۱۳۰ وابستگی معنی داری نشان دادند.

همچنین، ۲۴ مورد چند ریختی تک نوکلئوتیدی با غلظت گلیکو پروتئین شماره ی ۱۳۰ در سرم افراد مورد مطالعه وابستند.

بخش rs17688225 کروموزومی شماره ۱۴ نیز باعث غلظت گلیکو پروتئین شماره ی ۱۳۰ در سرم افراد مورد مطالعه وابستگی مثبت و از طرفی با ضخامت لایه ی اینتیمامدیای سرخرگ کاروتیدی وابستگی منفی داشت.

نتایج ما نشان می دهد که غلظت گلیکو پروتئین شماره ی ۱۳۰ توسط چند جایگاه ژنتیکی مختلف در ژنوم انسانی ساماندهی و تنظیم می شود. لازم به ذکر است که این جایگاه های ژنتیکی احتمالا با جایگاه های ژنتیکی تنظیم کننده ضخامت لایه ی اینتیمامدیای سرخرگ کاروتید همپوشانی دارند.

مطالعه شماره ۳-

تحلیل های مطالعه سوم بر اساس داده های دو گروه از بیماران سوئدی مبتلا به روماتیسم مفصلی می باشد.

گروه مطالعات همه گیر شناسی روماتیسم مفصلی موسوم به EIRA که شامل تعداد ۲۸۵۹ بیمار بوده و گروه بیماران مبتلا به روماتیسم مفصلی زودرس منطقه Umeå (e RA -Umeå) که شامل تعداد ۱۰۱۱ نفر می باشند.

نتایج ما بیانگر حضور همزمان و گسترده ی بسیاری از انواع مختلف پادتن های اختصاصی بیماری روماتیسم مفصلی در نمونه های بیماران می باشد. حضور این پادتن ها احتمالا نتیجه فرآیند گسترش و توزیع یا واکنش متقابل اپی توپها می باشد. بررسی انواع پادتن های موجود نشان می دهد که استعمال دخانیات با حضور پادتن های ضد CEP-1 , Cit-Fibα_{36-50} و همچنین ایمونوگلوبین نوع A ضد RF وابسته است.

در این مطالعه هیچ گونه ارتباطی میان مصرف کم مشروبات الکلی و افزایش نمایه ی توده ی بدن (BMI) با حضور نوع خاصی از پادتن های خود ایمن مشاهده نشد.

LIST OF SCIENTIFIC PAPERS

I. Moreno Velasquez I*, **Golabkesh Z***, Kallberg H, Leander K, de Faire U, Gigante B.

    Circulating levels of interleukin 6 soluble receptor and its natural antagonist, sgp130, and the risk of myocardial infarction.

    Atherosclerosis. 2015;240(2):477-81.

II. Bonomi A, Veglia F, Baldassarre D, Strawbridge RJ, **Golabkesh Z**, Sennblad B, Leander K, Smit AJ, Giral P, Humphries S, Tremoli E, Hamsten A, Faire U, Gigante B, on behalf of the IMPROVE study group

    Analysis of the genetic variants associated with circulating levels of sgp130. Results from the IMPROVE study.

    Genes Immun. 2020;21(2):100-8.

III. **Golabkesh Z**, Frumento P, Hansson M, Lundberg K, Arkema E, Mathsson-Alm L, Jakobsson P, Holmdahl R, Skriner K, Serre G, Rönnelid J, Dahlqvist SR, Klareskog L, Alfredsson L, Brynedal B.

    Manuscript

    * Contributed equally

CONTENTS

LIST OF ABBREVIATIONS

ACR	American college of rheumatology
anti-CCP	Anti-cyclic citrullinated peptide
ACPAs	Anti-citrullinated protein/peptide antibodies
AP	Attributable proportion
Bif	Bifurction
CAD	Coronory artery disease
CHD	Coronary heart disease
Chr	Chromosome
CI	Confidence interval
c-IMT	Carotid intima-media thickness
CC	Common carotid
CRP	C-reactive protein
CV	Cardiovascular
CVDs	Cardiovascular diseases
CVE	Cardiovascular events
EC	Endothelial cell
EIRA	Epidemiological Investigation of Rheumatoid Arthritis
ELISA	Enzyme-linked immunosorbent assay
eRA-Umeå	Early rheumatoid artheritis Umeå cohort
EULAR	European league against rheumatism
FDR	False discovery rate
Gp130	Glycoprotein 130
GTEx	Genotype-tissue expression
GWAS	Genome Wide Association Study
HDL	High-density lipoprotein
ICA	Internal carotid artery
I_CC	1st centimeter of common carotid
Ig	Immunoiglobolin
IL-	Interleukin
IL-6R	IL-6 receptor
IMPROVE	carotid **I**ntima **M**edia Thickness and c-IMT **PR**ogression and the risk of **V**ascular **E**vents

MDS	Multidimentional scaling
MI	Myocardial infarction
MSD	MesoScale Discovery
OR	Odds ratio
RA	Rheumatoid arthritis
RF	Rheumatoid factor
S	Synrgic index
sgp130	Soluble glycoprotein 130
SHEEP	Stockholm Heart Epidemiology Program
sIL-6R	Soluble interleukin-6 receptor
TNF-α	Tumor necrosis factor α
WHO	World Health Organization

1   BACKGROUND

Inflammation is a self-protection reaction of the body in response to the harmful stimuli like pathogens, if inflammation progress to a chronic state, this may cause diseases, such as atherosclerosis or rheumatoid arthritis (RA). The focus of this thesis was on inflammatory biomarkers and two chronic inflammatory diseases: cardiovascular diseases (CVDs), particularly Myocardial Infarction (MI) and atherosclerosis, as well as RA.

A biomarker usually imputes to a distinct biological indicator such as measurable molecules in bodily fluids and can be used to assess both normal or pathogenic processes and evaluation of treatment responses. To be more specific, the risk or progression of a disease or the effect of a given treatment for a disease can be evaluated by relevant biomarkers, such as changes in expression or state of a protein. They can also be used to infer the genetic causes of diseases (1). For instance, the presence of autoantibodies in the serum of patients in a very early stage of the disease is a typical biomarker of autoimmune diseases. Autoantibodies as biomarkers can help the clinicians to diagnose the disease at an earlier stage and also classify the patient and provide adequate medications.

1.1   CVD

CVDs are of interest for researchers because they are the primary source of mortality in the world. The World Health Organization (WHO) estimates that CVDs will be the leading cause of death in all continents apart from Africa until 2030 and therefore remain a center of attention within the field. The 2016 Global Burden of Disease reported 31% of all deaths globally (17.9 million), as attributed to CVD (2).

Based on European CVD statistics 2016, despite all the development in diagnosis and treatment of CVDs, still more than 4 million deaths occur every year due to CVD across the continent (3). According to the WHO, CVDs are defined as a class of disorders that involve heart and blood vessels and include (4):

1) Coronary Heart Disease (CHD)
2) Cerebrovascular diseases
3) Peripheral arterial disease
4) Congenital heart disease
5) Rheumatic heart disease
6) Deep vein thrombosis
7) Pulmonary embolisms

Risk factors contributing in the development of CVDs can be categorized as:

1) Modifiable risk factors: i.e. hypertension, smoking, raise blood glucose (diabetes), lack of physical activity, unhealthy nutritional habits, hyperlipidemia, being overweight and obese. These risk factors can be controlled or treated, that in turn prevents CVDs and reduces the number of deaths each year caused by CVDs.

2) Non-modifiable risk factors: i.e. age, gender and family history of CVD. These actualities cannot be changed, however regular checkups for high-risk groups of individuals is recommended (5).

1.1.1 CHD

CHD is the most common type of CVD. Alternative titles for CHD are coronary artery disease (CAD), ischemic heart disease and atherosclerotic heart disease. CHD consists of stable angina, unstable angina, MI and sudden cardiac death. These are the clinical manifestation of atherosclerosis, a chronic inflammatory disease of the coronary arteries.

Despite the importance of common risk factors for the onset of cardiovascular events (CVEs), it is commonly suggested that more than 50% of patients suffer from CHD have none of the traditional cardiovascular (CV) risk factors (6). This implies that other nontraditional risk factors have a significant role in the development of CHD. Therefore, it is essential to investigate the non-conventional risk factors and new biomarkers in addition to traditional risk factors to predict CHD and consequently CVDs. This would enhance the chance of preventing the disease onset and thereby reducing CVDs mortality.

1.1.1.1 MI

MIs usually occur due to acute ischemia in myocardium where thrombus blocking the coronary arteries. Artery occlusion happens because of stenosis of the coronary artery by an atherosclerotic plaque that causes acute ischemia, lack of blood flow to the myocardium, which in turn leads to oxygen deprivation in the cardiac muscles.

The definition of MI has developed gradually with the increase in sensitivity and specificity of available biomarkers. The universal definition of MI was originally published in 2007 (19). According to the international task force, the updated universal definition for MI (from 2014 (20)) concentrates on clinical criteria as well as biomarkers and consist of electrocardiogram changes and imaging. Acute MI is defined as myocardial cell death in response to extended myocardial ischemia.

1.1.1.2 Atherosclerosis

Atherosclerosis is a chronic and progressive inflammatory disease of the blood vessel wall and represents the biological lesion underlying atherosclerosis-related CVDs. The clinical manifestations of atherosclerosis are specific for different vascular beds and are related to the stability of focal atherosclerotic lesions, called plaque. Plaques usually contain loads of lipids, including cholesterol and fatty acids and white blood cells, particularly macrophages (7).

If atherosclerotic plaque forms in the coronary arteries, it is more likely to grow within the vascular wall and reducing the blood flow in cardiac tissue and consequently, facilitate the onset of chronic ischemia. Atherosclerotic lesions are categorized as stable or unstable (vulnerable) plaques. Stability of an atherosclerotic plaque is more important than its size and depends on its cellular and extracellular contents.

Stable plaques are characterized by a thick fibrous cap, small lipid cores, few inflammatory cells and many smooth muscle cells. Stable plaques usually result in clinical symptoms described as stable angina. At the other side of the spectrum are atherosclerotic plaques in the coronary arteries, which are characterized by large lipid cores, high inflammatory content (numerous macrophages) and pro-angiogenic activities. These are called unstable plaques. They have relatively few smooth muscle cells and a very thin fibrous cap that tends to rupture in response to different stimuli (8, 9).

When the cap rupture, is followed by activation of the coagulation cascade and thrombus formation. Existing platelets and coagulation factors in blood circulation will be faced by the plaque's extracellular matrix and lipid core that has thrombogenic characteristics. Afterwards, the disrupted plaque is used as scaffolding for platelet aggregation and coagulation, eventually thrombus formation.

Thrombus size depends on the plaque rupture area. When it is large enough, the thrombus can occlude the coronary vessel lumen. The blockage can be either partial or complete, thereby accelerate the occurrence of an acute coronary event. The clinical manifestation of plaque rupture or superficial erosion is often MI (10, 11).

Atherosclerosis might remain subclinical during years, which indicates the requirement of different methods to identify the presence of atherosclerosis. Available techniques such as intravascular ultrasonography and B-mode ultrasonography are used to measure the surrogate markers of atherosclerosis. According to the estimations, the different dosage of statin treatment can be prescribed to reduce, cease or even inverse the progression of atherosclerotic disease (12).

Atherosclerosis and inflammation

Several lines of evidence indicated the key role of inflammation in all phase of atherosclerosis. Various cytokines like IL-12, IL-6, IL-1, IL-2, IL-8, IL-10, IL-17, tumor necrosis factor α (TNF-α), and C-reactive protein (CRP) are associated with CVDs and involved in the inflammatory processes contributing to atherosclerosis and plaque formation in the vessel walls of human (13). However, the independent contribution of inflammatory biomarkers to the risk of CVD is still not fully known.

Subclinical atherosclerosis

Carotid intima-media thickness (c-IMT) is a surrogate marker for early atherosclerosis that reflects the extension of the thickness of the intima-media layer in carotid artery. c-IMT is used as an intermediate phenotype for diagnosis of early stages of atherosclerosis. Several large population-based studies have indicated c-IMT as a valid estimation of the risk for CVEs (14).

Based on the results of different epidemiological studies, c-IMT is associated with various CV risk factors like diabetes and impaired glucose tolerance, hypercholesterolemia (15, 16), high-density lipoprotein cholesterol and triglycerides (17). Using c-IMT as a biomarker of CVD is beneficial because it is a non-invasive, un-complicated and a low-cost method. Moreover, it is reproducible and radiation-free. This test can classify individuals as high or low risk groups for CVDs, and appropriate precautionary action plans can be implemented accordingly (12).

c-IMT usually is measured in far and near walls of four segments of both right and left carotid arteries: at the internal carotid arteries, at the carotid bifurcation, in the 1st cm of the common carotid, in the proximal artery (18).

Differences in c-IMT in different parts of the artery can reflect the variation in the local hemodynamic forces. Usually, c-IMT measurement of the deeper wall (far wall) is more reliable for diagnosis of atherosclerosis compared to the adjacent wall and is thus the most applied in clinical studies.

Based on guidelines presented jointly by European society of hypertension and European society of cardiology, the normal values for c-IMT is <0.9 mm, and c-IMT value between 0.9 mm and 1.5 mm is an indication of atherosclerosis and high risk of CVD. c-IMT values >1.5 mm demonstrate asymptomatic carotid plaque. Changes in c-IMT can reflect regression and or progression of atherosclerotic CVD (18). If the increased in c-IMT is detected in the early stage, there is a possibility of prevention or treatment before more serious damage to the vessel wall.

1.2   BIOMARKERS FOR CVDS

1.2.1   IL-6

IL-6 is a 26-kDa inflammatory cytokine with a four-helix bundle structure and consists of 184 amino acids (21). IL-6 is a pleiotropic cytokine that has pro-inflammatory as well as anti-inflammatory characters. This key cytokine has different effects on the immune system, liver, the endothelium and the vascular smooth cells. IL-6 influences the accumulation of platelets and proliferation of vascular smooth muscle cells in coronary arteries (22). In hepatocytes, IL-6 regulates the production and the release of the acute-phase proteins, for instance, CRP (23). In endothelial cells, IL-6 has a regulatory effect on the expression of adhesion molecules as well as other cytokines such as IL-1β and TNF-α. The secretion of these cytokines, along with IL-6 from endothelial cells creates a microenvironment that facilitates the initiation of an inflammatory process in the vessel wall. This represents one of the central mechanisms involved in the onset and progression of atherosclerosis (24).

1.2.2   IL-6R, sIL-6R, gp130 (Biology of IL-6 inflammatory signaling pathways)

IL-6 mediates its biological activities by binding to two membrane proteins: an 80-kDa glycoprotein called IL-6 Receptor (IL-6R) and a 130-kDa trans-membrane signal transducer protein named glycoprotein130 (gp130). IL-6R is expressed on leukocytes, monocytes and hepatocytes, where it regulates the release of acute-phase proteins and inflammatory cytokines (25). The soluble form of IL-6R (sIL-6R) is present in serum (ng/ml) and is a 50-55 kDa protein lacking the trans-membrane and cytoplasmic regions of IL-6R. sIL-6R is produced either by limited proteolytic shedding of the membrane-bound receptor or by translation from alternatively spliced mRNA (26) (Figure 2).

The IL-6 signaling pathway that is activated in the presence of membrane bound IL-6 receptor is called classic-signaling, which mostly regulate the beneficial regenerative and anti-bacterial roles of IL-6. The alternative signaling pathway that is mediated by soluble IL-6R is known as trans-signaling. It is suggested that trans-signaling is accounted for the majority of the detrimental IL-6 effects (27). In both signaling pathways, IL-6/IL-6R or IL-6/sIL-6R complex is followed by the homodimerization of gp130 subunits that are naturally expressed on all cell types. Thereafter the intercellular signaling cascade is initiated (Figure 1).
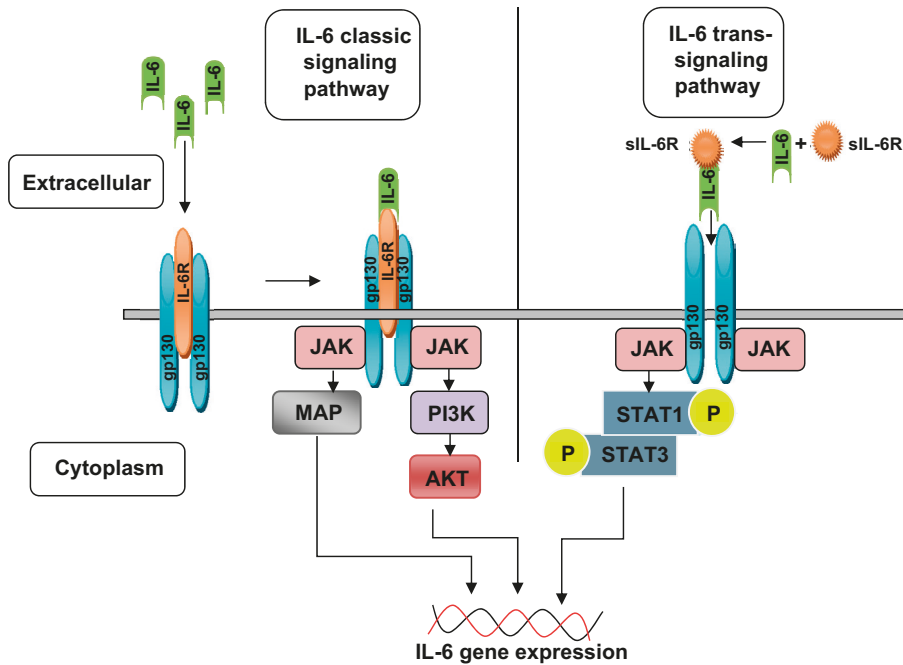
Figure 1. IL-6 classic-signaling pathway through IL-6R (left panel) and trans-signaling pathway through sIL-6R (right panel).

gp130 has no measurable affinity for IL-6 or IL-6R alone, but a high affinity for both IL-6/IL-6R and IL-6/sIL-6R complexes. Therefore, all cells that have gp130 present in their membranes can be responsive to IL-6, even in the absence of IL-6R, by the complex of IL-6 and sIL-6R (28).

1.2.3   sgp130

A soluble form of gp130 (sgp130) exists in human blood in three isoforms (50-110kDa); sgp130-RAPS, sgp130-E10 and full-length sgp130 (29-31). They are produced by proteolytic cleavage of gp130 and or alternative splicing of mRNA encoding the transmembrane protein (32). sgp130 represents a natural antagonist of the IL-6/sIL-6R pathway (33). sgp130 inhibits the trans-signaling through specific binding to the IL-6/sIL-6R complex with a high affinity (1mM). IL-6/sIL-6R complex coordinates the pro-inflammatory and the proatherogenic effects of IL-6 through trans-signaling (34, 35) and based on several animal and in vitro studies sgp130 circulation can prevent its binding to the membrane-bound gp130 and block the signaling pathway (36, 37) (Figure 2). In other words, sgp130 can behave as an anti-inflammatory factor by inhibiting inflammatory trans-signaling pathway and have a beneficial effect on inflammatory diseases and atherosclerosis,

thus can be an ideal pharmacological target for IL-6 signaling cause inflamma-
tory conditions. According to an in-vitro study, sgp130 play an inhibitory role
for IL-6 trans-signaling mostly in the full-length isoform (38). Note that it is not
possible to recognize which isoform of sgp130 is detected by biological assays.
The potential therapeutic application of sgp130 to prevent CVDs is of interest for
several research institutions.
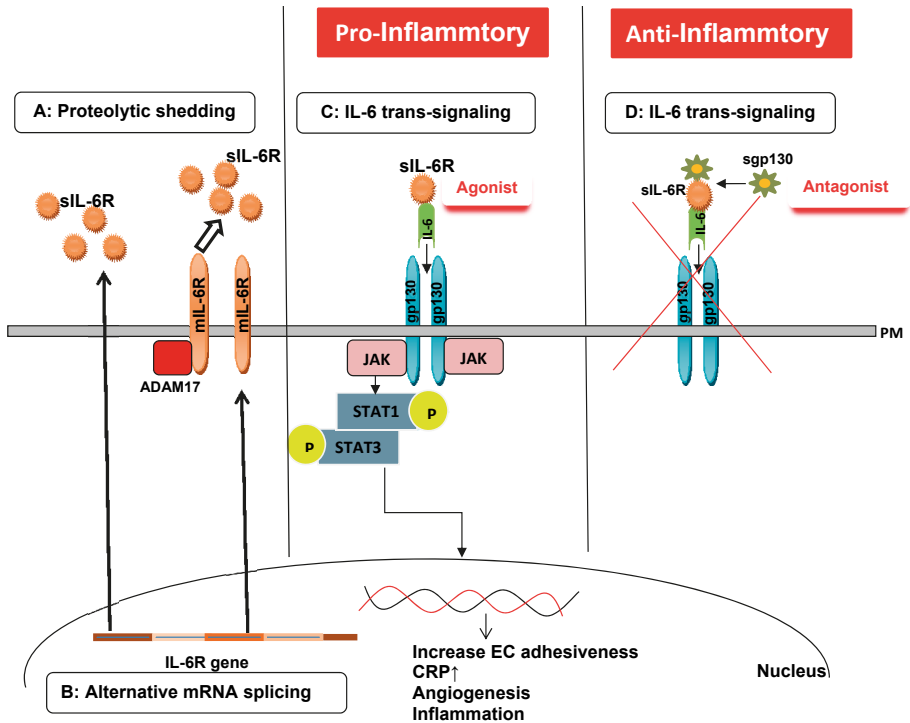
Figure 2. The IL-6 signaling pathways: soluble forms of IL-6R and gp130 are produced
by proteolytic cleavage (A) and or alternative splicing (B) of the full-length membrane-
bound forms. The pro-inflammatory effect of IL-6 occurs through trans-signaling (C)
and sgp130 can inhibit the trans-signaling (D).

1.3   IL-6, SIL-6R, SGP130 AND RISK OF CVDS

IL-6 is a known and independent risk indicator of various CVDs. In particular,
increased serum level of IL-6 is associated with elevated MI risk (39). One pro-
spective study of MI showed men with high IL-6 plasma levels had 2.3 times
higher risk of developing MI compare to those with low levels, still significant
after adjusting for traditional CV risk factors (40). Moreover, results from two
recent genetic meta-analyses of >200 000 subjects suggested a causal relationship
between IL-6 signaling and coronary heart disease (41).

sIL-6R seems to mediate the inflammatory effects of IL-6 via trans-signaling (42) (Figure 3). The role of IL-6 trans-signaling pathway (through IL-6/sIL-6R complex) has been emphasized in retaining chronic inflammation in systemic diseases like atherosclerosis (43). In a case-control study, patients with MI and CHD showed an elevated level of sIL-6R compared to controls, in the same population no difference in level of sgp130 was observed in cases and controls (44, 45). In other studies of the heart failure, an increased level of sgp130 has been reported in patients (46-48). The causal effect of IL-6 trans-signaling on development of atherosclerosis, as a key cause of CVDs, has been investigated by different clinical (49) and experimental studies (50, 51).

It has been studied if blocking IL-6 trans-signaling can prevent the inflammatory effects of IL-6 in different diseases. In this regard, a recombinant form of sgp130 (sgp130Fc) has been applied as IL-6 trans-signaling inhibitor in several inflammatory and degenerative disease models in the animal and in-vitro studies (52, 53). An experimental research performed on a mouse model indicated that the administration of sgp130Fc has a significant protective effect on atherosclerosis. It showed that sgp130 reduced the atherosclerotic plaque development and progression through nullifying the IL-6/sIL-6R trans-signaling (50). This suggests that sgp130 may antagonize the effect of IL-6/sIL-6R complex on the progression of atherosclerotic lesions. sgp130Fc is in fact now tested as an anti-inflammatory drug in phase 1 clinical trials for rheumatoid arthritis (54).

1.3.1   sgp130 genetic variants and risk of CVD

Genetic and environmental factors together define if an individual is susceptible to CVD. Genome Wide Association Study (GWAS) have provided a platform to explore the association between specific genetic variants and diseases.

The genes which regulate the level of sgp130 mainly have not been discovered yet. The association of polymorphisms in the gp130 gene with CVD risk has been investigated in a few studies. One single nucleotid polymorphism (SNP) (rs3729960) in GP130 at chromosome five is associated with a decline in the risk of MI, independently of other conventional CV risk factors (55). Furthermore, one study on two separate population, 546 subjects from OSLO high-risk population for CAD and 299 subjects from VIENA population with angiographically proven CAD, demonstrated a higher level of sgp130 in the individuals carrying the gp130 polymorphism G148C in comparison with wild type (56).

Increasing the knowledge on genetic variants regulating sgp130 is important, to understand the mechanisms underlying generation of sgp130 as an inhibitor of IL-6 trans-signaling and as a potential therapeutic marker in inflammatory diseases.

1.4 C-IMT AND INFLAMMATORY BIOMARKERS

Research on the association between inflammatory biomarkers and c-IMT as an indicator of CVD is essential for more precise risk prediction of CVDs as well as for effective prevention and treatment of atherosclerosis. Some previous studies have shown the association between c-IMT and inflammatory biomarkers. Wang et al. have indicated the association between c-IMT and CRP in women (57). Furthermore, an independent positive association between IL-6 level and c-IMT has been reported in patients with growth hormone deficiency who are at higher risk of CVDs (58). Moreover, results from one study in patients with obstructive sleep apnea has shown significant correlation between c-IMT and CRP, IL-6 and IL-18 serum levels. This proposes an association between systemic inflammatory biomarkers and elevated risk of atherosclerosis progression, thus increasing the risk of CVDs in patients with obstructive sleep apnea (59). Another finding from an epidemiological study with individuals at risk of CVDs (having at least three different traditional CV risk factors) showed an inverse association between the plasma IL-5 concentration and changes in c-IMT over 30 months follow-up in women (60).

1.5 RA

RA is the most common chronic inflammatory autoimmune disease, and a consequence of the faulty attacks of the immune system to the body tissue, specifically synovial tissue. The result is inflammation and stiffness of the joint linings, and unless successfully treated, it leads to bone erosion and loss. RA initially affects the wrist and small joints of the hands, such as middle joints of the fingers, and typically leads to painful and swollen joints (61). The disease involves symmetric pattern in pain, stiffness and swelling in peripheral joints on both sides of the body. Other symptoms such as fever, fatigue or low energy and reduced appetite can appear gradually. It can take weeks and months to verified diagnosis (62-64). The inflammation might expand to other parts of the body like lungs, heart, eyes and nerves. The cause of RA, like other autoimmune diseases is complex and involving both genetic and environmental factors (65). RA can appear in any age, but its incidence rate increases with age (up to age 55-80) and women have 2.5-3 times higher risk. It is estimated that RA affects nearly 0.5-1% of the whole population of the world (66). According to data from nationwide register-based study and Swedish Rheumatology Quality register in Sweden, RA incidence is 41 per 100,000 (67, 68). Various inflammatory cytokines like IL-6, TNF-α and IL-1 are highly expressed in RA. TNF-α is the main cytokine causing inflammation in the synovium of RA patients. Increased level of IL-6 is measurable in synovial fluids of RA patients with high disease activity. Synovial membrane express IL-6, and there is a correlation between the level of IL-6 and the level of radiological joint damage. Tocilizumab is used as a medication against the IL-6 receptor and inhibits the IL-6 trans-signaling and inflammatory effect of IL-6.

1.6 BIOMARKERS FOR RA

1.6.1 Rheumatoid factor (RF)

RF is an autoantibody that was originally found in RA patients. IgM-RFs are the most observed isotype, but IgA, IgG, IgE, IgD RFs may also be detected. RFs have been reported in other autoimmune and non-autoimmune diseases as well as in healthy individuals (69). The RF test has high sensitivity but low specificity for RA. RF is detected in about 75% of the RA patients, but it is also present in other autoimmune diseases and infectious diseases. Positive RF test is not sufficient for RA diagnosis but aids diagnosis. For being classified as a RA patient according to the 1987 American College of Rheumatology (ACR), the patients must fulfill at least four of seven criteria (Table 1), where RF positivity is one criterion.

Table 1. The 1987 revised criteria for the classification of RA*

The 1987 revised criteria for the classification of RA*
1.Morning stiffness
2. Arthritis of 3 or more joint areas
3. Arthritis of hand joints
4. Symmetric arthritis
5. Rheumatoid nodules
6. Serum rheumatoid factor
7. Radiographic changes

* The patients must have at least 4 of these 7 criteria to be classified as RA case

Source: The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis (70)

1.6.2 Anti-citrullinated protein/peptide antibodies (ACPAs)

Citrullination is a posttranslational protein modification where the unusual amino acid citrulline substitutes an arginine. Calcium-dependent peptidyl arginine deiminase (PAD) is an evolutionarily conserved protein that catalyzes the citrullination of proteins. Several isoforms for PAD are known in human (PAD1–4 and PAD6). PAD can citrullinate the extracellular proteins such as collagen and fibrinogen in tissues under inflammatory conditions (71).

Antibodies directed against citrullinated peptides are RA specific (72-74). They are routinely measurable using an anti-cyclic citrullinated peptide (anti-CCP) test. The recent generation of CCP (CCP-2) test is an ELISA based assay that captures reactivity towards either in vitro or synthetic citrullinated peptides. It is more discriminative than RF test, since it has RF-like sensitivity along with high specificity for RA. Around 40% of RF-negative patients with RA are positive for the anti-CCP test.

10

ACPAs are predictive marker used by clinicians for early RA diagnosis. The updated diagnostic criteria from 2010 from ACR and EULAR are shown in Table 2 (72). It is more likely that RF positive patients are also ACPA positive. Since anti-citrulline immunity and RF autoantibodies are expressed even years before the onset of any symptoms, it is believed that they are involved in the early process of joint destruction and the pathogenesis of RA.

Data from a longitudinal study showed that 75% of 318 undifferentiated inflammatory arthritis subjects with anti-CCP positive at baseline have classified as RA patients after one-year follow-up. This number increased to 93% after three years of follow-up. Meanwhile, only 25% of anti-CCP negative individuals had progressed to RA after three years. A similar study of 314 early RA patients reported that 90% of anti-CCP positive participants were classified as RA after one-year follow-up (75). This emphasizes on prognostic characteristics of anti-CCP antibodies as a biomarker for RA and can help patients to be prioritized for treatment.

Importantly, different RA patients display reactivity towards different specific citrullinated peptides (65, 76). Commercial CCP-2 tests are meant to identify any reactivity towards citrullinated peptides, but they do not specify which certain citrullinated peptides that the individual has reactivity against.

Table 2. The 2010 ACR/EULAR criteria for the classification of RA. ESR: Erythrocyte sedimentation rate.

Classification criteria for RA (score-based algorithm: add scores of categories A-D: a score of ≥6/10 is needed for classification of a patient as having definite RA	Score
A. Joint involvement	
1 large joint	0
2-10 large joints	1
1-3 small joints (with or without involvement of large joints)	2
4-10 small joints (with or without involvement of large joints)	3
>10 joints (at least 1 small joint)	5
B. Serology (at least 1 result is needed for classification)	
Negative RF and negative ACPA	0
Low-positive RF or low-positive ACPA	2
High-positive RF or high-positive ACPA	3
C. Acute phase reactants (at least 1 test result is needed for classification)	
Normal CRP and normal ESR	0
Abnormal CRP or abnormal ESR	1
D. Duration of symptoms	
<6 weeks	0
≥6 weeks	1

Source: 2010 Rheumatoid arthritis classification criteria: an American College of Rheumatology/European League Against Rheumatism collaborative initiative (77)

Recently a new multiplexed array has been developed that simultaneously captures reactivity towards multiple citrullinated antigens. This array includes more than 40 citrullinated autoantigens that were subdivided according to their "protein of origin". Table 3 summarizes the citrullinated peptide antigens on the multiplex chip array. Citrullinated peptide antigens on the multiplex microarray were named based on their protein source and the location of amino acid in the peptide chain that arginine residue was substituted by citrulline residue.

Table 3. Citrullinated peptide antigens on the multiplex microarray.

Protein source	Citrullinated Peptide
Fibrinogen	cit-Fibß$_{36-52}$, cit-Fibα$_{563-583}$, cit-Fibα$_{580-600}$, cit-Fibα$_{621-635}$, cit-Fibα$_{36-50}$, cit-Fibß$_{60-74}$, cit-Fibß$_{cit-R}$, cit-Fibß$_{R-cit}$, CCP-1
Vimentin	cit-Vim$_{60-75}$, cit-Vim$_{2-17}$
α-enolase	CEP-1
Collagen type II	cit-C1, cit-F4$_{R-cit}$, cit-F4$_{cit-cit}$, cit-F4$_{cit-R}$
filaggrin	CCP-1
hnRNP A3	cit-Pept-Z1, cit-Pept-Z2, cit-Pept-1, cit-Pept-5, cit-Pept-Bla26

Peptides on the array included in study III derived from:

1) vimentin, which is fibroblast intermediate filament found in the cytoskeleton in eukaryotic cells and present in around two-third of RA patients (78).

2) fibrinogen is a hexameric plasma glycoprotein central to coagulation. Around 50-60% of RA patients have immune reactivity towards citrullinated fibrinogen epitopes (79).

3) alpha-Enolase (α-Enolase): is a ubiquitously expressed glycolytic enzyme. Around 40% of RA patients express antibody against citrullinated-peptide epitope CEP-1, which is an immunodominant epitope in this group of peptides (80).

4) collagen type II are the triple-helical peptides that are the main component of hyaline cartilage. Anti-citrullinated collagen type II antibodies are found in about 40% of patients with RA (81).

5) filaggrin is a filament-associated protein that attaches to keratin fibers, mostly found in epithelial cells.

6) Heterogeneous Ribonuclear Protein A3 (hnRNP A3).

The presence of processes like cross-reactivity and epitope spreading among ACPA are widely known, which contribute to the co-occurrence of these RA specific citrullinated peptide antibodies. (82, 83).

The ability of an antibody to react with a similar epitope on different antigens call cross-reactivity. Some of the antibodies towards citrullinated peptides may react to more specific citrullinated antigens, but most of them tend to be widely cross-reactive (82, 83).

Epitope spreading is characterized as the development of immunity against diverse epitopes. This immune response can be directed towards a self or foreign antigen, to subdominant epitopes on that protein instead of the immunity reactivity against dominant specific epitope (84, 85). Co-occurrence of ACPAs from epidemiological aspect can be translated to mediating or colliding effect, meaning each antibody can mediate the presence of another antibody.

1.7   ENVIRONMENTAL RISK FACTORS FOR RA

Environmental factors can play an essential role in the origination of systemic inflammation and autoimmunity of RA prior to visible joint symptoms.

Smoking is a well-established exposure that associates with elevated risk of ACPA positive RA but not ACPA negative RA. It has been shown that smoking cause citrullination in the lung and has an association with the occurrence of anti-citrulline immunity in RA (86-88). Alcohol consumption has been shown to have a significant inverse association with risk of RA (89-93). A higher body mass index is associated with increased risk of ACPA negative RA (93-95). It is not known yet if RA risk factors have an association with specific anti-citrullinated antibodies and thereby can be potentially engaged in RA pathogenesis through inducing anti-citrulline immunity.

As it was mentioned, the effects of environmental risk factors differ between ACPA positive and ACPA negative RA patients (66). ACPA positive RA patients also show a more severe course of the disease compared to ACAP negative RA patients (96). Consequently, distinct phenotypic manifestations of RA have an association with distinct RA relevant risk profile. This implies ACPA positive and ACPA negative RA patients go through different pathogenic mechanisms to develop the disease. It is suggested that these antibodies, and the specific immunity they represent, are involved in the pathogenesis of RA. Since they are expressed many years before the onset of the disease, they have the prognostic ability.

Increasing the knowledge in specific anti-citrulline immunity in association with established RA related environmental risk factors is vital to understand the role of environmental risk factors in etiology and pathogenesis of RA.

2   AIM

2.1   OVERALL AIM

The aim of this thesis was to evaluate the role of new biomarkers in chronic inflammatory diseases focusing partly on atherosclerosis and its primary clinical manifestation MI, and RA.

2.2   SPECIFIC AIMS

The specific aims of this thesis were:

- To investigate the association between sIL-6R and sgp130, respectively, with MI and explore the potential interaction between these biomarkers.

- To explore the association between sgp130 serum levels and c-IMT and c-IMT progression after 30 months follow-up in a European population with high-risk profile for CVDs.

- To identify genetic variants associated with sgp130 serum levels and to investigate if those genetic loci are associated with c-IMT as a marker of subclinical atherosclerosis.

- To identify if environmental risk factors of RA are associated with the presence of RA specific autoantibodies.

3   MATERIALS AND METHODS

3.1   MATERIALS

3.1.1   SHEEP study

Study I was based on data from the Stockholm Heart Epidemiology Program (SHEEP), which is a large population-based case-control research designed to investigate genetic and environmental risk factors of relevance for the occurrence of MI. The study base was consisted of all Swedish citizens living in Stockholm county from 1992-1994, aged 45 to 70 without previous clinically diagnosed MI.

3.1.1.1   Case identification and control selection

Overall, 5452 participants were enrolled in the study, 2246 cases and 3206 controls. The cases were included in the SHEEP at the time of disease incidence at ten emergency hospitals within the Stockholm county. Cases were diagnosed according to the diagnosis criteria for MI, accepted by the Swedish Association of Cardiologists in 1991-1994. In this thesis, only non-fatal cases (n=1213) were included that are patients who survived at least 28 days post-MI and had no further MI before sample collection. Fatal cases (n=603) were excluded due to the absence of blood sampling.

Controls (n=1561) who matched on sex, age (five years interval) and hospital catchment area were randomly selected from the study base within two days from case incidence. In order to substitute potentially nonresponsive controls, five controls per case were selected simultaneously. Occasionally both the first and potential substitute controls were contacted and included in the study due to the late response of the initial control. Therefore, there are more controls than cases in the study.

Both cases and controls candidates were identified through the National Patient Registry and were investigated if they had previous MI (ICD9-codes 410 or ICD-10 I21).

Clinical investigations were undertaken on average three months after the index events, including blood samplings under fasting conditions with the collection of whole blood for DNA extraction, serum and plasma (EDTA and citrated). The serum samples were kept at -70°C until analyzed. To collect data on a large set of potential risk factors, cases and controls completed a questionnaire, which was complemented with a telephone interview, to complete missing information. Controls underwent a health examination as close as possible in time to the corresponding cases to avoid biases due to seasonal change in blood parameters. Non-fatal cases and controls had participation rate of 87% (n=1643) and 73% (n=2339) respectively.

Anthropometric measurements such as BMI (Kg/m^2), systolic and diastolic blood pressures were evaluated. Other biochemicals were also measured such as insulin, total cholesterol, triglycerides, HDL cholesterol, CRP, serum glucose, blood lipids and TNF-α.

3.1.1.2 Biomarker measurement

sIL-6R

The serum level of sIL-6R was assessed in 1785 serum samples from 682 cases and 1103 controls using MesoScale Discovery (MSD) Human Cytokine assay (Gaithersburg, MD, USA), following the manufacturer's assay protocol. Samples were diluted 1:75, and the calculated concentrations from the standard curve were expressed in (ng/mL). The minimum detectable value for sIL-6R was 0.1 pg/mL.

The intra-assay variability was asessed by running n=267 samples in duplicate within the same experiments, whereas the inter-assay variability was assessed by duplicating n=67 samples in separate experiments. The intra-and inter-assay coefficient variations were 6.1 % and 3.8% respectively. According to the manufacturer, the recommended threshold is 15% and 18% for mean intra-assay and mean inter-assay coefficient of variation, respectively.

sgp130

The serum level of sgp130 were measured using an assay from R&D systems®, Quantikine® ELISA according to the protocol instructions. Because of lack and or inadequacy of serum, in total, 1726 serum samples were evaluated for sgp130, 664 cases and 1062 controls. Samples were diluted 100 times, and serum concentrations (ng/mL) were derived from the standard curve. The intra variability (1.8%) and inter variability (12.1%) were calculated respectively by duplicating n=25 samples within a plate and n=37 samples in independent experiments. No specific threshold for intra- and inter-assay variability were suggested by the manufacturer for sgp130, although previous studies have reported an intra-assay variability <10–11% and inter-assay variability <10–16% (97, 98).

3.1.1.3 Ethical consideration

The SHEEP study was conducted in accordance with the Helsinki Declaration and was approved in 1991 by the Regional Ethical Review Board at Karolinska Institutet. Participants gave their oral informed consent since no written informed consent was in use.

3.1.2 IMPROVE study

IMPROVE (carotid **I**ntima-**M**edia Thickness and c-IMT **PR**ogression and the risk of **V**ascular **E**vents) is a multicenter prospective study designed to investigate the association of c-IMT and c-IMT progression with the risk of future CVEs. From March 2004 to April 2005, 3711 subjects (men, N= 1772 and women, N=1931) aged from 55 to 79 years free of any CVDs but with medical history for at least three conventional CV risk factors (e.g. hypertension, diabetes, dyslipidemia, smoking and family history of CVDs) were selected by seven recruiting centers from five European countries: France (n=501 from Paris), Sweden (n=533 from Stockholm), Finland (n=1050 from two centers in Kuopio), Italy (n=1095 from Milan and Perugia) and the Netherlands (n=532 from Groningen).

At baseline, every study participant completed an extensive questionnaire on lifestyle habits, former disease and treatment. Anthropometric measures were recorded, and a large biobank with whole blood, serum and plasma was established and stored at −80°C until used. For evaluating c-IMT changes overtime, subjects were followed for three years, and ultrasonographic measurements were repeated at two time-points, at 15 months and 30 months, using the same ultrasonographic protocol applied at baseline. Smoking was defined as current smoking. Hypertension was defined if self-reported and or diastolic blood pressure ≥90 mmHg and or systolic blood pressure ≥140 mmHg and or treatment with antihypertensive drugs; Diabetes was defined as self-reported and or blood glucose level ≥7 mmol/L and or treatment with insulin or oral hypoglycemic drugs. Hypercholesterolemia was defined as LDL cholesterol ≥4.13 mmol/L and or treatment with cholesterol-lowering drugs. More details about study design have been described elsewhere (99).

3.1.2.1 Ultrasonographic measures

Participants underwent c-IMT measurements by trained sonographers using a Technos system (Esaote, Genova, Italy), equipped with a 5-10 Mhz linear array probe. Ultrasounds data were collected from the far walls of the left and right common carotid (CC), the bifurcation (Bif), the internal carotid artery (ICA) and the 1st centimeter of common carotid (I_CC) in anterior, lateral, and posterior angles. All measurements were done in at least three different frames at three time-points: at baseline, after 15 months and after 30 months of follow-up. The baseline c-IMT ultrasonographic measurements selected for the study II were: 1) IMT_{mean}: the average of mean c-IMT for all the eight segments (left and right I_CC, CC, Bif and ICA); 2) IMT_{max}: the largest c-IMT value recorded among all eight the segments investigated; 3) $IMT_{mean-max}$: the average of the eight max c-IMT values recorded at each of the eight segments. c-IMT baseline values are reported in mm.

c-IMT was also measured after 15 and 30 months of follow-up and the progression of c-IMT was calculated at 15 months, by dividing the difference between the 15 months and corresponding baseline value by the length of intervening time period. c-IMT progression at 30 months calculated by linear regression model between three-point measurements and expressed in mm/year.

3.1.2.2 Measurement of serum sgp130 levels

Serum sgp130 levels were measured by DuoSet ELISA development kits of human sgp130 (DY228) provided by R&D Systems ® (R&D systems Minneapolis, MN, USA). Samples required a 100-fold dilution and the range of a standard curve was 20 ng/mL to 0.25 ng/mL. Briefly, 96-well plates were coated by diluted capture antibody in the working concentration of 4.0 μg/mL sealed and incubated overnight at room temperature. Then for blocking step, 2% Bovamin serum albumin was used (200 μL/well) and incubated for one hour at room temperature. Detection antibody in working concentration of 0.08 μg/mL was added and incubated for 2 hours at room temperature. To optimize the protocol for serum samples, different concentrations for capture antibody and detection antibody were tested. To validate the sample diluent (0.2% BSA in 1X PBS), linearity test was performed by adding (spiking) known amount of human recombinant sgp130 to the samples. Microplate reader set to 450 nm and a correction wavelength of 540 nm or 570 nm.

To calculate the intra- and inter-assay coefficient variation, a known concentration of 5 ng/uL from recombinant sgp130 was duplicated in both the same and two different plates. The intra- and inter-assay coefficients of variation were 1.88% and 12.1% respectively.

3.1.2.3 Genotyping; CardioMetaboChip 200k and the ImmunoChip

Genomic DNA from IMPROVE study participants was genotyped using two custom-made genotyping arrays; 1) CardioMetaboChip 200k: a custom Illumina iSelect genotyping array for the study of genetic variants associated with metabolic and CVDs, and 2) ImmunoChip: is a custom Illumina Infinium HD array containing approximately 200,000 variants mapping in genetic regions identified by GWAS as potentially relevant for immune-mediated diseases. More detailed information on these two arrays can be found (100, 101).

3.1.2.4 Ethical consideration

The IMPROVE study was funded by the Vth European Union program. The study was carried out in accordance with the Helsinki Declaration and approved by the IRB at each one of the seven recruiting centers: 1) the Regional Ethics Review Board at Karolinska Institutet, Stockholm Sweden, 2) IRB at the Groupe Hôpitalier Pitie-Salpetriere, Paris, France, 3) the IRB Comitato Etico delle Aziende Sanitarie della regione Umbria, Perugia and 4) the IRB at the Ospedale Niguarda

Ca´Granda, Milano, both in Italy, 5) the IRB at the University Hospital Groningen, Groningen, the Netherlands, 6) the IRB Hospital District of Northern Savo and 7) and the IRB at University of Eastern Finland, both in Kuopio, Finland. Each participant provided two different written consents one for general participation in the study and one for genotyping.

3.1.3   EIRA study

The EIRA (Epidemiological Investigation of Rheumatoid Arthritis) is an ongoing population-based case-control study since 1996 recruited subjects 18-79 years aged from defined (southern/central) regions of Sweden. Only cases, who were selected from May 1996 until November 2009, were included for the analysis in this study. The participation rate for the cases was 94%.

3.1.3.1   *Case identification and control selection*

Cases (n=2859) were defined as RA patients diagnosed according to the 1987 ACR criteria by rheumatologists within twelve months after the onset of joint disease symptoms. Sampling was done at the first visit before applying any RA specific medication.

Controls (n=581) were randomly selected through the Swedish national register and matched for age, sex and residential area, more details on study population can be found in (102). Data derived from the questionnaire on lifestyle-related risk factors and blood samples of all participants were collected at baseline for further genetic and serological analysis.

Subjects were defined regarding exposure to smoking to two categories: "smokers" and "never-smokers". Smokers are individuals that presently smoke, had smoked before, or those who occasionally smoke whereas never-smokers had not ever smoked cigarettes. Similarly, regarding alcohol intake, participants were classified either as "ever-drinker" or "never-drinker". Moreover, the label "high alcohol consumer" was assigned to men consuming at least 168 grams of alcohol per week, and to women having at least 108 grams per week. All others were classified as low alcohol consumers. BMI was classified as obese (≥30 kg/m^2) or not obese (<30 kg/m^2). For additional analysis, two other categories were defined: 1) overweight or obese (≥25 kg/m^2) and 2) normal or underweight (<25 kg/m^2).

3.1.3.2   *Measurement of serum level of IgG specific ACPA*

A microarray-based on the ImmunoCAP ISAC system (Phadia AB, Uppsala, Sweden) was customized to measure the level of antibodies against various citrullinated peptides. This array has been validated using ELISA-based technology. Details were described in (76). More than 40 different citrullinated peptides and their arginine-containing counterpart were immobilized in a microarray, and the reactivity towards

these peptides was measured based on detected fluorescence intensities. Reactivity towards 19 citrullinated peptides was included in study III (Table 3).

The cut-offs for the presence of antibody against each citrullinated peptide was calculated based on the 98th percentile in the 581 healthy controls. It is assumed, 2% of the general population would give reactivity toward any citrullinated peptide regardless of having RA, which can be because of assay noise or unspecific binding.

3.1.3.3 Measurement of serum level of RF isotypes

In EIRA serum level of IgM, IgG and IgA-RF were measured in RA patients using EliA immunoassay on Phadia 2500 (Phadia GmbH, Freiburg, Germany).

3.1.3.4 Ethical consideration

Participants were informed about the study by health care professionals at the rheumatology clinics, provided oral consent which was documented in their medical records. Ethical approval was obtained from the Regional Ethical Review Board at Karolinska Institutet, Stockholm, Sweden.

3.1.4 eRA-Umeå study

The early RA (eRA-Umeå) cohort consists of 1022 patients (692 women and 330 men) fulfilling the 1987 ACR criteria for RA diagnosed at the Departments of Rheumatology in the four most northern counties of Sweden, who were included in the National Register for early RA. All subjects were recruited to eRA-Umeå cohort between Jan 1996-April 2012. Data on lifestyle and environmental risk factors for RA were collected through a self-reported questionnaire. Plasma samples were collected at baseline and kept in -80°C freezers until use (103). Twenty-two patients lacked information regarding smoking.

3.1.4.1 Measurement of plasma level of IgG specific ACPA

Expression of IgG specific antibodies against citrullinated peptides were assessed in plasma of 1011 cases using the custom-made array chip (Thermo Fisher Scientific, ImmunoDiagnostics, Uppsala, Sweden). Citrullinated peptide antigens were as follow: α-enolase peptide (CEP-1), Collagen type II (C1, F4-$_{R\text{-}cit}$, F4-$_{cit\text{-}cit}$ and F4-$_{cit\text{-}R}$), fibrinogen (Fib$\alpha_{36\text{-}50}$, Fib$\alpha_{563\text{-}583}$, Fib$\alpha_{580\text{-}600}$, Fib$\alpha_{621\text{-}635}$, Fib$\beta_{36\text{-}52}$, Fib$\beta_{60\text{-}74}$), Filaggrin (CCP-1), vimentin (cit-Vim$_{2\text{-}17}$, cit-Vim$_{60\text{-}75}$) and hnRNP-A3 (Pept-Bla-26, Pept-1, Pept-5, PeptZ1and PeptZ2). The cut-off value for positivity was set at the 98th percentile of 477 healthy controls for all the antibodies (103).

3.1.4.2 Ethical consideration

The patients gave their written informed consent, and the Regional Ethics Committee at Umeå University Hospital approved the study.

3.2 STATISTICAL ANALYSIS

3.2.1 Study I

Differences between cases and controls were calculated by Kruskal–Wallis test for continuous variables and by chi-square (χ2-test) for the binary variables.

Levels of sIL-6R and sgp130 were categorized in quartiles according to the serum levels in the controls. sIL-6R quartile boundaries were (ng/mL) $Q_1 \leq 31.6$, $31.6 < Q_2 \leq 40.8$, $40.8 < Q_3 \leq 54.5$, $54.5 < Q_4$, and sgp130 quartile boundaries were (ng/mL) $Q_1 \leq 308.8$, $308.8 < Q_2 \leq 365.6$, $365.6 < Q_3 \leq 432.6$ and $432.6 < Q_4$.

Logistic regression models were applied to compute the odds ratio for MI in association with the high level of sIL-6R and sgp130, respectively. Exposure to high sIL-6R and sgp130 was defined according to the cut-off values of the 75th and 90th percentiles of the distribution of these biomarkers in controls. The association results reported as OR with 95% CI were calculated for the exposure to high levels of sIL-6R and sgp130, respectively, considering $\leq$75th and $\leq$90th percentiles as reference categories.

In the crude model (model 1), analysis was adjusted for sex, age and hospital catchment area. Based on previous literature additional potential confounders were included in model 2; hypertension (individuals on antihypertensive drug therapy or with blood pressure $\geq$140/90 mmHg), diabetes (subjects with a glucose value >6.7 mmol/l and or treatment with insulin and or other drug treatment), hypercholesterolemia (total cholesterol $\geq$6.4 mmol/l or receiving any lipid-lowering medication), current smoking and BMI.

To investigate if circulating sgp130 levels can change the association between sIL-6R and MI, first the association between high sIL-6R (>75th) and risk of MI was stratified based on the median value (365.6 ng/mL) for sgp130 using logistic regression. Results were expressed as OR with 95% CI. Due to the low number of study participants in the group having sIL-6R>75th and sgp130>90th, the median was used as the cut-off for the sgp130. However, resembling analysis was performed using the 90th percentile cut-off for the sgp130 distribution, and similar results were achieved. A model, including adjustments for the previously mentioned confounders was also applied.

When there is a biological interaction, the effect of the presence of two interacting factors together is higher than the additive effect of each one of them (104). Therefore, the interaction between serum levels of sIL-6R (low$\leq$75[th] /high>75[th]) and sgp130 (low$\leq$50th /high>50th) was modelled by defining four exposure groups:

1) the group exposed to sIL-6R>75th but sgp130>50th
2) the group exposed to sIL-6R>75th but sgp130$\leq$50th

3) the group exposed to sIL-6R$\leq$75th but sgp130$\leq$50th

4) the group exposed to sIL-6R$\leq$75th but sgp130>50th (this group had the lowest risk and was used as the reference group)

The relative risk of MI was computed for every exposure group and was compared with the reference group. Analysis were adjusted for sex, age, hospital catchment area, hypertension, diabetes, hypercholesterolemia, BMI and smoking.

Afterwards, the potential synergistic interaction between the two biomarkers was explored by calculating the attributable proportion (AP) and the synergy index (S) together with 95% CI. The AP>0 indicates the presence of an interaction. The S $\geq$1.0 indicates a positive interaction and synergistic effect, while S <1.0 represents a negative interaction and antagonistic effect. For the AP and the S, respectively, 95% confidence intervals were calculated.

All statistical analyses were performed using StataCrop LP (College Station, TX) version 13. The interaction analysis was calculated using the Epinet sheet.

3.2.2   Study II

Due to skewed distribution of sgp130 serum levels, the log-transformed values were used for the genetic association analysis. The quality control for genetic data has been carried out on both the individual genotyping chip and the combined dataset. More information on number of the subjects and including and excluding criteria in study II is illustrated in Figure 3.

After quality control procedure 360,842 SNPs contained in the combined CardioMetabo-Immuno chip from 3439 participants were assessed for association with serum sgp130 levels, by means of multivariable linear regression analysis under the assumption of an additive model of inheritance. Results were reported as beta (β) and standard error (SE) and *p-value* after adjustment for age, sex and population structure (using multidimensional scaling (MDS) components). *p-value*$\leq$1$\times$10^{-5} was set as the significance threshold and *p-values*>1$\times$10^{-5} but $\leq$ 1$\times$10^{-4} as the suggestive association threshold.

Data from a public source of tissue-specific gene expression and regulation was used to report the effect of SNP genotype on tissue expression (105).

The association between potentially relevant SNPs to sgp130 serum level and c-IMT baseline measures were analyzed using linear regression. The first model was adjusted for the potential confounding factors age, sex and latitude while the second model was also adjusted by sgp130.
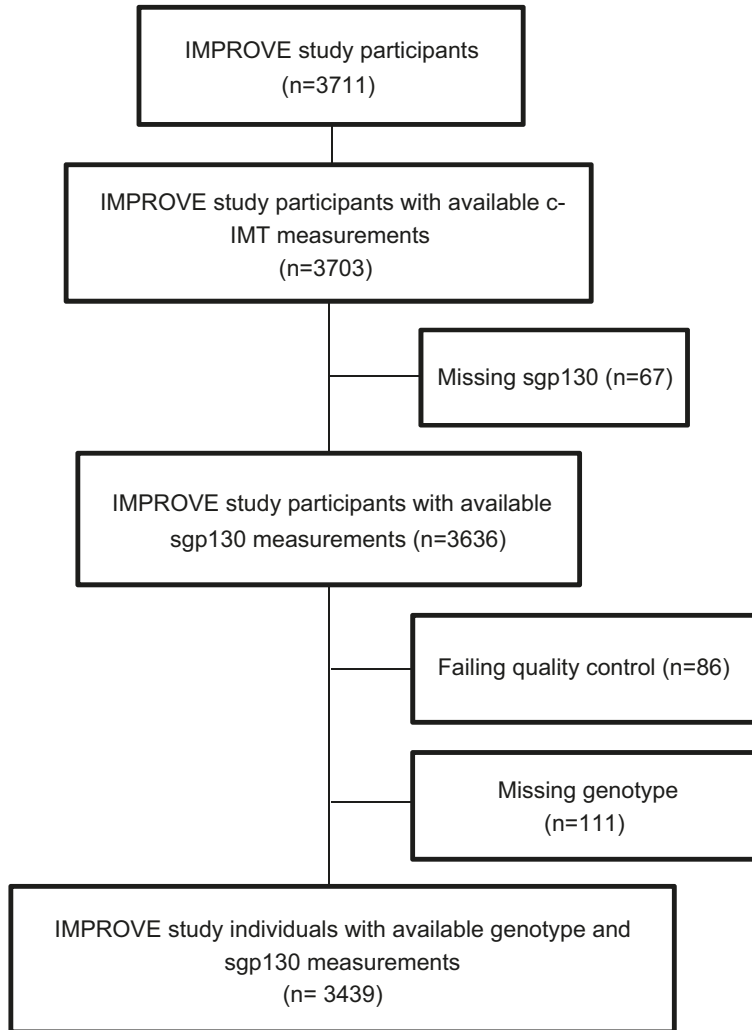
Figure 3. Flowchart summarizing the IMPROVE study participants included in study II.

General epidemiological analyses were calculated by using SAS version 9.4 (SAS Institute, Cary, NC) and SNPs association analysis was done using PLINK v1.07.

In the preliminary analysis performed on the IMPROVE, sgp130 serum levels differed significantly between men and women; therefore, the analyses stratified by gender. Since sgp130 serum levels (ng/mL) were not normally distributed, the measurements were categorized in quartiles based upon the distribution in the entire IMPROVE studies. Quartiles boundaries are $Q1 \leq 452.2$, $452.2 < Q2 \leq 566.6$, $566.6 < Q3 \leq 705.5$ and $705.5 \leq Q4$. Differences among quartiles were calculated by Kruskal-Wallis.

Multiple linear regression models were applied to investigate the association between level of sgp130 and c-IMT at baseline and after 30 months follow-up in two models. The first regression model was adjusted for sex, age and latitude, while the second model also included traditional CV risk factors as covariates (hypertension, hypercholesterolemia, diabetes, BMI and smoking).

p-value ≤ 0.05 was set as significant in all analysis. All statistical analyses were performed using StataCrop LP (College Station, TX) version 13.

3.2.3   Study III

To investigate the correlation between all pairs of antibodies in EIRA and eRA-Umeå, the Pearson correlation coefficient was calculated, which is a measure of the strength of the linear relationship between every two antibodies and varies from +1 to -1.

The χ2-test was used to compare the frequency of presence of each antibody in the two studied cohorts.

Initially, univariate logistic regression was used to model the probability of the presence of 22 RA specific antibodies in terms of being exposed to RA environmental risk factors (smoking, alcohol consumption and BMI), fitting a separate model for each antibody. To tackle the mediation and colliding effect of antibodies to the presence of each other, all antibodies were modelled jointly in the logistic regression analysis. The analysis was performed separately for smoking and alcohol and BMI in EIRA. Results were presented as OR and *p-value* and false discovery rate (FDR).

p-value less than 0.05 was taken as nominally significant. FDR was calculated by p.adjust function to correct for multiple testing and FDR ≤ 10% was considered as acceptable. To assess if the indicated significant associations are mediated by the presence of other antibodies, potential colliders were removed, and association was reanalyzed.

The RF isotype data in the eRA-Umeå cohort was not available, therefore the logistic regression model was performed for 19 antibodies and smoking. Data on alcohol consumption and BMI in eRA-Umeå were not available. Furthermore, this set of analysis was repeated in the merged EIRA and eRA-Umeå participants (n=3797).

Homogeneity between the two cohorts was tested using a likelihood ratio test to assess if the results differed significantly among the cohorts. All statistical analysis was performed in the R software v. 3.3.3 for Windows.

4 RESULTS AND DISCUSSION

4.1 STUDY I, II

4.1.1 Association of sIL-6R and sgp130 with the risk of MI

Results from study I showed that median levels of sIL-6R were higher in cases with MI than controls at inclusion in SHEEP. In line with our results, other studies have indicated increased levels of sIL-6R in MI cases (45) and patients with CHD (44). It has been shown that sIL-6R mediates the pro-atherogenic effect of IL-6 in endothelial cells via trans-signaling by stimulating the production of chemokine and increasing the expression of adhesion molecules (42, 106). Furthermore, elevated sIL-6R has been reported in association with endothelial dysfunction and arterial stiffness (107). Previous studies did, however, measure sIL-6R at the event, which likely mirroring the acute inflammatory phase. In SHEEP sIL-6R was measured three months after MI which likely reflect extended chronic inflammation.

Our data suggest that increased sIL-6R levels ($>75^{th}$) are associated with an increased risk of MI (OR=1.6, 95% CI 1.3-2.0), even though adjusting for potential confounders reduced the risk (OR=1.4, 95% CI 1.1-1.8). Different cut-off limits (75th and 90th percentile) were tested for sIL-6R and sgp130, based on distributions in controls. Using the 90th percentile as a threshold for sIL-6R as expected resulted in an even higher risk estimation (OR= 1.7, 95% CI 1.2-2.3). Results from conditional logistic regression models also gave comparable ORs in crude (OR= 1.5, 95% CI 1.2-1.9) and adjusted (OR= 1.4, 95% CI 1.0-1.8) models.

A lab in Germany highlighted a buffer system for IL-6 trans-signaling formed by sIL-6R and sgp130 (37). Based on this argument, sgp130 inhibits the trans-signaling through binding to the IL-6/sIL-6R complex with a high affinity (10 pM) after the complex was unified, this is 100 times higher than the affinity of binding sIL-6R to IL-6 (1nM) (34). Therefore, the presence of high levels of sgp130 prevents IL-6/sIL-6R to bind membrane-bound gp130 and thereby also prevents the activation of IL-6 trans-signaling.

According to the manufacturer, it is not distinguishable if unattached sIL-6R or sIL-6R in complex with IL-6 was measured. It could be theorized that the measured sIL-6R in the SHEEP cohort partly reflects the complex of sIL-6R/IL-6 leading trans-signaling pathway. Alternative interpretation can be the higher unattached sIL-6R in the patients is joined to IL-6 and initiating the trans-signaling.

No difference in the serum level of sgp130 was observed between cases with MI and controls in the SHEEP. In agreement with our results, other studies reported

no difference between sgp130 serum level in cases with MI, CHD and controls (45). In a study of IL-6 signaling in patients with MI, no correlation was observed between sgp130 and intensity of myocardial necrosis (108). However, higher sgp130 serum concentrations were reported as a marker of inflammation in other diseases, e.g. RA, inflammatory bowel disease (109) and colon cancer (110).

In the SHEEP no association was found between sgp130 and MI using the 50th and 75th percentile as a cut-off, while exposure to very high serum sgp130 (>90th percentile) was inversely associated with risk of MI when adjusting for potential confounders (OR=0.68, 95% CI 0.5-0.9). When adding covariates one by one to the model, diabetes decreased the ORs to a notable extent, suggesting the actual effect of diabetes as a confounder on this association. In line with this result, Italian data from older individuals (age ≥ 65 years) demonstrated an association between metabolic syndrome and high plasma levels of sgp130 (111). Moreover, in another study from Poland, an independent negative association between sgp130 and insulin resistance has been indicated in women with polycystic ovarian syndrome proposing an inhibitor role for sgp130 in IL-6 trans-signaling in insulin-resistant conditions (112).

An elevated level of sgp130 has been shown to block the IL-6 trans-signaling via IL-6/sIL-6R complex (113) and have a protective effect on the inflammatory disease progression (114). High sgp130 can dismiss retaining of chronic inflammation in systemic diseases like atherosclerosis by inhibition of the IL-6 trans-signaling via binding to IL-6/sIL-6R complex. Administration of recombinant sgp130 in the mouse model of atherosclerosis has indicated an inhibition of the rapid increase in the number of smooth muscle cells and decline in atherosclerotic plaque progression (50, 115, 116). This explains the reduction of the MI risk in the presence of high sgp130 serum level. Previous studies have indicated that only extreme levels of biomarkers might influence risk of disease (117, 118), which is in accordance with detecting an association only for the 90th percentile of sgp130 levels here.

Similar to sIL-6R, there was no possibility to recognize if measured levels of sgp130 in the SHEEP is unattached or attached to sIL-6R/IL-6 complex (sIL-6R/sgp130/IL-6). Thus, one can speculate sgp130 in either form can inhibit the trans-signaling pathway and deduce the susceptibility to MI.

4.1.2 Interaction analysis

Results from interaction analysis revealed an indication of a biological interaction between high sIL-6R (>75th percentile) and low sgp130 (<50th percentile) (AP= 0.19, 95% CI -0.2 – 0.5 and S index= 1.7, 95% CI 0.5-6.1). It is worthy of note that a larger population would be needed to conclude on the presence of interaction between sgp130 and sIL-6R. Study of biological interaction between

two biomarkers can provide an approach to understand the biological mechanism underlying development of CVD and specifically atherosclerosis.

As outlined earlier, binding of sgp130 and IL-6/sIL-6R complex can inhibit the progression of IL-6 trans-signaling pathway. Research performed on mice indicated that the administration of sgp130 has significant protective effect on atherosclerosis. It showed that sgp130 reduced the atherosclerotic plaque development and progression through nullifying the IL-6/sIL-6R trans-signaling independently of classic-signaling (50). This suggests that sgp130 may antagonize the effect of IL-6/sIL-6R complex on the progression of atherosclerotic lesions. A recombinant form of sgp130 is in fact now tested as an anti-inflammatory drug in phase 1 clinical trials (54). Nevertheless, the larger study material than SHEEP is required to achieve a proof on synergism or antagonism and provide more accurate estimates.

4.1.3   Genetic variants associated with serum sgp130 levels

In study II the association of genetic variants with the level of sgp130 was investigated in IMPROVE. Around 360,842 SNPs passed quality control and were analyzed for association with log-transferred sgp130. Although the commonly accepted *p-value* in GWA studies is $\leq 1\times10^{-8}$, this study utilised more relaxed *p-value* thresholds for significance ($\leq 1\times10^{-5}$) as well as for suggestive association ($\leq 1\times10^{-4}$). This can in part be motivated by the fact that this was not a GWA study, but a more in-depth investigation of the loci which are part of the two genotyping arrays. Only two SNPs, rs10935473 mapping at chromosome (Chr) 3 and rs1929666 at Chr 10 surpassed chosen *p-value* threshold for significant association with sgp130. 24 SNPs surpassed the suggestive association threshold level. The manhattan and locus plot of the association *p-values* does not show very distinct peaks of associations around these SNPs, although a few of the neighbouring SNPs also show suggestive associations.

The indicated SNPs on chromosome 3, rs10935473, have moderate correlation (r^2:0.67) to rs9858592, placed in the ST3GAL6-antisense RNA 1 (ST3GAL6AS1). According to the genotype-tissue expression (GTEx) project reports, the effect allele at both SNPs have association with reducing the expression of ST3GAL6AS1 in different tissues like adipose tissue, the heart and the arterial wall as well as inverse association with circulating sgp130.

ST3GAL6AS1 have a role in the regulation of cell adhesion molecules. Molecular cell adhesion intervenes in extravasating the inflammatory cells from the circulation and accumulate on the vascular endothelium in the early stage of atherosclerosis.

Besides, rs9858592 is in moderate correlation with rs865474, which is also located in ST3GAL6 and has a history of association with BMI.

Rs74760246 (β= -0.028, *p-value*= 1.21×10^{-5}) and rs3087409 (β= 0.029, *p-value*=2.70×10^{-5}) were the only two SNPs in this study that previously have shown association with risk of inflammatory diseases as well as CVDs. rs74760246 located on Chr 1 has a powerful correlation (r^2>0.8) with rs1421389 and rs10494757 placed at DENNB1, which is a gene known for the association with the chronic inflammatory diseases (119, 120). rs3087409 mapping at WRN on Chr 8 correlated with a SNP formerly associated with premature ageing as well as the risk for MI and stroke (121).

The genetic loci that some of the identified suggestive SNPs mapped at, have been formerly associated with regulating glucose and cholesterol metabolism, e.g. rs300624 on Chr 1 that recognised as liver receptor homolog 1 (122), rs3813774 on Chr 19 and rs73063812 on Chr 7 have all suggestive inverse association with serum levels of sgp130. Furthermore, rs16932962 on Chr 9 in TTC39B and rs1681503 on Chr 11 in ARAP1 have suggestive positive association with sgp130. TTC39B slightly correlated with mentioned suggestively associated SNPs has association with low HDL levels (122).

In total, disregarding the chosen significance threshold, these results propose that SNPs involving in regulating sgp130 are also regulating cardiometabolic phenotypes that have a minor inflammation phenomenon in common.

4.1.4 Association of sgp130 and c-IMT

Results from a preliminary analysis in IMPROVE showed women with higher sgp130 level had smaller c-IMT (markers of subclinical atherosclerosis) at baseline as well as the smaller progression of c-IMT after 30 months. Preliminary results from multiple linear regression indicated sgp130 has significant inverse relationships with c-IMT at baseline in women, but no significant relationships to c-IMT change over time in either men or women. The opposite direction of the association between c-IMT and sgp130 is in line with a therapeutic effect of sgp130 on atherosclerosis progression. Data from an animal study showed applying recombinant sgp130 led to regression of atherosclerotic plaques (50).

It must however be noted that no firm evidence of the association between c-IMT and sgp130 was observed in IMPROVE, therefore, further research is required to confirm or reject the weak associations detected here.

4.1.5 Association of the SNPs associated with sgp130 with c-IMT

SNPs that showed signs of association with circulating sgp130 levels were evaluated for association with c-IMT at baseline in IMPROVE. rs17688225 on Chr 14 was the only SNP inversely associated with c-IMT measures (c-IMT$_{mean}$: β=-0.010

SE=0.005, *p-value*=0.0251; c-IMT$_{mean–max}$: β=−0.010 SE= 0.005, *p-value*=0.0347; c-IMT$_{max}$: β=−0.025 SE=0.009, *p-value*=0.0049). Interestingly this SNP had a suggestive positive association with circulating sgp130 (β=0.030, *p-value*=4.77×10-5), which goes in line with the atheroprotective effect of sgp130 (123).

4.2   STUDY III

4.2.1   Correlation between ACPAs

The pairwise correlation between the presence of RA-specific antibodies against specific peptides or isotypes of RF were visualised in the two heatmaps plots (Figure 4). A similar pattern of correlation between different anti-citrullinated peptide antibodies was observed in the EIRA and eRA-Umeå cohorts. These biomarkers were positively correlated in different degrees, and they were not clusters by the protein of origin. One group of antibodies including antibodies against CEP-1, Cit-Pept-5, Cit-Pept-Z1, Cit-Pept-Z2, CCP-1, Cit-PeptBla26, Cit-Fibβ$_{36-52}$, Cit-Fibα$_{621-635}$ and Cit-Fibα$_{563-583}$ showed a very strong correlation to each other. Of note, these belong to the different source of citrullinated peptides: α-enolase, hnRNP A3, filaggrin and fibrinogen. Another set of antibodies including Cit-Fibα$_{36-50}$, Cit-C1, Cit-F4$_{Cit-R}$, Cit-Pept-1were less correlated to each other and the other reactivities against citrullinated peptides.

IgA-, IgG- and IgM-RF in EIRA demonstrated a strong positive correlation to each other but a rare co-occurrence with a highly correlated group of ACPAs. RF is less specific for RA compare to ACPAs and is also used as a serological marker for other autoimmune diseases. Thereby, two distinct molecular mechanisms for the production of RF and ACPAs are presumable (124).

The correlation between different reactivities can be due to cross-reactivity, epitope-spreading, shared causes, or isotype switching in terms of RF. Cross-reactivity is one of the reasons that can explain the co-occurrence of reactivity towards different citrullinated peptides. The existence of similar epitopes on two or more citrullinated peptides can eventuate binding of the antibody specific to one of these peptides to the other one.

It has been recognized during recent years that ACPA can also cross-react to another post-translationally modified motifs, which indicated that cross-reactivity is wide-spread (125, 126). The co-occurrence of antibodies reported in this study is in line with epitope spreading, the process where the antigens that are recognized increases or shifts during an immune response. Epitope spreading happens years before RA onset (84, 127). Jointly this indicated that the presence of reactivity towards one antigen often mediates the presence of reactivity against other antigens. Using epidemiological terms, this means that different reactivities mediate the presence of each other.
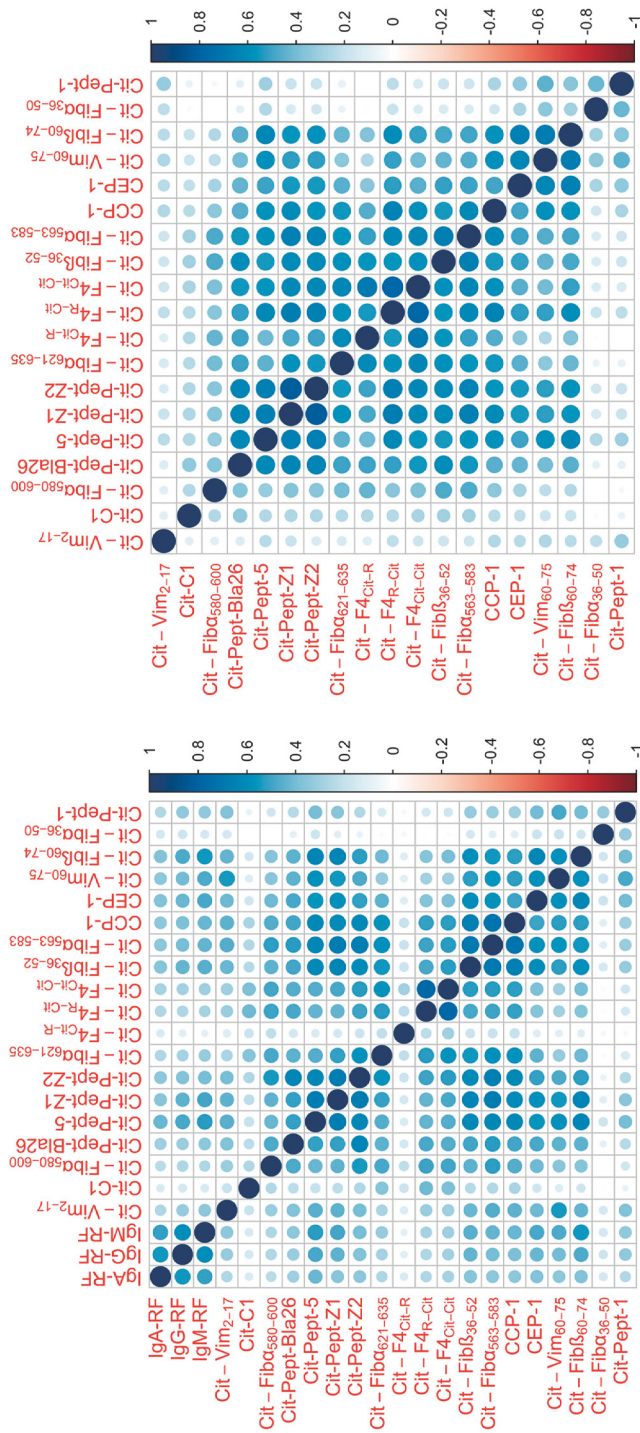
Figure 4. The Pearson coefficient correlation between 22 antibodies in EIRA (left panel) and 19 antibodies in eRA-Umeå (right panel). The colour intensity indicates the strength of the correlation (scale on the right).

4.2.2 Association between smoking and presence of ACPAs

For association analysis initially, a separate model for each antibody were fitted. Almost all antibodies in EIRA demonstrated a significant positive association with smoking except for anti-Cit-C1and anti-Cit-F4$_{Cit-R}$ (128). These results were in accordance with causal paths between smoking and antibody presence, and with extensive mediation due to cross-reactivity and or epitope spreading.

In this study, the interest was to assess whether environmental risk factors of RA cause RA by influencing the presence of specific antibodies. These antibodies are correlated to each other with different strength, but the knowledge about biological mechanisms of these relationships are limited. Thus, to avoid detecting associations driven by mediation, a logistic regression model was used, which evaluated all antibody jointly. That revealed in EIRA, being an ever smoker had a negative association with having antibodies towards Cit-Vim$_{60-75}$ and Cit-C1 as well as a positive association with having IgA-RF, anti-CEP-1 and anti-Cit-Pept-Bla26 antibodies (*p-value* ≤ 0.05).

Considering the FDR, only IgA-RF (OR=2.14, FDR=1.6*10-8 %) and anti-Cit-Vim$_{60-75}$ (OR=0.69, FDR=3.2%) remained significantly associated with smoking in EIRA. In eRA-Umeå, only anti-Cit-Fib$_{36-50}$ antibody was significantly associated with smoking after considering the multiple testing (OR=1.78, FDR=0.070).

Results from merged analysis, including both EIRA and eRA-Umeå cases, indicated smoking had a negative association with anti-Cit-C1(OR=0.71, FDR=3.8%) and anti-Cit-Vim$_{60-75}$ (OR=0.77, FDR=8.2%) antibodies as well as a positive association with anti-Cit-Fibα$_{36-50}$ (OR=1.32, FDR=3.8%) and anti-CEP-1 (OR=1.39, FDR=1.6%). The merged analysis thereby strengthened the confidence of association between antibodies toward CEP-1 and Cit-C1 and smoking but downplayed the association between anti-Cit-Vim$_{60-75}$ antibody and smoking, which was not observed in eRA-Umeå at all. This emphasized a requirement of more investigation to conclude on the true effect of anti-Cit-Vim$_{60-75}$ antibody and smoking.

Several studies have investigated the association between smoking and RA (129). It is been hypothesized that smoking contributes to RA development by inducing protein citrullination in the lung and thereby causes chronic inflammation and initiation of anti-citrullinated peptide immunity in the lungs of the individuals (130). The low level of ACPA has been detected in both non-RA inflammatory diseases affected the lung tissues and in bronchoalveolar lavage of non-smoker RA patients (131, 132). Lungs, as the second most affected organs by RA, can be the anti-citrullination immunity onset point (130).

The presence of mediator and collider may affect the association between environmental risk factors and the presence of RA specific ACPA (Figure 5).
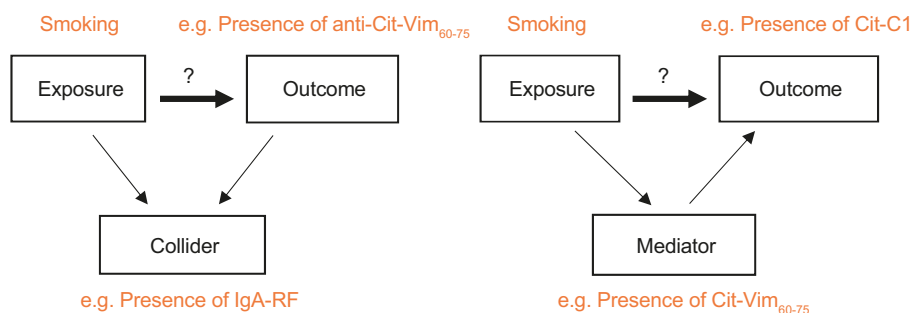
Figure 5. Collider (Left panel) and mediator (Right panel) scenarios in association with exposure and outcome. Examples are hypothetical.

According to the immunological literature, antibodies can mediate the presence of each other, which from the epidemiological point of view can be translated to giving both mediator and collider role to antibodies in the production process of each other (Figure 5) (104, 133). A Mediator is a variable that intervenes the direct relationship between exposure and outcome, whereas a collider is a variable that is causally influenced by exposure and outcome. As mentioned earlier, including colliders in the regression model can lead to biased results and false associations. Therefore, to understand the role of environmental risk factor in the pathogenesis of RA through immunity towards specific citrullinated peptide, in EIRA and merged data, potential colliders were excluded, and the association analysis was reassessed for remaining antibodies.

In this regard, to investigate if the association between IgA-RF and smoking in EIRA is a direct association, we excluded potential colliders (other four smoking-associated antibodies: Anti-Cit-Vim$_{60-75}$, anti-Cit-C1, anti-CEP-1 and anti-Cit-Pept-Bla26) thereafter analyzed the logistic model for 18 antibodies. The results from EIRA showed a significant positive association between IgA-RF and smoking; this association was robust when excluding potential colliders.

Association between smoking and IgA-RF was far stronger than the positive association of anti-CEP-1 IgG and anti-Cit-Pept-Bla26 IgG with smoking. Several studies have shown smoking has a strong association with RF compare to ACPA (131, 134-136). It was shown in this study, the correlation of IgA-RF with reactivity towards citrullinated peptides was not strong ($r2= 0.13-0.43$). This was in agreement with an earlier study performed in EIRA, indicating a difference between IgA- and IgG- class antibodies to CCP2 cyclic citrullinated peptides in association with smoking. In that study they found an association between smoking and IgA CCP2 but not between smoking and IgG CCP2 (137). This suggests that smoking can lead to citrullinating of autoantigens such as fibrinogen and α-enolase and vimentin in the lungs and cause inflammation in the lung's mucosal surfaces,

which leads to IgA ACPA responses towards the citrullinated peptides (131, 134). Increased IgA ACPA has been reported before in early RA patients, and it is known that IgA is the primary antibody of the mucosal immune system (138, 139). As a future perspective of ACPA studies, it will be of interest to assess the relationship between smoking and IgA response to ACPA.

The potential colliding effect of IgA-RF on the association between smoking and anti-Cit-Vim$_{60-75}$ antibody in EIRA also was avoided by excluding IgA-RF, anti-Cit-C1, anti-CEP-1 and anti-Cit-PeptBla26 antibodies from the model. The results confirmed a negative association between anti-Cit-Vim$_{60-75}$ and smoking among RA patients in EIRA. A similar analysis was done in the merged dataset to avoid the colliding effect. Every time three of anti-Cit-Vim$_{60-75}$, anti-Cit-Fibα$_{36-50}$, CEP-1, anti-Cit-C1 were excluded from the regression model and evaluated the effect of the association on the left one. After all, in the merged data, smoking showed a direct and independent association with anti-Cit-Fibα$_{36-50}$, anti-CEP-1 and anti-Cit-C1 antibodies but not with anti-Cit-Vim$_{60-75}$.

The negative association between anti-Cit-Vim$_{60-75}$ and smoking was not robust in our different analyses. Overall, it was unexpected to find a negative association between a citrullinated antigen and smoking. Therefore, another statistical approach was taken to avoid mediation effects in the found negative association. A stratified subset of the population that did not express any of the antibodies positively associated to smoking was therefore utilized to assess the association between smoking and anti-Cit-Vim$_{60-75}$ in a univariable model. By this approach, the negative association between smoking and anti-Cit-Vim$_{60-75}$ was corroborated in EIRA (OR=0.60, *p-value*=6.7*10^{-3}), but not in the merged dataset. The result from sensitivity analysis on the merged data was in line with the result of association analysis for anti-Cit-Vim$_{60-75}$ and smoking after removing the colliders effect.

The stratified analysis was also repeated to assess the real negative association between Cit-C1 and smoking in the merged dataset. No negative association remained (OR=0.72, *p-value*=0.11) after excluding antibodies towards anti-Cit-Vim$_{60-75}$, anti-Cit-Fibα$_{36-50}$ and CEP-1.

Results from previous studies and our own using univariate regression showed a weak positive association between smoking and anti-Cit-Vim$_{60-75}$ antibody. It is likely that due to the positive correlation between anti-Cit-Vim$_{60-75}$ antibody and other positively associated antibodies with smoking, the negative association has been covered up in the univariate regression model. Although in the merged analysis, the significant negative association between smoking and anti-Cit-Vim$_{60-75}$ was disappeared after removing collider effects. Hence further investigation is required to deduce the presence of a negative association between smoking and anti-Cit-Vim$_{60-75}$ antibody.

In EIRA, we had available data on irregular smokers who might have different pathophysiology of RA from regular smokers. The association analysis was repeated on individuals excluded irregular smokers, and the results did not alter.

The homogeneity of association signals between the two cohorts, EIRA and eRA-Umeå, was evaluated using a likelihood ratio test; results did not differ significantly among the cohorts (*p-value*=0.062).

In summary, our study indicated that smoking might give rise to antibodies against a few antigens, perhaps by increasing the exposure of these antigens in affected tissues. Trough processes such as epitope spreading, isotype switching and cross-reactivity this might after that develop into the co-expression of several antibodies as is often observed in RA patients.

4.2.3 Presence of ACPAs in association with alcohol and BMI

In EIRA, anti-Cit-F4$_{Cit-Cit}$ antibody was negatively associated with being an ever-drinker (OR=0.56, FDR=7.9%, 95% CI=0.37-0.85) while anti-Cit-F4$_{R-Cit}$ antibody indicated a suggestive association with being an ever-drinker (OR=1.56, *p-value*=0.046). The association of ACPAs and high consumption of alcohol also was investigated, and no significant association was detected. Merely a suggestive association between IgA-RF (OR=0.62, *p-value*=0.015) and anti-Cit-Fibα$_{580-600}$ (OR=1.83. *p-value*=0.016) and high alcohol consumption was observed.

The association between the RA ACPA-specificity and BMI was also investigated in the EIRA. Anti-Cit-F4$_{R-Cit}$ antibody (OR=1.60, *p-value*=0.032) had a suggestive positive association with obesity taking BMI>30 while anti-Cit-Vim$_{60-75}$ antibody (OR=0.65, *p-value*=0.011) showed a suggestive negative association with obesity (though FDR>10%). Thereafter, the association between 22 antibodies and being overweight taking BMI>25 was tested; only a stronger negative suggestive association was observed between the anti-Cit-Vim$_{60-75}$ antibody (OR=0.71, *p-value*=0.0049, FDR=11%) and being overweight.

Previous studies has shown alcohol consumption (89, 90) have a significant inverse association with risk of RA (89-93) while a higher body mass index is associated with increased risk of only ACPA negative RA (93-95). Since in this thesis, the focus was to assess the effect of environmental risk factors on ACPA fine-specificity, so it might be anticipated to observe no association between BMI and these antibodies. Our results indicated the contribution of alcohol consumption and BMI in the development of RA is not through the pathways involving ACPAs.

5 METHODOLOGICAL CONSIDERATION

Several strengths and limitations of the studies included in this thesis deserve to be mentioned:

5.1 STRENGTHS

The main strength of population-based studies included in this thesis, the SHEEP, the EIRA, the IMPROVE and the eRA-Umeå , is using the large number of individuals who have provided a wide range of information in lifestyle, environmental factors and biological tests at the time of recruitment in the study. Besides, the participation proportion of cases in all case-control and cohort studies included in this thesis were high, which allows the participants in the study to be representative of the study base.

Furthermore, the combination of two population of EIRA and eRA-Umeå provided a larger sample size and increased the power of association analysis, however, more in-vitro and epidemiological investigations on a larger population are required to draw a particular conclusion on the association between smoking and RA-specific antibodies.

Applying united and standard methods to measure exposure and outcome was one of the most important strengths of all four populations. c-IMT measurements were performed in all seven centers of IMPROVE using the same protocol on high-resolution B-mode ultrasound. In EIRA and eRA-Umeå also similar customized microarray were used to measure reactivity towards citrullinated peptides.

Another strength of IMPROVE that worth to mention is using custom made CardioMetaboChip and ImmunoChip collect the genetic data that increased the probability of finding SNPs associated with CV and inflammatory diseases. Moreover, in study III, when many concurrent statistical tests were involved in association analysis, to reduce the risk of detecting false-positive associations, the multiple-testing correction has been calculated (FDR).

5.2 CAUSALITY AND REVERSE CAUSALITY

To eventuate on a causal relationship between an exposure and outcome, the exposure sampling must be performed before outcome occurrence. In the SHEEP study, the serum of participants was collected minimum three months post-MI. The reason for late sampling was to wait for regaining stability in metabolic and inflammatory markers that caused by MI, and to avoid the influence of disease and medication on serum values. Hence, concluding on the causal relationship

between exposure and disease of the study I is not straight forward. There is a probability that measured biomarkers three months after MI not reflecting fully the true values before MI (140, 141). Although, there are some studies emphasizing on regaining metabolic stability three months after MI (140, 141). In addition, from a prospective study of other inflammatory biomarkers (IL-6 and TNF-α) in the SHEEP indicated comparable levels to baseline (39, 142, 143). Therefore, computed OR might be overestimated or underestimated, which cannot be corrected in association analysis.

There is also a possibility that cases change their lifestyle habits after having a heart attack like post-MI smoking cessation and physical activity, which may affect measured biomarkers circulation. Similarly, for EIRA and eRA-Umeå, where autoantibodies might have been present years before the diagnosis of RA, patients might have experienced symptoms that cause them to alter their lifestyles. It is not possible to determine if exposure precedes the outcome precisely. It is however very improbable that the presence of autoantibodies causes individuals to start smoking. Therefore, it is essential to keep in mind that retrospective data collection might be subjected to inherent bias. In addition, it is unachievable to get a deep intuitive comprehending of the biological mechanisms underlying the observed association in observational studies. The evidence of the casual effect of sgp130 on atherosclerosis in the IMPROVE was inconclusive.

Different source of errors in studies included in this thesis was discussed as follows.

5.3 MISCLASIFICATION BIAS

5.3.1 Misclassification of exposure

Recall bias

There is a possibility of introducing recall bias when participants are asked to recall exposures, as is the case with data on environmental and metabolic factors by questionnaires in study III (EIRA and eRA-Umeå cohorts). However, the short duration between onset of the first symptom and diagnosis of the disease (median: 195 days) as well as the short time between diagnosis and filling the questionnaire (within a year) in EIRA make recall bias less likely. Although data on main exposures in SHEEP and IMPROVE were not collected by questionnaires, information on some of the covariates were self-reported, which may lead to potential misclassification of confounders.

In general recall bias is an inherent restriction in all case-control study plans because there is a possibility that cases remember differently in comparison to controls when they answer the questionnaires.

Survival bias

In study I, the results cannot be genuinely generalized to fatality cases (n= 603) since only non-fatal MI cases were included in the analysis. This was due to impossibility of measuring biomarkers three months after the event in fatal cases. There is a possibility that measured exposure in non-fatal cases is different (might be lower) from the level of exposure in fatal cases. It would be therefore a possibility of underrating the effect of exposures (sIL-6R and sgp130) on the outcome (MI).

Biomarker measurements

In all the four population, collected serum and DNA samples were kept in biobank -80°C to -70°C freezers for several years until experiments. It is a common phenomenon in biomarker epidemiological studies nowadays. Biomarkers concentrations are likely affected by the long storage and samples might degrade. According to prior epidemiological studies on cytokines using fresh samples in comparison with SHEEP and IMPROVE samples, the direction and size of the association is not massively affected by storage time (45, 144-147). However, if sample degradation occurs, it would lead to non-differential misclassification since neither the exposure nor the outcome would differentially alter in cases and controls in the SHEEP (and in all participants of the IMPROVE).

Furthermore, the exposures were dichotomized taken in the analysis in the EIRA (presence or absence of the specific antibody) and in the SHEEP (sIL-6R and sgp130). Thus, non-differential misclassification of a dichotomous exposure is likely. If the continues values of exposure were used still the non-differential misclassification may change the OR, depending on in which strata the dichotomous exposure was misclassified. In addition, none of the studies aimed to establish on absolute values for the biomarkers in this thesis. One of the strategies to avoid misclassification of exposure in the SHEEP was to measure biomarkers in cases and controls blindly in every experiment. In the IMPROVE all ultrasonic scans also were read blindly by experts.

5.3.2   Misclassification of outcome

c-IMT is largely applied as surrogate marker for atherosclerosis, but still, it does not mirror the atherosclerosis alone. Thus, it is difficult to generalize the findings from c-IMT association in IMPROVE to atherosclerosis. However, several ultrasonic measurements were performed to obtain c-IMT variables, those that were selected (IMT_{mean}, IMT_{max}, $IMT_{mean-max}$) to be included in study II, were highly correlated to each other and thus yielded similar information.

The criteria for MI diagnosis have changed since the time of collecting SHEEP materials. The MIs included in the SHEEP might not be representative of all MIs

diagnosed with new criteria, considering more precise recent methods. Therefore, the results from the SHEEP might not be generalized to the more recent settings.

In study III, the presence of antibodies was derived from the 98th percentile in the healthy controls. Given the low frequency of each antibody among the healthy controls (2% by definition) the selection of controls and chance will have a considerable effect on the threshold value and consequently the frequency of the antibody among the cases. However, this process should be random and not lead to differential misclassification of outcome.

5.4 SELECTION BIAS

Non-participation is very important in the epidemiological studies and can be a source of selection bias when individuals participating in the study are different from non-participants. The participation rate was high in SHEEP, EIRA and eRA-Umeå cohorts, which gives a smaller chance of selection bias. If the exposure information differs among participants and non-participant, it might cause a differential non-participation bias, which can underestimate the association result. In the SHEEP, there were individuals that had not enough serum samples for biomarkers measurements (sIL-6R and sgp130). They were distributed amongst cases and controls at random, therefore, the selection bias is improbable. Furthermore, the baseline characteristics of excluded individuals were compared to the total population of the SHEEP. Most of the risk factors and anthropometric characteristics indicated comparable prevalence except prevalence of age and hypertension.

Individuals included in the IMPROVE were selected according to high-risk profile for CVE, with the presence of at least three vascular risk factors, from seven European centers. Thus, the selected population for IMPROVE are not representative of all population with European ethnicity at risk of CVD. Therefore, the results cannot be generalized to the general European population with less than three CV risk factors.
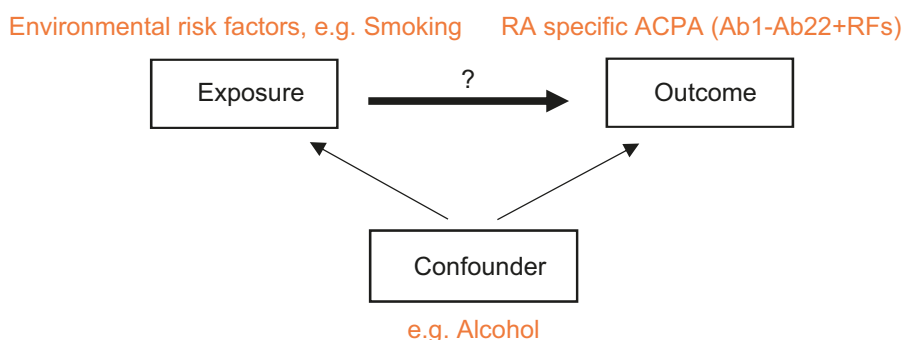
Cases in EIRA and eRA-Umeå were newly diagnosed included from all rheumatology units, from both public and private sectors. Due to the free health care system in Sweden, all individuals have access to medical services and would not avoid seeking medical care because of financial concerns. The fact that almost all public and private rheumatology units in the study area reporting the diagnosed RA patients to the EIRA database were linked to the general welfare system, therefore, reduces the chance of selection bias. However, investigations into non-participation in EIRA using the 1996-2005 part of the cohort (patients in study III were recruited later) showed that non-participant cases were older, had a lower income, shorter education and were more often born outside of Sweden (148).

No similar investigations of the later part of the cohort have been performed, but it is likely that the participation bias is similar. It is unclear at this point if age, income, education or ethnicity affects the presence of the investigated antibodies.

The revised diagnosis criteria of (EULAR/ACR) in 2010 included ACPA-antibodies as one criterion and enables the detection of patients earlier in their disease. Since both cohorts in study III were included based on the old 1987 diagnosis criteria, this might mean that the sample is slightly biased and includes a smaller proportion of ACPA positive patients. However, since patients usually express ACPA-antibodies several years before diagnosis, this bias is expected to be small.

5.5   CONFOUNDING BIAS

Presence of confounders may change the estimated strength of the association. By definition, the confounder must have an association with both the disease and the exposure (104). The models need to be adjusted for confounders to avoid bias in the estimates of the parameters describing the association of interest. Therefore, in EIRA and eRA-Umeå, the smoking association analysis were adjusted for alcohol and alcohol association analysis for smoking (Figure 6).

Environmental risk factors, e.g. Smoking   RA specific ACPA (Ab1-Ab22+RFs)

```
┌──────────────┐        ?         ┌──────────────┐
│   Exposure   │ ───────────────▶ │   Outcome    │
└──────────────┘                  └──────────────┘
         ▲                              ▲
          ╲                            ╱
           ╲      ┌──────────────┐    ╱
            ╲     │  Confounder  │   ╱
             ╲    └──────────────┘  ╱
```

e.g. Alcohol

Figure 6. potential confounding effect of alcohol consumption in the association between smoking and RA specific antibodies. Examples are hypothetical.

In SHEEP and IMPROVE, the biomarkers associations analyses have been adjusted for conventional CV risk factors, e.g. smoking, diabetes, hypercholesterolemia and hypertension. However, in the study of genetic factors of IMPROVE, fewer potential confounders were considered because the exposure itself is quite improbable to be affected by other factors.

5.5.1 Residual and unmeasured confounding

It is rational when evaluating inflammatory biomarkers, they could also reflect inflammatory statuses, which were not the aim of investigation when the study was designed. Association of cytokines and their receptors with different cancers and other chronic inflammatory diseases have been reported (149, 150). It was not possible to adjust for these inflammatory conditions in study I and II since the data was not available. Therefore, it is difficult to rule out the presence of uncontrolled confounders in those studies.

6 CONCLUSION AND FUTURE PERSPECTIVE

6.1 STUDY I, II

Increased serum level of sIL-6R had an association with increased risk of MI while the elevated serum level of sgp130 has an inverse association with the risk of MI, in a Swedish population aged 45-70 years old. A sign of interaction between sIL-6R and sgp130 with risk of MI was detected. More extensive population-based studies are required to increase the power to derive a conclusion on the additive interaction. However, our results highlighted the necessity of focusing on molecular pathways instead of only one biomarker when estimating the risk of CVDs.

Investigation of the SNPs associated with sgp130 serum level indicated a possible overlap on genetic variants regulating sgp130 level and c-IMT measures. Our results can shed light on several cardiometabolic pathways with the intervention of sgp130. Increasing the knowledge on genetic variants regulating sgp130 is important, to understand the mechanisms underlying generation of sgp130 as an inhibitor of IL-6 trans-signaling and as a potential therapeutic marker in inflammatory diseases. Thus, replication of these findings and more investigation on therapeutic role of high sgp130 may be of interest for future atherosclerosis research.

6.2 STUDY III

A high co-occurrence exists among a group of RA-specific antibodies while some other antibodies are expressed in RA patients unaccompanied of each other. Smoking is associated with antibodies against IgA-RF and a limited set of citrullinated peptides. This indicates that smoking might play a causal role in the development of RA through a mechanism involving antibodies reactivity towards these peptides. As a future perspective of ACPA studies, it will be of interest to assess the relationship between smoking and IgA response to ACPA. Further investigations on the biological mechanism of the associations between smoking and anti-citrullination immunity are also needed.

7   ACKNOWLEDGMENTS

I wrote this thesis during a challenging time of pandemic and this PhD literally would not have been accomplished without supports I have received from my husband, **Behrouz**. First, I would like to express my appreciation to you, for all the sacrifices that you have made on my behalf specially during the last months.

I also would like to pay my respect to all the **participants** in my studies. Thanks for your contribution to medicine and science.

I would like to express my sincere gratitude to my main supervisor, **Boel Brynedal**, you are brilliant, caring and the most responsible person that I have met at IMM! Thanks for sharing your experience, expertise and wisdom during the years we have worked together and while writing the thesis. I have learned a lot from your computational skills as well as your endless honesty. I am grateful for having you as my main supervisor.

To my co-supervisors, **Karin Leander** (for Epi-courses and for being kind and respectful), **Paolo Frumento** (for helping me with R and statistics, you are an excellent teacher!), **Lars Alfredsson** (for your positive attitude) and my mentor **Erik Melen** (for your continuous support and advice when I needed it).

To **Karolinska Institutet** for providing me with all the necessary funding and required facilities and enabling me to fully concentrate on my education and research.

To the study directors at the Institute of Environmental Medicine, **Lena Palmberg** and **Ralf Morgenstern**, for your assistance during hard times of my PhD.

My sincere thanks also go to my former colleagues from the unit of Cardiovascular Epidemiology, for sharing your knowledge and introducing me to new, and complex ideas that have stretched my brain and helped me acknowledge and adopt a different perspective. For sharing your culture and for all the fun that we had together: **Ilais, Cecilia, Dashti, Reza, German, Xia, Mohammad, Bahareh, Max, Hozan, Kori, Mohsen, Paolo, Ashwini, Sheikh Humayoon, Anna I, Pingling, Maral, Rebeca, Kornia, Lena, Anna P, Edit, Ida, Helga, Anita, Matteo, Eva, Hedley and Chiara.**

My former labmates from the unit of Immunology and Chronic Disease, thank you for showing me that I am not alone in my ways, losses, struggles, and wins: **Mizan, Karlhans, Emma, Dyvia, Elin, Imran, Sudir.**

Thanks to my lunch partners: **Mizan** (for all the lunches we had together and sharing thoughts. You have good taste in choosing restaurants!), **Maral** (you are a true

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