ADVANCES IN METABOLIC MECHANISMS OF AGING AND ITS RELATED DISEASES

EDITED BY: Katia Aquilano, Daniele Lettieri Barbato, Raffaella Faraonio and Natascia Ventura

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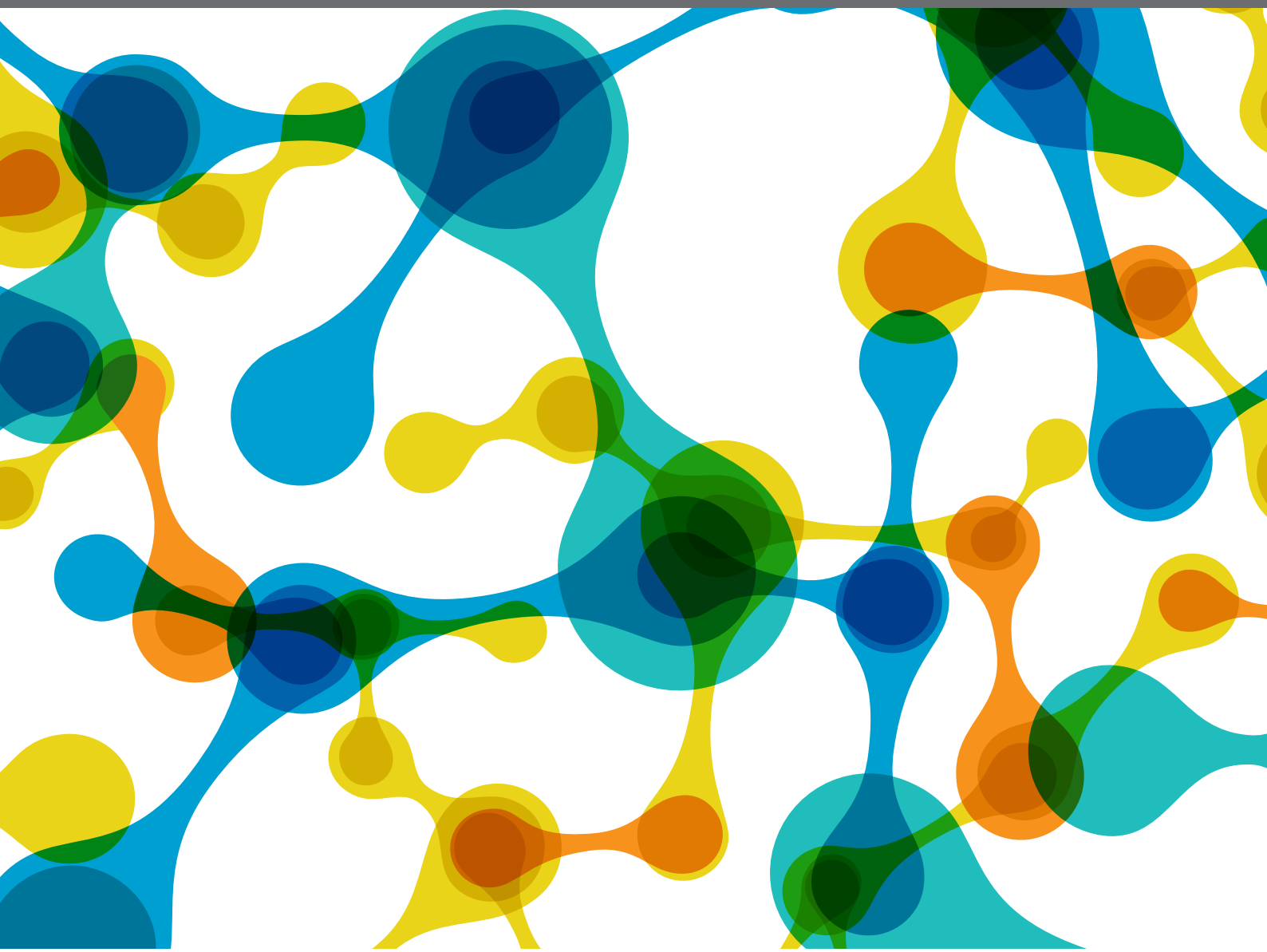

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ADVANCES IN METABOLIC MECHANISMS OF AGING AND ITS RELATED DISEASES

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EDITORIAL
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Editorial: Advances in Metabolic Mechanisms of Aging and Its Related Diseases

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Keywords: senescence, glucose metabolism, lipid metabolism, mitochondria, oxidative stress, aminoacid metabolism, signaling

Editorial on the Research Topic

Advances in Metabolic Mechanisms of Aging and Its Related Diseases

Aging is a dynamic and irreversible physiological process, which leads to the progressive decline of biological functions involving all tissues and organs, due to a variety of endogenous and environmental factors. Metabolic changes are some of the hallmarks of aging and the alteration of metabolic pathways may accelerate the process and the onset of age-related diseases.

The metabolic bases of aging are lesser-known and far from being explained. There is evidence, however, that points to the role of mitochondria as key modulators of the aging process. These organelles are involved in a plethora of biological functions that affect aging both directly and indirectly. On the one hand, aging is characterized by bioenergetic failure and is accompanied by the impairment of mitochondrial dynamics and mitochondrial quality control pathways, leading to excessive production of reactive oxygen species (ROS), which are harmful to cell proteins, lipids, and DNA. On the other hand, partial reduction of mitochondrial function, as well as mild oxidative stress, have been shown to, paradoxically, promote health span across species, indicating that mitochondrial activity must be finely tuned for optimization of the aging process.

Among other organs, the brain is the most sensitive to the aging process because it requires a high amount of energy. In this Research Topic, Messina et al. comprehensively review current knowledge about the involvement of mitochondria in age-related brain dysfunctions (e.g., dementia and neurodegenerative diseases), focusing on molecular mechanisms assuring mitonuclear communications, the integrity of the mitochondrial lattice, and the cross-talk with other cellular organelles. They describe how such mechanisms become dysfunctional and affect neuronal plasticity and long-term memory storage.

The brain is a unique organ with a highly heterogeneous cellular composition and a more complex bioenergetic system than peripheral tissues. Such complexity derives not only from the variety of the residing cell types but also from the intricate network of their cellular communications. In the context of brain aging, Qi et al. summarize the different metabolic phenotypes of brain cells and describe how their metabolism is mutually modulated to maintain brain bioenergetic homeostasis. In particular, they focus on the astrocyte-neuron metabolic interactions through the lactate shuttle and the coordination of the lipid metabolism.

Cellular senescence is a process characterized by a stable and irreversible cell growth arrest and represents another well-established hallmark of the aging process. Environmental challenges such as ionizing radiation induce cellular senescence and accelerate aging. For this reason, in recent years particular attention has been given to the study of the effects of space missions on astronauts as they

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are exposed to cosmic rays and are more prone to develop many age-related diseases (e.g., cataract, heart and brain diseases). In this regard, Giovanetti et al. describe the main sources of radiation in space and their deleterious effects and highlight the importance of considering space flights as a precious opportunity to study the mechanisms underlying aging in humans.

Mounting evidence shows that senescent endothelial cells (ECs) can play a role in instigating the morphological and biochemical changes that accompany the vascular dysfunction typical of many age-related diseases including cardiovascular diseases (CVD). After giving a comprehensive review of the EC metabolism and metabolic reprogramming in cellular senescence, in their review, Sabbatinelli et al. emphasize that the metabolic changes occurring in senescent ECs are critical for the setting of pro-inflammatory states characterizing CVD diseases and that a deeper understanding of the factors involved in the metabolic reshaping of ECs is pivotal for finding effective therapeutic or preventive strategies to treat age-related diseases. Nitti et al. provide an up to date and critical overview of the molecular pathways by which heme oxygenase 1 (HMOX1), an enzyme involved in heme catabolism, and bilirubin, the heme degradation product, protect vascular integrity. In particular, due to these antioxidant and anti-inflammatory roles, the authors underline the importance of moderately increasing the plasma concentration of bilirubin to counteract CVD diseases. Senescent cardiomyocytes show decreased NAD^+ levels and an increase of senescent cardiomyocytes positively correlates with cardiac diseases. Wang L.-F. et al. report that CD38, the major hydrolase for degradation of NAD^+, is causally involved in the senescence program as this enzyme is increased in senescent cardiomyocytes and its ablation alleviates the D-galactose-induced myocardial cell senescence and oxidative stress by the activation of the NAD^+/Siruin1 signaling pathway.

Brinkmann et al. and Faienza et al. focus their attention on the other two proteins that seem to play a key role in aging pathophysiology by coordinating the communication between the nucleus and mitochondria. In particular, Brinkmann et al. provide a systematic review of the existing literature about the Aryl-hydrocarbon Receptor (AhR), an evolutionarily conserved transcription factor involved in the regulation of biological responses to environmental planar aromatic hydrocarbons (e.g., dioxin, flavonoids), which was recently shown by the authors to have pro-aging effects across species. In their review, they try to reconcile the contradictory studies indicating either its pro- or anti-aging effects by a possible AhR-mitochondrial cross-talk to shed light on this factor as a means of developing anti-aging strategies. In their opinion paper, Faienza et al. draw the attention to TRAP1, a mitochondrial chaperone that is a member of the heat shock protein 90 family, involved in the maintenance of mitochondrial homeostasis. Advances in the comprehension of the post-translational modifications (i.e., *S*-nitrosylation) orchestrating TRAP1 activity and its effect on gene expression and mitochondrial physiology with relevance to aging and age-related diseases are described in detail.

In their original paper, Wang J. et al. give evidence of the involvement of altered branched-chain amino acid (BCAA) catabolism in dysfunctional glycemic control, which is typical of type 2 diabetes. By using mice with ablation of mitochondrial protein phosphatase 2C, which is involved in the BCAA degradation pathway, they show the occurrence of mild BCAA catabolic defects leading to a decrease in body weight and better tolerance of glucose in lean, but not in normal or obese animals, mainly due to amelioration of liver glucose metabolism.

The liver is a complex metabolic organ that is fundamental for maintaining metabolic health by regulating systemic lipid and glucose metabolism and by protecting from xenobiotic and endobiotic stress. For this reason, the alteration of liver function during aging increases our susceptibility to age-related diseases. Carotti et al. provide original contributions to this Research Topic, obtained both from experimental models and patients affected by non-alcoholic fatty liver disease. The authors show that liver steatosis is caused by the impairment of lipid degradation through lipophagy, which occurs to an extent that is tightly correlated with disease severity and progression.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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ORIGINAL RESEARCH
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CD38 Deficiency Alleviates D-Galactose-Induced Myocardial Cell Senescence Through NAD$^+$/Sirt1 Signaling Pathway

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Our previous research showed that CD38 played vital roles in Ang-II induced hypertrophy and high fat diet induced heart injury. However, the role of CD38 in heart aging is still unknown. In the present study, we reported that CD38 knockdown significantly protected cardiomyocytes from D-galactose (D-gal)-induced cellular senescence. Cellular senescence was evaluated by β-galactosidase staining, the expressions of genes closely related to aging including p16 and p21, and the ROS production, MDA content and the expressions of oxidant stress related genes were examined by biochemical analysis, Western blot and QPCR. Our results showed that the expression of CD38 was increased in H9c2 cells after D-gal treatment and the expressions of NAMPT and Sirt1 were downregulated in heart tissue from old mice. CD38 knockdown significantly reduced the number of SA-β-gal-positive cells and the expressions of p16 and p21 in H9c2 cells with or without D-gal treatment. The acetylation level of total protein was decreased in CD38 knockdown group, but the expression of Sirt3 was increased in CD38 knockdown group treated with D-gal. In addition, knockdown of CD38 significantly attenuated D-gal induced ROS production, MDA content and NOX4 expression in the cells. Inhibition Sirt1 partially reversed the effects of CD38 knockdown on D-gal induced senescence and oxidative stress. Furthermore, NAD$^+$ supplementation reduced D-gal induced cellular senescence, ROS production and MDA content. The expression of SOD2 was increased and the NOX4 expression was decreased in H9c2 cells after NAD$^+$ supplementation. Taken together, our results demonstrated that CD38 knockdown alleviated D-gal induced cell senescence and oxidative stress via NAD$^+$/Sirt1 signaling pathway.

Keywords: CD38, D-galactose, oxidative stress, heart senescence, NAD$^+$

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INTRODUCTION

Senescence is a state of irreversible cellular arrest which is regulated by heredity and environmental factors. The characteristics of aging are involved in various aspects including genomic instability, telomere attrition, mitochondrial dysfunction, ROS accumulation and so on (Lopez-Otin et al., 2013). In developed countries, aging is the biggest risk factor for the leading causes of death,

and the incidence of cardiac disease increases dramatically with age (Kaeberlein, 2013). The data from American Heart Association in 2014 showed that the incidence of cardiovascular diseases for 60 to 79 years of age is >70% and for >80 years of age is >80% in America (Go et al., 2014). The alternations associated with cardiac aging mainly included four levels: functional, structural, cellular and molecular (Steenman and Lande, 2017). Mitochondrial dysfunction and oxidative stress had been known as important factors in heart aging. Emerging evidences revealed that mitochondrial production of ROS was significantly increased in the heart with advanced age (Judge et al., 2005; Lesnefsky et al., 2016; Martin-Fernandez and Gredilla, 2016). Therefore, it is important to find efficient methods to inhibit oxidative stress and improve mitochondrial function, then delaying senescence of heart.

Nicotinamide dinucleotide (NAD^+) is a key cofactor for maintaining the cellular energy metabolism. Several studies reported that the content of NAD^+ was declined during aging and senescence (Mouchiroud et al., 2013; Verdin, 2015; Zhu et al., 2015). Accumulative evidences revealed that NAD^+ supplementation or other approaches that can restore NAD^+ levels were highly protective during aging (Belenky et al., 2007; Mouchiroud et al., 2013; Mills et al., 2016; Katsyuba et al., 2018). CD38 is a major hydrolase for degradation of NAD^+, and expressed in many tissues. It has been founded that the content of NAD^+ in heart and brain tissues was increased significantly in CD38 deficiency mice than wild-type mice (Aksoy et al., 2006). Recently, a study showed that CD38 gradually increased in tissues of liver, muscle and adipose during aging, and the involvement of CD38 in aging might be in part due to down-regulated Sirt3 activity (Camacho-Pereira et al., 2016). Besides, it was reported that several metabolic features of aging were mitigated with a potent and specific inhibitor of CD38 by reversing tissue NAD^+ decline (Tarrago et al., 2018). Sirtuins were class III histone deacetylases, which used NAD^+ as substrate. A number of evidences indicated that Sirtuin family had beneficial effects against age-associated damage and oxidative stress (Ho et al., 2009; Qiu et al., 2010; Someya et al., 2010; Tasselli et al., 2017). Our previous study showed that CD38 deficiency had protective roles in many cardiac diseases including ischemia/reperfusion injury, cardiac hypertrophy induced by Ang-II and lipid overload-induced heart injury (Guan et al., 2016; Guan et al., 2017; Wang et al., 2018a). However, whether CD38 affects heart aging and the mechanisms remains unknown.

In the current study, in order to test the roles of CD38 in heart aging, D-galactose (D-gal) was used to establish an aging cell model with CD38 knockdown H9c2 stable cell line. Our results showed that D-gal promoted cardiomyocytes senescence and increased ROS production, and the expression of CD38 was up-regulated in senescent cardiomyocytes, while the expressions of NAMPT and Sirt1 were down-regulated in old mice heart. CD38 knockdown could attenuate myocardial cell aging and oxidative stress induced by D-gal, while Sirt1 specific inhibitor EX-527 reversed the effects of CD38 deficiency on senescence and oxidative stress. In addition, our results revealed that the effects could be rescued by NAD^+, suggesting that the NAD^+/Sirt1 signaling may play important roles in myocardial cells aging induced by D-gal.

MATERIALS AND METHODS

Chemicals and Antibodies

D-Galactose (Cat. No: G5388), NAD^+ (Cat. No: N5755) and EX-527 (Cat. No: E7034) were purchased from Sigma-Aldrich (St. Louis, MO). Antibodies against p16, p21, NAMPT, NOX4, and GAPDH were purchased from Abcam. Acetylated-Lysine, LC3B, Sirt3, and SOD2 antibodies were purchased from CST. Antibody against Sirt1 was purchased from Millipore. CD38 antibody was from R&D Systems.

Animals

Male C57BL/6 mice were used in our experiment. The mice were fed with standard diet chow and housed in an animal room with a 12-h: 12-h light/dark cycle under controlled environment ($22 \pm 3°C$, 50–60% relative humidity). Two to 3 month-old mice were used as the young group and 11- to 12-month-old mice as old group. All animals were treated according to the "guidelines for the care and use of experimental animals" of Nanchang University. All experimental procedures were approved by the Ethics Committee of Nanchang University, and the experiments were conducted according to the approved guidelines.

Cell Culture and Treatment

H9c2 cells (ATCC) were regularly cultured in high-glucose DMEM containing 10% fetal bovine serum, 100 U/ml penicillin and 100 mg/ml streptomycin at 37°C in a humidified incubator under 5% CO2. The CD38 knockdown H9c2 cell line was prepared as previously described in reference (Guan et al., 2016). When the cells were about 80% confluence, the cells were treated with D-Galactose or combined with Sirt1 specific inhibitor EX-527 (25 μM) for 48 h, then the cells were harvested for further experiments. When it comes to NAD^+ treatment, the H9c2 cells were treated with D-Galactose for 24 h, then cells were incubated with NAD^+ (1.0 mM) for another 24 h before analysis. H9C2 stable cell line with knockdown of CD38 was prepared in our laboratory as previously described (Guan et al., 2016). The NC was defined as siRNA control H9C2 stable cells which were transfected with scramble control siRNA and selected with 1 μg/mL puromycin.

SA-β-Gal Staining

To evaluate myocardial cell senescence induced by D-galactose, senescence-associated β-galactosidase (SA-β-gal) staining was performed according to the instruction. Briefly, after D-galactose treated, the cells were washed with phosphate buffered saline (PBS) and fixed with 4% paraformaldehyde for 15 min at room temperature. After washing three times with PBS, the cells were incubated in SA-β-gal staining solution overnight at 37°C. Then the staining cells were observed under light microscope, and the senescent cells were blue color. The experiment was repeated three times in each group.

ROS Detection

The content of ROS production in cells was examined using H2DCF-DA (Sigma-Aldrich). Briefly, the cells underwent corresponding treatment, then were washed with PBS. About 1×10^6 cells were collected and incubated with 10 μM H2DCF-DA for 30 min at 37°C with an atmosphere of 5% CO_2 away from light. Then the cells were washed with PBS and the fluorescence was detected with automatic microplate reader at wavelengths of 488 nm (excitation) and 520 nm (emission).

Measurement of Peroxidation Levels

The content of MDA in cells was detected using MDA Assay Kit (Dojindo, Mashiki-machi, Japan) according to the manufacturer's instructions. Protein concentration in lysates was determined by BCA Protein Assay Kit (Pierce). All experiments were performed at least three times.

Total RNA Extraction and Real-Time RT-PCR

Total RNA from myocardial cells and heart tissue was isolated using Trizol reagent (Invitrogen) according to the manufacturer's protocol. The concentration of RNA was measured by Nano Drop 2000. Then the RNA was experienced reverse transcription with Takara high capacity cDNA synthesis kit. Relative expression of mRNAs was measured by real-time PCR with SYBR Premix Ex Taq^{TM} II (Takara) in the ABI-ViiA7 PCR machine. All PCR reactions were performed in triplicate. The following primer pairs were used: Rat CD38, 5-CTGCCAGGATAACTACCGACCT-3 (Forward) and 5-CTTTCCCGACAGTGTTGCTTCT-3 (Reverse); GAPDH, 5-AGCCAAAAGGGTCATCATCT-3 (Forward) and 5-GGGGCCATCCACAGTCTTCT-3 (Reverse); Mouse p16, 5- CGGGGACATCAAGACATCGT-3 (Forward) and 5- GCCGGATTTAGCTCTGCTCT-3 (Reverse); Mouse NAMPT, 5-TCGGTTCTGGTGGCGCTTTGCTAC-3 (Forward) and 5- AAGTTCCCCGCTGGTGTCCTATGT-3 (Reverse).

Western Blot Analysis

Total protein was prepared with RIPA Lysis Buffer (Thermo Fisher). Then the lysates were centrifuged at 12,000 rpm for 15 min at 4°C. The protein concentration was determined by BCA Protein Assay Kit (Pierce). A total of 30 μg of each protein sample was resolved by SDS-PAGE and transferred to PVDF membranes and then the membranes were blocked with 5% non-fat milk for 1 h and then incubated overnight at 4°C with indicated antibodies. The membranes were then washed three times with TBST and then incubated with horseradish peroxidase- (HRP-) conjugated anti-rabbit or anti-mouse secondary antibodies. At last, the proteins were

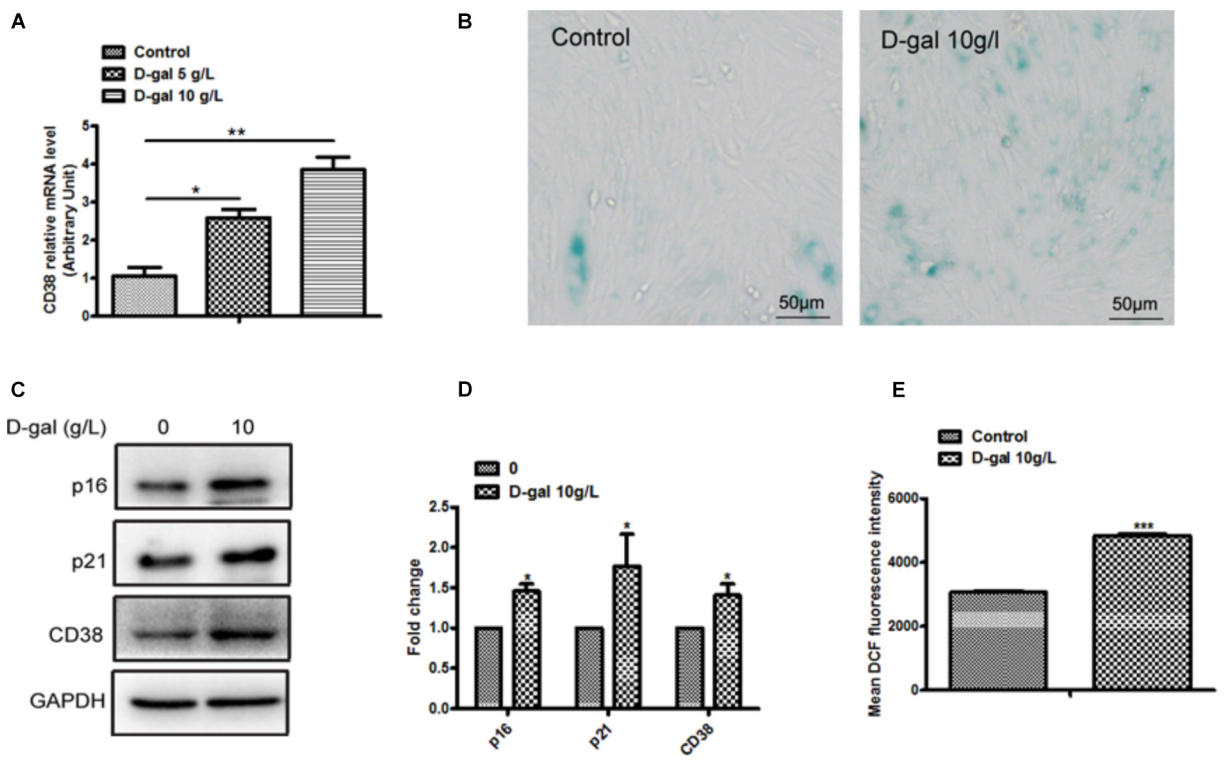

FIGURE 1 | Myocardial cells senescence and ROS production were increased by D-gal. **(A)** The expression of CD38 by real-time PCR analysis in H9c2 cells treated with different concentrations of D-gal. **(B)** SA-β-gal staining in H9c2 cells treated with D-gal (10 g/L). **(C)** The images of senescence marker p16, p21 and CD38 protein by Western blot analysis in H9c2 cells treated with D-gal (10 g/L). **(D)** Quantitative analysis of p16, p2,1 and CD38 protein level from western blot bands. **(E)** The mean fluorescence intensities of ROS production were quantitatively analyzed in H9c2 cells treated with D-gal. Data are shown as mean ± SEM, $^*p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$, $n = 3$ per group.

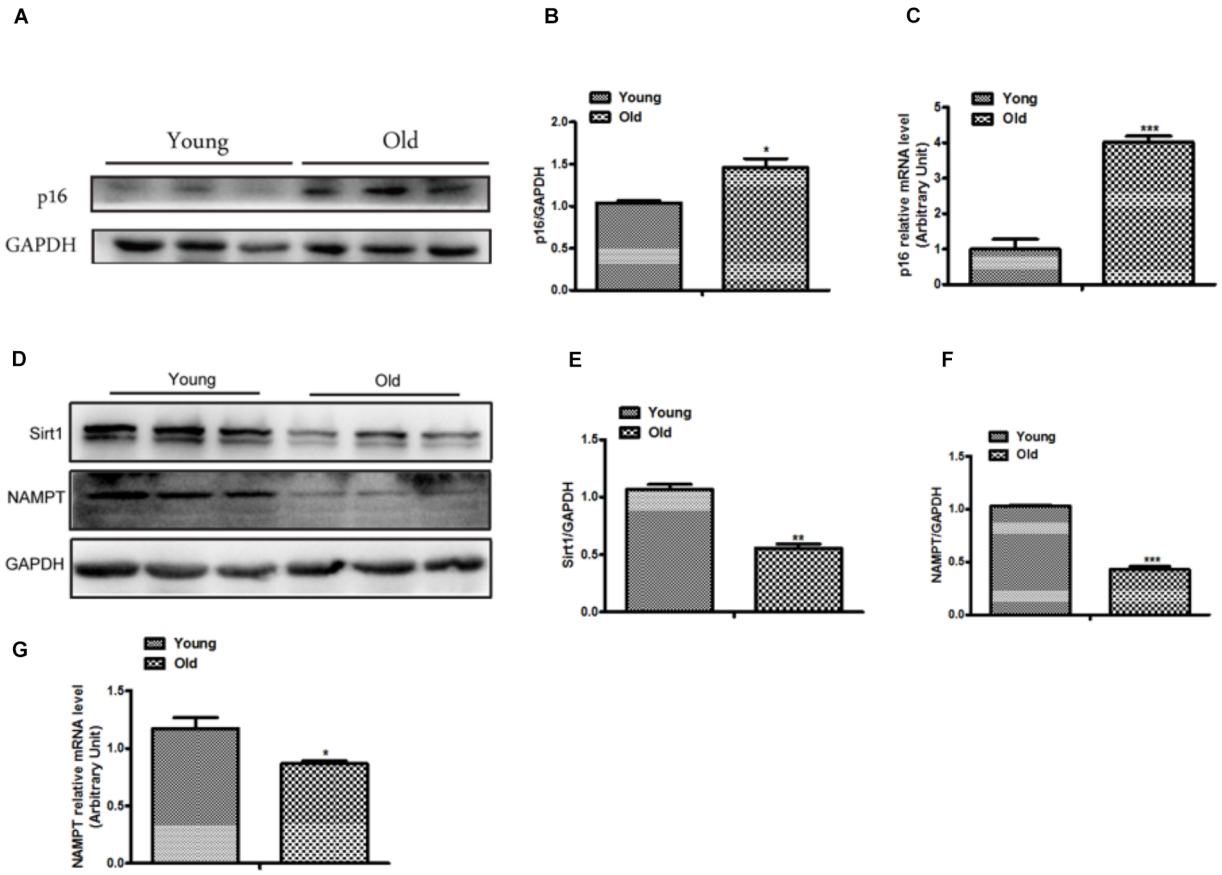

FIGURE 2 | The expression of p16 was increased and the expressions of Sirt1 and NAMPT were decreased in heart tissue from old mice. The western blot image **(A)** and the quantitative analysis **(B)** of p16 protein were determined in heart tissue from old and young mice. **(C)** The mRNA expression of p16 was determined by qPCR in in heart tissue from old and young mice. The Western blot images **(D)** and the quantitative analysis **(E,F)** of the Sirt1 and NMAPT proteins were determined in heart tissue from old and young mice. **(G)** The mRNA expression of NAMPT was determined by qPCR. The data are expressed as the mean ± SEM. from three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

detected with enhanced chemiluminescence (ECL) (Thermo Fisher). GAPDH was used as the internal control.

Statistical Analysis

All experiments were performed at least three times. Data are presented as means ± SE. And statistical analysis was performed with SPSS (Statistical Package for the Social Sciences) 19.0 software using Student's *t*-test. Statistical significance was set at $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

RESULTS

CD38 Expression and the Senescence Were Significantly Increased in D-Gal Induced Myocardial Cells

To explore the potential role of CD38 in heart aging, we first examined the CD38 expression in H9c2 cells treated with D-gal. The results showed that the expression of CD38 was markedly increased in H9c2 cells after D-gal treatment (**Figures 1A,C**). Moreover, to investigate the effects of D-galactose on myocardial cell senescence, we used SA-β-Gal staining to evaluate cellular senescence. The staining results showed that 10 g/L D-gal remarkably increased SA-β-gal-positive cells compared with control group (**Figure 1B**). Furthermore, we found the expressions of senescence marker including p16 and p21 were significantly increased in the H9c2 cells treated with D-gal (**Figures 1C,D**). These results suggested that 10 g/L D-gal could promote cellular senescence, and the CD38 expression was increased in D-gal treated cells. Besides, we also found that the content of ROS production has a significant increase in H9c2 treated with D-gal (**Figure 1E**). All the results indicated that D-gal also increased oxidative stress of myocardial cells.

Cellular Senescence Was Increased and Sirt1 Signaling Pathway Was Inhibited in Heart Tissue of Old Mice

To further confirm whether cellular senescence was increased in heart tissue of old mice, we evaluated the senescence in heart tissue from mice approximately one year old. The results

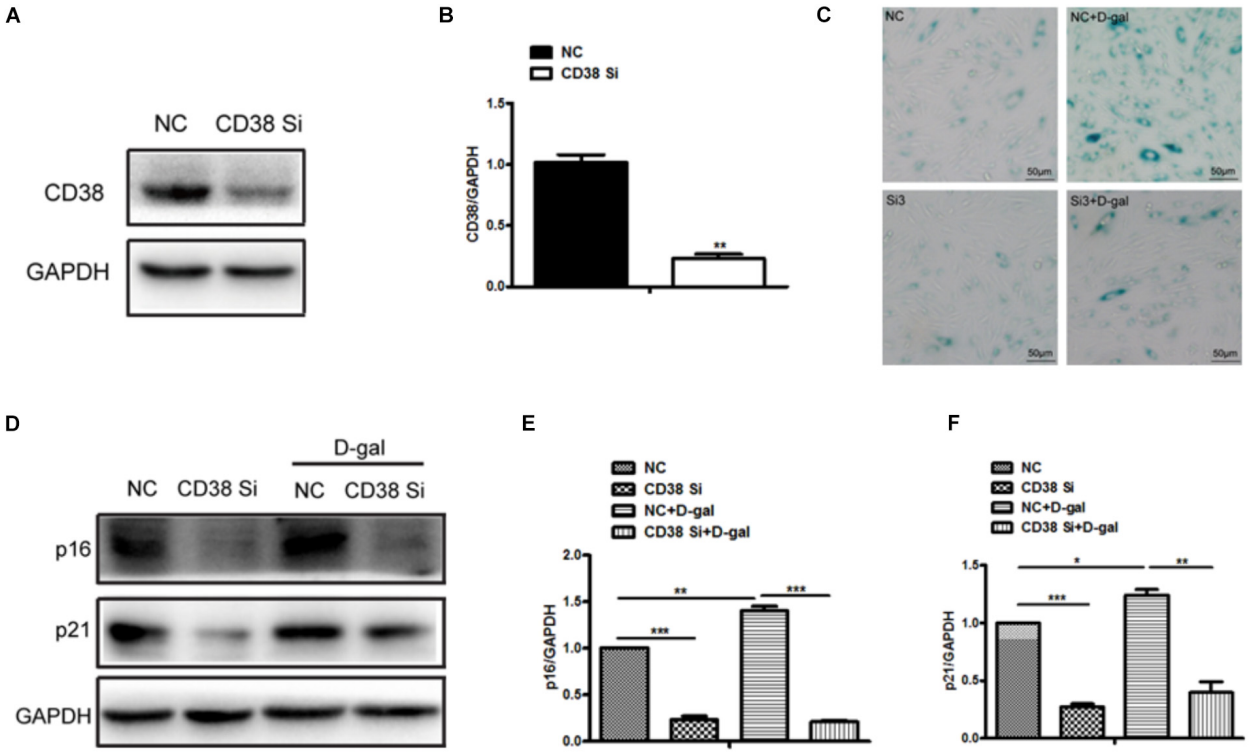

FIGURE 3 | CD38 knockdown decreased D-gal induced myocardial cells senescence. **(A)** The CD38 protein expression was determined by western blot **(A)** and the quantitative analysis **(B)** in the CD38 knockdown stable H9c2 cell lines. **(C)** SA-β-gal staining in CD38 knockdown H9c2 cells treated with or without with D-gal (10 g/L). **(D)** The Western blot images **(D)** and the quantitative analysis **(E,F)** of senescence marker p16 and p21 protein were determined in CD38 knockdown H9c2 cells treated with or without with D-gal (10 g/L). The data are expressed as the mean ± SEM. from three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

suggested that the expression of p16 has a remarkable increase in old group compared with young group (**Figures 2A,B**), and the mRNA level of p16 was consistent with the protein (**Figure 2C**). Moreover, the proteins related to Sirt1 signaling pathway were altered between old and young mice, as shown in **Figures 2D–G**, the expressions of Sirt1 and NAMPT which has been considered as the pivotal rate-limiting enzyme in the mammalian NAD^+ salvage pathway, were significantly decreased in old mice. Moreover, we also found the expression of Sirt1 was decreased in D-gal induced cellular senescence (**Supplementary Figure S1B**). Taken together, our results demonstrated that cellular senescence was increased and the Sirt1-mediated signaling pathway was inhibited in old mice.

CD38 Knockdown Attenuated D-Gal Induced Myocardial Cells Senescence

In order to further elucidate the roles of CD38 in myocardial senescence induced by D-gal *in vitro*, we examined the effects of CD38 on D-gal-induced senescence using CD38 knockdown H9c2 stable cell lines. The interference efficiency of CD38 was approximately 90% by western blotting analysis (**Figures 3A,B**). Furthermore, the SA-gal-positive cells were markedly decreased in CD38 knockdown group compared with control group after D-gal treatment using SA-β-Gal staining (**Figure 3C**). And in CD38 knockdown H9c2 cells, the senescence was aggravated after treated with D-gal combined with Sirt1 specific inhibitor EX-527 (**Supplementary Figure S1C**). In addition, we found the expressions of senescence marker p16 and p21 were reduced in CD38 knockdown H9c2 cells with or without D-gal stimulation (**Figures 3D–F**). These results indicated that CD38 knockdown improved D-gal induced myocardial cells senescence and inhibition of Sirt1 partially reversed the effects of CD38 deficiency on senescence *in vitro*.

CD38 Knockdown Decreased D-Gal Induced Myocardial Cells Oxidative Stress

Aging is often accompanied by an increase in oxygen free radicals. Besides senescence, we also found that oxidative stress was increased after D-gal stimulation. Then we further explore the roles of CD38 in D-gal induced oxidative stress. The results showed that the level of total protein acetylation increased in H9c2 cells under D-gal treatment, while CD38 knockdown reduced it with or without D-gal stimulation (**Figure 4A** and **Supplementary Figure S1E**), indicating that the sirtuin activity might be increased. Beisdes, the expression of Sirt3 which was mainly located in mitochondria was increased in CD38 knockdown cells after D-gal treatment

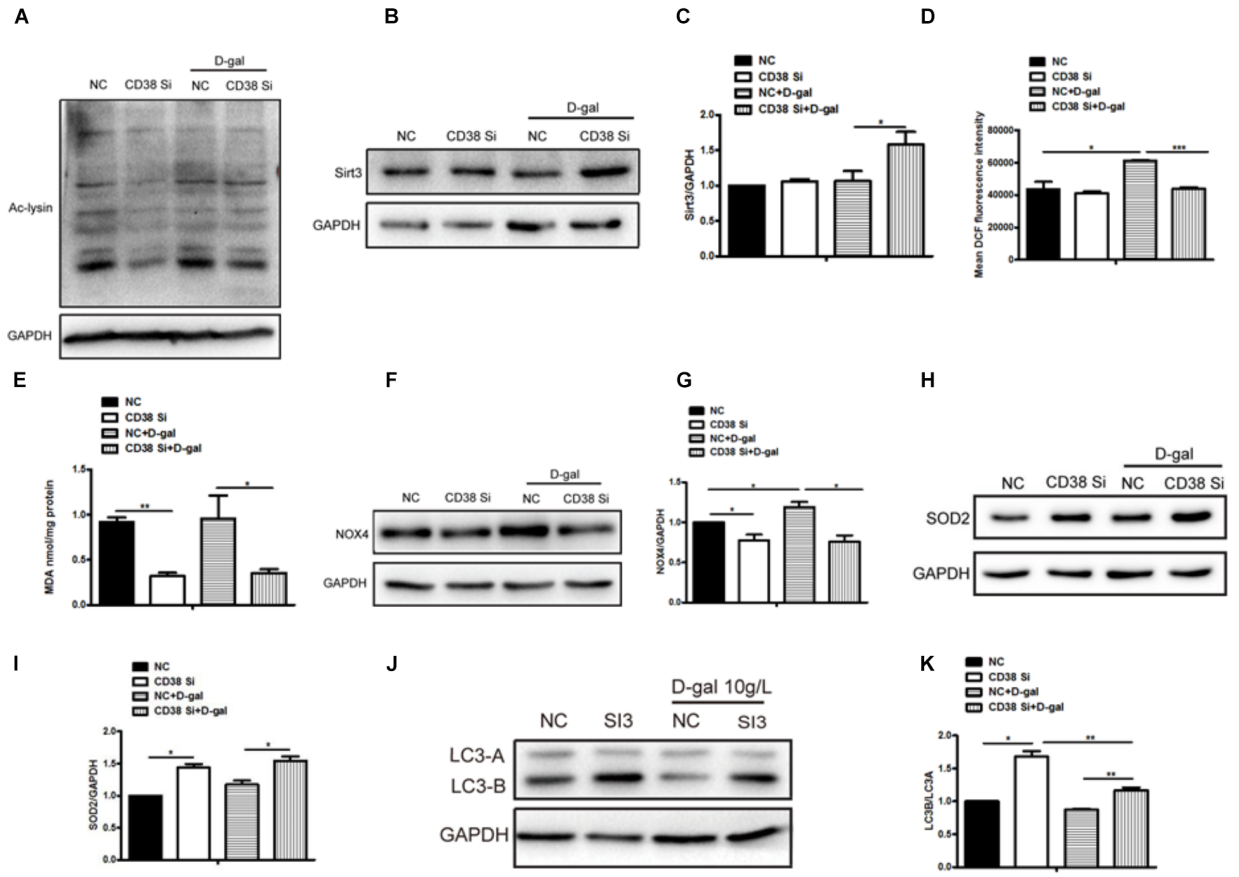

FIGURE 4 | CD38 knockdown decreased D-gal induced oxidative stress in cardiomyocytes. **(A)** The protein acetylation level of CD38 knockdown H9c2 cells treated with or without with D-gal (10 g/L) was analyzed by western blotting with antibody against acetylated-lysine. The western blot images **(B)** and quantitative analysis **(C)** of the protein level of Sirt3 were determined in CD38 knockdown H9c2 cells. **(D)** The mean fluorescence intensities of ROS production were quantitatively analyzed in CD38 knockdown H9c2 cells treated with or without with D-gal. **(E)** The content of MDA was measured in CD38 knockdown H9c2 cells treated with or without with D-gal. The western blot images **(F)** and quantitative analysis **(G)** of the protein level of the oxidative stress related gene NOX4 were determined in CD38 knockdown H9c2 cells. The western blot images **(H)** and quantitative analysis **(I)** of the protein level of the anti-oxidative stress related gene SOD2 were determined in CD38 knockdown H9c2 cells. The western blot images **(J)** and quantitative analysis **(K)** of the protein level of the autophagy associated gene LC3 were determined in CD38 knockdown H9c2 cells. The data are expressed as the mean ± SEM. from three independent experiments. $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$.

(**Figures 4B,C**). Moreover, the ROS production was increased by D-gal, but decreased by CD38 knockdown in cells treated with D-gal (**Figure 4D**), and EX-527 reversed the effects of CD38 deficiency on oxidative stress (**Supplementary Figure S1D**). In addition, we also detected the content of MDA in D-gal induced CD38 knockdown H9c2 cells, and the indicator could reflect the degree of oxidation damage. The results showed that the level of MDA in cells declined in the group of CD38 knockdown with or without D-gal treatment (**Figure 4E**). Besides, the expression of NOX4, which was associated with ROS production, was increased upon treatment with D-gal, but decreased by CD38 knockdown (**Figures 4F,G**). And we found the expression of antioxidant gene SOD2 was upregulated in CD38 knockdown group with or without D-gal stimulation (**Figures 4H,I**). Moreover, the expression of LC3B/LC3A was significantly increased in CD38 knockdown cells, suggesting that the autophagy might be increased (**Figures 4J,K**). The results above indicated that CD38 knockdown decreased D-gal induced oxidative stress, and Sirt1 was responsible for D-gal induced senescence and oxidative stress.

NAD^+ Partially Rescued D-Gal Induced Cellular Senescence and Oxidative Stress

The roles of NAD/Sirt signaling in D-gal induced cellular senescence were examined in H9c2 cells after supplement NAD^+ under D-gal treatment. The SA-β-Gal staining showed that the SA-β-gal-positive cells were reduced in cells treated with D-gal plus NAD^+ compared with D-gal alone (**Figure 5A**). Meanwhile, the ROS production was markedly decreased by supplement of NAD^+ in H9c2 cells treated with D-gal (**Figure 5B**). Besides that, the content of MDA was decreased in cells treatment with NAD^+ (**Figure 5C**). Furthermore, the results showed that NAD^+ increased the expression of SOD2 but decreased the expression of

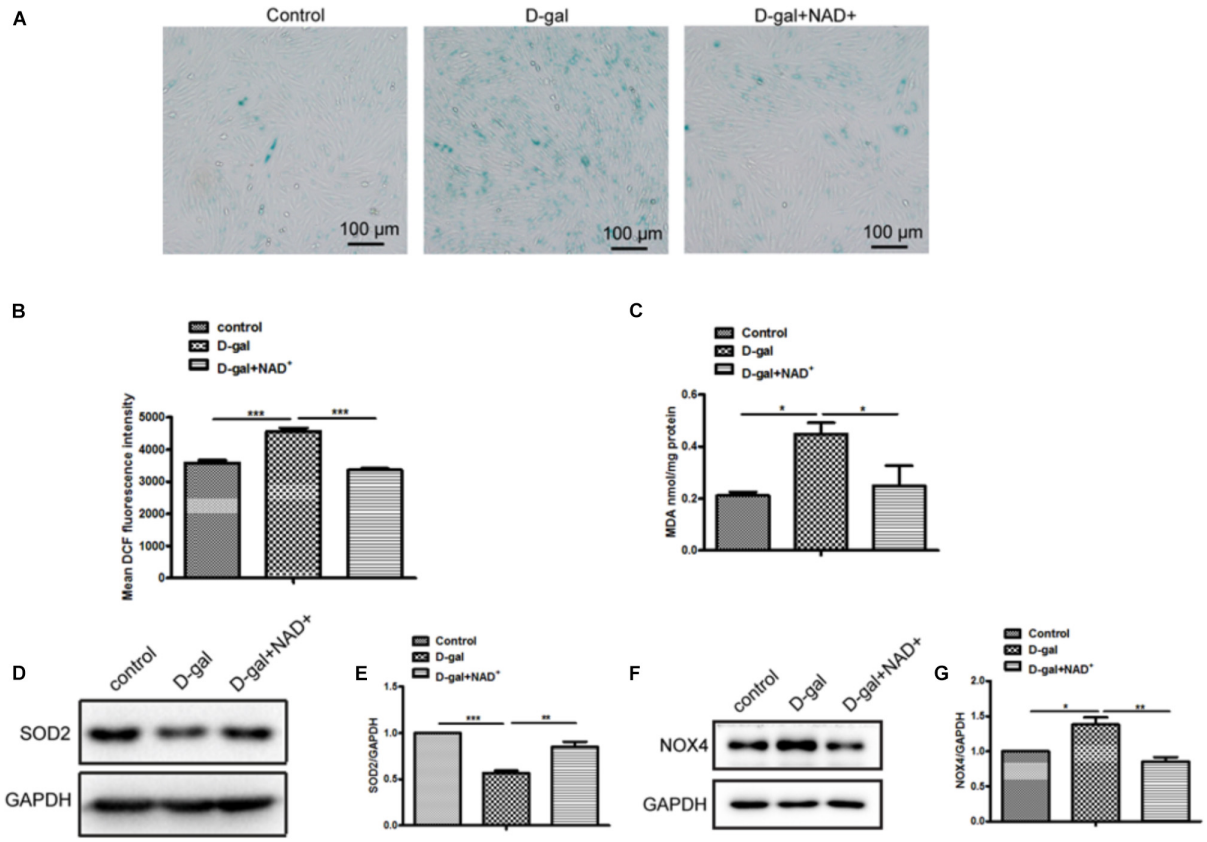

FIGURE 5 | NAD^+ supplement improved D-gal induced senescence and oxidative stress in cardiomyocytes. **(A)** SA-β-gal staining was performed in H9c2 cells treated with D-gal (10 g/L) or combined with NAD^+ (1.0 mM). **(B)** The mean fluorescence intensities of ROS production were quantitatively analyzed in H9c2 cells treated with D-gal (10 g/L) or combined with NAD^+ (1.0 mM). **(C)** The content of MDA was measured in H9c2 cells. The western blot images **(D)** and quantitative analysis **(E)** of the protein level of the oxidative stress related gene SOD2 were determined in H9c2 cells. The western blot images **(F)** and quantitative analysis **(G)** of the protein level of the oxidative stress related gene NOX4 were determined in H9c2 cells. The data are expressed as the mean ± SEM. from three independent experiments. $^*p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

NOX4 in H9c2 cells compared with D-gal treatment group alone (**Figures 5D–G**). Taken together, these results demonstrated that NAD^+ supplement could partially rescue D-gal induced cellular senescence and oxidative stress.

DISCUSSION

Accumulating studies reported that the amount of people who are over 65 years old will be double from 12% in 2010 to 22% in 2040 (Heidenreich et al., 2011). Cardiovascular disease (CVD) is the main reason causing death in older people and in turn age-associated changes in endothelial and cardiac cells may enhance the risk of CVD development (Olivieri et al., 2013). The main characteristics of cardiac aging include cardiac cell death, hypertrophy and fibrosis. The senescent cells usually expressed senescence-associated β-galactosidase and $p16^{INK4A}$, so SA-β-gal staining and the expression of p16 are most commonly used to evaluate aging. D-galactose is widely used for preparing mouse aging model (Li et al., 2016; Liao et al., 2016), and it has been reported that D-gal significantly decreased rat heart function (Chang et al., 2016). In addition, subcutaneous injection of galactose could impair cognitive performance in rodents (Sadigh-Eteghad et al., 2017), so D-gal was also used to examine brain aging. In the present study, we used D-gal to induce senescence in cardiomyocytes. The results showed that 10 g/L D-gal increased myocardial cell senescence which was reflected in increased SA-β-gal staining and the expressions of senescence marker p16 and p21. Meanwhile, the ROS production was also increased in H9c2 cells treated with D-gal. More importantly, the expression of CD38 was upregulated in H9c2 cells at mRNA and protein level after treated with D-gal, suggesting that CD38 may play a role in D-gal-induced cardiomyocytes senescence.

CD38 is an important hydrolase of NAD^+. The intracellular level of NAD^+ was associated with aging and related diseases (Zhang et al., 2016; Li et al., 2017). NAD^+ concentrations were decreased in animals during aging and senescence (Verdin, 2015). Our previous study also demonstrated that CD38 played vital roles in adipogenesis and high fat induced oxidative stress (Wang et al., 2018a,b). But the role of CD38 in heart aging was unknown. In this study, we did examine the expression of

CD38 in heart tissue from 2 to 3 month-old mice (young) and 11 to 12-month-old mice, unfortunately we did not observe the increase of CD38 expression in the heart tissue from old mice (11 to 12-month-old) compared to young mice (**Supplementary Figure S1A**). The possible reason might be due to that the 11 to 12-month mice were too young for aging research since it has been reported that CD38 was up-regulated in several tissues such as liver, adipose tissue, spleen and muscle at least in 24 month-old mice (Camacho-Pereira et al., 2016). But we showed that the expression of Sirt1, a key molecule involved in NAD^+ metabolism, was significantly down-regulated in old mice, and the expression of NAMPT, a rate-limiting enzyme in NAD^+ salvage pathway, was also decreased in heart tissue from old mice. These results further demonstrated the important roles of NAD^+ metabolism in heart aging.

To further explore the role of CD38 in myocardial cell senescence, a CD38 knockdown stable H9c2 cell line was used in the study. The interference efficiency was about 80 percent. Our results showed that CD38 knockdown markedly decreased the SA-β-gal-positive cells and the expressions of senescence marker p16 and p21 compared with control group. $P16^{INK4A}$ is a selective inhibitor of cyclin D-dependent CDK4 and CDK6 and commonly used as a marker for evaluating senescence. It was reported that p16 was markedly increased in almost all rodent tissues with advancing age (Krishnamurthy et al., 2004). However, since p16 also has apparent limitations as a biomarker of senescence *in vivo* the combination of SA-β-gal staining and p16 is more reliable in evaluating aging.

Oxidative stress is closely related to many diseases, including aging. Cardiac aging is often accompanied by accumulating damage of mitochondrial and increasing level of reactive oxygen species (ROS) production in myocardiocytes and heart tissues (Kong et al., 2014). Many papers have presented that D-gal can induce oxidative stress in aging and other models (Li et al., 2016; Qiu et al., 2017). In the current study, our results showed the intracellular protein level of acetylation, the production of ROS, the content of MDA, and the expression of NADPH Oxidase 4 which was an indicator of oxidative stress significantly declined in CD38 knockdown group treated with D-gal, indicating that CD38 knockdown efficiently inhibited oxidative stress induced by D-gal. And the ROS production was aggravated after treated with D-gal combined with Sirt1 specific inhibitor EX-527. In addition, study showed that autophagy played pivotal roles in the heart during the aging process (Shirakabe et al., 2016). Our results suggested autophagy level was significantly increased in the group of CD38 knockdown, manifested as an increased expression of LC3B/LC3A. However, the relationship between the CD38-mediated decreased autophagy and increased oxidative stress needs to be further study. Taken together, these findings indicated that CD38 knockdown inhibited the oxidative induced by D-gal and increased autophagy.

NAD^+ concentrations were decreased in animals during aging (Gomes et al., 2013), suggesting that NAD^+ supplementation might exert protective effects during aging (Zhang et al., 2016). In this study, NAD^+ supplementation reduced H9c2 cell senescence, the production of ROS and MDA content when treated with D-gal. On the contrary, the expression of antioxidant gene SOD2 was increased, but the expression of NOX4 was decreased after NAD^+ supplementation. These results indicated that NAD^+ supplementation could reduce senescence and oxidative stress induced by D-gal *in vitro*.

CONCLUSION

Our results demonstrated that NAD^+/Sirt signaling played important roles in heart aging. CD38 knockdown alleviated D-gal induced cell senescence and oxidative stress, and NAD^+ supplementation had the similar effects with CD38 knockdown. Obviously, this study will provide new insights in elucidating the mechanism of heart aging and finding the therapeutic targets for delaying or preventing heart aging.

DATA AVAILABILITY

All datasets for this study are included in the manuscript and the **Supplementary Files**.

ETHICS STATEMENT

The animal study was reviewed and approved by the Ethics Committee of Nanchang University.

AUTHOR CONTRIBUTIONS

L-FW and QC performed the experiments, participated in the design of the study, and carried out the animal model. KW, Y-FX, and X-HG participated in the data analysis. H-BX and K-YD conceived the study and participated in the design and coordination of the study. L-FW drafted the manuscript. H-BX revised the manuscript. All authors read and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2019.01125/full#supplementary-material

FIGURE S1 | Sirt1 was responsible for D-gal induced senescence and oxidative stress and reversed the effects of CD38 knockdown on it. **(A)** The western blot image of CD38 protein were determined in heart tissue from 1 month and 12 month old mice. **(B)** The western blot image of Sirt1 protein in H9c2 cells treated with D-gal (10 g/L). **(C)** SA-β-gal staining in CD38 knockdown H9c2 cells treated with or without with D-gal (10 g/L) or combined with Sirt1 specific inhibitor EX-527 (25 μM). **(D)** The mean fluorescence intensities of ROS production were quantitatively analyzed in H9c2 cells treated with D-gal (10 g/L) or combined with EX-527 (25 μM). **(E)** The protein acetylation level of CD38 knockdown H9c2 cells treated with or without with D-gal (10 g/L) was analyzed by western blotting with antibody against acetylated-lysine. Data are shown as mean ± SEM, $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$, $n = 3$ per group.

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frontiers in Physiology

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BCAA Catabolic Defect Alters Glucose Metabolism in Lean Mice

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Recent studies show branched-chain amino acid (BCAA) catabolic pathway is defective in obese animals and humans, contributing to the pathogenesis of insulin resistance and diabetes. However, in the context of obesity, various processes including the dysfunctional lipid metabolism can affect insulin sensitivity and glycemic regulation. It remains unclear how BCAA catabolic defect may exert direct impacts on glucose metabolism without the disturbance of obesity. The current study characterized the glucose metabolism in lean mice in which the genetic deletion of PP2Cm leads to moderate BCAA catabolic defect. Interestingly, compared to the wildtype control, lean PP2Cm deficient mice showed enhanced insulin sensitivity and glucose tolerance, lower body weight, and the preference for carbohydrate over lipids utilization. Metabolomics profiling of plasma and tissues revealed significantly different metabolic patterns in the PP2Cm deficient mice, featured by the marked alterations in glucose metabolic processes, including gluconeogenesis/glycolysis, glycogen metabolism, and tricarboxylic acid cycle. The metabolic changes of glucose were predominantly observed in liver but not skeletal muscle or white adipose tissue. The elevated branched-chain keto acids (BCKAs) resulted from the BCAA catabolic defect may play a critical role in regulating the expression of key regulators of glucose metabolic processes and the activity of respiratory Complex II/succinate dehydrogenase in TCA cycle. Together, these results show BCAA catabolic defect significantly alters glucose metabolism in lean mice with some impacts different or even opposite from those in obese mice, highlighting the critical role of BCAA catabolism in glycemic regulation and the complex interplay between macronutrients in lean and obese animals.

Keywords: branched-chain amino acids, glucose metabolism, catabolic defect, lean mice, liver

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Abbreviations: BCAA, branched-chain amino acid; BCAT, BCAA transaminase; BCKA, branched-chain keto acid; BCKD, branched-chain-α-ketoacid dehydrogenase; BCKDK, branched-chain-α-ketoacid dehydrogenase kinase; GTT, glucose tolerance test; ITT, insulin tolerance test; PP2Cm, mitochondrial protein phosphatase 2C; PPm1k, protein phosphatase 1K (PP2C domain containing); RER, respiratory exchange ratio; ROS, reactive oxygen species; T2DM, type 2 diabetes mellitus; TCA cycle, tricarboxylic acid cycle.

INTRODUCTION

In the past several years, insulin resistance and diabetes have been linked with disrupted branched-chain amino acids (BCAAs) homeostasis in obese animals and humans (Lynch and Adams, 2014). BCAAs, including leucine, isoleucine and valine, are essential amino acids. A number of observational studies found that elevated circulating levels of BCAAs are associated with type 2 diabetes mellitus (T2DM) and insulin resistance in humans and some rodent models (Shaham et al., 2008; Huffman et al., 2009; Tai et al., 2010; Xu et al., 2013; Lynch and Adams, 2014; Lian et al., 2015). Longitudinal and prospective studies in different cohorts have reported that increased BCAA level in blood is predictive for diabetes pathogenesis and change of plasma BCAA level is prognostic for intervention outcomes of diabetes (Wang et al., 2011; Melnik, 2012; Wang-Sattler et al., 2012; Floegel et al., 2013; Lu et al., 2013; McCormack et al., 2013). Lower BCAA level has been associated with improved insulin resistance after interventional procedures (Laferrere et al., 2011; Wang et al., 2011; Shah et al., 2012). The clear association has led to the speculation about a potential causative role of the disrupted BCAA homeostasis in T2DM (Lynch and Adams, 2014).

Branched-chain amino acid homeostasis is determined largely by their catabolic activities in tissues. The first two steps of BCAA catabolism are shared by all three BCAAs. The initial deamination step to produce branched chain keto acids (BCKAs) is catalyzed by BCAA transaminase (BCAT), which is followed by the oxidative decarboxylation to form CoA esters, a reaction catalyzed by BCKA dehydrogenase (BCKD) complex. The BCKD complex is the rate-limiting enzyme for BCAA catabolism and tightly regulated by inhibitory phosphorylation by BCKDK and activating dephosphorylation by mitochondrial phosphatase 2C (PP2Cm). Loss of PP2Cm in genetic model partially impairs BCAA catabolism, leading to the higher plasma BCAA and BCKA concentrations (Lu et al., 2009). Similarly, in obese animals and humans, BCAA catabolic genes are down-regulated and the BCAA catabolism is moderately defective, contributing to the elevated plasma BCAAs and BCKAs (She et al., 2007b, 2013; Pietiläinen et al., 2008; Herman et al., 2010; Lackey et al., 2013; Lu et al., 2013; Menni et al., 2013; Zimmerman et al., 2013).

The strong association between the elevated BCAA level and the obesity-associated T2DM indicates that disrupted BCAA homeostasis may contribute to the dysfunctional glycemic control. Indeed, recent studies show that BCAA catabolic defect contributes to the obesity-associated insulin resistance and diabetes (White et al., 2018; Zhou et al., 2019). However, in obese animals and humans, the dysregulated lipid metabolism and other processes dramatically affects insulin sensitivity and glucose metabolism. Thus, it remains challenging to distinguish the neat impacts of BCAA catabolic defect on glucose metabolism from the disturbance of obesity in obese animals. Using lean mice, the current study investigates the impacts of BCAA catabolic defect on glucose metabolic processes in a genetic mouse model in which PP2Cm is ablated to partially impair BCAA catabolism.

MATERIALS AND METHODS

Animals

Wild type C57BL/6 and PP2Cm knockout male, age-matched mice were on the same genetic background and maintained in the same facility. PP2Cm germ-line knockout mice were generated as previously described (Lu et al., 2009). All animals (at age of 10–14 weeks) were housed at 22°C with a 12-h light, 12-h dark cycle with free access to water and standard chow. All animal procedures were carried out in accordance with the guidelines and protocols approved by the Committee for Humane Treatment of Animals at Shanghai Jiao Tong University School of Medicine or the University of California at Los Angeles Institutional Animal Care and Use Committee.

Indirect Calorimetry Measurements

Measurements of oxygen consumption (VO_2) and carbon dioxide production (VCO_2) with indirect calorimetry were performed at ambient temperature using a Comprehensive Laboratory Animal Monitoring System (CLAMS, Columbus Instruments, OH, United States) according to the instructions of the manufacturer. Respiratory exchange ratio (RER) equals [volumes of CO_2 released]/[volumes of O_2 consumed]. Male mice were admitted to a CLAMS with free access to food and water and allowed to acclimatize in individual metabolic cages for 48 h before any measurements and the data were collected in the next 36 h.

Glucose and Insulin Tolerance Test

Male mice were fasted for 6 h starting at 8 am. For insulin tolerance test, mice were injected intraperitoneally with insulin (0.75 U/kg body weight; Sigma, United States). For glucose tolerance test, mice were injected intraperitoneally with D-glucose (1.5 g/kg body weight; Sigma, United States). Blood glucose concentrations were measured using a portable glucometer (Johnson & Johnson, United States) through tail bleeding at the times indicated after injection.

RNA Isolation and qRT-PCR

Total RNA was extracted from tissues or cells using the Trizol (Invitrogen, United States). Total RNA (2 μg) was reverse transcribed using random primers and MMLV (Promega, United States). Each cDNA sample was analyzed with the Applied Biosystems Prism7900HT Real-Time PCR System using Absolute SYBR Green (ABI, United States) with the following primers' sequences:

human GYS2_F:5′CTGTAACATCCCTGGGTGGG3′,
human GYS2_R:5′GCCTCCAACTTTATTGGTCACT3′,
mouse GYS2_F:5′CCAGACAAATTCCACCTAGAGC3′,
mouse GYS2_R:5′GGGCCTGGGATACTTAAAGC3′.
human PYGL_F:5′CACTTCAGTGGCAGATGTGGTG3′,
human PYGL_R:5′GCAGTGGAAATCTGCTCTGACAG3′,
mouse PYGL_F:5′GAGAAGCGACGGCAGATCAG3′,
mouse PYGL_R:5′CTTGACCAGAGTGAAGTGCAG 3′.

Metabolomic Analysis

The metabolomic analysis was carried out by Metabolon, Inc. (Durham, NC) using tissues from male wildtype or PP2Cm knockout mice at 14 weeks of age. After 6-h fasting starting at 8am, the animals were sacrificed by cervical dislocation. Tissue samples from white adipose tissue (epididymal fat), skeletal muscle (soleus/gastrocnemius), and liver were quickly harvested and frozen in liquid nitrogen and maintained at −80°C until processed. Samples were prepared using the automated MicroLab STAR® system from Hamilton Company. To extract metabolites from tissues, extraction solution based on methanol was added to each sample in identical weight to volume ratio. The tubes containing extraction mixtures were centrifuged to precipitate proteins, and the supernatants containing metabolites were recovered for metabolomics analysis. Several types of controls were analyzed in concert with the experimental samples. The LC-MS portion of the platform was based on a Waters ACQUITY ultra-performance liquid chromatography (UPLC) and a Thermo-Finnigan LTQ mass spectrometer operated at nominal mass resolution, which consisted of an electrospray ionization (ESI) source and linear ion-trap (LIT) mass analyzer. The samples destined for analysis by GC-MS were dried under vacuum prior to being derivatized under dried nitrogen using bistrimethyl-silyltrifluoroacetamide. Derivatized samples were separated on a 5% diphenyl/95% dimethyl polysiloxane fused silica column and analyzed on a Thermo-Finnigan Trace DSQ fast-scanning single-quadrupole mass spectrometer using electron impact ionization (EI) and operated at unit mass resolving power. Raw data was extracted, peak-identified and QC processed using Metabolon's hardware and software. Peaks were quantified using area-under-the-curve. A collection of information interpretation and visualization tools including Principal Component Analysis (PCA) and Random Forest (RF) analyses were used for data analysts. Welch's two-sample *t*-test is used to test whether two unknown means are different from two independent populations.

Cell Culture

HepG2 cells were cultured in Dulbecco's modified Eagle's medium (Hyclone, Beijing) supplemented with 10% fetal bovine serum (FBS, Sigma), penicillin (100 IU/mL) and streptomycin (100 μg/mL) in a humidified 5% CO_2-95% air incubator at 37°C. For stimulation by BCAA (800 μM) or BCKA (400 μM), the cells were incubated in serum-free DMEM for 12 h, and then incubated in BCAA-free DMEM for 1 h before the initiation of 12 h treatments. BCAA and BCKA were diluted in BCAA-free DMEM. Custom BCAA-free DMEM was provided by Invitrogen. BCAA and BCKA chemicals were purchased from Sigma.

Mitochondrial Assay

The isolation of mitochondria to measure oxygen consumption was performed as described elsewhere (Korge et al., 2011). Briefly, mitochondria were isolated from tissues and oxygen consumption was measured using an Ocean Optics fiber optic spectrofluorometer. Mitochondria (0.25 mg/ml) were added to the assay buffer (125 mM KCl, 10 mm HEPES-KOH, pH 7.4). The oxygen concentration in the buffer was continuously recorded via an Ocean Optics FOXY fiber optic oxygen sensor. Pyruvate, malate, and glutamate were added as free acids buffered with Tris (pH 7.4) for Complex I activity assay. Succinate was used for Complex II activity assay in presence of rotenone (1 μM). Addition of 0.2 mM ADP initiated oxygen consumption. NaCl or BCKA-Na mixture was added to the reaction system after the first pulse of ADP was consumed. Then the second pulse of ADP was added. The oxygen consumption rate (OCR) was calculated with each ADP addition. The relative rate of oxygen consumption was calculated by dividing the OCR of second pulse of ADP by the OCR of the first pulse of ADP. The presented data represented the average values of three independent experiments.

Statistics

Unless otherwise specified, statistical analyses were performed with two-sided Student's *t*-test or two-way ANOVA, followed by a Bonferroni *post hoc* test (tolerance tests) where appropriate using GraphPad Prism. Data were calculated as the mean ± SEM. A *p*-value of less than 0.05 was considered statistically significant.

RESULTS

Physiological Characterization of the Lean Mice With BCAA Catabolic Defect

In order to examine the effects of defective BCAA catabolism on glucose metabolism in lean mice, we characterized the metabolic phenotypes of PP2Cm deficient mice in which the gene encoding PP2Cm has been genetically disrupted (Lu et al., 2009). PP2Cm is the specific BCKD phosphatase that dephosphorylates BCKDE1a subunit at Ser293 in the presence of substrates. PP2Cm deficiency partially impairs BCAA catabolism, leading to elevated plasma BCAA and BCKA concentrations. PP2Cm deficient mice showed significantly lower body weight compared with wildtype control (**Figure 1A**) without food intake change (**Figure 1B**). In indirect calorimetry, PP2Cm deficient mice showed similar energy expenditure and physical activity compared with wildtype mice (data not shown). Interestingly, the RER in PP2Cm deficient mice was significantly higher compared to that in wildtype mice, indicating PP2Cm deficient mice had an overall preference for carbohydrates as metabolic substrate (**Figures 1C,D**). Furthermore, glucose tolerance test and insulin tolerance test demonstrated enhanced glucose clearance and insulin sensitivity in PP2Cm deficient mice (**Figures 1E–H**), accompanied with an unaffected fasting plasma insulin level (data not shown). Together, these data demonstrate clear alterations of glucose metabolism in lean mice with BCAA catabolic defect.

Metabolomics Profiling Reveals Distinguishable Metabolic Patterns of PP2Cm Deficient Mice

We next performed metabolomics profiling analyses of plasma to further characterize the biochemical changes in overall metabolism of fasted PP2Cm deficient mice. A total of 315

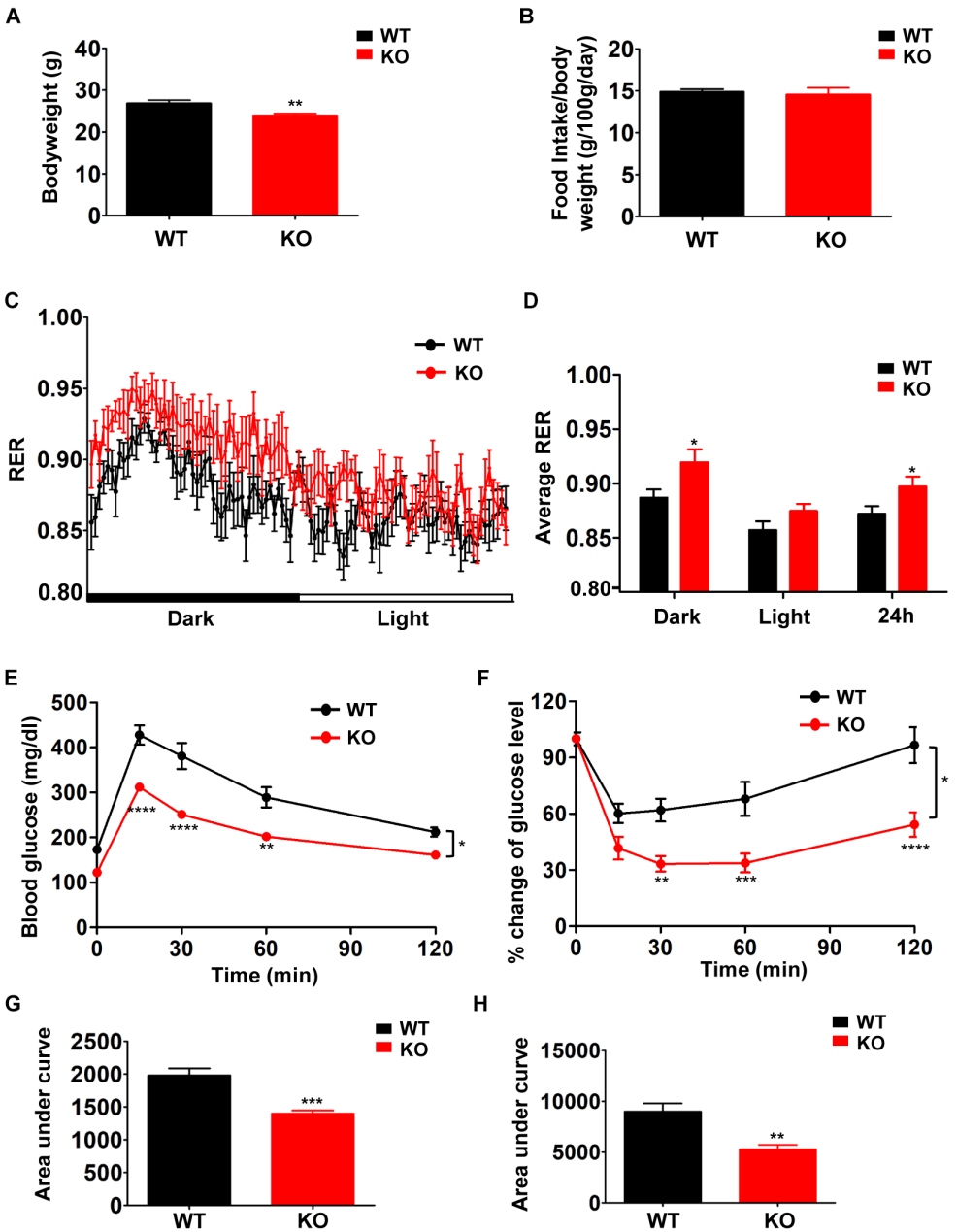
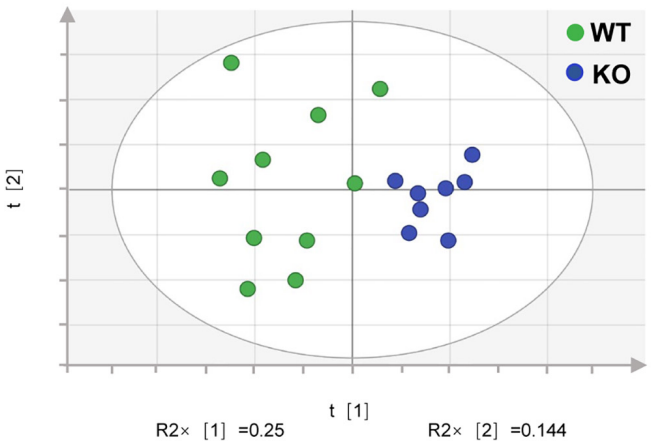


FIGURE 1 | Branched-chain amino acid (BCAA) catabolic defect reduces body weight with beneficial effects on glucose metabolism in PP2Cm KO mice. Body weight (**A**, n = 10–11), food intake (**B**, n = 10–11), respiratory exchange ratios (RER) (**C**, n = 9/genotype), average RER during light and dark cycles (**D**, n = 9/genotype), glucose tolerance test **(E,G)** and insulin tolerance test **(F,H)** of WT and PP2Cm KO male mice fed a normal diet (n = 8 for each group). Data are represented as means ± SEM. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ compared to WT.

named biochemicals were identified and measured in mouse plasma samples. The identities and metabolic pathways of these metabolites are provided in **Supplementary Table S1**. Statistical comparisons revealed a large number of statistically significant differences between groups. Principal component analysis (PCA) determines if samples from different groups can be segregated based on differences in their overall metabolic signature. The PCA results illustrated a clear differentiation of PP2Cm deficient and wildtype groups (**Figure 2A**). Meanwhile, Random Forest (RF) analysis bins individual samples into groups based on their metabolite similarities and differences, and also defines which metabolites contribute most strongly to the group binning. RF analysis of PP2Cm deficient and wildtype mouse plasma samples resulted in a 94% predictive accuracy for assignment of individual plasma into their proper groups (**Figure 2B**). BCAAs and their metabolites including



B

Random Forest Confusion Matrix

Actual Group \ Predicted Group	KO	WT	Class Error
KO	8	0	0.000
WT	1	9	0.100
Predictive accuracy = 94%			

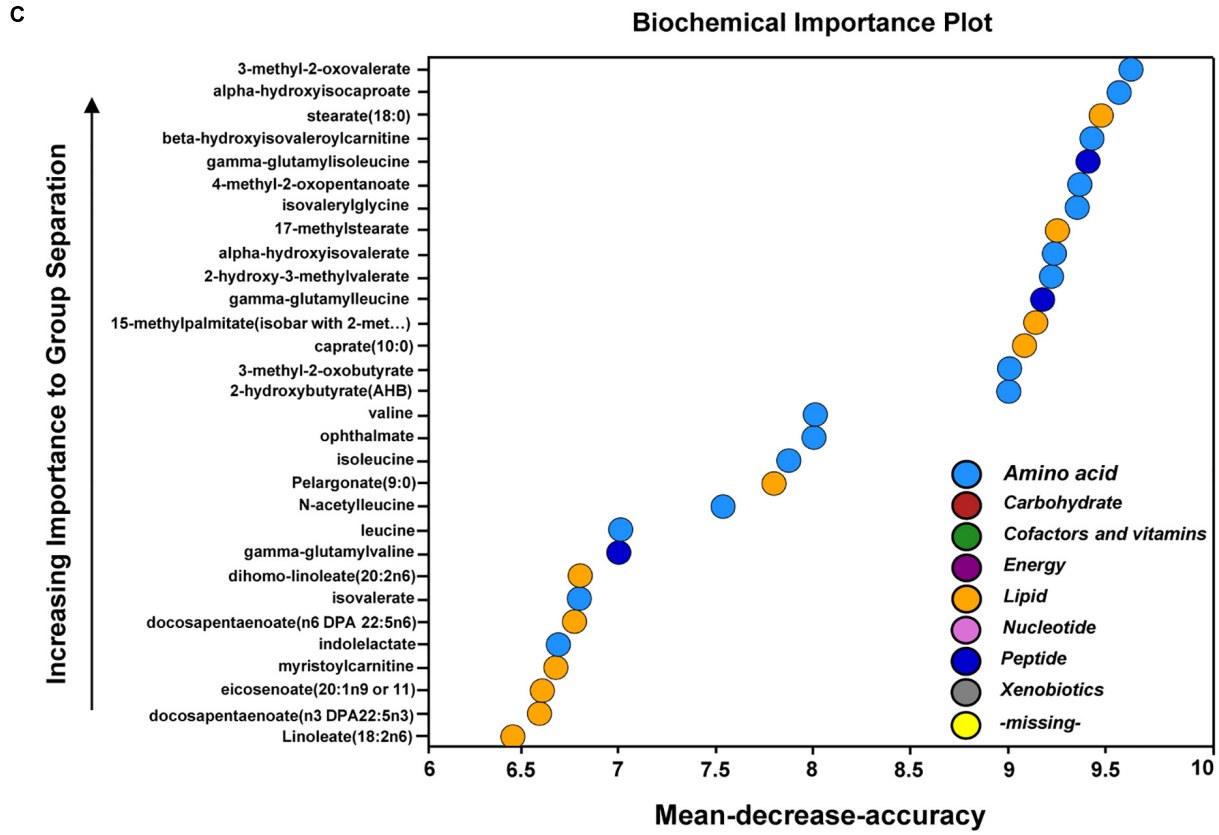


FIGURE 2 | Deletion of PP2Cm resulted in significant global metabolic perturbations. **(A)** Principal component analysis (PCA) of metabolomic profiles revealed a distinct genotype-based separation for the plasma samples (WT, *n* = 10; KO, *n* = 8). **(B)** Random Forest Confusion Matrix. Random Forest classification using named metabolites detected in the plasma of wildtype (WT) and PP2Cm knockout (PP2Cm KO) mice resulted in a predictive accuracy of 94% (WT, *n* = 10; KO, *n* = 8). **(C)** List of the top 30 biochemicals that separated different genotypes based on their importance.

the BCKAs 3-methyl-2-oxovalerate, 4-methyl-2-oxopentanoate, and 3-methyl-2-oxobutyrate were among the top 30 metabolites most strongly contributing to proper group binning (**Figure 2C**). Together, PCA and RF analyses showed clearly distinguishable changes in the plasma metabolites between PP2Cm deficient and wild-type control mice.

Systemic BCAA Catabolic Defect in PP2Cm Deficient Mice

Comparison of plasma global biochemical profiles for wildtype and PP2Cm deficient mice revealed several key signatures. As expected, the most dramatic effects of PP2Cm ablation was on metabolites of BCAA metabolic pathway (**Figure 3A**). Plasma levels of valine, leucine and isoleucine were elevated in PP2Cm deficient mice relative to wildtype mice (**Figure 3B**). The BCKAs, 3-methyl-2-oxobutyrate, 3-methyl-2-oxovalerate, and 4-methyl-2-oxopentanoate were also elevated in the plasma of PP2Cm deficient mice. The alpha-hydroxycarboxylic acids, 2-hydroxy-3-methylvalerate, alpha-hydroxyisocaproate, and alpha-hydroxyisovalerate, derived from reduction of the BCKAs, were all increased in the plasma of PP2Cm deficient mice (**Figure 3B**). Elevation of the BCAAs, BCKAs, and 2-hydroxycarboxylic acids was consistent with a decrease in BCKD activity, which was further supported by the lower abundance of beta-hydroxyisovaleroylcarnitine in the plasma of PP2Cm deficient mice (**Figure 3B**). Interestingly, some metabolites derived from products downstream of BCKD, including isovalerylglycine, isovalerate, 3-methylcrotonylglycine, 2-methylbutyrylcarnitine, and isobutyrylcarnitine, were increased in the plasma of PP2Cm deficient mice (**Figure 3B**), suggesting complexity of BCAA catabolism from different tissues (Hutson et al., 2005). Nevertheless, genetic ablation of PP2Cm clearly causes systemic BCAA catabolic defect in mice.

Tissue-Specific BCAA Catabolic Defect in PP2Cm Deficient Mice

To better understand how BCAA catabolic defect affects regional metabolism, we performed metabolomics analyses in liver, white adipose tissue, and skeletal muscle, the three key tissues in metabolic regulation, in fasted mice.

A total of 336 compounds of known identity were identified and analyzed in liver tissues (**Supplementary Table S2**). Compared to wildtype counterparts, PP2Cm deficient liver possessed an accumulation of BCKAs, 4-methyl-2-oxopentanoate and 3-methyl-2-oxovalerate (**Figure 4A**). Lower levels of downstream BCAA catabolites including isovalerylcarnitine and beta-hydroxyisovaleroylcarnitine were observed in PP2Cm deficient liver (**Figure 4A**). These changes reflected the impaired BCKD activity due to the ablation of PP2Cm. It was unexpected that the BCAA levels were not higher while 2-methylbutyrylcarnitine was elevated in the PP2Cm deficient liver (**Figure 4A**).

A total of 317 chemicals of known identity were detected in skeletal muscle samples (**Supplementary Table S3**). BCAA and BCAA catabolic products formed upstream of BCKD, including 3-methyl-2-oxovalerate, 4-methyl-2-oxopentanoate, alpha-hydroxyisovalerate, and allo-isoleucine, were elevated in the skeletal muscle from PP2Cm deficient mice (**Figure 4B**), consistent with defective BCKD activity. In addition to these changes, metabolites formed downstream of BCKD (isobutyrylcarnitine and 2-methylbutyrylcarnitine) were found with trends of lower levels in the skeletal muscles from PP2Cm deficient animals compared to wildtype counterparts (**Figure 4B**). These changes demonstrated the impaired BCKD activity and the defect in BCAA catabolism in PP2Cm deficient skeletal muscle.

A total of 180 compounds of known identity were detected in adipose tissue samples (**Supplementary Table S4**). PP2Cm deficient adipose tissue possessed trends of elevated BCAAs compared to wildtype counterparts (**Figure 4C**). Furthermore, the BCKD downstream catabolites isovalerylcarnitine and isobutyrylcarnitine showed trends of decrease in PP2Cm deficient adipose tissue compared to that of wildtype mice (**Figure 4C**).

Together with the plasma profiles, these data suggest that PP2Cm ablation leads to the whole-body BCAA catabolic defect, accompanied with tissue-specific patterns.

Impacts of BCAA Catabolic Defect on Glycogen Metabolism

Besides the defective BCAA catabolism in PP2Cm deficient mice, significant alterations were detected by metabolomics analyses in glucose metabolic processes including glycogenesis/glycogenolysis, glycolysis/gluconeogenesis, and TCA cycle in different tissues of fasted mice.

One major change was observed in glycogen metabolism in liver of fasted P2Cm deficient mice. Glycogen is the storage form of glucose in liver and skeletal muscle. Glycogen synthesis (glycogenesis) is important for blood glucose disposal in the fed state. During short-term fasting periods, the liver releases glucose mainly through glycogenolysis, contributing to the maintenance of blood glucose (**Figure 5A**). PP2Cm deficient liver possessed significantly higher level of the glycogen metabolite maltotriose and trends of elevated maltohexaose, maltopentaose, and maltotetraose compared to wildtype counterparts (**Figure 5B**). Meanwhile, PP2Cm deficient liver showed higher mRNA expression of glycogen phosphorylase (PYGL) and glycogen synthase (GYS2), the controllers of glycogenolysis and glycogenesis in liver, respectively (**Figure 5C**). Interestingly, BCKAs, but not BCAAs, induced PYGL expression in HepG2 cells (**Figure 5D**). The expression of GYS2 in HepG2 was low and the impacts of BCAAs and BCKAs on it remained unclear. These results indicate that BCAA catabolism defect alters glycogen metabolism in liver.

In contrast to liver tissue, glycogen metabolites maltose was markedly decreased in PP2Cm deficient skeletal muscle while maltotriose and maltotetraose showed no significant changes (**Figure 5E**), indicating different effects of BCAA catabolic defect on glycogen metabolism in liver and skeletal muscle.

Impacts of BCAA Catabolic Defect on Glycolysis/Gluconeogenesis

Glycolysis and gluconeogenesis are two central processes of glucose metabolism in reverse direction, sharing enzymes and metabolic intermediates (**Figure 6A**). The abundances of glucose, glucose-6-phosphate, and likely fructose-6-phosphate (upstream metabolites of glycolysis) were increased in PP2Cm deficient liver while fructose-1,6-biphosphate and 3-phosphoglycerate (downstream metabolites of glycolysis) showed lower levels

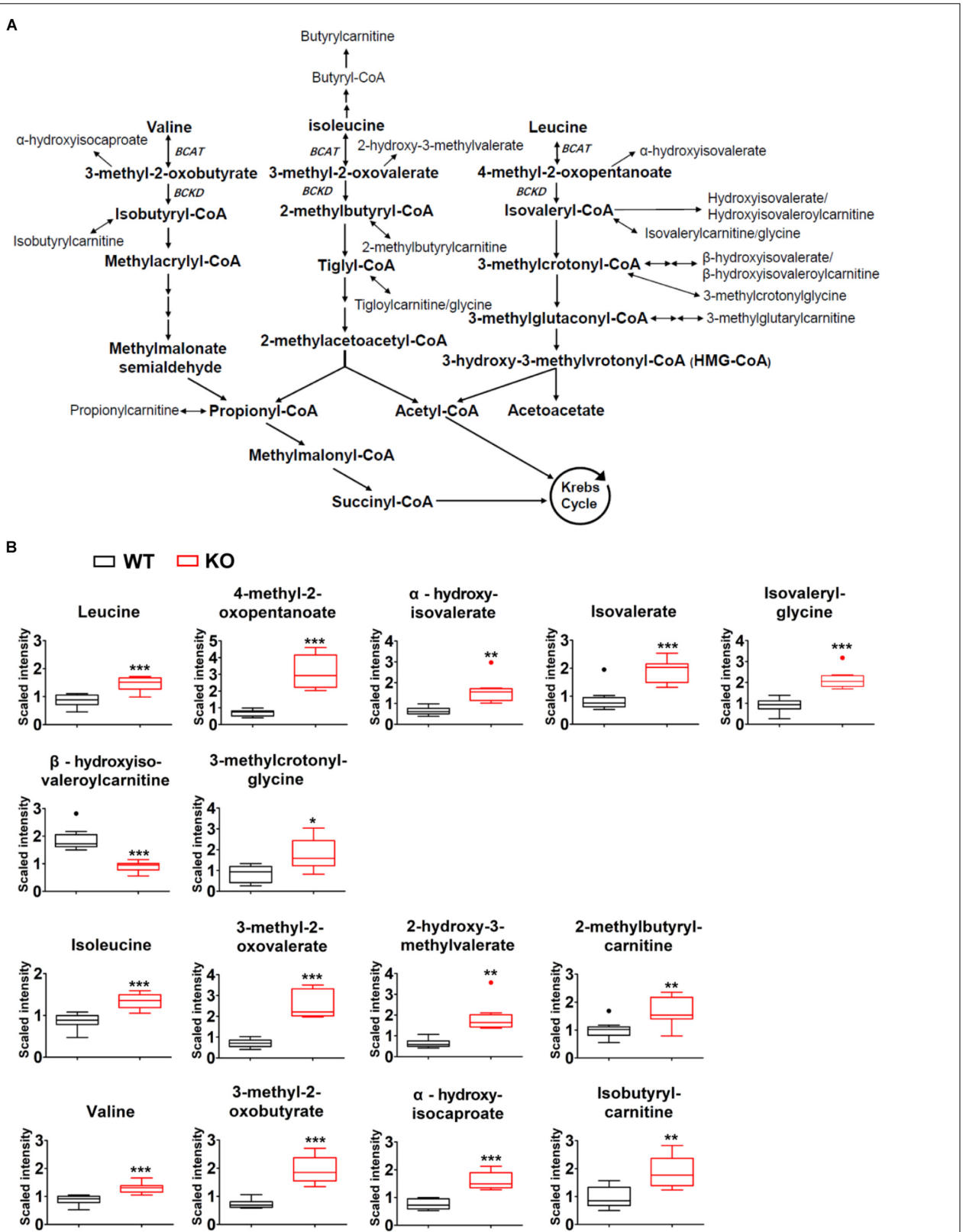


FIGURE 3 | Global BCAA catabolic defect in lean PP2Cm KO mice. **(A)** Illustration of BCAA catabolic process with enzymes, intermediates, and derivatives. **(B)** Relative abundances of BCAAs and their corresponding metabolites in the plasma of wildtype (WT) and PP2Cm knockout (PP2Cm KO) mice (WT, $n = 10$; KO, $n = 8$). $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

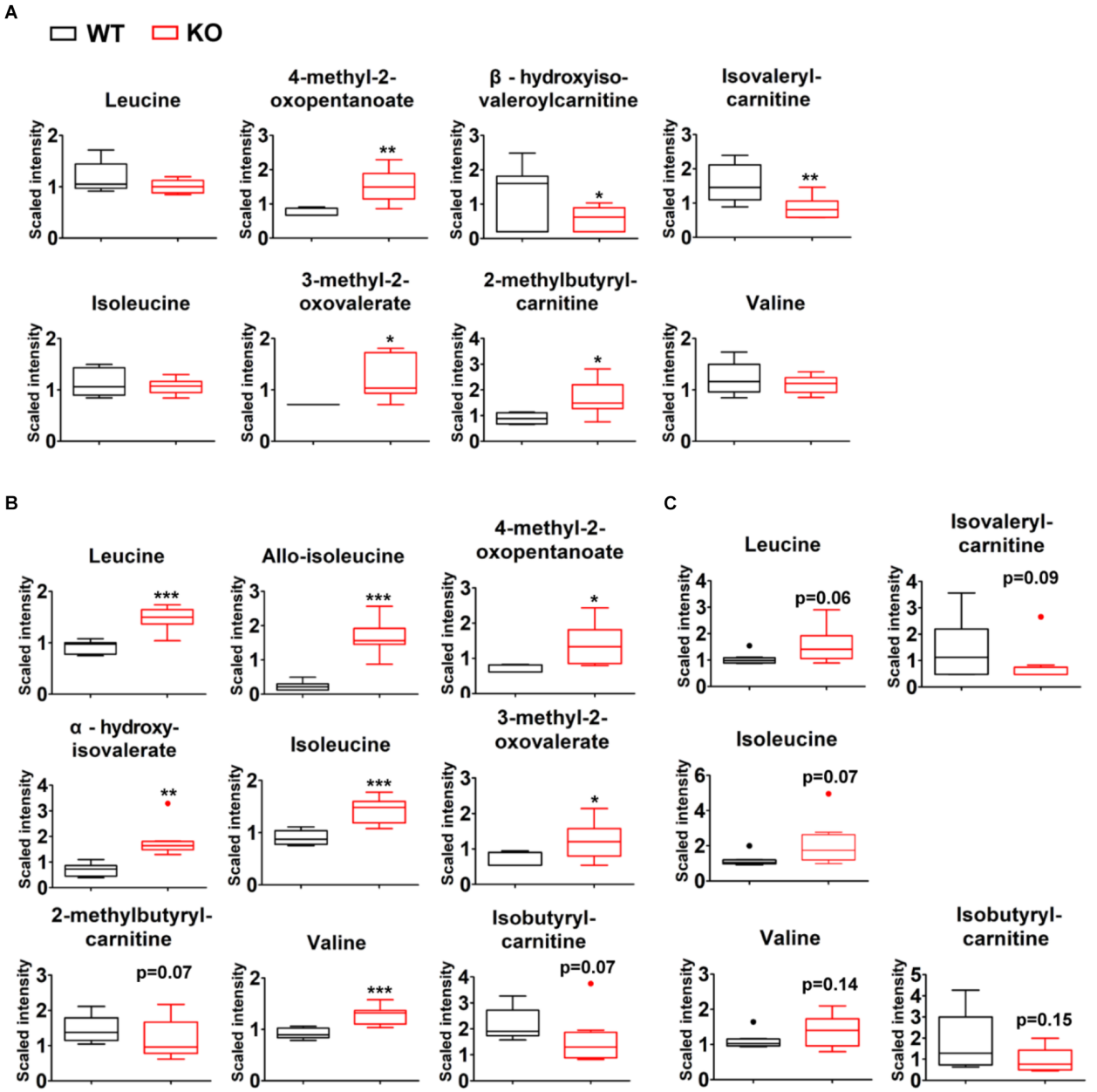


FIGURE 4 | Tissue-specific BCAA catabolic defect in lean PP2Cm KO mice. Relative abundances of BCAAs and their corresponding metabolites in the liver **(A)**, skeletal muscle **(B)**, and white adipose tissue **(C)** of wildtype (WT) and PP2Cm knockout (PP2Cm KO) mice (n = 8 for each group). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

(**Figure 6B**). No significant differences were detected for pyruvate, lactate, and dihydroxyacetone phosphate (**Figure 6B**).

Allosteric modulators of glycolysis/gluconeogenesis include acetyl-CoA, AMP, ADP, citrate, F-2,6-BP (**Figure 6C**). The metabolomics analyses detected lower citrate, unchanged acetyl-carnitine, unchanged ADP, and higher AMP in the PP2Cm deficient liver, which likely enhanced glycolysis but not gluconeogenesis (**Figure 6D**).

In contrast to liver, no significant differences in glycolic intermediates were observed between PP2Cm deficient and wildtype adipose tissues or skeletal muscle, respectively (**Supplementary Figure S1**).

Impacts of BCAA Catabolic Defect on TCA Cycle

Glycolysis generates pyruvate, which subsequently is oxidized in the tricarboxylic acid (TCA) cycle. The metabolomics analyses detected lower abundances of multiple TCA cycle intermediates including fumarate, malate, and citrate (**Figure 6D**) in PP2Cm deficient liver compared to wildtype counterpart (**Figure 7A**).

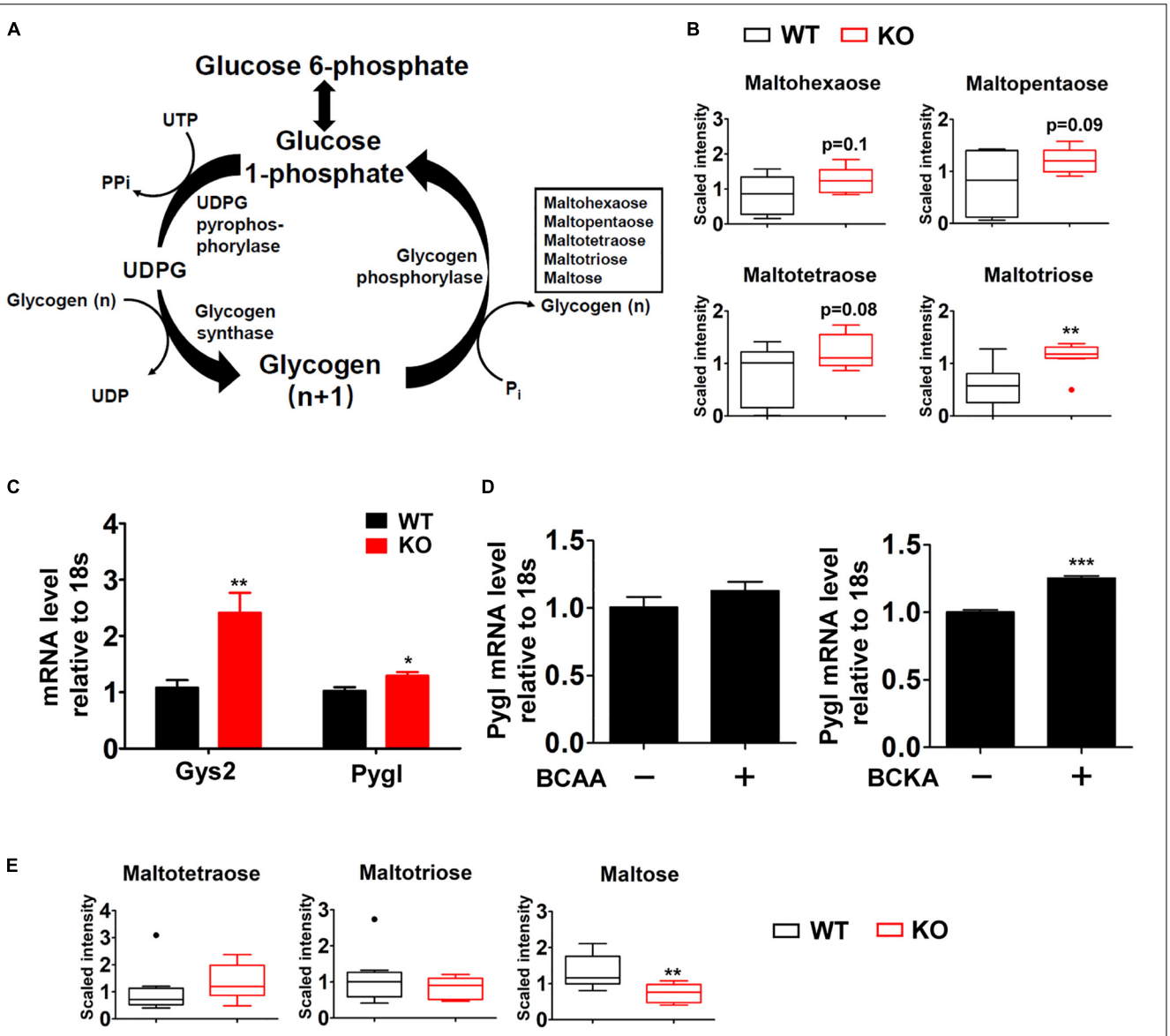


FIGURE 5 | Box plots of various metabolites in glycogen catabolic pathway altered in the PP2Cm KO mice. **(A)** Illustration of glycogen metabolic pathway with enzymes and intermediates. **(B)** Significantly altered metabolites in liver are shown as box plots. *$P < 0.05$; **(C)** Real-time RT-PCR result of specific genes using mRNA from liver of wildtype (WT) and PP2Cm knockout (PP2Cm KO) mice ($n \geq 9$ for each group). Data are represented as means ± SEM. *$p < 0.05$; **$p < 0.01$. **(D)** mRNA levels of PYGL in BCAA and BCKA treated HepG2 cells ($n = 3$ for each group). Data are represented as means ± SEM. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. **(E)** Significantly altered metabolite in skeletal muscle ($n = 8$ for each group). *$p < 0.05$; **$p < 0.01$.

It has been shown that BCKAs inhibited mitochondrial respiration in cardiac mitochondria (Sun et al., 2016). The enzyme that catalyzes the conversion from succinate to fumarate in TCA cycle is succinate dehydrogenase, which is also the respiration Complex II in the electron transfer chain in mitochondria (**Figure 7A**). The lower abundances of fumarate and malate but not succinate indicated the inhibition of succinate dehydrogenase/Complex II. BCKAs was elevated in the PP2Cm deficient liver (**Figure 4A**). We then examined the impacts of BCKAs on isolated liver mitochondria. Interestingly, BCKAs significantly inhibited Complex II but not Complex I mediated respiration in isolated liver mitochondria (**Figures 7B–E**), different from the impacts on heart mitochondria (**Supplementary Figure S2**; Sun et al., 2016). These results indicate that accumulated BCKAs due to BCAA catabolic defect may directly inhibit TCA cycle and mitochondrial oxidative phosphorylation in liver mitochondria.

The TCA cycle intermediates showed no alterations in PP2Cm deficient skeletal muscle (**Supplementary Figure S3A**). Citrate and *cis*-aconitate showed trends of increase in PP2Cm deficient adipose tissue compared to the wildtype counterpart

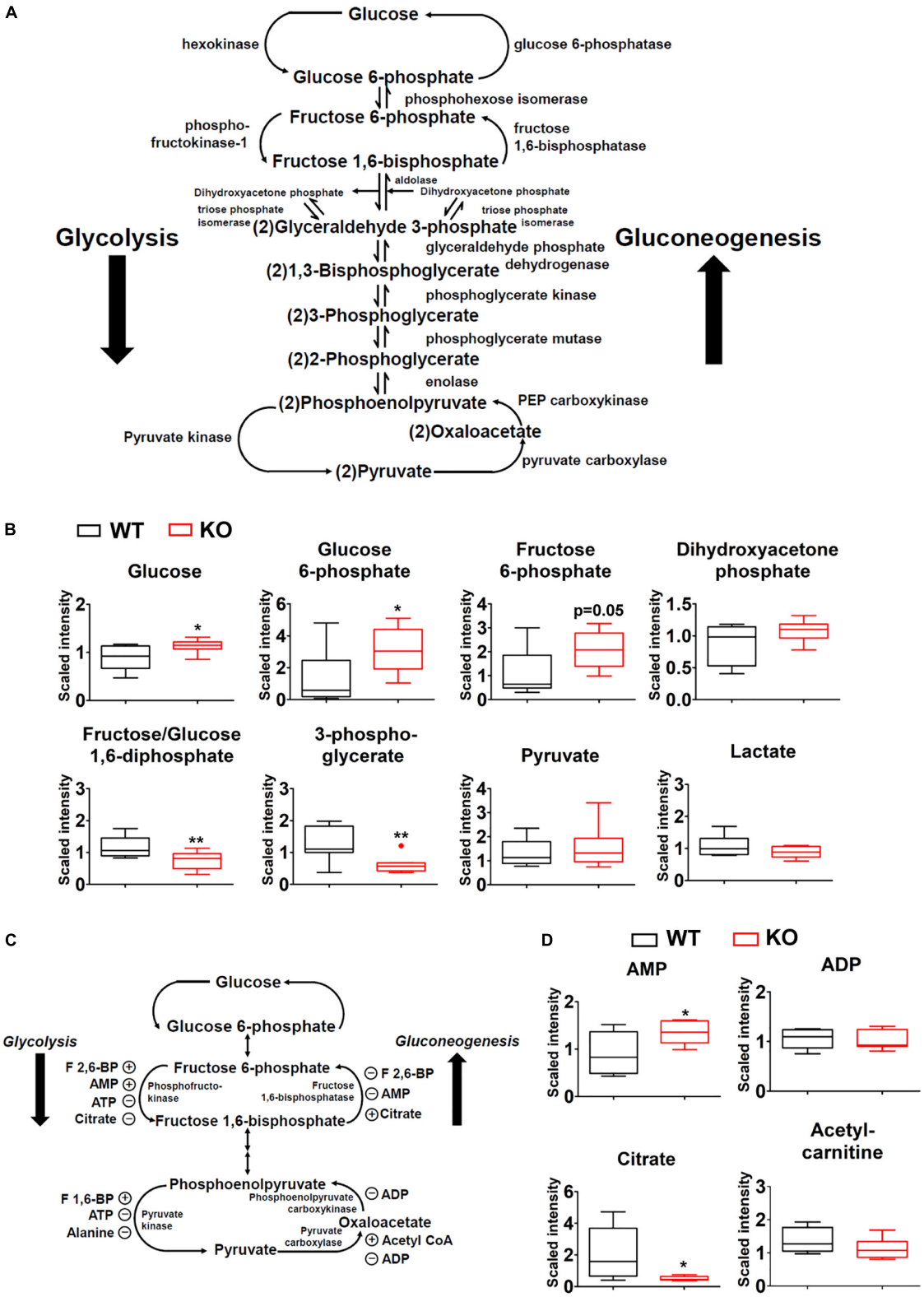


FIGURE 6 | Impacts of BCAA catabolic defect on glycolysis/gluconeogenesis in liver. **(A)** Illustration of glycolysis/gluconeogenesis with enzymes and intermediates. **(B)** Metabolomics analysis results showing the relative levels of glycolysis/gluconeogenesis intermediates in PP2Cm KO liver (n = 8 for each group). **(C)** Illustration of glycolysis/gluconeogenesis with enzymes and allosteric modulators. **(D)** Metabolomics analysis results showing the relative levels of allosteric modulators of glycolysis/gluconeogenesis in PP2Cm KO liver (n = 8 for each group). $^{*}p < 0.05$, $^{**}p < 0.01$.

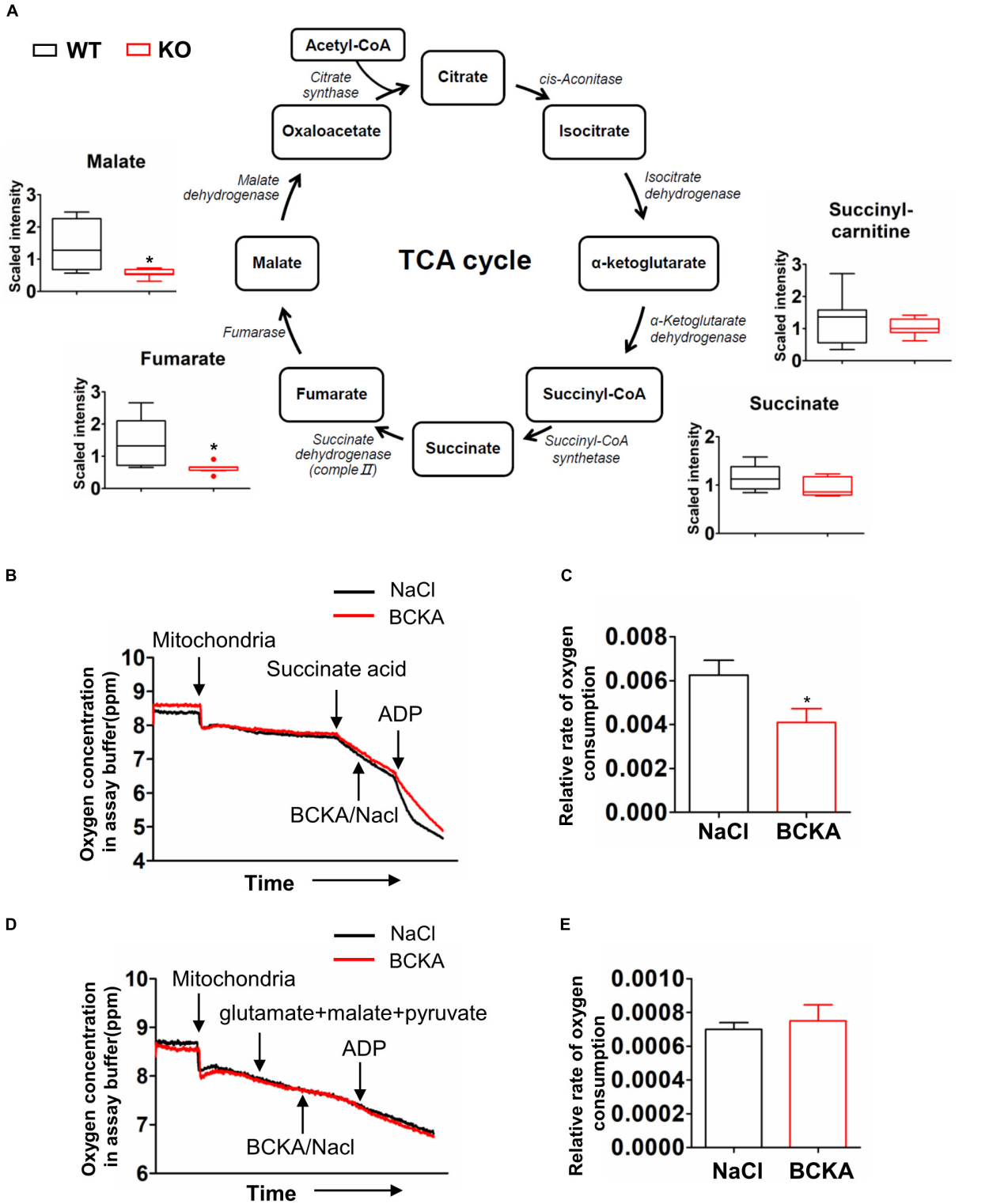


FIGURE 7 | Impacts of BCAA catabolic defect on TCA cycle. **(A)** Metabolomics analysis results showing the relative levels of metabolites mapped onto the TCA cycles in liver of wildtype (WT) and PP2Cm knockout (PP2Cm KO) mice (n = 8 for each group). **(B–E)** Oxygen consumption in mitochondria isolated from wildtype liver in absence or presence of BCKA-Na (500 μM each of KIC, KIV, KMV mixed) using Complex II **(B,C)** or Complex I **(D,E)** substrates, respectively. NaCl (1.5 mM) was used as control. Y-axis: oxygen concentration (ppm) in assay buffer. The assay was completed in ∼12 min. **(C,E)** Relative oxygen consumption rate in the absence or presence of BCKAs calculated based on results in panels (**B** or **D**) (n = 3 in each group; Data are represented as means ± SEM. *$p < 0.05$ vs. control), respectively.

(**Supplementary Figure S3B**), although this trend may primarily arise from an outlier data point.

DISCUSSION

In the present study, we investigated the impacts of BCAA catabolic defect on glucose metabolism in lean mice. Compared to wildtype mice on normal chow, the PP2Cm deficient mice with genetic defect in BCAA catabolism were leaner and favored carbohydrate over lipids for energy production, accompanied with enhanced glucose and insulin tolerance. Metabolomics profiling revealed distinct global metabolic patterns in PP2Cm deficient mice, characterized with tissue-specific BCAA catabolic defects and alterations in gluconeogenesis/glycolysis, glycogen metabolism, and TCA cycle. The predominant impacts of BCAA catabolic defect on glucose metabolism were observed in liver where BCAA catabolic defect led to the accumulation of BCKAs but not BCAAs. Interestingly, BCKAs regulated the gene expression of some key regulators of glucose metabolism and inhibited the succinate dehydrogenase/respiratory Complex II in isolated liver mitochondria, interrupting TCA cycle and mitochondrial oxidative phosphorylation. Together, these results clearly demonstrated the multiple impacts of BCAA catabolic defect on glucose metabolism in lean mice.

PP2Cm is a member of PP2C family and specific phosphatase of BCKD (Lu et al., 2009). Many metabolic phenotypes of PP2Cm deficient mice, such as lower body weight and enhanced insulin sensitivity, are similar to those of mice with BCAA dietary supplement or BCATm ablation (She et al., 2007a; Lynch and Adams, 2014). BCATm is the first enzyme in BCAA degradation pathway to catalyze the conversion of BCAA to BCKA. Ablation of BCATm leads to BCAA catabolic defect and dramatic BCAA accumulation. The similar phenotypes of these different BCAA experimental models strongly suggest that the altered glucose metabolism in PP2Cm deficient mice is caused by the BCAA catabolic defect. A recent study suggests PP2Cm and BCKDK also regulate the activity of a key lipogenic enzyme ATP-citrate lyase (ACL), showing BCKDK increases ACL phosphorylation and lipogenesis (White et al., 2018). Based on this observation, it can be speculated that PP2Cm ablation would enhance lipogenesis (White et al., 2018). However, the PP2Cm deficient mice are leaner compared with the wildtype control. In addition, the plasma levels of some free fatty acids, including stearate, are lower in PP2Cm deficient mice compared with those in control (data not shown). Although the biochemical processes of lipogenesis haven't been investigated in the current study, it is less likely that there is increased lipogenesis when the PP2Cm deficient mice are leaner. The details warrant further investigation.

Using metabolomics profiling, this study analyzed hundreds of metabolites involving in numerous major metabolic pathways in a high-throughput manner, generating a global view of the metabolic state of the PP2Cm deficient mice. One limitation of current metabolomics analyses is the limited number of metabolites. An analysis of more metabolites will certainly provide extra information for the metabolic alterations. Another limitation is the metabolomics results demonstrated the relative concentrations of metabolites, which can be further validated quantitatively. In the meanwhile, the relative plasma BCAA and BCKA levels in these metabolomics results corroborated the previous studies in which BCAA and BCKA levels were measured quantitatively (Lu et al., 2009). Finally, more mechanistic studies and dynamics metabolomics profiling of PP2Cm deficient mice following meal (or BCAA intake) would provide more insights into the metabolic alterations in PP2Cm deficient mice.

Liver, skeletal muscle, and adipose tissue contribute significantly to the whole body BCAA homeostasis, coordinating via the interorgan shuttling of BCAA and their metabolites (Hutson et al., 2005). Consistent with the global impairment of BCKD activity, BCAA and BCAA catabolic products formed upstream of BCKD are elevated in the plasma of PP2Cm deficient mice. Interestingly, some derivatives from products downstream of BCKD are also increased in PP2Cm deficient plasma, possibly resulting from the interorgan shuttling of BCAA catabolites. BCAAs are elevated in skeletal muscle and adipose tissue but not liver in the PP2Cm deficient mice. In contrast, BCKAs are significantly accumulated in skeletal muscle and liver in the PP2Cm deficient mice. The BCKD downstream catabolites are diminished in PP2Cm deficient skeletal muscle and white adipose tissues. In PP2Cm deficient liver, however, BCKD downstream catabolites are either elevated or diminished, indicating possible influence from the blood. Those data demonstrate great complexity in BCAA catabolism.

The major impacts of BCAA catabolic defect on glucose metabolism have been observed in liver but not adipose tissue or skeletal muscle. Liver is the key organ controlling global glucose metabolism via multiple metabolic pathways including, but not limited to, glycolysis and oxidation, gluconeogenesis, glycogenolysis and glycogenesis. Our data suggest that glycogen metabolism is enhanced in the PP2Cm deficient liver. The elevated glycogen catabolite levels may reflect increased degradation to replenish glucose and facilitate glycolytic metabolism in PP2Cm deficient liver, which is further supported by the elevated upstream glycolytic metabolites (glucose, glucose 6-phosphate and fructose 6-phosphate) in the PP2Cm deficient liver. Interestingly, the downstream glycolytic intermediates (fructose-1,6-biphosphate, 3-phosphoglycerate) are lower in the PP2Cm deficient liver. Since the upstream glycolytic metabolites are important compounds at the junction of several other metabolic pathways including the pentose phosphate pathway and the polyol pathway, it is possible that the elevated upstream metabolites in glycolysis enter these pathways, leading to the lower downstream metabolites.

Branched-chain keto acids may play a unique role in glucose metabolic regulation in liver. Higher BCKA, but not BCAA, abundances are detected in the PP2Cm deficient liver. BCKAs, but not BCAAs, markedly increases PYGL expression in HepG2 cell, consistent with the higher PYGL expression in PP2Cm deficient liver and indicating a direct regulation on glycogen metabolism by BCKAs. On the other hand, the metabolomics profile of TCA cycle in PP2Cm deficient liver is consistent with the inhibitory effects of BCKAs on respiration Complex II/succinate dehydrogenase in isolated mitochondria.

Interestingly, these impacts on liver glucose metabolism are not observed in PP2Cm deficient skeletal muscle or adipose tissue even BCKAs are elevated in these tissues. Thus, BCAA catabolism defect may affect glucose metabolism via BCKA accumulation in liver.

In the PP2Cm deficient mice, BCAA catabolic defect enhances insulin sensitivity in lean animals. Similarly, in another genetic model, inactivation of mitochondrial BCAT abolishes BCAA catabolism, resulting in lower adiposity and improved glucose tolerance in lean mice (She et al., 2007a). These beneficial impacts of BCAA catabolic defect on insulin sensitivity are opposite to its stimulatory effects on insulin resistance in obese mice, supporting the notion that BCAAs exert different metabolic effects depending on the catabolic and anabolic states (Bifari and Nisoli, 2017). The underlying mechanisms remain unclear. It has been suggested that tissue-specific alterations of BCAA catabolic flux may play a role in the obesity-associated insulin resistance. The reduced catabolism by some tissues, such as adipose tissue, shunts the BCAA oxidation toward other tissues, such as skeletal muscle, in obese mice, and promotes insulin resistance (Newgard, 2012; Neinast et al., 2018). In the genetic models of PP2Cm or BCATm knockout mice, however, the BCAA catabolism is likely impaired in all tissues. These observations indicate a critical role of the BCAA catabolism in skeletal muscle in determining insulin sensitivity. On the other hand, the reduced body weight (**Figure 1A**), adiposity, and white adipose mass (data not shown) in the lean PP2Cm KO mice may contribute to the enhanced insulin sensitivity. In addition, numerous studies have indicated that BCAA and BCKA can directly regulate pyruvate dehydrogenase complex activity (Li et al., 2017), mitochondrial respiration (**Figure 7**) (Sun et al., 2016), and insulin signaling and secretion (Lynch and Adams, 2014), which may all affect glucose metabolism in lean mice.

In summary, the current study shows BCAA catabolic defect affects glucose metabolism in lean mice. BCAA catabolic defect leads lower body weight and better glucose tolerance. These beneficial impacts are consistent with previous reports showing BCAA supplementation or BCAA-rich protein diets are associated with positive effects on body weight and glucose homeostasis (Lynch and Adams, 2014). On the other hand, recent studies show BCKD expression is reduced in obese and diabetic animals and humans, likely leading to BCAA catabolic defect and contributing to the development of insulin resistance and diabetes (She et al., 2007b, 2013; Pietiläinen et al., 2008; Herman et al., 2010; Lackey et al., 2013; Menni et al., 2013; Zimmerman et al., 2013; Cummings et al., 2018; White et al., 2018). Thus, the BCAA catabolic defect may show different even opposite impacts on glucose metabolism in lean and obese animals. While this discrepancy has been noted recently and the mechanisms remain unclear (Bifari and Nisoli, 2017), our results highlight the critical role of BCAA catabolism in glycemic regulation and the complex interplay among macronutrients.

DATA AVAILABILITY

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

Animal Subjects: The animal study was reviewed and approved by Committee for Humane Treatment of Animals at Shanghai Jiao Tong University School of Medicine or the University of California at Los Angeles Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

HS and MZ designed the research. JW, YL, XS, JS, and MC performed the research. YL, JW, KL, JF, MZ, and HS analyzed the data. YW helped to design the overall study and analyzed the data. HS, MZ, JW, and KL contributed to the manuscript preparation.

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SUPPLEMENTARY MATERIAL

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REVIEW
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Where Metabolism Meets Senescence: Focus on Endothelial Cells

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Despite the decline in their proliferative potential, senescent cells display a high metabolic activity. Senescent cells have been shown to acquire a more glycolytic state even in presence of high oxygen levels, in a way similar to cancer cells. The diversion of pyruvate, the final product of glycolysis, away from oxidative phosphorylation results in an altered bioenergetic state and may occur as a response to the enhanced oxidative stress caused by the accumulation of dysfunctional mitochondria. This metabolic shift leads to increased AMP/ATP and ADP/ATP ratios, to the subsequent AMPK activation, and ultimately to p53-mediated growth arrest. Mounting evidences suggest that metabolic reprogramming is critical to direct considerable amounts of energy toward specific activities related to the senescent state, including the senescence-associated secretory phenotype (SASP) and the modulation of immune responses within senescent cell tissue microenvironment. Interestingly, despite the relative abundance of oxygen in the vascular compartment, healthy endothelial cells (ECs) produce most of their ATP content from the anaerobic conversion of glucose to lactate. Their high glycolytic rate further increases during senescence. Alterations in EC metabolism have been identified in age-related diseases (ARDs) associated with a dysfunctional vasculature, including atherosclerosis, type 2 diabetes and cardiovascular diseases. In particular, higher production of reactive oxygen species deriving from a variety of enzymatic sources, including uncoupled endothelial nitric oxide synthase and the electron transport chain, causes DNA damage and activates the NAD^+-consuming enzymes polyADP-ribose polymerase 1 (PARP1). These non-physiological mechanisms drive the impairment of the glycolytic flux and the diversion of glycolytic intermediates into many pathological pathways. Of note, accumulation of senescent ECs has been reported in the context of ARDs. Through their pro-oxidant, pro-inflammatory, vasoconstrictor, and prothrombotic activities, they negatively impact on vascular physiology, promoting both the onset and development of ARDs. Here, we review the current knowledge on the cellular senescence-related metabolic changes and their contribution to the mechanisms underlying the pathogenesis of ARDs, with a particular focus on ECs. Moreover, current and potential interventions aimed at modulating EC metabolism, in order to prevent or delay ARD onset, will be discussed.

Keywords: cellular senescence, endothelial cells, age-related diseases, type 2 diabetes, metabolism, glycolysis

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INTRODUCTION

Aging is the leading single risk factor for the development of most, if not all, major age-related chronic diseases, such as neurodegenerative, cancer, metabolic and cardiovascular diseases (CVDs). Aging and age-related diseases (ARDs) share a common set of basic biological mechanisms, such as inflammation, the accumulation of macromolecular damage, adaptation to molecular and psychological stressors, epigenetic changes, metabolic dysfunction, loss of proteostasis, and defective stem cell function (Kennedy et al., 2014). Major ARDs include type 2 diabetes (T2DM), cardiovascular diseases (CVDs), osteoporosis and certain types of cancers (Olivieri et al., 2018; Prattichizzo, 2019). The low-grade, chronic, and systemic inflammation underlying the aging process and ARDs was called "inflammaging" (Franceschi et al., 2000; Fulop et al., 2018).

The notion that the lifespan of many species can be extended through reduction of energy intake (Speakman, 2005) suggests a critical role of macronutrient metabolism in the control of regulatory processes influencing proliferation, survival (Redman et al., 2018; Mitchell et al., 2019), and ARD development (Fontana et al., 2004). Accordingly, obesity is a risk factor for many ARDs and carries out a life-shortening action (Poirier et al., 2006). The onset of ARDs can be counteracted through overweight reduction by decreasing the energetic food and by increasing energy expenditure with physical activity (Stubbs and Lee, 2004; Everitt and Le Couteur, 2007; Fontana and Partridge, 2015). Notably, centenarians, individuals capable of reaching the extreme limit of human life and characterized by an exceptionally healthy phenotype, share features observed in human adult volunteers who followed caloric restriction (CR) regimens (Franceschi et al., 2018). All these evidences suggest that major pathways driving organismal aging are intimately connected with metabolism (Lopez-Otin et al., 2016), a hypothesis also confirmed by the fact that dysfunctional mitochondria are a common feature of aged cells and major ARDs (Lane et al., 2015; Correia-Melo et al., 2016).

Within the cells, energy is generated in the form of adenosine triphosphate (ATP), mainly through mitochondrial oxidative phosphorylation (OXPHOS) in the presence of oxygen, and through anaerobic glycolysis in its absence (Saraste, 1999). This "general rule" could be subjected to modifications during particular conditions, such as cellular senescence (Wiley and Campisi, 2016). Senescent cells (SCs) exhibit some peculiar characteristics, including growth arrest, telomere shortening, enhanced senescence associated (SA) β-Galactosidase activity, and the acquisition of a pro-inflammatory senescence-associated secretory phenotype (SASP), which is responsible for both inflammaging and the spreading of senescence to neighboring cells. Beyond the exhaustion of replicative potential, cellular senescence can also be triggered by different stressors, including hypoxia, endotoxin, and reactive oxygen species (ROS) (Campisi, 2013).

Endothelial cells (ECs) form the inner lining of blood vessels (Fishman, 1982). In adult organisms they are supposed to remain in a quiescent state, but they can be rapidly activated by a variety of stimuli (Schlereth et al., 2018). They provide a significant contribution to the transduction of signals between blood and tissues (Munoz-Chapuli et al., 2004); moreover, by the release of the gaseous mediator nitric oxide (NO), ECs play a crucial role in maintaining the vascular tone and in preventing platelet aggregation (Huang et al., 1995; Sabbatinelli et al., 2017). The integrity of the EC monolayer is a critical requisite to allow blood flow and avoid uncontrolled thrombosis (Giannotta et al., 2013). Growing evidence show that dysfunctional senescent ECs can play a key role in instigating the morphological and biochemical changes that accompany vascular dysfunction, thus not a mere epiphenomenon in pathogenesis of CVDs (Childs et al., 2014; Tian and Li, 2014). For this reason, senescence of ECs has become a central focus of the investigations on ARDs.

Here, we review the latest findings on the metabolic changes that occur during the aging process of endothelial cells. Moreover, due to EC strategic location in the human organism, here we support the hypothesis of a potential role of senescence-associated metabolic alterations of ECs in the onset and development of ARDs. Finally, the potential relevance of targeting specific EC metabolic features to counteract ARDs will be discussed.

METABOLIC REPROGRAMMING IN CELLULAR SENESCENCE

A peculiar feature of SCs is that they remain metabolically active, despite their growth arrest. Their high metabolic rate is intimately linked to SASP acquisition, but whether it is a cause or an effect of the inflammatory phenotype and altered proliferative status of SCs has yet to be established (Wiley and Campisi, 2016).

Recent literature confers to metabolic reprogramming a deterministic role in modulating the inflammatory responses of the innate immune cells (Van den Bossche et al., 2017): in macrophages, metabolic pathways not only provide energy but also regulate their phenotype and function. Lipopolysaccharide (LPS)(+IFNγ)-activated proinflammatory (i.e., M1) macrophages mediate host defense through an enhanced glycolytic metabolism and impaired mitochondrial OXPHOS. On the contrary, interleukin (IL)-4(13)-activated (i.e., M2) macrophages, which promote wound healing and Th2-mediated responses, mainly rely on OXPHOS for the synthesis of ATP. These data suggest that metabolic changes may also underlie acquisition of the SASP in senescent cells.

In the 1980s, the first attempts to analyze energy metabolism in aging cells showed that glucose consumption and lactate production are elevated in replicative senescent human diploid fibroblasts (HDFs) (Goldstein et al., 1982; Bittles and Harper, 1984). This "glycolytic state," accompanied by an imbalance of the activity of the glycolytic enzymes, results in a less energetic state, mirrored by the drop of ATP and GTP intracellular levels as cell cultures enter in replicative senescence (Zwerschke et al., 2003). More recently, metabolomic approaches on extracellular metabolites released by senescent fibroblasts confirmed a shift toward glycolysis compared to young cells (James et al., 2015). This general mechanism, however, is more or less pronounced depending on the cell type and shows some

exceptions, such as senescent human mammary epithelial cells where glucose consumption and lactate secretion do not increase (Delfarah et al., 2019).

The role of malic enzyme (ME) in senescent cells has been extensively investigated due to its role in maintaining cellular redox homeostasis. The two ME isoforms ME1 and ME2 catalyze the decarboxylation of malate to pyruvate with concomitant formation NADPH or NADH, respectively. Regeneration of reducing equivalents for anabolic processes in form of NADPH can be achieved either through the "malate oxidation shunt" or through the pentose phosphate pathway (PPP) (Fox et al., 1994). In the context of aging cell, p53 by binding to specific response elements, attenuates ME1 and ME2 transcription. At the same time ME depletion induces senescence by stabilizing p53, suggesting that also ME exerts an inhibitory effect on p53 (Jiang et al., 2013). Moreover, expression of both malate dehydrogenase (MDH)1, a mitochondrial tricarboxylic acid (TCA) cycle enzyme catalyzing malate oxidation to oxaloacetate, and MDH2, the cytosolic enzyme of malate-aspartate shuttle, declines during aging, leading to an impaired transfer of reducing equivalents into the mitochondria. In HDF, the subsequent decrease of cytosolic NAD^+/NADH ratio is accompanied by the induction of replicative senescence (Zwerschke et al., 2003; Lee et al., 2012; Wiley et al., 2016). The need to regenerate NAD^+ explains the upregulation of lactate dehydrogenase (LDH) in senescent HDFs, which release lactate to avoid the arrest of glycolysis due to both product accumulation and excessively low intracellular pH (Lang et al., 2003; Zwerschke et al., 2003) (**Figure 1**). These observations on the role of malate prompted the hypothesis that malate supplementation could delay senescence and extend lifespan (Edwards et al., 2013). Similar effects have been shown in *C. elegans* and *D. melanogaster*

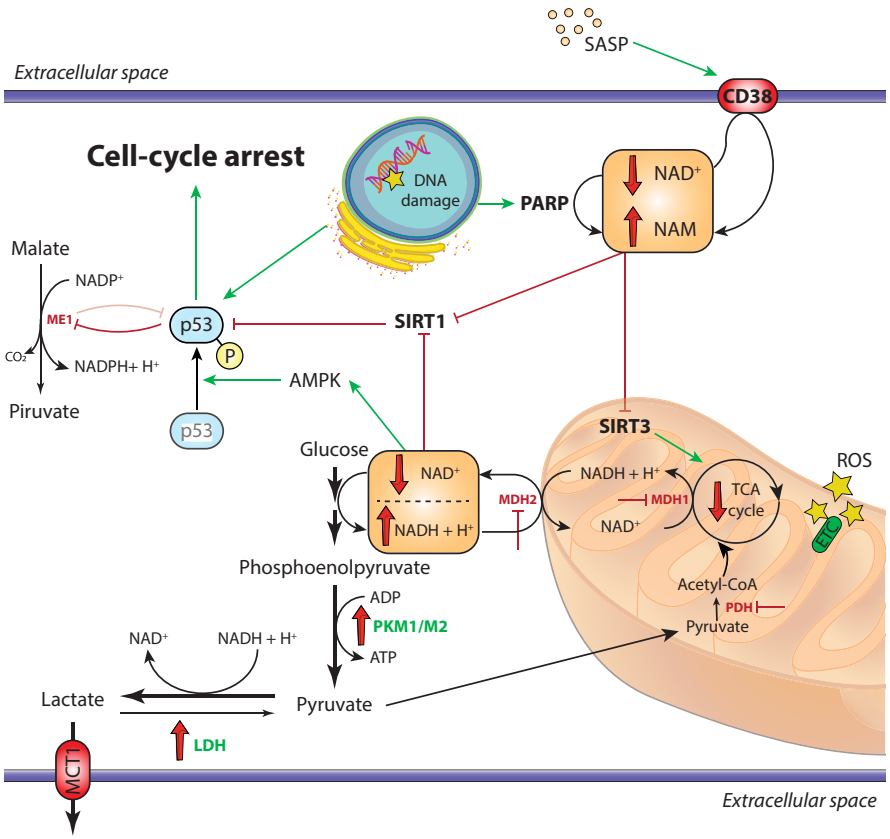

FIGURE 1 | Overview of the metabolic alterations driving cellular senescence. In cells undergoing replicative senescence, the upregulation of LDH and the inhibition of both PDH and the malate-aspartate shuttle (MDH1 and MDH2) result in the diversion of pyruvate away from oxidative phosphorylation and toward aerobic glycolysis. This leads to the decrease of cytosolic NAD^+/NADH ratio, which triggers the activation of the energy sensor AMPK. Moreover, the accumulation of DNA damage, also mediated by ROS in the dysfunctional mitochondria and the SASP, activate the NAD^+-consuming enzymes PARP1 and CD38, respectively. The subsequent impairment of SIRT1 deacetylase activity, coupled with the AMPK-mediated phosphorylation of p53, triggers the arrest of cell replication and the establishment of irreversible senescence. Moreover, activated p53 inhibits the activity of the malic enzymes ME1 and ME2, further impairing the cellular antioxidant mechanisms through reduction of NADPH levels. Downregulated enzymes are in red, upregulated enzymes are in green; red and green arrows indicate repression or induction, respectively. AMPK, adenosine monophosphate-activated protein kinase; ETC, electron transport chain; LDH, lactate dehydrogenase; MCT1, monocarboxylate transporter 1; MDH1/MDH2, malate dehydrogenase 1/2; ME1, malic enzyme 1; PARP, poly (ADP-ribose) polymerase; PDH, pyruvate dehydrogenase; PKM1/PKM2, pyruvate kinase M1/M2; ROS, reactive oxygen species; SASP, senescence-associated secretory phenotype; SIRT, sirtuin; TCA, tricarboxylic acid.

for α-ketoglutarate and oxaloacetate, two other TCA cycle intermediates (Williams et al., 2009; Chin et al., 2014; Su et al., 2019). However, such observation needs to be corroborated by evidence in mammals.

The role of PPP, the alternative source of NADPH, has been explored in cellular senescence. In fact, even if SCs have a lower demand of deoxyribonucleotides (dNTPs), they still require NADPH to allow the activity of the reactive oxygen species (ROS)-detoxifying thioredoxins, glutaredoxins and peroxiredoxins. Importantly, replicative senescence can be triggered by a lack of dNTPs due to reduced substrate availability. The activity of the first enzyme of the oxidative branch of PPP, namely glucose-6-phosphate dehydrogenase (G6PDH), is decreased during senescence. Accordingly, G6PDH-deficient cells exhibit accelerated oxidant-induced senescence (Ho et al., 2000; Cheng et al., 2004), a process that can be partially rescued by telomerase ectopic expression (Wu et al., 2009). Notably, transgenic mice overexpressing G6PDH display extended lifespan through increased NADPH levels (Nobrega-Pereira et al., 2016) and knockdown of the tumor suppressor ataxia-telangiectasia mutated (ATM) gene restores glucose flux throughout the PPP and allows cells to overcome senescence (Aird et al., 2015).

The findings on the metabolic features of replicative SCs have been only in part confirmed in oncogene-induced senescence (OIS). Seminal studies reported that cells undergoing OIS have high glycolysis rate, along with an elevated OXPHOS activity, when compared to proliferating cells (Quijano et al., 2012; Dorr et al., 2013; Kaplon et al., 2013; Takebayashi et al., 2015). In line with these findings, both pyruvate kinase (PK) isoforms, i.e., PKM1 and PKM2, and pyruvate dehydrogenase (PDH), two enzymes with a key role in glycolysis and in the conversion of pyruvate to acetyl-CoA, are upregulated in SCs. This leads to an enhanced use of pyruvate for the TCA cycle causing increased cellular respiration and redox stress (Zwerschke et al., 2003; Dorr et al., 2013; Kaplon et al., 2013). The TCA cycle could be also fueled by metabolites from fatty acid catabolism. Indeed, Ras-induced senescent cells manifest a decline in lipid synthesis and an increase in fatty acid oxidation (FAO), which results in a higher rate of basal oxygen consumption (Quijano et al., 2012). Inhibition of carnitine palmitoyltransferase 1A (CPT1A), the key rate-limiting enzyme for oxidation of free fatty acids (FFA) into the mitochondria, prevented senescence and SASP establishment (Quijano et al., 2012). In a recent work, Fafián-Labora et al. demonstrated that fatty acid synthase (FASN) activity is important for mitochondrial bioenergetics in the initial phases of senescence. FASN is an enzyme that catalyzes *de novo* synthesis of fatty acids by combining malonyl-CoA to the acetyl-CoA derived from glycolysis-produced pyruvate. Indeed, inhibition of FASN activity prevented the p53-mediated induction of senescence, the secretion of the canonical SASP factors IL-1α, IL-1β, IL-6, and the release of extracellular vesicles (EVs) mediating the spread of pro-senescence signals at the paracrine level (Borghesan et al., 2019; Fafian-Labora et al., 2019). Notably, other studies reported that p53 activation inhibits FASN, suggesting a negative feedback loop (Ford, 2010).

High lipogenesis would explain the progressive accumulation of membranous organelles during cell senescence (Kim et al., 2010). It is well known that lysosome mass expands during senescence, recently an increase in mitochondrial mass was also established (Correia-Melo et al., 2016; Fafian-Labora et al., 2019). A considerable number of senescent−associated changes are dependent on mitochondria. Indeed, mitochondrial dysfunction can elicit a specific type of proinflammatory phenotype, defined as mitochondrial dysfunction-associated senescence (MiDAS) (Wiley et al., 2016). MiDAS differs from the prototypical SASP for the lack of an IL-1/NF-κB-dependent mechanism. In MiDAS, a reduced NAD^+/NADH ratio is believed to trigger adenosine monophosphate-activated protein kinase (AMPK) and p53 activation (Wiley et al., 2016; Giuliani et al., 2017).

NAD^+/NADH ratio is one of the most reliable markers of the redox state of the cell. Its decrease has been reportedly linked to cellular senescence (Son et al., 2016; Zhang et al., 2016). NAD^+ decline in aging is extensively reviewed by Verdin (Verdin, 2015). Low levels of NAD^+ were also reported in several tissues (Massudi et al., 2012; Zhu et al., 2015), and supplementation of NAD^+ precursors increased life span in different species (Fang et al., 2016; Zhang et al., 2016). Interestingly, SCs were shown to induce the SASP-mediated expression of CD38 – an ectoenzyme with a high NADase activity – in non-senescent cells, such as endothelial cells and bone marrow-derived macrophages (Chini et al., 2019; Covarrubias et al., 2019). CD38 inhibitors rescued NAD^+ decline and ameliorated a number of age-related metabolic outcomes in mice (Tarrago et al., 2018). A similar beneficial effect on mouse lifespan has been described also for the EV-mediated cell-to-cell transfer of the extracellular isoform of nicotinamide phosphoribosyltransferase (NAMPT), the rate-limiting enzyme of NAD^+ salvage pathway (Yoshida et al., 2019). EVs are membrane-coated nanoparticles actively released by almost all cell types, including ECs (Jansen et al., 2017). EVs are usually categorized according to their size and surface markers, and are able to shuttle and deliver functional proteins and nucleic acids in a paracrine and systemic manner (Thery et al., 2018).

Recently, Nacarelli et al. (2019) demonstrated a direct link between intracellular NAD^+ levels and the SASP in OIS. High SA-chromatin remodeling is related to the upregulation chromatin-binding protein High-Mobility Group A1 (HMGA1) which binds genomic A + T reach regions to increase chromatin accessibility (Nacarelli et al., 2019). Interestingly, inhibition or silencing of NAMPT, one of the targets of HMGA1, decreased glycolysis, mitochondrial respiration, and oxygen consumption, along with NAD^+/NADH ratio, which is normally elevated in OIS (Nacarelli et al., 2019; Søgaard and Gil, 2019). The subsequent increase of ADP/ATP ratio activates AMPK, which leads to p53-mediated inhibition of p38MAPK. By activating NF-κB, p38MAPK acts as an important up-stream effector of the SASP (Freund et al., 2011). Therefore, in OIS a high NAD^+/NADH ratio finally results in the activation of the SASP. The apparently contrasting observation in different models of senescence lends support to the hypothesis that NAD^+ metabolism specifically controls distinct subsets of SASP factors. Indeed, MiDAS is triggered by a drop of NAD^+/NADH and leads

to IL-10 and TNF-α production (Wiley et al., 2016), whereas OIS boosts NAD^+ levels, in contrast with the general decline of NAD^+/NADH ratio observed in aging and confirmed in replicative senescence (Zwerschke et al., 2003).

We can conclude that dramatic alterations of carbohydrate and lipid metabolism occur in senescent cells, with divergent outcomes partially depending on senescence trigger. In general, senescent cells acquire a "glycolytic state," but the fate of the resulting pyruvate changes between replicative and oncogene/therapy induced senescence. Notably, the pyruvate "hub" has been promoted as a druggable target for treatment of many diseases, including diabetes, ischemic heart disease, and cancer (Roche and Hiromasa, 2007; Olenchock and Vander Heiden, 2013). By directing specific substrates to one pathway or another, the entire metabolic set-up of the cell can be affected, in some cases reaching a desirable effect.

METABOLIC FEATURES OF ENDOTHELIAL CELLS

Although they share common characteristics, ECs can exhibit several phenotypic differences, depending on the specific chemical and physical characteristics of the vascular districts in which they are living (Dejana et al., 2017). ECs in the microvasculature are involved in the bidirectional exchange of gases, macromolecules, and cells between tissues and blood, and can also perform enzymatic modifications of circulating mediators, such as lipoproteins and angiotensin I (Chi et al., 2003). ECs actively participate in angiogenesis, i.e., the generation of new capillaries from existing vessels; in the adult physiological angiogenesis occurs mainly in the female reproductive system and in wound healing, however it plays a significant role in promotion and progression of many pathological conditions, including cancer and chronic inflammation (Potente and Carmeliet, 2017). Angiogenesis implies a switch of selected ECs toward a proliferative and migratory phenotype (Jimenez-Valerio and Casanovas, 2017). These ECs are known as tip and stalk cells, the first guiding the migration of the latter to achieve the elongation of the developing vessel (Jakobsson et al., 2010). Angiogenesis is a high energy-demanding process, therefore, from their original quiescent state, ECs must undergo a substantial reprogramming of their metabolism.

Quiescent ECs rely mainly on glycolysis for their energy demanding, despite the high abundance of oxygen in the vascular compartment. Accordingly, even if the mitochondrial mass in ECs is variable depending to the vascular district; it is generally lower than other cell types (Eelen et al., 2018). During angiogenesis, vascular endothelial growth factor (VEGF) signaling induces in ECs a further boost of glycolysis, with the upregulation of the GLUT-1 transporter and an increased synthesis of the allosteric modulator fructose-2,6-bisphosphate (F2,6BP) by the enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3 (PFKFB3), a master regulator of glycolysis (De Bock et al., 2013). In this context, EC mitochondrial metabolism remains largely underexplored. While a substantial amount of evidence is available on the impairment of angiogenesis under glucose-deprivation conditions (He et al., 2013; Terashima et al., 2013; Garcia et al., 2015; Gu et al., 2019), only one very recent report demonstrated that also the inhibition of electron transport chain decreases EC proliferation. Interestingly, this effect is related to a lowered NAD^+/NADH ratio, rather than to a decreased availability of ATP (Diebold et al., 2019).

The notion that ECs exhibit different glycolytic rates according to their specialization or proliferation rate is quite intuitive, however, glycolysis is not only an energy supplier for these cells but can modulate their function and phenotype. Under hypoxic conditions, the hypoxia inducible factor 1-alpha (HIF-1α)-mediated upregulation of the glycolytic pathway, coupled with the downregulation of PDH, induces an accumulation of lactate, which in turn i) stabilizes HIF-1α and mediates a paracrine proangiogenic effect on neighboring ECs (Sonveaux et al., 2012; Lee et al., 2015), ii) affects the functional polarization of tumor-associated macrophages toward the pro-tumoral M2 phenotype (Colegio et al., 2014). EC phenotype can be changed through targeting enzymes or regulators of the glycolysis. The forkhead box O transcription factor 1 (FOXO1) plays a major role in maintaining ECs in a quiescent state by suppressing c-myc signaling and reducing glycolysis (Wilhelm et al., 2016). Similarly, the downregulation of hexokinase 2 (HK2) via the disruption of the fibroblast growth factor receptor (FGFR)/c-myc axis impairs proliferation and migration of ECs (Yu et al., 2017). The interplay between the two PK isoforms represents an important crossroad in determining the fate of the resulting pyruvate. In a model of pulmonary arterial hypertension, the increased PKM2 expression fosters aerobic glycolysis and induces a more proliferative state (Caruso et al., 2017). On the contrary, under physiological conditions, the laminar blood flow promotes the NO-mediated S-nitrosylation of PKM2, which results in a reduced glycolysis and in an enhanced funneling of substrates through the PPP (Siragusa et al., 2019). In cells with a low replication rate, like quiescent ECs, a balanced PPP activity promotes antioxidant responses through synthesis of reducing equivalents in form of NADPH.

The metabolic features of senescent ECs are still poorly explored. Moreover, whether the effects of EC senescence, including the SASP, could be mediated by metabolic changes is still being debated. An emerging role for SIRT3, a member of the sirtuin family mainly localized in the mitochondria, in cellular aging has been outlined by recent studies (Ansari et al., 2017). Through its NAD-dependent deacetylase activity, SIRT3 regulates the mitochondrial metabolic pathways, including FAO (Kanwal, 2018). Specifically, SIRT3 promotes the influx of substrates into the TCA cycle by promoting the activity of PDH and acyl-CoA dehydrogenase (Kincaid and Bossy-Wetzel, 2013). Moreover, SIRT3 seems to exert a positive regulator effect on the TCA cycle, even if results are still contradictory (Verdin et al., 2010). Similarly to the other members of the sirtuin family, depletion of SIRT3 has been shown to reduce human lifespan (Bellizzi et al., 2005; Brown et al., 2013). Notably, strategies aimed to upregulate SIRT3 in endothelial cells resulted in a higher protection against stress-induced premature senescence, an effect mediated by the deacetylation of FoxO3 (Liu et al., 2015; Xing et al., 2018). For some aspects, ECs seem to escape the

rules describing the senescence-associated metabolic shift that have been postulated from studies in other cell lineages. For example, the observation that HDF senescence is accompanied by an upregulation of the whole glycolytic machinery was not confirmed in HUVECs (Unterluggauer et al., 2008). Conversely, a recent report showed a senescence-associated decline in EC glycolysis, which is mediated by a reduced PFKFB3 activity. This trend, but not senescence, was reverted by nuclear factor erythroid 2-related factor 2 (NRF2) overexpression (Kuosmanen et al., 2018). Another study on EC replicative senescence revealed that a NAMPT/SIRT1/FoxO1-mediated slight increase in aerobic glycolysis exerts a protective effect by limiting ROS production (Borradaile and Pickering, 2009).

Glutamine represents another important metabolite for ECs. It can be used as an energy source, via deamination and subsequent transamination to form α-ketoglutarate which enters the TCA cycle, to produce the antioxidant peptide glutathione, or to provide substrates for nucleotide biosynthesis (Eelen et al., 2015). Glutamine was shown to be required for vessel sprouting, even if the impaired angiogenesis during glutamine starvation could be rescued by asparagine supplementation (Huang H. et al., 2017). The notion that senescent ECs strongly rely on glutaminolysis for their energy demand comes from the observation in these cells of an increased lactate synthesis independent from glycolysis. Remarkably, the inhibition of glutaminase 1 (GLS1) is able to induce apoptosis and senescence even in young ECs (Unterluggauer et al., 2008).

Although ECs obtain a minor amount of ATP from oxidative phosphorylation, they efficiently oxidize fatty acids: ECs are endowed with all the proteins required for the uptake and intracellular transport of fatty acids (Hagberg et al., 2013). Indeed, in the microcirculation, ECs can extract fatty acids from circulating lipoproteins through lipoprotein lipase (LPL). Fatty acids are then absorbed through the fatty acid transport proteins (FATP)3 and FATP4 and bound by the intracellular fatty acid binding protein FABP4. Fatty acids can then be oxidized to provide carbons to replenish TCA cycle, thus allowing the synthesis of dNTP precursors, or directed to the surrounding tissues (Mehrotra et al., 2014). In a recent report, Kalucka et al. showed that fatty acid oxidation is the only upregulated metabolic pathway in quiescent ECs. Notably, beta-oxidation is increased neither for bioenergetic purposes nor to meet the anabolic demands of the cells. Rather, fatty acids are oxidized to increase NADPH regeneration by the malic enzyme once their carbons enter the TCA cycle in form of acetyl-CoA. As a result, quiescent ECs have higher amounts of reduced glutathione and thus are more protected against oxidative stress (Kalucka et al., 2018). Interestingly, this beneficial effect is mediated by Notch1 signaling, which exerts also a central role during the earlier phases of senescence, by switching the secretome of ECs away from the pro-inflammatory SASP and toward the TGF-β-mediated release of immunosuppressive and fibrogenic factors (Hoare et al., 2016).

Cellular senescence also involves endothelial progenitor cells (EPCs), a population of circulating $CD34^+$ cells participating in new vessel formation and in vascular remodeling through their ability to differentiate into ECs. The age-related decline in EPC survival and regeneration could precipitate endothelial dysfunction, thus triggering the onset of CVDs (Olivieri et al., 2016). It has been demonstrated that the increased susceptibility of elderly individuals to ischemic disorders could be mediated, at least in part, by a blunted response of senescent EPCs to hypoxia, which fail to upregulate selected genes related to HIF-1 signaling and glucose uptake (Wu et al., 2018). To this regard, the upregulation of SIRT1 and NRF2 have both been shown to delay EPC senescence and to preserve the proliferative, migratory, and angiogenic activities in senescent EPCs (Lamichane et al., 2019; Wang et al., 2019).

Investigations on EC metabolic features could take advantage of their peculiar localization and lend important insight into the pathogenesis of CVDs. Targeted metabolomics of large cohorts of samples are required to get a more comprehensive point of view on mechanisms which were extensively characterized in *in vitro* models. While multiomics approaches proved useful to identify circulating signatures with a diagnostic and/or prognostic role for many conditions (Zierer et al., 2015; Correia et al., 2017; Niewczas et al., 2019), their role in providing *ex vivo* mechanistic clues is hampered by the lack of information on the relative contribution of various tissues to the circulating metabolome. To address this issue, the field is moving toward more specific approaches allowing the deconvolution of complex circulating signatures, including the application of machine learning tools and the study of EVs (Heitzer et al., 2019). A growing body of evidence is focusing on the characterization of endothelial EVs isolated from plasma (Igami et al., 2019; Mork et al., 2019; Oliveira et al., 2019; Utermohlen et al., 2019). Even if a consensus on the methods for isolation and characterization of these EVs is far from being reached, it is important to remark that the profiling of the cargo of circulating EVs sorted according to the parent cells allows enhanced specificity compared to single molecular markers in the blood.

Figure 2 provides an overview of the most relevant physiologic and pathologic pathways in ECs. Understanding the mutual influence between EC senescence and the complex network of metabolic pathways could provide valuable insight into the pathogenesis of many ARDs. This will be the focus of the next sections.

ENDOTHELIAL CELL METABOLISM IN AGE-RELATED DISEASES

Increasing evidence is suggesting a bidirectional interplay among EC metabolism and ARDs (Graupera and Claret, 2018). One of the most characterized metabolic alteration in ECs exposed to hyperglycemia, i.e., a known senescence-promoting stimulus (Prattichizzo et al., 2018a), is the diversion of glycolytic intermediates into alternate pathways (**Figure 2**). This is the result of the activation of the enzyme polyADP-ribose polymerase 1 (PARP1) following the ROS-induced DNA damage. PARP1 mediates the ribosylation of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and depletes NAD^+ intracellular levels, further inhibiting the glycolysis flux (Du et al., 2003). Moreover, entry of glucose 6-phosphate into the PPP is

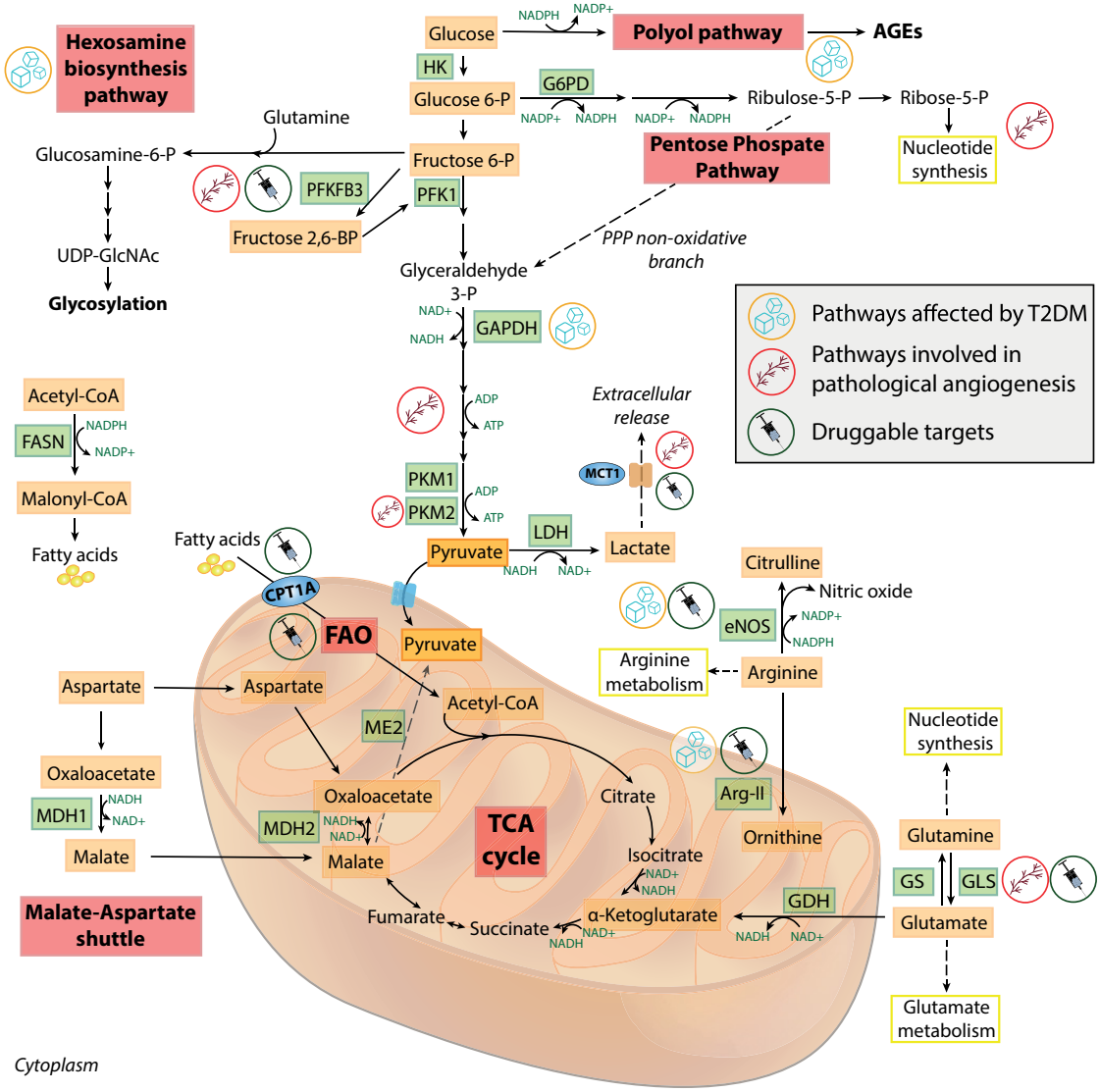

FIGURE 2 | Metabolic features of healthy and dysfunctional endothelial cells. Schematic overview of the most relevant metabolic pathways in endothelial cells. Pathways, enzymes and metabolites affected by T2DM or involved in pathological angiogenesis, and the druggable targets discussed in the text, are labeled by specific icons. AGEs, advanced glycation end-products; Arg-II, arginase II; CPT1A, carnitine palmitoyltransferase 1A; eNOS, endothelial nitric oxide synthase; FAO, fatty acid oxidation; FASN, fatty acid synthase; G6PD, glucose 6-phosphate dehydrogenase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GDH, glutamate dehydrogenase; GLS, glutaminase; GS, glutamine synthetase; HK, hexokinase; LDH, lactate dehydrogenase; MCT1, monocarboxylate transporter 1; MDH1/MDH2, malate dehydrogenase 1/2; ME2, malic enzyme 2; PFK1, phosphofructokinase 1; PFKFB3, 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3; PKM1/PKM2, pyruvate kinase 1/2; PPP, pentose phosphate pathway; T2DM, type 2 diabetes; TCA, tricarboxylic acid; UDP-GlcNAc, uridine diphosphate N-acetylglucosamine.

also restricted, due to a cAMP-mediated impairment of glucose 6-phosphate dehydrogenase during hyperglycemia (Zhang et al., 2010). The reduced PPP activity leads to lowered NADPH levels, causing both a reduced endothelial nitric oxide synthase (eNOS) activity and a blunted antioxidant response. In addition, excessive glucose is metabolized into the pathologic polyol and hexosamine biosynthetic pathways, which are the major contributors to the generation of advanced glycation end-products in diabetes (Berrone et al., 2006). The aberrant non-enzymatic glycation of circulating and intracellular proteins is a key determinant of T2DM cardiovascular complications (Mapanga and Essop, 2016). Endothelial dysfunction (ED) and senescence are "historically" considered two consequences of T2DM (Bonfigli et al., 2016; Prattichizzo et al., 2018a,d), possibly as a result of the overabundance of senescence-promoting, endothelium-damaging factors in the bloodstream of these patients, e.g., pro-oxidant molecules, glucose, and lipids (Prattichizzo et al., 2018c). In particular, the role of EC senescence has been emphasized in both the development of T2DM and of its CV complications. Indeed, EC senescence has been found in the adipose tissue of obese subjects preceding the development of T2DM (Minamino et al., 2009; Villaret et al., 2010), as well as

in other vascular districts in diabetic mice and humans (Orimo et al., 2009; Prattichizzo et al., 2018a; Yokoyama et al., 2019). On the other side, the observation that ED development often precedes the appearance of the T2DM phenotype in obese subjects (Hadi and Suwaidi, 2007), coupled by the intuitive assumption that the endothelium represents the biggest organ regulating nutrient availability across the whole organism, has prompted the research toward the study of the effect of selective gain/loss of function of metabolic genes in ECs (Graupera and Claret, 2018).

A seminal paper suggested that deletion of insulin receptor (IR) in ECs is sufficient to alter the expression of eNOS and endothelin-1 in mice treated with a high-salt diet, providing the first link among EC metabolism and general vascular tone (Vicent et al., 2003). Later, another group showed that EC-specific Irs2 KO mice have a blunted insulin-dependent glucose uptake in the muscles when fed with HFD (Kubota et al., 2011). Beyond insulin signaling, also the selective modulation in ECs of genes involved in angiogenesis, i.e., VEGF/VEGFR2, ANG2/TIE2, and DLL4/NOTCH1 (Robciuc et al., 2016; Seki et al., 2018), as well as in fatty acids transport, i.e., VEGF-B/NRP1 and CD36, affect whole body metabolism (Hagberg et al., 2010; Son et al., 2018). Interestingly, both promotion and inhibition of angiogenesis have been shown to foster insulin sensitivity, suggesting a context-dependent effect (Sun et al., 2012). Proposed mechanistic explanations include the induction of apoptosis in dysfunctional adipocyte (for restricted angiogenesis) and "healthy" expansion of adipose tissue (for enhanced angiogenesis) (Graupera and Claret, 2018).

While the role of senescent EC metabolism has not been studied in relation to specific ARDs, a key role for an alteration of metabolism in senescent ECs may be inferred by studies modulating key factors involved in the senescence process. Indeed, mice with EC-specific p53 deficiency fed a high fat diet (HFD) showed improvement of insulin sensitivity and less fat accumulation compared to WT mice (Yokoyama et al., 2014). Of note, p53 is a master transcription factor promoting senescence in virtually all cell types (Hafner et al., 2019). Similarly, NF-κB activation is held to be a cornerstone of SASP development in a plethora of different SCs, including ECs (Salminen et al., 2012). Transgenic mice expressing dominant-negative IκB under the Tie2 promoter/enhancer (thus with functional inhibition of NF-κB signaling specifically in ECs) were protected from the development of insulin resistance associated with both genetic and diet-induced obesity. Strikingly, these mice showed also an increase in lifespan, coupled by a decreased age-related insulin resistance and vascular senescence (Hasegawa et al., 2012). The relevance of these findings for human ARDs remains to be tested. However, an increased expression of p53, along with an increased activation of NF-κB has been observed in aged arteries from human subjects (Donato et al., 2008; Morgan et al., 2013).

Beyond T2DM and vascular function, also heart remodeling has been shown to be affected by metabolic alterations in ECs. Indeed, genetic ablation of Rbp-jκ (Notch signaling) in ECs promoted alterations in fatty acid metabolism in the whole organism, followed by heart hypertrophy and failure (Jabs et al., 2018). The deprivation of available FFAs as substrate prompted the use of glucose in cardiomyocytes and the consequent activation of the mTOR pathway, a master metabolic rheostat regulating both cellular senescence and organismal aging (Weichhart, 2018). Accordingly, a ketogenic diet was able to restore normal cardiac function in this mouse model (Jabs et al., 2018), suggesting the potential of dietary intervention to treat (and not only prevent) also life-threating ARDs. A similar framework has been proposed also to explain the cardioprotective properties of sodium-glucose cotransporter (SGLT)-2 inhibitors (i) (Ferrannini et al., 2016). SGLT-2i are a recently introduced class of glucose-lowering drugs inhibiting the reabsorption of glucose in the proximal convoluted tubule, thus promoting glucose elimination through the kidneys (Santos-Gallego et al., 2019). Clinical trials have shown a striking benefit in terms of CV mortality and worsening of heart failure in diabetic patients treated with SGLT-2i, an effect not ascribable to an improved glycemic control (Prattichizzo et al., 2018b). It has been hypothesized that the decreased availability of glucose, coupled by an increase in the circulating levels of ketone bodies (KB), improves the energetic function of the heart (Ferrannini et al., 2016; Prattichizzo et al., 2018b). This hypothesis has been recently tested in porcine, non-diabetic hearts, where the treatment with a SGLT-2i switches myocardial fuel utilization away from glucose toward KB, FFA, and branched chain amino acids, thereby improving myocardial energetics, enhancing left ventricular systolic function, and ameliorating adverse left ventricle remodeling (Santos-Gallego et al., 2019).

The promotion of myocardial function by shifting toward FFA utilization is likely dependent on the function of the liver, rather than EC metabolism. However, EC metabolism may influence the pathobiology of different organs by at least 3 mechanisms: (i) by releasing active secondary mediators, such as NO (Kubota et al., 2011; Yokoyama et al., 2014); (ii) by regulating the flow of lipids from bloodstream to organs (Hagberg et al., 2010; Jabs et al., 2018); (iii) by regulating vessel density, and thus indirectly nutrients availability and interstitial insulin levels (Robciuc et al., 2016). Beyond adipose tissue and muscle insulin sensitivity, the latter mechanism has been shown to be crucial for the development of age-induced osteoporosis (Ramasamy et al., 2016). Indeed, skeletal blood flow and endothelial Notch activity are reduced in aged mice, leading to decreased angiogenesis and osteogenesis, which can be reverted by genetic reactivation of Notch (Ramasamy et al., 2016). As mentioned above, Notch is regarded as a central metabolic sensor and regulator in multiple cell types.

Regarding possible secondary mediators released by ECs, the role of EVs deserves particular attention. The effects of EVs are now attracting intense interest also in the context of aging and ARDs (Prattichizzo et al., 2019). For instance, senescent ECs secrete miR-31 enriched EVs to inhibit mesenchymal stem cells osteogenic differentiation, possibly contributing to age-induced osteoporosis (Weilner et al., 2016). Of note, miR-31 is upregulated by high-glucose and inhibits cell differentiation also in other contexts (Zhen et al., 2017).

Overall, increasing evidence is suggesting a key role for EC metabolism in a plethora of ARDs, while less studies are available regarding the effect of the specific alterations of

senescent EC metabolism in the whole organisms. However, the emerging roles of endothelial p53 and NF-κB, two cornerstones of the senescence process, in the regulation of both cellular and organismal metabolism suggest that metabolic alterations in specific senescent cells, and in particular ECs, can affect whole body metabolism and ARD development, a hypothesis deserving exploration in the future.

THERAPEUTIC INTERVENTIONS TARGETING ENDOTHELIAL CELL METABOLISM

Thirty years ago, the identification and cloning of VEGF paved the way to the development of novel strategies aimed to treat those conditions in which angiogenesis plays a dominant role (Apte et al., 2019). Agents targeting members of the VEGF family and their receptors are currently routinely employed in the treatment of many solid malignancies, including colorectal cancer, renal cell carcinoma, and non-small cell lung carcinoma (Tirumani et al., 2015), and in non-neoplastic conditions with a recognized angiogenic component, such as proliferative diabetic retinopathy and age-related macular degeneration (Virgili et al., 2018). Investigations on the mechanism of action of anti-VEGF agents helped to highlight the aforementioned interesting connections between angiogenesis and EC metabolism and offered a number of novel druggable targets that could prove useful, for example, in the case of failure of anti-VEGF treatments.

Targeting the high-glycolytic state of ECs within tumor vessels is emerging as an anti-cancer therapeutic strategy (Fitzgerald et al., 2018). In a murine model, pharmacological inhibition of glycolysis activator PFKFB3 promotes the normalization of tumor vessels and facilitates delivery of chemotherapeutic drugs. Moreover, PFKFB3 tightened ECs by upregulating vascular endothelium (VE)-cadherin, thus reducing the passage of cancer cells across the EC monolayer (Cantelmo et al., 2016). Interestingly, inhibition of PFKFB3 lowered the expression of adhesion molecules in ECs treated with IL-1β, suggesting that limiting glycolysis could represent a feasible approach to prevent the SASP-mediated spreading of EC senescence. PFKFB3 inhibitors recently reached the clinical trial phase, with promising results from a phase 1 multi-center study conducted on patients with solid tumors (Redman et al., 2015).

Given the proangiogenic role of lactate in ECs, several studies focused on target the monocarboxylate transporter 1 (MCT1) to avoid lactate exchange across ECs. Lactate mediates angiogenesis through the activation of the NF-κB/IL-8 pathway and the stabilization of HIF-1α (Vegran et al., 2011; Sonveaux et al., 2012). Evidence on cell and animal models revealed that MCT1 inhibition in ECs can drive direct anti-angiogenic effects through the enhanced degradation of HIF-1α. Drugs targeting MCT1 in a non-selective manner are currently been tested in small-scale trials (Kershaw et al., 2015). On the other hand, administration of the telomerase activator TA-65 contributes to improve blood flow recovery through increasing expression of HIF-1α, VEGF-A, and peroxisome proliferator-activated receptor (PPAR)-γ coactivator 1-alpha (PGC-1α), indicating that telomerase activation could prove a valuable therapeutic option to rescue ischemic tissues in elderly individuals (Kokubun et al., 2019).

Targeting EC metabolism could prove useful in treating a plethora of conditions sharing endothelial dysfunction as a pathogenic mechanism. Evidence of an age-related impairment of endothelial function dates back to 1990s. The identification of eNOS as the enzyme responsible for NO synthesis prompted the supplementation of its precursor L-arginine to reverse endothelial dysfunction (Chauhan et al., 1996). However, the enthusiasm for this essential amino acid as an easy therapeutic option in the prevention of many CVDs was tempered by the observation that L-arginine could even exert detrimental effects on vascular function through an inductive effect on arginase, which competes with NOSs for their common substrate (Hayashi et al., 2006; Schulman et al., 2006; Caldwell et al., 2015). For this reason, attention has moved to arginase as a putative target to ameliorate age-related endothelial dysfunction. A major contribution of its activity in determining microvascular dysfunction and remodeling has been outlined in obesity (Chung et al., 2014; Masi et al., 2018), arterial hypertension (Michell et al., 2011), and T2DM (Shemyakin et al., 2012). Interestingly, knockout of the gene encoding for the mitochondrial arginase type II (Arg-II) has been shown to restore eNOS function, to counteract the SASP in senescent ECs (Wu et al., 2015), and to extend lifespan in mice through the inhibition of mTOR signaling (Xiong et al., 2017). Moreover, mTOR blockade with rapamycin decreases the expression of the arterial senescence marker p19 and ameliorates oxidative stress- mediated endothelial dysfunction in old mice, suggesting a possible role for the rapamycin analogs, i.e., rapalogs, in the treatment of age-related CVDs (Lesniewski et al., 2017).

Fenofibrate, a PPAR-α agonist, is a common lipid-lowering drug exerting a number of interesting pleiotropic effects. By increasing EC eNOS expression and lowering circulating oxidized LDL, fenofibrate ameliorated the age-related endothelial dysfunction in a cohort of healthy individuals (Walker et al., 2012). Further mechanistic studies on animal models revealed that the eNOS stimulation, along with other fenofibrate effects, is mediated by enhanced AMPK activity following liver kinase B1 (LKB1) translocation from the nucleus to the cytoplasm (Sohn et al., 2017; Xin et al., 2019; Xu et al., 2019). Additionally, fenofibrate lowered the cyclooxygenase 2-mediated production of vasoconstrictor prostaglandins (Xu et al., 2019). By restoring the balance between vascular relaxation and contractility, fenofibrate could represent a feasible preventive approach for the vascular complications of T2DM. Notably, fenofibrate ameliorated osteoarthritis in elderly patients by selectively clearing senescent chondrocytes (Nogueira-Recalde et al., 2019). The observation of a similar senolytic effect on ECs would provide additional benefits for this largely employed drug also in the treatment of a number of age-related vascular conditions.

Investigations into a variety of diseases highlighted a role for CPT1, the rate-limiting enzyme for FAO, as a druggable target (Dai et al., 2018; Melone et al., 2018). Inhibition of endothelial CPT1a impairs EC proliferation and activates the endothelial-to-mesenchymal transition, which plays a role in the

TABLE 1 | Summary of the interventions targeting endothelial cell metabolism with a potential role in the treatment of age-related diseases.

Pathway/ mechanism	Intervention	Experimental model	Outcome(s)	References	Progression to the clinical trial stage
Glycolysis	Genetic inhibition of PFKFB3	Tumor ECs from C57BL/6 mice livers	Tightening of the vascular barrier, decreased expression of cancer cell adhesion molecules in ECs, improved delivery of chemotherapeutic drugs	Cantelmo et al., 2016	Phase 1 NCT02044861
Hypoxia response	MCT1 inhibition	HUVECs, RJ:NMRI mice	Inhibition of HIF-1-dependent angiogenesis	Sonveaux et al., 2012	Phase 1 NCT01791595
	Administration of telomerase activator TA-65	C57BL/6 mice	Enhancement of collateral vascular flow recovery during age-related ischemia	Kokubun et al., 2019	Phase 1 NCT02766790, NCT02531334, NCT01753674, NCT02731807
Aminoacid metabolism	Arginase II knockout	HUVECs, C57BL/6J mice	eNOS recoupling, inhibition of EC SASP	Wu et al., 2015	Phase 1 NCT02009527, NCT02903914, NCT03314935, NCT03361228
		C57BL/6J mice	Extended lifespan *via* inhibition of $p16^{INK4a}$, $p66^{Shc}$, and S6K1 signaling pathways	Xiong et al., 2017	
	Glutamine administration	Sprague-Dawley rats	Attenuation of cardiopulmonary bypass-induced inflammatory response *via* regulation of NOSs activity	Hayashi et al., 2002	Commercially available as food supplement
		STZ diabetic C57BL/6 mice	Enhancement of circulating EPC mobilization *via* increase of plasma MMP-9, SDF-1, HIF-1 and VEGF levels	Su et al., 2017	
	Glutaminase overexpression	HUVECs	Delaying of EC senescence	Unterluggauer et al., 2008	No
	Glutaminase-1 inhibition	HUVECs, HAECs, HMECs	Inhibition of aberrant EC proliferation and migration	Peyton et al., 2018	Phase 1 and 2 (18 trials)
Fatty acid metabolism	Fenofibrate administration	Middle-aged/older men and women	Improvement of endothelium-dependent vasodilation, reduction of plasma oxLDL	Walker et al., 2012	Commercially available for the treatment of dyslipidemia
		HFD C57BL/6J mice	Inhibition of HFD-induced insulin resistance and kidney injury *via* AMPK activation	Sohn et al., 2017	
		MAECs, STZ diabetic C57BL/6 mice	Decreased intracellular O_2^- levels, improvement of endothelium-dependent relaxation *via* enhanced eNOS and AMPK phosphorylation	Xin et al., 2019	
		STZ diabetic C57BL/6 mice	Amelioration of vascular endothelial dysfunction, reversal of kidney injury	Xu et al., 2019	
	Genetic and pharmacological inhibition of CPT1A	HUVECs, C57BL/6 mice	Inhibition of pathological ocular angiogenesis	Schoors et al., 2015	No
	L-carnitine administration	Hypertensive Wistar Kyoto rats	Improvement of endothelial function *via* enhanced NO and PGI_2 bioavailability and upregulation of the antioxidant systems	Bueno et al., 2005; Miguel-Carrasco et al., 2010	Commercially available as food supplement
		HAECs	Stimulation of eNOS activity *via* AMPK/Src-mediated signaling	Ning and Zhao, 2013	
mTOR pathway	Rapamycin administration	B6D2F1 mice	Improvement of age-related endothelium-dependent vasodilation, amelioration of arterial senescence markers	Lesniewski et al., 2017	Commercially available as immunosuppressive drug

HAECs, human aortic endothelial cells; HMECs, human dermal microvascular endothelial cells; MAECs, mouse aortic endothelial cells; HUVECs, human umbilical vein endothelial cells; PGI_2, prostaglandin I_2; STZ, streptozocin; S6K1, ribosomal protein S6 kinase beta-1.

pathogenesis of pulmonary arterial hypertension, atherosclerosis, and tumor spreading (Schoors et al., 2015; Xiong et al., 2018). On the contrary, stimulation of CPT1 activity by chronic L-carnitine administration improved endothelial function in an animal model of congenital heart defect (Sharma et al., 2013). In light of the crucial role of FAO in maintaining EC

quiescence and redox balance, L-carnitine supplementation, by counteracting the age-related decline in CPT1a activity (Gomez et al., 2012), could prove useful to delay the onset of age-related endothelial dysfunction. The encouraging evidence from several *in vitro* and animal studies still needs to be supported by large clinical trials (Bueno et al., 2005; Miguel-Carrasco et al., 2010; Ning and Zhao, 2013).

Glutamine, the most abundant circulating amino acid, has been extensively studied in aging research. Its blood levels decline during acute illness are increased in healthy centenarians (Montoliu et al., 2014; Meynial-Denis, 2016). A seminal study showed that glutamine administration in rats improves eNOS activity and reduces the endothelial inflammatory response following cardiopulmonary bypass (Hayashi et al., 2002). The activity of glutamine synthetase is impaired during endothelial dysfunction, due to peroxynitrite-mediated nitration of its active site (Gorg et al., 2005). This could lead to reduced TCA cycle anaplerosis, which results in the impairment of EC antioxidant system (see above) (Addabbo et al., 2013). Of note, restoring the glutamine-dependent anaplerosis through GLS overexpression has already proved beneficial in ameliorating the age-related bone loss (Huang T. et al., 2017), and in delaying EC senescence (Unterluggauer et al., 2008). Finally, the evidence that glutamine metabolism is also involved in EC proliferation supports the hypotheses that targeting GLS1 could represent a feasible approach to treat diseases associated with an aberrant EC proliferation (Peyton et al., 2018), and that glutamine supplementation can foster endothelial progenitor cell mobilization and promote vascular endothelium repair in diabetes-related ischemic injury (Su et al., 2017).

The observations discussed in the present section are summarized in **Table 1**. Altogether, these evidences reinforce the notion that alterations in EC metabolism are rather primary drivers than consequences of disease (Goveia et al., 2014). The development of drugs capable of targeting specific enzymes or pathways at the endothelial level is still in its infant phase. However, their progression into large clinical trials is imminent and could represent a turning point in the treatment of CVDs.

CONCLUSION AND FUTURE PERSPECTIVES

Accumulating evidence is suggesting that SCs are characterized by a deep reshaping of metabolic pathways. The overall picture appears highly complex, considering that specific metabolic alterations characterize different cell types and various pro-senescence stimuli. However, a general trend toward a more glycolytic state in senescent cells has been reproduced with different stimuli and in a wide range of cell types, including ECs. The role of EC metabolism in the development of T2DM and CVDs is clearly emerging, mainly thank to specific mouse models with tissue-selective deletion of metabolic genes (Graupera and Claret, 2018). However, less information is available regarding the role of senescent EC metabolism in ARDs development, even though preliminary findings suggest a key role for senescence EC related genes, e.g., p53 and NF-κB, in the regulation of organismal aging (Hasegawa et al., 2012; Yokoyama et al., 2014). Disentangling the effective contribution of altered EC metabolism in humans remains challenging, as well as the impact of dietary or pharmacological intervention on this very specific process. Studies focusing on senescent EC metabolism are warranted to clarify the disease-modifying/preventive potential of both these approaches. In fact, interventions aimed at modifying diet and metabolism have already proven to be potentially effective strategies in the prevention and treatment of ARDs (Fontana and Partridge, 2015; Brandhorst and Longo, 2019). In particular, the preventive and curative roles of specific diets have now been demonstrated by placebo-controlled, randomized clinical trials showing a cardioprotective role for Mediterranean diet (Estruch et al., 2018) and the reduction of multiple cardiometabolic risk factors with CR (Kraus et al., 2019). While future studies will define to what extent these effects can be obtained with specific molecules/nutrients, the quality of evidence regarding these dietary patterns and ARD prevention should prompt the adoption of a Mediterranean-based, low-calories dietetic regimen at the population level.

AUTHOR CONTRIBUTIONS

JS, FP, and AG collected relevant literature and wrote the manuscript. JS and AG prepared the figures. FO, AP, and MR made a substantial, direct, and intellectual contribution through their experience in the field. MR critically advised and reviewed the manuscript.

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MINI REVIEW
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Cellular Specificity and Inter-cellular Coordination in the Brain Bioenergetic System: Implications for Aging and Neurodegeneration

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As an organ with a highly heterogenous cellular composition, the brain has a bioenergetic system that is more complex than peripheral tissues. Such complexities are not only due to the diverse bioenergetic phenotypes of a variety of cell types that differentially contribute to the metabolic profile of the brain, but also originate from the bidirectional metabolic communications and coupling across cell types. While brain energy metabolism and mitochondrial function have been extensively investigated in aging and age-associated neurodegenerative disorders, the role of various cell types and their inter-cellular communications in regulating brain metabolic and synaptic functions remains elusive. In this review, we summarize recent advances in differentiating bioenergetic phenotypes of neurons, astrocytes, and microglia in the context of their functional specificity, and their metabolic shifts upon aging and pathological conditions. Moreover, the metabolic coordination between the two most abundant cell populations in brain, neurons and astrocytes, is discussed regarding how they jointly establish a dynamic and responsive system to maintain brain bioenergetic homeostasis and to combat against threats such as oxidative stress, lipid toxicity, and neuroinflammation. Elucidating the mechanisms by which brain cells with distinctive bioenergetic phenotypes individually and collectively shape the bioenergetic system of the brain will provide rationale for spatiotemporally precise interventions to sustain a metabolic equilibrium that is resilient against synaptic dysfunction in aging and neurodegeneration.

Keywords: mitochondria, neuron, astrocyte, microglia, metabolic shift, metabolic coupling, brain aging, neurodegenerative diseases

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INTRODUCTION

Active metabolism is fundamental in maintaining the life and activity of organisms by mediating the exchange of material, energy, and information with the environment. Most organisms rely on the catabolism of organic molecules to obtain energy. The acquirement of mitochondria by eukaryotic cells *via* endosymbiosis is proposed to remarkably expand their genomic capacity, which energetically drives and enables the evolution from single- to multi-cell systems (Lane and Martin, 2010;

Lane, 2014). In higher organisms like the mammals, the production, storage, and utilization of fuels have been compartmentalized to, and distributed among, different organs. Organs such as liver, muscle, heart, and adipose tissue have adapted to possess specialized metabolic capacity and flexibility in using different fuels to meet their diverse functional needs.

Among all organs, brain is unique, not only because it consumes 20% of total glucose with its 2% of body weight (Bélanger et al., 2011), but also due to its unique metabolic profile and metabolite pool that are separated from the rest of the body by the blood–brain barrier (BBB). Although brain ultimately uses substrates from the periphery, its capacity to uptake and metabolize them varies from one fuel to another, with glucose being the predominant preference (Lundgaard et al., 2015). Within the brain, the highly heterogeneous cellular composition comes with diverse energetic capacity and fuel preference across cell types. While previous studies have established the vital role of bioenergetics system in brain aging and neurodegenerative diseases, the physiological and pathological roles of different cell types and their metabolic coordination are still being actively investigated. Accordingly, most strategies to restore brain bioenergetic homeostasis in neurodegenerative diseases either indistinguishably targeted all cell types or focused on neurons with limited consideration of the bioenergetic contributions from non-neuronal cells. In this review, we sought to discuss recent advances in revealing cell-type-specific bioenergetic role of brain cells with connection to their specialized functions. Moreover, inter-cellular metabolic coordination between neurons and astrocytes is reviewed in the context of brain aging and age-associated neurodegenerative disorders.

DIVERSE METABOLIC PHENOTYPES OF BRAIN CELLS

The mammalian brain is composed of diverse, specialized cell populations, including neurons, astrocytes, oligodendrocytes, microglia, and others. All of them together not only enable the highly refined electrophysiological activities, but also fulfill the organ's nutritional needs and its defense against pathogens (Saunders et al., 2018). Recently, high-throughput single-cell RNA sequencing has achieved an unprecedented resolution in distinguishing and clustering cell types and sub-cell types in both rodent and human brains (Zeisel et al., 2015; Lake et al., 2016; Saunders et al., 2018). Moreover, studies in aging and degenerating brains also suggested that bioenergetic genes in different cell types are differentially altered in aging or diseases. Comparative analysis of bulk tissue- and single-cell RNA-seq of Alzheimer's brains revealed distinct transcriptional changes across cell types, and a decline in mitochondrial genes at tissue level is only seen in neurons but not in glial cells from the same region of brain (Mathys et al., 2019).

Consistent with their transcriptomic profiles, different brain cell types have their unique metabolic phenotypes (Magistretti and Allaman, 2015; **Figure 1**). Both glia and neurons have the capacity to fully metabolize glucose, but their functional diversity differentiates their metabolic preference. Neurons, which generate and consume most brain ATPs, primarily rely on mitochondrial oxidative phosphorylation (OXPHOS) for energy transduction and possess low glycolytic capacity due to the suppression of a key glycolysis enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFKFB) (Schönfeld and Reiser, 2013; Zheng et al., 2016; **Figure 1**). For non-glucose substrates, neurons have a low capacity to utilize fatty acids (Schönfeld and Reiser, 2013) but can metabolize ketone bodies generated in non-neuronal cells from fatty acid β-oxidation.

As the most abundant cell type in the brain, astrocytes provide critical metabolic and structural support to neurons, including modulation of ion homeostasis, supply of nutrients, and control of BBB permeability (Suzuki et al., 2011; Bolaños, 2016; Boison and Steinhäuser, 2018). In contrast to neurons, astrocytes maintain lower OXPHOS activity but higher glycolysis rate (Bélanger et al., 2011; Bouzier-Sore and Pellerin, 2013; **Figure 1**). Large amount of glucose metabolized by glycolysis in astrocytes is released as lactate, which serves as important metabolic fuel for neurons (Bolaños, 2016). Astrocytes also store excessive fuels in glycogen that can rapidly regenerate glucose for glycolysis or glutamate synthesis (Suzuki et al., 2011). Multiple lines of evidence suggest that astrocytes can metabolize non-glucose substrates including fatty acids (Ioannou et al., 2019), glutamate (Schousboe et al., 2014), and ketone bodies (McNair et al., 2017).

Microglia are resident immune cells in the brain (Viader et al., 2015), and are closely related to host defense against pathogens and CNS disorders (Perry et al., 2010). The survival and activation of microglia depend on sufficient energy supply. Cell-type-specific RNA-seq analysis reveals that microglia express the full set of genes required for both glycolysis and OXPHOS (Zhang et al., 2014), but their bioenergetic phenotype is activation-state-dependent (Aldana, 2019). Resting microglia depend mainly on OXPHOS for ATP production, whereas activated microglia favor glycolysis as manifested by increased lactate production and decreased mitochondrial oxygen consumption (Ghosh et al., 2018; Aldana, 2019; **Figure 1**). Additionally, transcriptome data suggest that microglia express key enzymes for fatty acids mobilization and β-oxidation, which may alternatively meet their elevated energy demand upon activation (Zhang et al., 2014).

A byproduct of mitochondrial OXPHOS is the electron leak from respiratory chain to generate superoxide and other reactive oxygen species (ROS; Murphy, 2009). Increased ROS production and oxidized redox status characterizes brain aging and neurodegeneration (Markesbery, 1997; Cadenas and Davies, 2000; Lin and Beal, 2006; Yin et al., 2012a, 2014; Hwang, 2013). Of all brain cell types, neurons are particularly susceptible to redox changes due to their high metabolic rate and limited antioxidant capacity (Baxter and Hardingham, 2016). Astrocytes, in contrast, have greater antioxidative potential (Gupta et al., 2012). Multiple studies have demonstrated that the astrocytic support of neuronal antioxidant system is a key neuroprotective mechanism against oxidative damage (Magistretti and Allaman, 2013). Since the electrons used to reduce ROS are ultimately from NADPH, which is generated by the pentose phosphate pathways (PPP) from glucose or by mitochondrial enzymes including the transhydrogenase (Yin et al., 2012b), elevated oxidative stress could cause a switch in re-routing fuels toward

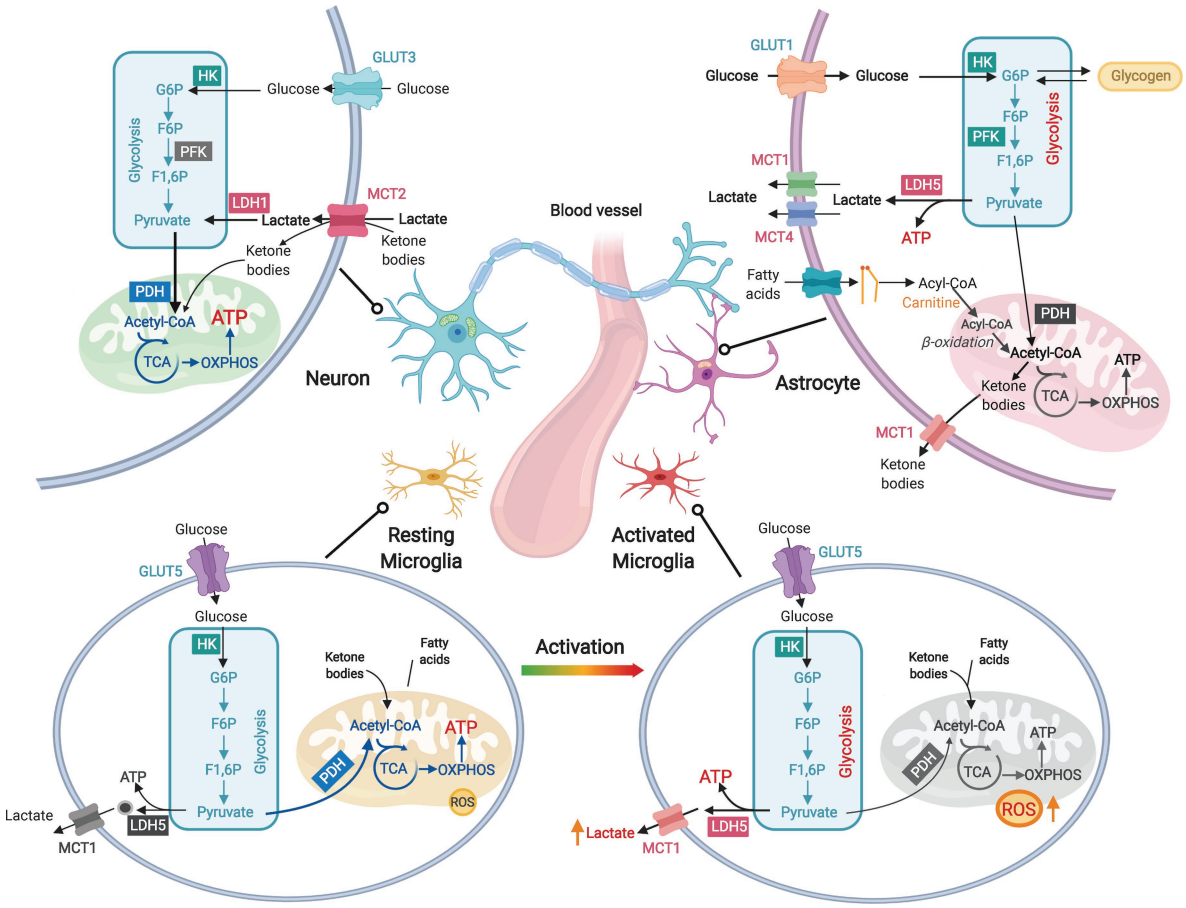

FIGURE 1 | Diverse metabolic phenotypes of brain cells. Neurons produce ATP primarily *via* mitochondrial oxidative phosphorylation (OXPHOS), but their glycolytic rate is less active than that of astrocytes due to suppressed PFK activity. Both lactate and ketone bodies can enter neurons *via* monocarboxylate transporter 2 (MCT2). Astrocytes are highly active in glycolysis to produce lactate but less dependent on OXPHOS due to low pyruvate dehydrogenase (PDH) activity. Lactate is generated by lactate dehydrogenase 5 (LDH5) in astrocytes and exported by MCT1 and MCT4. Astrocytes are capable to metabolize fatty acids through β-oxidation. Fatty acids transport into mitochondria is facilitated by carnitine and carnitine palmitoyltransferases (CPTs). Ketone bodies can be generated in astrocytic mitochondria *via* ketogenesis. Excessive astrocytic glucose can be stored as glycogen, which can be converted back to glucose. Resting microglia have high rate of OXPHOS and low production of reactive oxygen species (ROS), whereas activated microglia produce large amount of ROS and undergo a metabolic reprogramming from OXPHOS toward glycolysis and lactate production.

NADPH production rather than ATP production (Agarwal et al., 2013). Accordingly, astrocytic oxidative stress dampens glucose uptake and diverts glucose into PPP for NADPH production, which subsequently decreases lactate release from astrocytes to neurons and compromises the neuronal redox homeostasis (Steele and Robinson, 2012). The low capacity to metabolize fatty acids of neurons is another cause for its sensitivity to oxidative challenges: a recent study revealed that excess ROS trigger lipids synthesis in neuron and give rise to neurodegeneration if managed improperly (Liu et al., 2017).

INTRA-CELLULAR BIOENERGETIC SHIFTS

Glucose is brain's dominating fuel under physiological conditions. Upon limited glucose availability, the brain undergoes a metabolic shift to use ketone bodies – including acetate, acetoacetate, and β-hydroxybutyrate – as its alternative energy source (Cunnane et al., 2011). Upon fasting, starvation, extended exercise, pregnancy, or development, fatty acids are mobilized from adipocytes and transported to liver for conversion to ketone bodies, which are subsequently transported to the brain to generate acetyl-CoA and eventually ATP in mitochondria (Drenick et al., 1972; Puchalska and Crawford, 2017; Mattson et al., 2018). Ketogenic interventions were reported with beneficial effect on cases of inborn metabolic disorder involving genetic deficiency of GLUT1 or pyruvate dehydrogenase, suggesting ketones as brain fuels to bypass glucose hypometabolism (Falk et al., 1976; Klepper, 2004; Wang et al., 2005). Although liver is believed to be the predominant supplier of ketone bodies, it was found that ketone bodies could also be generated in astrocytes from fatty acids (Martinez-Outschoorn et al., 2012). In brain aging and a variety of neurodegenerative diseases, a decline in glucose metabolism has been well-established

(Ott et al., 1999; Fabre et al., 2002; Cunnane et al., 2011; Ding et al., 2013; Goyal et al., 2017). Under this situation, the brain may adapt a similar bioenergetic shift from exclusively relying on glucose toward using ketone bodies in response to energetic deficits (Ding et al., 2013; Klosinski et al., 2015; Camandola and Mattson, 2017). Beneficial effect of ketogenic diet on cognitive and cardiovascular outcomes has been observed in aging mice (Newman et al., 2017) and patients with mild cognitive impairment, a transition state toward AD (Krikorian et al., 2012).

While mitochondrial function in neurons is known to be impaired during aging, astrocytes exhibit elevated mitochondrial respiration in aging rat brains (Jiang and Cadenas, 2014), which is consistent with human studies showing increased astrocytic TCA activity with age (Boumezbeur et al., 2010). In parallel with these bioenergetic changes, rodent and human studies also demonstrated that the expression of glial fibrillary acidic protein (GFAP), a marker protein for activated astrocytes, increases with age (Nichols et al., 1993; Wu et al., 2005). Overall, age-associated activation of astrocytic OXPHOS could restrict their capacity to supply neurons with substrates such as lactate or glucose (Yan et al., 2013; Rodríguez-Arellano et al., 2016), and is connected with a switch from being neurotrophic to neurotoxic and to neuroinflammation (Jiang and Cadenas, 2014).

Similar to their peripheral counterparts macrophages, the bioenergetics of microglia also undergo a switch from OXPHOS toward glycolysis upon activation (Orihuela et al., 2016). LPS-activation of microglia-like BV-2 cell blocks mitochondrial oxygen consumption while activating anaerobic glycolysis and PPP (Volobouéva et al., 2013; Gimeno-Bayón et al., 2014). Consistently, glucose transporters GLUT1 and GLUT4, and hexokinase 2 (HK2), are upregulated in activated microglia (Gimeno-Bayón et al., 2014). Such a metabolic switch is critical to enable the fast response of activated microglia to energy-demanding processes such as proliferation, migration, cytokine secretion, and phagocytosis, because of the much faster rate in generating ATP by glycolysis than OXPHOS (Orihuela et al., 2016). Activation of microglia and chronic neuroinflammation are key features of aging and neurodegeneration. Upon LPS challenge, microglia from aged animals produce more ROS, compared to those isolated from young mouse brains (Tichauer et al., 2014; **Figure 1**). In a familial AD mouse model, exposure to amyloid-β triggers acute microglial activation in parallel with a metabolic shift from OXPHOS to glycolysis (Baik et al., 2019). A similar shift was also found in microglia from multiple sclerosis patients (Van Der Poel et al., 2019).

INTER-CELLULAR METABOLIC COMMUNICATIONS

Astrocyte-Neuron Lactate Shuttle

The diverse ability to perform OXPHOS and glycolysis between neurons and astrocytes is proposed to be related to inter-cellular coupling of glucose metabolism by the astrocyte-neuron lactate shuttle (Pellerin and Magistretti, 1994; Díaz-García et al., 2017; **Figure 2**). Glutamate released from active neurons activates astrocytic glycolysis and the production of lactate, which is used by neurons for synaptic activities (Mächler et al., 2016). Astrocytes take up more glucose than their energetic needs, which suggests their role in maintaining an extracellular lactate pool for neuronal use (Chuquet et al., 2010). In Drosophila, glial cells express glycolytic enzymes to produce lactate and alanine, which are then used to derive pyruvate for neuronal OXPHOS (Brooks et al., 1999; Volkenhoff et al., 2015). Lactate, pyruvate, and ketone bodies cross cell membranes through proton-linked monocarboxylate transporters (MCTs) (Halestrap, 2011; **Figure 2**). Knocking down astrocytic lactate transporter MCT4 in mouse hippocampus leads to memory retention, supporting the role of astrocytic lactate for long-term synaptic plasticity and memory (Suzuki et al., 2011). A recent *in vivo* study using a genetically encoded FRET sensor *Laconic* revealed a lactate gradient from astrocytes to neurons (Mächler et al., 2016). Astrocyte-to-neuron lactate shuttle was also found to be more functional in young hippocampi whereas aged neurons exhibit reduced dependence on astrocytic lactate with disrupted metabolic crosstalk (Drulis-Fajdasz et al., 2018). Perturbations to neuron-astrocyte metabolic coupling are seen in multiple neurodegenerative diseases (Rama Rao and Kielian, 2015). Decreased expression of astrocytic MCTs and defective neuron-astrocyte coupling of glucose metabolism was found in mouse models of AD and amyotrophic lateral sclerosis (ALS) (Ferraiuolo et al., 2011; Ding et al., 2013). A decline in levels of lactate, glucose, and other glycolytic intermediates was seen in the cerebrospinal fluid of Parkinson's patients (Ohman and Forsgren, 2015).

Neuron-Astrocyte Coordination of Fatty Acid Metabolism

Another metabolic coupling between neurons and astrocytes that was described recently is the coordination of fatty acid metabolism (Liu et al., 2015, 2017; Rambold et al., 2015; Ioannou et al., 2019; **Figure 2**). Lipids constitute 50% of the brain dry weight, mainly as fundamental component of membrane structures (Bruce et al., 2017). Since neurons are known to have minimum capacity to catabolize fatty acids (Schonfeld and Reiser, 2017), unused or recycled neuronal lipids are transported to astrocytes and stored in lipid droplets (LDs) before the metabolism in mitochondria (Liu et al., 2017; Ioannou et al., 2019). Sequestering of excessive fatty acids in LD could prevent lipotoxicity and mitochondrial dysfunction (Listenberger et al., 2003). In Drosophila, LD formation in niche glia under oxidative stress dampens intracellular ROS generation and oxidation of polyunsaturated fatty acids, which protects glia and neuroblasts from the peroxidation chain (Bailey et al., 2015). Upon nutrient depletion, LDs can deliver FAs to mitochondria for β-oxidation (Greenberg et al., 2011; Khor et al., 2013), and LD accumulation was found in olfactory bulb and vestibular nucleus of mice with dysfunctional mitochondria (Liu et al., 2015). Notably, apolipoprotein E-ε4 (APOE4), the strongest genetic risk factor for AD (Corder et al., 1993), reduces neuron-to-astrocyte transfer of fatty acids, which could be the underlying mechanism of the lipid dyshomeostasis seen in the disease (Liu et al., 2017). Additionally, LDs were also found to buildup in microglia of aged mouse or human brains, which then trigger ROS production

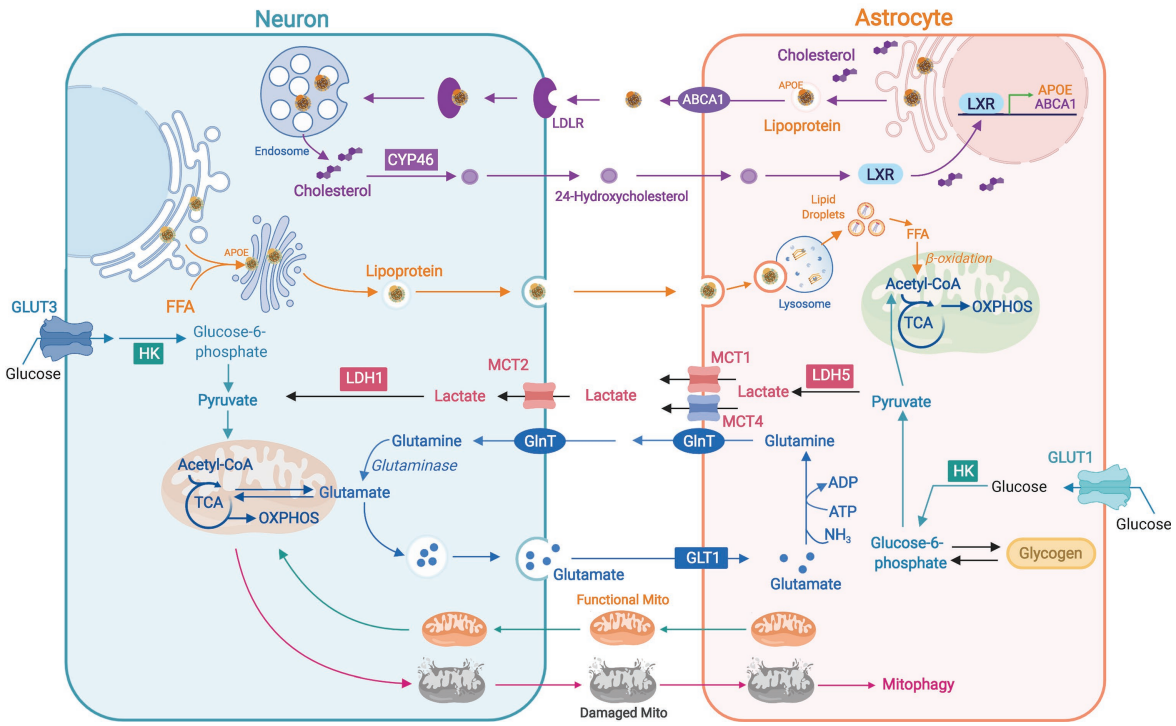

FIGURE 2 | Neuron-astrocyte metabolic coordination. Astrocyte-synthesized cholesterols are packaged in APOE-associated lipoproteins and exported by ATP-binding cassette transporter A1 (ABCA1); released lipoproteins bind to neuronal LDL receptors and are internalized for neuronal use or conversion to 24-hydroxycholesterol (24-OHC) by cholesterol 24-hydroxylase (CYP46). 24-OHC produced by neurons can activate astrocytic live X receptor (LXR) to induce the expression of APOE and ABCA1. Hyperactive neurons release excessive free fatty acids (FFAs) in APOE-associated lipid particles to astrocytes where FFAs are targeted to lipid droplets for subsequent degradation by mitochondrial β-oxidation. High glycolytic rate in astrocytes produces lactate that can be transported to neurons by MCTs and used for ATP production. Neurotransmitter glutamate released by neurons is uptaken by astrocytes through glutamate transporter 1 (GLT1) and converted to glutamine for recycle to neurons and the re-generation of glutamate. Neurons can transfer damaged axonal mitochondria to astrocytes for autophagic degradation (mitophagy) whereas astrocytes can transfer healthy mitochondria to adjacent neurons as a neuroprotective and neurorecovery mechanism after stroke.

and secretion of pro-inflammatory cytokines, and contribute to neurodegeneration (Marschallinger et al., 2019).

Cholesterol Metabolism Across Neuron and Astrocyte

Brain contains ~20% cholesterol of the body (Zhang and Liu, 2015), which is essential for membrane fluidity, vesicle formation, and synaptic transmission (Björkhem and Meaney, 2004). Brain synthesizes its own cholesterol due to the block of cholesterol-carrying lipoproteins across the BBB. Since neurons acquire extra cholesterol for synaptogenesis from astrocytes (Mauch, 2001), and brain cholesterol needs to be hydroxylated to 24-hydroxycholesterol in neurons before excretion (Bjorkhem et al., 1998; Meaney et al., 2002), cholesterol transport from astrocytes to neurons is vital for synaptic function and sterol homeostasis in the brain. Upon co-culture, astrocytes stimulate neuronal neurite outgrowth, which is impaired when astrocytes without SREBP2 (a cholesterol synthesis regulatory protein) are used. *In vivo*, SREBP2 knockout in astrocytes alters brain development and impairs behavioral and motor functions in mice (Ferris et al., 2017), suggesting astrocyte-to-neuron cholesterol trafficking is key for brain development and synaptic function. APOE is the primary apolipoprotein that transports cholesterol in the brain. APOE lipoproteins carrying cholesterol and phospholipids are effluxed of astrocytes and uptaken into neurons by binding to lipoprotein receptors (Pfrieger and Ungerer, 2011; **Figure 2**). APOE-deficient mice exhibit markedly reduced cholesterol levels in hippocampus, and AD-like behavioral and synaptic impairments (Champagne et al., 2002; Levi et al., 2005). Astrocytes and neurons expressing APOE4 have reduced capacity in secreting or binding to cholesterol and phospholipids (Mahley, 2016). LDL receptor and LRP1 from LDL receptor family are main receptors to uptake APOE-containing lipoprotein particles to neurons. LRP1 knockout in forebrain neurons disrupts lipid metabolism, and leads to neuroinflammation and synapse loss (Liu et al., 2010). During aging, mRNA levels of the rate-limiting enzyme of cholesterol synthesis HMGCR decrease in astrocytes (Boisvert et al., 2018), which could contribute to dendrite atrophy and synaptic dysfunction seen in the aging brain. A study in AD-patient iPSC-derived neurons suggests that neuron-specific activation of cholesterol degradation could be a potential therapeutic target to alle*via*te Aβ and Tau pathology in AD (Van Der Kant et al., 2019).

Additional Mechanisms of Neuron-Astrocyte Metabolic Interactions

Other than the coupling of glucose and lipid metabolism, additional metabolic interactions between neurons and astrocytes are essential for brain functions. Neuronal levels of reduced glutathione (GSH), the most abundant redox modulator eliminating ROS in the brain, are found to be synthesized using precursors amino acids, including glycine and cysteine, from astrocytes. GSH released from astrocytes is cleaved by γ-glutamyl transpeptidase on astrocyte surface to glycine and cysteine before being uptaken by neurons for GSH synthesis (Dringen, 2000). Inhibition of GSH biosynthesis in astrocytes triggers neuronal toxicity, and depletion of GSH during aging leads to microglia activation and increased neuronal susceptibility to cell death (Hirrlinger et al., 2002; Lee et al., 2010).

Intra-cellularly, damaged mitochondria are removed *via* mitophagy. Inter-cellularly, neurons release damaged axonal mitochondria to adjacent astrocytes for autophagic degradation (Chung-Ha et al., 2014). Conversely, astrocytes can transfer healthy mitochondria to adjoining neurons for ATP production and recovery from stroke (Hayakawa et al., 2016; **Figure 2**). When extracellular mitochondria are removed from astrocyte-conditional media, their neuroprotective effect is abolished (Hayakawa et al., 2016). At the molecular level, astrocyte-generated ATP, together with its degradation product adenosine, is involved in supporting synaptic transmission and neuronal excitability (Chen et al., 2013; Tan et al., 2017).

PERSPECTIVE

The high energy demand of the brain renders it sensitive to changes in bioenergetic capacity. Disruptions to substrate availability and/or mitochondrial function are well-known hallmarks of brain aging and a variety of age-associated neurodegenerative disorders. Distinguishing cell type-specific bioenergetic contributions to brain aging is particularly of significance because of previous findings linking aging with diverse metabolic changes between different brain cells. Elucidating the cellular specificity and inter-cellular coupling within the brain bioenergetic system is also critical to understand the mechanisms underlying selective cellular vulnerability in neurodegeneration. Mitochondrial enhancers targeting all brain cells, such as creatine, coenzyme-Q, and Mito-Q, have thus far exhibited limited efficacy for neurodegenerative diseases (Moreira et al., 2010; Chaturvedi and Flint Beal, 2013; Murphy and Hartley, 2018). While such a lack of success may be ascribed to multiple reasons including window of intervention and translatability of existing animal models (Murphy and Hartley, 2018), for neurodegenerative diseases that typically harbor complex and dynamic etiologies, precision strategies targeting specific cells or inter-cellular metabolic interactions may be needed at different disease stages. For stages when reactive astrocytes and microglia are playing a central role, interventions that facilitate their metabolic reprogramming could be more effective than direct OXPHOS enhancers (Baik et al., 2019; Polyzos et al., 2019). Recent methodological advances have started to enable targeted delivery of therapeutics to different brain cells *via* nanoparticles or viral carriers (Zhang et al., 2016; Sharma et al., 2018). Furthermore, emerging intra- and inter-cellular strategies targeting non-energetic facets of the mitochondria have manifested encouraging efficacy (Fang et al., 2019; Joshi et al., 2019), which suggest an necessity of combinational therapies for these currently incurable diseases.

AUTHOR CONTRIBUTIONS

All authors contributed to the discussion of content, generation of figures, and writing, review, editing of the manuscript.

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REVIEW
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The Aryl Hydrocarbon Receptor (AhR) in the Aging Process: Another Puzzling Role for This Highly Conserved Transcription Factor

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Aging is the most important risk factor for the development of major life-threatening diseases such as cardiovascular disorders, cancer, and neurodegenerative disorders. The aging process is characterized by the accumulation of damage to intracellular macromolecules and it is concurrently shaped by genetic, environmental and nutritional factors. These factors influence the functionality of mitochondria, which play a central role in the aging process. Mitochondrial dysfunction is one of the hallmarks of aging and is associated with increased fluxes of ROS leading to damage of mitochondrial components, impaired metabolism of fatty acids, dysregulated glucose metabolism, and damage of adjacent organelles. Interestingly, many of the environmental (e.g., pollutants and other toxicants) and nutritional (e.g., flavonoids, carotenoids) factors influencing aging and mitochondrial function also directly or indirectly affect the activity of a highly conserved transcription factor, the Aryl hydrocarbon Receptor (AhR). Therefore, it is not surprising that many studies have already indicated a role of this versatile transcription factor in the aging process. We also recently found that the AhR promotes aging phenotypes across species. In this manuscript, we systematically review the existing literature on the contradictory studies indicating either pro- or anti-aging effects of the AhR and try to reconcile the seemingly conflicting data considering a possible dependency on the animal model, tissue, as well as level of AhR expression and activation. Moreover, given the crucial role of mitochondria in the aging process, we summarize the growing body of evidence pointing toward the influence of AhR on mitochondria, which can be of potential relevance for aging.

Keywords: aryl hydrocarbon receptor, aging, mitochondria, *C. elegans*, mice, human

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1. AGING HALLMARKS AND ASSOCIATED CHANGES

Aging is defined as the time-dependent biological deterioration of structural, cellular and tissue components as well as physiological functions (e.g., stress resistance, immune system, ability to sense and move, decline of organ functionality). It is accompanied by an increased risk for the development of major life-threatening diseases such as cardiovascular disorders, cancer and neurodegenerative disorders (Lopez-Otin et al., 2013; Kaeberlein, 2016). The interest in aging research showed an increase in the past 60 years with a number of almost 7000 new PubMed entries in 2018. 60 years of research have advanced our knowledge on the molecular mechanisms of aging

and a number of theories of aging have been proposed (Harman, 1956; Hamilton, 1966; Kirkwood, 1977; Villeponteau, 1997). These theories primarily fall into two categories (i) programmed and (ii) non-programmed based theories. While programmed theories of aging propose that mainly changes in the activity of specific genes, hormones, or the immune system are accountable for the aging process, non-programmed theories consider cellular damage resulting from the interaction with toxicants from the environment or by-products of metabolism the primary factor contributing to aging (Jin, 2010). It seems however most likely that the interaction between genetic and environmental factors shapes the aging process (Kenyon, 2010; Dato et al., 2017). Although aging is a very complex phenomenon and a variety of factors can affect different cells/tissue/organs and their interconnectivity at the same time, different evolutionarily conserved aging hallmarks have been described: (1) genomic instability, (2) telomere attrition, (3) epigenetic alterations, (4) loss of proteostasis, (5) deregulated nutrient sensing, (6) mitochondrial dysfunction, (7) cellular senescence, (8) stem cell exhaustion, and (9) altered intercellular communication (Lopez-Otin et al., 2013; Tigges et al., 2014). Indeed, apart from telomere attrition, all mentioned hallmarks can be observed in vertebrate as well as invertebrate model organisms (Kaeberlein, 2013). Mitochondria clearly play a central role in the aging process and it is interesting to note that while mitochondrial dysfunction itself is one of the hallmarks of aging, severe mitochondrial dysfunction can also promote most if not all other hallmarks of aging.

1.1 Mitochondria and Aging

Mitochondria are highly interconnected organelles and are composed of two specialized membranes, an intermembrane space and a matrix containing a circular DNA, which reminds us of their bacterial origin. Mitochondria play a central role in cell and organismal homeostasis and beside their major role in energy metabolism, they also control additional crucial cellular functions ranging from iron and calcium homeostasis to cell death and survival pathways. Given the central importance of mitochondria, cells developed a variety of protective mechanisms to cope with, prevent and repair their damage and alterations thus ensuring cells with the appropriate amount of functional mitochondria in physiological as well as stressful conditions. The "Mitochondrial Free Radical Theory of Aging," MFRTA, which has taken central stage for several decades (Harman, 1956) in the aging field, states that during life reactive oxygen species (ROS) produced during mitochondria respiration gradually induce irreversible molecular and cellular damages with consequent functional decline ultimately playing a causal role in the aging process. It is therefore not surprising that failure of mitochondrial quality control pathways or severe, non-repairable mitochondrial damage, lead to a plethora of disorders and accelerate the aging process. More surprisingly, yet interestingly, is instead that the MFRTA theory has been recently questioned by the growing body of evidence showing that mild (as opposed to severe) increase in ROS and mild mitochondrial stress can actually promote healthy aging in an evolutionarily conserved manner (Ristow and Zarse, 2010; Ristow and Schmeisser, 2011; Munkacsy and Rea, 2014; Schiavi and Ventura, 2014). This provocative finding (in the field referred to as threshold effect or mitohormesis) has completely changed our classical view of the role of mitochondria in the aging process and stimulated the investigation of novel strategies to promote healthy aging. Taken MFRTA and mitohormesis together it is envisioned that mitochondria play a pivotal role in cell homeostasis and therefore in the aging process.

Dato et al. (2017) estimated that genetic factors only account for one quarter, while environmental and epigenetic factors account for three-quarters of age-associated changes. Considering the high influence of environmental factors on aging, we have recently investigated the role of a central environmental sensor, the highly conserved transcription factor Aryl-hydrocarbon Receptor (AhR) in the aging process (Eckers et al., 2016). Here, we will first review the conflicting evidence pointing to both pro- and anti-aging roles for AhR in aging. We will then try to reconcile these findings based on possible age-, tissue- or dose-dependent activation, and finally discuss pieces of evidence indicating a possible interaction between the AhR and mitochondria, which could be of relevance for the aging process.

2. AhR AND AGING

The AhR was discovered in 1976 by Poland et al. (1976) as a dioxin-binding protein. The activity of this highly conserved transcription factor is historically dependent on the binding of ligands to its ligand binding domain (LBD). The functionality of this transcription factor is shaped by its functional domains: a basic helix-loop-helix domain (bHLH), two Per-ARNT-Sim (PAS) domains and a transcriptional activation domain (TAD). The N-terminal bHLH domain is involved in DNA binding, binding of heat shock protein 90 (Hsp90), and dimerization with AhR nuclear translocator (Arnt) (Ashida et al., 2008; Abel and Haarmann-Stemmann, 2010). The PAS A domain is required for binding to the Arnt, while the PAS B domain carries the LBD and thus is relevant for ligand binding but also interaction with the AhR-interacting protein [Aip (also XAP2)] and Hsp90. Carboxy-terminal of the AhR is a TAD (Ashida et al., 2008; Abel and Haarmann-Stemmann, 2010). In the absence of ligands, the AhR resides in the cytoplasm bound to Hsp90, Aip, and p23 (Ikuta et al., 1998; Ashida et al., 2008). These co-factors stabilize the ligand-affine state of the AhR and prevent its degradation (Ma and Whitlock, 1997; Meyer and Perdew, 1999). The functions of these co-factors are crucial and AhR is degraded in the absence of Aip or Hsp90 (Hwang et al., 2016). Binding of a ligand causes conformational changes resulting in the exposure of the nuclear localization signal (NLS) and the dissociation from Hsp90, Aip, and p23. In this state, AhR can shuttle to the nucleus, where it dimerizes with Arnt. The AhR-Arnt heterodimer then binds to the xenobiotic responsive elements (XREs) (core sequence 5′-GCGTG-3′) of AhR target genes. These target genes include phase-I detoxification genes like cytochrome P450 (*cyp*) monooxygenase genes (e.g., *cyp1A1* or *cyp1B1*), phase-II detoxification genes like UDP glycosyltransferases (*ugts*) (e.g., *ugt1A1* or *ugt1A6*), and glutathione S-transferases (*gsts*) (e.g., *gstA1* or *gstA2*) (Yueh et al., 2003; Ashida et al., 2008; Xue et al., 2017). To avoid the constant activation of the AhR,

negative feedback loops regulate the AhR cascade pathway (Mulero-Navarro and Fernandez-Salguero, 2016; Xue et al., 2017). It is interesting to note that different AhR target genes (e.g., enzymes involved in glutathione synthesis and modulation) as well as genes regulating or regulated by AhR (e.g., Sirt1, p53, Hif1, p300, and HSP90) are involved in the aging process (Henry and Gasiewicz, 1993; Marlowe et al., 2004; Koizumi et al., 2014; Li et al., 2014; Ming et al., 2015; Panchanathan et al., 2015; Ajami et al., 2017; Janssens et al., 2019; Sutter et al., 2019).

Various compounds influence the activity of the AhR, but not all of them are direct ligands. In fact, for some of the compounds modulating the activity of the AhR the direct mechanism is not known. Other compounds modulate AhR activity through an indirect mechanism. For this reason, in this review, we will refer to AhR modulators rather than ligands unless their direct binding to the LBD has been verified. A very well studied group of AhR modulators are xenobiotics. Particularly 2,3,7,8-tetrachlorodibenzo-p-dioxin (dioxin) is a planar molecule that has been shown to bind to the binding pocket of the LBD of mammalian AhR (Poland et al., 1976). Other modulators of the AhR are polyphenols, which can be found in a variety of different fruits and vegetables (Amakura et al., 2003), but whether they really bind to the AhR or affect its activity through other mechanisms is largely elusive (see Xue et al., 2017 for a detailed review). Epigallocatechin gallate, for example, binds to HSP90 and by doing so inhibits AhR signaling (Palermo et al., 2005). Curcumin instead can directly bind the AhR (Ciolino et al., 1998) and inhibits its downstream signaling through inhibition of AhR phosphorylation by the protein kinase C (PKC) (Nishiumi et al., 2007). An *in silico* analysis of the interactions between quercetin and the LBD of human AhR showed that quercetin can bind to specific residues in the AhR binding pocket (Jin et al., 2018). Interestingly, many of the polyphenols modulating AhR activity also affect mitochondria (Sandoval-Acuna et al., 2014). Besides these exogenous modulators, endogenous compounds affect AhR activity as well. These endogenous modulators are mainly, but not solely tryptophan derivatives, like the high-affinity ligand 6-formylindolo[3,2-b]carbazole (FICZ), a photoproduct of tryptophan, which is produced in response to UVB light (Rannug et al., 1987; Fritsche et al., 2007). Another endogenous but low-affinity AhR modulator is kynurenine (Opitz et al., 2011). More recently, compounds produced by the microbiota have been identified as AhR modulators. These are, similarly to the endogenous AhR modulators, mostly derivatives of tryptophan such as indole, indoxyl-3-sulfate, indole-3-propionic acid, indole-3-aldehyde, indole-3-acetate, and tryptamine (Jin et al., 2014; Rothhammer et al., 2016).

It is interesting to note that many of these AhR modulators may affect aging or age-associated diseases. Dioxin exposure, for example, can cause cancer, and cardiovascular diseases (Mandal, 2005; Marinkovic et al., 2010). Exposure to the xenobiotic and AhR ligand benzo[*a*]pyrene, which directly binds to the AhR (Okey et al., 1984) and causes its nuclear localization, shortens the lifespan in mice (Sakakibara et al., 2005), and promotes neurodegeneration as well as Alzheimer's disease and Parkinson's disease-like phenotypes in zebrafish (Gao et al., 2015). However, the actual involvement of the AhR in these processes was not investigated in these studies. Instead, in another study, benzo[*a*]pyrene was shown to cause cancer in an AhR-dependent manner (Shimizu et al., 2000) and similarly, the endogenous AhR modulator kynurenine promotes tumor formation through the AhR (Opitz et al., 2011). Moreover, another work showed that mice carrying a low-affinity AhR allele are more susceptible to benzo[*a*]pyrene-induced lethality than mice with a high-affinity AhR allele, suggesting the importance of the degree of AhR activation (Kerley-Hamilton et al., 2012). Many of the plant-derived dietary AhR modulators, on the other hand, have life- and health-extending effects across species. Curcumin, for example, shows protective effects on age-related neurodegenerative diseases in different species (Lim et al., 2001; Alavez et al., 2011; Caesar et al., 2012). Also, the AhR modulator quercetin extends lifespan in *Caenorhabditis elegans (C. elegans)* (Kampkotter et al., 2008; Pietsch et al., 2009), *Drosophila melanogaster* (Drosophila) (Proshkina et al., 2016) and mice (Xu et al., 2018). Interestingly, indole produced by commensal *Escherichia coli* was found to extend the lifespan of *C. elegans*, Drosophila and mice in an AhR-dependent manner (Sonowal et al., 2017) but in all other studies it has not been investigated whether the compounds mediate healthspan in an AhR dependent manner. While all of these studies focus on the effect of specific compounds on aging, there are only a few studies directly linking AhR and aging. In fact, a search on the MEDLINE/PubMed database with the Medical Subject Headings (MeSH) terms "ah receptor" and "aging" gave only 29 results. Here, we want to review the current state of research on the role of the AhR in aging in different model organisms as well as humans. We have however deliberately decided not to describe the large body of association studies correlating AhR activity/expression to age-associated diseases in human, which, although very interesting, would require a separate review.

2.1 AhR and Aging in Invertebrates

When studying aging, invertebrate model organisms offer some advantages over vertebrates: they are small, easy to cultivate, cheap in maintenance and most importantly have a short lifespan. These characteristics allow the performance of aging studies with a large number of individuals in a short time. Moreover, the conservation of the major aging pathways, as well as aging features, have made invertebrates like the nematode *C. elegans*, and the fruit fly *Drosophila melanogaster* elected model organisms of aging research (Kenyon, 2010; Kaeberlein, 2013). The homologs of the AhR are *ahr-1* (Powell-Coffman et al., 1998) and *spineless* (Duncan et al., 1998) in *C. elegans* and Drosophila, respectively. AhR in *C. elegans* and Drosophila differ from mammalian AhR in their structure and their ligand binding ability. In fact, the classical AhR ligand dioxin does not bind AHR-1 (Powell-Coffman et al., 1998) and most likely neither Spineless (Duncan et al., 1998). Additionally, no direct binding of a ligand has ever been shown in *C. elegans* or Drosophila. *In vitro* studies showed that the Drosophila AhR is constitutively active which might result in the inability of ligand-dependent activation

(Kudo et al., 2009). Recent studies show however that indoles from commensal bacteria extend the lifespan of *C. elegans* and Drosophila in an AhR-dependent manner (Sonowal et al., 2017). This might suggest that microbiota-derived small molecules could act as evolutionarily conserved AhR modulators. For a long time, the main focus of AhR research has been on the response to xenobiotics and the inability of invertebrate AhR to bind dioxin might be one of the reasons why few is known about the function of invertebrate AhRs. However, a conserved function of the AhR in the regulation of developmental processes has been shown in *C. elegans* and Drosophila: *C. elegans ahr-1* mutants develop slightly slower than wild type (Aarnio et al., 2010) and have defects in neuronal development (Huang et al., 2004; Qin and Powell-Coffman, 2004; Qin et al., 2006; Smith et al., 2013). Similarly, Drosophila *spineless* mutants have defects in the development of antenna (Burgess and Duncan, 1990) and sensory neurons (Kim et al., 2006). Another conserved function of the AhR might be the regulation of fertility. *C. elegans ahr-1* mutants have a slightly reduced egg number and an increased embryonic lethality (Aarnio et al., 2010). Although no such function is described in Drosophila, reproduction is affected by AhR in mice (Baba et al., 2008). These studies suggest that the physiological functions of the AhR might be conserved during evolution. Most importantly, AhR has as well an evolutionarily conserved role in the regulation of aging (Eckers et al., 2016). In contrast to the detrimental effects of loss of AhR function in early life, during aging a decreased AhR expression is beneficial: in a cross-species study, we showed that the AhR promotes aging phenotypes in human, mice and *C. elegans* (Eckers et al., 2016) and thus present an evolutionarily conserved role of the AhR in the aging process. More specifically, *C. elegans* carrying a loss of function allele of *ahr-1* had a longer lifespan and an increase in physiological functions (e.g., motility and pharyngeal pumping) and stress resistance during aging (Eckers et al., 2016). Moreover, a higher spontaneous movement activity of *C. elegans*, *D. melanogaster* and humanized mice with reduced *AhR* expression or activity is reported (Williams et al., 2014). Although this is not a direct phenomenon in aging, a decreased movement can be considered a parameter for aging. A potential over-activation of the AhR during the aging process is further supported in *C. elegans* by the observation of increased *ahr-1* mRNA expression during aging (Sonowal et al., 2017).

2.2 AhR and Aging in Mice

Currently, four different strains of mice with a complete AhR deficiency (AhR−/−) exist, which have been generated by different laboratories. In two of these strains, the coding part of exon 1 of the AhR gene was replaced with either a neomycin resistance cassette (Fernandez-Salguero et al., 1995) or the bacterial β-galactosidase gene fused to a NLS (Mimura et al., 1997) (thereby deleting the translation start codon as well as a stretch of basic amino acids that may play a role in DNA binding. The third line was generated by deletion of exon 2, which encodes the bHLH domain. Deletion of this exon leads to out of frame splicing from exon 1 into exon 3 and translation termination in codon 24, such that no functional AhR is produced (Schmidt et al., 1996). The AhR-deficiency does not result in lethality during *in utero* development, as the pups in all lines show a Mendelian distribution of the different genotypes (AhR+/+, AhR+/−, AhR−/−). Recently, a fourth AhR knockout mouse model (C57BL/6-*Ahr*$^{TM1.2Arte}$) was created by Taconic[1]. These mice carry a deletion in exon 3, resulting in an out of frame splicing of exons 2 to exon 4. For simplicity, in this review the four different mice strains will be designated as AhR$^{\Delta 1neo/\Delta 1neo}$ (Fernandez-Salguero et al., 1995) AhR$^{\Delta 1gal/\Delta 1gal}$ (Mimura et al., 1997), AhR$^{\Delta 2/\Delta 2}$ (Schmidt et al., 1996) and AhR$^{\Delta 3/\Delta 3}$ (Taconic), respectively. These mouse strains show different phenotypes. On the one hand they exhibit common features like alterations in hepatic development, reproductive health, and retarded growth during the first 4 weeks compared to wild type mice. On the other hand, they show differences in immune system and reaction and susceptibility to infection, which is possibly due, at least in part, to differences in the genetic background (Fernandez-Salguero et al., 1995; Schmidt et al., 1996; Fernandez-Salguero et al., 1997; Lahvis et al., 2005; Baba et al., 2008; Esser, 2009; Butler et al., 2012).

Given that the AhR is heavily involved in detoxification it is not surprising that AhR deficiency has a profound effect on the hepatic system. All four AhR-deficient mice have reduced liver size, portal fibrosis, and a persistent intrahepatic porto-systemic shunt. Furthermore AhR−/− mice display an increased susceptibility to hepatocarcinogenesis and developed larger tumors (Moreno-Marin et al., 2017). In contrast, the AhR$^{\Delta 1neo/\Delta 1neo}$ improved the regenerative potential of the lung in response to the deleterious effects of acute toxin exposure (Morales-Hernandez et al., 2017).

AhR is not only involved in xenobiotic metabolism, but also in regulation of inflammation like macrophage M1/M2 polarization and cytokine secretion. It is discussed that AhR activation induces oxidative stress as a result of excessive generation of ROS. Recent studies indicate that AhR also affects several age-associated processes, such as vascular function or cellular senescence and age-associated macular degeneration (Hu et al., 2013; Singh et al., 2014; Eckers et al., 2016; Bravo-Ferrer et al., 2019). The recently generated AhR$^{\Delta 3/\Delta 3}$ mice showed a premature aging phenotype resulting in a reduced life span. Those mice display functionality decline in several organs (Fernandez-Salguero et al., 1995; Bravo-Ferrer et al., 2019). In contrast, the AhR$^{\Delta 2/\Delta 2}$ mice show a similar survival rate as wild type mice until 15 months of age and do not display a premature aging phenotype (Singh et al., 2014).

Atherosclerosis is assumed as an age-related, chronic inflammatory disease. Several studies have demonstrated that activation of AhR by dioxin or benzo[*a*]pyrene promotes atherosclerosis (Schmidt et al., 1996; Curfs et al., 2005; Wu et al., 2011). AhR overexpressing mice, which display a 10-fold higher affinity to benzo[*a*]pyrene, were crossed to apolipoprotein E deficient mice. Those mice display larger hearts under basal conditions. Moreover, mice showed increased numbers of atherosclerotic plaques in response to benzo[*a*]pyrene compared to a congenic mouse strain expressing an AhR with lower affinity (Kerley-Hamilton et al., 2012). We demonstrated the impact of AhR in the vascular system. By using the AhR$^{\Delta 2/\Delta 2}$ mice,

[1]https://www.taconic.com/mouse-model/ahr-knockout-mouse

TABLE 1 | Age-related phenotypes in different species and tissues.

Species	Observation	AhR regulation*	References
C. elegans ahr-1(ju145)	Increased lifespan, movement, heat stress resistance and Pharyngeal pumping	Promotes aging	Eckers et al., 2016
C. elegans and *Drosophila melanogaster*	Increased movement	Promotes aging	Williams et al., 2014
C. elegans ahr-1(ju145) & ahr-1(ia03)	Increased lifespan	Promotes aging	Sonowal et al., 2017
Drosophila melanogaster	Increased heat stress survival	Promotes aging	Sonowal et al., 2017
Mus musculus B6.129AhR$^{\Delta 1/\Delta 1G}$	Cardiac hypertrophy; macular degeneration; Pyloric hyperplasia; hepatocellular tumors; skin lesions	Prevents aging	Fernandez-Salguero et al., 1995; Fernandez-Salguero et al., 1997
Mus musculus B6.129AhR$^{\Delta 2/\Delta 2}$	Similar survival rate as wild type mice until 15 months of age	No aging phenotype	Singh et al., 2014
Mus musculus B6.129AhR$^{\Delta 1/\Delta 1F}$	Bladder cancer in older mice; regress of seminal vesicles	Prevents aging	Baba et al., 2008; Butler et al., 2012
Mus musculus AhR$^{\Delta 3/\Delta 3}$	Cardiac hypertrophy, liver fibrosis; kyphosis	Prevents aging	Bravo-Ferrer et al., 2019
Mus musculus and Human	Positive correlation between macular degeneration and AhR expression	Prevents aging	Hu et al., 2013
Mus musculus and Human	Positive correlation between AhR expression and vessel stiffness in the cardiovascular system	Promotes aging	Eckers et al., 2016
Human	Positive correlation between coronary arterial disease and AhR in the cardiovascular system	Promotes aging	Huang et al., 2015

**Prevents aging: AhR expression/activity prevents aging. Promotes aging: down-regulation of AhR expression/activity promotes aging.*

which display no phenotype in adulthood, we showed a decrease in vascular stiffness, which was accompanied by increased eNOS-activity and NO-bioavailability (Eckers et al., 2016). On the other hand, AhR$^{\Delta 1neo/\Delta 1neo}$ mice demonstrated cardiac hypertrophy, thickening of the arterial media and increased numbers of vascular smooth muscle cells in the arterial wall (Sauzeau et al., 2011).

In summary, the four AhR-deficient mice show different phenotypes with respect to aging (**Table 1**). However, since the AhR is needed during development and as a response to environmental, one has to consider that an early embryonic defect could result in a different outcome in adulthood. Therefore, it is maybe difficult to use mice, which are total AhR knockouts. One should rather use AhR conditional knockout mice to induce AhR deficiency in adulthood.

2.3 AhR and Aging in Humans

Evidence for the role of AhR in aging in human subjects or human cell culture systems is rare. Most of these studies focus on the effect of certain AhR modulators on aging or associated parameters. A recent study showed for example that activation of the AhR by airborne polycyclic aromatic hydrocarbons induced cell aging and the expression of aging-related genes in human skin cells (Qiao et al., 2017). Interestingly, the expression of aging-related genes was inhibited by the presence of an AhR antagonist (Qiao et al., 2017). While studies using AhR ligands/modulators are very valuable for finding treatments for the prevention of pollution-induced aging or disease phenotypes, the direct effect of AhR in these processes is elusive since even high-affinity ligands like dioxin also affect cells in an AhR-independent manner (Hossain et al., 1998). There are only very few studies on the role of AhR on aging in the absence of modulators. A study from 2013 shows that AhR activity decreases during aging in human retinal pigment epithelial cells (Hu et al., 2013). Moreover, AhR protein levels were lower in cells from old donors compared to young donors, while AhR mRNA levels remained unaltered (Hu et al., 2013). They verified their findings in a mouse model and associated decreased activity of the AhR to age-related macular degeneration-like pathology (Hu et al., 2013). In 2016 we found that AhR expression is positively correlated to cardiovascular aging in humans (Eckers et al., 2016). Pulse wave velocity as the up to now best marker for vascular aging is increased with age and is positively correlated with AhR mRNA levels. Thus, increased AhR expression seems to be associated with old age in humans, thus we propose that AhR expression level is an indicator for vessel functionality (Eckers et al., 2016). Along the same lines, AhR expression has been linked to the incidence of coronary arterial disease in an epidemiological study on a Chinese population (Huang et al., 2015). They found increased AhR mRNA expression in coronary arterial disease patients compared to controls and suggested AhR as a diagnostic biomarker for coronary arterial disease (Huang et al., 2015).

In summary, the few studies on the role of AhR in human aging, similar to mice studies, display a complex role of the AhR. It has to be noted that one should clearly separate the effects of AhR in the development and in the aging process from invertebrates to vertebrates. However, it could be possible that the effect of the AhR on aging is tissue-dependent (**Table 1**) as well as environment dependent. Thus, more research in adult and aged invertebrates, vertebrates and humans is needed to understand the pathophysiological role of AhR in aging in different tissues, organs, as well as in the whole organism.

3. AhR-MITOCHONDRIA CROSSTALK

Mitochondria play a central role in the aging process, are targeted by environmental pollutants and represent a central hub in nutrient metabolism. Interestingly, both environmental pollutants and dietary factors, such as polyphenols, can influence the transcriptional activity of the AhR. We, therefore,

envisioned a possible crosstalk between AhR and mitochondria. Surprisingly, while mitochondria are extensively studied (more than 150,000 publications on PubMed) and, there are many studies investigating the influence of different AhR modulators on mitochondria, there are only 32 publications directly linking mitochondria to the AhR.

The effects of polyphenols on AhR and mitochondria are complex (reviewed in Sandoval-Acuna et al., 2014; Xue et al., 2017). On the one hand natural polyphenols are ROS scavengers and thereby influence mitochondria, which are targeted by ROS, but also mitochondrial down-stream signaling through the scavenging of mitochondrial ROS. This ROS scavenging function might be as well important for the influence of polyphenols on AhR activity. ROS can activate AhR through the conversion of tryptophan to FICZ (Smirnova et al., 2016) and thus the ROS-scavenging properties of polyphenols might prevent this activation and connect AhR with mitochondria. On the other hand, additional ROS-scavenging independent functions of polyphenols are reported on mitochondria like regulation of mitochondrial biogenesis, mitochondrial membrane potential, and mitochondrial electron transport chain activity (Sandoval-Acuna et al., 2014). Given the central role of Sirt1 in the aging process, a very interesting ROS-scavenging independent mode of action of polyphenols on mitochondria is the induction of mitochondrial biogenesis through Sirt1. Several polyphenols indeed were shown to activate Sirt1 (reviewed in Ajami et al., 2017). Considering the interaction between AhR and Sirt1 (Koizumi et al., 2014; Ming et al., 2015; Sutter et al., 2019), the notion that ROS activates AhR (Smirnova et al., 2016), and the multiple roles polyphenols may exert on mitochondria, these findings provide indirect evidence for a possible crosstalk between AhR, Sirt1 and mitochondria of relevance for the aging process.

The first direct evidence of a link between mitochondria and the AhR was published in 2002 (Senft et al., 2002). In this study, Senft and co-workers investigated the role of AhR signaling in the increase of mitochondrial ROS upon dioxin treatment in the liver of mice. Specifically, they found that dioxin treatment induced mitochondrial ROS in wild type but not in AhR−/− mice (Senft et al., 2002). Interestingly, they noticed that the basal mitochondrial ROS levels were lower in AhR−/− mice (Senft et al., 2002), which might suggest that AhR has an impact on the mitochondria not only in the presence of ligands but also under normal conditions. Dioxin exposure was also shown to decrease mitochondrial membrane potential in spermatozoa of mice in an AhR-dependent manner (Fisher et al., 2005). Similarly, embryonic stem cells and beating cardiomyocytes from AhR−/− mice are protected against the dioxin-induced increase in markers of mitochondrial stress and of mtDNA damage (Wang et al., 2016). Together, these studies suggest that AhR mediates mitochondrial dysfunction in response to dioxin. Moreover, benzo[*a*]pyrene was shown to increase mitochondrial dysfunction and decrease the mitochondrial membrane potential, resulting in the depletion of ATP levels along with inhibition of the oxygen consumption rate in the human keratinocyte cell line (HACAT). In this study, it was shown that the removal of damaged mitochondria by mitophagy is reduced in AhR and CYP1B1 (an AhR target gene) knockdown but a direct link between AhR and mitophagy was not established (Das et al., 2017). In another study genetic ablation of the AhR resulted in reduced expression of Superoxide Dismutases (SODs) in fibroblasts. Thus, those fibroblasts are more sensitive to cigarette smoke resulting in increased cell death and reduced proliferation, which is accompanied by decreased mitochondrial membrane potential (Rico De Souza et al., 2011). The detrimental effect of loss of AhR function is further supported by studies in embryonic hearts of mice, where the disruption of AhR signaling leads to mitochondrial dysfunction (Carreira et al., 2015b). Similarly, female, but not male mice exposed to dioxin as embryos showed altered expression in genes of the canonical mitochondrial pathway and a higher number of mitochondria in the heart (Carreira et al., 2015a). In adult AhR−/− mice those changes were not observed (Carreira et al., 2015a). In both studies the effect of dioxin treatment was not investigated in AhR−/− mice.

AhR does not only influence mitochondrial function but two studies have recently suggested that the AhR is also localized within the mitochondria. Tappenden et al. (2011) were the first to identify an interaction between the AhR and the ATP5α1 subunit of the ATP synthase complex in different cell lines. Further analysis of the exact localization of the mitochondrial AhR in murine hepatoma cells showed that it localizes inside the intermembrane space (Hwang et al., 2016). Interestingly, when treated with dioxin, mitochondrial localization of the AhR and interaction with ATP5α1 were lost (Tappenden et al., 2011; Hwang et al., 2016), suggesting that AhR only localizes inside the mitochondria in the absence of ligands. Considering that AhR is bound by AIP in the absence of ligands and that AIP has been found to interact with the Mitochondrial import receptor subunit TOMM20 and to mediate preprotein transport in mitochondria (Yano et al., 2003), AIP could be the critical mediator of AhR localization into mitochondria. Indeed, siRNA against TOMM20

TABLE 2 | Tissue- and age-specific effects of AhR expression on mitochondrial function in mice.

Tissue/cells	Age	Observation	Effect of AhR−/−	References
Liver		Dioxin exposure increases mitochondrial ROS	Protective to dioxin exposure	Senft et al., 2002
Spermatozoa		Dioxin exposure decreases the mitochondrial membrane potential	Protective to dioxin exposure	Fisher et al., 2005
Fibroblasts		Reduced expression of SODs in AhR deficient cells	Detrimental to cigarette smoke	Rico De Souza et al., 2011
Heart	Embryo	AhR−/− induce mitochondrial dysfunction	Detrimental	Carreira et al., 2015b
	Adult	Dioxin treatment of embryos induces mitochondrial dysfunction in adults	No effect	Carreira et al., 2015a

reduces mitochondrial AhR, but not cytoplasmatic or nuclear AhR by 70% (Hwang et al., 2016). Thus, Hwang et al. proposed that AIP and HSP90 contribute to the mitochondrial localization of AhR by interacting with TOMM20, which imports AhR into the intermembrane space (Hwang et al., 2016).

Taken together, these studies strongly suggest that the effects of AhR on mitochondrial function are likely tissue-, age-, and maybe even sex-dependent (**Table 2**). Additionally, the presence of dioxin seems to have a strong impact on the outcome of the study. Nonetheless, these are mainly *in vitro* studies and causal-effect, as well as mechanistic studies in primary cells and model organisms in more physiological conditions, are required to clearly establish a possible crosstalk between AhR and mitochondria. Interestingly, in *C. elegans*, animals with reduced mitochondrial function and *ahr-1* mutants share some phenotypic features like slower larval development, alterations in fat metabolism and sensory neurons and most importantly lifespan extension (Rea et al., 2007; Aarnio et al., 2010; Schiavi et al., 2013; Smith et al., 2013; Maglioni et al., 2014; Eckers et al., 2016). Moreover, long-lived mitochondrial mutants have increased levels of *cyps*, *ugts*, and *gsts* (Cristina et al., 2009; Liu et al., 2014; Mao et al., 2019), which, at least in mammals are known target genes of the AhR. Investigating the potential AhR-mitochondrial crosstalk in appropriate *in vivo* model systems will certainly help revealing its potential causal role in different pathophysiological contexts including aging and associated pathologies.

AUTHOR CONTRIBUTIONS

NV and JH contributed to the conception of the review. VB and NA-A wrote the sections of the manuscript. All authors contributed to the manuscript revision, editing read, and approved the submitted version.

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REVIEW
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Heme Oxygenase Dependent Bilirubin Generation in Vascular Cells: A Role in Preventing Endothelial Dysfunction in Local Tissue Microenvironment?

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Among antioxidants in the human body, bilirubin has been recognized over the past 20 years to afford protection against different chronic conditions, including inflammation and cardiovascular disease. Moderate increases in plasma concentration and cellular bilirubin generation from metabolism of heme via heme oxygenase (HMOX) in virtually all tissues can modulate endothelial and vascular function and exert antioxidant and anti-inflammatory roles. This review aims to provide an up-to-date and critical overview of the molecular mechanisms by which bilirubin derived from plasma or from HMOX1 activation in vascular cells affects endothelial function. Understanding the molecular actions of bilirubin may critically improve the management not only of key cardiovascular diseases, but also provide insights into a broad spectrum of pathologies driven by endothelial dysfunction. In this context, therapeutic interventions aimed at mildly increasing serum bilirubin as well as bilirubin generated endogenously by endothelial HMOX1 should be considered.

Keywords: HMOX1, bilirubin, endothelial cells, diabetes, atherosclerosis, hypertension, obesity, tumor

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INTRODUCTION

Bilirubin is widely known as an end-product of the degradation of heme which occurs in the spleen primarily as part of red blood cell removal. Senescent or damaged red blood cells are degraded and the heme groups derived from hemoglobin are converted into biliverdin by the activity of heme oxygenases (HMOX1 and HMOX2) (Maines, 1988), and then converted into bilirubin via the

Abbreviations: 8-oxodGuo, 8-oxo-7,8-dihydro-2'-deoxyguanosine; ALA, 5-aminolevulinic acid; ABCA1, ATP-binding cassette transporter A; AngII, angiotensin II; AT1, angiotensin type 1 receptor; BBB, blood-brain barrier; BRT, bilirubin ditaurate; BLVRA, biliverdin reductase; CO, carbon monoxide; CoPPIX, cobalt protoporphyrin IX; CVD, cardiovascular disease; DM, diabetes mellitus; EDH, endothelial dependent hyperpolarization; EPC/cEPC, endothelial progenitor cells/circulating endothelial progenitor cells; FABPs, fatty acid binding proteins; GS, Gilbert syndrome; HFD, high fat diet; HG, high glucose; HMOX1, heme oxygenase 1; HMOX2 heme oxygenase 2; HNE, hydroxynonenal; MCAO, middle cerebral artery occlusion; MMP2, metalloproteinases; MS, multiple sclerosis; NOXs, NADPH oxidases; NSCLC, non-small cell lung cancer; OATP, organic anion transport binding protein; OVA, ovalbumin; PE, preeclampsia; PI, protease inhibitor; PMBCs, peripheral blood mononuclear cells; ROS, reactive oxygen species; SLE, systemic lupus erythematosus; UCB, unconjugated bilirubin; UTG1A1, uridine diphpspho-glucuronosyl transferase 1A1; VSMC, vascular smooth muscle cells; XO, xanthine oxidase.

activity of BLVRA. This form of bilirubin, known as UCB, is bound to albumin to enable its plasma transport to the liver where it is conjugated with glucuronic acid and excreted as main component of bile (Kapitulnik, 2004).

The elevation of bilirubin concentration in plasma is well known as a marker of hemolytic conditions, liver damage or bile-duct impairment. When the concentration of UCB exceeds the binding capability of albumin, the fraction of free bilirubin (unbound UCB) increases and can cause toxicity in the nervous system in particular, a serious condition known as bilirubin encephalopathy which has been the focus of excellent reviews (Ostrow et al., 2003; Shapiro, 2003; Kapitulnik, 2004). It is important to note that neuronal cells are particularly sensitive to UCB toxicity compared to other cell types (Silva et al., 2002). Indeed, neuronal toxicity is observed *in vitro* even at relatively low concentrations, e.g., 0.5 μM UCB is toxic to primary cells and acts to enhance glutamate-induced toxicity (Grojean et al., 2000). Moreover, mitochondrial damage is critically involved in neuronal damage induced by UCB especially in developing neurons (Rodrigues et al., 2002).

Nonetheless, different beneficial aspects linked to mild elevations of plasma bilirubin (up to 12 mg/dl) have been recognized. The anti-inflammatory effect of jaundice was already observed by Hench (1938) in patients affected by rheumatoid arthritis and by Engerman and Meyer (1959) in hyperbilirubinemic rats. More recent evidence comes from individuals with GS, where bilirubin affords cardiovascular protection (Vítek et al., 2002; Novotný and Vítek, 2003; Maruhashi et al., 2012; Gazzin et al., 2016). Furthermore, the degradation pathway of heme occurs in virtually all tissues, since heme-containing proteins are ubiquitously distributed and undergo physiological turnover. Notably, also the generation of bilirubin in peripheral tissues has been proposed to be protective (Sedlak et al., 2009; Takeda et al., 2015; Nam et al., 2018), and bilirubin derived from vascular cells could modulate tissue microenvironment playing both antioxidant and anti-inflammatory roles (Siow et al., 1999; He et al., 2015). We pointed out this aspect in the different contexts analyzed in the present review. Importantly, studies of cytoprotective pathways in endothelial cells is receiving considerable attention in view of potential therapeutic targets to maintain vascular health or to delay or treat vasculopathies (Kim Y.-M. et al., 2011; Calay and Mason, 2014; Mason, 2016).

BIOSYNTHESIS, METABOLISM, BIOAVAILABILITY AND ACTIVITY

Heme oxygenase (HMOX) is the first, rate-limiting enzyme in heme degradation pathway, with two major isoforms of HMOX identified. HMOX1 is inducible, expressed only under oxidative stress or when heme concentration increases; HMOX2 is constitutively present mainly in testis and neuronal cells (Maines et al., 1986) but has also been detected in vascular cells (Chen et al., 2014). HMOX catalyzes the opening of the prothoporphyrinic ring of heme, generating biliverdin, free iron (Fe^{2+}) and CO (Maines, 1988). The activity of BLVRA converts biliverdin into UCB. Heme degradation occurs in all cell types in order to complete the turnover of heme-containing proteins. Furthermore, HMOX1 induction crucially drives cell adaptive responses to stressors and its metabolic products exert potent biological activities (Siow et al., 1999; Otterbein et al., 2016; Kishimoto et al., 2019). CO has anti-apoptotic and anti-inflammatory activities (Ryter et al., 2002), both intracellularly and in the microenvironment (Morita and Kourembanas, 1995) of blood vessels by increasing cGMP concentration and activating the MAPK pathway (Siow et al., 1999; Ryter et al., 2002). The generation of free iron is potentially highly toxic but, under physiological conditions, a parallel induction of the heavy chain of ferritin, and the activation of membrane Fe-ATPase transporters occurs (Dulak et al., 2002; Loboda et al., 2008). This adaptation is critical in decreasing intracellular Fe^{2+} content and prevents the generation of ROS via the Fenton reaction, and thereby maintains endothelial function (Balla et al., 2007). Finally, bilirubin is a potent antioxidant, as highlighted by the pioneering studies of Stocker et al. (1987). It has been well demonstrated that bilirubin efficiently scavenges ROS, peroxynitrite and peroxyradicals (Neuzil and Stocker, 1993). Moreover, as it is lipophilic, bilirubin is able to prevent lipid peroxidation (Stocker et al., 1987). Indeed, in HEK293 cells, the ability of UCB to prevent lipid peroxidation has been well documented (Sedlak et al., 2009), e.g., in hepatoblastoma cells and plasma samples exposed to oxidative stressors and LPS (Zelenka et al., 2012). Notably, UCB was shown to prevent LDL oxidation, 20 times more efficiently than vitamin E (Wu et al., 1994).

In addition to HMOX1, modulation of other steps involved in bilirubin production, from the synthesis of heme rings to the reduction of biliverdin catalyzed by BLVRA, plays a role in cellular protection (**Figure 1**). Indeed, recent evidence from different cell lines suggests that a continuous *de novo* synthesis of heme occurs with the specific purpose of bilirubin generation in order to provide cytoprotection (Takeda et al., 2015). Indeed, by inhibiting the synthesis of ALA the synthesis of bilirubin is reduced, leading to increased cell damage in response to oxidative stress and demonstrating that endogenous bilirubin is highly efficient in preventing cell damage.

Furthermore, BLVRA contributes to the antioxidant properties of the HMOX1/bilirubin system, as pointed out by Jansen and Daiber (2012). Importantly, Nam et al. (2018) have recently discussed the physiological relevance of the conversion of biliverdin to bilirubin through an energy consuming reaction, highlighting that the reaction carried out by BLVRA dramatically increases the electrophilicity of bilirubin in comparison to biliverdin. This enables its binding to thiol reactive cysteines on Keap1, favoring Nrf2-dependent gene induction and thereby generating a positive feedback of cytoprotection. Moreover, the up-regulation of Nrf2 induced by bilirubin treatment has already been demonstrated in neuronal-like cells (Qaisiya et al., 2014) and is of relevance in the context of maintaining vascular health. Notably, bilirubin can be oxidized back to biliverdin and then be regenerated by the activity of BLVRA. The biliverdin-bilirubin cycle has been postulated to be highly active under reducing oxidative stress (Baranano et al., 2002) and, by cooperating with GSH (Sedlak et al., 2009), is able to

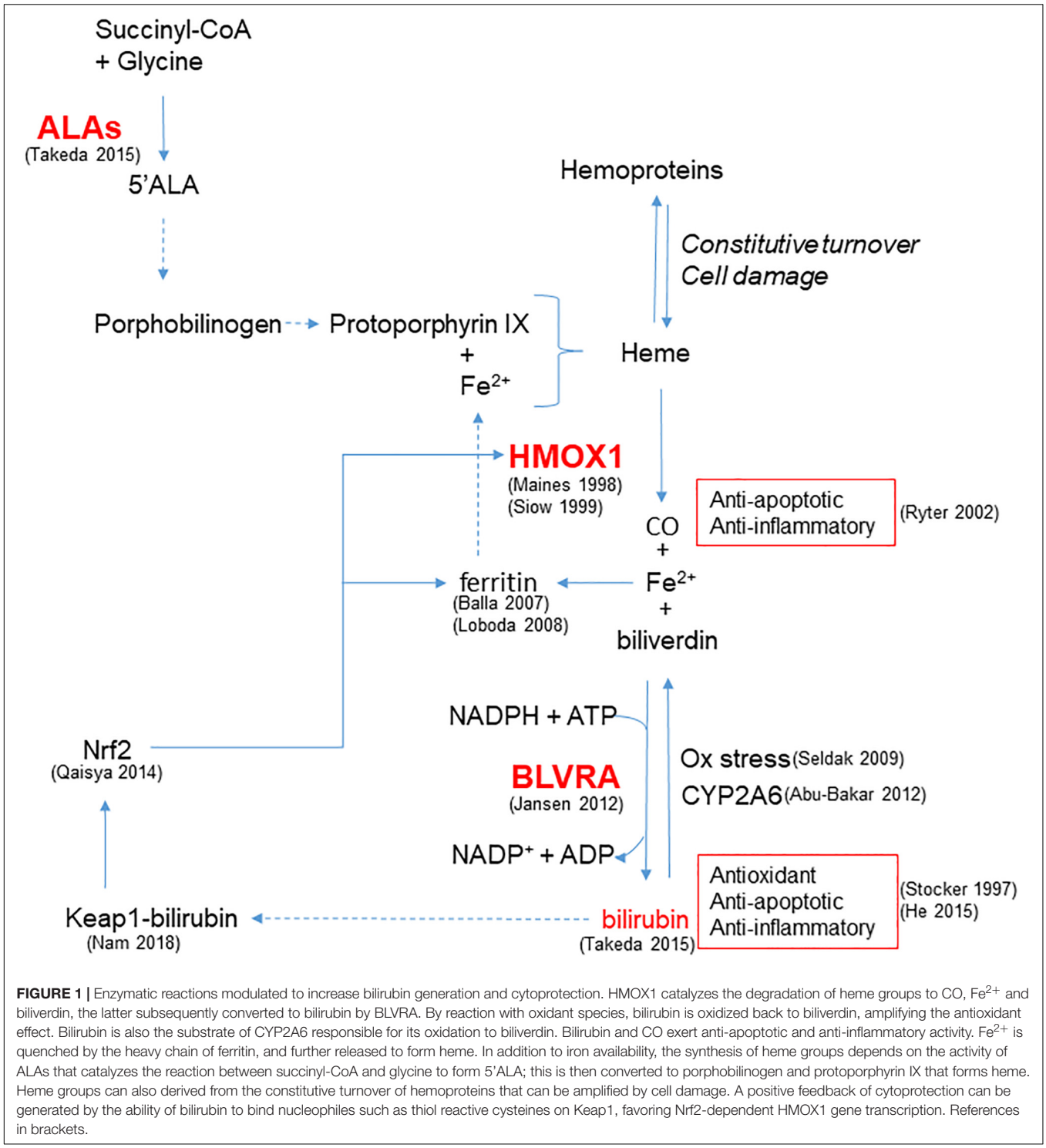

FIGURE 1 | Enzymatic reactions modulated to increase bilirubin generation and cytoprotection. HMOX1 catalyzes the degradation of heme groups to CO, Fe^{2+} and biliverdin, the latter subsequently converted to bilirubin by BLVRA. By reaction with oxidant species, bilirubin is oxidized back to biliverdin, amplifying the antioxidant effect. Bilirubin is also the substrate of CYP2A6 responsible for its oxidation to biliverdin. Bilirubin and CO exert anti-apoptotic and anti-inflammatory activity. Fe^{2+} is quenched by the heavy chain of ferritin, and further released to form heme. In addition to iron availability, the synthesis of heme groups depends on the activity of ALAs that catalyzes the reaction between succinyl-CoA and glycine to form 5'ALA; this is then converted to porphobilinogen and protoporphyrin IX that forms heme. Heme groups can also derived from the constitutive turnover of hemoproteins that can be amplified by cell damage. A positive feedback of cytoprotection can be generated by the ability of bilirubin to bind nucleophiles such as thiol reactive cysteines on Keap1, favoring Nrf2-dependent HMOX1 gene transcription. References in brackets.

counteract a 10,000-fold excess of H_2O_2 (Doré et al., 1999), affording protection in endothelial cells (Jansen et al., 2010). Moreover, mitochondrial CYP2A6 is able to perform the same oxidation of UCB back to biliverdin, highlighting the biological importance of biliverdin-bilirubin redox cycle to maintain cell homeostasis (Abu-Bakar et al., 2012).

UCB is able to bind different proteins to facilitate crossing plasma or intracellular membranes. As widely reviewed (Bellarosa et al., 2009), it can bind ABC transporters (ABCB1, ABCC1,2,3, ABCG2 and the OATP). In plasma, UCB binds to albumin and to enable uptake in the liver, it binds to transport proteins initially identified as ligandins (Litwack et al., 1971)

and subsequently recognized as members of GSH transferases (Habig et al., 1974). Especially in the liver, UCB is transformed to conjugated bilirubin by the activity of uridine diphosphoglucoronosyl transferase 1A1 (UGT1A1) in order to increase its water solubility. Hepatic bilirubin metabolism and transport have been widely investigated (Kamisako et al., 2000).

Interestingly, as far as the intracellular transport is concerned, it was postulated as early as 1969 (Levi et al., 1969) that UCB could bind FABPs which are involved in the intracellular transport of organic anions. In this context, the discovery of UnaG protein from freshwater eel (Kumagai et al., 2013), a protein belonging to FABPs that selectively binds bilirubin and generates a fluorescent signal, improved the detection of endogenously generated bilirubin enabling studies of its subcellular localization and transport. Endogenous UCB seems to be mainly located at ER cytosolic membranes, associated with HMOX1/BLVRA activities and has also been detected in the nucleus and mitochondria (Park et al., 2016). However, intracellular transport mechanisms of endogenous UCB and its role in subcellular compartments remain to be explored.

Among the different cell types, neuronal cells are highly sensitive to UCB toxicity especially during development, and undergo apoptosis at relatively low concentrations (0.25–5 μM) of UCB (Grojean et al., 2000; Rodrigues et al., 2002). In addition, as far as endothelial cells are concerned, different sensitivities to UCB have been postulated depending on the different tissues from which endothelial cells were derived. Moreover, immortalized endothelial cell lines, such as bEnd.3 cells used as a model of BBB, undergo oxidative stress and apoptosis when treated with 20–40 μM UCB, while no significant damage occurs in endothelial cells derived from pancreatic islets of Langerhans (Kapitulnik et al., 2012). The peculiar sensitivity of BBB-like endothelial cells has been confirmed by other authors (Palmela et al., 2012), underlining the marked sensitivity of brain endothelial cells and neurons to UCB, as previously reviewed (Ostrow et al., 2003; Brito et al., 2014).

However, regardless of the endothelial cell type, the protective, pro-survival activity of low concentrations of UCB (0.1–5 μM) has been widely documented and ascribed to its antioxidant properties (Kapitulnik et al., 2012). Thus, although the different cell types exhibit varying degrees of sensitivity toward bilirubin toxicity, they all benefit from bilirubin cytoprotection at low concentrations. This has clinical implications, since recent research has shown that BRT prevents ROS generation in *ex vivo* platelets, opening the possibility of screening the actions of acute BRT treatment to improve platelets storage and function (Pennell et al., 2019).

EFFECTS ON VASCULAR REDOX STATUS: IMPLICATIONS FOR HYPERTENSION, DIABETES AND ISCHEMIA/REPERFUSION INJURY

The molecular mechanisms underlying the antioxidant activity of UCB in endothelial cells have been investigated extensively in the context of various pathophysiological conditions and diseases. Cytoprotection afforded by UCB has been attributed to quenching of superoxide anions derived from NOXs (Datla et al., 2007). Indeed, HMOX1 induction by hemin prevents NOX2/4 activation in apolipoprotein E-deficient mice characterized by vascular oxidative stress, and the same result is observed following bilirubin treatment *in vitro* or HMOX1 overexpression via gene transfer in VSMC (Datla et al., 2007). Notably, UCB derived from HMOX1 in endothelial cells has been shown to be able to scavenge NADPH oxidase derived ROS, impairing leukocyte transmigration and inflammatory responses (Vogel and Zucker, 2016), as discussed in Section "Effects on Adhesion Molecule Expression: Implications for Obesity, Atherosclerosis and Chronic Inflammatory Diseases."

Protection afforded by bilirubin in endothelial cells has recently been reported Ziberna et al. (2016), who demonstrated that HMOX1 induction in response to CoPPIX or cell supplementation with a very low concentration of bilirubin ($\leq$50 nM) significantly elevates intracellular bilirubin levels, leading to increased antioxidant defenses. It is important to note that bilirubin derived from HMOX2 can also protect vascular cells. Notably, based on the co-localization of HMOX2 with NOX4 in cerebral microvascular endothelial cells, it has been hypothesized that bilirubin may scavenge ROS derived from TNFα induced NOX4 activation (Basuroy et al., 2009). Thus, there is accumulating evidence that low concentration of UCB, both plasma and endothelial cell derived, can affect endothelial redox balance and thereby attenuate cell damage in oxidative stress.

Bilirubin and Hypertension

Slight increases in plasma total bilirubin concentrations (1.53 ± 0.48 mg/dl) have been reported to preserve flow-mediated vasodilation compared to subjects with low levels of plasma bilirubin (0.40 ± 0.08 mg/dl) (Erdogan et al., 2006). As recently reviewed (Mortada, 2017), a negative correlation between serum level of bilirubin levels and hypertension has been established in different studies (Mortada, 2017). The antihypertensive activity of a moderate hyperbilirubinemia is related to an increase in NO bioavailability due to the reduction of AngII-dependent superoxide generation by endothelial cells (Pflueger et al., 2005; Vera et al., 2009). Indeed, bilirubin quenches superoxide anion derived from NOX in cultured vascular endothelial cells exposed to AngII and increases the bioavailability of NO, leading to vascular relaxation and lowering of blood pressure (Pflueger et al., 2005; Fujii et al., 2010).

Furthermore, HMOX1 derived UCB protects endothelial cells against nitrosative stress, induced by hemin pre-treatment, which is able to further increase HMOX1 dependent bilirubin generation to reduce endothelial apoptosis induced by peroxynitrite (Foresti et al., 1999). Indeed, NO and peroxynitrite are able to induce HMOX1 in endothelial cells, and bilirubin may directly scavenge NO to prevent excessive accumulation (Kaur et al., 2003) and the formation of peroxynitrite-dependent protein modification in human plasma (Minetti et al., 1998).

Thus, a positive loop of cytoprotection seems to exist between NO and HMOX1-derived bilirubin (**Figure 2**).

Moreover, the increased availability of NO due to UCB, positively affects the rate of glomerular filtration improving renal hemodynamics (Vera and Stec, 2010). However, it has also been hypothesized that other mechanisms in addition to superoxide scavenging can be involved in the ability of bilirubin to lower blood pressure and decrease hypertension, for instance the modulation of calcium and potassium channels (Stec et al., 2013). In fact, it has been shown that the induction of HMOX1, through the generation of bilirubin, favors vessel relaxation by acting on the transport of calcium and potassium, and thereby improving EDH in a rat model of spontaneous hypertension (Li et al., 2013).

Bilirubin and Diabetes

The role played by UCB in preventing endothelial dysfunction in chronic sequaele of diabetes has been widely demonstrated (Vítek, 2012). Indeed, the incidence of retinopathy, coronary artery disease, and cerebrovascular diseases is low in Gilbert subjects (i.e., bilirubin level 1.2 mg/dl) with diabetes (Inoguchi et al., 2007). Moreover, the Hisayama Study revealed that increased bilirubin levels counteract diabetic retinopathy (Yasuda et al., 2011), with total bilirubin concentration negatively correlated with the severity of retinopathy (Sekioka et al., 2015).

Furthermore, in diabetic rats, up-regulation of HMOX1 increases serum bilirubin, reduces superoxide anion and endothelial sloughing induced by hyperglycemia (Quan et al., 2004). Moreover, we have reported that activation of the HMOX1/bilirubin pathway protects endothelial cells against HG-induced damage by reducing HNE production and lipid peroxidation (He et al., 2015). In addition, as mentioned before, bilirubin acts by inhibiting NOX. This action, at the level of endothelial cells and mesangial cells in diabetic kidney, has been shown to ameliorate diabetic nephropathy (Fujii et al., 2010). An elegant study by Liu et al. (2015), in different animal models of diabetes, established that HMOX1 overexpression improves vascular function, but that BLVRA activity is also required, underlining that conversion of biliverdin into bilirubin is fundamental. These authors also established

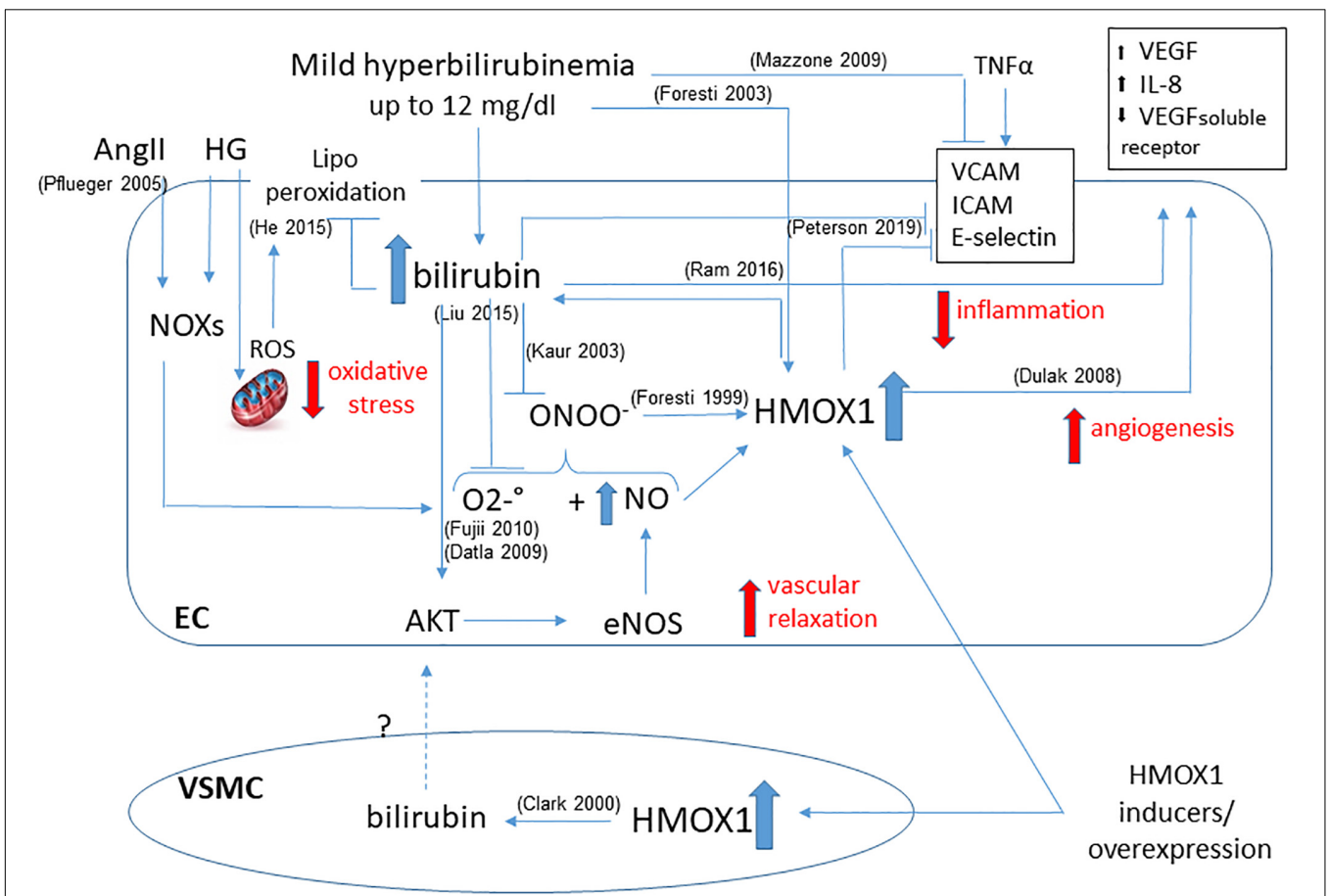

FIGURE 2 | Functional effects of bilirubin on endothelial cells. Positive loop between plasma- and HMOX1 derived bilirubin is shown highlighting the main activities in maintaining endothelial function. The ability to reduce oxidative stress by quenching both $O_2^{-\cdot}$ and $ONOO^-$ prevents the dysfunction due to HG or AngII. The increased bioavailability of NO favors vascular relaxation and further activates HMOX1. The direct activity of bilirubin and HMOX1 in preventing the expression of adhesion molecules (VCAM, ICAM, E-selectin) is showed as well as the generation of angiogenetic factors (VEGF, IL-8, VEGF soluble receptor). A further contribution from VSMC-derived bilirubin has been hypothesized. References in brackets.

that administration of exogenous bilirubin affords vascular protection by restoring AKT-dependent signal transduction (Liu et al., 2015). AKT seems to be a key target in bilirubin activity in endothelial cells, and the ability to restore the AKT-eNOS pathway is involved in not only maintaining endothelial redox balance and cell survival but also pro-angiogenic signals (see section "Effects on Angiogenesis: Implications for Wound Healing, Vascular Dysfunction in Pregnancy and Tumor Growth"). In addition, in a mouse model of obesity, bilirubin has been shown to activate AKT, reducing insulin resistance in skeletal muscle (Dong et al., 2014) and improving glucose tolerance. The role of HMOX1 and BLVRA in inflammation associated with diabetes has recently been reviewed (Rochette et al., 2018).

Bilirubin and Ischemia/Reperfusion Injury

Bilirubin has been shown to be protective against the development of ischemic damage associated with ischemic/reperfusion injury and endothelial activation (Yang et al., 2016). A recent cross-sectional study performed on 1121 healthy Japanese demonstrated that low levels of plasma bilirubin are associated with a high prevalence of ischemic modifications in brain white matter (Higuchi et al., 2018). In this context, a recent review form Thakkar et al. (2019) rigorously analyzed clinical studies highlighting a relationship between bilirubin levels and cerebral ischemic stroke. These authors provided evidence that in the large majority of the studies, a negative relationship exists between the level of serum bilirubin and prevalence of stroke. However, they also discussed limitations of some of these studies that only considered total bilirubin levels and ischemic stroke without considering hemorrhagic stroke or traumatic brain injury. Interestingly, recent evidence in a rat model of MCAO established that intraperitoneal administration of biliverdin significantly reduces the infarct size by modulating microRNA-mRNA network (Zou et al., 2019). It is important to note that at the level of the nervous system, up-regulation of HMOX1 can be both protective and damaging as reviewed recently (Nitti et al., 2018), but the role played by HMOX1 derived bilirubin in neurons and non-neuronal cells in neuroprotection has been highlighted (Hung et al., 2010).

In different models of cardiac ischemia/reperfusion damage, bilirubin administration protects against tissue damage. Indeed, *ex vivo* heart perfusion with BRT improves post-ischemic outcomes by reducing oxidative damage (Bakrania et al., 2016). Moreover, intraperitoneal administration of bilirubin has been shown to prevent cardiolipin oxidation and to reduce the infarct size in a rat model of coronary ischemia/reperfusion injury (Ben-Amotz et al., 2014). More recently, preconditioning performed using nanoparticles of pegylated bilirubin was highly effective in preventing hepatic ischemic damage (Kim et al., 2017). Furthermore, the critical importance of the endogenous HMOX1/bilirubin pathway has been demonstrated following supplementation cell culture media not only with UCB but also with hemin. Both reduce ROS generation induced by re-oxygenation of cardiomyocytes subjected to hypoxia, preventing cell injury (Foresti et al., 2001). Furthermore, hemin efficiently prevents oxidative damage in VSMCs by increasing bilirubin generation (Clark et al., 2000). Even though the majority studies performed *in vivo* or *ex vivo* evaluate whole tissue without considering the specific role played by endothelial cells, it is conceivable that bilirubin supplementation can directly protect the endothelium. It is important to note that UCB induces HMOX1 up-regulation in aortic endothelial cells, generating a positive loop that increases endothelial resistance to oxidative stress (Foresti et al., 2003). The specific anti-inflammatory activity exerted by bilirubin on endothelial cells plays a role in preventing ischemia/reperfusion damage by reducing leukocyte infiltration, as discussed in section "Effects on Adhesion Molecule Expression: Implications for Obesity, Atherosclerosis and Chronic Inflammatory Diseases."

Moreover, protection afforded by bilirubin against rejection of kidney transplantation has been demonstrated, and its antioxidant activity in preventing ischemia/reperfusion damage as well as its anti-inflammatory action that favors organ acceptance has been reviewed, highlighting the positive impact on renal hemodynamics (Sundararaghavan et al., 2018).

EFFECTS ON ADHESION MOLECULE EXPRESSION: IMPLICATIONS FOR OBESITY, ATHEROSCLEROSIS AND CHRONIC INFLAMMATORY DISEASES

The first evidence for the anti-inflammatory properties of bilirubin stems from Philip Hench who in 1938 described the remission of rheumatoid arthritis in the 94% of patients who developed jaundice (Hench, 1938). Even though Hench tried to correlate this phenomenon with an increased level of cortisol (Hench, 1949), this has never been demonstrated, and the anti-inflammatory effect of jaundice has been then proved to be due to UCB itself (Jangi et al., 2013). In this context, a negative correlation between bilirubin plasma concentration and clinical manifestations of other immune-mediated diseases such as MS (Peng et al., 2011) and SLE (Vítek et al., 2010) has been demonstrated. Moreover, an elevation of UCB between 2 and 12 mg/dl has been shown to reduce experimental autoimmune encephalomyelitis in an animal model of MS (Liu et al., 2008). However, it is important to note that UCB concentrations higher than 15–20 mg/dl always induce cytotoxicity.

The ability of UCB to modulate immunoglobulin production, native or acquired immunity as well as complement activity has been reviewed by others (Basiglio et al., 2009; Jangi et al., 2013), highlighting how UCB can impact neutrophil and macrophage phagocytosis, as well as antigen presenting functions to lymphocytes and the generation of pro-inflammatory cytokines. In this way, UCB exerts beneficial effects in different chronic inflammatory and autoimmune diseases, as discussed in section "Bilirubin and Chronic Inflammatory Diseases." However, we here focus on the role played by endothelial cells,

which are closely involved in the onset and development of inflammation.

Bilirubin and Obesity

Endothelial dysfunction and inflammation characterize the development and progression of obesity, and mild hyperbilirubinemia contributes to a reduction of obesity especially during aging. These findings were particularly significant when abdominal obesity, triglyceridemia and hip and waist circumference were considered (Seyed Khoei et al., 2018). The same authors have shown that in Gilbert individuals expression level of p-AMPK, PPAR-α and γ, and PGC1-α in PBMCs were significantly higher in comparison to age- and gender-matched control subjects (Mölzer et al., 2016), whilst others have shown that UCB limits lipid deposition in adipose cells by binding PPAR-α nuclear receptors (Stec et al., 2016). The effect of UCB toward PPARα is discussed subsequently with a focus on atherosclerotic lesions (see section "Bilirubin and Atherosclerosis").

Furthermore, a positive effect of CoPPIX-induced HMOX1 activation on reduction of body weight has been reported by Abraham et al. (2016). The same group, more recently focused on long-term endothelial HMOX1 activation in mice fed a HFD demonstrating the gene therapy to increase expression of HMOX1, reduced ICAM and VCAM expression, decreased serum markers of inflammation, such as IL-1 and TNFα, reduced the size of adipocytes and down-regulated PPARγ. Furthermore, these effects were prevented by using a specific inhibitor of HMOX1 activity, indirectly suggesting that the ability to restore a proper crosstalk between the vasculature and adipocytes depends on the metabolic products of endothelial HMOX1 activity (Peterson et al., 2019). Together these findings demonstrate that endothelial activation of HMOX1 could play an important role in the treatment/prevention of obesity, and further highlight the importance of AMPK-dependent signaling in mediating the anti-obesogenic effects of bilirubin.

Bilirubin and Atherosclerosis

Endothelial activation and recruitment of inflammatory cells are two pivotal steps in the development of atherosclerotic lesions (Moore et al., 2013). The negative correlation between plasma concentrations of bilirubin and onset and progression of CVD is clearly demonstrated by numerous studies and is evident in individuals with GS (Hopkins et al., 1996; Vítek et al., 2002; Novotný and Vítek, 2003; Maruhashi et al., 2012; Vítek, 2017).

UCB has been shown to impair leukocyte migration by affecting endothelial adhesion molecules. The activation of endothelial cells induced by pro-inflammatory cytokines such as TNFα leads to the expression of adhesion molecules (Young et al., 2002). *In vitro* studies demonstrated that UCB prevents TNFα-induced leukocyte adhesion to endothelial cells by reducing the expression of E-selectin, VCAM and ICAM (Mazzone et al., 2009a) by impairing NF-kB nuclear translocation (Mazzone et al., 2009b). However, others maintain that bilirubin does not modulate expression of adhesion molecules but instead impairs adhesion molecule-dependent intracellular signals. Indeed, the signaling cascade activated by the binding of leukocyte integrins to VCAM and ICAM is known to generate ROS from the activity of NOX and XO, favoring leukocyte transmigration following activation of MMP2 (Wang and Doerschuk, 2000, 2002). Vogel et al. (2017) have reported that the intraperitoneal administration of bilirubin in LdL$^{-/-}$ mice prevents atherosclerotic plaque formation by inhibiting leukocyte infiltration in vessel wall. These authors also confirmed *in vitro* that both exogenous bilirubin and HMOX1 derived bilirubin are highly efficient in reducing VCAM and ICAM dependent signals by quenching NOX4- and XO-derived ROS.

It has clearly been shown that UCB mimics the hypolipidemic activity of fenofibrate (Hinds and Stec, 2018). In addition, its ability to act as Selective PPAR Modulator (SPPARM) toward PPARα has been pointed out, confirming that, by enhancing lipid metabolism, bilirubin is able to reduce cholesterol deposition, decreasing plaque formation (Hinds and Stec, 2018). However, it has been also reported that UCB decreases the export of cholesterol from macrophages through the degradation of the ABCA1 (Wang et al., 2017). These data show that, in the vessel wall, the activity of bilirubin on lipid metabolism is complex and merits further investigation. However, serum UCB does exert a strong antioxidant activity at the level of atherosclerotic plaque by reducing the content of lipoperoxides in the lesions (Lapenna et al., 2018).

As recently reviewed, bilirubin prevent platelets aggregation due its ability to interfere with the surface expression of adhesion molecules and its antioxidant activity, thereby supporting a role played in the prevention of hypercoagulability and thrombosis (Kundur et al., 2015). Even though at high concentration (50–200 μM) UCB activates p38/MAPK favoring platelet apoptosis (NaveenKumar et al., 2015), the protective role of bilirubin against platelet aggregation has been confirmed in Gilbert individuals (Kundur et al., 2017), providing evidence for the inhibition of collagen-induced activation (Kundur et al., 2014).

Bilirubin and Chronic Inflammatory Diseases

As mentioned before, the beneficial role of UCB in autoimmune and chronic inflammatory diseases is widely accepted. Indeed, there is clinical evidence that higher total serum bilirubin levels reduce the risk of rheumatoid arthritis (Fischman et al., 2010), Gilbert's subjects are less likely to develop Crohn's disease (de Vries et al., 2012), and patients with MS have low levels of total, conjugated and UCB (Peng et al., 2011, 2012), as highlighted in a previous extensive review (Jangi et al., 2013).

Focusing on the role played by endothelial cells in the context of chronic inflammation, it has been shown that bilirubin impairs the expression and the activity of adhesion molecules as documented in animal models of airways inflammation and inflammatory colitis. Indeed, intraperitoneal administration of bilirubin blocks the influx of leukocytes into the lungs of mice with OVA-induced asthma (Keshavan et al., 2005), and bilirubin is able to counteract iNOS expression and activity in colonic tissue by preventing leukocytes infiltration (Zucker et al., 2015). In both these studies, the molecular mechanism involves the

ability of bilirubin to disrupt VCAM1-dependent signaling by quenching intracellular ROS.

EFFECTS ON ANGIOGENESIS: IMPLICATIONS FOR WOUND HEALING, VASCULAR DYSFUNCTION IN PREGNANCY AND TUMOR GROWTH

Pro-angiogenetic activity of bilirubin has been demonstrated and related both to vascular activation of HMOX1 and to an increased plasma concentration of UCB. Favorable or detrimental actions can be associated with increased angiogenesis, depending on the different pathological conditions involved. Indeed, pro-angiogenetic activity can be desirable in repairing tissue damage or in preventing placental dysfunction (Chen and Zheng, 2014) but becomes deleterious when is linked to retinal degeneration in diabetes (Wang and Lo, 2018) or in the context of tumor growth (Viallard and Larrivée, 2017).

At a molecular level, pro-angiogenetic activity of bilirubin is achieved by the modulation of different pathways, some of which are not directly related to its antioxidant activity. *In vivo* and *in vitro* studies have shown that exogenous bilirubin promotes angiogenesis in response to ischemia via direct modulation of the PI3K/AKT pathway, which favors eNOS activation (Ikeda et al., 2015). The activation of AKT pathway in response to bilirubin exposure has been also been confirmed in other studies (Liu et al., 2015).

Furthermore, vascular HMOX1 induction favors the production of angiogenic factors, such as VEGF and IL-8, and decreases antiangiogenic factors such as VEGF soluble receptor (Dulak et al., 2008; Kim Y.-M. et al., 2011), with all HMOX1 metabolic products seeming to mediate these proangiogenic activities (Dulak et al., 2008; Kim Y.-M. et al., 2011). Moreover, the angiogenic potential of endothelial cells exposed to intermittent HG is restored by CoPPIX-induced HMOX1 activation to reduce ER stress (Maamoun et al., 2017). In this context, the protective role of HMOX1 in maintaining endothelial function by preventing both oxidative and ER stress has recently been reviewed (Maamoun et al., 2019). VEGF, in addition, is able to activate endothelial HMOX1 favoring angiogenesis, leading to positive feedback. However, the role played by HMOX1 in the vasculature is more complex, since it has been shown in VSMC that HMOX1-derived CO is able to increase VEGF production, whilst HMOX1-derived iron can inhibit VEGF, thus affecting endothelial responses in different ways (Dulak et al., 2002) which are not completely understood.

Furthermore, constitutive HMOX2 has been proposed to play an important role in angiogenesis. In HMOX2 null mice, an increase in corneal angiogenesis has been observed, which is paralleled by a down-regulation of HMOX1 (Seta et al., 2006). However, in another study HMOX2 deletion favors angiogenesis but, in this case, a parallel up-regulation of HMOX1 was observed (Bellner et al., 2009). Thus, the interplay between HMOX1 and HMOX2 seems to be more complex and merits further investigation.

Bilirubin and Wound Healing

The process of wound healing is strictly dependent on tightly regulated angiogenesis. The role played by both HMOX1 (Lundvig et al., 2012) and HMOX2 (Halilovic et al., 2011) in wound healing has been well documented and related to the generation of CO and bilirubin. In particular, HMOX1 deficient mice exhibit less capability for wound healing compared to wild type mice due to a reduced ability to recruit endothelial progenitor cells (EPC) for capillary formation

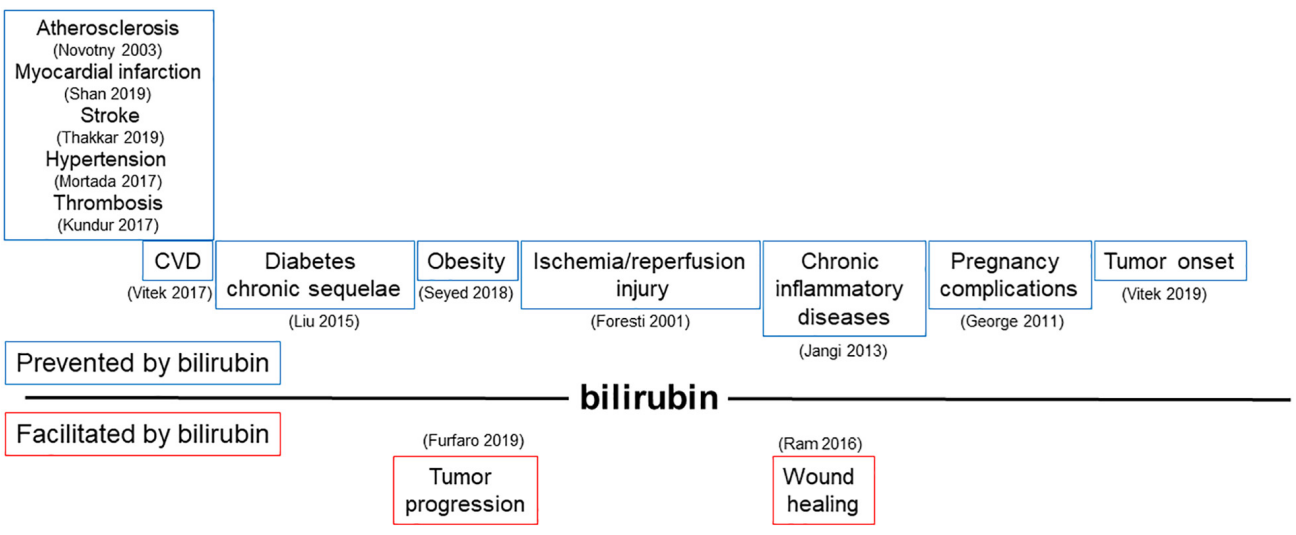

FIGURE 3 | Modulation of bilirubin production can affect a broad range of pathologies driven by endothelial dysfunction. Schematic representation of pathophysiological processes influenced by the ability of bilirubin to modulate endothelial function. In the upper part, diseases prevented by bilirubin are shown; in the lower part, conditions favored by locally generated bilirubin are indicated. References in brackets.

TABLE 1 | Bilirubin affects vascular cell functions: *In vivo* and *ex vivo* studies.

Experimental model	Plasma bilirubin concentration (mg/dl) or μM	Main findings	References
Hypertension			
C57BL/6J mice	0.9–1 mg/dl	Bilirubin i.v. infusion (37.2 mg/kg) or indinavir induce moderate hyperbilirubinemia and prevent AngII dependent hypertension	Vera et al., 2009
Gunn rats	117.9 ± 9.8 μM	Pathophysiological concentrations of UCB protects animals from hypertensive effects of AngII by reducing oxidative stress	Pflueger et al., 2005
Human plasma	15 μM	The addition of bilirubin to human plasma samples reduces peroxynitrite-induced protein modifications	Minetti et al., 1998
C57BL/6J mice	1–1.2 mg/dl	Moderate elevations in unconjugated bilirubin are able to preserve renal hemodynamics in AngII-dependent hypertension.	Vera and Stec, 2010
C57BL/6J mice	1–2 mg/dl	Mild hyperbilirubinemia induced by UGTA1A1 antisense decreases AngII-induced hypertension inhibition superoxide generation	Stec et al., 2013
Old male SHRs or WKY rats	Serum content not modified	Up-regulation of HMOX1, through the generation of bilirubin, improves endothelial function favors vessel relaxation in a rat model of spontaneous hypertension	Li et al., 2013
Diabetes			
Diabetic patients with or without GS	1.3–1.6 mg/dl	Patients with diabetes and GS show a lower prevalence of vascular complications than diabetic patients	Inoguchi et al., 2007
Diabetic patients (Hisayama study)	0.89–1.1 mg/dl	Patients with plasma bilirubin concentration in the highest quartile show a reduced prevalence of diabetic retinopathy	Yasuda et al., 2011
Diabetic patients (type 2 DM)	0.80 ± 0.39 mg/dl	Serum total bilirubin concentration negatively correlated with the severity of retinopathy	Sekioka et al., 2015
STZ-induced diabetic rats	3.3 mg/dl	Up-regulation of HMOX1 activity induced by CoPPIX by increasing bilirubin concentration reduces endothelial damage in diabetic animals	Quan et al., 2004
STZ-induced diabetic Gunn rats	7.01 ± 0.43 mg/dl	Hyperbilirubinemic rats are protects against renal diabetic complications	Fujii et al., 2010
db/db mice	Not significant increases	Biliverdin (5 mg/kg) protects diabetic mice toward renal complications	Fujii et al., 2010
db/db mice	200 nM	HMOX1 induction and BLVRA activation prevent endothelial damage in diabetes, through the modulation of Akt pathway	Liu et al., 2015
db/db and DIO mice	Not evaluated	HMOX1 activation (CoPPIX 10–20 mg/kg, i.p.) and Bilirubin (20 μmoli/kg i.p.) reduce hyperglycemia and increase insulin sensitivity	Dong et al., 2014
T2DM patients	64 ± 21 μM	Short-term treatment with atazanavir inhibits UGT1A1 activity induces a mild hyperbilirubinemia and ameliorates endothelial functions	Dekker et al., 2011
Ischemia/reperfusion damage			
Healthy human subjects	<0.5 mg/dl	Low levels of plasma bilirubin are associated with a high prevalence of ischemic modifications in brain white matter	Higuchi et al., 2018
MCAO rats	Not evaluated	Biliverdin administration (35 mg/kg i.p.) significantly reduces the cerebral infarct size	Zou et al., 2019
"*Ex vivo*" perfused rat hearts	Not applicable	Treatment with bilirubin ditaurate (BRT, 50 μM) prevents ischemia/reperfusion damage. The infarct size shows negative correlation with BRT tissue content	Bakrania et al., 2016
Rat model of coronary ischemia	Not evaluated	Intraperitoneal injection of bilirubin (10 mg/kg) decreases heart infarct size	Ben-Amotz et al., 2014
C57BL/6 mice	Not evaluated	Preconditioning performed using nanoparticles of PEG-bilirubin (10 mg/kg) attenuates IRI-associated hepatocellular injury	Kim et al., 2017

(Continued)

TABLE 1 | Continued

Experimental model	Plasma bilirubin concentration (mg/dl) or μM	Main findings	References
Obesity			
GS and age-/gender-matched healthy controls and obese female type 2 diabetes patients	>17.1 μM (1 mg/dl)	Mild hyperbilirubinemia protects from age-related weight gain and dyslipidaemia.	Seyed Khoei et al., 2018
GS subjects	35.3 ± 1.04 μM (male) 28.9 ± 6.8 μM (female)	GS subjects show higher expression level of p-AMPK, PPARα and γ and PGC1α in PMBCs in comparison to age- and gender –matched control subjects. They are less likely to contract metabolic diseases or die prematurely	Mölzer et al., 2016
PPARα knockout		Bilirubin (30 mg/kg) directly binds to activate PPARα and limits lipid deposition in adipose cells	Stec et al., 2016
C57 male mice		HMOX1 induction by CoPPIX (5 mg/100 gr subcutaneous) or HMOX1 endothelial overexpression by gene therapy in HFD mice reduce inflammation markers, down-regulate PPARγ and reduce adipocytes size	Peterson et al., 2019
Atherosclerosis			
GS subjects	29.2 ± 11.6 μM	Mild hyperbilirubinemia contribute to reduce oxidative stress and vascular complications in atherosclerotic patients with GS	Maruhashi et al., 2012
GS subjects	32.6 ±13.5 μM	Mild hyperbilirubinemia protects GS subjects from ischemic heart disease	Vítek et al., 2002
Male atherosclerotic patients	//	Meta-analysis of 11 published studies proves the inverse relationship between serum bilirubin levels and atherosclerosis	Novotný and Vítek, 2003
Early familial CAD patients	8.9 ± 6.1 μM(CAD) 12.4 ± 8.1 μM(ctr)	CAD risk is negatively correlated with bilirubin concentration	Hopkins et al., 1996
$Ldlr^{-/-}$ mice	1.7 mg/dl	Intraperitoneal injection of bilirubin reduces VCAM and ICAM signaling in endothelial cells preventing plaque formation	Vogel et al., 2017
Patients with carotid atherosclerotic plaques	13.6 ± 3 μM	Serum concentration of bilirubin (total, direct and indirect) is correlated with the reduction of lipoperoxides in the lesions	Lapenna et al., 2018
GS subjects	23 ± 5 μM	Increased levels of circulating bilirubin inhibits platelet aggregation and granule release	Kundur et al., 2014
Chronic inflammation			
MS patients	<13.97 μM	Patients with long duration MS show low levels of bilirubin plasma concentration	Peng et al., 2011
SLE patients	7.1 ± 5.3 μM	Low serum bilirubin represented a strong predictor of the manifestation of SLE symptoms Subjects with higher serum bilirubin levels, such as those with Gilbert's syndrome, might be protected from the development of SLE.	Vítek et al., 2010
RA patients	>11 μM	Higher serum total bilirubin level is negatively associated with RA	Fischman et al., 2010
Crohn's patients	>23 μM	GS confers a protective effect on the development of Crohn's disease	de Vries et al., 2012
SJL/J mice and Lewis rats	Not evaluated	Bilirubin treatment (2 × 100 mg/kg per day i.p.) successfully prevented the development of chronic EAE. Treatment with ZnPP exacerbates acute EAE	Liu et al., 2008
C57BL6/J mice	Not evaluated	Bilirubin (30 mg/kg i.p.) suppresses colonic inflammation, induced by the oral administration of DSS, by preventing leukocytes infiltration	Zucker et al., 2015
C57BL/6J	Not evaluated	Bilirubin treatment (30 mg/kg i.p.) blocks the influx of leukocytes into the lungs of mice with OVA-induced asthma	Keshavan et al., 2005

(Continued)

TABLE 1 | Continued

Experimental model	Plasma bilirubin concentration (mg/dl) or μM	Main findings	References
Wound healing			
C57BL/6J	0.55 ±0.04 mg/dl (after 1 h i.p.)	Bilirubin treatment (5 mg/kg i.p.) enhance blood flow recovery in response to ischemia by promoting angiogenesis through endothelial cells activation via Akt-eNOS-dependent manner	Ikeda et al., 2015
HMOX1$^{-/-}$ mice	Not evaluated	HMOX1null mice show less capability of wound healing compared to WT mice due to a reduced endothelial migration and neovascularization	Deshane et al., 2007
HMOX1$^{-/-}$ mice	Not evaluated	Gene therapy by using pHRE-HO-1 vector protects against oxidative stress, improves angiogenesis and favors tissue regeneration	Jazwa et al., 2013
Wistar rats	Not evaluated	Administration of Bilirubin (i.p. 30 mg/kg) favors cutaneous wound healing by a pro-healing modulation of pro-inflammatory/anti-inflammatory cytokines, adhesion molecule and favoring angiogenesis	Ahanger et al., 2016
STZ Wistar rats	Not applicable	Bilirubin treatment (0.3% ointment) accelerates the timely progression of wound healing by modulating expression of cytokines and growth factors (HIF-1α, VEGF, TGF-β1, SDF-1α, TNFα, IL-1β, IL-10 and MMP-9) promotes angiogenesis, reduces inflammation and improves maturation of wound	Ram et al., 2016
STZ Wistar rats	Not applicable	Bilirubin treatment (0.3% ointment) markedly improved the antioxidant profile of the diabetic wound and accelerates cutaneous wound healing	Ram et al., 2014
Pregnancy complications			
FVB pregnant mice	Not evaluated	Pharmacological inhibition of HMOX1 (induced by SnPP 30 μmoli/kg i.v.) is associated with pathogenesis of pregnancy complications and preeclampsia	Zhao et al., 2008
Tumor			
Cancer patients	>17 μM	UGT1A1 gene polymorfism is positively correlate with overall survival in cancer patients	Vitek et al., 2019b
GI and CRC patients	0.52 ±0.017 mg/dl	Increase in serum bilirubin level is associated with a markedly decreased prevalence of CRC	Zucker et al., 2004
CRC patients	9.8 μM	Patients with CRC have lower serum bilirubin levels: 1 μmol/L decrease in serum bilirubin is associated with a 7% increase in CRC risk. UGT1A1*28 allele carrier status is associated with a 20% decrease in risk of CRC	Jirásková et al., 2012
GS subjects	32 ± 13.6 μM	Older subjects with GS show decreased DNA damage in epithelial tissue. No correlation have been found with DNA damage in lymphocytes	Wallner et al., 2012
BALB/c nude mice	35–40 μM	Treatment with bilirubin (25 mg/kg i.p.) reduces colon cancer growth via activation of ERK1/2	Ollinger et al., 2007
BALB/c-nude mice	Not evaluated	Fasting-mimicking diet (FMD) or short-term starvation (STS) reduces HMOX1 expression in cancer and sensitizes cancer cells to chemotherapy	Di Biase et al., 2016
Aging			
12-month-old Gunn rats	75 ±18 μM	Mildly elevated levels of bilirubin improve anthropometric and metabolic parameters in hyperbilirubinemic old rats respect to normobilirubinemic	Zelenka et al., 2016
GS subjects	33.12 ±9.86 μM	GS subjects have on average longer telomeres compared to age- and gender-matched control	Tosevska et al., 2016
C57BL/6 mice	Not evaluated	Hemin-induced HMOX1 upregulation, through the generation of both CO and bilirubin, limits cardiomyocytes senescence	Shan et al., 2019

In vivo and ex vivo studies. AngII, angiotensin II; BRT, bilirubin ditaurate; BLVRA, biliverdin reductase; CAD, coronary artery disease; CO, carbon monoxide; CoPPIX, cobalt protoporphyrin IX; CRC, colorectal cancer patients; db/db, diabetic mice; DIO, diet-induced obese; DM, diabetes mellitus; DSS, dextran sodium sulfate; EAE, experimental autoimmune encephalomyelitis; FMD, fasting-mimicking diet; FVB, friend leukemia virus B; GI, gastro-intestinal; GS, Gilbert Syndrome; HFD, high fat diet; IRI, ischemia/reperfusion injury; Ldlr$^{-/-}$, low-density lipoprotein receptor-deficient mice; MCAO, middle cerebral artery occlusion; MS, multiple sclerosis; OVA, ovalbumin; PMBCs, peripheral blood mononuclear cells; RA, rheumatoid arthritis; SHR, spontaneously hypertensive rat; SJL, Swiss Jim Lambert mice; SLE, systemic lupus erythematosus; SnPP, tin protoporphyrin IX; STS, short-term starvation; STZ, streptozocin; UCB, unconjugated bilirubin; UTG1A1, uridine diphpspho-glucuronosyl transferase 1A1; ZnPP, zinc protoporphyrin IX; WKI, Wistar Kyoto rat.

TABLE 2 | Bilirubin affects vascular cell functions: *In vitro* studies.

Experimental model	Bilirubin concentration (mg/dl) or μM in cell culture media	Main findings	References
Human EC			
HUVECs	10–20 μM bilirubin	Bilirubin inhibits migration of THP-1 monocytes across activated HUVEC monolayers	Vogel et al., 2017
HUVECs	20 μM	Bilirubin markedly inhibited the migration of Jurkat cells across TNFα-stimulated HUVEC monolayers by impairing VCAM1 signaling	Zucker et al., 2015
HUVECs	Not evaluated	HMOX1 induction (10 μM CoPPIX) prevents high glucose-induced reduction in NO release and enhanced VEGF-A expression	Maamoun et al., 2017
HUVECs	50 μM	Bilirubin promoted proliferation of endothelial cells and also affects invasion capability of cells from trophoblast	Ha et al., 2015
HUVECs and VSMCs	Not evaluated	PPAR ligands induce HMOX1 and block the inflammatory response in vascular cells	Krönke et al., 2007
HUVECs, HAECs, HDMECs	10 μM	HIV protease inhibitors (PIs) induce HMOX1 that, by generating bilirubin, counteract the anti-proliferative and inflammatory actions of PIs	Liu et al., 2016
ECV304	Not evaluated	Statins increase HMOX1 expression	Grosser et al., 2004
HMEC-1	Not evaluated	Cell transfection with plasmid vector (pHRE-HO-1) carrying human HMOX1 driven by three hypoxia response elements (HREs) and cultured in 0.5% oxygen, by up-regulating HMOX1, effectively protects against oxidative stress and promotes angiogenesis	Jazwa et al., 2013
HBMECs	50 or 100 μM UCB in presence of HSA	Short-term exposure to UCB activates endothelial cells and late-term exposure to UCB increases paracellular permeability, overall increasing endothelial damage	Palmela et al., 2012
HAECs	1–10 μM	Bilirubin increases AKT dependent eNOS phosphorylation and favors leukocyte adhesion migration and tube formation	Ikeda et al., 2015
HCAECs	1, 5, and 10 μM bilirubin	Niacin increases HMOX1 expression and inhibits TNFα induced endothelial inflammation	Wu et al., 2012
HGEnCs	10 μM	HMOX1 induction (25 μM CoPPIX) as well as bilirubin supplementation directly reduce ET-1 generation	Bakrania et al., 2018
cEPCs	10–20 mg/dl	Progenitor endothelial cells induced to proliferate when exposed to 5 mg/dl bilirubin; higher concentrations (up to 20 mg/dl) induce cell death	Jabarpour et al., 2018
EA.hy926	0.5–100 μM	Exogenous bilirubin increases endothelial antioxidant activity as well as HMOX1-dependent bilirubin generation	Ziberna et al., 2016
Commercially EC	Not evaluated	EC-transfected with HMOX1 release substances that increase healthy adipocytes	Peterson et al., 2019
Human and non-human EC			
HUVECs and H5V cells	0.015 μM < Bf < 0.030 μM	UCB, at clinically relevant concentrations, limits over-expression of adhesion molecules and inhibits PMN-endothelial adhesion induced by pro-inflammatory cytokine TNFα, even though UCB itself does not alter expression of these adhesion molecules. Inhibition NF-kappaB transduction pathway	Mazzone et al., 2009a,b
HAECs and mAECs	Not evaluated	In HAECs and in primary mAECs from $HMOX1^{+/+}$ and $HMOX1^{-/-}$, SDF-1 (100-200 ng/ml) favors angiogenesis through the induction of HMOX1	Deshane et al., 2007

(Continued)

TABLE 2 | Continued

Experimental model	Bilirubin concentration (mg/dl) or μM in cell culture media	Main findings	References
Non-human EC			
mHEVa and mHEVc	10–20 μM	Bilirubin inhibits leukocyte transmigration across endothelial cell monolayer	Keshavan et al., 2005
CMVECs	1 μM	Bilirubin supplementation prevent endothelial apoptosis induced by TNFα by reducing NOX4-derived ROS	Basuroy et al., 2009
bEnd.3 and MS1	1–40 μM	Endothelial cells derived from BBB are more sensitive to UCB pro-apoptotic effect than endothelial cells from pancreas	Kapitulnik et al., 2012
mAECs	Not evaluated	Primary endothelial cells from HMOX2$^{-/-}$ mice show an increased oxidative stress, inflammation and excessive angiogenesis	Bellner et al., 2009
BAEC	1 μM	Bilirubin supplementation restores cell protection against acute high glucose treatment in endothelial cells exposed to HMOX1 inhibitor, preventing HNE production	He et al., 2015
Other cells from cardiovascular system			
Bovine vascular smooth-muscle cells	0.5–5 μM	Short term treatment with bilirubin as well as HMOX1 induction (25–200 μM hemin) protect against oxidant-mediated damage	Clark et al., 2000
H9c2	0.5 μM	Bilirubin treatment as well asHMOX1 induction (5 μM hemin) protects against hypoxia/reoxygenation	Foresti et al., 2001
Primary mice cardiomyocytes	20 μM	HMOX1 induction (10 μM hemin) as well as bilirubin supplementation limits senescence	Shan et al., 2019

BAEC, bovine aortic endothelial cells; b.End3, murine brain microvascular endothelial cell line; Bf, free bilirubin; BBB, blood-brain barrier; cEPCs, circulating endothelial progenitor cells; CMVECs, cerebral microvascular endothelial cells; COPPIX, cobalt protoporphyryn IX; EA.hy926, human endothelial cell line; EC, endothelial cells; ECV304, human endothelial cells derived from umbilical cord; ET-1, vascular endothelin-1; HAECs, human aortic endothelial cells; HBMECs, human brain microvascular endothelial cells; HCAECs, human coronary artery endothelial cells; HDMECs, human dermal microvascular endothelial cells; HGEnCs, immortalized human glomerular endothelial cells; HMEC-1, human microvascular endothelial cells; HREs, hypoxia response elements; HUVECs, human umbilical vein endothelial cells; H5V, murine heart endothelial immortalized cells; H9c2, rat cardiomyocytes; mAECs, mouse aortic endothelial cells; mHEVa/c, murine high endothelial venule-like cells; MS1, microvascular endothelial cell line; PIs, protease inhibitors; PMN, polymorphonuclear neutrophils; ROS, reactive oxygen species; SDF-1, stromal cell-derived factor 1; UCB, unconjugated bilirubin; VSMC, vascular smooth muscle cells.

(Deshane et al., 2007). Interestingly, gene therapy performed using an hypoxia-regulated vector encoding for HMOX1 protects against oxidative damage and favors tissue regeneration by improving angiogenesis both *in vitro* and *in vivo*, avoiding the potential toxic effects due to iron overload (Jazwa et al., 2013). In this context, it has recently been reported that hyperbilirubinemic infants have an increased number of circulating endothelial progenitor cells (cEPC) which also exhibit an increased ability to proliferate, migrate and repair wounds. Indeed, tissues treated with conditioned medium derived from these cells up-regulate the expression of VEGF and IL-10 and reduce TNFα (Jabarpour et al., 2018). The involvement of bilirubin has also been documented in a model of cutaneous wounds, where i.p. administration of bilirubin (30 mg/kg) increases contraction and extracellular matrix deposition, with a down regulation of pro-inflammatory markers such as TNFα (Ahanger et al., 2016). Further evidence for a vasoactive role of bilirubin was reported by Ram and colleagues, who demonstrated that the topical treatment with 0.3% bilirubin ointment reduces oxidative stress in the granulation tissue of wounds in diabetic rats to favor wound healing (Ram et al., 2014, 2016). Other studies have underlined that, in chronic inflammation, HMOX1 could also be involved in the inhibition of leukocyte infiltration thereby favoring angiogenesis to complete tissue repair (Bussolati and Mason, 2006).

Bilirubin and Vascular Dysfunctions in Pregnancy

The involvement of HMOX1 in placental development and pathology has been reviewed by Levytska et al. (2013) and also highlighted by others (George et al., 2011, 2013). HMOX1 deficiency is associated with the pathogenesis of pregnancy complications and PE (Zhao et al., 2008). In this context, studies with human umbilical vein endothelial cells and trophoblasts highlight the importance of bilirubin in angiogenesis, particularly in the regulation of spiral artery remodeling (Ha et al., 2015). Moreover, in choriocarcinoma cells, it has been shown that the up-regulation of HMOX1 or exposure to bilirubin prevents advanced glycation end-product induced generation of sFTL-1, the soluble decoy receptor for VEGF, known to play a critical role in the pathogenesis of PE (Jeong et al., 2014). However, it is worth noting that placenta-derived soluble factors involved in maternal hypertension act by increasing endothelin-1 production from

endothelial cells. In this context, it has been shown that HMOX1 expression prevents endothelin-1 production in glomerular endothelial cells (Bakrania et al., 2018) and pharmacological activation of HMOX1 or treatment with bilirubin has been proposed to attenuate PE (Ramma and Ahmed, 2014).

Bilirubin and Tumor Growth

In general, bilirubin is associated with anti-proliferative and anti-neoplastic activity. In fact, specific polymorphisms in UGT1A1 gene and the consequent hyperbilirubinemia are correlated positively with an increased overall survival in cancer patients (Vitek et al., 2019b), particularly considering colorectal (Jirásková et al., 2012), lung and breast cancers (Wagner et al., 2015). Moreover, reduced bilirubin levels are associated with increased cancer risk (Ching et al., 2002; Zucker et al., 2004). There are a few exceptions, for instance, a reported increase in the incidence of breast cancer in Gilbert individuals, which is related to specific polymorphisms of UGT1A1 promoter, as reviewed by Wagner et al. (2015).

At molecular level, an antigenotoxic activity of bilirubin has been hypothesized and a negative correlation between bilirubin concentration and DNA damage in epithelial cells has been documented (Wallner et al., 2012) even though, this has not been confirmed from an analysis of 8oxodGuo in PBMCs from GS individuals or Gunn rats (Wallner et al., 2013).

Thus, bilirubin not unlike other antioxidants, can counteract cancer onset and development by preventing DNA damage and the oxidative stress which plays a central role in carcinogenesis (Marengo et al., 2016).

Moreover, the anti-cancer proprieties of bilirubin have been documented, with bilirubin administration i.p. in mice increasing plasma bilirubin up to 40μM and drastically reducing colon cancer growth via activation of ERK1/2 (Ollinger et al., 2007). These findings have been confirmed by *in vitro* experiments showing that bilirubin concentrations >25μM exert pro-apoptotic activity whereas concentrations <25μM have no effect on cell viability or proliferation.

Nonetheless, it is important to note that once a tumor mass has developed, cancer cells can take advantage of antioxidants, especially when they derived from the activation of intrinsic adaptive pathways, and can be used for their growth and survival, favoring resistance to therapies and disease progression (Gorrini et al., 2013; Gill et al., 2016). This has been well documented for other antioxidants such as GSH (Traverso et al., 2013) and may apply to bilirubin. Indeed, role of HMOX1 in cancer progression has been highlighted and, even though some tissue specificity need to be considered, in many cancer types its expression correlates with tumor growth, aggressiveness, metastatic and angiogenetic potential, resistance to therapy, tumor escape, and poor prognosis (Furfaro et al., 2016; Nitti et al., 2017; Piras et al., 2017). It is important to note that HMOX1 and its metabolic byproducts can be involved in the generation of a permissive microenvironment, which is fundamental for cancer progression. In this context, a role for HMOX1 derived bilirubin has been implicated by Di Biase et al. (2016) and our group (Furfaro et al., 2019) in the progression of melanoma. Considering the role HMOX1 in reducing immune-surveillance, in favoring angiogenesis and invasiveness, it seems likely that some of these properties can be ascribed to the *in loco* generation of bilirubin.

Moreover, the involvement of HMOX1 in physiological angiogenesis in pregnancy and pathological angiogenesis in cancer has been proposed, since both share a necessity for a permissive microenvironment in which cell invasion, cytoprotection, angiogenesis and immune-escape are favored (Zhao et al., 2015). However, the specific role of bilirubin in this context has yet to be elucidated, providing a new field for investigation.

THERAPEUTIC PERSPECTIVES

Different therapeutic approaches aimed at elevating plasma bilirubin concentration as well as modulating cellular HMOX1 activity have been proposed for the treatment of many disorders, as widely reviewed by others (McCarty, 2007; Vitek et al., 2019b). Here, we focused on that ones in which the modulation of endothelial cell function is pivotal.

Increasing the Level of Plasma Bilirubin

Elevating bilirubin plasma concentration needs to be considered carefully due to bilirubin's well-known toxic effects. As discussed, bilirubin supplementation significantly decreases infarct size in animal models of ischemia damage (Ben-Amotz et al., 2014) as does BRT (Bakrania et al., 2016). Notably, in a mouse model of islet transplantation, bilirubin supplementation i.p. to a donor mouse as well as *in vitro* preconditioning of cells significantly suppresses the immune response and favors tolerance improving the outcomes (Adin et al., 2017). Furthermore, the use of nanoparticle of PEGylated bilirubin has been reported to protect liver tissue from ischemia-reperfusion damage (Kim et al., 2017) and to reduce lung inflammation (Kim et al., 2017). Notably, the use of bilirubin-coated stents reduces inflammation and endothelial activation preventing restenosis in porcine carotid arteries (Bae et al., 2018).

In humans, elevated plasma bilirubin levels, as side effects of drugs such as HIV PIs, have been proposed to improve endothelial function. For instance, 3-day atazanavir treatment in type II diabetes patients significantly ameliorates endothelial dysfunction (Dekker et al., 2011). Notably, three different HIV PIs, ritonavir, atazanavir and lopinavir, have been shown to be inducers of endothelial HMOX1. Moreover, HMOX1 induction is able to attenuate PI-dependent antiproliferative and inflammatory activity on endothelial cells primarily through the generation of bilirubin (Liu et al., 2016).

Importantly, therapeutic approaches leading to induce iatrogenic GS and to improve bioavailability of bilirubin might represent a valid strategy to prevent and ameliorate oxidative stress-related diseases, and McCarty (2007) already highlighted endothelial cells as crucially targeted by these therapies. More recently, the efficacy of different natural compounds which act by inhibiting UGT1A1 and elevate serum bilirubin has been confirmed, for instance silymarin, a seed extract of milk thistle (Silybum marianum), is able

to protect against lipoperoxidation (Šuk et al., 2019) as are other natural compounds and herbal extracts (Vitek et al., 2019a). In addition, it is important to note that many natural compounds can increase bilirubin generation by activating HMOX1, as demonstrated that curcumin inducing HMOX1 expression and elevating bilirubin serum level, leading to protection against acute vascular inflammation both *in vivo* and *in vitro* (Xiao et al., 2018).

Furthermore, bilirubin analogs or related compounds can exert anti-proliferative effects as discussed earlier. In this context it is important to note that administration of natural tetrapyrrolic compounds structurally related to bilirubin, such as molecules extracted from *Spirulina platensis*, exert *in vitro* and *in vivo* actions including antiproliferative effects in cancer cells (Konícková et al., 2014). In addition, nanoparticles of both biotinylated and PEGylated bilirubin have recently been developed as drug delivery system improving the anti-angiogenetic efficacy of target tumor therapy (Lee et al., 2018; Yu et al., 2019).

Modulation of Cytoprotective HMOX1

The role of HMOX1 activity in protection of endothelial cells against oxidative stress has been already highlighted (Kim Y.-M. et al., 2011) as has the opportunity to target HMOX1 to improve endothelial function and prevent CVD. However, induction of HMOX1 activity can be potentially harmful, mainly due to the generation of toxic amounts of iron. Thus, even though hemin is commonly used in experimental settings both *in vitro* and in animals to activate HMOX1, the use of the heme analogs such as heme arginate or hematin remains potentially toxic at the vascular level (Balla et al., 2000). However, other drugs such as niacin (Wu et al., 2012), statins (Grosser et al., 2004) and fenofibrates (Krönke et al., 2007) have been shown to be vasculoprotective via activation of HMOX1. Interestingly, valsartan induces HMOX1 expression in the aortic wall and increases serum bilirubin levels via down-regulation of AT1, leading to the reduction of intimal thickening (Li et al., 2014).

Finally, to achieve a more efficient up-regulation of HMOX1 and to avoid its pathological overexpression a molecular approach has been proposed: the use a hypoxia-regulated plasmid coding for HMOX1 to enhance vascular cell resistance *in vitro* and improve recovery from ischemia/reperfusion injury *in vivo* (Jazwa et al., 2013).

As far as tumor therapy is concerned, it has been reported that chlorophyll is able to inhibit HMOX1 expression and activity, exerting antiproliferative and antioxidative effects toward pancreatic cancer and inducing a significant reduction of pancreatic tumor size (Vanková et al., 2018). However, the efficacy of HMOX1 modulation specifically toward tumor angiogenesis has yet to be investigated.

CONCLUSION AND FUTURE RESEARCH

Bilirubin, far from being just a waste product highly toxic for neurons, has been recognized as a powerful antioxidant and anti-inflammatory molecule. The potential application of bilirubin for the treatment of human diseases is driven by its ability to prevent endothelial dysfunction, thus affecting not only the treatment and prevention of CVDs (Ayer et al., 2016; Bulmer et al., 2018; Maruhashi et al., 2019), but also many other pathologies in which the alterations of endothelial cells has been proved to play a role (**Figure 3** and **Tables 1**, **2**).

As both increasing plasma levels of bilirubin and up-regulating HMOX1 could lead to toxic effects, the discovery of new strategies to reduce its toxic potential to maintain therapeutic effectiveness will be key for the treatment/prevention of endothelial dysfunction. Finally, it is worth noting that senescence and aging processes, strongly associated with accumulation of oxidative damage, are reduced/slowed by bilirubin. Notably, has been recently highlighted the negative relationship among bilirubin concentration and cancer mortality, even though the relationship is missing among bilirubin and CVD mortality (Vitek et al., 2019b). It is conceivable that larger studies focused on older population are needed to explain this lack of association. Indeed, the inverse correlation between plasma UCB levels and the prevalence of obesity has be proved to be stronger in older subjects (Seyed Khoei et al., 2018).

Nonetheless, it has been proved that in Gilbert subjects and in Gunn rats both mitochondrial and cytosolic ROS production is reduced leading to a decrease of chronic inflammation markers and better anthropometric parameters (Zelenka et al., 2016). Furthermore, telomere shortening is slower in patients with mild hyperbilirubinemia, implicating a correlation with reduced oxidative stress and blood markers of inflammation (Tosevska et al., 2016). Furthermore, in senescent fibroblasts the activity of BLVRA is significantly reduced in comparison to cells derived from younger donors, suggesting that a deficit of bilirubin may be responsible for increased oxidative stress and DNA damage (Kim S. Y. et al., 2011). Moreover, hemin-induced HMOX1 up-regulation limits cardiomyocyte senescence both *in vitro* and in aged mice via the generation of both CO and bilirubin (Shan et al., 2019). Thus, the careful modulation of HMOX1 activity and bilirubin generation offers a promising target in the context of prevention and treatment of endothelial dysfunction but also with an aim to ensure healthy aging.

AUTHOR CONTRIBUTIONS

All authors conceived and wrote the manuscript, and revised the literature. MN and GM provided financial support.

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Do You Remember Mitochondria?

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Dementia is one among the consequences of aging, and amnesia is often one of the most common symptoms. The lack of memory, as a consequence of both "healthy" aging or neurodegenerative conditions, such as in Alzheimer's disease, has a dramatic impact on the patient's lifestyle. In fact, the inability to recall information made by a previous experience could not only alter the interaction with the environment, but also lead to a loss of identity. Mitochondria are key regulators of brain's activity; thanks to their "dynamic organelles" nature they constantly rearrange in the cell body and move along axons and dendrites, changing in dimension, shape, and location, accordingly to the cell's energy requirements. Indeed, the energy they can provide is essential to maintain synaptic plasticity and to ensure transmission through presynaptic terminals and postsynaptic spines. Stressful conditions, like the ones found in neurodegenerative diseases, seriously impair mitochondria bioenergetic, leading to both loss of proper neuronal interaction and of neuron themselves. Here, we highlighted the current knowledge about the role of mitochondria and mitochondrial dynamics in relation to neurodegenerative disorders linked to aging. Furthermore, we discuss the obstacles as well as the future perspectives aimed to enlarge our knowledge about mitochondria as target for new therapeutic strategies to slow down aging and neurodegenerative disease's symptoms.

Keywords: mitochondria, neurodegenerative diseases, mitophagy and mitochondrial dynamics, reactive oxygen species, aging

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INTRODUCTION

Aging is characterized by a time-dependent impairment of physiological functions due to an accumulation of changes, at both cellular and molecular level, which could result in pathological conditions such as cancer, neurodegeneration, obesity, and cardiovascular disorders (López-Otín et al., 2013).

Among other organs, the brain is most sensitive to the aging process, since neurodegenerative conditions leading to dementia, Alzheimer's, Parkinson's, and Huntington's disease exponentially increase with age progression (Mattson and Arumugam, 2018). One of the main causes of brain's sensitivity relies on its high energy requests. In fact, in order to perform neuro-electrical transmission, neurons require a high rate of ATP, as to maintain ionic gradient, preserve Ca^{2+} equilibrium, and regulate the synaptic vesicle recycling. These activities result in the consumption of up to 20% of the total ATP produced in the whole body. Glucose metabolism is the main energy source in the brain, and as a consequence neurons deeply rely on mitochondrial oxidative phosphorylation (OXPHOS) system to produce ATP. Furthermore, the neuronal network depends

upon synaptic plasticity, which is severely affected by lack of energy, resulting in impaired information transmission with consequent loss of memory and learning. Thus, it is not surprising that a disturbance of normal mitochondria physiology/functionality could lead to a decrease in neurons activity, and eventually cell death and neurodegeneration (Sun et al., 2016; Grimm and Eckert, 2017).

Nowadays, the involvement of mitochondrial dysfunction in the onset and development of neurodegenerative diseases has been extensively investigated (Beal, 2005; Cabral-Costa and Kowaltowski, 2019; Panchal and Tiwari, 2019). In Alzheimer's disease (AD) a reduced electron transport activity has been associated with increased production of reactive oxygen species (ROS). In addition, amyloid beta and Tau accumulation at mitochondria results in organelle's damage as well as in the impairment of mitochondrial trafficking and removal. Parkinson's disease (PD) is characterized by an impairment in the mitochondrial respiratory Complex I activity, leading to altered Ca^{2+} homeostasis and ROS generation, by the presence of mtDNA mutations, and by mutations in many of the genes involved in the removal of the damaged organelles through the mitophagy process, such as Parkin, PINK1, LRKK2, DJ-1. Huntington's disease (HD) is characterized by reduced Complex II activity, impaired Ca^{2+} uptake capacity, oxidative imbalance, and reduced mitochondria turnover (Beal, 2005; Cabral-Costa and Kowaltowski, 2019; Panchal and Tiwari, 2019). In addition to the disease-specific hallmarks, a commune feature of these disorders is the impairment of the mitochondrial quality control system, mainly due to a failure in the mitophagy process (Rodolfo et al., 2017). Nevertheless, at present there are not enough clues about the possible therapeutic advantages and/or applicability of mitophagy manipulation.

Another common feature of neurodegenerative conditions in the elders relies not only on the loss of neuronal plasticity but also of the neurons involved in the long term memory's storage, such as Engram cells (Roy et al., 2016). The role played by mitochondria and mitochondrial dynamics in the process of long term memory engravement and recall is not at all clear, but quite surely they should play an important one. Moreover, as mitochondria and mitochondrial dynamics, have been shown to be important in the establishment of cellular memory in muscle stem cells (Cheikhi et al., 2019), should we imagine a similar role for mitochondria in the neuron? Could we imagine to, somehow, interfere with these processes in order to sustain, recall, or restore memory in the elders? How the knowledge acquired during the last decade, about some of the main cellular processes involved in the maintenance of organelle's quality and homeostasis (Barbosa et al., 2019), would open a new scenario in the therapeutic approaches to diseases linked to aging?

ROS AND MITOCHONDRIA IN AGING PROGRESSION

Aging, seen as a phenomena of growth, decline and death, is the "natural" consequence of an impairment of cellular functions, in particular of the decline in mitochondrial function and metabolic rate. In the "free radical theory of aging," Harman postulated that aging is the consequence of the damage to cells and tissues, due to a progressive increase in the levels of free radicals (Harman, 1956). ROS are by-products of normal cellular activities, and mitochondria are one of the most important source of ROS (mtROS). ATP production by the electron transport chain, in particular complex I and III, generate superoxide radical ($O_2\bullet-$) and molecular oxygen (O_2), which is finally reduced to water (Grimm and Eckert, 2017). In physiological conditions, ROS participate in cell signaling and are involved in processes, such as immune response, inflammation, synaptic plasticity, memory, and learning (Kishida and Klann, 2007; Snezhkina et al., 2019). The unbalanced increase in ROS levels results in oxidative stress, which could perturb cell's homeostasis, structures, and functions, and in turn leading to pathological conditions, such as AD, PD, and cancer (Snezhkina et al., 2019). Cells and mitochondria protect themselves from ROS damage through the activity of a number of enzymatic defense systems, such as: glutathione peroxidases (GPXs), thioredoxin peroxidases (TRXPs), superoxide dismutases (SODs), peroxiredoxins (PRDXs), glutathione (GSH), thioredoxin 2 (TRX2), glutaredoxin 2 (GRX2), cytochrome c oxidase (complex IV); as well as antioxidants molecules, such as coenzyme Q, ascorbic acid, tocopherol, vitamin E, and carotene. Nevertheless, synaptic transmission, maintenance of membrane potential, functionality of ion channels, and synaptic plasticity in the brain, depends upon the energy provided by mitochondria, thus rendering this organ more sensitive to ROS accumulation and damage (Terman et al., 2010). In fact, during aging as well as in neurodegenerative diseases, the brain shows an increase in ROS production, coupled with a lowering of the antioxidant mechanisms, suggesting a direct correlation between oxidative stress and neuronal death (Venkateshappa et al., 2012). Maintenance of mitochondrial functionality is thus crucial not only for neuronal survival but could also guarantee the activities linked to memory engravement and recall.

On the other side, in the last decade, the free radical theory of aging has been challenged by numerous observations reporting the beneficial effect of mtROS on health and lifespan, in different animal models, ranging from worms to mammals (Lee et al., 2003; Dell'agnello et al., 2007; Rea et al., 2007; Copeland et al., 2009). In particular, it has been reported that mitochondrial stress-dependent mtROS generation, could result in a dual dose-dependent response, namely: a harmful one, when the levels of the mtROS generated are high; and a protective one, associated with increased health and lifespan, when the levels of mtROS generated are low (Schulz et al., 2007). These observations lead to the formulation of the "mitohormesis" theory, in which a mild and sub-lethal mitochondrial stress activates a broad and complex cytosolic and nuclear response (**Figure 1**), able to promote long-lasting metabolic and biochemical changes, and to improve health and viability (Tapia, 2006; Yun and Finkel, 2014; Barcena et al., 2018). Key players in this scenario are mitochondria generated/released molecules, such as mtROS and mitokines, the role and nature of the latter still to be fully addressed (Durieux et al., 2011; Deng and Haynes, 2016; Zhang et al., 2018; Conte et al., 2019; Klaus and Ost, 2020), able

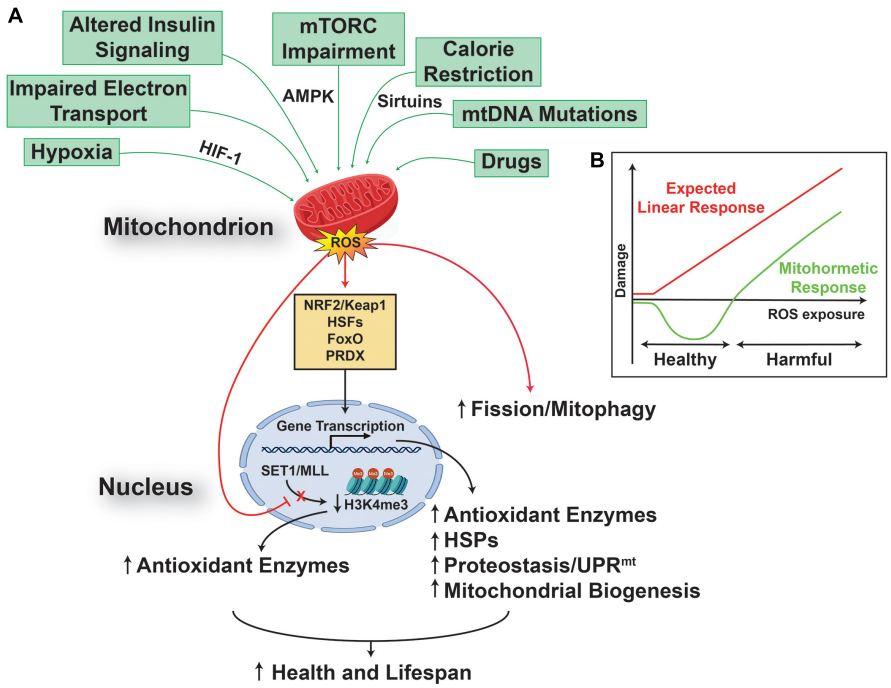

FIGURE 1 | Reactive oxygen species promote health and lifespan. **(A)** Multiple mitochondria converging stresses results in an increased production of mitochondrial reactive oxygen species (mtROS), which are able to activate a transcriptional retrograde response, between the organelle and the nucleus, linking mtROS to nuclear events. The result of this pathway is an increased cell stress resistance that can extend health and lifespan. **(B)** This effect relies on a mitochondrial process called mitohormesis, in which mtROS engages a non-linear response (green), instead of the linear response (red) postulated by the classical free radical theory of aging. The mitohormetic response (green) is characterized by health span-promoting effects at low mtROS doses, while cellular and systemic damage are the consequence of higher mtROS doses.

to induce and regulate a complex signaling network between mitochondria, the nucleus (Quiros et al., 2016), other organelles, such as lysosomes (Ramachandran et al., 2019), and the cytosol (D'Amico et al., 2017), resulting in increased cell stress resistance that can improve health and extend lifespan. Of particular interest is the recent observation that early developmental transient redox stress in *Caenorhabditis elegans* could indeed modify the methylation state of the genome, and lead to increased stress resistance, improved redox homeostasis, and prolonged lifespan (Bazopoulou et al., 2019).

In addition, the activation of the mitohormetic response has a huge impact not only on the regulation of pro-survival pathways, such as the ones controlling mitochondrial dynamics and quality (Palikaras et al., 2015; Palmeira et al., 2019), but also on cell killing ones, and could result in the preservation rather than elimination of post-mitotic cells, such as neurons (Hekimi et al., 2016).

MITOCHONDRIAL DYNAMICS IN AGING BRAIN

Mitochondria are dynamic organelles that form a complex cellular network, which is constantly subjected to fission and fusion processes, and whose "healthy state" is maintained through mitophagy. Mitochondrial network modifications allow the cells: to relocate the organelles where more energy is required, a feature of importance in neurons with extensive axons and numerous dendrites (Mandal and Drerup, 2019); to guarantee the right number of mitochondria in each daughter cell after division (Mishra and Chan, 2014); and are also involved in the quality control of the organelle. Indeed, mitochondrial fusion allows mitochondria to increase OXPHOS and ATP production, to exchange mitochondrial DNA (mtDNA), and to dilute possible organelle's damage (Sebastián et al., 2017). On the other hand, mitochondrial fission results into network fragmentation, leading to increased ROS production and signaling toward cell death by apoptosis, but also allow correct mitochondrial division during cell division, and the isolation of damaged organelles, which would then be degraded by mitophagy (Simula et al., 2017). The regulation of these processes is tightly regulated and relies on the action of several proteins. Fusion of the outer mitochondrial membrane (OMM) is mainly dependent on the GTPase Mitofusin 1 and 2 (Mfn-1 and -2) (Eura et al., 2003); whilst fusion of the inner mitochondrial membrane occurs thanks to the activity of the dynamin-related GTPase Optic-Athropy-1 (Opa-1) protein (Cipolat et al., 2004). Fission of the mitochondrial network occurs through the activity of the GTPase Dynamin-Related-Protein-1 (Drp1) (Oliver and Reddy, 2019; Qi et al., 2019). After an initial constriction of the OMM, due to the interaction with the endoplasmic reticulum and actin filaments, Drp-1 forms multimeric spirals around the OMM, which further constrict until they divide the membranes, using GTP hydrolysis

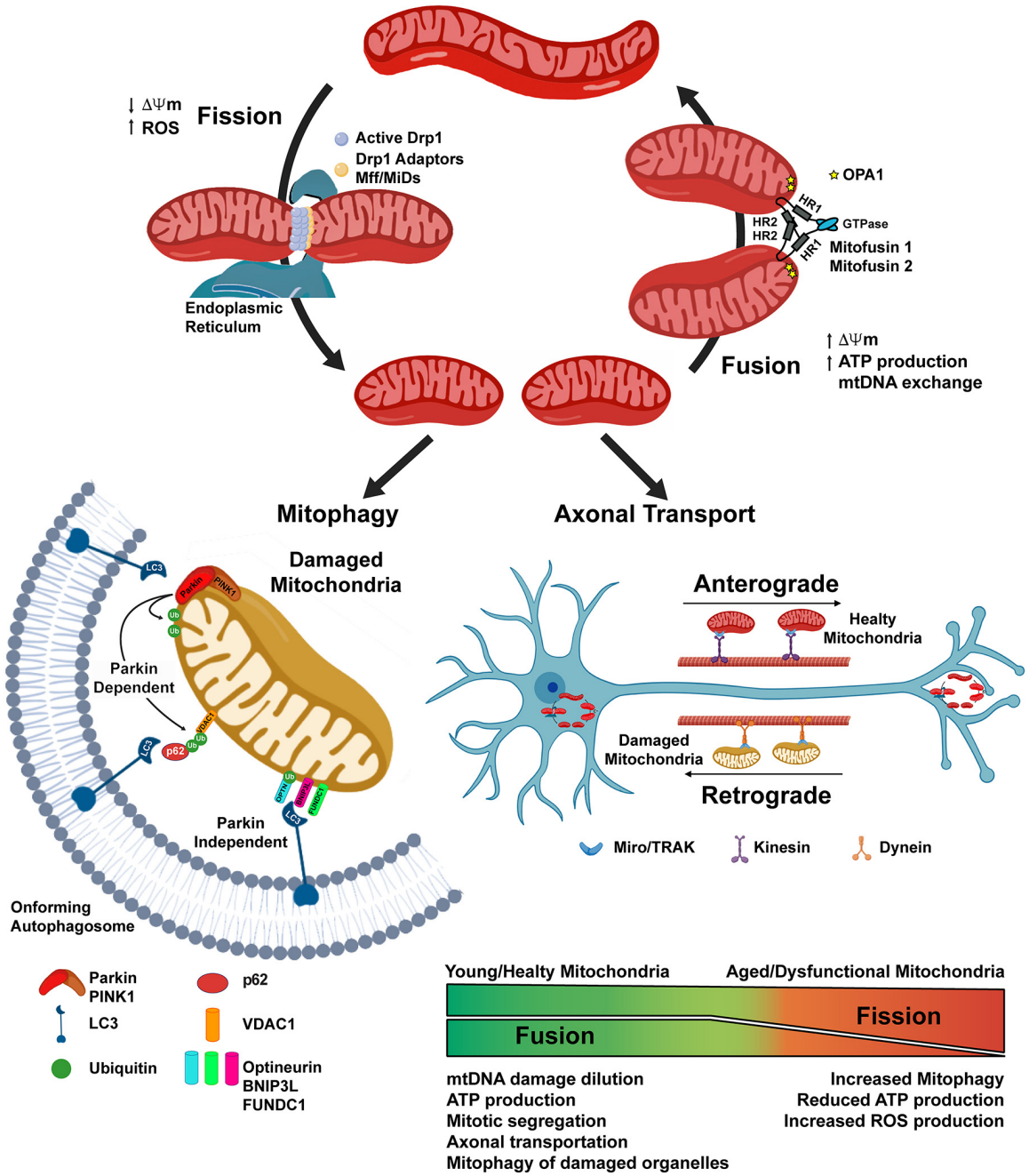

FIGURE 2 | Mitochondrial dynamics depends on the balance of Fission and Fusion processes. Mitochondrial fission is initiated through the interaction of mitochondria with the Endoplasmic Reticulum and the actin cytoskeleton. The activation of the Dynamin related protein-1 (Drp1) allows its translocation to the mitochondrial outer membrane, where with the help of the mitochondrial fission factor (Mff) and the mitochondrial dynamics proteins (MiDs), constrains the organelle until it divides into two separate ones. Mitochondrial fusion is dependent on the action of Mitufusin-1 and -2, on the outer mitochondrial membrane (OMM), and of OPA1, on the inner mitochondrial membrane. Fission is not only a physiological event, i.e., allowing the organelles to be transported along axons or correctly segregated in daughter cells during mitosis, but could also be a consequence of mitochondrial damage, such as the loss of the mitochondrial membrane potential ($\Delta\Psi m$), thus allowing the removal of damaged organelles through the mitophagy process. Parkin-dependent mitophagy requires the PINK1-dependent recruitment of the cytosolic Parkin on the OMM, where it could ubiquitinate mitochondrial resident protein, such as VDAC1. This protein signal is later recognized by the adaptor protein p62 which, through the interaction with LC3 on the membrane of the on-forming autophagosome, delivers the targeted mitochondria to the degradation. In the Parkin-independent mitophagy, the damaged organelle is recognized by LC3 through the interaction with other protein receptors, such as BNIP3L, FUNDC1, Optineurin, localized on the outer mitochondrial membrane.

to catalyze this process (Kraus and Ryan, 2017; van der Bliek et al., 2017) (**Figure 2**).

Mitochondrial dynamics are at the basis of healthy aging (Sharma et al., 2019) and their dysregulation, such as increased mitochondrial fission and reduced fusion are prominent features both in physiological aging and neurodegenerative conditions. Indeed, Drp1 hyperactivity has been reported for AD, PD, HD, Down syndrome, multiple sclerosis, and amyotrophic lateral sclerosis (ALS) (Oliver and Reddy, 2019). Moreover, fusion/fission imbalance has been proposed to cause impairment in memory and learning (Li et al., 2019; Yan et al., 2019), as mitochondrial network dynamics are pivotal in neurons, in order to localize the organelles in dendrites, axon, and synapses, located far away from the cell body. Mutations in the Drp1 and OPA1 proteins results in the lack of mitochondria on axon terminals, with consequent impairment of synaptic transmission, the main cause of cognitive deficits (Li et al., 2004).

The "health" of the mitochondrial network, relies on the action of a complex mitochondrial quality control program, involving different pathways that regulates: the elimination of improperly folded proteins, via the action of mitochondrial resident proteases and of a process termed OMM associated degradation (Pickles et al., 2018); the mitochondrial biogenesis, in which the coordination of nuclear and mitochondrial genome is crucial; the possible recovery and/or demise of damaged organelles, through the activation of the mitochondrial unfolded response (UPR^{mt}) (Shpilka and Haynes, 2018), and mitophagy (Pickles et al., 2018). Mitophagy is a specialized form of autophagy, in which damaged mitochondria are engulfed into autophagosomes, which then fuse with lysosomes allowing the degradation of the cargo (Galluzzi et al., 2017; Rodolfo et al., 2017). Damaged mitochondria are flagged for recruitment to the autophagosome thanks to the activation of the PTEN-induced putative kinase 1 (PINK1) protein in the OMM, which in turn allow the recruitment of the ubiquitin ligase Parkin from cytosol. Parkin activity on the OMM proteins, allows their ubiquitination and recognition by different protein receptors (optineurin, p62, NBR1 and others) located in the forming autophagosome (**Figure 1**). Mitophagy is an active process that requires energy, thus when ATP levels decrease, as with age, the process does not occur at the same rate, and the accumulation of dysfunctional mitochondria in the neuronal soma could inhibit mitochondrial axonal transport, thus leading to neurodegenerative disease onset as well as to cognitive deficit (Kerr et al., 2017).

FROM BENCH TO BEDSIDE

In the last decade, the search toward the development of new therapeutic and pharmacological strategies, able to counteract the progression of neurodegenerative diseases and/or to ameliorate the life of the elder people, pointed to the identification of molecules able to regulate mitochondrial dynamics and/or general and specific autophagy mechanisms. Mitochondrial fission depends upon the activation of the Drp1 protein, so different drugs able to inhibit this process have been identified and tested both *in vitro* and *in vivo* (for a complete review see Oliver and Reddy, 2019), among them: Mdivi-1, inhibits excessive mitochondrial fission and promotes fusion, and is being considered for clinical trials for human diseases (Cassidy-Stone et al., 2008; Reddy et al., 2018); the selective peptide inhibitor P110, proved to be effective on a PD mouse model (Qi et al., 2013); Dynasore has been discovered as a specific inhibitor of Dynamin-1 and -2 as well as of Drp1 (Macia et al., 2006), but there are just few reports on its efficacy on mitochondria (Gao et al., 2013); the small molecule DDQ [diethyl (3,4-dihydroxyphenethylamino) (quinolin-4-yl) methylphosphonate)], proved to be able not only to reduce mitochondrial fission and to enhance fusion, but also to induce mitochondrial biogenesis, and a reduction of both beta-amyloid and Drp1 proteins in APPSwe/Ind cells (Kuruva et al., 2017). The obtained results suggested these compounds as promising candidates able to reduce excessive mitochondrial fission, as to ameliorate neuronal mitochondrial functionality as well as the cognitive deficit, in AD, PD, and HD patients. Nevertheless, there is still a lot to be done before anyone of this drugs could be authorized for the treatment in humans.

Macroautophagy and mitophagy are highly regulated mechanisms and, as of today, any of the possible interventions, specifically aimed at modulating them, are not available for use in humans. This is mostly due to the fact that drugs licensed for the use in humans and able to activate or inhibit autophagy (i.e., rapamycin, chloroquine, hydroxychloroquine, etc.) were not developed for this purpose, they show limited specificity toward the autophagic process and/or the target cell, and most of their targets have also an autophagy-independent function. In addition, there is a lack of suitable and precise biomarkers, to monitor the efficacy of the treatment, as well as of more physiological *in vivo* models of autophagy deficiencies (Galluzzi et al., 2017). In neurodegenerative diseases, showing defective autophagy as a common feature, the identification of the precise point at which autophagy fails and the development of drugs able to target specific types and/or steps of the autophagic process, are the major points on which being focused in the future (Scrivo et al., 2018). In particular, there is growing interest in the possibility of modifying the number of mitochondria, their dynamics, or the mitochondrial quality-control system, in pathological instances in which any of these were impaired or abnormal. As of today, there are no really effective strategies to do so (Georgakopoulos et al., 2017), and it should be taken in account that these processes are the result of a complex interplay between different cellular pathways, and the alteration of one of them could severely affect the others (Strappazzon and Cecconi, 2015; Strappazzon et al., 2015). In this scenario, the increasing knowledge about this interplay could be beneficial to the development of combined therapeutic approaches, aiming to different aspects such as, ROS formation targeting, mitochondrial biogenesis stimulation, mitochondrial dynamics modulation (Elfawy and Das, 2019). For example, development of new delivery systems able to enhance the antioxidant bioavailability or administration of a diet enriched in natural compounds able to stimulate mitophagy, could be coupled to unique mitochondrial proteins identified as specific targets for the newly developed drugs (Palikaras et al., 2017; Di Rita et al., 2018;

Di Rita and Strappazzon, 2019). In this view, the future improvement of new technical approaches, such has optogenetic, could be of importance in the design and development of effective therapies (D'Acunzo et al., 2019).

Another important aspect to be taken in account, relies on the cellular heterogeneity of the central nervous system (CNS) and to cell specific response to a specific treatment, both in physiological and pathological conditions. We should foster a more complete picture of cell's interactions, mitochondrial heterogeneity, and autophagy regulation in the CNS, in order to develop new compounds, capable of targeting autophagy and/or mitophagy in specific cell types or brain region. In this scenario, the recent development of new and better analytical methods, to study mitochondria in normal and diseased states, took advantage from the development of software, such as MitoMo (Zahedi et al., 2018), in which machine learning was used to analyze data obtained by different mitochondrial imaging approaches, thus allowing a deep analysis of mitochondrial features in any cell type. This kind of development would be more and more important when coupled to the development of methods, able to target mitochondria *in vivo*, for both imaging and isolation. As an example, the use of the *MitoTag* mice, highlighted that in the brain, not only cells are of different types, but also mitochondria showed cell-type-specific functional and molecular diversity, with hundreds of differentially regulated mitochondrial proteins, which could be used as cell-type-specific mitochondrial markers in healthy vs. diseased CNSs (Fecher et al., 2019).

Restoration or amelioration of mitochondrial functionality in pathological settings, is not the only field to be explored and implemented in the future, as deeper knowledge of the mechanisms underlying memory engravement, storage, and recall could open a new scenario to ameliorate the life of the elders. In this scenario, we could envision the development and improvement of technology, allowing the labeling and manipulation of specific cell ensembles, responsible for memory engrams, which have been shown to be sufficient for memory recall (Tonegawa et al., 2015; de Sousa et al., 2019), also in an animal model of neurodegeneration (Roy et al., 2016). Nowadays, a direct link between mitochondrial activity and memory formation remains quite elusive, even if it has been reported a crucial role in the cannabinoid-induced amnesia (Hebert-Chatelain et al., 2016) and the interesting observation that long-term potentiation in neurons requires a rapid burst of dendritic mitochondrial fission as well as an increase in the concentration of mitochondrial matrix Ca^{2+} (Divakaruni et al., 2018).

Of great interest is the recent report about the existence of a phenomenon termed "mitoengram," in which transient stress results in enduring physio-chemical changes of the mitochondrial network and couple to key epigenetic modifications, in muscle stem cells (Cheikhi et al., 2019). Whether this role for mitochondria and mitochondrial dynamics in the balance between persistent and transient cellular memory could also happen in other cell types, such as neurons, as well as its potential exploitation as a therapeutic target, are a new research field worth to be investigated.

CONCLUSION

The last decade gave us a bunch of knowledge about cellular processes at the basis of aging and its linked pathologies. Our understanding is growing day by day and the continuous development of new technologies allow us to imagine a wide field of therapeutic approaches to ameliorate the life of "aged people" as well as to counteract the onset and progression of neurodegenerative diseases. Nevertheless, a lot remains to be discovered and big efforts would be necessary in order to effectively transfer the obtained knowledge from bench to the bedside.

AUTHOR CONTRIBUTIONS

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TRAP1: A Metabolic Hub Linking Aging Pathophysiology to Mitochondrial S-Nitrosylation

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TRAP1: A CHAPERONE WITH MANIFOLD METABOLIC EFFECTS

Tumor necrosis factor receptor-associated protein 1 (TRAP1), also known as heat shock protein 75 (Hsp75), is the mitochondrial member of the Hsp90 family of molecular chaperones, which acts as a key regulator of mitochondrial homeostasis and bioenergetics (Hoter et al., 2018). At variance with the other members, TRAP1 (i) shows a marked asymmetric conformation; (ii) can use both Ca^{2+} and Mg^{2+} as cofactors; and (iii) aids substrate folding without co-chaperone assistance, this making it unique among Hsp90 family members (Lavery et al., 2014; Masgras et al., 2017b; Elnatan and Agard, 2018).

To date, few TRAP1 clients have been discovered, most of which are proteins involved in different mitochondrial functions, such as apoptosis and metabolism control (Hoter et al., 2018). TRAP1 exerts a protective role in mitochondria and is able to prevent oxidative stress-induced cell death through the inhibition of the permeability transition pore (PTP) opening (Matassa et al., 2018). This effect can be both direct, through the inhibition of cyclophilin D (CypD), and indirect, through the modulation of reactive oxygen species (ROS) concentration (Amoroso et al., 2014; Matassa et al., 2018). Such ability is intimately linked to TRAP1 role in metabolism, i.e., the control of electron flow along the respiratory complexes of the electron transport chain (ETC) (Masgras et al., 2017b). In particular, TRAP1 is able to downregulate both Complex II and Complex IV activities, which occurs in concert with phosphorylation events mediated by mitochondria-localized kinases (Yoshida et al., 2013; Masgras et al., 2017a). This feature represents a key intersection point in TRAP1-mediated control of metabolism since Complex II takes part in the ETC but also acts as the succinate dehydrogenase (SDH) within the tricarboxylic acid (TCA) cycle (Sciacovelli et al., 2013). SDH catalyzes the conversion of succinate into fumarate; therefore, its inhibition causes an imbalance in the relative concentration of these metabolites, i.e., an increase of succinate (Sciacovelli et al., 2013; Rizza et al., 2016). It has been demonstrated that this accumulation enables the release of the hypoxia-inducible factor 1α (HIF1α) inhibition. This event stabilizes the active dimer HIF1α/β, leading to the activation of HIF1-mediated transcription of genes coding for glycolytic enzymes, and for BCL2 and adenovirus E1B 19-kDa-interacting protein 3 (Bnip3), which mediates mitochondrial selective removal by autophagy (mitophagy) (Singh et al., 2017; Zhang et al., 2018). In such a way, TRAP1 is able to rewire metabolism by downregulating mitochondrial oxidative phosphorylation (OXPHOS) and promoting glycolysis, a feature resembling the so-called "Warburg effect" distinctive of cancer cells (Yoshida et al., 2013). As a result, OXPHOS downregulation is associated with limited ROS production, increased mitochondrial tolerance to oxidative stress, and protection from apoptosis (Masgras et al., 2017b; Matassa et al., 2018).

TRAP1 IS A TARGET OF *S*-NITROSYLATION

S-nitrosylation is a redox posttranslational modification of cysteine residues induced by nitric oxide (NO) (Rizza and Filomeni, 2016). It is dynamically regulated by the amount of NO produced by NO synthases (NOSs) and exchanged between nitrosothiols (SNOs) and free sulfhydryls (SHs), as well as by the ability of denitrosylases to reduce SNO groups. *S*-nitrosoglutathione reductase (GSNOR) is the prototype of this class of enzymes. It contributes to regulate the levels of *S*-nitrosylated proteins (PSNOs) (Rizza and Filomeni, 2017); therefore, conditions of GSNOR deficiency are associated with a general increase of PSNOs. Recently, it has been demonstrated that *Gsnor*-null cells are characterized by mitochondrial dysfunction and metabolic changes (Rizza et al., 2018). Moreover, it has been reported that GSNOR-downregulating hepatocellular carcinoma (HCC) cells show reduced levels of TRAP1 (Rizza et al., 2016). Mass spectrometry analyses indicated that this phenomenon is associated with selective nitrosylation of Cys501 in TRAP1, and cell biology experiments provided the evidence that this modification induces loss of stability of TRAP1 and its accelerated degradation *via* the proteasome (Rizza et al., 2016).

Besides this regulation, it has also been very recently reported that *S*-nitrosylation of Cys501 produces a decrease of TRAP1 ATPase activity likely through an allosteric mechanism (Faienza et al., 2020). ATPase assays, together with molecular dynamics simulations, indicate that *S*-nitrosylation negatively impacts TRAP1 activity *i*) *directly*, through intra- and inter-protomer long-range communication events that exert distal effects on the active site, and *ii*) *indirectly*, through the regulation of open-to-close state transition, which is crucial for this class of chaperones to complete the ATPase cycle (Faienza et al., 2020). Cells expressing a mutant form of TRAP1, in which Cys501 is substituted by a serine (C501S), are more resistant to mitochondrial toxins (Rizza et al., 2016) and to staurosporine-induced apoptosis (Faienza et al., 2020), suggesting that *S*-nitrosylation of Cys501 impacts TRAP1 biology. Integrating these pieces of evidence, it is reasonable to propose that *S*-nitrosylation-induced degradation of TRAP1 is a direct consequence of its loss of activity.

Abbreviations: *5meC*, 5-methylcytosine; *Bnip3*, BCL2 and adenovirus E1B 19-kDa-interacting protein 3; *CypD*, cyclophilin D; ERK1/2, extracellular signal-regulated kinase 1 and 2; *GSNOR*, *S*-nitrosoglutathione reductase; *HCC*, hepatocellular carcinoma; *HIF1α*, hypoxia inducible factor 1*α*; *Hsp*, heat shock protein; *NO*, nitric oxide; *NOS*, nitric oxide synthase; *OXPHOS*, oxidative phosphorylation; *PINK1*, PTEN-induced kinase 1; *PSNOs*, *S*-nitrosylated proteins; *PTP*, permeability transition pore; *ROS*, reactive oxygen species; *SDH*, succinate dehydrogenase; *TCA*, tricarboxylic acid; *TET*, ten-eleven translocation; *TRAP1*, tumor necrosis factor receptor-associated protein 1; UPR^{mt}, mitochondrial unfolded protein response.

EFFECTS OF TRAP1 *S*-NITROSYLATION IN AGING

Various lines of evidence indicate that GSNOR downregulation, or loss, correlates with cell senescence and mammalian aging (Rizza et al., 2016, 2018). Based on what above reported, this allows speculating that TRAP1 expression could be also involved, or play a role, in aging physiopathology. Given the function of TRAP1 as mitochondrial chaperone and its importance in mitochondrial proteostasis, this hypothesis is credible. Aging is, indeed, accompanied by a progressive decline of mitochondrial functions and turnover, which, according to the mitochondrial free radical theory of aging, is considered causative of a number of age-associated pathologies, such as neurodegenerative diseases (Akbari et al., 2019) and cancer (López-Otín et al., 2013).

Studies conducted in fruit flies have revealed that TRAP1 overexpression benefits insect health by increasing fertility and locomotor ability (Baqri et al., 2014). The positive effects induced by TRAP1 overexpression in *Drosophila* are directly related to its ability to modulate heat and oxidative stress resistance through the regulation of mitochondrial proteostasis and the activation of the mitochondrial unfolded protein response (UPR^{mt}) (Baqri et al., 2014; Gumeni and Trougakos, 2016). The loss of proteostasis is a signature of aging and is closely related to chaperone functions (López-Otín et al., 2013; Sala et al., 2017). Interestingly, this is exacerbated during aging by PSNOs accumulation.

Parkinson's Disease

Due to the role of TRAP1 in the maintenance of mitochondrial homeostasis, it is conceivable that any dysregulation of its expression/activity may be associated with the onset of diseases related to mitochondrial dysfunctions. In agreement with this assumption, negative modulation of TRAP1 protein levels has been frequently reported in *in vitro* and *in vivo* models of Parkinson's disease (PD) (Pridgeon et al., 2007; Costa et al., 2013; Zhang et al., 2013; Fitzgerald et al., 2017). In particular, it has been demonstrated that ectopic expression of TRAP1 is able to counteract dysfunctional phenotypes in PD models, such as those induced by α-synuclein overexpression, preventing, or attenuating, mitochondrial defects and apoptosis (Butler et al., 2012; Fitzgerald et al., 2017; Hoter et al., 2018). Moreover, in *Drosophila* and mammalian models of PD, it has been reported that TRAP1 overexpression fully rescues mitochondrial impairments associated with phosphatase and tensin homolog (PTEN)-induced kinase 1 (PINK1) loss of function, but only partially those induced by Parkin mutation (Costa et al., 2013; Zhang et al., 2013), suggesting that TRAP1 acts downstream of PINK1 and in parallel to Parkin. Interestingly, TRAP1 loss of function in *Drosophila* phenocopies the effects induced by PINK1 deficiency, including mitochondrial and locomotor activity defects (Costa et al., 2013).

TRAP1 ability to rescue PINK1 deficiency goes through TRAP1 phosphorylation by PINK1 (Pridgeon et al., 2007), which enhances its ability to inhibit oxidative stress and to protect neuronal cells from death (Fitzgerald et al., 2017;

Hoter et al., 2018). Along the same line, a homozygous loss-of-function mutation of TRAP1, caused by a premature stop codon, has been identified in a sporadic PD patient. Studies performed in fibroblasts obtained from this patient revealed different molecular phenotypes distinctive of mitochondrial dysfunction, e.g.: i) high ROS levels; ii) impaired UPR^{mt}; iii) loss of mitochondrial transmembrane potential; iv) enhanced susceptibility to mitophagy and apoptosis (Fitzgerald et al., 2017). However, debates on the methods used in this study to perform genome datasets analyses, together with the low frequency of this mutation, call into question the general relevance of this finding in PD research (Fitzgerald et al., 2018; Gaare et al., 2018).

Mitochondrial functions, as well as PINK1 and Parkin activities, are affected by conditions of excessive *S*-nitrosylation, which are a distinctive signature of PD brains. How much this is a cause or consequence of PD, or contributes in a positive feedback loop to the establishment of the pathological state, is still a matter of debate. What is known is that *S*-nitrosylation at Cys658 in PINK1 inhibits PINK1 kinase activity and, in turn, compromises mitophagy and viability of PD cellular models (Oh et al., 2017). Likewise, Parkin *S*-nitrosylation has been proposed to affect Parkin E3-ligase activity, thereby causing accumulation of misfolded proteins and damaged mitochondria (Chung et al., 2004; Yao et al., 2004; Nakamura and Lipton, 2013; Ozawa et al., 2013). Based on these lines of evidence, it is conceivable that *S*-nitrosylation negatively affects TRAP1—and, in turn, mitochondrial homeostasis, metabolism, and apoptosis—at different levels, acting both directly (at Cys501) and indirectly (through the inhibition of its upstream regulator PINK1, or Parkin). Whatever the mechanism is, the hypothesis that TRAP1 *S*-nitrosylation plays any role in neuronal injury distinctive of PD and—at least in principle—in other neurodegenerative diseases is realistic and claims for further investigations, e.g., to identify TRAP1 clients downstream of PINK1 and understand their regulation following PINK1 and TRAP1 *S*-nitrosylation.

Cancer

By several reasons, cancer can be considered another age-related disease. Aging is, indeed, a major risk factor in cancer development. Former studies, aimed at investigating the role of TRAP1 in cancer, highlighted that it is overexpressed in several tumor tissues (if compared with peer non-tumor counterparts) and suggested that this phenomenon was linked to TRAP1 ability to rewire metabolism and favor the "Warburg effect" (Sciacovelli et al., 2013; Masgras et al., 2017a,b; Matassa et al., 2018). These assumptions have been redefined in the last few years as: *i*) some tumors are not fully glycolytic but, depending on environmental availability of nutrients, can interchangeably use OXPHOS to sustain their accelerated growth, and *ii*) TRAP1 has been found differentially expressed in different tumors—and in different stages within the same tumor—in order to adjust metabolism to tumor cell needs (Matassa et al., 2018).

A context-dependent effect in cancer is common also to *S*-nitrosylation, with NO being considered a "Janus faced" molecule in tumorigenesis, due to its ability to both promote or inhibit cancer cell growth (Di Giacomo et al., 2012). From this angle, TRAP1 nitrosylation at Cys501 can play the role of tuner of TRAP1 oncogenic role. Structurally, Cys501 is placed close to Ser511 and Ser568, which are the two residues indicated as preferential targets of phosphorylation by the extracellular signal-regulated kinase 1 and 2 (ERK1/2) (Masgras et al., 2017a). ERK1/2 binding and phosphorylation stimulate the formation of TRAP1/SDH multimeric complex and enhance the inhibitory activity of TRAP1 on SDH (Masgras et al., 2017a), thereby positively contributing to the TRAP1-induced metabolic reprogramming of cancer cells. Based on this evidence, it is reasonable to hypothesize that *S*-nitrosylation might interfere with ERK1/2-mediated phosphorylation, or, *vice versa*, phosphorylation impedes Cys501 accessibility to the solvent, and its propensity to be modified by NO. Whatever is the direction of this interplay, it is plausible that Cys501 nitrosylation and Ser511/568 phosphorylation are two mutually exclusive posttranslational modifications of TRAP1. How much this cross talk impacts TRAP1 allosteric regulation and its capability to act as a metabolic hub rather than its antioxidant or anti-apoptotic roles still wait to find answer. Nevertheless, given the effects of *S*-nitrosylation on TRAP1 oncogenic properties, the use of NO donors can be hypothesized as useful approaches in combined treatments aimed at selectively killing chemoresistant tumors where TRAP1 is overexpressed and contributes to apoptosis resistance (Costantino et al., 2009).

Is TRAP1 an Epigenetic Modulator of Aging and Aging-Related Diseases *via* *S*-Nitrosylation?

As previously described, TRAP1-mediated inhibition of SDH generates an accumulation of succinate that modulates HIF1 transcriptional activity. This phenomenon depends on the inhibitory effects that succinate exerts on α-ketoglutarate-dependent dioxygenases, a group of enzymes that hydroxylate different substrates, i.e., HIF1α (Laukka et al., 2016). This family of enzymes also includes several epigenetic regulators, such as histone demethylases and 5-methylcytosine (5meC) hydroxylases [also referred to as ten-eleven translocation (TET) proteins] (Xiao et al., 2012). Epigenetic modifications, such as those induced by alterations of TETs, have been included in the hallmarks of aging (López-Otín et al., 2013). Concerning this aspect, we have recently discovered a functional association between TET1 and GSNOR expression (Rizza and Filomeni, 2018; Rizza et al., 2018), with both proteins decreasing during aging. However, many aspects, mostly those aimed at understanding i) if the relationship between TET1 and GSNOR is biunivocal and regulated by a feedback loop; ii) how TET1/GSNOR signaling axis is initiated; iii) how it is fueled and kept sustained during life span, remain still unanswered. In regard to this, TRAP1 could represent the missing link underlying the close relationship between TET1 and GSNOR in aging. Namely, by affecting SDH activity, TRAP1 decrease by *S*-nitrosylation could result in a disbalance of succinate-to-fumarate ratio, which, consequently, i) inhibits TET1 activity, ii) leads to an increase in CpG island methylation in several promoters, and iii) causes a decrease of protein expression (as,

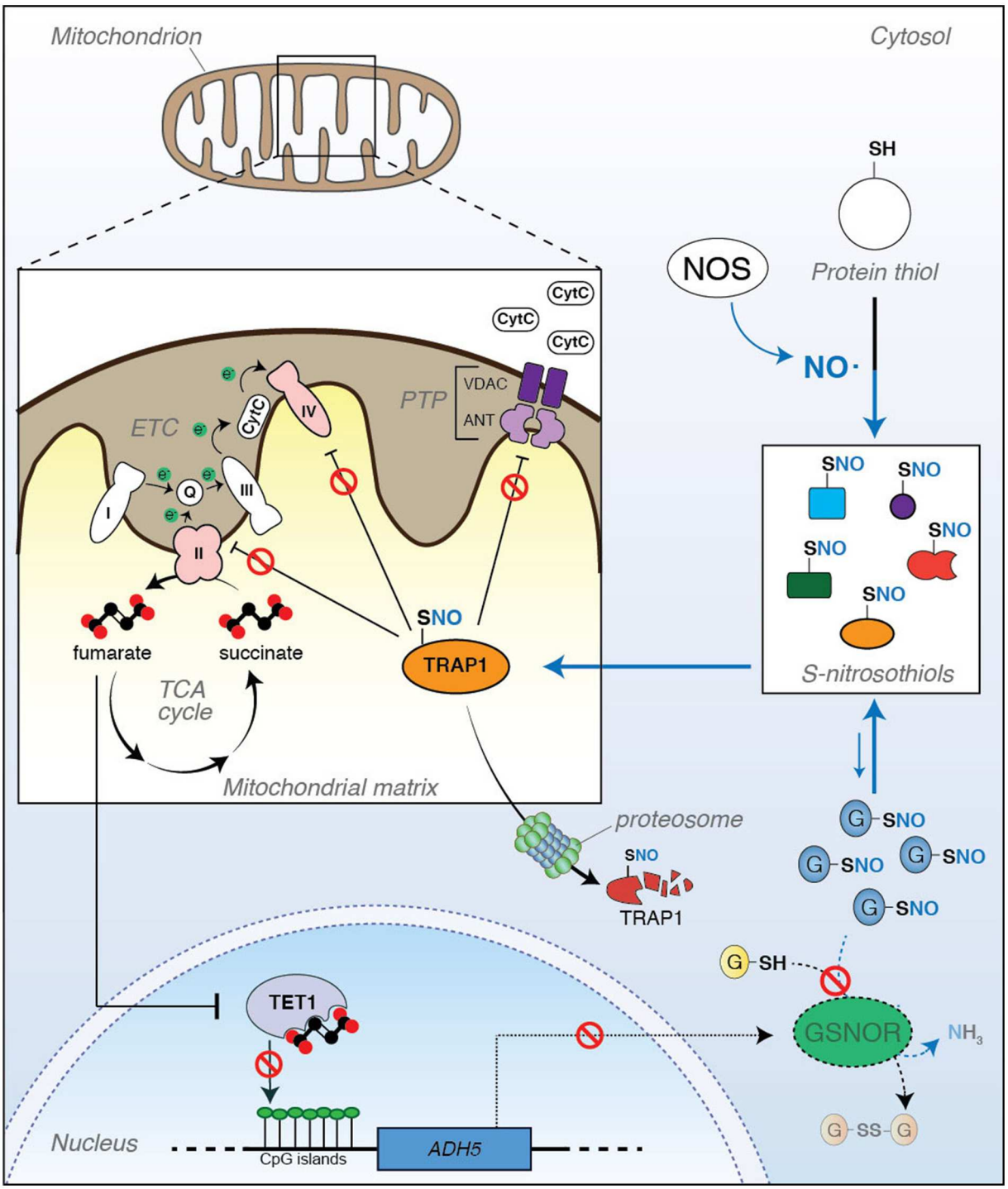

FIGURE 1 | Tumor necrosis factor receptor-associated protein 1 (TRAP1) is a metabolic hub linking *S*-nitrosylation to aging. (*Right*) *S*-nitrosylation is a posttranslational modification induced by nitric oxide (NO). It regulates the activity, localization, stability, and functions of a series of cysteine (SH)-containing proteins that, upon reaction with NO, become nitrosylated (*S*-nitrosothiols, or SNOs). Protein *S*-nitrosylation extent does not only depend on the rate of NO synthesis by NO synthase (NOS), but it is also controlled by the ability of a class of enzymes (denitrosylases) to catalyze the SNO-to-SH reduction. *S*-nitrosoglutathione reductase (GSNOR) is a well-known and, probably, the best characterized example of denitrosylase so far identified. However, it does not directly react with protein-SNOs. Actually, GSNOR catalyzes the reduction of *S*-nitrosoglutathione (GSNO) to glutathione (GSH). GSNO levels are in equilibrium with protein-SNOs through a spontaneous exchange reaction called *trans*-nitrosylation; therefore, by controlling GSNO levels, GSNOR indirectly regulates the extent of protein *S*-nitrosylation. (*Left*) It has been recently reported that TRAP1 undergoes S-nitrosylation, this impacting its stability. Inside the mitochondrion, TRAP1 antagonizes PTP opening and downregulates OXPHOS through

(Continued)

FIGURE 1 | the inhibition of Complexes II and IV of the electron transport chain (ETC), both these events preventing cytochrome *c* (CytC) release and apoptosis. However, when *S*-nitrosylated at Cys501, TRAP1 activity decreases, this probably inducing its degradation by the proteasome with a consequent increase in apoptosis susceptibility and Complex II activity. It can be speculated that this condition might result in a disbalance of succinate-to-fumarate ratio (i.e., an accumulation of fumarate levels), which can impact ten-eleven translocation (TET)1-dependent epigenetic activity and, in turn, produce a hyper-methylation of CpG islands in the promoter of several genes. Recently, it has been reported that *ADH5* (the gene coding for GSNOR) is among those genes which are epigenetically controlled by TET1, and its silencing contributes to cell senescence and mammalian aging. In this scenario, a new role of TRAP1 as an epigenetic factor of aging can be hypothesized, with *S*-nitrosylation acting as a modulatory event of this loop of regulation.

indeed, observed for GSNOR). Therefore, consistent with the establishment of a positive feedback loop, *S*-nitrosylation could target TRAP1 and keep TET1 inactive to initiate and sustain epigenetic silencing of GSNOR expression (**Figure 1**).

CONCLUDING REMARKS

Oxidative damage to cells and tissues has always been considered a major cause of aging. Being the main ROS production site inside the cells, mitochondria are usually indicated as the principal source of oxidative stress, with their dysfunction being causative of (or at least contributing to) aging and age-related diseases (Akbari et al., 2019).

Recently, it has been proposed that *S*-nitrosylation plays a role in the onset of aging. Several mitochondrial proteins found to be associated to neurodegeneration (e.g., Drp1, PINK1, Parkin) are, indeed, target of *S*-nitrosylation, and recent findings about the deleterious effects of GSNOR deficiency on mitochondrial functions and mammalian longevity further support these observations (Rizza et al., 2018). In this scenario, TRAP1 modulation by excessive *S*-nitrosylation could represent a new regulatory mechanism involved in aging and age-related pathophysiology, other than a means to reprogram cell metabolism.

In this Opinion, we have attempted to provide food for thought and elaborate on the potential impact of TRAP1 *S*-nitrosylation in mitochondrial physiology, with relevance to aging and age-related diseases (e.g., cancer and neurodegeneration), which are, *de facto*, pathological states associated with mitochondrial dysfunctions.

AUTHOR CONTRIBUTIONS

FF and GF wrote the paper. SR and FF conceived and drew up the figure. PG and all authors discussed, corrected, and critically read the paper.

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Lipophagy Impairment Is Associated With Disease Progression in NAFLD

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Non-alcoholic fatty liver disease (NAFLD) is the most common cause of chronic liver disease in Western countries and is associated with aging and features of metabolic syndrome. Lipotoxicity and oxidative stress are consequent to dysregulation of lipid metabolism and lipid accumulation, leading to hepatocyte injury and inflammation. Lipophagy consists in selective degradation of intracellular lipid droplets by lysosome and mounting evidence suggests that lipophagy is dysregulated in NAFLD. Here we demonstrate lipophagy impairment in experimental models of NAFLD and in a NAFLD patient cohort by histomorphological and molecular analysis. High fat diet-fed C57BL/6J male mice and high-fat/high-glucose cultured Huh7 cells showed accumulation of both p62/SQSTM1 and LC3-II protein. In 59 NAFLD patients, lipid droplet-loaded lysosomes/lipolysosomes and p62/SQSTM1 clusters correlated with NAFLD activity score (NAS) and with NAS and fibrosis stage, respectively, and levels of expression of lysosomal genes, as well as autophagy-related genes, correlated with NAS and fibrosis stage. An increased amount of lipid droplets, lipolysosomes and autophagosomes was found in subjects with NAFLD compared to healthy subjects at ultrastructural level. In conclusion, here we observed that NAFLD is characterized by histological, ultrastructural and molecular features of altered autophagy that is associated with an impaired lipid degradation. Impaired autophagy is associated with features of advanced disease. Lipopolysosomes, as individuated with light microscopy, should be further assessed as markers of disease severity in NAFLD patients.

Keywords: autophagy, high-fat diet, lipolysosomes, non-alcoholic steatohepatitis, lipophagy

Abbreviations: AFP, alpha-fetoprotein; ALB, albumin; ATG12, ubiquitin-like protein ATG12; ATG7, ubiquitin-like modifier-activating enzyme ATG7; ATP6V1B2, v-type proton ATPase subunit B, brain isoform.; BECN1, beclin-1; CTSD, cathepsin D; GABARAP, gamma-aminobutyric acid receptor-associated protein; HFD, high-fat diet; HIST1H3A, histone cluster 1H3 family member a; LAMP1, lysosome-associated membrane glycoprotein 1; LAMP2, lysosome-associated membrane glycoprotein 2; mTOR, serine/threonine-protein kinase mTOR; NAS, NAFLD activity score; NASH, non-alcoholic steatohepatitis; NPC1, NPC intracellular cholesterol transporter 1; nSAS, nSolver Analysis Software; p62/SQSTM1, sequestosome-1; RAB7A, ras-related protein Rab-7a; TBS, tris buffered saline; TEFB, transcription factor EB; TG5, autophagy protein 5; TTR, transthyretin; TUBB, tubulin-beta; ULK1, serine/threonine-protein kinase ULK1.

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is becoming the most common hepatic disorder in Western populations and is emerging as one of the principal causes for liver cirrhosis, hepatocellular carcinoma (HCC), and liver transplantation (Bedogni et al., 2014). NAFLD is significantly associated with conditions of hyperglycemia, dyslipidemia, obesity and other features that characterize the metabolic syndrome specially occurring in older individuals (Stefan et al., 2008). Recent studies suggest that advanced age leads to more severe histological changes and lower clinical response as hepatic lipids accumulate over time (Mofrad et al., 2003; Angulo, 2010; Bouchi et al., 2016; Hashimoto et al., 2017; Jinjuvadia et al., 2017; Leung et al., 2017; Sookoian and Pirola, 2017). Indeed, the main feature of NAFLD is steatosis, which consists in the overload of intracellular neutral lipids in the form of lipid droplets (LDs) due lipid homeostasis disruption (Gluchowski et al., 2017). In particular, the uptake and synthesis of lipids overwhelm the ability of the cell to degrade or secrete lipids. The majority of NAFLD patients have simple steatosis or steatosis with non-specific changes and do not progress or evolve very slowly to advanced liver disease (Vernon et al., 2011). Conversely, a subgroup of patients with NAFLD presents the histological criteria for non-alcoholic steatohepatitis (NASH) and has a significant risk of progression toward cirrhosis and HCC (Vernon et al., 2011).

Expanding the knowledge of factors involved in the development and progression of hepatic steatosis and NAFLD is mandatory in order to enhance the repertoire of tools for assessing disease severity and optimizing treatment. LD autophagy, the so-called lipophagy, is the main process responsible for lipid catabolism in the liver. It is a finely regulated step mechanism that consists in: (1) protein-mediated sequestration of cargo (portion or whole LD) within cytosolic double-membrane vesicles, and consequent formation of a phagosome; (2) tracking and transport of autophagosome to lysosome; (3) fusion between autophagosome and lysosome and formation of the autophagolysosome; (4) cargo degradation by lysosomal lipases.

In the early stage of the process, i.e., during the formation of autophagosomal membranes, cytosolic LC3 (LC3-I) is conjugated to phosphatidylethanolamine (PE) by the action of two consecutive ubiquitylation-like reactions catalyzed by the E1-like enzyme Atg7 and the E2-like enzyme Atg3, in order to form LC3-II. When autophagosomes fuse with lysosomes, LC3-II is degraded by lysosomal proteases. Therefore, the increase of LC3-II level is connected with its impaired turnover and is used as a marker of reduced autophagy (Klionsky et al., 2016). p62/sequestosome (SQSTM)1 is recruited as an autophagy receptor required for selective macroautophagy connecting polyubiquitinated cargo with autophagosomes. Consequently, p62/SQSTM1 accumulation represents another hallmark of autophagy blockage.

In the last years, an increasing number of studies have shown that dysregulation of lipophagy plays a pathogenetic role in the development of NAFLD, and that all the steps of this process can be affected (Patrick and Lake, 1969; Kwanten et al., 2014; Martinez-Lopez and Singh, 2015; Schulze et al., 2017). Notably, a previous study analyzed the accumulation of p62/SQTSM1 as marker of autophagy blockage in hepatocytes of NAFLD patients (Fukushima et al., 2018). Finally, lysosomes are crucial organelles for the final phase of fat disposal, and lipolysosomes represent LD-loaded lysosomes in which lipid catabolism takes place thanks to specific hydrolases. They can be individuated as vacuoles surrounded by lysosomal related-proteins, constituting an electron dense membrane at the ultrastructural analysis (Hayashi et al., 1977, 1983; Iancu et al., 2013).

In the present study, we have assessed autophagy both in *in vitro* and in *in vivo* models of NAFLD as well as in the liver of NAFLD patients. The results presented give effort to the notion that lipophagy/autophagy is impaired in NAFLD to an extent that is tightly correlated with disease severity and progression (Berson and Pessayre, 1997; Tandra et al., 2011).

MATERIALS AND METHODS

Patients

Fifty-nine patients who had undergone liver biopsy for suspected NASH at the Hepatology Unit of the University Hospital Campus Bio-Medico of Rome and whose paraffin-embedded liver tissue was available for further analysis, were included in the study. It was confirmed that there was no history of alcoholic intake >20 gr/day if woman and >30 gr/day if man or of use of drugs known to induce liver damage, and that the following results had been obtained: negative anti-HCV antibodies and HBsAg; antinuclear antibodies (ANA) $<$ 1:80 and negative anti-mitochondrial (AMA), anti-smooth muscle (ASMA) and anti-liver and kidney microsomal (anti-LKM) antibodies; normal transferrin saturation and serum levels of ceruloplasmin and alpha-1 antitrypsin. All patients had signed an informed consent in which the possible use of part of their hepatic tissue for research purposes was specified. The protocol of the study conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Ethics Committee of the University Campus Bio-Medico of Rome.

Liver biopsies from ten patients with normal transaminases, negative for HBsAg and anti-HCV antibodies tests, and presenting histologically normal liver with less than 5% steatosis were included in the study as control group for histological and immunohistochemical evaluations. These samples were from patients who had undergone liver surgery for metastatic tumors (liver tissue for immunohistochemistry was at least 0.5 cm from the metastatic lesion).

Animal Model, Treatment and Biochemical Analyses

Mouse experimentation was conducted in accordance with accepted standard of humane animal care after the approval by relevant local (Institutional Animal Care and Use Committee, Tor Vergata University) and national (Ministry of Health) committees. Adult C57BL/6J (3 months-age-old) male mice (purchased from ENVIGO, Italy) were randomly divided into four groups: mice fed with normal diet (ND: 3.85 kcal/g among

which 10% kcal from fat, 20% from protein and 70% from carbohydrate) for 4 months (group 1) or for 8 months (group 2); mice fed with high fat diet (HFD: 5.24 kcal/g among which 60% kcal from fat, 20% from protein, and 20% from carbohydrate) for 4 months (group 3) or for 8 months (group 4). ND (#D12450B) and HFD (#D12492) were from Research Diets, INC (New Brunswick, NJ, United States). ND contained 38% sugars, 4% saturated fats, 6% unsaturated fats and casein; HFD contained 20% sugars, 54% saturated fats, 6% unsaturated fats and casein. Mice were maintained at 23.0 ± 1.0°C and 55.0 ± 5.0% relative humidity under a 12/12 h light/dark cycle (lights on at 6:00 AM, lights off at 6:00 PM). At the end of treatment, mice were starved for 6 h before blood samples collections and body weight measurement; successively, mice were sacrificed by cervical dislocation, and liver tissues were explanted for the analyses. Blood glucose, cholesterol, alanine transaminase (ALT), aspartate transaminase (AST), and triglycerides were measured through the automatized KeyLab analyser (BPCBioSed, Italy) using specific colorimetric assay kits (BPCBioSed).

Cells and Treatments

The hepatocyte-derived human carcinoma cell line Huh7 was obtained from the American Type Culture Collection (Rockville, MD, United States) and cultured in low-glucose Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum, 1% penicillin-streptomycin, and 1% L-glutamine in a humidified incubator at 37°C with 5% CO_2. For treatments, cells were plated at 80% confluence and, after 24 h, culture medium was replaced with complete high-glucose DMEM and 400 mM of BSA-conjugated palmitic acid was added for 72 and 96 h.

Western Blot

Animal liver tissue was homogenized in RIPA buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 12 mM deoxycholic acid, 0.5% Nonidet P-40, and protease and phosphatase inhibitors). Ten μg proteins were loaded on SDS-PAGE and subjected to Western blotting. Nitrocellulose membranes were incubated with anti-heat shock protein-60 (Hsp60) (Abcam, ab46798), anti-p62/SQSTM1 (Abcam, Ab91526), anti-LC3 (Sigma-Aldrich, L7543) primary antibodies at 1:1000 dilution. Successively, membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies. Immunoreactive bands were detected by a FluorChem FC3 System (Protein-Simple, San Jose, CA, United States) after incubation of the membranes with ECL Selected Western Blotting Detection Reagent (GE Healthcare, Pittsburgh, PA, United States).

Liver Pathology

For both human and animal liver tissue, tissue sections were stained with haematoxylin and eosin and Sirius red and each case was assessed by a single operator. In patients, the severity of steatosis (0–3), features of NASH, including necroinflammation (0–3), hepatocellular ballooning (0–2), and fibrosis stage (0–4) were assessed according to the NAFLD clinical research network scoring system (Kleiner et al., 2005), and each case was diagnosed as simple steatosis or NASH according to Brunt et al. (1999).

Immunohistochemistry and Immunofluorescence

Each case was analyzed by immunohistochemistry for LAMP1, a lysosomal marker (ab24170, 1:50 titre, rabbit polyclonal, Abcam, Cambridge, United Kingdom) and for p62/SQSTM1 (ab56416, 1:100 titre, mouse monoclonal, Cambridge, United Kingdom), a marker of autophagic impairment. Immunohistochemistry was performed on sections obtained from formalin-fixed tissues embedded in paraffin. Immunohistochemical stained sections were scanned using the Hamamatsu NanoZoomer 2.0-RS.

LAMP1 was used to visualize vesicles of lysosomal origin (with a LAMP1-positive membrane). The number of large LAMP1-positive vesicles, with a visible lumen, i.e., lipolysosomes, was evaluated in five fields randomly chosen for each case with a magnification of 400×. A mean value was therefore derived in each sample for statistical analysis.

For the evaluation of p62/SQSTM1 expression, five fields randomly chosen were analyzed (magnification ×200) for each sample. The immunostaining was evaluated by using an open source plugin IHC Profiler for Image J (Varghese et al., 2014). All images analyzed were thresholded at the same intensity value. A scale that ranges from 0 to 3 was applied to describe the intensity of staining in each field, where 0 means negative and 3 strongly positive. The percentage of liver tissue with different score of intensity were recorded for each field and an immunohistochemistry score was derived as follows: [(3+%tissue) X3 + (2+%tissue) X2 + (1+%tissue) X1]. A mean value was then calculated for each sample (Potts et al., 2012).

Immunofluorescent staining was performed on sections from paraffin-embedded tissue and cell culture with the same antibodies used for immunohistochemistry. Secondary Alexa Fluor 488 and/or Alexa Fluor 568-conjugated donkey anti-rabbit, anti-mouse, anti-goat antibodies (Invitrogen, Carlsbad, CA, United States) were added for 30 min (1:200 titre), followed by another extensive washing step in TBS. Nuclear counterstaining was performed using Vectashield mounting medium (Vector Laboratories, Burlingame, CA, United States) containing 4,6-diamidino-2-phenylindole (DAPI). Fluorescence images were collected with a Nikon A1 Confocal Laser Microscope System (Nikon, Tokyo, Japan). Acquisition was carried out using the Imaging Software NIS-Elements (Nikon).

Ultrastructural Analysis

A subgroup (n = 5) of liver samples were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer for the ultrastructural study. After fixation, dehydration and impregnation, the samples were included in epoxy resins and acrylic, cut at the ultramicrotome and processed for the ultrastructural study of hepatocytes by electron microscopy. In particular, morphological aspects typical of lipid overload, lysosomal activation and lipophagy/autophagy process, such as the presence of lipid droplets, membrane-bound lipid vacuoles (lipolysosomes) and autophagosomes were evaluated in 5000× fields. The presence of lipolysosomes in hepatocytes of NAFLD patients by both light and electron microscopy was evaluated according to previous criteria by Iancu et al. (2013).

mRNA Analysis

For RT-qPCR analysis, total RNA was extracted by using Quick-RNA extraction kit (Zymo Research). RNA (3 μg) was retro-transcribed by using M-MLV (Promega, Madison, WI). qPCR was performed in triplicate by using validated qPCR primers (BLAST), Ex TAq qPCR Premix and the Real-Time PCR (Applied Biosystem). mRNA levels were normalized to Alpha-fetoprotein mRNA, and the relative mRNA levels were determined through the $2^{-\Delta\Delta Ct}$ method. Primers used for RT-qPCR are listed in **Table 1**.

Nanostring nCounter analysis was performed in a subgroup of patients and controls (n = 38). Total RNA was extracted using the High Pure FFPET RNA Isolation Kit (Roche) from 10 μm sections of FFPE liver biopsies of NAFLD patients (27 specimens) and 11 controls. The RNA was quantified with the BioPhotometer® D30 (Eppendorf) and 100 ng of total RNA was subjected to Nanostring nCounter Analysis System. This system utilizes a novel digital color-coded barcode technology that is based on direct multiplexed measurement of gene expression and offers high levels of precision and sensitivity (<1 copy per cell). The technology uses molecular "barcodes" and single molecule imaging to detect and count hundreds of unique transcripts in a single reaction.

We used a custom nCounter CodeSet containing 20 genes, selected in order to evaluate the expression levels (mRNA levels) of genes involved in autophagy and of other functional genes in NAFLD versus healthy liver samples. In particular, of these genes, 6 are lysosome related genes (LAMP1, LAMP2, CTSD, NPC1, ATP6V1B2, TFEB) and 9 are key genes involved in autophagy (mTOR, ULK1, BECN1, ATG12, ATG5, ATG7, p62/SQSTM1, RAB7A, GABARAP) (**Table 2**). We have also included in the panel 3 hepatocyte markers (AFP, ALB, and TTR) to normalize the values for the number of hepatocytes present in each sample and 2 housekeeping genes (TUBB and HIST1H3A), to normalize the values for total number of cells. Nanostring nCounter analysis was performed following the manufacturer's protocol. In brief, 100 ng of total RNA was hybridized in solution at 65°C for 16 h with specific pairs of ∼50 base probes for each gene set mRNA. The Reporter Probe carries the signal; the Capture Probe allows the complex to be immobilized for data collection. After hybridization, the probe excess was removed and the probe/target complexes aligned and immobilized in the nCounter Cartridge. Sample Cartridges were placed in the Digital Analyzer for data collection. Color codes on the surface of the cartridge will be counted and tabulated for each target molecule. Raw counts for each mRNA was normalized (for hepatocyte markers or for housekeeping genes) and analyzed using nSolver Analysis Software (nSAS) to obtain differential expression analysis of 44 genes in NAFLD samples versus control (healthy) samples.

TABLE 1 | Primer sequences used for RT-qPCR analyses.

Gene	Primers
Col1a1	fw: CGATGGATTCCCGTTCGAGT
	rv: GAGGCCTCGGTGGACATTAG
Col3a1	fw: GACCTAAGGGCGAAGATGGC
	rv: AAGCCACTAGGACCCCTTTCT
IL1B	fw: TTCGAGGCACAAGGCACAA
	rv: TGGCTGCTTCAGACACTTGAG
IL1b	fw: GCACTGGGTGGAATGAGACT
	rv: GGACATCTCCCACGTCAATCT
IL6	fw: GAACTCCTTCTCCACAAACATGTAA
	rv: TTGTTTTCTGCCAGTGCCTCT
Il6	fw: GGATACCACTCCCAACAGACC
	rv: GCCATTGCACAACTCTTTTCTCA
Mmp2	fw. TCTGGTGCTCCACCACATAC
	rv: CCATGGTAAACAAGGCTTCATGG
Mmp9	fw: TGGTCTTCCCCAAAGACCTG
	rv: AGCGGTACAAGTATGCCTCTG
TNFA	fw: GCCCATGTTGTAGCAAACCC
	rv: TATCTCTCAGCTCCACGCCA
Tnfa	fw: ATGGCCTCCCTCTCATCAGT
	rv: CTTGGTGGTTTGCTACGACG

Statistics

Depending on the parametric or nonparametric distribution, variables are expressed as mean ± SD or median and 25–75% interquartile range (25–75% IR), respectively. Differences were evaluated by the Kruskal–Wallis test. Correlations were carried out by the Spearman's rank correlation test. A $p < 0.05$ was considered statistically significant. SPSS software (version 22.00; SPSS Inc., Chicago, IL, United States) was used for statistical analyses.

RESULTS

Progressive Impairment of Lipophagy in Cell and Mouse Models of Liver Steatosis

In order to generate a preclinical model of NAFLD, C57BL/6J mice were fed with HFD for 4 or 8 months. Mice fed with normal diet (ND) served as controls. As expected, HFD mice underwent a progressive increase of body weight (**Figure 1A**) and alteration of biochemical parameters such as increased blood glucose, cholesterol and ALT (**Figure 1B**). An increase of liver weight (**Figure 1C**), and the acquisition of a steatotic phenotype (**Figure 1D**) were also observed, in association with enhanced expression of inflammatory cytokines (IL-6, TNF-α, IL-1β) and fibrosis markers (Col1a1, Mmp2) (**Figure 1E**), collectively recapitulating the occurrence of a NAFLD-like clinical phenotype. At the protein level, a significant increase of both p62/SQSTM1 and LC3-II/LC3-I ratio was noticed compared to ND group that was more marked after 8 months than after 4 months of HFD treatment (**Figures 1F,G**).

Similar results were observed in the *in vitro* model of hepatocellular fat overload. In particular, Huh7 cells were cultured in high-glucose DMEM supplemented with 400 μM palmitic acid (high-fat/high-glucose, HFHG). Starting at 72 h of treatment, up-regulation of cytokine mRNAs (IL-6, TNF-α,

TABLE 2 | Gene, Protein name and Function are derived from https://www.uniprot.org.

Gene	Protein name	Function
Lysosomes related genes		
LAMP1	Lysosome-associated membrane glycoprotein 1	It presents carbohydrate ligands to selectins.
LAMP2	Lysosome-associated membrane glycoprotein 2	It plays an important role in chaperone-mediated autophagy, a process that mediates lysosomal degradation of proteins in response to various stresses and as part of the normal turnover of proteins with a long biological half-life.
CTSD	Cathepsin D	Acid protease active in intracellular protein breakdown.
NPC1	NPC intracellular cholesterol transporter 1	Intracellular cholesterol transporter that acts in concert with NPC2 and plays an important role in the egress of cholesterol from the endosomal/lysosomal compartment.
ATP6V1B2	V-type proton ATPase subunit B	Non-catalytic subunit of the peripheral V1 complex of vacuolar ATPase. V-ATPase is responsible for acidifying a variety of intracellular compartments in eukaryotic cells.
TFEB	Transcription factor EB	It plays a central role in the expression of lysosomal genes. It acts as a positive regulator of autophagy by promoting expression of genes involved in autophagy.
Autophagy related gene		
MTOR	Serine/threonine-protein kinase mTOR	Serine/threonine protein kinase playing a central regulatory role in cellular metabolism, growth and survival in response to hormones, growth factors, nutrients, energy and stress signals.
ULK1	Serine/threonine-protein kinase ULK1	Serine/threonine-protein kinase involved in autophagy in response to starvation. It acts upstream of phosphatidylinositol 3-kinase PIK3C3 to regulate the formation of autophagophores, the precursors of autophagosomes.
BECN1	Beclin-1	It plays a central role in autophagy and acts as core subunit of the PI3K complex that mediates formation of phosphatidylinositol 3-phosphate.
ATG12	Autophagy-related protein 12	Ubiquitin-like protein involved in autophagy vesicle formation.
ATG5	Autophagy protein 5	Involved in autophagic vesicle formation.
ATG7	Autophagy-related protrein 7	E1-like activating enzyme involved in the 2 ubiquitin-like systems required for cytoplasm to vacuole transport and autophagy. It activates ATG12 for its conjugation with ATG5.
P62/SQSTM1	Sequestosome-1/ Ubiquitin-binding protein p62	Autophagy receptor required for selective macroautophagy (aggrephagy). It functions as a bridge between polyubiquitinated cargo and autophagosomes. It interacts directly with both the cargo to become degraded and an autophagy modifier of the MAP1 LC3 family.
RAB7A	Ras-related protein Rab-7a	Key regulator in endo-lysosomal trafficking. It plays a role in the fusion of phagosomes with lysosomes.
GABARAP	Gamma-aminobutyric acid receptor-associated protein	Ubiquitin-like modifier that plays a role in intracellular transport of GABA(A) receptors and its interaction with the cytoskeleton. Involved in apoptosis. Involved in autophagy. Whereas LC3s are involved in elongation of the phagophore membrane, the GABARAP/GATE-16 subfamily is essential for a later stage of autophagosome maturation.
Hepatocyte markers		
AFP	Alpha-fetoprotein	It binds copper, nickel and fatty acids as well as serum albumin, and bilirubin less well than.
ALB	Serum albumin	The main protein of plasma having a good binding capacity for water, Ca^{2+}, Na^{+}, K^{+}, fatty acids, hormones, bilirubin and drugs.
TTR	Transthyretin	Thyroid hormone-binding protein.
Housekeeping genes		
TUBB	Tubulin beta chain	Tubulin is the major constituent of microtubules.
HIST1H3A	Histone H3.1	Core component of nucleosome.

IL-1β), increase of both p62/SQSTM1 protein and LC3-II/LC3-I ratio (**Figures 2A,B**) as well as accumulation of lysosomal mass at confocal analysis with the lysosomal marker LAMP1 (**Figure 2C**) were observed, suggesting autophagy inhibition and lysosomal reaction.

Increased Lipolysosomes and Impaired Lipid Catabolism in NAFLD Patients

Based on the results obtained in the experimental models of NAFLD, we moved to a cohort of 59 NAFLD patients. The main characteristics of these patients are reported in **Table 3**.

By immunohistochemistry for LAMP1, lysosomes from healthy subjects were detected mainly near the hepatocyte biliary pole, and no LAMP1-positive vacuoles with visible lumens were detected. In NAFLD patients, the lysosomal compartment architecture was completely disarranged. In particular, lysosomes were no longer located at the biliary pole, and LAMP1-positive vacuoles were observed inside hepatocytes. Moreover, the number of LD-loaded lysosomes, the so-called lipolysosomes, positively correlated with the NAS score ($\rho = 0.375$, $p < 0.005$) (**Figure 3**).

The increase of lysosomal mass was supported by increased levels of expression of lysosomal genes (CTSD, LAMP1, LAMP2, NPC1, ATP6V1B2,TFEB), whose levels of expression were correlated with the NAS score ($r = 0.7$; $p < 0.001$;$r = 0.5$; $p < 0.005$;$r = 0.4$; $p < 0.01$;$r = 0.4$; $p < 0.05$; $r = 0.6$; $p < 0.001$; $r = 0.4$; $p < 0.01$, respectively), as well as with fibrosis stage

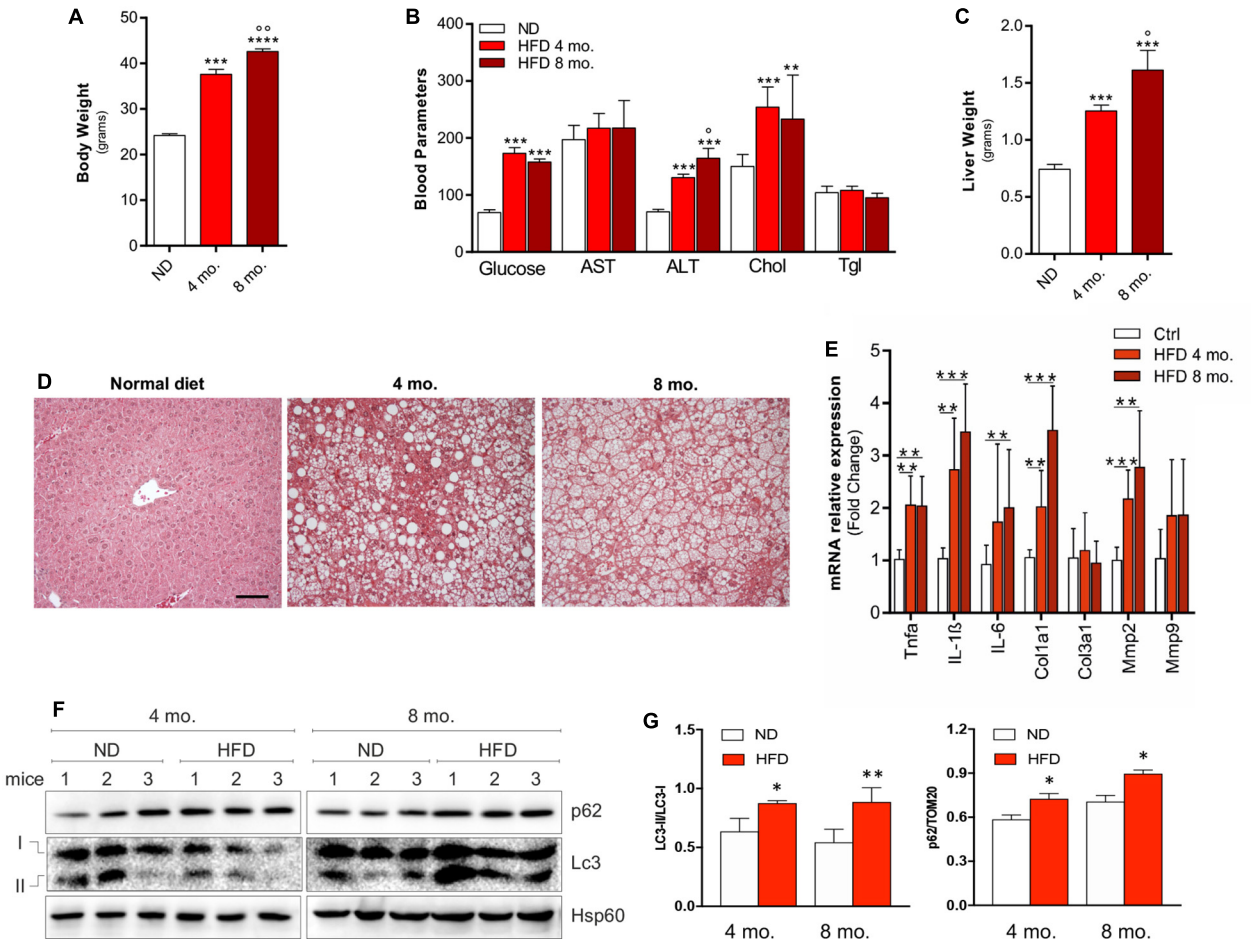

FIGURE 1 | C57BL/6J male mice were fed with high fat diet (HFD) or normal diet (ND) for 4 or 8 months. Increase of body weight **(A)**, alterations of biochemical parameters **(B)**, increase of liver mass **(C)** accompanied by histological steatosis **(D)** were observed in HFD mice compared to ND. The mRNA expression of inflammation- and tissue remodeling-related genes **(E)** and autophagy protein markers LC3-II/LC3-I ratio and p62/SQSTM1 **(F)** were progressively increased as demonstrated by densitometric analyses **(G)**, indicating autophagy inhibition. HSP60 was used as loading control and representative immunoblots are reported. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ vs. ND; °$p < 0.05$, °°$p < 0.01$ vs. 4 mo. ($n = 6$ mice per group). Original magnification: × 200. Calibration bar: 25 μm.

($r = 0.6$; $p < 0.001$; $r = 0.7$; $p < 0.00$; $r = 0.6$; $p < 0.001$; $r = 0.5$; $p < 0.005$, $r = 0.8$; $p < 0.001$; $r = 0.6$; $p < 0.001$, respectively) (**Figure 4**).

Impaired Autophagy in NAFLD Patients

Consistent with the results obtained in mice, p62/SQSTM1 protein levels as well as fibrosis were significantly increased in the liver of NAFLD patients. In particular, immunohistochemical positivity of p62/SQSTM1 increased with progression of fibrosis, accumulating in hepatocytes in the so-called Mallory-Denk bodies, and indicating autophagy blockage. Furthermore, by means of immunofluorescence, p62/SQSTM1 aggregates were observed outside the lysosomes, suggesting and impairment also of the first steps of autophagy (**Figure 5**). Moreover, significant correlations between levels of expression of autophagy-related genes (ATG12, ATG5, ATG7, BECN1, GABARAP, MTOR, RAB7A, P62/SQSTM1, ULK1) and the NAS score ($r = 0.4$; $p < 0.05$; $r = 0.4$; $p < 0.01$; $r = 0.5$; $p < 0.005$; $r = 0.5$; $p < 0.005$; $r = 0.3$; $p < 0.05$; $r = 0.6$; $p < 0.001$; $r = 0.3$; $p < 0.05$; $r = 0.5$; $p < 0.005$; $r = 0.3$; $p < 0.05$, respectively) and fibrosis stage were observed ($r = 0.7$; $p < 0.001$; $r = 0.6$; $p < 0.001$; $r = 0.7$; $p < 0.001$; $r = 0.7$; $p < 0.001$; $r = 0.5$; $p < 0.005$; $r = 0.7$; $p < 0.001$; $r = 0.5$; $p < 0.001$; $r = 0.5$; $p < 0.005$; $r = 0.4$; $p < 0.01$, respectively) (**Figure 6**).

Evaluation of autophagy was also performed at ultrastructural level in human biopsies processed for TEM experiment. An increased amount of lipid droplets, lipolysosomes and autophagosomes was found in subjects with NAFLD compared to healthy subjects ($p < 0.05$) (**Figure 7**).

NAFLD Progression and Aging in NAFLD Patients

Histological markers of NAFLD progression were associated with aging in NAFLD patients. Standard histopathological features of NAFLD severity, such as grading and staging of the disease (NAS and fibrosis score, respectively) were significantly different

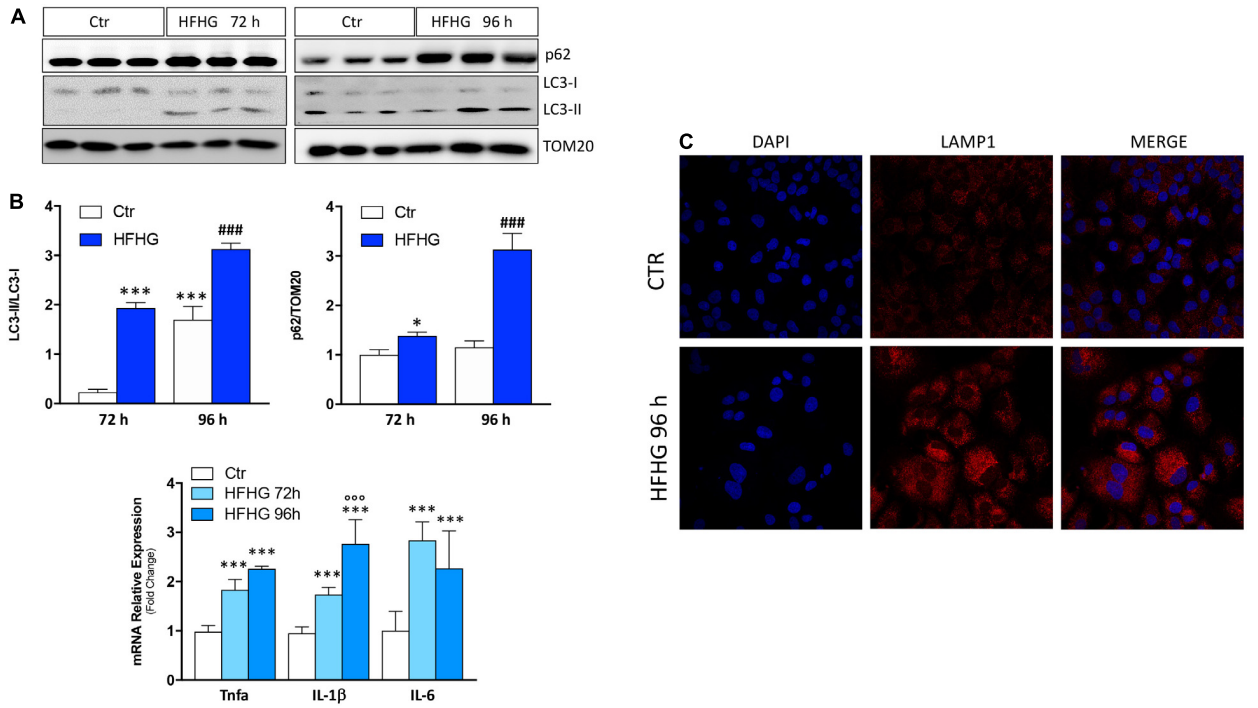

FIGURE 2 | Huh7 human cells treated with 400 μM palmitate and 4.5 g/L glucose (HFHG) show accumulation of p62/SQSTM1 protein and increase of LC3-II/LC3-I protein ratio **(A)** as well as up-regulation of inflammatory cytokines **(B)** and lysosomal mass **(C)** after 72 and 96 h of treatment. TOM20 was used as loading control. Densitometric analyses of p62/SQSTM1 and LC3-II/LC3-I are shown in the bottom panel **(A)**. $n = 3$; $^{***}p < 0.001$, $^{*}p < 0.05$ vs. Ctr; $^{°°°}p < 0.001$ vs. HFHG 72 h, $^{###}p < 0.001$ vs. Ctr 96 h.

TABLE 3 | Biological, anthropometric, and biochemical characteristics of the NAFLD patients.

N	59
Age (years)	49.9 ± 14.8
Sex (M / F)	35 / 24
BMI (kg/m^2)	29.4 ± 4.5
AST (value/u.n.l.)	1.3 ± 0.9
ALT (value/u.n.l.)	2.2 ± 1.9
GGT (value/u.n.l.)	2.1 ± 2.0
Glycemia (mg/dL)	102.3 ± 25.3
Total cholesterol (mg/dL)	204.3 ± 48.6
HDL cholesterol (mg/dL)	47.4 ± 12.6
Triglycerides (mg/dL)	170.9 ± 117.4
Diabetes (Yes/No)	25 / 34
Hypertension (Yes/No)	29 / 30

Data are expressed as mean ± standard deviation.

in NAFLD patients above and below 54 years (**Table 4**). Furthermore, immunohistochemical positivity of p62/SQSTM1 was correlated with aging in NAFLD patients ($r = 0.3$, $p < 0.05$).

DISCUSSION

Aging and obesity predispose to the development of NAFLD. Actually, the chronic overload of lipids has harmful effects in the liver, exposing resident hepatocytes to their cytotoxic action (lipotoxicity) and oxidative stress, the effect of which are more pronounced with aging (Zelber-Sagi et al., 2006; Chen et al., 2013; Xu et al., 2013a). Lipophagy represents the main lipid degrading system in the liver and its impairment was recently associated with the pathogenesis of NAFLD (Sinha et al., 2014; Zhang et al., 2018). In lipophagy, the neutral lipids are partially subtracted in autophagosomes and transported to lysosomes, which represent the final destination of lipids in this pathway.

In the present study, we have demonstrated that chronic lipid overload through HFD in mice and palmitic acid treatment in a human hepatocyte derived cell line, respectively, leads to a time-dependent inhibition of autophagy. Notably, in HFD-fed mice, the progression of NAFLD-mimicking features was strongly associated with the degree of autophagy inhibition (p62/SQSTM1 and LC3-II/LC3-I increase). Moreover, here we showed, for the first time, the presence of lipolysosomes in hepatocytes of NAFLD patients by both light and electron microscopy. In NAFLD, lipolysosomes likely represent the progressive impairment of the lysosomal function, and in particular of the capability of lysosomal hydrolases to catabolize fat. This is supported by the correlation between the increased number of lipolysosomes and disease activity in terms of necroinflammation.

The up-regulation of lysosomal genes, such as CTSD, LAMP1, LAMP2, NPC1, ATP6V1B2, TFEB, suggesting an increase of the overall lysosomal mass, should be well interpreted as an attempt to counteract lysosomal dysfunction. Among these genes,

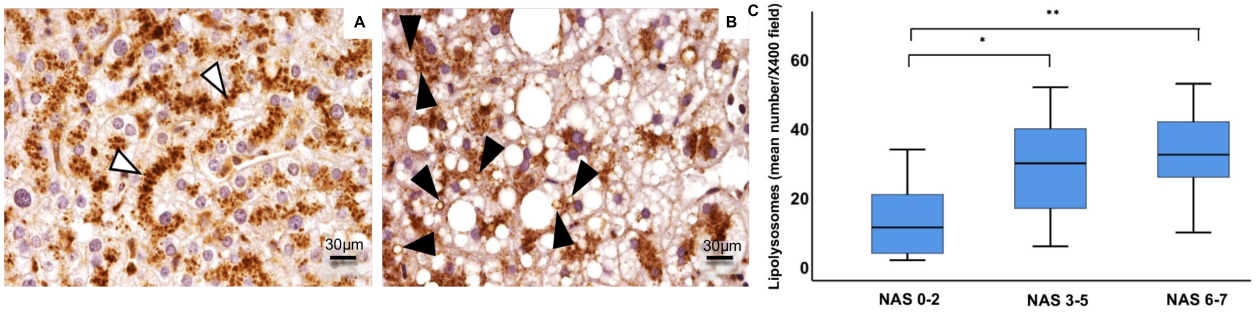

FIGURE 3 | Immunohistochemical expression of LAMP1 in human hepatocyte lysosomes (LAMP1-positive particles) were regularly disposed along the path of biliary canaliculi in healthy liver **(A)**, while they were patchy distributed in fatty liver **(B)**. In fatty liver of NAFLD patients (n = 59), enlarged fat-loaded lysosomes, namely lipolysosomes, could be individuated as intracellular droplets with lysosomal LAMP1-positive membrane and visible lumen (**B**, arrowheads). The number of lipolysosomes/field at light microscopy increased in NAFLD patients with higher NAS (Non-alcoholic fatty liver disease Activity Score) **(C)**. Original magnification: × 400 **(A,B)**. *$p < 0.05$, **$p < 0.01$.

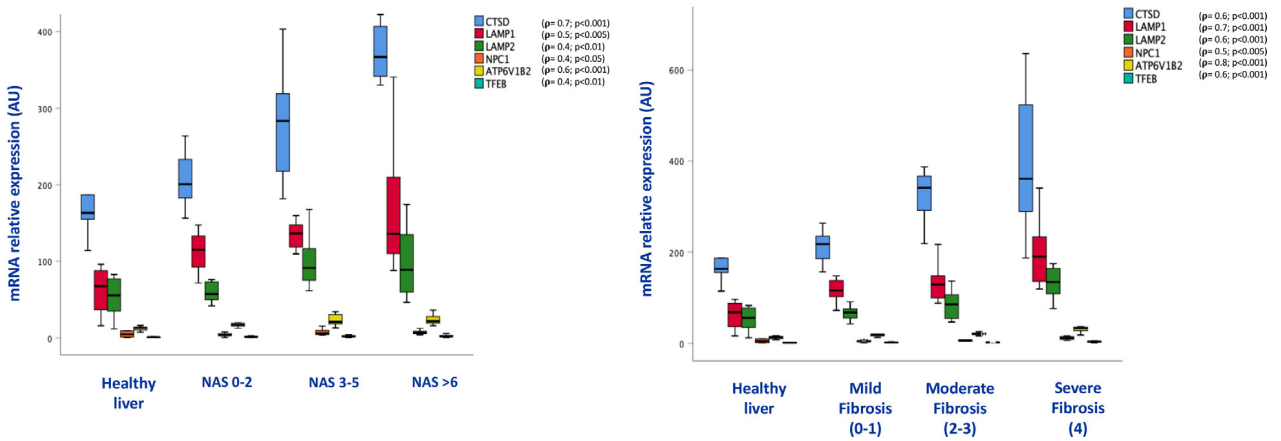

FIGURE 4 | Box plots of mRNA expression of lysosome related genes in association with NAS score (on the **left**) and fibrosis stage (on the **right**); (n = 38). For correlation purpose Spearman's **ρ** test was performed.

notably, CTSD has been already correlated with inflammation and impairment of lipid metabolism in NAFLD. Houben et al. (2017) have shown that inhibition of CTSD in mice results in reduced cholesterol and triglyceride levels in the liver. The group of Liao showed that CTSD is up-regulated as cholesterol accumulates in lysosomal compartments in an NPC1 knock-out model, demonstrating an increase of CTSD when lysosomes are engulfed (Liao et al., 2007). In the present study, CTSD expression was higher in NAFLD patient compared to controls, and a direct correlation was observed between CTSD expression and both NAS and fibrosis.

Lysosome engulfment by lipids has been shown to inhibit autophagosome turnover, and several studies in lysosomal storage diseases have demonstrated an impairment in the fusion between autophagosomes and fatty lysosomes that leads to defective autophagic clearance (Fukuda et al., 2006; Settembre et al., 2008; Sarkar et al., 2013). Consistent with this, as already described (Fukuo et al., 2014), we confirmed an increased expression of p62/SQSTM1 mRNA and protein in the liver of NAFLD patients that was significantly associated with disease activity and fibrosis stage. The increase of p62/SQSTM1 suggests that the autophagic cargos cannot fuse with lysosomes in order to be degraded, leading to cytosolic aggregates of p62/SQSTM1; accordingly, double labeling experiment revealed that p62/SQSTM1 clusters are detached from lysosomal-derived structure. Furthermore, in NAFLD patients, TEM analysis revealed an increased number of autophagosomes and lipolysosomes, likely representing the compensatory expansion of a bad-functioning autophagic machinery. Indeed, the expression of several genes involved in autophagy (Atg7, Atg5, GABARAB, and Rab7) was significantly correlated with NAS and fibrosis stage. These are key proteins for autophagic vesicle formation and lysosome maturation, and their deficit was found to suppress autophagic flux and to impair the autophagosome-lysosome fusion (Xu et al., 2013b). Moreover, autophagy could exert an anti-fibrogenic role since it promotes survival of hepatocytes and hepatocyte apoptosis is a central event in the fibrogenic process (Ni et al., 2014; Ruart et al., 2019; Hammoutene et al., 2020).

In conclusion, in the present study, we have observed histological, ultrastructural and molecular features of altered

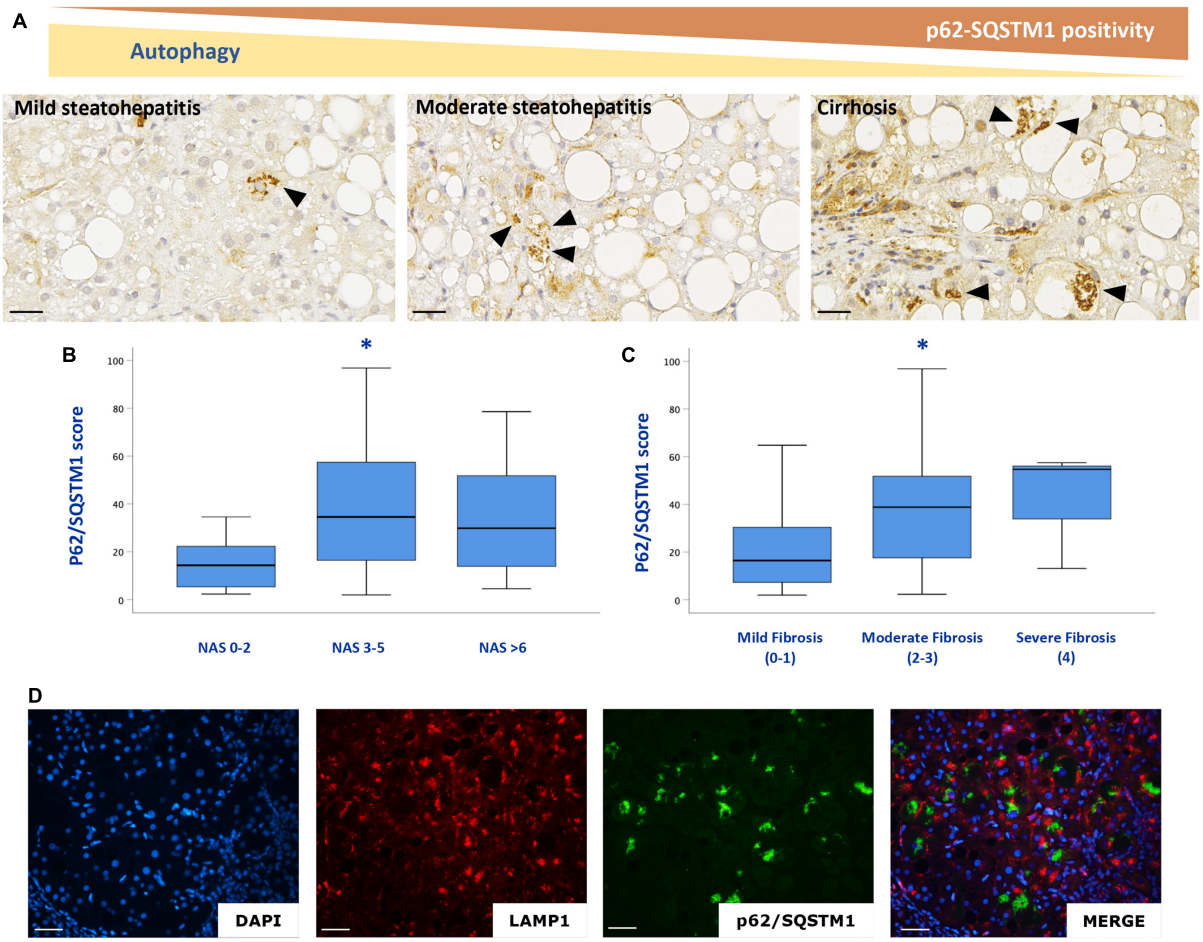

FIGURE 5 | p62/SQSTM1 accumulates with NAFLD progression **(A–C)**. P62/SQSTM1 positive Mallory-Denk bodies (arrowheads) were individuated in hepatocytes. Double labeling experiments with a lysosome marker (LAMP1, red) and autophagy impairment marker (p62/SQSTM1 green), confirmed that p62/SQSTM1 accumulates outside the lysosomes **(D)**. $n = 59$. Scale bar: 25 μm; * = $p < 0,05$ vs. NAS 0–2 or mild fibrosis.

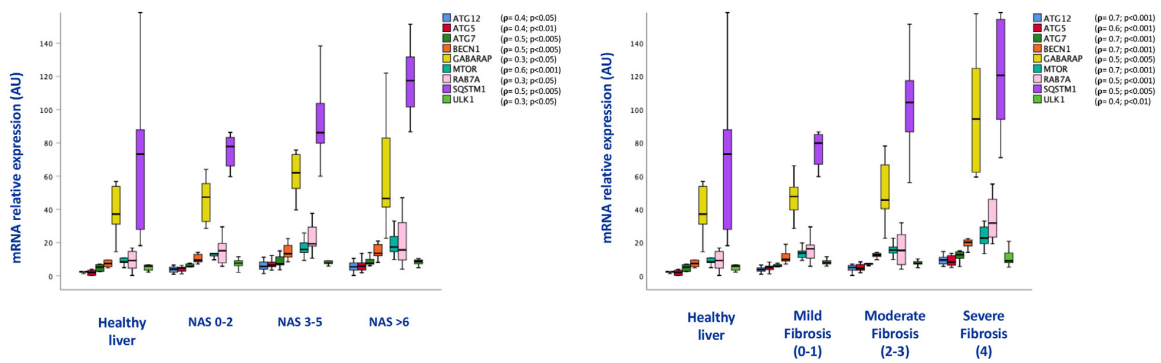

FIGURE 6 | Box plots of mRNA expression of autophagy related genes in association with NAS score (on the **left**) and fibrosis stage (on the **right**); ($n = 38$). For correlation purpose Spearman's **ρ** test was performed.

autophagy in NAFLD, leading to impaired lipid degradation. Impaired autophagy seems to be a pivotal factor determining the clinical progression of NAFLD also considering that a decrease in autophagic activity was previously described with aging, which is associated with NASH progression (Rubinsztein et al., 2011). Individuating molecular markers of lipophagy impairment could help to identify those patients at risk of progression. In particular, in this study, LAMP1-positive

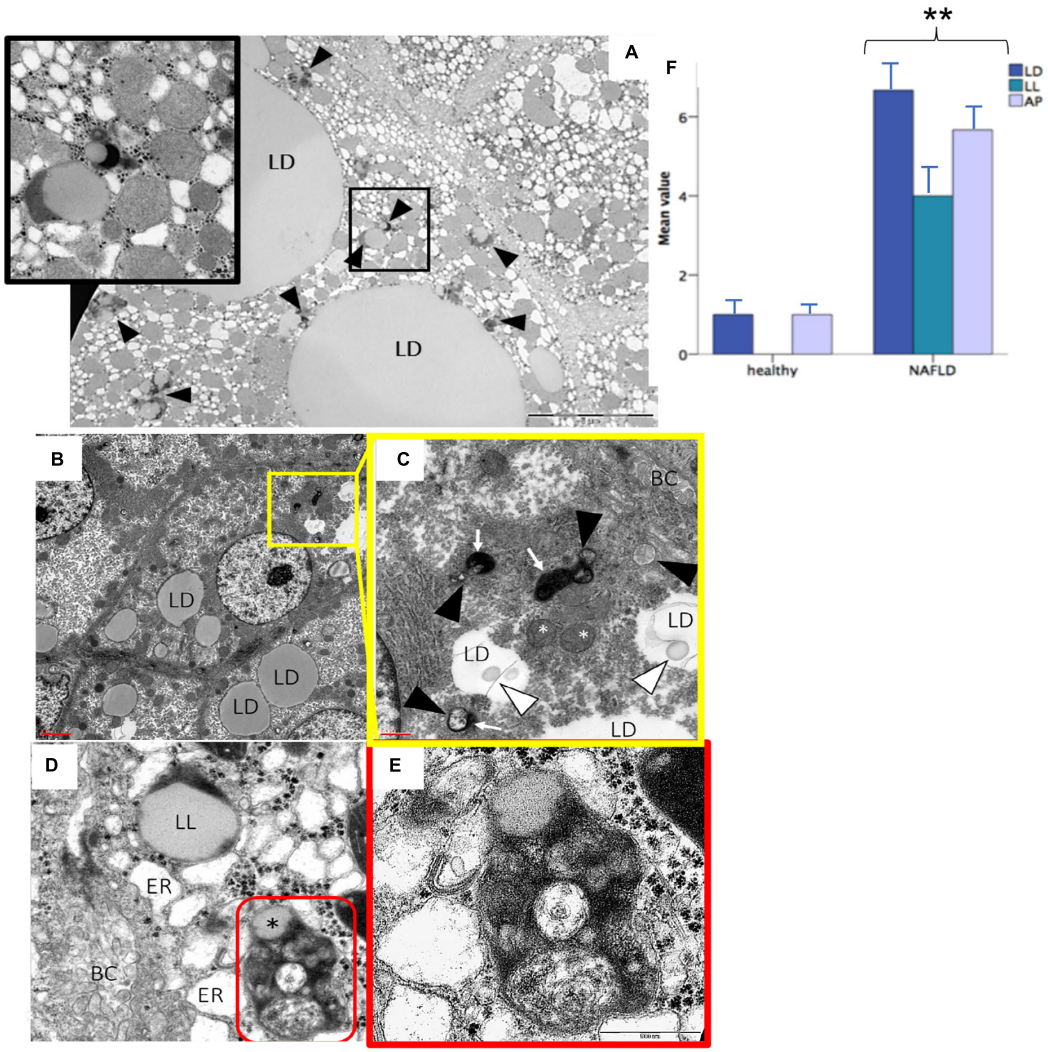

FIGURE 7 | Transmission electron microscopy analysis confirmed the presence of lipolysosomes with dense lipid core and typical electron-dense lysosomal membrane in fatty liver **(A–D)**. The different cellular organelles involved in the autophagic process phases are depicted in the high power fields **(C,E)**. Bars representing the number of autophagic organelles in NAFLD patients compared to healthy subjects (mean values, $n = 5$) **(F)**. LD, lipid droplet; LL, lipolysosome; AP, autophagosome; ER, stressed endoplasmic reticulum; BC, bile canaliculus; red circle, autolysosome; white arrowhead, phagophore; black arrowhead, immature autophagosome; white arrow, lysosome; white asterisk, stressed mitochondria; black asterisk, lipid component of autolysosome. Original magnification: 3900× **(A)**; 2300× **(B)**; 12000× **(C,D)**; 46000× **(E)**. $^{**}p < 0.05$.

vacuoles with visible lumen, known as lipolysosomes, were identified as morphological feature of altered lipophagy. Further studies are clearly awaited in order to verify if quantification of lipolysosomes could be a feasible and reproducible tool for assessing the severity of NAFLD and its propensity to progress.

TABLE 4 | Correlation of NAFLD characteristics with patients' age.

Age (years)	<54	>54	Mann-Whitney U Test
Number of patients	29	30	–
NAS	2 (1.5)	2.5 (1)	$p = 0.04$
Fibrosis	1 (1)	2 (0.75)	$p = 0.001$

Data are expressed as median (interquartile range).

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Comitato Etico dell'Università Campus Bio-Medico

di Roma with authorization n° 30/15 OSS ComEt CBM. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by the University Animal Welfare Committee-OPBA, Tor Vergata University with authorization no 378/2017-PR.

AUTHOR CONTRIBUTIONS

SC designed and supervised the study, performed the morphological experiments and the statistical analysis, and wrote the manuscript. FZ performed molecular experiments with Nanostring and gave an intellectual contribution. SR and FV performed the immunohistochemical experiments and provided intellectual contribution. KA designed molecular experiments, provided intellectual contribution, and helped writing the manuscript. MZ contributed to the design of the electron microscopy study. MF performed fluorescence microscopy experiments. GP provided tissue samples from UCBM Hospital. FA helped in recruiting patients' cohort from UCBM database. RA-I contributed to the critical revision of the manuscript. AP contributed to the study design and provided intellectual contribution. SM supervised the study and contributed to the critical revision of the manuscript. DL-B performed *in vivo* experiments, analyzed molecular data, and provided substantial intellectual contribution. UV-G obtained the patients informed consents, contributed substantially to the design of the study, to the discussion of the data, and to the writing of the manuscript. All authors contributed to the article and approved the submitted version.

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MINI REVIEW
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Why Do the Cosmic Rays Induce Aging?

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The increasing duration of space missions involves a progressively higher exposure of astronauts to cosmic rays, whose most hazardous component is made up of High-Atomic number and High-Energy (HZE) ions. HZE ions interact along their tracks with biological molecules inducing changes on living material qualitatively different from that observed after irradiation for therapeutic purposes or following nuclear accidents. HZE ions trigger in cells different responses initialized by DNA damage and mitochondria dysregulation, which cause a prolonged state of sterile inflammation in the tissues. These cellular phenomena may explain why spending time in space was found to cause the onset of a series of diseases normally related to aging. These changes that mimic aging but take place more quickly make space flights also an opportunity to study the mechanisms underlying aging. In this short review, we describe the biological mechanisms underlying cell senescence and aging; the peculiar characteristics of HZE ions, their interaction with living matter and the effects on the organism; the key role of mitochondria in HZE ion-induced health effects and aging-related phenomena.

Keywords: aging, cosmic rays, mitochondrion, DNA damage, cell signaling

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INTRODUCTION

In the hypothesis of exploration and settlement on the other planets, it is necessary to make long-duration space flights safer for astronauts. A detrimental effect often observed is indeed a state of frailty such as cardiovascular and cognitive disorders. The presence of these typical features of aging is very intriguing, and the cellular pathways involved in both cases are investigated not only to minimize the health risk of space missions but also to bring insight on the age-related mechanisms. While being aware that astronauts are subjected to numerous stress factors, this minireview is concerning the effects induced by the heavy ions' component of cosmic rays, that thanks to their high linear energy transfer (LET), they are able to induce biological effects that persist over time and are transmitted through the tissue.

We will focus mainly on the triggered oxidative imbalance and DNA damage that represent the common elements underlying both the detrimental effects induced by cosmic rays and the aging process.

AGING: DEFINITION AND CHARACTERISTICS

Aging is an inevitable decay due to the passage of time that involves a general progressive loss of homeostasis in various organs and in the whole body, causing the impairment of vital functions and

increased vulnerability to death. The loss of homeostasis leads, sooner or later, to the development of pathologies typical, such as diabetes, cancer, cardiovascular diseases, and alteration of cognitive functions (Khan et al., 2017).

In accordance with López-Otín et al. (2013), there are three families of distinctive signs of aging: (i) genomic instability caused by the deterioration of telomeres, epigenetic alterations, and loss of proteostasis; (ii) the accentuation of the compensatory responses, first of all the increase in apoptosis, cell senescence, and mitochondrial imbalance; (iii) finally, the exhaustion of stem cells and alteration in intercellular communication.

The theory that the accumulation of stochastic damage to DNA is the initial cause of almost all the listed aging processes is supported by various results (Niedernhofer et al., 2018). To avoid the onset of neoplasia, some of the DNA damage response (DDR) mechanisms, different according to the damage, intervene for the repair. In case damage is not repairable early, cells are initiated toward a programmed death or forced into a state of replicative quiescence typical of senescent cells. A close correlation between aging and persistence of unrepaired DNA damage is demonstrated by the observation that patients bearing DNA repair genetic defects such as xeroderma pigmentosum, ataxia telangiectasia, etc., show the hallmarks of premature aging (Kraemer and DiGiovanna, 2015). Likewise, exposure to environmental or therapeutic genotoxic compounds induces the early onset of aging features (Darby et al., 2013). The persistence of chromosomal damage is caused by reactive oxygen species (ROS) originated both by environmental factors such as radiation or mitochondrial imbalance (Sohal et al., 1994). In fact, the DNA damage from ionizing radiation has thus been attributed to the formation of radicals from the radiolysis of water molecules, as will be treated extensively in a later paragraph. More recently, also in light of the progress on understanding radicals as elements of intracellular and intercellular communication (Paladino et al., 2018), more and more weight is being given to biological pathways mediated by mitochondrial oxide-reduction while intermediate metabolic products can lead to oxidation, nitrosylation, and alkylation of DNA (Hekimi et al., 2011). Whatever their origin, ROS are able to induce DNA damage either directly or indirectly by interfering with the activity of the epigenetic regulatory factors and the DDR proteins. The most harmful DNA oxidation product directly induced by ROS imbalance is the 7,8-dihydro-8-oxoguanine (8-oxoG) lesion, the level of which correlates with age-related diseases such as cardiovascular diseases and neurodegeneration (Jacob et al., 2013). Silencing the OGG1 gene, which codes for the enzyme responsible for the removal of 8-oxoG, induced a massive amplification of oxidized DNA (Larsen et al., 2006), thus perpetuating chromosomal damage.

DNA repair capacity was found to decrease with aging (Imam et al., 2006); this, together with the increase in ROS production, causes an increased persistency. In many cases, the presence of unrepaired chromosomal damage does not trigger the mechanisms leading to programmed death but rather to senescence. All these processes seem to involve a high number of regulatory proteins, among which a pivotal role is played by p53 and p21, which, through the inhibition of some cyclin kinases, inhibit apoptosis and keep the cell in a replicative stasis (Regulski, 2017).

One of the characteristics of those senescent cells in which unrepaired chromosomal foci persist is the capacity to "spread" harmful information to healthy neighboring cells, increasing in that way the tissue's number of senescent cells. This phenomenon, generically called bystander effect, passes through the release by the secretory senescent cells [senescence-associated secretory phenotype (SASP)] of a complex secretome, which includes cytokines, growth factors, and microvesicles (Acosta et al., 2013; Urbanelli et al., 2016). The physiological aim of this process may be to "predispose" the tissue cells to respond to subsequent stress, avoiding the formation of neoplasia. The accumulation of senescent cells or their loss for apoptosis results in a progressive decrease of tissue functionality that is a typical feature of aging. According to this interpretation, aging would be nothing more than the consequences on cells of the signals triggered by the persistence of chromosomal damage and by the oxidative imbalance.

We will now explore how ionizing radiation in general but cosmic rays in particular may trigger this process faster by virtue of their peculiarities.

COSMIC RAYS INTERACTION WITH BIOLOGICAL MATTER

During long space missions, astronauts are subjected to a particularly harsh environment due to multiple factors as isolation, microgravity and macrogravity, and galactic cosmic rays (GCRs) and solar particle events (SPEs). While on Earth, the main component of natural radiation consists of radon, its decay products, and gamma rays from the decay of natural radioisotopes. GCRs contain a dynamic mixture of hydrogen (H) and helium (He) nuclei, and High-Atomic number and High-Energy (HZE) ions, as carbon (C), oxygen (O), neon (Ne), silicon (Si), calcium (Ca), and iron (Fe). The GCR energy spectra are very broad, ranging from 10 MeV/n to 50 GeV/n (Durante and Cucinotta, 2008). SPEs are composed of 90% protons, He ions, and a very small amount of HZE ions. About 21% of the dose equivalent to astronauts is constituted by HZE ions, 2% by Fe alone. HZE ions penetrate by many centimeters, producing along their track large ionizations' clusters, differently from the more uniform energy depositions by low LET radiation (Simonsen et al., 2020). In fact, despite their concentration of around 1%, HZE ions, due to their high penetration and strong oxidizing power, are the most effective components to threaten human health (Norbury et al., 2016). Furthermore, for their capacity of inducing metabolism perturbations not only in the directly hit cells and in the nearby bystander ones but also in their progeny, HZE ions were demonstrated to induce long-lasting effects mimicking those of aging (Li et al., 2014).

Unlike what happens for low LET radiation, the HZE ion entrance dose is relatively low, reaching its maximum at the end of their tracks (Bragg peak) (Katz and Cucinotta, 1999). The ability of HZE ions to deposit the maximum dose at precise depth is exploited for the treatments of tumors. Along their

track, HZE ions induce the generation of secondary radiations including photons, protons, neutrons, alpha particles, other heavy ions with lower velocity, and the energetic electrons d rays (Li et al., 2014). Due to their long range, the d rays may also hit cells far from the primary particle track, increasing by 30–40% the HZE ion dose (Yan et al., 2014). The concentration of radiolytic species induced in and around the particle track is very dense (Muroya et al., 2006; Cucinotta et al., 2011), causing extensive covalent modifications in targeted macromolecules. The persistence of DNA damage unrepaired, such as that induced by HZE ions, leads to the accumulation of DNA repair effectors ATM (ataxia-telangiectasia, mutated) and ATR (ATM and Rad3-related) at the sites of damage, originating DNA segments with chromatin alterations, hallmark of the cell's senescence (Rodier et al., 2009). These modifications lead to changes in signaling events that may affect proteins and genes involved in the oxidative metabolism (Li et al., 2014).

PHYSIOLOGICAL AND PATHOLOGICAL IMPACT OF COSMIC RAYS

The radiation dose absorbed by astronauts during the long-duration space travels could exceed the admissible doses (Cucinotta, 2001), raising concern about the early and late health risks. The uncertainties in estimating space radiation risks have been recognized by several reports from the National Council on Radiation Protection and Measurements (NCRP) -Report 160 (Bolus, 2013). This uncertainty is largely due to the insufficient information on the radiobiology of HZE ions that produce both quantitative and qualitative differences in biological effects compared to gamma or X-rays, but until now, no sufficient human data are available and it was difficult to exclude the impact of hereditary traits (Cucinotta et al., 2015).

The exclusion of genetic factors was achieved in a study conducted on two identical twins, one sent for a year on the International Space Station, and the other remained on Earth (Garrett-Bakelman et al., 2019). Several physiological and biological parameters were compared including body mass, bone density, telomere length, genetic damage, transcriptional and metabolic alterations, immune response, oxidative metabolism, and a battery of tests for cognitive performances. Most of these did not show any significant differences or returned to baseline values after the return, while changes in the expression of some genes, genomic instability, and a decline of cognitive efficiency persisted even 6 months from the conclusion of the mission.

Long-term decline in learning recall was observed in patients undergoing brain radiotherapy without sparing the hippocampus (Gondi et al., 2013), whereas impairment of the hippocampus-dependent learning and memory was observed in rodents exposed to low doses of HZE ions (Britten et al., 2017). Significant loss of neuronal progenitor cells occurred within a few hours after low radiation doses, and the effects extended over time because the differentiation of the surviving neuronal progenitor cells depends on the amount of the delivered dose (Volobouéva and Giffard, 2011). Smart (2017) demonstrated that the neurodegeneration is mainly due to the loss of neurons, but other factors could contribute to the onset of cognitive deficits, as well as damage to the microcirculation, decreased metabolic activity, degeneration of synapsis and myelin, and glia proliferation. Impairment of hippocampal neurogenesis and alteration of microglial functions have recently been proposed as main causes of the aging-related cognitive decline (Chowen and Garcia-Segura, 2020). Krukowski et al. (2018b) demonstrated that the development of neurodegenerative disorders in aging mice was associated with a prolonged state of sterile inflammation, characterized by increased microglial cell number and phagocytic activity. This process was initiated by the complement components C1q, C3, and CR3, which regulate the microglial–synapse interactions. A single exposure of simulated cosmic rays was proven to induce in male mice long-term cognitive and behavioral impairment with increase in microgliosis and synapse loss in the hippocampus, but, remarkably, the female cohorts did not display any cognitive or behavioral deficits and no microglia changes (Krukowski et al., 2018a). The explanation is in the embryonic development which is distinct between males and females; in fact, estrogen priming in male increases the microglia cells' reactivity, while in female, the hippocampal microglia cells are less reactive (Villa et al., 2018). A lower inflammatory response in female mice could explain the lack of hippocampal damage induced by HZE ions.

There is limited and sporadic epidemiologic data for long-term cardiovascular morbidity and mortality of United States astronauts. In a first study in astronauts who participated in the Apollo 11, 12, and 14–17 Moon missions, it was shown that heart attack was the second leading cause of death (Yan et al., 2014). Afterward, Delp et al. (2016) found that the astronauts who have traveled outside of the Earth's protective magnetosphere showed a higher mortality rate due to cardiovascular diseases compared to non-flight astronauts and those who flew only low Earth orbit missions. In the same study, in mice treated with HZE ions was observed an altered response to acetylcholine (ACh) in vasodilation of muscle feed arteries, causing dangerous pressure failure. Low and moderate doses of HZE ions may increase the risk of cardiovascular diseases by altering DNA methylation and the expression of genes related to cardiovascular function (Koturbash et al., 2016) or by inducing a pro-inflammatory state (Hughson et al., 2018).

Cancer is another main risk faced by astronauts on long-term space exploration missions. The risk estimate cannot be inferred from the knowledge we have about terrestrial radiation. HZE ions in fact affect DNA by inducing, differently from low-LET radiation, multiple damaged sites and clustered DNA damage not easily repairable with the DDR system (Asaithamby and Chen, 2011). Kennedy et al. (2018) treated immortalized human bronchial epithelial cells with high LET ^{56}Fe, ^{28}Si, and low-LET X-rays to assess the respective methylation patterns. These radiations induced DNA methylation by distinct mechanisms involving different chromatin regions. ^{56}Fe ions were found to affect the accessible chromatin region enriched of promoters and regulatory genes. These results were compared with those collected through the Genome Atlas Project, finding that the methylation of the CpG sites induced by ^{56}Fe ions showed

significant overlapping with the genes undergoing promoter hypermethylation in primary lung cancer. Thus, the DNA methylation pattern may be considered an enduring biomarker of exposure to HZE ions with the potential of long-term health impact, such as cancer.

INVOLVEMENT OF THE MITOCHONDRIA

In addition to regulating the production of ATP, mitochondria carry out multiple functions in the cell and their imbalance is associated with multisystem diseases, and many age-associated metabolic disorders are linked to defects of the mitochondrial respiratory chain (Alston et al., 2017). Notably, the brain and the heart, the organs most compromised in diseases related to mitochondrial dysregulation, are usually the most compromised also in astronauts after a long travel through space.

As reported in the previous paragraphs, the energy of high-LET radiation is deposited along a much smaller number of narrow tracks, therefore, when the HZE ions hit a mitochondrion, they are able to induce a strong oxidative imbalance, triggering persistent changes in the machines producing ROS and the activation of reactive nitrogen species (Datta et al., 2013). The first target of the radicals' increase induced by HZE ions is the mitochondrial DNA (mtDNA) because it is particularly subjected to oxidative stress and then is close to the electron transport chain where mitochondrial ROS are produced. Already Harman (1972) had suggested for mtDNA a key role in the aging process, and mutated mtDNA has been found in neurons during normal aging and in neurodegenerative diseases in the cerebral cortex (Nissanka and Moraes, 2018).

The mtDNA repair machinery is very poor compared to the nuclear one; therefore, the organelle homeostasis is maintained by means of a continuous process of fusion/fission, which, among other things, leads to the elimination of damaged elements through mitophagy. It has been observed that in cells treated with ionizing radiation, the fusion/fission cycle is compromised, and this consequently induces the maintenance of non-functional mitochondria in which both the dysregulation of energy processes and the production of radicals continue (Jin et al., 2018). Also, in xeroderma pigmentosum and the Cockayne's syndrome, mitochondria undergo an increase in mass and polarization, signs of imbalance of the remodeling process (Prates Mori and de Souza-Pinto, 2018). One hypothesis is that the malfunction of respiratory chain in radiation-damaged mitochondria together with nuclear signals not yet clarified could lead to a sharp decrease in NAD+ production, thus inhibiting mitophagy, a NAD + -dependent process (Fang et al., 2019).

Ultimately, damage to mtDNA can significantly affect cell function not only by decreasing the mitochondrial energy production but also by increasing the production of free radicals. In this way, the mitochondria continue to produce excess ROS which in turn participate in the formation of genomic damage, thus perpetuating the processes underlying senescence or alternatively of cell apoptosis. Increased oxidative stress and accelerated senescence signaling were observed in mice exposed to both γ and Fe^{56}; importantly, HZE ions were proven to be more effective in inducing oxidative damage and accelerating aging compared to γ rays (Suman et al., 2013). Furthermore, high and persistent ROS levels could influence, through the bystander effect, the behavior of the neighboring cells, spreading damage in the tissues (Li et al., 2015). The key role of oxidative stress is highlighted by recent National Aeronautics and Space Administration (NASA) translational research guidelines. One of the four areas of interest indeed concerns the effect of space flights irradiation on the generation of reactive oxygen and nitrogen species capable of impact on astronauts' health (Alwood et al., 2017).

But the increase in radicals does not seem to be the only factor capable of transferring the damage induced by HZE ions from the mitochondria to nuclear DNA. There also appears to be a two-way relationship between radiation-induced damage to nuclear DNA and mtDNA. On the one hand, it is known that the presence of persistent IR-dependent damage to nuclear DNA has an indirect effect on the mitochondrion's functioning through an unclear mechanism. On the other hand, exposure to IR induces the transfer of mtDNA to the nucleus (Singh et al., 2017). This transfer is triggered by the induction of double-strand breaks in mtDNA, the fragments are then subsequently incorporated into nuclear genomic coding regions causing the induction of oncogenes and permanent changes in gene expression (Ricchetti et al., 2004).

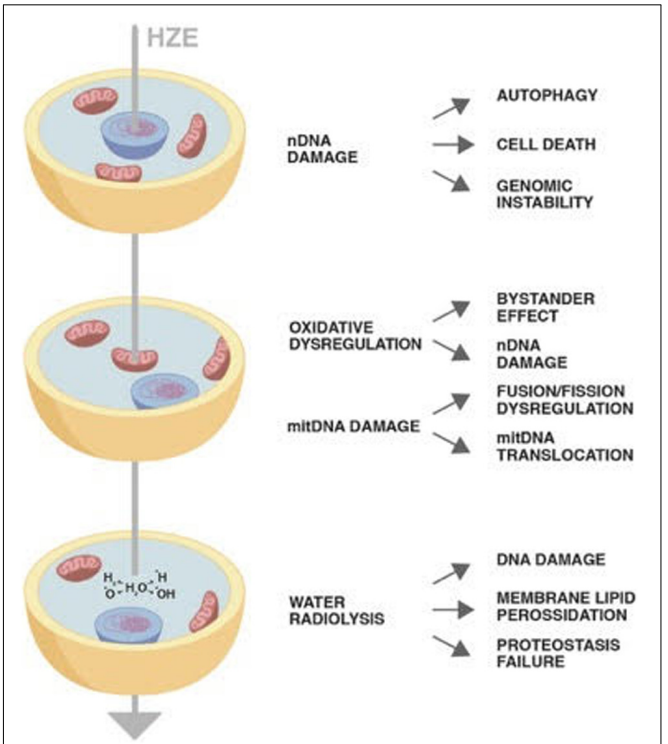

FIGURE 1 | The diagram shows the impact of a High-Atomic number and High-Energy (HZE) ion on cells. The HZE ion can hit a column of cells depositing energy at different cell compartments: nucleus, mitochondria, and cytoplasm. The type and persistence of the effects triggered by the interaction of the particle with the biological matter may vary as indicated in the text.

CONCLUSION

Despite their concentration in the cosmic rays being around 1%, HZE ions due to their high penetration and strong oxidizing power have been proven to induce permanent damages through horizontal and vertical transmission. The direct or indirect damage through radiolysis of mitochondria has as a consequence not only failure of its metabolic role but also establishment of a persistent oxidative imbalance. Once damaged, mtDNA may insert in nuclear chromosomes perpetuating genomic instability (**Figure 1**). The cells bearing DNA damage that cannot be quickly repaired with the DDR system enter in apoptosis or in the quiescent state typical of senescence. Final result is a decrease in the tissue functionality as is occurring in aging. Therefore, cosmic rays would mimic the effect of aging, inducing a persistent state of sterile inflammation damaging DNA, proteins, and lipids the consequences of which are aging-related disease such as cardiovascular diseases, neurocognitive impairment, and increase of cancer occurrence.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication. In particular AG treated the part concerning the nature and effects of cosmic rays. SR the biological processes related to senescence and aging. FT the part related to mitochondria.

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