Altered venous shear stress induce endothelial mechanosensitive ETS1-Notch4/Dll4 signaling in varicose veins

Presented by

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BACKGROUND

- Varicose veins are characterized by **hemodynamic instability** due to valvular incompetence and factors like orthostatism.
- The site of venous blood reflux is the lower extremities of the body, but the principal location is the great saphenous vein.
- Risk factors include a positive family history, Increase in age, female gender, pregnancy, obesity, and orthostatic lifestyle.
- Corrective treatments include sclerotherapy, compression stockings, vein stripping, endovenous ablations.
- Despite advances in treatment options for varicose veins, the recurrence rate remains very high.
- Identifying pharmacological drug targets is essential to develop more effective non-invasive therapeutics.

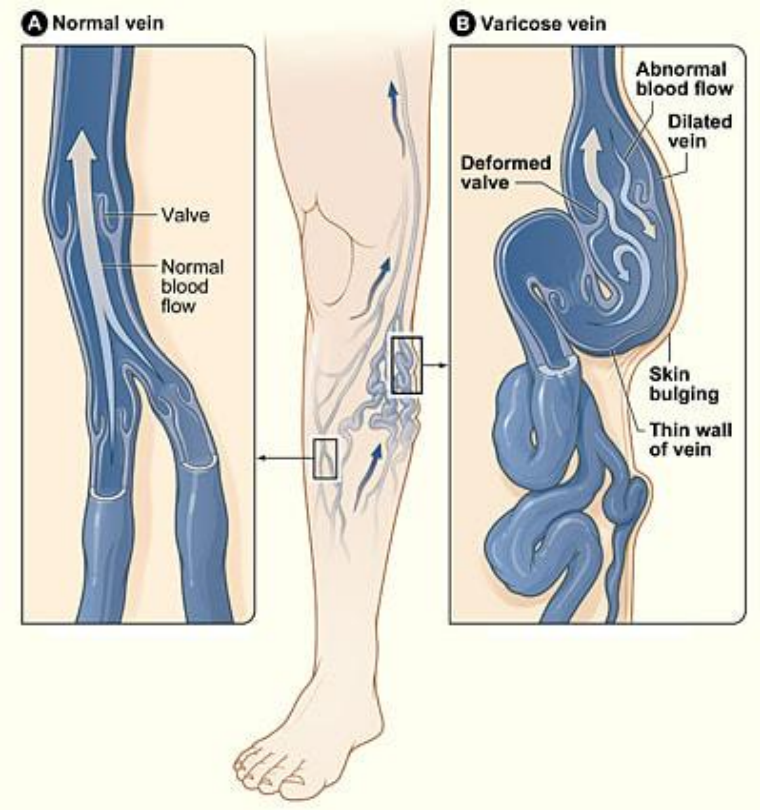


Image courtesy: Physiopedia

(A) A normal vein with a working valve and normal blood flow. **(B)** A varicose vein with a deformed valve, abnormal blood flow, and thin stretched walls.

Altered hemodynamics in the pathophysiology of varicose veins

- Fluctuations in hemodynamic forces in the vessel wall cause pathological gene expression and activation of downstream pathways, eventually causing vein wall remodeling.
- However, the mechanism by which altered biomechanical cues get translated into abnormal venous wall remodeling is unelucidated.

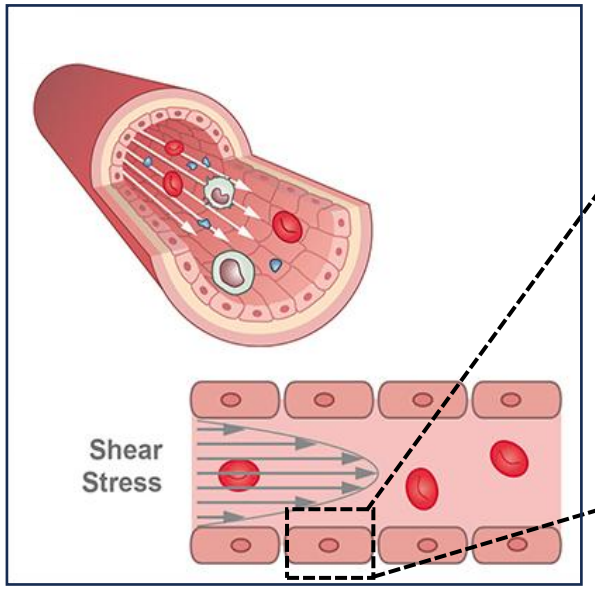


Image courtesy: Ibidi, Germany

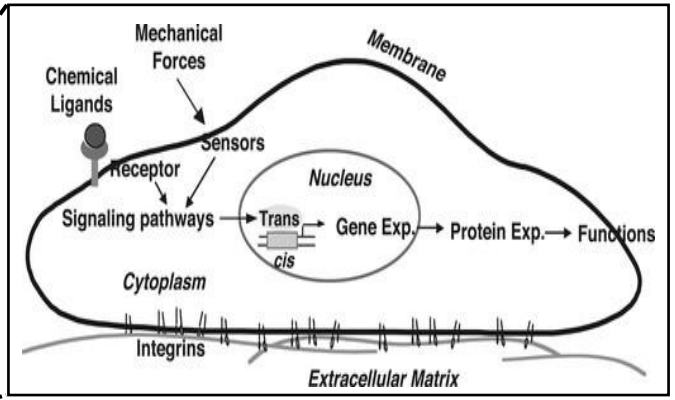
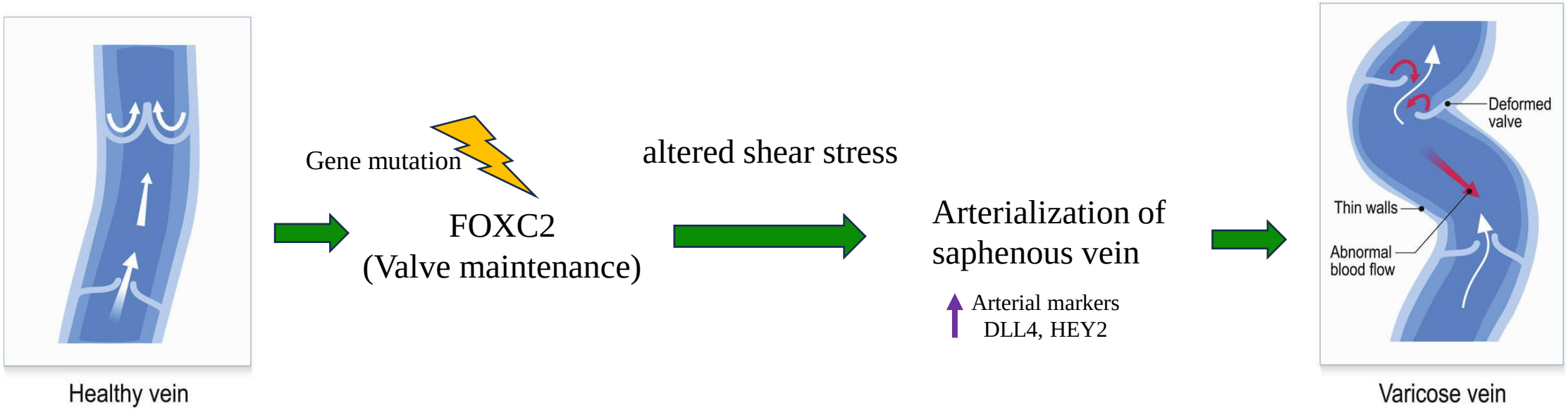


Image courtesy: Shu Chien., Am J Physiol Heart Circ Physiol, 2006

- Blood flow produces mechanical frictional forces, parallel to the blood flow exerted on the endothelial wall of the vessel called fluid shear stress. Represented in dyn/cm^2
- Two main types of flow exist in the vasculature: laminar and disturbed flow. Laminar flow occurs where vessel gemoetry is straight and uniform, whereas disturbed flow occurs where vessels bifurcate or curve highly.

Known:



- This indicates that Notch signaling is implicated in the pathological arterialization of the saphenous veins in varicosities.

- Notch signaling is a significant biological pathway that respond to fluid shear stress and regulates vasculogenesis, angiogenesis and arteriovenous differentiation.

- Studies in endothelial fluid shear stress model have identified several endothelial shear sensitive genes such as KLF2, ETS1, BMP4 etc (Sathanoori R et al.,2015).

- Moreover, ETS1 is found to be a transcriptional activator of Notch receptors1, 4 as well as ligand Dll4 (Yanjie Zhu et al.,2020).

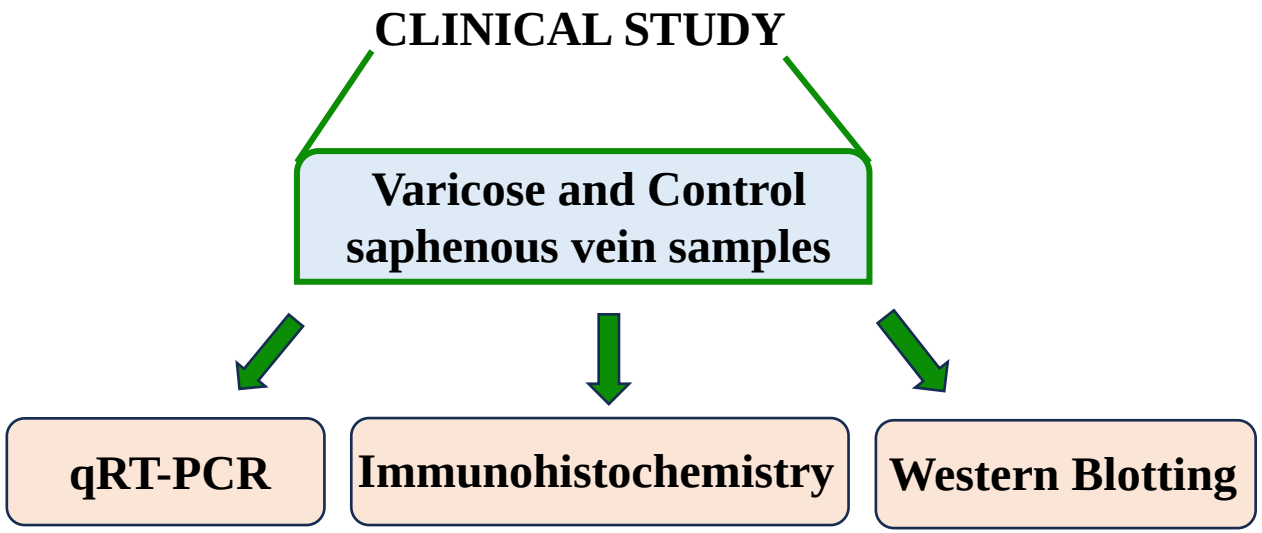
HYPOTHESIS

- Perturbations in hemodynamic forces in the venous system can initiate ETS1-Notch signaling in luminal endothelial cells that results in functional and structural changes in venous vasculature.

OBJECTIVES

- To analyze mRNA and protein expression of Notch receptors and their ligands in human varicose veins.
- To examine the expression pattern of mechanosensitive ETS1 in varicose veins at mRNA and protein level.
- To delineate ETS1-Notch4/Dll4 signaling in endothelial cells exposed to disturbed shear stress.

WORKFLOW



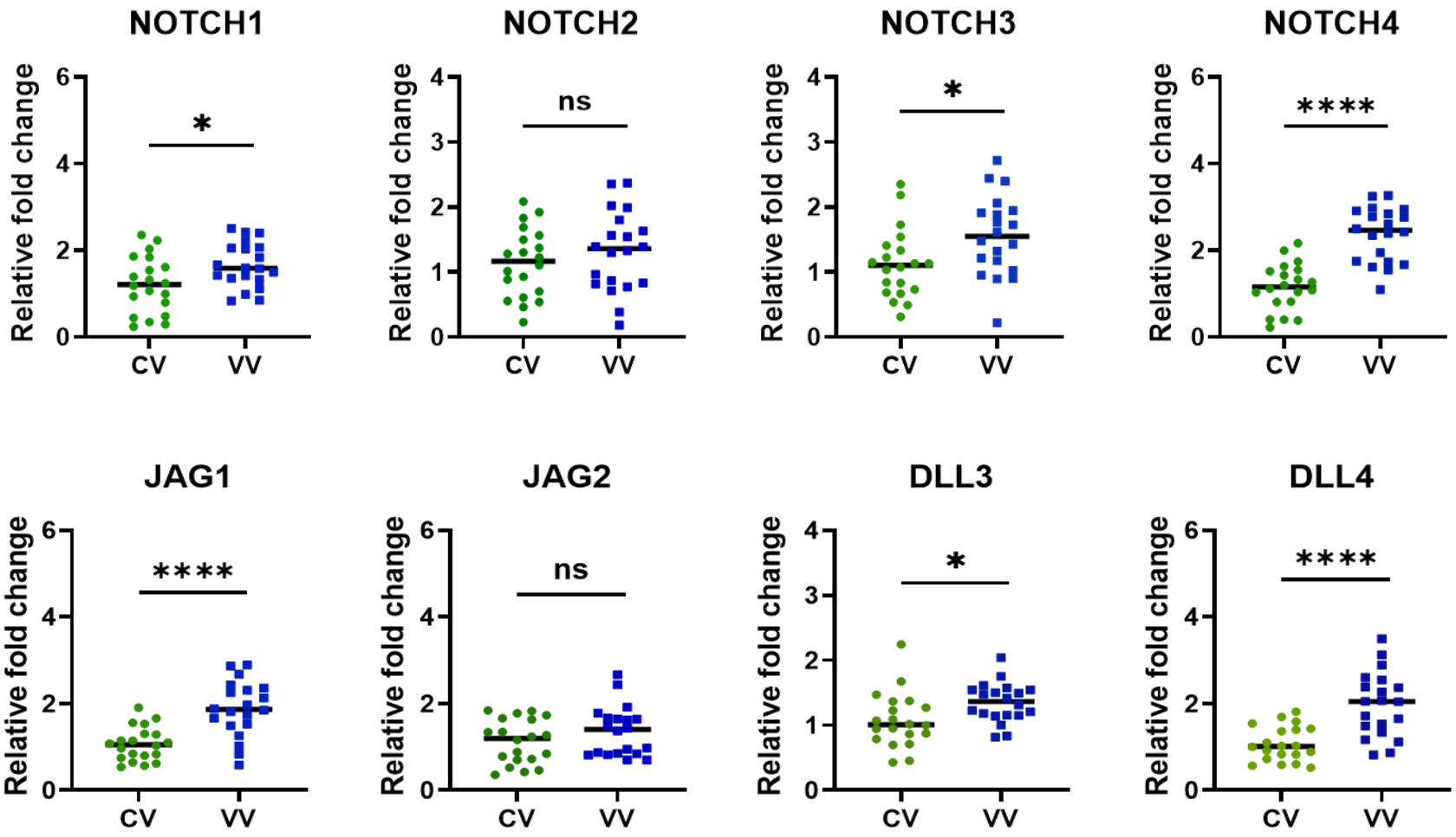
- Varicose vein samples were collected from patients (n=30) with CEAP Class 3 stage who underwent corrective surgery at Kempegowda Institute of Medical Science, Bangalore.
- Control saphenous veins were procured from 32 patients who had undergone coronary artery bypass grafting (CABG) at Sri Jayadeva Institute of Cardiovascular Sciences and Research, Bangalore.

Inclusion criteria → Diagnosis of varicose veins based on physical examination and doppler ultrasound

Exclusion criteria → Comorbidities, Individuals who had previous lower limb vascular surgery, deep vein thrombosis, varicosities due to neoplasms, pregnant women and minors

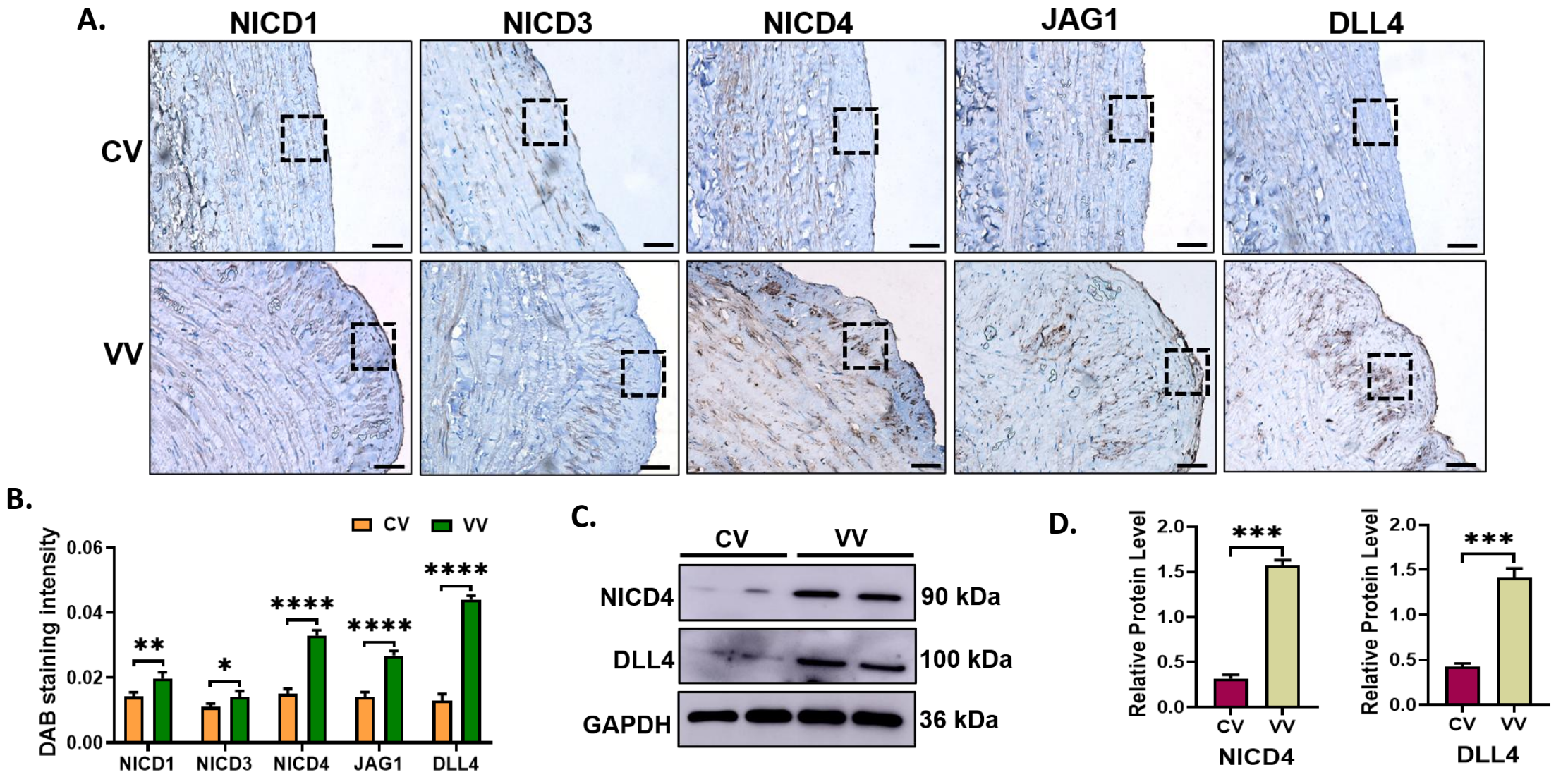
RESULTS

Notch signaling is upregulated in human varicose veins



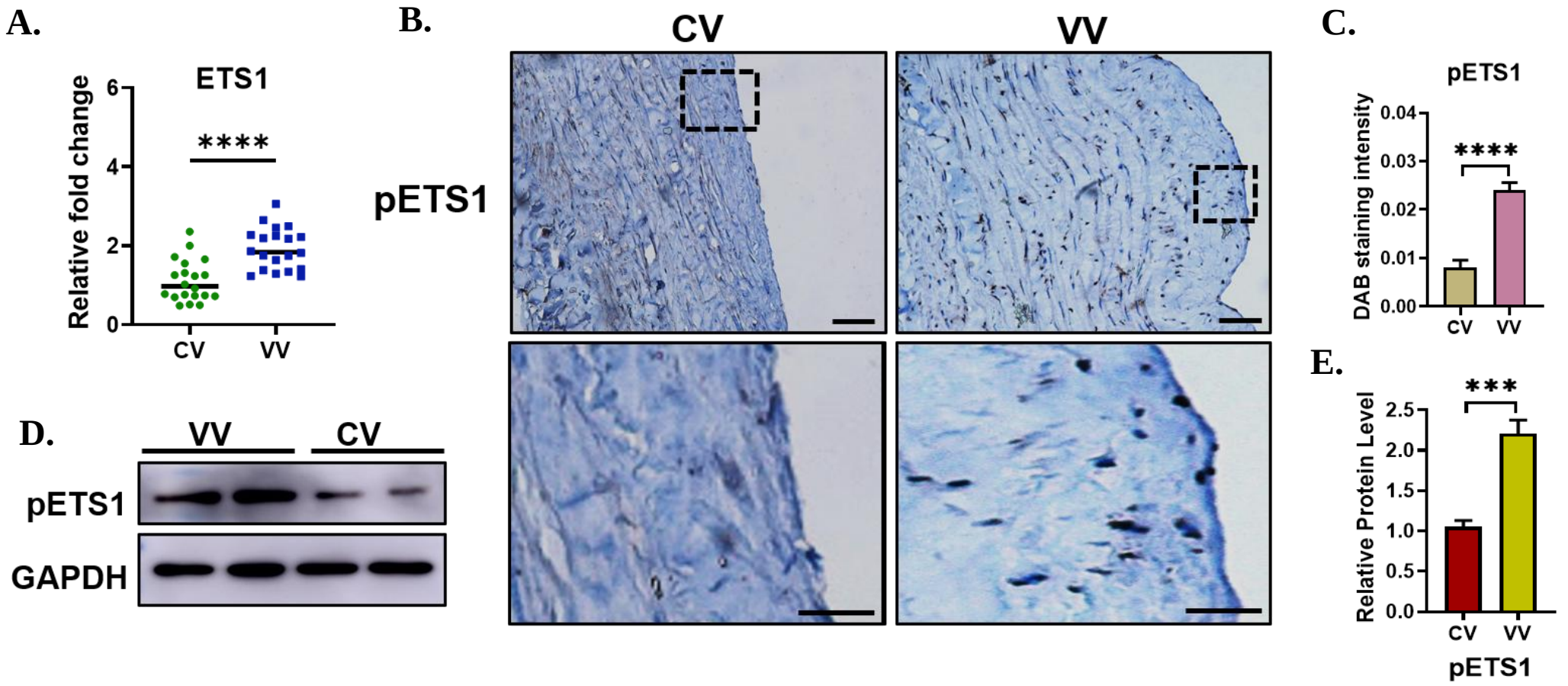
Scatter plots representing mRNA fold changes of Notch1-4, Jag1-2, Dll3-4 in 20 human varicose and control saphenous veins. Values are mean ± SD. *$p < 0.05$ vs control tissue, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. ns nonsignificant difference.

Notch4 and its ligand Dll4 is overexpressed in the neointima of varicose veins



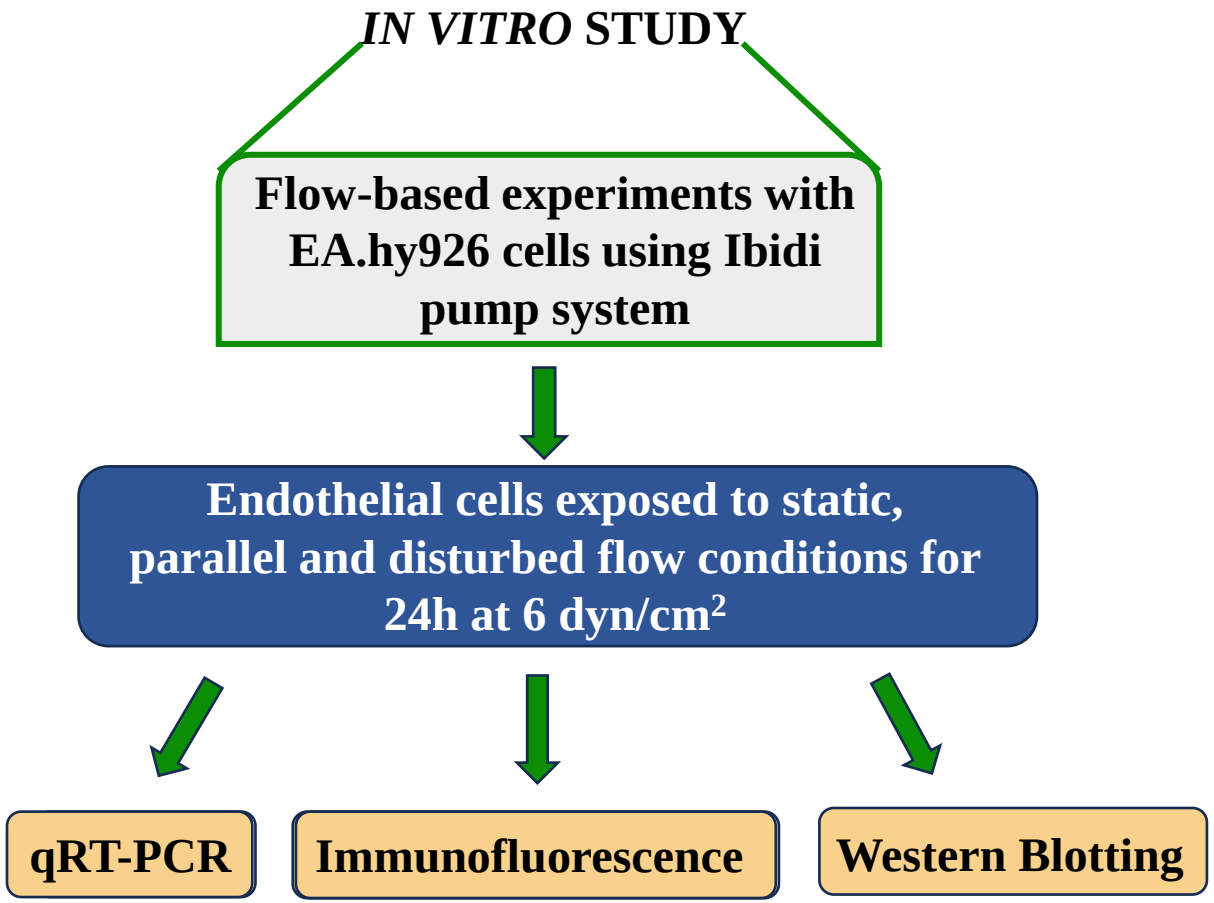
A. Immunostaining of Notch1,3,4, Jag1 and Dll4 in control(CV) and varicose vein(VV). Magnification 20X ,Scale bar 100 µm. **B.** Bar graph showing semiquantitative H score analysis. **C.** Representative western blots of NICD4 and Dll4 proteins. GAPDH was considered as loading control. **D.** Bar graph demonstrating densitometry analysis of immunoblots. *$p < 0.05$ vs control tissue, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. ns nonsignificant difference.

ETS1 is elevated at mRNA and protein levels in varicose veins



A. Scatter plot representing mRNA fold change of ETS1 in 20 human varicose(VV) and control saphenous veins(CV). **B.** Immunohistochemistry showing nuclear localization of phosphoETS1 in varicose veins. **C.** Bar graph representing semiquantitative H score Analysis. **D**. Representative western blot. **E.** Densitometry analysis of immunoblots. *$p < 0.05$ vs control tissue, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. ns nonsignificant difference.

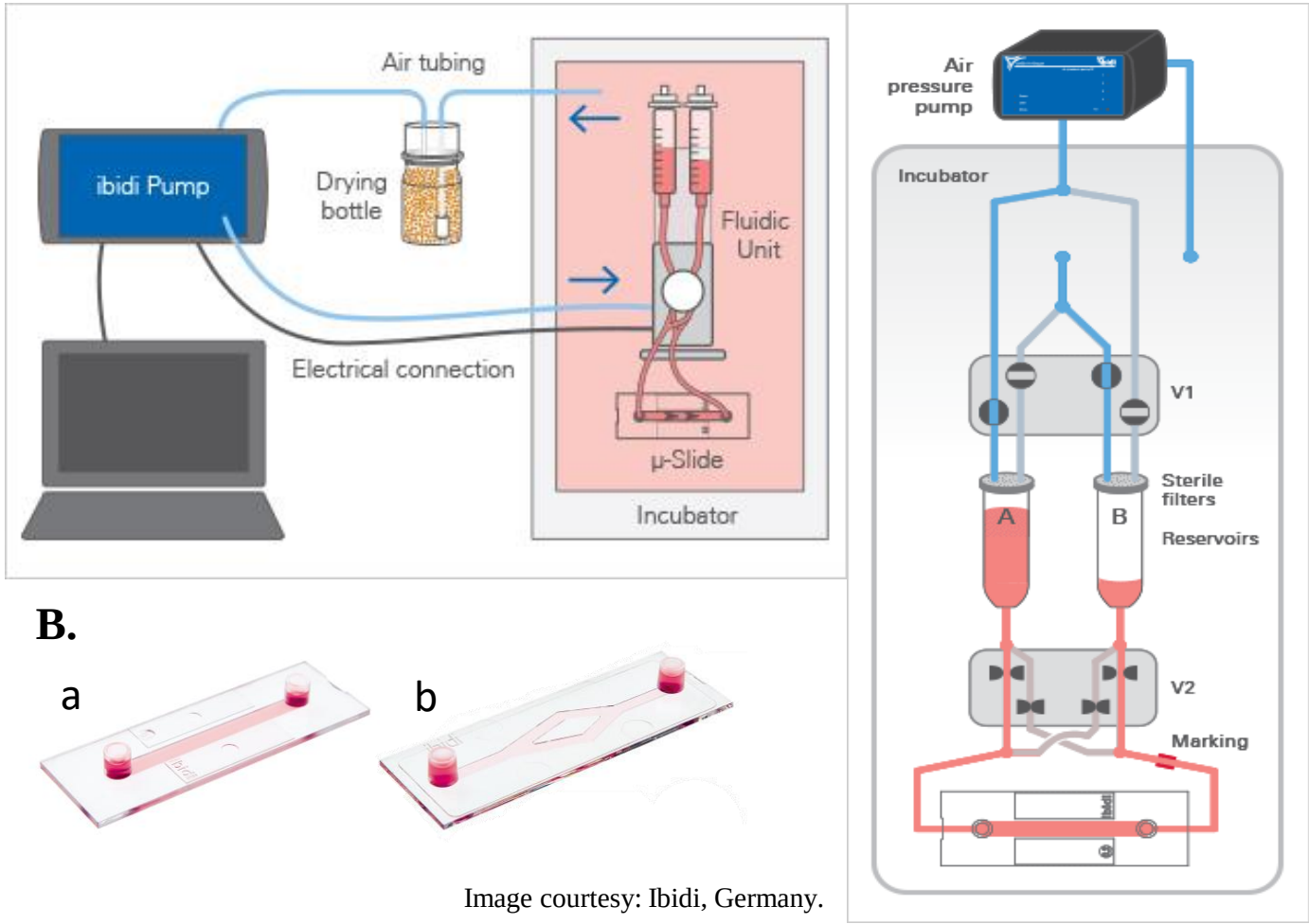
WORK DESIGN


IN VITRO STUDY
Flow-based experiments with EA.hy926 cells using Ibidi pump system
Endothelial cells exposed to static, parallel and disturbed flow conditions for 24h at 6 dyn/cm^2
qRT-PCR
Immunofluorescence
Western Blotting

Flow-based microfluidic Ibidi pump system

- The flow-based experiment was performed using an Ibidi pump system (Ibidi-Integrated BioDiagnostics, Germany) maintained in a humidified chamber containing 5% CO2.
- The Ibidi Pump System consists of two main components: the ibidi Pump and the Fluidic Unit.
- The Ibidi Pump applies pressurized air(in blue) to the reservoirs of the Fluidic Unit. Fluidic Unit performs valve-switching operations on the Perfusion Set (fluidic reservoirs and tubing) to generate unidirectional flow in a channel µ-slide.
- µ-slide I 0.4 Luer ibiTreat was used for performing uniform flow or to mimic normal venous flow, and µ-slide Y-shaped ibiTreat for non-uniform flow or to mimic disturbed shear stress.

A.

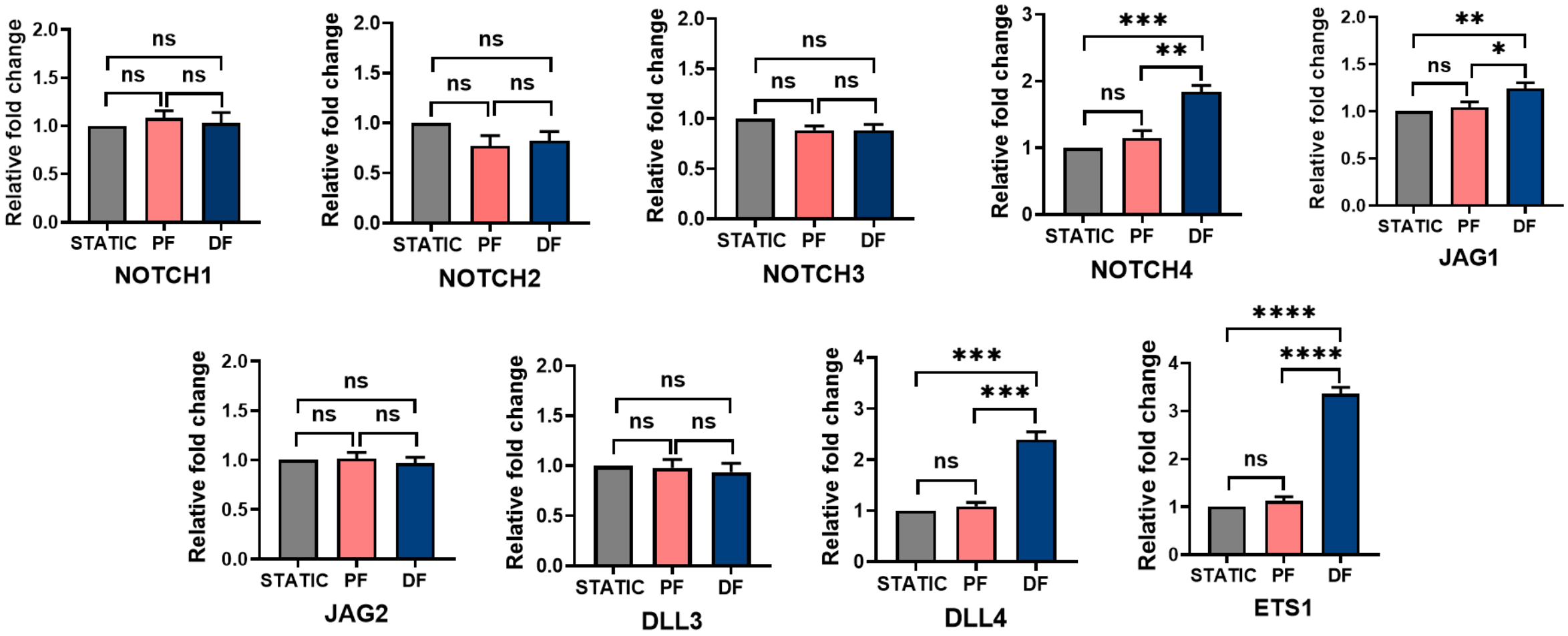


B.

Image courtesy: Ibidi, Germany.

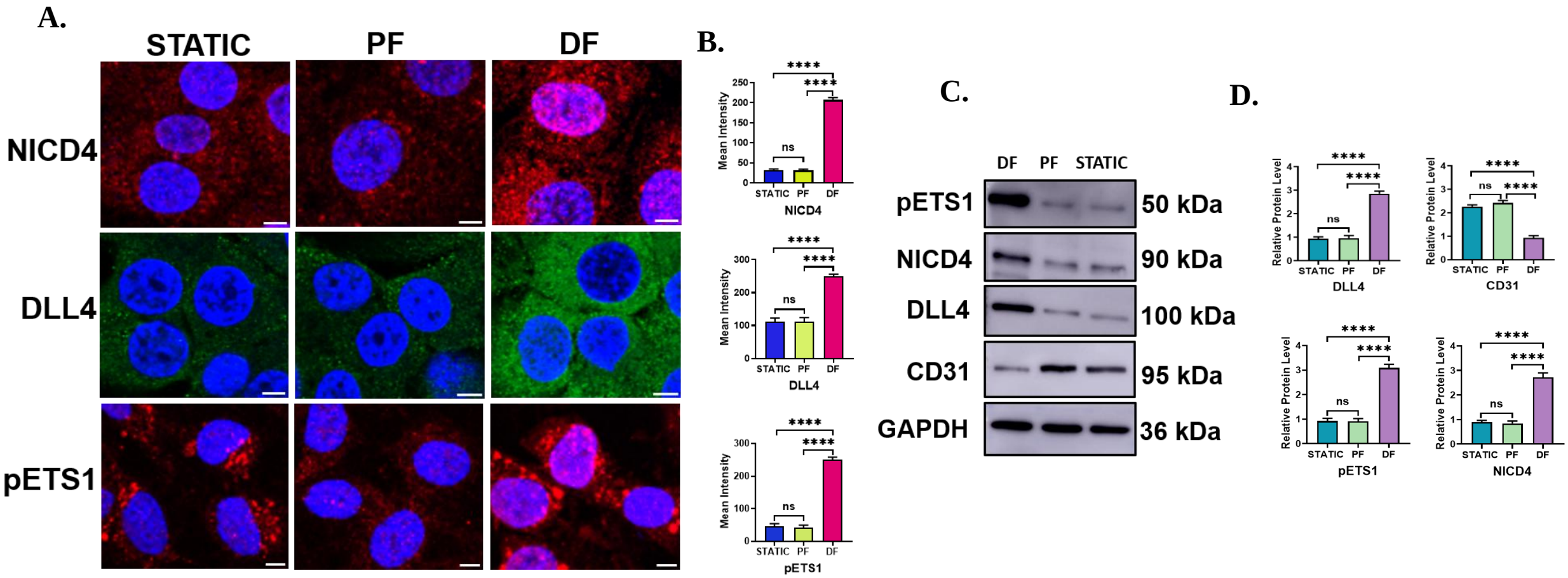
A. Schematic diagram representing flow-based microfluidic platform. **B.** Channel µ-slides: a. µ-slide I 0.4 Luer ibiTreat and b. µ-slide Y-shaped ibiTreat.

Disturbed venous flow induce pETS1-Notch4/Dll4 signaling in vein endothelial cells



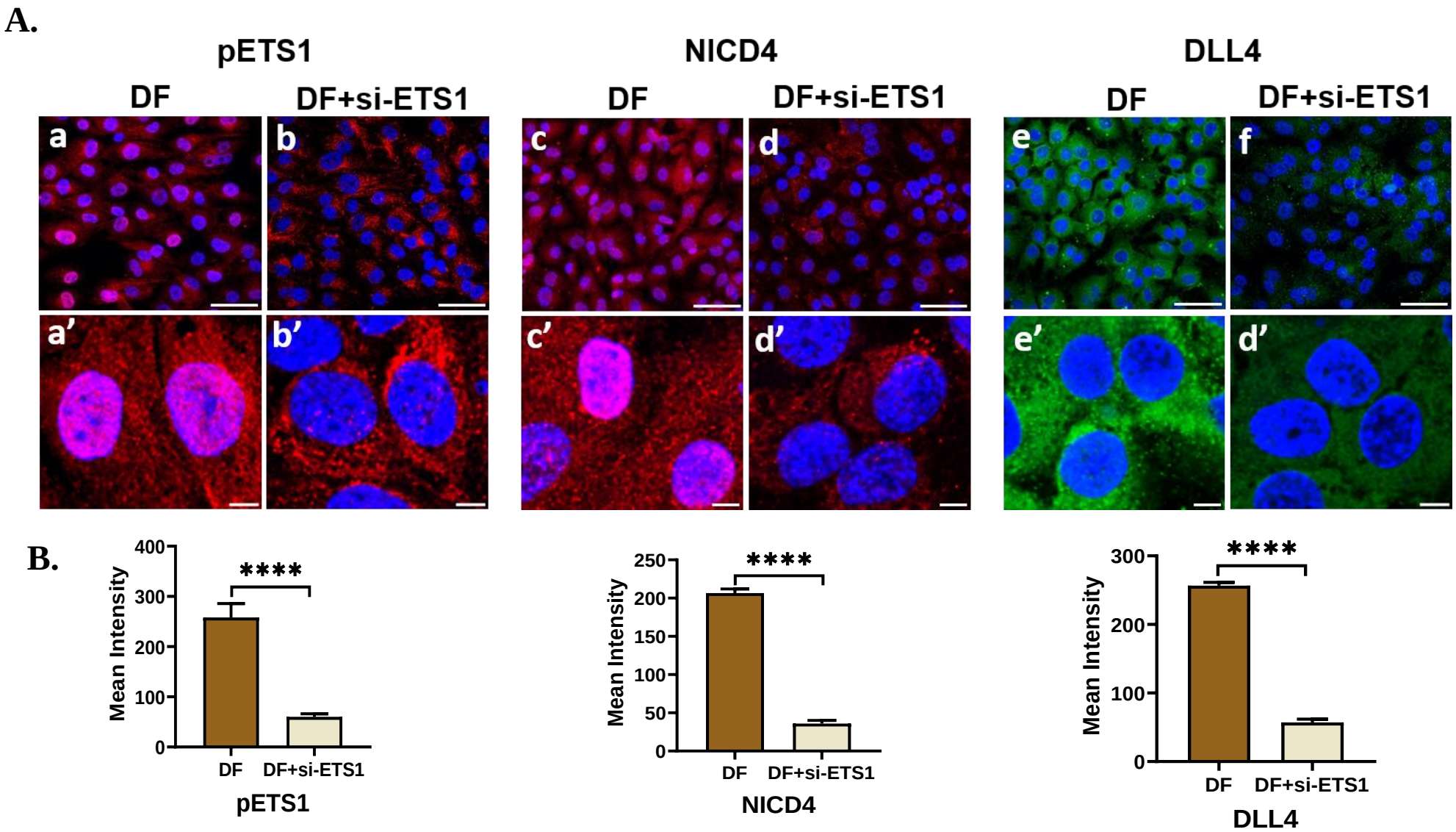
mRNA level expression of Notch receptors 1-4, ligands Jag1-2, Dll3-4 and ETS1 upon exposure of endothelial cells to disturbed fluid flow at 6 dyn/cm^2 for 24h (n=3). mRNA fold values in parallel and disturbed flow were calculated relative to the static control. All data were normalized with GAPDH expression and are given as relative to static control. *$p < 0.05$ vs control tissue, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. ns nonsignificant difference.

Disturbed venous flow induce pETS1-Notch4/Dll4 signaling in vein endothelial cells



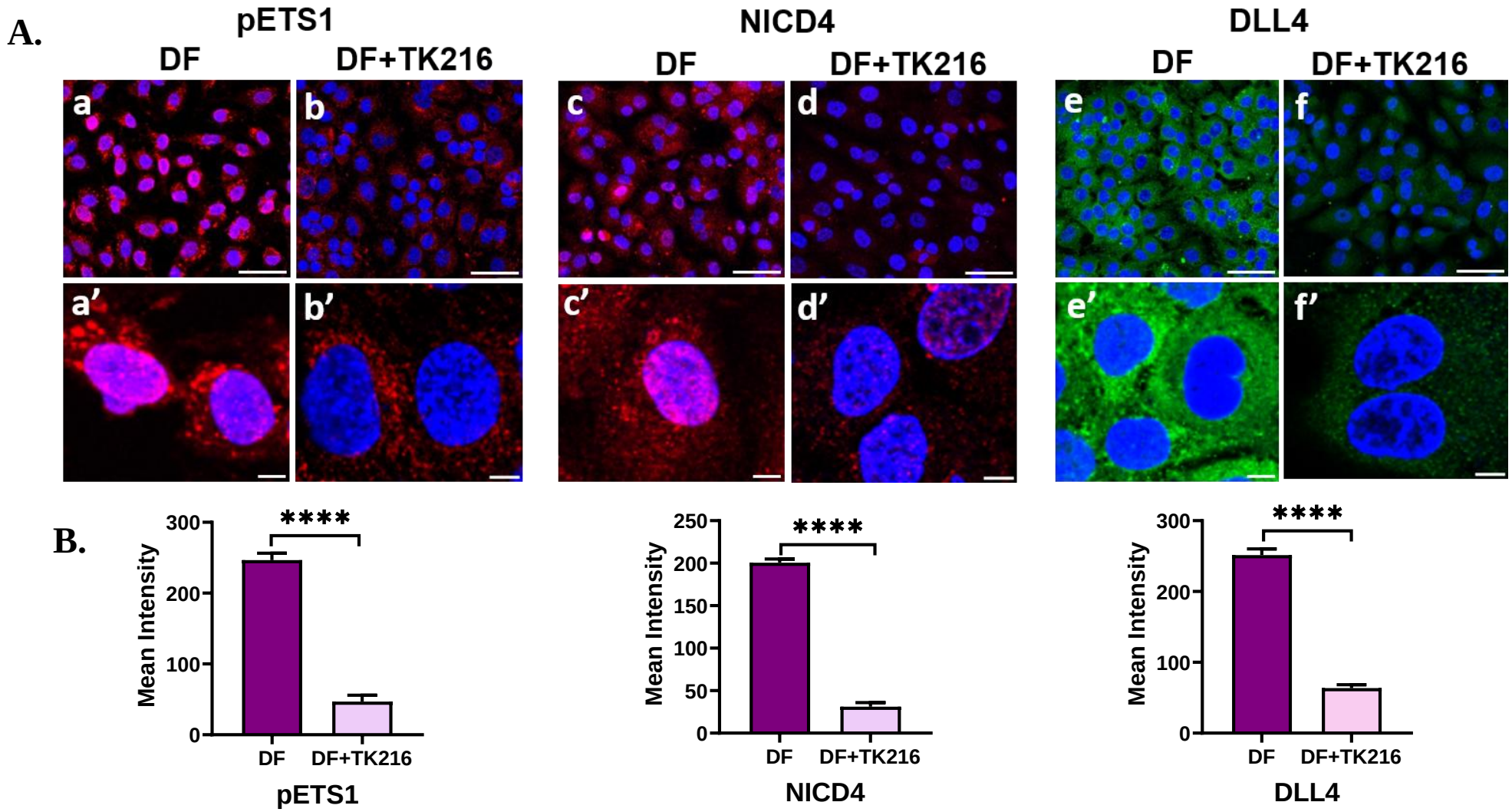
A. Immunofluorescence images of NICD4, DLL4 and phosphoETS1 in EA.hy926 cells exposed to Static, Parallel and for Disturbed flow at 6 dyn/cm^2 for 24h. High magnification, Scale bar - 20 µM. **B.** Mean fluorescence intensity bar graph PF- parallel uniform shear stress, and DF- disturbed shear stress. **C.** Representative western blots of pETS1, NICD4, Dll4 and CD31 proteins. GAPDH was considered as the loading control. **D.** Densitometry analysis of immunoblots. *$p < 0.05$ vs respective static or parallel uniform shear-treated groups, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. ns nonsignificant difference.

Knockdown of ETS1 downregulated Notch4/Dll4 protein expression



A. SiRNA mediated knockdown of pETS1 in endothelial cells exposed to disturbed flow significantly downregulated the expression profile of pETS1, NICD4 and Dll4 protein. (a-f scale bar 50 µM, magnification 40×, a'-f' scale bar 20 µM and high magnification) **B.** Bar graph showing Mean fluorescence intensity DF - disturbed shear stress, ****$p < 0.0001$.

Inhibition of ETS1 by TK216 significantly reduced the overexpression of NICD4 and Dll4



A. ETS1 inhibition by 1µm TK216 significantly reduced the overexpression of pETS1, NICD4 and Dll4 in endothelial cells exposed to disturbed flow for 24 h ($p < 0.0001$) (a-f scale bar 50 µM, magnification 40×, a'-f' scale bar 20 µM and high magnification). **B)** Mean fluorescence intensity bar graph.

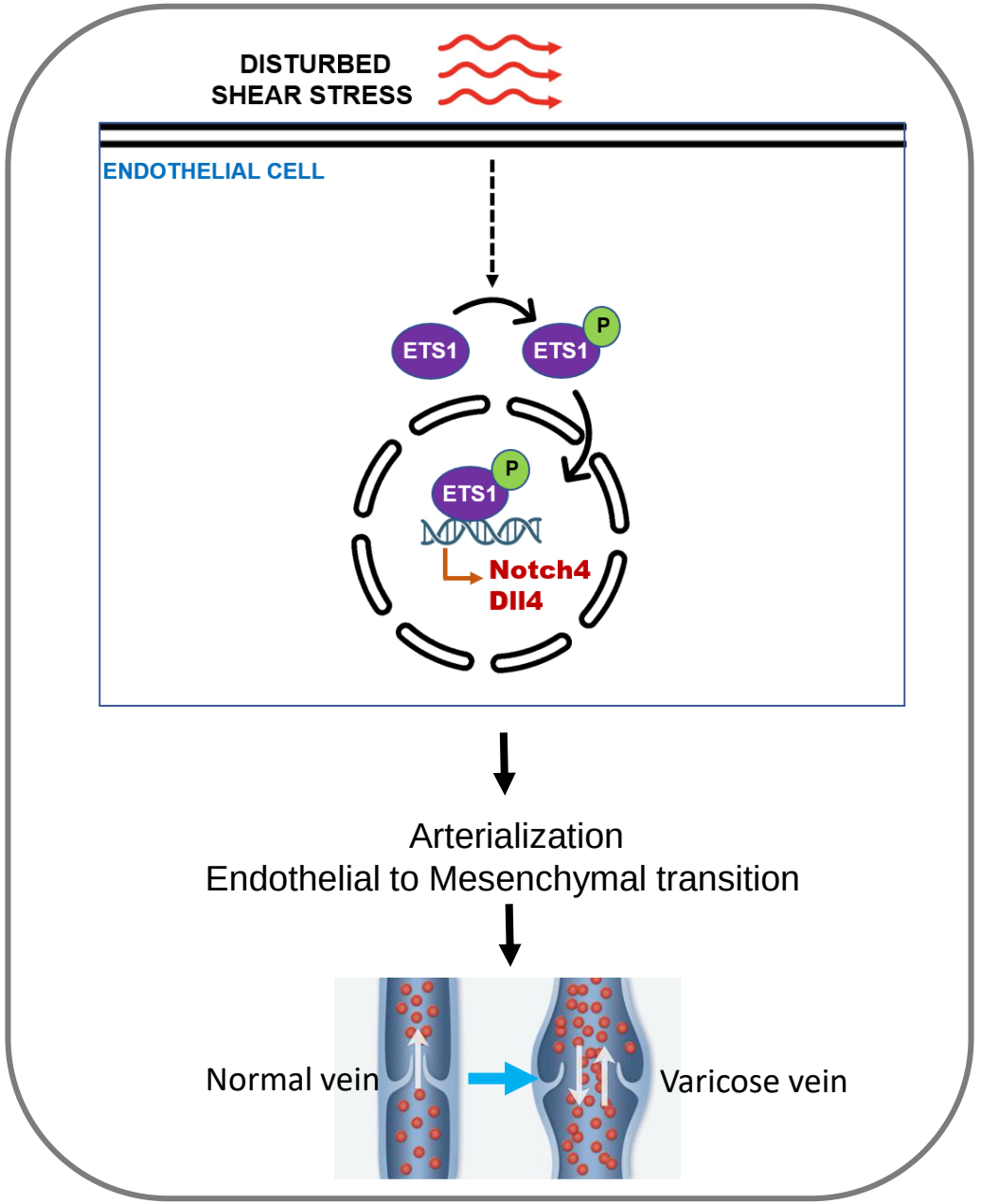
CONCLUSION

- Our study provides evidence for the role of disturbed fluid shear stress-mediated Notch4/Dll4 expression in the pathogenesis of varicose veins, presumably through ETS1.

- Targeting ETS1 rather than downstream Notch components may serve as an efficient strategy for varicose vein small molecular therapeutics.

FUTURE WORK

- To delineate the molecular mechanism by which fluid flow activate ETS1.



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