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Endotheliopathies: Current concepts and importance in clinical practice

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Endotheliopathies: Current concepts and importance in clinical practice

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Editorial: Endotheliopathies: Current concepts and importance in clinical practice

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KEYWORDS

endotheliopathies, endothelial dysfunction, endothelial injury syndromes, COVID-19, hematological disorders, cardiovascular disorders, hematopoietic cell transplantations

Editorial on the Research Topic
Endotheliopathies: Current concepts and importance in clinical practice

Over the last years, our understanding of endothelium has greatly evolved (1, 2) (Anyfanti et al.). Cardiovascular and hematological disorders, as well as hematopoietic cell transplantation, are considered key fields in which endothelial dysfunction has been studied. The list of endothelial injury syndromes is constantly updated, including not only toxicity syndromes but also novel entities, such as the coronavirus disease-19 (COVID-19) (3–5).

Despite the plethora of studies, the clinical significance of endothelial dysfunction remains under investigation. Better understanding of current concepts and significance in clinical practice emerges as extremely important to set the ground for the development of therapeutic approaches specifically targeting the endothelium. Several questions remain unanswered in this complex field.

This Research Topic gathered Original Research, Brief Research Report, and Mini Review articles, focusing on endothelial dysfunction or endothelial injury studies in the following areas:

- Novel entities recognized as endotheliopathies, such as COVID-19
- Cardiovascular disorders
- Hematological disorders
- Hematopoietic cell transplantation
- Chronic inflammatory disorders

All articles submitted to us for this Research Topic underwent a rigorous peer review process. Ultimately, eleven articles were published.

(i) In pre-eclamptic patients, phosphatidylserine exposing extracellular vesicles were increased and associated with global hemostatic parameters and fibrin clot properties (Lalic-Cosic et al.).

(ii) In systemic sclerosis, up-to-date knowledge of cellular and molecular aspects in vasculopathy, as well as therapeutic approaches were reviewed (Zanin-Silva et al.).

(iii) In essential hypertension, pathophysiological evidence of endothelial dysfunction in cardiovascular diseases and potential innovative therapeutic strategies were reviewed (Gallo et al.).

(iv) In pulmonary essential hypertension, vascular remodeling and its potential involvement of innate and adaptive immunity were reviewed (Tobal et al.).

(v) In systemic sclerosis, uric acid was significantly associated with the capillaroscopic patterns, reflecting a progressive microvasculopathy (Pagkopoulou et al.).

(vi) In the life-threatening field of thrombotic microangiopathies, complement-mediated damage was reviewed (Blacso et al.).

(vii) In COVID-19, hematological abnormalities were associated with type I interferon pathway activation and disease outcomes (Georgakopoulou et al.).

(viii) In Takayasu artiriitis, cardiovascular risk directly associated with diastolic dysfunction and inflammatory cell infiltration in the vessel wall (Cicco et al.).

(ix) In psoriasis, circulating and vascular biomarkers of endothelial dysfunction were summarized, and the impact of systemic psoriasis treatments on endothelial dysfunction and patients' cardiovascular risk was discussed (Anyfanti et al.).

(x) In secondary thrombotic microangiopathies, loss of glycocalyx integrity impaired complement factor H binding and cyclosporine-induced endothelial cell injury (Teoh et al.).

(xi) In thrombotic thrombocytopenic purpura, the PLASMIC score was applied in risk prediction of a real-world cohort (Lee et al.).

Taking into account the multi-disciplinary character of this Research Topic, we hope that it will inspire researchers to continue their explorations into novel advances in their fields.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Conflict of interest

EG has received honoraria from Alexion, Gilead, Sanofi, Sobi, and Omeros Pharmaceuticals.

The remaining author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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ORIGINAL RESEARCH
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Phosphatidylserine Exposing Extracellular Vesicles in Pre-eclamptic Patients

Sanja Lalic-Cosic[1], Violeta Dopsaj[1], Mirjana Kovac[2], Vesna Mandic-Markovic[3], Zeljko Mikovic[3], Fariborz Mobarrez[4†] and Aleksandra Antovic[5†]*

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Lalic-Cosic S, Dopsaj V, Kovac M, Mandic-Markovic V, Mikovic Z, Mobarrez F and Antovic A (2021) Phosphatidylserine Exposing Extracellular Vesicles in Pre-eclamptic Patients. Front. Med. 8:761453. doi: 10.3389/fmed.2021.761453

Background: Pre-eclampsia (P-EC) is associated with systemic inflammation, endothelial dysfunction and hypercoagulability. The role of extracellular vesicles (EVs) in coagulation disturbances affecting the development and severity of P-EC remains elusive. We aimed to evaluate the concentration of EVs expressing phosphatidylserine (PS) and specific markers in relation to the thrombin and fibrin formation as well as fibrin clot properties, in pregnant women with P-EC in comparison to healthy pregnant women of similar gestational age.

Methods: Blood samples of 30 pregnant women diagnosed with P-EC were collected on the morning following admission to hospital and after delivery (mean duration 5 days). The concentration of the PS-exposing EVs (PS+ EVs) from platelets ($CD42a^+$, endothelial cells ($CD62E^+$), and PS+ EVs expressing tissue factor (TF) and vascular cell adhesion molecule 1 (VCAM-1) were measured by flow cytometry. Further phenotyping of EVs also included expression of PlGF. Markers of maternal haemostasis were correlated with EVs concentration in plasma.

Results: Preeclamptic pregnancy was associated with significantly higher plasma levels of PS+ $CD42a^+$ EVs and PS+ VCAM-1^+ EVs in comparison with normotensive pregnancy. P-EC patients after delivery had markedly elevated concentration of PS+ $CD42a^+$ EVs, $CD62E^+$ EVs, TF^+ EVs, and VCAM-1^+ EVs compared to those before delivery. Inverse correlation was observed between EVs concentrations (PS+, PS+ TF^+, and $PlGF^+$) and parameters of overall haemostatic potential (OHP) and fibrin formation, while PS+ VCAM-1^+ EVs directly correlated with FVIII activity in plasma.

Conclusion: Increased levels of PS+ EVs subpopulations in P-EC and their association with global haemostatic parameters, as well as with fibrin clot properties may suggest EVs involvement in intravascular fibrin deposition leading to subsequent microcirculation disorders.

Keywords: extracellular vesicles, endogenous thrombin potential, overall haemostatic potential, fibrin structure, endothelial dysfunction, pre-eclampsia

INTRODUCTION

Pre-eclampsia (P-EC) is a pregnancy-specific multisystem disorder associated with high perinatal and maternal morbidity and mortality rates, and at the same time linked to long-term health consequences for mothers and their offspring (1–3). The root cause of P-EC is considered to be a defect in early placental development, followed by generalized inflammation and progressive endothelial damage predisposing to coagulation activation. Haemostatic equilibrium shifted toward a procoagulable state in normal pregnancy is even more pronounced in P-EC, resulting in enhanced thrombin generation, increased platelet activation and deposition of microthrombi in renal and placental vasculature (4, 5). Likewise, disseminated endothelial cell dysfunction and injury occurring in P-EC have been related to the release of placenta-derived factors and their effects on the maternal vasculature. The maternal circulating concentrations of anti-angiogenic proteins, soluble fms-like tyrosine kinase-1 receptor (sFlt-1), and soluble endoglin (sEng) are elevated in P-EC, whereas pro-angiogenic factors, vascular endothelial growth factor (VEGF), and placental growth factor (PlGF) are reduced. The resulting angiogenic imbalance causes a maternal syndrome characterized by hypertension and proteinuria (6, 7). Furthermore, upon stimulation the endothelium expresses tissue factor (TF) and allows exposure of sub-endothelial structures, suffering loss of its non-thrombogenic features (8). Additionally, activated endothelial cells express vascular cell adhesion molecule 1 (VCAM-1) and support leukocyte adhesion, contributing to the pathological endothelial dysfunction seen in P-EC (9).

Recently, numerous studies have also reported altered numbers and phenotype of extracellular vesicles (EVs), found as potentiating factors of the prothrombotic state identified in P-EC (10). Circulating EVs are small membrane vesicles with a diameter of 0.1–1 μm, produced by cytoplasmic membrane blebbing and shedding upon cell activation or apoptosis. The most abundant originate from platelets, but EVs from different cell types are found in the blood circulation under normal physiological conditions, acting as transporters and messengers in cell to cell communication (11). Expression of cell-derived EVs associated with gestational complications, such as P-EC and recurrent pregnancy loss or fetal growth restriction, is considered as a pathogenic factor due to their procoagulatory and proinflammatory potential (12–15). The effect of EVs on the coagulation system is thought to be related to EV exposure of phosphatidylserine (PS) alone or in combination with TF, the key initiator of the blood coagulation *in vivo*. By exposing negatively charged phospholipids, EVs provide a catalytic surface that facilitates the assembly of tenase and prothrombinase complexes leading to thrombin generation and subsequent fibrin production. Concomitant expression of TF enhances the procoagulant activity of EVs, up to 2-fold, although the mechanism of TF activation and its state (truncated or untruncated) are still a debated issue (16–18). Moreover, EVs are potent proinflammatory inducers, which interact with both endothelial and immune cells, and may contribute to the widespread intravascular inflammation (19).

The aim of the present study was to measure levels of EVs and their various phenotypes in the maternal circulation of healthy pregnant women and women with P-EC, and to relate these levels to maternal global haemostatic plasma markers of coagulation activation. Additionally, we analyzed the changes in PS+ EVs populations in plasma samples from P-EC patients before and after delivery.

MATERIALS AND METHODS

Patients

The study population consisted of 66 women at 25–39 weeks of gestation, including 36 women with a normal pregnancy and 30 women with P-EC. All investigated females were part of the larger study previously published by our group (20). We enrolled patients referred to a tertiary care maternity hospital (The Obstetrics and Gynaecology Clinic "Narodni Front") with a confirmed diagnosis of P-EC between April 2014 and November 2016, as previously described. According to the revised criteria of the International Society for the Study of Hypertension in Pregnancy, published in 2014, diagnostic criteria for P-EC include the development of hypertension in a woman with previously normal blood pressure accompanied with one or more of the following new onset conditions: proteinuria, other maternal organ dysfunction and uteroplacental dysfunction (intrauterine growth restriction—IUGR). If the woman with chronic hypertension also manifests one or more of the above features of P-EC, this is classified as chronic hypertension with superimposed P-EC (21). From each patient two blood samples were collected: (1) in the morning following admission to hospital and (2) 3–10 days after delivery (mean duration 5 days).

Healthy pregnant women of similar age and gestation with no previous history of thromboembolic events, cardiovascular diseases (CVD), and/or P-EC were included as the control group. Recruitment and blood sampling were carried out during their scheduled routine prenatal care visit, with no further follow-up of pregnancy outcome.

All patients and controls gave their written informed consent and underwent an interview on their smoking habits, ongoing medication and own or family history of pregnancy complications, venous thrombotic diseases, diabetes, and CVD. The study was approved by local Ethics Committee of Gynaecology and Obstetrics Clinic "Narodni Front" in accordance with the internationally accepted ethical standards.

Blood Sampling

Venous blood samples were collected into plastic tubes with 0.109 mol/L trisodium citrate (1 part trisodium citrate + 9 parts blood, pH 7.4). Platelet poor plasma (PPP) was obtained by double centrifugation at 2,600 g for 15 min at room temperature (with plasma supernatant harvesting in between). The final plasma supernatant was dispensed in aliquots of 500 μL and frozen at −70°C until analysis.

Analysis of Extracellular Vesicles

The 500 μL PPP samples were thawed at 37°C for 5 min and then prepared by sequential centrifugations at 2,000

× g for 20 min and at 13,000 × g for 2 min at room temperature (with plasma supernatant harvesting in between). All measurements were performed on a Beckman Gallios flow cytometer (Beckman Coulter, Brea, CA, USA), as previously described (22). After centrifugation, 20 μl of the supernatant was incubated in the dark with 5 μl lactadherin-FITC (Haematologic Technologies, Essex Junction, VT, USA), together with either 5 μL CD42a-PE (GPIX, Beckman Coulter, Brea, CA, USA), or 5 μL CD62E-APC (E-selectin, AH diagnostics, Stockholm, Sweden), or 5 μl CD142-PE (TF, BD, NJ, USA), or 5 μL CD106-PE (VCAM-1, AH diagnostics, Stockholm, Sweden). Further phenotyping included expression of PlGF (Anti-PlGF-FITC, Abcam, Cambridge, UK). Megamix-Plus FSC (Biocytex, Marseille, France), a mix of beads with diameters (0.1, 0.3, 0.5, and 0.9 μm), was used to determine the EV gate. EVs were defined as particles <1 μm in size and positive for the antibodies described above. Lactadherin was used to identify the initial population of phosphatidylserine exposing EVs, since it is more sensitive in detection of PS-rich EVs than annexin V. The platelet and endothelial components were confirmed by their expression of CD42a and CD62E, respectively. The results are presented as concentrations of detected EVs (EVs/μl plasma).

Global Haemostatic Assays

The EV concentrations were compared with the FVIII concentration and the results of global haemostatic assays, endogenous thrombin potential (ETP) and overall haemostatic potential (OHP), and turbidimetric parameters of fibrin clot formation, the polymerization rate (Vmax), and the number of protofibrils per fiber (Max Abs). All assays were carried out according to previously published methods (20).

Statistical Analysis

Statistical analyses were performed using SPSS 20.0 (IBM Corp. Released 2011. IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY: IBM Corp.) and R 3.4.2. (23). Depending on data distribution continuous variables are expressed as mean with standard deviation (SD) or median with inter quartile range (IQR), and compared using the parametric Student *t*-test and non-parametric Mann-Whitney *U*-test, as appropriate. Categorical variables are presented as count (%) and were compared by the Chi-square test. Pairwise comparisons were applied to compare the same index of one subject before and after delivery using the dependent samples *t*-test and the Wilcoxon Signed Rank test for variables with or without normal distribution, respectively. Correlations between independent variables were calculated using Spearman's rank correlation analysis. In order to control the analysis for confounding variables, logarithmic transformation of data was performed on several variables. Normally distributed variables were correlated using Pearson correlation analysis and partial correlation. Differences were considered significant for $p < 0.05$.

TABLE 1 | General characteristics of patients with P-EC ($n = 30$) and controls ($n = 36$).

	Controls	Patients with P-EC	*p*-value
Age (years)	30.6 ± 4.8	31.1 ± 6.2	0.753
BMI (kg/m^2)	25.6 ± 2.6	30.9 ± 6.2	<0.001
Smoking status (*n*)			
- Non-smokers	30 (83%)	26 (87%)	0.745[a]
- Smokers	6 (17%)	4 (13%)	
Gestational age (weeks)	33.5 ± 3.1	33.4 ± 3.7	0.900
Parity (*n*)			
- Primiparous	18 (50%)	14 (47%)	0.810[a]
- Multiparous	18 (50%)	16 (53%)	
Previous pregnancy complications (*n*)	5 (27%)	12 (75%)	0.005[a]
Family history of pregnancy complications (*n*)			
- Positive	2 (6%)	5 (17%)	0.041[a]
- Negative	34 (94%)	25 (83%)	
Family history of CVD (*n*)			
- Positive	10 (28%)	10 (33%)	0.112[a]
- Negative	26 (72%)	20 (67%)	

Data reported as the mean ± standard deviation or frequency n (%).
[a]*Chi-square test.*

RESULTS

Patient and Control Characteristics

The clinical characteristics of the study subjects are presented in **Table 1**. Pregnant women with P-EC and controls with uncomplicated pregnancies were of similar age, parity, and gestational age at blood sampling. There were no significant differences in smoking status or family history of CVD. However, rates of previous pregnancy complications and reported positive family history of pregnancy complications were higher in the P-EC group. Women with P-EC had a significantly higher body mass index (BMI) than control subjects ($p < 0.001$).

Extracellular Vesicles

PPP samples of 30 women with P-EC and 36 women with normal pregnancy were analyzed by flow cytometry and phenotyped according to protein expression. In total, 5 phenotypes of EVs were measured: PS+ CD42a$^+$, PS+ CD62E$^+$, PS+ CD142$^+$, PS+ CD106$^+$, and PlGF$^+$. **Figure 1** shows the gating strategy of EVs phenotyping by flow cytometry (**Figure 1A**) and representative dot-plots of PS+ platelet-derived EVs and PS+ VCAM-1$^+$ EVs in a healthy pregnant woman (**Figures 1B,C**) and a patient with pre-eclampsia before and after delivery (**Figures 1D–G**). The largest portion of PS-exposing EVs originated from platelets in all investigated groups (36.9% in normal pregnant women, 51.5 and 29.5% in women with pre-eclampsia before and after delivery, respectively).

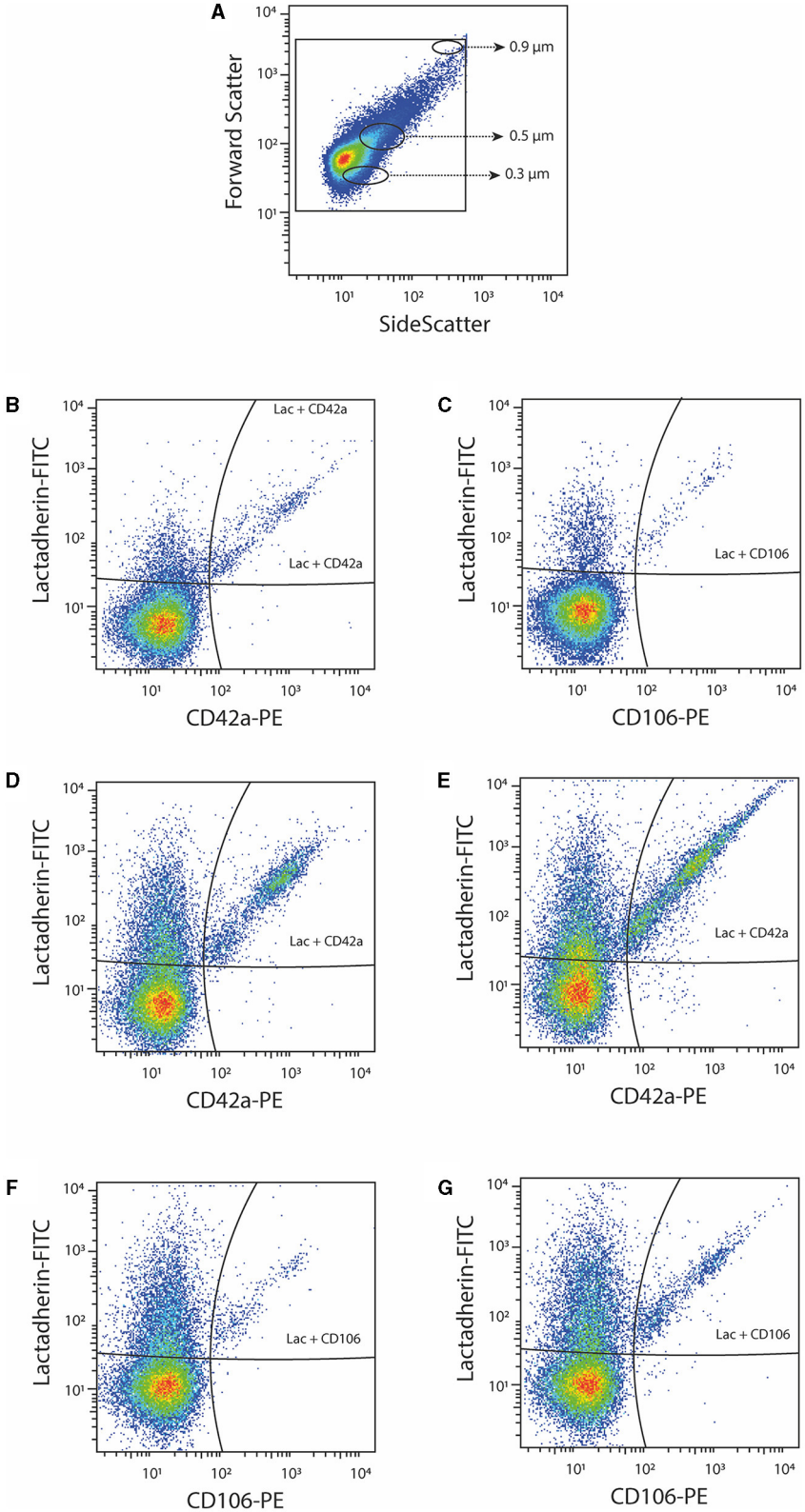

FIGURE 1 | Representative dot-plots of platelet-derived ($CD42a^+$) and VCAM-1^+ ($CD106^+$) EVs. **(A)** EV gating strategy based on beads with diameter 0.3, 0.5, and 0.9 µm; **(B,C)** platelet-derived ($CD42a^+$) EVs and VCAM-1^+ ($CD106^+$) EVs in a healthy pregnant woman; **(D,E)** platelet-derived ($CD42a^+$) EVs in a patient with pre-eclampsia before and after delivery; **(F,G)** VCAM-1^+ ($CD106^+$) EVs in a patient with pre-eclampsia before and after delivery.

TABLE 2 | Concentration of circulating extracellular vesicles and levels of investigated haemostatic parameters in patients with P-EC before and after delivery ($n = 30$) and in controls ($n = 36$).

Parameter	Controls	Patients with P-EC	
	Before delivery	Before delivery	After delivery
Extracellular vesicles			
PS+ EVs/μL	957 (586.5–2022.5)	905 (700–2100)	2445.5 (1727–4235)†††
PS+ CD42a$^+$ EVs/μL	353 (151.5–455)	466 (327–560)*	720.5 (585–1266)†††
PS+ CD62E$^+$ EVs/μL	108 (66–118)	91 (70–104)	136.5 (103–156)†††
PS+ CD142$^+$ EVs/μL	34 (15.5–69.5)	29 (15–59)	87 (20–189)†
PS+ CD106$^+$ EVs/μL	39.5 (27–47)	58 (40–80)***	102 (75–153)†††
PlGF$^+$ EVs/μL	295.5 (176.5–408.5)	198.5 (118–327)	263 (150–399)
Endogenous thrombin potential			
ETP (AUC, %)	93.5 (90.5–105.0)	112.5 (106.0–119.0)***	107.0 (101.5–118.0)
Peak height (%)	104.8 ± 10.3	115.2 ± 13.1**	128.4 ± 17.9††
Overall haemostatic potential			
OCP (Abs-sum)	247.0 ± 32.9	229.4 ± 44.5	246.9 ± 50.8
OHP (Abs-sum)	177.1 ± 38.1	198.7 ± 40.7*	219.3 ± 43.8
OFP (%)	27.3 (16.7–35.5)	12.2 (6.6–18.8)***	8.8 (5.3–14.4)†
Fibrin clot properties			
Vmax (AU/min)	0.47 ± 0.14	0.55 ± 0.10**	0.57 ± 0.16
Max Abs (AU)	1.36 ± 0.18	1.25 ± 0.21*	1.30 ± 0.24
Factor VIII activity			
FVIII (%)	189.1 ± 78.3	259.9 ± 115.4**	343.2 ± 103.6††

Data reported as the mean ± standard deviation or median with inter quartile range (IQR). EVs, extracellular vesicles; ETP, endogenous thrombin potential; OCP, overall coagulation potential; OHP, overall haemostatic potential; OFP, overall fibrinolysis potential; Vmax, polymerization rate; Max Abs, number of protofibrils per fiber. EV markers: CD42a, glycoprotein IX (platelet marker); CD62E, E-selectin (endothelial marker); CD142, Tissue Factor (TF); CD106, Vascular Cell Adhesion Molecule-1 (VCAM-1); PlGF, placental growth factor.

*Controls vs. P-EC: *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.*

P-EC before vs. after delivery: †$p < 0.05$, ††$p < 0.01$, and †††$p < 0.001$.

Phenotyping of EVs in Women With P-EC Compared to Healthy Pregnancy

Although the concentrations of PS+ EVs in women with P-EC and healthy pregnancy were revealed to be the same, comparing different phenotypes of PS-exposing EVs we demonstrated significantly higher concentrations of PS+ CD42a$^+$ platelet-derived and PS+ VCAM-1$^+$ EVs in women with P-EC (**Table 2**). However, no differences were observed in endothelial-derived (PS+ CD62E$^+$) EVs and TF-expressing PS+ EVs between P-EC patients and healthy pregnant women. Also, we found similar concentrations of PlGF-expressing EVs in the P-EC patients and healthy pregnant women (**Figures 2A,B**).

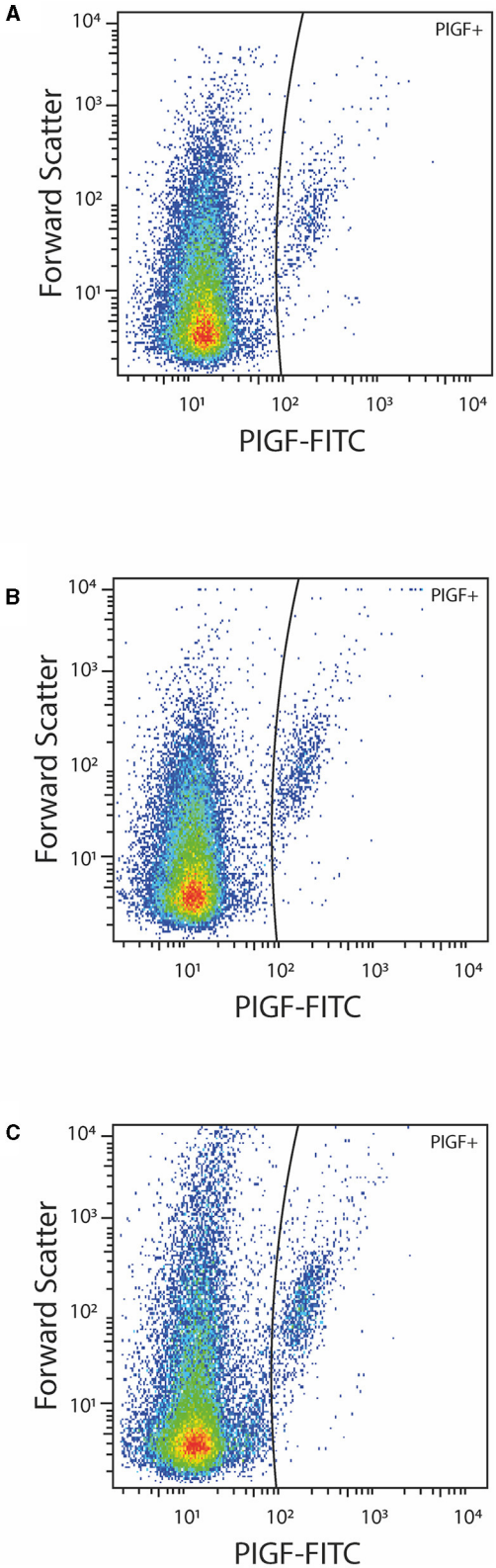

FIGURE 2 | Representative dot-plots of PlGF positive EVs in a healthy pregnant women **(A)** and a patient with pre-eclampsia before **(B)** and after **(C)** delivery.

TABLE 3 | Correlation between the results of haemostatic parameters and EV concentrations.

Variable	PS+ EVs/μL		PS+ CD42a$^+$ EVs/μL		PS+ CD62E$^+$ EVs/μL		PS+ CD142$^+$ EVs/μL		PS+ CD106$^+$ EVs/μL		PlGF$^+$ EVs/μL	
	Rho	*p*	Rho	*p*	Rho	*p*	Rho	*p*	Rho	*p*	Rho	*p*
ETP	−0.18	0.36	−0.07	0.71	−0.20	0.29	−0.32	0.09	−0.05	0.78	−0.32	0.09
Peak height	−0.01	0.99	0.22	0.24	−0.12	0.53	−0.27	0.14	0.11	0.56	−0.16	0.39
OCP	−0.47	**0.009**	−0.14	0.47	−0.24	0.20	−0.44	**0.016**	0.01	0.99	−0.35	0.058
OHP	−0.49	**0.007**	−0.17	0.39	−0.24	0.22	−0.49	**0.007**	−0.07	0.72	−0.36	0.053
OFP	−0.10	0.58	0.00	0.99	−0.12	0.51	0.23	0.22	−0.07	0.71	0.10	0.59
Vmax	−0.41	**0.031**	−0.20	0.31	−0.28	0.14	−0.46	**0.013**	−0.23	0.24	−0.52	**0.004**
Max Abs	−0.49	**0.011**	−0.28	0.16	−0.28	0.17	−0.46	**0.019**	0.07	0.74	−0.40	**0.045**
FVIII	0.12	0.54	0.18	0.33	0.21	0.27	0.03	0.88	0.39	**0.034**	0.09	0.63

All the data were assessed using Spearman's rank correlation analysis and are reported as the correlation coefficients (Rho) with the corresponding p-values (p). ETP, endogenous thrombin potential; OHP, overall haemostatic potential; OCP, overall coagulation potential; OFP, overall fibrinolysis potential; Vmax, polymerization rate; Max Abs, number of protofibrils per fiber; FVIII, factor VIII activity; EVs, extracellular vesicles. EV markers: CD42a, glycoprotein IX (platelet marker); CD62E, E-selectin (endothelial marker); CD142, Tissue Factor (TF); CD106, Vascular Cell Adhesion Molecule-1 (VCAM-1); PlGF, placental growth factor. All significant associations are bolded.

Phenotyping of EVs in Women With P-EC After Delivery

The concentration of EVs and their phenotypes were also analyzed in women with P-EC before and 3–10 days after delivery. It is reasonable to expect that concentrations of EVs increase after delivery, which is associated with the delivery itself. Indeed, pairwise comparisons of results obtained in women with P-EC before and after delivery showed an ∼2.5-fold increase in the concentration of PS+ EVs accompanied by the rise of all investigated EV phenotypes. As presented in **Table 2**, concentrations of PS+ CD42a$^+$, PS+ CD62E$^+$, PS+ CD142$^+$, and PS+ CD106$^+$ were significantly elevated in women with P-EC after delivery compared to the values before delivery. However, although slightly higher in the P-EC group after delivery, the concentration of EVs expressing PlGF did not differ significantly between P-EC patients before and after delivery (**Figures 2B,C**).

Haemostatic Parameters and Their Correlation to EVs Concentrations

Results of global haemostatic assays (ETP and OHP), turbidimetric measurements of fibrin clot formation (Vmax and Max Abs), and factor VIII activity assay are presented in **Table 2**. Compared to gestational age-matched controls women with P-EC showed significantly elevated ETP, peak height and OHP values associated with depressed fibrinolysis [decreased overall fibrinolysis potential (OFP) values; 12.2 [6.6–18.8] % vs. 27.3 [16.7–35.5] %, $p < 0.001$]. Furthermore, P-EC patients exhibited significantly higher Vmax and lower Max Abs values, indicating the faster formation of the fibrin clot composed of thinner fibers. In the P-EC group after delivery, a significant increase of the peak height and an additionally decreased rate of fibrinolysis were observed, without significant change in fibrin clot properties. FVIII activity was above the normal range for non-pregnant individuals with a significant difference in all groups ($p < 0.01$).

Since this study focused on EVs presenting negatively charged phospholipids on their outer leaflet and their impact on coagulation disturbances, we performed Spearman correlations between the detected concentration of particles and measured haemostatic parameters (**Table 3**). Our results demonstrated that PS+ EVs correlated with overall coagulation potential (OCP; $r = -0.47$, $p = 0.009$), overall haemostatic potential (OHP; $r = -0.49$, $p = 0.007$) and fibrin formation parameters: maximum absorbance (Max Abs; $r = -0.49$, $p = 0.011$) and polymerization rate (Vmax; $r = -0.41$, $p = 0.031$). The same coagulation parameters correlated also with the EVs copresenting TF and PS on their outer leaflet (OCP $r = -0.44$, $p = 0.016$; OHP $r = -0.49$, $p = 0.007$; Max Abs $r = -0.46$, $p = 0.019$; Vmax $r = -0.46$, $p = 0.013$, respectively). While PlGF exposing EVs also showed significant correlation with Max Abs and Vmax ($r = -0.40$, $p = 0.045$ and $r = -0.52$, $p = 0.004$, respectively), PS+ VCAM-1$^+$ EVs were correlated only with FVIII activity ($r = 0.39$, $p = 0.034$). Interestingly, PS+ platelet-derived and endothelial-derived EVs showed no significant correlation with any of the investigated coagulation parameters. However, the Pearson correlation analysis showed the similar strength of association between EVs concentrations and measured haemostatic parameters, except no association was found between concentration of EVs exposing PlGF and Max Abs. After adjustment for maternal age and BMI we observed a moderate correlation between CD62E$^+$ endothelial-derived EVs and ETP ($r = -0.42$, $p = 0.030$), while the association between PS+ CD106$^+$ EVs concentration and FVIII activity was no longer statistically significant. Regarding maternal complications (HELLP, renal complications, thrombocytopenia, placental abruption, and neurological disorders) and perinatal complications (IUGR and oligohydramnios) observed in our P-EC group we found no significant differences in the levels of investigated EVs between patients with and without complications. Correlation analysis revealed no association between the EVs concentrations and 1- or 5-min APGAR score.

DISCUSSION

Our findings indicate that pre-eclamptic pregnancy is associated with significantly higher plasma levels of PS+ platelet-derived EVs expressing CD42a and PS+ VCAM-1$^+$ EVs in comparison with normotensive pregnancy. Moreover, P-EC patients

after delivery had markedly elevated concentrations of PS+ $CD42a^+$ EVs, $CD62E^+$ EVs, TF^+ EVs, and VCAM-1^+ EVs compared to those before delivery, but there was no evidence of increased $PlGF^+$ EVs concentration. Haemostatic results confirmed the presence of the pronounced hypercoagulable state in P-EC patients in comparison with healthy pregnant women. Patients with P-EC exhibited enhanced thrombin generation, characterized by elevated ETP and peak height values, accompanied by elevated OHP and reduced OFP values implying reduced fibrinolytic capacity. Moreover, a higher polymerization rate (Vmax) and lower Max Abs values indicating the faster formation of condensed fibrin clots composed of thinner fibers revealed altered fibrin clot properties in this specific group of patients. Further increase in peak height combined with a decrease in OFP values was recorded in P-EC patients after delivery suggesting the presence of activated coagulation and diminished fibrinolysis despite the cessation of pregnancy. To address the role of PS+ EVs in the hypercoagulable state present in P-EC, we analyzed their association with global haemostatic assays (ETP and OHP), as well as with fibrin clot properties. A moderate inverse association was observed between EVs concentration (PS+, PS+ TF^+, and $PlGF^+$) and OHP, OCP, and fibrin formation parameters, while PS+ VCAM-1^+ EVs directly correlated with FVIII activity in plasma. Surprisingly, PS+ EVs originating from platelets and endothelial cells did not show a correlation with any of the investigated coagulation parameters, suggesting other possible contributions to the hypercoagulable state present in P-EC.

As expected and in line with previous reports, our results revealed no difference between women with P-EC and control subjects in concentration of PS+ EVs (24, 25). However, the focus of this study was to investigate the expression of procoagulant PS on EVs, and unlike most others that used annexin V to detect PS, we employed lactadherin, which has been shown to bind PS more efficiently in a calcium-independent manner (26). In our study, both absolute and relative levels of PS+ $CD42a^+$ platelet-derived EVs (GPIX, adhesive platelet marker) were significantly elevated in patients with P-EC, although showing no correlation to thrombin generation, fibrin formation and degradation, or increased fibrin network density. Studies evaluating the levels of platelet-derived EVs during pregnancy complicated by P-EC have given conflicting results, with either reduced or unchanged, and even elevated platelet-derived EVs levels between P-EC and healthy pregnant women, but none of them used the CD42a marker (14, 27, 28). Lack of direct association between PS+ $CD42a^+$ platelet-derived EVs and investigated coagulation parameters in our study suggests that PS alone may not be sufficient to facilitate thrombus formation. VanWijk et al. reported that EVs induced the TF/FVII-dependent coagulation activation pathway but did not enhance thrombin generation and therefore concluded that EVs were not directly involved in the increased coagulation activation in P-EC (29). Recent *in vitro* studies implied the participation of platelet-derived EVs in coagulation propagation via TF- or contact-dependent thrombin generation, but could not demonstrate their impact on fibrin network density or stability (30, 31). Nevertheless, in the inflammation setting, as seen in P-EC, TF derived from stimulated monocytes in the circulation may act as initiator. Moreover, the interaction of platelet-derived EVs with leukocytes or endothelial cells may activate these cells and induce their TF-dependent procoagulant activity as well as further increase inflammatory reactions (32). In addition, platelet-derived EVs, by complement activation, may be further involved in the regulation of clot structure and function. P-EC is associated with abnormal complement activation while an excessive activation of the terminal pathway has been described in P-EC complicated by IUGR. Therefore, systemic activation of complement system might have an important input to the coagulation and inflammation disturbances bridging them to the maternal syndrome of P-EC (33–35).

Hypercoagulability, platelet activation, and inflammation are systemic manifestations accompanying maternal hypertension and proteinuria, as clinical hallmarks of P-EC (36). However, the primary disorder has been related to placental ischemia and oxidative stress leading to endothelial activation and injury. Endothelial cell activation could be associated with elevated levels of endothelial-derived EVs and several studies found significantly elevated concentration of endothelial-derived EVs in patients with P-EC (27, 37, 38). Our results showed no difference between the P-EC and control groups in concentrations of PS+ endothelial-derived EVs or their association with investigated coagulation parameters. As endothelial-derived EVs could express procoagulant, anticoagulant, and fibrinolytic activity, these findings might reflect their ambivalent role in coagulation and fibrinolysis (39–41). However, here we report for the first time in P-EC patients, a significantly increased concentration of PS+ EVs expressing VCAM-1. VCAM-1 has been proposed as a pro-inflammatory marker, being expressed exclusively on cytokine-stimulated endothelium and promoting firm adhesion of mononuclear cells to endothelium. Increased levels of soluble VCAM-1 in the plasma have been previously reported in patients with P-EC, vascular and inflammatory diseases, while elevated VCAM-1^+ EVs were demonstrated only in the latter (42–46). Additionally, in our study PS+ VCAM-1^+ EVs levels correlated significantly with FVIII activity, suggesting that PS+ EVs with increased VCAM-1 exposure are more coagulable and potentially more prone to cell interaction. Nevertheless, further studies are needed to elucidate if an increase in PS+ VCAM-1^+ EVs could be attributed to changes in endothelium function that may potentiate a hypercoagulable state.

Upregulated TF expression on endothelial cells following endothelial stimulation or inflammatory-induced on the surface of circulating monocytes, tissue macrophages, and neutrophils has also been associated with release of TF-bearing EVs into the blood circulation and observed in different diseases (47). Concurrent expression of PS and TF on EVs potentiate the procoagulant effect of EVs. In line with previous studies, we found similar levels of PS+ TF^+ EVs in both the P-EC and the control group, while a significant increase was observed in the P-EC group after delivery (12, 29). A post-delivery rise in TF-dependent coagulation has been described (48). However, not MP-bound TF, but soluble TF was measured in this study.

Although numerous investigations showed that the endothelial dysfunction present in maternal tissue of P-EC

patients is associated with decreased plasma concentrations of PlGF, due to its binding to excessively released sFlt-1 (49), we observed no significant change in the concentration of $PlGF^+$ EVs in the investigated groups (**Figure 2**). A clear conclusion about our findings with regard to PlGF-exposing EVs and their associations with haemostatic parameters could not be drawn since we did not explore the cellular origin of these circulating $PlGF^+$ EVs, nor measure the PlGF concentration in plasma. Bearing in mind that PlGF is a more sensitive and precise predictor of P-EC than any other single biomarker, possible implications of such an observation remain to be determined.

Interestingly, concentrations of PS+, PS+ TF^+, and $PlGF^+$ EVs inversely correlated with OHP, OCP, and fibrin formation parameters assessed in the evaluation of haemostatic status in patients with P-EC. Our results are consistent with a recent report suggesting that EVs may be incorporated in the haemostatic plug and therefore inversely associated with haemostatic activation (50). Nevertheless, a significant increase of EVs concentration in P-EC patients after delivery, without further change in OHP, OCP, Vmax and Max Abs, may suggest a possible additional anticoagulant effect of high levels of PS+ TF^+ EVs through thrombin-catalyzed activation of protein C (APC) orchestrated by soluble thrombomodulin (TM). Increased levels of TM, released due to diffuse endothelial damage, have been observed in P-EC (51, 52). However, this presumption of the potential anticoagulant effect of EVs should be studied further. In the above-mentioned study (50), EV concentration further correlated directly with Ks (coefficient of fibrin clot permeability), indicating an inverse correlation to fibrin tightness demonstrated also (via Vmax and Max Abs) in our study.

The present investigation has certain limitations. The number of patients included in the evaluation was small. Additionally, we were not able to compare data obtained in P-EC patients after delivery with post-delivery data of healthy pregnant women due to the study design.

In conclusion, numbers of CD42a platelet and VCAM-1 positive PS-exposing EVs were increased in P-EC patients before and after delivery, accompanied by additional post-partum elevation of the total, endothelial and TF-bearing PS+ EVs. Inverse association of PS+ and TF^+ PS+ EVs with OHP and fibrin clot properties may suggest EVs involvement in intravascular fibrin deposition leading to the consequent microcirculation disorders found in P-EC. Furthermore, EVs contribution to unfavorable clot features combined with the engagement of PS+ VCAM-1^+ EVs in P-EC may be associated with subsequent CVD development in these patients.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by local Ethics Committee of Gynaecology and Obstetrics Clinic Narodni Front, Belgrade, Serbia. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

SL-C, VD, MK, FM, and AA were responsible for the study conception and design, interpreted the data, and critically revised and finalized the manuscript. SL-C performed global haemostatic assays, analyzed the data, and drafted the manuscript. FM designed the flow cytometric panels, analyzed the EV samples, and prepared figures. VM-M and ZM recruited participants, contributed to data collection, and interpretation of the results. All authors reviewed and approved the final version of the manuscript.

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REVIEW
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Vascular Remodeling in Pulmonary Arterial Hypertension: The Potential Involvement of Innate and Adaptive Immunity

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Pulmonary arterial hypertension (PAH) is a severe disease with high morbidity and mortality. Current therapies are mainly focused on vasodilative agents to improve prognosis. However, recent literature has shown the important interaction between immune cells and stromal vascular cells in the pathogenic modifications of the pulmonary vasculature. The immunological pathogenesis of PAH is known as a complex interplay between immune cells and vascular stromal cells, via direct contacts and/or their production of extra-cellular/diffusible factors such as cytokines, chemokines, and growth factors. These include, the B-cell—mast-cell axis, endothelium mediated fibroblast activation and subsequent M2 macrophage polarization, anti-endothelial cell antibodies and the versatile role of IL-6 on vascular cells. This review aims to outline the major pathophysiological changes in vascular cells caused by immunological mechanisms, leading to vascular remodeling, increased pulmonary vascular resistance and eventually PAH. Considering the underlying immunological mechanisms, these mechanisms may be key to halt progression of disease.

Keywords: pulmonary arterial hypertension, immunology, vascular remodeling, endotheliopathy, macrophage, histology, immunopathology, interleukin-6

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INTRODUCTION

Pulmonary arterial hypertension (PAH) is a progressive cardiovascular disease with high mortality and serious impact on the quality of life of affected patients. Due to increased pulmonary vascular resistance (PVR) with pressure overload on the right side of the heart, right sided heart failure and death could be the consequence. According to the recommendation of the 6th Pulmonary Hypertension (PH) world symposium of 2018, PH is diagnosed if mean pulmonary arterial pressure (mPAP) exceeds 20 mmHg, assessed by right heart catheterization (RHC) (1). For many years, the diagnosis of PH was based on mPAP values $\geq$ 25 mmHg, due to concerns of over-diagnosis and over-treatment. However, the main reason for over-diagnosis appeared the lack of confirmation by RHC. Clinically, it is important to distinguish pre-capillary PAH from post-capillary pulmonary hypertension. This can be determined by measuring the pulmonary arterial wedge pressure (PAWP) and PVR. PAH, also known as pre-capillary PH, is then defined by a

mPAP $\geq$ 20 mmHg and a PAWP $\leq$ 15 mmHg. The PVR is critical to distinguish post-capillary PH from combined pre- and post-capillary PH. Combined PH is defined as: mPAP $\geq$ 20 mmHg and PAPW $\geq$ 15 mmHg, and PVR must be $\geq$ 3 Wood units. Recognition of these subtypes of PH provides insight into the etiology. The classification of PH according to the most recent consensus meeting is shown in **Figure 1** (1). When studying the etiologies of PH, numerous different underlying diseases may contribute to the development of the typical vasculopathy or vascular remodeling that subsequently leads to increased pressure in the pulmonary circulation. Idiopathic PAH (IPAH) is known to feature this pre-capillary vasculopathy, however, without direct contribution of an underlying disease. It is hypothesized that triggers such as infections or toxins inducing endothelial cell injury and activation play an important causative role. PH could also be caused by left sided heart disease, chronic lung disease, chronic thrombo-embolic events (CTEPH) or caused by multifactorial triggers (**Figure 1**). Interestingly, PH is often associated with systemic autoimmune diseases and the role of the immune system in PH has been studied for decades. Examples of autoimmune diseases with increased risk of developing PAH are in particular systemic sclerosis (SSc) and mixed connective tissue disease (MCTD). However, PAH may also occur in a minority of systemic lupus erythematosus (SLE) and anti-phospholipid syndrome (APS) patients, the latter in the context of CTEPH (2). Involvement of the vasculature, inflammation and stronger clotting tendency, is core to the pathologic process in these conditions. However, the underlying mechanisms are very different. Remarkably, ANCA-associated vasculitis, a systemic disease that predominantly affects small and medium-sized vessels, is almost never complicated by PAH. These observations highlight the complexity of PAH and its association with very specific pathological mechanisms leading to vascular remodeling seen in PAH. Other factors also may contribute to the pulmonary risk in these systemic autoimmune patients. SLE and anti-phospholipid syndrome APS are associated with hyper coagulability, and can cause chronic thrombo-emboli and therefore may develop CTEPH (3). SSc can induce pulmonary fibrosis leading to hypoxic vasoconstriction with long term vascular remodeling. Patients with SSc are at risk to develop cardiac fibrosis or even pulmonary venous occlusive disease (PVOD), leading to PH in a pathophysiological different manner (4). In the PH classification (**Figure 1**) these patients are clustered as connective tissue disease associated PAH (CTD-aPAH). This cohort also includes numerous patients with the rare presentation of PAH and coexistent rheumatoid arthritis, giant cell arteritis, and myositis, either or not in combination with interstitial lung disease. Altogether, we must better understand how precapillary PAH develops, and thereto need to study the pathways of immune-activation, how autoimmunity develops, and how and by which effector mechanisms the early signals translate into long term downstream pulmonary vascular changes. Vasculopathy in PAH has been observed in all vessels of the pulmonary arterial circulation. Plexiform lesions are typical for PAH, and have a possible role in shunting between pulmonary and bronchial circulation. Other lesions, like intimal thickening, adventitial fibrosis and arterialization of non-muscular vessels, are also observed in PAH and contribute to increased PVR. Many studies have reported insights in the process of vascular remodeling in PAH, indicating that both innate and adaptive immune system are strongly involved. However, it is still not fully understood how cells of the immune system induce changes to the pulmonary vascular wall and how this could lead to PAH. The scope of this review is focused on the immunological mechanisms that contribute to vascular remodeling in PAH with a particular focus on idiopathic and CTD-aPAH. Additionally, we focus on the interaction between vascular stromal cells and immune cells. Finally, possibilities for future research and clinical implications will extensively be discussed.

HISTOPATHOLOGY OF VASCULAR REMODELING IN PULMONARY ARTERIAL HYPERTENSION

PAH is considered to be a pan-vasculopathic disease of the pre-capillary compartment of the pulmonary circulation. Nevertheless, histology shows that different phenotypes of vascular remodeling in PAH occur depending on the size and function of the different vessels. These vessels are the distal muscular arteries with a diameter of 70–500 μm, small pre-capillary pulmonary arterioles ranging from 20 to 70 μm, and small capillaries that have diameters smaller than 20 μm. Lesions that are observed in the distal muscular arteries consist of medial hypertrophy and hyperplasia, intimal and adventitial fibrosis and *in situ* thrombosis or plexiform lesions (**Figure 2**). Small arterioles and capillaries often show obliteration, muscularization and perivascular inflammation. Taking the different layers of the vascular wall as a starting point, we will elaborate on the possible mechanisms that underlie the different lesions that are seen in PAH.

Intimal Remodeling, Plexiform Lesions and Pericytes

The intimal layer primarily consists of an endothelial monolayer. In severe PAH, with mPAP pressures higher than 45–50 mmHg, the intimal fractional thickness is increased up to three fold as is observed in some 25% of the patients with PAH (5). This results in an increase of pulmonary vascular resistance (PVR) by 40 times. The thickened intimal layer consists predominantly of collagen and mucin rich matrix, fibroblast-like cells, endothelial cells, as well as pulmonary arterial smooth muscle cells (PASMCs). The common denominator for the development of irreversible vascular remodeling in PAH is an altered crosstalk between cells in the vascular wall, this concerns particularly the endothelial cells lining the intimal layer. The endothelial cell is known as a critical source of key mediators for vascular remodeling like growth factors [fibroblast growth factor (FGF)-2], serotonin (5-HT), angiotensin II (AngII), vasoactive peptides like nitric oxide (NO), prostaglandin I2 (PGI2), endothelin-1 (ET-1), cytokines like interleukin-1 (IL-1), IL-6 and chemokines (6–9). Overproduction of these paracrine mediators has a direct effect on the proliferation of other cells in the vascular wall,

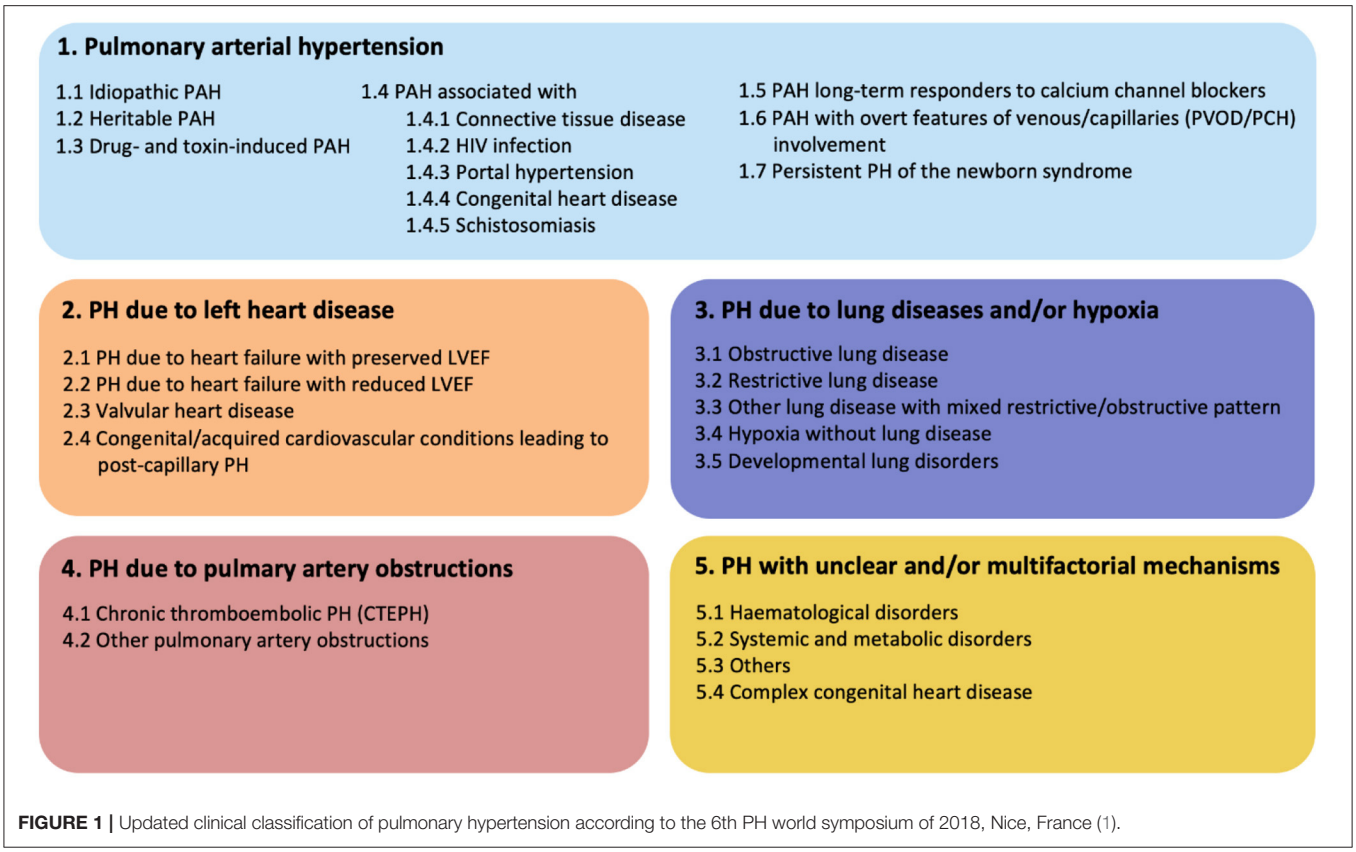

FIGURE 1 | Updated clinical classification of pulmonary hypertension according to the 6th PH world symposium of 2018, Nice, France (1).

like PASMC, pericytes or endothelial cells themselves (autocrine effects), contributing to intimal remodeling (10, 11).

When endothelial cells proliferate in an overshooting regenerative manner, they can form plexiform lesions. These lesions are the classic histological hallmark of PAH, and are mainly seen in severe or progressive PAH (12, 13). Plexiform lesions are often located at vascular branching points and contain vascular channels, that are highly ordered. The vascular channels in plexiform lesions are lined with intact endothelium, that is separated by intermediate PASMCs with in-between synthetic and contractile phenotypes. Both phenotypes are necessary for progressive vascular remodeling. Plexiform lesions often resemble glomeruloid-like lesions with sprouting of new blood vessels, and excessive expression of angiogenic markers like vascular endothelial growth factor (VEGF), and hypoxic inducible factor-1α (HIF-1α). This suggests a process of disordered angiogenesis (14). Plexiform lesions are more often seen in IPAH, but can also be found in some 50% of the CTD-aPAH cases, with similarities in composition, architecture and microenvironment (5). Inflammatory cells in these lesions are a mixture of T-cells ($CD3^+$), monocytes and macrophages ($CD68^+$) and tryptase positive mast-cells in both IPAH and CTD-aPAH (15). Later in this review, the role of these cells in PAH will be further discussed.

Plexiform lesions are typical features of long standing vascular remodeling in PAH. Moreover, they may also have functional implications in vascular remodeling. Based on findings of close association of plexiform lesions and dilated bronchial microvessels in patients who died due to severe IPAH, plexiform lesions are suggested to function as anostomotic structures between the pulmonary and bronchial circulation (16). Hemodynamic stress, caused by these anastomoses, could lead to vascular wall stretch in the bronchial circulation and expansion of the vasa vasorum of pulmonary arteries. This could provide a pathway for progenitor and inflammatory cells to participate in pulmonary arterial remodeling. Nevertheless, Ghigna et al. showed that bronchial artery hypertrophy and bronchopulmonary shunting was also associated with post-capillary remodeling (17). This occurred more frequently in patients with genetic BMPR2 mutations. They also described the newly found singular millimetric fibrovascular lesions (SiMFis), which were also associated with these genetic BMPR2 mutations. A direct relationship between the degree of bronchial vascular remodeling and disease severity, based on PVR, mPAP or cardiac index, was not observed in this study.

Pericytes are important supporting cells that maintain endothelial viability in angiogenesis, a process in which new vessels sprout from existing vessels. Recently, the role of pericytes in the systemic vascular changes of diabetic retinopathy and stroke has been investigated (18–22). In these cases, the loss of pericytes caused vascular dysfunction and loss of vessels. However, the role of pericytes in the pulmonary vasculature is less clear. Ricard et al. showed that increased pericyte coverage of the distal arterial vasculature in PAH correlated well with vascular

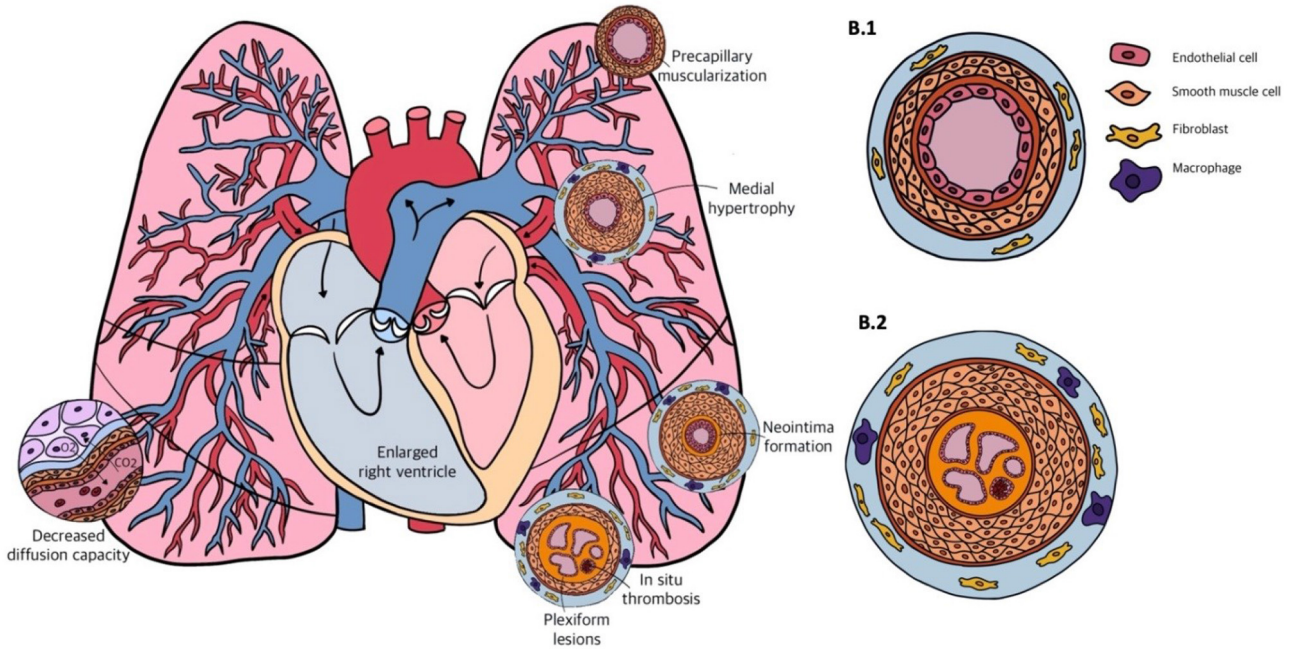

FIGURE 2 | (A) Schematic overview of the cardiopulmonary system in pulmonary arterial hypertension (PAH) and the different forms of vascular remodeling. **(B)** Transversal sections of the pulmonary arterial vasculature in a healthy person (B.1) and in a patient with end-stage PAH (B.2). Showing from inside to outside: multiple capillary channels typical for plexiform lesions, with *in-situ* thrombosis, intimal thickening, pulmonary artery smooth muscle cell (PASMC) proliferation in the medial layer, and infiltration of fibroblasts and macrophages in the adventitial layer.

remodeling and that this process was stimulated by FGF-2 and IL-6 (11). TGF-β in this study affected the capacity of pulmonary pericytes to differentiate into smooth muscle-like cells, thereby contributing to increased PVR and PAH development. Dierick et al. studied the role of pulmonary resident $PW1^+$ progenitor cells in hypoxia-induced PH (23). They showed that hypoxia-induced vascular muscularization is dependent on $PW1^+$ cells, and showed that some of these cells also express pericyte markers. Most likely mediated by stimulation by CXCR-4. This is mainly the case in neo-muscularization of non-muscularized vessels in PH. Nevertheless, Crnkovic et al. studied the involvement of other cell lineages that contribute to SMC differentiation and showed that resident SMCs are the major source of α-smooth muscle cell $actin^+$ cells that appear in the remodeled pulmonary arteries (24). Besides, endothelial cells were also covered in the lineage tracing experiments of Crnkovic et al., but due to the complexity and limitations of the current murine models, a definitive conclusion on the subject of endothelial mesenchymal transition (endoMT) remains absent. However, endothelial cells are substantially involved in the biology of SMCs in vascular remodeling in PAH. Sheikh et al. showed that enhanced HIF1-α upregulates endothelial cell secretion of platelet derived growth factor-B (PDGF-B), which induces priming of SMCs, making them more susceptible to induce distal arteriolar muscularization (25). Bordenave et al. also investigated the role of pericytes in distal arteriolar muscularization (26). They confirmed that pericytes are mobilized to muscularized distal arterioles in PAH and demonstrated that pulmonary pericytes are altered in patients with IPAH, overexpressing CXCR-7 and TGF-βRII. Although also other cell lineages may contribute to neo-muscularization and vascular remodeling in PAH, pulmonary pericytes orchestrate multiple critical functions that can directly and indirectly contribute to this process. Yuan et al. showed that the disturbed interaction between endothelial cells and pericytes leads to vascular remodeling and is mediated by pyruvate dehydrogenase kinase 4 (PDK4), which is a key player in mitochondrial metabolism and can therefore influence downstream effects of glycolysis (27). Targeting these pathways may resolve pericyte and endothelial cell interactions and contribute to rescuing vascular remodeling.

Medial Remodeling

The medial vascular wall is also known to play a major role in vascular remodeling in PAH and primarily consists of PASMCs (28). Stacher et al. have shown that in PAH the media fractional thickness may increase to 20%, and in combination with the increased intimal thickness this correlates well with the increase in mPAP and PVR (5). PASMC proliferation in PAH is a key feature of vascular remodeling and has been thoroughly investigated. Already in the early 80s, animal studies using either hypoxia or monocrotaline (MCT) gave insight into the course and specific anatomical location of PASMC proliferation. In a hypoxic rat model, the onset of proliferation was observed in the hilar vessels, predominantly in the intimal and adventitial

layer, but with minimal changes in the medial layer (29). In the MCT rat model similar observations were made (30). However, an important finding was the decrease of PASMC proliferation in the hypoxic models after 4–5 weeks, suggesting that active PASMC proliferation is a sign of early disease (31–33). This has also been observed in a pathologic study of human tissue of IPAH and hereditary PAH patients (HPAH), in which there was no active PASMC proliferation in end stage lung tissue (34). As mentioned before, endothelial cells contribute significantly to PASMC proliferation (10). However, many of the paracrine mediators remain still unknown. On top of the PASMC proliferation in vessels with an already existing medial layer, vessels without a distinct medial layer become muscularized (35). This is often accompanied by neointima formation. The distal extension of smooth muscle arteries into small peripheral, normally non-muscular, pulmonary arteries is commonly seen in vascular remodeling in PAH, leading to increased vascular tone and PVR. A new perspective on PASMCs in vascular remodeling in PAH is cellular senescence (36). Cellular senescence is defined as irreversible loss of cell growth and proliferation, which is mainly characterized by the cessation of cell replication. PASMC senescence in PAH is induced by hypoxia and plays a key role in hypoxia-induced PASMC proliferation (36). A fraction of the PASMCs become senescent which promotes paracrine IL-6 release, that is mediated by the mTOR/S6K1 pathway which accelerates PASMC senescence, and showing the important role of immune mediated mechanisms in vascular remodeling in PAH.

The Adventitial Layer as an Inflammatory Signaling Hub

The adventitial layer of the vessel wall is a fascinating compartment but its role in vascular remodeling is debated and somewhat controversial. The "inside-out" hypothesis of vascular remodeling, as was mentioned earlier, states that endothelial dysfunction, either caused by inciting factors coming from the blood, or by intrinsic endothelial cell abnormalities, results in intima fibrosis, PASMC proliferation and migration and the formation of neointima. However, the "outside-in" hypothesis recently claimed a more dominant role for the adventitial layer in vascular remodeling. Adventitial thickening in PAH is mild, or lacking, as shown in histopathological studies by Stacher et al. (5). In contrast, Chazova et al. reported two- to four-fold increase in adventitial thickness in an autopsy series of 19 IPAH patients compared to controls (28). The adventitial layer is physiologically complex and consists of numerous cell types such as fibroblasts, immune modulatory cells, resident progenitor cells, endothelial cells of the vaso-vasorum and even adrenergic nerves (37). In comparison, intimal and medial "monocellular" layers consist of endothelial cells, pericytes and PASMCs only. Although structural changes in the adventitia have less direct impact on PVR than thickening of intima and media, it plays a major role as inflammatory signaling hub and facilitates feed-forward interactions between incoming macrophages and resident fibroblasts (38, 39). The "outside-in" hypothesis will be extensively discussed in the next section.

The Histopathology of Vascular Remodeling in Summary

The aforementioned studies, all show some involvement of immune cells through the different layers of the vascular wall in vascular remodeling in PAH, and suggest a dynamic interaction with stromal vascular cells. The thickness of the vessel layers correlates well with increased PVR and thereby development of PAH, with maybe the exception of the adventitial layer. However, fractional thickness is not the only cause of PAH development. How the state and timing of activity of different immune cell types relate to vessel wall changes and increased PVR, is not fully clear from available studies. This will be of interest particularly to better understand (peri)-vascular inflammation and the interactive role between the vascular wall and immune system and how this leads to extensive vascular remodeling in development of PAH. This may also lead to novel targets of therapy for PAH.

PERIVASCULAR INFLAMMATION AND THE INTERPLAY BETWEEN IMMUNE CELLS AND VASCULAR STROMAL CELLS IN VASCULAR REMODELING IN PAH

Vascular infiltration of immune cells, consisting of both innate and adaptive origin, in PAH has been observed in many different studies. Marsh et al., investigated lungs of PAH patients with end-stage disease by using flow cytometry (40). They observed increased activated plasmacytoid dendritic cells (pDCs), macrophages, mast cells and T-cell receptor γδ (TCR-γδ) T-cells, which suggests a link between innate and adaptive immunity. TCR-γδ T-cells are important in tissue homeostasis and wound healing by releasing insulin-like growth factor-1. This enhances PASMC proliferation and, as mentioned before, dysregulated proliferative PASMCs are a hallmark of vascular remodeling in PAH (41). pDCs produce substantial amounts of type-1 interferon, which has been implicated in PAH pathogenesis as well. Type-1 interferon can also induce anti-inflammatory effects by activating Tregs (42, 43). Due to lack of cell subtype specific markers in the Marsh study, it is unclear what type of macrophages infiltrated into the vessel wall. However, this study extracted cells from whole lung tissue samples, thereby not only focusing on immune cells in pulmonary vessels, but also in parenchymal pulmonary tissue as well. Nevertheless, this may immunologically be as important. Perros et al. have shown the presence of pulmonary lymphoid neogenesis and composition of tertiary lymphoid tissue in IPAH (44). Colvin et al. have shown that in PAH, bronchus-associated lymphoid tissue expands and contributes to auto-immunity and auto-antibody development (45).

Although we would like to draft a clear overview of the main functions of each immunological cell in vascular remodeling in PAH, it is unfortunately not preferable. Due to the dynamic nature and complexity of both the immune system and vascular remodeling itself, we preferred to focus on the dynamic interplay between both the immune cells and vascular cells mediated by many different cytokines and chemokines altogether. However,

elucidating the mechanisms in this way may be more challenging. Nevertheless, by discussing these mechanisms in this way we preserve a more natural depiction of the patho-immunological mechanisms involved in vascular remodeling in PAH. Due to the both advancing clinical implications of interleukin-6 (IL-6) and it being the most discussed immunological mediator in PAH, we decided to discuss the role of this cytokine in a separate paragraph.

Fibroblasts and M2 Polarized Macrophages

El Kasmi et al. showed promising results regarding effects on vascular remodeling by cells in the adventitial layer of the vascular wall in PAH (46). This study showed an increase in $CD68^+$ cells (monocytes and macrophages) in the adventitial vascular layer in human IPAH, hypoxia induced PH in calves and MCT rats. These $CD68^+$ cells expressed CD163 and CD206, known to be M2-like macrophage subtypes. Interestingly, Signal Transducer And Activator Of Transcription 3 (STAT3) expression was upregulated in these cells, which suggests a different polarization mechanism than the conventional IL-4/IL-13 mediated pathway. The latter is usually induced via STAT6 expression. Their findings indicate that M2 polarization can also be mediated by IL-6. Kasmi et al. also showed that conditioned media of adventitial fibroblasts from different PH models, and from humans with PAH, in contact with macrophages induce a shift to the M2 subtype. Min Li et al. investigated the distinct phenotypical changes of these adventitial fibroblast after exposure to chronic hypoxia (47). This phenotype, that is also known as a pulmonary hypertension fibroblast (PHFib), was characterized by high expression levels of canonical pro-inflammatory cytokines (IL-1β, IL-6), macrophage chemo-attractant cytokines CCL2 (MCP-1), CXCL12 (SDF-1), CCL5 (RANTES), macrophage growth factor (GM-CSF), CD40L and VCAM-1, contributing to M2 macrophage subtype polarization. This study also showed that PASMCs that were harvested from the same vessels, do not show a different phenotype. However, fibroblasts seem to differentiate into pro-fibrogenic or PASMC-like subtypes, which suggests a broad involvement in vascular remodeling in PAH.

The role of other chemokines in PAH has also been studied. Amsellem et al. investigated the CX3CL1/CX3CR1 and CCL2/CCR2 (better known as MCP-1) chemokine systems in hypoxia induced PH (48). The impact on monocyte trafficking, macrophage polarization, and pulmonary vascular remodeling was addressed. $CX3CR1^{-/-}$ mice were protected against hypoxic PH compared to wild-type mice, whereas $CCL2^{-/-}$ mice and double $CX3CR1^{-/-}/CCL2^{-/-}$ mice exhibited similar PH severity as did wild-type mice. CX3CR1 deficiency showed an increase in both the number of lung monocytes as well as macrophage perivascular inflammation and most importantly, a shift from M2 to M1 macrophage polarization. The absence of M2 macrophages, therefore, seems to prevent development of PH. CX3CR1 deficient mice showed diminished PASMC proliferation, which was partly mediated by CX3CL1 secretion. This study suggests a role of M2 macrophages as effector cells in PH. Supernatant from hypoxia induced-M2 macrophages induces PASMC proliferation, and is also able to induce endothelial cell injury, mediated by leukotriene B4 (LTB-4), contributing to the outside-in hypothesis (49, 50). M2 macrophages also exhibit functions on the vascular wall that do not involve vascular stromal cells, for instance excreting extracellular matrix (ECM). Legumain is a newly discovered cysteine proteinase belonging to the C13 peptidase family and is primarily expressed in macrophages. Legumain increased the synthesis of extracellular matrix (ECM) proteins via matrix metalloproteinase-2 (MMP-2) activation, promoting vascular remodeling in PAH (51).

The study of Hashimoto-Kataoka et al. showed the dynamic interplay between cytokines, adaptive and innate immune cells and eventually PASMC proliferation (52). Firstly, they showed that IL-6 blockade ameliorated hypoxic pulmonary hypertension (HPH) in mice, which also prevented the hypoxia induced accumulation of Th17 cells and M2 macrophages in lungs. IL-17 blockade had no effects on HPH, though IL-21 knockout mice were resistant to HPH. These knockout mice also didn't accumulate M2 macrophages in their lungs, once again suggesting an important role for M2 macrophage in PH. IL-21 induced M2 macrophages were cultured and PASMCs were then incubated with conditioned medium of these macrophages. Interestingly this induced significant PASMC proliferation. Investigating the molecular mechanism behind proliferation, it was shown to be related to CXCL12, that normally acts as a chemokine that stimulates PASMC proliferation by binding to CXCR4. When CXCR4 was antagonized, PASMC proliferation did not occur after incubation of PASMCs with the conditioned media of the M2 macrophages. Hypoxia did also increase the number of Ki67 positive PASMCs, indicating increased proliferation. IL-21 knockout mice had significantly less Ki67 positive PASMCs. To validate the clinical significance, IL-21, Arg-1 and CD206 cells (M2 macrophage subtype markers) were stained in tissue of IPAH patients undergoing lung transplantation and controls. This data showed significant increase of both IL-21 producing cells, and M2 macrophages in the adventitial layer. Concluding, M2 macrophages play a major role as effector cells in vascular remodeling in PAH. M2 macrophages are known for their heterogeneity, and future studies should focus on specific M2 macrophage subtypes and their specific interactions with vascular stromal cells in PAH.

Mast-Cell—B-Cell Axis and Humoral Immunity in PAH

It is known that tissue resident mast cells, B- and T-cells are increased in patients with diverse forms of PAH. These different cell types have an important direct effect on the vasculature, especially by their involvement on the immunological micro-environment by secreting chemokines and cytokines that promote further vascular low-grade inflammation.

Breitling et al. studied the interaction between mast-cells and B-cells mediated by IL-6 in PH rat models (53). This study revealed an important link between mast cells and B-cells with an interplay of IL-6 in the MCT and aortic banding rat

models. They showed that mast-cells in PH produce substantial amounts of IL-6 which promotes B-cells to differentiate into plasma cells, which is in line with the observed up-regulation of immunoglobulin production (53). Aortic banding, showed a drastic increase in total IgG over a period of 9 weeks, and when IgG was purified and transferred to healthy animals, PH developed, suggesting a pathognomonic mechanism of auto-antibodies in PH. Upregulation of IL-6 also showed abundance of T-cells in an IL-6 dependent and mast-cell dependent manner, which suggests a broader role of IL-6 than the mast-cell—B-cell axis only (54). When rats were treated with mast-cell stabilizer ketotifen, IL-6 levels virtually returned to zero.

Different auto-antibodies against endothelial cells (55, 56), phospholipids (57), fibroblasts (58), and nuclear antigens have been described in PAH. Tamby et al. observed the presence of anti-endothelial cell antibodies (AECAs) in IPAH, which hinted at a possible role of the humoral immune system in the pathogenesis of PAH (55). However, not all PAH patients display circulating AECAs (56). AECA is an umbrella term for antibodies against many unknown endothelial antigens. Some groups have tried to identify AECA antigens, but it is still not known to what extent distinct AECAs may exhibit clinical significance. The group of Dib et al. identified targets in patients with SSc, with or without PAH, and in patients with IPAH (59). They observed AECA binding to lamin A/C and the tubulin β-chain, both intracellular proteins. However, hypothetically membrane surface antigens are the main antigens for AECA binding *in vivo*. It is still unknown if and at what stage of disease AECAs may play an important role. Our group has investigated the role of pathogenicity of AECAs (60). We showed that the presence of AECAs contributed to increased vascular inflammation by increased production of pro-inflammatory cytokines, like IL-6, chemokines IL-8 and CCL2 (MCP-1) and an increased expression of adhesion molecules like intercellular adhesion molecule 1 (ICAM-1) and VCAM-1. These molecules are known to promote vascular inflammation and vasculopathy, and could therefore contribute to vascular remodeling in PAH. However, when isolated human umbilical vein endothelial cells (HUVECs) are exposed to the AECAs of IPAH and SSc patients, there is no interplay with other immune cells in this model. The dynamic process of inflammation cannot be tested on isolated cell cultures. Therefore, it is of great importance that other *in vitro* models, besides already existing animal models, will be utilized. Tissue engineered vessel-on-a-chip, or so-called organoids to assess vascular remodeling by using different co-cultures of vascular stromal cells in contact with patient immune cells, sera or isolated immunoglobulins could be a viable option. Patient specific antibody—antigen matching by differentiating patient-derived pluripotent stem cells (iPSCs) into endothelial cells could help discovering more relevant antigens that may play a role in the immune mediated vascular remodeling in PAH.

Complement Mediated Vascular Remodeling

The complement system is a very complex and evolutionary old immunological protein cascade with 3 different pathways known as the mannose binding lectin, classical and alternative route. The complement system has multiple effector mechanisms e.g., chemotactic, opsonic, pro-inflammatory and membrane attack complex-mediated lytic mechanisms. The role of the complement system in PAH has not been investigated to great extent, especially in the case of local inflammation of the tissues. However, Stenmark and colleagues recently studied the role of the complement system in vascular remodeling in PH. They particularly looked at the role of complement in the early phase of disease in rat and mouse hypoxic models. Lungs of these mice and rats demonstrated C3 depositions both peri-bronchial as well as perivascular, with high levels of anaphylatoxin receptor expressing cells. Complement depositions were related to localized proliferating cells as measured by Ki67, and increased secretion of GM-CSF and MCP-1 stimulating macrophage inflammation. RNA sequencing suggested hypoxia induced alternative complement pathway activation, mediated by its activator complement factor b (Cfb). Analysis of serum complement markers, suggested the alternative complement cascade as a biomarker in PAH. Hypoxia also induced luminal and medial IgM depositions, whereas IgG deposits were found more perivascular. Immunoglobulin knockout models showed lack of complement activation, reduced perivascular accumulation of $CD68^{+}$ macrophages and decrease in both Ki67 positive cells and chemoattractant secretion. After immunoglobulin restitution, these effects were reversed, proposing that complement mediated perivascular lung inflammation in PAH is immunoglobulin mediated and, as such, involves the classical pathway. Other more chronic PAH hypoxia animal models and lung tissue of IPAH patients showed C3d (complement 3 degradation) depositions suggesting the longitudinal involvement of complement in PAH. Functional analyses of the complement system in a clinical setting, however, may be informative in pointing toward underlying antibody-mediated diseases such as SLE or active cryoglobulinemia.

The Role of IL-6 in Vascular Remodeling in PAH

Clinically, IL-6 serum levels in severe PAH have already been studied for over 25 years. Humbert et al. showed increased serum levels of IL-6 compared to controls and chronic obstructive pulmonary disease associated PAH (COPD-aPAH) patients, suggesting that IL-6 may be an important mediator in PAH (61). Since then, many other groups have confirmed this observation in mild PAH as well. However, most of these studies were conducted in small single center cohorts composed of primarily severe IPAH patients, making it unclear if this increase in IL-6 serum levels was due to critical illness or if it was a driving factor in vascular remodeling. Simpson et al. addressed this important issue, and investigated the association between IL-6 and its cellular sources in different clinical phenotypes of PAH (62). This study showed that IL-6 was produced by vascular cells, where after they investigated the role of IL-6 as a mechanistic biomarker in early disease in different subtypes of PAH. Simpson et al. showed that PASMCs are one of the major vascular cells that produce IL-6, besides the perivascular infiltrates of inflammatory

cells. This pathogenic role of IL-6 in PAH has been confirmed by others.

Steiner et al. showed that over-expression of IL-6 in transgenic mice under normoxic conditions leads to development of elevated right ventricular systolic pressure as well as vasculopathic changes seen in PAH (63). This effect was even larger under hypoxic circumstances. Vascular homeostasis is highly dependent on the balance between apoptosis and proliferation of the individual vessel wall cells. Up and down regulation of factors that maintain vascular homeostasis based on a correct balance between apoptosis and proliferation were dysregulated in these transgenic IL-6 mice. This suggests that IL-6 may induce development and progression of vascular remodeling in PH, independently of hypoxia, via pro-proliferative anti-apoptotic mechanisms. This was also seen in the new MRL/lpr autoimmune mouse model for PH, which develops hypergammaglobulinemia, produces various auto-antibodies (for instance anti-dsDNA), and spontaneously develops vasculitis and nephritis (64). Although, the pulmonary consequences were never well studied until now, this mouse model seems to induce PH with SLE-like characteristics. Interestingly, this occurred by inducing disturbed vasomotility via increased prepro-ET1 (prepro-endothelin 1) and decreased eNOS (endothelial nitric oxide synthase) activity. IL-6 was also upregulated and led to vascular remodeling with increased PASMC proliferation and decreased apoptosis, and eventually PAH. The latter is in concordance with other studies, like the study of Le Hiress et al. in which specific cytokines, including IL-6 and upregulation of ICAM, VCAM and E-cadherin were observed due to dysfunctional endothelial cells, without presence of a distinct immunological disease (65). Our clinical experience with immunology in PAH patients for over 15 years showed us that many PAH patients show signs of immune mediated disease, without confirmation to a specific auto-immune disease or clinical diagnosis. Biomarkers like soluble IL-2 receptor and IL-6 may be elevated, or auto-antibodies are present and may contribute to the development of PAH, without developing full symptoms as seen in connective tissue diseases. Immune activation or dysregulation may lead to increased IL-6 levels in pulmonary tissue and vessels and may, just as in animal models, contribute to development of PAH most likely in a multi-hit manner. Savale et al. showed that knock-out of IL-6 in mice, attenuates PH development induced by hypoxia (66). *In vitro* studies showed increased IL-6 secretion by PASMCs with a further increase after hypoxia. IL-6 knock-outs showed less perivascular infiltration of immune cells and less pronounced arterial muscularization, suggesting a role for IL-6 in modulating lung vessel inflammation and remodeling during hypoxic PH progression as well. Tamura et al. showed insights into the direct underlying mechanism of IL-6 on the vascular wall. They elucidated the role of ectopic membrane-bound IL-6 receptor upregulation in PASMCs in PH and showed that IL-6 induces overexpression of anti-apoptotic proteins like MCL-1 and BCL2. This led to excessive accumulation of PASMCs within the pulmonary arterial vasculature (67). They also showed that deletion of the IL-6 receptor or blocking via anti-IL-6 receptor antibodies (tocilizumab), leads to resistance to experimental PH development under hypoxic circumstances.

Inflammatory and Immunological Mechanisms in Summary

Traditionally, vascular inflammation has been considered an "inside-out" response centered on neutrophil granulocyte, monocyte and macrophage recruitment from the lumen to the intima of blood vessels after endothelial cell injury or activation. However, growing evidence supports a new paradigm of an "outside-in" hypothesis, in which vascular inflammation is initiated and/or perpetuated by adventitial fibroblasts. The aforementioned studies substantiate this "outside-in" hypothesis, that in combination with other findings may be triggered by changes of the intimal vascular layer. How these mechanisms develop over time and what the underlying trigger may be, like antibodies targeting cells of the vascular wall, should be investigated in an immunological approach. We tried to depict an overview of the many different immunological mechanisms involved in vascular remodeling in PAH in **Figure 3**. However, histological proof of active inflammation in vascular remodeling in PAH patients remains limited. This might be due to a relative contra-indication of video assisted thoracic surgery (VATS) procedures in PAH patients (68). Therefore, histological analysis is mostly performed on post-transplant and post-mortem material. However, a quenched representation of immune cell involvement due to end stage disease must be considered. The interaction of both stromal vascular cells and immune cells is dynamic and complex but can be studied in *in vitro* models like organoids, optionally made viable by inducible pluripotent stem cells (iPSCs). Eventually, these models may help uncover potential targets for therapeutic intervention on both stromal and immunological basis.

CLINICAL IMPLEMENTATION OF IMMUNE SUPPRESSION IN PAH

Over the last decades great progress was made on the development of novel therapies in PAH. Examples of these are the classes of endothelin-1 receptor antagonists (ERAs), phosphodiesterase-5 inhibitors (PDE5i), and the prostacyclins, which can now also be administered orally, or prostacyclin receptor agonists like selexipag. Interestingly, these therapies mainly focus on vasodilation of the pulmonary vasculature to lower intrapulmonary pressure, but despite their efficacy these are not true curative therapeutic options, and life expectancy and quality of life for PAH patients is still relatively low. It is also intriguing that it is unknown and not well studied how these therapies impact the vascular remodeling. The same holds true for the effects of these compounds on the immune phenomena that were involved in the disease process. As we extensively elucidated the role of the immune system in vascular remodeling in PAH throughout this review, we anticipate that the immune system may be a promising therapeutic target. This could be important, as immunological mechanisms contribute to

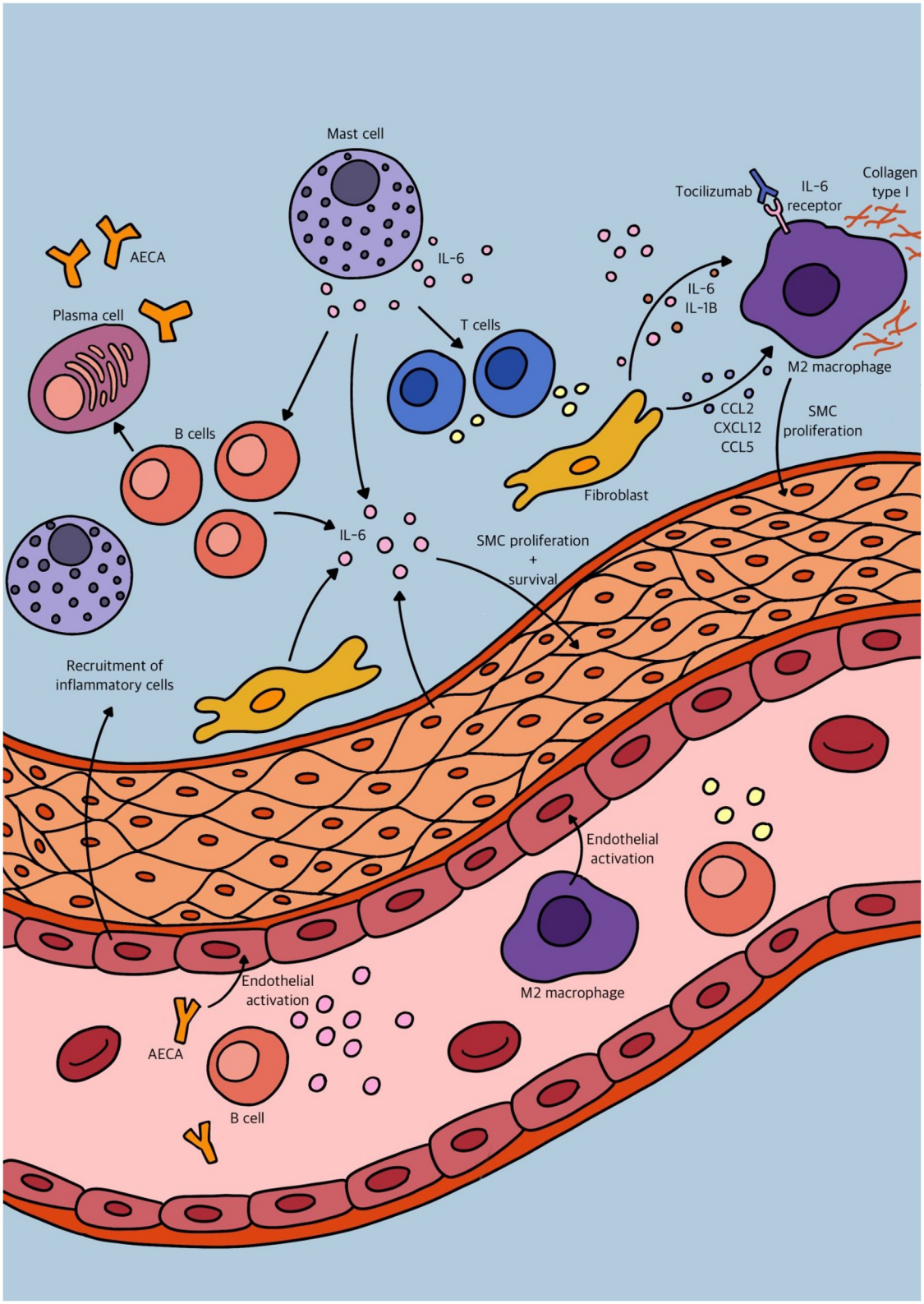

FIGURE 3 | Schematic overview of some of the different immunological mechanisms involved in vascular remodeling in pulmonary arterial hypertension (PAH).

irreversible vascular remodeling and its progression should be halted as early as possible.

Nearly all the aforementioned preclinical studies suggest a major role for IL-6 in vascular remodeling in PAH. This makes IL-6 an interesting potential therapeutic target. The use of tocilizumab (anti-IL-6-receptor blocker) has been investigated for itsefficacy in PAH, in a randomized phase 2 clinical trial (www.clinicaltrials.gov NCT02676947) (69, 70). Six months of treatment with tocilizumab, in this selection of patients, showed no significant improvement of PVR. However, this study had some major limitations. Simpson et al. showed that IL-6 levels vary in different subtypes of PAH, and other studies already showed that IL-6 in patients with for instance COPD are very low, compared to IPAH or CTD-aPAH (61, 62). Tocilizumab may therefore not be the drug of choice in patients without supportive evidence for underlying IL-6 mediated pathology. This randomized controlled trial (RCT) included world health organization (WHO) type 1 PAH patients, though CTD-aPAH patients with underlying disease like mixed connective tissue disease (MCTD), SLE and, especially, rheumatoid arthritis (RA) were excluded (69, 70). Toshner et al. argued that PAH in these patients may be rare and that they, in many cases, already used immunosuppression.

Studies on the use of immunosuppressive agents in PAH associated with SLE/MCTD exist but are limited in number (71–74). In the paradigm disease for PAH, namely SSc-aPAH and in rare diseases like the anti-synthetase syndrome associated with PAH, only a few case reports are published (75, 76). This is remarkable, as immune activity and autoimmunity is clearly present in these patients. One reason will be that in the majority of these patients the development of PAH is a late manifestation, and treatment is considered by many to be outside the window of opportunity. It is therefore important to identify as early as possible the still reversible immunological phase in the disease process. It is also still a challenge to identify and select the optimal target of immune suppression for the individual patient, depending on the dominant underlying mechanism. Selecting a broad range of patients with different underlying pathologies, be it immune mediated or not, may hamper the final results when studying the effects of, for instance, tocilizumab. Tocilizumab has already been investigated as a monotherapy in RA with favorable results (77). It therefore seems less logical to exclude patients from a trial in which a common pathogenic cytokine is targeted. In SSc, the paradigm vasculopathic disease for PAH, IL-6 serum levels were also increased when compared to healthy controls and correlated with disease severity (78). Nevertheless, due to the exclusion criteria, the major etiology of CTD-aPAH in the study of Toshner et al. was SSc (70). This study showed that more patients with CTD-aPAH had a >15% reduction in PVR after 6 months of tocilizumab treatment, in comparison to IPAH/HPAH patients. Therefore, tocilizumab could still be an interesting therapeutic option in SSc-aPAH, and should be specifically investigated in the future.

The effects of immunosuppression in PAH could be less easy to measure than the direct effect of vasodilators, but it will most likely contribute to the halting progression of the pathogenic vascular remodeling. Outcome measurements should focus on survival, maintenance of functional class (NYHA), diffusion capacity, or 6 min walking distance in addition to pulmonary vascular resistance or mean pulmonary arterial pressure (mPAP). Novel therapeutic options like sotatercept are currently under investigation and show promising results in decreasing PVR, by blocking activins and restoring anti-proliferative balance of the BMPR2 (bone morphogenetic protein receptor 2) pathway (79), suggesting reversibility in vascular remodeling. Research on these new agents should also focus on the effects of immune cells that contribute to vascular remodeling. Treatment for PAH in the future will most likely consist of a combination of medication that affects different mechanisms in the development of PAH. Vasodilative agents will quickly reduce right ventricular systolic pressure (RVSP), while immune modulation will halt or slow disease progression and vascular remodeling. In combination with agents that stimulate reversibility of the remodeled vessels a better outcome for the patient is anticipated.

DISCUSSION

Due to the elaborate dynamic interplay between different aspects of vascular stromal cells, chemokines, cytokines, complement proteins and immune cells of both the innate and adaptive immune system, it is hard to quickly summarize the underlying process in PAH. This review established a broad view of the many roles of the immune system in PAH, especially focusing on vascular remodeling. Endothelial injury may act as a trigger, inducing cytokine and chemokine responses, producing vasoconstrictive agents like endothelin-1 and decreased eNOS, and possibly activating the complement cascade leading to more inflammation. Damaged endothelial cells become dysfunctional and directly stimulate vascular stromal cells such as PASMCs to proliferate. Pericytes that regulate homeostasis of proliferation in vessels become dysfunctional, and differentiate into PASMCs themselves, losing their regulating function. Besides, local cells like fibroblasts and mast-cells are activated as well. These cells produce cytokines like IL-6 that have direct effects on vascular stromal proliferation and cell wall thickening, but also activate other immune cells like B-cells, stimulating more antibody-production and perivascular inflammation. Besides, macrophages polarize into a pro-fibrotic vascular repair type mediated by IL-6, that act in an overshooting manner excreting matrix protein, inducing vascular fibrosis and even stimulate proliferation of PASMCs. Fibroblasts may also differentiate into PASMC types and migrate into the medial layer inducing medial thickness, and neointima formation.

CONCLUSION

By definition, PAH is a progressive pan-vasculopathic cardiopulmonary disease caused by overshooting immunological and stromal "repair" mechanisms, eventually leading to decreased quality of life of patients, with risk of cardiac failure and premature death. PAH is known for its broad etiology and pathogenesis. However, in the clinical approach of patients with PAH the identification of underlying active immune

involvement is crucial, being one of the driving factors of disease progression. Immune activation, as evidenced by the presence of circulating autoantibodies, composition and phenotype of lymphocyte subsets, and other soluble biomarkers, should be explicitly investigated in clinical patients. Future studies should focus on the selective use of immunosuppressant's and immunomodulatory agents in PAH to stop disease progression. It would also be of interest to study the immunomodulatory effect of the present vasoactive agents as well novel agents like the activin antagonist sotatercept. Integration of models in which the patient's immune cells, serum and vascular stromal cells meet, could be essential for a better understanding of the process of a dynamic disease like PAH. Collaboration between clinicians and fundamental researchers is therefore key in the progress of treating this multi-factorial and devastating disease.

AUTHOR CONTRIBUTIONS

RT wrote the concept of the review which was adapted to the final version by the other authors. RY prepared the figures. The final version was approved by all authors. All authors contributed to the concept of the review.

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GLOSSARY

5-HT, 5 hydroxy-tryptophane
AECAs, Anti-endothelial cell antibodies
AngII, Angiotensin 2
Anti-dsDNA, Anti-double stranded DNA
APS, Anti-phospholipid syndrome
BMPR2, Bone morphogenetic protein receptor 2
C3d, Complement degradation product d
CCL2, C-C motif ligand 2
CCL5, C-C motif ligand 5
CD, Cluster of differentiation
Cfb, Complement factor b
COPD-aPAH, Chronic obstructive pulmonary disease associated pulmonary arterial hypertension
CTD-aPAH, Connective tissue disease associated pulmonary arterial hypertension
CTEPH, Chronic thromboembolic pulmonary hypertension
CXCL12, C-X-C motif ligand 12
ECM, Extracellular matrix
eNOS, Endothelial nitric oxide synthase
ERAs, Endothelin receptor antagonists
ET-1, Endothelin 1
FGF-2, Fibroblast growth factor 2
GM-CSF, Granulocyte macrophage colony stimulating factor
HPAH, Hereditary pulmonary arterial hypertension
HPH, Hypoxic pulmonary hypertension
HUVEC, Human umbilical vein endothelial cell
ICAM-1, Intercellular adhesion molecule-1
IL-1, Interleukin 1
IL-13, Interleukin-13
IL-17, Interleukin-17
IL-21, Interleukin-21
IL-4, Interleukin-4
IL-6, Interleukin 6
IPAH, Idiopathic pulmonary arterial hypertension
iPSC, Inducible pluripotent stem cell
LTB-4, Leukotriene B4
MCP-1, Monocyte chemoattractant protein 1
MCT, monocrotaline
MMP-2, Matrix metallo-proteinase 2
mPAP, Mean pulmonary arterial pressure
NO, Nitric Oxide
NYHA, New York heart association
PAH, Pulmonary arterial hypertension
PASMC, Pulmonary artery smooth muscle cell
PAWP, Pulmonary arterial wedge pressure
pDCs, Plasmacytoid dendritic cells
PDE5i, phosphodiesterase-5 inhibitors
PDGF-B, platelet derived growth factor-B
PDK4, Pyruvate dehydrogenase kinase 4
PGI2, Prostaglandin I2
PH, Pulmonary hypertension
PHFib, Pulmonary hypertension fibroblast
PVOD, Pulmonary venous occlusive disease
PVR, Pulmonary vascular resistance
RANTES, Regulated upon activation normal T cell expressed and presumably secreted
RCT, Randomized controlled trial
RHC, Right heart catheterization
SDF-1, Stromal cell derived factor 1
SiMFis, Singular millimetric fibrotic lesions
SLE, Systemic lupus erythematosus
SSc, Systemic sclerosis
STAT3, Signal Transducer And Activator Of Transcription 3
STAT6, Signal Transducer And Activator Of Transcription 6
TCR-γδ, T-cell receptor γδ
Th17, T-helper 17
VATS, Video assisted thoracic surgery
VCAM-1, Vascular cellular adhesion molecule 1.

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MINI REVIEW
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Management of Endothelial Dysfunction in Systemic Sclerosis: Current and Developing Strategies

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Systemic Sclerosis (SSc) is an autoimmune disease marked by dysregulation of the immune system, tissue fibrosis and dysfunction of the vasculature. Vascular damage, remodeling and inadequate endothelial repair are hallmarks of the disease. Since early stages of SSc, damage and apoptosis of endothelial cells (ECs) can lead to perivascular inflammation, oxidative stress and tissue hypoxia, resulting in multiple clinical manifestations. Raynaud's phenomenon, edematous puffy hands, digital ulcers, pulmonary artery hypertension, erectile dysfunction, scleroderma renal crisis and heart involvement severely affect quality of life and survival. Understanding pathogenic aspects and biomarkers that reflect endothelial damage in SSc is essential to guide therapeutic interventions. Treatment approaches described for SSc-associated vasculopathy include pharmacological options to improve blood flow and tissue perfusion and, more recently, cellular therapy to enhance endothelial repair, promote angiogenesis and heal injuries. This mini-review examines the current knowledge on cellular and molecular aspects of SSc vasculopathy, as well as established and developing therapeutic approaches for improving the vascular compartment.

Keywords: systemic sclerosis, vasculopathy, cellular therapy, endothelial cells, vasodilator agent

INTRODUCTION

Systemic sclerosis (SSc) is an autoimmune disease marked by diffuse vasculopathy, immunological dysregulation and fibrosis of the skin and internal organs. Vascular manifestations derive mostly from impaired blood flow and tissue ischemia, and are a challenge for the management of SSc patients (1–3). In this mini-review, we examine the current and developing therapeutic interventions with pharmacological agents and cellular therapy for SSc-associated vasculopathy.

PATHOPHYSIOLOGY OF THE VASCULAR ENDOTHELIUM IN SYSTEMIC SCLEROSIS

The endothelium is a metabolically active tissue that ensures regulation of vascular tone, coagulation and fibrinolysis, smooth muscle proliferation, cell adhesion and inflammation (4). Vascular injury is an early event in SSc, with damage and activation of endothelial cells (ECs) (5, 6) (**Figure 1**). Injured ECs in SSc produce increased levels of endothelin-1 (ET-1) and von Willebrand factor (vWF), and low levels of nitric oxide (NO) and endothelial nitric oxide synthase (5). The resulting imbalance between vasodilation and vasoconstriction modifies the vascular tone, contributing to tissue hypoxia. ET-1 also induces differentiation of fibroblasts into a myofibroblastic phenotype, promoting intimal hyperplasia, luminal narrowing, and vessel obliteration (7, 8). Myofibroblasts may also be originated through the endothelial-to-mesenchymal transition (EndoMT) (9), when ECs downregulate expression of markers such as CD31 and VE-cadherin, and assume a myofibroblast phenotype, characterized by fusiform morphology and expression of α-SMA (10). The abnormal vascular tonus and the increased expression of vWF stimulate platelet aggregation and hypercoagulation, leading to further vascular damage (11, 12). Reactive oxygen species contribute to further enhance the damage, participating in the initiation and progression of SSc (2, 5).

Cell adhesion molecules play an important role in promoting endothelial integrity, besides regulating leukocyte migration, vascular permeability and angiogenesis (13). Increased expression of adhesion molecules and their soluble levels, detected in early stages of SSc, correlate with disease severity and visceral involvement (14–18). Indeed, increased levels of E-selectin, vascular cell adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1) lead to activation of ECs, dysregulation of angiogenesis and, as consequence, chronic and progressive vascular damage (19).

IMPAIRED COMPENSATORY ANGIOGENESIS AND VASCULOGENESIS

In SSc, damage and apoptosis of ECs result in loss of capillaries that are not repaired by compensatory mechanisms of vasculogenesis and angiogenesis (20, 21). Vascular endothelial growth factor (VEGF) regulates blood vessel growth, with key role in the process of angiogenesis (22). Serum levels of VEGF and its receptor (VEGFR) are increased in SSc (16, 23–26). Exposure to high levels of VEGF causes an exaggerated angiogenic stimulus, with proliferation of ECs, resulting in chaotic architecture of vessels, as observed by capillaroscopy (19). An anti-angiogenic isoform, VEGF165, has been described in SSc patients (27), and platelet releases containing VEGF165 impair angiogenesis *in vitro* (28). In addition, function and frequencies of endothelial progenitor cells (EPCs) are compromised in SSc, playing a defective role in vasculogenesis (29). **Table 1** describes additional biomarkers associated with vascular damage in SSc.

CLINICAL MANIFESTATIONS OF SSC-ASSOCIATED VASCULOPATHY

Raynaud's phenomenon is one of the first manifestations of the disease (8, 73). Progressive structural damage of the vessels, followed by proliferative endarteritis and consequent tissue ischemia, leads to systemic involvement, characterizing SSc as a microvascular disease. Telangiectasias and digital ulcers are frequent vascular manifestations of SSc, and associate with poor prognosis (74, 75). Scleroderma renal crisis, a severe clinical condition characterized by poor renal cortical perfusion and rapidly progressive renal failure, was a leading cause of death until the 1970s, when use of angiotensin-converting enzyme inhibitors significantly improved patient management and outcomes (76–80). Primary and secondary cardiac involvements are described as frequent and probably underestimated in SSc (81–83), and from 5 to 15% of SSc patients develop pulmonary hypertension (79, 81). Less explored, but still frequent vascular manifestations of SSc are erectile dysfunction, vascular malformations of the gastro-intestinal mucosa (gastric antral vascular ectasia - GAVE) and, to some extent, myopathy (66, 84–86). Routine assessments for vascular involvement include clinical inspections, evaluation of organ function and, when required, right-heart catheterism. Such manifestations should be actively investigated and treated early, before advanced organ damage.

PHARMACOLOGICAL APPROACHES

Therapeutic strategies for vasculopathy in SSc aim to improve symptoms of Raynaud's phenomenon (RP), heal and prevent development of digital ulcers (DU), and decrease the ischemic damage to internal organs. Multiple pharmacological options, with different mechanistic approaches, are available and recommended in the management of SSc patients (**Figure 2**) (87). New strategies, including cell therapy, have been developed to further improve this aspect of the disease.

CALCIUM CHANNEL BLOCKERS

Calcium channel blockers reduce intracellular calcium concentrations, inducing relaxation of smooth muscle and vasodilation (88). Dihydropyridines are broadly recommended to attenuate severity and frequency of uncomplicated RP in SSc (87, 89). Short and long-term use of calcium channel blockers decreased plasma markers of oxidative stress (90), and *in vitro*, nicardipine protected ECs against oxidative injury (91). Calcium channel blockers also decreased serum concentrations of N-terminal pro-brain natriuretic peptide (NT-proBNP) in patients with SSc-associated PAH, indicating a possible antispastic and vasodilatory effect on the pulmonary circulation, not corroborated, however, by hemodynamic changes (92). In patients with <5 years of SSc, nifedipine and nicardipine improved myocardial perfusion and left ventricle function, respectively, supporting the hypothesis of myocardial Raynaud's phenomenon in SSc (93).

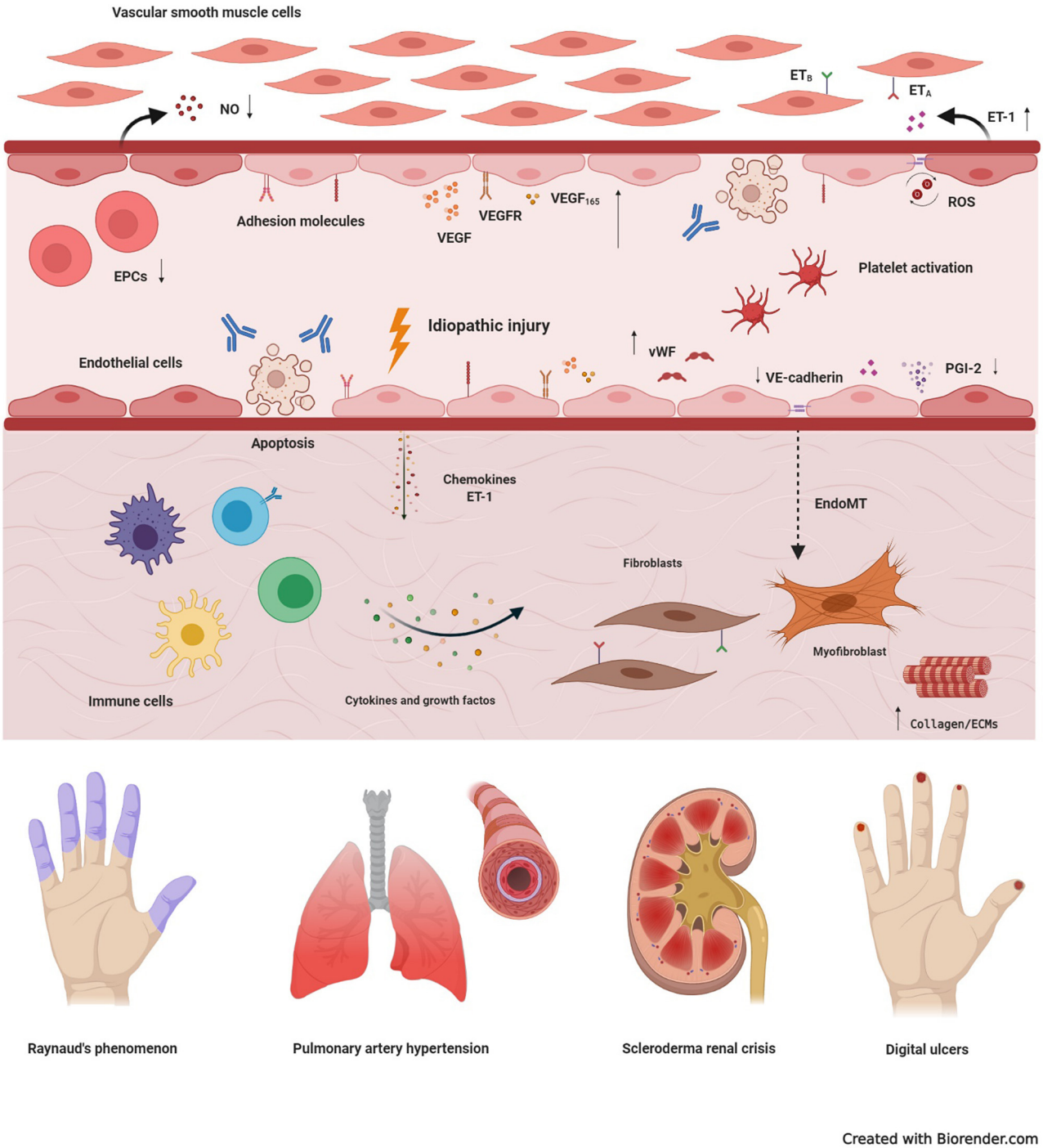

FIGURE 1 | Basic mechanisms of systemic sclerosis-related vasculopathy. Vascular injury is considered an initial event in the development of systemic sclerosis (SSc), and may be triggered by multiple factors, including autoantibodies, infectious agents, reactive oxygen species (ROS), or idiopathic stimuli. In the early stages of disease, vascular damage leads to activation of endothelial cells (ECs), with expression of adhesion molecules, production of chemokines, von Willebrand factor (vWF) and vasoconstrictor agents, such as endothelin-1 (ET-1). Molecules produced by the injured endothelium recruit immune cells, that generate a perivascular infiltrate. Prolonged inflammation leads to tissue fibrosis, with excessive activation of resident fibroblasts that transdifferentiate into myofibroblasts, the main cell type involved in excessive collagen production and other extracellular matrix components (ECMs). Myofibroblasts are also originated through the endothelial-to-mesenchymal transition (EndoMT). Dysfunction of endothelial progenitor cells (EPCs), antibody-induced ECs apoptosis, persistent platelet activation, decreased production of vasodilatory nitric oxide (NO) and prostaglandin I-2 (PGI-2), synthetized by ECs, also participate in the pathogenesis of SSc-vasculopathy. In addition, compensatory mechanisms of vasculogenesis and angiogenesis, including vascular endothelial growth factor (VEGF) and its receptor (VEGFR), are dysregulated and ineffective. High

(Continued)

FIGURE 1 | expression of VEGF165, an anti-angiogenic isoform, contributes to this scenario. Reactive oxygen species, further contribute to intensify damage and activation of the endothelium and, thus, increase tissue injury. Clinical manifestations of SSc-related vasculopathy include Raynaud's phenomenon, pulmonary arterial hypertension, scleroderma renal crisis, telangiectasias, digital ulcers and digital pitting scars, which severely affect quality of life and may compromise survival. ET_A, type A endothelin receptor; ET_B, type B endothelin receptor.

TABLE 1 | Biomarkers associated with endothelial activation or vascular damage in SSc and clinical correlates.

Biomarkers	Class/function	Clinical associations	References
Adhesion molecules (ICAM-1, VCAM-1, selectins)	Cell–cell interactions	Capillaroscopic abnormalities Disease severity Pulmonary fibrosis	(14, 15, 18, 30–35)
Angiopoietin system (ANG-Tie)	Angiogenesis	Disease activity Digital ulcers Esophageal dysmotility Microangiopathy Proliferative vasculopathy	(36–41)
Anti-centromere (ACA)	Autoantibodies	Microangiopathy Pulmonary arterial hypertension	(41–43)
Anti-AT1R and -ETAR	Autoantibodies	Digital ischemic Pulmonary arterial hypertension (PAH)	(44, 45)
Anti-endothelial cell (AECA)	Autoantibodies	Pulmonary fibrosis	(46)
Anti-RNA polymerase III	Autoantibodies	Gastric Antral Vascular Ectasia (GAVE) Scleroderma renal crisis Diffuse skin thickening Cardiopulmonary involvement Rapid disease progression	(34, 47–55)
Anti - topoisomerase I (anti-SCl70)	Autoantibodies	Digital ulcers Heart involvement Interstitial lung disease	(56)
Endoglin (CD105)	Type I membrane glycoprotein.	Digital ulcers	(57)
Endothelin-1	Vasoconstrictor molecule	Interstitial lung disease Right ventricle dysfunction	(58–62)
Endostatin	Angiogenesis	Digital vascular damage Skin and pulmonary fibrosis	(63, 64)
Thrombomodulin	Coagulation	Pulmonary hypertension	(65)
Thrombospondin-1 (TSP-1)	Antiangiogenic glycoprotein	Brachio-cervical inflammatory myopathy	(66)
Vascular endothelial cell growth (VEGF)	Angiogenesis	Diffuse skin subset Interstitial lung involvement Nailfold capillary loss Pulmonary Artery Hypertension (PAH)	(25, 67–72)

ETAR, endothelin receptor type A; AT1R, Ang receptor type-1.

ENDOTHELIN-1 RECEPTORS ANTAGONISTS

Endothelin-1 receptor antagonists target ET-1, a crucial mediator in SSc vasculopathy. Ambrisentan is a selective type A endothelin receptor antagonist, while bosentan and macitentan are dual antagonists, targeting both type A and B receptors (88). In two randomized clinical trials, bosentan prevented the development of new DU, but did not heal active DU (94, 95). Ambrisentan reduced the number of active and new DU in SSc patients, also decreasing pain and disability (96, 97).

Bosentan and ambrisentan improved hemodynamic parameters in patients with SSc-PAH (98, 99). Bosentan also decreased serum concentrations of endothelial activation markers ICAM-1, VCAM-1, P-selectin and PECAM-1 (100). *In vitro* experiments with preincubation of microvascular endothelial cells (MVECs) from SSc patients with bosentan or macitentan decreased the expression of mesenchymal markers, identifying a possible pharmacological interference pathway to prevent EndoMT (101).

PHOSPHODIESTERASE-5A INHIBITORS

Phosphodiesterase-5A (PDE-5A) hydrolyzes the cyclic guanosine-5-monophosphate (cGMP), associated to the nitric oxide (NO) vasodilator pathway. PDE-5A inhibitors reduce the metabolism of cGMP, intensifying the vasodilatory effects of NO (102). In SSc patients, PDE-5A inhibitors decreased frequency and duration of RP attacks, improved DU healing (103) and reduced disability and discomfort associated with RP (104). For SSc-PAH, sildenafil reduced pulmonary artery pressure, with beneficial effects on cardiopulmonary status (105).

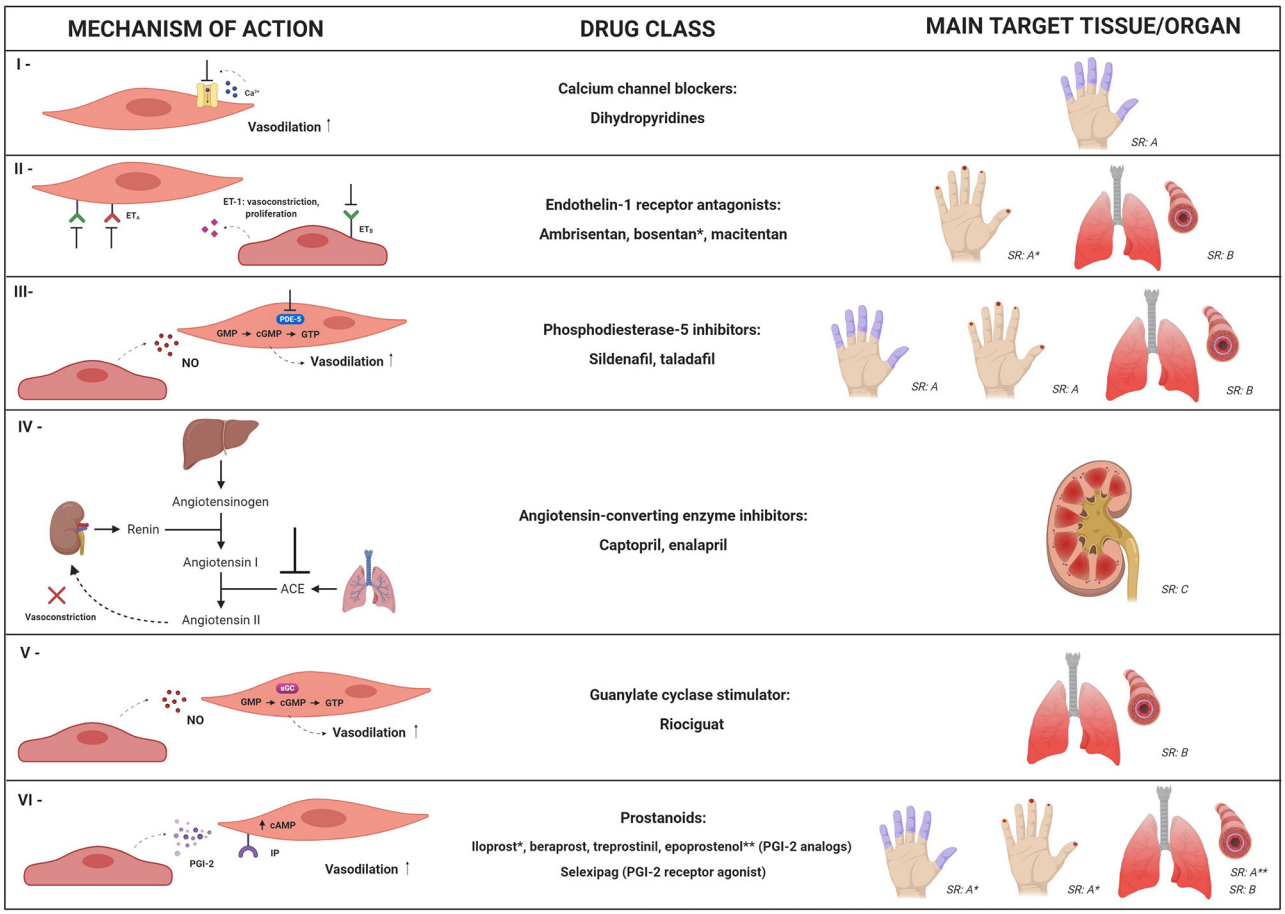

FIGURE 2 | Mechanistic targets of the main pharmacological approaches for systemic sclerosis-related vasculopathy. Multiple drug options, with different mechanistic approaches, can be used for management of vascular manifestations in SSc patients. This figure summarizes mechanisms and clinical indications for: calcium channel blockers (I); endothelin-1 receptor antagonists (II); phosphodiesterase-5 (PDE-5) inhibitors (III); angiotensin-converting enzyme (ACE) inhibitors (IV); guanylate cyclase stimulator – riociguat (V) and prostanoids (VI). SR: strength of recommendation; SR: *A*, SR: *B* or SR: *C* according to the European League against Rheumatism, in 2017 (87). Dihydropyridines, PDE-5 inhibitors and iloprost are recommended for SSc-associated Raynaud's phenomenon (SR: *A*); bosentan is recommended to reduce the number of new digital ulcers (SR: *A*); PDE-5 inhibitors and iloprost are recommended for active digital ulcers (SR: *A*); PDE-5 inhibitors, endothelin-1 receptor antagonists (bosentan, ambrisentan, and macitentan) and riociguat are recommended for treatment of SSc-related pulmonary artery hypertension (SR: *B*); epoprostenol (SR: *A*) and other prostacyclin analogs (SR: *B*) are recommended for severe SSc-related pulmonary artery hypertension; ACE inhibitors are recommended as treatment for scleroderma renal crisis (SR: *C*). * and ** indicate clinical conditions in which specific drugs from the class are recommended. NO, nitric oxide; ET-1, endothelin-1; ET_A, type A endothelin receptor; ET_B, type B endothelin receptor; cGMP, cyclic guanosine-5-monophosphate; sGC, soluble guanylate cyclase; cAMP, cyclic adenosine monophosphate; GTP, guanosine-5′-triphosphate; IP, prostacyclin receptor; PGI-2, prostaglandin I-2.

Combined therapy of tadalafil plus ambrisentan resulted in better responses for SSc-PAH than monotherapy with either agent (106). However, sildenafil did not affect the number of circulating EPCs or VEGF serum levels in SSc patients with vasculopathy (107–109). PDE-5 inhibitors have been also investigated as treatment for erectile dysfunction and, although SSc patients have poor response to on-demand administration, daily fixed doses may be effective (110).

PROSTANOIDS

Prostacyclin, also known as prostaglandin I-2 (PGI-2), is synthetized by vascular ECs, promoting vasodilation and decreasing platelet aggregation, inflammation and vascular smooth muscle proliferation (111). Prostacyclin analogs (iloprost, beraprost, treprostinil, and epoprostenol) and the prostacyclin receptor agonist (selexipag) are available pharmaceutical agents that enhance the prostacyclin pathway and thus promote vasodilation (88).

Iloprost was effective for treatment of RP, DU and PAH in SSc patients (112–117), also decreasing serum levels of ICAM-1, VCAM-1 and E-selectin, reflecting reduced activation of ECs (115). Iloprost and bosentan combinatory therapy increased the number of nailfold capillaries (118). Beraprost did not prevent development of DU (119) and had little effect on hemodynamic parameters in SSc-PAH (120). Conversely, epoprostenol improved clinical status and hemodynamic

parameters (121, 122), and increased serum levels of adiponectin (123), suggesting effects on vascular function (124) and on adipose tissue metabolic pathways (123). Treprostinil improved cutaneous blood flow (125, 126) and healing of DU (127), but recent studies failed to show changes in vascular, angiogenic and inflammatory biomarkers (128).

Prostacyclin agonists have short half-life, high frequency of administration and multiple side effects, and products with more convenient posology have been investigated. Selexipag is an oral selective prostacyclin receptor agonist that promotes vasodilation by increasing cyclic adenosine monophosphate concentrations (129) and has been effective for PAH (130). For the peripheral circulation, however, efficacy of this drug is still debated. While in a randomized, placebo-controlled study, selexipag failed to reduce the frequency of RP attacks (131), an open observational study showed considerable improvement of RP, also suggesting that selexipag may be effective for DU healing and resolution of DU related-pain (132).

ANGIOTENSIN-CONVERTING ENZYME INHIBITORS

Angiotensin-converting enzyme (ACE) inhibitors block the conversion of angiotensin I into the vasoconstrictor agent angiotensin II, promoting rapid control of the blood pressure (133, 134). Over the past decades, ACE inhibitors had a significant impact on outcomes of SSc patients with scleroderma renal crises (SRC), decreasing the need for dialysis and increasing survival (78, 135). Prophylactic use, however, did not reduce the incidence and was associated with poor prognosis and risk of death after onset of SRC (136, 137).

RIOCIGUAT

Riociguat is a soluble guanylate cyclase (sGC) stimulator that leads to strong vasodilator effects on the pulmonary arteries (138–144). Clinical trials in PAH patients, including SSc, showed improvements in pulmonary vascular resistance (145). An initial study failed to demonstrate significant reduction of active or painful DU, or changes in plasma levels of VEGF, E-selectin, VCAM-1 and ICAM-1, but long-term observations detected complete healing of the DU (146), and improvement of discomfort and disability associated with RP (147). Larger studies should determine the impact of riociguat on the peripheral vasculature (148, 149).

CYCLOPHOSPHAMIDE

Cyclophosphamide (CYC), an immunosuppressive drug mostly used for SSc-related interstitial lung disease (150), also affects the vascular compartment, both in experimental and clinical scenarios (5). Cyclophosphamide improved nailfold capillaroscopic patterns (151), increased the number of circulating EPCs and reduced serum levels of VEGF, E-selectin and thrombomodulin, markers of endothelial injury and activation (152, 153), indicating that CYC may affect pathogenic processes associated with lung damage and fibrosis, such as re-endothelialization and re-epithelialization of the alveolar-capillary barrier (154).

Dermal MVECs exposed to the serum of CYC-treated SSc patients had better proliferation and less apoptosis than those exposed to serum of untreated SSc patients. Additionally, serum levels of antiangiogenic factors pentraxin 3 (PTX3), matrix metalloproteinase (MMP)-12, endostatin and angiostatin were significantly reduced after CYC treatment in SSc patients, suggesting a therapeutic effect on peripheral microvasculopathy (155).

FLUOXETINE

Fluoxetine is a selective serotonin reuptake inhibitor that has been recommended as treatment for SSc RP attacks (87). Serotonin participates in Raynaud's phenomenon pathogenesis as a stimulator (156–158), but fluoxetine has paradoxical vasodilation effects, mediated by 5HT7 and 5HT2B receptors (159), that affect the NO and calcium pathways (160–162). Fluoxetine reduced the severity of RP attacks in SSc patients, with no impact on soluble P-selectin or wWF levels, however (163).

LESS TRADITIONAL THERAPEUTIC INTERVENTIONS

Statins have been studied in immune-mediated diseases, including SSc, due to their immunomodulatory effects (164–166). Rosuvastatin improved endothelial function in SSc patients, assessed by skin microcirculation and brachial artery flow (167). Atorvastatin improved the visual analog scale for RP and DU, and was associated with reduced plasma levels of endothelial activation markers ICAM-1, E-selectin and ET-1, oxidative stress and vWF activity (159, 168). Atorvastatin led to transient increase in numbers of circulating EPCs (159), but failed to induce maturation of EPCs into ECs *in vitro*, indicating a limited therapeutic potential on vascular repair (169). Topical nitrate application is also effective in the treatment of RP in SSc patients. Nitrates are degraded into NO, increase cGMP concentration in the vascular smooth muscle and lead to vasodilation (170). Nitroglycerine tapes improved the peripheral circulation in SSc patients (171). Likely, MQX-503, a novel compound of nitroglicerine, was well-tolerated, improving the cutaneous blood flow in SSc patients (172). Topical application of glyceryl trinitrate increased DU perfusion, indicating supplementation of the NO pathway by nitrates as a promising strategy (173).

More recently, pirfenidone, an antifibrotic drug considered for treatment of interstitial lung disease (174), has shown vasodilatory effects. In animal models, pirfenidone induced pulmonary artery relaxation, restored renal blood flow and stimulated the NO pathway involving voltage-gated KV7 channels (175, 176). Clinical studies should further evaluate potential effects of the drug on the vascular compartment.

Local therapies are also described for SSc-associated vasculopathy. Botulinum toxin (Btx) inhibits acetylcholine release from presynaptic nerve terminals, reducing vascular

smooth muscle contraction, and improving local circulation (177). A randomized controlled trial was inconclusive, since administration of Btx unexpectedly worsened blood flow in hands of SSc patients with RP, but patient perceptions of hand function and discomfort improved (178). Series of cases and one systematic review show healing of DU and reduction of pain in most patients after digital Btx applications (179, 180). Laser and intense pulsed light therapies have been investigated for digital ulcers and telagiectasies, with reports of safety and improvements of patient perception and blood flow (181, 182). In SSc patients with severe ischemic complications, especially vascular obstruction of the hands, peripheral or digital sympathectomy, microsurgical revascularization and digital artery reconstruction may be indicated. Besides limitations, these approaches are able to increase blood perfusion, decrease or eliminate pain, and may be recommended for selected cases (183).

CELLULAR THERAPIES FOR SSC-ASSOCIATED VASCULOPATHY

In the last two decades, different cellular therapy approaches have been investigated for SSc patients (184). Local applications of fat graft/adipose-derived stem cells (ADSCs) or bone marrow hematopoietic stem cells show the strongest potential for regeneration of damaged tissue and vascular remodeling.

FAT GRAFTING AND STROMAL VASCULAR FRACTION/ADIPOSE-DERIVED STEM CELLS-BASED THERAPY

Adipose-derived stem cells can be isolated from the stromal vascular fraction (SVF), located in the white adipose tissue (184), and show robust angiogenic activity (185–195). Patients with SSc treated with local administration of autologous fat grafts showed improvement of RP symptoms (188, 195), and complete healing of DU (189, 193). Treatment also led to significant increase of capillary density in fingers affected by DU (193) and enabled better pain control (189). Furthermore, autologous fat grafts increased mouth opening and vascularization in perioral areas of SSc patients (191).

Local injections of autologous SVF also improved RP, vascular flow, hand pain and finger edema in SSc patients (190, 192). Combination of autologous SVF and platelet-rich plasma, which is reported to enhance ADSC proliferation (194), also increased capillary density and decreased vascular ectasia in SSc patients, suggesting induction of neoangiogenesis (196). When locally implanted, ADSCs secrete VEGF and fibroblast growth factor, which may support local angiogenesis (197). These cells promote proliferation and inhibit apoptosis of ECs (198). Nevertheless, ADSCs isolated from SSc patients exhibit abnormal proliferation, metabolism, differentiation potential, and have a pro-fibrotic phenotype (194, 199–201), suggesting that despite beneficial effects, autologous ADSCs may not achieve full potential in tissue repair (185). More efforts are needed to investigate how they interfere with disease pathogenesis, and if there is potential for systemic therapy (185).

HEMATOPOIETIC STEM CELL TRANSPLANTATION

Over the past 25 years, hundreds of patients with severe and progressive SSc have undergone autologous stem cell transplantation (AHSCT) (202), with better outcomes regarding survival, disease control and quality of life, when compared to conventional treatment (203–206). Indications for AHSCT include mainly fibrosis-related manifestations of SSc, such as skin thickening and interstitial lung disease (202–206). Patients with severe vascular manifestations, especially those with pulmonary hypertension or scleroderma renal crisis are usually excluded (143–147) and extensive cardiac assessment is recommended to avoid inclusion of patients with asymptomatic cardiac involvement (207). The procedure resets the immune system and promotes better control of autoreactivity, inflammation and fibrosis processes (208, 209).

To date, little is known about the impact of AHSCT on SSc-associated vasculopathy. Stem cell transplantation did not change dermal vessel density evaluated by immunostaining for endothelial markers CD31, VE-cadherin and vWF (210). On the other hand, AHSCT partially restored the microvascular structure assessed by nailfold video capillaroscopy (211), increased capillary counts, normalized cutaneous expression of VE-cadherin and decreased the expression of Interferon α mRNA in the skin, which is known as a potent inhibitor of angiogenesis (212, 213). Serum levels of VEGF decreased after AHSCT (214), which can be interpreted as a good result, since disrupted VEGF upregulation is associated with abnormal vessel morphology in SSc (24). Mechanisms to possibly explain the positive influence of AHSCT on the vascular compartment of SSc patients include removal of cells associated with inhibitory effects on endothelial repair, mobilization of endothelial progenitor cells from the bone marrow (212), and other still unidentified mechanisms of angiogenesis (211).

OTHER CELL TYPES USED FOR SSC-VASCULOPATHY: BONE MARROW MESENCHYMAL STROMAL CELLS

Mesenchymal stromal cells (MSC) are potential tools to treat vascular dysfunction, due to their immunosuppressive, anti-fibrotic and proangiogenic properties (215–217). Although MSCs from SSc patients display reduced capacity to differentiate into ECs *in vitro* (218), intramuscular injections of autologous MSCs reduced necrotic areas in one SSc patient with critical limb ischemia (219). After treatment, angiographies showed important revascularization, and histological analyses showed strong expression of angiogenic factors possibly effective through paracrine mechanisms. A SSc patient with multiple active skin ulcers was treated with intravenous infusion of allogeneic MSCs, with improvement of pain and blood flow in hands and fingers (220). In five SSc patients treated with intravenous allogeneic

MSC infusions, there was healing of skin ulcers, and two of these patients also healed lesions of acral necrosis (221). An ongoing double-blind randomized placebo-controlled trial aims to evaluate safety and potential efficacy of intramuscular injections of allogeneic MSC as treatment for DU. In addition to clinical evaluations, such as DU healing and hand function, this study plans also analyze biomarkers in peripheral blood and skin biopsies (222).

CONCLUSIONS AND FUTURE DIRECTIONS

Treatment of SSc-related vasculopathy remains difficult, despite the multiple available therapeutic options and targeted pathways. So far, patients seem to present advanced vascular involvement since early periods of disease, with vessel disruption and ischemic lesions. The narrow therapeutic window, associated with multiple pathophysiological presentations, makes development of new strategies a challenge. There are no reliable biomarkers of vascular severity or extension, so identification of patients with disabling or life-threatening vascular involvement is often too late. Best therapeutic effects include healing of ulcers and improvement of blood flow in pulmonary, renal and peripheral vascular beds. Cell therapy has an important potential, and may be expanded and refined in the future to achieve more substantial goals. Besides subsiding inflammation, future strategies should aim to fully repair and reverse established vascular damage.

AUTHOR CONTRIBUTIONS

DZ-S and MS-G conceived the study. DZ-S, MS-G, and MK-V searched the literature and wrote the draft. DZ-S created the images. MO critically revised the final version of the manuscript and provided funding. All authors contributed to the article and approved the submitted version.

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BRIEF RESEARCH REPORT
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Association Between Uric Acid and Worsening Peripheral Microangiopathy in Systemic Sclerosis

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Objective: The key element in the pathogenesis of systemic sclerosis (SSc) is microcirculatory changes in several vascular beds. Uric acid is associated with endothelial dysfunction and therefore, microvascular damage. The aim of this study was to examine the association between uric acid (UA) and peripheral microvascular involvement in patients with SSc.

Methods: We included consecutive, consenting patients with SSc. Serum UA, urea and creatinine were measured, and glomerular filtration rate (GFR) was calculated with CKD-EPI. All participants underwent nailfold video-capillaroscopy (NVC) to evaluate the microcirculation.

Results: A total of 64 patients (95.3% women) were included in the study. UA levels were significantly associated with the number of avascular areas ($r = 0.290$; $p = 0.020$), whereas no correlation was shown for the GFR ($r = -0.065$; $p = 0.609$). A significant trend of UA in the three capillaroscopic patterns was shown (3.90 ± 1.52 vs. 4.15 ± 0.98 vs. 5.38 ± 2.26; for early, active, and late patterns respectively, $p = 0.028$). Multivariate analysis showed that male gender (β = 3.049; 95% CI = 0.997–5.101) and UA (β = 0.352; 95% CI = 0.117–0.588) were independently associated with the number of avascular areas.

Conclusion: These data suggest that UA levels are significantly associated with the capillaroscopic patterns, reflecting a progressive microvasculopathy.

Keywords: uric acid, nailfold video-capillaroscopy, microvasculopathy, systemic sclerosis, cardiovascular

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INTRODUCTION

In systemic sclerosis (SSc), inflammation and microvascular dysfunction appear to be the main events that progressively stimulate fibrotic process. The precise etiology of fibrotic changes remains partially understood and may include impaired communication between endothelial cells, epithelial cells and fibroblasts, lymphocyte activation, autoantibody production, inflammation, and connective tissue fibrosis (1). Alterations in microvasculature are considered the hallmark of

SSc vascular involvement and occur in the first stages of the disease. Given the heterogenicity of clinical symptoms and organ involvement, there is an ongoing effort to establish biomarkers for the evaluation of microvasculopathy (2), which represents one of the earliest clinical manifestations of SSc presented in various guises such as Raynaud's phenomenon, digital ulcers, and pulmonary arterial hypertension (PAH) (3).

Uric acid (UA) is the final oxidation product of purine metabolism. Elevated serum UA levels have been associated with endothelial dysfunction, possibly by decreasing nitric oxide availability (4) and stimulating vascular smooth muscle cell proliferation leading to arterial stiffness, and gradually, widespread microvascular damage (5–7). UA levels have been found elevated in SSc patients and have been associated with the presence of vascular complications including pulmonary arterial hypertension (PAH) (8) digital ulcers (9) and abnormal findings in nailfold video capillaroscopy (NVC) (10); the latter is a non-invasive and reproducible imaging technique of the capillary vascular bed, used for the assessment of peripheral microvascular damage in SSc. It is extensively used in the differentiation between primary and secondary Raynaud's phenomenon in daily practice. "Abnormal nailfold capillaries" (when referring to the "scleroderma pattern") are included in the 2013 American College of Rheumatology (ACR)/ European League Against Rheumatism (EULAR) classification criteria for SSc (11). Cumulative data suggest that NVC measurements can serve as a reliable marker of the extent and severity of microvasculopathy in different vascular districts such as pulmonary (12) and myocardial microcirculation (13) to the point that NVC is currently considered as a surrogate marker of SSc progression (14). However, the association between UA and NVC changes in SSc has not been well-established.

In this context, the aim of this study was to investigate the potential relationship between UA levels and microvascular alterations assessed by NVC in a large well-characterized cohort of SSc patients.

MATERIALS AND METHODS

Study Participants and Inclusion / Exclusion Criteria

The study included consecutive patients with SSc attending the Scleroderma Clinic of the Fourth Department of Internal Medicine, Hippokration General Hospital, Thessaloniki, Greece, between March 2018 and September 2020, who were screened for the study. All patients satisfied the revised EULAR/ACR criteria for the diagnosis of SSc (11). The exclusion criteria included past diagnosis of cardiovascular disease defined as coronary heart disease, stroke, or peripheral vascular disease, diabetes mellites, as well as patients with carotid artery surgical procedures. Patients on diuretics were also excluded. The study had ethics approval from the Ethics Committee of the School of Medicine, Aristotle University of Thessaloniki and written informed consent was obtained from all participants according to the Declaration of Helsinki.

Protocol Overview

All participants underwent a thorough physical examination and demographic data were collected by a questionnaire. Complete medical history was also recorded which included the duration of the disease, existence of pulmonary hypertension, pulmonary fibrosis, or esophageal motility disorders, as documented by imaging or endoscopic examination, respectively, as well as medication and cardiovascular risk factors (smoking, hypertension).

Parameters of Interest and Definitions

Various hematological and biochemical laboratory parameters such as routine biochemistry and hematology, lipid and bone profile tests, inflammatory markers such as erythrocyte sedimentation rate (ESR) and Creactive protein (CRP), immunological markers such as antinuclear antibodies (ANA), anti-centromeric antibodies (ACA), and anti-topoisomerase IIa (anti-scl-70) antibodies were tested. Serum UA and creatinine levels were measured with photometric measurement of the solution and GRF was calculated using the CKD-EPI equation (15). NVC assessment and blood sampling were performed the same day. Blood pressure was recorded according to 2018 ESC/ESH Guidelines for the management of arterial hypertension, with a validated oscillometric device (16). Arterial hypertension was defined on the basis of the patients' history (self-reposted hypertension) and/or antihypertensive medication intake.

NVC Assessment

Study participants underwent NVC using an Optilia Digital Capillaroscope and a 200× contact lens and the photos collected were analyzed with OptiPix Capillaroscopy (Sollentuna, Sweden) software system. Prior to performing the test, patients were placed in a quiet environment at a temperature between 20 and 25°C. A drop of cedar oil was placed on each fingernail prior to observation for better image analysis. The findings were classified in one of the following qualitative patterns: early, active, and late NVC pattern (17). The "early" pattern was characterized by a few enlarged or giant capillaries and relatively well-preserved capillary distribution; the "active" pattern was characterized by numerous giant capillaries, mildly disturbed capillary architecture, and moderate capillary loss; the "late" type was characterized by severe capillary loss with extensive vascular desertification, and disruption of normal capillary architecture. NVC parameters to be measured were capillary density (number of capillaries per 1 mm in the distal row of each finger), giant capillaries (homogeneously enlarged capillaries $>50\,\mu m$), enlarged capillaries ($>20\,\mu m$ and $\leq 50\,\mu m$), micro-bleeding, oedema, avascular areas (the normal range adopted was 9 capillaries per linear millimeter), ramified capillaries, bushy and tortuous capillaries. Capillary's density was evaluated in the distal row of each finger, based on the number of capillaries per 1 mm, and the mean capillaroscopic skin ulcer risk index (CSURI) was automatically calculated with software image analysis.

TABLE 1 | Baseline characteristics of the study participants.

Parameters	Value
N	64
Age (years)	57.54 ± 12.99
Gender	
Men, *n* (%)	3 (4.7)
Women, *n* (%)	61 (95.3)
Weight (kg)	64.25 ± 10.38
Height (cm)	162 [80]
BMI (m^2)	24.54 ± 3.87
Pulmonary fibrosis *n*, (%)	23 (35.94)
Pulmonary hypertension *n*, (%)	12 (18.75)
Esophageal involvement *n*, (%)	26 (40.63)
ESR (mm/h)	16 [17.5]
CRP (mg/dl)	0.76 [4.34]
Hemoglobin (gr/dl)	12.75 ± 1.14
Uric acid (mg/dl)	4.03 [2.13]
Ur (mg/dl)	33 [15.88]
Cr (mg/dl)	0.80 [0.26]
Potassium (mEq/L)	4.21 [0.75]
Sodium (mEq/l)	140 [2.3]
TChol (mg/dL)	194.86 ± 42.91
LDL(mg/dL)	117.61 ± 38.36
Tgl (mg/dL)	115 [56]
SBP (mmHg)	128.94 ± 19.8
DBP (mmHg)	76.72 ± 9.2
GFR (ml/min/1.73 m^2)	82.6 ± 24.07
ANA, *n* (%)	62 (96.88)
ACA, *n* (%)	19 (29.69)
Scl-70, *n* (%)	25 (39.01)
Febuxostat, *n* (%)	0 (0)
Allopurinol, *n* (%)	1 (1.56)
Omeprazole, *n* (%)	7 (10.94)
Losartan, *n* (%)	4 (6.25)

ESR, Erythrocyte sedimentation rate; CRP, C-Reactive Protein; GFR, Glomerular Filtration Rate; SBP, systolic blood pressure; DBP, diastolic blood pressure; ANA, Antinuclear antibodies; ACA, Anti-centromere antibodies; Scl-70, Anti–Scl-70 antibodies.

Statistical Analysis

Statistical analysis was performed with Statistical Package for Social Sciences 22 (SPSS Inc, Chicago, IL, USA). Continuous variables were expressed as mean values ± standard deviation (SD) or median [interquartile range] according to the normality of distribution tested with the Kolmogorov-Smirnov or the Shapiro-Wilk test. Categorical variables were presented as absolute frequencies and percentages (*n*, %). Comparisons for continuous variables were performed with the student's *t*-test or the Mann-Whitney *U* test, according to the normality of the distribution. Multiple comparison analysis was performed with the ANOVA or the Kruskal-Wallis tests, according to normality. Chi-square or Fishers exact test was used for comparisons of categorical variables. Uni- and multivariable linear regression analysis was performed to explore the parameters possibly associated with the number of avascular areas. We first tested for univariate associations and included in the multivariable model only variables with associations of $p < 0.2$ in univariate analysis. We report β-coefficients with 95% confidence intervals (CI). $P < 0.05$ (two-tailed) were considered statistically significant for all comparisons.

TABLE 2 | Correlation of urea, creatinine, GFR, and UR with NVC parameters.

		Ur	Cr	GFR	UA
Capillaries	r	−0.023	−0.127	0.087	−0.157
	p	0.859	0.318	0.492	0.216
Avascular	r	0.060	0.089	−0.065	0.290
	p	0.638	0.482	0.609	0.020
Edema	r	0.144	−0.084	0.051	−0.211
	p	0.255	0.509	0.691	0.094
Microbleeding	r	0.141	−0.069	0.011	0.094
	p	0.267	0.586	0.931	0.460
Enlarged	r	0.062	−0.187	0.198	−0.052
	p	0.624	0.139	0.116	0.681
Giant	r	0.087	0.083	−0.093	−0.037
	p	0.495	0.512	0.464	0.770
Ramified	r	0.036	0.182	−0.155	0.129
	p	0.778	0.150	0.220	0.312
Bushy	r	0.066	0.059	−0.040	0.139
	p	0.604	0.644	0.756	0.272
Tortous	r	−0.004	0.009	−0.051	0.137
	p	0.976	0.942	0.690	0.282
CSURI	r	0.019	0.071	−0.094	−0.041
	p	0.882	0.579	0.462	0.749

GFR, Glomerular Filtration Rate; UA, uric acid; CSURI, skin ulcer risk index.

RESULTS

Patient Characteristics

Table 1 depicts demographic, anthropometric, clinical, and laboratory characteristics of the study population. In total, 64 Caucasian patients with SSc (95.3% women) with mean age 57.54 ± 12.99 years were included in the study. The number of capillaries per mm were 6 [4], avascular areas were 2 [3] and CSURI index 3.25 [6.57].

Uric Acid and NVC Measurements

The correlation of UA, urea, creatinine, and GFR with the parameters of NVC is presented in **Table 2**. Serum UA levels were significantly associated with the number of avascular areas ($r = 0.290$; $p = 0.020$), whereas no correlation was shown for the eGFR ($r = -0.065$; $p = 0.609$). All the other NVC parameters measured showed no correlation with the levels of UA. There was no significant correlation with CSURI ($r = -0.041$; $p = 0.749$).

Within-groups comparisons revealed a significant trend of UA levels in the capillaroscopy patterns reflective of progressive micro-vasculopathy (3.90 ± 1.52 vs. 4.15 ± 0.98 vs. 5.38 ± 2.26; for early, active, and late patterns, respectively, $p = 0.028$) (**Figure 1**). Similarly, the comparison between different NVC

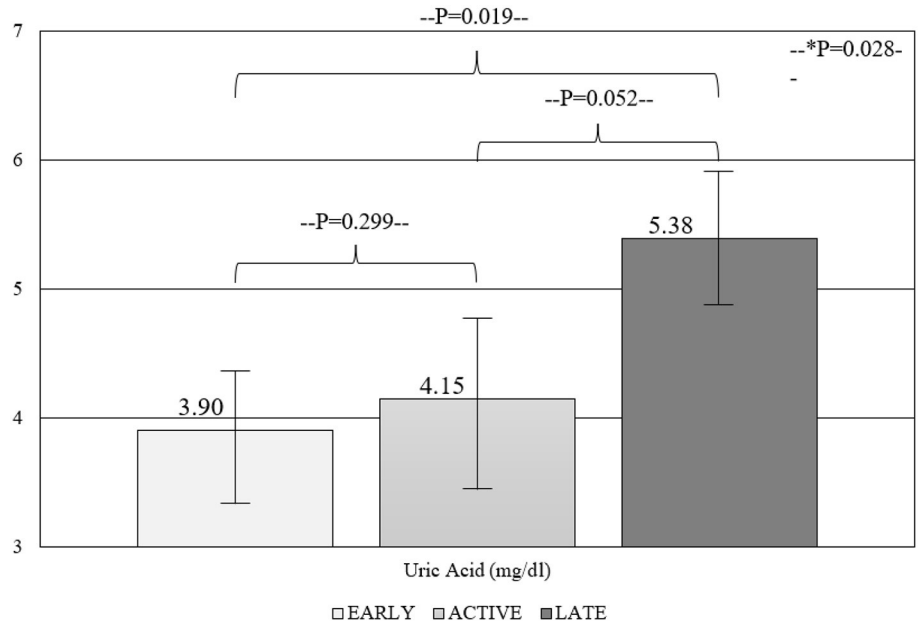

FIGURE 1 | Comparison of UA in the three NVC patterns.

TABLE 3 | Uni- and multivariable linear regression analysis of parameters possibly associated with the number of avascular areas as evidenced with the capillaroscopy.

Parameter	Univariate analysis				Multivariable analysis			
	Estimate	Std error	95% CI	*P* value	Estimate	Std error	95% CI	*P* value
Male gender	3.049	1.027	0.997–5.101	**0.004**	2.492	1.011	0.469–4.515	**0.017**
Age	0.016	0.018	−0.020–0.051	0.388				
BMI	−0.055	0.060	−0.175–0.065	0.362				
Smoking	−0.826	0.505	−1.836–0.184	0.107	−0.312	0.491	−1.295–0.671	0.528
Hypertension	0.373	0.498	−0.623–1.368	0.457				
eGFR	−0.003	0.010	−0.022–0.016	0.762				
UA	0.352	0.118	0.117–0.588	**0.004**	0.272	0.121	0.030–0.514	**0.028**
CRP	0.010	0.008	−0.007–0.026	0.240				
SBP	−0.008	0.012	−0.031–0.016	0.508				
DBP	−0.016	0.025	−0.066–0.035	0.540				

GFR, Glomerular Filtration Rate; UA, uric acid; CRP, C-Reactive Protein; SBP, systolic blood pressure; DBP, diastolic blood pressure. Bold letters represent $p < 0.05$.

pattern groups demonstrated higher UA levels in patients with late compared to early and active patterns ($p = 0.019$ and $p = 0.052$), while the degree of NVC changes was not associated with creatinine or eGFR levels.

Parameters Associated With Avascular Areas in NVC

In order to identify possible factors associated with the avascular areas in NVC, we have performed a uni- and multivariable linear regression analysis with a number of avascular areas being the dependent variable and several demographic, anthropometric, medical history, and laboratory parameters as the independent variables. As shown in **Table 3**, in multivariable analysis male gender (β = 3.049; 95% CI = 0.997–5.101) and UA (β = 0.352; 95% CI = 0.117–0.588) were shown to be independently associated with the number of avascular areas, whereas smoking was not.

DISCUSSION

The main finding of our study is the positive correlation between NVC structural alterations and serum UA concentrations in patients with SSc. In particular advancing stages of SSc-related microangiopathy as determined by both NVC "scleroderma patterns" and the number of avascular areas are associated with higher UA levels, indicating UA as a potential biomarker of peripheral vascular damage in SSc.

This possibility may reflect biologically relevant metabolic procedures involved in the pathogenesis and progression of SSc-related microvasculopathy. For example degradation of UA by the oxidative action of the enzyme xanthine oxidoreductase leads to the production of reactive oxygen species and the initiation of several detrimental procedures such as increased cytokine production, inflammation, and endothelial activation all of which contribute to vascular injury (18). Taking into account that SSc is regarded as a disease of increased oxidative stress (19), upregulation of oxygen free radicals and low antioxidant defense capacity driven by increased UA levels (20) may play a crucial role in the pathophysiology of microangiopathy.

Thus, it is not surprising that previous reports have demonstrated significant associations between high UA levels and various aspects of SSc vasculopathy. In fact elevated UA levels do not only confer a diagnostic value in the identification of SSc individuals at early, asymptomatic stages of PAH as indicated by DETECT study (21), but they also serve as a useful biochemical tool for the assessment of PAH severity (22) and outcomes as they appear to be associated with survival in these patients (23). Besides pulmonary microvasculature, high UA levels were independently associated with the occurrence of digital ulcers in a cross-sectional study including 71 persons with SSc (9) providing another link between peripheral microangiopathy and oxidative stress in this condition. In addition, UA levels have been linked with the extent of renal microvasculopathy in SSc defined as increased intrarenal stiffness (10).

Our findings are in line with a previous small study (10) and confirm the positive correlation between UA levels and peripheral microvascular damage in SSc individuals. Furthermore, we demonstrated that UA levels are increasing in correlation with progressive stages of SSc microangiopathy, particularly in the presence of late (worse) NVC pattern suggesting that UA may contribute to the evolution of microcirculatory abnormalities in SSc. However, the cross-sectional design of all the aforementioned studies—including the current one—does not allow the establishment of any temporal relationships between UA and rarefaction of digital arteries in SSc. Whether such observations indicate a reverse causality, implying that elevated UA levels may not have a direct crucial effect on endothelial derangement and subsequent vascular injury, but they rather represent an easily measurable byproduct of oxidative stress remains to be determined in longitudinal or experimental studies.

On the other hand, the relationship between high UA and various markers of microvascular dysfunction in different vascular beds namely retinal arteriolar narrowing (24), microalbuminuria (25) and coronary flow reserve (26) has been described in previous studies especially in patients with higher cardiovascular risk profile. Considering the increased rate of cardiovascular events such as stoke and myocardial infarction among SSc patients (27) and the well-established contribution of hyperuricemia to the occurrence and development of cardiovascular disease in both rheumatic diseases and general population (28–30), the results of our study provide further insights in the complex association between micro- and macro vascular involvement in SSc. A growing amount of evidence point toward significant correlations between higher grades of SSc microangiopathy and indices of cardiovascular disease such as arterial stiffness (31) and cardiomyopathy (13, 32). Interestingly enough, a recent study by our group indicated that worsening phases of NVC patterns were associated with higher cardiovascular risk scores (33) suggesting that excessive capillary loss and macrovascular endothelial dysfunction may be closely interrelated and promote cardiovascular disease in this population. In this regard, the demonstrated association between progressive microvascular injury and higher UA levels may indicate UA as a marker of generalized, widespread vasculopathy in SSc including both micro- and microvasculature.

The main limitation of our study is the single-center, cross-sectional design which precludes any causal relationships between UA levels and vasculopathy as discussed above. However, NVC was performed in all fingers except thumbs and the acquisition of two adjacent images from each finger according to the updated European League against Rheumatism (EULAR) recommendations (34). We took particular care to include individuals with SSc, with a broad spectrum of disease-related visceral involvement as well as comorbidities representative of a real-life population with SSc. To the best of our knowledge, this is the largest study to investigate the association between UA and microvascular injury by detailed qualitative and semi-quantitative assessment based on a validated algorithm.

In conclusion, serum levels of UA are significantly associated with progressive micro-vasculopathy based on the qualitative NVC pattern. These results provide evidence that UA may be a significant mediator of the microvascular damage in these patients.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of School of Medicine, Aristotle University Thessaloniki, Greece. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

EP collected the data and drafted the paper with support from TD. SS contributed to the perception of the study and the editing of the paper. ET collected the data. AM verified the analytical methods and performed the analysis. GK and AG were involved in planning and supervised the work and critically reviewed the paper. TD supervised the project and contributed to drafting the paper. All authors discussed the results and contributed to the final manuscript.

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Endothelial Dysfunction in Hypertension: Current Concepts and Clinical Implications

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Endothelium plays a fundamental role in the cardiovascular system, forming an interface between blood and adjacent tissues by regulating the vascular tone through the synthesis of nitric oxide, prostaglandins and other relaxing factors. Endothelial dysfunction is characterized by vasoconstriction, cell proliferation and shifting toward a proinflammatory and prothrombic state. In hypertension endothelial dysfunction may be involved in the initiation and development of vascular inflammation, vascular remodeling, and atherosclerosis and is independently associated with increased cardiovascular risk. Different conditions such as impaired vascular shear stress, inflammation and oxidative stress, activation of the renin angiotensin system have been described as important pathophysiological mechanisms involved in the development of endothelial dysfunction. The release of extracellular vesicles by neighboring cells in the vascular wall has emerged as an important regulator of endothelial function and with potential antihypertensive properties and beneficial effects by counteracting the hypertension mediated organ damage. Furthermore, macrovesicles are emerging as an innovative therapeutic approach for vascular protection, allowing the delivery of bioactive molecules, such as miRNA and drugs interacting with the renin angiotensin system. In this review we summarize the available evidence about the pathophysiological implications of endothelial dysfunction in cardiovascular diseases, focusing on hypertension and its sequelae, and the potential innovative therapeutic strategies targeting the endothelium with the aim to improve vascular function and remodeling.

Keywords: endothelium, inflammation, angiotensin II, vascular function, reactive oxygen species (ROS)

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INTRODUCTION

Vascular endothelium plays an important role in cardiovascular (CV) physiology, forming an interface between blood and adjacent tissues and it is involved in nutrients and metabolites transport as well as in the interaction with circulating cells, hormones, and cytokines (1). Endothelial cells regulate the vascular tone through the synthesis of nitric oxide (NO), prostaglandins and other relaxing factors. Moreover, healthy endothelium provides antioxidant, anti-inflammatory, and antithrombotic functions and contributes to the maintenance of vascular tone, serving as a gatekeeper for organ/tissue homeostasis and blood pressure control (2).

Endothelial dysfunction is characterized by a shift of the actions of the endothelium toward reduced vasodilation, cell proliferation, platelet adhesion and activation and proinflammatory

and prothrombic state. Endothelial dysfunction occurs in association with several CV risk factors, including hypertension, hypercholesterolemia and insulin resistance, contributing to inflammation in the vascular wall, of resistance arteries as well as to increased lipoprotein oxidation, smooth muscle cell proliferation, extracellular matrix deposition, cell adhesion, and thrombus formation in conducting arteries (3–5).

It should be noted that the manifestations of endothelial dysfunction may precede the development of hypertension (6). Essential hypertension is characterized by functional and structural alterations in resistance arteries which lead to increased peripheral vascular resistance (7). Endothelial dysfunction may contribute to the increased peripheral resistance by several mechanisms that leads to the enhanced constriction and vascular remodeling (i.e., structural, mechanical, and functional alterations) of resistance arteries, which is associated to the development and complications of hypertension (6, 8). In particular, endothelial dysfunction may participate to the increased myogenic tone of resistance arteries through the activation of the renin-angiotensin system (RAS), endothelin-1, catecholamines, and growth factors production, leading to vasoconstriction, vascular remodeling and then to increased resistance to blood flow and ultimately to increased peripheral blood pressure. The induction of inflammatory processes in the vascular wall may be associated to endothelial dysfunction and may contribute further to the remodeling of resistance arteries (6, 9), and conduit arteries which is associated with the increased risk of atherosclerosis and the development of CV disease (CVD) (6, 10–12).

In this review we will discuss the available evidence on the pathophysiological implications of endothelial dysfunction in hypertension, as well as the potential innovative therapeutic strategies targeting the endothelium.

MECHANISMS OF ENDOTHELIAL DYSFUNCTION IN HYPERTENSION, AND THERAPEUTIC INTERVENTION

Mechanical Stimuli

Endothelial function is tightly regulated by the activation of several mediators and systems including NO, prostaglandins and other relaxing factors as well as by mechanical stimuli including vascular shear that stimulates numerous downstream signaling pathways to maintain and regulate endothelial function and vascular tone (13, 14). A fundamental distinction should be made between steady laminar and oscillatory flow (**Figure 1**). It has been shown that laminar flow enhances the production of vasodilator factors such as NO, prostacyclin, tissue-type plasminogen activator by the activation of mechanosensors and mechanosensitive channels which have been proposed to regulate a broad range of endothelial and vascular functions (15). Laminar shear activates the glycocalyx mechanosensing which is transferred by the cytoskeleton to integrins that distribute the force via actin microfilaments, microtubules, and intermediate filaments through the focal adhesion of c-Src kinases (15) leading to the maintenance of endothelial integrity. Increased laminar shear stress results also in elevated concentration of endothelial cytosolic calcium, leading to the activation of NO synthase (eNOS) and the increased production of NO. Elevated cytosolic calcium levels also trigger the opening of calcium-activated potassium channels, which is associated to endothelial cells hyperpolarization and thereby to vasorelaxation. Moreover, platelet endothelial cell adhesion molecule-1 (PECAM-1) along with caveolin, tyrosine-specific phospho-transferase Fyn, vascular endothelial growth factor (VEGF)-receptor 2 and the vascular endothelial cadherin (VE-cadherin) forms a mechanosensory complex which confers an adequate responsiveness to the beneficial effects of shear stress in endothelial cells (16). In particular, in laminar flow the force exerted on PECAM-1 triggers the activation of VEGF- receptor 2 in the absence of its ligand, which in turn induces integrin-mediated signaling and ultimately leads to the suppression of inflammatory pathways (17).

In response to increased shear stress, AMPK-induced phosphorylation and sirtuin-1-mediated deacetylation promoted eNOS compartmentalization and activation with atheroprotective effects in an *in vivo* mouse model (18), thus contributing to vascular protection. Moreover, laminar flow increases JNK-mediated p53 phosphorylation, GADD45 and p21cip1, inhibiting endothelial cells growth and atherosclerotic plaque development (19, 20).

On the other hand, oscillatory flow reduces eNOS expression, promotes leukocyte infiltration, smooth muscle proliferation and the secretion of proinflammatory molecules, such as MCP-1 (monocyte chemotactic protein 1), PDGFs (platelet derived growth factor), and endothelin-1 leading to vasoconstriction, increased blood pressure (BP) and atherosclerosis development in larger arteries (21). These processes involve the activation of mechanosensitive genes in endothelial cells, inducing the increase of reactive oxygen species (ROS) and the activation of several transcription factors, such as Kruppel-like factor [KLF2/4], NF-κB, AP-1, early growth response-1, c-Jun, c-fos, and c-myc, as well as the activation of mitogen-activated protein kinases (MAPKs) and small ubiquitin-like modifier (SUMO) signaling (15, 22–24). Interestingly the SUMOylation process can downregulate the expression of the protective transcription factor p53 which in turn can be associated to the development of CV complications in hypertension. Furthermore, oscillatory flow induces the activation of the PI3Kinase-Akt pathway which leads to the assembly of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-2 and to the production of ROS (15), thus contributing to the vascular inflammation and remodeling. Other possible mechanisms involved in endothelial dysfunction triggered by oscillatory flow include the expression of the transcriptional factor Yes-associated protein (YAP) and its related coactivator PDZ binding motif (TAZ) that enhances cell cycle regulatory genes such as cyclin A1 (CNNA1) and E2F transcription factor 1 (E2F1) and increases inflammation

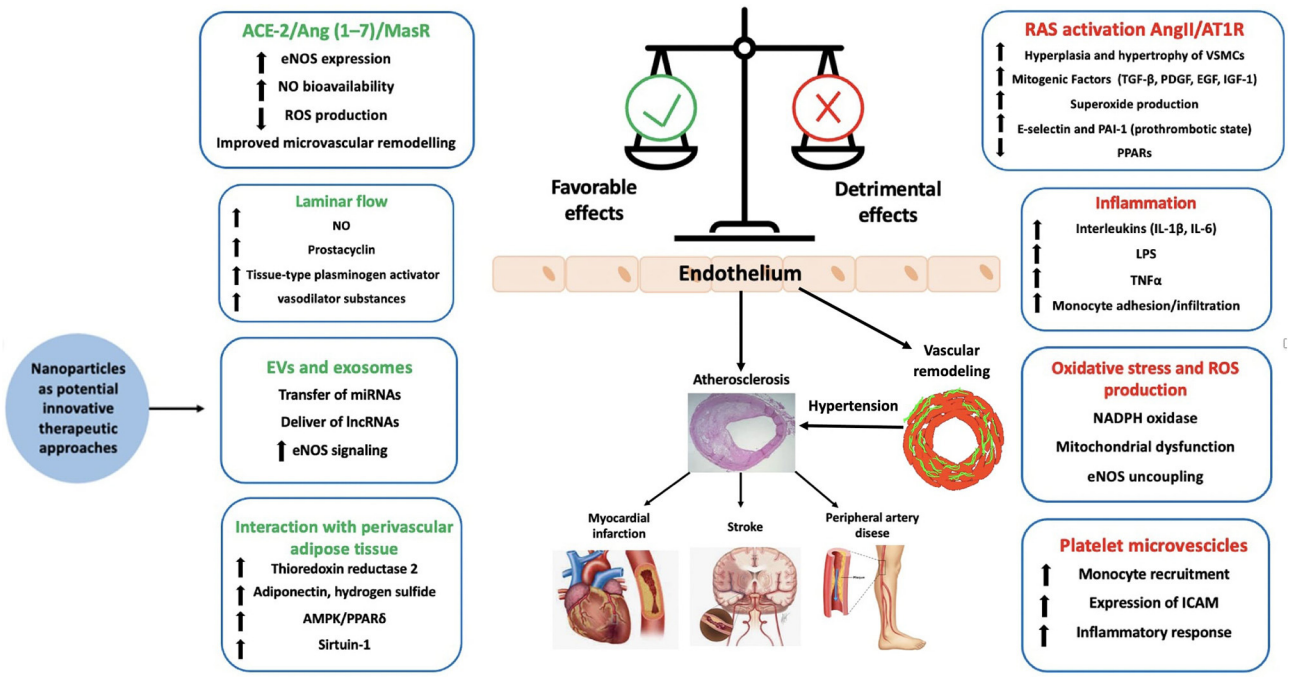

FIGURE 1 | Factor contributing to endothelial function and dysfunction. ACE-2, angiotensin converting enzyme-2; AMPK, AMP-activated protein kinase; Ang, angiotensin; AT1R, type 1 angiotensin II receptor; EGF, epidermal growth factor eNOS, endothelial nitric oxide synthase; EVs, extracellular vesicles; ICAM, intercellular adhesion molecule 1; IGF-1, insulin-like growth factor-1; lncRNA, long-non-coding RNA; LPS, lipopolysaccharide; MCP-1, monocyte chemotactic protein 1; NAPDH, nicotinamide adenine dinucleotide phosphate; NO, nitric oxide; PAI-1, plasminogen activator inhibitor-1; PDGF, platelet derived growth factor; PPAR, peroxisome proliferator receptor; ROS, reactive oxygen species; TGF-β, tumor growth factor-β; TNFα, tumor necrosis factor.

and monocyte attachment, contributing in turn to endothelial dysfunction (15).

Role of Oxidative Stress and Inflammation

A large body of evidence over the past years has shown that ROS are involved in endothelium dysregulation. In the vascular system the major source of ROS production is NADPH oxidase whose expression is increased in hypertensive conditions by several stimuli including shear stress alterations, renin angiotensin system (RAS) and endothelin activation (25).

ROS are key signaling molecules through which vasoactive agents such as angiotensin II (Ang II), endothelin-1 and prostanoids mediate effects at cellular level, and may modify cell function through highly regulated redox-sensitive signal transduction. This may occur through the alteration of intracellular calcium homeostasis contributing to vasoconstriction, cell growth and inflammation which lead to hypertension development and hypertension mediated organ damage (HMOD) (26, 27). ROS stimulate multiple signaling pathways involved in inflammation, cell growth and vascular remodeling. These pathways include the activation of NF-κB, MAPK, JAK-2, STAT, p21Ras, Pyk-2 (Proline-rich Tyrosine Kinase 2) and AKT, receptor tyrosine kinases such as EGFR (Epidermal Growth Factor Receptor), IGFR (Insulin-like Growth Factor Receptor 1) and PDGFR (Platelet Derived Growth Factor Receptor), protein tyrosine phosphatases and redox-sensitive transcriptor factors such as Activator Protein 1 (AP)-1 and Hypoxia-inducible factor 1 (HIF-1) (15, 22–24, 28–33).

In hypertension, oxidative stress promotes aberrant cell signaling and post-translational modification (oxidation and phosphorylation) of proteins and in turn cell and tissue damage (34). In particular, protein phosphatases such as tyrosine phosphatases and protein serine/threonine phosphatases are inactive in the oxidized state, resulting in increased phosphorylation and activation of downstream protein targets involved in cell growth and inflammation which may contribute to vascular remodeling and hypertension development (34, 35) (**Figure 1**). ROS can also inhibit SIRT1 activity through oxidative modifications on its cysteine residues. Decreased activity of SIRT1 enhances the NF-κB signaling, which supports inflammatory responses (36). Moreover, reduced SIRT1 activity is associated with a decreased AMP-activated protein kinase (AMPK) activation, which results in a reduced expression of antioxidant enzymes such as manganese superoxide dismutase, catalase, γ glutamylcysteine synthase, and thioredoxin (37).

Increased ROS concentration induces the reduction of NO bioavailability by the increased quenching (34). Furthermore, the ROS-dependent phosphorylation of ERK5 by phosphokinase-C-ζ (PKCζ) and the activation of tumor necrosis factor α (TNFα)- mediated pathway induces the degradation of eNOS leading to the reduced production of NO

concentration and in turn contributing further to endothelial dysfunction (34).

Role of Renin Angiotensin System and Its Antagonism

RAS and in particular its key effector Ang II play a fundamental role in the development of hypertension and its sequelae, contributing to endothelial dysfunction, cell growth, oxidative stress, vasoconstriction and inflammation. Ang II induces hyperplasia and hypertrophy of vascular smooth muscle cells (VSMC) in resistance arteries by modulating the endogenous production of mitogenic factors (including TGF-β (tumor growth factor-β), PDGF (platelet-derived growth factor), EGF (epidermal growth factor), IGF-1 (insulin-like growth factor 1) (38) and by enhancing basal superoxide production through the activation of cSrc, PKC, phospholipase A2 (PLA2) and phospholipase D (PLD) and increased NADPH oxidase and ROS generation (27, 39, 40). Moreover, Ang II stimulates the production of E-selectin and plasminogen activator inhibitor-1 (PAI-1), contributing to a prothrombotic state and to atherosclerotic plaque rupture (41). In addition, Ang II downregulates PPARs which have been largely demonstrated to reduce inflammatory response in experimental animals and to decrease serum markers of inflammation in humans (42). Through the stimulation of AT1 (Ang II type 1) receptors, Ang II also induces the synthesis of aldosterone which activates mineralocorticoid receptors enhancing inflammation, fibrosis, and endothelial damage (43).

As a matter of fact, RAS inhibitors and mineralocorticoid receptor antagonists have been demonstrated to reduce the proinflammatory and pro-fibrotic effects of Ang II and aldosterone, improving endothelial function and reducing oxidative stress (44).

Available evidence suggests that RAS blockade obtained by angiotensin converting enzyme (ACE) inhibitors or angiotensin receptor blockers (ARBs) is associated with improved function and structure of resistance arteries (6, 45, 46). The activation of complementary protective axes of the RAS may potentially contribute to the beneficial effects of RAS blockers. This includes the expression of angiotensin II type 2 receptor (AT2R) through the activation of a functional crosstalk between AT1R/AT2R during selective AT1R blockade (47–50). AT2R may contribute to improve endothelial dysfunction and arterial remodeling in hypertensive conditions, as its activation is linked to vasodilation, NO production and antiproliferative and anti-inflammatory effects (51). Thus, AT2R may participate to the mechanisms whereby therapeutic use of ARBs induces cardiovascular protection (52).

Experimental studies suggest that also the activation of ACE-2/Ang (1–7)/MasR axis may in part counteract the Ang II–induced actions in the cardiovascular system, including endothelial dysfunction, vasoconstriction and cell growth (53). In this regard, we have recently shown that ACE-2/Ang (1–7)/MasR axis plays an important role in arterial protection during selective AT1R blockade through the improvement of endothelial function and remodeling of resistance arteries *via* the reduction of ROS production and increased NO bioavailability (54) (**Figure 1**).

Effect of Other Antihypertensive Drugs and Endothelial Dysfunction

Other antihypertensive agents recommended in clinical practice have also shown vascular protective effects. Mineralocorticoid receptor antagonists have been demonstrated to reduce arterial stiffness and to improve endothelial function, measured by flow-mediated dilation (55). Calcium channel blockers have been demonstrated to have pleiotropic effects leading to the improvement of endothelial function and to the reduction of central aortic pressures (56). These effects are not directly linked to the antagonism of voltage-dependent calcium channels but rather are associated to the reduction of ET-1, monocyte chemoattractant protein-1 and C-reactive protein (57). Third generation beta-blockers with α1-adrenergic receptor antagonist activity have also been shown to improve endothelial function through antioxidant mechanisms and cause NO-dependent vasodilation (57) (**Figure 2**). Furthermore, endothelin receptor antagonists, might represent feasible future therapeutic agents to prevent endothelial dysfunction, vascular remodeling and organ damage in hypertension (58).

RELATIONSHIP BETWEEN ENDOTHELIUM AND EXTRACELLULAR VESICLES

Extracellular vesicles (EVs) are released in plasma from cells after the fusion of multivesicular bodies with the plasmic membrane and can deliver their cargo, including mRNA, microRNA (miRNA), small amounts of DNA, transcription factors, cytokines, and growth factors, to other cells in remote locations (59, 60). EVs can also be released into the extracellular space by neighboring cells through paracrine mechanisms along with the systemic release in plasma (61). Available findings have shown a correlation between endothelial dysfunction and circulating levels of EVs, particularly in patients with hypertension, coronary artery disease (CAD) and diabetes, although conflicting evidence exists with respect to their protective or harmful role (62–64). EVs have been identified as potential novel biomarkers and bioactivators in the development of hypertension affecting vascular tone in patients with endothelial dysfunction (65). It has been shown that EVs may reduce endothelial-dependent vasodilation and impair acetylcholine (ACh) induced vasorelaxation in a concentration-dependent manner. However, it has not been completely clarified how circulating EVs may affect resistance artery function during the basal state and when overt hypertension may occur (66). A recent study has demonstrated that an enriched EVs preparation from normotensive individuals (humans or rats) impair vasodilation in response to endothelial-dependent vasodilators, potentially through L-NAME inhibitory effects on eNOS. These findings support a paracrine/endocrine role of circulating EVs in the regulation of vascular tone in resistance

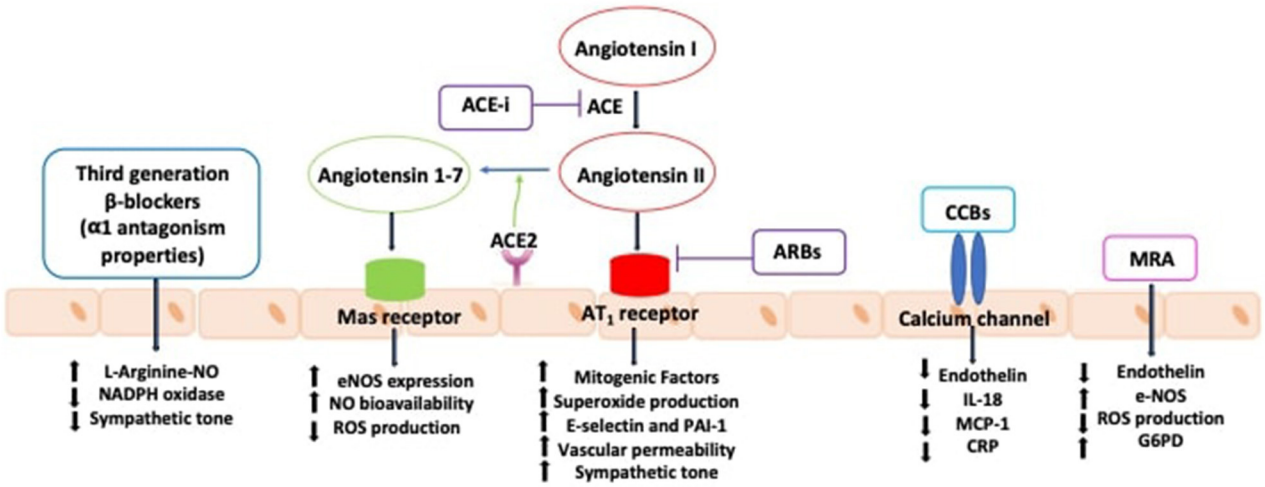

FIGURE 2 | Effects of antihypertensive agents in improving endothelial function. ACE, angiotensin converting enzyme; ACE-i, angiotensin converting enzyme inhibitors; ARBs: angiotensin receptor blockers; AT1R, type 1 angiotensin II receptor; CCBs, calcium channel blockers; CRP, C-reactive protein; eNOS, nitric oxide synthase; G6PD, glucose-6-phosphate dehydrogenase; IL-18, interleukin-18; MCP-1, monocyte chemotactic protein 1; MRA, mineralocorticoid receptor antagonists; NAPDH, nicotinamide adenine dinucleotide phosphate; NO, nitric oxide; PAI-1, plasminogen activator inhibitor-1; ROS, reactive oxygen species.

arteries (67). Other animal studies showed that the dilatation of mouse mesenteric arteries induced by shear stress was impaired by the infusion of EVs isolated from diabetic patients (68) and that endothelial derived EVs decreased NO and increased ROS production, impairing ACh-mediated vasorelaxation, at aortic ring level (69).

On the other hand, EVs have shown beneficial effects on endothelial cells by inhibiting hyperproliferative pathways, through the activation of eNOS signaling mediated by miRNA (70, 71). Moreover, MiR-143/miR-145 contained in EVs has been shown to reduce atherosclerotic lesion formation in the aorta of ApoE−/− mice (72). MiR-19a72 and miR-23b70 mediate the atheroprotective laminar shear stress-induced cell cycle arrest via a decrease in E2F1 and hypophosphorylation of retinoblastoma or directly targeting cyclin D1 (73).

Interestingly, an increasing body of evidence have shown that long non-coding RNA (lncRNAs) can be selectively packaged into EVs and may act as regulators of endothelial function which may represent a promising therapeutic tool, although further studies are required to clarify the specific targets (74, 75).

POTENTIAL FUTURE THERAPEUTIC STRATEGIES BASED ON NANOPARTICLES

Over the past decades several non-pharmacological (i.e., diet, antioxidants, and vitamin supplementation) and pharmacological approaches have been suggested in order to improve endothelial function as mentioned previously. Recently, selective, and more specific approaches such as nanoparticles have been proposed. Nanomedicine is emerging as an innovative approach with the aim to target specific endocytic pathways throughout the formulation of different nanoparticles, encapsulating therapeutic agents with enhanced bioavailability and ensuring treatment effectiveness. Indeed, the challenge to drug delivery in the endothelium consists in the selection of appropriate targets and in the design of nanoparticle-formulations with appropriate binding-properties to the vascular endothelium in micro, small, medium and in large vessels against continuous flowing blood (76, 77). In particular, the delivery through nanoparticles of many clinically used drugs such as antihypertensive agents, statins, antidiabetic drugs and interleukin 1β monoclonal antibodies may represent a potential target for treatment of endothelial dysfunction thus yielding potential new therapeutic approaches (78).

Future application may include the use of several types of small molecules that target complementary epigenetic pathways. More specifically, histone deacetylase inhibitors, DNA methyltransferase inhibitors, histone methyltransferases and demethylase inhibitors have been demonstrated to play an essential role in the regulation of endothelial stem/progenitor cell functions through modifying chromatin structure. In such a context, nanoparticles might be used to modulate the activities of epigenetic enzymes to enhance the vascular repair function of endothelial cells (79).

CONCLUSIONS

Several lines of evidence support the role of endothelium in physiology of peripheral arteries. Impairment of vascular flow, RAS activation, oxidative stress and inflammation have been demonstrated to play a fundamental role in the development of endothelial dysfunction in hypertensive patients, leading to vascular remodeling, atherosclerotic plaques progression and eventually increased risk of CV events (**Figure 1**).

The modulation of vascular inflammation through RAS blockers and other antihypertensive drugs is a well assessed therapeutic approach to improve vascular function and remodeling.

Recent evidence suggests that EVs have attracted increasing interest both as biomarkers or mediators of disease, as well as vehicles for delivering bioactive molecules, such as miRNA and drugs interacting with RAS, with potential beneficial effects on the endothelium. Hence EVs have emerged as important regulators of endothelial function and potentially as a promising novel therapeutic approach to improve endothelial dysfunction.

Moreover, a better molecular understanding of organ vasculature-bed heterogeneity and of organ/tissue microenvironment-governed endothelial cell phenotypic changes may represent the lead foundation for innovative tissue specific therapies (80).

AUTHOR CONTRIBUTIONS

GG, MV, and CS substantially contributed to the conception and design, drafted the article, and approved the final version to be published.

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Hematological Abnormalities in COVID-19 Disease: Association With Type I Interferon Pathway Activation and Disease Outcomes

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Increased expression of interferon (IFN)-stimulated genes (ISGs) in peripheral blood, has been previously reported in viral infections, as well as in autoimmune disorders, in association with reduced leukocyte and platelet counts. Though cytopenias are common in patients with COVID-19 disease and predict severe outcomes, the underlying mechanisms have not been fully elucidated. In the current study, we aimed to determine the prevalence of hematological abnormalities in the setting of active COVID-19 infection and to explore whether they associate with disease outcomes and activation of type I IFN pathway. One-hundred-twenty-three consecutive SARS-CoV2 infected patients were included in the study. Clinical and laboratory parameters were recorded for all study participants. In 114 patients, total RNA was extracted from whole peripheral blood and subjected to real time PCR. The relative expression of three interferon stimulated genes (ISGs; IFIT1, MX-1, and IFI44) was determined and a type I IFN score reflecting peripheral type I IFN activity was calculated. The rates of anemia, leukopenia, and thrombocytopenia were 28.5, 14.6, and 24.4%, respectively. Among leukocytopenias, eosinopenia, and lymphopenia were the most prominent abnormalities being found in 56.9 and 43.1%, respectively. Of interest, patients with either eosinopenia and/or thrombocytopenia but no other hematological abnormalities displayed significantly increased peripheral type I IFN scores compared to their counterparts with normal/high eosinophil and platelet counts. While eosinopenia along with lymphopenia were found to be associated with increased risk for intubation and severe/critical disease, such an association was not detected between other hematological abnormalities or increased type I IFN scores. In conclusion, hematological abnormalities are commonly detected among patients with COVID-19 infection in association with severe disease outcomes and activation of the type I IFN pathway.

Keywords: COVID-19, type I interferon, white blood cells, platelets, eosinophils, cytopenias, eosinopenia, lymphopenia

INTRODUCTION

Type I interferons (IFNα and IFNβ) are secreted by almost all cells in the body in response to the stimulation of cell surface and intracellular pattern recognition receptors (PRRs) by viral and microbial products. They are comprised of multiple IFN-alpha subtypes, including IFN-beta, IFN-delta, IFN-epsilon, IFN-kappa, IFN-tau, IFN-omega, and IFN-zeta, all of which have significant structural homology and bind to a common heterodimeric receptor (consisting of the IFN-alpha/beta RI and IFN-alpha/beta R2 subunits expressed on most cell types). IFNα and IFNβ are the best-defined and most broadly expressed IFNs. They act in an autocrine and paracrine manner to induce an antiviral state in both virus-infected cells and uninfected neighboring cells *via* the induction of interferon stimulated genes (ISG) (1, 2). It is well-appreciated that dysregulation of type I interferon (IFN) pathway has been implicated in the pathogenesis of autoimmune disorders such as Sjogren's Syndrome (SS) and systemic lupus erythematosus (SLE) (3, 4). In the setting of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection, dysregulation of host IFN responses has been known to be associated with severe disease outcomes (5).

Several studies have demonstrated increased frequency of hematologic abnormalities in viral infections in humans (6, 7). Lymphopenia, thrombocytopenia, and eosinopenia have been also reported in patients with SARS-CoV-2 infection, in association with disease severity and mortality (8–11). However, the pathogenetic mechanisms of the hematological alterations in patients with SARS-CoV-2 infection have not been fully explored.

Given that activation of the type I IFN pathway has been previously associated with reduced platelet or leukocyte counts in viral infections and systemic autoimmune diseases such as lupus (12, 13), we aimed to explore whether increased type I IFN inducible gene expression in peripheral blood of patients with COVID-19 infection, could contribute to the observed hematological abnormalities. Moreover, the prevalence of hematological abnormalities in the setting of active COVID-19 infection and the association with disease outcomes will be also explored.

MATERIALS AND METHODS

Study Population

Our study population is comprised of 123 consecutive newly diagnosed cases of COVID-19 infection (68 men; 47 men >50 years old, 21 men ≤50 years old and 55 women; 48 women >50 years old, 7 women ≤ 50 years old) who were evaluated to the outpatient department or hospitalized in COVID-19 unit of Laiko General Hospital, University of Athens School of Medicine, Greece, from October 2020 to February 2021. All patients were infected with the alpha variant (B.1.1.7).

Adults diagnosed with COVID-19 disease by a positive real-time (PCR) test in a nasopharyngeal sample were included in the study. Patients with a previous history of neoplastic disorders receiving steroids or immunosuppressive/chemotherapeutic agents or experienced a recent bacterial or parasitic infection were excluded. All patients gave written informed consent prior to participation in the study. The study conformed to the principles of the Declaration of Helsinki and was approved by the Ethics Committee of Laiko General Hospital (protocol number: 18954-14/12/2020). Patients unable to give informed consent because of impaired cognitive function were also excluded. A history of asthma was detected in five out of 123 patients (three women, two men, age range 20–58). None of them were intubated.

Detailed information about demographics, comorbidities, medication history, presenting COVID-19 related features and laboratory parameters upon initial evaluation or admission were recorded for all study participants as follows: age, sex, date of evaluation or admission, comorbidities and medications, symptoms, vital signs, date of illness, hematocrit, hemoglobin, white blood cells (WBC) count, lymphocyte, neutrophil, eosinophil and monocyte count, serum creatinine, blood urea nitrogen, aspartate transaminase (AST), alanine (ALT), gamma-glutamyl transferase (GGT), alkaline phosphatase, creatinine kinase (CK), lactate dehydrogenase (LDH), C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), troponin, fibrinogen, and D-dimers. Leukopenia was defined as a reduction of WBC< 4 K/μL, neutropenia as a reduction of neutrophils <1.5 K/μL, lymphopenia as a reduction <1 K/μL, eosinopenia is as a reduction of eosinophils <0.01 K/μL, and thrombocytopenia as a reduction of platelets<150 K/μL (14–16). According to the World Health Organization (WHO), anemia is defined as hemoglobin (Hb) levels <12.0 g/dL in women and <13.0 g/dL in men.

Classification of patients was based on their disease course and according to Center for Disease Control and Prevention (CDC) clinical spectrum of SARS-CoV-2 infection, to asymptomatic, mild, moderate, severe and critical illness.*https://www.covid19treatmentguidelines.nih.gov/overview/clinical-spectrum.

More specifically asymptomatic cases were defined as individuals with a positive virologic test (i.e., a nucleic acid amplification test or an antigen test) for SARS-CoV-2, but who have no symptoms that are consistent with COVID-19. Mild cases were defined as individuals manifesting any of the various signs and symptoms of COVID-19 (e.g., fever, cough, sore throat, malaise, headache, muscle pain, nausea, vomiting, diarrhea, and loss of taste and smell) but who do not have shortness of breath, dyspnea, or abnormal chest imaging. As moderate cases were classified individuals with evidence of lower respiratory disease during clinical assessment or imaging and who have an oxygen saturation (SpO_2) ≥94% on room air at sea level. As severe cases were classified individuals who have SpO_2 <94% on room air at sea level, a ratio of arterial partial pressure of oxygen to fraction of inspired oxygen (PaO_2/FiO_2) <300 mm Hg, a respiratory rate >30 breaths/min, or lung infiltrates >50%. As critical cases were defined individuals who have respiratory failure, septic shock, and/or multiple organ dysfunction.

On this basis, we further divided our patient population into high (severe, critical) vs. low severity score (asymptomatic, mild, and moderate) groups.

RNA Extraction—Quantitation of Type I IFN Score

Total RNA was extracted from whole peripheral blood derived from 114 patients with high quality RNA using the TRIzol Reagent (Invitrogen, USA) according to manufacturer's instructions and immediately stored at −80°C. The quantity and quality of RNA samples were spectrophotometrically tested (Biospec Nano, Japan).

Five hundred nanograms of total RNA was reverse transcribed using PrimeScript RT reagent Kit (Takara Japan) as per manufacturer's instructions on an Veriti cycler. All complementary DNAs were diluted 1:10 with nuclease free water (Qiagen, Germany) immediately after synthesis and stored at −20°C.

Quantitative real-time polymerase chain reaction (qRT-PCR) was implemented to quantify the expression of selected genes using the Sacace96 thermocycler and the KAPA SYBR FAST Mastermix (KAPA Biosystems, South Africa), as previously described (3). Briefly, genes preferentially induced by type I IFNs were selected, including IFN-induced protein with tetratricopeptide repeats 1 (IFIT1), interferon induced protein 44 (IFI44) and myxovirus (influenza virus) resistance 1 (MX1). Glyceraldehyde phosphate dehydrogenase (GAPDH) was used as an internal control and normalization gene (housekeeping gene). Primers specific sequences are presented in **Supplementary Table 1**. All reactions were performed in duplicate. The RNA from peripheral blood of confirmed healthy subjects served as reference sample and was included in each PCR plate, to ensure normalization across experiments. Type I IFN score was calculated as previously described (3, 4). The cut-off for high type I IFN scores among patient peripheral samples was defined as the mean plus 3 SD of the corresponding IFN scores in HC (cut-off for high type I score: 8).

Statistical Analysis

All statistical analyses were performed using SPSS v.25.0 (IBM, Armonk, NY, U.S.) and GraphPad Prism 9 (GraphPad Software, San Diego, CA, U.S.), with the level of statistical significance being set at 0.05 for univariate and 0.10 for multivariate analysis, respectively. Chi square or Fisher's exact test were performed to compare the frequencies of categorical variables and Mann-Whitney, or *t*-test were employed for detecting significant differences in numerical variables. Spearman's correlation coefficients were calculated to detect correlations between numerical variables. Backward stepwise logistic regression was applied to explore whether hematological abnormalities are independently associated with diverse COVID-19 related outcomes taking into consideration potential confounders such age and sex.

RESULTS

Hematological Abnormalities, Demographics, and Clinical Outcomes of Study Participants

Hematological abnormalities as well as demographics, other laboratory features and outcomes in the whole study cohort are displayed in **Table 1** and **Supplementary Table 2**, respectively. The rates of anemia, leukopenia and thrombocytopenia were 28.5, 14.6, and 24.4%, respectively. Among leukocytes, eosinopenia and lymphopenia were the most prominent abnormalities being found in 56.9 and 43.1%, respectively. The mean age of study participants was 62.9 ± 16.6 years, with 68 (55.3%) of the patients being males and 55 (44.7%) females. Regarding disease severity, 6 (4.9%) patients were asymptomatic, 21 (17.1%) patients had mild, 1 (0.8%) moderate, 61 (49.6%) patients severe, and 34 (27.6%) critical disease. One hundred and seven (87%) patients recovered, 13 (10.6%) patients were intubated, and 16 (13%) patients died. Nine out of 13 intubated patients died (69.2%) and four recovered (30.8%).

TABLE 1 | Prevalence of hematological abnormalities among the 123 study participants.

Hematological findings	(*n* = 123)
Hb (g/L) (mean ± SD)	13.3 ± 2.2
WBC (K/μL) (mean ± SD)	6.3 ±2.4
Neutrophils (K/μL) (mean ± SD)	4.6 ± 2.1
Lymphocytes (K/μL) (mean ± SD)	1.2 ± 0.7
Monocytes (K/μL) (mean ± SD)	0.5 ± 0.5
Eosinophils (K/μL) (mean ± SD)	0.4 ± 0.7
PLT (K/Ml)	226 ± 185
Anemia (%)	28.5
Leukopenia (%)	14.6
Neutropenia (%)	2.4
Lymphopenia (%)	43.1
Eosinopenia (%)	56.9
Thrombocytopenia (%)	24.4

Hb, Hemoglobin; PLT, platelets; WBC, white blood cells.

Association of Hematological Abnormalities With Distinct Demographic Characteristics and Disease Outcomes

We next sought to explore whether hematological abnormalities were associated with distinct demographic variables or disease outcomes. As shown in **Table 2**, patients with baseline anemia were older and more likely to be asymptomatic compared to those without anemia (age: 68.1 ± 17.2 vs. 60.8 ± 16.0, $p = 0.01$; rates of asymptomatic infection: 11.4 vs. 2.3%, $p = 0.03$, respectively). Moreover, compared to those with normal/high lymphocyte or eosinophil counts, patients with lymphopenia and /or eosinopenia at first evaluation were at higher risk for severe/critical disease (88.7 vs. 70% and 90 vs. 62.2%, *p*-values 0.01 and <0.001, respectively) and mechanical ventilation (17.3 vs. 5.7% and 15.7 vs. 3.8%, *p*-values 0.04 and 0.03, respectively). No significant associations were detected between age or adverse outcomes with decreased WBC, neutrophil or platelet counts. Of note, male sex was associated with the occurrence of thrombocytopenia, with only 20% of patients with thrombocytopenia being females compared to 52.7% with normal/high platelet counts, $p = 0.002$). No association between platelet count with fibrinogen or D-dimer levels was detected

TABLE 2 | Association between hematological abnormalities with demographic characteristics and COVID-19 related outcomes.

	Anemia	**Normal or elevated Hb levels**	***p*-value**
Age (mean ± SD)	68.1 ± 17.2	60.8 ± 16.0	0.01
Females (%)	48.6	43.2	0.59
Asymptomatic (%)	11.4	2.3	0.03
High severity scores (%)	80	77.3	0.74
Intubation (%)	11.8	10.2	0.80
Death (%)	17.1	11.4	0.39
	Leukopenia (*n* = 18)	**Normal or elevated WBC (*n* = 105)**	
Age (mean ± SD)	62.7 ± 14.5	63.0 ± 17.0	0.76
Females (%)	38.9	45.7	0.59
Asymptomatic (%)	11.1	3.8	0.18
High severity scores (%)	77.8	78.1	0.97
Intubation (%)	16.7	9.6	0.37
Death (%)	5.6	14.3	0.30
	Lymphopenia (*n* = 53)	**Normal or elevated lymphocytes (*n* = 70)**	***p*-value**
Age (mean ± SD)	64.8 ± 15.8	61.5 ± 17.2	0.43
Females (%)	35.8	51.4	0.08
Asymptomatic (%)	5.7	4.3	0.72
High severity scores (%)	88.7	70	0.01
Intubation (%)	17.3	5.7	0.04
Death (%)	18.9	8.6	0.09
	Neutropenia (*n* = 3)	**Normal or elevated neutrophils (*n* = 120)**	***p*-value**
Age (mean ± SD)	62.0 ± 14.8	62.9 ± 16.7	0.83
Females (%)	66.7	44.2	0.43
Asymptomatic (%)	0	5	0.69
High severity scores (%)	66.7	78.3	0.63
Intubation (%)	33.3	10.1	0.19
Death (%)	0	13.3	0.49
	Eosinopenia (*n* = 70)	**Normal or elevated eosinophils (*n* = 53)**	***p*-value**
Age (mean ± SD)	63.7 ± 15.6	61.9 ± 18.0	0.77
Females (%)	47.1	41.5	0.53
Asymptomatic (%)	1.4	9.4	0.04
High severity scores (%)	90	62.2	<0.001
Intubation (%)	15.7	3.8	0.03
Death (%)	15.7	9.4	0.30
	Thrombocytopenia (*n* = 30)	**Normal or elevated PLTs (*n* = 93)**	***p*-value**
Age (mean ± SD)	64.9 ± 13.4	62.3 ± 17.6	0.47
Females (%)	20	52.7	0.002
Asymptomatic (%)	6.7	2.3	0.60
High severity scores *n* (%)	90	74.2	0.06
Intubation (%)	13.3	9.8	0.58
Death (%)	10	14	0.57

Hb, Hemoglobin; PLT, platelets; WBC, white blood cells; High severity scores, severe and critical disease.

TABLE 3 | Independent associations between eosinopenia and lymphopenia with intubation risk and higher severity scores following adjustment for age and sex in a multivariate logistic regression model.

	***p*-value**	**OR 95%(CI)**[†]
Lymphopenia		
Intubation risk	0.04	3.9 [1.1–13.8]
Disease severity	0.03	5.3 [1.1–25.2]
Eosinopenia		
Intubation risk	0.04	2.90 [1.0–8.1]
Disease severity	≤0.0001	6.9 [2.4–19.8]

Odds ratios (ORs) and 95% confidence intervals (CIs) are displayed.
[†] *Adjusted for age and gender.*

($r = -0.12$, $p = 0.89$ for fibrinogen and $r = -0.016$, $p = 0.86$ for D-dimers, data not shown). In **Supplementary Figure 1**, the distribution of full blood counts according to severity status is displayed. Following multivariate regression analysis including potential confounders such as age and sex, we could not detect any independent association of hematological parameters with death (data not shown). Regarding severity scores and intubation risk, lymphopenia and eosinopenia but no other hematological abnormalities such as leukopenia, anemia, thrombocytopenia, or neutropenia were found to be independently associated with intubation risk or severe outcomes. ORs and corresponding 95% CI are presented in **Table 3**.

Association of Type I IFN Score and Hematological Abnormalities Among COVID-19 Patients

To identify any possible association of type I IFNs with hematological variables, correlation analysis was performed. As shown in **Figure 1**, a significantly inverse correlation was found between type I IFN score with WBC ($r = -0.237$, $p = 0.01$, panel B), platelet ($r = -0.34$, $p = 0.0002$, panel C) and absolute neutrophil count ($r = -0.206$, $p = 0.03$, panel D) among COVID-19 infected patients. In contrast, a weak positive correlation was observed between type I IFN score and Hb levels ($r = 0.186$, $p = 0.048$, panel A). No other significant associations between absolute lymphocyte ($r = -0.174$, $p = 0.07$, panel E) or eosinophil ($r = -0.163$, $p = 0.08$, panel F) count with peripheral blood type I IFN score were detected.

In view of the correlations detected between type I IFN score and blood cell counts we next sought to explore whether high type I IFN scores (see Methods) were associated with discrete hematological outcomes. Ninety-nine patients were classified in the high IFN score group and 15 patients were classified in the low IFN group. As displayed in **Table 4** and **Figures 2C,F**, patients displaying high type I IFN score in peripheral blood, had significantly higher rates of thrombocytopenia and eosinopenia compared to those with normal/high platelet or eosinophil numbers (29.3 vs. 0, $p = 0.015$ and 63.6 vs. 26.7%, $p = 0.007$, respectively). No other significant differences in the rates of anemia, leukopenia, neutropenia or lymphocytopenia were detected between high and low type I IFN groups (**Figures 2A,B,D,E**). No statistically significant differences in

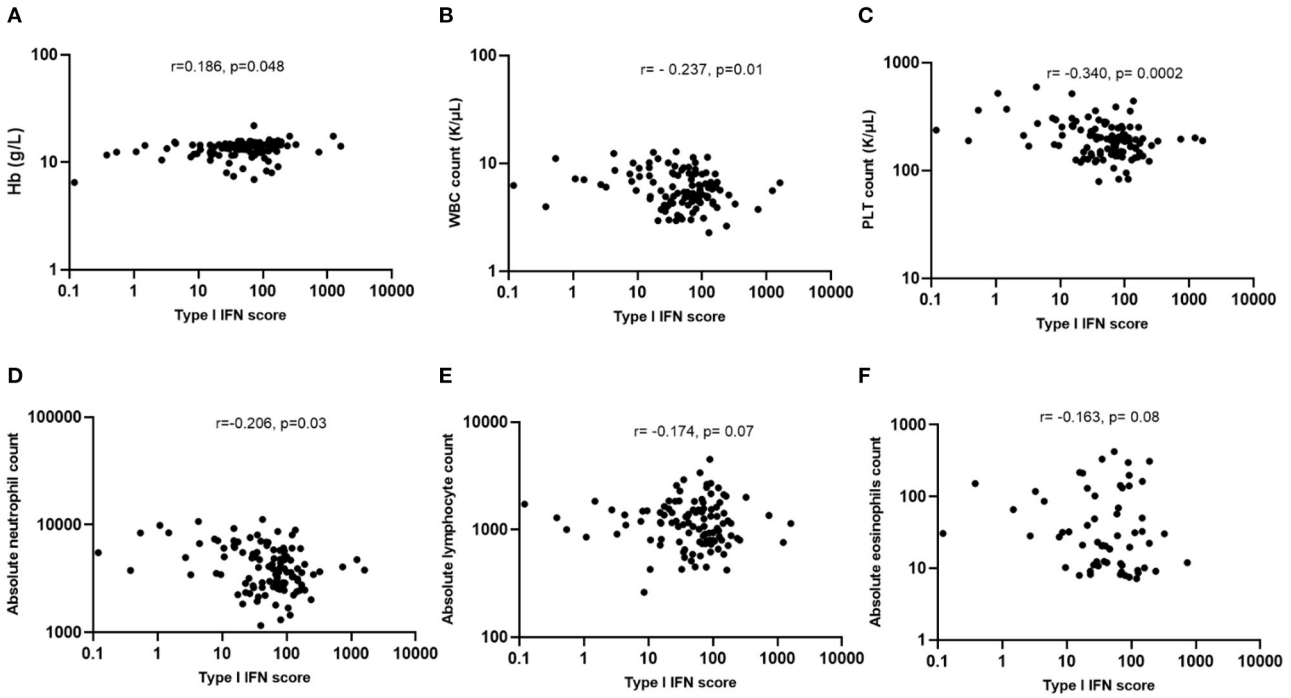

FIGURE 1 | Associations of type I IFN score with hematological features of COVID-19 patients. **(A)** Weak association between type I IFN score and Hb levels ($r = 0.186$, $p = 0.048$); **(B,C)** Statistically significant inverse correlation between type I IFN score with WBC ($r = -0.237$, $p = 0.01$) and PLT counts ($r = -0.340$, $p = 0.0002$); **(D–F)** Statistically significant inverse correlation between type I IFN score with absolute neutrophil ($r = -0.206$, $p = 0.03$), but not with lymphocyte ($r = -0.174$, $p = 0.07$) or eosinophil counts ($r = -0.163$, $p = 0.08$). Hb, hemoglobin; PTL, platelets; WBC, white blood cells; IFN, interferon; *r*, Spearman's correlation.

TABLE 4 | Hematological variables in low and high type I IFN score groups.

	Low type I IFN score ($n = 15$)	High type I IFN score ($n = 99$)	*p*-value
Hematological findings			
Hb (g/L)	12.6 ± 2.5	13.4 ± 2.2	0.33
WBC (K/μL) (mean ± SD)	7.9 ± 2.7	6.1 ± 2.4	0.01
Absolute neutrophil count (mean ± SD)	5,600 ± 2,300	4,500 ± 2,100	0.07
Absolute lymphocyte count (mean ± SD)	1,700 ± 1,100	1,100 ± 500	0.08
Absolute monocyte count (mean ± SD)	500 ± 200	400 ± 500	0.10
Absolute eosinophil count (mean ± SD)	900 ± 1,100	300 ± 700	0.002
PLT (K/Ml)	302 ± 127	216 ± 198	0.001
Anemia (%)	40.0	26.3	0.27
Leukopenia (%)	6.7	17.2	0.30
Neutropenia (%)	0	3	0.49
Lymphopenia (%)	26.7	47.5	0.13
Eosinopenia (%)	26.7	63.6	0.007
Thrombocytopenia (%)	0	29.3	0.015

CBC, complete blood count; Hb, hemoglobin; IFN, interferon; PTL, platelets; SD, standard deviation; WBC, white blood cells; High severity scores, severe and critical disease.

outcomes or other laboratory variables were detected between high and low IFN groups (**Supplementary Table 3**).

DISCUSSION

To the best of our knowledge, we report for the first time that patients with active SARS-CoV-2 infection with either eosinopenia and/or thrombocytopenia display increased peripheral type I IFN scores compared to their counterparts with normal/high eosinophil and platelet counts. These findings indicate a possible implication of type I IFN activation in hematological alterations commonly observed in COVID 19 patients. While heightened type I IFN scores along with thrombocytopenia were more prominent in male patients, they were not associated with increased risk for severe outcomes including intubation and death or other hematological abnormalities such as anemia, neutropenia or lymphopenia; of note, the latter along with eosinopenia were shown to be associated with more severe outcomes and higher intubation risk, in line with previous observations (10, 11, 17). Of note, patients with normal/high eosinophilic counts but lower hemoglobin levels were more likely to be asymptomatic.

Hematological abnormalities including leukopenia and thrombocytopenia have been previously described as common findings in SARS patients complicated (18) or not (19) by

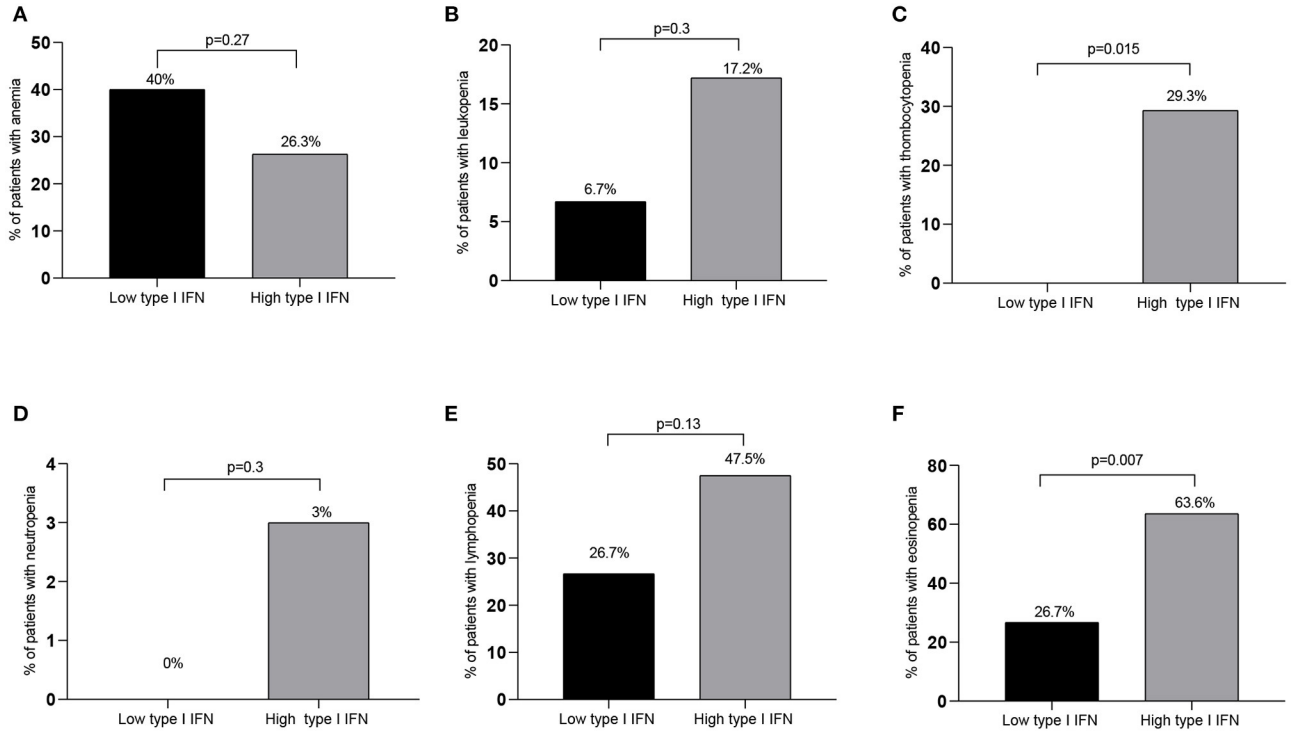

FIGURE 2 | Rates of hematological abnormalities among high vs. low IFN groups. While no significant differences between patient groups with high and low type I IFN peripheral blood scores were detected in the rates of anemia, leukopenia, neutropenia or lymphopenia [**(A,B,D,E)**, respectively], significantly higher rates of thrombocytopenia and eosinopenia were detected in the high compared to the low type I IFN subset **(C,F)**. Only significant differences are shown. IFN, interferon; $p \leq 0.05$ is considered significant.

pneumonia. As possible pathogenetic mechanisms, autoimmune destruction, viral or cytokine related insult of hematopoietic stem/progenitor cells, decreased production or increased consumption of platelets in the infected lungs and hypoxia induced mitochondrial disturbances leading to platelet activation and apoptosis, have been all postulated as potential mechanisms (20–23). In the current study, no association between D-dimer levels with platelet counts were detected implying that reduction of platelet numbers is rather independent of local consumption due to thrombotic state (24).

In the setting of SARS-CoV-2 infection, lymphopenia has been previously attributed to direct invasion of lymphocytes and bone marrow by the virus and destruction of the spleen or lymph nodes, to the deleterious effects of cytokine storm on T cells, as well as to elevated lactic acid levels known to reduce lymphocyte proliferation (22). Regarding eosinopenia, the pathophysiology remains ambiguous and multifactorial; inhibition of eosinophilopoiesis, at the level of bone marrow, eosinophilic exhaustion following antiviral enzyme release, and impairment of the interleukin-33 (IL-33) pathway, known to be implicated in eosinophil activation (25) are potential contributory mechanisms. In contrast with lymphocytic infiltration which is identified as a major feature in lung tissue biopsies derived from SARS-CoV-2 patients suffering by acute respiratory distress syndrome, eosinophil recruitment has not been so far reported (26, 27).

The observed association between heightened peripheral blood type I IFN scores with eosinopenia and thrombocytopenia could provide novel insights into underlying mechanisms of cytopenias in the setting of SARS-COV-2 infection. In patients with viral infections and autoimmune diseases a link between type I IFN activation with thrombocytopenia and leukopenia has been previously demonstrated (12, 13) with megakaryocytes expressing functional type I IFN receptors (28). In patients with multiple sclerosis administration of high-dose IFN-β therapy, as disease modifying therapy, has been shown to strongly associate with thrombocytopenia (29) and neutrophil-mediated type I IFN pathway activation in the bone marrow has been shown to affect B cell development in both human and murine lupus (30). Regarding the association of type I IFN and eosinophils, the data are conflicting. Type I IFN has been reported to reduce circulating levels of eosinophil granule proteins, to inhibit eosinophilopoiesis, and negatively regulate production of eosinophil activating cytokines *in vitro* (31). Besides, type I IFN restricts type 2 immune responses, which drive eosinophil recruitment, through the regulation of group 2 innate lymphoid cells (32). However, it should be noted that other studies have shown that type 2 immune responses are mediated by type I IFN in patients with multiple sclerosis and chronic eosinophilic

rhinosinusitis (33, 34). The current study, to the best of our knowledge, is the first to directly support a link between type I IFN activation in the setting of in SARS-CoV-2 infection with lower eosinophil and platelet counts, indicating that type I IFN response is a potential mechanism of these hematological alterations observed in COVID-19 disease.

It has been previously suggested that lupus patients with activated type I IFN activity have reduced complement levels (35) and are characterized by impaired endothelial function which in turn associates with increased platelet activation (36). Moreover, impairment of endothelial dysfunction, widespread coagulopathy and complement-induced thrombotic mechanisms have been postulated as major determinants of systemic microangiopathy and thromboembolism among severe cases of COVID-19 (37).

Asymptomatic patients with previous COVID-19 infection have been shown to have downregulated type I IFN inducible genes (38), with SARS-CoV-2 infection delaying the immune system from being activated (39). Recent studies demonstrate that immune cells fail to produce IFN or fail to activate the IFN-mediated response to SARS-CoV-2 infection, rendering the innate immune system unresponsive to viral replication and depriving the cells of the antagonistic action of IFN (40, 41). On the other hand, type I IFN may have an injurious pro-inflammatory effect in severe SARS-CoV-2 infection and therapy with type I IFNs could probably be harmful when administered later in the disease course (42).

Though inborn genetic errors in type I IFN pathway and neutralizing antibodies against type I IFNs leading to impaired type I IFN responses have been associated with adverse outcomes in the setting of SARS-CoV-2 infection (43–45), in the present study we did not detect a direct association between type I IFN peripheral blood score and adverse outcomes such as intubation risk or death. This observation is in accord with a recent meta-analysis by da Silva et al. reporting that plasma protein levels of type I IFN cannot be used as a severity marker for COVID-19 (46). Besides, activation of type I IFN pathway has been previously shown to upregulate the expression of angiotensin converting enzyme-2 receptors in a loop-back mechanism, resulting in further augmentation in intracellular viral load in COVID-19 disease (47).

While previous reports revealed prominent innate immune responses including type I IFN responses among premenopausal women in association with favorable outcomes (48–51), we paradoxically detected an association between type I IFN activation and male gender; this could be confounded by a prominent rate of thrombocytopenia -also shown to be associated with type I IFN responses- among male patients. Besides, premenopausal females are underrepresented in the present study possibly due to less severe disease, not requiring evaluation in a hospital setting. A recent study using artificial intelligence techniques revealed that genes involved in activation of immune pathways (IL-6, TNF, JAK2, IL-1B, SERPINE1, TGFB1, CD8A, and VWF) serve as major hubs in the COVID-19- thrombocytopenia interactomes. Decreased levels of thrombopoietin or its receptors (cMpl) possibly mediated by JAK2 have been postulated as plausible mechanisms (52). Therefore, sex biased immune responses either as a result of distinct sex chromosomes, hormonal exposure or epigenetic variations could partially explain the higher level of inflammation markers and severe cytopenias in SARS-CoV-2 infection observed in males (53, 54).

In view of the above data, JAK inhibitors, already included in the therapeutic armamentarium against COVID-19 (55), hold significant promise in the management of inflammatory and vasculothrombotic manifestations of SARS-CoV-2 infection (47).

Limitations of our single center study are the relatively small number of patients included along with the heterogenicity of disease outcomes. Moreover, beyond type I IFN inducible gene expression, no other proinflammatory cytokines -potentially relevant in COVID-19 related outcomes- were measured.

In conclusion, the current study suggests that hematological abnormalities are commonly detected among SARS-CoV-2 infected patients often in association with severe outcomes and activation of type I IFN pathway. Further studies are needed to confirm these observations.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of Laiko General Hospital (protocol number: 18954-14/12/2020). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

CM: conceptualization. CM, PL, CS, VG, and NS: methodology. CM, VG, PL, CS, AG, and NS: formal analysis, investigation, and manuscript review and editing. VG and PL: writing—original draft preparation. CM and NS: data and funding acquisition, resources, and supervision. All authors contributed to the article and approved the submitted version.

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MINI REVIEW
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Complement Mediated Endothelial Damage in Thrombotic Microangiopathies

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Thrombotic microangiopathies (TMA) constitute a group of different disorders that have a common underlying mechanism: the endothelial damage. These disorders may exhibit different mechanisms of endothelial injury depending on the pathological trigger. However, over the last decades, the potential role of the complement system (CS) has gained prominence in their pathogenesis. This is partly due to the great efficacy of complement-inhibitors in atypical hemolytic syndrome (aHUS), a TMA form where the primary defect is an alternative complement pathway dysregulation over endothelial cells (genetic and/or adquired). Complement involvement has also been demonstrated in other forms of TMA, such as thrombotic thrombocytopenic purpura (TTP) and in Shiga toxin-producing *Escherichia coli* hemolytic uremic syndrome (STEC-HUS), as well as in secondary TMAs, in which complement activation occurs in the context of other diseases. However, at present, there is scarce evidence about the efficacy of complement-targeted therapies in these entities. The relationship between complement dysregulation and endothelial damage as the main causes of TMA will be reviewed here. Moreover, the different clinical trials evaluating the use of complement-inhibitors for the treatment of patients suffering from different TMA-associated disorders are summarized, as a clear example of the entry into a new era of personalized medicine in its management.

Keywords: C5b-9 deposition, complement system activation, complement blockade, endothelia cells, membrane attack complex, thrombotic microangiopathies

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INTRODUCTION

Thrombotic microangiopathies (TMA) constitute a group of disorders characterized by micoangiopathic hemolysis, platelet consumption and systemic organ damage. The identification of the underlying etiology among TMA-associated disorders is a major challenge due to the variability of clinical presentation and the absence of pathognomonic histological findings. Despite these factors, its assessment is crucial for directed therapy (1).

The vascular endothelium plays a pivotal role in the regulation of the hemostatic balance. In this regard, endothelial cells (EC) provide a non-thrombogenic inner layer of the vascular wall which maintains the blood fluidity, prevent thrombosis through different anticoagulant, and antiplatelet

mechanisms, and regulate clot formation, limiting it to those areas without vascular integrity (2). Endothelial injury is the common underlying mechanism among different TMA, leading to the microvasculature thrombosis observed in these conditions (3) (**Figure 1**).

The complement system (CS) is a central component of innate immunity and bridges the innate to the adaptive immune response. Activation of complement pathways under physiological conditions facilitates the clearance of microbes, damaged cells and immune complexes from an organism, promotes inflammation, and attacks pathogen's cell membrane. To prevent undesirable activation and tissue damage, complement activation is strictly controlled by numerous regulators under normal circumstances (4).

During the last decades, the contribution of dysregulated complement activation to endothelial damage has been widely demonstrated in some TMA forms. Among them, the prototype is atypical hemolytic syndrome (aHUS), in which complement dysregulation occurs as the primary event (genetic and/or adquired alternative complement pathway dysregulation). Complement involvement has also been demonstrated in thrombotic thrombocytopenic purpura (TTP) and in Shiga toxin-producing *Escherichia coli* hemolytic uremic syndrome (STEC-HUS), but, in these cases, it occurs as a secondary event, triggered either by ADAMTS13-deficiency or toxin-mediated injury, respectively (5). Finally, the role of complement has also been suggested to be involved in other TMA-associated disorders, although it has not been completely elucidated (6).

Distinguishing among the different causes of TMA may be challenging, leading to a delay in etiologic diagnosis and, therefore, in the initiation of the most appropriate treatment. The development of diagnostic tools based on functional and genetic studies to assess the involvement of complement in the pathogenesis of the different clinical entities is crucial (1). In this regard, new management approaches of TMA are being evaluated, and complement-targeted therapies are gaining importance since they selectively block this inflammation pathway, thus avoiding the side effects of some traditional therapies (7).

This review addresses the relationship between endothelial damage and complement dysregulation among the main causes of TMA. We also summarize the clinical trials carried out in TMA-associated disorders treated with complement inhibitors (**Table 1**) and the need to consolidate and develop different biomarkers that may allow an individualized treatment in the near future.

COMPLEMENT ENDOTHELIAL DAMAGE IN THROMBOTIC MICROANGIOPATHIES

Atypical Hemolytic Uremic Syndrome

Dysregulation of the alternative complement pathway over EC surfaces is the main cause of TMA development in atypical hemolytic uremic syndrome (aHUS) (8, 9). Genetic variants in complement components (gain or loss function mutations) and/or acquired conditions (antibodies against complement factor H) have been identified in about 40–60% of patients (10–12). Coexistence of these predisposing factors with environmental triggers or complement amplifying conditions (i.e., infections, drugs, surgery, pregnancy) may lead to disease onset (13, 14). aHUS is an ultra-rare disease, that affects both pediatric and adult populations, of a systemic nature and with highly morbidity and mortality rates (14–16). These characteristics, together with the need for a clinical diagnosis (exclusion of all potential TMA causes), could delay an early etiological treatment with terminal complement blockers (17–22), decreasing organ function recovery outcomes (especially kidney function).

Endothelial activation and injury in aHUS occur mainly due to immune innate system dysregulation and the loss of protection at cell surfaces. Disease phenotype and severity will depend on pro- and anti-inflammatory cytokines balance and regulation of individual complement components (23, 24). Once the complement cascade is amplified, the terminal phase will play a key role, especially, the cleavage of C5 molecule into C5a and the subsequent generation of membrane attack complex (MAC), also called C5b-9 (16). C5a is a potent anaphylatoxin, promoting the recruitment of platelets and leukocytes to the endothelial surface. In addition, it causes endothelial retraction with the consequent exposure of the underlying basement membrane and overexpression of procoagulant tissue factor (25). MAC forms pores in pathogens or targeted cell membranes leading to osmolysis (cytolytic effector of innate and adaptative immunity). It can also induce cell activation and intracellular signaling (26), promoting a switch from an anti-coagulant and anti-inflammatory endothelial phenotype to a highly active, pro-coagulant phenotype. Although it may not be identified in all cases, aHUS patients present a defect of AP regulation over EC surfaces, allowing an abnormal formation of terminal complement phase (C5a and C5b-9) on the endothelium, which lead to cell apoptosis and TMA development, with a special predilection for the kidney vasculature (27).

Thrombotic Thrombocytopenic Purpura

ADAMTS-13 (a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13) deficiency is the key event in the pathophysiology of TTP (28). When functional, ADAMTS-13 cleaves the ultra-Large von Willebrand factor (ULVWF) multimers, which are highly reactive to platelets, before VWF is released to plasma. ADAMTS-13 deficiency, which can be congenital or acquired, leads to the accumulation of ULVWF secreted by the endothelium, causing platelet aggregation, formation of microthrombi and ultimately endothelial damage (29). However, an important factor in the pathogenesis of this disease is the different susceptibility of the vascular beds. The selective injury to dermal, renal, and cerebral microvasculature does not occur in EC from lung and liver (30). Notably, this different susceptibility is not appreciated in cells with a macrovascular origin. This phenomenon has not been described only *in vitro* experiments (31) but also in animal models (32), and offers the potential explanation for tissue distribution damage in TTP.

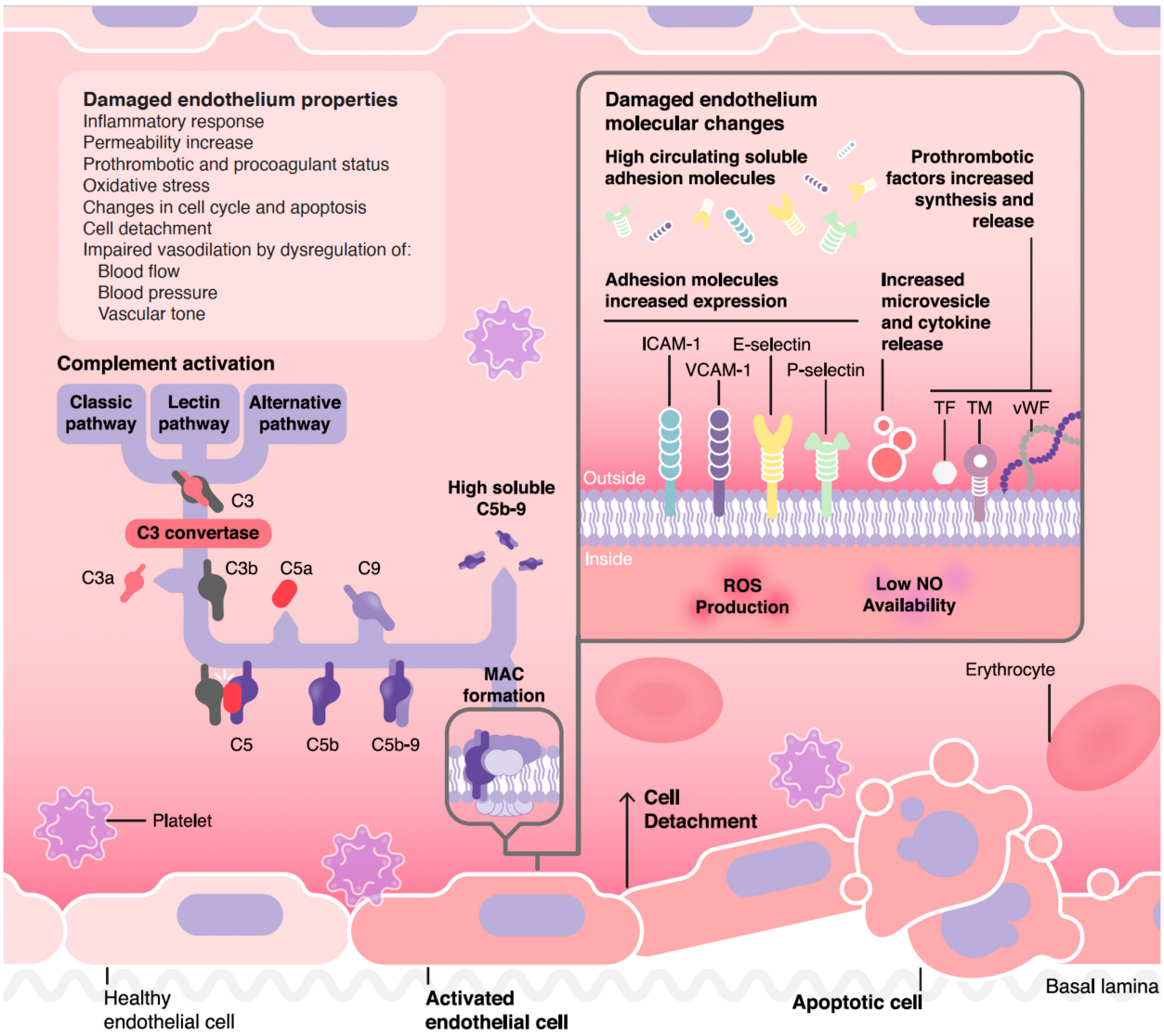

FIGURE 1 | Pathophysiology of complement mediated endothelial damage in TMA. ICAM-1, Intercellular Adhesion Molecule 1; VCAM-1, Vascular cell adhesion protein 1; TF, tissue factor; TM, thrombomodulin; vWF, von Willebrand factor; MAC, membrane attack complex.

Although the role of complement dysregulation in TTP is not as well-defined as it is in aHUS etiology, it has been demonstrated through several approaches. Increased levels of complement anaphylatoxins and soluble C5b-9 (sC5b-9), have been detected in patients with acute TTP when compared with remission and also with healthy controls (33, 34). Furthermore, significant decrease of C3a and sC5b9 has been observed during plasma exchange in TTP patients (34). Higher complement activity has been also reported in patients dying during an acute TTP episode compared to patients responding to treatment (35). From the three different pathways through which the CS can be activated, the classical is the one triggered by immunocomplexes. The majority of TTP cases are idiopathic and mediated by immunoglobulin G antibodies to ADAMTS-13 (29). Therefore, antigen-antibody complexes formed by ADAMTS-13 and anti-ADAMTS-13 IgG directly activate the classical complement pathway, leading to downstream activation of C3 and formation of C3a (34). Moreover, the increased thrombi formation in acute TTP could also accelerate complement activation (36) through its activity as C5 convertase by forming positive feedback loops (37). In keeping with this, it has been documented that TTP could trigger aHUS in susceptible individuals presenting mutations in CFH or other complement-related proteins (38). Finally, there is evidence showing that anti-C5 therapy is effective in refractory TTP unresponsive to plasma (39).

In addition, the role of the lectin complement pathway (LP) has recently been postuated in TTP through the finding

of mannose-binding lectin associated serine protease (MASP2) elevated levels in the sera from acute phase TTP patients. Moreover, *in vitro* experiments using plasma from PTT patients suggest a role of this pathway in microvascular endothelial cell injury through an specific caspase 8 activation that can be blocked by the anti-MASP2 human monoclonal antibody narsoplimab (40).

Shiga Toxin-Producing *Escherichia coli* Hemolytic Uremic Syndrome

Shiga toxin (Stx)-producing *Escherichia coli* (STEC) O157:H7 is the main cause of hemorrhagic colitis, occurring mainly in childhood. In 5–15% of cases it progresses to hemolytic uremic syndrome (HUS), and constitutes the leading cause of HUS worldwide (41).

Endothelial damage plays a central role in the underlying pathogenesis of STEC-HUS, and Stx (principally Stx subtype 2) is thought to be the key in this microangiopathic process through several mechanisms. *In vitro* studies have demonstrated that Stx upregulates the generation of adhesive molecules (E-selectin, ICAM-1, and VCAM-1) and chemokines (MCP-1, IL-8, fractalkine), promoting the adhesion of leukocytes to cultured human EC under flow conditions (42, 43). Stx also induces rapid release of ULVWF multimers from EC and inhibits the multimer cleavage by ADAMTS13, thus favoring platelet adhesion and clot formation in the microvasculature (41, 44). Moreover, Stx promote changes in gene expression, stimulating mRNA and protein production of chemokines and cytokines which may exacerbate endothelial damage (45). All these events generate changes in the endothelial phenotype, resulting in a prothrombogenic intravascular environment.

Another key element in STEC-HUS pathogenesis is dysregulation of the CS, and, likewise, it is mainly driven by Stx. Stx activates complement in the fluid phase, with reduced plasma levels of C3 and augmented levels of C3a, Bb and sC5b-9 during the active phase of the disease (46, 47). This evidence, along with the demonstration in murine models of disease that factor B knockout mices were protected from Stx induced renal damage (48), indicates that complement activation is produced via alternative pathway (AP) in STEC-HUS patients. Moreover, deposits of C3 and C9 have been detected on platelet-leukocyte heterotypic aggregates and microvesicles obtained from these patients (49, 50), thus indicating that complement AP might contribute to endothelial damage and thrombosis in STEC-HUS.

Two double-blinded placebo controlled trials with eculizumab (C5 inhibitor) has been performed to evaluate its efficacy for STEC-HUS treatment: ECULISHU (NCT02205541) in France looking at renal outcome in pediatric population, completed in 2019, and ECUSTEC (NCT01410916) in the United Kingdom looking at overall disease severity, completed in 2015 (**Table 1**). Until results become available from these prospective, randomized control trials, practitioners will continue to rely on small case series, single-center studies, and case reports to guide the eculizumab off-label indication for STEC-HUS treatment.

Transplantation Associated Thrombotic Microangiopathy

Transplantation associated thrombotic microangiopathy (TA-TMA) is a severe complication associated with allogeneic hematopoietic cell transplantation (HCT) that occurs when endothelial dysfunction triggers the CS inducing the formation of platelet enriched thrombi in the microvasculature and a consequent hemolytic anemia (51). This complication can constitute a mild self-limiting disease or be associated with multiorgan disorder leading to death (52). The incidence and mortality of TA-TMA varies widely among studies and hospitals due to heterogeneous diagnostic criteria and under-recognition (53).

The endothelial damage in HCT is multifactorial and cumulative (54, 55) due to the effect of the conditioning regimen, calcineurin inhibitors, infections, graft vs. host disease and processes inherent to HCT such as the engraftment syndrome, among others (56–59). This endothelial damage leads to a release of proinflammatory cytokines and procoagulant factors together with nitric oxide depletion and an increase in the expression of adhesion molecules at cell surface. All these phenomena promote further endothelial injury and leads to platelet aggregation and the initiation and propagation of the complement cascade (60, 61). There is evidence showing that neutrophil extracellular traps (NETs) could constitute the mechanistical link between endothelial damage and complement activation (62). Several approaches have identified increased levels of C3b and sC5b-9 (63, 64), being the last included in TMA diagnostic algorithm proposed by Jodele et al. (63). In this algorithm the authors assessed that TA-TMA should be suspected in HCT recipients with an acute elevation of LDH, proteinuria >30 mg/dL, and hypertension more severe than expected with calcineurin or steroid therapy, and that clinical interventions should be considered for patients with both proteinuria >30 mg/dL and elevated sC5b-9. All these phenomena have been included in the "Three-Hit Hypothesis" postulated by Dvorak et al. (65). This hypothesis includes the different risk factors for the development of TA-TMA in three potential consecutive events that culminate in a cycle of activation of endothelial cells: Hit 1, an underlying predisposition to complement activation (genetical susceptibility due to complement gene variants) or pre-existing endothelial injury; Hit 2, the direct effect on endothelium of toxic conditioning regimen; and Hit 3, the additional insults triggered by medications, alloreactivity, infections, and/or antibodies that occur post-transplant. Furthermore, the anti-C5 monoclonal antibody eculizumab has proven to be an effective therapeutic strategy for TA-TMA, both in pediatric (66) and adult (67) patients, supporting the involvement of CS in the pathogenesis of TA-TMA.

Kidney Transplantation Associated Thrombotic Microangiopathies

TMA associated with kidney transplantation may occur as a recurrence of aHUS or without previous history of thrombotic microangiopathy (*de novo* TMA). Kidney transplantation

TABLE 1 | Complement inhibitors clinical trials.

Drug	Function	Status	Phase	Description	Population	No. of participants	Identifier
SHUa							
Crovalimab	C5 inhibitor	Recruiting	3	Multicenter, single-arm study	Adults and adolescents	90	NCT04861259
Crovalimab	C5 inhibitor	Not yet recruiting	3	Multicenter, single-arm study	Pediatric patients	35	NCT04958265
Eculizumab	C5 inhibitor	Completed (May 30, 2017)	2	Open-label, Multi-center clinical trial	Adult, older adult	44	NCT01194973
Eculizumab	C5 inhibitor	Completed (April 29, 2015)	2	Open-label, multi-center clinical trial	Pediatric	22	NCT01193348
Eculizumab	C5 inhibitor	Completed (July 23, 2015)	2	Open-label, multi-center controlled clinical trial in patients with plasma therapy-resistance	Adults	16	NCT00844545
Iptacopan	Factor B inhibitor	Recruiting	3	Multicenter, single-arm, open label trial	Adult, older adult	50	NCT02604420
Ravulizumab	C5 inhibitor	Active, not recruiting	3	Single-arm study	Adults and adolescents	58	NCT02949128
Ravulizumab	C5 inhibitor	Active, not recruiting	3	Open-label, multicenter study	Children and adolescents	31	NCT03131219
MAT related with autoimmune disorders							
Ravulizumab	C5 inhibitor	Recruiting	2	Double-blind, randomized, placebo-controlled study in participants with proliferative lupus nephritis (LN) or immunoglobulin A nephropathy (IgAN)	Adults	120	NCT04564339
Shiga-toxin Related SHUa							
Eculizumab	C5 inhibitor	Completed (July 12, 2019)	3	Prospective randomized controlled therapeutic trial versus placebo	Pediatric	100	NCT02205541
Eculizumab	C5 inhibitor	Completed (April 5, 2013)	3	Open-label, multi-center trial	Child and adult	198	NCT01410916
TMA after hematopoietic stem cell transplantation							
Eculizumab	C5 inhibitor	Recruiting	2	Early intervention to treat TMA/aHUS-associated MODS	Children and young adults	21	NCT03518203
LFG316	C5 inhibitor	Terminated (January 5, 2021)	2	Randomized, open label, controlled, multiple dose study	Adult, older adult	7	NCT02763644
Ravulizumab	C5 inhibitor	Recruiting	3	Randomized, double-blind, placebo-controlled, multicenter study	Adults and adolescents	184	NCT04543591
Ravulizumab	C5 inhibitor	Recruiting	3	Open-label, single arm, multicenter study	Pediatric	40	NCT04557735
Infections: COVID							
AMY-101	C3 inhibitor	Not yet recruiting	2	Randomized, parallel assignment study	Adult, older adult	144	NCT04395456
Bradykinin	C1 inhibitor	Completed (August 18, 2021)	2	Prospective, randomized, double-blind, multicenter, prospective study	Adult, older adult	44	NCT05010876

(Continued)

TABLE 1 | (Continued)

Drug	Function	Status	Phase	Description	Population	No. of participants	Identifier
Conestat Alfa	rhC1-INH	Active, not recruiting	2	Randomized, parallel-group, open-label, multi-center pilot trial	Adult, older adult	129	NCT04414631
Icatibant	C1 inhibitor	Recruiting	2	Randomized, open, multicenter, proof of concept	Adult, older adult	120	NCT04978051
Eculizumab	C5 inhibitor	Recruiting	2	Bayesian open labeled randomized clinical trial (nested in the CORIMUNO-19 cohort)	Adult, older adult	120	NCT04346797
Ravulizumab	C5 inhibitor	Recruiting	3	Open labe, randomized study	Adult, older adult	32	NCT04570397
Ravulizumab	C5 inhibitor	Terminated	3	Open-label, randomized, controlled study	Adult, older adult	202	NCT04369469
Ruconest	rhC1-INH	Recruiting	2	Randomized, parallel-group, open-label, multi-center pilot trial	Adult, older adult	120	NCT04530136
Zilucoplan	C5 inhibitor	Completed (July 2, 2021)	2	Prospective, randomized, open-label study	Adult, older adult	81	NCT04382755
Infections: Sepsis							
CaCP29	C5 inhibitor	Completed (April 25, 2016)	2	Randomized, placebo-controlled, double-blind, dose controlled trial	Adult, older adult	72	NCT02246595
C1-esterase inhibitor	C1-esterase inhibitor	Completed (December 2, 2014)	3	A randomized controlled pilot study	Adults	20	NCT01766414
TMA associated with a trigger							
Ravulizumab	C5 inhibitor	Recruiting	3	Randomized, double-blind, placebo-controlled, multicenter study in participants who have thrombotic microangiopathy associated with a trigger	Adult, older adult	100	NCT04743804

For the resulting table several searches in clinicaltrial.gov were perform including all countries and interventional clinical studies that were completed, terminated, recruiting and not recruiting. Studies with less than 5 participants have been excluded. In those that are already completed the last update is included between parenthesis. The searches performed were: (1) combination of the concepts "thrombotic microangiopathy" and "complement inhibitors", (2) the pathologies included in the present review and "complement inhibitors", and (3) the search of several individual drugs.

recipients are exposed to many triggers that can damage the endothelium and produce TMA, which can have a significant impact on allograft survival (68). The incidence is concentrated in the first months after transplantation and the main causes are ischemia-reperfusion damage, acute humoral rejection, medications, and opportunistic infections (69). Unlike TMA that occurs in native kidneys, graft biopsy is very useful since it can offer an etiological diagnosis in most cases.

aHUS recurrence after transplantation depends on genetic predisposition and endothelial damage is mediated by a dysregulation of AC pathway (70, 71). Endothelium could also be injured by important number of factors in transplanted patients, triggering *de novo* TMA. Calcineurin inhibitors (CNI) can induce TMA through arteriolar vasoconstriction (increased synthesis of endothelin and thromboxane A2, and reduced expression of prostacyclin and prostaglandin E2) (72–74), platelet activation, anti-fibrinolytic and pro-coagulant effects (75) and, by activation of AC pathway (microparticles released by injured EC) (76). m-TOR inhibitors block cell cycle progression and proliferation, induce endothelial progenitor cells death, increase procoagulant status, reduce fibrinolytic state and decrease renal expression of endothelial growth factor (77–80). All these processes can contribute to the endothelial damage

that triggers *de novo* TMA onset, described with the use of CNI, m-TOR, or a combination of both (possibly related to elevated serum levels) (81, 82). Acute humoral rejection is an important cause of endothelial damage in kidney grafts, frequently associated with TMA development. Donor specific antibodies bind to major histocompatibility complexes on EC, fixing the CS and activating them through the over-expression of pro-inflammatory genes. Terminal complement phase (C5b-9) has been also related to allograft vasculopathy development in humoral response, since it can activate EC, favoring T cell recruitment and secretion of cytokines and interferon γ. Finally, ischemia-reperfusion injury is also associated with endothelial damage and TMA onset. The changes induced on EC produce complement activation that in turn accentuates and perpetuates endothelial damage at the expense of MAC (83). Animal models have shown a clear benefit of blocking the terminal phase of complement by different therapeutic strategies (84–86).

Thrombotic Microangiopathies Related to Autoimmune Disorders

Antiphospholipid syndrome (APS) and systemic erythematous lupus (SLE) are the autoimmune disorders most frequently associated with TMA, which can result in a life-threating complication in these contexts.

APS is a prothrombotic disorder characterized by thrombosis and/or pregnancy morbidity, which occurs in the presence of antiphospholipid antibodies (aPL). Among them, anti-β2-glycoprotein I (B2GPI) antibodies are one of the main drivers of the endothelial damage, since they upregulate the endothelial expression of prothrombotic factors and adhesion molecules. B2GPI also interacts with von Willebrand factor (vWF), leading to platelet activation (87). Moreover, murine models have demonstrated that increased complement activation plays a role in APS pathogenesis, and the interaction of C5a with its receptor C5aR leads to inflammation, placental insufficiency, and thrombosis (88, 89). APS can occur in isolation or in association with other autoimmune diseases, such as SLE.

SLE is a multisystem autoimmune disorder associated with the presence of autoantibodies against double-stranded DNA (ds-DNA), among others, which can produce endothelial damage, either directly binding to EC or forming circulating immunocomplexes that deposit on vessels. In this regard, it has been demonstrated that several indicators of endothelial dysfunction (Pentraxin 3, E-selectin, VCAM-1) are higher in patients with SLE compared to healthy controls (90). CS is also involved in SLE pathogenesis, since these immunocomplexes activate the classical complement pathway and cause tissue damage, as can be observed in kidney biopsies of patients with lupus nephritis, in which C1q, C3 and C4d deposition is common (90, 91). Moreover, there is increasing evidence that CS is linked with thrombotic events occurring is SLE. An example is the relationship that has been observed between the deposition of complement activation products (C1q, C3d, C4d) on platelets surfaces with an increased risk for venous thrombosis in SLE patients (92).

Thrombotic Microangiopathies Associated With Infections

Besides the above-mentioned STEC-HUS, TMA has been associated with a large number of infectious diseases, specially viral (6). Mechanisms of TMA-associated with infectious diseases are complex, and differ depending on the pathogens causing them.

Cytomegalovirus (CMV) can directly damage EC and cause platelet adhesion by inducing the expression of adhesion molecules and release of vWF (93). Moreover, CMV activates the complement classical pathway by binding of C1q to CMV infected cells (94).

TMA is also a known complication of HIV infection, with different forms of presentation. Cases of both classic TTP and aHUS have been described in HIV-infected patients (95, 96), although the exact mechanisms involved remain unclear. Most cases present without a severe reduction in ADAMTS-13 activity levels, which support the hypothesis that the underlying mechanism may be different from classic TTP. It has been suggested that endothelial cells can be infected by HIV (96).

Moreover, TMA induced by influenza A virus (H1N1) infection was reported during the 2009 pandemic. The neuraminidase produced by this virus, as well as by the bacteria *Streptococcus pneumoniae,* causes erythrocyte fusion and hemolysis, activation of platelets and generation of thrombin, leading to TMA in both cases. Furthermore, low levels of ADAMTS-13 and elevated sC5b-9 have been detected in patients with H1N1 infection (97).

More recently, the severe acute respiratory syndrome coronavirus 2 (SARS-COV-2) has emerged as another clear example of viral infection in which endothelial damage occurs in parallel to an overactivation of complement cascade. Patients with moderate and severe COVID-19 disease exhibit elevated C5a and sC5b-9 plasma levels compared with healthy controls, the later correlating with vWF plasma levels and disease severity (98). Moreover, COVID-19 patients were also found to have C5b-9 and C4d skin deposits, as well as mannose-binding lectin (MBL) deposits in lungs, suggesting the overactivation of alternative and lectin-complement pathways (99–101). These observations may reflect the close relationship between endothelial stress and complement dysregulation in this condition. In this regard, isolated experiences using complement inhibitors under a compassionate-use program, such as narsoplimab (102), or AMY-101, a compstatin-based C3 inhibitor (103) has been publicated. The former (narsoplimab) demonstrated rapid and sustained reduction of circulating endothelial cells, as well as with decreased circulating inflammatory cytokines, while the second (AMY-101) was associated with a favorable clinical evolution in a patient with COVID-19 severe pneumonia. All together, these results suggest that CS plays a central role in the pathophysiology of COVID-19-related lung injury.

Moreover, several clinical trials targeting the complement system are currently ongoing in patients with COVID-19

(**Table 1**). Among them, two have been completed: AntagoBrad-Cov (NCT05010876), a prospective, randomized, double-blind, multicenter study of three parallel groups of patients comparing the efficacy of human C1 inhibitor, administered alone or in combination with icatibant (a specific bradykinin B2 receptor antagonist) on the pulmonary manifestations of COVID-19 infections; and ZILU-COV (NCT04382755), a prospective, randomized, open-label, study to investigate the efficacy of a complement C5 inhibitor (Zilucoplan®) in improving oxygenation and short-and long-term outcome of COVID-19 patients with acute hypoxic respiratory failure. The results of the latter haven't been published yet.

Interestingly, samples from septic shock (SS) patients induced higher C5b-9 deposition on EC than those from COVID-19 patients, whereas there were no differences regarding sC5b-9 levels between both groups. Thus, COVID-19 endotheliopathy may differs from SS, in which endothelial damage and complement may also play an important pathogenic role (98). In this regard, two clinical trials have been performed (**Table 1**). The first one, completed in 2014, is VECTORII Study (NCT01766414), a randomized, controlled, pilot study to evaluate the role of a C1-esterasa inhibitor in the modulation of innate immune response in a human endotoxemia model. The inhibition of C1-esterase exerted anti-inflammatory effects in the absence of classic complement activation (104). The second one, completed in 2016, is SCIENS Study (NCT02246595), a phase II clinical trial conducted to study safety, tolerability, pharmacokinetics, and pharmacodynamics of Vilobelimab (IFX-1; CaCP 29), a recombinant monoclonal antibody against C5a, in patients with severe sepsis or septic shock. Vilobelimab demonstrated to neutralize selectively C5a in a dose-dependent manner without blocking formation of the membrane attack complex, and without resulting in detected safety issues (105).

DISCUSSION

Clinical TMA management (diagnostic-therapeutic process) is a great challenge due to its systemic nature (variable signs and symptoms) and high associated morbidity and mortality. Enhancing the etiological knowledge of the different clinical entities involved in TMA will allow the development of targeted therapies that may improve their poor prognosis. The cornerstone for thrombi development in the microvasculature is the endothelial cell damage, common to all TMA-associated disorders. However, these pathological processes exhibit differential patterns and mechanisms of endothelial injury, although occasionally there may be also a pathophysiological overlap.

CS, a key element of the innate immune system, requires precise regulation. Defects in the elements involved result in dysregulation and over-activation. In the case of TMA, an acquired or congenital dysregulation of the alternative complement pathway is primarily responsible for the endothelial damage that occurs in aHUS patients. Therefore, the development of monoclonal antibodies that block the terminal complement phase (C5) has led to a great advance in aHUS management, especially with early therapy introduction. The excellent response to these treatments has led to the research of the potential pathological role of the CS in other TMA forms with higher prevalence and without known etiological treatment.

Figure 1 summarizes the pathophysiological process through which complement could damage the endothelium in different TMA forms, as well as the resulting functional and molecular endothelial changes. One of the difficulties faced in making a diagnosis of complement mediated TMA is the lack of reliable markers of CS hyperactivation. For instance, in the case of the paradigmatic complement mediated TMA, aHUS, relatively few patients have consumption of complement factors, complement mutations are heterozygous-and the corresponding protein concentrations in blood are not consistently abnormal-and genetic screenings are slow and even uninformative in up to 50% of cases (106). In general, the quantification of levels of individual complement components in serum is not a straightforward approach in TMA diagnosis, as soluble levels are not reliable biomarkers of complement activation in any form of TMA (107), although the quantification of sC5b-9 has shown some usefulness in the management of patients with TA-TMA (64) and is available clinically (commercial kits). For this reason, new diagnostic tools are needed for TMAs management. In this regard, functional studies may help to demonstrate the role of complement activation not only at diagnosis, but also to monitor treatment response. Among them, the analysis of C5b-9 deposition on endothelial cells culture could be an attractive option, since it has shown a great correlation in different clinical stages of aHUS patients, as well as in certain secondary TMA forms by different research groups (108–112). However, there is still an open discussion about their utility, especially because it is not a quick procedure, requires specialized and trained personnel, and is not commercially available (1).

In section 2, we have reviewed the evidence generated regarding the participation of the CS in various TMA forms. Since the approval of eculizumab for the treatment of aHUS in 2011, the development of therapies that directly target CS at different levels has enabled a large number of clinical trials focused on complement blockers in patients with different TMA-associated disorders (**Table 1**). Most of them have evaluated the role of C5-inhibitors, mainly eculizumab and ravulizumab (a long-acting C5-inhibitor), in the treatment of various conditions. Among them, those performed with eculizumab in patients with aHUS and STEC-HUS have been already completed. In recent decades, a large number of potential complement-related targets have progressively emerged, such as C3, factor B and C1q. Probably, this fact may be related with the large amount of scientific evidence generated regarding the role of complement in the pathogenesis of the different conditions reviewed in this work. As shown in **Table 1**, several clinical trials evaluating these factors are ongoing, and their results may probably help to extend the therapeutic options for TMA-associated conditions in the near future.

In conclusion, endothelial damage in TMA can have different origins depending on the responsible pathological process. However, the potential role of complement system in many

of them represents a real and current treatment opportunity. Identifying the exact factors involved in each disease will allow an individualized patient management. This is a challenge that will require a great effort involving clinicians, immunologists, geneticists, and basic researchers.

AUTHOR CONTRIBUTIONS

MB reviewed the bibliography and wrote aHUS, kidney transplantation associated TMA and discussion sections. MP reviewed and wrote the TTP and TA-TMA sections, and designed **Table 1** and **Figure 1** with the support of MB and EG-O. EG-O reviewed and wrote introduction, STEC-HUS, TMA related to autoimmune disorders, and TMA associated with infections sections. MD-R contributed to the manuscript with her critical review and comments. All authors contributed to the article and approved the submitted version.

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Application of PLASMIC Score in Risk Prediction of Thrombotic Thrombocytopenic Purpura: Real-World Experience From a Tertiary Medical Center in Taiwan

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Thrombotic thrombocytopenic purpura (TTP) is a life-threatening disorder caused by severe ADAMTS13 (a disintegrin and metalloprotease with thrombospondin type 1 repeats, member 13) deficiency (activity <10%). Urgent intervention based on the timely evaluation of ADAMTS13 level is crucial to guide optimal therapy. The recently developed PLASMIC score based on seven items allows the rapid identification of patients at high risk for TTP due to severe ADAMTS13 deficiency. This retrospective study included 31 hospitalized patients with suspicious thrombotic microangiopathy in National Cheng Kung University Hospital from December 2016 to July 2021. Data on ADAMTS13 activity and medical and laboratory information were retrieved from medical records. The PLASMIC score could be calculated in 24 of the 31 patients with available data, and the final cohort was stratified according to the 7-point PLASMIC score. All patients with high PLASMIC score (6–7) exhibited severe ADAMTS13 deficiency (activity ≤10%). One patient with a brain tumor and a PLASMIC score of 6 did not have severe ADAMTS13 activity of ≤10%. The patients in the intermediate- and low risk groups (PLASMIC scores of 5 and 0–4, respectively) exhibited ADAMTS13 activities of above 10%. Given the role of prompt diagnosis in the timely delivery of appropriate therapy, these findings confirm and strengthen the predictive value of the PLASMIC score in patients at high risk for TTP due to severe ADAMTS13 deficiency.

Keywords: thrombotic thrombocytopenic purpura, thrombotic microangiopathy, ADAMTS13 (a disintegrin-like and metalloprotease with thrombospondin type 1 repeats), PLASMIC score, relapsed, refractory, systemic lupus erythematosus

INTRODUCTION

Thrombotic thrombocytopenic purpura (TTP), first described by Moschcowitz in 1924 (1), is a life-threatening disease with a mortality rate of 10–20% despite appropriate therapeutic management (2). TTP is a rare form of thrombotic microangiopathy (TMA) characterized by microangiopathic hemolytic anemia (MAHA) accompanied with severe thrombocytopenia and organ ischemia secondary to disseminated microvascular platelet-rich thrombi. For a particular underlying cause, the clinical features observed in patients with TMA are neither sensitive nor specific. Therefore, rigorously derived and easily deployable clinical diagnostic tools that can identify individuals with TTP are critical.

In 1998, deficiency of ADAMTS13 (a disintegrin and metalloprotease with thrombospondin type 1 repeats, member 13), a von Willebrand factor-cleaving protease, has been recognized as the cause of TTP (3–5). Since then, several studies in patients with TMA have demonstrated that an ADAMTS13 activity below 10% is a specific feature of TTP. The diagnosis of TTP requires prompt attention given that it is a fatal condition that requires urgent plasmapheresis. However, testing for ADAMTS13 activity is not widely available and has long turnaround times.

In the last two decades, major advances have facilitated our understanding of TTP and ADAMTS13. Several clinical scoring systems have been developed for the rapid identification of patients who are most likely to have severe ADAMTS13 deficiency. These diagnostic scores have been evaluated for their ability to improve diagnostic accuracy and to guide early treatment in patients with TTP (6–9). In 2010, Bentley et al. published the first clinical diagnostic tool for TTP. Total of five parameters (platelet count, D-dimer, reticulocytes, creatinine, and indirect bilirubin) were found to be predictive of severe ADAMTS13 deficiency (10). The French score with three-components, platelet count, creatinine and antinuclear antibody, described by Coppo et al. in 2010, is an alternative scoring system used to identify TTP (11). Encouragingly, the PLASMIC score, which was derived based on the data of 214 patients in the multi-institutional Harvard TMA Research Collaborative registry, was able to predict severe ADAMTS13 deficiency. The PLASMIC score is used to stratify patients with TMA according to their risk of severe ADAMTS13 deficiency based on the following seven items: platelet count $<30 \times 10^9$/L, hemolysis variable (elevated reticulocyte count, undetectable haptoglobin, or indirect bilirubin >2.0 mg/dL), no active cancer, no history of solid organ or stem cell transplant, mean corpuscular volume (MCV) <90 fL, international normalized ratio (INR) <1.5, and creatinine <2.0 mg/dL (7, 12, 13).

Therapeutic plasmapheresis (TPE) remains the cornerstone of TTP management (14, 15). Plasmapheresis, which usually starts as a 1.5-fold plasma volume exchange followed by a 1.0-fold plasma volume exchange thereafter, should be commenced immediately in patients undergoing diagnostic workup for suspicious TTP. Plasmapheresis is performed daily until the resolution of clinical manifestations related to organ involvement, stable recovery of platelet counts, and cessation of hemolysis (16).

Thrombotic thrombocytopenic purpura is a medical emergency, and its prompt recognition is imperative because of the high mortality rates in untreated or mismanaged patients. Disparities in early diagnosis and timely treatment remain a clinical unmet need.

MATERIALS AND METHODS

Study Cohort

This retrospective cohort included adult patients aged $\geq$20 years who were evaluated for TMA at National Cheng Kung University Hospital in Taiwan between December 2016 and July 2021. The diagnosis of TMA was based on the presence of thrombocytopenia (platelet count $<150 \times 10^9$/L) and MAHA (hemoglobin <10 g/dL in the presence of schistocytes). Patients with documented infection or disseminated intravascular coagulation were excluded. Patients with no laboratory data required before plasmapheresis and those with missing data were also excluded (**Figure 1**).

Patients were categorized as those with TTP and those with other forms of TMA. The diagnosis of TTP was based on a documented ADAMTS13 activity of $\leq$10%. All patients who did not meet the diagnostic criteria for TTP were classified as those with other forms of TMA, including atypical hemolytic uremic syndrome, systemic lupus erythematosus (SLE) associated TMA, drug-induced TMA, transplant-associated TMA, malignant hypertension-associated TMA, malignancy-associated TMA, and unexplained TMA. Relapsed TTP that was defined as disease recurrence after $\geq$30 days since TPE discontinuation (17). In patients who were refractory, it was defined as a failure of platelet response after 4–7 days of TPE or a clinical deterioration in a patient receiving standard therapy (18).

The ADAMTS13 activity was performed using a chromogenic enzyme-linked immunosorbent assay (TECHNOZYM® ADAMTS-13 activity ELISA; Technoclone). In the study institution, a hematology consult was required in cases where TMA was considered and ADAMTS13 activity could be determined only in patients with suspicious TMA.

Data Collection and Risk Stratification

The clinical and laboratory information were retrieved from the medical records. Data included age, sex, initial presentation, comorbidities, and treatment course. The study patients were stratified according to the 7-point PLASMIC score, which included platelet count $<30 \times 10^9$/L, hemolysis variable (elevated reticulocyte count, undetectable haptoglobin, or indirect bilirubin >2.0 mg/dL), no active cancer, no history of solid organ or stem cell transplant, MCV <90 fL, INR <1.5, and creatinine <2.0 mg/dL. The presence of each item is worth 1 point, with a maximum PLASMIC score of 7. The PLASMIC scores of 0–4, 5, and 6–7 indicate low, intermediate, and high risk for severe ADAMTS13 deficiency, respectively (7). The level of agreement between the PLASMIC score and ADAMTS13 activity was evaluated. To verify the predictive value of severe acquired

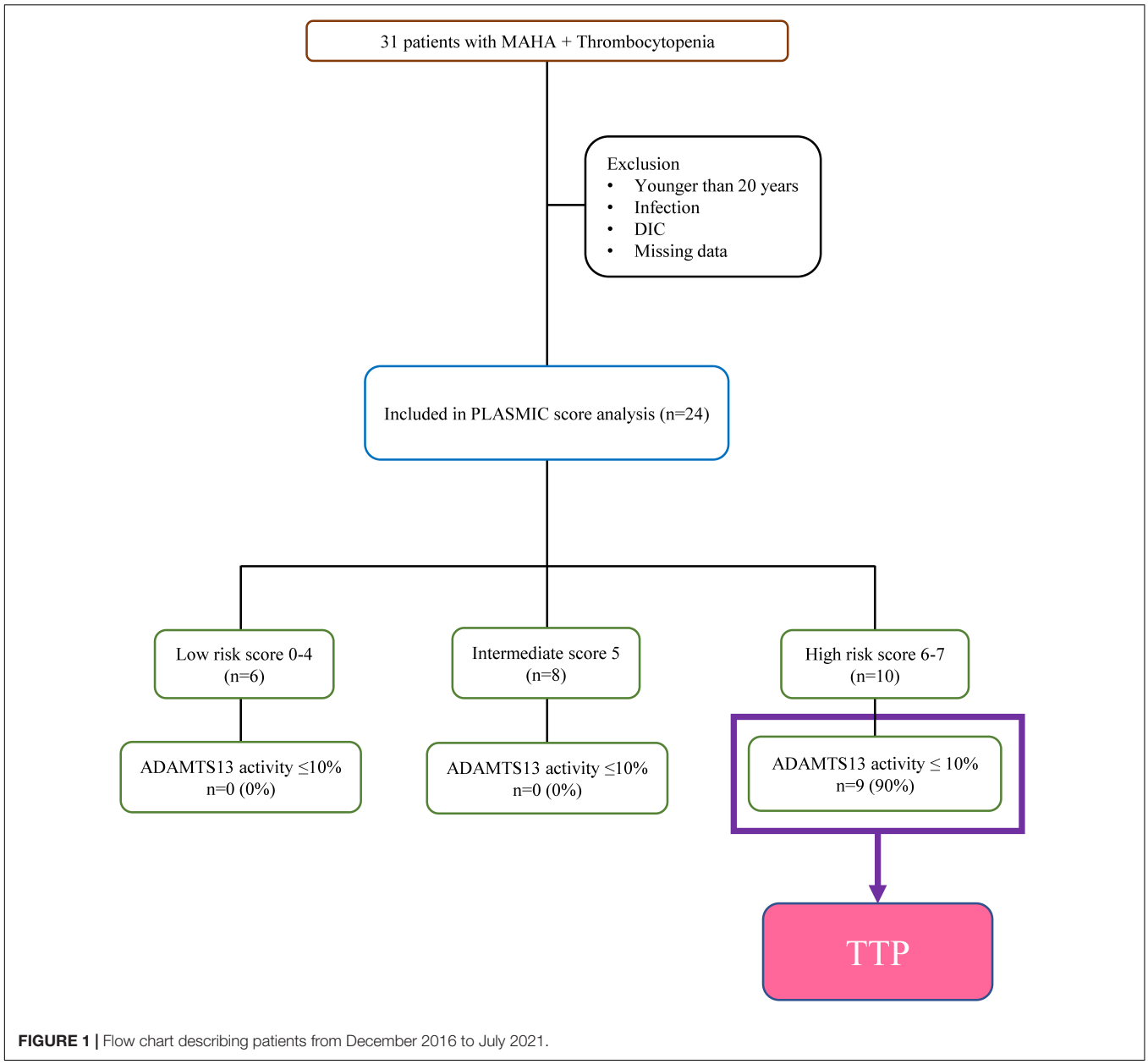

FIGURE 1 | Flow chart describing patients from December 2016 to July 2021.

ADAMTS13 deficiency at the time of diagnosis, the French score, which involves three items (platelet count $\leq 30 \times 10^9$/L, serum creatinine ≤ 2.26 mg/dL, and antinuclear antibody positivity), was also calculated. The presence of each item in the French score is worth 1 point, and French scores of 0, 1, and 2–3 indicate low, intermediate, and high risk of severe ADAMTS13 deficiency, respectively (11). The study was approved by the Institutional Review Board at the National Cheng Kung University Hospital (IRB no., B-ER-110-256).

Statistical Analysis

GraphPad Prism statistical software (version 9; GraphPad, San Diego, CA, United States) was used for all statistical analyses. Continuous data were presented as means ± SD or medians (interquartile range) depending on the distribution. Categorical data were presented as numbers and percentages. Comparisons of normally and abnormally distributed continuous variables were performed using Student's *t* and the Mann–Whitney *U* tests, respectively.

RESULTS

During the study period, 24 of the 31 consecutive patients who were evaluated fulfilled the criteria for TMA (**Figure 1**). The ADAMTS13 activity levels were ≤ 10 and $>10\%$ in 9 and 15 patients with TMA, respectively. The PLASMIC score could be determined in all 24 patients (100%) who had data available for

all seven items, and nine of the 24 patients with TMA were diagnosed with TTP according to the ADAMTS13 activity results.

Table 1 summarizes the clinical and laboratory characteristics and the treatment course of the patients with TTP and those with other forms of TMA (non-TTP TMA). Briefly, sex ($P = 0.9325$), age ($P = 0.4369$), hemoglobin ($P = 0.7585$), INR ($P = 0.4102$), and lactate dehydrogenase ($P = 0.1737$) were not significantly different between the patients with TTP and those with other TMA. Conversely, the patients with TTP had significantly higher MCV ($P = 0.0239$), lower platelet count ($P = 0.0007$), and lower creatinine ($P = 0.0017$) compared with other TMA. Because of the severity of symptoms and the need for plasmapheresis, all patients with TTP underwent more cycles of plasmapheresis than those with other TMA (median, 14 versus 3 days; $P < 0.0001$) (**Table 1**).

The PLASMIC score was used to stratify the entire study cohort ($n = 24$) into three risk categories (**Table 2**). The diagnosis of TTP based on an ADAMTS13 activity of ≤10% was confirmed in nine of the ten patients stratified into the high risk group (PLASMIC score, 6 or 7). The positive predictive value of the PLASMIC score was 90% (95% confidence interval, 57.53–98.35%). The intermediate risk group (PLASMIC score, 5) included one patient with atypical hemolytic uremic syndrome, four patients with SLE-associated TMA, one patient with malignant hypertension, and two patients with unexplained TMA. The low risk group (PLASMIC score, 0–4) included one patient with SLE-associated TMA, two patients with drug-induced TMA, and two patients with transplant-associated TMA. The negative predictive value of the PLASMIC score was 100% (**Supplementary Table 1**).

In the present study cohort, the positive predictive value of the French score, a simpler ADAMTS13 deficiency risk prediction tool, was 64.3%, which was lower than that of the PLASMIC score (**Supplementary Table 2**).

The median follow-up period was 18.5 months (range, 1–44 months). There was no mortality during the acute phase after TMA presentation in the TTP group. Nevertheless, three patients (patient nos. 1, 4, and 5) developed relapsed or refractory TTP (**Table 3**). These patients initially presented with neurological deficits, and patient no. 4 recovered after another cycle of consecutive plasmapheresis. Patient nos. 1 and 5 had a refractory disease and received anti-CD20 monoclonal antibody (rituximab) and other immunosuppressants in addition to the therapeutic plasma exchange.

In the high risk group, one of the 10 patients, a 38-year-old woman with ADAMTS13 activity level of >10%, initially presented with intermittent headache; the image showed brain tumor with hemorrhage. She exhibited an immediate drop in platelet count with MAHA, during the hospitalization. The

TABLE 1 | Characteristics of patients with TTP and those with other forms of TMA.

	TTP ($n = 9$)	Other TMA ($n = 15$)	*P*-value
Female sex	7 (77.8%)	12 (80%)	0.9325
Age, median (range)	46 (22–70)	42(19–87)	0.4369
ADAMTS13 activity, median, % (range)	0	49 (20–86)	<0.0001
Laboratory, median (range)			
Hemoglobin count (g/dL)	7.2 (5.4–9.9)	7.1 (5.3–10.3)	0.7585
MCV (fL)	90.9 (82.4–97.9)	87.2 (71.8–92.8)	0.0239
INR	1.08 (1.02–1.12)	1.19 (0.82–2.26)	0.4102
Platelet ($10^3/\mu L$)	1.1 (4–21)	5.7 (13–105)	0.0007
Creatinine (mg/dL)	0.69 (0.44–1.25)	4.27 (0.32–16.49)	0.0017
Lactate dehydrogenase (U/L)	930 (352–5065)	608 (373–2853)	0.1737
Plasmapheresis, *n* (%)	9 (100)	9 (60)	
Days of plasmapheresis			
Median days (range)	14 (8–25)	3 (0–19)	<0.0001
Immunosuppressant, *n* (%)	9 (100)	11 (73)	
Glucocorticoids	9 (100)	9 (60)	
Rituximab	4 (45)	3 (20)	
Cyclophosphamide	2 (22)	0	
Other treatments, *n* (%)			
Eculizumab	0	1 (7)	
Eltrombopag	0	1 (7)	

TTP, thrombotic thrombocytopenic purpura; TMA, thrombotic microangiopathy; ADAMTS13, a disintegrin and metalloprotease with thrombospondin type 1 repeats, member 13; MCV, mean corpuscular volume; INR, international normalized ratio.

TABLE 2 | PLASMIC score, ADAMTS13 activity, and distribution of clinical diagnoses.

	PLASMIC score risk prediction ($n = 24$)		
	Low risk[a] ($n = 6$)	Intermediate risk[b] ($n = 8$)	High risk[c] ($n = 10$)
ADAMTS13 activity ≤10%, *n* (%)	0	0	9 (90)
ADAMTS13 activity >10%, *n* (%)	6 (100)	8 (100)	1 (10)
Clinical diagnosis, *n* (%)			
TTP	0	0	9 (90)
Other TMA			
aHUS	0	1 (12.5)	0
SLE-associated TMA	1 (16.7)	4 (50)	0
DI-TMA	2 (33.3)	0	0
TA-TMA	2 (33.3)	0	0
Malignant hypertension	0	1 (12.5)	0
Unexplained TMA	1(16.7)	2 (25)	0
Malignancy	0	0	1(10)

[a] *Low risk: PLASMIC score of 0–4.*
[b] *Intermediate risk: PLASMIC score of 5.*
[c] *High risk: PLASMIC score of 6–7.*
ADAMTS13, a disintegrin and metalloprotease with thrombospondin type 1 repeats, member 13; TTP, thrombotic thrombocytopenic purpura; TMA, thrombotic microangiopathy; aHUS, atypical hemolytic uremic syndrome; SLE, systemic lupus erythematosus; DI-TMA, drug-induced thrombotic microangiopathy; TA-TMA, transplant-associated thrombotic microangiopathy.

TABLE 3 | Baseline characteristics and serial ADAMTS13 activity levels of patients with TTP.

No.	Sex	Age	Comorbidity	Initial presentation	PLASMIC score	ADAMTS13 (%)*	Relapsed/ refractory	ADAMTS13 (%)_DOF	ADAMTS13 (%)_DOF
1	F	51	Nil	Altered consciousness	6	0	Yes	0 4 days	0 15 days
2	F	40	Nil	Petechia	7	0	No		
3	F	22	Nil	Syncope	6	0	No		
4	M	70	T2DM HTN	Drowsiness	6	0	Yes	0 106 days	
5	F	33	SLE	Dizziness	7	0	Yes	0 57 days	24 384 days
6	M	61	HBV	Dizziness	6	0	No		
7	F	34	SLE	Headache	7	0	No		
8	F	62	HTN	Headache	6	0	No		
9	F	37	SLE	Petechia	6	0	No		

ADAMTS13, a disintegrin and metalloprotease with thrombospondin type 1 repeats, member 13; TTP, thrombotic thrombocytopenic purpura; T2DM, type 2 diabetes mellitus; HTN, hypertension; SLE, systemic lupus erythematosus; HBV, chronic hepatitis B infection; ADAMTS13(%): ADAMTS13 activity level at presentation; DOF: days of follow-up after first ADAMTS13 activity result.*

clinical course was fulminant, and she eventually succumbed to septic shock.

DISCUSSION

Timely identification of patients with TMA who require urgent treatment is a key unmet clinical need. The retrospective analysis of the current cohort of 24 patients based on risk stratification using the 7-point PLASMIC score confirmed the role of clinical assessment in the timely detection of severe ADAMTS13 deficiency in adult patients with TMA. In agreement with previous studies (8, 19, 20), none of the patients with low risk had severe ADAMTS13 deficiency. Additionally, 90% of the high risk patients with severe ADAMTS13 deficiency benefited from prompt therapeutic plasmapheresis. These patients received plasmapheresis and glucocorticoids treatment immediately once clinical judgment supports the diagnosis of TTP.

The French score, described by Coppo et al., is an alternative scoring system used to identify TTP (11). In the present study, the PLASMIC score with internal and external validation by the Harvard TMA registry, exhibiting superior performance in the prediction of ADAMTS13-deficient TTP compared with the French score (**Supplementary Tables 1, 2**). Data on antinuclear antibody levels were not collected at the initial presentation in most cases. Moreover, differences were observed in the TMA patient selection between the French and PLASMIC scores. The French Score relies on an increased level of clinical judgment from the provider to employ, whereas the PLASMIC score is intended for all-comers even when TTP does not have as high a degree of suspicion that the French score assumes at baseline. A recent brief report demonstrated a moderate correlation among three clinical TMA diagnostic scoring systems (PLASMIC, French, Bentley) and ADAMTS13 levels (21). Considering these instruments, two important caveats are identified: the exact definition of severe ADAMTS13 deficiency remains unclear, which may be assay dependent, and the cutoffs applied to generate these score systems varied across studies; no prospective research has been conducted on these prediction tools (22).

Acquired TTP, which is immunologically mediated, is associated with several autoimmune disorders such as SLE and Hashimoto's thyroiditis. The diagnosis of TTP in the setting of SLE may be difficult because of overlapping clinical symptoms. Likewise, the laboratory criteria of MAHA, which include the presence of schistocytes in the peripheral blood smear, are extremely subjective and rely on experienced laboratorians and hematologists. In the present study, 3 of the 10 patients (30%) in the high risk group were definitively diagnosed with ADAMTS13-deficient TTP associated with SLE. Patients with SLE are at a higher risk for SLE-associated TTP-like MAHA. The underlying pathophysiology is frequently heterogeneous and can be secondary to antiphospholipid syndrome or vasculitis (23). Five of the patients with TMA in the intermediate- and low risk groups had concurrent SLE. In a study of 1,203 SLE cases in Korea, Kwok et al. reported that 2.2% of the patients presented with the clinical features of TTP at the time of diagnosis (24). In two other studies including 40 and 105 patients, concurrent SLE was present in 12 and 45% of the patients with TTP, respectively (25, 26). Moreover, a review of the literature encompassing the period from 1968 to 2002 reported that the mortality was higher among 56 patients with concurrent SLE and TTP compared with those with idiopathic TTP despite optimal treatment (27).

Relapsed TTP is defined as disease recurrence after $\geq$30 days since TPE discontinuation (17). Refractory TTP is defined as a failure of platelet response after 4–7 days of plasmapheresis or a clinical deterioration despite standard therapy (18). In patients with relapsed or refractory TTP despite treatment with ADAMTS13 inhibitors, patient reevaluation is important to identify other potential etiologies of MAHA and thrombocytopenia. In the present cohort, three of the nine patients with TTP (34%) experienced relapsed or refractory TTP and one of these patients received 100 mg rituximab once and exhibited a good response. Emerging evidence suggests that weekly pulse rituximab can achieve a good response in patients with relapsed or refractory TTP (28–30).

The study cohort utilized for the derivation of the PLASMIC score was from the Harvard TMA registry, representing mostly Western ethnic groups (7). In their study, Tiscia et al. demonstrated the good diagnostic performance of the PLASMIC score in a cohort from Southern Italy (19). Another study of a small Chinese cohort reported that a modified PLASMIC score, which included lactate dehydrogenase in addition to the original seven items of the PLASMIC scoring system, might be more suitable for identifying patients with ADAMTS13 deficiency (31). To the best of our knowledge, few studies with larger cohorts have validated the PLASMIC score as a new prediction tool and this is the first study to verify the ability of the PLASMIC score to predict TTP risk in an East Asian cohort.

The present study has some limitations that should be acknowledged. First, this was a retrospective study; however, this approach was unavoidable because of the rarity of TMA with severe ADAMTS13 deficiency. Second, the cohort size was small because of the low rate of TTP.

Albeit rare, acquired TTP is associated with high mortality because of an aggressive clinical course in the setting of delayed diagnosis without optimal treatment. The present study results support the utility of the PLASMIC score as a rapid, feasible, and reliable clinical assessment tool to predict severe ADAMTS13 deficiency in adult patients with TMA.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

C-HL designed and conducted the study, collected the data, performed the statistical analyses, and interpreted and wrote the manuscript. Y-CH conducted the study and collected the data. S-SL, Y-TH, and Y-PC conducted the study. T-YC designed the study, analyzed and interpreted the data, and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2022.893273/full#supplementary-material

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Cardiovascular Risk in Patients With Takayasu Arteritis Directly Correlates With Diastolic Dysfunction and Inflammatory Cell Infiltration in the Vessel Wall: A Clinical, *ex vivo* and *in vitro* Analysis

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Background: Takayasu Arteritis (TAK) increases vascular stiffness and arterial resistance. Atherosclerosis leads to similar changes. We investigated possible differences in cardiovascular remodeling between these diseases and whether the differences are correlated with immune cell expression.

Methods: Patients with active TAK arteritis were compared with age- and sex-matched atherosclerotic patients (Controls). In a subpopulation of TAK patients, Treg/Th17 cells were measured before (T0) and after 18 months (T18) of infliximab treatment. Echocardiogram, supraaortic Doppler ultrasound, and lymphocytogram were performed in all patients. Histological and immunohistochemical changes of the vessel wall were evaluated as well.

Results: TAK patients have increased aortic valve dysfunction and diastolic dysfunction. The degree of dysfunction appears associated with uric acid levels. A significant increase in aortic stiffness was also observed and associated with levels of peripheral T lymphocytes. $CD3^+$ $CD4^+$ cell infiltrates were detected in the vessel wall samples of TAK patients, whose mean percentage of Tregs was lower than Controls at T0, but increased significantly at T18. Opposite behavior was observed for Th17 cells. Finally, TAK patients were found to have an increased risk of atherosclerotic cardiovascular disease (ASCVD).

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Conclusion: Our data suggest that different pathogenic mechanisms underlie vessel damage, including atherosclerosis, in TAK patients compared with Controls. The increased risk of ASCVD in TAK patients correlates directly with the degree of inflammatory cell infiltration in the vessel wall. Infliximab restores the normal frequency of Tregs/Th17 in TAK patients and allows a possible reduction of steroids and immunosuppressants.

Keywords: Takayasu arteritis (TAK), echocardiography, immune cell infiltration, vascular stiffness, T helper-like cells, Regulatory T Lymphocytes (Tregs)

INTRODUCTION

Vasculitis of large vessels is an inflammation of the wall of large and medium-sized arteries, including the aorta and its main branches and the pulmonary artery (1). The clinical signs and symptoms are due to both systemic inflammation and local vascular complications and are associated with elevated inflammatory markers (1). Among these diseases, Takayasu arteritis (TAK) is a chronic granulomatous vasculitis. The majority of TAK patients present with concurrent nonspecific inflammatory signs and symptoms, primarily associated with local stenosis (93%), occlusion (57%), dilatation (16%), and aneurysm formation (7%) (2).

Abnormal immune response is a crucial factor in the pathogenesis of TAK. Regulatory T lymphocytes (Tregs), a subset of $CD4^+$ T cells that express high levels of both CD25 (the α-chain of the high-affinity interleukin-2 receptor) and the transcription factor forkhead box protein P3 (Foxp3), are central mediators of peripheral tolerance (3). Under certain conditions, Tregs can differentiate into T helper (Th)-like cells, leading to a drastic change in their immune functions. Indeed, recent evidence suggests that Tregs can differentiate into Th1, Th2, or Th17 cells, leading to a shift from an immunosuppressive function to a role in the pathogenesis of autoimmune diseases (4). The potential role of Tregs and their associated cytokine secretion in TAK patients is under active investigation to expand the horizon for more effective therapies.

Because of the inflammatory nature of TAK, first-line treatment generally consists of high-dose steroids (usually prednisone). Commonly prescribed second-line agents include immunosuppressants (2). Although these traditional agents can be effective in inducing remission of TAK, relapses remain common when prednisone is discontinued (5, 6). Recent advances in the treatment of TAK patients have been based on the increased understanding of the pathophysiology of TAK and the concurrent availability of new biologic therapies (7). To date, the administration of anti-tumor necrosis factor-alpha (TNF-α) agents, such as infliximab, appears to be a valuable and safe alternative to standard therapy (4). By blocking TNF-α-induced activation of inflammatory signals, infliximab leads to long-term clinical improvement with significant benefits for patients' quality of life (8, 9).

TAK patients are likely to have lesions at different vascular levels, mostly at the subclavian and carotid levels, with either bilateral involvement or focal involvement confined to one carotid or subclavian artery, which can be monitored by ultrasonography (10, 11). At the onset, pathophysiological features are usually nonspecific and include systemic disturbances such as fever, anorexia, weight loss, night sweats, and fatigue. As the disease progresses, diffuse proliferation of the arterial tunica intima along with fibrotic and stenotic lesions may cause ischemic symptoms, whose clinical manifestations depend on the location of the affected arteries. Further progression of TAK leads to destruction of the tunica media, which is often accompanied by aortic regurgitation as well as aneurysm and vessel rupture (7).

Few data are available on ultrasound-based assessment of cardiac function in large vessel vasculitis, although features of early cardiac involvement have been described in other autoimmune diseases (12, 13). Nevertheless, an increase in left ventricular mass (14) and indirect involvement of the right ventricle, secondary to pulmonary hypertension, have been detected by echocardiography in TAK patients (15). Thus, as with several other diseases associated with inflammatory features in the arterial wall, such as hypertension and atherosclerosis (16–18), changes in cardiac structure and function may characterize the progression of TAK disease, increasing the overall risk of cardiovascular morbidity/mortality (19, 20). However, ultrasonographic parameters that can accurately characterize cardiovascular risk and correlations between inflammatory parameters and cardiovascular structural changes have not been studied in TAK patients.

Cardiovascular risk data are based on the assessment of vascular changes (21). The increased vascular resistance is the consequence of many phenomena associated with the development of atherosclerosis (21). The cornerstone of this evolution is inflammation, which is due to increased infiltration of immune cells into the vessel wall (21–23). In classic cardiovascular diseases, there are accepted parameters for assessing organ damage, which represent the referring values also in ultrasound assessment (24). The aim of this study was to compare vascular and cardiac ultrasonography parameters predictive of increased cardiovascular risk in TAK patients, with respect to values obtained in hypertensive and atherosclerotic patients. We also investigated the relationship between cardiac and carotid artery changes (by ultrasonography) and peripheral blood inflammatory cell concentrations. Finally, we investigated the potential role of biomarkers (frequency of Treg and Th17 cells) in TAK-refractory patients, and found that treatment with infliximab ameliorated the Tregs and Th17 ratio with

a concomitant clinical improvement. Overall, the study of Tregs and Th17 populations might represent an additional, novel therapeutic approach to identify cardiovascular risk in TAK patients.

MATERIALS AND METHODS

Patients and Study Design

This cross-sectional single-center study was conducted in conformity to the Good Clinical Practice Guidelines of the Italian Ministry of Health and the ethical guidelines of the Declaration of Helsinki (as revised and amended in 2004) and with the approval of the Ethics Committee of the University of Bari Medical School (Code number 06R76Y9-1).

Thirty TAK patients (22 female, 8 male, aged 49 ± 14 years) were consecutively enrolled and followed-up from January 2014 to December 2018 among patients with large vessel vasculitis admitted to our Center. Patients met the European League against Rheumatism criteria for large vessel vasculitides (25). Patients were in the active phase of the disease (ITAS-A > 6) and were clinically assessed before (time point T0) and 18 months after (T18) treatment with steroids and infliximab (5 mg/kg) therapy (26). However, steroids were tapered within 5-6 months and generally stopped. The Control group consisted of 30 age- and sex-matched patients (22 F, 8 M, 51 ± 12 years old) who received an ultrasound diagnosis of atherosclerosis according to ESC guidelines (24, 27) and had no history of chronic viral infection, autoimmune disease, immune-mediated disease, hematologic malignancies, or cancer. All patients underwent a comprehensive medical examination, including medical history (age, sex, smoking habits, drug treatment, and concomitant diseases) and physical examination.

Clinical and Laboratory Evaluation

Blood pressure values were measured with an electronic sphygmomanometer and were reported as the mean of three consecutive in-office measurements in the supine position after 15 min of rest. The 10-year estimation of the risk of cardiovascular events was performed for all patients using the ACC/AHA validated ASCVD score (28).

At patients admission, levels of erythrocyte sedimentation rate (ESR), creatinine, blood glucose, total cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL), triglycerides, uric acid, CRP, and C3 complement fraction were measured by commercial laboratory diagnostic kits. A standard lymphocytogram was performed in 10 TAK and 8 control patients.

Ultrasound and Imaging Evaluation

A standardized method included a transthoracic echocardiogram with Doppler evaluation (ETG) and ultrasonography Doppler evaluation (SAD) of the supra-aortic vessels (29). All ultrasound measurements were performed with a 2.5-MHz probe (MyLabSeven Doppler echocardiography device, Esaote, Italy) in the left lateral decubitus position; SAD was performed with a 12-MHz linear probe (MyLabSeven Doppler device, Esaote, Italy) on the relaxed neck in the supine position.

Echocardiogram and Cardiac Doppler

Using a previously standardized flow-chart (30), two-dimensional echocardiography was performed to measure aortic root diameter (Ao) in parasternal long-axis view during both systolic (AoS) and diastolic phases (AoD) by an ECG-guided point measurement. In the same position, one-dimensional echocardiography (M-mode) was used to obtain end-diastolic measurements of interventricular septum thickness (IVS), posterior wall thickness (PWD), and left ventricular internal diameter (LVD). According to the international guidelines, the left ventricular mass (LVM) was calculated using the Devereux formula for M-mode diameters. Relative wall thickness (RWT) was calculated using the international validated formula (31). An apical four-chamber view (A4C) was used to measure tricuspid annular plane systolic excursion (TAPSE) and right atrial area (RA). The A4C view was used to measure left ventricular end-diastolic volume (LVEDV), ejection fraction (EF), and left atrial volume (LAV). Sub-costal view (SC) was used to measure inferior vena cava diameter (CVD). Ao, LAV and LVM were indexed to body surface area (BSA) and height 2.7 (LVMi2.7) (24). Aortic root stiffness was assessed using the Aortic Stiffness Index (ASI = In (SBP/DBP)/[(AoS – AoD)/AoD] (32). Doppler measurements were performed in the A4C B-mode view. The velocity of tricuspid regurgitation (TRV) was determined using a continuous wave Doppler curve of tricuspid regurgitation (TR) trace. The peak value of TRV was used to measure the pressure difference between the right ventricle and right atrium (RA) according to the simplified Bernoulli equation ($P = 4$[TRmax]2). RA filling pressure was estimated from the diameter and respirophasic variability of the inferior vena cava during normal breathing. The pulmonary arterial pressure (PAP) estimate was a derived sum of RA filling pressure and TR pressure. In the same view, pulsed-wave Doppler was used to measure transmitral velocity E and A and septal velocity e' in tissue Doppler imaging (TDI) mode. Diastolic dysfunction was assessed as the combination of mitral ratio E/A and E/e' and were stratified as indicated in guidelines (31).

Carotid Ultrasound Doppler

This evaluation was performed to assess vascular damage. Extensive evaluation was performed in both groups, looking to carotid, upper and lower limbs arteries, as well as aorta, kidney, and splanchnic arteries. In TAK patients, the vessel wall dysregulated activity was investigated by evaluation of vascular dilation as well as vessel wall oedema or neovascularization using contrast-enhancement Doppler ultrasound. Carotid intima-media thickness (IMT) was considered as standard measure between the two groups and was sampled using basal B-mode imaging. Selecting the best view of the common carotid artery, radiofrequency analysis yields the mean IMT based on ten automated measurements taken on the posterior wall of the common carotid artery at a length of 1 and 1 cm distal to the vessel bifurcation. Blood flow was analyzed with PW Doppler at a standard angle of 60°, measuring the flow of the common and internal carotid arteries. The North American Symptomatic Carotid Endarterectomy Trial (NASCET) method was used to quantify arterial stenosis and atherosclerosis (33).

Advanced Imaging Technique

After the evaluation of ultrasound, Computerized Tomography angiography was performed in TAK patients. On the contrary, Controls underwent only ultrasound vascular evaluation according to ESC guidelines (27) and CT scan was performed only in critical vascular damage. Therefore, these data were not used in comparison.

Histological Analysis

Ten TAK patients and eight Controls underwent surgery for critical supraaortic vessel stenosis resolution. In these patients, surgical samples were collected to evaluate immune cell infiltration. Samples were fixed in neutral 10% buffered formalin, dehydrated and enclosed in paraffin. Five micrometer thick slices were taken from the paraffin-embedded blocks, deparaffinized, rehydrated and routinely stained with hematoxylin-eosin. Immunohistochemistry was then performed using antibodies for the following markers: monoclonal mouse anti-human CD4 (Agilent, DAKO Omnis, Carpinteria, CA, USA, Cat. M7310), monoclonal mouse anti-human CD8 (Novacastra Laboratories Ltd., Cat. NCL-L-CD8-4B11), polyclonal rabbit anti-human CD3 (Agilent, DAKO Omnis, Cat. GA503) and monoclonal mouse anti-human CD15 (Agilent, DAKO Omnis, Cat. GA062).

To study the infiltration of immune cells, the expression of HMGB1 (34) was assessed using polyclonal rabbit anti-HMGB1 serum (Ab18256, Abcam, Cambridge, USA). Blocks were pretreated on PT-LINK (DAKO) instrument with EDTA [EnVision Flex, Target Retrieval Solution, High Ph (50x), DAKO] for CD3, CD4, CD8 antibodies and Citrate [EnVision Flex, Target Retrieval Solution, Low Ph (50x), DAKO] for HMGB1 antibodies. Immunohistochemistry was performed to measure the density of $CD4^+$ and $CD8^+$ cells in 10 fields at 400× magnification of each sample. One field measured 140 micrometers in length and 110 micrometers in width, and the total amplitude was 15,400 micrometers squared. A Reichert Polyvar 2 microscope with a JTV digital telecamera and a Trinitron monitor (Sony) was used. HMGB1 expression was assessed by highlighting the chromogen signal on the plasma membrane, nucleus, cytoplasm or extracellular medium of the samples examined. The relative expression level was calculated by adding the degree of staining intensity (grade 0 = no staining; grade 1 = weak staining; grade 2 = moderate staining; grade 3 = intense staining) with the percentage of mass extension (score 0: <1%; score 1: 1-25%; score 2: 26-50%; value 3: 51-74%; score 4: ≥75%). The resulting final scores were rated as high (if > 3) or low (if ≤ 3). After processing, two different expert pathologists scored the samples. The final value reported represents the mean of the two values.

Biological Samples and Cell Preparations

Sera were obtained after centrifugation of clotted blood samples and stored at −20°C until further analysis. PBMCs were isolated from heparinized samples using Ficoll-Hypaque (GE Healthcare Life Sciences) gradient separation. $CD4^+CD25^+CD127^{-/dim}$ T cells were purified using the $CD4^+CD25^+CD127^{-/dim}$ regulatory T Cell Isolation Kit II (Miltenyi Biotec, Auburn, CA, USA). The obtained cell populations had a purity of 95% as shown by flow cytometry on immunostained cells.

Cell Cultures and Stimulation

PBMCs (6 x 10^5/well) were cultured in triplicate in 96-well round-bottom plates in 200 ml of Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% of heat-inactivated FBS, 2 mM of L-glutamine, 100 U/ml of penicillin, and 100 mg/ml of streptomycin (all from Sigma-Aldrich, St Louis, MN, USA). Cells were untreated and/or treated with 10 ng/ml of PMA and 1 mg/ml of ionomycin (all from Sigma-Aldrich) for 18 h in a humidified atmosphere containing 5% CO2. After 5 h, 3μM of monensin was added to block Golgi transport (Sigma-Aldrich). Cells were then harvested and immunostained.

Cytofluorimetric Staining

A set of commercial monoclonal antibodies (mAbs) have been used in flow cytometry to analyze the expression of Tregs. Peridinin-chlorophyll proteins cyanine 5.5 conjugated (Perce) anti-CD4 mAb, phycoerythrinCyanine7 conjugated (PECy7) anti-IL-17 mAb, and the alloficocianin- conjugated (APC) anti-Foxp3 mAb were all part of the Human Th17/Treg phenotyping kit (Cat. 560762, Becton Dickinson-BD Biosciences, San Jose, CA, USA). Fluorescein isothiocyanate (FITC) conjugated anti-CD4 mAb and FITC conjugated anti-CD3 mAb were purchased from Beckman Coulter (Brea, California, USA) (Cat. 345768 and Cat. 557851, respectively). Cells were incubated with mAbs to surface antigens for 30 min at 4°C and then washed twice in cold phosphate-buffered saline (PBS) (Sigma-Aldrich) containing 0.1% fetal bovine serum (FBS) (Sigma-Aldrich) before flow cytometry was performed. To determine Th17/Treg phenotype, cells were fixed, permeabilized, and stained according to the manufacturer's instructions for the Human Th17/Treg Phenotyping Kit (BD Biosciences). Stained cells were acquired using FACSCanto II cytofluorimetry (BD Biosciences) and analyzed using FACSDiva software (BD Biosciences).

Statistics

Data were analyzed using GraphPad Prism software (La Jolla, CA, USA) and expressed as means ± S.D. Chi-square test was performed to analyze the distribution of dichotomous values. The non-parametric Mann-Whitney test for comparisons and Spearman distribution for correlations were performed for non-normally distributed data. The parametric unpaired *t*-test for comparisons and Pearson distribution for correlations were performed for normally distributed data. A value of $p < 0.05$ was taken as an indication of statistical significance.

RESULTS

Baseline Features and Clinical Differences

Baseline history information and clinical parameters for all patients are summarized in **Tables 1**, **2**. While no significant difference was found in the incidence of overweight/obesity (**Table 1**), body mass index (BMI) (25.21 ± 5.21/28.44 ± 4.22) and body surface area (BSA) (1.73 ± 0.29/1.89 ± 0.23) values tended to be lower in TAK patients than in control

TABLE 1 | Baseline history information of enrolled patients.

Characteristic	Takayasu arteritis	Controls	*P*-value
Age (years)	49.27 ± 18.87	51.43 ± 12.51	Ns
F/M	22/8	22/8	Ns
BMI (kg/m^2)	25.59 ± 5.7021	28.44 ± 4.22	Ns#
BSA (m^2)	1.73 ± 0.31	1.89 ± 0.23	Ns#
Overweight/obesity (*n*°)	9/5	12/7	Ns
Arterial hypertension (*n*°)	12	30	0.001
Diabetes (*n*°)	3	4	Ns
Ischemic heart disease (*n*°)	4	3	Ns
Ischemic brain disease (*n*°)	3	2	Ns
Preserved EF heart failure (*n*°)	3	5	Ns
Smoke (*n*°)	5	7	Ns
Cigarettes (*n*/day) [IQR]	15 [7.5-18]	13 [7-20]	Ns
ASCVD (%)	21.69 ± 16.09	8.55 ± 7.63	0.001#
Medications			
Anti-hypertensives drugs (*n*°)	12	30	0.001
Antiplatelet (*n*°)	11	10	Ns
Statins (*n*°)	3	25	0.001
Oral antidiabetic drugs (*n*°)	3	3	Ns
Insulin (*n*°)	1	1	Ns
Steroids (*n*°)	22	-	
Prednisone equivalent (mg/day)	27.36 ± 17.86	-	
DMARDs (*n*°)	22	-	
Infliximab (*n*°)	16	-	

ASCVD, atherosclerotic cardiovascular disease; BMI, body mass index; BSA, body surface area; DMARDS, disease modified anti rheumatic drugs; EF, ejection fraction. Ns, not significant. Data were analyzed using Chi-Squared test or Student T-test (#).

TABLE 2 | Clinical and routine parameters of enrolled patients.

Characteristic	Takayasu arteritis	Controls	*P*-value
SBP (mmHg) right	125.30 ± 20.34	123.35 ± 10.27	Ns
SBP (mmHg) left	120.65 ± 30.31	125.11 ± 9.32	Ns
DBP (mmHg) right	70.71 ± 2.495	78.93 ± 6.10	0.0004
DBP (mmHg) left	72.35 ± 8.00	79.73 ± 5.01	0.0002
HR (bpm)	76.28 ± 13.99	67.80 ± 8.57	0.0076
Creatinine (mg/dl)	0.92 ± 0.44	0.81 ± 0.21	Ns
Creatinine Clearance (ml/min)	89.93 ± 34.32	87.17 ± 16.43	Ns
Glycaemia (mg/dl)	98.82 ± 32.16	91.47 ± 14.26	Ns
Total Cholesterol (mg/dl)	171.04 ± 43.66	192.01 ± 32.83	0.042
HDL (mg/dl)	55.96 ± 23.39	56.63 ± 11.24	Ns
LDL (mg/dl)	96.36 ± 34.03	113.82 ± 29.34	0.041
Triglyceride (mg/dl)	103.90 ± 37.95	107.91 ± 42.56	Ns
Uric Acid (mg/dl)	4.30 ± 1.98	4.65 ± 1.19	Ns

SBP, systolic blood pressure; DBP, diastolic blood pressure; HR, heart rate; HDL, high-density lipoprotein; LDL, low-density lipoprotein. Ns, not significant.

group. Total cholesterol (171.04 ± 43.66/192.01 ± 32.83) and LDL cholesterol (96.36 ± 34.03/113.82 ± 29.34) were slightly albeit significantly lower in the TAK group, while the values for HDL (55.96 ± 23.39/56.63 ± 11.24) or triglycerides (103.90 ± 37.95/107.91 ± 42.56) overlapped between the groups (**Table 2**). Accordingly, the estimated incidence of atherosclerosis was low in our TAK patients and treatment with statins was less frequent than in the control patients (**Table 1**).

Although the use of antihypertensive medications was less frequent in TAK patients (**Table 1**), no difference was observed in systolic blood pressure (SBP) values, either on the right arm (125.30 ± 20.3/4123.35 ± 10.27) or on the left arm (120.65 ± 30.31/125.11 ± 9.32) (**Table 2**). Instead, diastolic blood pressure (DBP) was significantly lower in both the right (70.71 ± 2.495/78.93 ± 6.10) and left arms (72.35 ± 8.00/79.73 ± 5.01) in TAK patients than in Controls (**Table 2**). Heart rate (HR) was significantly higher in TAK patients (76.28 ± 13.99/67.80 ± 8.57) than Controls (**Table 2**). Thus, there was no difference in individual cardiovascular risk factors between the two groups of enrolled patients.

Ultrasound Evaluation and Vascular Damage

Interventricular septum (IVS) (11.88 ± 2.03/11.88 ± 1.64) and left atrial volume (LAV) (65.16 ± 32.44/63.57 ± 16.21) were increased in patients with TAK, overlapping with values in Controls (**Table 3**). Also, left ventricular mass (LVM) (205.30 ± 63.11/205.91 ± 44.73), LVM indexed for BSA (LVMi) (118.70 ± 32.20/117.04 ± 21.89), and height2.7 (LVMi2.7) (56.07 ± 19.25/58.37 ± 12.01) were similar in both groups and always increased in Controls compared with the normal population (24, 25). Although most echocardiographic parameters were not significantly different between TAK and Controls (**Table 3**), TAK patients showed a trend to increase LAV indexed for BSA (LAVi) (37.42 ± 16.54/30.69 ± 16.54) (**Table 3**). These results suggest that, although similar cardiac remodeling was observed in both groups, TAK may have increased volume overload in the left heart.

Increased diastolic dysfunction and aortic regurgitation were also observed in TAK patients (**Table 3**). Consistent with the evidence that diastolic dysfunction is associated with increased uric acid levels (35), a marker of endothelial stress, we found that uric acid levels were significantly elevated in TAK patients with severe aortic regurgitation compared with TAK patients without dysfunction (5.06 ± 1.51/2.96 ± 0.79). Accordingly, TAK patients with moderate-severe aortic regurgitation had elevated uric acid levels compared with patients without regurgitation (6.03 ± 1.22/3.64 ± 1.21) or mild-to-moderate diastolic dysfunction (6.03 ± 1.22/3.18 ± 1.20) (**Figure 1A**). Although uric acid levels were not significantly different between TAK and patients (4.30 ± 1.98/4.65 ± 1.19) (**Table 2**), a correlation was observed between the severity of diastolic dysfunction and elevated uric acid levels in patients in the TAK group (**Figure 1B**). On the same line, an increase in arterial stiffness (ASI) (16.54 ± 7.88/13.28 ± 3.11) was observed in TAK patients compared with Controls

TABLE 3 | Echocardiographic parameters of enrolled patients.

Parameter	Takayasu arteritis	Controls	*P*-value
IVS (mm)	11.88 ± 2.03	11.88 ± 1.64	Ns
LvedD (mm)	47.02 ± 5.76	46.89 ± 4.48	Ns
PWT (mm)	11.38 ± 1.45	11.44 ± 1.25	Ns
LVedVol	87.24 ± 26.70	77.69 ± 15.62	Ns
LVM (gr)	205.30 ± 63.11	205.91 ± 44.73	Ns
LVMi (gr/m^2)	118.70 ± 32.20	117.04 ± 21.89	Ns
LVMi2.7	56.07 ± 19.25	58.37 ± 12.01	Ns
Aod (mm)	32.71 ± 4.56	31.57 ± 3.36	Ns
Aoi (mm/m^2)	19.26 ± 2.49	17.24 ± 2.15	Ns
RWT	0.48 ± 0.08	0.49 ± 0.07	Ns
LAV (ml)	65.16 ± 32.44	63.57 ± 16.21	Ns
LAVi (ml/m^2)	37.42 ± 16.54	30.69 ± 16.54	0.01
Ejection fraction (%)	61.42 ± 5.41	61.83 ± 2.73	Ns
E velocity (cm/s)	69.44 ± 16.45	56.43 ± 15.42	Ns
A velocity (cm/s)	65.63 ± 26.40	67.07 ± 15.63	Ns
e' velocity (cm/s)	8.20 ± 0.51	7.23 ± 2.41	Ns
E/e' ratio	10.82 ± 7.32	9.10 ± 2.89	Ns
IMT (mm)	1.93 ± 0.79	1.66 ± 1.32	Ns
Aortic wall thickness (mm)	3.96 ± 0.80	2.83 ± 0.57	0.001
Diastolic dysfunction (none/I/II/severe)	9/10/9/2	6/19/5/0	0.049#
Aortic regurgitation severity (none/mild-to-moderate/ moderate-to-severe)	17/8/5	26/3/1	0.033#

IVS, interventricular septum; LvedD, left ventricle end-diastolic diameter; PWT, posterior wall thickness; LVM, left ventricle mass; LVMi, LVM indexed for BSA; Aod, aortic diameted; RWT, relative wall thickness; LAV, left atrial volume; LAVi, LAV indexed for BSA; IMT, intima-media thickness. Ns, not significant. Data were analyzed using Student T-test or Chi-Squared test (#).

(**Figure 2A**). Moreover, TAK patients without an already known cardiovascular disease showed a significant positive correlation with diastolic dysfunction (**Figure 2B**) and with uric acid levels (**Figure 2C**). This suggests that TAK patients have an increased vessel remodeling.

A statistically significant increase in aortic wall thickness was measured in TAK patients compared with Controls (1.93 ± 0.79/1.66 ± 1.32) (**Table 3**). Standard carotid intima-media thickness (IMT) showed no difference between patients of both groups. Different branches involved into the two groups and ultrasound activity patterns are listed in **Supplementary Table 1**. Active wall remodeling in TAK patients was described as 12.5% vascular dilation, 46.7% of wall neovascularization, and 66.7% oedema. No differences were found in vascular involvement between the two groups (**Supplementary Table 1**). However, in those TAK patients who did not undergo surgical treatment, the thickness of the common carotid wall was inversely related to the aortic diameter when indexed with the BSA (Aoi), as well as to the aortic wall thickness (**Figures 2D,E**). Interestingly, IMT of the common carotid artery was significantly correlated with atherosclerotic cardiovascular disease (ASCVD) in TAK (**Figure 2F**) but not in controls. These data suggest that cardiovascular risk in TAK patients is promoted by enhanced vascular remodeling rather than by classic risk factors.

Immune Cells and Vascular Involvement

A significant decrease in peripheral blood lymphocyte count was observed in TAK patients compared to Controls (1584.4 ± 554.6/2178.6 ± 626.5, **Table 4**). No other significant differences were observed between the two groups. As expected, ESR (41.48 ± 31.49/10.15 ± 6.79) and C-reactive protein (CRP) values (46.30 ± 57.44/3.01 ± 0.45) were significantly higher in TAK patients than in Controls (**Table 4**).

Based on the immunological analysis, we found that ASI values were negatively correlated with peripheral total white blood cell counts (WBCs), neutrophils and $CD3^+CD8^+$, $CD3^+$HLA-DR^+ counts, and neutrophil-to-lymphocyte ratio (NLR). Conversely, ASI values showed a significant positive correlation with total lymphocyte count and total $CD3^+$ and $CD3^+CD4^+$ cell count (**Table 5**).

A positive significant correlation was found for aortic wall thickness with $CD3^+$HLA-DR^+ and $CD3^+CD4^+$ peripheral cells, while this vessel parameter had a significant negative correlation with the total number of $CD3^+$, $CD3^+CD8^+$ and NLR (**Table 6**). Finally, SBP levels were significantly correlated with ASI (**Table 5**) but negatively correlated with aortic wall thickness (**Table 6**).

Overall, these findings support the hypothesis that increased vascular inflammation in TAK patients is a major determinant for the reduced vascular compliance. No correlation was found to disease activity nor to different sites of disease (data not shown).

Immune Cell Infiltration in TAK and Atherosclerotic Patients

To confirm the possible relationship between infiltration by immune cells and the extent of vascular lesions in TAK patients, *ex vivo* experiments were performed on blood vessel sections from patients of both groups.

A significant increase in $CD3^+$, $CD4^+$, and $CD8^+$ cell infiltration was observed in samples from TAK patients compared with Controls (**Figure 3**). High-mobility group box 1 (HMGB1) staining, which labels immunological inflammatory cells, was also significantly higher in the TAK group (**Figure 3**).

To evaluate neutrophilic vessel wall infiltration, $CD15^+$ immune cells were stained and their level was found significantly higher in TAK-derived vessels than in samples from Controls (**Figure 3**). Moreover, in TAK vessels, there was a significant direct correlation between $CD4^+$ (T helper or Th) cell infiltration and $CD15^+$ cells ($r = 0.800$, $p = 0.038$), but not with the total number of $CD3^+$ or $CD8^+$ cells. Both $CD15^+$ and $CD4^+$ cells were directly correlated with HMGB1 ($r = 0.894$, $p = 0.029$) (**Figure 3**), suggesting an association with inflammation-related vascular damage. No significant histological correlation was observed in samples from atherosclerotic patients.

In line with previous observations, histology results support the concept that infiltration by immune cells is a key feature of vascular damage and chemotactic cytokines release.

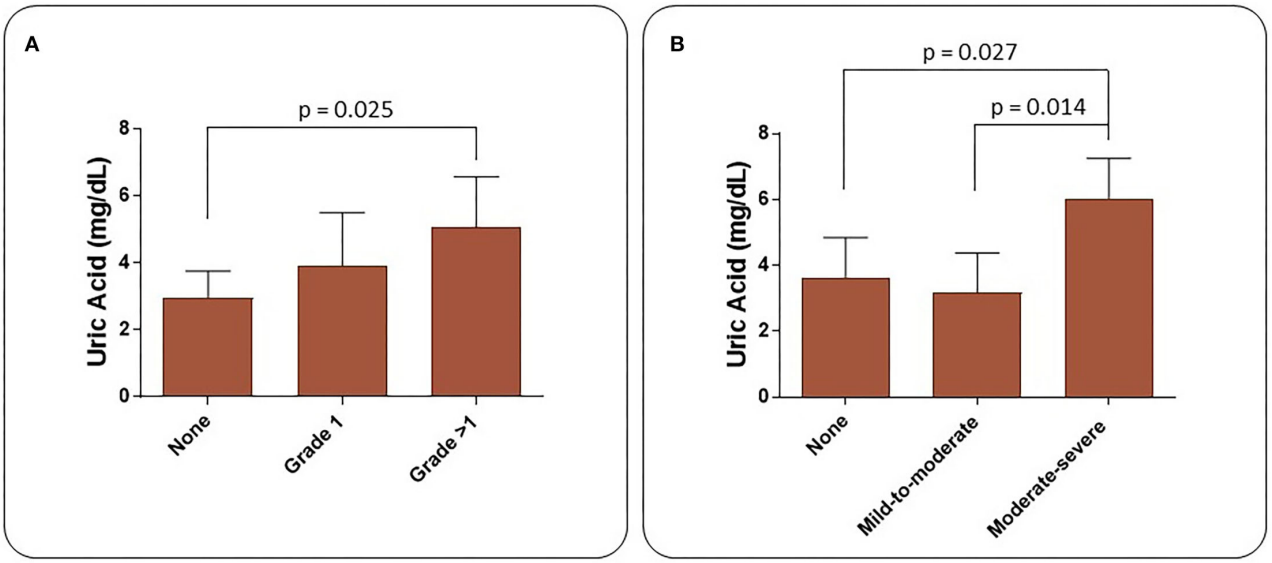

FIGURE 1 | Uric acid levels in TAK patients increase according to the severity of heart diastolic dysfunction **(A)** and aortic valve regurgitation **(B)**.

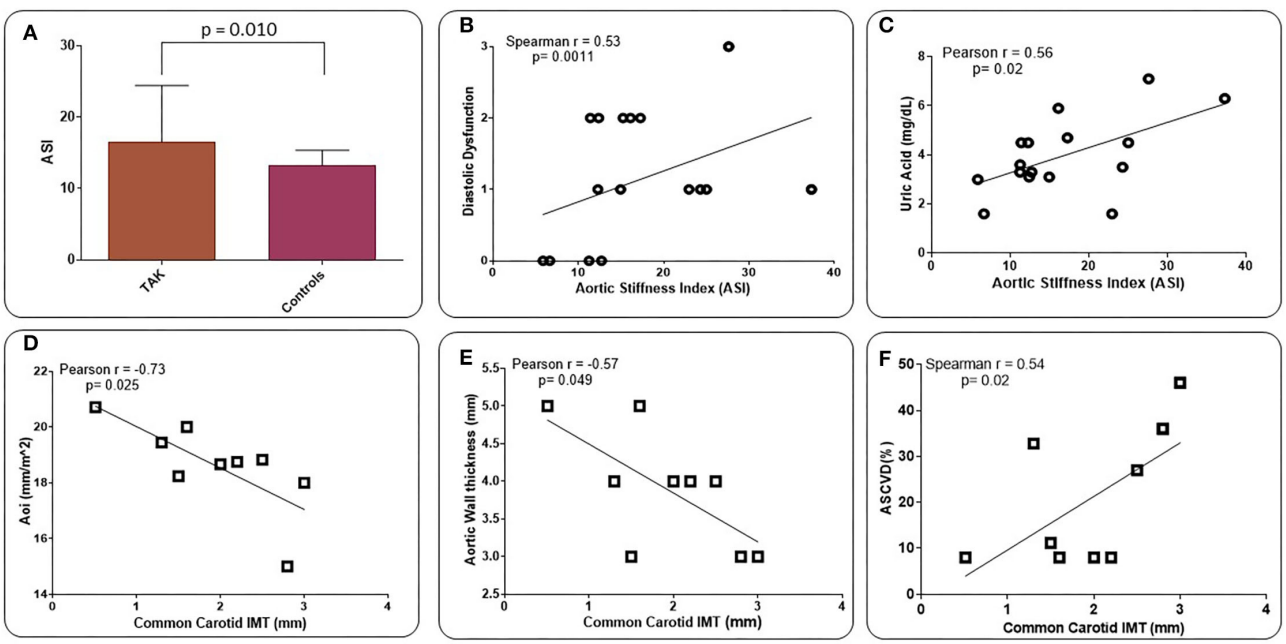

FIGURE 2 | Values of aortic vascular stiffness (ASI) in TAK patients compared with Controls (atherosclerotic patients) **(A)**. ASI values are directly related to the degree of LV diastolic dysfunction **(B)** and uric acid (UA) levels **(C)** in TAK patients. Common carotid intima-media thickness (IMT) shows an inverse correlation with aortic diameter indexed by body surface area (BSA) (Aoi) **(D)** and aortic wall thickness **(E)**, and a direct correlation with ASCVD 10-year risk rate **(F)** in TAK patients. Evaluation was performed excluding already known cardiovascular disease **(B,C)** and those who underwent to surgical treatment **(D-F)**.

Frequency of Treg and Th17 Cells

In a subpopulation of sixteen TAK patients treated with infliximab, the frequency of $CD4^+FoxP3^+$Tregs and $CD3^+CD4^+$ interleukin (IL)-17^+ cells were assessed before (time 0, T0) and after 18 months of treatment (time 18, T18) (**Figure 4A**). A subset of 15 Controls were included. Flow cytometry was performed on freshly isolated peripheral blood mononuclear cells (PBMCs) stimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycin (calcium ionophore), activating protein kinase C, bypassing the T cell

TABLE 4 | Peripheral blood immunological parameters of enrolled patients.

Characteristic	TAK	Controls	*P*-value
Total WBC count (cell/ml)	7,226.3 ± 1,823.4	8337.8 ± 3274.6	Ns
Lymphocytes (cell/ml)	1,584.4 ± 554.6	2,178.6 ± 626.5	0.01
$CD3^+$ (cell/ml)	1,331.1 ± 504.0	1,864.2 ± 784.6	Ns
$CD3^+$ $HLA\text{-}DR^+$ (%)	3.75 ± 3.59	3.50 ± 2.08	Ns
$CD3^+$ $CD4^+$ (cell/ml)	738.8 ± 301.8	1,064.3 ± 544.5	Ns
$CD3^+$ $CD8^+$ (cell/ml)	560.3 ± 250	719.0 ± 325.5	Ns
$CD3^+$ $CD16/56^+$ (cell/ml)	135.7 ± 98.86	226.9 ± 167.0	Ns
$CD19^+$ (cell/ml)	123.0 ± 159.7	156.7 ± 104.9	Ns
Neutrophils (cell/ml)	4,902.2 ± 2193.4	5,536.2 ± 2702.2	Ns
NLR	4.43 ± 4.17	2.94 ± 2.07	Ns
ESR (mm/h)	41.48 ± 31.49	10.15 ± 6.79	0.0001
CRP (mg/dl)	46.30 ± 57.44	3.01 ± 0.45	0.0001
C3 (g/L)	1.19 ± 0.26	1.24 ± 0.28	Ns

WBC, white blood cells; NLR, neutrophil to lymphocyte ratio; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; C3, complement component 3. Ns, not significant.

TABLE 5 | Pearson correlation analysis between Aortic Stiffness Index and peripheral blood immunological parameters and blood pressure of Takayasu patients.

Characteristic	R	*P*-value
Total WBC count	−0.85	0.007
Lymphocytes	0.91	0.002
$CD3^+$	0.97	0.001
$CD3^+$ HLA^-DR^+	−0.98	0.0005
$CD3^+$ $CD4^+$	0.75	0.03
$CD3^+$ $CD8^+$	−0.84	0.008
$CD3^+$ $CD16/56^+$	0.16	Ns
$CD19^+$	0.32	Ns
Neutrophils	−0.93	0.001
NLR	−0.97	0.001
ESR	0.21	Ns
CRP	0.02	Ns
Uric Acid	0.57	0.04
SBP	0.68	0.03

WBC, white blood cells; NLR, neutrophil-lymphocyte ratio; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; SBP, systolic blood pressure. Ns, not significant.

TABLE 6 | Pearson correlation analysis between Aortic wall thickness and peripheral blood immunological parameters and blood pressure of Takayasu patients.

Parameter	R	*P*-value
Total WBC count	0.009	Ns
Lymphocytes	−0.91	0.0007
$CD3^+$	−0.79	0.005
$CD3^+$ HLA^-DR^+	0.86	0.003
$CD3^+$ $CD4^+$	0.62	0.03
$CD3^+$ $CD8^+$	−0.87	0.003
$CD3^+$ $CD16/56^+$	−0.09	Ns
$CD19^+$	−0.09	Ns
Neutrophils	0.27	Ns
NLR	−0.97	0.001
ESR	−0.44	Ns
CRP	−0.31	Ns
Uric Acid	−0.44	Ns
SBP	−0.55	0.049

WBC, white blood cells; NLR, neutrophil-lymphocyte ratio; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; SBP, systolic blood pressure. Ns, not significant.

membrane receptor complex and leading to activation of several intracellular signaling pathways, resulting in T cell activation and production of a variety of cytokines (36). At T0, the mean percentage of Tregs was significantly reduced in TAK patients (0.22/0.67, $p < 0.0001$) compared to Controls and significantly increased at T18 (0.22/0.86, $p < 0.001$) (**Figure 4B**). At the same time, the frequency of $CD3^+CD4^+IL\text{-}17^+$ cells indicating IL-17 expression behaved in the opposite way. The higher number of Th17 cells observed in TAK patients at T0 (3.2/1.4, $p < 0.0001$) significantly decreased at T18 (3.2/2.2, $p < 0.001$) (**Figure 4C**), likely as a result of infliximab treatment. Experiments performed to investigate function and/or viability of Tregs are, at this time, too preliminary to perform a statistical analysis.

Taken together, these results support the idea that biologic therapy may be useful to achieve better control of TAK and achieve a Treg/Th17 score similar to that of Controls. Further prospective and larger studies should be useful to understand whether these results may allow a possible decrease in steroid and immunosuppressant therapy and help to protect against cardiovascular risk.

DISCUSSION

This cross-sectional, single-center study aimed to investigate the potential association between parameters of vascular and cardiac impairment, infiltration of immune cells into vessel tissue, and overall risk of cardiovascular events in TAK patients. Patients with atherosclerosis, whose cardiovascular risk factors are well known, were selected as a control group.

The development of atherosclerosis is a recognized cardiovascular risk factor, and steroid therapies can have significant side effects on the lipid spectrum, with a notable impact on total and LDL cholesterol levels (37). Although steroid treatment is expected to prolong the life expectancy of TAK patients, it may contribute to an increased risk of cardiovascular events through dysregulation of plasma lipid levels. In our study, TAK patients had lower levels of total and LDL cholesterol compared with atherosclerotic patients, with no significant difference in HDL cholesterol or triglyceride levels between the two groups.

Due to the disease-related pathophysiological changes in vascular compliance, blood pressure levels are often elevated in TAK patients (38). However, while systolic blood pressure (SBP)

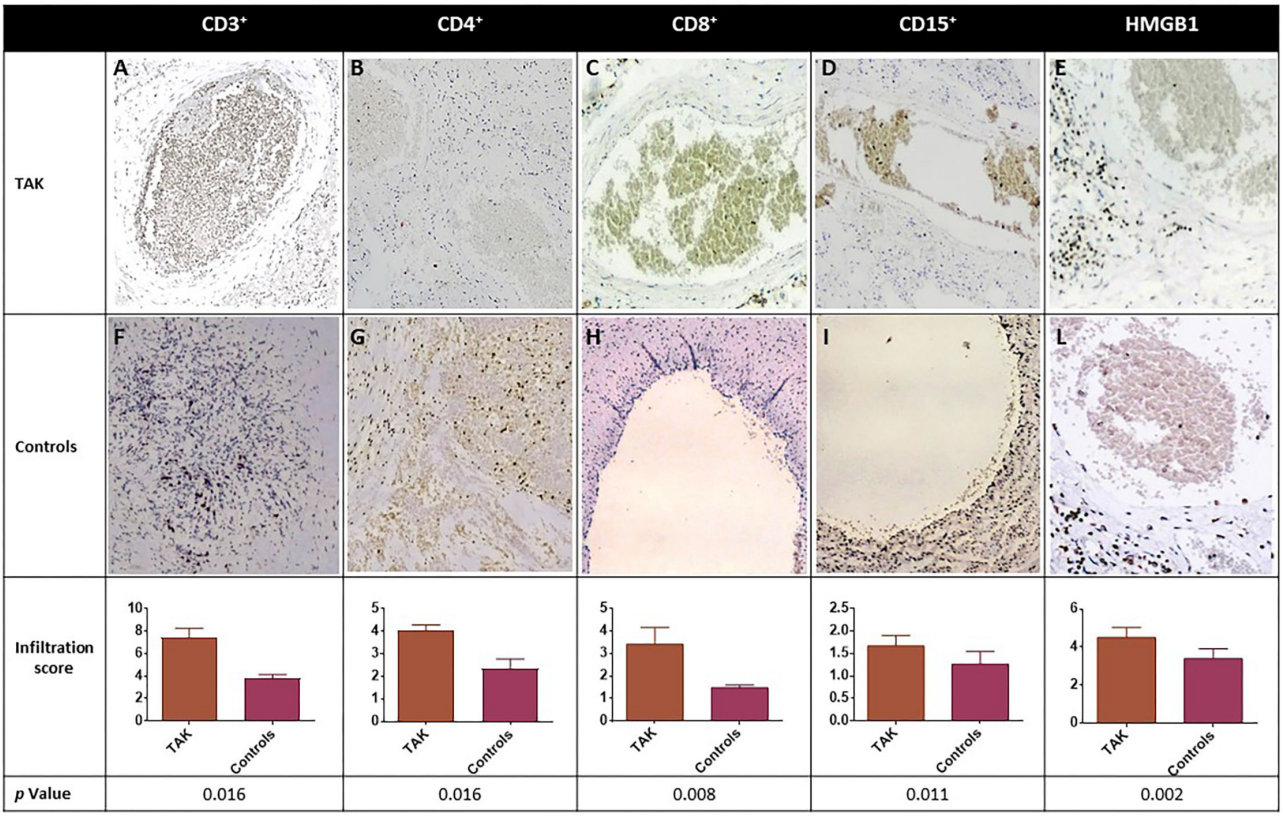

FIGURE 3 | Immune cell infiltration ($CD3^+$, $CD4^+$, $CD8^+$, and $CD15^+$) and expression level of inflammation-associated cytokines of vascular injury HMGB1 in biopsy samples from blood vessels of TAK patients **(A-E)** and Controls **(F-L)**. Representative immunohistochemistry (original magnification: 20X) is shown in the top and middle panels. The lower panel shows the results as mean ± S.D. of three independent experiments for each field.

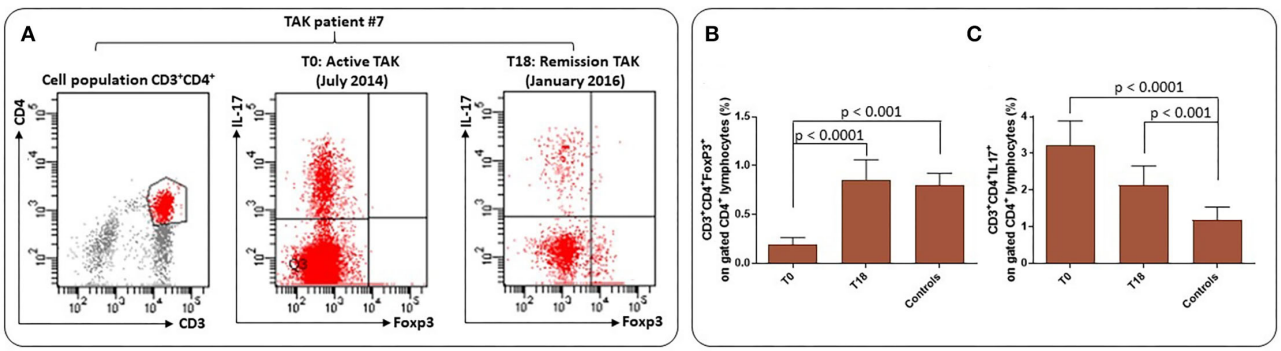

FIGURE 4 | Frequency of Treg and Th17 cells in TAK patients and atherosclerotic Controls. **(A)** Flow cytometry analysis: gating was performed on live $CD3^+CD4^+$ cells to identify Foxp3+Treg and IL-17^+ cells. Representative plots from one patient for each group. **(B)** Results are presented as mean ± S.D. of $CD3^+CD4^+Foxp3^+$ percentages and $CD3^+CD4^+$IL-17^+ T cells **(C)** in TAK patients before (T0) and after 18 months (T18) of infliximab treatment and in matched Controls (Wilcoxon signed-rank test and unpaired *t*-test, Mann-Whitney test).

values in TAK patients were similar to those in atherosclerotic patients, diastolic blood pressure (DBP) values in both left and right arms were significantly lower in TAK patients. As in Controls (38, 39), these results demonstrate that accelerated arterial stiffness and concomitant higher pulse wave velocity progression in our TAK patients may contribute to a significant increase in SBP and concomitant decrease in DBP values.

On echocardiographic ultrasound, the parameters of cardiovascular dysfunction in the TAK patients were comparable to those in the atherosclerotic patients: Both groups showed

similar increases in LVM, IVS, and LAV. Similar to Controls (40, 41), concentric left ventricular hypertrophy is observed in TAK patients (14).

Mild-to-moderate and moderate-to-severe aortic regurgitation were observed in our TAK patients, with the most severe conditions correlating with the highest serum uric acid levels. Aortic regurgitation may be the result of aortic root dilatation, the risk of which increases in parallel with the rise in serum uric acid levels (42). According to recent epidemiological studies, elevated uric acid levels have been associated with the development of hypertension (42) and dyslipidemia (43), leading to a predictive marker for cardiovascular events (44). Uric acid enhances the transcription of nuclear factor-KB (45), which has pro-inflammatory and pro-atherogenic effects in the vascular wall (46), as it stimulates the production of inflammatory cytokines, including TNF-α (47), and the release of C-reactive protein (48). This protein in turn enhances the expression of cellular adhesion molecules, promotes cellular apoptosis, and leads to endothelial dysfunction and arterial stiffness (49). In addition, high uric acid levels have a pro-oxidant effect, triggering the oxidation of lipoproteins (50) while limiting the bioavailability of nitric oxide in the arterial wall (51–54). The decreased bioavailability of nitric oxide promotes endothelial dysfunction, increases vascular tone and consequently arterial stiffness (54, 55). Interestingly, in our TAK patients, diastolic dysfunction worsened with the increase in uric acid levels. The positive correlation found between increased ASI, uric acid levels and the degree of diastolic dysfunction in TAK patients explains the LV remodeling process in these patients.

LAVi was higher in TAK patients than in atherosclerotic patients. In addition to pressure and volume overload (56), LAV increases in conditions with systemic inflammation such as autoimmune diseases (13, 57) or pneumonia (58). The potential prognostic value of LAV dimensions for cardiovascular risk in TAK patients is interesting but deserves further investigation.

Carotid intima/media thickness (IMT), a surrogate marker of cardiovascular risk factors and atherosclerotic cardiovascular disease (ASCVD), was equally increased in both groups; in TAK patients, IMT was inversely correlated with aortic wall thickness. In addition, in TAK patients but not in Controls, common carotid IMT had a positive significant correlation to ASCVD. This suggests that inflammatory burden in TAK patients may represent an independent risk factor for cardiovascular or atherosclerosis-related disease, similar to canonical risk factors in atherosclerotic patients.

According to other reports (59–61), a significant decrease in total lymphocytes in peripheral blood and considerable infiltration of $CD3^+$, $CD4^+$, and $CD8^+$ T cells in the arterial wall was observed in TAK patients during the active phase. The vessel wall also exhibited granulomatous infiltrates of $CD4^+$ T cells and macrophages, neovascularization, loss of smooth muscle cells in the tunica media with damage to elastic membrane lamellae and elastin fibers, and growth of a lumen-constricting neointima, which may explain the increased ASI in these patients. Further evidence for these data is that ASI was positively correlated with total peripheral lymphocyte count and total $CD3^+$ and $CD3^+CD4^+$ T-cell counts. While hypertension is associated with increased aortic stiffness independent of aortic wall thickness (21), we can therefore speculate that $CD3^+$, $CD4^+$, and $CD8^+$ T-cell infiltrates and the resulting fibrotic outcomes are behind the increased ASI and aortic wall thickness in our TAK patients. To our knowledge, these findings described in TAK patients. Previous reports in giant cell arteritis did not show any correlation between peripheral T lymphocytes and arterial stiffness, evaluated with pulse wave velocity (PWV) (62). Conversely, in other vascular diseases, a role of T lymphocytes in vascular stiffness has been suggested by the relationship with increased PWV values (63).

Inflammation of vessel walls, mechanical or immune-mediated injury, and ischemia/reperfusion of tissues lead to the release of HMGB1 (High Mobility Group Box 1), a small nuclear protein that promotes DNA bending and preferential assembly of transcription factors at specific DNA domains (34, 64). When HMGB1 is secreted by activated immune cells into the extracellular environment, it acts on all cell populations involved in vascular inflammation and functions as a candidate signaling protein for the transition from acute inflammation to self-sustaining chronic inflammation in large vessel vasculitis (34, 64). Injured endothelial cells release HMGB1 (65) and attract endothelial cell progenitors that promote neovascularization (66). In addition, HMGB1 activates dendritic cells and promotes their functional maturation and responsiveness to chemokines and regulates cell migration (64). HMGB1 also attracts myocyte precursors and vessel-associated stem cells (67), which are required for intimal hyperplasia/angiogenesis typical of vessel remodeling in large vessel vasculitides (68). The significant increase in HMGB1 immunostaining in the arterial wall of our TAK patients and the direct correlation between HMGB1 and infiltration of $CD15^+$ and $CD4^+$ cells support the existence of a vicious circle that enhances autocrine/paracrine release of HMGB1 (25, 34, 65, 69) and alters vascular morphology and hemodynamic balance in TAK patients.

Compared with atherosclerotic patients, TAK patients had a high frequency of $CD3^+CD4^+$IL-17^+ cells, indicating increased IL-17 expression, and lower levels of $CD4^+$FoxP3^+Treg cells in peripheral blood. Moreover, with respect to TAK patients receiving standard steroid therapy alone, infliximab co-treated patients showed significant improvement in clinical symptoms, allowing steroids to be discontinued more quickly. When these patients were assessed at T18 of combined therapy with infliximab, $CD3^+CD4^+$IL-17^+, $CD4^+$, and FoxP3^+Treg cells substantially overlapped levels found in atherosclerotic patients. These results suggest that biologic therapy is highly effective compared with standard treatment in both improving clinical symptoms and controlling disease pathophysiology, as demonstrated by the more rapid de-escalation of daily prednisone dose and normalization of Tregs and Th17 cell frequency, and that infliximab facilitates immunologic balance in TAK patients as in atherosclerotic patients. Although these results are still preliminary and limited, they may serve as first evidence for future, statistically powered studies to examine the impact of biologic treatments on clinical outcomes in larger

groups of TAK patients. In particular, it will be important to determine whether infliximab treatment can slow down, or even prevent the cardiovascular impairments associated with worsening of inflammatory status in TAK patients. This study has some limitations. Firstly, it was a single center study with small sample size, warranting future statistically powered studies in order to evaluate, in a proper prospective analysis, the impact on clinical outcome in terms of survival. All echocardiographic analyses could be significantly influenced by the quality of ultrasound images. Due to the disease studied, the hemodynamic state of right heart was evaluated only with ultrasound and no data on right heart catheterization were available. Additionally, all patients with comorbidities such as atrial fibrillation or lung disease were excluded, reducing the potential generalization of our results. Finally, the majority of TAK patients were under steroid treatment while Controls received anti-hypertensive treatment, and these two strategies may have different impact on vascular compliance. However, to our knowledge, there are no previous data analyzing cardiovascular risk in patients affected by TAK or the role of immune cells in cardiovascular risk in these patients.

CONCLUSIONS

Overall, our results suggest that the severity of large vessel damage is higher in TAK patients than in Controls. The histological data suggest different pathogenic mechanisms underlying TAK or atherosclerosis. Interestingly, the increased ASCVD risk in TAK patients seems to be directly related to infiltration of the vessel wall by inflammatory cells, and damage-associated molecular patterns may play a key role in this mechanism. This may suggest that in systemic inflammation and vasculitis, optimal disease control may also reduce the residual risk of cardiovascular disease. As shown by our preliminary results on infliximab treatment, this drug seems to be particularly effective in restoring the normal frequency of Tregs and Th17 cells in TAK patients. On this basis, assessment of Tregs and Th17 populations could serve as a potential biomarker to monitor treatment efficacy and as a novel therapeutic target to reduce cardiovascular risk in TAK patients.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of the University of Bari Medical School (Code number 06R76Y9-1). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

SC and VD contributed to the conception. SC, VD, CS, and GC contributed to design of the work. SC, VD, ACR, GC, SN, CS, AL, IS, and MP contributed to the acquisition of data. SC, VD, and AV contributed to data analysis and drafted the work. SC, VD, CS, AV, ACM, MF, and GI contributed to interpretation of data for the work. AS, GB, CC, LR, RR, and MM revised the manuscript critically for important intellectual content. All authors contributed to manuscript revision, read, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2022.863150/full#supplementary-material

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Endothelial Dysfunction in Psoriasis: An Updated Review

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Although psoriasis is predominantly a chronic inflammatory skin disorder, epidemiological data provide a solid link between psoriasis, especially in its more severe forms, and increased risk for cardiovascular morbidity and mortality. Apart from the increased prevalence of traditional cardiovascular risk factors, chronic inflammation appears to act synergistically with the underlying process of endothelial dysfunction toward the development of accelerated atherosclerosis, subclinical vascular injury and subsequently, clinically evident cardiovascular manifestations. Endothelial dysfunction is regarded as an early precursor of atherosclerosis with a predictive value for the development of future cardiovascular events. A thorough understanding of the mechanisms of endothelial dysfunction in psoriasis might pave the path for the development of more accurate cardiovascular risk prediction tools and possible therapeutic targets aiming to alleviate the increased cardiovascular burden associated with the disease. The present review summarizes the available evidence about the role of chronic inflammation and other important pathophysiological mechanisms involved in the development of endothelial dysfunction in psoriasis. An overview of studies implementing the most widely applied circulating and vascular biomarkers of endothelial dysfunction in psoriasis patients will be provided, and the impact of systemic psoriasis treatments on endothelial dysfunction and patients' cardiovascular risk will be discussed.

Keywords: endothelial dysfunction, psoriasis, cardiovascular risk, atherosclerosis, circulating biomarkers, vascular biomarkers

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INTRODUCTION

Psoriasis is a chronic inflammatory skin disease with a reported prevalence of approximately 2% in Europe and North America, triggered and preserved by dysregulated interactions between the innate and adaptive components of the immune system (1, 2). However, cutaneous manifestations are not the only clinical demonstration of the disease. Accelerated rates of cardiovascular complications and major adverse cardiovascular events have been consistently observed in patients with psoriasis (3). Although these patients are often characterized by an unfavorable cardiometabolic profile, increased cardiovascular risk in psoriasis appears to be directly associated with the disease *per se* (1, 2). Pathophysiologically, central to the inflammatory hypothesis of

atherosclerosis is the mutual interplay between inflammation and endothelial dysfunction, as has been described in other chronic inflammatory autoimmune disorders (4). Their synergism triggers and augments the sequence of accelerated atherosclerosis, subclinical target organ damage and eventually, clinically evident cardiovascular manifestations (4, 5).

Keeping in mind that endothelial dysfunction is considered as the earliest precursor of cardiovascular disease (CVD) (6), a thorough understanding of this process in psoriasis might commence interventions for the early identification and more effective monitoring of high-risk individuals. Therefore, the present mini review aims to provide an overall assessment of endothelial dysfunction in psoriasis, with emphasis placed on mechanisms that promote endothelial dysfunction and subsequently lead to the increased cardiovascular risk associated with the disease. Studies providing evidence of endothelial dysfunction using established as well as novel markers of endothelial dysfunction in psoriasis will be discussed. Lastly, data regarding effects of psoriasis-specific systemic treatments on endothelial dysfunction will be provided. To this end, a PubMed search was performed to identify relevant articles published in English, using the following medical terms: "psoriasis," "endothelial dysfunction," "cardiovascular," and "atherosclerosis."

CARDIOVASCULAR MORBIDITY AND COMORBIDITY IN PSORIASIS

Psoriasis is associated with multiple cardiometabolic diseases, which are divided into cardiovascular comorbidities and major adverse cardiovascular events (coronary heart disease, ischemic heart disease, myocardial infarction, congestive heart failure, and stroke) all of which contribute to increased cardiovascular morbidity and mortality (7, 8). The strength of this association appears to vary according to psoriasis disease severity (9). **Table 1** summarizes relevant systematic reviews and meta-analyses, all of them published within the past decade, supporting the association of psoriasis with conventional cardiovascular risk factors and adverse cardiovascular events.

One crucial point is whether patients with psoriasis have an increased risk of cardiovascular events independently of -versus mediated by- their less favorable risk factor profile. At present, there is evidence for both directions. Prevalence of traditional cardiovascular risk factors is increased among patients with psoriasis compared to non-psoriasis individuals, such as ischemic heart disease, peripheral vascular disease, diabetes, hypertension, dyslipidemia, obesity and metabolic syndrome (8). In addition, several systematic reviews and meta-analyses have established the association of psoriasis with increased rates of atherosclerotic CVD. In a recent meta-analysis by Raaby et al., psoriatic patients, especially those with severe psoriasis, had an increased risk of CVDs (stroke, myocardial infarction, cardiovascular death) (10). Consistent with the above, Dhana et al. showed increased all-cause and cardiovascular mortality risk in psoriasis patients, especially those with severe psoriasis, compared to those without (11). Nevertheless, those with mild disease did not present an increased risk of cardiovascular mortality (11). More recently, increased risk of coronary artery disease was demonstrated in psoriasis by Kaiser et al., assessed by computed tomography and coronary calcium score (CCS) (12). Consequently, available epidemiological data provide solid evidence for a strong link between psoriasis and atherosclerotic CVD, which is at least partially mediated by their aggravated cardiovascular risk profile. Further experimental and clinical studies have attempted to shed light on the primary underlying pathophysiological processes eventually resulting in the establishment of clinically overt CVD independently of traditional cardiovascular factors, as described below.

PATHOPHYSIOLOGY OF ENDOTHELIAL DYSFUNCTION IN PATIENTS WITH PSORIASIS: THE ROLE OF CHRONIC INFLAMMATION

Endothelial dysfunction is defined as loss of vascular dilation in response to biological and mechanical stimuli owing to the pathologic transition of the endothelium into a non-adaptive state secondary to decreased nitric oxide (NO) bioavailability (13). It is considered as a key step in the initiation and progression of atherosclerosis, the development of which involves complex interactions between the endothelium, circulating lipids, platelets, and the immune system (14–19). Several factors, including circulating proinflammatory cytokines, reactive oxygen species, oxidized LDL-C, autoantibodies and traditional cardiovascular risk factors directly and indirectly activate endothelial cells and impair their function, resulting in impaired vascular relaxation, increased leukocyte adhesion, increased endothelial permeability and generation of a pro-thrombotic state (15, 20). Importantly, all of these factors are dysregulated in psoriasis. Due to activation of immune-mediated mechanisms, the vascular endothelium presents a pro-inflammatory phenotype in psoriasis with upregulation of chemotactic, proatherogenic and vascular adhesion molecules, including tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), IL-6 and the IL-17 family of cytokines, interferon, and vascular cell adhesion molecule 1 (VCAM1). The downstream consequences result in vascular arterial inflammation and direct cytokine-induced injury (20, 21). Mechanisms of endothelial dysfunction in psoriasis are presented in **Figure 1**.

As the atherosclerotic procedure is being increasingly recognized as an inflammatory process, mechanisms of accelerated atherosclerosis in chronic autoimmune diseases have become a topic of growing interest (22). Pathogenetic mechanisms of CVD in psoriasis appear to be complex and have not yet been fully elucidated. However, psoriasis-triggered pathological pathways, in particular endothelial dysfunction, may activate or augment pre-existing atherosclerosis, which subsequently results in the development of clinically overt CVD (22–24).

Furthermore, chronic inflammation alters lipoproteins structurally and functionally in ways that cannot be captured

TABLE 1 | A synopsis of systematic reviews and meta-analyses investigating the association of psoriasis with conventional cardiovascular risk factors and adverse cardiovascular events.

References	Type of study	Cardiovascular comorbidities	Study population	Key findings
Kaiser et al. (12)	Systematic review and meta-analysis	CAD	14 eligible studies: 1,427 patients with psoriasis and 9,670 controls	Patients with psoriasis (RR = 1.14, 95% CI: 1.04–1.26; $p = 0.004$). For more severe CAD (CCS > 100) the risk was further increased (RR = 1.71, 95% CI: 1.28–2.30; $p < 0.001$)
Dhana et al. (11)	Systematic review and meta-analysis	Cardiovascular mortality	12 eligible studies: 5 studies including 285,675 psoriasis patients, 3 studies including 188,223 patients with mild psoriasis and 4 studies including 17,317 patients with severe psoriasis	Pooled RR = 1.15 (95% CI: 1.09–1.21, $I^2 = 65.9\%$, $P = 0.02$) in patients with psoriasis. Pooled RR = 1.05 (95% CI: 0.92–1.20, $I^2 = 90.3\%$, $P < 0.001$) for mild psoriasis. Pooled RR = 1.38 (95% CI: 1.09–1.74, $I^2 = 91.0\%$, $P < 0.001$) for severe psoriasis
Raaby et al. (10)	Systematic review and meta-analysis	Stroke MI	13 high-quality observational studies	Risk of stroke (HR = 1.10, 95% CI: 1.0–1.19) and risk of MI (HR = 1.20, 95% CI: 1.06–1.35), in patients with mild psoriasis. The risks of both stroke (HR = 1.38, 95% CI: 1.20–1.60), MI (HR = 1.70, 95% CI: 1.18–2.43) and cardiovascular death (HR = 1.37, 95% CI: 1.13–1.67) were increased in patients with severe psoriasis
Pietrzak et al. (22)	Review and meta-analysis	Cardiovascular events	Four case–control and 10 cohort studies.	Elevated risk for CV events in psoriasis patients compared with non-psoriasis controls (OR = 1.28; 95% CI: 1.18–1.38)
Armstrong et al. (97)	Systematic review and meta-analysis	Stroke MI	Nine eligible studies were included representing a total of 201.239 patients with mild and 17.415 patients with severe psoriasis	Risk of MI (RR = 1.29; 95% CI: 1.02–1.63) and stroke (RR = 1.12; 95% CI: 1.08–1.16) in mild psoriasis. Significantly increased risk of cardiovascular mortality (RR = 1.39; 95% CI: 1.11–1.74), MI (RR = 1.70; 95% CI: 1.32–2.18), and stroke (RR = 1.56 95% CI: 1.32–1.84) in severe psoriasis
Samarasekera et al. (98)	Systematic review and meta-analysis	Cardiovascular Disease MI Stroke	Of the 14 included studies, 10 were population-based cohorts, and sample sizes in the psoriasis group ranged from 462 to 130.976	RR relative to the general population was 1.37 (95% CI: 1.17–1.60) for CVD mortality, 3.04 (95% CI: 0.65–14.35) for MI, and 1.59 (95% CI: 1.34–1.89) for stroke
Miller et al. (8)	Meta-analysis	Cardiovascular disease Ischemic heart disease Vascular disease Atherosclerosis Cerebrovascular disease Cardiovascular mortality Diabetes Hypertension Dyslipidemia Obesity Metabolic syndrome	75 studies including up to 503.686 cases and 29.686.694 controls	Cardiovascular disease in total (OR = 1.4; 95% CI: 1.2–1.7), ischemic heart disease (OR = 1.5; 95% CI: 1.2–1.9), peripheral vascular disease (OR = 1.5; 95% CI: 1.2–1.8), and atherosclerosis (OR = 1.1; 95% CI: 1.1–1.2), cerebrovascular disease (OR = 1.1; 95% CI: 0.9–1.3) and cardiovascular mortality (OR = 0.9; 95% CI: 0.4–2.2). Diabetes (OR = 1.9 95% CI: 1.5–2.5), hypertension (OR = 1.8 95% CI: 1.6–2.0), dyslipidemia (OR = 1.5 95% CI: 1.4–1.7), obesity by body mass index (OR = 1.8 95% CI: 1.4–2.2), obesity by abdominal fat (OR = 1.6; 95% CI: 1.2–2.3), and the metabolic syndrome (OR = 1.8; 95% CI: 1.2–2.8)
Miller et al. (99)	Meta-analysis	Total cholesterol LDL Triglyceride Systolic blood pressure Diastolic blood pressure BMI Waist circumference Fasting glucose Non-fasting glucose HbA1c	59 studies with up to 18.666 cases and 50.724 controls	Psoriasis cases had a higher total cholesterol WMD = 8.83 mg dL^{-1}, 95% CI: 2.94–14.72, $P = 0.003$. Higher LDL WMD = 9.90 mg dL^{-1}, 95% CI: 1.56–18.20, $P = 0.020$. Higher triglyceride WMD = 16.32 mg dL^{-1}, 95% CI: 12.02–20.63, $P < 0.001$. Higher systolic blood pressure (WMD = 4.77 mmHg, 95% CI: 1.62–7.92, $P = 0.003$). Higher diastolic blood pressure (WMD = 2.99 mmHg, 95% CI: 0.60–5.38, $P = 0.014$). Higher BMI (WMD = 0.73 kg m^{-2}, 95% CI: 0.37–1.09, $P < 0.001$). Higher waist circumference (WMD = 3.61 cm, 95% CI: 2.12–5.10, $P < 0.001$). Higher fasting glucose (WMD = 3.52 mg dL^{-1}, 95% CI: 0.64–6.41, $P = 0.017$). Higher non-fasting glucose (11.70 mg dL^{-1}, 95% CI: 11.24–12.15, $P < 0.001$) (=0.65 mmol L^{-1} and a Higher HbA1c 1.09 mmol mol^{-1}, 95% CI: 0.87–1.31, $P < 0.001$)
Gaeta et al. (100)	Meta-regression analysis	MI Vascular disease Overall mortality Overall Cardiovascular Risk	13 studies. 1.684.032 person-year became available in the psoriasis group and 43.146.770 person-year in the control group	Patients with psoriasis showed an increase of the overall cardiovascular risk compared to the control group (RR = 1.24 [CI: 1.18–1.31]; $P < 0.00001$). Significantly higher risk of infarction (RR = 1.24 [1.11–1.39]; $P < 0.00001$), vascular disease (RR = 1.27 [1.12–1.43]; $P < 0.00001$) and overall mortality (RR = 1.41 [0.97–2.04]; $P < 0.00001$)

(Continued)

TABLE 1 | (Continued)

References	Type of study	Cardiovascular comorbidities	Study population	Key findings
Gu et al. (101)	Meta-analysis	Stroke MI Cardiovascular disease Coronary heart disease Peripheral vascular disease Cardiovascular mortality	15 cohort studies	Risk of stroke (RR = 1.26; 95% CI: 1.12–1.41; $p < 0.0001$). Risk of MI (RR = 1.32; 95% CI: 1.13–1.55; $p = 0.001$). Cardiovascular disease (RR = 1.47; 95% CI: 1.30–1.6; $p = 0.0001$). Combined RRs = 1.39 (95% CI: 1.03–1.86; $p = 0.03$) for coronary heart disease, 1.55 (95% CI: 1.02–2.34; $p = 0.04$) for peripheral vascular disease, and 1.33 (95% CI: 1.00–1.77; $p = 0.05$) for cardiovascular mortality
Xu and Zhang (102)	Meta-analysis	Stroke MI	Seven cohort studies	Psoriasis significantly increases the risk of stroke (RR = 1.21; 95% CI: 1.04–1.4) and MI (RR = 1.22; 95% CI: 1.05–1.42) separately. Substantial evidence of heterogeneity was also observed in both subgroup analyses ($P < 0.001$, $I^2 = 86.8\%$ and $P < 0.001$, $I^2 = 83.1\%$).

BMI, body mass index; CAD, coronary artery disease; CCS, coronary calcium score; CI, confidence intervals; LDL, low density lipoprotein; MI, myocardial infarction; OR, odds ratio; RR, rate ratio; WMD, weighted mean difference.

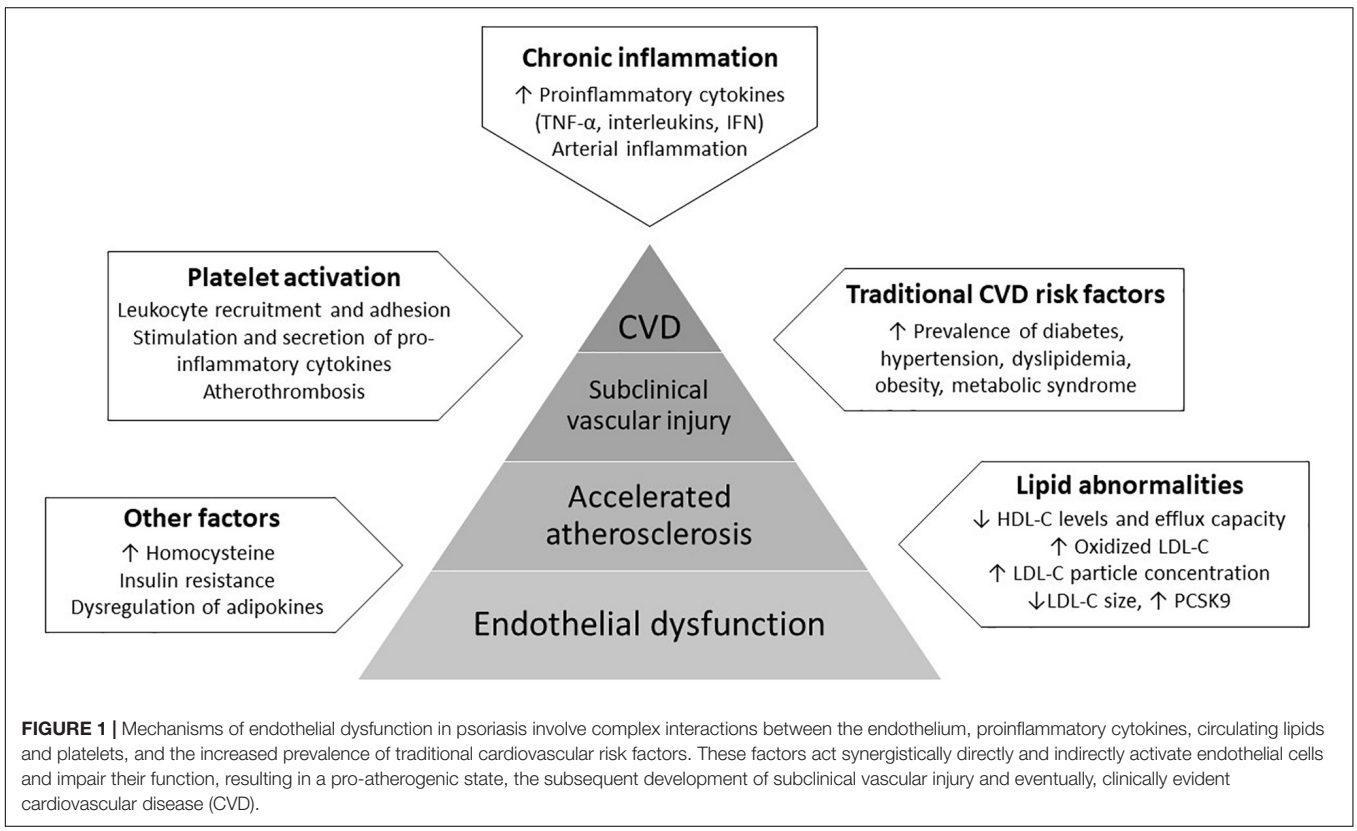

FIGURE 1 | Mechanisms of endothelial dysfunction in psoriasis involve complex interactions between the endothelium, proinflammatory cytokines, circulating lipids and platelets, and the increased prevalence of traditional cardiovascular risk factors. These factors act synergistically directly and indirectly activate endothelial cells and impair their function, resulting in a pro-atherogenic state, the subsequent development of subclinical vascular injury and eventually, clinically evident cardiovascular disease (CVD).

through standard lipid measurements (25). Inflammation drives modification of LDL-C into small, dense particles that are known to exert pro-atherogenic effects (25). Dyslipidemia is independently associated with endothelial dysfunction, while (26, 27) lipoproteins are implicated in the generation of oxidative stress (26, 28). Oxidized LDL-C modulates NO availability through reduction of eNOS activity or by enhanced metabolism of NO by asymmetric dimethylarginine (ADMA), an endogenous competitive eNOS inhibitor (27, 29, 30). Abnormalities in lipid profile are common in psoriasis and considered to contribute substantially to endothelial dysfunction (31). Patients with psoriasis present reduced levels and efflux capacity of HDL-C, increased LDL-C particle concentration and decreased LDL-C size, as well as elevated levels of circulating PCSK9 when compared to non-psoriasis individuals (31–33).

Platelet activation is regarded as another potent mediator of endothelial dysfunction in psoriasis (34). Apart from their contribution to psoriatic skin lesions, platelets are key regulators of inflammation, immune function, and atherothrombosis. Activated platelets contribute substantially to psoriasis associated inflammation by stimulation and secretion of pro-inflammatory cytokines (18, 35). They perpetuate leukocyte recruitment,

adhesion, and rolling along the activated endothelium, thereby promoting atherosclerosis and inducing endothelial dysfunction (36). Patients with psoriasis present increased circulating biomarkers of platelet activation, such as platelet-derived microvesicles, soluble p-selectin, platelet-lymphocyte and platelet-neutrophil aggregates (37). Platelets derived from psoriasis patients augment endothelial cell activation with up to a 20-fold increase in endothelial-derived cytokines such as IL-1β and IL-8 (35). In psoriasis, platelets promote IL-17 secretion from CD4+ lymphocytes, and induce *in vitro* endothelial injury and apoptosis when co-localized with neutrophil subtype granulocytes through the formation of neutrophil extracellular traps, a process known as NETosis (38, 39).

Lastly, although less established, other characteristic features of patients with psoriasis may contribute to the development of endothelial dysfunction. For instance, homocysteine levels are elevated in psoriasis and correlate with the severity of the disease (27). Increased homocysteine promotes oxidative stress and has been associated with the development of atherosclerosis and CVD (40, 41). In addition, insulin resistance is common in psoriasis. As a vasoactive hormone, insulin promotes vasodilation in a NO-dependent manner, increases vasodilation and presents anti-inflammatory effects (42). Likewise, adipokines appear dysregulated in patients with psoriasis, as a result of obesity which is a common comorbidity, but also independently, as they modulate cutaneous inflammation with a probable pathogenetic role in psoriasis (16). Pro-inflammatory adipokines in psoriasis may be released in the peripheral blood as a result of adipose tissue inflammation (16). Elevated adiponectin levels suppress inflammation and immune responses, whereas low adiponectin levels (including leptin, adiponectin, and resistin) are associated with endothelial dysfunction and the development of the metabolic syndrome or CVDs (16).

EVALUATION OF ENDOTHELIAL DYSFUNCTION IN PSORIASIS

Considering the pathophysiological and clinical significance of endothelial dysfunction, several studies have attempted to quantify the burden of endothelial dysfunction in psoriasis, using both circulating and vascular biomarkers.

Circulating Biomarkers of Endothelial Dysfunction in Psoriasis

Asymmetrical dimethylarginine (ADMA), oxidized LDL, endothelial progenitor cells (EPCs), endothelial glycocalyx and endothelial microvesicles (EMVs) represent reliable circulating biomarkers of endothelial dysfunction but are currently applied only as research tools (6).

Psoriasis patients present elevated levels of ADMA, a potent eNOS inhibitor of the L-arginine-NO pathway, that correlate with disease severity, suggesting an important role of endothelial dysfunction in the pathogenesis of psoriasis (27). By contrast, ADMA was not increased in a smaller study of mild-to-moderate plaque-type psoriatic patients with low-to-medium grade systemic inflammation (43). Few studies have examined oxidized LDL as a marker of endothelial dysfunction in psoriasis. In a large study of 252 psoriasis patients and controls, psoriasis subjects presented increased levels of lipoprotein (a), oxidized lipoprotein (a) and oxidized HDL (44), although a smaller study of 79 patients with psoriasis and 80 controls failed to reveal any differences in the levels of oxidized LDL (45). Remarkably, oxidized LDL in the former study was significantly associated with non-calcified coronary plaque burden assessed by coronary computed tomographic angiography (44).

Expressed on the endothelial cell surface within blood vessels, the endothelial glycocalyx regulates blood vessel permeability and homeostasis. In a large study of 297 psoriatic patients and 150 controls, glycocalyx thickness in sublingual microvessels was reduced among patients, and correlated with disease activity, carotid atherosclerosis, impaired coronary flow reserve and markers of myocardial deformation assessed by speckle-tracking imaging (46). EPCs are the progenitor cells that are able to differentiate into functional endothelial cells, sustain vasculogenesis and promote vascular repair in ischemic diseases. Significantly reduced levels of circulating EPCs have been measured in psoriasis patients compared to controls, and an inverse correlation with disease severity was observed (47). Likewise, another study recruiting plaque-type psoriasis patients demonstrated decreased EPC levels, as well as an independent association with pulse wave velocity, a well-established marker of arterial stiffness (48). By contrast, these results were not confirmed in a more recent study (17). MicroRNA (miRNA) expression and especially circulating miR-200s were positively correlated with markers of cardiovascular dysfunction such as left ventricular mass (49).

Microvesicles are small vesicles (0.1–1 μm) released from plasma membrane as a result of cellular activation or apoptosis. EMVs display multivalent important biological properties and contribute to vascular homeostasis (50). Their levels increase substantially in patients with CVDs such as hypertension, diabetes mellitus, acute and chronic coronary artery disease (51–53), but also in patients with chronic autoimmune inflammatory diseases (54). Increased levels of EMVs have been found in patients with psoriasis (14, 55). Notably, increased EMVs concentrations in psoriasis were observed beyond cardiometabolic risk factors (55). Another study showed higher ratio of EMVs/EPCs in psoriasis patients, which independently correlated with higher carotid intima-media thickness, an established marker of subclinical atherosclerosis (19, 56). These findings suggest that increased EMVs in psoriasis might not simply represent a consequence of endothelial cell activation, but may also have a role in psoriasis pathophysiology leading to accelerated atherosclerosis.

Vascular Markers of Endothelial Dysfunction in Psoriasis

The most widely applied, non-invasive vascular methods for the functional assessment of endothelial dysfunction include laser Doppler flowmetry and imaging (LDF/LDI), and the gold-standard flow-mediated dilation (FMD) of the brachial artery, which is currently considered as the gold-standard non-invasive

method, with a predictive value for future cardiovascular events especially in high-risk populations (57). Several studies have assessed endothelial dysfunction in psoriasis with FMD, summarized in a recent meta-analysis demonstrating lower FMD measurements among patients compared with controls (56). Although laser Doppler techniques have been mainly applied for the assessment of vascular perfusion within plaques rather than the evaluation of systemic microcirculation in psoriasis (58), NO-dependent vasodilation was attenuated in psoriasis patients in a small study using LDF, and correlated with the degree of psoriatic symptomatology (59). Other vascular methods that focus on the evaluation of endothelial function, such as quantitative coronary angiography and positron emission tomography (PET), are compromised by significant limitations including radiation, reproducibility and cost. However, a recent meta-analysis of 1,427 patients with psoriasis without prior coronary artery disease and 9,670 controls showed higher prevalence and burden of coronary artery disease among patients, detected by CCS with or without cardiac computed tomography angiography (12). Using ^{18}F-fluorodeoxyglucose positron emission tomography imaging, a randomized placebo-controlled pilot study showed higher vascular inflammation in ascending aorta of patients with moderate-to-severe psoriasis as compared to controls (60). Similarly, arterial inflammation was more pronounced in patients with mild psoriasis compared to controls by use of the same method (61).

Collectively, available data regarding the above circulating and vascular biomarkers appear in line with the hypothesis that endothelial dysfunction is implicated in the development of accelerated atherosclerosis in psoriasis. Further extending this notion, the potential improvement of endothelial function following successful control of psoriasis-related inflammation has been the subject of several studies over the past years.

THE IMPACT OF PHARMACOLOGICAL INTERVENTIONS ON ENDOTHELIAL DYSFUNCTION IN PSORIASIS

To date, topical therapies are the cornerstone for managing mild psoriasis which typically covers less than 5% body surface area (62). However, patients with moderate and severe disease, who are presumably at higher cardiovascular risk based on the above, are candidates for newer systemic therapies as first-line treatment (63, 64). The observation that these therapies could target the accompanying vascular dysfunction and ameliorate biomarkers of cardiovascular risk (65), has several therapeutic implications for cardiovascular risk prevention in psoriasis that are currently under vigorous investigation.

Tumor Necrosis Factor-α Inhibitors

Tumor necrosis factor-α inhibitors (adalimumab, certolizumab, etanercept, or infliximab) showed a protective cardiovascular profile in multiple, mainly observational studies of psoriasis. It has been hypothesized that this action is probably mediated by their beneficial effect on endothelial cell function (66). There is at present no strong, definite evidence for a significant beneficial effect of anti-TNF-α biologics on endothelial function in psoriasis (66, 67), although some promising data do exist. TNF-α blockade has led to improvement of vascular function assessed through resting endothelium-dependent vascular tone by low-flow-mediated constriction, yet FMD values remained unchanged (68). In this context, treatment with TNF-a inhibitors was associated with significant reductions in endothelial and platelet microvesicles levels (69). A small cohort study detected a significant improvement of endothelial dysfunction markers (serum intercellular adhesion molecule 1 and FMD) after a short-period treatment with adalimumab (70). Adalimumab has shown anti-inflammatory effects and improved FMD in patients with psoriasis (71). Conversely, a recent RCT study, that used 18F-FDG PET-CT to assess vascular inflammation, did not detect any superiority of adalimumab over phototherapy or placebo (72). In a recent meta-analysis of RCTs, there was no beneficial effect on imaging biomarkers (aortic vascular inflammation or FMD) of cardiovascular risk in patients exposed to adalimumab (73). Treatment with etanercept increased the EPC count in a small double blind, placebo-controlled, cross-over study, indicating improved endogenous endothelial regenerative capacity, but brachial artery flow-mediated and nitroglycerin-mediated dilation was not modified with treatment (74).

IL-17 and IL-23 Inhibitors

IL-17 inhibitors target either the IL-17 ligand (secukinumab, ixekizumab, and bimekizumab) or its receptor (brodalumab). Secukinumab did not show any clinically significant effect on aortic vascular inflammation (assessed by 18F-FDG PET/CT) in a placebo-controlled RCT either on short- or long-term follow-up (75). Nevertheless, the CARIMA (Evaluation of Cardiovascular Risk Markers in Psoriasis Patients Treated with Secukinumab) trial indicated the protective role of secukinumab on endothelial cell function measured with FMD (76). Recently, the potential benefit of a reduced dose interval has been examined in heavier patients and in those with suboptimal responses (77, 78). Ustekinumab inhibits both IL-12 and IL-23 by targeting their shared p40 subunit. Positive effects of ustekinumab impact on vascular inflammation were observed using 18F-FDG PET/CT (79). However, levels of circulating endothelial- and platelet-derived microvesicles remained unchanged in patients with psoriasis successfully treated with anti-IL-12/23, regardless of clinical improvement (80). In a recent meta-analysis of RCTs, ustekinumab, yet not secukinumab, induced short-term reductions in aortic vascular inflammation, whereas FMD remained unchanged. Nevertheless, these reductions were not sustained in the long-term (73). The hypothesis of cardiovascular risk reduction in psoriasis patients receiving ustekinumab (81) was further questioned by the observation of no substantially different risk of major adverse cardiovascular events among TNF-a inhibitors, ustekinumab or placebo therapy in several studies (82–85). The effect of newer IL-23 and other cytokines inhibitors recently approved (Guselkumab, Tildrakizumab, and Risankizumab) on endothelial dysfunction remains to be investigated (86–92).

Oral Systemic Treatments

In a prospective longitudinal pilot study, systemic therapy with fumaric acid esters resulted in an improvement of endothelial vasodilator function assessed by venous occlusion plethysmography (93). Methotrexate, apremilast, acitretin, and cyclosporine are oral available treatment options for psoriasis. Nevertheless, evidence regarding protective effects on cardiovascular disease in psoriasis exists mainly for methotrexate (94, 95), which has shown neutral short-term effects on endothelial function in patients with psoriasis (96). Further studies are warranted to determine the effects of these treatment modalities on endothelial dysfunction in patients with psoriasis.

CONCLUSION AND FUTURE PERSPECTIVES

Psoriasis, especially in its severe forms, is an independent risk factor for cardiovascular morbidity and mortality. Several psoriasis-induced mechanisms promote the development of endothelial dysfunction, which acts synergistically with chronic inflammation and activates or potentiates pre-existing atherosclerosis in psoriasis. Available human studies using the most widely applied circulating and vascular biomarkers of endothelial dysfunction provide clinical evidence of endothelial dysfunction in patients with psoriasis, that correlates with disease severity. However, the clinical utility of biomarkers of endothelial dysfunction in psoriasis patients in terms of cardiovascular risk prediction needs to be addressed further. Moreover, it is hypothesized that effective control of the disease might improve endothelial cell function and subsequently modulate cardiovascular risk. Although some encouraging data have been published, large, prospective, appropriately designed studies are urgently warranted to provide strong evidence regarding the possibility of sustained beneficial effects on endothelial function of such therapies in psoriasis. Last but not least, future studies need to investigate whether interventions specifically targeting at the improvement of endothelial function in patients with psoriasis might provide incremental benefits in the modulation of both chronic inflammation and risk of future CVD.

AUTHOR CONTRIBUTIONS

EG, EL, and AP contributed to the conception and design of the study. PA and AM performed the literature searching and wrote the first draft of the manuscript. KG and MG wrote the sections of the manuscript. KG prepared the table. PA designed the figure and edited the final draft of the manuscript. EG reviewed and supervised the final version of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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The loss of glycocalyx integrity impairs complement factor H binding and contributes to cyclosporine-induced endothelial cell injury

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Background: Calcineurin inhibitors (CNIs) are associated with nephrotoxicity, endothelial cell dysfunction, and thrombotic microangiopathy (TMA). Evolving evidence suggests an important role for complement dysregulation in the pathogenesis of CNI-induced TMA. However, the exact mechanism(s) of CNI-induced TMA remain(s) unknown.

Methods: Using blood outgrowth endothelial cells (BOECs) from healthy donors, we evaluated the effects of cyclosporine on endothelial cell integrity. Specifically, we determined complement activation (C3c and C9) and regulation (CD46, CD55, CD59, and complement factor H [CFH] deposition) as these occurred on the endothelial cell surface membrane and glycocalyx.

Results: We found that exposing the endothelium to cyclosporine resulted in a dose- and time-dependent enhancement of complement deposition and cytotoxicity. We, therefore, employed flow cytometry, Western blotting/CFH cofactor assays, and immunofluorescence imaging to determine the expression of complement regulators and the functional activity and localization of CFH. Notably, while cyclosporine led to the upregulation of complement regulators CD46, CD55, and CD59 on the endothelial cell surface, it also diminished the endothelial cell glycocalyx through the shedding of heparan sulfate side chains. The weakened endothelial cell glycocalyx resulted in decreased CFH surface binding and surface cofactor activity.

Conclusion: Our findings confirm a role for complement in cyclosporine-induced endothelial injury and suggest that decreased glycocalyx density, induced by cyclosporine, is a mechanism that leads to complement alternative pathway dysregulation *via* decreased CFH surface binding and cofactor activity. This mechanism may apply to other secondary TMAs—in which a role for complement has so far not been recognized—and provide a potential therapeutic target and an important marker for patients on calcineurin inhibitors.

KEYWORDS

thrombotic microangiopathy, calcineurin inhibitors, cyclosporine, endothelium, heparan sulfate, proteoglycans, complement, alternative pathway

Introduction

Thrombotic microangiopathies (TMAs) are defined by their common clinical features: microangiopathic hemolytic anemia (MAHA), non-immune thrombocytopenia, and end-organ injury (1–3). TMAs are systemic conditions with the potential for multi-organ involvement, including the kidneys, the brain, the gastrointestinal tract, the respiratory tract, and the skin. Crucial to the development of TMA is injury to the microvascular endothelium. Injuries to the endothelium post its activation lead to excessive platelet and neutrophil recruitment and eventually to thrombus formation, chronic inflammation, and organ failure (1, 4, 5). While complement cascades are critical to mounting appropriate immune responses, the regulation of their products is critical to maintaining host cell integrity, notably for the vascular endothelium. Indeed, the loss of complement regulation favors spontaneous complement activation, resulting in endothelial injury and the formation of (micro-)thrombi (5–7). Complement dysregulation is also increasingly recognized in the pathogenesis of TMAs and is found in patients with various forms of secondary comorbidities (i.e., TMA spectrum) (1, 8–11).

The alternative pathway (AP) of complement is constitutively active (spontaneous tick-over), resulting in a low, but constant, level of circulating C3b in the plasma, which can bind to either host cell or pathogen surfaces. Since C3b is free to coat and disrupt surfaces without distinction, there are regulatory mechanisms that tightly protect host cells from complement-mediated injury, including membrane-associated proteins like membrane cofactor protein (MCP/CD46), decay-accelerating factor (DAF/CD55), and protectin (CD59) as well as the secreted protein complement factor H (CFH), which circulates in human plasma at high (200–300 ug/mL) concentrations (8, 12, 13). The density and localization of these regulatory proteins represent one of the key principles of complement control and are critical to maintaining the integrity of self-surfaces such as the vascular endothelium. Genetic mutations in CD46 or CFH, as well as the expression of anti-CFH autoantibodies, result in excessive complement activation—in particular *via* the alternative pathway—and increase patient susceptibility to develop TMA *via* endothelial injury (14–19). A number of additional mutations in complement (modulator) genes, including C3 itself, complement factor B (CFB), factor I (CFI), and thrombomodulin (THBD/CD141), have also been linked to endothelial cell injury and TMAs. There is, however, variable penetrance described in patient families within a pedigree with complement mutations, implicating a contribution from the environment as being necessary to trigger TMA manifestations in a patient who is genetically susceptible ("multiple-hit" hypothesis) (1, 8, 15). Among the events that precede the onset of TMA, the most relevant are respiratory and gastrointestinal tract infections and pregnancy (16, 20). Secondary TMA can also occur post-transplant when it is associated with antibody-mediated rejection and immunosuppressive medications like calcineurin inhibitors (CNIs) (9, 21–24).

Calcineurin inhibitors (CNIs) such as cyclosporine and tacrolimus are highly effective immunosuppressive agents, which are widely used to prevent allograft rejection in solid organ and hematopoietic stem cell transplantation and to treat autoimmune disorders. Their use is also associated with adverse effects, such as hypertension, nephrotoxicity, vascular injury, and the development of CNI-induced arteriolopathy, which negatively impact patient and allograft survival (25–32). In addition, CNIs are known to trigger post-transplant TMA (28, 29, 31, 33). The possible cause for these adverse effects, in particular TMA, in endothelial injury associated with CNI use, secondary to vasoconstriction-associated ischemia, increased platelet aggregation, and the activation of prothrombotic factors (27).

Evolving evidence suggests an important role for complement dysregulation in the pathogenesis of CNI-induced microvascular endothelial cell injury, which is crucial for the development of TMA (34, 35). Recently, CNI-mediated endothelial injury—in particular in the glomerular capillaries—has been linked to complement activation *in vivo*, and a central role of the complement alternative pathway has been identified (34). The exact mechanism, by which CNI induces complement activation, however, remains poorly understood. Because cyclosporine use is associated with vascular injury, development of TMA, and nephrotoxicity, we examined whether cyclosporine exposure leads to complement-mediated endothelial cell injury and investigated the mechanism by which complement dysregulation is induced in an *in vitro* model utilizing blood outgrowth endothelial cells (BOECs).

Materials and methods

Patient samples

BOECs were isolated from the peripheral blood of two healthy adult volunteers. Normal human serum (NHS) was derived from three healthy adult volunteers.

Blood outgrowth endothelial cells

BOECs were isolated as previously described and cultured in endothelial cell growth medium (cEGM-2: Endothelial Basal Medium 2 [EBM-2] supplemented with growth factors [EGM-2 BulletKit]) (Lonza, Walkersville, MD), 10% fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO), and 1% antibiotic–antimycotic (Gibco, Invitrogen, Life Technologies, Carlsbad, CA) (36, 37). Cells were maintained at 37°C, in an environment with 5% CO_2. Passages 3–12 were used.

Cyclosporine treatment and complement fixation on endothelial cells

BOECs grown to confluence were exposed to cyclosporine 10, 20, 50, or 100 μg/ml in media for up to 24 h. Cyclosporine stock solution (Sandimmune IV, Novartis Pharmaceuticals Canada Inc., Dorval,

QC, 50 mg/ml) was kindly provided by the SickKids pharmacy. For experiments involving complement fixation on BOECs, cells were sequentially exposed to 50% NHS (1:1 in serum-free media [SFM]) for 30 min. In experiments utilizing an antibody specifically blocking the membrane-anchored complement regulator CD59 (BRIC229, IgG2b, International Blood Group Reference Laboratory, NHS Blood and Transplant, Bristol, UK), cells were incubated for 30 min with the blocking antibody (5 μg/ml) prior to exposure to 50% NHS (36, 38).

Detection of complement deposition on endothelial cells

C3b and C5b-9 deposition on BOEC surfaces were demonstrated by flow cytometry using a C3c antibody detecting the C3c portion of native C3 and C3b (C3c-FITC conjugated antibody, Abcam, ab4212, 1:50 dilution) and C9 (Complement Technologies Inc, TX, A226, 1:100 dilution). Cells were grown to confluence and exposed to cyclosporine treatment and complement fixation as described. Cells were washed with phosphate-buffered saline (PBS) and incubated with Fixable Viability Dye eFluor780 (eBioscience, San Diego, CA, 1:1,000 dilution reconstituted in PBS) at 4°C for 30 min. For flow cytometry, cells were harvested by scraping and washed with PBS before use (Supplementary material).

Assessment of Weibel–Palade body mobilization and von Willebrand factor release from endothelial cells

von Willebrand Factor release from BOECs was detected *via* immunofluorescence as described previously (36). BOECs treated with media for 24 h, followed by incubation with anti-CD59 blocking antibody for 30 min and 50% NHS/50% SFM for 30 min, were used as positive control and compared to cells kept in media (negative control). Cells were then washed and fixed with 2% paraformaldehyde and permeabilized with 0.2% Triton in PBS, followed by incubation with rabbit anti-VWF (Dako, Carpinteria, CA, A0082, 1:1,000) and goat anti-VE-cadherin (Santa Cruz Biotechnology, Dallas, TX, sc-6458, 1:250) for 4 h. Alexa Fluor 488- and Alexa Fluor 555-conjugated species-specific secondary antibodies were used at a dilution of 1:1,000. Nuclei of cells were stained with 0.12 μg/ml Hoechst stain (Thermo Fisher Scientific, Waltham, MA) for 5 min.

Characterization of membrane-anchored complement regulators

To determine the expression level of the membrane-anchored complement regulators MCP/CD46, decay-accelerating factor (DAF/CD55), and CD59 on BOECs, BOEC lysates were utilized for flow cytometry and Western blotting analysis (Supplementary material).

Detection of CFH binding to endothelial cell surfaces

The binding of CFH to BOEC surfaces was demonstrated by flow cytometry as described previously (39), using purified CFH (CSL Behring, Marburg, Germany) tagged with Alexa Fluor 488 succinimidyl ester (10 μg/mL, Life Technologies) for 1 h at room temperature before being dialyzed overnight in PBS. Cells exposed to 500 mU/mL neuraminidase (MilliporeSigma; N2876) were used as the positive control. Cells were washed two times with PBS and scraped off. Cells were then incubated with Fixable Viability Dye eFluor780 at 4°C for 30 min. They were then washed with PBS and resuspended in 100 μL PBS. Each sample was then incubated with 4 μg of Alexa Fluor 488-tagged CFH for 1 min, after which 500–1,000 μL of Attune focusing fluid (Thermo Fisher Scientific, 4449791) was added and assessed by flow cytometry (Supplementary material).

For immunofluorescence experiments, cells were cultured to a minimum of 80% confluency on collagenized coverslips and exposed to cyclosporine A as described. Cells exposed to 500 mU/mL Neuraminidase for 1 h and 0.5 U/mL Heparinase III (H8891-5UN, Sigma-Aldrich, St. Louis, MO) for 30 min were used as positive controls. Cells were washed and fixed with 4% paraformaldehyde, blocked for 1 h with 3% BSA, followed by incubation with goat anti-Factor H (1:100, Complement Technology Inc., TX; A237) and mouse anti-heparan sulfate (1:50, US Biological Life Sciences, Salem, MA; H1890) overnight at 4°C. Goat Alexa Fluor 488 and Mouse Alexa Fluor 555 secondary antibodies were used, respectively, at a dilution of 1:200 for 1 h at room temperature. The nuclei of the cells were stained with 0.12 μg/ml Hoechst stain (Thermo Fisher Scientific, Waltham, MA) for 5 min. Confocal microscopy was performed as detailed in Supplementary material, and total fluorescence intensity was measured using ImageJ software.

CFH surface cofactor activity assay

To determine CFH cofactor activity on BOEC surfaces, cells exposed to 500 mU/mL neuraminidase for 1 h (Millipore Sigma; N2876) were used as the positive control. Cofactor activity of surface-bound CFH was detected as previously described (40). Cells were incubated with 10 μg/ml CFH (CSL Behring, Marburg, Germany) at 37°C for 1 h, 10 μg/ml CFI (EMD Millipore Corp., MA, 341280) and with 3.3 μg/ml C3b (EMD Millipore Corp., MA, 204860). The supernatant was collected at baseline and various subsequent time points (up to 180 min), and the samples were transferred to a reduced sample buffer and separated by 10% SDS-PAGE. The appearance of C3b degradation fragments was detected by Western blotting (Figure 6). Primary goat anti-C3, 1:1,000 dilution (Complement Technology Inc., TX, A213) with corresponding secondary HRP-conjugated antibody at a dilution of 1:5,000 was used for detection.

Imaging of glycocalyx

To image the endothelial glycocalyx, an Alexa Fluor 594-conjugated wheat germ agglutinin (WGA, Thermo Fischer Scientific, W11262, dilution 2 μg/ml), anti-heparan sulfate antibody (Abcam,

Cambridge, UK, ab23418, 1:100), and peanut agglutinin (PNA, Vector Labs, Ontario, CA, FL-1071-5, 1:200) were used. Cells were cultured to confluence on coverslips and exposed to cyclosporine as described. Cells exposed to 500 mU/mL neuraminidase for 1 h were used as a positive control in WGA and PNA experiments. Cells exposed to 0.5 U/mL Heparinase III (H8891-5UN, Sigma-Aldrich, St. Louis, MO) for 30 min were used as a positive control in heparan sulfate experiments. Cells were incubated with Alexa Fluor 594-conjugated WGA for 5 min on ice and washed two times with ice-cold HBSS, and the coverslips were mounted in a Chamlide magnetic chamber (Life Cell Instrument, Seoul, Korea) and overlaid with media. Confocal microscopy was performed as detailed in Supplementary material, and total fluorescence intensity was measured using ImageJ software. For experiments using anti-heparan sulfate and PNA, cells were washed and fixed with 2% paraformaldehyde, followed by incubation with mouse anti-heparan sulfate (1:100) and anti-PNA (1:100) for 1 h. Alexa Fluor 488-conjugated species-specific secondary antibodies were used at a dilution of 1:1,000. Nuclei of cells were stained with 0.12 μg/ml Hoechst stain (Thermo Fisher Scientific, Waltham, MA) for 5 min.

Statistics

Figures were generated with GraphPad Prism (Version 6.0c; GraphPad Software, La Jolla, CA) and displayed as the mean and standard deviation. Statistical analysis was performed *via* paired *t*-test or two-way ANOVA with *post-hoc* analysis. A $p < 0.05$ was considered statistically significant. In the figure legends, *p*-values are presented as follows: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$.

Results

Cyclosporine causes endothelial cell injury and complement deposition

The use of cyclosporine is associated with a vascular injury in pathophysiological situations. We, therefore, tested whether cyclosporine treatment of cultured BOECs caused endothelial cell toxicity using an established lactate dehydrogenase (LDH) assay for lytic cell death. We found that cyclosporine caused cytotoxicity of BOEC cultures in a dose- and time-dependent fashion (Supplementary Figure S1). Specifically, the acute (1 h) treatment of BOECs with low concentrations (<50 μg/mL) of cyclosporine did not cause cell lysis, while a 24-h treatment of the cells with cyclosporine used above 250 μg/mL led to lysis of nearly the entire culture. Intermediate concentrations of cyclosporine (50 μg/mL) caused ∼60% of the cells to rupture, and lower concentrations of 10 μg/ml did not lead to any detectable LDH release (Supplementary Figure S1). We, therefore, chose to treat BOECs within the range of 10 μg/mL (non-lethal) and 50 μg/mL (∼half-maximal lysis) concentrations of cyclosporine in subsequent experiments.

To that end, confluent monolayers of BOECs were treated with these concentrations of cyclosporine in medium containing 10% fetal bovine serum (FBS) for 24 h and subsequently exposed to 50% NHS in serum-free medium (SFM) for 30 min as established in Supplementary Figure S1. Under these conditions, we found that the treatment of BOECs with 50 μg/ml of cyclosporine caused a significant increase in complement C3 deposition (Figure 1A, MFI: cyclosporine 50 μg/mL 441.1 ± 67.1 vs. control 265.8 ± 50.1, $n = 4$, $p = 0.023$). Using lower doses of cyclosporine (10 μg/ml), we determined that the increased deposition of C3c was enhanced in the absence of serum. Factors in the serum prevented C3c deposition on the cyclosporine-treated BOEC cultures: >2.5% FBS prevented C3c deposition, while at <0.5% FBS, significantly increased C3c was detected on the surface of cyclosporine-treated cells (Figure 1B, MFI: cyclosporine 10 μg/mL in serum-free media 622.5 ± 32.72 vs. control 343.1 ± 65.84, $n = 6$, $p < 0.01$).

Inhibiting the function of CD59, a membrane-anchored complement regulator, is an established means of sensitizing complement fixation on endothelial cells. Blocking CD59 with antibodies has the dual effect of complement induction mainly *via* sensitization (classical pathway) but also through complement amplification (alternative pathway) (36, 38, 41–43). We were interested to examine whether cyclosporine had general effects on the membrane topology that impact C3c deposition or if its effect was *via* CD59. Using the same flow cytometry approach used in Figure 1B, we found that blocking CD59 indeed led to a large increase in C3c associated with the endothelial cells (Figure 1C). However, cyclosporine treatment further increased C3 deposition ∼2-fold beyond the level achieved by blocking CD59 alone. This effect was also observed for C5b-9 to an even greater extend fold increase (Figure 1D). Thus, BOECs treated with cyclosporine had a dose-dependent injury concomitant with increased complement deposition that could be enhanced by the removal of serum or complement regulators.

Cyclosporine induces von Willebrand factor release from endothelial cells

Weibel–Palade bodies (WPBs) are endothelial storage granules containing pro-hemostatic and pro-inflammatory molecules, including VWF, P-selectin, interleukin-8, endothelin-1, and angiopoietin-2 (44–46). As previously demonstrated by us and others, WPBs are exocytosed upon endothelial cell injury and activation to release their contents, which potentiates inflammatory responses, vascular leakage, and leukocyte adhesion (36, 45, 47). Given that cyclosporine resulted in endothelial cell injury and complement deposition, we hypothesized that cyclosporine treatment may also lead to the endothelial release of VWF.

Using the previously established protocol, we first showed that complement activation indeed caused the release of intracellular VWF (Supplementary Figure S2—positive control using anti-CD59 sensitization) (36). We then found that BOECs treated with cyclosporine 10 μg/mL had less intense staining of intracellular VWF (Figure 2). Taken together, our results showed that cyclosporine induces VWF release from BOECs.

Cyclosporine treatment leads to the increased expression of membrane-associated complement regulators

The regulation of the alternative pathway of complement activation is executed by a combination of fluid-phase (CFH and CFI)

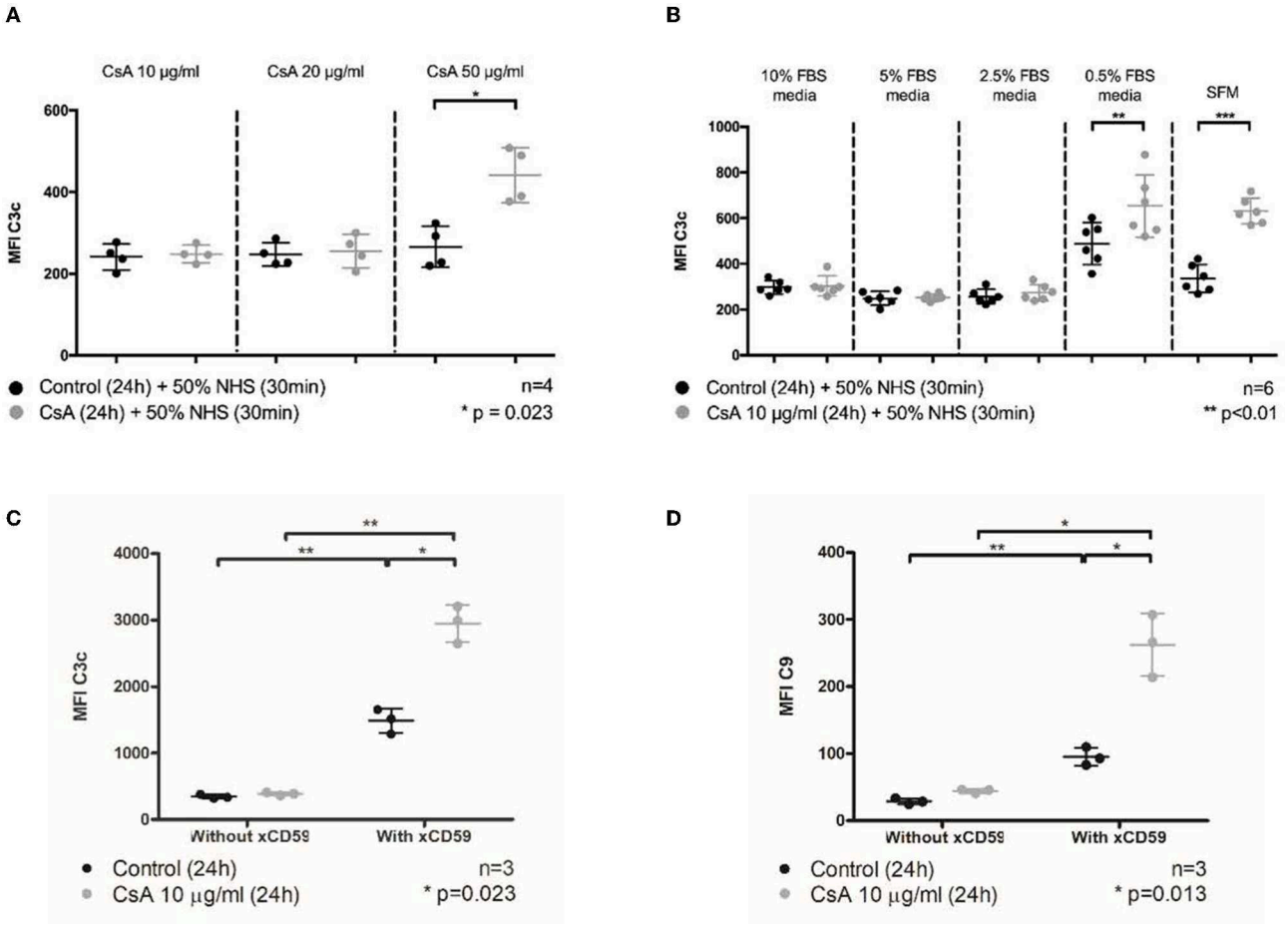

FIGURE 1
Cyclosporine causes complement deposition on endothelial cell surfaces, enhanced by serum starvation and anti-CD59 sensitization. Blood outgrowth endothelial cells (BOECs) were incubated in cyclosporine (CsA) for 24 h, followed by 50% NHS for 30 min. Unless specified, cyclosporine was reconstituted with media/10% FBS. C3c and C9 deposition on BOEC surfaces was detected by flow cytometry. Non-viable cells were excluded from analysis with Fixable Viability Dye eFluor 780. **(A)** Incubating BOECs with cyclosporine 50 µg/ml resulted in significantly higher C3c deposition ($n = 4$, $p = 0.023$, paired, two-tailed t-test). **(B)** Incubating BOECs with cyclosporine 10 µg/ml reconstituted in media supplemented with decreasing amounts of FBS resulted in significantly higher C3c deposition ($n = 6$, $p < 0.01$, paired, two-tailed t-test). **(C)** Addition of anti-CD59 antibody enhanced cyclosporine-induced C3c deposition. Incubation of BOECs with cyclosporine 10 µg/ml for 24 h, followed by anti-CD59 antibody incubation for 30 min, prior to 50% NHS for 30 min caused a significantly increased C3c deposition ($n = 3$, $p = 0.023$, two-way ANOVA, Sidak's multiple comparison test). **(D)** Addition of anti-CD59 antibody also enhanced cyclosporine-induced C9 deposition ($n = 3$, $p = 0.013$, two-way ANOVA, Sidak's multiple comparison test). In keeping with previous data, no increase in C9 deposition was detected when BOECs were incubated with media or cyclosporine 10 µg/ml alone. This *** signifies the degree of statistical significance as denoted by the p value in the figure and in the "Statistics" section in Materials & Methods.

and membrane-bound regulators (mainly MCP/CD46, DAF/CD55, and CD59) that maintain the balance between complement activation and inhibition (8, 13). Given that cyclosporine caused an increase in complement activation on the surface of BOECs, it was conceivable that cyclosporine decreased the expression of membrane-bound complement regulators. We, therefore, assessed the expression of MCP/CD46, DAF/CD55, and CD59 on the surface of BOECs after their treatment with cyclosporine using flow cytometry, and the total cell expression of these regulators by probing cell lysates with Western blotting. We found that treatment of the BOECs with low concentrations (10 µg/mL) of cyclosporine resulted in the increased surface and total cell expression of MCP/CD46, DAF/CD55, and CD59 (Figure 3). Incubation with cyclosporine at higher concentrations of cyclosporine (50 µg/mL) resulted in a similar effect (data not shown). Thus, the increased complement deposition on the surface of cyclosporine-treated cells was not the result of the lost expression of membrane-bound complement regulators.

Cyclosporine treatment leads to impaired CFH binding and regulation on endothelial cells

Since enhanced complement deposition induced by cyclosporine occurred in the context of *increased* expression of membrane-bound complement regulators, we hypothesized that cyclosporine may instead impair CFH-mediated complement regulation. CFH is the central circulating alternative pathway inhibitor, which competitively prevents C3b deposition on cell surfaces, acts as a cofactor to CFI to cleave surface-bound C3b, and accelerates the decay of the C3bBb complex (48–50). To exert these functions, CFH is known to be closely associated with endothelial surfaces *via* its multiple glycosaminoglycan/sialic acid-binding domains (51–55).

To test whether cyclosporine impaired CFH binding, we pre-treated BOECs with cyclosporine and then assessed the ability of the cells to secure Alexa Fluor 488-conjugated CFH from the culture

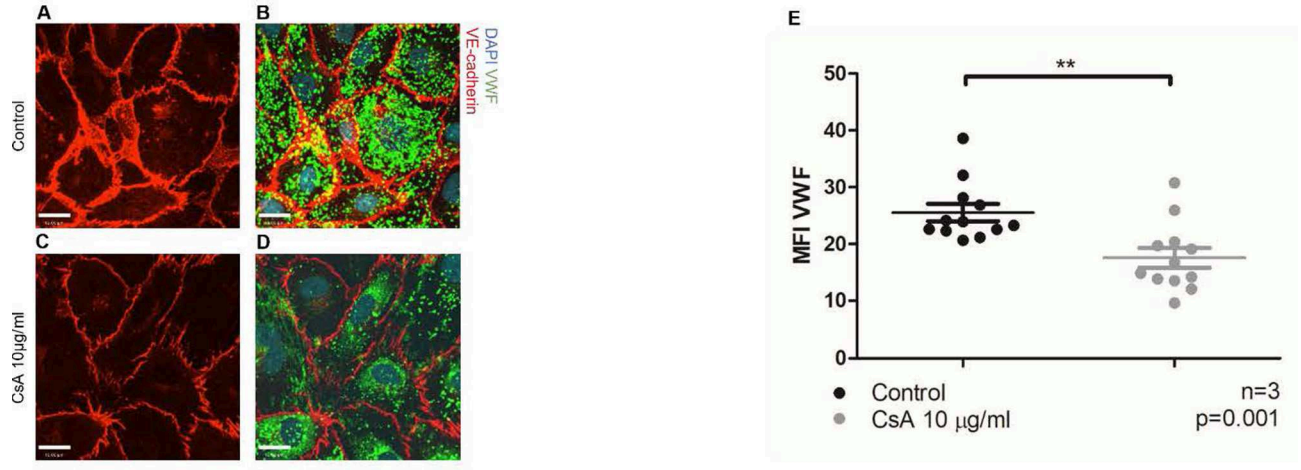

FIGURE 2
Cyclosporine causes endothelial cell release of Weibel–Palade bodies and its contents. **(A–E)** Von Willebrand factor (VWF) release from blood outgrowth endothelial cells (BOECs) was detected *via* immunofluorescence. Control BOECs were treated with media for 24 h. The experimental condition was with BOECs treated with cyclosporine (CsA) 10 μg/ml for 24 h. BOECs were fixed with 2% paraformaldehyde and permeabilized with 0.2% Triton in phosphate-buffered saline, followed by incubation with rabbit anti-VWF (1:1,000) and goat anti-VE-cadherin (1:250) for 4 h. Alexa Fluor 488 (green)- and 555 (red)-conjugated species-specific secondary antibodies were used at dilution of 1:1,000. Nuclei were stained with 0.12 μg/ml Hoechst stain for 5 min. Images were taken using an IX81 inverted microscope (Olympus Corp., Tokyo, Japan) with a 60/1.35 oil immersion objective and a C9100-13 back-thinned EM-CCD camera (Hamamatsu Photonics, Hamamatsu City, Shizuoka Pref., Japan) with a CSU X1 spinning disk confocal scan head (Yokogawa, Yokogawa Canada Inc., AB). Bar = 22 μm. Treatment with cyclosporine 10 μg/ml for 24 h led to less intracellular VWF and less intense staining of VE-cadherin **(A–E)** ($n = 3$, $^{**}p = 0.001$, two-tailed *t*-test).

medium. The Alexa Fluor 488-labeled CFH was added for 1 min to live cells before their analysis by flow cytometry. We found that incubation of BOECs with cyclosporine at 10 μg/mL for 24 h caused a significant reduction in CFH binding (Figures 4A, B, MFI control 386.3 ± 97.8 vs. cyclosporine 10 μg/mL 78.3 ± 45.8, $n = 3$, $p = 0.0078$). A brief (1 h) treatment of the cells with neuraminidase used at 500 mU/mL, an enzyme that cleaves terminal sialic acid groups from glycoproteins, was used as a positive control. The functionality of neuraminidase in cleaving sialic acid was confirmed by live imaging with wheat germ agglutinin (WGA; see the section below) and by CFH binding. Removal of sialic acids inhibited CFH binding to the endothelium to nearly the same extent as cyclosporine treatment (Figure 4B). This reduction in CFH binding on cells treated with cyclosporine 10 μg/mL for 24 h was also confirmed by immunofluorescence (Figures 4C–L, MFI control 6.43 ± 0.44 vs. cyclosporine 10 μg/mL 3.03 ± 0.26, $n = 3$, $p < 0.001$).

Locally concentrating CFH to the membrane of the vascular endothelium is critical for the protection of the membrane from complement deposition. The activity of the CFH, once docked to the endothelial surface, can subsequently be measured by assays that determined the degradation of complement. We assessed the functional consequences of the cyclosporine-induced reduction in CFH binding to BOECs by employing a previously established CFH surface cofactor activity assay (40). In this assay, endothelial cell-bound CFH was used as the sole source of CFH. The incubation of C3b with CFI and CFH results in C3b degradation with the appearance of C3b fragments with molecular weights of 68 kDa (C3b α'1), 43/46 kDa (C3b α'2), all of which can be detected *via* the same Western blotting approach. We first assessed the endogenous cofactor activity of the membrane-bound complement regulator MCP/CD46 in the absence of CFH when exposed to media (control) and various concentrations of cyclosporine (10, 50, and 100 μg/mL). Degradation products were detectable after 90 min, with no detectable significant differences between cyclosporine concentrations (Figure 5A, Supplementary Figures 3A, C, E, G, I). Pre-incubation of BOECs with CFH resulted in the appearance of C3b degradation products after 15 min (Figure 5B), demonstrating the expected significantly higher cofactor activity of CFH on endothelial surfaces. However, when BOECs were pre-incubated with neuraminidase followed by incubation with CFH and subsequently with C3b and CFI in the absence of additional CFH, degradation products were detectable only after 60 min. This result was in keeping with a lack of surface CFH in cells devoid of sialic acids (Figure 5C).

We then assessed the effect of cyclosporine exposure on the cofactor activity of surface-bound CFH. BOECs exposed to increasing doses of cyclosporine (10, 50, and 100 μg/mL for 24 h) demonstrated a dose-dependent decrease in CFH cofactor activity as evidenced by the later appearances of C3b degradation products: cyclosporine 10 μg/mL after 45 min, cyclosporine 50 μg/mL after 45 min, and cyclosporine 100 μg/mL after 90 min (Figures 5D–F). Taken together, we found decreased cofactor activity of CFH on BOECs pre-treated with cyclosporine (Figure 5G).

Cyclosporine treatment weakens the endothelium glycocalyx with reduced CFH surface binding

CFH has been reported to bind to endothelial surfaces *via* its glycosaminoglycan/sialic acid-binding domains (51–55). Since removing sialic acids with neuraminidase ablated CFH binding to the same extent as cyclosporine treatment, we assessed whether cyclosporine exerted its inhibitory effects on CFH binding *via* remodeling of the glycocalyx. We first stained

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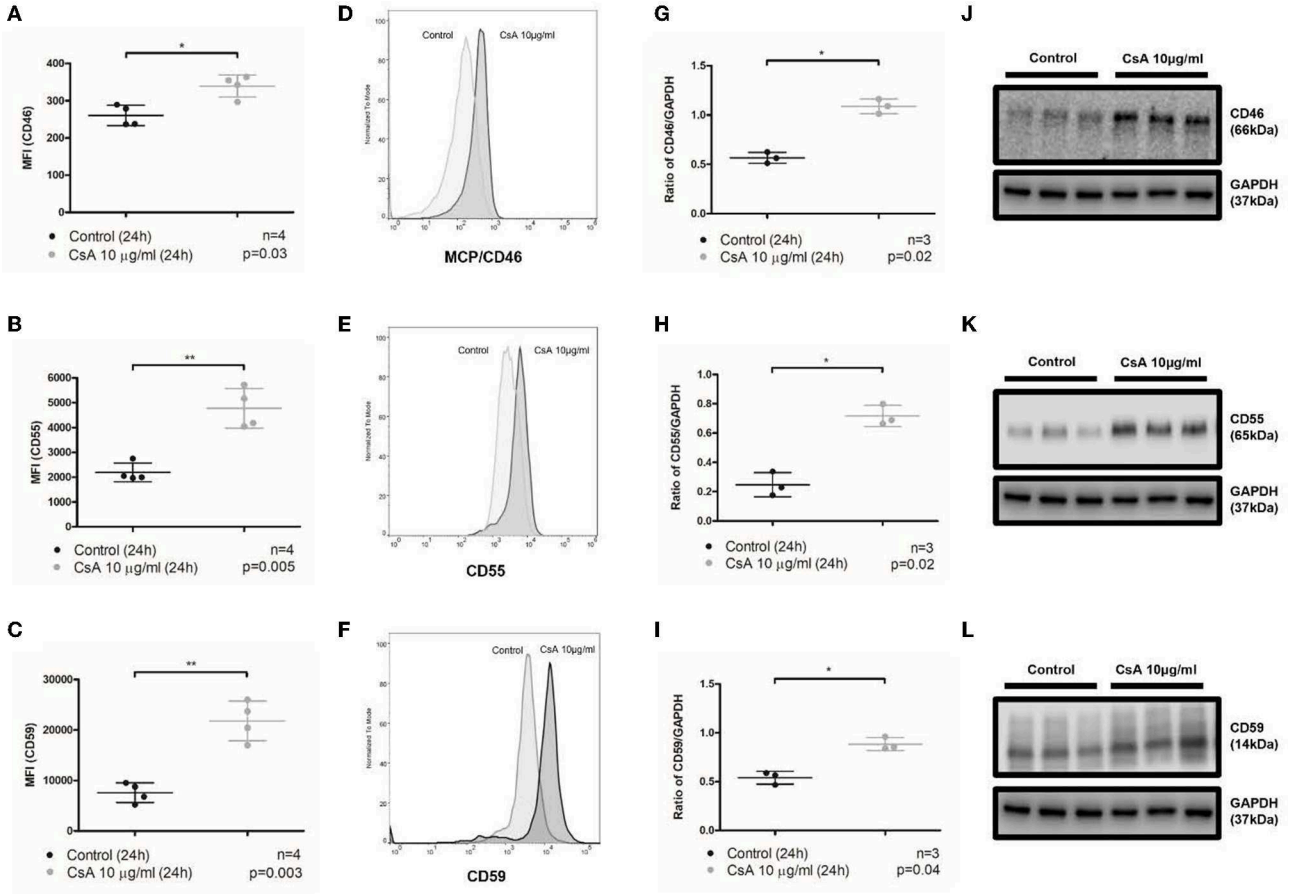

FIGURE 3
Cyclosporine causes increased expression of membrane-bound complement regulators MCP/CD46, DAF/CD55, and CD59 on endothelial cell surfaces. **(A–F)** Blood outgrowth endothelial cell (BOEC) surface membrane-bound complement regulators detected by flow cytometry. Non-viable cells were excluded from analysis with Fixable Viability Dye eFluor 780. Graphical summary of median fluorescence intensity (MFI) from separate experiments **(A–C)** with representative histograms **(D–F)**. Compared to control, incubating BOECs in cyclosporine (CsA) 10 μg/ml for 24 h resulted in increased expression of membrane-bound complement regulators on BOEC surfaces: **(A, D)** MCP/CD46 ($n = 4$, $p = 0.03$, paired, two-tailed *t*-test), **(B, E)** DAF/CD55 ($n = 4$, $p = 0.005$, paired, two-tailed *t*-test), and **(C, F)** CD59 ($n = 4$, $p = 0.003$, paired, two-tailed *t*-test). **(G–L)** Membrane-bound complement regulators MCP/CD46, DAF/CD55, and CD59 in cell lysates by Western blot. Graphical summary of mean gray area of specified protein band normalized to loading control from separate experiments **(G–I)** and Western blots **(J–L).** Compared to media, incubating BOECs in cyclosporine 10 μg/ml for 24 h resulted in increased BOEC protein content of membrane-bound complement regulators: **(G, J)** MCP/CD46 ($n = 3$, $p = 0.02$, paired, two-tailed *t*-test), **(H, K)** DAF/CD55 ($n = 3$, $p = 0.02$, paired, two-tailed *t*-test), and **(I, L)** CD59 ($n = 3$, $p = 0.04$, paired, two-tailed *t*-test). This * and ** signifies the degree of statistical significance as denoted by the p value in the figure and in the "Statistics" section in Materials & Methods.

glycans/polysaccharides containing sialic acid and N-acetyl-D-glucosamine using the lectin wheat germ agglutinin (WGA) conjugated to Alexa Fluor 594. Of note, we imaged the cells live as fixation resulted in a dramatic decrease in overall fluorescence. To prevent endocytosis of the lectin, incubation with Alexa Fluor 594-WGA was performed in the cold (4°C). We determined a decrease in Alexa Fluor 594-WGA staining in BOECs treated with neuraminidase used at 500 mU/mL for 1 h, with conditions identical to those that inhibited CFH binding (Figures 5C, 6A, B: MFI neuraminidase 18,459 ± 6,154 vs. control 32,525 ± 8,990, $p < 0.0001$). Treatment with cyclosporine at 10 μg/mL also resulted in less intense staining with Alexa Fluor 594-WGA when compared to control (Figures 6C, D: MFI cyclosporine 10 μg/mL 18,752 ± 6,154 vs. control 32,525 ± 8,990, $p < 0.0001$). The decrease in the WGA signal in cyclosporine was more apparent in the clusters on the apical surface of the endothelial cells and less visible at cell–cell junctions (Figures 6A, C).

We further assessed whether cyclosporine had additional effects on the endothelial glycocalyx, specifically on the surface density of heparan sulfates. Heparan sulfates are covalently attached to the proteoglycans process in the Golgi apparatus (e.g., syndecans and glypicans). These side chains can be detected by immunostaining: While the polysaccharides may not be immunogenic on their own, in the context of proteoglycans, good antibodies have been generated and made commercially available. We, therefore, immunostained non-permeabilized control or cyclosporine-treated endothelial cells with anti-heparan sulfate antibodies. When compared to control, treatment with cyclosporine used at 10 μg/mL resulted in an ∼60% decrease in the intensity of heparan sulfate per cell (Figures 6E–N: MFI cyclosporine 10 μg/mL 3.01 ± 0.36 vs. control 9.30 ± 1.04, $p < 0.0001$). Heparinase III, a polysaccharide lyase that degrades heparan sulfate, was used as the positive control, which led to a similar decrease in the intensity of heparan sulfate (Figures 6H–J, N: MFI heparinase III 3.19 ± 0.41 vs. control 9.30 ± 1.04, $p < 0.0001$).

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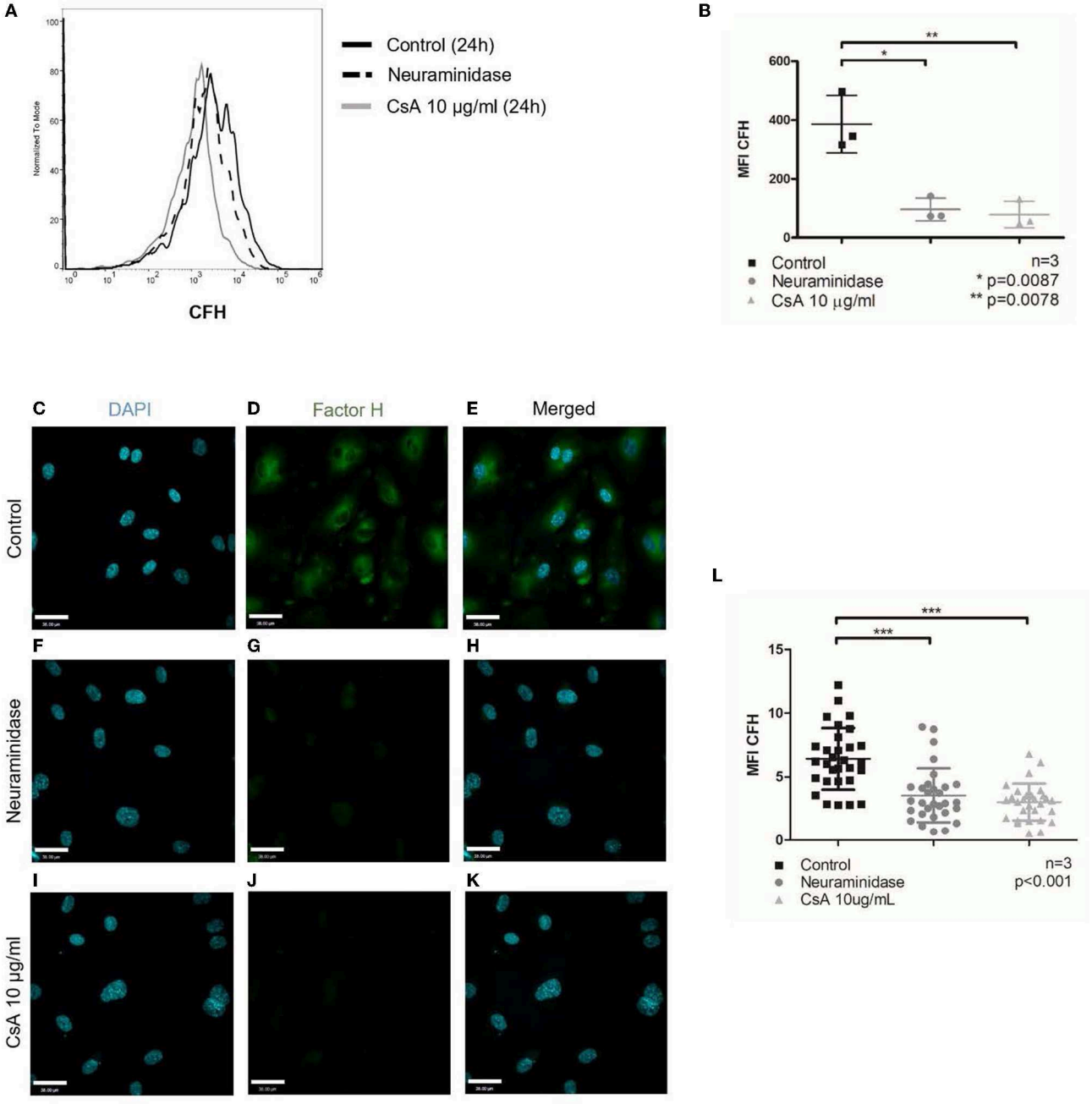

FIGURE 4
Cyclosporine causes reduced complement factor H binding on surfaces of endothelial cells. Alexa Fluor 488-conjugated complement factor H (CFH) binding on blood outgrowth endothelial cell (BOEC) surface was assessed by flow cytometry **(A, B)**. Non-viable cells were excluded from analysis with Fixable Viability Dye eFluor 780. Representative histogram **(A)** and graphical summary of mean fluorescence intensity from separate experiments **(B)**. Compared to control (no cyclosporine), incubating BOECs in cyclosporine (CsA) 10 µg/ml for 24 h resulted in reduced CFH binding on BOEC surface ($n = 3$, $^{**}p = 0.0078$, paired, two-tailed t-test). Treatment with neuraminidase 500 mU/ml, which cleaves sialic acid groups from glycoproteins, also resulted in reduced CFH binding on BOEC surface ($n = 3$, $^{*}p = 0.0087$, paired, two-tailed t-test). CFH was also assessed by immunofluorescence **(C–L)**. Representative images **(C–K)** and mean fluorescence intensity from three sets of experiments with 10 representative images taken per condition (each dot represents 1 image) were measured with ImageJ and summarized **(L)**. Compared to control, incubating BOECs in CsA 10 µg/ml for 24 h resulted in reduced CFH binding on BOEC surface ($n = 3$, $^{***}p < 0.001$, paired, two-tailed t-test)

Treatment with cyclosporine 10 µg/mL and heparinase III led to a similar decrease in CFH (Supplementary Figure S4: MFI cyclosporine 10 µg/mL 3.03 ± 0.26 vs. heparinase III 4.11 ± 0.20 vs. control 6.43 ± 0.44, $p < 0.0001$).

Finally, the modifications to the glycocalyx upon cyclosporine treatment could be the result of overactive hydrolases (i.e., glycosidases or proteases) or the result of mistrafficking and expression of proteoglycans and glycoproteins. To determine whether surface glycoproteins in cyclosporine-treated cells were devoid of sialic acids, we used a lectin, peanut agglutinin (PNA), that recognizes exposed, terminal galactose sugars. We found that cyclosporine-treated cells did not have cleaved sialic acids from

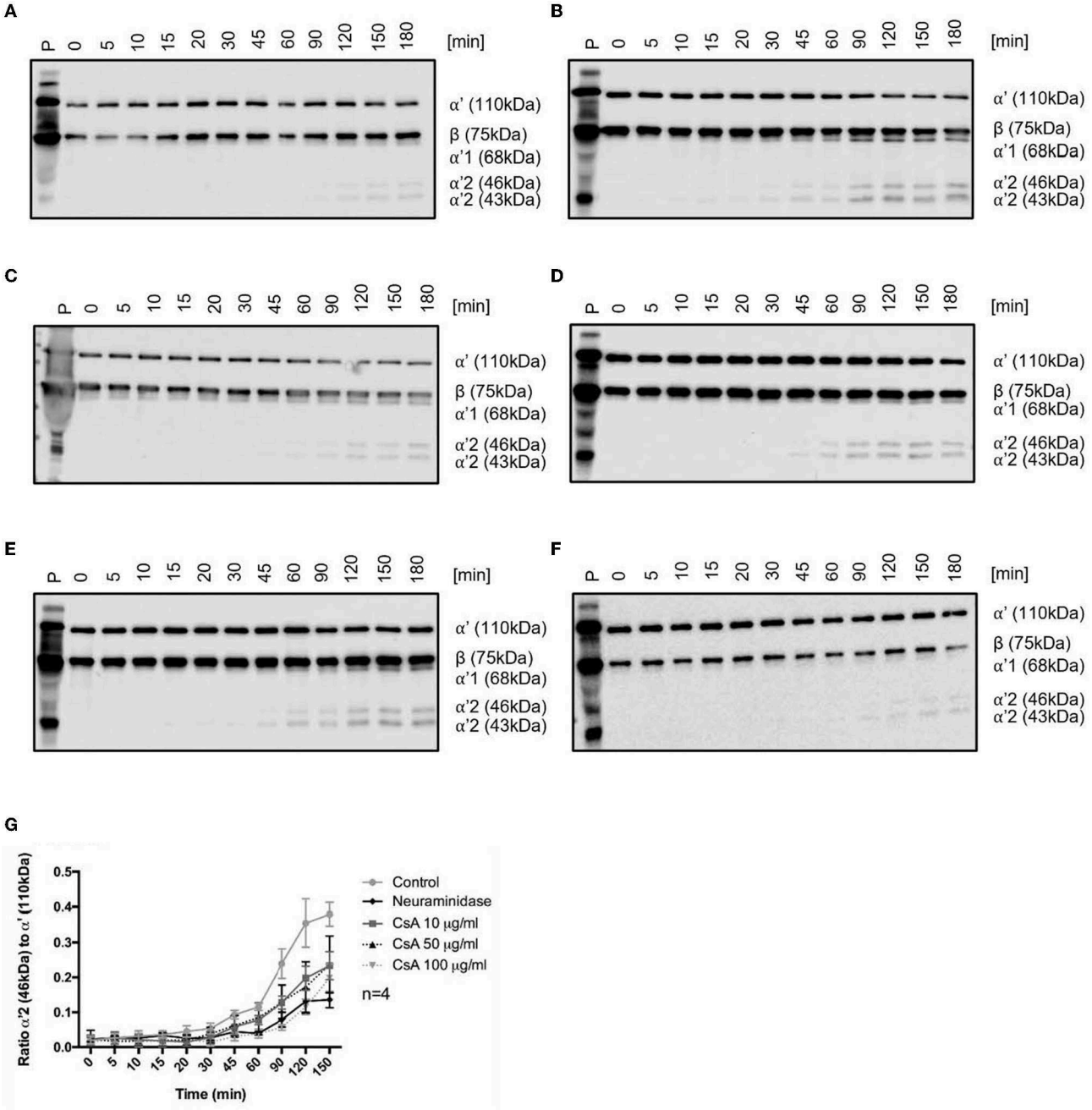

FIGURE 5
Cyclosporine causes impaired complement factor H regulation on surfaces of endothelial cells. **(A–F)** Cyclosporine (CsA) leads to impaired complement factor H (CFH) surface cofactor activity detected by a CFH surface cofactor activity assay. Blood outgrowth endothelial cells (BOECs) were incubated with C3b 3.3 μg/ml and complement factor I (CFI) 10 μg/ml at 37 degrees Celsius, with or without pre-incubation with CFH 10 μg/ml at 37 degrees Celsius. The appearance of C3b degradation fragments was analyzed by Western blotting (representative Western blots are shown in **(A–F)**. **(A)** Endogenous cofactor activity on BOEC without CFH. BOECs were incubated with C3b and CFI at 37 degrees Celsius. Degradation products (α'68, α'46, and α'43 kDa fragments of the C3b α' chain) were detectable after 90 min and increased with time. **(B)** Cofactor activity of CFH on the surface of BOEC. BOECs were pre-incubated with CFH for 1 h at 37 degrees Celsius and thoroughly washed, prior to incubation with C3b and CFI at 37 degrees Celsius. Degradation products were detectable after 15 min. **(C)** Cofactor activity of CFH on the surface of neuraminidase-treated BOEC. Neuraminidase cleaves sialic acid groups from cell surfaces. BOECs were pre-incubated with neuraminidase 500 mU/ml for 1 h followed by CFH for 1 h at 37 degrees Celsius, prior to being thoroughly washed and incubated with C3b and CFI at 37 degrees Celsius. Degradation products were detectable after 60 min. **(D–F)** Cofactor activity of CFH on the surface of cyclosporine-treated BOEC. BOECs were pre-incubated with **(D)** cyclosporine 10 μg/ml, **(E)** cyclosporine 50 μg/ml, and **(F)** cyclosporine 100 μg/ml for 24 h. They were then incubated with CFH for 1 h at 37 degrees Celsius, and C3b degradation products were detectable: **(D)** cyclosporine 10 μg/ml after 45 min, **(E)** cyclosporine 50 μg/ml after 45 min, and **(F)** cyclosporine 100 μg/ml after 90 min. These results suggest that cyclosporine causes impaired CFH binding and regulation on surfaces of BOECs. **(G)** Graphical presentation of CFH surface cofactor activity assay experiments. For statistical analysis, we formulated a ratio of the mean gray value of the α'2 46 kDa band with the mean gray value of the α'110 kDa band. An increased ratio indicates that the α' chain was cleaved into its split products, indicative of C3b inactivation. There was a significant reduction in CFH cofactor activity on the surfaces of BOECs treated with cyclosporine when compared with control ($n = 4$, $p < 0.005$ for control vs. cyclosporine 10 μg/ml from 90 min onwards; $p < 0.04$ for control vs. cyclosporine 50 μg/ml from 90 min onwards; $p < 0.03$ for control vs. cyclosporine 100 μg/ml from 45 min onwards; $p < 0.001$ for control vs. neuraminidase 500 mU/ml from 60 min onwards paired, two-tailed t-test).

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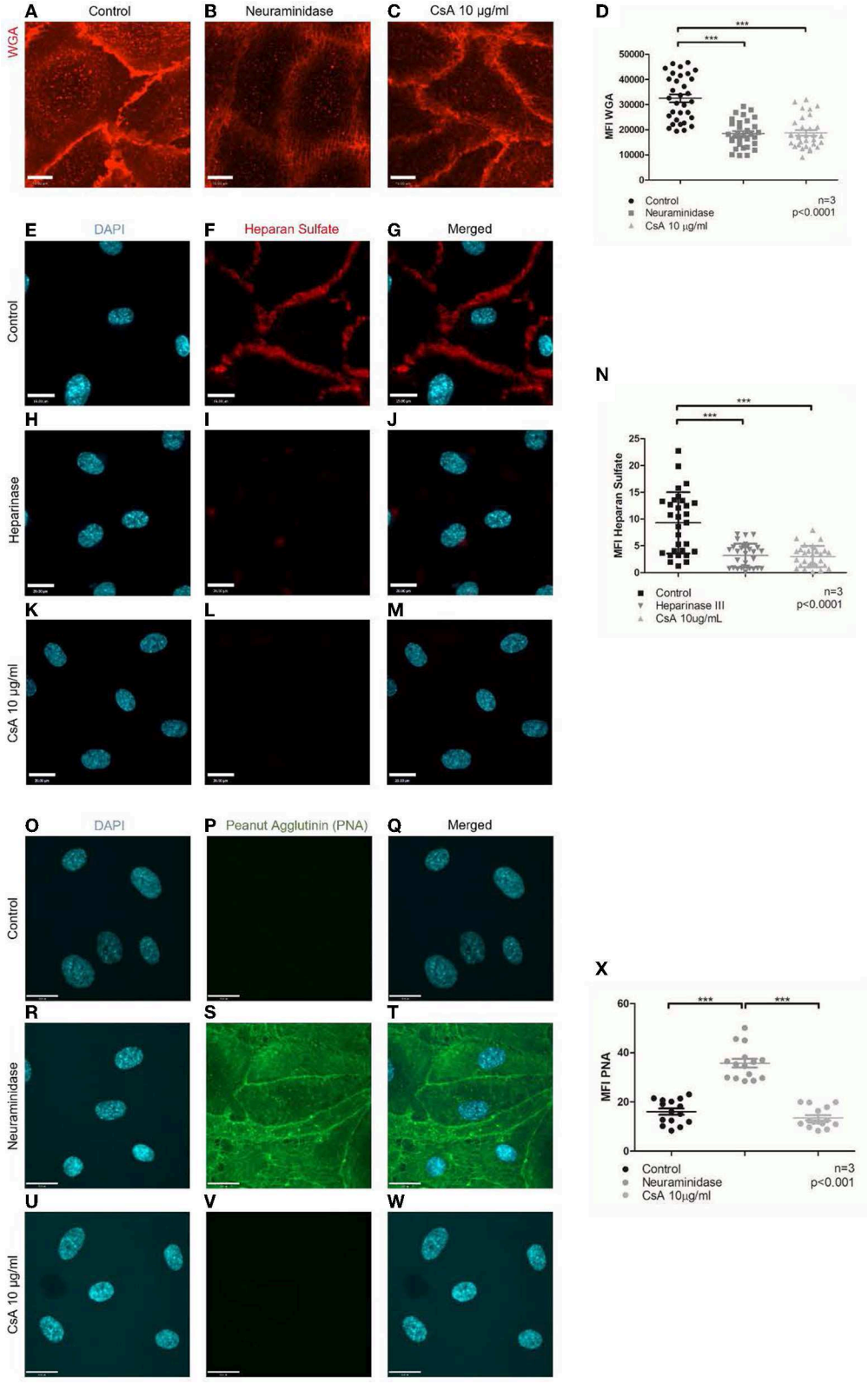

FIGURE 6
Cyclosporine causes breakdown of endothelial cell glycocalyx. **(A–D)** Live cell imaging of blood outgrowth endothelial cell (BOEC) glycocalyx with Alexa Fluor 594-conjugated wheat germ agglutinin (WGA). When compared to BOECs treated with **(A)** media for 24 h (control), BOECs treated with **(B)** neuraminidase 500 mU/ml for 1 h and **(C)** cyclosporine (CsA) 10 μg/ml for 24 h were less intensely stained with WGA. Mean fluorescence intensity from three sets of experiments with 10 representative images taken per condition (each dot represents 1 image) was measured with ImageJ and summarized in **(D)**. There was a significant reduction in mean fluorescence intensity of WGA staining on BOECs treated with cyclosporine ($n = 3$, $^{***}p < 0.0001$, two-tailed *t*-test). Bar = 22 μm. **(E–X)** Heparan sulfate and PNA were detected *via* immunofluorescence. Control BOECs were treated with media for 24 h, BOECs treated with neuraminidase 500 mU/ml for 1 h were used as positive control for PNA experiments, and BOECs treated with heparinase III 0.5 U/ml for 30 min were used as positive control for heparan sulfate experiments. BOECs were fixed with 2% paraformaldehyde, followed by incubation

(Continued)

FIGURE 6 (Continued)
with mouse anti-heparan sulfate (green) and anti-PNA (green). Images were taken using an IX81 inverted microscope (Olympus Corp., Tokyo, Japan) with a 60/1.35 oil immersion objective and a C9100-13 back-thinned EM-CCD camera (Hamamatsu Photonics, Hamamatsu City, Shizuoka Pref., Japan) with a CSU X1 spinning disk confocal scan head (Yokogawa, Yokogawa Canada Inc., AB). Bar = 22 μm. When compared to control, treatment with cyclosporine 10 μg/ml for 24 h led to reduction in heparan sulfate **(E–N)** ($n = 3$, 10 representative images taken per condition, ***$p < 0.0001$, two-tailed t-test) but similar staining with PNA **(O–X)** ($n = 3$, 5 representative images taken per condition, ***$p < 0.001$, two-tailed t-test). Cells treated with neuraminidase 500 mU/ml (positive control) confirmed increased PNA staining **(R–T)**.

surface glycoproteins as evidenced by no PNA signal observed on the surface of the cells (Figures 6 O–X: MFI cyclosporine 10 μg/mL 13.6 ± 4.1 vs. control 16.1 ± 4.9, p = ns). As a positive control, we showed increased PNA staining in BOECs treated with neuraminidase (Figures 6R–T, X: MFI neuraminidase 35.8 ± 6.7 vs. control 16.1 ± 4.9, $p < 0.001$). The findings with PNA were also confirmed on flow cytometry (Supplementary Figure S5).

Taken together, our findings suggest that cyclosporine treatment results in endothelial glycocalyx breakdown *via* the loss of surface glycoproteins and heparan sulfates, which leads to impaired CFH surface binding.

Discussion

Calcineurin inhibitor use is associated with acute and chronic tubulo-interstitial, arteriolar, and glomerular injury (27, 32). While possible mechanisms of injury relate to vasoconstriction-associated ischemia, increased platelet aggregation, activation of prothrombotic factors, and disruption of vascular endothelial growth factor (VEGF) regulation of angiogenesis (56), evolving evidence also suggests the involvement of the complement system (34). The association between CNI use and the development of TMA in patients (28, 30, 31) and the observation of complement deposition in areas of endothelial injury in kidney biopsy specimens affected by CNI toxicity hint the involvement of complement (57). Animal models of CNI toxicity implicate the complement system and offer explanations of how further complement-mediated injury can be propagated (34, 35). However, the exact mechanism by which CNIs induce complement activation is still unknown.

Our findings shed light on the pathogenesis of CNI toxicity and specifically identify complement activation on the vascular endothelium as a mechanism. To our knowledge, we are the first to establish an *in vitro* model utilizing BOECs to study the effect of cyclosporine and complement activation on endothelial cells. We found that cyclosporine treatment causes complement deposition and endothelial cell injury, which results in VWF release from Weibel–Palade bodies.

Our findings suggest a role for complement-mediated endothelial cell injury induced by cyclosporine and, for the first time, implicate CFH surface dysregulation in cyclosporine-induced complement activation on endothelial cells. CFH, a plasma protein acting as a cofactor to CFI-mediated cleavage of C3b, must recognize and bind to endothelial cell glycocalyx glycosaminoglycans and terminal sialic residues *via* short consensus repeats (SCRs) 6–8 and 19–20 (48, 51–54). Adapting a previously described flow cytometry protocol of quantifying the binding of CFH and a previously established method of assessing the surface cofactor activity of CFH (39, 40), we found that cyclosporine treatment led to decreased CFH binding to endothelial cell surfaces and impaired CFH surface cofactor activity. In these assays, we also treated BOECs with neuraminidase to test whether the absence of sialic acid on the glycocalyx of endothelial cells affected the binding and surface cofactor activity of CFH. The neuraminidase used (derived from Clostridium perfringens) primarily targets sialic acids in α2,3 (to a lesser extent α2,6 and α2,8) configuration and can cleave terminal sialic acid from O-linked glycans, N-linked glycans, and glycolipids. Of particular interest, we found that neuraminidase treatment led to a similar impairment of CFH surface binding and cofactor activity, suggesting the possibility that cyclosporine affects CFH binding to endothelial cell surfaces by reduction of the glycocalyx.

Utilizing live cell imaging of endothelial cells stained with wheat germ agglutinin (WGA) that binds to sialic acid and N-acetylglucosaminyl residues within the endothelial cell glycocalyx, we found that cyclosporine and neuraminidase treatment significantly diminished the endothelial cell glycocalyx. Furthermore, we found that cyclosporine-induced endothelial cell glycocalyx breakdown occurred mainly through the loss of heparan sulfate. Taken together, these findings suggest that cyclosporine treatment leads to the shedding of heparan sulfate in the endothelial cell glycocalyx, leading to impaired CFH recognition of and binding to host endothelial cell surfaces, which impairs its surface regulation of the alternative pathway. The inability of CFH to inactivate C3b covalently bound to endothelial cell surfaces results in an uninhibited amplification loop that allows for the full activation of the complement cascade. This mechanism leading to alternative pathway dysregulation by CFH could potentially be generalized to other forms of TMA where endothelial cell glycocalyx injury is involved.

Contrary to our initial hypothesis, we found that cyclosporine treatment caused increased expression of the surface membrane-bound complement regulators MCP/CD46, DAF/CD55, and CD59, a possible compensatory cellular response to cyclosporine treatment and the resultant impaired CFH regulation of the alternative pathway. MCP/CD46 aids in the inactivation of C3b as a cofactor in the CFI-catalyzed cleavage of C3b, DAF/CD55 accelerates the disintegration of the C3 and C5 convertases, and CD59 prevents the formation of the membrane attack complex (C5b-9) by binding to C8. The failure of CFH to bind to endothelial cell surfaces and exert its function that is induced in our model by cyclosporine leads to an increased C3b load, which, when not tightly regulated, will be amplified with the formation of the C3 convertases and even more C3b, eventually leading to the activation of the terminal pathway. In this context, we speculate that increasing the expression of the other complement regulatory armamentarium would be in the host endothelial cells' best survival interest.

When cyclosporine was reconstituted in standard endothelial growth medium, there was increased complement deposition (C3 and C9) with cyclosporine 50 μg/ml or higher. When reconstituted in serum-free media, increased complement deposition occurred with cyclosporine 10 μg/ml, suggesting that serum-starved BOECs were

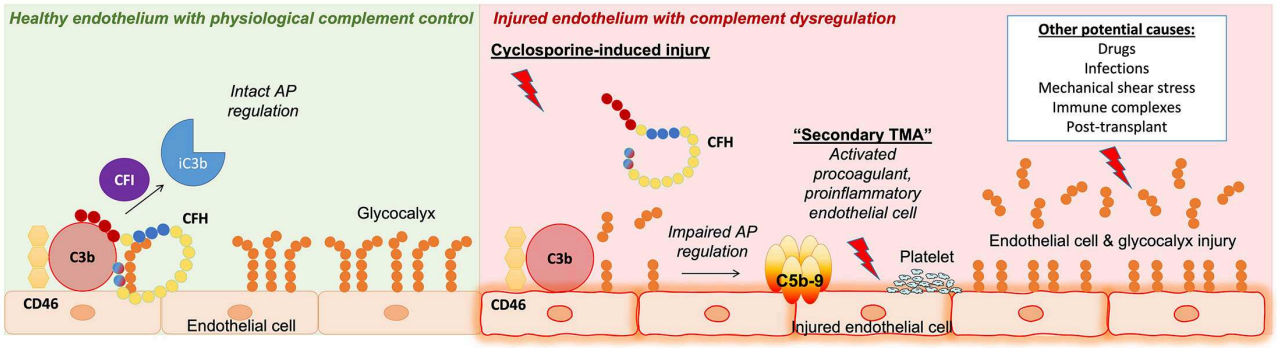

FIGURE 7
Summary of key findings and concepts presented in our work. **(Left)** Healthy endothelium with physiological complement control where the regulation of the complement alternative pathway (AP) is intact. Complement factor H (CFH) acts as a cofactor with complement factor I (CFI) and membrane cofactor protein (CD46) to inactivate C3b on endothelial cell surfaces. **(Right)** Injured endothelium with complement dysfunction. Our work presented in this study identified a role for complement in cyclosporine-induced endothelial cell injury. We showed that endothelial cells exposed to cyclosporine had decreased glycocalyx density, leading to complement AP dysregulation *via* decreased CFH surface binding and cofactor activity. This mechanism of endothelial cell and glycocalyx injury leading to complement AP dysfunction could potentially be applicable to other forms of secondary thrombotic microangiopathy (TMA).

more susceptible to cyclosporine-induced complement deposition. Incubating cells with an anti-CD59 blocking antibody, an established model to induce complement deposition on endothelial cells (36, 41–43), led to further enhancement of cyclosporine-induced complement deposition on endothelial cells. Given the ∼2-fold increase in surface expression of CD59 after exposure to cyclosporine, the fact that the anti-CD59 is a monoclonal IgG2b antibody—an isotype that activates complement *via* the classical pathway—and the fact that anti-CD59 inhibits the action of the surface-bound complement regulator CD59, the increased complement deposition on endothelial cells induced by cyclosporine is likely due to anti-CD59 antibody-initiated activation of the classical pathway, exacerbated by a reduced capacity to regulate the amplification propagated *via* the alternative pathway (36, 58).

Within our model, we found an optimal balance of endothelial cell survival and CNI effect with cyclosporine doses between 10 and 100 μg/ml for up to 24 h. In the clinical setting, the therapeutic target trough range for cyclosporine is maintained between 100 and 400 ng/ml but varies depending on the indication of its use, the type of transplant, the use of concomitant immunosuppression, and time post-transplant. Suggested target 2-h post-dose levels could be as high as 2 μg/ml (59). *In vitro* experimental studies of cyclosporine effect on various endothelial cell lines used a wide range of drug concentrations ranging from 0.1 μg/ml to 4000 μg/ml over varying exposure durations (up to 72 h) (25, 34, 60–64). Although the levels of cyclosporine maintained clinically are lower than those used in experimental *in vitro* studies, they are not directly comparable. It is a limitation of *in vitro* models of disease, and the differences reflect different susceptibility of various endothelial cell lines and inter-species differences. The duration of exposure used in *in vitro* models is also limited to 24–72 h, whereas many patients are on life-long immunosuppression. To our knowledge, we are the first to study the effect of cyclosporine utilizing BOECs.

In conclusion, we found that cyclosporine leads to injury of the endothelial cell glycocalyx and breakdown of heparan sulfate that negatively impacts CFH regulation of the alternative pathway of complement *via* decreased CFH binding to the endothelial cell surface (Figure 7). Enhanced susceptibility to complement-mediated injury secondary to impaired regulation of the alternative pathway might represent a shared mechanism of endothelial injury applicable to various forms of (secondary) TMA, including those caused by toxic agents, mechanical stress, and autoantibodies, which warrants further elucidation.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material. Further inquiries can be directed to the corresponding author.

Ethics statement

The study was approved by the Research Ethics Board of the Hospital for Sick Children (SickKids), Toronto, ON. Signed written informed consent was obtained from all volunteers whose samples were used in the study. The study was performed in keeping with the Declaration of Helsinki.

Author contributions

CWT designed and coordinated the project, performed experiments, interpreted the results, and wrote the initial and subsequent revised versions of the manuscript. MR designed the project, performed experiments, interpreted the results, and reviewed the manuscript. CO-S performed experiments, interpreted the results, and reviewed the manuscript. SF designed experiments, interpreted the results, and reviewed the manuscript. JP, JL, AB-H, VB, and EB performed experiments and reviewed the manuscript. LR interpreted the results and reviewed the manuscript. CL designed and coordinated the project, interpreted the results, and reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2023.891513/full#supplementary-material

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