ONLINE APPENDIX

Downregulation of the Longevity-Associated Protein SIRT1 in Insulin Resistance and Metabolic Syndrome. Potential Biochemical Mechanisms.

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Analytical methods. Plasma glucose was measured with the glucose oxidase method on a glucose analyzer (Beckman, Albertville, MN). Plasma insulin and C-peptide were measured by conventional RIA. Total cholesterol, triglyceride and FFA concentrations were assayed with an enzymatic assay, and high-density lipoprotein (HDL) cholesterol was measured using polyethylene glycol precipitation with enzymatic quantification.

Circulating peripheral blood mononuclear cell/monocytes preparation. PBMC were collected from 20 mL of heparinized blood over Histopaque-1077 (Sigma-Aldrich, Milano, Italy). PBMC were washed three times with saline phosphate buffer (PBS) and suspended in OMNIzol (Euroclone, Lugano, Switzerland) for RNA extraction and in hypotonic lysis buffer (20 mmol HEPES, 2 mmol EGTA, 10 mmol β-glycerophosphate, 1 mmol dithiothreitol (DTT), 2 mmol vanadate, 10 ug/mL phenylmethylsulfonylfluoride (PMSF), 1 μg/mL leupeptin, 5 μmol aprotinin) for protein extraction. Because PBMC are composed of a mixed cell population, the two main components of which being monocytes/macrophages and lymphocytes, some experiments were performed in the monocyte/macrophage population. For this aim, PMBC were isolated from patients and incubated in tissue culture dishes at 37°C for 1 h. The monocyte/macrophage cells adhered to the dish were washed five times with sterile PBS, where the nonadherent cells were collected and washed with sterile PBS. More than 90% of adherent cells were monocytes by morphologic examination and by staining with Diff-Quik.

Cell Culture. THP-1 human monocytes obtained from American Type Culture Collection (Manassas, VA, USA) were cultured in RPMI 1640 medium with 2 mmol/L L-glutamine, 4,5 mmol/L glucose, 10 mmol/L HEPES, 1.0 mmol/L sodium pyruvate, 0.05 mmol/L 2-mercaptoethanol, and 10% fetal bovine serum in a humid atmosphere containing 5% $\mathbf{CO_2}$ at 37°C. Cells density never exceeded $1x10^6$/mL and culture medium was refreshed every other day. For all experiments, THP-1 cells were cultured in serum-reduced RPMI-1640 medium (2% FBS).

Assessment of cell viability. At the end of each treatment, the number of live and total cells was counted with trypan blue staining. Cell viability was assessed by calculating the percentage of live cells using trypan blue exclusion.

Quantitative Real Time RT-PCR (Q-PCR). Total RNA extracted was resuspended in RNAse-free water and its quality and quantity were determined by LabChip technology (Agilent Bioanalyzer 2100; Agilent Technologies, Waldbroom, Germany) and by the ratio from the absorbance readings at 260 nm and 280 nm with a spectrophotometer (Perkin-Elmer, Foster City, California, USA). To eliminate DNA contamination, 1 μg of total RNA was treated with DNase I, Amp Grade (Invitrogen, Carlsbad, California, USA). First-strand cDNA was synthesized with 1 μg of RNA extracted using iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. Quantitative real-time polymerase chain reaction assay was performed in a Thermal Cycler (iCycler iQ5, Bio-Rad,

Hercules, CA). In brief, 2 µl cDNA was amplified in a real-time PCR reaction containing 400 nmol of each primer and 5X SYBR Green SuperMix (Bio-Rad, Hercules, CA). All reactions were performed in 96-well plates, in triplicate. A negative control containing all reagents but no cDNA template was included in all runs. Real-time PCR was performed following the thermal protocol: 95°C for 3 min to denature, 45 cycles of 95°C for 30 s for denaturing and 60°C for 1 min for annealing and extension. Primers were designed from sequences derived from the GenBank database using Primer 3 (provided by the Whitehead Institute, Cambridge, Massachusetts, USA) and Operon's Oligo software (Operon Technologies Inc., Alameda, California, USA) and were purchased from Eurofins MWG (Ebersberg, Germany). Primer sequences are reported in Supplemental table I. Validation of the specificity of Q-PCR assay was performed by melt-curve analysis and by agarose gel analysis. β-actin and RPLP0 ribosomal protein were used as housekeeping genes. Data analyses were performed with the iQ™ Optical System Software (Bio-Rad, Hercules, CA). The comparative cycle threshold method (ΔΔCt), which compares the difference in cycle threshold values between groups, was used to obtain the relative fold change in gene expression. No difference was found in the gene expression of these two housekeeping genes in PBMC.

Western blot (WB) analysis. PMBC were disrupted with a Dounce homogenizer (20 strokes; Kontes Co., Vineland, NJ) on ice for 1 min. The homogenate was then centrifuged at 14000 x g for 15 min at 4°C in an Eppendorf centrifuge. The resultant supernatant was removed and stored at -80°C. Protein concentration was determined by Lowry's method, using bovine serum albumin as the standard. Protein samples (30 µg) were separated by SDS-PAGE electrophoresis using a 10% polyacrylamide gel, as previously described (18). Proteins separated in the gel were electroblotted onto nitrocellulose membrane (Hybond ECL, Amersham Biosciences, UK) in blotting solution containing 48 mmol/L Tris, 39 mmol/L glycine, 0.037% SDS, and 20% v/v methanol for 3 h at 100 V at 4°C, using a Transblot cell system (Elettrofor, Padova, Italy). The membranes were blocked overnight at 4°C in T-PBS containing PBS, 0.05% v/v Tween and 5% bovine serum albumin (BSA). Then, membranes were exposed to anti-SIRT1 (1:2000 dilution), anti-GAPDH (1:5000) primary antibody overnight at 4°C (Santa Cruz Biotechnology, Santa Cruz, CA), anti-JNK (1:2000) and anti-phospho (p)-JNK (1:1000) (Cell Signalling Tech., EuroClone, Milan, Italy). The membranes were washed (4 times, 20 min each) in T-PBS and then incubated with horseradish peroxidase-conjugated secondary antibody (1:10000). Chemiluminescent signal was developed using Super Signal West Pico substrate (Pierce, EuroClone, Milan, Italy). The blots were scanned and quantified using a chemiluminescence molecular imaging system (Versa Doc 3000, Bio-Rad, Hercules, CA). The results were expressed relative to the control(s), on the same blot, defined as 100%, and by the protein of interest/GAPDH densitometric ratio.

p53-Acetylation. Immunoprecipitations of Acetylated-p53 was carried out by incubating 2 µg of p53 antibody with 1 mg of cell lysate overnight, followed by 50 µl of protein A/G Plus-agarose (Santa Cruz Biotechnology, Santa Cruz, CA) slurry for 4 hours at 4°C. After washing, immunoprecipates were boiled in SDS-PAGE loading buffer, subjected to SDS-PAGE, transferred to nitrocellulose filter, and probed with the specified primary antibody Acetylated-lysine Ab (Cell Signalling Tech., EuroClone, Milan, Italy) and the appropriate peroxidase-conjugated secondary antibody (Amersham Biosciences, UK). Western blot of 50 µg of whole cell lysates was similarly performed using appropriate primary and secondary antibodies. Chemiluminescent signal was developed using Super Signal West Pico Substrate (Pierce, EuroClone, Milan, Italy), and blots were imaged and quantified using a chemiluminescence molecular imaging system (Versa Doc 3000, Bio-Rad, Hercules, CA). The results were expressed relative to the control(s), on the same blot, and the values are expressed as fold increase after normalization with total p53.

Sirt1 silencing. THP-1 were cultured until 5th passage and were placed in 12-wells dishes. The cells were grown in normal growth medium (RPMI-1640) with 10% FBS at 37°C with 5% CO_2 until 60% of confluence. The ON-target plus SMART pool human siRNA for Sirt1and ON-target plus Non-targeting siRNA (Dharmacon, Celbio, Italy) were diluted at the concentration of 100 μM with an appropriate siRNA buffer. The siRNA was added to the appropriate Accell siRNA delivery media to obtain final concentration 1 μM of siRNA. Cell suspension was removed from the growth media and was incubated with the mix (siRNA plus Accell media) for 72 h without changing or adding medium. Then, cells were collected for RNA and protein extraction, as previously described. The ON-target plus SMART pool siRNA were the followings: GCAAAGGAGCAGAUUAGUA; GCGAUUGGGUACCGAGAUA;GGAUAGGUCCAUAUACUUU; CCACCUGAGUUGGAUGAUA; CCACCUGAGUUGGAUGAUA.

Measurement of intracellular ROS. The intracellular production of ROS was detected using the fluorescent probe carboxy-dihydro-2′,7′-dichlorohydrofluorescein diacetate (H_2DCFDA) (Invitrogen, Milan, Italy). Briefly, THP-1 cells were seeded in 6 well plates at 1×10^5 cells/well and were stimulated with indicated agonists. Then, cells were incubated with 50 μM H_2DCFDA for 30 min at 37 °C. Fluorescence was detected on a Shimadzu RF-1501 fluorescence reader. Rate of ROS production over a 40 min period was determined and normalized to untreated cells.

NAD+ determination. We added 0.5 M ice-cold $HClO_4$ to the cells. After 2 min, we collected 100 μl of supernatants by centrifugation at 2000 g for 5 min, added 20 μl K_2HPO_4 (1 M) with cooling on ice and adjusted pH 7.2-7.4 with KOH. We added 50 μl of supernatant to the reaction mixture containing 0.1M sodiumpyrophosphate-semicarbazid (Sigma) pH 8.8, absolute ethanol and H_20. We assed NAD^+ spectrophotometrically at 339 nm at 25°C as a mean difference in absorbance before and 6 min after addition of alcohol deydrogenase (Roche).

Supplemental table I. Primer sequences of gene expression.

Gene	For Sequence	Rev Sequence	Tm	Amplificate (bp)
SIRT1	taccgagataaccttctgttcg	gttcgaggatctgtgccaat	60°C	176
SIRT2	agaagcagacatggacttcct	ctcccaccaaacagatgac	60°C	152
SIRT3	cattccagacttcagatcgc	agcagccggagaaagtagt	60°C	184
SIRT4	tgggatcatccttgcaggtat	tggtcagcatgggtctatca	60°C	173
SIRT5	gccaagttcaagtatggcaga	cgccggtagtggtagaa	60°C	213
SIRT6	caagtgtaagacgcagtacgt	atgtacccagcgtgatggac	60°C	221
SIRT7	atgagcagaagctggtgc	ctgtctggtgtctgtgga	60°C	198
NAMPT	tcgctgaccacagatacagg	ccaagagactgctggcat	60°C	224
β-Actin	agagctagctgcctgac	ggatgccacaggactcca	60°C	111
RPL0	gcgacctggaagtccaacta	catcagcaccacagcctt	60°C	147

Supplemental table II. Demographics and clinical characteristics of the study subjects by insulin sensitivity.

Abbreviations: IS (insulin sensitive), IR (insulin resistant). Data expressed as mean ± SE. * $p<0.05$ versus IS.

Characteristics	All (n=54)	IS (n=28)	IR (n=26)
Sex (Male/Female)	37/17	17/11	20/6
Age (years)	46.0±1.0	44.1±1.5	47.9±1.1*
Body mass index (kg/m^2)	26.7±0.8	23.3±0.6	30.3±1.0*
Waist (cm)	93.5±2.0	84.1±1.7	103.7±2.7*
Systolic blood pressure (mmHg)	121.7±2.0	111.5±2.4	133.7±5.4*
Diastolic blood pressure (mmHg)	80.1±1.0	77.8±2.7	84.3±2.5
Fasting plasma glucose (mg/dl)	85.7±2.0	81.5±2.4	90.1±2.9*
Fasting plasma insulin (μU/ml)	10.5±1.1	5.9±0.8	15.6±1.7*
Fasting plasma C-peptide (ng/ml)	1.6±0.1	1.2±0.1	2.1±0.2*
Plasma triglyceride (mg/dl)	120.2±12.8	88.7±6.8	154.2±23.1*
Plasma total cholesterol (mg/dl)	195.7±5.0	187.7±5.4	204.3±6.7
Plasma HDL cholesterol (mg/dl)	49.4±2.2	51.1±2.3	45.3±2.3
Plasma LDL cholesterol (mg/dl)	126.1±4.0	118.5±5.1	133.4±5.5
Plasma free fatty acids (μmol/l)	592±59	534±49	654±53

Supplemental table III. Demographics and clinical characteristics of study subjects by carbohydrate metabolism status upon OGTT.
Abbreviations: NGT (normal glucose tolerance), IFG impaired fasting glucose, IGT impaired glucose tolerance). Data expressed as mean ± SE. * p<0.05 versus NGT.

Characteristics	All (n=54)	NGT (n=41)	IGT/IFG (n=13)
Sex (Male/Female)	37/17	26/15	11/2
Age (years)	46.0±1.0	44.3±1.1	51.0±1.4*
Body mass index (kg/m^2)	26.7±0.8	25.0±0.7	32.1±1.5*
Waist (cm)	93.5±2.0	89.7±2.2	105.6±3.3*
Systolic blood pressure (mmHg)	121.7±2.0	117.2±3.4	130.7±6.0
Diastolic blood pressure (mmHg)	80.1±1.0	79.5±2.6	83.3±1.7
Fasting plasma glucose (mg/dl)	85.7±2.0	80.2±1.5	102.9±3.7*
Fasting plasma insulin (µU/ml)	10.5±1.1	8.6±1.0	16.8±2.9*
Fasting plasma C-peptide (ng/ml)	1.6±0.1	1.5±0.1	2.1±0.3*
Plasma triglyceride (mg/dl)	120.2±12.8	100.4±7.8	182.6±41.8*
Plasma total cholesterol (mg/dl)	195.7±5.0	190.6±4.4	211.9±10.8*
Plasma HDL cholesterol (mg/dl)	49.4±2.2	49.6±1.8	44.3±3.7
Plasma LDL cholesterol (mg/dl)	126.1±4.0	120.7±3.8	142.9±10.1*
Plasma free fatty acids (µmol/l)	592±59	586±44	609±64

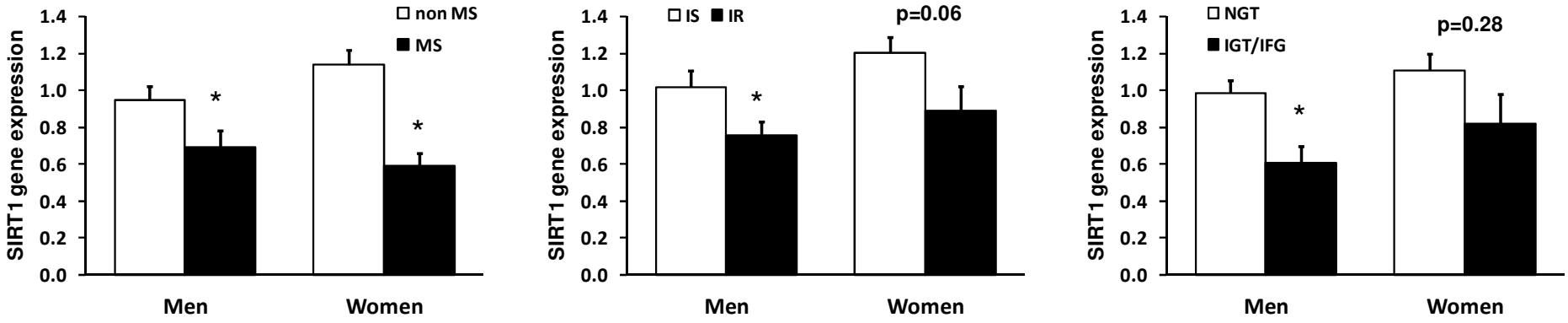
Supplemental table IV. Multiple stepwise regression analysis to identify variables independently associated with SIRT1 genomic expression, dependent variable.

VARIABLE	STANDARDIZED BETA	P
Step 1 (r^2=0.242)		
No. of MS components	-0.492	<0.0001
Step 2 (r^2=0.309)		
Age	-0.266	0.032
No. of MS components	-0.430	0.001

Variables which did not enter the final model: waist, age, HDL, triglycerides, plasma glucose, systolic and diastolic blood pressure, and Si.

Supplemental Figure I. SIRT1 gene expression analysis stratified by gender when subjects were divided into non-MS and MS or into IR and IS, or into NGT and prediabetes (IGT/IFG).

Abbreviations: MS, metabolic syndrome; IR, insulin resistant; IS, insulin sensitive; NGT, normal glucose tolerance; IGT, impaired glucose tolerance; IFG, impaired fasting glucose. Data expressed as mean ± SE. *p<0.05 versus the gender matched group.



Supplemental Figure II. SIRT1 protein expression normalized by GAPDH protein in THP-1 cells treated with glucose or mannitol 10 mM or 20 mM. Mannitol, used as osmotic control for glucose, exerted no effect. Data expressed as mean of 3 experiments ± SE. *p<0.05 versus control.