Evaluation of VEGF-A in platelet and microRNA-126 in serum after coronary artery bypass grafting

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Abbreviation list: CABG, coronary artery bypass grafting; VEGF-A, vascular endothelial growth factor-A; IP-VEGF-A, intra-platelet vascular endothelial growth factor-A; S-VEGF-A, serum vascular endothelial growth factor-A; IP-miR-126, intra-platelet miR-126; IL-6, interleukin-6; TPO, thrombopoietin

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Abstract

Objectives: Platelet functions are thought to contribute to clinical outcomes after heart surgery. This study was conducted to assess the pivotal roles of vascular endothelial growth factor-A (VEGF-A) and microRNA-126 (miR-126) during coronary artery bypass grafting (CABG). **Materials and Methods:** Whole blood was collected for platelet isolation from 67 patients who underwent CABG surgery between July 2013 and March 2014. VEGF-A and miR-126 levels in serum, plasma, and platelets were measured at various time points and compared with clinical characteristics. **Results:** The platelet count was decreased at 3 days after CABG. This dynamic change in platelet count was larger after conventional coronary artery bypass (CCAB) than off-pump coronary artery bypass (OPCAB). VEGF-A in the same number of platelets (IP-VEGF-A) was increased at 3 day after CABG, followed by an increase of VEGF-A in serum (S-VEGF-A) at 7 days after surgery. The miR-126-3p level in serum (S-miR-126-3p) increased rapidly after CABG and then decreased below preoperative levels. The IP-VEGF-A level on day 7 after CABG in patients with peripheral artery disease (PAD), who suffered from endothelial dysfunction, was higher compared with patients without PAD. Conversely, S-miR-126-3p on day 7 after surgery was lower in patients with PAD than in patients without PAD. **Conclusions**: Low levels of S-miR-126-3p due to endothelial dysfunction may lead to high IP-VEGF-A, which is closely related to complications after CABG.

Introduction

Coronary artery bypass grafting (CABG) is the standard treatment for coronary artery diseases. CABG improves the lives of patients with left ventricular dysfunction caused by severe coronary artery stenosis [1]. Prolonged bleeding for several days after surgery is a complication of CABG, which is primarily caused by extensive surgery, extracorporeal circulation, and hemodilution. However, anti-platelet and anti-coagulant treatments are necessary after CABG to prevent graft restenosis [2]. Therefore, improving the post-operative course and understanding the hematopoietic dynamics, especially of platelets, before and after CABG are crucial.

Platelet dysfunction worsens the surgical prognosis [3]. Platelets have adhesive functions to create blood clots and interact with coagulation systems [4]. They also release various secretory proteins, such as von Willebrand factor, interleukin-8 (IL-8), and endothelin [5,6]. Vascular endothelial growth factor-A (VEGF-A), a proangiogenic factor, is stored and released by activating platelets [7]. Among VEGF family members, VEGF-A is the principal regulator of angiogenesis, vascular permeability, vascular repair, and wound healing [8]. VEGF-A in platelets is a biomarker for several diseases [9]. For example, intra-platelet VEGF-A (IP-VEGF-A) is a valuable marker for liver regeneration after hepatocellular carcinoma resection [10]. Although several studies have reported alterations in the platelet count and platelet aggregation in patients who undergo CABG, postoperative chronological changes in platelets and IP-VEGF-A are unknown.

MicroRNAs (miRNAs) are a class of small noncoding RNAs that suppress expression of multiple target genes, which are implicated in various

diseases such as cancer and cardiovascular disease [11,12]. MiR-126 is a disease-associated miRNA expressed specifically in endothelial cells and platelets [13]. It regulates vasculogenesis, angiogenesis, and vascular inflammation [14], and depletion of miR-126 in endothelial cells leads to severe endothelial cell dysfunction [15], which suggests that miR-126 plays an important role in vasculature homeostasis. In general, a miRNA duplex (premature miRNA) produces two mature miRNAs, namely guide and passenger strands. Mature miR-126 consists of miR-126-5p and miR-126-3p, both of which are involved in regulating angiogenetic activity of endothelial cells in vitro and in vivo [16].

Many miRNAs released from cells circulate through the bloodstream and are detectable in blood [17]. These extracellular miRNAs in blood (circulating miRNAs) are clinically recognized as biomarkers and therapeutic targets. MiR-126-5p and miR-126-3p levels are elevated in the blood of patients with acute myocardial infarction [18]. The importance of these circulating miRNAs in patients with atherosclerotic diseases has been partially described [19]. However, the significance of miR-126-5p and miR-126-3p in blood after CABG surgery remains unclear.

In this study, we investigated changes in serum VEGF-A (S-VEGF-A), IP-VEGF-A, serum miR-126 (S-miR-126), and intra-platelet miR-126 (IP-miR-126) after CABG and analyzed the relationship between these markers and clinical outcomes.

Materials and Methods

Prospective Study Cohorts

From July 2013 to March 2014, 98 patients underwent elective CABG surgery at Kagoshima University Hospital and the National Hospital Organization Kagoshima Medical Center. The research ethics committees of Kagoshima University Hospital (25-131) and the National Hospital Organization Kagoshima Medical Center approved this study (25-62). All participants had provided written informed consent before inclusion in the study. The study was conducted in accordance with the ethical standards of the Committee on Human Experimentation of the institution at which the experiments were performed or in accordance with the ethical standards of the Helsinki Declaration of 1975. Administration of oral aspirin to all patients was stopped at 7 days before surgery and 100 mg oral aspirin was administered within 24 hours after the operation. Patients with hemodialysis, liver dysfunction, or who had received platelet transfusion within the perioperative period were excluded. Under the exclusion criteria, 67 cases were analyzed. Thirty-six cases had undergone off-pump CABG (OPCAB) and 31 cases had undergone conventional CABG (CCAB) by a cardiopulmonary bypass with mild hypothermia (32°C) and cardiac arrest.

Blood Sample Collection

After inducing general anesthesia, whole blood samples were obtained from the central venous cannula preoperatively (Tpre) and on postoperative days 0 (T0) and 1 (T1). Subsequently, venous blood was collected with a 21 G needle on postoperative days 3 (T2), 7 (T3), 14 (T4), 21 (T5), and 28 (T6).

The complete blood count was measured using an automated hematology analyzer (Sysmex XE-5000; Sysmex Corporation, Kobe, Japan).

Serum and Plasma Preparation

Blood samples were divided into three tubes: a serum-separating tube, 5.0-mL citrate tube containing 0.5 mL of 3.2% sodium citrate for plasma, and an EDTA-2K tube (Venoject II; Terumo Corp., Tokyo, Japan). Serum tubes were incubated for at least 30 minutes at room temperature to allow clotting. Both serum and plasma tubes were centrifuged at 1,710 × *g* for 10 minutes and then the supernatants were carefully aliquoted and stored at -80°C. The EDTA-2K tube was used to measure the complete blood count (CBC) by the XE-5000 automated hematology analyzer.

Platelet Isolation

Two citrate tubes were centrifuged at 90 × *g* for 15 minutes to prepare platelet-rich plasma (PRP). Platelets in the PRP were counted using the XE-5000 automated hematology analyzer. Then, 200 µL PRP was centrifuged at 2,810 × *g* for 20 minutes to form pellets. The supernatant (platelet-poor plasma) was carefully removed and the platelet pellets were carefully washed with 300 µL Tyrode's solution (Sigma-Aldrich, St. Louis, MO, USA) with 6.7 mM citrate and stored at -80°C before use.

Quantification of Growth Factors and Cytokines

Serum, plasma, and platelet samples were analyzed using enzyme-linked immunosorbent assay kits for human VEGF-A, interleukin-6, and

thrombopoietin (Quantikine; R&D Systems, Minneapolis, MN, USA) by the manufacturer's guidelines. Platelet pellets isolated from each 200-µL aliquot of PRP were lysed in 200 µL lysis buffer (150 mM sodium chloride, 25 mM Tris-HCl pH 7.6, 1% Tergitol-type NP-40, 0.1% sodium dodecyl sulfate, and 1% sodium deoxycholate). The lysate solution was pipetted and vortexed until the pellets were completely dissolved in the lysis buffer. IP-VEGF-A/1 × 10^6 platelets was calculated using a previously reported equation [10].

RNA preparation

Each 150-µL aliquot of serum and platelet pellets was mixed in 750 µL Qiazol Lysis Reagent (Qiagen, MD, USA). Total RNA was extracted from the serum and platelets using miRNeasy Serum/Plasma and miRNeasy Mini kits (Qiagen), respectively, following the manufacturer's protocols. To normalize the sample validation in the RNA extraction step, a Spike-In control (cel-miR-39) (Qiagen) was added to the serum samples. RNAs were eluted with 14 µL RNase-free water. Platelet miRNAs were diluted with RNase-free water as per the PRP platelet count to adjust for the same platelet count (1 × 10^4/µL).

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

All reagents and primers were obtained from Applied Biosystems (Foster City, CA, USA). cDNA was synthesized from 2.5 µL serum RNA and 5 µL platelet RNA using a TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems). The mixture was incubated at 16°C for 30 minutes, 42°C for 30 minutes, and then 85°C for 5 minutes. qPCR was performed using the 7300 Real-Time PCR system (Applied Biosystems). MiR-126-3p

and miR-126-5p expression levels were normalized to the Spike-In control level and calculated using the $2^{-\Delta\Delta Ct}$ method.

Definitions of postoperative complications

Prolonged mechanical ventilation was defined as a duration of postoperative mechanical ventilation, which exceeded 48 hours. Wound complications were defined as superficial or deep sternal wound complications. Superficial wound complications included sterile dehiscence or persistent drainage that required prolonged dressing changes for more than 2 weeks. Deep wound complications included sternal osteomyelitis and wound infections that involved the sternum, mediastinum, or deep organs. Pleural effusion was defined as prolonged chest tube drainage for more than 1 week after surgery or the need for a postoperative puncture. Postoperative atrial fibrillation was diagnosed by an electrocardiogram monitored for 24 hours within a week.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 8 (GraphPad Prism Software, Inc., San Diego, CA, USA). The paired t-test was used to compare transition of the same patient and the Mann–Whitney U-test was used to compare two groups. A p-value of <0.05 was considered significant.

Results

Clinical characteristics and outcomes

Table 1 lists the clinical characteristics of all patients. The study population was predominantly male (95%) with a mean age of 66 years. Approximately half the population was diagnosed with diabetes mellitus and the mean HbA1c level was 6.5 ± 0.7%. Of the patients, 59% were former smokers and all smokers had stopped smoking at least 1 month before surgery. Patients with chronic obstructive pulmonary disease (5%), cerebral vascular disease (21%), and peripheral arterial disease (12%) were included. The operative procedure was chosen by the surgeon and off-pump CABG (OPCAB) was selected for 54% of patients (Table 2). Table 2 lists other operative data. Operation time and cardiopulmonary bypass (CPB) perfusion time was thought to be within the appropriate length. Blood transfusion was performed regularly. Platelet transfusion was not performed in all cases. No patients had died during the perioperative period until 30 days after surgery. The major clinical complications included postoperative paroxysmal atrial fibrillation (30%), pleural effusion (18%), wound complications (10%), and respiratory failure with mechanical ventilation for >48 hours (12%; Table 2).

Postoperative changes in the platelet count and volume

The platelet count was significantly decreased around T0–T2 postoperation ($p < 0.001$) and then increased from T3 (Figure 1A). The peak median platelet count ($44.1 \times 10^4/\mu L$) at T4 was higher than the preoperative count ($18.2 \times 10^4/\mu L$; Figure 1A). The postoperative mean platelet volume (MPV) and platelet large cell ratio (P-LCR) were changed slightly. However,

these values were significantly increased on T2 ($p < 0.001$), which was slightly earlier than the time at which the platelet count had increased (Figure 1B and 1C). Conversely, the MPV and P-LCR were significantly decreased on T0 ($p < 0.001$) and T4 ($p < 0.001$; Figure 1 B and 1C). To investigate the effect of cardiopulmonary bypass on the platelet count and volume, patients were divided into two groups: patients who underwent conventional CABG (CCAB) and patients with off-pump CABG (OPCAB). The platelet count was lower at T0 and T1 on CCAB than OPCAB. Conversely, MPV and P-LCR were higher in patients with CCAB than those with OPCAB at T1–T2 and T2, respectively (Figure 1D–1F). The platelets count was similarly increased, and MPV and P-LCR were equally decreased at T4 on CCAB and OPCAB (Figure 1D–F). These results suggest that CCAB reduces the platelet count and increases immature platelets during the early postoperative period compared with OPCAB and there was no difference in the increased level of postoperational platelet production around T4 between CCAB and OPCAB.

Postoperative thrombocytosis factors

To examine regulation of platelet biogenesis, we measured the proinflammatory cytokine IL-6 and thrombopoietin (TPO) in serum. IL-6 levels were significantly higher at T0, T1, and T2 than at Tpre (preoperative value; $p < 0.001$) (Figure 2A). The TPO level was also increased postoperatively ($p < 0.001$; Figure 2B). The peak level of TPO was slightly delayed compared with that of IL-6. Because TPO promotes megakaryocyte growth and platelet production, the increase in platelets after surgery might be induced by TPO.

TPO levels were not significantly different between CCAB and OPCAB groups (Figure 2D). IL-6 was higher in patients with CCAB only at T0 (Figure 2C).

Postoperative changes in S-VEGF-A and IP-VEGF-A

The preoperative level (Tpre) of the median S-VEGF-A was 244.5 pg/mL. No groups differed significantly in terms of the clinical parameters in Table 1 (data not shown). S-VEGF-A was significantly decreased postoperatively (T0, $p < 0.001$) and then dramatically elevated from T3 after CABG (Figure 3A). S-VEGF-A peaked at T4 (1003.6 pg/mL) and then decreased rapidly (Figure 3A). Because S-VEGF-A is mainly derived from platelets, we next measured IP-VEGF-A. The median IP-VEGF-A level before CABG (Tpre) was 0.81 pg/1 × 10^6 platelets, which was significantly decreased postoperatively ($p < 0.001$) and then increased from T2 (Figure 3B). The median IP-VEGF-A (2.79 pg/1 × 10^6 platelets) peaked at T3. In OPCAB, S-VEGF was higher than CCAB throughout the course with a significant difference at T0 and IP-VEGF was not significantly different between the two groups (Figure 3C and 3D).

Relationship between S-VEGF-A or IP-VEGF-A and PAD or postoperative complications

To examine the effect of the increased S-VEGF-A and IP-VEGF-A after CABG, the relationships between S-VEGF or IP-VEGF-A and the clinical conditions were analyzed at T3. Patients with peripheral artery disease (PAD) had significantly increased IP-VEGF-A compared with those without PAD ($p = 0.049$; Figure 4A), but not S-VEGF-A. IP-VEGF-A in patients with

postoperative treatment-resistant wound complications, but not respiratory failure, was significantly higher (p = 0.04), but no relationships were detected between S-VEGF-A and these two complications (Figure 4B and 4C).

Postoperative changes in miR-126-3p and miR-126-5p expression

Next, we measured the level of miR-126 that is mainly produced and released from endothelial cells. Precursor miR-126 consists of two mature miRNAs, miR-126-3p (guide strand) and miR-126-5p (passenger strand), which are detectable in serum. Therefore, the miR-126-3p and miR-126-5p levels in the serum and platelets were measured by qPCR. Serum miR-126-3p (S-miR-126-3p) and serum miR-126-5p (S-miR-126-5p) were increased postoperatively and then decreased to below preoperative levels after T2 (Figure 5A). MiR-126-3p and miR-126-5p in the same number of platelets (IP-miR-126-3p and IP-miR-126-5p/5 × 10^4 platelets, respectively) were slightly increased at T1 and T2 after CABG and then decreased to the same level as that at Tpre (Figure 5B).

Relationship between S-miR-126-3p or S-miR-126-5p and PAD or postoperative complications

Next, we examined the relationships between miR-126-3p or miR-126-5p and PAD or clinical complications at T3 (Figure 6 and Supplementary Figure 1). S-miR-126-3p in patients with PAD (p=0.001) was significantly lower than that in patients without PAD (Figure 6A). S-miR-126-3p in patients with respiratory failure (p=0.001) and wound complication (p=0.02) was significantly lower than that in patients without the two complications (Figure

6B and 6C). However, the levels of IP-miR-126-3p/5 × 10^4 platelets were not significantly different between patients with or without PAD, respiratory failure, and wound complications (Figure 6). In measurements of S-miR-126-5p and IP-miR-126-5p/5 × 10^4 platelets, only the S-miR-126-5p level in patients with PAD was significantly lower compared with that in patients without PAD (Supplementary Figure 1).

Discussion

We found dynamic changes in the platelet count associated with changes in IL-6 and TPO during the perioperative period of CABG. VEGF-A also changed in response to changes in the platelet count and IP-VEGF-A/1 × 10^6 platelets had changed before S-VEGF-A. Moreover, IP-VEGF-A/1 × 10^6 platelets was more significantly increased in patients with PAD and wound complications. Conversely, S-miR-126-3p had decreased in patients with PAD, respiratory failure, and wound complications. However, IP-miR-126-3p/5 × 10^4 platelets showed no significant change in these patients.

In CABG, vascular anastomoses cause intimal damage and expose subendothelial collagen tissues, which induce platelet activation and aggregation, leading to graft stenosis. Therefore, anti-platelet treatments are clinically recommended to prevent graft stenosis early after surgery. However, the detailed mechanism by which platelets are involved in causing graft stenosis is unclear. Immature platelets have increased MPV and high platelet activity, and an association has been reported between coronary artery diseases, such as myocardial infarction, and MPV [20]. In this study, higher platelet activity was expected because of the increased MPV at T2. Although the platelet count was low at T0 and T1, surgical stress may induce IL-6 production, followed by increased TPO, thereby producing more immature platelets after T2. Thereafter, the graft was strongly exposed to activated platelets and was affected by factors in these platelets. Etulain et al. reported that platelet-mediated angiogenesis is independent of VEGF-A and fully inhibited by aspirin [21]. If aspirin was not administered orally in our cases, the VEGF-A levels in platelets and serum would have been even higher and the

effects of cytokines on angiogenesis may have been much stronger. Therefore, administering anti-platelet agents during the very early stage (before T2) may facilitate prevention of graft stenosis [22]. Additionally, the platelet count was lower and MPV was higher in patients with CCAB than in those with OPCAB, strongly indicating an effect of cardiopulmonary bypass on platelets during the early postoperative period, which might contribute to operative outcomes.

VEGF-A activates vascular smooth muscle cells (VSMCs) in the intima-deficient part of vessels, which causes graft stenosis by promoting VSMC migration and proliferation [23]. Inflammatory responses and hypoxic conditions increase serum VEGF-A derived from white blood cells and platelets after CABG [24]. In this study, IP-VEGF-A/1 × 10^6 platelets showed a maximum value at T2 when MPV had increased, which suggests a relationship between platelet maturity and IP-VEGF-A. Avci et al. showed that a high MPV was associated with the occurrence of AMI [20]. This indicates that VEGF-A might be involved in the high MPV and frequency of circulatory diseases. Additionally, S-VEGF-A levels corresponded with IP-VEGF-A levels to some extent, which suggests that VEGF-A in blood is mostly derived from platelets. Our data demonstrated that IP-VEGF-A/1 × 10^6 platelets more accurately predicted clinical changes, such as wound complications and PAD, than S-VEGF-A, which suggests that IP-VEGF-A can better determine clinical outcomes. Platelets that contain more VEGF-A might adhere to the intima-depleted portion and transmit VEGF-A directly to vascular smooth muscle cells, thereby causing graft stenosis.

MiRNAs are expected to be biomarkers to diagnose diseases and predict prognoses. For example, miR-1 and miR-133 are thought to be prognostic

biomarkers after coronary diseases, and miR-22 and miR-126 are elevated in patients with atrial fibrillation [25]. Regarding CABG, myocardial-specific miRNAs from blood samples and auricles have been investigated to evaluate CABG-associated myocardial injury [26]. Our current study investigated miR-126 levels at multiple time points over 4 weeks to determine the effects during the CABG perioperative period.

MiR-126-3p and miR-126-5p are abundant in the vascular endothelium and associated with angiogenesis [14]. Our findings revealed that the dynamics of miR-126-3p and miR-126-5p in serum or platelets were similar on pre- and post-operative days (Figure 5). We examined the levels of miR-126-3p and miR-126-5p in patients with PAD because they are expected to have systemic endothelial dysfunction due to atherosclerosis. The serum levels of miR-126-3p and miR-126-5p in patients with PAD were lower compared with those in patients without PAD (Figure 6 and Supplementary Figure 1). IP-miR-126-3p/5 × 10^4 platelets and IP-miR-126-5p/5 × 10^4 platelets showed no statistical alterations between patients with or without PAD (Figure 6 and Supplementary Figure 1). These results suggest that S-miR-126-3p and S-miR-126-5p influence endothelial conditions more than IP-miR-126-3p/5 × 10^4 platelets and IP-miR-126-5p/5 × 10^4 platelets.

Respiratory failure and wound healing delay are common postoperative complications after CABG. Our study found that respiratory failure had occurred in 12% of patients (Table 2). S-VEGF-A levels and IP-VEGF-A/1 × 10^6 platelets tended to be slightly higher in patients with respiratory failure (Figure 4B). The reason for this might be that patients with poor respiratory status would be exposed to hypoxic conditions that promote systemic VEGF-A production.

Patients with respiratory failure had low S-miR-126-3p (Figure 6B), which suggested that a decrease of miR-126-3p in serum might affect high VEGF-A. Patients with wound complications had high VEGF-A/1 × 10^6 platelets and low S-miR-126-3p (Figures 4C and 6C). Wound healing requires angiogenesis, which may increase VEGF production in platelets. Delayed wound healing caused by endothelial cell dysfunction might decrease miR-126-3p in serum, following by a high level of VEGF-A in platelets. Further study should address this point.

MiR-126-3p modulates intracellular VEGF-A signaling by downregulating PIK3R2 and SPRED1, which suppresses PIK3 and ERK1/2 pathways [27]. In this study, miR-126-3p in serum and platelets were elevated during the early postoperative period, which was likely due to acute inflammation induced by surgical stress (Figure 5). VEGF-A was also upregulated at 7 days after surgery (T3), which would promote wound healing (Figure 3). These phenomena contributed to vascular inflammation after CABG. Prolonged inflammation increases miR-126-3p production in endothelial cells and VEGF-A production in blood cells [13]. In general, systematic inflammation due to cardiac surgery likely increases miR-126-3p and miR-126-5p in serum and VEGF-A in platelets derived from megakaryocytes, which would promote angiogenesis and wound healing. Then, continuously increasing serum miR-126-3p and miR-126-5p might inhibit VEGF-A production in megakaryocytes within 7 days after surgery, which resolves vascular inflammation (Figure 7). In PAD patients, postoperative VEGF-A in platelets was less suppressed by miR-126-3p and miR-126-5p, which sustained a relatively high VEGF-A level in platelets, which may promote vascular inflammation. Further studies may reveal the close relationship and

pathological role of serum miR-126 and VEGF-A in platelets of patients who undergo CABG.

Limitation

Because the number of patients was not sufficient to perform subanalysis between the groups of surgical complications, the statistical significance in this study might be contradicted by a future larger study. Our data demonstrated that patients with PAD had high IP-VEGF-A and low S-miR-126-3p/5p, which suggested a negative association with S-miR-126-3p/5p and IP-VEGF-A, although a correlation analysis was not performed because of the small number of patients. In a future study, the number of patients should be increased to elucidate the relationship between miR-126-3p/5p and IP-VEGF-A in patients after CABG.

Conclusion

We found a dynamic change in platelet-related clinical markers, which might contribute to the operative outcome. The increase of S-miR-126 was earlier than that of IP-VEGF-A after CABG. IP-VEGF-A at 7 days was higher and S-miR-126 was lower in patients with PAD than those in patients without PAD. Low levels of S-miR-126-3p due to endothelial dysfunction may lead to high IP-VEGF-A, which is closely related to complications after CABG.

Declaration of Conflicting Interests

The authors declare no potential conflicts of interest.

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Figure legends

Figure 1. Dynamic change of platelet factors after CABG. (A–C) Total cases (n=67) and (D–F) cases divided into two groups, conventional CABG (CCAB) group (n=31) and off-pump CABG (OPCAB) group (n=36). (A and D) platelet count, (B and E) mean platelet volume (MPV), and (C and F) P-LCR were measured on 8 different days (Tpre-T6). Black box: CCAB; Gray box: OPCAB. Lines in the middle of the boxes indicate median values. The lower and upper edges of the boxes are the first and third quartiles, respectively. ***$p < 0.001$, **$p < 0.01$, and *$p < 0.05$ versus Tpre (A–C) and CCAB vs OPCAB (D–F). The two-tailed paired *t*-test (A–C) and Mann–Whitney *U*-test (D–F) were performed.

Figure 2. Dynamic change of IL-6 and Thrombopoietin (TPO) after CABG. (A and B) Total cases (n=67) and (C and D) cases divided into two groups, conventional CABG (CCAB) group (n=31) and off-pump CABG (OPCAB) group (n=36). (A and C) IL-6 and (B and D) TPO were measured on 8 different days (Tpre-T6). Black box: CCAB; Gray box: OPCAB. Lines in the middle of the boxes indicate median values. The lower and upper edges of the boxes are the first and third quartiles, respectively. ***$p < 0.001$, **$p< 0.01$, and *$p < 0.05$ versus Tpre (A and B) and CCAB vs OPCAB (C and D). The two-tailed paired *t*-test (A and B) and Mann–Whitney *U*-test (C and D) were performed.

Figure 3. Dynamic change of VEGF-A in serum and in platelets after CABG. (A and B) Total cases (n=67) and (C and D) cases divided into two groups,

conventional CABG (CCAB) group (n=31) and off-pump CABG (OPCAB) group (n=36). (A and C) Serum VEGF (S-VEGF-A) and (B and D) intra-platelet VEGF (IP-VEGF-A)/1 × 10^6 platelets were measured on 8 different days (Tpre-T6). Black box: CCAB; Gray box: OPCAB. ***p < 0.001, **p < 0.01, and *p < 0.05 versus Tpre (A and B) and CCAB vs OPCAB (C and D). The two-tailed paired *t*-test (A and B) and Mann–Whitney *U*-test (C and D) were performed.

Figure 4. Serum VEGF-A (S-VEGF-A) and intra-platelet VEGF-A (IP-VEGF-A)/1 × 10^6 platelets divided by the patient background and surgical complications. S-VEGF-A and IP-VEGF-A at T3 with or without (A) peripheral artery disease (PAD) (p=0.801 and p=0.005), (B) postoperative complications, respiratory failure (p=0.081 and p=0.07), and (C) wound complications (p=0.391 and p=0.04). (+) indicates patients with PAD (A), respiratory failure (B) and wound complications (C). (-) indicates patients without PAD (A), respiratory failure (B), or wound complications (C). Lines in the middle of the boxes indicate median values. The lower and upper edges of boxes are the first and third quartiles, respectively. The Mann–Whitney *U*-test was performed for all analyses.

Figure 5. Dynamic change of miR-126-3p and miR-126-5p in serum and in platelet after CABG. (A) Serum miR-126-3p (S-miR-126-3p), serum miR-126-5p (S-miR-126-5p), (B) intra-platelet miR-126-3p (IP-miR-126-3p)/5 × 10^4 platelets, and intra-platelet miR-126-5p (IP-miR-126-5p)/5 × 10^4 platelets were measured on 8 different days (Tpre-T6). Lines in the middle of the boxes

indicate median values. The lower and upper edges of the boxes are the first and third quartiles, respectively. ***p < 0.001, **p < 0.01, and *p < 0.05 versus T1. The two-tailed paired *t*-test was performed for all experiments.

Figure 6. Serum miR-126-3p (S-miR-126-3p) and intra-platelet miR-126-3p (IP-miR-126-3p) /5 × 10^4 platelets divided by the patient background and surgical complications. S-miR-126-3p and IP-miR-126-3p /5 × 10^4 platelets were measured at T3 with or without (A) peripheral artery disease (PAD) (p=0.001 and p=0.27), (B) postoperative complications, respiratory failure (p=0.001 and p=0.09), and (C) wound complications (p=0.02 and p=0.25). (+) indicates patients with PAD (A), respiratory failure (B), and wound complications (C). (-) indicates patients without PAD (A), respiratory failure (B), or wound complications (C). Lines in the middle of the boxes indicate median values. The lower and upper edges of the boxes are the first and third quartiles, respectively. The Mann–Whitney *U*-test was performed.

Figure 7. Schematic presentation of the predictive role of serum miR-126-3p and miR-126-5p (S-miR-126-3p/5p) and intra-platelet VEGF-A (IP-VEGF-A) in regulating endothelial functions. Arrows represent our experimental data. Dotted lines show our hypothesis.

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Table 1
Clinical Characteristics of the Study population (n=67)

Clinical Parameter	
Age, year	66 ± 3
Male, %	95
BMI, kg/m^2	24 ± 3
LVEF, %	58 ± 12
Casual Factors	
Hypertension, %	85
Diabetes mellitus, %	50
Dyslipidemia, %	55
Smokers, %	59
COPD, %	5
CVD, %	21
PAD, %	12
Laboratory data	
HbA_{1c}, %	6.5 ± 0.7
CRP, mg/dL	0.2 ± 0.4
Triglyceride, mg/dL	144 ± 88
Total cholesterol, mg/dL	163 ± 43
HDL-C, mg/dL	43 ± 12
LDL-C, mg/dL	100 ± 38

Values are mean ± SD BMI; body mass index, LVEF; left ventricular ejection fraction, CRP; C-reactive protein,HDL-C; high density lipoprotein cholesterol, LDL-C; low density lipoprotein cholesterol, COPD; chronic obstructive pulmonary diseases, CVD; cerebrovascular disease, PAD; peripheral artery diseases

Table 2

Operative data	
Off-pump CABG, %	**54**
Bypass number	**4 ± 1**
Operation time, min	**317 ± 64**
CPB perfusion time, min	**70 ± 81**
Cardiac arrest time, min	**51 ± 60**
Intraoperative blood loss, mL	**779 ± 1327**
Transfusion volume, mL	**607 ± 757**
RCC , mL	**607 ± 757**
FFP , mL	**674 ± 525**
Postoperative complication	
Death until 30 days after surgery, %	**0**
Postoperative atrial fibrillation, %	**30**
Pleural effusion, %	**18**
Graft failure, %	**9**
PMI, %	**0**
Respiratory failure with mechanical ventilation (>48h), %	**12**
Wound complication, %	**10**

Values are mean ± SD, CPB; cardiopulmonary bypass, RCC; red cells concentrate, FFP; fresh frozen plasma PMI; postoperative myocardial infarction

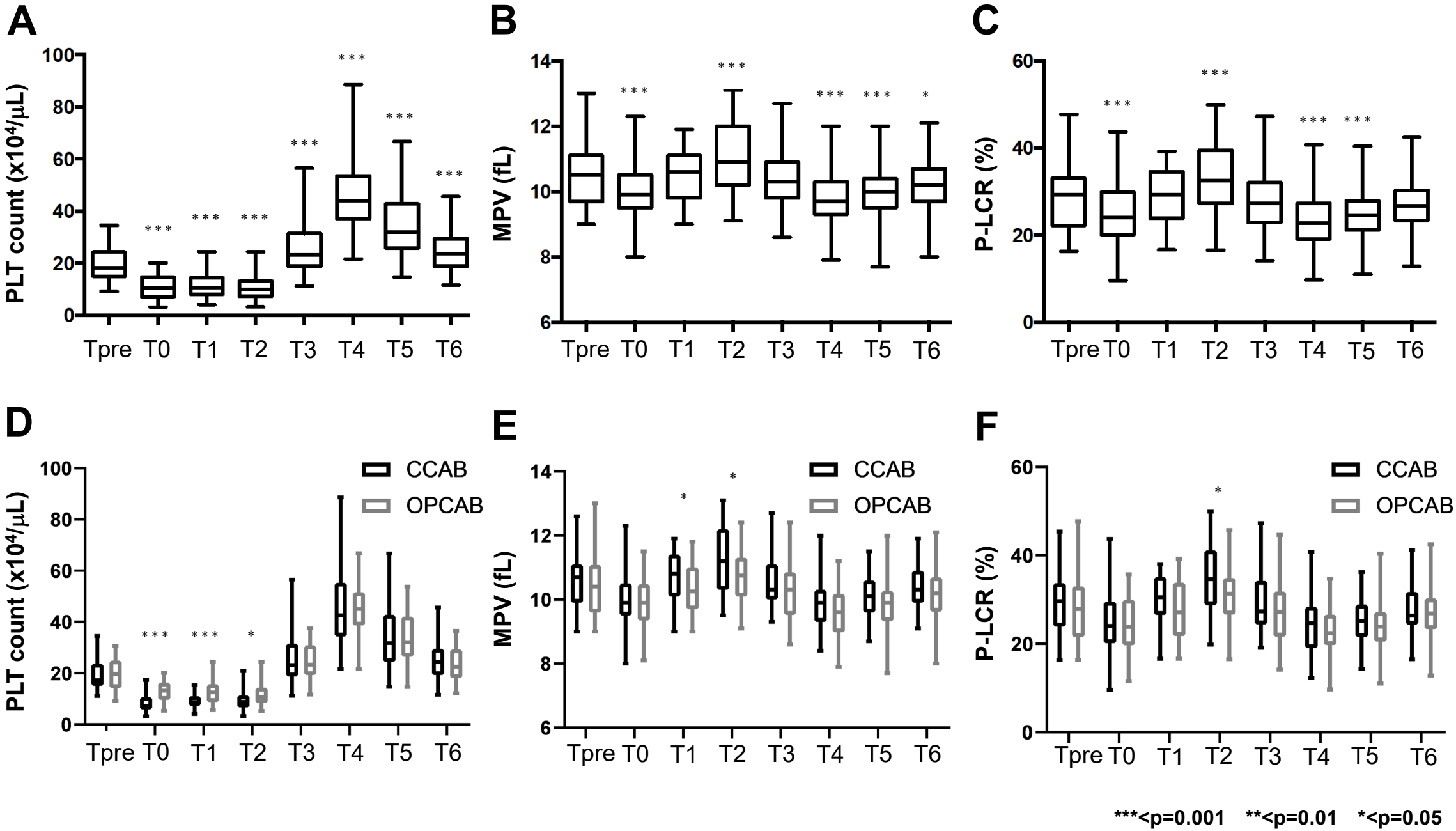
A
B
C
D
E
F
PLT count (x10⁴/μL)
MPV (fL)
P-LCR (%)
Tpre T0 T1 T2 T3 T4 T5 T6
CCAB
OPCAB
***<p=0.001 **<p=0.01 *<p=0.05

Figure 1

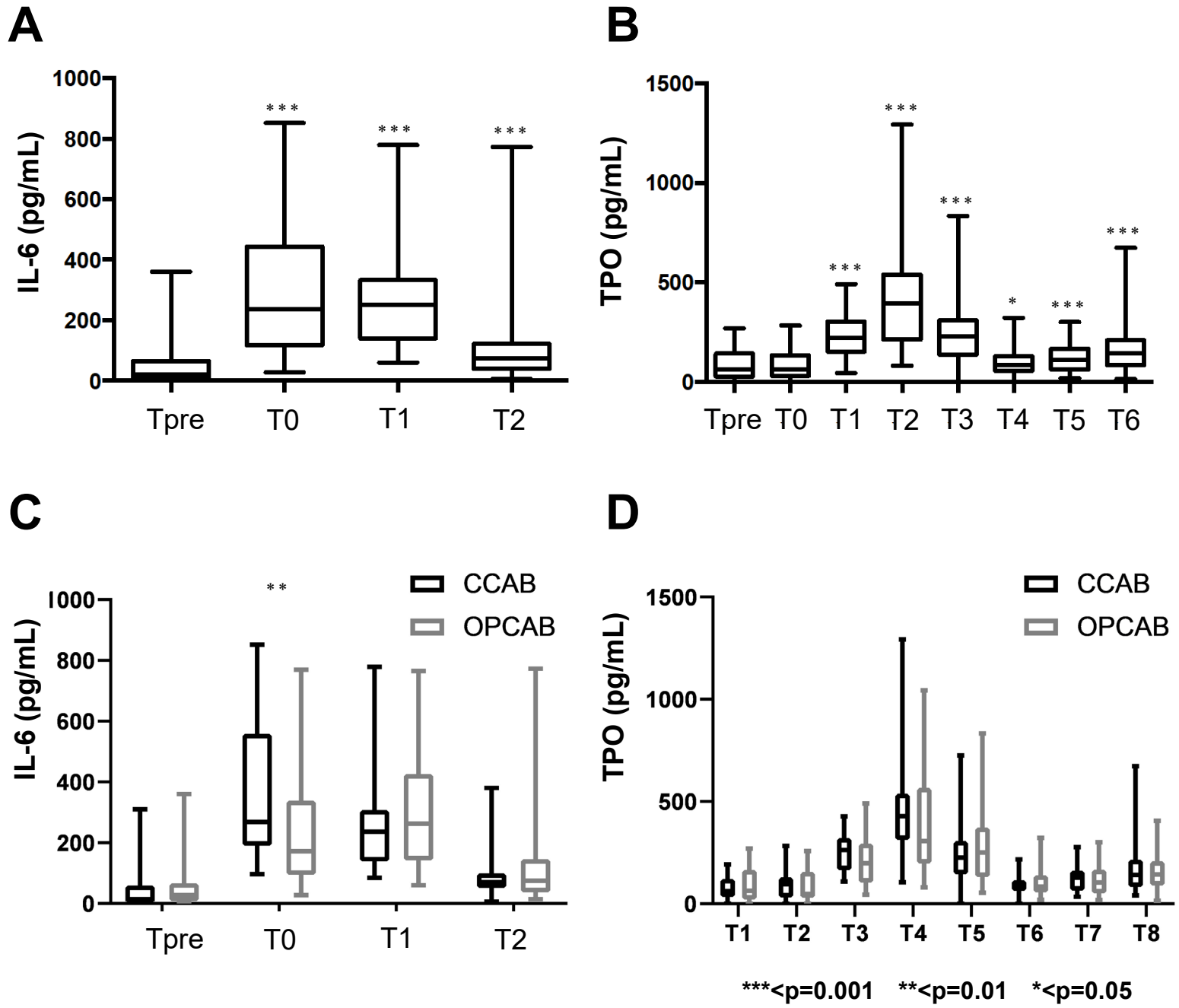
A
IL-6 (pg/mL)
1000
800
600
400
200
0
***
***
***
Tpre
T0
T1
T2
B
TPO (pg/mL)
1500
1000
500
0
***
***
***
*
***
***
Tpre
T0
T1
T2
T3
T4
T5
T6
C
IL-6 (pg/mL)
1000
800
600
400
200
0
**
CCAB
OPCAB
Tpre
T0
T1
T2
D
TPO (pg/mL)
1500
1000
500
0
CCAB
OPCAB
T1
T2
T3
T4
T5
T6
T7
T8
***<p=0.001 **<p=0.01 *<p=0.05

Figure 2

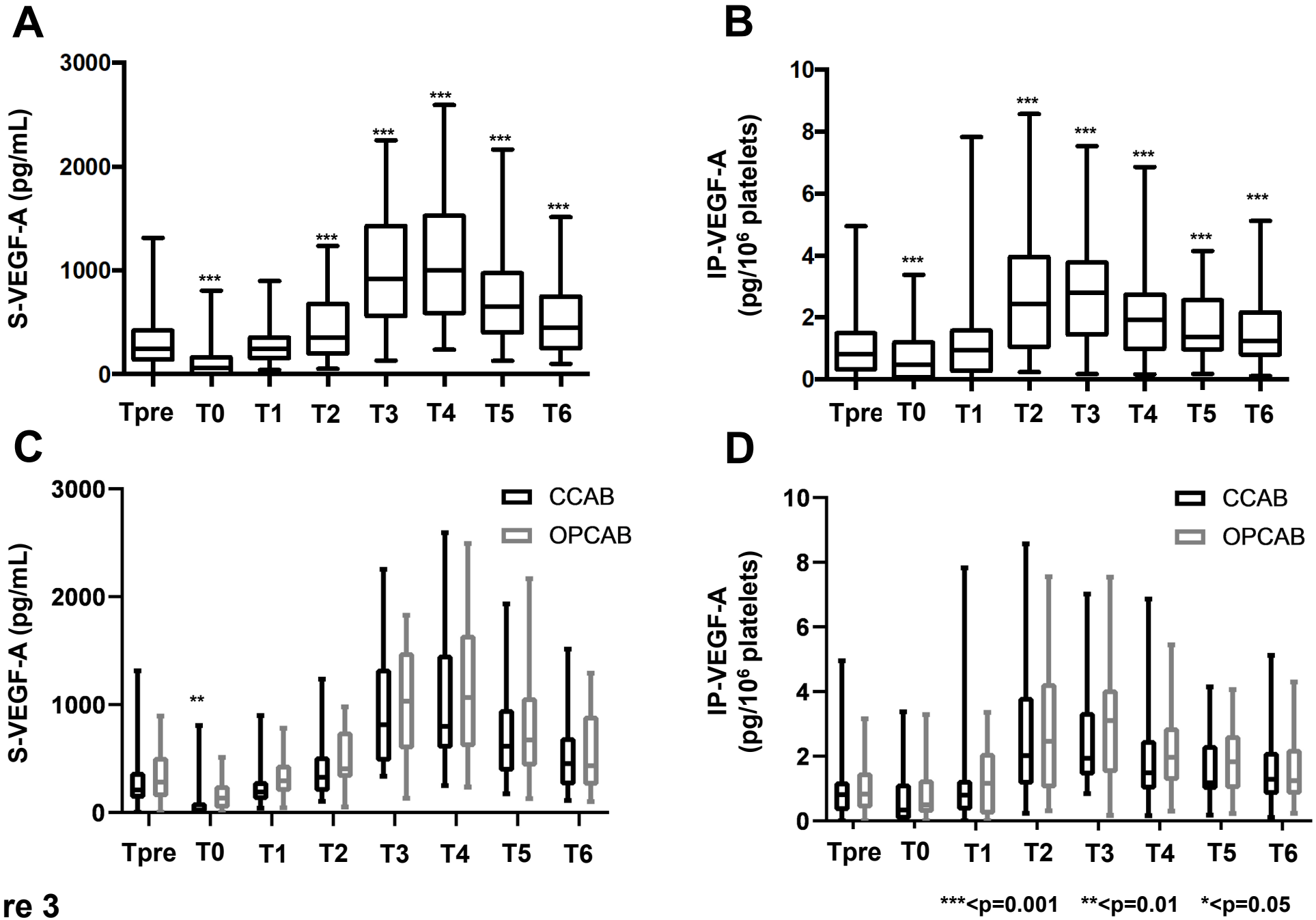
A
S-VEGF-A (pg/mL)
3000
2000
1000
0
***
Tpre T0 T1 T2 T3 T4 T5 T6
B
IP-VEGF-A (pg/10⁶ platelets)
10
8
6
4
2
0
***
Tpre T0 T1 T2 T3 T4 T5 T6
C
S-VEGF-A (pg/mL)
3000
2000
1000
0
**
CCAB
OPCAB
Tpre T0 T1 T2 T3 T4 T5 T6
D
IP-VEGF-A (pg/10⁶ platelets)
10
8
6
4
2
0
CCAB
OPCAB
Tpre T0 T1 T2 T3 T4 T5 T6
***<p=0.001 **<p=0.01 *<p=0.05

Figure 3

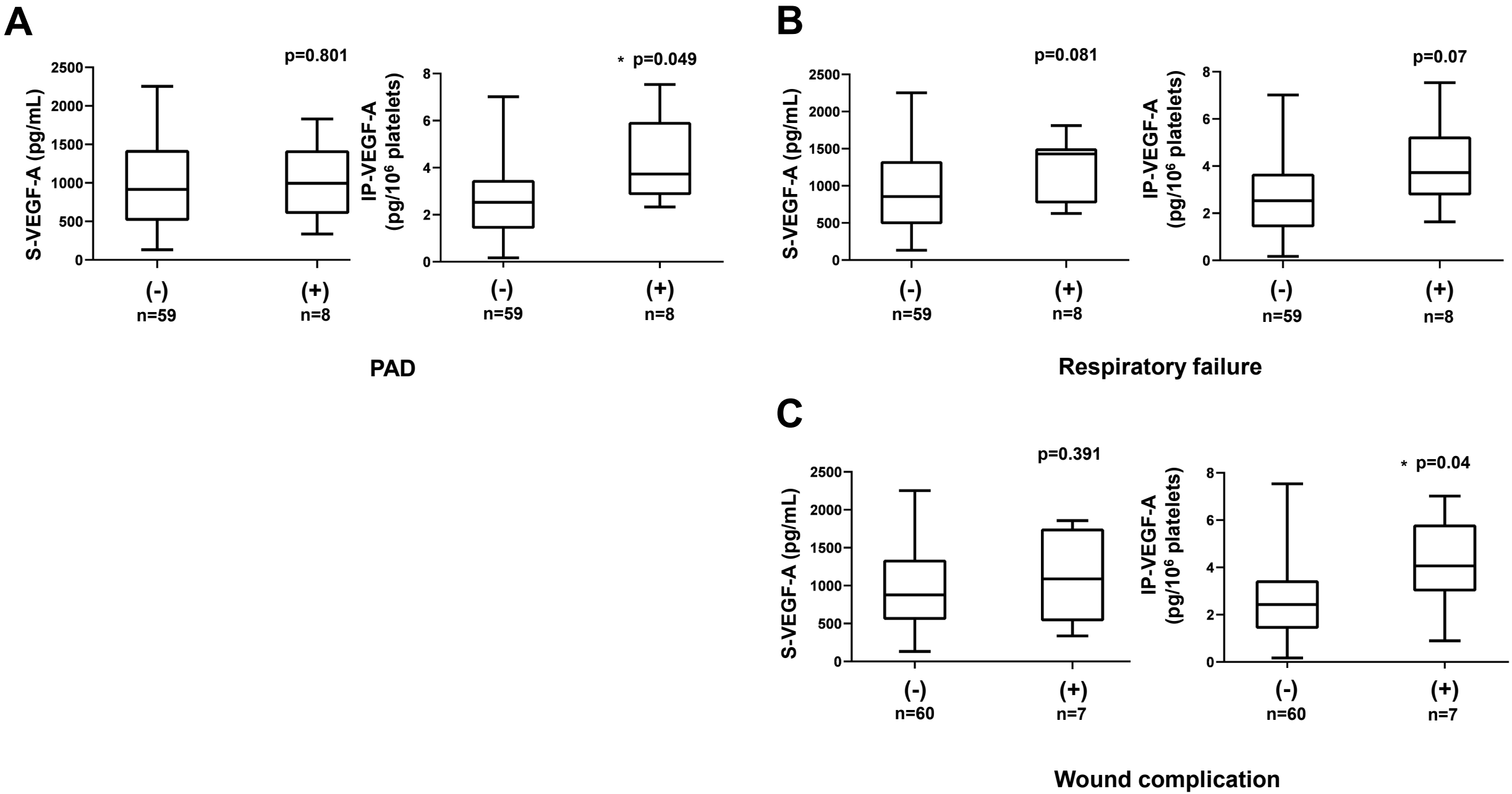
A
p=0.801
S-VEGF-A (pg/mL)
2500
2000
1500
1000
500
0
(-)
n=59
(+)
n=8
* p=0.049
IP-VEGF-A (pg/10^6 platelets)
8
6
4
2
0
(-)
n=59
(+)
n=8
PAD
B
p=0.081
S-VEGF-A (pg/mL)
(-)
n=59
(+)
n=8
p=0.07
IP-VEGF-A (pg/10^6 platelets)
(-)
n=59
(+)
n=8
Respiratory failure
C
p=0.391
S-VEGF-A (pg/mL)
(-)
n=60
(+)
n=7
* p=0.04
IP-VEGF-A (pg/10^6 platelets)
(-)
n=60
(+)
n=7
Wound complication

Figure 4

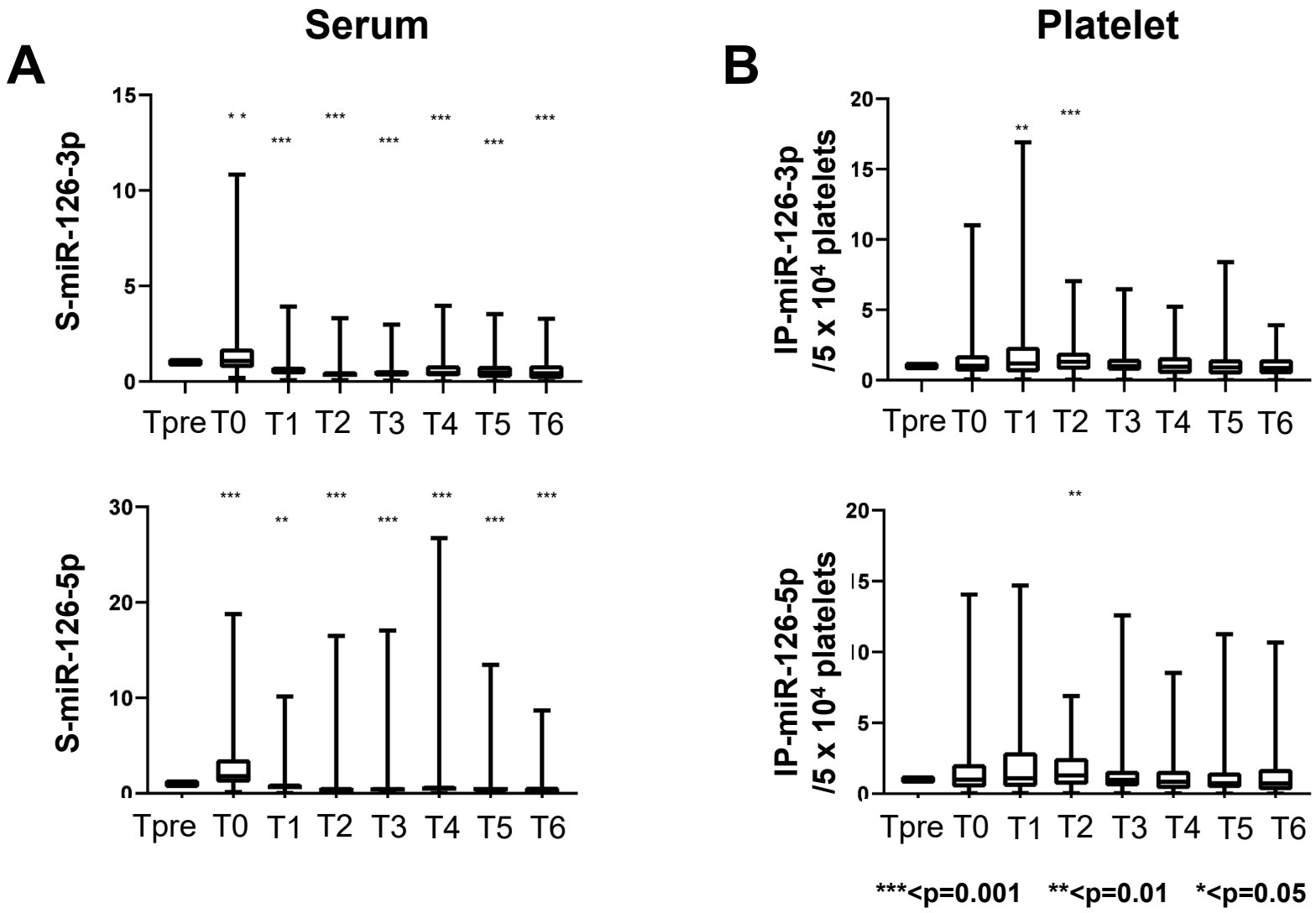
A
Serum
S-miR-126-3p
15
10
5
0
Tpre T0 T1 T2 T3 T4 T5 T6
S-miR-126-5p
30
20
10
0
Tpre T0 T1 T2 T3 T4 T5 T6
B
Platelet
IP-miR-126-3p /5 x 10⁴ platelets
20
15
10
5
0
Tpre T0 T1 T2 T3 T4 T5 T6
IP-miR-126-5p /5 x 10⁴ platelets
20
15
10
5
0
Tpre T0 T1 T2 T3 T4 T5 T6
***<p=0.001 **<p=0.01 *<p=0.05

Figure 5

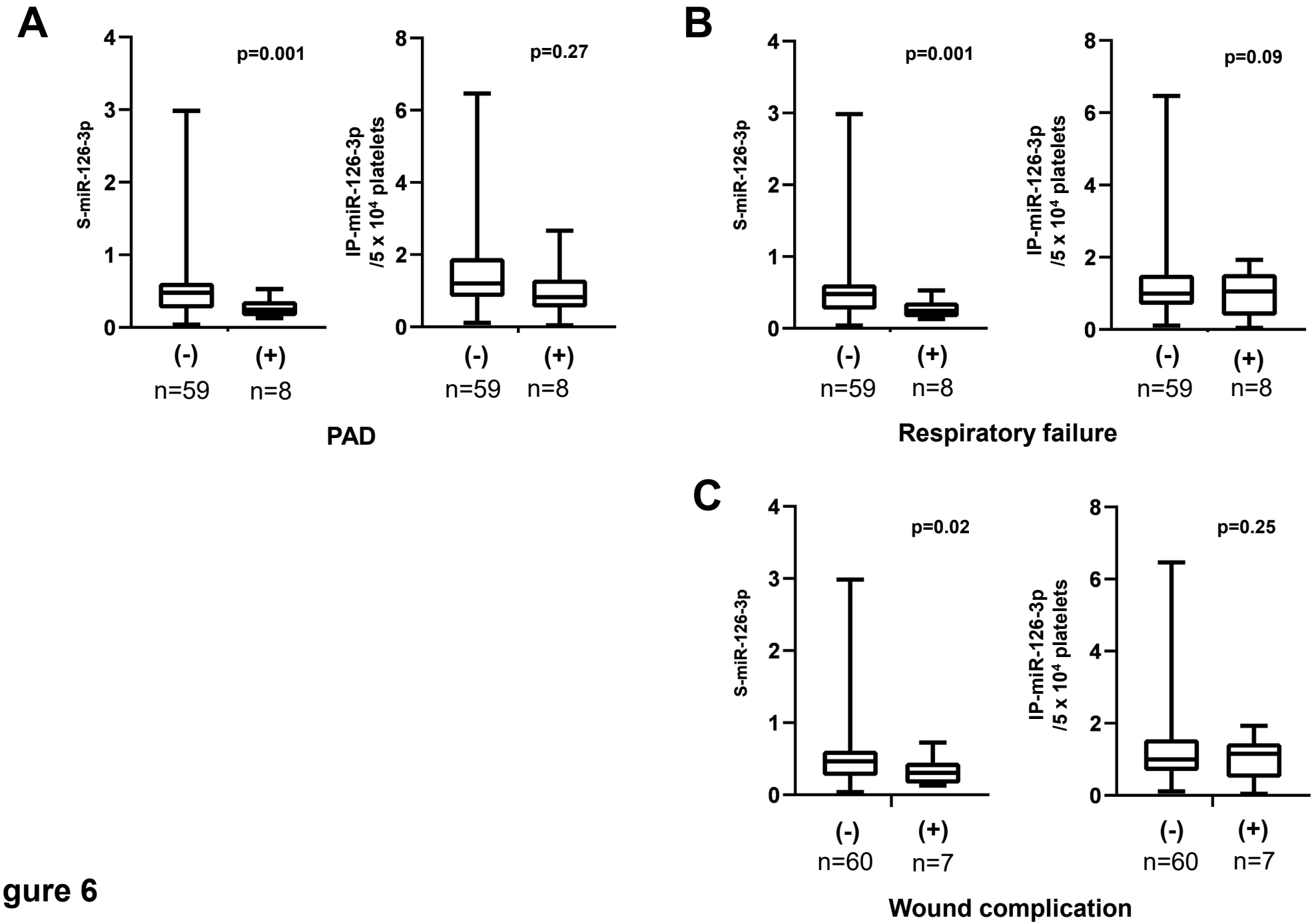
A
S-miR-126-3p
p=0.001
IP-miR-126-3p
/5 x 10⁴ platelets
p=0.27
(-)
(+)
n=59
n=8
n=59
n=8
PAD
B
S-miR-126-3p
p=0.001
IP-miR-126-3p
/5 x 10⁴ platelets
p=0.09
(-)
(+)
n=59
n=8
n=59
n=8
Respiratory failure
C
S-miR-126-3p
p=0.02
IP-miR-126-3p
/5 x 10⁴ platelets
p=0.25
(-)
(+)
n=60
n=7
n=60
n=7
Wound complication

Figure 6

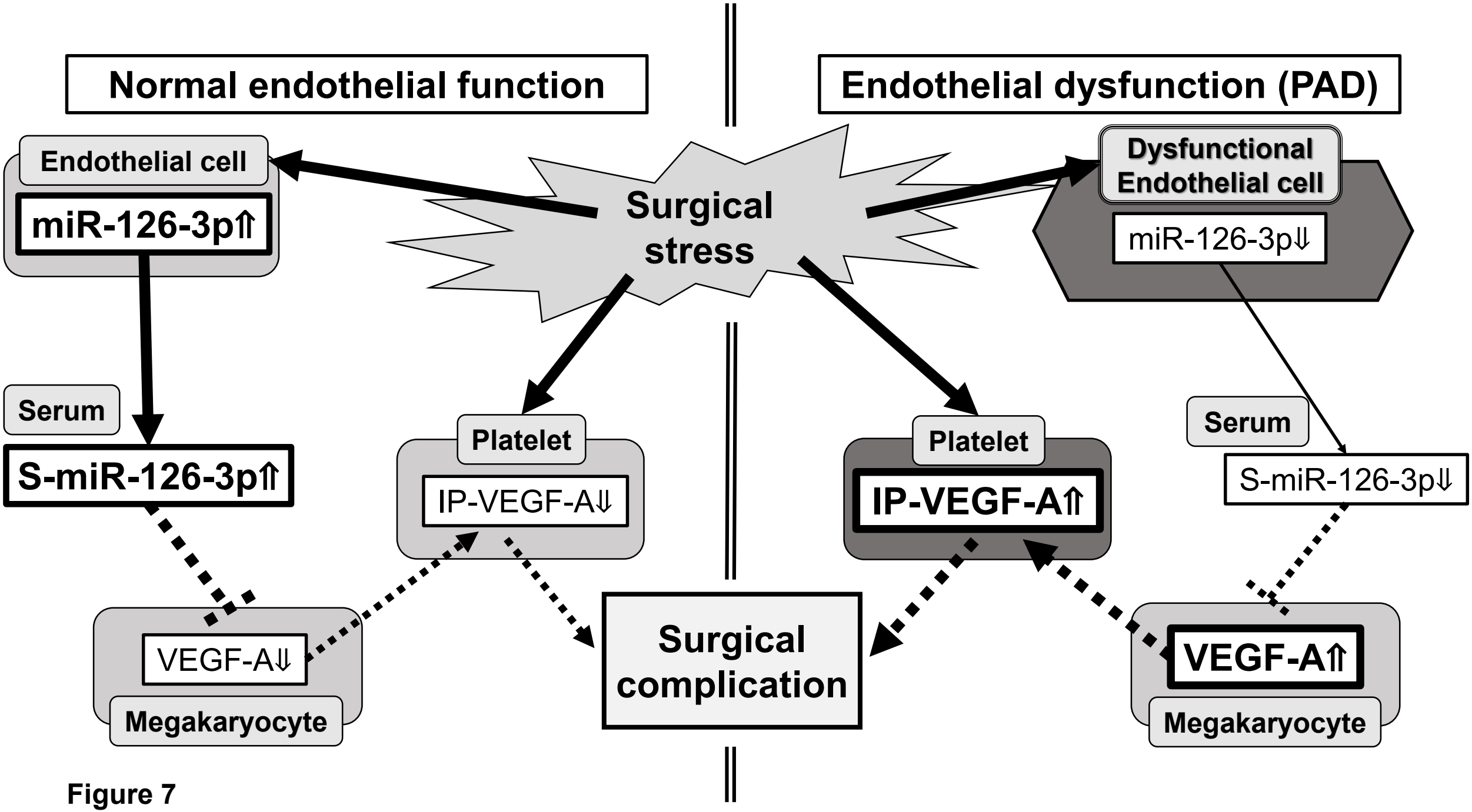
Normal endothelial function
Endothelial dysfunction (PAD)
Endothelial cell
miR-126-3p⇑
Surgical stress
Dysfunctional Endothelial cell
miR-126-3p⇓
Serum
S-miR-126-3p⇑
Platelet
IP-VEGF-A⇓
Platelet
IP-VEGF-A⇑
Serum
S-miR-126-3p⇓
VEGF-A⇓
Megakaryocyte
Surgical complication
VEGF-A⇑
Megakaryocyte

Figure 7