# Endothelial Autophagy Does Not Influence Venous Thrombosis in Mice

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Macroautophagy, herein referred to as 'autophagy', is essential for cellular homeostasis and stress adaptation and constitutes a process that facilitates the lysosomal degradation of intracellular material sequestered within autophagosomes. Autophagy becomes insufficient in aging organisms, threatening their functionality and survival.[1] Moreover, autophagy fails to maintain cellular functions in multiple chronic diseases including (but not limited to) infectious, neoplastic and neurodegenerative diseases.[1]

Several recent studies have shed light on the role of autophagy in haemostasis and thrombosis (►**Fig. 1A**).

In platelets, autophagy machinery is constitutively active under resting conditions.[2] Mice with platelet-specific deletion of Atg7, a key protein of the autophagy cascade, have normal platelet numbers and size distributions, but exhibit a robust bleeding diathesis in the tail-bleeding assay and a prolonged occlusion time in the $FeCl_3$-induced carotid injury model, a model of arterial thrombosis.[2]

Autophagy also contributes to haemostasis in endothelial cells. Indeed, autophagy regulates endothelial secretion of von Willebrand factor from intracellular organelles, known as Weibel–Palade bodies. Mice with endothelial-specific deletion of Atg7 exhibit impaired epinephrine-stimulated von Willebrand factor release, reduced levels of high-molecular weight von Willebrand factor multimers and a corresponding prolongation of the bleeding time.[3] The role of endothelial autophagy in arterial thrombosis has also been investigated. When compared with wild-type animals, mice with endothelial-specific deletion of Atg7 exhibit prolonged time to carotid and mesenteric artery occlusion following $FeCl_3$ injury. Furthermore, these mice deficient in endothelial autophagy are characterized by smaller thrombi in laser-injured cremasteric arterioles.[4] Altogether, these results show that a defect in the autophagy protein Atg7 in platelets or in endothelial cells induces a bleeding tendency including prolonged bleeding time and increased time to occlusion following arterial injury. The role of autophagy in venous thrombosis is unknown.

We recently reported that deficiency in endothelial autophagy in cultured cells and in transgenic mice led to a pro-inflammatory, pro-senescent and pro-apoptotic phenotype of endothelial cells exposed to high shear stress areas.[5] Endothelial apoptosis can result in exposure of the subendothelium to blood flow, which would then promote platelet adhesion and thrombosis.[6] This prompted us to analyse in vivo venous thrombus formation in mice deficient in endothelial autophagy.

We obtained mice deficient in endothelial autophagy by crossing *VE-cadherin-Cre* transgenic mice with *Atg5*$^{lox/lox}$ mice. We first performed an in vivo tail-bleeding assay, as described.[2] To measure bleeding, tails of 7- to 10-week-old sedated (isoflurane) male and female mice were transected 3 mm from the tip and immersed in saline at 37°C. The time required for bleeding cessation was recorded, and the mice were observed for an additional minute. Experiments were terminated at 10 minutes. Then, another group of mice was

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| | Bleeding time | Occlusion time in arterial models of thrombosis | Thrombus weight in venous model of thrombosis |
|---|---|---|---|
| **Deficiency in platelet autophagy** | Prolonged in Atg7 deficient mice (2) | Prolonged in Atg7 deficient mice (2) | Not tested |
| **Deficiency in endothelial autophagy** | Prolonged in Atg7 (3) and Atg5 deficient mice (present study) | Prolonged in Atg7 deficient mice (4) | No impact in Atg5 deficient mice (present study) |

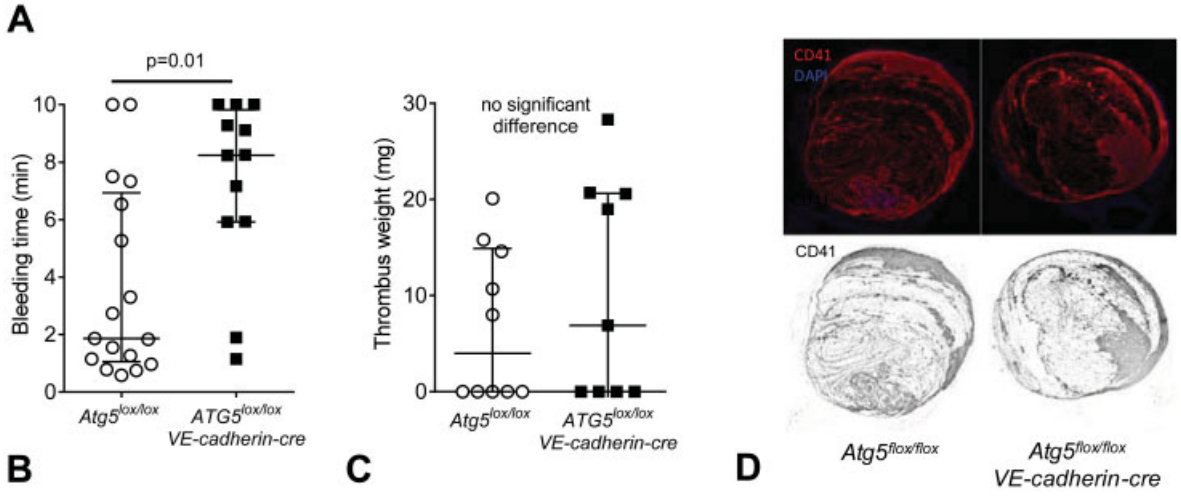


**Fig. 1** A defect in endothelial autophagy does not modulate thrombus formation. (**A**) Summary of the studies investigating the role of autophagy in haemostasis and thrombosis. (**B**) Bleeding times of 7- to 10-week-old male and female *Atg5*$^{lox/lox}$ ($n = 17$) versus *Atg5*$^{lox/lox}$*; VE-cadherin-Cre* ($n = 12$). (**C**) Quantification of the thrombus weight developed in 10 weeks old *Atg5*$^{lox/lox}$ ($n = 10$) versus *Atg5*$^{lox/lox}$; *VE-cadherin-Cre* ($n = 9$) mice 48 hours after inferior vena cava stenosis. (**D**) Representative CD41 staining of thrombi (original magnification: ×20). Data are given as median (horizontal bar) and interquartile range (error bar). Comparison between groups was performed using the Mann–Whitney test.

subjected to a mouse model of venous thrombosis based on flow restriction in the inferior vena cava. This model was previously reported to respect endothelial cell integrity, unlike the laser and $FeCl_3$ injury models, and to take into account the exposure of von Willebrand factor.[5,7,8] Mice were anaesthetized by intraperitoneal injection of a solution of midazolame (5 mg/kg body weight; Ratiopharm), medetomidine (0.5 mg/kg body weight; Pfizer) and fentanyl (0.05 mg/kg body weight; CuraMed Pharma GmbH). After a median laparotomy the inferior vena cava was exposed by atraumatic surgery and a space holder (FloppyR II Guide Wire 0.014, Guidant Corporation) was positioned on the vessel followed by a narrowing ligature below the left renal vein. Subsequently, the wire was removed to avoid complete vessel occlusion. Side branches were not ligated or manipulated. The median laparotomy was immediately sutured. For thrombus weight measurement 48 hours after flow reduction, the inferior vena cava was excised just below the renal veins and proximal to the confluence of the common iliac veins.[7]

As shown in the ►**Fig. 1B**, we observed that *Atg5*$^{lox/lox}$ mice had a normal bleeding time, whereas *Atg5*$^{lox/lox}$*-VE-cadherin-Cre* had a significant increase in the bleeding time, as previously reported in *Atg7*$^{lox/lox}$*-VE-cadherin-Cre* (►**Fig. 1A**).[3]

We then analysed in vivo venous thrombus formation using the mouse model of flow restriction in the inferior vena cava in mice deficient in endothelial autophagy. We observed no effect of the deficiency in endothelial autophagy on thrombus size or composition (►**Fig. 1C**, **D**).

This lack of effect of a deficiency in endothelial autophagy on venous thrombus formation might result from the counterbalance of pro-thrombotic factors, such as increased endothelial apoptosis induced by the defect in autophagy,[5] by antithrombotic factors, including the reduced levels of high-molecular weight von Willebrand factor multimers in mice deficient in endothelial autophagy.[3] A defect in platelet function might also occur in constitutive *Atg7*$^{lox/lox}$*- VE-cadherin*-cre or *Atg5*$^{lox/lox}$*-VE-cadherin*-cre mice, like in *Atg7*$^{lox/lox}$*- PF4-cre* mice.[2] Indeed, expression of the Cre-recombinase in *VE-cadherin*-cre mice is not restricted to endothelial cells, but is also present in around 50% of myeloid cells, since early embryonic endothelial and haematopoietic cells arise from a common embryonic precursor called the haemangioblast.[9]

The contrast between the prolonged time to occlusion reported with arterial models of thrombosis using FeCl3 or laser injury in *Atg7*$^{lox/lox}$*-VE-cadherin*-cre mice and the absence of effect on thrombus size we observed in our venous model of thrombosis in *Atg5*$^{lox/lox}$*-VE-cadherin*-cre mice might be explained by the various impacts of these models on endothelial cells. Indeed, FeCl3 or laser injury induce endothelial damages likely masking changes in endothelial phenotype

induced by endothelial mutation, while the contribution of platelets remains obvious.[10] Conversely, the venous model of thrombosis we used respects endothelial integrity, allowing the analysis of the effect of the deficiency in autophagy on endothelial phenotype and function.[7]

### Authors' Contributions

P.E.R. and C.M.B. wrote the manuscript. J.B. performed experiments. M.K. and A.C.V. generated the mouse model. P.E.R., C.M.B. and K.S. analysed the data. All authors read and critically reviewed the manuscript.

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### Conflict of Interest

None.

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