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# Tocilizumab improves oxidative stress and endothelial glycocalyx: A mechanism that may explain the effects of biological treatment on COVID-19

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## ABSTRACT

We investigated the effects of tocilizumab on endothelial glycocalyx, a determinant of vascular permeability, and myocardial function in rheumatoid arthritis (RA). Eighty RA patients were randomized to tocilizumab ( $n = 40$ ) or conventional synthetic disease-modifying antirheumatic drugs (csDMARDs) and glucocorticoids (GC) ( $n = 40$ ) for 3 months. Forty healthy subjects with similar age and sex served as controls. We measured: (a) perfused boundary region (PBR) of the sublingual arterial microvessels (increased PBR indicates reduced glycocalyx thickness), (b) pulse wave velocity (PWV), (c) global LV longitudinal strain (GLS), (d) global work index (GWI) using speckle tracking echocardiography and (e) C-reactive protein (CRP), malondialdehyde (MDA) and protein carbonyls (PCs) as oxidative stress markers at baseline and post-treatment. Compared to controls, RA patients had impaired glycocalyx and myocardial deformation markers ( $P < 0.05$ ). Compared with baseline, tocilizumab reduced PBR ( $2.14 \pm 0.2$  versus  $1.97 \pm 0.2 \mu\text{m}$ ;  $P < 0.05$ ) while no significant differences were observed post-csDMARDs + GC ( $P > 0.05$ ). Compared with csDMARDs + GC, tocilizumab achieved a greater increase of GLS, GWI and reduction of MDA, PCs and CRP ( $P < 0.05$ ). The percent improvement of glycocalyx thickness (PBR) was associated with the percent decrease of PWV, MDA, PCs and the percent improvement of GLS and GWI ( $P < 0.05$ ). Tocilizumab improves endothelial function leading to a greater increase of effective myocardial work than csDMARDs + GC through a profound reduction of inflammatory burden and oxidative stress. This mechanism may explain the effects of tocilizumab on COVID-19.

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## 1. Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory systemic disease characterized by increased risk of cardiovascular disease (CVD) (López-Mejías et al., 2016; Lazou et al., 2020). Beyond conventional risk factors, the mechanisms linking RA and CVD are not fully understood, but systemic inflammatory burden, increased oxidative stress and microvascular endothelial dysfunction are contributing factors to

accelerated early atherogenesis (England et al., 2018; Bordy et al., 2018). Endothelial glycocalyx is a complex layer composed of glycoproteins, proteoglycans and other soluble components on the luminal side of the blood vessels. It prevents the direct contact of circulating blood cells with endothelial surface (Lekakis et al., 2011). Pathophysiological conditions, such as inflammation and oxidative stress, are associated with glycocalyx impairment (Yilmaz et al., 2019). In particular, reactive oxygen species (ROS) induce an acute but rapidly reversible impairment of glycocalyx structure after antioxidant

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Nomenclature	
ACPA	anti-citrullinated protein antibodies
AI	augmentation index
bDMARDs	biological disease-modifying antirheumatic drugs
COVID-19	coronavirus disease 2019
CRP	C-reactive protein
cSBP	central systolic blood pressure
csDMARDs	conventional synthetic disease-modifying antirheumatic drugs
CVD	cardiovascular disease
DAS28	disease activity score in 28 joints
eGFR	estimated glomerular filtration rate
GC	glucocorticoids
GCS	global circumferential strain
GCSR E	global circumferential strain rate E
GCW	global constructive work
GLS	global longitudinal strain
GLSR E	global longitudinal strain rate E
GRS	global radial strain
GRSR E	global radial strain rate E
GWE	global work efficiency
GWI	global work index
GWW	global wasted work
IL-6	interleukin-6
LDL-C:	low-density lipoprotein cholesterol
LV	left ventricular
MDA	malondialdehyde
MMPs	metalloproteinases
NSAIDs	non-steroidal anti-inflammatory drugs
PBR	perfused boundary region
PCs	protein carbonyls
PWV	pulse wave velocity
RA	rheumatoid arthritis
RF	rheumatoid factor
ROS	reactive oxygen species
SDF	Sidestream Darkfield imaging
SM22 $\alpha$	smooth muscle protein 22- $\alpha$
sPLA2-IIA	group IIA secretory phospholipase A2
TGF- $\beta$ 1	transforming growth factor- $\beta$ 1
TNF- $\alpha$ :	tumor necrosis factor- $\alpha$
VAS	visual analogue score
VLDL-C:	very low-density lipoprotein cholesterol
$\alpha$ -SMA	$\alpha$ -smooth muscle actin

treatment (Singh et al., 2013). Non-invasive visualization techniques have permitted the accurate estimation of the sublingual microvasculature glycocalyx thickness (Lekakis et al., 2011).

Moreover, left ventricular (LV) systolic function is impaired in RA patients (Cioffi et al., 2017). Interleukin-6 is increased in RA patients and has direct negative effects on myocardial function (Lazou et al., 2020). Myocardial deformation by speckle tracking echocardiography permits detection of subclinical myocardial dysfunction despite the presence of a normal ejection fraction (Sitia et al., 2012; Schroeder et al., 2016). In addition, myocardial work index is a novel marker of ventricular-arterial interaction, which is derived by LV pressure-myocardial strain loop acquired by speckle tracking echocardiography (Ikonomidis et al., 2019a).

According to the standard of care of RA, in the absence of unfavorable prognostic markers, such as autoantibodies, high disease activity, or early erosions, conventional synthetic disease-modifying antirheumatic drugs (csDMARDs) with addition of short-term glucocorticoids (GC) would be used to start therapy (Smolen et al., 2017). Conversely, failure of  $\geq 2$  csDMARDs with GC at maximum tolerated doses and/or presence of autoantibodies, high disease activity, or early erosions are indication for the use of novel biological agents either as monotherapy or in combination with csDMARDs (e.g. methotrexate). Tocilizumab, a biological disease-modifying antirheumatic drugs (bDMARDs), is a recombinant humanized monoclonal antibody against the interleukin-6 (IL-6) receptor which is used for the treatment of moderate to severe RA (Shetty et al., 2014). The side effects of tocilizumab include opportunistic infections such as tuberculosis, skin and soft tissue infections, infusion reactions, increase in lipid levels, transient neutropenia and elevation of liver enzymes especially in combination with methotrexate (Jones and Ding, 2010). Previous studies have shown that treatment with tocilizumab leading to an improvement of endothelial function, arterial elasticity and LV myocardial function (Protogerou et al., 2011; Kobayashi et al., 2016; Ikonomidis et al., 2019b).

However, it is not clear whether IL-6 inhibitors have a favorable effect on endothelial glycocalyx and myocardial work.

In the present study, we hypothesized that IL-6 inhibition by tocilizumab would have a greater benefit on endothelial glycocalyx and myocardial work than treatment with csDMARDs and GC. Thus, the primary endpoint was the differential effects of tocilizumab in comparison to the respective effects of csDMARDs and GC on glycocalyx and

LV myocardial work index as assessed by speckle tracking echocardiography. Another endpoint was the association of changes of endothelial glycocalyx with the respective changes in myocardial function, arterial elastic properties and biomarkers of inflammation and oxidative stress.

## 2. Material and methods

### 2.1. Study population and protocol

We examined 80 patients (mean age  $64 \pm 9$  years, 61 females) with rheumatoid arthritis (ACR/EULAR classification criteria for RA) (Ale-taha et al., 2010) who had not adequately responded to csDMARDs and GC or cannot tolerate other approved drug classes for RA. All patients were under methotrexate 7.5 mg once per week, leflunomide 20 mg once daily and prednisolone 5 mg once daily before inclusion in the study.

Exclusion criteria were stable coronary artery disease (CAD) or acute coronary syndrome, familial hyperlipidemia, type-1 diabetes mellitus, history of steroid induced diabetes, moderate or severe valvular heart disease, chronic obstructive pulmonary disease, chronic renal disease (estimated glomerular filtration rate [eGFR] less than  $60 \text{ mL/min/1.73 m}^2$ ) and malignancies. Coronary artery disease was excluded by the absence of clinical history, angina and reversible myocardial ischemia, as assessed by thallium myocardial scintigraphy or dobutamine stress echocardiography. None of the study patients were on treatment with non-steroidal anti-inflammatory drugs (NSAIDs) within the past year.

We calculated the composite inflammatory disease activity score in 28 joints (DAS28), which includes C-reactive protein (CRP), the visual analogue score (VAS) of wellbeing and the number of tender and swollen joints from a total of 28 joints using the formula:  $\text{DAS28} = \sqrt{(0.56 \times \text{number of tender joints}) + \sqrt{(0.28 \times \text{number of swollen joints})} + [0.70 \times \ln(\text{CRP})] + (0.014 \times \text{VAS})$  (Sokka et al., 2000).

Patients were randomized to receive tocilizumab treatment (160 mg subcutaneous once a week) ( $n = 40$ ) or were treated with an increase of their initial dose of methotrexate (rapid escalation to 25 mg once per week) and an increase of prednisolone by 5 mg with addition of leflunomide at the same dose (20 mg once daily) according to standard clinical practice as previously published (Ikonomidis et al., 2008) ( $n = 40$ ) for 3 months. Randomization was applied by an attending rheumatologist (P.K.) using a table of random numbers as reproduced from

the online randomization software <http://www.graphpad.com/quickcalcs/index.cfm>. Several multicenter clinical trials have shown that tocilizumab acts quickly and effectively either as monotherapy or in combination with other agents in RA and its toxicity profile is manageable (Jones and Ding, 2010). Moreover, several studies have demonstrated the beneficial effects of this biological agent in endothelial and LV myocardial function in patients with RA (Protogerou et al., 2011; Kobayashi et al., 2016; Ikonomidis et al., 2019b).

All patients were re-evaluated as outpatients every 15 days to estimate clinical status and CRP. To examine the patients' compliance with treatment, we asked these patients to provide the used ampoules of tocilizumab and the used cartridges of methotrexate, leflunomide and prednisolone tablets at each visit.

The current study conforms to the principles outlined in the Declaration of Helsinki. The study protocol was approved by the Ethics Committee of University General Hospital "Attikon" and written informed consents were obtained from all participants.

## 2.2. Endothelial glycocalyx

We measured the perfused boundary region (PBR) of the sublingual arterial microvessels with a lumen diameter ranged from 5 to 25  $\mu\text{m}$  using Sidestream Darkfield (SDF) imaging (Microscan, Glycocheck, Microvascular Health Solutions Inc., Salt Lake City, Utah, USA). This technique provides a fast and non-invasive assessment of the endothelial glycocalyx thickness. The PBR represents the depth of penetration of red blood cells (RBC) into endothelial glycocalyx. The normal value of PBR is  $2 \pm 0.2 \mu\text{m}$  according to our measurements performed in healthy subjects (Laboratory of Preventive Cardiology and Echocardiography Department, Attikon Hospital, National and Kapodistrian University of Athens, Greece). Higher PBR values indicate a greater penetration of RBC towards the endothelium and reflect a damaged endothelial glycocalyx. In contrast, lower PBR values reflect a preserved endothelial glycocalyx thickness. Glycocalyx damage may be an early manifestation of endothelial dysfunction in atherosclerosis, such that measuring glycocalyx thickness in these patients may help to stratify their future cardiovascular risk. The assessment of glycocalyx thickness using SDF imaging lasts 3 min, provides recording and automated analysis of >3000 microvessel segments of sublingual microcirculation and has satisfactory reproducibility (Ikonomidis et al., 2019c). Hence, this technique was proposed as a valid method to assess endothelial function by the European Society of Cardiology Working Group on Peripheral Circulation (Lekakis et al., 2011). The PBR values are independent of hematocrit and heart rate, because the software only records vessel segments that have a filling percentage of more than 50%. Thus, vessel segments are only selected when at least 11 of the total 21 marker lines have a positive signal for the presence of a RBC. Hence, PBR measurements are independent of RBC filling of the vessel segments which is affected by hematocrit or heart rate.

## 2.3. Arterial stiffness

Carotid-femoral pulse wave velocity (PWV-m/s), augmentation index (AI-%) and central aortic systolic and diastolic pressures were measured using tonometry by Complior (Alam Medical, Vincennes, France). Normal values were PWV <10 m/s (Williams et al., 2018). AI was defined as  $[(P2 - P1)/\text{pulse pressure}] \times 100$ , where P2 is the late backward systolic wave and P1 is the early forward systolic wave (Ikonomidis et al., 2015).

## 2.4. Echocardiography

Studies were performed using a Vivid E95 (GE Medical Systems, Horten, Norway) ultrasound system. All studies were digitally stored in a computerized station (EchoPac GE 202, Horten, Norway) and analyzed by two observers, who had no access to clinical and laboratory data.

### 2.4.1. 2D strain and strain rate analysis

In all patients we measured global LV longitudinal systolic strain (GLS-%) and peak diastolic strain rate (GLSR E-1/s) from standard two-dimensional (2D) echocardiography images obtained with a frame rate of 70–80/s, from the apical 4, 2, and 3 chamber views using 17 LV segment model and utilizing a dedicated software (EchoPac PC 203, GE Healthcare, Horten, Norway), as previously published (Ikonomidis et al., 2019c, 2015). The normal value for GLS is considered to be  $-22.5 \pm 2.7\%$  (Sugimoto et al., 2017; Ikonomidis et al., 2014). Additionally, we measured global circumferential (GCS-%) and global radial strains (GRS-%) for the 6 mid-LV segments of parasternal short axis at the level of papillary muscles as previously published. (Sugimoto et al., 2017; Ikonomidis et al., 2014). The normal value for GCS and GRS is considered to be  $-31.9 \pm 4.5\%$  and  $37.4 \pm 8.4\%$ , respectively. The intra- and inter-observer reproducibility for LV strain and strain rate were 8% and 9% respectively.

Additionally, we assessed the ratio of carotid-femoral PWV to global longitudinal strain (PWV/GLS-m/s%) as an index of ventricular-arterial interaction, as previously published (Ikonomidis et al., 2019a, 2019d). The ratio had negative values because of negative GLS values and the more negative the value, the more normal.

### 2.4.2. Myocardial work index

We measured the myocardial global work index (GWI-mmHg%) as the area under the curve from mitral valve closure to mitral valve opening using dedicated software (EchoPac PC 203, GE Healthcare, Horten, Norway) to construct pressure-LV longitudinal myocardial strain loop by speckle tracking echocardiography (Ikonomidis et al., 2019a; Manganaro et al., 2020). Furthermore, the software calculated the global constructive work (GCW-mmHg%) as work performed during shortening in systole adding negative work during lengthening in isovolumetric relaxation and the wasted myocardial work (GWW-mmHg%) as negative work performed during lengthening in systole adding work performed during shortening in isovolumetric relaxation. The constructive work divided by the sum of constructive and wasted work provides the global work efficiency (GWE-%) (Manganaro et al., 2020). The inter- and intra-observer reproducibility for GWI were  $\leq 8\%$  and  $\leq 9\%$  respectively.

## 2.5. Laboratory assays

Rheumatoid factor (RF; positivity  $\geq 15$  IU/mL) was determined by nephelometry (Siemens Healthcare Diagnostics, Germany). Anticitrullinated protein antibodies (ACPA; positivity  $\geq 5$  U/mL) were measured using ELISA (Diatest, Axis-Shield Diagnostics Ltd., UK) (Sokolove et al., 2014). C-reactive protein (CRP) was determined by high-sensitivity particle-enhanced immunonephelometry (Dade Behring, Marburg, Germany; measurements range: 0.175–1100 mg/L) as previously published (Ikonomidis et al., 2019b). Malondialdehyde (MDA) and protein carbonyls (PCs) blood levels were measured spectrophotometrically with a commercial kit (Oxford Biomedical Research, Rochester Hills, Michigan, USA) of colorimetric assay for lipid peroxidation (measurements range 1–20 nmol/L) (Ikonomidis et al., 2019b).

## 2.6. Statistical analysis

All comparisons were performed with the Statistical Package for Social Sciences 22.0 for Windows (SPSS Inc., Chicago, Illinois, USA). Normally distributed variables are given as mean  $\pm$  standard deviation. Data with a not normally distribution are expressed as median with interquartile range (25th – 75th percentile) and were analyzed after transformation into ranks. Differences in mean values for each of the measured variables were compared by *t*-test or paired *t*-test for continuous variables with normal distributions, by Mann-Whitney test for continuous variables with parametric distribution, and by a chi-squared or Fisher's exact test for categorical variables. We used parametric

(Pearson) or nonparametric (Spearman) correlation coefficients to examine simple correlations.

Analysis of variance (ANOVA, general linear model, SPSS 22, SPSS Inc.) for repeated measurements was performed (a) for measurements of the examined markers at baseline and 3 months after treatment used as a within-subject factor and (b) for the effects of treatment (tocilizumab versus csDMARDs + GC) as a between-subject factors. The F and P values of the interaction between time of measurement of the examined markers and type of treatment were calculated. Furthermore, the F and P values of the comparison between treatments were calculated. The Greenhouse-Geisser correction was performed when the sphericity assumption, as assessed by Mauchly's test, was not met. Age, sex, cardiovascular risk factors, DAS28 and mean blood pressure were included in multivariate models as covariates. The percentage changes of the examined variables post-treatment between the treatment groups and the comparisons between healthy control subjects and each treatment group at baseline or at 3 months were also analyzed by factorial ANOVA. A p-value of less than 0.05 ( $P < 0.05$ ) was considered statistically significant.

We planned to study the percent change ( $\Delta$ ) of GWI after treatment from independent control (patients on csDMARDs + GC) and experimental subjects (patients on tocilizumab) with 1 control per experimental subject. In a pilot study of 10 patients treated with csDMARDs + GC and 10 treated with tocilizumab, the response within each group was normally distributed with standard deviation 10%. The true difference between patients treated with csDMARDs + GC and those treated with tocilizumab in the means of  $\Delta$ GWI was 10%. Therefore, we would need to study 40 patients treated with csDMARDs + GC and 40 treated with tocilizumab, to be able to reject the null hypothesis that the population means for  $\Delta$ GWI post-treatment of the csDMARDs + GC and tocilizumab groups are equal with probability (power) 0.8 and type-I error probability 0.05.

### 3. Results

Clinical characteristics such as age, BMI, sex, cardiovascular risk factors and medications were similar between the study groups (Table 1). At baseline, all patients had similar values of disease activity score in 28 joints (DAS28), biomarkers, blood lipid levels and markers of endothelial, vascular and LV myocardial function (Table 2;  $P > 0.05$  for all comparisons). None of the patients were withdrawn from the study owing to adverse effects or inadequate response to treatment.

DAS28 was similarly improved in all patients 3 months post-treatment (Table 2;  $P = 0.697$ ). Total cholesterol, low-density lipoprotein cholesterol (LDL-C) and triglycerides increased in patients treated with tocilizumab compared to those under csDMARDs + GC ( $P < 0.05$  for all comparisons, data not shown).

#### 3.1. Effects of treatment on endothelial glycocalyx thickness and vascular function

A significant interaction between the type of treatment and the change of PBR, brachial systolic and diastolic blood pressure, central systolic blood pressure and PWV was observed post-treatment ( $F = 6.417$ ,  $P$  for interaction = 0.012,  $F = 4.306$ ,  $P = 0.043$ ,  $F = 4.117$ ,  $P = 0.047$ ,  $F = 4.613$ ,  $P = 0.038$  and  $F = 5.395$ ,  $P = 0.028$ , respectively) in a model including age, sex, cardiovascular risk factors and DAS28 (and mean blood pressure for the models analyzing PBR and PWV). Compared with baseline, the percent decrease in PBR (indicating improved endothelial glycocalyx), brachial systolic and diastolic pressure, central systolic blood pressure and PWV was greater after tocilizumab than after csDMARDs + GC ( $-8.5\%$  versus  $+1.3\%$ ,  $-4.6\%$  versus  $-1.4\%$ ,  $-4.8\%$  versus  $-2.3\%$ ,  $-4.2\%$  versus  $-1.8\%$ , and  $-7\%$  versus  $+2.2\%$ , respectively;  $P < 0.05$  for all comparisons; Table 2, Fig. 1A).

Compared with baseline, all patients had reduced augmentation index (AI) post-treatment ( $P = 0.019$ ). However, there was a significant

**Table 1**

Baseline characteristics of the study population.

	Tocilizumab (n = 40)	csDMARDs + GC (n = 40)	Controls (n = 40)	P-value
Disease duration, y	11 (1–26)	10 (1–25)	–	0.892
RF, IU/mL	14.7 ± 2.3	14 ± 2.2	–	0.662
ACPA, U/mL	4.5 ± 2	4.8 ± 1.9	–	0.428
Age, y	64 ± 10	63 ± 8	63 ± 9	0.916
BMI, Kg/m <sup>2</sup>	30 ± 5	29 ± 4	29 ± 5	0.659
Female sex, n (%)	31 (77.5)	30 (75)	31 (77.5)	0.932
Risk factors, n (%)				
Smoking	12 (30)	13 (32.5)	12 (30)	0.828
Hypertension	19 (47.5)	21 (52.5)	20 (50)	0.252
Dyslipidemia	13 (32.5)	13 (32.5)	13 (32.5)	0.982
Diabetes mellitus	8 (20)	10 (25)	8 (20)	0.495
Medication, n (%)				
ACE inhibitors	9 (22.5)	10 (25)	9 (22.5)	0.793
β-Blockers	5 (12.5)	5 (12.5)	5 (12.5)	0.925
Ca <sup>2+</sup> channel blockers	9 (22.5)	10 (25)	10 (25)	0.878
Diuretics	12 (30)	11 (27.5)	11 (27.5)	0.792
Statins	5 (12.5)	5 (12.5)	4 (10)	0.789
Antidiabetics	3 (7.5)	3 (7.5)	4 (10)	0.857

Data are expressed as median values (first quartile - third quartile), mean values ± SD or number (%). Continuous variables were compared with the paired Student *t*-test. Binary variables were compared with the chi-square test. csDMARDs: conventional synthetic disease-modifying antirheumatic drugs; GC: glucocorticoids; RF: rheumatoid factor; ACPA: anti-citrullinated protein antibodies; BMI: body mass index; ACE: angiotensin-converting enzyme. P-value: P of model of the ANOVA for comparisons between groups.

interaction between the type of treatment and the change of AI post-treatment ( $F = 4.717$ ,  $P$  for interaction = 0.036; Table 2) after adjusting for age, sex, mean blood pressure, cardiovascular risk factors and DAS28. Thus, patients under tocilizumab showed a greater improvement of AI ( $-112\%$  versus  $-10\%$ ,  $P = 0.037$ ), compared to those under csDMARDs + GC.

#### 3.2. Effects of treatment on LV myocardial deformation

Compared with baseline, all patients had improved GLS ( $P = 0.011$ ), GLSR E ( $P = 0.041$ ), GCS ( $P = 0.039$ ) and GRSR E ( $P = 0.042$ ) after 3 months treatment. However, there was a remarkable interaction between the type of treatment and the change of GLS, PWV/GLS, GCS and GRS ( $F = 4.926$ ,  $P$  for interaction = 0.031,  $F = 6.213$ ,  $P = 0.017$ ,  $F = 4.967$ ,  $P = 0.031$  and  $F = 5.061$ ,  $P = 0.030$ , respectively) after adjusting for age, sex, cardiovascular risk factors, DAS28 and mean blood pressure. Treatment with tocilizumab caused the greatest improvement of GLS, GLSR E, GCS, GRS and GRSR E ( $+10.2\%$  versus  $+1.7\%$ ,  $+9\%$  versus  $+4\%$ ,  $+13.8\%$  versus  $+1.5\%$ ,  $+8.7\%$  versus  $+2.9\%$  and  $+12.7\%$  versus  $+7.4\%$ , respectively;  $P < 0.05$  for all comparisons). Compared with baseline, patients treated with tocilizumab had lower PWV/GLS ratio ( $P = 0.015$ ) post-treatment, while no significant changes were evident for PWV/GLS in patients that received csDMARDs + GC (Table 2;  $P = 0.933$ ).

#### 3.3. Myocardial work index

Compared with baseline, all patients had increased GWI ( $P = 0.002$ ), GCW ( $P = 0.003$ ), GWE ( $P = 0.035$ ) and reduced GWW ( $P = 0.028$ ) after 3 months treatment. Nevertheless, there was a significant interaction between the type of treatment and the change of GWI and GCW ( $F = 4.607$ ,  $P$  for interaction = 0.039 and  $F = 4.892$ ,  $P = 0.033$ , respectively) after adjusting for age, sex, cardiovascular risk factors, DAS28 and mean blood pressure. Patients on tocilizumab achieved a greater increase of



**Table 2**

Changes in disease activity markers, oxidative stress markers, endothelial glycocalyx thickness, vascular markers and echocardiographic markers of LV myocardial function in the study population during the study period.

	Tocilizumab (n = 40)		csDMARDs + GC (n = 40)		P-value	Controls (n = 40) Baseline
	Baseline	3 months	Baseline	3 months		
<b>DAS28</b>	6.1 (5.2–6.8)	3.7 (3.4–4.3)	5.6 (3.8–7)	4.1 (3.1–5.1)	0.241	–
<b>CRP, mg/L</b>	13.7 (7.2–40)	2.3 (0.1–5.5)	11.6 (6.5–36.2)	5.9 (2.1–10.2)	0.003	1.4 (0.01–2.0)*†
<b>MDA, μM/L</b>	1.8 (1.2–2.5)	1.0 (0.6–1.4)	2.0 (1.8–3.2)	1.9 (1.9–3.2)	0.003	0.97 (0.84–1.30) *†
<b>PCs, nmol/mg</b>	0.0136 (0.011–0.014)	0.0108 (0.008–0.014)	0.0129 (0.008–0.014)	0.0126 (0.007–0.013)	0.004	0.0093 (0.007–0.101) *†
<b>PBR, 5–25 μm</b>	2.14 ± 0.2	1.97 ± 0.2	2.2 ± 0.2	2.23 ± 0.3	0.012	2.04 ± 0.2*†
<b>SBP, mmHg</b>	130 ± 15	125 ± 14	132 ± 17	131 ± 16	0.043	129 ± 9
<b>DBP, mmHg</b>	81 ± 9	76 ± 9	82 ± 9	80 ± 8	0.047	80 ± 6
<b>Central SBP, mmHg</b>	134 ± 18	129 ± 16	135 ± 19	134 ± 18	0.038	131 ± 11*†
<b>PWV, m/s</b>	11 ± 3	10.3 ± 2	11.2 ± 2	11.5 ± 3	0.028	9.7 ± 2*†
<b>AI, %</b>	20 (12–26)	9.4 (4–25)	23 (14–32)	21 (6–24)	0.036	12.3 (1–27)*†
<b>LVEF, %</b>	65.5 ± 12	65.9 ± 12.5	64.6 ± 16	64.2 ± 15	0.463	66.2 ± 9
<b>Sm, cm/s</b>	8.9 ± 1.6	9.5 ± 2.1	8.5 ± 1.6	8.4 ± 1.9	0.574	9.4 ± 1.7*†
<b>Em/Am</b>	0.80 ± 0.4	0.86 ± 0.4	0.81 ± 0.3	0.85 ± 0.3	0.452	0.96 ± 0.3*†
<b>E/Em</b>	9.7 ± 3.9	8.7 ± 2.7	9.1 ± 3.3	9.5 ± 4.1	0.160	7.4 ± 2.4*†
<b>GLS, %</b>	–16.1 ± 2.9	–17.6 ± 2.5	–16.3 ± 3.2	–16.7 ± 2.8	0.031	–22.5 ± 2.2*†
<b>PWV/GLS, m/s%</b>	–0.68 ± 0.25	–0.58 ± 0.19	–0.68 ± 0.22	–0.69 ± 0.26	0.017	–0.45 ± 0.13*†
<b>GLSR E, 1/s</b>	0.91 ± 0.3	1 ± 0.3	0.95 ± 0.23	0.99 ± 0.26	0.118	1.2 ± 0.29*†
<b>GCS, %</b>	–17.5 ± 4.7	–20.3 ± 4.9	–17.6 ± 5	–17.9 ± 5.2	0.031	–24.9 ± 4.6*†
<b>GCSR E, 1/s</b>	1.09 ± 0.4	1.2 ± 0.4	1.17 ± 0.45	1.19 ± 0.43	0.084	1.35 ± 0.35*†
<b>GRS, %</b>	29.8 ± 9	32.3 ± 10	30.6 ± 10	31.6 ± 11	0.030	37.2 ± 9*†
<b>GRSR E, 1/s</b>	–1.91 ± 0.6	–2.19 ± 0.9	–2.1 ± 0.97	–2.3 ± 0.71	0.349	–2.6 ± 0.51*†
<b>GWI, mmHg%</b>	1769 ± 466	2014 ± 402	1752 ± 476	1802 ± 394	0.039	1935 ± 423*†
<b>GCW, mmHg%</b>	2073 ± 551	2312 ± 477	2014 ± 370	2142 ± 389	0.033	2231 ± 445*†
<b>GWW, mmHg%</b>	126 ± 74	97 ± 53	122 ± 62	108 ± 72	0.026	94 ± 42*†
<b>GWE, %</b>	92 ± 5	94 ± 4	93 ± 3	93 ± 4	0.910	95 ± 5*†

Data are expressed as median values (first quartile - third quartile) or mean values ± SD. csDMARDs: conventional synthetic disease-modifying antirheumatic drugs; GC: glucocorticoids; DAS28: disease activity score in 28 joints; CRP: C-reactive protein; MDA: malondialdehyde; PCs: protein carbonyls; PBR: perfused boundary region of the sublingual arterial microvessels ranged from 5 to 25 μm; SBP: systolic blood pressure; DBP: diastolic blood pressure; PWV: pulse wave velocity; AI: augmentation index; LVEF: left ventricular ejection fraction; Sm, Em, and Am by Tissue Doppler imaging; GLS: global longitudinal strain; PWV/GLS: ventricular-arterial interaction (pulse wave velocity to global longitudinal strain ratio); GLSR E global longitudinal early diastolic strain rate; GCS: global circumferential strain; GCSR E global circumferential early diastolic strain rate; GRS: global radial strain; GLSR E global radial early diastolic strain rate; GWI: global work index; GCW: global constructive work; GWW: global wasted work; GWE: global work efficiency. P-value: P for time × treatment interaction obtained by repeated measures ANOVA. \*P < 0.05 for controls versus tocilizumab at baseline; †P < 0.05 for controls versus prednisolone at baseline.

GWI, GCW and the greatest reduction of GWW compared with those under csDMARDs + GC post-treatment (+12.2% versus +2.7%, +10.3% versus +5.9% and –30% versus –13%, respectively; P < 0.05 for all comparisons; Table 2, Fig. 1B–D).

### 3.4. Effects of treatment on inflammation burden and oxidative stress

All patients had reduced CRP, MDA and PCs post-treatment (P < 0.05; Table 2). Compared with csDMARDs + GC, tocilizumab achieved a greater reduction of CRP (–83% versus –58%; F = 8.9, P = 0.004), MDA (–39% versus –11%; F = 13.1, P < 0.001) and PCs (–26% versus –2%; F = 11.2, P < 0.001) and (Table 2).

### 3.5. Interrelation of endothelial glycocalyx, vascular, myocardial function and oxidative stress markers

In the whole study population, the percent decrease of AI was positively correlated with the percent decrease of PBR (r = 0.34, P = 0.026) and inversely associated with the percent increase of GWI (r = –0.38, P = 0.018) and GCW (r = –0.29, P = 0.036).

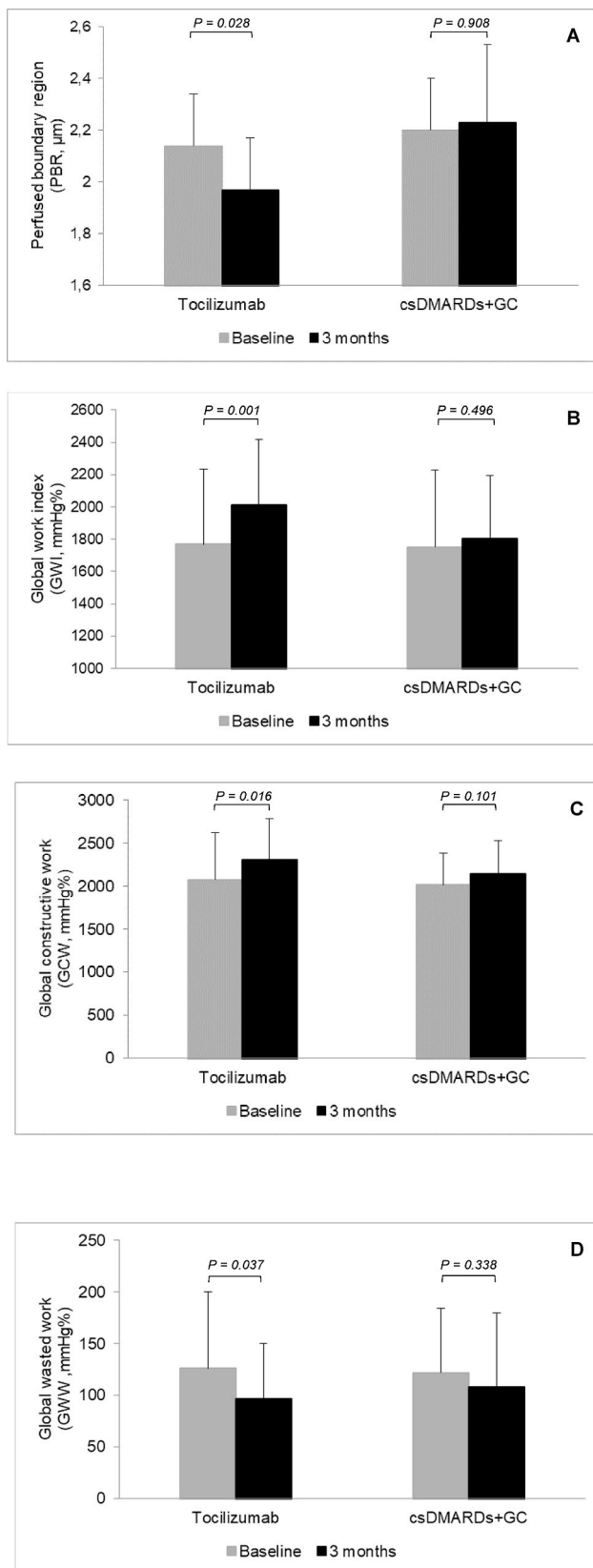
In patients under tocilizumab, the percent decrease of PBR (improved glycocalyx) was associated with the percent decrease of cSBP (r = 0.46, P = 0.037), PWV (r = 0.37, P = 0.026), AI (r = 0.38, P = 0.039), MDA (r = 0.41, P = 0.012), PCs (r = 0.38, P = 0.024), GLS (r = 0.43, P = 0.029), GCS (r = 0.58, P = 0.011) and inversely related with the percent improvement of GRS (r = –0.49, P = 0.047), GWI (r = –0.39, P = 0.041) and GCW (r = –0.42, P = 0.032).

## 4. Discussion

In the present study, we have shown that inhibition of IL-6 activity by tocilizumab improved endothelial glycocalyx thickness, as assessed by reduced PBR, and achieved a greater reduction of arterial stiffness, as estimated by PWV and AI of the aortic pulse wave, inflammatory burden as assessed by C-reactive protein (CRP) and oxidative stress markers compared to csDMARDs + GC treatment in patients with rheumatoid arthritis (RA). Furthermore, patients treated with tocilizumab showed a greater increase of LV myocardial deformation markers and myocardial work index attributed to an increase in constructive and decrease of wasted myocardial work than those treated with csDMARDs + GC after 3 months treatment.

Additionally, in the present study, tocilizumab treatment was associated with increased blood lipid levels. This finding is in line with previous studies showing that IL-6 inhibition increases total cholesterol and LDL-C by reducing various receptor surface levels and sPLA2-IIA expression leading to both decrease LDL-C and VLDL-C uptake in tissues (Ikonomidis et al., 2019b; McInnes et al., 2015; Gabay et al., 2016). However, this increase in blood lipid levels did not appear to counter-balance the beneficial effects of IL-6 inhibition on vascular and myocardial function as all examined vascular and myocardial deformation markers were improved post-tocilizumab treatment compared to csDMARDs + GC treatment in our study. In line with our findings, the risk of major adverse cardiovascular events during tocilizumab therapy was found to be associated with the control of disease activity but not lipid changes (Rao et al., 2015).

Interleukin-6 inhibition by tocilizumab has been shown to improve endothelial function as evaluated by flow mediated dilation (FMD), in



**Fig. 1.** (A) Perfused boundary region (PBR,  $\mu\text{m}$ ), (B) global work index (GWI, mmHg%), (C) global constructive work (GCW, mmHg%), and (D) global wasted work (GWW, mmHg%) in the two study groups pre- and post-treatment. The T lines on bars of the figures indicate standard deviation of the mean value.

RA patients (Kotani et al., 2017). In the present study, patients under tocilizumab had a remarkable improvement of endothelial glycocalyx, as assessed by PBR. The glycocalyx is the basis for endothelial cells - plasma interaction. A functioning glycocalyx promotes endothelium-dependent dilation by mechano-transducing shear stress (Yao et al., 2007). Inflammatory-mediated damage of glycocalyx by pro-inflammatory cytokines such as IL-1, IL-6, tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), and IL-8 leads to alterations in vascular permeability with associated interstitial fluid shift and generalized edema (Chelazzi et al., 2015). Furthermore, IL-6 is linked with its circulating soluble receptor forming a complex which binds to the membrane glycoprotein130 (Tanaka et al., 2016). This binding increases vascular endothelial growth factor production from endothelium causing loss of endothelial glycocalyx barrier properties. Glycocalyx damage increases vascular permeability to proteins and enhances contact of inflammatory cells to endothelium. Thus, glycocalyx degradation can be responsible for a number of several clinical condition including severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Tocilizumab blocks effectively both soluble and the cell membrane IL-6-receptor (Tanaka et al., 2016) restoring glycocalyx integrity and resistance to transcapillary water and protein permeability. On the other hand, certain clinical features of the COVID-19 infection can imitate those observed in systemic autoimmune diseases. Indeed, in a recent study, 68.7% of patients with COVID-19 were positive for any kind of systemic autoantibody. This finding suggests a post-SARS-CoV-2 or para-SARS-CoV-2 infectious autoimmune reactivation. The authors suggested that the presentation of cytokines, such as IL-6, in the cytokine storm, can lead to autoinflammatory activation and autoimmunity, probably through natural B-cells or molecular mimicry (Vlachoyiannopoulos et al., 2020). Tocilizumab is being used in patients with COVID-19, mostly in the advanced disease stage, on the basis of the previous experience with chimeric antigen receptor (CAR) T-cell immunotherapy-induced cytokine release syndrome (Zhang et al., 2020). Although, several phase II and III clinical studies of anti-IL-6 receptor antibodies are ongoing and will elucidate the beneficial effects of these drugs in COVID-19, earlier studies suggest that immunomodulatory therapy with tocilizumab may have a favorable role during the hyperinflammatory state of SARS-CoV-2 infection (Fernández-Ruiz et al., 2020). High costs and safety risks, including opportunistic infections, may be a barrier for its extensive use in COVID-19 (Zhao, 2020).

The greater reduction of inflammation and oxidative stress after tocilizumab treatment, as shown in a previous study (Ikonomidis et al., 2019b) and also confirmed in the present study, may explain the greater improvement of endothelial glycocalyx properties, as estimated by reduced PBR compared to csDMARDs + GC treatment in the present study. Indeed, in our study, the percent change of PBR was associated with a greater reduction of oxidative stress markers, namely MDA and PC. The prompt reduction of oxidative stress has been shown to cause a rapid glycocalyx cadherin externalization and gap junction restoration (Singh et al., 2013), restoring the perturbed glycocalyx permeability. Moreover, the percent reduction of PBR was correlated with a significant decrease of central systolic blood pressure and arterial stiffness post-tocilizumab treatment suggesting that glycocalyx impairment is associated with abnormal aortic elastic properties (Ikonomidis et al., 2018) in RA.

Previous studies have shown that IL-6 promotes vascular remodeling, fibrosis and increased arterial stiffness via increased transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1)-mediated matrix metalloproteinases (MMPs) 2 and 3 signaling (Kossakowska et al., 1999; Zhang et al., 2005; Wang et al., 2016). Furthermore, an experimental study has demonstrated that IL-6 blockade prevents degradation of aortic vascular smooth muscle cell contractile proteins  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) and smooth muscle protein 22- $\alpha$  (SM22 $\alpha$ ) and thus improves aortic wall properties (An et al., 2017). Indeed, in our study, the effects of IL-6 inhibition both on endothelial glycocalyx as well as on vascular smooth muscle and collagen turnover may have resulted in the greater improvement of

arterial elastic properties as showed by the significant decrease of PWV, augmentation index and reducing central and brachial blood pressure, confirming previous findings (Protogerou et al., 2011; Ikonomidis et al., 2019b). However, it is the first time to our knowledge that improvement of endothelial glycocalyx by IL-6 inhibition has been linked to improved arterial wall properties. On the other hand, increased CRP levels can promote arterial hypertension through up-regulation of the expression of angiotensin type 1 receptors in vascular smooth muscle (Wang et al., 2003) and vasoactive factor endothelin-1 (Verma et al., 2002). Thus, in the present study IL-6 inhibitor tocilizumab may have improved to a greater extent arterial stiffness and central arterial haemodynamics than treatment with csDMARDs + GC due to the greater reduction of inflammatory burden as assessed by CRP, oxidative stress and greater improvement of endothelial glycocalyx.

Deformation analysis by speckle tracking echocardiography revealed early development of myocardial dysfunction despite normal ejection fraction in RA (Løgstrup et al., 2017) while diastolic dysfunction is also over-represented in RA patients and is associated with higher circulating IL-6 levels (Liang et al., 2010). An impaired microcirculation is shown to reduce myocardial deformation (Løgstrup et al., 2012) and together with elevated oxidative stress and a cytokine-induced myocardial collagen deposition and fibrosis, it may lead to LV dysfunction (Ikonomidis et al., 2008). In line with the above findings, in our study, patients under tocilizumab had improved LV myocardial strain and strain rate (longitudinal, circumferential and radial) and showed more effective cardiac work, as estimated by an increase of global myocardial work index, related to an increase in constructive and decrease in wasted work, compared to the group of csDMARDs + GC. These findings may be interpreted both by the decrease of oxidative stress and by the reduction of arterial stiffness and central arterial haemodynamics, as observed in this study post-tocilizumab treatment. Modulation of afterload through decrease of arterial stiffness and vascular resistance, in addition to reduction in blood pressure, may lead to reduced myocardial oxygen demand, improved coronary perfusion in diastole, and thus improvement of subendocardial blood flow and LV performance (Ikonomidis et al., 2019a). In the present study, improved LV myocardial function markers post-tocilizumab treatment, were associated with the respective reduction of abnormal wave reflection, as assessed by AI and the improvement of endothelial glycocalyx. These findings suggest that IL-6 inhibition has beneficial effects on both vascular and myocardial function, improves ventricular-arterial interaction (improved PWV/GLS ratio), leading to a more efficient myocardial work and LV performance and thus prevents wasting of myocardial energetics and reserves in RA.

#### 4.1. Study limitations

The study design does not permit to investigate the causality for the changes in endothelial glycocalyx thickness and LV myocardial effective work post-tocilizumab treatment. Moreover, our study was a single-center trial, not blinded to patients. Further prospective large-scale clinical trials are required to determine whether and to what extent improvement of endothelial glycocalyx arterial elasticity and LV myocardial performance are maintained over time.

#### 5. Conclusions

IL-6 inhibition by tocilizumab improves endothelial glycocalyx thickness and arterial elastic properties leading to a greater increase of effective myocardial work compared to csDMARDs + GC likely through a profound reduction of inflammatory burden and oxidative stress after 3 months treatment in patients with RA. The favorable effects of tocilizumab on vascular permeability as assessed by endothelial glycocalyx and myocardial function may at least partly explain the reported beneficial effects of this treatment on diseases with excess IL-6 release such as SARS-CoV-2.

#### CRediT authorship contribution statement

**Ignatios Ikonomidis:** Conceptualization, Methodology, Formal analysis, Writing - review & editing, Supervision. **George Pavlidis:** Formal analysis, Writing - original draft, Visualization. **Pelagia Katsimbri:** Investigation, Resources. **Vaia Lambadiari:** Conceptualization, Supervision. **John Parissis:** Investigation. **Ioanna Andreadou:** Investigation. **Maria Tsoumani:** Investigation. **Dimitrios Boumpas:** Supervision. **Dimitrios Kouretas:** Investigation. **Efstathios Iliodromitis:** Supervision, Resources.

#### Declaration of competing interest

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

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