Longitudinal determination of skin collagen glycation and glycoxidation rates predicts early death in C57BL/6NNIA mice

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ABSTRACT **In 1988, the National Institute on Aging launched a 10-year program aimed at identification of biomarkers of aging. Previous results from our laboratory showed that pentosidine, an advanced glycation product, formed in skin collagen at a rate inversely related to maximum life span across several mammalian species. As part of the Biomarkers Program, we investigated the hypothesis that longitudinal determination of glycation and glycoxidation rates in skin collagen could predict longevities in *ad libitum*-fed (AL) and caloric restricted (CR) mice. C57BL/6NNia male mice were biopsied at age 20 months and at natural death. Glycation (furosine method) was assessed by gas chromatography/mass spectrometry (GC/MS) and the glycoxidation products carboxymethyllysine (CML) and pentosidine were determined by GC/MS and HPLC, respectively. CR vs. AL significantly ($P<0.0001$) increased both mean (34 vs. 27 months) and maximum (47 vs. 31 months) life spans. Skin collagen levels of furosine (pmol/μmol lysine) were ~2.5-fold greater than CML levels and 100-fold greater than pentosidine. Individual accumulation rates modeled as linear equations were significantly ($P<0.001$) inhibited by CR vs. AL for all parameters and in all cases varied inversely with longevity ($P<0.1$ to <0.0001). The incidence of three tissue pathologies (lymphoma, dermatitis, and seminal vesiculitis) was found to be attenuated by CR and the latter pathology correlated significantly with longevities ($r=0.54$, $P=0.002$). The finding that markers of skin collagen glycation and glycoxidation rates can predict early deaths in AL and CR C57BL/6NNia mice strongly suggests that an age-related deterioration in glucose tolerance is a life span-determining process.—Sell, D. R., Kleinman, N. R., Monnier, V. M. Longitudinal determination of skin collagen glycation and glycoxidation rates predicts early death in C57BL/6NNIA mice. *FASEB J.* 14, 145–156 (2000)**

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FOR THE PAST > 15 years, our laboratory has focused on elucidating the role of the Maillard reaction in aging and diabetes. In this reaction, sugars react nonenzymatically with proteins such as collagen to form protein adducts and cross-links, which can be fluorescent and yellow-colored and contribute to protein insolubilization (1). Glucose and other sugars, or their degradation products, have been implicated in the aging process by formation of such irreversible molecules (2). In support of this hypothesis, studies show that glucose tolerance progressively declines with age (3, 4) whereas advanced glycation and glycoxidation products increase with age in the extracellular matrix (5–7). In contrast, the anti-aging intervention caloric restriction, which is known to increase life span (8), improves glucose tolerance in rats (9, 10) and monkeys (11–13), and decreases glycoxidation in rodents (5, 14). Conversely, diets high in sugars increase glycoxidation (15) and decrease longevities in rats (16) and mice (17).

In the late 1980s, the National Institute on Aging (NIA) launched a 10-year program aimed at identifying biomarkers of aging, i.e., biological or biochemical markers that would predict the onset and progression of age-related processes and longevity (18). Rodents were chosen as models because their life spans are considerably shorter than those of humans and with the hope of using the biomarkers in future studies of aging processes and their interventions in humans (18, 19). However, the concept of the actual existence of markers of aging has been controversial (20), with many differences in opinions about the exact criteria and paradigm that make good candidate markers (18–21). Some authors suggested that because of the complexity of aging phenomena, not just one, but many diverse markers, or a panel of markers, would be necessary in order to achieve success if, indeed, such a paradigm could be identified (20).

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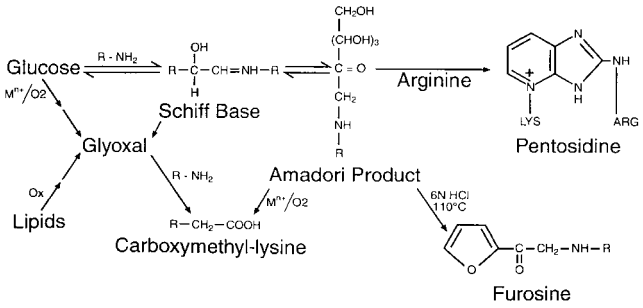

Figure 1. Biochemical relationship between the glycation and glycoxidation markers determined in the study. Note that N^{ε}-(carboxymethyl)lysine (CML) can also potentially originate from lipid peroxidation (45) and pentosidine from ascorbate (1).

In this prospective, previous results from this (5–7) and other laboratories (14, 22) have shown that glycoxidation markers correlate strongly with chronological age in cross-sectional studies, suggesting that they have strong potential as candidate biomarkers for practical application in longevity and intervention studies. Subsequently, it was also shown that glycoxidation rate determined by pentosidine in skin collagen was inversely related to maximum life span across several mammalian species (5). In this latter study, pentosidine (**Fig. 1**) was related to longevity not by absolute level, but by the rate of formation represented by slopes, i.e., its rate of change over time. However, data in these previous studies were obtained cross-sectionally such that inference to individual longevities could not be made. Thus, in the present study we have investigated three markers of aging longitudinally in mice (Fig. 1): furosine, i.e., a measure of glycation; and N^{ε}-(carboxymethyl)lysine (CML) and pentosidine, i.e., measures of glycoxidation. The hypothesis tested is that longitudinal determination of glycation and glycoxidation rates can predict individual longevities in *ad libitum* (AL) and caloric restricted (CR) mice and that CR will retard this rate. We have further investigated the relationship of these rates to major tissue pathologies present at death. In short, these results show that these rates modeled as linear equations were significantly inhibited by CR vs. AL for all parameters, and in all cases were found to vary inversely with longevity. Furosine, i.e., glycated lysine residues, surprisingly emerged as the most important factor in explaining life span of these mice.

MATERIALS AND METHODS

Animals care and housing

A total of 31 AL and 32 CR male mice of the C57BL/6NNia strain were received at age 508 days from NIA rodent colonies located at the National Center for Toxicological Research (NCTR, Jefferson, Ark.). Animals were transferred to a Micro-Isolator PLUS air/water system (Model No. 59015, Lab Products, Maywood, N.J.) and individually housed in these cages using a 12 h light-dark cycle at 24°C according to regulations and approval set forth by the CWRU Institutional Animal Care and Use Committee. Mice were maintained throughout the study in specific pathogen-free (SPF) conditions using sterilized cages, feed, and bedding, the latter of which consisted of reprocessed ground corncobs (The Andersons, Maumee, Ohio). All procedures, including transferring of mice in and out of cages and feeding, were done under a class 100 Stay-Clean L/F-B laminar flow hood/workbench (model No. 30909B, Lab Products). All mice were continuously fed a diet that was shipped throughout the study by NCTR to CWRU in sealed plastic bags, which were autoclaved before shipment. These bags were open under the hood after spraying with Alcide sterilant/disinfectant (Exspor, Alcide Corp., Redmond, Wash.) and stored inside a sterilized 19 × 10.5 × 8" micro-isolator cage that was open and closed under the hood. AL mice were continuously fed the NIH-31 diet in pellet form as described (23). CR mice were fed a modified version of this diet that was made nutritionally adequate in minerals and vitamins by supplementation but deficient in calories by restricting the diet to 60% of the average calorie intake of AL mice, as described (23). This consisted of pellets cut according to known weight and daily feeding according to a predetermined pellet weight and age schedule provided by the NCTR as described (24). Each mouse was weighed approximately once a month until death on an electronic balance. The histories of body weights of these mice prior to shipment were obtained from the NCTR.

The assessment of SPF status of these mice was done biannually by in-house disease surveillance monitoring using CF1 sentinel mice. This consisted of transferring dirty bedding from cages of experimental to those of the sentinel mice, which were later killed and examined for tissue serology, bacteriology, and parasitology by Charles River Laboratories (Wilmington, Mass.).

Skin procurement by biopsy

Skin biopsies were obtained surgically from mice at age 605 days. Cages were moved to a location under a laminar flow/fume hood, the surface of which was kept moist during surgery with Alcide sprayed onto a layer of paper towels. Each mouse was anesthetized by Metofane (Schering-Plough Animal Health Corp., Omaha, Nebr.). The continuous maintenance of the anesthetic during surgery was provided by inserting the nose of the mouse into the plugger end of a 10 cc syringe containing gauze moisten with Metofane. An approximate 4 mm diameter skin biopsy was taken from the dorsal region near the neck after shaving the region with surgical clippers and prepping with iodine solution. The incision was closed with a surgical clip applied by the Autoclip Wound Closing System (Stoelting, Wood Dale, Ill.). Each biopsy was washed in saline and stored frozen at −70°C. When the incision healed, the clip was removed by an autoclip remover utensil (Stoelting).

Postmortem handling of mice

During this study, mice were carefully examined daily for vitality and sickness. At death, mice were weighed and evaluated at necropsy for gross pathological diagnoses by the attending veterinarian (Dr. N. Kleinman). Their skin was removed starting from the neck down and stored frozen at −70°C.

Skin processing and analytical assays

Hair was removed from skin by scraping frozen tissue samples with the blade of a scalpel as described previously (7). Likewise, samples were homogenized and extracted (7). In all analyses described below, specimens belonging to the same animal were processed consecutively in order to minimize experimental error.

Pentosidine was determined in acid hydrolysates of processed skin samples by high-performance liquid chromatography (HPLC). Acid hydrolysates equivalent to 50 μg hydroxyproline (hyp) contained in volumes of either 152–350 μl (tissue at biopsy) or 57–163 μl (tissue at death) were pipetted into the bottom of 12 × 75 mm borosilicate glass tubes, followed by addition of the internal standard, 50 μl of 1 μg/ml pyridoxamine (Sigma Chem. Co., St. Louis, Mo.). Contents of the tubes were then dried using a Speed-Vac Rotary Centrifuge (Savant Instruments, Holbrook, N.Y.), followed by reconstituting each tube with 200 μl of water containing 0.1 M heptafluorobutyric acid. Totals of 160 μl equivalent to 40 μg hyp—i.e., equivalent 256 μg collagen based on 14% hyp of collagen by weight (5)—were injected onto a HPLC containing a 4.6 × 250 mm 218TP54 C18 Vydac, 5 μ column equilibrated and eluted as described (7). Pyridoxamine and pentosidine eluted at ~11 and 33 min.

Lysine, furosine, and CML were determined in acid hydrolysates of processed skin samples derivatized as their trifluoroacetyl methyl esters by selected ion-monitoring gas chromatography/mass spectrometry (SIM-GC/MS) using modified procedures of Dunn et al. (25) and Glomb and Monnier (26). Acid hydrolysates equivalent to amounts previously described for pentosidine were pipetted into the bottom of 13 × 100 mm screw-type cap culture tubes, followed by the addition of 100 μl of 4 nmol/ml deuterated CML (*d*4-CML) as the internal standard. The contents of each tube were then dried by Speed-Vac (Savant). One milliliter of anhydrous methyl alcohol containing 0.2 M thionyl chloride (Aldrich, Milwaukee, Wis.) was pipetted into the tube, followed by sealing with a Teflon liner screw cap. The tube was placed into a 110°C oven for ~30 min, followed by evaporating contents with a stream of nitrogen. A total of 0.5 ml anhydrous dichloromethane and 1 ml of trifluoroacetic anhydride (Aldrich) was added and the tube was left to sit at room temperature for 1 h. After evaporation of organic solvents (nitrogen), the contents of each tube were transferred to 100 μl glass inserts to autosampler vials (Hewlett-Packard, Wilmington, Del.) with three separate 100 μl washes with dichloromethane. The solvent in the insert vial was dried between washes with a stream of nitrogen. After the last wash, the solvent was dried and the contents in the vial were reconstituted with 25 μl of dichloromethane. For each sample, 2 μl was injected onto a Hewlett-Packard GC/MS equipped with a 5890 Series II Gas Chromatograph, 5971 Series Mass Selective Detector, and a 6890 Series Automatic Injector. Separations were made on a 25 m X 0.2 mm X 0.33 μM Ultra 2 column cross-linked with 5% PH ME siloxane (Hewlett-Packard) using the temperature program described by Dunn et al. (25). Lysine, D4-CML, CML, and furosine were monitored at ions m/z = 320, 396, 392, and 110, which eluted at 23, 29.4, 29.4, and 36.2 min, respectively. Quantitation was by comparison to calibration curves using standards prepared and run under similar conditions.

Diagnosis and management of dermatitis

Mice were assessed for absence or presence of dermatitis. Cases were categorized into being mild, moderate, or severe. A case was designated mild if scratching occasionally occurred but was cured by a single clip of toenails. Moderate cases were diagnosed when scratching was chronic but held in check by multiple clips of toenails over time (i.e., ≥ two clips). More severe cases involving episodes of both scratching and chewing, causing skin lesion ulcerations, were held in check by multiple toenail clipping and application of a mouse Elizabethan collar (Stoelting) until healing.

Evaluation at necropsy for gross pathology and major diagnosis at death

Each mouse was evaluated at death for major pathological diagnosis. The thorax cavity was examined first. Cardiomegaly was diagnosed upon observing unilateral or bilateral heart enlargement. Cardiopathy/cardiomyopathy was diagnosed if inflammation or other apparent abnormalities such as chamber dilations, blood clots, masses, or valve nodules were present. Lungs were examined for collapse, atelectasis, congestion, effusions, edema, and masses. In severe cases of cardiomegaly and cardiopathy, congestive heart failure was diagnosed.

The abdominal cavity was examined next. The internal contents of the stomach and the remainder of the intestines were examined for the presence of melena. If melena was present, it was diagnosed as ulcerative gastroenteritis. In some cases, melena existed only in the stomach; however, most frequently it extended to portions of the small intestines down to the level of the cecum and colon.

The liver was examined for size, color, architecture, infiltrations, and growths. Liver enlargement, or hepatomegaly, was frequently accompanied by infiltrative hepatopathy involving discoloration, loss of normal architecture, and lobular accentuation. However, in other cases, especially in CR mice, infiltrates observed as white or red foci on lobes were not necessarily accompanied by increased liver size. In contrast, in some mice, especially those of the CR cohort, the liver appeared to be smaller than normal and frequently had a yellowish discolored appearance. Mice that showed chronic weight loss and emaciation were diagnosed with hepatic degeneration/insufficiency. However, whether this diagnosis actually caused death or was secondary to other pathology was not investigated.

The entire spleen or one of its portions was diagnosed upon enlargement as splenomegaly. Enlargement was frequently homogenous whereas others showed dark or white colored foci. Other spleens showed accentuation of white pulp, growths, cysts, or blood-filled hemangiomas. Similar to the liver, many CR mice had enlarged spleens as well; however, others had very small spleens, appearing below normal in size.

Renal pathology was diagnosed upon observing unilateral or bilateral enlargement of kidneys, tumor-appearing masses, growths, or nodules on the kidney surface, and dilations of renal tract parts (i.e., dilations in renal pelvis).

Seminal vesiculitis was diagnosed and graded for severity as either mild, moderate, severe, or extremely severe. Mild cases involved unilateral or bilateral enlargement (i.e., hypertrophy or dilatation) of the seminal vesicles with opacity. Moderate cases, which were most numerous, involved greater enlargement, yellow discoloration, opacity, and turgidity. Severe cases were similar to moderate ones, but also showed unilateral or bilateral internal hemorrhaging and/or suppuration. Extreme conditions further showed red and black discoloration, torsion, ischemia, necrosis, and, in one case, rupture of the vesicle contents into the peritoneal cavity.

Statistical methods

Statistical methods have been described elsewhere (5–7) and thus are only summarized here. Data were tested for normal-

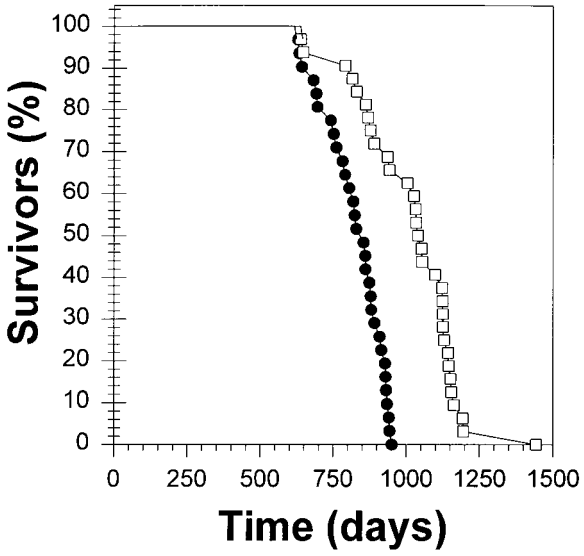

Figure 2. Survival curve for C57BL/6NNia male mice used in study of longitudinal rates of skin collagen glycation and glycoxidation. Percent surviving members of *ad libitum* (AL) and caloric restricted (CR) cohorts as a function of life span starting from time zero at birth. Each point represents death of single mouse shown for AL-fed mice as filled circles and CR mice as open squares.

ity by the W test and homogeneity of variance by either the Burr-Foster Q test or the Levene's test by SPSS software (SPSS, Inc., Chicago, Ill.). Pearson correlation tables, regression analyses, and ANOVA were done by SPSS. Age adjustment of data, outlier test, and comparison of regression equations for slopes were done according to methods previously described (5–7). The Kruskal-Wallis one-way ANOVA was computed by SPSS.

RESULTS

Survival and body weights of mice

AL and CR mice were shipped and received from the NCTR at 508 days of age. Survival was significantly ($P<0.0001$) related to diet: AL vs. CR, $r=0.58$ (**Fig. 2**). The first mouse in the AL cohort to die was 629 days old, which also coincided with the rapid rate phase of death in the survival curve (Fig. 2). Likewise, the first death in the CR cohort occurred at the age of 638 days; however, mortality acceleration did not occur until the death of the third mouse at age 791 days (Fig. 2). The median and maximum life spans for cohorts were as follows: AL, 842 and 951 days; CR, 1040 and 1442 days. The tailing effect frequently observed in survival curves due to the delay of death in the oldest living individuals of the population was not observed for the AL cohort. The tail observed for CR mice was essentially due to a single mouse that lived to age 1442 days, superseding the death of the previous CR mouse at age 1195 days (Fig. 2).

Mean body weights as shown in **Fig. 3** were significantly ($P<0.0001$) affected by diet ($r=0.83$) and age ($r=0.42$). Body weights for AL mice increased during growth and maturation, reached a peak between the ages of 364 and 504 days, and thereafter progressively declined into senescence (Fig. 3). Body weights for CR mice were consistently lower than AL mice of similar age and showed considerably less variation (Fig. 3). The maximum body weights attained for AL and CR mice were 41 ± 5 and 28 ± 2 g, which occurred at 504 days of age. Thereafter, the progressive decline observed in body weights was considerably more rapid for AL vs. CR mice (Fig. 3).

Results for survival of AL and CR mice, including median and maximum life spans and the degree of curve triangulation (Fig. 2), are similar to previous results published for this strain by the NIA/NCTR, i.e., AL vs. CR: median, 840 vs. 966 days; maximum, 1050 vs. 1246 days (8, 28). The observed lack of a distinct tail in survival curves (Fig. 2) is probably due to fewer mice represented in the present study compared with the previous survival data published for these mice (8, 28). Similarly, the patterns in mean body weights for this strain over life span (Fig. 3), including maximums achieved and the observed middle age peaking for AL mice prior to late age decline, also agree with previously reported data (8, 28). The further observations of a lack of a peaking effect in body weights for CR mice, but with greater weight uniformity compared with AL mice, have also been previously observed (8, 28).

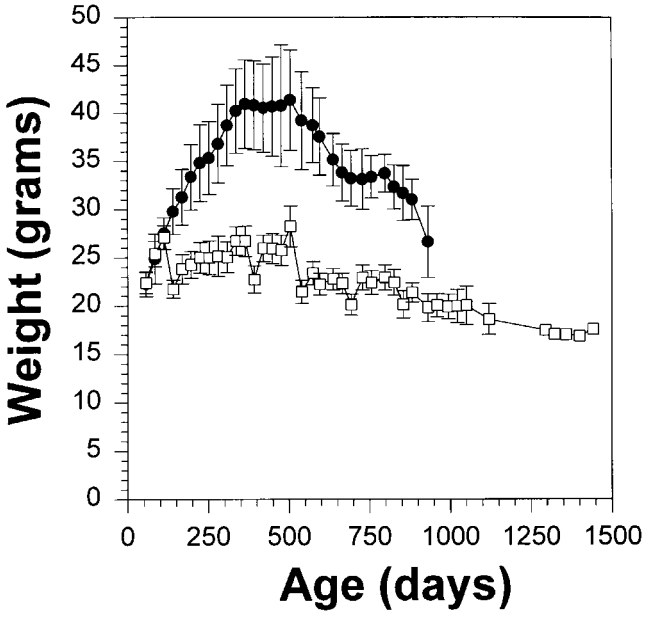

Figure 3. Body weights (grams) of mice used in longitudinal study from Fig. 1 as a function of life span starting from time zero at birth. Each point represents mean ±SD of surviving mice. See Fig. 2.

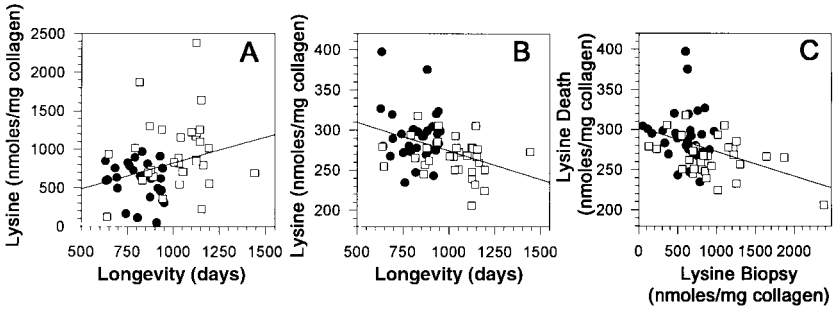

Figure 4. Lysine content of mouse skin collagen as a function of individual longevities of mice in a longitudinal study. Each point represents level expressed as nmol per milligram collagen for a single mouse. *A)* level at biopsy vs. longevity; *B)* level at time of death vs. longevity; *C)* level at biopsy vs. time of death. See Fig. 2. Regression equations: *A)* y=153 + 0.67x, n=63, r=0.29, P=0.023; *B)* y=345 − 0.071x, n=63, r=−0.39, P=0.002; *C)* y=303 − 0.03x, n=63, r=−0.38, P=0.002.

The effects of diet, age, and longevity on lysine content of mouse skin collagen

The biochemical relationships among markers assayed in this study are shown in Figs. 4–7. Mean content of lysine residues, a common denominator used in many studies of the Maillard reaction *in vivo* (14, 25, 27, 29), was found to be significantly (P<0.0001) greater at biopsy than at the time of death: 773 vs. 280 nmol/mg collagen (compare scale of *y* axis for **Fig. 4*A*** vs. Fig. 4*B*). The results at biopsy were significantly (P=0.001) less for AL vs. CR, 598 vs. 942 nmol/mg collagen. In contrast, lysine content at death was significantly (P<0.0001) but moderately greater for AL vs. CR, 293 vs. 266 nmol/mg collagen.

Additional relationships were noted among the variables lysine, sampling time, and longevity when data from mice were pooled across diets for regression analyses (i.e., AL+CR, n=63). At the time of biopsy (Fig. 4*A*), lysine correlated weakly but positively (r=0.29) with longevity (P=0.023). In contrast, a significant (P<0.002) inverse relationship (r=−0.39) was noted at the time of death between lysine and longevity (Fig. 4*B*). In these analyses, the effect of diet on lysine level of skin was significant at biopsy (r=0.43, P=0.0004) and at death (r=0.43, P=0.0005). However, none of these aforementioned relationships were significant (P>0.05) when data were analyzed separately within diets, i.e., AL (n=31) vs. CR (n=32) (Fig. 4*A, B*). In further analyses, lysine levels of skin collagen at biopsy were correlated with levels at death (Fig. 4*C*). When mice were analyzed across diets (n=63), a significant (P<0.002) inverse relationship (r=−0.38) was noted between levels at biopsy vs. death. When analyzed within diets, however, the relationship was significant for CR (r=−0.40, P<0.025, n=32) but not AL (r=−0.10, P>0.05, n=31). Overall, these results suggest that the fewer the lysines modified by the aging process, the greater the longevity of the animal.

Because of the differences noted in lysine levels with tissue sampling time and longevity, skin parameters of glycation (i.e., furosine) and glycoxidation (i.e., CML and pentosidine) were expressed separately both per milligram collagen and per μmoles lysine. Pearson correlation analyses in **Table 1** show that two methods for expression of parameters were highly correlated with each other at the time of death (r=0.90 to 0.97, P<0.0001), but correlated considerably less at the time of biopsy (r=0.13 to 0.67, Table 1). However, overall composite relation-

TABLE 1. *Correlations between methods of expressing results*[a]

Tissue sampling time	Parameter								
	Furosine			CML			Pentosidine		
	AL	CR	AL+CR	AL	CR	AL+CR	AL	CR	AL+CR
	(pmol/mg collagen vs. pmol/μmol lysine)								
At biopsy (r):	0.67	0.15	0.24	0.18	0.45	0.19	0.13	0.45	0.38
(n)	(31)	(32)	(63)	(31)	(32)	(63)	(31)	(32)	(63)
Prob. (P):	0.0003	NS	NS	NS	0.037	NS	NS	0.01	0.003
At death (r):	0.91	0.92	0.90	0.91	0.86	0.91	0.94	0.93	0.97
(n)	(31)	(32)	(63)	(31)	(32)	(63)	(31)	(32)	(63)
Prob. (P):	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Pooled (r):	0.82	0.72	0.72	0.21	0.66	0.38	0.73	0.68	0.72
(n)	(62)	(64)	(126)	(62)	(64)	(126)	(62)	(64)	(126)
Prob. (P):	<0.0001	<0.0001	<0.0001	NS	<0.0001	0.0001	<0.0001	<0.0001	<0.0001

[a] Pearson correlation coefficients of relationship between expressing results as parameter per milligram of collagen vs. per μmoles of lysine.

TABLE 2. *Correlations between longevity and parameters of glycation and glycoxidation*[a]

	Parameter								
	Furosine			CML			Pentosidine		
Tissue sampling time	AL	CR	AL+CR	AL	CR	AL+CR	AL	CR	AL+CR
	(Level expressed as pmol/μmol lysine vs. longevity)								
At biopsy (r):	−0.34	+0.04	+0.24	+0.15	−0.39	−0.09	+0.13	−0.36	−0.13
(n)	(31)	(32)	(63)	(31)	(32)	(63)	(31)	(32)	(63)
Prob. (P):	NS	NS	NS	NS	NS	NS	NS	0.05	NS
	(Level expressed as pmol/mg collagen vs. longevity)								
At biopsy (r):	−0.41	+0.33	+0.22	−0.50	+0.15	+0.35	−0.19	−0.03	+0.32
Prob. (P):	0.021	NS	NS	0.005	NS	0.005	NS	NS	0.010
	(Level expressed as pmol/μmol lysine vs. longevity)								
At death (r):	+0.40	+0.44	+0.33	+0.18	+0.26	+0.44	+0.23	+0.62	+0.81
(n)	(31)	(32)	(63)	(31)	(32)	(63)	(31)	(32)	(63)
Prob. (P):	$NS_{(0.09)}$	0.013	0.035	NS	NS	<0.002	NS	0.0002	<0.0001
	(Level expressed as pmol/μmol lysine vs. age)								
Biopsy + death (r):	+0.74	+0.82	+0.75	+0.21	+0.74	+0.46	+0.57	+0.75	+0.73
(n)	(62)	(64)	(126)	(62)	(64)	(126)	(62)	(64)	(126)
Prob. (P):	<0.0001	<0.0001	<0.0001	NS	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

[a] Pearson correlation coefficients of the relationship between longevity (or age) to glycation (furosine) or glycoxidation (CML and pentosidine) in mouse skin collagen at the times of biopsy and death.

ships (n=126) between the two methods of expression were significantly correlated for all parameters as follows: furosine (r=0.72, P<0.0001), CML (r=0.38, P=0.0001), and pentosidine (r=0.72, P<0.0001). Except where noted, the results below express measured parameters on the basis of μmoles lysine.

Relationship between longevity and markers of glycation and glycoxidation

Pearson correlation analyses of levels of furosine, CML, and pentosidine at the time of biopsy and at death vs. longevity are summarized in **Table 2**. Levels at biopsy when expressed per μmoles lysine correlated weakly with longevity within and across diets. Only in one of these comparisons did the results reach significance (P<0.05): pentosidine in CR mice vs. longevity, r=−0.36 (Table 2). These correlations improved somewhat when levels at death were analyzed (Table 2), and were especially significant and positively correlated with longevity (r=0.33, 0.44, 0.81) when results were pooled across diets for furosine (P<0.035), CML (P<0.002), and pentosidine (P<0.0001), respectively. There was a significant (P<0.02) trend for these parameters measured at biopsy in AL mice to correlate inversely with longevity when results were expressed as per milligram of collagen. As shown in Table 2 and plotted in **Fig. 5**, the results reached significance for furosine (r=−0.41, P=0.021) and CML (r=−0.50, P=0.005), but not pentosidine (r=−0.19, P=0.39). Further multifactor analysis consisting of levels at biopsy vs. longevity in mice showed that prediction (r=0.55) reached significance (P=0.019) with the equation: longevity = 1032 -1.28 (furosine) - 0.18 (CML) - 6.95 (pentosidine).

In another study, levels at the time of biopsy and death were plotted vs. age (**Fig. 6**). Initial analyses showed no significant (P>0.05) effect of AL vs. CR on parameters at the time of biopsy: mean furosine, 548 vs. 481 pmol/μmol lysine (P=0.14); CML, 274

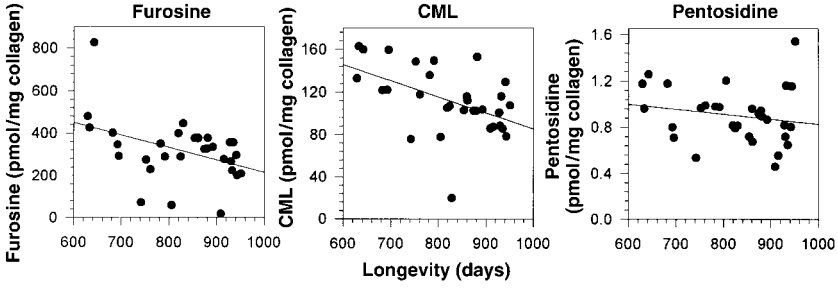

Figure 5. Furosine, CML, and pentosidine content of mouse skin collagen at time of biopsy as a function of respective longevities of AL mice used in a longitudinal study. Each point represents level expressed as pmoles per milligram collagen for a single mouse. Regression equations: furosine, y=803 - 0.59x, n=31, r=−0.41, P=0.021; CML, y=237 − 0.15x, n=31, r=−0.50, P=0.005; pentosidine, y=1.26 − 4.4×10^{-4}x, n=31, r=−0.19, P=0.31.

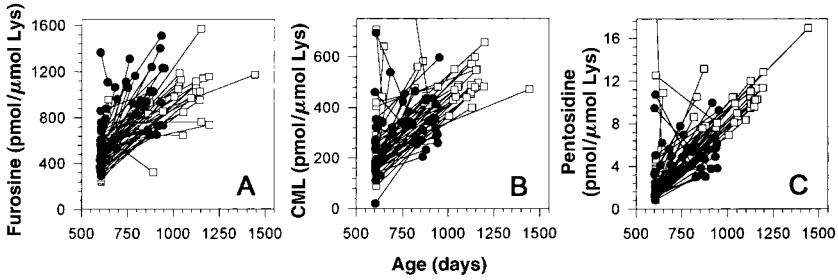

Figure 6. Level of glycation and glycoxidation at times of biopsy and death connected by a line representing a longitudinal accumulation rate in parameter for each mouse in the study. Levels are expressed as pmoles per μmoles lysine: *A)* furosine, *B)* CML, *C)* pentosidine. See Fig. 2. For each line within a parameter, a slope is determined representing rate of change of parameter (*dP/dt*). These slopes are plotted in Fig. 7.

vs. 225 (P=0.42); pentosidine, 2.2 vs. 2.9 (P=0.39) (data not shown). Further correlations across sampling times and diets (n=126, Table 2) showed significant (P<0.0001) relationships with age for furosine (r=0.75), CML (r=0.46) and pentosidine (r=0.73) (Table 2). As shown in Fig. 6, a line was drawn connecting each mouse's level at the time of biopsy to its level at the time of death. Thus, each line represented a slope or rate of change of the parameter (*dP/dt*) from the period starting at biopsy (i.e., age 605 days) to the time of death. These slopes were in turn plotted vs. individual longevities, as shown in **Fig. 7**.

Figures 6 and 7 show that the slopes are negative for some mice in the study (Fig. 7), which is due to the level at biopsy being greater than the level at the time of death. Second, some mice showed slopes that deviated from those of the main group. As proved by a statistical test, such deviations were found to be outliers that occurred particularly in mice dying soon after biopsy. These outliers, as listed in **Table 3**, were not included in the further statistical analyses. Third, mice with early demise, especially those of the AL cohort, showed a rapid increase in rate over the time period. This is most pronounced for furosine levels of AL mice shown in Fig. 6*A*. This figure also shows an age-related, accelerated spreading of furosine levels, which is elevated in AL vs. CR mice.

An inverse relationship between longevity and rate of change for each parameter is evidenced by the regression lines drawn for AL and CR cohorts of Fig. 7. Regression equations for these lines are shown in Table 3. For AL mice, a highly significant (P<0.0001) inverse relationship is noted between longevity of mice and rates of change in furosine (r=−0.70) and pentosidine (r=−0.70), whereas that of CML (r=−0.33) approached significance (P=0.067). The same inverse relationship noted for AL mice holds true for CR mice. However, the relationship is attenuated by CR as evidenced by the lack of decline of these lines shown in Fig. 7 and equated in Table 3, being significant for furosine (r−0.39, P=0.028) and CML (r−0.56, P=0.0015), but approaching significance for pentosidine (r−0.31, P=0.09). A comparison of these equations for slopes shows that for each parameter the overall rate of change is significantly (P<0.001) less for CR vs. AL mice (i.e., slopes for furosine, 0.006 vs. 0.016; CML, 0.001 vs. 0.005; pentosidine, 2×10^{-5} vs. 2×10^{-4}). In contrast, prediction of these slopes by testing against life span and diet as the independent variables during regression analyses (data not shown) shows life span to be significant for all three parameters: furosine (P=0.0001), CML (P=0.03), and pentosidine (P=0.0002). However, the effect of diet was marginal: furosine (P=0.38), CML (P<0.10), and pentosidine (P<0.05).

In further evaluation, slopes of Figs. 6 and 7 were regressed on longevities by using stepwise multiple regression analysis in order to indicate the most important factors in determining the life spans of mice in this study. Diet (P<0.0001) and furosine (P=0.0002) as *dFur/dt* were selected with the equation (r=0.75): longevity = 748 + 169(diet) - 34(*dFur/dt*). Further proof of the importance of furosine in explaining life spans was by age adjustment of levels for all parameters at the time of death (data not shown). Statistical comparison of AL vs. CR data by the Kruskal-Wallis one-way ANOVA for levels age-adjusted to 950 days showed significance for furosine (P=0.003), but not CML (P=0.65) or pentosidine (P=0.49).

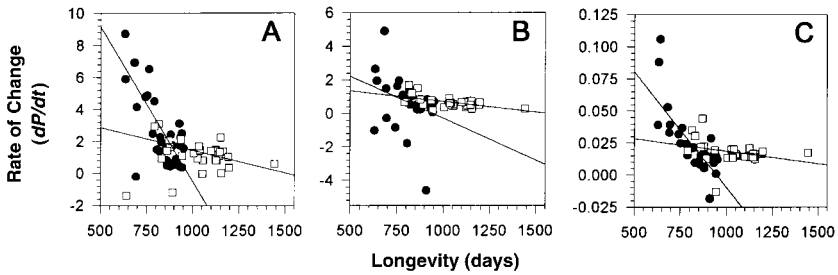

Figure 7. Longitudinal accumulation rates of glycation or glycoxidation parameters (*dP/dt*) as a function of individual longevities of mice: *A)* furosine, *B)* CML, *C)* pentosidine. See Fig. 2. Regression lines were determined for data points within diets for each parameter. Equations for these lines are shown in Table 3.

TABLE 3. *Relationships between longevity and rate of change for parameters (Fig. 7)*[a]

Dietary regimen	Parameter		
	Furosine	CML	Pentosidine
	(x = longevity vs. y = rate of change of parameter as slope)		
Ad libitum (AL)	$y = 16 - 0.016x$ $r = -0.70(1)$[b] $n = 30$ $P < 0.0001$	$y = 4.7 - 0.005x$ $r = -0.33$ $n = 31$ $P = 0.067$	$y = 0.17 - 1.81 \times 10^{-4}x$ $r = -0.70$ $n = 31$ $P < 0.0001$
Calorie restricted (CR)	$y = 7.8 - 0.006x$ $r = -0.39$ $n = 32$ $P = 0.028$	$y = 2 - 0.001x$ $r = -0.56(2)$[b] $n = 30$ $P = 0.0015$	$y = 0.04 - 1.96 \times 10^{-5}x$ $r = -0.31(2)$[b] $n = 30$ $P = 0.09$

[a] Regression equations and correlation coefficients expressing the relationship between longevity and rate of change (i.e., slope) of glycation (furosine) or glycoxidation (CML and pentosidine) between the times of biopsy (age = 605 days) and death in mouse skin collagen. [b] Number of outliers that have been excluded in the statistical analyses are given in parentheses. Coordinates (x, y) of outliers where x = longevity and y = rate of change are as follows (Fig. 6): furosine AL, (642, −6.61); CML CR, (638, −8.20), (646, 11.7); pentosidine CR, (638, −0.468), (646, 0.237). Coordinates with common longevities are from the same mouse.

Relationship between diet and tissue pathology at death

Specific pathological changes were found either decreased, increased, or unaffected by CR vs. AL (**Fig. 8**). As previously reported (30–33), many pathological changes were decreased by CR vs. AL including the percent incidences of seminal vesiculitis (22 vs. 83%, r=0.65, P<0.0001), ulcerative dermatitis (3 vs. 58%, r=0.60, P<0.0001), splenomegaly (22 vs. 42%, r=0.34, P=0.006) and enlarged mesenteric lymph nodes (6 vs. 26%, r=0.27, P=0.034). Furthermore, there was also a nonsignificant (P>0.05) trend for CR to reduce both heart (r=0.15) and renal (r=0.11) pathologies, although the incidence of the latter pathology was minor in this study (6 vs. 13%). In contrast, several pathologies were increased by CR, which included hepatic degeneration/insufficiency (41 vs. 13%, r=0.31, P=0.013) and minor problems with tooth loss/malocclusion observed only in CR mice (13% vs. ND, r=0.26, P=0.043). Finally, a large incidence of ulcerative gastroenteritis was observed in both CR vs. AL mice (78 vs. 87%, P>0.05). Incidences of infiltrative hepatopathy (38 vs. 48%) and subcutaneous (s.c.) edema (22 vs. 16%) were not significantly (P>0.05) different between dietary cohorts (Fig. 8).

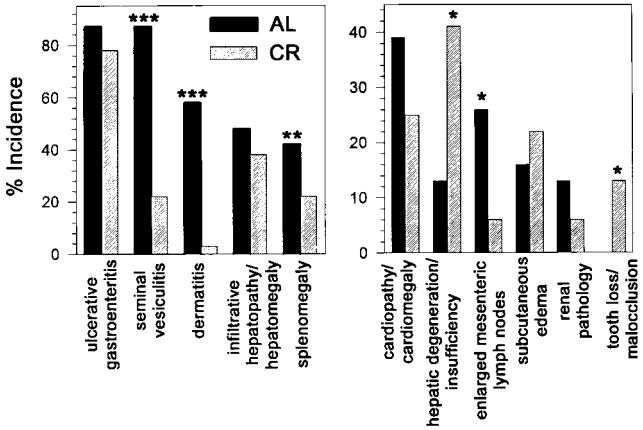

Figure 8. Percent incidences of specific pathologies of AL and CR mice during the longitudinal study. AL vs. CR: *P<0.05, **P<0.01, ***P<0.001.

Correlations between pathologies and associations with longevity

Because postmortem pathology varied widely between AL and CR cohorts, further analyses were conducted within diets with regard to correlations between pathologies and their relationship to age. To summarize, seminal vesiculitis was the only pathology that correlated with age, but only in AL mice. Incidences (r=0.54, P=0.002) and severities (r=0.54, P=0.002) were positively correlated with longevities: older AL mice tended to have more severe cases. Furthermore, seminal vesiculitis mildly but negatively correlated with slopes representing rates of glycation and glycoxidation as follows: furosine (r−0.29, P=0.11); CML (r−0.38, P=0.038); and pentosidine (r−0.45, P=0.012). Finally, the most noteworthy and consistent observation in both AL and CR cohorts was the interrelationships between s.c. edema and pathologies involving both the spleen and liver. In short, infiltrative hepatopathy was positively correlated with hepatomegaly (AL, r=0.71, P<0.0001; CR, r=0.58, P=0.001), splenomegaly (AL, r=0.44, P=0.013; CR, r=0.49, P=0.004) and s.c. edema (AL, r=0.45, P=0.011; CR, r=0.68, P<0.0001). These observations are most likely related to metastatic neoplasia causing sequential hepatic dysfunction, hypoproteinemia, and edema.

TABLE 4. *Summary of the most probable cause of death of mice in the longitudinal study*

Group	Major diagnosis	Total number
Ad libitum (AL):	*Hepatoma/neoplasia* [liver (7), stomach tumor with metastases to liver (1), hemorrhage of splenic tumor with metastases to liver (1), angiosarcoma with metastases to liver (1)]	10
	Lymphoma [enlarged spleen (5), enlarged liver (5), metastases to liver (2), enlarged inguinal lymph node (2), enlarged sublumbar lymph node (1), enlarged mesenteric lymph node (3), enlarged thymus (1), mass near adrenal area of kidney (1), duodenal mass with partial obstruction (1)]	9
	Unknown/not apparent [liver insufficiency and degeneration (1)]	9
	Congestive heart failure [secondary to left atrial mass (1)]	2
	Renal disease [dilated renal pelvis with possible obstruction of kidney, enlarged urinary bladder]	1
		Total: Σ 31
Calorie restricted (CR):	*Unknown*/not apparent [liver insufficiency and degeneration (6)]	13
	Hepatoma/neoplasia [processes involving liver (1), both spleen and liver (5), spleen and kidney (1), angiosarcoma of spleen with metastases to liver (1), loss of lobular structure and modelling of liver associated with hypoproteinemia and pleural infusion (1)]	10
	Congestive heart failure	3
	Starvation secondary to eating problem [malocclusion (1), missing incisor (2)]	3
	Lymphoma [enlarged mesenteric (2) and sublumbar (1) lymph nodes, associated with neoplasia present in liver and kidneys (2), enlarged kidneys (1)]	2
	Intestinal obstruction	1
		Total: Σ 32

Major diagnoses as probable cause of death

From necropsy examinations and previous pathological analyses, major diagnoses were evaluated as to the most probable cause of death for each mouse. These results are summarized in **Table 4**. As observed, the causes of death were not determined in 13 CR and 9 AL mice. Neoplasia, including both hepatoma (n=10) and lymphoma (n=9), was the major diagnosis made in AL mice. Even though hepatoma (n=10) was major in CR mice, the incidence of lymphoma (n=3) was suppressed in this cohort.

DISCUSSION

To our knowledge, this study provides the first evidence that markers of glycation and glycoxidation can predict individual longevity in a rodent strain. The results are most likely related to glucose tolerance in the aging mouse. In humans, it has generally been concluded that glucose tolerance progressively deteriorates with chronological age even without regard to nutritional and physical states (4). The significance of this impaired tolerance is important and has profound significance for the elderly population. The impairment can either contribute, cause, or catalyze a wide range of diseases such as diabetes and various cardiovascular anomalies including hypertension, atherosclerosis, and coronary artery disease (34, 35). A further concern is for nondiabetic or borderline diabetic individuals who are unaware or apathetic to its diagnosis, risks, and ramifications (34–36). In rodents, however, experimental data concerning impairment of glucose tolerance with advancing age have depended on species, strain, and gender examined (6). However, it has generally been concluded that an insulin-resistant state exists in aging rodents similar to that observed in humans (3).

In previous cross-sectional studies with C57BL/6 mice, it was found that glycohemoglobin levels increased until ~12 months of age and declined thereafter, thereby reflecting the body weight data in Fig. 2. This suggests that glycohemoglobin measurement is not a reliable indicator of cumulative glycemia at late age in this strain (6). Cross-sectional comparisons of CR vs. AL subsequently showed no difference in pentosidine levels in skin and tendon collagens at ages 6, 12, 18, or 24 months (7). Conversely, CR vs. AL was found to significantly inhibit furosine formation in skin collagen at 24 months, but not at earlier ages (6). Taken together, these results suggest that impairment of glucose tolerance probably starts some time late in the life span of these mice—soon after the age of 20 months.

In three separate studies of the accumulation of AGEs and intervention paradigms on Type I diabetes in humans it was found that the degree of glycemic

control strongly modulated the levels of glycation and AGE formation in skin collagen (27, 39, 40). In general, these results showed that the more intensive the therapy, the slower rate of the accumulation. Most noteworthy was that glycation (Fig. 1) was reversed with improved glycemic control in all studies (27, 39, 40). However, the hypothesis of whether AGE formation can be reversed with diabetic intervention remains questionable (27, 39). In one study comparing long-term intensive vs. conventional therapy in participants to the Diabetes Control and Complications Trial, collagen glycation as furosine emerged as the parameter most consistently associated with diabetic complications in subjects not under intensive glycemic control (40) even when all parameters (including glycated hemoglobin and measures of collagen cross-linking, glycation, and glycoxidation) were evaluated. In this study, it was observed that despite the reversibility of glycation (Fig. 1), glycated residues in collagen surprisingly reflected cumulative glycemia over 12 months and more. Thus, steady-state levels of glycemia tend to change little in diabetic individuals over long periods of time (40). A similar conclusion was reached in the studies of age-related glycemic patterns in healthy individuals (41).

In the present investigation, the finding that furosine formation rates (*dFur/dt*) are better predictors of individual longevities in mice than those of CML and pentosidine (Figs. 6 and 7) may best be explained by the biochemical origins of these products (Fig. 1). Whereas furosine can originate only from glucose (42) and reflect cumulative glycemia (40, 41), CML and pentosidine can originate from multiple sugars and are influenced by oxidative events that are catalyzed by transition metals and inhibited by antioxidative factors intrinsic and extrinsic to tissues (43). Furthermore, CML has multiple origins, including serine myeloperoxidase (44) and lipid peroxidation (45) reactions. In contrast, pentosidine is specific for the Maillard reaction (6). Indeed, superimposed on these events is the fact that collagen turnover decreases with age (1). Undoubtedly, the reversibility of the glycation reaction shown in Fig. 1 may be minimized by this factor.

Previous studies by Masoro et al. (46) showed that CR maintains lower plasma glucose and insulin levels than AL control rats. Such results suggest a primary role of an altered carbohydrate metabolism by CR in influencing aging processes and enhanced life span. Based on these findings (46), it was subsequently postulated by Parr (47) that insulin exposure and sensitivity control the rate of mammalian aging. The enhancement by CR was speculated to be mediated through insulin-induced activation of tyrosine kinase receptor and subsequent signaling pathways. Studies with the nematode model of aging, *C. elegans*, showed that mutations in the *daf-2* gene that encodes an insulin receptor-like molecule, DAF-2, increases life span (48). The fact that components of DAF-2 show strong structural and functional homology to the mammalian insulin receptor, including a tyrosine kinase domain, suggests involvement in the insulin signaling pathway that controls both metabolic and diapause events in this worm. Thus, it was concluded by Kimura et al. (48) that mutations in the *daf-2* gene mimic the effects of CR on life extension observed in rodents by a similar mechanism. However, a more recent study of CR in *C. elegans* suggests this mechanism is distinct from that of CR. *Daf-2* mutations control diapause events, leading to a distinct phenotypic dark appearance due to fat accumulation in the intestine. In contrast, mutations in other genes such as *eat-2*, which controls food intake, present a totally different phenotype; namely, emaciation and a very pale appearance due to decreased food intake and low intestinal stores of fat (49).

Since the discovery of longevity genes that are associated with these glycation and glycoxidation reactions is of considerable interest for mammalian aging, collaborative efforts with Drs. Richard Miller and David Burke (University of Michigan) are in progress to map specific gene loci markers with measured parameters of this study in a population of genetically heterogeneous mice, i.e., progeny of (BALB/cJ X C57BL/6J) F1 mothers crossed to (C3H/HeJ X DBA/2J) F1 fathers. Preliminary results (unpublished observations) suggest genes on mouse chromosomes 1, 2, and 8 may have associations with CML, furosine, and pentosidine, respectively. Preliminary data, though not yet fully conclusive, suggest that a locus on chromosome 12 may influence both longevity and low levels of furosine in collagen.

In the present investigation, specific pathologies were followed for major diagnoses at death in mice and associations with measured parameters. Incidences of neoplasia, dermatitis, and seminal vesiculitis (Fig. 8) have been observed for this strain by other investigators as well (32, 50–53). This is in addition to the noted delay/suppression by CR on these pathologies (30–32, 54). Of particular interest is that in a previous study, CR was able to retard the development of diethylnitrosamine-induced tumors in the livers of mice (55). In this study, age-related insulinaemia reflecting resistance of tissues to the effects of insulin was much greater for AL vs. CR mice and correlated with the number of liver foci. In consideration of the known mitogenic effects of insulin on cultured hepatocytes, these investigators hypothesize that insulin may contribute to the promotion of these tumors *in vivo*. In light of the findings of Barzilai and Rossetti (3) showing the association between hepatic insulin resistance and

age in rodents, it is intriguing that numerous studies describing cell surface molecules known to be involved in the signaling cascades for oncogene activation are characterized as insulin receptors (56–58). These discoveries are significant for three reasons. First, the overexpression or mutation of oncogenes such as *Ras* genes is a common cause of cancer especially liver neoplasia in mice (57). Second, dysregulation of the signaling pathways at the level of the receptor or other key elements in the cascade has been shown to result in a variety of neoplastic disorders such as hepatoma, lymphoma, and other carcinomas (58). Third, recent epidemiological evidence points to a link between non-insulin-dependent Type II diabetes and various cancers in humans, suggesting an association between neoplasia and insulin resistance (58).

In conclusion, the results of this longitudinal study show that the skin collagen glycation rate measured as furosine could predict early death in AL and CR mice (Table 3). Similarly, pentosidine and CML when used as indices of glycoxidation could predict early death in AL and CR mice, respectively (Table 3). However, further stepwise regression analysis selected diet and rate of glycation of skin collagen as the most important factors in explaining longevity of mice. Hence, this latter result further supports the proposition made in a previous study (6) that glucose intolerance of yet unknown etiology probably occurs at a late age in these mice and plays an important role in catalyzing pathological lesions in tissues. FJ

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REFERENCES

1. Sell, D. R., and Monnier, V. M. (1995) Aging of long-lived proteins: extracellular matrix (collagens, elastins, proteoglycans) and lens crystallins. In *Physiology of Aging* (Masoro, E. J., ed) pp. 235–305, Oxford University Press, New York
2. Cerami, A. (1985) Hypothesis: glucose as a mediator of aging. *J. Am. Geriat. Soc.* **33,** 624–626
3. Barzilai, N., and Rossetti, L. (1996) Age-related changes in body composition are associated with hepatic insulin resistance in conscious rats. *Am. J. Physiol.* **270,** E930–E936
4. Paolisso, G., Gambardella, A., Ammendola, S., D'Amore, A., Balbi, V., Varricchio, M., and D'Onofrio, F. (1996) Glucose tolerance and insulin action in healthy centenarians. *Am. J. Physiol.* **270,** E890–E894
5. Sell, D. R., Lane, M. A., Johnson, W. A., Masoro, E. J., Mock, O. B., Reiser, K. M., Fogarty, J. F., Cutler, R. G., Ingram, D. K., Roth, G. S., and Monnier V. M. (1996) Longevity and the genetic determination of collagen glycoxidation kinetics in mammalian senescence. *Proc. Natl. Acad. Sci. USA* **93,** 485–490
6. Sell, D. R. (1997) Ageing promotes the increase of early glycation Amadori product as assessed by ε-N-(2-furoylmethyl)-L-lysine (furosine) levels in rodent skin collagen. The relationship to dietary restriction and glycoxidation. *Mech. Ageing Dev.* **95,** 81–99
7. Sell, D. R., and Monnier, V. M. (1997) Age-related association of tail tendon break time with tissue pentosidine in DBA/2 vs. C57BL/6 mice: The effect of dietary restriction. *J. Gerontol. Biol. Sci.* **52A,** B277–B284
8. Sprott, R., and Austad, S. N. (1996) Animal models for aging research. In *Handbook of the Biology of Aging* (Schneider, E. L., and Rowe, J. W., eds) pp. 3–23, Academic Press, New York
9. Kalant, N., Stewart, J., and Kaplan, R. (1988) Effect of diet restriction on glucose metabolism and insulin responsiveness in aging rats. *Mech. Ageing Dev.* **46,** 89–104
10. Feuers, R. J., Casciano, D. A., Shaddock, J. G., Leakey, J. E. A., Duffy, P. H., Hart, R. W., Hunter, J. D., and Scheving, L. E. (1991) Modifications in regulation of intermediary metabolism by caloric restriction in rodents. In *Biological Effects of Dietary Restriction* (Fishbein, L., ed) Chap. 19, pp. 198–206, Springer-Verlag, 1991, New York
11. Kemnitz, J. W., Roecker, E. B., Weindruch, R., Elson, D. F., Baum, S. T., Bergman, R. N. (1994) Dietary restriction increases insulin sensitivity and lowers blood glucose in rhesus monkeys. *Am. J. Physiol.* **266,** E540–E547
12. Bodkin, N. L., Ortmeyer, H. K., and Hansen, B. C. (1995) Long-term dietary restriction in older-aged rhesus monkeys: effects on insulin resistance. *J. Gerontol. Biol. Sci.* **50A,** B142–B147
13. Lane, M. A., Ball, S. S, Ingram, D. K., Cutler, R. G., Engel, J., Read, V., and Roth, G. S. (1995) Diet restriction in rhesus monkeys lowers fasting and glucose-stimulated glucoregulatory end points. *Am. J. Physiol.* **268,** E941–E948
14. Cefalu, W. T., Bell-Farrow, A. D., Wang, Z. Q., Sonntag, W. E., Fu, M.-X., Baynes, J. W., and Thorpe, S. R. (1995) Caloric restriction decreases age-dependent accumulation of the glycoxidation products, N^{ε}-(carboxymethyl)lysine and pentosidine, in rat skin collagen. *J. Gerontol. Biol. Sci.* **50A,** B337–B341
15. Levi, B., and Werman, M. J. (1998) Long-term fructose consumption accelerates glycation and several age-related variables in male rats. *J. Nutr.* **128,** 1442–1449
16. Murtagh-Mark, C. M., Reiser, K. M., Harris, R., and McDonald, B. B. (1995) Source of dietary carbohydrate affects life span of Fischer 344 rats independent of caloric restriction. *J. Gerontol. Biol. Sci.* **50A,** B148–B154
17. Mlekusch, W., Lamprecht, M., Öttl, K., Tillian, M., and Reibnegger, G. (1996) A glucose- rich diet shortens longevity of mice. *Mech. Ageing Dev.* **92,** 43–51
18. Baker, G. T., and Sprott, R. L. (1988) Biomarkers of aging. *Exp. Gerontol.* **23,** 223–239
19. McClearn, G. G. (1997) Biomarkers of age and aging. *Exp. Gerontol.* **32,** 87–94
20. Miller, R. A., Bookstein, F., Van der Meulen, J., Engle, S., Kim, J., Mullins, L., and Faulkner, J. (1997) Candidate biomarkers of aging: age-sensitive indices of immune and muscle function covary in genetically heterogeneous mice. *J. Gerontol. Biol. Sci.* **52A,** B39–B47
21. Mooradian, A. D. (1990) Biomarkers of aging: do we know what to look for? *J. Gerontol. Biol. Sci.* **45,** B183–B186
22. Dyer, D. G., Dunn, J. A., Thorpe, S. R., Bailie, K. E., Lyons, T. J., McCance, D. R., and Baynes, J. W. (1993) Accumulation of Maillard reaction products in skin collagen in diabetes and aging. *J. Clin. Invest.* **91,** 2463–2469
23. Witt, W. M., Sheldon, W. G, and Thurman, J. D. (1991) Pathological endpoints in dietary restricted rodents—Fischer 344 rats and B6C3F1 mice. In *Biological Effects of Dietary Restriction* (Fishbein, L., ed) pp. 73–86, Springer-Verlag, New York
24. Hart, R. W., Hass, B. S., Turturro, A., and Lewis, S. M. (1996) The use of nutrition to implement refinement in animal bioassays. *Lab Animal* (March Issue) 29–34
25. Dunn, J. A., McCance, D. R., Thorpe, S. R., Lyons, T. J., and Baynes, J. W. (1991) Age-dependent accumulation of N^{ε}-(carboxymethyl)lysine and N^{ε}-(carboxymethyl)-hydroxylysine in human skin collagen. *Biochemistry* **30,** 1205–1210
26. Glomb, M. A., and Monnier, V. M. (1995) Mechanism of protein modification by glyoxal and glycolaldehyde, reactive intermediates of the Maillard reaction. *J. Biol. Chem.* **270,** 10017–10026
27. Lyons, T. J., Bailie, K. E., Dyer, D. G., Dunn, J. A., and Baynes, J. W. (1991) Decrease in skin collagen glycation with improved glycemic control in patients with insulin-dependent diabetes mellitus. *J. Clin. Invest.* **87,** 1910–1915
28. Sprott, R. L. (1997) Diet and calorie restriction. *Exp. Gerontol.* **32,** 205–214
29. Wells-Knecht, M. C., Lyons, T. J., McCance, D. R., Thorpe, S. R., and Baynes, J. W. (1997) Age-dependent increase in ortho-

tyrosine and methionine sulfoxide in human skin collagen is not accelerated in diabetes. *J. Clin. Invest.* **100,** 839–856
30. Weindruch, R., and Walford, R. L. (1982) Dietary restriction in mice beginning at one year of age: effect on life-span and spontaneous cancer incidence. *Science* **215,** 1415–1418
31. Bronson, R. T., and Lipman, R. D. (1991) Reduction in rate of occurrence of age related lesions in dietary restricted laboratory mice. *Growth Dev. Aging* **55,** 169–184
32. Turturro, A., and Hart, R. (1992) Dietary alteration in the rates of cancer and aging. *Exp. Gerontol.* **27,** 583–592
33. Masoro, E. J. (1993) Dietary restriction and aging. *J. Am. Geriatr. Soc.* **41,** 994–999
34. Harlan, Y. C., Harlan, W. R., Landis, J. R., and Goldstein, N. G. (1987) Factors associated with glucose tolerance in adults in the United States. *Am. J. Epidemiol.* **126,** 674–684
35. Matts, J. P., Buchwald, H., Fitch, L. L., Campos, C. T., Varco, R. L., Campbell, G. S., Pearce, M. B., Yellin, A. E., Smink, R. D., Jr., Sawin, H. S., Jr., and Long, J. M. (1995) Subgroup analyses of the major clinical endpoints in the program on the surgical control of the hyperlipidemias (POSCH): Overall mortality, atherosclerotic coronary heart disease (ACHD), and ACHD mortality or myocardial infarction. *J. Clin. Epidermiol.* **48,** 389–405
36. Gerstein, H. C., Pais, P., Pogue, J., and Yusuf, S. (1999) Relationship of glucose and insulin levels to the risk of myocardial infarction: A case-control study. *J. Am. Coll. Cardiol.* **33,** 612–619
37. Monnier, V. M., and Cerami, A. (1983) Nonenzymatic glycosylation and browning of proteins in vivo. *Am. Chem. Soc.* **215,** 431–449
38. Monnier, V. M. (1989) Toward a Maillard reaction theory of aging. In *The Maillard Reaction in Aging, Diabetes, and Nutrition* (Baynes, J. W., and Monnier, V. M., eds) pp. 1–22, Alan R. Liss, New York
39. Beisswenger, P. J., Moore, L. L., and Curphey, T. J. (1993) Relationship between glycemic control and collagen-linked advanced glycosylation end products in Type I diabetes. *Diabetes Care.* **16,** 689–694
40. Monnier, V. M., Bautista, O., Kenny, D., Sell, D. R., Fogarty, J., Dahms, W., Cleary, P. A., Lachin, J., Genuth, S., and DCCT Ancillary Group. (1999) Skin collagen glycation, glycoxidation, and crosslinking are lower in subjects with long-term intensive versus conventional therapy of Type 1 diabetes. Relevance of glycated collagen products versus HbA_{1c} as markers of diabetic complications. *Diabetes* **48,** 870–880
41. Baynes, J. W., Watkins, N. G., Fisher, C. L., Hull, C. J., Patrick, J. S., Ahmed, M. U., Dunn, J. A., and Thorpe, S. R. (1989) The Amadori on protein: structure and reactions. In *The Maillard Reaction in Aging, Diabetes, and Nutrition* (Baynes, J. W., and Monnier, V. M., eds) pp. 43–67, Alan R. Liss, New York
42. Furth, A. J. (1988) Methods for assaying nonenzymatic glycosylation. *Anal. Biochem.* **175,** 347–360
43. Fu, M.-X., Wells-Knecht, K. J., Blackledge, J. A., Lyons, T. J., Thorpe, S. R., and Baynes, J. W. (1994) Glycation, glycoxidation and crosslinking of collagen by glucose: kinetics, mechanisms and inhibition of late stages of the Maillard reaction. *Diabetes* **43,** 676–683
44. Anderson, M. M., Hazen, S. L., Hsu, F. F., and Heinecke, J. W. (1997) Human neutrophils employ the myeloperoxidase-hydrogen peroxide-chloride system to convert hydroxy-amino acids into glycolaldehyde, 2-hydroxypropanal, and acrolein. A mechanism for the generation of highly reactive alpha-hydroxy and alpha, beta-unsaturated aldehydes by phagocytes at sites of inflammation. *J. Clin. Invest.* **99,** 424–432
45. Fu, M.-X., Requena, J. R., Jenkins, A. J., Lyons, T. J., Baynes, J. W., and Thorpe, S. R. (1996) The advanced glycation end product, N^{ϵ}(carboxymethyl)lysine, is a product of both lipid peroxidation and glycoxidation reactions. *J. Biol. Chem.* **271,** 9982–9986
46. Masoro, E. J., McCarter, R. J. M., Katz, M. S., and McMahan, C. A. (1992) Dietary restriction alters characteristics of glucose fuel use. *J. Gerontol. Biol. Sci.* **47,** B202–B208
47. Parr, T. (1996) Insulin exposure controls the rate of mammalian aging. *Mech. Ageing Dev.* **88,** 75–82
48. Kimura, K. D., Tissenbaum, H. A., Liu, Y., and Ruvkun, G. (1997) *Daf-2*, an insulin receptor-like gene that regulates longevity and diapause in *C. elegans. Science* **277,** 942–946
49. Lakowski, B., and Hekimi, S. (1998) The genetics of caloric restriction in *C. elegans. Proc. Natl. Acad. Sci. USA* **95,** 13091–13096
50. Finch, C. E., and Girgis, F. G. (1974) Enlarged seminal vesicles of senescent C57BL/6J mice. *J. Gerontol. Biol. Sci.* **29,** 134–138
51. Parkening, T. A., Collins, T. J., and Au, W. W. (1988) Paternal age and its effects on reproduction in C57BL/6NNia mice. *J. Gerontol. Biol. Sci.* **43,** B79–B84
52. Witt, W. M. (1989) An idiopathic dermatitis in C57BL/6N mice effectively modulated by dietary restriction. *Lab Anim. Sci.* **39,** 470
53. Andrews, A. G., Dysko, R. C., Spilman, S. C., Kunkel, R. G., Brammer, D. W., and Johnson, K. J. (1994) Immune complex vasculitis with secondary ulcerative dermatitis in aged C57BL/6NNia mice. *Vet. Pathol.* **31,** 293–300
54. Perkins, S. N., Hursting, S. D., Phang, J. M., and Haines, D. C. (1998) Calorie restriction reduces ulcerative dermatitis and infection-related mortality in p53-deficient and wild-type mice. *J. Invest. Dermatol.* **111,** 292–296
55. Lagopoulos, L., Sunahara, G. I., Würzner, H., Dombrowsky, I., and Stalder, R. (1991) The effects of alternating dietary restriction and ad libitum feeding of mice on the development of diethylnitrosamine-induced liver tumors and its correlation to insulinaemia. *Carcinogenesis* **12,** 311–315
56. Martin-Zanca, D., Barbacid, M., and Parada, L. T. (1990) Expression of the *trk* proto- oncogene is restricted to the sensory cranial and spinal ganglia of neural crest origin in mouse development. *Genes Dev.* **4,** 683–694
57. Pearline, R. V., Lin, Y.-Z., Shen, K. J., Brunt, E. M., Bowling, W. M., Hafenrichter, D. G., Kennedy, S., Flye, M. W., and Ponder, K. P. (1996) Alterations in enzymatic functions in hepatocytes and hepatocellular carcinomas from *Ras*-transduced livers resemble the effects of insulin. *Hepatology* **24,** 838–848
58. Morris, S. W., Naeve, C., Mathew, P., James, P. L., Kirstein, M. N., Cui, X., and Witte, D. P. (1997) *ALK*, the chromosome 2 gene locus altered by the t(2;5)in non-Hodgkin's lymphoma, encodes a novel neural receptor tyrosine kinase that is highly related to leukocyte tyrosine kinase (LTK). *Oncogene* **14,** 2175–2188

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