

METHODS FOR MEASURING METABOLIC DYSFUNCTION OR RISK OR PRESENCE OF AN AGE-ASSOCIATED DISEASE

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of priority of U.S. Provisional Application No. 63/509,964 filed June 23, 2023, and U.S. Provisional Application No. 63/602,871 filed November 27, 2023, each of which is hereby incorporated by reference in its entirety.

BACKGROUND

[0002] Chronological age is well understood to be the single greatest risk factor for nearly every major cause of mortality and morbidity in living organisms, including humans and companion dogs. Even before the development of observable disease, the physiology and metabolic function of organ systems and tissues progressively declines throughout life. Accordingly, there remains an unmet need for safe and effective products and methods that promote longevity and extend lifespan and methods to measure the decline in systemic physiological and metabolic function.

BACKGROUND

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SUMMARY

[0004] In some embodiments is a method for promoting health or treating metabolic dysfunction or risk or presence of an age-associated disease in a subject, the method comprising: administering a nutraceutical composition or a pharmaceutical composition to a subject, wherein the administering is based on an assessment of metabolic dysfunction or risk or presence of an age-associated disease in the subject, and wherein the assessment comprises: (a) measuring a fatty acid level within the subject using a high-fidelity fatty acid assay, or measuring an insulin level and/or insulin sensitivity within the subject using a high-fidelity insulin assay; and (b) evaluating the subject's quality of life, frailty, or multimorbidity.

[0005] In some embodiments is a method for promoting health or treating metabolic dysfunction or risk or presence of an age-associated disease in a subject, the method comprising: administering a nutraceutical composition or a pharmaceutical composition to a subject, wherein the administering is based on an assessment of metabolic dysfunction or risk or presence of an age-associated disease in the subject, and wherein the assessment comprises: (a) measuring a fatty acid level within the subject using a high-fidelity fatty acid assay, or measuring an insulin level and/or insulin sensitivity within the subject using a high-fidelity insulin assay; or (b) evaluating the subject's quality of life, frailty, or multimorbidity.

[0006] In some embodiments is a method for increasing lifespan, the method comprising treating metabolic dysfunction or risk or presence of an age-associated disease in a subject, the method comprising: administering a nutraceutical composition or a pharmaceutical composition to a subject, wherein the administering is based on an assessment of metabolic dysfunction or risk or presence of an age-associated disease in the subject, and wherein the assessment comprises: (a) measuring a fatty acid level within the subject using a high-fidelity fatty acid assay, or measuring an insulin level and/or insulin sensitivity within the subject using a high-fidelity insulin assay; and/or (b) evaluating the subject's quality of life, frailty, or multimorbidity.

[0007] In some embodiments is a method for detecting and/or treating metabolic dysfunction or an age-associated disease in a subject, the method comprising: (a) measuring a fatty acid level within the subject using a high-fidelity fatty acid assay, or measuring an insulin level and/or insulin sensitivity within the subject using a high-fidelity insulin assay; and/or (b) evaluating the subject's quality of life, frailty, or multimorbidity.

[0008] In some embodiments, the method comprises measuring the fatty acid level within the subject and measuring the insulin level within the subject. In some embodiments, the measuring of the fatty acid level and/or the insulin level in biological matrices (whole blood, serum, plasma, CSF, synovial fluid, urine, fecal matter, saliva, tissue homogenates including: muscle, liver, heart, pancreas, brain), and the evaluating of the quality of life, frailty, or multimorbidity provide a correlation for detecting the metabolic dysfunction or risk or presence of an age-associated disease.

[0009] In some embodiments is a method for identifying a subject in need of treatment for metabolic dysfunction or risk or presence of an age-associated disease, the method comprising: (a) measuring a fatty acid level within the subject using a high-fidelity fatty acid assay, or measuring an insulin level and/or insulin sensitivity within the subject using a high-fidelity insulin assay; and/or (b) evaluating the subject's quality of life, frailty, or multimorbidity.

[0010] In some embodiments, the method comprises measuring the fatty acid level within the subject and measuring the insulin level within the subject. In some embodiments, the measuring of the fatty acid level and/or the insulin level, and the evaluating of the quality of life provide a correlation for detecting the metabolic dysfunction or risk or presence of an age-associated disease. In some embodiments, the quality of life, frailty, or multimorbidity is measured by Health-Related Quality of Life (HRQL) or Canine Frailty Index (CFI).

[0011] In some embodiments is a method of health screening for the subject, comprising: measuring a fatty acid level within the subject, wherein the fatty acid level is measured using a high-fidelity fatty acid assay, or measuring an insulin level within the subject, wherein the insulin level is measured using a high-fidelity insulin assay, wherein the subject is a companion mammal. In some embodiments, the method comprises evaluating the subject's quality of life, wherein the quality of life is measured by Health-Related Quality of Life (HRQL) or the subject's frailty or multimorbidity using the Canine Frailty Index (CFI).

[0012] In some embodiments, the subject has metabolic dysfunction or risk or presence of an age-associated disease. In some embodiments, the method further comprises comparing the fatty acid level within the subject with a pre-determined fatty acid level, or comparing the insulin level within the subject with a pre-determined insulin level. In some embodiments, the method further comprises promoting or maintaining health of the subject by administering a nutraceutical composition to the subject. In some embodiments, the nutraceutical composition comprises quercetin, azelaoyl phosphatidylcholine, or 4-oxodocosahexaenoic acid.

[0013] In some embodiments, the method further comprises treating the metabolic dysfunction or risk or presence of the age-associated disease in the subject by administering a pharmaceutical composition to the subject. In some embodiments, the pharmaceutical composition comprises troglitazone, troglitazone derivatives, rosiglitazone, lobeglitazone, or a combination thereof. In some embodiments, the pharmaceutical composition comprises troglitazone, rosiglitazone, lobeglitazone, or a combination thereof. In some embodiments, the pharmaceutical composition comprises rosiglitazone, lobeglitazone, or a combination thereof. In some embodiments, the pharmaceutical composition comprises a thiazolidinedione moiety.

[0014] In some embodiments, the high-fidelity fatty acid assay is a gas chromatography-mass spectrometry (GCMS) assay, a ultra performance liquid chromatography (UPLC) assay, or a liquid chromatography mass spectrometry (LCMS) assay. In some embodiments, the high-fidelity fatty acid assay is the GCMS assay. In some embodiments, the high-fidelity fatty acid

assay is the UPLC assay. In some embodiments, wherein the high-fidelity fatty acid assay is the LCMS assay.

[0015] In some embodiments, the high-fidelity insulin assay is an enzyme-linked immunoassay (ELISA). In some embodiments, a sample for the high-fidelity insulin assay is obtained using an oral glucose tolerance test (OGTT).

[0016] In some embodiments, the metabolic dysfunction or risk or presence of an age-associated disease is related to adipose dysfunction. In some embodiments, the risk or presence of the age-associated disease is cancer, sarcopenia, cardiovascular disease, obesity, diabetes, cognitive dysfunction, cancer/neoplasia, liver disease, renal disease, degenerative orthopedic disease, immunosuppressive disease, hyperlipidemia, metabolic dysfunction/disease, hepatic lipidosis, metabolic syndrome, pancreatitis, hypertension or a combination thereof.

[0017] In some embodiments, the insulin level is measured from serum insulin level or plasma insulin level. In some embodiments, the fatty acid level is the fatty acid concentration. In some embodiments, the fatty acid level is evaluated by a fatty acid in a biological matrix. In some embodiments, the serum fatty acid is free fatty acids (FFA), saturated fatty acids (SFA), palmitic acid (PA), oleic acid (OA), linoleic acid (LA), alpha linoleic acid, gamma linoleic acid, palmitoleic acid, margaric acid, stearic acid, myristic acid, pentadecanoic acid, arachidic acid, behenic acid, lignoceric acid or a combination thereof. In some embodiments, the serum fatty acid is free fatty acids (FFA), saturated fatty acids (SFA), palmitic acid (PA), oleic acid (OA), or linoleic acid (LA), or a combination thereof. In some embodiments, the serum fatty acid is palmitic acid (PA) and oleic acid (OA). In some embodiments, the serum fatty acid is palmitic acid (PA) and linoleic acid (LA). In some embodiments, the serum fatty acid is oleic acid (OA) and linoleic acid (LA).

[0018] In some embodiments, the serum fatty acid is palmitic acid (PA), oleic acid (OA) and linoleic acid (LA). In some embodiments, the serum fatty acid is free fatty acids (FFA) and saturated fatty acids (SFA). In some embodiments, the serum fatty acid is free fatty acids (FFA) and palmitic acid (PA). In some embodiments, the serum fatty acid is free fatty acids (FFA) and oleic acid (OA). In some embodiments, the serum fatty acid is free fatty acids (FFA) and linoleic acid (LA). In some embodiments, the serum fatty acid is palmitic acid (PA). In some embodiments, the serum fatty acid is oleic acid (OA). In some embodiments, the serum fatty acid is linoleic acid (LA). In some embodiments, the serum fatty acid is free fatty acids (FFA). In some embodiments, the serum fatty acid is saturated fatty acids (SFA).

[0019] In some embodiments, the method further comprises measuring the serum insulin level. In some embodiments, the serum fatty acid is evaluated from the subject's blood sample.

[0020] In some embodiments, the subject is fasted. In some embodiments, the subject is fasted for at least 4 hours. In some embodiments, the subject is fasted for at least 6 hours. In some embodiments, the subject is fasted for at least 10 hours.

[0021] In some embodiments, the serum insulin level is evaluated from the subject's blood sample. In some embodiments, the quality of life is measured by HRQL. In some embodiments, the frailty or multimorbidity is measured by CFI.

[0022] In some embodiments, the subject is a mammal. In some embodiments, the mammal is a dog, cat, horse, cow, pig, rabbit, rodent, sheep, non-human primate, or human. In some embodiments, the mammal is a dog. In some embodiments, the mammal is a mouse or rat. In some embodiments, the mammal is a human.

[0023] In some embodiments, an increase of the fatty acid level and increase in CFI indicates the metabolic dysfunction or risk or presence of the age-associated disease. In some embodiments, the fatty acid level is increased by at least 10% compared to a subject without metabolic dysfunction or risk or presence of the age-associated disease. In some embodiments, the fatty acid level is increased by at least 20% compared to a subject without metabolic dysfunction or risk or presence of the age-associated disease.

[0024] In some embodiments, an increase of the insulin level and increase in CFI indicates the metabolic dysfunction or risk or presence of the age-associated disease. In some embodiments, the insulin level is increased by at least 10% compared to a subject without metabolic dysfunction or risk or presence of the age-associated disease. In some embodiments, the insulin level is increased by at least 20% compared to a subject without metabolic dysfunction or risk or presence of the age-associated disease.

[0025] It shall be understood that different aspects and/or embodiments of the disclosure can be appreciated individually, collectively, or in combination with each other. Various aspects and/or embodiments of the disclosure described herein may be applied to any of the uses set forth below and in other methods for increasing lifespan in a mammal. Any description herein concerning a specific composition and/or method apply to and may be used for any other specific composition and/or method as disclosed herein. Additionally, any composition disclosed herein is applicable to any herein-disclosed method. In other words, any aspect or embodiment described herein can be combined with any other aspect or embodiment as disclosed herein.

INCORPORATION BY REFERENCE

[0026] All publications, patents, and patent applications herein are incorporated by reference to the same extent as if each individual publication, patent, or patent application was specifically and individually indicated to be incorporated by reference. In the event of a conflict between a term herein and a term in an incorporated reference, the term herein controls.

BRIEF DESCRIPTION OF THE DRAWINGS

[0027] **FIG. 1** illustrates FFA, SFA, PA, OA and LA concentrations are associated with increasing age and higher CFI scores. Scatterplot showing FFA, SFA, PA, OA and LA serum concentrations ($\mu\text{g/mL}$) increasing with age and CFI in dogs. The fitted line shows the association between each grouped or individual fatty acid with age, insulin, CFI and HRQL (left to right) without covariate adjustment, estimated using linear regression.

[0028] **FIG. 2** illustrates the plot showing the relationship between CFI and chronological age, with a linear regression relationship.

[0029] **FIG. 3** illustrates that FFA, SFA, PA, OA, and LA are associated with CFI after adjusting for the effects of BCS. Scatterplot of observed and predicted FFA, SFA, PA, OA and LA serum concentrations ($\mu\text{g/mL}$) with CFI scores in dogs. The fitted regression line shows the estimated association between the given fatty acid and CFI adjusting for BCS. Mean BCS and ages 4, 8, 12, and 16 used when estimating predicted CFI scores. Colored dots represent dogs of different age groups (2-6 yellow, 6-10 teal, 10-14 blue, 14-18 purple).

[0030] **FIG. 4** illustrates FFA, SFA, PA, OA and LA are associated with fasting insulin after adjusting for the effects of weight. Scatterplot of observed FFA, SFA, PA, OA and LA serum concentrations ($\mu\text{g/mL}$) with fasting insulin concentrations (mIU/L) in dogs. The fitted regression lines within each panel show the estimated association between the given fatty acid and insulin concentrations adjusting for weight based on model fit. Mean weight and ages 4, 8, 12, and 16 used when estimating predicted insulin ($\mu\text{g/mL}$). Colored dots represent dogs of different age groups (2-6 yellow, 6-10 teal, 10-14 blue, 14-18 purple).

[0031] **FIG. 5** illustrates the histogram of the fatty acid analysis cohort ($n=61$) compared to the full Healthspan cohort ($n=451$).

[0032] **FIG. 6** illustrates quantile-quantile normality plots of residuals generated from models testing interactions of fatty acids and age against CFI seen in Table 3.

[0033] **FIG. 7** illustrates quantile-quantile normality plots of residuals generated from models testing interactions of fatty acids and age against CFI seen in Table 4.

[0034] **FIG. 8** illustrates scatterplot of sensitivity analysis of the interaction of FFA, SFA, PA, OA and LA serum concentrations (ug/mL) with fasting insulin concentrations (mIU/L) in dogs. The fitted regression line shows the estimated association between the given fatty acid and CFI, adjusting for weight (kgs). Mean weight and ages 4, 8, 12, and 16 used when estimating predicted insulin (mIU/L). Colored dots represent dogs of different age groups (2-6 yellow, 6-10 teal, 10-14 blue, 14-18 purple).

[0035] **FIG. 9** illustrates a pairwise scatter and individual density plots of all variables considered in this report: insulin, age, weight, BCS, HRQL total score, HRQL domains (E/E, H/C, A/C, C/R) and Canine Frailty Score (CFI).

[0036] **FIG. 10** illustrates a scatterplot showing standardized log(insulin) blood levels decrease with age in dogs. The fitted line shows the positive association between insulin and age without covariate adjustment, estimated using quantile (median) regression. This positive association held after adjusting for weight and body condition score (BCS) (slope=0.07, 95% CI=(0.04, 0.10), $p<0.001$) and is reflected in the text printed on the figure. Colored dots represent dogs of different weight groups (<50, >50, >100 and >150lb).

[0037] **FIG. 11** illustrates insulin blood levels associated with HRQL Total, and certain HRQL domains, adjusting for age, weight, and BCS. Scatterplot showing standardized log(insulin) blood levels associations with HRQL Total, HRQL domains (Energetic/Enthusiastic, Happy/Content, Active/Comfortable, Calm/Relaxed), and CFI in dogs. The fitted quantile regression line shows the unadjusted association between insulin and median values of these measures. Text within each panel shows the covariate-adjusted associations between insulin blood levels and median values of each measure in models containing age, weight, and BCS as covariates. Insulin is significantly negatively associated with median HRQL Total, Energetic/Enthusiastic, and Active/Comfortable domains after adjusting for age, weight, and BCS. Colored dots represent dogs of different age groups (<3, 3-6, 6-9, 9-12, 12-15, >15 years).

[0038] **FIG. 12** illustrates the inverse relationship between insulin blood levels and HRQL A/C domain scores is stronger in older dogs, adjusting for weight and BCS. Scatterplot showing standardized log(insulin) blood levels associations Active/Comfortable (A/C) domain in young (<6 years old) and old (>7 years old) dogs. The fitted line shows the unadjusted association between insulin and A/C, estimated using quantile (median) regression models stratified by old and young dogs. Text within each panel shows the covariate-adjusted associations between insulin blood levels and median A/C scores in a model adjusting for age, weight, and BCS as covariates. Insulin is significantly negatively associated in both young ($\beta=-0.06$, 95% CI=(-

0.11, -0.01), $p=0.014$) and old dogs ($\beta=-0.17$ 95% CI= $(-0.29, -0.05)$, $p=0.005$), however, this effect is over two times as strong in old dogs. Colored dots represent dogs of different age groups (<3 , 3-6, 6-9, 9-12, 12-15, >15 years).

[0039] **FIG. 13** illustrates the predicted median HRQL Total scores for insulin levels (lowest, middle, highest), adjusting for age, weight, and BCS. Predicted median and 95% confidence intervals (CI) of HRQL Total from a quantile regression model fit with insulin levels as primary predictor, and covariates age, weight, and BCS in the model. Points represent the predicted median value for each insulin group. Vertical lines reflect the 95% CI around the estimated median. Text within the figure shows the pairwise comparisons across insulin levels, specifically the contrast between the two groups, the 95% CI, and the p-value associated with the analysis. P-values are adjusted for multiple comparison correction using Tukey method. Insulin groups were defined as sample tertiles, resulting in the following assignments: low= $[2.53 \text{ mU/L}, 12 \text{ mU/L}]$, middle= $[12 \text{ mU/L}, 20.9 \text{ mU/L}]$, and high= $[20.9 \text{ mU/L}, 107 \text{ mU/L}]$. The overall effect of insulin was statistically significant ($p=0.005$).

[0040] **FIG. 14** illustrates the pairwise scatter and individual density plots of all variables considered: insulin, age, weight, and BCS.

[0041] **FIG. 15** illustrates the scatterplot of observed and predicted Canine Frailty Index (CFI) values as a function of interactions between fasting insulin (mU/L) and age in dogs. The fitted regression line shows the covariate adjusted predictions for dogs of a given age across insulin levels, adjusting for weight (kgs) and body condition score (BCS). Mean values for weight (kgs) and BCS and ages 4, 7, 10, 15, and 18 used when estimating predicted CFI.

[0042] **FIG. 16** illustrates total HRQL scores and age within the longitudinal and non-longitudinal datasets. The top panel shows all data points in gray and those in the target age range (> 10 years) in red. The lower panel shows the trajectories of dogs in this target age range for which have longitudinal data ($n = 159$). At the population level, there exists a similar decline in quality of life within the longitudinal data.

DETAILED DESCRIPTION

Introduction

[0043] The proximate cause of death for most dogs is euthanasia and owners frequently cite discomfort, disability, poor quality of life, and advanced age as reasons for electing euthanasia. Higher Canine Frailty Index (CFI) scores are associated with lower Health Related Quality of

Life (HRQL) scores, implying that the development of multimorbidities and increased frailty as assessed by veterinarians reflects the owners' perceptions of reduced quality of life.

[0044] New analysis shows that fasted insulin levels increase with age and are associated with a decline in quality of life. Specifically, it is seen that for dogs of the same age, weight, and body condition, a 58.8% increase in serum insulin levels is associated with the equivalent of a 1 year additional decline in HRQL total scores in dogs 14 lb and over and 10 years or older, and 66.7% for dogs 40 lb and over and 7 years and over.

[0045] New analysis demonstrates that fatty acids, such as but not limited to, free fatty acids, saturated fatty acids, palmitic acid, oleic acid and linoleic acid increase with age. Every year increase in age is associated with a 0.09 to 0.11 standard deviation (SD) increase in mean fatty acid concentrations, adjusting for the effect of body condition score. Increased fatty acid levels are linked with progressive frailty/multimorbidity in dogs in the Healthspan Study, independent of weight and body condition score. Increased fatty acid concentrations are also associated with higher fasted insulin levels. Thus, by using a high fidelity method of correlating the fatty acids and/or insulin levels, it will be useful for detecting the presence of metabolic dysfunction which predisposes the subject to age-associated diseases. These methods will identify a subject in need of treatment for the underlying metabolic dysfunction and predisposition to age-associated diseases.

[0046] Efficient detection of metabolic dysfunction and therefore predisposition to age-associated diseases using the new method described herein will be useful for treating and preventing the age-associated diseases in a timely manner, which will result in prolonged lifespan and healthspan in the subject.

[0047] Several core mechanisms that drive aging are highly conserved across species including worms, flies, rodents, and humans. In humans, aging mechanisms have been associated with excess visceral adiposity resulting in adipose dysfunction and systemic metabolic consequences. Likewise, in dogs it has been demonstrated that adiposity increases with age independent of nutritional status and is correlated with reduced lifespan. The increase in whole body adiposity with age is primarily driven by an expansion of visceral adipose tissue, which is associated with poor metabolic outcomes due its anatomical location and high rate of lipolytic activity in comparison to subcutaneous adipose tissue.

[0048] Due to the high lipolytic activity of visceral adipose tissue, fatty acids are released into the circulation to be utilized by other tissues, such as the liver and skeletal muscle.

Importantly, chronically elevated FFAs are correlated with obesity, insulin resistance, and are predictive of all-cause mortality.

[0049] Fasting insulin levels, along with glucose levels, are often used to clinically determine degree of metabolic dysfunction within humans and dogs. When a metabolic stressor is present, such as overnutrition or advanced age, more insulin is secreted in order to regulate glucose homeostasis and lipid metabolism. Chronic elevation of insulin to combat metabolic stressors leads to hyperinsulinemia, which inevitably desensitizes the peripheral tissues including liver and skeletal muscle to insulin itself, causing an overall state of whole-body insulin resistance. Both hyperinsulinemia and insulin resistance are well described phenotypes of aging-related diseases like sarcopenia, and are also signs of advanced age itself.

[0050] These findings from a large scale study in clinically healthy dogs support the role of insulin and its relevance to metabolic dysfunction and physiological aging. Higher body condition scores (BCS) were associated with higher serum insulin levels. By comparing low, medium, and high insulin groups, the data revealed that higher insulin levels were associated with a decline in HRQL total score of 2-3 points. Longitudinal studies of HRQL show that HRQL roughly declines 1.04 points for each year of age. Based on an internal analysis from a large-scale source of HRQL data from several independent studies, this change in score is roughly equivalent to the magnitude of decline in HRQL over 3 years of aging. When the insulin data are expressed as a continuous variable (at the most granular level of measurement), insulin levels show a significant negative association with HRQL total ($\beta=-1.42$, 95% CI = (-2.19, -0.64)). These results are interpreted as every standard deviation increase in $\log(\text{insulin})$ is associated with a 1.42 point lower median HRQL total scores, independent of the effects of age, weight or BCS.

[0051] Both fatty acid and fasting insulin measurements, taken individually and together, are sufficient to inform whether metabolic dysfunction is present. Studies using caloric restriction (CR) in dogs have demonstrated that fasting insulin and fatty acid levels are lower in the CR compared to the group fed *ad libitum*. The dogs in the CR group were also observed to have longer lifespans.

Methods

[0052] In one aspect, provided herein is a method for detecting metabolic dysfunction and treating a propensity to age-associated disease in a subject, the method comprising administering a nutraceutical composition or a pharmaceutical composition to a subject, wherein the administering is based on an assessment of metabolic dysfunction and/or a propensity to age-

associated disease in the subject. In some embodiments, the assessment comprises (a) measuring a fatty acid level within the subject using a high-fidelity fatty acid assay, or measuring an insulin level and/or insulin sensitivity within the subject using a high-fidelity insulin assay; and (b) evaluating the subject's quality of life, frailty, or multimorbidity status. In some embodiments, the subject is a dog.

[0053] In one aspect, provided herein is a method for promoting health or treating metabolic dysfunction or risk or presence of an age-associated disease in a subject. In some embodiments, the method comprises administering a nutraceutical composition or a pharmaceutical composition to a subject.

[0054] In some embodiments, the administering is based on an assessment of metabolic dysfunction or risk or presence of an age-associated disease in the subject. In some embodiments, the assessment comprises: (a) measuring a fatty acid level within the subject using a high-fidelity fatty acid assay, or measuring an insulin level and/or insulin sensitivity within the subject using a high-fidelity insulin assay. In some embodiments, the assessment further comprises (b) evaluating the subject's quality of life, frailty, or multimorbidity. In some embodiments, the subject has an age-associated disease. In some embodiments, the subject is a dog.

[0055] In one aspect, provided herein is a method for detecting metabolic dysfunction and treating a propensity to age-associated disease in a subject, the method comprising administering a nutraceutical composition or a pharmaceutical composition to a subject, wherein the administering is based on an assessment of metabolic dysfunction and/or a propensity to age-associated disease in the subject. In some embodiments, the assessment comprises (a) measuring a fatty acid level within the subject using a high-fidelity fatty acid assay, or measuring an insulin level and/or insulin sensitivity within the subject using a high-fidelity insulin assay; or (b) evaluating the subject's quality of life, frailty, or multimorbidity status. In some embodiments, the subject is a dog.

[0056] In one aspect, provided herein is a method for detecting metabolic dysfunction and treating a propensity to age-associated disease in a subject, the method comprising: administering a nutraceutical composition or a pharmaceutical composition to a subject, wherein the subject has been assessed as having metabolic dysfunction. In some embodiments, the subject has been assessed as having metabolic dysfunction by a method comprising measuring a fatty acid level within the subject using a high-fidelity fatty acid assay, or measuring an insulin level and/or insulin sensitivity within the subject using a high-fidelity insulin assay. In some embodiments,

the method comprises evaluating the subject's quality of life, frailty, or multimorbidity. In some embodiments, the subject is a dog.

[0057] In one aspect, provided herein is a method for promoting or maintaining health in a subject, the method comprising administering a nutraceutical composition to a subject, wherein the administering is based on an assessment of metabolic function in the subject. In one aspect, provided herein is a method for treating metabolic dysfunction in a subject, the method comprising administering a pharmaceutical composition to a subject, wherein the administering is based on an assessment of metabolic dysfunction in the subject. In some embodiments, the assessment comprises measuring a fatty acid level within the subject using a high-fidelity fatty acid assay, or measuring an insulin level and/or insulin sensitivity within the subject using a high-fidelity insulin assay. In some embodiments, the subject is a dog.

[0058] Provided herein is a method for detecting and/or treating metabolic dysfunction in a subject, the method comprising: (a) measuring a fatty acid level within the subject, wherein the fatty acid level is measured using a high-fidelity fatty acid assay, or measuring an insulin level within the subject, wherein the insulin level is measured using a high-fidelity insulin assay; and (b) evaluating the subject's quality of life, frailty, or multimorbidity status, thereby detecting the age-associated disease.

[0059] Provided herein is a method for identifying a subject in need of treatment for metabolic dysfunction, the method comprising: (a) measuring a fatty acid level within the subject, wherein the fatty acid level is measured using a high-fidelity fatty acid assay, or measuring an insulin level within the subject, wherein the insulin level is measured using a high-fidelity insulin assay; and/or (b) evaluating the subject's quality of life, or evaluating the subject's frailty or multimorbidity status, thereby identifying a subject in need of treatment for metabolic dysfunction and age-associated diseases.

[0060] Provided herein is a method for identifying a subject in need of monitoring, in need of management of, or in need of intervention for metabolic dysfunction and/or age-associated disease, the method comprising: (a) measuring a fatty acid level within the subject, wherein the fatty acid level is measured using a high-fidelity fatty acid assay, or measuring an insulin level within the subject, wherein the insulin level is measured using a high-fidelity insulin assay; and (b) evaluating the subject's quality of life, frailty, or multimorbidity status, thereby monitoring a subject in need of treatment for the age-associated disease.

[0061] The methods can be useful for assigning and validating a new reference range for fatty acid and insulin, for identifying abnormal fatty acid levels, for identifying abnormal insulin

levels, for identifying predispositions for frailty or reduced HRQL due to fatty acid levels and/or insulin levels, and for identifying successful intervention points. The methods can also include measuring the fatty acid and/or insulin level and evaluating quality of life multiple time points. This may be useful to improve determination of metabolic dysfunction and age-associated disease, and methods to treat or monitor metabolic dysfunction and age-associated disease.

[0062] Provided herein is a method of health screening for a subject, comprising: measuring a fatty acid level within the subject, wherein the fatty acid level is measured using a high-fidelity fatty acid assay, or measuring an insulin level within the subject, wherein the insulin level is measured using a high-fidelity insulin assay, wherein the subject is a companion mammal. In some embodiments, the method further comprises evaluating the subject's quality of life, wherein the quality of life is measured by Health-Related Quality of Life (HRQL) or Canine Frailty Index (CFI). In some embodiments, the subject has an age-associated disease.

[0063] The subjects described herein may be at risk of developing an age-related disease, in need of intervention for, and/or in need of an appropriate intervention for the age-related disease. The methods described herein are useful for identifying, treating, monitoring, and managing the age-associated diseases. The methods described herein are also useful for identifying a subject predisposed to an age-associated disease. The methods described herein are also useful for identifying an intervention for the age-associated disease.

[0064] The methods described herein can identify abnormal FFA levels, identify abnormal insulin levels, identify predispositions for frailty or reduced HRQL due to FFA and insulin levels, and can identify successful intervention. The method described herein may be useful for assigning and validating new reference ranges for FFA and insulin.

[0065] Quality of life can be measured indicators including, but not limited to the frailty of the subject, physical and mental health, physical and mental health perceptions, and future outlook. In some embodiments, the quality of life can be measured by Health-Related Quality of Life (HRQL) In some embodiments, frailty, physical health and multimorbidity can be measured by Canine Frailty Index (CFI).

[0066] Measuring the fatty acid level and/or insulin level and/or insulin sensitivity in conjunction with evaluating the subject's quality of life can be a novel and new diagnostic method for determining if the subject has an age-associated disease. In some embodiments, the method comprises measuring the fatty acid level within the subject and measuring the insulin level within the subject. In some embodiments, the measuring of the fatty acid level and/or the

insulin level, and the evaluating of the quality of life provide a correlation for detecting metabolic function and predisposition to age-associated disease.

[0067] In some embodiments, the method comprises measuring the fatty acid level within the subject and measuring the insulin level within the subject over time. In some embodiments, measuring the subject over time can comprise longitudinal assessment. In a non-limiting example, the measurement can be performed over weeks, months, or years. In some embodiments, the measurement can be performed at least one a week, at least twice a week, at least once a month, at least twice a month, at least once every two months, at least once every three months, at least once every four months, at least once every five months, or at least once every six months.

[0068] The methods described herein can further comprise comparing the fatty acid level within the subject with a pre-determined fatty acid level as a standard for determining whether the subject is at risk for or has an age-associated disease. The ranges and levels would be unique to the different assays. The comparison between the fatty acid level within the subject and with a pre-determined fatty acid level would be evaluated based on the unique assays to determine if there is a statistical significance to the change in the fatty acid level.

[0069] The fatty acid level can refer to the concentration of the fatty acid, or any other measurement of fatty acid known by one of skill in the art. In some embodiments, the fatty acid level is the fatty acid concentration.

[0070] In some embodiments, the method described herein further comprises promoting or maintaining health of the subject by administering a nutraceutical composition to the subject. The nutraceutical composition can be a useful supplement for treating the subject. In some embodiments, the nutraceutical composition comprises quercetin, azelaoyl phosphatidylcholine, or 4-oxodocosahexaenoic acid. In some embodiments, the method described herein further comprises treating the age-associated disease in the subject by administering a pharmaceutical composition to the subject. In some embodiments, the pharmaceutical composition is a thiazolidinedione. In some embodiments, the pharmaceutical composition comprises rosiglitazone, lobeglitazone, or a combination thereof.

[0071] The method described herein can be useful for monitoring a subject treated for the metabolic dysfunction, for example, treatment with the nutraceutical composition or pharmaceutical composition. In a non-limiting example, the methods can provide useful metrics for identifying the subject's response to the treatment method. In some embodiments, the subject

being treated is monitored for appropriate intervention for, or at risk of developing age-associated diseases.

[0072] Various assays are useful for measuring the fatty acid level and insulin level of the subject. One of skill in the art can select a useful assay for measuring the fatty acid level and/or insulin level for the methods described herein. In some embodiments, the fatty acid assay is a high-fidelity fatty acid assay. In some embodiments, the high-fidelity fatty acid assay is a gas chromatography-mass spectrometry (GCMS) assay, ultra performance liquid chromatography (UPLC) assay, or a liquid chromatography mass spectrometry (LCMS) assay. In some embodiments, the high-fidelity fatty acid assay is the GCMS assay. In some embodiments, the high-fidelity fatty acid assay is the UPLC assay. In some embodiments, the high-fidelity fatty acid assay is the LCMS assay.

[0073] In some embodiments, the insulin assay is a high-fidelity insulin assay. In some embodiments, the high-fidelity insulin assay is an enzyme-linked immunoassay (ELISA). In some embodiments, the high-fidelity insulin assay is used to quantify insulin derived from an oral glucose tolerance test (OGTT).

[0074] The biomarkers can be related to an age-associated disease. In some embodiments, the age-associated disease is related to adipose dysfunction.

[0075] In some embodiments, the age-associated disease is cancer, sarcopenia, obesity, diabetes, cognitive dysfunction, cancer/neoplasia, cardiovascular disease, liver disease, renal disease, degenerative orthopedic diseases, immunosuppressive diseases, hyperlipidemia, metabolic dysfunction/disease, hepatic lipidosis, metabolic syndrome, pancreatitis, hypertension or a combination thereof.

[0076] In some embodiments, the age-associated disease is cancer. In some embodiments, the age-associated disease is sarcopenia. In some embodiments, the age-associated disease is cardiovascular disease. In some embodiments, the age-associated disease is obesity. In some embodiments, the age-associated disease is diabetes. In some embodiments, the age-associated disease is cognitive dysfunction. In some embodiments, the age-associated disease is cancer/neoplasia. In some embodiments, the age-associated disease is liver disease. In some embodiments, the age-associated disease is renal disease. In some embodiments, the age-associated disease is degenerative orthopedic disease. In some embodiments, the age-associated disease is immunosuppressive disease. In some embodiments, the age-associated disease is hyperlipidemia. In some embodiments, the age-associated disease is metabolic dysfunction/disease. In some embodiments, the age-associated disease is hepatic lipidosis. In

some embodiments, the age-associated disease is metabolic syndrome. In some embodiments, the age-associated disease is pancreatitis. In some embodiments, the age-associated disease is hypertension.

[0077] The insulin level of a subject can be measured from any suitable sample of the subject from any suitable biological matrix. In some embodiments, the insulin level is measured from the serum insulin level or plasma insulin level. In some embodiments, the insulin level is measured from the serum insulin level. In some embodiments, the insulin level is measured from the plasma insulin level. In some embodiments, the insulin level is evaluated from the subject's blood sample. In some embodiments, the serum insulin level is evaluated from the subject's blood sample. In some embodiments, the insulin level is evaluated from the subject's whole blood, serum, plasma, CSF, synovial fluid, urine, fecal matter, saliva, or tissue homogenates (e.g., muscle, liver, heart, pancreas, brain).

[0078] The fatty acid level of a subject can be measured from any suitable sample of the subject from any suitable biological matrix. In some embodiments, the fatty acid level is evaluated by serum fatty acid or plasma fatty acid. In some embodiments, the fatty acid level is evaluated by serum fatty acid. In some embodiments, the fatty acid level is evaluated by plasma fatty acid. In some embodiments, the serum fatty acid is evaluated from the subject's blood sample. In some embodiments, the fatty acid level is the fatty acid concentration. In some embodiments, the fatty acid level is evaluated from the subject's whole blood, serum, plasma, CSF, synovial fluid, urine, fecal matter, saliva, or tissue homogenates (e.g., muscle, liver, heart, pancreas, brain).

[0079] Fatty acids are suitable biomarkers for age-associated diseases. In some embodiments, the serum fatty acid is free fatty acids (FFA), saturated fatty acids (SFA), palmitic acid (PA), oleic acid (OA), linoleic acid (LA), alpha linolenic acid, gamma linolenic acid, palmitoleic acid, margaric acid, stearic acid, myristic acid, pentadecanoic acid, arachidic acid, behenic acid, lignoceric acid or a combination thereof. In some embodiments, the serum fatty acid is free fatty acids (FFA), saturated fatty acids (SFA), palmitic acid (PA), oleic acid (OA), or linoleic acid (LA), or a combination thereof. In some embodiments, the serum fatty acid is a C₁₂-C₂₄ fatty acid. In some embodiments, the serum fatty acid is a C₁₈-C₂₄ fatty acid. In some embodiments, the serum fatty acid is a C₁₂-C₂₉ fatty acid. In some embodiments, the serum fatty acid is a saturated fatty acid. In some embodiments, the serum fatty acid is an unsaturated fatty acid.

[0080] In some embodiments, the serum fatty acid is palmitic acid (PA) and oleic acid (OA). In some embodiments, the serum fatty acid is palmitic acid (PA) and linoleic acid (LA). In some

embodiments, the serum fatty acid is oleic acid (OA) and linoleic acid (LA). In some embodiments, the serum fatty acid is palmitic acid (PA), oleic acid (OA) and linoleic acid (LA). In some embodiments, the serum fatty acid is free fatty acids (FFA) and saturated fatty acids (SFA). In some embodiments, the serum fatty acid is free fatty acids (FFA) and palmitic acid (PA). In some embodiments, the serum fatty acid is free fatty acids (FFA) and oleic acid (OA). In some embodiments, the serum fatty acid is free fatty acids (FFA) and linoleic acid (LA).

[0081] In some embodiments, the serum fatty acid is palmitic acid (PA). In some embodiments, the serum fatty acid is oleic acid (OA). In some embodiments, the serum fatty acid is linoleic acid (LA). In some embodiments, the serum fatty acid is free fatty acids (FFA). In some embodiments, the serum fatty acid is saturated fatty acids (SFA).

[0082] Free fatty acid can refer to any lipid fatty acid species that is released from adipose tissue. In some embodiments, the free fatty acid can be an aggregate of any fatty acid known by one of skill in the art. In some embodiments, the free fatty acid can be an aggregate of palmitic acid, palmitoleic acid, margaric acid, stearic acid, oleic acid, linoleic acid, gamma-linolenic acid, and alpha-linoleic acid. Free fatty acids can refer to a group of essential fatty acids that can only be obtained through diet.

[0083] In some embodiments, the method further comprises measuring the serum fatty acid and measuring the serum insulin levels.

[0084] The subject may be fasted or fed before evaluation of the biomarkers in the subject, which can depend upon the assay being used. In some embodiments, the subject is fed. In some embodiments, the subject is fed no more than 2 hours before evaluation.

[0085] In some embodiments, the subject is fasted. In some embodiments, the subject is fasted for at least 4 hours. In some embodiments, the subject fasted for at least 6 hours. In some embodiments, the subject fasted for at least 8 hours. In some embodiments, the subject fasted for at least 10 hours. In some embodiments, the subject fasted for at least 12 hours.

[0086] The subject described herein is a mammal. In some embodiments, the mammal is a dog, cat, horse, cow, pig, rabbit, rodent, sheep, non-human primate, or human. In some embodiments, the mammal is a dog. In some embodiments, the mammal is a mouse or rat. In some embodiments, the mammal is a human.

[0087] Increase of fatty acid level and increase in CFI indicates metabolic disease or the risk or presence of age-associated disease.

[0088] In some embodiments, the fatty acid level is increased by at least 10% compared to a subject without metabolic dysfunction or risk or presence of an age-associated disease. In some

embodiments, the fatty acid level is increased by at least 20% compared to a subject without metabolic dysfunction or risk or presence of an age-associated disease. In some embodiments, the fatty acid level is increased by at least 30% compared to a subject without metabolic dysfunction or risk or presence of an age-associated disease. In some embodiments, the fatty acid level is increased by at least 40% compared to a subject without metabolic dysfunction or risk or presence of an age-associated disease. In some embodiments, the fatty acid level is increased by at least 50% compared to a subject without metabolic dysfunction or risk or presence of an age-associated disease.

[0089] In some embodiments, the fatty acid level is increased by at least 10% compared to the subject's previously measured fatty acid level. In some embodiments, the fatty acid level is increased by at least 20% compared to the subject's previously measured fatty acid level. In some embodiments, the fatty acid level is increased by at least 30% compared to the subject's previously measured fatty acid level. In some embodiments, the fatty acid level is increased by at least 40% compared to the subject's previously measured fatty acid level. In some embodiments, the fatty acid level is increased by at least 50% compared to the subject's previously measured fatty acid level.

[0090] In some embodiments, the insulin level is increased by at least 10% compared to a subject without metabolic dysfunction or risk or presence of an age-associated disease. In some embodiments, the insulin level is increased by at least 20% compared to a subject without metabolic dysfunction or risk or presence of an age-associated disease. In some embodiments, the insulin level is increased by at least 30% compared to a subject without metabolic dysfunction or risk or presence of an age-associated disease. In some embodiments, the insulin level is increased by at least 40% compared to a subject without metabolic dysfunction or risk or presence of an age-associated disease. In some embodiments, the insulin level is increased by at least 50% compared to a subject without metabolic dysfunction or risk or presence of an age-associated disease.

[0091] In some embodiments, the insulin level is increased by at least 10% compared to the subject's previously measured insulin level. In some embodiments, the insulin level is increased by at least 20% compared to the subject's previously measured insulin level. In some embodiments, the insulin level is increased by at least 30% compared to the subject's previously measured insulin level. In some embodiments, the insulin level is increased by at least 40% compared to the subject's previously measured insulin level. In some embodiments, the insulin level is increased by at least 50% compared to the subject's previously measured insulin level.

[0092] The ranges for the fatty acid levels can vary, dependent upon which type of assay is used. A mammal without metabolic dysfunction or risk or presence of an age-associated disease can be used as the reference level for comparison with the subject described herein. In some non-limiting embodiments, the fasting insulin range and fatty acid range may be described herein.

[0093] In some embodiments, the free fatty acid range is from about 200 ug/mL to about 80000 ug/mL. In some embodiments, the free fatty acid range is from about 1000 ug/mL to about 15000 ug/mL. In some embodiments, the free fatty acid range is from about 2000 ug/mL to about 10000 ug/mL. In some embodiments, the free fatty acid range is from about 2000 ug/mL to about 9000 ug/mL. In some embodiments, the free fatty acid range is from about 2000 ug/mL to about 8000 ug/mL. In some embodiments, the free fatty acid is least 20 ug/mL, at least 50 ug/mL, at least 100 ug/mL, at least 200 ug/mL, at least 500 ug/mL, at least 1000 ug/mL, at least 1500 ug/mL, or at least 2000 ug/mL. In some embodiments, the free fatty acid is at most 200 ug/mL, at most 500 ug/mL, at most 1000 ug/mL, at most 1500 ug/mL, at most 2000 ug/mL, or at most 5000 ug/mL.

[0094] In some embodiments, the saturated fatty acid range is from about 100 ug/mL to about 10000 ug/mL. In some embodiments, the saturated fatty acid range is from about 300 ug/mL to about 8000 ug/mL. In some embodiments, the saturated fatty acid range is from about 500 ug/mL to about 5000 ug/mL. In some embodiments, the saturated fatty acid range is from about 800 ug/mL to about 4000 ug/mL. In some embodiments, the saturated fatty acid range is from about 900 ug/mL to about 3500 ug/mL. In some embodiments, the saturated fatty acid is at least 20 ug/mL, least 50 ug/mL, at least 100 ug/mL, at least 200 ug/mL, at least 500 ug/mL, at least 1000 ug/mL, at least 1500 ug/mL, or at least 2000 ug/mL. In some embodiments, the saturated fatty acid is at most 200 ug/mL, at most 500 ug/mL, at most 1000 ug/mL, at most 1500 ug/mL, at most 2000 ug/mL, at most 3000 ug/mL, or at most 10000 ug/mL.

[0095] In some embodiments, the palmitic acid range is from about 50 ug/mL to about 10000 ug/mL. In some embodiments, the palmitic acid range is from about 100 ug/mL to about 8000 ug/mL. In some embodiments, the palmitic acid range is from about 200 ug/mL to about 6000 ug/mL. In some embodiments, the palmitic acid range is from about 300 ug/mL to about 4000 ug/mL. In some embodiments, the palmitic acid range is from about 400 ug/mL to about 3000 ug/mL. In some embodiments, the palmitic acid range is from about 400 ug/mL to about 2000 ug/mL. In some embodiments, the palmitic acid is at least 20 ug/mL, least 50 ug/mL, at least 100 ug/mL, at least 200 ug/mL, at least 500 ug/mL, at least 1000 ug/mL, at least 1500 ug/mL, or at least 2000 ug/mL. In some embodiments, the palmitic acid is at most 200 ug/mL, at most 500

ug/mL, at most 1000 ug/mL, at most 1500 ug/mL, at most 2000 ug/mL, at most 3000 ug/mL, or at most 10000 ug/mL.

[0096] In some embodiments, the oleic acid range is from about 50 ug/mL to about 10000 ug/mL. In some embodiments, the oleic acid range is from about 100 ug/mL to about 8000 ug/mL. In some embodiments, the oleic acid range is from about 200 ug/mL to about 6000 ug/mL. In some embodiments, the oleic acid range is from about 300 ug/mL to about 4000 ug/mL. In some embodiments, the oleic acid range is from about 400 ug/mL to about 2500 ug/mL. In some embodiments, the oleic acid range is from about 400 ug/mL to about 2000 ug/mL. In some embodiments, the oleic acid is at least 20 ug/mL, least 50 ug/mL, at least 100 ug/mL, at least 200 ug/mL, at least 500 ug/mL, at least 1000 ug/mL, at least 1500 ug/mL, or at least 2000 ug/mL. In some embodiments, the oleic acid is at most 200 ug/mL, at most 500 ug/mL, at most 1000 ug/mL, at most 1500 ug/mL, at most 2000 ug/mL, at most 3000 ug/mL, or at most 10000 ug/mL.

[0097] In some embodiments, the linoleic acid range is from about 50 ug/mL to about 10000 ug/mL. In some embodiments, the linoleic acid range is from about 100 ug/mL to about 8000 ug/mL. In some embodiments, the linoleic acid range is from about 200 ug/mL to about 6000 ug/mL. In some embodiments, the linoleic acid range is from about 400 ug/mL to about 4000 ug/mL. In some embodiments, the linoleic acid range is from about 500 ug/mL to about 3000 ug/mL. In some embodiments, linoleic acid range is from about 700 ug/mL to about 3000 ug/mL. In some embodiments, the linoleic acid range is from about 800 ug/mL to about 3000 ug/mL. In some embodiments, the linoleic acid is at least 20 ug/mL, least 50 ug/mL, at least 100 ug/mL, at least 200 ug/mL, at least 500 ug/mL, at least 1000 ug/mL, at least 1500 ug/mL, or at least 2000 ug/mL. In some embodiments, the linoleic acid is at most 200 ug/mL, at most 500 ug/mL, at most 1000 ug/mL, at most 1500 ug/mL, at most 2000 ug/mL, at most 3000 ug/mL, or at most 10000 ug/mL.

[0098] In some embodiments, the fasting insulin range can be from about 0.02 mU/L to about 1000 mU/L. In some embodiments, the fasting insulin range can be from about 0.2 mU/L to about 500 mU/L. In some embodiments, the fasting insulin range can be from about 2 mU/L to about 100 mU/L. In some embodiments, the fasting insulin level is at least 0.01 mU/L, at least 0.1 mU/L, at least 1 mU/L, at least 5 mU/L, at least 10 mU/L, at least 100 mU/L, at least 200 mU/L, or at least 500 mU/L. In some embodiments, the fasting insulin level is at most 0.1 mU/L, at most 1 mU/L, at most 5 mU/L, at most 10 mU/L, at most 100 mU/L, at most 200 mU/L, or at most 500 mU/L.

[0099] In some embodiments, the subject has a Health-Related Quality of Life (HRQL) Total score ranging from about 20 to about 100. In some embodiments, the subject has a HRQL Total score ranging from about 30 to about 90. In some embodiments, the subject has a HRQL Total score ranging from about 40 to about 80. In some embodiments, the subject has a HRQL Total score ranging from about 30 to about 60. In some embodiments, the subject has a HRQL Total score of at most 30, at most 40, at most 50, at most 60, at most 70, or at most 80. In some embodiments, the subject has a HRQL Total score of at most 30, at most 40, at most 50, at most 60, or at most 70. In some embodiments, the subject has a HRQL Total score of at most 30, at most 40, at most 50, or at most 60. In some embodiments, the subject has a HRQL Total score of at least 10, at least 20, at least 30, at least 40, at least 50, or at least 60. In some embodiments, the subject has a HRQL Total score of at least 10, at least 20, at least 30, at least 40, or at least 50.

[0100] In some embodiments, the subject has a Canine Frailty Index (CFI) score ranging from 0 to 0.7. In some embodiments, the subject has a CFI score ranging from 0 to about 0.1. In some embodiments, the subject has a CFI score ranging from 0 to about 0.2. In some embodiments, the subject has a CFI score ranging from 0 to about 0.3. In some embodiments, the subject has a CFI score ranging from 0.01 to about 0.1. In some embodiments, the subject has a CFI score ranging from 0.01 to about 0.2. In some embodiments, the subject has a CFI score ranging from 0.01 to about 0.3. In some embodiments, the subject has a CFI score ranging from 0.01 to about 0.4. In some embodiments, the subject has a CFI score ranging from about 0.1 to about 0.2. In some embodiments, the subject has a CFI score of about 0.1 to about 0.7. In some embodiments, the subject has a CFI score of about 0.1 to about 0.7. In some embodiments, the subject has a CFI score of about 0.2 to about 0.7. In some embodiments, the subject has a CFI score of about 0.4 to about 0.7. In some embodiments, the subject has a CFI score of at least 0.01, at least 0.1, at least 0.2, at least 0.3, at least 0.4, at least 0.5, at least 0.6, or at least 0.7. In some embodiments, the subject has a CFI score of at most 0.1, at most 0.2, at most 0.3, at most 0.4, at most 0.5, at most 0.6, at most 0.7, or at most 0.8.

Subjects

[0101] In some embodiments of the methods described herein, the subject is a mammal. In some embodiments of the methods described herein, the mammal is a dog, cat, horse, cow, pig, rabbit, rodent, sheep, non-human primate, or human. In some embodiments of the methods described herein, the mammal is a non-rodent. In some embodiments of the methods described herein, the mammal is a dog. In some embodiments of the methods described herein, the mammal is a human. In some embodiments of the methods described herein, the mammal is a

cat. In some embodiments of the methods described herein, the mammal is a horse. In some embodiments of the methods described herein, the mammal is a cow. In some embodiments of the methods described herein, the mammal is a pig. In some embodiments of the methods described herein, the mammal is a rabbit. In some embodiments of the methods described herein, the mammal is a rodent. In some embodiments of the methods described herein, the rodent is a mouse or a rat. In some embodiments of the methods described herein, the mammal is a sheep. In some embodiments of the methods described herein, the mammal is a non-human primate.

[0102] In some embodiments, the subject is a mammal, *e.g.*, a human, mouse, rat, guinea pig, dog, cat, horse, cow, pig, rabbit, sheep, or non-human primate, such as a monkey, chimpanzee, or baboon. In some embodiments, the mammal is a non-rodent. In some embodiments, the mammal is a dog.

[0103] In some embodiments, the mammal has reached maturity. As used herein, the term mature or maturity, and the like, refers to a mammal that is capable of sexual reproduction and/or a mammal that has achieved its adult height and/or length.

[0104] In some embodiments, the mammal is administered a herein-disclosed composition as it is nearing or once it has reached halfway to its expected lifespan for the mammal's species, strain, breed, sex, and/or age. It is known that small dog breeds (*e.g.*, Chihuahua) have longer expected lifespans than larger dog breeds (*e.g.*, Great Dane). Accordingly, a Chihuahua, which has an expected lifespan of 15 years, will reach halfway to its expected lifespan at about 7 years (or earlier); thus, a Chihuahua may be administered a composition beginning around 7 years of age. On the other hand, a Great Dane, which has an expected lifespan of 7 years, will halfway to its expected lifespan at about 3 years; thus, a Great Dane may be administered a composition beginning around 3 years of age (or earlier). As disclosed herein, a mammal may be administered a composition once it has reached maturity; thus, either dog breed may be administered a composition about its first-year birthday.

[0105] Any dog breed can be administered a composition of the present disclosure and treated by a herein-described method. Lists of the most common dog breeds, identified by year, are maintained by the American Kennel Club. See, the World Wide Web (*www*) at akc.org/expert-advice/news/most-popular-dog-breeds-full-ranking-list; the lists of dog breeds, published at the time of the present application's filing, are incorporated by reference in their entireties. Illustrative common dog breeds include Retrievers (Labrador), German Shepherd Dogs, Retrievers (Golden), French Bulldogs, Bulldogs, Beagles, Poodles, Rottweilers, Pointers (German Shorthaired), Yorkshire Terriers, Boxers, Dachshunds, Pembroke Welsh Corgis,

Siberian Huskies, Australian Shepherds, Great Danes, Doberman Pinschers, Cavalier King Charles Spaniels, Miniature Schnauzers, Shih Tzu, Boston Terriers, Bernese Mountain Dogs, Pomeranians, Havanese, Shetland Sheepdogs, Brittanys, Spaniels (English Springer), Pugs, Mastiffs, Spaniels (Cocker), Vizslas, Cane Corso, Chihuahuas, Miniature American Shepherds, Border Collies, Weimaraners, Maltese, Collies, Basset Hounds, and Newfoundlands. In some embodiments, the subject is a one-year old dog. In some embodiments, the subject is a two-year old dog. In some embodiments, the subject is a three-year old dog. In some embodiments, the subject is a four-year old dog. In some embodiments, the subject is a five-year old dog. In some embodiments, the subject is a six-year old dog. In some embodiments, the subject is a seven-year old dog. In some embodiments, the subject is a eight-year old dog. In some embodiments, the subject is a nine-year old dog. In some embodiments, the subject is a ten-year old dog. In some embodiments, the subject is an eleven-year old dog. In some embodiments, the subject is a twelve-year old dog. In some embodiments, the subject is a thirteen-year old dog. In some embodiments, the subject is a fourteen-year old dog. In some embodiments, the subject is a fifteen-year old dog. In some embodiments, the subject is a sixteen-year old dog. In some embodiments, the subject is a seventeen-year old dog. In some embodiments, the subject is a eighteen-year old dog. In some embodiments, the subject is a nineteen-year old dog. In some embodiments, the subject is a twenty-year old dog.

[0106] In some embodiments, the mammal has an age in a range of from about 1 to about 15 years old, from about 15 to about 20 years old, from about 20 to about 25 years old, from about 25 to about 30 years old, from about 30 to about 35 years old, from about 35 to about 40 years old, from about 40 to about 45 years old, from about 45 to about 50 years old, from about 50 to about 55 years old, from about 55 to about 60 years old, from about 60 to about 65 years old, from about 65 to about 70 years old, from about 70 to about 75 years old, from about 75 to about 80 years old, from about 80 to about 85 years old, from about 85 to about 90 years old, from about 90 to about 95 years old or from about 95 to about 100 years old, or older. In some embodiments, the mammal is 5 to 12 years old. In some embodiments, the mammal is 5 to 15 years old. In some embodiments, the mammal is 10 to 20 years old. In some embodiments, the mammal is 8 to 15 years old. In some embodiments, the mammal is 10 to 20 years old. In some embodiments, the mammal is 8 to 12 years old.

[0107] In some embodiments, the subject is a non-human animal, and therefore the disclosure pertains to veterinary use. In some embodiments, the non-human animal is a household pet, *e.g.*, a dog. In some embodiments, the non-human animal is a livestock animal.

[0108] As used herein, the term healthspan refers to the part of a subject's life during which they are generally in good health or a period of life spent in good health, free from chronic diseases and disabilities/conditions of aging. Accordingly, a herein-disclosed composition or method that treats, prevents, reduces the severity of, and/or delay the onset of various aging-associated conditions, as mentioned above, improves the healthspan of a mammal. In some embodiments, the chronic disease is a cancer; thus, improving the healthspan of the mammal comprises treating a cancer and/or delaying or preventing the onset of the cancer in the mammal.

Definitions

[0109] The terminology used herein is for the purpose of describing particular cases only and is not intended to be limiting.

[0110] As used herein, unless otherwise indicated, the terms “a”, “an” and “the” are intended to include the plural forms as well as the single forms, unless the context clearly indicates otherwise.

[0111] The terms “comprise”, “comprising”, “contain”, “containing”, “including”, “includes”, “having”, “has”, “with”, or variants thereof as used in either the present disclosure and/or in the claims, are intended to be inclusive in a manner similar to the term “comprising.”

[0112] By preventing is meant, at least, avoiding the occurrence of a disease and/or reducing the likelihood of acquiring the disease. By treating is meant, at least, ameliorating or avoiding the effects of a disease, including reducing a sign or symptom of the disease.

[0113] The term “about” or “approximately” means within an acceptable error range for the particular value as determined by one of ordinary skill in the art, which will depend in part on how the value is measured or determined, *e.g.*, the limitations of the measurement system. For example, “about” can mean 10% greater than or less than the stated value. In another example, “about” can mean within 1 or more than 1 standard deviation, per the practice in the given value. Where particular values are described in the application and claims, unless otherwise stated the term “about” should be assumed to mean an acceptable error range for the particular value.

[0114] Reference herein to “one embodiment,” “one version,” or “one aspect” can include one or more such embodiments, versions or aspects, unless otherwise clear from the context.

[0115] Any aspect or embodiment described herein can be combined with any other aspect or embodiment as disclosed herein.

[0116] While preferred embodiments of the present disclosure have been shown and described herein, it will be obvious to those skilled in the art that such embodiments are provided by way of example only. Numerous variations, changes, and substitutions will now occur to

those skilled in the art without departing from the disclosure. It should be understood that various alternatives to the embodiments described herein may be employed. It is intended that the following claims define the scope of the disclosure and that methods and structures within the scope of these claims and their equivalents be covered thereby.

[0117] The section headings used herein are for organizational purposes only and are not to be construed as limiting the subject matter described.

EXAMPLES

[0118] The following illustrative examples are representative of embodiments of the stimulation, systems, and methods described herein and are not meant to be limiting in any way.

Example 1. Quantification of Fatty Acids and Insulin Along with Quality of Life Indicators Assays and Methods Used

[0119] Fatty Acid Quantification. Quantified by Metabolon Inc (Morrisville, NC). Canine plasma samples were analyzed by gas chromatography-mass spectrometry (GC/MS; Metabolon, Inc) for the determination of the total content of SFAs (myristic acid, pentadecanoic acid, palmitic acid, stearic acid, arachidic acid, behenic acid, lignoceric acid), MUFAs (myristoleic acid, palmitoleic acid, oleic acid, vaccenic acid, cis-11-eicosaenoic acid, nervonic acid), PUFAs (linoleic acid, gamma-linolenic acid, alpha-linolenic acid, mead acid, cis-11,14-eicosadienoic acid, eicosatetraenoic acid, eicosapentaenoic acid, adrenic acid, osbond acid, docosapentaenoic acid, docosahexaenoic acid), and FFAs (palmitic acid, palmitoleic acid, margaric acid, stearic acid, oleic acid, linoleic acid, gamma linolenic acid, alpha-linolenic acid), after conversion into their corresponding fatty acid methyl esters. In addition, dihomogamma-linolenic acid and erucic acid were analyzed but not discussed in this report. Aliquots of plasma were pipetted into tubes and lyophilized. Internal standard solution was added to the lyophilized plasma samples. The solvent was removed by evaporation under a stream of nitrogen. The dried sample was subjected to methylation/transmethylation with methanol/sulfuric acid, resulting in the formation of the corresponding methyl esters of free fatty acids and conjugated fatty acids. The reaction mixture was neutralized and extracted with hexanes. An aliquot of the hexanes layer was injected onto a 7890A/5975C GC/MS system (Agilent Technologies, CA). Mass spectrometric analysis was performed in the single ion monitoring positive mode with electron ionization. Quantitation was performed using both linear and quadratic regression analysis generated from fortified calibration standards prepared immediately prior to each run. Raw data was collected and processed using Agilent MassHunter GC/MS Acquisition B.07.04.2260 and Agilent MassHunter

Workstation Software Quantitative Analysis for GC/MS B.09.00/ Build 9.0.647.0 (Agilent Technologies, CA). Data reduction was performed using Microsoft Office 365 ProPlus Excel (Microsoft, WA).

[0120] Insulin Quantification. Quantified by Mercodia SV (Sweden). Fasting serum insulin concentrations were measured using a commercially available sandwich ELISA (10-1203-01) by Mercodia (Sweden) per the kit manufacturer's instructions. A precoated microplate with an immobilized antibody for insulin was loaded with serum samples or concentration standards for binding. After washing away unbound substances, an HRP conjugated antibody for insulin was added to the wells. Incubation was performed for 2 hours at 18-25°C on a plate shaker (700-900rpm). The excess unbound enzyme-labeled antibody was washed again and the remaining conjugate was allowed to react with a 3,3',5,5'-tetramethylbenzidine solution. The assay was terminated by adding an acidic solution and read at 450 nm wavelength on a microplate reader. A curve was generated from the known concentration standards and the unknown serum samples were determined using that curve.

[0121] List of potential fatty acids. Combinations paired with CFI (canine frailty index).

1. Palmitic Acid + Oleic Acid
2. Palmitic Acid + Linoleic Acid
3. Oleic Acid + Linoleic Acid
4. Palmitic Acid + Oleic Acid + Linoleic Acid
5. FFA + SFA
6. FFA + Palmitic Acid
7. FFA + Oleic Acid
8. FFA + Linoleic Acid
9. Insulin + Palmitic Acid
10. Insulin + Oleic Acid
11. Insulin + Linoleic Acid
12. Insulin + FFA
13. Insulin + SFA

*FFA includes the aggregated concentrations of palmitic acid, palmitoleic acid, margaric acid, stearic acid, oleic acid, linoleic acid, gamma linoleic acid, alpha-linoleic acid.

*SFA includes the aggregated concentrations of myristic acid, pentadecanoic acid, palmitic acid, stearic acid, arachidic acid, behenic acid, lignoceric acid.

[0122] **Adipose dysfunction in aging, disease, and lifespan**. Increases in visceral fat are associated with metabolic dysfunction in dogs. There is a positive correlation between insulin levels and abdominal visceral to subcutaneous fat volume ratio. Laboratory models that increase or decrease visceral fat mass in dogs have been shown to decrease or increase insulin sensitivity,

respectively. Metabolic and cardiovascular impairments are also strongly correlated with visceral fat in an obese dog model.

[0123] A direct relationship between visceral fat and cardiovascular disease has been established in companion dogs by Thengchaisri and colleagues. 87 dogs were evaluated of which 43 were physically healthy and 44 had existing heart disease. The amount of intra-abdominal fat of dogs in the heart disease group was significantly higher than that in the healthy group ($23.5 \pm 1.5\%$ vs. $19.4 \pm 1.2\%$; $P = 0.04$). Their body condition scores were not significantly different, nor were the levels of subcutaneous fat.

[0124] Fatty acids in aging and disease. Adipose tissue provides an energy source by breaking down triacylglycerol and releasing fatty acids and glycerol into the circulation. Increases in visceral adiposity correlate to increases in circulating fatty acids, attributable to the hyper-lipolytic nature of this adipose compartment. There are three main species of fatty acids released from adipose tissue: saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs). Generally, most MUFAs and PUFAs are considered metabolically beneficial with the notable exceptions of oleic and linoleic acid, which are associated with states of metabolic dysfunction. Conversely, SFAs are consistently associated with metabolic dysfunction and insulin resistance in humans and have been linked to clinically significant changes in serum bile acid concentrations and liver enzyme activities in dogs. Palmitic acid, one of the most abundant individual lipid species that contributes to the SFA pool, is known to directly impair glucose homeostasis in the liver and muscle in humans.

[0125] There is evidence to support the relationship between circulating lipids, metabolic dysfunction and longevity in dogs. Infusion of exogenous lipids induces whole body and peripheral insulin resistance as measured by a hyperinsulinemic euglycemic clamp. These findings agree with human studies where fatty acids have been used as predictive markers of metabolic dysfunction and insulin resistance in seemingly healthy individuals.

[0126] Serum fatty acid levels in previously collected blood samples from a subset of the client-owned dogs enrolled in the Healthspan Study were collected and evaluated for their correlation with age, body weight, and body condition. Furthermore, individual and grouped fatty acid levels were correlated with Health-Related Quality of Life using the VetMetrica® HRQL owner-questionnaire and frailty/multimorbidity using a veterinarian-assessed Canine Frailty Index (CFI).

Fatty acid levels increase with age in dogs in the Healthspan Study

[0127] This study was an unblinded pilot observational clinical study conducted at 11 sites across the United States. It included one study visit and had no interventions. The owner completed an HRQL assessment prior to the single study visit. On day 0, each enrolled dog received all scheduled study events including a Physical Examination, Body Condition Score, Muscle Condition Score, Complete Blood Count, biochemical profile, serum T4 measurement, urinalysis, and CFI. Additional blood samples were collected that were used to assess fasted insulin, and fatty acid levels.

[0128] The study enrolled 451 eligible, mature, adult dogs, consisting of 43.6% mixed breeds and 56.4% purebred dogs. Dogs were enrolled based on target age and size groups. The total evaluable population was n=451, with n=450 for analyses involving CFI and n= 448 for HRQL.

[0129] Fatty acid quantification was performed using banked samples from the study. There were only 61 samples remaining with sufficient volume to allow for testing to be performed. Levels of free fatty acids (FFA), saturated fatty acids (SFA), palmitic acid (PA; a 16:0 saturated fatty acid), oleic acid (OA; a 18:1n9 monounsaturated fatty acid) and linoleic acid (LA: a 18:2n6 polyunsaturated fatty acid) were measured using gas chromatography-mass spectrometry (GC/MS; Metabolon, Inc).

[0130] One dog of the 61 had missing insulin concentration values and is not included in any analyses or models involving insulin.

[0131] **Results.** Age was significantly positively correlated with free fatty acids, saturated fatty acids, palmitic acid, oleic acid and linoleic acid ($p < 0.05$, **Table 1**, FIG. 1). Age was also significantly negatively correlated with BCS (body condition score; $p < 0.01$).

Table 1: Pearson's correlation coefficients evaluating bivariate relationships between FFA, SFA, PA, OA and LA with age, weight, BCS, fasting insulin concentrations, HRQL and CFI scores

Variable	Age (yrs)	Weight (kgs)	BCS	Insulin (mU/L)	HRQL Total	CFI	FFA	SFA	Palmitic acid (16:0) (µg/mL)	Oleic acid (18:1n9) (µg/mL)	Linoleic acid (18:2n6) (µg/mL)
Age (yrs)	--										
Weight (kgs)	-0.16	--									
BCS	-0.36**	0.41***	--								
Insulin (mU/L)	0.26*	0.22	-0.07	--							

HRQL Total	-0.36**	0.07	0.22	-0.22	--					
CFI	0.67***	-0.12	-0.31*	0.38**	-0.38**	--				
FFA	0.30*	0.18	0.14	0.35**	-0.07	0.26*	--			
SFA	0.26*	0.21	0.22	0.31*	0.04	0.23	0.95***	--		
Palmitic acid (16:0) (µg/mL)	0.34**	0.07	0.04	0.31*	-0.16	0.31*	0.92***	0.87***	--	
Oleic acid (18:1n9) (µg/mL)	0.29*	0.06	0.01	0.43***	-0.25	0.32*	0.88***	0.75***	0.88***	--
Linoleic acid (18:2n6) (µg/mL)	0.26*	0.19	0.13	0.24	-0.01	0.20	0.94***	0.87***	0.84***	0.73*** --

Cell values reflect Pearson's correlation coefficient and significance level. *--p<0.05, **--p<0.01, ***--p<0.001

[0132] To test if associations between fatty acid concentrations and age remained, above and beyond any potential effects of BCS, a multiple regression model of age and BCS were fit against each fatty acid concentration. Age ($p < 0.05$) was significantly positively associated with free fatty acids, saturated fatty acids, palmitic acid, oleic acid and linoleic acid (**Table 2**). As each individual and grouped fatty acid has been standardized to mean = 0 and SD = 1, it can interpret the effects for age as every year increase is associated with a 0.09 to 0.11 SD increase in mean fatty acid concentrations, adjusting for the effects of BCS. BCS was significantly positively associated with free fatty acid and saturated fatty acid concentrations, after adjusting for age. These data demonstrate fatty acid concentrations' association with age independent of body weight and BCS scores.

Table 2: Multiple regression estimating main effects of age and BCS in association with FFA, SFA, P

Standardized Outcome	Variable	Coefficient (95% CI)	P value	Overall P value	R ²
FFA	Intercept	-2.32 (-3.96, -0.67)	0.007**	0.007**	0.16
	Age (yrs)	0.10 (0.04, 0.17)	0.003**		
	BCS	0.26 (0.02, 0.50)	0.033*		
SFA	Intercept	-2.72 (-4.34, -1.10)	0.001**	0.003**	0.18
	Age (yrs)	0.10 (0.04, 0.17)	0.003**		
	BCS	0.33 (0.10, 0.57)	0.006**		

Palmitic acid (16:0)	Intercept	-1.84 (-3.50, -0.19)	0.030*	0.011*	0.14
	Age (yrs)	0.11 (0.04, 0.18)	0.003**		
	BCS	0.17 (-0.07, 0.41)	0.155		
Oleic acid (18:1n9)	Intercept	-1.44 (-3.14, 0.25)	0.094	0.044*	0.10
	Age (yrs)	0.09 (0.02, 0.16)	0.013*		
	BCS	0.13 (-0.12, 0.37)	0.310		
Linoleic acid (18:2n6)	Intercept	-2.13 (-3.80, -0.46)	0.013*	0.018*	0.13
	Age (yrs)	0.09 (0.03, 0.16)	0.008**		
	BCS	0.24 (0.00, 0.48)	0.051		

Each fatty acid measure is standardized by mean centering and scaling by standard deviation (SD); units = 1 SD

[0133] Conclusion. Concentrations of free fatty acids, saturated fatty acids, palmitic acid, oleic acid and linoleic acid increase with advancing chronological age in dogs in the Healthspan Study. Every year increase in age is associated with a 0.9 to 0.11 SD increase in mean fatty acid concentrations, adjusting for the effect of BCS. These data suggest that age is the primary factor driving the increase in fatty acid concentrations, irrespective of BCS.

Canine Frailty Index (CFI) scores increase with age in the Healthspan Study

[0134] CFI is based on a series of 33 questions relating to the medical history and physical examination, performed by a veterinarian. The CFI is calculated by summing the question responses (0, 0.5, or 1) and dividing by the total number of questions (33). The total CFI therefore lies between 0 and 1, where a frailty index of 0 denotes a dog that is not at all frail. Frailty scores in the evaluable population of dogs in the Healthspan Study ranged from 0.0 to 0.59. Lower frailty scores indicate fewer health deficits (lower frailty/multimorbidity). Higher scores indicate more points tallied due to health deficits (higher frailty/multimorbidity). The CFI incorporates diagnoses of specific age-associated diseases, including those most commonly associated with natural death and euthanasia in dogs: neoplasia, musculoskeletal disease, cardiac diseases, chronic kidney disease, and CNS dysfunction.

[0135] The frailty score in the Healthspan Study was significantly different between the old (N=269; mean=0.10) and young (N=181; mean=0.01) age groups, with a p-value < 0.001 (Table

9). The age and CFI were plotted for each dog as displayed in **FIG. 2**. These data were used to evaluate the expected change in CFI with each additional year of age.

[0136] When these data are plotted against age, the average increase in CFI per year is 0.016. This is roughly the same as the 0.017 coefficient found by Banzato et al. in his study of 401 dogs. The CFI tool has therefore shown consistent results across two countries, 12 veterinarians and 852 dogs, indicating that it is robust and reliable.

Fatty acid concentrations are associated with CFI scores in the Healthspan Study

[0137] To estimate the covariate-adjusted association between fatty acids and CFI scores, a multiple regression model was used to estimate the relationship between fatty acids and CFI accounting for effects of age and BCS. First, test the main effects of each fatty acid adjusting for age and BCS against CFI, as well as a model testing the interaction effects between each fatty acid and age (**Table 6**). Fatty acid measures were not significantly associated with CFI in main effects models adjusting for age and BCS, however, significant interaction effects between all fatty acids (free fatty acids, saturated fatty acids, palmitic acid, oleic acid and linoleic acid) and age ($p < 0.05$, **Table 3**, **FIG. 3**) was detected. All coefficients for the interactions between age and fatty acid measures were > 0 , suggesting that the effects appear to be stronger in older dogs.

FIG. 3 provides a visualization of the observed and predicted interaction between fatty acids and age on CFI.

Table 3: Multiple regression testing the interactions between FFA, SFA, PA, OA and LA age on CFI scores.

Model	Variable	Coefficient (95% CI)	P value	Overall P value	R ²
Model 1	Intercept	-0.05 (-0.19, 0.10)	0.513	<0.001***	0.54
	Standardized FFA	-0.07 (-0.12, -0.01)	0.019*		
	Age (yrs)	0.02 (0.01, 0.02)	<0.001***		
	BCS	0.00 (-0.03, 0.02)	0.664		
	Standardized FFA x Age (yrs)	0.01 (0.00, 0.01)	0.004**		
Model 2	Intercept	-0.02 (-0.17, 0.12)	0.749	<0.001***	0.53
	Standardized SFA	-0.05 (-0.10, 0.00)	0.041*		
	Age (yrs)	0.02 (0.01, 0.02)	<0.001***		
	BCS	-0.01 (-0.03, 0.01)	0.466		
	Standardized SFA x Age (yrs)	0.01 (0.00, 0.01)	0.007**		
Model 3	Intercept	-0.03 (-0.17, 0.12)	0.721	<0.001***	0.52
	Standardized Palmitic acid	-0.05 (-0.11, 0.00)	0.070		
	Age (yrs)	0.02 (0.01, 0.02)	<0.001***		
	BCS	-0.01 (-0.03, 0.01)	0.477		
	Standardized Palmitic acid x Age (yrs)	0.01 (0.00, 0.01)	0.018*		

Model 4	Intercept	-0.03 (-0.17, 0.11)	0.705	<0.001***	0.53
	Standardized Oleic acid	-0.05 (-0.11, 0.00)	0.074		
	Age (yrs)	0.02 (0.01, 0.02)	<0.001***		
	BCS	-0.01 (-0.03, 0.01)	0.544		
	Standardized Oleic acid x Age (yrs)	0.01 (0.00, 0.01)	0.012*		
Model 5	Intercept	-0.07 (-0.21, 0.07)	0.350	<0.001***	0.54
	Standardized Linoleic acid	-0.08 (-0.14, -0.02)	0.008**		
	Age (yrs)	0.02 (0.01, 0.02)	<0.001***		
	BCS	0.00 (-0.02, 0.02)	0.802		
	Standardized Linoleic acid x Age (yrs)	0.01 (0.00, 0.01)	0.003**		

Each fatty acid measure is standardized by mean centering and scaling by standard deviation (SD); units = 1 SD

[0138] In the univariate model examining the relationship between grouped and individual fatty acids and CFI scores, increasing free fatty acids, palmitic acid and oleic acid concentrations were positively correlated with increasing CFI scores. When adjusting for BCS in a multiple regression interaction model with age, increases in grouped and individual fatty acid concentrations (free fatty acids, saturated fatty acids, palmitic acid, oleic acid and linoleic acid) were associated with higher CFI scores, an association which appears to be stronger in the older dogs.

Fatty acid concentrations are associated with fasting insulin in the Healthspan Study

[0139] To the covariate-adjusted association between fatty acids and fasting insulin, a multiple regression model was used to estimate the relationship between fatty acids and insulin accounting for effects of age and weight. It was first tested the main effects of each fatty acid adjusting for age and weight against insulin (**Table 7**), as well as a model testing the interaction effects between each fatty acid and age (**Table 4**). In main effects models, oleic acid showed a significant positive association with insulin, while free fatty acids showed a trending positive association. There were also significant interaction effects between all fatty acids measured and age ($p < 0.05$, **Table 4**, **FIG. 4**). All coefficients for the interactions between age and fatty acid measures were > 0 , suggesting that effects of fatty acids on insulin concentrations appear to be stronger in older dogs. **FIG. 4** provides a visualization of the observed and predicted interaction between fatty acids and age on insulin concentration.

Table 4: Multiple regression testing the interactions between FFA, SFA, PA, OA and LA age on fasting insulin.

Model	Variable	Coefficient (95% CI)	P value	Overall P value	R ²
Model 1	Intercept	5.98 (-3.46, 15.42)	0.22	<0.001***	0.45

	Standardized FFA	-14.93 (-22.74, -7.12)	<0.001***	
	Age (yrs)	0.78 (-0.03, 1.59)	0.066	
	Weight (kgs)	0.23 (0.04, 0.41)	0.022*	
	Standardized FFA x Age (yrs)	1.83 (1.12, 2.55)	<0.001***	
Model 2	Intercept	6.99 (-2.53, 16.52)	0.156	<0.001*** 0.43
	Standardized SFA	-13.32 (-20.46, -6.19)	<0.001***	
	Age (yrs)	0.77 (-0.05, 1.59)	0.07	
	Weight (kgs)	0.20 (0.01, 0.39)	0.045*	
	Standardized SFA x Age (yrs)	1.75 (1.06, 2.45)	<0.001***	
Model 3	Intercept	5.34 (-4.33, 15.02)	0.284	<0.001*** 0.4
	Standardized Palmitic acid	-14.92 (-23.38, -6.46)	0.001**	
	Age (yrs)	0.76 (-0.09, 1.61)	0.084	
	Weight (kgs)	0.25 (0.06, 0.44)	0.013*	
	Standardized Palmitic acid x Age (yrs)	1.71 (0.97, 2.46)	<0.001***	
Model 4	Intercept	8.32 (-0.55, 17.20)	0.072	<0.001*** 0.48
	Standardized Oleic acid	-12.30 (-19.96, -4.64)	0.003**	
	Age (yrs)	0.46 (-0.33, 1.25)	0.256	
	Weight (kgs)	0.24 (0.06, 0.42)	0.010**	
	Standardized Oleic acid x Age (yrs)	1.65 (0.98, 2.32)	<0.001***	
Model 5	Intercept	3.22 (-6.72, 13.15)	0.528	<0.001*** 0.38
	Standardized Linoleic acid	-16.50 (-24.99, -8.00)	<0.001***	
	Age (yrs)	1.09 (0.24, 1.93)	0.015*	
	Weight (kgs)	0.25 (0.05, 0.44)	0.019*	
	Standardized Linoleic acid x Age (yrs)	1.85 (1.05, 2.65)	<0.001***	

Each fatty acid measure is standardized by mean centering and scaling by standard deviation (SD); units = 1 SD

[0140] In the univariate model examining the relationship between grouped and individual fatty acids and fasting insulin, increasing concentrations of free fatty acids, saturated fatty acids, palmitic acid and oleic acid levels are significantly associated with increasing fasting insulin concentrations. When adjusting for weight in a multiple regression interaction model with age, increases in all grouped and individual fatty acids measured (free fatty acids, saturated fatty acids, palmitic acid, oleic acid and linoleic acid levels) were significantly associated with higher fasting insulin concentrations, an association which appears to be stronger in the older dogs.

Example 2. High fat diet feeding leads to an increase in lipid species associated with metabolic dysfunction in dogs

[0141] The consumption of a high fat diet (HFD) results in increased adiposity, free fatty acid (FFA) levels and insulin resistance leading to poor metabolic outcomes. While preclinical studies investigating HFD-induced metabolic dysfunction are well-described in rodents, HFD feeding in dogs may represent a more translationally relevant model that mimics

pathophysiological changes occurring in humans with metabolic dysfunction. Therefore, the impact of chronic HFD feeding on fasting metabolic markers, lipidomic profiles and insulin sensitivity in two studies in dogs was investigated.

[0142] Study 1: Twenty-one male dogs (mixed beagle and mongrels) aged 3-7 years old were sampled at baseline while on a normal diet (ND) and again 10-12 weeks after ND (n=10) or HFD-feeding (n=11) for fasting parameters, lipidomic profiling and insulin sensitivity assessed by intravenous glucose tolerance test (IVGTT). HFD was achieved by augmenting the maintenance diet with pork lard to achieve a 74% final fat content diet.

[0143] Study 2: Twenty-four male dogs (beagles) aged 3-7 years old were sampled after 9 weeks while on a ND (n=12) or HFD (n=12) and again 17 weeks after ND or HFD-feeding for fasting parameters and lipidomic profiling. Additionally, insulin sensitivity was assessed by oral glucose tolerance test (OGTT) at 5 weeks of ND or HFD feeding and again after 15 weeks after ND or HFD feeding.

[0144] In both studies, feeding a HFD produced an increase in body weight compared to ND-fed dogs ($p>0.001$). HFD-feeding also led to increases in fasting insulin and leptin, while impairing insulin sensitivity measured by IVGTT(SI) ($p<0.05$) in Study 1 and OGTT (insulin AUC) ($p>0.05$) in Study 2.

[0145] Lipidomic profiling revealed that HFD-feeding resulted in significant increases in lipid species associated with metabolic dysfunction compared to dogs fed a normal diet when measured via gas chromatography-mass spectrometry (GC-MS) ($p>0.05$). In both studies FFA was also quantified via a clinically used non-esterified fatty acids (NEFA) assay. Neither study demonstrated any change in NEFA levels at all time points measured. Moreover, there was minimal agreement between the GC-MS FFA quantification and the clinically used NEFA quantification.

[0146] These data confirm that HFD-feeding in beagle and mongrel dogs recapitulates metabolic dysfunction seen in other preclinical species, as well as aspects of metabolic dysfunction in human populations. Additionally, clinically used NEFA assays may be insufficient to detect biologically relevant changes in FFA levels in response to metabolic stressors such as HFD.

Example 3. Evaluation of Fatty Acid Species as Biomarkers of Health-Related Quality of Life and Frailty in Dogs

[0147] Adipose dysfunction is well-characterized in the pathophysiology of obesity, insulin resistance and aging in humans. In metabolically dysfunctional states, fatty acid release from the adipose tissue is accelerated and utilization by other tissues is impaired leading to elevated

concentrations in circulation, making them attractive biomarkers of disease. While fasting fatty acid levels have been correlated with many disease states in humans, and is a predictor of all-cause mortality, these substrates have not been well characterized in other species. There have been no previous investigations interrogating the changes in individual or grouped fatty acid levels with age in dogs. Moreover, there have been no investigations examining fatty acid levels in the context of declining quality of life and progressive frailty in aging dogs. While the use of high-fidelity measurements, such as gas chromatography-mass spectrometry (GC-MS), to directly quantify lipid species in humans has become more widespread, there have been relatively few published studies using GC-MS to directly quantify and profile fatty acid distribution patterns in dogs.

[0148] This study was an unblinded pilot observational clinical study conducted at 11 sites across the United States. It included one study visit and had no interventions. The owner completed an HRQL assessment prior to the single study visit. On day 0, each enrolled dog received all scheduled study events including a Physical Examination, Body Condition Score, Muscle Condition Score, Complete Blood Count, biochemical profile, serum T4 measurement, urinalysis, and CFI. Additional blood samples were collected that were used to assess fasted insulin, and fatty acid levels.

[0149] To this end, findings from additional analysis of samples collected for the study is reported. Blood collected from a subset (61) of 451 client-owned dogs of various ages and sizes (≥ 2 and < 6 (young dogs) or ≥ 7 years of age (old dogs), < 25 (small dogs) or > 50 lbs (large dogs) was measured for 28 fatty acid species. Individual species and groupings of fatty acids have been demonstrated to be correlated, and potentially causal, to different aging-related disease states in other species via adipose expansion and dysfunction. For this reason, palmitic acid (PA), oleic acid (OA), linoleic acid (LA) and the aggregated fatty acid groups free fatty acids (FFA) and saturated fatty acids (SFA) that are reported in the human literature and demonstrated in house to be associated with a metabolic aging phenotype in dogs was chosen for assessment.

[0150] The aims of this study were threefold: 1) Evaluate grouped and individual fatty acid concentrations with age 2) evaluate the potential of fatty acid concentrations as biomarkers of owner-reported Health-Related Quality of Life (HRQL) and/or clinician-assessed Canine Frailty Index (CFI) scores and 3) evaluate the relationship between fatty acid concentrations and fasting insulin concentrations. Pearsons' correlation and multiple regression were used to evaluate fatty acid concentrations in association with age, weight, BCS, insulin concentrations, HRQL, and CFI scores.

[0151] The resulting analysis demonstrated that FFA, SFA, PA, OA and LA concentrations are positively correlated with increasing chronological age but not body weight or body condition score (BCS). For every year increase in age there was a 0.9 to 0.11 SD increase in mean fatty acids. Conversely, when adjusting for age there was a significant positive association for FFA and SFA with BCS. None of the fatty acids measured were associated with HRQL, however it was not appropriately powered to see an association with this metric.

[0152] For the first time, provided is real world evidence that the elevation of grouped and individual fatty acids are associated with increased CFI scores and progressive frailty in companion dogs. Moreover, these observed changes in fatty acid concentrations are reflective of clinically relevant and importantly, *quantifiable*, changes in dogs. This positions certain fatty acids (FFA, SFA, PA, OA and LA) as potential predictors of CFI, an outcome measure that is broadly accessible for usage in clinical practice.

[0153] Elevations in fasting insulin concentrations were previously associated with reductions in quality of life (HRQL), making it a potential predictor of a clinically available quality of life metric. It is also demonstrated for the first time that a significant association exists between grouped and individual fatty acids (FFA, SFA, PA, OA and LA) with fasting insulin, suggesting that fatty acid concentrations may still be associative with HRQL.

[0154] Collectively these data describe for the first time that fatty acids are elevated with age, suggesting that adipose dysfunction is increased with age. Additionally, it was demonstrated that the elevation of these fatty acids are associated with fasting insulin and progressive frailty in dogs. Thus, that adipose dysfunction is contributing to decreasing quality of life and increased frailty with advanced age in dogs. In the univariate model examining the relationship between grouped and individual fatty acids and CFI scores, increasing free fatty acids, palmitic acid and oleic acid concentrations were positively correlated with increasing CFI scores. When adjusting for BCS in a multiple regression interaction model with age, increases in grouped and individual fatty acid concentrations (free fatty acids, saturated fatty acids, palmitic acid, oleic acid and linoleic acid) were associated with higher CFI scores, an association which appears to be stronger in the older dogs.

[0155] Herein is the aim to determine whether certain fatty acids, namely FFA, SFA, PA, OA and LA are related to age, fasting insulin, quality of life, and/or frailty in dogs. To this end, serum fatty acid levels (28 species total, see **methods**) from previously collected blood samples was measured from a subset of the client-owned dogs enrolled in the study and evaluated their correlation with age, body weight, and body condition. Furthermore, it can be correlated

individual and grouped fatty acid levels with Health-Related Quality of Life and frailty using two validated tools, the VetMetrica Health-Related Quality of Life (HRQL) owner-questionnaire and a veterinarian-assessed Canine Frailty Index (CFI), respectively.

Methods

[0156] Study population. A total of 451 client-owned, clinically stable adult dogs, aged ≥ 2 and <6 (young dogs) or ≥ 7 years of age (old dogs), < 25 (small dogs) or > 50 lbs (large dogs), were recruited for study LOY001-CLD-EFF1-PIL. Of the 451 dogs, 406 have complete data across age, weight, BCS, HRQL scores, CFI scores and insulin measures.

[0157] Due to limited sample volume, fatty acid quantification was performed on 61 dog serum samples. These samples had sufficient sample volume remaining for the assay. This sample of 61 dogs are those included in the analyses of the present report. One dog had missing insulin concentration values, and is not included in any analyses or models including insulin.

Descriptive statistics and visual distributions from this cohort can be found in **Table 5** and **FIG. 5**.

Table 5. Descriptive statistics of demographic variables, FFA, SFA, PA, OA, LA, insulin, and HRQL and CFI scores for Healthspan dogs with fatty acid concentrations measured.

	Young (N=23)	Old (N=38)	Total (N=61)
Age (years)			
N	23	38	61
Mean	4.1 (1.3)	10.6 (2.5)	8.2 (3.8)
Median (25 th , 75 th)	4.0 (3.0, 5.2)	10.2 (9.0, 11.9)	8.1 (4.8, 11.1)
Min - Max	2.3 – 5.9	7.10 – 17.7	2.3 – 17.7
Weight (kgs)			
N	23	38	61
Mean	27.5 (16.5)	24.3 (15.4)	25.5 (15.8)
Median (25 th , 75 th)	31.3 (8.7, 40.6)	27.8 (8.2, 33.2)	28.7 (8.2, 34.0)
Min - Max	1.4 – 62.1	1.3 – 54.6	1.3 – 62.1
Sex			
Female	15 (65.2%)	17 (44.7%)	32 (52.5%)
Male	8 (34.8%)	21 (55.3%)	29 (47.5%)
Intact status			
Intact	3 (13.0%)	2 (5.3%)	5 (8.2%)
Desexed	20 (87.0%)	36 (94.7%)	56 (91.8%)
Purebred			
Yes	18 (78.3%)	30 (78.9%)	48 (78.7%)
No	5 (21.7%)	8 (21.1%)	13 (21.3%)
Body Condition Score			
N	23	38	61
Mean	5.9 (1.1)	5.4 (1.1)	5.6 (1.1)
Median (25 th , 75 th)	6.0 (5.0, 7.0)	5.0 (5.0, 6.0)	5.0 (5.0, 6.0)
Min - Max	4.0 – 8.0	3.0 – 8.0	3.0 – 8.0
Insulin			
N	23	37	60
Mean	17.9 (11.3)	21.6 (16.2)	20.2 (14.5)
Median (25 th , 75 th)	13.9 (9.6, 23.7)	20.2 (11.4, 27.0)	17.8 (10., 25.7)
Min - Max	5.7 – 54.0	2.5 – 100.1	2.5 – 100.1
HRQL Total			

N	23	38	61
Mean	50.7 (3.7)	47.7 (7.4)	48.9 (6.4)
Median (25 th , 75 th)	51.5 (47.5, 53.0)	46.7 (42.1, 52.8)	48.5 (45.6, 53.1)
Min - Max	42.6 – 58.4	35.7 – 69.2	35.7 – 69.2
Canine Frailty Score			
N	23	38	61
Mean	0.0 (0.0)	0.1 (0.1)	0.1 (0.1)
Median (25 th , 75 th)	0.0 (0.0, 0.1)	0.1 (0.0, 0.1)	0.1 (0.0, 0.1)
Min - Max	0.0 – 0.1	0.0 – 0.6	0.0 – 0.6
Free Fatty Acids			
N	23	38	61
Mean	3902.6 (959.6)	4427.0 (1532.9)	4229.3 (1361.0)
Median (25 th , 75 th)	3729.3 (3152.8, 4430.8)	3809.4 (3298.8, 5556.7)	3785.1 (3238.5, 5039.2)
Min - Max	2341.5 – 6215.2	2380.5 – 8497.9	2341.5 – 8497.9
Saturated Fatty Acids			
N	23	38	61
Mean	1676.5 (424.2)	1849.6 (557.5)	1784.3 (514.6)
Median (25 th , 75 th)	1618.5 (1399.8, 1946.7)	1630.8 (1409.3, 2292.8)	1628.2 (1405.8, 2149.5)
Min - Max	943.3 – 2718.1	977.1 – 3363.3	943.3 – 3363.3
Palmitic acid (16:0)			
(µg/mL)			
N	23	38	61
Mean	751.0 (215.7)	855.7 (354.2)	816.2 (311.5)
Median (25 th , 75 th)	724.3 (615.2, 865.2)	724.9 (620.6, 1035.3)	724.3 (616.7, 974.4)
Min - Max	412.7 – 1329.6	453.6 – 2093.7	412.7 – 2093.7
Oleic acid (18:1n9)			
(µg/mL)			
N	23	38	61
Mean	737.4 (266.1)	869.8 (442.9)	819.9 (388.7)
Median (25 th , 75 th)	667.9 (565.1, 801.9)	687.9 (556.0, 1010.5)	678.8 (554.0, 948.8)
Min - Max	471.4 – 1445.8	394.2 – 2210.7	394.2 – 2210.7
Linoleic acid (18:2n6)			
(µg/mL)			
N	23	38	61
Mean	1401.7 (342.3)	1573.2 (536.4)	1508.5 (476.9)
Median (25 th , 75 th)	1452.7 (1161.0, 1550.6)	1397.0 (1135.7, 1914.4)	1415.5 (1128.4, 1809.7)
Min - Max	798.9 – 2013.5	765.2 – 3036.0	765.2 – 3036.0

HRQL = Health-Related Quality of Life; Young = dogs <7 years old; Old = dogs ≥ 7 years old.

[0158] Standards applied. Owner Informed Consent was obtained for all dogs. Good Clinical Practice (GCP) Standards were followed. Approval by an independent IACUC was obtained on December 13, 2020 through Veterinary & Biomedical Research Center, Inc (VBRC).

[0159] Sample collection. Fasted blood samples were collected via venipuncture in a serum separator tube. The protocol required at least 6 hours of fasting. Samples were left to clot at room temperature for no more than 60 minutes prior to centrifugation at 2800-3200 rpm for 10 minutes at ambient temperature. From each sample, the resultant serum was divided into aliquots of approximately 500-1000µL using 1.8 mL cryovials and stored at approximately -80°C until shipment on dry ice to Metabolon, Inc (Morrisville, North Carolina) for analyses.

[0160] Fatty acid quantification. Canine serum samples were analyzed by gas chromatography-mass spectrometry (GC/MS; Metabolon, Inc) for the determination of the total content of FFAs (palmitic acid, palmitoleic acid, margaric acid, stearic acid, oleic acid, linoleic

acid, gamma linoleic acid, alpha-linoleic acid); SFAs (myristic acid, pentadecanoic acid, palmitic acid, stearic acid, arachidic acid). Behenic, nervonic and lignoceric acids were also quantified, however they did not pass assay QC so their data is not included. Aliquots of plasma were pipetted into tubes and lyophilized. Internal standard solution was added to the lyophilized plasma samples. The solvent was removed by evaporation under a stream of nitrogen. The dried sample was subjected to methylation/transmethylation with methanol/sulfuric acid, resulting in the formation of the corresponding methyl esters of free fatty acids and conjugated fatty acids. The reaction mixture was neutralized and extracted with hexanes. An aliquot of the hexanes layer was injected onto a 7890A/5975C GC/MS system (Agilent Technologies, CA). Mass spectrometric analysis was performed in the single ion monitoring positive mode with electron ionization. Quantitation was performed using both linear and quadratic regression analysis generated from fortified calibration standards prepared immediately prior to each run. Raw data was collected and processed using Agilent MassHunter GC/MS Acquisition B.07.04.2260 and Agilent MassHunter Workstation Software Quantitative Analysis for GC/MS B.09.00/ Build 9.0.647.0 (Agilent Technologies, CA). Data reduction was performed using Microsoft Office 365 ProPlus Excel (Microsoft, WA).

[0161] HRQL Scores. Scores were available for four HRQL sub-domains: Energetic/Enthusiastic (E/E), Happy/Content (H/C), Active/Comfortable (A/C), and Calm/Relaxed (C/R). Individual HRQL domain scores range from 0 to 6, with larger scores indicating higher quality of life in that domain. Following Davies et al, HRQL total score was calculated as the sum of all four domains, which was then standardized and rescaled such that total scores ranged from 0 to 100 with a mean of 50 and a standard deviation of 10. The HRQL total score represents a dog's overall quality of life, where higher scores indicate better quality of life and lower scores lower quality of life.

[0162] CFI Scores. The CFI was developed as a tool to measure frailty through the assessment of multimorbidity and functional deficits associated with aging in dogs (Banzato et al., 2019). The CFI was developed as an analog to the frailty index used in humans or rodents, where frailty is assessed across multiple dimensions and reflects a general state of poor health. The CFI contains 33 discrete clinical health items, ranging from yes/no answers on functional ability (e.g., "Assistance for standing up", with responses as "Yes" = 1 or "No" = 0) to specific disease states (e.g. "Disease of the oral category", with responses as "No" = 0, "Mild" = 0.5, "Severe" = 1). These items were assessed by a veterinarian. Scores were then totaled (maximum value of 33) and then divided by

the total number of items, such that CFI scores range from 0 to 1, with 1 indicating the highest frailty.

[0163] Data analysis. Each individual dog served as the experimental unit. Missing data points were not imputed and only dogs with available fatty acid concentrations were included in analyses ($n = 61$). All available data were used where possible.

[0164] Descriptive statistics. Descriptive statistics (N , mean, standard deviation, median, 25th and 75th percentiles, minimum and maximum) of demographic features (age, weight, BCS, sex, desexed status, % pure or mixed breed), insulin (mU/L)), fatty acid species and groupings (FFA, SFA, PA, OA and LA) outcome variables (HRQL total and CFI scores) were summarized for the entire sample and stratified by old (>7 years) and young dogs (<7 years).

[0165] Pairwise Pearson's Correlations. To explore simple correlations between fatty acid species, age, weight, BCS, healthspan outcome measures (HRQL and CFI) and insulin (mU/L), pairwise correlations using Pearson's correlation was performed. These correlations provided an estimate of the strength and direction of the relationships among variables of interest, without consideration of the effects of other covariates. Simple linear regression was also used to visualize any associations of interest.

[0166] Significant associations detected in correlation analyses were then tested further using multiple linear regression. Pearson correlation results were also used to identify potential covariates of interest.

[0167] Multiple regression. To estimate associations between age and each fatty acid, independent of the effects of covariates, a multiple linear regression model was fit for each fatty acid species as the outcome, respectively, treating age as the primary predictor.

[0168] To estimate the relationship between fatty acid species and clinical outcomes (HRQL total, CFI scores, and insulin), a similar approach was followed. For each outcome, a multiple linear regression model was fit to individually assess the main effect of each fatty acid, adjusting for age and other covariates. Next, potential interaction effects between each fatty acid species and age on clinical outcomes by adding in an interaction term with age into each model was explored. All covariates and interactions were treated as continuous variables and were selected based on results of Pearson's correlations. To facilitate coefficient interpretation and comparisons of effect sizes across regression models, individual and grouped fatty acid measures were normalized ("standardized") via mean centering and dividing by their standard deviation. Model assumptions were evaluated by visualizing model residuals.

[0169] Coefficient estimates along with 95% confidence intervals, coefficient and global model p values, and goodness of fit measures (R^2) are reported for all models. Type I error was set to $\alpha=0.05$.

Results

[0170] A total of 61 dogs were included for FFA analyses. On average, animals were 8.2 years old, 25.5 kg and 52.5% female. See **Table 5** for information and descriptive statistics for age, sex, desexed status, purebred (yes/no), weight, BCS, HRQL total scores, HRQL domain scores, CFI scores, insulin (mU/L) concentrations and fatty acid concentrations ($\mu\text{g/mL}$).

[0171] Unadjusted Pairwise Pearson's Correlations. To explore simple correlations between fatty acids, age, potential covariates like weight and BCS, and health outcome measures (insulin, HRQL total and CFI), pairwise correlations using Pearson's correlation were estimated. These analyses are unadjusted, in that they do not account for any joint effects between variables of interest. However, these correlations provide a first look at the direction and strength of variables of interest. All pairwise correlations between fatty acids, ages, weight, BCS, HRQL, CFI, and insulin are reported in **Table 1**.

[0172] All fatty acids analyzed (FFA, SFA, PA, OA and LA) were statistically significantly positively correlated with age ($p < 0.05$; **FIG. 1**; **Table 1**). Therefore, all fatty acids are candidates for further testing in a multiple regression context. BCS also shows a significant negative association with age ($p < 0.01$; **Table 1**), revealing it as a potential covariate in models exploring relationships between age and fatty acids.

[0173] When assessing the correlation of fatty acids to frailty (CFI), increases in the concentrations of 2 individual fatty acids (PA, OA) and 1 group of fatty acids (FFA) significantly correlated with increased CFI scores ($p < 0.05$; **FIG. 1**; **Table 1**). SFA and LA, while not reaching significance, were observed to have trending correlations with CFI ($p < 0.10$; **FIG. 1**; **Table 1**). While not all fatty acids reached a statistically significant association with CFI, due to trends and observed relationships in **FIG. 1**, they were further tested using multiple regressions. Both age and BCS were significantly correlated with CFI ($p < 0.001$ and $p < 0.05$, respectively; **Table 1**), making them candidate covariates in models exploring relationships between fatty acids and CFI.

[0174] Insulin regulates serum concentration of fatty acids in response to nutrient availability. Previously, fasting insulin has been demonstrated to increase with age and display a negative correlation with total HRQL scores. It is therefore assessed whether fasting insulin concentrations were correlated with fatty acid concentrations. Increasing fasting insulin concentrations were significantly positively correlated with increasing FFA, SFA, PA and OA

concentrations ($p < 0.05$; **FIG. 1**; **Table 1**), and LA showed a trending association ($p = 0.06$; **FIG. 1**). All relationships between fatty acids and insulin were further tested using multiple regression. Age was significantly positively associated with insulin ($p < 0.05$; **Table 1**) while weight showed a trending positive correlation ($p < 0.10$), suggesting them as covariates in models testing relationships between insulin and fatty acids.

Fatty acid concentrations increase with age independent of body weight and BCS

[0175] To test if associations between fatty acid concentrations and age remained, above and beyond any potential effects of BCS, a multiple regression model of age and BCS were fit against each fatty acid concentration. Age ($p < 0.05$) was significantly positively associated with FFA, SFA, PA, LA and OA (**Table 2**). BCS was significantly positively associated with FFA and SFA concentrations, after adjusting for age. As each individual and grouped fatty acid has been standardized to mean = 0 and SD = 1, the effects for age as every year increase is associated with a 0.9 to 0.11 SD increase in mean fatty acid concentrations, adjusting for the effects of BCS can be interpreted. These data demonstrate fatty acid concentrations’ association with age independent of body weight and BCS scores.

Fatty acids association with CFI scores

[0176] To estimate the covariate-adjusted association between fatty acids and CFI scores, a multiple regression model was used to estimate the relationship between fatty acids and CFI accounting for effects of age and BCS. It was first tested the main effects of each fatty acid adjusting for age and BCS against CFI (**Table 6**) as well as a model testing the interaction effects between each fatty acid and age (**Table 3**). Fatty acid measures were not significantly associated with CFI in main effects models adjusting for age and BCS (**Table 6**), however, significant interaction effects between all fatty acids (FFA, SFA, PA, OA and LA) and age ($p < 0.05$, **Table 3**, **FIG. 3**) was detected. All coefficients for the interactions between age and fatty acid measures were > 0 , suggesting that the effects of fatty acids appear to be stronger in older dogs. **FIG. 3** provides a visualization of the observed and predicted interaction between fatty acids and age on CFI. Model diagnostics can be found in **FIG. 6**.

Table 6. Multiple regression testing the main effects of FFA, SFA, PA, OA and LA on with CFI scores, adjusting for age and BCS

Model	Variable	Coefficient (95% CI)	P value	Overall P value	R ²
Model 1	Intercept	0.00 (-0.15,0.15)	0.999	<0.001***	0.46
	Standardized FFA	0.01 (-0.01,0.03)	0.358		
	Age (yrs)	0.02 (0.01,0.02)	<0.001***		

Model 2	BCS	-0.01 (-0.03,0.01)	0.317	
	Intercept	0.01 (-0.15,0.16)	0.942	<0.001*** 0.46
	Standardized SFA	0.01 (-0.01,0.03)	0.344	
	Age (yrs)	0.02 (0.01,0.02)	<0.001***	
Model 3	BCS	-0.01 (-0.03,0.01)	0.289	
	Intercept	0.00 (-0.15,0.15)	0.981	<0.001*** 0.47
	Standardized Palmitic acid	0.01 (-0.01,0.03)	0.282	
	Age (yrs)	0.02 (0.01,0.02)	<0.001***	
Model 4	BCS	-0.01 (-0.03,0.01)	0.333	
	Intercept	0.00 (-0.15,0.14)	0.985	<0.001*** 0.47
	Standardized Oleic acid	0.02 (-0.01,0.04)	0.15	
	Age (yrs)	0.02 (0.01,0.02)	<0.001***	
Model 5	BCS	-0.01 (-0.03,0.01)	0.33	
	Intercept	-0.01 (-0.16,0.14)	0.862	<0.001*** 0.46
	Standardized Linoleic acid	0.01 (-0.02,0.03)	0.642	
	Age (yrs)	0.02 (0.01,0.02)	<0.001***	
	BCS	-0.01 (-0.03,0.01)	0.386	

Each fatty acid measure is standardized by mean centering and scaling by standard deviation (SD); units = 1 SD

Fatty acids are associated with fasting insulin

[0177] To estimate the covariate-adjusted association between fatty acids and fasting insulin, a multiple regression model was used to estimate the relationship between fatty acids and insulin accounting for effects of age and weight. Weight was included as a covariate to account for any metabolic signature confounding due to body size. First tested was the main effects of each fatty acid adjusting for age and weight against insulin (**Table 7**) as well as a model testing the interaction effects between each fatty acid and age (**Table 4**). In main effects models, OA showed a significant positive association with insulin, while FFA showed a trending positive association. Significant interaction effects between all fatty acid measures and age ($p < 0.05$, **Table 4**, **FIG. 4**) was detected. All coefficients for the interactions between age and fatty acid measures were > 0 , suggesting that effects of fatty acids on insulin concentrations appear to be stronger in older dogs. **FIG. 4** provides a visualization of the observed and predicted interaction between fatty acids and age on insulin concentrations. Model diagnostics can be found in **FIG. 7**.

Table 7. Multiple regression testing the main effects of FFA, SFA, PA, OA, and LA on with Insulin (mU/L) scores, adjusting for age and weight.

Model	Variable	Coefficient (95% CI)	P value	Overall P value	R ²
Model 1	Intercept	8.45 (-2.81,19.70)	0.147	0.008**	0.19
	Standardized FFA	3.53 (-0.16, 7.21)	0.066		

Model 2	Age (yrs)	0.84 (-0.13, 1.81)	0.094	0.014*	0.17
	Weight (kg)	0.19 (-0.03, 0.42)	0.102		
	Intercept	7.67 (-3.67,19.01)	0.19		
	Standardized SFA	2.87 (-0.84, 6.58)	0.135		
	Age (yrs)	0.93 (-0.05, 1.90)	0.067		
Model 3	Weight (kg)	0.20 (-0.03, 0.42)	0.1	0.011*	0.18
	Intercept	7.56 (-3.60,18.71)	0.19		
	Standardized Palmitic acid	3.08 (-0.60, 6.75)	0.106		
	Age (yrs)	0.87 (-0.11, 1.85)	0.089		
	Weight (kg)	0.22 (0.00, 0.44)	0.058		
Model 4	Intercept	9.14 (-1.36,19.64)	0.094	<0.001***	0.25
	Standardized Oleic acid	5.23 (1.77, 8.69)	0.004**		
	Age (yrs)	0.71 (-0.21, 1.64)	0.135		
	Weight (kg)	0.20 (-0.01, 0.41)	0.064		
	Intercept	6.37 (-5.06,17.81)	0.279		
Model 5	Standardized Linoleic acid	1.73 (-2.01, 5.47)	0.368	0.027*	0.15
	Age (yrs)	1.02 (0.04, 2.00)	0.046*		
	Weight (kg)	0.22 (-0.01, 0.45)	0.072		

Each fatty acid measure is standardized by mean centering and scaling by standard deviation (SD); units = 1 SD

[0178] Due to a single outlier in insulin concentrations, a sensitivity analysis was also performed excluding any insulin concentration values > 90 mU/L, and interaction models were re-fit. **Table 8** and **FIG. 8** show results of these sensitivity analyses. With removal of the outlier, all interactions except OA and PA remained statistically significant, with PA showing trending significance (p=0.087).

Table 8. Sensitivity analysis of interaction between FFA, SFA, PA, OA, LA and Insulin (mU/L), excluding insulin values ≥ 90 mU/L.

Model	Variable	Coefficient (95% CI)	P value	Overall P value	R ²
Model 1	Intercept	10.81 (2.59,19.02)	0.013*	0.063	0.12
	Standardized FFA	-5.94 (-13.49,1.61)	0.129		
	Age (yrs)	0.27 (-0.45,0.98)	0.464		
	Weight (kg)	0.21 (0.05,0.37)	0.014*		
	Standardized FFA x Age (yrs)	0.76 (0.02,1.51)	0.049*		
Model 2	Intercept	11.04 (2.96,19.12)	0.010**	0.079	
	Standardized SFA	-5.72 (-12.35,0.92)	0.097		
	Age (yrs)	0.27 (-0.43,0.98)	0.451		
	Weight (kg)	0.20 (0.04,0.36)	0.018*		
	Standardized SFA x Age (yrs)	0.75 (0.05,1.45)	0.040*		
Model 3	Intercept	10.63 (2.44,18.82)	0.014*	0.076	
	Standardized Palmitic acid	-5.58 (-13.35,2.20)	0.166		
	Age (yrs)	0.25 (-0.47,0.97)	0.502		

	Weight (kg)	0.22 (0.06,0.38)	0.009**		
	Standardized Palmitic acid x Age (yrs)	0.65 (-0.08,1.38)	0.087		
Model 4	Intercept	11.96 (4.02,19.90)	0.005**	0.029*	0.15
	Standardized Oleic acid	-3.00 (-10.96,4.96)	0.463		
	Age (yrs)	0.15 (-0.56,0.85)	0.683		
	Weight (kg)	0.21 (0.05,0.37)	0.011*		
	Standardized Oleic acid x Age (yrs)	0.53 (-0.23,1.32)	0.177		
Model 5	Intercept	9.42 (1.21,17.63)	0.029*	0.09	0.11
	Standardized Linoleic acid	-7.81 (-15.22,-0.40)	0.044*		
	Age (yrs)	0.39 (-0.33,1.11)	0.291		
	Weight (kg)	0.22 (0.06,0.38)	0.009**		
	Standardized Linoleic x Age (yrs)	0.85 (0.12, 1.57)	0.025*		

Discussion

[0179] Under normal physiological conditions, fatty acids are used as a substrate for energy production and signaling molecules affecting different metabolic processes. The storage and release of fatty acids from the adipose tissue is regulated by nutrient availability and the presence of insulin, which signals to the adipose tissue to stop releasing fatty acids by shutting down lipolysis. With increasing chronological age, the visceral adipose compartment expands and becomes resistant to the anti-lipolytic effects of insulin resulting in an increase in certain fatty acids in circulation. Chronic elevation of these fatty acids can negatively impact peripheral tissues leading to lipotoxicity, hyperinsulinemia and whole body insulin resistance. This phenomenon implicates the expansion and dysfunction of the visceral adipose as a driver of the metabolic dysfunction and insulin resistance observed with advanced chronological age.

[0180] *Relationships of age, BCS, and weight on fatty acid levels.* In a univariate model examining the relationship between the fatty acids and age without any other covariates, all grouped and individual fatty acids analyzed increased with advancing chronological age ($p < 0.05$). This is the first investigation in companion dogs to show FFA, SFA, PA, OA and LA all are progressively elevated as dogs age. Additionally, when assessing fatty acid levels in relation to BCS and weight independently, there was no association between fatty acid concentrations and BCS or weight, suggesting their elevation is independent of these measures. Multiple regression models confirm the observations in the univariate models, with the exception of FFA and SFA which were revealed to have associations with BCS, while PA, OA and LA continued to have no significant association with BCS.

[0181] These data suggest that chronological age is a predictor of FFA, SFA, PA, OA and LA concentrations, irrespective of both BCS and weight. These data validate more limited cross

sectional studies seen in laboratory beagles which demonstrated that FFA concentrations are significantly increased in aged dogs. Moreover, these data recapitulate phenotypes in humans with insulin resistance and expanded/dysfunction visceral adipose, which are more prevalent in aged populations. Given the independent associations with age, these data suggested that FFA, SFA, PA, OA and LA have utility as potential biomarkers for aging in dogs.

[0182] In the univariate model examining the relationship between grouped and individual fatty acids and CFI scores, increasing FFA, PA and OA concentrations were positively correlated with increasing CFI scores. When adjusting for BCS in a multiple regression interaction model with age, increases in grouped and individual fatty acid concentrations (FFA, SFA, PA, OA and LA) were associated with higher CFI scores. These data suggest that certain fatty acids may be predictive of frailty in dogs and speak to the importance of proper adipose function in regulating fatty acid concentrations in dogs as they age. While the human literature has just now begun to associate specific fatty acid species to frailty, no such comparable analysis previously existed in dogs.

[0183] For the first time, these data provide real world evidence that the elevation of grouped and individual fatty acids are associated with the progressive frailty in companion dogs. Moreover, these observed changes in fatty acid concentrations are reflective of clinically relevant and importantly, *quantifiable*, changes in dogs. This positions certain fatty acids (FFA, SFA, PA, OA and LA) as potential predictors of CFI, an outcome measure that is broadly accessible for usage in clinical practice.

[0184] *Relationships between fatty acids and fasting insulin.* In the univariate model examining the relationship between grouped and individual fatty acids and fasting insulin, increasing concentrations of FFA, SFA, PA and OA are significantly associated with increasing fasting insulin concentrations. When adjusting for weight in a multiple regression interaction model with age, increases in all grouped and individual fatty acids measured (FFA, SFA, PA, OA and LA) were significantly associated with higher fasting insulin concentrations, an association which appears to be stronger in the older dogs.

[0185] As described previously, the interaction between fatty acid concentrations and insulin concentrations is well-described in the human literature. A consequence of adipose dysfunction with age is the loss of insulin sensitivity within the adipose itself, which leads to increased lipolysis and thus increased concentrations of fatty acids in circulation. The association of these grouped and individual fatty acids with both fasting insulin and with age suggests that dogs with advanced age display the dual hallmarks of adipose dysfunction and insulin resistance.

[0186] Elevations in fasting insulin concentrations were associated with reductions in quality of life (HRQL), making it a potential predictor of a clinically available quality of life metric. Thus, adipose dysfunction is contributing to decreasing quality of life and increased frailty with advanced age in dogs.

[0187] **Conclusion.** Multiple regression testing demonstrated increased serum FFA, SFA, PA, OA and LA concentrations in this population companion dogs were significantly associated with increasing chronological age. Additionally, increasing FFA, SFA, PA, OA and LA concentrations were significantly associated with progressive frailty and fasting insulin concentrations in dogs as they age. Taken together, these data strongly support fatty acids as a marker of aging, adipose dysfunction and frailty in dogs regardless of size and body condition.

Example 4. Evaluation of Insulin as Biomarkers of Health-Related Quality of Life and Frailty in Dogs

[0188] Metabolic dysfunction is well-characterized in the pathophysiology of obesity, cardiovascular disease, type II diabetes and aging in humans. Fasted insulin is an integral in maintaining proper metabolic function and has been shown to be dysregulated in metabolically perturbed states, making it an attractive biomarker of disease. Previous investigations have evaluated the effect of age on insulin concentrations in dogs, as well as in dogs with cardiac disease, obesity, and type II diabetes, but have not examined them in the context of declining quality of life and progressive frailty in aging dogs.

[0189] To this end, findings from a continuation of the study is reported, in which blood collected from a subset of 451 client-owned dogs of various ages and sizes (≥ 2 and < 6 (young dogs) or ≥ 7 years of age (old dogs), < 25 (small dogs) or > 50 lbs (large dogs)) was measured for serum insulin concentrations. The aim of this study was to evaluate and recapitulate expected relationship of serum insulin concentrations with age, while assessing the effects of both weight and Body Condition Score (BCS) as covariates in the analysis. Furthermore, it is aimed to evaluate the potential of serum insulin concentration as biomarker of owner-reported Health-Related Quality of Life (HRQL) and clinician-assessed Canine Frailty Index (CFI) scores. Regression analyses and Spearman's correlation tests were used to evaluate insulin concentrations in association with age, weight, BCS, HRQL, and CFI score.

[0190] Consistent with other studies, serum insulin increased with age, independent of weight and BCS. These results bolster the evidence that blood levels of these metabolic health markers are likely associated with the (patho)physiological process of aging and are potentially useful candidates for markers of biological age.

[0191] Furthermore, while serum insulin levels did not show a significant association with CFI after accounting for the effects of age and BCS, they did show a significant inverse relationship with total and individual HRQL scores above and beyond the effect of age, weight, and BCS. Taken together, these data provide supportive evidence that serum insulin may be a valuable, stand-alone indicator of metabolic health and HRQL in dogs regardless of age, size, and body condition.

[0192] Herein, it was aimed to determine if, similar to humans, changes in insulin over various ages also occur in dogs and if they could be predictive of the age, quality of life or frailty state of the animal. To this end, serum insulin levels from previously collected blood samples from a subset of the client-owned dogs enrolled in the study were measured and evaluated for their correlation with age, body weight, and body condition. Furthermore, serum insulin levels with Health-related quality of life and frailty was correlated using two validated tools, the VetMetrica Health-Related Quality of Life (HRQL) owner-questionnaire and a veterinarian-assessed Canine Frailty Index (CFI), respectively.

Methods

[0193] Study population. 451 client-owned, clinically stable adult dogs, aged ≥ 2 and <6 (young dogs) or ≥ 7 years of age (old dogs), < 25 (small dogs) or > 50 lbs (large dogs), of which 403 had age, weight, BCS, HRQL scores, CFI scores, and insulin measures observed. 399 had available insulin readings. 335 dogs had complete data on all measure.

[0194] Standards Applied. Owner consent was obtained for all dogs. Good Clinical Practice (GCP) Standards were followed. Approval by an independent IACUC was obtained on December 13, 2020 through Veterinary & Biomedical Research Center, Inc (VBRC).

[0195] Sample collection. Fasted blood samples were collected via venipuncture in a serum separator tube for evaluation of insulin. The protocol required at least 6 hours of fasting, although this was not confirmed at the time of collection. Samples were left to clot at room temperature for no more than 60 minutes prior to centrifugation at 2800-3200 rpm for 10 minutes at ambient temperature. From each sample, the resultant serum was divided into aliquots of approximately 500-1000 μ L using 1.8 mL cryovials and stored at approximately -80°C until shipment on dry ice to Eve Technologies (Calgary, Canada) for analyses.

[0196] Insulin. Fasting serum insulin concentrations were measured using a commercially available sandwich ELISA (10-1203-01) by Mercodia (Sweden) per the kit manufacturer's instructions. A precoated microplate with an immobilized antibody for insulin was loaded with serum samples or concentration standards for binding. After washing away unbound substances,

an HRP conjugated antibody for insulin was added to the wells. Incubation was performed for 2 hours at 18-25°C on a plate shaker (700-900rpm). The excess unbound enzyme-labeled antibody was washed again and the remaining conjugate was allowed to react with a 3,3',5,5'-tetramethylbenzidine solution. The assay was terminated by adding an acidic solution and read at 450 nm wavelength on a microplate reader. A curve was generated from the known concentration standards and the unknown serum samples were determined using that curve.

[0197] Quality control. Quality control was performed for insulin on a standard curve and 2 to 4 wells on each plate to evaluate the coefficients of variability (CV) between assays. Samples with CV > 15% or insufficient sample volume were excluded. Following quality control and exclusion due to unmet screening criteria such as incomplete data or unverified age, there were 399 available insulin measurements. Of these measurements, 396 insulin measurements had complete data on age, weight, body condition score (BCS), HRQL, and CFI scores.

[0198] Data analysis. Each individual dog served as the experimental unit. Missing data points were not imputed and only dogs with available insulin concentrations were included in analyses (n = 403).

[0199] Descriptive statistics. Descriptive statistics (N, mean, standard deviation, median, 25th and 75th percentiles, minimum and maximum) of demographic features (age, sex, desexed status, % pure or mixed breed), key biomarker insulin (mU/L) and outcome variables (HRQL total, individual HRQL domain scores, CFI scores) were summarized for the entire dataset as well as stratified by old (≥ 7 years of age) and young (≥ 2 and < 6 years of age). Due to the non-normality of these data, differences across old and young dogs were tested using Wilcoxon Rank Sum tests for continuous variables and Chi-squared tests for categorical variables.

[0200] HRQL Scores. Scores were available for four HRQL sub-domains: Energetic/Enthusiastic (E/E), Happy/Content (H/C), Active/Comfortable (A/C), and Calm/Relaxed (C/R). Individual HRQL domain scores range from 0 to 6, with larger scores indicating higher quality of life in that domain. Following Davies et al, HRQL total score was calculated as the sum of all four domains, which was then standardized and rescaled such that total scores ranged from 0 to 100 with a mean of 50 and a standard deviation of 10. The HRQL total score represents a dog's overall quality of life, where higher scores indicate better quality of life and lower scores with lower quality of life.

[0201] CFI Scores. The CFI was developed as a tool to measure frailty through the assessment of multimorbidity and functional deficits associated with aging in dogs. The CFI was developed as an analog to the frailty index used in humans or rodents, where frailty is assessed

across multiple dimensions and reflects a general state of poor health. The CFI contains 33 discrete clinical health items, ranging from yes/no answers on functional ability (e.g., “Assistance for standing up”, with responses as “Yes” = 1 or “No” = 0) to specific disease states (e.g. “Disease of the oral category”, with responses as “No” = 0, “Mild” = 0.5, “Severe” = 1). These items were assessed by a veterinarian. Scores were then totaled (maximum value of 33) and then divided by the total number of items, such that CFI scores range from 0 to 1, with 1 indicating the highest frailty.

[0202] Testing for normality. Normality of insulin, HRQL total, HRQL domain, and CFI scores were assessed visually using histograms and normal quantile-quantile plots. All variables showed deviations from normality.

[0203] Spearman’s correlation to evaluate bivariate associations. As the vast majority of variables were non-normally distributed, Spearman’s correlation was used to estimate the bivariate relationships between 1) insulin and age, 2) and between each biomarker (insulin) and HRQL total, HRQL domain (E/E, A/C, H/C, and C/R), and CFI scores individually. Spearman’s correlation is a non-parametric analog to Pearson’s correlation providing an estimated correlation coefficient (ρ) ranging from -1 to 1, where coefficients farther from 0 indicate stronger associations. Spearman’s correlations were calculated using the function `cor.test()` from the R Studio `stats` library.

[0204] Median regression. Covariate-adjusted associations between 1) biomarkers and age, and 2) biomarkers and HRQL total, HRQL domains and CFI scores were evaluated using quantile regression. Quantile regression is an extension of linear regression and is recommended when assumptions for linear regression are not met (e.g., non-normality and heteroscedasticity). One advantage of quantile regression over linear regression is that it is more robust to the effects of outliers, as quantile values (e.g., conditional expectations of medians) are estimated instead of conditional means. While quantile regression can estimate any conditional quantile, the conditional median to understand associations among the majority of dogs was estimated.

[0205] To estimate associations between age and each biomarker, independent of the effects of covariates, the following full main effects model was fit treating insulin as the outcome, respectively: age was treated as the primary predictor and weight and BCS scores were considered covariates. Potential interactions between age and weight or age and BCS in association with insulin by adding these interaction terms individually to the full main effects model were also explored.

[0206] To estimate the relationship between each biomarker and healthspan measures, a similar approach was followed. For each healthspan measure (HRQL total, EE, HC, AC, CR, and CFI), a full main effects model treating insulin as the primary predictor, and age, weight, and BCS as covariates was fit. For any models of healthspan for which a biomarker and one or more covariates were significant predictors, the interactions between those covariates and the biomarker were tested. Significant interaction effects could indicate that associations between each biomarker and the relevant healthspan measure could differ by the level of another covariate. Understanding potential interaction effects helps generate hypotheses and enhances understanding of mechanisms of association.

[0207] Standard errors around regression coefficients were estimated using the Huber sandwich estimator, allowing for robustness to non-independent errors. Coefficient estimates along with 95% confidence intervals are presented for all models, along with R^1 , a measure of model goodness of fit similar to R^2 (0 = worst fit, 1 = best fit). All variables in covariate adjustments and interactions were treated as continuous variables.

[0208] All analyses and data presentations were performed using R version 4.1.2.

Results

[0209] Of 451 dogs who completed the Healthspan study, 403 had age, weight, BCS, HRQL scores, CFI scores and insulin measures observed. 399 had available insulin readings. 335 dogs had complete data on all measures. **Table 9** shows descriptive statistics of age, sex, desexed status, purebred (yes/no), weight, BCS, HRQL total scores, HRQL domain scores, CFI scores, and insulin (mU/L) concentrations.

Table 9. Descriptive statistics of demographic variables, insulin, and HRQL and CFI scores for Healthspan dogs or insulin levels measured

	7+ years old (N=244)	< 6 years old (N=159)	Total (N=403)	p value
Age (years)				< 0.001 ^a
N	244	159	403	
Mean (SD)	10.38 (2.47)	3.89 (1.08)	7.82 (3.77)	
Median (Q1, Q3)	10.00 (8.30, 12.10)	3.90 (3.10, 4.80)	7.90 (4.20, 10.60)	
Min - Max	7.00 - 17.70	2.00 - 5.90	2.00 - 17.70	
Weight (lbs)				0.021 ^a

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N	244	159	403	
Mean (SD)	50.56 (33.81)	58.57 (31.92)	53.72 (33.27)	
Median (Q1, Q3)	58.30 (16.30, 73.60)	64.20 (23.40, 76.30)	61.60 (17.85, 75.00)	
Min - Max	2.80 - 164.00	3.00 - 176.40	2.80 - 176.40	
Body Condition Score				0.945 ^a
N	244	159	403	
Mean (SD)	5.41 (0.98)	5.43 (0.88)	5.42 (0.94)	
Median (Q1, Q3)	5.00 (5.00, 6.00)	5.00 (5.00, 6.00)	5.00 (5.00, 6.00)	
Min - Max	3.00 - 9.00	4.00 - 8.00	3.00 - 9.00	
Sex				0.903 ^b
Female	132 (54.1%)	87 (54.7%)	219 (54.3%)	
Male	112 (45.9%)	72 (45.3%)	184 (45.7%)	
Intact				< 0.001 ^b
No	9 (3.7%)	29 (18.2%)	38 (9.4%)	
Yes	235 (96.3%)	130 (81.8%)	365 (90.6%)	
Purebred				0.231 ^b
No	59 (24.2%)	47 (29.6%)	106 (26.3%)	
Yes	185 (75.8%)	112 (70.4%)	297 (73.7%)	
Insulin (mU/L)				0.010 ^a
N	240	159	399	
Mean (SD)	20.49 (14.43)	17.81 (12.64)	19.42 (13.79)	
Median (Q1, Q3)	17.58 (11.16, 25.19)	14.15 (9.36, 21.00)	15.97 (10.32, 23.95)	
Min - Max	2.53 - 107.00	2.55 - 76.37	2.53 - 107.00	
Standardized log(Insulin)				0.010 ^a
N	240	159	399	
Mean (SD)	20.49 (14.43)	17.81 (12.64)	19.42 (13.79)	
Median (Q1, Q3)	17.58 (11.16, 25.19)	14.15 (9.36, 21.00)	15.97 (10.32, 23.95)	
Min - Max	2.53 - 107.00	2.55 - 76.37	2.53 - 107.00	
Canine Frailty Index Score				< 0.001 ^a
N	244	158	402	
Mean (SD)	0.10 (0.09)	0.02 (0.02)	0.07 (0.08)	
Median (Q1, Q3)	0.08 (0.03, 0.14)	0.00 (0.00, 0.02)	0.05 (0.02, 0.09)	
Min - Max	0.00 - 0.59	0.00 - 0.09	0.00 - 0.59	
HRQL Total Score				< 0.001 ^a
N	243	158	401	
Mean (SD)	48.27 (8.04)	53.28 (7.52)	50.24 (8.20)	

Median (Q1, Q3)	47.47 (42.61, 53.00)	52.43 (48.47, 55.10)	50.11 (45.13, 54.19)	
Min - Max	31.62 - 83.02	41.68 - 83.02	31.62 - 83.02	
Energetic/Enthusiastic				< 0.001 ^a
N	243	158	401	
Mean (SD)	2.91 (0.73)	3.57 (0.47)	3.17 (0.71)	
Median (Q1, Q3)	3.00 (2.43, 3.49)	3.59 (3.30, 3.98)	3.31 (2.71, 3.70)	
Min - Max	0.84 - 4.15	2.19 - 4.15	0.84 - 4.15	
Happy/Content				< 0.001 ^a
N	243	158	401	
Mean (SD)	5.56 (0.49)	5.86 (0.23)	5.68 (0.43)	
Median (Q1, Q3)	5.71 (5.39, 6.00)	6.00 (5.80, 6.00)	5.82 (5.52, 6.00)	
Min - Max	3.98 - 6.00	4.62 - 6.00	3.98 - 6.00	
Active/Comfortable				< 0.001 ^a
N	243	158	401	
Mean (SD)	4.96 (0.80)	5.61 (0.49)	5.22 (0.76)	
Median (Q1, Q3)	5.16 (4.40, 5.63)	5.80 (5.47, 5.90)	5.47 (4.81, 5.80)	
Min - Max	2.59 - 6.00	3.34 - 6.00	2.59 - 6.00	
Calm/Relaxed				0.004 ^a
N	243	158	401	
Mean (SD)	3.28 (0.68)	3.07 (0.74)	3.20 (0.71)	
Median (Q1, Q3)	3.38 (2.84, 3.84)	3.01 (2.48, 3.68)	3.22 (2.65, 3.72)	
Min - Max	1.13 - 4.26	1.20 - 4.26	1.13 - 4.26	
a.	Wilcoxon Rank Sum test			
b.	Chi-Squared test			

[0210] Pairwise Spearman's Correlations. To explore simple correlations between biomarkers (insulin), age, covariates (weight, BCS), and healthspan outcome measures (HRQL and frailty), pairwise correlations using Spearman's correlation due to violations of normality assumptions was estimated. These correlations provided an estimate of the strength and direction of the relationships among variables of interest, without consideration of the effects of other covariates. **FIG. 9** shows a pairwise scatterplot and correlations of all variables included presented in this report. To explore simple correlations between biomarkers (insulin), age, covariates (weight, BCS), and healthspan outcome measures (HRQL and frailty), pairwise correlations using Spearman's correlation due to violations of normality assumptions were estimated. These correlations provided an estimate of the strength and direction of the relationships among

variables of interest, without consideration of the effects of other covariates. **FIG. 9** shows a pairwise scatterplot and correlations of all variables included presented herein.

Table 10. Spearman’s correlation coefficients evaluating bivariate relationships between insulin with age, weight, BCS, and HRQL and CFI scores

	Variable	Insulin	
		rho	p-value
Biomarkers	Insulin	--	
Demographics	Age (yrs)	0.18	<0.001
	Weight (lbs)	0.05	0.278
	Body Condition Score	0.26	<0.001
Health Outcomes	HRQL Total	-0.25	<0.001
	Energetic/Enthusiastic	-0.27	<0.001
	Happy/Content	-0.17	0.001
	Active/Comfortable	-0.26	<0.001
	Calm/Relaxed	-0.04	0.378
	Canine Frailty Score Index	0.10	0.053

* **Bold** values indicate statistical significance (p-value <0.05)

Table 11. Multiple median regression estimating the joint main and interaction effects of age, weight and BCS in association with insulin. Three models are presented for insulin: 1) a full main effects model, 2) an interaction model with age x weight, 3) an interaction model with age and BCS.

Model	Variables	Effect on standardized log(Insulin)		
		Coefficient (95% CI)	p-value	R ₁
Full main effects model	(Intercept)	-2.20 (-3.00, -1.41)	<0.001	0.02
	Age (yrs)	0.07 (0.04, 0.10)	<0.001	
	Weight (lbs)	0.00 (0.00, 0.00)	0.711	
	BCS	0.30 (0.16, 0.43)	<0.001	

Age x weight (Intercept)	-	(-3.04, -1.22)	<0.001	0.07
Age (yrs)	2.13	(0.00, 0.12)	0.069	
Weight (lbs)	0.06	(-0.01, 0.01)	0.686	
BCS	0.00	(0.17, 0.44)	<0.001	
Age x weight	0.30	(0.00, 0.00)	0.666	
Age x BCS (Intercept)	-	(-4.73, -1.86)	<0.001	0.07
Age (yrs)	3.29	(0.06, 0.36)	0.005	
Weight (lbs)	0.21	(0.24, 0.76)	<0.001	
BCS	0.50	(0.00, 0.00)	0.737	
Age x BCS	0.00	(-0.05, 0.00)	0.072	

* **Bold** variable names indicate statistical significance (p -value <0.05); R^1 is a measure of model goodness of fit, where 0 is the worst fit and 1 is the best fit.

Insulin increases with age after adjusting for weight and body condition

[0211] Spearman's correlation found insulin was positively correlated with age ($\rho=0.18$, $p<0.001$) and positively correlated with BCS ($\rho=0.26$, $p<0.001$), but not correlated with weight ($\rho=0.05$, $p=0.278$) (**Table 10**).

[0212] A main effects quantile regression model including age, weight and BCS in association with insulin was fit, to estimate the joint effects of these variables. Age ($\beta=0.07$, 95% CI=(0.04, 0.10), $p<0.001$, **FIG. 10**) and BCS ($\beta=0.30$, 95% CI=(0.16, 0.43), $p<0.001$), but not weight ($\beta=0.00$, 95% CI=(0.00, 0.00), $p=0.711$), were significantly associated with insulin (**Table 11**). The coefficient for age as, every unit increase in age is associated with a 0.07 SD increase in median log(insulin) values, adjusting for the effects of weight and BCS can be interpreted. For every point increase in BCS, there is a 0.30 SD increase in median log(insulin) values.

[0213] The main effects model demonstrated that age was significantly positively associated with insulin levels. To evaluate if this relationship between age and insulin was dependent on the other covariates in the model (weight and BCS), the interactions between 1) age and weight, and 2) age and BCS were tested. No significant interactions were found between age and weight ($\beta=0.00$, 95% CI=(0.00, 0.00), $p=0.666$) or age and BCS ($\beta=-0.03$, 95% CI=(-0.05, 0.00), $p=0.072$), indicating that the relationship between age and insulin is not dependent on weight or BCS.

Insulin is associated with HRQL total and certain HRQL domains

[0214] Spearman's correlation indicated that insulin was negatively associated with HRQL Total ($\rho=-0.25$, $p<0.001$), E/E ($\rho=-0.27$, $p<0.001$), H/C ($\rho=-0.17$, $p<0.001$) and A/C ($\rho=-$

0.26, $p < 0.001$) scores (**Table 10**). However, these associations did not take into account the effects of covariates like age, weight and BCS. To estimate the covariate-adjusted association between insulin and HRQL, five multiple median regression models were used to estimate the relationship between insulin and HRQL total, and HRQL domains (E/E, H/C, A/C, C/R). In each of these models, standardized $\log(\text{insulin})$ was treated as the primary variable with age, weight and BCS treated as covariates. After adjusting for age, weight, and BCS, standardized $\log(\text{insulin})$ blood levels were significantly associated with HRQL Total ($\beta = -1.49$, 95% CI = (-2.31, -0.67), $p < 0.001$), and Energetic/Enthusiastic ($\beta = -0.16$, 95% CI = (-0.23, -0.08), $p < 0.001$) and Active/Comfortable domains ($\beta = -0.09$, 95% CI = (-0.17, -0.01), $p = 0.023$) (**FIG. 11, Table 12**). The coefficient estimate for HRQL Total can be interpreted as every SD increase in $\log(\text{insulin})$ levels is associated with a -1.49 decrease in median HRQL Total scores.

Table 12. Multiple median regression estimating the association between insulin blood levels and HRQL (total and individual), after adjusting for age, weight and BCS. Six models are presented for each outcome: 1) HRQL Total, 2) Energetic/Enthusiastic, 3) Happy/Content, 4) Active/Comfortable, 5) Calm/Relaxed. Each model contains the same main effects: standardized $\log(\text{insulin})$ as the primary predictor and age, weight and BCS as covariates.

Outcome	Variables	Coefficient (95% CI)	p-value	R ¹
HRQL Total	(Intercept)	55.94 (50.72, 61.16)	<0.001	0.12
	Standardized $\log(\text{Insulin})$	-1.49 (-2.31, -0.67)	<0.001	
	Age (yrs)	-0.79 (-1.00, -0.58)	<0.001	
	Weight (lbs)	-0.02 (-0.04, 0.01)	0.200	
	BCS	0.09 (-0.84, 1.01)	0.853	
Energetic/Enthusiastic	(Intercept)	4.18 (3.69, 4.66)	<0.001	0.19
	Standardized $\log(\text{Insulin})$	-0.16 (-0.23, -0.08)	<0.001	
	Age (yrs)	-0.11 (-0.13, -0.09)	<0.001	
	Weight (lbs)	0.00 (0.00, 0.00)	0.086	
	BCS	0.00 (-0.08, 0.09)	0.966	
Happy/Content	(Intercept)	6.09 (5.90, 6.28)	<0.001	0.09
	Standardized $\log(\text{Insulin})$	-0.02 (-0.05, 0.01)	0.121	
	Age (yrs)	-0.04 (-0.05, -0.03)	<0.001	
	Weight (lbs)	0.00 (0.00, 0.00)	0.235	
	BCS	0.00 (-0.03, 0.04)	0.868	

Active/Comfortable	(Intercept)	6.61 (6.12, 7.10)	<0.001	0.19
	Standardized log(Insulin)	-0.09 (-0.17, -0.01)	0.023	
	Age (yrs)	-0.10 (-0.12, -0.07)	<0.001	
	Weight (lbs)	0.00 (0.00, 0.00)	0.026	
	BCS	-0.06 (-0.15, 0.03)	0.159	
	Calm/Relaxed	(Intercept)	2.74 (2.04, 3.44)	<0.001 0.01
		Standardized log(Insulin)	-0.07 (-0.18, 0.03)	0.170
		Age (yrs)	0.02 (-0.01, 0.05)	0.122
		Weight (lbs)	0.00 (0.00, 0.00)	0.883
		BCS	0.07 (-0.05, 0.18)	0.251

* **Bold** variable names indicate statistical significant (p-value<0.05); R¹ is a measure of model goodness of fit, where 0 is the worst fit and 1 is the best fit.

[0215] Potential interaction effects between insulin and other covariates were tested by adding in interaction terms into models for which insulin and other covariates were significant. This included testing the following interactions in the following models: 1) insulin x age in association with HRQL Total, 2) insulin x age in association with E/E domain, 3) insulin x age in association with A/C domain, and 4) insulin x weight in association with A/C domain. Interaction between age and insulin in association with A/C scores was detected ($\beta=-0.03$, 95% CI=(-0.05, -0.01), p=0.001), indicating that negative associations between insulin and A/C scores were stronger for every year increase in age. No other interaction effects were statistically significant.

[0216] As interpreting and visualizing interaction effects between continuous measures can be challenging, this relationship was further explored by fitting a model of A/C scores including standardized log(insulin), age, weight and BCS but stratified by young (ages <6 years) and old (ages >7 years) dogs (**FIG. 12**). Insulin was significantly negatively associated in both young ($\beta=-0.06$, 95% CI=(-0.11, -0.01), p=0.014) and old dogs ($\beta=-0.17$, 95% CI=(-0.29, -0.05), p=0.005), however, this effect was over two times as strong in old dogs.

Post-hoc analysis of HRQL total and Insulin levels

[0217] To further aid in the interpretation of the negative association between fasting insulin and HRQL total scores (**Table 12**), an additional post-hoc analysis was performed in which the same main effects model were fit but instead treated insulin levels as categories “low”, “medium”, and “high”. These categories were defined as the insulin levels of the lowest third, middle third,

and highest third of the sample (i.e., tertile splits of insulin levels). Identical to the previous main effects models, covariates included age, weight and BCS.

[0218] Of the 399 dogs with insulin measures in the healthspan sample, insulin tertiles were defined as: lowest insulin levels (n=133, range=[2.53 mU/L, 12 mU/L]), highest insulin (n=133, range=[20.8 mU/L, 107 mU/L]), and medium levels (n=133, range=[12 mU/L, 20.8 mU/L]). 2 dogs (1 in the lowest and highest insulin groups, respectively) had missing HRQL Total scores, thus the analytic sample size included 397 dogs.

[0219] Results of the median regression showed that insulin level was significantly associated with HRQL total score (**Table 13**, Likelihood ratio p=0.003), independent of the effects of age, weight and BCS. Compared to dogs in the lowest insulin group, the estimated median HRQL Total score for dogs in the highest insulin group was 3.32 lower (**Table 13**, p=0.002), and the estimated median HRQL Total score for dogs in the medium insulin group was 2.29 (**Table 13**, p=0.006).

FIG. 13 shows the predicted median HRQL Total score for each insulin level group, adjusting for covariates, along with pairwise group comparisons between estimated HRQL Total scores for each group. **FIG. 14** shows a pairwise scatterplot and correlations of selected covariates.

Table 13. Multiple quantile (median) regression model showing effects of insulin tertile group (low, medium, high), adjusting for age, weight, and BCS as covariates (n = 397).

Outcome	Variables	Coefficient (95% CI)	p-value	LRT ¹ p-value
HRQL Total	(Intercept)	56.67 (51.91, 61.43)	<0.001	0.003
	Age (yrs)	-0.76 (-0.97, -0.56)	<0.001	
	Weight (lbs)	-0.01 (-0.04, 0.01)	0.229	
	BCS	0.24 (-0.63, 1.12)	0.583	
	Insulin tertile			
	Low: [2.53 mU/L, 12 mU/L])	referent		
	Middle: (12 mU/L, 20.8 mU/L]	-2.29 (-3.93, -0.67)	0.006	
	High: (20.8 mU/L, 107 mU/L]	-3.32 (-5.42, -1.23)	0.002	

1. LRT = likelihood ratio test, testing the joint addition of all insulin tertiles into a model without

* **Bold** variables indicate statistically significant associations with HRQL total scores

Elevated insulin is associated with higher CFI score in dogs and higher disease burden

[0220] Multiple linear regression was used to estimate the relationship between insulin and CFI. Main effects model included insulin along with covariates age (years), weight (kgs), body condition score (BCS) as main effects. The interaction between age and insulin was also tested to explore that age may moderate the effect of insulin on frailty. Robust standard errors were used for significance testing.

[0221] Multiple linear regression was used to estimate the relationship between insulin levels and CFI, testing main effects as well as interaction effects with age. Results of this analysis show

a significant interaction effect between age and insulin (**Table 14**, $p=0.028$), such that higher insulin is associated with increased frailty, an effect that becomes stronger with age. As insulin and CFI scales differ dramatically, standardized regression coefficients are reported in **Table 14**. **FIG. 15** provides a visualization of the observed and predicted CFI values across both insulin levels and age on their natural scales.

[0222] The canine frailty index (CFI) is a broad multimorbidity assessment incorporating criteria such as disease diagnoses, physical examination and clinical laboratory abnormalities, physical performance measures, and others. Frailty is then defined as a set proportion of elements present. Therefore, this index is not only reflective of frailty through its composite score, but also is representative of disease burden. The relationship between elevated fasting insulin and increased scores on the CFI therefore indicates that increased fasting insulin is associated with higher disease burden.

Table 14. Multiple linear regression of interaction effect between age and standardized insulin on standardized CFI.

Variable	Coefficient (95% CI)	p-value
Intercept	-0.72 (-1.16, -0.28)	0.001**
Age (yrs)	0.18 (0.16, 0.20)	<0.001***
Standardized(Insulin)	-0.10 (-0.23, 0.04)	0.161
Weight (kg)	0.00 (0.00, 0.01)	0.222
BCS	-0.14 (-0.22, -0.06)	<0.001***
Age (yrs) x Standardized(Insulin)	0.02 (0.00, 0.04)	0.028*

CFI and insulin are standardized (mean centered and scaled by standard deviation) to facilitation coefficient interpretation.
Standard errors and p-values estimated using Huber-White robust standard errors.

Discussion

[0223] All living organisms possess biochemical processes that convert chemical substrates into energy necessary to sustain life. The inability to maintain homeostatic metabolic processes and concurrent susceptibility to metabolic stressors has been shown to be a primary characteristic of many human and canine pathologies, such as obesity, type II diabetes, sarcopenia, cachexia, cancer, and chronological age. Insulin has been described as having an associative relationship, and in some cases a causal relationship, with certain aging-related diseases.

[0224] The physiological role of insulin is maintaining glycemic homeostasis by facilitating glucose uptake, blunting endogenous glucose production and inhibiting lipolysis. Chronically elevated insulin levels lead to a condition known as hyperinsulinemia, which can lead to

peripheral insulin resistance in the skeletal muscle, liver, and adipose tissue, and prelude conditions like metabolic syndrome and type II diabetes. In human health, the homeostatic model for insulin resistance (HOMA-IR), which represents a calculated parameter based on fasted insulin and glucose levels, is a useful tool for diagnosing insulin resistance and pre-diabetes.

[0225] *Relationships of age, BCS, and weight on serum insulin levels.* Fasted serum insulin levels showed the opposite relationship and were observed to increase with advancing chronological age ($p < 0.001$). These findings are consistent with a previous large scale study in clinically healthy dogs and further support the role for insulin and its relevance to physiological aging. Additionally, higher BCS scores were associated with higher serum insulin levels ($p < 0.001$). No interaction effects were observed between age*BCS nor age*weight in explaining serum insulin levels, indicating that although BCS does positively associate with serum insulin, the effect of age on insulin is not modulated by BCS.

[0226] These data suggest that age is a predictor of fasted insulin levels, irrespective of both BCS and weight. Furthermore, these data validate cross-sectional investigations in the canine literature which suggest that aged dogs experience hyperinsulinemia. Given the independent association with age, these data and previous investigations suggest that fasted insulin levels have utility as a potential biomarker for aging in dogs.

[0227] *Relationships between fasted insulin and healthspan outcome measures: HRQL.* Fasted insulin was inversely correlated with HRQL total ($p < 0.001$) and the HRQL domains E/E, H/C, A/C ($p < 0.001$). When accounting for age, BCS and weight, a significant inverse association with HRQL total ($p < 0.001$) and the domains E/E and A/C ($p < 0.001$; $p = 0.023$) was still present. Additionally, an interaction between age and insulin levels was associated with the HRQL domain score Active/Comfortable (A/C), where the inverse relationship between insulin and A/C scores in *old dogs* was found to be over two times as strong as in *young dogs*. This suggests that insulin levels may be more predictive of a dog's physical activity and comfort as age increases and speak to the importance of metabolic health in influencing physical activity, function, and wellbeing. Interestingly, the finding that the relationship between fasted serum insulin and an owner assessed outcome measure for health-related quality of life parallels human studies where individuals with higher insulin resistance report poorer HRQL.

[0228] To further aid interpretation of the negative relationship between fasted insulin and HRQL, insulin tertiles as categorical groups were compared. By comparing low, medium, and high insulin groups, the data revealed that higher insulin levels were associated with a decline in HRQL total score of 2-3 points. Based on an internal analysis from a large-scale source of HRQL

data from several independent studies, this change in score is roughly equivalent to the magnitude of decline in HRQL over 3 years of aging. Therefore, changes in fasted insulin levels are reflective of clinically meaningful changes in the dog.

[0229] Together these data demonstrate that like humans, dogs show a relationship between hyperinsulinemia and accelerated aging.⁵¹ For the first time in dogs, these data position fasted insulin levels as a potential predictor of health-related quality of life, an outcome measure that is broadly accessible for usage in clinical practice.

[0230] **Conclusion.**

[0231] Fasted insulin concentrations in this population of clinically stable, companion dogs were significantly associated with age, independent of weight and BCS. Insulin concentrations increased with age.

[0232] Insulin remained significantly associated with HRQL total and HRQL sub domains (E/E, H/C, A/C) independent of age, body weight, and BCS. Increased insulin concentrations corresponded with decreased health-related quality of life. Taken together, these data strongly support the use of insulin as a marker of aging in dogs, and identify serum insulin as a valuable, standalone indicator of metabolic health and HRQL in dogs regardless of age, size, and body condition.

Example 5. Health-Related Quality of Life declines with age

[0233] 1308 data points are provided from dogs with longitudinal scores from within unpublished VetMetrica datasets. 159 dogs are greater than 10 years of age for which there is longitudinal data (**FIG. 16**). Over a short time scale, these dogs show a variety of HRQL trajectories. It is noted that within these longitudinal data, the HRQL scores still decline with age on the whole (**FIG. 16**, bottom panel).

[0234] A linear mixed effects model is fit to the empirical meta-analysis data for dogs 10 years of age and older in this study. Covariates for age, sex, and gonadectomized status are included (**Table 15**, model 1) as well as the rough weight class in model 2. This model helps anchor the assumptions about the magnitude of a meaningful difference in HRQL, described as a 6-12 month delay in decline in quality of life. The data are transformed such that sex is 0 for Male dogs and 1 for Female dogs, gonadectomized is 0 for intact dogs and 1 for those spayed or neutered, respectively. Size classification in model 2 (**Table 15**) is based on grouping rough AKC breed sizes together with weight criteria in the Healthspan study. Dogs indicated as Small or Medium in Rodger (Rodger 2021) are considered to have a small size, corresponding to 0, and

dogs indicated as Large or Extra Large are considered to be of large size, corresponding to 1. HRQL total scores normalized via the Davies transformation are the dependent variable for interest. Random effects capture within-dog correlation in score. The coefficients found through these linear mixed effects models are presented in Table 3 below. The coefficient for age in model 2, 1.04 units on the HRQL Davies scale, corresponds to the difference in HRQL total scores that is believed to be most representative of a one-year delay in the decline in quality of life scores.

Table 15. Coefficients recovered from linear mixed effects models with covariates for Age, Sex, Gonadectomized status and random effects by subject. Random effects are fit by individual dogs.

Model 1: Age, Sex, and Gonadectomized			Model 2: Addition of small/large size class		
	Coef (SE)	P-value		Coef (SE)	P-value
(Intercept)	54.7 (1.68)	<0.001	(Intercept)	55.6 (1.72)	<0.001
Age (yrs)	-1.00 (0.133)	<0.001	Age (yrs)	-1.04 (0.134)	<0.001
Sex	0.153 (0.470)	0.744	Large Size	-1.15 (0.486)	0.018
Gonadectomy	1.052 (0.521)	0.043	Sex	0.176 (0.469)	0.708
Group Var	26.51 (0.779)		Gonadectomy	1.13 (0.521)	0.030
			Group Var	26.31 (0.774)	

[0235] While preferred embodiments of the present invention have been shown and described herein, it will be obvious to those skilled in the art that such embodiments are provided by way of example only. Numerous variations, changes, and substitutions will now occur to those skilled in the art without departing from the invention. It should be understood that various alternatives to the embodiments of the invention described herein may be employed in practicing the invention. It is intended that the following claims define the scope of the invention and that methods and structures within the scope of these claims and their equivalents be covered thereby.

CLAIMS

What is claimed is:

1. A method for promoting health or treating metabolic dysfunction or risk or presence of an age-associated disease in a subject, the method comprising:
administering a nutraceutical composition or a pharmaceutical composition to a subject, wherein the administering is based on an assessment of metabolic dysfunction or risk or presence of an age-associated disease in the subject, and wherein the assessment comprises:
 - (a) measuring a fatty acid level within the subject using a high-fidelity fatty acid assay, or measuring an insulin level and/or insulin sensitivity within the subject using a high-fidelity insulin assay; and
 - (b) evaluating the subject's quality of life, frailty, or multimorbidity.
2. A method for promoting health or treating metabolic dysfunction or risk or presence of an age-associated disease in a subject, the method comprising:
administering a nutraceutical composition or a pharmaceutical composition to a subject, wherein the administering is based on an assessment of metabolic dysfunction or risk or presence of an age-associated disease in the subject, and wherein the assessment comprises:
 - (a) measuring a fatty acid level within the subject using a high-fidelity fatty acid assay, or measuring an insulin level and/or insulin sensitivity within the subject using a high-fidelity insulin assay; or
 - (b) evaluating the subject's quality of life, frailty, or multimorbidity.
3. A method for increasing lifespan, the method comprising treating metabolic dysfunction or risk or presence of an age-associated disease in a subject, the method comprising:
administering a nutraceutical composition or a pharmaceutical composition to a subject, wherein the administering is based on an assessment of metabolic dysfunction or risk or presence of an age-associated disease in the subject, and wherein the assessment comprises:
 - (a) measuring a fatty acid level within the subject using a high-fidelity fatty acid assay, or measuring an insulin level and/or insulin sensitivity within the subject using a high-fidelity insulin assay; and/or
 - (b) evaluating the subject's quality of life, frailty, or multimorbidity.

4. A method for detecting and/or treating metabolic dysfunction or an age-associated disease in a subject, the method comprising:

(a) measuring a fatty acid level within the subject using a high-fidelity fatty acid assay, or measuring an insulin level and/or insulin sensitivity within the subject using a high-fidelity insulin assay; and/or

(b) evaluating the subject's quality of life, frailty, or multimorbidity, thereby detecting the metabolic dysfunction or risk or presence of an age-associated disease.

5. The method of any one of claims 1 to 4, comprising measuring the fatty acid level within the subject and measuring the insulin level within the subject.

6. The method of any one of claims 1 to 5, wherein the measuring of the fatty acid level and/or the serum insulin level, and the evaluating of the quality of life, frailty, or multimorbidity provide a correlation for detecting the metabolic dysfunction or risk or presence of an age-associated disease.

7. A method for identifying a subject in need of treatment for metabolic dysfunction or risk or presence of an age-associated disease, the method comprising:

(a) measuring a fatty acid level within the subject using a high-fidelity fatty acid assay, or measuring an insulin level and/or insulin sensitivity within the subject using a high-fidelity insulin assay; and/or

(b) evaluating the subject's quality of life, frailty, or multimorbidity, thereby identifying a subject in need of treatment for the age-associated disease.

8. The method of claim 7, comprising measuring the fatty acid level within the subject and measuring the insulin level within the subject.

9. The method of claim 7 or 8, wherein the measuring of the fatty acid level and/or the insulin level, and the evaluating of the quality of life provide a correlation for detecting the metabolic dysfunction or risk or presence of an age-associated disease.

10. The method of any one of claims 1 to 9, wherein the quality of life, frailty, or multimorbidity is measured by Health-Related Quality of Life (HRQL) or Canine Frailty Index (CFI).

11. A method of health screening for a subject, comprising:
measuring a fatty acid level within the subject, wherein the fatty acid level is measured using a high-fidelity fatty acid assay, or
measuring an insulin level within the subject, wherein the insulin level is measured using a high-fidelity insulin assay,

wherein the subject is a companion mammal.

12. The method of claim 11, further comprising evaluating the subject's quality of life, wherein the quality of life is measured by Health-Related Quality of Life (HRQL) or the subject's frailty or multimorbidity using the Canine Frailty Index (CFI).

13. The method of claim 11 or 12, wherein the subject has metabolic dysfunction or risk or presence of an age-associated disease.

14. The method of any one of claims 1 to 13, further comprising comparing the fatty acid level within the subject with a pre-determined fatty acid level, or comparing the insulin level within the subject with a pre-determined insulin level.

15. The method of any one of claims 3 to 14, further comprising promoting or maintaining health of the subject by administering a nutraceutical composition to the subject.

16. The method of claim 15, wherein the nutraceutical composition comprises quercetin, azelaoyl phosphatidylcholine, or 4-oxodocosahexaenoic acid.

17. The method of any one of claims 3 to 9 or 13 or 14, further comprising treating the metabolic dysfunction or risk or presence of the age-associated disease in the subject by administering a pharmaceutical composition to the subject.

18. The method of claim 17, wherein the pharmaceutical composition comprises rosiglitazone, lobeglitazone, or a combination thereof.

19. The method of any one of claims 1 to 18, wherein the high-fidelity fatty acid assay is a gas chromatography-mass spectrometry (GCMS) assay, a ultra performance liquid chromatography (UPLC) assay, or a liquid chromatography mass spectrometry (LCMS) assay.

20. The method of claim 19, wherein the high-fidelity fatty acid assay is the GCMS assay.

21. The method of claim 19, wherein the high-fidelity fatty acid assay is the UPLC assay.

22. The method of claim 19, wherein the high-fidelity fatty acid assay is the LCMS assay.

23. The method of any one of claims 1 to 22, wherein the high-fidelity insulin assay is an enzyme-linked immunoassay (ELISA).

24. The method of any one of claims 1 to 23, wherein a sample for the high-fidelity insulin assay is obtained using an oral glucose tolerance test (OGTT).

25. The method of any one of claims 1 to 24, wherein the metabolic dysfunction or risk or presence of an age-associated disease is related to adipose dysfunction.

26. The method of any one of claims 1 to 9 or 13 to 25, wherein the risk or presence of the age-associated disease is cancer, sarcopenia, cardiovascular disease, obesity, diabetes, cognitive dysfunction, cancer/neoplasia, liver disease, renal disease, degenerative orthopedic disease, immunosuppressive disease, hyperlipidemia, metabolic dysfunction/disease, hepatic lipidosis, metabolic syndrome, pancreatitis, hypertension or a combination thereof.

27. The method of any one of claims 1 to 26, wherein the insulin level is measured from serum insulin level or plasma insulin level.

28. The method of any one of claims 1 to 27, wherein the fatty acid level is the fatty acid concentration.

29. The method of any one of claims 1 to 28, wherein the fatty acid level is evaluated by a serum fatty acid.

30. The method of claim 29, wherein the serum fatty acid is free fatty acids (FFA), saturated fatty acids (SFA), palmitic acid (PA), oleic acid (OA), linoleic acid (LA), alpha linoleic acid, gamma linoleic acid, palmitoleic acid, margaric acid, stearic acid, myristic acid, pentadecanoic acid, arachidic acid, behenic acid, lignoceric acid or a combination thereof.

31. The method of claim 30, wherein the serum fatty acid is free fatty acids (FFA), saturated fatty acids (SFA), palmitic acid (PA), oleic acid (OA), or linoleic acid (LA), or a combination thereof.

32. The method of claim 31, wherein the serum fatty acid is palmitic acid (PA) and oleic acid (OA).

33. The method of claim 31, wherein the serum fatty acid is palmitic acid (PA) and linoleic acid (LA).

34. The method of claim 31, wherein the serum fatty acid is oleic acid (OA) and linoleic acid (LA).

35. The method of claim 31, wherein the serum fatty acid is palmitic acid (PA), oleic acid (OA) and linoleic acid (LA).

36. The method of claim 31, wherein the serum fatty acid is free fatty acids (FFA) and saturated fatty acids (SFA).

37. The method of claim 31, wherein the serum fatty acid is free fatty acids (FFA) and palmitic acid (PA).

38. The method of claim 31, wherein the serum fatty acid is free fatty acids (FFA) and oleic acid (OA).

39. The method of claim 31, wherein the serum fatty acid is free fatty acids (FFA) and linoleic acid (LA).
40. The method of claim 31, wherein the serum fatty acid is palmitic acid (PA).
41. The method of claim 31, wherein the serum fatty acid is oleic acid (OA).
42. The method of claim 31, wherein the serum fatty acid is linoleic acid (LA).
43. The method of claim 31, wherein the serum fatty acid is free fatty acids (FFA).
44. The method of claim 31, wherein the serum fatty acid is saturated fatty acids (SFA).
45. The method of any one of claims 40 to 44, further comprising measuring the serum insulin level.
46. The method of any one of claims 29 to 31, wherein the serum fatty acid is evaluated from the subject's blood sample.
47. The method of any one of claims 1 to 46, wherein the subject is fasted.
48. The method of claim 47, wherein the subject fasted for at least 4 hours.
49. The method of claim 47, wherein the subject fasted for at least 6 hours.
50. The method of claim 47, wherein the subject fasted for at least 10 hours.
51. The method of any one of claims 47 to 50, wherein the serum insulin level is evaluated from the subject's blood sample.
52. The method of any one of claims 1 to 9 or 12 to 51, wherein the quality of life is measured by HRQL.
53. The method of any one of claims 1 to 9 or 12 to 51, wherein the frailty or multimorbidity is measured by CFI.
54. The method of any one of claims 1 to 53, wherein the subject is a mammal.
55. The method of claim 54, wherein the mammal is a dog, cat, horse, cow, pig, rabbit, rodent, sheep, non-human primate, or human.
56. The method of claim 55, wherein the mammal is a dog.
57. The method of claim 55, wherein the mammal is a mouse or rat.
58. The method of claim 55, wherein the mammal is a human.
59. The method of any one of claims 1 to 58, wherein an increase of the fatty acid level and increase in CFI indicates the metabolic dysfunction or risk or presence of the age-associated disease.

60. The method of claim 59, wherein the fatty acid level is increased by at least 10% compared to a subject without metabolic dysfunction or risk or presence of the age-associated disease.

61. The method of claim 60, wherein the fatty acid level is increased by at least 20% compared to a subject without metabolic dysfunction or risk or presence of the age-associated disease.

62. The method of any one of claims 1 to 61, wherein an increase of the insulin level and increase in CFI indicates the metabolic dysfunction or risk or presence of the age-associated disease.

63. The method of claim 62, wherein the insulin level is increased by at least 10% compared to a subject without metabolic dysfunction or risk or presence of the age-associated disease.

64. The method of claim 63, wherein the insulin level is increased by at least 20% compared to a subject without metabolic dysfunction or risk or presence of the age-associated disease.