UNIVERSITÀ DEL PIEMONTE ORIENTALE

DEPARTMENT OF HEALTH SCIENCES

PhD MEDICAL SCIENCES AND BIOTECHNOLOGY

XXXII PROGRAM

Cancer, metabolic diseases, ageing

LIPOPROTEIN-ASSOCIATED PHOSPHOLIPASE A_2 AS CARDIOVASCULAR RISK FACTOR AND THERAPEUTIC TARGET IN NEPHROPATIC PATIENTS

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LIPOPROTEIN-ASSOCIATED PHOSPHOLIPASE A_2 AS CARDIOVASCULAR RISK FACTOR AND THERAPEUTIC TARGET IN NEPHROPATIC PATIENTS.

SUMMARY

Background. Lipoprotein-associated phospholipase A_2 (Lp-PLA_2) is a serine lipase associated with Low-Density Lipoprotein (LDL), that enters into the vessel wall, increases the oxidative stress in atherosclerotic lesions and makes the plaque instable. Lp-PLA_2 is recognized to be a strong inflammatory, oxidant and atherogenic promoter in the general population, but very few data are available for subjects with renal failure.

The aim of the present PhD thesis was to investigate the role of Lp-PLA_2 among patients with advanced renal failure.

Chapter 1. In 102 hemodialyzed patients in Novara, it has been demonstrated that Lp-PLA_2, activity is increased and associated with a more inflammatory phenotype than in healthy subjects (Rolla R. J Nephrol 2015;69:749-55).

Chapter 2. In healthy subjects and cardiac patients, Lp-PLA_2 positively correlates with the risk of acute cardiovascular morbidity and mortality, but no studies are available for renal subjects. With this PhD research project, it was demonstrated for the first time that Lp-PLA_2 is an independent risk factor for cardiovascular morbidity and mortality in 102 hemodialyzed subjects, followed for 3 years. (De Mauri A. J Nephrol 2019; 32(2): 283-288).

Chapter 3. Lp-PLA_2 is recognized to be associated with peripheral artery disease in healthy subjects and cardiac patients, but no data are available for nephropathic patients. In this PhD research project, Lp-PLA_2 was recognized, for the first time, as independently associated with peripheral artery disease and lower limb ischemic ulcers, in dialyzed subjects followed for 5 years. (De Mauri A. Epub ahead of print in Therapeutic Apheresis and Dialysis 2019 Dec 20, DOI 10.1111/1744-9987.13465).

Chapter 4. The interest of clinicians for Lp-PLA_2 is due both to pharmacological as well as non pharmacological interventions. Here, it is reported a personal experience, demonstrating that lipoprotein apheresis reduces serum Lp-PLA_2 and improves lower limb ulcers, just confirming that this approach could represent an additional therapeutic chance (abstract presented as invited communication at "11^{th} Congress of Therapeutical Apheresis", Pesaro, $8\text{-}9^{th}$, November, 2018; manuscript in preparation).

Chapter 5. In recent years, the scientific literature confirmed the beneficial role of the nutritional therapy in several chronic diseases. In this PhD project, it was demonstrated that: first, the Mediterranean diet is associated with lower levels of Lp-PLA_2 in 41 subjects with advanced renal failure. Second, the low protein diet, a strategy to reduce uremic symptoms and delay the progression of renal disease, is effective in decreasing Lp-PLA_2, in 28 subjects with advanced renal failure. (These investigations were presented as abstracts at 56th ERA-EDTA Congress, Budapest,

13-16th, June, 2019 and at 60th Congress of Italian Society of Nephrology, Rimini 2-5th, October 2019, manuscripts in preparation).

In **conclusion** with this PhD project it was demonstrated that, among renal patients as in the general population, Lp-PLA_2 could be considered a risk factor for acute cardiovascular morbidity and mortality and peripheral artery disease. Lp-PLA_2 could also represent a useful target for pharmacological and non-pharmacological approaches in renal subjects (lipoprotein-apheresis). Moreover, Lp-PLA_2 serum levels could be reduced by means of an adequate nutritional therapy and by a safe, low-cost and kidney-friendly lifestyle.

Finally, the role of Lp-PLA2 has been recently postulated in non-atherosclerotic diseases as well as in metastatic cancer, thus it is mandatory to extend its study in chronic kidney disease patients.

SOMMARIO

La fosfolipasi A_2 associata alle lipoproteine (Lp-PLA_2) è una serin lipasi, associata per lo più alle LDL, che, una volta penetrata nella parete vascolare, trasforma il core lipidico della placca aterosclerotica in core necrotico, rendendolo instabile. Mentre è scientificamente riconosciuta l´attività proinfiammatoria e proaterosclerotica di Lp-PLA_2 nella popolazione generale e nei pazienti cardiopatici e dismetabolici, i dati sui pazienti con insufficienza renale sono pochi e incerti. Lo scopo del presente progetto di ricerca è stato quello di studiare la Lp-PLA_2 nei pazienti con insufficienza renale avanzata.

Capitolo 1. E´stato dimostrato, in 102 pazienti emodializzati di Novara, che la Lp-PLA_2, è presente in concentrazioni maggiori, è associata ad un profilo più aterogeno delle lipoproteine e correla con gli eventi cardiovascolari nel breve termine.

Capitolo 2. La Lp-PLA_2 è stata irrevocabilmente associata a morbidità e mortalità cardiovascolari nella popolazione generale, diabetica e cardiopatica, ma non ci sono dati per i nefropatici. Con uno studio osservazionale è stato dimostrato per la prima volta che la Lp-PLA_2 è un predittore indipendente di eventi cardiovascolari e correla con la mortalità anche nei 102 dializzati arruolati e seguiti per 3 anni (De Mauri A. J Nephrol 2019; 32(2): 283-288).

Capitolo 3. La Lp-PLA_2 è stata associata all´arteriopatia periferica nella popolazione generale e cardiopatica, ma non ci sono studi su soggetti con insufficienza renale. Seguendo i 102 pazienti precedentemente arruolati per un follow-up di 5 anni, è stato dimostrato che la Lp-PLA_2 predice le ulcere ischemiche degli arti inferiori nei dializzati (De Mauri A. Epub ahead of print in Therapeutic Apheresis and Dialysis 2019 Dec 20, DOI 10.1111/1744-9987.13465).

Capitolo 4. Siccome l´interesse per la Lp-PLA_2 si estende alle possibilità terapeutiche, farmacologiche e non, è stata qui riportata la mia esperienza personale, che dimostra che la lipoprotein-aferesi (trattamento riconosciuto per l´arteriopatia periferica) reduce i livelli serici di Lp-PLA_2, migliorando la perfusione tissutale e conducendo a guarigione le ulcere, in un soggetto dializzato affetto da arteriopatia periferica avanzata. (abstract presentato come invited communication al "11^o Congresso di Aferesi Terapeutica", Pesaro, 8-9 Novembre, 2018; testo in preparazione).

Capitolo 5. La letteratura scientifica sta sempre più conferendo un ruolo terapeutico alla dieta e allo stile di vita. Con due studi prospettici inclusi in questa tesi di dottorato, è stato riscontrato che la Dieta Mediterranea si associa a livelli inferiori di Lp-PLA_2, in 41 soggetti con insufficienza renale avanzata non in dialisi. È stato dimostrato, inoltre, che la dieta ipoproteica, da tempo raccomandata per contenere i sintomi uremici e la progressione di malattia, riduce, dopo soli 2 mesi, l´attività di Lp-PLA_2 in 28 pazienti con insufficienza renale grave. (Questi studi sono stati presentati come

abstract al 56° Congresso della Società Europea di Dialisi e Trapianto, Budapest, 13-16 Giugno 2019 e al 60° Congresso della Società Italiana di Nefrologia, Rimini 2-5 Ottobre 2019, testi in stesura).

In **conclusione,** in questo progetto di Dottorato è stato dimostrato, per la prima volta, che la Lp-PLA_2 è elevata e ha un profilo maggiormente pro-aterosclerotico e pro-infiammatorio nei pazienti con insufficienza renale avanzata; che in questa popolazione la Lp-PLA_2 correla con gli eventi cardiovascolari, la mortalità e l´arteriopatia periferica in fase avanzata, in un follow-up di 3-5 anni.

La Lp-PLA_2 è, inoltre, un target terapeutico per le terapie farmacologiche e non, quali la lipoprotein-aferesi. Infine, anche nei nefropatici, i livelli di Lp-PLA_2 possono essere contenuti e rimodulati da una dieta e stile di vita adeguati, quali la Dieta Mediterranea e la dieta ipoproteica.

Siccome si stanno scoprendo nuovi ruoli per la Lp-PLA2, ad esempio in patologie non aterosclerotiche e nel cancro metastatico, è doveroso estendere la ricerca e le eventuali terapie anche ai pazienti affetti da insufficienza renale.

BACKGROUND

General features on Lp-PLA_2

Lipoprotein-associated phospholipase A_2 (Lp-PLA_2) is a serine lipase mainly produced by activated monocytes and macrophages in an inflammatory milieu; in the blood stream Lp-PLA_2 circulates bound to High-Density Lipoproteins (HDLs) by one third and to the Low-Density Lipoproteins (LDLs) by two-thirds and protein B100 is the mediator of this link[1]. The Lp-PLA_2 gene is located on chromosome 6 q21.2-p12.

Initially, in the early '80, Lp-PLA_2 was recognized by its action on hydrolyzing platelet-activating factor (PAF) so that it was named "platelet –activating factor acetylhydrolase (PAF-AH)[2]": it was supposed to remove oxidized phospholipids and reduce the highly oxidized lipids productions just acting as an antioxidant agent. Later, it was clarified that PAF-HA is a member of the PLA_2 family, but specific for the breakdown of PAF and oxidized fatty acid residues and its name was changed into "Lipoprotein-associated phospholipase A_2".

Lp-PLA_2 enters into the vessel wall and catalyses the hydrolysis of the phospholipids on the surface of the LDLs, thus releasing lysophosphatidylcholine, oxidized fatty acids (OxLDL) and oxidized non esterified fatty acids (OxNEFAS). These mediators trigger the inflammatory cascade, the production of TNFα-, IL-6 and induce the chemotaxis of leucocytes into the sub-intimal space of the arterial wall, converting them into foam cells (Figure 1 and 2). As a consequence, Lp-PLA_2 enhances the growth and the conversion of the atherosclerotic plaque from a lipid to a necrotic core[3,4,5,6], making the plaques instable and promoting acute cardiovascular events. Lp-PLA_2 is also recognized to be a strong inflammatory, oxidant and atherogenic promoter.

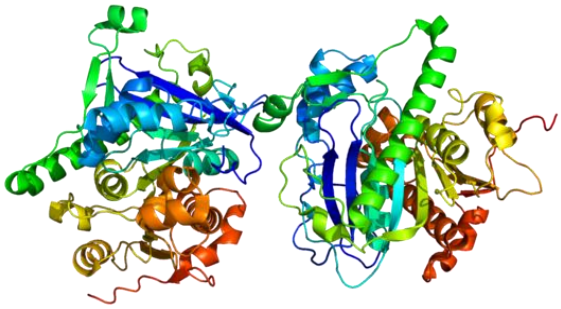

Figure 1. Structure of the molecule

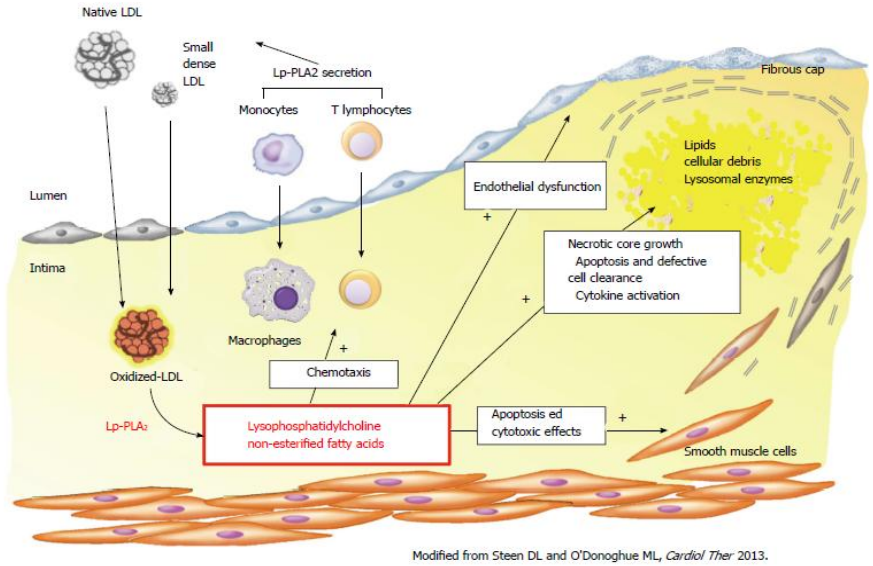

Figure 2 Pathogenic role of lipoprotein-associated phospholipase A2 in atherosclerosis development. LDL: Low-density lipoprotein; Lp-PLA2: Lipoprotein-associated phospholipase A2.

Modified from Steen DL and O'Donoghue ML, *Cardiol Ther* 2013.

Figure 2

To determine the serum levels of Lp-PLA_2 two methods are available[7]:

The assay for the mass (ng/ml) quantifies the primary accessible molecule on lipoproteins surface:

- Enzyme-linked immunosorbent assay kit (R&D Systems, Inc., Minneapolis, MN);
- PLAC test, enzyme-linked immunoadsorbent assay (diaDexus, Inc., south San Francisco, CA, USA).

The assay for the activity (ng/ml/min) assess the complete Lp-PLA_2 activity:

- Enzymatic PAF-acetylihydrolase Assay kit (Abcam, Cambridge, MA);
- PLAC Test, enzymatic assay (diaDexus, Inc., San Francisco, CA, USA).

Both are recognized as useful tool to evaluate Lp-PLA_2 in experimental studies and in clinical practice.

In this thesis the PLAC enzymatic assay test was used (diaDexus, Inc., San Francisco, CA, USA). (See methods).

Hypothesis of the research project

Renal dysfunction is associated with several perturbations in lipid metabolism, leading to dyslipidaemia and accumulation of atherogenic particles. Despite patients with renal failure can show higher as well as lower levels of total-cholesterol, LDLs and HDLs, the composition of these lipoproteins is modified. For instance, the increased levels of Apoprotein B enhances the circulation of atherogenic particles; the reduction or dysfunction of lipoprotein lipase induces an increased level of triglyceride-rich lipoproteins (VLDL, IHDL); the dysfunction of the lecithin-cholesterol acyl-transferase causes a dysfunction in HDLs that cannot exert their anti-inflammatory effects.

In addition, the imbalance between pro-oxidant and anti-oxidant agents in favour of a burden of oxidative stress, increases the levels of oxidized fatty acids and oxidized non esterified fatty acids, representing a bridge between dyslipidaemia, oxidative stress and inflammation[8,9].

In this contest, the hypothesis of the present PhD thesis was that Lp-PLA_2 could represent the expression of inflammatory and atherogenic disorders and could play a role in vascular damage in nephropatic patients.

AIM

The aim of the present PhD thesis was to investigate the role of Lp-PLA_2 in patients with advanced renal failure and in particular:

- to evaluate serum levels of Lp-PLA_2 in patients with end stage renal disease, compared to healthy subjects;
- to evaluate a possible relationship between Lp-PLA_2 and cardiovascular morbidity and mortality in hemodialyzed patients;
- to evaluate a possible relationship between Lp-PLA_2 and peripheral artery disease and lower limb ischemia in hemodialyzed patients;
- to investigate some interventional approaches to reduce serum Lp-PLA_2 in order to improve the clinical outcome of lower limb ulcers;
- to investigate the role of the nutritional therapy in reducing serum Lp-PLA_2 levels.

METHODS AND PATIENTS

Patients

5 clinical observational studies were performed, recruiting 102 haemodialyzed patients (HDP) for study 1, 2 and 3 (see Chapters 1, 2, and 3), only one HDP as a case report in Chapter 4, and 41 and 28 patients with advanced renal failure not on dialysis for study 4 and 5, respectively (see Chapter 5). All patients were enrolled in the "Nephrology and Dialysis Unit", Maggiore della Carità University Hospital, in Novara.

Baseline demographic and clinical data as well as comorbidities such as diabetes mellitus and cardiac disease, and therapy were obtained by reviewing medical records, clinical summaries and patients interviews. As general accepted in clinical practice, diabetes mellitus was defined as the current or past use of oral hypoglycaemic agents or insulin; coronary artery disease was defined by history of myocardial infarction or angina and/or instrumental evidence of ischemic heart disease (electrocardiogram, echocardiogram, stress test angiography/angioplasty, coronary artery bypass grafting). During the follow-up, we recorded every acute cardiac, peripheral and cerebral vascular event as well as death. Personal and clinical data were collected in a database in an anonymous form.

We also selected, as controls, forty non renal subjects, matched for gender, age, presence of diabetes and cardiac disease and statin therapy.

Samples

After the long-interdialytic interval, predialysis venous whole blood samples were collected. Routine laboratory measurements were performed in the Clinical Chemistry Laboratory, Department of Health Sciences, Amedeo Avogadro University of Eastern Piedmont, Novara, Italy. The residual plasma was collected in tubes identified by a code number and the date and stored in access-controlled refrigerators (-80 °C) at the Clinical Chemistry Laboratory for further evaluation of biochemical parameters investigated in this project.

Control patients' blood samples were also analysed in our Clinical Chemistry Laboratory. All of them showed normal blood count, normal C reactive protein levels, normal plasma glucose, normal renal and liver indices.

Routine biochemical parameters and, in particular, LDL, HDL, triglycerides, Apo A, Apo B, high sensitivity C-Reactive Protein and Lp-PLA_2 were analyzed using the ADVIA® 1800 Clinical Chemistry Analyzer (Siemens Healthcare Diagnostics).

For the quantitative determination of Lp-PLA_2 activity, the new PLAC® test was used (diaDexus, Inc., San Francisco, CA, USA).

Lp-PLA_2, in serum or plasma, hydrolyzes the *sn*-2 position of the substrate, 1-myristoyl-2-(4 nitrophenylsuccinyl) phosphatidylcholine, producing a coloured reaction product, 4-nitrophenol. The rate of formation of 4-nitrophenol is followed spectrophotometrically and the Lp-PLA_2 activity is calculated from the rate of change in absorbance. A set of five known Lp-PLA_2 calibrators is used to generate a standard curve to plot the change in absorbance against Lp-PLA_2 activity levels in nmol/min/mL and deriving Lp-PLA_2 activity levels in our samples.

A clinical cut-point of Lp-PLA_2 activity level > 194 nmol/min/mL was chosen to consider a patient at higher risk of cardiovascular disease as suggested by the producer.

STATISTICAL ANALYSIS

Statistical analyses were performed using the SPSS statistical software v.15.0 (SPSS Inc., Chicago, IL, USA). Normal distribution was preliminary assessed by q-q plot, Kolmogorov–Smirnov and Shapiro–Wilk tests. Differences between two groups were estimated by Unpaired T test and Mann–Whitney U-test for parametric and non-parametric data, respectively. Results are expressed as median and interquartile ranges. Variables were analysed with univariate Cox logistic regression and, if statistically significant, entered in a multivariate logistic model.

A p values < 0.05 was considered statistically significant.

The study was approved by the Ethical Committee of our Institution (981/CE).

CHAPTER 1

Lp-PLA_2 in patients with renal failure

Very few studies investigated Lp-PLA_2 levels in subjects with renal failure.

In 1999, Milionis[10] found that PAF-HA is higher in patients with renal failure than in healthy subjects, as well as in peritoneal dialysis patients compared to hemodialysis ones, maybe because of the higher levels of all other lipids. The same Author did not find any differences in Lp-PLA_2 levels before or after the dialysis session or according to the type of dialyzer membrane used. Tektas[11] confirmed these results in a small sample of HDP (43) and controls (15): Lp-PLA_2 mass was increased in hemodialysis patients compared to controls and positively associated with total and LDL-cholesterol and triglycerides and negatively correlated with HDL and nitrite levels, because of impaired arginase/NO pathway.

Wang[12] et Coll. observed an increased Lp-PLA_2 mass and activity in patients with renal failure compared with non-renal patients, but they did not find any differences among different stages of kidney disease, suggesting that the alteration of Lp-PLA_2 levels occurs early in the course of kidney disease and contributes to the pathogenesis and progression of renal dysfunction.

STUDY 1

The aim of the first clinical observational study was to evaluate the serum levels of Lp-PLA_2 in HDP and to determine whether there is any correlation with cardiovascular events, during a short follow-up.

102 unrelated Caucasian prevalent HDP were enrolled in June 2013 and followed for the subsequent 6 months. They were under chronic haemodialysis, three times a week, from at least 3 months. No other exclusion criteria were applied.

Among enrolled subjects, there were 64 (63%) males, the mean age was 68±15 years, with a dialytic vintage of 29 (13-53) months; the prevalence of diabetes mellitus, hypertension and coronary artery disease were 35%, 54% and 40%, respectively (Table 1).

Lp-PLA_2 serum levels were 187±44 nmol/min/ml (median and IQR 184 and 156-214 nmol/min/ml, respectively); 42% of HDP had Lp-PLA_2 activity higher than the normal cut-off of 194 nmol/min/ml. In contrast, healthy controls had Lp-PLA_2 activity of 158±26 nmol/min/ml and 4 (10%) of them had Lp-PLA_2 activity higher than the normal cut-off ($p < 0.001$).

Every acute cardiac, peripheral and cerebral vascular event, as well as death were occurred during the 6 months before and after the enrolment.

TABLE 1. Characteristic of the patients.

VARIABLE	Values
Gender	
Male N (%)	64 (63%)
Female N (%)	38 (37%)
Age (years)	71 (59-78)
Time of Dialysis (months)	29 (13-53)
Diabetes Mellitus N (%)	36 (35%)
Coronary Artery Disease N (%)	41 (40%)
Total Cholesterol (mg/dl)	158 (127-191)
LDL-cholesterol (mg/dl)	79 (63-102)
HDL-cholesterol (mg/dl)	41 (33-51)
Triglycerides (mg/dl)	139 (92-205)
Statin therapy N (%)	26 (25%)
ApoB/ApoA1 (ratio)	0.72 (0.58-0.89)
C-Reactive Protein (mg/dl)	0.4 (0.1-0.9)
Lp-PLA_2 activity (nmol/min/ml)	184 (156-214)
Lp-PLA_2 > 194 nmol/min/ml	43 (42%)

It was found that subjects with end stage renal disease on dialysis had higher Lp-PLA_2 levels than healthy volunteers (187± 44 vs 158±26 nmol/min/ml, p =0.001) and most of them had a Lp-PLA_2 activity higher than the normal cut-off (42% vs 10%). HDP had significantly lower levels of total, LDL and HDL cholesterol and significantly higher levels of triglycerides. In regard to apolipoproteins, Apo A1 concentration was significantly lower in HDP than in controls, whereas Apo B was comparable, resulting in a higher ApoB/ApoA1 ratio among uremic subjects (Table 2). Moreover, an altered composition in HDL was also shown: levels of Apo A1, the main component of HDL, largely responsible for reverse cholesterol transport through the macrophage ATP-binding cassette transporter ABCA1, were significantly lower in uremic patients. This phenomenon might impact on the physiological activity of HDLs, impairing their ability to promote cholesterol efflux, as demonstrated in several studies on uremic serum in humans and animals[13,14].

TABLE 2. Comparison in lipoprotein profile between HDP and healthy subjects. A p value <0.05 was considered statistically significant.

	HDP	**Controls**	**p**
Total Cholesterol (mg/dl)	159 ± 39	201 ± 31	P < 0.001
LDL cholesterol (mg/dl)	86 ± 31	124 ± 27	P < 0.001
HDL cholesterol (mg/dl)	43 ± 16	58 ± 12	P < 0.001
Apoprotein A1 (g/L)	1.04 ± 0.21	1.23 ± 0.17	P < 0.001
Apoprotein B (g/L)	0.74 ± 0.20	0.76 ± 0.16	P < 0.05
ApoB/ApoA1(ratio)	0.7 ± 0.2	0.6 ± 0.1	P < 0.001
Lp-PLA_2activity (nmol/min/mL)	187 ± 44	158 ± 26	P < 0.001
Lp-PLA_2/ApoB (ratio)	265 ± 75	214 ± 36	P < 0.005

During the follow-up 48 acute cardiovascular events occurred: 27 cardiac, 12 peripheral arterial, 5 cerebral and 4 mesenteric artery accidents. In particular, 19 of the 33 subjects with cardiovascular events (58%) and 24 of the 69 subjects without cardiovascular events (35%) had Lp-PLA_2 levels above the threshold ($p < 0.05$). Moreover, HDP with Lp-PLA_2 plasma levels > 194 nmol/min/mL were more likely to have adverse cardiovascular events (OR 2.54; 95 % (CI) 1.09, 5.95) compared to patients with Lp-PLA_2 plasma levels < 194 nmol/min/ml.

In conclusion, this study it was demonstrated that, in HDP, serum activity of Lp-PLA_2 is increased and associated with a more atherogenic milieu. Again, in HDP, Lp-PLA_2 correlated with acute cardiovascular events even during a short follow-up.

The present study was published in 2015: Rolla R, De Mauri A, Valsesia A, Vidali M, Chiarinotti D, Bellomo G. Lipoprotein profile, lipoprotein-associated phospholipase A_2 and cardiovascular risk in hemodialysis patients. J Nephrol 2015 Dec;28(69:749-55)"[15].

J Nephrol (2015) 28:749–755
DOI 10.1007/s40620-015-0194-0

ORIGINAL ARTICLE

Lipoprotein profile, lipoprotein-associated phospholipase A_2 and cardiovascular risk in hemodialysis patients

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CHAPTER 2

Lp-PLA$_2$ and cardiovascular disease.

Several studies confirmed the role of Lp-PLA$_2$ as a predictor of cardiovascular morbidity and mortality in apparently healthy subjects[16,17], in diabetic and dysmetabolic patients[18,19] and in subjects with cardiac disease[20,21,22,23].

Based on these evidences, the guidelines of three major heart international societies, the American College of Cardiology, the American Heart Association and the European Society of Cardiology, included the Lp-PLA$_2$ activity measurement in the charts used to stratify the cardiovascular risk of asymptomatic patients in order to optimize the lipid lowering therapy[24] (Figure 3).

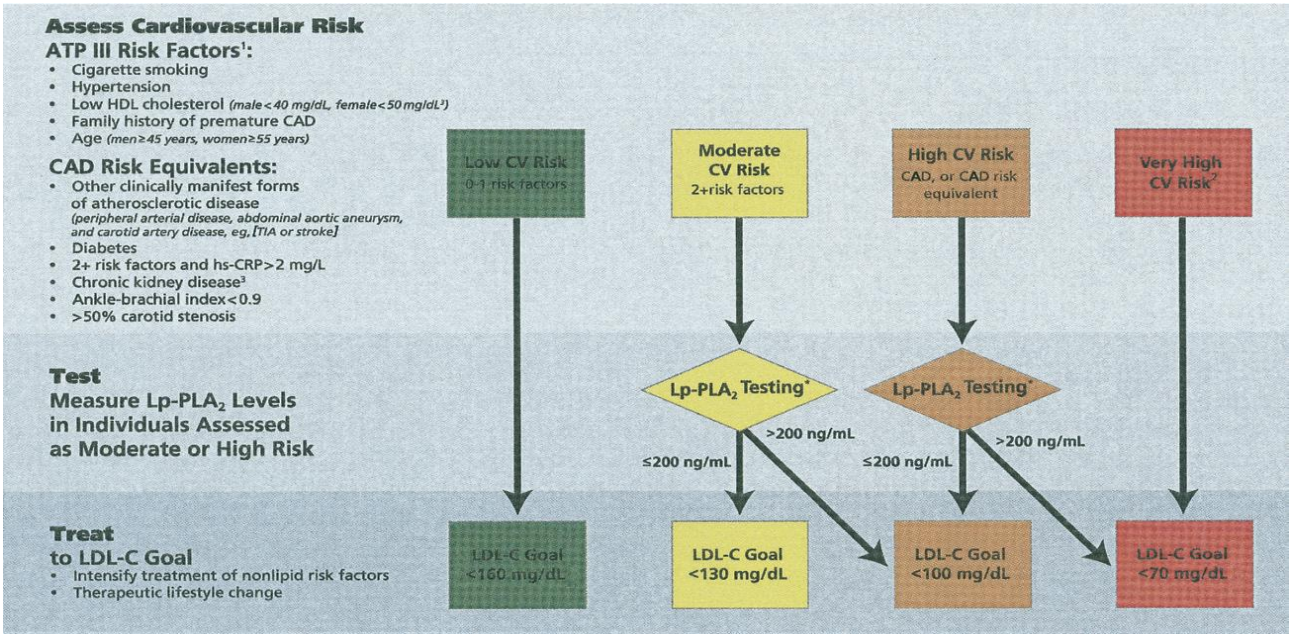


Figure 3 from "2008. American Journal of Cardiology (www.AJConline.org).

Chronic Kidney Disease is recognized as a chronic inflammatory disease, with a large production of proinflammatory cytokines that enhance atherosclerosis and the progression of renal and cardiovascular dysfunction[25,26].

Due to its biological activity[2-4], Lp-PLA$_2$ could be considered a bridge between inflammatory and atherosclerotic disease, but few studies investigated the behaviour of Lp-PLA$_2$ in the renal population.

With regard to HDP, Winkler[27] found a relationship between Lp-PLA$_2$ and cardiovascular outcomes during a mean follow-up of 4 years, but it was a post-hoc analysis of the 4D Study, restricted only to diabetic subjects.

Despite these evidences in the general and cardiac population[28,29], the literature lacks of studies investigating the role of Lp-PLA$_2$ in predicting cardiovascular events and death in uremic subjects.

STUDY 2

A second clinical observational study was performed in order to evaluate a possible relationship between serum Lp-PLA_2 activity and long-term cardiovascular events and death among haemodialyzed patients[30], followed for three years.

Patients enrolled in Study 1[15] were further followed until June 2016, (Table 1).

Lp-PLA_2 and cardiovascular events in haemodialysis patients.

At the end of the follow up, the total and cardiovascular-related mortality was 42% and 26%, respectively. During the observation time, 49 subjects (50%) experienced cardiovascular accidents, 51% of them developed at least one event and 9% more than three events. Overall, we observed 66 cardiovascular events: 33 peripheral arterial, 28 cardiac, 12 cerebral and 3 other acute accidents.

HDP with Lp-PLA_2 higher than the 194 nmol/min/ml cut-off had higher cholesterol and higher ApoB/ApoA1 ratio [0.83 (0.71-0.97) vs 0.61 (0.54-0.79), $p<0.0005$], while 0.7 is the level to determine a high cardiovascular risk according to the literature.

In the group with higher Lp-PLA2 activity levels, more patients experienced cardiovascular accidents (74% vs 29%, $p<0.005$) as well as cardiovascular death (42% vs 20%, $p=0.033$) (Table 3).

Table 4. Comparison between subjects with Lp-PLA_2 lower and higher than the cut-off (a p value <0.05 was considered statistically significant.).

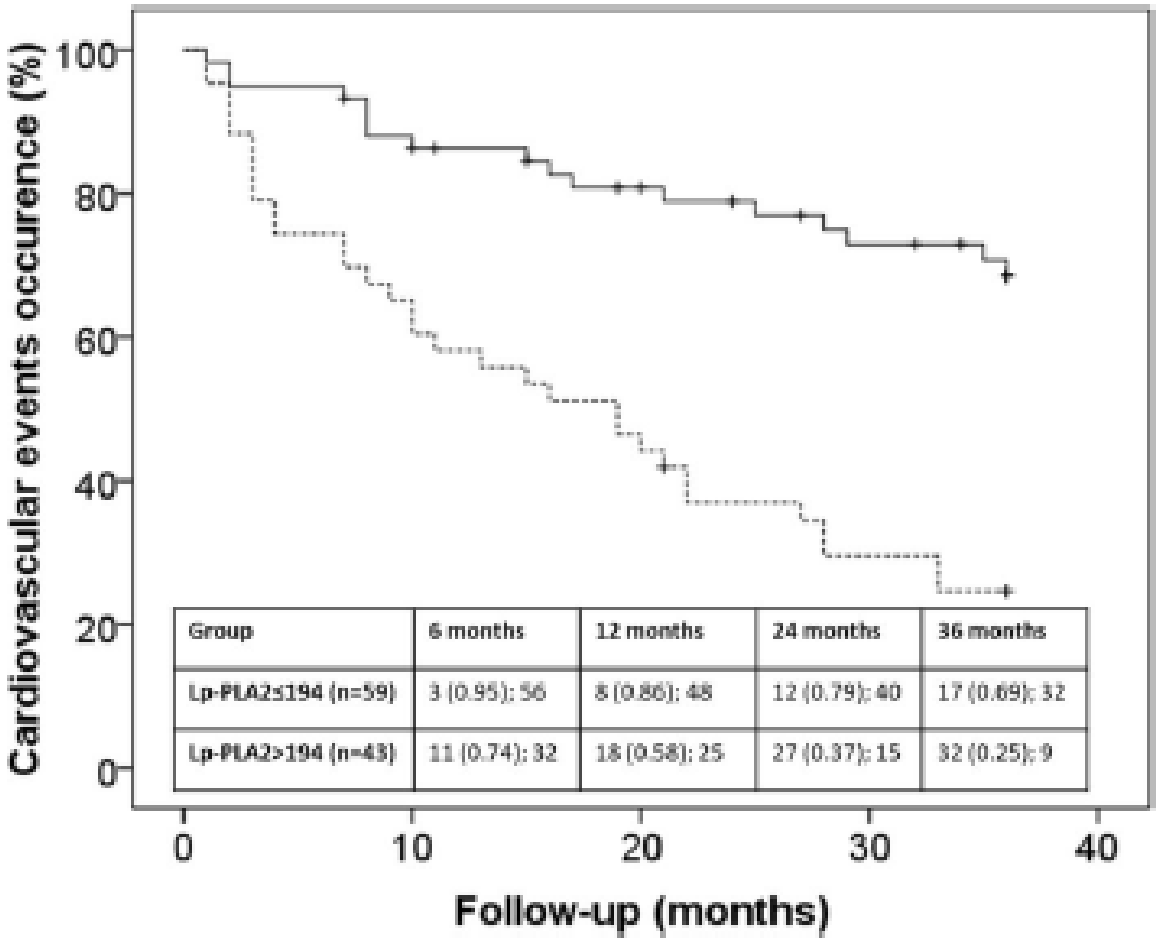
Variables	**PLA_2≤194 nmol/min/ml (N=59)**	**PLA $_2$>194 nmol/min/ml (N=43)**	**p**
Age, years (median, IQR)	69 (58-76)	73 (60-825)	0.120
Time of Dialysis, months (median, IQR)	22 (11-49)	34 (21-68)	0.072
BMI, kg/m^2 (median, IQR)	24.0 (20.8-26.6)	23.8 (21.2-25.8)	0.607
Diabetes Mellitus, N (%)	22 (37%)	14 (33%)	0.678
Hypertension, N (%)	33 (56%)	22 (51%)	0.690
Coronary Artery Disease, N (%)	26 (44%)	15 (35%)	0.416
Lower limb ischemic lesions, N (%)	15 (25%)	17 (40%)	0.138
CRP, mg/dl (median, IQR)	0.28 (0.06-0.75)	0.54 (0.08-1.18)	0.168
Total Cholesterol, mg/dl (median, IQR)	142 (118-187)	171 (140-195)	**0.018**
LDL cholesterol, mg/dl (median, IQR)	67 (51-93)	92 (73-115)	**<0.0005**
HDL cholesterol, mg/dl (median, IQR)	41 (34-53)	40 (32-47)	0.418
Triglycerides, mg/dl (median, IQR)	148 (90-204)	133 (92-209)	0.730
ApoB/ApoA1 ratio (median, IQR)	0.61 (0.54-0.79)	0.83 (0.71-0.97)	**<0.0005**
Statin therapy N (%)	14 (24%)	12 (28%)	0.652
Overall mortality, N (%)	22 (37%)	21 (49%)	0.276
Cardiovascular-related mortality, N (%)	12 (20%)	18 (42%)	**0.033**
Presence of cardiovascular events, N (%)	17 (29%)	32 (74%)	**<0.0005**

In the univariate logistic regression analysis, age (OR=1.06, p=0.001), Lp-PLA_2 (OR=1.02, p=0.004), C-Reactive Protein (OR 1.36, p= 0.028), LDL-cholesterol (OR=1.01, p=0.044), ApoB/ApoA-I ratio (OR=27.6, p=0.003), diabetes mellitus (OR=13.2, p<0.001), hypertension (OR= 3.67, p=0.002) and coronary artery disease (OR= 3.3, p=0.005) were significantly associated with cardiovascular events. Lp-PLA_2 (OR=1.02, p=0.008), age (OR=1.06, p=0.017), diabetes mellitus (OR=24.3, p<0.001) and hypertension (OR=3.7, p=0.025) remained independently associated with cardiovascular events in the multivariate analysis (Table 4).

Table 4 Cox regression analysis for cardiovascular event occurrence (a p value <0.05 was considered statistically significant).

	Univariate Cox Regression		**Multivariate Cox Regression**	
Predictor of CV events	**OR (95% CI)**	**p**	**OR (95% CI)**	**p**
Sex (M vs F)	1.50 (0.82-2.75)	0.194		
Age (ys)	1.05 (1.02-1.08)	**<0.005***	1.04 (1.01-1.07)	**0.008***
BMI	0.99 (0.94-1.06)	0.845		
Dialytic age	1.00 (0.99-1.01)	1.000		
Lp-PLA_2 activity	1.01 (1.01-1.02)	**<0.005***	1.01 (1.00-1.02)	**0.017***
C-Reactive Prtein	1.36 (1.03-1.78)	**0.028***	1.10 (0.78-1.54)	0.587
Total Cholesterol	1.01 (1.01-1.01)	0.125		
HDL-Cholesterol	0.99 (0.97-1.01)	0.225		
LDL-Cholesterol	1.01 (1.01-1.02)	**0.002***	1.00 (0.99-1.02)	0.450
Triglycerides	0.99 (0.99-1.00)	0.693		
apoB/apoA-I ratio	31.4 (7.1-138.3)	**<0.005***	3.60 (0.38-33.95)	0.264
Diabetes Mellitus	2.9 (1.7-5.2)	**<0.005***	2.72 (1.43-5.18)	**0.002***
Coronary artery disease	1.84 (1.05-3.22)	**0.034***	0.85 (0.44-1.63)	0.623
Hypertension	2.62 (1.42-4.82)	**0.002***	2.56 (1.32-4.93)	**0.005***

Figure 4. Comparison between patients with Lp-PLA_2 activity higher (dashed line) or lower (continuous line) than 194 nmol/min/ml.



Lp-PLA_2 and cardiovascular mortality in haemodialysis patients.

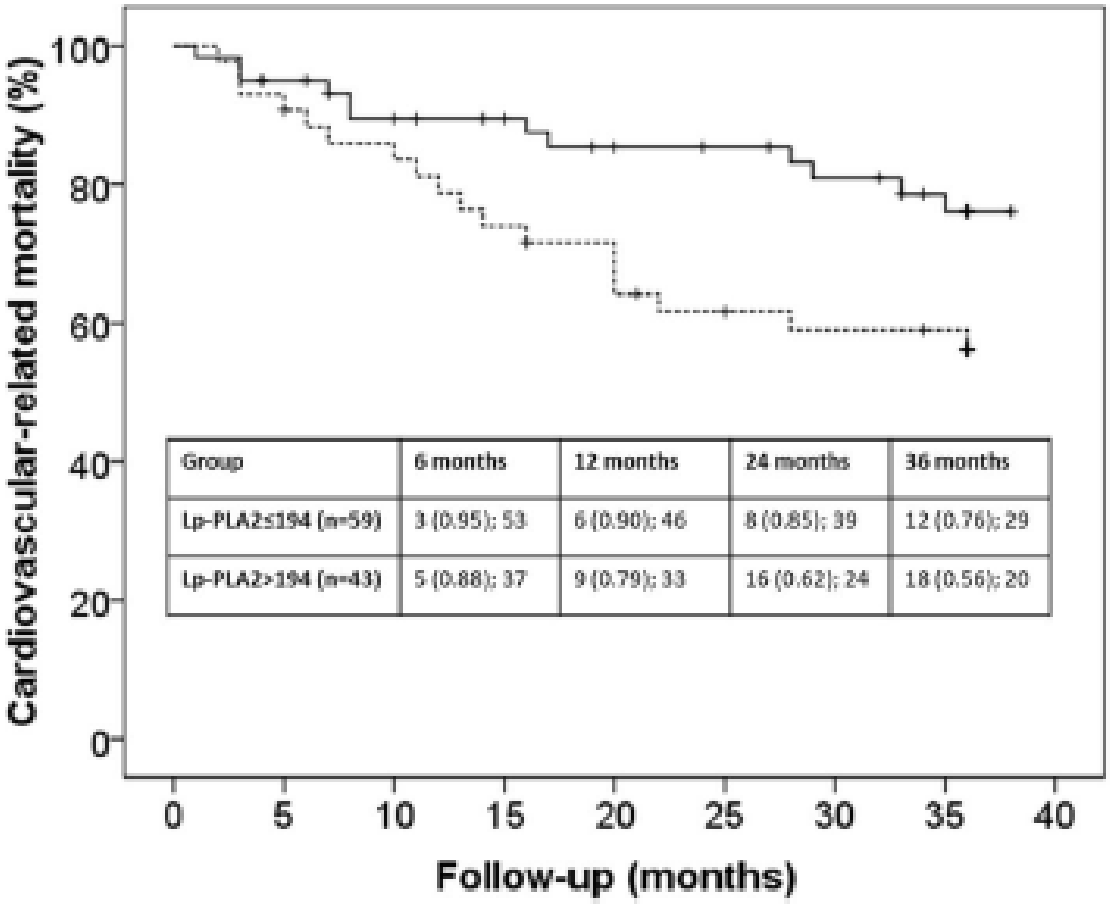
In univariate logistic regression analysis, C-Reactive Protein (OR=1.92, p=0.019), LDL-cholesterol (OR=1.02, p=0.04), ApoB/ApoA-I ratio (OR=33.8, p=0.004), diabetes mellitus (OR=4.48, p<0.001), hypertension (OR= 7.17, p=0.002), previous cardiovascular accidents (OR=45.4, p<0.001) and coronary artery disease (OR= 6.6, p<0.001) were significantly associated with cardiovascular death. In the multivariate logistic regression, the independent predictors of cardiovascular mortality were: cardiac events occurred during the follow-up (OR= 29.1, p=0.007), hypertension (OR=7.8, p=0.014) and coronary artery disease (OR=6.2, p=0.012). Lp-PLA_2 was only marginally associated with cardiovascular mortality (p=0.078) (Table 5).

The Kaplan-Meyer cardiovascular death free survival curve is showed in Figure 5.

Table 5 Logistic regression analysis for death (a p value <0.05 was considered statistically significant).

	Univariate Logistic Regression		**Multivariate Logistic Regression**	
Predictor of CV death	**OR (95% CI)**	**p**	**OR (95% CI)**	**p**
Sex (M vs F)	0.54 (0.20-1.43)	0.211		
Age (ys)	1.04 (0.99-1.08)	0.059		
BMI	0.99 (0.90-1.08)	0.768		
Dialytic age	0.99 (0.98-1.00)	0.231		
Lp-PLA_2 activity	1.01 (0.99-1.02)	0.078		
C-Reactive Protein	1.92 (1.12-3.29)	**0.019***	1.29 (0.56-2.98)	0.555
Total Cholesterol	1.01 (0.99-1.02)	0.433		
HDL-Cholesterol	0.99 (0.95-1.02)	0.381		
LDL-Cholesterol	1.02 (1.00-1.03)	**0.040***	1.00 (0.97-1.03)	0.863
Triglycerides	0.99 (0.99-1.00)	0.451		
apoB/apoA-I ratio	33.78 (3.08-369.98)	**0.004***	24.99 (0.20-3079.29)	0.190
Diabetes Mellitus	4.48 (1.75-11.48)	**0.002***	1.56 (0.38-6.48)	0.541
Coronary artery disease	6.66 (2.46-18.08)	**<0.001***	6.25 (1.50-26.11)	**0.012***
Cardiovascular events	45.37 (5.82-353.58)	**<0.001***	29.19 (2.55-333.78)	**0.007***
Hypertension	7.17 (2.25-22.81)	**0.001***	7.85 (1.52-40.48)	**0.014***

Figure 5. Comparison between patients with Lp-PLA_2 activity higher (dashed line) or lower (continuous line) than 194 nmol/min/ml.



In conclusion, in this study it was for the first time demonstrated that Lp-PLA_2 correlates with acute cardiovascular events in dialyzed patients during a mean follow up of three years.

However, other studies are needed to confirm these findings and to elucidate the potential role of Lp-PLA_2 as risk stratification marker and as treatment target in uremic subjects.

The present study was published as "De Mauri A, Vidali M, Chiarinotti D, Bellomo G, Rolla R. Lipoproteine-associate phospholipase A_2 predicts cardiovascular events in hemodialysis patients. J Nephrol 2019; 32(2): 283-288".

Journal of Nephrology (2019) 32:283–288
https://doi.org/10.1007/s40620-018-0521-3

ORIGINAL ARTICLE

Lipoprotein-associated phospholipase A2 predicts cardiovascular events in dialyzed patients

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Received: 3 May 2018 / Accepted: 30 July 2018 / Published online: 27 August 2018

CHAPTER 3

Lp-PLA_2 and peripheral arterial disease in dialyzed patients.

Peripheral artery disease (PAD) is a manifestation of atherosclerotic vascular disease, as well as coronary artery and cerebrovascular disease. The term PAD is sometimes used to summarize diseases affecting all extracoronary and extracranial arteries, including upper and lower limbs but also carotid, vertebral, mesenteric and renal arteries. More often, and also in the present thesis, the term PAD is used as a synonymous of lower limb arterial disease[31,32].

The fate of patients suffering from PAD mainly depends on the clinical presentation: patients with intermittent claudication rarely (less than 5%) progress to critical limb ischemia, but they are more likely to experience a cardiac or cerebrovascular events; patients with initial critical limb ischemia, pain at rest, foot ulcers, instead, show an amputation rate and a one year mortality rate of 30%.

PAD affects 15 to 40% of hemodialysis patients. Despite diabetes mellitus is the main determinant of PAD, advanced renal failure represents an independent risk factor for foot ulcers. In the uremic milieu, PAD quickly shows an aggressive behavior, with a high prevalence of ulcerations and amputations, about 30% and 15%, respectively, among diabetic dialyzed patients, compared to 15% and 7% among diabetic non-dialyzed ones[33-34]. In addition, renal patients undergo fewer revascularization procedures but experience more perioperative complications and worse outcomes after interventions[35,36].

Due to its biological activity, Lp-PLA_2 has been identified as a predictor of cardiovascular morbidity and mortality in several studies[3-7,28,29].

The role of Lp-PLA_2 in determining PAD remains controversial, with both positive[37,38] and negative[39] observations. In about 6000 elderly (73 years) adults of the CHS Study[37], Lp-PLA_2 mass and activity were associated with both incident low ankle-brachial index and incident PAD during a mean follow-up of 3 and 6 years. These findings were confirmed by the ARIC study[38], in which Lp-PLA_2 correlated with incident PAD among about 10000 younger patients (62 years) during a mean follow-up of 14 years. On the contrary, in the MESA study[39] (4600 patients, follow-up 9.2 years) no association was found between Lp-PLA_2 and incident subclinical PAD. These discrepant results may be explained by younger age (61 years) of the cohort, with low prevalence of comorbidities (10 to 20%) and with lower Lp-PLA_2 levels.

The burden of this severe disease is due to several traditional (diabetes mellitus, hypertension, smoke, hyperlipemia) and uremia-related (inflammation, vascular calcifications) risk factors, but probably also to other unknown vascular toxins. As a consequence, understanding the relationship between PAD and its etiological factors might allow a strict surveillance and, probably, an early treatment of this higher risk population.

STUDY 3

The aim of the third study was to evaluate the relationship between Lp-PLA_2 activity and the development of chronic limb ischemia in hemodialyzed patients.

As described in the Methods section, one hundred and two unrelated Caucasian chronic three-weekly HDP recruited in June 2013 were followed until June 2018 (Table 1).

The endpoint was the incidence of lower limb ischemia (LLI), defined as de-novo occurrence of foot ulcers, with or without soft tissue infection, ischemia or gangrene, amputation of the limb upon or below the ankle, acute occlusive ischemia and the worsening of the pre-enrollment condition needing endovascular or surgical revascularization. Traumatic injuries or calciphylaxis were not included in the aforementioned definition.

The102 HDP enrolled in Study 1 (Table 1) were followed for five years. The prevalence of hypertension, coronary heart disease (CAD), diabetes mellitus and previous PAD was 54%, 40%, 35% and 31%, respectively. Twenty-nine subjects (28%) developed lower limb ischemic lesions in a median time of 26 (9-60) months.

Interestingly, patients with abnormal Lp-PLA_2 activity had higher occurrence of lower limb lesions during the follow up (44% vs 17%, p=0.003) (Table 6 and Figure 6). However, no association was found between presence of previous PAD and higher Lp-PLA_2 levels (p=0.138).

In this study no correlations were found between Lp-PLA_2 and C-Reactive Protein or between LLI and C- Reactive Protein, but a marginal relationship between Lp-PLA_2 and LDL-cholesterol levels was evidenced, maybe because the LDL-particles are the main vehicle of Lp-PLA_2 in the bloodstream.

During the follow-up, 56 patients died, 11 (20%) for reasons directly or indirectly linked to peripheral artery disease and the related complications.

TABLE 6 Comparison between subjects with Lp-PLA_2 activity lower and higher than 194 nmol/min/ml(a p value <0.05 was considered statistically significant).

Variables	**PLA_2≤194 nmol/min/ml (N=59)**	**PLA $_2$>194 nmol/min/ml (N=43)**	**p**
Age, years (median, IQR)	69 (58-76)	73 (60-825)	0.120
Time of Dialysis, months (median, IQR)	22 (11-49)	34 (21-68)	0.072
BMI, kg/m^2 (median, IQR)	24.0 (20.8-26.6)	23.8 (21.2-25.8)	0.607
Hypertension, N (%)	33 (56%)	22 (51%)	0.690
Coronary Artery Disease, N (%)	26 (44%)	15 (35%)	0.416
Diabetes Mellitus, N (%)	22 (37%)	14 (33%)	0.678
HDP with previous PAD, N (%)	15 (25%)	17 (40%)	0.138
HDP with de novo ulcers, N (%)	10 (17%)	19 (44%)	**0.003**
Lp-PLA2 activity, nmol/min/mL (median, IQR)	162 (141.5-178)	222 (203.5-250.5)	**<0.001**
Total cholesterol, mg/dl (median, IQR)	142 (118-187)	171 (140-195)	**0.018**
LDL cholesterol, mg/dl (median, IQR)	67 (51-93)	92 (73-115)	**<0.001**
HDL cholesterol, mg/dl (median, IQR)	41 (34-53)	40 (32-47)	0.418
Triglycerides, mg/dl (median, IQR)	148 (90-204)	133 (92-209)	0.730
ApoB/ApoA1 ratio (median, IQR)	0.61 (0.54-0.79)	0.83 (0.71-0.97)	**<0.001**
CRP, mg/dl (median, IQR)	0.28 (0.06-0.75)	0.54 (0.08-1.18)	0.168

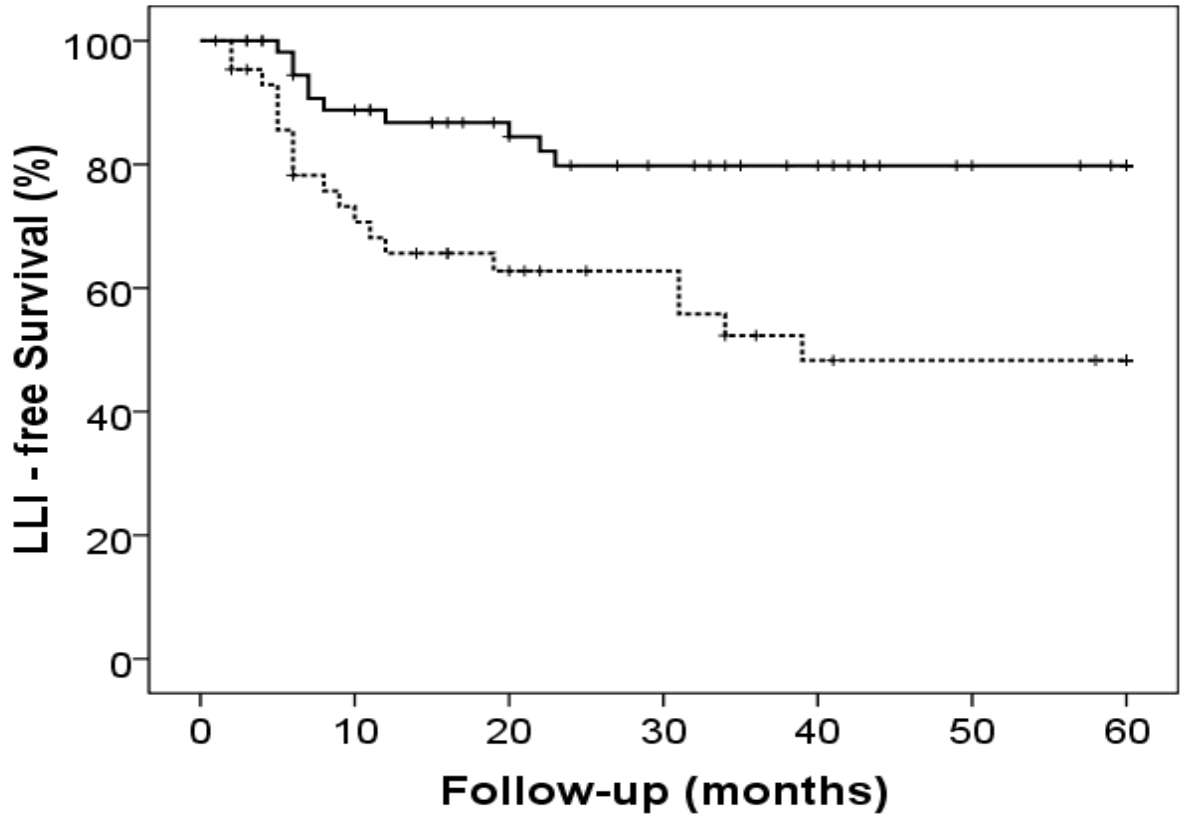

Figure 6. Lower limb ischemia free survival in patients with abnormal (dashed line) and normal (solid line) Lp-PLA_2 activity

In univariate Cox regression analysis, age (HR=1.04, p=0.027), diabetes mellitus (HR=7.6, p<0.001), coronary artery disease (HR=2.37, p=0.021), Lp-PLA_2 (HR=1.01, p=0.001), LDL-cholesterol (HR=1.02, p=0.004) and ApoB/ApoA-I ratio (HR=38.9 p<0.001) were significantly associated with reduced time to lower limb lesions (Table 7). However, in multivariate Cox regression analysis, only diabetes mellitus (HR=7.94, p<0.001) and Lp-PLA_2 activity (HR=1.01, p=0.018) remained independently associated with time to lower limb ischemia (Table 6). When the presence of previous PAD was added to the multivariate model, only previous PAD (HR=14.54, 95%CI 4.38-48.31; p<0.001) and Lp-PLA_2 activity (HR=1.01, 95%CI 1.01-1.02; p=0.003) remained associated with the development of lower limb lesions, whereas diabetes mellitus lost statistical significancy (HR=2.44, 95%CI 0.95-6.27; p=0.064).

Table 7 Cox regression analysis for time to lower limb ischemia (a p value <0.05 was considered statistically significant).

	Univariate Cox Regression		**Multivariate Cox Regression**	
Predictor of LLI	**HR (95% CI)**	**p**	**HR (95% CI)**	**p**
Sex (M vs F)	1.1 (0.52-2.33)	0.801		
Age (years)	1.04 (1.00-1.07)	**0.027***	1.02 (0.98-1.05)	0.345
Dialytic age	1.00 (0.99-1.00)	0.244		
BMI	1.00 (0.93-1.08)	0.991		
Hypertension	2.17 (0.99-4.77)	0.055		
Coronary artery disease	2.37 (1.13-4.95)	**0.021***	0.92 (0.41-2.11)	0.851
Diabetes Mellitus	7.6 (3.3-17.4)	**<0.001***	7.94 (3.07-20.55)	**<0.001***
Lp-PLA_2 activity	1.01 (1.01-1.02)	**0.001***	1.01 (1.00-1.03)	**0.018***
Total Cholesterol	1.01 (1.00-1.02)	0.075		
HDL-Cholesterol	0.99 (0.97-1.02)	0.537		
LDL-Cholesterol	1.02 (1.01-1.03)	**0.004***	1.01 (0.99-1.02)	0.358
Triglycerides	1.00 (1.00-1.00)	0.725		
ApoB/apoA-I ratio	38.9 (5.2-290.9)	**<0.001***	2.28 (0.16-32.73)	0.545
C-Reactive Protein	1.22 (0.84-1.76)	0.299		

In this study, higher levels of Lp-PLA_2 activity were associated with lower limb ischemia in HDP, independently of the traditional risk factors. This positive association might be explained by several reasons: first, HDP enrolled were older than in other studies and with a higher prevalence of traditional comorbidities; second, the uremic milieu could enhance the biological activity of Lp-PLA_2; third, only clinically evident lower limb ischemia was evaluated, representing an advanced stage of PAD, suggesting that Lp-PLA_2 might worsen previous subclinical damages, as also demonstrated by the fact that previous PAD obviously predicted *de novo* PAD. Other traditional risk factors, such as physical activity and smoking status, were not included in the analysis and this could be considered as a major limitation of this work. Moreover, with additional analyses, diabetes mellitus lost the statistical significancy maybe because of the overlap between previous PAD and diabetes mellitus itself in determining *de novo* ulcers.

In conclusion, it was demonstrated, for the first time, that Lp-PLA_2 is an independent predictor of lower limb ischemia in dialyzed patients. Larger controlled randomized trials are needed to confirm these findings and to elucidate the potential role of Lp-PLA_2 either as a pathogenic factor and a therapeutic target in peripheral artery disease.

The present study was published as "De Mauri A, Vidali M, Chiarinotti D, Bellomo G, Rolla R. Lipoprotein-associate phospholipase A_2 predicts lower limb ischemia in hemodialysis subjects. Epub ahead of print in Therapeutic Apheresis and Dialysis 2019 Dec 20, DOI 10.1111/1744-9987.13465".

ORIGINAL ARTICLE

Therapeutic Apheresis and Dialysis | ISFA | WILEY

Lipoprotein-associated phospholipase A2 predicts lower limb ischemia in hemodialysis subjects

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CHAPTER 4

Lp-PLA_2 as a target of intervention

The interest of clinicians and researchers for Lp-PLA_2 is not only due to its prognostic role, but mainly to its pathogenetic role in the development of atherosclerosis and as a potential therapeutic target.

Statins and hypolipemic drugs play a marginal role in reducing Lp-PLA_2 levels, about 10 to 30%, through an indirect effect on the overall lipid concentration.

Therefore, several efforts are ongoing to find Lp-PLA_2 inhibitors to be used as anti-atherosclerotic drugs, among which the most studied is Darapladib[40,41]. Despite in experimental models[42] and in human second phase III study[43], Darapladib has been associated to a reduction of the lipid necrotic core in carotid and coronary plaques, in large controlled randomized trials, it failed to reduce the primary endpoint of cardiovascular mortality and events in patients with stable[44] or instable[45] coronary artery disease, even if it showed some beneficial effects on the secondary endpoints. Despite someone argues that Lp-PLA_2 is not useful as a pharmacological target[46,47], other Authors believe that Lp-PLA_2 remains a very important treatment target, even if therapeutic molecules and their dose are still to be optimized.

However, no data are available in patients with renal failure. Only one study investigated the pharmacokinetic and pharmacodynamic of Darapladib in subjects with severe renal impairment, confirming an increased exposure to the drug in these patients, that, however, was well tolerated[48].

The investigation of a possible pharmacological therapy in HDP is beyond the scope of this research project.

HDP show a high prevalence and a very aggressive PAD[31,32] and undergo fewer revascularization procedures, with more perioperative complications and worse outcomes[33,34]: for instance, percutaneous transluminal angioplasty suffers from high restenosis rates in HDP. In HDP, PAD does not benefit from medical therapy and it is closely associated with an increased risk of hospitalization, morbidity and mortality[49,50].

Among non-surgical interventions, lipoprotein-apheresis (LA), a treatment consisting in the selective removal of atherogenic apo-B containing lipoproteins (VLDL-, LDL-cholesterol, Lp(a) lipoprotein) with negligible effect on atheroprotective HDL blood levels[51], is officially recognized for the treatment of atherosclerotic vascular diseases such as familial hypercholesterolemia, lipoprotein (a) hyperlipoproteinemia and PAD[52,53].

LA has been successfully used in the management of PAD in HDP: it has been demonstrated to reduce LDL, C-Reactive Protein and fibrinogen and to improve the absolute walking distance and ankle-brachial index[54].

Several mechanisms are involved in the therapeutic effects of LA in PAD:

First, the reduction of atherogenic lipoproteins serum concentrations, because they are cleared by the filters (Figure 7).

Second, several pleiotropic mechanisms, including (Figure 8 and 9):

a) increased production of anti-inflammatory and anti-atherogenic cytokines;
b) improvement of red blood cell deformability;
c) endothelial vascular cell modulation with the reduction of adhesion factors (ICAM, ELAM) and increased HGF and endothelial progenitor cells;
d) increased vasodilatation (PG, bradikinine, NO).

The final effect is a vessel dilatation, an improvement of macro and microcirculation and, in turn, tissue perfusion. For all these reasons, LA is now considered an important therapeutic option and a rescue therapy for nephropathic patients with resistant PAD[55,56].

Figure 7. Percent reduction of lipoprotein serum levels in different LA modalities. (Bambauer R et al, The Scientif World J, 2012). **The last row represents our experience, as no data are available in the literature about the clearance of Lp-PLA_2.**

	Cascade Filtration	**Immuno-adsorption**	**HELP**	**LDL-adsorption Liposorber**	**DALI**	**Liposorber D**
Cholesterol	35 – 50	30	50	45	60	55
LDL	30 – 45	35	45	35 – 40	60 – 75	60 – 75
HDL	35 – 50	20	10 – 20	-	16 - 29	5 – 13
Lp(a)	60 – 70	60	46	60	ca 40	60 – 75
Triglycerides	60	60	60	70	16	ca 66
Fibrinogen	50	10 – 20	50	30	21	20 – 40
IgM	35	10 – 20	-	-	-	14
IgA	55	10 – 20	-	-	-	-
Factor VIII	-	10 – 20	10 – 20	20	-	-
C3	-		50	-	-	-
C4	-		50	-	-	-
Plasminogen	-		50	-	-	-
Lp-PLA_2	**50**	**?**	**?**	**?**	**?**	**?**

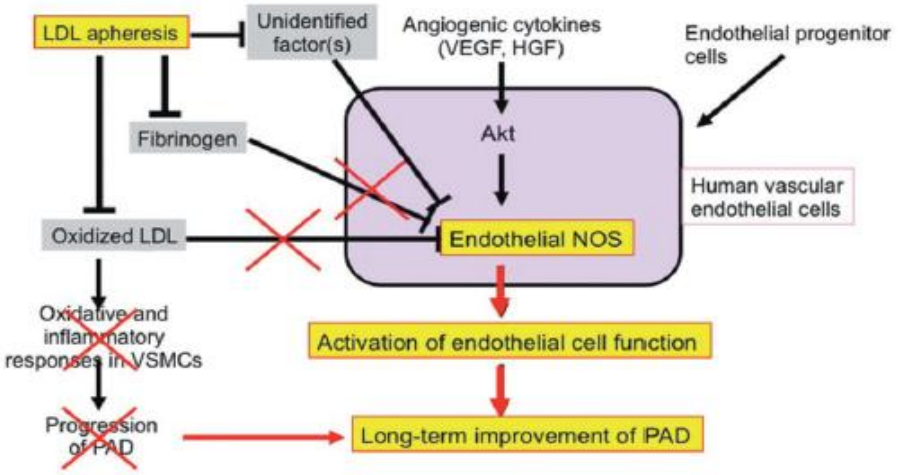
Figure 8 from Tamura K. Ther Apher Dial 2013; 17:185-192

TABLE 2. *Proposed effects of low density lipoprotein (LDL) apheresis*

- Reduction of whole blood and plasma viscosity
- Improvement of red blood cells (deformability)
- Increase of vasodilation factors (bradykinin, NO, PGI_2)
- Reduction of coagulation factors (fibrinogen etc.)
- Reduction of cell adhesion factors (ICAM-1, ELAM-1etc.)
- Reduction of CRP and MMP-9
- Inhibition of platelet activation
- Increase of HGF and endothelial progenitor cells

CRP, C-reactive protein; ELAM-1, endothelial leukocyte adhesion molecule-1 (E-selectin); HGF, hepatocyte growth factor; ICAM-1, intercellular adhesion molecule-1; MMP-9, matrix metalloproteinase-9; NO, nitric oxide; PGI_2, prostacyclin.

Figure 9 from Tamura K. Ther Apher Dial 2013; 17:185-192



Despite only one study[57] and our personal experience (case report, not published) demonstrated that apheresis sessions are able to reduce Lp-PLA_2 serum concentration, we truly believe that this approach could represent an additional therapeutic option for high risk patients with severe PAD and abnormal levels of Lp-PLA_2. While traditional surgery and endovascular procedures are able to restore circulation up to medium size arteries, lipoprotein apheresis could restore microcirculation, by reducing the aggregation of macromolecules that might occlude small vessels and capillaries and by increasing vascular permeability.

Case report

In Novara, 5 HDP (4 males, 1 female), mean age 66±9 years, dialytic vintage 5 years (1,5 to 9 years), BMI 24.8±6.3 kg/m^2, underwent lipoprotein apheresis. Two patients dropped out, one for an allergic reaction and one for an acute coronary event. Three patients successfully completed the therapy.

The type of lipoprotein apheresis performed was the Double-Filtration Plasmapheresis (DFPP): first, cells are separated from whole blood and are reinfused into the patient, then the plasma is selectively filtered to remove lipoproteins and, finally, the purified plasma is reinfused into the patient (Figure 10 and 11).

Each LA treatment consists of 2 bi-weekly sessions followed by 8 weekly sessions, treating a fluid volume equal to 1,5-2 plasma volumes during each session.

PR was a male, 66 year-old, smoker patient, suffering from hypertension, diabetes mellitus and end stage renal disease, on three-weekly hemodialysis from April 2014. In November 2016, he developed lower limb infected (S. aureus and E. coli) ischemic lesions at the left leg and at the second toe of the left foot. He underwent a percutaneous transluminal angioplasty of the left iliac-femoral arterial axis and the surgical amputation of toe (see Figure 13, panel A and B).

Between December 2016 and February 2017, an entire cycle, 12 sessions, of LA was performed.

During LA, lower limb perfusion dramatically improved and the wounds completely healed as shown in Figure 13, panel C to H.

For every session, serum levels of fibrinogen and Lp-PLA_2 were measured (other lipids were not tested because their clearance is well known in the literature). Fibrinogen was tested to monitor the safety of the treatment and avoid coagulation disorders.

As expected, serum levels of fibrinogen decreased to 41±11%, before and after LA; Lp-PLA_2 activity decreased to 47±9%, before and after DFPP (Figure 14 and 15).

To our knowledge, this is the first time that Lp-PLA_2 activity is tested during DFPP sessions (Table 7, last row), demonstrating a significant reduction of about 53%.

This case report and review of the literature were presented as an oral communication at the X Congress of Therapeutic Apheresis, Naples, 4-5th November 2016.

Figure 10. Explicative schedule of DFPP.

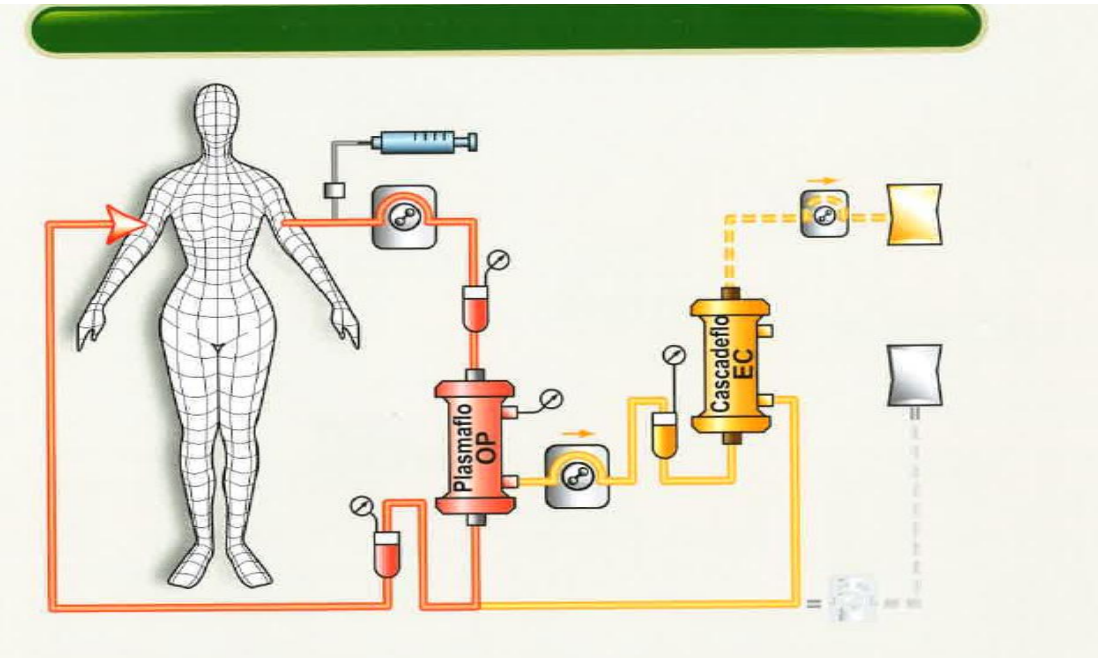


Figure 12. Details for the plasma separation process.

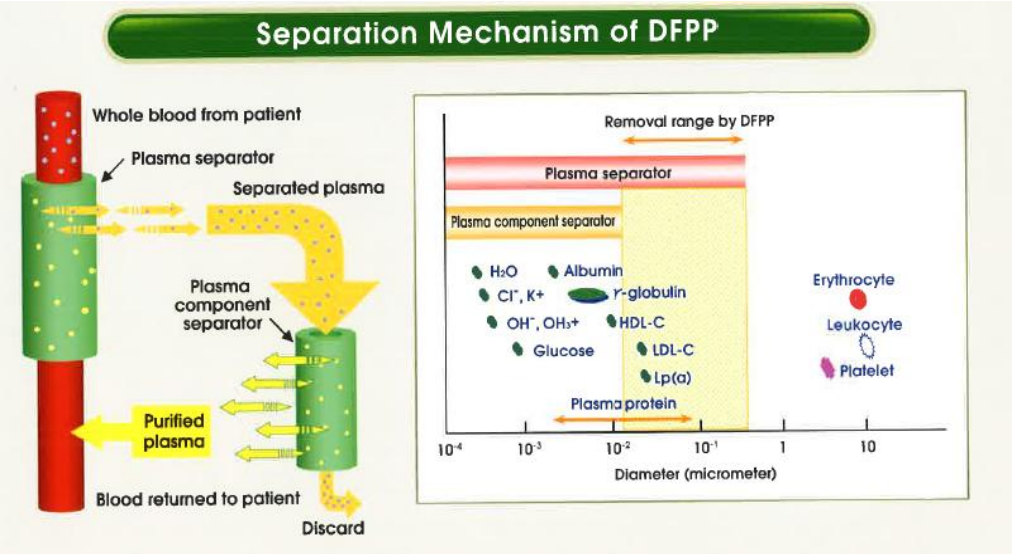
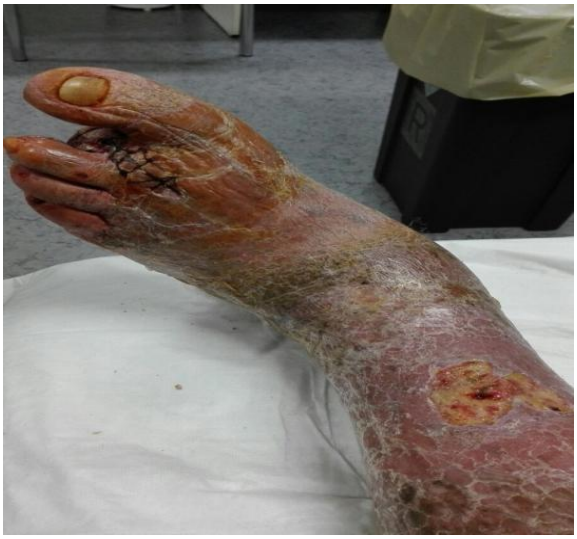


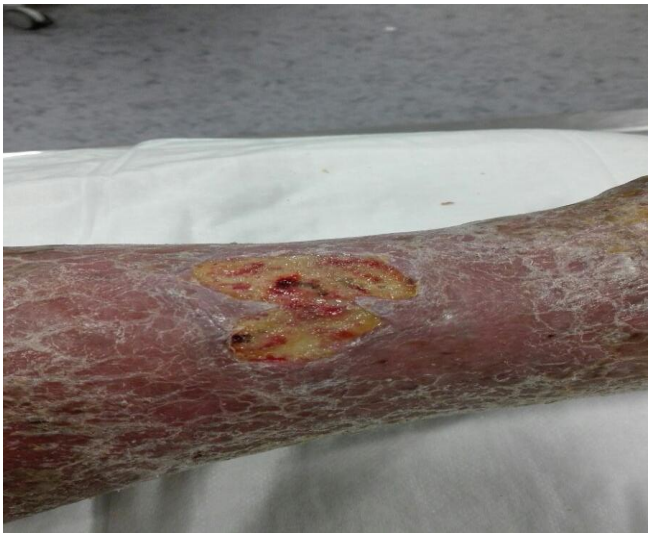
Figure 13 Wound follow-up during the 12 DFPP sessions.

Before DFFP (panel A and B)

Panel A

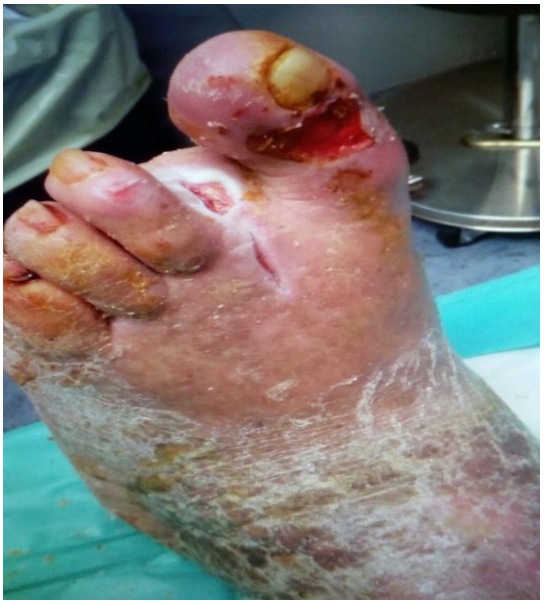


Panel B

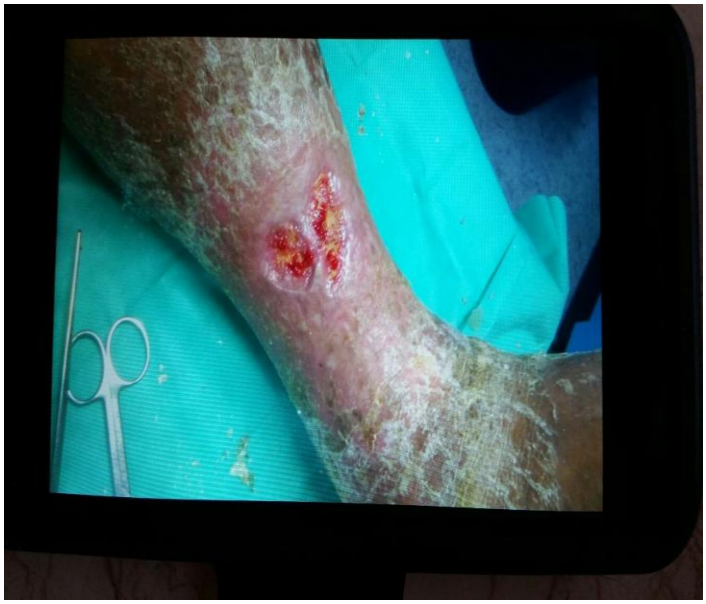


After 3 DFPP sessions (panel C and D)

Panel C

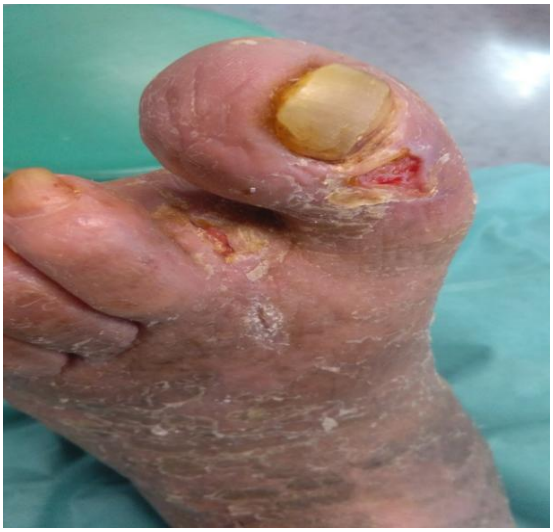


Panel D

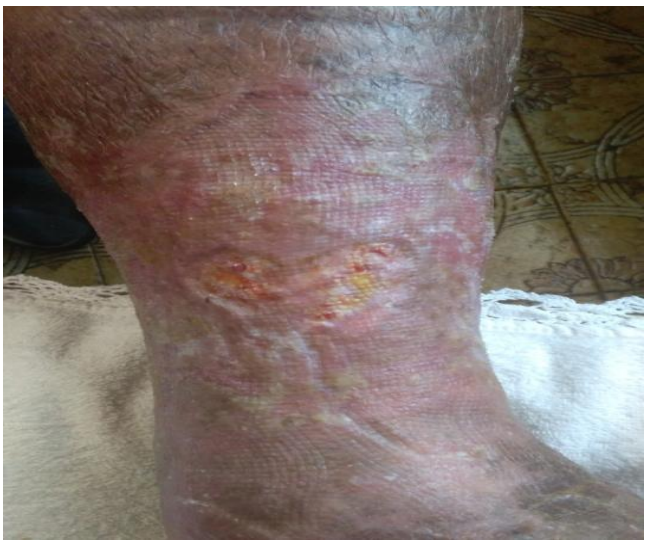


After 8 DFPP sessions (panel E and F)

Panel E

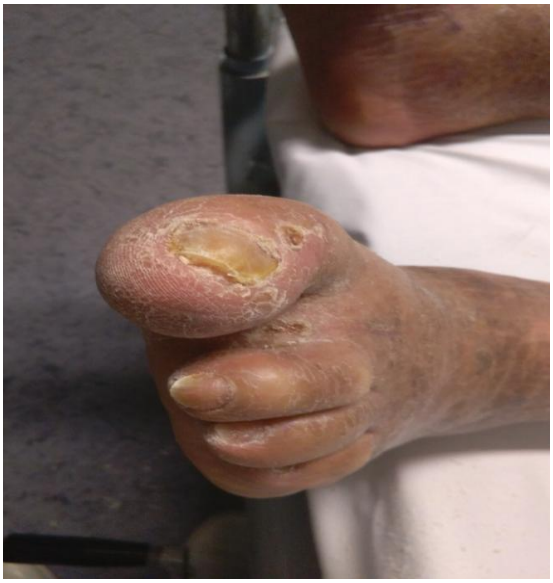


Panel F



After 12 DFPP sessions, end of the treatment (panel G and H)

Panel G



Panel H

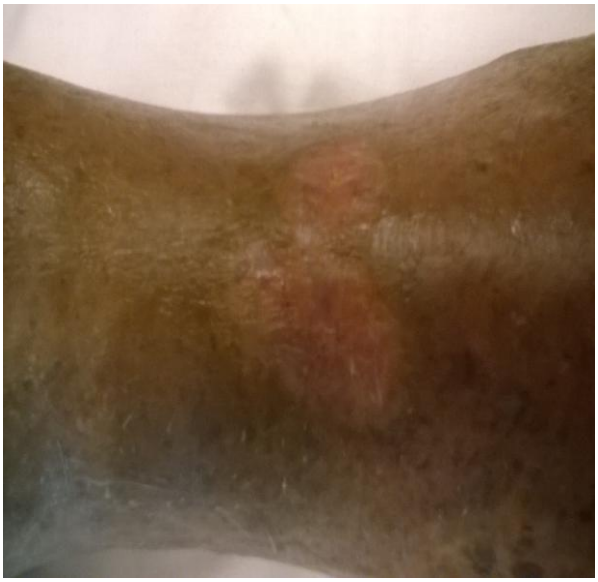


Figure 14: Percentage reduction of Lp-PLA_2 levels in every DFPP session.

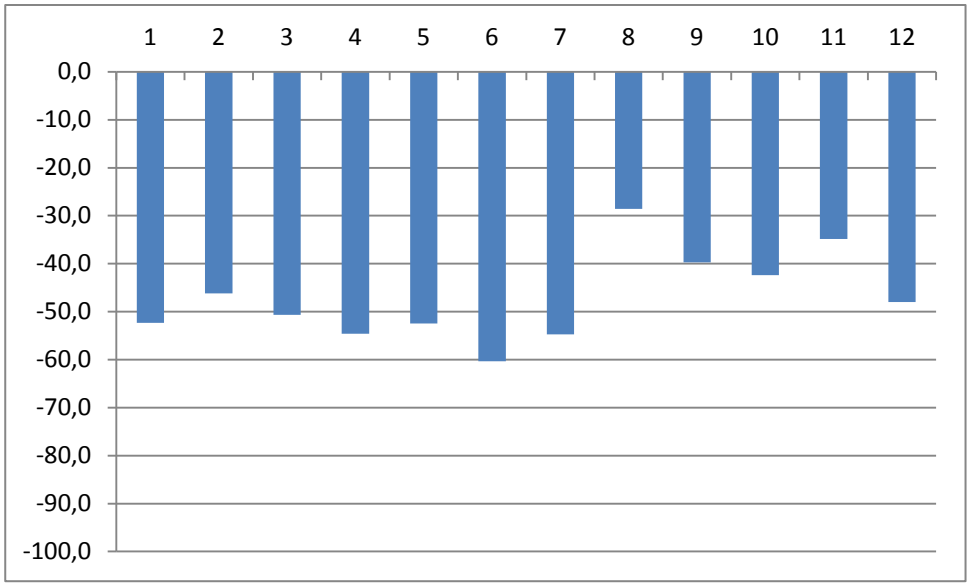
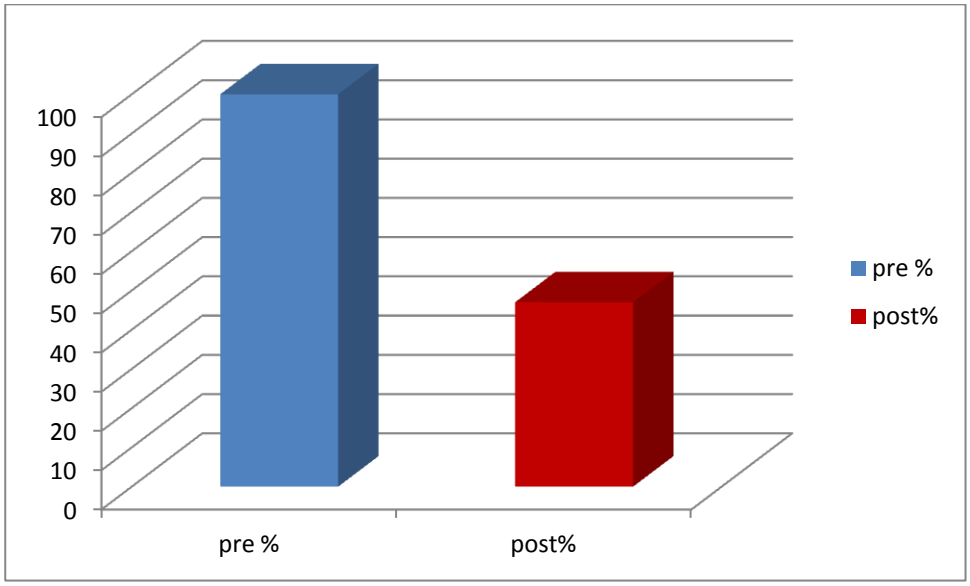


Figure 15: Mean percent reduction of Lp-PLA_2 levels with DFPP.



CHAPTER 5

Relationship between Lp-PLA$_2$ and diet

Very few studies investigated the role of the diet and the lifestyle in reducing Lp-PLA$_2$ levels and activity.

One study found that whole grains and legumes reduce LpPLA$_2$ in plasma and peripheral blood mononuclear cells in subjects with diabetes[58] with normal renal function.

The literature agrees that plant-based diets reduce atherogenic lipids and cardiovascular risk[59,60] in the general population. In particular, Lp-PLA$_2$ was reduced of 16% after only 4 weeks of plant-based diet, in dysmetabolic patients with normal renal function[61].

It is now recognized that the best dietary pattern with the strongest evidence for its ability to prevent chronic diseases is the Mediterranean diet. Mediterranean diet is rich in healthy components such as cereals, legumes, vegetables, fish and wine that can favourably modulate the pro-inflammatory and pro-atherogenic profile of several molecules[62].

Because of its high beneficial effects on the public health and its characteristic "skills, knowledge, rituals, symbols and traditions concerning crops, harvesting, fishing, animal husbandry, conservation, processing, cooking, and particularly the sharing and consumption of food", the Mediterranean diet was listed in 2010 and registered in 2013 as "Intangible Cultural Heritage of Humanity" (Document 8 COM 8.10) by UNESCO.

The Mediterranean Adequacy Index (MAI) was formulated to objectively and simply asses the food intake of several European, American and Japanese cohorts of subjects enrolled in the Seven Countries Study and longitudinally examined between 1965 and 2001[63]. MAI values range from about 1 in the United States of America to 9 in Japan and are inversely correlated with chronic diseases and mortality[64]. In Italy, MAI decreased in the recent years and the first value of 7.5 in Nicotera in 1960 decreased to 2.5-3 in Perugia in 1987[65], but its validity as a predictor tool has been confirmed[66].

MAI is calculated by dividing the sum of the percentage of total energy deriving from typical Mediterranean foods by the sum of the percentage of total energy deriving from non-typical Mediterranean foods. A MAI score > 5 indicates a good adherence to the Mediterranean diet.

$$MAI = \frac{(\%\text{en cereals} + \text{legumes} + \text{potatoes} + \text{vegetables} + \text{fruit fresh and dry} + \text{fish} + \text{wine} + \text{virgin olive oil})}{(\%\text{en milk} + \text{cheese} + \text{meat} + \text{eggs} + \text{animal fats and margarines} + \text{sweet beverages} + \text{cakes/pies} + \text{cookies})}$$

Despite these evidences across different populations, no data are available for nephropathic subjects.

STUDY4

Lp-PLA$_2$ and Mediterranean Adequacy index (MAI) in patients with advanced renal failure

The aim of the present perspective study was to evaluate MAI in patients with advanced renal failure and to investigate whether any correlation exists with Lp-PLA$_2$, cardiovascular events and renal death. All patients signed an informed consent to participate in the study.

41 adult patients with an eGFR calculated according to the MDRD formula <25 ml/min/m^2, afferent to the outpatient clinic of the Nephrology Unit in Novara, from March 2017 to March 2018 were enrolled. Of these 41 patients, 20 were males, the mean age was 63±13 years, 9 (22%) had diabetes mellitus and 7 (17%) had coronary artery disease (Table 8). Enrolled subjects, that were eating 0.78±0.55g of proteins/kg of body weight/day, received a low protein diet (LPD) prescription (protein load 0.6 g/kg of body weight/day, energy intake 30-35 kcal/kg/day, salt less than 6 g/day, phosphorus load less than 800 mg/day, low content of saturated fats and cholesterol, high content of fibres; calcium, vitamin D, folic acid, vitamin B12, iron and erythropoietin supplementation according to the usual clinical indications at the discretion of the healthcare professionals).

Patients were asked about their previous food habits to calculate the MAI index and the biochemical parameters and Lp-PLA$_2$ activity were determined, as described in the Methods section. Cardiovascular events or dialysis initiation were recorded. The study was approved by the Ethical Committee of our Institution (215/CE n. CE 30\17).

Paramaters	**41 Patients**
Age (years)	62.8±12.4
Male/female	20/11
Hypertension n (%)	37 (90)
Diabetes mellitus n (%)	9 (22)
Coronary artery disease n (%)	7 (17)
Causes of ESRD	
Hypertension	19
Diabetes mellitus	5
Genetics	8
Others	9
Baseline dietary protein content (g/kg/day)	0.78±0.55
Baseline dietary energy intake (Cal/g/day)	22.5±5.8

Table 8. Characteristics of the patients (a p value <0.05 was considered statistically significant).

The mean±sd and median MAI were 3±3.3 and 2, respectively, with higher mean MAI (7.8±5.8) among the 6 (15%) foreign patients coming from Mediterranean area than among Italians (2.2±1.8, p<0.001).

The group with a MAI≥2 (h-MAI: 4.9±3.8, close to the recommended target) was compared with the group with a MAI<2 (l-MAI: 1±0.5, close to the Northern America value).

The two groups did not differ in most parameters; the foreign subjects, mainly from the Mediterranean area, were all in the h-MAI group (30% vs 0%, p<0.01).

Of note, Lp-PLA_2 levels were significantly lower in h-MAI group than in l-MAI (139±42 vs 179±52 ng/ml/min, p=0.02) (Table 9).

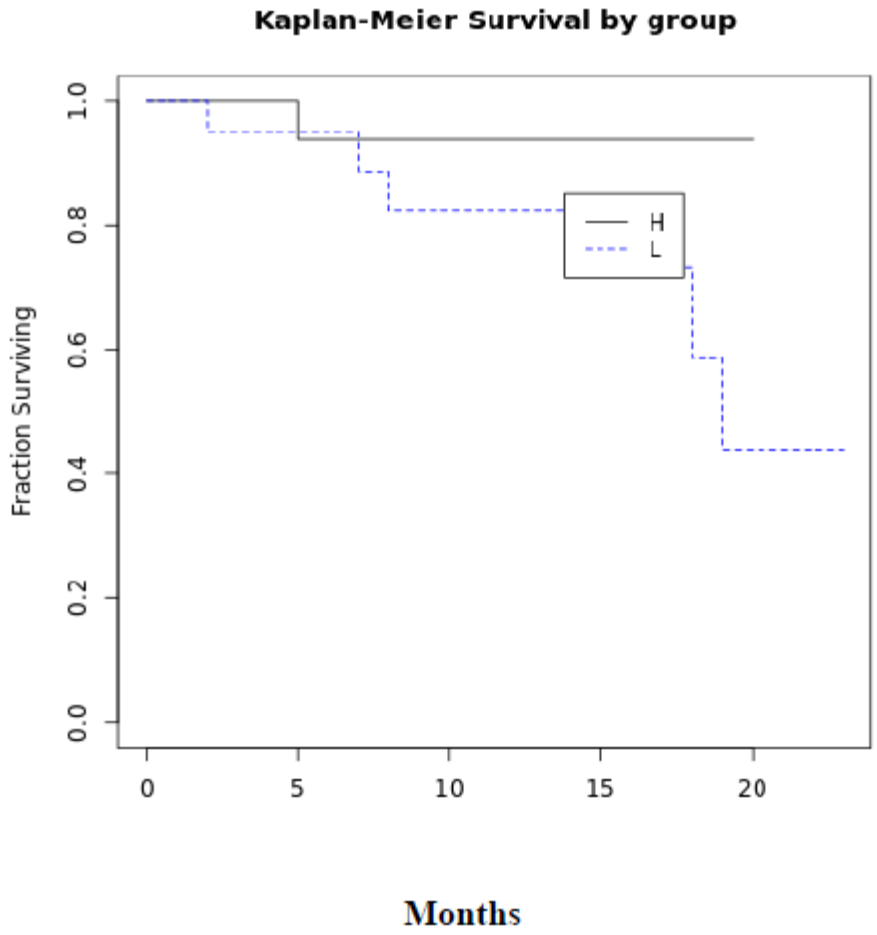
Despite boths groups started a kidney-friendly diet, in the h-MAI group more patients experienced cardiovascular events or started dialysis (0% vs 10% and 5% vs 20%, respectively) and the difference became statistically significant with regard to the combined endpoint (5% vs 30% of patients, p=0.05 and 0.04±0.21 vs 0.4±0.67 per patient, p= 0.03, respectively), in the subsequent 12±7 months after MAI calculation (Figure 16).

In conclusion, this study demonstrated, for the first time in patients with advanced renal failure, that: first, among Italian people MAI is similar than previous studies; second, MAI predicts the cardiovascular events and renal death in the subsequent year of follow-up; third, the higher the MAI the lower the Lp-PLA_2 activity, suggesting some role of the healthy diet in determining the levels of this cardiovascular marker.

Table 9. Comparison between subjects with MAI higher and lower than 2 (a p value <0.05 was considered statistically significant).

Paramteters	**MAI ≥2 (21 pz)**	**MAI<2 (20 pz)0**	**p**
MAI	4.9±3.8	1±0.5	NS
Baseline protein intake (g/kg/day)	0.68±0.16	0.81±0.23	**0.07**
Age (years)	62.3±11.5	63.3±13.6	NS
Male/female	16 (76%)	14 (70%)	NS
Hypertension n (%)	19 (90%)	18 (90%)	NS
Diabetes Mellitus n (%)	8 (38%)	1 (5%)	**0.05**
Coronary artery disease n (%)	5 (24%)	2 (20%)	NS
Foreign patients n (%)	6 (30%)	0	**0.001**
MDRD-eGFR (ml/min)	19.3±3.8	19.6±4.5	NS
Urine proteins (g/24h)	1.3±0.9	1.6±1.7	NS
Haemoglobin (g/dl)	12.3±1.2	12.3±1.9	NS
Blood Urea Nitrogen (mg/dl)	53±16	46±11	NS
Uric acid (mg/dl)	6.3±1.8	5.7±0.9	NS
Albumin (g/dl)	4.4 ±0.3	4.2±0.3	NS
Calcium (mg/dl)	9.4±0.5	9±0.5	NS
Phosphorus (mg/dl)	3.6±0.8	3.6±0.6	NS
Total cholesterol (mg/dl)	185±31	191±52	NS
HDL (mg/dl)	42±13	45±14	NS
Triglycerides (mg/dl)	216±161	200±168	NS
LDL (mg/dl)	107±27	115±43	NS
C-Reactive Protein (mg/dl)	0.4±0.6	0.5±0.6	NS
$HCO3^-$ (mmol/l)	22.9±4.1	22.9±2.8	NS
Parathyroid hormone (ng/ml)	99.9±96.3	76.9±56.7	NS
Lp-PLA_2 (nmol/min/ml)	**139±42**	**179±52**	**0.02**

Figure 16. Combined cardiovascular event and renal death free survival (months) in h-MAI group (continuous line) or l-MAI group (dashed line)



This study was presented as a poster at the 56th ERA-EDTA Congress, Budapest, 13-16/06/19 and as an oral communication at the 60th Congress of Italian Society of Nephrology, Rimini 2-5/10/19, (manuscript in preparation).

STUDY 5

Lp-PLA$_2$ and low protein diet (LPD) in subjects with advanced renal failure

Chronic kidney disease (CKD) is a condition characterized by a burden of toxins that promote systemic inflammation and atherosclerosis.

Among CKD patients low protein diet (LPD), with a protein intake of 0.8-0.6 g/kg/body weight/day, mainly from vegetables, is a useful and historically pursued option to reduce uremic symptoms, hypertension, hyperphosphatemia, proteinuria, cardiac complications, malnutrition and to delay the progression of renal failure towards the end stage[67,68.] In addition, it is a safe and low cost therapy, reducing morbidity and mortality[69,70,71].

Despite these evidences, the role of LPD in reducing Lp-PLA$_2$ activity has not been investigated.

The aim of the present perspective study was to evaluate whether the LPD could reduce Lp-PLA$_2$ serum activity.

50 adult patients with an eGFR calculated according to the MDRD formula <25 ml/min/m^2 afferent to the outpatient clinic of the Nephrology Unit, in Novara, from March 2017 to March 2018 were evaluated: 22 were excluded (5 did not sign the informed consent, 8 had instable conditions, 9 refused the LPD). 28 were enrolled (19 male, mean age 63±14 years), 6 with diabetes mellitus and 5 with coronary artery disease. Enrolled subjects, that were eating 0.78±0.55 g of proteins/kg of body weight/day (Table 10), received the following diet prescription: protein load 0.6 g/kg of body weight/day, energy intake 30-35 kcal/kg/day, salt less than 6 g/day, phosphorus load less than 800 mg/day, low content of saturated fats and cholesterol, high content of fibres; calcium, vitamin D, folic acid, vitamin B12, iron and erythropoietin supplementation according to the usual clinical indications at the discretion of the attending physician. Diet adherence was tested through dietary interviews and measuring urinary nitrogen excretion.

Lp-PLA$_2$ activity and routine biochemical parameters were measured as described in the Methods section. Bioelectrical impedance analysis (BIA) was performed to estimate total body water (TBW), fat-free body mass (kg), fat mass (kg), phase angle through an Akern model 101 (Akern Srl); hand grip and arm circumference were also measured.

Serum and bioelectrical parameters were compared before (T0) and after two months (T2) of LPD.

All patients signed an informed consent to participate in the study. The study was approved by the Ethical Committee of our Institution (215/CE n. CE 30\17)

Table 10. Baseline characteristics of enrolled patients

Parameters	**Patients 28**
Age (years)	63.5±13.7
Male/female (n)	19/9
Hypertension (n)	25
Diabetes Mellitus (n)	6
Coronary artery disease (n)	5
Baseline dietary protein intake (g/kg of body weight/day)	0.78±0.55
Baseline energy dietary intake (Cal/kg of body weight/day)	22.6±5.8

The renal function, measured as the mean of creatinine and urea clearance and with the MDRD and CKD-EPI equations, was 19.4±6.7, 18.9±4.3 and 17.5± 4.2 ml/min, respectively at T0 and remained stable during the follow-up, as well as the urinary protein excretion (about 1.3±1.5 g/24h). Total Urinary Nitrogen (TUN) significantly decreased, confirming a good adherence to the nutritional therapy (Table 11).

Table 11comparison between the period before and after 2 months of LPd (a p value <0.05 was considered statistically significant).

Parameters	**T0**	**T2**	**P**
Mean creatinine-urea clearance (ml/min)	19.4±6.7	17.7±5.4	NS
MDRD-eGFR (ml/min/1.73m^2)	18.9±4.3	19.2±4.9	NS
CKD-EPI-eGFR (ml/min/1.73m^2)	17.5±4.2	17.8±4.8	NS
Urinary protein excretion (g/24h)	1.3±1.5	1.6±2.2	NS
Total Urea Nitrogen g/24h/kg	11±3.4	9.2±2.9	**0.01**

After only 2 months of LPD, a significant decrease in total cholesterol (188±48 mg/dl vs 177±40 mg/dl, p=0.02) and a clinically, even if not statistically, significant decrease in triglycerides (224±189 vs 169±69 mg/dl, p= 0.07) were observed. Blood Urea Nitrogen, haemoglobin, uric acid, calcium, phosphorus, HDL, LDL, C-Reactive Protein, $HCO3^-$ and parathyroid hormone did not change. Interestingly, doses of epoetin Z and furosemide significantly decreased (1464±3108 to 893±2330 UI per week, p =0.019, and 40±72 to 31±42 mg per day, p=0.007, respectively). BMI

(28.4±5.8 kg/m^2), fat mass (23.2±9.8 kg), free fat mass (48.5±15.4 kg), hand grip (33±10 kg), arm circumference (31.2±4.8 cm) and phase angle (4.7±1.4 degrees) did not change, but the triceps skinfold significantly decreased (18.6±8.4 vs 17.1±7.6 mm, p=0.04).
Of note, LPD significantly reduced the Lp-PLA_2 activity (161±51 to 151±50 nmol/mil/min, p=0.02).

Table 10 Changes in parameters before and after low protein diet(a p value <0.05 was considered statistically significant).

Parameters	**T0**	**T2**	**p**
Haemoglobin (g/dl)	12.4±1.7	12.1±1.7	NS
BUN (mg/dl)	51.7±15.9	46.9±15.1	NS
Uric acid (mg/dl)	6.1±1.4	6±1.4	NS
Albumin (g/dl)	4.3 ±0.3	4.1±0.4	**0.02**
Calcium (mg/dl)	9.1±0.5	9±0.6	NS
Phosphorus (mg/dl)	3.6±0.8	3.7±0.9	NS
Total cholesterol (mg/dl)	188±46	177±40	**0.02**
HDL (mg/dl)	44±14	44±13	NS
Triglycerides (mg/dl)	224±189	169±69	NS
LDL (mg/dl)	107±46	98±35	NS
C-Reactive Protein (mg/dl)	0.6±0.7	0.6±0.7	NS
$HCO3^-$ (mmol/l)	22.9±3.8	23.7±3.1	NS
Parathyroid hormone (ng/ml)	85.1±55.7	96±55	NS
Epoetin (UI/week)	1464±3108	893±2330	**0.019**
Furosemide (mg/day)	40± 72	31±42	**0.007**
Lp-PLA_2 (nmol/mil/min)	**161.2±51**	**151±50**	**0.02**

This study demonstrated, for the first time, that a LPD reduces serum Lp-PLA_2; further studies are needed to elucidate the role of LPD in reducing the cardiovascular risk associated to Lp-PLA_2 in renal subjects.

This study was presented as a poster at the 56th ERA-EDTA Congress, Budapest, 13-16/06/19 and at the 60th Congress of Italian Society of Nephrology, Rimini 2-5/10/19, (manuscript in preparation).

CONCLUSIONS

In conclusion, with this PhD research project, it was demonstrated that Lp-PLA_2 activity is elevated and associated with a more atherogenic profile in patients with end stage renal disease on dialysis. Lp-PLA_2 correlates with acute and chronic cardiovascular events and mortality; Lp-PLA_2 also correlates with peripheral artery disease and lower limb ulcers in HDP.

In subjects with advanced renal failure Lp-PLA_2 may be considered not only a risk factor but also an independent predictor and an aetiological determinant of atherosclerotic disease.

Finally, Lp-PLA_2 might represent an important therapeutic target for pharmacological (Lp-PLA_2 inhibitors), non-pharmacological (lipoprotein apheresis) and nutritional interventions in nephropathic patients.

FUTURE PERSPECTIVES

Received: 28 November 2018 | Revised: 20 April 2019 | Accepted: 30 April 2019
DOI: 10.1002/med.21597

REVIEW ARTICLE

WILEY

Lipoprotein-associated phospholipase A2: The story continues

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As described in the present thesis, Lp-PLA_2 is recognized to exert an important role in atherosclerosis, mainly by mediating the vascular inflammation through the regulation of oxidized lipids metabolism.

However, new insights into Lp-PLA_2 biology are available and new investigations are elucidating new roles for the same actor[72].

Alteration in the levels and in the activity of Lp-PLA_2 is found in several diseases, such as diabetes mellitus, diabetic macula oedema, Alzheimer´s disease, obstructive sleep apnea, obesity, and above all, metastatic breast, colon, liver, lung and kidney cancers.

As a consequence, new efforts are ongoing to pharmacologically reduce the Lp-PLA_2 serum or tissue concentration and activity. In addition to the first clinically tested Lp-PLA_2-inhibitor by GlaxoSmithKline (Darapladib), other novel inhibitors are under consideration as "anti-Lp-PLA_2 drugs".

In this context, it is mandatory for scientists, especially for nephrologists, to include subjects with advanced renal failure in their experimental and clinical researches, in order to grant to all patients all the new therapeutic options.

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