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ORIGINAL ARTICLE

Oxidative stress–induced fibrinogen modifications in liver transplant recipients: unraveling a novel potential mechanism for cardiovascular risk

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Abstract

Background: Cardiovascular events represent a major cause of non–graft-related death after liver transplant. Evidence suggest that chronic inflammation associated with a remarkable oxidative stress in the presence of endothelial dysfunction and procoagulant environment plays a major role in the promotion of thrombosis. However, the underlying molecular mechanisms are not completely understood.

Objectives: In order to elucidate the mechanisms of posttransplant thrombosis, the aim of the present study was to investigate the role of oxidation-induced structural and functional fibrinogen modifications in liver transplant recipients.

Stefano Gitto and Claudia Fiorillo share first authorship.

Fabio Marra and Matteo Becatti share last authorship.

Methods: A case-control study was conducted on 40 clinically stable liver transplant recipients and 40 age-matched, sex-matched, and risk factor–matched controls. Leukocyte reactive oxygen species (ROS) production, lipid peroxidation, glutathione content, plasma antioxidant capacity, fibrinogen oxidation, and fibrinogen structural and functional features were compared between patients and controls.

Results: Patients displayed enhanced leukocyte ROS production and an increased plasma lipid peroxidation with a reduced total antioxidant capacity compared with controls. This systemic oxidative stress was associated with fibrinogen oxidation with fibrinogen structural alterations. Thrombin-catalyzed fibrin polymerization and fibrin resistance to plasmin-induced lysis were significantly altered in patients compared with controls. Moreover, steatotic graft and smoking habit were associated with high fibrin degradation rate.

Conclusion: ROS-induced fibrinogen structural changes might increase the risk of thrombosis in liver transplant recipients.

KEYWORDS
cardiovascular risk, fibrinogen, lipid peroxidation, liver transplant, oxidative stress

Essentials

- Cardiovascular events are the main causes of death after liver transplant.
- We investigated the role of oxidation-induced fibrinogen modifications after transplant.
- Transplanted patients showed systemic oxidative stress associated with fibrinogen alterations.
- The fibrinogen modifications might explain the high rate of posttransplant thrombosis.

1 | INTRODUCTION

Cardiovascular (CV) events are a leading cause of non–graft-related death in liver transplant (LT) recipients [1] and represent the third cause of late mortality [2], accounting for 19% to 42% of non–graft-related mortality [3]. Previous studies have shown that after LT, patients are at a significantly higher risk of CV disease than the general population, largely because of the high prevalence of the metabolic syndrome [4] and of the use of immunosuppressants [5,6]. In fact, calcineurin inhibitors tend to impair endothelial function and integrity contributing to a chronic allograft vasculopathy [7]. Moreover, calcineurin inhibitors lead to toll-like receptor 4–mediated endothelial inflammation, activation, and dysfunction [8]. This agrees with the observation that a chronic low-grade inflammation is present after solid organ transplantation [9–11] and chronic inflammation itself represents a well-known nontraditional risk factor for the development of CV events [12–15]. The incidence of thrombosis in LT recipients varies depending on several factors, including the type of thrombosis (eg, hepatic artery thrombosis, portal vein thrombosis, and venous thromboembolism), the population studied, and the duration of follow-up [16–18]. However, the pathogenetic mechanisms are not fully elucidated.

Fibrinogen is a 340-kDa hexameric glycoprotein containing 2 each of the 3 polypeptide chains $(A\alpha B\beta\gamma)_2$ primarily synthesized in hepatocytes. It has a central role in clot formation, particularly in the assembly of the fibrin network and in platelet aggregation [19]. Several chronic inflammatory conditions, including cirrhosis and solid organ transplantation, are associated with oxidative stress, characterized by an imbalance between oxidant and antioxidant agents [20–28]. Reactive oxygen species (ROS) has been shown to induce fibrinogen structural modifications, resulting in alterations in its biological activity [29,30] and prothrombotic phenotype characterized by a clot resistant to plasmin-induced degradation [31–33], which significantly contributes to vascular occlusion and thrombus development [34–36]. We hypothesized that fibrinogen oxidation and its functional alterations may have a role in thrombosis and CV events in LT recipients as demonstrated in another context of chronic inflammation [29].

On these bases, the present study investigates ROS-induced structural and functional fibrinogen alterations as a possible mechanism of inflammation-induced thrombosis in patients after LT. Furthermore, the correlations between fibrin resistance to plasmin-induced lysis (FR) rate and many clinical patterns of both LT recipients and donor have been also explored.

2 | METHODS

2.1 | Study design

This cross-sectional case-control study was developed in accordance with the ethical standards reported in the 1964 Declaration of Helsinki and its later amendments [37] and was approved by the local Ethics Committee ("Comitato Etico Area Vasta Centro"; approval number: 22028). All enrolled patients, before participating, signed an informed consent. The reporting of this study conforms to the Strengthening the Reporting of Observational Studies in Epidemiology guidelines [38]. The study sample included 40 LT patients and 40 age-matched, sex-matched, and risk factor–matched healthy control subjects.

Inclusion criteria were age of >18 years and LT performed not earlier than 6 months before enrollment. Exclusion criteria were therapy with antidiabetic or lipid-lowering medications; presence of chronic systemic diseases or unstable conditions in the 6 months before enrollment, including hospital admission except for prescheduled surgery, pregnancy, or lactation; adherence to a weight loss treatment program in the previous 6 months; retransplantation; multiorgan transplantation; HIV infection; or coinfection.

Forty LT recipients (92.5% White; 77% males; age, 62 ± 9 years) were consecutively enrolled from October 2022 to June 2023 in the outpatient transplant clinic of Azienda Ospedaliero-Universitaria Careggi, Florence, Italy. A control group of 40 subjects without any liver diseases (100% White; 77% males; age, 56 ± 15 years) was identified and matched 1:1 with patients, based on sex, age, body mass index (BMI), total cholesterol, and levels of low-density lipoprotein, triglycerides, glucose, and fibrinogen. Exclusion criteria were as described above for patients. Demographic and clinical characteristics of the population studied are summarized in the Table.

2.2 | Experimental procedures

Patients underwent general medical examination, including detailed medical and family history and anthropometric data registration, during one of the prescheduled follow-up visits. Personal and clinical data were collected in anonymized folders dedicated to the study. At the time of visit, blood was drawn from an antecubital vein for planned biohumoral tests requested for follow-up of the transplanted patients, and additional blood was drawn for the specific tests of the present study and processed as described below.

Enrolled patients underwent measurement of liver stiffness and controlled attenuation parameter by vibration-controlled transient elastography and carotid Doppler ultrasound with calculation of intima-medial thickness and detection of plaques.

2.3 | Sample collection and fibrinogen purification

Blood samples were collected in BD Vacutainer blood collection tubes (Becton, Dickinson and Company) containing trisodium citrate (1:10) or EDTA (0.17 mM). After centrifugation (1500× *g* for 15 minutes at 4 °C), aliquots of sodium citrate plasma were used for experiments or stored at −80 °C for further analysis. Another aliquot of sodium citrate plasma was used for fibrinogen purification using ethanol precipitation, as previously described [29,39]. Fibrinogen concentration was determined by UV/Vis Spectrophotometer (ONDA UV-20) at a wavelength of 280 nm, assuming an extinction coefficient of 1.51 mg/mL. EDTA blood samples were promptly used for fluorescence-activated cell sorting (FACS) analysis.

2.4 | Measurement of ROS production and glutathione content in peripheral leukocytes

Intracellular ROS production in leukocytes was assessed according to previously described procedures [40–42]. Briefly, 100 μL of EDTA-anticoagulated blood samples were suspended in 2 mL of Red Blood Cell Lysis buffer (BioVision). The mixture was gently agitated and then incubated at room temperature in darkness for 10 minutes, in accordance with the manufacturer's instructions. Next, the samples were centrifuged, the supernatant was discarded, and cells were washed twice in phosphate buffered saline (PBS).

To assess leukocyte ROS production and glutathione (GSH) content, cells were incubated with H_2DCFDA (2.0 μmol/L; Invitrogen) or GSH detection reagent, ThiolTracker Violet (10 μmol/L; Invitrogen) in RPMI medium (Merck KGaA) devoid of serum and phenol red. After labeling, the cells were rinsed, resuspended in PBS, and then promptly analyzed using a FACSCanto flow cytometer (Becton, Dickinson and Company). Data analysis was conducted using the BD FACSDiva software (Becton, Dickinson and Company).

2.5 | Plasma lipid peroxidation assay

Plasma lipid peroxidation was measured using an ALDetect Lipid Peroxidation assay (BML-AK170-Enzo Life) as previously reported [43]. The results are expressed as equivalent of malondialdehyde (MDA; nanomoles per milliliter).

2.6 | Plasma nitrate/nitrite and plasma GSH content assays

Plasma GSH and plasma nitrate/nitrite concentration were determined using fluorometric and spectrophotometric methods, respectively, according to the manufacturer's instructions (Cayman Chemicals).

2.7 | Determination of plasma total antioxidant capacity

Plasma total antioxidant capacity (TAC) was assessed by oxygen radical absorbance capacity assay based on the fluorescence decay of

TABLE Main patterns of enrolled transplanted patients and matched controls.

Variables	Cases (40)	Controls (40)	*P* values[a]
Anagraphic			
Sex (M/F), *n*	31/9	31/9	1.00
Age (y), mean ± SD	62 ± 9	56 ± 15	.06
White race, *n* (%)	37 (92.5)	N/A	
Time from LT (y), median (range)	4 (0-25)	N/A	
BMI (kg/m^2), mean ± SD	27.5 ± 2.4	26.8 ± 2.4	.87
Abdominal circumference (cm), mean ± SD	102.4 ± 9.9	N/A	
Anamnesis			
Hypertension, *n* (%)	26 (66.7)	N/A	
Past HCV, *n* (%)	22 (55)	N/A	
Past HCC, *n* (%)	26 (65)	N/A	
Past CVE, *n* (%)	1 (2.5)	N/A	
Familiarity for CVE, *n* (%)	13 (32.5)	N/A	
mTOR inhibitor, *n* (%)	18 (45)	N/A	
Lifestyle			
Tobacco use, *n* (%)	33 (82.5)	N/A	
Alcohol use, *n* (%)	8 (20)	N/A	
MEDI-LITE, mean ± SD	11.6 ± 1.9	N/A	
Degree of PA (low/moderate/high), *n*	24/9/7	N/A	
Laboratory			
Total cholesterol (mg/dL), mean ± SD	200 ± 35	194 ± 61	.64
LDL (mg/dL), mean ± SD	120 ± 29	114 ± 35	.87
Triglyceride (mg/dL), mean ± SD	127 ± 56	114 ± 41	.43
HDL (mg/dL), mean ± SD	56 ± 15	N/A	
Glycemia (mg/dL), mean ± SD	88 ± 16	95 ± 15	.15
Fibrinogen (mg/dL), mean ± SD	361 ± 101	342 ± 87	.63
Uric acid (mg/dL), mean ± SD	5 ± 1	N/A	
Creatinin (mg/dL), mean ± SD	0.97 ± 0.27	N/A	
TNF-alfa (pg/mL), median (range)	8.67 (4.97-46.31)	N/A	
IL-1 beta (pg/mL), median (range)	1.99 (1.24-25.15)	N/A	
IL-6 (pg/mL), median (range)	2.62 (1.33-235.71)	N/A	
IL-17A (pg/mL), median (range)	5.78 (4.65-19.29)	N/A	
Instrumental			
Liver elastography (KPa), mean ± SD	6.80 ± 3.40	N/A	
Liver elastography (CAP), mean ± SD	216 ± 46	N/A	
Right carotid IMT (μm), mean ± SD	699.88 ± 157.37	N/A	
Left carotid IMT (μm), mean ± SD	728.68 ± 179.08	N/A	
Carotid plaque, *n* (%)	16 (44)	N/A	

(Continues)

TABLE (Continued)

Variables	Cases (40)	Controls (40)	*P* values[a]
Donor patterns			
Donor age (y), mean ± SD	72 ± 13	N/A	
Donor BMI (kg/m^2), mean ± SD	26.2 ± 3.6	N/A	
Donor steatosis, *n* (%)	16 (44)	N/A	

BMI, body mass index; CAP, Controlled Attenuation Parameter; CVE, cardiovascular event; F, female; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HDL, high-density lipoprotein; IL, interleukin; IMT, intima-media thickness; LDL, low-density lipoprotein; LT, liver transplant; M, male; MEDI-LITE, adherence to the Mediterranean diet score; N/A, not applicable, not assessed; PA, physical activity; TNF, tumor necrosis factor.
[a]Wilcoxon's signed-rank test.

fluorescein upon oxidation by peroxyl radicals generated by the thermal decomposition of azo compounds such as 2,2′-azobis(2-amidinopropane) dihydrochloride. A 6-nM fluorescein solution in 75-mM sodium phosphate buffer (pH 7.4) and 250-μM Trolox (Merck KGaA) (as a standard), a water-soluble analog of vitamin E, were used. After preincubation of samples for 30 min at 37 °C with 100 μL of fluorescein, a 19-mM (final concentration) 2,2′-azobis(2-amidinopropane) dihydrochloride solution was added to start the reaction. Fluorescence was measured setting excitation at 485 nm and emission at 537 nm on a microplate fluorometer (BioTek Synergy H1). Results were expressed as Trolox equivalents (nanomoles per milliliter).

2.8 | Fibrinogen oxidation assessment

ROS-induced fibrinogen oxidation was assessed by measuring dityrosine content on purified fibrinogen fractions, using a PerkinElmer LS 55 spectrofluorometer equipped with a thermostated cell holder attached to a Haake F8 water bath, as previously described [29].

2.9 | Fibrinogen structure determination

Fibrinogen secondary structure was assessed by circular dichroism (CD) spectra of purified fibrinogen recorded on a Jasco Fluorometer (Jasco 810). CD is an excellent tool for rapid determination of protein secondary structure. CD is defined as the unequal absorption of left-handed and right-handed circularly polarized light. When molecules interact with light, they may absorb right- and left-handed circularly polarized light to different extents (hence the term CD). The result is that different structural elements display characteristic CD spectra [44]. In particular, CD spectra were recorded at 25 °C in 0.2 cm quartz cells from 280 to 180 nm (far UV) using a protein concentration of 0.5 mg/mL. Samples were filtered through 0.22 μM filters and 3 spectra recorded for each sample. Molar ellipticity values (*q*) were calculated according to the equation: $[\theta]\ (\text{deg}\cdot\text{cm}^2\cdot\text{dmol}^{-1}) = [\theta\ (\text{MRW})]/[10(\text{l})(\text{c})]$, where θ is the displacement from the baseline value *X* to the full range in degrees; MRW is the mean residue weight of the amino acids; (l) is the path length of the cell (centimeters); and (c) is protein concentration (grams and milliliter). Moreover, intrinsic fibrinogen fluorescent spectra were acquired to assess structural changes in its spatial conformation [29]. Protein intrinsic fluorescence is dependent on the presence of aromatic amino acids like phenylalanine, tyrosine, and tryptophan. In particular, tryptophan fluorescence is used to monitor structural changes in the protein as its emission maximum is highly dependent on the polarity of the environment. The intensity of the emission and the wavelength of maximum intensity for tryptophan fluorescence are indicators of the average residue microenvironment and will be monitored to provide information on protein tertiary structure. Fibrinogen intrinsic fluorescence max intensity (347 nm) was recorded at a protein concentration of 0.5 mg/mL and 25 °C in PBS using a PerkinElmer LS 55 spectrofluorometer equipped with a thermostated cell holder attached to a Haake F8 water bath. The excitation wavelength was 280 nm. A 2 mm × 10 mm quartz cuvette was used.

2.10 | Determination of 3-dimensional fibrin structure by confocal microscopy

Fibrin clots were also analyzed by confocal microscopy. Two hundred forty microliters of fibrinogen (1 mg/mL) in 100 mmol/L Tris/HCl, 5 mmol/L $CaCl_2$, and pH 7.4 were pipetted onto glass coverslips. The polymerization reaction was started by adding 60 μL of thrombin (final concentration, 0.25 U/mL) at 25 °C. After 90 minutes, samples were stained with rabbit antifibrinogen β-chain (1:100) primary antibodies (HPA001900, Sigma-Aldrich) and Alexa Fluor 555-goat anti-rabbit immunoglobulin G (H + L) secondary antibodies (1:500, Life Technologies). Glycerol was used as the mounting medium. Confocal images (ie, *z*-stacks acquired along 3 directions: *x*, *y*, and *z* axes) were acquired with a SP8 confocal microscope (Leica) equipped with Leica plan apo 63× oil immersion objective. Collected images were analyzed with Leica Application Suite X (LAS X) software. No deconvolution was applied. Fibrin deposition (surface plot) was analyzed using ImageJ software (National Institutes of Health). Fiber diameter and pore size were quantified using LAS X software. Pore size measurements were taken using a line tool to determine the average diameter of the pores on 2-dimensional slices (single stack) of the clots. In each image, at least 30 pores were measured. Although pore sizes are inherently 3-dimensional (3D), studies have demonstrated that 2-dimensional pore size measurements can accurately represent the 3D structure

of the pores [45–47]. Fiber thickness was measured by analyzing cross-sectional views of the fibers using LAS X line tool. The diameter of each fiber was measured at several points along its length to obtain an average thickness. In each image, at least 50 fibers were measured.

2.11 | Fibrinogen functional analysis

Fibrinogen functional analysis encompassed the evaluation of thrombin-catalyzed fibrin polymerization kinetics and fibrin susceptibility to plasmin-induced lysis. Thrombin-catalyzed fibrin polymerization was induced and monitored as previously described [29]. The absorbance curves were characterized in terms of the following:

i) Maximum slope (Vmax) representing the steepest part of the curve.
ii) Lag phase, quantified as the time (in minutes) elapsed until an increase in absorbance was observed.
iii) Maximum absorbance (Max Abs) of the growing clot, recorded 120 minutes after the initiation of polymerization.

In addition, we monitored the digestion of fibrin induced by plasmin as previously reported [29]. Fibrin clots were prepared in microcentrifuge tubes by incubating human thrombin (12 units/mL final concentration) with 10 μg fibrinogen in 20 μL of 100 mM Tris/HCl, 5 mM $CaCl_2$, and pH 7.4 for 2 hours at 25 °C. Plasmin was then added (5 μL of 100 μg/mL), and the fibrin clots were digested over a period of 6 hours at 37 °C. The digestion reaction was terminated by adding 10 μL of lithium dodecyl sulfate gel electrophoresis sample buffer. The same lot of thrombin and of plasmin were used for all experiments. Samples were heated at 70 °C for 10 minutes under reducing conditions (50 mM dithiothreitol). Then, aliquots from each digest (equivalent to 10 μg of fibrin) were loaded onto 4% to 12% Bis-Tris gels. After electrophoresis, gels were stained with Coomassie blue. Band intensities of stained gels were quantified by densitometry using the ChemiDoc system and Quantity-One software (Bio-Rad). Data were expressed as FR, which represents the percentage of the densitometric reading of the fibrin beta chain after 6 hours of plasmin digestion with respect to the beta band densitometric reading of the undigested protein (time 0 for incubation with plasmin). A high FR value indicates a low fibrin degradation level (and potentially a higher CV risk).

2.12 | Statistical analysis

Descriptive statistics, such as frequencies, percentages, median (range), and mean (±SD), were used to describe the sample's characteristics as appropriate. For comparison between patients and controls, we used the Wilcoxon signed-rank test. Data distribution was checked using the Shapiro–Wilk test. All experiments were performed in triplicate and, for each subject, the mean of the 3 experiments was considered, after testing the low intraexperiment and interexperiment variability and the reproducibility of measures using analysis of variance Bonferroni test. For all analyses, P values of <.05 were considered statistically significant.

We examined the relationships between FR and various patient and donor characteristics by computing correlations and performing independent samples t-tests. Specifically, Spearman's rho correlations were used investigating the relationships between FR and patient and donor's age and donor's BMI. Patients' posttransplant weight, waist circumference, BMI, time from transplant, adherence to the Mediterranean diet score, liver elastography (both stiffness and controlled attenuation parameter values), total cholesterol, high-density lipoprotein, low-density lipoprotein, triglycerides, glycemia, uric acid, creatinine, fibrinogen, tumor necrosis factor alfa, interleukin (IL)-1beta, IL-6, IL-17A, and carotid ultrasound data (in particular, left and right carotid intima-medial thicknesses) were assessed.

To explore difference in FR, t-test comparisons were used for sex, CV familiarity, history of hepatitis C virus infection and/or hepatocellular carcinoma, donor patterns (age, BMI, steatosis, and death cause), use of mTOR inhibitors, hypertension, metabolic syndrome criteria, International Physical Activity Questionnaire score (active vs no active), smoking (never vs currently/in the past, pack/years), and alcohol consumption (no vs yes), carotid ultrasound data about carotid plaque (presence or absence). As measures of effect size [48], Cohen's d was used for t-test. Values from 0.2 to 0.5 are indicators of a small effect, values from 0.5 to 0.8 represent a medium effect, and values from 0.8 represent a large effect.

Statistical analyses were performed using the Graph Pad Prism 5 software (GraphPad Software), STATA (StataCorp LLC, version 14), and SPSS (IBM, version 28). For all analyses, P values of <.05 were considered statistically significant.

3 | RESULTS

3.1 | Patients' characteristics

LT patients and matched controls were comparable in terms of demographic features and CV risk profile. Demographic and clinical characteristics of the population studied are summarized in the Table.

Enrolled patients had normal liver function tests, including prothrombin time, albumin, and bilirubin. Notably, the study criteria excluded patients with significant alterations in liver tests, including both function tests and transaminases. None of the enrolled patients were on anticoagulant therapy.

3.2 | Intracellular leukocyte redox status

Data on intracellular-derived ROS production and GSH content in leukocyte subpopulations in LT patients and matched controls are reported in Figure 1. LT patients showed a significant increase in ROS levels in all the 3 leukocyte fractions as compared with controls. Specifically, lymphocyte ROS levels were 1288 (1131-1584) vs 663

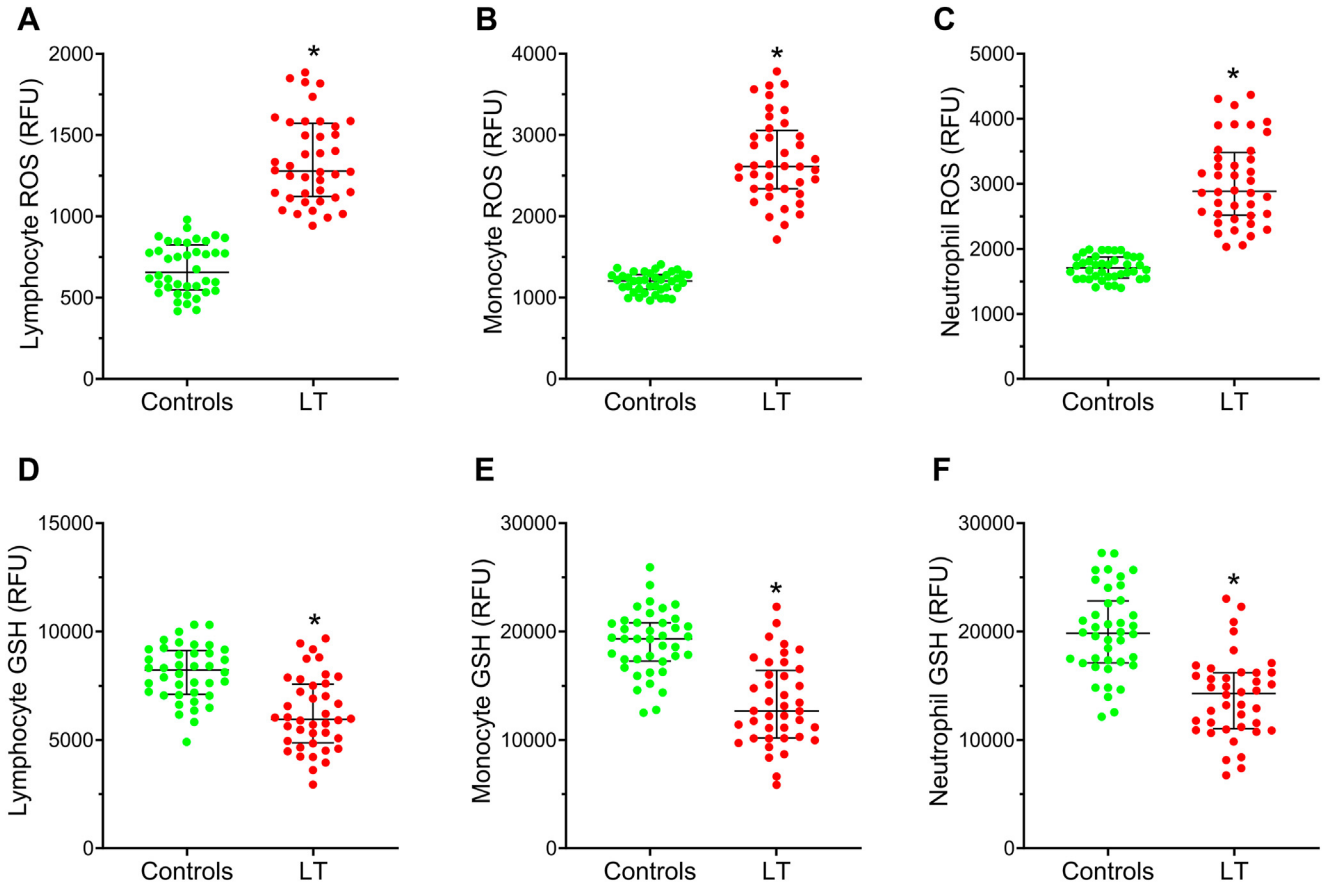

FIGURE 1 Increased leukocyte reactive oxygen species (ROS) production and decreased leukocyte glutathione (GSH) content in liver transplant (LT) patients compared with controls. Flow cytometry analysis shows significant alteration in (A–C) lymphocyte-, monocyte-, and neutrophil-derived ROS and (D–F) lymphocyte, monocyte, and neutrophil GSH content in LT patients vs controls. *Statistical significance ($P < .001$). RFU, relative fluorescence units.

(553-833) relative fluorescence units (RFU) for LT and controls, respectively ($P < .001$; Figure 1A), monocyte ROS levels were 2610 (2335-3052) vs 1208 (1106-1284) RFU ($P < .001$; Figure 1B), and neutrophil ROS levels were 2899 (2530-3495) vs 1721 (1564-1887) RFU ($P < .001$; Figure 1C). Regarding intracellular GSH content, lymphocyte GSH levels were 5916 (4837-7549) vs 8190 (7074-9090) RFU for LT and controls, respectively ($P < .001$; Figure 1D), monocyte GSH levels were 12,616 (10,140-16,327) vs 19,233 (17,193-20,716) RFU ($P < .001$, Figure 1E), and neutrophil GSH levels were 14,277 (10,999-16,195) vs 19,836 (17,098-22,831) RFU ($P < .001$, Figure 1F). These results indicate the presence of oxidative stress in leukocytes from LT patients.

3.3 | Systemic redox status

Systemic redox status was assessed by quantifying lipid peroxidation marker (MDA), TAC, nitrate/nitrite, and GSH content in plasma. A significantly enhanced lipid peroxidation was found in the plasma of LT patients as compared with controls (1.83 [1.66-1.96] vs 0.35 [0.30-0.38] MDA nmol/mL; $P < .001$), paired by a significantly reduced TAC (16.7 [15.1-18.5] vs 23.1 [19.2-25.6] mM Trolox equivalent; $P < .001$; Figure 2A, B, respectively). Moreover, a significantly increase in plasma nitrate/nitrite content was found in LT patients as compared with controls (11.99 [7.70-16.45] vs 5.39 [3.66-6.83] nmol/mL; $P < .001$), paired by a significantly reduced GSH plasma content (12.84 [8.35-23.02] vs 22.49 [17.92-26.97] nmol/mL; $P < .001$; Figure 2C, D, respectively). These results indicate the presence of systemic oxidative stress in LT patients.

3.4 | Fibrinogen structural alterations and fibrinogen oxidation

Protein function is directly related to protein structure. A protein specific shape determines its function. Even slight alterations in protein structure can exert significant effects on their biological functions. To assess the impact of impaired redox status on fibrinogen structure, we qualitatively compared far-UV CD spectra of fibrinogen purified from LT patients and matched controls. Fibrinogen purified from controls showed a typical α-helix secondary structure with minima at 208 and 222 nm, whereas a decreased negative peak in the 215 nm to 225 nm region was observed in LT, suggesting a reduced α-helical content (Figure 3A). This result

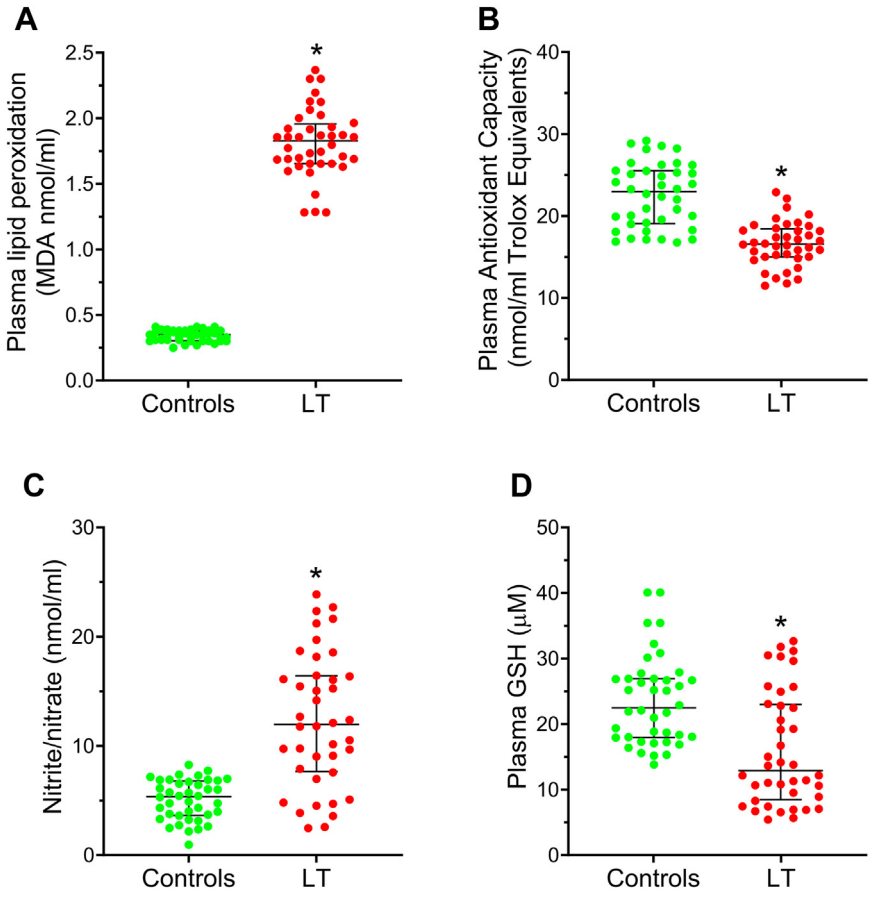

FIGURE 2 Signs of oxidative stress in plasma from liver transplant (LT) patients. (A, B) Plasma lipid peroxidation and total antioxidant capacity in LT patients and controls. (C, D) Nitrate/nitrite and glutathione (GSH) levels in LT patients vs controls. *Statistical significance ($P < .001$). MDA, malondialdehyde.

demonstrates fibrinogen structural alterations in LT patients. The exploration of fibrinogen tertiary structure was extended through a comprehensive analysis of the intrinsic fluorescence characteristics exhibited by purified fibrinogen samples. These characteristics are primarily influenced by the extent to which hydrophobic amino acid residues are exposed to the surrounding solvent environment. This

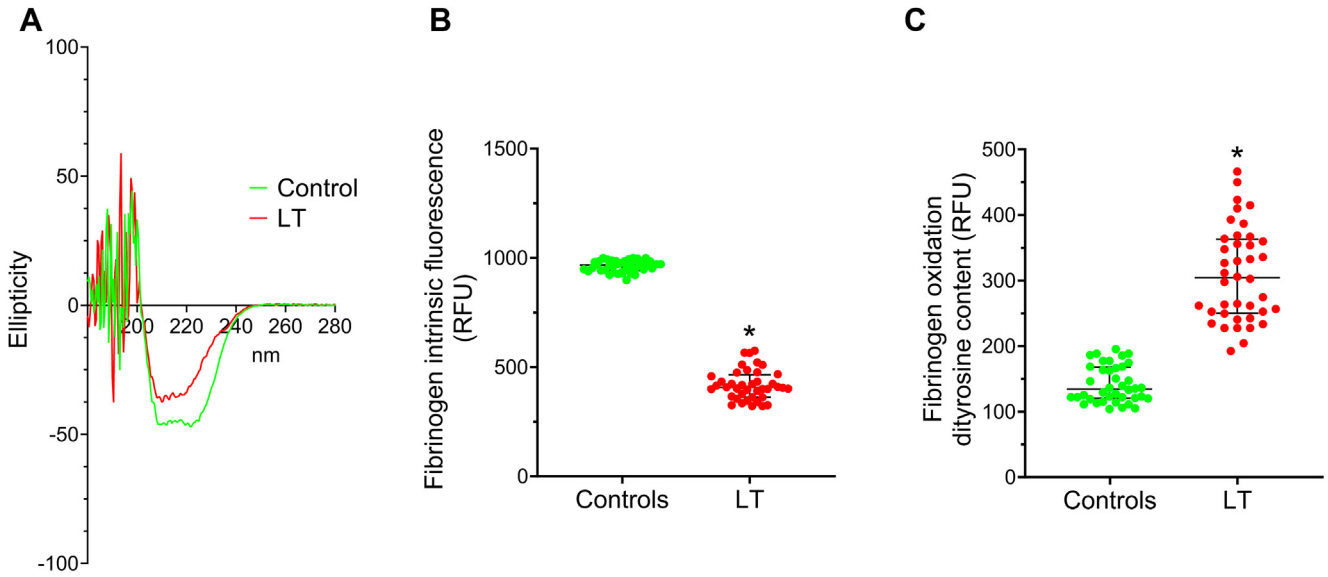

FIGURE 3 Fibrinogen from liver transplant (LT) patients displays structural alterations and oxidation with respect to controls. Fibrinogen secondary structure was evaluated by circular dichroism spectroscopy. (A) Representative circular dichroism spectra of fibrinogen oxidation, (B) fibrinogen tertiary structure by intrinsic fibrinogen fluorescence, and (C) fibrinogen oxidation analysis via dityrosine content assessment in LT patients and controls. *Statistical significance ($P < .001$). RFU, relative fluorescence units.

investigation aimed to shed light on the 3D molecular arrangement of fibrinogen. The quantification of the fibrinogen fluorescence intensity revealed significant differences in LT patients with respect to controls (407 [363-465] vs 968 [942-984] RFU, respectively; $P < .001$; Figure 3B).

Fibrinogen structural alteration can be related to fibrinogen oxidation. To assess the impact of systemic oxidative stress on fibrinogen oxidation, we assessed the ROS-induced fibrinogen oxidation by measuring the dityrosine content on purified fibrinogen fractions. A significant increase in dityrosine content was found in fibrinogen from LT as compared with controls (304 [250-362] vs 134 [120-168] RFU, respectively; $P < .001$; Figure 3C).

These results clearly indicate that patients with LT exhibit structurally altered fibrinogen, probably due to fibrinogen oxidation.

3.5 | Fibrin 3D structure

The major structural component of a blood clot is a mesh of fibrin fibers. Fibrin clots were analyzed using 3D confocal microscopy (Figure 4). The 3D confocal image of the control group depicts a fibrin network that is relatively open with distinct, well-distributed fibers. The network structure facilitates efficient fibrinolysis. In contrast, the fibrin clot from the LT patient is noticeably denser with closely packed fibers. This suggests altered fibrin formation, which could impact clot physical properties, potentially making it less susceptible to lysis and increasing the risk of thrombotic complications. Images quantification confirms that while control fibrin gels are characterized by large pores and thick fibers, those from LT patients were considerably denser, with narrow pores and thin fibers (Figure 4B, C). Supplementary Figure S1 confirmed these data.

3.6 | Fibrinogen functional analysis

To assess the impact of structural alterations on fibrinogen function, we assessed thrombin-catalyzed fibrin polymerization (Figure 5) and fibrin susceptibility to plasmin-induced lysis (Figure 6) in LT patients and matched controls.

Fibrinogen purified from LT patients showed a reduced ability to polymerize into fibrin, displaying significant differences in the main parameters of the polymerization kinetics (lag phase, Vmax, and Max Abs) as compared with controls. Particularly, in LT patients, lag phase value increased (6.8 [5.6-7.5] vs 3.4 [2.9-4.2], $P < .001$), whereas a reduction in Vmax (0.0054 [0.0038-0.0064] vs 0.0087 [0.0067-0.0101], $P < .001$) and Max Abs (0.128 [0.904-0.151] vs 0.206 [0.196-0.228], $P < .001$) compared with controls was observed (Figure 5B–D). To evaluate the effect of fibrinogen oxidation on fibrin formation, an *in vitro* fibrinogen oxidation experiment was performed (Supplementary Figure S2). These results demonstrate the causal relationship between fibrinogen oxidation and polymerization kinetic parameters. In particular, in the presence of increasing fibrinogen oxidation levels, Vmax and Max absorbance progressively and significantly decreased, whereas lag time increased in a dose-dependent manner.

When evaluating fibrin susceptibility to plasmin-induced lysis by monitoring the degradation rate of the fibrin β chain after 0 to 6 hours of plasmin digestion (Figure 6A), a significantly higher content of residual (undigested) fibrin was found in samples from LT patients as compared with controls (77 [49–72] vs 24 [19–29]; $P < .0001$), thereby indicating a remarkable fibrin resistance to lysis in LT (Figure 6B).

3.7 | Correlations between FR rate and main cohort patterns

The correlations between FR and categorical variables relevant to the patients under study are shown in Supplementary Table S1. FR was significantly increased in patients who had received an organ from donors with steatosis (2-tailed $P < .05$). Moreover, FR was higher in past or active smokers than no-smokers (2-tailed $P = .06$; 1-tailed test $P = .032$). Concerning the continuous variables, we found no significant correlations with FR (data not shown).

4 | DISCUSSION

CV events currently represent a major cause of concern in patients receiving a LT, and in the coming decades, they will become progressively more relevant given the increase in the average age of recipients and the continuous rise of pre- and post-LT metabolic disorders [73]. On this basis, it is necessary to understand the pathogenic mechanisms underlying the increased CV risk after LT. Thrombosis is a critical event in determining the appearance and outcome of CV events; however, its underlying molecular mechanisms are not fully elucidated. It has been shown that LT recipients are at increased risk of oxidative stress due to preexisting liver failure, ischemia-reperfusion injury, graft functional impairment, rejection episodes, and the use of immunosuppressive medications [74–76]. Here, we investigated the possible involvement of oxidative-mediated structural and functional fibrinogen alterations in LT recipients as a potential mechanism of thrombosis in LT.

Our data demonstrate that LT recipients exhibit a pronounced systemic redox imbalance, which is closely linked to a substantial increase in fibrinogen oxidation. Oxidation is a well-known factor responsible for inducing changes in protein conformation, potentially resulting in the generation of (auto)antigens. This, in turn, can trigger inflammation, tissue damage, alterations in the immune profile, autoimmune responses, and thrombosis [30,31,77,78]. Consequently, our findings suggest a significant association between fibrinogen oxidation and modifications in its structural and functional properties. Our far-UV CD spectroscopy data revealed compromised secondary structures in LT fibrinogen samples, indicating that fibrinogen oxidation contributes to the reduction of α-helix–rich regions. Moreover, intrinsic fluorescence analysis unveiled substantial modification in the

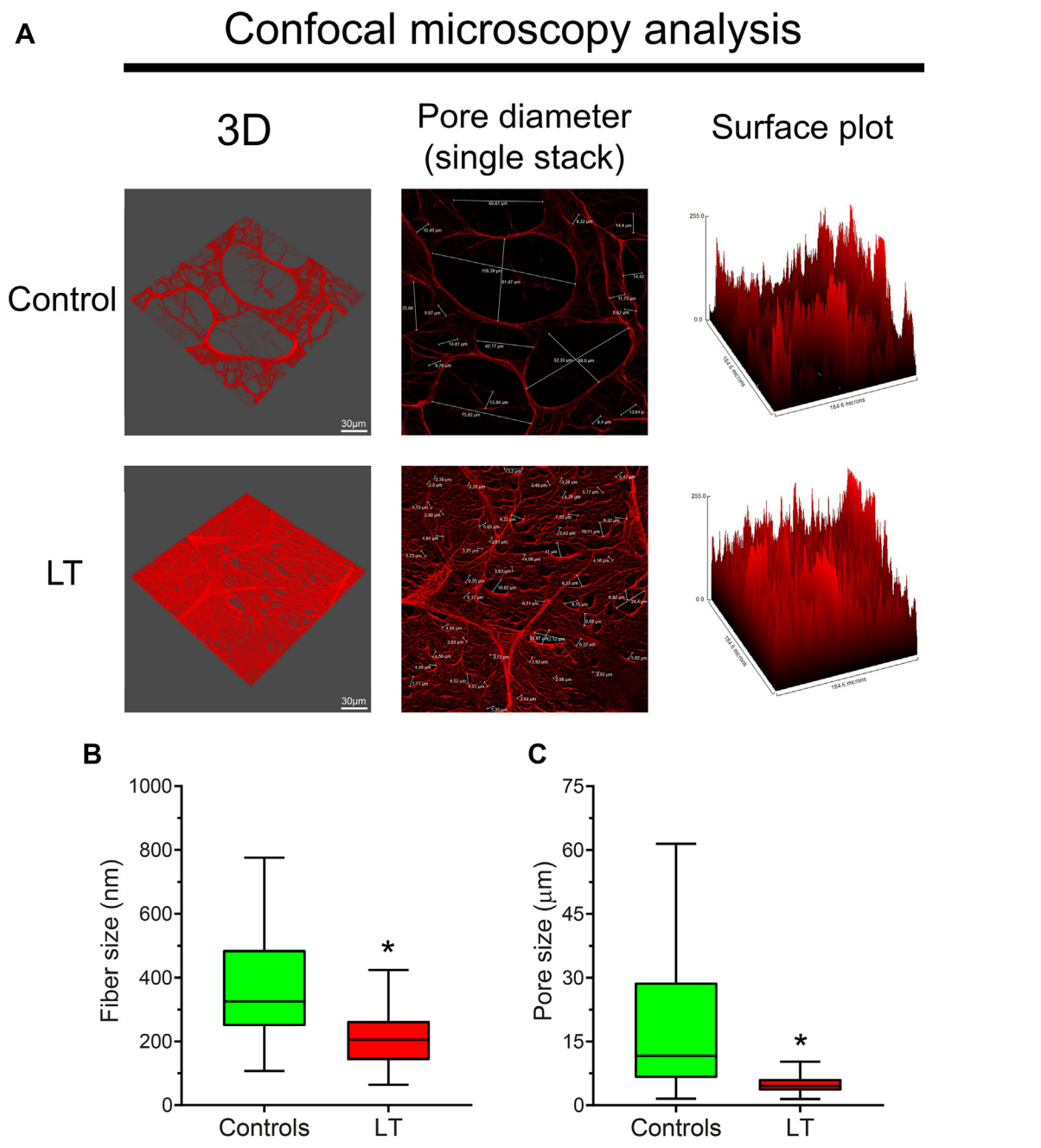

FIGURE 4 Fibrin clot from liver transplant (LT) patients is denser than that from controls. Three-dimensional (3D) confocal microscopy analysis (630× magnification) of fibrin clot from purified fibrinogen purified from LT patients and controls. The fibrin clot from LT patient is noticeably denser with closely packed fibers (A). The surface plot of the LT sample is more uniform and peaks sharply, suggesting a denser and more uniform fibrin deposition. This may contribute to a stiffer clot, which could be less effective at accommodating physical stress or strain. The fibrin fibers in the control are thicker than those in the LT sample (B). Thicker fibers typically indicate a more robust and elastic network. Consistent with the single stack analysis, LT fibrin clot shows a significant decrease in pore diameter compared with the control (C). This aligns with the observations of a denser, potentially more rigid network in LT patients. Single stacks are 184.6 µm × 184.6 µm. Values are represented as median and IQR. *Statistical significance ($P < .001$).

tertiary structure of fibrinogen in LT recipients. These characteristics are primarily influenced by the exposure of hydrophobic amino acid residues to the surrounding environment and the obtained results indicate an evident alteration in fibrinogen tertiary structure.

Our 3D confocal images of fibrin derived from controls show a relatively open fibrin network with distinct, well-distributed fibers, facilitating efficient fibrinolysis. In contrast, fibrin clot from LT patients appears noticeably denser with closely packed fibers, suggesting

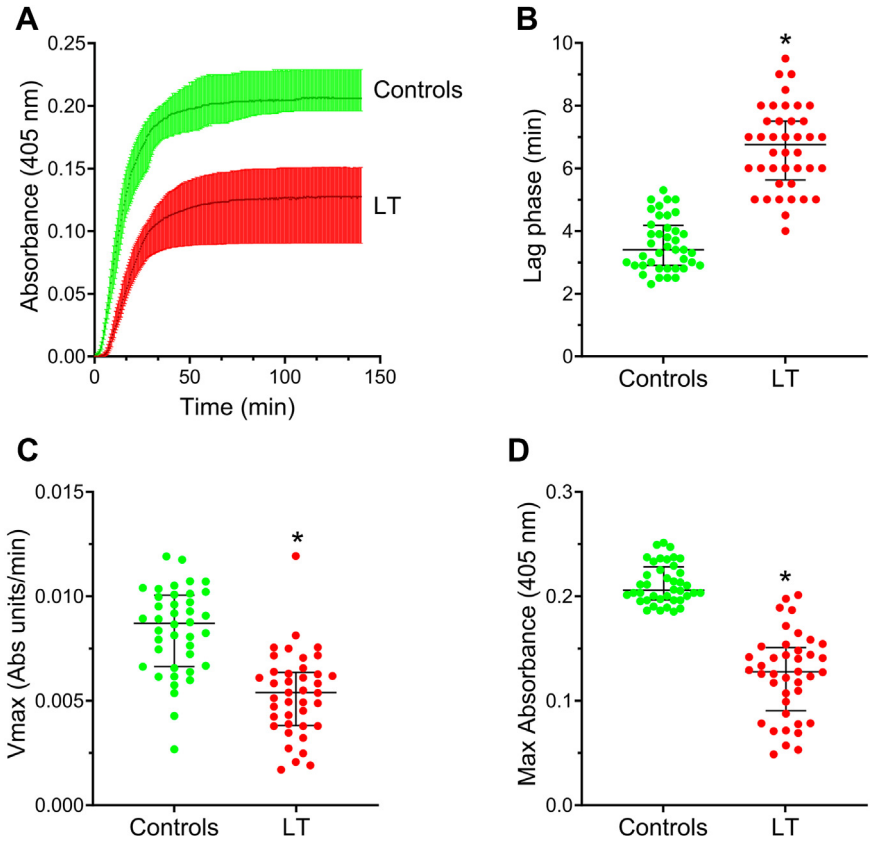

FIGURE 5 Fibrinogen from liver transplant (LT) patients displays functional alterations with respect to controls. (A) The ability of fibrinogen to polymerize into fibrin in LT patients and controls. (B–D) Variation in lag phase, maximum slope (Vmax), and maximum (Max) absorbance (Abs) of fibrinogen polymerization curves in LT patients vs controls. These alterations are related to a different fibrinogen structure in LT fibrinogen with respect to controls. *Statistical significance ($P < .001$).

altered fibrin formation. This denser structure could impact clot physical properties, potentially making it less susceptible to lysis and increasing the risk of thrombotic complications. Quantification of images confirms that control fibrin gels have large pores and thick fibers, whereas those from LT patients are considerably denser, with narrow pores and thin fibers. These findings are in keeping with observations obtained in other prothrombotic conditions, where clots characterized by dense networks exhibit resistance to plasmin-induced lysis [29,79], features that are closely linked to inflammation and oxidative stress [31]. Functionally, clots composed of fine fibers and small pores possess a higher tendency to thrombosis [31,80–82], suggesting that oxidative fibrinogen modifications could have a role in the onset of thrombosis in LT patients.

The above considerations are further supported by evidence that fibrinogen structural alterations affect the fibrin polymerization process and fibrin susceptibility to plasmin-induced lysis [49–51]. Indeed,

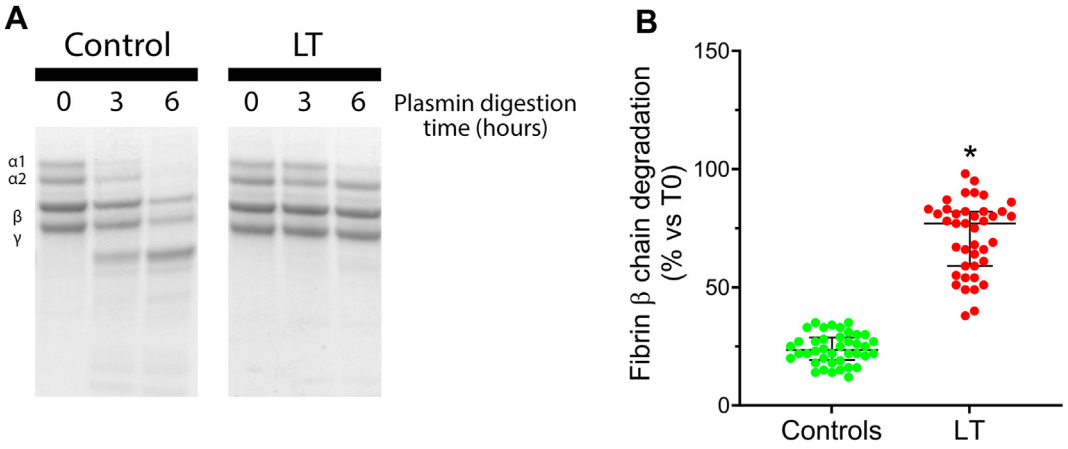

FIGURE 6 Fibrin from liver transplant (LT) patients is resistant to plasmin-induced lysis. (A) Representative gel of fibrin degradation after 0, 3, and 6 hours of plasmin digestion using fibrinogen purified from LT patients and controls. (B) Degradation rate of the fibrin β chain. *Statistical significance ($P < .001$).

the process of fibrin formation was considerably altered in LT recipients, consistent with our earlier *in vitro* studies demonstrating that carbonylation of fibrinogen leads to oxidation-dependent decrease in its clotting ability, which could be reversed by the antioxidant Trolox [39]. Furthermore, the presence of amino acid arginine, which is highly susceptible to oxidation, in the thrombin-cleavage site of fibrinogen may provide an explanation, at least in part, for the altered kinetics of fibrin polymerization observed in LT recipients [52]. Decreased Vmax and final turbidity do not necessarily indicate diminished clotting. Unlike many fibrin polymerization studies, our experiment uses purified fibrinogen instead of plasma samples. The observed differences in polymerization kinetics between LT patients and controls are due solely to variations in the fibrinogen substrate. This straightforward enzyme-substrate reaction highlights structural differences in fibrinogen, affecting its interaction with thrombin. To remove the fibrinopeptides, thrombin binds to the central region of fibrinogen not only through the active site cleft but also through its fibrinogen-binding exosite I [53]. According to our data, most *in vitro* and *ex vivo* studies report differences in fibrinogen polymerization kinetics between oxidized fibrinogen and nonoxidized fibrinogen. Specifically, the lag phase is consistently prolonged, while the Max Abs and maximum velocity, as measured in turbidity tests, are consistently decreased [30,31,39,50,54–64,79]. In contrast, some *in vitro* and *ex vivo* investigations have shown an increase in the polymerization rate [63–65].

We also investigated whether the increased resistance of fibrin to lysis observed in LT recipients was correlated with clinical and laboratory characteristics in these patients. A correlation between smoking or donor steatosis and the rate of FR was observed. These data confirm the significant influence of smoking on the outcomes of LT recipients, particularly regarding CV risk. It aligns with findings in the general population, highlighting tobacco's role in intensifying oxidative stress [66]. Accordingly, tobacco use has been recognized as a consolidated CV risk factor also in the specific context of LT [67]. The association with donor steatosis underscores the relevance of metabolic dysfunction in conferring an increased CV risk. Further studies are needed to explore the pathophysiologic mechanisms responsible for this association and to promote an optimal donor-recipient matching according to the specific long-term CV risk of transplanted patients. Along these lines, it is well known that transplantation with moderate-severe steatotic grafts represents a significant risk factor for poor graft outcomes [68].

Our study has several limitations. First, our study does not explore a direct association between fibrinogen oxidative alterations and thrombotic events. This should be the objective of future prospective studies with an adequately long follow-up. Second, the number of patients enrolled was limited. Studies with a larger population are necessary to confirm these intriguing results. Notably, it is well known that patients with liver disorder show an increased oxidative stress and it persists in the post-LT period. Oxidative stress is due to preexisting hepatic impairment, ischemia-reperfusion injury, immunosuppression, and functional graft rejection [69]. In particular, Aydin et al. [69] demonstrated that levels of end-products of lipid peroxidation are higher during cirrhosis and after transplantation compared with those in healthy subjects.

Notably, the immunosuppressive drugs seem to have a chief role in the maintenance of oxidative stress. In fact, both tacrolimus and the "old" cyclosporine induce oxidative stress [70,83,84].

In conclusion, we first provided novel lines of evidence indicating the presence of ROS-induced, structural, and functional fibrinogen modifications in LT recipients. Our data increase the understanding of the complex mechanisms underlying the increased risk of thrombotic events after LT. The present study potentially opens new possibilities for future investigation directed toward assessment of new diagnostic tools to better assess the CV risk and innovative therapeutic approaches to restore the redox balance.

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AUTHOR CONTRIBUTIONS

S.G., C.F.: concept and design, writing of article. F.R.A., E.F., S. Borghi, D.R., S. Baldi: experiments and procedures. M.F., E.L.: clinical revision. R.L.D., L.L., T.Z., A.I., S. Palladino: data collection. P.F., D.G., P.D.S.: revision of study design and final draft. F.C., C.L.: statistical analysis. F.V., S.A., G.L., S. Petruccelli, P.C., F.S., L.S., A.A., A.T.: critical revision of the final draft. F.M. and M.B.: concept and design, coordination.

RELATIONSHIP DISCLOSURE

There are no competing interests to disclose.

DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPLEMENTARY MATERIAL

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