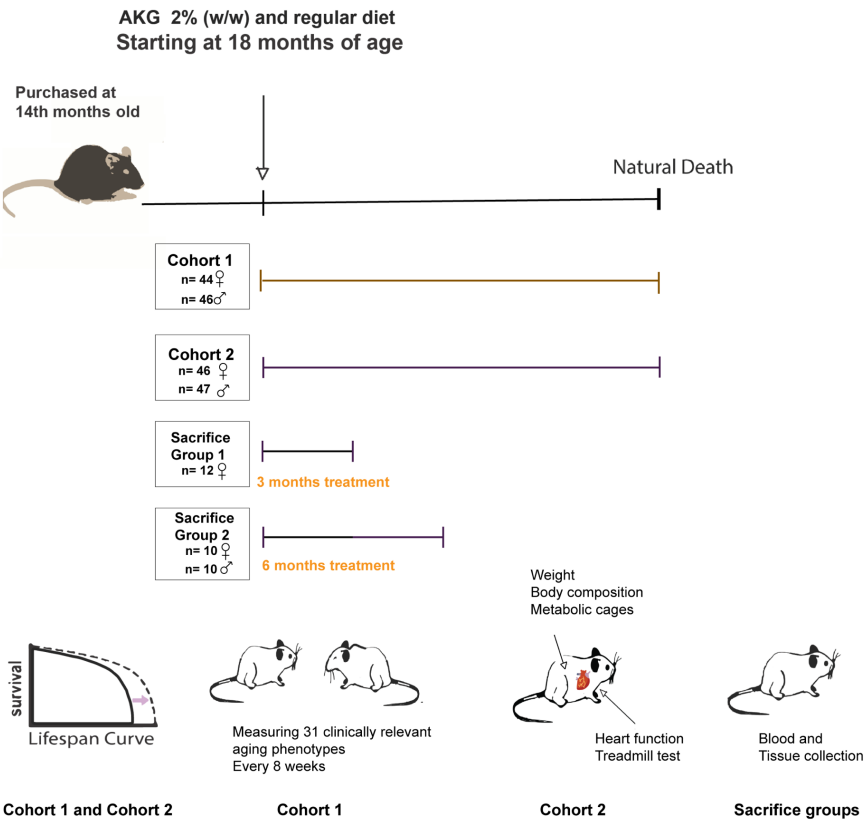
Cell Metabolism, Volume *32*

Supplemental Information

Alpha-Ketoglutarate, an Endogenous Metabolite, Extends Lifespan and Compresses Morbidity in Aging Mice

Azar Asadi Shahmirzadi, Daniel Edgar, Chen-Yu Liao, Yueh-Mei Hsu, Mark Lucanic, Arash Asadi Shahmirzadi, Christopher D. Wiley, Garbo Gan, Dong Eun Kim, Herbert G. Kasler, Chisaka Kuehnemann, Brian Kaplowitz, Dipa Bhaumik, Rebeccah R. Riley, Brian K. Kennedy, and Gordon J. Lithgow



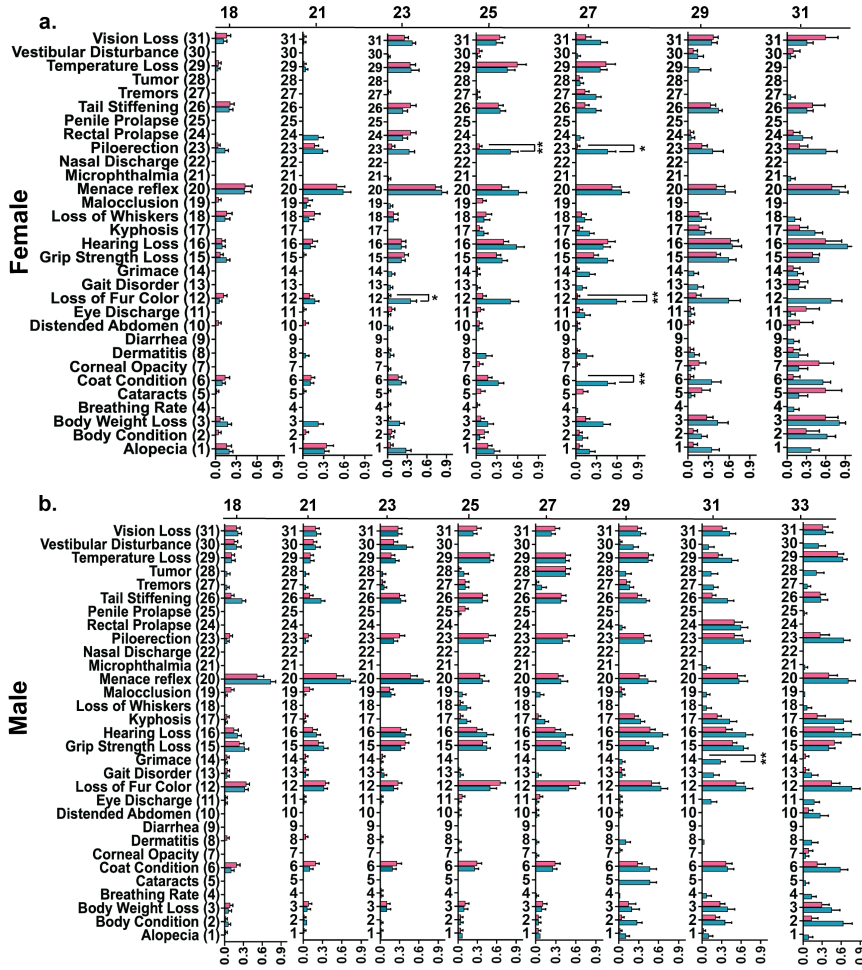
List of assessed deficits in aged mice- Frailty index

Integument:	Physical/Musculoskeletal:	Vestibulocochlear/Auditory:	Digestive/Urogenital:
1. Alopecia	6. Tumors	14. Vestibular disturbance	23. Malocclusions
2. Loss of fur color	7. Distended abdomen	15. Hearing loss	24. Rectal prolapse
3. Dermatitis	8. Kyphosis	16. Cataracts	25. Vaginal/uterine/ Penile prolapse
4. Loss of whiskers	9. Tail stiffening	17. Eye discharge/swelling	26. Diarrhea
5. Coat condition	10. Gait disorders	18. Microphthalmia	**Respiratory system:**
	11. Tremor	19. Corneal opacity	27. Breathing rate/depth
	12. Forelimb grip strength	20. Vision loss	**Discomfort and others:**
	13. Body condition score	21. Menace reflex	28. Mouse Grimace
		22. Nasal discharge	29. Piloerection
			30. Temperature
			31. Weight

Figure S1. Experimental Design, Related to Figure 1-4. The mice were fed AKG or Standard diet at 18 months of age. The study consists of two cohorts (n=183 animal total) and two sacrifice groups. Cohort-1 consists of female control (n=24), female AKG (n=20), male control (n=24) and male AKG (n=22) animals. Cohort-2 consists of female control (n=23), female AKG (n=23), male control (n=24) and male AKG (n=23) animals. All cohorts and sacrificed groups were started on diet at the same age (18 months). Cohort-1 mice were used for all the frailty index measurements and lifespan. Cohort-2 mice were used for replication of survival, metabolic studies and complementary aging studies. List of assessed 31 clinically relevant frailty is provided. All the phenotypes were scored as described by Whitehead, J.C., et al., except for temperature and weight for which the new scaling scores were used. **Temperature and Weights Scoring:** Briefly, average and standard deviations (STDEV) were calculated sex specifically using our own baseline data sets (data collected before the start of the treatment-mice at 18th month old). A decrease in temperature or weight within one STDEV is scored as a (0), a decrease bigger than one STDEV but smaller than 2 STDEV will be scored as a (0.5) and any decrease more than 2 STDEV is scored as a (1)

		Age (days) at 50% mortality		Age (days) at 90% mortality		Age (days) at 100% mortality	
		Cohort-1	Cohort-2	Cohort-1	Cohort-2	Cohort-1	Cohort-2
Female	Control	876	847	1015	931	1019	948
	AKG	932	875	1109	962	1116	975
	Percentage survival increase from inception of study (AKG vs. Cntrl)	16.6%	10.5%	19.7%	8%	21%	6.6%
	Percentage survival increase from birth (AKG vs. Cntrl)	6.3%	3.3%	9%	3.3%	10%	2.8%
Male	Control	955	812	1068	969	1109	987
	AKG	995	847	1155	987	1173	1008
	Percentage survival increase from inception of study (AKG vs. Cntrl)	9.6%	12.8%	16.4%	4.2%	11.2%	4.7%
	Percentage survival increase in survival from birth (AKG vs. Cntrl)	4.2%	3.7%	8.1%	1.8%	5.7%	2.1%

Table S1. The effect of AKG treatment on lifespan, Related to Figure 1.



Female			
Phenotypes	**Age**	**P-value (nonparametric t-test)**	**P-value (Bonferroni correction) P<0.0016**
(1) Alopecia	23 mo	0.012*	ns
(3) Body Weight loss	23 mo	0.0397*	ns
(6) Coat Condition	29 mo	0.042*	ns
	31 mo	0.039*	ns
(12) Loss of fur color	23 mo	0.0008***	*
	25 mo	0.0052**	ns
	27 mo	<0.0001****	**
	29 mo	0.0219*	ns
	31 mo	0.021*	ns
(13) Gait Disorder	27 mo	<0.0001****	**
(14) Grimace	27 mo	0.0149*	ns
(23) Piloerection	18 mo	0.0021**	ns
	23 mo	0.0053**	ns
	25 mo	0.0002***	**
	27 mo	0.0010**	*
(24) Rectal prolapse	21 mo	0.0036**	ns

Male			
Phenotypes	**Age**	**P-value (nonparametric t-test)**	**P-value (Bonferroni correction) P<0.0016**
(2) Body Condition	29 mo	0.0092**	ns
	33 mo	0.0071**	ns
(11) Eye discharge	33 mo	0.035*	ns
(14) Grimace	31 mo	0.0002***	*
(26) Tail stiffening	21 mo	0.0214*	ns
(27) Tremors	31 mo	0.0084**	ns
(30) Vestibular disturbance	29 mo	0.0135*	ns
	31 mo	0.0135*	ns

Figure S2. AKG treatment extends health span and alleviates frailty phenotypes, Related to Figure 2. All individual frailty phenotypes (total of 31 phenotypes), separately graphed (**a**) female and (**b**) male. Data are mean ±s.e.m. of the group, n= all animals alive at each measurement time. *P <0.0016, **P < 0.00032 (Non-parametric two tailed t-test, Bonferroni correction). As in almost all longitudinal studies, missing values have caused data fluctuations during the course of our study. The main contributor to this fluctuation is the death of the frail animal between measurement time points. When the deceased animal is omitted from the group, the total frailty score of the group is improved (lower scores). The remaining mice in the study might exhibit the phenotype, which again change the total score of the group.

Frailty Phenotypes (Female)	Trend test (Mann-Kendall)		Frailty Phenotype (Female)	Trend test (Mann-Kendall)	
	S statistics	P-Value		S statistics	P-Value
1. Alopecia	7	0.3675207	**17. Kyphosis**	18	0.0076802**
2. Body Condition	21	0.0026666*	**18. Loss of Whiskers**	6	0.7595545
3. Body Weight	13	0.0715054	**19. Malocclusion**	-4	0.45325471
4. Breathing Rate	9	0.1282753	**20. Menace Reflex**	7	0.3675207
5. Cataracts	11	0.0572793	**21. Microphthalmia**	5	0.4469742
6. Coat Condition	19	0.0068637**	**22. Nasal Discharge**	0	NaN
7. Corneal Opacity	11	0.0572793	**23. Piloerection**	15	0.0354981*
8. Dermatitis	17	0.0162609*	**24. Rectal Prolapse**	13	0.0567382
9. Diarrhea	6	0.2112995	**25. Tail Stiffening**	13	0.0715054
10. Distended Abdomen	9	0.1803918	**26. Temperature Loss**	5	0.4523703
11. Eye Discharge	16	0.0226870*	**27. Tremors**	9	0.1803918
12. Loss of Fur color	20	0.00389**	**28. Tumors**	2	0.8025873
13. Gait Disorder	18	0.0098091**	**29. Vestibular Disturbance**	14	0.0482861*
14. Grimace	11	0.1331284	**30. Vision Loss**	5	0.5480055
15. Grip Strength Loss	17	0.0162609*	**31. Vaginal Prolapse**	0	NaN
16. Hearing Loss	19	0.0068637**			

Frailty Phenotypes (Male)	Change with Age		Frailty Phenotype (Male)	Change with Age	
	S statistics	P-Value		S statistics	P-Value
1. Alopecia	22	0.009374**	**17. Kyphosis**	23	0.00609055**
2. Body Condition	17	0.046063*	**18. Loss of Whiskers**	5	0.38273309
3. Body Weight	26	0.0019817**	**19. Malocclusion**	4	0.706197
4. Breathing Rate	13	0.1077560	**20. Menace Reflex**	4	0.71052302
5. Cataracts	10	0.197012	**21. Microphthalmia**	11	0.10017829
6. Coat Condition	26	0.0019817**	**22. Nasal Discharge**	-7	0.3093073
7. Corneal Opacity	10	0.197012	**23. Piloerection**	22	0.00937477**
8. Dermatitis	19	0.021895**	**24. Rectal Prolapse**	3	0.66252058
9. Diarrhea	0	NaN	**25. Tail Stiffening**	12	0.17354622
10. Distended Abdomen	12	0.1439428	**26. Temperature Loss**	22	0.00937477**
11. Eye Discharge	20	0.0169649*	**27. Tremors**	12	0.17354622
12. Loss of Fur color	22	0.0093747**	**28. Tumors**	23	0.00508587**
13. Gait Disorder	14	0.0107762*	**29. Vestibular Disturbance**	0	1
14. Grimace	4	0.6671689	**30. Vision Loss**	16	0.06348653
15. Grip Strength Loss	19	0.0248218*	**31. Penile Prolapse**	4	0.66716899
16. Hearing Loss	28	0.000836***			

Table S2. Results of statistical analysis that test for a possible monotonic trend for each frailty phenotype with aging (time), Related to Figure 3. P values are the result of Mann-Kendall Trend Test for changes in frailty over time for females (Table 3) and males (Table 4), *P <0.05, **P <0.01 and ***P <0.001. The absolute value of S statistics indicate the direction and magnitude of the trend (S >0 an increasing trend, S<0 a decreasing trend, S=0 no trend).

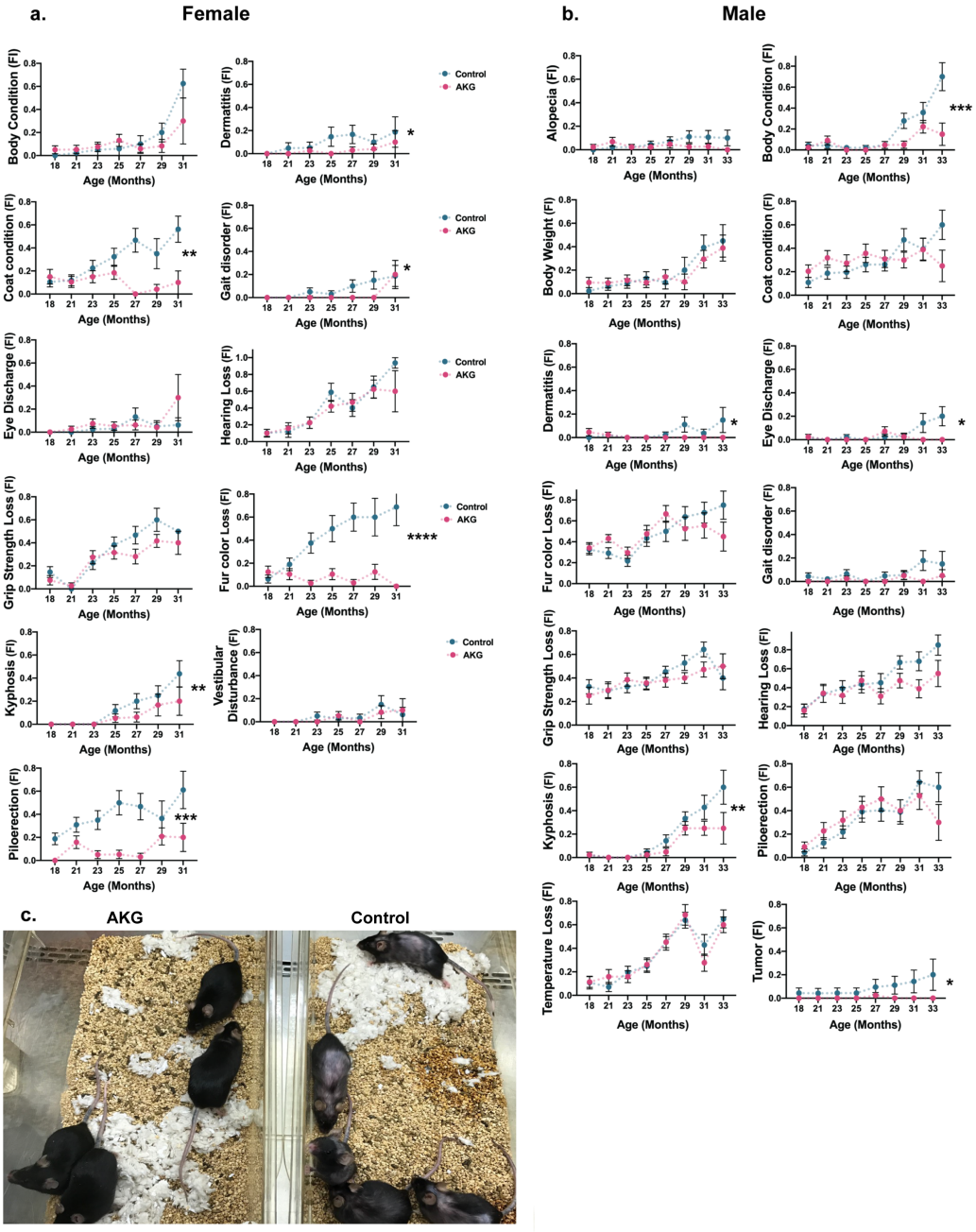
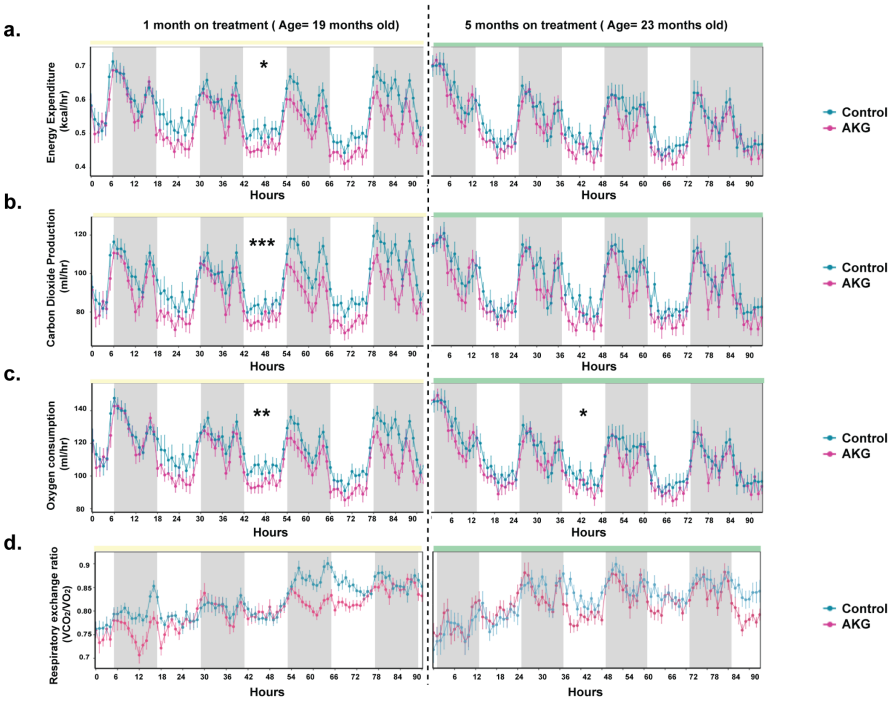


Figure S3. Age-associated frailty phenotypes (including only those that have significant monotonic trend with time), Related to Figure 3. Individually graphed frailty phenotypes that significantly increase with aging, comparing control with AKG treated mice for (**a**) female and (**b**) male. Data are mean ±s.e.m. of the group. Mixed Models was used to test if each age dependent phenotype was affected by AKG supplementation for female (**c**) and male (**d**) in the study. *P <0.05, **P <0.01, ***P<0.001, ****P<0.0001. As in almost all longitudinal studies, missing values have caused data fluctuations during the course of our study. The main contributor to this fluctuation is the death of the frail animal between measurement time points. When the deceased animal is omitted from the group, the total frailty score of the group is improved (lower scores). The remaining mice in the study might exhibit the phenotype, which again change the total score of the group. (**c**) A picture of two cages of female mice side by side at the age of 28 months; on the left are AKG treated mice and on the right are control mice. As presented by the picture female AKG mice have better coat condition more black hair and less alopecia.



P value	1 month on treatment (19 months old)			5 months on treatment (23 months old)		
Energy Expenditure	Light = 0.11	*Dark =0.021	*Full Day= 0.0121	Light = 0.12	Dark= 0.304	Full Day=0.058
Carbon Dioxide	*Light =0.014	*Dark=0.011	*** Full Day<0.001	*Light=0.04	*Dark=0.037	*Full Day=0.019
Oxygen Consumption	*Light=0.045	*Dark=0.014	**Full Day= 0.006	Light =0.16	Dark=0.38	Full Day= 0.25
Respiratory Exchange Ratio	Light=0.217	Dark=0.117	Full Day= 0.128	Light=0.296	Dark=0.822	Full Day= 0.536

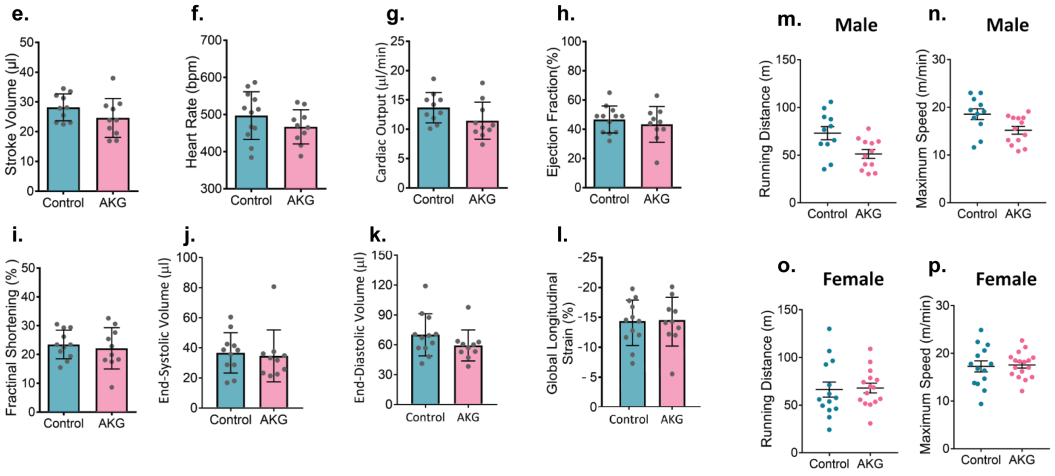


Figure S4. Metabolic rate and heart function of aged mice (Cohort-2 data), Related to Figure 3. (**a**) Energy expenditure, (**b**) Carbon dioxide production and (**c**) Oxygen consumption decrease upon AKG administration in female mice. However (**d**) Respiratory exchange ratio remains the same. Animals were monitored for about four consecutive days (92 hrs).The measurements were done at two separate time points during lifespan (19 and 23 months old) using same animals for both runs. Plots were generated using CalR, the data is adjusted to bodyweight. Control (n=5) and AKG (n=5). Data are mean±s.e.. *$p < 0.05$ and ***$p < 0.001$ (Two way ANOVA tests). (**e-l**) Echocardiography test was performed to measure cardiovascular function close to animal median life, age=29 months old, n= all female animals alive at the time of study, data are mean±s.e.m. No significant change was observed for any of the measurements (t-test two tailed). Treadmill exhaustion tests were performed to measure cardiovascular system and motor function for (**m**,**n**) male and (**o**,**p**) female, age= 29 months old. n= all animals alive at the time of study. No significant change (t-test two tailed).

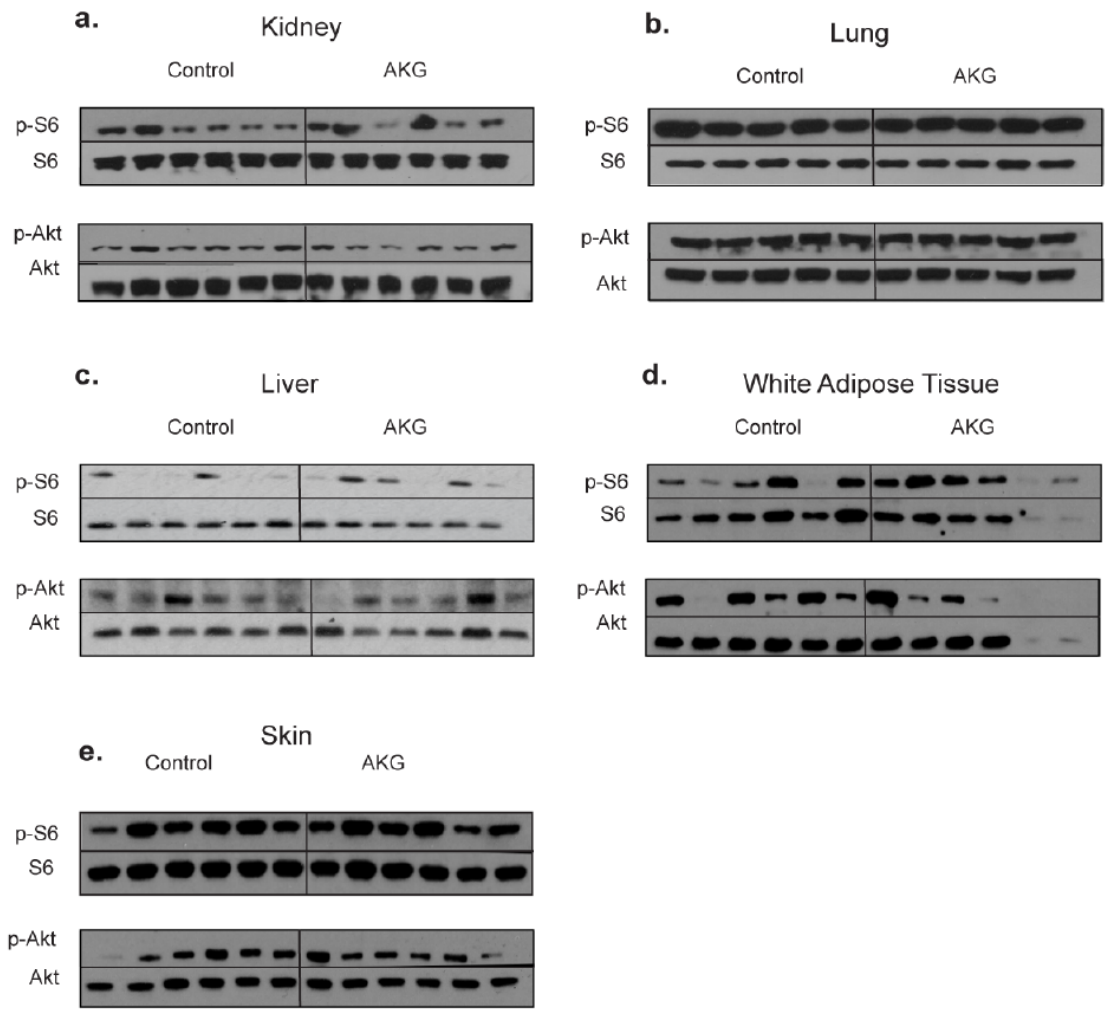
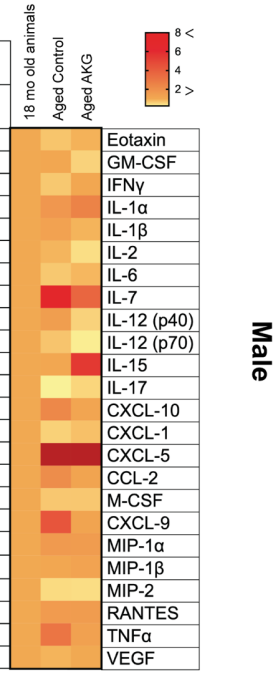


Figure S5. mTOR activity is not changed in tissues of mice treated with AKG, Related to Figure 4. (**a-f**) Western blots of mTORC1 (indicated by p-S6/S6) and mTORC2 (indicated by p-Akt/Akt) activities in multiple tissues of female mice treated with AKG for 3 months. Overall three months AKG treatment does not change mTORC1 and mTORC2 activities

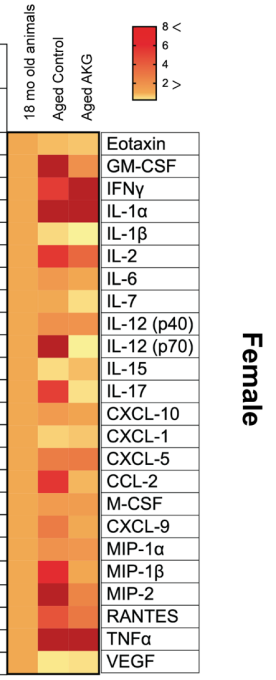
a.

	18 mo old animals		Aged Control		Aged AKG	
	Mean (pg/ml)	STDEV	Mean (pg/ml)	STDEV	Mean (pg/ml)	STDEV
Eotaxin	591.3	104.9	408.4	101.7	537.4	90.5
G-CSF	261.3	44.3	277.22	70.7	139.9	27.1
IFNy	2.6	2.9	1.8	1.97	2.8	2.5
IL-1a	140.1	113.1	246.7	227.2	357.8	80.3
IL-1B	3.3	0.8	4.4	3.15	2.9	1.15
IL-2	5.5	2.0	4.7	4.3	2.3	1.53
IL-6	11.9	6.5	7.9	4.2	10.0	14.3
IL-7	4.1	3.7	29.6	35.7	15.2	23.4
IL-12	7.0	6.1	10.4	9.98	3.7	3.8
IL-12	23.6	29.7	16.5	14.4	6.4	NaN
IL-15	23.0	12.9	23.4	11.0	126.2	232.5
IL-17	6.1	3.3	1.39	0.95	3.1	1.1
CXCL-10	69.2	3.3	162.4	110.9	80.5	22.7
CXCL-1	179.1	46.1	102.0	71.4	131.1	93.9
CXCL-5	469.4	413.9	7302.9	6745.7	9755.5	7505.9
CCL-2	16.2	5.3	35.3	24.1	20.1	1.5
M-CSF	3.3	1.6	2.2	1.1	2.2	0.9
CXCL-9	25.0	28.3	108.5	146.8	30.8	11
MIP-1α	59.1	48.7	97.0	60.3	91.8	39.4
MIP-1β	86.2	19.4	89.5	5.7	107.9	44.5
MIP-2	32.6	10.8	14.8	13.2	13.8	4.7
RANTES	21.4	13.4	34.2	23.7	33.7	10.7
TNFa	1.9	NaN	5.8	3.8	2.5	2.5
VEGF	0.6	0.3	0.6	0.1	0.6	0.3



b.

	18 mo old animals		Aged Control		Aged AKG	
	Mean (pg/ml)	STDEV	Mean (pg/ml)	STDEV	Mean (pg/ml)	STDEV
Eotaxin	479.2	13.7	374.6	52.7	319.1	137.9
G-CSF	338.1	47.9	375.0	252.0	304.2	175.1
IFNy	1.0	NaN	3.1	3.3	5.4	8.7
IL-1a	22.0	1.3	113.1	114.9	117.3	149.1
IL-1B	18.8	28.6	7.7	7.0	2.9	1.9
IL-2	2.0	1.0	6.5	2.4	4.6	5.2
IL-6	9.3	9.9	12.5	11.8	9.7	6.5
IL-7	3.39	1.2	3.3	3.5	1.3	0.5
IL-12	8.3	NaN	12.4	9.3	11.8	8.1
IL-12	27.2	NaN	193.7	Nan	4.5	3.8
IL-15	45.2	41.7	17.9	10.7	34.0	24.4
IL-17	4.7	3.4	14.6	22.9	1.6	0.9
CXCL-10	57.4	11.6	74.6	13.3	63.9	15.2
CXCL-1	264.8	NaN	142.1	29.8	174.4	57.5
CXCL-5	832.4	NaN	1549.6	1566.5	1585.0	904.7
CCL-2	15.9	0.8	52.5	37.3	13.3	4.4
M-CSF	2.3	1.5	3.0	1.4	2.9	2.4
CXCL-9	24.0	10.4	45.2	26.9	23.6	9.5
MIP-1α	76.6	10.5	112.6	105.1	105.7	125.5
MIP-1β	85.1	3.3	291.4	308.8	85.6	62.0
MIP-2	9.1	11.4	279.9	459.5	15.8	14.6
RANTES	11.6	NaN	31.5	47.8	22.9	14.1
TNFa	0.8	NaN	33.1	39.2	7.0	1.2
VEGF	2.5	3.8	0.7	0.08	0.9	0.5



Supplemental Figure 6. AKG reduces inflammation more robustly in females than in males, Related to Figure 4. The mean plasma concentration of 24 inflammatory cytokines and chemokines of middle aged (age=18 months, n=5), aged control and AKG fed (24 months old, n=5) animals for (**a**) Male and (**b**) Female. The heatmap of each sex has been presented alongside their value chart. For graphing the heatmap, the fold changes of cytokines and chemokines were calculated for (**a**) males and (**b**) females using the untreated 18 months old animals as reference for each sex.

Table S3. qRT-PCR primers used in this study, Related to Star Method (Oligonucleotides)

Genes	Forward primers (5'-3')	Reverse primers (5'-3')	Probe
Mouse $p16^{INK4A}$	AACTCTTTCGGTCGTACCCC	TCCTCGCAGTTCGAATCTG	Custom designed UPL probe: 5'-/56-FAM/AGG TGA TGA/ZEN/TGAT GGGCAACGTT CAC/3IABkFQ -3
Mouse p21	TTGCCAGCAGAATAAAAGGTG	TTTGCTCCTGTGCGGAAC	UPL Probe #9
Human IL-1-α	GGTTGAGTTTAAGCCAATCCA	TGCTGACCTAGGCTTGATGA	UPL Probe #6
Human IL-1-β	CTGTCCTGCGTGTTGAAAGA	TTGGGTAATTTTTGGGATCTACA	UPL Probe #78
Human IL-6	GCCCAGCTATGAACTCCTTCT	GAAGGCAGCAGGCAACAC	UPL Probe #45
Human IL-8	AGACAGCAGAGCACACAAGC	ATGGTTCCTTCCGGTGGT	UPL Probe #72
Human CCL2	AGTCTCTGCCGCCCTTCT	GTGACTGGGGCATTGATTG	UPL Probe #40
Human CXCL-1	GCTGAACAGTGACAAATCCAAC	CTTCAGGAACAGCCACCAGT	UPL Probe #52
Human MMP-3	CAAAACATATTTCTTTGTAGAG GACAA	TTCAGCTATTTGCTTGGGAAA ,	UPL Probe #36
Human actin	CCAACCGCGAGAAGATGA	TCCATCACGATGCCAGTG	UPL Probe #58
Human p21	TCACTGTCTTGTACCCTTGTGC	GGCGTTTGGAGTGGTAGAAA	UPL Probe #32