SUPPLEMENTAL MATERIAL

SUPPLEMENTAL METHODS

TR-LIFS Instrumentation

The tissue autofluorescence (time-resolved fluorescence pulse transients) was measured at wavelengths ranging from 360 nm to 550 nm (10 nm increments) following excitation with a pulsed nitrogen laser (700 ps, 337 nm). A bifurcated fiber-optic probe held perpendicularly to the intimal surface of the carotid plaque sample was used for the excitation and collection of the fluorescence light. TR-LIFS data for each point was recorded in approximately 30 seconds. Specimens were kept moist with saline (intermittently delivered drops) prior to and during TR-LIFS investigation.

Histopathological Analysis

Each histological slide was visualized with light microscopy and digitized. Based on studies in our lab that examined sequential tissue slices over a 4 mm region, it was determined that the tissue was homogeneous enough to use one slice of tissue as an estimate of the composition of the entire ROI. The ROI was measured from the digitized images of the slides using AxioVision image processing software (Carl Zeiss Inc, Germany). Digitized H&E and trichrome-stained sections were further analyzed to quantify, by percentage, the relative content of collagen, elastin, SMCs, calcification, and necrosis within each ROI. Necrosis was defined as morphologically distinct extracellular tissue spaces composed of cholesterol clefts and/or necrotic debris of foam cells. Macrophage and leukocyte content within the ROI was quantified by percentage from the digitized CD68 and CD45 stained sections, respectively. CD68 also stains for other lipid-laden cells, but for this study, all lipid-laden cells stained with CD68 have similar

de-stabilizing effects as lipid-laden macrophages. The picrosirius red slides were imaged using circularly polarized light and the collagen content (thick and thin fibers) within each ROI was quantified using an algorithm developed in MATLAB (The MathWorks, Inc). The script was based on published studies using similar methods [1-3]. The SMC actin staining was quantified with an automatic measurement program involving segmentation and thresholding (Carl Zeiss Inc, Germany).

Statistical Analysis

Provided here is the full set of Pearson R and P values for correlations between plaque biochemical constituents and MMP level (supplemental table 1) and the full set of Pearson R and P values for significant correlations between TR-LIFS variables and MMP-9 and -2 level changes (supplemental table 2). Supplemental figure 1 displays the MMP-9 information from supplemental table 4 and supplemental figure 2 displays the MMP-2 information from supplemental table 4.

The carotid samples used for this study were part of a larger subset of data and thus one additional set of correlations was computed to determine that the results of this study were in agreement with the previously reported time-resolved data[4]. The biological constituents at each investigated location were correlated with the corresponding TR-LIFS parameters (τ_λ, I_λ, and LEC_λ) for each subset of data associated with MMP-9 0-to-1, 1-to-2, and 0-to-2 level changes (supplemental table 3) and MMP-2 1-to-2, and 0-to-2 level changes (supplemental table 4).

SUPPLEMENTAL DISCUSSION

MMP Level vs. Plaque Composition

MMP-9 and -2 are secreted by macrophages and SMCs and perform a variety of functions including disrupting the basement membrane surrounding SMCs to allow migration, assisting to organize collagen formation and angiogenesis, as well as signaling apoptosis in SMCs, macrophages, and ECM components [5-12]. Thus MMPs are capable of 1) controlling geometrical remodeling by regulating collagen degradation and organization [10], and 2) increasing plaque vulnerability by degrading ECM components in the fibrotic cap, enabling growth of vasa plaquorum [13] and increasing necrotic core formation via leakage of erythrocytes and inducing apoptosis [5]. Despite this complex process, our results indicated that increased expression of both MMP-2 and -9 was consistently associated with signs of weakening of plaque structure, such as increased levels of macrophages and necrosis and decreased collagen and SMCs (figure 3). Interestingly, another study aiming to detect MMP -2 and -9 content using an activatable near-infrared fluorescence (NIRF) probe detected by molecular optical imaging found that levels of both MMPs were elevated in macrophage-rich lesions [14], similar to our findings.

MMP-9

The loss of elastin and slight ($P = 0.07$, data not shown) increase in collagen between MMP-9 levels 0-to-1 was attributed to the TR-LIFS signal coming from a volume of tissue with a higher ratio of collagen to elastin as collagenous plaque develops in the intima. Degradation of the fibrotic cap and an increase in inflammatory cells are two markers of plaque vulnerability [15] that appear as MMP-9 increases from level 1-to-2. Thus a low level of MMP-9 identifies an IT specimen, a medium level of MMP-9

identifies an FP or FA specimen and a high level of MMP-9 identifies an FA-inf or TCFA specimen.

Our finding that MMP-9 is associated with a slight increase in collagen between IT and FP plaques, but with an otherwise decrease in collagen is consistent with reports in the literature that find MMP-9 involved in assisting SMCs with creation of collagen fibrils [10] and in degrading denatured collagens [16]. MMP-9 also cleaves collagens I, III, IV, and V and degrades insoluble collagens and collagen I cross-links [7, 17] that are a major contributor to the fluorescence of atherosclerotic arteries [18]. The association of MMP-9 with macrophages is consistent with previous studies, showing that MMP-9 is produced and secreted by macrophages [19] and often found co-localized with macrophages in vulnerable regions of atherosclerotic plaque [20].

MMP-2

The main difference reported here between MMP-2 and -9 is that MMP-2 is associated with both IT plaques and atherosclerotic artery whereas MMP-9 is associated with only atherosclerotic artery. A variety of pathologic changes have similar associations with MMP-2 and -9 expression. For instance, our results show that increased levels of MMP-2 and -9 are associated with increased numbers of macrophages and necrosis, consistent with literature that reports MMP-2 and -9 to be upregulated in regions of vulnerable atherosclerotic plaque [8, 21-23]. MMP-2 and -9 have also been shown to be upregulated in plaques with outward remodeling [11] and high shear and circumferential stress, that also can be indicators of vulnerability [22, 23]. The association in our data of loss of elastin with MMP-2 and -9 is also confirmed in the literature by studies that show MMP-2 and -9, which are generally known as gelatinases for their capability of degrading

gelatin (the cleaved pieces of collagen helix), are also capable of degrading elastin [5, 12].

SMCs

Previous reports have shown SMCs and MMPs to be positively correlated [5, 9, 10, 17] except for in an inflamed fibrous cap where they are found to be negatively correlated [19]. Because our results come only from the fibrotic cap, we found a negative correlation between SMC and MMP content.

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SUPPLEMENTAL FIGURE LEGENDS

Figure S1. Pearson R values ($P < 0.05$) for correlations between TR-LIFS parameters and MMP-9 levels for collagen (solid black), elastin (black outline, white fill), necrosis (solid gray), and macrophages (black outline, gray fill). Circles: level 0-to-1, triangles: level 1-to-2, squares: level 0-to-2.

Figure S2. Pearson R values ($P < 0.05$) for correlations between TR-LIFS parameters and MMP-2 levels for collagen (solid black), elastin (black outline, white fill), necrosis (solid gray), and macrophages (black outline, gray fill). Triangles: level 1-to-2, squares: level 0-to-2.

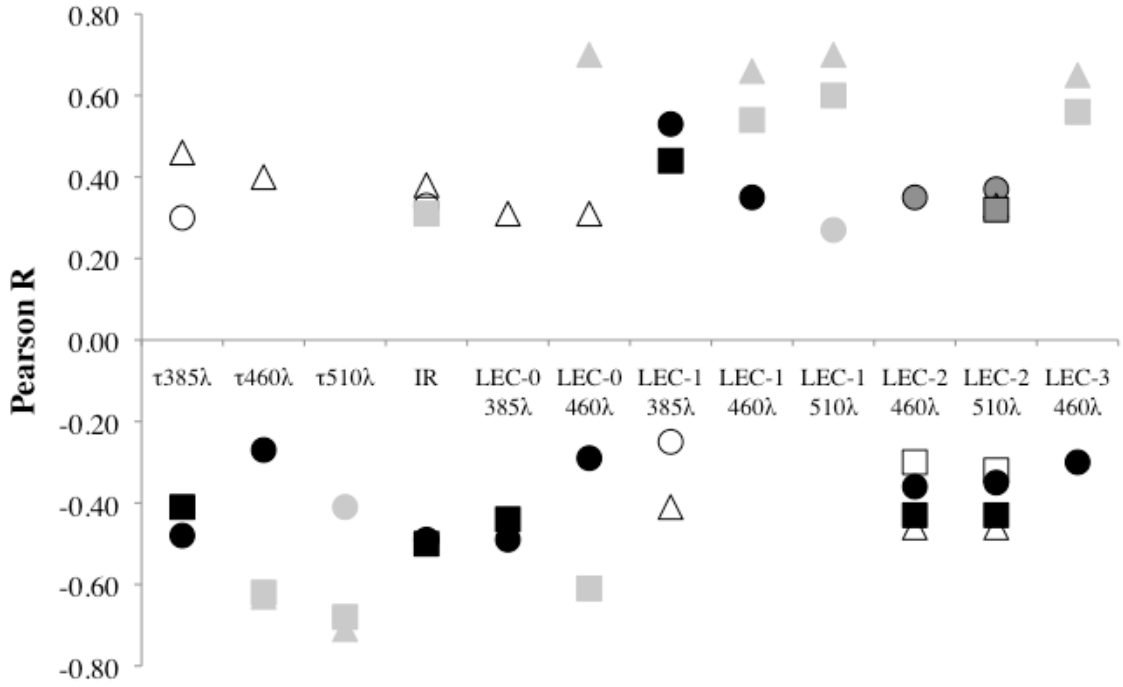

Figure S1.

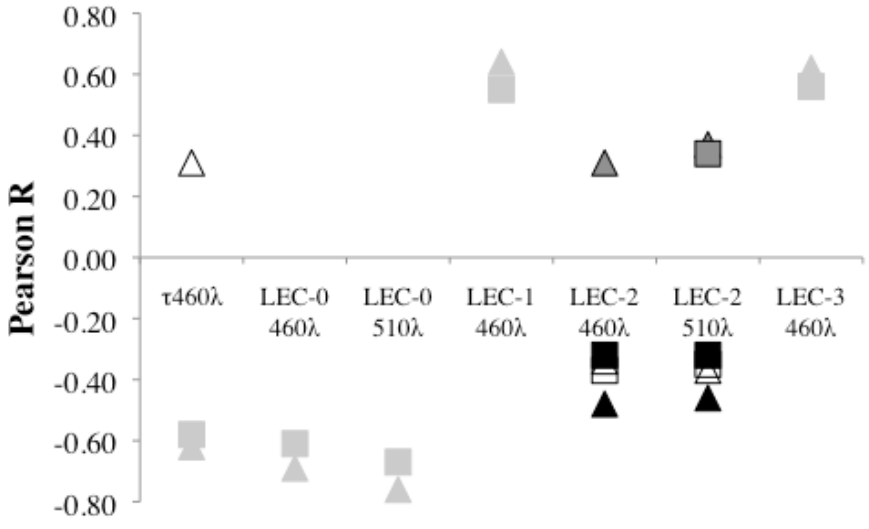

Figure S2.

SUPPLEMENTAL TABLES

Supplemental Table 1. Pearson R and P values for correlations between plaque biochemical constituents and MMP-2 and -9 level changes. Significant correlations ($P<0.05$) in bold.

	Level 0-to-1		Level 1-to-2		Level 0-to-2	
MMP-9	R	P	R	P	R	P
collagen	0.25	0.07	**-0.33**	**0.03**	-0.10	0.45
elastin	**-0.28**	**0.04**	-0.20	0.19	**-0.39**	**0.00**
SMC	-0.08	0.58	**-0.33**	**0.03**	**-0.42**	**0.00**
macrophages	0.19	0.16	**0.36**	**0.02**	**0.52**	**0.00**
necrosis	0.03	0.83	**0.36**	**0.02**	**0.37**	**0.00**
MMP-2	R	P	R	P	R	P
collagen	0.08	0.55	**-0.29**	**0.04**	-0.21	0.14
elastin	0.06	0.65	**-0.42**	**0.00**	-0.28	0.05
SMC	-0.03	0.85	**-0.47**	**0.00**	**-0.45**	**0.00**
macrophages	-0.04	0.76	**0.55**	**0.00**	**0.47**	**0.00**
necrosis	-0.09	0.52	**0.45**	**0.00**	**0.37**	**0.01**

Supplemental Table 2. Pearson R and P values for TR-LIFS parameters correlated with MMP-2 and -9 level changes. Significant correlations (P<0.05) in bold.

	Level 0-to-1		Level 1-to-2		Level 0-to-2	
MMP9	R	P	R	P	R	P
$\tau_{375\lambda}$	**0.74**	**0.02**	-0.06	0.88	-0.14	0.74
$\tau_{460\lambda}$	0.51	0.16	**-0.92**	**<.001**	**-0.91**	**0.001**
$\tau_{510\lambda}$	0.02	0.97	**-0.79**	**0.01**	**-0.91**	**0.002**
I_R	**0.87**	**0.002**	-0.58	0.08	0.63	0.05
LEC-$0_{385\lambda}$	**0.68**	**0.04**	-0.16	0.7	0.25	0.48
LEC-$0_{460\lambda}$	0.16	0.65	**-0.89**	**0.003**	**-0.87**	**0.01**
LEC-$1_{385\lambda}$	**-0.76**	**0.01**	0.62	0.05	-0.46	0.18
LEC-$1_{460\lambda}$	-0.55	0.1	**0.82**	**0.01**	**0.76**	**0.02**
LEC-$1_{510\lambda}$	**-0.83**	**0.003**	**0.68**	**0.04**	0.23	0.56
LEC-$2_{460\lambda}$	0.48	0.19	**0.80**	**0.01**	**0.82**	**0.004**
LEC-$2_{510\lambda}$	0.29	0.44	**0.78**	**0.01**	0.71	0.05
LEC-$3_{460\lambda}$	-0.44	0.21	**0.84**	**0.01**	**0.87**	**0.01**
MMP2	R	P	R	P	R	P
$\tau_{460\lambda}$	**-0.82**	**0.01**	**-0.87**	**0.005**	**-0.95**	**<.001**
LEC-$0_{460\lambda}$	0.36	0.35	**-0.82**	**0.01**	**-0.90**	**0.003**
LEC-$0_{510\lambda}$	-0.41	0.27	**-0.76**	**0.03**	**-0.81**	**0.01**
LEC-$1_{460\lambda}$	-0.34	0.34	**0.82**	**0.01**	**0.83**	**0.01**
LEC-$2_{460\lambda}$	0.29	0.44	**0.80**	**0.01**	**0.68**	**0.03**
LEC-$2_{510\lambda}$	0.04	0.92	0.67	0.05	**0.74**	**0.01**
LEC-$3_{460\lambda}$	0.01	0.98	**0.81**	**0.02**	**0.81**	**0.02**

Supplemental Table 3. Statistically significant ($P < 0.05$) Pearson R and P values for correlations between spectroscopic parameters at distinct MMP-9 level changes vs. plaque biochemical constituents.

		collagen		elastin		necrosis		macrophages	
MMP-9		**R**	**P**	**R**	**P**	**R**	**P**	**R**	**P**
$\tau_{385\lambda}$	0-to-1	0.30	0.030	-0.48	<.001	-	-	-	-
	1-to-2	0.46	0.002	-	-	-	-	-	-
	0-to-2	-	-	-0.41	0.002	-	-	-	-
$\tau_{460\lambda}$	0-to-1	-	-	-0.27	0.049	-	-	-	-
	1-to-2	0.40	0.010	-	-	-0.63	<.001	-	-
	0-to-2	-	-	-	-	-0.62	<.001	-	-
$\tau_{510\lambda}$	0-to-1	-	-	-	-	-0.41	0.002	-	-
	1-to-2	-	-	-	-	-0.71	<.001	-	-
	0-to-2	-	-	-	-	-0.68	<.001	-	-
I_R	0-to-1	0.33	0.010	-0.49	<.001	-	-	-	-
	1-to-2	0.38	0.010	-	-	-	-	-	-
	0-to-2	-	-	-0.50	<.001	0.31	0.020	-	-
LEC-$0_{385\lambda}$	0-to-1	-	-	-0.49	<.001	-	-	-	-
	1-to-2	0.31	0.040			-	-	-	-
	0-to-2	-	-	-0.44	<.001	-	-	-	-
LEC-$0_{460\lambda}$	0-to-1	-	-	-0.29	0.030	-	-	-	-
	1-to-2	0.31	0.040	-	-	0.70	<.001	-	-
	0-to-2	-	-	-	-	-0.61	<.001	-	-
LEC-$1_{385\lambda}$	0-to-1	-0.25	0.060	0.53	<.001	-	-	-	-
	1-to-2	-0.41	0.010	-	-	-	-	-	-
	0-to-2	-	-	0.44	<.001	-	-	-	-
LEC-$1_{460\lambda}$	0-to-1	-	-	0.35	0.010	-	-	-	-
	1-to-2	-	-	-	-	0.66	<.001	-	-
	0-to-2	-	-	-	-	0.54	<.001	-	-
LEC-$1_{510\lambda}$	0-to-1	-	-	-	-	0.27	0.040	-	-
	1-to-2	-	-	-	-	0.70	<.001	-	-
	0-to-2	-	-	-	-	0.60	<.001	-	-
LEC-$2_{460\lambda}$	0-to-1	-	-	-0.36	0.010	-	-	0.35	0.010
	1-to-2	-0.46	0.002	-	-	-	-	-	-
	0-to-2	-0.30	0.030	-0.43	0.001	-	-	-	-
LEC-$2_{510\lambda}$	0-to-1	-	-	-0.35	0.010	-	-	0.37	0.005
	1-to-2	-0.46	0.002	-	-	-	-	0.33	0.030
	0-to-2	-0.32	0.020	-0.43	0.001	-	-	0.32	0.020
LEC-$3_{460\lambda}$	0-to-1	-	-	-0.30	0.030	-	-	-	-
	1-to-2	-	-	-	-	0.65	<.001	-	-
	0-to-2	-	-	-	-	0.56	<.001	-	-

Supplemental Table 4. Statistically significant ($P < 0.05$) Pearson R and P values for correlations between spectroscopic parameters at distinct MMP-2 level changes vs. plaque biochemical constituents.

		collagen		elastin		necrosis		macrophages	
MMP-2		**R**	**P**	**R**	**P**	**R**	**P**	**R**	**P**
$\tau_{460\lambda}$	1-to-2	0.31	0.030	-	-	-0.62	<.001	-	-
	0-to-2	-	-	-	-	-0.58	<.001	-	-
LEC-$0_{460\lambda}$	1-to-2	-	-	-	-	-0.69	<.001	-	-
	0-to-2	-	-	-	-	-0.61	<.001	-	-
LEC-$0_{510\lambda}$	1-to-2	-	-	-	-	-0.76	<.001	-	-
	0-to-2	-	-	-	-	-0.67	<.001	-	-
LEC-$1_{460\lambda}$	1-to-2	-	-	-	-	0.64	<.001	-	-
	0-to-2	-	-	-	-	0.55	<.001	-	-
LEC-$2_{460\lambda}$	1-to-2	-0.34	0.010	-0.48	<.001	-	-	0.31	0.030
	0-to-2	-0.37	0.010	-0.32	0.030	-	-		
LEC-$2_{510\lambda}$	1-to-2	-0.37	0.010	-0.46	<.001	-	-	0.37	0.008
	0-to-2	-0.35	0.010	-0.32	0.020	-	-	0.34	0.020
LEC-$3_{460\lambda}$	1-to-2	-	-	-	-	0.62	<.001	-	-
	0-to-2	-	-	-	-	0.56	<.001	-	-