

Long-term NMN treatment increases lifespan and healthspan in mice in a sex dependent manner

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Long-term NMN treatment increases lifespan and healthspan in mice in a sex dependent manner

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ABSTRACT

Nicotinamide adenine dinucleotide (NAD⁺) is essential for many enzymatic reactions. During aging, levels of NAD⁺ decrease by up to 50%, the repletion of which provides a range of health benefits in both mice and humans. It remains unknown, however, whether the NAD⁺ precursor nicotinamide mononucleotide (NMN) extends lifespan in mammals. Here, we investigate the effect of long-term NMN on the healthspan and lifespan of male and female mice. In both sexes, NMN reduced frailty, an effect that was associated with increased abundance of *Anaerotruncus colihominis*, a butyrate-producing gut bacterium. In females, but not males, NMN increased median lifespan by 8.5%, and upregulated mitochondrial function markers in metabolic tissues. In males, but not females, NMN increased physical activity and boosted metabolic health, whilst also upregulating inflammatory pathways, likely as a result of sex-specific increased expression of NAD⁺ consumers including CD38. These results highlight the potential of NAD⁺ boosters for treating age-related conditions.

Keywords: NMN, NAD⁺, frailty, healthspan, lifespan, mouse models, sex differences, males, females, longevity, metabolomics, microbiome, metabolism

INTRODUCTION

Nicotinamide adenine dinucleotide (NAD) plays a critical role in almost all major biological processes, including energy metabolism, DNA repair, and gene expression regulation¹. The oxidized version, NAD⁺, is an essential cosubstrate for many enzymes, including sirtuin deacylases² and the DNA damage sensing role of PARPs³, both of which are proposed to play roles in aging. During aging in humans and mice, levels of NAD⁺ decline by up to 50%⁴⁻¹¹, contributing to pseudohypoxia, impaired energy metabolism, loss of epigenetic information, and decreased cell survival¹².

Previous studies have demonstrated that NAD⁺ levels can be increased *in vivo* by supplementing the diet with NAD⁺ boosters, most commonly nicotinamide riboside (NR) and nicotinamide mononucleotide (NMN)¹. These supplements, when given orally to mice and humans, increase NAD⁺ levels 2-5 fold in serum, liver and kidney within 1-4 hours of dosing, with maximum levels reached in humans after 10 days of dosing¹³⁻¹⁵.

Animal studies have demonstrated a wide range of health benefits associated with NAD⁺ boosters, including improved glucose and lipid metabolism, decreased fatty liver, protection against acute kidney injury, increased endurance, enhanced mitochondrial function and reduced inflammation^{1,6,16}. In the context of aging, NMN and NR have been shown to have benefit in mouse models of many age-related diseases including Type 2 diabetes, cardiovascular disease, kidney function, and cancer^{17,18}. The effect of NR on mouse lifespan has shown mixed results, with positive effects seen in a premature aging mouse model¹⁹, and a late-life treatment study of male mice²⁰, whilst a large-scale NIA study of both sexes, using a lower dose, saw no effect²¹. The effect of NMN on lifespan has not yet been tested in mice, although several functional measures of aging are reduced with chronic NMN treatment in older male mice^{6,13}.

The health effects of long-term NAD⁺ boosters in aging female mice have not been as well characterized, although one study in high fat diet fed mice suggested greater benefit of short term NMN treatment in female than male mice²² and short term NMN feeding can restore fertility in reproductively aged female mice²³. Additionally, several studies using mouse models of either overexpression or knock-out of NAMPT (one of the rate limiting enzymes for production of NAD⁺) found an effect of these manipulations on NAD⁺ levels and physiological outcomes in female but not male mice^{24–26}. The authors suggest that female mice may have higher sensitivity to changes in NAMPT and/or NAD⁺ levels than males. More work is needed to understand sex differences in response to NAD⁺ boosters.

Strong preclinical evidence, at least in males, for the wide-ranging health benefits of NAD⁺ boosters¹ has prompted fast growing interest in NMN and NR as potential therapeutics for age-related disease and functional decline. In particular, NMN has at least 34 registered clinical trials as of October 2025 (clinicaltrials.gov). Published clinical results have demonstrated that NMN can increase NAD⁺ levels in blood in humans, and there are initial reports of health benefits including decreased arterial stiffness and cholesterol levels, lower tryglycerides, increased insulin sensitivity, greater endurance, and reduced hypertension^{14,27–35}.

Despite the extensive body of data on NMN supplementation in health and disease from both preclinical and clinical studies, the effect of long-term NMN supplementation on lifespan is not known. Here, we report the effects of chronic NMN treatment started in middle-life on health and lifespan in both male and female mice. Our study reveals that long-term NMN treatment increases lifespan and healthspan in a sex-dependent manner, with both sexes showing delayed frailty and significant increases in the abundance of a putatively healthy, butyrate-producing gut bacterial species, *A. colihominis*, female mice showing an increase in lifespan, and male mice

showing improvements in metabolic health and physical activity. We also show sex-specific effects of NMN on metabolic tissue gene expression and NAD⁺ metabolism, which may explain the differences in lifespan extension observed between the sexes.

RESULTS AND DISCUSSION

Long-term NMN treatment delays frailty in both sexes.

To test the effect of long-term exposure of mice to NMN, we dosed male and female C57BL/6NIA mice with ~550 mg/kg/day of NMN in their drinking water, from 13 months of age (Figure 1A; Supplementary Figure 1A,B). This high dose is the equivalent of approx. 3000mg/day for a 70kg person, 2-3 times the dose usually used in clinical studies^{14,27–35}. The majority of mice were followed for their natural lifespan, with prospectively selected subsets either regularly assessed for health measures or used for tissue collection at 24 months of age (Figure 1A). We assessed frailty using the mouse clinical frailty index³⁶, a well validated non-invasive assessment of 31 age-related health deficits. Frailty index (FI) scores were assessed at baseline and, combined with body weight, used to assign treatment and control groups of mice to ensure an even distribution between groups.

At 18.5 and 21 months of age, both male and female mice exposed to long-term NMN treatment had lower FI scores (Figure 1B, C) indicating a delayed onset of frailty in these mice compared to untreated controls. Analysis of specific deficits indicated that NMN-treated male mice had, among other differences, reduced vision loss, less distended abdomen, maintained fur color, and better breathing rate than controls (Figure 1D). In treated females, there was greater fur color maintenance, better coat condition, less kyphosis and less tail stiffening (Figure 1E). NMN treatment did not accelerate any deficits in either sex (Figure 1D, E).

We recently developed frailty-based algorithms that predict age (FRIGHT age) and remaining lifespan (AFRAID score) in male mice³⁷. Application of these tools to the male mice at 21 months showed that NMN-treated mice had lower predicted age, indicating lower biological age, and higher predicted remaining lifespan than untreated mice (Figure 1F,G). Another commonly used measure of biological age, DNA methylation age, assessed using either blood or liver-based clocks, was not significantly affected by NMN treatment (Supplementary Figure 1C-E). Other measures of systemic function and health in aging were unchanged with NMN treatment, including grip strength (Supplementary Figure 1F,I), nesting and burrowing abilities (Supplementary Figure 1G,H,J,K) and estrous cycling regularity for the female mice (Supplementary Figure 1L).

Long-term NMN treatment increases median lifespan in female mice.

The delayed onset of frailty in the NMN-treated mice was consistent with an extension of healthspan. Next, we assessed whether the treatment also resulted in an extension of lifespan. Two cohorts of NMN-treated mice were assessed and the results were consistent between them (Supplementary Figure 1M-P) and with reported lifespans for wildtype C57BL/6NIA mice, with female mice being shorter lived than the males³⁸. In the NMN-treated females, median lifespan was increased by 8.5% and maximal (90%) lifespan by 7.9%, compared to controls (Figure 1I). Although the male mice treated with NMN appeared to have an early protection against mortality, as indicated by the increased AFRAID scores at 22.5 months and apparent separation of the survival curves up to 24 months (Figure 1H), overall there was no significant increase in either maximum or median lifespan for males (Figure 1H). Interestingly, this is one of the few reported lifespan interventions to have a greater effect in female than male wild-type aging mice, with many

other interventions showing the opposite effect^{39,40}.

For cohort 1, bodies were collected at time of death and, where possible, underwent a thorough blinded histopathological analysis for pathology and disease across 17 tissues. Despite the increased lifespan of the female mice, there was no difference in disease burden (Figure 1J) or morbidity index (Supplementary Figure 1Q) between treated and untreated mice, implying a delay in the onset of age-related diseases generally with NMN treatment, rather than the targeting of a specific disease. In fact, the only histopathological outcome where we saw an effect of treatment was glomerulonephritis grade, a measure of inflammation in the kidneys, which was slightly elevated in the NMN-treated mice compared to controls (Supplementary Figure 1R), despite their increased longevity. This is consistent with observations that centenarians and naked mole-rats have increased uremic toxins (possibly microbial in origin⁴¹), despite their exceptional longevity^{41,42}, and may be related to the high dose of NMN used in this study. Female mice, regardless of treatment, had higher tumor burden and index than male mice (Figure 1K, Supplementary Figure 1S), which may have contributed to the observed sex differences in lifespan in the control mouse cohorts. A recent study observed increased metastasis with the NAD⁺ booster NR in an immune-compromised cancer mouse model⁴³. In this study of wildtype mice, however, there was no effect of NMN on tumor counts or severity across any tissue (Figure 1K, Supplementary Figure 1S). This is consistent with the whole blood count data, where we observed age and sex effects, but no effect of NMN treatment on white blood cell, red blood cell or platelet counts (Supplementary Figure 1T-Y).

Long-term NMN treatment improves metabolic health in male mice.

We tracked body weight every two weeks across the lifespan of the mice in the cohort 1

longevity study. In male control mice we saw the characteristic gain of weight in middle-life, with a decrease in weight in later life (Figure 1L). This curve was attenuated, however, in the NMN-treated mice, with significantly less weight gain between 18 and 24 months of age (Figure 1L), consistent with previous studies¹³. This body weight effect was independent of food intake (Supplementary Figure 2A). Body composition analysis at 24 months of age using DEXA imaging showed that, for NMN-treated male mice compared to controls, this weight difference was a result of a reduction in fat gain, rather than a loss of muscle mass (Figure 1M-P).

To further examine the effects on NMN on metabolism, we assessed fasting glucose levels across four time points and saw the expected decline with age in both untreated controls⁴⁴, and NMN-treated mice (Supplementary Figure 2B). At 24 months, we also measured a panel of metabolic-related hormones in plasma and observed no difference between control and NMN-treated groups (Supplementary Figure 2C-N). Previous studies have reported subtle effects of NMN on glucose metabolism in mice, but at younger ages¹³.

At 25 months of age, a subset of body-weight matched mice were assessed for metabolic flexibility and locomotor activity during the night and day. Respiratory exchange ratio (RER) is the ratio of carbon dioxide produced to oxygen consumed (Supplementary Figure 2O-R) and gives an indication of the source of fuel for energy metabolism in the body. Values closer to 1 indicate mostly carbohydrate metabolism, those closer to 0.7 indicate fat metabolism, and in healthy young mice we typically see a circadian cycling of this ratio from higher in the dark cycle (when mice are active and consume more food) to lower in the light cycle (when mice are less active and feed less). Compared to controls, NMN-treated mice had greater metabolic flexibility than controls, with more pronounced circadian changes in RER (Figure 1Q,R).

The activity of mice is well known to decrease as they age. In the dark cycle, when mice

are most active, NMN-treated male mice showed greater activity compared to the untreated controls (Figure 1S,T). In open-field arenas at 24 months of age, there was no difference between treatment groups for total distance moved in this test (Supplementary Figure 2S), although these assessments were completed during the light cycle only.

To further understand the metabolic changes fueled by NMN, we measured the global metabolome in plasma of male mice at two timepoints post treatment (18.5 and 21 months of age). Besides NAD⁺ metabolites, which we describe below, 137 other metabolites were changed in NMN-treated plasma samples compared to control mice, with 25 up- and 112 down-regulated (Supplementary Figure 3A-B). Enrichment analysis for plasma metabolites down-regulated with NMN treatment showed enrichment for several pathways including glycine and serine metabolism, phospholipid biosynthesis, methylhistidine metabolism and methionine metabolism (Supplementary Figure 3C). Many of these pathways are linked with one-carbon metabolism, the dysregulation of which is known to be associated with age-related diseases including cancer and neurodegeneration^{45,46}, and the manipulation of which is linked to longevity⁴⁷⁻⁴⁹.

In female mice, the protective metabolic effects of NMN seen in the males were not observed. There was a significant effect of age on body weight, with some gain in mid-life, but this was not affected by NMN treatment (Supplementary Figure 2T-U). In females, there was also no difference between lean and fat mass, (Supplementary Figure 2V-X), respiratory exchange ratio or activity in the metabolic cages (Supplementary Figure 2Y-Z) or openfield (Supplementary Figure 2AB). Consistent with whole-body energy expenditure data, female mice showed minimal changes to the plasma metabolome, with only nine non-NAD pathway metabolites significantly changed in NMN compared to control mice (Supplementary Figure 3D).

NMN metabolism differs between males and females after acute and chronic NMN treatment.

Based on previous work suggesting possible greater sensitivity to changes in NAD^+ levels in females than males²⁴⁻²⁶, we hypothesized that the sex-specific effect of NMN treatment on lifespan and metabolic health may have been due to differences in how NMN is metabolized (Figure 2A). To explore this, we completed an acute labelled-NMN treatment experiment to comprehensively characterize NMN metabolism in aged animals across both sexes for the first time (Figure 2B). We dosed 12 and 24 month male and female C57BL/6NIA mice with a bolus of 600mg/kg M+8 NMN isotopically labeled on the [2,4,5,6-D4]-nicotinamide moiety and [5,5-D2]-ribose ring (Supplementary Figure 4A) via oral gavage. To mimic the long-term health and lifespan experiment we also pre-dosed the mice orally with unlabelled NMN for 10 days (Figure 2B). We collected plasma, liver and skeletal muscle 6 hours after the delivery of labeled NMN. We supplemented this with analysis of NAD^+ metabolomics after long-term NMN treatment, using plasma at two timepoints (18.5 and 21 months of age), as well as liver and skeletal muscle at 24-25 months of age.

As expected, long-term NMN treatment led to increased levels of plasma NAD^+ metabolites, specifically NAM, NR, meNAM and NAR (Figure 2C). NAD^+ itself was undetectable in plasma, and NMN levels were low across all groups and unaffected by treatment (Figure 2C). Levels of NAM, meNAM and NAR were higher in NMN-treated females than males (Figure 2C), suggesting possible greater metabolism of NMN in plasma for females across both the NAM salvage and Preiss-Handler pathways. For the acute experiments, NMN and NAD^+ could not be detected in plasma, consistent with rapid metabolism of orally delivered NMN reported previously⁵⁰. NAM levels were increased to a similar extent in both sexes (Supplementary Figure 4B,C). For NA, however, young females had higher levels six hours post-treatment than young

males (Figure 2D, Supplementary Figure 4D,E), supporting the finding of possibly greater Preiss-Handler pathway metabolism of NMN in females. NR supplementation has been shown to cause a similar increase in serum NA levels, although only male mice were assessed⁵¹.

As has been reported previously¹¹, we observed a small but significant decrease in NAD⁺ levels with age in both the liver and gastrocnemius muscle across both sexes (Figure 2E,F), but no baseline differences in NAD⁺ levels between the sexes. In liver, acute NMN treatment also resulted in expected increases in NAD⁺ levels (Figure 2E) and NAAD, a metabolic intermediate in the Preiss-Handler pathway (Figure 2G, Supplementary Figure 4F-G). Although higher NAAD levels were seen with acute NMN treatment in old females than old males (Figure 2G), higher NAD⁺/NADH ratio values were observed in old males than old females (Supplementary Figure 4G). Additionally, after long-term NMN treatment, liver NAD⁺ (Figure 2H), NADH and meNAM (Supplementary Figure 4H-I) levels increased in males, whilst only NAD⁺/NADH ratio increased in females (Figure 2I). This data suggests the opposite of the female upregulation that was seen in the plasma and perhaps hints at different metabolism kinetics across tissues in the two sexes.

In skeletal muscle, acute NMN treatment raised NAD⁺ levels (Figure 2F) but this effect was not as pronounced as in the liver, and no increase in NAD⁺/NADH ratio was seen across sexes (Supplementary Figure 4J). Increases in NAM were observed across all groups (Supplementary Figure 4K), and for NAAD, at least in young males (Figure 2J). No clear sex effects were observed in the muscle after acute NMN treatment. Similar results were seen in gastroc muscle after long-term NMN treatment (Supplementary Figure 4L-O).

Overall, these experiments show that the conversion of NMN to NAD⁺, and other key metabolites, is generally well maintained with age in C57BL/6 mice, with some sex and tissue differences. The plasma data, in particular, suggests possibly greater metabolism via the Preiss-

Handler pathway in females than males, but the inconsistency in liver and gastroc muscle, highlights that these findings are likely timing-, dose- and tissue-specific. Further experiments are required to determine the specific implications of these findings for NAD⁺ precursor dosing, but overall these results suggest that sex differences in NMN metabolism are not the main contributor to our observed sex specific lifespan effects.

Microbial metabolism of NMN via the Preiss-Handler pathway in vivo

Previous work has suggested an important role of the gut microbiome in the metabolism of NMN (and NR) via de-amidation into NA and incorporation into NAD⁺ via the *Preiss-Handler pathway*^{15,51–53} (Figure 2A). Mammalian cells do not contain deamidase enzymes, so microbes have a unique role in this metabolic pathway, but the extent to which NMN is metabolized in this way *in vivo*, rather than directly incorporated into host cells and metabolised via the Salvage pathway, remains unknown. The acute labelled-NMN treatment experiment (Figure 2B) outlined above allows us to address this.

As expected, increased NAD⁺ levels in liver and gastroc with M+6 labelled NMN treatment were almost entirely attributable to the incorporation of exogenous labelled NMN, as indicated by M+3 labelled NMN (Figure 2E, F). In addition, for both tissues, we observed a striking increase in M+4 labelled NAAD (Figure 2G, J), a key intermediate in the Preiss-Handler pathway, but not the salvage pathway (Figure 2A)⁵⁴. Importantly, the lack of detectable M+6 NAD⁺ or NAAD suggests that the majority of NMN underwent hydrolysis into free nicotinamide before its deamidation into nicotinic acid^{49,52}, and eventual metabolism to NAAD and then NAD⁺ (Figure 2A). Thus, in these experiments, the direct incorporation of NMN to NAD⁺ was not a major route of NAD⁺ synthesis after NMN treatment. However, the amount of labelled-NMN that we used in

the current study was low, so these results do not preclude the direct incorporation of some NMN into cells for metabolism via the salvage pathway^{55,56}.

Overall, these results support previous work for both both NR^{15,51} and NMN^{52,57} suggesting that the majority of orally delivered NMN undergoes microbial deamidation by bacteria in the gut, and metabolism via the Preiss-Handler pathway.

Long term NMN-treatment leads to a sustained increase of Anaerotruncus colihominis in the gut microbiome of both sexes

In C57BL/6J mice, NMN and NR have previously been shown to increase the abundance of some commensal bacterial taxa (*Ruminococcae* and *Prevotellaceae*) while decreasing the abundance of others (*Bilophila* and *Oscillibacter*)⁵⁸. To identify potential causes of increased health and lifespan, we examined the gut microbiome features associated with NMN treatment across the lifespan by taking feces from four timepoints and subjecting 358 samples to metagenomic shotgun sequencing. Age was the largest observed contributor to bacterial species-level beta-diversity, explaining 15% of the variance in Bray-Curtis distances in female and male mice alike (PERMANOVA $p=0.001$). NMN-treatment was strongly associated with beta-diversity in female mice and weakly in male mice (Figure 3A,B). Two specific species were increased with NMN treatment after adjusting for sex and age, including *Bacteroides caecimuris* (Supplementary Figure 5A,B), a mouse gut microbe known to decrease in aging⁵⁶. Investigation of the association of *B.caecimuris* with plasma metabolites, identified positive correlations with indole products (Figure 3C), suggesting a possible anti-inflammatory role of this microbe.

The largest observed shift in species abundance with NMN, however, was observed for *Anaerotruncus colihominis*, a butyrate-producing bacterium in the *Oscillospiraceae* family, which

has been shown to suppress neuroinflammation in mice and is enriched in human centenarians^{59–61}. Long-term NMN supplementation induced a more than 10-fold increase in *A. colihominis* abundance in female and male mice alike, independent of age (Figure 3D,E). In particular, even though *A. colihominis* declined steadily with age in both the NMN-treated and control mice, its NMN-induced increase was maintained across the lifespan. *A. colihominis* levels were also associated with lower FI scores (Figure 3F) and longer lifespan across male and female mice, adjusting for age (Figure 3G). Mediation analysis across all detected microbes, adjusting for age and treatment, and controlling for the false discovery rate (FDR $q < 0.05$), suggests *A. colihominis* is a complete mediator of the effect of NMN on frailty for both males and females (Figure 3H, Supplementary Figure 5C), and is a partial mediator of the effect of NMN on lifespan for males only (Figure 3I, Supplementary Figure 5D).

Together this provides evidence that the NMN-induced increase in *A. colihominis* is likely contributing to the health effects of NMN in the host. Interestingly, this association appears to be stronger in female mice, supporting the findings of increased plasma NMN Preiss-Handler metabolites in females, and possibly contributing to the female-specific lifespan increase observed with NMN treatment.

Anaerotruncus colihominis metabolises NMN into NAD^+

Given the strong association between NMN treatment and *A. colihominis*, we sought to clarify the mechanisms of this interaction. When we explore the association between *A. colihominis* and plasma metabolites we see a strong correlation with NAD^+ metabolites including methylnicotinamide, nicotinamide and NR (Figure 3C), suggesting a direct role of *A. colihominis* in metabolizing NMN in the gut. This is supported by metagenomic analysis of the microbial genes

differentially expressed in our samples with NMN treatment (Supplementary Figure 5E). Enrichment analysis identifies significant upregulation of nicotinate and nicotinamide metabolism-related genes in both males and females with NMN treatment compared to controls, and these genes are largely from the Clostridia class (Figure 3J), of which *A. colihominis* is a member.

These results, combined with our labelled NMN experiment finding that microbial deamidation by bacteria in the gut is the main route of NMN metabolism *in vivo*, suggested a possible role of *A. colihominis* in directly metabolizing NMN. To test this hypothesis, we anaerobically cultured *A. colihominis* with and without M+12 labeled NMN (see methods), and directly measured cell growth, and NAD⁺ metabolite content. Bacterial growth curves were largely unaffected by treatment, with OD values comparable between treated and untreated cultures throughout the exponential phase. A slight but consistent increase in OD was observed selectively at stationary phase in treated cultures, suggesting a possible effect of NMN during nutrient deprivation conditions (Figure 3K). At 6 hrs, we found M+12 labeled NMN within bacterial cells, indicating direct uptake of the labelled NMN (Figure 3L). By 20 hours, NMN levels had dropped but there was a large increase in M+3 labelled NAD⁺ (Figure 3M), indicating that the labelled NMN had been deamidated before incorporation into NAD⁺ within the *A. colihominis* cells.

These data suggest that *A. colihominis* does metabolise NMN, which results in increased intracellular NAD⁺ and increased growth of this microbe, which may directly or indirectly contribute to increased health after NMN treatment. Labelled NA was not detected in the media after treatment (Supplementary Figure 5F), indicating it is unlikely *A. colihominis* is directly providing this deamidated metabolite for uptake in the gut. *A. colihominis* may provide other potentially beneficial metabolites, such as butyrate⁶² and acetate, which could indirectly exert a

health effect in the host. More specific exploration of these mechanisms is needed.

Overall these findings suggest a potential opportunity to optimize the microbiome, such as with an *A. colihominis* probiotic, to synergistically boost the benefits of orally-delivered NAD⁺ precursors.

Long-term NMN treatment prevents some age-related gene expression changes in muscle and liver, in a sex-specific manner

Given the mixed effects of sex on NMN metabolism, we sought clearer explanations for these observed sex differences in metabolic health and lifespan with NMN treatment. We measured the transcriptomes of key metabolic tissues - gastrocnemius muscle, liver and white adipose tissue (WAT) - from 24 month-old NMN-treated mice and compared them to untreated age-matched controls, and 6 month old untreated mice.

We first completed an untargeted transcriptome-wide analysis of differentially expressed genes in 24 month old NMN compared to control mice, and found that NMN treatment had variable effects on gene expression across tissues and sexes (Supplementary Figure 6A). The strongest effect of NMN treatment was observed in the skeletal muscle of males, and surprisingly, there was almost no overlap between groups (Supplementary Figure 6A), implying that there are tissue and sex specific effects of NMN, rather than a conserved set of genes affected by NMN treatment in all contexts. We focused on the male muscle effects, and applied hierarchical clustering to the top 400 genes changed with NMN treatment, across all samples. Interestingly, the young and NMN-treated groups were clustered separately from the old controls (Supplementary Figure 6B), perhaps indicating that NMN treatment prevents some age-related gene expression changes in males, consistent with previous studies in skeletal muscle, liver¹³, aorta⁶³, and brain

vasculature⁶⁴. As far as we are aware, gene expression changes in aging females with NMN treatment have not previously been characterized, and it is interesting that, at least in this untargeted analysis, the effects of NMN were much less clear in female liver, muscle and WAT than in males (Supplementary Figure 6A).

To quantitatively assess the effect of NMN treatment on the transcriptomic age of old mice, we applied mouse multi-tissue transcriptomic clocks of chronological age (tAge)⁶⁵ across tissues, sexes, and treatment groups (Figure 4A, Supplementary Figure 6C,D). In female mice, the clock showed a trend towards decreased tAge in NMN-treated WAT relative to controls (Figure 4A), whereas no significant NMN-induced changes were detected in females for muscle or liver (Supplementary Figure 6C,D). In males, the clock indicated a slight increase in tAge across tissues of old NMN-treated mice compared to controls (Figure 4A, Supplementary Figure 6C,D).

To assess qualitative gene expression changes associated with the pro- and anti-aging effects of NMN, we performed gene set enrichment analysis (GSEA) and compared functional signatures of NMN with established transcriptomic signatures of aging and lifespan-extending interventions^{66,67} (Supplementary Figure 6E). Consistent with the lifespan data, NMN-associated changes in females clustered with signatures of lifespan-extending interventions, including calorie restriction and rapamycin, and displayed anti-aging features such as upregulation of genes involved in oxidative phosphorylation, respiratory electron transport, mitochondrial translation, and DNA repair, along with downregulation of genes associated with inflammatory responses and complement and coagulation cascades (Supplementary Figure 6E). In males, however, we observed the opposite pattern. NMN-induced gene expression changes were characterized by upregulation of inflammatory responses and interferon signaling pathways, together with downregulation of fatty acid metabolism, mitochondrial translation, and oxidative

phosphorylation, thereby recapitulating aging-associated changes (Supplementary Figure 6E). These pro-aging functional changes were especially pronounced in male WAT and liver. Together, these findings support sex-specific effects of NMN and point to potentially maladaptive molecular effects in males (Figure 4A, Supplementary Figure 6C-E), which may contribute to the absence of lifespan extension in male mice following NMN treatment.

Long-term NMN treatment upregulates mitochondrial activity, DNA repair and inflammation pathways in muscle and liver in a sex-specific manner

To dig further into these complex findings and understand what is driving the sex-specific effects of NMN on muscle and liver gene expression, we completed GSEA comparing gene expression in old NMN versus control groups for both sexes (Figure 4B,C). This analysis shows that in both liver and muscle from female NMN-treated mice, compared to age-matched controls, there is upregulation of mitochondria-related pathways, including mitochondrial translation and oxidative phosphorylation (Figure 4B,C). To confirm this enrichment we assessed mtDNA copy number and saw increases in female NMN-treated muscle and liver (Figure 4D,E), adding additional evidence of increased mitochondrial function with NMN treatment in female mice. For male NMN-treated mice, enrichment of mitochondrial pathways was not observed (Figure 4B,C), and there was no change in mtDNA copy number with NMN treatment in either liver, muscle or WAT (Figure 4D,E, Supplementary Figure 6F). The findings in the female mice support previous work showing that NMN increases mitochondrial function in brain⁶⁸, hepatocytes⁶⁹ and ovaries^{70,71}, although an effect of sex was not previously observed. This data provides a possible sex-specific mechanism contributing to the increased healthspan and lifespan observed with NMN treatment in female mice.

In muscle, comparing NMN-treated to control samples, we also see enrichment of DNA repair pathways and other cell cycle and proliferation related pathways (Figure 4C). To further explore this, we focused on specific genes within these pathways and completed a targeted differential expression analysis (Figure 4F, Supplementary Figure 6G). This analysis shows increased expression of many of these genes, including *Xrcc1*, *Gadd45a* and *Trp53* with NMN treatment in liver and muscle of male mice (Figure 4F, Supplementary Figure 6G), but not female mice (Figure 4F, Supplementary Figure 6G). *Trp53*, in particular, is a tumor suppressor protein that plays a critical role in DNA repair and cell cycle maintenance⁷². We confirmed that protein levels of p53 also increase with NMN treatment in male (but not female) liver (Figure 4G). These findings are consistent with previous work showing increased DNA repair with NMN treatment⁷³, and this may contribute to the observed beneficial health effects of long-term NMN treatment, especially in male mice.

In concert with the increased DNA repair signatures seen with NMN treatment in male mice, we also see upregulation of inflammation and immune response pathways in the liver and muscle (Figure 4B,C). Targeted analysis of immune and inflammation-related genes confirms increased expression in both liver and muscle, of specific genes associated with several immune cell types including neutrophils and T cells in NMN treated males, but not females, compared to controls (Supplementary Figure 7A,B). We also confirmed that protein levels of NF- κ B, a central regulator of inflammation, were increased in liver from male, but not female, NMN-treated mice (Figure 4H).

Interestingly, this upregulation of inflammatory and immune pathways with NMN treatment appears to have both beneficial and maladaptive consequences across tissues for male mice. When we look directly at muscle fiber histology, we observe less ECM fibrosis, and larger

fiber size for NMN-treated males compared to controls (Figure 4I-L), which may be a result of increased immune function reducing fibrotic build up, and preventing age-related fiber atrophy^{74,75}. No histological changes with NMN treatment were observed in female muscle (Supplementary Figure 7C,D). Additionally, we measured circulating inflammatory cytokines in plasma at 24 months, and see a moderate systemic anti-inflammatory effect of NMN across both sexes (Supplementary Figure 7E-H). Out of 31 cytokines measured, four showed a significant effect of treatment (Supplementary Table 1), with reduced levels in NMN-treated mice compared to controls (Supplementary Figure 7E-H), especially for males. Analyses of whole brain tissue also show a trend towards reduced GFAP, a marker of neuroinflammation, with NMN treatment across males and females (Supplementary Figure 7I-L). Although these findings require further exploration in future work, together they suggest a complex, and potentially tissue specific, inflammatory phenotype with NMN treatment, especially in males.

Overall, these sex-specific findings imply a balance of beneficial and adaptive effects of high dose NMN treatment across tissues, particularly in males. Increased mitochondrial activity with NMN treatment in female mice may be contributing to their increased lifespan. However, for NMN treated male mice, increased DNA repair may be contributing to metabolic health benefits, but this is balanced by an increased inflammatory response that may explain the lack of increased lifespan compared to females.

NAD⁺ consuming enzymes upregulated in muscle from male, but not female mice, with high dose NMN treatment

NAD⁺ consuming enzymes including PARPs, CD38 and sirtuins are known mediators of both inflammation and DNA repair, involved in immune cell activation and recruitment⁷⁶⁻⁷⁸, and

linked to age-related declines in NAD⁺ levels^{4,11}. Given this, we sought to determine if the observed sex differences in gene expression outcomes, especially the increased inflammatory phenotype observed in males but not females, may be related to sex differences in expression of NAD⁺ consuming genes.

We used targeted analysis to look at the differential gene expression of 20 known NAD⁺ consumers after long term NMN treatment in muscle and liver across males and females (Figure 4M,N). We found consistent increased expression of many of these NAD⁺ consuming genes after NMN treatment in males, but not females (Figure 4M,N). In particular, CD38, PARP4 and 6 were increased in male liver (Figure 4M) and PARP10, 12 and 14 in male muscle (Figure 4N), all of which have known pro-inflammatory effects^{78,79}. However, in addition to these inflammation-related NAD⁺ consumers, we also saw an increase in possibly beneficial sirtuin enzymes including SIRT2, 3 and 7 in muscle (Figure 4N), and SIRT2 and 4 in liver (Figure 4M), which are associated with anti-fibrotic and anti-inflammatory effects^{80–82}. The balance of these enzymes may be contributing to the balance of beneficial and maladaptive effects observed across tissues in males with long-term NMN treatment.

These sex-specific findings provide a possible explanation for the male-specific upregulation of DNA repair, with the accompanying increase in inflammation, that is not observed in females after NMN treatment, which may contribute to the sex specific health and lifespan findings of our study. We can hypothesize that NMN-treated males have greater CD38 and PARP activity than females (possibly as a result of estrogen receptor-PARP interactions^{83,84}), which consume the NMN-derived NAD⁺ (and induce DNA repair and inflammatory pathways) whilst in females more NAD⁺ is available for redox reactions which results in increased mitochondrial health. This is supported by previous work including a study of long-term CD38 inhibitor treatment

in mice, that observed increased lifespan in males, but not in females⁸⁵, and previous studies of PARP inhibitors in stroke and infection models have also observed benefits in males, but not females⁸⁶. These findings suggest the possible use of a CD38 (or PARP) inhibitor in combination with NMN treatment in aging males to maximize health benefits. More research is needed, however, especially in the context of menopause and reduced estrogen in aging for women⁸⁷, in order to identify optimal synergistic interventions to boost NAD⁺ in aging for both males and females.

CONCLUSION

This is the first study to explore the sex-specific and long-term effects of NMN treatment on frailty and lifespan in mice. NMN treatment delayed frailty and increased both median and maximum lifespan in female, but not male, mice. Although we did not observe a lifespan extension in male mice, NMN was well tolerated, and the male mice showed delayed frailty and improved metabolic health. Additionally, in both sexes NMN-treatment led to a sustained increase in the gut microbe *A. colihominis*, which we show incorporates NMN-derived NA into NAD⁺ to support growth, and may contribute to the beneficial health effects of NMN. Our detailed exploration of NMN metabolism in both sexes, suggests tissue-specific sex differences in the Preiss-Handler metabolic pathway, which may contribute to sex differences in health outcomes. Sex-specific gene expression outcomes provide possible explanations for sex differences in health and lifespan, with female muscle and liver showing upregulation of mitochondrial function with long-term NMN treatment. Males, however, show increased expression of genome stability genes, along with upregulation of inflammation and immune responses pathways, which appear to provide a balance of both beneficial and maladaptive changes with high dose NMN treatment. Male-specific

upregulation of NAD⁺ consumers, especially CD38 and PARP14, with NMN treatment may contribute to these sex differences, and suggest the opportunity to pharmacologically inhibit these enzymes to maximize the benefits of NMN supplementation in males. Overall, this work adds to the promising preclinical evidence for NMN as a therapeutic to increase health in aging and highlights the importance of exploring sex differences in aging studies.

Data Availability

The RNA-seq data have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE345519. The metabolite data used in this study are archived in Zenodo at <https://doi.org/10.5281/zenodo.22239891>. The microbiome metagenome data have been deposited in NCBI's Sequence Read Archive (SRA) and are accessible through BioProject accession number PRJNA1522491.

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Author Contributions

D.A.S., M.B.S., L.R., M.S.B., and A.E.K. initiated and conceived of the project. A.E.K. performed most of the experiments, analyzed data, and co-wrote the manuscript. K.C, R.T.S., N.R.J. and R.A.Z. completed tissue metabolomics and microbiome experiments and analysis. M.B.S., M.A, J.L., J.A., S.M., X.T., R.C., K.W., L.S., C.D., K.L., and M.M. assisted with or performed mouse experiments. Y.G, M.Z, A.T and D.Z. analyzed RNA-seq data, A.C, C.D and S.M.G. analyzed microbiome data. H.T-W., S.O., M.A, M.Z., K.R., J.L., J.A., P.G., X.T., C.P., T.D.M., C.O, N.I., A.M. and M.M. assisted with tissue processing, experiments and analysis. L.W provided labelled NMN, and Y.I did histopathology analysis. J.M., M.B.S., M.S.B., R.d.C., L.W., Y.I., V.G., J.B., J.M.R., G.C., L.R. and D.A.S provided advice and assistance with experiments. D.A.S. supervised the projects execution and co-wrote the manuscript. All authors reviewed and edited the manuscript.

Declaration of Interests

D.A.S. is a founder, equity owner, advisor to, board member of, and inventor on patents licensed to MetroBiotech, a company developing NAD⁺ boosters to treat diseases. He is also founder and chair of Life Biosciences, an epigenetic reprogramming company. Additional info on D.A.S. affiliations can be found at <https://sinclair.hms.harvard.edu/david-sinclairs-affiliations>. J.A.B reports receiving research funding and materials from Pfizer, Elysium Health, Calico Life Sciences, and Metro International Biotech; and consulting fees from Pfizer, Elysium Health, Altimmune, and Cytokinetics; he holds a patent for using NAD⁺ precursors in liver injury. S.M.G. is on the scientific advisory board of Thorne, Inc., which is a consumer supplement company.

Thorne was not involved in the funding, design, implementation, or publication of this work. L.E.W. is a co-founder, equity holder, scientific advisor and inventor on patents licensed to MetroBiotech. He is also a founder and scientific advisor to Jumpstart Fertility, and his lab at UNSW is supported by Proto Axiom. The other authors declare no competing interests.

FIGURE LEGENDS

Figure 1. Long-term NMN treatment delays frailty in both sexes, increases median lifespan in female mice and improves metabolic health in male mice.

(A) Experimental design schematic. (B) Frailty index (FI) scores for male and (C) female mice. Mixed effects model p value results for age, treatment (NMN) and the interaction (a*N) shown in boxes, and posthoc Sídák's multiple comparisons p values shown on graphs if $p < 0.1$. Males $n=80$ controls, $n=79$ NMN; females $n=20$ controls, $n=20$ NMN at 13.5 months. (D) Mean scores ($\pm$ SEM) for individual frailty index items for males and (E) females. * indicates $p < 0.05$ for unpaired t-test between NMN-treated mice and controls. (F) FRIGHT Age (model for chronological age), and (G) AFRAID scores (model for remaining lifespan) for male mice at 21 months of age. Unpaired t-test p-values show on graph, $n=67$ NMN, $n=71$ controls. (H) Kaplan-meier survival curves for NMN-treated and control males and (I) females. The X-axis starts at 13 months of age when NMN treatment was started. Log-rank test p-value show in boxes. (J) Disease burden (sum of diseases as determined from post-mortem histopathology analysis across 17 tissues) and (K) tumor burden (count of tumors from post-mortem histopathology analysis) for male ($n=78$ NMN, $n=88$ control) and female ($n=15$ NMN, $n=20$ control) mice. Two way ANOVA results for treatment (NMN), sex and the interaction (s*N) shown in boxes, and posthoc Tukey's multiple comparisons p-values shown on graphs if $p < 0.1$. (L) Mean body weight change (as a % of baseline weight at 13 months) for male mice (control $n=109$, NMN $n=109$ at baseline). (M) Representative DEXA imaging scans of control or NMN-treated male mice at 24 months of age. (N) Mean lean mass (as percent of total body mass), (O) mean fat mass (as percent of total body mass) or (P) lean/fat ratio for male mice ($n=7$ per group) at 24 months of age. (Q,R) Respiratory exchange ratio and (S,T) activity as determined from indirect calirometry for male mice at 24 months of age ($n=6$

control, n=7 NMN). For (R) and (T), mean values shown for each 12 hour period of either light or dark. Two way ANOVA p-value results for time, treatment (NMN) and the interaction (t*N) shown in boxes, and posthoc Sídák's multiple comparisons p values shown on graphs if $p < 0.15$. See also Supplementary Figures 1-3.

Figure 2. NMN metabolism is affected by sex, but not age, in mice

(A) Schematic showing NAD^+ metabolism via the salvage and Preiss-Handler pathways (NAD, Nicotinamide adenine dinucleotide; NMN, Nicotinamide mononucleotide; NR, Nicotinamide riboside; NAM, nicotinamide; meNAM, methyl-NAM; ADP, Adenosine diphosphate; NAR, nicotinic acid riboside; NAMN, nicotinic acid mononucleotide; NA, nicotinic acid; NAAD, Nicotinic acid adenine dinucleotide). (B) Schematic of acute labelled NMN experiment. Male young (12 months) n=5 controls, n=5 NMN; females young (12 months) n=4 controls, n=6 NMN; Male old (24 months) n=4 controls, n=6 NMN; females old (24 months) n=4-5 controls, n=4-5 NMN. (C) Heatmap of NAD^+ metabolites in plasma of mice treated with NMN from 13 months of age, or control male (n=22 control, n=23 NMN) and female mice (n=20 control, n=19 NMN). For the y axis labels, *italics* indicates a significant effect of sex (using a linear model, controlling for age and treatment), **bold** indicates a significant effect of treatment (using a linear model, controlling for age and sex). (D) Levels of NA in plasma from young (Y) and old (O) male and (I) female mice 6 hrs post oral gavage with 600mg/kg of NMN. 3-way ANOVA significant p-value results for treatment (NMN), age, sex and interaction terms shown in boxes, and posthoc Sídák's multiple comparisons p values shown on graphs if $p < 0.10$. (E) Total ion counts (TIC) of NAD^+ in liver or (F) gastroc muscle or (G) NAAD in liver, from young and old male and female mice 6 hrs post oral gavage with 600mg/kg of NMN. 3-way ANOVA significant p-value results for treatment

(NMN), age, sex and interaction terms shown in boxes, and posthoc Sídák's multiple comparisons p values shown on graphs if $p < 0.15$. Proportions of labelled NAD^+ or NAAD levels are indicated by colored bars. M+0 indicates endogenous NAD^+ . (H) Relative levels (data mean-centered and divided by the standard deviation of each variable) of NAD^+ , or (I) NAD^+/NADH ratio in liver of male (n=7 control, n=8 NMN) and female (n=7 control, n=8 NMN) mice treated with NMN from 13 months of age or controls. Adjusted p-values from multiple t-tests shown on figures if $p < 0.1$. (J) TIC of NAAD in muscle, from young and old male and female mice 6 hrs post oral gavage with 600mg/kg of NMN. 3-way ANOVA significant p-value results for treatment (NMN), age, sex and interaction terms shown in boxes, and posthoc Sídák's multiple comparisons p values shown on graphs if $p < 0.15$. See also Supplementary Figure 4.

Figure 3. Long-term NMN treatment increases levels of *A. colihominis*, a direct metabolizer of NMN.

(A) Principal coordinate analysis of Bray-Curtis distances of stool microbial communities from NMN-treated and control female and (B) male mice. P values for PERMANOVA corrected for age shown on graph. (C) Biclustered heatmap displaying correlations between microbiome and metabolite features that were significantly correlated with NMN treatment following FDR correction. Regression r values are displayed using blue-to-yellow heatmap coloring. All regressions included age and sex as covariates as well as animal ID as a mixed effect. (D) CLR normalized relative abundances of *A. colihominis* across treatment in females and (E) males. Two-way ANOVA significant p-value results for treatment (NMN), age and interaction term shown in boxes, and posthoc Sídák's multiple comparisons p values shown on graphs if $p < 0.10$. (F) Correlation of frailty score and (G) lifespan with *A. colihominis* across sexes. Ordinary least

squares fits displayed across sexes, line shading represents 95% confidence interval. (H) Mediation of NMN treatment (X) on frailty score (Y) by *A. colihominis* (M) in female mice. (I) Mediation of NMN treatment (X) on lifespan (Y) by *A. colihominis* (M) in male mice. For both mediation results the average causal mediation effect (ACME) p-value is displayed on the curved arrow connecting X to M to Y, the average direct effect (ADE) p-value is the arrow connecting X to Y. The p-values for the effect of X on M and M on Y are also reported and derived from their corresponding linear mixed effects models. The marginal R^2 (R^2_m) for the model $Y \sim X + M + (1|Animal)$ is reported for each median result. All regressions included age and sex as covariates, unless stratified by sex, as well as animal ID as a mixed effect. (J) Barplot displaying cumulative gene abundances at the pathway level grouped by predicted organism taxonomy for the KEGG nicotinate and nicotinamide path. (K) Growth curves over 20 hours for cultured *A. colihominis* +/- 250 uM labeled NMN. (L) Total ion counts (TIC) of NMN or (M) NAD^+ in *A. colihominis* cells cultured for 6 or 20 hrs +/- 250 uM labeled NMN. T-tests comparing control and NMN at each timepoint shown on graphs if $p < 0.10$. Proportions of labelled NMN or NAD^+ are indicated by colored bars. M+0 indicates endogenous NAD^+ . See also Supplementary Figure 5.

Figure 4. Long-term NMN treatment prevents age-related gene expression changes in male muscle and liver.

(A) Biological tAge calculated based on gene expression data from liver for young (6 month), old (24 month) control and old NMN-treated male and female mice. (B) Heatmap showing the Normalized Enrichment Scores (NES) of selected biological pathways from GSEA (Hallmark, GO, and Reactome) in male and female old NMN treated compared to control mouse liver or (C) gastroc muscle. Statistical significance is indicated by asterisks based on nominal p-values (* $p <$

0.05, ** $p < 0.01$, *** $p < 0.001$). (D) Relative mtDNA copy number (ratio of mtDNA to nDNA) in liver or (E) muscle from female NMN treated or control mice at 24 months of age. $n=5-6$ mice per group. Student's t-test p-value results shown on graphs if $p < 0.10$. (F) Log2 fold change (Log2FC) of NMN compared to control genome stability-related gene counts in 24 month old male or female liver. * indicates p-value < 0.05 using Welch's t-test based on the raw count distributions of NMN compared to control. (G) Normalised expression levels of p53 and (H) NF- κ B in liver from 24 month old NMN treated or control mice. 2-way ANOVA significant p-value results for treatment (NMN), sex and interaction terms shown in boxes, and posthoc Fisher's LSD multiple comparisons p values shown on graphs if $p < 0.10$. (I) Representative images of 24 month male control or NMN treated TA muscle stained with Mason's Trichrome, where blue staining indicates fibrosis, or (J) stained with H&E. (K) Quantification of percent fibrosis in the extracellular matrix or (L) muscle fibre cross-sectional area across control or NMN treated 24 month male TA muscle ($n=6-8$ per group). T-test p-value results shown on graph. (M) Log2 fold change (Log2FC) of NMN compared to control NAD⁺ consuming enzyme gene counts in 24 month old male or female liver or (N) muscle. * indicates p-value < 0.05 using Welch's t-test based on the raw count distributions of NMN compared to control. See also Supplementary Figures 6 and 7.

METHODS

Mice

Male and female C57BL/6NIA mice were obtained from the National Institute on Aging (NIA) Aging Rodent Colony. Mice were housed at Harvard Medical School in ventilated microisolator cages with a 12 hour light cycle, at 71°F with 45-50% humidity. For the duration of the

experiments mice were fed AIN-93G Purified Rodent Diet (Dyets Inc, PA). Mice were group housed (3-4 mice per cage) to start, although over the lifespan experiment cage-mates died and mice were left singly housed. Mice were checked daily for health and survival. Mice were euthanized if determined to be moribund (likely to die in the next 48 hours) by an experienced researcher or veterinarian based on exhibiting at least two of the following: inability to eat or drink, severe lethargy or persistent recumbence, severe balance or gait disturbance, rapid weight loss (>20% in one week), an ulcerated or bleeding tumor, and dyspnea or cyanosis. In these cases, the date of euthanasia was used as an estimate of date of death. 16.9% of mice were censored as either their cause of death/euthanasia was determined to not be age-related (eg. dermatitis, fighting, injury, n=66) or they were used for tissue collection (n=28). For the duration of the experiments all cage changes and food/water replacements were completed by the research team, such that mice were only handled by a limited number of people. All animal experiments were approved by the Institutional Animal Care and Use Committee of the Harvard Medical Area.

NMN preparation and treatment

Mice were allocated by cage at baseline (12 $\pm$ 1 month of age) to NMN treatment or control groups based on their frailty index (FI) scores and body weights. NMN was sourced from GeneHarbor Biotechnologies Ltd (Hong Kong) and stored at -20°C. Purity was assessed with LC-MS, and Endotoxin contamination was ruled out with an LAL Endotoxin Assay Kit (Genscript). Once per week NMN was made up in reverse osmosis water at a concentration of 4.8 g/L, filter sterilized and provided to mice in their regular water bottles. For Cohort 1 mice (n=218 males, n=40 females) water was weighed each week in order to calculate the total water consumed per cage per week and thus the average NMN dose ingested per mouse per day.

Mouse health assessments

For all Cohort 1 mice (n=218 males, n=40 females) every two weeks body weights were taken for each mouse, and food intake and water intake were measured per cage. For a subset of Cohort 1 mice, regular assessments of health and function were also completed:

Frailty was assessed with the mouse clinical frailty index as previously described³⁶. FRIGHT and AFRAID scores were calculated as previously described³⁷ using frailty index items and body weight changes over time.

Grip strength was assessed using a Bioseb grip strength meter. Mice were held by the tail base and allowed to grip the meter. Mice were slowly pulled back from the meter until they naturally release their paws, and the maximum force recorded. Mice underwent 5 consecutive trials with at least 15 minutes rest between trials. The highest grip strength value was excluded, and the average of the remaining 4 trials used as the final grip strength.

Nest building was assessed using the protocol published by Deacon et al (2006)⁸⁸. Briefly, one hour before the dark phase mice were singly housed in clean cages with nestlet material (2 g). After approximately 16 h, nests were photographed and scored from 1-5 by two blinded scorers, based on how much of the nestlet was torn and the shape and depth of the nest.

Burrowing was assessed as previously described⁸⁹. Briefly, 3 hours before the dark phase mice were singly housed with a custom made burrow (plastic tubing raised at one end) containing 200 grams of standard mouse chow. After 2 hrs, and then 16 hrs, the amount of food (by weight) remaining in the burrow was assessed.

Estrous cycling was determined in the female mice using previously published methods^{90,91}. Briefly, at the same time each day for 10 d, the vagina of each mouse was flushed with 75 μ l of sterile saline, and the lavage collected. Immediately, a drop was placed onto a slide and the cell

visualized using a light microscope. An experienced scorer determined the cycle (diestrous, estrous, metestrous or proestrous) based on the cell composition of the sample. Mice were scored as either irregularly (scored 1) or regularly (scored 0) cycling according to three methods^{87–89} based on the length and order of the stages. The average of the three scores was taken to give a final irregularity index.

Open field assessment was used to assess activity of mice⁹². Mice were placed individually in the openfield arena for 10 min and the total distance (mm) was recorded using CleverSys software.

Mouse metabolic assessments

For male mice, body composition was determined using Dual Energy X-ray Absorptiometry (DEXA) on anaesthetized mice (3% isoflurane). For female mice, body composition was measured by magnetic resonance imaging using the EchoMRI™-100H (Echo Medical Systems) on awake mice.

Metabolic outcomes were measured in male and female mice by indirect calorimetry in open circuit oxymax chambers using the Comprehensive Lab Animal Monitoring System (CLAMS; Columbus Instruments, Columbus, OH, USA). Mice were singly housed with water and food available ad libitum under a 12-12-hour light-dark cycle at approximately 24°C. The mice were kept in the monitoring cages for 3 d, and the first 24 hrs was excluded from analysis. Hourly summary data was plotted and summarized using CalR (Version 1.3)⁹³.

Mouse blood collection and analysis

Mice were fasted for 5-6 hours in the early morning. Blood samples (150-300 µl) were collected in anesthetized mice (3% isoflurane) from the submandibular vein into tubes containing approximately 10% by-volume of 0.5M EDTA. Blood was spun at 1500 x g for 10 minutes and

plasma removed. Blood cell pellets were stored frozen at -80°C. A 30 µl sample of whole blood was stored on ice and processed within 4 hrs with the Hemavet 950 (Drew Scientific) to give whole blood count parameters.

Fasting glucose, insulin and HOMA-IR

Fasting glucose was measured in freshly collected blood using a standard glucometer and strips.

Tissue collection

Between 23 and 25 months of age a subset of the lifespan mice were euthanized for tissue collection. Mice were injected i.p with sodium pentobarbitol, anesthesia was established, then mice were exsanguinated from the inferior vena cava. Blood was collected into Eppendorf tubes containing approximately 10% by volume of 0.5M EDTA and processed as above. Tissues were collected and flash frozen in liquid nitrogen before long-term storage at -80°C.

Epigenetic age assessment with TIMEseq

PBMC samples collected from mice at 13.5 (baseline), 18 and 22 months of age were used, plus flash-frozen liver samples collected at 23-25 months of age. DNA was extracted from samples using a magnetic bead-based method. For blood, red blood cells were first lysed in RBC lysis buffer (155 mM NH₄Cl, 12 mM NaHCO₃, 0.1 mM EDTA, pH 7.3) with shaking, then centrifugation and removal of the supernatant x 3. Remaining blood cells, or frozen liver was then incubated with TESR buffer (10 mM Tris-HCl, 25 mM EDTA, 0.5% SDS, 20 µg/mL RNase) for 30 minutes at 37°C, with regular douncing with a plastic mortar to break up tissue. Proteinase-K (20 mg/ml) was added at the solution incubated for 16 hrs at 65°C. Tubes were spun, supernatant removed and NGS magnetic beads added at a 0.6:1 ratio. Dna was allowed to bind beads, beads were separated on a magnetic rack, washed x 2 with 80% ethanol, and DNA eluted in 10 mM Tris-

HCl. DNA concentration was determined with PicoGreen, then diluted to approx. 20 ng/μl, requantified and rediluted to 10 ng/μl.

DNA was then processed using the TIMEseq technique⁹⁴. Blood samples were pooled with other experimental samples and sequenced on a NextSeq using a High output kit (150 cycles R1, 5 cycles R2). Liver samples were sequenced on a NovaSeq flowcell (100 cycles R1, 100 cycles R2). TIME-Seq pools were demultiplexed using sabre, cutadapt (version 2.5) was used to trim adaptors, reads were mapped with bowtie2 (version 2.3.4.3) using Bismark50 (version v0.22.3; options -N 1 --non_directional) to bisulfite converted genome (bismark_genome_preparation) mm10, and reads were subsequently filtered using the bismark function filter_non_conversion (option --threshold 11). bismark_methylation_extractor was used to call methylation for each sample with options to avoid overlapping reads (--no-overlap) in PE sequencing and to ignore the first 10 bps of each read (if SE: --ignore 10 and --ignore_3prime 10; if PE: --ignore_r2 10 --ignore_3prime_r2 10 as well). Reads that failed to map as pairs but that mapped individually were processed with the same pipeline and joined to methylation data using bismark2bedGraph. The blood and liver specific mouse clocks developed by Griffin et al.⁹⁴ were then applied to each sample to calculate an epigenetic age in months. Delta age was calculated as the epigenetic age – actual age.

Inflammatory Cytokines and Metabolic Hormones

Frozen plasma collected at euthanasia from 23-25 month old male and female mice was used for the analysis of inflammatory cytokines using the mouse 32-plex discovery assay and the 12-plex metabolic hormone discovery assay with Eve Technologies.

Histopathology

For Cohort 1, where possible, bodies were collected at time of death and the abdominal, thoracic

and cranial cavities opened and exposed. Bodies were then stored in Bouin's solution long term before being processed for histopathological analysis by pathologist Yuji Ikeno, M.D., Ph.D at The University of Texas Health Science Center at San Antonio. A total of 79 NMN and 88 control males and 15 NMN and 20 control females were processed (others were excluded for being too decomposed, cannibalized or for fixative spillage during transportation).

After gross inspection, fixed tissues were processed conventionally, embedded in paraffin, sectioned at 5 μ m (except the kidneys; 3 μ m), and stained with hematoxylin and eosin (H&E), Congo red or periodic acid-Schiff (PAS). A complete pathological analysis of 17 tissues (brain, pituitary gland, heart, aorta, lungs, trachea, esophagus, stomach, small intestine, colon, liver, gallbladder, pancreas, spleen, urinary bladder, thyroid/parathyroid gland, adrenal glands, muscles (iliopsoas, gastrocnemius, and quadriceps), sternum, spinal cord, vertebrae, knee joint, nasal passage, thymus, ventral abdominal skin, and gonadal tissues) was completed using gross and microscopic analysis. All pathological lesions were identified for each animal, including both neoplastic and non-neoplastic diseases. The severity of each lesion was assessed using the grading system we have developed for aging mice⁹⁵. Tumor burden was calculated as the sum of different types of tumors, disease burden as the sum of the histopathological changes in each animal, morbidity index as the number of fatal diseases per mouse, and tumor index as the number of neoplastic (both benign and malignant) lesions per mouse. All pathological analyses were accomplished by a double blind procedure without knowledge of the animal's identity.

Gut microbiome

Mouse stool was collected by individually placing mice in clean transfer containers for 5-10 minutes. 2-3 pellets were then collected per mouse in Eppendorf tubes, flash frozen and stored at -80°C. Samples were processed with the ZymoBIOMICS® Shotgun Metagenomic Sequencing

Service for Microbiome Analysis (Zymo Research, Irvine, CA). Genomic DNA was extracted using the Zymo-96 MagBead DNA Kit and libraries were prepared using the Illumina Nextera DNA Flex Library Prep Kit. Libraries were quantified with TapeStation (Agilent Technologies), pooled and then sequenced on a NovaSeq (Illumina) to provide at least 20M reads for each sample. Quality control and trimming of raw sequencing data was conducted with FASTP⁹⁶. Taxonomic quantification was conducted with Kraken2⁹⁷ using a confidence threshold of 0.3 and a custom database based on the Kraken2 standard database, where the human reference genome was switched out for the Genome Reference Consortium Mouse Build 39 (GRCm39). Taxa abundances were obtained using Bracken⁹⁸ with an abundance cutoff of 10 reads prior to reassignment. Statistical analyses were conducted using species assigned reads rarified to the smallest observed value across samples and normalized by centered log ratio (CLR). Species detected in fewer than 25% of samples were removed prior to analysis. Univariate correlations were performed using the function “mixedlm” from the Python “statsmodels” package. For all correlation analyses, age and sex were included as covariates and animal ID was treated as a random effect. For correlations of frailty and lifespan with microbiome and metabolite features treatment was also included as a covariate. Univariate correlation p-values were adjusted for multiple hypothesis testing using the Benjamini-Hochberg false rate discovery. Mediation analysis was performed using the function “mediate” from the R package “mediation”. The function “lmer” from the package “lme4” was used to incorporate animal ID as a random effect in the mediation analysis. Treatment and age were included as covariates and separate analyses were performed for each gender to assess sex specific differences. For beta-diversity analyses, Bray-Curtis distances were calculated from the rarified species reads. Contributions to beta-diversity were assessed with PERMANOVA using the “adonis2” function from the “vegan” R package (<https://github.com/vegandevs/vegan>) and

using veterinary-requested euthanization, day of sampling, treatment, group, age at death, and frailty index as sequential covariates. PERMANOVA p-values were obtained from 1,000 random permutations.

A. Colihominis experiments

Anaerotruncus colihominis (DSM 17241; DSMZ, Braunschweig, Germany) was cultured in DSMZ Medium 58 (Bifidobacterium Medium; DSMZ) at 37°C in an anaerobic chamber (Sheldon Manufacturing) and maintained under an atmosphere of 90% N₂, 5% CO₂, and 5% H₂. Prior to each experiment, a working stock was expanded by overnight liquid culture in DSMZ Medium 58 at 37°C.

To assess the effect of nicotinamide mononucleotide (NMN) on bacterial growth, overnight liquid culture of *A. colihominis* was diluted to OD of 0.1 in DSMZ Medium 58 with vehicle (sterile water) or 250 uM NMN in sterile flat-bottom 96-well plates (200 µL per well) and grown in an anaerobic chamber. Each condition was tested with n = 5-6 replicate wells per plate, and the experiment was performed in at least two independent biological replicates on separate days. Bacterial growth was monitored continuously over 24 hours by measuring OD₆₀₀ at 15-minute intervals using a Cerillo Stratus microplate reader operated inside the anaerobic chamber with the Labrador software (Cerillo Bio). Labelled NMN was provide by MetroBiotech (Cambridge, MA), and the specific structure is proprietary and confidential.

Culture samples were collected at 6 hours and 20 hours post-inoculation by centrifugation at 10,000 × g for 5 minutes at 4°C to separate the cell pellet from the conditioned medium. The supernatant (media fraction) and cell pellet were placed immediately on dry ice following centrifugation and subsequently stored at -80°C until metabolite extraction.

A. Colihominis metabolite detection

Polar metabolites were extracted from the culture media and cell pellet fractions and run on LC/MS as previously described⁹⁹. Metabolites were extracted from culture media using cold 100% methanol, and from bacterial pellet using a cold 40:40:20 (v/v/v) acetonitrile:methanol:water, vortexed and incubated on dry ice for 10-20 min and then centrifuged at 16,000 g for 30 minutes at 40C. Extracts were immediately transferred to a mass spectrometry vial and analyzed on a liquid chromatography coupled to a mass spectrometer. Metabolite separation and analysis was performed on a Vanquish Horizon UHPLC system (Thermo Fisher Scientific) coupled to the Orbitrap Exploris 240 Mass Spectrometer (Thermo Fisher Scientific) as reported previously⁹⁹.

Metabolite separation and analysis was performed on a Vanquish Horizon UHPLC system (Thermo Fisher Scientific) coupled to the Orbitrap Exploris 240 Mass Spectrometer (Thermo Fisher Scientific) as reported previously⁹⁹. The hydrophilic interaction chromatography (HILIC) LC separation was performed on a XBridge BEH Amide column (2.5 μ m, 2.1 mm X 150 mm, Waters, Milford, MA). Solvent A is 95% water in 5% acetonitrile with 40mM ammonium hydroxide and 20mM acetic acid and solvent B is 80% acetonitrile in 20% water with 40mM ammonium hydroxide and 20mM acetic acid. The gradient was 0 min, 100% B; 3 min, 100% B; 3.2 min, 90% B; 6.2 min, 90% B; 6.5 min, 80% B; 10.5 min, 80% B; 10.7 min, 70% B; 13.5 min, 70% B; 13.7 min, 45% B; 16 min, 45% B; 16.5 min, 100% B; and 22 min, 100% B. The flow rate was 300 μ L/min. The column temperature was set to 30 °C. The autosampler temperature was set to 4 °C, and the injection volume was 5 μ L. MS scans were obtained in both negative and positive ion modes with a resolution of 120,000 at m/z 200, in addition to an automatic gain control target of 3 x 10⁶ and m/z scan range of 72 to 1000.

Raw LC/MS data were converted to mzML format using the command line “msconvert” utility¹⁰⁰.

Data were analyzed via MAVEN software and the annotations of the metabolites was confirmed using our in-house library generated using the Mass Spectrometry Metabolite Library (Millipore Sigma, supplied by IROA Technologies).

RNA extraction and RNAseq

Gastrocnemius muscle, liver and white adipose tissue from male and female mice collected at 23-25 months of age, plus young control C57BL/6NIA samples (6 months) were processed for RNA extraction. Briefly, 30-40 mg of tissue was pulverized using the Covaris manual pulverizer (CP01). Powdered tissue was transferred to an Eppendorf tube, and 500-750ul Trizol added, vortexed and allowed to sit at room temp for 10 mins. 100-150ul of chloroform was added, vortexed and incubated for 10 mins. Solution was spun at 12000 g for 15 mins at 4°C, and the top aqueous phase was transferred to a new tube. RNA was cleaned up using the NucleoSpin RNA kit (Macherey-Nagel), following the standard protocol. RNA quality and concentration was determined using a 4200 Tapestation System (Agilent). Only samples with a RIN score > 7.5 were used for further analysis. mRNA library preparations and sequencing were done by Novogene using a Poly A enrichment-based library prep, and a Novoseq PE150 kit for sequencing. Sequencing quality was checked with fastqc, reads mapped with hisat2 (samtools) using mm10 (UCSC) reference genome. Resulting sam files were used to make raw feature counts matrices. Gene count data were subjected to DEG analysis using the 'limma' pipeline. Genes that had a counts less than 5 across all the samples were excluded, leaving 24,545 genes for analysis.

Differential expression analysis

Gene counts for each sample were then log transformed and the mean-variance relationship in the data were estimated, with a weight assigned to each observation (voom method). Design matrix

for differential expression analysis included age/treatment, sex and tissue. The data with weight information along with the multi-factor design matrix were then subjected to linear modeling with empirical bayes smoothing of gene-wise standard deviations. For comparisons between two conditions, for instance, NMN vs. control samples in the old gastroc group, we defined the DEG by the criteria that *fdr* method adjusted p -value < 0.1 .

Transcriptomic clock analysis

Raw RNA-seq count data were filtered separately for each tissue by retaining genes with at least 10 counts in at least 20% of samples from that tissue. Filtered data were processed using Relative Log Expression (RLE) normalization, log transformation, and YuGene transformation. Missing values for clock genes not detected in a given tissue were imputed using precomputed mean expression values. Normalized gene expression profiles were centered to the median profile of young control samples. Transcriptomic age (tAge) for each sample was estimated using Bayesian Ridge multi-tissue transcriptomic clocks of chronological age⁶⁵. Pairwise tAge differences between groups within each tissue and sex were assessed using mixed-effects ANOVA models implemented with the *rma.uni* function from the *metafor* package in R. P -values were adjusted for multiple testing with the Benjamini-Hochberg approach.

Transcriptomic signatures analysis

To characterize functional changes induced by NMN in old animals across sexes and tissues in terms of their association with aging and longevity, we performed gene set enrichment analysis (GSEA) on sex- and tissue-specific NMN-induced changes and transcriptomic signatures of aging and lifespan extension derived through meta-analysis. These reference signatures included aging-associated changes within individual organs (liver, brain, and kidney) and across tissues in multiple

species (mouse, rat, and human)^{66,67}, as well as signatures associated with mouse median and maximum lifespan and with established individual lifespan-extending interventions, including caloric restriction and rapamycin⁶⁶. For each signature, we ranked genes using a signed log-transformed p-value metric estimated through differential expression analysis:

$$-\log(pv) \times \text{sgn}(lfc),$$

where pv and lfc are p-value and logFC of a certain gene, respectively, and sgn is the signum function (equal to 1, -1 and 0 if value is positive, negative or equal to 0, respectively). Ranked gene lists were then subjected to GSEA using the fgsea package in R, with 10,000 permutations and multilevel Monte Carlo sampling. Gene sets were drawn from the HALLMARK, KEGG, and REACTOME collections of the Molecular Signatures Database (MSigDB).

Enrichment analysis and targeted gene analysis

Gene set enrichment analysis (GSEA) was performed to identify biological pathways differentially regulated by NMN treatment in male and female mice. Analysis was conducted using GSEA software (v4.3.3, Broad Institute, <https://www.gsea-msigdb.org/gsea/downloads.jsp>) with 1,000 permutations performed against the gene set null distribution. Mouse gene sets used in analysis, including Hallmark (MH), BioCarta (M2), Reactome (M2), and Gene Ontology Biological Process (M5-GOBP), were retrieved from the Molecular Signatures Database (<https://www.gsea-msigdb.org/gsea/msigdb/mouse/genesets.jsp>). Normalized Enrichment Scores (NES) and nominal p-values were visualized using heatmaps generated with the ggplot2 R package. Only results with absolute NES > 1 and p-value < 0.05 were considered statistically significant and labeled with asterisks in the heatmap.

The NAD⁺-consuming, immune cell marker, and genome stability gene lists were manually curated based on literature searches and publicly available information. NAD⁺-consuming genes

were selected from known NAD⁺-metabolizing enzyme families, including PARPs, SIRTs, and NADases like CD38. Immune cell marker genes were identified by searching for established marker genes of major immune cell types (B cells, T cells, NK cells, macrophages, monocytes, and dendritic cells) using published literature. Genome stability genes included key regulators of DNA repair, replication, and damage checkpoints, compiled from Gene Ontology (GO) terms “DNA repair,” “genome stability,” and “DNA damage response.” All curated lists were manually reviewed for relevance and to ensure non-overlapping classification among categories.

Selected NAD⁺-consuming genes, immune cell marker genes, and genome stability genes were extracted from raw gene count matrices derived from RNA-seq experiments. Genes with low expression (mean raw count < 20) were excluded. For each gene, the log₂ fold change (Log₂FC) was calculated between NMN-treated and untreated old groups. The p-value was determined using Welch’s t-test based on the raw count distributions. Filtered datasets were visualized in Python (matplotlib v3.9) as bar plots displaying Log₂FC values for each gene. Genes with p < 0.05 were indicated by asterisks.

mtDNA quantification

Total DNA from snap-frozen liver and muscle tissues was isolated using the Quick-DNA Miniprep Kit (Zymo Research) according to the manufacturer’s instructions. The mtDNA copy number was quantified in triplicate using LightCycler 480 SYBR Green I Master Mix (Roche) on a QuantStudio 5 Real-Time PCR System (Thermo Fisher Scientific). The relative mtDNA content was assessed using primers for the mitochondrial gene (Cytb), and the nuclear gene beta-2-microglobulin (B2m) served as an internal control for normalization. The primer sequences were as follows: mouse Cytb: 5'-CCCTAGCAATCGTTCACCTC-3' (forward) and 5'-TGGGTCTCCTAGTATGTCTGG-3' (reverse); mouse B2m: 5'-

ATGGGAAGCCGAACATACTG-3' (forward) and 5'-CAGTCTCAGTGGGGGTGAAT-3' (reverse). The relative fold change was calculated using the 2- $\Delta\Delta C_t$ method.

Simple Western (Jess) Assay

Frozen liver tissues from untreated old mice and NMN-treated old mice were homogenized in RIPA buffer (4 μ L/mg tissue) on ice. Lysates were stored at -20 °C until analysis. For each Simple Western assay, 1 μ g of total protein lysate was loaded and probed with the antibodies listed below. Simple Western chemiluminescence assays were performed using the Jess system (ProteinSimple) according to the manufacturer's Separation Module protocol.

Antibody	Company	Dilution or Concentration
p53 (mouse)	Abcam, ab246223	12 μ g/ml (1:50 dilution)
NF- κ B p65 (rabbit)	Abcam, ab32536	26 μ g/ml (1:20 dilution)
GAPDH (mouse)	R&D Systems, MAB5718	0.1 μ g/ml
Vinculin (rabbit)	Cell Signaling Technology, 13901S	1.7 μ g/ml (1:300 dilution)
Anti-mouse secondary HRP	ProteinSimple, 042-205	As recommended
Anti-rabbit secondary HRP	ProteinSimple, 042-206	As recommended

Data were analyzed using Compass for SW software. For NF- κ B p65 + Vinculin assay, peak areas were determined using Gaussian Fit method. For the p53 + GAPDH assay, peak areas were determined manually using dropped lines. Normalized protein expression levels were calculated by dividing the peak area of the protein of interest by that of the corresponding loading control. Normalized values were visualized in GraphPad Prism (v10). Statistical significance was assessed using two-way ANOVA, and multiple comparison significance was determined by Fisher's least significant difference (LSD) test.

Muscle histology quantification

Tibialis anterior or quadriceps muscle was dissected from mice, frozen in Tissue-Tek® O.C.T. Compound (Sakura), sectioned via cryostat, and stained with either hematoxylin and eosin (H&E) or Masson's Trichrome. Brightfield imaging was performed on Agilent Biotek Cytation (20x magnification). All muscle image analysis was performed in ImageJ. H&E image analysis was completed using MyoSAT, an ImageJ macro (Cheetham Research Group at the Cornell University Veterinary College, https://github.com/CheethamLab/MyoSAT_ImageJ_Macro/tree/master/Documentation). For Masson's Trichrome image analysis, a custom macro was used to deconvolute staining, isolate muscle fibrosis and ECM area, and quantify overlap.

Brain GFAP immunohistochemistry

Flash frozen brains were cryosectioned at -21°C (Leica BioSystems, CM1950, Wetzlar, Germany) at 14 µm and mounted directly onto slides (VWR Colorfrost Plus, Radnor, PA, USA). Sections were post-fixed in 4% formaldehyde, washed three times with TBS, and blocked in 3% horse serum in TBS + 0.3% Triton-X-100 solution for 1 h. Sections were then washed again three times with TBS and incubated in primary antibody (GFAP: 1:1000, PA514358, Thermo Scientific, Waltham, MA, USA) overnight at 4 °C, washed three times with TBS, and incubated in secondary antibody at RT for 1 h (Alexa 488: 1:500, 711-545-152, Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA). After washing two times with TBS, nuclei were stained with Hoechst (1:2000, H1399, Invitrogen, Waltham, MA, USA) incubated in PBS for 15 min and coverslipped (CS-24X50, AmScope, Irvine, CA, USA) using antifade aqueous mounting medium. Fluorescence imaging were conducted to visualize and record immunofluorescence and staining results (Leica THUNDER DMI8 3D inverted epifluorescence microscope and LAS X 3D Analysis Software v.

2018.7.3, Leica Microsystems, Wetzlar, Germany). Images were processed using appropriate software¹⁰¹ (FIJI v2.1.0/1.53c, Madison, WI, USA).

Plasma metabolomics

Flash frozen plasma was processed for global metabolomics by Metabolon. Briefly, samples were prepared using the automated MicroLab STAR® system from Hamilton Company and proteins were precipitated with methanol under vigorous shaking for 2 min (Glen Mills GenoGrinder 2000) followed by centrifugation. Samples were analyzed using Ultrahigh Performance Liquid Chromatography-Tandem Mass Spectroscopy (UPLC-MS/MS). All methods utilized a Waters ACQUITY ultra-performance liquid chromatography (UPLC) and a Thermo Scientific Q-Exactive high resolution/accurate mass spectrometer interfaced with a heated electrospray ionization (HESI-II) source and Orbitrap mass analyzer operated at 35,000 mass resolution. The sample extract was dried then reconstituted in solvents compatible to each of the four methods. Each reconstitution solvent contained a series of standards at fixed concentrations to ensure injection and chromatographic consistency. One aliquot was analyzed using acidic positive ion conditions, chromatographically optimized for more hydrophilic compounds. In this method, the extract was gradient eluted from a C18 column (Waters UPLC BEH C18-2.1x100 mm, 1.7 µm) using water and methanol, containing 0.05% perfluoropentanoic acid (PFPA) and 0.1% formic acid (FA). Another aliquot was also analyzed using acidic positive ion conditions, however it was chromatographically optimized for more hydrophobic compounds. In this method, the extract was gradient eluted from the same afore mentioned C18 column using methanol, acetonitrile, water, 0.05% PFPA and 0.01% FA and was operated at an overall higher organic content. Another aliquot was analyzed using basic negative ion optimized conditions using a separate dedicated C18 column. The basic extracts were gradient eluted from the column using methanol and water,

however with 6.5 mM Ammonium Bicarbonate at pH 8. The fourth aliquot was analyzed via negative ionization following elution from a HILIC column (Waters UPLC BEH Amide 2.1x150 mm, 1.7 μ m) using a gradient consisting of water and acetonitrile with 10 mM Ammonium Formate, pH 10.8. The MS analysis alternated between MS and data-dependent MS_n scans using dynamic exclusion. The scan range varied slightly between methods but covered 70-1000 m/z. Raw data files are archived and extracted as described below.

Raw data was extracted, peak-identified and QC processed using Metabolon's hardware and software. Compounds were identified by comparison to library entries of purified standards or recurrent unknown entities. Peaks were quantified using area-under-the-curve. Peak areas were then batch-normalized (divided by the median of the samples within that batch), normalized to volume of sample extracted, re-scaled to median 1, missing values imputed with the minimum observed value for each compound and the data log transformed. Data was split into two subsets for ongoing analysis: NAD⁺-related metabolites (methylnicotinamide, quinolinate, ADP-ribose, nicotinamide, NMN, N-methylnicotinate, nicotinamide riboside, nicotinate ribonucleoside, N1-Methyl-pyridone-carboxamide, nicotinamide-N-oxide) and non- NAD⁺ remaining metabolite (the remaining metabolites), and analysed per sex. Analysis was completed with the online version of MetaboAnalyst 6.0^{102,103}. Features that were significantly different between treatment groups were determined using linear mixed models with treatment as the primary outcome and age as a covariate, with an FDR adjusted p value < 0.1. Enrichment analysis was completed using MetaboAnalyst and the Small Molecule Pathway Database (SMPDB) with a p value < 0.1, and at least two metabolites present in each pathway.

Acute labelled NMN experiment

Labelled NMN was synthesized by GeneHarbor Biotechnology through an enzymatic synthesis as

described previously⁵², feeding in [2,4,5,6-D₄]-nicotinamide (Cambridge Isotopes Laboratories, DLM-6883) and [5,5-D₂]-ribose (Cambridge Isotope Laboratories DLM-778). This resulted in mixed NMN labelling of M+6, M+5 and M+4 (61.3%, 30.9% and 7.7%), however in line with other studies^{51,56}, only nicotinamide labelling of NAD⁺ could be detected *in vivo* (Figure 4K,P). Once incorporated into NAD⁺, the M+4 nicotinamide ring undergoes a shift to M+3 due to the loss of a deuterium labelling during the transition from NAD⁺ to NADH⁵⁴.

12 month and 20 month old male and female C57Bl6J/NIA mice were procured from National Institute of Aging rodent colony and allowed to adapt for 2 weeks in an animal housing room at the University of Pennsylvania. Mice were orally gavaged with either PBS (vehicle) or unlabeled 600 mg/kg NMN once a day for 10 days. On the 11th day mice were gavaged with a mixture of unlabeled and labeled NMN in PBS (7.3% labelled NMN). Serum samples were collected before dosing, 3h and 6h following NMN gavage. Mice were sacrificed at the 6 hr time point by cervical dislocation and decapitation and tissues samples were collected and snap frozen and stored at -80°C. The ratio of labeled to unlabeled NMN in gavage solution was verified by mass spectrometry as 1:13 and used to correct the relative contribution to the labeling of NAD⁺. This method can accurately predict the labeling pattern of single molecule such as NAM and NA, however, could lead to underestimation of larger molecules that are generated by combining two labeled molecules such as coupling of NAM and ribose to ultimately generate NMN and NAD⁺, or NA and ribose to produce NAR.

Tissue NAD⁺ metabolomics

Water soluble metabolites from the serum samples collected in the NMN labeling experiment were extracted using methanol. Briefly, 50 µl of 100% methanol was added to 5ul of serum, vortexed and incubated on ice for a minimum of 10 minutes. The samples were vortexed and centrifuged at

16,000 x g for 30 min. 40 μ l of extract was mixed with 10 μ l of water for metabolite measurement. For other tissues, frozen samples were cut, weighed, ground on a cryomill (Retsch), and extracted using ice-cold 40:40:20 acetonitrile:methanol:water (25 mg tissue per ml extraction buffer). Tissue extracts were vortexed and incubated on wet ice for a minimum of 10 mins and centrifuged at 16,000 x g for 30 min. The clear supernatant transferred to a new Eppendorf tube and centrifuged again to remove debris and used for metabolomics analysis.

Hydrophilic interaction liquid chromatography (HILIC) separation of metabolites extracted from serum and tissues was performed with an XBridge BEH amide column (150 mm X 2.1 mm, 2.5 particle size, Waters Corporation, Milford, MA) on a Vanquish Horizon UHPLC system (Thermo Fisher Scientific, Waltham, MA) as described previously (PMID: 32125145). Solvent A comprised of 95% acetonitrile and 5% water with 20mM acetic acid and 40mM ammonium hydroxide and pH adjusted to 9.4, and solvent B contained 80% acetonitrile, 20% water, 20mM acetic acid and 40mM ammonium hydroxide, pH 9.4. The gradient 0 min, 100% B; 3 min, 100% B; 3.2 min, 90% B; 6.2 min, 90% B; 6.5 min, 80% B; 10.5 min, 80% B; 10.7 min, 70% B; 13.5 min, 70% B; 13.7 min, 45% B; 16 min, 45% B; 16.5 min, 100% B; and 22 min, 100% B. Other LC parameters include a flow rate of 300 μ l/min, column temperature of 250°C, 5 μ l sample injection volume, and autosampler temperature maintained at 40°C. Separated metabolites were analyzed on a Thermo Q exactive PLUS instrument with a HESI source to detect molecules. The parameters for mass spectrometry were set as follows: spray voltage of -2.7 kV in a negative mode and 3.5 kV in positive mode on HESI source, capillary temperature of 3000C, auxiliary gas heater at 3600C, and S-lens RF level of 45. The m/z scan range of 72-1000 in either positive or negative ionization mode, AGC target of 3e6, and maximum IT of 200 ms were used to collect data on mass spectrometry.

Raw data files were converted into mzxml files using msconvert command-line utility. Isooptomer labeling and untargeted metabolomics data analysis were done using open-source LC-MS data processing software El-maven and in-house metabolite library established from authentic standards. Isotope labeling patterns were corrected for natural isotope abundance in NMN labeling experiment using AccuCor. Standard curves of NAM and NA were used to calculate the absolute concentration of these metabolites in circulation. For the long-term NMN tissue experiments data was mean-centered and divided by the standard deviation of each variable so relative levels were compared. For the acute NMN experiment data is plotted as Total ion counts (TIC).

Statistics

Data was analyzed and graphed using either GraphPad Prism (Version 10.1.1) or R (4.2.2). Specific statistical tests used for each comparison are outlined in the figure legends. Figures show data as mean +/- SEM unless otherwise indicated. P value of <0.05 was considered significant unless otherwise indicated.

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SUPPLEMENTARY INFORMATION

Supplementary Figure 1. Additional healthspan and lifespan data.

NMN dose calculated based on water intake for (A) male and (B) female mice in Cohort 1 across the course of the study. (C) Epigenetic delta age calculated using TIMEseq-based blood clock for male and (D) female mice at four ages. (E) Epigenetic delta age calculated using TIMEseq-based liver clock for male and female mice at 24 months of age. (F) Grip strength/body weight, (G) nesting scores and (H) food remaining after an overnight burrowing assessment for male mice across the study. (I) Grip strength/body weight, (J) nesting scores and (K) food remaining after an overnight burrowing assessment for female mice across the study. (L) Mean irregular estrous cycling score for female mice across three timepoints. (M-P) Kaplan-meier survival curves for NMN-treated and control males and females from Cohorts 1 and 2 plotted separately. X axis starts at 13 months of age when NMN treatment was started. Log-rank test p-value show in boxes. (Q) Morbidity index, (R) glomerulonephritis grade and (S) tumor index for male (n= 78 nmN, n=88 control) and female (n= 15 nmN, n=20 control) mice. Two way ANOVA results for treatment (NMN), sex and the interaction (s*N) shown in boxes, and posthoc Tukey's multiple comparisons p-values shown on graphs if p<0.1. (T) Platelet (PLT) concentration, (U) White blood cell (WBC) count and (V) red blood cell (RBC) count for male mice across the study. (W) Platelet

concentration, (X) WBC count and (Y) RBC count for female mice across the study. For (F)-(L) and (T)-(Y) mixed effects model p value results for age, treatment (NMN) and the interaction (a*N) shown in boxes. Males n=25 controls, n=24 NMN at 13.5 months; females n=20 controls, n=20 NMN at 13.5 months. Mean +/- SEM shows on figures.

Supplementary Figure 2. Additional metabolic health data.

(A) Food intake (mean grams/day) for male mice (control n=4 cages, NMN n=45 cages at baseline). (B) Blood fasting glucose levels (mean +/- SEM) for male mice across the study. N=25 controls, n=24 NMN at 13.5 months. For (A) and (B) mixed effects model p value results for age, treatment (NMN) and the interaction (a*N) shown in boxes. (C) Concentration (pg/ml) of amylin, (D) C-peptide 2, (E) Ghrelin, (F) PYY, (G) GIP, (H) GLP-1, (I) Glucagon, (J) Restin, (K) insulin, (L) leptin, (M) PP and (N) secretin in plasma from male and female mice at 24 months of age (n=8 per group). Two way ANOVA results for treatment (NMN), sex and the interaction (s*N) shown in boxes, and posthoc Tukey's multiple comparisons p values shown on graphs if $p < 0.1$. (O-P) Oxygen consumption and (Q-R) carbon dioxide production as determined from indirect calimetry for male mice at 24 months of age (n=6 control, n=7 NMN). (S) Total distance moved over 10 minutes in the openfield arena (mean +/- SEM) for male mice across the study. Mixed effects model p value results for age, treatment (NMN) and the interaction (a*N) shown in boxes. N=25 controls, n=24 NMN at 13.5 months. (T) Mean body weight change (as a % of baseline weight at 13 months) for female mice (control n=20, NMN n=20 at baseline). (U) Food intake (mean grams/day) for female mice (control n=5 cages, NMN n=5 cages at baseline). For (T) and (U) mixed effects model p-value results for age, treatment (NMN) and the interaction (a*N) shown in boxes. (V) Mean lean mass (as percent of total body mass), (W) mean fat mass (as percent of

total body mass) and (X) lean/fat ratio for female mice (n=7 control, n=8 NMN) at 24 months of age. (Y) Respiratory exchange ratio and (Z) activity as determined from indirect calorimetry for female mice at 24 months of age (n=5 control, n=8 NMN). Mean values shown for each 12 hour period of either light or dark. Two way ANOVA p-value results for time, treatment (NMN) and the interaction (t*N) shown in boxes. (AB) Total distance moved over 10 minutes in the openfield arena (mean +/- SEM) for female mice across the study. Mixed effects model p value results for age, treatment (NMN) and the interaction (a*N) shown in boxes. N=20 controls, n=20 NMN at 13.5 months.

Supplementary Figure 3. Plasma metabolomics data.

(A) Heat map of all plasma metabolites down-regulated, or (B) up-regulated, with adjusted p value < 0.1 and fold change > 0.6 (linear model) comparing NMN treatment with controls for male mice at 18.5 and 21 months of age (n=22-23 per group). (C) Metabolism pathways identified through enrichment analysis for the metabolites from (A). (D) Heat map of all plasma metabolites up or down-regulated with adjusted p value < 0.1 and fold change > 0.6 (linear model) comparing NMN treatment with controls for female mice at 18.5 and 22.5 months of age (n=17-20 per group).

Supplementary Figure 4. Additional NMN metabolites data.

(A) Labelled NMN diagram, labelled atoms shown in green. (B-C) Levels of NAM, or (D-E) NA in plasma from young (Y) and old (O) male (B,D) and female (C,E) mice 0, 3 and 6 hrs post oral gavage with 600 mg/kg of NMN. Mixed effects model significant p value results for treatment (NMN), time post gavage, age and interaction terms shown in boxes, and posthoc Sidák's multiple comparisons p values shown on graphs if p<0.15. (F) Levels of NAM, or (G) NAD⁺/NADH ratio

in liver from young (Y) and old (O) male and female mice 6 hrs post oral gavage with 600 mg/kg of NMN. Mixed effects model significant p value results for treatment (NMN), age, sex and interaction terms shown in boxes, and posthoc Sídák's multiple comparisons p values shown on graphs if $p < 0.05$. (H) Relative levels (data mean-centered and divided by the standard deviation of each variable) of NADH (nicotinamide adenine dinucleotide) or (I) meNAM (methyl-nicotinamide) in liver of male (n=7 control, n=8 NMN) and female (n=7 control, n=8 NMN) mice treated with NMN from 13 months of age or controls. Adjusted p-values from multiple t-tests shown on figures if $p < 0.1$. (J) Levels of NAD^+/NADH ratio, or (K) NAM in gastroc from young (Y) and old (O) male and female mice 6 hrs post oral gavage with 600 mg/kg of NMN. Mixed effects model significant p value results for treatment (NMN), age, sex and interaction terms shown in boxes, and posthoc Sídák's multiple comparisons p values shown on graphs if $p < 0.1$. (L) Relative levels of NAD^+ , (M) NAD^+/NADH ratio, (N) meNAM or (O) NADH in gastroc muscle of male (n=7 control, n=8 NMN) and female (n=7 control, n=8 NMN) mice treated with NMN from 13 months of age or controls. Adjusted p-values from multiple t-tests shown on figures if $p < 0.1$.

Supplementary Figure 5. Additional microbiome data.

(A) CLR normalized relative abundances of *B. caecimuris* across treatment in females and (B) males. Two-way ANOVA significant p-value results for treatment (NMN), age and interaction term shown in boxes, and posthoc Sídák's multiple comparisons p values shown on graphs if $p < 0.10$. (C) Mediation of NMN treatment (X) on frailty score (Y) by *A. colihominis* (M) in male mice. (D) Mediation of NMN treatment (X) on lifespan (Y) by *A. colihominis* (M) in female mice. For both mediation results the average causal mediation effect (ACME) p-value is displayed on

the curved arrow connecting X to M to Y, the average direct effect (ADE) p-value is the arrow connecting X to Y. The p-values for the effect of X on M and M on Y are also reported and derived from their corresponding linear mixed effects models. The marginal R^2 (R^2_m) for the model $Y \sim X + M + (1 | \text{Animal})$ is reported for each median result. All regressions included age and sex as covariates, unless stratified by sex, as well as animal ID as a mixed effect. (E) Microbial genes differentially expressed in male and female mice with chronic NMN treatment compared to controls.

Supplementary Figure 6. Additional transcriptomic data.

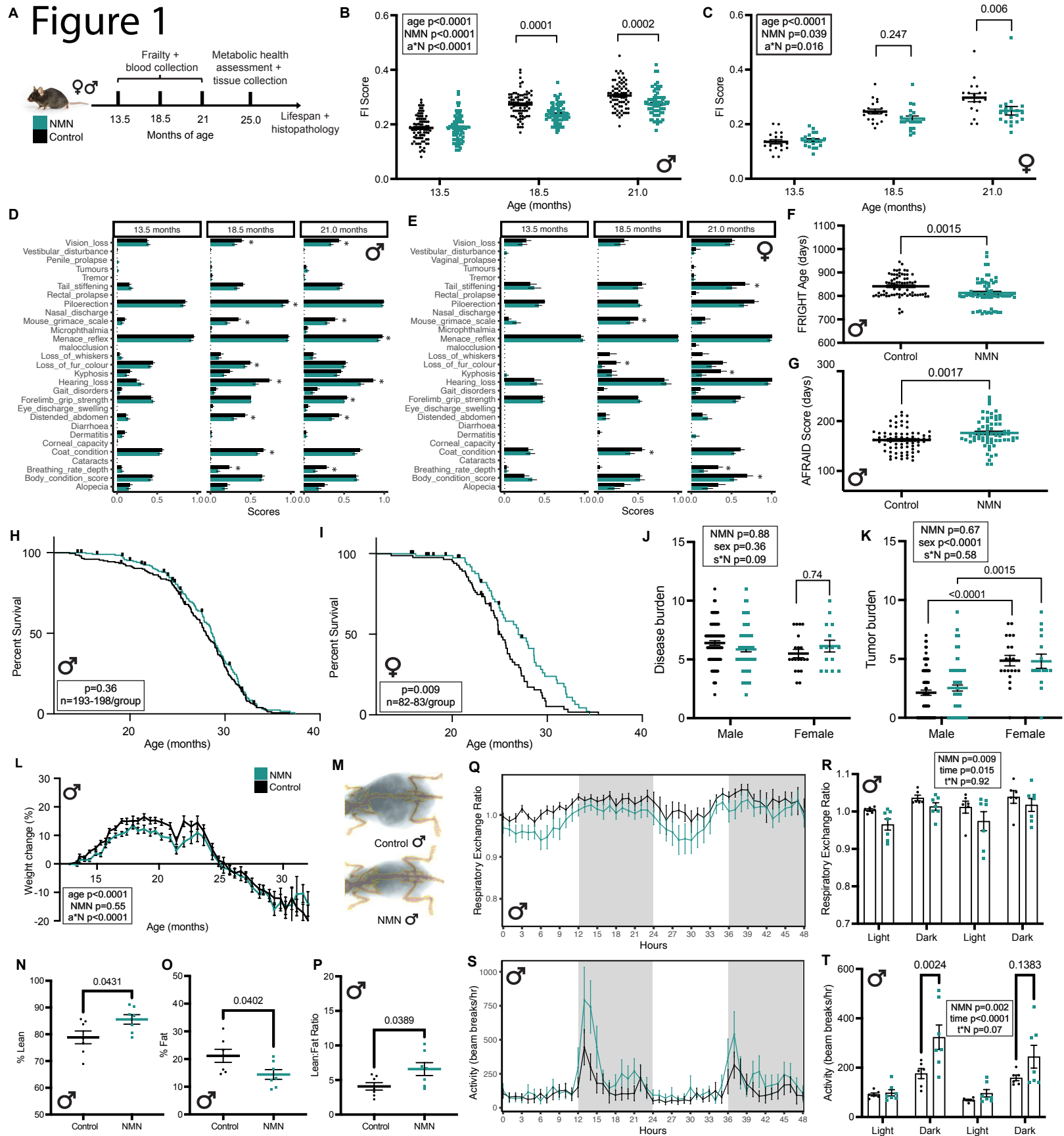
(A) UpSet plot of number of differentially expressed genes (DEGs) between NMN-treated and age-matched control groups, across sexes and tissues (W, white adipose tissue; L, liver; G, gastrocnimius muscle) (B) Heatmap of DEGs in NMN vs control for male gastroc muscle. Expression levels scaled, rows and columns are automatically ordered based on row/column means. Samples (x axis) are labelled based on their group (young, old, nmn) and their ID (1-6). (C) Biological tAge calculated based on gene expression data from gastroc muscle or (D) white adipose tissue (WAT), for young (6 month), old (24 month) control and old NMN-treated male and female mice. (E) Heatmap showing Normalized Enrichment Scores (NES) and p values (represented by asterisks) for known transcriptomic signatures of aging (shown with red text), lifespan extension (shown with green text), and signatures calculated for liver, muscle, WAT or all tissues (pooled) from male and female 24 month old NMN treated or control mice. Pathways, shown on the y axis, are based on the HALLMARK, KEGG, and REACTOME collections of the Molecular Signatures Database (MSigDB). Only results with absolute NES > 1 and p-value < 0.05 were considered statistically significant and labeled in the heatmap. (F) Relative mtDNA copy

number (ratio of mtDNA to nDNA) in WAT from male or female NMN treated or control mice at 24 months of age. n=5-6 mice per group. (G) Log2 fold change (Log2FC) of genome stability-related gene counts in 24 month old male and female muscle with NMN treatment or control. * indicates p-value <0.05 using Welch's t-test based on the raw count distributions.

Supplementary Figure 7. Additional data focused on inflammation and immune outcomes.

(A) Log2 fold change (Log2FC) of immune marker gene counts in 24 month old male and female muscle, or (B) liver, with NMN treatment or control. * indicates p-value <0.05 using Welch's t-test based on the raw count distributions. (C) Quantification of percent fibrosis in the extracellular matrix, or (D) muscle fibre cross-sectional area across control or NMN treated 24 month female quad muscle (n=6-8 per group). T-test p-value results shown on graph. (E) Log-normalised concentrations of cytokines interleukin (IL)-2, (F) IL-15, (G) IL-12p40 and (H) Leukemia inhibitory factor (LIF) in plasma of male and female mice at 24 months of age (n=8 per group). Two way ANOVA results for treatment (NMN), sex and the interaction (s*N) shown in boxes, and posthoc Tukey's multiple comparisons p values shown on graphs if p<0.1. (I) Representative fluorescent immunohistochemistry from male control and NMN treated mice of GFAP (green), and Hoechst (blue) in coronal brain sections of cortex, dentate gyrus, hypothalamus, and cerebellar (N=1 per group) (20x, scale bar=100 μ m). (J) Quantification of whole-brain lysate western blots across male and female control and NMN treated mice (N=5-7 per group). Two way ANOVA results for treatment (NMN), sex and the interaction (s*N) shown in box, and posthoc Tukey's multiple comparisons p values shown on graphs if p<0.1. (K) Representative western blot from male and (L) female samples of whole-brain lysates stained for GFAP and beta-Actin.

Figure 1



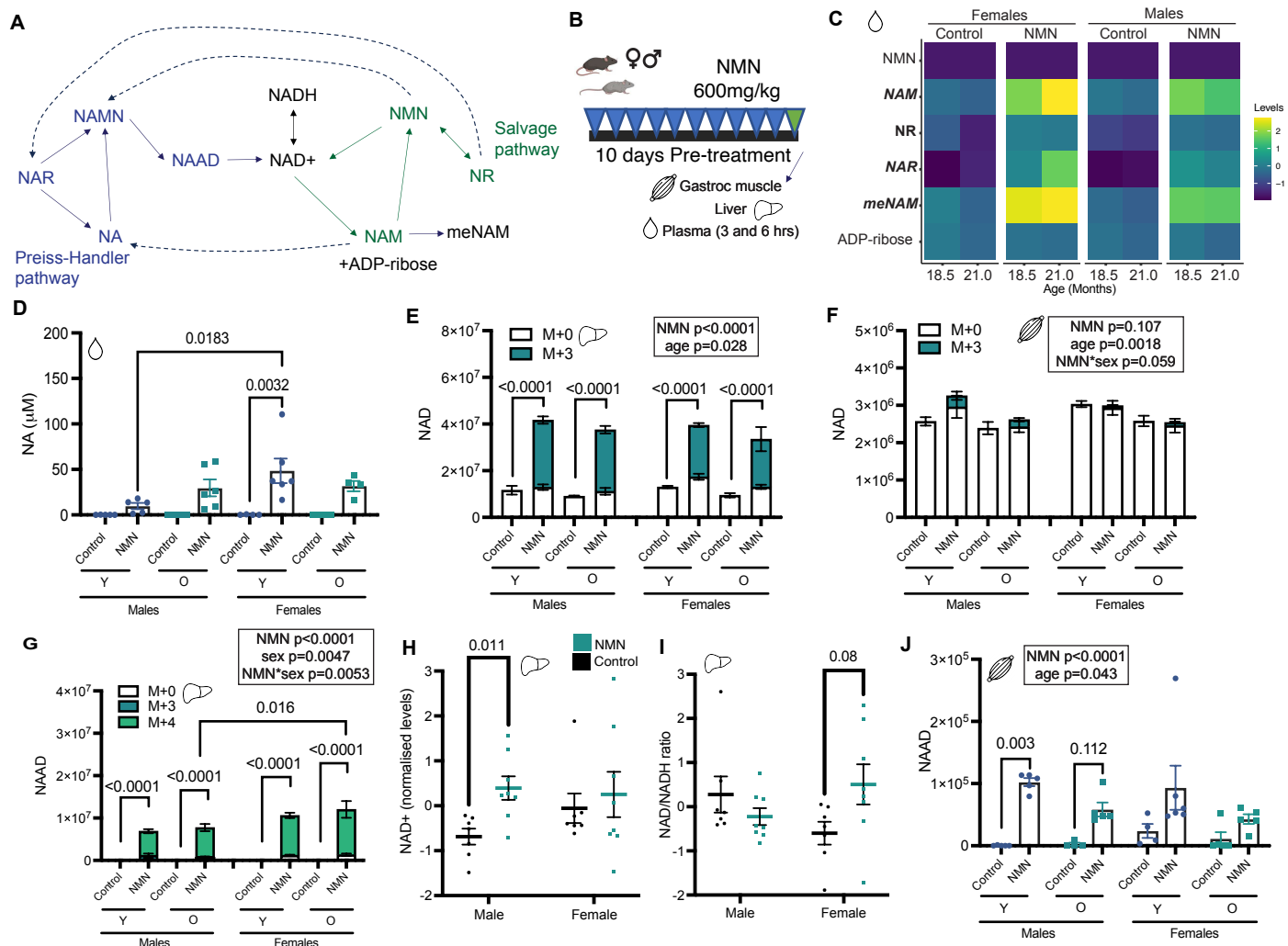


Figure 2

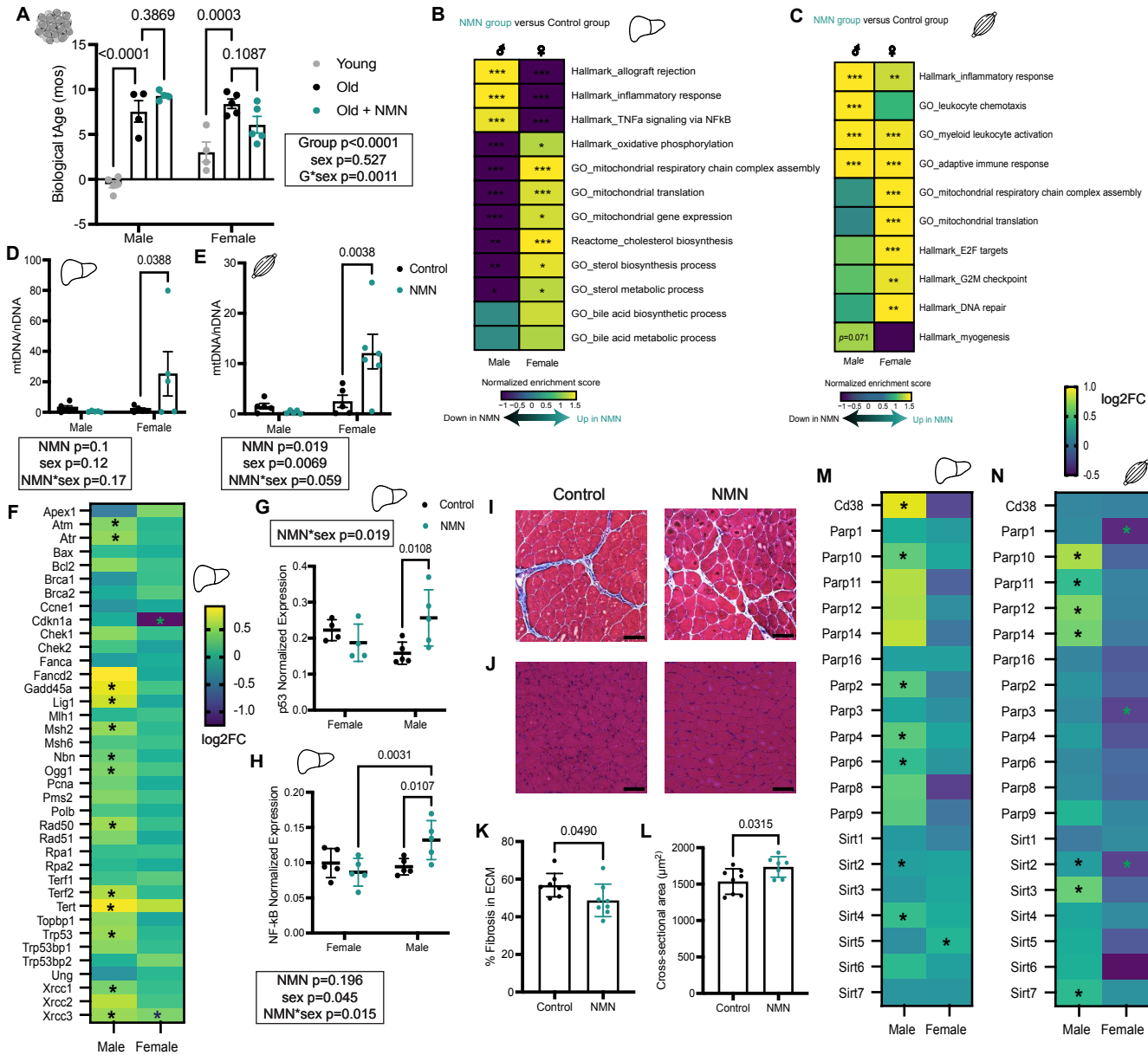
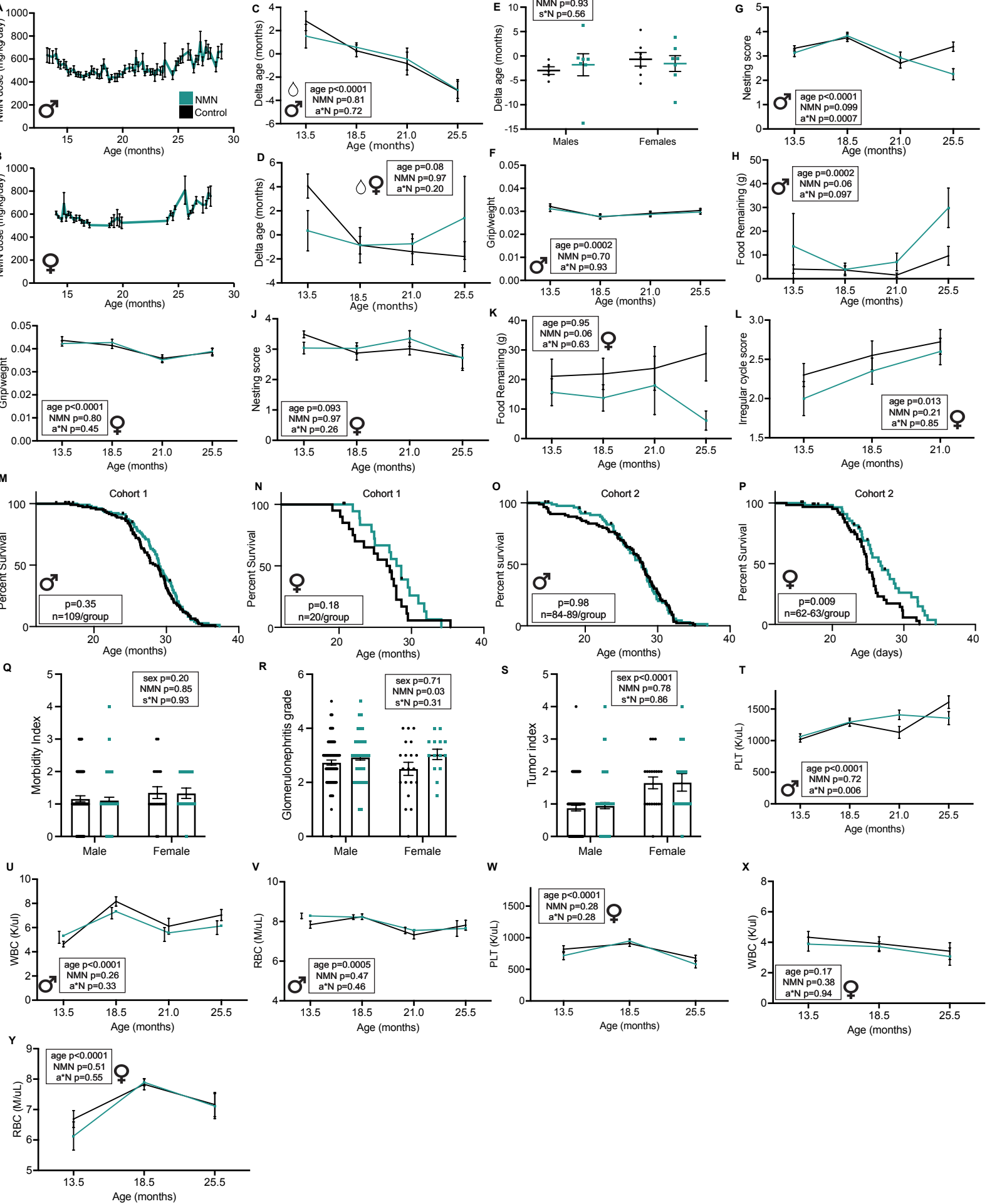
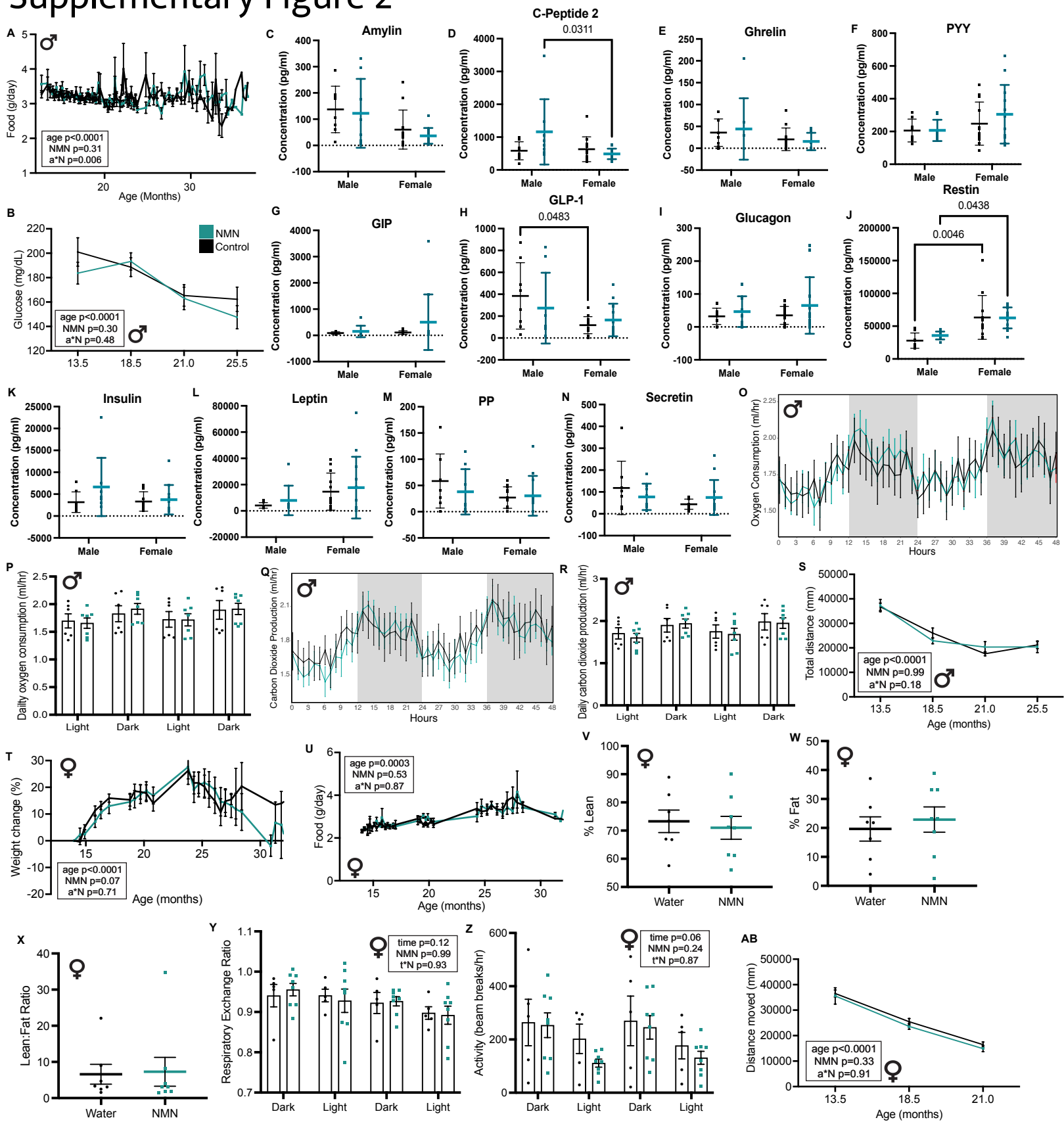


Figure 4

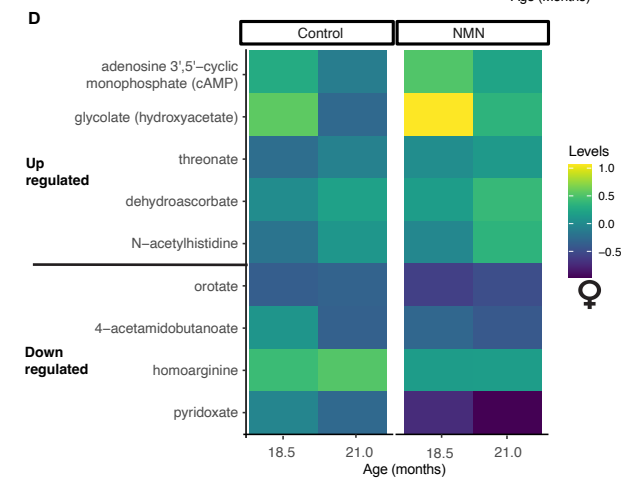
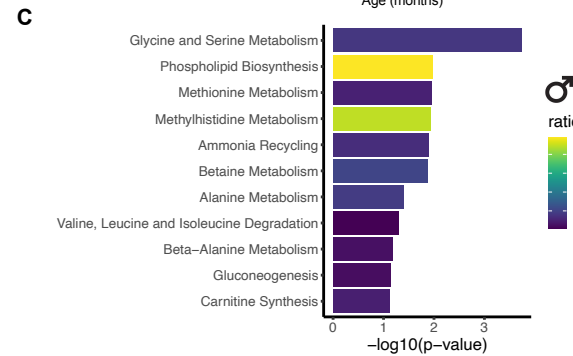
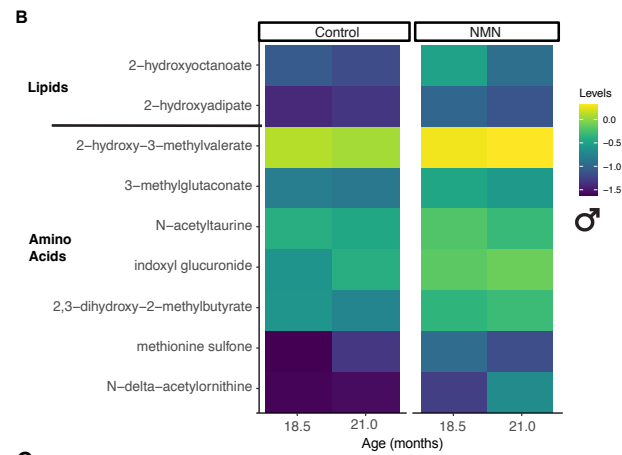
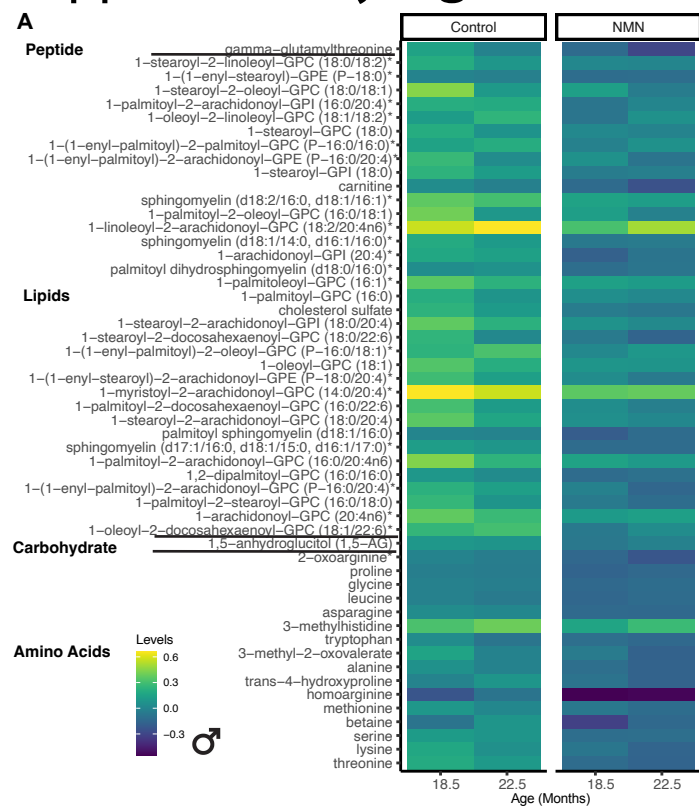
Supplementary Figure 1

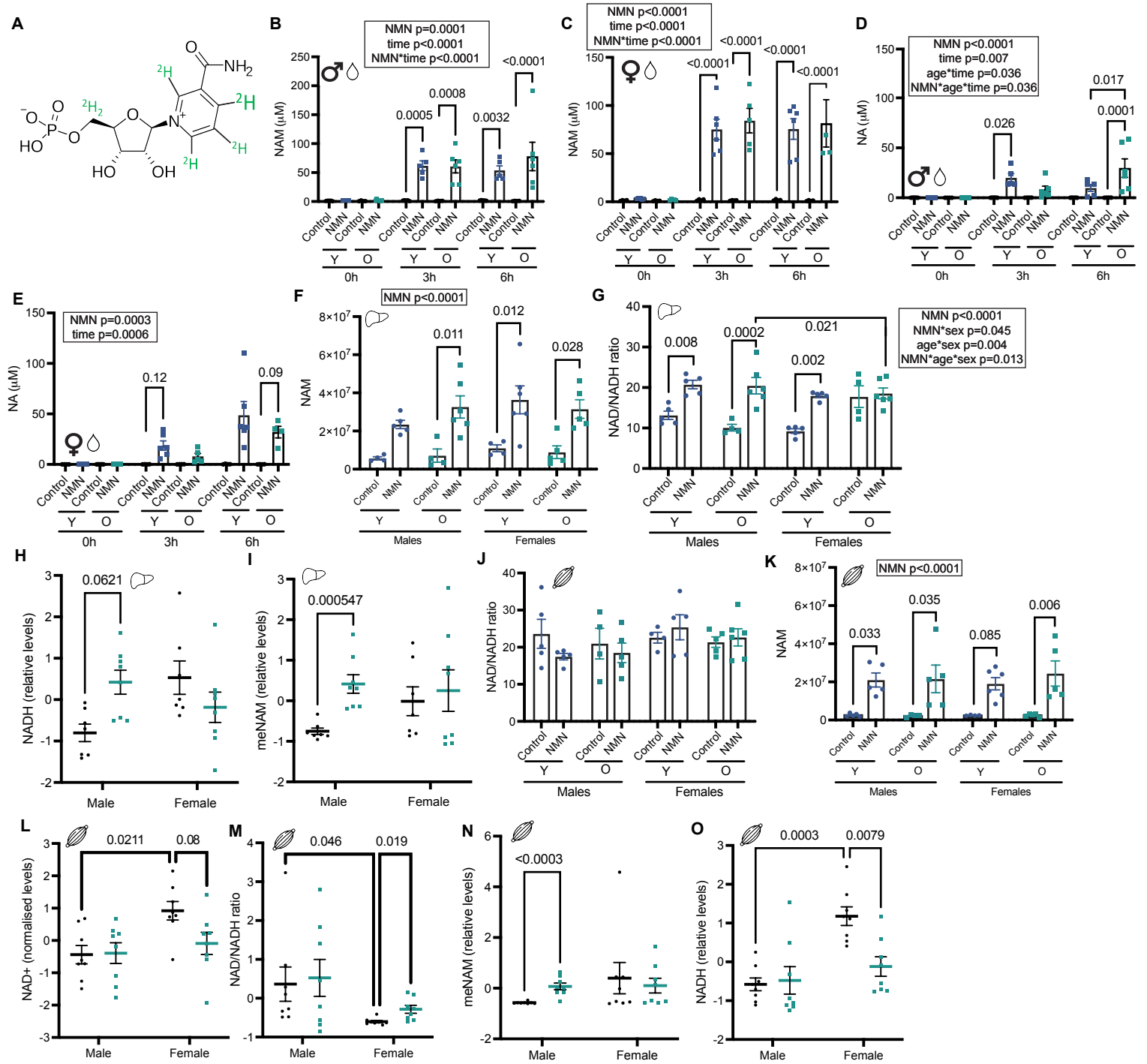


Supplementary Figure 2



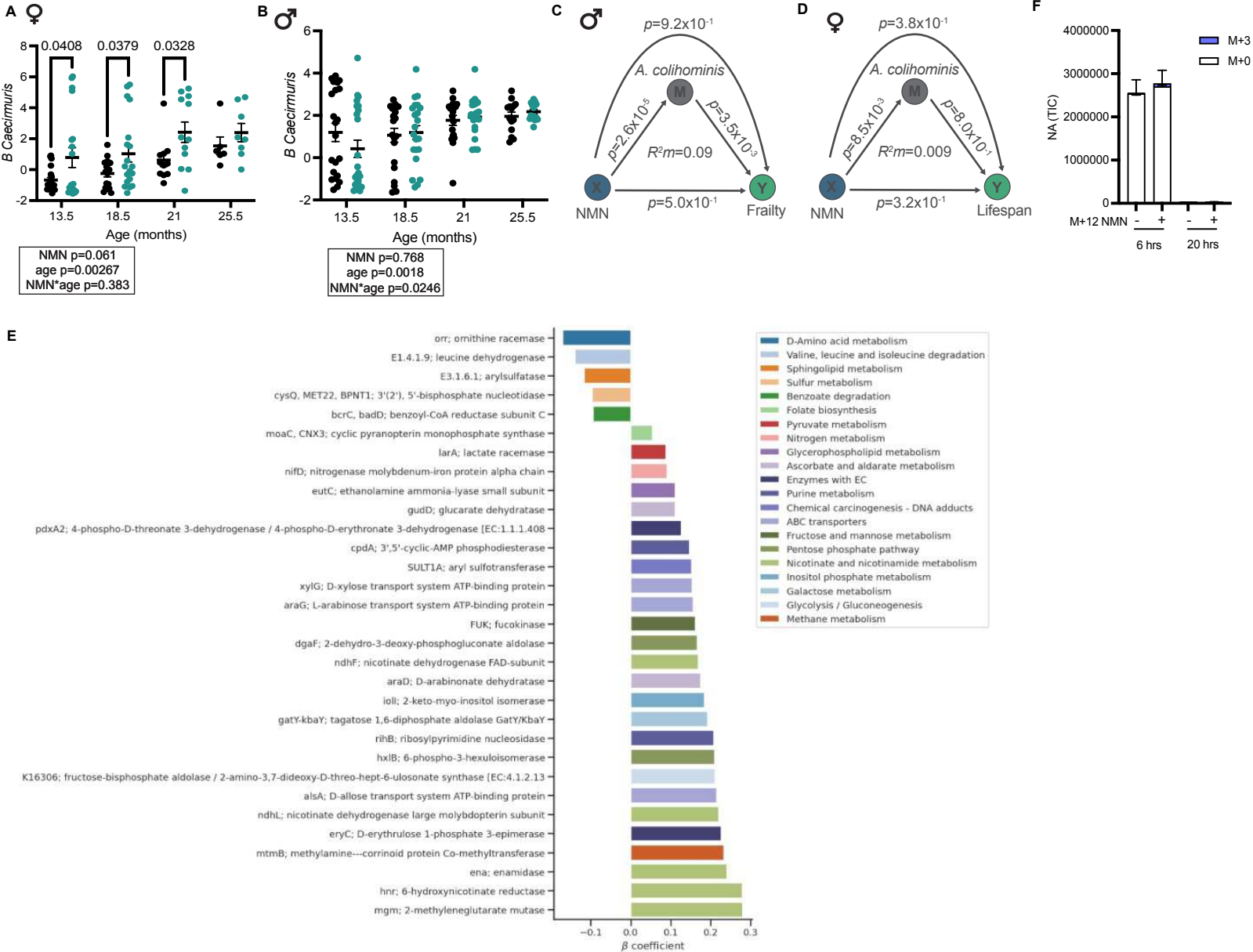
Supplementary Figure 3



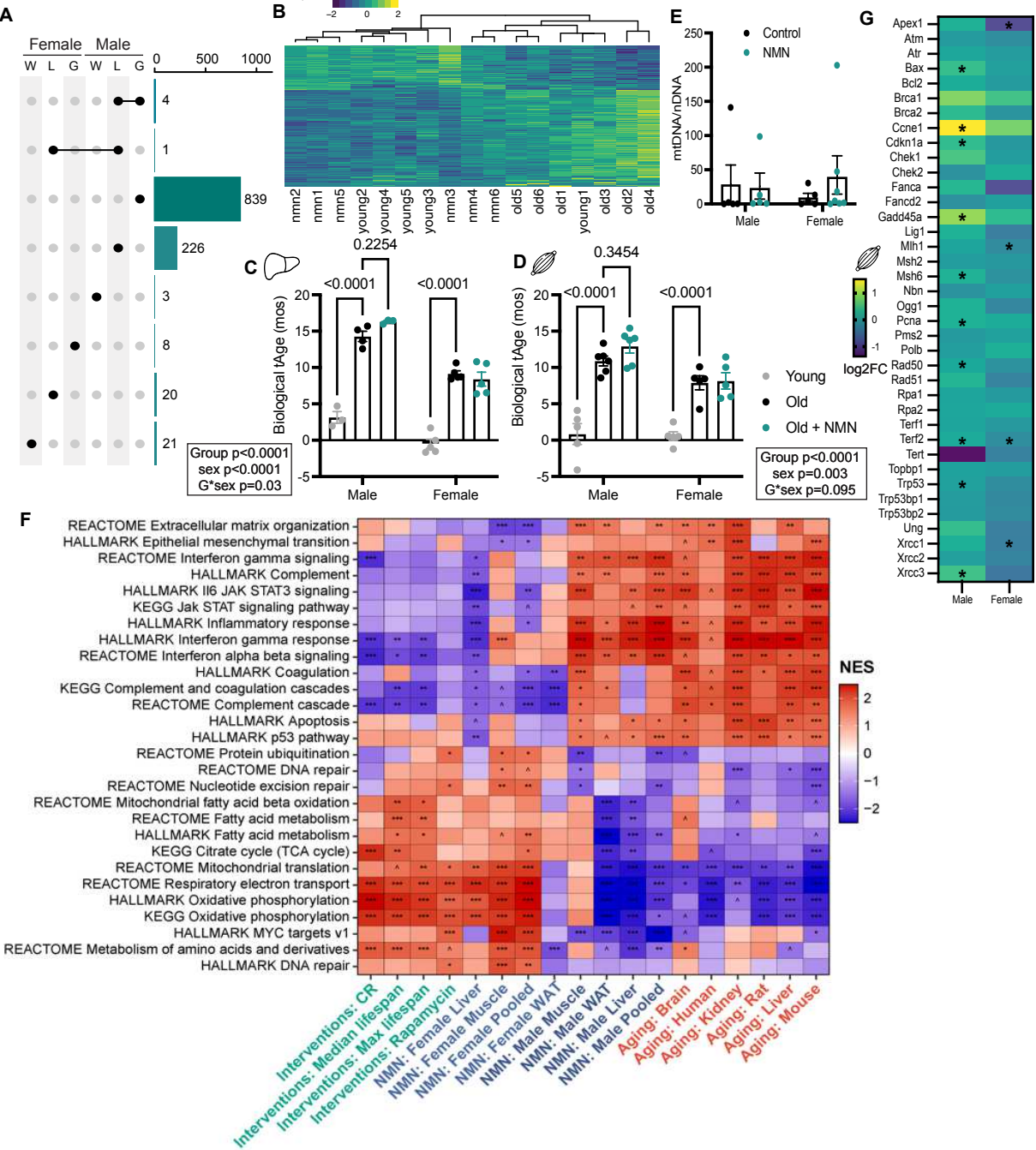


Supplementary Figure 4

Supplementary Figure 5



Supplementary Figure 6



Supplementary Figure 7

