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Mortality, cardiac and cerebral damages reduction by IL-1 inhibition in a murine model of TTP

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Abstract:
Thrombotic thrombocytopenic purpura (TTP), a rare but fatal disease if untreated, is due to alteration in Von Willebrand factor cleavage resulting in capillary microthrombi formation and ischemic organ damage. Interleukin-1 (IL-1), has been shown to drive sterile inflammation following ischemia and could play an essential contribution to post-ischemic organ damage in TTP. Our objectives were to evaluate IL-1 involvement during TTP and to test the efficacy of the recombinant IL-1 receptor antagonist, anakinra, in a murine TTP model. We retrospectively measured plasmatic IL-1 concentrations in TTP patients and controls. TTP patients exhibited elevated plasma IL-1α and β concentrations, which correlated with disease course and survival. In a TTP mouse model, we administered anakinra (IL-1 inhibitor) or placebo for 5 days and evaluated the efficacy of this treatment. Anakinra significantly reduced mortality of mice (P<0.001). Anakinra significantly decreased TTP-induced cardiac damages as assessed by blood troponin concentrations, evaluation of left ventricular function by echocardiography, [18F]FDG PET of myocardial glucose metabolism, and cardiac histology. Anakinra also significantly reduced brain TTP-induced damages, evaluated through blood PS100b concentrations, nuclear imaging and histology. We finally showed that IL-1α and β trigger endothelial degranulation in vitro, leading to the release of Von Willebrand factor. In conclusion, Anakinra significantly reduced TTP mortality in a pre-clinical model of the disease by inhibiting both endothelial degranulation and post-ischemic inflammation, supporting further evaluations in humans.

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Abstract

Thrombotic thrombocytopenic purpura (TTP), a rare but fatal disease if untreated, is due to alteration in Von Willebrand factor cleavage resulting in capillary microthrombi formation and ischemic organ damage. Interleukin-1 (IL-1), has been shown to drive sterile inflammation following ischemia and could play an essential contribution to post-ischemic organ damage in TTP.

Our objectives were to evaluate IL-1 involvement during TTP and to test the efficacy of the recombinant IL-1 receptor antagonist, anakinra, in a murine TTP model.

We retrospectively measured plasmatic IL-1 concentrations in TTP patients and controls. TTP patients exhibited elevated plasma IL-1α and β concentrations, which correlated with disease course and survival. In a TTP mouse model, we administered anakinra (IL-1 inhibitor) or placebo for 5 days and evaluated the efficacy of this treatment. Anakinra significantly reduced mortality of mice ($P<0.001$). Anakinra significantly decreased TTP-induced cardiac damages as assessed by blood troponin concentrations, evaluation of left ventricular function by echocardiography, [18F]FDG PET of myocardial glucose metabolism, and cardiac histology. Anakinra also significantly reduced brain TTP-induced damages, evaluated through blood PS100b concentrations, nuclear imaging and histology. We finally showed that IL-1α and β trigger endothelial degranulation in vitro, leading to the release of Von Willebrand factor.

In conclusion, Anakinra significantly reduced TTP mortality in a pre-clinical model of the disease by inhibiting both endothelial degranulation and post-ischemic inflammation, supporting further evaluations in humans.

Key points:

- Plasmatic IL-1 levels are raised in TTP patients and correlate with mortality and severity of cardiac injury in patients
- In a mouse model of TTP, IL-1 inhibition with anakinra significantly reduced mortality as well as cerebral and cardiac injury

Main Text

Introduction

Immune mediated thrombotic thrombocytopenic purpura (TTP) is a rare and life-threatening thrombotic microangiopathy caused by anti-ADAMTS13 autoantibodies and severe ADAMTS13 deficiency, the enzyme involved in cleavage of pro-thrombotic Von Willebrand factor (VWF) multimers[1]. The disease is characterized by formation of systemic microthrombi, resulting from a two-step mechanism, namely inhibition of ADAMTS13, and endothelial degranulation leading to exocytosis of ultra-large VWF multimers[2]. TTP is characterized by severe thrombocytopenia, hemolytic anemia and organ ischemia, mainly affecting heart and brain. Despite numerous treatments, including plasma exchanges, corticosteroids, rituximab and more recently caplacizumab, TTP remains a fatal disease for approximately 5% of patients[3], cardiac ischemia representing the primary cause of death [4]. Cardiac and cerebral ischemia may also be associated with long-term sequels [5,6].

The interleukin-1 (IL-1) pathway is known to drive sterile inflammation following ischemia[7]. This pathway relies on 2 cytokines, IL-1α and β, which bind to the same signaling receptor (IL-1R1). IL-1α behaves as a damage-associated molecular pattern (DAMP) since it is constitutively present in the cytoplasm of cells and can be released into the extracellular compartment following ischemia-induced cell death[8]. IL-1β is mainly produced by leukocytes and its transcription and processing may be activated by various DAMPs released by cell death. These two cytokines, which are released after ischemia, contribute significantly to tissue damage[9–12]. The size of experimental infarcts is reduced in IL-1α and IL-1β-KO mice as well as in IL-1R1-KO mice[13–15]. Anakinra, a recombinant form of the natural IL-1R antagonist, inhibits both biological effects of IL-1α and IL-1β and thus reduces both infarct size and post-ischemic impaired cardiac function in various in vivo studies[16–18]. Anakinra as an inhibitor of the IL-1 signaling pathway is therefore a promising target for the treatment of organ ischemia, which has recently been investigated in human clinical trials during acute coronary syndrome [19–22].

TTP is an ischemic disease in which the involvement of IL-1 pathway has not been well demonstrated to date. Only one study reported slightly higher levels of IL-1β at diagnosis than during remission in 13 patients[23]. Given recent reports demonstrating the key function of IL-1

111 in ischemia[24,25], we aimed to study the implication of IL-1 pathway in cardiac and brain
112 ischemia-induced injury during TTP.
113
114 First, we evaluated activation of IL-1 pathway in patients with TTP based on plasma levels of
115 circulating IL-1α and β. Then, we inhibited IL-1 with anakinra in a severe mouse model of
116 TTP. Finally, we tested the ability of IL-1 to trigger endothelial degranulation and thus VWF
117 release, a critical step in TTP pathogenesis.
118

Methods

Patients

Inclusion protocol

A prospective study was conducted in France between 2008 and 2011 consisting in a National Clinical Research Project (#2007/23) approved by the Ethical Committee of the "Assistance Publique-Hôpitaux de Marseille" and following informed consent according to the Declaration of Helsinki. Diagnostic criteria for TTP and then for patient classification (survivor, non-survivor) are described in supplement data. For each patient in the acute phase, platelet poor plasma was obtained from the first therapeutic plasma exchange (PEX) and thus prior to treatment of the disease. Plasma from the first PEX of patients with autoimmune neurological diseases (myasthenia gravis or polyradiculoneuritis) formed the negative controls. The control patients therefore have 2 decisive characteristics in common with the TTP patients: an autoimmune disease and a plasma collection that was carried out at the time of the first plasma exchange. Plasma from TTP patients was also collected during remission by venipuncture into sodium citrate.

Blood analysis

Plasma IL-1α concentrations from TTP and control patients were measured by ELISA (IL-1α human ELISA kit, Invitrogen). IL-1β levels in plasma from TTP and control patients were determined by ProQuantum High-Sensitivity Immunoassays (IL-1β human ProQuantum Immunoassay Kit, Thermofisher). Troponin T and PS-100b concentrations were measured in TTP plasma by electrochemiluminescence technique (Cobas 8000, module e602, Roche).

Murine studies

Mouse characteristics and ethical aspects

Male and female ADAMTS13 KO mice (B6.129-ADAMTS13tm1Dgi[26]) on C57Bl6xCASA background (with elevated VWF plasma levels) were kept and raised in our facilities on a 12h light/dark cycle, with free access to food and water. At the time of the experiments the mice were 12-15 weeks and had a body weight of 18–26 g. The mice were examined clinically on a daily basis, paying particular attention to their general condition, weight and stress levels. All experiments were performed in accordance with National Institutes of Health guidelines and were approved by the ethics committee in charge of animal experimentation of our institution (national agreement: D1305520, APAFIS#30226-2021050614583963).

Experimental protocols

Murine model of VWF-induced TTP was driven in ADAMTS13 KO mice by intravenous injection in the retro-orbital sinus of 1500IU/kg body weight of recombinant human VWF (Veyvondi, Takeda) on days (D) 0, 1 and 2. Control mice received daily retro-orbital injections of 0.9% NaCl of equivalent volume (vehicle). Treated mice received daily intraperitoneal injection of anakinra (Sobi) at 100mg/kg from day 0 to day 5, whereas untreated mice received intraperitoneal injection of 0.9% NaCl (placebo) of equivalent volume. Anakinra or placebo were injected 5 minutes after VWF at D0, D1, and D2. Four groups of mice were thus constituted: "Control" (injection of Vehicle and placebo), "Anakinra" (injection of Vehicle and anakinra), "TTP" (injection of VWF and placebo) and "TTP + anakinra" (injection of VWF and anakinra).

To test the efficacy of the dosage of Anakinra at 100mg/kg, two protocols have been designed. The first included blood samples at D3 and a survival study up to D6 (Protocol No.1: Survival study, Supplemental Figure S1A). The second included imaging (ultrasound and isotope imaging) at D0 and D2 prior to cervical dislocation at D2 and a histologic study (Protocol No.2: Isotope imaging and histologic studies, Supplemental Figure S1B). Each outcome was analyzed by one-way ANOVA on rank (Kruskall Wallis test), then in case of p-value<0.05, by a further comparison of each group to the reference group ("TTP") performing Dunn's correction.

Murine model of IL-1-induced TTP was driven in ADAMTS13 KO and WT mice by intraperitoneal injections of 10ng/g human recombinant IL-1α or IL-1β (Biotechne) every hour for 9 hours (Supplemental Figure S1C).

Blood tests

Blood samples were collected from the facial vein on anesthetized mice with 1-2% isoflurane. Hematocrit, hemoglobin and platelet counts were performed on EDTA-treated blood diluted to $1/3000^{th}$ using a veterinary hematology analyzer (Leytemed). Blood smear was stained with May-Grünwald-Giemsa for manual assessment of schistocytes, and remaining blood was centrifuged at 2000g for 15 minutes for plasma extraction. Troponin I levels in plasma from TTP and control mice was determined by ELISA (high sensitivity mouse cardiac troponin-I ELISA, Life Diagnostic). IL-1α and β concentrations in murine plasma were determined by ELISA (IL-1α ELISA kit, Invitrogen, and IL-1β ProQuantum Immunoassay Kit, Thermofisher, respectively). PS-100b levels were determined by ELISA (Mouse S100B ELISA Kit, Mybiosource). D-dimer concentrations were determined by ELISA (Mouse D-2D, MyBio Source). VWF antigen levels were measured in mouse plasma with an in-house enzyme-linked immunosorbent assay essentially as previously described using a pair of

polyclonal rabbit anti-VWF antibodies (Agilent Technologies)[27]. Normal citrated pooled plasma obtained from wild-type C57B6-mice was used as reference. VWF multimer profile was performed essentially as previously described in 2% SDS-agarose gels[28]. VWF was detected with an in-house alkaline phosphatase-conjugated polyclonal anti-VWF and colorimetric alkaline phosphatase-substrate kit (Bio-Rad Laboratories). Membranes were imaged with a G:BOX Chemi XT16 Image Systems (Syngene). Multimer profiles were analyzed using the Gel Analyzer tool of ImageJ. High molecular weight multimers were defined as all peaks beyond the 10th peak.

Heart imaging

In vivo heart structure and function were evaluated at D0 and D2 using a high-frequency scanner (Vevo2100 VisualSonics). Briefly, mice were anesthetized with 1-2% isoflurane inhalation and placed on a heated platform to maintain temperature during the analysis. Two-dimensional imaging was recorded with a 22-55 MHz transducer (MS550D) to capture long- and short-axis projections with guided M-Mode and B-Mode and analysis with VevoLab software (VisualSonics). A target heartrate of 450 ± 100 beats per minute was used to record the M-mode. Ejection fraction (EF) was defined as the difference between the telediastolic and telesystolic volume, divided by the telediastolic volume. Shortening Fraction (SF) was defined as the difference between the end-diastolic and telesystolic diameters, divided by the end-diastolic diameter.

MicroPET/CT acquisitions were performed at D0 and D2 on a NanoscanPET-CT camera (Mediso). [18F]FDG (^{18}F-Fluorodesoxyglucose) tracer was injected intraperitoneally. Mice were maintained under 1-2% isoflurane anaesthesia during acquisition. Static PET imaging was performed 1 hour after radiotracer injection, during 20 min. Quantitative region-of-interest (ROI) analysis of the PET signal was performed using Invicro VivoQuant 4.0 software (Mediso) and tissue uptake values were expressed as a mean percentage of the injected dose per gram of tissue (%ID/g) for [18F]FDG. [18F]FDG was purchased as a ready-to-use radiopharmaceutical (Gluscan, Advanced Accelerator Applications).

Heart histology

Methods for heart histology are described in supplemental methods.

Brain and in vitro studies

Methods for brain imaging and brain histology and in vitro endothelial cell studies are described in supplemental methods.

Statistical analysis

Methods for statistical analysis are described in supplemental methods.

All experiments in humans were conducted within the framework of a national clinical research project approved by the ethics committee of Assistance Publique-H  pitaux de Marseille (#2007/23). All experiments in mice were carried out in accordance with National Institutes of Health guidelines and were approved by our institution's animal experimentation ethics committee (national agreement: D1305520, APAFIS#30226-2021050614583963).

Results

Increased IL-1α and β plasma concentrations correlate with disease prognosis

30 plasmas from acute phase TTP patients (20 survivors and 10 non-survivors), 10 plasmas from control patients and 10 plasmas from TTP patients in remission were first analyzed (detailed information of TTP and control patients in Table 1). IL-1α and β concentrations were significantly higher in non-survivor TTP patients compared with survivors (P=0.044, Figure 1A-B). IL-1α and β concentrations were significantly higher in the surviving TTP patients compared with the control group (P=0.028 and P<.001 respectively, Figure 1A and 1B). In patients tested during remission, plasma IL-1α and IL-1β concentrations were significantly lower than those observed in the acute phase of the disease (*P=0.006* and *P=0.004* respectively, Figure 1A-B). IL-1α and β plasma concentrations correlated during the acute phase (r^2= 0.692, *moderate correlation*, P<.001, Supplemental Figure S2A). The ratio of IL-1α to IL-1β was significantly higher in TTP patients who did not survive than in survivors (P=0.002, Supplemental Figure S2B).

Ischemic damages in TTP primarily affect heart and brain. We retrospectively assessed involvement of these two major organs by measuring troponin T and PS-100b concentrations in plasma of TTP patients. Plasma troponin levels were significantly higher in non-survivors compared to survivors (P=0.031, Supplemental Figure S2C), as were PS-100b levels (P=0.022, Supplemental Figure S2D). Plasma troponin and PS-100b concentrations strongly correlated with IL-1α and β concentrations in TTP patients (troponin and IL-1α: r^2=0.714, *strong correlation*, P<.0001; troponin and IL-1β: r^2=0.758, *strong correlation*, P<.0001; PS-100b and IL-1α: r^2=0.538, *moderate correlation*, P<.0001; PS-100b and IL-1β: r^2=0.546, *moderate correlation* P<.001, Figure 1C to F). We found no correlation between IL-1α and IL-1β plasma levels and anti-ADAMTS13 immunoglobulin G (IgG) concentrations in TTP patients (P=0.85 and P=0.54 respectively, r^2=0.03 and r^2=0.12 *weak correlation* for both, Supplemental Figure S2E and F).

Anakinra reduces mortality in a murine model of TTP

Based on these preliminary observations, we next asked whether IL-1 may be involved in a TTP murine model. We first explored a model of TTP using daily intravenous injection of VWF (1500IU/kg) for 3 days in ADAMTS13 KO mice. This protocol (Supplemental Figures S1A and S1B) induced acute TTP characterized by severe thrombocytopenia, mechanical hemolytic anemia (schistocytes) and capillary microthrombi (Supplemental Figure S3A-E). This protocol showed high mortality rate with 70% of mice died at D4 (Supplemental Figure S3F). Plasma IL-1α and IL-1β concentrations were significantly elevated (Supplemental Figure S3G-H).

We then assessed the efficacy of anakinra in this murine model. We first determined the dose of anakinra to study, based on dose-response evaluation. TTP was induced in 36 mice randomly assigned to 6 groups treated with 1, 10, 25, 50, 100, 200 mg/kg/day anakinra respectively (Figure 2A). Plasma troponin and PS-100b concentrations were measured and compared at the peak disease severity (D3). The 100mg/kg dose was the lowest dose to achieve a statistically effect for both changes in plasma troponin and PS-100b levels (Figure 2B and 2C). The lowest median survival rate was observed in the group with the lowest dose (1mg/kg), while the 2 highest doses (100 and 200mg/kg) had the highest survival rate, but these results were not statistically significant (P=0.07, Figure 2D). We therefore selected the 100mg/kg dose of anakinra.

A survival study was then conducted on male mice divided into 4 groups: with or without TTP and with or without treatment by 100mg/kg/day anakinra (protocol No.1, Supplemental Figure S1A). TTP induced a significant excess mortality compared to the two control groups which was significantly reduced by the administration of anakinra (*P*=0.004, Figure 2E).

Anakinra reduces cardiac and cerebral damages in a murine model of TTP

To further understand underlying mechanisms of beneficial effect of anakinra on mortality, additional male mice were included to evaluate specific cardiac and brain damages (protocol No.2, Supplemental Figure S1B).

Cardiac involvement was assessed using multimodality approach, including troponin assays, echocardiography, PET imaging and histology. TTP-mice exhibited a significant increase of troponin concentrations compared to control mice (P<.001, Figure 3A) which were significantly reduced by anakinra treatment (P=.038). TTP also resulted in significant alterations SF compared to controls (P<.001), which were significantly reduced by anakinra (P=0.035, Figure 3B, Figure 3C). Moreover, TTP induced a significant increase of [18F]FDG cardiac uptake compared to controls (P<.001, Figure 3D-E), which was significantly reduced by anakinra (P<.001). Evaluation of ischemic myocardial lesions based on histological scale was consistent with previous assessments: significant histological lesions in the TTP group compared to the control group (P=0.003, Figure 3F-G) were reduced by anakinra (P=0.043).

Evaluation of brain involvement included analysis of PS-100b levels, [18F]FDG brain and [99mTc]Tc-DTPA uptake, and histological assessment. Mice from the TTP group exhibited a significantly higher increase of PS-100b plasma levels than the control group (P=0.016, Figure 4A) which was significantly reduced by anakinra treatment (P=0.041). TTP also resulted in significant increase in [18F]FDG brain uptake compared to controls (P=0.036, Figure 4B-C), which was significantly reduced by anakinra (P=0.023). Moreover, TTP was associated with significant [99mTc]Tc-DTPA brain uptake increase compared to control (P<.001, Figure 4D-E), which was significantly reduced by anakinra (P=0.037). Analysis of brain tissue from mice showed that TTP caused significant neuronal damage compared to controls (P<.001, Figure 4F-G), which was significantly reduced by anakinra (P<.001).

Ischemia-induced IL-1 triggers endothelial degranulation leading to amplification loop of VWF release in TTP

During TTP, thrombocytopenia is known to be the result of platelet aggregation on high molecular weight VWF multimers previously degranulated by endothelial cells. Then, microthrombi resulting from this process are responsible for mechanical red blood cell lysis and anemia. Hematological parameters (anemia and thrombopenia) are therefore indirect markers of the occurrence of capillary microthrombi. Unexpectedly, in our TTP murine model, we observed that anakinra significantly improved the number of red blood cells (P=0.029, Supplemental Figure S4A) and platelets (P=0.035, Supplemental Figure S4B). Analyze of myocardial capillary microthrombi by immunofluorescence on myocardial sections from 18 hearts of mice with or without TTP, treated or not with anakinra

(Supplemental Figure S4C-D) demonstrated that treated mice had fewer capillary microthrombi than those from non-treated mice (P=0.008).

Given the decrease in the number of capillary microthrombi in mice treated with an IL-1 receptor antagonist, and since preliminary studies show no effect of anakinra on VWF reactivity (Supplemental Figure S5A-E), we wondered whether IL-1 itself is involved in endothelial cell degranulation. We then stimulated HMVEC-c with PBS, IL-1α or IL-1β for 1 hour and observed significantly higher VWF concentrations in IL-1α and β-stimulated cells supernatants (P=0.008 and P=0.008 for IL-1α and β, respectively, Figure 5A). IL-1α and β-induced VWF release was detectable as early as 15 minutes and did not change significantly after 30 minutes of stimulation, in favor of a rapid VWF release mechanism (Figure 5B-C). The rapid release of VWF by endothelial cells is known to be calcium dependent. We then blocked intracellular calcium signalization with MAPTAM, a cell-permeable calcium chelator. MAPTAM significantly decreased IL-1-induced VWF release (IL-1α: P=0.036 and IL-1β: P=0.036, Figure 5A), confirming that IL-1-induced endothelial degranulation was calcium-dependent. We also tested IL-1α and β on intracellular calcium flux using a fluorescent calcium probe. In response to both cytokines, HMVEC-c generated significantly greater calcium flux than PBS (Figure 5D). TTP plasma has been shown to trigger calcium-dependent endothelial degranulation[29]. To assess whether IL-1 present in the plasma from TTP patient induced endothelial degranulation, we stimulated HMVEC-c with TTP or control plasma, in the presence or absence of anakinra. Anakinra decreased the release of VWF in the supernatants of HMVEC-c stimulated by TTP plasma (P=0.03, Figure 5E). Consistent results were obtained from the measurement of intracellular calcium flux (Figure 5F).

Since IL-1α and β could trigger endothelial degranulation in vitro, we wondered whether these cytokines could induce TTP in ADAMTS13 KO mice. We injected ADAMTS13 KO and the WT control mice with high concentrations of IL-1α, IL-1β or equivalent volume of PBS and assessed the development of TTP using previously reported read-out. IL-1α and β resulted in significant thrombopenia compared to PBS only in ADAMTS13 KO mice (P<.001 for both, Figure 6A). To differentiate between thrombocytopenia due to microangiopathy or possible intravascular disseminated coagulation mechanism, D-dimer concentrations were measured. D-dimer increased after IL-1α and β injection (P<.001, Supplemental Figure S6A) but this increase was independent of KO or WT genotype (P=0.08), rendering unlikely an underlying intravascular disseminated coagulation mechanism in ADAMTS13KO mice. As expected, given the short delay (H10), we did not observe significant anemia in the mice

(Supplemental Figure S6B), but schistocytes were observed in all IL-1α and IL-1β injected KO mice (Supplemental Figure S6C). Moreover, IL-1α and IL-1β injections significantly increased VWF plasma levels only in KO mice ($P<.001$ for both, Figure 6B), and as expected this increase was associated with a loss of high-molecular-weight VWF multimers in electrophoresis (Supplemental Figure S6D). IL-1α and β injections increased troponin levels only in KO mice ($P<.001$ for both, Figure 6C). Histologically, cardiac ischemic lesions were only observed in KO mice injected with IL-1α or IL-1β ($P=0.006$ and $P=0.03$, Figure 6D). Finally, immunofluorescence analysis of cardiac capillary microthrombi revealed that only ADAMTS13 KO mice injected with IL-1α or IL-1β exhibited significant increase in the amount of intra-capillary VWF thrombi ($P=0.006$ and $P=0.03$, Figure 6E).

Discussion

TTP is characterized by an ADAMTS13 deficiency and systemic tissue ischemia resulting from capillary microthrombi accumulation. The two main organs affected are heart and brain. Cardiac ischemia is assessed in humans by blood troponin assay and echocardiographic evaluation of EF[30]. It is a major risk factor for disease mortality. Assessment of neurological ischemia is not standardized to date, but recent studies have highlighted a possible alteration of the blood-brain barrier (BBB) in TTP[31], which may be associated with later development of cognitive impairment and depression[32,33].

IL-1α and IL-1β are two main proinflammatory cytokines involved in pathogenesis of tissue lesions secondary to an ischemic process[34]. Indeed, the release of multiple DAMPs by ischemic dying cells induces IL-1α and β transcription and IL-1β processing through NLRP3 inflammasome assembling and caspase-1 activation, in a large part through binding to TLR4 and TLR2[35]. In mice, TLR2/4 inhibition[36] has been shown to reduce both post-ischemic myocardial leukocyte infiltration and the size of myocardial infarctions [37]. Thus, the role of IL-1α and β in ischemia due to TTP deserved investigations.

First, we observed that mostly IL-1α but also IL-1β concentrations were elevated during the acute phase of the disease, normalized during remission, and correlated with disease mortality and morbidity evaluated by troponin T and PS-100b concentrations. Troponin is an important prognosis biomarker during TTP, known to correlate with cardiac involvement and mortality[30]. Although not previously evaluated in TTP, PS-100b is considered as a good biomarker of brain ischemia[38], predicting clinical stroke status in patients during the early phase of the disease[39]. Circulating concentrations of PS-100b appeared to correlate with TTP-induced mortality in our study. The higher IL-1α/β ratio observed in non-surviving TTP patients in our study may indicate a greater contribution of IL-1α in determining the severity of TTP. IL-1α is an intracellular cytokine, constitutively present in epithelial and endothelial cells which is released in apoptotic bodies or during cell necrosis[40]. The elevated concentrations of IL-1α observed in TTP patients may indicate a necrotic endothelial origin of this cytokine, which is not directly related to the immune mechanism of the disease (anti-ADAMTS13 antibodies) but rather to its ischemic features. However, the present study was not designed to determine the original cell source of IL-1α or β.

The increase in IL-1α and IL-1β levels observed in our study may seem modest. However, extremely low serum levels of these cytokines are sufficient to elicit clinically relevant pro-inflammatory effects (a few nanograms injected into humans are sufficient to induce fever)[41]. IL-1α and IL-1β exert mainly local effects (para- and autocrine), so serum levels are often undetectable. In a highly inflammatory disease such as severe sepsis with multi-organ failure, for example, only a few studies have succeeded in detecting a very small increase in IL-1β in the order of a few pg/mL[42]. Similar limitations of the assays have been observed in organ ischemia such as stroke[43,44] or myocardial infarction[45,46]. So, to assess the potential pathogenic role of IL-1α and β during TTP, we then evaluated the effect of the recombinant human IL-1 receptor antagonist, anakinra, in a murine model of TTP. The classic murine model of TTP is based on a single injection of 2000 IU/Kg VWF[47]. This model is slightly severe (0% mortality) and failed to prove favorable effect of anakinra. We therefore adapted it by performing 3 daily injections of VWF at 1500 IU/kg, resulting in a model with high mortality much closed to that observed in humans. The dose of anakinra (100mg/kg/day) was selected after a preliminary dose-response study. Although it was much higher than that used in humans (100mg/day), it was consistent with doses commonly used in mouse models of ischemia[48–50] or inflammatory diseases[51]. This may be explained by a lower affinity of anakinra for the mouse IL-1 receptor. Anakinra injections decreased cardiac lesions, as assessed by troponin concentrations, echocardiography, PET imaging and histological analysis. In addition, anakinra also reduced cerebral ischemia, demonstrated by circulating PS-100b concentrations, cerebral glucose metabolism and BBB analysis. The cerebral and cardiac hypermetabolism ([18F]FDG uptake) observed in untreated TTP mice by PET suggests a local inflammatory response triggered by ischemia leading to vascular leakage. This was associated with a rupture of the BBB, as evidenced by capillary leakage of [99mTc]Tc-DTPA. These results are thus in support of an important role for IL-1 in TTP pathogenesis, and a protective effect of IL-1 inhibition.

Anakinra-treated mice exhibited less severe hematological disturbance than untreated mice suggesting an unexpected role of IL-1 in endothelial VWF degranulation. We confirmed this effect in vitro using microvascular endothelial cells treated by IL-1. These findings were also observed in vivo since it was possible to induce TTP in ADAMTS13 KO mice by injection of IL-1α and β alone, although at supraphysiological concentrations. It should be noted that IL-1 can cause inflammatory lesions in the heart independently of ADAMTS-13 deficiency. However, in this case, the histologic lesions occur only after several days[18,52], compared to 10

hours in our study. Therefore, ADAMTS-13 deficiency sensitizes cardiac cells early to IL-1 toxicity through VWF degranulation, followed by the production of micro-thrombi that consume platelets and high-molecular-weight VWF multimers and generate TTP[53].

Our results suggest that IL-1 may be involved in an amplification loop of VWF endothelial degranulation in TTP. Several such amplification loops have already been reported in TTP, involving complement[54], heme[55], or NETs[56]: TTP-induced ischemia and hemolysis trigger complement activation, free heme production and NET generation which promote subsequent endothelial degranulation and VWF release, amplifying micro-thrombi formation and constituting a harmful amplification loop increasing TTP severity[57]. IL-1α and β may also participate in such a amplification loop, since these two cytokines are released in response to ischemia[8]. Thus blocking IL-1 may not only decrease the consequences of ischemia-induced inflammation but also inhibit endothelial degranulation, a well-recognized mechanism in TTP pathogenesis[58], but not targeted by current treatments of the disease.

This study has several limitations. Cardiac and neurological outcomes in TTP patients were assessed retrospectively and through biological markers only. In the murine model, we injected anakinra rapidly after the onset of TTP possibly artificially increasing its effectiveness. However, given the high mortality rate in our model (80% at D3 without treatment), it appears difficult to start IL-1 inhibition more lately. In addition, we did not investigate the optimal duration of anakinra treatment. Here again, we are limited by the model, since TTP is "active" only as long as VWF injections are given. As soon as these injections are stopped, surviving mice get better and better and platelets rise spontaneously. It therefore appears difficult to study the effect of longer durations of anakinra treatment on remitting mice after stopping injections. To modulate the onset and duration of anakinra treatment, and possibly evaluate more time points after TTP with non-invasive Doppler and PET scans, we need to establish less severe models of microangiopathy that last several days. So far, such models are not available. Another limitation of our study is that brain [18F]FDG uptake may have been affected by BBB alteration due to TTP. Finally, the induction of TTP by IL-1 injection in mice required the administration of very high doses of IL-1. However, induction of endothelial degranulation and TTP in mice has been shown to be challenging, only one such model has been reported to date, driven by large concentrations of shigatoxin[26,59].

In conclusion, our work reports consistent results on the role of IL-1α and β in TTP. Released in response to ischemia, these 2 cytokines contribute to organ damages through induction of sterile inflammation and triggering VWF endothelial degranulation. Anakinra improves hematologic parameters of thrombotic microangiopathy, reduces cardiac and cerebral and significantly decreases TTP-induced mortality in mice. The relevance of this work should be evaluated in humans.

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Authorship contributions:

RM, RC, ML, YK, MM and ET conducted in vitro and in vivo experiments. RM, ML, SR and GC conducted the histological analyses. RM, SF, GH and BG performed functional imaging analyses. PL performed and interpreted VWF antigen and multimers analysis. PP and GK monitored the multi-center clinical study. RM, ML and ET wrote the first draft of the manuscript. RC, GH, BG, SF, FDG and GK revised it critically. RM, ET and GK managed the overall research enterprise.

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The authors declare no competing interests

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Tables

Table 1

TTP patients	
Demographics	
Age in year (IQR)	45 (29-53)
Male, n (%)	12 (40)
Laboratory features	
Platelets, G/L (IQR)	13 (9-30)
Hemoglobin, g/L (IQR)	73 (60-98)
LDH, IU/L (IQR)	1348 (871-1715)
Presence of schistocytes, n (%)	30 (100)
ADAMTS13 activity, % (IQR)	5 (0-5)
Anti-ADAMTS13 IgG, IU/mL	71 (51-112)
Clinical features	
Mortality, n (%)	10 (33)
Control patients	
Demographics	
Age in year (IQR)	54 (53-58)
Male, n (%)	5 (50)
Neurologic disease	
Myasthenia gravis, n (%)	8 (80)
Acute polyradiculoneuritis, n (%)	2 (20)

Table 1. Demographics, clinical and biological data of the 30 TTP and the 10 control patients (median)
IQR: interquartile range; LDH: lactate deshydrogenase, n: number

Figure legends

Figure 1. IL-1α and β concentrations in the plasma of TTP patients; correlations with troponin and PS-100b concentrations

(A) IL-1α and (B) IL-1β plasma levels quantified by ELISA in TTP non-survivors (n=10), TTP survivors (n=20), TTP remission (n=10) and controls (n=10). *IL-1α plasma levels in non-survivors: 6.65[4.39;13.64] pg/mL, in survivors: 1.72[0.86;4.88]pg/mL, in control: 0.08[0;1.64]pg/mL and in remission: 0.72[0.36;1.19]pg/mL. IL-1β plasma levels in non-survivors: 3.51[3.20;3.93]pg/mL, in survivors :3.34[3.19;3.45]pg/mL, in control: 2.55[0.87;3.22]pg/mL and in remission 2.31[0.82;3.12]pg/mL.* (C) Correlation between troponin T and IL-1α or (D) between troponin T and IL-1β plasma levels in TTP patients. (E) Correlation between PS-100b and IL-1α or (F) between PS-100b and IL-1β plasma levels in TTP patients. (**P<.05, **P<.01, ***P<.001).*

Figure 2. Dose-response study of anakinra in mouse model of TTP; survival curve at the selected dose of 100mg/kg

(A) Protocol for dose-response study. (B) PS-100b levels at D3 among the 6 dosage groups. *One-Way ANOVA: P*<.001. *Multiple comparisons with Dunn's correction: 100mg/kg/day: 1570[1280;1880]ng/mL vs 10mg/kg/day: 2750[2340;2930]ng/mL, P*=0.04 *and 1mg/kg/day: 3125[3060;3190] ng/mL, P*=0.02. (C) Plasma troponin I levels at D3 among the 6 dosage groups. *One-Way ANOVA: P*<.001. *Multiple comparisons with Dunn's correction: 100mg/kg/day: 2.4[2.1;4.5]ng/mL, 50mg/kg/day: 11.0[10.3;12.3]ng/mL, P*=0.003, *25mg/kg/day: 11.6[10.7;12.7]ng/mL, P*=0.003, *10mg/kg/day: 19.9[14.7;25.6]ng/mL, P*<.001 *and 1mg/kg/day: 27.5[26.5;28.5]ng/mL, P*<.001. (D) Probability of survival of the 6 dosage groups. *Log-rank test: P=0.07. (*ns: *P*>.05, **P*<.05, ***P*<.01, ****P*<.001). (E) Probability of survival of the 4 groups of mice: TTP (VWF+placebo, n=28), TTP + anakinra (VWF+anakinra 100g/kg, n=14), and two control groups (control [vehicle+placebo], n=5 and anakinra [vehicle+anakinra] 100mg/kg, n=5) (****P*<.001). The curves for the two control groups overlap (no deaths in either group).

Figure 3. Anakinra reduces TTP-induced myocardial damage

(A) Blood troponin I levels in mice at day 3 for the 4 groups of mice: TTP (VWF+placebo, n=10), TTP+anakinra (VWF+anakinra, n=10), and two control groups (control [vehicle+placebo], n=5 and anakinra [vehicle+anakinra], n=5). *One-way ANOVA: P<.001. Troponin concentrations in TTP: 21.15[16.50;25.78]ng/mL, control: 0.41[0.24-0.43]ng/mL, anakinra: 0.24[0.14;0.42]ng/mL, and TTP+anakinra: 4.53[2.26;6.85]ng/mL.* (B) Change in fraction shortening in mice between day 0 and day 2 for the 4 groups of mice: TTP, n=12; TTP+anakinra, n=13, control, n=10 and anakinra, n=10. *One-way ANOVA: P<.001. Change in SF in TTP:-11.8[-18.9;-9.3]%, controls: -2.8[-12.3;4.4]%, anakinra: -0.8[-1.6;1]% and TTP+anakinra:-5[-9.5;-2.5]%.* (C) Measurement of the shortening fraction on long axis echocardiographic view. (D) Change in [18F]FDG cardiac uptake between D0 and D2 for the 4 groups of mice: TTP, n=5; TTP+anakinra, n=6, control, n=6 and anakinra, n=6. (E) Measurement of [18F]FDG cardiac PET signal. *One-Way ANOVA: P*=0.008. *Change in 18F]FDG cardiac uptake in TTP:2.8[2.5;3.5]x10^{-3}%ID/g, control: 0.03[-0.07;0.1]x10^{-3}%ID/g, anakinra 0.8[-0.2;1.2]x10^{-3}%ID/g and TTP+anakinra: 0.9[0,24-1.2]x10^{-3}%ID/g.* (F) Semi-quantitative assessment of myocardial damage in the 4 groups of mice, n=5 for each group. *One-way ANOVA: P<.001; Score in TTP: 5[4;5.88], control: 1[0.56;1.25], anakinra:1[0.8;2.1] and TTP+anakinra: 2.75[2;3.38].* (G) Cardiac histology in HE staining. *(***P*<.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001).

Figure 4. Anakinra reduces TTP-induced cerebral damage

(A) PS-100b levels at D3 for the 4 groups of mice: TTP (VWF+placebo, n=10), TTP + anakinra (VWF+anakinra, n=10), and two control groups (control [vehicle + placebo] and anakinra [vehicle + anakinra], n=5 for both). *One-way ANOVA: P*<.001*; PS100b levels in TTP: 2975[2619;3098]pg/mL, controls: 1080[1075;1085]pg/mL, anakinra 1080[1070;1080]pg/mL and TTP+anakinra: 2035[1558;2018]pg/mL.* (B) Change in [18F]FDG brain uptake in mice between D0 and D2 for the 4 groups of mice: TTP, n=5; TTP+anakinra; n=6, control; n=6 and anakinra, n=6. *One-way ANOVA: P*<.001*; Change in [18F]FDG brain uptake in TTP: 1.4[0.99;1.67]x10^{-3}%ID/g, control: 0.25[0.02;0.69]x10^{-3}%ID/g, anakinra: 0.1[0.0;0.3]x10^{-3}%ID/g and TTP+anakinra: 0.1[-0.1;0.09]x10^{-3}%ID/g.* (C)Measurement of [18F]FDG brain PET signal. (D) Change in [99mTc]Tc-DTPA brain uptake between D2 and D0 for the 4 groups of mice: TTP, n=5; TTP+anakinra, n=6; control, n=6 and anakinra, n=6. *One-way ANOVA: P*<0.001*; Change in [99mTc]Tc-DTPA*

brain uptake in TTP: 16.3[9.89;27.65]x10^{-5}%ID/g, control: 0.72[0.28;1.51]x10^{-5}%ID/g, anakinra: 1.4[0.6;1.8]x10^{-5}%ID/g and TTP+anakinra:3.08[2.06;5.21]x10^{-5}%ID/g.
(E) Measurement of [99mTc]Tc-DTPA brain SPECT signal. (F) Histological assessment of brain ischemic damage, n=3 for each group. *Percentage of healthy Purkinje cells in TTP:44%, controls:65%, anakinra: 70% and TTP+anakinra 61%*. (G) Brain histology in HE staining. *(*P*<.05, ***P*<.01, ****P*<.001).

Figure 5. IL-1α and β induce endothelial degranulation and contribute to WVF exocytosis in TTP

(A) VWF concentrations in cell supernatants after stimulations with IL-1α (10ng/mL), IL-1β (10ng/mL) and PBS +/- MAPTAM (1µmol/L). Thrombin (4IU/mL) is used as positive control. *IL-1α:10.7[8.5-15.5]ng/mL; IL-1β: 10.7[6.6-16.3]ng/mL; PBS: 4.8[1.6-5.6]ng/mL; IL-1α+MAPTAM: 1.2[0.97;1.4]ng/mL; IL-1β+MAPTAM: 0.7[0.23;1.2]ng/mL.* (B) VWF concentrations in cell supernatants after stimulations with IL-1 α (10ng/mL), or (C) β (10ng/mL) for 15, 30 and 60 minutes. (D) Calcium flux after stimulation with IL-1α (10ng/mL), β (10 ng/mL) and PBS during 20 seconds. *AUC of IL-1α: 62.2[95%CI: 60.7-63.7]x10^{10}AU, IL-1β: 64.5[95%CI: 63.3-65.7]x10^{10}AU and PBS: 8.0[95%CI: 7.9- 8.2]x10^{10}AU.* (E) VWF concentrations in cell supernatants after stimulations with TTP plasma (1%, n=6), control plasma (1%, n=6) and PBS, +/- anakinra (10µg/L). *TTP:105.0[95.0-140.5]ng/mL and TTP+anakinra: 65.0[53.5-84.5]ng/mL.* (F) Calcium flux after stimulations with TTP plasma (1%), control plasma (1%) and PBS, +/- anakinra (10µg/L) during 20 seconds. *AUC of TTP: 13.6[95%CI:12.9-14.2]x10^{10}AU, TTP+anakinra: 5.9[95%CI:5.8-6.1]x10^{10}AU, control 3.4[95%CI:3.3-3.6]x10^{10}AU and control+anakinra :1.4[95%CI:1.3-1.4]x10^{10}AU. (*ns: *P*>.05, **P*<.05, ***P*<.01).*

Figure 6. IL-1 α and β induce TTP in ADAMTS13KO mice

(A) Platelet levels between at H10 in ADAMTS13 KO mice (KO) or wild type (WT) mice injected with IL-1α, β or PBS (n=5 per group). *Two-way ANOVA for genotype: P*<.001 *and for injected compound: P*<.001. *Platelets levels in KO/IL-1α: 205[140;271]G/L, KO/IL-1β: 189[166;246]G/L and KO/PBS: 763[700;995]G/L.* (B) VWF levels between at H10 in ADAMTS13 KO mice (KO) or wild type (WT) mice injected with IL-1α, β or PBS (n=5 per group). *VWF levels in KO/IL-1α: 3040[270;4610]%, KO/IL-1β: 3050[1500;8670]% and KO/PBS: 120[60;130]ng/mL. VWF levels in WT/IL-1α: 180[160;220]ng/mL, WT/IL-1β: 170[140;180]ng/mL and WT/PBS: 70[50;80]ng/mL.* (C) Blood troponin I levels between H0 and H10 in ADAMTS13 KO mice (KO) or wild type (WT) mice injected with ILα, β or PBS (n=5 per group). *Two-way ANOVA for genotype: P*<.001 *and for injected compound: P*<.001. *Troponin I levels in KO/IL-1α: 4.7[4.0;5.3]ng/mL, KO/IL-1β: 5.9[3.5;8.9]ng/mL and KO/PBS: 0.7[0.4;0.9]ng/mL.* (D) Semi-quantitative assessment of myocardial damage. *Two-way ANOVA for genotype: P*<.001 *and injected compound: P*=0.008. *Scores in KO/IL-1α: 5.0[2.3;6.4], KO/IL-1β: 3.8[2.5;5.3] and KO/PBS: 1.7[2.5;7.3].* (E) Immunofluorescence measurement of intravascular Von Willebrand Factor; green = anti-VWF antibodies, blue = DAPI). *Two-way ANOVA for genotype: P*<.001 *and injected compound: P*=0.005. *Capillary fluorescence: KO/IL-1 α: 26[25;28]x10^{5}UA vs KO/IL-1β: 27[25:29]x10^{5}UA and KO/PBS: 20[18;22]x10^{5}UA.* (ns: *P*>.05, **P*<.05, ***P*<.01, ****P*<.001, *****P*<.0001)

FIGURE 1

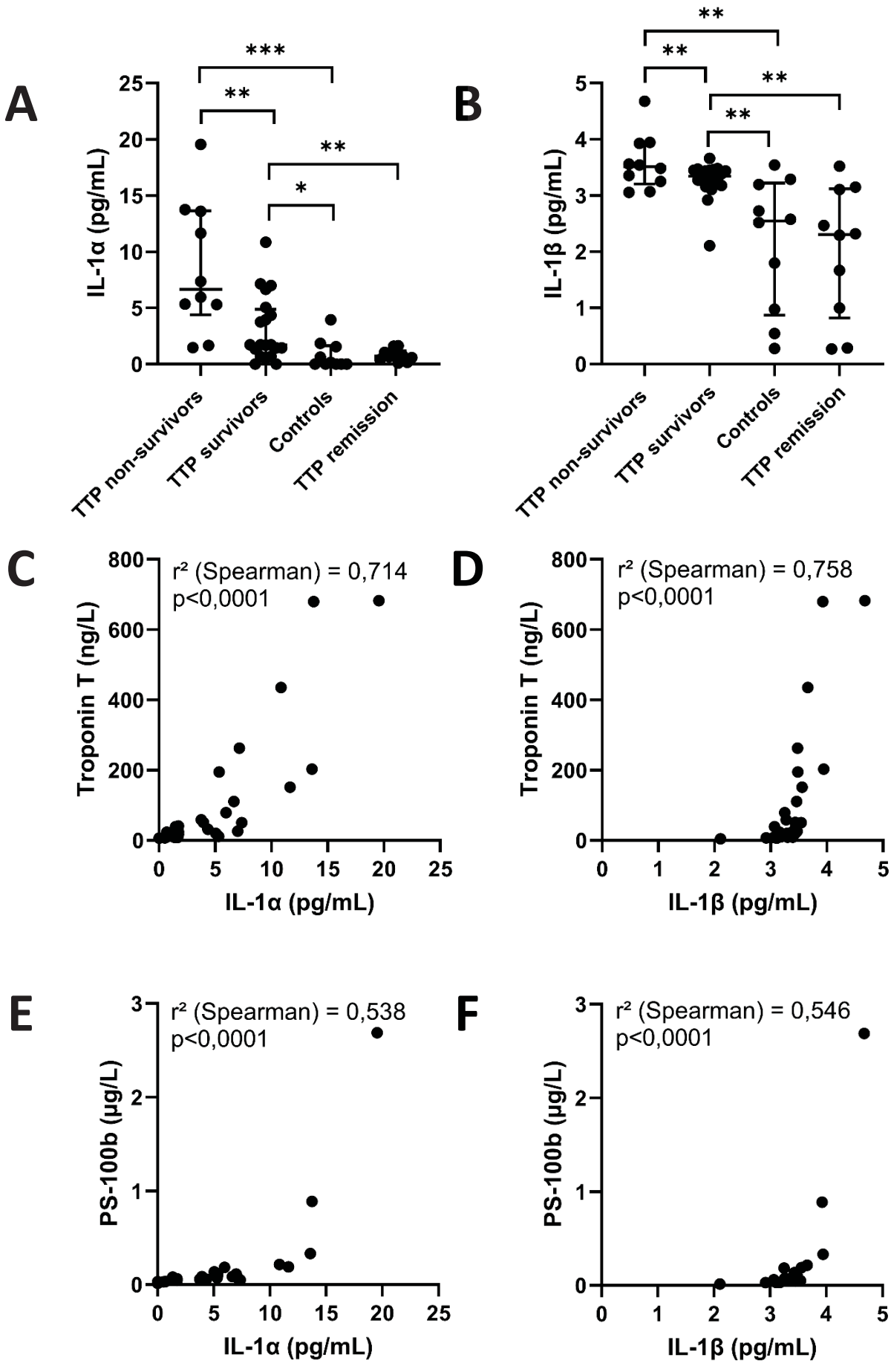


FIGURE 2

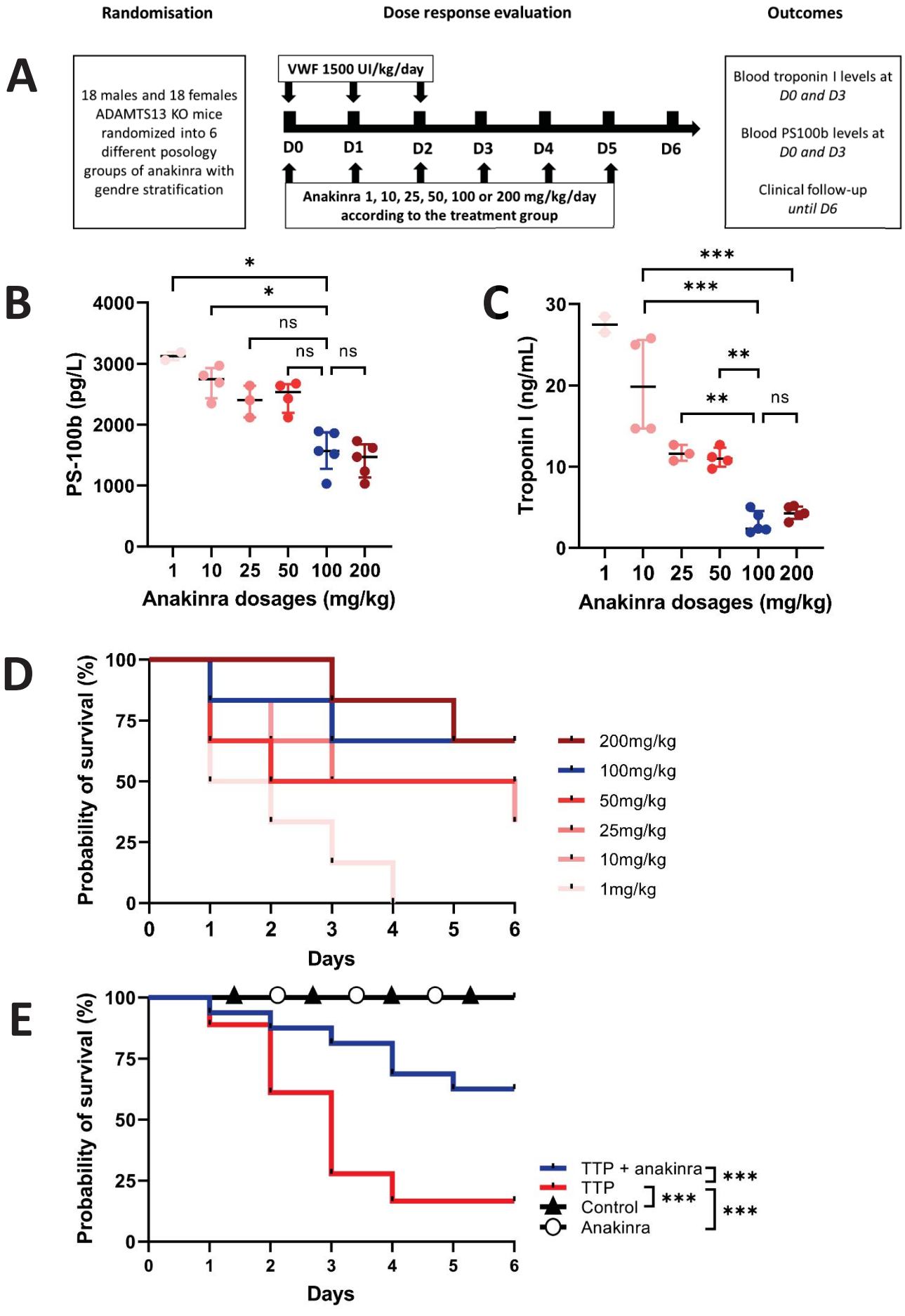


FIGURE 3

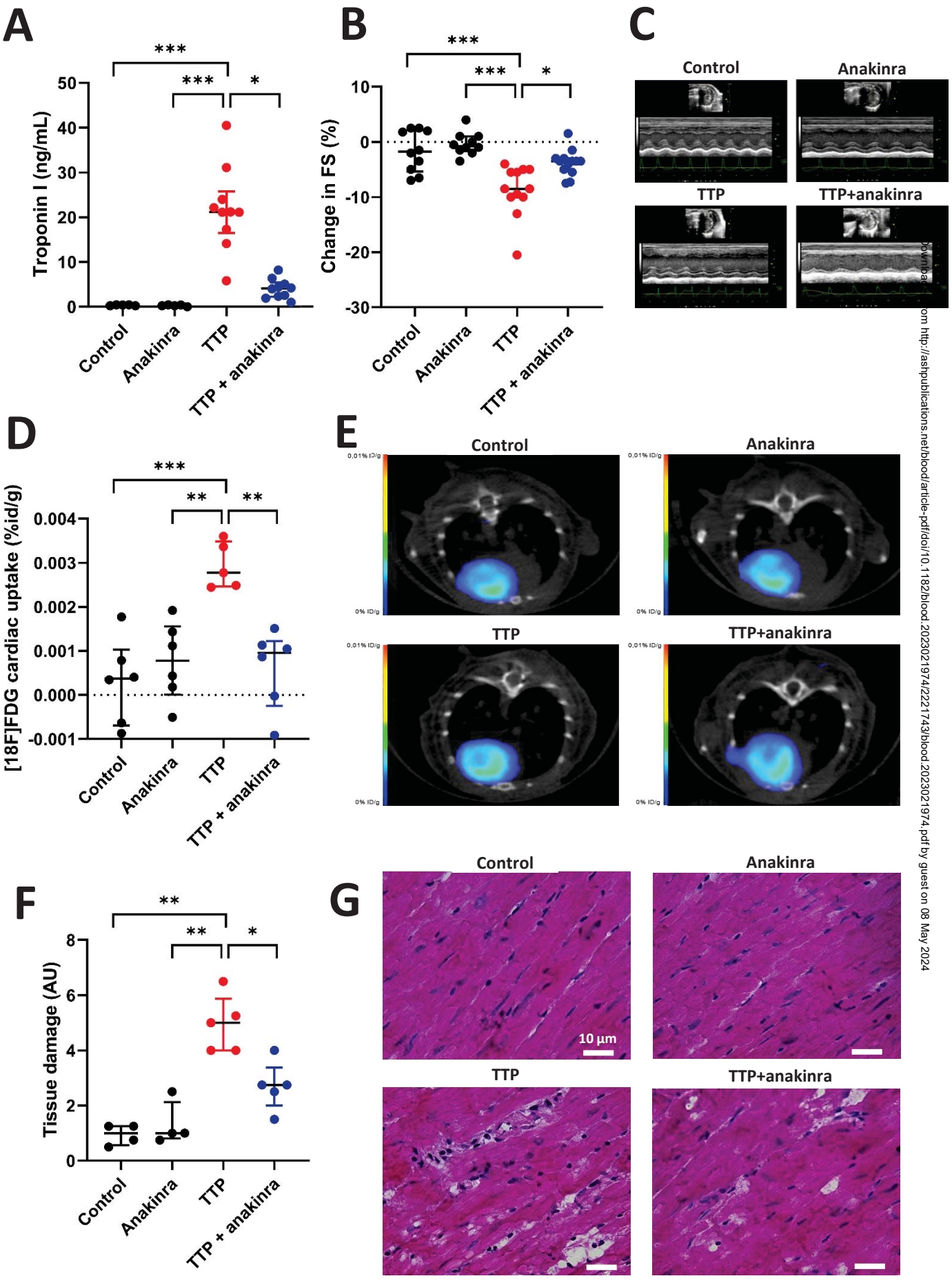


FIGURE 4

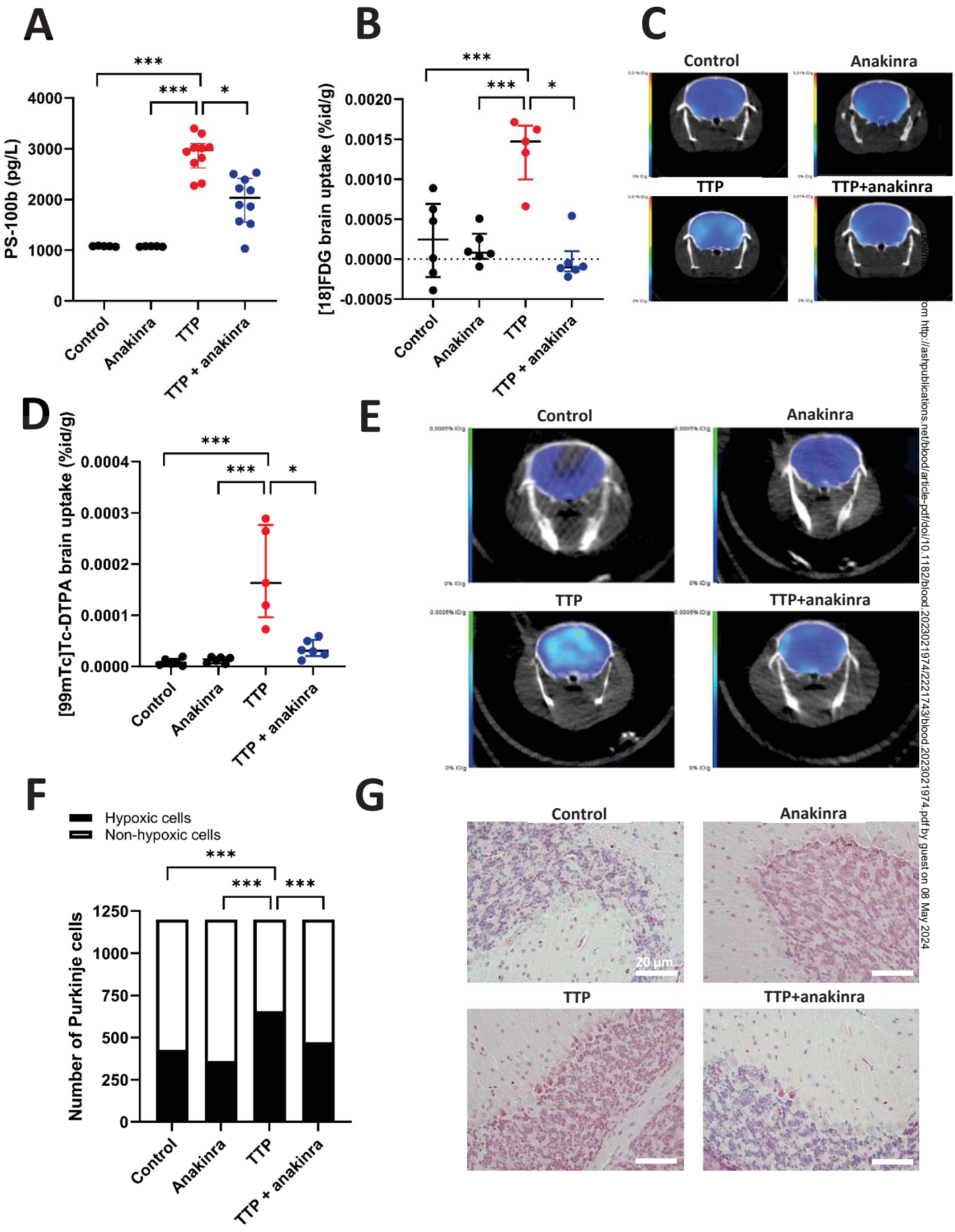


FIGURE 5

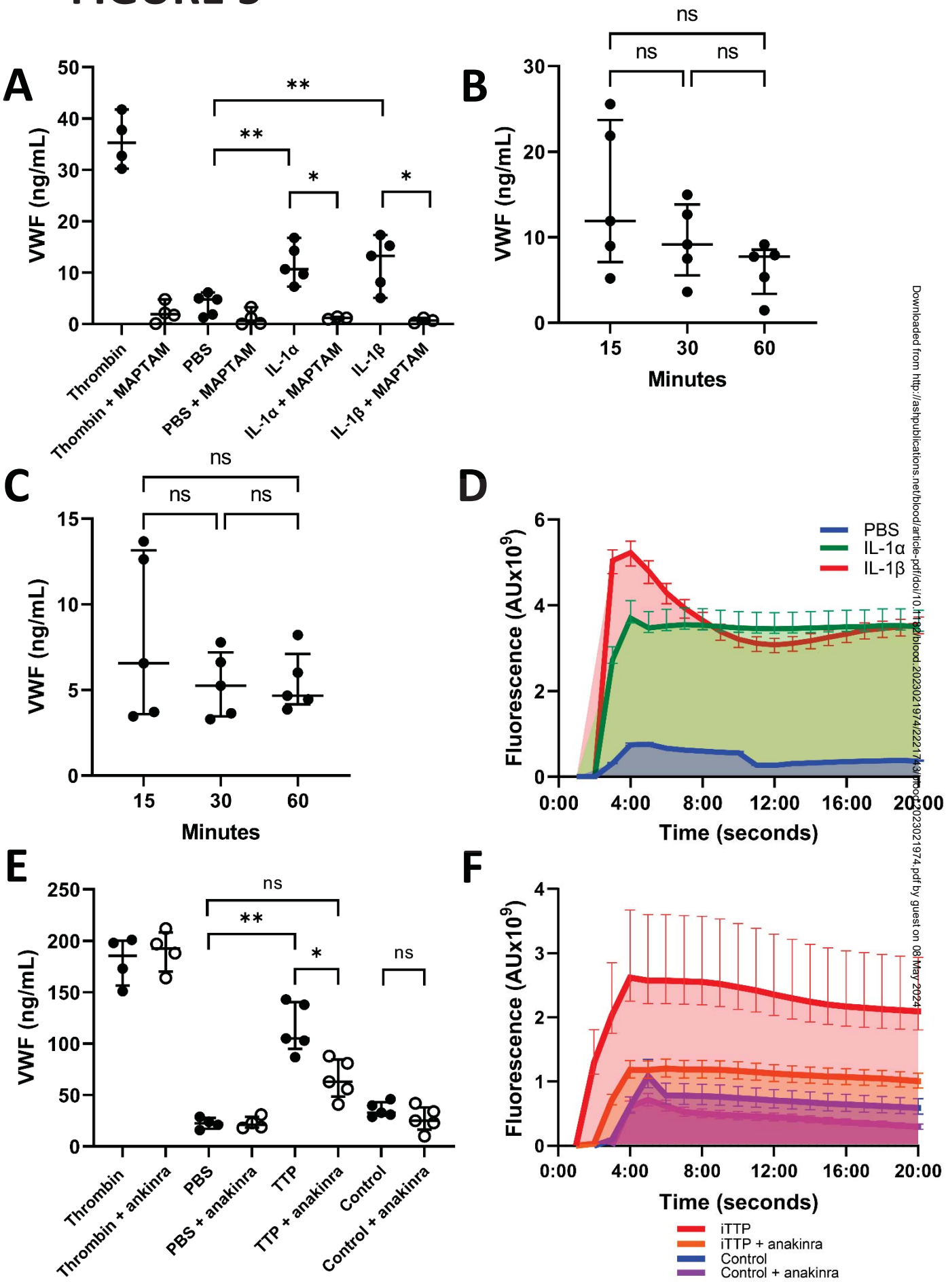
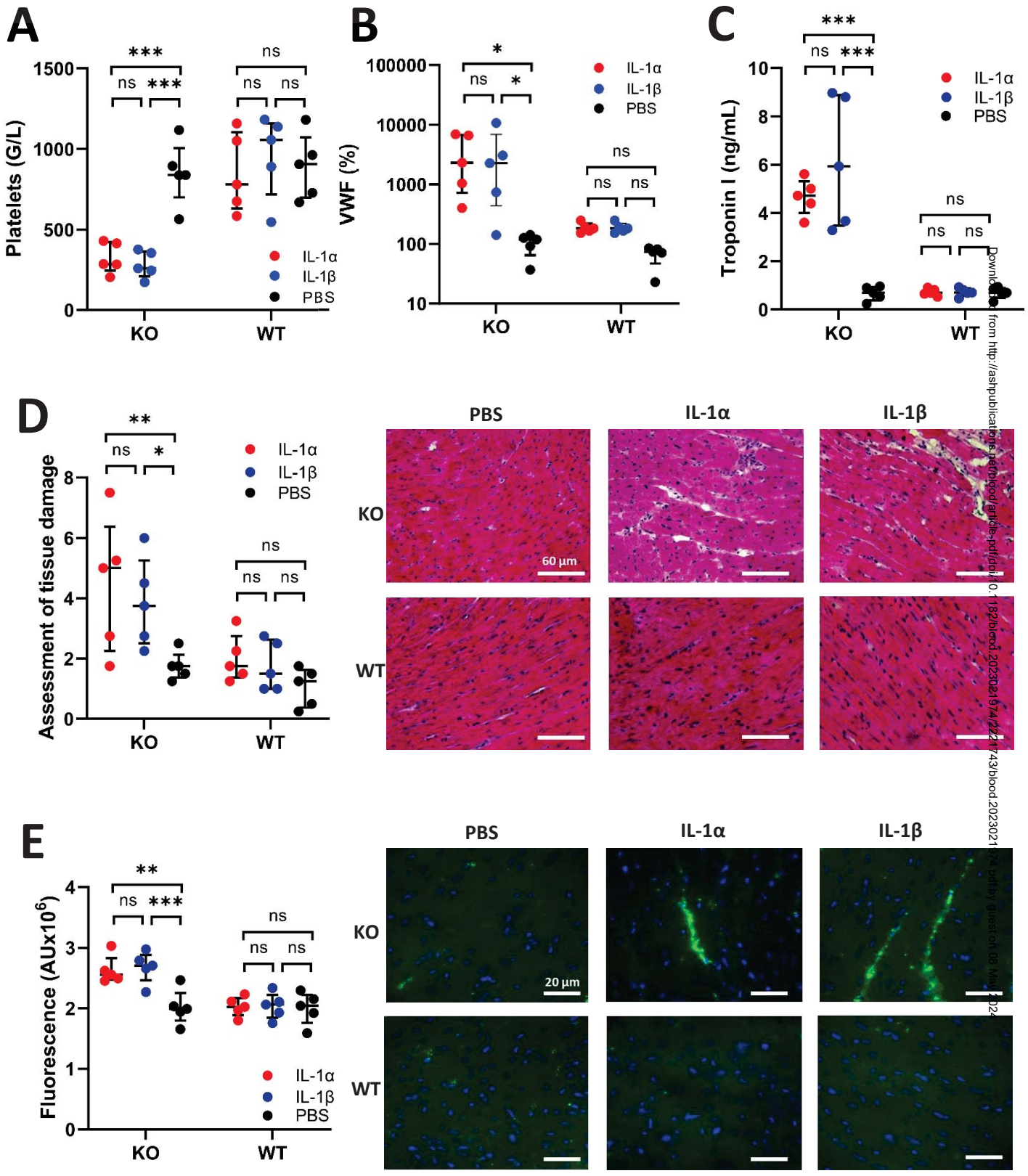
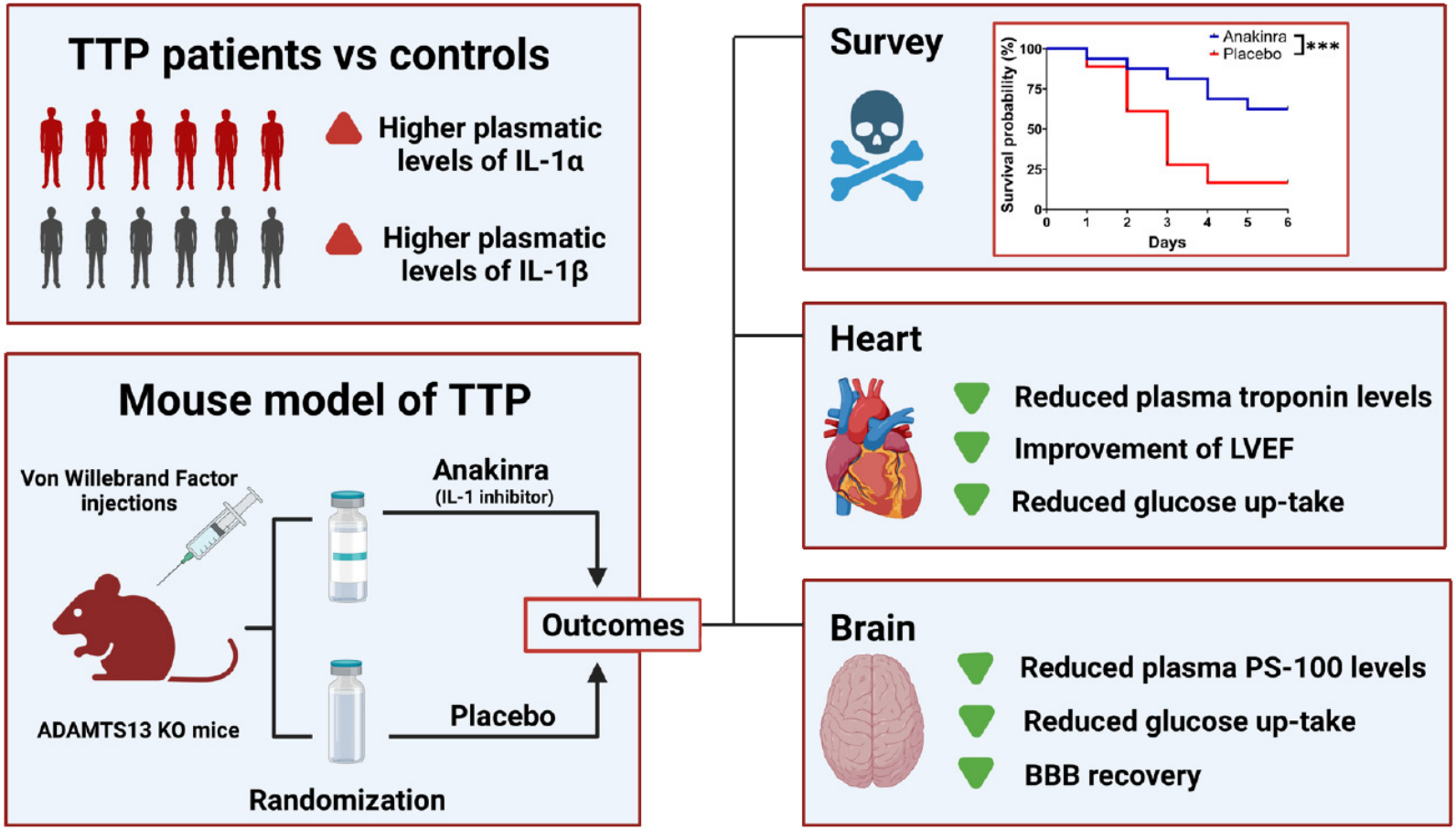


FIGURE 6



Effect of IL-1 Inhibition With Anakinra in a Mouse Model of Thrombotic Thrombocytopenic Purpura (TTP)



TTP: Thrombotic thrombocytopenic purpura; LVEF: Left Ventricular Ejection Fraction; PS-100: protein S 100; BBB: Blood-Brain Barrier

Conclusions: **In a mouse model of TTP, IL-1 inhibition with anakinra significantly reduced mortality as well as cerebral and cardiac injury.**

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***Blood* Visual Abstract**