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## **Author's manuscript to:**

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### **6 Cardioprotection and lifespan extension by the natural polyamine spermidine.**

7 Eisenberg T, Abdellatif M, Schroeder S, Primessnig U, Stekovic S, Pendl T, Harger A, Schipke J,  
8 Zimmermann A, Schmidt A, Tong M, Ruckstuhl C, Dammbroeck C, Gross AS, Herbst V, Magnes  
9 C, Trausinger G, Narath S, Meinitzer A, Hu Z, Kirsch A, Eller K, Carmona-Gutierrez D, Büttner S,  
10 Pietrocola F, Knittelfelder O, Schrepfer E, Rockenfeller P, Simonini C, Rahn A, Horsch M, Moreth K,  
11 Beckers J, Fuchs H, Gailus-Durner V, Neff F, Janik D, Rathkolb B, Rozman J, de Angelis MH,  
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14 Kroemer G, Sedej S, Madeo F.

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## Cardioprotection and lifespan extension by the natural polyamine spermidine

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## 28 **Abstract**

29 Aging is associated with an increased risk of cardiovascular disease and death. Here we  
30 show that oral supplementation of the natural polyamine spermidine extends the lifespan of  
31 mice and exerts cardioprotective effects, reducing cardiac hypertrophy and preserving  
32 diastolic function in old mice. Spermidine feeding enhanced cardiac autophagy, mitophagy  
33 and mitochondrial respiration, and it also improved the mechano-elastical properties of  
34 cardiomyocytes in vivo, coinciding with increased titin phosphorylation and suppressed  
35 subclinical inflammation. Spermidine feeding failed to provide cardioprotection in mice that  
36 lack the autophagy-related protein Atg5 in cardiomyocytes. In *Dahl* salt-sensitive rats that  
37 were fed a high-salt diet, a model for hypertension-induced congestive heart failure,  
38 spermidine feeding reduced systemic blood pressure, increased titin phosphorylation and  
39 prevented cardiac hypertrophy and a decline in diastolic function, thus delaying the  
40 progression to heart failure. In humans, high levels of dietary spermidine, as assessed from  
41 food questionnaires, correlated with reduced blood pressure and a lower incidence of  
42 cardiovascular disease. Our results suggest a new and feasible strategy for the protection  
43 from cardiovascular disease.

## 1 **Introduction**

2 Cardiovascular disease has reached epidemic proportions in the elderly and remains the  
3 worldwide leading cause of death. Human aging is typically accompanied by cardiac  
4 hypertrophic remodeling and a progressive decline of left ventricular (LV) diastolic function<sup>1,2</sup>.  
5 Abnormal diastolic function is present in >20% of the population >65 years of age<sup>3</sup>. Although  
6 less than half of all patients with diastolic dysfunction show clinical signs of congestive heart  
7 failure, even patients not meeting the diagnostic criteria are at increased risk to develop heart  
8 failure<sup>4</sup>. No treatment has yet been shown to convincingly target and prevent age-associated  
9 diastolic dysfunction or heart failure, likely because our understanding of the fundamental  
10 mechanisms underlying progressive deteriorations in the (ultra-)structure and function of the  
11 aging heart is incomplete.

12 Recent studies have revealed that autophagy, a major cellular quality control  
13 mechanism, may be able to minimize the functional decline of aging cardiomyocytes by  
14 degrading and recycling long-lived proteins, which are potentially toxic if damaged, as well as  
15 cytoplasmic components and dysfunctional organelles (in particular, damaged  
16 mitochondria)<sup>5,6</sup>. Clearance of dysfunctional mitochondria through a specific type of selective  
17 autophagy, termed mitophagy, may be beneficial for cardiac function, because mitochondria  
18 can overproduce reactive oxygen species if they are functionally impaired and ignite lethal  
19 signalling pathways if they are permeabilized. In view of the established longevity-extending  
20 effects of enhanced cytoprotective autophagy in model organisms, it seems plausible that  
21 autophagy might also be able to counteract cardiac aging<sup>7</sup>. We previously discovered that  
22 the natural polyamine spermidine, a dietary compound, extends lifespan and health span  
23 through induction of autophagy in yeast, flies and worms<sup>8,9</sup>. Dietary supplementation of  
24 spermidine delayed age-associated memory impairment in flies<sup>10</sup>, prevented motor  
25 impairment in flies elicited by transgenic expression of human  $\alpha$ -synuclein<sup>11</sup>, and protected  
26 mice from TDP-43-associated proteinopathies<sup>12</sup>, in line with a general neuroprotective action  
27 of this polyamine. In several model organisms, the lifespan extending and neuroprotective  
28 effects of spermidine were abolished upon inactivation of essential autophagy-related  
29 genes<sup>8,10</sup>. Here, we explored the potential cardioprotective effects of spermidine in rodent  
30 models of physiological cardiac aging (mice) and high salt-induced congestive heart failure  
31 (rats). We also provide evidence that dietary spermidine intake in humans inversely  
32 correlates with cardiovascular disease.

33

34

## 1 **Results**

### 2 **Spermidine extends the lifespan of wild-type C57BL/6 mice**

3 In view of the life prolonging effects of spermidine in model organisms<sup>8,9</sup>, we tested the long-  
4 term survival effects of specific polyamines in C57BL/6J wild-type female mice, which had a  
5 *life-long* (Fig. 1a) access to drinking water supplemented with distinct polyamines. Strikingly,  
6 spermidine- or spermine-supplemented mice had a significantly extended median lifespan as  
7 compared to control (receiving normal drinking water) or putrescine-supplemented mice (Fig.  
8 1b, c and Supplementary Tables 1 and 2). To enhance the translational potential of these  
9 findings, we administered spermidine *late-in-life*, (a regimen more applicable to humans) to  
10 pre-aged male and female mice (Fig. 1a). Again, we found that spermidine feeding  
11 significantly prolonged median lifespan by ~10% (Fig. 1d and Supplementary Fig. 1).  
12 Spermidine-fed animals displayed increased circulating spermidine levels, confirming its  
13 systemic bioavailability (Fig. 1e). Food and water consumption, body weight and lean/fat  
14 mass composition were similar in spermidine-fed and control groups (Supplementary Fig. 2),  
15 excluding the possibility that polyamine supplementation extends lifespan by inducing a  
16 calorically-restricted state<sup>13</sup>.

17

### 18 **Dietary spermidine delays cardiac aging by improving diastolic function**

19 Tumor burden and cardiac aging are significant predictors of mortality in C57BL/6 mice and  
20 humans<sup>14,15</sup>. Comprehensive pathological characterization of tissues collected from mice at  
21 an advanced age (28 months), as well as from old mice that became moribund and were  
22 sacrificed as “end-of-life” animals<sup>16</sup>, revealed similarly high tumor frequencies in spermidine-  
23 treated and control mice (Supplementary Fig. 3 and Supplementary Tables 3, 4). This finding  
24 suggests that the potential ability of spermidine to inhibit tumor formation, which has been  
25 observed after chemo-induction of tumors<sup>17</sup>, does not explain its life prolonging effects.

26 Since only minor histopathological abnormalities were observed in cardiac tissue  
27 obtained from 28-month-old or from “end-of-life” animals (Supplementary Tables 3, 4), we  
28 next subjected aged mice with *late-in-life* spermidine supplementation to structural and  
29 functional cardiac phenotyping. Spermidine reversed age-associated (23 months)  
30 echocardiography-detectable hypertrophy, as indicated by a reduction in tibia length-  
31 normalized left ventricular mass (LV mass/TL) and posterior wall thickness (PW/TL) to values  
32 below those observed in middle-aged (18 months) wild-type mice (Fig. 1f, Supplementary  
33 Table 5). Hypertrophic remodeling is the most common age-related myocardial abnormality  
34 that is associated with diastolic and/or systolic dysfunction, eventually leading to heart failure

1 in humans<sup>18</sup>. Evaluation of cardiac function by invasive hemodynamic pressure-volume  
2 measurements revealed that compared with age-matched control mice, mice fed spermidine  
3 *late-in-life* had significantly enhanced diastolic properties, as reflected by a reduction of LV  
4 end-diastolic pressure (EDP; Fig. 1g, h) with a trend towards improved active relaxation  
5 (shortened time constant of LV pressure decay  $\tau$ ; Supplementary Table 6), as well as  
6 significantly reduced LV passive stiffness, as reflected by decreased myocardial stiffness  
7 constant  $\beta$  (Fig. 1i), with a downward shift of the end-diastolic pressure-volume relationship  
8 [EDPVR] obtained by transient *vena cava* occlusion for load-independent cardiac function  
9 assessment (Supplementary Fig. 4). The systolic properties of aged hearts were less  
10 affected by spermidine. Load-dependent parameters, such as ejection fraction (EF) and  
11  $dP/dt_{max}$  as indicators of LV contractility, were comparable in all tested groups (Fig. 1j,  
12 Supplementary Tables 5, 6). However, ventricular-vascular coupling (VVC), a parameter that  
13 describes the interaction of the LV with the arterial system, is positively correlated with  
14 cardiovascular performance and is associated with prognosis in heart failure patients<sup>19</sup>, was  
15 increased in mice fed spermidine *late-in-life* and was similar to the value observed in young  
16 mice (Fig. 1k). Notably, spermidine did not affect systemic systolic and diastolic blood  
17 pressure (Fig. 1l), indicating that reduced hypertrophic remodeling, improved VVC and  
18 enhanced cardiac function were independent of arterial afterload. Moreover, 24-month-old  
19 control mice displayed a moderate but significant increase in relative lung weight (LW/TL), a  
20 sign of pulmonary congestion that results from abnormal diastolic function, as compared to  
21 that in young animals (Supplementary Table 7). This age-dependent increase in relative lung  
22 weight was less pronounced and was non-significant in spermidine-treated animals  
23 (Supplementary Table 7). Despite the evidence for pulmonary congestion, a typical  
24 complication in heart failure, in physiologically aged C57BL/6 mice, these mice are not  
25 considered to represent an experimental model of heart failure<sup>20</sup>. Old C57BL/6 mice exhibit  
26 diastolic dysfunction with an increased risk for the development of heart failure, thus closely  
27 recapitulating human cardiac aging in the absence of hypertension and associated co-  
28 morbidities<sup>20</sup>.

29

### 30 **Cardiomyocyte composition and function are improved by spermidine**

31 In the elderly patient, cardiac hypertrophy is related to structural and functional remodeling  
32 that may involve (i) changes in the composition and structure of the extracellular matrix,  
33 mainly characterized by fibrotic (collagen-rich) tissue; (ii) altered coronary microvascular  
34 rarefaction; and/or (iii) effects on cardiomyocytes themselves<sup>21</sup>. To test if spermidine  
35 reverses age-induced cardiac fibrosis and decreased coronary microvascular density, we  
36 subjected the hearts of aged mice fed spermidine *late-in-life* to ultrastructural analysis by

1 design-based stereology. Electron microscopy did not reveal changes in the volume fraction  
2 or absolute volume of collagen, interstitium, capillaries or cardiomyocytes in the LV  
3 (Supplementary Fig. 5a and Supplementary Table 8). However, age-related effects on  
4 subcellular cardiomyocyte composition were reversed by spermidine, as reflected by  
5 increased relative mitochondrial and myofibrillar volumes and a reduced (mitochondria- and  
6 myofibril-free) sarcoplasmic volume (Fig. 2a, b, Supplementary Fig. 5b and Supplementary  
7 Table 8). These results suggest that spermidine has cardiomyocyte-intrinsic effect. We  
8 hypothesized that the increased myocardial compliance (i.e. myocardial elasticity) induced by  
9 spermidine originates from improved contractile apparatus and cardiomyocyte function<sup>22</sup>.  
10 Consistent with this idea, both transcriptome and proteome analyses of cardiac tissue  
11 extracts (Supplementary Fig. 6 and Supplementary Tables 9, 10) revealed a rejuvenated  
12 molecular phenotype with respect to components of the cytoskeletal apparatus (i.e. myosin  
13 heavy chain proteins, ankyrins, integrins, dystonin), inflammatory processes and  
14 mitochondrial respiratory chain complex I proteins (i.e. members of the Nduf protein family),  
15 all of which are essential for cardiomyocyte mechano-elasticity<sup>23</sup> and healthy  
16 cardiac aging<sup>24,25</sup>. Accordingly, the respiratory competence of cardiac mitochondria through  
17 respiratory chain complex I was increased in mice supplemented with spermidine as  
18 compared to control mice (Fig. 2c, Supplementary Fig. 7a, b); thus, spermidine reversed an  
19 age-induced decline in mitochondrial respiratory function<sup>26</sup>. Furthermore, spermidine  
20 reversed the age-associated decline of the mitochondria-related metabolite levels, including  
21 that of NADPH and mevalonate (Supplementary Fig. 7c-e), of which the latter has been  
22 linked to mitochondrial surveillance<sup>27</sup> and cardiac health<sup>28</sup>. Moreover, determination of the  
23 (chronic) low-grade inflammatory status of aged mice (*see Methods*) revealed that  
24 spermidine reduced the age-dependent rise in plasma levels of the pro-inflammatory  
25 cytokine tumor necrosis factor- $\alpha$  (TNF $\alpha$ ) (Fig. 2d, Supplementary Fig. 8). The passive  
26 stiffness of cardiomyocytes is determined primarily by titin-related mechanisms<sup>22</sup>, which are  
27 negatively affected by inflammatory conditions, in part mediated by TNF $\alpha$ <sup>29</sup>. Cardiomyocytes  
28 co-express a larger (more compliant) and a smaller (stiffer) isoform of titin, termed N2BA and  
29 N2B, respectively. While the isoform composition of titin, as assessed by the  
30 N2BA/[N2B+N2BA] ratio, was unchanged (Fig. 2e, f), spermidine enhanced the levels of both  
31 total and serine 4080 phosphorylation of the N2B isoform (Fig. 2e, g, Supplementary Fig. 5c).  
32 Phosphorylation of N2B on serine 4080 is known to reduce cardiomyocyte stiffness via  
33 cGMP/PKG-dependent signalling<sup>22</sup>.

34



## 1 **Spermidine enhances cardiomyocyte autophagic flux in both young and aged mice**

2 We previously identified spermidine as a potent inducer of autophagy<sup>8,9</sup>, a cellular process  
3 crucial for general proteostasis as well as mitochondrial and cardiomyocyte function<sup>5</sup>.  
4 Therefore, we next tested whether spermidine supplementation improves autophagic flux in  
5 aging cardiomyocytes. To assess basal autophagic flux, we treated *ad libitum*-fed 13-month-  
6 old C57BL/6J wild-type mice supplemented with spermidine for the final four weeks with the  
7 vacuolar protease inhibitor leupeptin, which blocks autophagosome turnover, and quantified  
8 levels of the autophagosomal marker LC3-II<sup>30</sup>. Treatment with leupeptin induced a significant  
9 increase of LC3-II levels in hearts from spermidine-supplemented mice, whereas age-  
10 matched controls showed a reduced (and non-significant) elevation of this marker (Fig. 3a,  
11 Supplementary Fig. 9e), indicating that spermidine increases cardiac autophagic flux *in vivo*.  
12 Cellular spermidine content in cardiac tissue was significantly increased in spermidine-  
13 supplemented animals as compared to controls (Fig. 3b).

14 The capacity of orally supplemented spermidine to induce autophagic flux *in vivo* in  
15 cardiomyocytes was corroborated by using transgenic cardiomyocyte-specific tandem-  
16 fluorescent mRFP-GFP-LC3 mice<sup>31</sup>. These mice serve as an autophagy reporter strain,  
17 carrying labeled autophagosomes; both red (mRFP) and green (GFP) fluorescence, as well  
18 as labeled autolysosomes; red (mRFP) fluorescence only. In this experiment, chloroquine  
19 was used to block autophagosome turnover for assessment of autophagic flux. Spermidine  
20 substantially increased the number of autophagosomes and autolysosomes under both  
21 vehicle- and chloroquine-treated conditions (Fig. 3c, d). Moreover, spermidine stimulated  
22 mitophagy in cardiomyocytes of both young and aged mice, as assessed in mice expressing  
23 the mitochondrial-targeted form of the fluorescent biosensor Keima (Mito-Keima). Mito-Keima  
24 fluorescence shows pH-dependent excitation characteristics, shifting excitation maxima to a  
25 higher wavelength after mitochondria come into contact with the acidic milieu of lysosomes in  
26 the context of mitophagy<sup>32</sup>. Thus, the ratio of 561 nm to 457 nm excited Keima fluorescence  
27 (referred to as Mito-Keima positive area) increases with a drop in pH (Online Methods).  
28 Spermidine treatment clearly increased the Mito-Keima-positive area in cardiomyocytes—  
29 indicative of increased mitophagy (Fig. 3e and Supplementary Fig. 9a-d). Together, these  
30 results suggest that autophagy may contribute to the improved cardiomyocyte structure and  
31 function induced by spermidine.

32

## 33 **Autophagy is required for spermidine-mediated cardioprotection**

34 To determine whether the *in vivo* cardioprotective effects of spermidine depend on  
35 autophagy, we took advantage of mice that have a cardiomyocyte-specific autophagy defect,

1 *Atg5<sup>fl/fl</sup>-MLC2a-Cre<sup>+</sup>* mice (*Atg5<sup>-/-</sup>*)<sup>5,6</sup>. We first verified that cardiomyocytes in these mice lack  
2 LC3-II and show increased levels of p62/SQSTM1, a direct target/substrate and cargo-  
3 receptor of autophagy known to increase in autophagy-deficient cardiomyocytes  
4 (Supplementary Fig. 10a-c). As these mice develop severe systolic impairment and heart  
5 failure early in life and do not reach the same age as wild-type animals<sup>5,6</sup>, we assessed  
6 cardiac function at 16 weeks of age, when *Atg5<sup>-/-</sup>* animals showed no echocardiography-  
7 detectable cardiac abnormalities under control conditions (Fig. 3f, g and Supplementary  
8 Table 11) and had systolic and diastolic properties that were comparable to those of *Atg5<sup>fl/fl</sup>-*  
9 *MLC2a-Cre<sup>-</sup>* control mice (*Atg5<sup>+/+</sup>*), as evaluated by invasive hemodynamics (Fig. 3h-k;  
10 Supplementary Fig. 10d, e and Supplementary Table 11-13). Strikingly, the spermidine-  
11 induced reduction of LV hypertrophy (i.e. reduction of LVmass/TL and PW/TL) observed in  
12 *Atg5<sup>+/+</sup>* mice was not detected in *Atg5<sup>-/-</sup>* mice, in which spermidine actually aggravated LV  
13 hypertrophy (Fig. 3f and Supplementary Table 11). This increased LV hypertrophy in  
14 spermidine-treated *Atg5*-deficient mice was associated with reduced diastolic function, as  
15 documented by a significantly elevated EDPVR  $\beta$ , indicative of increased LV passive  
16 stiffness (Fig. 3i; Supplementary Fig. 10d and Supplementary Table 12). Notably, significant  
17 increases in LV contractility, as indicated by a higher end-systolic elastance (Ees, the slope  
18 of end-systolic pressure–volume relationship (ESPVR)) and VVC were observed in  
19 spermidine-treated *Atg5<sup>+/+</sup>* but not *Atg5<sup>-/-</sup>* mice (Fig. 3j, k, Supplementary Fig. 10e and  
20 Supplementary Table 12). Hence, spermidine-treated *Atg5<sup>-/-</sup>* mice showed impaired systolic  
21 function, as indicated by a reduced ejection fraction (Fig. 3g). Collectively, these data  
22 indicate that spermidine prevents typical age-related cardiac deterioration in an autophagy-  
23 dependent manner, reducing LV hypertrophic remodeling and improving diastolic function,  
24 contractility and ventricular-vascular coupling.

## 25 **Spermidine reduces blood pressure and delays progression to heart failure in *Dahl*** 26 **rats**

27 From a clinical perspective, hypertension represents one of the most important risk factors  
28 for the development of heart failure<sup>33</sup> and occurs in the majority of elderly patients suffering  
29 from cardiovascular disease<sup>33</sup>. Because hypertension and a manifest heart failure phenotype  
30 are absent in physiologically aging wild-type mice<sup>14</sup>, we employed *Dahl* salt-sensitive rats fed  
31 a high-salt diet, which constitute a clinically relevant animal model of hypertension-induced  
32 hypertrophy, diastolic dysfunction and heart failure<sup>34</sup>. These rats also exhibit phenotypic traits  
33 observed in hypertension-associated diseases in humans, including comorbidities such as  
34 renal dysfunction<sup>35</sup>. *Dahl* salt-sensitive rats fed a high-salt diet had progressively increased  
35 mean arterial blood pressure, an effect that was delayed by 4 weeks when spermidine was  
36 co-administered with high-salt (Fig. 4a, b and Supplementary Fig. 11). Spermidine

1 supplementation increased the plasma levels of spermidine in *Dahl* rats and led to  
2 significantly decreased plasma levels of ornithine, the substrate for the rate-limiting enzyme  
3 in polyamine biosynthesis, compared to control animals (Fig. 4c). This effect on ornithine  
4 levels may connect polyamine metabolism to the bioavailability of arginine (Supplementary  
5 Fig. 12a), the only source for the generation of the vasodilator nitric oxide (NO)<sup>36</sup>, which has  
6 been shown to abrogate salt-sensitive hypertension in *Dahl* salt-sensitive rats<sup>37</sup>. Therefore,  
7 the anti-hypertensive effect of spermidine might be explained by effects on arginine  
8 metabolism. Indeed, spermidine increased arginine bioavailability, as determined by an  
9 elevated global arginine bioavailability ratio (GABR, defined as arginine/[ornithine+citrulline])  
10 (Fig. 4c), and increased the arginine/ornithine ratio, while also decreasing the cumulative  
11 level of ornithine and citrulline (Supplementary Fig. 12c, d). These findings suggest the ability  
12 of spermidine to improve NO production/bioavailability. Elevation of the GABR and the  
13 arginine/ornithine ratio, as well as decreased levels of ornithine plus citrulline (indicative of  
14 diminished arginine catabolism) have been associated with reduced cardiovascular risk<sup>38,39</sup>.

15 To explore whether spermidine attenuates hypertension-induced hypertrophic remodeling  
16 and the progression to heart failure in this model, we assessed cardiac dimensions and  
17 function. Spermidine treatment reduced tibia length-normalized LV mass, posterior wall  
18 thickness and heart weight, indicating that it attenuated the increase in cardiac hypertrophy  
19 observed in controls (Fig. 4d and Supplementary Table 14, 15). Furthermore, spermidine  
20 enhanced diastolic function, as reflected by a reduction in the E/E' ratio, a parameter that  
21 strongly correlates with mean LV filling pressure<sup>40</sup> (Fig. 4e). Indeed, LV-EDP was reduced  
22 (Fig. 4f, g) along with a reduction in LV stiffness, as reflected by a decreased myocardial  
23 stiffness constant for indexed volumes  $\beta_i$  (Fig. 4h, Supplementary Table 16) with a downward  
24 shift of the EDPVR (Supplementary Fig. 13) as well as an increase in the levels of total and  
25 S4080 phosphorylation of the N2B titin isoform (Supplementary Fig. 14a, b). Comparable to  
26 our findings in aging mice, enhanced diastolic function in rats was accompanied by a  
27 significant reduction of circulating TNF $\alpha$  levels (Supplementary Fig. 14c), a pro-inflammatory  
28 marker with increased levels in heart failure patients<sup>41</sup>.

29 In control animals fed a high-salt diet, relative lung and liver weights (normalized to  
30 tibia length) increased from 7 weeks of age to 14 or 19 weeks of age (Fig. 4i). Spermidine  
31 treatment significantly delayed the increases in relative lung and liver weights (Fig. 4i and  
32 Supplementary Table 15), suggesting that spermidine reduces pulmonary and systemic fluid  
33 accumulation, respectively, which are characteristic of heart failure. Ejection fraction was  
34 preserved (>70%) in all groups (Fig. 4j), implying that spermidine delays the progression  
35 from hypertension-induced hypertrophy to a phenotype that resembles heart failure with  
36 preserved ejection fraction (HFpEF). Control animals fed a high-salt diet showed higher

1 arterial elastance (i.e. arterial stiffness) for indexed volumes ( $E_{a_i}$ ), a surrogate of arterial  
2 load<sup>42</sup>, at 14 or 19 weeks of age, compared to 7 weeks of age. These animals appeared to  
3 compensate for this increased arterial elastance by increasing LV contractility, as indicated  
4 by an increase in end-systolic elastance for indexed volumes ( $E_{e_s}$ ; Supplementary Fig. 13b  
5 and Supplementary Table 16), leading to comparable VVC values in the control groups of  
6 different ages (Fig. 4k and Supplementary Table 16). Notably, spermidine administration  
7 decreased arterial stiffness (Supplementary Table 16), resulting in a significantly improved  
8 VVC (Fig. 4k and Supplementary Table 16), similar to the effects we observed in both young  
9 and old *Atg5*-competent mice treated with spermidine.

10 Renal abnormalities are commonly observed in chronic arterial hypertension<sup>43</sup> and  
11 contribute to the pathogenesis of heart failure in humans<sup>44</sup> as well as in *Dahl* rats<sup>45</sup>, which  
12 have impaired renal salt metabolism leading to water retention and, thus, systemic volume-  
13 overload. Spermidine treatment of high-salt fed *Dahl* rats delayed the appearance of several  
14 signs of hypertensive renal injury, namely arterial hyalinosis with fibrosis, glomerulosclerosis  
15 and thrombotic microangiopathy (Fig. 4l and Supplementary Fig. 15a-c). Measurement of  
16 urinary lipocalin-2 (Lcn-2) levels, a sensitive marker of acute renal damage<sup>46</sup>, corroborated  
17 the protective action of spermidine on renal function (Fig. 4m). Induction of autophagy by  
18 spermidine<sup>8,9</sup> may contribute to renal tissue homeostasis and contribute to the anti-  
19 hypertensive effects of spermidine supplementation. Compared to control animals,  
20 spermidine-supplemented animals showed a significant increase in renal spermidine content  
21 (Supplementary Fig. 15d) and a significant decrease in the levels of SQSTM1/p62, a specific  
22 autophagy substrate whose levels decrease when autophagic flux is enhanced  
23 (Supplementary Fig. 15e). These findings suggest that autophagic processes might play a  
24 role in spermidine-induced kidney protection.

25

## 26 **Dietary spermidine inversely correlates with cardiovascular disease in humans**

27 Finally, we evaluated the association of dietary spermidine intake with cardiovascular  
28 diseases (including heart failure) and blood pressure in human subjects. In a prospective,  
29 population-based cohort (Bruneck Study<sup>47</sup>), dietary intake of spermidine (as assessed by  
30 food questionnaires) was inversely associated with the risk of both fatal heart failure (a ~40%  
31 reduction in risk in the high compared to low spermidine intake groups) and clinically overt  
32 heart failure; both risks were more pronounced in men (Fig. 5a, b). Intake of spermidine was  
33 also inversely related to the risk of other cardiovascular diseases, as assessed by a  
34 composite of acute coronary artery disease, stroke and death due to vascular disease (Fig.  
35 5c), and to systolic and diastolic blood pressures (Fig. 5d), which were significantly lower in

1 the high compared to low spermidine intake groups. High intake of spermine or of spermine  
2 and spermidine combined showed similar associations as high intake of spermidine  
3 (Supplementary Fig. 16). In contrast, putrescine intake did not show these associations (Fig.  
4 5a-c) and tended to be associated with an increase in blood pressure (Fig. 5d). Notably,  
5 spermidine intake showed a significant inverse association with plasma levels of soluble N-  
6 terminal pro-B type natriuretic peptide (NT-proBNP), the key clinically-used biomarker for  
7 heart failure ( $r = -0.115$ ,  $p=0.001$ ). Moreover, in an exploratory approach, we tested whether  
8 spermidine intake correlated with the levels of 131 plasma proteins (data not shown). This  
9 analysis revealed strong inverse associations for proteins implicated in cardiac disease,  
10 including chitinase-3-like protein 1 (CHI3L1), which is implicated in plaque inflammation,  
11 matrix degeneration, and plaque rupture ( $r=-0.19$ ,  $P=1.2\times 10^{-6}$ , FDR  $q=2.7\times 10^{-4}$ ); and  
12 growth/differentiation factor 15 (GDF-15), which is implicated in heart failure, atrial fibrillation,  
13 chronic kidney disease, and possibly vascular calcification ( $r=-0.13$ ,  $P=1.0\times 10^{-3}$ , FDR  
14  $q=4.7\times 10^{-2}$ ).

15

## 16 **Discussion**

17 This study reveals that spermidine treatment in mice ameliorates hypertrophic remodeling of  
18 the aged heart, blocks age-related changes in cardiomyocyte composition and functionality,  
19 enhances diastolic function independently of effects on systemic blood pressure and extends  
20 lifespan. It thus appears plausible that lifespan prolongation by spermidine is due to  
21 suppression of death from cardiac-related causes; however, to what degree the effects of  
22 spermidine on the heart account for its lifespan prolonging effects is a highly challenging  
23 question and remains to be investigated in a suitable experimental setting. Other protective  
24 effects of spermidine (including anti-tumorigenic effects<sup>17</sup>) may also contribute to its lifespan-  
25 extending effects, although we did not detect a reduced cancer incidence in aged  
26 spermidine-treated C57BL/6 mice. Notably, unlike other longevity-promoting agents<sup>48,49</sup>,  
27 spermidine had no detectable effects on glucose and insulin metabolism (Supplementary Fig.  
28 17). Our data extend previous findings on the ability of spermidine to reduce arterial stiffness  
29 in aged mice<sup>50</sup>.

30 The cardioprotective effects of spermidine may be due to several underlying  
31 mechanisms, including both direct cardiac effects as well as extracardiac (systemic and  
32 renal) effects (Fig. 6). Systemic effects by spermidine may involve anti-inflammatory  
33 processes as well as blood pressure lowering effect (in the setting of salt-induced  
34 hypertension). Both chronic low-grade inflammation and hypertension reportedly cause  
35 mechano-elastic impairments and mitochondrial dysfunction of cardiomyocytes<sup>29</sup>. Oral  
36 supplementation of spermidine promotes basal autophagic flux in cardiac tissue, and the

1 direct protective effects of spermidine on the heart appear to require cardiomyocyte  
2 autophagy. Re-activation of basal autophagy by spermidine has recently been reported to  
3 maintain the regenerative function of skeletal muscle stem cells in aging mice<sup>51</sup>. It remains to  
4 be tested whether spermidine has a similar effect on other adult stem cells, including the  
5 putative cardiac ones that have been described to produce new and functional  
6 cardiomyocytes. Spermidine may otherwise facilitate rejuvenation of aged cardiomyocytes  
7 through increasing their mitochondria and myofilament content (as we observed in this  
8 study).

9         Additionally, we found that spermidine reduces salt-induced hypertension, left  
10 ventricular hypertrophy and delays the progression to heart failure in *Dahl* salt-sensitive rats.  
11 Comparable with our findings in aged mice, spermidine-fed *Dahl* rats showed increased  
12 ventricular-vascular coupling (denoting improved cardiovascular efficiency) and enhanced  
13 titin phosphorylation. A mechanistic link between spermidine and titin phosphorylation is  
14 suggested by the increased global arginine bioavailability ratio in spermidine-treated *Dahl*  
15 salt-sensitive rats. As arginine is the precursor to NO, which activates soluble guanylyl  
16 cyclase, increased arginine bioavailability should lead to increased activity of PKG, one of the  
17 main kinases that phosphorylates titin. PKG phosphorylates titin in a cardiac-specific domain  
18 (N2-Bus), and we found that spermidine treatment led to increased phosphorylation of N2-  
19 Bus both globally and at the PKG-specific residue (S4080). The functional consequence of  
20 titin phosphorylation is a reduction in titin-based myocardial passive stiffness<sup>22</sup>, possibly  
21 explaining how spermidine preserves the diastolic properties of the heart, which are  
22 disturbed in heart failure. In addition, the reduction of subclinical levels of circulating TNF $\alpha$   
23 may contribute to spermidine-induced titin-phosphorylation by further increasing NO  
24 bioavailability through the reduction of oxidative stress<sup>29</sup> (Fig. 6). It remains to be elucidated  
25 whether a crosstalk between autophagy and titin phosphorylation exists.

26         In line with our experimental findings, epidemiological analyses corroborate the novel  
27 concept that spermidine-rich diets are preventive against cardiovascular disease and reduce  
28 the risk of cardiac death in humans. Interventional studies are warranted to test the  
29 therapeutic potential of dietary spermidine. It has to be acknowledged that estimation of  
30 dietary spermidine intake was based on food frequency questionnaires, which is the standard  
31 method in nutritional epidemiology, yet an indirect way of quantification that does not  
32 consider differences in food processing and preparation.

33         In summary, spermidine intake reduces cardiovascular pathologies, including  
34 hypertension and cardiac dysfunction associated with heart failure. In the aging population,  
35 the incidence and prevalence of heart failure are increasing in association with comorbidities  
36 such as obesity, diabetes and renal abnormalities. Our study paves the way for prospective

1 clinical trials to evaluate the potential cardiovascular- and other health-promoting effects of  
2 spermidine-enriched diets.

3

#### 4 **Accession Codes**

5 Array data are available via Gene Expression Omnibus (GEO) database with identifier  
6 GSE86882. Proteome data are available via ProteomeXchange with identifier PXD004916.

7

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## 1 **Author Contributions**

2 T.E., S. Sedej, G.K. and F.M. designed and supervised the study; T.E., M.A., G.K., S. Sedej, and F.M.  
3 wrote the manuscript. T.E., M.A., S. Schroeder, U.P., S. Stekovic, T. Pendl, A.H., J. Schipke, A.Z.,  
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5 P.R., C.S., A.R., M.H., F.N., D.J., B.R., J.R, T.M., M.M., P.W., M.v.F.-S., R.P. and S.Sedej performed  
6 experiments, analyzed and discussed data. K.E., K.M., J.B., H.F., V.G.-D., M.H.d.A., G.H., B.P., L.S.,  
7 T. Pieber, J.W., S.J.S., W.A.L., C. Mühlfeld, J. Sadoshima., J.D., and S.K. discussed & analyzed data  
8 and gave conceptual advice.

9

## 10 **Competing Financial Interests**

11 F.M., T.E., D.C-G., S.J.S. and S. Stekovic. have equity interests in TLL, a company founded in 2016  
12 that will develop natural food extracts.

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- 27

1 **Figure Legends**

2

3 **Figure 1. Spermidine extends lifespan and improves cardiac diastolic function in mice.**

4 **(a)** Schematic overview of spermidine administration to aging wild-type C57BL/6J mice.  
5 Spermidine was supplemented to drinking water starting from the age of 4 months (*life-long*)  
6 or 18 months (*late-in-life*) and cardiovascular parameters (Fig. 1f-l) or molecular phenotypes  
7 (Figs. 2 and 3) were analyzed at the indicated time points (M, months).

8 **(b-d)** Kaplan-Meier survival analyses. In the *life-long* supplementation experiment using  
9 C57BL/6J female mice (**b**, **c**), the same control group was used. The *late-in-life*  
10 supplementation experiment (**d**) used C57BL/6J male and female mice. Dashed lines depict  
11 median lifespans. N=40/41 (**b**, control/spermidine), N=40/20/17 (**c**,  
12 control/putrescine/spermine), N=91/86 (**d**, control/spermidine) mice (see Supplementary  
13 Tables 1 and 2 for more details). P-values, calculated using Breslow test, represent pairwise  
14 comparisons of survival curves between the groups *Spermidine vs. Control* (**b**), *Spermine vs.*  
15 *Control* (**c**), and *Spermidine vs. Control* (**d**).

16 **(e-k)** Effects of *late-in-life* supplementation of spermidine in C57BL6/J male mice, analyzed  
17 at the indicated ages (M, months). Shown are whole blood polyamine levels (**e**), left  
18 ventricular mass-to-tibia length ratio (LVmass/TL), indicative of cardiac hypertrophy (**f**),  
19 representative hemodynamic pressure-volume loops (**g**), left ventricular end-diastolic  
20 pressure (EDP) (**h**), myocardial stiffness constant (end-diastolic pressure-volume relationship  
21 [EDPVR]  $\beta$  obtained from exponential fits presented in Supplementary Fig. 4a) (**i**), ejection  
22 fraction, as determined by echocardiography (**j**) and ventricular-vascular coupling (VVC) (**k**).  
23 N=15/18 (**e**, 24M/24M+S), N=10/14/20/20 (**f**, **j**, 4M/18M/23M/23M+S), N=10/8/10/10 (**h**, **i**, **k**,  
24 4M/18M/24M/24M+S) mice.

25 **(l)** Systolic and diastolic arterial blood pressures in mice analyzed in (**e**). N=8/11 (**l**,  
26 24M/24M+S) mice.

27 \*\*\* $p < 0.001$ , \*\* $p < 0.01$ , \* $p < 0.05$  and # $p < 0.06$  (ANOVA with post-hoc Tukey, ANCOVA (panel i)  
28 or Welch's *t*-test (panel e), see Methods). For box-and-whisker plots, whiskers show minima  
29 and maxima within 1.5 interquartile range.

30

1 **Figure 2. Spermidine improves cardiomyocyte composition and mitochondrial**  
2 **function in mice.**

3 C57BL/6J male mice were supplemented with spermidine (+S) *late-in-life* (see Fig. 1a for the  
4 feeding scheme) and hearts were subjected to molecular and biochemical analyses at the  
5 indicated ages (M, months).

6 **(a, b)** Representative transmission electron micrographs **(a)** and quantification of left  
7 ventricular cardiomyocyte composition using design-based stereology **(b)**. Relative volumes  
8 of mitochondria  $Vv(mi/myo)$ , myofibrils  $Vv(mf/myo)$  and mitochondria- and myofibril-free  
9 sarcoplasm  $Vv(sp/myo)$  per cardiomyocyte are shown. *mi*, mitochondria; *mf*, myofibrils; *myo*,  
10 cardiomyocyte; *nu*, nucleus; *sp*, sarcoplasm. Scale bar represents 2  $\mu\text{m}$ . N=10/15/14  
11 (4M/24M/24M+S) mice.

12 **(c)** Oxygen consumption (complex I-mediated respiration) of isolated cardiac mitochondria  
13 using high-resolution respirometry. N=8 (left), N=5 (right) mice/group.

14 **(d)** Plasma TNF $\alpha$  levels after *late-in-life* spermidine supplementation of C57BL6/J male mice.  
15 Mice that had a possibly acute inflammatory condition were excluded (see Supplementary  
16 Fig. 8 and Methods). N=12/12/13/9/10 (5M/21M/21M+S/23M/23M+S) mice.

17 **(e)** Titin isoform composition and phosphorylation. Representative *Western Blots* probed with  
18 a pan-phospho-serine/threonine-antibody for detection of total N2B phosphorylation (P-N2B,  
19 quantified in g), Coomassie-stained *PVDF* membrane for detection of total N2B (*Total-N2B*),  
20 and a *Coomassie Blue*-stained gel for detection of N2BA and N2B isoforms quantified in f  
21 (see Methods for details on isoform identification).

22 **(f)** The N2BA/(N2BA+N2B) ratio was quantified by densitometry using normalization  
23 standards for inter-gel comparisons (see Methods). (N=12 mice/group).

24 **(g)** Quantification of total N2B phosphorylation (left) and serine 4080 (S4080)-specific N2B  
25 phosphorylation (right, from densitometry of western blots representatively shown in  
26 Supplementary Fig. 5c). (N=12 mice/group).

27 Panels b,c, f,g: \*\*\*p<0.001, \*\*p<0.01 and \*p<0.05 (ANOVA with post-hoc Tukey, Welch's test  
28 with post-hoc Games-Howell or *t*-test (paired in panel c) as appropriate, see Methods). Panel  
29 d: P-value represent factor (T, treatment; A, age) comparisons by two-way ANOVA including  
30 21M and 23M groups followed by simple main effects (\*\*p<0.01, \*p<0.05 vs. age-matched  
31 control); +++p<0.001 (ANOVA post-hoc Tukey comparing controls). For box-and-whisker plots,  
32 whiskers show minima and maxima within 1.5 interquartile range.

1 **Figure 3. Spermidine ameliorates cardiac function through induction of autophagy.**

2 **(a, b)** Cardiac autophagic flux assessed by the LC3-II/GAPDH ratio, determined using  
3 western blot analysis (Supplementary Fig. 9e) **(a)** and cardiac tissue levels of spermidine **(b)**  
4 50 min after intraperitoneal injection of leupeptin or vehicle in 13-month-old C57BL/6J male  
5 mice with or without 4 weeks of spermidine supplementation to the drinking water. N=7/13  
6 (Vehicle/Leu) mice/group. Co, Control; Spd, Spermidine; Leu, Leupeptin.

7 **(c, d)** Young (3-month-old) transgenic mice harboring cardiac-specific expressed tandem-  
8 fluorescence mRFP-GFP-LC3 (tf-LC3) were subjected to spermidine treatment for 2 weeks  
9 and hearts were analyzed by confocal microscopy 4 hours after intraperitoneal injection of  
10 chloroquine (CQ) or vehicle to assess autophagic flux. Representative RFP/GFP/*Hoechst*  
11 overlays **(c)** and quantification of autophagosomes (orange puncta in **c, arrows**) and  
12 autolysosomes (red puncta in **c, arrowheads**) **(d)** are shown. Scale bars represent 50  $\mu$ m.  
13 N=3 mice/group.

14 **(e)** Mitophagy was assessed in young (6-month-old) or aged (18-month-old) C57BL/6J wild-  
15 type mice treated with or without 2 weeks of spermidine supplementation and injected with  
16 AAV9-Mito-Keima. The positive ratiometric area (see Supplementary Fig. 9b-d) of Mito-  
17 Keima fluorescence (561 nm/457 nm excitation), indicative of mitophagy, was quantified.  
18 N=3 mice/group.

19 **(f-k)** Cardiomyocyte-specific *Atg5*-deficient mice (*Atg5*<sup>-/-</sup>) were analyzed at 16 weeks of age  
20 and compared to age-matched *Atg5*<sup>+/+</sup> littermates, with or without 12 weeks of spermidine  
21 supplementation (see Supplementary Fig. 10). Shown are tibia length-normalized left  
22 ventricular mass (LVmass/TL) **(f)**, ejection fraction (EF) **(g)**, representative pressure-volume  
23 loops **(h)**, myocardial stiffness constant (end-diastolic pressure-volume relationships  
24 [EDPVR]  $\beta$  obtained from exponential fits, Supplementary Fig. 10d) **(i)**, end-systolic  
25 elastance (Ees, slope of the end-systolic pressure-volume relationship, Supplementary Fig.  
26 10e) **(j)** and ventricular-vascular coupling (VVC) **(k)**. N=16/15 and N=12/14 **(f, g, Co/Spd)**  
27 mice, *Atg5*<sup>+/+</sup> and *Atg5*<sup>-/-</sup>, respectively. N=10/10 and N=9/11 **(i-k, Co/Spd)** mice, *Atg5*<sup>+/+</sup> and  
28 *Atg5*<sup>-/-</sup>, respectively.

29 P-values represent factor (T, treatment; G, genotype) comparisons by two-way ANOVA  
30 (ANCOVA in panel i) followed by simple main effects (\*\*\*p<0.001, \*\*p<0.01, \*p<0.05 and  
31 #p<0.06). Dot-plots show means  $\pm$  s.e.m. For box-and-whisker plots, whiskers show minima  
32 and maxima within 1.5 interquartile range.

33

34

1 **Figure 4. Spermidine ameliorates salt-induced hypertension and heart failure in *Dahl***  
2 **salt-sensitive rats.**

3 **(a)** Schematic overview of spermidine administration (starting from the age of 7 weeks) to  
4 *Dahl* salt-sensitive rats fed a high-salt diet (8% NaCl) *ad libitum*. Cardiovascular parameters  
5 **(b-k)** and renal tissue characteristics **(l, m)** were recorded at the indicated ages.

6 **(b)** Mean arterial blood pressure (MBP) by the non-invasive tail-cuff method. (N=10  
7 rats/group). Red symbols denote the first time point at which there is a non-significant  
8 difference in MBP compared to the peak MBP of the group.

9 **(c)** Plasma spermidine and ornithine content and global arginine bioavailability ratio (GABR).  
10 N=12/12/12, 11/12/12, 11/12/10 (7wk/9wk/9wk+S) rats, left, center and right sub-panels,  
11 respectively.

12 **(d, e)** Echocardiographic assessment of tibia length-normalized LV mass (LVmass/TL) **(d)**  
13 and the ratio of peak early Doppler transmitral flow velocity (E) to the corresponding  
14 myocardial tissue Doppler velocity (E') **(e)**. N=10 rats/group.

15 **(f-k)** Representative pressure-volume loops **(f)**, LV end-diastolic pressure (EDP) **(g)**,  
16 myocardial stiffness constant for indexed volumes (end-diastolic pressure-volume  
17 relationship [EDPVR]  $\beta$ ) **(h)**, tibia length (TL)-normalized lung and liver weights **(i)**, ejection  
18 fraction (EF) **(j)**, and ventricular-vascular coupling (VVC) **(k)**. N=10/9/9/10/10  
19 (7wk/14wk/14wk+S/19wk/19wk+S) rats.

20 **(l)** Representative micrographs of 9 rats/group analyzed for renal fibrosis in 19-week-old rats,  
21 as assessed by picrosirius red collagen staining .

22 **(m)** Urinary lipocalin-2 (Lcn-2) levels. N=12/10/10 (7wk/19wk/19wk+S) rats.

23 \*\*\* $p < 0.001$ , \*\* $p < 0.01$  and \* $p < 0.05$  (ANOVA with post-hoc Tukey in panel c, m, Kruskal-Wallis  
24 with corrected multiple-comparisons in panel d). Panels b, e, g-k: p-values represent factor  
25 (T, treatment; A, age) comparisons by two-way ANOVA (mixed-design in b and e) including  
26 14wk and 19wk groups followed by simple main effects (\*\* $p < 0.01$ , \* $p < 0.05$  vs.  
27 age-matched control); \*\*\* $p < 0.001$ , \*\* $p < 0.01$ , \* $p < 0.05$  (ANOVA with post-hoc Tukey  
28 comparing controls). Dot- and line-plots show means  $\pm$  s.e.m. For box-and-whisker plots,  
29 whiskers show minima and maxima within 1.5 interquartile range.

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32

1 **Figure 5. Dietary spermidine intake inversely correlates with human cardiovascular**  
2 **disease.**

3 **(a-c)** Associations of polyamine intake (spermidine or putrescine) in human subjects with  
4 death due to heart failure **(a)**, clinically overt heart failure **(b)** and incident cardiovascular  
5 disease (CVD, a composite of acute coronary artery disease, stroke and death due to  
6 vascular disease) **(c)**. Hazard ratios **(a and c, time-to-event analysis)** and odds ratios **(b,**  
7 **cross-sectional analysis)** are indicated for one standard deviation higher intake of the given  
8 polyamine. Models were unadjusted (U) or had multivariable adjustment (M) for age, sex,  
9 total caloric intake, current smoking, diabetes, alcohol consumption, and diastolic blood  
10 pressure. \*Death due to heart failure was defined according to the International Statistical  
11 Classification of Diseases and Related Health Problems, 10th revision (ICD-10) codes I50.x,  
12 I13.0, I13.2, I11.00, I11.01, or I97.1, and the results represent sub-distribution hazard ratios  
13 based on the Fine and Gray model and account for the competing risk of death due to  
14 causes unrelated to heart failure. \*\*Diagnosis of clinically overt heart failure (ascertained in  
15 2010) relied on gold standard Framingham criteria. \*\*\*See methods for incident CVD criteria.

16 **(d)** Association of polyamine (spermidine or putrescine) intake with systolic (BP<sub>sys</sub>) and  
17 diastolic (BP<sub>dia</sub>) blood pressures repeatedly assessed in 829 participants of the Bruneck  
18 Study (1995-2010). The effects shown represent the average difference in blood pressure  
19 (mmHg) associated with one standard deviation higher intake of the given polyamine (M1) or  
20 between the first and third tertile groups (M2) under adjustment for age, sex, and total caloric  
21 intake.

22

23 **Figure 6. Mechanistic model of spermidine-mediated cardioprotection in aging and**  
24 **hypertensive heart failure.** Oral (dietary) supplementation of spermidine improves cardiac  
25 function by (i) promoting protective autophagy and mitophagy in cardiomyocytes; (ii) reducing  
26 subclinical, chronic inflammation (circulating TNF $\alpha$  levels) that impinges on cardiomyocyte  
27 function; (iii) improving systemic arginine bioavailability that may favor the production of the  
28 vasodilator nitric oxide (NO), and thus decrease systemic blood pressure; and (iv) inhibiting  
29 kidney damage through induction of autophagy. Improved renal function by spermidine  
30 treatment may additionally contribute to reduced arterial blood pressure and cardioprotection  
31 in the setting of salt-induced hypertension. In conjunction with spermidine's anti-inflammatory  
32 action, its autophagy-dependent effects on cardiomyocytes lead to enhanced mitochondrial  
33 volume and function, increased titin phosphorylation and reduced hypertrophy, which in turn  
34 result in improved mechano-elastic properties of cardiomyocytes.

## 1 **Online Methods**

2

### 3 **Animal housing and polyamine supplementation**

4 **Wild-type C57BL/6 mice** were purchased from Charles River (C57BL/6J:Crl females),  
5 Envigo, former Harlan Laboratories (C57BL/6N:Hsd females) or Janvier Labs, France  
6 (C57BL6/J:Rj males). Mice used for the *late-in-life* longevity experiments (Fig. 1d,  
7 Supplementary Tables 1, 2) were obtained from our in-house animal facility (C57BL/6J mice  
8 derived from various breeding lines were divided equally to spermidine-supplemented or  
9 control groups). These mice originated from more than 20 breeding pairs originally used for  
10 backcrossing transgenic strains. We used only wild-type mice (confirmed by PCR-based  
11 genotyping) that originated from at least five backcrosses to the C57BL/6J strain. The  
12 C57BL/6J colony established in house for backcrossing is renewed (purchased from Janvier  
13 labs) at least every 18 months to reduce genetic drift. Supplementation of polyamines (see  
14 Fig. 1a and Methods below) was conducted either *life-long* (starting at age 4 month) or *late-*  
15 *in-life* (starting at age 18 month). Analysis of lifespan, 'omics' and blood parameters,  
16 ultrastructural analyses, mitochondrial function as well as cardiac parameters  
17 (hemodynamics and echocardiography, non-invasive blood pressure measurements) were  
18 performed in independent cohorts of animals. **Cardiomyocyte-specific Atg5-deficient**  
19 **male mice** ( $Atg5^{flox/flox}-MLC2a-Cre^+$  mice;  $Atg5^{-/-}$ ) were generated from  $Atg5^{flox/flox}$  mice  
20 (obtained from Riken BRC, Japan, with the kind consent of Dr. Noboru Mizushima)<sup>52</sup> crossed  
21 with knock-in mice expressing Cre recombinase driven by the cardiomyocyte-specific  $\alpha$ -  
22 myosin light chain ( $MLC2a-Cre$ ) promoter<sup>53</sup>. After weaning,  $Atg5^{flox/flox}-MLC2a-Cre^+$  ( $Atg5^{-/-}$ )  
23 and  $Atg5^{flox/flox}-MLC2a-Cre^-$  ( $Atg5^{+/+}$ , controls) mice were each randomly divided into two  
24 treatment groups (spermidine-treated and non-treated controls). Treatment was initiated in  
25 young one-month-old mice that were treated for up to 12-14 weeks of age before they were  
26 subjected to cardiac functional analyses (echocardiography and hemodynamics). This time  
27 point was selected based on a previous study<sup>6</sup>, showing that cardiac restricted  $Atg5^{-/-}$  mice  
28 have no cardiac phenotype at 3 months of age. PCR analysis of genomic DNA from ear  
29 biopsies was performed for the assessment of genotypes using published primers "exon3-1",  
30 "short 2" and "check 2"; *loxP*-flanked *Atg5* allele<sup>52</sup> and primers "MLC2a-1",  
31 5'-GGATCTATGTGGAGCCCTGTCT-3' and MLC2a-2", 5'-GCACACAAGTCCCTGGCTCTGT-3'  
32 (Cre allele)<sup>53</sup>. Adult ventricular cardiomyocytes were isolated from 12-16-week-old  $Atg5^{-/-}$   
33 male mice and their age-matched control littermates using a previously reported protocol<sup>54</sup>.  
34 Isolated cardiomyocytes were then subjected to immunoblotting of LC3, p62 and Atg5 to  
35 confirm Atg5-deficiency and impaired autophagy (see Supplementary Fig. 10). **Dahl salt-**  
36 **sensitive male rats** (N = 60 in total), an experimental model of hypertensive heart failure<sup>45</sup>,



1 were purchased at the age of 4 weeks from the Charles River Laboratories (USA). Low salt  
2 laboratory chow containing 0.3% NaCl (AIN-76A, Research Diets, Inc. USA) was fed to 60  
3 weaning *Dahl* rats until the diet was switched to a high-salt diet (AIN-76A with 8% NaCl,  
4 Research Diets, Inc. USA) at 7 weeks of age. Twelve rats fed the low-salt diet were  
5 sacrificed at the age of 7 weeks and served as a young control group. The remaining 48 rats  
6 were randomly divided into two cohorts each consisting of two groups (N=12 animals per  
7 group): group 1 received regular drinking water without spermidine (non-treated control),  
8 whereas group 2 received regular drinking water supplemented with spermidine for a period  
9 of 7 (short-term cohort) or 12 (long-term cohort) weeks (see Fig. 4a). Blood pressure was  
10 assessed independently in the short- and long-term cohorts, with comparable results at  
11 similar ages (i.e. 7, 9, 11 weeks). Hemodynamic cardiac assessment was conducted as a  
12 terminal procedure independently in the two cohorts, yielding also comparable results with  
13 respect to spermidine effects, but at different ages (14 and 19 weeks).

14 Animals were housed under SPF (mice) or conventional (rats) conditions in a 12h light/dark  
15 cycle with access to food (standard chow for mice, *Ssniff* V1534) and water *ad libitum*.  
16 Autoclaved nest material and paper houses served as cage enrichment for mice. The  
17 polyamines spermidine (3 mM in all experiments, with the exception of mouse *life-long*  
18 supplementation for lifespan estimation, 0.3 mM; mouse pathology analysis, glucose  
19 tolerance test, metabolic parameters, and body composition, 0.3 and 3 mM), spermine (3  
20 mM) and putrescine (3 mM) were administered orally via drinking water prepared from  
21 aqueous stock solutions as described elsewhere<sup>8</sup> Control animals received regular drinking  
22 water. Food and water consumption was recorded twice a week at indicated ages (averaged  
23 over a time period of 4 weeks) by weighing water bottles and food pellets. Animal cages  
24 were always randomly assigned to treatment or control groups.

25 With the exception of animal care and housing as well as experiments performed on the 7-  
26 week-old rats cohort, the experimenters were blinded to the age and treatment of the  
27 animals. All animal experiments were performed in accordance with national and European  
28 ethical regulation (Directive 2010/63/EU) and approved by the responsible institutional  
29 (Rutgers-New Jersey Medical School's Institutional Animal Care and Use Committee) or  
30 government agencies (District Government of Upper Bavaria, Germany, and  
31 Bundesministerium für Wissenschaft, Forschung und Wirtschaft, BMWFW, Austria: BMWF-  
32 66.010/0161-II/3b/2012, BMWF-66.007/0011-II/3b/2013, BMWF-66.010/0053-WF/II/3b/2014;  
33 BMWF-66.010/0160-WF/V/3b/2014, BMWFW-66.007/0002-WF/V/3b/2015, BMWFW-  
34 66.007/0024-WF/V/3b/2015).

35

## 1 **Mouse lifespan analysis**

2 For lifespan experiments, the housing group size was maintained at two or more animals per  
3 cage. This was assured by joining single-housed animals (i.e. last survivor in a cage) to other  
4 cages of the same treatment group. Regular sentinel observation was used to ascertain the  
5 SPF health status of the animals. Animals were inspected daily for their general health  
6 status. Whenever the health condition of an animal indicated that its welfare was  
7 compromised, the mouse was euthanized and classified as either (i) an *end-of-life* (EOL)<sup>16</sup>  
8 animal likely dying within the next 24-48 hours, or (ii) a censored animal (due primarily to  
9 severe bite wounds, obvious tumors or skin lesions after excessive grooming). EOL animals  
10 were defined by established “estimation of death” criteria similar to the *Interventions Testing*  
11 *Program* (ITP) program guidelines of the *National Institute on Aging* (NIA)<sup>55</sup>, with the  
12 exception that any one of these criteria was considered sufficient to indicate close natural  
13 death. The majority of animals were simply found dead during daily inspections  
14 (Supplementary Table 1). Of note, the overall lifespan observed in the present study was  
15 slightly shorter compared to other studies using C57BL/6 mice<sup>15,56</sup>, a difference that may be  
16 due to differences in housing conditions, estimation-of-death criteria or the highly stringent  
17 criteria we used to identify mice in severe discomfort, which must be euthanized, thus  
18 affecting the number of censored mice. Survival data were analyzed by Kaplan-Meier  
19 method and significant differences in survival distribution curves between the groups were  
20 determined using the Breslow test. OriginPro 2016 software (OriginLab) was used for  
21 survival data analysis, including also the calculation of lifespan estimates. Censored animals  
22 were still counted as live individuals before the time at which they were euthanized.  
23 However, during the *life-long* supplementation experiment (C57BL/6J:Crl female mice), we  
24 observed an unusually high incidence of C57BL/6-typical alopecia<sup>57</sup> with signs of ulcerative  
25 dermatitis early in life (between the age of 200 and 300 days), which required us to euthanize  
26 about 40% of all mice. We decided not to censor these mice, but rather to completely  
27 exclude them from the survival analysis. Notably, in this experiment, the fraction of animal  
28 loss early during the experiment was similar in the spermidine-supplemented and control  
29 groups.

30

## 31 **Analysis of body composition and metabolic parameters in aging mice**

32 Body composition (relative lean- and fat mass) was determined by *in vivo* NMR spectroscopy  
33 using the Minispec mq NMR analyser (Brucker Optics, USA), according to the  
34 manufacturer's instructions. General activity (assessed by the total number of beam breaks,  
35 *XT+YT counts*) as well as the respiratory exchange rate (*RER*, expressed as the volumetric

1 quotient of carbon dioxide elimination of the animal divided by oxygen consumption,  
2  $V_{CO_2}/V_{O_2}$ ) was determined using *PhenoMaster*<sup>TM</sup> cages (TSE-Systems). Mice were singly  
3 housed and the first complete 12 hour dark- and light-cycles were recorded for parameter  
4 analysis after an initial adaptation phase of 8 hours.

5

#### 6 **Intraperitoneal glucose tolerance test**

7 Intraperitoneal glucose tolerance tests were performed as previously described<sup>58</sup>. Briefly, the  
8 food was removed for 16 to 18 hours overnight. During the experiment, mice were singly  
9 housed in empty cages without food, water or bedding. The basal fasting blood glucose level  
10 was determined using a drop of blood collected from the tail vein, using an Accu-Chek Aviva  
11 glucose analyzer (Roche/Mannheim). Thereafter mice were injected intraperitoneally with 2 g  
12 of glucose/kg fasting body mass and blood glucose levels were determined 15, 30, 60 and  
13 120 minutes after glucose injection.

14

#### 15 **Fasting insulin determination**

16 Fasting insulin was measured in plasma obtained from 3 hour fasted mice (water supplied *ad*  
17 *libitum*) using an ultra-sensitive mouse insulin ELISA Kit (Crystal Chem, Downers Grove,  
18 Illinois, USA). The wide range assay protocol was performed according to the manufacturer's  
19 protocol. Insulin concentrations of the samples were calculated using a semi-logarithmic  
20 4-parameter fit standard curve in Prism 6 (GraphPad Software Inc, La Jolla, California, USA).

21

#### 22 **Non-invasive blood pressure measurements**

23 Systolic, mean and diastolic blood pressures, as well as heart rate, were non-invasively  
24 measured in conscious animals by the tail-cuff method using the CODA<sup>TM</sup> system (Kent  
25 Scientific Corporation, USA). Animals were placed in a cylindrical holder on a temperature-  
26 controlled platform (kept at 37 °C) and recordings were performed in steady-state conditions.  
27 Blood pressure values were averaged from three consecutive measurements.

28

#### 29 **Echocardiography**

30 Transthoracic echocardiography was performed similarly as described<sup>59</sup>. Briefly, lightly  
31 anaesthetized mice (0.5% isoflurane and 99.5% O<sub>2</sub>) and rats (2% isoflurane and 98% O<sub>2</sub>)

1 were placed on a temperature-controlled warming pad (kept at 37 °C) and imaged in the  
2 supine position using a high-resolution micro-imaging system equipped with a 30-Mhz and  
3 17.5-Mhz linear array transducer (Vevo770™ Imaging System, VisualSonics Inc., Canada),  
4 respectively. Parasternal long-axis M-mode tracings of the left ventricle (LV) were recorded  
5 at the level just above the papillary muscles and LV end-diastolic diameter (LVEDD), LV end-  
6 systolic diameter (LVESD), interventricular septum thickness (IVS) and LV posterior wall  
7 thickness (PW) were measured. Fractional shortening was calculated using the equation:  
8  $100 \times [(LVEDD - LVESD) / LVEDD]$ . Left ventricular end-systolic and end-diastolic volumes as  
9 well as ejection fraction were calculated according to the Teichholtz formula and the LV mass  
10 was calculated according to the Troy formula<sup>60</sup>. The ratio of peak early filling velocity of  
11 transmitral flow (E) to the corresponding mitral valve annulus velocity (E') was evaluated  
12 using pulsed-wave and tissue Doppler imaging, respectively. All measures were averaged  
13 from three consecutive cardiac cycles under stable conditions.

14

#### 15 **Hemodynamic pressure-volume measurements**

16 Invasive hemodynamic measurements and analysis of pressure-volume (PV) loops were  
17 performed as a terminal procedure according to established protocols<sup>61</sup>. Mice and rats were  
18 anaesthetized (induction: 3-4% isoflurane with 96-97% O<sub>2</sub>; maintenance: 1-2% isoflurane  
19 with 98-99% O<sub>2</sub>), intubated and mechanically ventilated (rats, SAR 1000, CWE, Inc.; mice,  
20 Harvard Mini-Vent (type 845), Harvard Apparatus). The animals were placed on a  
21 temperature-controlled heating platform (TC-1000, CWE, Inc.) and their core temperature  
22 was maintained at 37.5 °C. Heart rate (HR) was continuously monitored using an  
23 electrocardiogram (Animal Bio Amp, FE136; ADInstruments). A mouse 1.4 F or rat 2.0 F  
24 pressure-conductance catheter (SPR-839 and SPR-838, respectively; Millar instruments)  
25 was inserted into the right carotid artery and advanced into the ascending aorta. After  
26 recording aortic (systemic) blood pressure, the catheter was advanced through the aortic  
27 valve into the LV where PV signals were continuously obtained (MPVS ultra, Millar  
28 Instruments) and recorded in a digital-form (MPVS PL3508 PowerLab 8/35, ADInstruments)  
29 at the acquisition rate of 2 kHz for later offline analysis (LabChart 8 pro, ADInstruments).  
30 Animals were allowed to stabilize for 5 minutes, then baseline load-dependent parameters of  
31 systolic and diastolic function, including LV end-systolic pressure (ESP), LV end-diastolic  
32 pressure (EDP), LV end-systolic volume (ESV), LV end-diastolic volume (EDV), stroke  
33 volume (SV), cardiac output (CO), arterial elastance (Ea), ejection fraction (EF), maximal  
34 slope of LV systolic pressure increment ( $dP/dt_{max}$ ), maximal slope of diastolic pressure  
35 decrement ( $dP/dt_{min}$ ) and time constant of LV pressure decay ( $\tau$ ) were measured and  
36 averaged from 10 consecutive beats, with ventilation suspended at end-expiration. After

1 baseline measurements, transient occlusion of the inferior *vena cava* (again with ventilation  
2 suspended) was performed and used to calculate multibeat-derived load-independent  
3 measures of cardiac systolic and diastolic functions: linear end-systolic pressure-volume  
4 relationship (ESPVR), calculated as  $ESP = \text{end-systolic elastance (Ees)} \times \text{ESV} + V_0$ , was used  
5 for the evaluation of cardiac contractility, while exponential end-diastolic pressure-volume  
6 relationship (EDPVR), calculated as  $EDP = \alpha \times \exp^{\beta \times \text{EDV}}$ , was implemented in assessing end-  
7 diastolic stiffness. In addition, ventricular-vascular coupling (VVC), indicative of  
8 cardiovascular efficiency, was calculated as the ratio between Ees and Ea. To correct for the  
9 large differences in body (and heart) size between young (7-week-old) and older (14- and 19-  
10 week-old) *Dahl* salt-sensitive rats, volumes were indexed to body surface area<sup>63</sup> as defined  
11 by  $9.1 \times (\text{body weight})^{2/3}$ . A polyethylene catheter was inserted into the right external jugular  
12 vein for hypertonic saline (10% NaCl) injection (10  $\mu\text{l}$  in mice and 40  $\mu\text{l}$  in rats) to calculate  
13 parallel conductance at the end of the experiment. Due to detected discrepancies of  
14 conductance- and echocardiography-derived left ventricular (LV) volumes in *Atg5*-deficient  
15 mice (not shown), the slope factor  $\alpha$  (a factor that is used to correct conductance-based  
16 estimation of ventricular volumes) was calculated using echocardiography-derived stroke  
17 volume, which correlates with the gold-standard method of volume estimation using Doppler  
18 flow-probes<sup>62</sup>. After assessment of hemodynamic parameters, animals were sacrificed and a  
19 gravimetric analysis of different organs, including heart, lungs, liver, spleen and kidneys, was  
20 performed.

21

## 22 **Histological evaluation**

23 Tissues were fixed in 4% neutral-buffered formaldehyde and paraffin-embedded. Kidneys  
24 were cut into 4  $\mu\text{m}$  thick sections followed by periodic acid-Schiff (PAS) staining (Merck,  
25 Darmstadt, Germany). The extent of glomerular injury was evaluated by assigning a  
26 semiquantitative PAS score as described previously<sup>64</sup>. Furthermore, we evaluated the extent  
27 of arterial hyalinosis and fibrosis semiquantitatively using a similar scoring system as above.  
28 The number of tubular casts in 6 adjacent high-power fields (magnification 400x) was  
29 counted. To assess the extent of renal fibrosis and damage, picrosirius red staining was  
30 performed. Renal tissue was first stained with Gill's hematoxylin (Merck), then with 1% Sirius  
31 red (Sigma-Aldrich, St. Louis, MO, USA) in a saturated aqueous solution of picric acid and  
32 then differentiated in acidified water. To investigate the cardiac death and cancer  
33 development phenotype (pathology analysis), 4  $\mu\text{m}$  thick sections of heart, liver, brain,  
34 spleen and kidney were stained with hematoxylin/eosin stain as follows: after rehydration,  
35 sections were stained in Mayers' acid hemalum for 2 min, blued in tap water for 2 min,  
36 contrasted with Eosin Y for 15 sec, rinsed in tap water for 5 sec, dehydrated in an increasing

1 ethanol series and placed in xylene before mounting with Entellan® (all chemicals from  
2 Merck, Germany). Slides were read using an Axioplan® brightfield microscope (Zeiss,  
3 Germany) by two pathologists independently (D.J., F.N.).

4

#### 5 **Assessment of urinary Lcn-2 levels**

6 Urinary levels of Lcn-2 protein were assessed using the Rat Lcn-2/NGAL DuoSet (R&D  
7 Systems, Abingdon, UK) kit according to the manufacturer's instructions.

8

#### 9 **Transcriptome expression profiling**

10 Expression profiling was done from hearts of four treated (3 mM *life-long* spermidine  
11 supplemented, see Fig. 1a) and four untreated mice at the age of 30-32 months, as well as  
12 from four six months old untreated control mice. Mice were sacrificed between 9 and 12 p.m.  
13 (noon). Hearts were dissected, immediately frozen in liquid nitrogen and stored at -80 °C. For  
14 total RNA isolation using RNeasy Midi kits (Qiagen), hearts were thawed in Trizol Reagent  
15 (Sigma) and homogenized using a Polytron homogenizer (Heidolph). 500 ng of total RNA  
16 was amplified in a single round using the Illumina TotalPrep RNA Amplification Kit (Ambion).  
17 750 ng of amplified RNA was hybridized to Illumina MouseRef8 v2.0 Expression Bead Arrays  
18 covering 25,600 annotated RefSeq transcripts. Staining and scanning (Illumina HiScan Array  
19 reader) were done according to the Illumina expression protocol. Illumina Genomestudio  
20 software was used for background correction and normalization (cubic spline algorithm).  
21 Significant gene regulation was analysed using SAM (Significant Analysis of Microarrays)  
22 included in the TM4 software package<sup>65</sup>. False discovery rates (FDRs) were calculated by  
23 1000 random permutations. The selection of the top differentially expressed genes with  
24 reproducible up- or down-regulation includes genes with an FDR below 10% and a mean fold  
25 change above 1.5 fold. Over-represented functional annotations (Gene Ontology) were  
26 identified using the Ingenuity Pathway Analysis. Genes and samples were clustered for heat  
27 map representation using the hierarchical clustering function (complete linkage) of Genesis  
28 software (release 1.7.6)<sup>66</sup>.

29

#### 30 **Proteomics analysis**

31 Heart specimens were obtained from three aged, three young and three aged mice treated  
32 with spermidine. Whole tissue lysates (50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM EGTA,

1 1% Triton X-100 supplemented with 1x *Complete*<sup>®</sup> protease inhibitor cocktail (Roche)) were  
2 prepared in SDS loading buffer, samples were reduced with 1 mM DTT (Sigma-Aldrich) for 5  
3 min at 95 °C and alkylated using 5.5 mM iodoacetamide (Sigma-Aldrich) for 30 min at 20 °C.  
4 Protein mixtures were separated by 4-12% gradient SDS-PAGE (NuPAGE, Invitrogen). The  
5 gel lanes were cut into 6 equal slices, the proteins were in-gel digested with trypsin  
6 (Promega)<sup>67</sup>, and the resulting peptide mixtures were processed on STAGE tips<sup>68</sup> and  
7 analyzed by LC-MS/MS. Mass spectrometric (MS) measurements were performed on an  
8 LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific) coupled to an Agilent 1200  
9 nanoflow-HPLC (Agilent Technologies GmbH, Waldbronn, Germany) as described<sup>69</sup>. MS  
10 raw data files were uploaded into MaxQuant software (version 1.4.1.2 (ref. 70)), which was  
11 used for identification of proteins and protein ratio assignment for peak detection, generation  
12 of peak lists of mass error corrected peptides and for database searches. A full-length  
13 UniProt mouse database additionally containing common contaminants such as keratins and  
14 enzymes used for in-gel digestion (based on UniProt mouse FASTA version July 2014) was  
15 used as a reference. Carbamidomethylcysteine was set as a fixed modification; methionine  
16 oxidation and protein amino-terminal acetylation were set as variable modifications and label-  
17 free was chosen as the quantitation mode. Three miscleavages were allowed, enzyme  
18 specificity was trypsin/P, and the MS/MS tolerance was set to 0.5 Da. The average mass  
19 precision of identified peptides was in general less than 1 ppm after recalibration. Peptide  
20 lists were further used by MaxQuant to identify and relatively quantify (label-free  
21 quantification, LFQ) proteins using the following parameters: peptide and protein false  
22 discovery rates, based on a forward-reverse database, were set to 0.01; the minimum  
23 peptide length was set to 7; the minimum number of peptides for identification and  
24 quantitation of proteins was set to two, of which one must be unique; the minimum ratio  
25 count was set to two; and identified proteins were requantified. The 'match-between-run'  
26 option (1 min) was used. The 10% most reproducible changes based on TTEST analysis  
27 between aged control and aged spermidine groups (yielding a p-value cut-off  $p < 0.15$ ) were  
28 considered as potential *hits*. To visualise age- or spermidine-induced changes in protein  
29 abundance (*heat map presentation*), the LFQ protein intensities of each sample were divided  
30 by the mean of respective aged control intensities (LFQ *Ratios*). Proteins and samples were  
31 clustered for heat map representation using the hierarchical clustering function (complete  
32 linkage) of Genesis software (release 1.7.6)<sup>66</sup>.

33

34

1 **Metabolite analysis by high performance liquid chromatography (HPLC) and mass**  
2 **spectrometry (MS)**

3 Tissue metabolite analysis was performed on whole tissue lysates. Tissues were snap frozen  
4 in liquid nitrogen and stored at -80 °C until metabolite extraction. For metabolite extraction,  
5 tissues were pulverized on dry ice using mortar and pestle. A quantitative HPLC-MS/MS-  
6 based determination of polyamines (ornithine, putrescine, spermidine and spermine) was  
7 performed essentially as described<sup>71</sup> in plasma or from whole blood and cardiac tissue acid  
8 extracts as indicated. Plasma was prepared by centrifugation of EDTA-collected blood at  
9 2,500 g for 20 min. 10 µl EDTA-collected blood or 10-15 mg pulverized tissue was used to  
10 generate whole blood- or tissue-extracts, respectively, with a final extract volume of 750 µl.

11 For metabolomic analysis, mice (12 animals per group) were fasted overnight (12-16 hours)  
12 and hearts were removed and snap frozen (liquid nitrogen) immediately after sacrificing the  
13 animals. Cardiac tissue extracts using 20-40 mg tissue wet weight were prepared by cold-  
14 methanol extraction as described<sup>72</sup>. A boiling ethanol extract from yeast grown aerobically on  
15 <sup>13</sup>C-glucose as the carbon source served as an internal standard for MS analysis<sup>73</sup> and was  
16 spiked into tissue samples before extraction. Metabolomics samples were measured with a  
17 LC/MS system from Thermo Fisher Scientific<sup>TM</sup>. A Dionex Ultimate 3000 HPLC setup  
18 equipped with an Atlantis T3 C18 pre- and analytical column (Waters, USA) was used for  
19 compound separation prior to mass spectrometric detection with an Exactive<sup>TM</sup> Orbitrap  
20 system. The method was adapted from *Buescher et al.*<sup>74</sup>. The injection volume was 10 µL  
21 per sample and negative ionization of metabolites was carried out via heated electrospray  
22 ionization. For the online detection of the metabolites a full scan of all masses between 70  
23 and 1,100 m/z with a resolution of 50,000 (at m/z 200) was used. LC/MS-data acquisition  
24 was conducted with Xcalibur software (version 2.2 SP1, Thermo Fisher Scientific (Waltham,  
25 USA)), automated peak integration with TraceFinder<sup>TM</sup> software (version 3.2, Thermo Fisher  
26 Scientific (Waltham, USA)). Screening and manual correction of peak integrations were done  
27 within the TraceFinder<sup>TM</sup> package. <sup>12</sup>C-peak area/<sup>13</sup>C-peak area ratios were normalized to  
28 the respective tissue sample wet weight. Age- or spermidine-induced changes in metabolite  
29 abundance were identified by dividing <sup>12</sup>C-peak area/<sup>13</sup>C-peak area ratios of each sample to  
30 the mean of respective aged control samples (Aged control-normalized *Ratios*) and the  
31 median was used for *heat map* representation. Principal Component Analysis (PCA) was  
32 applied to the complete dataset using the R-function `prcomp` (stats-package) with R version  
33 3.1.1 in order to detect potential clustering and distances between samples.

34

35



## 1 **Plasma arginine and citrulline determination**

2 Arginine and citrulline concentrations were measured in serum with modifications of  
3 previously described chromatographic methods<sup>75,76</sup>. Briefly, after precipitation of serum with  
4 perchloric acid following neutralization of the supernatant with sodium carbonate, the  
5 extracted amino acids were derivatized online with o-phthalaldehyde and separated on a  
6 reverse phase column with gradient elution. Quantification was performed with ratios of  
7 fluorescence signals of the relevant amino acids to the internal standard norvaline in  
8 comparison to the appropriate calibration curves. Intra-assay and inter-assay CV's were all  
9 below 10%.

10

## 11 **Isolation of cardiac mitochondria and high-resolution respirometry**

12 Isolation of cardiac mitochondria and high-resolution respirometry was performed similarly to  
13 published methods<sup>26</sup>, using an isolation buffer containing 0.2% BSA and 5 mg/ml bacterial  
14 protease (Sigma-Aldrich, P8038). Hearts were quickly excised immediately after terminal  
15 blood collection under isoflurane anaesthesia and processed as described<sup>26</sup>. Optical density  
16 at 600 nm (OD600) of the final mitochondrial suspension (isolation buffer including BSA, but  
17 excluding protease) was determined by serial dilutions in a TECAN GeniusPro plate reader  
18 and served as an estimate of mitochondrial mass used for normalization. Oxygen  
19 consumption was assayed at 37 °C with an Oxygraph-2k high-resolution respirometer  
20 (Oroboros Instruments, Austria) according to the manufacturer's recommendations. OD600  
21 equivalents of isolated myocardial mitochondria corresponding to 10-20 µg mitochondrial  
22 protein were diluted in 2 ml equilibrated measurement medium (100 mM sucrose, 20 mM K+-  
23 TES (pH=7.2), 50 mM KCl, 2 mM MgCl<sub>2</sub>, 1 mM EDTA, 4 mM KH<sub>2</sub>PO<sub>4</sub>, 3 mM malate and  
24 0.1% (v/v) BSA) within a closed and calibrated system with constant stirring. For  
25 measurement of complex I activity, 5 mM pyruvate and 10 mM glutamate were added as  
26 reduced substrates after initially recording residual oxygen consumption (*ROX*) resulting in  
27 leak respiration (*LEAK*), followed by sequential additions of 450 µM ADP (*OXPHOS*) and 10  
28 µM cytochrome c (*OXPHOS + CytC*). 1.25 µM oligomycin were finally used to monitor the  
29 residual respiration (proton leak) followed by titration with FCCP (0.5 µM steps) to assess  
30 maximum respiration in uncoupled state and subsequent inhibition of respiratory activity  
31 through antimycin A. For each oxygraphic protocol (see Supplementary Fig. 7a), two mice  
32 were always processed in pairs (one aged control (24M) combined with either one aged  
33 spermidine-supplemented (24M+S) or one young control (5M)). The absolute oxygen  
34 concentration remained above 100 nmol/ml throughout all recordings.

35

## 1 **Plasma cytokine determination**

2 Plasma cytokine levels of apparently healthy mice or rats (i.e. subclinical cytokine levels)  
3 were assessed by electrochemiluminescence-based immunoassays using the MSD V-Plex  
4 Plus Proinflammatory Panel 1 (mouse) assay kit or a customized TNF $\alpha$  / IL-10 V-Plex Rat  
5 cytokine kit (Meso Scale Diagnostics, USA). 25  $\mu$ l of plasma derived from EDTA-collected  
6 whole blood by centrifugation (20 min, 2500 g) was processed according to the  
7 manufacturer's instructions. Whole blood was obtained from isoflurane-anesthetized animals  
8 at the age of 21 (non-fasted) or 23 (overnight-fasted before sacrifice) months by terminal  
9 bleeding (mice) or directly from the heart immediately at the end of hemodynamic  
10 assessment (rats). In addition to analyzing the complete inflammatory cytokine dataset  
11 (subclinical inflammatory status) of aged mice (Supplementary Fig. 8a), we performed an  
12 additional analysis, in which mice with *potentially* acute inflammatory conditions were  
13 excluded (Supplementary Fig. 8b), due to the fact that such mice, though lacking overt  
14 clinical manifestations, may strongly confound interpretation of the age-associated (chronic)  
15 low-grade inflammation status. Animals with two or more statistically-identified outlier  
16 cytokines were considered as animals with an acute inflammatory condition. Animals with a  
17 single outlier cytokine were included in the analysis, but the respective value was winsorized  
18 (see Supplementary Fig. 8). An outlier cytokine was identified using the 2.2-fold inter-quartile  
19 range (IQR) labeling rule applied to the respective group. Of note, the total number of  
20 excluded and winsorized values was similar in all aged groups.

21

## 22 **Immunoblotting and assessment of autophagic flux**

23 Measurements of the protein levels of APG5L/ATG5 and the autophagy-substrate  
24 p62/SQSTM1, as well as measurements of LC3 lipidation (LC3-II/GAPDH ratio), were  
25 performed on tissue extracts using lysis buffer containing 50 mM Tris-HCl, pH 7.4, 1 mM  
26 EDTA, 1 mM EGTA, 1% Triton X-100 supplemented with 1x *Complete*<sup>®</sup> protease inhibitor  
27 cocktail (Roche). Immunoblotting on polyvinylidene difluoride (PVDF) membranes was performed  
28 by standard procedures and probed with antibodies recognizing APG5L (ATG5) (dilution:  
29 1:1000, ab018327, Abcam), p62 (SQSTM1) (dilution: 1:1000, PM045, MBL), LC3B (dilution:  
30 1:1000, #2775, Cell Signaling Technologies), GAPDH (dilution: 1:1000, #2118, Cell Signaling  
31 Technologies), and horseradish peroxidase (HRP)-linked anti-rabbit IgG (dilution: 1:3000,  
32 #7074, Cell Signaling Technologies). Data were analyzed and quantified by densitometry  
33 with ImageLab software (Bio-Rad laboratories). Full scans of representative blots are shown  
34 in Supplementary Fig. 18. Autophagic flux in wild-type animals was assessed using  
35 leupeptin-based inhibition of LC3-II turnover according to a published protocol<sup>30</sup>. Following 4

1 weeks spermidine supplementation, 13-month-old C57BL6/JRj male mice (Janvier, France)  
2 were subjected to intraperitoneal (i.p.) injection of leupeptin (40 mg/kg body weight, Sigma,  
3 Austria) or vehicle (0.9% sterile sodium chloride solution). Fifty minutes after the i.p. injection,  
4 animals were sacrificed and hearts were excised. Age-matched mice that did not receive  
5 spermidine were used as controls. Cardiac tissues were subjected to immunoblot analysis of  
6 LC3 (see above).

7

## 8 **Autophagic flux using transgenic animals**

9 Transgenic mice with cardiac-specific expression of tandem-fluorescent mRFP-GFP-LC3  
10 (Tg-tf-LC3) generated on a C57/BL6 background with the mouse  $\alpha$ -Myosin Heavy Chain  
11 promoter were used<sup>31</sup>. In order to evaluate autophagic flux *in vivo*, 3-month-old Tg-tf-LC3  
12 mice were supplemented with 3 mM spermidine for 2 weeks (mice receiving normal drinking  
13 water served as controls). In some mice, chloroquine (10 mg/kg) was injected 4 hours before  
14 euthanasia and the number of fluorescent LC3 dots (indicative of autophagosomes and  
15 autolysosomes) was determined by confocal microscopy. Fresh heart slices were embedded  
16 with tissue-TEK OCT compound (Sakura Finetechnical Co., Ltd.) and frozen at -80 °C.  
17 Sections 10  $\mu$ m in thickness were obtained from the frozen tissue samples using the Leica  
18 Biosystems CM3050 S Research Cryostat (Leica), air-dried for 30 min, fixed with 10%  
19 formalin for 10 min, mounted using a reagent containing DAPI, and viewed under a  
20 fluorescence microscope.

21

## 22 **Mitophagy evaluation**

23 Evaluation of mitochondrial autophagy was achieved by monitoring Mito-Keima and Lamp1–  
24 YFP fluorescence as previously described<sup>32</sup>. Briefly, 6-month-old (young) and 18-month-old  
25 (aged) C57BL/6J mice were injected with AAV-Mito-Keima<sup>77</sup> and AAV9-Lamp1-YFP<sup>77</sup> 4  
26 weeks preceding analyses of Mito-Keima fluorescence and Lamp1–YFP fluorescence using  
27 confocal microscopy. Mice were treated with spermidine (3 mM) or vehicle (normal drinking  
28 water) for 3 weeks. Ratiometric images of Mito-Keima fluorescence (561 nm/457 nm  
29 excitation) were calculated and visualized in blue color. High ratiometric signals were defined  
30 as Mito-Keima positive areas, indicating mitochondrial autophagy (mitophagy). Mito-Keima  
31 fluorescence detected after excitation at 561 nm is shown in red color, while fluorescence  
32 after excitation at 457 nm is shown in green color. Merged images of Mito-Keima  
33 fluorescence (after 561 nm excitation) and Lamp1–YFP (shown in blue color) confirmed  
34 lysosomal localization of Mito-Keima-positive areas.

## 1 **Titin isoform separation and phosphorylation assays**

2 Homogenized myocardial samples were analyzed by 1.8% SDS–PAGE. Protein bands were  
3 visualized using Coomassie blue, scanned, and measured densitometrically, as described<sup>78</sup>.  
4 In place of a protein size marker in the titin range, human heart and diaphragm extracts  
5 (Source: Biobank at Dept. of Cardiovascular Physiology, Bochum, Germany) were used as  
6 *standards* for N2B/N2BA and N2A isoforms identification<sup>79</sup>, respectively. Western blotting  
7 was performed using anti-phosphoserine/threonine antibodies (Cat. No. PP2551, Biotrend  
8 Chemicals, Cologne, Germany, former ECM Biosciences) to measure global titin  
9 phosphorylation, and with affinity-purified phosphosite-specific anti-titin antibodies against  
10 phospho-S4080 in the N2Bus domain of mouse titin (custom-made against the peptide  
11 LFS(PO3H2)EWLRNI; dilution 1:500; Eurogentec, Brussels, Belgium)<sup>79</sup>. As a secondary  
12 antibody, we used horseradish peroxidase-conjugated IgG (Acris Antibodies, Herford,  
13 Germany). For signal amplification we used the Enhanced Chemoluminescence Western blot  
14 detection kit (GE Healthcare, Little Chalfont, UK). Staining was visualized using the LAS-  
15 4000 Image Reader (Fuji Science Imaging Systems), and densitometry was performed using  
16 Quantity One 1-D Analysis software (Bio-Rad laboratories). Equal protein loading and  
17 transfer was confirmed by densitometry of the signal on Coomassie-stained  
18 polyvinylidene fluoride (PVDF) membranes and western blot signals were normalized to the  
19 corresponding PVDF signals. Full scans of representative blots are shown in Supplementary  
20 Fig. 18. Two samples per group were processed in parallel on the same blot and the  
21 *standards* (always using the same identical extracts) were used for intra-gel normalization,  
22 allowing for quantitative inter-gel comparisons.

23

## 24 **Design-based stereology**

25 Mouse hearts were fixed by vascular perfusion with 4% neutral buffered formaldehyde and  
26 kept in the fixative for at least 24 h. Then the left ventricle (including the interventricular  
27 septum) was isolated, weighed and randomly sampled for electron microscopy (EM). The  
28 samples were post-incubated in a fixative containing 1.5% paraformaldehyde and 1.5%  
29 glutaraldehyde in 0.15 M HEPES buffer. Subsequently, the samples were post-fixed with 1%  
30 osmium tetroxide, stained *en bloc* with a half-saturated uranyl acetate/water solution,  
31 dehydrated in an ascending acetone series and finally embedded in epoxy resin. From the  
32 embedded samples, ultrathin sections were generated, mounted on EM support grids and  
33 post-stained with lead citrate and uranyl acetate. The sections were analysed with a  
34 Morgagni transmission electron microscope (FEI, Eindhoven, Netherlands) and test fields for  
35 morphometry were assessed using a digital camera according to a systematic uniform

1 random sampling scheme<sup>80</sup>. To estimate the volume fractions of cardiomyocytes and their  
2 organelles (myofibrils, mitochondria, sarcoplasm, nuclei, lipofuscin granules) as well as  
3 interstitium and its subcompartments (collagen fibrils, capillaries), point grids were projected  
4 onto the test fields and the number of points hitting the structures of interest and the  
5 reference volume were counted. The volume fraction of a structure was calculated by  
6 dividing the number of points hitting the structure by the number of points hitting the  
7 reference volume<sup>81</sup>. The total volume was then calculated by multiplying the volume fraction  
8 of a structure with the reference volume, e.g. the volume of the left ventricle. The latter was  
9 estimated by dividing the ventricular weight by the density of muscle tissue, 1.06 g/cm<sup>3</sup>  
10 (ref. 82).

11

## 12 **Human study subjects, quantification of nutritional intakes and data analyses**

13 Study subjects belonged to the Bruneck Study, a prospective, population-based survey on  
14 the epidemiology and pathogenesis of atherosclerosis and cardiovascular disease (CVD)<sup>47</sup>.  
15 Extended study details and risk factor assessment using validated standard procedures can  
16 be found online in the *Supplementary Note* (Section on *Information on Human Data*  
17 *Analysis*). Long-term average dietary intakes were ascertained by a dietitian-administered  
18 118-item food frequency questionnaire (FFQ). This questionnaire was based on the gold  
19 standard FFQ by Willett and Stampfer<sup>83</sup> and modified to better fit the dietary peculiarities in  
20 the survey area. Special nutrient data were compiled for polyamines using published data  
21 (see online Table at the *Supplementary Information on Human Data Analysis*). **Death due to**  
22 **heart failure** was defined according to the *International Statistical Classification of Diseases*  
23 *and Related Health Problems, 10th revision* (ICD-10) diagnosis codes I50.x, I13.0, I13.2,  
24 I11.00, I11.01 and I97.1 (considering heart failure following cardiac surgery or due to  
25 presence of cardiac prosthesis). **Clinically overt heart failure** was defined according to gold  
26 standard Framingham criteria (presence of at least 2 major criteria or 1 major criterion in  
27 conjunction with 2 minor criteria)<sup>84</sup> and assessed as part of the 2010 re-examination of the  
28 Bruneck Cohort. The primary composite CVD (**incident CVD**) endpoint included vascular  
29 death (from myocardial infarction [MI], ischemic stroke, or sudden cardiac death), acute  
30 coronary artery disease (consisting of nonfatal MI, new-onset unstable angina defined as  
31 angina at rest, crescendo angina or new-onset severe angina, and acute coronary  
32 interventions), and ischemic stroke. MI was defined by the World Health Organization's  
33 criteria for definite disease status. Stroke was classified according to the criteria of the  
34 National Survey of Stroke. All other revascularization procedures (percutaneous intervention,  
35 bypass, and surgery) were carefully recorded. Ascertainment of events or procedures did not  
36 rely on hospital discharge codes or the patient's self-report but rather on a careful review of

1 medical records provided by the general practitioners and files of the Bruneck Hospital and  
2 the extensive clinical and laboratory examinations performed as part of the study protocols.  
3 Incident CVD events were ascertained from 1995 through 2010, and 100% follow-up was  
4 achieved<sup>85</sup>. Causes of death as well as clinically overt heart failure were categorized by a  
5 senior researcher who was unaware of the dietary data. Dietary intakes were cumulatively  
6 averaged over follow-up visits to capture long-term dietary behaviour and to reduce within-  
7 subject variability. Polyamine intake was log-transformed and adjusted for total caloric intake  
8 by using the residual method<sup>86</sup> in a log-log simple linear model. Polyamine intake was then  
9 scaled to unit variance such that effects were estimated for a one-standard deviation  
10 increase in intake.

11 Plasma protein levels were measured by the Olink Proseek Multiplex Inflammation I (n=92  
12 proteins) and Olink Proseek Multiplex CVD I (n=92 proteins) proximity extension assays<sup>87</sup> in  
13 samples from participants of the Bruneck 2000 assessment (n=658). For proteins measured  
14 in both assays (n=23 proteins), measurements from the newer Inflammation I assay were  
15 used, and proteins with more than 25% non-detects (n=30) were excluded, leaving 131  
16 proteins for analysis. Proteins with skewness exceeding 1 were log-transformed. Skewness  
17 was calculated using the e1071 package for R, which used a formula  $m_3/s^3$ , where  $m_3$  is  $\sum_i (x_i$   
18  $- \mu)^3/n$ ,  $\mu$  the sample mean,  $n$  the sample size,  $s$  the sample standard deviation, and  $x_i$  the  
19 individual data values. Polyamine intake was averaged over the assessments made in 1995  
20 and 2000, calorie-adjusted and log-transformed. Association of dietary polyamine intake to  
21 the plasma protein levels was tested using Pearson correlation partial to age, sex, and  
22 caloric intake, and in this analysis multiple testing was accounted for by the Benjamini-  
23 Hochberg procedure (particularly suitable for high-dimensional data).

24 The numbers of unique subjects and of diet records used for this analysis were 829 and  
25 2540, respectively. Statistical procedures are detailed in the Supplementary Note. All P  
26 values are two-sided and an  $\alpha$  level of 0.05 is used throughout. Analyses were conducted  
27 with R 3.1.1. See *Supplementary Note on Human Data Analysis* for more details on outcome  
28 and methodologies.

29

### 30 **Statistical analysis of the experimental data.**

31 Data are presented either as dot-plots and line graphs showing mean  $\pm$  s.e.m., or as box-  
32 plots, showing mean (dot), median (center line) and interquartile range (IQR), along with  
33 whiskers showing minima and maxima within 1.5 or 2.2 IQR as indicated. Sample sizes were  
34 chosen based on literature (i.e. lifespan analyses<sup>55</sup>) or using standard power analysis  
35 (statistical power:  $\geq 0.8$  and  $\alpha$  value:  $< 0.05$ ) based on our preliminary echocardiographic

1 data obtained from young and aged animals yielding 9 or 12 animals per group (Student's *t*-  
2 test or ANOVA, respectively). For some measurements in mice, sample size was adapted to  
3 the observed effect size and numbers increased to 15-20 animals per group. Indicated  
4 sample size (see figure legends) always refers to biological replicates (independent animals).  
5 If not otherwise stated, statistical testing was performed using IBM SPSS statistics software  
6 (Version 23). Student's *t*-test (paired or unpaired, as appropriate) and analysis of variance  
7 (ANOVA) with Tukey's post-hoc tests were used for comparisons between two or multiple  
8 groups, respectively. Where appropriate, a two-way ANOVA was applied (independent or  
9 mixed design that was Greenhouse-Geisser-corrected in case of sphericity violation as  
10 tested by Mauchly's test) followed by testing simple main effects (i.e. multiple comparisons of  
11 different levels of each factor that were Bonferroni-corrected if the factor had more than 2  
12 levels) in case of main factor<sup>88</sup> or interaction significance. Myocardial chamber stiffness  
13 constant ( $\beta$ ) and end-systolic elastance (Ees) were compared between the groups including  
14 other parameters in the fitting equation ( $\alpha$  in case of exponential end-diastolic pressure-  
15 volume relationship and  $V_0$  in case of linear end-systolic pressure-volume relationship) as co-  
16 variates to account for their influence using analysis of co-variance (ANCOVA)<sup>89</sup> after  
17 confirming homogeneity of regression slopes between the compared groups.

18 The reported significance values are always two-sided. Overall normal distribution of data  
19 (residuals) was confirmed using Shapiro-Wilk's test. Homogeneity of variance was tested  
20 using Levene's test. Data violating these assumptions were transformed to meet the  
21 assumptions or tested as follows: non-normally distributed data were tested by non-  
22 parametric Kruskal-Wallis test after confirming equality of ranks variances (tested by non-  
23 parametric Levene's test) and followed by multiple comparisons using Mann-Whitney *U* test  
24 controlling for family-wise error rate by adjusting the significance level ( $\alpha$ ) according to the  
25 number of multiple comparisons ( $n$ ) ( $\alpha=0.05/n$ ). Whenever heterogeneous variances were an  
26 issue, Welch's *t*-test or Welch's test with *Games-Howell*-corrected post-hoc comparisons  
27 were applied.

28 To compare tumor incidence in aged mice, binomial logistic regression was conducted.  
29 Details of statistical analysis applied to *Human* data, *Omics* data or lifespan analyses by  
30 Kaplan-Meier method are indicated in their respective sections in the *Methods*.

31

32

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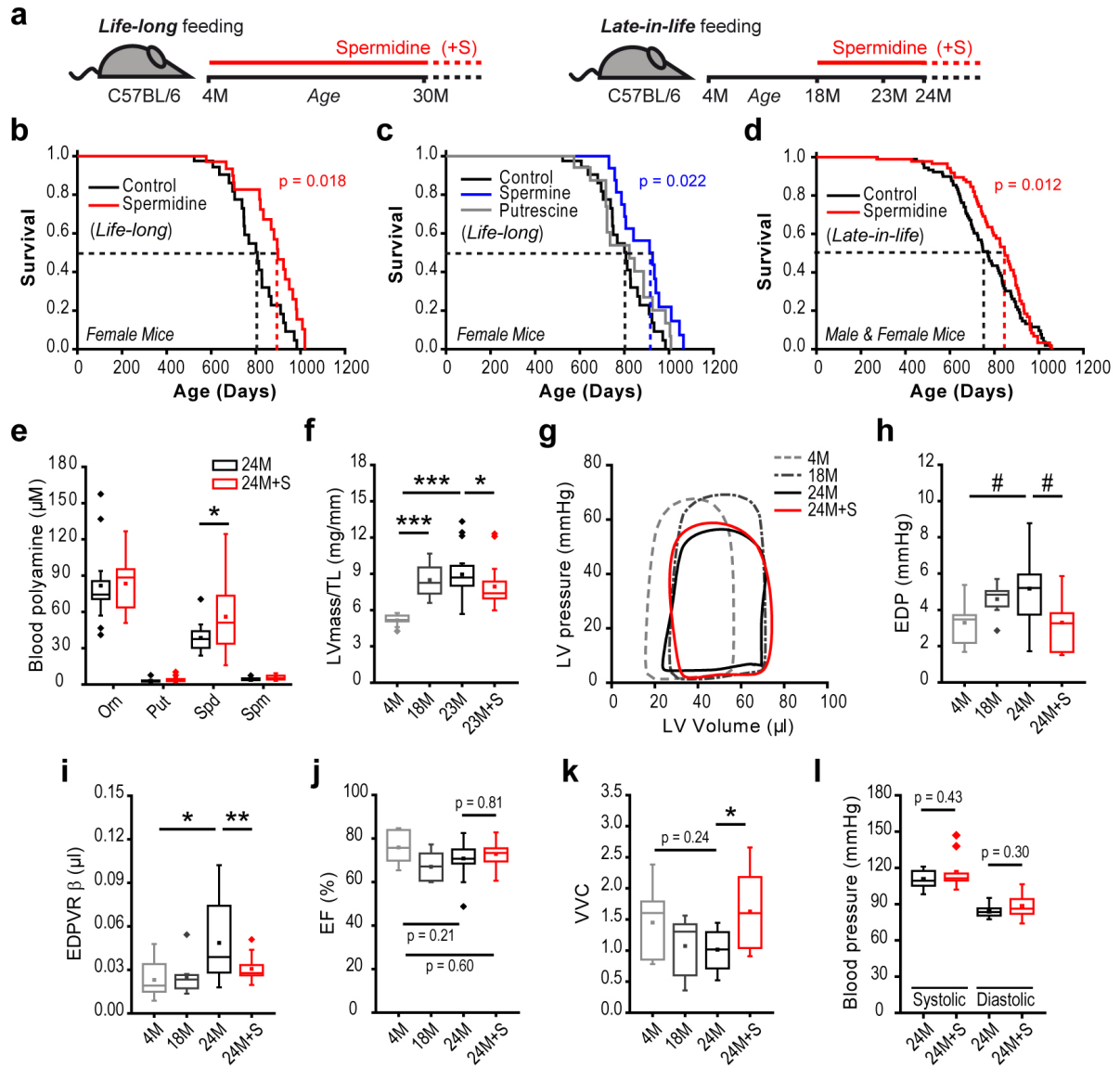


Figure 1

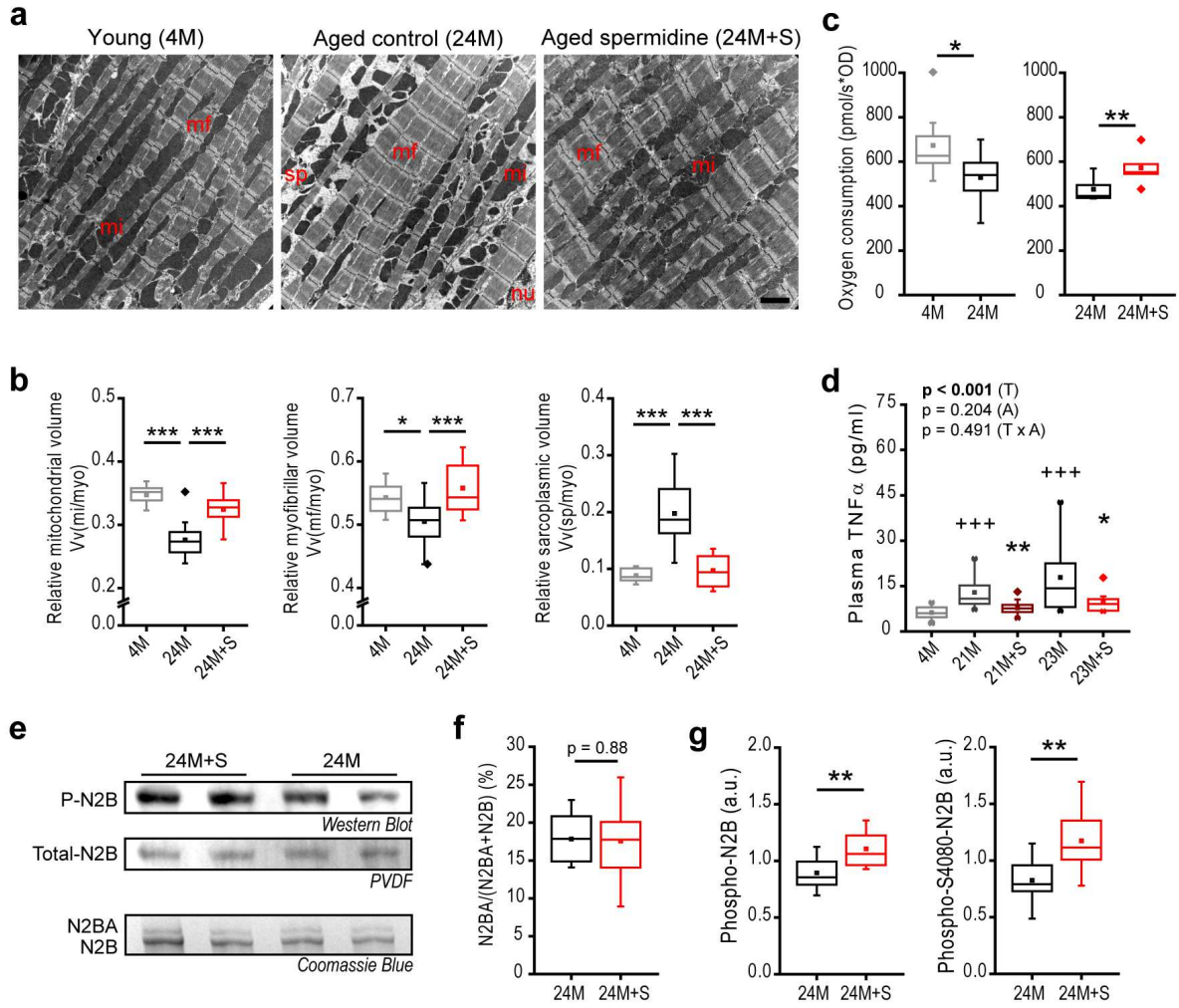


Figure 2

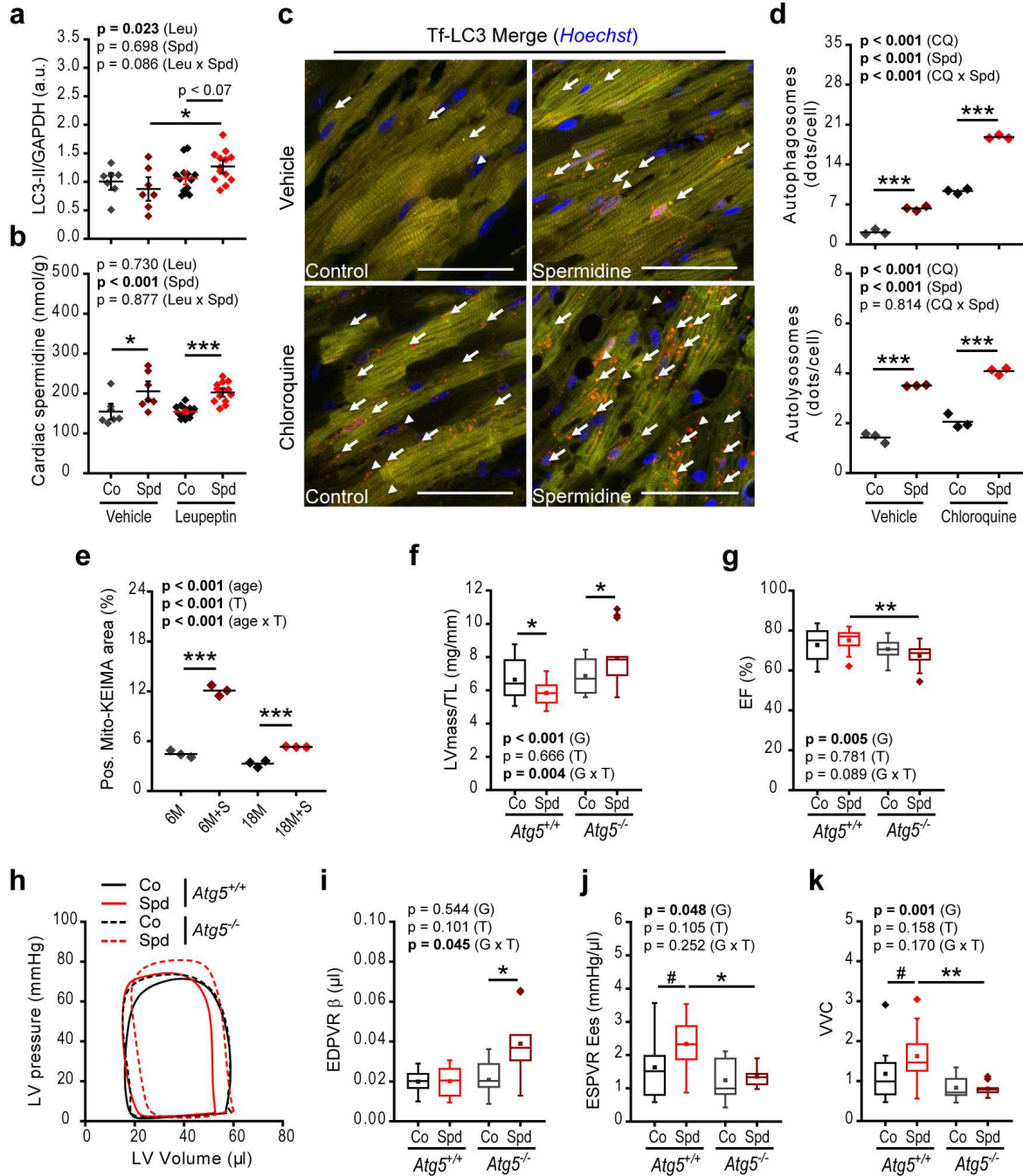


Figure 3

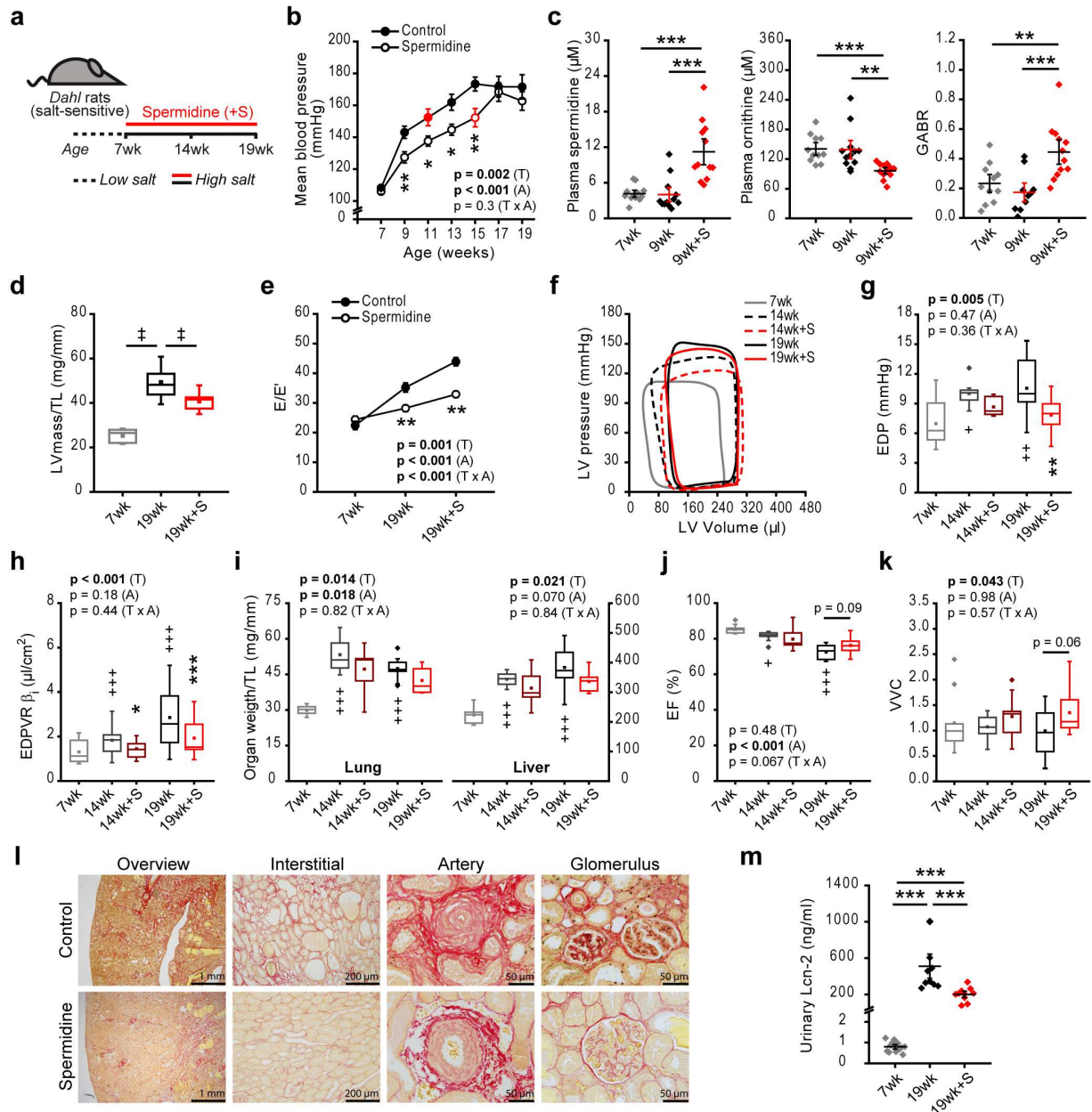


Figure 4

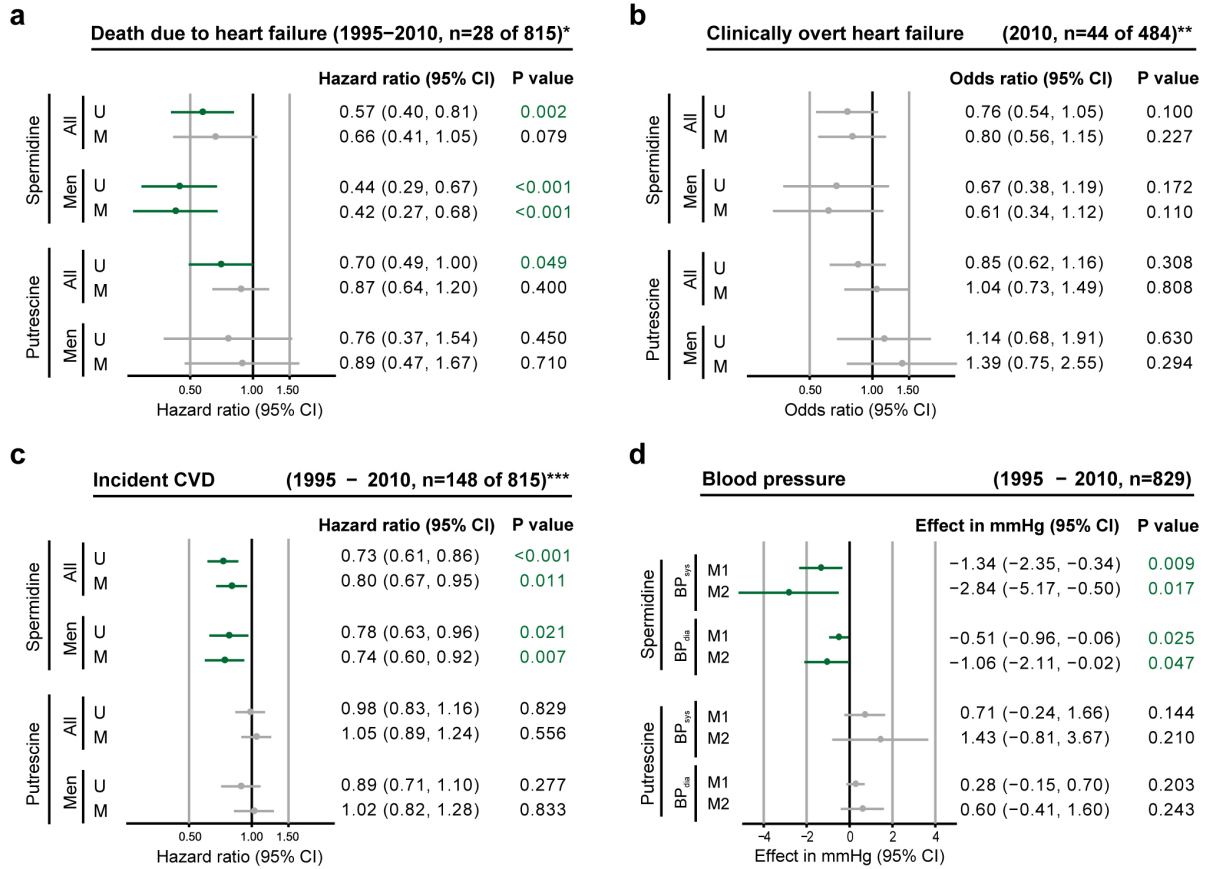


Figure 5

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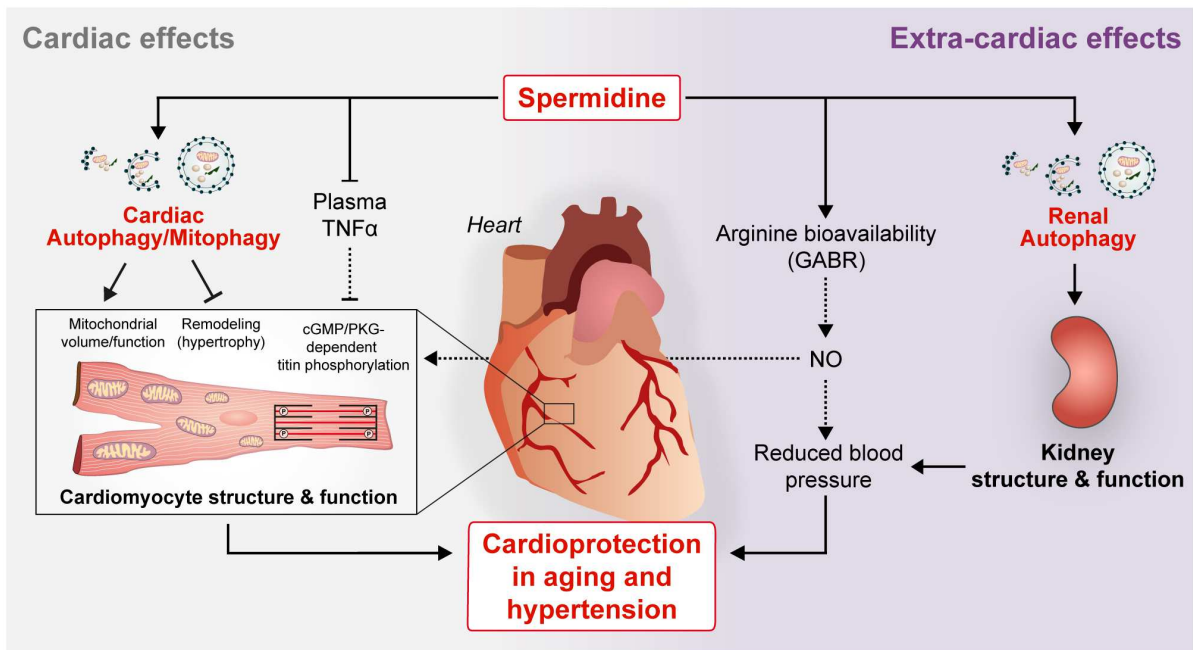


Figure 6

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