Lipoprotein-associated phospholipase A2 (Lp-PLA2) Impact and role as cardiovascular risk marker

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2008

Department of Clinical Sciences in Malmö

Cardiovascular Epidemiology and Internal Medicine Research Groups

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Faculty of Medicine

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Doctoral Dissertation

Akademisk avhandling som, med vederbörligt tillstånd av Medicinska fakulteten vid Lunds Universitet för avläggande av doktorsexamen i medicinsk vetenskap, kommer att offentligen försvaras i aulan, medicinska kliniken, ingång 35, Universitetssjukhuset MAS, Malmö, lördagen den 25 oktober 2008 kl. 09.15

Fakultetsopponent: Professor Olle Wiklund, Göteborgs Universitet

Handledare: Professor Bo Hedblad

Abstract

Lipoprotein-associated phospholipase A2 (Lp-PLA_2) is today considered a novel specific vascular inflammatory biomarker. The general aim of this thesis was to study the role and impact of Lp-PLA_2 as cardiovascular (CV) risk marker in an epidemiologic perspective. Specific aims were to explore the cross-sectional association of Lp-PLA_2 with traditional CV risk factors, to asses the genetic influence of PLA2G7 on plasma levels of Lp-PLA_2, and to study the morbidity and mortality of cardiovascular disease (CVD) in relation to Lp-PLA_2. Data from the population-based "Malmö Diet and Cancer" CV cohort (n=6103) was used. Information on Lp-PLA_2 was available on 5393 subjects (41 % men) with a mean follow-up of 10.6 years. National and local registers were used to retrieve the incidence of coronary events (CE), ischemic stroke and mortality.

Lp-PLA_2 (assessed as activity or mass) increases with age, is higher in males and in current smokers. Lp-PLA_2 is strongly correlated with blood lipids (especially LDL-cholesterol), however, is weakly correlated to glucose and to the extent of carotid asymptomatic atherosclerosis (i.e. intima-media thickness and plaque).

Genetic variation at the PLA2G7 gene locus significantly influences plasma Lp-PLA_2 activity and mass levels, in a position and sex-specific manner.

Both elevated levels of Lp-PLA_2 activity and mass, respectively, are independent of blood lipids, hs-CRP and other traditional cardiovascular risk factors, associated with an increased risk for incident ischemic stroke. No similar independent relationship was observed between Lp-PLA_2 and CE.

Lp-PLA_2 activity, compared with mass, is more strongly correlated to all five components constituting the metabolic syndrome (MetS) and increased more linearly with number of MetS components. Elevated levels of Lp-PLA_2 activity was related to increased risk for incident CVD regardless of MetS. High Lp-PLA_2 levels and presence of MetS were additive predictors of those who experienced a CV event.

It is concluded that both genetic and life-style factors are related to elevated levels of Lp-PLA_2. Lp-PLA_2 is independently associated with increased CVD risk, in particular ischemic stroke. There is an additive effect of Lp-PLA_2 to presence of MetS on incident CVD risk, which may identify an especially high risk individual.

Till alla dom som hjälpte mig att göra det möjligt!

Var dig själv. Alla andra är redan upptagna.

Oscar Wilde

Contents

List of publications

I. **Persson M**, Nilsson JÅ, Nelson JJ, Hedblad B, Berglund G. The epidemiology of Lp-PLA_2: Distribution and correlation with cardiovascular risk factors in a population-based cohort. *Atherosclerosis 2007;190:388-396*.

II. **Persson M**, Nelson JJ, Berglund G, Hedblad B. Lp-PLA_2 activity and mass are associated with increased incidence of ischemic stroke. A population-based cohort study from Malmö, Sweden. *Atherosclerosis 2008;200:191-198.*

III. **Persson M**, Nelson JJ, Hedblad B, Berglund G. Elevated Lp-PLA_2 levels add prognostic information to the Metabolic Syndrome on Incidence of Cardiovascular Events among middle-aged non-diabetic subjects. *Arterioscler Thromb Vasc Biol. 2007; 27;1411-1416.*

IV. **Persson M**, Stirnadel H, Carlsson J, Melander O, Berglund G, Hedblad B. In a population-based cohort study; variations in the PLA2G7 gene are associated with Lp-PLA_2 activity and mass. Submitted Manuscript.

Abbreviations

apo	apolipoprotein
ARIC	Atherosclerosis Risk in Communities Study
ATPIII	Adult Treatment Programme III
BMI	body mass index
CAD	coronary artery disease
CI	confidence interval
CE	coronary events
CHD	coronary heart disease
CV	cardiovascular
CVD	cardiovascular disease
DNA	deoxyribonucleic acid
FDA	Food and Drug Administration
HbA_1C	Haemoglobin A1C
HDL	high density lipoprotein cholesterol
HOMA	Homeostasis Model Assessment
HRT	hormone replacement treatment
hsCRP	high sensitive C-reactive protein
ICD	International Classification of Diseases
IMT	intima-media thickness
LDL	low-density lipoprotein cholesterol
Lp-PLA_2	lipoprotein-associated phospholipase A2
Lyso-PC	lyso-phosphatidylcholine
MDCS	Malmö Diet and Cancer Study
MetS	Metabolic syndrome

MI	myocardial infarction
NCEP	National Cholesterol Education Programme
oxFFA	oxidized free fatty acid
oxLDL	oxidized low density lipoprotein cholesterol
PROVE IT TIMI 22	PRavastatin Or atorVastatin Evaluation and Infection Therapy-Thrombosis In Myocardial Infarction Trial
ROC	receiver operator characteristic
RR	relative risk
SD	standard deviation
sdLDL	small-dense LDL
SNP	single nucleotide polymorphism
SPSS	Statistical Package for Social Sciences
STROMA	Stroke registry of Malmö
T2DM	Type 2 diabetes mellitus
US	United States
VA-HIT	Veteran Affairs HDL Intervention Trial
WHS	Women Health Study
WOSCOP	West of Scotland Coronary Prevention Study

Introduction

Cardiovascular disease still a challenge

Although mortality from cardiovascular disease (CVD) has decreased markedly over the last decades [1] coronary hearth disease (CHD) and stroke still remain the leading cause of death in developed countries [2, 3]. CVD accounts for nearly half of all deaths in developed countries [2] and CVD are expected to be the leading cause of death worldwide [4]. About 50% of all CHD and strokes in the population occurs among individuals with normal cholesterol levels [5-7]. Although the importance of conventional risk factors such as smoking, diabetes, hypertension, and hypercholesterolemia for CVD is clearly documented, a large proportion (62%) of subjects with established coronary artery disease (CAD) do have none or only one of these risk factors [8]. It has been suggested that even after accounting for established risk factors, one-half of all coronary events remains unexplained [9, 10].

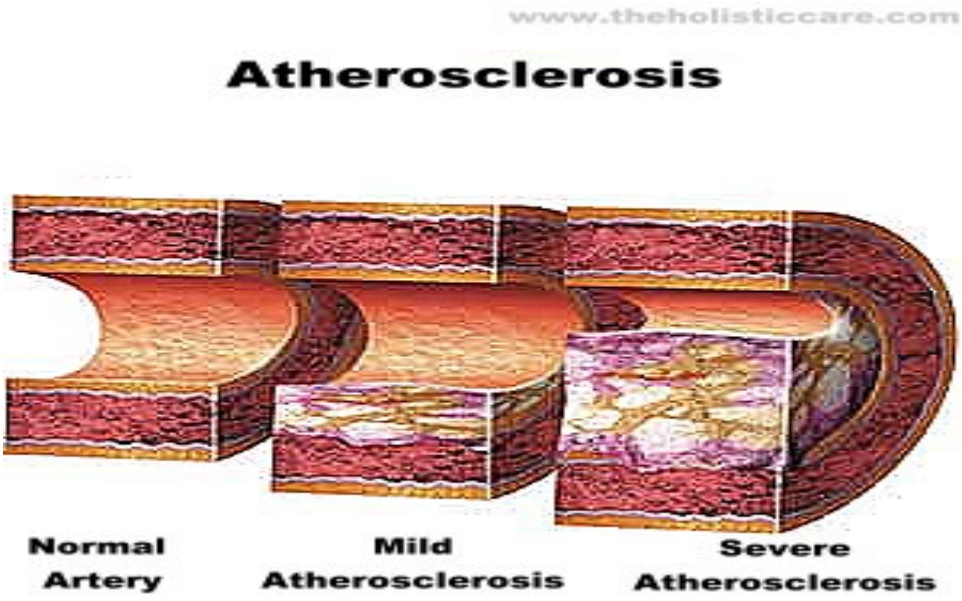
Throughout the world, stroke is the second-leading cause of death [11] and in United States (US) as well as in Sweden the third most common cause of death [3, 12]. Stroke is also the leading cause of adult disability, and women are at higher risk of stroke than men [13]. Almost 85-90% of all strokes are of ischemic origin [13, 14].

The CVD risk factors that have been targeted most aggressively over the last decades are elevated blood pressure and cholesterol, particularly low-density lipoprotein (LDL)-cholesterol [4]. It is well-documented that lowering LDL-cholesterol with statins reduces the likelihood of CVD for patients at various levels of risk [15-19]. A decreased prevalence of smoking together with an aggressive pharmacological treatment of blood lipids and blood pressure during the last decades has been associated with a decreasing incidence of CVD. However due to a growing aging population CVD will still remain as the leading cause of

premature death [2]. Some authors suggest that LDL-cholesterol is less predictive of CHD than in earlier trials [20]. Even if target LDL-cholesterol goal is achieved there remains a significant residual risk for CHD events [20]. Although elevated levels of LDL-cholesterol contribute to the acceleration of atherosclerosis, it provides little information about the state of the artery wall. In a clinical trial, including post acute syndrome patients, almost every fifth patient had a recurrent cardiovascular event within 2 years despite an aggressive lipid-lowering statin therapy that was associated with markedly reduced LDL-cholesterol level [21]. Furthermore, the initial presentation of CAD has been reported to be myocardial infarction (MI) or sudden cardiac death among 62% men and 42 % among women [22]. Thus, it has been argued that we do need better and more effective tools for identification of persons at high cardiovascular risk [23].

Risk factors for stroke include age, gender, ethnicity, hypertension, history of CAD or atrial fibrillation, diabetes mellitus, metabolic syndrome (MetS), smoking, alcohol, physical inactivity, etc [24]. The predictive value of blood lipids for incident stroke has been questioned. In the population-based Atherosclerosis Risk in Community (ARIC) study, including almost 13,000 subjects, aged 45-64 years, LDL-cholesterol levels were found to be similar between stroke cases and controls [25]. Furthermore, several clinical trials have shown the reduction in stroke in association with statin therapy [17, 26, 27]. The mechanism is not clearly demonstrated and it has been speculated whether stroke reduction observed in these trials might be explained by statin pleiotropic anti-inflammatory actions [28]. However, recently high levels of apolipoprotein (apo)-B, and low levels of apoA-1 have been reported to be related to increased risk of ischemic stroke [29, 30].

Today it is widely recommended to use CVD risk assessment tools to identify subjects who should be targeted for intervention. Additional information, beyond traditional CV risk factors, from cardiovascular imaging techniques and biomarkers has been discussed to enhance CAD and stroke risk prediction and possibly the adequacy of risk factor modification.



Atherosclerosis

The role of atherosclerosis has resulted in a paradigm shift; it is now recognised as a consequence of inflammatory processes and not only an accumulation of lipids in the artery wall. Atherosclerosis is today widely considered as a chronic inflammatory process, with evidence of inflammation at all stages of disease, from initial plaque formation to destabilisation and subsequent rupture [31-33].

Vascular inflammation starts with an endothelial dysfunction and migration of leukocytes and LDL-cholesterol. When the LDL particle enter the intimal space it may be oxidized, induces the expression of adheshion molecules on endothelial cells, which allows monocyte-derived macrophages and T-lymphocytes to gather in the subendothelial space. These macrophages

have a high affinity for oxidized LDL (oxLDL), and the ingestion of oxLDL particles by macrophages results in the formation of foam cells. The foam cells progress to fatty streaks and atherosclerotic plaques. If excess influx of lipids into the subendothelial space exists the atherosclerotic process proceeds and results in a necrotic lipid rich plaque covered by a fibrous cap. A thin fibrous cap can easily rupture and expose the thrombogenic core to the blood stream with subsequent thrombosis or occlusion of the artery [31]. Many patients suffer from ischemic heart disease despite treatment with lipid-lowering drugs and with LDL-cholesterol levels below target value [7].

Mechanistic studies have identified blood borne inflammatory cells (e.g monocytes, machrophages, T-lymphocytes) and their products as primary drivers of the inflammatory process [34-36]. Today there is much evidence supporting their role for inflammation in all phases of atherosclerosis [34]. There is documentation that inflammation markers are associated to development of traditional CV risk factors and atherosclerosis [34, 37, 38].

Several population-based studies were initiated to examine the relation between various inflammatory cells, mediators, markers and incident CVD [10, 35, 39-41]. One inflammatory marker, high sensitivity C-reactive protein (hs-CRP), an acute-phase reactant that reflects low-grade systemic inflammation, has been studied in a variety of cohorts and CVD complications. There are numerous prospective studies providing consistent results of the relationship between elevated baseline levels of hsCRP and increased risk of CVD [42]. In patients with acute coronary syndrome hsCRP has been demonstrated to be associated with increased long-term mortality and coronary heart failure [43].

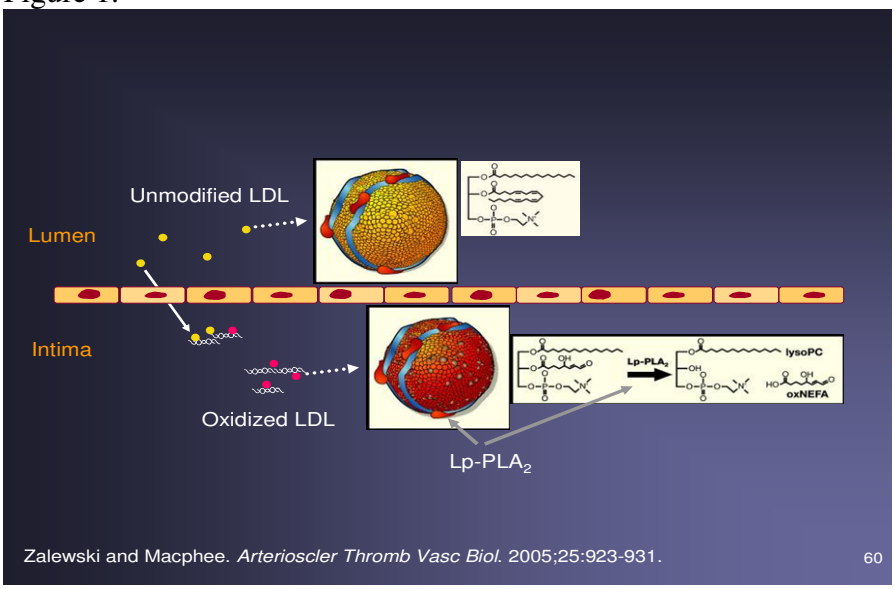
It has been stated that a biomarker of atherosclerosis should be directly involved in the causal pathway of plaque formation and inflammation and been shown to have high specificity and low biologic variability [44, 45]. Most markers reflecting a systemic inflammation (i.e. acute

phase reactants as hsCRP) are depending on the presence of infections, rheumatologic disorders, obesity, etc. Furthermore, hsCRP has been shown to have a great biologic fluctuation within an individual and thus questionable for repeated measurements over time due to its high variability. Another emerging inflammatory biomarker with a vascular specificity is lipoprotein-associated phospholipase A2 (Lp-PLA_2). This biomarker has been considered relative unique in its high specificity for and part of a causal pathway of plaque inflammation [46]. This together with a low biologic variability of Lp-PLA_2 has led to the suggestion that Lp-PLA_2 could be a promising biomarker for atherosclerosis [47-49].

Pathophysiology of Lp-PLA_2 and mechanism of action

Lp-PLA_2 and its role as a novel biomarker for atherosclerosis and vascular inflammation have been explored for several years. Lp-PLA_2, a 45.4 kDa protein, is a calcium-independent member of the phospholipase A_2 family. Monocytes, macrophages, T-lymphocytes, mast and liver cells are the main sources producing the enzyme Lp-PLA_2 [50-52] and these cells are involved in the atherogenesis and progression of atherosclerosis [53]. In humans, Lp-PLA_2 is bound predominantly to LDL-cholesterol and to minor extent to high-density lipoprotein (HDL) cholesterol and very low-density lipoprotein cholesterol [54, 55]. Oxidative modification to the phospholipids component of LDL particle provides the substrate for the enzyme [56, 57]. When phospholipids are oxidized on the LDL particle, Lp-PLA_2 acts rapidly by cleaving one of the fatty acids on the *sn*-2 position of the glycerol moiety and generates two potent mediators, lysophosphatidylcholine (lyso-PC) and oxidized free fatty acid (oxFFA) [58 53] (Figure 1).

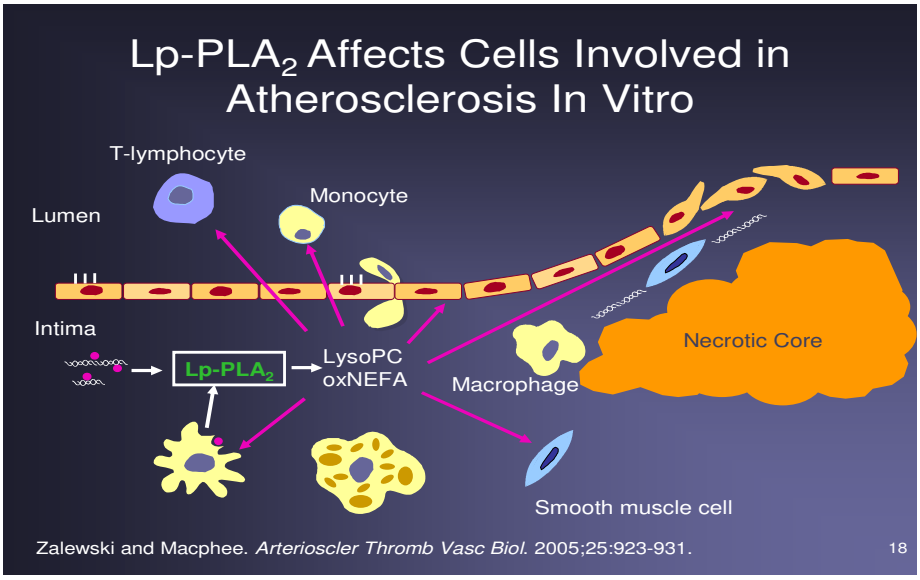
Figure 1.



Lp-PLA_2 acts only on oxLDL and hydrolysis can be carried out solely by Lp-PLA_2 [53, 56, 58]. Both the substrate for Lp-PLA_2, oxLDL and the product lysoPC, have been associated with pro-apoptotic effects on macrophages [58, 59]. Lyso-PC and oxFFA are highly soluble, diffuse throughout the atheroma, and effect the various cell types involved in atherosclerosis [56]. Lyso-PC is a potent chemoattractant for monocytes and T-cells promote endothelial dysfunction, stimulate macrophages proliferation and induce apoptosis in smooth muscle cells [56, 60, 61]. Activated macrophages and foam cells produce more Lp-PLA_2 and Lp-PLA_2 is released by plaques into the circulation [62]. As Lp-PLA_2 is produced within the atherosclerotic plaque, Lp-PLA_2 is more likely to reflect vascular instead of systemic inflammation [63]. LysoPC plays an important role in the effect of Lp-PLA_2 on endothelial dysfunction [62, 64] (Figure 2). In plaques, Lp-PLA_2 was expressed mainly in the necrotic core and surrounding vulnerable and ruptured plaques and to a minor extent in less advanced lesions [65] which suggest that Lp-PLA_2 could be a mediator of plaque progression. An inflammatory process in association with atherosclerosis may take place locally in the vessel wall, however reflected by increased levels of inflammatory biomarkers in the systemic circulation [66]. It has been shown that the expression of Lp-PLA_2 is increased in human aortic atherosclerotic plaques [67].

Lp-PLA_2 is more expressed in macrophages of vulnerable and ruptured plaques, and within the necrotic core, compared to less advanced plaques [65].

Figure 2.



Epidemiologic evidence of Lp-PLA_2 as a cardiovascular risk marker

The association between Lp-PLA_2 and CVD has been studied in clinical settings, clinical trials as well as in cohort studies [68-72]. Recently it was shown that Lp-PLA_2 was associated with coronary endothelial dysfunction independently of other CV risk factors [73]. In that study patients with elevated levels of Lp-PLA_2 had an odds ratio (OR) of 3.3 for having coronary endothelial dysfunction compared with patients with normal Lp-PLA_2 levels. In year 2000, a case-control study in association with the West of Scotland Coronary Prevention Study (WOSCOPS) demonstrated that high levels of Lp-PLA_2 were associated with a two-fold increased risk of CHD. This relationship remained after taking traditional CV risk factors and other inflammatory markers (including hsCRP) into account [68]. This finding has been

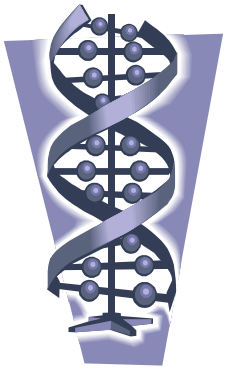
supported by many population-based studies [70-72] including only healthy individuals. On the contrary, one study, including only women, did however not show the independent association of Lp-PLA_2 with incident CVD [69]. In addition, many clinical epidemiological studies, including patients with established CAD and stroke, has shown the independent association of increased Lp-PLA_2 levels with recurrent CHD and ischemic stroke [74-78]. Assessment of Lp-PLA_2, in terms of activity and mass, are different in most previous studies. None of these previous studies included simultaneously both Lp-PLA_2 activity and mass assessment within the same study population. Furthermore, some studies included only men or women [72] or only the determination of Lp-PLA_2 mass [68].

Metabolic Syndrome (MetS)

The MetS is a clustering of metabolic risk factors which increase the risk of developing type 2 diabetes mellitus (T2DM) and CVD [79, 80]. The syndrome has been known since the beginning of 1920 but it was not until 1988 when Gerald Reaven showed an interest in the syndrome and showed its link to insulin resistance [81, 82] that the scientific interest was growing. Today there are several definitions used to define MetS and there is a debate regarding which components should be included [83]. Essential components of MetS are abdominal obesity, elevated blood

pressure, dyslipidemia and glucose intolerance. The World Health Organization (WHO), European Group for the Study of Insulin Resistance (EGIR), the National Cholesterol Education Program (NCEP)/Adult Treatment Panel III (ATPIII), American Association of Clinical Endocronologists, and International Diabetes Federation (IDF) have all offered somewhat different definitions regarding risk factors and cut-off levels of included components [84]. Nevertheless, all definitions are predictors of CVD but recently reports have indicated a leading role for the ATPIII definition [80, 85-87].

With an increasing prevalence of MetS and obesity in the general population [88-90] there is an emerging public health problem. Subjects with MetS have higher oxLDL concentrations and more small sized LDL-particles (sdLDL) [91]. Inflammation markers, in particular hsCRP, have also been linked to MetS [92], and consequently put individuals with MetS at higher risk for CVD. Lp-PLA_2 activity has been shown correlated to sdLDL, [93] to systolic blood pressure, HDL-cholesterol and triglycerides [68, 70, 72], i.e. all components constituting the MetS. These correlations could be of interest to explore in the association between MetS and Lp-PLA_2. In addition, in a study of almost 80 healthy women there was no relationship between Lp-PLA_2 mass levels and insulin resistance as assessed by the insulin suppression test [94]. Individuals with MetS are demonstrated to progress towards diabetes [95] and considered to be at moderate to high risk for CVD [89, 90]. Thus, the inclusion of inflammatory biomarkers (i.e. hsCRP or Lp-PLA_2) could be of clinical importance when assessing cardiovascular risk prediction in patients with MetS [45].



Lp-PLA$_2$ and genetic influences

The gene for Lp-PLA$_2$ (*PLA2G7*) has 12 exons and is located on chromosome 6p21.2 to 12. A large number of single nucleotide polymorphisms (SNPs) have been described, many in small studies, and some variants noted mainly in certain ethnic groups. The most frequently studied SNPs are R92H (rs1805017), I198T (rs1805018), V279P and A379V (rs1051931) [96, 97]. The V279P variant is common in Japanese and Turks but absent in Caucasians [98, 99]. The missense polymorphisms I198T and A379V, identified mainly in Caucasians, are thought to decrease the substrate affinity of Lp-PLA$_2$, possibly prolonging the activity of platelet activating factor, which in turn is associated with many inflammatory diseases [97]. Furthermore, the most studied polymorphism A379V has shown to be associated with higher levels of Lp-PLA$_2$ activity and lower risk of MI in two European studies [100, 101]. On the contrary, in a Taiwanese study the V allele polymorphism in A379V was associated with lower Lp-PLA$_2$ activity and more complex coronary atherosclerosis [102]. Several other polymorphisms have been identified, but however, so far little is known about their role in affecting the regulation or production of Lp-PLA$_2$ assessed as activity and mass, respectively.

Aims of the thesis

The general aim of this thesis was to study the role and impact of Lp-PLA_2 as a CV risk marker using a population-based cohort study.

Specific aims

- To explore the effect of genetic variation in *PLA2G7* gene on plasma Lp-PLA_2 activity and mass levels.

- To examine the cross-sectional associations of demographic and anthropometic characteristics and other CVD risk factors with plasma levels of Lp-PLA_2 activity and mass, respectively.

- To explore whether Lp-PLA_2 activity and mass, respectively, are associated with incidence of CHD or ischemic stroke, respectively.

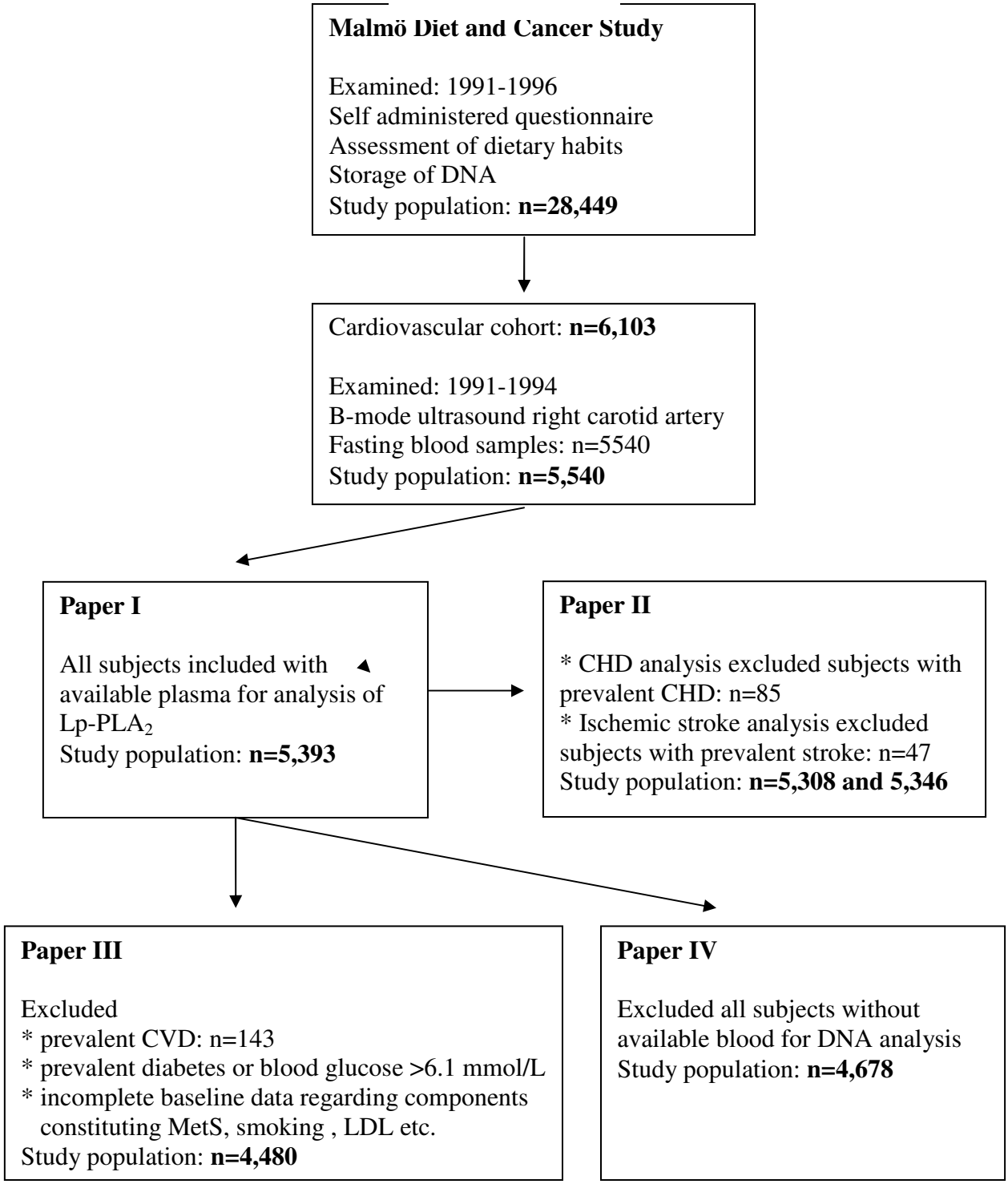
- To study the relationship between Lp-PLA_2 and MetS and to assess the independent contribution of MetS and Lp-PLA_2 on incident CVD.

Material and methods

Subjects

The Malmö Diet and Cancer Study (MDCS) is a population-based prospective cohort study designed to explore the associations between dietary habits and cancer [103]. All men, aged 49-73 years, and women, aged 45-73 years, living in the city of Malmö, Sweden were eligible for the study. The only exclusion criteria were mental incapacity or inadequate language skills in Swedish. Recruitment was performed by public advertisement with posters and pamphlets [104]. Participation was voluntary and without any financial compensation. In all 28,449 individuals were enrolled and the participation rate was 41%. Detailed information on non-participants has been presented previously [105]. At baseline examination, each subject was seen by a nurse for anthropometrics, supine blood pressure measurement, non-fasting blood sampling and administration of a questionnaire including hereditary, medical condition, dietary, and life style factors [106]. Between October 1991 and February 1994, every other subject was randomly invited to take part in a sub-study of the epidemiology of the carotid artery disease [106-109] hereto known as the "Cardiovascular cohort". This cohort consisted of 6,103 subjects (60% women) aged between 46-69 years (mean 58 years). In all subjects included in the cardiovascular cohort a B-mode ultrasound examination of the right carotid artery was performed [108]. Fasting blood samples were not collected at the baseline visit due to logistic reasons. Thus, participants were asked to return for a subsequent visit (median time of 8.6 months after the baseline visit) to donate whole blood samples in a fasting condition. A total of 5,540 of the 6,103 subjects returned and plasma for analysis of lipids and glucose was obtained as well as plasma for storage at minus 80 degree [109]. Of these participants sufficient stores of plasma were available from 5,393 for the purpose of measuring Lp-PLA_2 activity and mass.

Flowchart of study population in each substudy on Lp-PLA$_2$



Methods

All subjects were seen by a nurse for standardized anthropometric and supine blood pressure measurement. Weight was measured to the nearest 0.1 kilogram using balance-beam scale wearing light in-door clothing and without shoes. Height was measured in standing position to the nearest 1 centimetre and without shoes. Body mass index (BMI) was calculated as kg/m^2. Waist circumference (centimetres) was measured in the standing position midway between the lower rib margin and the iliac crest. Supine blood pressure (mm Hg) was measured once after 10 minutes rest using a three-cuff manometer.

Ultrasound examination of right carotid artery was performed by specially trained and certified sonographers. The examination has been described in detail previously [108]. In short, the carotid bifurcation was scrutinised for the existence of atherosclerotic plaque, defined as a focal thickening of the intima-media layer. Intima media thickness (IMT) was determined in the far wall of the distal common carotid artery (IMT-cca) and in carotid bifurcation (IMT-bulb), according to the leading edge principal and using a specially designed computer-assisted image analysis system [108, 110]. Plaque occurrence was defined as a focal thickening of intima-media wall more than 1.25 mm [111]. Plaque score (values of 0, 1 or 2) was constructed in which 0 corresponded to no visual plaque, 1 corresponded to a visual plaque less than 10 mm^2, and 2 corresponded to a plaque equal or greater than 10 mm^2 [111].

Information obtained from the self-administered questionnaire

Smoking habits; classified as current smoker, ex-smoker and never smoker.

Education; classified into three groups: ≤ 9 years of education, 9 to 12 years of education, or more than 12 years of formal education. In some analyses, a 2-grade scale was used for classifying education which included 10 years or less, or more than 10 years of education.

Physical activity; calculated from questions adapted from the Minnesota Leisure Time Physical Activity Questionnaire including 18 different physical activities, separately for the four seasons. The number of minutes per week of each activity was multiplied with an intensity coefficient [112]. Low level of physical activity was defined as the lowest quartile of the score revealed by this questionnaire.

Alcohol consumption; was based on a menu-book in which the subjects filled in their meals and drinks for seven consecutive days. High alcohol consumption was characterized as consumption more than 30g alcohol per day for women and more than 40g alcohol per day for men [113].

Hypertension; was defined as self-reported physician-diagnosed or current hypertensive treatment (paper I), in paper II to IV, hypertension included those subjects with a blood pressure ≥ 140 mm Hg systolic or ≥ 90 mm Hg diastolic [114].

Diabetes mellitus; was defined as self-reported physician-diagnosed or current diabetic treatment (paper I). In paper II to IV, diabetes mellitus included subjects with fasting whole blood glucose equal or above 6.1 mmol/L, self-reported physician diagnosed diabetes mellitus or current treatment with anti-diabetic drugs.

Laboratory analyses

All participants were instructed to refrain from smoking, alcohol and food intake, over night fasting or at least 10 hours before sample drawing. Blood samples were drawn for blood glucose (mmol/L), insulin (iu/L), HbA_1C (%), triglycerides (mmol/L), total and HDL-cholesterol (mmol/L), and measured according to standard procedures at the Department of Clinical Chemistry, University Hospital Malmö [115]. The LDL cholesterol (mmol/L)

concentration was calculated according to Friedewald's formula [116]. Homeostasis model assessment (HOMA) value was calculated as (p-insulin x p-glucose)/22.5 [117].

The analysis of hsCRP (mg/L) was performed using the Tina-quant® CRP latex high sensitivity assay (Roche Diagnostics, Basel, Switzerland) on an ADVIA® 1650 Chemistry System (Bayer Healthcare, NY, USA). The principle of the assay is a particle-enhanced immunoturbidimetric assay, where anti-CRP antibody coated latex particles react with the CRP-antigen in the samples to form antigen/antibody complexes. The resulting agglutination can be measured turbidimetrically. Study samples were analysed as discrete samples and results were read in 6 second intervals for a 1 minute time period following 5 minutes incubation. The mean value of these measurements was the reported result. The assay was calibrated using C.f.a.s® protein (Roche Diagnostics, Basel Switzerland) within a two-point calibration curve and 5 quality control samples (Precipath®, Precinorm® both Roche Diagnostics, Basel, Switzerland; Assayed Human Sera Level 2 and Level 3, both Randox Laboratories, Crumlin, UK; pooled in-house human plasma) were used to monitor the performance of the assay. The average coefficient of variation was 4.6%.

Genetic analyses

Deoxyribonucleic acid (DNA) was extracted from granulocyte or buffy coat suspensions, maintained at –80°C from the time of enrolment. Samples were thawed rapidly at 37°C; a 200 µL aliquot was subjected to QiaAmp mini-preps in 96-well format (Qiagen, Hilden, Germany) according to the manufacturers' instructions. SNPs were selected from the dbSNP database to include all non-synonymous coding SNPs with heterozygosity > 0.05. In addition, one promoter SNP (rs1421378), one SNP near the poly A attachment site of the 3'utr (rs974670) and one SNP far distal to this point (rs2216464) were selected to provide

haplotype information. In all, 2.5 ng DNA was used for each SNP assay on the Applied Biosystems 7900HT instrument using SNP genotyping assays C_7582939_10 for rs1421378 (5'A>G), C_7582933_10 for rs1805017 (R92H), C_2032803_1 for rs1805018 (I198T), C_2032800_20 for rs1051931 (A379V), C_7582925_10 for rs974670 (3'utr C>T) and C_15858042_10 for rs2216464 (far 3'T>C) and TaqMan Mastermix No UNG, in a total reaction volume of 6 µL in 384 microtiter plates, according to manufactures instructions.

Measurement of Lp-PLA$_2$ activity and mass

Plasma aliquots prepared from fasted blood samples were collected and stored at -80°C. The mass of Lp-PLA$_2$ in the study samples was quantified using the PLAQ™ Test, i.e. a second-generation assay (diaDexus Inc., South San Francisco, CA, USA). The test resembles a sandwich enzyme immunoassay with two specific monoclonal antibodies as described by Caslake *et. al.* [118] combined with a horseradish-peroxidase – tetramethylbenzidine detection system. The change in absorbance resulting from the enzymatic turnover of the substrate was measured spectrophotometrically (Wallac, now Perkin Elmer Inc, Boston, MA, USA) and is directly proportional to the concentration of Lp-PLA$_2$ present in the study sample. A 6-point standard curve with known Lp-PLA$_2$ quantities, provided by the manufacturer, was employed to determine Lp-PLA$_2$ concentration of the study samples. All samples were analysed in duplicates and a duplicate was expected to show a coefficient of variation of less than 20% and if not the samples were reanalyzed. The average coefficient of variation was 4.26% on a random of 50 first subjects in the MDCS. The performance of the assay was monitored with two sets of three quality control samples, two provided by the manufacturer and one in-house quality control sample consisting of pooled plasma from four healthy donors. For an assay plate to be accepted, 4 out of the 6 quality control samples were expected to pass. Results

were calculated from the raw data, by point-to-point fit of the standard samples using Multicalc software (Wallac, now Perkin Elmer Inc, Boston, MA, USA).

Lp-PLA_2 activity was measured using [3H]PAF (platelet activating factor) as substrate. Briefly, plasma (5µL) or assay buffer (for determination of background and total dpm) were transferred into a 96 well flat-bottomed polystyrene plate (Costar) and allowed to equilibrate to room temperature. A 100µL aliquot of [3H]PAF substrate working solution (prepared fresh daily), consisting of 100µM [3H]PAF (0.4M [3H]PAF (Specific Activity 21.5 Ci/mmol, Perkin Elmer Life Sciences) plus 99.6M C16-PAF (Avanti Polar Lipids Inc) in assay buffer (100mM HEPES, 150mM NaCl, 5mM EDTA, pH7.4) was added to each well and the plate was vortexed and incubated at room temperature for 5 min. The reaction was terminated by addition of 50µL ice-cold aqueous bovine serum albumin solution (50mg/mL) followed by vortex mixing and incuabtion for 5min at 4C. Ice-cold trichloroacetic acid (56% aqueous solution; 25µL) was added to each well, vortexed and incubated for 15min at 4C. Plates were then sealed and centrifuged at ~6,000 x g for 15 min at 4°C, and aliquots of supernatant (45µL) were transferred to a picoplate (Perkin Elmer). In order to determine total dpm added, 10µL [3H]PAF substrate working solution was added to wells containing buffer instead of plasma. Some wells were left blank to determine background hydrolysis. Microscint-20 (200µL; Perkin Elmer Life Sciences) was added to all wells, plates were sealed and vortex mixed for 10 min. The plates were counted in a Topcount liquid scintillation counter (Perkin Elmer Life Sciences) and Lp-PLA_2 activity values (nmol PAF hydrolysed/min/mL) were derived from the raw data according to the formula below:

LpPLA2 activity = 160(CPM45µL-test - CPMBlanks)/(CPM10µL-spiking - CPMBlanks)*

Where; CPM45µL-test is the mean dpm value for a test plasma

CPMBlanks is the mean dpm of wells without plasma of the blanks

CPM10µL-spiking is the mean dpm of wells containing [3H]PAF substrate

The range of detection was 8-150 nmol/min/mL. All samples were tested in duplicate. Samples were retested if the replicate coefficient of variation was >20%. The average coefficient of variation was 5.78%.

Plasma-EDTA samples are stable for Lp-PLA_2 activity and mass measurements within 7 days of collection for refrigerated samples and for more than 10 years from collection when stored at -70ºC (data on file from dia Dexus). All Lp-PLA_2 measurements were performed by diaDexus in San Francisco US in 2005.

Definition of the Metabolic Syndrome (MetS)

The MetS was defined in accordance to the current NCEP/ATPIII criteria [119, 120]. Subjects who had three or more of the following criteria were considered to have MetS.

- Abdominal obesity: waist ≥ 102 cm for men and ≥ 88 cm for women
- Hypertriglyceridemia: triglyceride levels ≥ 1.70 mmol/L or, on drug treatment for elevated triglycerides.
- Low HDL-cholesterol levels: HDL < 1.03 mmol/L for men and < 1.30 mmol/L for women, or on drug treatment for decreased HDL.
- High blood pressure: Systolic blood pressure ≥130 mmHg or diastolic blood pressure ≥85 mmHg, or current treatment with blood pressure-lowering treatment.
- Elevated blood glucose: fasting whole blood glucose ≥ 5.6 to 6.0 mmol/L or established diabetes.

In paper III subjects with blood glucose >6.1 mmol/L or established diabetes were excluded.

Classification of cardiovascular events

Record linkage with the Swedish Hospital Discharge Register, the Malmö Myocardial Register [121], the Stroke register of Malmö (STROMA) [122, 123], and the Swedish Causes of Death Register. The ascertainment of cases and validity of these registries has been shown to be high [124]. The Swedish Hospital Discharge Register is a national register of all inpatients at all hospitals in Sweden since 1987, kept by the Centre for Epidemiology at the Swedish national Board of Health and Welfare [124, 125]. STROMA was established in 1989 with the purpose to monitor the incidence of stroke in Malmö [123]. Each case of suspected stroke, among both inpatients and outpatients, was assessed by a specialised research nurse, with supervision of a senior physician. Underlying causes of death and hospitalization diagnosis, respectively, were coded in accordance with the 9th version of the International Classification of Diseases (ICD-9). All subjects were followed from baseline examination until first occurring CHD, stroke, emigration from Sweden, or death until December 31st 2003.

A CHD event was defined as non-fatal MI (ICD-code 410) or death due to ischemic hearth disease (ICD-codes 410-414). Stroke was defined as fatal and non-fatal stroke (ICD-codes 430,431,434). Ischemic stroke (ICD-code 434) was diagnosed when computed tomography, magnetic resonance imaging or autopsy could verify the infarction and/or exclude haemorrhage and non-vascular disease. By definition subjects with transient ischemic attacks were not included. In order to find cases who moved out from the city after screening procedure, we also used the National Hospital Discharge Register and the Swedish Cause of Death Register, using the same diagnosis validation procedures as for STROMA.

Statistics

All the statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) 11.0 in paper I, SPSS 13.0 in Paper II-III and SPSS 16.0 in paper IV. Probability values less than 0.05 were considered statistically significant for non-interaction terms and $P<0.10$ was considered significant for interaction terms.

Paper I

The distributions of triglycerides, glucose, insulin, HOMA, HbA1C, hsCRP and physical activity were markedly skewed and therefore log-transformed. Means with standard deviation (SD) [medians with inter quartile range for skewed variables] and percentages for baseline demographic and clinical characteristics were computed for the entire cohort and by sex. For continuous variables, we assessed correlation of Lp-PLA_2 with variables three ways with use of Pearson's or Spearman's correlation coefficient: partially adjusted for age and sex, partially adjusted for age, sex and LDL-cholesterol level, and partially adjusted for age, stratified by sex. Continuous factors were divided into tertiles and computed mean Lp-PLA_2 level within each tertile, stratified by sex. Mean Lp-PLA_2 level was computed and adjusted for age and sex by analysis of covariance for key categorical risk factors. Differences between categories were calculated and expressed with 95% confidence interval (CI). A general linear model was used to examine the incremental influence (cumulative R^2) of risk factors for the degree of explanation of the variation in Lp-PLA_2.

Paper II

T-test for continuous variables and chi-square for dichotomous variables was used to test differences between subjects without or with incident CHD and ischemic stroke, respectively. Kaplan-Meier survival plots were used to study the cumulative event-free survival in relation

to tertiles of Lp-PLA_2, a log-rank test was used to evaluate statistical differences between groups. Cox regression was used to investigate the incidence for CHD and ischemic stroke, respectively, in relation to tertiles (using the lowest tertile as referent) of Lp-PLA_2 activity and mass, respectively, with adjustment for confounding factors. Tolerance was calculated in order to assess collinearity between the independent variables. Possible interactions were analyzed by including interaction terms in the final model. To test if LDL-cholesterol levels modified the association between Lp-PLA_2 mass and CHD and ischemic stroke, we split the cohort by the median LDL- cholesterol level (i.e. 4.1 mmol/L).

Paper III

Kappa statistics (ê) was used to assess the level of agreement between Lp-PLA_2 activity and mass (in tertiles). The incidence (per 1000 person-years) was standardized for sex and age (5-year groups) using direct standardization, and weighted for age-distribution of the present cohort. A general linear model was used to adjust the relations for age, sex, and LDL-cholesterol and to test the linear effects of Lp-PLA_2 levels across the number of components involved in the MetS. Age- and sex-adjusted c statistics, analogous to the area under the receiver operator characteristic (ROC) curve, were used to assess the discrimination of CVD prediction model based on high Lp-PLA_2 alone versus those having the MetS alone. Kaplan-Meier survival analysis was used to assess the relation of Lp-PLA_2 activity and mass, respectively, and presence of MetS with CVD events during follow-up. Cox regression model was used to evaluate the potential additive effect of both elevated Lp-PLA_2 and presence of MetS association with incident CVD.

Paper IV

Frequency differences and deviation from Hardy-Weinberg equilibrium were analyzed by CHI-2 test. For the genotype-Lp-PLA_2 plasma levels association analyses, we assumed an additive model of inheritance. *T*-test was used to compare mean plasma Lp-PLA_2 activity and mass, respectively, levels in different SNPs polymorphisms. A Spearman rho correlation test was used to assess degree of association between different SNPs. We conducted multiple linear regression analyses to test if genotypic effects on plasma levels of Lp-PLA_2 activity and mass, respectively, were independent of covariates. Three different models were used all with Lp-PLA_2 as the dependent variable. In addition, in model 2, an interaction term was included to assess possible interactions between sex and genotype on plasma levels of Lp-PLA_2. Finally, highly correlated SNPs (r^2>0.5) were simultaneously included in the model 2 to assess the independent contribution of each genotype on Lp-PLA_2 levels.

Results and manuscript specific conclusion

Paper I: The epidemiology of Lp-PLA_2: Distribution and correlation with cardiovascular risk factors in a population-based cohort

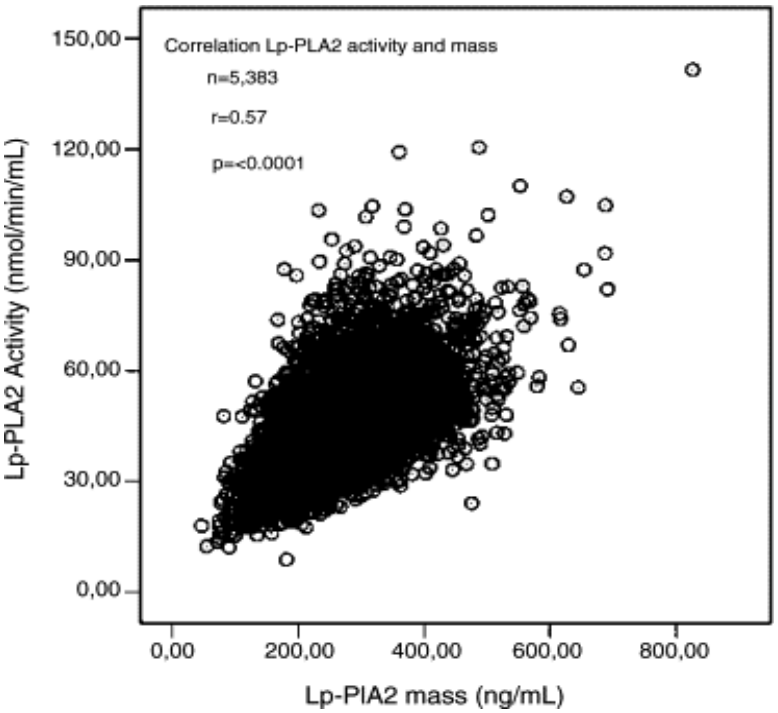
Aim

To examine the cross-sectional associations of Lp-PLA_2 with anthropometric, demographic and other CV risk factors with plasma levels of Lp-PLA_2.

Results

Mean (SD) Lp-PLA_2 activity was 45.5 (13.1) nmol/min/mL and mean Lp-PLA_2 mass was 269.8 (80.7) ng/mL. The correlation between Lp-PLA_2 activity and mass was r=0.57(Figure 1).

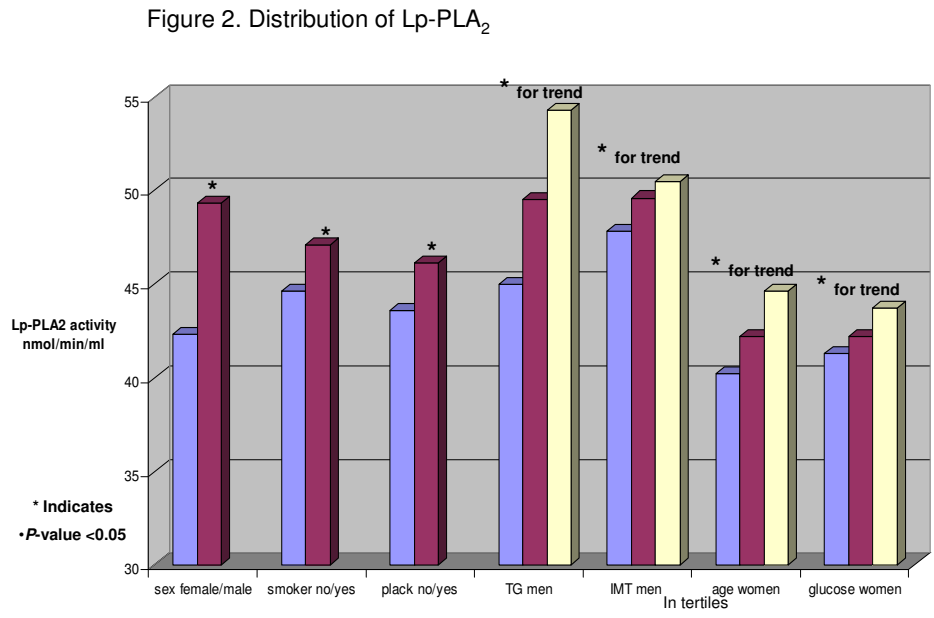
Figure 1. Correlation between Lp-PLA_2 activity and mass. n=number; r=correlation coefficient; *p*=*p*-value



Lp-PLA_2 activity and mass were related to age, a correlation that was stronger in women than in men. Mean level of Lp-PLA_2 activity and mass, were significantly higher in men than in

women, 49.6 versus 42.5 nmol/min/mL, and 287.7 versus 257.2 ng/mL, respectively. Lp-PLA_2 was strongly correlated with total cholesterol, LDL, LDL/HDL ratio, HDL and triglycerides. Besides the lipids, the strongest associations in both genders to levels of Lp-PLA_2 were observed for fasting glucose, insulin and HOMA. In men, Lp-PLA_2 was also weakly associated with BMI and blood pressure. Lp-PLA_2 mass, but not Lp-PLA_2 activity, was weakly associated with hsCRP (r=0.10 and r=0.02, respectively). Lp-PLA_2 increased with a greater extent of ultrasound-determined atherosclerosis, from 41 in the lowest tertile of IMT-cca to 42 and 44 nmol/min/mL in the second and third IMT-cca tertile, respectively (Figure 2).

Figure 2. Distribution of Lp-PLA_2 activity in different sub-groups.



Plack: a focal thickening of intima-media, more than 1.25mm, TG: triglycerides, IMT: intima-media thickening.

Current smokers, subjects with low educational level and subjects with presence of plaque in right carotid artery had statistically significant higher levels of Lp-PLA_2 activity compared to non-smokers, better educated subjects and subjects without plaques (Figure 2).

To assess how known CV risk factors explained the variability of Lp-PLA_2, parameters were fit in a generalized linear model and the R^2 was calculated. The 12 measured variables explained significantly more of the variation in Lp-PLA_2 activity than in Lp-PLA_2 mass (cumulative R^2=0.34 versus R^2=0.19). The strongest factors explaining the variation in the Lp-PLA_2 activity were LDL, HDL and sex. The corresponding factors for Lp-PLA_2 mass were LDL, sex, and smoking status. Seven percent of the variation in Lp-PLA_2 activity and 5% of the variation in Lp-PLA_2 mass was explained by gender.

Conclusion

Plasma Lp-PLA_2 levels increases with age, and are higher in males and in smokers. Lp-PLA_2 is positively correlated with LDL-cholesterol and triglycerides and inversely correlated with HDL-cholesterol. Lp-PLA_2 mass, but not LP-PLA_2 activity, is to a minor degree correlated with hsCRP. Both Lp-PLA_2 activity and mass are associated with carotid asymptomatic atherosclerosis (i.e. presence of plaques and CIMT). The variation of Lp-PLA_2 activity and mass was only explained to 35% and 19%, respectively, by measured variables.

Paper II: Lp-PLA_2 activity and mass are associated with increased incidence of ischemic stroke. A population-based cohort study from Malmö, Sweden.

Aim

To explore whether Lp-PLA_2 activity and mass, respectively, are associated with incidence of CHD and ischemic stroke, respectively.

Results

Subjects with incident ischemic stroke and CHD, respectively, had compared to event-free subjects significantly higher mean levels of blood pressure (142, 152 and 152 mmHg, respectively), Lp-PLA_2 activity (45.5, 49.9 and 50.7 nmol/min/mL, respectively) and Lp-PLA_2 mass (268.2, 290.4 and 291.6 ng/mL, respectively). Noticeable, subjects who experienced a CHD had compared to those with incident ischemic stroke and event-free subjects much higher LDL-cholesterol levels (4.4, 4.2 and 4.2 mmol/mL, respectively). During a mean follow-up time of 10.6 years there were 347 incident CV events, 195 subjects had CHD of which 44 were fatal and 152 had an ischemic stroke of which 12 were fatal.
In an age- and sex-adjusted Cox regression model, the upper compared to the bottom tertile of both Lp-PLA_2 activity and mass, were statistically significantly related to an increased risk of ischemic stroke (relative risk (RR); 1.79, 95% CI 1.16-2.76 and 1.71; 1.12-2.62, respectively). The corresponding figures for incident CHD were RR: 2.11; 95% CI 1.42-3.14 for Lp-PLA_2 activity and 1.34; 95% CI 0.94-1.90 for Lp-PLA_2 mass. The RR:s for ischemic stroke and CHD after further adjustment for potential confounders are presented in Table 1.

Table 1. Adjusted RR (95% CI) of first incident ischemic stroke or first CHD during 10 years follow-up by baseline Lp-PLA_2 activity and mass levels (in tertiles, T1-T3). Adjustment was made for age, sex LDL, HDL, use of lipid lowering treatment, BMI, hsCRP, smoking status, diabetes, SBP and high alcohol consumption.

	T1	T2	T3
Lp-PLA_2 activity			
Ischemic stroke	1.0 (ref)	1.44 (0.88-2.37)	1.94 (1.15-3.26)
CHD	1.0 (ref)	1.24 (0.79-1.96)	1.48 (0.92-2.37)
Lp-PLA_2 mass			
Ischemic stroke	1.0 (ref)	1.65 (1.02-2.65)	1.92 (1.20-3.10)
CHD	1.0 (ref)	0.84 (0.56-1.26)	0.95 (0.65-1.40)

Conclusion

Elevated levels of LP-PLA_2 activity and mass, respectively, are associated with an increased risk of ischemic stroke, independent of other cardiovascular risk factors. No similar independent relationship was observed between Lp-PLA_2 and CHD.

Paper III: Elevated Lp-PLA$_2$ levels add prognostic information to the Metabolic Syndrome on incidence of cardiovascular Events among middle-aged non-diabetic subjects.

Aim

To study the relationship between Lp-PLA$_2$ and MetS, and to assess the independent contribution of MetS and Lp-PLA$_2$, on incident CVD.

Results

In this non-diabetic cohort (n=4480) 16.4% (i.e.14.0% in women and 20.5% in men) had MetS. Subjects with MetS had significantly higher mean level of Lp-PLA$_2$ activity (51.3 versus 43.8 nmol/min/mL) and Lp-PLA$_2$ mass (280.9 versus 266.5 ng/mL) compared to subjects without MetS. Lp-PLA$_2$ is associated with all five metabolic components involved the syndrome. Both mean level of Lp-PLA$_2$ activity and mass, respectively, increased by increasing number of metabolic components (Table 3).

TABLE 3. Mean Levels of Lp-PLA$_2$ Activity and Lp-PLA$_2$ Mass In Relation to No. of Components Involved in the Metabolic Syndrome (MetS)

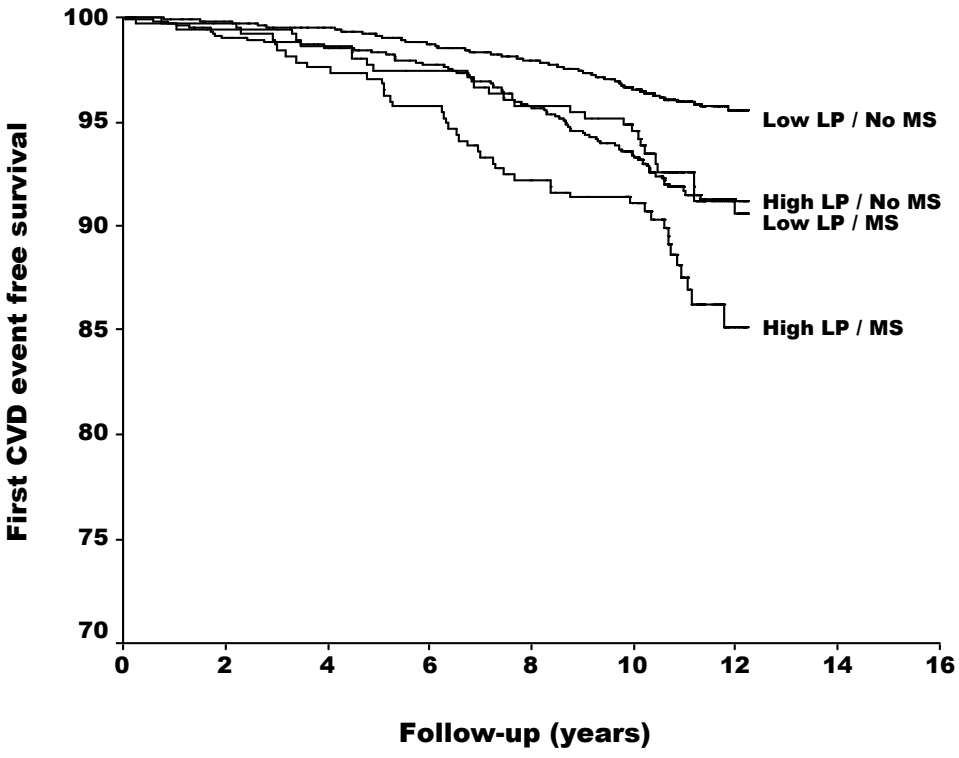
No. of MetS Components	No. of Subjects	Lp-PLA2 Activity (nmol/min/mL)	Lp-PLA2 Mass (ng/mL)
0	657	40.8±11.3	252.0±73.5
1	2082	43.5±11.8	266.3±77.6
2	1007	46.1±12.9	275.4±82.2
3	531	50.0±13.6	281.3±84.5
4 or 5	203	52.5±14.8†‡	276.9±85.5†‡

†P for trend <0.001, unadjusted. ‡P for trend <0.001 for activity and 0.472 for mass, respectively, after adjustment for age, sex, and LDL-cholesterol.

During mean follow-up time of 10.6 years a total of 261 incident CVD events occurred. Elevated levels of Lp-PLA$_2$ activity was statistically significantly associated with incident CVD (RR; 1.46: 95% CI 1.01-2.13) in a multivariate adjusted model including MetS. Both elevated levels of Lp-PLA$_2$ and presence of MetS were, independently of traditional CV, risk

factors, associated with incident CVD. To evaluate the potential additive effect of both markers we divided the cohort in four groups, i.e. low to mid (tertile 1 and 2) versus high Lp-PLA_2 activity in combination with and without MetS. The referent group consisted of subjects with low to mid Lp-PLA_2 activity and no MetS. The RR for incident CVD associated with a combination of both elevated Lp-PLA_2 activity and MetS was, after adjustment for age, sex, LDL-cholesterol, lipid lowering treatment, smoking, hsCRP, physical activity and high alcohol consumption 1.97; 95% CI 1.34-2.90. Corresponding RR:s for high Lp-PLA_2 activity alone and for the presence of MetS alone were 1.40; 95% CI 1.03-1.92 and 1.46; 0.94-2.27, respectively.

Figure 2**.** Kaplan-Meier curves showing the incidence of first CVD events (MI or ischemic stroke) in relation to absence or presence of high Lp-PLA_2 and MetS in non-diabetic middle-aged subjects.



Conclusion

Lp-PLA_2 is associated with MetS. Elevated levels of Lp-PLA_2 activity were related to increased risk for incident CVD regardless of MetS. There is an additive effect of Lp-PLA_2 to MetS on future CVD risk, which may identify an especially high risk individual.

Paper IV: In a population-based cohort study, variations in the PLA2G7 gene are associated with Lp-PLA$_2$ activity and mass.

Aim

To assess the effect of genetic variation in PLA2G7 gene on plasma Lp-PLA$_2$ activity and mass concentration levels.

Results

Allele frequencies ranged from 20-41%, except for I198T (5%). Subjects who possess the minor allele for rs1051931 (A379V) and rs2216464 (far3CT) had significantly higher plasma Lp-PLA$_2$ activity, with a mean difference of 3.2 nmol/min/mL; 95% CI: 1.3-5.1, p<0.001 and 3.3; 1.4-5.2 nmol/min/mL, p<0.001, respectively (Figure 2a). Lp-PLA$_2$ mass plasma levels were 32.2 (23.0-41.3, p<0.001) ng/mL and 17.9 (10.9-24.9, p<0.001) ng/mL that was significantly higher in subjects who possess the minor alleles for SNPs rs1805017 (R92H) and rs1421378 (5'AG) compared to subjects homosygot for the major alleles, respectively (Figure 2b). These associations remained statistical significant after taking age, HDL-, LDL-cholesterol and lipid lowering medication into account. Highly correlated SNPs were rs1051931 with rs2216464 (r^2=0.55) and rs1805017 with rs1421378 (r^2=0.98). Including both rs1805017 and rs1421378 simultaneously into the multivariate model, the association between Lp-PLA$_2$ mass and rs1805017 was strengthened, and the association between Lp-PLA$_2$ mass and rs1421378 turned inverse and non-statistically significant.

A sex-specific association between rs1051931 (i.e. A379V) and Lp-PLA$_2$ mass was observed (p for interaction 0.04), showing opposite relationships of Lp-PLA$_2$ mass levels and this polymorphism in men compared to women.

Figure 2a

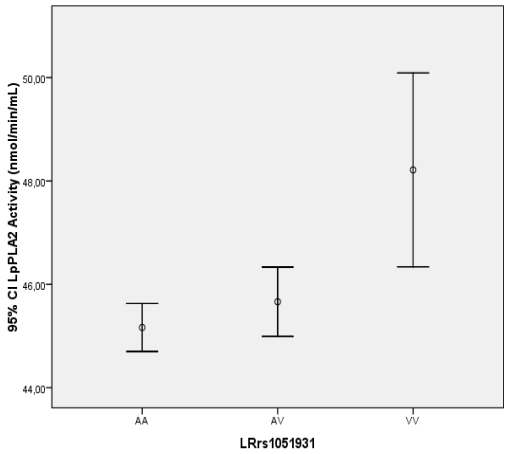
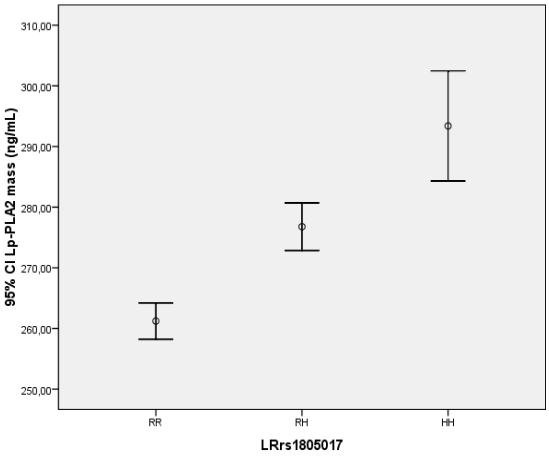


Figure 2b



Conclusion

In middle-aged Caucasians, genetic variation at the PLA2G7 gene locus significantly influences plasma Lp-PLA_2 activity and mass levels, in a position and sex-specific manner.

Identification of the biological effects of specific genetic variants may further increase their future value as biomarkers and potential therapeutic targets.

General Discussion

There is global public health problem with an aging population, increasing number of individual's with obesity and MetS, and a remaining high incidence of CVD [13, 89, 90]. Some author argue that there is a need for a more intensive risk stratification in intermediate- and high risk patients to improve treatment for CVD, i.e. coronary events and stroke [44]. It has also been suggested that Lp-PLA_2 could be a novel biomarker, which represent a non-invasive tool to assess plaque stability [62, 65]. Accurate classifications of patients with atherosclerotic vascular disease is an important task in order to choose appropriate risk reducing therapy, which includes lifestyle modification and anti-hypertensive and lipid-lowering treatment [126]. It is widely recommended to use established current guidelines to help professionals to identify patients in order to reduce the occurrence of CHD, stroke and peripheral artery disease and their complications [127-129]. At present, inflammatory markers are not recommended for use in low-risk populations as a screening tool. Lp-PLA_2 is however suggested to be used in subjects to be at moderate or high risk by risk assessment [126].

Lp-PLA_2: correlation with other cardiovascular risk factors

Lp-PLA_2 is higher among men than women

The finding that both Lp-PLA2 activity and mass levels are higher in males compared to women (Paper 1) has been a consistent finding in other population-based and clinical studies [70-72, 130]. In the ARIC study the mean plasma levels of Lp-PLA_2 mass were 421µg/L in men and 339 µg/L in women [70]. In the Rotterdam study (which included assessment of Lp-PLA_2 activity) men had significantly higher levels than women, i.e. 46.8 compared to 43.0 nmol/min/mL [72]. Similar findings have also been reported from two other recently published

studies [130, 131]. The Dallas Heart Study; which included a multiethnic population, Lp-PLA_2 activity was reported to be higher in men compared to women, 161 versus 134 nmol/min/mL [130]. The US population-based Cardiovascular Health Study, including individuals aged 65 or older, also showed higher Lp-PLA_2 activity levels for men compared to women 42.7 vs 37.3 nmol/min/mL [131]. Although Lp-PLA_2 mass was measured in these studies no sex-specific data of Lp-PLA_2 mass has been currently reported. There is evidence that estrogen down-regulates Lp-PLA_2 expression [132]. In our study, users (i.e. 14%) compared to non-users of hormone replacement therapy (HRT) at baseline had lower levels of both Lp-PLA_2 activity and mass, i.e. 40.5 vs 43.0 nmol/min/mL and 248.4 vs 259.1 ng/mL, respectively. This finding is in accordance with the Women Health Study (WHS) [69] and the Dallas Heart Study [130]. In both studies levels of Lp-PLA_2 mass and Lp-PLA_2 activity, respectively, was significantly lower in HRT users compared to non-users (0.98 vs 1.23 mg/L and 126 vs 136 nmol/min/mL, respectively). However, excluding current HRT users in our cohort, the significant sex-specific difference in mean Lp-PLA_2 levels (as measured by mass or activity) remained. Further, research on the sex-specific difference in plasma Lp-PLA_2 is warranted to better understand the different physiological regulation of Lp-PLA_2 in men and women.

Lp-PLA_2 is highly correlated with blood lipids but not with hsCRP

A consistent finding, and in accordance with other studies, is that both Lp-PLA_2 activity and mass are strongly associated to blood lipids [68-70, 118, 130, 131, 133, 134]. This is not a surprising finding as Lp-PLA_2 is mainly bound to LDL particles [118]. In the present study almost 25% of the variation in both Lp-PLA_2 mass and activity could be explained by the variation of LDL-cholesterol. Furthermore, we found a significant but modest inverse correlation to HDL-cholesterol (Paper 1), a finding consisted with many [69, 70, 130] but not in all other studies [68, 71].

The inverse correlation between Lp-PLA_2 and HDL-cholesterol in our study was stronger for Lp-PLA_2 activity than for mass (r=-0.24 and r=-0.09, respectively). Differences between studies, however, could be explained by different design, study populations and methods.

Most studies, supporting our findings, have reported a non-significant correlation between Lp-PLA_2, assessed as mass or activity, and hsCRP [68, 70-72, 131]. To our knowledge there are today only few studies reporting a significant however modest association between Lp-PLA_2 and hsCRP [130, 135]. One study included patients with established T1DM (n=92) and in another study the association between hsCRP and Lp-PLA_2 mass was found only in women. The inflammatory marker hsCRP has been demonstrated as a independent predictor for incident CVD in many clinical and population-based studies [42, 43, 136, 137]. Numerous other studies have also demonstrated that Lp-PLA_2 is independently, including hsCRP, associated with incidence of CAD and stroke [68, 70, 72, 138]. This finding indicates that these two biomarkers may reflect distinctively different mechanism on the atherosclerotic process. There are also some studies which have explored the additive effect of Lp-PLA_2 to hsCRP in predicting CHD and stroke [70, 71, 139]. All these studies have clearly demonstrated that the combination of elevated levels of hsCRP and Lp-PLA_2 was associated with a substantially increased risk of CHD or stroke. In addition in the HELICOR study, which included 312 patients with CAD and 479 age- and sex-matched controls, the correlation between Lp-PLA_2 mass and more than 15 different inflammatory and haemostatic markers was assessed [134]. In that study the top versus the bottom quartile of Lp-PLA_2 concentration was associated with an almost two-fold OR for severe angiographic CAD, which was independent of many inflammatory and hemostatis markers, i.e. hsCRP, serum amyloid A, plasminogen-activator-inhibitor-1, interleukin-6, tumor necrosis factor-alfa, intercellular adhesion molcule-1, white blood cell count, fibrinogen, D-dimer and lipo protein(a). Together these results, including ours, supports Lp-

PLA_2 as a novel specific vascular inflammatory biomarker for CAD and ischemic stroke risk which is independent of other biomarkers reflecting systemic inflammation and haemostasis.

Lp-PLA_2 and endothelial dysfunction

In symptomatic compared to asymptomatic carotid artery plaques increased levels of Lp-PLA_2 and lyso-PC have been found [46]. In a study by Lavi et al. it was demonstrated that the local production of Lp-PLA_2 and lyso-PC, which correlated with endothelial dysfunction, was higher in patients with early coronary atherosclerosis compared to healthy control subjects [62]. In our cross-sectional study we have showed that plasma Lp-PLA_2 activity levels increased with a greater extent of carotid ultrasound-determined atherosclerosis, i.e. IMT-cca, and Lp-PLA_2 was also associated with the amount of plaque (Paper 1). The association between Lp-PLA_2 and carotid IMT and plaque was however modest, and one possible explanation could be that this non-invasive imaging technique does not separate vulnerable from stable plaques. It is also been shown that Lp-PLA_2 staining is intense in rupture-prone plaques, however minimal staining was detected in early stable plaques [65]. In that study Lp-PLA_2 also co-localized with apoptotic macrophages. This finding suggests that Lp-PLA_2 may be closely linked with the progression and vulnerability of human coronary atheroma [65]. Coronary events do not occur only from severe luminal narrowing. Many acute CHD events seem to occur from atheroma showing less than 50% occlusion [65, 140]. This circumstance has shifted our focus of atherosclerosis as a focal disease caused by severe stenosis to a systemic disease characterized by endothelial dysfunction and plaque inflammation, and its consequence cardiovascular events by plaque rupture and thrombosis mainly at the sites of mild to moderate stenosis. Thus, some authors have even argued that inflammatory biomarker, as the

vascular specific Lp-PLA_2, enzyme should be included in current risk stratification models in order to better identify risk patients with unstable plaques [23, 46, 65].

Correlation between Lp-PLA_2 activity and mass

The correlation between levels of Lp-PLA_2 activity and mass in the MDCS was rather high r=0.57 (Paper 1). Only few clinical studies, and some population-based studies, have reported correlation between Lp-PLA_2 activity and mass. In summary, the reported correlation between activity and mass in these studies varies between r=0.35 to r=0.86. In a small case-control study of male patients with CAD, the correlation was reported to be very high (r=0.86) [118]. In the PROVE IT- TIMI 22 study, a clinical trial involving patients with recent acute coronary syndrome (n=3625 and mostly men), there was only a modest correlation between Lp-PLA_2 activity and mass (r=0.35) [138]. In another study of patients with CHD, the correlation between Lp-PLA_2 activity and mass was r=0.57 [76]. Similar or even higher correlations between Lp-PLA_2 activity and mass have been demonstrated from population-based studies including asymptomatic subjects [130, 131]. In the Dallas Heart Study [130], there was a strong correlation (r=0.69) between Lp-PLA_2 activity and mass, and in the Cardiovascular Health Study [131] the corresponding correlation coefficient was 0.51. The difference in association between Lp-PLA_2 activity and mass in different studies could have several explanations, i.e. differences in design, study populations or methods for measuring Lp-PLA_2. Many present studies have used different Lp-PLA_2 assays. The Malmö, PROVE IT-TIMI 22, and Cardiovascular Health studies have all used a radiometric method to assess Lp-PLA_2 activity, while a calorimetric method was used in the Dallas Heart Study. To our knowledge no study has reported data on agreement between these two methods assessing Lp-PLA_2 activity within the same population. A first generation PLAC™ Test by diaDexus was initially used to measure Lp-

PLA_2 mass concentration in some studies at the beginning of year 2000. A second-generation diaDexus PLAC™ Test has been used in many other studies including the MDCS. Data from the AIRGENE study have demonstrated considerable stability and good reproducibility of serial Lp-PLA_2 mass measurements using this second-generation PLAC™ Test [48].

Variations in plasma levels of Lp-PLA_2 activity and mass, are modestly explained by other measured cardiovascular risk factors

Variation in Lp-PLA_2 activity and mass levels, respectively, was in our study explained by measured lifestyle and biological variables only to 35% and 19%, respectively (Paper 1). This finding regarding Lp-PLA_2 activity is in agreement with a recent publication from the Cardiovascular Health Study [131]. In that study, including subjects >65 years, the total percentage variability (i.e. 29%) of Lp-PLA_2 activity was explained by age, gender, race, smoking, diabetes, blood pressure, blood lipids, BMI, CRP, creatinine, haemoglobin, platelets, fibrinogen, factor VII, white blood cell count and albumin. Furthermore, similar to our study LDL- and HDL-cholesterol were substantially the most important factors when explaining the Lp-PLA_2 activity variability. No other study, except MDCS, has to our knowledge examined factors accounting for the variability of Lp-PLA_2 mass. More studies are needed to further explore the remaining variability in terms of genetic determinants and the fact that Lp-PLA_2 expression has been shown to be dependent on leukocyte activation and is particularly high within the lipid core of the atheroma [56, 65].

Association between Lp-PLA_2 and cardiovascular events

Lp-PLA_2 activity and mass are associated with increased incidence of ischemic stroke

In Paper II it was concluded that elevated levels of Lp-PLA_2 activity and mass, respectively, was associated with increased incident of ischemic stroke. To our knowledge, the present study is the first prospective population-based cohort study exploring simultaneously Lp-PLA_2 activity and mass and their independent relationship to incident CHD and ischemic stroke. We found an age-and sex adjusted relationship between Lp-PLA_2 activity and risk of incident CHD. This was however attenuated and did not remain statistically significant when blood lipids were included into the model. No association was observed between Lp-PLA_2 mass and incident CHD. Many primary- [68, 70, 72, 141] as well as secondary-based [74, 133, 138] prevention studies have demonstrated a significant positive association between Lp-PLA_2 levels, measured as activity or mass, with incident CHD. However, in the ARIC study [70], including almost 13,000 healthy middle-aged subjects with a follow-up period of 6 years, the association between Lp-PLA_2 and incidence of CHD (i.e. non-fatal and fatal MI) was, after taking traditional cardiovascular risk factors and hsCRP into account, observed only in subjects with an LDL-cholesterol below 130 mg/dL (i.e. 3.37 mmol/L). In the WHS (a rather small case-control study) which included only healthy middle-aged women, the univariate significant association between Lp-PLA_2 mass and incident CHD was attenuated and became non-statistically significant when adjustment for blood lipids was included in a multivariate analysis [69]. Furthermore, in the Veterans Affairs Trial (VA-HIT), an intervention trial with gemfibrozil treatment or placebo in almost 1500 post-MI men with low HDL-cholesterol and low LDL-cholesterol, Lp-PLA_2 activity has been found to be associated with incident coronary events (CE) but not with incident stroke [142]. As previously mentioned, this converse finding could be due to different study design, , i.e. case-control, nested case-control or total population-based cohort, study populations including differences in mean levels of blood

lipids, and different methods and assay to measure Lp-PLA_2. One possible explanation for the different association between Lp-PLA_2 and incident CHD and ischemic stroke, respectively, then in our study might be related to the high mean LDL-cholesterol, or to the fact that total- and LDL cholesterol is not as strongly correlated to stroke as it is for CHD [19, 27]. Furthermore, in our study Lp-PLA_2 activity was more strongly than Lp-PLA_2 mass related to incident CHD. One possible explanation could be that enzyme activity but not mass, is related to sdLDL particles [93], secondly that sdLDL particles are more atherogenic than large buoyant LDL-cholesterol [143, 144].

Most studies are looking at a combined endpoint of CVD, in terms of CHD and stroke. Few studies have explored the relation between Lp-PLA_2 levels and the incidence of stroke. Our finding regarding a two-fold increased risk for incident ischemic stroke associated with elevated levels of Lp-PLA_2, as measured as activity and mass, is consistent with three other population-based studies, i.e. the ARIC, [139], the Rotterdam Study [72] and a recently published study of postmenopausal women [145]. The ARIC study, a case-cohort study, including men and women with a mean age of 58 years and racially mixed, found that increased levels of Lp-PLA_2 mass, predicted stroke [139]. An increased level of Lp-PLA_2 activity was associated with incident stroke in the Rotterdam study including predominantly older women (mean age 69) [72]. The third study of 1,874 postmenopausal women, of whom 61% were non-users of hormone replacement therapy (HRT), increased levels of Lp-PLA_2 mass was in non-users related to increased risk of ischemic stroke [145]. No increased stroke risk was observed for elevated Lp-PLA_2 among HRT-users.

Elevated levels of Lp-PLA$_2$ activity add prognostic information to the MetS on incidence of CVD

To our best knowledge the MDC is the first study exploring the interaction between Lp-PLA$_2$ activity and mass, respectively, and MetS on incidence of CVD in a large population-based non-diabetic cohort (Paper III). All metabolic components constituting the syndrome, according to algorithms proposed by the NCEP/ATPIII [119, 120], were related to Lp-PLA$_2$ but the association was stronger for activity than for mass. In addition, with increased number of components there was a significant increase in plasma Lp-PLA$_2$ levels. Some smaller studies have shown that Lp-PLA$_2$ activity is related to established diabetes, i.e. in T2DM and T1DM patients. In a cross-sectional study including 92 T1DM patients, of whom 77 met the criteria of MetS, patients with in comparison to without MetS had significantly higher plasma levels of Lp-PLA$_2$ mass [135]. Another finding in that study, and supporting ours, was that Lp-PLA$_2$ mass levels increased linearly by increased number of MetS components. Lp-PLA$_2$ activity has also been shown associated with T1DM in another study, which included 42 T1DM patients and 48 control subjects [146]. Furthermore, in a case-control study by Serban et al, including 50 T2DM patients, 50 patients with dyslipedemia, and 50 controls, Lp-PLA$_2$ activity levels was found significantly higher in diabetic and dyslipidemic patients, respectively, compared to the control subjects [147]. Our findings are also consistent with the report from the Intermountain Heart Collaborative Study [148]. That study included almost 1500 angiographically patients, of whom 67% had CAD and 42% MetS, all followed for 7.5 years. Lp-PLA$_2$ mass levels above the median was more predictive of angiographic CAD in patients with than without MetS, and a subset of patients with MetS and elevated levels of Lp-PLA$_2$ had higher risk for CHD death (odd ratio [OR]: 2.14) compared to those with only elevated Lp-PLA$_2$ levels and absence of MetS (OR: 1.64). Subjects with MetS have been shown to have a high degree of oxidative stress and inflammation [91, 149]. Adipose cells present in

visceral fat generate inflammatory cytokines, which in turn can trigger hepatic production of CRP, and an association between CRP and endothelial dysfunction has been demonstrated in various experimental settings [150]. The MetS is a constellation of low-grade inflammatory components, and MetS individuals are at an increased risk for T2DM and CVD [120]. Subjects with presence as compared to absence of MetS have been shown to have significantly higher levels of different inflammatory marker, e.g. hsCRP, tumor necrose factor-R1and R2, interleukin-6, intercellular adhesion molecule, and fibrinogen, all important factors contributing to a significantly increased risk of CVD [42, 92, 149]. However, the causality between hsCRP and components constituting the MetS has been questioned [151]. Furthermore, although hsCRP has been shown independent of MetS associated with incident CHD in many studies [152-154], a recent report from the Nurses Health Study and the Health Professionals Study showed that most inflammatory markers did not add further information beyond MetS for prediction of CHD [149].

In summary, firstly, as systemic inflammation markers, i.e. hsCRP, is not or only weakly related to Lp-PLA_2 [68, 70, 72, 130], and secondly Lp-PLA_2, opposite to many other inflammatory markers, seems not or weakly to be unrelated to BMI or insulin resistance [70, 94], together with our findings that elevated Lp-PLA_2 levels increases cardiovascular risk beyond the risk of having MetS in non-diabetic subjects, this together indicates a unique potential of Lp-PLA_2 as a vascular specific inflammatory marker. Thus, as subjects with MetS are considered to be at intermediate risk for CVD, some authors have even suggested that Lp-PLA_2 testing could presently be recommended as an adjunct to traditional risk assessment in patients at moderate and high 10-year risk of CVD [126].

Variations in PLA2G7 gene are associated with Lp-PLA$_2$ activity and mass.

To our knowledge the MDCS is the first large study, including almost 4700 middle-aged Caucasians, exploring the association between *PLA2G7* gene polymorphisms and plasma levels of Lp-PLA$_2$ activity and mass, respectively. In Paper IV we found that VV (i.e. rs1051931) and CC (i.e. rs2216464) carriers, respectively, had significantly higher Lp-PLA$_2$ activity levels. Furthermore, we also found that subjects possessing the minor allele HH (i.e. rs1805017) and GG (i.e. rs141348) had significantly higher Lp-PLA$_2$ mass levels. At present there are a only some smaller studies, or from selected patients or ethnic groups, who have reported information on the association between 379VV allele polymorphism and Lp-PLA$_2$ activity levels [97, 101, 155]. As ethnicity was the third most important predictor of Lp-PLA$_2$ variability in the Cardiovascular Health study, an analysis that included 18 lifestyle and biological factors [131], together with the current knowledge that genetic influence may differs substantially between ethnic groups [98, 99, 101], more studies are needed to further explore the clinically relevant impact of Lp-PLA$_2$ polymorphisms on plasma LP-PLA$_2$ levels and future CVD risk.

Clinical implications

Data from a large number of population-based, as well as primary and secondary preventive studies (Figure 1) confirm that Lp-PLA$_2$ is associated with an increased risk of incident CVD [47]. In addition, Lp-PLA$_2$ may represent a novel specific vascular inflammatory biomarker for CVD risk assessment. In contrast to many other inflammatory biomarkers (i.e. CRP), Lp-PLA$_2$ does not reflect systemic inflammation, and thus is not affected by common infections and arthritis [49]. As CVD is still the leading cause of death and disability in many countries [13],

together with the fact that traditional CV risk factors only account for about 50-90% of variability of CVD risk [9, 10, 156]. Almost 6 out of 10 US patients with CAD have none or only one of conventional risk factor (i.e. hypertension, smoking, hypercholesterolemia and diabetes mellitus) [8], and the shift in understanding that inflammation participates in atherosclerosis has emerged the need of inflammatory biomarkers in risk prediction and other applications including guide for therapy, etc [9, 10]. In a statement for healthcare professionals from the Centers for Disease Control and Prevention and American Heart Association the use of hsCRP has been already recommended as part of global risk prediction in asymptomatic individuals, particularly those considered to be at intermediate risk for CVD by traditional CV risk factors [44]. Whether the same recommendations is valid for Lp-PLA_2 remains to be elucidated, although the US Food and Drug Administration (FDA) recently has approved Lp-PLA_2 blood testing for assessing patients for risk of CHD and ischemic stroke. Worldwide, prevalence of MetS and obesity are increasing with an increasing number of subjects at intermediate to high CVD risk [88-90]. Some have already suggested that Lp-PLA_2, which seems to be a reliable CVD predictor in studies across different ethnic populations [47, 157], "is to be recommended as diagnostic test for vascular inflammation to better identify patients at high or very high risk who will benefit from intensification of lipid-modifying therapies" [126]. However, further clinical validation in well-designed observational and interventional studies is needed before these recommendations can be properly evaluated in order to include them in the clinical diagnostic algorithms.

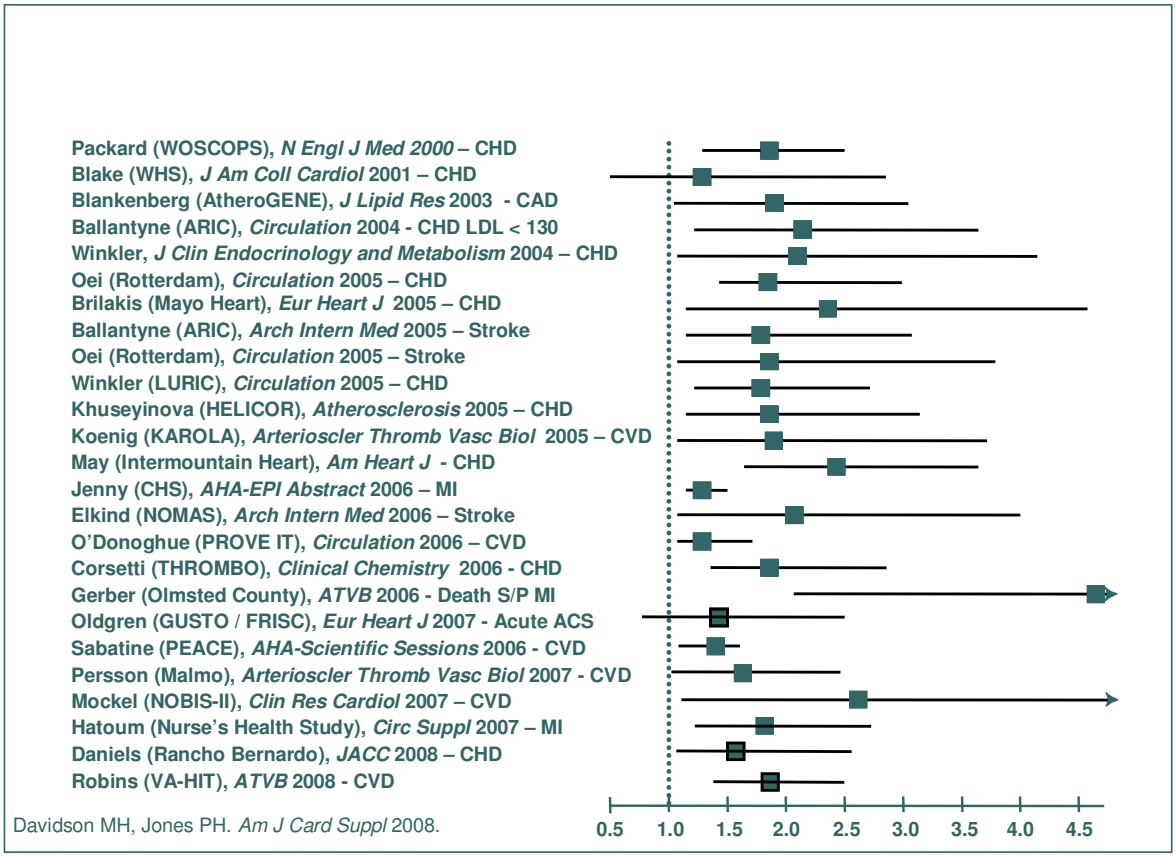


Figure 1. A summary primary- and secondary preventive studies showing the association between elevated Lp-PLA_2 and incidence of CVD.

What criteria are needed to determine the clinical significance of a risk marker/ factor?

According to ATP III guidelines [9] an emerging risk factor should significantly, and independently of other major traditional risk factors, predict an increased risk. Secondly, there should be a relatively high prevalence of the risk factor in the general population. Third, the risk factor should be stable with respect to diet and diurnal variation. Fourth, it should be easy to measure, inexpensive and there should be an available well-standardized commercial assay. Finally, preferably modification of the risk factor should in a clinical trial be associated with a risk reduction.

Does Lp-PLA_2 meet all these criteria? There are over 25 prospective population-based studies showing consistently that elevated Lp-PLA_2 levels is significantly associated with an increased incidence of CVD, independently of other established major cardiovascular risk factors [47]. In recent meta-analysis by Garza et al., the authors concluded that Lp-PLA_2, whether assessed as activity or mass, resulted in almost the same magnitude of risk (i.e. a two-fold) for incident CVD [47]. Lp-PLA_2 has a low biologic variability and fluctuation, which is similar to blood lipids [48, 49], which makes Lp-PLA_2 suitable to be assessed serially. Lp-PLA_2 levels could be measured both as activity or mass but there is no consensus today which of these is most valuable in a clinical setting. In our study, Lp-PLA_2 activity was, in comparison to mass, more strongly associated with presence of MetS and incident CVD. It has also been demonstrated that Lp-PLA_2 activity but not mass is associated with sdLDL [93, 158], a lipid fraction highly related to the MetS [91, 159]. However, in paper III we also found that 40% of non-diabetic subject with high levels of Lp-PLA_2 activity also had low or medium levels of Lp-PLA_2 mass and vice versa. Furthermore, when interpreting different results from studies reporting Lp-PLA_2 mass it is of importance to know what generation of assay was used, i.e. so far mostly first and second generation of PLAC test from diaDexus. In fact, in a letter to the Editor of Clinical Chemistry, McConnell and Jaffe commented on the results from the AIRGENE study [48] and pointed out that second generation assays are no longer commercially available. They also suggested that the third in comparison to the second Lp-PLA_2 mass generation assay has much greater variability [160]. Recently, a fourth generation assay has been evaluated and cleared by US FDA. This assay is considered to be more easy to use in clinical practise; however there is yet no available information on validation or reproducibility for this forth-generation assay.

Several clinical studies have demonstrated the efficacy of lipid-lowering drug treatment (i.e. statins, fenofibrate, niacin, ezetimibe and omega-3 fatty acids) in association with reduction of Lp-PLA_2 plasma levels; this effect is mainly explained by a reduction in LDL-cholesterol [68, 161-163]. Whether modification of lifestyle factors in terms of smoking cessation, change of dietary habits, weight reduction and physical activity, etc, are related to reductions in Lp-PLA_2 levels remains to be evaluated. Today, there are several ongoing clinical trials evaluating the efficacy of Lp-PLA_2 inhibitors. One new compound under investigation (i.e. darapladib, SB-480848) has been shown to inhibit most of Lp-PLA_2 activity in atherosclerotic plaque from rabbits [164]. Oral administration of this compound to healthy volunteers has demonstrated a marked reduction in Lp-PLA_2 activity [165, 166]. Furthermore, recently the effect of darapladib on coronary plaque deformability composition and size was evaluated (using intravascular ultrasound imaging and palpography) in 330 patients with established CAD and with a follow-up of 12 months [167]. In contrast to placebo, and in addition to adherence to high-level of standard-care treatment, Lp-PLA_2 inhibition with darapladib prevented necrotic core expansion, a key determinant of plaque vulnerability. The authors suggested that Lp-PLA_2 inhibition may represent a novel therapeutic approach for CVD prevention. Future ongoing studies are underway to answer the question whether lowering Lp-PLA_2 levels by an inhibitor is associated with a reduced incidence of CVD.

Methodological aspects

Representativity

The participation rate in the MDCS was only 41% and it is common problem that attendance rate in cohort studies have declined during the last decades [168]. In a study of non-attendees in MDCS, the all-cause mortality was 2-3 times higher in non-participants compared to participants [105]. The increased mortality associated with non-participation could probably be explained by a higher prevalence of smoking, high alcohol consumption and poorer socio-economic circumstances among non-attendees as been demonstrated in previous population-based studies from Malmö [168]. Participants in the MDCS were recruited through community invitation. A previous study from the MDCS, which compared community against personal invitation showed favour in terms of socio-demographic and lifestyle factors for the community recruited approach [104]. Results from that study also strengthen the "healthy cohort" effect which could underestimate the results found for Lp-PLA_2 in the four studies included in this thesis.

Follow-up and Endpoints

A common problem in long-term prospective studies is change of exposure over time. It is mostly unknown what happens between the baseline examination and during the follow-up period in terms of endpoints. In MDCS individuals with baseline-detected hypertension, T2DM, hyperlipedemia, etc, were referred to their private physician or to physicians within the primary health care organization in the city of Malmö. One can assume that many patients were subsequently initially and during the follow-up period treated for their detected CV risk factors which may consequently have reduced their forthcoming CVD risk. Furthermore, many moderate to high CVD risk MDCS subjects have also been involved in clinical trials [169,

[170], a circumstance that also might have changed their initial CV risk during the follow-up period. All these circumstances could influence the observed associations for Lp-PLA_2, i.e. it is reasonable to assume that the observed risk increase for incident CVD, and in particular ischemic stroke, associated with elevated Lp-PLA_2 in MDCS might therefore be underestimated.

All individuals in MDCS were followed to first incident CHD, ischemic stroke or death or until 31 December 2003 by data linkage with regional and national registers. Several studies have documented the validity and completeness of these registers [107, 121, 171]. A validation study performed on the Swedish Hospital Discharge Register regarding the diagnosis MI found that an MI was false in only 5 percent of the cases [124]. A major strength of the stroke diagnosis is that STROMA register has continuously searched for patients with symptoms of stroke during the entire follow-up period and included both hospitalized and non-hospitalized patients. National registers were used to find those who moved away from the city. The diagnosis of stroke or subtype classification was verified by computed tomography scan, autopsy, or lumbar puncture and verified by a specialist research nurse under supervision of a senior physician. By definition, patients with transient ischemic attacks were excluded. Routine hospital discharge registries poorly reflect the incidence of stroke in the population, among patients discharged alive from hospital nearly 30 percent of the stroke diagnoses could be false-positive and 6 percent false-negative. A validation study from the Swedish Hospital Discharge Register on the diagnosis stroke has so far not been performed, but only 5% of all incident strokes in the MDCS occurred outside Malmö.

Conclusions

Inflammation is today regarded as an important factor in the initiation and progression of atherosclerosis. Lp-PLA_2 as a novel biomarker of vascular inflammation may add information beyond traditional cardiovascular risk factors when predicting an individual's risk for CVD events.

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This thesis shows that:

- In middle-aged Caucasians, genetic variation at the *PLA2G7* gene locus significantly influences plasma Lp-PLA_2 activity and mass levels, in a position and sex-specific manner.
- Plasma Lp-PLA_2 levels increases with age, and are higher in males and in smokers. Lp-PLA_2 is positively correlated with LDL-cholesterol and triglycerides and inversely correlated with HDL-cholesterol. Variation of Lp-PLA_2 activity and mass was only explained to 35% and 19%, respectively, by 12 measured lifestyle and biological variables.
- Our studies support previous evidence that high levels of LpPLA_2 activity and mass, respectively, independently of traditional CV risk including LDL-cholesterol and other blood lipids, increase the risk for incident CVD, especially for ischemic stroke.
- Lp-PLA_2 is associated with all components involved in the MetS according to NCEP/ATP III definition. Results from our study show that both Lp-PLA_2 and MetS are independently associated with incident CVD. Simultaneous presence of Lp-PLA_2 activity and MetS may identify an especially high risk individual.

Populärvetenskaplig sammanfattning

Hjärtkärlsjukdom är fortfarande den vanligaste orsaken till för tidig död trots att insjuknandet under de senaste årtionden minskat och behandlingen blivit bättre. Befolkningen blir allt äldre, fler har övervikt/fetma, vilket medför att en ökande andel har metabola rubbningar. Metabola rubbningar och övervikt/fetma medför en ökad risk för hjärtinfarkt och stroke (”slaganfall”). Hjärtinfarkt är i västvärlden och Sverige den vanligaste, och stroke den tredje vanligaste, orsaken till för tidig död. Många av riskfaktorerna för hjärtkärlsjukdom som rökning, övervikt/fetma, fysisk inaktivitet, höga blodfetter, högt blodtryck och diabetes är livstilsrelaterade och därmed förändringsbara. Under de senaste decennierna har vissa av dessa riskfaktorer behandlats framgångsrikt. Rökningen har minskat, samt det finns idag effektiva mediciner som sänker blodtrycket och blodfetterna. Dessutom har vi ett bättre omhändertagande av bland annat diabetiker. Trots en intensiv behandling kvarstår hjärtkärlsjukdom fortfarande som den vanligaste dödsorsaken. I USA har populationsbaserade studier visat att mer än hälften av patienterna med etablerad kranskärlsjukdom har ingen eller endast en riskfaktor i termer av högt blodtryck, rökning, högt kolesterol eller diabetes. Dessutom är hjärtinfarkt eller plötslig hjärtdöd den första manifestationen av kranskärlsjukdom hos 6 av 10 män och 4 av 10 kvinnor. Många har ansett det behövs nya biomarkörer för att bättre kunna hitta de individer med hög risk att insjukna i hjärtkärlsjukdom och dess komplikationer.

Hjärtkärlsjukdom är framför allt en aterosklerotisk (”åderförkalknings”) åkomma med manifestationer, företrädesvis i stora kroppspulsådern, hals- ben- och hjärtats kranskärl, som utvecklas under lång tid utan några symtom. Viktiga komponenter i aterosklerosprocessen är åderförfettning och inflammation. Anrikningen av LDL-kolesterol (”farligt blodfett”) som förhärdas (”oxideras”) är grunden till det aterosklerotiska placket i kärlväggen. Olika inflammatoriska processer gör att placket kan bli instabilt vilket medför ökad risk för ruptur

och därmed komplikationer såsom akut hjärtinfarkt eller stroke. Under de senaste decennierna har flertalet populationsbaserade studier genomförts med avsikt att beskriva de inflammatoriska markörer förenade med hjärtkärlsjukdom. Ett antal inflammationsmarkörer, som t.ex. CRP (”snabbsänka”) och speglande en systemisk inflammation, har oberoende av traditionella riskfaktorer visats vara relaterade till ökat insjuknade i hjärtkärlsjukdom. En annan biomarkör, dock mer specifikt kärlrelaterad, är Lp-PLA_2 (lipoprotein-associerat fosfolipas A2). Lp-PLA_2 är ett enzym i blodbanan som företrädesvis (ca 80 %) är bundet till LDL partikeln. Experimentella studier har visat att Lp-PLA_2 är involverade i kärlväggens aterosklerostiska process och finns i hög koncentration i det instabila placket.

Syftet med detta avhandlingsarbete var att studera huruvida Lp-PLA_2, mätt som aktivitet respektive massa, är och till vilken grad förenat med omgivnings-, livsstils- och andra biologiska riskfaktorer för hjärtkärlsjukdom (Delarbete 1). Dessutom utforska huruvida förhöjda nivåer av Lp-PLA_2 är förenat med en ökad risk för insjuknande i hjärtinfarkt och stroke (Delarbete 2). Vidare studera hur och om Lp-PLA_2 är associerat med faktorer som ingår i det metabola syndromet (anhopning av metabola riskfaktorer för hjärtkärlsjukdom) samt utvärdera om förhöjda nivåer av Lp-PLA_2 kan modifiera risken för hjärtkärlsjukdomsinsjuknande hos individer med syndromet (Delarbete 3). Avslutningsvis vill vi utforska om det finns genetiska polymorfier (genetiska varianter) som påverkar blodkoncentrationen och produktionen av Lp-PLA_2 aktivitet och massa (Delarbete 4).

För samtliga delarbeten användes deltagare i ”Malmö Kost och Cancer” studiens hjärtkärlkohort, vilken består av 6103 män och kvinnor mellan 45-69 år. Samtliga individer undersöktes oblodigt med hjälp av ultraljud över halskärlen för förekomst och kvantifiering av ateroskleros. Fastande blodprov togs för analys av bland annat blodfetter, blodsocker samt

plasma och blodceller som lagrades. Lp-PLA_2 nivåer av aktivitet och massa i blodet analyserades på 5393 individer, vilket utgör studiepopulationen i denna avhandling. Delarbete 1 visade att Lp-PLA_2 är högre hos män jämfört med kvinnor samt att kvinnor med substitutionsbehandling med östrogen har ännu lägre nivåer. Plasma nivån av Lp-PLA_2 är högre hos rökare, högre hos individer med förhöjda blodfetter samt högre hos individer med plackförekomst i halspulsådern. Både Lp-PLA_2 aktivitet och massa är starkt relaterat till blodnivån av totalkolesterol och framför allt LDL, samt måttligt omvänt till HDL (”ofarligt”). Sambandet mellan Lp-PLA_2 nivån och ultraljudsdetekterad intima-media tjocklek (”indikerande ateroskleros”) är svagt. Inget samband fanns mellan Lp-PLA_2 och den systemiska inflammationsmarkören CRP vilket indikerar olika patofysiologiska vägar av den inflammatoriska kaskaden i kärlväggen. Blodnivån av Lp-PLA_2 aktivitet och massa förklarades endast till 35 respektive 19 % av 12 studerade omgivnings-, livsstils- och biologiska variabler vilket antyder att Lp-PLA_2 har andra patofysiologiska mekanismer än traditionella riskfaktorer.

I delarbete II fann vi att individer med förhöjda nivåer av Lp-PLA_2 aktivitet eller massa i blodet har en fördubblad risk att insjukna i stroke. Riskökningen kvarstår även efter att man tagit hänsyn till effekten av andra riskfaktorer, såsom ålder, kön, rökning, blodfetter och blodtryck. Ett liknande samband kunde inte påvisas mellan förhöjda nivåer av Lp-PLA_2 aktivitet och massa och insjuknande i hjärtinfarkt

I samstämmighet med andra tidigare publikationer visade vi i delarbete III att individer med ett metabolt syndrom har en ökad risk för insjuknande i hjärtkärlsjukdom. Dessutom att Lp-PLA_2 nivån är starkt korrelerat till alla komponenter (högt blodtryck, lågt HDL-kolesterol, höga triglycerider, bukfetma och rubbad sockertolerans) som ingår i syndromet. Vidare fann

via att ju fler metabola komponenter individen har desto högre är Lp-PLA_2 nivån. Studien visade även att förhöjda nivåer av Lp-PLA_2 aktivitet, oberoende av förekomsten av metabolt syndrom, var associerat med ökad risk för insjuknande i hjärtkärlsjukdom. Samtidig närvaro av metabolt syndrom och stegrade Lp-PLA_2 nivåer ökar risken ytterligare, vilket kan identifiera individer med en högre risk.

Genetikens betydelse för blodnivån av Lp-PLA_2 studerades i delarbete IV. I genen som kodar för Lp-PLA_2 (*PLA2G7*) finns det ett antal genetiska varianter beskrivna. I vårt arbete studerade vi sex olika varianter och deras effekt på plasma nivåer av Lp-PLA_2 aktivitet och massa. Homozygot (samma uppsättning ifrån båda föräldrarna) för två genetiska varianter (minor allele i A379V samt R92H) påverkade 7-12 % av nivån av Lp-PLA_2 i blodet

Då hjärtkärlsjukdom är den vanligaste dödsorsaken och de traditionella riskfaktorerna förklarar i olika populationer mellan 50-90 % av orsaken, är det av största vikt att vi hittar andra biomarkörer som kan identifiera fler individer med hög risk att insjukna i hjärtkärlsjukdom och dess komplikationer. Lp-PLA_2 kan vara en ny viktig biomarkör som indikerar lokal inflammation i kärlen.

Slutsatsen i denna avhandling är att flera faktorer, såväl genetiska som livsstilsfaktorer påverkar nivån av Lp-PLA_2 i blodet. Förhöjda nivåer av Lp-PLA_2 är associerat med en ökad risk för insjuknande i hjärtkärlsjukdom speciellt i stroke. Lp-PLA_2 adderar information om risk för framtida hjärtkärlinsjuknande hos individer med ett metabolt syndrom.

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