ORIGINAL RESEARCH ARTICLE

Neutrophils pro-inflammatory and anti-inflammatory cytokine release in patients with heart failure and reduced ejection fraction

Diana Chaar[1,2], Benjamin Dumont[1,3], Branka Vulesevic[1], Paul-Eduard Neagoe[1], Agnes Rakel[2,4], Martin G. Sirois[1,3]* and Michel White[1,2]*

[1]Research Center, Montreal Heart Institute, 5000 Belanger Street, Montreal, Quebec H1T 1C8, Canada; [2]Department of Medicine, Université de Montréal, Montreal, Quebec, Canada; [3]Department of Pharmacology and Physiology, Faculté de Medicine, Université de Montréal, Montreal, Quebec, Canada; [4]Research Center, Centre Hospitalier de l'Université de Montréal (CHUM), Université de Montréal, Montreal, Québec, Canada

Abstract

Aims   Heart failure with reduced ejection fraction (HFrEF) is characterized by sub-clinical inflammation. Changes in selected biomarkers of inflammation concomitant with the release of pro-inflammatory and anti-inflammatory cytokines by neutrophils have not been investigated in patients with HFrEF.
Methods and results   Fifty-two patients, aged 68.8 ± 1.7 years, with HFrEF and left ventricular ejection fraction 28.7 ± 1.0%, and 21 healthy controls (CTL) were recruited. Twenty-five HF patients had type 2 diabetes. Venous blood samples from HF and CTL were collected once. Neutrophil-derived pro-inflammatory and anti-inflammatory cytokine levels were assessed in plasma by ELISA. Plasma biomarkers assessed included: C-reactive protein (CRP), vascular endothelial growth factor (VEGF), interleukins (IL)-6, -8, -1 receptor antagonist (-1RA), nitric oxide (NO), soluble intercellular adhesion molecule-1 (sICAM-1), vascular cell adhesion molecule 1 (sVCAM-1) and E-Selectin (sE-Sel). Neutrophils were isolated and stimulated with various agonists to promote VEGF, IL-6, IL-8, and IL-1RA release. Compared with CTL, HFrEF patients showed a marked decrease in circulating VEGF [178.0 (interquartile range; IQR 99.6; 239.2) vs. 16.2 (IQR 9.3; 20.2) pg/mL, $P \leq 0.001$] and NO [45.2 (IQR 42.1; 57.6) vs. 40.6 (IQR 30.4; 47.1) pg/mL, $P = 0.0234$]. All other circulating biomarkers were significantly elevated. Neutrophils isolated from patients with HFrEF exhibited a greater IL-8 release in response to LPS [1.2 ± 0.1 (CTL); 10.4 ± 1.6 ng/mL (HFrEF) and 12.4 ± 1.6 ng/mL (HFrEF and DM), $P \leq 0.001$]. IL-6 release in response to LPS was not changed in HFrEF patients without diabetes, whereas it was significantly increased in patients with HFrEF and diabetes [46.7 ± 3.9 (CTL) vs. 165.8 ± 48.0 pg/mL (HFrEF), $P = 0.1713$ and vs. 397.7 ± 67.4 pg/mL (HFrEF and DM), $P \leq 0.001$]. In contrast, the release of VEGF and IL-1RA was significantly reduced in HFrEF (VEGF; TNF-α: 38.6 ± 3.1 and LPS: 25.3 ± 2.6 pg/mL; IL1RA; TNF-α: 0.6 ± 0.04 and LPS: 0.3 ± 0.02 ng/mL) compared with CTL (VEGF; TNF-α: 60.0 ± 9.4 and LPS: 41.2 ± 5.9 pg/mL; IL1RA; TNF-α: 3.3 ± 0.2 and LPS: 2.3 ± 0.1 ng/mL).
Conclusions   Patients with HFrEF exhibit a significant decrease in circulating VEGF. The release of VEGF and both pro-inflammatory and anti-inflammatory cytokines from the stimulated neutrophils is markedly altered in these patients. The clinical significance of these findings deserves further investigation.

Introduction

Heart failure (HF) is characterized by a broad-spectrum increase in biomarkers related to neuro-humoral activation, extracellular matrix turnover, and myocardial remodelling.[1,2] Similarly, patients with HF exhibit a significant elevation of markers related to subclinical inflammation and oxidative stress.[1,2] The increase in many of these

markers has been related to an adverse outcome in these patients.[3,4]

Neutrophils are leukocytes acting as the first line of host defense against pathogens but also in inflammation-mediated injury. More recently, neutrophilia (higher neutrophil count), lymphopenia (lower lymphocyte count), and a greater neutrophil to lymphocyte ratio (NLR) have been associated with HF severity, complications following LVAD insertion, and overall mortality in patients with various aetiologies of HF.[5–8] An increase in neutrophil blood count has not only been shown to correlate with the severity of coronary damage in patients with coronary artery diseases (CAD)[9,10] but also with the presence of heart failure.[11,12] Despite the prominent role of neutrophils in inducing the chronic inflammatory response in the pathogenesis of many diseases,[13] their role in the pathophysiology of heart failure and the impact of neutrophils targeted therapy remain largely unknown.

Several clinical and biochemical parameters characterize higher risk HF patients. Among the clinical parameters, the presence of diabetes mellitus (DM) has been associated with an increase in early mortality following acute decompensated heart failure, overall hospitalizations, and long-term events in patients with heart failure.[14,15] High-risk patients, such as those with diabetes and HF, exhibit an increased activation of the innate system.[16,17] Despite the mounting evidences regarding the significant role of diabetes on the pathophysiology of heart failure and more specifically on inflammation, the changes in circulating biomarkers with the concomitant assessment of neutrophils-mediated pro-inflammatory and anti-inflammatory cytokine release have not been reported in HF patients with or without diabetes nor compared with healthy controls (CTL).

The primary objective of this study was to investigate the changes in circulating biomarkers and selected cytokines related to sub-clinical inflammation and their release from stimulated neutrophils in patients with HF and reduced ejection fraction (HFrEF) compared with CTL. The secondary objective was to explore the effects of diabetes and HF on these responses.

Methods

Population

The design of this mechanistic clinical study was a prospective non-randomized non-interventional investigation. A total of 27 patients with HF and 25 patients with HF and DM were prospectively studied at the Montreal Heart Institute (MHI). The blood collection from all patients (52) and 21 CTL was performed once. This study was approved by the Scientific Research Committee and the Ethics Committee of the MHI

(ethics No. ICM #01-406 and No. ICM #12-1374) and conforms to the principles outlined in the Declaration of Helsinki. Donors were informed about the procedures and signed a written informed consent before participating in the study.

Selection criteria for controls and for patients with heart failure

The CTL recruited for this study were eligible assuming they had no significant medical conditions nor were treated by any anti-inflammatory medication for at least 14 days prior to blood collection. Patients with HF and with NYHA classification of 2 or 3 were recruited from the MHI Heart Failure clinic. These patients were classified as HFrEF if their LVEF was ≤40%,[18,19] as documented by contrast ventriculography, magnetic resonance imaging, radionuclide ventriculography, or echocardiography assessed within the previous 12 months if no significant cardiac events occurred because the initial LVEF assessment. The patients had to be optimally treated with A-II modulating agents, beta-blocker, and mineralocorticoid antagonist agents unless not tolerated or contra-indicated. In addition to the previous inclusion criteria outlined above, patients with HF and DM required an HbA1c < 10% (13.4 mmol/L) and good glycaemic control by any available hypoglycaemic medications and treated with secondary preventive medication as per current guidelines. The most significant exclusion criteria were the presence of severe chronic pulmonary disease, chronic active inflammatory disease, severe renal failure (creatinine >250 μmol/L), liver dysfunction (blood transaminases ≥3-fold upper normal values), and ongoing malignancy. Other exclusion criteria included a recent myocardial infarction, stroke, or cardiac surgery (<3 months), HF NYHA class IV and/or unstable clinical condition. Patients with HF and DM, and CTL having ongoing and/or recent infection within 2 weeks prior to the study (as this would affect neutrophil counts) or had CRP values higher than 15 mg/L (suggesting some acute inflammatory status) were excluded from this study.

Study protocol—plasma, serum, and neutrophil collection

Venous blood samples from CTL and patients with HF with or without DM were collected in one SST serum separation tube (3.5 mL blood volume) and in 30 mL syringes pre-filled with the anticoagulant citrate Dextrose solution USP (ACD) Formula A (ratio ACD : blood 1:5; 45 mL blood volume). Following a 200 *g* centrifugation of the anticoagulated blood, 4 mL of platelet-rich plasma (PRP in ACD) were re-centrifuged (11 000 *g*, 2 min, 4°C) to obtain citrate platelet-free plasma (PFP). The SST tube was also centrifuged (1500 *g*, 15 min, RT) to obtain serum, and all serum and plasma samples were aliquoted and frozen at −80°C. Neutrophils were

isolated by Dextran sedimentation followed by Ficoll-Paque density gradient and re-suspended in RPMI medium supplemented with 25 mM HEPES and 1% penicillin/streptomycin as described previously.[20] Contamination with peripheral blood mononuclear cells was <0.1% as determined by morphological analysis and flow cytometry (data not shown), and viability was >98% (Trypan blue dye exclusion).[20] Pure neutrophil population (>99% purity) was used for all *in vitro* studies.

Biomarkers quantification

Plasma levels of interleukin (IL)-1 receptor antagonist (IL-1RA), IL-6, IL-8 and total nitric oxide (NO) and serum level of vascular endothelial growth factor (VEGF) were analysed by Quantikine ELISA (R&D Systems, Minneapolis, MN). The plasmatic levels of soluble intercellular adhesion molecule-1 (sICAM-1), vascular cell adhesion molecule 1 (sVCAM-1) and E-Selectin (sE-Sel) were analysed by a Luminex Assay multiplex kit using the Bio-Plex 200 analyser (Bio-Rad Laboratories, Saint-Laurent, QC). High-sensitivity CRP (hsCRP) in serum samples was quantified by nephelometry by the Clinical Biochemistry Laboratory at the MHI.

Neutrophil stimulation and treatments

Purified neutrophils (5×10^6/mL, 500 μL) were incubated in RPMI − 1640 solution (Gibco, Carlsbad, CA) supplemented with 5% fetal bovine serum (PAA laboratories, Etobicoke, ON) 1% penicillin/Streptomycin/Glutamax (P/S) (Gibco) and 25 mM HEPES (Sigma, Oakville, ON), and named RPMI (for complete RPMI-1640 solution). Neutrophils were then stimulated for 2 h with control vehicle (PBS), tumour necrosis factor-α (TNF-α; 10 ng/mL) (Peprotech, RockyHill, NJ) or bacterial lipopolysaccharide (LPS; *Escherichia coli* 0111:B4; 1 μg/mL) (Sigma) at 37°C, 5% CO_2. Upon stimulation, neutrophils were centrifuged at 900 *g* for 7 min and supernatants stored at −80°C. The selected aforementioned agonists (LPS or TNF-α) were used based on their corresponding capacity to promote VEGF, IL-1RA, IL-6, and IL-8 release by the neutrophils.[21,22]

Statistical analysis

Continuous variables are presented as mean ± SEM (normally distributed data) or as medians and interquartile range (IQR) and categorical values as proportions (%). Statistical comparisons were made using a two-way analysis of variance (ANOVA), followed by a Tukey's multiple comparison test (normally distributed data) or using a Kruskal–Wallis test, followed by a Dunn's multiple comparisons test. For *Table 1*, continuous variables were compared using either a one-way ANOVA or Student's *t*-test, whereas categorical variables were compared using a χ^2 test or a Fisher's exact test. Analyses were performed using GraphPad Prism 9.1.0 and differences were considered significant at $P \leq 0.05$.

Results

The clinical characteristics of the study population are presented in *Table 1*. The study population consisted of 21 CTL, 27 patients with HF without DM (HFrEF) and 25 HF patients with HF and DM (HFrEF + DM). Most patients had a HF caused by ischaemic heart disease. There was a higher proportion of patients with hypertension among patients with diabetes. There were no significant differences in LV size and ejection fraction between patients with or without diabetes. Patients with diabetes presented a higher use of A-II modulating agents and/or the combination of both ACE inhibitor and angiotensin receptor blockade. No patients were chronically treated by an angiotensin receptor/neprilysin inhibitor (ARNI). The levels of NT-proBNP were significantly elevated in patients with HF. By the time of recruitment in this study no patient were treated with a SGLT2 inhibitor. Both HF groups were significantly older than the CTL group. Pearson correlation analysis showed no significant correlation between all circulating biomarkers (except for CRP) and age for CTL.

The results for the circulating levels of selected biomarkers are presented in *Figures 1 and 2*. HF patients yielded a profound decrease (>90% reduction) in the circulating levels of VEGF. Similarly, circulating levels of nitric oxide (NO) were significantly reduced in patients with HF regardless of the presence or absence of diabetes. Patients with HF exhibited significant increased levels of hsCRP, sICAM-1, sVCAM-1, sE-Sel (HFrEF + DM only), IL-6, and IL-8, as well as an elevation of the circulating levels of the anti-inflammatory cytokine IL-1RA. In addition, there was a significant increase in the circulating levels of IL-6, IL-8, and IL-1RA ($P < 0.001$) and a non-significant decrease ($P = 0.0617$) in circulating metabolites of NO in patients with HFrEF + DM as compared with patients with HFrEF without diabetes.

Effect of pro-inflammatory agonists on the release of selected cytokines by neutrophils

We assessed the capacity of selected pro-inflammatory agonists to induce the release of cytokines known to be expressed and released by human neutrophils.[22–24] We assessed the effect of a 2 h treatment with either TNF-α (10 ng/mL) and/or LPS (1 μg/mL) compared with PBS-control vehicle to induce the release of VEGF, IL-1RA, IL-8, and IL-6 by neutrophils (5×10^6/mL, 500 μL) (*Figure 3*).

Table 1 Patient's demographics

	CTL (n = 21)	HFrEF (n = 27)	HFrEF + DM (n = 25)	P value
Age (years)	44.9 ± 2.71	68.89 ± 2.51	68.68 ± 2.18	˂0.001
Male	14 (66.6%)	19 (70.4%)	21 (84.0%)	0.36
NYHA class				0.39
II	-	11 (40.7%)	7 (28.0%)	
III	-	16 (59.3%)	18 (72.0%)	
HFrEF duration (months)		77.00 ± 15.52	88.28 ± 16.70	0.62
Aetiology				0.87
Ischaemia	-	20 (74.1%)	18 (72.0%)	
Cardiomyopathy	-	4 (14.81%)	2 (8.0%)	
Valvular	-	1 (3.7%)	1 (4.0%)	
Others	-	2 (7.4%)	4 (16.0%)	
LVEF	-	30.15 ± 1.39	27.08 ± 1.21	0.10
LVIDD	-	60.96 ± 1.75	61.12 ± 1.71	0.95
LVIDS	-	50.25 ± 2.36	49.71 ± 2.01	0.86
Hypertension	-	15 (55.5%)	24 (96.0%)	˂0.001
Dyslipidaemia	-	21 (77.8%)	22 (88.0%)	0.47
Stroke	-	3 (11.1%)	4 (16.0%)	0.70
Haematology & Biochemistry				
Haemoglobin (g/L)	-	132.19 ± 2.09	127.52 ± 2.89	0.19
Leucocytes (×10^9/L)	-	7.75 ± 0.43	7.96 ± 0.34	0.71
Na (mmol/L)	-	137.62 ± 0.61	137.12 ± 0.63	0.57
K (mmol/L)	-	4.34 ± 0.07	4.48 ± 0.09	0.22
Urea (mmol/L)	-	11.58 ± 1.10	11.36 ± 0.90	0.88
Creatinine (μmol/L)	-	139.22 ± 11.4	128.76 ± 8.20	0.47
NT-proBNP	-	2754 ± 567	2490 ± 651	0.76
Medication				
ACEi	-	16 (59.3%)	16 (64.0%)	0.78
ARB	-	9 (33.3%)	15 (60.0%)	0.09
Beta-blockers	-	27 (100%)	24 (96.0%)	0.48
Diuretic agent	-	27 (100%)	23 (92.0%)	0.23
Statin	-	19 (70.4%)	20 (80.0%)	0.53
Anti-platelets	-	24 (88.9%)	24 (96.0%)	0.61
Metformin	-	-	8 (32.0%)	
Sulfonylureas	-	-	8 (32.0%)	
α-glucosidase inhibitors	-	-	-	
DPP-4 inhibitor	-	-	6 (24.0%)	
GLP-1 agonist	-	-	1 (4.0%)	
SGLT2 inhibitor	-	-	-	
Insulin	-	-	7 (28.0%)	

ACEi, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker; CTL, healthy controls; DM, type-2 diabetes mellitus; DPP-4, dipeptidyl peptidase 4; GLP-1, glucagon-like peptide 1; HFrEF, heart failure with reduced ejection fraction; LVEF, left ventricular ejection fraction; LVIDD, left ventricular internal diameter end diastole; LVIDS, left ventricular internal diameter end systole; NYHA, New York Health Association; SGLT2, sodium-glucose co-transporter-2.
Continuous variables are shown as mean ± standard error mean and categorical variables as number (%). Anti-platelets included aspirin, clopidogrel, prasugrel, or ticagrelor or a combination of these agents.

At baseline (PBS), we observed a non-significant increase of the neutrophils secretion of pro-inflammatory biomarkers (VEGF, IL-6, and IL-8) and a significant decrease of the anti-inflammatory IL-1RA in both HFrEF and HFrEF + DM patients compared with CTL (*Figure 3A–D*). No significant differences were observed in the basal biomarkers secretion between HFrEF and HFrEF + DM.
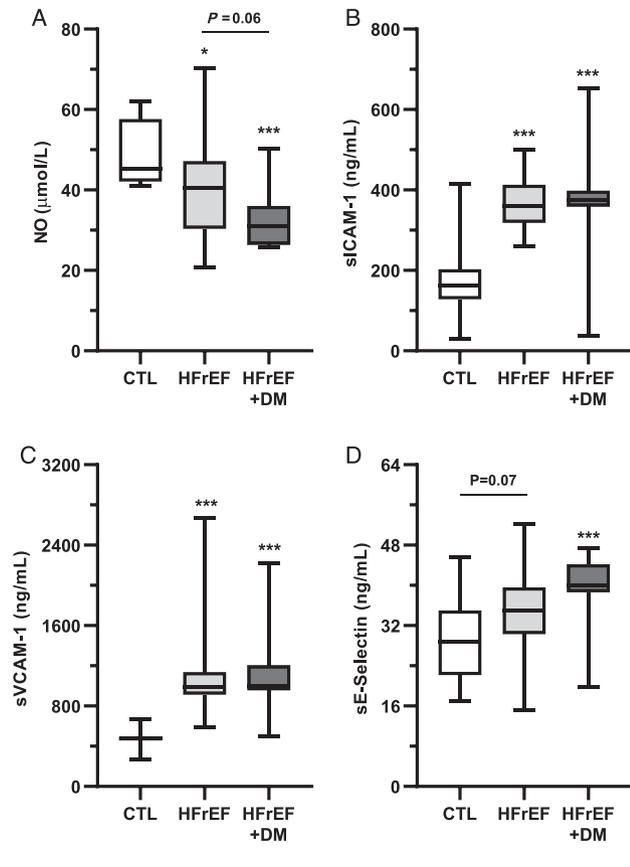
Upon stimulation with either TNF-α (10 ng/mL) or LPS (1 μg/mL), all secreted biomarkers from CTL neutrophils were increased, with VEGF and IL-1RA being statistically significant (*Figure 3A–D*). VEGF release following TNF-α and LPS stimulation of isolated neutrophils from HFrEF patients was significantly reduced by 35.7% and 38.6% respectively while isolated neutrophils from patients with HFrEF + DM yielded a 38.7% (TNF-α) and 38.3% (LPS; not significant) decrease in VEGF compared with CTL (*Figure 3A*). No

significant differences were observed in either TNF-α- or LPS-induced biomarkers secretion between HFrEF and HFrEF + DM.

Upon TNF-α and LPS stimulation, IL-1RA secretion from HFrEF neutrophils was significantly reduced by 81.8% and 87.0% respectively, and by 69.7% and 73.9% in HFrEF + DM compared with CTL neutrophils (*Figure 3B*). There was a higher but a non-significant increase of IL-1RA secretion from HFrEF + DM, compared with HFrEF neutrophils, independently from the agonist used.

Neutrophils from patients with HFrEF and HFrEF + DM were significantly more potent than those from CTL to secrete IL-8 by a magnitude of 10-fold following LPS stimulation (*Figure 3C*). No significant differences were observed in either TNF-α- or LPS-induced biomarkers release between HFrEF and HFrEF + DM.

Figure 1 Measure of circulating endothelial biomarkers. Plasma from all three groups [healthy volunteers (CTL), HFrEF without DM (HFrEF) and HFrEF with DM (HFrEF + DM)] was analysed by ELISA for NO (Nitrite/Nitrate) and by Luminex multiplex for sICAM-1, VCAM-1, and E-Selectin. (A) Plasmatic NO from all HFrEF patients decreased significantly, compared with healthy volunteers (CTL). On the other hand, all soluble adhesion molecules [sICAM-1 (B), sVCAM-1 (C), and sE-Selectin (D)] increased in all HFrEF patients, compared with CTL. Data are presented as medians and IQR. $^*P < 0.05$ and $^{***}P < 0.001$ as compared with CTL.

Figure 2 Measure of circulating inflammation biomarkers. Plasma from all three groups [healthy volunteers (CTL), HFrEF without DM (HFrEF) and HFrEF with DM (HFrEF + DM)] was analysed by ELISA for VEGF, IL-8, IL-6, and IL-1RA and by nephelometry for CRP. (A) Plasmatic VEGF from all HFrEF patients decreased significantly, compared with healthy volunteers (CTL). (B) Plasmatic CRP, (C) IL-8 and (D) IL-6 were significantly increased in both HFrEF and HFrEF + DM patients compared with CTL, whereas plasmatic IL-1RA was increased significantly only in patients with HFrEF + DM compared with CTL. HFrEF + DM yielded higher plasmatic concentration of (C) IL-8, (D) IL-6, and (E) IL-1RA, compared with HFrEF. Data are presented as medians and IQR. $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with CTL. $^{†††}P < 0.001$ compared with HFrEF.
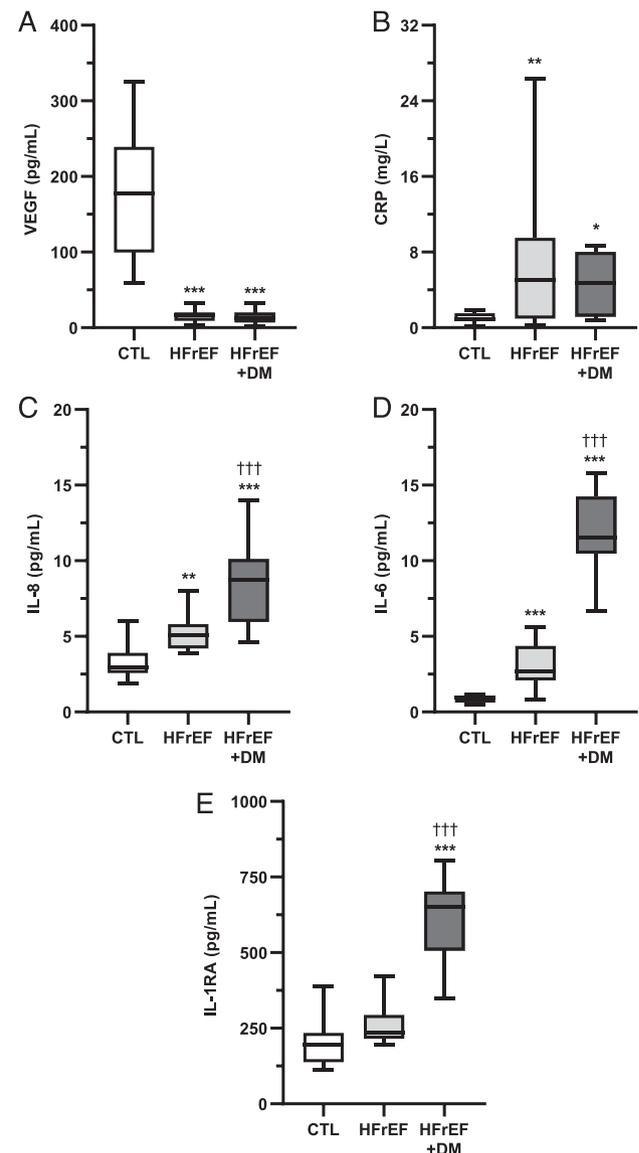

Because TNF-α is unable to promote IL-6 release[25] we only used LPS as agonist. LPS-induced IL-6 secretion from HFrEF and HFrEF + DM neutrophils was increased by 4- and 8.5-fold respectively compared with neutrophils from CTL (*Figure 3D*). Neutrophils from HFrEF + DM secreted a significantly higher amount of IL-6 (2.4-fold) compared with those isolated isolated from patients with HFrEF without diabetes.

Discussion

In this clinical mechanistic study, we reported a significant increase in the markers of vascular inflammation (sICAM-1, sVCAM-1, and sE-Sel), pro-inflammatory (IL-6 and IL-8) and anti-inflammatory (IL-1RA) interleukins in patients with HF. In contrast we observed a marked decrease in both circulating VEGF, a pro-angiogenic marker, and NO, a vascular relaxing factor in these patients. Under basal conditions, neutrophils from HF patients were primed to release VEGF, IL-6, and IL-8 in excess, while the release of the anti-inflammatory cytokine IL-1RA was significantly reduced. Neutrophils from

Figure 3 Effect of TNF-α and LPS on the release of pro-inflammatory biomarkers by human neutrophils. Isolated neutrophils from all three groups [healthy volunteers (CTL), HFrEF without DM (HFrEF), and HFrEF with DM (HFrEF + DM)] were stimulated with TNF-α (10 ng/mL) or LPS (1 μg/mL) for 2 h and released VEGF, IL-1RA, IL-8, and IL-6 were quantified by DuoSet ELISA. Neutrophils from CTL exhibited a significant increase in (A) VEGF, (B) IL-1RA, and (C) IL-8 following TNF-α and LPS stimulation. In all HFrEF groups, (A) VEGF and (B) IL-1RA release was lower, whereas (C) IL-8 release was significantly increased, compared with CTL. The release of IL-6 from neutrophils (D) was only achieved by LPS stimulation and was significantly increased in HFrEF + DM, compared with CTL and HFrEF. Data are presented as mean ± SEM. $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with respective PBS. $^{†††}P < 0.001$ compared with CTL. $^{###}P < 0.001$ compared with HFrEF.
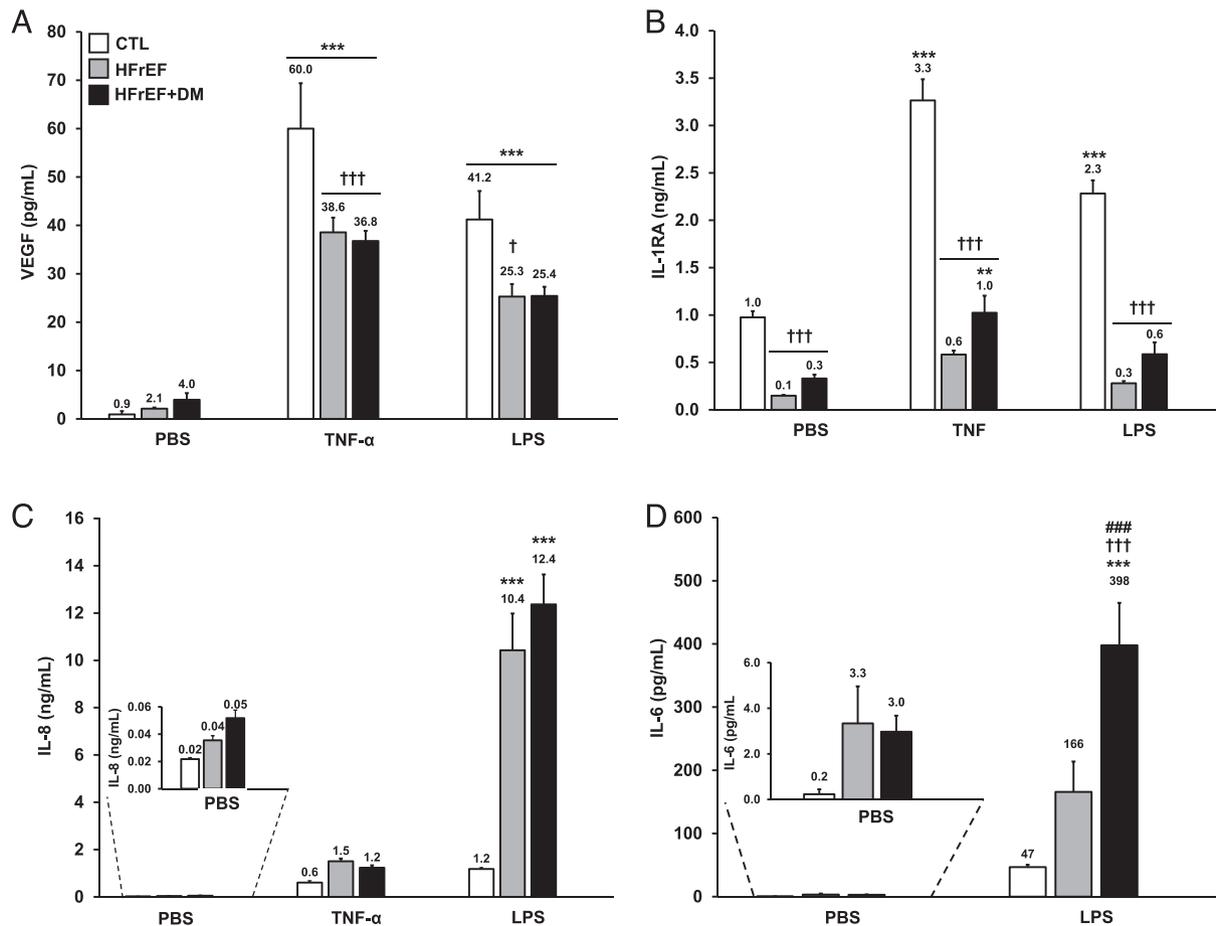
patients with both HF and diabetes exhibited a greater response to LPS for IL-6 and IL-8 release, whereas they were less efficient to release VEGF and IL-1RA following LPS or TNF-α stimulation.

Systemic inflammation has been recognized as a pathobiologic feature of both acute and chronic HF. The presence of sub-clinical inflammation has been associated with the development, progression, and complication of HF, and has been predictive of poor outcome independently of other clinical parameters such as left ventricular ejection fraction or New York Heart Association functional class.[26] In addition, in patients with chronic HF, intestinal congestion and ischaemia increase the number of pathogenic bacteria in the intestine and contribute to further promote low grade-inflammation.[27,28]

A significant and a broad spectrum increase in circulating neuro-hormones and pro-inflammatory and pro-thrombotic biomarkers have been reported in HF patients.[3] Herein, we report a profound decrease in the circulating levels of VEGF in HF patients with advanced, yet stable disease. VEGF plays a significant role on angiogenesis upon vascular and tissue injury in humans.[29,30] In addition, VEGF may contribute to the growth of cardio-myofibroblasts, and has been reported to exert cytoprotective, anti-oxidative, and anti-apoptotic effects on the cardiomyocytes.[31,32] A decrease of circulating VEGF has been reported in animal models of advanced heart failure.[32,33] In the initial phases of HF, an increase of VEGF is observed in the myocardium, however, the exhaustion of VEGF release during prolonged hypoxia has been proposed as an important underlying factor contributing to a decrease

of myocardial capillary density and the transition to decompensated heart failure.[32,34] Our data are also in agreement with previous clinical studies reporting a decrease of circulating VEGF in patients with HF.[30,35]

The haemodynamic stress of HF such as an increase in wall tension and a decrease in peripheral blood flow trigger the release of an array of pro-inflammatory cytokines by the cardiomyocytes, cardiac fibroblasts, endothelial cells and circulating leukocytes. In addition, bacteria and bacterial cell component such as LPS are transferred into the blood because of the abnormality of intestinal barrier function and an increase in central venous pressure.[28] The combination of low circulating VEGF and increased inflammation of the coronary microvascular endothelium most likely lead to a dysregulation of NO generation, and an increase in the production of reactive oxygen species (ROS).[36] These latter observations are in agreement with the data reported here showing a reduction of circulating NO levels in patients with HF.

As previously reported, we observed a marked increase of soluble endothelial adhesion molecules (sICAM-1, sVCAM-1, and sE-Sel) in patients with HF.[2] This could be explained in part by an impaired synthesis and release of the VEGF-NO complex, as a decrease in endothelial NO synthesis induces the expression and translocation of endothelial adhesion molecules (ICAM-1, VCAM-1, and E-Sel).[37,38] This effect can also be exacerbated by the ability of CRP at concentrations known to be associated with an increased risk of cardiovascular events (>3 mg/L) to attenuate NO production.[39] In addition, CRP inhibits the expression of eNOS and NO synthesis and upregulates the expression of adhesion molecules.[39,40]

Herein, we report a significant increase of both circulating pro-inflammatory interleukins (IL-6 and IL-8). Circulating levels of IL-6 and IL-8 were significantly increased in patients with HF and diabetes. These observations are in agreement with our previous published work and other independent studies, supporting the concept of a vascular and systemic inflammation in HF.[26,41–43] Both IL-6 and IL-8 are known to play a significant role in both HF and diabetes.[26,42,43] In this study, we reported some small and inconsistent changes in the pro-inflammatory markers between diabetic and non-diabetic patients. These results may be explained by the differences in the aetiology, gender, and the severity of heart failure. Furthermore, once the main disease (HF) has evolved to a certain point, the impact of DM on these biomarkers may be significantly attenuated. Together, these data, along with a decrease in VEGF and NO, are supportive of a significant impairment in vascular and microvascular function in HF patients, which is most likely intensified by the presence of diabetes.[44,45]

IL-1RA, a member of the IL-1 family, is an anti-inflammatory and anti-proliferation cytokine.[46] Here we reported a significant increase in circulating IL-1RA only in patients with HF and diabetes. The reason for this remains unclear. Nevertheless, this may be associated with an increase in inflammatory status in these high-risk patients and consequently with a reactive increase in some counteracting anti-inflammatory mediators such as IL-1RA. As such, the increased inflammatory status in patients with HF and DM may be associated with a parallel reactive increase in IL-1RA levels by the neutrophils.[45,47]

Patients with HF have an increasingly activated innate immune system.[48] Data from large clinical studies and some smaller mechanistic investigations have shown some abnormalities of leucocytes and neutrophils in human heart failure.[5,48,49] Beyond circulating biomarkers, here we reported changes in the capability of the stimulated neutrophils to release some selected pro-angiogenic, and pro-inflammatory and anti-inflammatory biomarkers in patients with HF with or without DM. Basal neutrophil-mediated VEGF release was slightly higher in HF patients when compared with CTL. However, upon stimulation with pro-inflammatory agonists, VEGF release from the stimulated neutrophils was markedly decreased in patients with HF. This latter observation is in agreement with previous reports showing an attenuation of VEGF release by diabetic cardiomyopathic animals[50,51] and by the neutrophils in human cardiac transplant recipients.[52]

In this study, we further explore the impact of diabetes on neutrophils pro-inflammatory and anti-inflammatory responses. Although neutrophil-mediated IL-8 release was not modulated by the presence of diabetes, HF patients with DM exhibited a higher pro and anti-inflammatory leukocyte response. Although IL-8 release was similar in both groups, LPS-induced IL-6 release was greater in patients with HF and DM. IL-6 plays a pivotal role in the inflammatory response through its synthesis and secretion from the liver. In contrast, IL-8 is mostly produced by monocytes, endothelial cells and neutrophils. Because the most relevant cytokines involved in diabetes include IL-6, IL-1, IL-18, and TNF-α, the observations reported here are in agreement with a significant perturbation of IL-6 release in patients with both HF and diabetes.

Despite a significantly higher level of circulating IL-1RA, we observed a marked decrease in its release from the neutrophils harvested from patients with HF with or without DM either in basal condition or following stimulation. One explanation could be that the neutrophils from these patients are less efficient for promoting IL-1RA release, suggesting that the observed increase of circulating IL-1RA in these patients is not due to the contribution of neutrophils but most likely release by other cells such as monocytes, hepatocytes, epithelial cells and adipocytes.[53,54] In a previous study, we reported that the neutrophil basal release of IL-1RA from CTL was between 2% and 10% from its total cellular content, and even under LPS challenge the release of IL-1RA was only 35% of its total content. Thus, it is plausible that neutrophils are less efficient to induce IL-1RA synthesis or release in HF.

The sub-cellular events involved with these observations would be a matter for further investigations.

Study limitations

This clinical mechanistic study consisted of a small sample size of patients with various duration and aetiologies of HF. In addition, specific information on the level of congestion and haemodynamic changes and other relevant information such as the duration of diabetes and glycaemic control were not readily available. The patients were enrolled only once, and the lack of follow-up did not allow for the assessment of the effects of dynamic changes in biomarkers as well as the clinical consequences of these findings. As such, this study does not support the routine use of these biomarkers in the clinical setting at the present time. Neutrophils-mediated release in selected pro-inflammatory and anti-inflammatory cytokines was limited to a few agonists and selected cytokines. Other pro and anti-inflammatory cytokine such as IL-1, CCL-5 and IL-10, IL-30 and IL-33 are likely to play a significant role in this patient population and deserve further investigations.

Conclusions

HF with reduced ejection fraction is characterized by a significant increase in various pro-inflammatory markers and a decrease in circulating VEGF and IL-1RA. The release of VEGF and other pro-inflammatory and anti-inflammatory cytokines by the neutrophil is markedly abnormal in patients with chronic yet stable HF when compared with healthy controls. These observations support the significant role of neutrophils in HF and justify further studies to better understand the sub-cellular events and the clinical impacts of these findings in patients with various disease severity and different HF phenotypes.

Conflict of interest

The authors have no conflicts of interest to declare.

References

1. Dick SA, Epelman S. Chronic Heart Failure and Inflammation. *Circ Res* 2016; **119**: 159–176.
2. White M, Cantin B, Haddad H, Kobashigawa JA, Ross H, Carrier M, Pflugfelder PW, Isaac D, Cecere R, Whittom L, Ali IS, Wang S-H, He Y, Groulx A, Touyz RM. Cardiac signaling molecules and plasma biomarkers after cardiac transplantation: Impact of tacrolimus versus cyclosporine. *J Heart Lung Transplant* 2013; **32**: 1222–1232.
3. Welsh P, Kou L, Yu C, Anand I, van Veldhuisen DJ, Maggioni AP, Desai AS, Solomon SD, Pfeffer MA, Cheng S, Gullestad L, Aukrust P, Ueland T, Swedberg K, Young JB, Kattan MW, Sattar N, McMurray JJV. Prognostic importance of emerging cardiac, inflammatory, and renal biomarkers in chronic heart failure patients with reduced ejection fraction and anaemia: RED-HF study. *Eur J Heart Fail* 2018; **20**: 268–277.
4. Bachschmid MM, Dupuy AM, Curinier C, Kuster N, Huet F, Leclercq F, Davy JM, Cristol JP, Roubille F. Multi-Marker Strategy in Heart Failure: Combination of ST2 and CRP Predicts Poor Outcome. *Plos One* 2016; **11**: e0157159.
5. Arruda-Olson AM, Reeder GS, Bell MR, Weston SA, VrL R. Neutrophilia Predicts Death and Heart Failure After Myocardial Infarction. *Circ Cardiovasc Qual Outcomes* 2009; **2**: 656–662.
6. Durmus E, Kivrak T, Gerin F, Sunbul M, Sari I, Erdogan O. Neutrophil-to-Lymphocyte Ratio and Platelet-to-Lymphocyte Ratio are Predictors of Heart Failure. *Arq Bras Cardiol* 2015; **105**: 606–613.
7. Hassan A, Zughul R, Villines D. Prognostic significance of Absolute Neutrophil Count in Patients with Heart Failure with Preserved Ejection Fraction. *Asian Pac J Health Sci* 2016; **3**: 104–108.
8. Uthamalingam S, Patvardhan EA, Subramanian S, Ahmed W, Martin W, Daley M, Capodilupo R. Utility of the Neutrophil to Lymphocyte Ratio in Predicting Long-Term Outcomes in Acute Decompensated Heart Failure. *Am J Cardiol* 2011; **107**: 433–438.
9. Strassheim D, Dempsey EC, Gerasimovskaya E, Stenmark K, Karoor V. Role of Inflammatory Cell Subtypes in Heart Failure. *J Immunol Res* 2019; **2019**: 1–9.
10. Sharma K, Patel AK, Shah KH, Konat A. Is Neutrophil-to-Lymphocyte Ratio a Predictor of Coronary Artery Disease in Western Indians? *Int J Inflam* 2017; **2017**: 1–8.
11. Woitas RP, Schwab S. Neutrophil counts and their potential prognostic value in

cardiovascular diseases. *J Lab Precis Med* 2017; **2**: 30.

12. Pfister R, Sharp SJ, Luben R, Wareham NJ, Khaw KT. Differential white blood cell count and incident heart failure in men and women in the EPIC-Norfolk study. *Eur Heart J* 2011; **33**: 523–530.

13. Mårdh CK, Root J, Uddin M, Stenvall K, Malmgren A, Karabelas K, Thomas M. Targets of Neutrophil Influx and Weaponry: Therapeutic Opportunities for Chronic Obstructive Airway Disease. *J Immunol Res* 2017; **2017**: 1–13.

14. Kristensen SL, Preiss D, Jhund PS, Squire I, Cardoso JS, Merkely B, Martinez F, Starling RC, Desai AS, Lefkowitz MP, Rizkala AR, Rouleau JL, Shi VC, Solomon SD, Swedberg K, Zile MR, McMurray JJV, Packer M. Risk Related to Pre–Diabetes Mellitus and Diabetes Mellitus in Heart Failure With Reduced Ejection Fraction. *Circ Heart Fail* 2016; **9**: e002560.

15. Gerstein HC. The Hemoglobin A1c Level as a Progressive Risk Factor for Cardiovascular Death, Hospitalization for Heart Failure, or Death in Patients With Chronic Heart Failure. *Arch Intern Med* 2008; **168**: 1699.

16. Maack C, Lehrke M, Backs J, Heinzel FR, Hulot J-S, Marx N, Paulus WJ, Rossignol P, Taegtmeyer H, Bauersachs J, Bayes-Genis A, Brutsaert D, Bugger H, Clarke K, Cosentino F, De Keulenaer G, Dei Cas A, González A, Huelsmann M, Iaccarino G, Lunde IG, Lyon AR, Pollesello P, Rena G, Riksen NP, Rosano G, Staels B, van Laake LW, Wanner C, Farmakis D, Filippatos G, Ruschitzka F, Seferovic P, de Boer RA, Heymans S. Heart failure and diabetes: metabolic alterations and therapeutic interventions: a state-of-the-art review from the Translational Research Committee of the Heart Failure Association–European Society of Cardiology. *Eur Heart J* 2018; **39**: 4243–4254.

17. McHugh K, DeVore AD, Wu J, Matsouaka RA, Fonarow GC, Heidenreich PA, Yancy CW, Green JB, Altman N, Hernandez AF. Heart Failure With Preserved Ejection Fraction and Diabetes. *J Am Coll Cardiol* 2019; **73**: 602–611.

18. Grodin JL, Philips S, Mullens W, Nijst P, Martens P, Fang JC, Drazner MH, Tang WHW, Pandey A. Prognostic implications of plasma volume status estimates in heart failure with preserved ejection fraction: insights from TOPCAT. *Eur J Heart Fail* 2019; **21**: 634–642.

19. Pitt B, Pfeffer MA, Assmann SF, Boineau R, Anand IS, Claggett B, Clausell N, Desai AS, Diaz R, Fleg JL, Gordeev I, Harty B, Heitner JF, Kenwood CT, Lewis EF, O'Meara E, Probstfield JL, Shaburishvili T, Shah SJ, Solomon SD, Sweitzer NK, Yang S, McKinlay SM, Investigators T. Spironolactone for heart failure with preserved ejection fraction. *N Engl J Med* 2014; **370**: 1383–1392.

20. Lavoie SS, Dumas E, Vulesevic B, Neagoe P-E, White M, Sirois MG. Synthesis of Human Neutrophil Extracellular Traps Contributes to Angiopoietin-Mediated In Vitro Proinflammatory and Proangiogenic Activities. *J Immunol* 2018; **200**: 3801–3813.

21. Cassatella MA, Östberg NK, Tamassia N, Soehnlein O. Biological Roles of Neutrophil-Derived Granule Proteins and Cytokines. *Trends Immunol* 2019; **40**: 648–664.

22. Neagoe P-E, Dumas E, Hajjar F, Sirois MG. Angiopoietin-1 but not angiopoietin-2 induces IL-8 synthesis and release by human neutrophils. *J Cell Physiol* 2012; **227**: 3099–3110.

23. Mantovani A, Cassatella MA, Costantini C, Jaillon S. Neutrophils in the activation and regulation of innate and adaptive immunity. *Nat Rev Immunol* 2011; **11**: 519–531.

24. Haddad LE, Sirois MG. Angiopoietin-1 upregulates de novo expression of IL-1beta and Il1-Ra, and the exclusive release of Il1-Ra from human neutrophils. *PLoS One* 2014; **9**: e88980.

25. Zimmermann M, Aguilera FB, Castellucci M, Rossato M, Costa S, Lunardi C, Ostuni R, Girolomoni G, Natoli G, Bazzoni F, Tamassia N, Cassatella MA. Chromatin remodelling and autocrine TNFa are required for optimal interleukin-6 expression in activated human neutrophils. *Nat Commun* 2015; **6**: 6061.

26. Murphy SP, Kakkar R, McCarthy CP, Januzzi JL. Inflammation in Heart Failure. *J Am Coll Cardiol* 2020; **75**: 1324–1340.

27. Pasini E, Aquilani R, Corsetti G, Dioguardi FS. Malnutrition and Gut Flora Dysbiosis: Specific Therapies for Emerging Comorbidities in Heart Failure. *Biomed Res Int* 2015; **2015**: 1–5.

28. Pasini E, Aquilani R, Testa C, Baiardi P, Angioletti S, Boschi F, Verri M, Dioguardi F. Pathogenic Gut Flora in Patients With Chronic Heart Failure. *JACC Heart Fail* 2016; **4**: 220–227.

29. Abraham D, Hofbauer R, Schäfer R, Blumer R, Paulus P, Miksovsky A, Traxler H, Kocher A, Aharinejad S. Selective Downregulation of VEGF-A165, VEGF-R1, and Decreased Capillary Density in Patients With Dilative but Not Ischemic Cardiomyopathy. *Circ Res* 2000; **87**: 644–647.

30. Arakawa H. Decreased serum vascular endothelial growth factor concentrations in patients with congestive heart failure. *Heart* 2003; **89**: 207–208.

31. Xu XH, Xu J, Xue L, Cao HL, Liu X, Chen YJ. VEGF attenuates development from cardiac hypertrophy to heart failure after aortic stenosis through mitochondrial mediated apoptosis and cardiomyocyte proliferation. *J Cardiothorac Surg* 2011; **6**: 54.

32. Taimeh Z, Loughran J, Birks EJ, Bolli R. Vascular endothelial growth factor in

heart failure. *Nat Rev Cardiol* 2013; **10**: 519–530.

33. Tao Z, Chen B, Tan X, Zhao Y, Wang L, Zhu T, Cao K, Yang Z, Kan YW, Su H. Coexpression of VEGF and angiopoietin-1 promotes angiogenesis and cardiomyocyte proliferation reduces apoptosis in porcine myocardial infarction (MI) heart. *Proc Natl Acad Sci USA* 2011; **108**: 2064–2069.

34. Carmeliet P, Ferreira V, Breier G, Pollefeyt S, Kieckens L, Gertsenstein M, Fahrig M, Vandenhoeck A, Harpal K, Eberhardt C, Declercq C, Pawling J, Moons L, Collen D, Risau W, Nagy A. Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. *Nature* 1996; **380**: 435–439.

35. Morine KJ, Paruchuri V, Qiao X, Mohammad N, McGraw A, Yunis A, Jaffe I, Kapur NK. Circulating multimarker profile of patients with symptomatic heart failure supports enhanced fibrotic degradation and decreased angiogenesis. *Biomarkers* 2015; **21**: 91–97.

36. Paulus WJ, Tschope C. A novel paradigm for heart failure with preserved ejection fraction: comorbidities drive myocardial dysfunction and remodeling through coronary microvascular endothelial inflammation. *J Am Coll Cardiol* 2013; **62**: 263–271.

37. Gimbrone MA, García-Cardeña G. Endothelial Cell Dysfunction and the Pathobiology of Atherosclerosis. *Circ Res* 2016; **118**: 620–636.

38. De Caterina R, Libby P, Peng HB, Thannickal VJ, Rajavashisth TB, Gimbrone MA, Shin WS, Liao JK. Nitric oxide decreases cytokine-induced endothelial activation. Nitric oxide selectively reduces endothelial expression of adhesion molecules and proinflammatory cytokines. *J Clin Invest* 1995; **96**: 60–68.

39. Sproston NR, Ashworth JJ. Role of C-Reactive Protein at Sites of Inflammation and Infection. *Front Immunol* 2018; **9**: 754.

40. Eisenhardt SU, Thiele JR, Bannasch H, Stark GB, Peter K. C-reactive protein: How conformational changes influence inflammatory properties. *Cell Cycle* 2014; **8**: 3885–3892.

41. Vulesevic B, Lavoie SS, Neagoe PE, Dumas E, Rakel A, White M, Sirois MG. CRP Induces NETosis in Heart Failure Patients with or without Diabetes. *Immunohorizons* 2019; **3**: 378–388.

42. Swirski FK, Nahrendorf M. Cardioimmunology: the immune system in cardiac homeostasis and disease. *Nat Rev Immunol* 2018; **18**: 733–744.

43. Nymo SH, Hulthe J, Ueland T, McMurray J, Wikstrand J, Askevold ET, Yndestad A, Gullestad L, Aukrust P. Inflammatory cytokines in chronic heart failure: interleukin-8 is associated with adverse outcome. Results from CORONA. *Eur J Heart Fail* 2014; **16**: 68–75.

44. Anker SD. Inflammatory mediators in chronic heart failure: an overview. *Heart* 2004; **90**: 464–470.

45. Bajpai A, Tilley DG. The Role of Leukocytes in Diabetic Cardiomyopathy. *Front Physiol* 2018; **9**: 1547.

46. Dinarello CA. Overview of the IL-1 family in innate inflammation and acquired immunity. *Immunol Rev* 2018; **281**: 8–27.

47. Hatanaka E, Monteagudo PT, Marrocos MSM, Campa A. Neutrophils and monocytes as potentially important sources of proinflammatory cytokines in diabetes. *Clin Exp Immunol* 2006; **146**: 443–447.

48. Ghaffari S, Nadiri M, Pourafkari L, Sepehrvand N, Movasagpoor A, Rahmatvand N, Rezazadeh Saatloo M, Ahmadi M, Nader ND. The predictive Value of Total Neutrophil Count and Neutrophil/Lymphocyte Ratio in Predicting In-hospital Mortality and Complications after STEMI. *J Cardiovasc Thorac Res* 2014; **6**: 35–41.

49. Silvestre-Roig C, Braster Q, Ortega-Gomez A, Soehnlein O. Neutrophils as regulators of cardiovascular inflammation. *Nat Rev Cardiol* 2020; **17**: 327–340.

50. Chou E, Suzuma I, Way KJ, Opland D, Clermont AC, Naruse K, Suzuma K, Bowling NL, Vlahos CJ, Aiello LP, King GL. Decreased Cardiac Expression of Vascular Endothelial Growth Factor and Its Receptors in Insulin-Resistant and Diabetic States. *Circulation* 2002; **105**: 373–379.

51. Han B, Baliga R, Huang H, Giannone PJ, Bauer JA. Decreased cardiac expression of vascular endothelial growth factor and redox imbalance in murine diabetic cardiomyopathy. *Am J Physiol Heart Circ Physiol* 2009; **297**: H829–H835.

52. Vitiello D, Chaar D, Neagoe P-E, Ducharme A, Carrier M, Pelletier GB, Racine N, Liszkowski M, Sirois MG, White M. Decreased circulating and neutrophil mediated VEGF-A165 release in stable long-term cardiac transplant recipients. *Vasc Cell* 2015; **7**: 4.

53. Arend WP. Biological role of interleukin 1 receptor antagonist isoforms. *Ann Rheum Dis* 2000; **59**: i60–i64.

54. Perrier S, Darakhshan F, Hajduch E. IL-1 receptor antagonist in metabolic diseases: Dr Jekyll or Mr Hyde? *FEBS Lett* 2006; **580**: 6289–6294.