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

Diana Chaar1,2, Benjamin Dumont1,3, Branka Vulesevic1, Paul-Eduard Neagoe1, Agnes Rakel2,4, Martin G. Sirois1,3* and Michel White1,2*

1Research Center, Montreal Heart Institute, 5000 Belanger Street, Montreal, Quebec H1T 1C8, Canada; 2Department of Medicine, Université de Montréal, Montreal, Quebec, Canada; 3Department of Pharmacology and Physiology, Faculté de Medicine, Université de Montréal, Montreal, Quebec, Canada; 4Department of Pharmacology and Physiology, Faculté de Medicine, Université de Montréal, Montreal, Quebec, Canada; 5Research 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), \( P = 0.0234 \)]. 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.

Keywords Heart failure; Diabetes; Neutrophil; Inflammation; Cytokines

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 biomarkers is thought to be driven by the release of cytokines by neutrophils. However, the role of neutrophils in the inflammatory process in patients with HFrEF is not well understood.
markers has been related to an adverse outcome in these patients. 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. 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) but also with the presence of heart failure. Despite the prominent role of neutrophils in inducing the chronic inflammatory response in the pathogenesis of many diseases, 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. High-risk patients, such as those with diabetes and HF, exhibit an increased activation of the innate system. 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%, 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 medication for at least 14 days prior to blood collection. Patients with HF and with 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 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.\textsuperscript{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).\textsuperscript{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 × 10⁶/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% CO₂. 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.\textsuperscript{21,22}

### Statistical analysis

Continuous variables are presented as mean ± SEM (normally distributed data) or 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 \( \chi^2 \) 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.\textsuperscript{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 × 10⁶/mL, 500 μL) (Figure 3).
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.

**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%)       |                     |         |
| Valvar             | 1 (3.7%)    | 1 (4.0%)       |                     |         |
| Others             | 2 (7.4%)    | 4 (16.0%)      |                     |         |
| **LVEF**           | 30.15 ± 1.39| 27.08 ± 1.21   | 0.10                |
| **LVDD**           | 60.96 ± 1.75| 61.12 ± 1.71   | 0.95                |
| **LVDS**           | 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 (×10⁹/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; LVDD, left ventricular internal diameter end diastole; LVDS, 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.
Because TNF-α is unable to promote IL-6 release 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). 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.

Because TNF-α is unable to promote IL-6 release, 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
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. Our data are also in agreement with previous clinical studies reporting a decrease of circulating VEGF in patients with HF.

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. 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). 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. 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). 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. In addition, CRP inhibits the expression of eNOS and NO synthesis and upregulates the expression of adhesion molecules.

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. Both IL-6 and IL-8 are known to play a significant role in both HF and diabetes. 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.

IL-1RA, a member of the IL-1 family, is an anti-inflammatory and anti-proliferation cytokine. 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.

Patients with HF have an increasingly activated innate immune system. Data from large clinical studies and some smaller mechanistic investigations have shown some abnormalities of leucocytes and neutrophils in human heart failure. 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 and by the neutrophils in human cardiac transplant recipients.

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 leucocyte 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. 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-driven 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.

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**Conflict of interest**

The authors have no conflicts of interest to declare.

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