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ROS-driven structural and functional fibrinogen modifications are reverted by interleukin-6 inhibition in Giant Cell Arteritis

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ABSTRACT

Background: Cranial and extra-cranial vascular events are among the major determinants of morbidity and mortality in Giant Cell Arteritis (GCA). Vascular events seem mostly of inflammatory nature, although the precise pathogenetic mechanisms are still unclear. We investigated the role of oxidation-induced structural and functional fibrinogen modifications in GCA. The effects of the anti-IL6R tocilizumab in counteracting these mechanisms were also assessed.

Materials and methods: A cross-sectional study was conducted on 65 GCA patients and 65 matched controls. Leucocyte reactive oxygen species (ROS) production, redox state, and fibrinogen structural and functional features were compared between patients and controls. In 19 patients receiving tocilizumab, pre vs post treatment variations were assessed.

Results: GCA patients displayed enhanced blood lymphocyte, monocyte and neutrophil ROS production compared to controls, with an increased plasma lipid peroxidation and a reduced total antioxidant capacity. This oxidative impairment resulted in a sustained fibrinogen oxidation (i.e. dityrosine content 320 (204–410) vs 136 (120–176) Relative Fluorescence Units (RFU), $p < 0.0001$), with marked alterations in fibrinogen secondary and tertiary structure [intrinsic fluorescence: 134 (101–227) vs 400 (366–433) RFU, $p < 0.001$]. Structural alterations paralleled a remarkable fibrinogen functional impairment, with a reduced ability to polymerize into fibrin and a lower fibrin susceptibility to plasmin-induced lysis. In patients receiving tocilizumab, a significant improvement in redox status was observed, accompanied by a significant improvement in fibrinogen structural and functional features ($p < 0.001$).

Conclusions: An impaired redox status accounts for structural and functional fibrinogen modifications in GCA, suggesting a potential role of tocilizumab for cardiovascular prevention in GCA.

1. Introduction

Giant Cell Arteritis (GCA) is a rare, systemic, large vessel vasculitis, typically affecting people over the fifth decade of life [1]. GCA mainly involves the medium and large arteries of the external cranial branches of the aorta, with cranial and extracranial manifestations [2]. Among the most disabling manifestations, sudden irreversible sight loss, mostly of ischemic nature, major cardiovascular events (namely acute

Abbreviations: CD, circular dichroism; GCA, Giant Cell Arteritis; IL, interleukin; IQR, interquartile range; Max Abs, maximum absorbance; MDA, malondialdehyde; NO, nitric oxide; ORAC, Oxygen Radical Absorbance Capacity; RFU, Relative Fluorescence Units; ROS, Reactive Oxygen Species; TAC, Total Antioxidant Capacity; TBARS, Thio-Barbituric Acid Reactive Substances; Vmax, maximum velocity.

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myocardial infarction and stroke) and venous thromboembolic events, are associated with the highest burden of morbidity and mortality in GCA [3–5].

Similar to other systemic vasculitis [6,7], the pathogenesis of thrombosis in GCA seems to be partly of inflammatory nature. Infiltrating CD4+ T lymphocytes, macrophages and monocytes are traditionally considered among the major mediators of vascular damage in GCA, via the production of various proinflammatory cytokines (such as IFN-γ, interleukin (IL)-17 and 6). [8]. On their turn, these cytokines stimulate vascular smooth muscle cells to produce chemokines - which further contribute to the recruitment of additional CD4 and CD8 T cells, and to migrate to the intima, proliferate and differentiate into myofibroblasts, leading to vascular remodelling and stenosis [9]. Among others, interleukin (IL)-6, mostly produced by activated macrophages, is a pleiotropic cytokine that has been linked to GCA activity [10]. Accordingly, the IL-6 receptor inhibitor tocilizumab is recognized as a valid therapeutic option for GCA [11–13].

More recently, also immature neutrophils have been found to surround temporal arteries of GCA patients, contributing to an inflammatory *milieu* via an enhanced production of reactive oxygen species (ROS) [14]. On this line, in a preliminary study by our group, we showed that GCA is associated with an impaired oxidative status, mainly characterized by a hyperproduction of ROS by all three leucocyte populations of lymphocytes, monocytes and neutrophils [15].

Moreover, we showed in different inflammatory and non-inflammatory conditions [6,7,16–26], that oxidative stress directly induces structural and functional alteration to fibrinogen, a key protein of blood coagulation cascade.

On these bases, this study primary investigated ROS-induced structural and functional fibrinogen modifications as a possible mechanism of inflammation-induced thrombosis in GCA. Moreover, in a subgroup of patients receiving tocilizumab according to routine clinical practice, this study aimed to evaluate the potential effects of IL-6 blockade in counteracting these prothrombotic mechanisms. This should include introductory information that lays out the clinical problem addressed by the research and that explains other background necessary for understanding the study.

2. Materials and methods

2.1. Design of the study

A cross-sectional study was conducted on a cohort of 65 consecutive patients diagnosed with GCA, and meeting the 2022 American College of Rheumatology/European Alliance of Associations for Rheumatology classification criteria for GCA [2] and referring to the Vasculitis Centre of the Careggi University Hospital (Florence, Italy). Sixty-five controls, matched to GCA patients by age, sex and main traditional cardiovascular risk factors (i.e. smoke, hypertension, dyslipidaemia, diabetes) or pre-existing cardiovascular events were also included. Subjects with other systemic vasculitis/autoimmune diseases, active infections or neoplastic conditions were excluded from this study. Moreover, GCA patients already included in our preliminary study by Ianni et al. [15] and those receiving biologic immunosuppressants agents at time of enrolment were excluded from this cohort.

A secondary prospective evaluation was conducted in a subgroup of 19 GCA patients (included in the main cohort) who started treatment with tocilizumab: in these patients, blood samples were re-collected after a median follow-up of 8 ± 2 months after treatment initiation. The study was conducted in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans and received approval by the local ethics committee (ref n. 12804). All subjects provided written informed consent before inclusion in the study.

2.2. Clinical assessment

At time of inclusion in the study, all patients underwent blood sample collection as well as a review of their demographic data, medical history, presence of traditional cardiovascular risk factors (i.e., smoke, hypertension, dyslipidaemia, diabetes) and pre-existing cardiovascular events. Also, a comprehensive assessment of clinical manifestations at time of GCA diagnosis, current disease activity at time of blood sample collection, and ongoing pharmacological therapies, was performed.

2.3. Fibrinogen purification

All analyses on fibrinogen structural and functional features were performed on purified fibrinogen fractions. At time of inclusion in the study, blood samples were collected in Vacutainer tubes containing 0.109 mM buffered trisodium citrate (1:10) or EDTA (0.17 mM). After centrifugation (1500 × *g* for 15 min at 4 °C), fibrinogen was purified using the ethanol precipitation method, as described in our previous works [16–18]. 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.

2.4. Assessment of intracellular ROS in peripheral leukocytes and plasma redox status

Intracellular leukocyte ROS production and blood oxidative parameters (namely lipid peroxidation and total antioxidant capacity, TAC) were assessed according to the laboratory protocols described in our previous works [16–18]. Briefly, FACSCanto flow cytometry (Becton-Dickinson, San Jose, CA, USA) was used to assess intracellular ROS production in lymphocytes, monocytes, and neutrophils. Plasma lipid peroxidation was estimated by quantifying plasma Thio-Barbituric Acid Reactive Substances (TBARS) levels using a TBARS Assay Kit (TBARS-Cayman) following the manufacturer's instructions, and Microplate Fluorometer (Biotek Synergy H1) was used for detection. Results were expressed in terms of malondialdehyde, MDA (nmol/mL).

Plasma TAC was assessed by the ORAC method (Oxygen Radical Absorbance Capacity), using Microplate Fluorometer (Biotek Synergy H1) for detection, and results were Trolox Equivalents (μM).

2.5. Fibrinogen structural analysis

ROS-induced fibrinogen oxidation was assessed by measuring dityrosine content on purified fibrinogen fractions, using a PerkinElmer LS 55 spectrofluorometer (Waltham, MA, USA) equipped with a thermostated cell holder attached to a Haake F8 water bath (Karlsruhe, Germany), as previously described [16]. Fibrinogen secondary structure was assessed via circular dichroism (CD) spectra of purified fibrinogen (0.5 mg/mL) recorded on a Jasco Fluorimeter (Jasco 810), as previously described [16].

Moreover, intrinsic fibrinogen fluorescent spectra were acquired to assess structural changes in its spatial conformation [16]. Fibrin clots were further determined by confocal microscopy, using a Leica plan apo 63× oil immersion objective, according to the protocol reported in [19].

2.6. In vitro assay: AAPH-treated fibrinogen

Increasing concentrations (0.5–1 mM) of AAPH were incubated at 37 °C for 12 h with 1 mg of purified human fibrinogen (Sigma, Milan, Italy) dissolved in 1 mL phosphate buffered saline pH 7.4. To eliminate any AAPH residues in the samples, fibrinogen was recovered and dialyzed against PBS before the assay was conducted. To evaluate the potential preventive effect of an antioxidant on the afore-mentioned AAPH-induced oxidation reaction, 1 mg of purified human fibrinogen (Sigma, Milan, Italy) dissolved in 1 mL phosphate buffered saline pH 7.4 was incubated with 1 mM AAPH in the presence of 0.1 mM Trolox at

37 °C for 12 h. Then, fibrinogen was recovered and dialyzed against PBS.

2.7. Fibrinogen functional analysis

Fibrinogen functional analysis included the assessment of thrombin-catalysed fibrin polymerization kinetics, and fibrin susceptibility to plasmin-induced lysis. Thrombin-catalysed fibrin polymerization was induced and monitored as previously described (15), and the kinetics of the absorbance curves were characterized in terms of i) *maximum slope* (Vmax) of the steepest part of the curve, ii) the *lag phase*, measured as the minutes elapsed until an increase in absorbance was recorded, and iii) *maximum absorbance* (Max Abs) of the growing clot, recorded 120 min after polymerization induction (15). Moreover, monitoring of plasmin-induced fibrin digestion was performed by quantifying the *ratio* between the fibrin beta-chain densitometric reading of the remaining protein at 6 h of plasmin digestion and that of the undigested protein (time 0 for incubation with plasmin) (15).

2.8. Effect of tocilizumab on blood redox status and fibrinogen oxidative modifications

A subgroup of GCA patients (n = 19) starting tocilizumab according to routine clinical practice were included in a secondary analysis. In these patients, blood samples were collected at time of tocilizumab beginning and following a median time of 8 ± 2 months of treatment, to investigate any variation in blood redox status and fibrinogen oxidative alterations following anti-IL6 treatment.

2.9. Statistical analysis

Categorical variables were reported as absolute frequencies and percentages, and were compared between GCA patients and controls using the Fisher exact test. Continuous variables were described as median values and interquartile range (IQR), and were compared between GCA patients and controls using the Mann Whitney test for unpaired data. 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 intra-experiment and inter-experiment variability and the reproducibility of measures using ANOVA Bonferroni Test. Correlations between oxidative stress biomarkers and fibrinogen structural and functional parameters were analyzed using Spearman's test, and rho correlation coefficient was calculated. In in vitro experiments assessing the causal relationship between ROS production and fibrinogen modifications, the Shapiro-Wilk test was used to assess normal distribution; then one-way ANOVA followed by Tukey's multiple comparisons tests were performed.

In the secondary analysis on patients receiving tocilizumab, variations in continuous parameters before vs after tocilizumab treatment were assessed by using the Wilcoxon signed-rank test for paired data. For all analyses, p values <0.05 were considered statistically significant. Statistical analyses were performed using the Graph Pad Prism 5 Software and the software STATA version 14.

3. Results

3.1. Redox status, fibrinogen structural and functional parameters, and their correlation in the cohort study

3.1.1. Clinical and demographic features of the study population

The demographic, clinical and therapeutic features of the 65 GCA patients and matched controls are reported in Table 1.

Forty-nine GCA patients were female (75.4 %), with a median age at GCA diagnosis of 74 (IQR 68–79) years and a median age at inclusion in the study of 76 (71–82) years. At time of GCA diagnosis, most patients had headache, constitutional symptoms and/or jaw claudication. Forty-four (67.7 %) patients had concomitant polymyalgia rheumatica. Regarding the cardiovascular profile, traditional risk factors, namely hypertension, dyslipidaemia, and diabetes, were reported in 38 (58.5 %), 32 (49.2 %) and 13 (20 %) of patients, respectively, while ten (15.4 %) were smokers. Fifteen patients (23.1 %) had pre-existing cardiovascular events, and three had acute vascular events at GCA diagnosis (4.6 %, including one stroke and two venous thrombosis).

Table 1
Main demographic and clinical features of the GCA patients included in the study. GCA: giant cell arteritis; IQR: interquartile range.

	GCA patients (n = 65)	Controls (n = 65)
Demographic data		
Female sex	49 (75.4 %)	49 (75.4 %)
Age at diagnosis, median (IQR)	74 (68–79)	–
Age at blood sample collection, median (IQR)	76 (71–82)	75 (69–82)
Clinical involvement		
Disease duration, median (IQR) years	2.5 (1–5)	–
Concomitant PMR	44 (67.7 %)	–
Clinical manifestations at diagnosis		
Headache	43 (66.2 %)	–
Constitutional	40 (61.5 %)	–
Jaw claudication	33 (50.8 %)	–
Scalp tenderness	15 (23.1 %)	–
Visual impairment	15 (23.1 %)	–
Vascular events	3 (4.6 %)	–
Active GCA manifestations at blood sample collection	24 (36.9 %)	–
Cardiovascular risk factors		
Hypertension	38 (58.5 %)	40 (61.5 %)
Dyslipidemia	32 (49.2 %)	33 (50.8 %)
Diabetes	13 (20 %)	12 (18.5 %)
Smoke	10 (15.4 %)	12 (18.5 %)
Pre-existing cardiovascular events	15 (23.1 %)	13 (20 %)
Ongoing therapies		
Glucocorticoids	53 (81.5 %)	–
Daily prednisone dosage (mg), median (IQR) months	7.5 (5–12)	–
Traditional immunosuppressants (methotrexate)	13 (20 %)	–
Cardioprotective therapy	37 (56.9 %)	36 (55.4 %)

At time of inclusion in the study, the median disease duration was of 2.5 (1–5) years and 24 patients (36.9 %) had active GCA. As for ongoing therapies, most patients (53; 81.5 %) were receiving glucocorticoids, at a median daily dosage of 7.5 (5–12) mg, and 13 (20 %) were receiving methotrexate. Thirty-seven patients (56.9 %) were on active cardioprotective therapy with statins, antihypertensive, anticoagulant and/or antiplatelet therapy. GCA patients and matched controls were comparable in terms of demographic features and cardiovascular risk profile (Table 1).

3.1.2. Intracellular leukocyte ROS production

Data on intracellular lymphocyte, monocyte, and neutrophil-derived ROS production in GCA patients and matched controls are reported in Fig. 1. GCA patients showed a significant increase in ROS levels in all the three leukocyte fractions as compared to controls. Namely, lymphocyte-derived ROS levels were 1225 (989–1457) vs 706 (578–873) RFU for GCA cases and controls, respectively, $p < 0.001$ (Fig. 1A); monocyte ROS levels were 2151 (1838–2598) vs 1222 (1109–1337) RFU, $p < 0.001$ (Fig. 1B), and neutrophil ROS were 2694 (2156–3531) vs 1784 (1643–1996) RFU, $p < 0.001$ (Fig. 1C). Notably, leukocyte ROS production in GCA patients seemed to be independent from the disease activity and from ongoing glucocorticoid therapy at time of blood sample collection (Tables A.1 and A.2).

3.1.3. Plasma redox status

Plasma redox status was assessed by quantifying lipid peroxidation markers and TAC. A significantly enhanced lipid peroxidation was found in the plasma of GCA patients as compared to controls [2.78 (1.79–3.69)

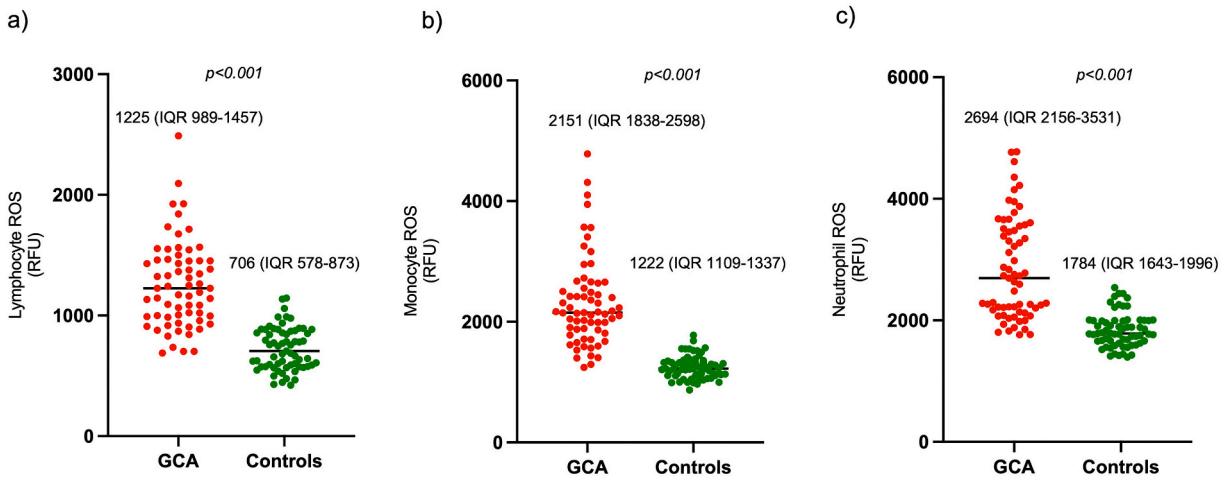

Fig. 1. Lymphocyte (A), monocyte (B) and neutrophil (C) ROS production in GCA patients and matched controls.

vs 0.36 (0.32–0.41) MDA nmol/mL, $p < 0.001$], paired by a significantly reduced TAC [15.0 (IQR 12.3–18.2) vs 20.9 (IQR 19.4–25.1) mM Trolox equivalent, $p < 0.001$] (Fig. 2A and B, respectively). Among GCA patients, a lower TAC was found in those with active disease at time of blood sample collection, although with borderline statistical significance [13.03 (10.92–17.34) vs 16.38 (13.42–18.55) mM Trolox equivalent in GCA patients with vs without active disease, $p = 0.049$] (Table A.1), whereas an increased lipid peroxidation was found in GCA patients not receiving glucocorticoids at time of blood sample collection [3.39 (2.64–3.87) vs 2.46 (1.62–3.5) MDA nmol/mL for patients receiving vs not-receiving glucocorticoids; $p = 0.032$].

3.1.4. Fibrinogen structural alterations

To assess the impact of the impaired redox status on fibrinogen structure, we qualitatively compared far-UV CD spectra of purified fibrinogen from GCA patients and matched controls. Fibrinogen purified from controls showed a typical α-helix secondary structure with minima at 208 nm and at 222 nm, whereas a decreased negative peak in the 215 nm to 225 nm region was observed in GCA, suggesting a reduced α-helical content (Fig. 3A). Moreover, ROS-induced fibrinogen oxidation was assessed by measuring dityrosine content on purified fibrinogen fractions. A significant increase in dityrosine content was found in fibrinogen from GCA as compared to controls (320 (204–410) vs 136 (120–176) RFU, respectively, $p < 0.001$) (Fig. 3b), irrespectively of disease activity at time of blood sample collection (Table A.1), with particularly increased levels in GCA patients receiving glucocorticoids (Table A.2). Fibrinogen tertiary structure was further investigated by analyzing the intrinsic fluorescence properties of purified fibrinogen samples, which are determined by the different exposure of hydrophobic amino acid residues to the solvent. The quantification of the fibrinogen fluorescence intensity revealed significant differences in GCA patients with respect to controls (134 (101–227) vs 400 (366–433) RFU, respectively, $p < 0.001$) (Fig. 3C). To evaluate the effect of fibrinogen oxidation on fibrinogen tertiary structure, an in vitro fibrinogen oxidation experiment was performed. Fibrinogen intrinsic emission fluorescence decreased in an oxidation-dependent manner, and Trolox treatment prevented these changes, demonstrating the key role of oxidation in fibrinogen tertiary structure modification (Table A.3).

Fibrin clots were further analyzed using confocal microscopy (630× magnification), which clearly showed that fibrin gels from GCA patients were denser, with narrow pores and thin fibers when compared to those from controls, which presented larger pores and thicker fibers (Fig. 3D).

3.1.5. Fibrinogen functional analysis

To assess the impact of fibrinogen structural alteration on fibrinogen function, we assessed thrombin-catalysed fibrin polymerization and fibrin susceptibility to plasmin-induced lysis in GCA patients and matched controls (Fig. 4A). Fibrinogen purified from GCA patients

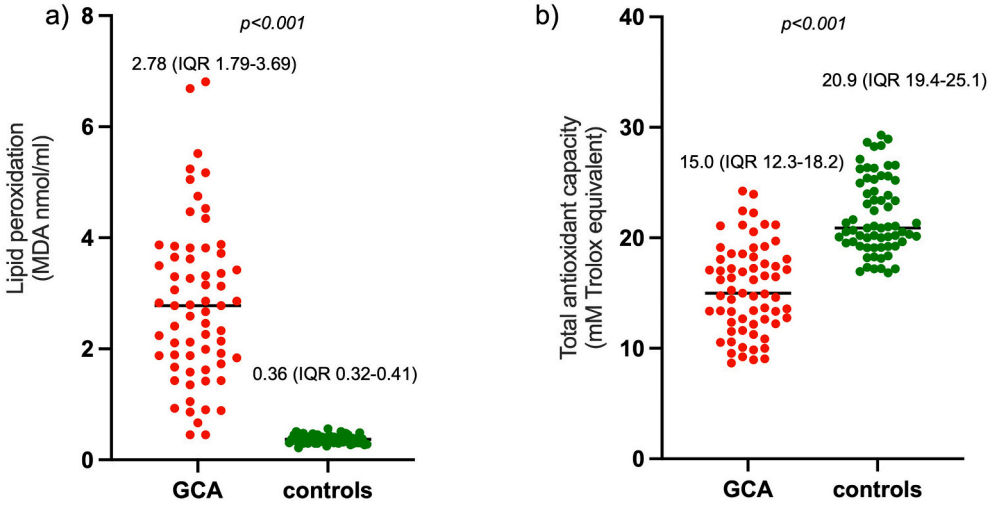

Fig. 2. Plasma lipid peroxidation (A) and total antioxidant capacity (B) in GCA patients and matched controls.
MDA: malondialdehyde (nmol/mL); ORAC: oxygen radical absorbance capacity.

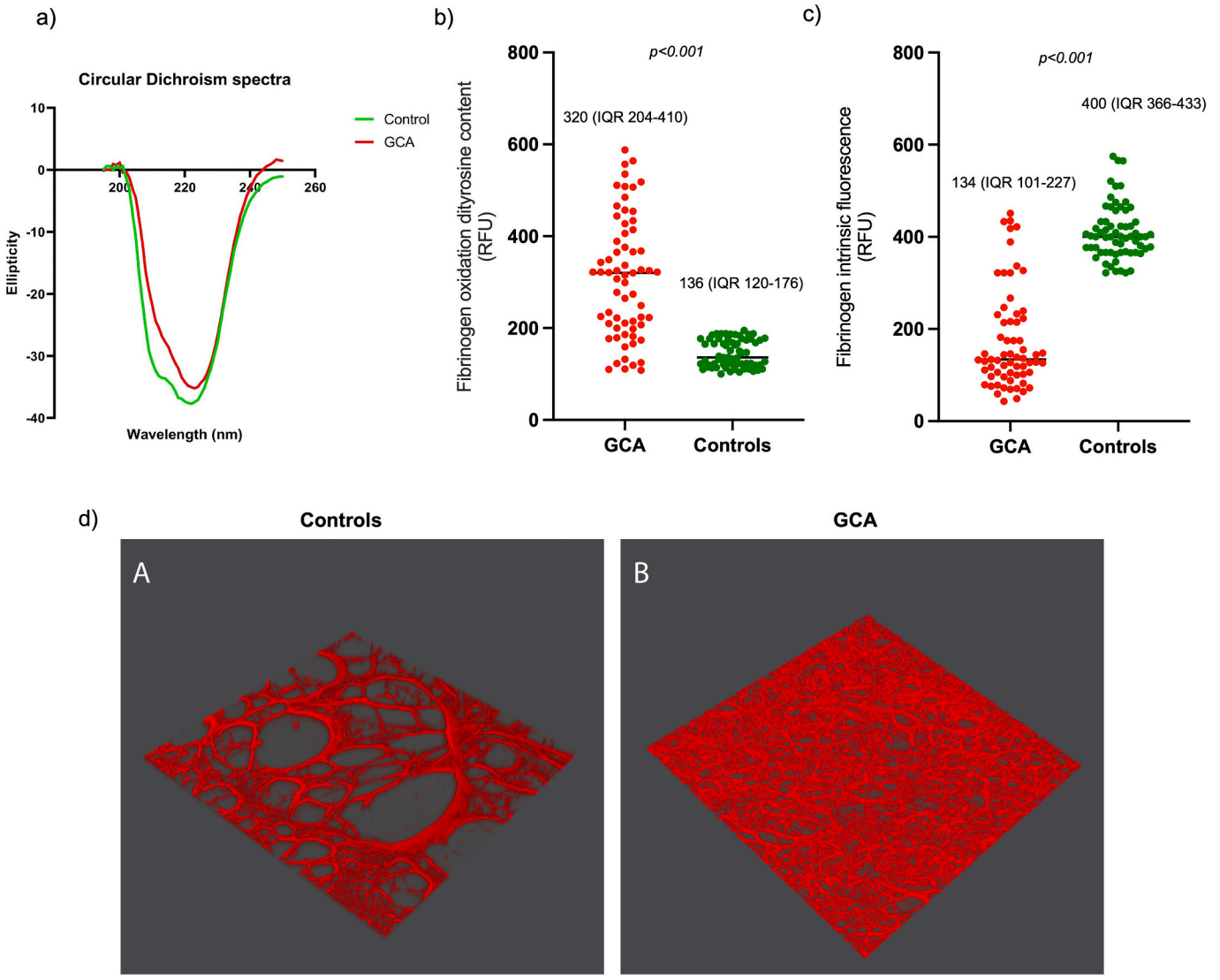

Fig. 3. Fibrinogen structural analyses. Representative circular dichroism spectra of fibrinogen oxidation (A), fibrinogen oxidation analysis via dityrosine content assessment (B) and intrinsic fibrinogen fluorescence (C) in GCA patients and matched controls. (D) Confocal microscopy analysis (630× magnification) of fibrin gels of fibrinogen purified from GCA patients and controls.

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 to controls. Particularly, in GCA patients, Lag phase value resulted increased [9.3 (6.7–11.3) vs 3.7 (3.0–4.5), $p < 0.001$], whereas a reduction in Vmax [0.004 (0.003–0.006) vs 0.008 (0.006–0.010), $p < 0.001$] and Max Abs [0.12 (0.10–0.13) vs 0.21 (0.20–0.22), $p < 0.001$] was observed. Interestingly, Max Abs was significantly lower among patients with active disease [0.11 (0.09–0.13) vs 0.13 (0.118–0.14), $p = 0.020$] (Table A.1), while no differences emerged when considering ongoing glucocorticoid therapy (Table A.2).

To evaluate the effect of fibrinogen oxidation on fibrin formation, an in vitro fibrinogen oxidation experiment was performed. Increasing concentrations of AAPH affected thrombin-induced polymerization of pure fibrinogen (Fig. A.1). In particular, in the presence of increasing AAPH concentrations, Vmax and Max absorbance progressively and significantly decreased, whereas lag time increased in a dose-dependent manner. To evaluate whether an antioxidant treatment could prevent the observed alterations, 0.1 mmol/L Trolox was added to the AAPH incubation reactions and thrombin-catalysed polymerization of fibrinogen was performed. As Fig. A.1 reports, the simultaneous incubation of AAPH with antioxidant Trolox was able to prevent the observed changes (Table A.3), demonstrating the effect of fibrinogen oxidation on fibrin formation.

When evaluating fibrin susceptibility to plasmin-induced lysis by monitoring the degradation rate of the fibrin β chain after 0 to 6 h of plasmin digestion, a significant higher content of residual (undigested) fibrin was found in samples from GCA patients as compared to controls [68 (50–82) vs 24 (18–29), $p < 0.0001$], thereby indicating a remarkable fibrin resistance to lysis in GCA (Fig. 4B and C). Notably, an impaired fibrin susceptibility to lysis was confirmed both in GCA patients with vs without active disease and with vs without ongoing glucocorticoid therapy at time of inclusion in the study (Tables A.1 and A.2).

To evaluate the effect of fibrinogen oxidation on fibrin degradation, an in vitro fibrinogen oxidation experiment was performed. Fibrin β-chain degradation at 0, 3, and 6 h after plasmin digestion in human purified fibrinogen treated with AAPH (in the absence or presence of Trolox) and quantification of residual β-chain intensity after 6 h of plasmin digestion are reported (Fig. A.1 and Table A.3). Fibrin clots obtained with pure fibrinogen after incubation with increasing AAPH concentrations showed reduced susceptibility to plasmin-induced lysis,

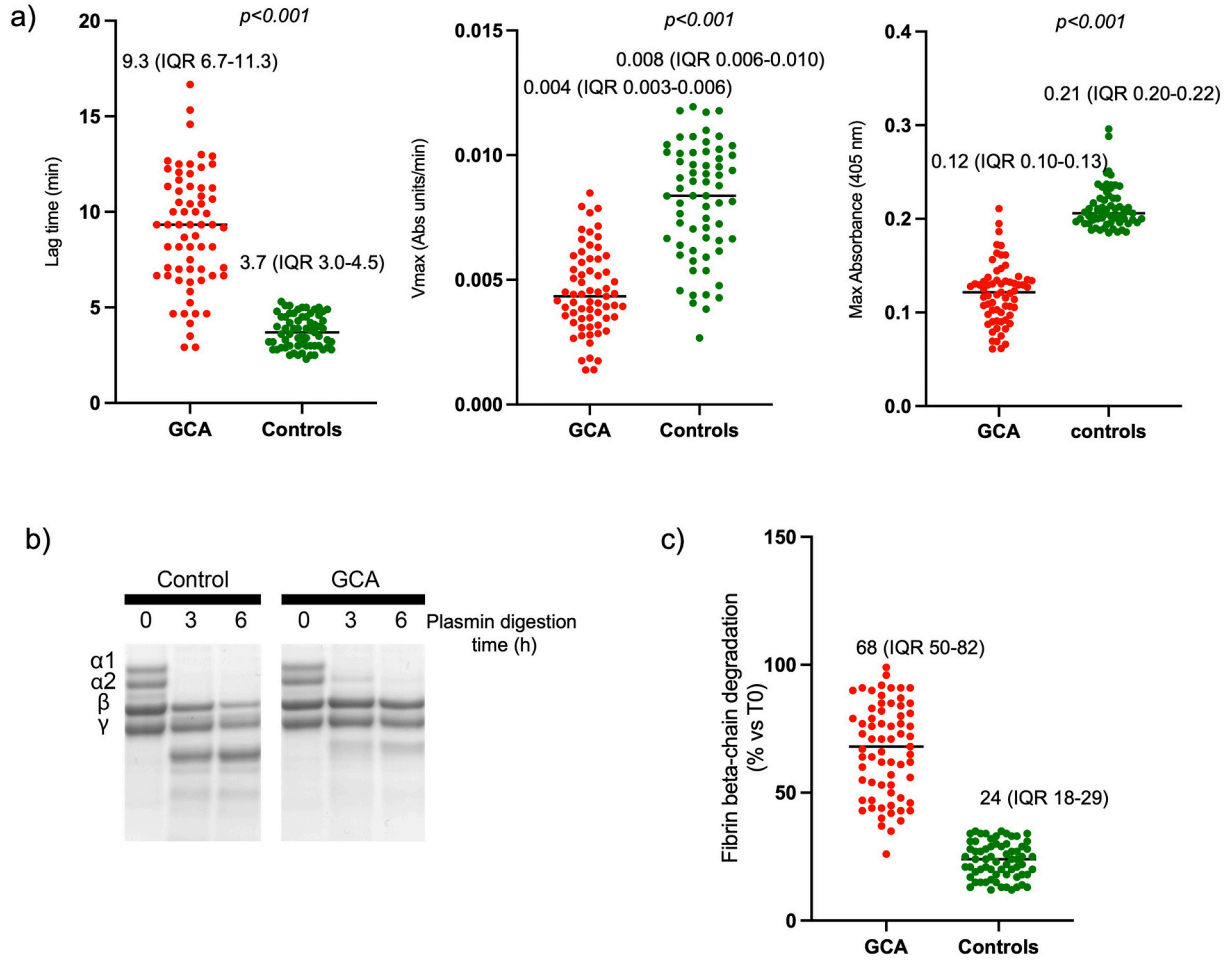

Fig. 4. (A) Lag phase, Vmax and Max Abs of thrombin-catalysed fibrin polymerization curves in fibrinogen purified from GCA patients. (B) Representative gel of fibrin lysis after 0-6 h of plasmin incubation with fibrinogen purified from GCA patients and controls. (C) Quantification of residual fibrin β chain after 6 h of plasmin digestion in GCA patients and controls.

at each considered time of plasmin digestion (Table A.3). As shown in Fig. A.1, the simultaneous incubation of AAPH and Trolox was able to prevent the observed changes in fibrin digestion by plasmin. These results clearly demonstrated the effect of oxidation on fibrin degradation.

3.1.6. Correlation between redox status and fibrinogen structural and functional alterations

In GCA patients, we assessed the correlation between fibrinogen oxidation, and structural and functional modifications (Fig. A.2). Fibrinogen degradation positively correlated with lymphocyte, monocyte and neutrophil ROS, as well as with markers of structural (dityrosine content and intrinsic fluorescence) and functional fibrinogen modifications (fibrin polymerization parameters). Moreover, a significant correlation emerged between parameters related to structural and functional fibrinogen features, and among the different fibrin polymerization parameters.

3.2. Variation in redox status and fibrinogen structural and functional parameters following tocilizumab treatment

In a subgroup of 19 patients newly treated with the anti-IL6 tocilizumab, the variation in redox status and fibrinogen structural and functional parameters following this treatment was investigated.

After 8 ± 2 months of treatment with tocilizumab, a significant reduction in intracellular leukocyte ROS levels was observed (Fig. 5A-C): namely, lymphocyte ROS decreased from 1144 (887–1465) to 852 (716–967) RFU, monocyte ROS from 2045 (1656–2661) to 1470 (1336–1585) RFU and neutrophil ROS from 2432 (2055–3877) to 1927 (1662–2210) RFU ($p < 0.001$ for all three comparisons). This was paired to a significant reduction in plasma lipid peroxidation markers [from 2.14 (1.35–3.50) to 0.69 (0.55–0.90) MDA nmol/mL, $p < 0.001$] and an increase in TAC [from 13.66 (12.77–16.83) to 17.63 (16.75–19.88) Mm Trolox equivalent, $p < 0.001$] (Fig. 5D and E). This variation in the overall redox status was accompanied by a remarkable improvement in fibrinogen structural parameters, as shown by the reduction in the dityrosine content [from 343 (210–457) to 189 (130–272) RFU, $p < 0.001$] and by the increase in fibrinogen intrinsic fluorescence [from 134 (100−211) to 311 (251–387) RFU, $p < 0.001$] (Fig. 5F–G).

Accordingly, confocal microscopy analysis showed that, after tocilizumab treatment, fibrin gels of fibrinogen purified from GCA patients displayed larger pores and thicker fibers as compared to those obtained before treatment (Fig. 5H).

Concomitantly, also fibrinogen functional parameters significantly improved, with a remarkable variation in all polymerization parameters (Lag phase, Vmax and MaxAbs, Fig. 6) and in the susceptibility to plasmin-induced lysis [residual fibrin β chain after 6 h of plasmin digestion: from 68 (48–83) to 46 (39–52); $p < 0.001$].

4. Conclusions

GCA is a medium-and-large vessel granulomatous vasculitis with a relatively high prevalence among the elderly [2,27], likely due to age-

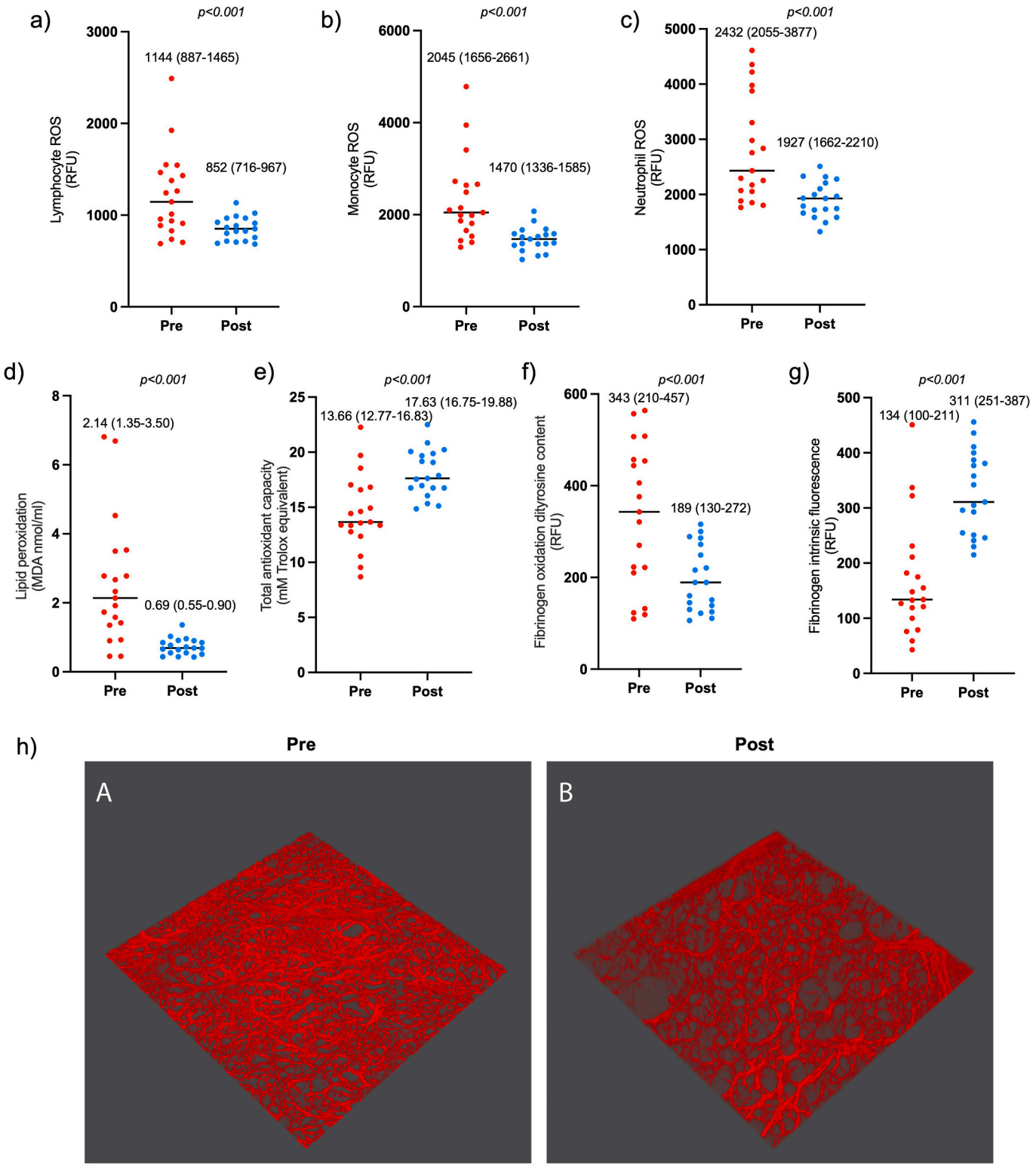

Fig. 5. Variation in lymphocyte (A), monocyte (B) and neutrophil (C) ROS production, in plasma lipid peroxidation (D) and total antioxidant capacity (E) in GCA patients before vs after treatment with tocilizumab. Variation in dityrosine content (F) and intrinsic fibrinogen fluorescence (G) before vs after treatment with tocilizumab. (H) Confocal microscopy analysis (630× magnification) of fibrin gels of fibrinogen purified from GCA patients before vs after treatment with tocilizumab.

MDA: malondialdehyde (nmol/mL); RFU: relative fluorescence units.

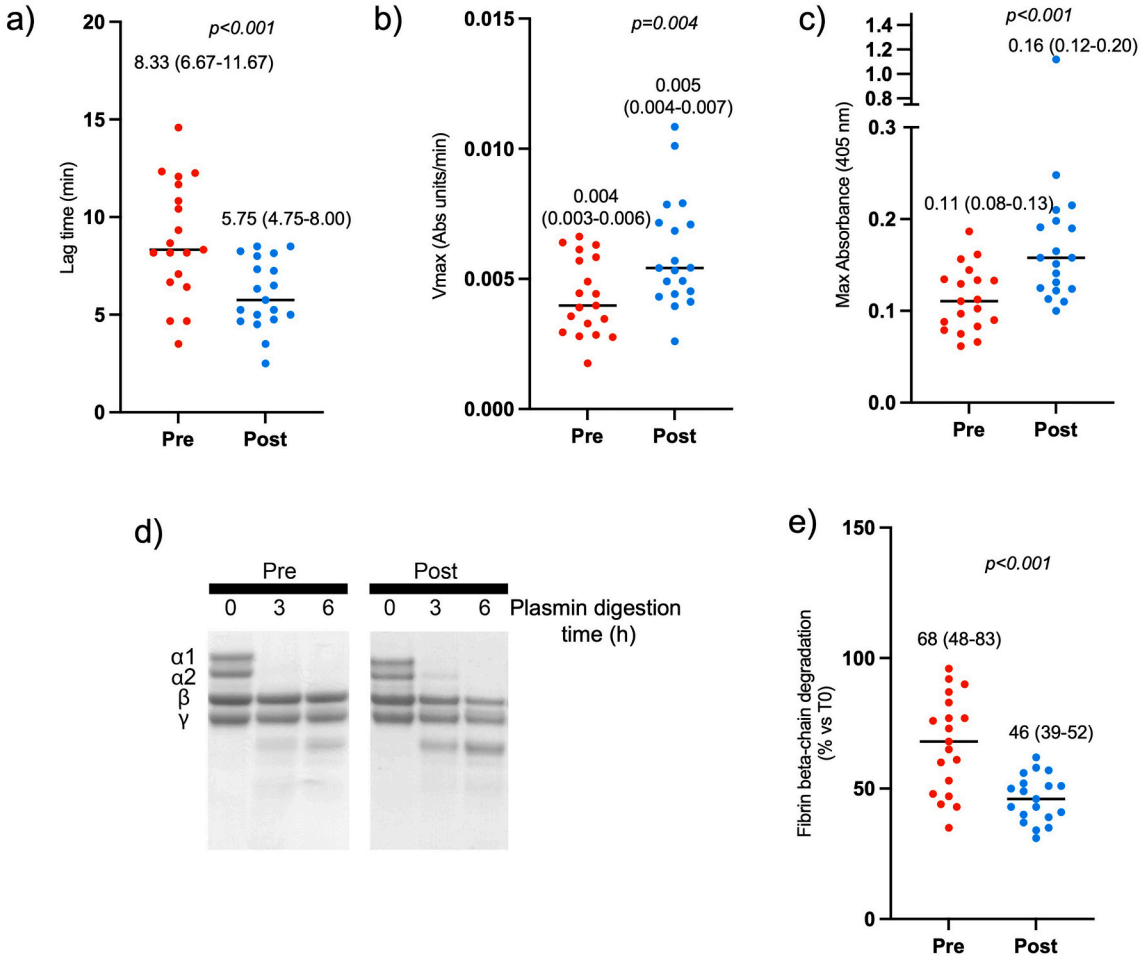

Fig. 6. Variation in (A–C) Lag phase, Vmax and Max Abs of the fibrin polymerization curves in GCA patients before vs after treatment with tocilizumab. (D) Representative gel of fibrin lysis after 0-6 h of plasmin incubation with fibrinogen purified from GCA patients before vs after treatment with tocilizumab. (E) Quantification of residual fibrin β chain after 6 h of plasmin digestion in GCA patients before vs after treatment with tocilizumab.

related changes in the blood vessels and in the immune system [28]. In GCA patients we recently showed an impaired expression of the histone/protein deacetylase SIRT1 - an antiaging enzyme with anti-inflammatory properties [15]- resulting in a significant increase in neutrophil-derived ROS production [15]. ROS originating from immature neutrophils induce a sustained protein oxidation and an increased permeability of the endothelial barrier [14]. Moreover, macrophage-derived ROS are known to directly contribute to vascular remodelling in GCA, with the destruction of the internal elastic lamina and the proliferation and migration of vascular smooth muscle cells into the intima [9]. It is known that, in GCA, vascular remodelling and inflammation lead to luminal occlusion and tissue ischemia, resulting in cranial ischemic events such as blindness and cerebrovascular accidents, and extra-cranial vascular manifestations [2], with an increased risk of venous thromboembolic events [5,29] [30].

In this study, we deepened our investigation on the role of oxidative stress as a possible mechanism sustaining thrombotic events in GCA.

Similarly to other autoimmune and inflammatory diseases [6,7,16–26], we showed for the first time that, in GCA, leukocyte-derived ROS directly induce pro-thrombotic structural and functional alterations to fibrinogen, a key blood coagulation molecule particularly responsible to ROS attack [31,32] and critical for clot formation, fibrin network arrangement and platelet aggregation [6,16,17,19–21,24–26,33–36]. Specifically, our results indicate that ROS are responsible for fibrinogen oxidation, and for an altered secondary and tertiary fibrinogen structure, as assessed by CD spectra and intrinsic fluorescent spectra. Of major note, we showed that fibrinogen structural alterations significantly and inversely correlated with fibrinogen functional features, namely thrombin-catalysed fibrin polymerization rate. In this basic enzyme-substrate reaction, a different polymerization kinetic is indicative of a structurally modified substrate (fibrinogen). In particular, the obtained kinetic parameter Max Abs correlated with blood redox status and fibrinogen carbonyl content, suggesting a key role of oxidation on fibrin polymerization.

Importantly, it is known that thrombin efficiently cleaves the consensus sequence (LTPRGVRL) [37], and that fibrinogen carries amino acids as proline and arginine, highly susceptible to oxidation, in its thrombin-cleavage site [37], which might, at least in part, account for the impaired fibrin polymerization kinetic observed in GCA. Our previous data and the current in vitro experiments clearly demonstrated that fibrinogen oxidation induces an oxidation-dependent decrease in fibrinogen clotting ability [19]. In addition, the antioxidant (Trolox) treatment reverted this effect, confirming the role of oxidation on fibrinogen function [19]. This is in line with other reports showing that oxidation impairs the clotting ability of fibrinogen when incubated with thrombin [38,39]. In agreement, oxidized fibrinogen showed a reduced susceptibility to plasmin-induced lysis, which significantly correlated with leukocyte ROS production, dityrosine content, intrinsic fluorescence, and polymerization parameters. Notably, these results were confirmed both in GCA patients with and without active disease manifestations or ongoing glucocorticoid therapy at time of inclusion in the study, suggesting that GCA is associated per se to oxidative imbalance

and clot alterations.

Of major note, our prospective analysis on GCA patients receiving tocilizumab revealed that inhibition of the IL-6 pathway effectively reduced oxidative stress biomarkers. Accordingly, a remarkable improvement in fibrinogen structural and functional parameters was observed following tocilizumab treatment. IL-6 is a pro-inflammatory cytokines whose levels correlate with disease activity in GCA [40]. Its role in cardiovascular manifestations in GCA is controversial [41]. IL-6 signalling is known to reduce nitric oxide (NO) bioavailability and to increase vascular superoxide, thus promoting oxidative stress and inducing vascular smooth muscle cells activation, immune cell recruitment, vascular dysfunction and hypertrophy [42]. On its turn, an increased ROS production increases IL-6 expression in vascular cells, further potentiating these effects [42]. In other autoimmune conditions characterized by a sustained IL-6 production, such as rheumatoid arthritis, treatment with the anti-IL6 receptor tocilizumab was found not only to effectively control disease activity, but also to significantly reduce serum ROS levels [43]. Similar results were confirmed in patients receiving tocilizumab for the treatment of COVID-19 [44]. Here, we confirmed the role of tocilizumab in counteracting oxidative stress in GCA, while clearly showing its effects also in terms of restoration of fibrinogen structural and functional features.

Some limitations in this study must be acknowledged. First, further studies are needed to demonstrate the association between fibrinogen oxidation and thrombotic events in GCA. Secondly, the impact of ongoing pharmacological therapies administered for GCA cannot be excluded; ideally, only newly diagnosed patients, naïve to any treatment (particularly glucocorticoids) should have been included. Considering that GCA is a rare disease, it is not feasible to prospectively enroll a cohort of newly diagnosed patients. However, our results suggested that ROS production as well as fibrinogen structural and functional features in GCA were not influenced by ongoing glucocorticoid therapy. Third, genetic polymorphisms responsible for fibrinogen structure modifications were not investigated. Moreover, γ' fibrinogen - originating from alternative mRNA processing – was not analyzed. Indeed, higher plasma concentrations of γ' fibrinogen yield fibrinolysis-resistant thrombi [45,46]. Another limitation of our study includes the lack of direct functional experiments demonstrating the role of IL-6 in leukocyte redox abnormalities. Although we observed enhanced leukocytes ROS production in GCA patients, we cannot establish a direct causative relationship between IL-6 and leukocyte redox imbalance based on our findings alone. However, it is important to note that the existing literature supports the notion that IL-6 can contribute to oxidative stress and redox imbalance by activating pathways involved in ROS production [47–50]. Therefore, while our study provides evidence of leukocyte oxidative stress in GCA patients, further studies specifically targeting IL-6 and its impact on leukocyte redox status are warranted to elucidate the underlying pathogenetic mechanisms.

Taken together, these results show, for the first time, that ROS induce structural and functional fibrinogen modifications in GCA, suggesting a possible role of this mechanism in the pathogenesis of vascular events in GCA. Pending future preclinical and clinical trials, these results suggest that tocilizumab might be considered as a candidate redox-balancing treatment which potential effect for cardiovascular prevention in patients with GCA.

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CRediT authorship contribution statement

Conceptualization: M. Becatti, C. Fiorillo, G. Emmi, N. Taddei and D. Prisco.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data and materials availability

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

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All people who contributed to this study are listed as co-authors.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.thromres.2023.08.011.

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