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Altered hematological, biochemical and immunological parameters as predictive biomarkers of severity in experimental myocardial infarction

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ABSTRACT

Preclinical studies in cardiovascular medicine are necessary to translate basic research to the clinic. The porcine model has been widely used to understand the biological mechanisms involved in cardiovascular disorders for which purpose different closed-chest models have been developed in the last years to mimic the pathophysiological events seen in human myocardial infarction.

In this work, we studied hematological, biochemical and immunological parameters, as well as Magnetic resonance derived cardiac function measurements obtained from a swine myocardial infarction model. We identified some blood parameters which were significantly altered after myocardial infarction induction. More importantly, these parameters (gamma-glutamyl transferase, glutamic pyruvic transaminase, red blood cell counts, hemoglobin concentration, hematocrit, platelet count and plateletcrit) correlated positively with cardiac function, infarct size and/or cardiac enzymes (troponin I and creatine kinase-MB).

Thus several blood-derived parameters have allowed us to predict the severity of myocardial infarction in a clinically relevant animal model. Therefore, here we provide a simple, affordable and reliable way that could prove useful in the follow up of myocardial infarction and in the evaluation of new therapeutic strategies in this animal model.

1. Introduction

Preclinical studies in cardiovascular medicine are mandatory in order to effect the translation of basic research to clinical practice (bench to beside. There are many anatomical and physiological differences between small animal models and humans limit the extrapolation of research results to the clinical scenario. In contrast, large animal models such as swine display similarities to humans in terms of anatomy, physiology and biochemical parameters (van der Spoel et al., 2011). In the field of cardiovascular medicine, the porcine model has been widely accepted by researchers and regulatory agencies as representative of the human disease, and is considered mandatory for understanding the biological mechanisms involved in cardiovascular disorders and to evaluate new therapies. In the setting of myocardial

Abbreviations: CRP, C-reactive protein; EF, ejection fraction; GGT, gamma-glutamyl transferase; GOT, glutamic oxaloacetic transaminase; GPT, glutamic pyruvic transaminase; HCT, hematocrit; HGB, hemoglobin concentration; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; MPV, mean platelet volume; MR, magnetic resonance; PCT, plateletcrit; PDW, platelet distribution width; PLT, platelet count; RBC, red blood cell count; RDW-CV, red blood cell distribution width coefficient of variation; RDW-SD, red blood cell distribution width standard deviation; WBC, total white blood cells count

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infarction, clinically-relevant animal models have permitted the identification of pathophysiological changes with clinical relevance. Additionally, these animal models are a valuable tool in the evaluation of safety aspects and efficacy of new therapeutic approaches and ideas prior to clinical application.

The vast majority of swine animal models have been used to mimic myocardial ischemia, either acute or chronic (Qu et al., 2012; Varga-Szemes et al., 2014), but they have also prooved useful for studying tachycardia and the anatomical substrate of arrhythmias (Tschabrunn et al., 2016).

Different surgical procedures have been developed in the last 40 years to model acute or chronic myocardial infarction. Early studies used open chest surgery ligating left anterior descending coronary artery (Lichtig et al., 1975). This surgical procedure has been used recently to study the effects of the ligation at different levels and diagonal branches (Huang et al., 2010). This approach, however, presents several disadvantages, mostly related to its invasive nature, that adversely affect the homogeneity of the injury obtained between animals. Moreover, cardiac parameters are influenced by the thoracotomy and high mortality rate and complications related to the surgical trauma are common (Munz et al., 2011).

In the last 20–30 years, the closed-chest model of myocardial infarction has been widely described in swine (Biondi-Zoccai et al., 2013; Crisóstomo et al., 2013; Pérez de Prado et al., 2009) and it is widely recognized as a more appropriate approach for cardiovascular research as it mimics the pathophysiological events in human myocardial infarction in absence of surgical influences (de Waard et al., 2016; Ishikawa et al., 2011). Variations of this model using different occlusion times that range from 40 min (Fernández-Jiménez et al., 2017), 60 min (McCall et al., 2012), 90 min (Koudstaal et al., 2014) or 150 min (Sasano et al., 2009) have been successfully developed in the last years.

Conventional evaluation methods such as electrocardiography and cardiac enzymes have been widely used in large animal model. New techniques developed in the last years include: modified electrocardiograms such as epicardial electrograms and body surface electrocardiograms in dogs subjected to occlusion of coronary artery (Mor-Avi et al., 1987), endocardial electromechanical mapping in a porcine acute infarction model (Odenstedt et al., 2003) and PET imaging in canine myocardial infarction models (Zalutsky et al., 1992). Although very different methods have been proposed to evaluate and monitor myocardial infarction, the anatomopathological analysis using different histological staining such as tetrazolium chloride (TTC) is still considered the most accurate approach for measuring infarct size.

To the best of our knowledge, there is a lack of immunological, hematological and biochemical studies in swine myocardial infarction models. In the present study, we aimed to identify any significant changes to commonly measured parameters that occur during the acute phase of myocardial infarction. Moreover, hematological/biochemical parameters were correlated with biomarkers widely used to quantify myocardial infarction such as cardiac enzymes (troponin I and creatine kinase-MB) and cardiac magnetic resonance-derived data (ejection fraction and infarct area).

The novelty of this work relies on the identification of biochemical or cellular parameters altered after myocardial infarction in the porcine model. We have identified several biomarkers useful for the assessment of myocardial infarction. These biomarkers are closely correlated with cardiac function or cardiac enzymes and may provide a simple, fast and accurate quantification of myocardial infarction in this animal model.

2. Materials and methods

2.1. Animals and experimental design

Nineteen Large White pigs were housed in the animal facility at the Jesús Usón Minimally Invasive Surgery Centre and used for all experimental procedures. Animals aged 3 months and weighing 30–35 kg at the beginning of the study were used. All experimental protocols were approved by the Animal Welfare Ethical Committee of the Jesús Usón Minimally Invasive Surgery Centre and fully complied with recommendations outlined by the local government (Junta de Extremadura) and by the Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes.

Blood sampling was performed before myocardial infarction model creation, at 24 h and 7 days after myocardial infarction.

2.2. Myocardial infarction model creation

A closed chest reperfused myocardial infarction was created as previously described (Crisóstomo et al., 2013). Briefly, anesthetized animals were subjected to a percutaneous right femoral access using a 7 Fr Introducer sheath (Terumo, Tokyo, Japan), and a Hockey Stick 6 Fr guiding catheter (Mach 1, Boston Scientific Corporation, Natick, MA, USA) was navigated under fluoroscopic guidance to the origin of the left coronary artery. Coronary angiograms were obtained in the 40° left anterior oblique projection to better demonstrate the length of the left anterior descending artery. A coronary balloon catheter (typically 3 mm x 8 mm, Ryujin Plus, Terumo, Tokio, Japan) was inserted over a 0.014″ coronary guidewire and advanced to below the origin of the first diagonal branch, where it was inflated to occlude flow to the distal myocardium. Occlusion was maintained for 90 min to assure transmural infarct creation. Upon balloon deflation, the coronary artery was checked for patency by repeating the angiogram. During the procedure, animals were fully monitored, including blood pressure, electrocardiogram, O_2 saturation, and end tidal CO_2. Continuous infusion of lidocaine at rate of 1 mg/kg/h (Lidocaine, Braun Medical, Barcelona, Spain) was used through the procedure. Systemic heparin (Heparina Rovi 5%, Laboratorios farmaceuticos Rovi, Madrid, Spain) was injected intravenously (150 UI/kg) prior to percutaneous sheath placement.

2.3. Biochemical analysis

Blood samples were collected, centrifuged to eliminate cellular debris and processed in the random access clinical analyzer Metrolab 2300 (Metrolab S.A., Buenos Aires, Argentina) to determine their biochemical composition (bilirubin, creatinine, glucose, urea, gamma-glutamyl transferase or GGT, glutamic oxaloacetic transaminase or GOT, glutamic pyruvic transaminase or GPT and C-reactive protein or CRP).

2.4. Hematological analysis and phenotypic characterization of peripheral blood lymphocytes

Blood samples were collected in EDTA containing tubes and leukocyte, red blood cells and platelets-related parameters were determined in an automatic hematology analyzer (Mindray BC-5300 Vet, Hamburg, Germany). Total white blood cells (WBC) were counted: neutrophils, lymphocytes, monocytes, eosinophils and basophils. Red blood cells (RBC) were also counted and additional parameters were also determined: hemoglobin concentration (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red blood cell distribution width coefficient of variation (RDW-CV), red blood cell distribution width standard deviation (RDW-SD). Finally, platelets count (PLT) was also quantified together with mean platelet volume (MPV), platelet distribution width (PDW) and plateletcrit (PCT).

For flow cytometry analysis, peripheral blood lymphocytes were isolated by centrifugation over Histopaque-1077 (Sigma, St. Louis, MO). The cells were washed twice with DMEM containing 10% FBS and stained with fluorescent-labelled monoclonal antibodies against porcine CD3 (Clone: CD3-12), CD8α (Clone: MIL12), CD16 (Clone: G7), CD27 (Clone: B30C7) and CD45RA (Clone: MIL13) from AbD Serotec

(Kidlington, UK) and CD4 (Clone: 74-12-4) from BD Biosciences (San Jose, CA, USA). For cytometric analysis, 2×10^5 cells were incubated for 30 min at 4 °C with appropriate concentrations of monoclonal antibodies and were then washed and re-suspended in PBS. The flow cytometric analysis was performed in a FACScalibur cytometer (BD Biosciences) after acquisition of 10^5 events. Cells were primarily selected using forward and side scatter characteristics. Multiparametric flow cytometry was performed on CD4 + T cells and CD8 + T cells (gated as CD4+ CD8α– and CD4- CD8α+ respectively). The co-expression of CD27 and CD45RA on CD4 + T cells and CD8 + T cells allowed us to distinguish three different T cell subsets with distinct functional properties: naïve T cells (CD45RA + CD27+), effector T cells (CD45RA–CD27+) and effector/memory T cells (CD45RA–CD27–). Fluorescence was analyzed using CellQuest software (BD Biosciences, San Jose, CA, USA) and isotype-matched negative control antibodies were used in all the experiments.

2.5. Cardiac enzyme analysis

Blood samples were collected in EDTA containing tubes for troponin I and creatine kinase-MB immunoassay (AQT90 Flex, Radiometer Iberica SL, Madrid, Spain). Results are given as μg/l.

2.6. Cardiac magnetic resonance

Cardiac MR studies (Intera 1.5 T, Philips Medical Systems. Best, The Netherlands) were performed 7 days after infarction. Retrospective cardiac triggering was used. A 4 elements phase array coil was placed around the animals' chest. Images were acquired in the intrinsic cardiac planes: short axis, vertical long axis and horizontal long axis views. For measurement of left ventricular function and mass breath hold gradient echo cine images were obtained over the entire left ventricle. For infarct size measurements, short axis images were acquired 5–15 min after the injection of 0.2 mmol/kg of a gadolinium-based contrast agent (Gadobutrol. Gadovist 1.1 mmol/l, Bayer Schering Pharma AG, Berlin, Germany) using a breath-hold 3D gradient-echo inversion-recovery sequence.

2.7. Statistical analysis

Statistical analysis was performed using the SPSS-21 software (SPSS, Chicago, IL, USA). Normality was assessed using a Shapiro-Wilk test. Paired comparisons were performed using a Student *t*-test for parametric data or Wilcoxon sign test for non-parametric data. Correlations were calculated using the Pearson coefficient correlation and "r" value was interpreted as follows: -1.0 to -0.5 or 0.5–1.0: Strong correlation, -0.5 to -0.3 or 0.3 to 0.5: Moderate correlation, -0.3 to -0.1 or 0.1 to 0.3: Weak correlation, -0.1 to 0.1: None or very weak correlation. All *p*-values ≤ 0.05 were considered statistically significant.

3. Results

3.1. Biochemical parameters in peripheral blood from infarcted swine

A total of 19 animals were used for this study. Two animals died from refractory arrhythmias during infarct creation. Peripheral blood samples were collected from the remaining 17 pigs before model creation (basal), at 24 h and 7 days after myocardial infarction. Biochemical parameters are shown in Fig. 1. Our results firstly demonstrated a significant decrease in bilirubin ($p \leq 0.001$) at 24 h and 7 d when compared to basal. In the case of urea, a significant decrease ($p \leq 0.05$) was found at day 7 when compared to 24 h. Interestingly, highly significant increases ($p \leq 0.001$) both in GOT and GPT were found at 24 h when compared to basal or 7 d. Moreover, significant increases were also observed on GOT ($p \leq 0.05$) and GPT ($p \leq 0.001$) at day 7 when compared to baseline. Finally, significant differences were also found on CRP showing a decrease at day 7 when compared to 24 h ($p \leq 0.05$) and basal ($p \leq 0.05$). Variations seen in creatinine, glucose and GGT, did not reach statistical significance.

3.2. Hematological parameters in peripheral blood from infarcted swine

The analysis of hematological parameters was performed on the same peripheral blood samples as above, collected before myocardial infarction (basal), and post-myocardial infarction at 24 h and 7 days.

We performed the quantitative analysis of different leukocyte subsets (lymphoid and myeloid). Leukocyte subsets were counted in an automatic hematology analyzer. Results are shown in Fig. 2. Fig. 2A shows a highly significant increase ($p \leq 0.001$) in WBC at 24 h when compared to basal and 7 d. Regarding neutrophils, a significant increase was also observed at 24 h when compared to basal and 7 d ($p \leq 0.001$ and $p \leq 0.01$, respectively). In the case of lymphocytes, a significant increase ($p \leq 0.05$) was only found at 7 d when compared to basal. Monocytes showed a significant increase at 24 h ($p \leq 0.05$) when compared to basal. Eosinophils displayed a significant increase at 24 h ($p \leq 0.01$) and 7 d ($p \leq 0.01$) when compared to basal. Finally, basophils showed a significant increase at 24 h and 7 days ($p \leq 0.001$ and $p \leq 0.05$, respectively) when compared to basal.

On the other hand, the hematological analysis was also focused on red blood cells and hemoglobin. This analysis, represented in Fig. 2B showed a significant decrease ($p \leq 0.01$) in RBC, HGB and HCT when comparing 7 d to 24 h. In terms of MCV, a significant decrease was found at 24 h ($p \leq 0.05$) and 7 d ($p \leq 0.05$) when compared to basal. No differences were found in MCH but a significant decrease was found in MCHC at 24 h and 7 d when compared to basal ($p \leq 0.05$). Regarding RDW-CV, several changes were found, with the highest significant difference ($p \leq 0.001$) seen between 7 d and basal. Finally, RDW-SD was significantly reduced at day 7 ($p \leq 0.05$) when compared to basal.

The hematologic analysis was completed with the evaluation of different platelet parameters. This analysis, represented in Fig. 2C, showed only a significant decrease in PLT ($p \leq 0.05$) and PCT ($p \leq 0.05$) at 24 h when compared to basal.

3.3. Phenotypic analysis of peripheral blood lymphocytes from swine myocardial infarction model

Lymphocytes from the 17 study pigs were isolated from blood samples by gradient centrifugation and phenotypically analyzed by flow cytometry at pre-specified time points: before myocardial infarction (basal) and at 24 h and 7 days post-infarction. The percentage of CD4 + T cells (gated as CD4+ CD8α–), CD8 + T cells (gated CD4-CD8α+), NK cells (gated as CD3-CD8α-CD4-CD16+) as well as the CD4/CD8 ratio was determined. This analysis of lymphocyte subsets, shown in Fig. 3, did not reveal any statistically significant difference. Only, a slight but not significant decrease in the percentage of NK cells was observed at 7d when compared to 24 h and basal samples.

Regarding the activation/differentiation status of lymphocytes after myocardial infarction, a deep analysis of activation/differentiation markers was performed on CD4+ and CD8 + T cell subsets. The CD45RA and CD27 co-expression was analyzed on peripheral lymphocytes and the percentages of naïve T cells (CD45RA + CD27+), effector T cells (CD45RA–CD27+) and effector/memory T cells (CD45RA–CD27–) were compared at different time points. No significant change was observed between different time points.

3.4. Myocardial infarction model assessment

Infarction was successfully induced in all surviving animals as demonstrated by a significant increase ($p \leq 0.001$) in cardiac enzymes at 24 h post-infarction (troponin I 27.41 ± 10.46 μg/l and CK-MB 15.02 ± 7.96 μg/l), compared to basal levels (troponin I 0.02 ± 0.02 μg/l and CK-MB 3.80 ± 1.34 μg/l) (Supplementary File 1).

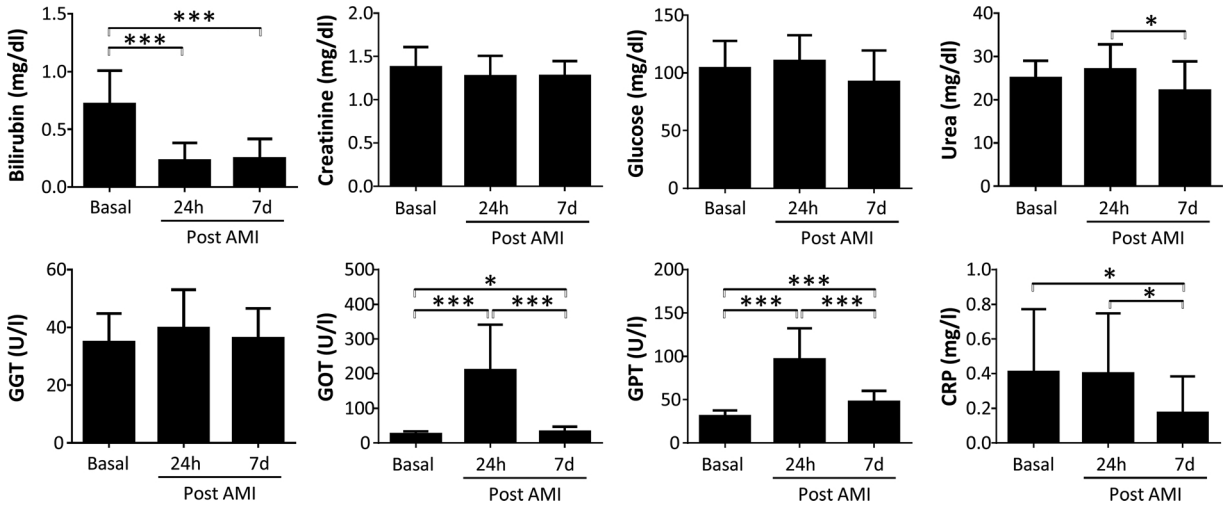

Fig. 1. Levels of biochemical parameters in peripheral blood. Blood samples were collected before acute myocardial infarction model creation (basal) 24 h and 7 d after. Blood samples were collected, centrifuged and processed to determine their biochemical composition. Normality was assessed using a Shapiro-Wilk test. Paired comparisons were performed using a Student *t*-test for parametric data or Wilcoxon sign test for non-parametric data. Graphs show the mean ± SD (n = 17). Horizontal lines show significant differences. *p ≤ 0.05; ***p ≤ 0.001.

Cardiac function was evaluated by Cardiac Magnetic Resonance at day 7 after myocardial experimental myocardial infarction. Ejection Fraction (% EF) as well as the size of myocardial infarction expressed as percentage of the Left Ventricle (% Infarct) were calculated from the MR studies. As shown in Table 1, EF ranged from 14% to 38% with a mean ± SD of 23.5% ± 8.1%. The % Infarct ranged from 14% to 33% with a mean ± SD of 23.2% ± 5.5%. Fig. 4 shows the individual MR analysis in the study group (n = 17) with the myocardial infarct area depicted on a selected representative short axis image.

3.5. Correlation analysis between cardiac function and blood-derived parameters

In order to identify biochemical and hematological biomarkers that could be useful for the evaluation and follow-up of myocardial infarction in this animal model, we performed a correlation analysis between MR-derived % EF and % Infarct at day 7 and blood-derived parameters (biochemical and hematological) obtained 24 h and 7 d post-infarction. This paired sample correlation was also evaluated on cardiac enzymes obtained at 24 h and day 7 post-infarction.

As expected, most of the blood-derived parameters analyzed did not show any significant correlation with cardiac function parameters and cardiac enzymes (Supplementary File 2). Fig. 5A shows the only significant correlations observed on biochemical parameters: strong positive correlation between GGT and % EF (r = 0.529; p = 0.029) and strong positive correlation between GPT and troponin I (r = 0.545; p = 0.024).

Similarly, Fig. 5B represents the significant correlations observed on red blood cells or hemoglobin: strong positive correlation between RBC and % Infarct (r = 0.514; p = 0.035), strong positive correlation between HGB and % Infarct (r = 0.536; p = 0.027) and moderate positive correlation between HCT and % Infarct (r = 0.482; p = 0.050).

Finally, regarding platelet parameters, Fig. 5C shows the correlations found between PLT and % Infarct (r = –0.504; p = 0.039), PLT and troponin I (r = –0.554; p = 0.021) and PCT and % Infarct (r = –0.493; p = 0.044).

Additionally, as expected, significant correlations were observed for cardiac enzymes and % EF (r = -0.482; p = 0.050 for CK-MB at 24 h) as well as between cardiac enzymes and % Infarct (r = -0.563; p = 0.019 and r = 0.623; p = 0.008 for troponin I at 24 h and 7 d, respectively).

4. Discussion

Clinically relevant animal models are essential to evaluate new therapeutic strategies in myocardial infarction. It is widely accepted that the anatomical and physiological parallelism between humans and pigs makes this animal model a valuable tool to mimic biological and adverse events that occur during myocardial ischemia and reperfusion. This paper aimed to analyze biochemical parameters, hematological values and lymphocyte subsets parameters during the acute phase of myocardial infarction in a closed chest porcine model. Additionally, changes in these blood-derived parameters were correlated to cardiac function measurements obtained by MRI.

Our biochemical analysis firstly demonstrated a significant decrease of bilirubin (heme oxygenase-1 metabolite) within 24 h post-infarction, which is normal considering that animals were fasted for 48 h prior to infarct induction, which causes an important physiological increase in this parameter (Baetz and Mengeling, 1971). The significant decrease observed after infarction corresponds to the normalization of bilirubin levels after the fasting period.

We also evidenced highly significant changes in GOT and GPT 24 h post-infarction as well as a direct correlation between GPT–troponin I and GGT–EF. These results are in agreement with recent clinical studies which demonstrated a correlation between serum transaminases and cardiac parameters (Killip classification, infarct-related coronary artery and troponin I) in patients with ST-segment elevation myocardial infarction (Gao et al., 2017). Additionally, in another study, transaminases quantified in 167 patients during the acute phase of percutaneous coronary intervention demonstrated a direct correlation with systolic dysfunction detected by MRI (Reinstadler et al., 2015). As in those clinical observations, in our animal model the correlation between hepatic enzymes and MRI was observed at day 7 post-infarction.

In terms of hematological parameters quantified in our preclinical infarct model, we observed a significant increase of WBC, neutrophils, eosinophils, monocytes and basophils at 24 h post-infarction when compared to basal samples. This leukocytosis is triggered by myocardial infarction through the activation of bone marrow, hematopoietic cells and leukocyte production (Dutta et al., 2015). In our animal model, the increase of myeloid cells at 24 h exhibits strong similarities with results previously described in patients (Engström et al., 2009; Sager et al., 2016). Moreover, Ferrari et al. have recently demonstrated that WBC counts in hospitalized patients at 72 h post-admission are predictive biomarkers that correlate with the size of ST segment elevation

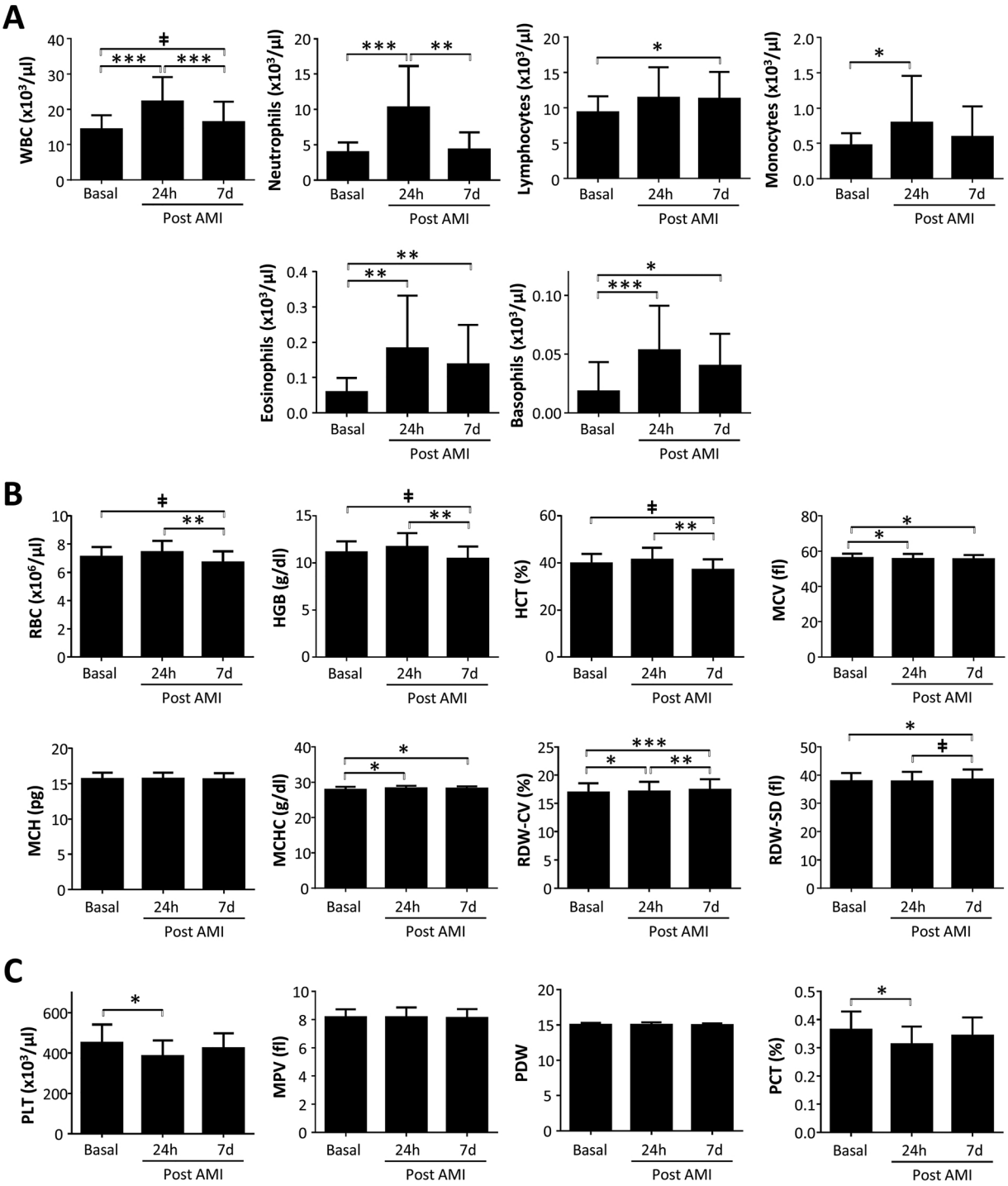

Fig. 2. Levels of hematological parameters in peripheral blood. Blood samples were collected before acute myocardial infarction model creation (basal) 24 h and 7 d after. Blood samples were collected in EDTA containing tubes and leukocyte subsets (A), red blood cells (B) and platelets (C) were counted in an automated hematology analyzer. Normality was assessed using a Shapiro-Wilk test. Paired comparisons were performed using a Student *t*-test for parametric data or Wilcoxon sign test for non-parametric data. Graphs show the mean ± SD (n = 17). Horizontal lines show significant differences. ǂp ≤ 0.1; *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001.

myocardial infarction (Ferrari et al., 2016).

Among the different subsets of WBC, neutrophils play a key role in myocardial infarction. A recent paper in neutrophil-depleted mice has demonstrated that neutrophils are involved in macrophages polarization towards an anti-inflammatory and reparative phenotype (Horckmans et al., 2017). Moreover, a clinical study with 701 patients showed that the percentage of neutrophils in patients with ST-segment elevated myocardial infarction was a predictor for long-term mortality (Men et al., 2015).

Our hematological characterization of porcine myocardial infarction model was also focused on RBC, HGB and HCT and platelets. Our results showed a significant decrease of RBC, HGB and HCT at day 7 when compared to 24 h. Interestingly, our analyses revealed not only significant differences but also a positive correlation between RBC, HGB and HCT with the percentage of infarcted area. Our results are related with clinical studies where the hematocrit has been used as a prognostic

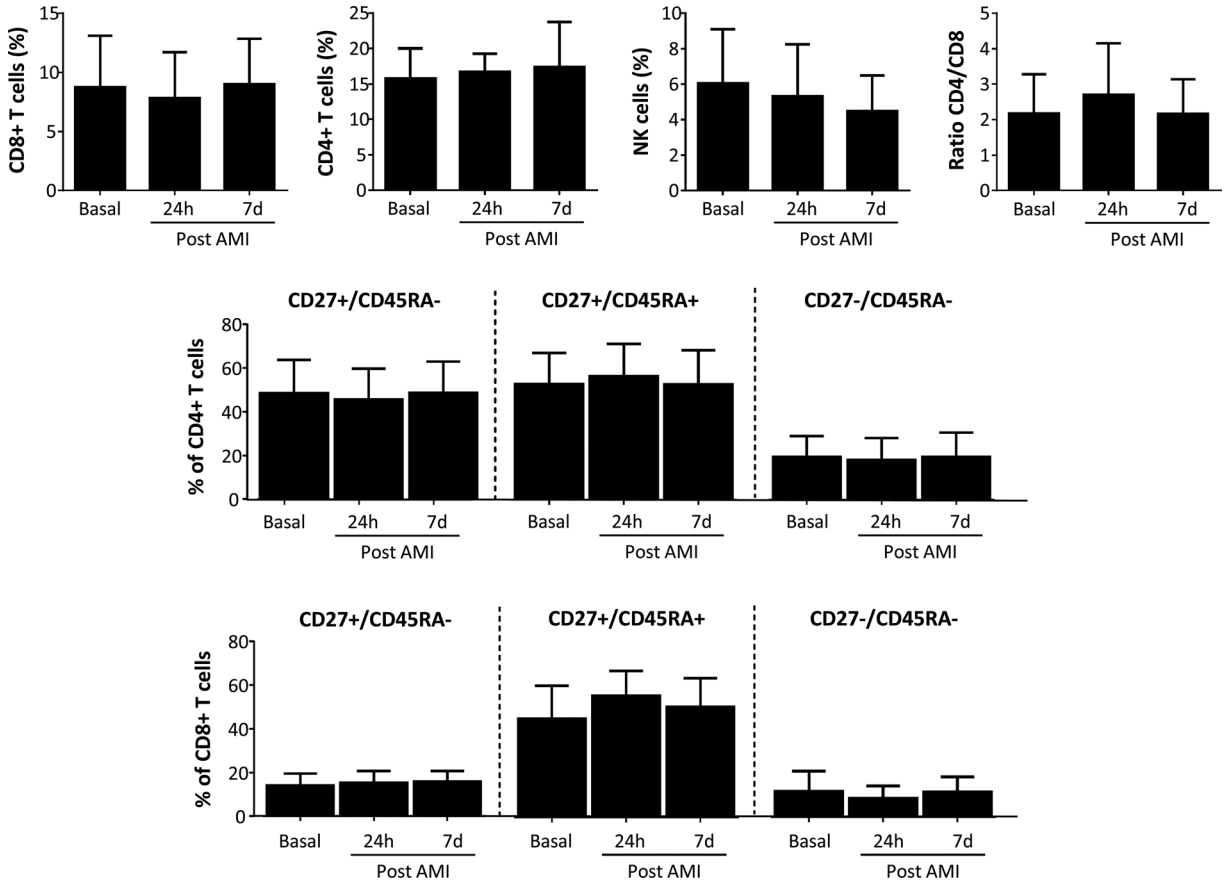

Fig. 3. Lymphocyte subsets distribution in peripheral blood. Peripheral blood lymphocytes were isolated from blood samples collected before acute myocardial infarction model creation (basal) 24 h and 7 d after. Peripheral blood lymphocytes were stained with fluorescent-labelled monoclonal antibodies against porcine CD3, CD4, CD8α, CD16, CD27 and CD45RA and different lymphocyte subpopulation were analyzed by flow cytometry. Normality was assessed using a Shapiro-Wilk test. Paired comparisons were performed using a Student *t*-test for parametric data or Wilcoxon sign test for non-parametric data. Graphs show the mean ± SD (n = 17). No significant differences ($p \leq 0.05$) were found.

Table 1
Cardiac assessment parameters derived from Magnetic Resonance in terms of percentage of myocardial infarction and ejection fraction for the different animals included in the study.

	CARDIAC ASSESSMENT	
Animal number	MI (%)	EF (%)
#1	33	23
#2	28	22
#3	25	34
#4	31	38
#5	18	16
#6	14	26
#7	24	38
#8	18	30
#9	23	18
#10	20	19
#11	16	24
#12	26	14
#13	27	15
#14	25	18
#15	20	17
#16	25	26
#17	25	27

biomarker in patients with ST-segment elevation myocardial infarction (Greenberg et al., 2010). Based on that, here we suggest that these blood parameters (RBC, HGB and HCT) could be considered as early biomarkers to predict the severity of surgically-induced myocardial infarction.

In the case of platelets, a significant decrease was observed 24 h post-infarction when compared to basal measurements. Platelets level has been considered a very helpful tool for the classification and monitoring of patients with ST-segment elevation (Paul et al., 2010). Additionally, platelet count has been associated with the outcomes of ST-elevation myocardial infarction (Ly et al., 2006) and unstable angina (Mueller et al., 2006). In our animal model, the significant decrease in platelets was accompanied by a significant increase in inflammatory cells which is in agreement with clinical results published by Järemo et al. who described that patients with acute myocardial infarction displayed an elevated inflammatory response associated to lower platelet counts (Järemo et al., 2000). Additionally, the correlation analysis in our animal model demonstrated that PLT counts and PCT at 24 h post-infarction were inversely correlated with the size of myocardial infarction at day 7. These findings support the use of PLT and PCT at 24 h as blood derived biomarkers which are inversely correlated with the severity of induced myocardial infarction. Moreover, the correlation observed between troponin I and PLT counts further supports the usefulness of this parameter in the follow-up of this animal model.

Finally, considering that myocardial infarction is an inflammatory process (acute or chronic depending on the etiology), here we analyzed the different lymphocyte subsets and differentiation/activation markers on CD4 + and CD8 + T cells. Although clinical studies monitoring these T cell subsets are somewhat ambiguous, it seems that there is a significant decrease of CD4 + /CCR7 + T cells after myocardial infarction (Hoffmann et al., 2012). The reason behind this change is still unclear in patients: redistribution of CD4 + T cells in periphery vs accumulation in myocardium. Experimental animal models are providing very

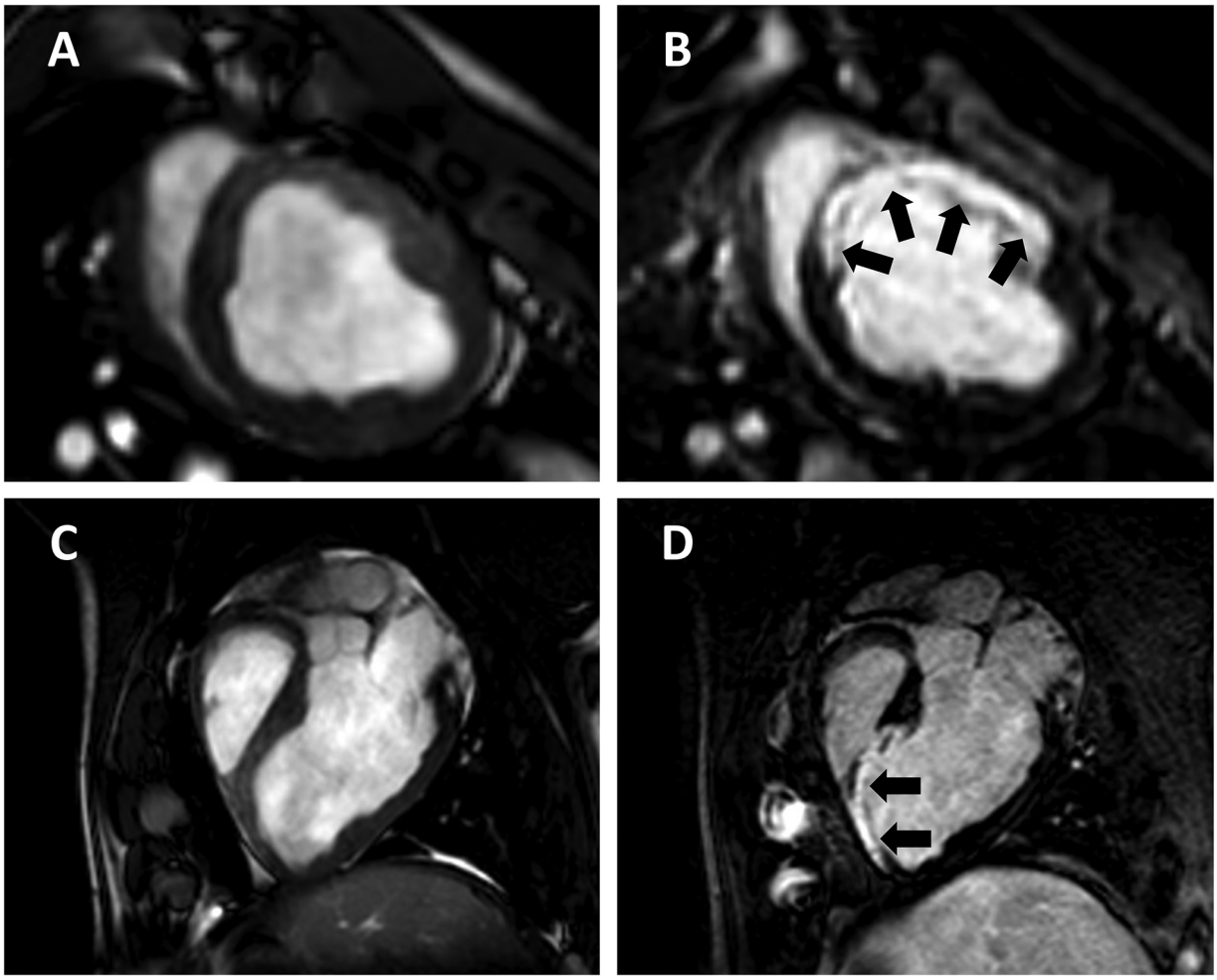

Fig. 4. Representative images of Cardiac Magnetic Resonance. Cardiac Magnetic Resonance studies were acquired at day 7 after myocardial infarction. Representative images were obtained from animal #12. A and B: short axis images. C and D: Four-chamber views. B and D depict delayed enhancement images obtained 10–15 min after gadolinium administration. The infarcted area is shown in white (arrows) and healthy myocardium in black.

relevant information regarding the role of CD4 + T cells. Firstly, a significant decrease in myocardial infarct size has been observed in CD4-depleted mice (Yang et al., 2006). Secondly, CD4+ regulatory T cells could reduce the pro-inflammatory environment shifting it towards a pro-healing phenotype in a rat myocardial infarction model (Tang et al., 2012). In the case of circulating CD8 + T cells in myocardial infarction, animal studies have demonstrated that CD8-deficiency did not result in any significant clinical phenotype after experimental myocardial infarction (Hofmann and Frantz, 2016).

In our study, no significant difference was observed when comparing different time points. However we should not discard hypothetical differences at short term follow-up. Indeed, our current studies are being focused in the very acute phase of myocardial infarction which have shown significant differences in the CD4/CD8 ratio and percentage of CD4+ and CD8 + T cells (manuscript in preparation).

Here we hypothesize that the absence of differences in CD4+ and CD8 + T cells after myocardial infarction may be related to the immunological status of our animal model. This idea is supported by clinical studies in cytomegalovirus-infected patients. These patients have an early and long-term decrease of terminally differentiated effector-memory CD8 + T cells after myocardial infarction (Hoffmann et al., 2015) and a direct relation between CD8 + T cell activation status and the pathophysiology of myocardial infarction has been suggested (Savva et al., 2013). Obviously, the immunological status and T-cell activation status of patients with acute coronary syndromes is completely different to the immunological status of young animals housed under pathogen-controlled conditions and without any co-morbidities. Based on that, we consider that the non-significant decrease of CD8 + T cells at 24 h post-AMI could be the consequence of using an animal model with an immune system under a naïve or resting state. However, we should highlight that studies performed under controlled conditions and in a homogeneous experimental group may provide a valuable tool to identify immunologically relevant biomarkers following the onset of ischemia.

In summary, we have performed a hematological, biochemical and immunological characterization of acute myocardial infarction in a clinically relevant animal model. We have identified blood derived parameters which are significantly altered after myocardial induction, and more importantly, which are significantly correlated with cardiac functional parameters and/or cardiac enzymes. For that reason, the *in vitro* determination of these parameters could be used as early markers to predict the severity of myocardial infarction. Moreover, it is a simple affordable and reliable way for the follow up of myocardial infarction and the evaluation of therapeutic products in preclinical settings.

5. Conclusions

The novelty of this paper lies in the identification of blood-derived biomarkers in a large animal model that closely resembles the pathophysiological progression of myocardial infarction. The identification of biomarkers is very useful for bridging the gap between preclinical studies in large animal models and clinical trials. It provides relevant

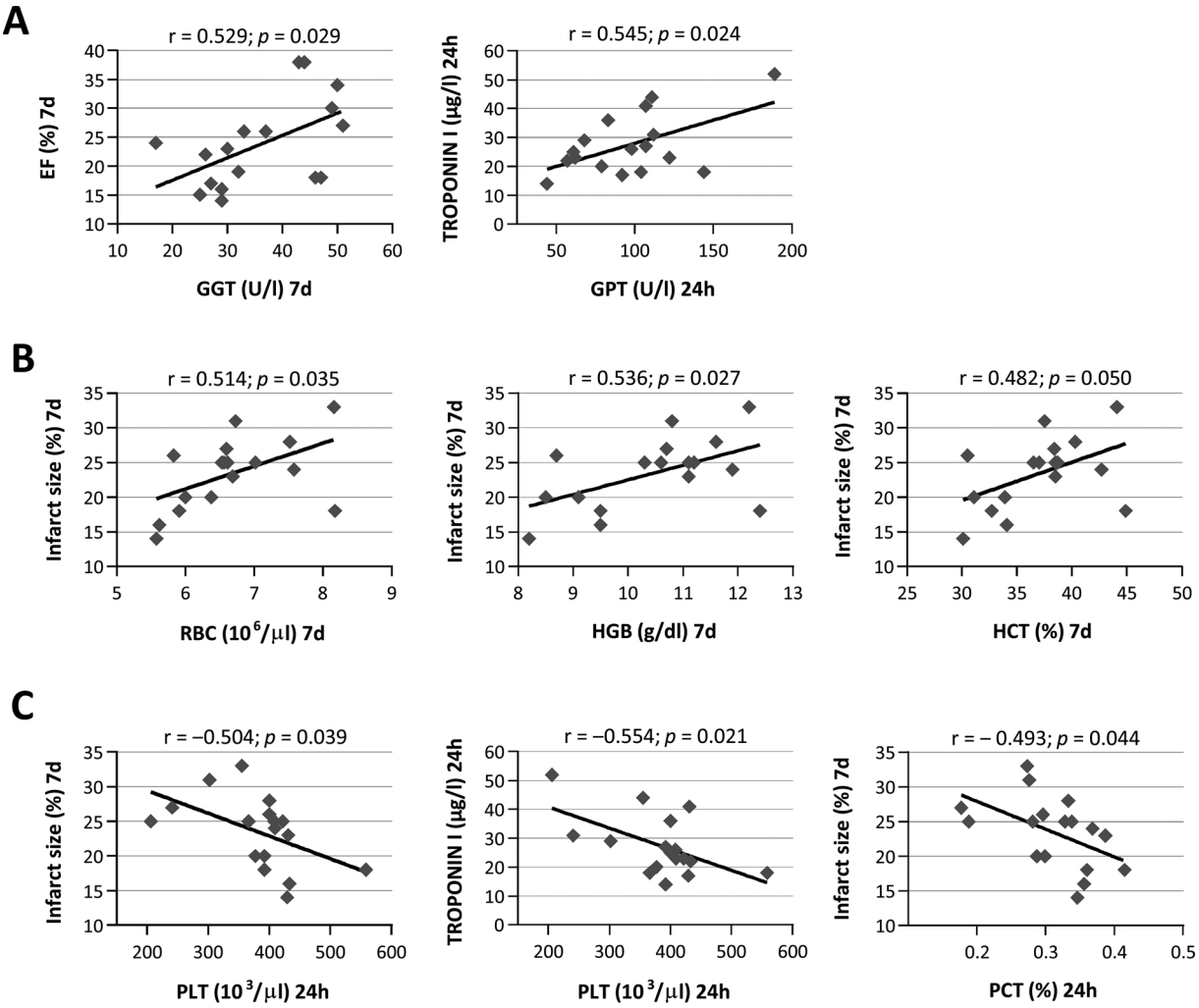

Fig. 5. Correlation analysis between cardiac assessment and blood-derived parameters. Correlations were calculated using the Pearson coefficient correlation. Graphs show significant correlations observed between cardiac assessment (percentage of ejection fraction and infarct area) and biochemical parameters (A) red blood cells (B) and platelets (C).

information in the evaluation of new therapeutic strategies for the treatment of myocardial infarction.

Declarations of interest

None.

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