No prominent role for terminal complement activation in the early myocardial reperfusion phase following cardiac surgery

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Abstract

OBJECTIVES: Complement activation is considered an important mediator of myocardial ischaemia/reperfusion (I/R) injury. Although complement inhibitors are highly effective in animals, clinical trials fail to show a substantial benefit in humans. This raises questions on the role of complement activation in human myocardial I/R injury.

METHODS: Soluble C5b-9, i.e. terminal complement complex, and C5a were assessed in patients with non-ischaemic ($n = 10$) and ischaemic heart failure ($n = 10$), and patients without heart failure ($n = 10$) undergoing cardiac surgery. To study the pathophysiology of human I/R injury, a model of arteriovenous measurements over the reperfused heart was applied at consecutive time points during the early reperfusion phase. Furthermore, C3d and C5b-9 depositions in pre-reperfusion myocardial and endomyocardial tissue were evaluated and compared to pre-transplantation tissue from myocardial allografts.

RESULTS: Simultaneous assessment of soluble C5b-9 and C5a in systemic and myocardial venous blood samples revealed the absence of net release from the reperfused heart in all three patient groups. Biopsies of patients with non-ischaemic heart failure showed the most abundant myocardial depositions of C3d and C5b-9: 4.8 times more C3d ($P = 0.008$) and 4.7 times more C5b-9 ($P = 0.004$) than donor tissue. Also C3d was abundantly present in endomyocardial tissue of both heart failure groups compared to donors (both $P = 0.02$).

CONCLUSIONS: No evidence was obtained that terminal complement activation is involved in the acute phase following myocardial reperfusion. Since complement deposition was already present before reperfusion, human complement inhibition might be more beneficial in the preoperative phase than during reperfusion.

Keywords: Immunology · Inflammation · Ischaemia · Reperfusion · Surgery

INTRODUCTION

Ischaemia/reperfusion (I/R) injury is considered a major contributor to reversible but also to irreversible cellular damage in multiple clinical situations such as heart transplantation, myocardial infarction and cardiovascular surgery. The pathophysiology of I/R injury is complex and incompletely understood. The innate immune system has been suggested to provide short-term adaptive responses to tissue injury. If the innate immune signalling becomes sustained or excessive, the activation of these beneficial homeostatic pathways is abolished by deleterious effects of inflammatory signalling [1].

The complement system is one of the basal defense mechanisms of the innate immune system consisting of a complex cascade of plasma proteins. The complement system is activated via three distinct pathways: the classical, alternative and lectin pathway. The end-result is activation of C5b-9, the cell-lysing membrane attack complex or terminal complement complex. When well-balanced and controlled, complement activation supports the defense against infections and facilitates wound healing. However, if the complement cascade is excessively activated it contributes to organ dysfunction leading to increased morbidity and mortality [2].

Complement activation is considered one of the key mediators of myocardial I/R-induced tissue damage [3–7]. During cardiac surgery, the complement cascade is activated as a result of the surgical trauma, use of cardiopulmonary bypass (CPB) and I/R injury.

Complement inhibitors are highly effective in reducing tissue damage in animal studies of myocardial I/R injury [4, 8, 9]. However, anti-complement therapy in human myocardial I/R injury has lead to differential results without clinical relevant benefits [10–12]. Whether the pathophysiology of human
myocardial I/R injury is based on complement activation may therefore be questioned. The limited human studies concerning complement activation in myocardial I/R injury have shown that there is an increased systemical activation of complement components the first hours after reperfusion [5, 6]. Those systemic levels show a biphasic pattern with inhibitory mechanisms from 8 h postoperatively onward [7]. Those previous studies have used systemic values as read-out of local myocardial complement activation.

In this clinical study, an integrative approach was used to measure simultaneously activated complement components in coronary sinus and radial artery blood for up to 24 h after reperfusion. This set-up allows to specifically measure the myocardial uptake or release of circulating complement components such as C5a and soluble (s)C5b-9 instead of only measuring systemic values. In addition to these simultaneous collected blood samples, tissue samples were taken from the left ventricle during surgery and stained for C3d and C5b-9. The purpose of the present study was to examine the role of complement activation in the early myocardial reperfusion phase following cardiac surgery.

MATERIALS AND METHODS

Patient characteristics

To investigate the role of local complement activation in early I/R injury, 30 patients scheduled for elective cardiac valve surgery with the use of CPB were studied. Ten patients with idiopathic non-ischaemic heart failure were scheduled for an external constraint device. In addition, 10 patients with ischaemic heart failure were scheduled for a ventricular reconstruction after a large infarction (n = 5) or a coronary artery bypass grafting procedure (n = 5). Ten patients without heart failure undergoing cardiac surgery were also included. Their valve regurgitation was caused by organic pathology, with a predominance of prolapse disease. Heart failure was defined as an inadequate pump function of the heart with an echocardiographically estimated ejection fraction biplane below 35% [13] and the presence of one or more clinical symptoms such as dyspnoea—reflected by their NYHA class [14]—oedema and fatigue existing for more than 3 months. All patients without heart failure had a moderate to normal pump function of the heart. These patients had no clinical signs of heart failure by physical examination and did not use high doses of diuretics. Exclusion criteria were perioperative corticosteroid treatment, minimal invasive surgical procedures, emergency operations and previous cardiac surgery. All included patients did not receive heparin, anti-complement therapy or immunosuppressive drugs before surgery, known to influence the measurements of complement activation markers. This single centre study was approved by our local ethics committee and all patients provided written informed consent.

Anaesthesia and surgical procedures

All participating patients received standardized anaesthetic procedures, according to a fast-track protocol. Patients were premedicated with oral lorazepam 1–2 mg the evening and morning before surgery. The anaesthetic regimen consisted of a continuous target controlled infusion of propofol with targets adjusted between 1.6 and 2 µg/ml to maintain a bispectral index value between 40 and 60. Sufentanil was infused at 0.1 µg/kg/h and remifentanil target controlled infusion was guided by haemodynamic response on stimuli. If necessary, inotropics—dobutamine, norepinephrine, enoximone or a combination—were used. Cardiac surgery was performed according to the local standardized protocol. All surgical procedures were performed via a midline sternotomy under normothermic CPB (Jostra Maquet, Maquet, Hirrlingen, Germany) with intermittent antegrade warm-blood cardioplegia. The CPB system was coated with a heparin softline coating. Exclusion criteria related to the use of this CPB system were autologous priming and ultrafiltration, influencing the measurements of inflammatory mediators. In all patients, a mitral valve annuloplasty—repair—procedure was performed. There is the possibility of introducing a coronary sinus catheter during this surgical procedure, since the transeptal approach of the mitral valve is the standard procedure in our hospital.

Biopsies

Five patients were selected from each patient group to obtain at two time points pre-reperfusion biopsies from the left ventricle (Fig. 1). These patients were selected by a blinded student based on their aortic cross-clamp time and on the time-frame between the collection of the biopsies and the start of aortic cross-clamping. This method was applied to create comparability for the immunohistochemical analyses between the groups. In specific, the ischaemic heart failure group contained two biopsies of patients scheduled for a ventricular reconstruction procedure and three biopsies of patients scheduled for a coronary artery bypass procedure. The early pre-reperfusion biopsies were taken through the mitral valve early after aortic cross-clamping. In other words, these biopsies were taken directly after opening of the heart and are therefore a good measure for preoperative involvement of complement activation. The late pre-reperfusion biopsy was obtained from the same location just before releasing the aortic cross-clamp, the moment of reperfusion. Only

Figure 1: Schematic representation of the coronary sinus catheter and location of the biopsy. This figure shows the position of the coronary sinus catheter in situ to collect myocardial venous blood samples. Moreover, the location of the left ventricular biopsy is depicted in this illustration showing endomyocardial and myocardial tissue. (© ManonProject.com).
pre-reperfusion biopsies could be collected since most of the included heart failure patients were haemodynamically instable in the early reperfusion phase. Lifting the heart, to take a post-reperfusion biopsy from the left ventricle, was considered unsafe. Biopsies were immediately snap frozen in liquid nitrogen and stored at −70°C until analysis. To compare the tissue of our pre-reperfusion biopsies, pre-transplantation tissue was collected from myocardial allografts of four brain death donors.

**Arteriovenous measurements**

Arterial (radial artery, 10 ml) and myocardial venous blood samples (coronary sinus, 10 ml) were collected over the reperfusion phase: 0, 15, 30 and 60 min after reperfusion. Two additional blood samples were drawn on venapunction the day before surgery (baseline) and 24 h after reperfusion from the brachial vein (both 10 ml) and arterial catheter, respectively. All samples were collected in pre-cooled tubes containing ethylenediamine-tetraacetic acid (BD Vacutainer, Plymouth, UK) and immediately placed on melting ice. Blood samples were centrifuged (1550 g, 10 min, 4°C) and plasma was re-centrifuged (10 000 g, 4 min, 4°C) to obtain leucocyte and thrombocyte free plasma. Aliquots were stored at −70°C until analysis.

**Immunohistochemistry**

Sections (5 µm) of snap-frozen biopsies were air dried and acetone-fixed for 10 min. C3d and C5b-9 deposition was assessed using a monoclonal antibody to a neo-epitope on C3d (Quidel, San Diego, CA, USA) or C5b-9 (aE11; Hycult Biotechnology, Uden, The Netherlands), respectively. Antibody binding was detected with horseradish peroxidase (HRP)-labelled goat antimouse Ig (DAKO, Glostrum, Germany). After washing, sections were incubated with tyramide-fluorescein isothiocyanate in tyramide buffer (NENTM Life Science Products, Boston, MA, USA), washed and incubated with HRP-labelled rabbit anti-fluorescein isothiocyanate (DAKO, Glostrum, Germany) and developed with DAB (Sigma, St Louis, MO, USA). Sections were counterstained with haematoxylin (Merck, Darmstadt, Germany) and mounted with imsol (Klinipath, Duiven, the Netherlands). Quantification of immunohistochemistry was performed in a blinded manner by assessing 10 consecutive high power fields (magnification, ×200) of myocardial and endomyocardial tissue on each section. Using Image J software, the positive area in each image expressed in pixels was determined.

**Plasma measurements**

sC5b-9 and C5a levels were assessed by sandwich ELISA. In brief, 96-wells ELISA plates (Nunc Bioscience, Belgium) were coated with a monoclonal antibody to a neo-epitope on C5b-9 (aE11; Hycult Biotechnology, Uden, The Netherlands) or monoclonal antibody to C5a (HM2079b; Hycult Biotechnology, Uden, The Netherlands), respectively. Plasma was incubated in the coated well and bound sC5b-9 or C5a was detected with a biotinlabelled monoclonal anti-C6 antibody (9C4; Hycult Biotechnology, Uden, The Netherlands) or anti-C5a antibody (CL.561; Hycult Biotechnology, Uden, The Netherlands), respectively, followed by detection with streptavidin–poly-HRP (Sanquin, Amsterdam, The Netherlands). Enzyme activity was detected using 2,2-azino-bis3-ethylbenzthiazoline-6-sulphonic acid (Sigma Chemical Co., St Louis, MO, USA). The optical density was measured at 415 nm using a microplate reader (Model 680; Biorad, Philadelphia, PA, USA). The detection limit for C5a was 1.95 ng/ml and for C5b-9 0.01 U/ml.
Statistical analysis

Differences between patient groups were analysed using the Kruskal–Wallis test with post hoc analyses with Mann–Whitney U-tests whenever appropriate. Categorical variables were compared using the Chi-square test. Comparisons between baseline values and values at 24 h after reperfusion were analysed with a Wilcoxon signed ranks test. The area under the curve (AUC) was calculated for the arterial and venous curves of the plasma measurements for the early (0–60 min) reperfusion phase. A delta AUC was calculated (venous minus arterial) and the null-hypothesis (delta AUC is 0) was tested by a paired t-test. Statistical analysis was performed using SPSS statistical analysis software version 17.0 (SPSS Inc, Chicago, IL, USA). A P-value less than 0.05 was considered significant (two-sided).

RESULTS

Patient population

Patient characteristics and perioperative details are summarized in Table 1. Two patients died in hospital: one patient with non-ischaemic heart failure died 8 days after surgery as a result of ventricular fibrillation and the other patient with ischaemic heart failure died 41 days after surgery as a result of therapy-resistant heart failure after a ventricular reconstruction.

Immunohistochemistry

To evaluate whether local complement deposition plays a role in myocardial I/R injury, presence and localization of C3d and

Figure 2: C3d and C5b-9 depositions in pre-reperfusion myocardial tissue. Early ischaemic myocardial tissue of patients with (A) non-ischaemic heart failure and (B) ischaemic heart failure, (C) non-heart failure patients and (D) donors were stained for C3d and C5b-9. Representative images are shown. Original magnifications ×200.
C5b-9 were studied in human left ventricular tissue collected in the early phase of the ischaemic period of the heart, i.e. the pre-reperfusion phase. No differences in deposition of C3d and C5b-9 were observed between the early and late pre-reperfusion biopsies (Supplementary data). Therefore, only the early pre-reperfusion biopsies were used, as read-out for preoperative involvement of complement activation. Immunohistochemical stainings showed deposition of C3d and C5b-9 in the vascular wall of small arteries in all pre-reperfusion biopsies (Fig. 2).

**C3d and C5b-9 deposition in myocardial tissue.** Patients with non-ischaemic heart failure showed the most abundant myocardial depositions of C3d and C5b-9: 4.8 times more C3d ($P = 0.008$) and 4.7 times more C5b-9 ($P = 0.004$) than donors. C3d deposition was accompanied by extensive C5b-9 deposition in patients with non-ischaemic heart failure, which was less pronounced in patients without heart failure (Fig. 3).

Unexpectedly, a distinct difference in C3d deposition was observed in pre-reperfusion endomyocardial tissue of the various patient groups (Fig. 4). C3d deposition was abundantly present in endomyocardial tissue of both patients with non-ischaemic and ischaemic heart failure in comparison to donor biopsies (both $P = 0.02$, Fig. 5). This C3d deposition was, however, not correlated to preoperative NTproBNP values (data not shown). No significant differences in endomyocardial C5b-9 deposition were observed between the groups ($P = 0.06$). Overall, endomyocardial tissue showed less C5b-9 deposition in all groups compared to myocardial tissue.

**Plasma measurements**

Assessment of complement depositions of C3d and C5b-9 in pre-reperfusion biopsies indicated clearly that the complement system was activated in the very early stages of ischaemia. To assess also plasma complement activation in the reperfusion phase, terminal complement complex activation was evaluated by measuring the release of sC5b-9 from the heart. This sC5b-9 release was assessed in the simultaneously collected systemical and myocardial venous blood samples. In addition, C5a was measured to assess whether the more upstream complement cascade is activated without causing the formation of the sC5b-9 complex (Fig. 6).

**No myocardial release or uptake of C5a or sC5b-9 in the early reperfusion phase.** No significant differences in sC5b-9 or C5a were measured over the heart, indicating the absence of net release of both complement activation markers from the reperfused heart during the first 60 min of reperfusion (Table 2). This suggests that the observed complement deposition in myocardial tissue was generated already in the preoperative phase.

**Similar levels of C5a and sC5b-9 at baseline and 24 h after reperfusion.** Not only arteriovenous concentration differences were studied. Similar baseline systemic levels of C5a ($P = 0.31$) and sC5b-9 ($P = 0.75$) were observed between the three patient groups the day before surgery. Also at 24 h after reperfusion, similar systemic levels of C5a ($P = 0.20$) and sC5b-9 ($P = 0.92$) were observed between the three patient groups. C5a and sC5b-9 returned even to baseline levels within 24 h after reperfusion in all patient groups with the exception of slightly lower sC5b-9 values in patients with ischaemic heart failure ($P = 0.03$).

**DISCUSSION**

It is generally accepted that complement plays a major role in myocardial I/R injury. However, since clinical trials consistently fail to show clinical relevant benefits of complement inhibitors, the role of complement activation in human myocardial I/R injury should be considered carefully. The present clinical study shows that myocardial C3d and C5b-9 depositions are already present in early left ventricular pre-reperfusion biopsies. Most abundant deposition was observed in patients with non-ischaemic heart failure. Endomyocardial biopsies, on the other hand, show clear depositions for C3d in both patients with non-ischaemic and ischaemic heart failure. These abundant early endomyocardial C3d depositions might be an underlying mechanism of heart failure, since these biopsies were used as read-out for preoperative involvement of complement activation. In addition, when comparing the heart failure groups with the donor group, also early endomyocardial C5b-9 deposition seems higher in the heart failure groups although not significantly different. Using our model of arteriovenous measurements, our results strongly suggest that terminal complement activation is not involved in the acute phase following myocardial reperfusion.

The observed myocardial and endomyocardial depositions of C3d and C5b-9 in our study are in line with previous human studies. However, these studies used autopsy or allograft tissue to investigate myocardial complement deposition. To our best knowledge, this is the first time that viable myocardial tissue is used to study myocardial and endomyocardial complement deposition. Nijmeijer et al. [15] used autopsy tissue of patients who died as a result of an acute myocardial infarction. Depositions of C3d and C5b-9 in patients treated with reperfusion or suffering from reinfarction were compared with depositions of both markers in patients who had no reperfusion or reinfarction. Patients with a reinfarction or with reperfusion therapy showed more depositions of these two complement activation markers. Unfortunately, no comparisons between their and our study
could be made for activation of complement components in plasma, since no plasma measurements were performed in addition to immunohistochemistry. In a study of Jenkins et al. [16], myocardial tissue, obtained by autopsy, was stained for C4d and C9 after myocardial infarction. Normal myocytes were non-reactive, whereas necrotic myocytes reacted strongly with the C4d and C9 antibodies. In our study, allograft tissue from donor hearts stained also negative for C3d and C5b-9.

As a result of the clear complement depositions in pre-reperfusion biopsies in our study, we were interested in the myocardial release of complement components upon myocardial reperfusion. By measuring arteriovenous differences over the reperfused organ, we were able to obtain data on local release of sC5b-9 and C5a from the heart. However, arteriovenous concentration differences did not indicate a myocardial release of C5a or sC5b-9.

Although no sC5b-9 or C5a release was observed in any of the studied groups, there was a remarkable increase in systemical sC5b-9 observed upon reperfusion in comparison to baseline, indicating activation of the immune system. This increase in complement components is in line with previous studies [5, 17, 18]. Remarkably, C5a levels were relatively high in patients with ischaemic heart failure (e causa ignota). There might be a less effective uptake and removal of C5a from plasma by granulocytes via cellular receptors such as leucocyte C5a receptors. There also might be a difference in carboxypeptidase N activity between

**Figure 4:** C3d and C5b-9 deposition in pre-reperfusion endomyocardial tissue. Early ischaemic endomyocardial tissue of patients with (A) non-ischaemic heart failure and (B) ischaemic heart failure, (C) non-heart failure patients and (D) donors were stained for C3d and C5b-9. Representative images are shown. Original magnifications ×200.
the patient groups. This enzyme cleaves the COOH-terminal arginine from C5a forming C5a des arginine. This C5a/C5a (desArg) complex has been measured with our C5a assay. The systemic increase in C5a and C5b-9 is not related to release of both activation markers from the heart, since our arteriovenous measurements over the heart did not show such a release. The systemic increase might be explained by the use of CPB and the surgical injury itself. However, the exact source remains to be unravelled. Baseline levels were similar between heart failure patients and non-heart failure patients in our study. This is in contrast to a study of Yasuda et al. [19], which showed increased levels of C3 split-products and sC5b-9 in patients with ischaemic heart failure. The increased baseline levels in their study might be explained by the fact that their patients suffered an acute myocardial infarction before the plasma measurements. Our patients did not have an acute myocardial infarction prior to cardiac surgery and were classified as chronic heart failure patients.

Despite the fact that in animal studies of myocardial I/R injury beneficial effects are observed following complement inhibition [4, 8, 9], only few complement inhibitors reached clinical trials such as anti-C5 antibodies [20], soluble complement receptor 1 (TP10) [21] and C1-esterase inhibitor [11]. However, all consistently show minute and inconsistent effects of anti-complement therapy in humans. These disappointing results raise the question whether animal models are sufficiently comparable to the human situation with relation to innate and adaptive immunity [22]. Another explanation might be that there is a narrow window of clinical opportunity in humans when treatments can be therapeutically effective [23], depending on timing, tissue penetration, patient characteristics, single vs. multiple cycle administration and many others. Based on our results, it may be more beneficial to use complement inhibitors during the chronic state of heart failure by contributing to anti-inflammatory processes instead of during reperfusion. Furthermore, it might be that in humans complement is not a key mediator of early reperfusion injury. This may well explain the unsuccessful outcomes of anti-complement therapy during the early reperfusion phase so far.

**Study limitations**

Our results show that terminal complement activation is not involved in the initiating phase of myocardial I/R injury. However, we only focused on the first 60 min of reperfusion and complement activation may result from other processes later in the cascade of I/R injury. This is confirmed in an animal study following renal I/R injury in which C3 deposition was present 2h after reperfusion and C6 and C9 depositions...
were present 12 h after reperfusion [24]. Complement might therefore have a role in repair or removal of damaged cells in the late reperfusion phase instead of an initiating role in the acute reperfusion phase. This concept matches the observed depositions of C5a and C5b-9 in patients with heart failure in our present study, who are already exposed to damage before surgery. In this study, only ischaemic pre-reperfusion biopsies could be collected since most of the included heart failure patients were haemodynamically instable in the early reperfusion phase. Myocardial tissue obtained during human autopsy is known for excessive deposition of C5b-9 [15]. Therefore, biopsies of human myocardial allografts were preferred as control tissue. However, also in these biopsies, some C5b-9 deposition might be observed as a result of the brain death situation of the donor [25], but due to ethical concerns no left ventricular biopsies were taken in healthy people. Therefore, biopsies of patients without heart failure were valuable as additional controls, although we cannot exclude cellular changes based on their valvular disease. As the goal of this study was to assess the basic pathophysiological role of complement activation in the process of I/R injury instead of correlating findings to the clinical outcome, small patient numbers were sufficient. However, some results were close to significant values which might be related to the small sample size. At last, aortic cross-clamping times and CPB times were different between the patient groups, although no correlations were observed between these durations and the measured complement components (data not shown).

**CONCLUSION**

Our findings do not support a prominent role for terminal complement activation in the acute phase following myocardial reperfusion. This is of major importance for the focus on therapeutic interventions currently being explored for human myocardial I/R injury, since clinical anti-complement therapies have not lead to appreciable clinically relevant beneficial effects so far. However, complement depositions of C3d and sc5b-9 were present before reperfusion in myocardial tissue, especially in patients with heart failure. These probably preoperative depositions might be an underlying mechanism of the syndrome of heart failure. Therefore, it may be more beneficial to use complement inhibitors during the chronic state of heart failure instead of during the acute phase of reperfusion.

**Table 2:** No arteriovenous concentration differences in all three patient groups

<table>
<thead>
<tr>
<th>Patient Group</th>
<th>Arterial AUC</th>
<th>Coronary sinus AUC</th>
<th>Delta AUC</th>
<th>P-value</th>
</tr>
</thead>
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<tr>
<td>NHF C5a (μg/ml*min)</td>
<td>2.20 (1.35–3.15)</td>
<td>2.29 (1.39–3.12)</td>
<td>0.09</td>
<td>0.20</td>
</tr>
<tr>
<td>NHF C5b-9 (U/ml*min)</td>
<td>407.47 (242.44–536.83)</td>
<td>406.85 (181.22–621.05)</td>
<td>−0.62</td>
<td>0.30</td>
</tr>
<tr>
<td>NHF C5a (μg/ml*min)</td>
<td>4.83 (3.20–6.24)</td>
<td>4.35 (2.84–6.90)</td>
<td>−0.48</td>
<td>0.051</td>
</tr>
<tr>
<td>NHF C5b-9 (U/ml*min)</td>
<td>271.32 (138.71–410.24)</td>
<td>269.24 (126.65–390.05)</td>
<td>−2.08</td>
<td>0.08</td>
</tr>
<tr>
<td>NHF C5a (μg/ml*min)</td>
<td>3.31 (2.30–3.65)</td>
<td>3.22 (2.03–4.19)</td>
<td>0.01</td>
<td>0.12</td>
</tr>
<tr>
<td>NHF C5b-9 (U/ml*min)</td>
<td>287.18 (247.76–332.85)</td>
<td>300.92 (222.07–355.85)</td>
<td>13.74</td>
<td>0.51</td>
</tr>
</tbody>
</table>

No release by the myocardium was observed for C5a or C5b-9 in patients with non-ischaemic heart failure (NIHF), ischaemic heart failure (IHF) and non-heart failure patients (NHF). A positive delta AUC indicates a myocardial release of C5a or C5b-9, whereas a negative delta AUC represents a myocardial uptake. Means of the AUC and interquartile ranges are shown.

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**SUPPLEMENTARY MATERIAL**

Supplementary material is available at *EJCTS* online.

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**Conflict of interest:** none declared.

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