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Smoking cessation only partially reverses cardiac metabolic and structural remodeling in mice

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Abstract

Aims: Active cigarette smoking is a major risk factor for chronic obstructive pulmonary disease that remains elevated after cessation. Skeletal muscle dysfunction has been well documented after smoking, but little is known about cardiac adaptations to cigarette smoking. The underlying cellular and molecular cardiac adaptations, independent of confounding lifestyle factors, and time course of reversibility by smoking cessation remain unclear. We hypothesized that smoking negatively affects cardiac metabolism and induces local inflammation in mice, which do not readily reverse upon 2-week smoking cessation.

Methods: Mice were exposed to air or cigarette smoke for 14 weeks with or without 1- or 2-week smoke cessation. We measured cardiac mitochondrial respiration by high-resolution respirometry, cardiac mitochondrial density, abundance of mitochondrial supercomplexes by electrophoresis, and capillarization, fibrosis, and macrophage infiltration by immunohistology, and performed cardiac metabolome and lipidome analysis by mass spectrometry.

Results: Mitochondrial protein, supercomplex content, and respiration (all $p < 0.03$) were lower after smoking, which were largely reversed within 2-week smoking cessation. Metabolome and lipidome analyses revealed alterations in

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mitochondrial metabolism, a shift from fatty acid to glucose metabolism, which did not revert to control upon smoking cessation. Capillary density was not different after smoking but increased after smoking cessation ($p = 0.02$). Macrophage infiltration and fibrosis ($p < 0.04$) were higher after smoking but did not revert to control upon smoking cessation.

Conclusions: While cigarette-impaired smoking-induced cardiac mitochondrial function was reversed by smoking cessation, the remaining fibrosis and macrophage infiltration may contribute to the increased risk of cardiovascular events after smoking cessation.

KEYWORDS

cardiac metabolism, cardiac remodeling, fibrosis, inflammation, macrophages, smoking cessation

1 | INTRODUCTION

Cigarette smoking is a major risk factor for the development of chronic obstructive pulmonary disease (COPD)[1] and cardiovascular diseases,[2] all contributing to the higher mortality rate of current smokers. The risk for cardiovascular complications is even higher for ex-smokers and could be related to irreversible cumulative changes induced by exposure to cigarette smoke.[3,4] For instance, former heavy smokers have a higher prevalence of type 2 diabetes mellitus, left ventricular systolic dysfunction, coronary artery disease, and peripheral arterial disease, as well as higher circulating levels of serum C-reactive protein and interleukin-6 compared to never smokers.[3] Skeletal muscle adaptations, such as increased fatigue and cachexia, are often reported in patients with COPD,[5–7] but the link with smoking-induced cardiovascular disease is not well understood. Smokers often exhibit lower physical activity levels and a poor diet, lifestyle factors that in themselves are significant risk factors for cardiovascular complications.[8] Therefore, these confounding factors blur how smoking itself affects intracellular cardiac structure and function.

Many substances in cigarette smoke are known to impair oxygen transport to tissue and cellular metabolism.[4,7,9,10] In addition, smoking reduced mitochondrial respiration in murine skeletal muscles, caused skeletal muscle atrophy,[9] and increased macrophage infiltration in bronchoalveolar lavage fluid that all reverted to control values after 2 weeks of smoking cessation.[11,12] Smoking has also been associated with capillary rarefaction.[13] It is most likely that the effects of smoking on mitochondrial function and capillaries are not restricted to skeletal muscle but will have a similar impact on mitochondria in any tissue, including the brain and heart. Likewise, smoke-induced circulating inflammatory markers from lungs may also cause an altered cardiac metabolism, local inflammation, and fibrosis, which may contribute to the elevated risk of developing cardiovascular diseases[11,12] and systolic dysfunction in smokers.[14] However, whether cardiac muscle metabolism, inflammation, and fibrosis are affected by cigarette smoking and to what extent these are reversible by smoking cessation has not yet been explored.

Therefore, the objective of the study was to comprehensively assess the impact of exposure to cigarette smoking and smoking cessation of up to 2 weeks on cardiac muscle structure, local inflammation, and cardiac metabolism in mice. We hypothesized that smoking negatively affects cardiac metabolism and induces local inflammation and fibrosis. To investigate this, metabolome and lipidome analyses were performed to assess changes in cardiac metabolism upon smoking and smoking cessation.

2 | MATERIALS AND METHODS

2.1 | Animals and study design

The supplemental datasheet contains an extended Materials and Methods section, which is in line with good publishing practice in physiology.[15] All experimental procedures were approved by the Leuven Ethical Committee. The study design was as previously described.[11] Eight-week-old male C57Bl/6JolaH mice (n = 44) were randomly divided into four groups: one exposed to cigarette smoke for 14 weeks (SM); a group exposed to cigarette smoke for 13 weeks and 1-week cessation (SC1W); a group exposed to cigarette smoke for 12 weeks and 2-week cessation (SC2W); and a control group (CON) exposed to room air.[11]

2.2 | (Immuno-)histochemistry and western blotting

Connective tissue, capillary, nuclear densities, succinate dehydrogenase (SDH) activity, macrophage infiltration, GLUT4 translocation and cardiomyocyte cross-sectional area were assessed in cardiac sections. In a subgroup of 20 animals, protein content of subunits of all five OXPHOS complexes, DRP1, OPA1, and GLUT4 was determined.

2.3 | Mitochondrial respiration and supercomplex content

Mitochondrial respiration was measured in the apex using high-resolution respirometry. Mitochondrial supercomplexes were visualized by blue native gel electrophoresis.

2.4 | Metabolomic and lipidomic analysis

Metabolome and lipidome analyses were performed in the apex, and data were normalized to internal standards and tissue weight.

2.5 | Statistical analysis

Results are expressed as the mean ± SEM. Statistical analysis consisted of two parts because the smoking cessation groups had two interventions (smoking + cessation). First, statistical differences between control and smoking were assessed with an independent *t*-test, and the effect of smoke cessation compared to smoking was tested with a one-way analysis of variance (ANOVA). To test whether 2 weeks of smoke cessation was significantly different from control, an independent *t*-test was performed. A $p < 0.05$ was considered significant.

3 | RESULTS

3.1 | Body and heart mass and cardiomyocyte size

Body mass was lower after smoking (SM) compared to control (CON; Figure 1A), and gradually increased after smoking cessation.[11] Heart mass was not significantly different between smoking and controls (Figure 1B) but was higher after 2 weeks of smoking cessation (SC2W) compared to smoking animals. The heart-to-body mass ratio was higher after smoking (5.2 ± 0.1 vs. 4.1 ± 0.2 mg·g^{-1} in control; $p < 0.001$) but did not change after smoking cessation. Cardiomyocyte cross-sectional area tended to be lower after smoking but did not increase after smoking cessation (Figure 1C,D).

3.2 | Cardiac fibrosis and capillary supply

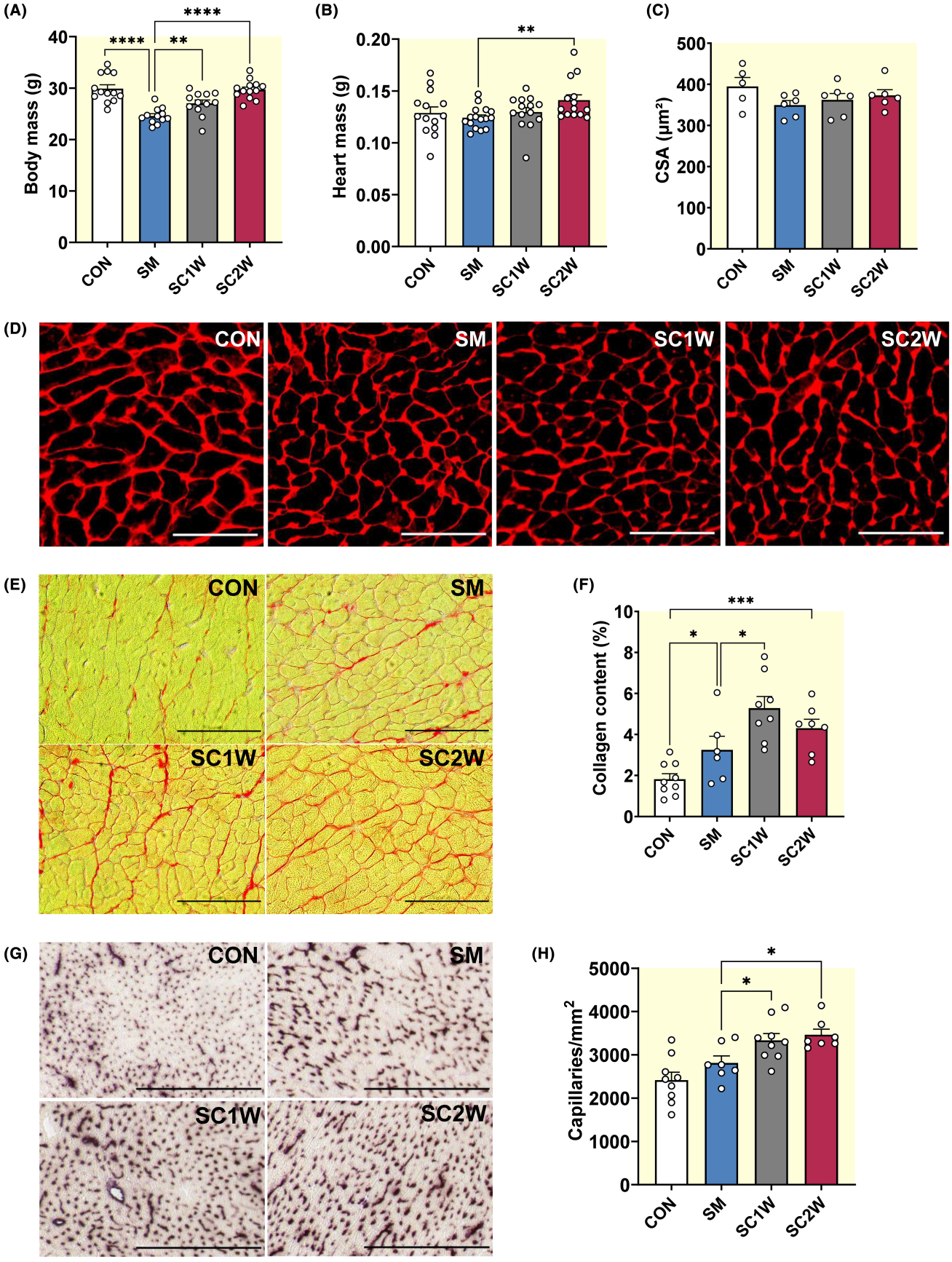
Collagen content was higher in smoking than control and further increased after 1-week smoking cessation (Figure 1E,F). Although collagen content was not different between 2 weeks of smoking cessation (SC2W) and smoking, collagen content was still higher in SC2W compared to control. Capillary density was not different after smoking compared to control, but significantly increased after smoking cessation, and remained higher than control (Figure 1G,H).

3.3 | Nuclear density and infiltration of immune cells

We performed a hematoxylin and eosin stain to visualize cells and nuclei. There were no significant differences in cardiac nuclear density between smoking and control, but nuclear density was higher after 1 week of smoking cessation, but not after 2 weeks, compared to smoking (Figure 2A,B). We reasoned that these additional nuclei could be immune cells such as macrophages, and indeed, cigarette smoke exposure increased the concentration of infiltrated macrophages compared to control (Figure 2C,D), which remained elevated above control after smoking cessation ($p < 0.0001$). No signs of cardiac cell necrosis or cell death were observed.

3.4 | Mitochondrial function

To understand smoking-induced alterations in cardiac metabolism, we assessed mitochondrial respiration in the apex. Leak respiration with NADH substrates (Figure 3A), NADH (complex I)-linked respiration (Figure 3B) and complex II-linked respiration (Figure 3C) were not significantly different between groups. Oxidative phosphorylation capacity tended to be lower after smoking compared to control ($p = 0.056$), and significantly increased to control values after smoking cessation (Figure 3D). Uncoupling respiration was significantly lower after smoking and returned to control level after smoking cessation (Figure 3E). Normalized leak respiration was significantly higher after smoking, and returned to control levels after smoke



cessation, indicative of a relatively higher mitochondrial uncoupling during smoking. Excess capacity (oxidative phosphorylation / electron transport capacity) was significantly higher after smoking (0.91 ± 0.06) versus control (0.84 ± 0.08, $p = 0.018$), which tended to remain higher after 2 weeks of smoke cessation (0.90 ± 0.06, $p = 0.068$).

FIGURE 1 Body mass, heart mass, cross-sectional area of cardiomyocytes, fibrosis, and capillary density after smoking and smoking cessation in mice. (A) Body (CON $n=13$, SM $n=12$, SC1W $n=12$, SC2W $n=13$) and heart mass (B) of mice exposed to cigarette smoke (SM $n=16$), and after 1–2 weeks of smoking cessation (SC1W $n=14$, SC2W $n=14$), compared to control (CON $n=14$). (C) Cardiomyocyte cross-sectional area was not significantly different between groups (CON $n=5$, SM $n=6$, SC1W $n=6$, SC2W $n=6$). (D) Representative images of cell membranes (wheat germ agglutinin antibody). (E) Representative images of collagen. (F) Collagen content was higher after smoking and further increased after smoking cessation (CON $n=9$, SM $n=6$, SC1W $n=8$, SC2W $n=7$). (G) Representative images of cardiac capillaries. (H) Capillary density increased after 1 and 2 weeks of smoking cessation (CON $n=9$, SM $n=7$, SC1W $n=9$, SC2W $n=7$). Results are expressed as mean ± SEM. The corresponding significant p values are shown in the figures. Scale bar represents 100 μm (D, E) or 250 μm (G). SM versus CON and SC2W versus CON (unpaired two-tailed t-test); SC1W versus SM and SC2W versus SM (one-way ANOVA).

Mitochondrial complex I subunit NDUFB8 content was lower after smoking compared to control (Figure S1D,E) and remained lower after 2-week smoke cessation than in control ($p=0.011$). No group differences in protein level of subunits for complexes II and III and ATP synthase were observed (Figure S1F–I), although complex IV subunit MTCO1 level was lower after smoking compared to control (Figure S1H). SDH activity (Figure 3F,G) was reduced after smoking, and continued to decrease after 1-week smoke cessation, but recovered to smoking levels after 2-week cessation. No significant group differences were observed in the protein content of key players of mitochondrial fusion (OPA1) and fission (DRP1; Figure S1A–C).

We next tested whether mitochondrial supercomplex formation was affected by smoking and normalized after smoking cessation. For this, we isolated mitochondria and performed blue-native electrophoresis. Independent of mitochondrial content, smoking was associated with a lower content of high-molecular-weight mitochondrial supercomplexes, which was restored to control after 2 weeks of smoke cessation (Figure 3H). Moreover, the content of free mitochondrial complexes tended to be lower after smoking and were significantly lower after smoking cessation (Figure 3I). The normalized mitochondrial supercomplex content (relative to total complexes) was not significantly different between smoking and control (Figure 3J), indicating a proportional decrease in supercomplexes and free complexes after smoking. The normalized mitochondrial supercomplex content increased after smoke cessation.

3.5 | Metabolomics

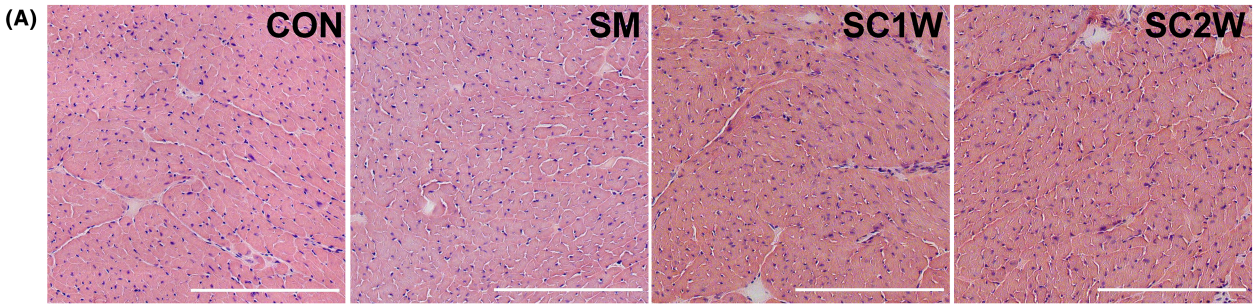
Metabolomics was performed to understand how cigarette smoking and cessation affected cardiac metabolism. Metabolites with variable importance in projection (VIP) score >1.4 were used for enrichment pathway analysis (Table S1, Figure 4). Enrichment pathway analysis revealed alterations in nicotinate and nicotinamide metabolism, glycolysis, pentose phosphate metabolism and gluconeogenesis, branched-chain amino acids degradation and nucleotide metabolism, mitochondrial beta-oxidation, and other lipid metabolism pathways.

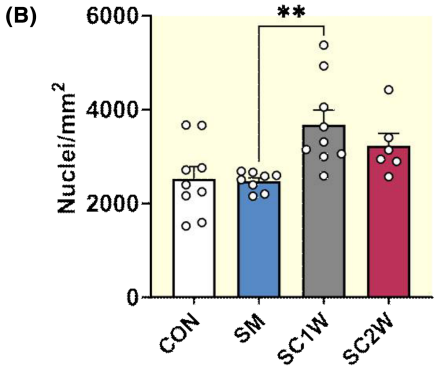
Smoking and smoking cessation decreased the reduced form of nicotinamide mononucleotide (NMNH) in the nicotinamide adenine dinucleotide (NAD^+) pathway (Figure S2). Two-week smoking cessation increased NAD^+ and NADH levels compared to smoking. Higher metabolite concentrations related to glycolysis and gluconeogenesis suggested a higher glucose breakdown after 2-week smoking cessation compared to control (Figure S3). Metabolites in the pentose phosphate pathway (ribose 5-phosphate and sedoheptulose 7-phosphate) were lower after 2 weeks of smoke cessation than in control. Ophthalmic acid, a biomarker of oxidative stress, was higher after smoking and 2 weeks of smoke cessation (Figure S3).

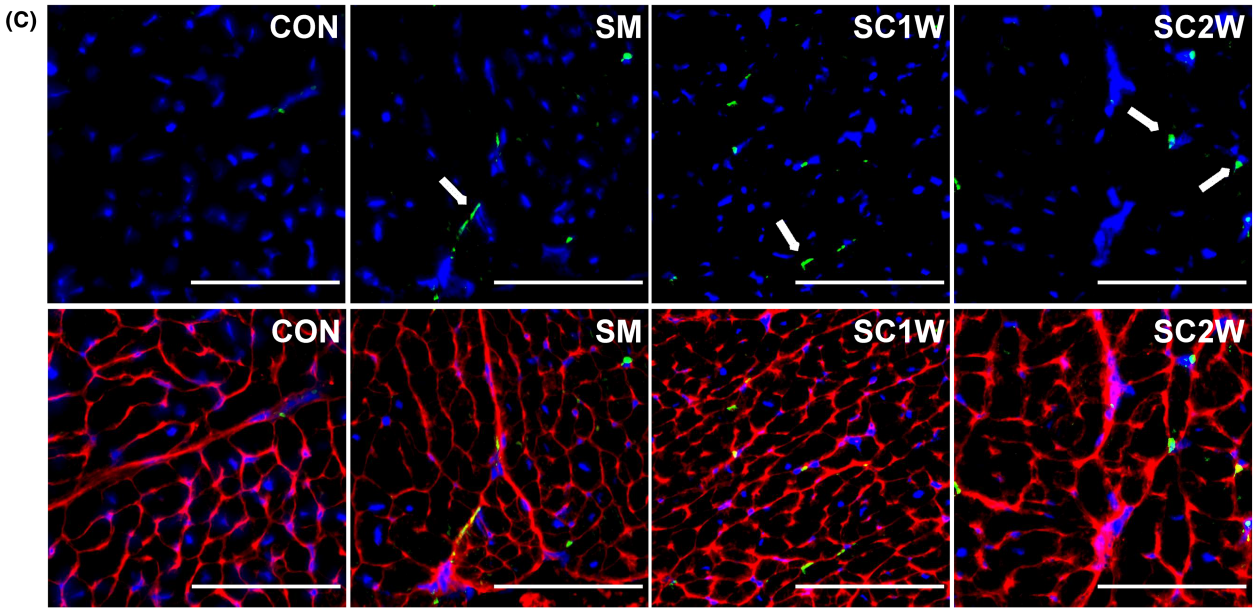
After 1-week smoking cessation, the branched-chain amino acids, isoleucine, leucine, valine, and allantoin (Figure S4), and ADP-ribose (Figure S2) were decreased compared to smoking. Branched-chain amino-acid content did not restore to control level after 2 weeks of smoke cessation, apart from allantoin and ADP-ribose. Cardiac purine and pyrimidine contents were not significantly changed by smoking but were higher after 2-week smoke cessation compared to control (Figure S5). Metabolites of purine biosynthesis (inosinic acid (IMP) and hypoxanthine) were not altered by smoking but were lower after 2-week smoke cessation ($p=0.0208$ and $p=0.0059$, respectively).

3.6 | Lipidomics

Lipid profiling was performed on the apex of the heart to study whether smoking caused alteration in the lipid content and species (Figure S6A–D). Enrichment analysis identified various lipid classes to be differentially abundant between groups (Figures S6A–D and S7A–D). Although total triacylglycerol concentration in heart tissue was not significantly altered after smoking and smoking cessation, long-chain and very-long-chain highly unsaturated triacylglycerol concentrations were higher after 2 weeks of smoke cessation compared to control and smoking







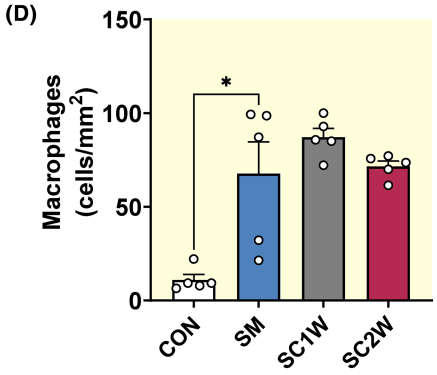


FIGURE 2 Nuclear density and macrophage infiltration of cardiac tissue after smoking and smoking cessation in mice. (A) Representative examples for hematoxylin and eosin staining in control (CON), smoking (SM), and 1–2 weeks of smoking cessation (SC1W and SC2W). (B) Nuclear density in cardiac tissue increased after smoking cessation (CON $n=9$, SM $n=8$, SC1W $n=9$, SC2W $n=6$). (C) Representative examples of macrophages (green), membranes (red), and nuclei (blue). Upper panel shows staining of cardiomyocyte nuclei and macrophages, and lower panel shows staining of cardiomyocyte membranes and nuclei and macrophages. White arrows show macrophages (upper panel). (D) Macrophage density was higher after smoking and smoking cessation. (CON $n=5$, SM $n=5$, SC1W $n=5$, SC2W $n=5$). Results are expressed as the mean ± SEM. Scale bar is 250 (A) or 50 μm (C). SM versus CON and SC2W versus CON (unpaired two-tailed *t*-test); SC1W versus SM and SC2W versus SM (one-way ANOVA).

(Figure 5), indicative of a lower breakdown or higher production of triacylglycerols after smoke cessation.

3.7 | Glucose transport

Our metabolome and lipidome analyses indicated that smoking cessation shifted metabolism from fatty acid oxidation toward glucose oxidation, possibly via an altered translocation of glucose transporter type 4 (GLUT4) from the cytosol to the cell membrane (Figure 6A). We therefore next determined whether there were differences in the membrane-associated GLUT4 in the mice that were fasted for >3 h. Smoking did not alter the fraction of GLUT4 associated with the cell membrane, but relatively more GLUT4 was translocated toward the cell membrane after smoking cessation (Figure 6C,D). No differences were observed in the amount of cell membrane that contained GLUT4. With western immunoblotting, we tested the hypothesis that GLUT4 protein concentration was different, but no group differences in overall GLUT4 protein concentration were observed (Figure 6C).

4 | DISCUSSION

We performed a detailed assessment of changes in cardiac structure and metabolism upon cigarette smoking and smoking cessation for up to 2 weeks in a mouse model of COPD. Cigarette smoking caused macrophage infiltration and fibrosis in the heart, which only partially recovered after smoking cessation. Surprisingly, smoking cessation—but not smoking itself—induced extensive capillary proliferation resulting in an increased capillary density. Smoking reduced maximal mitochondrial capacity that returned to control values after 2 weeks of smoking cessation. This was associated with quantitative and qualitative decrements in mitochondrial protein content, SDH activity, and mitochondrial supercomplex formation. Metabolome and lipidome analyses indicated a shift from fatty acid to glucose oxidation upon smoking cessation, which confirmed that cardiac metabolism only partially returns to control after smoking cessation.

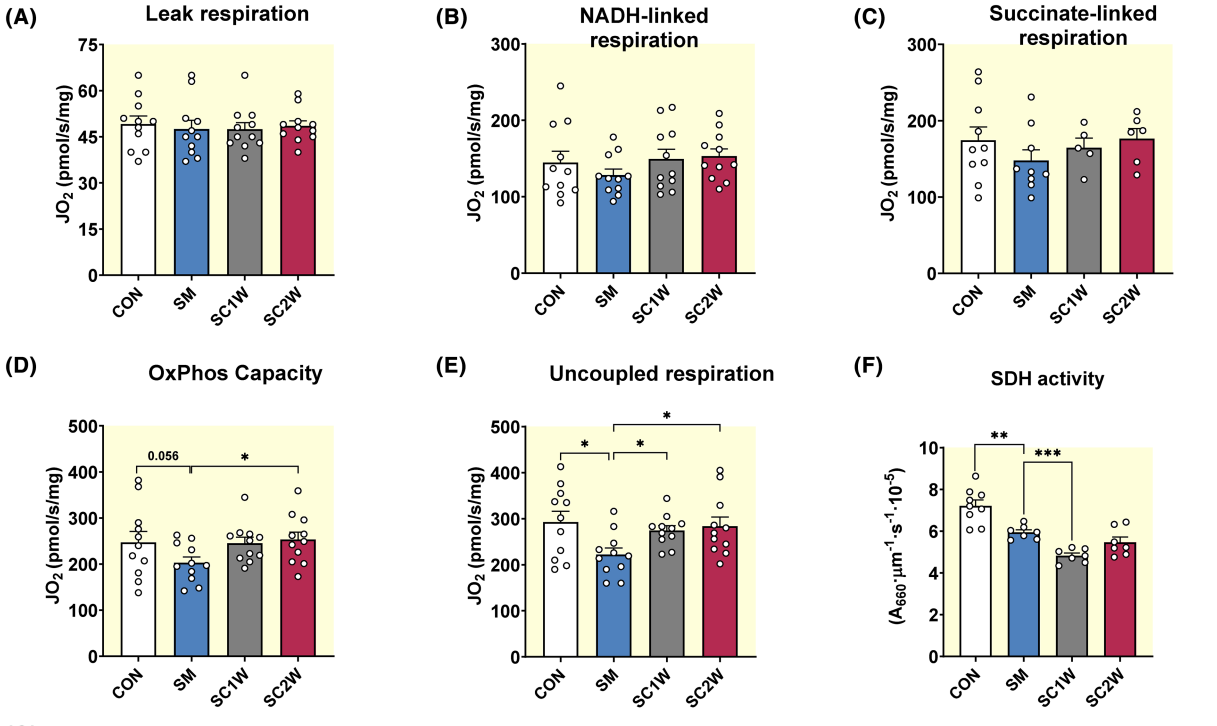
4.1 | Cardiac atrophy, local inflammation, and fibrosis

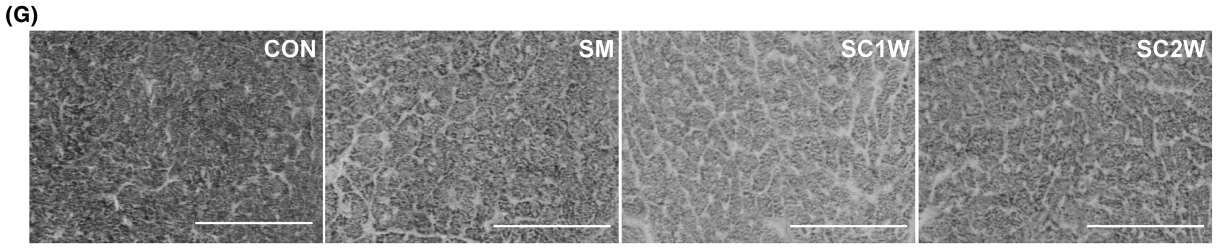
We observed a lower cardiac weight and tendency for a lower fibre cross-sectional area after cigarette smoking. Extrapulmonary manifestations are often observed in COPD, and these results suggest that besides skeletal muscle atrophy and cachexia,[4–6] the cardiac muscle is not spared from smoking-induced atrophy. Currently, it is unclear what the cellular and molecular overlap is between the cardiac and skeletal muscle atrophy upon cigarette smoking and whether cardiac atrophy is similar to cachexia. We propose that the term "cardiac cachexia" should refer to cardiac alterations, rather than skeletal muscle cachexia in patients with chronic heart failure.[16]

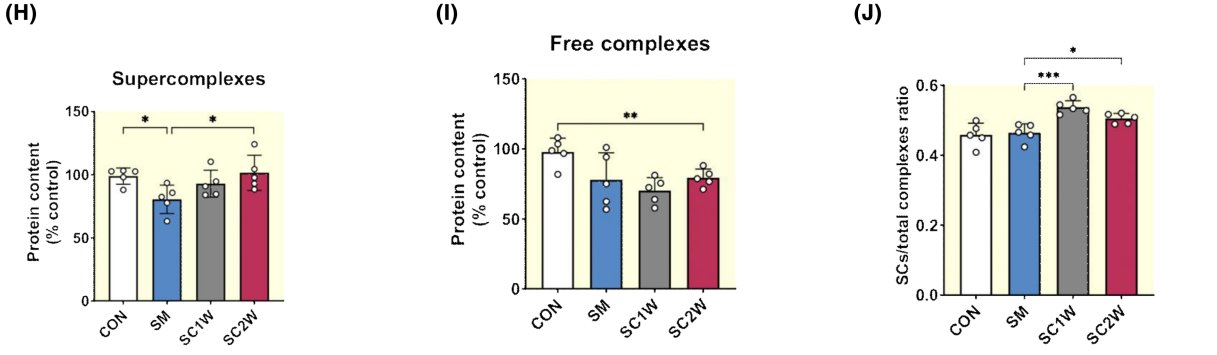
Local and systemic inflammation likely contribute to the altered cardiac structure and function. Smoking was accompanied by macrophage infiltration into the heart which did not revert to control after smoking cessation. The infiltrations occurred predominantly around endothelial cells. This local endothelial inflammation may induce a higher endothelial permeability in cigarette smoke-exposed mice.[17] These tissue-infiltrating macrophages are involved in the clearance of dead, dysfunctional, and dying cells, as well as tissue remodeling and angiogenesis.[18]

Macrophage infiltration is also associated with a higher local concentration of pro-inflammatory cytokines, such as TNF-α, various interleukins, and TGF-β, negatively affecting cardiac metabolism,[19,20] and can also promote collagen production and fibrosis.[21] Indeed, the smoking-induced fibrosis did not recover after cigarette smoking cessation. Cardiac macrophage infiltration, higher fibrosis, and capillary density after smoking and smoking cessation indicate that local inflammation contributed to the structural changes in the heart, linked to a stiffened heart and diastolic dysfunction,[22] typical for heart failure with preserved ejection fraction often observed in COPD patients.

The lower cytosolic concentrations of branched-chain amino acids (BCAA; leucine, isoleucine, and valine) in the heart after smoking cessation can be related to an increased protein synthesis[23] upon smoking cessation, but







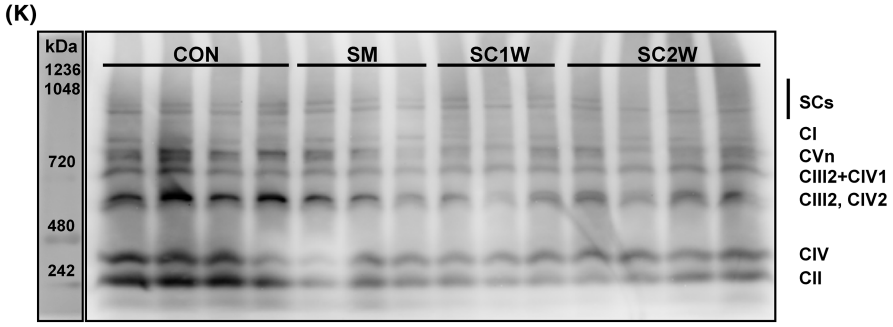


FIGURE 3 Cardiac maximal mitochondrial capacity is reduced with smoking and restored after smoking cessation. Succinate dehydrogenase activity depression in smoking and smoking cessation groups and supercomplex formation in the heart after smoking and smoking cessation in mice. (A) Leak respiration, (B) NADH (complex I) linked with pyruvate, malate, and glutamate, and (C) complex II-linked respiration (with succinate/rotenone) were not significantly different among control (CON), smoking (SM), and after 1–2 weeks smoking cessation (SC1W and SC2W). (D) Oxidative phosphorylation and (E) uncoupled respiration were lower in smoking compared to 2-week smoking cessation (CON $n=11$, SM $n=11$, SC1W $n=11$, SC2W $n=11$). (F) Lower succinate dehydrogenase (SDH) activity after smoking and both groups of smoking cessation compared to control (CON $n=9$, SM $n=7$, SC1W $n=7$, SC2W $n=7$). (G) Representative images of succinate dehydrogenase (SDH) activity staining. (H) Protein content of high-molecular-weight supercomplexes was lower after smoking and recovered after smoke cessation (CON $n=5$, SM $n=5$, SC1W $n=5$, SC2W $n=5$). (I) Free or low-molecular-weight complexes of CI-IV and ATPase synthase were lower in SC1W and SC2W. (J) Normalized supercomplex relative to total complex increased after smoke cessation. (K) Representative example of free mitochondrial complexes and complexes assembled in the supercomplex (SCs: $I+III_2+II_n$, $I+III_2+IV_1$). Results are expressed as mean ± SEM. Scale bar is 100 μm (G). SM versus CON and SC2W versus CON (unpaired two-tailed *t*-test); SC1W versus SM and SC2W versus SM (one-way ANOVA).

the fibre cross-sectional area did not enlarge. The discrepancy between the gains in heart weight without a concomitant change in cardiomyocyte size at smoking cessation is likely due to other factors than an elevated protein synthesis. Possibly, the increased cell infiltration and blood volume contribute to elevated heart weight after smoking and smoking cessation. Circulating inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), also increase BCAA catabolism and leucine oxidation.[24] A declined BCAA content may therefore also be a consequence of continued elevated local inflammation rather than a reflection in protein synthesis.

We observed increased levels of purines and pyrimidines after 2-week smoking cessation. These changes in nucleotide abundance may contribute to metabolic derangements and ventricle dysfunction as similar changes are also seen in pulmonary arterial hypertension[25] and myocardial hypertrophy.[26] Metabolites connected to the uridine metabolism are involved in protein O-GlcNAcylation, which in turn is directly linked to insulin resistance.[27] Indeed, GLUT4 translocation to the cell membrane was altered, but future studies are required to fully understand how smoking and smoking cessation alter whole-body insulin resistance.

4.2 | Angiogenesis

While smoking has been linked to endothelial damage and a lower capillary-to-fibre ratio in skeletal muscle,[13] we did not observe a decreased capillary density in the heart of mice exposed to cigarette smoke. This discrepancy between smoking-induced changes in capillary density in skeletal muscle and cardiac muscle is perhaps due to the unceasing demand for oxygen and nutrients in the beating heart, while skeletal muscles are intermittently active. Surprisingly, capillary density increased after smoking cessation. We speculate that a smoking cessation-induced increase in the number of circulating endothelial progenitor cells[28] could stimulate neovascularization, further facilitated by the sustained local inflammation and release of cytokines by local macrophages.

4.3 | Cardiac mitochondrial structure and function

Uncoupled respiration was lower after cigarette smoking but returned to control values after 2 weeks of smoking cessation. Likely, these results are explained by a combination of a lower mitochondrial protein content (evidenced by lower mitochondrial complexes I and IV content and SDH activity), less supercomplexes, and intrinsic impairments in the convergent electron flow within the mitochondria, indicated by the higher normalized NADH-linked respiration. Cigarette smoking directly impairs mitochondrial respiration and electron transport via inhibition of complex III and IV activity by carbon monoxide and other cigarette smoke compounds,[9,11] which may explain the impairments in the convergent electron flow into complex III. Smoking cessation alleviated this direct inhibition of mitochondrial respiration and improved maximal electron transport system capacity, likely due to alterations in the mitochondrial supercomplex formation, rather than adding more mitochondria per se, as mitochondrial protein content was only marginally affected by smoking and cessation. Interestingly, the time course of some adaptations, such as SDH activity and the protein content of some free complexes, was different from the smoking cessation-induced alterations in uncoupled respiration or amount of supercomplexes. The underlying reason for this is currently unclear.

We hypothesize that the intricate balance between disassembly and re-assembly of mitochondrial supercomplexes and novel protein synthesis from individual subunits is affected by smoking and smoking cessation, which partly explains the alteration of supercomplex content and overall mitochondrial respiration. This could explain the increased mitochondrial respiration, without obvious mitochondrial biogenesis. Nollet et al. (2023) indeed recently

showed that enhanced incorporation of complex I into respiratory supercomplexes improved the capacity to oxidize NADH and thereby increased NADH-linked respiration.[29] The formation of mitochondrial supercomplexes may reflect an adaptation to cope with oxidative stress, also inferred from our results of a higher ophthalmic acid (a marker of oxidative stress) observed in smoking and smoking cessation.[30]

The nicotinamide pathway was affected after smoking, as the precursor NMNH was lower after smoking despite similar NAD^+ levels. Lower NAD^+ levels are observed in several cardiovascular diseases, including atherosclerosis and heart failure with reduced and preserved ejection fraction.[31] NMNH, NAD^+, and NADH significantly increased after smoking cessation, indicative of an increased synthesis or a decreased degradation, since ribose-5-phosphate (a breakdown product of NAD^+[32]) was lower. These improvements in NAD^+ metabolism contribute to improved mitochondrial function and redox signalling[33] after smoking cessation.

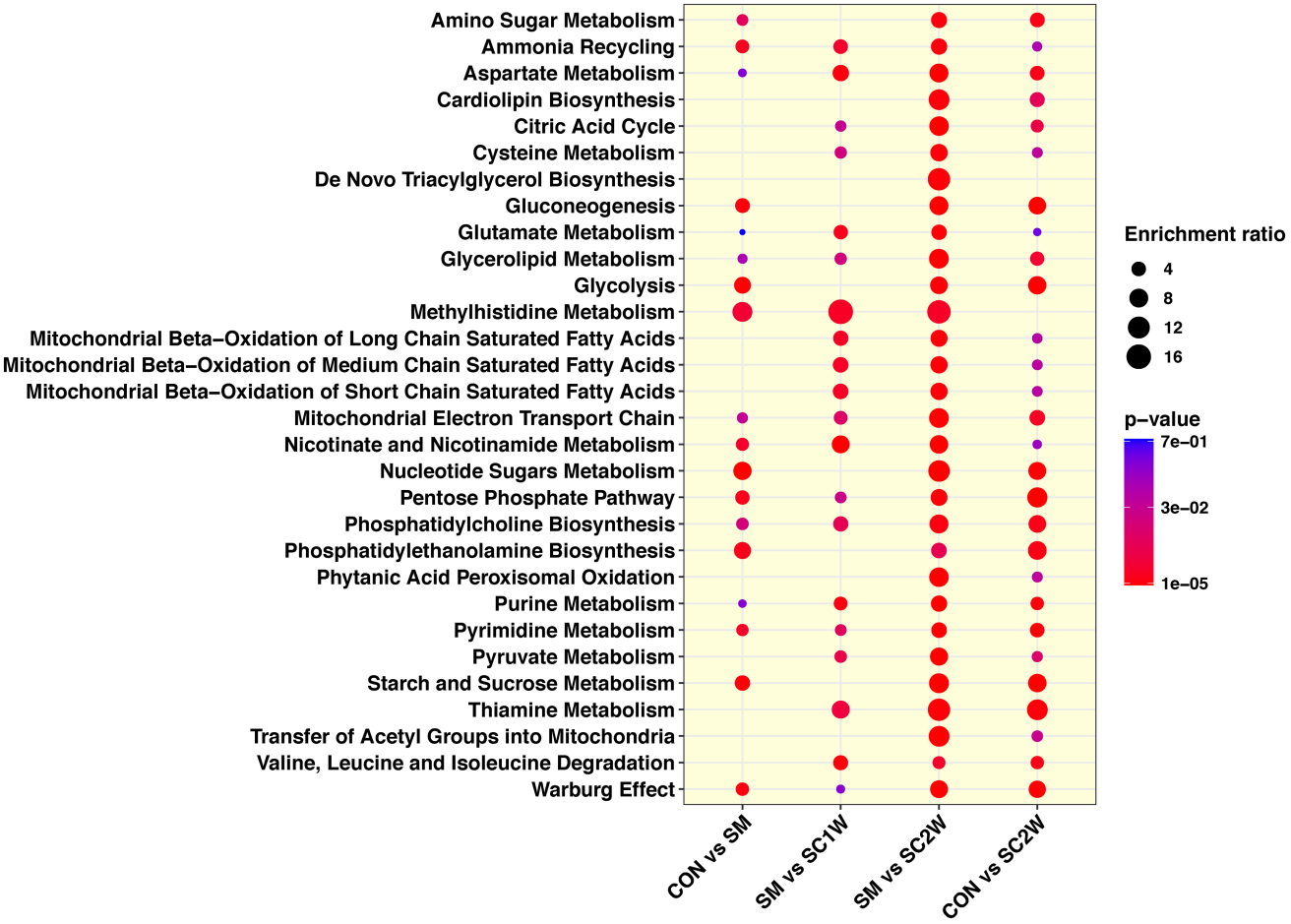
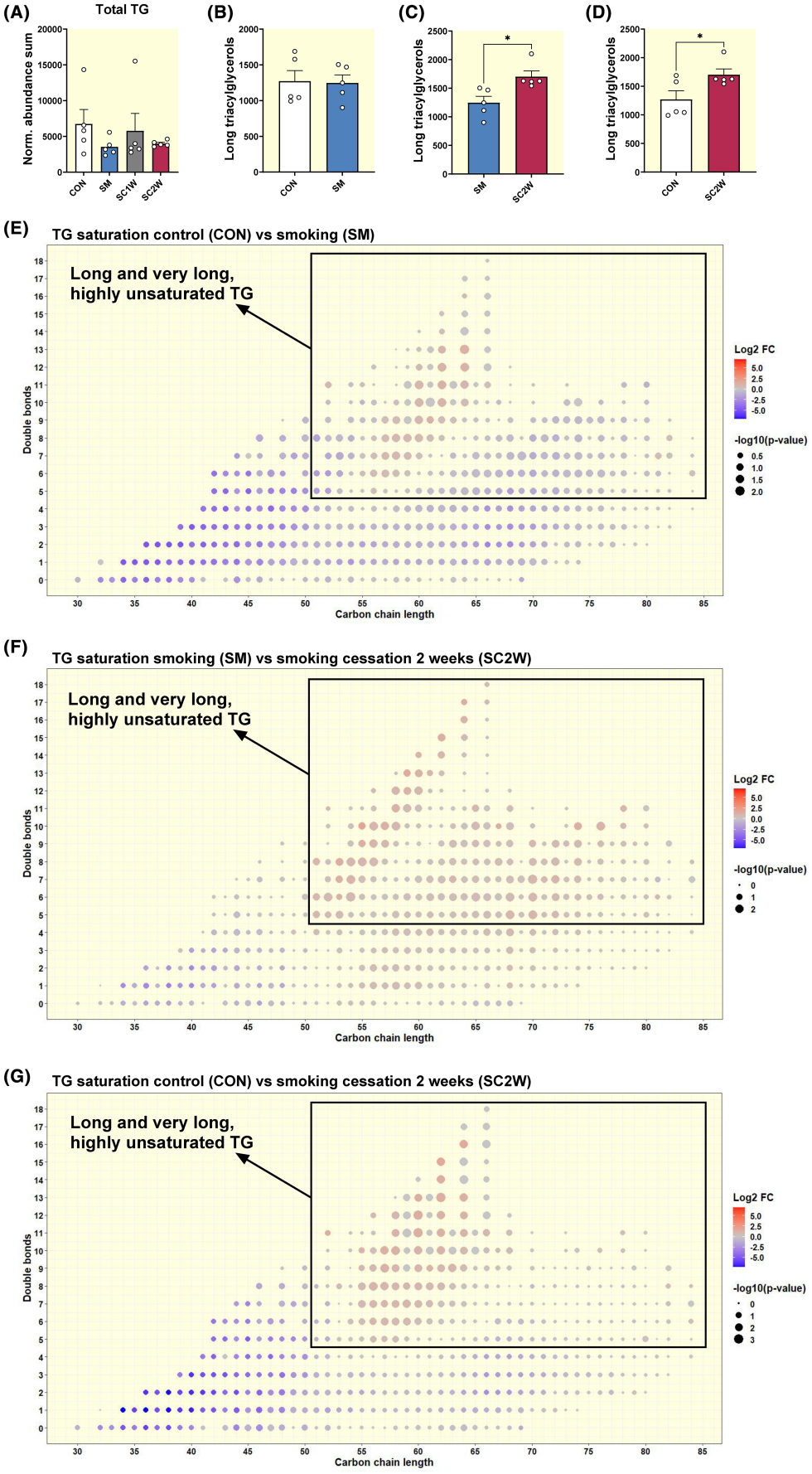


FIGURE 4 Top 30 metabolic pathways altered after smoking and smoking cessation in mice. Enrichment plot depicts several metabolic pathway alterations induced by smoking and smoking cessation. Metabolites were used with variable importance in projection (VIP) score >1.4 for control (CON $n = 5$), smoking (SM $n = 5$), and 1 to 2 weeks of smoking cessation (SC1W $n = 5$ and SC2W $n = 5$). The size of the dot represents enrichment ratio (metabolite count enriched in the pathway), and the color presents significance.

FIGURE 5 Effect of smoking and smoking cessation on triacylglycerols abundance in mice cardiac tissue. (A) Total triacylglycerols concentration in control (CON $n = 5$), smoking (SM $n = 5$), and 1- or 2-week smoking cessation groups (SC1W $n = 5$ and SC2W $n = 5$, respectively). SM versus CON and SC2W versus CON (unpaired two-tailed *t*-test); SC1W versus SM and SC2W versus SM (one-way ANOVA). (B-D) Comparison of long- and very-long-chain highly unsaturated triacylglycerols among control, smoking, and 2 weeks of smoking cessation. Triacylglycerols TG (51:5)–TG (84:18) were chosen for comparison. CON versus SM, SM versus SC2W, and CON versus SC2W (unpaired two-tailed *t*-test). (E-G) Dot-plot illustration of triacylglycerol saturation among control, smoking, and two weeks of smoking cessation. Results are expressed as mean ± SEM.



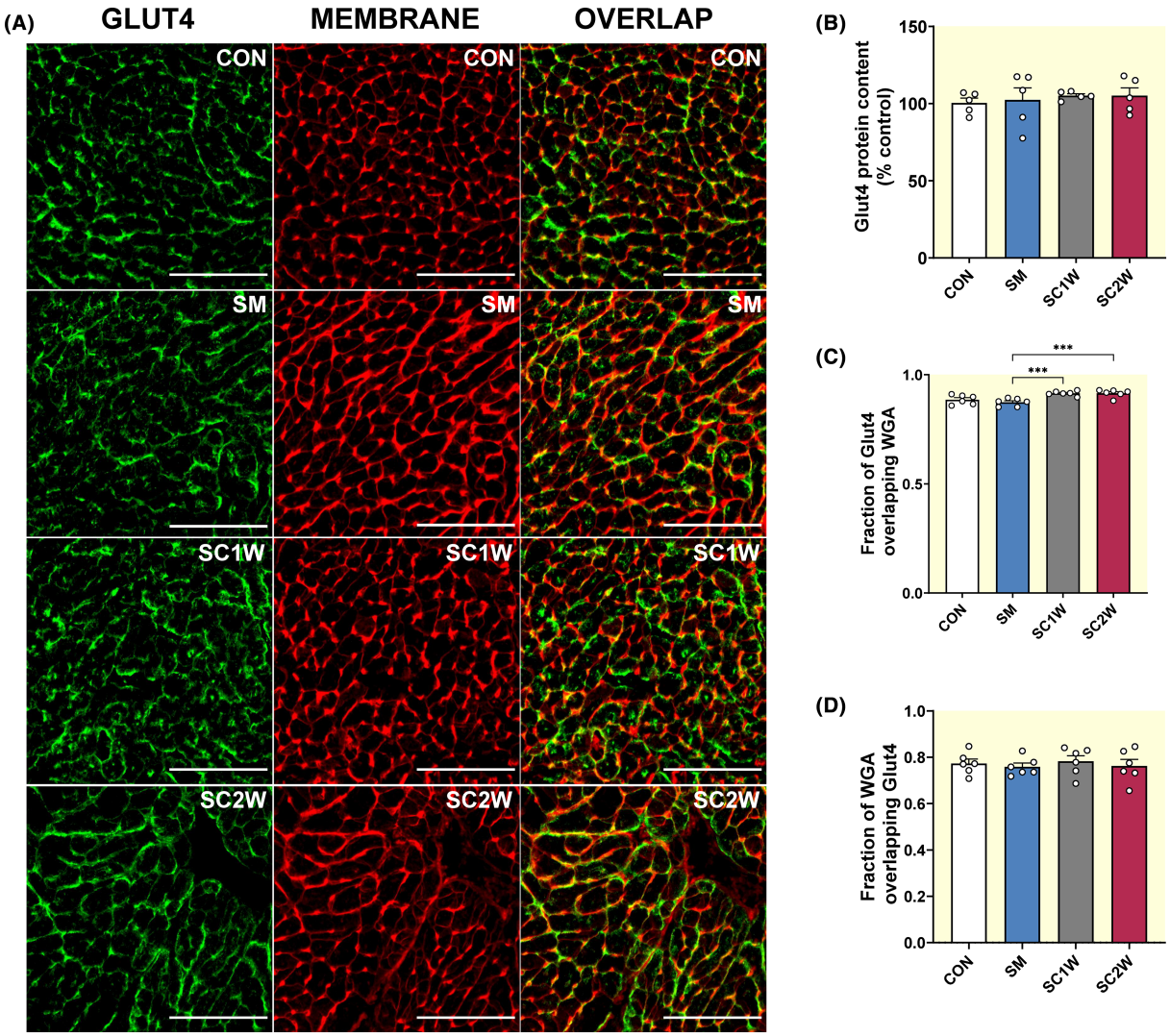


FIGURE 6 More glucose transporter type 4 (GLUT4) translocation at the cell membrane after smoking and smoking cessation in mice. (A) Representative images of heart sections stained with GLUT4 antibody. Left panel shows GLUT4 protein staining with GLUT4 antibody, middle panel shows membrane staining with WGA antibody, and right panel shows overlapping of GLUT4 and membrane staining. Scale bar is 100 μm. (B) Western blot analysis was used to measure protein content of GLUT4 (CON $n=5$, SM $n=5$, SC1W $n=5$, SC2W $n=5$). (C) Fraction of GLUT4 localized at the cell membrane increased after smoke cessation. (D) Fraction of membrane overlapping with GLUT4 in control (CON $n=6$), smoking (SM $n=6$), and 1- or 2-week smoking cessation (SC1W $n=6$, SC2W $n=6$). Results are expressed as mean ± SEM. SM versus SM versus CON and SC2W versus CON (unpaired two-tailed *t*-test); SC1W versus SM and SC2W versus SM (one-way ANOVA).

4.4 | Alterations in intracellular metabolism

Cardiomyocytes exhibit metabolic flexibility[34] and predominantly use fatty acids and glucose for ATP production.[34,35] Our metabolomics results are indicative of a shift away from fatty acid to glucose oxidation during smoking cessation, similar to that observed in cardiovascular diseases,[36] where metabolic inflexibility is observed.[34,35] We observed increased levels of various glycolytic intermediates, suggestive of a larger reliance on glucose oxidation after smoking cessation.[37] This was associated with a higher colocalization of GLUT4 at the plasma membrane after smoking cessation. Our lipidomic analysis confirmed a systematic increase in concentration of various long-chain highly unsaturated fatty acids after 2 weeks of smoking cessation, indicative of reduced fatty acid oxidation. These data confirm a cellular shift in metabolism toward glucose oxidation away from fatty acid oxidation. Why this particularly occurred during smoking cessation remains unknown but is in line with anecdotal evidence of glucose craving after smoking cessation. Whether this is a cardiac-specific adaptation or is associated with changes in whole-body change toward glucose oxidation deserves further study.

We observed long-chain and very-long-chain highly unsaturated triacylglycerols concentrations after 2 weeks of smoke cessation compared to control and smoking. Excess storage of triacylglycerols is associated with insulin resistance and mitochondrial abnormalities in skeletal muscle,[38,39] cardiac hypertrophy,[35] and ultimately a reduced ventricular function.[40] Serum triacylglycerol concentration is also higher in patients with COPD,[41] and serum diacylglycerols and triacylglycerols were negatively associated with skeletal muscle oxidative capacity in patients with severe COPD.[42] Since severity of COPD is negatively associated with plasma sphingolipid concentrations,[43] we also observed lower tissue concentrations of sphingomyelin SM(t33:0) and ceramide Cer(d44:3) in smoking compared to control. Furthermore, there were several ceramides and hexosylceramides with significantly lower concentrations after smoking cessation in comparison to control.

5 | Limitations of the study

Here, we highlight some limitations of this study that could be addressed in future studies. First, we did not measure systolic and diastolic function,[44] or local cardiac blood flow, to link our cellular and molecular alterations to cardiac function. The assessment of whole-body insulin sensitivity or glucose tolerance tests, as well as isotope-labelled substrate analyses, would have provided more depth into the whole-body and cardiac metabolic alterations upon smoking. Electron microscopy imaging of mitochondrial cristae and fragmentation was not performed[39] but could have added important new insights into the structural changes in mitochondrial morphology and function upon cigarette smoking and smoking cessation. Lastly, the contribution of the immune system was understudied, as the time course and exact nature of immune cell infiltration in the heart were something that we could not assess with the current study design.

6 | CONCLUSION

Here, we provide an in-depth analysis of the structural and metabolic alterations in the heart of mice exposed to cigarette smoke and follow the adjustments after smoking cessation, which were not enough to completely recover to the control level. Our cellular and molecular analyses show that smoking reduces maximal mitochondrial respiratory capacity and causes a metabolic shift away from fatty acid oxidation to glucose metabolism. These cellular adaptations are associated with alterations in antioxidant signalling, NAD^+ metabolism, and accumulation of long-chain unsaturated triacylglycerols. Smoking cessation normalized maximal mitochondrial capacity, partly due to increased assembly of mitochondrial supercomplexes. The local infiltration of macrophages confirms a local inflammatory environment that does not normalize after smoking cessation. The observation that cardiac fibrosis, local inflammation, and metabolic alterations remained after 2 weeks of smoking cessation could help to explain the increased risk for atherosclerosis, myocardial infarction, and arrhythmias in ex-smokers. These results contribute to a better understanding of the increased risk of cardiovascular diseases in patients with COPD and open the potential for finding new therapies to protect the heart during smoking and immediately after smoking cessation, as well as to develop new quit-smoking programs.

AUTHOR CONTRIBUTIONS

Rob C. I. Wüst: Conceptualization; investigation; funding acquisition; writing – original draft; writing – review and editing; project administration; supervision; visualization; formal analysis; methodology. **Jekaterina Aid:** Conceptualization; methodology; software; investigation; formal analysis; funding acquisition; visualization; writing – original draft; writing – review and editing. **Ajime Tom Tanjeko:** Conceptualization; investigation; writing – review and editing; project administration. **Jef Serré:** Conceptualization; investigation; writing – review and editing. **Moritz Eggelbusch:** Investigation; formal analysis; visualization; writing – review and editing; methodology. **Wendy Noort:** Investigation; visualization; formal analysis; methodology; writing – review and editing. **Gerard M. J. de Wit:** Methodology; investigation; writing – review and editing. **Michel van Weeghel:** Writing – review and editing; visualization; investigation. **Marju Puurand:** Funding acquisition; writing – review and editing; supervision; conceptualization. **Kersti Tepp:** Funding acquisition; writing – review and editing; supervision; conceptualization. **Ghislaine Gayan-Ramirez:** Conceptualization; methodology; investigation; supervision; project administration; writing – review and editing; funding acquisition. **Hans Degens:** Conceptualization; investigation; methodology; writing – review and editing; project administration; supervision. **Tuuli Käämbre:** Writing – review and editing; funding acquisition; supervision; conceptualization.

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CONFLICT OF INTEREST STATEMENT

The authors do not declare conflicts of interest.

DATA AVAILABILITY STATEMENT

Data and material are available upon request from the corresponding author.

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REFERENCES

1. Lundback B, Lindberg A, Lindstrom M, et al. Not 15 but 50% of smokers develop COPD?—Report from the obstructive lung disease in Northern Sweden studies. *Respir Med*. 2003;97(2):115-122.
2. Axson EL, Ragutheeswaran K, Sundaram V, et al. Hospitalisation and mortality in patients with comorbid COPD and heart failure: a systematic review and meta-analysis. *Respir Res*. 2020;21(1):54.
3. Ahmed AA, Patel K, Nyaku MA, et al. Risk of heart failure and death after prolonged smoking cessation: role of amount and duration of prior smoking. *Circ Heart Fail*. 2015;8(4):694-701.
4. Degens H, Gayan-Ramirez G, van Hees HW. Smoking-induced skeletal muscle dysfunction: from evidence to mechanisms. *Am J Respir Crit Care Med*. 2015;191(6):620-625.
5. Sanders KJ, Kneppers AE, van de Bool C, Langen RC, Schols AM. Cachexia in chronic obstructive pulmonary disease: new insights and therapeutic perspective. *J Cachexia Sarcopenia Muscle*. 2016;7(1):5-22.
6. Wüst RCI, Degens H. Factors contributing to muscle wasting and dysfunction in COPD patients. *Int J Chron Obstruct Pulmon Dis*. 2007;2(3):289-300.
7. Wüst RCI, Morse CI, de Haan A, Rittweger J, Jones DA, Degens H. Skeletal muscle properties and fatigue resistance in relation to smoking history. *Eur J Appl Physiol*. 2008;104(1):103-110.
8. Booth FW, Gordon SE, Carlson CJ, Hamilton MT. Waging war on modern chronic diseases: primary prevention through exercise biology. *J Appl Physiol (1985)*. 2000;88(2):774-787.
9. Gosker HR, Langen RC, Bracke KR, et al. Extrapulmonary manifestations of chronic obstructive pulmonary disease in a mouse model of chronic cigarette smoke exposure. *Am J Respir Cell Mol Biol*. 2009;40(6):710-716.
10. Wüst RC, Jaspers RT, van der Laarse WJ, Degens H. Skeletal muscle capillarization and oxidative metabolism in healthy smokers. *Appl Physiol Nutr Metab*. 2008;33(6):1240-1245.
11. Ajime TT, Serre J, Wüst RCI, et al. Two weeks of smoking cessation reverse cigarette smoke-induced skeletal muscle atrophy and mitochondrial dysfunction in mice. *Nicotine Tob Res*. 2021;23(1):143-151.
12. Darabseh MZ, Maden-Wilkinson TM, Welbourne G, et al. Fourteen days of smoking cessation improves muscle fatigue resistance and reverses markers of systemic inflammation. *Sci Rep*. 2021;11(1):12286.
13. Nogueira L, Trisko BM, Lima-Rosa FL, et al. Cigarette smoke directly impairs skeletal muscle function through capillary regression and altered myofibre calcium kinetics in mice. *J Physiol*. 2018;596(14):2901-2916.
14. Hendriks T, van Dijk R, Alsabaan NA, van der Harst P. Active tobacco smoking impairs cardiac systolic function. *Sci Rep*. 2020;10(1):6608.
15. Jensen BL, Persson PB. Good publication practice in physiology 2021. *Acta Physiol (Oxf)*. 2022;234(1):e13741.
16. Loncar G, Springer J, Anker M, Doehner W, Lainscak M. Cardiac cachexia: hic et nunc. *J Cachexia Sarcopenia Muscle*. 2016;7(3):246-260.
17. Mazzone P, Tierney W, Hossain M, Puvenna V, Janigro D, Cucullo L. Pathophysiological impact of cigarette smoke exposure on the cerebrovascular system with a focus on the blood-brain barrier: expanding the awareness of smoking toxicity in an underappreciated area. *Int J Environ Res Public Health*. 2010;7(12):4111-4126.
18. Oishi Y, Manabe I. Macrophages in inflammation, repair and regeneration. *Int Immunol*. 2018;30(11):511-528.
19. Shi A, Hillege MMG, Wüst RCI, Wu G, Jaspers RT. Synergistic short-term and long-term effects of TGF-beta1 and 3 on collagen production in differentiating myoblasts. *Biochem Biophys Res Commun*. 2021;547:176-182.
20. Eggelbusch M, Shi A, Broeksma BC, et al. The NLRP3 inflammasome contributes to inflammation-induced morphological and metabolic alterations in skeletal muscle. *J Cachexia Sarcopenia Muscle*. 2022;13(6):3048-3061.
21. McDonald LT, Zile MR, Zhang Y, et al. Increased macrophage-derived SPARC precedes collagen deposition in myocardial fibrosis. *Am J Physiol Heart Circ Physiol*. 2018;315(1):H92-H100.
22. Gonzalez A, Lopez B, Ravassa S, San Jose G, Diez J. The complex dynamics of myocardial interstitial fibrosis in heart failure. Focus on collagen cross-linking. *Biochim Biophys Acta Mol Cell Res*. 2019;1866(9):1421-1432.
23. Petersen AM, Magkos F, Atherton P, et al. Smoking impairs muscle protein synthesis and increases the expression of myostatin and MAFbx in muscle. *Am J Physiol Endocrinol Metab*. 2007;293(3):E843-E848.
24. Holecek M. Branched-chain amino acids in health and disease: metabolism, alterations in blood plasma, and as supplements. *Nutr Metab (Lond)*. 2018;15:33.

25. Prisco SZ, Rose L, Potus F, et al. Excess protein O-GlcNAcylation links metabolic derangements to right ventricular dysfunction in pulmonary arterial hypertension. *Int J Mol Sci*. 2020;21(19):7278.
26. Swain JL, Sabina RL, Peyton RB, Jones RN, Wechsler AS, Holmes EW. Derangements in myocardial purine and pyrimidine nucleotide metabolism in patients with coronary artery disease and left ventricular hypertrophy. *Proc Natl Acad Sci U S A*. 1982;79(2):655-659.
27. Yki-Jarvinen H, Virkamaki A, Daniels MC, McClain D, Gottschalk WK. Insulin and glucosamine infusions increase O-linked N-acetyl-glucosamine in skeletal muscle proteins in vivo. *Metabolism*. 1998;47(4):449-455.
28. Kondo T, Hayashi M, Takeshita K, et al. Smoking cessation rapidly increases circulating progenitor cells in peripheral blood in chronic smokers. *Arterioscler Thromb Vasc Biol*. 2004;24(8):1442-1447.
29. Nollet EE, Duursma I, Rozenbaum A, et al. Mitochondrial dysfunction in human hypertrophic cardiomyopathy is linked to cardiomyocyte architecture disruption and corrected by improving NADH-driven mitochondrial respiration. *Eur Heart J*. 2023;44(13):1170-1185.
30. Anwar MR, Saldana-Caboverde A, Garcia S, Diaz F. The Organization of Mitochondrial Supercomplexes is modulated by oxidative stress in vivo in mouse models of mitochondrial encephalopathy. *Int J Mol Sci*. 2018;19(6):1582.
31. Tong D, Schiattarella GG, Jiang N, et al. NAD(+) repletion reverses heart failure with preserved ejection fraction. *Circ Res*. 2021;128(11):1629-1641.
32. Dolle C, Rack JG, Ziegler M. NAD and ADP-ribose metabolism in mitochondria. *FEBS J*. 2013;280(15):3530-3541.
33. Yaku K, Okabe K, Nakagawa T. NAD metabolism: implications in aging and longevity. *Ageing Res Rev*. 2018;47:1-17.
34. Smith RL, Soeters MR, Wüst RCI, Houtkooper RH. Metabolic flexibility as an adaptation to energy resources and requirements in health and disease. *Endocr Rev*. 2018;39(4):489-517.
35. Makrecka-Kuka M, Liepinsh E, Murray AJ, et al. Altered mitochondrial metabolism in the insulin-resistant heart. *Acta Physiol (Oxf)*. 2020;228(3):e13430.
36. McGarrah RW, Crown SB, Zhang GF, Shah SH, Newgard CB. Cardiovascular Metabolomics. *Circ Res*. 2018;122(9):1238-1258.
37. Tulen CBM, Wang Y, Beentjes D, et al. Dysregulated mitochondrial metabolism upon cigarette smoke exposure in various human bronchial epithelial cell models. *Dis Model Mech*. 2022;15(3):dmm049247.
38. Kelley DE, Goodpaster BH. Skeletal muscle triglyceride. An aspect of regional adiposity and insulin resistance. *Diabetes Care*. 2001;24(5):933-941.
39. Eggelbusch M, Charlton BT, Bosutti A, et al. The impact of bed rest on human skeletal muscle metabolism. *Cell Rep Med*. 2024;5(1):101372.
40. Ge F, Hu C, Hyodo E, et al. Cardiomyocyte triglyceride accumulation and reduced ventricular function in mice with obesity reflect increased long chain fatty acid uptake and de novo fatty acid synthesis. *J Obes*. 2012;2012:205648.
41. Xuan L, Han F, Gong L, et al. Association between chronic obstructive pulmonary disease and serum lipid levels: a meta-analysis. *Lipids Health Dis*. 2018;17(1):263.
42. Li R, Adami A, Chang CC, Tseng CH, Hsiai TK, Rossiter HB. Serum Acylglycerols inversely associate with muscle oxidative capacity in severe COPD. *Med Sci Sports Exerc*. 2021;53(1):10-18.
43. Bowler RP, Jacobson S, Cruickshank C, et al. Plasma sphingolipids associated with chronic obstructive pulmonary disease phenotypes. *Am J Respir Crit Care Med*. 2015;191(3):275-284.
44. Wüst RCI, Coolen BF, Held NM, et al. The antibiotic doxycycline impairs cardiac mitochondrial and contractile function. *Int J Mol Sci*. 2021;22(8):4100.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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