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Higher lactate and purine metabolite levels in erythrocyte-rich fresh venous thrombus: Potential markers for early deep vein thrombosis

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ABSTRACT

Background: Thrombolytic therapy is effective in fresh deep vein thrombosis (DVT) although the benefit may fall below the risk of bleeding in non-fresh thrombosis. Markers reflecting fresh DVT have not been established. The present study aims to identify metabolites reflecting fresh venous thrombus and their role in thrombus formation.

Methods: Metabolites of rabbit venous blood and jugular venous thrombus 4 h after thrombus induction were analysed using electrophoresis-time of flight mass spectrometry. The effects of the altered metabolites on blood coagulation and platelet aggregation were assessed by using rotation thromboelastometry and platelet aggregometer. Cellular contents and glucose transporter (Glut)-1 expression in aspirated human DVT samples were pathologically analysed.

Results: Metabolome analysis identified 226 metabolites (133 cationic and 93 anionic metabolites). Largely altered 18 metabolites (thrombus/blood ratio: > 5 or < 0.5) included glycolytic metabolites, redox-related metabolites, purine nucleotides and tryptophan metabolites. Among the metabolites with > 5-fold increase, lactic acid was most abundant and guanine modestly enhanced whole blood clotting with thromboelastometry. Lactic acid and adenosine monophosphate inhibited collagen-induced platelet aggregation. Human DVTs were rich in erythrocytes expressing Glut-1. The erythrocyte content and Glut-1 expression were negatively correlated with the time after onset of DVT.

Conclusions: Glycolysis-, purine-, and redox-related metabolites may reflect fresh erythrocyte-rich venous thrombus, and altered metabolites may affect venous thrombus formation. An increased level of lactate may reflect active glycolysis of thrombus cellular components, predominantly erythrocytes.

1. Introduction

Venous thromboembolism (VTE) is comprised of deep venous thrombosis (DVT) and pulmonary embolism (PE). Estimated incidence rates of VTE range from 1 to 2 per 1000 person-years, and the 1-year fatality rates after DVT alone or DVT with cancer are 23% or 47%, respectively [1]. The incidence of VTE has remained stable over the past decade, despite an increase in risk factor prevalence and implementation of thromboprophylaxis [2]. Up to half of patients with proximal DVT develop post-thrombotic syndrome despite optimal anticoagulant therapy [3]. Additional treatment with catheter-directed thrombolysis reduced the development of post-thrombotic syndrome at 2- and 5-year follow-up compared to standard anticoagulation therapy; however, 8% of patients suffered from major or clinically relevant bleeds [4,5]. Therefore, it is important to develop laboratory or imaging tools that can predict the responses to thrombolysis therapy.

We previously reported that magnetic resonance imaging can visualize the early phase of DVT as high or isosignal intensity on T2 or T1 weighted images, respectively, in a rabbit jugular vein thrombosis model. The findings from T2 or T1 weighted images indicate the effectiveness of thrombolysis therapy [6]. Additionally, erythrocytes, fibrin, and platelet contents in DVT time-dependently decreased with cell lytic changes and were replaced with fibrous tissue with endothelium, myofibroblasts, and macrophages [6]. Human venous thrombus mainly

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consists of erythrocytes, fibrin, platelets, and leukocytes [7]. Animal studies have also shown that leukocytes and platelets cooperate to initiate and propagate venous thrombus formation [8]. Thus, venous thrombus formation is a multicellular dynamic process involving erythrocytes, platelets, and leukocytes, and the early phase of DVT involves all types of blood cells.

Previous studies showed that cellular metabolism is altered in aggregated platelets and diseased or hypoxic erythrocytes. *In vitro* analysis showed that both platelet nucleotide release and mitochondrial respiration depend on adenosine triphosphate supplied either from glycolysis or oxidative phosphorylation [9]. A recent metabolomics analysis of sickle cell erythrocytes detected accumulation of glycolytic intermediates, ascorbate metabolism intermediates, and alteration of glutathione pathway metabolites [10]. The allostery-dependent haemoglobin interaction with Band III in erythrocytes accelerated the consumption of upstream glycolytic metabolites such as glucose-6 phosphate and increased downstream metabolites such as phosphoenolpyruvate under hypoxic conditions [11]. The results suggest that cellular metabolism in the thrombus differs from that in the blood. Clinical studies revealed reduced acylcarnitine levels in the plasma of patients with VTE, and plasma metabolites were associated with a high risk of PE [12,13]. A mouse model of DVT revealed alterations in carnitine, sphingolipid, lipid, and adenosine metabolism in the serum and vein wall [14]. However, comprehensive metabolic changes in the venous thrombus *in vivo* remain unknown.

We analysed the metabolic alterations in rabbit venous blood and venous thrombus using capillary electrophoresis-time of flight mass spectrometry (CE-TOFMS) to identify metabolic differences between these samples and investigated the cellular components and glucose transporter 1 (Glut1) expression, as well as their changes over time in aspirated human DVT.

2. Materials and methods

Full description of this section is available in the Supplemental file.

2.1. Blood sampling and venous thrombosis model in rabbit jugular veins

The Animal Care Committee of the University of Miyazaki approved the study protocols (No. 2010-511). Blood samples were collected from the right jugular veins before thrombus formation. After blood correction, thrombi were induced in the right jugular veins by endothelial denudation and vessel ligation (blood stasis) [15].

2.2. Metabolite extraction and metabolome analysis

Metabolite extraction and metabolome analysis were conducted at Human Metabolome Technologies, Inc. (HMT) (Tsuruoka, Yamagata, Japan). Metabolome analysis was conducted by Basic Scan package of HMT using CE-TOFMS based on the methods described previously [16,17]. Briefly, CE-TOFMS analysis was carried out using an Agilent CE capillary electrophoresis system equipped with an Agilent 6210 time-of-flight mass spectrometer, Agilent 1100 isocratic HPLC pump, Agilent G1603A CE-MS adapter kit, and Agilent G1607A CE-ESI-MS sprayer kit (Agilent Technologies, Waldbronn, Germany). The spectrometer was scanned from m/z 50 to 1000 [17]. Peaks were extracted using MasterHands, automatic integration software (Keio University Tsuruoka town campus, Tsuruoka, Yamagata, Japan) in order to obtain peak information including m/z, peak area, and migration time (MT) [18]. Hierarchical cluster analysis and principal component analysis were performed by HMT's proprietary software, PeakStat and SampleStat, respectively. Detected metabolites were plotted on metabolic pathway maps using VANTED software [19].

2.3. Histological analysis of rabbit jugular vein thrombus

For histological analysis of cellular components, the removed thrombi from the jugular veins were fixed and embedded in paraffin. Areas of eosin positive erythrocytes, integrin αIIbβ3 immunopositive platelets (Affinity Biologicals, Inc., Ancaster, CA, USA), and haematoxylin-positive nuclear cells (leukocytes) were analysed using a colour imaging morphometry system (WinROOF, Mitani, Fukui, Japan) [20].

2.4. Thromboelastogram assay of rabbit whole blood

Effects of adenosine monophosphate (AMP), guanine, hypoxanthine, lactic acid (Sigma-Aldrich, St. Louis, MO, USA) on whole blood coagulation were measured using a ROTEM analyser (Pentapharm GmbH, Munich, Germany) [21].

2.5. Measurement of rabbit platelet aggregation

Effects of AMP, guanine, hypoxanthine, lactic acid (Sigma-Aldrich) on collagen-induced platelet aggregation were measured using an aggregometer (Aggregation Analyser PA-20, Kowa, Nagoya, Japan).

2.6. Histological analysis of aspirated thrombi from patients with DVT

The Ethics Committees at the participating institution approved the study protocol (Approval No. 427). Fifteen thrombi were obtained from 15 patients with DVT. The immunopositive areas for glucose transporter-1 (Glut-1, Acris, Herford, Germany), erythrocyte glycophorin A (DAKO), lymphocytes (CD45, DAKO), monocyte/macrophage CD68 (DAKO), neutrophil CD66b (BioLegend, San Diego, CA, USA), and platelet integrin αIIbβ3 (Affinity Biologicals, Inc.) were analysed using a colour imaging morphometry system (WinROOF, Mitani) [20].

2.7. Statistical analysis

All data are presented as the means and standard deviation. Differences between or among groups were tested using unpaired *t*-test or the one-way analysis of variance with Bonferroni's multiple comparison test (GraphPad Prism 5, GraphPad Software, Inc., San Diego, CA, USA). Relationships between factors were evaluated using Spearman's test and $P < 0.05$ was considered statistically significant.

3. Results

3.1. Cellular components in venous blood and thrombus in rabbit jugular vein

Cell count of the rabbit venous blood collected from jugular vein was calculated with an automated haematology analyser. The number of erythrocytes, platelets, and leukocytes and haematocrit value in the rabbit venous blood were $586.6 \times 10^4 \pm 33.5 \times 10^4$/μL, $43.9 \times 10^4 \pm 5.5 \times 10^4$/μL, 5600 ± 1200/μL, and 39.0 ± 1.8%, respectively. Cellular components of the rabbit venous thrombus were analysed using HE stain or immunohistochemistry. Percentages of erythrocyte-, platelet-, and leukocyte-areas in the venous thrombus were 64.1 ± 6.7%, 15.8 ± 5.7%, and 0.5 ± 0.5%, respectively.

3.2. Metabolic differences in rabbit venous blood and thrombus

To evaluate comprehensive metabolic status in the venous blood and thrombus, we performed metabolomic analysis with CE-TOFMS in the jugular venous blood and venous thrombus 4 h after endothelial denudation and blood stasis. Metabolomics analysis identified 226 metabolites (133 cationic and 93 anionic metabolites). Fig. 1A shows the principal component analysis results of the rabbit venous blood and thrombus. Metabolites in the venous blood and thrombus were

distinctly separated along with the first principal components (PC1) axis. Metabolites with highest absolute factor loading values in PC1 were taurine, ethanolamine phosphate, glycerol 3-phosphate, cysteic acid, uric acid, allantoin, fructose 6-phosphate, glutamine, and tryptophan, which are mainly involved in amino acid metabolism (Supplementary Table 1). Fig. 1B and Supplementary Table 2 show the hierarchical clustering analysis results of metabolites in the rabbit venous blood and thrombus. The hierarchical tree indicates the relative distance among the metabolite peaks. The red or green densities indicate high or low concentrations of metabolites, respectively. Principal component analysis and hierarchical clustering analysis revealed a remarkable metabolic difference between the venous blood and thrombus.

Fig. 2 shows thrombus to blood ratio of metabolites which showed significantly > 5-fold increase (12 metabolites) or less than half (6 metabolites) in the venous thrombus compared with the venous blood (Supplementary Table 3). The altered metabolites belonged to purine metabolism [hypoxanthine, guanine, AMP, guanine monophosphate (GMP)], tryptophan metabolites (serotonin, 3-hydroxykynurenine, tryptophan), redox reaction-related metabolite (glutathione, nicotinamide adenine dinucleotide phosphate^{+}, methionine sulfoxide, cysteine glutathione disulphide), central carbon metabolites (lactic acid, citric acid, glucose 6-phosphate), a lipid membrane component (choline, ethanolamine phosphate), taurine, and hypotaurine.

3.3. Quantification of altered metabolites

Supplementary Table 4 shows quantified levels of the 108 metabolites. To compare metabolite levels between the rabbit venous blood and venous thrombus, a volume of 100 μL rabbit blood was considered as 100 mg. Abundant metabolites in the venous thrombus were lactic acid, glycine, glutamate, cysteine glutathione disulphide, glutamine, and lysine, in descending order. Abundant metabolites in the venous blood were lactic acid, glycine, glutamate, cysteine glutathione disulphide, 3-hydroxybutyric acid, and lysine, in descending order. The levels of lactic acid, AMP, choline, hypoxanthine, guanine, glutathione, and GMP were significantly increased by at least 5-fold in the venous thrombus compared to in the venous blood. In contrast, the levels of citric acid, glucose 6-phosphate, nicotinamide adenine dinucleotide phosphate^{+}, tryptophan, and fructose 6-phosphate were significantly decreased to less than one-half in the venous thrombus compared to in the venous blood (Fig. 3).

3.4. Metabolites on whole blood coagulation and platelet aggregation

Because the levels of lactic acid and purine metabolites significantly increased in the venous thrombus compared to in the venous blood, we evaluated effect of the metabolites on blood coagulation and platelet aggregation. Fig. 4A shows clotting parameters assessed by thromboelastometry. Lactic acid and guanine modestly shortened clotting time, and lactic acid increased the alpha-angle. Hypoxanthine and AMP did not affect whole blood coagulation. Fig. 4B shows the inhibitory effect on collagen-induced platelet aggregation by AMP or lactic acid.

3.5. Cellular contents and Glut-1 expression in human aspirated DVT

Because lactic acid was an abundant metabolite in the thrombus and can affect thrombus formation or stabilization, we examined the cellular content, glucose transporter expression and its content in human DVTs which were aspirated 5 to 60 days after onset. Fig. 5A shows the histological contents of erythrocytes, platelets, neutrophils, lymphocytes, and monocytes/macrophages in the aspirated DVT. Among them, the erythrocyte content was most abundant in the thrombus and leukocytes accounted for < 10% of the thrombus. Fig. 5B and C show microphotographs of the aspirated thrombus 5 days after onset. The thrombus was rich-in erythrocytes and Glut-1 closely localized to the

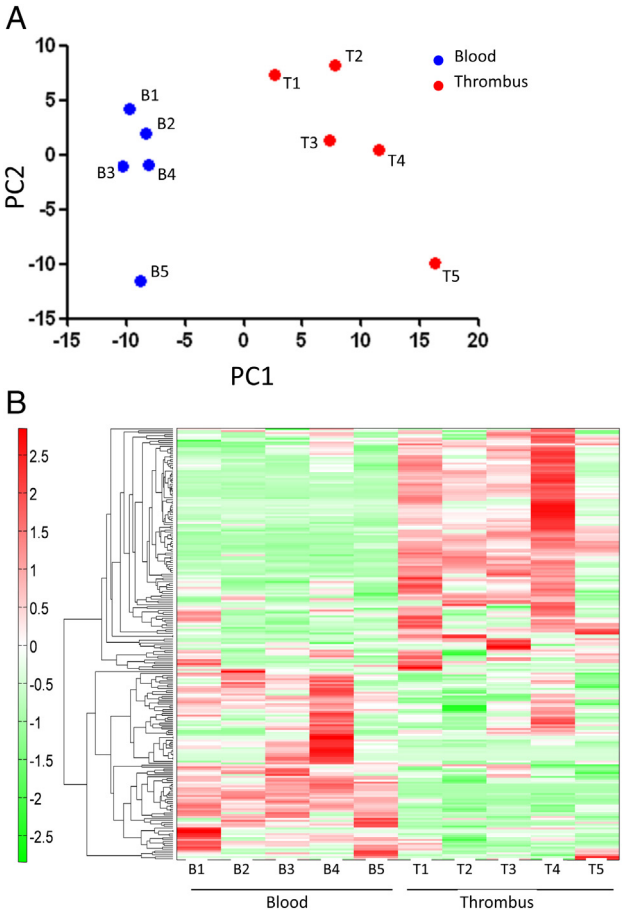

Fig. 1. Metabolomic analysis of venous blood and thrombus metabolites in rabbits.
A. Principal component analysis discriminates metabolic features in rabbit venous blood (B1–5, blue) and jugular vein thrombus (T1–5, red). Supplementary Table 1 shows factor-loading values for PC1 and PC2 on score plots.
B. Representative heatmap assessed using hierarchical clustering analysis shows metabolic differences between groups. Supplementary Table 2 shows original data for each metabolite. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

erythrocytes but not to the platelets. Neutrophils were more frequent than macrophage/monocytes in this case at 5 days after onset. The immunopositive areas for erythrocytes ($r = -0.73$, $p < 0.01$, $n = 15$) and Glut-1 ($r = -0.55$, $p < 0.05$, $n = 15$) negatively correlated with the time after onset. The immunopositive areas for monocytes/macrophages ($r = 0.58$, $p < 0.05$, $n = 15$) and CD45 ($r = -0.54$, $p < 0.05$, $n = 15$) positively correlated with the time after the onset. The immunopositive area for platelets ($r = 0.35$, $p = 0.20$, $n = 15$) or neutrophils ($r = 0.13$, $p = 0.64$, $n = 15$) was not correlated with the time after onset.

4. Discussion

We showed that glycolysis, purine, tryptophan, and redox-related metabolites in the fresh venous thrombus differed from those in the venous blood from rabbits. Lactic acid, guanine, or AMP can affect blood coagulation or platelet aggregation *in vitro*. Additionally, Glut-1 expression on erythrocytes was negatively correlated with the time after onset of human DVT.

The difference between blood and thrombus cellular and serum components may partly affect the metabolic difference between them. To exclude the effect of concentration in the thrombus, we selected metabolites that were 5-fold greater or less than one-half in the venous

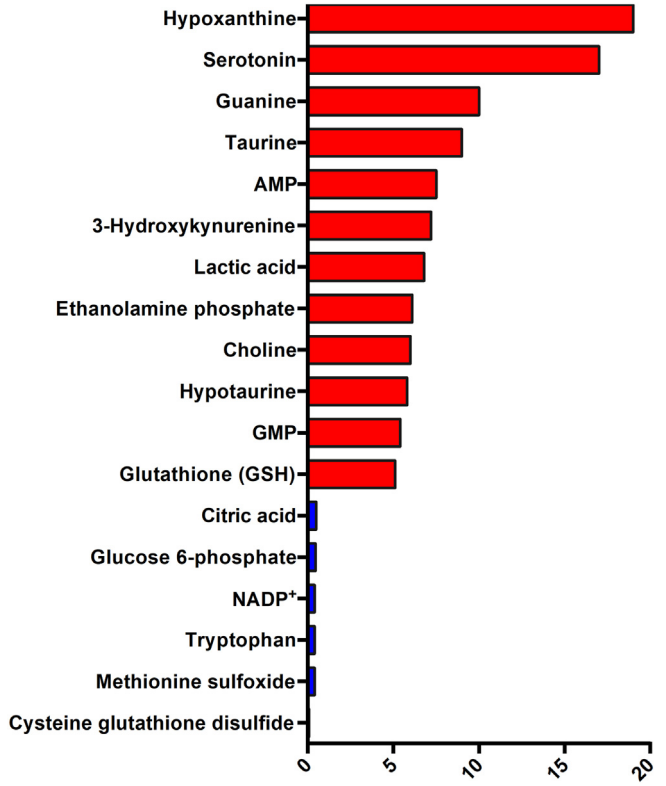

Fig. 2. Ratio of selected metabolite levels in jugular vein thrombus to levels in venous blood.
Relative levels of 12 metabolites or 6 metabolites are significantly higher than 5-fold or less than one-half in venous thrombus, compared to in venous blood. Supplementary Table 3 shows original data for each metabolite. AMP, adenosine monophosphate; GMP, guanine monophosphate, $NADP^+$, nicotinamide adenine dinucleotide phosphate.

thrombus compared to in the whole blood. Although cellular metabolism is not always similar among blood cells, the cellular contents in the thrombus suggest that thrombus metabolites are derived from erythrocytes, platelets, and leukocytes in descending order. Deguchi et al. [12] compared plasma metabolites between patients with VTE and control subjects and identified low plasma levels of acylcarnitines in patients with VTE. Zeleznik et al. [13] compared serum metabolites in patients with PE with different severities, and identified significant differences in the tricarboxylic acid cycle, fatty acid, and purine metabolite pathway between low- and intermediate/high risk PE patients. Serum from DVT mice showed different concentrations of purine metabolites, tricyclic acid cycle intermediates, L-carnitine, and sphingolipid metabolites [14]. Alterations in purine metabolites in previous and our studies suggest that the serum metabolite change in patients with VTE partly reflect metabolic alterations in intrathrombus cells.

In erythrocyte metabolism, glucose consumption is higher in deoxygenated erythrocytes than in oxygenated erythrocytes although they lack a nucleus and mitochondria [22]. Band III, a major transmembrane protein of erythrocytes, has a greater affinity for deoxyhaemoglobin, than oxyhaemoglobin. The band III also shows high affinity for glycolytic enzymes, such as phosphofructokinase, aldolase, glyceraldehyde-3-phosphate dehydrogenase, and lactate dehydrogenase, resulting in inhibition of the glycolysis pathway [23]. Competition between deoxyhaemoglobin and glycolytic enzymes for the cytoplasmic domain of Band III is responsible for erythrocyte metabolic modulation under hypoxia conditions [24]. Although we did not measure the oxygen levels in the venous thrombus, occlusive thrombus formation may have limited the oxygen supply in intrathrombus cells. The increase in lactate levels and decrease in glycolytic metabolite levels may be related to enhanced glycolysis in intrathrombus erythrocytes.

Platelets were the second most abundant cellular components in the venous thrombus. Previous studies detected accelerated ATP production in glycolysis and oxidative phosphorylation in agonist-stimulated platelets [25,26]. Thrombin but not adenosine diphosphate (ADP) caused a shape change and secretion of α-granules, as well as enhanced glucose uptake and translocation of Glut-3 on the cell membrane [27]. Fukami et al. examined lactate production and O_2 consumption in resting and thrombin-stimulated platelets [26]. The results suggest that platelet central carbon metabolism contributes to platelet function, which is compatible with the increase in lactate levels and decrease in glycolytic metabolite levels in the venous thrombus.

Lactic acid was the most abundant metabolite in the venous blood and thrombus, and modestly enhanced whole blood coagulation and inhibited platelet aggregation. Crowell et al. [28] demonstrated that lactic acid infusion led to marked shortening of the whole blood clotting time *in vitro* and *in vivo*. Acidaemia induced by hydrochloric acid impaired whole blood coagulation measured by thromboelastography [29]. These results suggest that lactic acid rather than acidaemia enhanced whole blood coagulation. Foley et al. [30] examined platelet aggregation in response to ADP in umbilical-vein blood from term infants and reported that acidosis induced by lactic acid or carbon dioxide impaired platelet aggregation in these subjects. Our results are comparable to those of previous reports. The net effects of lactate on venous thrombus formation and stabilization require further examination. In addition to a metabolic marker of venous thrombus, lactate level is a prognostic predictor of patients with PE. Patients with lactate level > 2 mM showed higher mortality than patients with a lower level [31].

The levels of purine metabolites, AMP, hypoxanthine, and guanine were higher in the venous thrombus than in the blood. Adenosine was detectable only in the venous thrombus. Zeleznik et al. performed plasma metabolomic analysis of 92 PE patients and identified elevated levels of hypoxanthine and xanthosine in intermediate/high-risk PE groups compared to in the low-risk PE group [13]. A mouse DVT model showed a 2.2-fold increase in the adenosine level in the vein wall and 9.5-fold decrease in the adenosine level in the serum compared to the control vein wall and serum [14]. Previous studies and our results suggest alterations of purine metabolism in DVT. Uptake and release of purines by erythrocytes are sensitive to changes in pH, inorganic phosphate, and oxygen concentration. Enhanced uptake was observed with low pH, decreased oxygen concentration, and high organic phosphate level [32]. Hypoxic incubation of washed platelets increased hypoxanthine release into the medium [33]. In addition to the catabolism of purine nucleotides, an increased lactate level and reduced oxygen supply in the intrathrombus environment may enhance the accumulation of purine metabolites in the venous thrombus. Fuentes et al. [34] showed that AMP inhibits platelet adhesion and aggregation *via* the adenosine A2 receptor and increase in cyclic AMP level. The present results are comparable to those of the previous study. AMP accumulation in the venous thrombus may destabilize aggregated platelets after venous thrombus formation.

Interestingly, the levels of tryptophan metabolites, such as serotonin and kynurenine pathway metabolites, were higher in the venous thrombus than in the blood. In contrast, the tryptophan level decreased in the venous thrombus. Because serotonin is a dense granule present in platelets, its elevation in thrombus is likely. Kynureine pathway metabolites have the potential to modulate immune reactions and inflammation [35]. Indoleamin 2, 3-dioxygenase and kynurenine 3-monoxygenase are enzymes that catalyse tryptophan to kynurenine and kynurenine to 3-hydroxykynurenine, respectively. Indoleamin 2, 3-dioxygenase 1 expressed in coronary atherosclerotic plaque macrophages, can enhance thrombus formation *via* thrombogenic tissue factor expression in cytokine stimulated macrophages [36]. Among peripheral blood cells, monocytes expressed these kynurenine pathway enzymes, which were upregulated in monocytes stimulated with interferon-γ [37]. Intrathrombotic interferon-γ levels were progressively elevated in

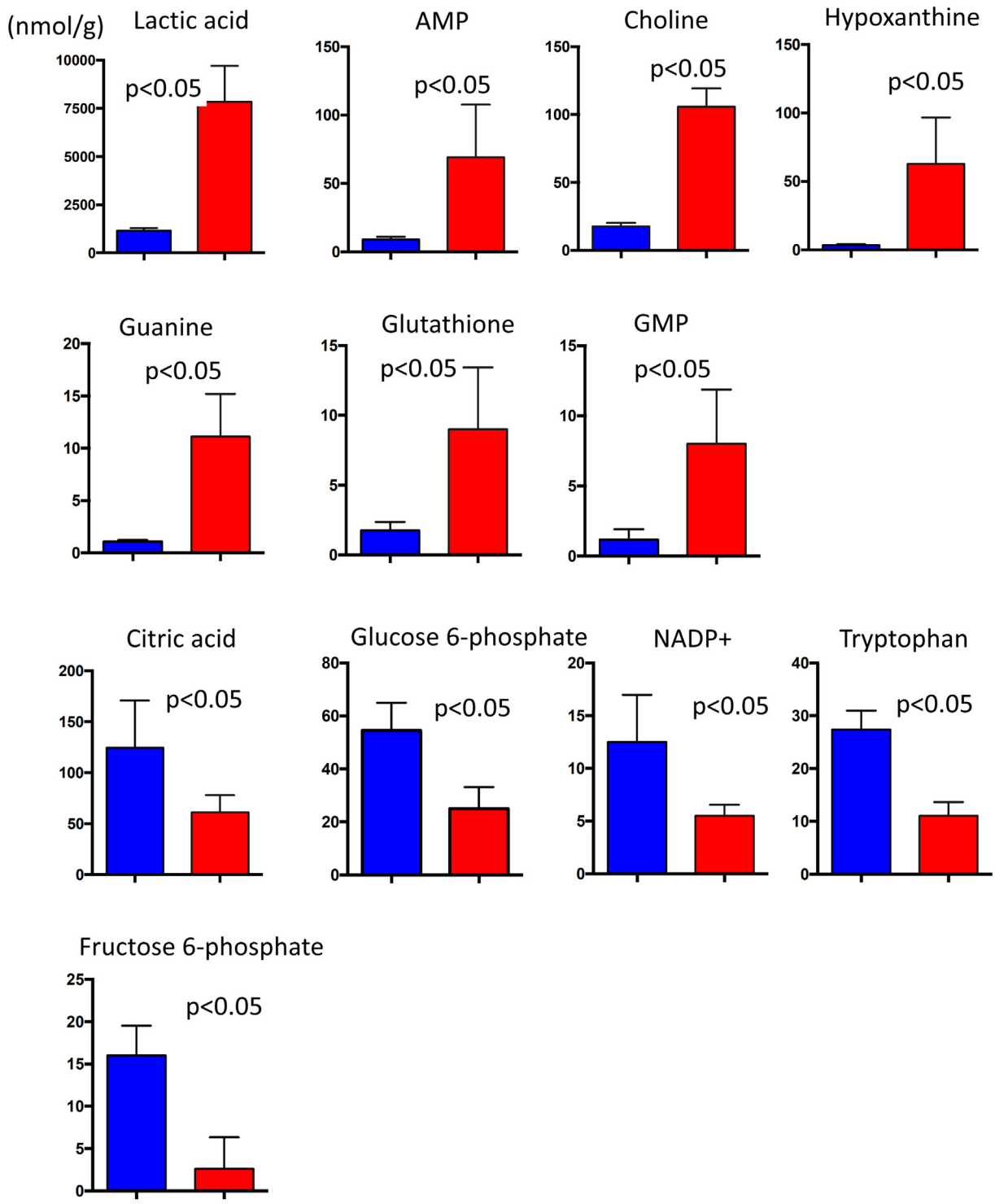

Fig. 3. Selected metabolite levels in jugular vein thrombus (red) and venous blood (blue).
Levels of 7 or 5 metabolites were significantly higher than 5-fold or less than one-half in venous thrombus, compared to in venous blood. Supplementary Table 4 shows original data for each metabolite. AMP, adenosine monophosphate; GMP, guanine monophosphate, $NADP^+$, nicotinamide adenine dinucleotide phosphate. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

ligation-induced DVT model mice [38]. *N*-(3,4-dimethoxycinnamoyl)-anthranilic acid, a derivative of a kynurenine pathway metabolite, reduced the haemorrhagic area and inflammation and increased CD163 macrophage contents after pulmonary radiofrequency ablation in rabbits [39]. CD163 is a scavenger receptor for the haemoglobin-haptoglobin complex, and CD163 macrophages show erythrocyte phagocytosis in human DVT and are closely localized along newly formed vessels [40]. Although the roles of kynurenine metabolites in the pathophysiology of DVT remain unknown, these metabolites may affect thrombus formation and its organizing reaction by modulating the thrombotic or reparative function of monocytes and macrophages.

Erythrocyte and Glut-1 expression was time-dependently decreased in the aspirated human DVT. A few clinical studies reported the use of ^{18}F-gluorodeoxyglucose (FDG)-positron emission tomography for

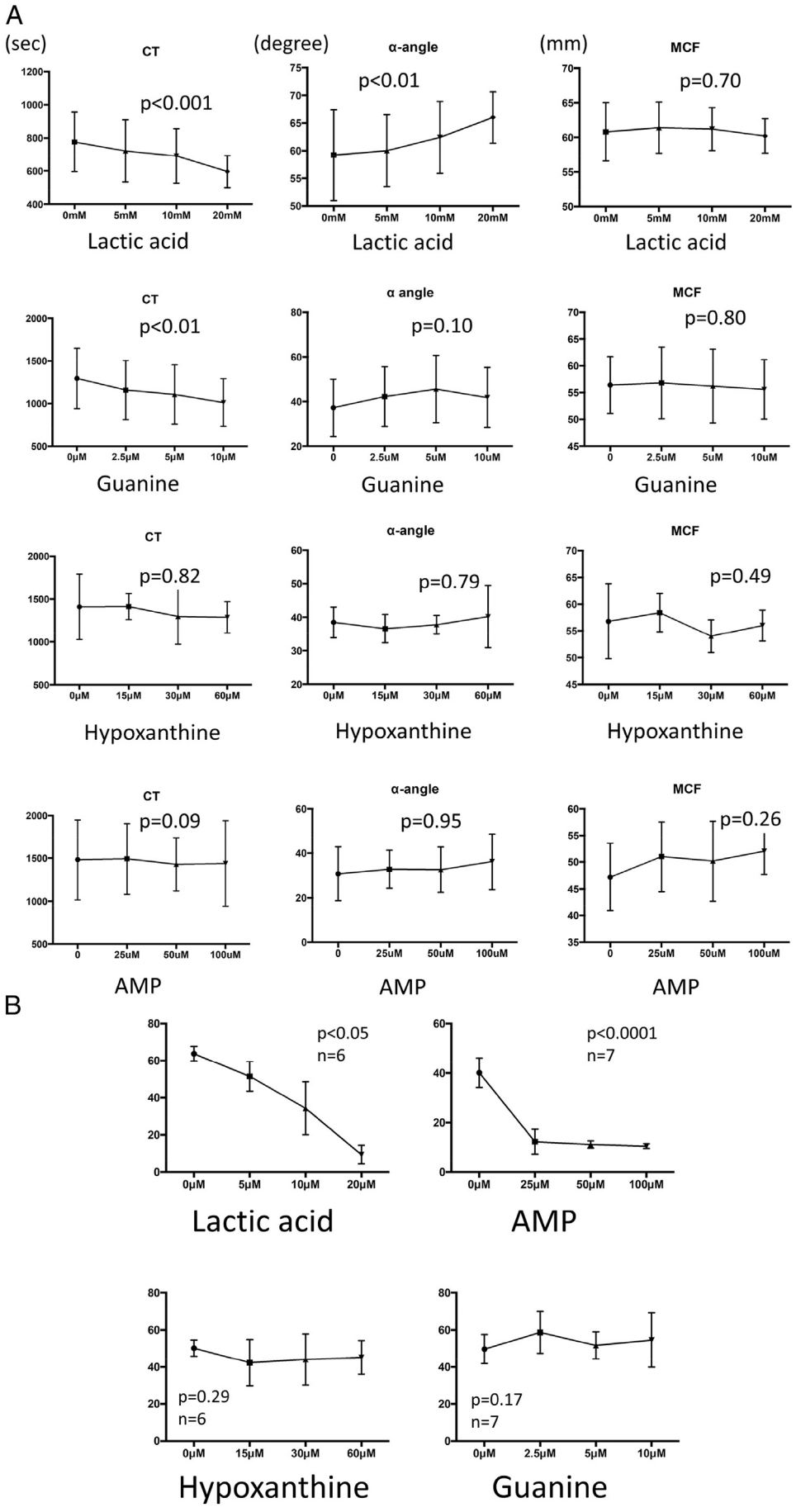

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Fig. 4. Effect of metabolites on whole blood coagulation and platelet aggregation.
A. Effect of lactate, guanine, hypoxanthine, and adenosine monophosphate on $CaCl_2$-induced whole blood coagulation. CT, clotting time; MCF, maximum clot firmness (n = 5 in each, one-way ANOVA).
B. Effect of lactate, guanine, hypoxanthine, and adenosine monophosphate on collagen induced platelet aggregation (one-way ANOVA).

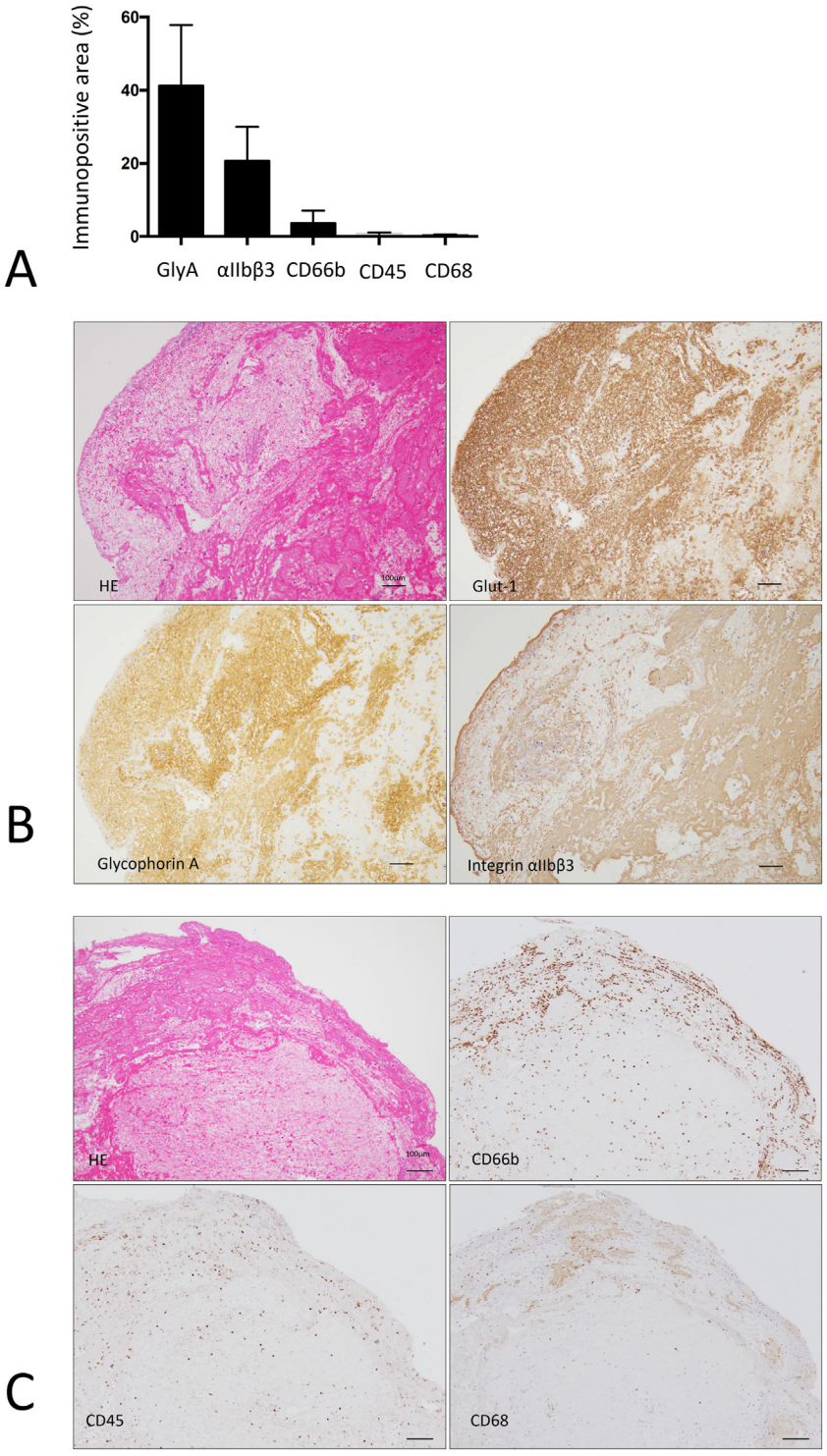

Fig. 5. Cellular contents and histological images of human deep venous thrombus.
A. Immunopositive areas for glycophorin A (GlyA, erythrocyte), integrin αIIbβ3 (platelet), CD66b (neutrophil), CD45 (lymphocytes), and CD68 (monocyte/macrophage) in human venous thrombus (n = 15).
B and C. Representative histological and immuohistochemical images of aspirated DVT 5 days after onset. Glut-1 immunopositive area is closely localized in glycophorin A immunopositive area, but not in integrin αIIbβ3 (B). CD66-immunopositive neutrophils are more than CD45-immunopositive lymphocytes or CD68-positive macrophage/monocytes (C).

diagnosing of DVT. FDG uptake in thrombosed veins was significantly greater than that in non-thrombosed veins in patients with DVT and time-dependently decreased within DVTs [41,42]. In a stasis-induced mouse DVT model, the neutrophil but not the macrophage content in the thrombi was correlated with FDG uptake in the thrombi [42]. In this study, neutrophils accounted < 10% of the human DVT. The Glut-1 expression suggests that erythrocytes play an important role in glucose uptake in human DVTs. Our results support that FDG uptake reflects the acuity of DVT onset.

There were several limitations to this study. First, we did not assess metabolic changes over the time in the rabbit model of venous thrombus. Because the venous thrombus is gradually replaced with endothelial cells, macrophages, myofibroblast/smooth muscle cells, and extracellular matrix [6], the metabolite levels may differ from those in the fresh thrombus in the jugular vein. Our study suggested that some metabolites in the thrombus affect whole blood coagulation and platelet function. Further studies are required to reveal the exact mechanisms and *in vivo* significance. Second, effect of therapeutic anticoagulants could affect the compositions of human DVT.

5. Conclusion

Increased levels of lactate, purine metabolite, and redox-related metabolites may reflect the fresh venous thrombus, and some altered metabolites may affect venous thrombus stabilization. An increased level of lactate may reflect active glycolysis of thrombus component cells, predominantly erythrocytes.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.thromres.2019.03.011.

Contributions

K. Maekawa analysed data and prepared the manuscript; A. Yamashita designed the study and contributed to collecting and analysing data; C. Sugita, S. Moriguchi-Goto, E. Furukoji, T. Sakae, and T. Gi participated in collecting data. T. Hirai co-chaired the study. Y. Asada chaired the study and oversaw the writing of the manuscript.

Disclosure of conflict of interests

The authors state that they have no conflict of interest.

Acknowledgements and disclosure

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References

[1] V. Tagalakis, V. Patenaude, S.R. Kahn, S. Suissa, Incidence of and mortality from venous thromboembolism in a real-world population: the Q-VTE Study Cohort, Am. J. Med. 126 (2013) (832.e13-21).
[2] L.J.J. Scheres, W.M. Lijfering, S.C. Cannegieter, Current and future burden of venous thrombosis: not simply predictable, Res. Pract. Thromb. Haemost. 2 (2018) 199–208.
[3] M.J. Baldwin, H.M. Moore, N. Rudarakanchana, M. Gohel, A.H. Davies, Post-thrombotic syndrome: a clinical review, J. Thromb. Haemost. 11 (2013) 795–805.
[4] T. Enden, Y. Haig, N.E. Kløw, C.E. Slagsvold, L. Sandvik, W. Ghanima, G. Hafsahl, P.A. Holme, L.O. Holmen, A.M. Njaastad, G. Sandbæk, P.M. Sandset, CaVenT Study Group, Long-term outcome after additional catheter-directed thrombolysis versus standard treatment for acute iliofemoral deep vein thrombosis (the CaVenT study): a randomised controlled trial, Lancet 379 (2012) 31–38.
[5] Y. Haig, T. Enden, O. Grøtta, N.E. Kløw, C.E. Slagsvold, W. Ghanima, L. Sandvik, G. Hafsahl, P.A. Holme, L.O. Holmen, A.M. Njaaastad, G. Sandbæk, P.M. Sandset, CaVenT Study Group, Post-thrombotic syndrome after catheter-directed thrombolysis for deep vein thrombosis (CaVenT): 5-year follow-up results of an open-label, randomised controlled trial, Lancet Haematol. 3 (2016) e64–e71.
[6] Y. Kuroiwa, A. Yamashita, T. Miyati, E. Furukoji, M. Takahashi, T. Azuma, H. Sugimura, T. Asanuma, S. Tamura, K. Kawai, Y. Asada, MR signal change in venous thrombus relates organizing process and thrombolytic response in rabbit, Magn. Reson. Imaging 29 (2011) 975–984.
[7] M. Takahashi, A. Yamashita, S. Moriguchi-Goto, K. Marutsuka, Y. Sato, H. Yamamoto, C. Koshimoto, Y. Asada, Critical role of von Willebrand factor and platelet interaction in venous thromboembolism, Histol. Histopathol. 24 (2009) 1391–1398.
[8] M.L. von Brühl, K. Stark, A. Steinhart, S. Chandraratne, I. Konrad, M. Lorenz, A. Khandoga, A. Tirniceriu, R. Coletti, M. Köllnberger, R.A. Byrne, I. Laitinen, A. Walch, A. Brill, S. Pfeiler, D. Manukyan, S. Braun, P. Lange, J. Riegger, J. Ware, A. Eckart, S. Haidari, M. Rudelius, C. Schulz, K. Echtler, V. Brinkmann, M. Schwaiger, K.T. Preissner, D.D. Wagner, N. Mackman, B. Engelmann, S. Massberg, Monocytes, neutrophils, and platelets cooperate to initiate and propagate venous thrombosis in mice in vivo, J. Exp. Med. 209 (2012) 819–835.
[9] E.H. Mürer, Release reaction and energy metabolism in blood platelets with special reference to the burst in oxygen uptake, Biochim. Biophys. Acta 162 (1968) 320–326.
[10] D. Darghouth, B. Koehl, G. Madalinski, J.F. Heilier, P. Bovee, Y. Xu, M.F. Olivier, P. Bartolucci, M. Benkerrou, S. Pissard, Y. Colin, F. Galacteros, G. Bosman, C. Junot, P.H. Roméo, Pathophysiology of sickle cell disease is mirrored by the red blood cell metabolome, Blood 117 (2011) e57–e66.
[11] A. Kinoshita, K. Tsukada, T. Soga, T. Hishiki, Y. Ueno, Y. Nakayama, M. Tomita, M. Suematsu, Roles of hemoglobin Allostery in hypoxia-induced metabolic alterations in erythrocytes: simulation and its verification by metabolome analysis, J. Biol. Chem. 282 (2007) 10731–10741.
[12] H. Deguchi, Y. Banerjee, S. Trauger, G. Siuzdak, E. Kalisiak, J.A. Fernández, L. Hoang, M. Tran, S. Yegneswaran, D.J. Elias, J.H. Griffin, Acylcarnitines are anticoagulants that inhibit factor Xa and are reduced in venous thrombosis, based on metabolomics data, Blood 126 (2015) 1595–1600.
[13] O.A. Zeleznik, E.M. Poole, S. Lindstrom, P. Kraft, A. Van Hylckama Vlieg, J.A. Lasky-Su, L.B. Harrington, K. Hagan, J. Kim, B.A. Parry, N. Giordano, C. Kabrhel, Metabolomic analysis of 92 pulmonary embolism patients from a nested case-control study identifies metabolites associated with adverse clinical outcomes, J. Thromb. Haemost. 16 (2018) 500–507.
[14] Y. Sung, K. Spagou, M. Kafeza, M. Kyriakides, B. Dharmarajah, J. Shalhoub, J.A. Diaz, T.W. Wakefield, E. Holmes, A.H. Davies, Deep vein thrombosis exhibits characteristic serum and vein wall metabolic phenotypes in the inferior vena cava ligation mouse model, Eur. J. Vasc. Endovasc. Surg. 55 (2018) 703–713.
[15] M. Takahashi, A. Yamashita, S. Moriguchi-Goto, C. Sugita, T. Matsumoto, S. Matsuda, Y. Sato, T. Kitazawa, K. Hattori, M. Shima, Y. Asada, Inhibition of factor XI reduces thrombus formation in rabbit jugular vein under endothelial denudation and/or blood stasis, Thromb. Res. 125 (2010) 464–470.
[16] Y. Ohashi, A. Hirayama, T. Ishikawa, S. Nakamura, K. Shimizu, Y. Ueno, M. Tomita, T. Soga, Depiction of metabolome changes in histidine-starved Escherichia coli by CE-TOFMS, Mol. BioSyst. 4 (2008) 135–147.
[17] T. Ooga, H. Sato, A. Nagashima, K. Sasaki, M. Tomita, T. Soga, Y. Ohashi, Metabolomic anatomy of an animal model revealing homeostatic imbalances in dyslipidaemia, Mol. BioSyst. 7 (2011) 1217–1223.
[18] M. Sugimoto, D.T. Wong, A. Hirayama, T. Soga, M. Tomita, Capillary electrophoresis mass spectrometry-based saliva metabolomics identified oral, breast and pancreatic cancer-specific profiles, Metabolomics 6 (2010) 78–95.
[19] B.H. Junker, C. Klukas, F. Schreiber, VANTED: a system for advanced data analysis and visualization in the context of biological networks, BMC Bioinf. 7 (2006) 109.
[20] A. Yamashita, S. Matsuda, T. Matsumoto, S. Moriguchi-Goto, M. Takahashi, C. Sugita, T. Sumi, T. Imamura, M. Shima, K. Kitamura, Y. Asada, Thrombin generation by intimal tissue factor contributes to thrombus formation on macrophage-rich neointima but not normal intima of hyperlipidemic rabbits, Atherosclerosis 206 (2009) 418–426.
[21] C. Sugita, A. Yamashita, Y. Matsuura, T. Iwakiri, N. Okuyama, S. Matsuda, T. Matsumoto, O. Inoue, A. Harada, T. Kitazawa, K. Hattori, M. Shima, Y. Asada, Elevated plasma factor VIII enhances venous thrombus formation in rabbits: contribution of factor XI, von Willebrand factor and tissue factor, Thromb. Haemost. 110 (2013) 62–75.
[22] J.R. Murphy, Erythrocyte metabolism. II. Glucose metabolism and pathways, J. Lab. Clin. Med. 55 (1960) 286–302.
[23] P.S. Low, Structure and function of the cytoplasmic domain of band 3: center of erythrocyte membrane-peripheral protein interactions, Biochim. Biophys. Acta 864 (1986) 145–167.
[24] I. Messana, M. Orlando, L. Cassiano, L. Pennacchietti, C. Zuppi, M. Castagnola, B. Giardina, Human erythrocyte metabolism is modulated by the O2-linked transition of hemoglobin, FEBS Lett. 390 (1996) 25–28.
[25] E.H. Mürer, A.J. Hellem, M.C. Rozenberg, Energy metabolism and platelet function, Scand. J. Clin. Lab. Invest. 19 (1967) 280–282.
[26] M.H. Fukami, H. Holmsen, L. Salganicoff, Adenine nucleotide metabolism of blood platelets. IX. Time course of secretion and changes in energy metabolism in thrombin-treated platelets, Biochim. Biophys. Acta 444 (1976) 633–643.
[27] H.F. Heijnen, V. Oorschot, J.J. Sixma, J.W. Slot, D.E. James, Thrombin stimulates glucose transport in human platelets via the translocation of the glucose transporter GLUT-3 from alpha-granules to the cell surface, J. Cell Biol. 138 (1997) 323–330.
[28] J.W. Crowell, B. Houston, Effect of acidity on blood coagulation, Am. J. Phys. 201 (1961) 379–382.

[29] M. Engström, U. Schött, B. Romner, P. Reinstrup, Acidosis impairs the coagulation: a thromboelastographic study, J. Trauma 61 (2006) 624–628.
[30] M.E. Foley, G.P. McNicol, An in-vitro study of acidosis, platelet function, and perinatal cerebral intraventricular haemorrhage, Lancet 1 (1977) 1230–1232.
[31] S. Vanni, G. Viviani, M. Baioni, G. Pepe, P. Nazerian, F. Socci, M. Bartolucci, M. Bartolini, S. Grifoni, Prognostic value of plasma lactate levels among patients with acute pulmonary embolism: the thrombo-embolism lactate outcome study, Ann. Emerg. Med. 61 (2013) 330–338.
[32] P.A. Berman, D.A. Black, L. Human, E.H. Harley, Oxypurine cycle in human erythrocytes regulated by pH, inorganic phosphate, and oxygen, J. Clin. Invest. 82 (1988) 980–986.
[33] M. Akahori, Y. Uedono, K. Yamagami, N. Takeyama, Y. Kitazawa, T. Tanaka, Hypoxia alters the energy metabolism and aggregation of washed human platelets, Haematologia (Budap) 26 (1995) 191–198.
[34] E. Fuentes, L. Badimon, J. Caballero, T. Padró, G. Vilahur, M. Alarcón, P. Pérez, I. Palomo, Protective mechanisms of adenosine 5′-monophosphate in platelet activation and thrombus formation, Thromb. Haemost. 111 (2014) 491–507.
[35] Q. Wang, D. Liu, P. Song, M.H. Zou, Tryptophan-kynurenine pathway is dysregulated in inflammation, and immune activation, Front. Biosci. (Landmark Ed.) 20 (2015) 1116–1143.
[36] Y. Watanabe, S. Koyama, A. Yamashita, Y. Matsuura, K. Nishihira, K. Kitamura, Y. Asada, Indoleamine 2,3-dioxygenase 1 in coronary atherosclerotic plaque enhances tissue factor expression in activated macrophages, Res. Pract. Thromb. Haemost. 2 (2018) 726–735.
[37] S.P. Jones, N.F. Franco, B. Varney, G. Sundaram, D.A. Brown, J. de Bie, C.K. Lim, G.J. Guillemin, B.J. Brew, Expression of the kynurenine pathway in human peripheral blood mononuclear cells: implications for inflammatory and neurodegenerative disease, PLoS One 10 (2015) e0131389.
[38] M. Nosaka, Y. Ishida, A. Kimura, Y. Kuninaka, M. Inui, N. Mukaida, T. Kondo, Absence of IFN-γ accelerates thrombus resolution through enhanced MMP-9 and VEGF expression in mice, J. Clin. Invest. 121 (2011) 2911–2920.
[39] H. Nakada, A. Yamashita, M. Kuroki, E. Furukoji, N. Uchino, T. Asanuma, Y. Asada, S. Tamura, A synthetic tryptophan metabolite reduces hemorrhagic area and inflammation after pulmonary radiofrequency ablation in rabbit nonneoplastic lungs, Jpn. J. Radiol. 32 (2014) 145–154.
[40] T. Gi, E. Furukoji, A. Yamashita, S. Moriguchi-Goto, M. Kojima, C. Sugita, T. Sakae, Y. Sato, T. Hirai, Y. Asada, CD163 macrophage and erythrocyte contents in aspirated deep vein thrombus are associated with the time after onset: a pilot study, Thromb. J. 14 (2016) 46.
[41] M.T. Rondina, U.T. Lam, R.C. Pendleton, L.W. Kraiss, N. Wanner, G.A. Zimmerman, J.M. Hoffman, C. Hanrahan, K. Boucher, P.E. Christian, R.I. Butterfield, K.A. Morton, (18)F-FDG PET in the evaluation of acuity of deep vein thrombosis, Clin. Nucl. Med. 37 (2012) 1139–1145.
[42] T. Hara, J. Truelove, A. Tawakol, G.R. Wojtkiewicz, W.J. Hucker, M.H. MacNabb, A.L. Brownell, K. Jokivarsi, C.W. Kessinger, M.R. Jaff, P.K. Henke, R. Weissleder, F.A. Jaffer, 18F-fluorodeoxyglucose positron emission tomography/computed tomography enables the detection of recurrent same-site deep vein thrombosis by illuminating recently formed, neutrophil-rich thrombus, Circulation 130 (2014) 1044–1052.