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Mature Enzymatic Collagen Cross-Links, Hydroxylysylpyridinoline and Lysylpyridinoline, in the Aging Human Vitreous

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Purpose. The vitreous body of the human eye undergoes progressive morphologic changes with aging. Since the enzymatic collagen cross-links hydroxylysylpyridinoline (HP) and lysylpyridinoline (LP) are known to be important for the integrity of the collagen matrix, the presence in the vitreous on aging was studied.

Methods. Vitreous bodies (VBs; $n = 143$) from 119 donors (age 4–80 years; mean ± SD, 54.3 ± 17.0 years) were carefully dissected. After weighing and freeze-drying, all samples were analyzed by high performance liquid chromatography. Left and right eyes of 24 donors were compared and, for age-related phenomena, 119 single eyes were used.

Results. Within one donor, no significant differences were found between left and right eyes. On aging, VB wet weight (4.42 ± 0.84 g) accumulates until 35 years and decreases thereafter. Collagen content (0.30 ± 0.14 mg), HP per triple helix (TH; 0.55 ± 0.18), and (HP plus LP)/TH (0.61 ± 0.19) increase until 50 years followed by a decrease, whereas LP/TH (0.057 ± 0.018) accumulates until 50 years and remains constant thereafter. The ratio between HP and LP (range, 0.42–31.0; median, 10.0) is constant over time.

Conclusions. The accumulation of enzymatic collagen cross-links until 50 years is consistent with collagen maturation and possible collagen synthesis in the human vitreous body. The decline of collagen cross-links after 50 years is consistent with collagen breakdown. (*Invest Ophthalmol Vis Sci.* 2009;50:1041–1046) DOI:10.1167/iovs.08-1714

The vitreous body of the human eye is the transparent and highly hydrated (98%–99% water) extracellular matrix (ECM) located behind the lens and surrounded by and attached to the retina. Its structure is maintained by heterotypic collagen fibrils, which contain collagen types II, V/XI, and IX, with type II predominating.[1] Types II, V, and XI collagen belong to the family of the fibril-forming collagens that assemble into fibrils and can form stable cross-links; type IX collagen belongs to the family of the fibril-associated collagens that is covalently linked to the surface of collagen fibrils.[2] Collagen fibrils in their turn can aggregate into collagen fibers. Enzymatic collagen cross-links are essential for the physical and mechanical properties of the collagen fibers.[3]

The formation of enzymatic collagen cross-links is preceded by collagen synthesis (Fig. 1). Synthesis of fibril-forming collagens (e.g., type II) starts with the transcription of the gene within the cell nucleus followed by its translation. After translation, procollagens are formed; these undergo multiple post-translational modifications (e.g., the hydroxylation of specific proline and lysine residues and the glycosylation of hydroxylysine residues) before their secretion into the ECM. The hydroxylation of lysine residues within the triple helix, as well as the C- and N-telopeptides, is catalyzed by lysyl hydroxylases.[4] In the ECM, the C- and N-terminal propeptides are removed by proteinases, enabling the molecules to aggregate into fibrils.[5–8] Subsequently, collagen fibrils are stabilized by the formation of enzymatic intermolecular and/or intramolecular cross-links. The formation of cross-links starts with the oxidative deamination of the ε-amino group of specific lysine and hydroxylysine residues within the C- and N-terminal telopeptides, leading to the formation of reactive aldehydes. The conversion of lysine and hydroxylysine into the respective aldehydes allysine and hydroxyallysine is catalyzed by the enzyme lysyl oxidase. The reactive aldehyde condensates either with hydroxylysine or lysine within an adjacent collagen molecule to form the stable intermolecular cross-links hydroxylysylpyridinoline (HP) and lysylpyridinoline (LP).[4,7,9,10]

With aging, the human vitreous undergoes a progressive increase in liquefied spaces (synchisis[11–14]) as well as an increase in optically dense structures (syneresis).[15,16] The first evidence of liquefaction has been observed at age 4 years.[13] Synchisis and syneresis progress slowly, and these processes can be followed by a posterior vitreous detachment (PVD), which is a separation between the vitreous cortex and the retina.[11,12,14,17] Postmortem studies reported that 45% of persons aged 60 to 69 years had at least 50% liquefaction,[14] that PVD is first seen in the sixth decade of life, and that 50% to 60% of persons aged 80 to 90 years had a PVD.[17] Posterior vitreous detachment in itself is not a serious condition, although it may lead to local interference with the passage of light and cause symptoms referred to as 'mouches volantes' or floaters. However, it may induce more serious pathology, such as retinal tears, retinal detachment, and intravitreal hemorrhage.[18] The pathophysiologic mechanisms underlying synchisis and syneresis have not yet been clarified. Currently, two possible mechanisms are discussed in the literature. Generally, synchisis is supposed to start with changes in the noncollagenous components of the matrix and to result in an aggregation of collagen

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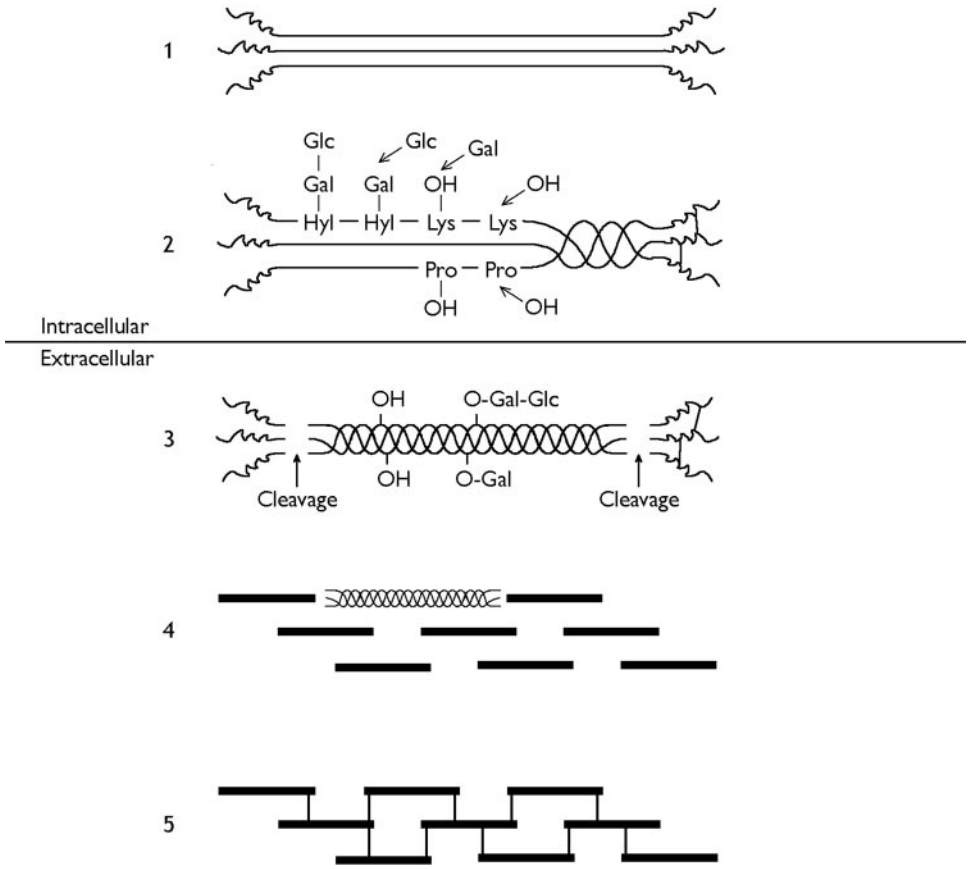

FIGURE 1. The synthesis of collagen. (1) Collagen is synthesized as pre-pro-α-chains. After translocation, the signal peptides are removed and the individual procollagen α-chains will associate through the C-peptides. (2) Procollagens undergo multiple posttranslational modifications such as the hydroxylation of specific lysine (Lys) and proline (Pro) residues as well as the glycosylation of hydroxylysyl residues. (3) The procollagen is excreted and is converted extracellularly into collagen by cleaving the propeptides. (4) Subsequently, collagen molecules assembly into ordered fibrils. (5) These are finally stabilized by the formation of intra- and/or intermolecular cross-links. Reprinted with permission from van der Slot-Verhoeven AJ. Telopeptide lysyl hydroxylase: a novel player in the field of fibrosis. Leiden: University of Leiden; 2005. Thesis.

fibrils.[1,16,19–22] Following this theory, synchisis and syneresis are the structural manifestations of a destabilization of the vitreous matrix.[13,16,19] More recent studies find evidence of an alternative hypothesis, in which a breakdown of the vitreous matrix leading to synchisis[23] would coincide with the synthesis of vitreous collagen,[24–29] leading to an increase in optically dense structures on aging (syneresis). In this theory, synchisis and syneresis can occur at different locations within the matrix and by different physiologic and pathophysiologic mechanisms.

In this study, we measured the contents of both HP and LP cross-links in whole human vitreous with aging, since the role of enzymatic collagen cross-links has not specifically been studied in the aging process of the vitreous. We show the presence of HP and LP cross-links, which appear to reach their maximum before the general onset of liquefaction.[14]

METHODS

Vitreous Preparation

Human eyes (n = 143) from 119 donors (80 men and 39 women) with ages varying from 4 to 80 years (mean, 54.3 ± 17.0 years) and with no known ophthalmic disorders were obtained from the Cornea Bank (Amsterdam, The Netherlands). Twelve donors (18 eyes) had diabetes mellitus and only one donor (age 75) had a complete PVD, defined as complete posterior detachment of the vitreous cortex from the retina until the vitreous base. Vitreous bodies (VBs) were prepared under a dissection microscope within 1 to 14 days postmortem (mean, 5.6 ± 2.6 days) according to a standard protocol previously described by Worst.[30] In short, eyes were placed in an eye holder filled with sodium chloride 0.9% and remained below the surface. Sclera, choroid, lens, and iris were removed. After blunt cleaving, almost all retina and ciliary body parts were dissected from the vitreous except for the strong interconnections around the pars plana, which were initially left in place to prevent damage to the vitreous cortex. Then, the lens capsule was carefully dissected with most fibers of the zonula from the vitreous base. The final step was the dissection of pars plana remnants consisting largely of ciliary body fragments still adhering to the vitreous.[31] Some vitreous base could have been removed during the latter procedure. All VBs were weighed and stored at −20°C before freeze-drying. In this study, VBs were divided in 24 pairs of left and right eyes, and 119 single eyes.

Freeze-drying

To reduce vitreous volume, VB samples were freeze-dried by a freeze-dryer (Christ Alpha 1-4; Salm en Kipp, Breukelen, The Netherlands). Before the drying process, the samples were put in liquid nitrogen. The lyophilization was performed using a shelf temperature of –30°C, a condenser temperature of –53°C and a pressure of 0.220 millibar (mbar) for 18 hours. Then, the shelf temperature and pressure were gradually increased to 20°C and 0.520 mbar, respectively, during 6 hours. Finally, the drying process was continued for another 20 hours under these conditions. In a separate pilot analysis, we confirmed by repetitive freeze-drying cycles that there was no loss of dry weight sample (data not shown).

HPLC Analyses

Analyses of HP, LP, and amino acid content were performed by HPLC, as described previously.[32,33] The HPLC system (Separations Analytical Instruments, Hendrik Ido Ambacht, The Netherlands) consisted of a multisolvent delivery system (Model 480 pump; Gynkotek, Germering, Germany), an autosampler (Triathlon; Spark Holland, Emmen, The Netherlands), a fluorometer (Model 821-FP; Jasco Benelux, IJsselstein, The Netherlands), and a degasser (Laboratory-Quatec Model Gastorr Gt-103; Omnilab, Milan, Italy). Calibration of amino acids was per-

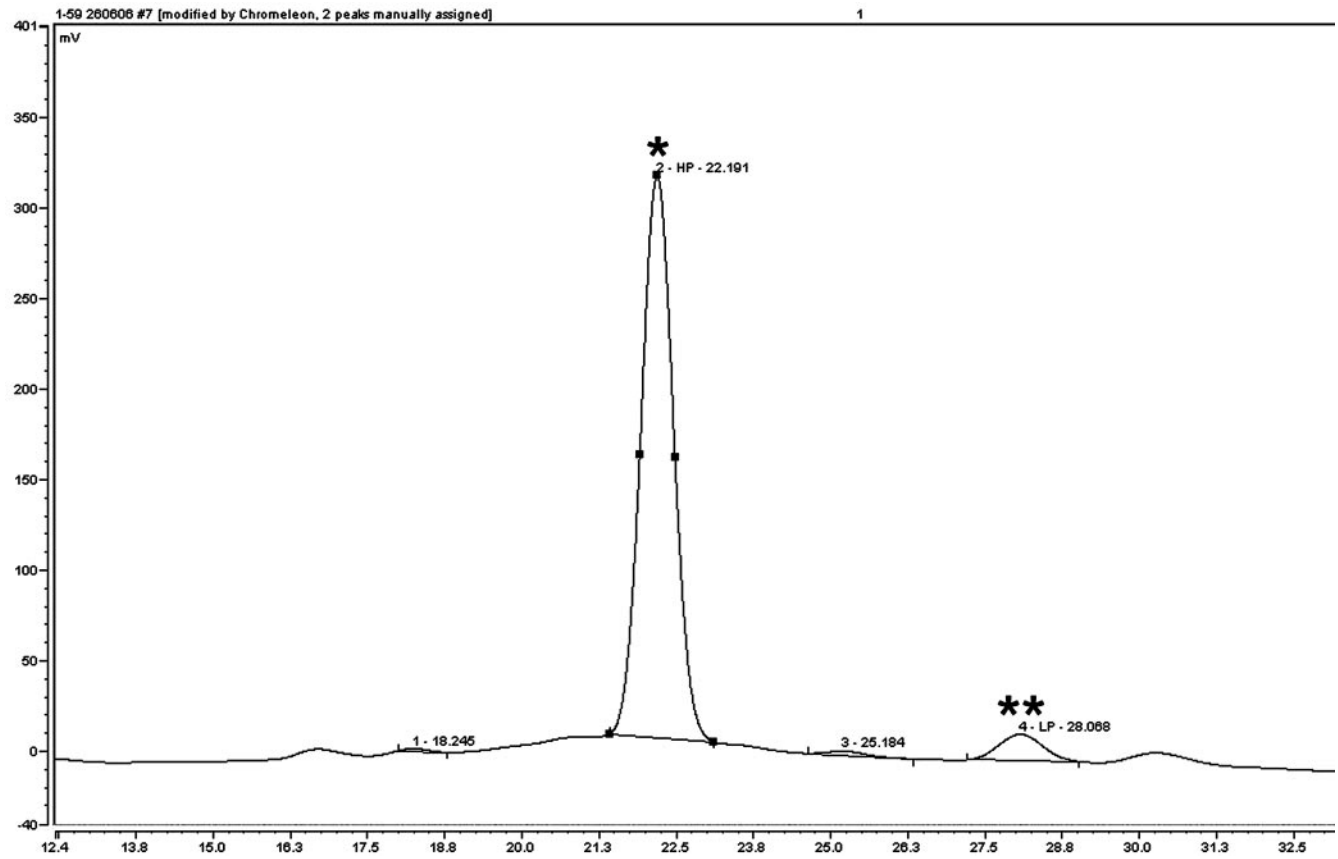

FIGURE 2. An example of HPLC output. The first peak (*) represents HP; the second peak (**) represents LP.

formed with the amino acid standard for collagen hydrolysates (A-9531; Sigma, St. Louis, MO).

Amino acids (hydroxyproline and proline) and cross-links were determined after acid hydrolysis, as described previously.[33] All freeze-dried samples were hydrolyzed in 6 M HCl at 110°C for 20 to 24 hours. After drying (Speed Vac SC 110; Savant, Farmingdale, NY), the specimens were dissolved in 200 μL water containing 10 μM pyridoxine, the internal standard for cross-link analysis and 2.4 mM homoarginine, the internal standard for amino acid analysis (both from Sigma).

For cross-link analysis (Fig. 2), the samples were diluted in 0.5% (v/v) heptafluorobutyric acid (HFBA; Fluka AG, Buchs, Switzerland) in 10% (v/v) acetonitrile (Rathburn, Walkerburn, Scotland). Separation was performed on a 4.6 mm $\times$ 150 mm reverse-phase column (Micropak ODS-80TM; Varian, Sunnyvale, CA). The column was equilibrated with 0.15% (v/v) HFBA in 24% (v/v) methanol (solvent A). Elution of pyridinolines and the internal standard pyridoxine was achieved at ambient temperature at a flow-rate of 1.0 mL/min in two isocratic steps: time 0 to 17 minutes solvent A; time 17 to 30 minutes 0.05% (v/v) HFBA in 40% methanol (solvent B). The column was washed with 0.1% (v/v) HFBA in 75% (v/v) acetonitrile (solvent C) for 10 minutes and equilibrated for 10 minutes with solvent A, resulting in a total analysis time of 50 minutes per sample. Fluorescence was monitored with a programable fluorometer: 0 to 22 minutes 295/400 nm (pyridoxine and pyridinolines).

For amino acid analysis, aliquots of the hydrolyzed samples were diluted in 0.1 M sodium borate buffer, pH 8.0, and derivatized at room temperature for 5 minutes with 6 mM 9-fluorenylmethyl chloroformate in acetone. Termination of the reaction and removal of excess reagent and acetone was performed by extraction with 600 μL pentane. After two additional extractions, 400 μL 25% acetonitrile in 0.1 M borate buffer, pH 8.0, was added. A 50 μL aliquot of the diluted sample was injected into the HPLC system, after which separation was performed on the above-mentioned reversed-phase column. Chromatography was carried out at a column temperature of 40°C; fluorescence was monitored at 254/630 nm. Solvent composition and the ternary gradient have been described in detail, previously.[32]

Collagen cross-links are expressed as mol per mol collagen, assuming 300 hydroxyproline residues per triple helical collagen molecule (TH).[34]

Statistical Analysis

The HPLC results were analyzed by Student's *t*-tests for differences between two groups. Age-related phenomena were studied by linear regression analysis and by curve estimation, a form of non-linear regression using a quadratic model, by which a reversal or top of the curve can be determined (an increase followed by a decrease for example). The age at the reversal point (top of the curve) was used as a cutoff point in the subsequent non-linear regression analyses. Right and left eyes were compared by paired Student's *t* tests. All analyses were performed with data analysis software (SPSS, version 14.0 for Windows; SPSS, Chicago, IL). When needed, the data were normalized using log transformation. $P < 0.05$ was considered to represent statistically significant differences.

RESULTS

Right versus Left Eye

The 24 right and left VBs showed no significant differences between right and left eyes in all test variables: (dry) weight, hydroxyproline per proline, percentage of collagen, mg collagen in total VB, HP/TH, LP/TH, (log) HP/LP, and (HP plus LP)/TH (data not shown). Thus, VBs of one donor showed a high correspondence between right and left eyes. Therefore,

only one (randomly chosen) eye of each donor was used in further analyses.

VB of Single Eyes

To detect age-related phenomena, single eyes of 119 donors were analyzed with linear regression and curve estimation followed by non-linear regression (Figs. 3A–F). Macroscopically, elder VBs appeared much smaller. By curve estimation analysis, vitreous wet weight (mean ± SD, 4.42 ± 0.84 g; Fig. 3A) appeared to increase until 35 years ($P = 0.048$) and to decrease thereafter ($P < 0.001$), while dry weight (40.1 ± 9.8 mg) and VB collagen content (0.30 ± 0.14 mg) only declined significantly after 35 years and 50 years, respectively (both $P < 0.001$; data not shown). By linear regression analysis, the hydroxyproline per proline ratio (0.17 ± 0.069; Fig. 3B), which is the ratio between collagenous and non-collagenous proteins, diminished markedly with aging ($P < 0.001$), whereas the percentage of collagen (percentage of the dry weight; 0.75 ± 0.33%, Fig. 3C) remained constant over time ($P = 0.111$). The ratio between HP and LP (range 0.42 to 31.0, median 10.0) did not change significantly (log transformed data: $P = 0.087$). Curve estimation analysis showed reversal points for HP/TH (0.55 ± 0.18; Fig. 3D), LP/TH (0.057 ± 0.018; Fig. 3E), and (HP plus LP)/TH (0.61 ± 0.19; Fig. 3F) at approximately age 50 years. Both HP/TH and (HP plus LP)/TH accumulated until 50 years (both $P < 0.001$) and decreased significantly thereafter ($P = 0.020$ and $P = 0.010$, respectively). LP/TH increased until 50 years ($P = 0.003$) and remained constant thereafter ($P = 0.355$). In our dataset, a few outliers were found and we did not remove them, since they had no effect on our results (not shown).

VBs showed no significant differences in the enzymatic collagen cross-links in our sub analysis of diabetics ($n = 12$) versus non-diabetics ($n = 107$); causes of death such as chronic alcohol intoxication ($n = 5$), vascular cause ($n = 73$), pulmonary cause ($n = 13$), malignancy ($n = 19$), and trauma ($n = 9$);

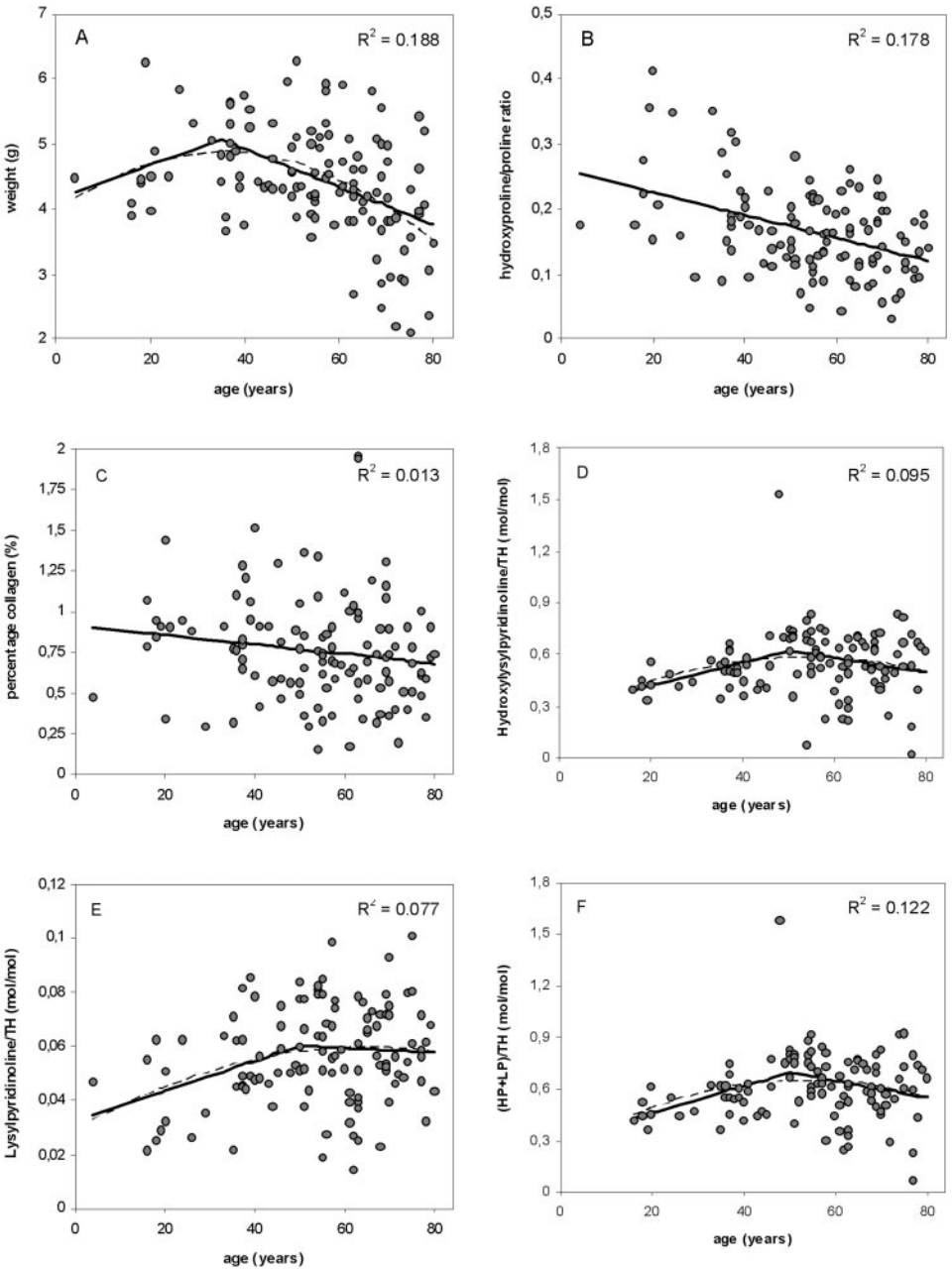

FIGURE 3. The effect of age on the vitreous body (VB). (**A**) VB weight increased ($P = 0.048$) until 35 years and decreased thereafter ($P < 0.001$). (**B**) On aging, the hydroxyproline/proline (Hyp/Pro) ratio declined ($P < 0.001$). (**C**) The collagen percentage remained constant over time ($P = 0.111$). (**D**) Until 50 years, hydroxylysylpyridinoline (HP) per TH rose ($P < 0.001$), whereas it decreased thereafter ($P = 0.020$). (**E**) Lysylpyridinoline per TH (LP/TH) increased until 50 years ($P = 0.003$), followed by a constant level ($P = 0.355$). (**F**) (HP plus LP)/TH showed an increase ($P < 0.001$) up to 50 years and diminished thereafter ($P = 0.010$).

and time interval (1 to 12 days) between death and preparation (data not shown). Finally, possible differences between sex (80 men and 39 women) were studied, since female sex is a risk factor for the development of PVD,[12,35,36] but no clear differences were found between men and women (data not shown).

Discussion

This study shows an increase in the mature enzymatic collagen cross-links HP and LP in the human vitreous body from childhood until 50 years, at which time decline (HP) or stabilization (LP) occurs. For the VB cross-link composition, the influence of LP appears to be limited, since HP is the most abundant collagen cross-link and the HP/LP ratio does not change significantly on aging. Overall, the enzymatic cross-links (HP plus LP) per TH increase until 50 years and decline thereafter.

The pyridinoline cross-links form the last enzymatic step in collagen maturation. They provide physical and mechanical strength to the collagen network and thus contribute to its integrity.[3] In this study, we cannot show a direct relationship between age-related morphologic changes and enzymatic collagen cross-linking. The direct relationship between enzymatic cross-links and morphologic changes with aging has not been studied in the VB.

In the VB, we find an increase in the enzymatic cross-links per TH until 50 years. A possible explanation for the increase could be the formation of enzymatic cross-links from the processing of type II procollagen present in the VB.[28] At this moment, it is not known whether the amount of procollagens in the vitreous changes with aging. Furthermore, the presence of procollagens may indicate that collagen synthesis, and thus cross-link formation, continuously take place with aging. This latter hypothesis is supported by the finding of immature cross-links in adult bovine vitreous.[26] In older cartilage and bone, immature cross-links decline in parallel with an increase in mature enzymatic cross-links.[37,38] In this study, we were only able to measure the mature enzymatic cross-links. Therefore, a direct relationship between increasing mature and decreasing immature cross-links could not be demonstrated. In non-mineralized tissues (such as cartilage and vitreous) maturation of immature collagen cross-links is probably a quick process taking only one to four weeks.[37,39–41] This is in contrast to mineralized ECMs (e.g., bone and dentine) in which maturation of enzymatic cross-links is a slower process because of the abundant presence of mineral.[42]

In addition to the increase in enzymatic cross-links per TH until 50 years, we found a decline thereafter. This decrease occurs at an age at which morphologic changes in the VB become more prominent.[14,17] A reasonable explanation could be a breakdown or loss of collagen cross-links, which is supported by the decrease in collagen content after 50 years and the morphologic presence of collagen fragments near liquefied spaces.[23] Results found in the VB are globally in agreement with other human ECMs (bone, cartilage, meniscus, and intervertebral disc) in which the maximum amount of enzymatic cross-links is often reached in adolescence or midlife.[10,38,43–46]

At this moment, the only study concerning HP and LP in the human VB, and thus our only reference for mature enzymatic cross-links, is based on vitrectomy samples.[47] However, this study failed to detect age-related changes in HP and LP cross-links. Possible explanations for this difference with our study include a limited age range (38–77 years versus 4–80 years); the use of vitrectomy samples (sampling error since the VB is not a homogenous structure[1,15,17,48]); and the expression of cross-links in nanograms per milliliter versus amounts per triple helix.

In the present study, the apparent increase in VB weight and VB collagen weight until 35 and 50 years, respectively, and the significant decrease thereafter was partly in agreement with a previous study which showed a maximum total VB weight around 40 years and a constant VB collagen content from the third decade onward, preceded by a possible increase.[13] Because we found higher amounts of total vitreous collagen weight (mean, 0.30 mg vs. 0.22 mg by estimation[13]), it was not likely that we lost collagens during preparation, although theoretically, a loss of macromolecules during preparation in elder VBs is more likely than in younger VBs. The increase followed by the decrease could be explained by a net collagen synthesis followed by a net collagen breakdown (and removal from the VB). In our opinion, it is impossible to explain this phenomenon by the assumption that collagen once formed never changes and only aggregates with aging. Our hypothesis can be supported by morphologic studies that showed an age-related loss of type IX collagen[49] and found evidence of collagen fragmentation near liquefied spaces.[23]

The hydroxyproline per proline ratio showed a significant decrease with aging, implying a higher increase in the amount of non-collagenous proteins than in the amount of collagens. Non-collagenous proteins in the vitreous include glycoproteins (such as opticin), proteoglycans (e.g., chondroitin sulfate), and other structural proteins (e.g., fibrillin).[1] Since glycoproteins and proteoglycans are the most abundant non-collagenous proteins of the VB,[1] the change in hydroxyproline per proline ratio could reflect an increase in these proteins. Alternatively, an increase in total protein concentration with aging has been described and found to be related to a progressive leakage of serum proteins into the VB.[50]

Our results on enzymatic collagen cross-links can contribute to the insight in the age-related processes of synchisis and syneresis in the concept of collagen turnover. The accumulation of collagen cross-links until 50 years is an indication of (ongoing) collagen maturation, which in its turn can be the result of collagen synthesis. At the age of 50 years, when striking morphologic changes in the VB are evident,[14,17] the enzymatic collagen cross-links start to diminish. This decline can be caused by collagen breakdown— but more importantly, the decline itself can contribute to the instability of the collagen network resulting in an increase in morphologic changes in the elder VB.

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