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A Systematic Evaluation of Collagen Crosslinks in the Human Cervix

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Abstract

Objective—The mechanical strength of the cervix relies on crosslinking of the tissue's collagen network. Clinically, the internal os is functionally distinct from the external os. We sought to detect specific collagen crosslinks in human cervical tissue and determine if crosslink profiles were similar at the internal and external os.

Study Design—Transverse slices of cervical tissue were obtained at the internal and external os from 13 non-pregnant, premenopausal women undergoing a benign hysterectomy. To understand how crosslinks were distributed throughout the entire cervix and at the internal and external os, biopsies were obtained from three circumferential zones in four quadrants from each slice.

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Biopsies were pulverized, lyophilized, reduced with sodium borohydride, hydrolyzed with hydrochloric acid and reconstituted in heptafluorobutyric acid buffer. Hydroxyproline was measured by ultra-performance liquid chromatography-electrospray ionization tandem mass spectrometry (UPLC-ESI-MS/MS), converted to total collagen, and normalized by dry weight. Collagen crosslinks pyridinoline (PYD), deoxypyridinoline (DPD), dihydroxylysinoxonorleucine (DHLNL), and the nonenzymatic advanced glycation end product pentosidine [PEN] were measured by UPLC-ESI-MS/MS and reported as crosslink density ratio (crosslink:total collagen). Generalized estimated equation analysis was used to compare results between the internal and external os and to compare quadrants and zones within slices from the internal and external os to determine if crosslink profiles were similar.

Results—592 samples from 13 patients were analyzed. Collagen crosslinks are detectable in the human cervix by UPLC-ESI-MS/MS. When comparing all samples from the internal and external os, similar levels of collagen content, PYD, DHLNL and DPD were found but PEN density was higher at the external os (0.005 vs 0.004, $P=0.001$). When comparing all internal os samples, significant heterogeneity was found in collagen content and crosslink densities across zones and quadrants. The external os exhibited heterogeneity only across zones.

Conclusion—Collagen crosslinks (PYD, DPD, DHLNL, and PEN) are detectable by UPLC-ESI-MS/MS in the human cervix. The internal os exhibits significant collagen crosslink heterogeneity compared to the external os. Further studies are needed to evaluate how collagen crosslink heterogeneity correlates to the mechanical strength and function of the human cervix.

Keywords

Cervix; collagen; crosslinking; preterm labor

INTRODUCTION

During pregnancy, the cervix undergoes dramatic changes in order to allow for successful parturition.¹ Ultrasound studies have shown that cervical ripening at term starts with dilation of the internal os and then progressive funneling towards the external os until total effacement is achieved.² A similar sequence of cervical changes occurs in pregnancies complicated by cervical insufficiency.³ During sterile vaginal exam, it is also common to note that the external os is open while the internal os remains closed. Although cervical tissue architecture was traditionally thought to be homogenous, these clinical findings fuel the following questions: Is the internal os functionally distinct from the external os? If so, are there differences in cervical tissue properties at the level of the internal os that influences its mechanical performance? The answers to these questions remains elusive as studies evaluating cervical tissue strength at the internal and external os are lacking.

Human cervical tissue is a hydrated connective soft tissue composed mainly of a collagen-rich extracellular matrix (ECM).^{4–8} Similar to other load-bearing tissues in the body (i.e. bone, tendon), the mechanical strength of cervical ECM relies in part on the type and degree of collagen crosslinking in its collagen network. Collagen crosslinks stabilize collagen molecules – a vital requirement to building strong, organized collagen networks. During collagen crosslink formation, immature crosslinks such as dihydroxylysinoxonorleucine

(DHLNL) are formed between a telopeptide residue and a helical residue. Subsequently, another telopeptide residue is formed between three collagen molecules resulting in “mature” crosslinks, deoxypyridinoline (DPD) and pyridinoline (PYD).⁹

Previously, the degree of collagen crosslinking was indirectly determined by measuring collagen extractability, where increased extractability correlated to decreased collagen crosslinking. Studies which used this method showed that collagen extractability significantly increases in a normal mouse cervix during pregnancy.¹⁰ Myers et al demonstrated similar results on human non-pregnant and term pregnant tissue samples.^{8,11} Recently, Akins et al measured mature collagen crosslinks in mouse cervical tissue by reversed phase high performance liquid chromatography and found cervical ripening in rodents is characterized by a decrease in pyridinoline (PYD) and deoxypyridinoline (DPD).¹²

Given that 1) mature collagen crosslinks provide stability and thus strength to the ECM, 2) collagen crosslink profiles are altered in cervical remodeling/softening and 3) the internal os appears to “soften” or “weaken” first during parturition and premature cervical remodeling, the goal of this study was to determine if specific collagen crosslinks are detectable in the human cervix and if the internal os has a different collagen crosslink profile compared to the external os.

MATERIALS AND METHODS

This study was approved by the Columbia University Medical Center Institutional Review Board (IRB #AAAI0337). Non-pregnant, premenopausal women undergoing a total hysterectomy for benign indications were identified and consented. Women were excluded if they were over 50 years of age, had an abnormal pap smear, or had prior cervical surgery. Demographic information (age, race, body mass index, obstetric history) and indication for procedure were collected. Uterine weight was obtained from pathology reports. (Table 1)

Immediately following the hysterectomy, 2–3mm transverse slices of the cervix were obtained at the level of the internal and external os. Anterior/posterior orientation of the tissue was maintained. One slice from the internal and external os was frozen and stored at –80°C until collagen content and crosslinks analysis was performed. An adjacent slice from each area was fixed in 10% neutral buffered formalin for 24h, paraffin embedded and sectioned for H&E staining. The H&E stained slides were reviewed with a pathologist to confirm the location in the cervix and exclude pathology. The formal pathology report from the surgical specimen was also reviewed and we proceeded with further tissue processing if the findings were benign.

Since early studies evaluating collagen structure in the cervix reported that the stroma contains “zones” of preferentially-aligned collagen^{13,14} (Figure 1A), our goal was to evaluate if collagen content and collagen crosslink densities differed depending on the “zone” or quadrant of the cervix. Using a 2mm punch biopsy (Miltex, #33-31), biopsies were obtained in duplicate from each “zone” from four distinct quadrants. (Figure 1B) Samples were lyophilized and dry weights recorded. Dehydrated samples were reduced in

sodium borohydride (NaBH₄) and then hydrolyzed in 12M hydrochloric acid in vacuo at 110°C for 18–24 hours. The hydrolysate was lyophilized and resuspended in heptafluorobutyric acid (HFBA) buffer. The molar content of hydroxyproline (HYP), PYD, DPD, DHLNL and PEN was measured using ultra performance liquid chromatography - electrospray ionization tandem mass spectrometry (UPLC-ESI-MS/MS) as previously described.¹⁵ Total collagen content was determined by using a mass ratio of collagen (molecular weight = 131g/mol) to hydroxyproline of 7.14:1 and the concentration of collagen was determined by dividing by the dry weight. Collagen crosslink density was determined for each type of collagen crosslink by dividing by the collagen content of the tissue sample. Therefore all collagen crosslink densities are reported on a mole per mole basis with collagen content.

ANALYSIS

Statistical Methods

An assumption of regression models is that observations or in this case the biopsy samples are independent of one another. Since we obtained multiple samples from the *same* patient, this violates the statistical assumption of “independence”. Failure to adjust for the intra-cluster dependence will result in biased variance estimation. Therefore, to account for this intra-cluster dependence, we fit all regression models based on the method of generalized estimating equations procedure.¹⁶

Collagen Content and Crosslink Analysis

A priori contrast procedures were performed. Using a linear regression model based on the methods of generalized estimating equations, we initially established the collagen content and collagen crosslink densities in the “total cervix” (internal os samples + external os samples from all 13 women). We then evaluated for any heterogeneity with respect to location (quadrants and zones). Next, we evaluated heterogeneity in collagen content and collagen crosslinks between samples from the internal os and the external os. We also analyzed differences in quadrants and zones between the internal and external os (ie quadrant or zone 1 from internal os to quadrant or zone 1 of external os). Lastly, we analyzed the tissue heterogeneity *within* the internal and external os (comparing quadrants and zones within each slice from the internal and external os). Since all comparisons were planned *a priori* before the beginning of the experiments, we did not correct for multiple testing¹⁷. All regression models were fit based on the GENMOD procedure in SAS version 9.4 (SAS Institute, Cary, NC).

RESULTS

Thirteen patients were consented and cervical tissue collected. A total of 624 biopsies were collected. Biopsies were excluded if an error was made during processing or if the collagen content was higher than 100%. This left a total of 592 samples for analysis. The average age was 45.2 years, average BMI 28.8, and the mean uterine weight was 1011g. (Table 1)

Total Cervix

The “total cervix” collagen content and specific collagen crosslink values reported in Table 2 represent a combination of all internal and external os samples from the 13 patients. PYD was the most common crosslink followed by DHLNL then DPD, with average densities of 0.123, 0.094 and 0.045 mol/mol, respectively. PEN was present in the cervix but in small amounts compared to PYD. (Table 2) DHLNL was the only crosslink density affected by parity with higher levels in nulliparous women (0.126 vs 0.080, $P=0.045$). There was no difference in the collagen content, PEN, DPD, and PYD densities between nulliparous and multiparous subjects. (Table 3)

When comparing collagen content by quadrants (Table 4, Figure 1C), there was a significantly lower collagen content in quadrant 4 (Q4) compared to quadrant 1 (Q1) (32.7% vs 34.7%, $P=0.034$). There were no significant differences in collagen crosslink densities between quadrants. (Table 4) When analyzing the zones in the “total cervix” (Table 5, Figure 1D) significant heterogeneity was noted. The mid-stromal zone (Z2) had higher levels of collagen content, PYD, DPD, and DHLNL densities and lower PEN density when compared to the inner region (Z1). The outer zone (Z3) had higher amounts of all collagen crosslinks compared to the inner zone (Z1). (Table 5)

Comparison of Internal Os to External Os

In order to determine if the internal and external os were similar with respect to collagen content and crosslink densities, we compared collagen content and crosslink densities from all the internal os samples to all of the external os samples. There were no significant differences in collagen content, PYD, DPD and DHLNL densities between the internal and external os. PEN density was significantly higher at the external os than the internal os (0.004 vs 0.005, $P=0.001$) (Table 6).

When comparing quadrants from the internal os to the quadrants of the external os, PEN density was higher in the external os for quadrant 1 (Q1) (0.006 vs 0.004, $P=0.033$) and quadrant 4 (Q4) (0.006 vs 0.004, $P=0.005$) When comparing the internal and external os by zones, there was significantly less PEN density at the internal os in the middle zone (Z2) (0.003 vs 0.004, $P<0.001$).

Analysis of the Internal Os

In order to determine if collagen content and crosslinks were evenly distributed at the level of the internal os, we analyzed all samples from the internal os and found significant heterogeneity in collagen content and all crosslink densities with respect to quadrants and zones. Specifically, there was significantly less collagen content in the quadrant 4 (Q4) and higher DPD and PYD densities in quadrant 3 (Q3). (Table 7) Significant heterogeneity was also noted with respect to zones as shown in Table 8.

Analysis of the External Os

In order to determine if collagen content and crosslinks were evenly distributed at the level of the external os, we analyzed all samples from the external os and found no significant differences in collagen content and all crosslink densities across the four quadrants. (Table

9) Significant heterogeneity was noted, however, in collagen content and all crosslink densities with respect to zones as shown in Table 10.

COMMENT

This study establishes that specific immature and mature collagen crosslinks are present in detectable amounts in the human cervix. The most abundant collagen crosslink is PYD followed by DHLNL then DPD. PEN is present but in smaller amounts compared to the other crosslinks. This trend appears to persist despite location in the cervix.

PEN was shown to be present in higher amounts at the external os compared to the internal os. PEN is a non-enzymatic advanced glycation product that is a modified protein which has been glycated and oxidized after coming into contact with sugars. The production of PEN increases in hyperglycemic environments and during aging and is implicated as the cause of atherosclerosis in diabetic patients¹⁸. Additionally, they form crosslinks between collagen, altering the extracellular matrix and resulting in increased tissue stiffness¹⁹. Studies on bone show that increased amounts of PEN serves as a marker for fracture risk whereas the formation of mature crosslinks results in increased bone strength⁹. The presence of PEN has never been demonstrated in the human cervix and its significance is unknown. Perhaps PEN tends to accumulate in the periphery of the cervix, where there may be less collagen turnover, resulting in higher levels at the external os.

We found significant heterogeneity in the human cervix with respect to collagen content and crosslinks. In particular, the internal os exhibited greater heterogeneity when compared to the external os. One explanation for this observation may be that the internal os is closer to the uterus and may contain cells (ie smooth muscle cells) which may influence collagen content and collagen crosslink density. As Danforth et al reported, the transition from uterine tissue to cervix varies and there may be more smooth muscle cells dispersed in cervical tissue that is closer to uterus.²⁰

When analyzing whole slices from the internal and external os, the most impressive heterogeneity was seen between zones of the cervix. Collagen content was higher in zone 2 at both the internal and external os and PYD (a mature collagen crosslink) was the most abundant collagen crosslink in this zone. Early and recent studies reported that the cervix may contain zones of preferentially-aligned fibers, including a central circumferential region. Given that crosslinks are known to stabilize and strengthen the collagen network and a shift in the dominance of immature to mature crosslinks are associated with increased tissue strength²¹, it is conceivable that the circumferential orientation in zone 2 or the “midstromal” region may provide the strength necessary to keep the cervix closed as the pressure of a growing pregnancy increases. Currently, we are testing tissue strength in relation to collagen directionality, which may provide further clues to the role of collagen crosslinks and zones of collagen in cervical tissue strength.

This study utilized whole transverse slices of cervical tissue from non-pregnant women for two reasons. First, to understand local differences in the cervical architecture requires the ability to evaluate intact sections. Secondly, approaches using smaller biopsies will be unrevealing until the structural relationships of each segment are understood. Although

evaluation of samples from pregnant patients would be ideal, obtaining sufficient whole tissue sections is impractical.

This study is limited by the number of subjects and their demographics. For instance, in our sample of 13 patients, the majority were Hispanic. To date, no studies have evaluated the effect of race on collagen content and crosslinking. It is also possible that the heterogeneity of the cervix may be affected by other factors such as mode of previous deliveries and type and approach of hysterectomy. Further studies with larger and more diverse patient demographics are needed to analyze the effect of these factors.

Another potential limitation of our study is the biopsy size. We used 2mm wide biopsies that were 2–3mm in length for two reasons. First, small punch biopsies were used to obtain samples from each quadrant and zone of the cervix. (Figure 1B) Secondly, we are presently evaluating the reliability of small biopsies in ongoing pregnancies. Although the size of the small biopsies may have influenced the range of the confidence intervals reported in this study, they have established the baseline variability of collagen content and crosslinks when utilizing small tissue samples. This information will be necessary for any study that plans to process small cervical tissue samples.

This study provides several important insights into cervical structure and novel approaches to understanding the cervix. First, we used UP LC-ESI-MSMS to measure the individual collagen crosslinks as well as collagen content. Previously, this was measured indirectly by measuring collagen extractability, with the amount of crosslinks being inversely proportional to the extractability.⁷ A direct measurement provides more detailed information and UP LC-ESI-MSMS has been used in other disciplines to quantify and identify specific crosslinks in other supportive human tissue such as bone, cartilage, and tendon.⁹

Secondly, this is the first study to measure and map the collagen content and crosslink densities in a systematic manner. The previous assumption based on samples obtained from the external os that cervical tissue is homogenous appears to be inaccurate and also does not correlate with clinical findings, as evidenced by “funneling” at the internal os in patients at risk for preterm labor or in cases evaluated by digital exam in which the external os is dilated but the internal os is closed. If the internal and external os are functionally unique, there may be differences in tissue structure which may ultimately affect the mechanical strength and performance of the tissue. As this study shows, there is significant heterogeneity in human cervical tissue which must be taken into consideration, particularly when using small biopsies. As such, our results in the non-pregnant cervix can serve as a foundation for further studies which aim to understand both normal and abnormal conditions of the cervix, such as cervical insufficiency. Lastly, our results will be important for future studies which will establish the correlation of collagen crosslink density to mechanical strength of human cervical tissue.

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Figure 1.

A) Traditional view of zones of preferential collagen fibers (adapted from [9]). B) Sampling location on transverse cervical slices, Q=quadrant and Z=zone. C) Sample locations for Table 3. D) Sample locations for Table 4.

Table 1

Patient demographics

Patient	Age	Race	BMI	Parity	Obstetrical History	Type of Hysterectomy	Uterine + Cervix Weight (g)
1	44	H	32.6	0	None	TAH	2994
2	49	H	23.3	0	None	TAH	1150
3	46	AA	34.9	0	None	TAH	6100
4	40	AA	37.9	0	None	TAH	1005
5	49	C	28.3	1	NSVD	TRH	267
6	48	H	27.8	1	NSVD	TLH	113
7	42	C	25.3	1	NSVD	TRH	225
8	41	AA	25.1	1	NSVD	TLH	192
9	42	C	28.1	1	NSVD	TLH	75
10	49	C	29.9	2	NSVD ×2	TVH	175
11	44	H	21.2	4	NSVD ×4	TLH	223
12	46	H	30.9	4	NSVD ×3, CS ×1	TRH	178
13	48	H	30.2	5	NSVD ×5	LAVH	250

Legend: H = Hispanic; AA = African-American; C = Caucasian; NSVD = normal spontaneous vaginal delivery; CS = cesarean section; TAH = total abdominal hysterectomy; TLH = total laparoscopic hysterectomy; TVH = total vaginal hysterectomy; LAVH = laparoscopic-assisted vaginal hysterectomy; TRH = total robotic hysterectomy

Table 2

Collagen content and specific collagen crosslinking density in the “total cervix” (average of samples from internal and external os from all 13 patients). Crosslink density is quantified on a mole per mole basis with collagen.

	Mean n=592*	Std Dev
Collagen content [mg/mg dry weight]	0.344	0.163
PEN [mol/mol]	0.005	0.003
DPD [mol/mol]	0.045	0.042
DHLNL [mol/mol]	0.094	0.072
PYD [mol/mol]	0.123	0.073

* Samples are from 13 patients

Table 3

Comparison of collagen content and specific collagen crosslinking density in the “total cervix” between nulliparous and multiparous subjects. Crosslink density is quantified on a mole per mole basis with collagen.

	Nulliparous n=173* (95% CI)	Multiparous n=419* (95% CI)	P value
Collagen content [mg/mg dry weight]	0.391 (0.366,0.416)	0.325 (0.31,0.34)	NS
DHLNL [mol/mol]	0.126 (0.112,0.140)	0.080 (0.075,0.086)	0.045
PEN [mol/mol]	0.004 (0.004,0.005)	0.005 (0.005,0.005)	NS
DPD [mol/mol]	0.053 (0.048,0.058)	0.041 (0.037,0.046)	NS
PYD [mol/mol]	0.145 (0.131,0.158)	0.114 (0.108, 0.120)	NS

* Samples are from 13 patients

Comparison of collagen content and specific crosslinks in the “total cervix” (average of samples from internal and external os from all 13 patients) by quadrants. Crosslink density is quantified on a mole per mole basis with collagen. Q1 = anterior quadrant, Q2 = left quadrant, Q3 = posterior quadrant, Q4 = right quadrant.

Table 4

	“Total Cervix” – Quadrant Comparison				
	Q1 n=142* (95% CI)	Q2 n=151* (95% CI)	Q3 n=150* (95% CI)	Q4 n=149* (95% CI)	P value
Collagen content [mg/mg dry weight]	0.347 (0.323,0.371)	0.345 (0.318,0.372)	0.357 (0.329,0.386)	0.327 (0.302,0.353)	0.034 for Q1 v Q4
PEN [mol/mol]	0.005 (0.005, 0.006)	0.005 (0.004,0.005)	0.005 (0.005,0.006)	0.005 (0.004,0.006)	NS
DPD [mol/mol]	0.045 (0.038, 0.052)	0.042 (0.036,0.049)	0.049 (0.042, 0.056)	0.043 (0.037,0.050)	NS
DHLNL [mol/mol]	0.090 (0.079,0.100)	0.091 (0.079, 0.103)	0.106 (0.093,0.119)	0.088 (0.077,0.099)	NS
PYD [mol/mol]	0.122 (0.110,0.134)	0.118 (0.106,0.131)	0.131 (0.120, 0.143)	0.119 (0.108,0.131)	NS

* Samples are from 13 patients

Table 5

Comparison of collagen content and specific crosslinks in the “total cervix” (average of samples from internal and external os from all 13 patients) by zones. Crosslink density is quantified on a mole per mole basis with collagen. Z1 = inner zone, Z2 = middle zone, Z3 = outer zone

	“Total Cervix” – Zone Comparison			
	Zone 1 n=197* (95% CI)	Zone 2 n=196* (95% CI)	Zone 3 n=199* (95% CI)	P value
Collagen content [mg/mg dry weight]	0.297 (0.276,0.317)	0.434 (0.412,0.456)	0.303 (0.282,0.324)	<0.001 for Z1 v Z2 NS for Z1 v Z3
PEN [mol/mol]	0.005 (0.005,0.005)	0.004 (0.004,0.004)	0.006 (0.005, 0.006)	0.002 for Z1 v Z2 0.008 for Z1 v Z3
DPD [mol/mol]	0.020 (0.018,0.022)	0.079 (0.072,0.087)	0.035 (0.032,0.038)	<0.001 for Z1 v Z2 and Z2 v Z3
DHLNL [mol/mol]	0.069 (0.060,0.077)	0.099 (0.090,0.108)	0.113 (0.101,0.125)	<0.001 for Z1 v Z2 and Z1 v Z3
PYD [mol/mol]	0.091 (0.082,0.099)	0.117 (0.109,0.124)	0.160 (0.149,0.172)	0.020 for Z1 v Z2 <0.001 for Z1 v Z3

* Samples are from 13 patients

Table 6

Comparison of collagen content and specific crosslinks between the internal and external os (average of all samples from internal os compared to average of all samples from external os). Crosslink density is quantified on a mole per mole basis with collagen.

	Internal Os vs External Os		
	Internal Os n=290* (95% CI)	External Os n=302* (95% CI)	P value
Collagen content [mg/mg per dry weight]	0.345 (0.326,0.364)	0.343 (0.325,0.362)	NS
PEN [mol/mol]	0.004 (0.004,0.005)	0.005 (0.005,0.006)	0.001
DPD [mol/mol]	0.045 (0.040,0.050)	0.044 (0.040,0.049)	NS
DHLNL [mol/mol]	0.090 (0.083,0.096)	0.098 (0.088,0.107)	NS
PYD [mol/mol]	0.123 (0.115,0.131)	0.122 (0.114,0.131)	NS

* Samples are from 13 patients

Table 7

Determining Internal Os Heterogeneity - Comparison of collagen content and specific collagen crosslinking density at the level of the internal os by quadrants. The analysis represents averages of all samples from the internal os from the 13 patients. Crosslink density is quantified on a mole per mole basis with collagen. Q1 = anterior quadrant, Q2 = left quadrant, Q3 = posterior quadrant, Q4 = right quadrant.

	Internal Os – Quadrant Comparison				P value
	Q1 n=71* (95% CI)	Q2 n=74* (95% CI)	Q3 n=74* (95% CI)	Q4 n=71* (95% CI)	
Collagen content [mg/mg per dry weight]	0.355 (0.320,0.389)	0.362 (0.322,0.401)	0.352 (0.309,0.395)	0.311 (0.272,0.349)	0.014 for Q1 v Q4
PEN [mol/mol]	0.004 (0.004,0.005)	0.004 (0.004,0.005)	0.005 (0.004,0.005)	0.004 (0.003,0.005)	NS
DPD [mol/mol]	0.043 (0.034,0.051)	0.046 (0.035,0.057)	0.050 (0.039,0.061)	0.042 (0.032,0.052)	0.022 for Q1 v Q3
DHLNL [mol/mol]	0.089 (0.074,0.103)	0.086 (0.072,0.101)	0.100 (0.086,0.114)	0.083 (0.070,0.096)	NS
PYD [mol/mol]	0.120 (0.104,0.136)	0.120 (0.102,0.138)	0.137 (0.120,0.154)	0.114 (0.099,0.130)	0.031 for Q1 v Q3

* Samples are from 13 patients

Table 8

Determining Internal Os Heterogeneity - Comparison of collagen content and specific collagen crosslinking density at the level of the internal os by zones. The analysis represents averages of all samples from the internal os from the 13 patients. Crosslink density is quantified on a mole per mole basis with collagen. Z1 = inner zone, Z2 = middle zone, Z3 = outer zone

	Internal Os – Zone Comparison			
	Zone 1 n=97* (95% CI)	Zone 2 n=97* (95% CI)	Zone 3 n=96* (95% CI)	P value
Collagen content [mg/mg per dry weight]	0.299 (0.270,0.327)	0.431 (0.397,0.465)	0.305 (0.274,0.336)	0.0023 for Z1 v Z2 NS for Z1 v Z3
PEN [mol/mol]	0.004 (0.004,0.005)	0.003 (0.003,0.004)	0.006 (0.005,0.006)	0.0102 for Z1 v Z2 <0.0001 for Z1 v Z3
DPD [mol/mol]	0.020 (0.017,0.023)	0.08 (0.069,0.091)	0.036 (0.031,0.041)	<0.0001 for Z1 v Z2 and Z1 v Z3
DHLNL [mol/mol]	0.067 (0.057,0.078)	0.094 (0.085,0.104)	0.107 (0.092,0.121)	0.0011 for Z1 v Z2 <0.0001 for Z1 v Z3
PYD [mol/mol]	0.093 (0.080,0.106)	0.117 (0.107,0.126)	0.160 (0.144,0.177)	0.0207 for Z1 v Z2 <0.0001 for Z1 v Z3

* Samples are from 13 patients

Table 9

Determining External Os Heterogeneity - Comparison of collagen content and specific collagen crosslinking density at the level of the external os by quadrants. The analysis represents averages of all samples from the external os from the 13 patients. Crosslink density is quantified on a mole per mole basis with collagen. Q1 = anterior quadrant, Q2 = left quadrant, Q3 = posterior quadrant, Q4 = right quadrant.

	External Os – Quadrant Comparison				P value
	Q1 n=71* (95% CI)	Q2 n=77* (95% CI)	Q3 n=76* (95% CI)	Q4 n=78* (95% CI)	
Collagen content [mg/mg per dry weight]	0.339 (0.304,0.375)	0.329 (0.292,0.367)	0.363 (0.324,0.401)	0.342 (0.307,0.378)	NS
PEN [mol/mol]	0.006 (0.005,0.006)	0.005 (0.004,0.005)	0.005 (0.005,0.006)	0.006 (0.005,0.007)	NS
DPD [mol/mol]	0.047 (0.036,0.058)	0.039 (0.031,0.047)	0.047 (0.039,0.056)	0.044 (0.035,0.053)	NS
DHLNL [mol/mol]	0.091 (0.074,0.107)	0.096 (0.077,0.114)	0.112 (0.090,0.133)	0.092 (0.074,0.110)	NS
PYD [mol/mol]	0.124 (0.105,0.142)	0.117 (0.098,0.135)	0.125 (0.110,0.141)	0.124 (0.108,0.141)	NS

* Samples are from 13 patients

Table 10

Determining External Os Heterogeneity - Comparison of collagen content and specific collagen crosslinking density at the level of the external os by zones. The analysis represents averages of all samples from the external os from the 13 patients. Crosslink density is quantified on a mole per mole basis with collagen. Z1 = inner zone, Z2 = middle zone, Z3 = outer zone

	External Os – Zone Comparison			
	Zone 1 n=100* (95% CI)	Zone 2 n=99* (95% CI)	Zone 3 n=103* (95% CI)	P value
Collagen content [mg/mg per dry weight]	0.295 (0.265,0.325)	0.437 (0.409,0.465)	0.301 (0.272,0.329)	<0.001 for Z1 v Z2 NS for Z1 v Z3
PEN [mol/mol]	0.006 (0.005,0.007)	0.004 (0.004,0.005)	0.006 (0.005,0.007)	0.003 for Z1 v Z2 NS for Z1 v Z3
DPD [mol/mol]	0.020 (0.018,0.023)	0.079 (0.069,0.088)	0.035 (0.031,0.039)	<0.001 for Z1 v Z2 and for Z1 v Z3
DHLNL [mol/mol]	0.070 (0.056,0.084)	0.104 (0.089,0.118)	0.119 (0.101,0.137)	<0.001 for Z1 v Z2 <0.001 for Z1 v Z3
PYD [mol/mol]	0.089 (0.077,0.101)	0.117 (0.105,0.129)	0.160 (0.144,0.177)	0.042 for Z1 v Z2 <0.001 for Z1 v Z3

* Samples are from 13 patients