# ORIGINAL PAPERS

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Vilma Vuohelainen[1, A, B, D, F], Timo Paavonen[2, A, E, F], Mari Hamalainen[3, B–F], Eeva Moilanen[3, A, C–F], Ari A. Mennander[1, A–F]

# C4d Deposition Reveals Myocardial Infarction After Cardiac Arrest – Experimental Study*

[1] Heart Hospital, Tampere University Hospital, Tampere, Finland
[2] Department of Pathology, Tampere University Hospital, Tampere, Finland
[3] Department of Immunopharmacology, Tampere University, Tampere, Finland

**A** – research concept and design; **B** – collection and/or assembly of data; **C** – data analysis and interpretation; **D** – writing the article; **E** – critical revision of the article; **F** – final approval of article; **G** – other

## Abstract

**Background**. The diagnosis of regional myocardial infarction (MI) after cardiac arrest and ischemia-reperfusion injury (IRI) is a major clinical challenge.
**Objectives.** We evaluated in a rat cardiac transplantation model whether IRI alone or with MI would induce complement C4d deposition.
**Material and Methods.** Isogenic heterotopic cardiac transplantation was performed in 16 Fischer 344 rats to induce IRI, of which 9 rats also underwent ligation of the left anterior coronary artery (LAD) of the heart to yield MI. Histology and qRT-PCR for endothelial nitric oxide synthase (eNOS), inducible nitric oxide synthase (iNOS) and transforming growth factor β (TGFβ) were performed after cessation of heart beat. C4d was evaluated by immunohistochemistry.
**Results.** Myocardial inflammation and C4d deposition was increased in grafts with IRI+MI as compared with IRI (0.71 vs. 0.14, PSU, respectively, $p < 0.04$ and 80.13 vs. 20.29, PSU, respectively, $p < 0.02$). The expression of eNOS decreased in grafts with IRI + MI as compared with IRI ($p < 0.05$). Receiver operating characteristic (ROC) curve analysis showed that IRI + MI was associated with C4d deposition (AUC 0.837; S.E. 0.116; $p = 0.035$; 95% C.I. 0.610–1.000).
**Conclusions.** Increased C4d deposition may be amenable to identify early MI after cardiac arrest. Early treatment aimed towards complement activation may provide a novel means for induced MI after cardiac arrest (**Adv Clin Exp Med 2015, 24, 3, 393–399**).

**Key words:** myocardial infarction, C4d, complement, heterotopic rat cardiac transplantation.

The definitive diagnosis of regional myocardial infarction (MI) after cardiac arrest and ischemia-reperfusion injury (IRI) is a major clinical and forensic challenge. Uncontrolled IRI after cardiac arrest may lead to permanent ongoing ischemia eventually developing MI. When feasible, the heart can be temporarily assisted by inserting a left ventricular assist device to reduce the myocardial workload. Even though prompt revascularization is performed to stenosed culprit coronary arteries, irreversible myocardial damage may ensue [1]. After cardiac dysfunction and surgery, interpretation of MI from the release of traditional markers- such as creatinine kinase, troponin T and pro-BNP may be blurred by the presence of inflammation and tissue destruction [2, 3].

IRI and MI may be investigated by the widely used heterotopic rat cardiac transplantation model [4]. This model provides the possibility to investigate the cardiac graft in a non-working state simulating

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the presence of a left ventricle assist device, while the recipient heart keeps the animal alive. Instead of endangering the recipient heart, in order to achieve MI, it is practical to ligate permanently the left anterior descending coronary artery (LAD) of the heterotopically transplanted cardiac graft. This animal model mimics the clinical concept of having an area of local and persisting MI after complete revascularization of the globally ischemic heart. Using this experimental model, we have recently demonstrated that MI after IRI has a remote myocardial impact after cardiac arrest [4].

Few *postmortem* evidence [1] and experimental studies [5, 6] suggest that complements are activated after MI. Complement activation is an early marker of tissue destruction [7–13]. The end-production of the complement activation cascade C4d represents a stable molecule suitable for evaluation. It is not known whether complement activation occurs after cardiac arrest, least to say whether C4d reveals the developing MI after IRI. We therefore tested the power of complement activation, as evidenced by C4d deposition, to detect MI after cardiac arrest and IRI in our heterotopic rat cardiac transplantation model. Histology and qRT-PCR for endothelial nitric oxide synthase (eNOS), inducible nitric oxide synthase (iNOS) and transforming growth factor β (TGFβ) were performed after cessation of heartbeat to verify the effect of IRI and MI on the remote myocardium by looking for delicate changes in gene expressions associated with the activation of C4d. Nitric oxide synthases and TGFβ mirror the molecular cascade activation related to recovery from IRI [14].

# Material and Methods

## Ethics

The study was approved by the State Provincial Office. Thirty two inbred Fischer 344 rats (F344/ /NHsd, Harlan Laboratories, The Netherlands) weighing 200–270 g, served as donors (n = 16) and recipients (n = 16). The rats were kept *in vivarium* and received humane care in compliance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH publication No. 86–23, revised 1996).

## Surgical Procedure

The rats were anesthetized with sevoflurane (Baxter, USA) for inhalation, and a mixture of ketamine (Ketalar®; Orion Pharma Oy, Espoo, Finland; 7.5 mg/100g) and medetomidine (Dormitor®; Pfizer Oy Animal Health, Espoo, Finland; 0.05 mg/100 g) intraperitoneally. A modified heterotopic transplantation of the heart was performed to all 16 grafts, as previously described [4]. Briefly, before harvesting, cold 4ºC infusion with cardioplegia fluid (Custodiol®; Bretschneider HTK solution for cardioplegia and miltiorgan protection, Germany) was infused into the donor aorta in order to arrest the heart and maximize myocardial protection. After harvesting, the cardiac graft was immersed into cold (4ºC) temperature physiologic saline fluid. The graft aorta was joined to the aorta and the graft pulmonary artery to the inferior *vena cava* of the recipient. From the recipient aorta, the transplanted heart received oxygenated blood that was introduced into the coronary arteries of the graft. *Via* the coronary sinus, this blood circulated into the right atrium and eventually the right ventricle, from where deoxygenated blood recirculated to the recipient rat throughout the pulmonary artery. Therefore, the nutritional flow of the myocardium consisted of oxygenated blood, and the transplanted heart was not ischemic after reperfusion upon transplantation. Since the aortic valve was competent, oxygenated blood was not allowed to fill the left ventricle, and therefore the transplanted heart simulated a non-working resting state of the left side of the graft. This heterogenous transplantation model allowed us to study IRI *in vivo* without interferences of myocardial stress factors. Total ischemia time before total graft reperfusion was 30 min after cardiac arrest. After the procedure, carprofen (Rimadyl®; Pfizer Oy Animal Health, Helsinki, Finland) 0.1~0.15 mL was given subcutaneously for pain relief.

## Experimental Groups

The rats were randomized into 2 groups. Seven grafts underwent transplantation only to serve as controls with IRI. In 9 grafts, the LAD was also ligated permanently at its proximal part with a single 7–0 suture yielding a confined local MI (IRI + MI); the ligation knot for LAD obstruction was placed immediately before the bifurcation of the 1st diagonal artery branch of the LAD.

## Graft Patency

Graft patency was achieved by means of palpation using a score from 0 to 6; 0 indicated no pulse, 2 indicated weak pulsation, and 6 meant normal contractility and strong pulsation. The palpation score, as a direct measure of cardiac vitality and effective contractility, proved to be a reliable and convenient test for definition of the end point

for graft survival, with no variability or bias in the evaluations of independent observers [15].

## Tissue Samples

The recipient rats were sacrificed when palpation score of the cardiac graft decreased to less than 2 out of 6. The basal part of the cardiac graft was snap frozen in liquid nitrogen and stored at –70ºC for further analysis. The middle half of the graft was embedded in paraffin and 5 μm sections were cut and stained with hematoxylin-eosin.

## Histology

Evaluation of histology was performed blinded to the study protocol. The following variables were evaluated: presence of myocardial edema, hemorrhage and inflammation. Periadventitial inflammation was graded according to an arbitrary scale from 0 to 2 and expressed as point score units (PSU): 0, no inflammation; 1, presence of occasional inflammatory cells; 2, groups of inflammatory or proliferating cells. Vacuolated nuclei of the media layer of intra-myocardial arteries reflected edema and were counted in a representative cross-sectional intra-myocardial artery chosen randomly from the left anterior ventricular wall. Round and smooth-edged blue nuclei of the media cells were defined as normal and expressed as PSU. The number of vacuolated nuclei was divided by the number of round smooth-edged media cell nuclei to obtain the relative number of vacuolated nuclei.

## Immunohistochemistry for C4d

Immunohistochemistry was performed using Ventana Lifesciences Benchmark XT© Staining module. The paraffin-embedded slides were deparaffinized with 3 changes of xylene, and rehydrated in a series of graded ethanol, and rinsed well under running distilled water. Slides were placed in a preheated retrieval buffer, 0.1 mmol EDTA, pH 8.0, for 30 min, then cooled in the buffer for 5 min, followed by a 5-min rinse under running distilled water. After heat-induced epitope retrieval, slides were placed on an autostainer (DAKO Corp, Carpinteria, California, USA). Sections were incubated with 3% hydrogen peroxide in ethanol for 5 min to inactivate the endogenous peroxides and incubated in C4d (dilution 1:50) (Biomedica Gruppe) for 30 min, followed by rinsing with Tris-buffered saline solution with Tween 20 (TBST) wash buffer. Secondary incubation was with DUAL-labeled polymerhorseradish peroxidase (K4061; DAKO Corp) for 15 min. The slides were rinsed with TBST wash buffer. Sections were then incubated in 3,3-diaminobenzidine (K3467, DAKO Corp) for 5 min, counterstained with modified Schmidt hematoxylin for 5 min, and rinsed for 3 min in tap water to blue sections, dehydrated with graded alcohols, and cleared in 3 changes of xylene before mounting. Positively stained C4d deposition was counted in a representative cross-sectional intra-myocardial artery chosen randomly from the left anterior ventricular wall. The total number of myocardial C4d deposition was calculated accordingly and expressed as PSU.

## Quantitative RT-PCR Analysis

The frozen tissue of the base of the heart was homogenized and RNA extraction was carried out with GenElute™ Mammalian Total RNA Miniprep kit (Sigma-Aldrich, St. Louis, MO, USA) with proteinase K treatment. Total RNA was then reverse-transcribed to cDNA using TaqMan® Reverse Transcription reagents and random hexamers (Applied Biosystems, Foster City, CA, USA). cDNA obtained from the RT reaction (amount corresponding to approximately 1 ng of total RNA) was subjected to quantitative PCR using QuantiTect® Primer Assays (Qiagen, Valencia, CA, USA) for eNOS, iNOS, TGFβ and GAPDH, Maxima® SYBR Green/ROX qPCR Master Mix (Thermo Scientific, Waltham, MA, USA) and ABI PRISM 7000 Sequence detection system (Applied Biosystems, Foster City, CA, USA). PCR reaction parameters for SYBR® Green detection were as follows: incubation at 50ºC for 2 min, incubation at 95ºC for 10 min, and thereafter 40 cycles of denaturation at 95ºC for 15 s and annealing and extension at 60ºC for 1 min. Each sample was determinated in duplicate. Ct values were determined, and the relative quantification was calculated using the $2^{-\Delta\Delta Ct}$ method [16]. Values of control samples were used as a calibrator, and the expression levels of eNOS, iNOS and TGFβ were normalized against GAPDH.

# Statistical Analysis

Statistical analysis was performed using SPSS for Windows (v. 20.0). Data is presented as median or mean ± standard error of the mean (SEM). Data was analyzed with Mann-Whitney *U* test when appropriate. Pearson correlation was performed to investigate for the relation between continuous variables. The predictive value of C4d deposition to identify remote immunological reactivity associated with myocardial infarction after IRI was assessed by Receiver operating characteristic (ROC) curve analysis. P-values $< 0.05$ were considered significant.

# Results

## Heart Graft Patency

One heart with IRI + MI and IRI were non-palpable one day after reperfusion. All other hearts with IRI + MI and with IRI had a palpation score less than 2 out of 6 after 2 days of reperfusion (Table 1).

**Table 1.** Number of dysfunctional hearts after reperfusion with palpation score < 2 out of 6

| Group, number | 1 day | 2 day |
|---|---|---|
| IRI, n = 7 (%) | 1 (14%) | 6 (86%) |
| IRI + MI, n = 9 (%) | 1 (11%) | 8 (89%) |

IRI = hearts with ischemia-reperfusion injury;
IRI + MI = hearts with ischemia-reperfusion injury and myocardial infarction.

## Histology

Two days after operation, there were no hemorrhagic or major remote ischemic myocardial differences between the hearts with IRI and IRI + MI (1.29 vs. 0.86, PSU, respectively, p = 0.59 and 1.57 vs. 1.43, PSU, respectively, p = 0.83). Global myocardial edema and epicardial inflammation did not differ among IRI and IRI + MI (2.29 vs. 2.14, PSU, respectively, p = 0.79 and 0.57 vs. 0.43, PSU, respectively, p = 0.94). A global ischemic area was faintly observed in the left anterior ventricular wall of the myocardium in IRI + MI; this was recorded as mild myocardial edema in the area corresponding to the developing infarction. However, there was no statistical difference in the number of sharp-edged dark media cell nuclei of intramyocardial arteries in hearts with IRI and IRI + MI (10.86 vs. 13.40, PSU, respectively, p = 1). There was also no difference in the number of clear smooth-edged media cell nuclei of remote intramyocardial arteries in hearts with IRI as compared with IRI + MI (24.83 vs. 17.50, PSU, respectively, p = 0.688). Myocardial inflammation increased in hearts with IRI + MI as compared with IRI (0.71 vs. 0.14, PSU, respectively, p = 0.04).

## Immunohistochemistry for C4d

Statistically, increased myocardial staining for C4d was observed in hearts with IRI + MI as compared with IRI (80.13 vs. 20.29, PSU, respectively, p = 0.02, Fig. 3A). Increased number of C4d deposition was also specifically observed in the media of intramyocardial arteries situated along the left anterior wall corresponding to the infarction

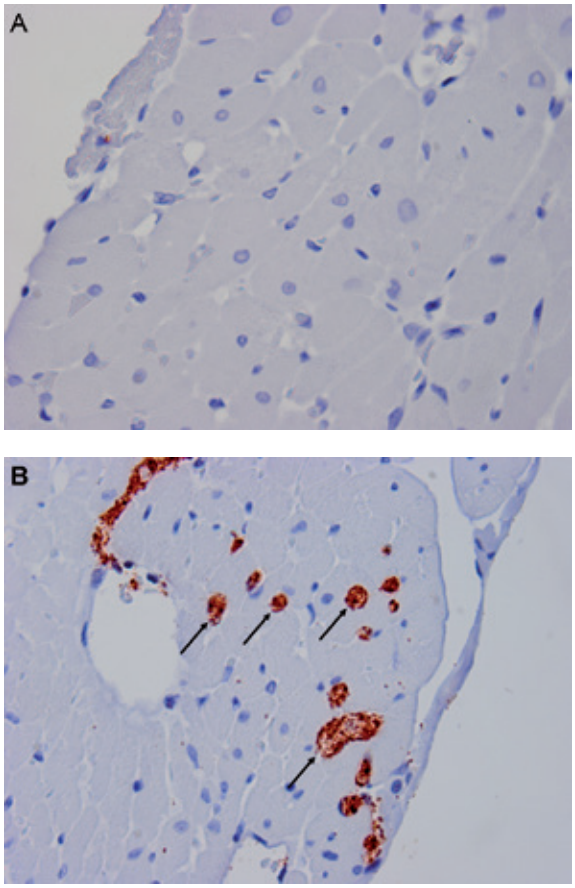


**Fig. 1.** Representative immunohistochemistry for myocardial C4d deposition of a heart with ischemia--reperfusion injury only (IRI; A), and a graft with ischemia-reperfusion injury and myocardial infarction (IRI + MI; B) 2 days after reperfusion. X40. Note intensive positive C4d staining (arrows) in remote myocardium of the heart in B

area in hearts with IRI + MI in contrast to only few staining in hearts with IRI (2.75 vs. 0.86, PSU, respectively, p = 0.01, Fig. 3B). The relative number of C4d deposition, that is the total number of C4d deposition divided by the number of observed intramyocardial arteries, remained elevated in hearts with IRI + MI as compared with hearts with IRI (1.73 vs. 1.25, PSU, respectively, p = 0.03).

## eNOS, iNOS and TGFß Expressions

The expression of eNOS decreased in hearts with IRI + MI as compared with IRI (0.51 vs. 1.58, respectively, p = 0.05). There were no differences in the expressions of iNOS and TGFβ in hearts with IRI + MI as compared with IRI (2.06 vs. 5.98, respectively, p = 0.64 and 1.03 vs. 2.69, respectively, p = 0.23). The Pearson correlation between C4d staining of the myocardium and the expression of eNOS was –0.357 (p = 0.156), and –0.017 (p = 0.481)

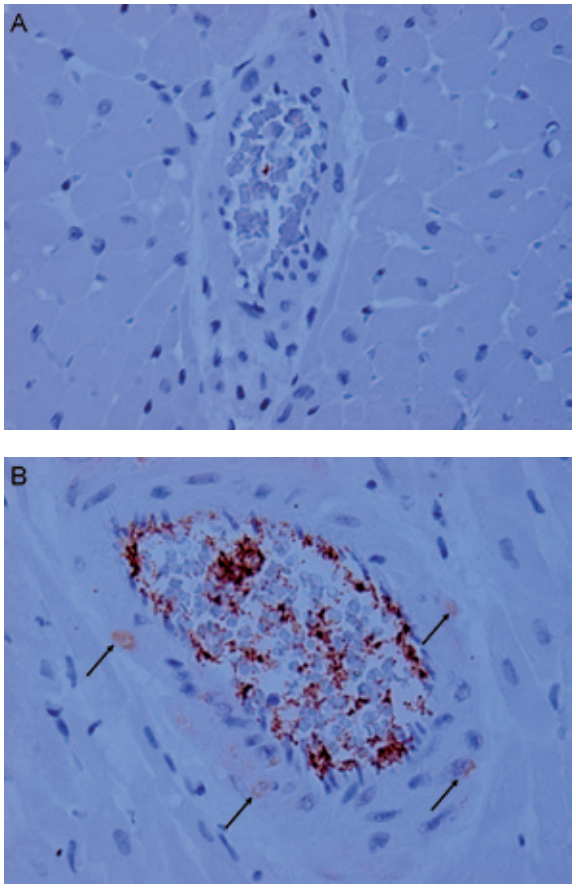


**Fig. 2.** Representative immunohistochemistry of an intramyocardial artery for C4d deposition of a heart with ischemia-reperfusion injury only (IRI; A), and a graft with ischemia-reperfusion injury and myocardial infarction (IRI + MI; B) 2 days after reperfusion. X40. Note positive C4d staining (arrows) in an intramyocardial artery of the heart in B

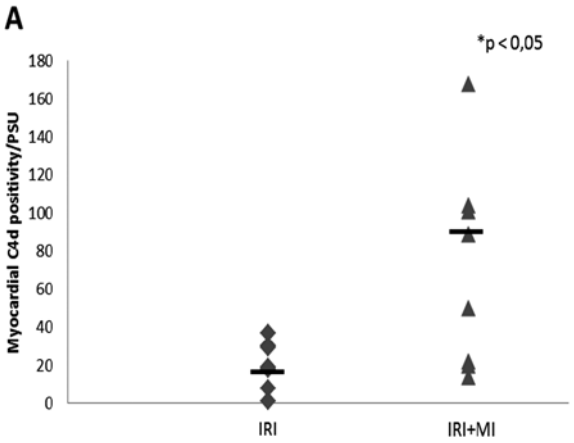


**Fig. 3A.** Number of myocardial C4d deposition in hearts with ischemia-reperfusion injury only (IRI, diamonds), and grafts with ischemia-reperfusion injury and myocardial infarction (IRI + MI, triangles). Note increased myocardial C4d deposition 2 days after reperfusion in hearts with IRI + MI as compared with IRI. *p < 0.05, Mann-Whitney. Horizontal bars indicate median

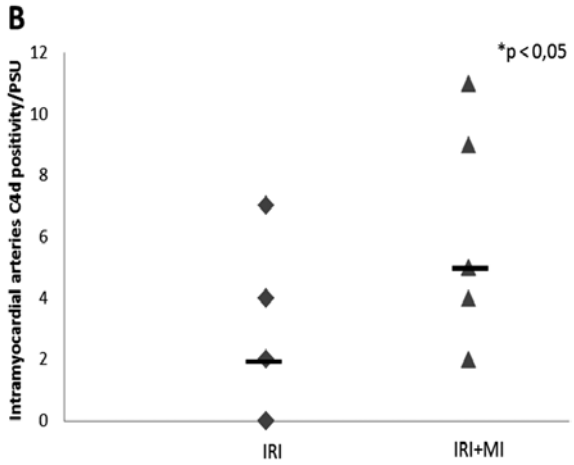


**Fig. 3B.** Number of intramyocardial artery wall C4d deposition in hearts with ischemia-reperfusion injury only (IRI, diamonds), and grafts with ischemia-reperfusion injury and myocardial infarction (IRI + MI, triangles). Note increased intramyocardial artery wall C4d deposition 2 days after reperfusion in hearts with IRI + MI as compared with IRI. *p < 0.05, Mann-Whitney. Horizontal bars indicate median

between C4d staining of intramyocardial arteries and the expression of eNOS. Statistically, these correlations were not significant.

## ROC Curve Analysis

The predictive value of C4d positive staining to myocardial infarction after IRI was assessed by Receiver operating characteristic (ROC) curve analysis. ROC analysis showed that myocardial infarction was associated with C4d deposition (AUC 0.837; S.E. 0.116; p = 0.035; 95% C.I. 0.610–1.000), but not with presence of inflammation per se (AUC 0.786; SE 0.131; p = 0.074; 95 % CI 0.529–1.000).

# Discussion

We demonstrate with this heterotopic rat cardiac transplantation model simulating the clinical concept of total cardiac arrest that C4d reveals MI in hearts that ceased to beat 2 days after IRI despite myocardial inflammation. In contrast, IRI alone did not suffice to induce increased C4d deposition.

A major challenge to the clinician is to define the presence of MI in hearts lost after IRI. It has previously been shown that complement activation occurs in the infarction area in otherwise healthy hearts [1, 5, 6, 17]. In our study, C4d was found in the myocardium remote to the infarction after IRI, and C4d was not only encompassed in the infarction area. C4d deposition was present

specifically in the wall of the intramyocardial arteries remote to the infarction. This is in accordance to previous observation stating that complement deposition was particularly prominent in the arterial wall indicating subsequent development of arteriopathy [18]. Importantly, complement C3d and C5b-9 depositions were significantly increased in larger areas of the hearts of patients with a reinfarction than in patients with single infarcts [1]. An immunological effect of MI in ischemic hearts undergoing IRI seems noteworthy and determines the outcome of the remote myocardium.

In contrast to some [1, 5, 6, 19], we did not observe increased C4d deposition in hearts with IRI per se. Reperfusion alone has not been demonstrated convincingly in humans to induce C4d deposition [1]. It is plausible that though C4d may be considered rather stable, a wash-out effect may occur after complete reperfusion in grafts with IRI *in vivo* [20]. On the other hand, it has been demonstrated that C4d deposition is a controversial indicator- at least of humoral rejection in ABO--compatible liver allografts. Diagnostic interpretation, based alone on the complement split product C4d, should be cautiously done [21]. The utility of C4d deposition to detect early myocardial infarction may help in the interpretation of perioperative ischemic injury [11, 22]. The intensity of microvascular C4d staining may differ during humoral rejection as compared with myocardial infarction alone [22].

In our study, we did not specifically investigate for the size of MI, since all grafts with IRI + MI had occlusion of the LAD exactly at the same anatomic site. As previously observed, complement activation together with C3d and C5b-9 depositions did not correlate with infarction size alone [1]. To verify the presence of MI during IRI, we analyzed eNOS expression of the myocardium to confirm the remote myocardial effect of MI. We also investigated the expressions of iNOS, TGFβ and studied intramyocardial artery histology. Nitric oxide synthases mirror the molecular cascade activation related to recovery from IRI [14], and these parameters were chosen to confirm the effect of IRI on the remote myocardium by seeking for delicate changes in gene expressions influencing the vascular endothelium. Decreased eNOS was observed in grafts with IRI + MI as compared with grafts with IRI alone. Importantly, myocardial inflammation was eminent during MI, but in contrast to C4d, it was not possible to identify the hearts with MI by the presence of inflammation per se since tissue destruction to some extension is also involved during IRI and cardiac dysfunction alone. Both iNOS and TGFβ were not capable to distinguish between IRI + MI and IRI as the presence of inflammation after cardiac dysfunction was imminent.

There is a strong need for specific markers of infarction independent of inflammation. Complement activation predicts concomitant inflammation. As an indicator of ongoing inflammation, activated complement deposition was increased in diseased aortic valves [23]. This study hints that myocardial C4d deposition due to MI after IRI and cardiac dysfunction occurs concomitantly with inflammation. It was beyond the scope of this study to investigate for the involvement of the alternative vs. classical complement pathway, and both may be present [6, 7]. We did not evaluate for gene expressions of C4d since the detection of C4d deposition by immunohistochemistry is relatively accurate and proved comparable at least to immunofluorescence methods [24].

In summary, C4d is a powerful indicator of the impact of MI to the remote myocardium after IRI. This observation may serve as an important adjunct in the diagnosis of MI after IRI. The inhibition of C4d deposition and the investigation of peripheral blood for complements may further elucidate the impact of complement activation during MI and IRI.

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## Address for correspondence:

Ari A. Mennander
Heart Hospital
Tampere University Hospital
SDSKIR
PL2000
Tampere
Finland
E-mail: ari.mennander@hotmail.com

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