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Organs-on-Chips: a new paradigm for safety assessment of drug-induced thrombosis

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Abstract

Blood hypercoagulability and thrombosis have been observed in patients during clinical trials of candidate drugs, yet these safety risks are seldom identified during preclinical testing, leading to increased mortality and morbidity, and increased attrition rates in the clinic. Current preclinical models — standard cell cultures, flow chambers, and animal models — are often ill-equipped to predict thrombosis in the clinic. *In vitro* models are typically assembled without critical biomechanical forces, such as shear stress and mechanical strain, or relevant cytoarchitecture, such as interactions between different tissue types, which are essential to physiological function. In addition, animal models not only are expensive and costly but also possess inherent cross-species biological differences that are difficult, if not impossible, to reconcile for accurate human predictions. As a preclinical platform with a potentially higher predictive value, organs-on-chips are fluidic systems that reproduce organ-level function via cellular components of human origin, tissue–tissue interfaces, and dynamic mechanical forces. Compared with other current preclinical models, organs-on-chips combine the advantages of tunability and ease of biochemical, histological, and image analysis, while bypassing difficulties in cross-species translation. In this review, we delineate the limitations of current preclinical models, which are often unable to predict drug-induced thrombosis, and report some recent advancements in Organs-on-Chips technology that represent a promising alternative for modeling tissue-specific thrombotic events and derisking next-generation drug discovery.

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Drug induced thrombosis, Safety assessment of therapeutics, Organ-on-Chip, Blood vessel-chip.

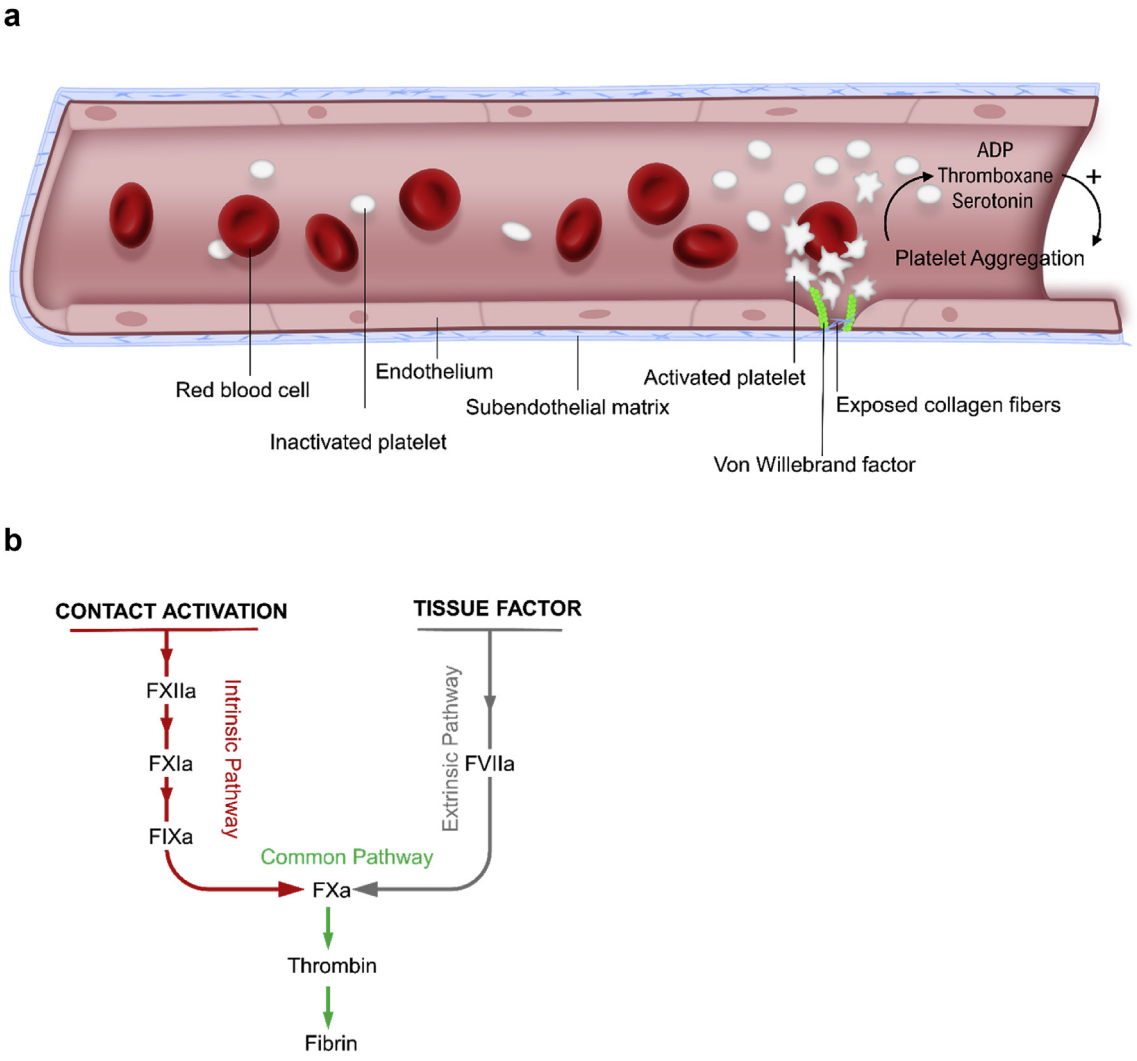
An overview on hemostasis and thrombosis

Scientific advancements in vascular biology over the past two decades have reshaped our understanding of the vascular wall, which was once conceptualized as an inert nucleated monolayer and is now regarded as a key, regulated component of physiological and pathological hemostasis [1]. With only one-cell thickness, the vascular endothelium senses and reacts to small variations in hemodynamic forces or blood-borne signals through a multitude of receptors and glycoproteins that project outward into the vascular lumen [2]. In healthy conditions, the endothelium acts as a physical barrier that confines blood within the systemic circulation (Figure 1 a). Expressed by endothelial cells, specific extracellular receptors, such as endothelial protein C receptor, thrombin receptor (thrombomodulin), and glycocalyx [3], actively suppress the coagulation cascade, as well as the deposition of prothrombotic factors and adhesion of blood cells or platelets under physiological conditions [4]. However, as part of the natural response to tissue injury, the composition of the endothelial surface shifts from a quiescent state to an active, prothrombotic state that promotes platelet recruitment and the formation of a blood plug, which limits hemorrhage. This process is referred to as 'primary hemostasis.' The transition from a blood plug to a 'thrombus' depends on fibrin deposition over the initial blood plug, an event tightly regulated by the coagulation cascade (Figure 1 b) and generally referred to as 'secondary hemostasis.' Under physiological conditions, the thrombotic event resolves: the fibrin clot dissolves via fibrinolysis, and the endothelium undergoes tissue repair, which is largely mediated by leukocytes, chemokines, and cytokines [5].

Thrombosis-associated safety risks

Cardiovascular-related complications are often cited as a primary driver of drug attrition, often occurring during late-stage clinical trials or postmarket phases [6–8]. Such cardiovascular liability can occur secondary to

Figure 1



Injury of a vascularized tissue or organ frequently results in physical disruptions of the endothelium, loss of barrier function, and subsequent extravasation of blood cells into the interstitial space. Initiation of primary hemostasis serves as a rapid response to what could otherwise be a lethal injury. **(a)** Hemostasis is generally initiated by exposure of the subendothelial extracellular matrix (ECM), a potent platelet agonist that is capable of engaging the von Willebrand factor (vWF), which promotes platelet adhesion to the subendothelium. vWF serves as a bridge between the wounded tissue and platelets, binding to both the exposed collagen at sites of injury and glycoprotein Ib-V-IX (GPIb-V-IX) on the platelet membrane. Once activated, platelets undergo morphological alterations and release procoagulant soluble factors, such as ADP, thromboxane, and serotonin, which cause further platelet adhesion and aggregation and vasoconstriction via the activation of smooth muscle cells. **(b)** The coagulation cascade has two initial pathways that lead to fibrin formation: the intrinsic pathway and extrinsic pathway. The intrinsic pathway, also called the contact activation pathway, is typically initiated by the activation of factor XII by negatively charged surfaces, such as collagen and glass. The extrinsic pathway is referred to as the tissue factor (TF) pathway. TF is a potent procoagulant effector that is expressed on the surface of activated endothelial cells and circulating leukocytes under pathological conditions [55]. TF is also released as microvesicles from activated endothelial cells or platelets [56].

drug-induced venous or arterial thromboembolism, which are poorly predicted in preclinical studies, ultimately resulting in postmarket black box labeling or outright market withdrawal of drugs [9,10]. The need for thrombogenic risk assessment first gained widespread attention after studies showed an association between hormonal contraceptives and venous thrombosis [11], which led the Food and Drug Administration (FDA) to recommend limitations to the duration of hormone replacement therapy [12]. In 2004, Vioxx (rofecoxib), a COX-2 inhibitor, was recalled from global markets owing to increased risk of myocardial infarction and stroke, both of which are adverse cardiovascular atherothrombotic events [13,14]. In 2007, erythropoiesis-stimulating agents, which combat anemia, were similarly tagged with black box warnings for safety concerns related to thrombosis [15]. Furthermore, many anticancer agents, such as

thalidomide (in combination with dexamethasone or chemotherapy), cisplatin, and ponatinib, have been shown to increase thrombogenic risks, compelling clinicians to use caution when prescribing these therapies for patients [16,17]. Given the unnecessary mortality and morbidity arising from these examples and countless more (Table 1), there is a significant demand for drug developers to identify and mitigate thrombotic safety risks as early as possible. Although the reasons underlying late-stage identification of these hazards are various, inadequate preclinical studies, which preferentially screen for bleeding risks but not for hypercoagulable states, are particularly suitable for re-evaluation and change [18].

Standard preclinical methods and their limitations

Over the past decade, studies have shown that nearly 90% of drugs fail during clinical trials. The likelihood of approval by the FDA of a drug entering phase 1 trials is 10.4–11% [18,19]. Moreover, despite increasing R&D spending, the number of innovative drugs or new molecular entities approved by the FDA has also been declining [7]. Although the causes of high attrition are diverse and vary between drug classes and therapeutic areas, approximately 60% of failures are due to unacceptable toxicity (preclinical toxicology and clinical safety) and insufficient efficacy [19]. In particular, poor toxicology and safety results have been the largest sources of oral small-molecule drug attrition [20]. Better human-relevant alternatives to current models are needed to improve the current paradigms of translating preclinical data to the clinic. Some studies have identified the preclinical stage of early translational medicine as a key time window for resource investments [19–21]. For example, it has been estimated that inadequate preclinical (*in silico*, *in vitro*, and *in vivo*) screening of safety signals accounts for 40% of safety failures [22]. As such, improved target selection and prediction of safety and efficacy profiles via higher fidelity preclinical models earlier on in the process will help address some of the challenges faced by the industry.

Animal models

Preclinical studies are primarily conducted in animals, specifically dogs, rats, and nonhuman primates. Mice are typically excluded from hemostasis testing, owing to blood volume constraints and difficult phlebotomy. Most preclinical evaluations of hemostasis are conducted on healthy animals, although some use animal models of induced thrombosis, such as rats or nonhuman primates infused with lipopolysaccharide (LPS), rabbits with electric current–induced thrombosis, or rats with laser-induced thrombosis [18]. Rats and rabbits have

Table 1 Drugs that have been reported to cause thrombosis.

Type of drug	Name	Mechanism	Reference
Antithrombotic	Tissue-type plasminogen activator	Interfere with the fibrinolytic pathway and endothelial function	[28,30]
Antithrombotic	Heparin	Formation of immunocomplexes, activation of the endothelial surface	[57]
Antidepressant	Serotonin	Platelet aggregation	[58]
Antipsychotic	Chlorpromazine	Platelet aggregation	[59]
Angiogenesis inhibitors	Thalidomide, lenalidomide	Venous thromboembolism caused by endothelial tissue damage	[60]
Chemotherapy	Bleomycin	Lung endothelial damage leading to pulmonary thrombosis and fibrosis	[37]
Antipsychotics	Clozapine	Platelet aggregation	[31]
Glucocorticoid	Prednisone	Platelet aggregation, decrease in fibrinogen and plasminogen, increase in prothrombin antithrombin and von Willebrand factor (vWF)	[61]
Chemotherapy	L-asparaginase	Impaired protein synthesis	[62]
Tumor necrosis factor (TNF)-blocking agent/ immunosuppressive	Infliximab	Arterial thrombosis	[63]
Protein-based therapy	Multiferon (interferon alpha)	Leukocyte entrapment in retinal microcirculation	[24]
Antipyretic	Phenylhydrazine	Induces phosphatidylserine externalization leading to vascular thrombosis	[64]
Antineoplastic (chemotherapy)	Mitomycin C	Renal endothelium damage	[65]
Topoisomerase inhibitor antineoplastic agent (chemotherapy)	Doxorubicin	Downregulation of the endothelial cell protein C anticoagulant pathway, altering the hemostatic balance of endothelial cells	[66]
Protein (growth factor)-based therapy	Erythropoietin	Increase in chronic inflammation and thrombin activatable fibrinolytic inhibitor	[67,68]

Numerous approved drugs disrupt the physiological hemostatic balance, causing pathological hypercoagulability and increasing thrombotic risks. In brief, drugs can induce thrombotic events through six primary pathways: (1) endothelial damage, which either mechanically exposes the subendothelium or alters the endothelial expression of coagulant and anticoagulant factors; (2) increasing platelet adhesion and/or aggregation; (3) altering red blood cell morphology or membrane properties; (4) increasing white blood cell adherence to the endothelium; (5) altering the coagulation system's balance between procoagulation (tissue factor and so on) and anticoagulation (fibrinolysis and so on), and (6) changes in blood flow via vasoconstriction or blood stasis [69].

been used to model thrombotic events triggered by contrast media, 5-fluorouracil (5-FU), carbon nanoparticles, and interferon alpha [23–26]. Despite the historic reliance on animal models for preclinical evaluations of compounds and therapies, a survey of scientists from small-, medium-, and large-sized pharmaceutical companies, academia, contract research organizations, and governmental or regulatory agencies showed that 80% of respondents lacked confidence in hemostasis test results in one or more preclinical species [18]. The reasons are multifaceted, including a lack of industry-standardized assays to assess thromboembolic risks in animals and fundamental limitations in cross-species translatability.

In vitro systems

Owing to the ease of use and high-throughput nature of *in vitro* cell culture systems, they have served as a mainstay tool of preclinical studies. Coupled with biomolecular assays and high-resolution imaging, these models can facilitate insights into basic biological phenomena. *In vitro* models have revealed prothrombotic mechanisms for various compounds and therapeutics. Human umbilical vein endothelial cells (HUVECs) cultured with contrast media, tissue plasminogen activator, and cyclosporine, which are all known to increase thrombotic risk, demonstrated endothelial damage, suggesting that subendothelial exposure *in vivo* triggers the coagulation cascade [27–30].

Paired with platelet aggregometry methods and flow cytometry, *in vitro* experiments with blood from human donors also identified possible mechanisms of platelet aggregation from clozapine, an antipsychotic, and carbon nanoparticles, which are being engineered for purposes of selective imaging and drug delivery [25,31]. Despite these important findings, cell culture systems take individual biological components — the endothelium, blood cells, and platelets — out of their natural context and do not incorporate the biomechanical dynamic microenvironment or the mechanical forces that are strongly involved in the delicate *in vivo* balance of hypocoagulable and hypercoagulable states. Moreover, conventional cell culture systems are most often deployed *a posteriori* to explain drug-induced thrombosis only after they are observed clinically and thus have limited predictive value.

Parallel-plate flow chambers and cone-and-plate chambers address some of the limitations of conventional cell culture systems. Most parallel-plate flow or cone-and-plate chambers feature compartments coated with extracellular matrix components and perfused with whole human blood at tunable shear rates. Eventually, some flow chambers include endothelialized surfaces, which have helped illustrate how endothelial cells change morphologically, mechanically, and biochemically to shear stress [32–34]. Although parallel-plate and cone-and-plate flow chambers have become quite advanced in recreating the rheology and dynamics of hypercoagulability, these systems do not include the relevant cytoarchitecture of the tissue, the complex tissue–tissue interactions that regulate the organ function in the body, the dynamic nature of the microenvironment created by blood flow, or the mechanical forces created by shear stress that are critical drivers of *in vivo* physiology and pathobiology [35]. Therefore, these systems are frequently unable to predict the organ-specific thrombotic events observed *in vivo*.

Organ-on-Chip technology

Despite the value of *in vitro* platforms, such as cell culture dishes and parallel-plate flow chambers, cells do not naturally exist in isolation or in static systems. Drug-induced thrombosis involves biological interactions that go beyond endothelial blood/platelet reactions and are localized to specific regional tissues and organs. For example, gemtuzumab ozogamicin, a drug-linked monoclonal antibody, and busulfan, an alkylating agent, are known to cause hepatic veno-occlusive disease; cisplatin, a chemotherapeutic agent, induces renal tubular injury, and bleomycin, another chemotherapeutic agent, causes pulmonary thrombosis [36–39]. As for studies in rats, dogs, rabbits, and nonhuman primates, animal models have long been questioned not only for their cross-species translatability but also for their high costs and ethical concerns centered around animal rights. In addition, the animal models can sometimes preclude mechanistic understanding of the cellular mechanisms driving the adverse drug reaction. This is critical to avoid this safety liability in future compounds but also to enable improved risk assessment. To accelerate the insights garnered from preclinical investigations of thrombosis and more generally systemic blood-related disorders, we need platforms that can combine the benefits of cell-based systems and flow chamber technologies, while also harnessing cells of human origin. An ideal system would reconstitute multiple variables that influence hypercoagulability — the endothelium, platelet interactions, tissue–tissue interfaces, laminar flow, shear stress (mechanical forces induced by flow through the vasculature), blood rheology, blood cell reactivity — in a manner that is physiologically relevant to specific organ-level niches. Organ-on-Chips are microengineered fluidic systems fabricated using microfabrication techniques, such as soft lithography or 3D printing, and populated with human cells in the dynamic microenvironment that emulates tissue–tissue interactions and organ-level function. Organ-on-Chip hold great promise in advancing the translation of preclinical data and reducing attrition rates in the clinic.

Blood vessel-Chips

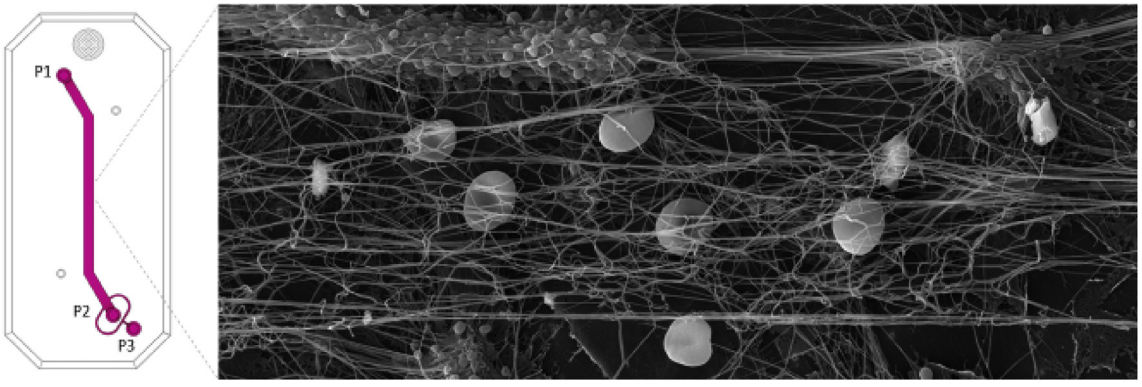
Blood vessel-Chips are the most basic Organ-Chip models, focusing on blood–endothelium interactions,

yet they hold great promise in safety and efficacy drug testing [40]. The Blood vessel-Chip reported by Barrile et al. [41] comprises a HUVEC-lined microchannel that allows for directional flow of media or whole blood and incorporates the mixing of an anticoagulant with blood at the outlet (Figure 2). This enables the model to provide both morphological end points assessing deposition of fibrin and platelets, as well as biochemical end points assessing specific cytokines and biochemical markers for thrombosis in the spent blood. This system incorporates endothelial–blood interactions and shear stress and has been used to evaluate endothelial activation, platelet adhesion, platelet aggregation, fibrin clot formation, and the presence of thrombin antithrombin (TAT) complexe in the effluent when the Blood vessel-Chip was exposed to a monoclonal antibody (Hu5c8) previously reported to cause cardiovascular complications in human patients. Interestingly, this prothrombotic effect was absent when the Blood vessel-Chip was exposed to Hu5c8-IgG2r, an antibody designed with an Fc domain that does not bind the human FcγRIIa receptor. While this finding remains to be confirmed clinically, these results suggest this platform might provide greater human-specific predictability than other existing cell-based models and animal models. The same platform has also been used with fixed HUVECs [42] and upon perfusion with whole blood, platelet adhesion, aggregation, and fibrin deposition can be measured on the fixed Blood vessel-Chip as well. This approach could potentially enable greater scalability and perhaps simplifies the use of a Blood vessel-Chip as a point-of-care diagnostic tool using patient-specific blood for personalized safety studies. Other investigators have developed 3D bioprinting methods to engineer their Organs-Chips with circular cross-sectional architectures, which could improve the fidelity of the system [43,44]. In contrast to the aforementioned models, where endothelial cells are seeded onto microengineered channels, some groups have developed methods for coaxing gel-embedded endothelial cells to sprout into capillary networks via mechanical and chemical factors [45,46]. While these vascular systems are physiological in their self-assembly, one major caveat of this approach is limited controllability regarding flow parameters, which is crucial in studying thrombosis.

Alveolus lung-Chip

As mentioned, drug-induced thromboembolism is a tissue-specific event, dependent on the biomechanical and biochemical milieus of the different tissues involved. In their 2018 publication, Jain et al. [47] recapitulated pulmonary thrombosis *in vitro*. The authors used primary epithelial cells juxtaposed to primary endothelial cells to reconstitute the human alveolar–capillary interface. This model was reported to be stable for more than 12 days in culture with a tight monolayer, demonstrating low permeability, and whole blood could flow for up to 20 min without any significant platelet aggregation or fibrin deposition under nonstimulated (control) conditions. LPS, a gram-negative bacterial endotoxin, has been shown to cause acute lung injury and induce pulmonary thrombosis *in vivo*. Using this Alveolus lung-Chip model, the authors were able to test the effects of LPS on pulmonary thrombus formation and assess whether *in vivo* biology could be reconstituted within the chip. Interestingly, they only observed a large increase in permeability of the tissue–tissue interface and corresponding increase in platelet binding when the LPS was added to the alveolar epithelium in the Alveolus lung-Chip, and not

Figure 2



The Blood vessel-Chip is typically made of a transparent elastomer (Polydimethylsiloxane, PDMS) with one main fluidic chamber (the vascular channel) coated with extracellular matrix and lined with endothelial cells. Whole blood or specific blood components, including platelets, red blood cells, or immune cells, are perfused from the inlet (P1) to the outlet (P2) through the vascular channel at a physiological relevant shear rate to reconstitute the mechanical forces and cell–cell interactions that regulate vascular hemostasis in the body. Morphological end points assessing deposition of fibrin and platelets can be captured through different imaging methods, including Scannin Electron Microscopy (SEM, image on the right was obtained from organs-on-chips exposed to 10 ng of tumor necrosis factor (TNF) overnight and then perfused with whole blood for 12 min). The specific design of the blood vessel-on-a-chip reported by Barrile et al. [41] incorporates a secondary fluidic inlet (P3) that allows the mixing of an anticoagulant with blood at the outlet (P2) and collection of soluble blood samples for detection of clinically relevant coagulation biomarkers.

when it was added directly into the vascular channel. Exposure of the alveolar epithelial cells to LPS correlated with disruption of endothelial cell—cell junctions, as demonstrated by immunofluorescence microscopic analysis of Vascular Endothelial-cadherin (VE-cadherin), and endothelial activation measured by increased expression of the Intercellular Adhesion Molecule 1 (ICAM-1). These results serve as a proof of principle for organ-specific pathophysiology and provide evidence of pulmonary thrombosis induced when relevant cell types — in this case, the alveolar epithelium — were perturbed, as opposed to HUVECs, which were not lung specific.

Conclusions

Thrombogenic events contribute substantially to cardiovascular safety risks and cause sizable mortality, morbidity, therapeutic development costs, and drug candidate attrition. One approach to addressing approach and speeding up drug discovery process to enable new therapeutics to reach patients faster is by improving the predictive nature of preclinical safety testing with more *in vivo* relevant models. Organs-Chips reconstitute organ-level function using human cells, emulating the appropriate microenvironment and key factors that drive gene expression and differentiation *in vivo*. Compared with current *in vitro* systems — conventional cell culture models, parallel-plate/cone-and-plate flow chambers, and animal models, Organ-on-Chip technology combines the advantages of *in vitro* tunability and ease of biochemical, histological, and image analysis, while bypassing difficulties in cross-species translation. As such, Organs-on-Chips have the potential to accelerate and improve the drug discovery and development process. A Blood vessel-Chip study conducted by Barrile et al. [41] demonstrated the applicability of Organs-on-Chips in identifying biologics (e.g. different versions of a monoclonal antibody) with mitigated thrombotic risk, although as with any preclinical finding, results must be confirmed through clinical studies. Moreover, the field continues to develop advancements in fabrication, such as 3D bioprinting, which has enabled groups to render vasculatures with circular lumens. Jain et al. [47] helped pave the way for tissue-specific modeling of thrombosis by using the Alveolus lung-Chip to recapitulate LPS-induced pulmonary thrombosis.

The studies discussed in this review provide evidence that we can apply Organ-on-Chip technology to better determine thrombosis-associated safety risks. Furthermore, the trend of induced Pluripotent Stem Cells (iPSC) derived Organs-on-Chips heralds an exciting transition away from less biologically relevant systems, based on immortalized cell lines or animal cells, and toward patient-specific platforms [48—53]. These innovations in stem cell and Organ-on-Chip technology, coupled with flow of whole blood from both healthy individuals and patients with genetic mutations (e.g. Factor V Leiden and sickle cell anemia) and with advanced analytical techniques such as automated image analysis of clots and tissues, will increase the sensitivity and specificity of thrombosis risk assessment [41,54]. These technology platforms open up the potential for personalized safety. Organs-on-chips are poised to help close the gap between preclinical prediction and ultimate clinical benefit.

Conflict of interest statement

P.N. is a shareholder of Emulate. C.B., S.B., G.H., and R.B. are employees and shareholders of Emulate Inc. A.D.v.d.M. is an external consultant for Emulate Inc.

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- • of special interest
- •• of outstanding interest

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