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Plasma Lysophosphatidylcholine and Phospholipase A_2 Activity in Chagas Disease Patients: A Comparative Analysis

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Abstract

Chagas disease (CD) affects 21 countries in the Americas and is caused by the parasite *Trypanosoma cruzi*. A key molecule involved in CD is lysophosphatidylcholine (LPC), which has been studied in various contexts: in the saliva of insect vectors, during the establishment of infection in the vertebrate host, and for the parasite itself. This lipid can be produced by the action of phospholipases A_2 (PLA_2), enzymes that catalyze the hydrolysis of phospholipids releasing fatty acids and lysophospholipids, such as LPC. This study investigates LPC levels and PLA_2 activities in the plasma of CD patients and compares these levels with those in healthy individuals and patients with idiopathic dilated cardiomyopathy (IDCM). Plasma from 64 CD patients, 54 healthy individuals, and 16 IDCM patients were analyzed. LPC levels and the activity of two types of phospholipase A_2: secreted ($sPLA_2$) and lipoprotein-associated (Lp-PLA_2) were measured. LPC levels and $sPLA_2$ activity were similar between CD patients and the control groups. However, there were notable differences in LPC levels and $sPLA_2$ activity between subgroups of CD patients and IDCM patients. This study is the first to identify LPC in patients with CD across various stages of the disease. It also offers new insights into the biochemical changes observed in the plasma of patients with IDCM.

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Keywords

Lysophosphatidylcholine, Phospholipase A_2, Plasma, Chagas Disease, Idiopathic Dilated Cardiomyopathy

1. Introduction

Chagas disease (CD) is endemic in 21 countries across the Americas, where it has an annual incidence of 30,000 new cases on average and 12,000 deaths yearly [1]. CD is caused by *Trypanosoma cruzi*, a protozoan that can be transmitted by over 40 identified species in Latin America [2], including blood-sucking insects of the Hemiptera order, subfamily Triatominae (commonly referred to as 'kissing bugs'). Despite the numerous vectors transmitting *T. cruzi*, *Triatoma infestans*, *Triatoma dimidiata*, *Triatoma brasiliensis*, *Rhodnius prolixus*, and *Panstrogylus megistus* are the ones most common [3] [4].

When an infected vector feeds on a mammalian host, it eliminates infective metacyclic trypomastigotes in its feces near to the bite wound. Subsequently, the host may scratch the region, contaminating the bite wound. Once in the bloodstream, parasites invade cells, differentiating into amastigote forms that multiply and further differentiate into intracellular trypomastigotes. This process results in lysis of the invaded cells, initiating numerous cycles of cellular invasion and release of trypomastigotes [5]. During acute infection, most patients are asymptomatic. If left untreated, it can progress to chronic infection, primarily affecting the cardiovascular and digestive systems. Chagas cardiomyopathy is the predominant cause of nonischemic cardiomyopathy in Latin America, impacting approximately 30% of individuals infected with the disease [6].

In our pursuit to identify a molecule critical for *T. cruzi* infection, our group dedicated its efforts to studying a bioactive lipid known as lysophosphatidylcholine (LPC). LPC is a lysophospholipid produced by the action of phospholipases A_2 (PLA_2), which are enzymes that can catalyze the hydrolysis of phospholipids at the *sn-2* position, releasing fatty acids and lysophospholipids, such as LPC. Phospholipases A_2 can be divided into different families, including secreted phospholipases A_2 ($sPLA_2$) and lipoprotein-associated phospholipases A_2 ($Lp\text{-}PLA_2$). $sPLA_2$ are secreted and act on extracellular phospholipids in a Ca^{2+} depedent-manner. Alternatively, $Lp\text{-}PLA_2$ is a specific form of PLA_2 associated with lipoproteins, primarily low-density lipoprotein (LDL), and plays a role in the hydrolysis of phospholipids found in LDL particles, potentially generating LPC as a product [7].

The role of LPC has been described across different aspects of CD. Firstly, in 2003, Golodne and colleagues [8] showed that PC and LPC are found in the lumen of the salivary glands of *R. prolixus* and that LPC can inhibit platelet aggregation, stimulated by *α*-thrombin, in a dose-dependent manner. LPC has also been detected in the salivary glands of *Triatoma infestans*, a vector of Chagas

disease in Brazil [9]. Regarding the infection of the vertebrate host, studies show that *R. prolixus* saliva and LPC induce cell chemotaxis and enhance *T. cruzi* infection *in vivo* [10]. Also, both saliva from *T. infestans* and purified LPC have shown to increase parasitemia and result in a higher mortality rate in animals infected by *T. cruzi* [9]. Moreover, it has been reported that *T. cruzi* can produce LPC [11], and *in vitro* studies show that the parasite proliferates and differentiates more effectively under its influence in culture [12].

While studies have shown that LPC is present in various stages of CD transmission, whether is vector's saliva or produced by the parasite itself, it is not known if this molecule has any role in patients with CD. Therefore, the aim of this study was to investigate whether CD patients have circulating LPC in the plasma and whether there are significant differences in the concentration of LPC and the activity of phospholipases A_2 when comparing samples from CD patients with those from healthy volunteers and patients with a cardiopathy unrelated to CD. To achieve this, we analyzed samples from CD patients at various stages of cardiac involvement (IA, IB, II, and III of the Modified Los Andes Classification) [13].

CD is a tropical neglected disease for which there is no cure. In the chronic phase of the disease, patients can be medicated to treat symptoms developed in the various organs compromised by *T. cruzi* infection. Therefore, studies that provide us with a better understanding of the infection mechanisms and the strategies adopted by the parasite to increase the efficiency of infection and development of CD are of utmost importance.

2. Materials and Methods

2.1. Patient Selection

Blood samples were provided by patients under treatment and by volunteers attending the Cardiology Service of the Clementino Fraga Filho University Hospital (HUCFF), Rio de Janeiro, Brazil, since January 1990. The serological diagnosis of *T. cruzi* infection was confirmed by indirect hemagglutination, indirect immunofluorescence, or ELISA. A positive result in two different tests confirmed the diagnosis of CD. We evaluated sixty-four patients with CD during the years 2010 and 2013, divided into four groups according to the modified Los Andes Classification, aged between 40 and 75 years, living in endemic regions for more than 20 years. Additionally, 54 healthy individuals and 16 patients with cardiopathy with an etiology distinct from CD (Idiopathic Dilated Cardiomyopathy, IDCM) also participated in the study. The study was approved by the institution's Research Ethics Committee (number: 053/07), and all participating patients signed the informed consent form, agreeing to participate in the research. Patients with IDCM present volume overload of valvular etiology and congestive heart failure that is similar to group III of patients with CD. Volunteer individuals without CD or IDCM were selected through the HUCFF blood bank. To be included as a volunteer, the individual could not have heart disease, had to be seronegative for

Chagas disease, hepatitis B and C, VDRL (Venereal Disease research laboratory), HIV (human immunodeficiency virus), and not having undergone organ transplantation. Patients were excluded from this study if they presented any of the following events: pre-treatment for CD with Benznidazole; any heart disease associated with or without CD (cardiac ischemia, hypertension, alcoholism, diabetes) confirmed after investigation; patients pre-treated with carvedilol; abusive use of alcohol or illicit drugs; creatinine > 2.5 mg/dL or previously undergoing dialysis; evidence of acute systemic infection; chronic obstructive pulmonary disease with continuous use of bronchodilators or steroids; liver diseases; neoplastic disease or hematological disorder; inflammatory or infectious diseases; changes in eating habits or changes in medication followed by worsening of the clinical condition. Patients under medication treatment (for example: digitalis, diuretics, and/or vasodilators) had their medications suspended 48 hours before laboratory and clinical examinations, without experiencing problems during the treatment interruption. However, the use of amiodarone was maintained.

2.2. Sample Collection and Preparation

Blood samples were collected in tubes containing EDTA (ethylenediaminetetraacetic acid), transferred to microtubes and centrifuged at 277 g for 18 minutes at 24˚C. After centrifugation, the plasma was separated and frozen at −80˚C for future analyses.

2.3. Quantification of Plasma LPC

A total of 5 µl of plasma was used for the determination of plasma LPC concentration using the Azwell LPC Assay kit (Cosmo Bio Co. Ltd.), according to the manufacturer's protocol.

2.4. $sPLA_2$ Enzymatic Assay

The protein concentration in the plasma samples was determined using the Lowry Method [14] and 100 µg were used for the assay. $sPLA_2$ enzimatic activity was determined using the fluorogenic substrate 1-acyl-2-{12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl}-sn-glycero-3-phosphocholine (12:0 NBD-PC; Avanti Polar Lipids Inc.). Plasma samples were incubated at 37˚C with the standard reaction medium composed of 20 mM Tris-HCl buffer, pH 7.5 and 2 mM $CaCl_2$. The reaction was triggered by the addition of 20 µM 12:0 NBD PC substrate and the fluorescence was measured by a plate-reading fluorimeter (PerkinElmer Multilabel Reader fluorimeter) for one hour. The excitation and emission wavelengths are 460 nm and 534 nm, respectively.

2.5. Lp-PLA_2 Enzymatic Assay

The plasma activity of Lp-PLA_2 was determined using the PAF-Acetylhydrolase Assay kit (Cayman Chemical Company, USA). The assay involves incubating 10 µl of plasma with the substrate 2-thio-PAF. Upon hydrolysis of the acetylthioe-

ster bond at the *sn-2* position, free thiols are released, which are detected using 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB; Ellman's reagent). The activity of Lp-PLA_2 was determined using an ELISA reader calibrated at 405 nm, as recommended by the manufacturer.

2.6. Statistical Analysis

All experiments were performed in experimental triplicates. The data were analyzed using PRISM software (GraphPad Software, San Diego, CA). Since a variable was assessed in three or more groups, the statistical test employed was ANOVA followed by Tukey's post-test for comparisons. Values of $p \leq 0.05$ were considered significant.

3. Results

3.1. Characterization of Study Participants

The 134 individuals participating in this study were divided into three groups: healthy control, CD patients, and IDCM patients. Within the CD patient group, there were four subgroups, that referred to the clinical subdivision of disease progression according to the Modified Los Andes Classification (IA, IB, II, and III) [13]. **Table 1** presents the number of individuals, and sex in each group.

Table 1. Number of individuals and sex in each group and subgroups of CD according to the Modified Los Andes Classification.

	Control	IA	IB	II	III	IDCM
	(n = 54)	(n = 16)	(n = 17)	(n = 16)	(n = 15)	(n = 16)
Male	25	8	8	8	7	8
Female	29	8	9	8	8	8

Next, we classified these individuals according to age, body mass index (BMI), biochemical parameters (hemoglobin, creatinine, TSH, and leukocytes), and the different medications they used to verify if there were significant differences between the CD subgroups. **Table 2** presents the mean values of demographic and laboratory characteristics of 64 patients with CD (IA, IB, II, and III).

The age range of participants in the CG group was between 59 and to 63 years. The BMI of a healthy individual should be between 18.5 and 25 while individuals with a BMI between 25.1 and 30 are considered overweight ("BMI Classification," World Health Organization). The mean BMI of participants in subgroup IA of the Los Andes classification was the only BMI value that could be considered overweight. The mean BMI of individuals in subgroups IB, II, and III was considered healthy. The mean plasma hemoglobin concentrations of all subgroups were borderline but did not characterize anemia. The reference values for TSH ranged from 0.4 to 4.5 mU/L, and according to the mean TSH

Table 2. Mean age and mean laboratory values of 64 patients with CD (Modified Los Andes Classification).

	IA (n = 16)	IB (n = 17)	II (n = 16)	III (n = 15)
Age (mean ± sd)	60.38 ± 6.34	61.63 ± 11.07	63.73 ± 5.06	59.93 ± 10.79
BMI (kg/m^2)	25.5 ± 4.2	24.1 ± 3.1	24.3 ± 4.3	23 ± 3.8
Hemoglobin (g/dL ± sd)	13.05 ± 0.22	13.09 ± 0.32	12.92 ± 0.21	12.96 ± 0.43
Creatinine (mg/dL ± sd)	0.86 ± 0.16	0.91 ± 0.14	0.91 ± 0.18	0.91 ± 0.16
TSH (mU/L ± sd)	2.85 ± 0.3	2.67 ± 0.35	2.78 ± 0.42	2.67 ± 0.56
Leucocytes (10^3/mm^3 ± sd)	13.12 ± 0.3	12.98 ± 0.38	13.12 ± 0.36	13.08 ± 0.35
Captopril	7	17	15	15
Amiodarone	0	0	1	15
Spironolactone	0	0	1	15

BMI: body mass index; TSH: thyroid stimulating hormone, sd: standard deviation.

concentrations of the participating individuals, all had proper normal thyroid function. Depending on the disease stage, some individuals may be prescribed medications such as the angiotensin-converting enzyme inhibitor Captopril, the class III antiarrhythmic and vasodilator Amiodarone, and Spironolactone, which serves as a potassium-sparing diuretic and antihypertensive.

3.2. sPLA$_2$ Activity in Plasma

Phospholipases A$_2$ are a superfamily of enzymes that promote the release of LPC, an important bioactive lipid in CD. Therefore, we firstly compared the activity of sPLA$_2$ among individuals in the control group and those with CD or IDCM. **Figure 1(a)** shows no significant differences between the control and CD groups, but significant differences were observed between the control and IDCM groups, as well as between the CD and IDCM groups. We next analyzed the data by distributing CD patients according to the Modified Los Andes Classification (**Figure 1(b)**). No significant differences were observed between the subgroups IA, IB, II, and III and the control group. However, when comparing the subgroups of individuals with CD with those with IDCM we found a significant difference between subgroups IB and III and those with IDCM (**Figure 1(b)**). It is important to emphasize that individuals in group III of the Modified Los Andes Classification have the same degree of cardiac involvement as those with idiopathic cardiomyopathy and use the same medications; however, the pathologies have different etiologies.

3.3. Lp-PLA$_2$ Activity in Plasma

We next measured the activity of another PLA$_2$ family, Lp-PLA$_2$, in the patients' plasma. Comparison between groups showed no significant difference (**Figure**

2(a)). When we distribute the CD group into the four subgroups according to the Los Andes Classification, we still did not detect any significant difference in the activity of this enzyme among groups (Figure 2(b)).

3.4. Quantification of LPC Concentration in Plasma

We also determined the plasma concentration of LPC in patients. The median concentration in the control group was not significantly different from those in patients with CD (red line) (Figure 3(a)). When we distribute the group with CD according to the Modified Los Andes Classification, we do not see any significant difference among the subgroups either (Figure 3(b)). However, when comparing the median concentration of plasma LPC in patients with CG and those with IDCM, we observe significant differences between subgroups IB and II and individuals with IDCM.

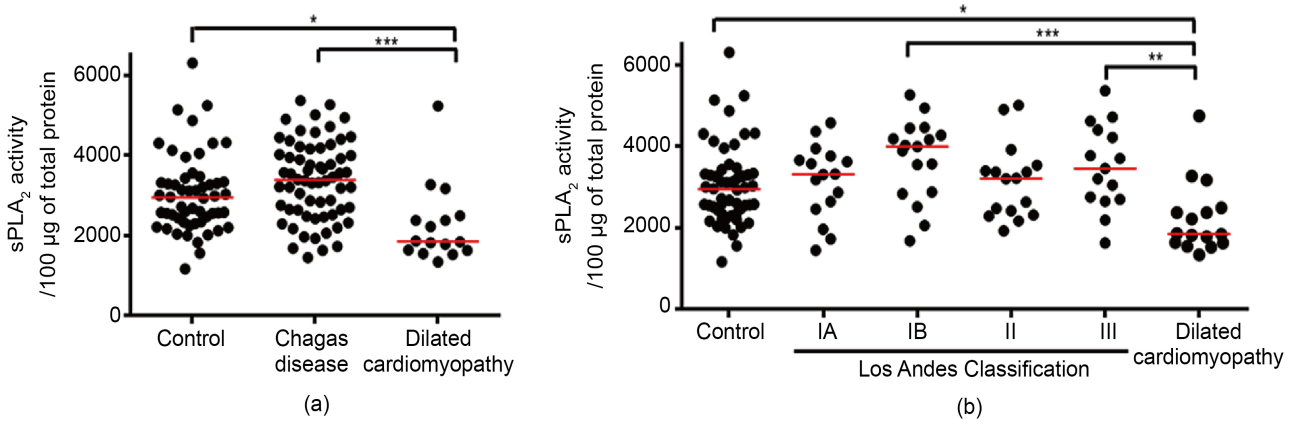

Figure 1. $sPLA_2$ activity in the plasma of control, CD and IDCM patients. $sPLA_2$ activity was measured using a fluorogenic substrate, 12:0 NBD-PC, in an enzymatic assay using 100 µg of plasma total protein. Statistical analysis: ANOVA, Tukey's post-test.

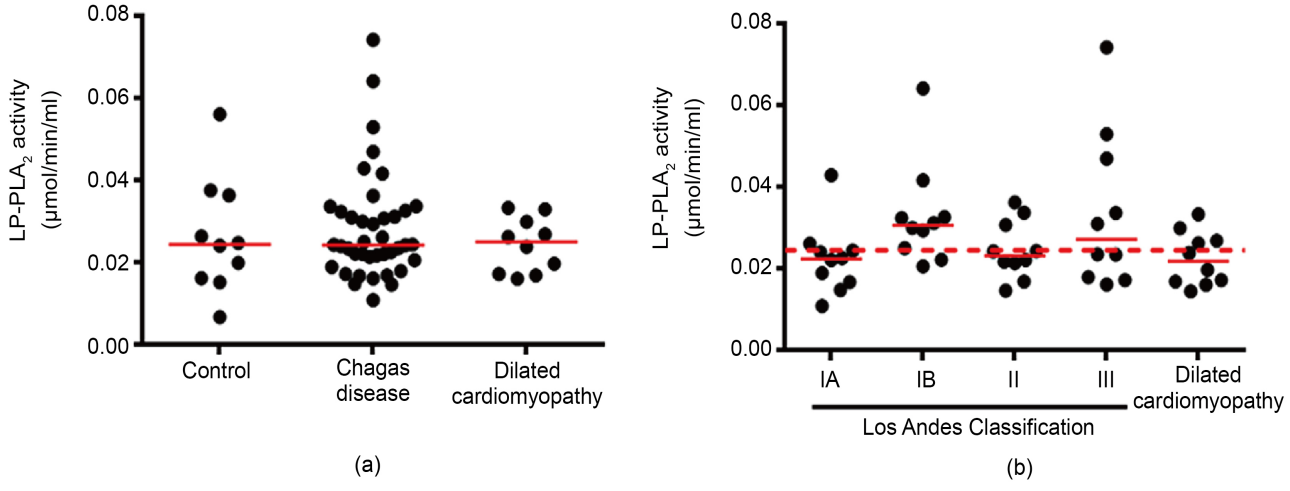

Figure 2. Lp-PLA_2 in the plasma of control, CD and IDCM patients. The plasma activity of the Lp-PLA_2 enzyme was measured using the PAF-Acetylhydrolase Assay kit (Cayman Chemical Company, USA). The dashed line in (b) corresponds to the median concentration value of the control group. Statistical analysis: ANOVA, Tukey's post-test.

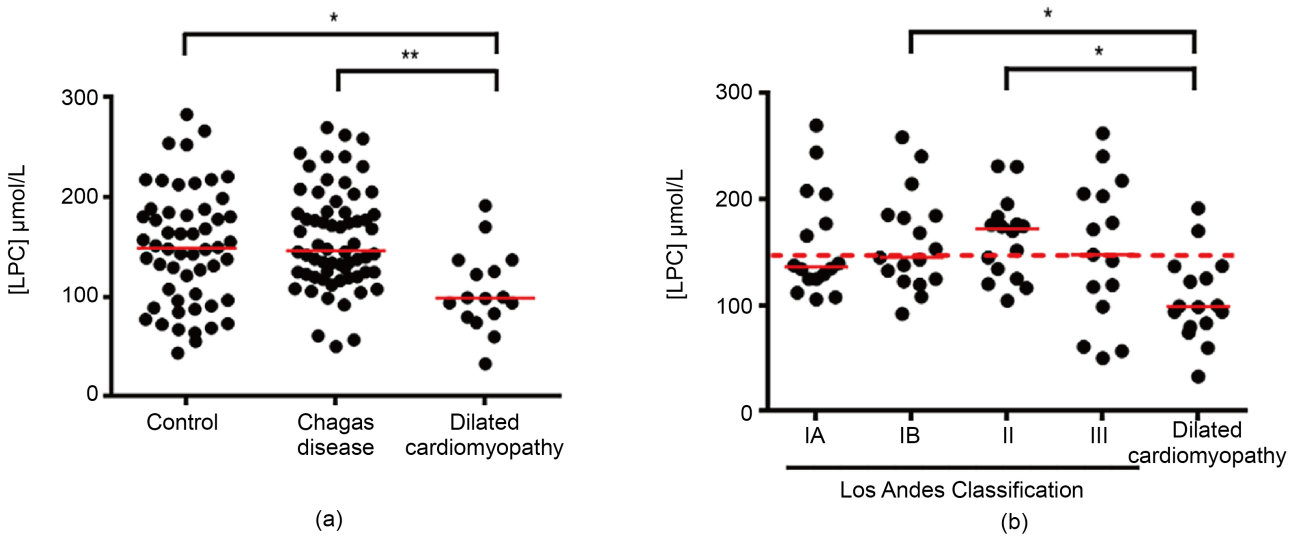

Figure 3. Concentration LPC in the plasma of control, CD and IDCM patients. A total of 5 µl of plasma were used to measure plasma LPC concentration using the Azwell LPC Assay kit (Cosmo Bio Co. Ltd.). Statistical analysis: ANOVA, Tukey's post-test.

4. Discussion

Bioactive lipids, such as lysophospholipids, are now recognized as more than mere structural molecules and components of cell membranes. They play a crucial role in cellular signaling processes, contributing significantly to the overall homeostasis of the organism [15]. In recent years, the role of bioactive lipids, including LPC, in the pathogenesis of various human diseases has been increasingly recognized.

Circulating LPC binds to albumin and lipoproteins, stimulating the modulation of the inflammatory response by blood cells. LPC is also implicated in the etiology of chronic inflammatory diseases, such as coronary artery disease and atherosclerosis development [16].

LPC can be produced by the hydrolysis of the fatty acid at *sn-2* position of phosphatidylcholine, catalyzed by PLA_2. These enzymes are divided into families based on their structure, enzymatic properties, and evolutionary relationship [17]. Among these, $sPLA_2$s and Lp-PLA_2s were studied in this work. $sPLA_2$s are calcium-dependent enzymes that exhibit unique tissue or cellular distributions and enzymatic properties, indicating distinct biological roles. Lp-PLA_2s, on the other hand, belong to the family of platelet-activating factor acetylhydrolase (PAF-AH) and are found associated with LDL in human plasma [7]. Both of these enzymes are capable of producing LPC.

Our group has extensively investigated this lipid in various aspects of CD, including its presence in the saliva of insect vectors [8] [9], its role in parasite establishment in the vertebrate host [10] and its impact on the proliferation and differentiation of *T. cruzi* [12]. However, it remained unclear whether individuals with CD exhibit alterations in LPC levels or in the activity of enzymes involved in its formation, such as PLA_2.

Therefore, this study demonstrated for the first time that LPC is present in the plasma of patients with different stages of CD. LPC has already been implicated in a number of diseases [16]. For example, a significant reduction in a number of LPC species in obese individuals with type 2 diabetes was observed [18]. Another study explored the relationship between plasma LPC concentration, weight loss, and inflammatory processes in cancer patients. LPC levels in these patients show an inverse correlation with C-reactive protein levels and may serve as a robust candidate marker for tumor progression, appearing to be an indicator of disease severity and malignancy [19].

However, plasma LPC concentration (**Figure 3(a)** and **Figure 3(b)**) and $sPLA_2$ activity (**Figure 1(a)** and **Figure 1(b)**) did not show significant differences when comparing the control group and patients with CD. Analyzing these same parameters between the subgroups of individuals with CD, according to the modified Los Andes Classification, and those with IDCM, we found a significant difference in both the LPC concentration and the $sPLA_2$ activity. Interestingly, even when comparing patients with similar levels of cardiac involvement, such as those in Group III and patients with IDCM, significant differences were evident. These findings suggest that the observed differences may be attributed to the distinct etiologies of each disease. It will be important, through lipidomic analysis, to investigate which LPC types predominate in the blood plasma of these patients, since the kit used for LPC measurement does not allow us to determine the predominant LPC species in these patients. Additionally, future assays with cultured cardiomyocytes and with knockout animals for different PLA_2 may help us understand the role of LPC in the pathogenesis of CD in patients.

IDCM is characterized by the dilation of the left ventricle and systolic dysfunction without an identifiable cause. Over 50% of patients with dilated cardiomyopathy have an idiopathic form, and in the absence of specific etiological treatments, management primarily focuses on symptom control, leading to a poor prognosis [20]. Our results show a significant reduction in LPC concentration and $sPLA_2$ activity in the plasma of patients with DCM compared to the control group. These findings could provide insights into new potential interventions for treating this disease.

As for the activity of Lp-PLA_2, while it is known to be associated with diseases such as atherosclerosis and type 2 diabetes [21], it remained remarkably consistent across all individuals analyzed, regardless of the stage of CD progression or IDCM (**Figure 2(a)** and **Figure 2(b)**).

To assess whether LPC levels and PLA_2 activity can be altered in patients with CD or IDCM, further research is warranted. Future studies should involve longitudinal collection of plasma samples from patients at multiple time points. By analyzing LPC levels and PLA_2 activity in relation to clinical progression and disease outcomes, it will be possible to elucidate any potential correlations and better understand the dynamics of them in the context of these diseases. Additionally, future analyses could include a larger sample size and other types of

cardiomyopathies for comparison beyond CD and IDCM.

As proposed by [22], the present study supports the idea that LPC is a ubiquitous lipid in host-parasite interactions. Exploring its role during infection could lead to the development of novel strategies for controlling vector transmission, managing parasite infection and understanding pathogenesis.

5. Conclusion

We examined whether CD patients have LPC in their plasma and if there are notable differences in LPC levels and phospholipase A_2 activity between CD patients, healthy volunteers, and patients with heart conditions unrelated to CD. We found that plasma LPC concentration and $sPLA_2$ activity do not change significantly between the control group and patients with CD whereas significant changes are, were observed between the subgroups of individuals with CD and those with IDCM. Lp-PLA_2 activity was consistently similar in all individuals, regardless of CD progression stage or IDCM. This is the first study to show that LPC is indeed present in patients with various degrees of CD, as this molecule was identified in different clinical stages of the disease.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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