Univerzita Karlova

Lékařská fakulta v Hradci Králové

Lipoprotein-asociovaná fosfolipáza A2 u diabetických pacientů ve stáří

DIZERTAČNÍ PRÁCE

Autor práce: MUDr. Joao Fortunato

Školitel: prof. MUDr. Vladimír Blaha, CSc.

Školitel konzultant: prof. MUDr. Luboš Sobotka, CSc.

Hradec Králové

2020

Charles University

Faculty of Medicine in Hradec Kralove

Lipoprotein-associated phospholipase A2 in geriatric diabetic patients

PhD Thesis

Author: MUDr. Joao Fortunato

Tutor: prof. MUDr. Vladimir Blaha, CSc.

Consultant: prof. MUDr. Lubos Sobotka, CSc.

Hradec Kralove

2020

Author's declaration

I hereby declare that this dissertation thesis is my own original work and that I indicated in the references all used information sources. I also agree with saving my dissertation thesis in the Medical Library of the Charles University, Faculty of Medicine in Hradec Kralove making it available for study and educational purposes provided that anyone who will use it for his/her publication or lectures is obliged to refer to or cite my work properly.

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Hradec Kralove, 2020 MUDr. Joao Fortunato

Acknowledgements

I would like to thank my tutor prof. MUDr. Valdimír Blaha, CSc. for his guidance, interest and time spent during my postgraduation.

I also would like to express by gratitude to my consultant prof. MUDr. Luboš Sobotka, CSc. for his given comments, motivation and theoretical knowledge during my postgraduation.

The data collection would not be possible without the help of the doctors from the 1st internal cardioangiologic clinic, University Hospital in Hradec Kralove. The study population was intervened under the care of these doctors and the blood samples were taken during the stay at this clinic.

I am also extremely grateful to MUDr. Božena Jurašková, Ph.D. for the inclusion of a control group to this work. Without this control group, most of the statistical analyses and conclusions taken from them would not be possible.

Sample measurements would not be possible without the unconditional support of prof. RNDr. Ctirad Andrýs to whom I also woe an enormous thank you.

For his motivation, comments and inspiration I would like to thank my fellow colleague MUDr. Pavel Skořepa.

Finally, I would like to thank my wife MUDr. Michaela Fortunato for her patience, inspiration, encouragement and motivation to finish this postgraduation.

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Used abbreviations

ADP	Adenosine diphosphate
apoB	Apolipoprotein B
BV	Balloon valvuloplasty
cGMP	Cyclic guanosine monophosphate
CHD	Coronary heart disease(s)
CVD	Cardiovascular disease(s)
HDL	High-density lipoprotein
HDL-C	High-density lipoprotein concentration
IL-1	Interleukin-1
IL-1β	Interleukin-1-beta
INF- γ	Interferon gamma
LDL	Low-density lipoprotein
LDL-C	Low-density lipoprotein concentration
Lp(a)	Lipoprotein (a)
Lp-PLA2	Lipoprotein-associated phospholipase A2
NEFAs	Nonesterified fatty acids
NO	Nitric oxide
PAF	Platelet-activating factor
PDGF	Platelet-derived growth factor
PECAM-1	Platelet-endothelial-cell adhesion molecule-1
PLA2G7	Phospholipase A2 group 7
ROS	Reactive oxygen species
SAVR	Surgical aortic valve replacement
sPLA2	Secreted phospholipases A2
TAVI	Transcatheter aortic valve implantation
TCFA	Thin-cap fibroatheroma
TGF-β	Transforming growth factor beta
TNF-α	Tumor necrosis factor alpha
VEGF	Vascular-endothelial growth factor
VLDL	Very low-density lipoprotein

Background

Cardiovascular diseases (CVDs) are the number one cause of overall mortality in developed countries. Despite a significant decrease in the mortality rate attributed to coronary heart disease in the past 10-15 years, the healthcare costs related to CVD, the associated morbidity and the prevalence of risk factors remain extremely high. It is therefore, of paramount importance, any contributions to the field.

The prevalence of atherosclerosis is ubiquitous in today's society and, again, any measures or interventions to prevent or reverse its development, decrease the risk of plaque rupture and ultimately decrease the incidence of cardiovascular events are most welcomed in the medical community. According to the World Health Organization, CVDs represented around 30% of the global deaths in 2010 and estimates that by 2030 more than 23.3 million persons will die annually from CVDs.

Phospholipase A2 enzymes are a diverse class of esterases able to recognize and catalyze the hydrolysis of the *sn*-2 ester bond of glycerophospholipids yielding nonesterified fatty acids (NEFAs), such as arachidonic acid; and lysophospholipids. This superfamily of enzymes is then divided into six families based on their structure, catalytic mechanism, localization and evolutionary relationships. These include 1) intracellular cytosolic Ca^{2+}-dependent, 2) intracellular cytosolic Ca^{2+}-independent, 3) secretory phospholipase A2 (sPLA2), 4) lysosomal phospholipase A2, 5) adipose specific phospholipase A2, and 6) Lipoprotein-associated phospholipase A2 (Lp-PLA2) (Ramanadham *et al.*, 2015). Most of their products function as and generate signaling molecules, such as prostaglandins, leukotrienes and platelet-activating factor (PAF), that intervene in host defense, inflammation and innate immunity (Stafforini, 2009).

Lp-PLA2 is also known as platelet activating factor acetylhydrolase (PAF-AH) due to the fact that it was discovered because of its ability to catalyze the hydrolysis of PAF; or as phospholipase A2 group 7 (PLA2G7, because of the gene in which it is encoded). It is thought to have considerable physiologic and pathophysiologic roles being an active participant and mediator in states of

oxidative stress, inflammation and atherosclerosis plaque development and progression. It is, therefore, not surprising that substantial investigations have been performed in the last years exploring the functions, involved metabolic pathways and potential pharmacological interventions altering the Lp-PLA2 mass concentration or activity.

Lp-PLA2 biochemistry

Lp-PLA2 is an extracellular Ca^{2+}-independent 45 KDa secreted enzyme formed by 441 amino acids, that circulates in plasma in the active form. It is encoded in the PLA2G7 gene located on chromosome 6p12-21.1. Chemically, by possessing a serine/aspartate/histidine catalytic triad (or Gly-X-Ser-X-Gly motif), the enzyme has more in common with neutral lipases and esterases than with other members of the PLA2 superfamily (which have a serine/aspartate dyad) (Cao *et al.*, 2011). It has a canonical tertiary fold arrangement with binding sites for low- and high-density lipoproteins (LDL and HDL, respectively). Hematopoietic stem cell-derived cells, such as monocytes, macrophages, mast cells, and T-lymphocytes, are the main sources of the Lp-PLA2 protein.

Lp-PLA2 function

Lp-PLA2 hydrolyzes several types of short-chained (up to 5 carbons long) and oxidized phospholipids that harbor acyl groups at the second position of the glycerol backbone. Due to this unique substrate specificity, the enzyme can circulate freely in the plasma in the active form without hydrolyzing native cellular phospholipids (it only hydrolyzes phospholipids where the fatty acid on the *sn*-2 position became shortened or oxidized). Oxidized phospholipids are thought to induce macrophage recruitment and to contribute to the initiation and progression of chronic inflammation characteristic for atherosclerosis. Lp-PLA2 might have a protective function in this setting by hydrolyzing these bioactive, proinflammatory, oxidatively fragmented phospholipids produced during the oxidation of LDL.

On the other hand, as the burden of oxidized phospholipids increases with disease progression, there is an increased expression of proinflammatory, procoagulant and proatherogenic products. The reaction catalyzed by Lp-PLA2

yields lysophosphatidylcholine, oxidized NEFAs and short NEFAs, molecules that have been shown to be highly effective proatherogenic inflammatory mediators.

Lysophosphatidylcholine, through its action on the G-protein-coupled receptor G2A, modulates endothelial activation, suppresses nitric oxide generation, inhibits cell migration and proliferation, and inhibits the phagocytic clearance of apoptotic cells (Mallat, Lambeau and Tedgui, 2010). These effects may contribute to the initiation and progression of atherosclerosis. The concentration of lysophosphatidylcholine in the plasma of healthy individuals usually ranges from 200 to 400 μmol/l. A scheme depicting pathophysiologic effects of oxidized phosphatidylcholine is shown in Figure 1. In summary, Lp-PLA2 seems to be a promoter in the development and progression of inflammation, primarily due to its proinflammatory products, lysophosphatidylcholine and oxidized NEFA.

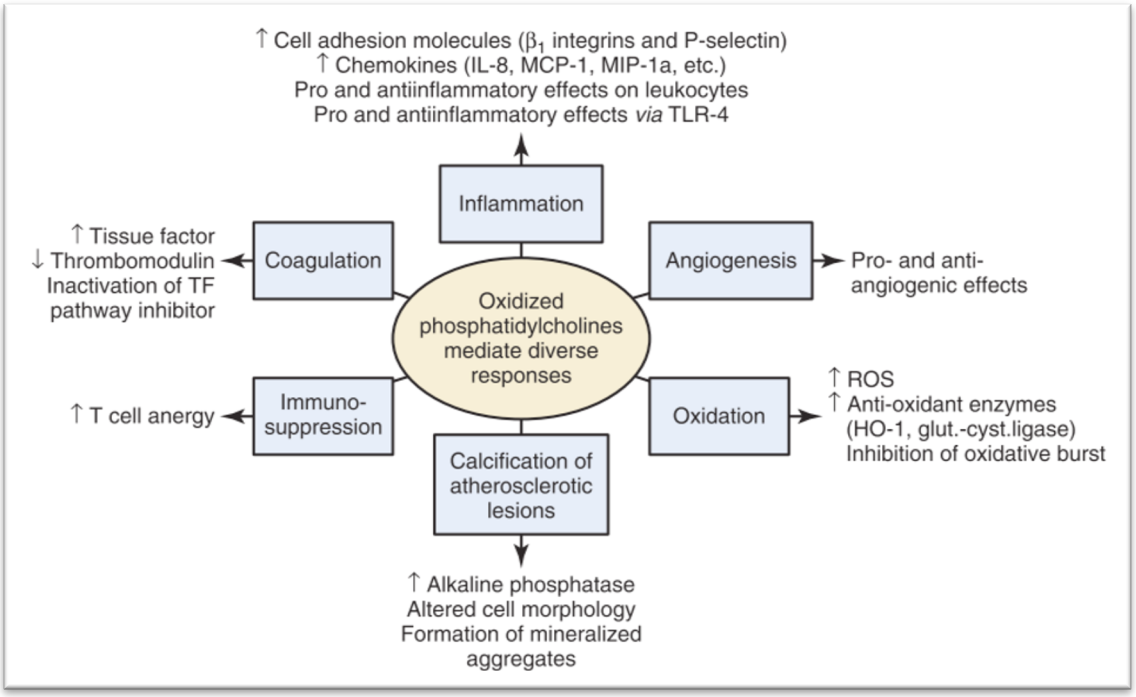

Figure 1 – Pathophysiologic effects of oxidized phosphatidylcholines

IL = interleukin, MCP-1 = monocyte-chemoattractant protein-1, MIP-1a = macrophage inflammatory protein-1a, ROS = reactive oxygen species, TF = tisuue factor (from Clinical Lipidology, a Companion to Braunwald's Heart Disease, 2nd edition, Elsevier Sauders, Christie M. Ballantyne, M.D., 2015)

Non-esterified long-chain fatty acids increase the adhesion of monocytes to endothelial cells, enhance cyclooxygenase-2 expression, increase the production of tumor necrosis factor, and others (Oestvang and Johansen, 2006). These fatty acids, however, are structurally different from those generated by Lp-PLA2, which yields oxidized short to medium chain fatty acids.

Short-chain oxidized NEFAs may act as endogenous ligands of nuclear receptors that inhibit inflammatory gene expression and promote the differentiation of monocytes to become anti-inflammatory (M2) (Litvinov *et al.*, 2010). Succinctly, substrates and products of Lp-PLA2 have been shown to have both pro- and anti-inflammatory and pro- and antioxidant effects.

Secretory phospholipase A2 (sPLA2) requires high calcium concentration to properly function and hydrolyzes a wide range of phospholipids substrates depending on the enzyme isoform. Like Lp-PLA2, sPLA2 may exert proatherogenic effects in the arterial wall (Webb, 2005). Phospholipid hydrolysis by sPLA2 yields potentially bioactive lipids, including nonesterified fatty acids (most notably, arachidonic acid) and lysophospholipids (Figure 2).

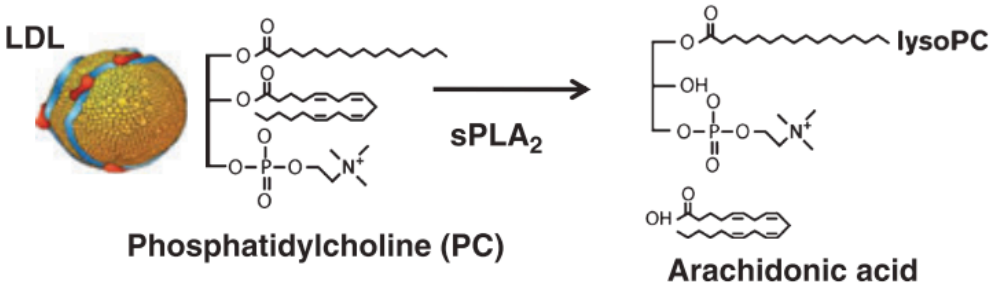

Figure 2 – secretory phospholipase A2 (sPLA2) action

lysoPC = lysophosphatidylcholine (Macphee, Nelson and Zalewski, 2006)

Note that, in contrast to Lp-PLA2, sPLA2 hydrolyses non-oxidized phospholipids contained in non-oxidized LDL particles. Besides this function, sPLA2 has been recently shown to play an important role in allergic reactions, being a regulator of mast cells maturation (Murakami and Taketomi, 2015), in the local mediation of lipid metabolism in response a specific microenvironmental cues (Sato *et al.*, 2014), and in the immune defense, by

hydrolyzing phosphatidylethanolamine and phosphatidylglycerol, major components of bacterial membranes (Nevalainen, Graham and Scott, 2008). Some isoforms of sPLA2 are overexpressed during inflammatory processes such as arthritis, atherosclerosis and sepsis while other isoforms are overexpressed in a variety of cancers (Dong *et al.*, 2010).

Lp-PLA2 binding to lipoproteins

In humans, 70-80% of the circulating Lp-PLA2 is non-covalently bound to LDL, 15-20% is contained in HDL and the remainder is found in very low-density lipoprotein (VLDL). It has greater affinity for the smallest, densest, most electronegative LDL particles, probably because of the conformation apoB100 adopts in these particles (which is distinct from that seen in larger LDL), facilitating Lp-PLA2 binding (Stafforini *et al.*, 1999). However, only a small fraction of circulating LDL particles has Lp-PLA2 attached to it (about 1%), which means that most of the LDL particles do not contain this enzyme at all. The binding site on Lp-PLA2 molecule differs according whether the carrier lipoprotein is LDL or HDL. Lp-PLA2 bound to HDL has a much lower specific activity than that bound to LDL. The LDL/HDL ratio for the enzyme may depend on the extent of its glycosylation.

From all circulating lipoproteins it is estimated that only approximately 0.1% are laden with Lp-PLA2. It also binds to the apoB-containing Lp(a), which transports oxidized phospholipids. When Lp-PLA2 acts within its normal physiological levels it may have beneficial effects because it hydrolysis these oxidized phospholipids, decreasing their proinflammatory potential.

In other mammalian species, because of different lipoprotein profiles when compared to humans, Lp-PLA2 may be exclusively bound to HDL. This is true for guinea pigs, rats and mice, and can have significant repercussions when studying the enzyme function. In this case Lp-PLA2 works solely in an antiatherogenic manner.

Because of this dual role it was proposed that the type of lipoprotein to which Lp-PLA2 associates affects its function. When it binds to LDL, it mainly acts in a proatherogenic manner, but when it associates with HDL, it may exhibit antiatherogenic properties.

Furthermore, the homeostasis of Lp-PLA2 is influenced by the ratio and distribution of the different lipoprotein classes. In primary hypercholesterolemia, LDL-bound Lp-PLA2 increases with the severity of the disease. In homozygous familial hypercholesterolemia there are very high levels of LDL-bound Lp-PLA2. Other types of mixed dyslipidemias, such as seen in diabetes mellitus, may also alter the function and distribution of Lp-PLA2.

In summary, LDL particles provide a circulating reservoir for the Lp-PLA2 enzyme, which remains inactive until LDL undergoes oxidation. After LDL oxidation within the arterial wall, a short acyl group at the *sn*-2 position of phospholipids becomes susceptible to hydrolysis by Lp-PLA2. From this reaction two potent pro-inflammatory and pro-atherogenic mediators are generated, namely lysophosphatidylcholine and oxidized fatty acid. These reactions are depicted in Figure 3.

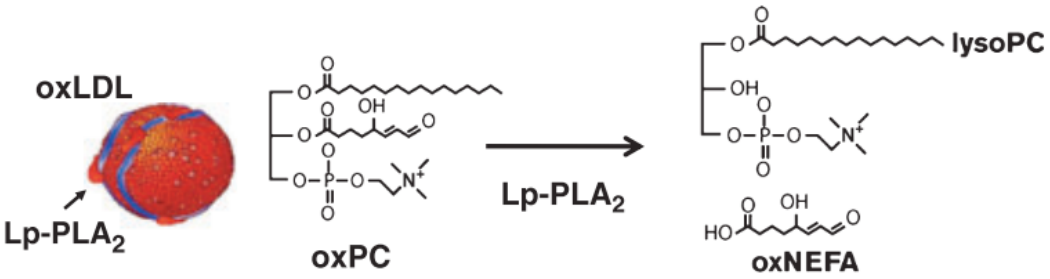

Figure 3 – Lipoprotein associated phospholipase A2 action

Oxidized phospholipids (oxPC) are hydrolyzed into lysophosphatidylcholine (lysoPC) and oxidized non-esterified fatty acids (oxNEFA) (Macphee, Nelson and Zalewski, 2006)

Lp-PLA2 gender, age and race

Kosaka *et al.* measured Lp-PLA2 activity in more than 3000 healthy Japanese individuals. Women expressed significantly lower Lp-PLA2 activity in comparison to men (Kosaka *et al.*, 2001). This finding was then confirmed in the AtheroGene study, whose population is composed by patients from Europe, North America and Australia (Blankenberg *et al.*, 2003).

Similarly to gender differences, Lp-PLA2 activity varies with age. It increases with age (Kosaka *et al.*, 2001). However, only women under 50 years of age had lower Lp-PLA2 activity as compared with men. Reasons for this difference may be related to hormonal status, as hormone replacement therapy reduces Lp-PLA2 levels.

Race-ethnic differences in Lp-PLA2 distribution have been assessed in population-based studies. In the Dallas Heart Study, a cohort of 3332 healthy, multiethnic participants, mean Lp-PLA2 activity was lower in African-Americans and Hispanics than in Caucasians (Brilakis *et al.*, 2005).

Lp-PLA2 and cardiovascular diseases

Since (Hakkinen *et al.*, 1999) demonstrated the presence of Lp-PLA2 mRNA transcripts in macrophage-rich atherosclerotic plaque by in-situ hybridization and immunohistochemical analysis, suggesting that Lp-PLA2 was locally synthetized, hundreds of studies were then published assessing the relationship between the Lp-PLA2 mass concentration or activity with the presence, prediction or prognostication of cardiovascular diseases.

One of these studies elucidated that Lp-PLA2 increases in advanced coronary atherosclerotic plaques (thin-cap fibroatheromas or ruptured plaques) when comparing to more stable coronary plaques (Kolodgie *et al.*, 2006) suggesting an association between Lp-PLA2 and plaque instability. This finding was then confirmed for atherosclerotic plaques in the carotid arteries, suggesting an association between Lp-PLA2 and stroke (Mannheim *et al.*, 2008).

The association between Lp-PLA2 levels and traditional cardiovascular risk factors including age was then extensively studied. Associations were found for age (Koenig *et al.*, 2004; Daniels *et al.*, 2008), LDL, HDL, LDL/HDL ratio, total cholesterol, triglycerides (Ballantyne, Hoogeveen and Bang, 2004; Koenig *et al.*,

2004; Persson *et al.*, 2007; Daniels *et al.*, 2008; Tsimikas *et al.*, 2009), small density LDL (Gazi *et al.*, 2005), obesity and the metabolic syndrome (Persson, Hedblad and Nelson, 2007).

The West of Scotland Coronary Prevention Study (WOSCPS) was the first study to propose Lp-PLA2 as an independent risk predictor for cardiovascular disease. The investigators found that increased baseline levels of Lp-PLA2 were strongly associated with cardiovascular events even after adjustment for traditional risk factors and inflammatory markers, including high-sensitivity C-reactive protein (hs-CRP) (Packard *et al.*, 2000). There was an approximate 20% increase in risk for every standard deviation increase in Lp-PLA2. The study population was composed of middle-aged hypercholesterolemic men in Scotland with high prevalence of other risk factors.

In a case-cohort analysis from the Women's Health Study (Blake *et al.*, 2001) Lp-PLA2 mass was measured in 28 263 women. The Lp-PLA2 levels were higher among women who later developed cardiovascular events but were not associated with increased coronary heart disease (CHD) risk after adjustment for traditional risk factors and hs-CRP. The study population was composed of middle-aged American women who were mostly professionals and had a lower event rate.

The AtheroGene study was designed to compare Lp-PLA2 activity between healthy subjects and patients with coronary artery disease (CAD). CAD patients were recruited on the occasion of a diagnostic coronary angiography. Individuals within the highest quartile of Lp-PLA2 activity had a 1.8-fold increase in CAD risk compared with those in the first quartile after adjusting for clinical and metabolic factors (Blankenberg *et al.*, 2003).

In the Atherosclerosis Risk in Communities (ARIC) study (Ballantyne *et al.*, 2004) Lp-PLA2 mass concentration and hs-CRP were measured in 1348 middle-aged American men and women (including a substantial number of African Americans) with a wide range of LDL-C. Lp-PLA2 and hs-CRP were associated to explore the increased risk for incident CHD. Lp-PLA2 and hs-CRP levels were higher in individuals who subsequently developed CHD than in those who remained free of CHD. They also divided the study population in those with LDL-C greater or lesser than 3.367 mmol/l (at that time, American guidelines

recommended a LDL-C cut-off of 130 mg/dl or 3.367 mmol/l to initiate therapy with statins) and concluded that Lp-PLA2 mass and the risk for CHD was substantially attenuated in individuals with LDL-C >3.367 mmol/l after adjustment for other risk factors. This means, that Lp-PLA2 mass independently predicted risk of incident CHD only among individuals with LDL-C <3.367 mmol/l. Furthermore, the association with other traditional risk factors was modifiable by C-reactive protein.

In the Monitoring of Trends and Determinants in Cardiovascular Disease (MONICA)-Augsburg cohort composed of 934 men aged 25 to 65 years and with a follow-up period of 14 years (Koenig *et al.*, 2004) the risk of CHD was increased by 21% for each standard deviation increase in Lp-PLA2 mass concentration.

Similarly, the Rotterdam Study (Oei *et al.*, 2005) found a 20% increased risk of CHD for each standard deviation increase in Lp-PLA2 activity within a median follow up of 6.4 years in a case-cohort study including 2238 men and women ≥55-years of age after adjustment for traditional cardiovascular risk factors. Of interest, no LDL cholesterol levels were available, so the investigators adjusted Lp-PLA2 activity for non-HDL cholesterol levels.

From the Coronary Artery Risk Development in Young Adults (CARDIA) study, 266 black and white young individuals aged 18-30 years were examined for the association of Lp-PLA2 mass and activity with the presence of calcified coronary plaque ascertained by cardiac computed tomography (CT). Lp-PLA2 mass was found to be independently associated with the presence of calcified coronary plaque and the amount of coronary calcium (Iribarren, Gross, Darbinian, Jr, *et al.*, 2005). Lp-PLA2 activity lost statistical significance when adjusted for other risk factors.

The Rancho Bernardo Study was another case-cohort study that evaluated Lp-PLA2 as an independent cardiovascular risk factor in 1077 Caucasian individuals from a southern California community with a median age of 72 years and without known previous coronary heart disease (CHD). After a mean follow-up of 16 years study participants were classified as incident CHD cases if at any time during the follow-up period they had a fatal or nonfatal myocardial infarction, coronary revascularization, or angina (grades 1 or 2 by Rose criteria). Baseline data for these analyses were obtained in 1984 to 1987

and the blood samples were analyzed for Lp-PLA2 mass concentration in 2005. Lp-PLA2 mass concentrations were significantly higher at baseline in those who developed CHS compared with those who did not.

More recently, another case-cohort study tested the population of the Prospective Study of Pravastatin in the Elderly at Risk (PROSPER) for the Lp-PLA2 association with cardiovascular diseases. PROSPER was a randomized control trial of pravastatin 40 mg daily versus placebo in 5804 men and women aged 70-82 years with a mean follow-up of 3.2 years, and with the primary endpoint of CHD and stroke. LDL was not a predictor of CHD or stroke in this trial. Lp-PLA2 activity and mass concentration were measured in 2804 men and 3000 women and they found a moderately association for mass concentration, but not for activity, with CHD and stroke after adjustment for other risk factors (Caslake *et al.*, 2010). Of note, this cohort included selected elderly individuals that previously have had a stroke or a myocardial infarction, or were at high risk for such an event, which gave an endpoint of new or recurrent cardiovascular events.

One more relevant case-cohort study took information from The Cardiovascular Health Study (CHS) to assess Lp-PLA2 as an independent risk factor for incident myocardial infarction, stroke and CVD death. The aim of the initial study was the identification of risk factors for CHD and stroke. From the initial study population composed of 5888 men and women ≥65 years of age at baseline, Lp-PLA2 activity and mass concentration were measured in 3949 participants. Lp-PLA2 mass concentration was associated with risk of myocardial infarction and stroke, but the results were attenuated after adjustment for traditional risk factors and statistical significance was lost for stroke and CVD death. Lp-PLA2 activity was associated with increased risk of myocardial infarction, stroke and CVD death. After adjustment to traditional risk factors, it remained significantly associated with myocardial infarction and CVD death, but nor with stroke (Jenny *et al.*, 2010).

A summary of some of the mentioned studies is depicted in Figure 4.

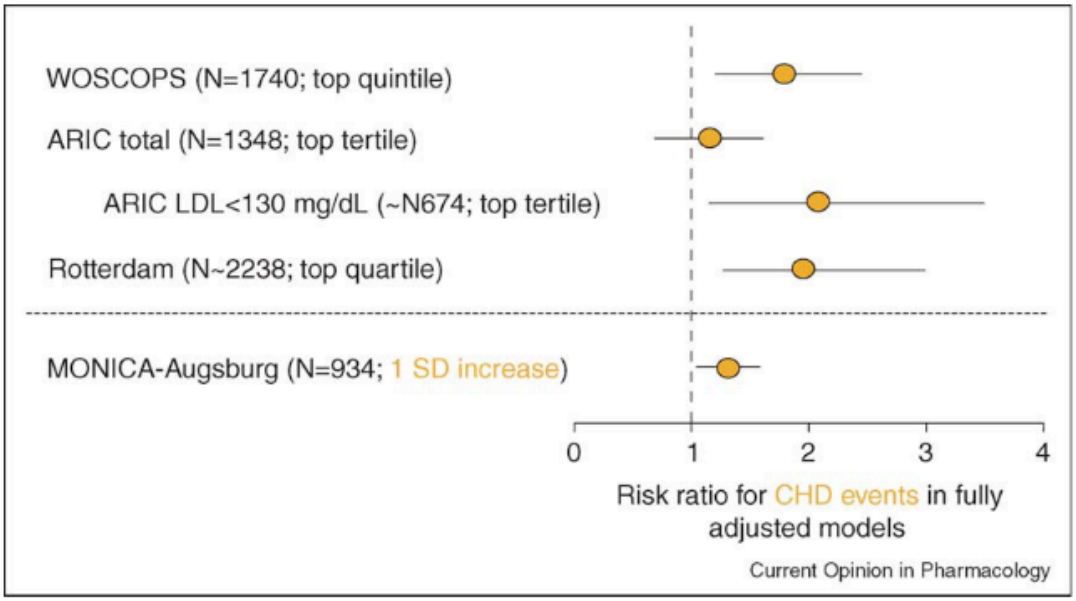

Figure 4 – Summary of studies Lp-PLA2 as a predictive marker of adverse cardiovascular events in individuals without prior coronary heart disease (ARIC = Atherosclerosis Risk in Communities, MONICA = Monitoring of Trends and Determinants in Cardiovascular Disease, WOSCOPS = West of Scotland Coronary Prevention Study. Taken from (Macphee, Nelson and Zalewski, 2006)

The results of the Justification for the Use of Statins in Prevention: an Intervention Trial Evaluating Rosuvastatin (JUPITER) were published in 2009. This randomized, double-blind, placebo-controlled trial compared rosuvastatin 20 mg daily to placebo in 17 802 men and women without previous cardiovascular disease or diabetes at study entry, and with a baseline LDL-C <3.37 mmol/l and a hs-CRP ≥2 mg/l. The trial was stopped early after a median follow-up of 1.9 years because of a 44% reduction in the primary endpoint of all vascular events, a 54% reduction in myocardial infarction, a 48% reduction in stroke, a 46% reduction in the need for arterial vascularization, and a 20% reduction in all cause mortality. A sub-analysis from the JUPITER trial evaluated the relationships of Lp-PLA2 activity and mass concentration with the risk of future vascular events. At baseline Lp-PLA2 mass and activity correlated with each other and with LDL-C, and the magnitude of those correlations increased after statin therapy. Rosuvastatin reduced Lp-PLA2 mass concentration by 33.8%, Lp-PLA2 activity by 33.2%, and LDL-C by 48.7%. Levels of Lp-PLA2

activity, but not mass, were associated with cardiovascular risk. However, Lp-PLA2 no longer predicted risk or modified clinical outcomes when individuals were treated with rosuvastatin 20 mg daily (Ridker *et al.*, 2012). This trial included more incident and carefully adjudicated CVD events than nearly all prior epidemiologic studies combined. It is also very important in understanding the role of Lp-PLA2 in predicting the risk of cardiovascular events after pharmacological reduction of LDL-C, non-HDL-C, and apoB. Reductions in Lp-PLA2 have been attributable in part to concomitant reductions in LDL-C or apoB.

The ARIC study was again mentioned in 2015 with the expansion of the cohort to 15 792 participants. From those 11 656 were investigated for the relationship of Lp-PLA2 activity with apoC3 loss-of-function (LOF) variants and the risk for incident cardiovascular disease in whites and African-Americans over a mean follow-up of 11 years. apoC3 LOF variants are associated with lower triglycerides and small dense LDL-C levels and higher HDL-C, reduced postprandial lipemia and reduced CHD risk. Lp-PLA2 activity was associated with increased risk of CHD after adjusting for traditional atherosclerosis risk factors (Pokharel *et al.*, 2015).

The Multi-Ethnic Study of Atherosclerosis (MESA) is a more recent cohort that included 6814 adults aged 45 to 84 years without previous cardiovascular events. It divided the participants with and without subclinical atherosclerosis assessed by measuring the coronary artery calcium content by CT scan and by the carotid intimal medial thickness. 5456 participants were included for the assessment of Lp-PLA2 activity and mass concentration as a predictor of cardiovascular events. The mean follow-up period was 10.2 years. Higher Lp-PLA2 activity and mass concentration were associated with increased risk of cardiovascular disease and CHD in both, individuals with and without subclinical atherosclerosis (Garg *et al.*, 2015).

The overwhelming majority of epidemiologic studies designed to investigate the relationship between Lp-PLA2 and coronary events in patients without prior history of CVD have demonstrated an association. This emerging body of evidence suggests that Lp-PLA2 may offer added value to established risk factors in assessing cardiovascular risk.

However, there are still some questions regarding the methodology and interpretation of these positive results, meaning the statistically significant association of Lp-PLA2 mass or activity with CVD. The strength of this association depends substantially on apoB or its associated lipid moieties as noted by the Lp-PLA2 Studies Collaboration meta-analysis.

Lp-PLA2 and stroke

Lp-PLA2 has been shown to be an independent predictor of future increased risk for stroke. Most of the available literature is based on case-cohort studies.

The above mentioned Rotterdam Study (Oei *et al.*, 2005) found Lp-PLA2 activity to be associated with the risk of ischemic stroke even after adjustment of traditional cardiovascular risk factors. Subjects in the highest quartile had an almost doubled risk of a future ischemic stroke compared to those in the lowest quartile. It was the first study to propose Lp-PLA2 activity as an independent risk factor for ischemic stroke. Of note, associations of total cholesterol, LDL-C, non-HDL cholesterol and HDL cholesterol as risk factors for ischemic stroke are much weaker than for myocardial infarction.

The Malmö Diet and Cancer Study (MDCS) was designed to explore the effects of diet on cancer risk in the urban area of Malmö, Sweden. From this cohort, 5393 individuals (3162 women and 2231 men) were selected to evaluate the associations of Lp-PLA2 activity and mass concentration with the incidence of CHD and ischemic stroke within a mean follow-up period of 10.6 years (Persson *et al.*, 2008). Both, Lp-PLA2 activity and mass concentration were statistically significantly associated with increased risk for incident ischemic stroke. In this study, once again, LDL-C did not modify the association of Lp-PLA2 activity or mass concentration with the risk for ischemic stroke. However, they did not find any statistically significant independent relationship between Lp-PLA2 activity and incident CHD (after adjusting for other traditional cardiovascular risk factors, statistical significance was lost for CHD).

From the Bruneck study (which aimed at studying the epidemiology and pathogenesis of atherosclerosis), a cohort of men and women aged 45-84 years comprising 765 individuals was evaluated for Lp-PLA2 activity within a 10 years

follow-up period. They concluded that Lp-PLA2 activity was associated with increased risk of future cardiovascular events, including stroke or transient ischemic attack and myocardial infarction (Tsimikas *et al.*, 2009). Interestingly, the ratio of oxidized phospholipids (detected by a monoclonal antibodies) to apoB levels predicted 10-year CVD event rates independently of traditional risk factors in a previous study from the Bruneck's cohort (Kiechl *et al.*, 2007).

The Northern Manhattan Stroke (NOMAS) study is prospective, population-based cohort consisting of 3298 stroke-free multiethnic individuals living in New York, USA, and the primary goals of the study were to describe the prevalence of vascular risk factors. Lp-PLA2 mass concentration was available for 1946 participants. Lp-PLA2 mass concentration was associated with the risk of large artery atherosclerotic stroke and this association was greater among non-Hispanic White participants, but not in other ethnic groups. There was no association of Lp-PLA2 with overall ischemic stroke risk (Katan *et al.*, 2014).

As for the prediction of coronary events it seems, that the results concerning the role of Lp-PLA2 in predicting incident ischemic stroke are also inconclusive, or at least, inconsistent.

Lp-PLA2 and recurrent cardiovascular diseases

Lp-PLA2 also appears to be a risk factor for recurrent cardiovascular events among patients with established cardiovascular disease (i.e. secondary prevention populations). A study examining individuals undergoing clinically indicated angiography demonstrated that higher levels of Lp-PLA2 were associated with a higher incidence of death or cardiovascular event independently of traditional CHD risk factors and C-reactive protein over a four-year follow-up period (Brilakis *et al.*, 2005). Later, a prospective study included 1051 patients aged 30 to 70 years with a diagnosis of coronary heart disease (84.9% of them were males) and participating in an in-hospital rehabilitation program. After adjusting for a wide range of established risk factors, there was still a two-fold increased risk for future CVD events in patients in the upper two tertiles of Lp-PLA2 mass concentration compared with those in the lowest tertile (Koenig *et al.*, 2006).

Lp-PLA2 levels were measured in patients with acute coronary syndrome at presentation in three large epidemiologic studies, the PROVE-IT, the FRISC II, and the GUSTO IV with the aim of evaluating Lp-PLA2 as a predictor of increased risk for a future cardiovascular event.

From the PROVE IT-TIMI 22 (PRavastatin Or atorVastatin Evaluation and Infection Therapy-Thrombolysis In Myocardial Infarction) trial Lp-PLA2 activity was available from 3648 patients (87.7% of the initial trial population) at the baseline, and was measured in 3265 participants (78.4% of the initial trial population) after a 30 days' follow-up. They found an association between Lp-PLA2 and the risk of recurrent cardiovascular events by comparing patients with Lp-PLA2 activity in the highest quintile at 30 days with patients with Lp-PLA2 activity in the lowest quintile. The main result, however, was an expecting decline in overall mean Lp-PLA2 activity (by 12.7%) after initiating treatment with a statin (atorvastatin 80 mg daily or pravastatin 40 mg daily). However, when separating the two groups, the changes in Lp-PLA2 activity were much smaller for patients treated with pravastatin, a result only partially explained by the change in LDL-C. The authors concluded that Lp-PLA2 is not useful for risk stratification when measured in the early days after an acute coronary syndrome and suggested that intensive statin therapy lowers Lp-PLA2 levels independent of LDL-C (Donoghue *et al.*, 2006). The initial trial (Cannon *et al.*, 2004), composed of 4162 patients, compared the effects of intensive (atorvastatin 80 mg daily) versus moderate (pravastatin 40 mg daily) statin therapy for the prevention of major adverse cardiac events after acute coronary syndrome.

The 2 other studies (FRISC II and GUSTO IV) were analyzed together. FRISC II was a Scandinavian prospective, randomized, multicenter study designed to evaluate early non-invasive or invasive strategies and long-term treatment with dalteparin or placebo in patients with acute coronary syndrome. Out of 2457 patients, 1362 were analyzed for Lp-PLA2 mass concentration. The GUSTO IV was a prospective, randomized, multicenter study involving 24 countries and 7800 patients with non-ST-elevation acute coronary syndrome assigned to receive abciximab bolus and subsequent infusion for 24 or 48 h or corresponding placebo infusion. Lp-PLA2 mass concentration was obtained from 904 randomly selected patients. Lp-PLA2 mass concentration did not predict the

increased risk of future myocardial infarction or death at up to one year in these cohorts (Oldgren *et al.*, 2007).

In a later study that included all (*N*=766) patients from the Thrombogenic Factors and Recurrent Coronary Events (THROMBO) postinfarction study, LpPLA2 activity doubled the risk for recurrent coronary events in the highest quartile (Corsetti *et al.*, 2006). The investigators concluded that Lp-PLA2 activity is a significant and independent predictor of risk for recurrent coronary events and that it performed better than apoB when included in multivariable models.

The Veterans Affairs HDL Intervention Trial (VA-HIT) was a placebo-controlled, 5 year intervention trial with gemfibrozil 1.2 grams daily to determine whether raising a low HDL-C (mean at baseline 0.83 mmol/l) in men with known, stable CHD and a low LDL-C (mean at baseline 2.87mmol/l) would reduce the major cardiovascular events of nonfatal myocardial infarction and CHD death. From the included 2175 men in this trial, 1451 (725 taking placebo, 726 taking gemfibrozil) were selected for measurements of Lp-PLA2 activity. In multivariate analyses, adjusted for major cardiovascular risk factors, elevated Lp-PLA2 activity at baseline, as well as after 6 to 7 months in the study, predicted a significant increase in the combined endpoint of myocardial infarction, CHD death and stroke over the 5-year period of trial (Robins *et al.*, 2008). These results supported the hypothesis that Lp-PLA2 can be used as a risk stratification tool in patients with stable coronary diseases.

From The Heart Protection Study Collaborative Group (HPSCG) another case-cohort analysis measured Lp-PLA2 activity and mass concentration in 19 037 individuals in a randomized trial of 40 mg simvastatin daily versus placebo and within a mean follow-up period of 5 years. They included participants with a previous diagnosis of cerebrovascular disease, CHD, other occlusive disease of non-coronary arteries, diabetes mellitus (type 1 or 2), and men aged 65 or older being treated for hypertension. Lp-PLA2 activity and mass concentration were positively correlated with each other, age, male sex, proatherogenic lipids, N-terminal pro-brain natriuretic peptide and CRP. Lp-PLA2 activity was associated with occlusive coronary events, but this association became non-significant after adjustment for apolipoproteins. By contrast, the association of apoB with a range

of occlusive vascular diseases remained highly significant and changed little after adjustment for risk factors, including Lp-PLA2 (Parish *et al.*, 2010).

At this stage we should analyze the results of the well conducted and robust JUPITER (for primary cardiovascular events) and HPSCG (for secondary cardiovascular events) trials. Together they evaluate almost 37 000 (17 802 in the JUPITER and 19 037 in the HPSCG trials) individuals taking statins. Once patients were treated with effective statin doses, the relationship between Lp-PLA2 and CVD changed significantly. Given that Lp-PLA2 is mostly bound to apoB, it is not surprising that statins, did not only decreased LDL-C, but also Lp-PLA2 activity and mass concentration in both studies. This raises the questions whether Lp-PLA2 is indeed an independent risk factor for CVDs, whether it adds value in risk stratification and whether it alters treatment decisions. Of importance is that the reduction in CVD in these trials was not modified by baseline Lp-PLA2 values and any apparent relationship of Lp-PLA2 to CVD risk was ameliorated by the statins (Stein, 2012). This makes the measurements of Lp-PLA2 activity or mass concentration of very limited use in patients being treated with statins, meaning all patients after a cardiovascular event (as secondary prevention) and in a considerable proportion of patients in primary prevention.

The Ludwigshafen Risk and Cardiovascular Health study included 2298 patients with and 661 patients without angiographically confirmed CAD in a follow-up period of 8 years. Lp-PLA2 mass concentration was, independently from other traditional risk factors, associated with the risk for total and cardiovascular mortality (Kleber *et al.*, 2011).

The Long-term Intervention with Pravastatin in Ischemic Disease (LIPID) study, published in 1998, randomized 9014 patients after myocardial infarction or unstable angina to receive placebo or pravastatin 3 to 36 months after the initial event. From the study population, 7863 patients had baseline measurement of Lp-PLA2 activity, which was positively associated with CHD events in a follow-up period of 6 years, but not after adjustment for 23 baseline factors. Of note, Lp-PLA2 activity was reduced after 1 year on pravastatin by 16%. This reduction accounted for at least as much of the pravastatin treatment effect on reducing CHD dead and myocardial infarction, as did LDL-C reduction.

The investigators suggest that statins could reduce CHD risk in part by decreasing Lp-PLA2 activity (White *et al.*, 2013).

Lp-PLA2 and recurrent stroke

From the Northern Manhattan Stroke (NOMAS) study, mentioned above, were initially evaluated 655 individuals with incident ischemic stroke. From the moment of diagnosis, they were followed-up to determine predictors of stroke recurrence and prognosis. Lp-PLA2 activity was available for 467 participants at baseline and the median follow-up was 4.0 years. After adjustment for traditional cardiovascular risk factors, Lp-PLA2 activity continued to have an increased risk of recurrent stroke (Elkind *et al.*, 2009). Patients in the top quartile had approximately 2.5 times increased risk of stroke recurrence as those in the first quartile, event after adjusting for other risk factors.

A subset of 3201 participants enrolled in the Clopidogrel in High-Risk Patients with Acute Non-disabling Cerebrovascular Events (CHANCE) trial were investigated for Lp-PLA2 activity and its association with recurrent vascular events in the acute period (up to 90 days) after a transient ischemic attack or a minor stroke. Lp-PLA2 activity was associated with the primary endpoint, even after adjustment for traditional risk factors. Patients with Lp-PLA2 activity above 225 nmol/min/mL had a 30% increased short-term risk of recurrent vascular events (Lin *et al.*, 2015).

A more recent prospective study measured Lp-PLA2 mass concentration in 179 cases of acute cerebral infarction confirmed by magnetic resonance imaging. Lp-PLA2 mass concentration was significantly elevated in these individuals at presentation comparing to 149 individuals without acute cerebral infarction. However there was no long term follow-up despite the conclusion made by the investigators, that the serum level of Lp-PLA2 may be a predictive factor for the occurrence and recurrence of acute cerebral infarction (Wei *et al.*, 2017).

Current state of the art – the meta-analyses

Because of the inconclusive results of previous epidemiologic individual studies several recent meta-analyses tried to find consensus in assessing Lp-PLA2 activity or mass concentration as a predictor of cardiovascular diseases.

The LpPLA2 Studies Collaboration group performed the first relevant meta-analysis. They gathered information from 32 prospective studies including a total of 79 036 individuals and concluded that elevated Lp-PLA2 activity and mass concentrations were associated with increased risks for CHD events, ischemic stroke and vascular and non-vascular mortality in a continuous manner within a follow-up period of 6 years (Thompson *et al.*, 2010). However, they also conclude that since Lp-PLA2 is physically linked, through apoB, with LDL, the validity of statistical attempts to distinguish the effects of Lp-PLA2 from those of proatherogenic lipids remains uncertain. However, this meta-analysis included both participants with and without a history of vascular disease. A subgroup analysis among individuals without prior vascular disease showed no such associations. Furthermore, not all the assays used for the measurement of Lp-PLA2 mass perform similarly.

In the end of 2016, a second meta-analysis was published. It included fifteen prospective observational studies comprising a total of 30 857 participants with CHD. Lp-PLA2 was independently associated with long-term cardiovascular events in patients with CHD, but not with long-term all-cause mortality in patients with CHD. The investigators did not find the prognostic value of Lp-PLA2 for predicting cardiovascular events in patients with acute coronary syndrome. They further suggested that differences in methodology (manual versus automated ELISA assays) could in part explain the results (Li, Zhao, *et al.*, 2017). This meta-analysis was focused on the prediction of secondary cardiovascular events.

Later, in 2017 the same group published a meta-analysis for the general population (prediction of primary cardiovascular and ischemic stroke events). 12 studies comprising 44 187 participants were included. Individuals had an average age of 63 years, 48.2% of them were males, and the mean follow-up period was 10 years. Higher Lp-PLA2 activity or mass concentration was independently associated with an increased risk of CHD and ischemic stroke. Lp-

PLA2 activity was shown to be superior to Lp-PLA2 mass concentration for this prediction (Li, Wei, *et al.*, 2017).

Further on, a meta-analysis included 11 studies comprising a total of 20 284 Asian participants, of which 4045 individuals have had a transient ischemic attack or were after a first ischemic stroke and 16 239 were residents in the general population. The follow-up period varied from 30 days to 11 years. Patients with transient ischemic attack and/or primary ischemic stroke who exhibited elevated blood levels of Lp-PLA2 activity were at higher risk of developing recurrent vascular events (Tian *et al.*, 2017). For the general population Lp-PLA2 mass concentration, but not activity, was associated with the risk of stroke.

Worthful mentioning is yet another Asian meta-analysis that included 8 studies comprising 46 034 participants. Neither Lp-PLA2 activity or mass concentration were associated with ischemic stroke and Lp-PLA2 could not be considered a risk factor for ischemic stroke (Xia, Hu and Song, 2017)

Finally, the newest available meta-analysis is the most complete at this time. 22 studies (prospective cohort studies or randomized controlled trials with data on Lp-PLA2 mass concentration and/or activity at baselines) were included, comprising a total population of 157 693 individuals. After adjusting for multiple conventional risk factors, elevated Lp-PLA2 levels of both activity and mass were associated with increased risk of stroke (7% and 11%, respectively, for each standard deviation higher value of Lp-PLA2). For patients with baseline ischemic stroke, minor stroke, or transient ischemic attack, and with 1 standard deviation higher Lp-PLA2 activity, the risk of recurrent vascular diseases was increased by 16%. The stroke risk was not increased in patients with baseline CHD. The investigators proposed Lp-PLA2 levels as a factor to predict stroke in high-risk individuals (Hu *et al.*, 2019).

Genome variations

Due to these inconclusive results mentioned above we need a better understanding of the Lp-PLA2 function. Lp-PLA2 effects may substantially differ whether it is bound to LDL or to HDL particles. Lp-PLA2 activity and HDL levels are often inversely associated, but this is likely explained by the fact that small dense LDL (with high Lp-PLA2 activity) is more abundant in subjects with low levels of HDL. Studying how genetic variations change Lp-PLA2 expression, properties and functions may help understanding these unanswered questions. Heritability studies revealed that approximately 62% of the variation in Lp-PLA2 activity was deemed to genetic factors.

The first such study evaluated a Japanese population and identified an association between a missense mutation in exon 9 of the Lp-PLA2 gene (G^{994} to T) with myocardial infarction in men, but not in women (Yamada *et al.*, 1998). This nucleotide change results in the substitution of valine by phenylalanine at the amino acid residue 279, which induces loss of Lp-PLA2 catalytic activity. The authors proposed that the loss of function of Lp-PLA2 is associated with increased risk for myocardial infarction. Later on, it was discovered that such mutation is commonly found in East Asians and affects about 25% of the Japanese population. The prevalence of such mutation declines when moving towards the West, with intermediate frequencies in China and Korea, substantially lower frequencies in the Middle East, and an almost completely absence in the European population. Thus, the results from the association studies on this V279F variant and coronary artery disease have been inconclusive.

The pursuit for more mutations changing Lp-PLA2 expression lead to the publication of many genome-wide association studies. The first of such studies used data from 6668 Caucasian subjects included in the Framingham Heart Study and identified polymorphisms at several loci that may contribute to inter-individual variations in Lp-PLA2 activity and mass concentration. For Lp-PLA2 activity, polymorphisms at 4 independent loci reached genome-wide significance including *APOE/APOC1* region on chromosome 12, *CELSR2/PSRC1* on chromosome 1, *SCARB1* on chromosome 12 and *ZNF259/BUD13* in the *APOA5/APOA1* gene region on chromosome 11. For Lp-PLA2 mass, 12 single

nucleotide polymorphisms (SNPs) achieved genome-wide significance, all clustering in a region on chromosome 6p12.3 near the *PLA2G7* gene, which encodes for Lp-PLA2 (Suchindran *et al.*, 2010).

Grallert *et* al. who made a meta-analysis with 4 additional cohorts, achieving a population of 13 664 subjects, followed these investigations. This meta-analysis revealed the association of PLA2G7 loci variants with Lp-PLA2 mass, while genetic variants involved in lipid metabolism (APOC1, CELSR2, LDL, ZNF259, SCARB1) were strongly associated with Lp-PLA2 activity (Grallert *et al.*, 2012). It is possible that genes that alter lipoprotein metabolism may also alter the binding of Lp-PLA2 to subspecies of lipoproteins.

For Caucasians, the missense polymorphisms I198T and A379V were identified. In the later, alanine is substituted by valine and this mutation was shown to be involved in changes in Lp-PLA2 activity. However, some studies related this variant with an increase (Ninio *et al.*, 2004; Hoffmann *et al.*, 2009) and some with a decreased (Liu *et al.*, 2006) in Lp-PLA2 activity not allowing for generalizations. Further studies evaluated this mutation for associations with cardiovascular diseases and, again, the results were very contradictory, and mainly influenced by the ethnicity of the study population (A379V mutation was associated with increased severity of coronary atherosclerosis in a Taiwanese population and with decreased atherosclerotic risk in Caucasians). In addition, another SNP, the R92H was found to be associated with CAD risk in a USA population (Sutton *et al.*, 2008).

Finally, a more recent meta-analysis included 14 studies including 22 603 individuals (12 432 cases and 10 171 controls). The majority of the 11 studies examining V279F variants were based on Asian populations and there was a significant inverse association with clinical atherosclerosis. Consequently, lifelong lower Lp-PLA2 activity in this population due to V279F polymorphism is a protective factor for clinical atherosclerosis. Concerning A379V polymorphism 9 studies were examined, 6 consisting of Asian populations and 3 of Caucasians (however comprising 50% of the total population). No associations with clinical atherosclerosis were found. Concerning R92H polymorphism 6 studies were included for analysis. Caucasians comprised 57% of the population. There was a significant positive association of R92H polymorphism with clinical

atherosclerosis. This association has been translated into increased levels of plasma Lp-PLA2 (gain of function). Lastly, 4 studies were including for the I198T polymorphism. Caucasians comprised 50,7% of the population, the remaining were Asians. No significant association with atherosclerosis was found (Santoso *et al.*, 2017).

In conclusion, despite genome studies extensively evaluated several tenths of polymorphisms, their expression is concealed to specific ethnic populations and, depending on their effect on the Lp-PLA2 mass or activity, some can be considered as risk factors for atherosclerosis (e.g. R92H polymorphism), and some as protective factors (e.g. V279F polymorphism).

Lp-PLA2 inhibitors

Due to the immense body of evidence shown in this paper, pharmacological inhibition of Lp-PLA2 is a natural step to keep on researching Lp-PLA2 and its role in CVD.

In 2003 a potent and reversible inhibitor of Lp-PLA2 (darapladib) was identified and tested in human whole plasma, in Watanabe hereditable hyperlipidemic rabbit plasma and then in rats as an oral formulation (Blackie *et al.*, 2003). Darapladib was then tested in the diabetes mellitus and hypercholesterolemia porcine model of accelerated atherosclerosis. After a period of induction (4 weeks) Lp-PLA2 activity was increased by approximately 230% compared to the levels at baseline. Treatment with darapladib was initiated at 4 weeks and resulted in a significant reduction of Lp-PLA2 activity, lead to a reduction in the development of coronary atherosclerosis (treated lesions were less severe, contained fewer macrophages, and showed smaller necrotic cores), and inhibited the subsequent progression to advanced lesions (Wilensky *et al.*, 2008). We have to stress out, that cholesterol concentration is not affected by Lp-PLA2 inhibition.

Following the study in a porcine model, a trial using peroral darapladib at a dose of 160 mg during 1-year period on 330 humans with newly documented coronary disease angiographically was performed (Serruys *et al.*, 2008). Darapladib prevented the expansion of the necrotic core when compared to

patients receiving placebo, where, despite a high level of standard-of-care treatment, the necrotic core continued to expand.

After these promising results a double-blind trial, the STABILITY trial (STabilization of Atherosclerotic plaque By Initiation of darapLadIb TherapY), followed. It involved 15,828 patients with stable coronary heart disease defined as previous myocardial infarction, previous percutaneous coronary intervention or coronary-artery bypass grafting, or as multivessel coronary artery disease. In addition, the presence of at least one more risk factor was required (age of 60 years or older, diabetes mellitus requiring pharmacotherapy, HDL-C of less than 1,03 mmol/L, smoker, moderate renal dysfunction or polyvascular arterial disease). Patients were given either 160 mg of darapladib daily or a matching placebo. After a median follow-up of 3,7 years there was no significant effects on the primary end points (cardiovascular death, myocardial infarction, or stroke) or on all-cause mortality (Harvey D. White *et al.*, 2014).

Shorter after, another double-blind, placebo-controlled trial, the SOLID-TIMI 52 (The Stabilization Of pLaques usIng Darapladib-Thrombolysis In Myocardial Infarction 52) involving 13,026 patients revealed no reduction in major coronary events when darapladib was added to standard of care after an acute coronary syndrome (Donoghue *et al.*, 2015).

These disappointing results might be, in part, related with a lack of fully understanding of the Lp-PLA2 physiology and function. Performing multiple correlation studies before developing this knowledge, might have led to misconceptions that were further passed on the literature.

Atherosclerosis

Before assessing LpPLA2 in our study population we have to do a review on the current proposed concepts for the pathogenesis and development of atherosclerosis leading to the associated clinical syndromes. Firstly, atherosclerotic lesions are ubiquitous in our society and the very first stages of this process can be observed at very young ages. This means, that most of these lesions are asymptomatic and may never cause clinical manifestations. Secondly, being ubiquitous, it is not a surprise, that atherosclerosis is the main cause of death and premature disability worldwide. Thirdly, the thought of this process to be caused by mere fatty deposits causing obstructions in the arterial bed is long ago completely refuted. Nowadays, atherosclerosis is seen as a complex and chronic inflammatory process involving highly specific cellular and molecular interactions that may culminate with the rupture of a plaque and the development of an acute clinical event, such as myocardial infarction, stroke, or sudden cardiac death. This model is also called the response-to-injury hypothesis and LpPLA2 definitely plays a considerable role in this process. Fourthly, atherogenesis occurs over a period of decades and the growth of plaques is not linear, rather there are periods of relative quiescence and periods of rapid evolution. This heterogeneous expression of the disease can be in part explained by exposure to risk factors such as smoking, hypertension, diabetes mellitus, and dyslipidemias.

It should be emphasized, that atherosclerosis is the leading cause of death and disability worldwide and it is more prevalent in developing countries.

Fatty streaks

The “fatty streak” (sometimes also called xanthoma) represents the earliest lesion of atherosclerosis. This lesion appears at very young age and is present in virtually all children older than 10 years, regardless of genetic, clinical, or dietary risk factors. Although “fatty streaks” can evolve into atherosclerotic plaques, not all progress. They seem to arise from focal endothelial dysfunction or injury within a morphological intact endothelium and are characterized by local increases in the amount of lipoproteins that bind to

components of the extracellular matrix, such as glycosaminoglycans, proteoglycans (previously known as mucopolysaccharides), and glycoproteins. This can impair lipoprotein function and slow down the ability to clear lipid particles from the arterial wall. By staying longer time, lipids are prone to oxidative modifications, yielding hydroxiperoxides, lysophospholipids, oxysterols and aldehytic breakdown products of fatty acids and phospholipids. These products trigger a local inflammatory response characterized by leukocyte recruitment.

Adhesion molecules such as selectins, integrins, and members of the immunoglobulin superfamily, are expressed on the surface of the arterial wall in regions of dysfunctional or injured endothelium. They capture leukocytes on the arterial wall and its expression is regulated not only by modified products from lipoproteins, but also by cytokines, such as interleukin-1 (IL-1) and tumor necrosis factor α (TNF-α). Chemoattractant cytokines then direct the migration of leukocytes into the arterial wall.

Normal laminar blood flow in a pulsatile, ordered manner increases the production of nitric oxide (NO) from L-arginine by endothelial cells through the action of NO synthase. NO is known by its vasodilator properties, by increasing cyclic guanosine monophosphate (cGMP) levels, but it also acts as a local anti-inflammatory autacoid (i.e. a local hormone), limiting the expression of local adhesion molecules and thus, inhibiting leukocyte adhesion and activation, platelet aggregation and smooth muscle proliferation.

On the other hand, disturbed blood flow decreases the production of NO and is encountered in branch points, making these predisposed sites for the development of atherosclerotic lesions (e.g. the carotid bifurcation). The presence of atherosclerotic risk factors also impairs NO vasodilator properties.

The recruitment of leukocytes is, thus, a highly organized process that can be disrupted or better, further potentiated, by free cholesterol and oxidized LDL particles, major components of the atherosclerotic plaque. Myeloperoxidases released from these inflammatory cells yield chlorinated species such as chlorotyrosyl moieties, which can also chlorinate HDL particles. This impairs the reverse cholesterol transport resulting in the inability to clear lipid particles

from the “fatty streak”. The oxidative stress is potentiated by the LpPLA2, as explained above, and further developed in the discussion section.

Finally, platelets also play a crucial role in atherogenesis, being important, not only in the formation of thrombi (*atherothrombosis*), but also in perpetuating the inflammatory environment by secreting several vasoactive chemokines and cytokines, such as CD40L, thrombospondin, and PAF (Pant *et al.*, 2014). Furthermore, they promote fibrosis of the atherosclerotic plaque by releasing platelet-derived growth factor (PDGF) and transforming growth factor β (TGF-β), and increase smooth muscle migration and proliferation (acting on protease-activated receptors). They are recruited in large amounts once microscopic breaches in endothelial integrity occur. These breaches expose highly thrombogenic extracellular matrix forming platelet-rich microthrombi. Oxidized LDL also stimulates platelet aggregation in response to adenosine diphosphate (ADP) and thrombin.

The inflammatory process is further amplified by the release of large amounts of reactive oxygen species (ROS), such as superoxide and hydrogen peroxide, in all stages of atherosclerosis. ROS have multiple important consequences. They denaturate NO, activate platelets, induce smooth muscle proliferation, oxidize proatherogenic lipids including LDL, and act as chemoattractants. Oxidized LDL then induces the release of more proinflammatory cytokines, activates proteases and leads to cell apoptosis, completing a vicious cycle of *inflammation induced by inflammation*.

The process of diapedesis

After recruitment of leukocytes into the arterial wall the transendothelial migration phase follows. This process is also known as diapedesis. It requires rapid disassembly of the leukocyte cytoskeleton on the apical surface of the endothelium and its reassembly on the abluminal side (Muller, 2003). Junctional adhesion molecules, platelet-endothelial-cell adhesion molecule-1 (PECAM-1, CD31), CD99, vascular-endothelial cadherin, cadherin 5 and CD144 are the responsible molecules aiding this process. Each one of them has a crucial role in different steps of diapedesis, and they have counterparts (sometimes the same

molecule) at the leukocyte surface. The endothelial tight junctions are breached by transmigrating leukocytes, but the vascular integrity is maintained.

The formation of foam cells

Once in the subintimal space, monocytes mature into macrophages, start to engulf oxidized LDL and transform into foam cells. This process is mediated by endocytosis through scavenger receptors and it is not regulated by LDL receptors as explained by the fact that individuals with homozygous familial hypercholesterolemia have also foam cells in their arterial walls.

Foam cells and reverse cholesterol transport provided by intact HDL may delay the formation of an atherosclerotic plaque. Once the amount of lipid entering the arterial wall exceeds that removed, foam cells accumulate and expand the intimal lesion. Some of them may then die by apoptosis resulting in the formation of a necrotic core, a feature of more advanced atherosclerotic plaques.

The atheroma

In predisposed individuals and by continuous exposure to the risk factors for atherosclerosis (e.g. dyslipidemias, hypertension, diabetes mellitus, smoking, obesity, inactive lifestyle, age) the formation of the atheroma (here synonymous with atheromatous or atherosclerotic plaques) ensues.

Morphologically, atheromas are patchy white to yellow raised lesions up to 1.5 cm in diameter that can coalesce to form larger masses. As referred earlier, most of the lesions are found at arterial bifurcations and they are more common in the infrarenal aorta, coronary arteries, popliteal arteries, internal carotid arteries, and in the circle of Willis.

The main components of the atherosclerotic plaques are 1) cells, including macrophages, T lymphocytes, and smooth muscle cells; 2) extracellular matrix, including collagen, elastic fibres, and proteoglycans; and 3) intracellular and extracellular lipid depositions. These components form a complex organized structure covered by a fibrous cap under which there is smooth muscle cell migration and proliferation induced by cytokine production (by macrophages, foam cells, lymphocytes, and by smooth muscle cells themselves). Of crucial importance are IL-1 and TNF-α, which induce the local production of growth and

differentiation factors, such as PDGF and TGF-β. This feature marks the transition from a simple accumulation of foam cells to a fibrofatty lesion.

Apoptosis of foam cells releases cholesterol to the vessel wall and, more importantly, prothrombotic molecules and metalloproteinases.

Not all recruited macrophages however exert in a proinflammatory fashion. As mentioned earlier, the main goal of inflammatory cells recruitment is to resolve the lipid accumulation at the arterial wall. Because of this notion, some macrophages will express an M2-like phenotype, favoring the resolution of inflammation by secreting factors such as TGF-β. While others, driven by binding of modified (e.g. oxidized) LDL to pattern-recognition receptors (e.g. Toll-like receptors) will express an M1-like phenotype, secreting proinflammatory cytokines (e.g. TNF-α and IL-1β), reactive oxygen species, myeloperoxidases, etc. Lp-PLA2 expression is increased by M1 (but not by M2) macrophages, which emphasizes the important role of inflammation in the regulation of Lp-PLA2 secretion.

Lymphocytes appear in the atherosclerotic lesion with the formation of foam cells. T helper 1 lymphocytes secrete proinflammatory cytokines (e.g. TNF-α and interferon-γ, INF- γ), while regulatory T cells, and possible B cells, have an anti-inflammatory function. Increased levels of Lp-PLA2 and lysophosphatidylcholine are found in thin-cap fibroatheromas and ruptured plaques but are almost absent in stable lesions.

The formation of the necrotic core

The development of a necrotic core marks the transition from a xanthoma to a fibroatheroma. The term necrotic core may be misleading (at least in the initial stages) because it is characterized by the presence of acellular, lipid-rich material in the intima and not necessarily by the morphological presence of death cells from previously living tissue or cells. These become only part of the necrotic core when the removal of apoptotic remnants by M2 macrophages is impaired. If these M2 macrophages are not quickly able to remove apoptotic remnants, they accumulate in the M2 macrophage endoplasmatic reticulum promoting their own apoptosis. This leads to the release of lipids, pro-inflammatory mediators and metalloproteinases. The apoptotic remnants

undergo secondary necrosis. As the plaque grows, apoptotic foam cells and smooth muscle cells are mainly seen at its margins, whereas at the core there is a necrotic mass of debris, containing large amounts of oxidized phospholipids (because of low clearance and auto-oxidation). These contribute to the expansion of the necrotic core, neovessel injury, intraplaque hemorrhages and plaque instability.

Angiogenesis

As atherosclerotic lesions advance, abundant plexuses of microvessels develop in connection with the artery's adventitial vasa vasorum. They provide an alternative entry pathway for the recruitment of more inflammatory cells, increase the flow of nutrients and oxygen, and may thereby promote plaque progression and remodeling. Like neovessels in the diabetic retina, neovessels of the atherosclerotic plaque are very fragile, lack supporting cells and easily leak resulting in intraplaque focal hemorrhages, which can provoke thrombosis in situ, yielding local thrombin generation, which in turn can activate smooth muscle and endothelial cells through ligation of protease-activated receptors. Once again, there is a perpetuation of the inflammatory vicious cycle because specialized macrophages (of the hemoglobin-induced phenotype) are recruited into the plaque to remove the haptoglobin-bound hemoglobin protecting the plaque against the cytotoxic effects of free hemoglobin. Individuals with impairment of the haptoglobin function (e.g. caused by the common Hp2-2 genotype) have an increased inflammatory response to intraplaque hemorrhage, which may increase the risk for cardiovascular events in this group (Finn *et al.*, 2012).

The inflammatory process and relative hypoxic conditions within the atherosclerotic plaque induce the expression of angiogenic factors, such as vascular-endothelial growth factor (VEGF), hypoxia-inducible factor, angiopoietin, PDGF, etc. (Camaré *et al.*, 2017). Lipids (e.g. cholesterol), oxidized lipids (e.g. phospholipids), and oxidized lipoproteins (e.g. oxidized LDL) promote the release of angiogenic factors, in part, explaining why neovascularization is not halted if the oxygen delivery is restored.

Fibrosis and calcification

The migration and proliferation of smooth muscle cells from the media into the intima of the arterial wall, where the necrotic core is located, is associated with the secretion of collagen, elastin, and proteoglycans. These synthetic smooth muscle cells have abundant rough endoplasmatic reticuli and Golgi complexes, and may even lack contractile proteins (making them difficult to identify using routine methods that detect smooth muscle α–actin and smooth muscle myosin heavy chain). Initially, the newly formed connective tissue is mainly loose and fibrocellular. Then, it is replaced by collagen-rich fibrous tissue, which, as the plaque advances, becomes its main component. This relative paucity of smooth muscle cells in advanced atheromata may result from the predominance of cytostatic mediators such as TGF-β and INF-γ, and from the apoptosis of smooth muscle cells.

Apoptotic cells, extracellular matrix, and necrotic core material may act as nidus for microscopic calcium granules, which subsequently expand to form larger lumps and plates of calcium deposits (Bentzon *et al.*, 2014). Calcification of the atherosclerotic plaque shares most of the features of bone mineralization; hence many proteins found in bone are also located in the plaque (e.g. osteocalcin, osteopontin, and bone morphogenic proteins).

The evolution of the atherosclerotic plaque results from the balance between 1) leukocytes and lipoproteins entering and exiting the lesion, 2) cell proliferation and cell death, and 3) extracellular matrix production and remodeling, calcification, and neovascularization. Multiple and often competing signals regulate these various events that are continuously influenced by the exposure to risk factors for atherosclerosis. The summarized schematic process is depicted on Figure 5.

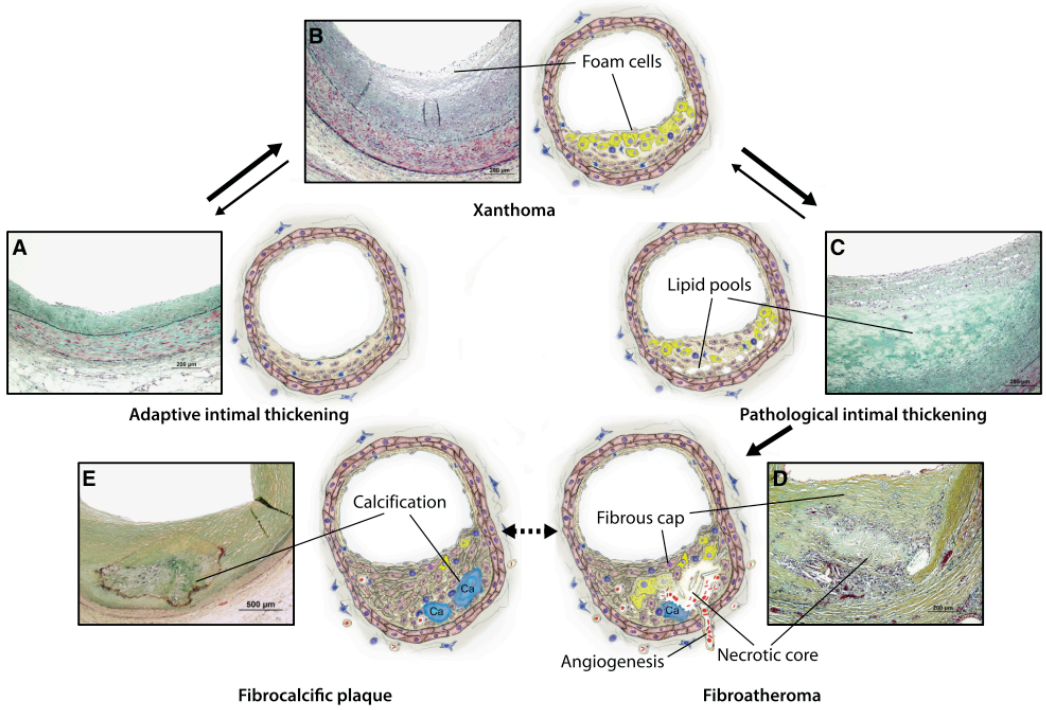

Figure 5 - Histological classification according to Vermani (Virmani *et al.*, 2000)

Plaque rupture

Before revising the mechanisms leading to plaque rupture, it is important to keep in mind, that until the burden of the atherosclerotic plaque does not exceed approximately 40% of the area encompassed by the internal elastic lamina, the plaque does not reduce the arterial lumen. In other words, in most of its life history the growth of the atheroma is abluminal (outwards), preserving the caliber of the lumen. This phenomenon accounts in part for the underestimation power of arteriografies in the diagnosis of atherosclerotic plaques.

If the growth of the plaque is steady and chronic, the lumen of the vessel eventually becomes stenotic, leading to the development of the clinical syndromes of stable angina pectoris or intermittent claudications of the lower extremities. The repeated local hypoxic events caused by the inability to increase the delivery of oxygen during increased physical work promotes the formation of collateral vessels (for example in the myocardium), mitigating the consequences of an acute occlusion. If, on the other hand, the growth is fast and erratic, there is no time for the formation of collateral vessels, and the fibrous cap of the atherosclerotic plaque ruptures, exposing the highly thrombogenic collagen and

tissue factor, and leading to thrombosis of the vessel with devastating consequences (such as acute coronary syndromes and sudden cardiac death).

A *ruptured plaque* is defined as a "plaque with deep injury with a real defect or gap in the fibrous cap that had separated its lipid-rich atheromatous core from the flowing blood, thereby exposing the thrombogenic core of the plaque" (Schaar *et al.*, 2004). No other confusing terminology, such as disruption or fissuring should be used. When thrombosis is present, but there are no microscopic signs of plaque rupture, the term *plaque erosion* is used (Quillard *et al.*, 2017). This erosion is characterized by the loss and/or dysfunction of the endothelial cells leading to thrombosis without plaque rupture. Vasospasm may be involved in the development of plaque erosions.

One of the most important factors leading to plaque rupture and consequent thrombosis is the thickness of the fibrous cap. Because thinner fibrous caps are more prone to rupture the term *thin-cap fibroatheroma* (TCFA) was introduced, denoting the atherosclerotic plaques with the highest risk for rupture. In fact, TCFAs are likely precursors of the majority of fatal coronary plaque ruptures (Falk *et al.*, 2013). Fibrous caps become thin with the loss of smooth muscle cells and collagen, which is potentiated by enzymes secreted by foam cells, such as proteases, plasminogen activators, and metalloproteinases.

In summary, the plaques with the highest risk for rupture, the more vulnerable, or the most thrombosis-prone plaques are the ones with a large acellular, lipid-rich necrotic core with an overlying fibrous cap infiltrated by inflammatory cells and diffuse calcification. The schematic representation of plaque rupture and healing is depicted at Figure 6. Finally, a schematic representation of the entire atherosclerotic process, including the representation of Lp-PLA2 molecule is shown in Figure 7.

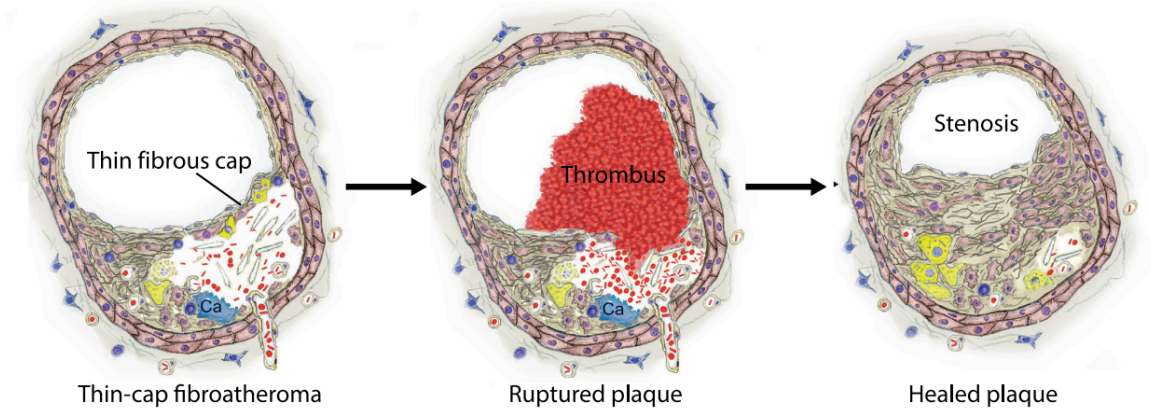

Figure 6 - Atherosclerotic plaque rupture and healing (Bentzon *et al.*, 2014)

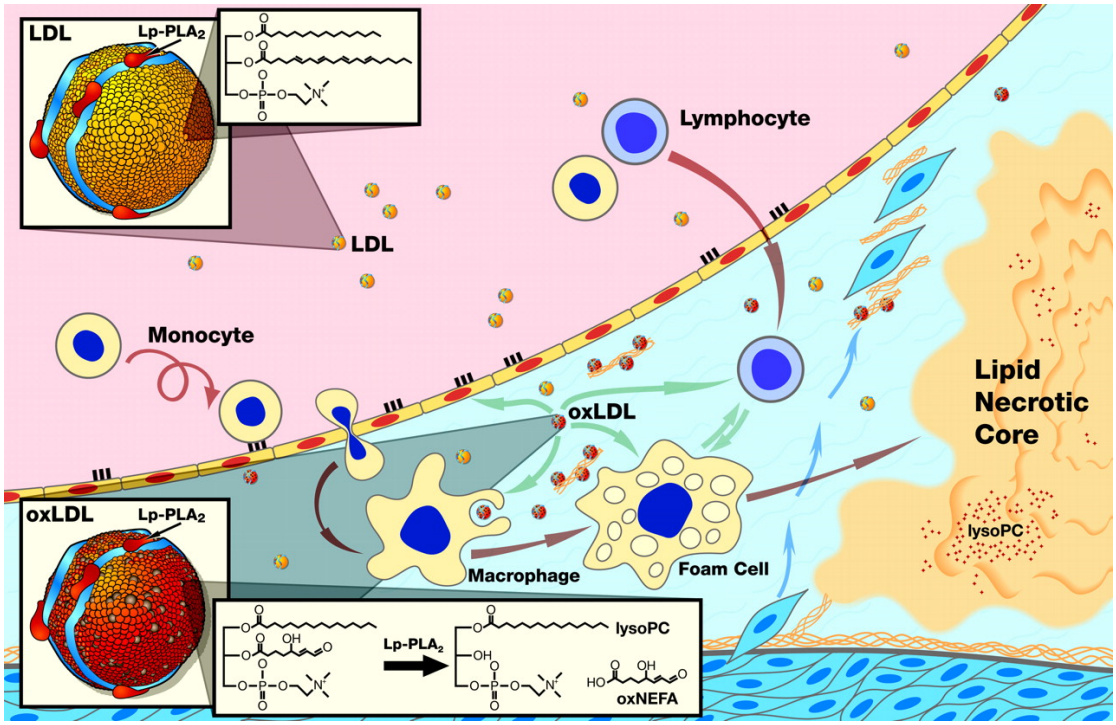

Figure 7 – Schematic representation of the proposed pro-atherogenic mechanism of Lp-PLA2 in the vessel wall. Lp-PLA2 binds to apoB on LDL, which delivers Lp-PLA2 to lesion-prone segments. Subsequent LDL oxidation leads to formation of truncated phospholipid in the *sn*-2 position, which is susceptible to enzymatic hydrolysis by Lp-PLA2. This results in the generation of lysophosphatidylcholine (lysoPC) and oxidized nonesterified fatty acids (NEFA), proinflammatory mediators that are also cytotoxic to macrophages and facilitate the formation of a necrotic lipid core in advanced atherosclerotic lesions (Zalewski and Macphee, 2005).

Aortic stenosis and transcatheter aortic valve implantation

Aortic stenosis, the narrowing of the aortic valve opening, is a severe and very common primary valve disease leading to surgery or catheter intervention in Europe and North America. Its prevalence increases with age affecting 1,3% of individuals aged 65-74 years, and 2,8-4,6% of patients >75 years (Iung and Vahanian, 2014). Pathophysiologically, degenerative calcific aortic stenosis, like atherosclerosis, represents an active, proliferative and inflammatory process caused by endothelial damage due to mechanical stress and lipid penetration leading to a progressive obstruction of the left ventricle outflow due to fibrosis, leaflet thickening and, finally calcification (Joseph *et al.*, 2017).

This progressive obstruction is initially asymptomatic (over decades), but once the symptoms of dyspnea, angina, heart failure or syncope develop, 50% of patients die within 2-3 years. This makes early diagnosis and intervention of paramount importance. However, quite often, the diagnosis is made upon the development of symptoms, i.e. in an already advanced age, and in individuals otherwise at high surgical risk due to other concomitant comorbidities. In these situations transcatheter aortic valve implantation (TAVI) is recommended (Baumgartner *et al.*, 2017). Of note, asymptomatic patients with severe aortic stenosis diagnosed by echocardiography are also indicated for intervention.

Aortic stenosis is classified into 4 categories taking into consideration the flow through the valve, the transvalvular gradient and the ejection fraction of the left ventricle. Low-flow, low-gradient aortic stenosis with reduced ejection fraction is the typical phenotype for elderly people and may be secondary to left ventricular systolic dysfunction or diminished ventricular volumes in a hypertrophied, stiffed left ventricle. They have the worse prognosis, even after surgical aortic valve replacement (SAVR) or TAVI and, thus, require further investigations (e.g. distinguish between true and pseudo-stenosis) before choosing the adequate treatment modality.

Currently, TAVI is only indicated in patients who are not suitable for SAVR. Nevertheless, recent clinical trials show a possible superiority over surgery, if the transfemoral (and not the apical) access is used (Reardon *et al.*,

2017), in elderly patients and even in adults at low to intermediate risk (Gargiulo *et al.*, 2016). The main reasons for these positive results are the technological advancements in the prostethic valves aiming at reducing local complications such as paravalvular leaks, valve dislocation or embolization.

Balloon valvuloplasty (BV) is used for palliation in patients in whom aortic valve replacement cannot be performed because of comorbid conditions. It can also be used as a bridge to SAVR or TAVI in unstable patients. Of note, the hemodynamic benefit of VA is only transient, lasting for only about 6 months (Ben-Dor *et al.*, 2013).

In summary, the 2017 ESC/EACTS Guidelines for the management of valvular heart disease (Baumgartner *et al.*, 2017) recommend SAVR in patients at low surgical risk - assessed by the surgical risk calculator from the Society of Thoracic Surgeons or from the European System for Cardiac Operative Risk Evaluation (STS or EuroSCORE II <4% or logistic EuroSCORE I <10%) and no other risk factors not included in these scores, such as frailty, porcelain aorta, or sequelae of chest radiation. The decision between SAVR and TAVI for any other patient not included in this group should be made by the Heart Team, being TAVI preferred in elderly patients suitable for transfemoral access.

Diabetic dyslipidemia

Diabetic dyslipidemia is a set of lipid abnormalities found in people with diabetes mellitus that are metabolically interrelated. It is typically characterized by an increase in plasma concentration of triglycerides (TG), decreased concentration of HDL (HDL-C) and a qualitative change in the LDL fraction due to increased amounts of small and dense LDL (Arca, Pigna and Favoccia, 2012). This triad can even be detected years before the diagnosis of type 2 diabetes mellitus (T2DM). There is increasing evidence that patients with T2DM have increased liver synthesis of large VLDL particles (Adiels *et al.*, 2005). VLDL production rate is the main determinant of TG concentration in plasma.

In the adipose tissue, insulin suppresses lipolysis by inhibiting the activity of hormone sensitive lipase, the enzyme responsible for the mobilization of free fatty acids from stored TGs. This means that insulin regulates the amount of circulating fatty acids and fails to inhibit the hormone sensitive lipase in states of insulin resistance. The ensuing higher availability of free fatty acids leads to a decrease degradation of apoB causing an overproduction of VLDL by the liver (the synthesis of VLDL starts with the assembling of TGs with apoB).

In addition, elevated free fatty acids can impair the activity of lipoprotein lipase (by detaching it from the endothelium), resulting in a decreased clearance rate of VLDL. Both, the overproduction and the decreased clearance of VLDL contribute for the hypertriglyceridemia seen in diabetic patients. Lipoprotein lipase is also inhibited by apoC-III. The expression of apoC-III is induced by glucose and inhibited by insulin. Thus, high apoC-III concentrations prevent the clearance of TG-rich lipoproteins and remnants, resulting in a further elevation of TGs.

Not only due to the features described above, but also due to changes in incretins secretion observed in states of insulin resistance, there will be an increase in postprandial TG-rich chylomicrons. These compete for the same clearance pathway as VLDL, which means that they cannot be effectively removed. In summary, T2DM is characterized by both fasting and postprandial hypertriglyceridemia.

Hypertriglyceridemia stimulates the activity of cholesteryl ester transfer protein (CETP), which facilitates the transfer of TG from TG-rich lipoproteins to HDL and LDL. TG-enriched HDLs are subjected to increased catabolism, which decreases their half-life and, thus their concentration. Decreased concentrations of HDL may result or exacerbate abnormal glucose homeostasis. TG-enriched LDLs are hydrolyzed by lipoprotein lipase or hepatic lipase, which results in a reduction of the particle size, giving rise to the small dense LDLs characteristic for diabetic dyslipidemia (Wu and Parhofer, 2014).

The small dense LDL particles are more atherogenic than normal sized LDL. They are more prone to be removed by scavenger receptors and increase the binding affinity of Lp-PLA2 for apoB. Note that the total amount of LDL particles does not necessarily reflect the LDL concentration (LDL-C), because the amount of cholesterol each particle carries varies. If we would want to measure the number of particles, apoB concentration would be the surrogate marker for the total number of atherogenic lipoprotein particles since each LDL, VLDL, intermediate-density lipoprotein and lipoprotein (a) contains one apoB.

Because Lp-PLA2 binds preferentially to these small dense LDL particles, which are characteristic for DM, improving glycemic control may reduce Lp-PLA2 activity. It has been estimated that the amount of Lp-PLA2 in small, dense LDL or electronegative LDL is 5 to 10 times higher than in normal-sized LDL particles. Furthermore, the fraction of Lp-PLA2 bound to HDL is reduced (there is a relatively lower fraction of HDL particles in diabetic dyslipidemia) and improving glycemic control will increase the proportion of Lp-PLA2 bound to HDL, probably potentiating its atheroprotective properties. Finally, the amount of lysophosphatidylcholine in circulation LDL is also increased in diabetic patients (Sonoki *et al.*, 2009) and this may be related to the increase Lp-PLA2 activity.

DM *per se* increases CVD risk two to four-fold (Cho *et al.*, 2002). This risk is strongly determined by the presence of end-organ damage (including nephropathy, neuropathy and retinopathy). However, most of the times, concomitant comorbidities, such as hypertension, dyslipidemia, abdominal obesity, and non-alcoholic fatty liver disease, increase this risk many folds.

Objectives

a) to determine whether Lp-PLA2 mass concentration is increased in diabetic geriatric patients comparing to non-diabetic geriatric patients
b) to evaluate whether Lp-PLA2 concentrations change after a clinical intervention (manipulation with heart valves)
c) to determine whether these changes vary between diabetic and non-diabetic geriatric patients
d) to discuss the current expert views on this molecule

Methods, statistical analysis

We performed a cross-sectional analysis on 44 geriatric patients aged 79.6 ± 5.6 years that had undergone TAVI or ballon valvuloplasty (BV) for the treatment of severe aortic stenosis. The patients were admitted to the Cardioangiologic clinic of the University Hospital of Hradec Králové, Czech Republic, between January 2009 and January 2011. The subject characteristics are described on Table 1. Most of the patients suffered from multiple comorbidities, but the reason for admission was related to their heart problems. Two of them had a BV before TAVI (one 8 months before and the second patient 1 month before); one of them needed BV 3 months after TAVI due to a perivalvular leak. The sample was then further divided into diabetic and non-diabetic patients. The local ethical committee approved the study and all patients gave a written consent.

Total of subjects enrolled	44
Male	19 (43.2 %)
Age (years)	79.6 ± 5.6
BMI (kg/m^2)	27.6 ± 4.7
Systolic BP (mmHg)	133 ± 18
Diastolic BP (mmHg)	75 ± 10
Total patients after TAVI	35
a) apical route	8
b) femoral route	27
Total patients after BV	9
Total patients with DM	21 (47.7 %)
a) on insulin therapy	9
b) on per oral antidiabetics	3
c) DM controlled by diet	9

Table 1 – Characteristics of subjects

Measurements are expressed as mean ± standard deviations or as the absolute number. BMI = body mass index, BP = blood pressure, TAVI = transcatheter aortic valve implantation, DM = diabetes mellitus, BV = balloon valvuloplasty.

Plasma Lp-PLA2 mass concentrations were obtained using an enzyme-linked immunosorbent assay (ELISA) kit (Cloud-Clone Copr., USA) before and 3 days after the procedure. The recommended instructions from the producer were thoroughly followed. Intraassay variations were <10%, and interassay variations were <12%. The detection range of the assay was 0.625-40ng/mL. Colored emission was then measured using the Multiskan Microplate Spectrophotometer from Thermofisher Scientific.

Succinctly, a specific antibody against Lp-PLA2 is applied and fixed to the internal side of the microtitre wells. The blood sample is then applied and Lp-PLA2 binds to the antibody. After washing out unbounded components from the microtitre wells, a second antibody (labeled with biotin) against Lp-PLA2 is added. A second washout follows before adding the enzyme horseradish peroxidase labeled with avidin. Avidin reacts with the biotin forming an avidin-biotin complex. After a third washout an indicator is added. The indicator changes color in the presence of horseradish peroxidade. The intensity of the

color is then proportional to the amount of the horseradish peroxidase in the complex, and consequently to the amount of Lp-PLA2 in the sample. It is measured by a special photometer (an ELISA reader) and analyzed by an appropriate software, which evaluates the relation between the color emission and the amount of Lp-PLA2 in the sample in relation to a standard calibration samples.

Fasting blood samples were obtained immediately before and then 3 days after the cardiac procedure from every patient. Blood was drawn and centrifuged immediately. The serum was then aliquoted and stored at -80°C. Total cholesterol, HDL-C and TGs were measured by enzymatic methods; LDL-C was measured by routine methods.

For statistical analysis the open source software PSPP (Free Software Foundation, Inc., USA) was used. A *p*-value less than 0.05 denoted the presence of a statistically significant difference. For comparison of the Lp-PLA2 mass concentration before and after the valve intervention, the paired *t*-test was used. The correlations between the levels of Lp-PLA2 mass and other variables were calculated using the Person's correlation coefficient. To calculate the differences between 2 groups (diabetic and non-diabetic, TAVI and BV), independent (unpaired) *t*-tests were used.

We included a control group using 48 healthy subjects aged 82.9 ± 4,2 years, living at a nursing home. The average Lp-PLA_2 mass concentration on the control group was 788.23 ± 210.95 ng/ml. The age of the subjects within study groups did not statistically differ. Moreover, we provided control values for age-matched healthy individuals, and thus normalization according to the age of the subjects was not included into the results.

Results

Concerning demographic and clinical characteristics at baseline, we examined 44 patients aged 79.6 ± 5.6 years (average age ± standard deviation). From those 43.2% were males. Overall average BMI (calculated as the actual weight in kilograms divided by the squared height in meters) fell into the category of overweight (27.6 ± 4.7). Measured total cholesterol was 4.16 ± 1.17 mmol/l, LDL-C 2.48 ± 0.96 mmol/l, HDL-C 1.25 ± 0.44 mmol/l, and TG concentration was 1.36 ± 0.66 mmol/l as depicted on table 2.

Total cholesterol (mmol/l)	4.16 ± 1.17
HDL-C (mmol/l)	1.25 ± 0.44
LDL-C (mmol/l)	2.48 ± 0.96
Triglycerides (mmol/l)	1.36 ± 0.66
CRP (mg/l)	20.8 [0.5 – 75.1]
Lp-PLA_2 (ng/ml)	1320 ± 342
a) Lp-PLA_2 on TAVI group	1296 ± 358
b) Lp-PLA_2 on BV group	1413 ± 268

Table 2 – Results of relevant parameters in the study population

Measurements are expressed as mean ± standard deviations or as the absolute number, with the exception of CRP (median [range]). HDL-C = high-density lipoprotein concentration, LDL-C = low-density lipoprotein concentration, CRP = C-reactive protein, Lp-PLA2 = lipoprotein-associated phospholipase A2, BV = balloon valvuloplasty.

According to the 2019 ESC/EAS guidelines for the management of dyslipidemias, aortic valve sclerosis (calcification of the aortic leaflets without significant transvalvular gradient) is associated with an increased risk CAD even in the absence of increased risk profiles. However, statin therapy in these patients failed to reduce the clinical progression of aortic stenosis, and a recommendation against the initiation of lipid-lowering therapy in patients with aortic valve stenosis without CAD was issued. Even though the lipid profile

measured in our study may be considered to be within normal range, the study population suffered from severe aortic stenosis, many of the patients had suffered a myocardial infarction in the past, had a medical history of hyperlipidemia and 62% of them were on statin therapy. They could be then considered to be at high or very-high cardiovascular risk, and the measured LDL-C would be above the recommended target (>1.8 or >1.4 mmol/l, respectively).

From a total of 44 patients, 21 (47.7%) had a medical history of diabetes mellitus and were either on insulin therapy (N=9), on peroral antidiabetics (N=3) or not medicated (N=9).

The main objective of study was the assessment of Lp-PLA2 mass concentration in a set of geriatric patients. However, we followed up the study population during 2 years and we monitored for in-hospital periprocedural combined safety endpoints (i.e. all-cause mortality, major stroke, myocardial infarction, life-threatening bleeding, major vascular complications, and acute kidney injury). One patient (3%) died due to a cerebrovascular event after TAVI. During the two-year follow-up, five (11%) deaths were noted (2 from stroke, 2 from infectious complications and 1 in a car accident).

Lp-PLA2 mass concentration was significantly correlated with relevant cardiovascular risk factors (as show in Table 3). As predicted, a strong correlation was found between Lp-PLA_2 mass and LDL-C (coefficient of 0.40, P = 0.01) as LDL particles are the main carrier for Lp-PLA2 (Figure 9). However, the strongest correlation was observed between Lp-PLA2 mass concentration and TG concentration (coefficient of 0.43, P = 0.01). The enzyme mass concentration was not correlated with body mass index (BMI), neither with HDL-C.

Parameters	Coefficient	*P*
Age	-0.14	NS
Total cholesterol	0.35	*0.04*
HDL-C	-0.14	0.41
LDL-C	0.40	*0.01*
Total cholesterol to HDL-C ratio	0.37	*0.03*
LDL-C to HDL-C ratio	0.36	*0.03*
Triglycerides	0.43	*0.01*
BMI	-0.30	0.87

Table 3 – Pearson correlation between Lp-PLA2 and other parameters

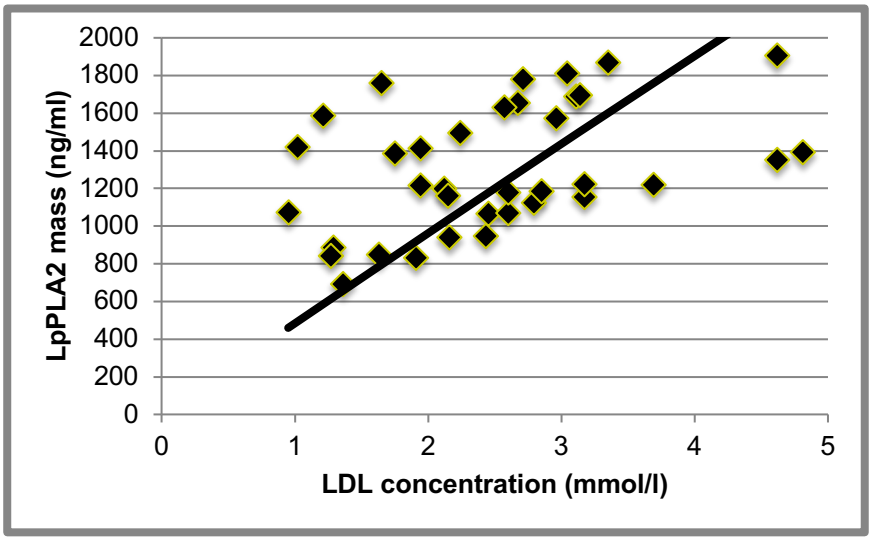

Figure 9 – Pearson correlation between Lp-PLA2 and LDL-C

In the present study, the population was divided into diabetic and non-diabetic patients to compare Lp-PLA2 mass concentration between them. The diagnosis of diabetes mellitus was taken from the available medical record. We did not take into account whether diabetic patients were on diet, peroral antidiabetics or on insulin therapy. Using the independent samples *t*-test, we concluded that Lp-PLA2 mass concentration is statistically significantly increased (p=0.03) in the diabetic group (1433.75 ± 353.58 ng/ml) comparing to the non-diabetic group (1215.7 ± 301.64 ng/ml). These results were obtained before the intervention at the aortic valve and they are shown in Figure 10.

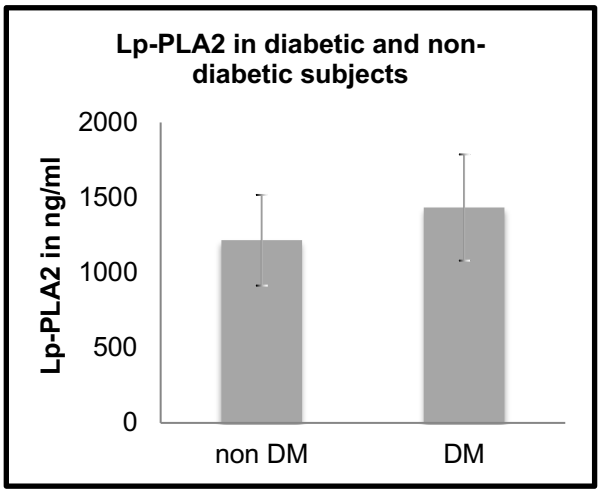

Figure 10 - Lp-PLA$_2$ mass was significantly increased on diabetic subjects

DM / non DM = patients with/without diabetes mellitus

There were no statistical differences between the two groups concerning total cholesterol concentration, HDL-C, LDL-C, TG, BMI, and systolic and diastolic blood pressures. There were no statistical differences concerning the level of Lp-PLA2 mass concentration after the intervention at the aortic valve between the diabetic and non-diabetic groups, meaning that the observed results after the procedure did not change differently between diabetic and non-diabetic individuals.

It was then hypothesized that the Lp-PLA2 mass concentration rises after the mentioned interventions at the aortic valve, using TAVI (either through the apical or the transfemoral approach) or BV. Using paired *t*-tests we confirmed this increment, obtaining a statistically significant $p<0.01$ on both groups: 1) for TAVI 1296 ± 358 ng/mL before the procedure and 1604 ± 437 ng/mL after it, and 2) for BV 1413 ± 268 ng/ml before and 1808 ± 303 ng/mL after the procedure. These results are shown in Figure 11.

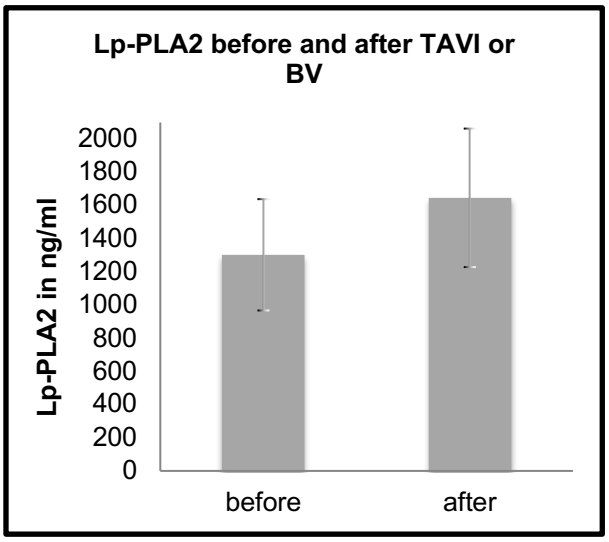

Figure 11 - Lp-PLA2 mass concentration significantly increased after the intervention at the aortic valve

(both diabetic and non-diabetic patients are included for calculations)

Using the independent samples *t*-test we noticed that Lp-PLA2 mass concentrations before and after the intervention at the aortic valve do not change differently whether TAVI or BV was performed. We obtained a *p* value of 0.36 when comparing the 2 sets of patients before the respective intervention and a *p* value of 0.15 after the intervention. This observation concludes that the enzyme mass concentration significantly increased in the same proportion after manipulation with the aortic valve, regardless of the interventional method (TAVI or BV).

Finally, a control group of 48 healthy subjects was included in the study. This allows a better understanding of the observed changes in the Lp-PLA2 mass concentration. Using independent samples *t*-tests, we conclude that the mass concentration of Lp-PLA2 is significantly increased in the study population (1319.77 ± 341.82 ng/ml) when compared to the control group (788.23 ± 210.95 ng/ml). For this observation we obtained a *p* value <0.01 as shown in Figure 8.

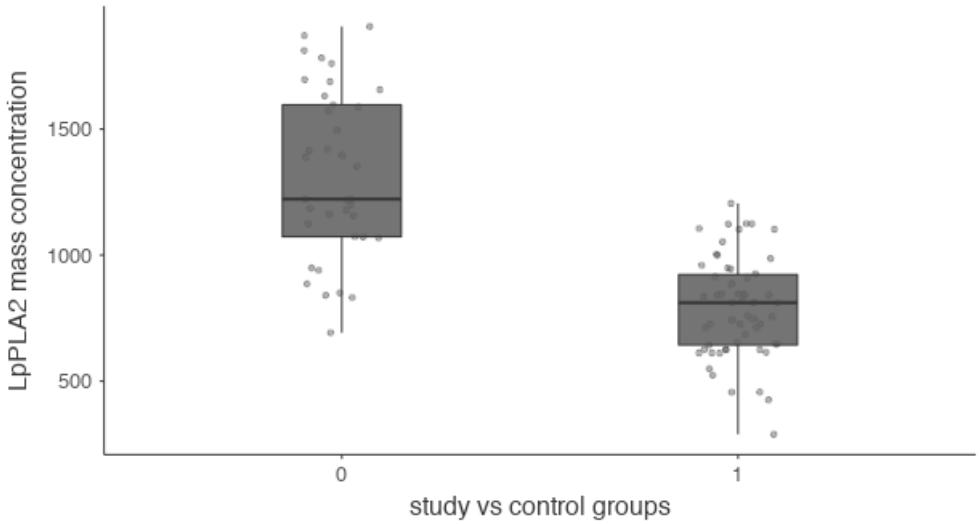

Figure 8 – LpPLA2 mass concentration measured in the study population (0) and in the control group (1) showing a significant difference.

Discussion

In the past years, Lp-PLA2 has been extensively studied as an independent risk factor for CVDs and as an active participant in the process of atherosclerotic plaque development. Data from more than 50000 patients revealed an association between increased Lp-PLA2 activity or mass concentration and an increased risk of cardiac death, myocardial infarction, acute coronary syndromes and ischemic stroke (Wilensky and Macphee, 2009). The latter is of importance because LDL and other markers of lipid metabolism have not been shown to be consistent predictors of stroke risk (Gorelick, 2008).

The present study shows that Lp-PLA2 mass concentration is increased in elderly patients when comparing to the adult population. According to previous studies the normal range for Lp-PLA2 mass concentration for men was set at 120-342 ng/ml (90^{th} percentile)(Colley, Wolfert and Cobble, 2011). In our control group the measured average Lp-PLA2 mass concentration was 788.23 ± 210.95 ng/ml. This control group consisted of 48 healthy elderly individuals living at a nursing home. We assume that, even though these individuals were categorized as "healthy", they were affected by subclinical atherosclerosis. We emphasize that, in real practice, when studying such advanced aged individuals it is almost impossible to find completely healthy subjects. This terminology was used to describe fully independent seniors able to walk without any aid or limitations, and without cognitive impairments.

The study population consisted of elderly individuals that underwent catheter-based interventions at the aortic valve. TAVI and BV are procedures reserved for high-risk patients with severe aortic stenosis. From this premise we can presume that the study population was affected by generalized atherosclerosis. This could explain the elevated Lp-PLA2 mass concentrations (1319.77 ± 341.82 ng/ml) measured in the study population before any intervention or subgrouping. Of note, the pathophysiological processes leading to calcific aortic valve degeneration are common to atherosclerosis, representing an active, proliferative and inflammatory process caused by endothelial damage due to mechanical stress and lipid penetration, leading to leaflet thickening and calcification.

Lp-PLA2 mass concentrations measured before any intervention were plotted against commoner markers of lipid metabolism. We obtained a strong correlation with LDL-C, and this is easily explained by the fact that LDL particles are the main carrier for Lp-PLA2, so they must increase or decrease in the same proportions. The association of Lp-PLA2 mass concentration with LDL, HDL and total cholesterol has been established in several investigations (Tselepis and Chapman, 2002; Ballantyne, Hoogeveen and Bang, 2004; Iribarren, Gross, Darbinian, Jacobs, *et al.*, 2005; Dohi *et al.*, 2011; Epps and Wilensky, 2011). Our results are in accordance with these previous studies. The reason why Lp-PLA2 preferably binds to small dense and more electronegative LDL may be related to the altered conformation of apoB100 in these particles. In addition, the presence of oxidized phospholipids in LDL is predicted to regulate the recruitment and the catalysis of Lp-PLA2.

We did not find a correlation between Lp-PLA2 and HDL-C. The first studies in mice, rats and guinea pigs suggested a protective function for Lp-PLA2. These species express lower concentrations of LDL and Lp-PLA2 is exclusively carried by HDL, conveying it antiatherosclerotic functions, for example by the inhibition of macrophage infiltration in the subendothelial space (Theilmeier *et al.*, 2000). We can assume that the reason why we did not find a correlation between Lp-PLA2 and HDL-C is connected with the study population, a high-risk group of patients with severe aortic stenosis and generalized atherosclerosis. Interestingly, N-linked glycosylation of plasma Lp-PLA2 hinders its binding to HDL (Tselepis *et al.*, 2001), meaning that the removal of glycosylation enhances its association with HDL. This could be explored in the future for new pharmacological interventions.

Three days after the intervention on the aortic valve (TAVI or BV) we measured the Lp-PLA2 mass concentration in all 44 elderly patients included in the study. The results showed a significant increase in the enzyme mass concentration. Because the pathophysiology processes for aortic calcific stenosis and atherosclerosis are the same, we can speculate a higher amount of this enzyme in the injured valve (as an analogy to an atherosclerotic plaque). Whether the finding of a higher Lp-PLA2 mass concentration after TAVI or BV is a direct consequence of the mechanical stress induced to the aortic valve during

the procedure or due to other unknown processes is just pure speculation because this biomarker was not tested before in this setting (in patients undergoing TAVI or BV). The impact of these results is not known, but it could be considered during the follow-up of these high-risk patients.

Finally, we divided the study population in two groups concerning the presence or absence of diabetes mellitus in their medical history. For further statistical analysis we did not take into account the type of diabetic control used by these patients. 42% of them were on a diabetic diet, 14% on peroral antidiabetics and 42% on insulin therapy. However, the results of our study showed a statistically significant higher Lp-PLA2 mass concentration on admission in diabetic patients when comparing to individuals without this disease. These results are in agreement with other previous studies (Noto, Chitkara and Raskin, 2006; Corsetti *et al.*, 2007). We did not find other statistically significant differences between diabetic and non-diabetic patients, including no differences in LDL-C, HDL-C and TG. This can partly be explained by the fact that 62% of diabetic and most of non-diabetic patients were being treated with a statin at the time of the study. It has been demonstrated that statins reduce the activity of Lp-PLA2 in patients with diabetes (Winkler *et al.*, 2004).

As described in the background section, diabetic patients exhibit a typical set of lipid abnormalities (often termed diabetic dyslipidemia), consisting of increased TG concentration, decreased HDL-C and a qualitative change in the LDL fraction towards smaller and denser particles. These particles can be easily taken up by macrophages in extravascular spaces contributing and resulting in atherogenesis. Furthermore, Lp-PLA2 binds preferentially to these small dense LDL particles, which can explain the abnormal distribution of Lp-PLA2 (with a relatively lower fraction in HDL particles), in diabetes mellitus (Kujiraoka *et al.*, 2006). Improving glycemic control may reduce Lp-PLA2 activity, mainly by increasing the relative proportion of Lp-PLA2 bound to HDL, which has been suggested to be atheroprotective (Sánchez-Quesada *et al.*, 2012). It has been estimated that the amount of Lp-PLA2 in small, dense LDL or electronegative LDL is 5 to 10 times higher than in normal-sized or electropositive LDL particles (Benítez *et al.*, 2003; Gazi *et al.*, 2005). The amount of lysophosphatidylcholine in

circulating LDL is also increased in diabetic patients (Sonoki *et al.*, 2009) and this may be related to the increased Lp-PLA2 activity. A prospective study showed that higher levels of Lp-PLA2 activity were associated with increased risk of incident coronary heart disease among diabetic subjects (Hatoum *et al.*, 2010). However, a comparison between diabetic and non-diabetic individuals was not made.

Still concerning diabetic dyslipidemia, we did not obtain a statistically relevant increased TG concentration in our diabetic patients, despite a graphical trend towards higher TG concentrations comparing to non-diabetic individuals. There was, however, a strong statistical correlation between Lp-PLA2 mass and TG concentrations. In states of insulin resistance, insulin fails to inhibit the hormone sensitive lipase, leading to the mobilization of free fatty acids from the adipose tissue. These may impair the activity of lipoprotein lipase and lead to a decrease in the catabolism of triglyceride-rich apoB lipoproteins, causing an overproduction of VLDL. Low concentrations of Lp-PLA2 can bind to VLDL (Srinivasan and Bahnson, 2010), the main carrier of TG into the tissues. This could explain the positive correlation between Lp-PLA2 mass and TG concentrations found in this study.

Of note, elevated LDL-C, elevated TG concentrations and low levels of HDL-C are strongly associated with increased risk for macrovascular (e.g. myocardial infarction, ischemic stroke, and coronary mortality) and microvascular (retinopathy, neuropathy, and nephropathy) among patients with T2DM (Toth *et al.*, 2012).

Levels of measured Lp-PLA2 mass concentration in this population are extremely elevated compared with other studies. Pre-analytical and analytical errors are a main concern. The temperature of the samples may induce biochemical coupling and uncoupling of Lp-PLA2 to the binding molecule (for example LDL). Because of this process, it is recommended that samples undergo immediate separation and freezing at -70 degrees Celsius for at least 18-24h prior to analysis. They should then be thawed to 4 degrees Celsius prior to the assay (Oliver *et al.*, 2011). In our study blood samples were drawn from patients to serum clot sample tubes and immediately centrifuged upon arrival at the same hospital laboratory. The serum was then aliquoted and stored at -80

degrees Celsius. Due to logistic and financial issues the samples were then thawed to 4 degrees Celsius and examined by the ELISA assay once there was a significant number of samples to be analyzed. We cannot prove if this approach influenced the results of our analysis, even though it is the standard methodology of our and most of other laboratories.

The same methodology was used to measure Lp-PLA2 mass concentration in the control group. This reduces the eventual pre-analytical problem described in the previous paragraph.

Another possible explanation for our results is related to the lithium-heparin sample tubes. This type of preservative can alter the results of Lp-PLA2 mass concentration, resulting in higher measured values by ELISA.

In addition, the results of commercially available assays for the measurement of Lp-PLA2 mass concentration, the PLAC assays are often not enough reproducible for statistical purposes. It has been suggested, that measures of Lp-PLA2 activity might serve as a more representative marker of the enzyme function.

From the studied literature there are some postulations worth mentioning.

The vast available studies tried to find correlations between Lp-PLA2 mass concentration and activity with cardiovascular events. Meaning that, most of them are epidemiological studies. Most found a positive correlation between Lp-PLA2 and cardiovascular diseases, but some did not. As mentioned earlier, the oxidation of LDL particles is one of the first steps in the pathogenesis of atherosclerosis. With an increase in oxidized LDL the innate immune system is activated and mobilizes monocytes and macrophages to the arterial wall. At this stage the amount of Lp-PLA2 rises exponentially and targets glycerophospholipids containing short and/or oxidized functionalities at the *sn*-2 position, generating lysophosphatidylcholine and NEFAs, which have been reported to have proinflammatory and pro-oxidative activities. The hypothesis that Lp-PLA2 might actively contribute to inflammation during atherosclerosis led to the development of reversible inhibitors. Of which, darapladib has been extensively tested and failed to reduce the incidence of cardiovascular death, myocardial infarction, stroke or all-cause mortality in patients with stable CVD, and failed to reduce the incidence of major coronary events after an acute

coronary syndrome. These findings raise the question whether the elevated enzyme levels might reflect a response to the proinflammatory/pro-oxidative stress that is typical for atherosclerosis (Stafforini and Zimmerman, 2014).

Furthermore, the strong positive correlation of Lp-PLA2 with LDL-C demonstrates that elevated circulating levels of Lp-PLA2 is associated with atherosclerosis, but such observations do not show a causal role for Lp-PLA2 in the disease process.

Another key unanswered question is whether Lp-PLA2 functions in the circulation, in atherosclerotic plaques and other tissues, or both. Transport to the intracellular compartment may be required before hydrolysis, and the relationship between circulating and tissue Lp-PLA2 and oxidized phospholipids has not been critically evaluated.

Conclusion

Based on the results from this study showed in the previous sections, we can postulate the following conclusions:

1) Lp-PLA2 mass concentration is increased in elderly healthy subjects living in a nursing home when comparing to the accepted normal range interval for the adult population.
2) Lp-PLA2 mass concentration is increased in elderly patients with aortic valve stenosis before TAVI or BV. The measured values for the study population are almost 2-fold higher than the values for the control group.
3) There were strong correlations of Lp-PLA2 mass concentration with LDL-C, total cholesterol and TG.
4) A statistically significant increase in Lp-PLA2 mass concentration occurs after TAVI or BV.
5) There is a higher baseline Lp-PLA2 mass concentration in diabetic patients comparing to non-diabetic patients.
6) Lp-PLA2 mass concentration increased in the same proportion in diabetic and non-diabetic patients after TAVI or BV.

The study was supported by the projects IGA MH CR no. NT/12287-5, and PRVOUK P37/12.

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