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BRIEF REPORT

Inferior vena cava branch variations in C57BL/6 mice have an impact on thrombus size in an IVC ligation (stasis) model

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Summary. *Background:* Animal models of venous thrombosis (VT) are critical tools for those investigating the VT mechanism. Recently, inferior vena cava (IVC) branches have been subject to debate, causing controversy in the field. *Objectives:* To understand how the variability of IVC branches, in commonly used C57BL/6 mice, have an impact on thrombus formation in the IVC ligation model. *Methods:* C57BL/6 male mice (n = 46), 20–25 g, were subjected to the IVC ligation model with various interruptions of the IVC branches. Control animals (n = 50) had all branches interrupted. Two days after IVC ligation, thrombus weight (TW), as a parameter of thrombus size, was assessed. *Results:* We found four different anatomical patterns. Side branches were more prevalent on the mouse's right side (34%) compared with the left (20%). In mice where side branches were absent (21%), back branches appeared larger. Also, 25% of mice had both side branches. Controls that had all IVC branches interrupted had the most consistent and largest TW (32.6 mg to 34.7 mg) while groups that had no back branches interrupted had the smallest TW (3.6–9.7 mg), a 4 to 9-fold decrease. All groups with open back branches had significantly smaller TW ($P < 0.05$) than controls. *Conclusions:* Variations in TW were observed based on different branch interruption patterns, compared with the fully ligated controls. Having two back branches was the most consistent anatomy and open back branches had the largest negative impact on thrombus size. This work confirms that the IVC branches significantly affect thrombus burden in C57BL/6 mice and further studies should be conducted in order to standardize this and other animal models of VT.

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Background

Animal models of venous thrombosis (VT) are key tools for those investigating the mechanism of VT because of the limitations involved when studying human subjects [1,2]. The initial description of the 'inferior vena cava (IVC) ligation (stasis) model' involved interruption of all IVC branches (side and back) to prevent blood flow re-entry into the area where the thrombi form, facilitating stasis [2,3]. Recently, the issue of interruption or no interruption of the IVC branches in mouse models of VT has been subject to debate, causing controversy in the field [4]. It is important to have consistent thrombus size in our animal controls and particularly critical in our experimental animals when comparing new drugs and known drugs, such as statins, for treatment of VT [5].

Objective

The objective of this study is to understand how the IVC branches in the commonly used mouse strain C57BL/6 impact thrombus formation in the IVC ligation model. Our hypothesis is that avoiding interruption of the branches will decrease thrombus size.

Methods

In order to test our hypothesis, we used C57BL/6 male mice (n = 96), 20–25 g, that were subjected to a laparotomy. The infra-renal IVC was then visualized and photographed and the IVC ligation model performed. We used the IVC ligation model involving the interruption of all side and back branches in the control groups (back branches were cauterized while side branches and the main IVC were ligated with 7-0 Prolene) [2,3]. Proximal back branches usually occur 1–2 mm below the left renal vein (LRV) while distal back branches are usually

5–6 mm below the LRV. Side branches, if present, are predominantly mid-IVC with a range of 2–4 mm below the LRV. Various interruption patterns were designed to test our hypothesis, and these groups are described in Fig. 1. Two days after IVC ligation, wet thrombus weight (TW), which includes the thrombus and associated IVC wall, as a parameter of thrombus size, was assessed in mg.

The IVC sample is taken just distal to the left renal vein down to the iliac bifurcation and the length is approximately 9–10 mm long in 20–25 g C57BL/6 mice. All sutures were removed from IVC samples before wet weight was determined. This is an acute time period when the IVC wall does not contain any significant collagen deposition or increased weight caused by VT, as seen in chronic VT vein wall samples. 'Wet IVC vein wall only' weights for these ages of animals range from 3 to 5 mg. All statistical analyses were completed using GraphPad Prism version 6.02 (San Diego, CA, USA). Differences between groups were determined by an unpaired *t*-test with Welch's correction. A one-way ANOVA analysis using the Tukey's multiple comparison test was performed for additional comparisons. A P value of ≤ 0.05 was considered significant. This research protocol was approved by the University's Committee on Use and Care of Animals (UCUCA).

Results and discussion

In this study, we documented four types of anatomical variations for the side branches (Fig. 1). Back branches were the most consistent vessels, with two back branches present in 98% of the mice. In the two animals out of 96 that did not have two back branches, one had one back branch and the other had none. Side branches were more prevalent on the mouse's right side (34%), compared with

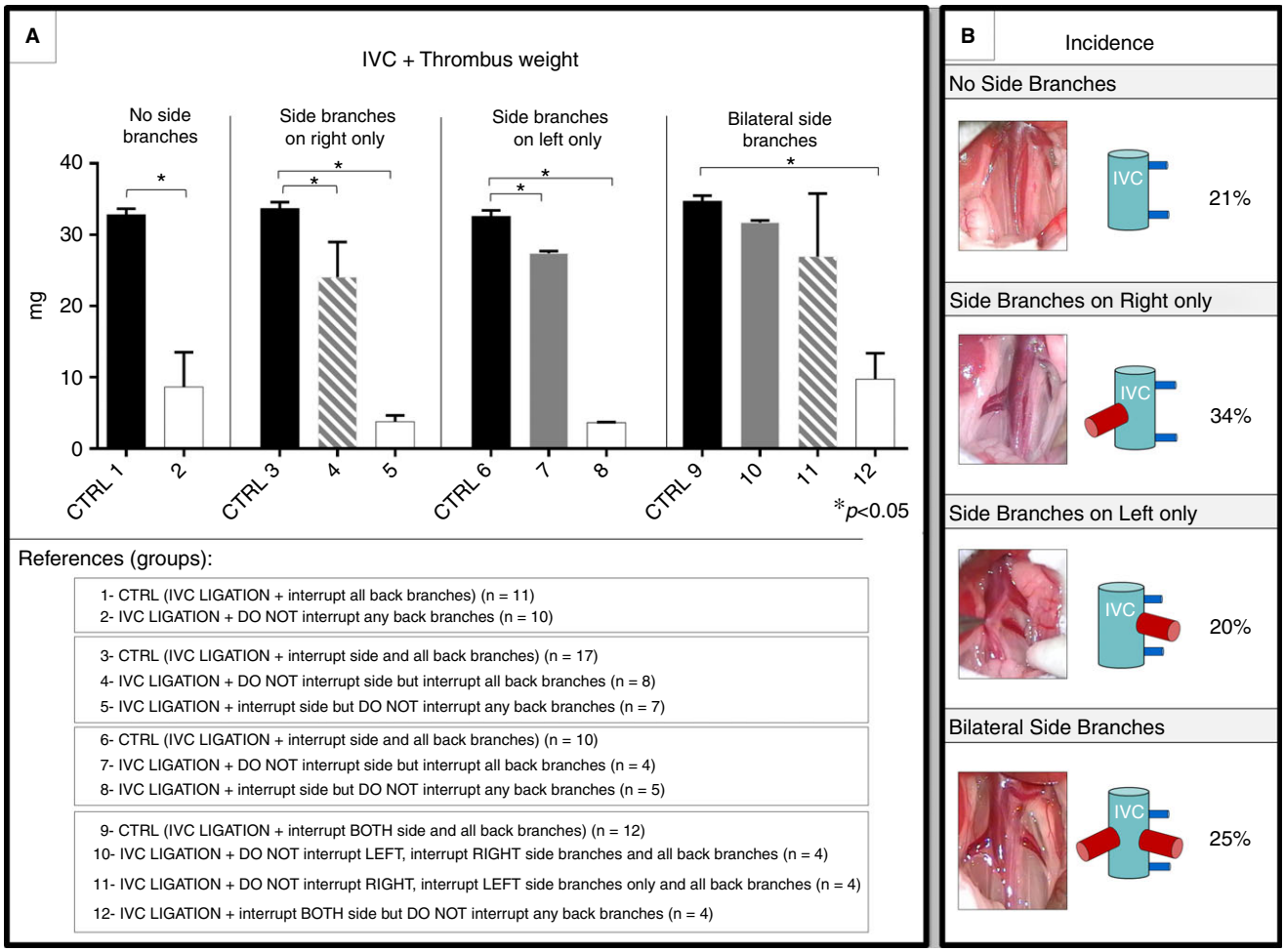

Fig. 1. Results. (A) IVC ± Thrombus weight. Bar graphs of the findings, grouped by presence of side branches, and significant *P*-value comparisons are indicated. All controls (represented by black) involved IVC and side branch ligations and back branches interrupted by cautery (1, 3, 6, 9). All animals with open back branches are in white and on the far right in each group (2, 5, 8, 12). All animals with open right side branches are in grey and white crosshatch (4, 11). All animals with open left side branches are in grey (7, 10). All weights are expressed in mg. Of note, none of the non-interrupted branch groups reached the thrombus size of the controls. Also, note that avoiding back branch interruptions (2, 5, 8, 12) creates the lowest thrombus weight in all groups. (B) Incidence of side branch patterns from a total of 96 mice and representative photographs and drawings of each group. Data were reported as mean ± standard error of the mean (SEM). IVC = inferior vena cava; mg = Milligrams; * = $P < 0.05$.

the left (20%). Also, 25% of the mice had both side branches and in mice where side branches were completely absent (21%), back branches appeared larger. Total interruption of all branches had the most consistent and largest TW (32.6 –34.7 mg) while groups that had no branches interrupted had the smallest TW (3.6–9.7 mg), a 4 to 9-fold decrease (Fig. 1). All groups with open back branches had significantly smaller TW ($P < 0.05$) than controls. Complete results for all groups are shown in Fig. 1.

C57BL/6 background mice are used extensively in research. This strain offers a variety of gene-deleted mice that help researchers understand diseases, including VT [6–8]. It is well known that in humans, and various animal species, the venous system is inconsistent, causing wide variability. By definition, the IVC is the 'great collector vein' and the blood from the lower limbs, pelvis and lower abdominal cavity, in most cases, enters the infra-renal IVC. However, the anatomy of the venous system varies between individuals, and mice are no exception (Fig. 2). Importantly for animal models of VT, this variation could represent a challenge for consistent thrombus burden. The infra-renal IVC is the area where thrombus formation is induced in all rodent IVC models of VT, including the IVC ligation (stasis) model (Fig. 3). Once the IVC is ligated, we observed that the thrombus grows caudally from the main IVC ligature up to the point of entry of the first branch that was not interrupted, including back branches, side branches or iliac veins (Fig. 3). In this current study, the IVC total ligation model, where every branch is interrupted, served as controls and, as importantly, all of the surgeries and harvests were done by the same surgeon so as to avoid technical variability. The thrombus size for this group was very consistent, with a low variability between animals, representing a trustable control (Fig. 1). Then, we alternately avoided the interruption of branches following a protocol as shown in (Fig. 1).

The results showed that not interrupting branches decreased thrombus size. When the side branches were not ligated, the thrombi were statistically smaller (Fig. 1), compared with controls. When the back branches were not interrupted, the thrombi were statistically the smallest (Fig. 1), versus controls, emphasizing the importance of cauterizing back branches. Thrombi size variability in a model can negatively affect data interpretation. Understanding the anatomy and how it influences data outcome is critical. An additional anatomical variation occurs between genders of the same species/strain. The ovarian veins drain either into the renal veins or the IVC, where the thrombus

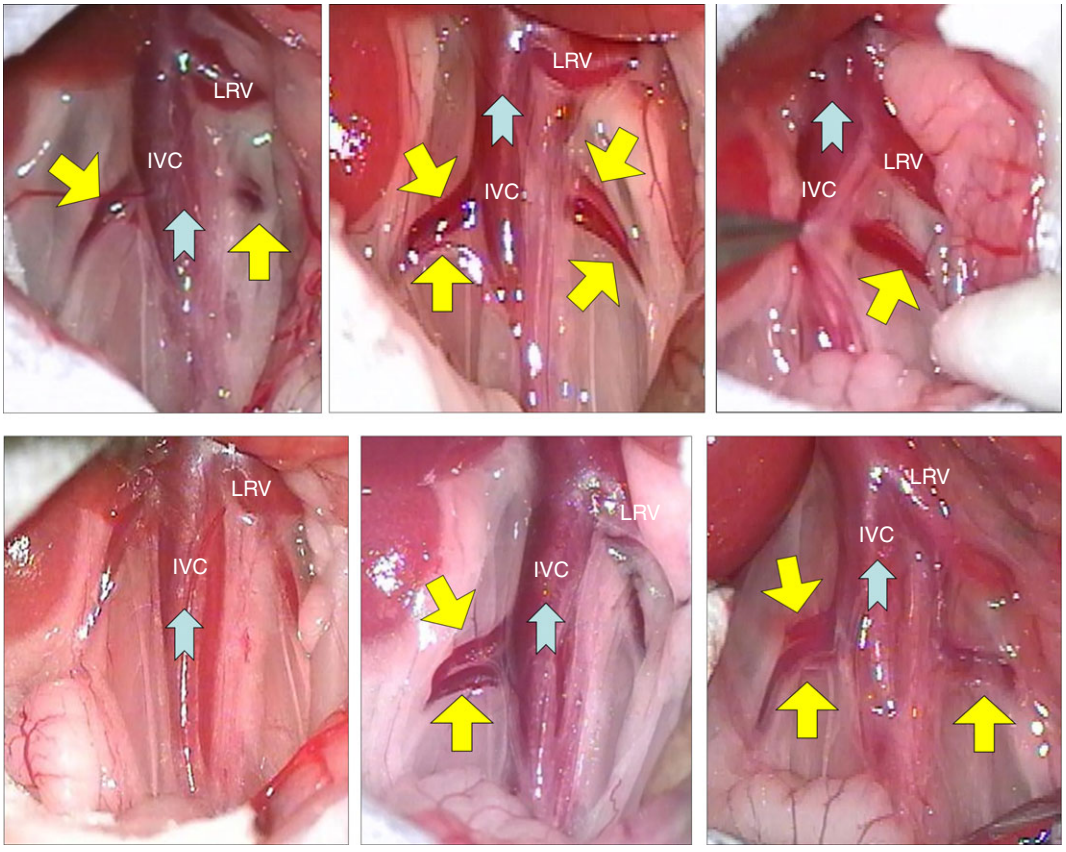

Fig. 2. Anatomical variations. Selected pictures showing side branch anatomical variations. Despite the fact that the animals were placed in four groups, variability in the number of branches per side increases the variability. IVC = inferior vena cava; LRV = left renal vein; blue arrows = blood flow; yellow arrows = side branches.

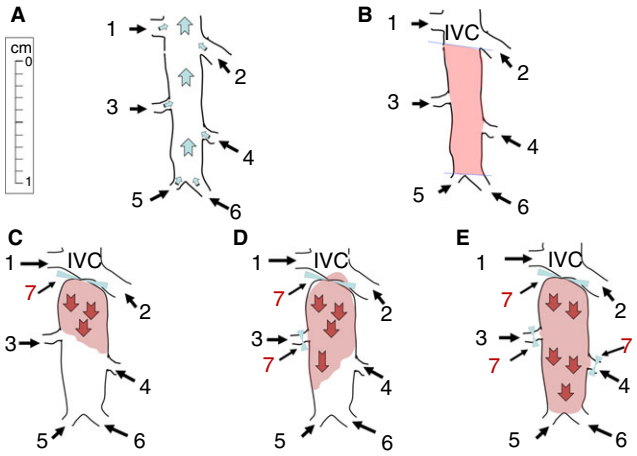

Fig. 3. Area and direction of thrombus formation in the ligation model. Schematic representation of the infra-renal inferior vena cava (IVC). (A) IVC with all open branches and its blood flow direction. (B) Area where the thrombus forms. (C) Thrombus forms from the ligature down to the first open side branch (branch identified with 3). (D) Thrombus forms from the ligature down to the first open side branch (branch identified with 4). (E) Thrombus consistently forms in the IVC from the ligature area, below the left renal vein to the iliac bifurcation, upon interruption of all side branches. A ruler was added to provide size comparison and all pictures were drawn to scale. IVC = inferior vena cava; 1 = right renal vein; 2 = left renal vein; 3 = side branch; 4 = side branch; 5 = right iliac vein; 6 = left iliac vein; 7 = ligature; blue arrows = blood flow; red arrows = thrombus growth direction; light blue lines in panels C, D and E indicate ligation points.

forms in this model. Ligation of the ovarian vein leads to organ necrosis and systemic inflammation that may negatively affect the venous thrombotic process that we are trying to investigate. Thus, the extra challenge that investigators face when using females is not only the hormonal differences, but additional branch variations as well.

This work emphasizes the importance of total stasis with respect to the mouse IVC ligation model. The IVC ligation model differs from the IVC stenosis model because in the latter the IVC is not totally occluded. From the surgical approach, these two models are very similar, although flow models tend to produce smaller thrombi than stasis models. The results of this study show that thrombus weight variability, due to avoiding branch ligations, is related to the venous system variations of the C57BL/6 mice (Fig. 2) rather than the particular model. Overall, we identified consistently four different patterns of side branches, and primarily two back branches. The influence of the IVC side branches on thrombus formation was recently studied in an IVC only ligation model in which no IVC branches were interrupted [4]. Modifications of the IVC branch scenarios presented in this work resulted in a wide range of thrombus weights. Thus, the result of this work clearly demonstrates that neglecting a single branch interruption, not just the side branches but particularly the back branches, has a negative impact on thrombus burden and introduces a cause of variability in thrombus weights (Fig. 1).

We agree that modifications of the original surgical technique may be necessary for individual protocols, but we have also demonstrated in this work that changing the ligation patterns of IVC branches introduces variability in thrombus sizes and may have an adverse effect on data interpretation. Whether or not these findings could be applied to other IVC models of VT, and mice of various backgrounds, remains to be seen. However, investigators recently identified that avoiding interruption of branches introduces variability in thrombus size in one of the variants of the mouse IVC stenosis model [9]. Finally, the authors would like to emphasize that if investigators change the original technique of the total ligation IVC stasis model, for any justifiable reason, this should be clearly stated in the material and methods section of the publication, to avoid introducing confusion into the field and increase the reproducibility of data among laboratories.

Addendum

Conception and design: J. Diaz, D. M. Farris, S. K. Wrobleski and T. W. Wakefield. Analysis and interpretation: J. Diaz, D. M. Farris, S. K. Wrobleski and T. W. Wakefield. Data collection: D. M. Farris. Writing the article: J. Diaz, D. M. Farris, S. K. Wrobleski, D. D. Myers Jr. and T. W. Wakefield. Critical revision of the article: J. Diaz, D. M. Farris, S. K. Wrobleski, D. D. Myers Jr. and T. W. Wakefield. Final approval of the article: J. Diaz, D. M. Farris, S. K. Wrobleski, D. D. Myers Jr. and T. W. Wakefield. Statistical analysis: J. Diaz and S. K. Wrobleski, Obtained funding: T. W. Wakefield. Overall responsibility: J. Diaz and T. W. Wakefield.

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Disclosures of Conflict of Interest

The authors state that they have no conflicts of interest.

References

1 Humphries J, Gossage JA, Modarai B, Burnand KG, Sisson TH, Murdoch C, Smith A. Monocyte urokinase-type plasminogen activator up-regulation reduces thrombus size in a model of venous thrombosis. *J Vasc Surg* 2009; **50**: 1127–34.
2 Aleman MM, Byrnes JR, Wang JG, Tran R, Lam WA, Di Paola J, Mackman N, Degen JL, Flick MJ, Wolberg AS. Factor XIII activity mediates red blood cell retention in venous thrombi. *J Clin Invest* 2014; **124**: 3590–600.
3 Wojcik BM, Wrobleski SK, Hawley AE, Wakefield TW, Myers DD Jr, Diaz JA. Interleukin-6: a potential target for post-thrombotic syndrome. *Ann Vasc Surg* 2011; **25**: 229–39.
4 Brandt M, Schonfelder T, Schwenk M, Becker C, Jackel S, Reinhardt C, Stark K, Massberg S, Munzel T, von Bruhl ML, Wenzel P. Deep vein thrombus formation induced by flow reduction in mice is determined by venous side branches. *Clin Hemorheol Micro* 2014; **56**: 145–52.
5 Patterson KA, Zhang X, Wrobleski SK, Hawley AE, Lawrence DA, Wakefield TW, Myers DD, Diaz JA. Rosuvastatin reduced deep vein thrombosis in ApoE gene deleted mice with hyperlipidemia through non-lipid lowering effects. *Thromb Res* 2013; **131**: 268–76.
6 Diaz JA, Ballard-Lipka NE, Farris DM, Hawley AE, Wrobleski SK, Myers DD, Henke PK, Lawrence DA, Wakefield TW. Impaired fibrinolytic system in ApoE gene-deleted mice with hyperlipidemia augments deep vein thrombosis. *J Vasc Surg* 2012; **55**: 815–22.
7 Sood V, Luke CE, Deatrick KB, Baldwin J, Miller EM, Elfline M, Upchurch GR Jr, Wakefield TW, Henke PK. Urokinase plasminogen activator independent early experimental thrombus resolution: MMP2 as an alternative mechanism. *Thromb Haemost* 2010; **104**: 1174–83.
8 Baldwin JF, Sood V, Elfline MA, Luke CE, Dewyer NA, Diaz JA, Myers DD, Wakefield T, Henke PK. The role of urokinase plasminogen activator and plasmin activator inhibitor-1 on vein wall remodeling in experimental deep vein thrombosis. *J Vasc Surg* 2012; **56**: 1089–97.
9 Geddings J, Aleman MM, Wolberg A, von Bruhl ML, Massberg S, Mackman N. Strengths and weaknesses of a new mouse model of thrombosis induced by inferior vena cava stenosis: communication from the SSC of the ISTH. *J Thromb Haemost* 2014; **12**: 571–3.