

Original Article

Distinct collagen resolution dynamics in early versus advanced liver fibrosis: a quantitative SHG/TPEF study in mice

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Abstract: Background & Aims: Liver fibrosis regression occurs after the source of injury is removed, but its dynamics and histologic features remain poorly characterized. Second-harmonic generation/two-photon excited fluorescence (SHG/TPEF) microscopy enables quantitative, high-resolution collagen assessment. We employed SHG/TPEF to characterize spatiotemporal collagen remodeling during spontaneous regression in carbon tetrachloride (CCl₄)-induced mouse models. Methods: Early- and advanced-stage liver fibrosis were established by intraperitoneal CCl₄ injection for 8 or 12 weeks. After peak injury, mice recovered for 4 or 12 weeks. Liver sections were analyzed by Sirius red staining and SHG/TPEF imaging, which quantifies 100 collagen measurements. Results: SHG/TPEF imaging showed strong positive correlations with collagen proportionate area (CPA) quantified by Sirius red staining in both early- (P8: $r = 0.96$, $P < 0.001$) and advanced-stage (P12: $r = 0.87$, $P < 0.001$) fibrosis models. In early-stage fibrosis, both CPA and SHG percentage decreased significantly after 4 weeks of recovery (CPA: 1.21 ± 0.30 vs. 0.51 ± 0.15 , $P < 0.001$; SHG: 2.13 ± 0.68 vs. 1.18 ± 0.13 , $P = 0.008$), with no further reduction by 12 weeks ($P = 0.651$ and $P = 0.778$, respectively). In contrast, advanced-stage fibrosis showed progressive declines, with significant reductions in SHG percentage between 4 and 12 weeks (1.91 ± 0.10 vs. 1.36 ± 0.35 , $P = 0.023$). Quantitative collagen measurements analysis revealed that 35 measurements, particularly collagen fiber number-related metrics, regressed significantly slower in P12R4 than in P8R4 mice ($P < 0.05$, fold change > 1.5). Notably, early-stage regression predominantly occurred within the first 4 weeks, involving total, portal, and septal collagen, whereas advanced-stage regression was delayed, occurring mainly between 4-12 weeks and affecting total, septal, and fibrillar collagen measurements. Conclusions: SHG/TPEF provides a quantitative platform for monitoring regression dynamics in preclinical settings. Advanced fibrosis resolves with a delay, underscoring stage as a critical determinant in anti-fibrotic evaluation.

Keywords: Liver fibrosis, extracellular matrix, collagen, regression, SHG/TPEF microscopy

Introduction

Liver fibrosis, characterized by the excessive accumulation of extracellular matrix components, represents a common pathologic outcome of various chronic liver diseases. Currently, the primary method to treat liver fibrosis is etiological control, since there are no specific anti-fibrotic drugs available. Over the past decade, numerous clinical and preclinical

studies have shown that removing or suppressing the injurious stimulus can promote liver fibrosis regression; however, a subset of patients still fails to achieve fibrosis regression [1-4]. Our previous study indicates that patients whose liver fibrosis does not regress after long-term etiological therapy face a significantly higher risk of liver-related clinical endpoints compared to those who achieve regression [5, 6]. Consequently, there is an urgent need to

develop targeted anti-fibrotic therapies that can complement etiological treatment and improve the rate of fibrosis regression.

Due to inherent individual variability in human subjects and ethical constraints, animal models, particularly the carbon tetrachloride (CCl₄)-induced mouse models, are extensively used to study the pathogenesis of liver fibrosis and evaluate potential anti-fibrotic agents [7]. In clinical trials, histologic assessment of liver biopsies remains the gold standard for evaluating therapeutic response. Although several semi-quantitative scoring systems (e.g., Knodell, Scheuer, Ishak, and Metavir) are routinely used in clinical practice [8], their application in CCl₄-induced mouse models has not been widely validated. Moreover, different therapeutic agents may target distinct components of scar tissue, reflecting specific cellular and molecular events [9]. Therefore, beyond traditional cross-sectional staging, there is an increasing need for precise and sensitive quantitative tools to assess liver fibrosis regression accurately in CCl₄-induced mouse models.

In recent years, a fully quantitative, label-free imaging system based on second-harmonic generation/two-photon excited fluorescence (SHG/TPEF) microscopy has been developed [10]. This reproducible platform enables precise visualization and quantification of collagen architecture and has been successfully applied to assess fibrotic progression in human liver biopsies from various etiologies, including virus infections [1, 11, 12], metabolic dysfunction-associated steatotic liver disease [13-15], and primary biliary cholangitis [16]. Similarly, the system has been validated for staging experimental liver fibrosis in rodents, showing strong correlation with collagen proportionate area (CPA) and hydroxyproline content [17, 18].

However, the ability of SHG/TPEF microscopy to detect subtle, progressive changes in collagen morphology during fibrosis regression, which is critical for accurately reflecting therapeutic response, has yet to be thoroughly established in experimental animal models. Therefore, this study aimed to quantitatively characterize fine-grained histologic alterations during spontaneous regression of CCl₄-induced liver fibrosis in mice using the SHG/TPEF platform. Our goal was to evaluate the sensitivity of this system in monitoring regressive changes and to provide a

detailed dynamic atlas of histologic remodeling during fibrosis regression in CCl₄-intoxicated mouse models.

Materials and methods

Mouse models of CCl₄-induced spontaneous regression of liver fibrosis

Eight-week-old male collagen $\alpha 1(I)$ -GFP mice (kindly provided by Prof. David A. Brenner, University of California-San Diego) [19], weighing 23.9 ± 0.7 g, were housed under a 12-h light/dark cycle with ad libitum access to food and water. After one week of acclimation, liver fibrosis was induced by intraperitoneal injection of 12.5% CCl₄ in olive oil (1:7, v/v) at a dose of 0.01 mL/g body weight, twice weekly. Two fibrotic stages were established: an early-stage after 8 weeks of CCl₄ intoxication (P8) and an advanced-stage after 12 weeks of CCl₄ intoxication (P12). Following injury, P8 and P12 mice were allowed to undergo spontaneous recovery for 4 (R4) or 12 (R12) weeks (n = 5/group). At the end of the intoxication periods (P8 and P12) and after the recovery periods (R4 and R12), mice were euthanized by intraperitoneal injection of tribromoethanol (2.5% solution) at a dose of 0.2 mL/10 g body weight (equivalent to 500 mg/kg). Liver specimens from the left lateral lobe were fixed in formalin, paraffin-embedded, and sectioned at 7 μ m thickness for Sirius red staining (Solarbio, China) and SHG/TPEF imaging. All mouse procedures were approved by the Animal Care and Use Committee of Beijing Friendship Hospital, Capital Medical University (approval No. 15-2004), and carried out according to the ARRIVE guidelines.

SHG/TPEF microscopy image acquisition and quantification

Unstained liver sections were imaged using a Genesis200™ system (HistoIndex Pte. Ltd., Singapore) employing SHG/TPEF technology as previously described [11]. Collagen fibers were visualized using SHG, while cellular structures were captured via TPEF. Imaging was performed with a resolution of 512 × 512 pixels. One to three random, non-overlapping tile-scanned images were acquired per section. Image analysis included automated identification and extraction of 100 collagen morphological features (Supplementary Table 1) describ-

ing numbers, length, width, and crosslinks of collagen fibers from portal, septal, and fibrillar regions based on established algorithms [20]. Additionally, raw SHG/TPEF images were processed using a dedicated digital segmentation pipeline to identify and quantify septal area, enabling precise morphometric assessment, as previously described [20]. The normalized values were averaged for further analysis.

Statistical analysis

The quantification of CPA from Sirius red-stained sections was performed using Image-Pro Plus 6.0 software (Media Cybernetics, MD, USA). All data were expressed as mean $\pm$ standard deviation. Differential abundance of collagen morphologic features between groups was assessed using Student's *t*-test. Given the limited sample size ($n = 5$ per group) and to avoid an overly conservative estimate that might obscure biologically relevant differences, we did not apply multiple comparison corrections. Instead, a $P < 0.05$ combined with a fold change (FC) > 1.5 was adopted as the threshold for significance for determination of differential abundance of collagen morphologic features. For comparisons among three groups, one-way ANOVA followed by Tukey's multiple comparisons test was conducted using GraphPad Prism version 9.0.0 (GraphPad Software, CA, USA), with an adjusted $P < 0.05$ considered significant. Additionally, Pearson correlation plots, heatmaps, uniform manifold approximation and projection (UMAP) plots, and volcano plots were generated and visualized using the SangerBox software [21].

Results

Comparison of SHG/TPEF microscopy and conventional histologic imaging in evaluating liver fibrosis regression in CCl₄-induced mouse models

The dynamics of spontaneous liver fibrosis regression (R4 and R12) were evaluated in two CCl₄-induced mouse models representing early- and advanced-stage liver fibrosis (P8 and P12) (**Figure 1A**). Histologic changes during regression were assessed using Sirius red staining and SHG/TPEF imaging. The SHG percentage, analogous to CPA, quantifies the collagen-to-tissue area ratio by means of SHG/TPEF, enabling clear differentiation be-

tween hepatocytes (TPEF, red) and collagen (SHG, green) (**Figure 1B**). Strong positive correlations were observed between CPA and SHG percentage in both the P8 ($r = 0.96$, $P < 0.001$) and P12 ($r = 0.87$, $P < 0.001$) model series (**Figure 1C**), demonstrating good concordance between these two independent histologic quantification methods. Notably, during regression in the early-stage model, both CPA and SHG percentage decreased markedly after 4 weeks of recovery (CPA: 1.21 ± 0.30 [P8] versus 0.51 ± 0.15 [P8R4], $P < 0.001$; SHG: 2.13 ± 0.68 [P8] versus 1.18 ± 0.13 [P8R4], $P = 0.008$), with no further reduction observed after an additional 8 weeks (CPA: 0.51 ± 0.15 [P8R4] versus 0.40 ± 0.08 [P8R12], $P = 0.651$; SHG: 1.18 ± 0.13 [P8R4] versus 1.00 ± 0.07 [P8R12], $P = 0.778$), indicating that fibrosis regression reaches a plateau by 4 weeks in this model (**Figure 1D**). In the advanced-stage model, however, both CPA and SHG percentage exhibited a continuous decline over the entire regression period. A significant decrease in SHG percentage was observed between R4 and R12 (1.91 ± 0.10 vs. 1.36 ± 0.35 , $P = 0.023$), whereas the reduction in CPA was not significant (0.89 ± 0.06 vs. 0.54 ± 0.05 , $P = 0.061$) (**Figure 1D**). Overall, these results highlight that the regression pattern is critically dependent on the pre-existing fibrotic burden.

Quantitative SHG/TPEF imaging reveals delayed regression of the number of collagen fibers in advanced liver fibrosis following short-term recovery

We next employed SHG/TPEF imaging to assess quantitative changes in both the quantity and morphology of collagen during regression of early and advanced liver fibrosis. These 100 collagen morphologic measurements were categorized into four groups: total collagen (total, 16 measurements), portal tract collagen (portal, 28 measurements), septal collagen (septal, 28 measurements), and fibrillar collagen (fibrillar, 28 measurements) (**Figure 2A** and **Supplementary Table 1**). At peak fibrosis (P8 and P12), heatmap analysis showed comparable levels of these fine collagen measurements between the two models (**Figure 2B**, left panel). After 4 weeks of recovery (P8R4 and P12R4), these measurements were observably downregulated in both models; however, the degree of reduction was notably more pro-

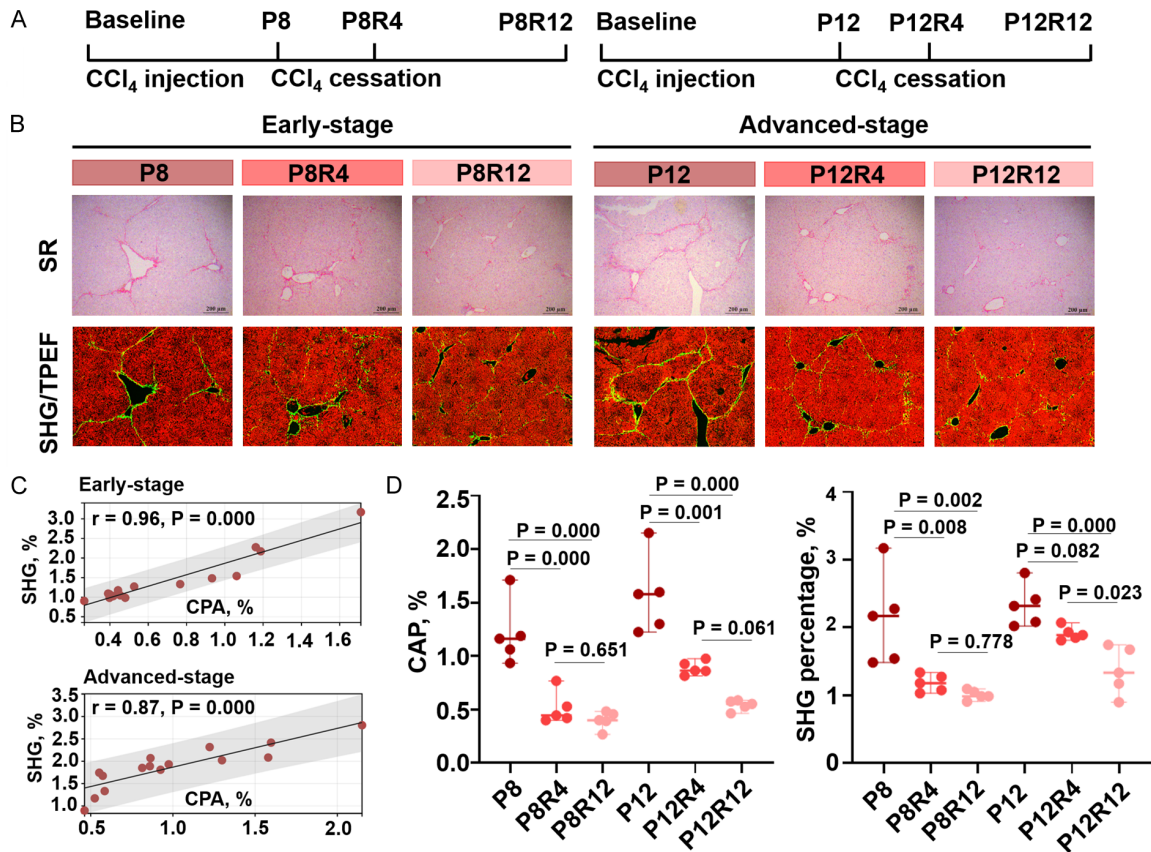


Figure 1. Comparative assessment of liver fibrosis regression using SHG/TPEF microscopy and conventional histology. **A.** Schematic of the experimental design. Two mouse models of CCl₄-induced liver fibrosis were established, representing early-stage (8 weeks, P8) and advanced-stage (12 weeks, P12) fibrosis. After CCl₄ withdrawal, spontaneous regression was assessed over 4-week (R4) or 12-week (R12) recovery periods, generating four groups: P8R4, P8R12, P12R4, and P12R12 (where P denotes injury duration and R denotes recovery duration). **B.** Representative Sirius red (SR) staining images and SHG/TPEF images are shown, depicting hepatocytes (red, TPEF signal) and collagen fibers (green, SHG signal). **C.** Pearson's correlation analysis between CPA and SHG percentage in the early-stage and advanced-stage liver fibrosis models. **D.** Quantification of liver fibrosis during regression using conventional Sirius red staining (expressed as collagen proportionate area [CPA], left) and SHG/TPEF imaging (expressed as SHG percentage, right).

nounced in the early-stage fibrosis model (P8) compared to the advanced-stage model (P12) (Figure 2B, middle panel). After 12 weeks of recovery, collagen measurement profiles in both the early- and advanced-stage groups (P8R12 and P12R12) became largely similar (Figure 2B, right panel). Additionally, UMAP analysis, incorporating the 100 collagen measurements as a combined signature, clearly distinguished the P8R4 group from the P12R4 group, highlighting divergent resolution (Figure 2C). Subsequent comparative analysis revealed that 35 collagen measurements, particularly the number of collagen fibers, regressed at a significantly slower rate in P12R4 mice compared to their P8R4 counterparts ($P < 0.05$

and $FC > 1.5$) (Figure 2D, 2E). These results suggest that advanced-stage fibrosis exhibits a slower regression rate for key collagen micro-architectural features following injury cessation. However, with prolonged recovery, the histologic outcome in advanced-stage fibrosis can approach that observed in early-stage fibrosis regression.

Divergent dynamics of collagen resolution in early and advanced liver fibrosis depicted by quantitative SHG/TPEF imaging analysis

We further used quantitative SHG/TPEF imaging to delineate the dynamics of spontaneous regression in early- and advanced-stage liver

Collagen resolution dynamics in liver fibrosis

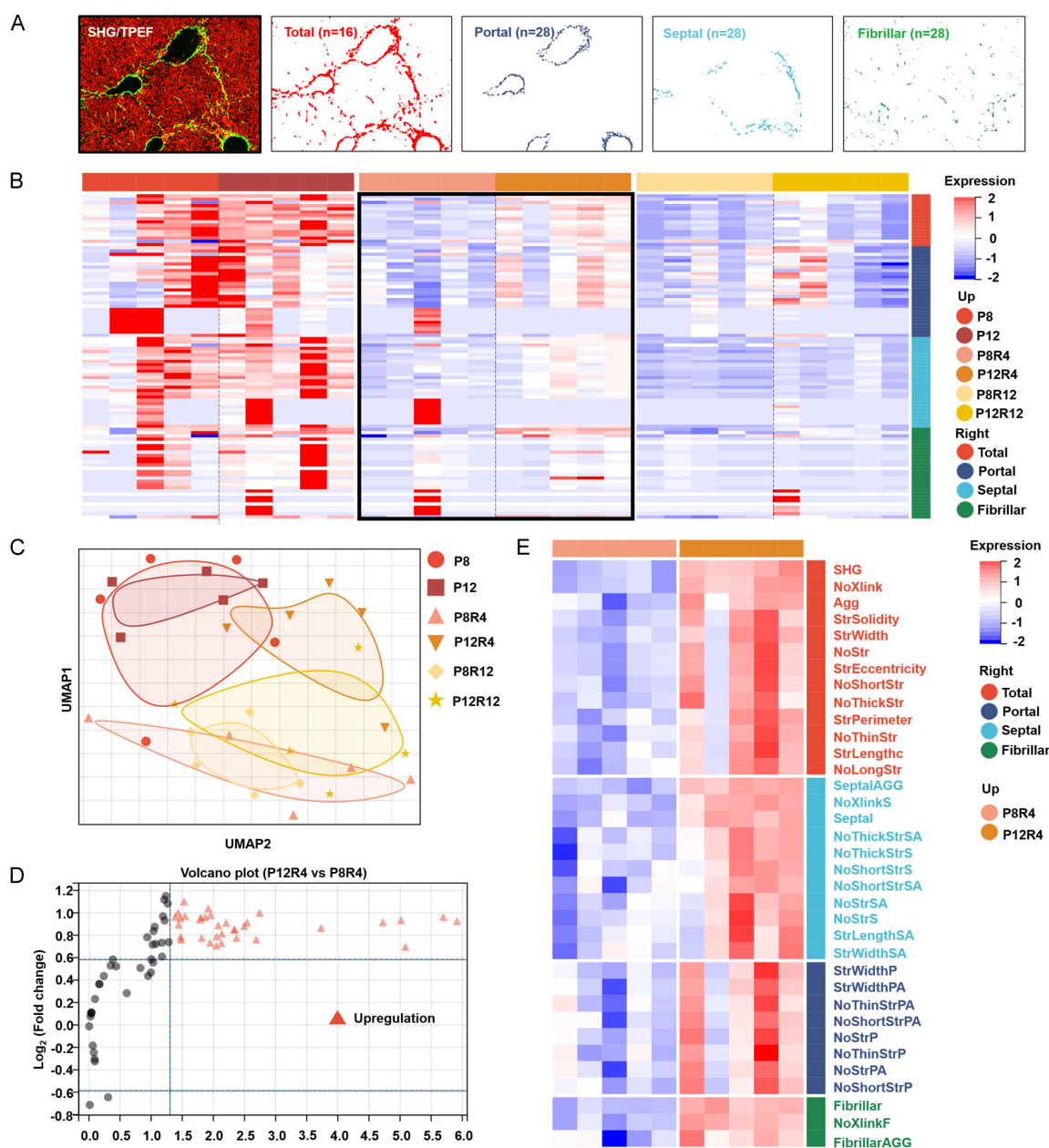


Figure 2. Quantitative SHG/TPEF imaging reveals differential regression dynamics of collagen microarchitecture between early- and advanced-stage liver fibrosis. **A.** Representative images of the four categories of collagen measurements analyzed by SHG/TPEF imaging: total (red), portal (dark blue), septal (light blue), and fibrillar (green) collagens. **B.** Heatmap displaying the 100 collagen measurements at peak fibrosis (P8 and P12, left panel), after 4 weeks of recovery (P8R4 and P12R4, middle panel), and after 12 weeks of recovery (P8R12 and P12R12, right panel). Expression values are Z score-normalized (red, high; blue, low). Mouse groups and collagen measurement categories are indicated by color bars. **C.** UMAP visualization based on the 100 collagen measurements, with mouse groups color-coded. **D.** Volcano plot highlighting 35 collagen measurements that exhibited significantly slower reversal rates in the advanced-stage model (P12R4) compared to the early-stage model (P8R4). **E.** Heatmap showing the 35 collagen measurements. Expression values are Z score-normalized (red, high; blue, low). Mouse groups and collagen measurement categories are color-coded.

fibrosis. As shown in **Figure 3A**, after 4 weeks of regression in early-stage fibrosis (P8 versus

P8R4), significant decreases were observed in 13 total, 17 septal, 8 portal, and 1 fibrillar col-

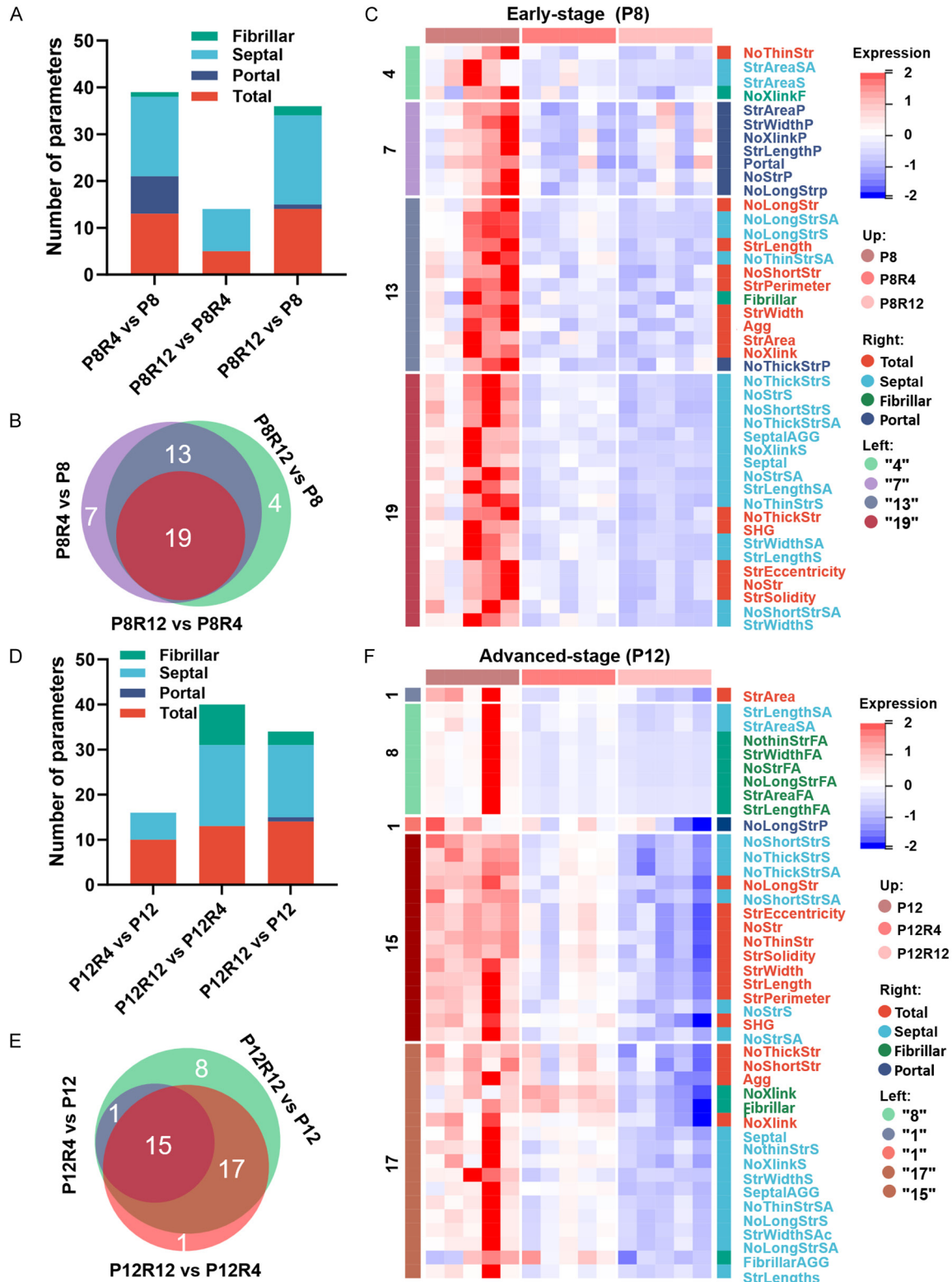


Figure 3. Divergent dynamics of collagen resolution during spontaneous regression in early-stage and advanced-stage liver fibrosis. (A) Schematic summary showing the number of collagen measurements (total, septal, portal, fibrillar) that were significantly downregulated during the early (0-4 weeks) and late (4-12 weeks) phases of regression in the early-stage fibrosis model (P8). Collagen measurement categories are color-coded. (B) Venn diagram illustrating the overlap and distinct sets of collagen measurements was significantly reduced at 4 weeks (P8R4) and

12 weeks (P8R12) of recovery in the early-stage model. (C) Heatmap displaying the expression patterns of collagen measurements with significant reductions in the overlapping and distinct sets shown in (B). Expression values are Z score-normalized (red, high; blue, low). Mouse groups and collagen measurement categories are indicated by color bars. (D) Schematic summary showing the number of collagen measurements significantly downregulated during the early (0-4 weeks) and late (4-12 weeks) phases of regression in the advanced-stage fibrosis model (P12). Collagen measurement categories are color-coded. (E) Venn diagram illustrating the overlap and distinct sets of collagen measurements significantly reduced at 4 weeks (P12R4) and 12 weeks (P12R12) of recovery in the advanced-stage model. (F) Heatmap displaying the expression patterns of collagen measurements with significant reductions in the overlapping and distinct sets shown in (E). Expression values are Z score-normalized (red, high; blue, low). Mouse groups and collagen measurements categories are indicated by color bars.

lagen measurements ($P < 0.05$ and $FC > 1.5$). However, between 4 and 12 weeks of regression (P8R4 versus P8R12), only 5 total and 9 septal measurements showed further significant reductions ($P < 0.05$ and $FC > 1.5$). Venn analysis indicated that 7 portal measurements (e.g., StrAreaP, StrWithP, NoXlinkP, StrLengthP, Portal, NoStrP, and NoLongStrP) were significantly downregulated within the first 4 weeks ($P < 0.05$ and $FC > 1.5$) and remained stable thereafter. In contrast, 19 measurements, predominantly septal (e.g., NoThickStrS, NoStrS, NoShortStrS, NoThickStrSA, SeptalAGG, NoXlinkS, Septal, NoStrSA, StrLengthSA, NoThinStrS, StrWidthSA, StrLengthS, NoShortStrSA, StrWidthS), exhibited progressive reductions throughout the regression period ($P < 0.05$ and $FC > 1.5$) (**Figure 3B, 3C**). In advanced-stage fibrosis, after 4 weeks of regression, significant decreases were noted in 10 total and 6 septal p measurements ($P < 0.05$ and $FC > 1.5$). Between 4 and 12 weeks, however, 13 total, 18 septal, and 9 fibrillar measurements showed significant reductions ($P < 0.05$ and $FC > 1.5$) (**Figure 3D, 3E**). In contrast, only one portal measurement (NoLongStrP) was significantly downregulated during this later interval ($P < 0.05$ and $FC > 1.5$). Among fibrillar measurements, NoThinStrFA, StrWidthFA, NoStrFA, NoLongStrFA, StrAreaFA, and StrLengthFA exhibited significant decreases only after 12 weeks of regression ($P < 0.05$ and $FC > 1.5$) (**Figure 3E, 3F**). Collectively, these findings demonstrate that spontaneous regression in early-stage liver fibrosis occurs predominantly within the first 4 weeks of recovery, primarily involving total, portal, and septal collagen measurements. In contrast, regression in advanced-stage fibrosis is delayed, occurring mainly between 4 and 12 weeks of recovery and predominantly affecting total, septal, and fibrillar collagen measurements.

Discussion

Our study employed SHG/TPEF microscopy to conduct high-resolution, quantitative characterization of collagen morphologic changes during spontaneous regression in the CCl_4 -induced mouse model of liver fibrosis. The robust correlation between SHG percentage and CPA corroborates SHG as a valid surrogate for total collagen; however, its broader dynamic range implies greater sensitivity to incremental alterations, a critical advantage for monitoring therapeutic responses where changes may be modest. Using this system, we found that the regression rate was fundamentally governed by the initial fibrotic stage, with advanced fibrosis exhibiting delayed regression of key microarchitectural collagen measurements. Overall, our study established SHG/TPEF microscopy as a powerful tool for evaluating novel therapeutic candidates in pre-clinical settings, while also providing a detailed kinetic atlas of collagen remodeling during regression.

The central finding of our study was the temporal dissociation in collagen resolution between early- and advanced-stage fibrosis. In the early-stage model (P8), regression is rapid and biphasic: a broad reduction across total, portal, and septal collagen measurements occurs within the first 4 weeks, followed by a more targeted resolution of septal parameters thereafter. In contrast, advanced fibrosis (P12) displays a delayed but ultimately effective regression, with minimal changes during the initial 4 weeks and substantial remodeling concentrated between 4 and 12 weeks. Critically, the most discriminative feature of this delayed kinetics is the behavior of fibrillar collagen measurements, which represent relatively mature, highly crosslinked collagen fibers, exhibiting no appreciable decline within the first 4 weeks but decreasing significantly between

weeks 4 and 12. This temporal pattern directly aligns with the unique resolution bottleneck in advanced disease, where the bulk of newly deposited collagens may be cleared early, but the densely crosslinked scaffold resists immediate proteolysis.

Focusing specifically on the degradation kinetics of mature/crosslinked collagen, we propose that the lag phase in advanced fibrosis reflects the time required for the endogenous remodeling machinery to overcome the physicochemical barriers posed by a stabilized extracellular matrix. Mature fibrillar collagen, owing to its extensive intermolecular crosslinks (mediated by enzymes such as lysyl oxidase) [22], is inherently more resistant to proteolytic cleavage than newly deposited, less crosslinked collagen [23, 24]. The delayed reduction of fibrillar measurements (e.g., NoThinStrFA, StrWidthFA, NoStrFA, NoLongStrFA, StrAreaFA, and StrLengthFA) observed exclusively between weeks 4 and 12 is therefore consistent with a model in which the advanced fibrotic scar must first undergo a “priming” phase, potentially involving enzymatic modification or partial decoring of the matrix, before efficient fragmentation and removal of the core crosslinked fibrils can occur. This interpretation is further supported by the observation that portal collagen measurements, which likely accumulate highly stable crosslinks as the initial injury nidus, remain largely unchanged during regression in the advanced model, echoing our previous findings in chronic hepatitis B patients [25].

While matrix metalloproteinases (MMPs) [26, 27], macrophage polarization [28, 29], and altered crosslinking enzyme activity [22, 30] are all plausible contributors to this delayed degradation kinetics, it is essential to emphasize that none of these molecular or cellular mechanisms were directly investigated in the present study. Our SHG/TPEF platform provides exquisite morphologic and architectural detail, but it does not offer direct information on enzymatic activity, cellular phenotypes, or biochemical crosslink density. Therefore, the proposed involvement of MMPs, collagen crosslinking, and macrophage function remains a working hypothesis derived from the kinetic signature we observed. Future studies integrating SHG/TPEF with complementary

approaches, such as immunohistochemistry for crosslinking enzymes (e.g., LOXL2) [30–32], zymography for MMP activity, and immunophenotyping of hepatic macrophages are warranted to definitively establish the biological drivers of the differential regression kinetics identified here. Functional perturbation experiments, including selective inhibition or knock-out of key crosslinking enzymes, would be particularly valuable to test whether the observed lag phase can be shortened, thereby accelerating regression in advanced fibrosis.

The clinical implications of these kinetic distinctions are substantial. Our data provide a pre-clinical correlate to the clinical observation that a subset of patients fails to achieve fibrosis regression despite effective etiological therapy. The 12-week CCl₄ model, which exhibits a delayed and prolonged remodeling phase, may better recapitulate the therapeutic challenge in patients with extensive, crosslinked scars. This supports the concept of a fibrotic threshold [33], beyond which the matrix acquires intrinsic stability and becomes less susceptible to endogenous resolution. For drug development, our findings highlight the need to evaluate anti-fibrotic candidates in both early and advanced models, as an agent that accelerates the initial rapid phase may not necessarily promote the complex, late-stage remodeling required in advanced disease. The specific fibrillar collagen measurements that demonstrated exclusive late-phase changes could serve as sensitive, quantitative pharmacodynamic biomarkers to differentiate the mechanisms of action of various therapeutic candidates, particularly those targeting mature matrix turnover.

Several limitations of this study should be acknowledged. First, we employed the CCl₄ model of toxic injury, which may not fully capture the complex matrix remodeling observed in metabolic dysfunction-associated or cholestatic liver fibrosis. Validation in additional etiological models is therefore required. Second, as noted above, the lack of direct molecular and cellular data precludes definitive conclusions about the mechanisms underlying the delayed fibrillar collagen regression. Third, although our sample size was sufficient to detect robust differences using stringent thresholds, it may have limited our power to

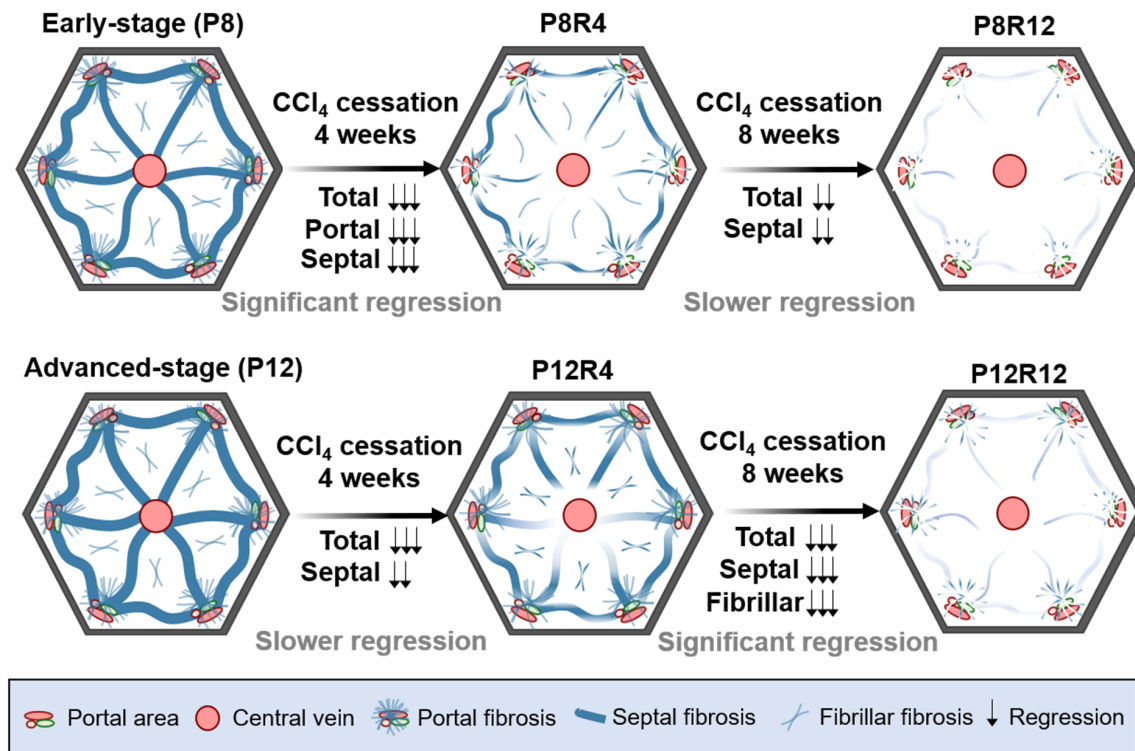


Figure 4. Schematic summary of differential collagen regression dynamics between early-stage and advanced-stage liver fibrosis. In early-stage fibrosis (up panel), significant regression of collagen deposits occurs rapidly within the first 4 weeks of recovery, primarily affecting total, portal, and septal collagen, with minimal changes thereafter. In contrast, advanced-stage fibrosis (down panel) exhibits a delayed regression rate, with collagen resolution occurring predominantly between 4 and 12 weeks of recovery and involving total, septal, and fibrillar collagen compartments more extensively.

identify more subtle changes in individual measurements. Fourth, we did not apply multiple-comparison corrections in our analysis of the 100 collagen measurements; this was a deliberate choice to avoid overly conservative estimates that could obscure biologically relevant signals in this exploratory study, but it increases the risk of false-positive findings and should be considered a limitation. Nevertheless, the strong temporal and categorical consistency across multiple measurements strengthens the reliability of our main conclusions.

Conclusion

Our study establishes SHG/TPEF microscopy as a sensitive, quantitative tool for dissecting collagen remodeling dynamics and provides a refined kinetic framework that distinguishes early regression of labile matrix from the delayed degradation of mature, crosslinked fibrils in advanced liver fibrosis (**Figure 4**). This framework not only advances our understand-

ing of fibrosis regression biology but also offers practical endpoints for preclinical evaluation of next-generation anti-fibrotic therapies.

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Disclosure of conflict of interest

None.

Abbreviations

CCl₄, carbon tetrachloride; CPA, collagen proportionate area; MMP, matrix metalloproteinase; SHG/TPEF, second-harmonic generation/

two-photon excited fluorescence; UMAP, uniform manifold approximation and projection.

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Supplementary Table 1. Collagen morphological parameters and their abbreviations

NO.	Abbreviation	Description
1	SHG	SHG Percentage
2	Agg	Aggregated Collagen Percentage
3	Dis	Distributed Collagen Percentage
4	NoStr	Number of Strings
5	NoShortStr	Number of Short Strings
6	NoLongStr	Number of Long Strings
7	NoThinStr	Number of Thin Strings
8	NoThickStr	Number of Thick Strings
9	StrArea	String Area
10	StrLength	String Length
11	StrWidth	String Width
12	StrEccentricity	String Eccentricity
13	StrSolidity	String Solidity
14	StrPerimeter	String Perimeter
15	StrOrientation	String Orientation
16	NoXlink	Number of Crosslinks
17	Portal	Portal Collagen Percentage
18	PortalAGG	Portal Aggregated Collagen Percentage
19	PortalDIS	Portal Distributed Collagen Percentage
20	NoStrP	Number of Strings (Portal)
21	NoShortStrP	Number of Short Strings (Portal)
22	NoLongStrP	Number of Long Strings (Portal)
23	NoThinStrP	Number of Thin Strings (Portal)
24	NoThickStrP	Number of Thick Strings (Portal)
25	StrAreaP	String Area (Portal)
26	StrLengthP	String Length (Portal)
27	StrWidthP	String Width (Portal)
28	NoStrPA	Number of Strings (Portal and Aggregated)
29	NoShortStrPA	Number of Short Strings (Portal and Aggregated)
30	NoLongStrPA	Number of Long Strings (Portal and Aggregated)
31	NoThinStrPA	Number of Thin Strings (Portal and Aggregated)
32	NoThickStrPA	Number of Thick Strings (Portal and Aggregated)
33	StrAreaPA	String Area (Portal and Aggregated)
34	StrLengthPA	String Length (Portal and Aggregated)
35	StrWidthPA	String Width (Portal and Aggregated)
36	NoStrPD	Number of Strings (Portal and Distributed)
37	NoShortStrPD	Number of Short Strings (Portal and Distributed)
38	NoLongStrPD	Number of Long Strings (Portal and Distributed)
39	NoThinStrPD	Number of Thin Strings (Portal and Distributed)
40	NoThickStrPD	Number of Thick Strings (Portal and Distributed)
41	StrAreaPD	String Area (Portal and Distributed)
42	StrLengthPD	String Length (Portal and Distributed)
43	StrWidthPD	String Width (Portal and Distributed)
44	NoXlinkP	Number of Crosslinks (Portal)
45	Septal	Septal Collagen Percentage
46	SeptalAGG	Septal Aggregated Collagen Percentage

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47	SeptalDIS	Septal Distributed Collagen Percentage
48	NoStrS	Number of Strings (Septal)
49	NoShortStrS	Number of Short Strings (Septal)
50	NoLongStrS	Number of Long Strings (Septal)
51	NoThinStrS	Number of Thin Strings (Septal)
52	NoThickStrS	Number of Thick Strings (Septal)
53	StrAreaS	String Area (Septal)
54	StrLengthS	String Length (Septal)
55	StrWidthS	String Width (Septal)
56	NoStrSA	Number of Strings (Septal and Aggregated)
57	NoShortStrSA	Number of Short Strings (Septal and Aggregated)
58	NoLongStrSA	Number of Long Strings (Septal and Aggregated)
59	NoThinStrSA	Number of Thin Strings (Septal and Aggregated)
60	NoThickStrSA	Number of Thick Strings (Septal and Aggregated)
61	StrAreaSA	String Area (Septal and Aggregated)
62	StrLengthSA	String Length (Septal and Aggregated)
63	StrWidthSA	String Width (Septal and Aggregated)
64	NoStrSD	Number of Strings (Septal and Distributed)
65	NoShortStrSD	Number of Short Strings (Septal and Distributed)
66	NoLongStrSD	Number of Long Strings (Septal and Distributed)
67	NoThinStrSD	Number of Thin Strings (Septal and Distributed)
68	NoThickStrSD	Number of Thick Strings (Septal and Distributed)
69	StrAreaSD	String Area (Septal and Distributed)
70	StrLengthSD	String Length (Septal and Distributed)
71	StrWidthSD	String Width (Septal and Distributed)
72	NoXlinkS	Number of Crosslinks (Septal)
73	Fibrillar	Fibrillar Collagen Percentage
74	FibrillarAGG	Fibrillar Aggregated Collagen Percentage
75	FibrillarDIS	Fibrillar Distributed Collagen Percentage
76	NoStrF	Number of Strings (Fibrillar)
77	NoShortStrF	Number of Short Strings (Fibrillar)
78	NoLongStrF	Number of Long Strings (Fibrillar)
79	NoThinStrF	Number of Thin Strings (Fibrillar)
80	NoThickStrF	Number of Thick Strings (Fibrillar)
81	StrAreaF	String Area (Fibrillar)
82	StrLengthF	String Length (Fibrillar)
83	StrWidthF	String Width (Fibrillar)
84	NoStrFA	Number of Strings (Fibrillar and Aggregated)
85	NoShortStrFA	Number of Short Strings (Fibrillar and Aggregated)
86	NoLongStrFA	Number of Long Strings (Fibrillar and Aggregated)
87	NoThinStrFA	Number of Thin Strings (Fibrillar and Aggregated)
88	NoThickStrFA	Number of Thick Strings (Fibrillar and Aggregated)
89	StrAreaFA	String Area (Fibrillar and Aggregated)
90	StrLengthFA	String Length (Fibrillar and Aggregated)
91	StrWidthFA	String Width (Fibrillar and Aggregated)
92	NoStrFD	Number of Strings (Fibrillar and Distributed)
93	NoShortStrFD	Number of Short Strings (Fibrillar and Distributed)
94	NoLongStrFD	Number of Long Strings (Fibrillar and Distributed)

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95	NoThinStrFD	Number of Thin Strings (Fibrillar and Distributed)
96	NoThickStrFD	Number of Thick Strings (Fibrillar and Distributed)
97	StrAreaFD	String Area (Fibrillar and Distributed)
98	StrLengthFD	String Length (Fibrillar and Distributed)
99	StrWidthFD	String Width (Fibrillar and Distributed)
100	NoXlinkF	Number of Crosslinks (Fibrillar)
