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SPECIAL ARTICLE

Choosing a mouse model of venous thrombosis: a consensus assessment of utility and application

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Summary. Murine models are widely used valuable tools to study deep vein thrombosis (VT). Leading experts in VT research came together through the American Venous Forum to develop a consensus on maximizing the utility and application of available mouse models of VT. In this work, we provide an algorithm for model selection, with discussion of the advantages, disadvantages, and applications of the main mouse models of VT. Additionally, we provide a detailed surgical description of the models with guidelines to validate surgical technique.

Keywords: deep vein thrombosis; electrolytic inferior vena cava model; femoral electrolytic model; ferric chloride model; inferior vena cava (IVC); mouse models; recurrent venous thrombosis model; stasis model; stenosis model; venous thromboembolism (VTE); venous thrombosis.

Introduction

Venous thromboembolism, consisting of deep vein thrombosis (VT) and pulmonary embolism, is increasingly seen as a major preventable or treatable disease. In 2008, the United States Surgeon General sent out a "Call to Action to Prevent Deep Vein Thrombosis and Pulmonary Embolism", which led to increased NIH funding and stronger efforts to find therapies for both prevention and treatment of VTE [1]. Despite moderate success with some of the newer therapies, there is still much room for improvement in terms of prevention in high-risk patients, reduction of bleeding complications, and in treatment of recurrent VT.

A major preclinical approach for understanding the biology of VT and testing new therapeutics is the use of murine models. Many of these models have shown utility in discriminating a number of factors that can influence thrombosis—from genetic effects to therapeutic interventions—leading to a better mechanistic understanding of VT development and resolution. However, model selection for addressing specific questions is still a poorly defined area, with investigators all too frequently applying one model where another might have been more appropriate. Furthermore, there is a lack of detailed descriptions for many applications of presumed "standardized" models, making comparisons among published results using the assumed "same" models problematic at best and potentially misleading in the interpretation of the findings. Indeed, lack of animal model standardization is a widespread problem beyond the field of VT, and careful model selection and characterization should be performed for all disease models.

The following overview is designed as a guide for how to perform, select, apply, and report seven of the major VT models that have been developed in mice. These VT models were chosen based on their use and thorough characterization to enable reproducibility between laboratories. Beyond the models discussed in this work, other models have been reported in publications that are less widely used. This work was compiled by experts following detailed discussion of the merits, limitations, and applications of commonly used murine models of VT during the American Venous Forum Meetings in 2014 and 2015, and continued discussions during the writing process of this

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manuscript. Consideration is given to the details of the techniques, reporting of outcome measures, and how a given model and the experimental measures derived from its application might best be interpreted in terms of our understanding of clinical VT development and resolution.

Mouse models of venous thrombosis

The murine system has several relatively large and accessible veins. The most popularly used vein for hosting thrombosis is the infrarenal inferior vena cava (IVC) distal to its junction with the left renal vein. This region should not be confused with the more proximal region of the vena cava which lies just below the heart and is only accessible via thoracotomy. The IVC yields sample sizes sufficient for the study of both vein wall tissue and thrombus. The absence of valves in the IVC is a drawback of IVC models when comparing to venous thrombi in humans. The other easily accessible large veins are the jugular, the femoral and the saphenous veins. The jugular vein is less commonly used for thrombosis models in mice because of its highly branching anatomy, variable diameter with dissection or manipulation, and because it is rarely the site of VT in humans. Both the femoral and saphenous veins are directly accessible with a simple skin incision and can be used without any dissection, leaving the vein essentially in its natural bed. Other venous systems that have been used in thrombosis research are the venules of thin-tissue structures, such as the cremaster muscle, the mesenteric microvasculature, and the ear. Although much has been gained from these venule models, they are qualitatively different, and are not categorically large veins. The relevance of venule-based models to large-vein thrombosis needs further clarification, and thus these models will not be discussed in this work.

Thrombosis is induced by altering/stopping blood flow, or by free radical endothelial cell activation. Thrombus size is commonly used as a metric for understanding the phase of thrombogenesis, thrombus amplification, recanalization, and eventual thrombus resolution. It is important to note that these models have almost 100% survival, and to our knowledge none of the models cause pulmonary embolism. A summary of the well-characterized models is presented in Table 1, with suggested guidelines for validation of surgical technique. A detailed description of the surgical procedure for each model is provided in Data S1.

Ligature based IVC models

Ligature models, which include stasis and stenosis models, involve the placement of a ligating or stenosing ligature around the IVC just caudal to the left renal vein to achieve stasis or low flow induction of thrombosis, respectively. The selection of this site and the various manipulations are based on the capacity to generate a sizable thrombus that can be weighed with a standard microbalance and subsequently evaluated with Western blotting or ELISA, or processed for histology or immune-based detection of specific antigens. Surgery can be performed in 20–30 min, with limited surgical setup (anesthesia machine and surgical microscope). In ligature-based models, the thrombus forms in the upstream distal direction, whereas clinical VTs are generally seen to form downstream from a nidus, such as a valve pocket. This reverse direction of growth may not be trivial and requires more investigation. However, resulting thrombi have been shown to be structurally similar to human thrombi [2–5].

The stasis model, also known as the ligation model, seeks to achieve complete stasis in the IVC, mimicking the clinical scenario of an occlusive thrombus. Any lateral or lumbar branches draining into the IVC between the left renal vein and the iliac bifurcation are interrupted, and the IVC is completely ligated. This total stasis leads to a severe vein wall reaction and nearly a 100% incidence of thrombosis. This widely used and well-characterized model produces thrombi highly consistent in size, with 10% variability [6]. A consolidated thrombus (with consistency transitioning between liquid and solid) is observed beginning 2 h post ligation, with reported outcomes extending to 28 days in most mouse strains. It has proven valuable in the study of interactions between the vein wall and thrombus during the progression from acute (first 2–3 days) to chronic inflammation and remodeling of the vein wall [7,8]. Retraction of fibrosed thrombus into the vessel wall is observed as a consequence of a total vein wall/thrombus retraction at chronic time points, specifically 21 days post thrombosis or further. Avoiding interruption of side and/or back branches, even due to surgical challenge, significantly reduces thrombus size and introduces variation in thrombus size between mice [9].

The St. Thomas stenosis model, named for its development and standardization at St. Thomas Hospital in London, involves a combination of reduced blood flow and endothelial damage (two out of three components of Virchow's triad) [10]. Posterior branches are left intact, while lateral branches are ligated, if present. The ligature, placed immediately distal to the left renal vein, is tied over a spacer such as a small length of 5–0 Prolene, which is subsequently removed to generate a ~95% reduction in blood flow. A neurovascular clamp is then applied to the vena cava wall at two locations in the upstream area for 15–20 s on each location sequentially, which causes apparent "bruising" and an additional thrombogenic stimulus to the vein. This model results in formation of a thrombus under low flow conditions with 90% incidence in the Balb/c mouse strain, with moderately lower rates of 75–80% incidence for the C57BL/6 strain (unpublished observational data).

The St. Thomas stenosis model has been subjected to several surgical modifications that are all broadly recognized under the stenosis model. Modifications avoid the endothelial damage step, and further vary in their

Table 1 A listing of the well-characterized murine DVT models, guidelines for surgical validation, and their advantages and disadvantages. Guidelines for surgical validation are provided for models with predictable outcomes in thrombus size, intended for training purposes on data reproducibility.

Model	Brief description	Incidence of thrombus induction	Thrombus size (Coefficient of variation); Guidelines for validating surgical technique	Advantages	Disadvantages	References
Stasis Model (Ligation Model)	Lateral branches are ligated and posterior branches are cauterized. The IVC is ligated with a nonreactive suture ligature placed just distal to the left renal vein	95–100%	Large (< 10% variation); In 9 to 10-week-old male C57BL/6 mice weighing 25 g, thrombus weights (IVC + thrombus at harvest) should be approximately 33 mg at day 2, 29 mg at day 6, and 18 mg at day 14.	Highly consistent thrombus size	Lack of blood flow may inhibit the maximal effect of administered systemic therapeutic agents on the thrombus and vein wall; thrombus forms against direction of blood flow	[6,33–37]
St. Thomas Model	Partial ligation of IVC, lateral branches interrupted, vascular clamp applied to IVC in two locations	*Strain dependent*—Balbc > 90%; C57BL6 75–80%	Moderate to large (20% variation); In a 25 g male BALB/C mouse, thrombus weight should be approximately 20 mg at 24 h with a volume of 18 mm [3]. C57BL/6 mice produce slightly smaller thrombi	Thrombus develops in the presence of blood flow, combines stasis and endothelial injury	Induction incidence is strain dependent; relevance of clamp injury is unclear; thrombus forms against direction of blood flow	[10,38–44]
Stenosis Model Modifications	Partial ligation of IVC without an endothelial damage step	40–100% (variable)	Small to large (85% variation)	Thrombus develops in the presence of blood flow; variability mimics that seen in humans	Variable incidence of thrombus induction; large variation in thrombus size; variation of branching phenotype draining into IVC introduces variability if branches are left open	[3,4,13,45]
Electrolytic IVC Model (EIM)	Small (≤ 250 μA) direct current applied to copper wire with the end inserted into a 25-gauge needle. Needle is placed inside the IVC on the anterior wall for ≤ 15 min. Lateral branches are ligated.	100%	*Current and time dependent* – moderate to large (< 10% variation); 10-week-old 25 g C57BL/6 mice show approximate thrombus weights (IVC + thrombus at harvest) of 17 mg at 2 days, 12 mg at 6 days, and 8 mg at 14 days when induced with 250 μA for 15 min.	Thrombus forms in direction of blood flow; highly consistent thrombus size; ability to modify current or application time to fit research question	Longer surgery time required (~30+ min)	[21–23,25]
IVC Ferric Chloride ($FeCl_3$) Model	A piece of filter paper satured in a solution of $FeCl_3$ is placed on the IVC	100%	*Concentration and time dependent*—Small (20% variation); In 20–25 g male C57BL6 mice, the wet thrombus weight (separated from the IVC in saline) should be approximately 1.3 mg at 30 min following induction with 3.5% $FeCl_3$ applied for 3 min.	Thrombus forms in direction of blood flow; ability to modify concentration or application time to fit research question	Only early time points (≤ 1 h) can be studied; small thrombus size requires microbalance and may not be adequate for biochemical assays	[17,46]

Table 1 (Continued)

Model	Brief description	Incidence of thrombus induction	Thrombus size (Coefficient of variation); Guidelines for validating surgical technique	Advantages	Disadvantages	References
Femoral Vein Electrolytic Model	A 1.5–3-volt direct current is applied to the exposed but un-dissected femoral vein (standard application of 30 s; modifications can range from 1 s up to 2 min)	100%	*Current and time dependent*—Small (25% variation); In 12–16 week old male C57BL6 mice, thrombus histomorphometric volume should be 0.015 mm [3] at day 1 when induced with 1.5 V for 30 s, or 0.140 mm [3] at day 1 when induced with 3 V for 90 s.	Presence of valves in femoral vein is clinically relevant; thrombus forms in direction of blood flow; ability to modify current or application time to fit research question; vein un-dissected in natural bed	Small thrombus size may not be adequate for biochemical assays	[20,47–53]
Recurrent VT	EIM is performed, and 21 days later a secondary thrombus is induced using either EIM or a ligature-based IVC model	100%	Moderate to large (20% variation) – the EIM and surgical procedure for the secondary method to be used should be validated using the guidelines above	Ability to study recurrent VT biology; clinically relevant; model of secondary induction may be varied for comparisons; initial thrombus becomes incorporated into the vein wall	Requires two surgeries on the same mouse	[28]

treatment of side branches as well as the chosen spacer. One variation leaves all branches open and uses a 0.36 mm wire spacer [4], while other variations interrupt side branches and use either a 30-gauge needle spacer [3,11–14], or a 0.26 mm wire spacer [15]. Experts in the field have noted that these modifications which avoid the endothelial damage step open a different and often variable thrombotic process in the model. The incidence and size of the resulting thrombus is largely dependent on the variation used; ligating side branches draining into the IVC between the left renal vein and iliac bifurcation can increase incidence of thrombus induction, and the spacer used has a direct effect on the degree of stenosis. Stenosis models result in thrombi that are smaller than those induced with the stasis model. The variability of this model mirrors the variability seen in humans and this may be useful in translating findings to the clinic. Variations on the stenosis model may be useful for studying pro-thrombotic conditions [16]. This model has been used to study early thrombotic events based on the apparent simulation of the clinical low-flow risk state [4].

Up to today, models performed with stenosis in the IVC have been collectively known as "stenosis models" despite the large degree of variation in surgical technique. Due to the amount of variation combinations, it is difficult to provide independent names for each surgical technique. Therefore, when using a stenosis model, the authors highly suggest listing either "St. Thomas Stenosis Model" or "Stenosis Model" with a detailed description of the surgical technique in the methods section of the publication. This will clarify to the field the actual surgical approach and serve as a comparative tool between laboratories. We also encourage reviewers to ensure thorough descriptions of the specific surgical modification used in addition to citing previous papers. Importantly, thrombus variability among mice subjected to the same surgical procedure, as is common in the variations of the stenosis model, are welcomed if the research question involves variability in thrombus size, but should be reported.

Free radical thrombosis models

Murine models of free radical induced thrombosis are achieved by either application of ferric chloride to the surface of the vein, or an electrolytic application of free radicals using non-injurious direct current to deposit metal cations (ferric ions) onto or within the vein. Importantly, free radical models induce thrombus formation in the direction of blood flow, as is observed in humans. Additionally, the surgical procedures are not as technically difficult compared to ligature-based models as no dissection is required between the IVC and aorta.

Free radical induced thrombosis may be performed chemically, using the ferric chloride ($FeCl_3$) model. This model has been widely used in murine carotid artery models, and has been adapted for the IVC. A small piece

of filter paper soaked in a solution of 3.5% ferric chloride by weight in water is applied to the IVC for 3 min [17]. This model may only be used to study very early time points (< 1 h), as the mouse cannot be safely recovered with the presence of $FeCl_3$ in the abdominal cavity. If used in the jugular or sapheno-femoral vein, however, chronic time points may be studied if the site of application is thoroughly irrigated [18]. The $FeCl_3$ applied to the IVC induces a thrombus that is typically very small, requiring an ultrasensitive micro-balance (error of measurement should be < 0.05 mg on the balance, as the thrombi are typically 1–2 mg) and offering little thrombus material for evaluation.

The femoral vein electrolytic model was designed to combine a simple method of thrombus induction commonly used in arterial models with intravital fluorescence microscopy. A 1.5-volt direct current is delivered to the surface of the exposed but un-dissected femoral vein for 30 s. Modifications in time—as short as 1–2 s and as long as 90 s—as well as current—up to 3 volts—can be used if a smaller or larger thrombus is desired, respectively. Thrombus can be studied in both acute and chronic time points, with resolution extending out to 28 days in larger thrombi [19]. The size of the thrombus is generally too small for weight measurements or subsequent protein analysis. The advantage is that the relatively small diameter (~0.6 mm) allows application of intravital fluorescence microscopy imaging for quantitative analysis of the development of a variety of thrombotic elements at the site of thrombus induction [20]. Additionally, the murine femoral vein has valves, which are thought to play an important role in human thrombogenesis.

The electrolytic IVC model (EIM) induces thrombosis by constant current application to a copper wire inserted into the IVC [21–23]. This generates free radicals in the wire which activate endothelial cells without injuring or destroying them. Prior to needle insertion, lateral branches are ligated while posterior branches remain open. Thrombi develop quickly, forming in the direction of maintained blood flow (without causing occlusion), with consistent size which can be evaluated for weight and subsequent protein/cellular composition [23]. Electrolysis does not alter intravascular temperature, but does require a longer operative time for current application and some damage to the vein wall at the needle insertion site. It is important to note that the needle insertion causes a 0.5 mm tear in the endothelial layer, which represents 14% of the vessel perimeter [24]; insertion of the needle without current application does not induce thrombosis [23]. The EIM is recommended for the study of anti-thrombotic, thrombolytic agents or any new agents in VT treatment and prophylaxis. Recently the EIM application was expanded for the study of prothrombotic conditions [25,26]. Further, imaging studies recently demonstrated the consistent thrombus size is both current and time dependent, enabling researchers to modify these parameters to create thrombi of consistent size to study both pro-thrombotic and anti-thrombotic phenotypes, supporting the modification introduced in 2015[27]. Additionally, a new approach was recently introduced to address lack of equipment availability and high cost, following the same principles but significantly reducing equipment cost [27].

Model of recurrent venous thrombosis

Among VT patients, 30% will suffer a recurrent episode within 10 years, with 45% occurring in the ipsilateral leg leading to an increased risk of post thrombotic syndrome. The IVC recurrent VT model was thus developed to investigate this biology by producing two episodes of VT in the same animal. Because of the high rate of recurrent thrombosis in humans, this model is a novel and direct application to the human condition. The initial thrombus is induced using the electrolytic IVC model (EIM), and a secondary episode is induced 21 days later using either the EIM or a ligature-based model. At the time of induction of the secondary episode, the primary thrombus has become incorporated into the vein wall and the lumen has been recovered. Using EIM as the primary insult allows for continuous blood flow and the opportunity to induce a second episode without needing to remove any ligature. This clinically relevant model has proven valuable in the study of recurrent VT biology [28].

Comparison and critique of the models

An ideal model would recapitulate the disease onset and pathology that are seen clinically. Because of our relative lack of understanding about VT development and progression, current animal models represent our "best guess", and thus, may lack critical features of clinical VT. A likely scenario for VT development, frequently presented by many in the field, is a low-flow or stasis state within the vein, often in a valve pocket, which can be compounded by genetic or acquired systemic prothrombotic propensity, an inflammatory state, or other factors that may enhance thrombogenesis. As such, none of the models mimic thrombogenesis in its natural form, but we can use them to study the stages of VT once a thrombus has formed.

Each model has its own unique benefits and limitations. For example, ligature-based models use slow flow or an occlusion to generate the thrombus. This is an opposite scenario to clinical VT development, for which the thrombus generates occlusion of the vein, with the exception of May-Thurner syndrome and portions of thrombus that develop distally following total occlusion. The free-radical-based models induce rapid thrombus growth and are more attuned to testing acute thrombogenesis. The maintained flow in the vein, with the possibility of thrombus

regression or embolization, provides a level of relevance not available with the ligature-based models. It should be noted that while stenosis models are often thought of as blood flow models, the resulting thrombus often leads to full stasis as the thrombus occludes the site of stenosis.

While most models may be used to study both pro-thrombotic and anti-thrombotic phenotypes, the research question may lead investigators to consider the sensitivity required for detecting differences between experimental groups. For example, the IVC stasis (ligation) model creates a close to peak growth in control conditions, so it is mostly relevant for evaluating anti-thrombotic phenotypes, i.e. experimental conditions expected to reduce thrombus size. In the case of pro-thrombotic phenotypes, a large effect should be expected for adequate sensitivity when using the stasis model [5,29]. In contrast, the stenosis model variations have a lower thrombus incidence and smaller thrombus size under control conditions, so these may be applied to pro-thrombotic phenotypic testing, with attention paid to rates of incidence between experimental groups. The EIM produces thrombi sufficiently large to test anti-thrombotic therapeutics, and may also be adjusted (decreasing the current or duration of current application) to study pro-thrombotic conditions [25].

It is of the utmost importance that publications give clear and detailed descriptions of the model with justification of any modifications used in the materials and methods section. Additionally, for rigorous science we recommend reporting data regardless of whether a thrombus developed in all mice, as variation in thrombus incidence could have important research implications. Further, robust end-point measures should be taken into consideration dependent on the model chosen during experimental design [30]. Researchers should continually strive to balance accurate and rigorous science with the highest standards of animal care, with the principles of the 3Rs (refinement, replacement and reduction) firmly in mind. The authors are therefore opposed to increasing the number of animals to match a representative or significant number, unless this is highly justified towards a specific research question. An initial power calculation should be carried out based on the variability of the end-point measure chosen in order to determine how many mice will be required [31].

Choosing a model for your research question

There is no single "best" model to represent this complex disease in all its stages. Therefore, the choice of model should be based on the specific research question. Investigators should consider the clinical scenario the model should represent, including relevant time points, what

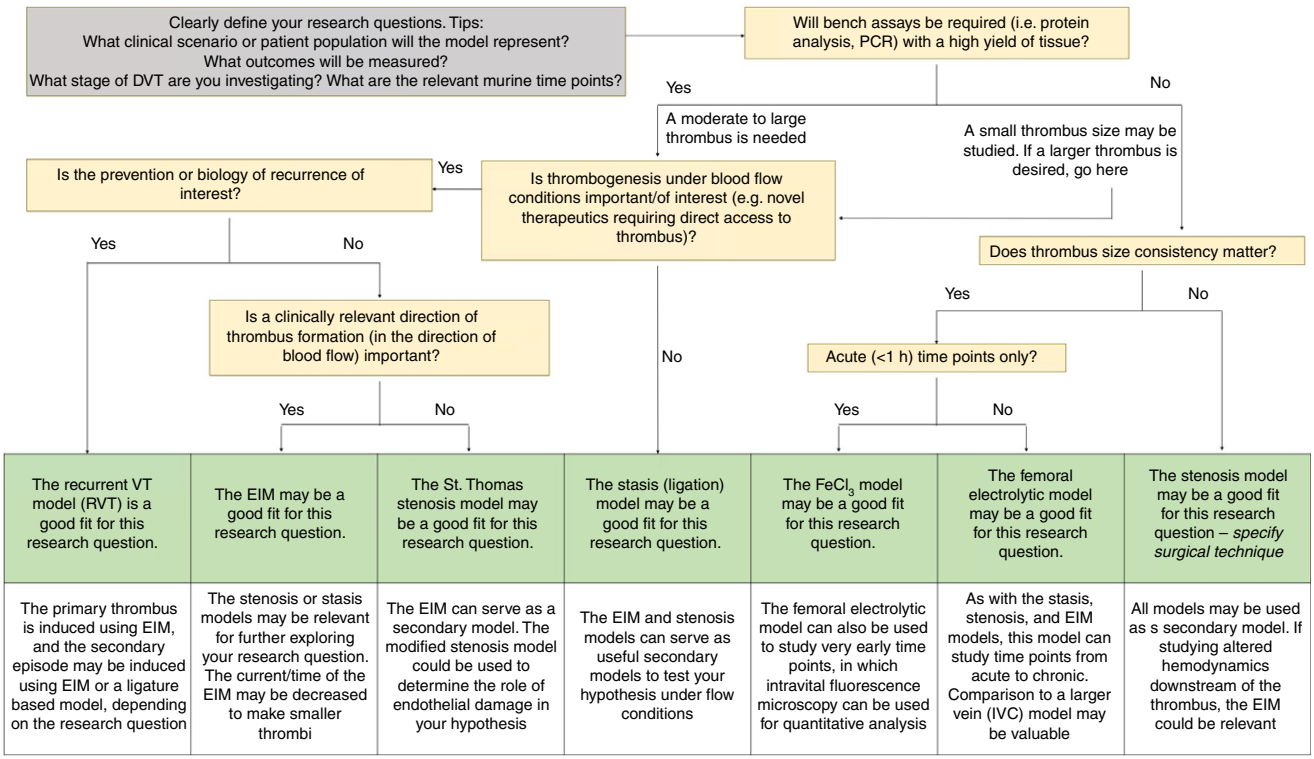

Fig. 1. Model selection algorithm. This flow chart may serve as a starting point for considerations in model selection. No single model is adequate to capture the complexity of VT; thus we recommend alternatives and secondary model options below each model. Mouse strain and sex should also be considered in model selection, as induction rates are strain dependent in stenosis models, and lateral branches draining into the IVC (common in mice from a C57 background) may not be interrupted in female mice.

outcomes will be measured, the strain of mouse that will be used, and available resources. All surgical models require a surgical microscope and anesthesia. Given the faster metabolic rate of mice compared to humans, thrombi ≤ 3 days old are considered acute, and the thrombi naturally resolve and become incorporated into the vein wall by day 28. When studying chronic VT, it can become increasingly difficult to remove the thrombus from the vein wall from 14 days following induction, which can hamper analysis.

Once a research question has been clearly defined, Fig. 1 may provide an aid in model selection. Because a clear "best murine model" for simulating VT does not exist, a practical and more generalizable approach to experimental investigations of VT is to apply two or more different models under similar conditions, such as in evaluating new transgenic/knockout mouse lines or novel pharmaceutical therapies [14]. By using multiple mechanisms for inducing thrombus growth in a vein, a broader representation of thrombogenesis is presented for testing a potential phenotype. Relevant options for secondary models are listed under each model in Fig. 1. The selected models should have a response range that is commensurate with the anticipated or hypothesized response.

In choosing a mouse strain, it is important to note the anatomical differences between mice from BALBc vs. C57BL/6 backgrounds. BALB/c mice have very few if any lateral branches compared with C57BL/6. Although posterior branches in BALB/c mice are increased in number, they are of a smaller caliber than in C57BL/6 mice. Thrombus size is generally larger and less variable in BALBc compared to C57BL/6 mice (Table 1). There are, however, far more genetically-modified mice available on a C57BL/6 background.

An important note for any model requiring the interruption of lateral branches is that in female mice from the C57BL/6 background, the right uterine vein commonly drains into the IVC, with ligation resulting in necrosis of the reproductive organs [32]. Thus, if an IVC model is being used, either the stenosis model variation that does not interrupt branching vessels or the use of BALB/c mice (which typically do not present lateral branches) is recommended to study VT in females. If mice from the C57BL/6 background are used, the branching phenotype should be noted as a variable influencing thrombus size and larger numbers of animals may be required.

In conclusion, there is no single "best" model to study VT, as areas of peri-thrombus blood flow and total stasis occur in human deep vein thrombosis. Model selection should be determined by the specific research question, and an additional model should be implemented to examine the experimental conditions under more than one setting. While no single mouse model can capture the complexity of VT, this method brings us closer to a rigorous understanding of the disease.

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The authors state that they have no conflict of interest.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article:

Data S1. Detailed surgical technique.

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Highlights

- This consensus details step-by-step surgical procedures in the supplemental material, providing a one-stop shop for investigators to understand how to surgically perform the models.
- Specific guidelines on thrombus size are provided to enable researchers performing these techniques to validate their surgical technique against this standard.
- The advantages and disadvantages of each model are discussed in detail with consideration to potential application of each model.
- An algorithm for model selection is provided which guides researchers in selecting a model to match the specific research question, and further suggests useful secondary models to explore the research question in more than one setting.