Quantification of Septa in Liver Fibrosis using Second Harmonic Generation Imaging

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ABSTRACT

Background

Liver fibrosis is associated with excessive accumulation of extracellular matrix in response to persistent injury, inflammation, and abnormal wound healing. A common method to assess liver fibrosis is by conventional staining and histopathological evaluation using i.e. Metavir Scoring. This assessment has limitation as it uses a narrow range scoring, a qualitative septa counts, and it is also prone to observer variations. Collagen quantitation in the conventional method refers to only total collagen and lacks description on the fibrous septa. Here we use second harmonic generation (SHG) together with two-photon excitation fluorescence (2PE) imaging and machine-learning image analysis algorithm to provide a more in depth look at the changes in the fibrous septa as fibrosis development advances or regresses from a stained-free histological tissue section.
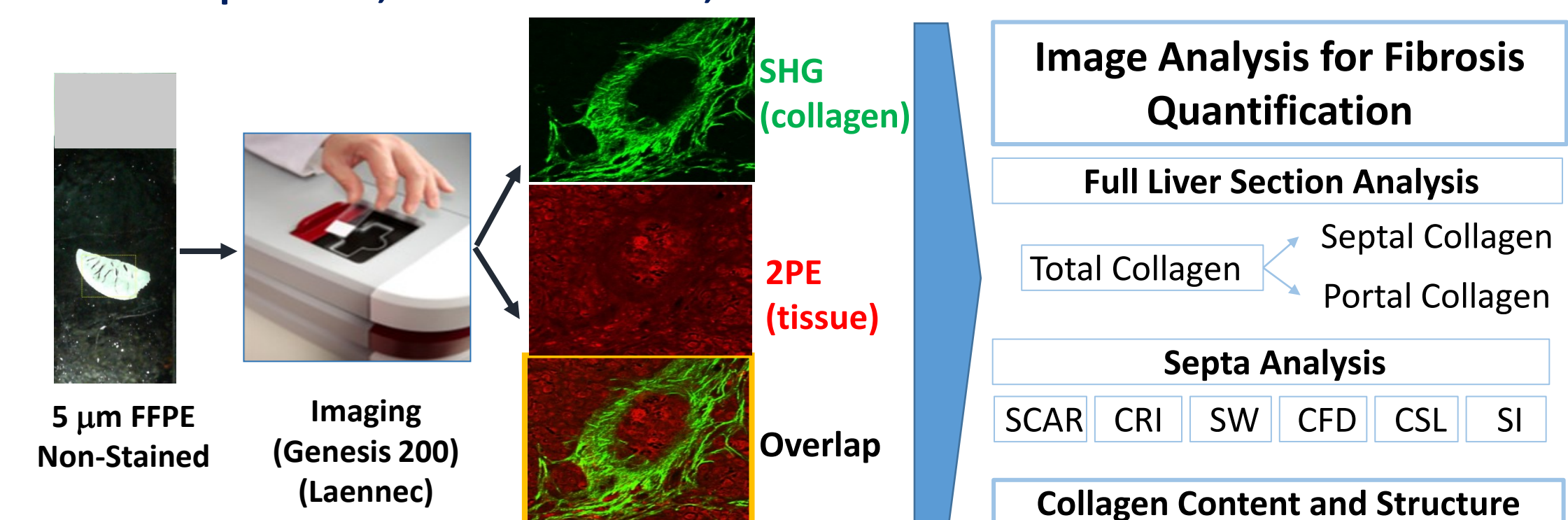
Method

In this study, mice are treated with olive oil (control) or carbon tetrachloride (CCl4) with or without an anti-fibrotic drug, Pirfenidone. CCl4 is a hepatatoxicant used to induce liver fibrosis. FFPE liver histological sections (5µm) were imaged (without staining) using Genesis 200[R], a multi-photon optical digital imaging system, which combines SHG to produce collagen specific images (collagens I and III) and 2PE to delineate tissue morphology. We developed a robust, semi-automatic image analysis algorithm to extract information on the fibrous septa. Full liver section analysis metrics include Total, Vasculature (portal and central vein), and Septal Collagen. Metrics for fibrous septa include Septal Collagen Area, Reticulation index, Fiber Density, Thickness (width), Branches, and Segment Length.

Results and Conclusion

The study result revealed that CCl4 induces liver fibrosis by not only increasing total collagen levels but also causes septa (quantifiable) characteristic changes. This induction was ameliorated with Pirfenidone. As fibrosis progress, there are significant changes as short thin fibrous septa developed into advanced bridging fibrosis. Majority of the septa show increase in collagen area accompanied by increase in collagen structure, which is a potential indicator of tissue stiffness. Fibrous septa are also thicker with more but shorter "tree" branches. SHG/ 2PE imaging is a novel stained-free imaging technique combined with a robust image analysis system to provide an semi-automated and fully quantitative extraction of information for liver fibrosis and the septa. This capability may help evaluate anti-fibrotic therapy efficacy during fibrosis progression and regression.

METHOD

Tissue Preparation, Instrumentation, and Workflow

5 µm FFPE Non-Stained

Imaging (Genesis 200) (Laennec)

SHG (collagen)
2PE (tissue)
Overlap

Image Analysis for Fibrosis Quantification

Full Liver Section Analysis

Total Collagen — Septal Collagen / Portal Collagen

Septa Analysis

SCAR | CRI | SW | CFD | CSL | SI

Collagen Content and Structure

- 5-200µM FFPE or Frozen sections
- Stain-Free and label- free imaging
- Fully quantitation of collagen and septa
- High Resolution (0.4 uM @ 20x)
- Non-destructive (tissue resusable)

- **2-Photon Excitation (2PE):** auto fluorescence for tissue morphology depict cellular structure and injury
- **Second Harmonic Generation (SHG):** Collagens I and III
- **Machine-** learning Image Analysis Software optimized and validated with pathologists

CCl4 Liver Fibrosis Model

Balb/c mice were treated (i.p. injection) with olive oil (**Crtl**) or **CCl4**- olive oil mixed solution for 4wk and 6wk with or without Pirfenidone (**PFD**), an anti-fibrotic therapeutic drug.

| Group 1	Group 2	Group 3	Group 4
Control	CCl4 (4wk)	CCl4 (6wk)	CCl4 (6wk) + Pirfenidone

Liver Fibrosis Image Analysis

1. Full Liver Section
 -Regional segmentation (histology image and graph)
 Total (Vasculature + Septa), Vasculature (Portal), Septal Collagen
 -2D Fibrosis Chart

2. Fibrous Septa Analysis
 -Septal Collagen Area Ratio% (SCAR): area occupy by collagen in the region of interest
 -Collagen Reticulation Index (CRI): measures collagen fiber network
 -Septal Width (SW): width at 1/3rd, 2/3rd length of septa
 -Septal Intersection(SI): # branches or intersections at 1/3rd, 2/3rd length of septa
 -Collagen Segment Length (CSL): average length of the septal branches
 -Collagen Fiber Density (CFD): collagen density within fiber, intensity directly proportional to amount collagen within fiber
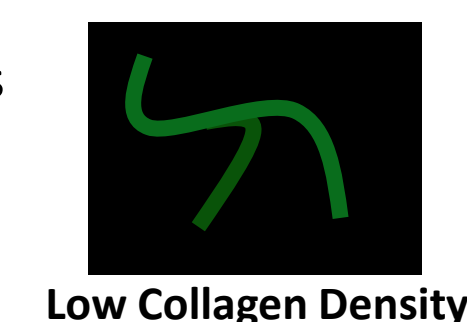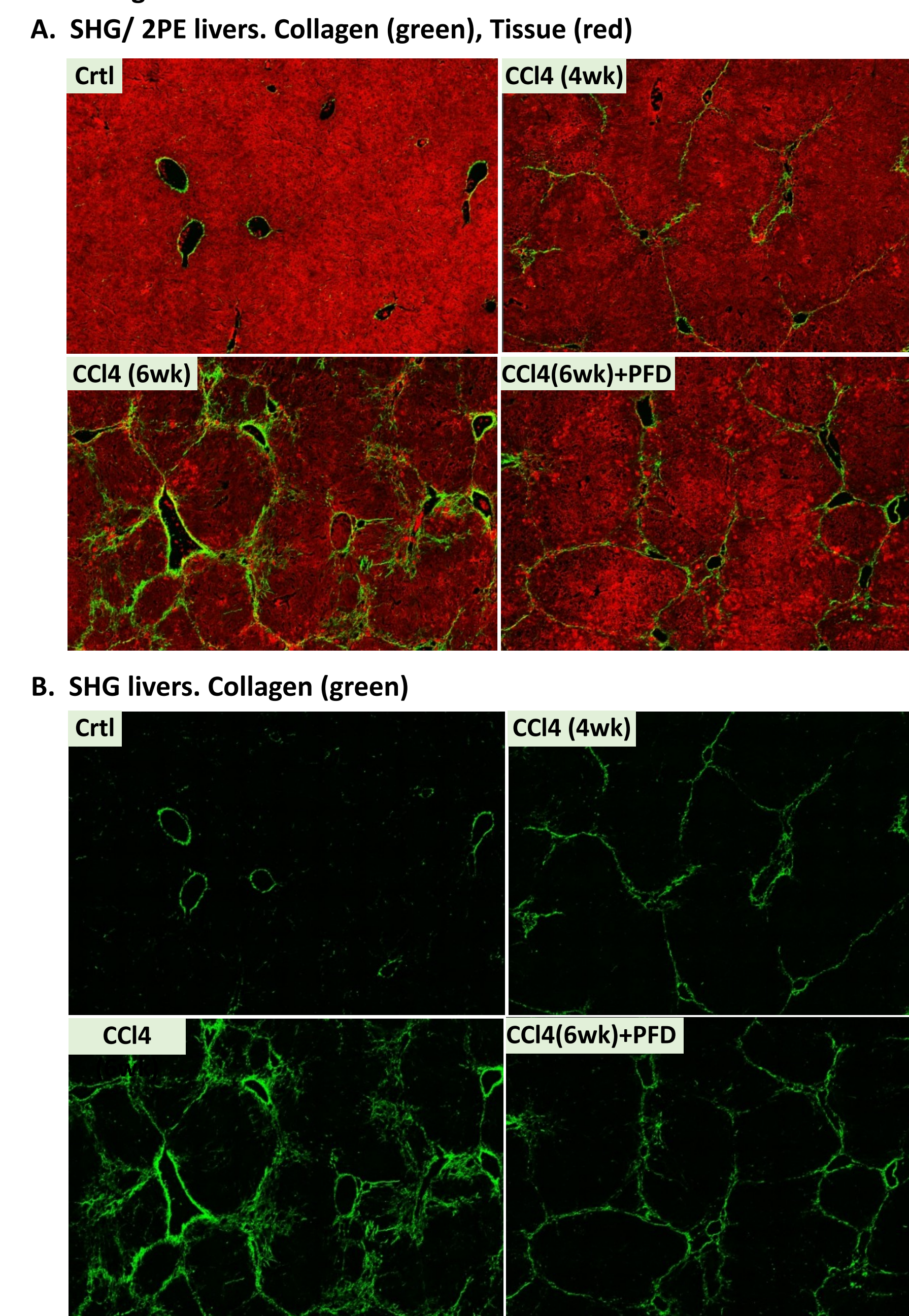
Reticulation Index
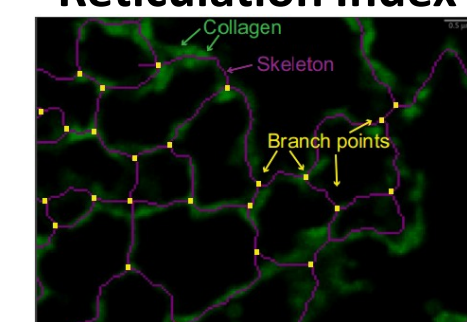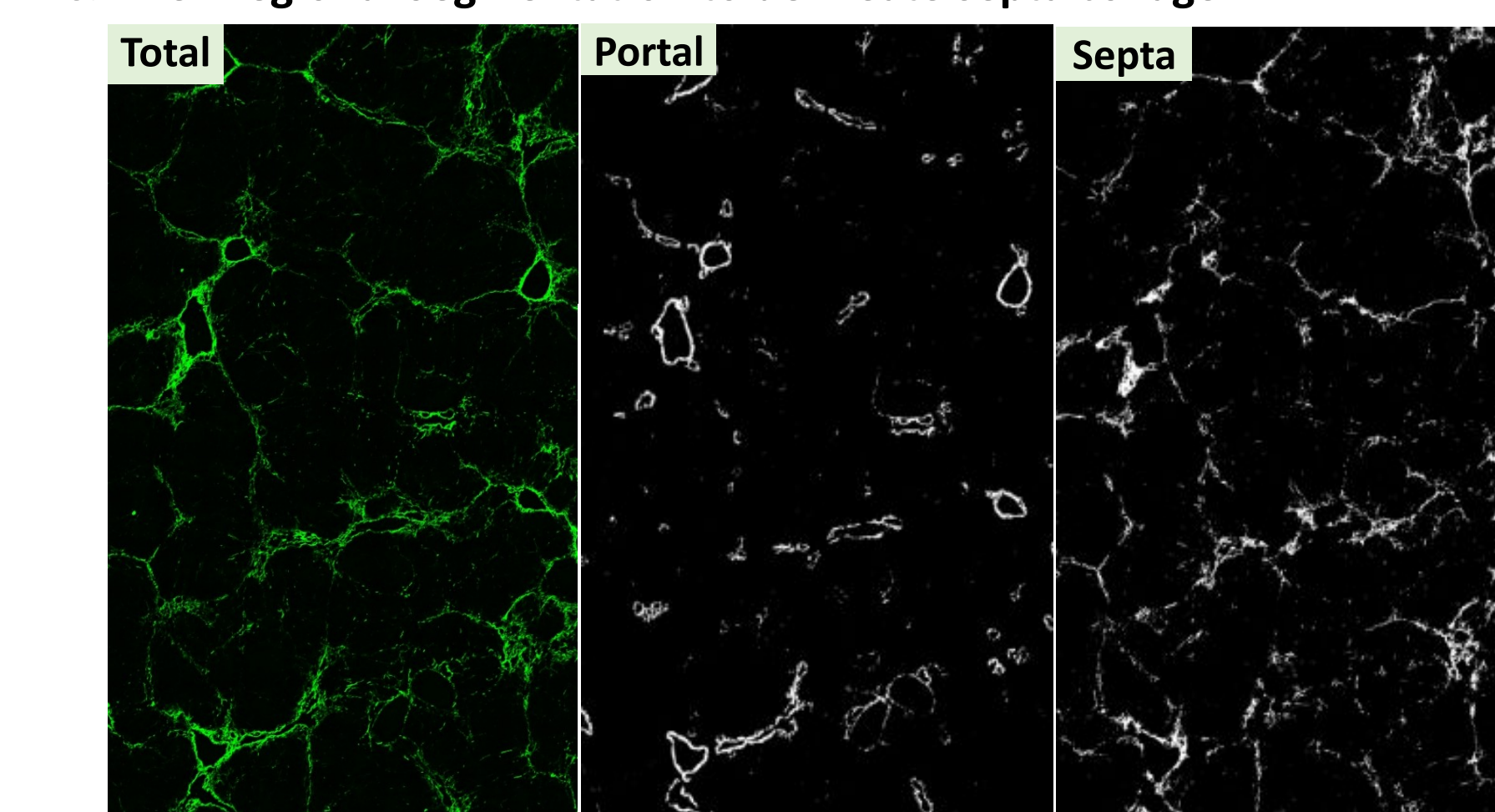

Same Collagen Area

High Collagen Density

Low Collagen Density

RESULT

Figure 1. CCl4-treated mice induces hepatic fibrosis and septa development in histological sections.

A. SHG/ 2PE livers. Collagen (green), Tissue (red)

Crtl | CCl4 (4wk) | CCl4 (6wk) | CCl4(6wk)+PFD

B. SHG livers. Collagen (green)

Crtl | CCl4 (4wk) | CCl4 | CCl4(6wk)+PFD

C. Liver Regional Segmentation to delineate septa collagen

Total | Portal | Septa

D. Quantitation of Liver Fibrosis

Regional Segmentation

	Total	Portal	Septal
Ctrl	1.95	0.76	1.18
CCl4 (4wk)	3.77	1.54	2.23
CCl4 (6wk)	8.84	3.53	5.31
CCl4+PFD(6wk)	5.18	2.25	2.93

2D Fibrosis Chart (Content vs Structure)
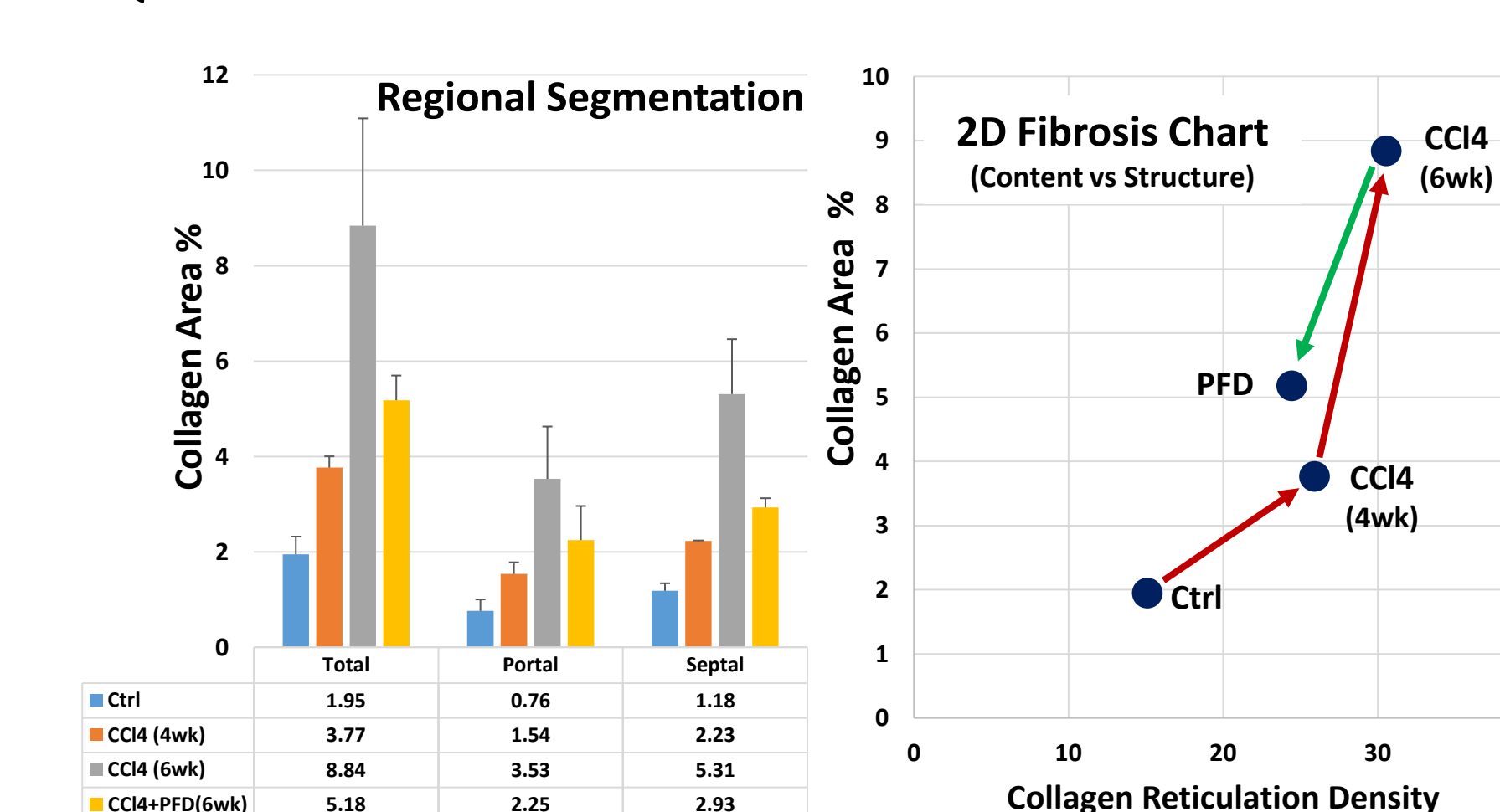
Figure 2. Liver Septal Analysis

A. Selection of ROIs

Multiple Selections | Skeleton | Septa | Cropped ROI

Septal Width

Region-of-Interest (ROI) AR = 0.5

B. Examples of various septa types and their respective value gradient

	Septal Bridge Characteristics				
	Width	Branches	Segment Length	Collagen Reticulation Index	Collagen Area
	at 1/3 & 2/3 intersection				
A					
B					
C					
D					
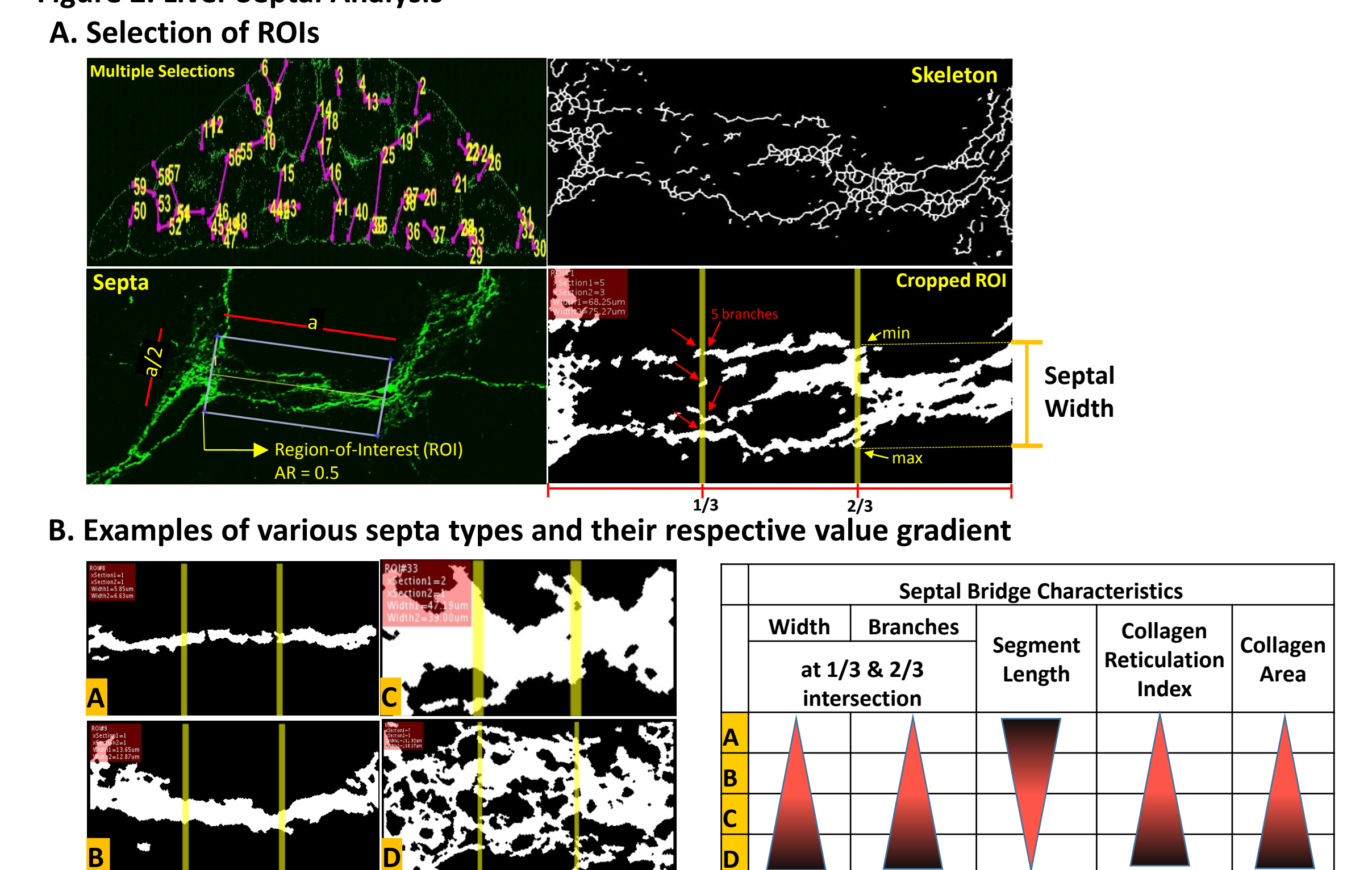
C. Fibrous septa development in CCl4-treated mice with or without Pirfenidone (PFD).

CCl4 (4wk) | CCl4 (6wk) | CCl4+ PFD (6wk)
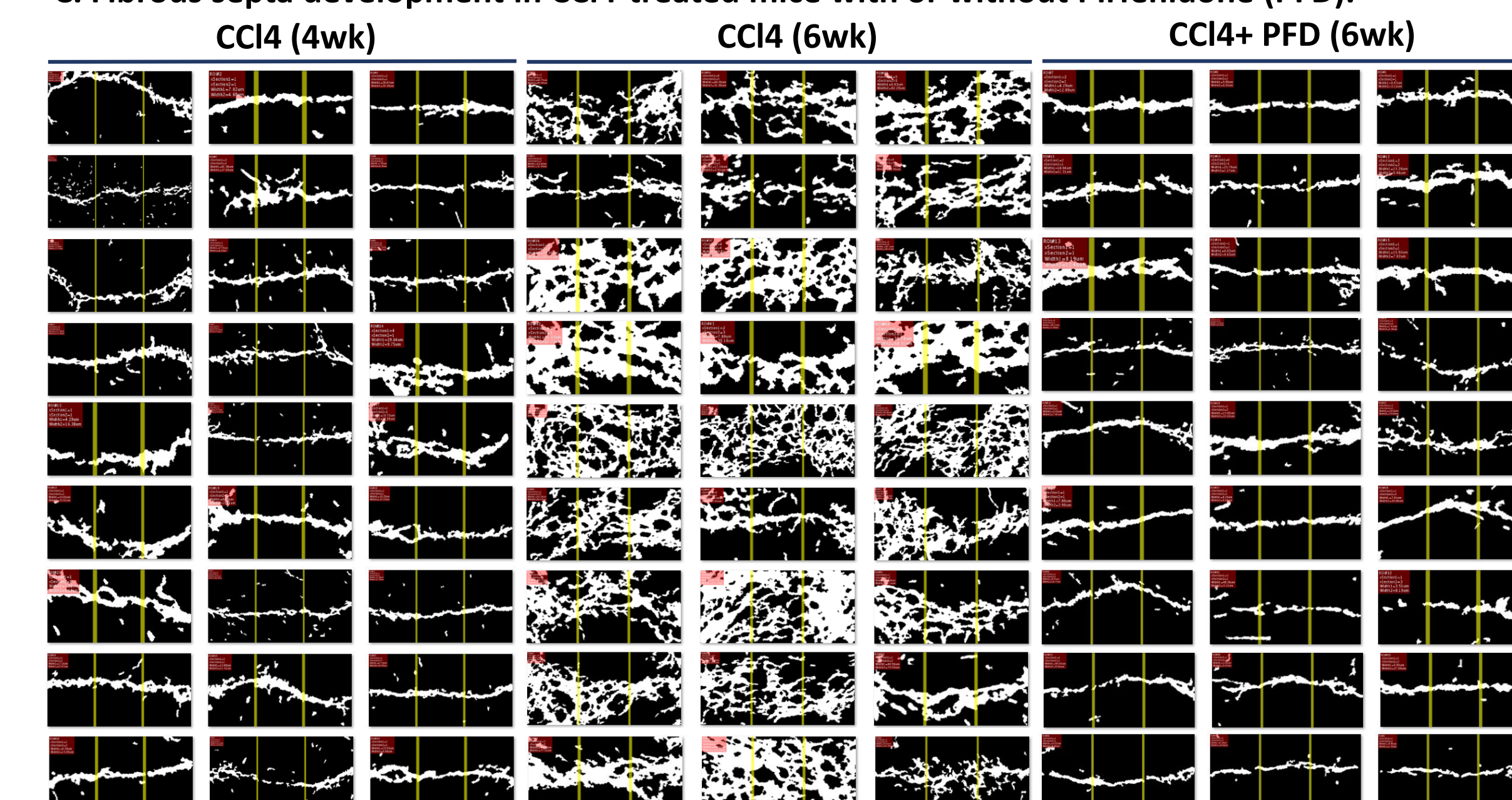
D. Quantitation of Septal features

Septal Collagen Area% | Collagen Reticulation Index | Septal Width
Collagen Fiber Density | Septal Branches | Collagen Segment Length
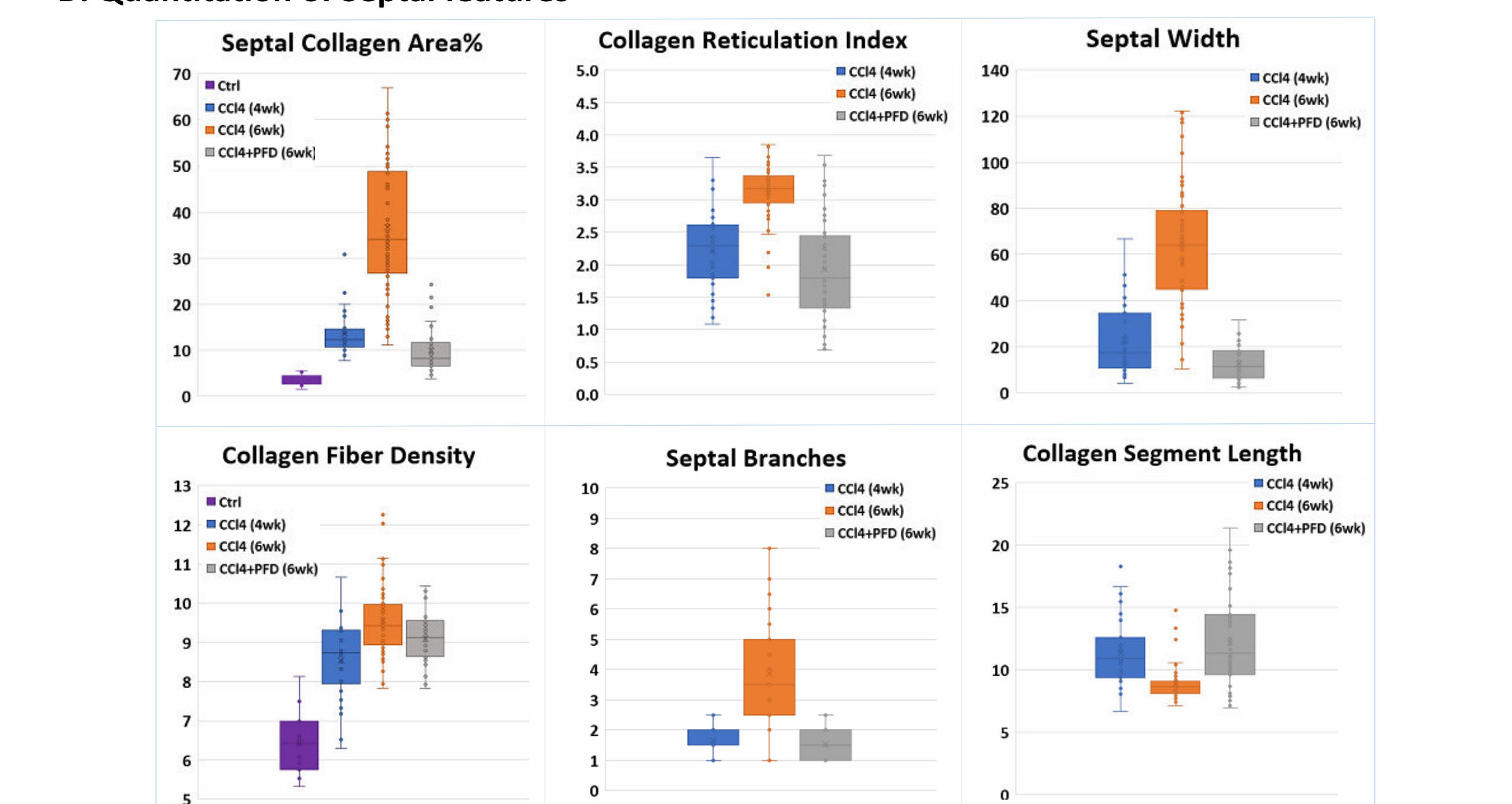
Conclusion

- SHG/2PE imaging is a sensitive stain-free novel technique which when combined with semi-automated image analysis for liver fibrosis and septa helps to evaluate anti-fibrotic therapy efficacy during fibrosis progression and regression.

- In addition to basic metrics for total collagen content quantification, we offer novel analysis capabilities that conventional method are not able to provide such as collagen network structure, liver regional segmentation (segmenting vasculature and septa collagens from total tissue collagen), and fibrous septa analysis.

- Our Septa Analysis Tool delivers a more in-depth understanding of septa development and changes during liver remodeling using quantifiable metrics. These metrics maybe the key to better liver fibrosis scoring and staging.