

Racemized and Isomerized Proteins in Aging Rat Teeth and Eye Lens

Rebecca A. Warmack,¹ Eduardo Mansilla,² Rodolfo G. Goya,³ and Steven G. Clarke¹

Abstract

The quantification of aspartic acid racemization in the proteins of nonmetabolically active tissues can be used as a measure of chronological aging in humans and other long-lived organisms. However, very few studies have been conducted in shorter-lived animals such as rodents, which are increasingly used as genetic and metabolic models of aging. An initial study had reported significant changes in the ratio of D- to L-aspartate in rat molars with age. Using a sensitive HPLC method for the determination of D- and L-aspartate from protein hydrolysates, we found no accumulation of D-aspartate in the molars of 17 rats that ranged in age from 2 to 44 months, and the amount of D-aspartate per molar did not correspond with molar eruption date as had been previously reported. However, developing an alternate approach, we found significant accumulation of isomerized aspartyl residues in eye lens proteins that are also formed by spontaneous degradation processes. In this study, we used the human protein L-isoaspartate/D-aspartate *O*-methyltransferase (PCMT1) as an analytical reagent in a sensitive and convenient procedure that could be used to rapidly examine multiple samples simultaneously. We found levels of isomerized aspartyl residues to be about 35 times higher in the lens extracts of 18-month-old rats versus 2-month-old rats, suggesting that isomerization may be an effective marker for biological aging in this range of ages. Importantly, we found that the accumulation appeared to plateau in rats of 18 months and older, indicating that potentially novel mechanisms for removing altered proteins may develop with age.

Introduction

THE USE OF RODENT MODELS in aging research can provide information on how drugs, diet, or environment affect longevity and health.¹⁻⁴ This research often involves transgenic or knockout rodent lines for the analysis of a specific gene, protein, or phenotype. For these studies, it is useful to correlate age, genetic alterations, or therapeutic interventions with changes in molecular markers of aging.

L-Aspartate and L-asparagine residues in aging proteins are subject to a variety of spontaneous chemical reactions, including racemization, isomerization, and deamidation.^{5,6} The accumulation of proteins containing these altered residues has been correlated with the decline in physiological function in the aging process.⁷ These amino acid residues are particularly prone to degradation due to the electrophilicity of the γ -carbonyl carbon in their side chains. In less metabolically active biological tissues, such as bone, teeth, or eye lens,

structural proteins may have lifetimes nearly as long as the organism itself and can accumulate these spontaneous modifications.⁸ In organisms with long life spans such as humans, aspartic acid racemization has been a commonly used measure for age determination in forensic science.^{9,10} The accuracy of age validation by D-aspartate accumulation for human tooth dentin has been estimated to be ± 3 years.¹¹ Similar studies have investigated the aspartic acid racemization in human bones and blood stains.^{12,13}

However, very little work has been done to optimize these racemization dating methods for smaller shorter-lived animals, such as rats. Only two studies have been done within rodents investigating the correlation of aspartic acid racemization in teeth with the age of the animal. In 1995, Ohtani et al. reported significant differences between D-aspartate in rat molar enamel from rats ranging in age from 55 to 250 days.¹⁴ They also reported that D/L-aspartate ratios corresponded with the eruption dates of the individual

¹Department of Chemistry and Biochemistry, The Molecular Biology Institute, University of California, Los Angeles, Los Angeles, California.

²Tissue Engineering, Regenerative Medicine and Cell Therapies Laboratory, CUCAIBA, Buenos Aires Province Ministry of Public Health, Buenos Aires, Argentina.

³Institute for Biochemical Research (INIBIOLP)-Cathedra of Histology B, Cathedra of Pathology B, School of Medicine, National University of La Plata, La Plata, Argentina.

molars. Interestingly, the recorded D/L ratios showed aspartate racemization rates some ten times faster than those observed in humans, possibly reflecting a species-specific rate of racemization.^{12,15} The second study was performed in senescence-accelerated mice using 2-, 6-, 12-, and 16-month-old animals. The D/L ratios reported from these whole molar samples also were shown to correlate with age.¹⁶

Human eye lens has also been analyzed for the accumulation of racemized aspartate derivatives. Like the mineralized collagens and associated proteins in teeth, the long-lived proteins of the eye lens, including α -, β - and γ -crystallin, can accumulate damaged residues. Masters et al. (1977) reported significant racemization rates within the human eye lens, with a linear correlation between age and the D/L aspartate ratio.¹⁷ In the case of crystallins, these modifications are associated with insolubilization and aggregation.¹⁸ The isomerization of aspartate and asparagine residues, resulting in the formation of L-isoaspartate and D-isoaspartate residues and linked with racemization through a common succinimide intermediate,^{19,20} has been linked to cataract formation.²¹ Although quantification of aspartate stereoisomers has been studied as another potential age marker in various other tissues,^{22–24} this has only been indirectly studied in the eye lens.^{21,25,26}

The studies described above demonstrate the time-dependent accumulation of aspartic acid derivatives and its correlation with age, particularly in long-lived species. With the prevalent use of mice and rats in aging and disease research, the development and optimization of methods used to investigate aging markers in these organisms are important. The goal of the work reported here was to explore the use of two age-related markers of aging in rats, racemization in molars and isomerization in the lens. Our results show that significant increases in racemization of aspartate/asparagine residues with age cannot be detected in molars, but that increases in the isomerization of these residues with age can be readily quantified in the lens.

Experimental Procedures

Collection of eye lens and teeth from aged rats

Sprague-Dawley (SD) rats were raised in the INIBIOLP rat colony at the National University of La Plata, Argentina. Animals were housed in a temperature-controlled room (22°C \pm 2°C) on a 12-hour light/12-hour dark cycle. Food and water are available *ad libitum*. In this rat colony, the average 50% survival time for females, studied in groups of 50–60 animals, is approximately 32 months, provided mammary tumors are systematically removed when detected by palpation. The 2-month-old, 18-month-old, and some of the 29-month-old animals received no treatment. However, some of the 29-month-old animals received, 3 months before sacrifice, an intracerebroventricular injection of a placebo adenovector expressing the reporter gene for the DsRed2 red fluorescent protein and were fixed as described below. Assessment of spatial memory by the Barnes Maze test²⁷ as well as immunohistochemical analysis of glial fibrillary acidic protein (for astrocytes) and doublecortin immunoreactive cells in the hippocampus (for neuroblasts) revealed no significant differences between these placebo control adenovector-treated and untreated animals. Furthermore, the incidence of cataracts in both groups at the time of sacrifice was comparable (unpublished data). Thus, it is unlikely that the

placebo adenovector treatment had a significant effect on the rate of eye lens or tooth protein racemization and isomerization. One rat (44 months old, designated SO) was intravenously injected with human bone marrow-derived mesenchymal stem cells as described.²⁸ Although the mesenchymal stem cell treatment seems to have extended the life span of the 44-month-old rat, it did not prevent the development of strong bilateral cataracts,²⁸ which again makes it improbable that the treatment had a significant impact on eye lens and tooth protein metabolism.

All animals used here were virgin females. Animals were sacrificed by rapid decapitation, except for the group of 29-month-old animals that were fixed. These animals were anesthetized with ketamine hydrochloride (70 mg/kg, i.p.) and xylazine (15 mg/kg, i.m.) and perfused with phosphate-buffered 4% paraformaldehyde (pH 7.4). After removal of the brain, the heads were frozen at -80°C and shipped to UCLA packed in dry ice. All experiments with animals were performed in accordance with the Animal Welfare Guidelines of NIH (INIBIOLP's Animal Welfare Assurance No. A5647-01); IACUC approved on April 3, 2007, extended December 19, 2011.

At UCLA, heads were thawed at 4°C for 3 days in 300 mL of deionized water. The jaws were separated and molars were removed using a burlisher. Excess soft tissue was removed and the molars were then cleaned in an ultrasonic water bath for 20 min in deionized water, followed by 20 min in ethanol, and vacuum-dried. Eyes were excised using surgical scissors and lenses were extracted under a Zeiss Stemi 2000 light microscope. Lenses were washed gently in 500 μL of deionized water in 1.5-mL microcentrifuge tubes on a rotator for 20 minutes at 4°C. Rat heads for lens extraction were not subjected to any tissue fixation.

Determination of D/L-aspartate ratios in molar hydrolysates

After drying, intact individual molars were acid hydrolyzed in 500 μL of 6 N HCl at 110°C for 6 hours under vacuum in a Waters PicoTag apparatus. Samples were vacuum-dried, resuspended in 50 μL of deionized water, and any remaining particulate material was removed by centrifugation. Analysis of *o*-phthalaldehyde/*N*-acetyl-L-cysteine (OPA-NAC) derivatives of aspartic acid was performed after the method of Aswad.²⁹ After a 20-fold dilution in water, 5 μL of the hydrolysate was added to 20 μL of the OPA-NAC reagent containing 4 mg *o*-phthalaldehyde (MP Biomedicals, LLC #102648) dissolved in 300 μL methanol, 250 μL 0.4 M potassium borate pH 10.3, 390 μL deionized water, and 60 μL 1 M *N*-acetyl-L-cysteine and was incubated for 3 minutes at room temperature. The reaction was stopped with the addition of 40 μL of 0.2 M sodium citrate, pH 5.5, and the solution analyzed on an HP Series II 1090 HPLC, using a 5 μm Agilent Eclipse C18 reverse-phase column (150 mm in length, 4.6 mm inner diameter) at 25°C. Solvent A was 50 mM Na acetate, pH 5.4. Solvent B was 80% (v/v) methanol and 20% (v/v) solvent A. Isocratic elution with 90% solvent A: 10% solvent B was carried out for 5 minutes. Then, solvent B was increased linearly to 100% over 5 minutes and maintained at 100% solvent B for 15 minutes. The flow rate was 1.0 mL/min throughout. Fluorescent derivatives were monitored using a Gilson Model 121 fluorimeter with a 50 watt halogen EPZ

projector lamp, an excitation filter of 305–395 nm (Gilson catalog number 095312), and a 450 wide-band emission filter (Gilson catalog number 095442). The RFU sensitivity was set at 0.05. The D-aspartate/L-aspartate ratio was calculated by integrated area under the appropriate peaks after correction for the background.

Determination of L-isoaspartate levels in eye lens proteins

Lenses from unfixed rat heads were thawed as described above, weighed, and then 20 μ L of T-PER Total Protein Extraction Reagent (Life Technologies 78510) was added per mg wet weight of lens at room temperature. Sodium EDTA, pH 7.5, and phenylmethanesulfonyl fluoride dissolved in isopropanol were added to final concentrations of 1 mM as protease inhibitors. Lenses were homogenized at room temperature in a 1.5-mL microcentrifuge tube with 200 strokes of a form-fitting plastic pestle (Kimble-Chase Kontes pellet pestle 7495150000; Fisher Scientific). Samples were centrifuged at 10,000 g for 5 minutes at room temperature to pellet unbroken cells and membrane debris. The supernatant was collected and stored at -20°C before further analyses.

The human recombinant protein L-isoaspartate/D-aspartate O-methyltransferase (PCMT1)—an enzyme that recognizes L-isoaspartate residues with high affinity³⁰—was used as an analytical reagent to quantify L-isoaspartate levels in the lens extract proteins. In a final volume of 100 μ L, 2.5 to 5 μ g of lens extract protein (as determined by a Lowry assay after precipitation with trichloroacetic acid) was incubated for 2 hours at 37°C with 5 μ g PCMT1 (purified as a His-tagged enzyme from *Escherichia coli* containing the expression plasmid #34852 available from Addgene.com as described by Patananan et al.,³¹ with a specific activity at 37°C of 3,361 pmol of methyl esters formed on ovalbumin/min/mg of enzyme) and final concentrations of 135 mM Bis-Tris-HCl, pH 6.4, and 10 μ M S-adenosyl-L-[methyl- ^3H]methionine ($[(^3\text{H})\text{AdoMet}]$) (prepared by a 1600-fold isotopic dilution of a stock of 72 Ci/mmol $[(^3\text{H})\text{AdoMet}]$ (PerkinElmer Life Sciences, NET155H00) with nonisotopically labeled AdoMet (*p*-toluenesulfonate salt; Sigma-Aldrich A2408)). The reaction was stopped by adding 10 μ L of 2 M sodium hydroxide, and 100 μ L of the 110 μ L mixture was transferred to a 9×2.5 cm piece of folded thick filter paper (Bio-Rad; catalog number 1650962), wedged in the neck of a 20-mL scintillation vial above 5 mL scintillation reagent (Safety Solve, Research Products International, catalog number 121000), tightly capped, and incubated at room temperature. After 2 hours, the folded filter papers were removed, the caps replaced, and the vials were counted thrice for 3 minutes each in a Beckman LS6500 scintillation counter. Background radioactivity in a no substrate blank was determined by incubating the recombinant human PCMT1, 135 mM Bis-Tris-HCl buffer, and 10 μ M $[(^3\text{H})\text{AdoMet}]$ as described above and was subtracted from the value obtained in experimental samples. Samples were analyzed in triplicate.

Rat eye lens endogenous protein L-isoaspartyl methyltransferase activity assays

In a final volume of 100 μ L, 10 μ L of lens extract protein was incubated for 2 hours at 37°C with final concentrations of 100 μ M KASA(isoD)LAKY peptide, 125 mM Bis-Tris-HCl,

pH 6.4, and 10 μ M $[(^3\text{H})\text{AdoMet}]$, as prepared above. The reaction was stopped by adding 10 μ L of 2 M sodium hydroxide, and 100 μ L of the 110 μ L mixture assayed for volatile radioactivity, as described in the section above. Background radioactivity was determined in a control lacking the lens extract and was subtracted from the value obtained in samples containing the lens extracts. Assays were performed in duplicate.

Results and Discussion

D-Aspartate accumulation in teeth cannot be used to determine rat age

Tooth enamel is 96% mineralized and contains only 4% protein.³² Most of the protein that is present is trapped in the solid matrix of hydroxyapatite and lasts for years with little to no metabolic turnover. Dentin is similarly solid and is approximately 70% mineralized. Given that the entrapped proteins can accumulate spontaneous chemical modifications, enamel and dentin can be useful for age determination through the quantification of protein damage.

D-Aspartate accumulation has been well correlated with age in human tooth enamel and dentin.^{33,34} Human teeth are relatively large, and the separation of enamel and dentin is a well-documented procedure.^{32,35} Ohtani et al.¹⁴ reported the successful application of these techniques to rat molar tooth enamel, a potentially exciting finding as rats live much shorter lives than humans, and rat teeth (4–10 mg) are much smaller than those in humans (500 to 2500 mg). In this article, the enamel was apparently separated from the rest of the molar with a rotary saw, and after hydrolysis, samples were analyzed for D- and L-aspartate by gas chromatography.

To optimize rodent age determination using reverse-phase liquid chromatography, attempts were made to replicate the removal of the dental crowns from the molars as described in Ohtani et al.¹⁴ However, the very small size of the rat teeth and the distribution of the enamel prevented separation in our hands. Personal communication with Ohtani revealed that in fact whole molars were used for their analyses as they also found that the teeth were too small for the separation of enamel and dentin and that the description of the separation was incorrect in their article. Thus, we also analyzed whole molars in this study.

After extraction and acid hydrolysis of the whole molar, OPA-NAC derivatives of amino acids were detected and quantified through RP-HPLC. Representative chromatographs can be seen in Figure 1, with D- and L-aspartate eluting at approximately 7 and 8 minutes, respectively, with baseline resolution (Fig. 1D). The D-aspartate peak was confirmed by running a hydrolysate alone (Fig. 1A), followed by a run containing half the amount of hydrolysate with added D-aspartate standard. In this study, the D-aspartate peak increased in area by sixfold, while all other peak areas were halved (Fig. 1B). Unhydrolyzed D- and L-Asp standards were also run to verify the elution times of the peaks of interest (Fig. 1C). These results demonstrate the reproducible separation and identification of D- and L-aspartate levels within the rat teeth with RP-HPLC analysis of OPA-NAC amino acid derivatives.

Respective levels of D- and L-aspartate were then quantified to calculate the ratios shown in Figure 2A, representing the amounts of aspartate racemization found in the individual

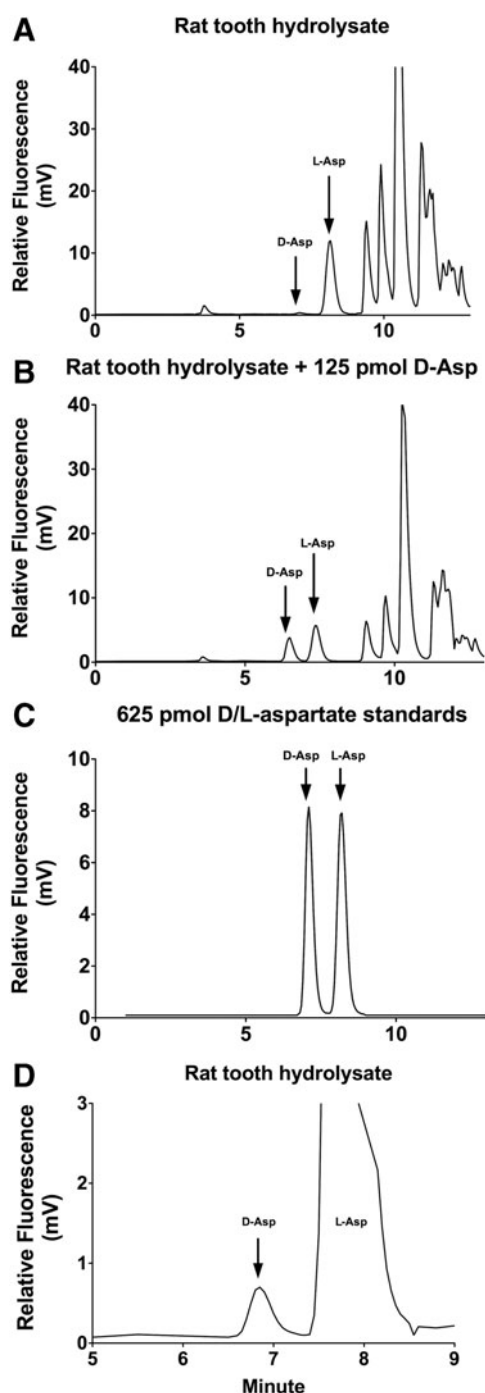


FIG. 1. Determination of the D-aspartate/L-aspartate ratio in rat tooth molar hydrolysates. (A) A representative chromatogram from reverse-phase HPLC analysis of OPA-NAC amino acid derivatives with fluorescence detection from a tooth hydrolysate of the first molar of a 29-month-old rat that was processed as described in the Experimental Procedures section. The position of D-Asp and L-Asp was ascertained by comparison of the elution positions of standards, chromatographed separately (not shown). (B) HPLC analysis of a mixture of half of the amount of tooth hydrolysate chromatographed in (A) with 125 pmol of a D-Asp standard. (C) An unhydrolyzed mixture of 625 pmol of D-Asp and 625 pmol of L-Asp was analyzed as described above. (D) An enlarged view of a portion of (A) showing the separation of the derivatives of D- and L-aspartate.

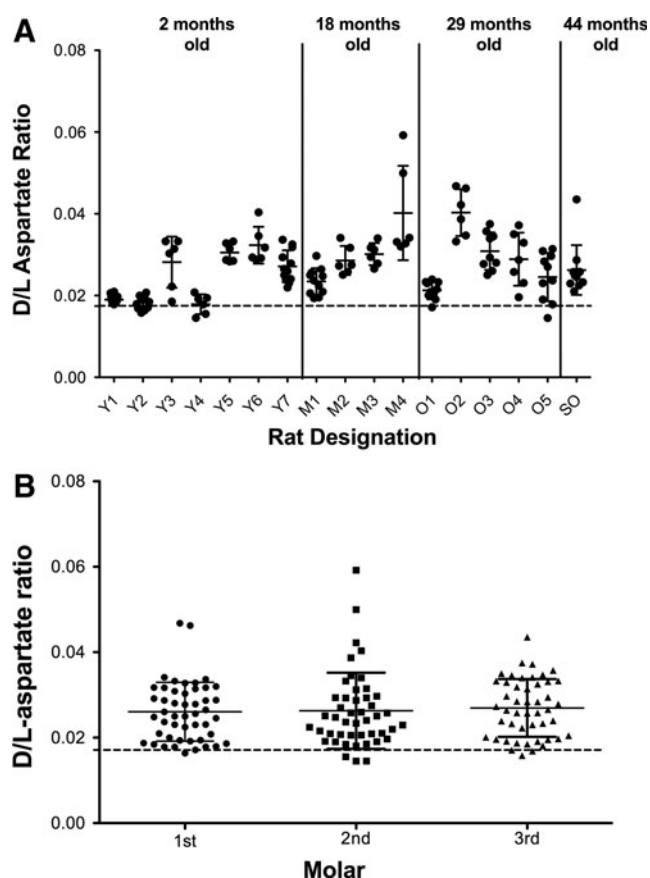


FIG. 2. D-Aspartate/L-aspartate ratios in rat molars do not significantly correlate with rat age or with the molar position. (A) Determination of D/L aspartate ratios of rat molar hydrolysates was performed as described in the Experimental Procedures section and Figure 1. Sets of first, second, and third molars were analyzed from seven young rats (Y1 to Y7; 2 months old), four middle-aged rats (M1 to M4; 18 months old), five old rats (O1 to O5; 29 months old), and one exceptionally old rat (S01; 44 months old). For some rats, independent analyses were made of each first, second, and third molar from the upper and lower jaw, as well as the left and right side, giving 12 samples per rat. In other cases, only molars from the right or left side were analyzed, giving 6 samples per rat. In a few cases, teeth were unavailable due to shattering during extraction from the jaw. Error bars indicate the standard deviation of the D/L aspartate ratio from all of the molars of an individual rat. A two-tailed Student's *t*-test with unpaired samples and unequal variance between the D/L-aspartate ratios of the groups of 2-, 18-, 29-, and 44-month-old samples revealed no significant differences in the D/L aspartate ratio (p -value >0.05). The dashed horizontal line shows the background racemization observed (D/L-aspartate ratio = 0.0174) when a sample of L-aspartic acid was acid hydrolyzed and analyzed under our conditions. (B) D/L-aspartate ratios plotted for each of the first, second, and third molars for the data shown in (A). Error bars indicate the standard deviation.

rat molars. Statistical analysis between the different age groups did not reveal significant differences in the D/L-aspartate ratios in contrast to what had been reported previously.¹⁴ In these experiments, four of the rat heads from the group at 29 months (designated O2, O3, O4, and O5 in Fig. 2A) had been fixed with 4% paraformaldehyde, while the

other rat head (designated O1) was simply frozen before analysis. We saw no differences in the degree of racemization of this group and the other age groups, suggesting that fixation did not affect this process.

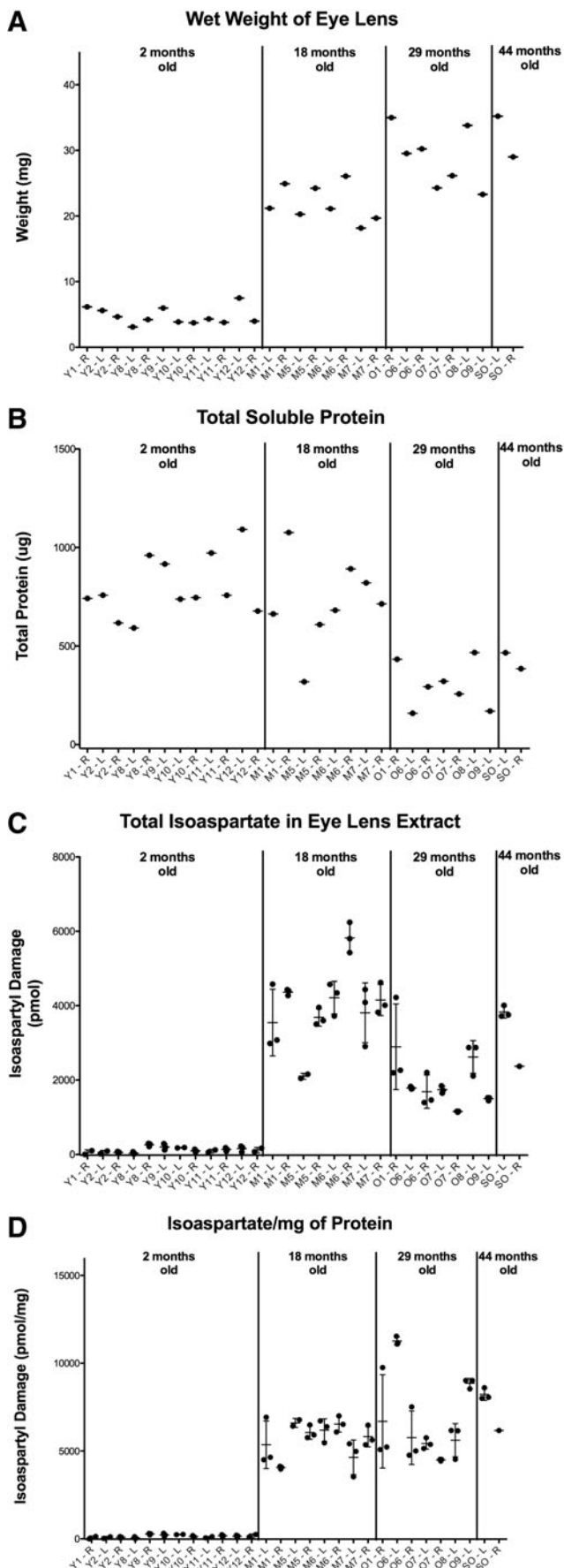
Rat molars are known to develop in a predictable manner, with the front molar erupting at approximately 19 days after birth, the second set on the 21st day, and the last set 35–40 days after birth.³⁶ Thus, in addition to exploring a relationship to age, we investigated the link between racemization and eruption date. Data for individual teeth were plotted according to the particular molar, shown in Figure 2B, and demonstrated that aspartate racemization does not significantly correspond with molar age relative to other molars in the mouth, contrary to the results reported by Ohtani et al.¹⁴

The discrepancies seen between our results and those in Ohtani et al.¹⁴ may have several origins. Our sampling included multiple rats for each age group excepting the 44-month-old rat, including seven 2-month-old rats, four 18-month-old rats, and five 29-month-old rats. The Ohtani group analyzed five individual rats of different ages ranging from about 2 months to 8 months. The increase in sample size may accurately represent the variability between rats of the same age group. Our age groups also spanned a much longer length of time.

It is also possible that there was some confusion in the Ohtani et al. publication¹⁴ since the techniques reported for the separation of molar enamel and dentin are not possible under the methods given. However, we demonstrate that the variability between rats of the same age prevents this method from reliably identifying rat age or from using D-aspartate levels as a marker of aging. Finally, our results suggest that the rates of racemization of rat molar proteins are probably not in fact markedly higher than in human teeth, as was indicated by the data of Ohtani et al.¹⁴

These results indicate that the rate of racemization of rat tooth proteins is slow enough that we cannot distinguish the

FIG. 3. L-isoaspartate accumulates in the rat eye lens after 2 months, but plateaus after 18 months of age. Rat eye lenses were extracted from seven young rats (2 months), four middle-aged rats (18 months), five old rats (29 months), and one exceptionally old rat (44 months), including several of the animals whose teeth were analyzed in Figure 2. Rats were identified as in Figure 2; R and L represent the left and right lenses. Cytosolic extracts were made and L-isoaspartate content measured as described in the Experimental Procedures section. (A) Wet weight of eye lens. (B) Data are shown for the total average amount of soluble protein from homogenized lens extract determined by triplicate Lowry protein assays after precipitation with 10% trichloroacetic acid. Student's *t*-test performed as in Figure 2 shows a *p*-value of 0.0007 for the averages of the technical replicates of the 18-month-old versus 29-month-old samples. (C) Data are shown for triplicate replicates of the total L-isoaspartate content in each extract. Error bars indicate standard deviation. Student's *t*-test shows a *p*-value of 0.0005 for the average values of the 18-month-old versus 29-month-old samples. (D) L-isoaspartate per mg protein. Error bars indicate standard deviation. No significant difference was found in the average values for the 18-month-old and 29-month-old samples (*p* > 0.05).



age-dependent accumulation above the background of spontaneous racemization resulting from the acid hydrolysis procedure. The acid hydrolysis conditions have been optimized to ensure near complete hydrolysis of peptide bonds linked to aspartic acid and asparagine residues while minimizing the background racemization.²³

L-isoaspartate levels in rat eye lens correspond with increased age

The degradation of L-aspartate and L-asparagine residues proceeds mainly through an L-succinimide intermediate.^{5,6,19} The rate of L-isoaspartyl formation from this intermediate is much greater than the rate of D-succinimide formation, resulting in a much faster accumulation of isomerized residues than racemized residues.¹⁹ Thus, damage to proteins at these residues may be better approached by analyzing L-isoaspartate.

We took advantage of the availability of an enzyme that specifically methylates both L-isoaspartate and D-aspartate residues, the human protein carboxyl methyltransferase 1 (PCMT1).²⁰ This enzyme recognizes L-isoaspartate residues with great preference over D-aspartate residues, resulting in its ability to serve as an analytical reagent for L-isoaspartyl residues.^{30,31,37-39} Although the methyltransferase has not been used in mineralized tissues such as tooth, it has been utilized to detect abnormal aspartyl residues that accumulate with age in mouse brain, heart, and testis proteins.⁴⁰ Given the slow protein turnover rates of the eye lens tissue and the higher abundance of proteins than in rat teeth, we hypothesized that L-aspartate isomerization to L-isoaspartate would be a better marker of age than protein racemization to D-aspartate.

Previous reports have indicated that the rat eye lens grows throughout life, but at a declining rate over the lifetime of the organism,^{41,42} a situation distinct from that seen in the human eye lens where most of the development occurs before one year of age and only increases very slowly thereafter.⁴³ In fact, lens wet weight has been used as a marker of rat age.^{42,44-46} From the results seen in Figures 3A and 4A, we also demonstrated an age-dependent increase in eye lens wet weight. However, it seems that as the lens grows, the amount of soluble protein decreases, suggesting the formation of damaged and aggregated proteins, dropping down from about 1000 μg in a 2-month-old rat to just below 500 μg in the 44-month-old rat (Figs. 3B and 4B).

Figures 3C, 3D, 4C, and 4D show the quantification of the levels of L-isoaspartate in proteins of the soluble extracts of lens from the vapor diffusion assay. From 2 months of age to 44 months, the level of isoaspartate per mg of protein (Figs. 3D and 4D) increases from an average of about 140 to 7200 pmol isoaspartate/mg eye lens protein, corresponding to about 0.00018 to 0.0090 residues of isoaspartate per residues of aspartate and asparagine. The largest differences occur between 2 months and 18 months of age. While the total levels of isoaspartate appear to be maximal at 18 months of age and decreasing at older ages following the decreases in soluble protein (Figs. 3B, C and 4B, C), the amount of isoaspartyl residues/mg protein appear to plateau after 18 months of age (Fig. 3D and 4D). Thus, as the eye lens ages, there is a significant increase in the isoaspartate level from 2 to 18 months, followed by decreases in both soluble protein and total isoaspartate in soluble proteins in

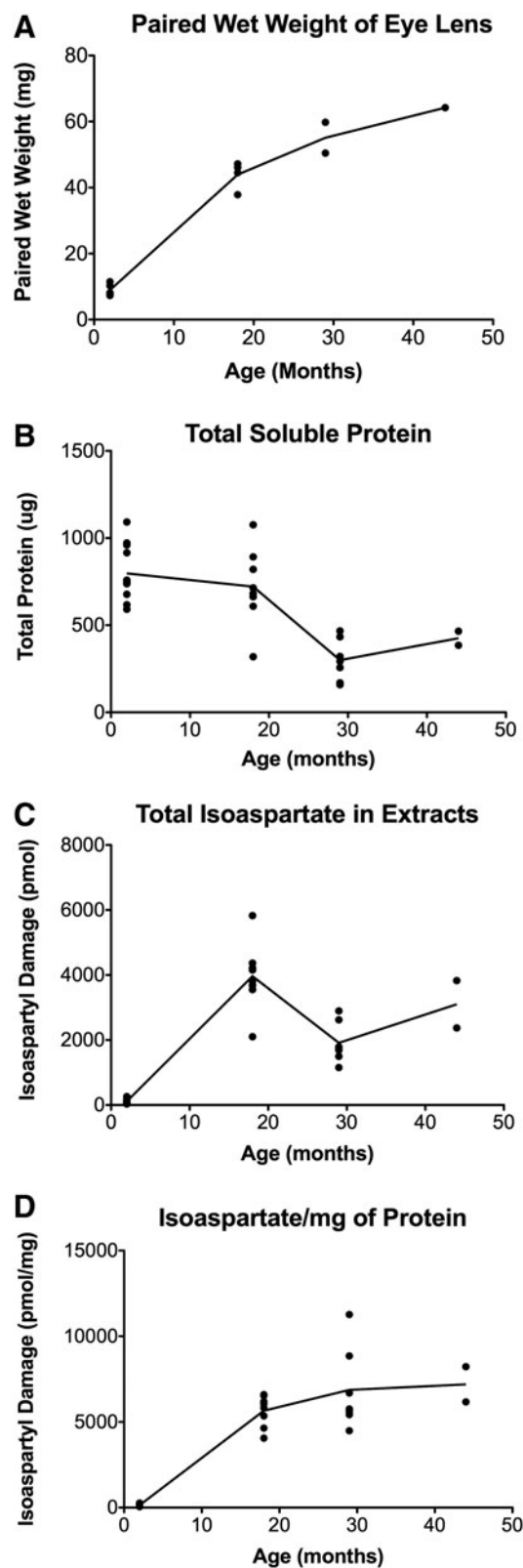


FIG. 4. The data from Figure 3 are plotted as a function of the age of the individual rats. In (A), the wet weights of the left and right lenses were added for each rat where both lenses were available. In (B–D), only the average values are shown.

the 29-month-old and 44-month-old animals. These results lead to a plateau in the amount of isoaspartate/mg protein by 18 months of age. The link between the amount of soluble protein and isoaspartate is not clear, but it is possible that isomerization itself may lead to aggregation. Interestingly, a similar situation may occur in human eye lens, where the endogenous PCMT1 enzyme is much less saturated with endogenous substrates before 30 years of age than after 50 years of age.²⁶

The plateau/decrease in total L-isoaspartate levels in soluble proteins and in the ratio of L-isoaspartate to total protein seen in the data of Figures 3C, 3D, 4C, and 4D by 18 months of age could have several origins. In the first place, lens tissues are known to contain endogenous PCMT1.²⁶ This enzyme catalyzes the first step of a pathway by which L-isoaspartate residues are converted to L-aspartate residues.^{20,30} It is possible that the activity of this enzyme is significantly increased in the older lenses, thus maintaining L-isoaspartate at a constant level.

To investigate this possibility, the specific activity of the endogenous PCMT1 enzyme was assayed in the eye lens extracts using saturating amounts of an L-isoaspartyl-containing peptide, KASA(isoD)LAKY (Fig. 5). Although we found a slight increase in the specific activity between the 2-month-old and 18-month-old samples, there was no further increase after 18 months and perhaps a decrease. Thus, it is unlikely that the plateau in the level of isoaspartate within the eye lens is due to increasing repair activity.

Since we observed that the levels of total soluble protein decreased over the lifetime of the rats, a second possibility is that the formation of aggregates may prevent the accumulation of L-isoaspartate residues. Finally, it is possible that other repair or maintenance pathways compensate in older animals by removing proteins with L-isoaspartate residues. The yeast *Saccharomyces cerevisiae*, for example, has no known homolog of the PCMT1 repair enzyme and yet is able to maintain low levels of L-isoaspartate, suggesting the

presence of other repair or degradation mechanisms, including a possible role of metalloproteases.³¹ It is conceivable then that proteases in the eye lens may recognize the high levels of aggregates or isomerized proteins and degrade these species to peptides and or amino acids.

In mice, it was similarly observed that in heart and testis extracts the amount of isoaspartate initially accumulates rapidly, and then levels off, suggesting again possible compensation mechanisms.⁴⁰ It is possible that the number of proteins with labile aspartate/asparagine residues is limited and that the plateau is due to all possible sites being racemized or isomerized. However, within an mg of protein, there are approximately 800 nmols of asparagine and aspartate residues, which may racemize or isomerize. The maximum level of damage that was observed in our study was about 12 nmol isoaspartate/mg protein (Fig. 3D), suggesting that we are only observing approximately 1.5% of sites forming aberrant derivatives.

As stated previously, whole tooth analysis for D-aspartate can validate a human individual's age to within 3 years. This variability exists, in part, due to the relatively slow rate of racemization and the background of spontaneous racemization that occurs during the acid hydrolysis step. In humans, the long life span can overcome most of these issues, simply because the age groups span across a greater length of time, which allows for the development of discernable levels of D-aspartate in teeth. Within rodents, however, where the life span is typically 2.5–3.5 years, a range of ± 3 years would not allow for accurate age determination. However, by taking advantage of L-isoaspartate's much more rapid rate of accumulation and measuring the accumulated levels of L-isoaspartate within eye lens extracts, we have shown clear differences between young and old rats. The relative ease of the preparation for these samples, as well as the more distinct differences in the measurements made, makes this technique an attractive alternative to previously reported methods.

Acknowledgments

The authors thank Dr. Aubrey D. N. J. de Grey of the SENS Research Foundation (Mountain View, CA) for initiating this work and for providing guidance throughout. The authors thank Dr. Shane White of the UCLA School of Dentistry for his help in preparing tooth samples and Drs. Rajendra Gangalum and Suraj Bhat of the UCLA Jules Stein Eye Institute for assistance and advice on the preparation of lens samples. They thank Dr. Jonathan Lowenson for his guidance in this work and for his help in preparing the manuscript. This work was supported by a grant from the Life Extension Foundation, Inc., and funds from the Elizabeth and Thomas Plott Chair in Gerontology of the UCLA Longevity Center. R.A.W. was supported by the Ruth L. Kirschstein National Research Service Award GM007185.

R.G.G. and E.M. provided the animal material; E.M. took responsibility for monitoring the health of the 44-month-old rat; R.G.G., R.A.W., and S.G.C. planned the study; R.A.W. performed the analytical experiments; R.A.W. and S.G.C. wrote the article and analyzed the data; and R.A.W., E.M., R.G.G., and S.G.C. reviewed and revised the article.

Author Disclosure Statement

No competing financial interests exist.

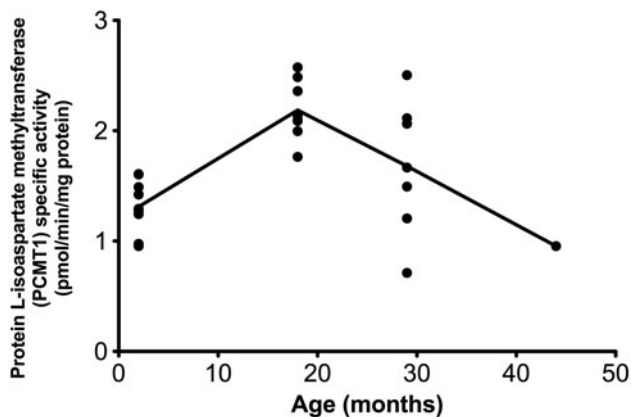


FIG. 5. Age dependence of the specific activity of endogenous protein L-isoaspartate methyltransferase in rat eye lens. Enzyme activity with an L-isoaspartyl-containing peptide was performed as described in the Experimental Procedures section. Each point represents the average of duplicate measurements. The connecting line shows the mean value of each age group. Student's *t*-test performed as in Figure 2 indicated that the only significant difference in the age groups was between the 2-month-old and 18-month-old groups ($p=4 \times 10^{-6}$).

References

1. Lebel M, Picard F, Ferland G, Gaudreau P. Drugs, nutrients, and phytoactive principles improving the health span of rodent models of human age-related diseases. *J Gerontol A Biol Sci Med Sci* 2012;67:140–151.
2. Spindler SR, Mote PL, Flegal JM, Teter B. Influence on longevity of blueberry, cinnamon, green and black tea, pomegranate, sesame, curcumin, morin, pycnogenol, quercetin, and taxifolin fed iso-calorically to long-lived, F1 hybrid mice. *Rejuvenation Res* 2013;16:143–151.
3. Foster TC, DeFazio RA, Bizon JL. Characterizing cognitive aging of spatial and contextual memory in animal models. *Front Aging Neurosci* 2012;4:12.
4. Stefanova NA, Kozhevnikova OS, Vitovtov AO, Maksimova KY, Logvinov SV, Rudnitskaya EA, Korbolina EE, Muraleva NA, Kolosova NG. Senescence-accelerated OXYS rats: A model of age-related cognitive decline with relevance to abnormalities in Alzheimer disease. *Cell Cycle* 2014;13:898–909.
5. Radkiewicz JL, Zipse H, Clarke S, Houk KN. Neighboring side chain effects on asparaginyl and aspartyl degradation: An *ab initio* study of the relationship between peptide conformation and backbone NH acidity. *J Am Chem Soc* 2001;123:3499–3506.
6. Wakankar AA, Borchardt RT. Formulation considerations for proteins susceptible to asparagine deamidation and aspartate isomerization. *J Pharm Sci* 2006;95:2321–2336.
7. Nowotny K, Jung T, Grune T, Höhn A. Accumulation of modified proteins and aggregate formation in aging. *Exp Gerontol* 2014;57:122–131.
8. Sakuma A, Ohtani S, Saitoh H, Iwase H. Comparative analysis of aspartic acid racemization methods using whole-tooth and dentin samples. *Forensic Sci Int* 2012;223:198–201.
9. Ritz-Timme S, Rochholz G, Schütz HW, Collins MJ, Waite ER, Cattaneo C, Kaatsch HJ. Quality assurance in age estimation based on aspartic acid racemization. *Int J Legal Med* 2000;114:83–86.
10. Arany S, Ohtani S, Yoshioka N, Gonmori K. Age estimation from aspartic acid racemization of root dentin by internal standard method. *Forensic Sci Int* 2004;141:127–130.
11. Waite ER, Collins MJ, Ritz-Timme S, Schutz HW, Cattaneo C, Borrman HI. A review of the methodological aspects of aspartic acid racemization analysis for use in forensic science. *Forensic Sci Int* 1999;103:113–124.
12. Collins MJ, Waite ER, van Duin AC. Predicting protein decomposition: The case of aspartic-acid racemization kinetics. *Philos Trans R Soc Lond B Biol Sci* 1999;354: 51–56.
13. Arany S, Ohtani S. Age estimation of bloodstains: A preliminary report based on aspartic acid racemization rate. *Forensic Sci Int* 2011;212:e36–e39.
14. Ohtani S, Matsushima Y, Ohhira H, Watanabe A. Age-related changes in D-aspartic acid of rat teeth. *Growth Dev Aging* 1995;59:55–61.
15. Ohtani S, Yamamoto T. Age estimation by amino acid racemization in human teeth. *J Forensic Sci* 2010;55: 1630–1633.
16. Ohtani S, Yamada Y, Yamamoto I, Marumo T, Sugeno H, Sugimoto H, Ogasawara A, Yamagishi M. Estimation of age from teeth using the racemization of aspartic acid (racemization method). *Nihon Hoigaku Zasshi* 2000;54:207–218.
17. Masters PM, Bada JL, Zigler JS Jr. Aspartic acid racemization in the human lens during ageing and in cataract formation. *Nature* 1977;268:71–73.
18. Vasavada AR, Praveen MR, Jani UD, Shah SK. Pre-operative prediction of posterior capsule plaque in eyes with posterior subcapsular cataract. *Indian J Ophthalmol* 2006;54:169–172.
19. Geiger T, Clarke S. Deamidation, isomerization, and racemization at asparaginyl and aspartyl residues in peptides. Succinimide-linked reactions that contribute to protein degradation. *J Biol Chem* 1987;262:785–794.
20. Johnson BA, Murray ED Jr., Clarke S, Glass DB, Aswad DW. Protein carboxyl methyltransferase facilitates conversion of atypical L-isoaspartyl peptides to normal L-aspartyl peptides. *J Biol Chem* 1987;262:5622–5629.
21. Fujii N, Sakaue H, Sasaki H, Fujii N. A rapid, comprehensive liquid chromatography-mass spectrometry (LC-MS)-based survey of the Asp isomers in crystallins from human cataract lenses. *J Biol Chem* 2012;287:39992–40002.
22. Ghandour H, Lin B, Choi S, Mason JB, Selhub J. Folate status and age affect the accumulation of L-isoaspartyl residues in rat liver proteins. *J Nutr* 2002;132:1357–1360.
23. Brunauer LS, Clarke S. Age-dependent accumulation of protein residues which can be hydrolyzed to D-aspartic acid in human erythrocytes. *J Biol Chem* 1986;261: 12538–12543.
24. Fonseca MI, Head E, Velazquez P, Cotman CW, Tenner AJ. The presence of isoaspartic acid in β -amyloid plaques indicates plaque age. *Exp Neurol* 1999;157:277–288.
25. Fujii N, Takata T, Fujii N. Quantitative analysis of isomeric (1- α -, 1- β -, d- α -, d- β -) aspartyl residues in proteins from elderly donors. *J Pharm Biomed Anal* 2015; 116: 25–33.
26. McFadden PN, Clarke S. Protein carboxyl methyltransferase and methyl acceptor proteins in aging and cataractous tissue of the human eye lens. *Mech Ageing Dev* 1986;34: 91–105.
27. Barnes CA. Memory deficits associated with senescence: A neurophysiological and behavioral study in the rat. *J Comp Physiol Psychol* 1979;93:74–104.
28. Mansilla E, Roque G, Sosa YE, Tarditti A, Goya RG. A Rat treated with mesenchymal stem cells lives to 44 months of age. *Rejuvenation Res* 2016;19:318–321.
29. Aswad DW. Determination of D- and L-aspartate in amino acid mixtures by high-performance liquid chromatography after derivatization with a chiral adduct of o-phthalaldehyde. *Anal Biochem* 1984;137:405–409.
30. Lowenson JD, Clarke S. Structural elements affecting the recognition of L-isoaspartyl residues by the L-isoaspartyl/D-aspartyl protein methyltransferase. Implications for the repair hypothesis. *J Biol Chem* 1991; 266:19396–19406.
31. Patananan AN, Capri J, Whitelegge JP, Clarke SG. Non-repair pathways for minimizing protein isoaspartyl damage in the yeast *Saccharomyces cerevisiae*. *J Biol Chem* 2014; 289:16936–16953.
32. Nanci A. *Ten Cate's Oral Histology*. St. Louis: Elsevier Mosby, 2013.
33. Helfman PM, Bada JL. Aspartic acid racemization in tooth enamel from living humans. *Proc Nat Acad Sci USA* 1975;72:2891–2894.
34. Helfman PM, Bada JL. Aspartic acid racemization in dentine as a measure of ageing. *Nature* 1976;262:279–281.
35. Griffin RC, Chamberlain AT, Hotz G, Penkman KE, Collins MJ. Age estimation of archaeological remains using amino acid racemization in dental enamel: A comparison of morphological, biochemical, and known ages-at-death. *Am J Phys Anthropol* 2009;140:244–252.

36. Schour I, Massler M. The teeth. In: Farris EJ, Griffith, JQ Jr. (eds): *The Rat in Laboratory Investigation*. Philadelphia: Lippincott Co., 1949.
37. Johnson BA, Aswad DW. Optimal conditions for the use of protein L-isoaspartyl methyltransferase in assessing the isoaspartate content of peptides and proteins. *Anal Biochem* 1991;192:384–391.
38. Lowenson JD, Clarke S. Recognition of D-Aspartyl Residues in Polypeptides by the Erythrocyte L-Isoaspartyl/D-Aspartyl Protein Methyltransferase—Implications for the Repair Hypothesis. *J Biol Chem* 1992;267:5985–5995.
39. Qin Z, Dimitrijevic A, Aswad DW. Accelerated protein damage in brains of PIMT+/- mice; a possible model for the variability of cognitive decline in human aging. *Neurobiol Aging* 2015;36:1029–1036.
40. Lowenson JD, Kim E, Young SG, Clarke S. Limited accumulation of damaged proteins in l-isoaspartyl (D-aspartyl) O-methyltransferase-deficient mice. *J Biol Chem* 2001;276:20695–20702.
41. Bours J, Hockwin O, Fink H. Biochemistry of the ageing rat lens: Lens wet weight and lens dry weight with respect to sex differences. *Ophthalmic Res* 1983;15:198–203.
42. Augusteyn RC. Growth of the eye lens: I. Weight accumulation in multiple species. *Mol Vis* 2014;20:410–426.
43. Augusteyn RC. Growth of the human eye lens. *Mol Vis* 2007;13:252–257.
44. Hardy AR, Quay RJ, Huson LW. Estimation of age in the Norway rat (*Rattus norvegicus* Berkenhout) from the weight of the eyelens. *J Appl Ecol* 1983;20: 97–102.
45. Vejaratpimol R, Liat LB. Age estimation of *Rattus argentiventer* from eye lens weight. *J Sci Soc Thailand* 1983;9: 107–118.
46. Tanikawa T. An eye-lens weight curve for determining age in black rats, *Rattus rattus*. *J Mamm Soc Jpn* 1993;18: 49–51.

Address correspondence to:

Steven G. Clarke
Department of Chemistry and Biochemistry
The Molecular Biology Institute
University of California
607 Charles E. Young Drive East
Los Angeles, CA 90095-1569

E-mail: clarke@mbi.ucla.edu

Received: September 22, 2015

Accepted: December 2, 2015