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Myocardial interleukin-6 in the setting of left ventricular mechanical assistance: relation with outcome and C-reactive protein

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Abstract

Background: In left ventricular assist device (LVAD) recipients, plasma levels of interleukin (IL)-6 are associated with Interagency Registry for Mechanically Assisted Circulatory Support (INTERMACS) profiles, reflecting post-operative risk. However, it is not clear how the cardiac level of IL-6, detectable on the tissue samples at the time of implantation, can contribute to predict the post-operative outcome.
Methods: In 40 LVAD recipients, blood and myocardial samples from LV-apex were collected at the time of implantation to assess plasma and cardiac IL-6 levels. Serum C-reactive protein (CRP) levels were considered as inflammatory variable routinely used in LVAD-based therapy.
Results: Cardiac IL-6 levels did not correlate with either plasma IL-6 levels (R=0.296, p=0.063) and tissue IL-6 mRNA expression (R=−0.013, p=0.954). Contrary to what happened for the plasma IL-6 and CRP, no differences were observed in cardiac IL-6 levels with respect to INTERMACS profiles (p=0.090). Furthermore, cardiac IL-6 concentrations, unlike IL-6 and CRP circulating levels, were not correlated with the length of intensive care unit stay and hospitalization.
Conclusions: Cardiac IL-6 levels do not contribute to improve risk profile of LVAD recipients in relation to clinical inpatient post-implantation. Instead, plasma IL-6 and serum CRP concentrations are more effective in predicting the severity of the clinical course in the early phase of LVAD therapy.

Keywords: C-reactive protein; interleukin-6; mechanical circulatory support; myocardium.

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Introduction

In the last decades, the left ventricular assist device (LVAD) implantation has become an adequate therapy for end-stage heart failure (ESHF) patients waiting for heart transplantation (HT). However, favorable outcomes still depend on proper patient selection, strategic timing of implantation, peri-operative risk and long-term clinical management [1].

Indeed, the patients characterized by the worst hemodynamic condition, as defined by Interagency Registry for Mechanically Assisted Circulatory Support (INTERMACS) profiles, are associated with poorer outcome [2].

As a result of the interaction within blood cellular component and artificial surfaces of mechanical circulatory support (MCS), a significant change in systemic immunologic and thrombostatic functions occurs when the device was implanted. LVAD implantation results in an altered state of monocyte and T-cell activation and an increased production of proinflammatory cytokines [3]. These alterations may cause the early onset of LVAD complications, such as multi-organ failure (MOF) [4, 5], highlighting the importance of inflammatory status monitoring during the clinical management of patients needing MCS.

The C-reactive protein (CRP) is the main inflammatory variable used routinely in the setting of LVAD patients,

although it represents a non-specific inflammatory marker [6]. CRP levels were found comparable between LVAD patients either with different outcome and clinical course [7].

Interleukin (IL)-6 is a cytokine involved in the progression of HF that plays an important role in prognosis of LVAD patients; in fact, signals depending on IL-6 levels may trigger monocyte activation, a crucial mechanism in the development of MOF [5, 6]. Circulating IL-6 levels, measured at pre- and post-LVAD implantation, increased in patients with the worst INTERMACS profiles with respect to patients hemodynamically more stable [8], suggesting that plasma IL-6 levels correlated with a poor prognosis and the post-operative outcome in LVAD candidates.

However, it is still unclear how myocardial pro-inflammatory cytokine might influence the early outcome of LVAD patients, compared to the systemic inflammation. Previous studies showed an increased expression of myocardial IL-6 and tumor necrosis factor-α (TNF-α) in deteriorating patients who required LVAD insertion [9, 10]. The aim of our study was to determine whether pre-implant cardiac IL-6 levels, as plasma IL-6 concentrations, may improve the prediction of post-implant outcome. Moreover, we would verify if IL-6 concentrations, both at cardiac or plasma levels, may be more effective in risk stratification compared to serum CRP levels.

Materials and methods

Patients and study design

Forty consecutive ESHF patients who underwent LVAD implantation as a bridge to HT were enrolled [11] between May 2006 and December 2012. Thirty-nine patients were supported by axial continuous flow devices (such as Heart Mate II Thoratec, Pleasanton, CA, USA; Incor, Berlin Heart AG, Berlin, Germany; De Bakey, MicroMed Technology, Inc., Houston, TX, USA), and one patient was supported by centrifugal continuous flow device (HeartWare International Inc., Framingham, MA, USA). The INTERMACS classification was applied by agreement between cardiologists and cardiac surgeons: nine patients were classified as INTERMACS profile 1, eight as profile 2, 22 as profile 3, and one as profile 4.

MOF was monitored pre-operatively and up to 2 weeks after intervention, calculating the total Sequential Organ Failure Assessment (tSOFA) score [12]. Length of intensive care unit (ICU) stay and hospitalization were considered as end-points, while ICU and 1-year survivals were taken into account as main adverse outcomes.

Blood samples were collected at pre-implant for determination of plasma IL-6 and serum CRP levels. Myocardial specimens were obtained from LV apex biopsy, produced by inflow cannula positioning during standard surgical procedure of LVAD implantation.

The study protocol conformed to the principles outlined in the Declaration of Helsinki and was approved by the Local Ethics Committee of the Niguarda Cà Granda Hospital. All subjects gave written informed consent to participate in the study.

Cardiac and circulating determinations

Deep frozen myocardial specimens, placed in a pre-cooled Teflon shaking flask, were ground using a Mikro-Dismembrator II (B. Braun Biotech International GmbH, Melsungen, Germany). Frozen tissue powder was treated with Tris-based lysis solution and IL-6 levels were assessed in the supernatant.

Cardiac and plasma IL-6 levels were measured using enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN, USA). Cardiac IL-6 levels were normalized by total protein value.

CRP levels were measured by high-sensitive immuno-nephelometric method (Roche Diagnostic GmbH, Mannheim, Germany).

Cardiac IL-6 mRNA expression

Total RNA was extracted from myocardial specimens and RNA concentration and purity were evaluated [13]. Starting from total RNA, first-strand cDNA was synthesized by iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA, USA). Primer Express Version 2.0 (Applied Biosystems Inc., Drive Foster City, CA, USA) was used for designing IL-6 primers. Real-time PCR was performed in duplicate using the Bio-Rad C1000TM thermal cycler (CFX-96 real-time PCR detection systems; Bio-Rad, Hercules, CA, USA). IL-6 relative quantification was obtained by ΔΔCt method using previously selected reference genes [13].

Histology and immunohistochemistry

In nine patients, the proximal portion of surgically excised LV-apex was placed in 5% formalin solution (pH 7.0) for traditional histological staining (hematoxylin & eosin). Masson's trichrome was performed for collagen staining and total and interstitial fibrosis according to Drakos SG [14]. Immunohistochemistry of IL-6 (mouse, multifunctional protein Santa Cruz Biotechnology, diluted 1:100), macrophage marker CD68 (macrophage marker Ab-4 mouse, Thermo Scientific, Inc., Waltham, MA, USA, diluted 1:50), and macrophage migration inhibitory factor (MIF, rabbit polyclonal, Santa Cruz Biotechnology, Inc. Dallas, TX-USA, diluted 1:250) was also made.

Histology and immunohistochemistry staining were performed on 10 adjacent sections (5 μm thick) of paraffin-embedded surgical specimens mounted on positively charged glass slides. Treatment for primary and secondary antibody binding was followed according to standardized protocol for immunohistochemistry of paraffin-embedded sections.

Immunoperoxidase detection systems were Vectastain elite ABC reagent and slides were counterstained with Mayer's hematoxylin. Human tonsil was used as positive control in each staining batch and samples from the same series without primary antibody served as negative controls.

Histological and immunohistochemical analysis of consecutive sections was carried out under a light microscope (Olympus BX43) at 10× to 40× original magnification and digitized by a video system (Olympus, Tokyo, Japan, D70 camera) interfaced to a computer with dedicated software (Olympus CellSens Dimension). Between 10 and 20 digitized microscopic fields (20× and 40× magnification) of each section were analyzed by CellSens Dimension color imaging software by two experienced observers (GP and FV) and pixel threshold for blue positive area (Masson trichrome stain of fibrosis) and for dark brown area (positive immunostained cell area) applied under the same microscope light settings. Average values of each section and

of each case were thus obtained for blue-stained areas and expressed as percentage of positive/total observed myocardial area. The same procedure was applied to dark brown-positive areas of IL-6, CD68 and MIF immunostained sections as an indirect index of positive cell number for each antibody, assuming a similar distribution of each marker in all strongly positive cells. A qualitative analysis of MIF and IL-6 distribution in CD68 positive cells was also accomplished.

Statistics

Data are expressed as median and interquartile range (I–III) or frequency (percentage). Normality was analyzed with the Kolmogorov-Smirnov test. Differences between baseline and post-LVAD implantation were assessed by paired Student's t-test or Wilcoxon signed-rank test, where appropriate, for continuous variables and χ^2 or Fisher's exact tests for categorical variables. Differences with INTERMACS profiles were tested by non-parametric Kruskal-Wallis test, followed by Bonferroni post-hoc test for pairwise comparisons. Spearman's correlation was used to analyze the relationship between variables. To assess the influence of tested parameters, backward stepwise multiple linear regression analysis was used. A p-value <0.05 was considered statistically significant. The SPSS 17 statistical software package (SPSS Inc, Chicago, IL, USA) was used for all calculations.

Results

Patient characteristics and outcome

The clinical characteristics of patients are summarized in Table 1. Patients were distributed among more

Table 1 Clinical characteristics.

	INTERMACS group		
	1 (n=9)	**2 (n=8)**	**3 (n=23)**
Age, years	54 (44–66)	59 (52–62)	51 (48–60)
Male gender, n (%)	8 (89)	7 (88)	21 (91)
NYHA class, n (%)			
III	1 (11)	2 (25)	10 (44)
IV	8 (89)	6 (75)	13 (56)
Etiology, n (%)			
IDC	5 (56)	4 (50)	15 (65)
IHD	4 (44)	4 (50)	8 (35)
Length of stay, days	42 (26–102)	45 (31–54)	48 (28–60)
ICU stay	21 (12–29)	13 (8–14)	11 (11–17)
Mortality rate, %	3 (33)	0 (0)	5 (22)
Treatments, n (%)			
ACEi+ARB	4 (44)	4 (50)	17 (74)
β-Blocker	2 (22)	5 (63)	22 (96)
Statins	2 (22)	1 (13)	7 (30)
Diuretics	7 (78)	5 (63)	23 (100)
Inotropic	7 (78)	4 (50)	11 (48)
IABP, n (%)	8 (89)	2 (33)	2 (9)
CI, L/min/m^2	2.09 (1.88–2.45)	1.50 (1.30–1.70)	1.70 (1.37–1.90)
RAP, mmHg	5 (3–14)	6 (4–10)	6 (3–9)
PCWP, mmHg	15 (9–24)	24 (17–33)	26 (22–33)
MAP, mmHg	81 (76–84)	90 (90–91)	75 (70–81)
LVEF, %	20 (17–24)	26 (19–28)	23 (20–27)
LVEDV, mL	248 (183–321)	190 (170–335)	265 (180–315)
LVEDD, mm	68 (58–76)	68 (55–81)	70 (65–76)
eGFR, mL/min/1.73 m^2	80 (55–102)	78 (50–119)	79 (54–89)
Total bilirubin, μmol/L	12.48 (9.06–49.59)	14.19 (9.06–17.10)	20.35 (10.26–30.44)
tSOFA score, n	6 (5–9)	3 (2–4)	4 (2–5)
C-reactive protein, nmol/L	78.06 (12.38–131.38)	7.14 (3.33–19.04)	11.42 (1.90–20.94)
NT-proBNP, ng/L	1058 (447–4693)	4185 (2296–5690)	4294 (1378–6219)

Values are presented as mean (±SD) or number (percentage). ACEi, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blockers; CI, cardiac index; eGFR, estimated glomerular filtration rate; IABP, intra-aortic balloon pump; IDC, idiopathic dilatative cardiomyopathy; IHD, ischemic heart disease; LVEDD, left ventricular end-diastolic diameter; LVEDV, left ventricular end-diastolic volume; LVEF, left ventricular ejection fraction; MAP, mean arterial pressure; NT-proBNP, N-terminal pro-hormone brain natriuretic peptide; NYHA, New York Heart Association; PCWP, pulmonary capillary wedge pressure; RAP, right atrial pressure; tSOFA, total Sequential Organ Failure Assessment.

advanced New York Heart Association (NYHA) classes, with idiopathic dilatative cardiomyopathy (IDC) as main etiology.

Eight patients died during ICU stay [16 (11–26) days] with MOF as main or secondary cause of death (3 patients with INTERMACS profile 1, and 5 with profile 3). At 1 year the survival rate was 77%. The pre-implant tSOFA score was 4 (2–6) and significantly worsened to 9 (4–10) at 1-week post-operative implantation ($p<0.001$). After 2 weeks post-operative implantation, tSOFA score returned to pre-implant value [1 (0–6), $p=0.063$].

Among survivors, length of ICU stay was 13 (8–17) days, while hospitalization was 49 (41–72) days. Ten patients underwent HT after LVAD support, while one patient was weaned from device due to successful cardiac recovery during LVAD-based therapy.

Cardiac and circulating IL-6 levels

The cardiac IL-6 level was 1.54 (0.42–3.50) pg/mg, ranging from 0.09 to 39.7 pg/mg, while pre-operative plasma IL-6 level was 8.15 (3.02–25.42) pg/mL, ranging from 0.4 to 500.5 pg/mL. Cardiac IL-6 levels did not correlate with either plasma IL-6 levels ($R=0.296$, $p=0.063$) and cardiac IL-6 mRNA expression [0.015 (0.007–0.039), ranging from 0.002 to 0.094, $R=-0.013$, $p=0.954$].

Plasma IL-6 concentrations, but not cardiac levels or expression, were correlated to pre-operative serum CRP concentrations ($R=0.762$, $p<0.001$) and circulating leukocytes ($R=0.441$, $p=0.005$).

Relationship with INTERMACS profiles

Patients were divided into three groups: Group 1 included patients with life threatening hypotension and critical organ hypoperfusion, despite rapidly escalating inotropic support (INTERMACS profile 1, n=9), Group 2 included hemodynamically unstable patients despite optimized intensive medical therapy (INTERMACS profile 2, n=8), while Group 3 included patients who were hemodynamically stable although requiring inotropic therapy in the majority of the cases (INTERMACS profiles ≥3, n=23).

Pre-implant plasma IL-6 levels and serum CRP concentrations differed among LVAD candidates of Group 1, 2 or 3 ($p=0.008$ and $p=0.044$, respectively). By post-hoc comparison test, patients in Group 1 had higher plasma IL-6 levels compared to patients in Groups 2 and 3, while serum CRP levels were higher only in patients of Group 1 with respect to those of Group 3 (Figure 1). No differences were instead observed in cardiac IL-6 levels with respect to INTERMACS profiles ($p=0.090$).

Relationship with pre-implant tSOFA score

Pre-implant tSOFA score was directly related with plasma IL-6 ($R=0.607$, $p<0.001$) and serum CRP ($R=0.586$, $p<0.001$) levels, while no correlation was found with cardiac IL-6, either as protein levels and mRNA expression ($R=0.255$, $p=0.112$ and $R=0.049$, $p=0.830$, respectively).

Both plasma IL-6 and CRP variables were tested by stepwise multiple linear regression. Only levels of CRP were independently associated with pre-implant tSOFA score ($\beta=0.0264$, $p=0.015$).

Relationships with survival

No difference in pre-implant cardiac and plasma levels of IL-6 and serum CRP concentrations were found between survivors and non-survivors both at 1 year from implantation or during ICU stay.

Relationships with ICU stay, hospitalization and post-operative tSOFA score

Pre-implant plasma IL-6 levels and CRP concentrations were related with tSOFA score at 2 weeks ($R=0.522$, $p=0.001$, and $R=0.366$, $p=0.028$, respectively) and with ICU stay ($R=0.410$, $p=0.009$ and $R=0.449$, $p=0.004$, respectively), but not with hospitalization ($R=0.149$, $p=0.358$, and $R=0.189$, $p=0.243$, respectively). Instead, cardiac IL-6 values did not correlate with any of the above clinical outcome.

The length of ICU stay was not different among LVAD candidates with different INTERMACS profiles ($p=0.111$).

Myocardial histology and immunohistochemistry

The median value of overall blue-stained collagen area, expressed as percentage of total observed myocardial area, was 8.2 (5.8–14.7)%, and was considered an index of total fibrosis; 5.6 (4.7–6.8)% was attributed to interstitial fibrosis following exclusion of perivascular and dense focal positive areas.

The average percentage of dark brown-positive area of each antibody tested was comparable among IL-6, CD68

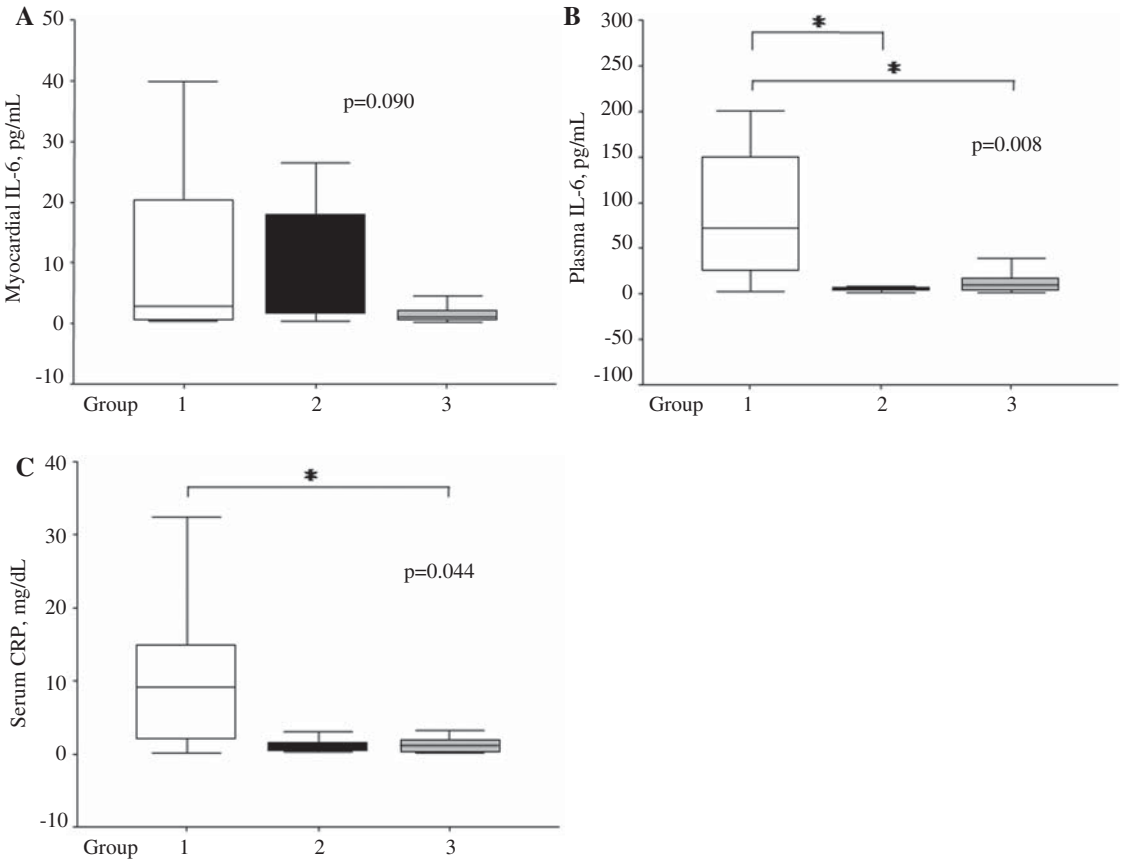

Figure 1 Cardiac (A) and plasma IL-6 (B) and serum CRP (C) levels according to INTERMACS profiles of LVAD recipients.
Group 1 (white bars): INTERMACS profile 1; Group 2 (black bars): INTERMACS profile 2; Group 3 (gray bars): INTERMACS profiles ≥3. *p<0.05 by post-hoc Bonferroni test.

and MIF, although a much broader variability was found for the two latter ones (Figure 2A). Qualitative analysis revealed that cells positively immunostained by MIF and IL-6 antibodies were mostly CD68 positive macrophages and, at a lesser extent, endothelial cells of neoformed microvessels (Figure 2B).

Discussion

To our knowledge this is the first study to compare IL-6 amounts, both at plasma and cardiac level, with peri-operative risk and outcome of LVAD recipients. The study shows that the apical content of IL-6 is not related to cytokine concentrations in bloodstream, and with the peri-operative risk assessed by INTERMACS profiles. On the contrary, plasma IL-6 content correlates with pre-operative hemodynamic status, post-operative tSOFA score and length of ICU stay, thus with the degree of severity of clinical course. Also, serum CRP levels predict the degree of post-operative severity but it correlates less with the pre-operative risk assessed by INTERMACS profile than circulating IL-6 concentrations.

Cytokines members of the IL-6 superfamily are expressed in the myocardium of failing heart, more in ESHF patients than in those with recent onset of symptoms [15], or less severe HF [9, 16]. Although characterized as ESHF patients, LVAD candidates are a heterogeneous population with different peri-operative risk grades and levels of inflammation [2, 8]. As previously reported [8] also in this cohort, the circulating levels of IL-6 were differently distributed according to INTERMACS value, with plasma IL-6 levels higher in patients with profile 1. On the contrary, IL-6 cardiac protein and expression were not associated with INTERMACS profiles.

In LVAD candidates, the peripheral levels of IL-6 probably derive from other organs in addition to the damaged heart. Indeed, plasma IL-6 concentrations of our LVAD patients were found related with pre-implant tSOFA score. Kidney and liver dysfunctions are present in several LVAD recipients, as evidenced by low pre-implant

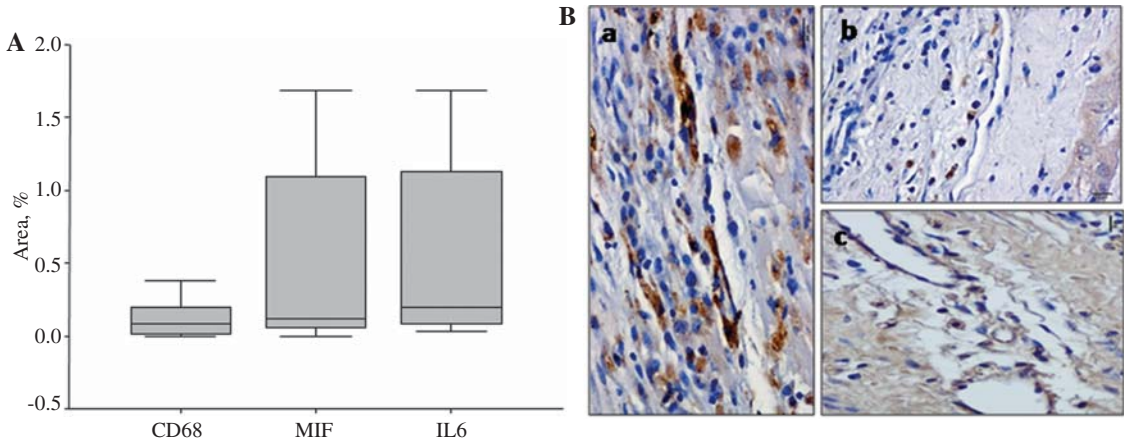

Figure 2 (A) Percent values of total section area of IL-6, CD68, and MIF positivity. (B): LV-apex immunohistochemical stained for CD68 (a), MIF (b), and IL-6 (c) in adjacent sections of LVAD recipient.
Original magnification 40× scale, bar 20 μm.

glomerular filtration rate and high bilirubin levels found in our patients. Pro-inflammatory cytokines are released into the bloodstream from distal sites during liver and kidney dysfunctions to orchestrate inflammatory reparative responses against injury [17]. Otherwise, cardiac content and expression of IL-6 may reflect an in situ inflammation due to the local infiltration of macrophages; this finding is also supported by myocardial immunostaining results that show a co-expression of IL-6 and MIF, a known factor promoting monocytes and T cells recruitment [18]. However, previous studies reported that myocardial IL-6 expression was not different between ESHF patients and donors, and not correlated with the circulating levels [15, 16, 19].

Pre-implant plasma IL-6 levels were positively associated also to tSOFA score at 2 weeks and to ICU stay, showing that this marker may earlier identify patients with a complicated clinical course.

Likewise to plasma IL-6, pre-implant CRP levels were higher in patients with INTERMACS profile 1 and were associated to tSOFA score and ICU stay, supporting a relationship between hemodynamic worsening and inflammatory pathways depending on circulating IL-6. CRP is produced by hepatocytes predominantly under transcriptional control of this cytokine [20]. CRP is synthesized in the liver as a result of increased IL-6 stimulation, while IL-6 is known to play a key role in controlling monocyte and endothelial activations [21].

The use of CRP as prognostic marker of adverse outcome in LVAD patients remains still uncertain and controversial, although this molecule has been considered a good indicator of post-operative adverse events in surgical patients during stay in the ICU [22, 23]. However, the evaluation of pre-operative CRP levels could help clinicians to predict the clinical course of LVAD patients using a laboratory tool, routinely present in the biochemical assessment of these subjects. Our results showed that pre-operative CRP levels were more associated with indicators of critical course in LVAD patients than IL-6, highlighting the better efficiency of CRP in predicting clinical outcome.

The availability of apical cardiac tissue, excised during LVAD implantation, may better integrate clinical and instrumental diagnosis with pathophysiological findings obtained from the tissue. However, the most interesting use of cardiac biopsies, in the clinical practice of LVAD therapy, is the identification of accurate prognostic cardiac markers, able to provide additional information with respect to those in blood. Although a previous work reported an increase in myocardium IL-6 levels [9] in deteriorating patients who require LVAD insertion, in our cohort we did not find any difference. Moreover, cardiac levels of IL-6 are less associated with the hemodynamic status of LVAD recipients, and do not contribute to improved risk prediction in relation to clinical course, compared to the circulating levels of IL-6 and CRP.

Further studies to evaluate the predictive power of the combined assessment of the CRP and IL-6 are warranted.

Study limitations

Four different models of LVAD were used during the study period; 29 HeartMate II, six Incor, four De Bakey and one HeartWare. Although different pump types do not show

differential effects on hemodynamic support [24], clinical complications associated to different device models may differ [25, 26]. For these reason, we compared incidence of the main adverse clinical problems (wound infection, liver and renal dysfunction, right ventricular failure, stroke, VAD malfunction and aortic insufficiency) in our four LVAD groups of patients. No significant differences were observed (data not shown).

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