# Changes in Phospholipid Composition of Blood Cell Membranes (erythrocyte, platelet, and polymorphonuclear) in Different Types of Diabetes—Clinical and Biological Correlations

S. Labrouche, G. Freyburger, H. Gin, M.R. Boisseau, and C. Cassagne

**A variety of disorders of erythrocyte, platelet, and polymorphonuclear leukocyte (PMN) functions have been described in diabetes. The phospholipid composition of erythrocyte, platelet, and PMN membranes from controls and from type I and II diabetics was investigated in this study. Phospholipids were determined by densitometry using the molybdenum blue reagent. In diabetics, the relative abundance of phosphatidylethanolamine (PE) increased in all cell types studied, whereas those of sphingomyelin (Sph) and phosphatidylcholine (PC) were decreased in platelets and PMN. The percentage of phosphatidylserine (PS) was reduced in erythrocytes but increased in platelets. The level of Sph in PMN was significantly lower in type I than in type II diabetics. Moreover, the longer the duration of diabetes and the poorer the metabolic control, the greater the decrease in Sph. Rheological parameters, which reflect the behavior of red blood cells (RBC), were correlated with the alteration in PE/PS ratio in these cells.**



THE ACTIVITIES of blood cells are controlled by a complex series of events mediated via the plasma membrane. Lipid components of biomembranes play a key role in membrane fluidity, and by affecting the conformation of membrane proteins, they govern exposure and diffusion of membrane components. In this study, we examined the phospholipid composition of blood cell membranes from diabetic patients to determine if they bore any relationship to the clinical condition.

The increased survival of diabetics, largely due to therapeutic improvement in metabolic control, unfortunately allows time for development of complications affecting small arteries and capillaries (microangiopathy) and large arteries (macroangiopathy or atherosclerosis). Active participation of blood cells is thought to be involved in these vascular complications, and diabetics present disorders in the function of blood cells, summarized as follows: Erythrocytes: Increased blood viscosity,[1] altered red blood cells (RBC) aggregation,[2] and increased adhesion of erythrocytes to endothelial cells.[3] Platelets: An increase in spontaneous platelet aggregation,[4] and hypersensitivity to aggregating agents of platelets from human insulin-dependent or non–insulin-dependent diabetes.[5-7] In addition, there is some evidence for an increased release of proteins from platelet α-granules in vivo in diabetic patients (platelet factor 4 and β-thromboglobulin).[6] Moreover, there are numerous reports of an increased activation of blood coagulation, which follows platelet activation.[8] Polymorphonuclear leukocytes (PMN): Bacterial infections in diabetic patients are an important cause of morbidity and mortality.[9] PMN play an important role in host defense, and impaired PMN functions have been observed in poorly controlled diabetics.[10] A decrease in leukocyte adherence in diabetics has also been reported by numerous investigators.[11,12] Alterations in random migration, chemotaxis phagocytosis, and bacterial killing have all been detected in leukocytes obtained from patients with diabetes.[13] Respiratory burst activity has been shown to be reduced[14,15] and dysregulated[16] in diabetic children. In the latter study, the resting activity of isolated PMN was significantly higher than that of PMN from controls, and the ratio of phorbol myristate acetate–stimulated activity to basal activity was found to be significantly reduced in diabetics relative to controls.

The phospholipid compositions of erythrocyte, platelet, and PMN membranes from 32 controls and 62 diabetics were determined. Particular attention was paid to patient selection and collection of clinical data. There are numerous reports on fatty acid composition of blood cell membranes in diabetes and their unsaturated to saturated ratios,[17,18] but few studies have been devoted to phospholipid composition. Phospholipids and their subclasses were determined independently of the degree of fatty acid saturation, which shows interindividual variation. The molybdenum blue reagent was chosen for its specificity toward phosphorus-containing compounds and for its independence from the degree of saturation of fatty acid moieties.

## SUBJECTS AND METHODS

### *Patients and Controls*

The control group consisted of blood donors selected at random from our blood bank. The absence of diabetes was checked in these individuals before each blood withdrawal, and none of these volunteers had taken medication for at least 10 days before the study (22 men and 10 women; mean age, 45 ± 10 years).

The 62 diabetic patients (30 men and 32 women) included 38 type I diabetics (onset of diabetes before age 21, postglucagon C-peptide–negative, insulin-treated; mean age, 52 ± 20 years; mean duration of follow-up study, 14 ± 10 years) and 24 type II diabetics (late onset, oral drug–treated, and for some, oral drug plus insulin; mean age, 65 ± 11 years; mean duration of follow-up study, 11 ± 9 years).

### *Clinical Follow-up Evaluation*

For each patient, a clinical questionnaire was completed including metabolic and biochemical parameters (glucose, hemoglobin

*From Unité 8, INSERM, Pessac-Bordeaux; CNRS-URA 1811, Université de Bordeaux II, Bordeaux; and Clinique Médicale et des Maladies infectieuses, Hôpital Pellegrin, Bordeaux, France.*

*Submitted December 24, 1994; accepted April 18, 1995.*

*Address reprint requests to S. Labrouche, PhD, Laboratoire d'Hematologie, Hôpital Pellegrin, 33076 Bordeaux Cedex, France.*



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$A_{1c}$ [$HbA_{1c}$], cholesterol, triglycerides, fibrinogen, total proteins, and albumin) and hematological (hematocrit, mean corpuscular volume, and RBC, white blood cell, and platelet counts), rheological (whole-blood viscosity and RBC aggregation), and clinical (body weight, age, diabetes duration, hypertension, alcohol consumption, and smoking habits) data, and assessment of any complications of diabetes such as retinopathy, arteriopathy, nephropathy, and neuropathy.

### *Analysis of Membrane Phospholipids*

Blood was collected by venipuncture in Vacutainer tubes (Becton Dickinson, Rutherford, NJ) containing EDTA.

*Isolation of erythrocytes.* The plasma and buffy coat were removed after centrifugation at 1,200 × *g*. RBC were washed three times by resuspending them in 5 vol 0.15-mol/L sodium chloride. Remnants of the buffy coat were removed after each wash; 0.5 mL erythrocyte "pellet" was resuspended in 0.5 mL 0.15-mol/L sodium chloride for cell counting (Coulter, Hialeah, FL).

*Isolation of platelets.* Platelet-rich plasma was collected by centrifugation at 150 × *g* for 20 minutes, and platelets were isolated by centrifugation at 1,200 × *g*. The cell pellet was washed three times by resuspending the cells in 5 vol EDTA buffer (containing 9.25 mmol/L Tris, 154 mmol/L NaCl, 6.4 mmol/L EDTA, and 5.6 mmol/L glucose, pH 7.4). The platelet pellet was resuspended in 1 mL EDTA buffer for cell counting.

*Isolation of PMN.* RBC were sedimented at 37°C on a mixture of dextran 9%–metrizoate 38% (7.2:3) according to the method of Duque and Ward.[19] The white blood cell–rich supernatant was centrifuged at 400 × *g* for 10 minutes, and residual RBC were removed by hypotonic lysis. White blood cells were then carefully layered onto a Percoll gradient (in Hanks balanced salt solution, d = 1.075) and centrifuged at 26,000 × *g* for 30 minutes at 4°C, according to the method of Hjorth et al.[20] The PMN ring was collected, washed twice in Hanks balanced salt solution, and resuspended in 1 mL Hanks balanced salt solution for cell counting (Coulter). Cell purity was greater than 97% using this procedure.

After vortexing the cells in cold distilled water, lipids were extracted using a method derived from the procedure described by Rose and Oklander for RBC,[21] with an isopropanol/chloroform (11:7 vol/vol) solvent system. The extracts were stored in chloroform/methanol (2:1 vol/vol) at −20°C and analyzed according to the method of Heape et al.[22] Briefly, lipid extracts were spotted onto 10 × 10-cm HPTLC plates (Silicagel 60F254; Merck, Darmstadt, Germany). Polar lipids were resolved by chromatography using the solvent system methyl acetate: *n*-propanol:chloroform: methanol:0.25% aqueous potassium chloride (25:25:28:10:7) for 7.5 cm of the plate. After air-drying for 5 minutes, phospholipid bands were visualized by spraying with molybdenum blue reagent[23,24] and scanned within 30 minutes at 760 nm (tungsten lamp) using a photodensitometric scanner (CAMAG TLC/HPTLC/ 76510) operating in the reflectance mode, coupled to a computing integrator (SP4100; Spectra-Physics, San Jose, CA). The scanning rate was 0.5 mm/s.

Phospholipid bands were identified by comigration with standard phospholipids, sphingomyelin ([Sph] from bovine erythrocytes), phosphatidylcholine ([PC] from egg yolk), phosphatidylserine ([PS] from bovine brain), phosphatidylinositol ([PI] from bovine liver), and phosphatidylethanolamine ([PE] from bovine brain), which were purchased from Sigma (St Louis, MO). Phospholipids were quantified by comparison with reference curves obtained with different known amounts of the different standard phospholipids run under identical conditions.

Student's *t* test was used to compare phospholipid levels between controls and patients. Clinical and biochemical parameters were related to phospholipid levels by linear regression analysis. In some cases, ANOVA was used to complete the statistical analysis by determining patient groups on either side of a threshold value (eg, duration of diabetes < 20 years and ≥ 20 years).

### *Rheological Tests*

Whole-blood viscosity was measured at 37°C in a Contraves LS30 rotational viscometer fitted with a 2T-2T bob-cup system (Contraves, Zurich, Switzerland) at seven shear rates from 0.2 to 128.5 $s^{-1}$. Plasma was examined at a single shear rate (128.5 $s^{-1}$). Due to the dependence of whole-blood viscosity on hematocrit, no normal range can be expressed for this variable. Viscosity thus must be interpreted by taking hematocrit, plasma viscosity, protein concentration, and RBC aggregation into account.

An erythroaggregometer (SEFAM, Vandoeuvre-les-Nancy, France) was used to measure erythrocyte aggregation at 37°C.[25,26] This apparatus measures changes in back-scattered light observed when RBC suspensions at different shear rates are abruptly stopped. The decrease in the optical signal reflects the formation of RBC aggregates, and a number of variables can be derived from the curve of light intensity as a function of time. They include the following: primary aggregation time (PAT), corresponding to the reverse of the slope of the curve between 0.5 and 2 seconds after cessation of maximal shear. This value decreases with increasing erythroaggregation; structure index, determined by the ratio of the intensity of back-scattered light 60 seconds after shear stop to the maximum back-scattered intensity (recorded just after the shear stress is abruptly stopped). It reflects the three-dimensional structure of the aggregates. It decreases when the shape of the aggregates changes from reticular to a more clustered form; and partial (PDT) and total (TDT) disaggregation thresholds, an estimate of the shear resistance of RBC aggregates.

The normal ranges of our laboratory were established in samples taken from 20 normal volunteers (aged 20 to 60 years): PAT, 3 ± 0.9 seconds; structure index, 0.57 ± 0.04; and PDT and TDT, 50 ± 10 and 140 ± 35, respectively.

Hematocrit was measured by microcentrifugation (Autocrit II, Becton Dickinson). Since some proteins can interfere with hemorheologic test results, levels of the following plasma proteins were measured: fibrinogen (Von Claus method), total proteins (Lowry method), and albumin (serum electrophoresis).

## RESULTS

Table 1 lists clinical and laboratory data of the population studied. The diabetic group included 32 patients presenting with no evidence of microvascular disease, whereas the remaining patients showed the following signs of diabetic complications: retinopathy (20 patients), arteriopathy (11 patients), nephropathy (four patients), and neuropathy (14 patients). The group exhibited high fasting glucose levels (mean, 12.7 ± 13.5 mmol/L) and poor metabolic control as evidenced by high $HbA_{1c}$ levels (mean, 12% ± 3%; normal, <7.5%).

Tables 2 lists, respectively, the phospholipid distribution of erythrocyte, platelet, and PMN membranes from controls, all diabetics, and patients belonging to each subgroup of diabetes. The values are expressed as percentages of the total area under the densitometric scan recording. In diabetics, PE was increased in all cell types studied. PS was decreased in RBC, leading to an increased PE/PS ratio. On the other hand, Sph and PC were significantly decreased in platelets and PMN from diabetic patients, whereas PS from platelets was higher in diabetics than in controls. Apart

**Table 1. Clinical and Laboratory Data of Type I and Type II Diabetic Subjects**

| Parameter | Type I (n = 38) | Type II (n = 24) |
|---|---|---|
| Clinical data | | |
| Age (yr) | 52 ± 20 | 65 ± 11 |
| Diabetes duration (yr) | 14.3 ± 10 | 11 ± 9 |
| Body weight (kg) | 66 ± 16 | 75 ± 21 |
| Hypertension (n) | 11 | |
| Alcohol consumption (n) | 4 | |
| Smoking habit (n) | 10 | |
| Diabetes complications (n) | | |
| Retinopathy | 20 | |
| Arteriopathy | 11 | |
| Nephropathy | 4 | |
| Neuropathy | 14 | |
| Biochemical data | | |
| Glucose (mmol/L) | 12.7 ± 13.5 | |
| $HbA_{1c}$ (%) | 12 ± 3 | |
| Cholesterol (mmol/L) | 5.7 ± 1.4 | |
| Triglyceride (mmol/L) | 1.7 ± 1.1 | |
| Fibrinogen (g/L) | 4 ± 1.8 | |
| Total proteins (g/L) | 64 ± 5 | |
| Albumin (g/L) | 40 ± 4 | |

NOTE. Results are the mean ± SD.

from Sph in PMN (see below), no significant differences were observed between the different diabetic subgroups (types I and II) for all cell types studied.

We then attempted to discern relationships between membrane phospholipid levels and various clinical parameters of the patients, including type and duration of diabetes, hypertension, complications (retinopathy, nephropathy, arteriopathy, or neuropathy), metabolic control (glucose and $HbA_{1c}$ levels), and cholesterol and triglyceride levels. In fact, alterations in membrane phospholipids were poorly correlated with the clinical data. None of the platelet or erythrocyte phospholipid alterations were found to bear any relationship to the clinical data. In PMN, the percentage modifications of PE bore no relationship to the clinical data, although there appeared to be some link between Sph and diabetes type, diabetes duration, and metabolic control ($HbA_{1c}$). Figure 1 shows that PMN Sph, as a percentage of total phospholipids, was significantly lower in type I than in type II diabetics (9.2% ± 1.6% *v* 12.4% ± 2.3%, $P < .0002$). Moreover, the longer the duration of diabetes and the poorer the metabolic control, the greater the decrease in Sph. Thus, PMN Sph was the only phospholipid found to be significantly correlated with the clinical findings.

**Table 2. Phospholipid Composition (%) of the Different Blood Cells Studied**

| Blood Cell Type | Controls | Diabetics | | |
|---|---|---|---|---|
| | | All | Type I | Type II |
| | n = 28 | n = 35 | n = 22 | n = 13 |
| Erythrocytes | | | | |
| Sph | 31 ± 2 | 32 ± 4 | 32 ± 5 | 32 ± 4 |
| PC | 20 ± 4 | 20 ± 3 | 21 ± 3 | 20 ± 3 |
| PS | 16 ± 3 | 13 ± 2† | 13 ± 2† | 13 ± 2† |
| PE | 33 ± 3 | 35 ± 4* | 35 ± 5 | 35 ± 3* |
| PE/PS | 2.2 ± 0.5 | 2.7 ± 0.5† | 2.7 ± 0.6† | 2.7 ± 0.36† |
| | n = 29 | n = 26 | n = 15 | n = 11 |
| Platelets | | | | |
| Sph | 25 ± 4 | 17 ± 4† | 18 ± 4† | 16 ± 3† |
| PC | 33 ± 5 | 28 ± 7* | 30 ± 7 | 27 ± 7† |
| PS | 13 ± 3 | 16 ± 4† | 15 ± 4* | 17 ± 4† |
| PI | 7 ± 3 | 9 ± 4 | 8 ± 4 | 10 ± 4* |
| PE | 22 ± 4 | 30 ± 3.5† | 30 ± 3† | 31 ± 4† |
| | n = 23 | n = 28 | n = 16 | n = 12 |
| PMN | | | | |
| Sph | 16 ± 3 | 11 ± 2† | 9 ± 2† | 12 ± 2† |
| PC | 33 ± 4 | 30 ± 6* | 31 ± 5 | 28 ± 5† |
| PS | 14 ± 4 | 14 ± 3 | 14 ± 3 | 15 ± 3 |
| PI | 9 ± 3 | 10 ± 3 | 9 ± 3 | 11 ± 4 |
| PE | 28 ± 6 | 35 ± 6† | 36 ± 6† | 34 ± 5† |

NOTE. Results are the mean ± SD.
*$P < .05$, †$P \leq .005$: controls *v* patients.

The possible relationship between alterations in phospholipids and disorders of cellular function was examined in RBC. Rheological parameters, which reflect the behavior of RBC, were correlated with alterations in the PE/PS ratio observed in RBC. The PE/PS ratio in RBC membranes was related to high shear rate viscosity, which is a reflection of cell deformability. Figure 2 shows the progressive decrease in the influence of the PE/PS ratio on whole-blood viscosity at decreasing shear rates. Surprisingly, the high shear rate viscosity decreased with an increase in the PE/PS ratio, indicating that high shear rate viscosity tends to be lower in diabetic patients with an altered membrane phospholipid composition.

The PE/PS ratio was not found to be correlated with low shear rate viscosity or any of the erythroaggregometer parameters. The alterations in phospholipids thus appear to have more influence on membrane deformability than on aggregation. However, erythrocyte aggregation was highly correlated with glucose levels (PAT, $P = .016$; PDT, $P = .003$; TDT, $P = .03$; low shear rate whole-blood viscosity, $P = .014$) and with fibrinogen (correlation between fibrinogen and structure index, $P = .016$).

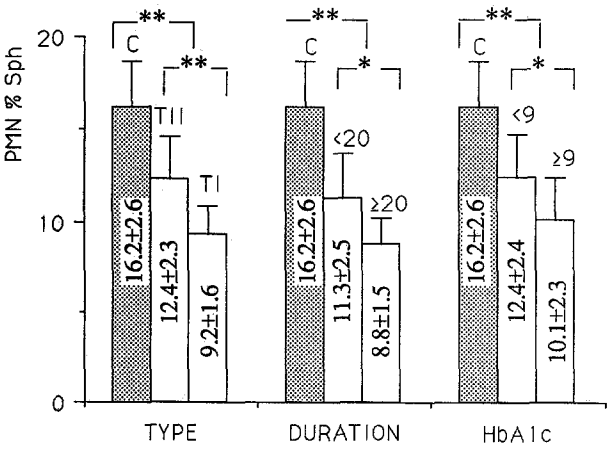


**Fig 1. Histograms showing the link between PMN Sph (expressed as % of total phospholipids) and type, duration, and metabolic control of diabetes. Significant differences between controls and patients and between each subgroup are indicated: *$P < .05$, **$P \leq .005$.**

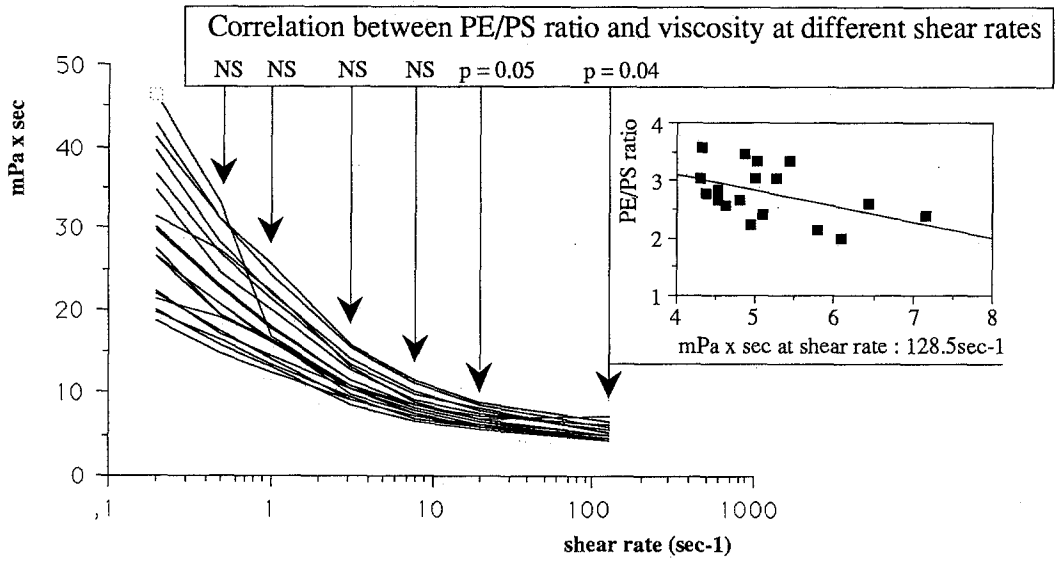


**Fig 2. Whole-blood viscosity curves of blood from diabetic patients whose RBC phospholipid composition was determined. Whole-blood viscosity increases at lower shear rates where erythroaggregation is predominant. At high shear rates, aggregates are dissociated and deformability becomes predominant. The PE/PS ratio is correlated with whole-blood viscosity at these high shear rates.**

## DISCUSSION

The phospholipid contents of erythrocytes, platelets, and PMN from normal volunteers, determined using a specific reaction for phosphorus-containing compounds, were in agreement with results reported in the literature.[18,27-39]

The increase in the PE/PS ratio of erythrocytes from diabetics (Table 2) supports the results of a previous study[39] of phospholipid and fatty acid composition of erythrocytes from type I and II diabetics. The new finding was a relationship between the RBC PE/PS ratio and whole-blood viscosity at high shear rate, which is governed largely by cell deformability. A direct link between membrane phospholipid composition and RBC behavior was thus evidenced. However, it does not explain the typical rheological impairment in diabetes, namely an enhanced RBC aggregation. Nevertheless, this diabetic rheological impairment was found in patients with altered erythroaggregation (PAT, 2.4 ± 0.5 *v* 3 ± 0.9 in controls, $P < .01$).

Marked alterations in phospholipids were observed in both platelets and PMN from diabetic patients. Sph and to a lesser extent PC were decreased, whereas platelet PS (a negatively charged phospholipid) was elevated. Neutral phospholipids were decreased in platelets, whereas the most negatively charged phospholipid, PS, was increased. It is of interest that negatively charged phospholipids like PS are known to be required for maximal clot-promoting activity. Apart from their structural functions, phospholipids form part of the cellular interface to interacting coagulation factors. Chap et al[30] have demonstrated that phospholipids in the plasma membrane of human platelets are not uniformly distributed between the two halves of the membrane bilayer. In resting, nonactivated platelets, PE, PS, and PI are primarily distributed in the inner leaflet of the bilayer. It has been found that activation of platelets by simultaneous action of thrombin and collagen alters the distribution of lipids in the plasma membrane so that a substantial amount of negatively charged PS and PE becomes exposed at the outer surface of the membrane. To balance this process, Sph appears to move from the outer to the inner leaflet of the plasma membrane.[33] Increased exposure of PS on activated platelets is involved in the hemostatic process, since negatively charged phospholipids markedly enhance conversion of factor X to factor Xa by a factor IX–factor VIIIa and $Ca^{2+}$ complex, as well as conversion of prothrombin to thrombin by a complex of factor Xa–factor Va and $Ca^{2+}$.

Although we did not determine distribution of the different lipids between inner and outer layers, we found an increase in PS and PE associated with a decrease in Sph in diabetics. The increased amount of PS may thus increase exposure of PS at the outer leaflet of activated platelets, and thus enhance activation of blood coagulation in diabetic patients, but this possibility will need to be assessed in further investigations.

As in platelets, we also observed a decrease in Sph in PMN from diabetics. There was also a relationship with the type and, interestingly, the duration and metabolic control of diabetes. Sph was dramatically decreased in type I diabetics, and to a lesser extent in type II. Sph was decreased in poorly controlled diabetics and in patients with a greater than 20-year history of the disease. Although there are many descriptions of PMN dysfunctions, their relationships with the metabolic control of diabetes have yet to be established. In a study of the relationship between PMN phagocytic functions and metabolic control parameters in diabetic patients, Marhoffer et al[40] have suggested that $HbA_{1c}$ might impair the PMN respiratory burst, resulting in an attenuated microbicidal activity. It would thus be

worth analyzing PMN phagocytic functions and membrane lipid composition in poorly controlled diabetics. The possible link between phospholipid composition and PMN behavior was supported by results of a study showing that modification of the phospholipid and fatty acid composition of PMN membranes in vitro induced an alteration of adherence, phagocytosis, and metabolic activation.[41] Subcellular fractionation and isolation of the different subcellular fractions should be performed to specify the relative contribution of the plasma membrane (~25% of the total membrane area of PMN) and subcellular organelles (~75% of the total membrane area of PMN).[42]

The exact relationships between the phospholipid alterations observed here and the functional behavior of different blood cell types will need to be elucidated in further studies.

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