CLINICAL RESEARCH
Vascular Biology and Medicine

Tubulin-folding cofactor E deficiency promotes vascular dysfunction by increased endoplasmic reticulum stress

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See the editorial comment for this article 'Identification of a novel therapeutic target in vascular dysfunction: a showcase of reverse and forward translational research linking bench to bedside', by Hiroshi Iwata and Tohru Minamino, https://doi.org/10.1093/eurheartj/ehab263.

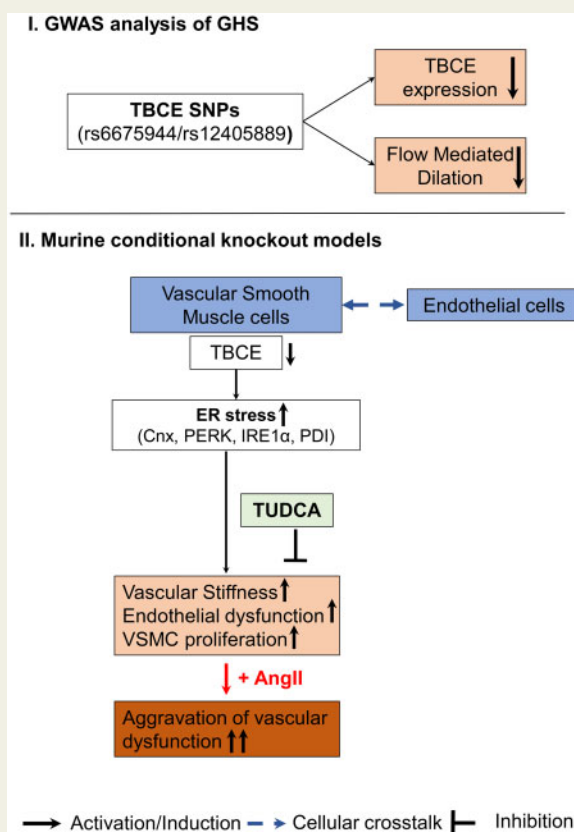|	
Aims	Assessment of endothelial function in humans by measuring flow-mediated dilation (FMD) risk-stratifies individuals with established cardiovascular disease, whereas its predictive value is limited in primary prevention. We therefore aimed to establish and evaluate novel markers of FMD at the population level.
Methods and results	In order to identify novel targets that were negatively correlated with FMD and investigate their contribution to vascular function, we performed a genome-wide association study (GWAS) of 4175 participants of the population based Gutenberg Health Study. Subsequently, conditional knockout mouse models deleting the gene of interest were generated and characterized. GWAS analysis revealed that single-nucleotide polymorphisms (SNPs) in the tubulin-folding cofactor E (TBCE) gene were negatively correlated with endothelial function and TBCE expression. Vascular smooth muscle cell (VSMC)-targeted TBCE deficiency was associated with endothelial dysfunction, aortic wall hypertrophy, and endoplasmic reticulum (ER) stress-mediated VSMC hyperproliferation in mice, paralleled by calnexin up-regulation and exacerbated by the blood pressure hormone angiotensin II. Treating SMMHC-ERT2-Cre^{+/−}TBCE^{fl/fl} mice with the ER stress modulator tauroursodeoxycholic acid amplified Raptor/Beclin-1-dependent autophagy and reversed vascular dysfunction.
Conclusion	TBCE and tubulin homeostasis seem to be novel predictors of vascular function and offer a new drug target to ameliorate ER stress-dependent vascular dysfunction.

Graphical Abstract

Schematic representation of the main findings regarding (i) the genome-wide association study (GWAS) analysis of the Gutenberg Health Study (GHS), and (ii) the translational approach in the transgenic vascular smooth muscle cell-specific tubulin-folding cofactor E (TBCE) knockout murine model. Arrows represent activation/induction pathways, T lines present inhibition pathways, while dotted arrows present cellular crosstalk between endothelial and smooth muscle cells as observed *in vivo*. Cnx, calnexin; ER, endoplasmic reticulum; IRE1α, inositol-requiring enzyme 1α; PDI, protein disulfide-isomerase; PERK, protein kinase RNA-like endoplasmic reticulum kinase; SNP, single-nucleotide polymorphism; TBCE, tubulin-folding cofactor E; TUDCA, tauroursodeoxycholic acid.

Introduction

Vascular dysfunction is a hallmark of cardiovascular disease (CVD). The specific properties of endothelial, smooth muscle and immune cells converge on the vasculature and determine vascular tone and homeostasis. Endothelial and/or smooth muscle cell dysfunction as well as activated immune cells contribute to progression of CVD,[1,2] the leading cause of mortality worldwide.[3]

Disruption of endothelial homeostasis leads to disturbed nitric oxide-dependent regulation of the vascular tone.[4] Myeloid cells are important contributors to the initiation of vascular dysfunction, inflammation, and remodelling,[5] in part by their ability to secrete chemokines and cytokines.[5] Vascular smooth muscle cells (VSMCs) are mural cells continually exposed to biochemical and mechanical stress generated in the circulation. They adapt to hemodynamic stimuli by switching to a hypertrophic, proliferative phenotype, and by secreting extracellular matrix proteins.[6] Thereby, they participate in the regulation of vascular tone and blood pressure.[7]

Numerous non-invasive techniques including flow-mediated dilation (FMD), venous occlusion plethysmography, or peripheral arterial tone/pulse waveform analysis have been developed for assessment of endothelial function.[8] FMD as the most frequently used technique, is useful for risk stratification in cohorts afflicted by CVD,[9,10] whereas data from various population-based studies have yielded equivocal results on the prognostic value of FMD concerning future

cardiovascular events.[9,11,12] The Gutenberg Health Study (GHS) is a population-based prospective cohort study and has previously shown that mid-regional pro-adrenomedullin and mid-regional pro-atrial natriuretic peptide were associated with FMD.[13]

In the GHS, FMD was strongly associated with age, sex, body mass index, and biomarkers of arterial hypertension or heart failure,[13] but classical risk factors accounted for only 15.4% of the variability of the reflective hyperaemic response in FMD.[14] The scope of our current study was to discover novel single-nucleotide polymorphisms (SNPs) that correlate with FMD through genome-wide association study (GWAS) at the population level. Furthermore, we aimed to identify putative mechanisms by the generation and vascular phenotyping of cell-specific conditional knockout mouse models.

Materials and methods

Full methods are available in the Supplementary material online.

Genome-wide association study

SNP information was gained from the genome data available from the GHS as previously described,[15] employing Affymetrix Genome-Wide Human SNP 6.0 arrays (Affymetrix, Santa Clara, CA, USA); after quality control, genetic data of 4,175 individuals were available for analysis. Correlation analysis by linear regression was performed in order to identify SNPs that were associated with FMD.

Animals

Three different strains of cell-specific, conditional knockout mouse models of tubulin-folding cofactor E (TBCE) were generated by serial crossings. $TBCE^{tm1c}$ mice were first generated according to EUCOMM® instructions.[16,17] $LysMCre^{+/-}TBCE^{fl/fl}$ mice were generated for myeloid cell-specific deficiency of TBCE,[18] $Tie2\text{-}ERT2Cre^{+/-}TBCE^{fl/fl}$ mice as a conditional knockdown model in endothelial cells (ECs),[19] and $SMMHC\text{-}ERT2\text{-}Cre^{+/-}TBCE^{fl/fl}$ as a conditional knockdown model in smooth muscle cells.[20] For all the above-mentioned strains, age-matched $Cre^{+/-}TBCE^{wt/wt}$ littermates were used as controls in all experiments. A total of 60 $LysMCre^{+/-}TBCE^{fl/fl}$, 80 $Tie2\text{-}ERT2\text{-}Cre^{+/-}TBCE^{fl/fl}$, and 140 $SMMHC\text{-}ERT2\text{-}Cre^{+/-}TBCE^{fl/fl}$ mice were used in the experiments. All animals were inbred and received proper care in compliance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1985) and were approved by the Ethical Committee of the Prefecture of Rheinland-Pfalz (G17-1-031). All animal experiments were performed in line with the ARRIVE guidelines.[21] The breeding strategy, genotyping, and workflow are described in Supplementary material online, Figure S2. Animals that did not meet the wellbeing criteria regarding the 3Rs were excluded from the experiments, while no outliers were removed from the experiments. The TBCEtm2c (e.g. TBCEfl/fl) strain has been accepted for archiving in the INFRAFRONTIER/EMMA repository (EMMA ID: EM13235) and is available for research purposes (https://www.infrafrontier.eu/search).

Vascular relaxation and constriction studies

To assess vasodilator potential of isolated aortic segments (3 mm), they were mounted to force transducers in organ chambers to test their response to acetylcholine (ACh) and nitroglycerine (GTN). The aortic rings were pre-constricted with prostaglandin F2α (PGF2α, 3 nM), a VSMC-dependent constrictor[22] to reach 50–80% of the tone induced by KCl. Concentration–relaxation curves were recorded in response to the endothelium-dependent vasodilator ACh (1 nM to 3 mM) as well as

GTN (1 nM to 30 mM).[23] Aortic tissue was snap frozen for further molecular and histological analyses.

Statistical analysis

Data are presented as means ± standard deviation. Continuous variables were compared among groups using one-way analysis of variance (ANOVA) and post hoc comparisons were made using Tukey's test, while comparisons concerning the ACh and GTN relaxation and tension data were analysed by two-way ANOVA and Tukey's test. No assumption of equal variability of differences was performed and data were corrected with Greenhouse-Geisser correction. A P-value of <0.05 was considered statistically significant. All statistical analyses and graph preparation were performed using GraphPad Prism 8.5 analysis software (GraphPad Software, Inc., La Jolla, CA, USA). No outliers due to biological diversity were excluded. Samples that did not meet our technical criteria were not included into the analyses a priori. The absence of outlying values was confirmed by GraphPad Prism analysis software, using ROUT method and $Q = 1\%$. Complete statistical analysis is included in the Supplementary material online.

Results

Single-nucleotide polymorphisms rs6675944 and rs12405889 are negatively correlated with TBCE expression and flow-mediated arterial dilation

GHS revealed that SNPs rs6675944 (T→C) and rs1205889 (T→G) in chromosome 1 were negatively correlated with FMD of the brachial artery (rs6675944 $\beta = -0.4841$, $P = 5.9e^{-27}$, $R^2_{tot} = 0.006$ and $\beta = -0.4841$, $P = 2.3e^{-12}$, $R^2_{tot} = 0.006$; rs12405889 $\beta = -0.5028$, $P = 8.8e^{-14}$, $R^2_{tot} = 0.006$ and $\beta = -0.5028$, $P = 1.0e^{-25}$, $R^2_{tot} = 0.006$) (Table 1). Moreover, rs6675944 was associated with decreased reflection index pre occlusion and post occlusion and increased intima-media thickness (IMT; mm), showing an increased arterial stiffness phenotype in the SNP-bearing individuals[24] (Table 1).

Both SNPs were found in the TBCE (tubulin folding cofactor E, or tubulin-specific chaperone E) gene region. mRNA expression analysis of peripheral blood mononuclear cells obtained from individuals that were enrolled in GHS revealed that SNPs were negatively associated with TBCE expression (β estimate: -0.4841, $R^2_{tot} = 0.010$) (Figure 1A and B). GTEx Portal[25] and NIH U.S. National Library of Medicine[26] data analysis showed that both SNPs have been identified in various human population studies, with a high occurrence in the general population (Supplementary material online, Figure S1). In a pooled human tissue expression analysis—as performed from TBCE gene expression data in various analysed tissues and attained from the GTEx Portal—both homozygous and heterozygous genotypes were associated with down-regulation of TBCE expression, being more pronounced in the homozygous genotype (Figure 1E and F; GTEx Portal). Concerning the individual tissue analysis, skeletal muscle TBCE expression appeared to be mostly affected by the presence of SNPs (logP = -12.6 and -15.2, respectively), while SNPs were also found to negatively affect TBCE expression in aortic and tibial arterial tissue (Figure 1C and D; GTEx Portal). Conclusively, our GWAS as well as GTEx human data show that the presence of TBCE SNPs is correlated with decreased transcripts of the gene in

Table 1 Linear regression model representing the correlation of the single-nucleotide polymorphisms (SNPs) rs6675944 and rs12405889 in the tubulin-folding cofactor E (TBCE) gene with measures of vascular function and morphology from 4175 individuals in the Gutenberg Health-Study

SNP	Gene	bp	Phenotype	R^2	Beta	P-value
rs6675944	TBCE (intron) Chr1	233639292	FMD	0.006	−0.4841	**5.92E−27**
			Augmentation index	0.255	−0.135	0.61
			Stiffness index pre occlusion (m/s)	0.242	−0.0055	0.84
			Stiffness index post occlusion cc (m/s)	0.3	−0.0241	0.38
			Reflection index pre occlusion	0.145	−0.359	0.066
			Reflection index post occlusion	0.206	−0.46	**0.028**
			IMT (mm)	0.34	0.00262	0.053
rs12405889	TBCE (intron) Chr1	233663875	FMD	0.006	−0.5028	**8.78E−25**
			Augmentation index	0.257	0.0541	0.84
			Stiffness index pre occlusion (m/s)	0.242	0.00133	0.96
			Stiffness index post occlusion (m/s)	0.3	0.0176	0.53
			Reflection index pre occlusion	0.146	0.308	0.12
			Reflection index post occlusion	0.206	0.34	0.11
			IMT (mm)	0.34	−0.0022	0.12

bp, base pair; Chr1, chromosome 1; FMD, flow-mediated dilation; IMT, intima-media thickness.
Bold values indicate significant values with $P < 0.05$.

various tissues. This decreased gene expression is negatively associated with FMD, leading to the assumption that TBCE downregulation might be causally involved in vascular dysfunction.
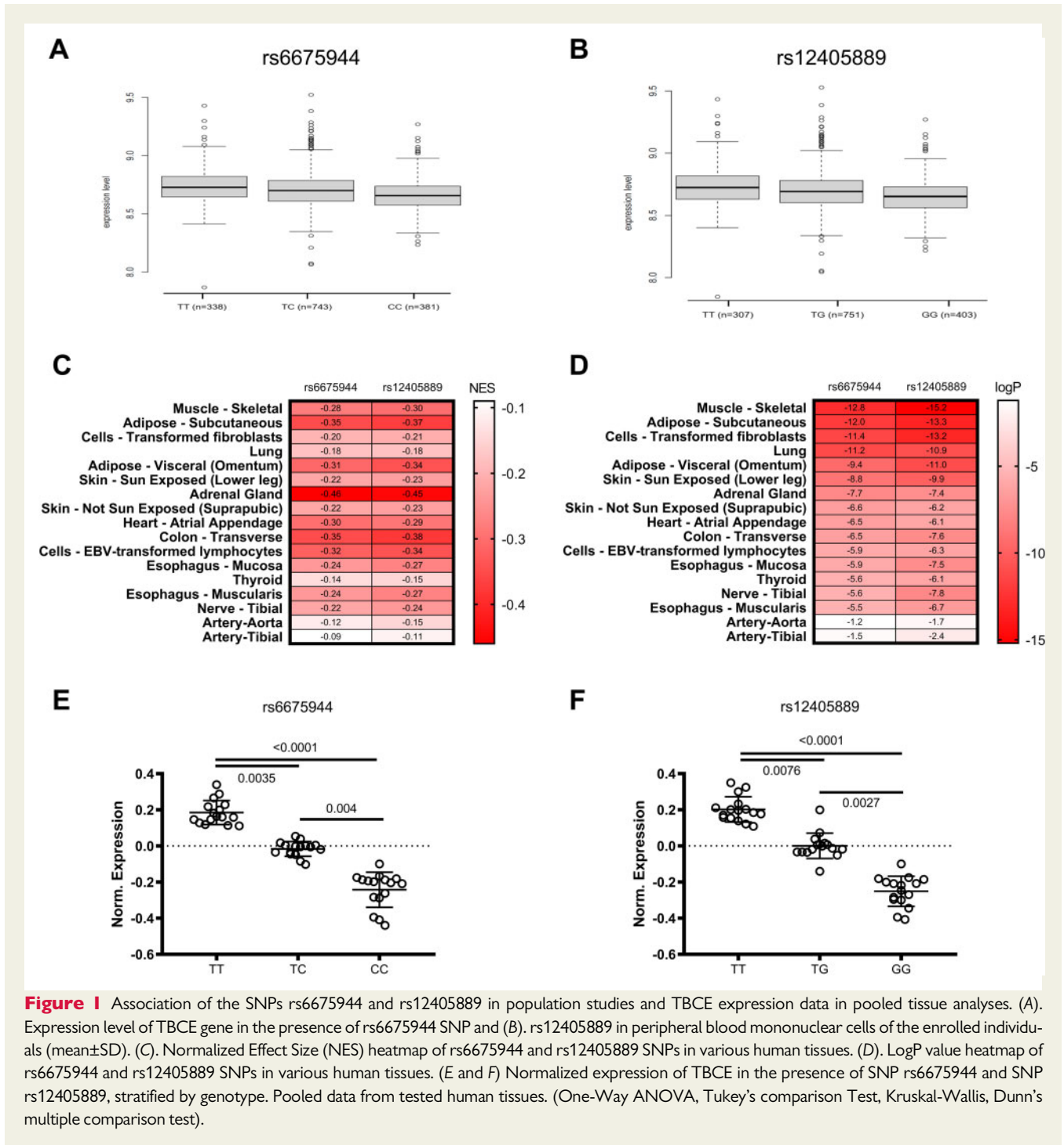
Conditional knockout of TBCE in smooth muscle cells leads to endothelial dysfunction, vascular stiffness, and increased vessel wall thickness

As the GWAS analysis was performed in the peripheral blood mononuclear cells of the enrolled individuals, we initially generated the LysMCre$^{+/−}$TBCE$^{fl/fl}$ mice (Supplementary material online, Figure S2) to check the phenotype of mice with myeloid cell-specific TBCE deficiency. LysMCre$^{+/−}$TBCE$^{fl/fl}$ mice did not show any signs of altered body weight and serum postprandial blood glucose, triglycerides and total cholesterol compared to control mice (Supplementary material online, Figure S3). Vascular relaxation and tension in response to ACh (endothelium-dependent) and GTN (endothelium-independent) were not significantly different between LysMCre$^{+/−}$TBCE$^{fl/fl}$ mice and their controls. Moreover, angiotensin II (AngII)-derived increase in systolic blood pressure, media thickness, and collagen/media thickness ratio were not significantly different between LysMCre$^{+/−}$TBCE$^{wt/wt}$ and LysMCre$^{+/−}$TBCE$^{fl/fl}$ mice (Supplementary material online, Figure S4). These data suggest a minor role of TBCE in lysozyme M$^+$ cells in vascular (dys)function.

To further elucidate the mechanisms of TBCE-associated endothelial dysfunction, we generated Tie2-ERT2-Cre$^{+/−}$TBCE$^{fl/fl}$ mice (Supplementary material online, Figure S2) in which endothelial-specific TBCE deletion was confirmed both in microcirculation and in the aortas (Supplementary material online, Figures S5 and S6). Tie2-ERT2-Cre$^{+/−}$TBCE$^{fl/fl}$ mice presented a decreased body weight compared to Tie2-ERT2-Cre$^{+/−}$TBCE$^{wt/wt}$, both being lower than C57BL/6J controls (Supplementary material online, Figure S3). These

mice had a decreased vascular tone both at baseline and in response to infusion with AngII. Vascular relaxation in response to ACh and GTN were comparable between Tie2-ERT2-Cre$^{+/−}$TBCE$^{fl/fl}$ mice and controls (Supplementary material online, Figure S7A–G). Although the vascular resistance and pulsatility indexes as measures of vascular stiffness did not differ between groups (Supplementary material online, Figure S7H–J), a significant increase in wall thickness was detected only in Tie2-ERT2-Cre$^{+/−}$TBCE$^{fl/fl}$+AngII mice, independently of changes in (relative) collagen content (Supplementary material online, Figure S7K–M).

CC-chemokine ligand 2 (Ccl2), interleukin-6 (Il6), and tumour necrosis factor alpha (Tnfα) mRNA expression was significantly up-regulated in Tie2-ERT2-Cre$^{+/−}$TBCE$^{fl/fl}$ mice compared to controls and in part amplified by AngII (Supplementary material online, Figure S8A). Staining of CD45$^−$/CD31$^+$ pulmonary ECs isolated from the Tie2-ERT2-Cre$^{+/−}$TBCE$^{fl/fl}$ animals revealed an up-regulation of NACHT, LRR and PYD domains-containing protein 3 (NLRP3, Supplementary material online, Figure S8B). NLRP3 inflammasome activation in Tie2-ERT2-Cre$^{+/−}$TBCE$^{fl/fl}$ was confirmed by immunoblotting for NACHT, LRR, FIIND, CARD domain, and PYD domains-containing protein 1 (NALP1), NLRP3 and their downstream target cleaved caspase 1 which was increased by AngII (Supplementary material online, Figure S8C). Endoplasmic reticulum (ER) stress markers calnexin, CHOP and protein kinase RNA-like endoplasmic reticulum kinase (PERK) as well as cleaved caspase-1 were increased in aortas and in ECs isolated from Tie2-ERT2-Cre$^{+/−}$TBCE$^{fl/fl}$ mice, in part augmented by AngII (Supplementary material online, Figure S8D and E). The slightly increased susceptibility of Tie2-ERT2-Cre$^{+/−}$TBCE$^{fl/fl}$ to AngII-induced hypertension (Supplementary material online, Figure S9) may be related to this pro-inflammatory vascular phenotype of Tie2-ERT2Cre$^{+/−}$TBCE$^{fl/fl}$ mice, without a significant accumulation of inflammatory cells at baseline (Supplementary material online, Figure S10). These findings suggest that endothelial-specific deletion of

Figure 1 Association of the SNPs rs6675944 and rs12405889 in population studies and TBCE expression data in pooled tissue analyses. (*A*). Expression level of TBCE gene in the presence of rs6675944 SNP and (*B*). rs12405889 in peripheral blood mononuclear cells of the enrolled individuals (mean±SD). (*C*). Normalized Effect Size (NES) heatmap of rs6675944 and rs12405889 SNPs in various human tissues. (*D*). LogP value heatmap of rs6675944 and rs12405889 SNPs in various human tissues. (*E* and *F*) Normalized expression of TBCE in the presence of SNP rs6675944 and SNP rs12405889, stratified by genotype. Pooled data from tested human tissues. (One-Way ANOVA, Tukey's comparison Test, Kruskal-Wallis, Dunn's multiple comparison test).

TBCE is associated with endothelial inflammasome activation/ER stress, which may explain the increased wall thickness via a possible cross-talk between ECs and VSMCs.

rs6675944 polymorphism was associated with increased carotid IMT in our GWAS (*Table 1*), suggesting a putative role of TBCE in VSMCs. We therefore sought to elucidate the role of TBCE in VSMCs in more detail by generating the SMMHC-ERT2-Cre$^{+/}$

$^{-}$TBCE^{tm2c} mice (Supplementary material online, *Figure S2*). The VSMC-specific TBCE deficiency was confirmed in microcirculation and in the aortas (Supplementary material online, *Figure S11*). In contrast to myeloid and EC-specific knockouts, the SMMHC-ERT2-Cre$^{+/-}$TBCE$^{fl/fl}$ mice presented a decreased survival rate compared to their SMMHC-ERT2-Cre$^{+/-}$TBCE$^{wt/wt}$ controls (Supplementary material online, *Figure S3C*).

Compared to controls, SMMHC-ERT2-Cre$^{+/-}$TBCE$^{fl/fl}$ aortas showed reduced response to PGF2α induced constriction with an even lesser response in SMMHC-ERT2-Cre$^{+/-}$TBCE$^{fl/fl}$+AngII group (*Figure 2A*). SMMHC-ERT2-Cre$^{+/-}$TBCE$^{fl/fl}$ mice were characterized by increased aortic stiffness, further exacerbated by AngII *in vivo* (*Figure 2B and C*). SMMHC-ERT2-Cre$^{+/-}$TBCE$^{fl/fl}$ aortas had endothelial dysfunction at baseline compared to their controls (*Figure 2D–G*). Vascular dysfunction was accompanied by increased left common carotid artery (LCCA) resistance and pulsatility indexes (*Figure 2H–J*). Histological evaluation of the aortas showed that wall thickness was increased in SMMHC-ERT2-Cre$^{+/-}$TBCE$^{fl/fl}$ mice, independent of AngII treatment. AngII infusion induced collagen deposition both in SMMHC-ERT2-Cre$^{+/-}$TBCE$^{wt/wt}$ and SMMHC-ERT2-Cre$^{+/-}$TBCE$^{fl/fl}$ mice, but more prominently in the SMMHC-ERT2-Cre$^{+/-}$TBCE$^{fl/fl}$ + AngII group (*Figure 2K–M*). Taken together, VSMC-targeted TBCE ablation leads to vascular dysfunction and stiffness, which is exacerbated by AngII.

AngII infusion led to a significant increase in LCCA wall thickness in the SMMHC-ERT2-Cre$^{+/-}$TBCE$^{fl/fl}$ mice reflective of increased vascular stiffness. It increased transverse aortic diameter in both SMMHC-ERT2-Cre$^{+/-}$TBCE$^{wt/wt}$ and SMMHC-ERT2-Cre$^{+/-}$TBCE$^{fl/fl}$ mice by trend (Supplementary material online, *Figure S12*). Moreover, AngII led to a significant increase in systolic and diastolic blood pressure in both SMMHC-ERT2-Cre$^{+/-}$TBCE$^{wt/wt}$ and SMMHC-ERT2-Cre$^{+/-}$TBCE$^{fl/fl}$ mice, while only the SMMHC-ERT2-Cre$^{+/-}$TBCE$^{fl/fl}$+AngII animals had a significant increase in heart rate, left ventricular ejection fraction and left ventricular posterior wall thickness (Supplementary material online, *Figure S13*).

VSMC-specific deficiency of TBCE modulates vascular inflammatory gene expression, promotes augmentation of endoplasmic reticulum stress and causes a proliferative phenotype paralleled by calnexin up-regulation

Expression analysis of the aortas confirmed down-regulation of the *Tbce* mRNA in SMMHC-ERT2-Cre$^{+/-}$TBCE$^{fl/fl}$ mice. AngII led to an up-regulation of vascular inflammation markers, such as *Ccl2*, *Il6*, *Il1β*, *Tnfα* and *Vcam1* in controls and was significantly attenuated in the SMMHC-ERT2-Cre$^{+/-}$TBCE$^{fl/fl}$ mice (*Figure 3A*). This phenotype was not accompanied by a significant immune cell infiltration in the SMMHC-ERT2-Cre$^{+/-}$TBCE$^{fl/fl}$ aortas (Supplementary material online, *Figure S14*). Interestingly, AngII treatment of the floxed mice led to a 28-fold increase of *Lcn2*, encoding for lipocalin-2 or neutrophil gelatinase-associated lipocalin, a growth factor and iron sequester involved in innate immune responses.[27] Immunofluorescence staining showed an increased abundance of calnexin in the aortic vasculature of SMMHC-ERT2-Cre$^{+/-}$TBCE$^{fl/fl}$ mice, indicating increased ER stress (*Figure 3B*). This was confirmed in VSMCs isolated from the murine aortas, showing a significant increase in calnexin, protein-disulfide isomerase (PDI) and CHOP in response to AngII (100 nM *in vitro*) and more pronounced in the SMMHC-ERT2-Cre$^{+/-}$TBCE$^{fl/fl}$ group, showing signs of ER stress already at baseline. Inositol-requiring enzyme 1α (IRE-1α) is an intracellular sensor protein of misfolded protein response and was decreased in both AngII-treated and sham-treated VSMCs from SMMHC-ERT2-Cre$^{+/-}$TBCE$^{fl/fl}$ mice, compatible with ER stress (*Figure 3C*). In aortic lysates, immunoblotting revealed an AngII-mediated increase in calnexin, CHOP and PDI expression, which was more pronounced in SMMHC-ERT2-Cre$^{+/-}$TBCE$^{fl/fl}$ mice (*Figure 3D*).

To further pinpoint putative mechanisms of TBCE-related vascular dysfunction, primary VSMCs were isolated from aortas and analysed by immunofluorescence confocal microscopy. Decreased TBCE expression along with double-nucleated cells positive for α-tubulin staining indicating a proliferative phenotype were observed in SMMHC-ERT2-Cre$^{+/-}$TBCE$^{fl/fl}$ mice (*Figure 4A, C and D*). Furthermore, immunofluorescence staining revealed up-regulated calnexin in VSMCs from SMMHC-ERT2-Cre$^{+/-}$TBCE$^{fl/fl}$ mice, independently of AngII treatment (*Figure 4B and E*) and in line with augmented ER stress. We found significantly higher proliferation of VSMCs from SMMHC-ERT2-Cre$^{+/-}$TBCE$^{fl/fl}$ mice compared to controls at baseline which was increased by AngII (100 nM) in VSMCs from both groups (*Figure 4F*). Using RNA silencing, we confirmed in human aortic smooth muscle cells (HAoSMCs), that decreased TBCE expression is associated with increased α-tubulin expression (*Figure 4G–J*). AngII treatment caused an increase in calnexin expression in control-transfected HAoSMCs, while the TBCE-silenced HAoSMCs had an increased calnexin expression at baseline, which was further up-regulated by AngII (*Figure 4H and K*). AngII increased proliferation of HAoSMCs in both the control and TBCE-siRNA group, while siRNA-transfected VSMCs exhibited a proliferative phenotype even in the absence of AngII (*Figure 4L*). Taken together, these data support a calnexin-associated increase in ER stress in VSMC-specific TBCE deficiency, which is linked to vascular inflammation and proliferation.

TUDCA reverses vascular dysfunction in mice with VSMC-specific deficiency of TBCE and decreases VSMC proliferation by amplification of Raptor/Beclin-1-mediated autophagy

To test whether the phenotype of TBCE-deficient mice could be modulated pharmacologically, we administered the chemical chaperone and ER stress modulator tauroursodeoxycholic acid (TUDCA, 300 mg/kg/day) to SMMHC-ERT2Cre$^{+/-}$TBCE$^{fl/fl}$ mice. TUDCA reversed vascular dysfunction, as presented by restored ACh-mediated relaxation (*Figure 5A and B*). TUDCA administration decreased PGF2a induced maximal constriction both in SMMHC-ERT2-Cre$^{+/-}$TBCE$^{fl/fl}$ and SMMHC-ERT2-Cre$^{+/-}$TBCE$^{wt/wt}$ mice (*Figure 5C*). Western blot analysis revealed that TUDCA decreased the ER stress markers Bip and CHOP in the SMMHC-ERT2-Cre$^{+/-}$TBCE$^{fl/fl}$ mice, while it led to an enhancement of the Raptor/Beclin-1 pathway (which plays an important role in autophagy[28]) independently of microtubule-associated proteins 1A/1B light chain 3B (LC3B, *Figure 5D and E*). *In vitro*, TUDCA treatment reversed the hyper-proliferative phenotype of SMMHC-ERT2-Cre$^{+/-}$TBCE$^{fl/fl}$ VSMCs, decreasing their proliferation and cellular confluency (*Figure 5F and G*). Protein expression analysis revealed that TUDCA decreased CHOP expression and caused an up-regulation of Raptor/Beclin-1, independently of LC3B *in vitro* (*Figure 5H and I*). Therefore, TBCE deficiency in VSMCs drives vascular dysfunction, which can be attenuated by TUDCA-induced ER stress modulation and amplification of Raptor/Beclin-1-mediated autophagy.
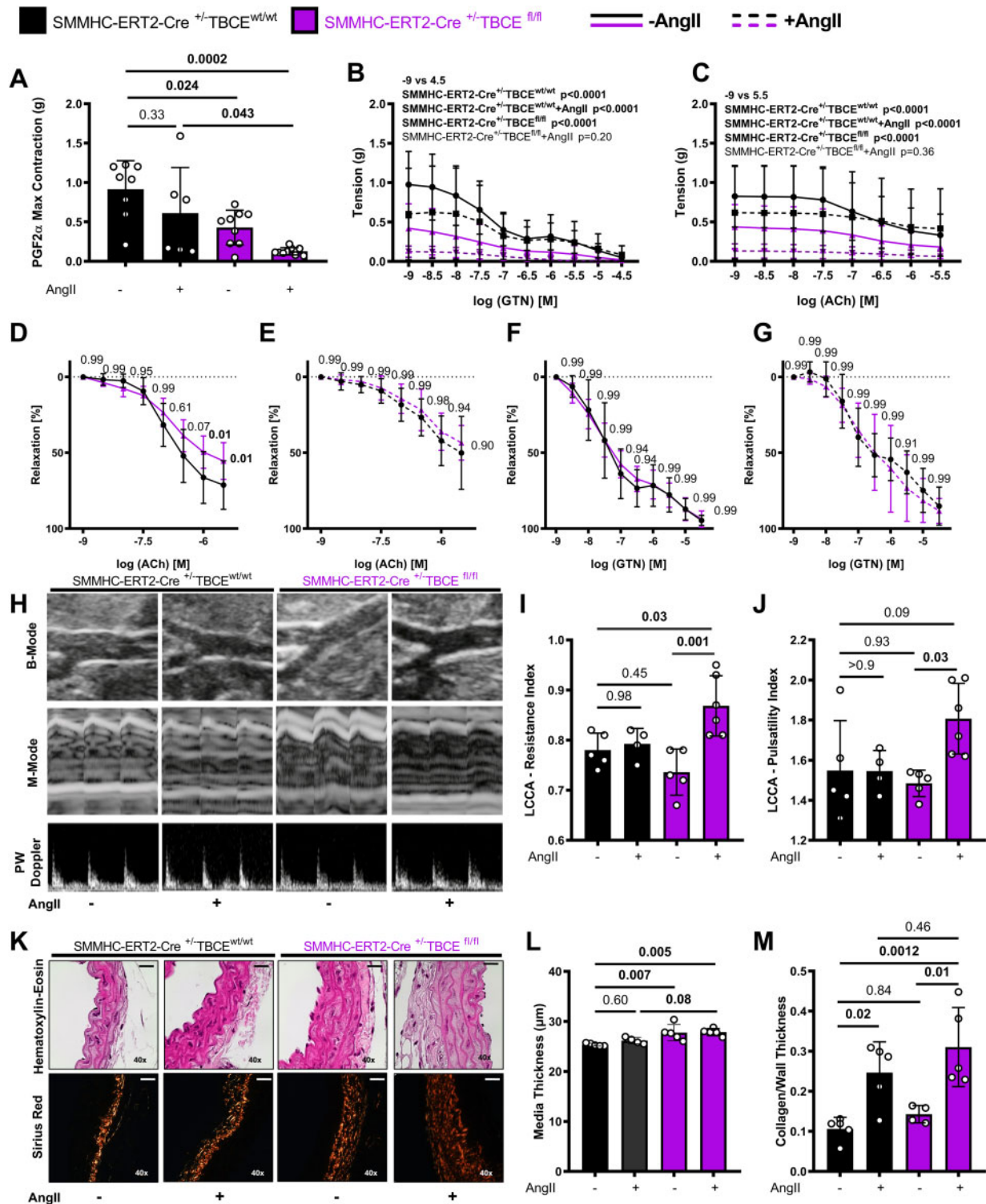
Figure 2 Smooth muscle-specific TBCE deficiency leads to endothelial dysfunction, vascular stiffness and increased vessel wall thickness. (*A–G*) Concentration/relaxation curves of isolated aortic rings. (*A*) Maximal contraction (g) in response to PGF2α. (*B and C*) Vascular tension (g) curves in response to ACh and GTN (Two-way ANOVA, Tukey's multiple comparison test). Vascular concentration-relaxation curves in response to ACh and GTN of sham-operated (*D and F*) and AngII infused (*E and G*; 1 mg/kg/day) mice (*n* = 6–8 per group) (two-way Student's *t*-test). (*H*) Representative ultrasound images of B-mode, M-mode and pulsed-wave Doppler of the murine aortas and graphs of left common carotid artery (LCCA). (*I*) Resistance index and (*J*) Pulsatility index (*n* = 5–6 per group). (*K*) Sirius red (collagen) and haematoxylin–eosin staining of murine aortas and graphs of *L.* Media thickness (μm) and (*M*) Collagen/media thickness (*n* = 4–5 per group); bars represent 20 μm and images were gained at a 40× magnification (*n* = 3–5 per group) (one-way ANOVA, Tukey's multiple comparison test).
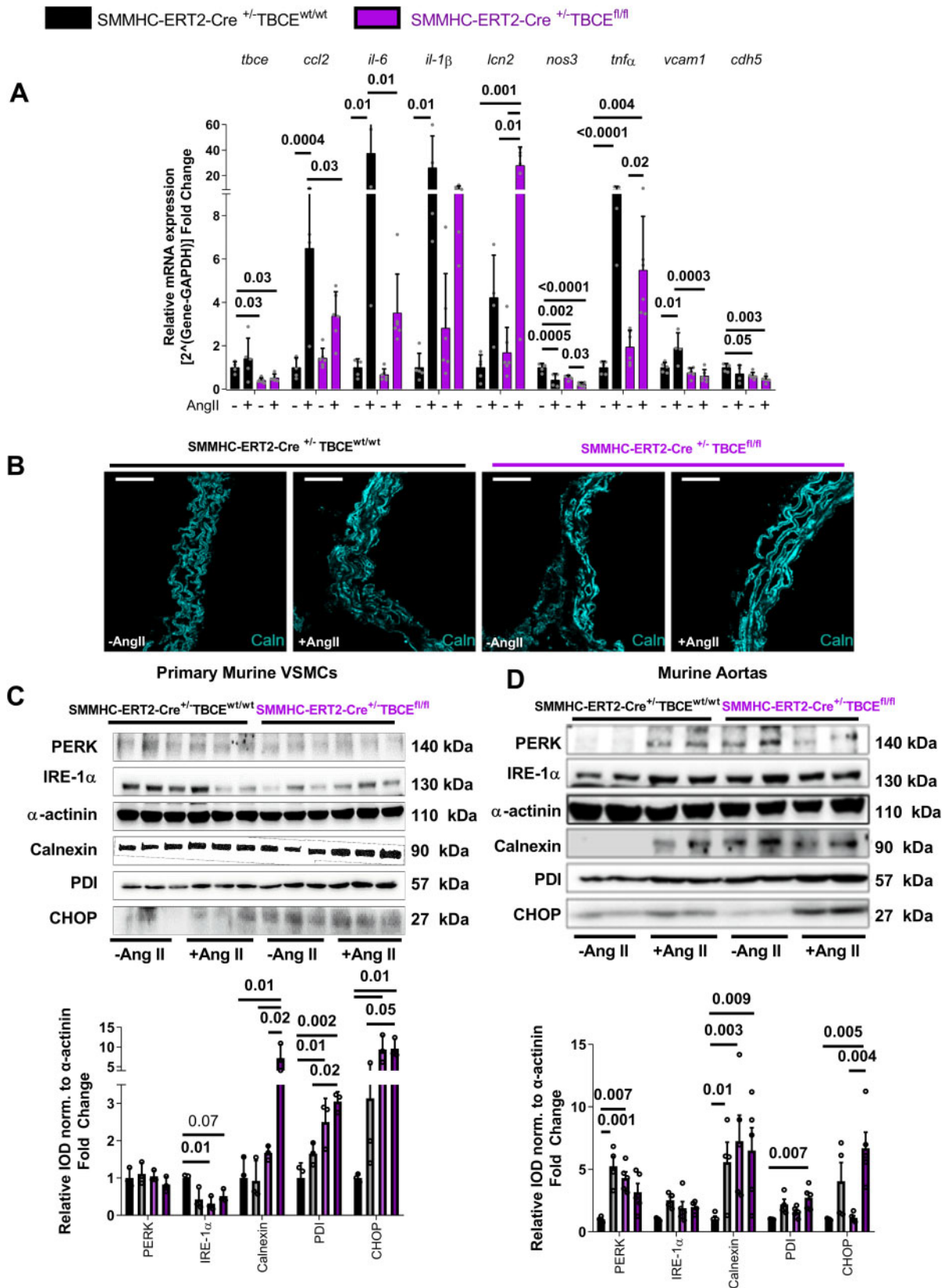
Figure 3 Pivotal role of ER stress for the vascular phenotype of SMMHC-ERT2-Cre^{+/−}TBCE^{fl/fl} mice. (*A*) RT-PCR data of mRNA expression of the differentially regulated genes from the murine aortas (*n* = 5–6 per group). (*B*) Immunofluorescent images of SMMHC-ERT2-Cre^{+/−}TBCE^{fl/fl} aortas stained against DAPI (blue) and Calnexin (cyan) (64× magnification, white bar represents 20 μm). Protein expression analysis of ER stress markers in vascular smooth muscle cells (*C*, *n* = 3 per group) and murine aortas (*D*, *n* = 4–5 per group). Top: Representative Western blot images; Bottom: Relative densitometry analysis of proteins normalized to α-actinin. Tukey's multiple comparison test; One-way ANOVA.
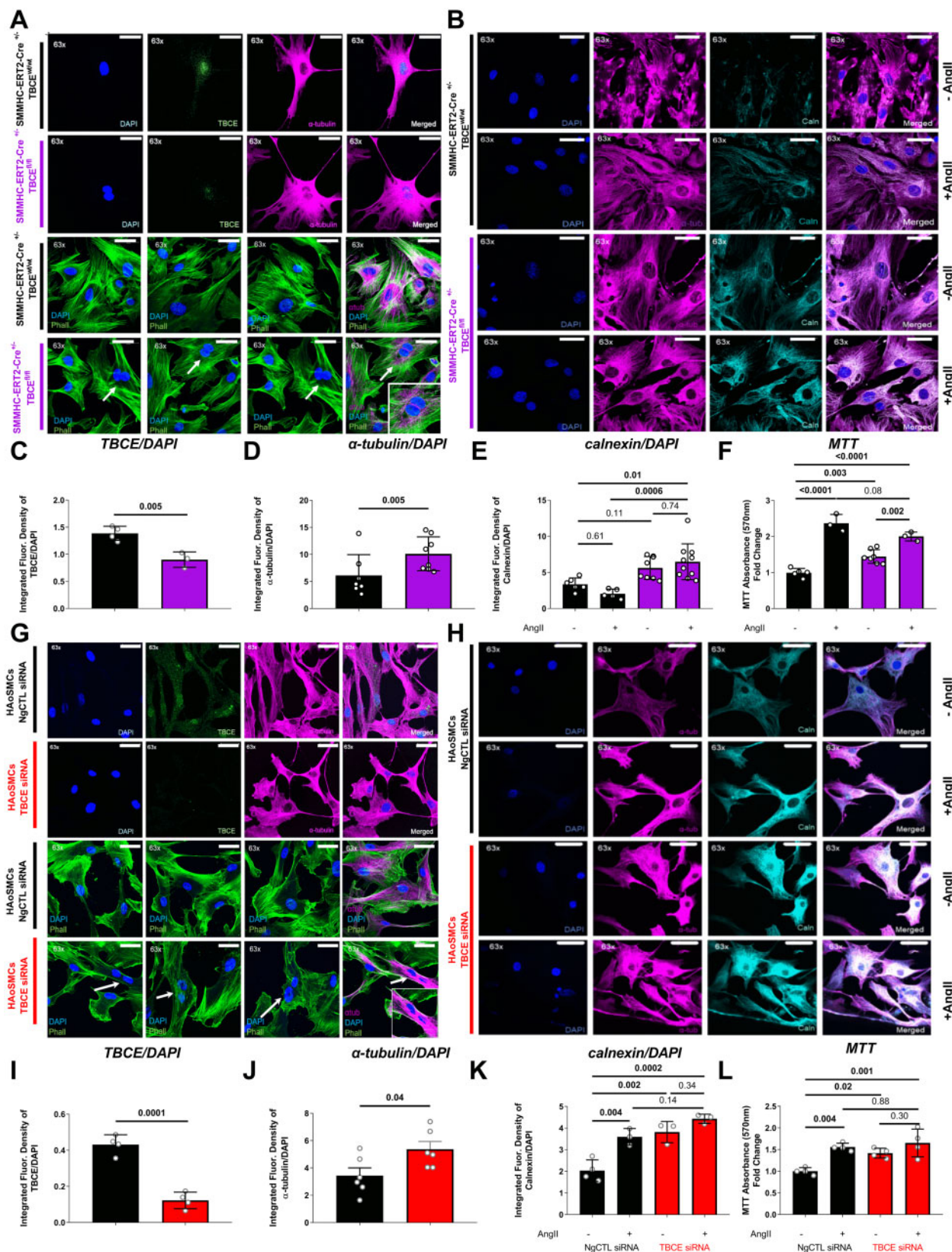
Figure 4 TBCE knockdown led to a proliferating phenotype in both primary murine VSMCs and in TBCE-silenced HAoSMCs. Confirmation of ER stress induction *in vitro*. Representative immunofluorescence images of (*A*) primary VSMCs isolated from murine aortas, stained for DAPI (blue), TBCE (green) and α-tubulin (magenta) (upper panel) or phalloidin (green, F-actin; *n* = 3 representative images per group), α-tubulin (magenta) and DAPI (blue) (lower panel) as cellular morphology markers; and (*B*) primary VSMCs isolated from murine aortas (±AngII treatment) were stained for DAPI (blue), α-tubulin (magenta) and calnexin (cyan). Graphs of integrated fluorescence intensity of (*C*) TBCE/DAPI (*n* = 3–4 per group) (*D*) α-tubu-lin/DAPI (*n* = 7–8 per group) (*E*) Calnexin/DAPI (*n* = 5–9 per group) (*F*) MTT proliferation assay of primary VSMCs (±AngII treatment) (*n* = 3–7 per

Discussion

In the current study, we present novel evidence that SNPs in the TBCE gene and TBCE down-regulation are important determinants of vascular dysfunction both in humans and mice. While endothelial-targeted TBCE deficiency induces a moderate inflammatory phenotype in the aorta, VSMCs-targeted TBCE deficiency leads to exacerbated vascular stiffness and inflammation, smooth muscle cell proliferation, and endothelial dysfunction associated with ER stress.

GWAS analyses have provided an invaluable tool to identify novel targets and mediators of CVD, including acute and chronic coronary syndrome.[29] Among those studies, novel non-coding SNPs,[30] as well as genetic mutations[31] have been associated with vascular dysfunction and hypertension in the general population.[32] GWAS on the FMD axis are scarce, however, some state-of-the-art GWAS analyses have identified an association of cardiac and vascular functional parameters with distinct genomic profiles.[33] It is noteworthy that throughout these genomic associations molecular targets that are already known to effect vascular homeostasis, such as endothelial nitric oxide synthetase or vascular endothelial growth factor have emerged as leading targets of interest.[29] The GWAS analysis derived from the GHS presented here is the first that reveals an association of the SNPs rs6675944 and rs12405889 in the TBCE gene with vascular function. rs6675944 polymorphism was additionally associated with increased carotid wall thickness showing an increased susceptibility of the SNP-bearing individuals to arterial stiffness.[24]

Both rs6675944 and rs12405889 SNPs are found in intron domains of the TBCE gene and lead to the generation of TBCE intron variants. Mutations at a specific site at which intron splicing occurs can cause genetic insertion, deletion or frame shift. Mutations in these sequences may lead to retention of large segments of intronic DNA during mRNA maturation, or to entire exon removal. These changes can result in the generation of a non-functional protein and result in the manifestation of diseases.[34] Alternative Splice Site Predictor (ASSP) software[35] revealed that the rs6675944 site is a highly probable alternative isoform/cryptic 5′ splice site (activation score: 0.879; confidence: 0.908) while rs12405889 is a highly probable alternative isoform/cryptic 3′ splice site (activation score: 0.674; confidence: 0.533). Therefore, SNPs are highly probable to mediate alternative or cryptic splicing of the TBCE gene. Cryptic splice sites are usually not spliced during transcription leading to the accumulation of intronic DNA and therefore might lead to the generation of a non-functional protein.[36] Finally, the Ensembl genome database[37] revealed that rs6675944 SNP can result in 37 transcripts that undergo nonsense-mediated decay and 6 non-coding transcript exon variants out of the

total of 61 transcripts, indicating that rs6675944 has a 70% probability of leading to a non-functional or truncated protein. rs12405889 can result in 39 transcripts that undergo nonsense-mediated decay and 8 non-coding transcript exon variants out of the total of 61 transcripts, indicating that rs12405889 has a 77% probability of leading to a non-functional or truncated protein.

TBCE specifically modulates α-tubulin incorporation in microtubule networks, which are responsible for cellular integrity, protein transportation and signal transduction.[38] Deletion and truncation of TBCE in humans are known to lead to the Sanjad-Sakati or Kenny-Caffey syndrome, which is characterized by congenital hypoparathyroidism, mental retardation, facial dysmorphism and extreme growth deficits,[39] while it is also associated with the prevalence of amyotrophic lateral sclerosis.[40] So far, research on TBCE has focused on the central nervous system,[41] where it has been shown that down-regulation of TBCE leads to Golgi microtubule deficits, which in turn leads to accumulation of ER-Golgi SNAREs.[40,42] In addition, it was shown in yeast that TBCE can secure the connection between microtubules and proteasome, protecting cells against misfolded protein stress in a cAMP-independent regulatory protein (PAC2)-dependent manner.[43]

Conclusively, *in silico* data show that both rs6675944 and rs12405889 SNPs—despite the fact that they are intronic SNPs—are characterized by an epigenetic regulatory trait, while having high probability to result in the generation of a truncated or dysfunctional protein. These findings prompted us to investigate TBCE-associated vascular effects in a translational approach *in vivo*.

With the present studies, we identified that endothelial-specific knockdown of TBCE leads to increased vessel wall thickness. Mechanistically, endothelial-specific TBCE knockdown was found to be associated with an inflammatory phenotype of the aortas and induction of ER stress. ER stress, a physiological response to unfolded protein accumulation, was associated with NLRP3 activation in the endothelium[44] and could act as a mediator of CVDs and hypertension.[45] In our model, activation of ER stress may be responsible for activation of NLRP3 inflammasome, which leads to production and release of cytokines, such as interleukin-6. The aforementioned inflammatory stimuli may therefore orchestrate the crosstalk between ECs and VSMCs[46] and induce VSMC proliferation and hypertrophy causing the thickened vessel wall[47] as seen in the Tie2-ERT2Cre^{+/−}TBCE^{fl/fl} transgenic mice.

The VSMC-targeted transgenic mice showed vascular dysfunction and stiffness already at baseline, which was aggravated by AngII treatment. Lipocalin-2 (LCN2) was up-regulated in the SMMHC-ERT2-Cre^{+/−}TBCE^{fl/fl} aortas, especially after AngII treatment (*Figure 3A*).
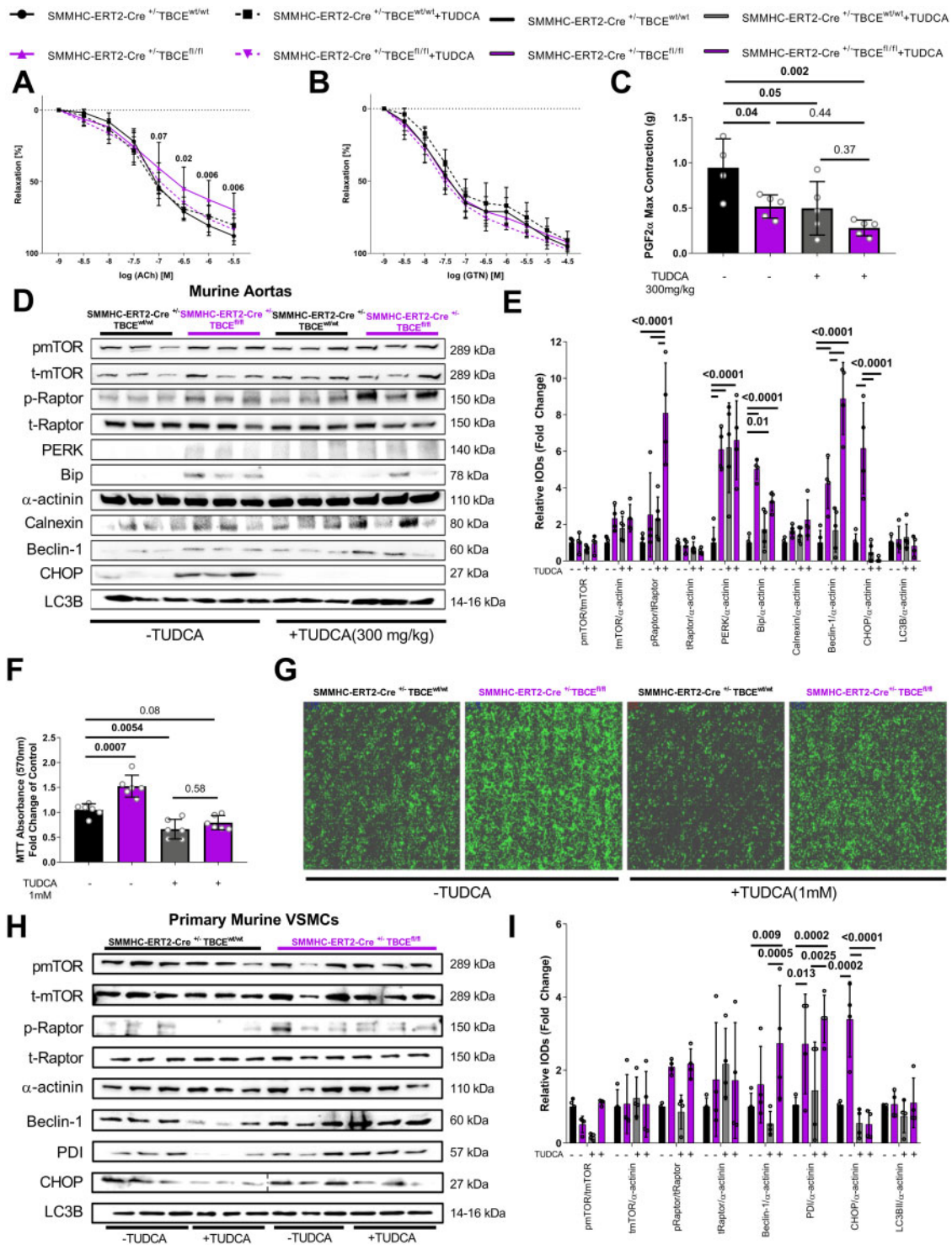
Figure 5 The chemical chaperone TUDCA reverses vascular dysfunction and VSMCs proliferation in mice with SMC-specific TBCE deficiency. Concentration/relaxation curves of isolated aortic rings, in response to (*A*) ACh and (*B*) GTN (Two-Way ANOVA, Tukey's multiple comparison test) (*C*) Maximal contraction (g) in response to PGF2α (*D*) Representative western blot images and (*E*) Graph of relative densitometry analysis of ER stress and autophagy markers of the murine aortas. (*F*) MTT absorbance (570 nm) graph of the SMMHC-ERT2-Cre$^{+/-}$TBCE$^{wt/wt}$ and SMMHC-ERT2-Cre$^{+/-}$TBCE$^{fl/fl}$ VSMCs with/without TUDCA treatment (1 mM) (*G*) Confluency images of the primary VSMCs in culture with/without TUDCA treatment (1 mM). (*H*) Representative Western blot images and *I.* graph of relative densitometry analysis of ER stress and autophagy markers of the primary VSMCs normalized to α-actinin. (Tukey's multiple comparison test; 1 way ANOVA; Different n numbers in the in vitro experiments refer to individual experiments; Dotted line on western blot images represent a readjustment of the representative image).

LCN2 is associated with increased VSMC proliferation and migration[48] or pro-fibrotic effects[49] and is correlated with symptomatic carotid atherosclerosis in humans.[50] LCN2 is a key inducer of ER stress in human pulmonary arterial smooth muscle cells and augments the progression of pulmonary hypertension.[51]

By investigating aortas and VSMCs isolated from the transgenic mice, we found that vascular stiffness and dysregulation were related to ER stress. Both in vivo and in vitro, ER chaperones and indicators of ER stress such as calnexin and PDI[52] were up-regulated when TBCE was diminished in VSMCs, leading to increased VSMC proliferation.

To confirm that ER stress is involved in mediating the vascular phenotype of SMMHC-ERT2-Cre$^{+/-}$TBCE$^{fl/fl}$ mice, we used the chemical chaperone TUDCA, shown to mitigate ER stress in multiple cell types[53] including VSMCs.[54–56] Oral administration of TUDCA reversed vascular dysfunction at the selected dose of 300 mg/kg. Furthermore, it decreased PGF2α-induced contractility, paralleled by the induction of Raptor/Beclin-1 related autophagy.[57] Evidence on TUDCA-induced autophagy are manifold; it may both inhibit and induce ER stress-mediated autophagy in nerve cells.[58] However, it is also established that TUDCA is capable of modulating the autophagy machinery and induce/amplify autophagy signalling in familial amyloidotic polyneuropathy.[59] It may also lead to Beclin-1-dependent autophagic flux,[58] supporting our in vivo and in vitro data.

The role of TUDCA in the treatment of vascular dysfunction is currently addressed by ongoing clinical studies [NCT03331432: Effects of Dietary Supplement Tauroursodeoxycholic Acid on Vascular Function (completed); NCT04001647: Targeting ER Stress in Vascular Dysfunction (recruiting); data from https://ClinicalTrials.gov]. TUDCA has shown to be beneficial in patients with amyotrophic lateral sclerosis in terms of cumulative survival and Amyotrophic Lateral Sclerosis Functional Rating Scale Revised score[60] and in obese-diabetic patients in terms of insulin reactivity,[61] apparently with little side effects. It therefore seems to be a safe dietary supplement and may be a promising agent against arterial hypertension. This is supported by the notion that in translational murine models, TUDCA has anti-hypertensive effects. In ob/ob mice, TUDCA mitigated arterial hypertension and cardiac hypertrophy.[62] In spontaneously hypertensive rats and in type 2 diabetic mice, TUDCA exhibited anti-hypertensive potential and improved coronary arterial function, at least in part by ameliorating ER stress.[63,64]

Conclusively, TBCE down-regulation leads to a multi-faceted vascular dysfunction in mice and humans. Most significantly, down-regulation of the chaperone TBCE in VSMCs leads to severe vascular dysfunction and stiffness characterized by an aberrant ER response and VSMC hyperplasia. Modulation of ER stress by the chemical chaperone TUDCA, attenuated the phenotype through enhancement of Beclin-1-induced autophagy. The effectiveness of TUDCA (approved in the USA for the treatment of primary biliary cirrhosis[53]) suggests putative translational value of our findings.

This is the first study investigating the role of TBCE from bedside to bench, highlighting the role of microtubule dynamics and ER stress in the vascular homeostasis (see Graphical Abstract). It represents a translational approach to identify novel targets of vascular dysfunction through a GWAS analysis of the general population, leading to the discovery of up-to-now unknown contributors to vascular dysfunction.

Limitations of the study

Despite the fact that our study extensively investigated the role of TBCE in vascular function in a translational manner, there are some study limitations. It should be noted that the data presented here may be limited by small sample sizes; a non-significant difference cannot be interpreted as a lack of association. Blood pressure in vivo was assessed by a non-invasive methodology, namely tail-cuff measurements and not by the invasive telemetry catheter implantation regarded as gold standard of blood pressure monitoring. Moreover, ER stress is a general term, describing more complex pathophysiological processes than we assessed in this work. More functional experiments will be needed to define the role of TBCE in ER stress in more detail.[65] Even though TUDCA has a well-established effect on ER stress, it has a multifaceted mechanism and we cannot rule out that its protective effects against vascular dysfunction are mediated—at least in part—by other modes of action that are not related to TBCE deficiency. To strengthen the findings from our GWAS study, it should be considered to validate our associations in an independent cohort in order to increase the translational value of TBCE SNPs as mediators of vascular dysfunction.

Supplementary material

Supplementary material is available at European Heart Journal online.

Data availability

The data underlying this article are available in the article and in its online supplementary material.

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Translational perspective

With this translational research project, we report the hitherto unknown relation between tubulin-folding cofactor E (TBCE) and vascular function discovered in a genome-wide association study of 4175 individuals and further investigated the putative mechanisms in three cell-specific conditional knockout animals. This is the first bedside-to-bench study correlating TBCE with vascular dysfunction, establishing endoplasmic reticulum (ER) stress as a key-mediator of the phenotype. Importantly, administration of the clinically relevant ER stress modulator tauroursodeoxycholic acid reversed vascular dysfunction *in vivo*. Our work establishes the grounds for further evaluating the role of TBCE and tubulin homeostasis in vascular dysfunction in both the pre-clinical and clinical setting.