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Mortality and Biomarkers of Aging in Heterogeneous Stock (HS) Mice

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A longitudinal study was undertaken to evaluate the relationships among a battery of aging biomarkers and subsequent survival time in 319 genetically heterogenous stock (HS) mice. The biomarker variables chosen were selected from the broad domains of behavior, homeostatic physiology, oxidative defense, and immune function; biomarkers were measured at 45, 90, 360, 630, and 900 days of age. Sex differences were found in the survivor and mortality functions, with a mortality rate crossover occurring at about 525 days and a survival curve crossover at about 750 days of age. Females experienced lower initial mortality but had more sharply increasing mortality with age than did males. Survival analysis using Gompertz parametric models with biomarkers as time-varying covariates yielded significant biomarkers from each domain. Following backward elimination procedures, the final set of independent mortality predictors included headpokes in the File activity apparatus, maximum cord drop time, weight, hematocrit, urine concentration, natural killer cell activity, and concanavalin A response.

A MAJOR gerontological emphasis in recent years has focused on the identification of biological markers, or biomarkers, of aging. Although variously defined, the term biomarker is generally accepted to refer to a variable that is capable of measuring some dimension of physiological or functional age. As Baker and Sprott (1) noted, a key assumption in biomarker-related research is that there are biological parameters that perform better than chronological age in predicting physiological or functional age. Implicit in this assumption is the acknowledgement that individuals of the same chronological age may vary in their physiological age. Biomarkers of physiological age, therefore, can offer great utility to studies on aging, both in the elucidation of basic and individual processes involved in senescence and in the evaluation of the effects of interventions. The theoretical and methodological issues central to biomarker research have been reviewed by previous authors, including Reff and Schneider (2), Baker and Sprott (1), Ingram (3), and McClearn (4–6).

A number of necessary attributes for biomarkers of aging have been discussed (3,6–8). Several key expectations are that biomarkers should (*i*) change with time in a manner that reflects the rate of physiological aging, (*ii*) be capable of differentiating age-related change from disease processes, (*iii*) demonstrate adequate reliability of measurement, and (*iv*) be nonlethal and able to be measured noninvasively, with minimal trauma to animal or human subjects.

In contrast to the general consensus regarding many requirements of biomarkers and biomarker-related research, the expectations regarding relationships between biomarkers of aging and longevity are less clear. In an early discussion, Brown and Forbes (9) suggested two important conditions for biomarker validity: biomarkers should reflect the probability of death at a given chronological age, and individuals who have died should have abnormal levels relative to living individuals of the same chronological age (9,10).

The expectation that biomarkers should predict longevity is reasonable in terms of classic theories of aging that relate declining physiological functioning, or vitality, to aging and death (11–13). In these models, population mortality rates are expressed as joint functions of external challenges, which vary in their severity, and declines in vitality, which may be conceptualized as energy required to overcome the environmental challenges. When individuals encounter challenges that exceed their vitality, death occurs. It has been demonstrated that Gompertz rates of mortality can result from such theoretical functions of vitality and external challenges (11,13).

To assume that, because aging ultimately leads to death, biomarkers of aging should predict age at death may be simplistic, however. Age at death may be confounded with disease or external factors that are largely independent of age-related effects (1). In addition, although vitality is theoretically conceptualized as monotonically declining, it is likely that individuals fluctuate in response to disease or stressors. Despite an overall declining trend in vitality, local excursions downward may be followed by compensatory increases in vitality due to recovery or adaptation to stress (6). If an individual is sufficiently distant from some lethal threshold, then recovery is probable, but if close to the threshold, compensatory recovery may be impossible. Under this conceptual framework, the expectation of age at death may be obtained from the mean trajectory in vitality loss, but there are a number of possible death dates depending on individual fluctuation (6). Thus, although correlation with longevity is a desirable characteristic for a biomarker, the timing of death may be relatively imprecise in relation to physiological age.

Aside from these conceptual issues, studies relating biomarkers to survival are also subject to practical limitations in measuring the effects of biomarkers on longevity. There is little question that methods appropriate to the

analysis of survival data, such as those incorporating parametric (e.g., Gompertz) or semiparametric (e.g., Cox) models should be used to evaluate the effects of biomarker levels within the context of age-associated increases in mortality rates. Other questions remain, however, including: (*i*) Will the effects of biomarkers be assessed independently or in the aggregate? (*ii*) When will biomarkers be measured? (*iii*) Will biomarkers be treated as time-constant or time-varying covariates? (*iv*) Is a given level of a biomarker assumed to have the same impact on mortality risk regardless of age? In considering these questions it becomes clear that, for any aging study, there are a number of alternative approaches that may be undertaken to investigate relationships between biomarkers and mortality.

Although several major human epidemiological studies have examined the associations among physiological variables and longevity (14,15), relatively few studies using animal models have examined relationships among multiple physiological or behavioral biomarkers of aging with subsequent longevity in genetically heterogeneous animals. Most animal studies to date linking biomarker values with mortality have involved (*i*) inbred or selected strains, such as the senescence-accelerated mouse strain SAM (16,17); (*ii*) associations of specific genetic markers or single traits (e.g., coat color, histocompatibility haplotype, body weight) with longevity (18–23); or (*iii*) the evaluation of specific aging interventions, most notably dietary restriction (24–26).

The primary goal of the present study was to evaluate the relationships among a battery of putative aging-related biomarkers and survival time in a sample of genetically heterogenous mice. To do so, we evaluated the independent effects of each biomarker and identified the most parsimonious set of candidate biomarkers that best predict survival. Because data on biomarker levels were available from five repeated occasions of testing, updated biomarker values were used as time-varying covariates in our survival models. Finally, within the constraints of the methods used in the present study, a given level of a biomarker is assumed to have a constant effect on mortality risk regardless of the age of measurement. For example, a specific hematocrit level is assumed to confer the same degree of risk or protection regardless of the age of the subject. These caveats and assumptions should be kept in mind throughout the following discussion.

Methods

Animals

The house mouse, *Mus musculus*, has proven useful as an animal model for many aging processes (27), and a number of candidate biomarkers have been proposed based on studies of inbred mice (28,29). Because of their genetic homogeneity, inbred mouse strains have proven invaluable in gerontological research by providing a solid, replicable basis for animal models of aging (4). However, designs employing only genetically identical animals suffer from limitations in assessing relationships among variables because all variance is environmental in origin (30,31). An alternative approach is to use genetically heterogeneous mice, such as the heterogeneous stock (HS) (30) which allows evaluation of genetic as well as environmental covariation in the mediation of relationships among biomarker variables. The present study was conducted as part of a larger investigation of biomarkers of aging in two generations of HS mice (32).

All mice were of the genetically heterogeneous HS strain, which was originally established by intercrossing eight inbred strains (30). The HS stock has been systematically maintained by random mating, with approximately 40 mating pairs in each generation. For the current study, breeding pairs were imported from the Institute for Behavioral Genetics (IBG) in Boulder, CO, to a specific pathogen-free (SPF) barrier facility at The Pennsylvania State University. The breeding generation was introduced to the barrier facility by caesarean-derivation and cross-fostering onto gnotobiotic Swiss females. Subsequently, these mice were mated by using the same systematically random procedures used in the maintenance colony at IBG (random matings with the restriction that cousin matings are avoided), yielding two generations for study—a parental generation and a second offspring generation. All mice were housed individually (except during breeding) in the barrier facility with a 12-h light/dark cycle. Food (NIH 31, open formula autoclavable mouse ration) and water were provided ad libitum; cages were changed weekly.

Study Design and Biomarker Measures

The design for this study was longitudinal, with five testing occasions at 45, 90, 360, 630, and 900 days of age. Biomarker variables were drawn from several broad domains: behavior, homeostatic physiology, oxidative defense, and immune functioning. Biomarkers were chosen on the basis of their ability to be measured nonlethally and noninvasively and on the basis of associations with age and physiological functioning as demonstrated in previous work done in our laboratory and by others (2,4,33). The biomarker variables included in the test battery are described in Table 1. Most biomarkers were measured at all five occasions. Exceptions include tail tendon fiber break time (LMAXTT), which was measured at occasions at 90, 360, 630, and 900 days, and the arachidonic acid metabolites thromboxane B2 (LTXB) and 6-keto-PGF1-α (LKETO), which were measured at 45, 90, 360, and 630 days. Variables showing significant deviation from normality were log-transformed prior to statistical analysis. Details regarding the study design and measures have been provided elsewhere (32).

Mice were checked daily for morbidity or death. Moribund animals were evaluated by appearance, posture, activity, behavior, and weight loss. All dead animals were necropsied, and pathology reports were prepared that evaluated cause of death where possible. Age at death was recorded in days for all animals.

Analyses

Nonparametric survival analyses.—Life tables and survival curves were constructed using the SAS LIFEREG procedure. Kaplan–Meier survival probability estimates were computed separately for male and female mice. Equality of survival curves for males and females was tested with log-

Table 1. Biomarker Variables Used in Survival Analysis

Biomarker Variable	Description of Measure
Behavior	
File Activity (FACTIV)	Number of squares entered in File exploratory apparatus in 3 min
File Rears (LFREARS)*	Number of rearings (vertical movements) in File exploratory apparatus in 3 min
File Headpokes (FHDPK)	Number of headpokes through holes in floor of File exploratory apparatus in 3 min
File Boli (FBOLI)	Number of boli deposited in File apparatus during 3-min period
File Urine (FDURN)	Dichotomous measure of urination in File apparatus during 3-min period; 0 = no urine; 1 = any urine
Cord Drop Time (MAXCDT)	Amount of time mouse successfully hangs by forepaws on a tight wire; maximum score from three 60-sec trials
Homeostatic Physiology	
Body weight (WT)	Body weight in grams
Tail tendon fiber break time (LMAXTT)*	Time for tail tendon fibers to break with 2-g weight attached while suspended in 7 M urea at pH 7.5 and 45°C; maximum of 4 fibers
Glucose 0 (GLUC0)	Baseline plasma glucose level expressed in mg/dl following a 12-h fast
Glucose 40 (RGLUC40	Change in plasma glucose level (mg/dl) 40 min after an intraperitoneal injection of 20 mg percent solution of glucose (2 g/kg body weight); regression residual score from baseline
Hematocrit 0 (HEMA0)	Percent of red blood cells in a blood sample following a 12-h fast
Hematocrit 40 (RHEMA40)	Percent of red blood cells 40 min after the glucose injection (for Glucose 40); regression residual score from baseline
Urine 0 (URINE0)	Baseline urine osmolality expressed in mOsml/kg
Urine 24 (RURIN24)	Urine osmolality following a 24 h period of water deprivation expressed in mOsml/kg (a measure of urine concentrating ability); regression residual score from baseline
Immune System	
Concanavalin A (LCONA)*	Proliferative response of lymphocytes to the mitogen Con A, expressed in counts/min
Conjugates (CONJ)	Percent of lymphocytes attached to target cells (tumor cell line YAC-1)
Dead Targets (DTAR)	Percentage of target cells (tumor cells) identified as dead by trypan blue exclusion test
Lymphocytes (LYMPHS)	Percentage of lymphocytes in whole blood
White Blood Cells (WBC)	White blood cell (leukocyte) count in whole blood, expressed as total cells/μL
Natural Killer 25:1 (LNK25)*	Natural killer cell activity against a tumor target, ratio of 25 lymphocytes to 1 target
Oxidative Defense	
Glutathione Peroxidase (GSHPX)	Selenium-dependent glutathione peroxidase levels in blood expressed as (micromoles H_2O_2 consumed/min)/mg hemoglobin
Thromboxane (LTXB)*	Plasma thromboxane B2 levels, expressed as pg/ml plasma
Prostacycline (LKETO)*	Plasma prostacycline metabolite: 6-keto-PGF1-α (levels expressed as pg/ml plasma)
Thromboxane:Prostacycline Ratio (TKRAT)	Ratio of TXB:6-keto-PGF1-α

*Log-transformed.

rank and Wilcoxon statistics. Hazard functions for males and females were also estimated using Kaplan–Meier methods and subjected to kernel-smoothing (34) using a SAS macro written by Paul Allison (35).

For the survival analyses, mice were characterized as either censored or noncensored. Censored animals were those who experienced accidental, testing-related, or reproductive-related deaths. The procedures used for the survival analyses enabled the use of information for censored animals up until the time they experienced death.

Parametric survival models.—Although the relationship between biomarker variables and survival may be addressed by using either parametric or semiparametric (36) survival models, parametric methods were chosen to allow examination of the time dependence of mortality in the study population. The Gompertz distribution, which has been the most widely used model in mortality studies, describes mortality as an exponentially increasing function of time,

$$\mu(t) = \alpha e^{\theta t},$$

in which $\mu(t)$ is the mortality rate at time t, α is the initial mortality rate (e.g., mortality rate at reproductive maturity), and θ is the Gompertz exponent, which describes the rate of acceleration of mortality with age. Many studies have demonstrated the failure of the Gompertz to describe mortality at very late ages, where mortality rates cease to increase exponentially or become constant (37–39); these failures of the Gompertz have been most often attributed to genetic heterogeneity (40,41). Despite these disadvantages, the Gompertz distribution offers two key advantages: it fits mortality, particularly all-cause mortality, well at most ages

for many populations, and its parameters have been well characterized theoretically in terms of thermodynamic descriptions of aging (11,13,14,42,43).

To examine the mortality kinetics of the study population, graphical, parameter-free methods were initially used to visually inspect the correspondence between survival or mortality functions and several parametric distributions, including exponential, Weibull, Gompertz, and log-logistic functions (44). Exponential and Gompertz parametric survival models were tested by using maximum-likelihood model fitting with the BMDP Statistical Software program P3R for nonlinear regression, in conjunction with the Fortran subroutine program P3RFUN written by Trond Petersen (44; T. Petersen, PhD, personal communication, November 1996). The fits of parametric models were evaluated using log-likelihood criteria.

Time-varying covariates.—Because the longitudinal design included five test occasions throughout the life span, the biomarker variables were used as time-varying covariates in the survival analysis. Problems may arise when event times (deaths) are measured more accurately than time-varying covariates are measured, as in the present case, in which age at death was measured in precise days, whereas biomarker levels were only assessed at regular intervals. Allison (35) and others (44,45) have discussed various strategies to handle such data. For our analyses, we chose to allow the biomarker covariates to vary over time as step functions, in which levels at each testing occasion were assumed to remain as such throughout the entire interval until the next test occasion. Because testing took place over several weeks and some mice either became moribund or died prior to completing all testing, deaths around the testing times could confound the analysis, resulting in the comparison of some mice's prior test data with other mice's current values. To avoid this problem, the covariate values were lagged by 21 days. For example, covariate values at 45 days were used to predict deaths up through 110 days, 90-day data were used for times between 111 and 380 days, etc. To facilitate the analysis of the biomarkers as time-varying covariates, a mouse-occasion, or subepisode, file was constructed that contained 1150 mouse-occasion records and included test occasion, interval start and end times, censoring (e.g., accidental death) information, and biomarker values (44,46). In this manner the file consisted of subepisodes encompassing biomarker measurements from one to five separate occasions of measurement over each mouse's lifespan.

Missing data procedures.—Even with lagged covariates, missing data values were observed for 2.6% of all possible values. Missing data analysis was conducted, and no consistent patterns were observed regarding the nature of variables with missing data or for the specific mice with missing data. Missing data were most often due to nonmeasurement of specific mice or occasional problems with the sample or assay procedures. We concluded that the missing data showed the pattern of being missing completely at random (MCAR) (47). Because the survival analytic procedures used require complete cases, severe sample size reduction and loss of power occurs with the deletion of all cases with any missing data, even though the total number of missing data points was small. Various mechanisms for adjusting the missing values have been proposed, some of which have been shown to result in severe bias (48). Maximum-likelihood procedures, such as the Expectation-Maximization (EM) algorithm (47), result in less bias than traditional procedures (48). The EMCOV procedure (49) is an iterative imputation procedure in which missing values are imputed by using all other variables and standard errors are obtained by bootstrapping. To accommodate the present study design, we imputed data within each occasion for mice completing the occasion, using the closest prior occasion as additional predictors.

Model specification.—Time-varying biomarker covariates were included in Gompertz models by relating the α parameter log-linearly with the covariate vector so that

$$\mu(t) = \exp(x'k\beta) \bullet \exp(\theta t).$$

The constant and time-dependent nonlinear regression coefficients obtained from the BMDP3R procedure and subroutine program correspond to $\ln(\alpha)$ and θ, respectively. Exponentiating the constant coefficient yields α, whereas θ is estimated directly.

All parameter estimates in the survival analysis were derived using maximum likelihood, a general method of finding estimators that maximize the probability, or likelihood, of the observed sample data. The total sample likelihood, L, is obtained as the product of individual probabilities for each observation as defined by the probability density function and survivor function for the specific parametric distribution used. Iterative procedures are then used to identify parameter values maximizing the likelihood or its log. Higher or less negative values of the log-likelihood correspond to better fits. The log-likelihood and associated likelihood ratio statistic can be used to assess goodness of fit and to compare nested models. For comparison of nested models, twice the difference between log-likelihoods is distributed as a χ^2 statistic. For a more detailed discussion of maximum likelihood estimation procedures applied to survival data, see Kalbfleisch and Prentice (50), Blossfeld et al. (44), or Allison (35).

Results

Table 2 presents mean survival and selected percentile levels of age at death for censored and noncensored mice by sex. For noncensored mice, variance for age at death was much greater in males than females ($F = 2.24$, df = 137, 131, $p < .0001$). The mean survival times for male and female noncensored mice were not significantly different ($t = .109$, $p = .91$).

The survival curves for the male and female mice in this study are shown in Figure 1. Some differences in the two survival curves are apparent. Male mice experienced greater mortality than female mice at younger ages but appeared to have enhanced survival at advanced ages. The two survival curves cross over at about 750 days of age, close to the median age at death for both sexes. The two curves were compared using the log-rank test and found to

Table 2. Mean and Percentile Levels of Survival Time (in Days) of HS Mice

	Male	Female	Total
Noncensored			
N	138	132	270
Mean	769.3	772.5	770.9
Standard deviation	283.6	189.6	241.8
Percentiles			
10%	375	552	461
25%	565	651	618
50%	781	770	775
75%	995	892	909
90%	1138	1021	1083
Maximum life span	1330	1278	1330
Censored*			
N	22	27	49
Mean	543.7	368.3	447.1
Standard deviation	333.2	279.0	313.8
Percentiles			
10%	101	75	75
25%	162	160	162
50%	641	184	394
75%	918	647	654
90%	919	660	918
Maximum life span	924	918	924

*Accidental, test-related, and reproductive-related deaths.

be significantly different (χ^2 = 4.64, 1 df, p = .031). The log-rank test emphasizes factors in the tail of the survival curve where equal weight is given to each failure time (51).

The significant difference in survival curves for males and females suggests a violation of proportionality. In proportional-hazards models, an important assumption is that individuals experience proportional hazards over time. Although males or females may experience higher hazard rates as a function of sex, resulting in a ratio of hazards for males and females, this ratio should remain proportional (i.e., it should not change over time). We tested the proportionality assumption for males and females using Cox proportional hazards regression by including an interaction term for sex with time; its significance verified the nonproportionality suggested by the survival curves. Stratified survival models may be used with methods such as Cox proportional hazards regression. Such methods, which allow the hazards across strata to be nonproportional but constrain parameter estimates of other covariates to be equal across the strata, may not be appropriate in the context of aging if some biomarkers have different meanings in males and females. For our parametric survival models, we tested models for males and females separately, although sexes were pooled for some analyses, with sex included as a covariate.

Figure 2 presents the hazard or mortality functions for

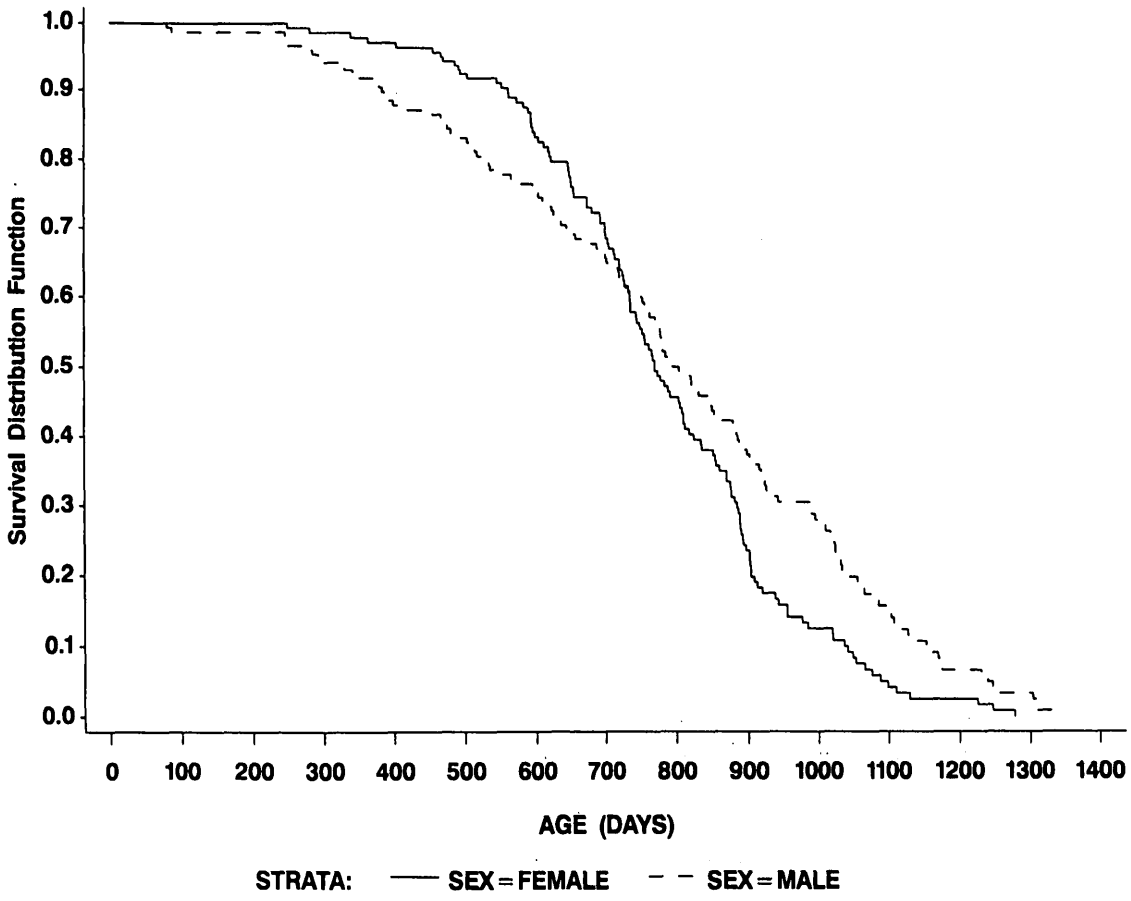

Figure 1. Survival curves for noncensored male (dashed line) and female (solid line) mice, showing the percent surviving at time points measured in days.

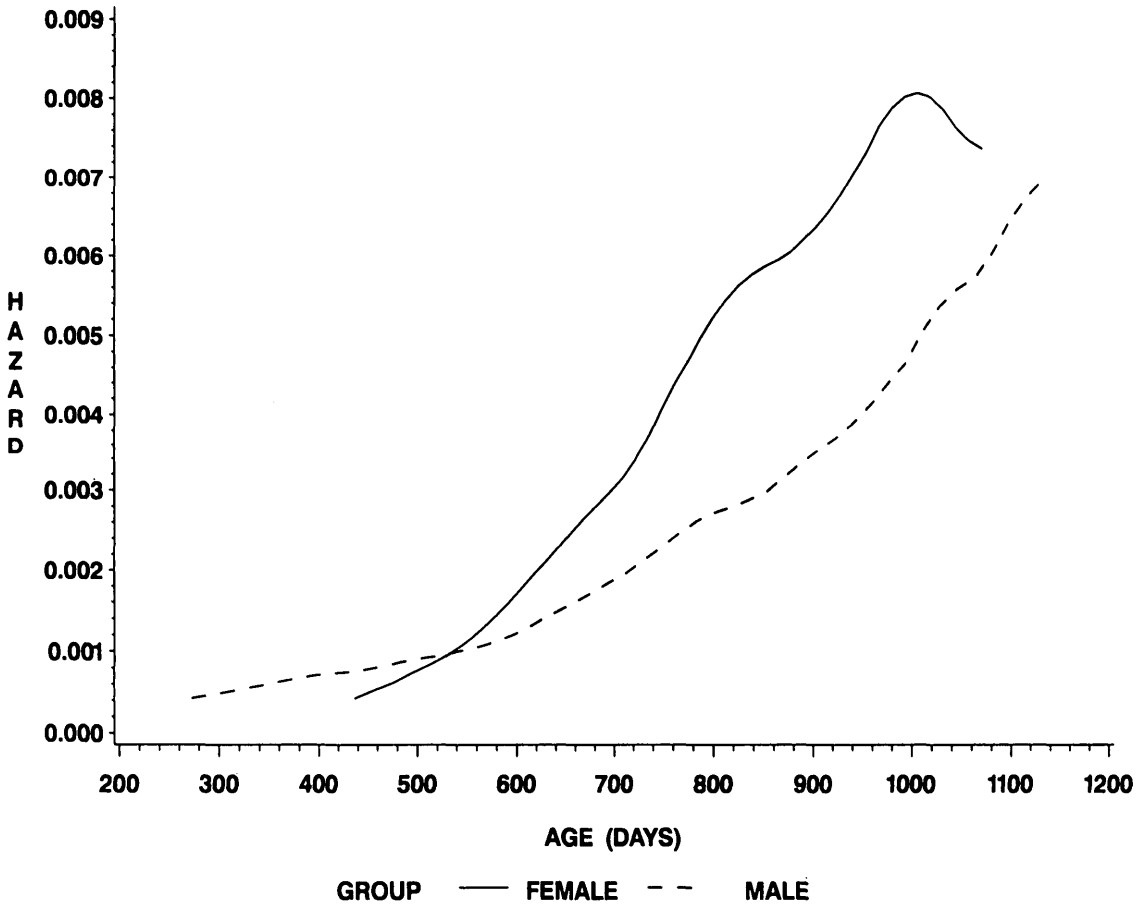

Figure 2. Smoothed hazard (mortality) functions for male (dashed line) and female (solid line) mice, with time measured in days.

male and female mice following kernel smoothing (35). The mortality crossover for males and females occurs at around 525 days of age, approximately 225 days prior to survival curve convergence, in keeping with mortality kinetic studies (52). It is clear from Figure 2 that females, although experiencing low early mortality, have more sharply increasing mortality than males. Although the kernel smoothing makes it difficult to interpret the hazard shape at late ages, it suggests that, at least for females, mortality acceleration declines very late in life, as many other studies have found.

Table 3 presents log-likelihood statistics and model parameters for exponential and Gompertz models with and without biomarker covariates, for sexes separately and together. Not surprisingly, Gompertz models fit significantly better than exponential models: for all groups, Gompertz models had less negative log-likelihood values and likelihood ratio χ^2 tests (twice the difference in log-likelihoods between Gompertz and exponential models) were very highly significant. For Gompertz models without biomarker covariates, the Gompertz initial mortality constant α was estimated as .00014 for males and .00006 for females. Initial estimates of θ, in Gompertz models without covariates, were .00360 for males and .00520 for females. The Gompertz parameters estimated without covariates confirm that males may experience higher initial mortality rates, but females experience greater age acceleration in mortality.

The addition of the initial set of 12 biomarker covariates found significant in univariate models resulted in a significant decrease in the log-likelihood function, relative to Gompertz models without biomarkers (likelihood-ratio χ^2 = 23.04, 12 df, p = .027 for males; χ^2 = 38.10, 12 df, p = .0002 for females). Final Gompertz models with reduced sets of biomarker covariates (following stepwise procedures) did not produce significantly worse fits than the initial Gompertz biomarker models (χ^2 = 6.63, 8 df, p = .577 for males; χ^2 = 3.05, 6 df, p = .803 for females).

The parameter estimates and significance levels for each biomarker tested singly in univariate Gompertz models are presented in Table 4. Estimates for males and females separately, as well as for sexes pooled using sex as a covariate, are shown. For each biomarker, the sign of the parameter coefficient indicates its direction on the hazard function, not survival. For covariates with p-values less than .10, hazard ratios are also presented that demonstrate the relative difference in hazards associated with an increment of two standard deviations in biomarker level. For females, univariate analyses identified 12 biomarkers as significant predictors of survival time: File activity, File headpokes, maximum cord drop time, body weight, baseline hematocrit, baseline urine concentration, linear and quadratic

Table 3. Model Statistics for Survival Models

Model	Male (n = 581) Log-Likelihood	Male (n = 581) Model Parameters	Female (n = 569) Log-Likelihood	Female (n = 569) Model Parameters	Total (n = 1150) Log-Likelihood	Total (n = 1150) Model Parameters
Exponential, no covariates (1 parameter)	L = −1060.58	λ = .0012	L = −1012.78	λ = .0013	L = −2073.37	λ = .0013
Gompertz, no covariates (2 parameters)	L = −981.83	α = .00014 θ = .00360	L = −893.20	α = .00006 θ = .00520	L = −1884.59	α = .00010 θ = .00417
χ^2, relative to exponential	χ^2 = 157.51, 1 df, p < .0001		χ^2 = 239.16, 1 df, p < .0001		χ^2 = 377.55, 1 df, p < .0001	
Gompertz, sex as covariate (3 parameters)	—	—	—	—	L = −1881.14	α = .00008 θ = .00429
χ^2, relative to exponential	—	—	—	—	χ^2 = 384.46, 2 df, p < .0001	
χ^2, relative to Gompertz without sex	—	—	—	—	χ^2 = 3.46, 1 df, p = .062	
Gompertz, initial multivariate (14–15 parameters)	L = −970.31	α = .00501 θ = .00368	L = −874.15	α = .00063 θ = .00531	L = −1860.85 (15 parameters)	α = .00299 θ = .00388
χ^2, relative to exponential	χ^2 = 180.55, 13 df, p < .0001		χ^2 = 227.26, 13 df, p < .0001		χ^2 = 384.46, 14 df, p < .0001	
χ^2, relative to Gompertz without covariates	χ^2 = 23.04, 12 df, p = .027		χ^2 = 38.10, 12 df, p = .0002		χ^2 = 40.57, 12 df, p < .0001	
Gompertz, final multivariate (6–10 parameters)	L = −973.6 (6 parameters)	α =.00019 θ = .00368	L = −875.68 (8 parameters)	α = .00071 θ = .00531	L = −1862.99 (10 parameters)	α = .00103 θ = .00388
χ^2, relative to exponential	χ^2 = 173.92, 5 df, p < .0001		χ^2 = 274.20, 7 df, p < .0001		χ^2 = 420.76, 9 df, p < .0001	
χ^2, relative to Gompertz with no covariates	χ^2 = 16.41, 4 df, p = .003		χ^2 = 35.04, 6 df, p < .0001		χ^2 = 36.30, 7 df, p < .0001	
χ^2, relative to Gompertz with 12 biomarker covariates	χ^2 = 6.63, 8 df, p = .577		χ^2 = 3.05, 6 df, p = .803		χ^2 = 4.28, 4 df, p = .369	

Note: n = number of mouse-occasion subepisodes.

terms for lymphocytes, concanavalin A (Con A) response, thromboxane levels, thromboxane:prostacycline ratio, and glutathione peroxidase activity. Far fewer variables were univariately significant for males: maximum cord drop time, change in hematocrit after glucose challenge, and natural killer cell activity. The results of the univariate models pooling the sexes largely reflect the results of both the univariate male and female models, with the addition of linear and quadratic effects of white blood cell count as significant predictors.

Somewhat different findings emerged when the initial multivariate model, which included all biomarkers significant in one or more univariate models, was tested. Results are shown in Table 5. For females, File headpokes, maximum cord drop time, weight, baseline urine concentration, and Con A response were significant. For males, maximum cord drop time, change in hematocrit following glucose challenge, natural killer cell activity, and Con A response were significant predictors of survival. In the total sample, weight, both hematocrit measures, baseline urine concentration, natural killer cell activity, and Con A response were associated with survival.

The results of final Gompertz multivariate models, following backward elimination of covariates, are shown in Table 6. The final set of predictors for females included File headpokes, maximum cord drop time, weight, baseline hematocrit, baseline urine concentration, and Con A response. For males, the final set of predictors included only four measures: maximum cord drop time, hematocrit change following glucose challenge, natural killer cell activity, and Con A response. For the pooled sample, File headpokes emerged as significant in the final model, along with weight, both hematocrit measures, baseline urine concentration, natural killer cell activity, and Con A response. As in the univariate case, the sex-combined model reflected some of the significant elements in each of the sex-separate models.

To evaluate the relationship between the biomarker covariates and age in the study population, repeated measures analysis of variance (ANOVA) was carried out using all available subepisode data from occasions two through five, which had equal time intervals between their measurement. Linear trends were tested using F tests. Individual values for each biomarker were also subjected to linear regression on age to obtain standardized regression coefficients to demonstrate the direction of linear change in biomarkers. The results are shown in Table 7. For both males and females, about two-thirds of the variables examined showed significant linear associations with age.

Generalized R^2 statistics were computed for all models from likelihood-ratio statistics (35,53) using the formula

$$R^2 = 1 - \exp\{-G^2/n\},$$

where G^2 is the likelihood-ratio χ^2 statistic for the null hypothesis that all covariates are equal to zero, and n is the number of mice (35). Although the generalized R^2 cannot be strictly interpreted as a proportion of variance accounted for, it has been shown in many cases to be similar in magni-

Table 4. Results of Univariate Gompertz Survival Analyses

Variable	Males		Females		Total	
	Parameter Estimate[a]	Hazard Ratio[b]	Parameter Estimate[a]	Hazard Ratio[b]	Parameter Estimate[a]	Hazard Ratio[b]
Behavior						
FACTIV	−.00086		−.01464‡	.72	−.00496	
LFREARS	.00970		−.07122		−.01838	
FHDPK	−.00123		−.05130*	.50	−.02372‡	.72
FBOLI	.06708		−.03080		.03690	
FDURN	−.17250		.19790		−.11092	
MAXCDT	−.00801‡	.75	.01150**	1.50	−.00005	
Homeostatic physiology						
WT	.00667		.05374*	1.62	.02727*	1.38
LMAXTT	.07151		−.06480		.03567	
HEMA0	−.03203		−.05701**	.64	−.05390***	.65
RHEMA40	−.08251*	.70	.00411		−.05716*	.79
GLUC0	.00065		−.00380		−.00053	
RGLUC40	−.00015		−.00104		−.00002	
URINE0	−.00002		−.00050**	.55	−.00019	
RURINE24	−.00009		.00006		−.00009	
Immune system						
LYMPHS	−.02108		−.06942‡	.17	−.03802*	.38
LYMPHS^2	.00014		.00063‡	7.84	.00030‡	2.60
WBC100	−.01511		−.00566		−.01417*	.45
WBC100^2	.00008		.00004		.00007‡	2.00
CONJ	.00540		−.00560		.00052	
DTAR	.00700		.00698		.00821	
LNK25	.22450*	1.59	.01420		.14709*	1.33
LCONA	−.06240		−.12510**	.68	−.08142**	.78
Oxidative defense						
LTXB	−.02222		−.19570*	.64	−.09783	
LKETO	.06520		−.01080		.02465	
TKRAT	−1.02370		1.38490‡	1.42	−1.08500‡	.77
GSHPX	.12015		1.99380‡	1.20	1.32354	
Sex and model parameters						
FEMALE					.32820**	1.39
CONST(lnα)	−8.88680***		−9.78076***		−9.19462***	
TDEP(θ)	.00360***		.00520***		.00417***	

[a]Significance levels: ‡$p < .10$; *$p < .05$; **$p < .01$; ***$p < .001$.

[b]For variables other than sex, the reported hazard ratios are those associated with two standard deviations of increment in the reported variable.

tude to R^2 values from ordinary least squares (OLS) regression methods (35). The generalized R^2 values for the Gompertz initial multivariate models were estimated as .13 for males and .21 for females. For the final multivariate models, the generalized R^2 estimates were .10 for males and .20 for females. For the total sample, generalized R^2 was estimated as .12 and .10, respectively, for initial and final multivariate models. Similar methods for demonstrating the percentage of variance accounted for are offered by Manton et al. (14), who measured reductions in the Gompertz time-dependent parameter θ following the addition of time-varying covariates. In our models, values of θ did not decrease within sex, but values for the total-sample θ changed from .00429 to .00388, a reduction of approximately 10%. Clearly, addition of the biomarker variables adds significant information to the Gompertz models, but they only explain a portion (10–20%) of age-related mortality.

Discussion

Our results suggest a difference in the survival experience of male and female mice as reflected in the survival curves. Mortality and survivor function crossovers have been the topic of recent discussions (54,55). Although early work on mortality crossovers between human sub-populations assumed that the crossovers were due to data error [see Nam (54) for review], a considerable body of evidence has accumulated relating crossovers to the effects of selective survival and unobserved heterogeneity (41,56). The basic premise of selective effects as an explanation for mortality crossovers is that if one population experiences a harsher environment, it will experience greater early mortality as frailer individuals die, resulting in a hardier population of survivors. Liu and Witten (52) recently demonstrated that, in the presence of a genetically predetermined maximum life span, if one group is advantaged over an-

Table 5. Results of Initial Multivariate Gompertz Survival Analyses

Variable	Males		Females		Total	
	Parameter Estimate[a]	Hazard Ratio[b]	Parameter Estimate[a]	Hazard Ratio[b]	Parameter Estimate[a]	Hazard Ratio[b]
Behavior						
FACTIV						
LFREARS						
FHDPK	.00521		−.04423*	.55	−.02144	
FBOLI						
FDURN						
MAXCDT	−.00895‡	.73	.01567**	1.74	.00071	
Homeostatic physiology						
WT	.00992		.06210*	1.75	.03045*	1.43
LMAXTT						
HEMA0	−.02544		−.03506		−.03371‡	.77
RHEMA40	−.08680‡	.69	−.02700		−.06697*	.75
GLUC0						
RGLUC40						
URINE0	−.00013		−.00035‡	.66	−.00021‡	.78
RURINE24						
Immune system						
LYMPHS			−.05447		−.02928	
LYMPHS^2	.00013		.00056		.00024	
WBC100						
WBC100^2						
CONJ						
DTAR						
LNK25	.28845**	1.82	.01110		.16149*	1.37
LCONA	−.08075‡	.78	−.10346*	.73	−.11661**	.70
Oxidative defense						
LTXB						
LKETO						
TKRAT	−1.27509		−.57530		−.89748	
GSHPX	−.97322		1.34212		.12983	
Sex and model parameters						
FEMALE					.48841***	1.63
CONST(lnα)	−5.29593**		−7.37442***		−5.81347***	
TDEP(θ)	.00368***		.00531***		.00388***	

[a]Significance levels: ‡$p < .10$; *$p < .05$; **$p < .01$; ***$p < .001$.

[b]For variables other than sex, the reported hazard ratios are those associated with two standard deviations of increment in the reported variable.

other group and experiences lower mortality early in life, then the advantaged population must experience mortality rate acceleration later in order for the two groups' survival curves to converge at the maximum life span.

Although other studies have reported sex differences in mortality in inbred strains of mice, very few studies have demonstrated mortality curve crossovers between sexes of any species (41,57). The present study's findings differ somewhat from previous studies in that the mortality and survival function crossovers occur relatively early in life (at around the median age) rather than at very advanced ages (52), and that the longest lived individuals were male rather than female. Using medflies, Carey and Liedo (57) presented the first solid evidence for sex crossovers in nonhuman species, but found that even with very large samples, it was difficult to classify one sex as longer lived due to inconsistent sex differences in several measures of longevity. Their study emphasized the need for large numbers in any study of sex differences in mortality kinetics. The present study's findings should be interpreted with caution due to the limited sample size for the study of mortality kinetics.

With regard to the prediction of mortality by the candidate biomarkers, significant predictors of survival were found from each of the domains represented in this study. From the battery of behavioral measures, greater levels of File activity and headpokes, which may measure curiosity and willingness to explore novel environments, were associated with decreased hazards of death for females. Female mice who had longer cord drop times also experienced lower hazards of death, although males, in univariate analyses, showed the opposite effect. Low cord drop times may be indicative of lesser strength, but, because many mice jump off the cord quickly, may also measure either greater excitability or willingness to act in novel situations.

Table 6. Results of Final Multivariate Gompertz Survival Analyses

Variable	Males		Females		Total	
	Parameter Estimate[a]	Hazard Ratio[b]	Parameter Estimate[a]	Hazard Ratio[b]	Parameter Estimate[a]	Hazard Ratio[b]
Behavior						
FACTIV						
LFREAR						
FHDPK			−.04661*	.53	−.02618*	.70
FBOLI						
FDURN						
MAXCDT	−.00762‡	.76	.00918‡	1.38		
Homeostatic physiology						
WT			.05310*	1.61	.02936*	1.41
LMAXTT						
HEMA0			−.04123‡	.73	−.04243**	.71
RHEMA40	−.07156‡	.74			−.06653*	.76
GLUC0						
RGLUC40						
URINE0			−.00042*	.61	−.00022‡	.77
RURINE24						
Immune system						
LYMPHS						
LYMPHS^2						
WBC100						
WBC100^2						
CONJ						
DTAR						
LNK25	.26233**	1.72			.15469*	1.35
LCONA	−.07892‡	.79	−.12678*	.78	−.12391***	.69
Oxidative defense						
LTXB						
LKETO						
TKRAT						
GSHPX						
Sex and model parameters						
FEMALE					.50103***	1.65
CONST(lnα)	−8.59508***		−7.24657***		−6.87502***	
TDEP(θ)	.00386***		.00469***		.00379***	

[a]Significance levels: ‡$p < .10$; *$p < .05$; **$p < .01$; ***$p < .001$.

[b]For variables other than sex, the reported hazard ratios are those associated with two standard deviations of increment in the reported variable.

From the domain of homeostatic physiology, baseline hematocrit was the most consistent predictor of mortality; higher hematocrit was significantly associated with reduced hazard in the total sample and for females. In this sample, baseline hematocrit declined linearly and dramatically with age. Age-related declines in hematocrit levels have been found in many prior studies, including studies of rats (58,59), mice (60,61), and monkeys (62). Similarly, baseline urine concentration also showed age-related declines, and higher values were associated with decreased mortality for females. For males, greater change (residual) in hematocrit scores following glucose challenge was also associated with decreased mortality. Fasting glucose and glucose tolerance, however, did not emerge as significant predictors in any survival model, and only baseline glucose showed strong age associations, and only for males. The lack of association of the glucose measures with mortality is difficult to reconcile with well-accepted theories regarding the role of nonenzymatic glycosylation in many aging processes (63,64). Studies in rats (65) have suggested that the longevity-extending effects of dietary restriction may in part be mediated through lower glucose levels, although Sell et al. (66) found only weak correlations between glycemia and species longevity and Leiter et al. (67) found no impairment of glucose tolerance in aged C57BL/6J mice. Another biomarker measure that is probably related to glycosylation processes is tail tendon fiber break time, a measure of collagen cross-linking. Collagen crosslinking has been associated with mechanistic theories of aging (68), but its effects probably occur largely through glycosylation processes (66,69). In the present study, tail tendon fiber break time showed the strongest association of any biomarker with age (standardized $\beta = +.92$ for males and +.90 for females), but did not emerge as a significant pre-

Table 7. Associations of Biomarkers with Age: Summary of Repeated Measures ANOVA and Regression Results

	Males			Females		
Variable	Repeated Measures ANOVA F (linear)	$p(F)$ (df_1 1, df_2 152)	Standardized Regression Coefficient	Repeated Measures ANOVA F (linear)	$p(F)$ (df_1 1, df_2 152)	Standardized Regression Coefficient
Behavior						
FACTIV	7.13	.0081	+.9267	10.16	.0013	+.9832
LFREARS	16.84	.0001	+.7038	22.00	.0001	+.5186
FHDPK	29.38	.0001	−.9603	24.60	.0001	−.9092
FBOLI	13.72	.0003	−.6685	.26	.6110	+.5158
FDURN	6.96	.0088	−.0197	.52	.4725	+.5821
MAXCDT	1.56	.2132	+.2875	.21	.6465	−.8119
Homeostatic physiology						
WT	416.32	.0001	+.3099	674.34	.0001	+.7520
LMAXTT	1036.53	.0001	+.9997	1017.52	.0001	+.9985
HEMA0	253.70	.0001	−.9894	404.56	.0001	−.9925
RHEMA40	.42	.5164	+.7530	.64	.4247	−.8975
GLUC0	5.70	.0176	−.9684	.52	.4733	−.6534
RGLUC40	.11	.7386	−.7080	.30	.5831	+.7301
URINE0	.73	.3943	−.6353	6.07	.0144	−.7632
RURIN24	5.29	.0222	+.9673	6.81	.0096	−.9430
Immune system						
LYMPHS	68.77	.0001	−.9833	101.92	.0001	−.9713
WBC100	7.97	.0051	+.5581	23.03	.0001	−.3016
CONJ	74.55	.0001	−.9644	59.07	.0001	−.9893
DTAR	46.15	.0001	−.9738	46.05	.0001	−.5147
LNK25	.89	.3467	−.5824	.01	.9191	−.7891
LCONA	.77	.3825	−.1180	.00	.9615	+.4054
Oxidative defense						
LTXB	53.63	.0001	−.6884	33.36	.0001	−.6404
LKETO	20.13	.0001	−.7686	25.88	.0001	−.7765
TKRAT	2.74	.0990	−.4392	.33	.5636	+.7934
GSHPX	104.53	.0001	+.9384	105.86	.0001	+.9492

dictor for either sex in the survival analyses. Because tail tendon fiber break time is so strongly associated with age, however, it is confounded in the survival analyses with the time-dependent Gompertz parameter θ. This is a problem shared by any biomarker strongly associated with chronological age, but is experienced to the greatest extent with this measure.

Another key area believed to be associated with aging mechanisms involved with free radical generation (70) is that of oxidative defenses, represented in the present study by glutathione peroxidase (GSHPX). In the HS study population, GSHPX showed apparent age-related increases, and higher values were marginally associated with increased hazard rates in females. In the present study, two arachidonic acid metabolites—thromboxane B2 and the prostacycline metabolite 6-keto-PGF1-α—were considered part of the oxidative defense domain due to the roles of eicosanoids as mediators in inflammation and in oxidative stress (71). These biomarkers were only measured from 45 to 630 days, and their levels decreased with age over that time period. Higher thromboxane levels were associated with enhanced survival in females. Eicosanoids have been shown to have complex interactions with other systems, such as T cell-mediated immunity (72).

The biomarkers chosen for the present study to evaluate immune function reflect diverse functional parameters. Of the immune measures, Con A, a measure of lymphocyte mitogen responsiveness, was the most consistent predictor of mortality and was associated with reduced hazard rates in most models. Blastogenic responses to mitogens have been shown in many studies to decline as a function of age; the significant reduction in hazard rates observed here may reflect individual differences in immunocompetence as a function of age (73,74). Many studies have also found age-related declines in natural killer cell activity (75,76), and it emerged as a significant independent predictor for survival in males and in the total sample. Conjugated tumor target cells and dead target cells, although age-related in the study mice, were not significant predictors of mortality.

In addition to examining the relationships of each biomarker to age and mortality in this sample, we explored the overall effect of the addition of biomarker covariates on Gompertz parameters (14), and also examined the ability of the biomarkers to explain mortality in the sample by using generalized R^2 methods (35). The results suggest that the addition of time-varying biomarker variables to Gompertz survival models adds significant information, but explains only a relatively small proportion of the variance in age at

death in this sample. Manton and colleagues (14,41) were able to explain a much greater proportion of variability in two longitudinal aging studies, Framingham and the National Long Term Care Survey, but attributed the greatest increases to the addition of functional status measures rather than physiological risk factors alone.

Although most of the biomarkers assessed in the present study showed clear changes with age, far fewer predicted mortality reliably. In interpreting these findings, several considerations should be addressed. First, to achieve significance in the present analysis, it is not sufficient for a biomarker to change with age if it does not also distinguish survivors from nonsurvivors of the same chronological age. Thus, the set of significant biomarkers should reflect biological functioning that is independent of chronological age, but will not necessarily include all biomarkers showing age-associated change. For example, as discussed previously, tail tendon fiber break time, though strongly related to age, does not do better than chronological age in predicting the hazard of death in this sample.

It should also be kept in mind that the procedures used in the present study emphasize the identification of variables that contribute, independently of other measures, to the prediction of survival. It is unlikely, however, that many biomarkers function independently; some biomarkers may be directly affected by other biomarkers, and others may covary together due to age-related changes in underlying physiological systems. Indeed, there is growing consensus that a comprehensive view of aging includes the concept of a complex nexus of interrelated variables constituting a system (6). Alternative systems-based approaches that may prove useful in survival analysis with biomarkers include the identification of underlying factors or principal components (62) and the inclusion of specific interaction terms between biomarkers (14). Such approaches, however, provide challenges in longitudinal studies if attrition over multiple occasions severely curtails the available sample size. For example, although the present study sample is large (n = 319) in comparison to some animal studies, sample size reduction at later ages nevertheless limits the scope of multivariate analyses that can be used to address specific factors differentiating late deaths.

A final consideration that should be kept in mind in evaluating the roles of biomarkers in predicting survival is that the present analysis does not explicitly model intraindividual changes in biomarkers. Biomarker values were replaced with updated levels following each occasion of measurement. Mice were therefore assessed on the basis of short- to moderately long-term (≤270 days, the maximum interval between occasions) prediction. It is possible that modeling individual differences in trajectories of biomarker change may provide more meaningful information regarding relationships between biomarkers and survival. In addition to long-term age trajectories, short-term intraindividual variability in biomarker values may also provide additional information. For example, a recent study of retirement community residents by Nesselroade and colleagues found that intraindividual variability in biomarkers measured repeatedly explained a substantial proportion of mortality over a 5-year period, whereas actual biomarker levels explained relatively little (77). The present analysis also assumes that a given biomarker value confers the same advantage or disadvantage regardless of the age of measurement. It may be more reasonable to assume that the significance and meaning of specific biomarkers may change depending on age, but allowing covariate parameter coefficients to vary over different portions of the life span requires large samples because statistical power is decreased with the introduction of many additional parameters.

In conclusion, this study confirmed hypothesized associations with age for a number of candidate biomarkers, but questions remain about the relationships between biomarkers of aging and longevity. Although several biomarkers emerged as significant independent predictors of mortality in our analyses, it is clear that much more remains to be learned about the complex interrelationships among biomarkers and their effects on aging systems over time. Future multivariate efforts addressing these complex issues have the potential to contribute greatly to our understanding of how biomarkers of aging explain mortality risk across the life span.

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