

Short Communication

Targeting Cellular Senescence as a Therapeutic Strategy to Attenuate Pulmonary Fibrosis Associated with Metabolic Syndrome

Michaela Rennerova^{1,2}, Tereza Havrlantova¹, Igor Simonik³, Renata Pavlovicova¹, Miloslava Cechova¹, Anna Cinkajzlova⁴, Dan-Diem Thi Le¹, Jana Klouckova⁴, Jiri Fronek⁵, Filip Thieme⁵, Jiri Neuzil^{2,6,7,8}, Lukas Werner⁴, Jan Stursa⁴, Milos Mraz⁴, Martin Haluzik^{1,4,9*}, Sona Stemberkova Hubackova^{1,6*}

¹Centre for Experimental Medicine, Institute for Clinical and Experimental Medicine, Prague, Czech Republic; ²Faculty of Science, Charles University, Prague, Czech Republic; ³St. Zdislava Hospital, Mostiste, Czech Republic; ⁴Diabetes Centre, Institute for Clinical and Experimental Medicine, Prague, Czech Republic; ⁵Transplant Surgery Department, Institute for Clinical and Experimental Medicine, Prague, Czech Republic; ⁶Institute of Biotechnology, Czech Academy of Sciences, Prague-West, Czech Republic; ⁷First Faculty of Medicine, Charles University in Prague, Prague, Czech Republic; ⁸School of Pharmacy and Medical Science, Griffith University, Southport, Qld, Australia; ⁹Institute of Medical Biochemistry and Laboratory Diagnostics, First Faculty of Medicine, Charles University and General University Hospital, Prague, Czech Republic.

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ABSTRACT: Metabolic dysfunction, in particular obesity and type 2 diabetes mellitus, increases susceptibility to pulmonary fibrosis via chronic inflammation, oxidative stress, and the accumulation of senescence. Senescent cells promote secretion of pro-fibrotic mediators that impair lung structure and repair. Here, we investigated whether reducing senescent cell burden using the mitochondria-targeted senolytic agent MitoTam, or weight-loss-induced improvement after bariatric surgery, can attenuate metabolic-associated pulmonary injury. In mice with diet-induced obesity, MitoTam improved glucose homeostasis, reduced adiposity, and markedly decreased senescence markers in lungs. This was accompanied by reduced inflammatory and fibrotic gene expression, diminished myofibroblast accumulation, and alleviated lung hypoxia. MitoTam also lowered senescence and expression of key pro-fibrotic factors, including GDF15, FGF2, TGF β , and endothelin-1 (EDN1) in subcutaneous adipose tissue, indicating both direct pulmonary and systemic endocrine effects. In patients with class III obesity, laparoscopic sleeve gastrectomy induced substantial body weight loss, improved metabolic parameters, and significantly reduced adipose tissue senescence and pro-fibrotic signaling, including EDN1 expression and its level in the circulation. These findings identify cellular senescence as a modifiable driver linking metabolic dysfunction to pulmonary fibrosis and highlight mitochondria-targeted senolysis with MitoTam as a promising therapeutic approach.

Keywords: Senescence, pulmonary fibrosis, metabolic disease, senolysis, mitochondria

INTRODUCTION

Metabolic dysfunction, particularly linked to obesity and type 2 diabetes mellitus (T2DM), is highly prevalent and

continues to rise globally. Over 650 million adults are affected by obesity and more than 500 million by T2DM, which often coexist as components of metabolic syndrome. These disorders are major contributors to

***Correspondence should be addressed to:** Sona Stemberkova Hubackova, Experimental Medicine Centre, Institute for Clinical and Experimental Medicine, Videnska 1958/9, 140 21 Prague, Czech Republic, e-mail: hubs@ikem.cz and prof. Martin Haluzik, Diabetes Centre, Institute for Clinical and Experimental Medicine, Videnska 1958/9, 140 21 Prague, Czech Republic, e-mail: halm@ikem.cz.

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morbidity and mortality, driving not only cardiovascular and renal diseases but also pulmonary complications.

Accumulating evidence indicates that metabolic dysfunction directly promotes a distinct form of lung remodeling characterized by pro-fibrotic features. Experimental studies demonstrate that obesity, insulin resistance, and hyperglycemia can induce structural and functional alterations in the lung independently of classical pulmonary diseases. High-fat diet-induced insulin resistance promotes pulmonary fibrosis through activation of transforming growth factor- β 1 (TGF- β 1) signaling, while diabetes similarly enhances TGF- β -dependent extracellular matrix deposition. Moreover, hyperglycemia exacerbates oxidative stress and activates pro-fibrotic pathways, further contributing to lung injury and inflammation [1]. Importantly, pulmonary impairment may in turn aggravate metabolic dysfunction by promoting muscle wasting and reducing physical activity. Recognizing this bidirectional relationship is essential, as management of metabolic components can improve both pulmonary outcomes and overall health.

Cellular senescence, a permanent arrest of cell division triggered by stressors such as DNA damage, oxidative stress, and chronic inflammation, plays a key pathogenic role in linking metabolic dysfunction to pulmonary disease. Although initially protective, the accumulation of senescent cells drives tissue dysfunction and chronic disease progression. Senescent cells secrete a pro-inflammatory mix of cytokines, chemokines, and matrix-remodeling enzymes, collectively known as the senescence-associated secretory phenotype (SASP). These SASP factors contribute to the persistent low-grade inflammation and tissue remodeling observed in respiratory disorders [2].

In metabolic disorders such as obesity and T2DM, SASP, systemic oxidative stress and mitochondrial dysfunction accelerate senescence in pulmonary epithelial, endothelial, and mesenchymal cells. Impaired clearance of senescent cells further amplifies their accumulation. Adipose tissue dysfunction, insulin resistance, and hyperglycemia create a pro-senescent and pro-fibrotic milieu that promotes lung remodeling, enhances extracellular matrix deposition, and impairs tissue repair, thereby contributing to fibrosis-like changes and compromised gas exchange [1], which may predispose to the development of metabolic dysfunction-associated pulmonary remodeling.

Bariatric surgery, especially laparoscopic sleeve gastrectomy (LSG), remains the most effective intervention for obesity and T2DM, achieving sustained body weight loss and metabolic improvement. Recent evidence indicates that bariatric surgery also reduces premature aging markers and systemic inflammation by decreasing adipose tissue mass, the main reservoir of

senescent cells in obesity. However, as surgery is not suitable for all patients, pharmacological strategies are being developed to target senescence directly. Senolytic agents, which selectively eliminate senescent cells, have shown promise in preclinical studies, improving metabolic control and reducing lung inflammation in models of metabolic and pulmonary disease [3].

Since the mitochondrial function is an important determinant of the aging process, we have recently reported that targeting mitochondria by mitochondria-targeted tamoxifen (MitoTam), an anti-cancer agent with strong senolytic effect, presents a plausible way to eliminate these cells in the context of pathological senescence as well as senescence-associated diseases. Elimination of senescent cells by MitoTam via activation of ferroptosis [4] considerably improves glucose control, decreases body weight, adipose tissue mass as well as systemic inflammation and reduces diabetic markers and diabetic comorbidities [5].

Together, these findings point to senescent cells as one of the drivers linking metabolic dysfunction to pulmonary remodeling. We therefore examined whether reducing cellular senescence, either pharmacologically with the MitoTam or through bariatric surgery, can reduce systemic and pulmonary pro-fibrotic pathways.

METHODS

Study Subjects

This was an open-label, non-randomized study performed in a cohort consisting of 59 subjects. 17 subjects underwent conservative treatment consisting of lifestyle interventions and a dietary regimen developed in collaboration with an obesitologist and a nutrition therapist to control diabetes and reduce body weight. 42 subjects were indicated to LSG by a multidisciplinary team consisting of a bariatric surgeon, internist, nutritionist, anesthesiologist, and psychologist. All subjects included in the study met the inclusion criteria of BMI ≥ 35 kg/m² and the age range of 18 to 75 years. Exclusion criteria included prior gastrointestinal surgery interfering with LSG, active malignancy, acute myocardial infarction, unstable angina pectoris, acute stroke 3 months prior to enrolment, acute liver or kidney failure, transplantation of insulin producing tissue, gravidity and mental disorders. Patients who participated in the study were not compensated financially. All participants were followed for a period of six months after intervention or initiation of conservative treatment.

All participants signed written informed consent prior to the enrolment into the study. The study was approved by the Human Ethics Review Board, Institute for Clinical and Experimental Medicine (IKEM), Prague, Czech

Republic (ethical approval code G-20-41) and by the Human Ethics Review Board, st. Zdislava Hospital, Mostiště, Czech Republic (ethical approval code S-18-01) and was performed in agreement with the principles of WMA Declaration of Helsinki - Ethical Principles for Medical Research Involving Human Subjects.

Patient blood and tissue sampling

At baseline (V1), blood and subcutaneous adipose tissue (SAT) samples were obtained intraoperatively during LSG in surgical patients and by biopsy in subjects undergoing conservative treatment. At the six-month follow-up (V2), blood and SAT samples were collected by biopsy in both groups. At the same time, the anthropometric data of the patients were re-evaluated.

Blood samples were taken after overnight fasting (6–12 h) and centrifuged for 10 min at 3000 x g within 30 min after withdrawal. Blood samples for the evaluation of hormone and incretin levels were collected into special blood tubes with inhibitor cocktail consisting of a dipeptidyl peptidase 4, esterase and other protease inhibitors (BD P800, Becton, Dickinson and Company, Franklin Lakes, NJ, USA). Aliquots were stored at -80 °C.

The samples of SAT were taken perioperatively from the surgical wound or under sterile conditions under local anesthesia with 1% mesocaine in the area of the front abdominal wall around the navel from a mini-incision after 6-12 h of fasting. SAT samples were harvested from approximately the same location in all patients. Aliquots were kept in RNAlater™ Stabilization solution (Thermo Scientific, Waltham, Massachusetts, USA), and stored at -80 °C.

Hormonal and biochemical assays

The patient's circulatory levels of leptin (sensitivity 52 pg/mL), insulin (sensitivity 57 pg/mL), C-peptide (sensitivity 17 pg/mL), GLP-1 (active) (sensitivity 2.9 pg/mL), ghrelin (active) (sensitivity 15 pg/mL), GIP (active) (sensitivity 1 pg/mL), glucagon (sensitivity 10 pg/mL) and PP (sensitivity 1 pg/mL) were determined using the multiplex assay kit Milliplex® Human Metabolic Hormone Panel V3 (HMH3-34K, Merck KGaA, Darmstadt, Germany). ELISA kit was used for detection of adiponectin level (sensitivity 0.2 ng/mL, Merck KGaA, Darmstadt, Germany) BioVendor R&D, Brno, Czech Republic and for the detection of mouse and human endothelin 1 (DET100; Bio-Techne, Minnesota, USA). The intra- and interassay variabilities for all assays were between 10.0 and 20.0 %. Mouse circulatory levels of leptin (sensitivity 82 pg/mL) and insulin (sensitivity 137 pg/mL) were determined using the multiplex assay kit

Milliplex® Mouse Metabolic Hormone Panel (MMHE-44K-11, Merck KGaA, Darmstadt, Germany).

Patient biochemical parameters (blood glucose, glycated hemoglobin – HbA1c, uric acid, creatinine, albumin, alanine aminotransferase (ALT), aspartate amino-transferase (AST), alkaline phosphatase (ALP), γ -glutamyltransferase (GGT), hs-CRP, total cholesterol, HDL cholesterol, LDL cholesterol, triglycerides) were determined at the Laboratory Methods Division, IKEM, Prague, Czech Republic or at the Laboratory AeskuLab, St. Zdislava Hospital, Mostiště, Czech Republic, by standard laboratory methods.

Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

Tissue samples (70 – 90 mg) were mechanically homogenised using the MagNA Lyser Instrument (1 x 10 s at 5 500 rpm) (Roche Diagnostics GmbH, Mannheim, Germany). Subsequently, RNA isolation from homogenised tissue was carried out, using the Qiagen RNeasy Lipid Tissue Mini Kit (Qiagen, Hilden, Germany). The RNA concentration and quality were determined using the NanoDrop™ 2000/2000c spectrophotometer (Thermo Scientific, Waltham, Massachusetts, USA). Reverse transcription was performed, employing random hexamer primers according to the protocol of the High-Capacity cDNA Reverse Transcription Kits (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Gene expression was determined using the QuantStudio 7 Pro Real-Time PCR System (Thermo Fisher Scientific, Waltham, Massachusetts, USA), by means of specific primers (see below) (Sigma, St. Louis, MO, USA). Relative amount of cDNA was evaluated by the $2^{-\Delta\Delta CT}$ method; data were normalized to the housekeeping β -actin gene (Table 1).

Detection of senescence-associated beta-galactosidase activity

For detection of SA- β -galactosidase activity, tissue samples were cut into small pieces (2 – 3 mm³) and fixed with 0.2% glutaraldehyde solution (in PBS; pH 7.2) for 1 h at 4 °C. Tissue was washed with PBS supplemented with 1 mM MgCl₂ (pH 5.5) and incubated with X-gal solution (1 mg/mL X-gal, 0.12 mM K₃Fe(CN)₆, 0.12 mM K₄Fe(CN)₆, 1 mM MgCl₂ in PBS at pH 5.5) at 37 °C for 3 h. Samples were washed with PBS and the colorimetric changes were quantified using the Photoshop (Adobe Inc., San José, CA, USA) and ImageJ (NIH, Bethesda, MD, USA) programmes.

Table 1. List of primers for RT-qPCR.

Gene	Forward	Reverse
Mouse		
<i>ACTA2</i>	TGCTGACAGAGGCACCACTGAA	CAGTTGTACGTCCAGAGGCATAG
<i>CDKN1A (p21)</i>	TCCAGACATTCAGAGCCACAG	AAAGTTCACCCGTTCTCGGG
<i>CDKN2A (p16)</i>	TGGTCACTGTGAGGATTACAGC	TTGCCATCATCATCACCTGG
<i>Col1a</i>	CCTCAGGGTATTGCTGGACAAC	CAGAAGGACCTTGTGTTGCCAGG
<i>EDN1</i>	GGCCCAAAGTACCATGCAGA	GATGGCCTCCAACCTTCGTA
<i>FBN1</i>	GCTGTGAATGCGACATGGGCTT	TCTCACACTCGCAACGGAAGAG
<i>FGF1</i>	CCAAGGAAAACGTCCACAGTCAG	ACGGCTGAAGACATCCTGTCTC
<i>FGF2</i>	AAGCGGCTCTACTGCAAGAACG	CCTTGATAGACACAACCTCCTCTC
<i>GDF15</i>	AGCCGAGAGGACTCGAACTCAG	GGTTGACGCGGAGTAGCAGCT
<i>HIF1a</i>	CCTGCACTGAATCAAGAGGTTGC	CCATCAGAAGGACTTGCTGGCT
<i>IL6</i>	TACCACTTCACAAGTCGGAGGC	CTGCAAGTGCATCATCGTTGTC
<i>PLIN2</i>	GACAGGATGGAGGAAAAGACTGC	GGTAGTCGTCACCCACATCCTTC
<i>TGFa</i>	TGGAAATCAACGGGATCAGC	AGTTGGTATCCAGGGCTCTCC
<i>ACTB</i>	CATTGCTGACAGGATGCAGAAGG	TGCTGGAAGGTGGACAGTGAGG
Human		
<i>CDKN1A (p21)</i>	TCACTGTCTTGTACCCTTGTGC	GGCGTTTGGAGTGGTAGAAA
<i>EDN1</i>	CTACTTCTGCCACCTGGACATC	TCACGGTCTGTTGCCTTTGTGG
<i>FGF2</i>	AGCGGCTGTACTGCAAAAACGG	CCTTTGATAGACACAACCTCCTCTC
<i>GDF15</i>	CAACCAGAGCTGGGAAGATTCTG	CCCGAGAGATACGCAGGTGCA
<i>PLAUR</i>	CCACTCAGAGAAGACCAACAGG	GTAACGGCTTCGGGAATAGGTG
<i>ACTB</i>	CCAACCGCGAGAAGATGA	CCAGAGGCGTACAGGGATAG

ACTB: β -actin; ACTA2: actin alpha 2; CDKN1A (p21): cyclin-dependent kinase inhibitor 1A; CDKN2A (p16): cyclin-dependent kinase inhibitor 2A; Col1a: collagen 1 alpha; EDN1: endothelin 1; FBN1: fibrillin 1; FGF1: fibroblast growth factor 1; FGF2: fibroblast growth factor 2; GDF15: growth differentiation factor 15; HIF1a: hypoxia inducible factor 1 alpha; IL6: interleukin 6; PLAUR: plasminogen activator urokinase receptor; PLIN2: perilipin 2; TGF β : transforming growth factor β .

Animal studies

Obese and prediabetic model: C57BL/6J mice (8 weeks old males; purchased from Velaz, Prague, Czech Republic) were fed a high fat diet (HFD) for 6 months. HFD comprised 60 % of ground rat/mouse maintenance Sniff V1530 (ssniff-Spezialdiäten GmbH, Ferdinand - Gebriel-Weg 16, D-59494, Soest), Sunar Complex 3 (Hero Czech, Prague, Czech Republic) (34 %), corn starch (1 %), and pork lard (25 %). Control mice were fed a standard diet (SD) containing rat/mouse maintenance ssniff® V1534-727 (10 mm pellets) (ssniff-Spezialdiäten GmbH). A total of 53 mice were divided into 3 groups, 17-18 mice each. The animals were fed SD or HFD; at the end of the 6-month period, HFD group was treated either with MitoTam (2 mg/kg of body weight) dissolved in 4 % ethanol in corn oil or with the vehicle (corn oil), both administered i.p. twice per week for 4 weeks. The animals fed SD were treated with the vehicle. Body weight was taken once per week during the 4 weeks of treatment. Food consumption was evaluated twice per week, daily food intake was estimated as kcal per day during the study. At the end of the experiment, animals were sacrificed between 9 and 11 am by anesthesia: Zoletil 100 at 5 mg/100 g b.w. (Virbac, Carros, France) and Rometar 20 at 2 mg/100 g b.w. (Rometar 20; Bioveta, Ivanovice, Czech Republic), and plasma/serum and organs were

collected and frozen at -80 °C until further analysis. The data presented come from two independent experiments.

Detection of hypoxia: HFD mice were prepared and treated as described above. At the end of the experiment, pimonidazole (60 mg/kg in 100 μ l of physiological solution) was injected intravenously through the tail vein. After 90 min, the animals were sacrificed as described above.

Pair-feeding study in HFD-fed mice: C57BL/6J mice (10 months old males; purchased from Velaz) were fed HFD (as described above) for 9 months to induce obesity and prediabetes. Mice were single-housed and divided into 3 groups: group HFD+MT (n=7) was treated with MitoTam (2 mg/kg of body weight) dissolved in 4 % ethanol in corn oil, groups HFD+PF (n=7) and HFD+CO (n=6) were treated with the vehicle by i.p. administration twice per week for 4 weeks. At the end of the experiment, animals were sacrificed by anesthesia: Zoletil 5 mg/100 g b.w. (Zoletil 100; Virbac) and Rometar 2 mg/100 g b.w. (Rometar 20; Bioveta), and plasma/serum and organs were collected and frozen at -80 °C until further analysis. Baseline values were taken by measuring food intake every day in single-housed mice for two weeks. With the onset of treatment with MitoTam or vehicle, we started with pair feeding. HFD+MT mice were provided with a significant excess of food in the cage top hopper. HFD+PF mice were fed the average amount of food that

HFD+MT mice consumed the previous day. One quarter of amount for the pair-feed group were given to mice at 8 a.m. and rest of food (3/4) were given at 4 p.m. HFD+CO control mice were provided with a significant excess of food in the cage top hopper.

All mice were maintained at 22 °C, relative humidity of 60 +/- 10 %, and 12 h/12 h light/dark regimen with water and food provided ad libitum, unless stated otherwise. Male mice were used in the experiments containing HFD because of their better hormonal stability and ability to develop obesity and prediabetes. Experiments were performed in agreement with the Animal Protection Law of the Czech Republic and were approved by the Ethics Committee of the Institute for Clinical and Experimental Medicine (IKEM), Prague, Czech Republic.

Oral glucose tolerance test (oGTT)

oGTT was performed during the last week of the experiment. Prior to the test, mice were fasted for 16 h. Baseline glucose was evaluated in blood samples taken from a tail cut (by removing the distal 2 mm of the tail) using the Accu-chek glucometer (Roche, Basel, Switzerland). The mice then received 2 g/kg body mass of a 400 g/L glucose solution (Ardeanutrisol G 40, Ardeapharma, Ševětín, Czech Republic) in sterile water delivered by oral gavage. Glucose levels were assessed at 0, 15, 30, 60, 120 and 180 min after its administration.

Indirect immunofluorescence

Lungs were snap-frozen immediately after resection from sacrificed animals. Tissue was then fixed overnight with 2% formaldehyde (inflation-fixation was not performed), washed extensively with PBS, incubated in dimethyl sulfoxide for 1 hour at RT and subsequently in 30% sucrose overnight at 4 °C. The tissue was then incubated in increasing concentration of the OCT mounting medium (20%, 50%, 70%) for 30 min and frozen in 100% OCT (Sigma, St. Louis, MO, USA). Cryo-sections (6 µm) were permeabilized by combination of dimethyl sulfoxide (15 min at RT) and saponin (0.5%, 15 min at RT; ThermoFisher Scientific, Waltham, MA, USA). After washing with PBS, sections were incubated in 10% FBS (diluted in PBS) for 30 min to block unspecific signal, and further incubated with diluted primary antibody anti-α-SMA (#19245, Cell Signaling, Massachusetts, USA), anti-γH2AX antibody (ab81299, Abcam) or anti-F4/80 antibody (ab300421, Abcam) (all antibodies were diluted 1:100 in PBS; overnight incubation at 4 °C), washed extensively with PBS, and incubated with goat anti-rabbit Alexa Fluor 488 (A11008; Thermo Fisher Scientific, Waltham, MA, USA) (1:1000; 1h at RT) or with the

hypoxyprobe FITC Mab (HP FITC Mab-1, Hypoxyprobe, MA, USA) (1:100 in PBS; overnight incubation at 4 °C). To counterstain nuclei, coverslips were mounted in Mowiol containing 4',6-diamidino-2-phenylindole (DAPI; Sigma, St. Louis, MO, USA). The signal was detected using the Leica SP8 FLIM confocal microscope (Leica Microsystems, Mannheim, Germany).

Histology

For paraffin embedding, tissue was fixed overnight in 2 % PFA (inflation-fixation before histology was not performed), placed in 70 % EtOH, and processed using the Leica ASP6025 autoprocessor. The program was set to 12 h standard dehydration and paraffin saturation protocol. After processing, tissue samples were embedded into formalin-fixed paraffin-embedded blocks upside down by cutting their surface and orienting the blocks transversally. Masson's Trichrome staining, PicroSirius Red staining, dehydration and placing the samples on coverslips were accomplished using the Leica ST5020 automated staining instrument in combination with the Leica CV5030 cover-slipper.

Pulmonary fibrosis was quantified on PicroSirius Red-stained sections using the Ashcroft scoring system (0–8 scale). Scores were assigned by blinded observer in randomly selected fields (n=10, magnification 100x) per lung section and averaged for each animal.

Accumulation of collagen and fibrotic tissue was assigned by blinded observer in randomly selected fields showing parenchymal tissue (n=4, magnification 100x) per lung section and averaged for each animal.

Evaluation of respiration and complex IV activity

The high-resolution Oxygraph-2k respirometer (Oroboros Instruments, Innsbruck, Austria) was used to assess uncoupled respiration from the frozen tissue [6]. Tissue (10–20 mg) was homogenized in tissue grind tube (Kimble, 885303-0002) in the 2 ml Mir05 medium (0.5 mM EGTA, 3 mM MgCl₂, 60 mM K-lactobionate, 20 mM taurine, 10 mM KH₂PO₄, 110 mM sucrose, 1 g/L essentially fatty acid-free bovine serum albumin, 20 mM Hepes, pH 7.1 at 30 °C) and transferred to the chamber of the Oxygraph-2k instrument. Respiration evaluation was performed at 37 °C as follows. After signal stabilization, chamber was closed. To measure complex I (CI) dependent uncoupled respiration, malonate (10 µg/ml) was added to inhibit CII. Level of CI uncoupled respiration after the addition of nicotinamide adenine dinucleotide (reduced form – NADH; 60 µg/ml) was determined. After OXPHOS inhibition by antimycin A (2 µg/ml) the activity of complex IV was measured by tetramethyl-*p*-phenylene diamine (0.5 mM) in the

presence of ascorbate (2 mM). Data were evaluated using DatLab8 software (Oroboros Instruments, Innsbruck, Austria).

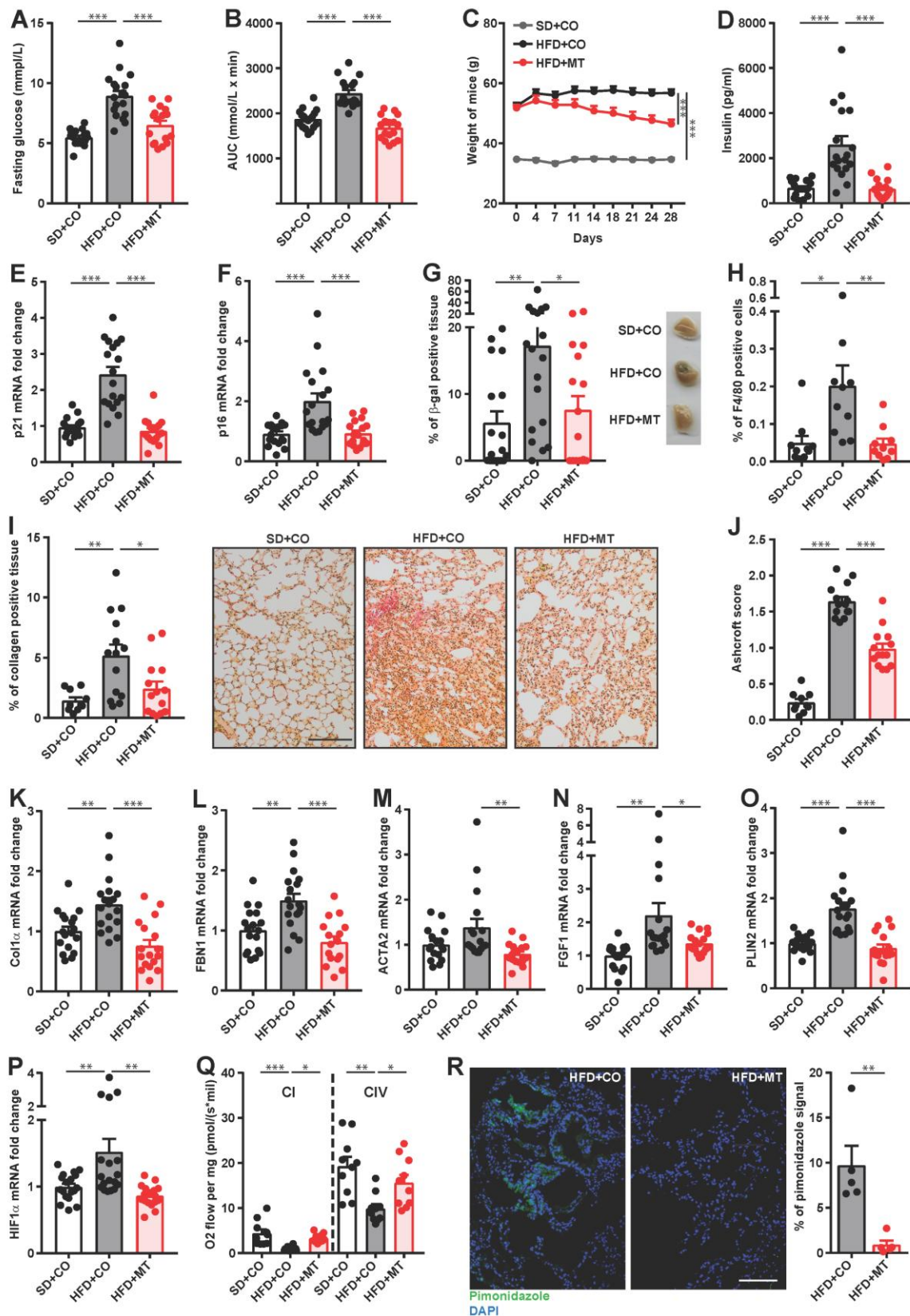


Figure 1. MitoTam ameliorates metabolic dysfunction and pulmonary fibrosis in HFD-induced obese mice. C57BL/6J mice (8 months old) on standard (SD) or high fat diet (HFD) were divided into three groups, i.e. SD+CO: standard diet + corn oil; HFD+CO: high fat diet + corn oil; HFD+MT: high fat diet + MitoTam (n=17-18 per group). The mice were treated with MitoTam (2 mg/kg body weight) dissolved in 4 % ethanol in corn oil or the vehicle given i.p. twice per week for a period of 4 weeks. Fasting blood glucose was determined at the end of the experiment after 16 hours of fasting (A), and area under the oGTT curve (AUC) was estimated (B). Total body weight was taken twice per week throughout the experiment (C). Postprandial serum insulin concentration was assessed by Luminex (D). The level of *p21^{Cip1/Waf1}* (E) and *p16^{Ink4a}* (F) mRNA in lungs was assessed by qRT-PCR. SA- β -gal positive tissue in lungs (%) is shown as bar graph (left) and representative tissue is presented (right; blue colour) (G). Macrophage infiltration in the lungs was assessed by immunohistochemistry using F4/80 staining (H). Histological samples of lungs were stained for collagen using PicroSirius Red staining. The bar indicates 150 μ m. (I). Ashcroft score was assessed (J). The level of *Colla* (K), *FBNI* (L), *ACTA2* (M), *FGF1* (N) *PLIN2* (O) and *HIF1a* (P) mRNA in lungs was assessed by qRT-PCR. Respiration via mitochondrial complex I (CI) and activity of mitochondrial complex IV (CIV) in lungs was measured by Oxygraph O2k (Q). C57BL/6J mice (8 months old; n=5-6) on high fat diet (HFD) were treated with MitoTam as described above. At the end of the experiment, pimonidazole (60 mg/kg in 100 μ l of physiological solution) was injected intravenously via the tail vein for 90 min. Hypoxic regions were documented by immunofluorescent staining using hypoxyprobe detecting incorporated pimonidazole. DAPI denoting cell nuclei. The bar indicates 100 μ m (R). Data are presented as mean $\pm$ SEM. Statistical significance was assessed using one-way ANOVA followed by Tukey's multiple comparisons test for panels A–Q. For panel R (n < 6), a non-parametric Mann–Whitney test was used. *p < 0.05; **p < 0.005; ***p < 0.001.

Statistics

Statistical analysis was performed using GraphPad Prism 10.1.0 (GraphPad software, Boston, MA, USA). In mouse experiments, One-way ANOVA followed by Tukey's multiple comparisons test presented as mean $\pm$ standard error of mean (SEM) were used to assess statistical significance. For the experiment shown in Figure 1R, due to the small sample size (n < 6), a non-parametric Mann–Whitney test was used for comparisons between two groups.

For parametric patients' data, results are expressed as mean $\pm$ SEM. A paired t-test was used to assess differences between visits, accounting for the within-subject design of the study. Data normality was assessed using the Shapiro–Wilk test. Statistical significance of all tests was assigned to p < 0.05.

RESULTS

MitoTam ameliorates obesity and prediabetes-associated lung remodeling and metabolic impairment

To evaluate whether reduction of senescent cells could alleviate pulmonary fibrosis-associated pathways in metabolic dysfunction, we employed a mouse model of long-term HFD-induced obesity and prediabetes, which is associated with pulmonary remodeling and pro-fibrotic changes. Consistent with our previous research [5], MitoTam improved fasting glucose (Fig. 1A) as well as glucose uptake (Fig. 1B), and reduced body weight (Fig. 1C and Suppl. Fig. 1A) and adipose tissue mass, with the most pronounced reduction of subcutaneous (SAT) and visceral (VAT) adipose tissue (Suppl. Fig. 1B, C). Glucose-lowering effect of MitoTam was linked to improvement of T2DM-related hormones profile (Fig. 1D; Suppl. Fig. 1D).

Table 2. Biochemical parameters in subjects before (V1) and 6 m (V2) after conservative procedure or LSG.

	Conservative treatment		p value	Bariatric surgery		p value
	V1	V2		V1	V2	
Number of subjects (n)	17			42		
Gender (m/f)	7/10			16/26		
Diabetics (n; %)	2; 11.8			17; 40.5		
Age (year)	44.7 $\pm$ 6.2			46.0 $\pm$ 9.0		
Body weight (kg)	128.11 $\pm$ 25.44	125.16 $\pm$ 24.39	0.182	131.36 $\pm$ 25.11	98.29 $\pm$ 19.76***	< 0.001
BMI (kg/m ²)	44.60 $\pm$ 8.82	43.34 $\pm$ 6.93	0.134	44.48 $\pm$ 6.47	33.22 $\pm$ 5.46***	< 0.001
Glycemia (mmol/L)	5.95 $\pm$ 1.56	5.80 $\pm$ 1.31	0.232	6.37 $\pm$ 1.85	5.15 $\pm$ 0.78***	< 0.001
HbA1c (mmol/L)	41.00 $\pm$ 9.17	41.33 $\pm$ 11.52	0.746	44.31 $\pm$ 13.60	36.23 $\pm$ 7.77***	< 0.001

hs-CRP (mg/L)	6.17 ± 4.33	5.70 ± 5.02	0.709	5.31 ± 4.78	2.42 ± 3.23***	< 0.001
AST (μkat/L)	0.43 ± 0.19	0.43 ± 0.12	0.867	0.53 ± 0.30	0.31 ± 0.12***	< 0.001
ALT (μkat/L)	0.65 ± 0.27	0.63 ± 0.20	0.390	0.79 ± 0.45	0.43 ± 0.18***	< 0.001
GGT (μkat/L)	0.54 ± 0.33	0.56 ± 0.28	0.620	0.68 ± 0.53	0.41 ± 0.42**	0.004
ALP (μkat/L)	1.43 ± 0.32	1.41 ± 0.29	0.672	1.25 ± 0.32	1.27 ± 0.36	0.496
Total cholesterol (mmol/L)	4.75 ± 0.88	4.76 ± 0.89	0.893	4.88 ± 0.93	4.62 ± 0.68*	0.047
TAG (mmol/L)	1.36 ± 0.80	1.32 ± 0.89	0.755	2.47 ± 1.25	1.33 ± 0.51***	< 0.001
HDL cholesterol (mmol/L)	1.12 ± 0.21	1.21 ± 0.26*	0.014	1.03 ± 0.32	1.31 ± 0.36***	< 0.001
LDL cholesterol (mmol/L)	3.01 ± 0.58	2.96 ± 0.59	0.550	3.14 ± 0.89	2.95 ± 0.74	0.084
Creatinine (μmol/L)	65.7 ± 12.5	68.1 ± 14.4	0.161	72.3 ± 13.9	66.3 ± 13.5**	0.002
Uric acid (μmol/L)	354.8 ± 82.4	361.3 ± 78.3	0.547	408.5 ± 92.8	327.0 ± 86.7***	< 0.001
Albumin (g/L)	45.7 ± 2.1	45.7 ± 1.9	0.990	43.1 ± 2.8	43.9 ± 2.9	0.069

Normally distributed data are presented as mean ± SEM. * p<0.05, ** p<0.01, *** p<0.001 V1 vs. V2, Paired t-test. ALT: Alanine Aminotransferase; ALP: Alkaline Phosphatase; AST: Aspartate Aminotransferase; BMI: Body Mass Index; hs-CRP: High Sensitive C-Reactive Protein; GGT: Gamma-glutamyl Transferase; HbA_{1c}: Haemoglobin A_{1c}; HDL: High-Density Lipoprotein; LDL: Low-Density Lipoprotein; TAG: Triglycerides.

Treated HFD mice showed significantly lower expression of senescence markers *p21^{Cip1/Waf1}* and *p16^{Ink4a}* in lung tissue (Fig. 1 E, F), accompanied by reduced senescence-associated β-galactosidase (SA-β-gal) activity (Fig. 1G) and DNA damage (Suppl. Fig. 1E), relative to HFD controls. This was paralleled by attenuation of obesity-induced inflammatory signaling, including decreased macrophage infiltration, as evidenced by reduced F4/80-positive cells (Fig. 1H; Suppl. Fig. 1G) and reduced expression of interleukin-6 compared to untreated HFD mice (Suppl. Fig. 1F).

In the lungs, untreated HFD-fed mice developed pathological changes consistent with fibrosis (Fig. 1I), including thickened alveolar septa, which are markedly ameliorated by MitoTam treatment (Fig. 1J). This was

confirmed by a significant reduction in overall fibrosis severity demonstrated by Masson's trichrome staining (Supplementary Fig. 1H). Accordingly, the abundance of αSMA-positive myofibroblasts in lung tissue was significantly decreased following MitoTam treatment (Supplementary Fig. 1I), indicating reduced fibrotic remodeling. This was accompanied by decreased expression of key extracellular matrix-related transcripts, including collagen 1a (*Colla*) and fibrillin 1 (*FBNI*) (Fig. 1K, L). Similarly, expression of actin alpha 2 (*Acta2*), encoding αSMA, fibroblast growth factor 1 (*FGF1*) and perilipin 2 (*Plin2*) was reduced in MitoTam-treated mice (Fig. 1M-O), further supporting diminished myofibroblast activity.

Table 3. Circulating gut hormones and adipokines analysed in subjects before (V1) and 6 m (V2) after LSG.

	Conservative treatment		p value	Bariatric surgery		p value
	V1	V2		V1	V2	
Leptin (ng/mL)	20.6 ± 9.2	18.2 ± 7.6	0.191	22.9 ± 14.8	8.4 ± 6.8***	< 0.001
Adiponectin (mg/L)	12.0 ± 9.8	12.9 ± 4.7	0.439	11.5 ± 5.7	18.0 ± 7.8***	< 0.001
Insulin (pg/mL)	1299.5 ± 727.3	1089.3 ± 730.7	0.399	1384.3 ± 831.0	1021.1 ± 990.8*	0.048
C-peptide (ng/mL)	1.9 ± 1.0	1.8 ± 0.9	0.827	2.5 ± 1.6	2.1 ± 2.1	0.348
Glucagon (pg/mL)	76.5 ± 40.8	71.8 ± 34.0	0.539	66.2 ± 51.0	55.6 ± 39.3	0.207
GLP-1 active (pg/mL)	24.0 ± 28.3	59.6 ± 120.1	0.235	55.5 ± 102.3	28.0 ± 29.3	0.098
GIP total (pg/mL)	20.8 ± 12.3	19.0 ± 12.1	0.643	35.2 ± 20.7	89.3 ± 97.7**	0.002
Ghrelin active (pg/mL)	143.5 ± 5.8	157.7 ± 136.3	0.645	127.5 ± 95.8	48.5 ± 22.1***	< 0.001
PP (pg/mL)	44.9 ± 34.6	68.4 ± 89.7	0.244	77.7 ± 63.4	176.2 ± 183.9***	< 0.001

Normally distributed data are presented as mean ± SEM. * p<0.05, ** p<0.01, *** p<0.001 V1 vs. V2, Paired t-test.

GIP: Glucose-dependent Insulinotropic Polypeptide; GLP-1: Glucagon-like Peptide-1; PP: Pancreatic Polypeptide.

Finally, MitoTam markedly reduced *Hif1α* expression in lung tissue (Fig. 1P), which was corroborated by improved mitochondrial respiration (complex I and IV activity) in lung tissue (Fig. 1Q), indicating improved tissue oxygenation and metabolic

function. This was consistent with the results of pimonidazole staining, which showed a substantial decrease in hypoxic areas in MitoTam-treated lungs compared with untreated HFD-fed animals (Fig. 1R).

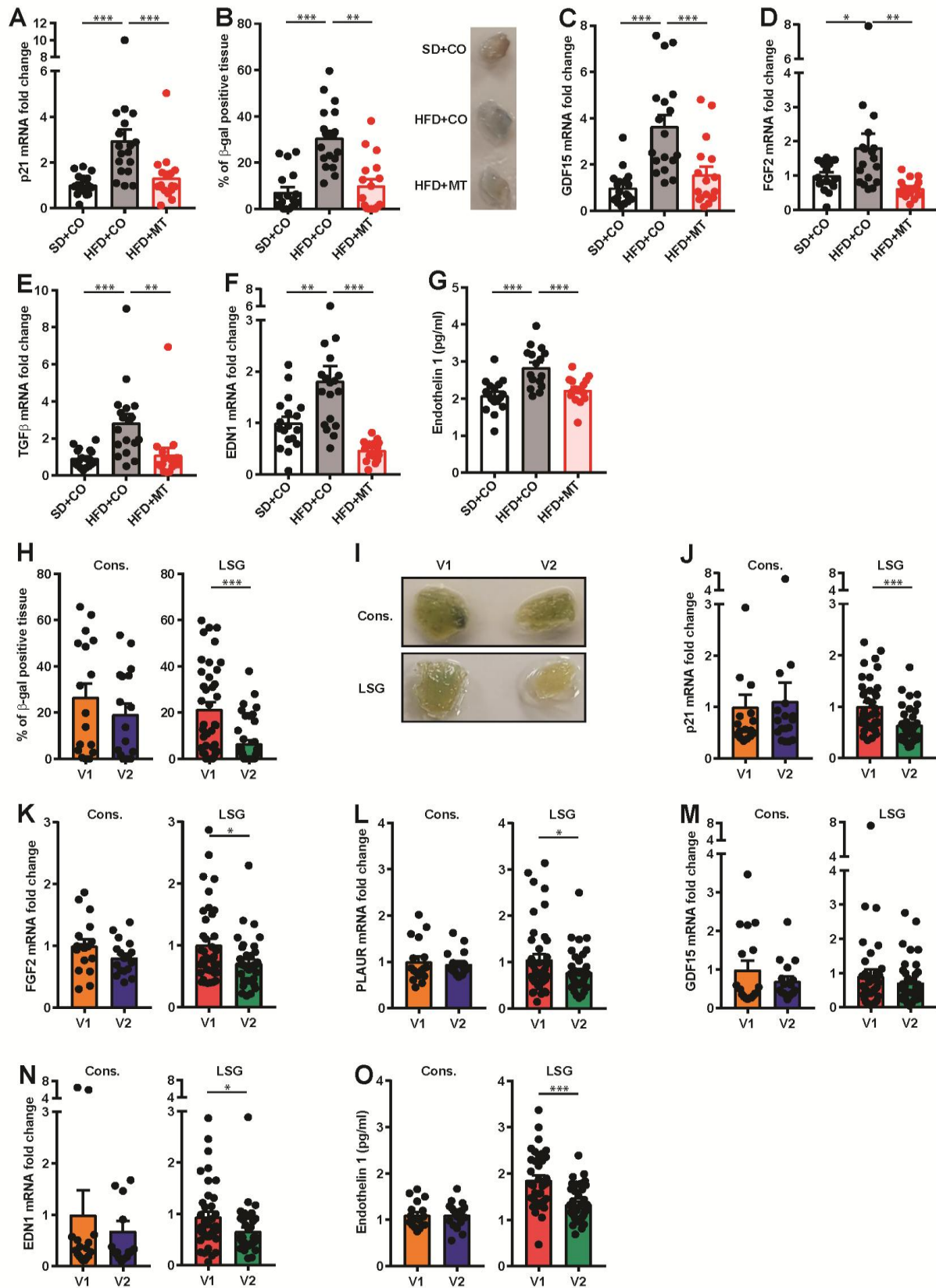


Figure 2. Senescence reduction in adipose tissue limits pro-fibrotic and Endothelin-1 signaling. C57BL/6J mice (8 months old) on standard (SD) or high fat diet (HFD) were divided into three groups, i.e. SD+CO: standard diet + corn oil; HFD+CO: high fat diet + corn oil; HFD+MT: high fat diet + MitoTam (n=17-18 per group). The mice were treated with MitoTam (2 mg/kg body weight) dissolved in 4 % ethanol in corn oil or the vehicle given i.p. twice per week for a period of 4 weeks. The level of *p21^{Cip1/Waf1}* mRNA in SAT was assessed by qRT-PCR (A). SA- β -gal positive tissue in SAT (%) is shown as bar graph (left) and representative tissue is presented (right; blue colour) (B). The level of *GDF15* (C), *FGF2* (D), *TGF β* (E) and *EDN1* (F) mRNA in SAT was assessed by qRT-PCR. Serum endothelin-1 concentration was assessed by ELISA (G). SAT samples from patients with class III obesity undergoing either conservative treatment (Cons.) or laparoscopic sleeve gastrectomy (LSG) were collected before intervention (V1) and 6 months after treatment (V2). SA- β -gal positive tissue in SAT (%) is shown as bar graph (H) and representative tissue is presented (right; blue colour) (I). The level of *p21^{Cip1/Waf1}* mRNA in SAT was assessed by qRT-PCR (J). The level of *FGF2* (K), *PLAUR* (L), *GDF15* (M) and *EDN1* (N) mRNA in SAT was assessed by qRT-PCR. Serum endothelin-1 concentration was assessed by ELISA (O). Data are presented as mean $\pm$ SEM. For mouse experiments (A–G), statistical significance was assessed using one-way ANOVA followed by Tukey's multiple comparisons test. For human data (H–O), paired t-test was used to compare values between V1 and V2 within the same subjects. *p < 0.05; **p < 0.005; ***p < 0.001.

To distinguish the contribution of reduced caloric intake from direct drug effects, we analyzed a pair-feeding (PF) cohort. Despite matched caloric intake and partial metabolic improvement (see [5]), PF mice did not reproduce the full extent of MitoTam-induced improvements. Specifically, reductions in senescence (SA- β -gal), macrophage infiltration, Ashcroft score, collagen deposition, fibrotic tissue area, α SMA-positive myofibroblasts, and mitochondrial respiration were significantly more pronounced in MitoTam-treated animals than in PF animals (Suppl. Fig. 2).

Decreased senescence and adiposity in subcutaneous adipose tissue limit pro-fibrotic signaling and protect against lung injury

Growing evidence indicates that adipose tissue-derived inflammatory and fibrotic signals can influence progression of pulmonary fibrosis [7]. HFD markedly increased senescence in SAT, as demonstrated by elevated *p21^{Cip1/Waf1}* mRNA expression (Fig. 2A) and a significant rise in β -gal positive tissue (Fig. 2B). Treatment with MitoTam substantially reduced both markers. This reduction was accompanied by a strong decrease in expression of *GDF15*, *FGF2* and *TGF β* (Fig. 2C–E), key drivers of pro-fibrotic signaling. Transcript levels of endothelin-1 (*Edn1*), a potent vasoconstrictor and pro-fibrotic mediator known to drive fibroblast activation and extracellular matrix accumulation in lung tissue, were similarly reduced following MitoTam treatment (Fig. 2F). Consistent with these transcriptional changes, circulating endothelin-1 concentrations were significantly lower in MitoTam-treated mice relative to HFD controls (Fig. 2G). Collectively, these data indicate that MitoTam diminishes adipocyte senescence and their pro-fibrotic secretory profile, reducing systemic risk factors for pulmonary fibrosis development.

Bariatric surgery-induced body weight loss suppresses adipose senescence and pro-fibrotic signaling

To determine whether similar senescence-related mechanisms operate in humans, we next examined the effects of LSG on adipose tissue remodeling in patients with severe obesity. All enrolled subjects met the criteria for class III obesity (BMI > 40 kg/m²), reflecting a cohort with very high metabolic risk. 17 patients underwent conservative treatment based on lifestyle modification, whereas 42 patients underwent LSG (Table 2). Six months after surgery, LSG patients experienced profound body weight loss, with body weight decreasing from 131.4 $\pm$ 25.1 kg to 98.3 $\pm$ 19.8 kg (p < 0.001) and BMI dropping from 44.5 $\pm$ 6.5 to 33.2 $\pm$ 5.5 kg/m² (p < 0.001), corresponding to class II obesity. In contrast, conservatively managed patients exhibited only minor, non-significant changes in both parameters. Metabolic improvements strongly paralleled body weight reduction after LSG: fasting glucose and HbA1c decreased significantly, and substantial reductions were observed in triglycerides, liver transaminases (AST, ALT, GGT), uric acid, and hs-CRP, collectively indicating enhanced glycemic control, improved liver function, and reduced systemic inflammation (Table 2). Conservative therapy resulted in only modest effects, with a small increase in HDL cholesterol as the most notable change.

LSG also induced marked shifts in circulating metabolic hormones and adipokines (Table 3). Leptin levels dropped by more than 60 %, accompanied by a significant rise in adiponectin, consistent with improved adipose tissue function. Postoperative decrease in insulin, C-peptide, and active ghrelin further reflected improved insulin sensitivity and appetite regulation. Conversely, pancreatic polypeptide (PP) and GIP rose significantly, demonstrating postoperative changes in gut hormone secretion. These hormonal adaptations were absent or minimal in conservatively treated subjects.

To evaluate the effect of body weight loss on senescence, stress signaling, and endothelial activation

within adipose tissue, we analyzed paired samples collected before (V1) and 6 months after intervention (V2) in both treatment groups. LSG induced a pronounced reduction in adipose tissue senescence, as evidenced by a significant decrease in the proportion of β -gal positive cells (Figure 2H, I). This decline in cellular senescence was accompanied by a substantial downregulation of *p21^{Cip1/Waf1}* mRNA expression (Figure 2J), whereas no significant changes were observed in conservatively treated subjects.

Markers involved in tissue remodeling and cellular stress responses, many of which also indirectly participate in pathways implicated in increased risk of pulmonary damage, were favorably altered following LSG. *FGF2*, *PLAUR* and *GDF15* decreased significantly in subcutaneous adipose tissue following LSG (Figure 2K–M). In contrast, none of these transcripts exhibited meaningful changes after conservative treatment, underscoring the specific impact of surgical body weight loss on these remodeling pathways. Expression of *EDN1* was significantly reduced after LSG (Figure 2N). Importantly, this transcriptional suppression was mirrored by a pronounced decline in circulating endothelin-1 levels (Figure 2O), indicating a systemic reduction in endothelial stress. No comparable improvements were observed in the conservative group.

DISCUSSION

While obesity and T2DM have long been recognized as risk factors for impaired lung function, emerging literature suggests that senescent cells may serve as a common upstream driver, linking metabolic and pulmonary pathology. Our findings support a model in which reducing senescent burden alleviates pulmonary fibrosis via both direct and indirect mechanisms. Directly, MitoTam appears to target senescent cells within the lung itself, lowering expression of canonical senescence markers, diminishing hypoxia-related signaling, and limiting myofibroblast activation, which is consistent with prior studies showing that senolysis reduces fibrotic remodeling in experimental lung injury [2]. At the same time, senescence reduction in adipose tissue may indirectly benefit the lung by lowering systemic pro-fibrotic and inflammatory mediators derived from dysfunctional adipose tissue. Importantly, although MitoTam markedly improves metabolic parameters and reduces adiposity, our pair-feeding experiments indicate that caloric restriction alone does not fully recapitulate its anti-senescent and anti-fibrotic effects, supporting the contribution of weight loss-independent mechanisms. This dual action suggests that senolytic interventions, such as MitoTam, could mitigate pulmonary fibrosis not only by improving the local pulmonary microenvironment

but also by reshaping the endocrine crosstalk between adipose tissue and the lung.

The patient data presented here reinforce the concept that reducing metabolic load can normalize senescence-associated pathways with potential downstream pulmonary benefits. Bariatric surgery has been previously associated with reduced systemic inflammation, improved mitochondrial function, and lower biological aging markers [8]. The marked decrease in adipose tissue senescence and pro-fibrotic gene expression observed in our patient cohort aligns with this report, suggesting that body weight loss-induced remodeling of adipose tissue may translate into reduced endocrine drivers of lung fibrosis. The pronounced decline in circulating endothelin-1 after LSG is particularly notable given the well-established role of endothelin signaling in pulmonary vasoconstriction and fibroblast activation. Our data therefore indicate a potential to reduce the risk or progression of pulmonary remodeling in patients with metabolic syndrome, rather than providing direct evidence of improved pulmonary fibrosis.

Taken together, these observations support a broader and unifying hypothesis: interventions that lower senescent cell burden, whether through metabolic improvement or through direct senolytic action, may serve as upstream modifiers of pulmonary fibrosis risk in individuals with obesity or prediabetes. MitoTam uniquely strengthens this concept by demonstrating simultaneous benefits in metabolic tissues and the lung. By directly disrupting mitochondrial function in senescent cells [4], MitoTam provides a targeted means of reducing pro-fibrotic signaling at both the systemic and pulmonary levels. This effect is supported by its pharmacokinetic profile, characterized by rapid tissue distribution and accumulation following administration [9], together with selective vulnerability of senescent cells linked to their low *ANT2* expression, which limits their ability to maintain mitochondrial integrity under MitoTam-induced stress [4, 10]. However, further studies are required to establish the translational relevance of this approach, including long-term safety assessment, and deeper mechanistic characterization.

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Author's contributions

SSH and MR conceived and proposed the research topic. SSH designed the study protocol. MR, TH, DDTL and SSH, performed in vivo experiments and patient analysis. IS, JF and FT performed bariatric surgery and a biopsy. RP performed ELISA and Luminex analysis. MC isolated RNA from the samples. AC and JK provided a sample management: handled patient sample acquisition, processing, storage, and cataloguing. JN, JS and LW synthesized MitoTam. MM and MH selected patients for the study and provided financial support for the study. SSH, MR, AC, JN and MH contributed to writing, revising and proofreading of the manuscript. All authors read and approved the final manuscript.

Declaration of Competing Interest

JN, JS and LW are co-owners of the MitoTam intellectual property. SSH, JN, JS and LW are co-inventors of MitoTam as a senolytic agent. JN, JS and LW are also co-inventors of MitoTam as an oncolytic agent. Other authors declare no conflict of interest.

Ethics approval

The study was approved by the Human Ethics Review Board, Institute for Clinical and Experimental Medicine (IKEM), Prague, Czech Republic (ethical approval code G-20-41) and by the Human Ethics Review Board, st. Zdislava Hospital, Mostiště, Czech Republic (ethical

approval code S-18-01). Animal experiments were performed in agreement with the Animal Protection Law of the Czech Republic and were approved by the Ethics Committee of the Ministry of Health, Czech Republic.

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Supplementary Materials

The Supplementary data can be found online at: www.aginganddisease.org/EN/10.14336/AD.2026.0150.