Circulation Research

ORIGINAL RESEARCH

Targeting SIRT1 Rescues Age- and Obesity-Induced Microvascular Dysfunction in Ex Vivo Human Vessels

Alessandro Mengozzi*, Sarah Costantino*, Francesco Paneni, Emiliano Duranti, Monica Nannipieri, Rudj Mancini, Michele Lai, Veronica La Rocca, Ilaria Puxeddu, Luca Antonioli, Matteo Fornai, Marco Ghionzoli, Georgios Georgiopoulos, Chiara Ippolito, Nunzia Bernardini, Frank Ruschitzka, Nicola Riccardo Pugliese, Stefano Taddei, Agostino Virdis*, Stefano Masi*

BACKGROUND: Experimental evidence suggests a key role of SIRT1 (silent information regulator 1) in age- and metabolic-related vascular dysfunction. Whether these effects hold true in the human microvasculature is unknown. We aimed to investigate the SIRT1 role in very early stages of age- and obesity-related microvascular dysfunction in humans.

METHODS: Ninety-five subjects undergoing elective laparoscopic surgery were recruited and stratified based on their body mass index status (above or below 30 kg/m^2) and age (above or below 40 years) in 4 groups: Young Nonobese, Young Obese, Old Nonobese, and Old Obese. We measured small resistance arteries' endothelial function by pressurized micromyography before and after incubation with a SIRT1 agonist (SRT1720) and a mitochondria reactive oxygen species (mtROS) scavenger (MitoTEMPO). We assessed vascular levels of mtROS and nitric oxide availability by confocal microscopy and vascular gene expression of SIRT1 and mitochondrial proteins by qPCR. Chromatin immunoprecipitation assay was employed to investigate SIRT1-dependent epigenetic regulation of mitochondrial proteins.

RESULTS: Compared with Young Nonobese, obese and older patients showed lower vascular expression of SIRT1 and antioxidant proteins (FOXO3 [forkhead box protein O3] and SOD2) and higher expression of pro-oxidant and aging mitochondria proteins p66^{Shc} and Arginase II. Old Obese, Young Obese and Old Nonobese groups endothelial dysfunction was rescued by SRT1720. The restoration was comparable to the one obtained with mitoTEMPO. These effects were explained by SIRT1-dependent chromatin changes leading to reduced p66^{Shc} expression and upregulation of proteins involved in mitochondria respiratory chain.

CONCLUSIONS: SIRT1 is a novel central modulator of the earliest microvascular damage induced by age and obesity. Through a complex epigenetic control mainly involving p66^{Shc} and Arginase II, it influences mtROS levels, NO availability, and the expression of proteins of the mitochondria respiratory chain. Therapeutic modulation of SIRT1 restores obesity- and age-related endothelial dysfunction. Early targeting of SIRT1 might represent a crucial strategy to prevent age- and obesity-related microvascular dysfunction.

GRAPHIC ABSTRACT: A graphic abstract is available for this article.

Key Words: aging ■ endothelial cells ■ microcirculation ■ mitochondria ■ obesity ■ sirtuins

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Cardiovascular morbidity and mortality burden health care systems worldwide and are deemed to worsen in the following years.[1] Two are the primary culprits: age and obesity. Increased lifespan often leads to unhealthy aging,[2] while obesity, whose prevalence also is expected to increase,[3] ignites the cardiovascular risk,[4] inflicting permanent

Correspondence to: Alessandro Mengozzi, MD, Department of Clinical and Experimental Medicine, University of Pisa, Italy, Via Savi 10. Email alessandro.mengozzi@medmcs.unipi.it
*A. Mengozzi, S. Costantino, A. Virdis, and S. Masi contributed equally.
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Novelty and Significance

What Is Known?

- The increasing prevalence of ageing and obesity fuels cardiovascular mortality and morbidity.
- Microcirculation is one of the earliest sites on which age and metabolic diseases exert their damage.
- SIRT1 (silent information regulator 1) mediates the benefits of calorie restriction in models of impaired ageing or metabolism.

What New Information Does This Article Contribute?

- SIRT1 is a central modulator of the earliest microvascular damage induced by age and obesity.
- Targeting SIRT1 restores endothelial dysfunction to a degree directly proportional to the age- and obesity-related damage.
- SIRT1 modulation of endothelial function is mediated by epigenetic changes mainly involving $p66^{Shc}$ and Arginase II and promoting an improved mitochondrial redox state.

The steadily increasing cardiovascular disease prevalence requires novel, effective therapeutic strategies. Research must observe cardiovascular risk from a new perspective: age and metabolic-related damage. Consistent with this premise, we focused on the earliest impairment site: microcirculation. To the best of our knowledge, this is the first time that the direct contribution of vascular SIRT1 to the restoration of microvascular endothelial function in both ageing and obesity has been explored in humans. We demonstrate a novel and crucial role in rescuing the human microcirculatory endothelial dysfunction, consistent across the whole spectrum of age- and metabolism-related impairment. Our findings depict SIRT1 as a novel central regulator of a homeostatic axis that connects vascular phenotype to systemic metabolism and ageing and to exposure to external adverse *stimuli*. SIRT1 capacity to protect and reverse the harmful epigenetic signature inflicted by ageing and obesity required further dedicated research. The vascular-specific protective effect described in humans might be translated into therapeutic strategies to preserve or restore SIRT1 in the human microcirculation, ultimately relieving healthcare systems.

Nonstandard Abbreviations and Acronyms

ATP6	ATP synthase 6
BMI	body mass index
Cytb	cytochrome b
FOXO3	forkhead box protein O3
mtROS	mitochondria reactive oxygen species
ND2	NADH dehydrogenase 2
ND5	NADH dehydrogenase 5
SIRT1	silent information regulator 1
SOD2	superoxide dismutase-2

damage which persists despite the optimal disease control.[5] It is becoming clear how, to challenge the age- and obesity-related cardiovascular risk, we must target the earliest site of damage: microvascular endothelial dysfunction.[6–8]

Sirtuins have raised substantial attention.[9] The mammalian SIRT1 (silent information regulator 1) is a histone deacetylase overexpressed during calorie restriction.[10] It promotes beneficial effects on lifespan and cardiometabolic homeostasis. In vitro and in vivo observations have demonstrated the downregulation of SIRT1 during aging[9] and obesity[11] and that, in murine models, restoring the activity of SIRT1 might improve endothelial function.[12] This evidence might suggest a relevant role for this histone deacetylase in linking the aging process and the metabolic damage to vascular health.[9,11] Mitochondria have been, at least in part, appointed as putative mediators of these effects.[13] Indeed, mitochondria dysfunction has been identified as a hallmark of several age-related[14] and metabolic diseases,[15,16] being in a tight relationship with endothelial function.[17] SIRT1 influences the activity of mitochondria by reducing the levels of mitochondrial reactive oxygen species (mtROS),[18] potentially preserving their oxidative phosphorylation efficiency and dynamics.[19–21]

In humans, some indirect observation with resveratrol (a SIRT1 activator)[22] shows encouraging findings, and some trials involving SIRT1 agonists are currently ongoing.[9] However, evidence is limited and, in part, controversial.[9] No study by far has assessed the effect of SIRT1 across aging and metabolic disease directly on the vascular phenotype.

We aimed to explore the contribution of SIRT1 to obesity- and age-related microvascular dysfunction in humans, focusing on its very early stages. To conduct a comprehensive and exhaustive exploration, we also investigated the mechanisms of the potential influence of SIRT1 on microvascular dysfunction, focusing on mitochondria oxidative stress signaling.

METHODS

Data Availability

All data are available to qualified investigators upon reasonable request.

Population

In a case-control design, 47 obese patients and 48 nonobese were consecutively recruited among patients referring to the Department of Clinical and Experimental Medicine of the University of Pisa for laparoscopic bariatric surgery and among consecutive patients referring to the Department of Surgery of the University of Pisa to undergo elective inguinal hernia repair. The population inclusion and exclusion criteria are reported in the Supplemental Material.

Microvessels (150–300 μm) were isolated from subcutaneous adipose tissue and mounted on a pressurized myograph to assess the vascular phenotype. Vessels were also processed for gene expression profiling, in situ mtROS and NO, Western blot, mitochondrial swelling and chromatin immunoprecipitation assays (Supplemental Material). The procedures of the study were in accordance with the institutional guidelines. The protocol was approved by the local Ethical Committee (protocol n. 12589), and each participant gave written informed consent to the study. All human studies and handling of human material were in accordance with the declaration of Helsinki.

Statistical Analysis

Continuous variables were tested for normality by the Shapiro-Wilk test. Non-normal variables were natural log-transformed when used in regression models or parametric tests. Data were presented as mean±SD for continuous parametric variables, as median (IQR) for continuous nonparametric variables and as percentages for binary variables. Participants were stratified based on their body mass index (BMI) status (≤ or >30 kg/m^2) and age (≤ or >40 years) into 4 groups: Young Nonobese, Young Obese, Old Nonobese and Old Obese. Pearson correlation was used to assess association, as appropriate. Independent samples Student *t* test or 2-way ANOVA using age and BMI categories as fixed factors and Holm-Sidak as a post hoc test was used to determine significant differences between groups, as appropriate. ANOVA for repeated measures, followed by a post hoc test with Holm-Sidak correction, was used to evaluate differences in the vasodilation obtained after incubation of the vessel with different drugs. When nonparametric tests were needed, the Kruskal-Wallis test with Dwass-Steel-Critchlow-Flinger post hoc test or Scheirer-Hare-Ray test using age and BMI categories as fixed factors followed by Dunn post hoc correction were used to compare independent groups. Within the same group, data were compared by Friedman test with Durbin-Conover as a post hoc test. No experiment-wide/across-tests multiple test correction was applied. A value of $P<0.05$ was considered statistically significant. All analyses were performed using SPSS software (IBM, version 20.0), GraphPad Prism (GraphPad Software, version 7.04), and jamovi (The Jamovi project 2022, version 2.3).

RESULTS

Biochemical Characterization and Vascular Phenotype

Table reports the studied population's clinical characteristics, stratified by age and BMI groups. Sex distribution was not statistically different across the 4 groups. There was no significant difference in age between Young Obese and Young Nonobese groups, while the age of the Old Nonobese was higher than the Old Obese ($P<0.001$). BMI was not different between Young Obese and Old Obese and between Young Nonobese and Old Nonobese. As expected, fasting insulin and the homeostatic model assessment for insulin resistance index were higher in Old Obese and Young Obese than in Old Nonobese and Young Nonobese. Also, eGFR was different between the groups, influenced by age-related decline and obesity-related hyperfiltration.[23]

Vessel structural parameters changed across the 4 groups. Figure 1A shows the predicted media-to-lumen ratio by age in obese versus nonobese. A 5-fold steeper slope was observed in the obese group, and a significant age*BMI group interaction ($P<0.01$) was shown. Endothelium-dependent vasodilation showed a graded decline from the Young Nonobese to the Old Obese groups. The Old Nonobese and Young Obese groups presented a similar vasodilatory response to acetylcholine (Figure 1B), while Old Nonobese and Old Obese showed a similar inhibition to L-NAME (Figure 1C). The nonendothelial dependent vasodilatory response was not different between groups (Figure 1D). The impaired endothelial function observed across the 4 groups was associated with substantial changes in intracellular mtROS and NO levels. There was a graded increase in the levels of mtROS with age and obesity (Figure 1E), also confirmed by the use of more specific fluorescent probes in a small number of patients (n=3 for each group; Figure S1). NO availability followed a reverse pattern across the 4 groups (Figure 1F; Figure S1). These features are in keeping with previously reported data from our group[8] and confirm the role of obesity in promoting early small vessel aging.[24]

SIRT1 Expression and Modulation of Genes Involved in the Control of Intracellular mtROS

Gene expression analysis revealed substantial changes in the expression of SIRT1 and several mitochondrial genes regulating NO production/degradation across the 4 groups. SIRT1 was significantly lower in Young Obese and Old Obese groups than in Young Nonobese and Old Nonobese. Old Nonobese also showed reduced SIRT1 expression when confronted with Young Nonobese (Figure 2A). Pro-oxidant enzyme p66^{Shc}, a mitochondria respiratory chain uncoupling factor,[25] followed a reverse trend (Figure 2B). This pattern was consistent for all the explored genes. FOXO3 (Forkhead box protein O3) and superoxide dismutase-2 (SOD2), mitochondrial antioxidant enzymes,[26] were downregulated in Old Obese, Young Obese and Old Nonobese (Figure 2C and 2D). Arginase II, known to impair NO availability during aging through competition with the eNOS for its substrate

Table. Characteristics of the Study Population

	Young non-obese (n=12)	Young obese (n=15)	Old nonobese (n=36)	Old obese (n=32)	*P* (age) / *P*(BMI)
Male sex, %	45%	53%	48%	46%	5.92×10^{-4}
Age, y	36±5‡§	32±6‡§	62±11*†§	55±8*†‡	6.12×10^{-21}
					9.62×10^{-3}
BMI, kg/m^2	23.71±3.17†§	46.67±7.42*‡	24.38±5.08†§	48.68±4.58*‡	NS
					4.22×10^{-38}
Systolic blood pressure, mm Hg	115±12	120±8	124±17	123±9	NS
					NS
Diastolic blood pressure, mm Hg	74±9	73±10§	79±7	80±8	2.25×10^{-3}
					NS
Fasting plasma glucose, mmol/L	5.2±0.7	5.0±0.9	5.7±0.9	5.7±1.2	1.42×10^{-2}
					NS
Fasting plasma insulin, pmol/L	30.9±0.8†§	88.6±2.4*‡	35.6±0.5†§	91.7±1.7*‡	1.57×10^{-2}
					2.87×10^{-53}
HOMA-IR	1.18±0.1†§	3.30±0.2*‡§	1.51±0.1†§	3.83±0.2*†‡	4.75×10^{-3}
					1.64×10^{-25}
eGFR, mL/min per 1.73 m^2	101±10†‡§	117±10*‡§	83±7*†§	87±9*†‡	9.35×10^{-4}
					9.48×10^{-7}
Total cholesterol, mmol/L	4.7±0.4	4.9±0.5	4.7±0.3	4.7±0.5	NS
					NS
Media-to-lumen ratio, %	5.94±0.48†§	9.27±1.71*‡§	7.26±0.39†§	10.14±1.54*†‡	8.97×10^{-5}
					7.41×10^{-26}
Media cross-sectional area, μm^2	10862±2784†§	15077±4963*‡	12824±2406†§	16609±3862*‡	NS
					2.08×10^{-7}

Patients did not assume any chronic therapy, nor did neither suffer from any cardiometabolic diseases except for obesity (in the Young Obese and Old Obese groups). All patients recruited were non-Hispanic white. Results are expressed as mean±SD or percentage values for categorical variables. Shapiro-Wilk test was adopted to assess normality. Data are presented as mean±SEM and were compared by 2-way ANOVA using age and BMI categories as fixed factors followed by Holm-Sidak post hoc correction. χ^2 was used to assess differences between groups for the male sex. BMI indicates body mass index; eGFR, estimated glomerular filtration rate; and HOMA-IR, the homeostatic model assessment for insulin resistance.

*$P<0.05$ vs Young Nonobese.

†$P<0.05$ vs Young Obese.

‡$P<0.05$ vs Old Nonobese.

§$P<0.05$ vs Old Obese.

arginine,[8] was increasingly expressed moving from Young Nonobese to Old Obese (Figure 2E).

SRT1720 Rescues Age- and Obesity-Related Endothelial Dysfunction

Incubation of the small arteries with SRT1720 improved endothelial function in Old Obese, Young Obese and Old Nonobese groups but not in the Young Nonobese group (Figure 3A through 3D). This improvement was more remarkable in the group with the most impaired phenotype (Old Obese) and of a similar magnitude between Old Nonobese and Young Obese; no statistical difference was reached between Young Obese and Old Obese (Figure 3E and 3F). Restoration of L-NAME inhibition to ACh response followed a similar trend across the 4 groups. In this case, Old Obese improvement was not different to the one observed for neither Young Obese nor Old Nonobese. The restoration of both ACh and L-NAME responses were related to age, BMI, and vascular remodelling (for Ach: $P<0.001$ for each; for L-NAME: $P<0.001$ for age, $P=0.0107$ for BMI, $P<0.001$ for media-to-lumen ratio; Figure 4). This relationship was also confirmed after adjustment for age, BMI, sex, mean blood pressure, creatinine and homeostatic model assessment for insulin resistance (for Ach: $P<0.001$ for age, $P=0.028$ for BMI, $P<0.001$ for media-to-lumen ratio; for L-NAME: $P<0.001$ for age, $P=0.003$ for media-to-lumen ratio; BMI was nonsignificant). Notably, sex was not related to the rescue of endothelial function in unadjusted or adjusted models.

Selective SIRT1 Inhibition in Young Nonobese Subjects Blunts Microvascular Endothelial Function

To explore the mechanisms underpinning SIRT1 restored endothelial function, we first aimed to assess selective

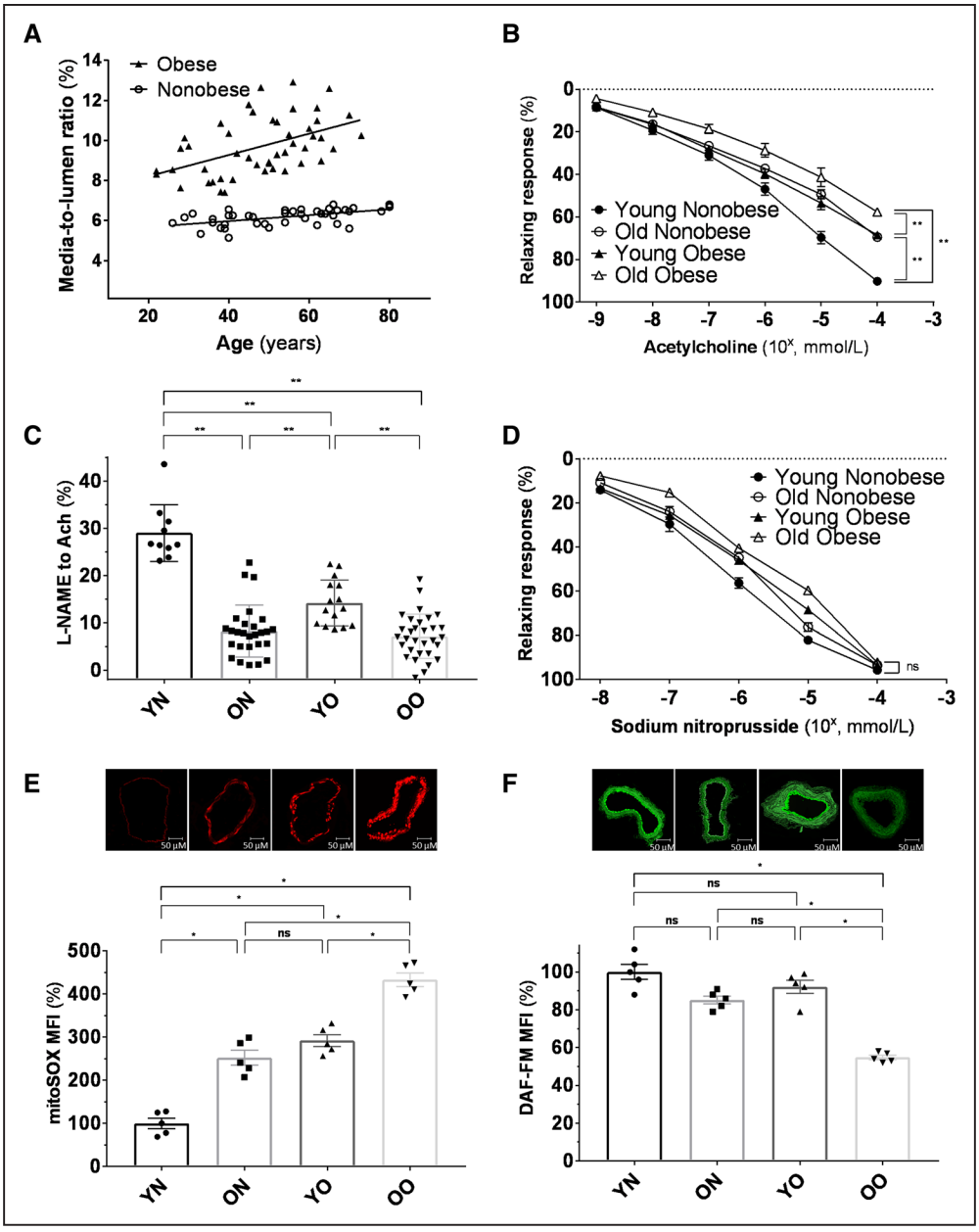

Figure 1. Microvascular characterization of the study population.
A, Media-to-Lumen (M/L) ratio per year of age in nonobese (white circle; n=42) and obese (black triangle; n=47) subjects. Regression lines for each group are shown. M/L ratio is expressed as percentage (%). Age and M/L are tightly related in both groups (obese: r=0.487, $P<0.01$; nonobese: r=0.555, $P=0.001$). The slope was 5-fold steeper in the obese group. In detail, the regression coefficients (x=0.05 in obese, x=0.01 in nonobese) depict how M/L increase of 0.5%/10 years in obese vs 0.1%/10 years in nonobese. Shapiro-Wilk test was adopted to assess normality. Linear regression analysis was conducted by adopting the M/L ratio as dependent variable, age as a covariate and BMI group (nonobese vs obese) as a factor. Interaction between terms was tested ($P<0.01$). **B**, Vasorelaxation to cumulative concentration of ACh in vessels precontracted with norepinephrine in the 4 groups (Young Nonobese: n=12; Old Nonobese: n=31; Young Obese: n=15; Old Obese: n=32). Vasodilatory response is expressed as % of the maximal diameter (Young Nonobese: black circle, Old Nonobese: white circle, Young Obese: black triangle, Old Obese: white triangle). **C**, Inhibition of ACh dilation by L-NAME expressed as the difference between the maximal vasodilatory response to ACh alone vs co-incubated with L-NAME. **D**, Vasorelaxation to cumulative concentration of SNP in vessels precontracted with norepinephrine in the 4 groups (young nonobese: n=12; old nonobese: n=31; young obese: n=15; old obese: n=32). Vasodilatory response is expressed as % of the maximal diameter (young nonobese: black circle, old nonobese: white circle, young obese: black triangle, old obese: white triangle). **E** and **F**, Differences in mtROS (**E**) and NO levels (**F**) assessed by mitoSOX and DAF-FM fluorescence in the 4 groups (n=5 for each group). Fluorescence is calculated as mean fluorescence intensity (MFI) and expressed as % of the Young Nonobese group. Original magnification is 40×. Shapiro-Wilk test was adopted to assess normality. Data are presented as mean±SEM and were compared by compared by 2-way ANOVA using age and BMI categories as fixed factors followed by Holm-Sidak post hoc correction (**B**–**D**) and Scheirer-Hare-Ray test using age and BMI categories as fixed factors followed by Dunn post hoc correction (**E** and **F**). A $P<0.05$ was considered significant. *$P<0.05$. **$P<0.01$. Ach indicates acetylcholine; M/L, media-to-lumen ratio; MFI, mean fluorescence intensity; ON, old nonobese; OO, old obese; SNP, sodium nitroprusside; YN, young nonobese; and YO, young obese.

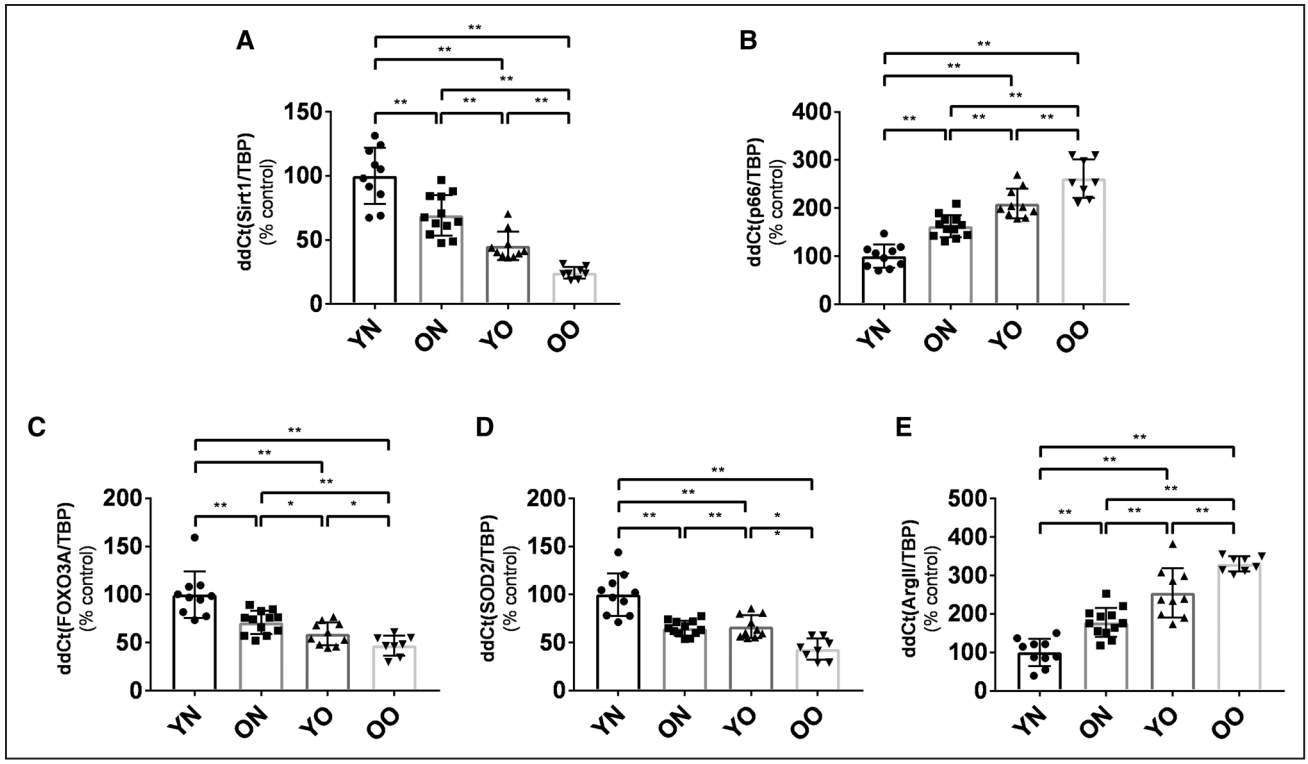

Figure 2. Expression of SIRT1 (silent information regulator 1) and SIRT1-related genes in the vascular wall of the 4 groups. **A**, Expression of SIRT1. **B**, Expression of the pro-oxidant enzyme p66^{Shc}. **C**, Expression of the antioxidant FOXO3 (Forkhead box protein O3). **D**, Expression of the SOD2 (superoxide dismutase-2). **E**, Expression of Arginase II. Results are expressed as % with respect to the Young Nonobese group. Shapiro-Wilk test was adopted to assess normality. FOXO3 and SOD2 were natural log-transformed for the means of the analyses. Young Nonobese: n=10, Old Nonobese: n=12, Young Obese: n=10, Old Obese: n=8. Data are presented as mean±SD and were compared by 2-way ANOVA followed by Holm-Sidak post hoc correction. A $P<0.05$ was considered significant. $^{*}P<0.05$;$^{**}P<0.01$. ArgII indicates Arginase II; FOXO3, Forkhead box protein O3; ON, old nonobese; OO, old obese; SOD-2, superoxide dismutase-2; YN, young nonobese; and YO, young obese.

SIRT1 effects on the microvascular phenotype. Thus, we tested selective SIRT1 inhibition on Young Nonobese vessels (n=5). The Young Nonobese group, that is, individuals with the most preserved vascular phenotype, was chosen for both the expected more significant difference in effect magnitude and the avoidance of underlying confounding impairment. Vasodilatory response to ACh and L-NAME of Young Nonobese small vessel transfected with SIRT1 siRNA sc-40986 was similar to the one observed at baseline for the Young Obese group (Figure 5A). mtROS and NO availability were as well impaired (Figure 5B and 5C). Western blot and qPCR assays were used to confirm the abrogation of SIRT1 levels in siRNA-treated vessels (Figure S2).

SIRT1 Inhibition on p66^{Shc} and Arginase II Is Attenuated in Small Visceral Arteries of Old Obese

To explore SIRT1 epigenetic control on p66^{Shc} and Arginase II, we performed a chromatin immunoprecipitation assay in the Old Obese and Young Nonobese groups, reflecting the most deranged and preserved phenotypes, respectively. We observed that the binding of SIRT1 on p66^{Shc} and Arginase II promoters is markedly reduced in Old Obese (Figure S3). This marked difference shows that in Old Obese the downregulated SIRT1 cannot prevent, by epigenetic modulation of p66^{Shc} and Arginase II promoters, the overexpression of these 2 pro-oxidant and pro-aging factors, as shown in Figure 2B and 2E.

SRT1720 Overnight Incubation Reduces mtROS Levels and Increases NO Availability in Small Visceral Arteries of Old Obese

To support the potential short-term influence of SIRT1 on the control of mtROS in obesity and to gather further information on the possible mechanisms underlying these effects, segments of small resistance arteries of Old Obese patients (n=5) were studied before or after incubation for 24 hours with SRT1720. Baseline Young Nonobese vessels used for the siRNA experiments were used as controls to compare fluorescence intensity. The segments studied after incubation with SRT1720 showed a significant reduction in the levels of mtROS and a substantial improvement in the NO availability compared with the segments studied

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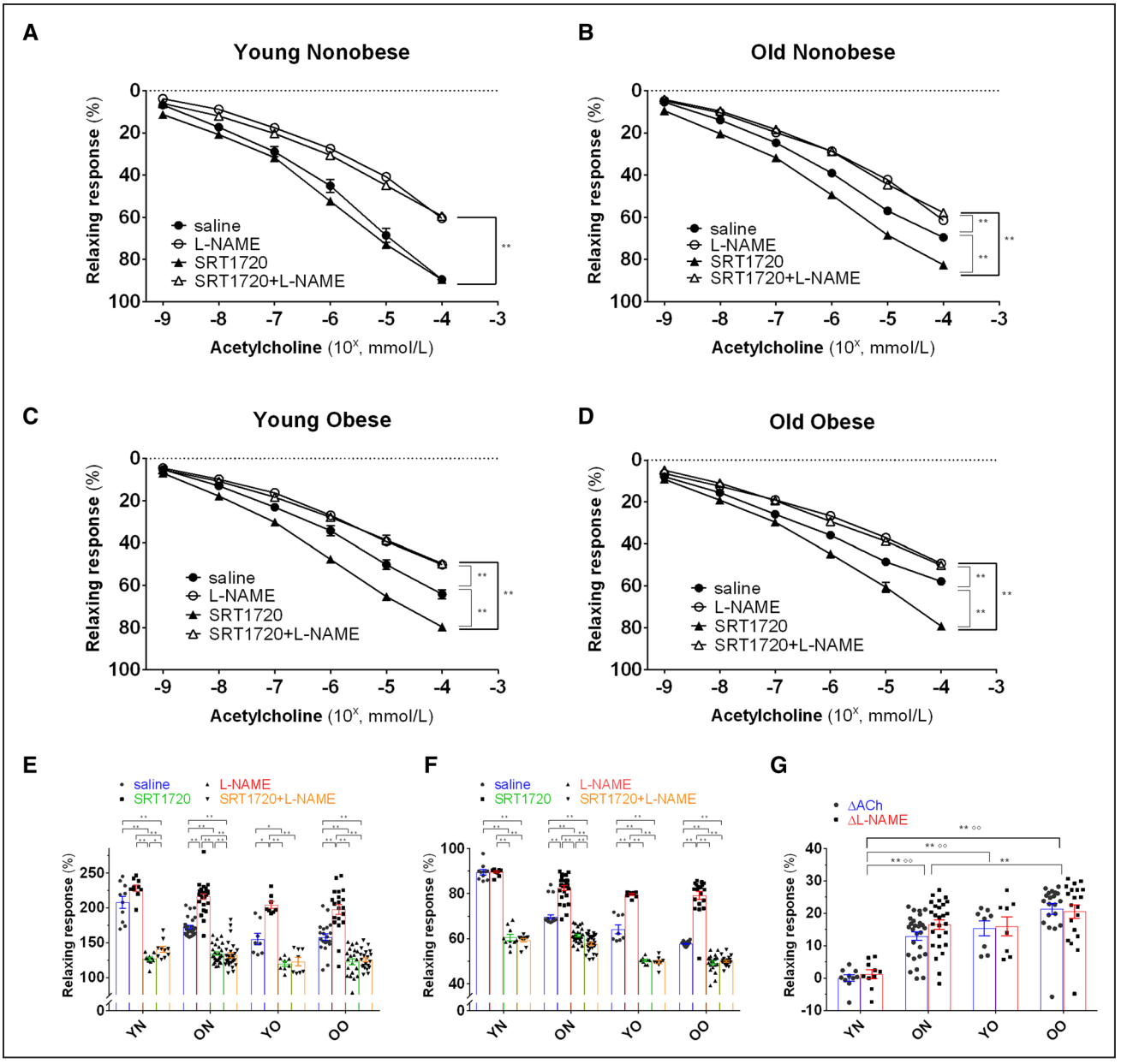

Figure 3. SRT1720 rescues relaxing response to Ach and L-NAME inhibition in the 4 groups.
A–**D**, Relaxing response to cumulative concentration to Ach in vessels precontracted with norepinephrine in the 4 groups (young nonobese: n=10; old nonobese: n=27; young obese: n=8; old obese: n=20). Vasodilatory response is expressed as % of the maximal diameter. The experiment was repeated 4 times for each patient by incubating the vessel with saline (black circle), L-NAME (white circle), SRT1720 (black triangle), SRT1720+L-NAME (white triangle). **E** and **F**, AUC (**E**) and maximal (**F**) vasodilatory response to Ach expressed as % of the maximal diameter in the 4 groups (young nonobese: n=10; old nonobese: n=27; young obese: n=8; old obese: n=20) in vessel precontracted with norepinephrine in the 4 groups. Vessels were incubated with saline (blue bars), SRT1720 (green bars), L-NAME (red bars) and SRT1720+L-NAME (orange bars). Baseline AUC and maximal vasodilatory response (blue bars) differ between the 4 groups ($P<0.01$), as expected. **G**, Maximal improvement in vasodilatory response to ACh (ΔACh, blue bars) or inhibitory response to L-NAME (ΔL-NAME, red bars) after SRT1720 incubation in the 4 groups (young nonobese: n=10; old nonobese: n=27; young obese: n=8; old obese: n=20). Shapiro-Wilk test was adopted to assess normality. Data are presented as mean±SEM and were compared by ANOVA for repeated measures followed by a post hoc test with Holm-Sidak post hoc correction. Baseline AUC and maximal vasodilatory response and maximal improvement in vasodilatory response to ACh (ΔACh) or inhibitory response to L-NAME in the 4 groups were compared by 2-way ANOVA followed by Holm-Sidak post hoc correction. A $P<0.05$ was considered significant. *$P<0.05$.**$P<0.01$. In the last panel (**G**): **$P=0.01$ or less for ΔACh response, °°$P=0.01$ or less for ΔL-NAME response. Ach indicates acetylcholine; ON, old nonobese; OO, old obese; YN, young nonobese; and YO, young obese.

immediately after isolation though inferior to the Young Nonobese (Figure 5B and 5C). These findings were also confirmed in a smaller sample of patients (n=3) by adopting ENZ-53013 and mitoPY1, more specific fluorescent probes for mtROS and NO availability, respectively (Figure S1).

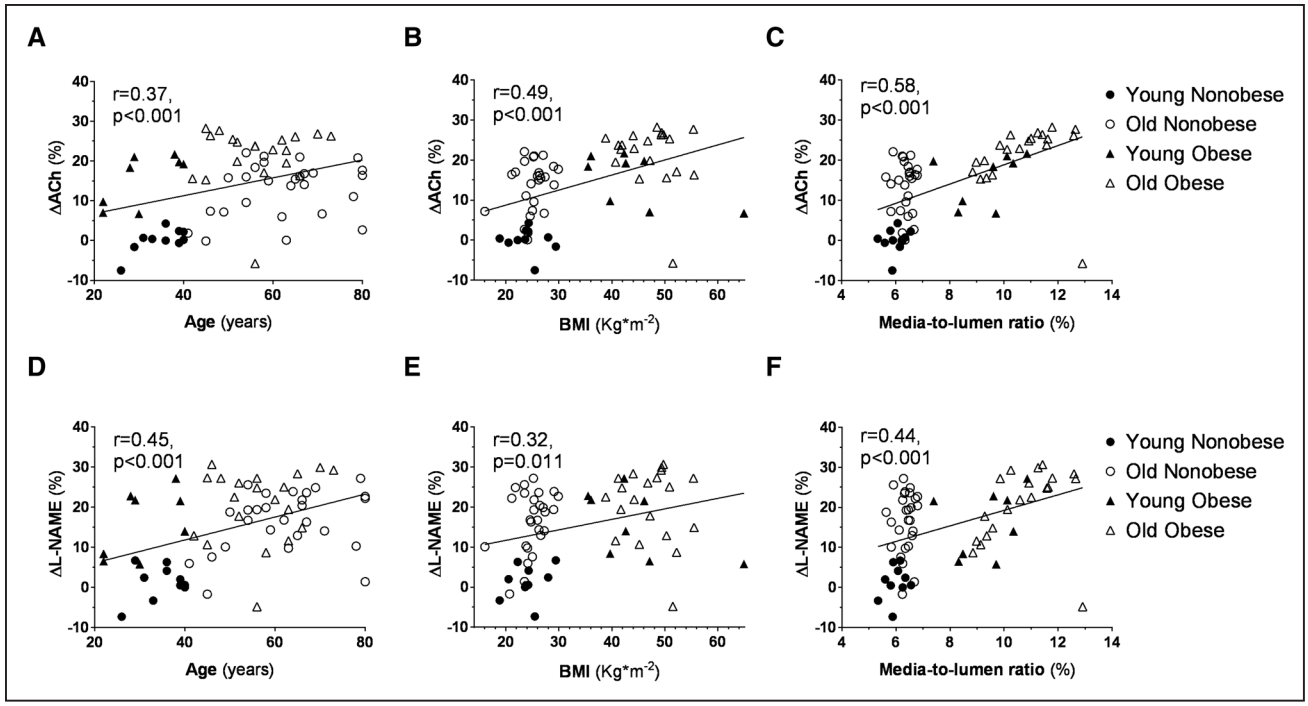

Figure 4. Relationship between the improvement observed with SRT1720 in ACh and L-NAME response and age, body mass index (BMI), and vascular remodeling.

SRT1720 induced improvement in vasodilatory response to ACh (**A**–**C**) and inhibitory response to L-NAME (**D**–**F**) shows a direct relationship with age, BMI and M/L in the 4 groups (young nonobese: black circle, old nonobese: white circle, young obese: black triangle, old obese: white triangle; young nonobese: n=10; old nonobese: n=27; young obese: n=8; old obese: n=20). The improved response is expressed as the improvement in maximal vasodilatory response to ACh (ΔACh) or inhibitory response to L-NAME (ΔL-NAME) after SRT1720 incubation. Shapiro-Wilk test was adopted to assess normality. Linear regression was used to model the relationship between the factors. Age, BMI and M/L were used as continuous variables in the model. Data are shown unadjusted. A $P<0.05$ was considered significant. Ach indicates acetylcholine; and M/L, media-to-lumen ratio.

Comparison of SRT1720 With mtROS and NADPH-Oxidase ROS Scavengers

The independent and additive contribution of mtROS and NADPH-oxidase ROS scavenging was tested in small resistance arteries of Old Obese patients (n=6). We explored the impact of these factors on vessels from the Old Obese as this group represents an example of an extreme dysfunctional phenotype, therefore, ensuring enough power to observe significant potential improvement. As expected by the other experiments showing the SRT1720-mediated reduction of mtROS, SRT1720 and mitoTEMPO effects are superimposable. Instead, gp91dstat alone impact is smaller (Figure 5D and 5E). These experiments highlight how SIRT1 modulation ameliorates endothelial functions in markedly deranged subjects (Old Obese group) with no additive contribution from the mitochondrial- and NADPH-derived ROS scavenging.

SRT1720 Restores Old Obese Small Vessel-Derived Mitochondria Integrity and Respiratory Chain Genes Expression

Mitochondria swelling assay was performed to characterize further the protection induced by a restored SIRT1 function on the mitochondria functional and structural integrity. Mitochondria extracted from segments of small resistance arteries from Old Obese patients (n=5) were studied before and after incubation for 24 hours with SRT1720. The rationale for the choice of the Old Obese group was the same as above. The incubation with SRT1720 improved the resistance of the mitochondria to the calcium load, suggesting an increased resistance of their membrane to fragmentation mainly related to oxidative damage (Figure 5F through 5I). Vessels from the same Old Obese patients (n=5) before and after overnight incubation with SRT1720 were processed to analyze the changes in gene expression and epigenetic patterns related to mitochondria ROS levels. Incubation with SRT1720 caused a downregulation in the expression of the $p66^{Shc}$ ($P=0.02$ versus baseline, Figure 6A) and Arginase II ($P=0.02$ versus baseline, Figure 6B) associated with increased binding of SIRT1 to $p66^{Shc}$ and Arginase II promoters (Figure 6D and 6E). SIRT3 expression was also increased after SRT1720 incubation ($P=0.02$ versus baseline, Figure 6C); however, chromatin immunoprecipitation assays did not show an interaction of SIRT1 with SIRT3 promoter. Mitochondria respiratory chain genes, namely ATP6 (ATP synthase 6), cytochrome b (Cytb), ND2 (NADH dehydrogenase 2)

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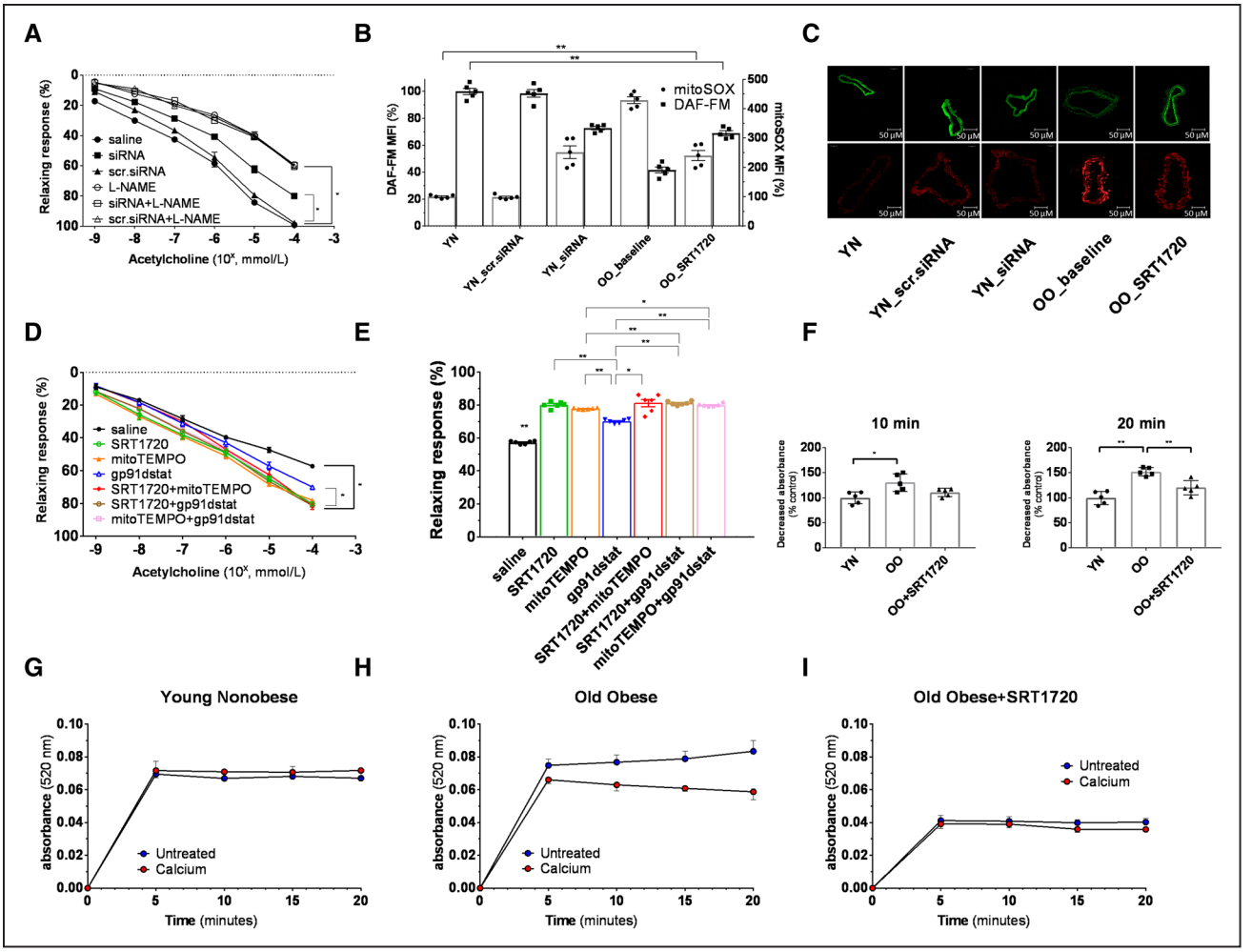

Figure 5. Exploration of the effect and mechanism of the SRT1720-induced rescuing of age- and obesity-related endothelial dysfunction.

A, Vasorelaxation to cumulative concentration of ACh in vessels precontracted with norepinephrine. Vasodilatory response is expressed as % of the maximal diameter. The experiment was conducted on vessels from Young Nonobese (n=5) at baseline (saline group), after overnight resting in transfection culture medium with scrambled siRNA (scr.siRNA group), and after overnight transfection with SIRT1 siRNA sc-40986 (siRNA group). The curves were repeated 2 times for each vessel, the first with ACh alone and the second after co-incubation with L-NAME (white triangle). Data are presented as mean±SEM and were compared by the Kruskal-Wallis test with Dwass-Steel-Critchlow-Flinger post hoc test. Within the same group, data were compared by the Friedman test. **B** and **C**, Differences in mtROS (red staining) and NO (green staining) levels assessed by mitoSOX and DAF-FM fluorescence between Young Nonobese vessels (n=5) at baseline (Young Nonobese), Young Nonobese vessels (n=5) after overnight resting in transfection culture medium with scrambled siRNA (Young Nonobese_scr.siRNA), Young Nonobese vessels (n=5) after overnight transfection with SIRT1 siRNA sc-40986 (Young Nonobese_siRNA) and between Young Nonobese vessels (n=5) at baseline (Young Nonobese), Old Obese vessels (n=5) before overnight incubation with SRT1720 (Old Obese_baseline), Old Obese vessels (n=5) after overnight incubation with SRT1720 (Old Obese_SRT1720). Data are presented as mean±SEM and were compared by the Kruskal-Wallis test with Dwass-Steel-Critchlow-Flinger post hoc test. Fluorescence is calculated as mean fluorescence intensity (MFI) and expressed as % of the Young Nonobese group. Original magnification is 40×. **D**, Relaxing response to cumulative concentration to Ach in vessels from Old Obese group (n=6) precontracted with norepinephrine. Vasodilatory response is expressed as % of the maximal diameter. The experiment was repeated 7 times for each patient, respectively by incubating the vessel with saline alone (black line), SRT1720 (green line), mitoTEMPO (orange line), gp91dstat (blue line), SRT1720+mitoTEMPO (red line), SRT1720+gp91dstat (brown line), mitoTEMPO+gp91dstat (pink line). Shapiro-Wilk test was adopted to assess normality. Data are presented as mean±SEM and were compared by ANOVA for repeated measures followed by a post hoc test with Holm-Sidak post hoc correction. **E**, Maximal vasodilatory response to Ach expressed as % of the maximal diameter in vessels from the Old Obese group (n=6) precontracted with norepinephrine. The experiment was repeated 7 times for each patient, respectively by incubating the vessel with saline alone (black bar), SRT1720 (green bar), mitoTEMPO (orange bar), gp91dstat (blue bar), SRT1720+mitoTEMPO (red bar), SRT1720+gp91dstat (brown bar), mitoTEMPO+gp91dstat (pink bar). Data are presented as mean±SEM and were compared by ANOVA for repeated measures followed by a post hoc test with Holm-Sidak post hoc correction. **F**–**I**, Mitochondria swelling assay of small resistance arteries of Young Nonobese (n=5) and Old Obese group (n=5). Mitochondria were tested before (blue dots) and after (red dots) the calcium load at baseline for Young Nonobese (**G**) and Old Obese groups (**H**) and, for the Old Obese group, after 24-hour incubation with SRT1720 (**I**). After the incubation with SRT1720, the reduction of absorbance was attenuated both at 10 and 20 minutes (**F**) and expressed as % of the Young Nonobese group, used as a control. Data are presented as mean±SEM and were compared by the Kruskal-Wallis test with Dwass-Steel-Critchlow-Flinger post hoc test. *P<0.05.**P<0.01. OO indicates old obese; and YN, young nonobese.

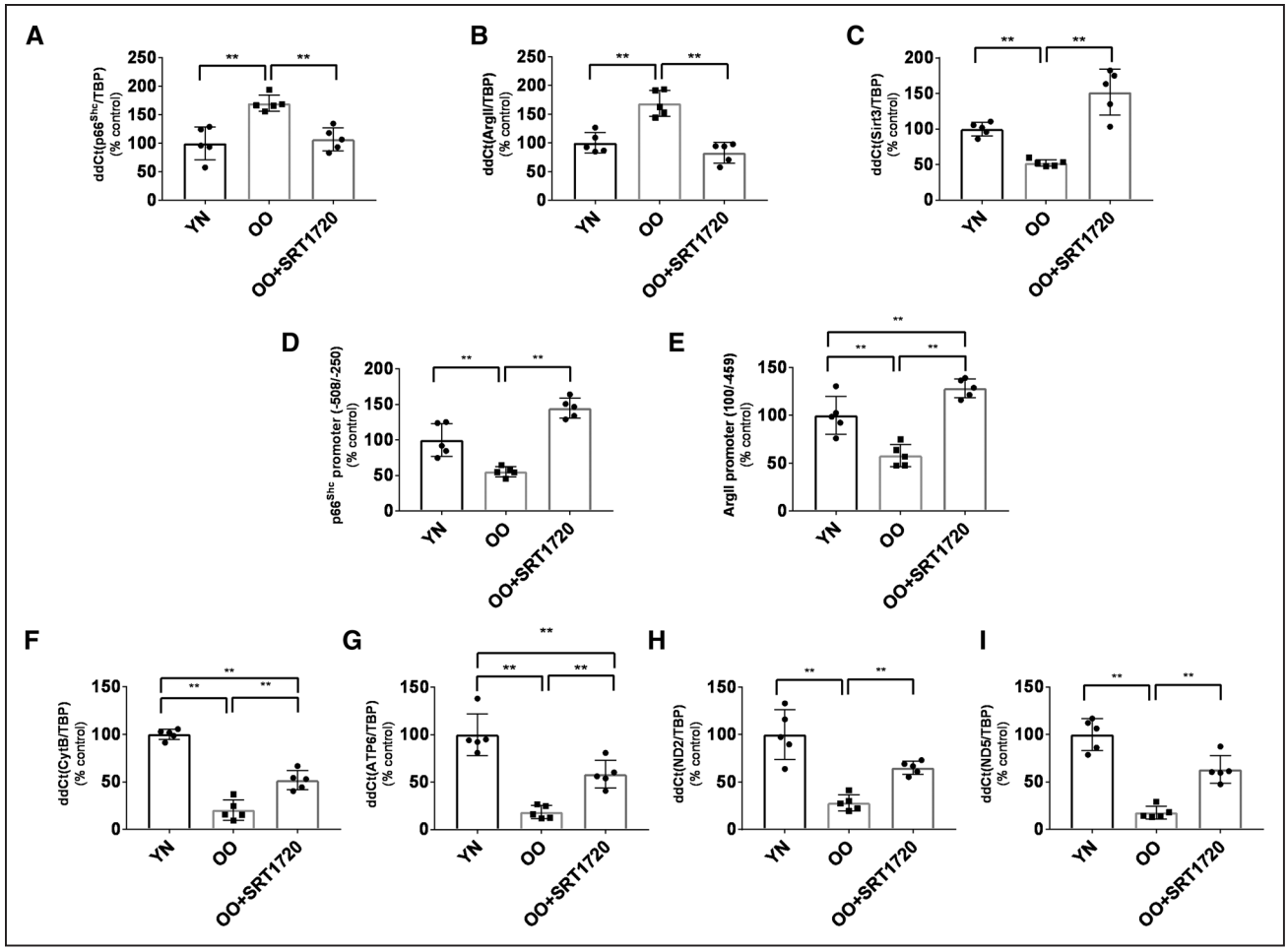

Figure 6. Genic profile of several proteins in the vessel wall of Old Obese subjects (n=5) before and after incubation with SRT1720.

A, Expression of p66^{Shc}. **B**, Expression of Arginase II. **C**, Expression of Sirt3. Notably, the ChIP assay did not detect the binding of SIRT1 (silent information regulator 1) on the Sirt3 promoter. **D**, qPCR after ChIP assay showing binding of SIRT1 on the promoter region of p66^{Shc} before and after incubation with SRT1720. **E**, qPCR after ChIP assay showing binding of SIRT1 on the promoter region of Arginase II before and after incubation with SRT1720. **F**–**I** Expression of mitochondria respiratory chain enzymes: ATP synthase 6 (**F**), Cytochrome b (**G**), NADH dehydrogenase 2 (**H**) and NADH dehydrogenase 5 (**I**). Data are presented as mean±SD and were compared by the Kruskal-Wallis test with Dwass-Steel-Critchlow-Flinger post hoc test. A $P<0.05$ was considered significant. **$P<0.01$. ArgII indicates arginase II; ATP6, ATP synthase 6; Cytb, Cytochrome b; ND2, NADH dehydrogenase 2; OO, old obese; and YN, young nonobese.

and ND5 (NADH dehydrogenase 5; Figure 6F through 6I), were found upregulated. These results suggest that SIRT1 activity can protect against mitochondria' increased ROS generation.

SRT1720 and mitoTEMPO Effect Is Consistent Across Age- and Obesity-Related Endothelial Dysfunction

MitoTEMPO induced a significant increase in the endothelial-dependent vasodilatory response to the Ach in the Young Obese, Old Obese and Old Nonobese groups ($P<0.001$) but not in the Young Nonobese group (Figure 7A through 7D). Intriguingly, the extent to which the endothelial-dependent vasodilation was rescued with MitoTEMPO was comparable to the effect obtained with SRT1720. In a small subpopulation (n=12, 3 subjects for each group), we compared the impact of SRT1720 on the vasodilatory response also with low-dose rotenone, an electron transport chain complex I inhibitor,[27] and tempol, a nontargeted SOD mimetic, confirming the superimposable effect of SIRT1 and an inhibitor of mtROS generation (Figure S4). These finding indirectly confirms that SRT1720 acts on pathways involving mtROS mitigation to control endothelial function across the whole spectrum of aging and obesity.

DISCUSSION

We report for the first time that (1) human microvascular age- and obesity-related endothelial dysfunction are similarly characterized by a progressive reduction in vascular SIRT1 expression; (2) this decrease in microvascular SIRT1 expression is paired with reduced NO availability

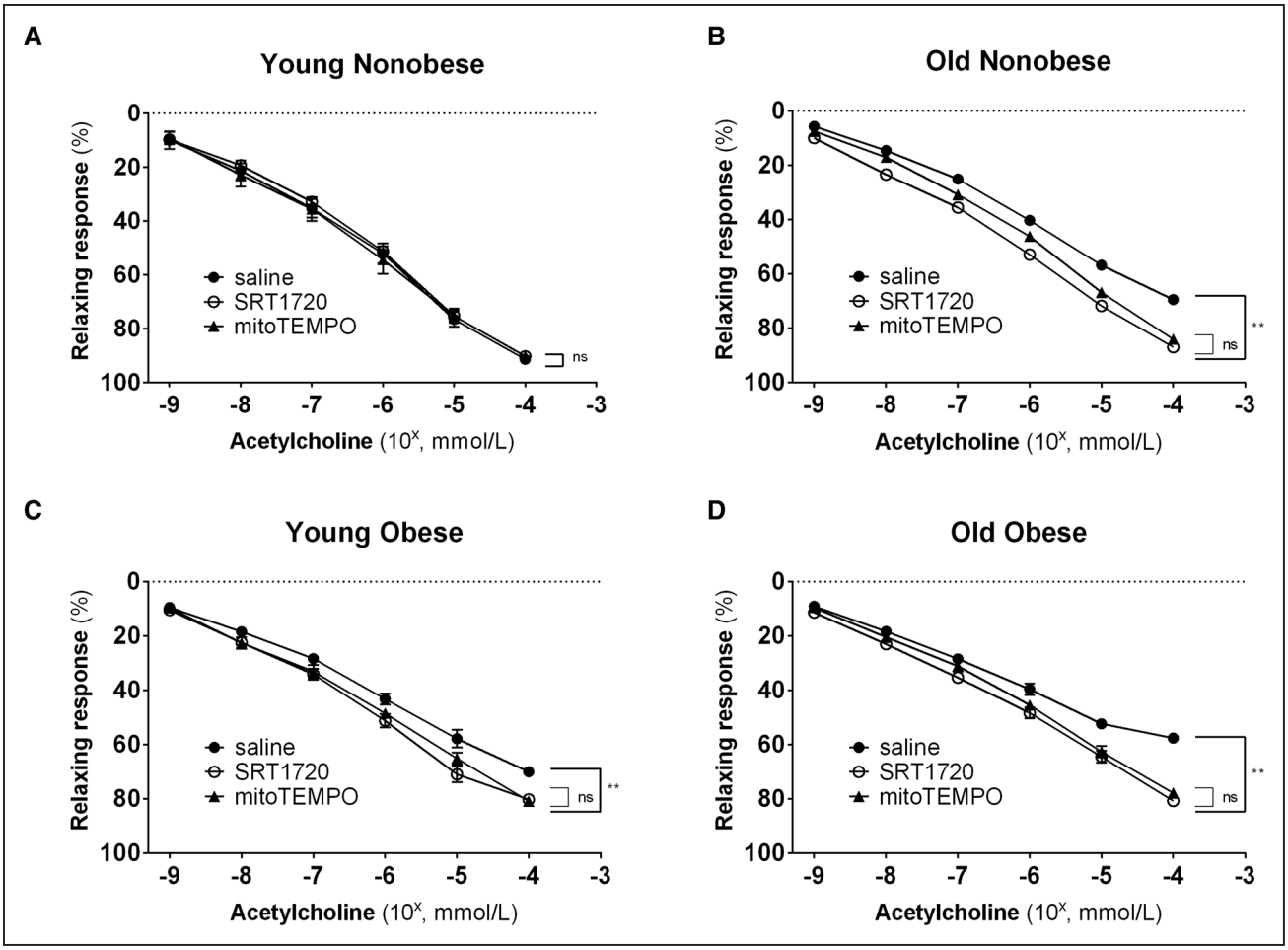

Figure 7. Comparison of SRT1720 and mitoTEMPO rescuing microcirculatory dysfunction in the 4 groups.
A–**D**, Relaxing response to cumulative concentration to Ach in vessels precontracted with norepinephrine in the 4 groups (young nonobese: n=5; old nonobese: n=16; young obese: n=8; old obese: n=22). Vasodilatory response is expressed as % of the maximal diameter. The experiment was repeated 3 times for each patient by incubating the vessel with saline (black circle), SRT1720 (white circle), and mitoTEMPO (black triangle). Shapiro-Wilk test was adopted to assess normality. Data are presented as mean±SEM and were compared by the Friedman test followed by Durbin-Conover as a post hoc test (**A**) and ANOVA for repeated measures followed by a post hoc test with Holm-Sidak correction (**B**–**D**). A $P<0.05$ was considered significant. **$P<0.01$.

and increased vascular p66^{Shc} and mtROS levels; (3) specific SIRT1 modulation significantly restores endothelial dysfunction in Old Obese, Young Obese and Old Nonobese individuals; (4) SRT1720 restored endothelial function by epigenetic changes, mainly involving p66^{Shc} and Arginase II, leading to a marked reduction of mtROS levels with a consistent effect across the whole age- and obesity spectrum. This is the first study to document a central involvement of SIRT1 in human aging- and obesity-related microvascular dysfunction underpinned by its significant modulation of mtROS levels.

Several observations have linked SIRT1 to aging and obesity.[9,11] Lower SIRT1 expression and activity are found in obese mice[28,29] and within the peripheral circulation and adipose tissue of obese subjects.[30,31] The tight regulation of SIRT1 by the availability of its metabolic cofactor, NAD^+, and the related intermediates, NADH and nicotinamide, might account for the negative effects of obesity on SIRT1 activity/expression.[10,32,33] Similarly, SIRT1 is downregulated in tissues of older mice[34] and peripheral T-cells of older human donors.[34] To our knowledge, this is the first evidence of a reduced expression of SIRT1 directly on the human microvasculature. Only a previous study explored the expression of SIRT1 in endothelial cells[35] but was limited to the aging phenotype and adopted cells from a large vessel (brachial artery). We investigated SIRT1 relationship with the microvascular phenotype, an acknowledged driver of age- and metabolic-related disease.[6,24] In mice, SIRT1 protects from age-related endothelial dysfunction.[36] In gain/loss-of-function murine models, SIRT1 exerts a protective effect on metabolic-related endothelial dysfunction.[9] Selective endothelial SIRT1 deficiency rarefies glycocalyx in mice.[37] In humans, systemic lower circulating SIRT1 in childhood is associated with microvascular dysfunction during adulthood.[38] Our findings confirm the tight relationship between vascular SIRT1 and age- and metabolic-related microvascular endothelial function in

humans. They promote the hypothesis of SIRT1 as a crucial biological link between cellular energy availability, metabolism, and aging.[9] We describe how in obesity, a condition of excessive nutrient availability ultimately accelerating aging,[39] the downregulation of SIRT1 is associated with the acquisition of a vascular aging phenotype. This is characterized by reduced epigenetic control on p66^{Shc},[40] reduced NO availability, increased mtROS levels and the consequent onset of endothelial dysfunction. In the long term, these alterations might lead to faster arteriolar remodeling with aging[41] (Figure 1A). Obesity considerably accelerates alterations related to attenuation of SIRT1 signaling, as these are already present in the Young Obese group.

Exogenous stimulation of SIRT1 activity has been proposed as a promising cardiovascular therapeutic target.[42,43] In vitro, SRT1720 protects microvascular endothelial cells from pro-inflammatory stimuli.[44] In mice, SRT1720 reverses vascular endothelial dysfunction, excessive superoxide production, inflammation,[12] and arterial stiffness[28] related to aging. In humans, results on SIRT1 agonists are scarce, and their therapeutic potential is yet to be explored.[9] Resveratrol improves endothelial dysfunction in patients with obesity.[22,45] SRT2104 showed a trend in improving the estimated mitochondrial oxidative capacity in humans.[46] However, evidence is poor, and both resveratrol and SRT1720 partially fail to achieve cardiometabolic relevant effect due to their demonstrated lack of vascular selectivity, instead exhibiting an organ preference for liver and white adipose tissue.[47] Here, we report for the first time a direct effect of SRT1720 on the human microcirculatory function, accurately measured through a complex ex vivo setting. The magnitude of the impact is consistent on different vascular phenotypes. It leverages a significant contribution of NO availability, as witnessed by the concordant improvement of L-NAME inhibition to ACh. The direct and strong relationship with age, BMI and vascular remodeling points out the crucial contribution of SIRT1 in rescuing metabolic-related endothelial dysfunction. Our choice to investigate the role of SIRT1 in a nondiabetic microvascular phenotype stresses the relevance of our findings, placing SIRT1 signaling-related impairment at the beginning of the health-to-disease phenotyping translation.

The control of SIRT1 on endothelial cell function involves several mechanisms. Besides the well-established capacity of SIRT1 to influence the expression of the eNOS[48] through modulation of the FOXO factor axis,[49] we found that vessels from obese patients had a reduced binding of SIRT1 on the promoter region of Arginase II, accompanied by an increased expression of this enzyme. We recently showed that arginase II contributes to obesity- and aging-related endothelial dysfunction by competing with the eNOS for the arginine substrate.[8] However, the influence of SIRT1 on NO availability is not limited to its activity on the eNOS. Indeed, it affects the expression of other enzymes with essential roles in regulating the mitochondria oxidative stress balance. First, we document an epigenetic control of SIRT1 on the expression of the p66^{Shc}. The lifespan determinant p66^{Shc} is involved in obesity-induced oxidative stress, mitochondrial dysfunction and vascular inflammation.[50,51] p66^{Shc} is responsible for vascular detrimental epigenetic signature related to excessive availability of metabolic substrates.[25,52] While subsequent reports confirmed the role of p66^{Shc} in promoting endothelial dysfunction in human obesity,[53] no studies explored, by far, its control by SIRT1 nor the similar alteration of the SIRT1/p66^{Shc} axis in aging and obesity in humans. The SIRT1 binding on the p66^{Shc} promoter was paired to higher p66^{Shc} gene expression. It should be noted that some studies report that only phosphorylated p66^{Shc} levels, not total p66Shc, are higher in the aging vasculature.[54] However, other observations in vitro and in vivo showed that total, mitochondrial-specific and phosphorylated p66^{Shc} were all found upregulated in different models of metabolic damage.[25,40,55] Although this is the first study to explore the SIRT1/p66^{Shc} axis in aging and obesity microvasculature in humans, an increase of p66^{Shc} mRNA expression has also been found in humans in peripheral blood in other models of aging[56] and metabolic damage.[53,57] This provides further consistency to our results and supports the hypothesis that both total and phosphorylated p66^{Shc} affect microvascular homeostasis.

The documented effect of SIRT1 on Arginase II and p66^{Shc} might be crucial in mediating its beneficial impact on vascular homeostasis. Our findings also did not exclude a possible post-translational role of SIRT1 in regulating Arginase II and p66^{Shc} functions. Second, we document that a restored SIRT1 activity can increase the production of mitochondria enzymes with key antioxidant capacities, such as SIRT3 and SOD2. We did not find evidence of direct binding of SIRT1 on the promoter region of SIRT3. However, SIRT3 higher levels after vessel exposure to SRT1720 suggest the involvement of post-translational SIRT1-mediated regulation. This is consistent with previous experiments reporting that endothelial cells exposed to a short incubation with resveratrol show upregulation of the AMPK-PGC-1α-ERRα pathway, indirectly increasing SIRT3.[58]

The upregulation of several mitochondrial antioxidant genes paired with the increased expression of genes involved in the respiratory chain might be one of the mechanisms explaining the positive modulation of SIRT1 on mitochondria ROS levels. An increased expression of the various complexes involved in oxidative phosphorylation in the electron transport chain improves the ATP synthesis derived from oxidative metabolism.[59] The mitigation of ROS levels by SIRT1 is acknowledged, repeatedly described in vitro,[18] also in human microvascular endothelial cells.[44] However, it is still debated[60] whether its primary target is NADPH-oxidase[61] or mtROS.[62] We

compared the endothelial function-specific impact of NADPH-oxidase inhibition and mtROS scavenging to SRT1720. The different magnitude of the effect and several indirect proofs of improved mitochondria homeostasis, as the restored resistance to mitochondria swelling assays, support a predominant mtROS-driven SIRT1 targeting action. This has been proposed but never reported in human vasculature.[63,64] We here describe it in the human small vessel arteries and shows its consistence across the whole range of age- and obesity-related microvascular impairment.

Our study has some weaknesses. The myograph-microvessel system is an ex vivo technique to assess microvascular function and structure on isolated vessels. Consequently, differences in local flow and mechanical forces imposed by the remodeling of large vessels in obesity might attenuate in vivo the benefits obtained through restoring the SIRT1 activity described in our experiments. However, micromyography remains the gold standard method for assessing endothelial microvascular function and structure in humans.[65] We focused on mitochondrial pathways that influence mitochondria and endothelial function, potentially connected with SIRT1 as documented in animal or in vitro experiments.[22,50] Therefore, we cannot exclude the presence of other epigenetic mechanisms through which SIRT1 might influence mitochondria oxidative stress and endothelial functions in humans. It should be noted that qPCR assays were performed on isolated microvessels composed of smooth muscle and endothelial cells. Thus, we did not investigate the contribution of SIRT1 to smooth muscle cell (SMC) homeostasis. Indeed, a recent observation in mice reported a positive effect of endothelial SIRT1 to SMC vasodilator response. However, this held true only in eNOS knockout mice.[36] We focused on endothelium because of its very early involvement during age- and obesity-related microvascular dysfunction, being acknowledged as one of the first actors involved in the healthy-to-disease transition.[6,7,24,41] Moreover, the preserved vasodilation to sodium nitroprusside indirectly excludes a nonendothelial substantial contribution to the phenotype. Also, the specificity of the fluorescent probes DAF-FM and mitoSOX is partial. However, both are still widely adopted for investigating tissue mtROS and NO levels.[66] Moreover, we have confirmed our results with more specific probes,[27,67–69] even though in a small subpopulation (Figure S1). The potential metabolic implications of our findings remain to be elucidated. Indeed, we have demonstrated the influence of SIRT1 on the expression of mitochondria proteins involved in the electron transport system. Still, we have not directly measured the mitochondria respiratory chain efficiency nor potential changes induced by a restored SIRT1 activity. Nonetheless, some observations described the potential benefits on the mitochondria oxidative phosphorylation induced by the changes in protein expression described in our study.[70–74] We have also to report that we did not measure protein levels, as we focused on mRNA. However, it should be noted that mRNA levels are acknowledged as informative of protein expression and are currently widely employed, especially in in-human studies.[53,56,57] Finally, it should be noted that the recruited obese population reflect a population of patients with a mild degree of disease, as we intended to investigate metabolic microvascular dysfunction from its very early stages. Although this is a strength in terms of pathophysiologic exploration of the health-to-disease transition, it might limit the generalization of the results to the standard population of patients with obesity. Finally, it should be clarified that even if SRT1720 can modulate the activity of SIRT3, this effect is dosage-dependent as SRT1720 EC_{50} is 0.16 µmol/L for SIRT1 instead of >300 µmol/L for SIRT3.[75] Thus, the dosage adopted in our study did not directly modulate SIRT3 activity.

In conclusion, SIRT1 represents a significant pathway influencing obesity- and aging-related microvascular dysfunction since its earliest stages through a complex epigenetic control on mtROS (Figure S5). Given the centrality of the microvascular system in response to metabolic demand,[6,7] SIRT1 endothelial-specific regulation influences the vascular phenotype and impacts systemic metabolism and aging, placing SIRT1 at the center of the crosstalk between substrates availability, cellular metabolism, and vascular phenotype. The microcirculatory endothelium is the earliest organ damaged by cardiovascular risk factors[6] which, since childhood,[76] inflict a permanent vascular-specific epigenetic wound.[7] Our findings strongly support early treatments to preserve the SIRT1 expression/activity. For instance, a recent long-term observation showed how high dietary intake of NAD^+ reduces the incidence of heart failure with preserved ejection fraction,[77] that is, the metabolic phenotype of heart failure.[78] Similarly, ketogenic states mitigate cardiometabolic damage[79] and preserve microvascular aging.[80] SIRT1 targeting intervention might thus represent crucial strategies to alleviate health care systems from the currently unbearable cardiovascular burden.

ARTICLE INFORMATION

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Affiliations

Department of Clinical and Experimental Medicine (A.M., E.D., M.N., I.P., L.A., M.F., C.I., N.B., N.R.P., S.T., A.V., S.M.) and Retrovirus Center and Virology Section, Department of Translational Research and New Technologies in Medicine and Surgery (M.L., V.L.R.), University of Pisa, Italy. Scuola Superiore Sant'Anna, Pisa, Italy (A.M., V.L.R., N.B.). Center for Molecular Cardiology, University of Zürich, Switzerland (S.C., F.P.). Department of Cardiology, University Heart Center (F.P., F.R.) and Department of Research and Education (F.P.), University Hospital Zurich, Switzerland. Unit of Bariatric Surgery, Azienda Ospedaliero-Universitaria Pisana, Pisa, Italy (R.M.). Paediatric Surgery Unit, Meyer Children's Hospital, Florence, Italy (M.G.). School of Biomedical Engineering and Imaging Sciences, King's College London, United Kingdom (G.G.). Department of Clinical Therapeutics, School of Medicine, National and Kapodistrian University of Athens, Greece (G.G.). Institute of Cardiovascular Science, University College London, United Kingdom (S.M.).

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Disclosures

None.

Supplemental Material

Detailed Methods
Figures S1 through S5
Table S1
References[81–94]

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