European Heart Journal (2015) **36**, 817–828
doi:10.1093/eurheartj/ehu179

BASIC SCIENCE

Targeting prolyl-isomerase Pin1 prevents mitochondrial oxidative stress and vascular dysfunction: insights in patients with diabetes

Francesco Paneni[1,2,3†], Sarah Costantino[1,2†], Lorenzo Castello[4], Rodolfo Battista[5], Giuliana Capretti[4], Sergio Chiandotto[4], Domenico D'Amario[6], Giuseppe Scavone[7], Angelo Villano[6], Alessandra Rustighi[8], Filippo Crea[6], Dario Pitocco[7], Gaetano Lanza[6], Massimo Volpe[3,4], Giannino Del Sal[8], Thomas F. Lüscher[1], and Francesco Cosentino[1,2*]

[1]Cardiology and Cardiovascular Research, Institute of Physiology and University Hospital, Zürich, Switzerland; [2]Cardiology Unit, Department of Medicine, Karolinska University Hospital, Solna, 171 76 Stockholm, Sweden; [3]IRCCS Neuromed, Pozzilli, Italy; [4]Cardiology, Department of Clinical and Molecular Medicine, University of Rome 'Sapienza', Rome, Italy; [5]Internal Medicine Unit, Civil Hospital, Sora, Italy; [6]Department of Cardiovascular Medicine, Catholic University, Rome, Italy; [7]Diabetes Care Unit, Internal Medicine, Catholic University, Rome, Italy; and [8]Laboratorio Nazionale CIB, AREA Science Park and Department of Life Sciences, University of Trieste, Trieste, Italy

Received 14 January 2014; revised 12 March 2014; accepted 31 March 2014; online publish-ahead-of-print 6 May 2014

This paper was handled by Stephanie Dimmeler (Prof. Johan-Wolfgang Goethe Universität, dimmeler@em.uni-frankfurt.de).

Aim Diabetes is a major driver of cardiovascular disease, but the underlying mechanisms remain elusive. Prolyl-isomerase Pin1 recognizes specific peptide bonds and modulates function of proteins altering cellular homoeostasis. The present study investigates Pin1 role in diabetes-induced vascular disease.

Methods and results In human aortic endothelial cells (HAECs) exposed to high glucose, up-regulation of Pin1-induced mitochondrial translocation of pro-oxidant adaptor $p66^{Shc}$ and subsequent organelle disruption. In this setting, Pin1 recognizes Ser-116 inhibitory phosphorylation of endothelial nitric oxide synthase (eNOS) leading to eNOS–caveolin-1 interaction and reduced NO availability. Pin1 also mediates hyperglycaemia-induced nuclear translocation of NF-κB p65, triggering VCAM-1, ICAM-1, and MCP-1 expression. Indeed, gene silencing of Pin1 in HAECs suppressed $p66^{Shc}$-dependent ROS production, restored NO release and blunted NF-kB p65 nuclear translocation. Consistently, diabetic $Pin1^{-/-}$ mice were protected against mitochondrial oxidative stress, endothelial dysfunction, and vascular inflammation. Increased expression and activity of Pin1 were also found in peripheral blood monocytes isolated from diabetic patients when compared with age-matched healthy controls. Interestingly, enough, Pin1 up-regulation was associated with impaired flow-mediated dilation, increased urinary 8-iso-prostaglandin $F_{2\alpha}$ and plasma levels of adhesion molecules.

Conclusions Pin1 drives diabetic vascular disease by causing mitochondrial oxidative stress, eNOS dysregulation as well as NF-kB-induced inflammation. These findings provide molecular insights for novel mechanism-based therapeutic strategies in patients with diabetes.

Keywords Oxidative stress • Endothelial function • Inflammation • Diabetes mellitus

* Corresponding author. Tel: +46 851775398, Fax: +46 8344964, Email: francesco.cosentino@ki.se

† F.P. and S.C. contributed equally to this work.

Translational Perspective

The present study demonstrates that Pin1 is a common activator of key pathways involved in diabetic vascular disease in different experimental settings including primary human endothelial cells, knockout mice, and diabetic patients. Gene silencing and genetic disruption of Pin1 prevent hyperglycaemia-induced mitochondrial oxidative stress, endothelial dysfunction, and vascular inflammation. Moreover, we have translated our findings to diabetic patients. In line with our experimental observations, Pin1 up-regulation is associated with impaired flow-mediated dilation, increased oxidative stress, and plasma levels of adhesion molecules. In perspective, these findings may provide the rationale for mechanism-based therapeutic strategies in patients with diabetes.

Introduction

Risk of cardiovascular complications is extremely high in patients with diabetes mellitus.[1] In this setting, hyperglycaemia is a key player in the development of atherosclerotic disease. Accumulation of reactive oxygen species and inflammation are major features of diabetic vascular phenotype.[2] However, the underlying mechanisms remain to be elucidated. A better understanding of the pathways involved in hyperglycaemia-induced vascular damage may provide the basis for novel therapeutic strategies to reduce the burden of cardiovascular disease in patients with diabetes.

Phosphorylation of proteins on serine or threonine residues preceding proline is emerging as a key signalling mechanism in several physiological and pathological processes.[3] The peptidyl-prolyl *cis-trans* isomerase Pin1 recognizes specific phosphorylated Ser/Thr-Pro-peptide bonds and regulates their conformational changes with high efficiency.[3] Pin1-catalysed isomerization modulates function of proteins involved in cellular homoeostasis. On the other hand, impaired expression and activity of Pin1 is implicated in the pathogenesis of cancer and Alzheimer's disease.[4] Indeed, Pin1 is overexpressed in most human cancers and correlates with prognosis.[5–7] This isomerase activates numerous oncogenes/growth enhancers and inhibits crucial tumour suppressors.[4] Moreover, Pin1 is required for the activation of NF-κB signalling in cancer cells[8–10] and participates to mitochondrial localization of oxidant proteins $p66^{Shc}$ and p53.[11–14]

Recent work has suggested that Pin1 may also play a role in the vascular endothelium.[15] In bovine endothelial cells, Pin1 negatively modulates endothelial nitric oxide synthase (eNOS) activity via isomerization of the phosphorylated Ser-116 residue.[15] In hyperglycaemic conditions, $p66^{Shc}$, eNOS, and NF-κB are key players triggering vascular complications.[2,16–18] The mitochondrial adaptor $p66^{Shc}$ is involved in the generation of reactive oxygen species leading to cellular apoptosis and vascular damage. Although the role of $p66^{Shc}$ as a regulator of lifespan in mammals has been challenged by recent controversial findings,[19] its importance is well-established in the setting of diabetic vascular complications. Genetic deletion of $p66^{Shc}$ protects against ROS-dependent endothelial dysfunction in diabetic mice.[16] Notably, $p66^{Shc}$ expression is increased in peripheral monocytes of patients with type 2 diabetes (T2DM) and correlates with oxidative stress.[20] Nitric oxide bioavailability is a key marker of vascular health and preservation of eNOS function warrants endothelial homoeostasis and prevents atherothrombosis.[2,21,22] In addition, nuclear translocation of NF-κB p65 leads to endothelial up-regulation of adhesion molecules critically involved in the diabetic atherosclerotic phenotype.[23–25] Hence, Pin1-dependent isomerization may alter function of proteins involved in the diabetic vascular phenotype.[18,26]

In experimental and human diabetes, we show that Pin1 is up-regulated by hyperglycaemia and triggers detrimental pathways leading to vascular complications. Targeting Pin1 protects against hyperglycaemia-induced mitochondrial oxidative stress, endothelial dysfunction, and vascular inflammation and may provide novel therapeutic insights in patients with diabetes.

Methods

A detailed description of the methods is provided in Supplementary material online.

Cell culture

Human aortic endothelial cells (HAECs, passages 5–7) were exposed for 3 days either to normal glucose (5 mmol/L) or high glucose concentrations (25 mmol/L). Mannitol (25 mmol/L) was used as an osmotic control.

Animals

Four- to six-month-old male C57BL/6 mice, Pin1 WT mice and $Pin1^{-/-}$ mice were used in all the experiments. Animal experiments were conducted in accordance with the guidelines approved by the Institutional Animal Care Committee of the University of Zürich, Switzerland, University of Rome 'Sapienza' and University of Trieste, Italy.

Study population

Thirty-seven patients with T2DM and 20 age-matched healthy subjects were consecutively recruited at the Cardiology Units of Sant'Andrea Hospital, University 'Sapienza' and Catholic University (Rome, Italy). The study protocol was approved by Local Ethics Committee and, in accordance with institutional Guidelines, all the participants were aware of the investigational nature of the study and gave written consent for their participation.

Statistical analysis

All data are presented as means ± SEM. Statistical comparison were made by using Student's *t*-test for unpaired data and one-way ANOVA, followed by Bonferroni's *post hoc* test, when appropriate. The between-variable correlations were measured by Spearman's analysis. Probability values <0.05 were considered statistically significant. All analyses were performed with GraphPad Prism (version 5.0) and SPSS (version 20) softwares.

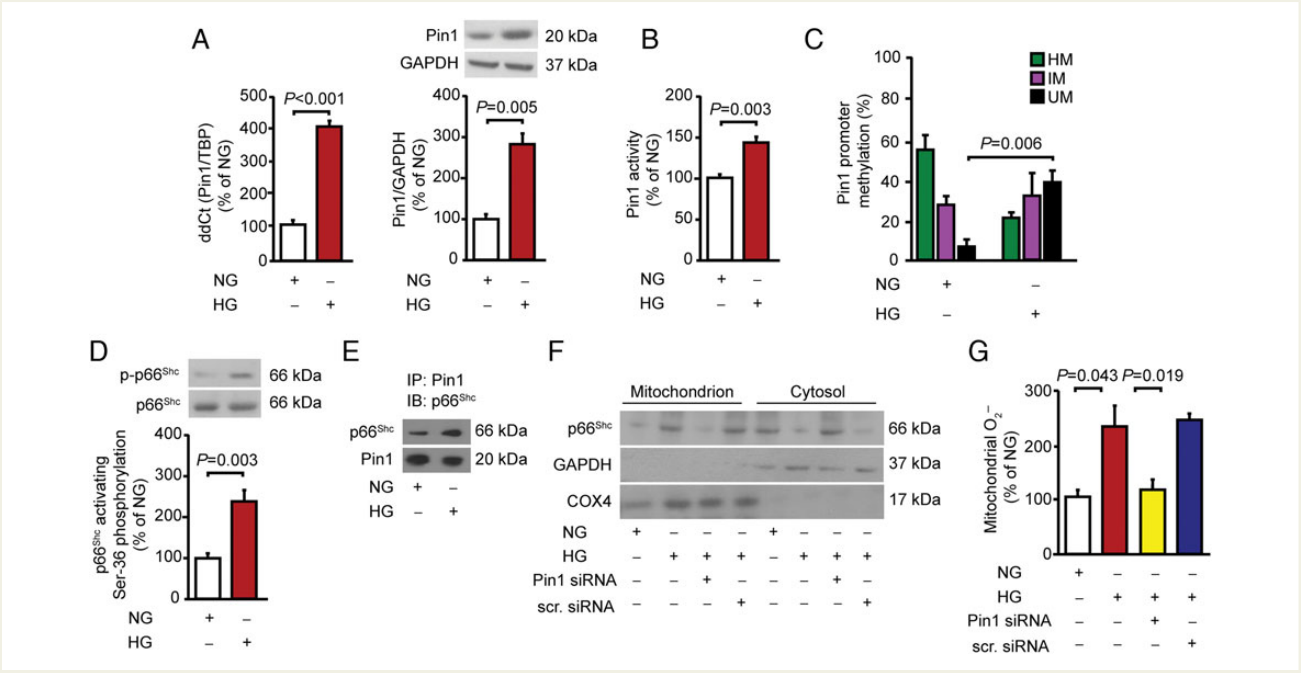


Figure 1 Hyperglycaemia-induced Pin1 up-regulation mediates mitochondrial oxidative stress in human aortic endothelial cells. (*A* and *B*) Pin1 gene ($n = 5$) and protein ($n = 6$) expression, as well as Pin1 activity ($n = 7$) in human aortic endothelial cells exposed to normal and high glucose concentrations. (*C*) Quantitative analysis of Pin1 promoter methylation in human aortic endothelial cells ($n = 6$). UM, unmethylated; IM, intermediately methylated; HM, hypermethylated CpG dinucleotides. (*D*) Western blot and densitometric quantification of $p66^{Shc}$ activating Ser-36 phosphorylation in human aortic endothelial cells exposed to normal and high glucose concentrations ($n = 4$). (*E*) Western blot showing the interaction of Pin1 with the mitochondrial adaptor $p66^{Shc}$ upon high glucose exposure. IB, immunoblotting; IP, immunoprecipitation. (*F*) Western blot showing $p66^{Shc}$ mitochondrial translocation in cells exposed to normal and high glucose in the presence or in the absence of Pin1 siRNA. Scrambled siRNA was used as a control. GAPDH and COX4 indicate loading controls for cytosolic and mitochondrial fractions, respectively. (*G*) ESR spectroscopy analysis of mitochondrial O_2^- generation ($n = 7$). Results are presented as means ± SEM. NG, normal glucose; HG, high glucose, O_2^-, superoxide anion.

Results

High glucose increases Pin1 expression and activity in human endothelial cells

To investigate the effects of hyperglycaemia on Pin1 expression, HAECs were exposed to high (HG, 25 mmol/L) and normal (NG, 5 mmol/L) glucose concentrations for 72 h. High glucose levels caused a significant up-regulation of Pin1 both at the mRNA and protein level (*Figure 1A*). Moreover, HG increased enzyme activity (*Figure 1B*). Interestingly, we found that Pin1 up-regulation is modulated by DNA-related epigenetic changes. Indeed, Pin1 promoter methylation, an important repressor of gene transcription, was significantly reduced in HAECs exposed to high glucose when compared with normal glucose (*Figure 1C*). Mannitol, used as an osmotic control, did not exert any effect on Pin1 expression or activity (data not shown).

Pin1 mediates $p66^{Shc}$-dependent mitochondrial ROS production

The molecular link between the isomerase Pin1 and mitochondrial adaptor $p66^{Shc}$ was investigated by pull-down experiments performed in HAECs exposed to HG and NG conditions. We observed that Pin1 recognizes Ser-36 phosphorylation of $p66^{Shc}$ induced by high glucose (*Figure 1D* and *E*). Interestingly, Pin1-dependent isomerization is able to induce $p66^{Shc}$ mitochondrial translocation and subsequent O_2^- generation (*Figure 1F* and *G*). Indeed, gene silencing of Pin1 blunted mitochondrial translocation of $p66^{Shc}$, preventing hyperglycaemia-induced ROS generation (*Figure 1F* and *G*).

Knockdown of Pin1 prevents mitochondrial network derangement and cytochrome *c* release

Loss of mitochondrial integrity is emerging as a determinant of endothelial dysfunction in diabetes.[27] In this regard, we investigated whether targeting Pin1 protects against hyperglycaemia-induced mitochondrial network disruption. Under control conditions, HAECs showed a complex network of thread-like mitochondria while exposure to high glucose induced a marked loss of mitochondrial networks characterized by smaller punctuate mitochondria, as shown by a confocal microscopy (*Figure 2A*). Interestingly, Pin1 knockdown prevented mitochondrial rupture and DNA fragmentation (*Figure 2A* and *B*). Moreover, isolated mitochondria were challenged with calcium overload and the rate of swelling determined by light scattering. Organelles from HAECs exposed to normal glucose showed stable absorbance throughout the 20-min time-course. In contrast, HG-treated cells displayed mitochondrial

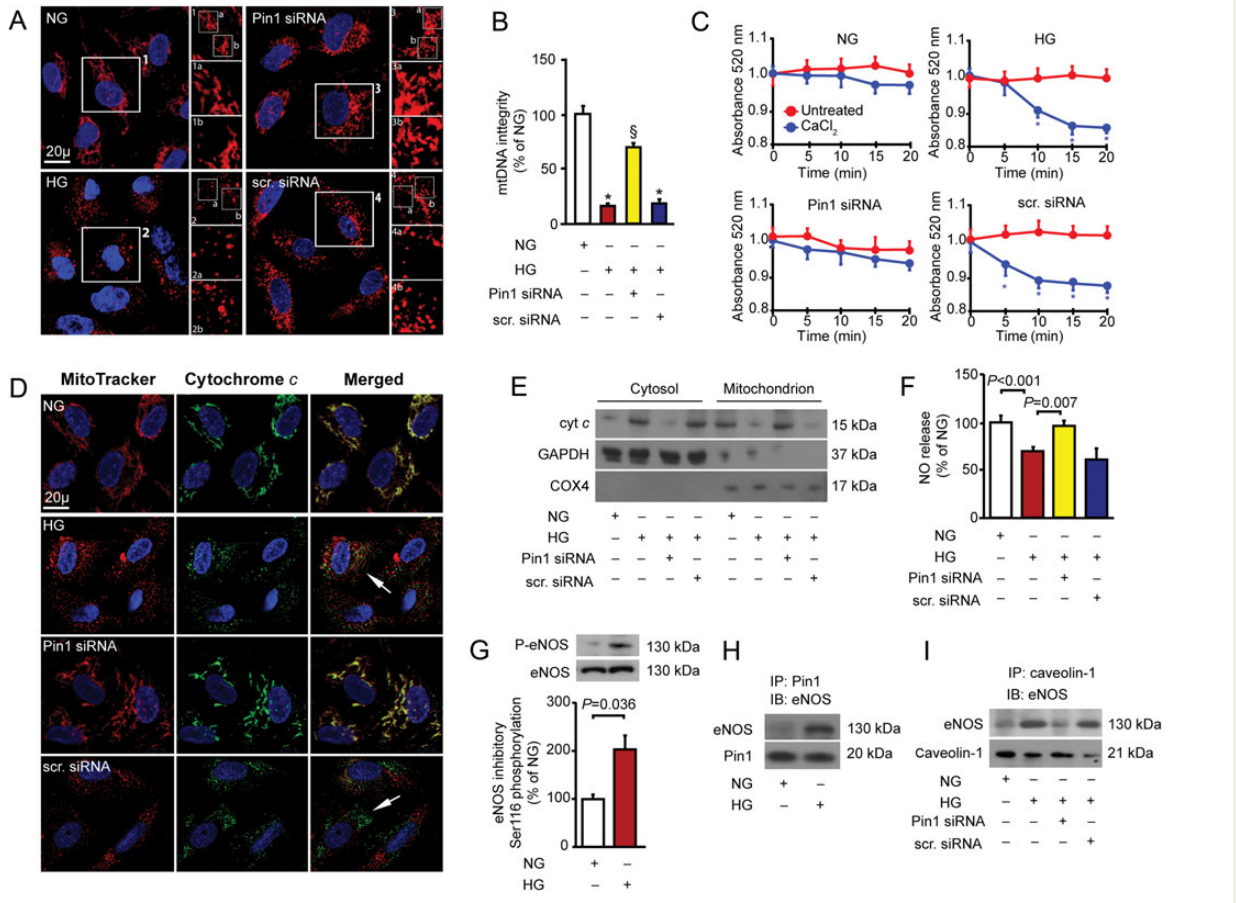


Figure 2 Targeting Pin1 rescues hyperglycaemia-induced mitochondrial disruption and endothelial nitric oxide synthase dysregulation. (*A*) Confocal microscopy images of mitoTracker-based mitochondrial staining showing alteration of an organelle network in human aortic endothelial cells exposed to high glucose in the presence or in the absence of Pin1 siRNA. Under normal glucose concentrations, human aortic endothelial cells show thread-like mitochondria (*Figure 1A* and *B*) while exposure to high glucose leads to smaller punctuate mitochondria (*Figure 2A* and *B*). Pin1 knockdown significantly attenuated mitochondrial disruption (*Figure 3A* and *B*). Scrambled siRNA was used as a control (*Figure 4A* and *B*). (*B*) Mitochondrial DNA integrity by real-time PCR ($n = 6$). *$P < 0.01$ vs. NG; §$P < 0.01$ vs. HG. (*C*) Mitochondrial swelling assay. Line graphs represent time-course of light absorbance decrease with (blue line) and without (red line) calcium overload ($n = 6$). *$P < 0.05$ vs. untreated. (*D*) Confocal microscopy images showing cytochrome *c* (green) release (arrows) from mitochondria stained with organelle marker MitoTracker (red). (*E*) Representative western blot (WB) showing cytochrome *c* release from mitochondria. (*F*) ESR spectroscopy analysis of nitric oxide release across the experimental conditions ($n = 6$). (*G*) Western blot and densitometric quantification of endothelial nitric oxide synthase inhibitory Ser-116 phosphorylation in human aortic endothelial cells exposed to normal and high glucose concentrations ($n = 4$). (*H*) Immunoprecipitation showing endothelial nitric oxide synthase–Pin1 association in cells exposed to normal or high glucose concentrations. (*I*) Endothelial nitric oxide synthase–caveolin-1 interaction in high glucose-treated human aortic endothelial cells with or without Pin1 siRNA. IB, immunoblotting; IP, immunoprecipitation. Results are presented as means ± SEM. NG, normal glucose, HG, high glucose.

swelling which was prevented by Pin1 down-regulation (*Figure 2C*). Silencing of Pin1 also protected against glucose-induced cytochrome *c* release, as assessed by double staining and western blot (*Figure 2D* and *E*).

Pin1 inhibits endothelial nitric oxide synthase activity

On the basis of previous studies showing that Pin1 regulates eNOS activity, we investigated whether Pin1 may affect eNOS in the presence of HG concentration. We found that endothelial NO release was impaired after exposure to HG, whereas concomitant silencing of Pin1 restored NO availability (*Figure 2F*). Pull-down experiments demonstrated that Pin1 recognizes high glucose-induced Ser-116 eNOS inhibitory phosphorylation, favouring association of eNOS with caveolin-1, an important repressor of eNOS catalytic activity[28] (*Figure 2G–I*). Accordingly, Pin1 knockdown prevented eNOS–caveolin-1 interaction, contributing to the preservation of NO availability in our setting (*Figure 2I*).

Pin1 mediates hyperglycaemia-induced up-regulation of adhesion molecules

Since Pin1 is a critical mediator of inflammation in cancer, we investigated whether this isomerase contributes to endothelial inflammation in hyperglycaemic conditions. Exposure to HG caused a

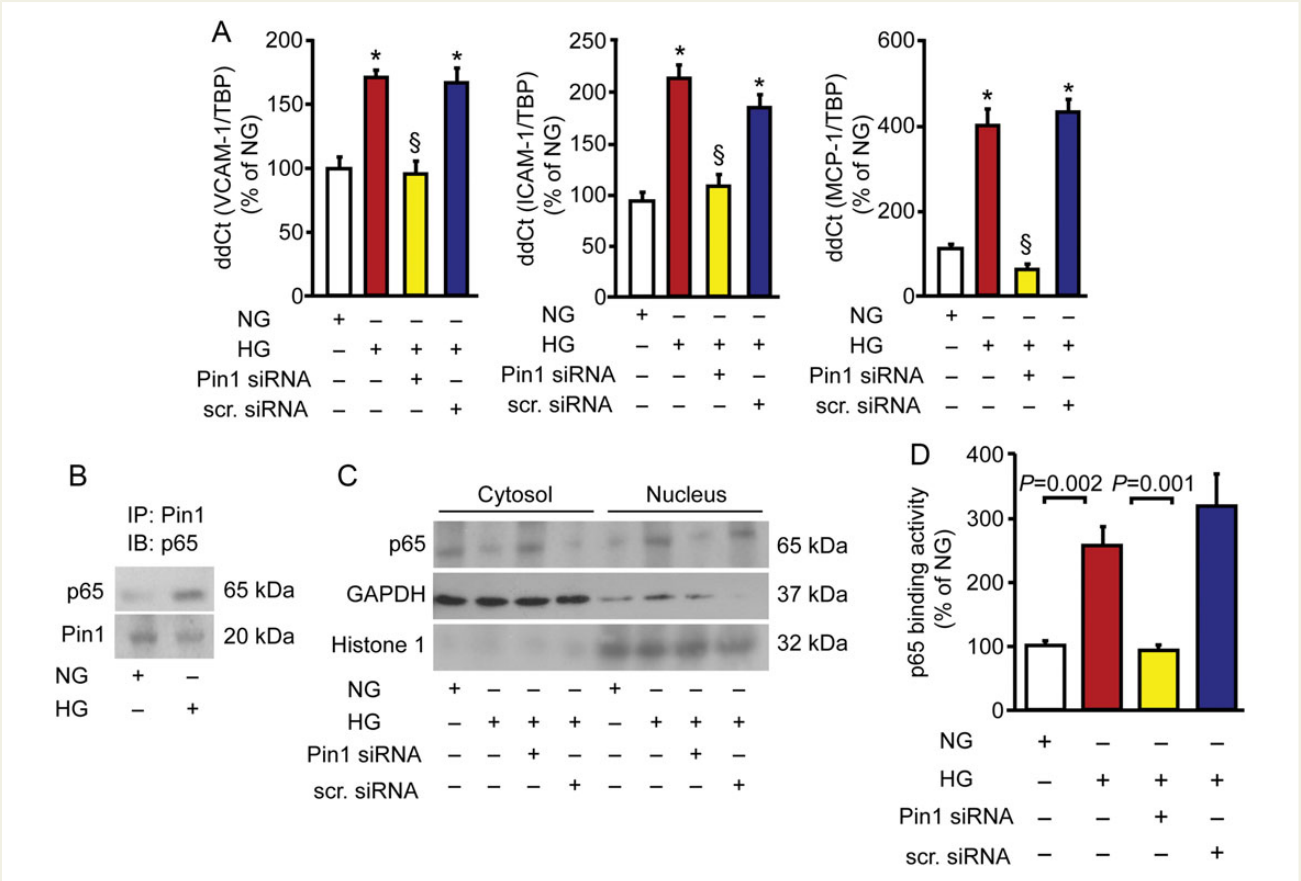


Figure 3 Pin1 triggers up-regulation of inflammatory adhesion molecules via nuclear factor kappa-B signalling. (*A*) Gene expression of inflammatory adhesion molecules vascular adhesion cell molecule-1, intercellular cell adhesion molecule-1, and MCP-1 in human aortic endothelial cells exposed to normal and high glucose concentrations in the presence or in the absence of Pin1 siRNA ($n = 6$). *$P < 0.01$ vs. NG§; $P < 0.01$ vs. HG. (*B*) Immunoprecipitation showing the interaction between Pin1 and NF-κB p65 in cells treated with high or normal glucose. IB, immunoblotting; IP, immunoprecipitation. (*C*) Representative western blot showing NF-κB p65 nuclear translocation. GAPDH and Histone 1 indicate loading controls for cytosolic and nuclear fractions, respectively. (*D*) NF-κB p65 binding activity ($n = 5$). Results are presented as means ± SEM. NG, normal glucose, HG, high glucose. VCAM-1, vascular adhesion cell molecule-1; ICAM-1, intercellular cell adhesion molecule-1; MCP-1, monocyte chemoattractant protein-1, NF-kB, nuclear factor kappa-B.

significant up-regulation of the adhesion molecules vascular adhesion cell molecule-1 (VCAM-1), intercellular cell adhesion molecule -1 (ICAM-1), and MCP-1 in HAECs (*Figure 3A*). In this setting, Pin1 recognizes NF-κB p65 favouring its nuclear translocation and increased binding activity (*Figure 3B–D*). Interestingly, Pin1 down-regulation by siRNA prevented NF-κB p65 activation and subsequent overexpression of adhesion molecules (*Figure 3A, C* and *D*).

Diabetic *Pin1*$^{-/-}$ mice are protected against endothelial dysfunction

Diabetes induced a significant increase of Pin1 expression in mouse aorta (*Figure 4A*). WT and *Pin1*$^{-/-}$ mice were studied to investigate whether Pin1 contributes to hyperglycaemia-induced endothelial dysfunction *in vivo*. Diabetic animals did not differ for body weight, glucose levels, blood pressure, and lipids (Supplementary material online, *Table S1*). Endothelial function was normal in control WT and *Pin1*$^{-/-}$ mice (*Figure 4B*). Interestingly, enough, endothelium-dependent relaxation to acetylcholine (10^{-9}–10^{-6} mol/L) was impaired in WT diabetic mice but not in *Pin1*$^{-/-}$ mice (*Figure 4B*). In contrast, endothelium-independent relaxation to sodium nitroprusside (10^{-10}–10^{-5} mol/L) did not differ across the experimental groups (data not shown).

Genetic deletion of Pin1 prevents mitochondrial oxidative stress

In line with our *in vitro* findings, mitochondrial ROS generation was abolished in *Pin1*$^{-/-}$ diabetic vessels (*Figure 4C*). Accordingly, genetic disruption of the isomerase prevented diabetes-related mitochondrial DNA fragmentation and swelling, two important hallmarks of mitochondrial dysfunction (*Figure 4D* and *E*). To further investigate the link between Pin1 and vascular dysfunction *in vivo*, knockdown of Pin1 was achieved by using siRNA technology in WT diabetic mice. Interestingly, Pin1 silencing rescued endothelial dysfunction when compared with scrambled-treated WT diabetic mice (*Figure 4F*).

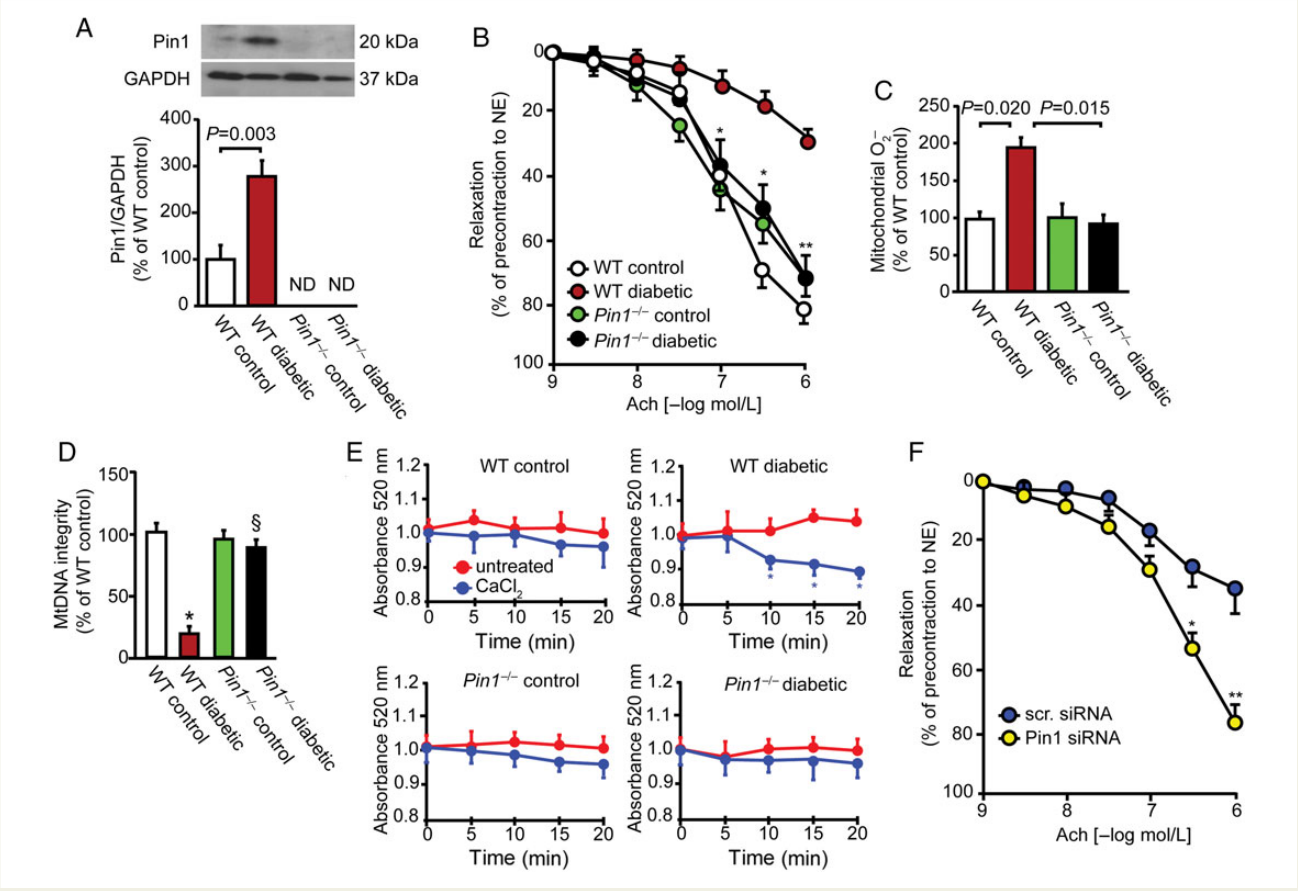


Figure 4 Genetic deletion of Pin1 protects against diabetes-related endothelial dysfunction and mitochondrial oxidative stress. (*A*) Representative western blot and densitometric quantification of Pin1 expression in the aorta of WT and *Pin1*$^{-/-}$ mice, with or without diabetes ($n = 5$). ND, not detectable. (*B*) Isometric tension studies in aortic rings isolated from the four experimental groups (WT control = 10, WT diabetic = 12, *Pin1*$^{-/-}$ control = 8, *Pin1*$^{-/-}$ diabetic = 7) per group. *$P < 0.05$ and **$P < 0.01$ vs. WT diabetic. NE, norepinephrine. (*C*) ESR spectroscopy analysis of mitochondrial superoxide anion (O_2^-) generation ($n = 6$). (*D*) Mitochondrial DNA integrity assessed by real-time PCR in the four experimental groups ($n = 7$). *$P < 0.01$ vs. WT control[§]; $P < 0.01$ vs. WT diabetic. (*E*) Mitochondrial swelling assay ($n = 6$). *$P < 0.05$ vs. untreated. (*F*) Endothelium-dependent relaxation in diabetic mice treated with scramble or Pin1 siRNA. *$P < 0.05$ and **$P < 0.01$ vs. scrambled siRNA. Results are presented as means ± SEM.

Suppression of diabetes-induced vascular inflammation in *Pin1*$^{-/-}$ mice

To investigate the effects of Pin1 on vascular inflammation, the expression of VCAM-1 and ICAM-1 was assessed in the aorta of WT and *Pin1*$^{-/-}$ diabetic mice. Aortas isolated from WT diabetic mice displayed a significant up-regulation of these adhesion molecules, as shown by immunofluorescence and real-time PCR (*Figure 5A* and *B*). Such an effect was explained by increased NF-κB p65 nuclear translocation and binding activity (*Figure 5C* and *D*). Interestingly, we found that diabetic mice lacking Pin1 were protected against vascular inflammation (*Figure 5A–D*).

Pin1 expression and activity in patients with diabetes

Pin1 gene expression and activity were assessed in peripheral blood monocytes of 37 patients with T2DM and 20 age-matched healthy controls. Type 2 diabetes mellitus subjects had higher BMI, waist circumference, blood pressure, and lower HDL levels (Supplementary material online, *Table S2*). Expression and activity of Pin1 were increased in T2DM and correlated with HbA_{1c} as well as fasting plasma glucose (FPG, *Figure 6A–C*).

Importantly, FPG ($\beta = 0.39$, $P = 0.021$) and HbA_{1c} ($\beta = 0.31$, $P = 0.034$) were independently associated with Pin1 gene expression, as shown by linear regression analysis adjusted for age, gender, waist circumference, blood pressure, HDL, and medications.

Pin1 up-regulation, endothelial dysfunction, and oxidative stress

Endothelial function, assessed by flow-mediated dilation (FMD), was significantly impaired in T2DM subjects when compared with controls (Supplementary material online, *Table S3*). Nitroglycerine-mediated dilatation was comparable in the two groups (Supplementary material online, *Table S3*) and no differences were observed in arterial diameter as well as resting or hyperaemic flow (data not shown). In agreement with an impairment of endothelial function, diabetic patients showed higher urinary levels of 8-iso$PGF_{2\alpha}$, *in vivo*

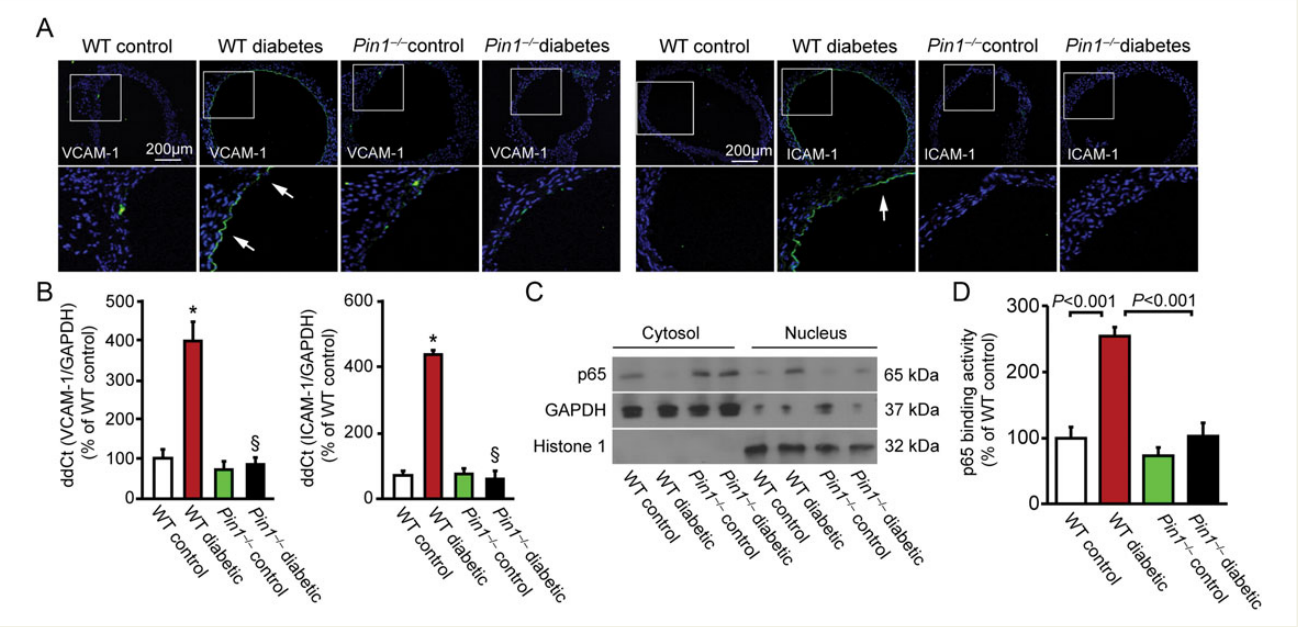


Figure 5 Loss of Pin1 suppresses vascular inflammation in diabetic mice. (*A*) Fluorescence microscopy images showing the expression of adhesion molecules vascular adhesion cell molecule-1 and intercellular cell adhesion molecule-1 (green) in aortic cross sections from WT and *Pin1*$^{-/-}$ mice with or without diabetes. Nuclei stained blue with DAPI. Higher magnification panels (bottom) show vascular adhesion cell molecule-1 and intercellular cell adhesion molecule-1 signals only in WT diabetic mice (arrows). (*B*) Gene expression of vascular adhesion cell molecule-1 and intercellular cell adhesion molecule-1 by real-time PCR ($n = 8$). *$P < 0.01$ vs. WT control[§]; $P < 0.01$ vs. WT diabetic. (*C*) Representative western blot of NF-κB p65 nuclear translocation in aortas isolated from the different experimental groups. (*D*) NF-κB p65 binding activity ($n = 5$). Results are presented as means ± SEM.

marker of oxidative stress (Supplementary material online, *Table S3*). Interestingly enough, Pin1 gene expression and activity significantly correlated with endothelial dysfunction and oxidative stress (*Figure 7A* and *B*). This finding was confirmed by linear regression analysis adjusted for confounding factors (Supplementary material online, *Table S4*).

Pin1 correlates with plasma adhesion molecules

We also investigated the correlation between Pin1 and plasma adhesion molecules. Vascular adhesion cell molecule-1, ICAM-1, and MCP-1 were significantly higher in T2DM when compared with controls (Supplementary material online, *Table S4*). Of interest, a significant association was found between Pin1 expression/activity and adhesion molecules, as shown by correlation and linear regression analysis (*Figure 8A*, Supplementary material online, *Table S4*). Collectively, these findings show that Pin1 may be critically involved in the vascular disease phenotype observed in diabetic patients (*Figure 8B*).

Discussion

Here, we show that Pin1 is up-regulated by hyperglycaemia and orchestrates pivotal molecular events triggering diabetic vascular disease. Several lines of evidence support our conclusions. Pin1 expression and activity are significantly increased in human endothelial cells exposed to high glucose and in aortas of diabetic mice. Activatory Ser-36 phosphorylation of adaptor protein p66^{Shc} is specifically recognized by Pin1 and this leads to p66^{Shc} mitochondrial translocation, ROS production, and organelle disruption. Pin1 also recognizes Ser-116 eNOS inhibitory phosphorylation contributing to impaired NO availability. Furthermore, this isomerase is required for hyperglycaemia-induced nuclear translocation of NF-κB p65, leading to up-regulation of inflammatory adhesion molecules. Importantly, genetic deletion of Pin1 prevented vascular oxidative stress, endothelial dysfunction, and NF-κB-driven inflammation in diabetic mice. In addition, the expression and activity of Pin1 are increased in peripheral blood monocytes of T2DM patients and correlate with brachial artery FMD, urinary levels of the oxidative marker 8-isoPGF$_{2\alpha}$, and plasma adhesion molecules.

Pin1 is emerging as a novel regulator of cellular function via modifications of protein structure upon recognition of a specific phosphorylation motif (Ser/Thr-Pro).[29] Pin1-dependent isomerization leads to stabilization of proteins in active configuration and enhances their degradation or accessibility for further modifications by other enzymes.[3] The biological relevance of such a signalling mechanism is supported by the notion that Pin1 dysregulation may contribute to diverse pathological conditions such as cancer and Alzheimer's disease.[3,4] Indeed, this isomerase is overexpressed in 38 out of 60 different human cancers and predicts prognosis.[5–7] In cancer cells, Pin1 recognizes a phosphorylated Thr-254-Pro motif of NF-κB p65 and inhibits its binding to IkBα, resulting in increased nuclear translocation.[8] Moreover, Pin1 controls mitochondrial trafficking of pro-oxidant proteins.[11,13,30] In murine

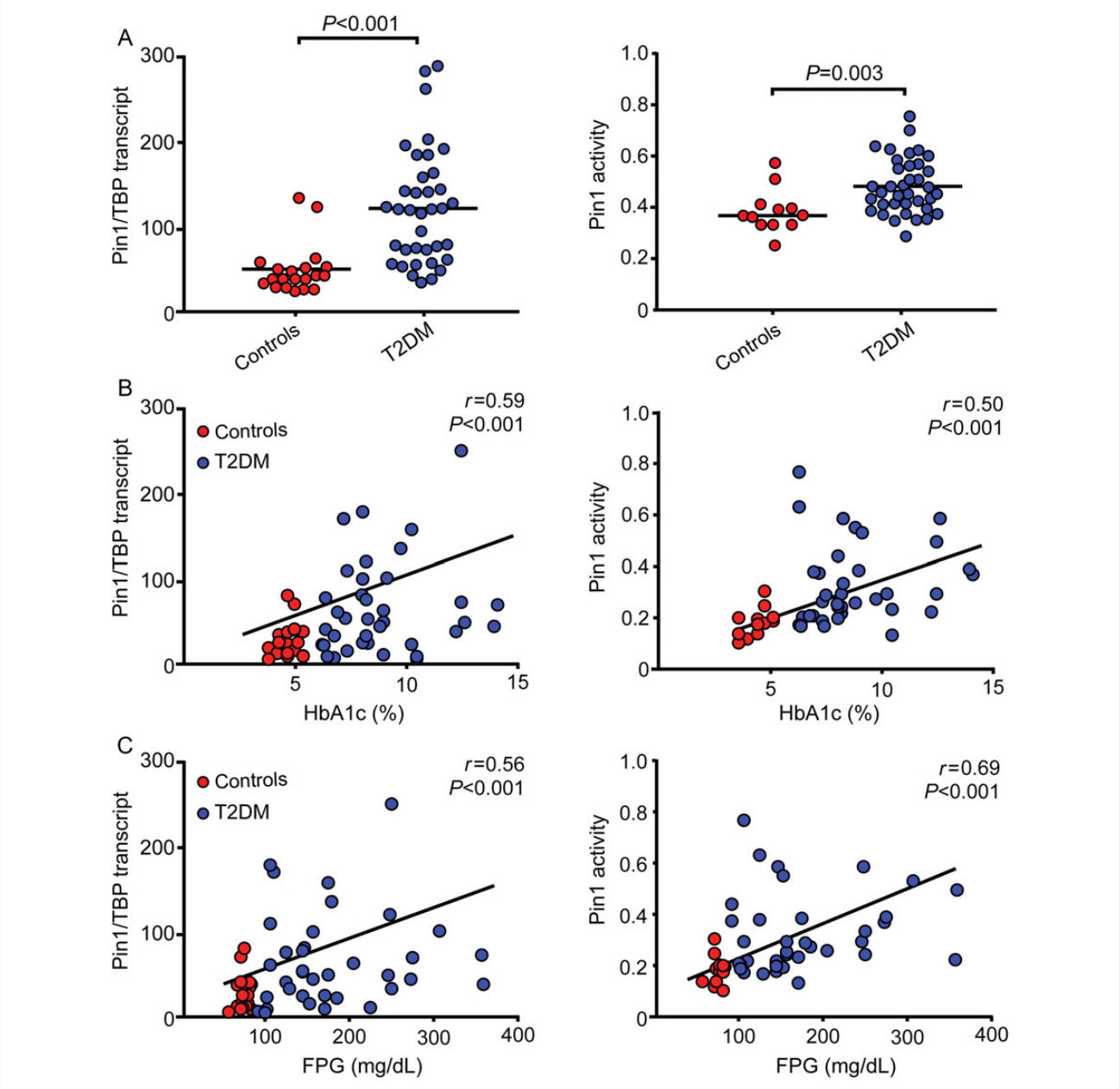


Figure 6 Pin1 expression and activity are increased in diabetic patients and correlate with glycaemic markers. (*A*) Scatter plots show Pin1 mRNA (controls = 20, T2DM = 37) and activity (controls = 12, T2DM = 37) in diabetic subjects and healthy controls. Results are presented as means ± SEM. (*B* and *C*) Correlation of Pin1 expression and activity with HbA_{1c} and FPG. *r* = Spearman's correlation coefficient. HbA_{1c} indicates glycosylated haemoglobin; FPG, fasting plasma glucose.

fibroblasts, Pin1 blockade prevents translocation of $p66^{Shc}$ to the mitochondria and oxidative stress.[11] Although Pin1 is an emerging trigger of proliferative, inflammatory, and pro-apoptotic signalling in cancer, its role in the pathogenesis of vascular disease remains to be elucidated. A recent work in bovine endothelial cells showed that Pin1 recognizes Ser-116 eNOS inhibitory phosphorylation and blunts NO release.[15] Indeed, pharmacological blockade of Pin1 induced a 30% increase in NO production.[15] Since mitochondrial oxidative stress, reduced NO availability, and vascular inflammation are major hallmarks of diabetic vascular disease,[18] we were prompted to investigate whether Pin1 is affected by hyperglycaemia and mediates vascular damage in this setting. Our findings show that Pin1 is up-regulated by high glucose levels and is responsible for mitochondrial translocation of $p66^{Shc}$ in the vascular endothelium. We and others have demonstrated that $p66^{Shc}$ is a key mediator of ROS generation and, hence, a major contributor of diabetic vascular complications.[16,17,31,32] Of note, $p66^{Shc}$ gene expression is increased in mononuclear cells obtained from patients with T2DM and correlates with oxidative stress.[20] The present study demonstrates that Pin1 is the upstream regulator

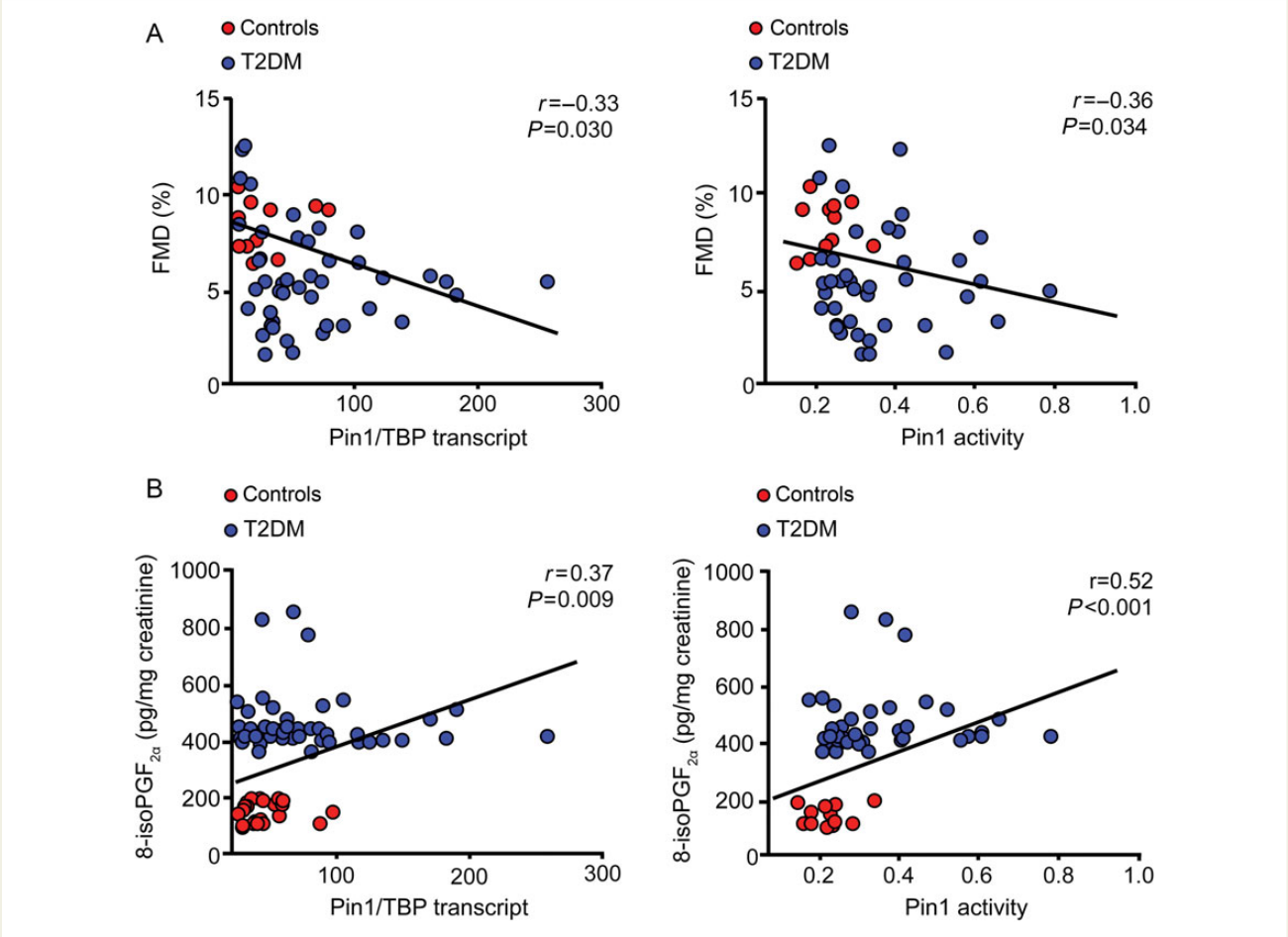


Figure 7 Pin1 correlates with endothelial function and oxidative stress. (*A* and *B*) Correlation of Pin1 expression and activity with flow-mediated vasodilation and urinary 8-isoPGF$_{2\alpha}$ levels. Red and blue circles indicate healthy controls ($n = 12–20$) and T2DM patients ($n = 37$), respectively. FMD, flow-mediated dilation; 8-isoPGF$_{2\alpha}$, 8-iso-prostaglandin F$_{2\alpha}$. r = Spearman's correlation coefficient.

of p66^{Shc} and its inhibition prevents oxidative stress and mitochondrial disruption in human endothelial cells and in mice under hyperglycaemic conditions. The relevance of our results is strengthened by recent work suggesting that loss of mitochondrial integrity may affect endothelial function in diabetic patients.[27,33]

We also found that in hyperglycaemic conditions Pin1 impairs eNOS activity via isomerization of its inhibitory Ser-116 residue. Proline-directed phosphorylation, requiring a proline at the $P + 1$ position, is a pre-requisite for Pin1 interaction and subsequent isomerization of its substrates. Of the five known serine/threonine phosphorylation sites in human eNOS, only Ser-116 conforms to the $P + 1$ proline requirement.[34] In our study, Pin1 isomerizes Ser-116 favouring eNOS interaction with caveolin-1, an important repressor of eNOS catalytic activity in the endothelium.[28,35] Accordingly, Pin1 deletion suppressed eNOS trafficking to the plasmalemmal caveolae and restored NO availability. This finding is supported by another study showing that transient Pin1 overexpression blunts NO release in unstimulated endothelial cells.[15] Furthermore, silencing of Pin1 prevents hyperglycaemia-induced oxidative stress in renal tubular cells[12] and neointima formation in mice by affecting proliferation of vascular smooth muscle cells.[12,36,37] These latter studies provided insights on the role of Pin1 in the vessel wall. However, our work demonstrates for the first time that Pin1 activation is a key driver of vascular damage in diabetes. Indeed, this study clearly shows that hyperglycaemia-induced Pin1 up-regulation leads to a deleterious vascular phenotype which can be prevented by targeting Pin1. Accordingly, *Pin1*$^{-/-}$ diabetic mice are protected against hyperglycaemia-induced endothelial dysfunction and mitochondrial oxidative stress.

We also provide strong evidence that Pin1 drives vascular inflammation in the setting of diabetes. Although it was recently shown that Pin1 promotes NF-κB p65 signalling in cancer cells,[8] no previous work investigated whether this isomerase mediates inflammation in the cardiovascular system. In our study Pin1-induced NF-κB p65 nuclear translocation and up-regulation of adhesion molecules *in vitro* and *in vivo*. We show here that deletion of Pin1 suppresses NF-κB p65 and expression of VCAM-1, ICAM-1, and MCP-1. These findings deserve attention since NF-κB activation has been reported in the endothelium of humans with T2DM.[23]

In contrast with our results, a previous study showed that *Pin1*$^{-/-}$ mice exhibit a significant impairment of endothelium-dependent relaxation and high blood pressure values.[38] Here, we

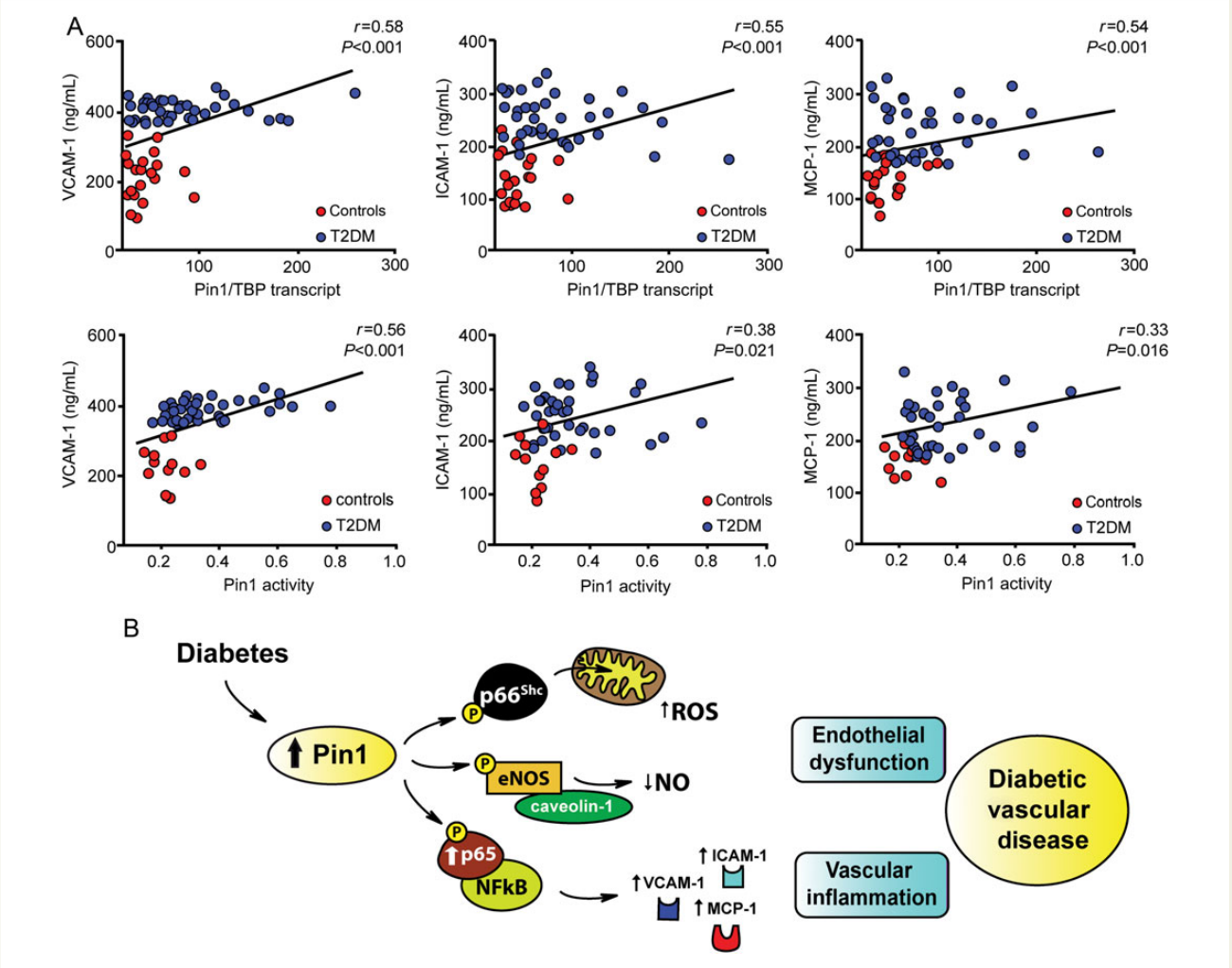


Figure 8 Pin1 correlates with inflammatory adhesion molecules in patients with diabetes. (*A*) Correlation of Pin1 expression and activity with plasma adhesion molecules vascular adhesion cell molecule-1, intercellular cell adhesion molecule-1, and monocyte chemoattractant protein-1. Red and blue circles indicate healthy controls ($n = 12–20$) and diabetic patients ($n = 37$), respectively. r = Spearman's correlation coefficient. VCAM-1, vascular adhesion cell molecule-1; ICAM-1, intercellular cell adhesion molecule-1; MCP-1, monocyte chemoattractant protein-1. (*B*) Schematic representation of the Pin1 role in diabetic vascular disease. Diabetes causes up-regulation of Pin1 favouring its interaction with phosphorylated serine and threonine residues of adaptor protein $p66^{Shc}$, endothelial nitric oxide synthase, and NF-κB p65. Pin1-$p66^{Shc}$ interaction is required for translocation of $p66^{Shc}$, mitochondrial ROS generation, and disruption. Phosphorylated endothelial nitric oxide synthase at Ser-116 is recognized by Pin1 leading to endothelial nitric oxide synthase–caveolin-1 interaction, blunted endothelial nitric oxide synthase activity and reduced NO release. Pin1 also triggers diabetes-induced nuclear translocation of NF-κB p65 and subsequent up-regulation of inflammatory adhesion molecules. All together these findings indicate that Pin1-induced conformational changes are critically involved in the diabetic vascular disease phenotype.

do not observe any endothelial dysfunction or hypertension in mice lacking Pin1. Different age of animals and experimental conditions may contribute to explain such discrepancy. In line with our findings, it was recently reported that blood pressure values and cardiac phenotype are comparable in WT and *Pin1*$^{-/-}$ mice.[39] Additional experimental observations suggest that silencing of Pin1 prevents angiotensin II-dependent oxidative stress, indicating that Pin1 blockade may rather be protective against arterial hypertension.[12] Another study also confirmed our results by showing that Pin1 overexpression impairs endothelium-dependent vasorelaxation in mice.[15]

In the present study, we have also translated our experimental findings to diabetic patients. Indeed, we found a significant up-regulation of Pin1 in subjects with T2DM when compared with age-matched healthy controls. Moreover, Pin1 correlated with brachial artery FMD, urinary 8-isoPGF$_{2\alpha}$, and plasma levels of adhesion molecules. Linear regression analyses showed that Pin1 was associated with oxidative stress, endothelial dysfunction, and vascular inflammation regardless of confounding factors. FPG and HbA_{1c} were also independent predictors of Pin1 up-regulation. Although the assessment of Pin1 expression in peripheral monocytes cannot be directly related to endothelial dysfunction, these findings are in

accordance with our experiments in HAECs demonstrating a key role of the isomerase in the human endothelium. The strength of our work is the observation of Pin1 as a key mediator of hyperglycaemia-related vascular damage across different experimental settings including knockout mice, primary human endothelial cells, and T2DM patients. Another novel finding is that hyperglycaemia reduces methylation of the Pin1 promoter. Since methylation is an important repressor of gene transcription,[40,41] our findings suggest that this epigenetic signature may contribute to Pin1 up-regulation in this setting. Undoubtedly, further studies are needed to better characterize epigenetic-driven Pin1 transcription in disease states.

In conclusion, we have shown that Pin1-dependent isomerization modulates key proteins involved in diabetic vascular disease (*Figure 8B*). Targeting Pin1 may restore vascular health by preventing such deleterious events. These findings have important implications for future mechanism-based therapeutic strategies in patients with diabetes.

Supplementary material

Supplementary material is available at *European Heart Journal* online.

Funding

This study was supported by grants from the Swiss Heart Foundation, Italian Ministry of Education, University and Research, PRIN 2010-2011 (to F.C.), and the Swiss National Research Foundation to T.F.L (3100-06811802/1). AIRC Special Program Molecular Clinical Oncology '5 per mille' and Italian Ministry of University and Research (RBAP10XKNC_003 and PRIN 2009- 2009YP9AE5) to G.D.S. F.P was the recipient of a PhD programme in Experimental Medicine at the University of Rome 'Sapienza'.

Conflicts of interest: none declared.

References

1. Beckman JA, Paneni F, Cosentino F, Creager MA. Diabetes and vascular disease: pathophysiology, clinical consequences, and medical therapy: part II. *Eur Heart J* 2013;**34**:2444–2452.
2. Paneni F, Beckman JA, Creager MA, Cosentino F. Diabetes and vascular disease: pathophysiology, clinical consequences, and medical therapy: part I. *Eur Heart J* 2013;**34**:2436–2443.
3. Lu KP, Zhou XZ. The prolyl isomerase PIN1: a pivotal new twist in phosphorylation signalling and disease. *Nat Rev Mol Cell Biol* 2007;**8**:904–916.
4. Lee TH, Pastorino L, Lu KP. Peptidyl-prolyl cis-trans isomerase Pin1 in ageing, cancer and Alzheimer disease. *Expert Rev Mol Med* 2011;**13**:e21.
5. Girardini JE, Napoli M, Piazza S, Rustighi A, Marotta C, Radaelli E, Capaci V, Jordan L, Quinlan P, Thompson A, Mano M, Rosato A, Crook T, Scanziani E, Means AR, Lozano G, Schneider C, Del Sal G. A Pin1/mutant p53 axis promotes aggressiveness in breast cancer. *Cancer Cell* 2011;**20**:79–91.
6. Tan X, Zhou F, Wan J, Hang J, Chen Z, Li B, Zhang C, Shao K, Jiang P, Shi S, Feng X, Lv N, Wang Z, Ling Y, Zhao X, Ding D, Sun J, Xiong M, He J. Pin1 expression contributes to lung cancer: prognosis and carcinogenesis. *Cancer Biol Ther* 2010;**9**:111–119.
7. Fukuchi M, Fukai Y, Kimura H, Sohda M, Miyazaki T, Nakajima M, Masuda N, Tsukada K, Kato H, Kuwano H. Prolyl isomerase Pin1 expression predicts prognosis in patients with esophageal squamous cell carcinoma and correlates with cyclinD1 expression. *Int J Oncol* 2006;**29**:329–334.
8. Ryo A, Suizu F, Yoshida Y, Perrem K, Liou YC, Wulf G, Rottapel R, Yamaoka S, Lu KP. Regulation of NF-kappaB signaling by Pin1-dependent prolyl isomerization and ubiquitin-mediated proteolysis of p65/RelA. *Mol Cell* 2003;**12**:1413–1426.
9. Atkinson GP, Nozell SE, Harrison DK, Stonecypher MS, Chen D, Benveniste EN. The prolyl isomerase Pin1 regulates the NF-kappaB signaling pathway and interleukin-8 expression in glioblastoma. *Oncogene* 2009;**28**:3735–3745.
10. Wang J, Ray PS, Sim MS, Zhou XZ, Lu KP, Lee AV, Lin X, Bagaria SP, Giuliano AE, Cui X. FOXC1 regulates the functions of human basal-like breast cancer cells by activating NF-kappaB signaling. *Oncogene* 2012;**31**:4798–4802.
11. Pinton P, Rimessi A, Marchi S, Orsini F, Migliaccio E, Giorgio M, Contursi C, Minucci S, Mantovani F, Wieckowski MR, Del Sal G, Pelicci PG, Rizzuto R. Protein kinase C beta and prolyl isomerase 1 regulate mitochondrial effects of the life-span determinant p66Shc. *Science* 2007;**315**:659–663.
12. Sun L, Xiao L, Nie J, Liu FY, Ling GH, Zhu XJ, Tang WB, Chen WC, Xia YC, Zhan M, Ma MM, Peng YM, Liu H, Liu YH, Kanwar YS. p66Shc mediates high-glucose and angiotensin II-induced oxidative stress renal tubular injury via mitochondrial-dependent apoptotic pathway. *Am J Physiol Renal Physiol* 2010;**299**:F1014–F1025.
13. Sorrentino G, Mioni M, Giorgi C, Ruggeri N, Pinton P, Moll U, Mantovani F, Del Sal G. The prolyl-isomerase Pin1 activates the mitochondrial death program of p53. *Cell Death Differ* 2013;**20**:198–208.
14. Grison A, Mantovani F, Comel A, Agostoni E, Gustincich S, Persichetti F, Del Sal G. Ser46 phosphorylation and prolyl-isomerase Pin1-mediated isomerization of p53 are key events in p53-dependent apoptosis induced by mutant huntingtin. *Proc Natl Acad Sci USA* 2011;**108**:17979–17984.
15. Ruan L, Torres CM, Qian J, Chen F, Mintz JD, Stepp DW, Fulton D, Venema RC. Pin1 prolyl isomerase regulates endothelial nitric oxide synthase. *Arterioscler Thromb Vasc Biol* 2011;**31**:392–398.
16. Camici GG, Schiavoni M, Francia P, Bachschmid M, Martin-Padura I, Hersberger M, Tanner FC, Pelicci P, Volpe M, Anversa P, Luscher TF, Cosentino F. Genetic deletion of p66(Shc) adaptor protein prevents hyperglycemia-induced endothelial dysfunction and oxidative stress. *Proc Natl Acad Sci USA* 2007;**104**:5217–5222.
17. Paneni F, Mocharla P, Akhmedov A, Costantino S, Osto E, Volpe M, Luscher TF, Cosentino F. Gene silencing of the mitochondrial adaptor p66(Shc) suppresses vascular hyperglycemic memory in diabetes. *Circ Res* 2012;**111**:278–289.
18. Giacco F, Brownlee M. Oxidative stress and diabetic complications. *Circ Res* 2010;**107**:1058–1070.
19. Ramsey JJ, Tran D, Giorgio M, Griffey SM, Koehne A, Laing ST, Taylor SL, Kim K, Cortopassi GA, Lloyd KC, Hagopian K, Tomilov AA, Migliaccio E, Pelicci PG, McDonald RB. The Influence of Shc Proteins on Life Span in Mice. *J Gerontol A Biol Sci Med Sci* 2013. doi:10.1093/gerona/glt198.
20. Pagnin E, Fadini G, de Toni R, Tiengo A, Calo L, Avogaro A. Diabetes induces p66shc gene expression in human peripheral blood mononuclear cells: relationship to oxidative stress. *J Clin Endocrinol Metab* 2005;**90**:1130–1136.
21. Forstermann U, Munzel T. Endothelial nitric oxide synthase in vascular disease: from marvel to menace. *Circulation* 2006;**113**:1708–1714.
22. Tabit CE, Chung WB, Hamburg NM, Vita JA. Endothelial dysfunction in diabetes mellitus: molecular mechanisms and clinical implications. *Rev Endocr Metab Disord* 2010;**11**:61–74.
23. Tabit CE, Shenouda SM, Holbrook M, Fetterman JL, Kiani S, Frame AA, Kluge MA, Held A, Dohadwala MM, Gokce N, Farb MG, Rosenzweig J, Ruderman N, Vita JA, Hamburg NM. Protein kinase C-beta contributes to impaired endothelial insulin signaling in humans with diabetes mellitus. *Circulation* 2013;**127**:86–95.
24. Kouroedov A, Eto M, Joch H, Volpe M, Luscher TF, Cosentino F. Selective inhibition of protein kinase Cbeta2 prevents acute effects of high glucose on vascular cell adhesion molecule-1 expression in human endothelial cells. *Circulation* 2004;**110**:91–96.
25. Baker RG, Hayden MS, Ghosh S. NF-kappaB, inflammation, and metabolic disease. *Cell Metab* 2011;**13**:11–22.
26. Paneni F, Volpe M, Luscher TF, Cosentino F. SIRT1, p66Shc, and Set7/9 in vascular hyperglycemic memory: bringing all the strands together. *Diabetes* 2013;**62**:1800–1807.
27. Shenouda SM, Widlansky ME, Chen K, Xu G, Holbrook M, Tabit CE, Hamburg NM, Frame AA, Caiano TL, Kluge MA, Duess MA, Levit A, Kim B, Hartman ML, Joseph L, Shirihai OS, Vita JA. Altered mitochondrial dynamics contributes to endothelial dysfunction in diabetes mellitus. *Circulation* 2011;**124**:444–453.
28. Li C, Ruan L, Sood SG, Papapetropoulos A, Fulton D, Venema RC. Role of eNOS phosphorylation at Ser-116 in regulation of eNOS activity in endothelial cells. *Vascul Pharmacol* 2007;**47**:257–264.
29. Lu KP. Phosphorylation-dependent prolyl isomerization: a novel cell cycle regulatory mechanism. *Prog Cell Cycle Res* 2000;**4**:83–96.
30. Napoli M, Girardini JE, Piazza S, Del Sal G. Wiring the oncogenic circuitry: Pin1 unleashes mutant p53. *Oncotarget* 2011;**2**:654–656.
31. Fadini GP, Albiero M, Menegazzo L, Boscaro E, Pagnin E, Iori E, Cosma C, Lapolla A, Pengo V, Stendardo M, Agostini C, Pelicci PG, Giorgio M, Avogaro A. The redox enzyme p66Shc contributes to diabetes and ischemia-induced delay in cutaneous wound healing. *Diabetes* 2010;**59**:2306–2314.
32. Menini S, Amadio L, Oddi G, Ricci C, Pesce C, Pugliese F, Giorgio M, Migliaccio E, Pelicci P, Iacobini C, Pugliese G. Deletion of p66Shc longevity gene protects against experimental diabetic glomerulopathy by preventing diabetes-induced oxidative stress. *Diabetes* 2006;**55**:1642–1650.
33. Kluge MA, Fetterman JL, Vita JA. Mitochondria and endothelial function. *Circ Res* 2013;**112**:1171–1188.

34. Mount PF, Kemp BE, Power DA. Regulation of endothelial and myocardial NO synthesis by multi-site eNOS phosphorylation. *J Mol Cell Cardiol* 2007;**42**:271–279.
35. Frank PG, Woodman SE, Park DS, Lisanti MP. Caveolin, caveolae, and endothelial cell function. *Arterioscler Thromb Vasc Biol* 2003;**23**:1161–1168.
36. Ki SH, Lee JW, Lim SC, Hien TT, Im JH, Oh WK, Lee MY, Ji YH, Kim YG, Kang KW. Protective effect of nectandrin B, a potent AMPK activator on neointima formation: inhibition of Pin1 expression through AMPK activation. *Br J Pharmacol* 2013;**168**:932–945.
37. Lv L, Zhang J, Zhang L, Xue G, Wang P, Meng Q, Liang W. Essential role of Pin1 via STAT3 signalling and mitochondria-dependent pathways in restenosis in type 2 diabetes. *J Cell Mol Med* 2013;**17**:989–1005.
38. Chiasson VL, Munshi N, Chatterjee P, Young KJ, Mitchell BM. Pin1 deficiency causes endothelial dysfunction and hypertension. *Hypertension* 2011;**58**:431–438.
39. Toko H, Konstandin MH, Doroudgar S, Ormachea L, Joyo E, Joyo AY, Din S, Gude NA, Collins B, Volkers M, Thuerauf DJ, Glembotski CC, Chen CH, Lu KP, Muller OJ, Uchida T, Sussman MA. Regulation of cardiac hypertrophic signaling by prolyl isomerase Pin1. *Circ Res* 2013;**112**:1244–1252.
40. Paneni F, Costantino S, Volpe M, Luscher TF, Cosentino F. Epigenetic signatures and vascular risk in type 2 diabetes: a clinical perspective. *Atherosclerosis* 2013;**230**:191–197.
41. Handy DE, Castro R, Loscalzo J. Epigenetic modifications: basic mechanisms and role in cardiovascular disease. *Circulation* 2011;**123**:2145–2156.

CARDIOVASCULAR FLASHLIGHT

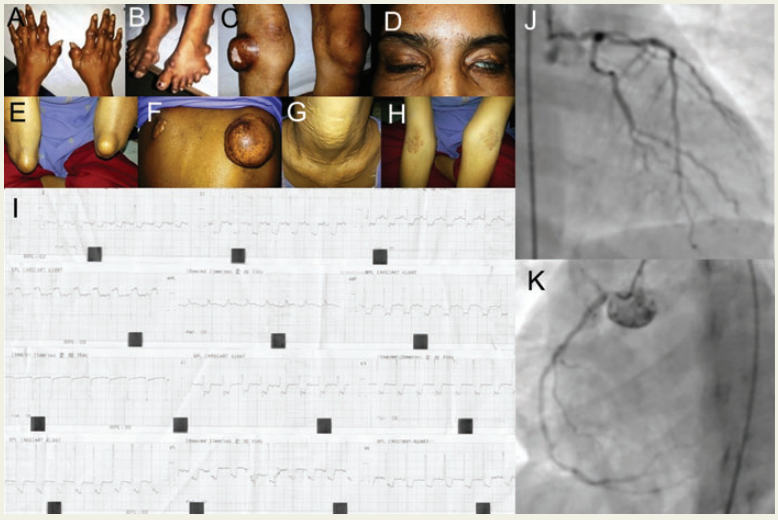
doi:10.1093/eurheartj/ehu468
Online publish-ahead-of-print 17 December 2014

Extensive xanthomas with severe coronary artery disease in a young patient with familial hypercholesterolemia

Vikas Agrawal* and Bhupendra Kumar Sihag

Department of Cardiology, Institute of Medical Sciences, Banaras Hindu University, Varanasi 221005, India

* Corresponding author. Tel: +91 9453300628, Email: vikky25@yahoo.com



A 20-year-old female presented to us with recent onset unstable angina. She had a strong family history of premature coronary artery disease (two elder sisters dying at a young age due to CAD). She had large tendinous xanthomas over extensor tendons of metacarpophalangeal joints and patella (*Panels A–C*). She also had xanthelesma, gaint tuberous xanthomas over extensor aspects of limbs and buttocks and Plane xanthoma involving the neck (*Panels D–H*). The ECG showed deep ST depression in all leads with ST elevation in avR (left main pattern, *Panel I*). Laboratory tests showed total cholesterol of 810 mg/dL (20.9 mmol/L), low-density lipoprotein cholesterol (LDL) of 733 mg/dL (18.9 mol/L), triglycerides (TG) of 195 mg/dL (2.2 mmol/L), and high-density lipoprotein cholesterol of 39 mg/dL (1.0 mmol/L). Her coronary angiography revealed severe left main disease involving ostium and mid shaft and also ostial RCA disease (*Panels I, J, and K*). Genetic study showed homozygotic mutation in LDL receptor gene. The patient is being planned for urgent PCI with intensive medical therapy.

Panel A: Large tendinous xanthomas over extensor tendons of metacarpophalangeal joints.
Panel B: Large tendinous xanthomas over extensor tendons of metatarsophalangeal joints.
Panel C: Large tendinous xanthomas over patella.
Panel D: Xanthelesma.
Panel E: Gaint tuberous xanthomas over extensor aspects of elbow.
Panel F: Gaint tuberous xanthomas buttocks.
Panel G: Plane xanthoma involving the neck.
Panel H: Xanthoma involving extensor aspect of forearm.
Panel I: ECG showing deep ST depression in all leads with ST elevation in avR (left main pattern).
Panel J: Coronary angiography showing severe left main disease involving ostium and mid shaft.
Panel K: Coronary angiography showing severe ostial RCA disease.