Improving Characterization, Staging & Management of Ocular Disease

Ocular Proteomics, LLC (OPL)

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Causes of Blindness

Age-related Macular Degeneration (AMD)

- Dry AMD
- Wet AMD

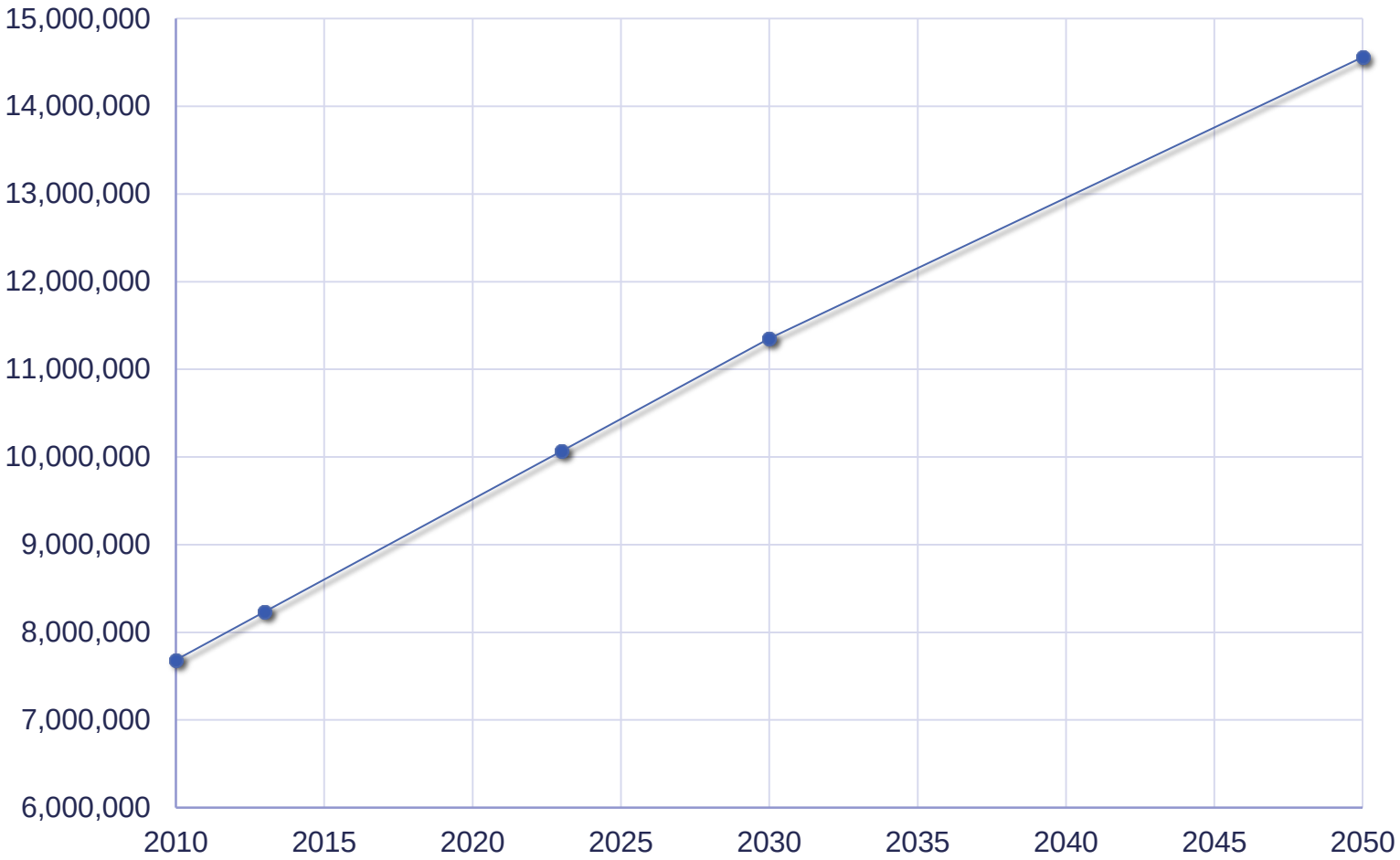
Diabetic Retinopathy (DR)

- Non-proliferative DR
- Proliferative DR

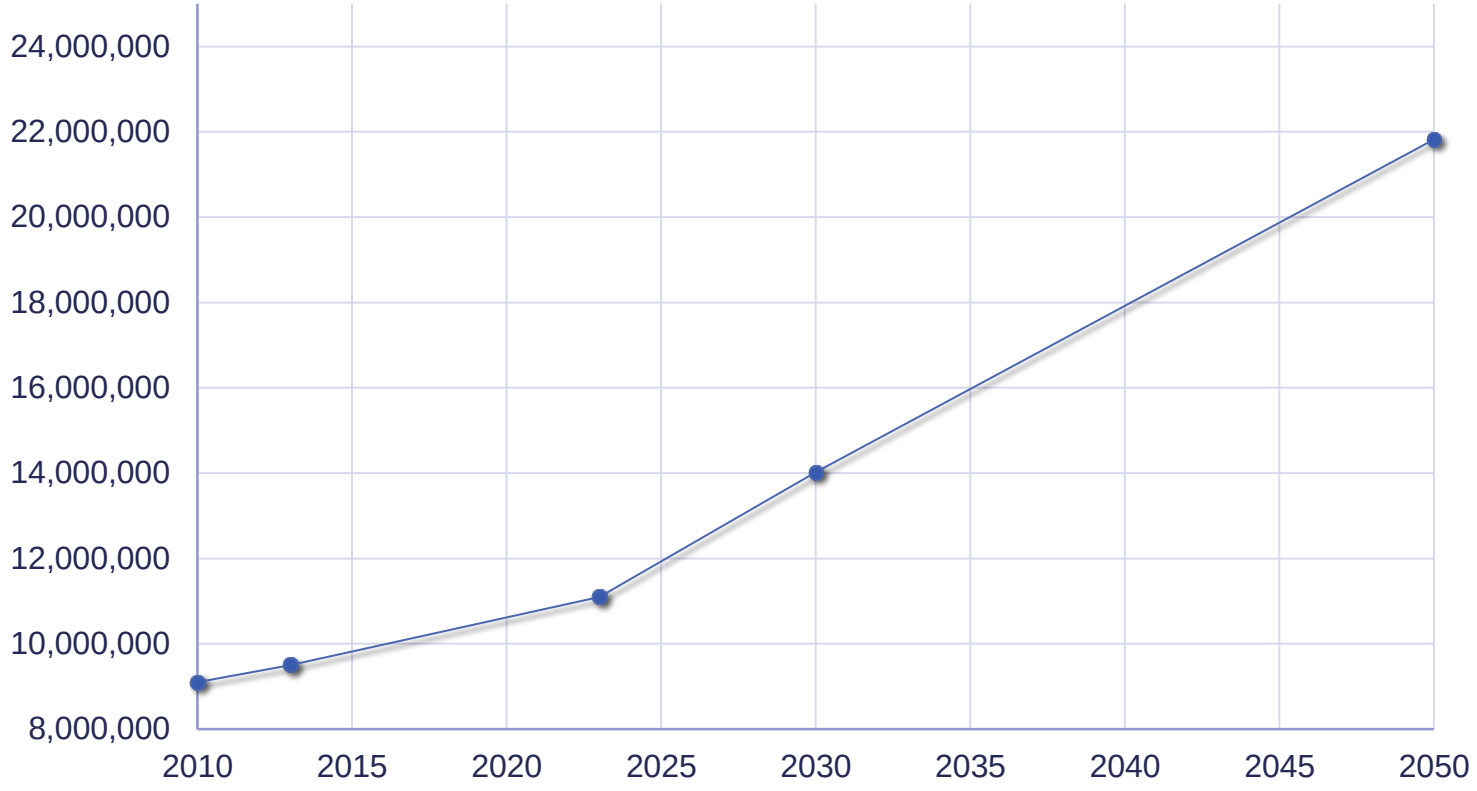
Retinal Vein Occlusions (RVO)

- Branch Retinal Vein Occlusion (BRVO)
- Central Retinal Vein Occlusion (CRVO)

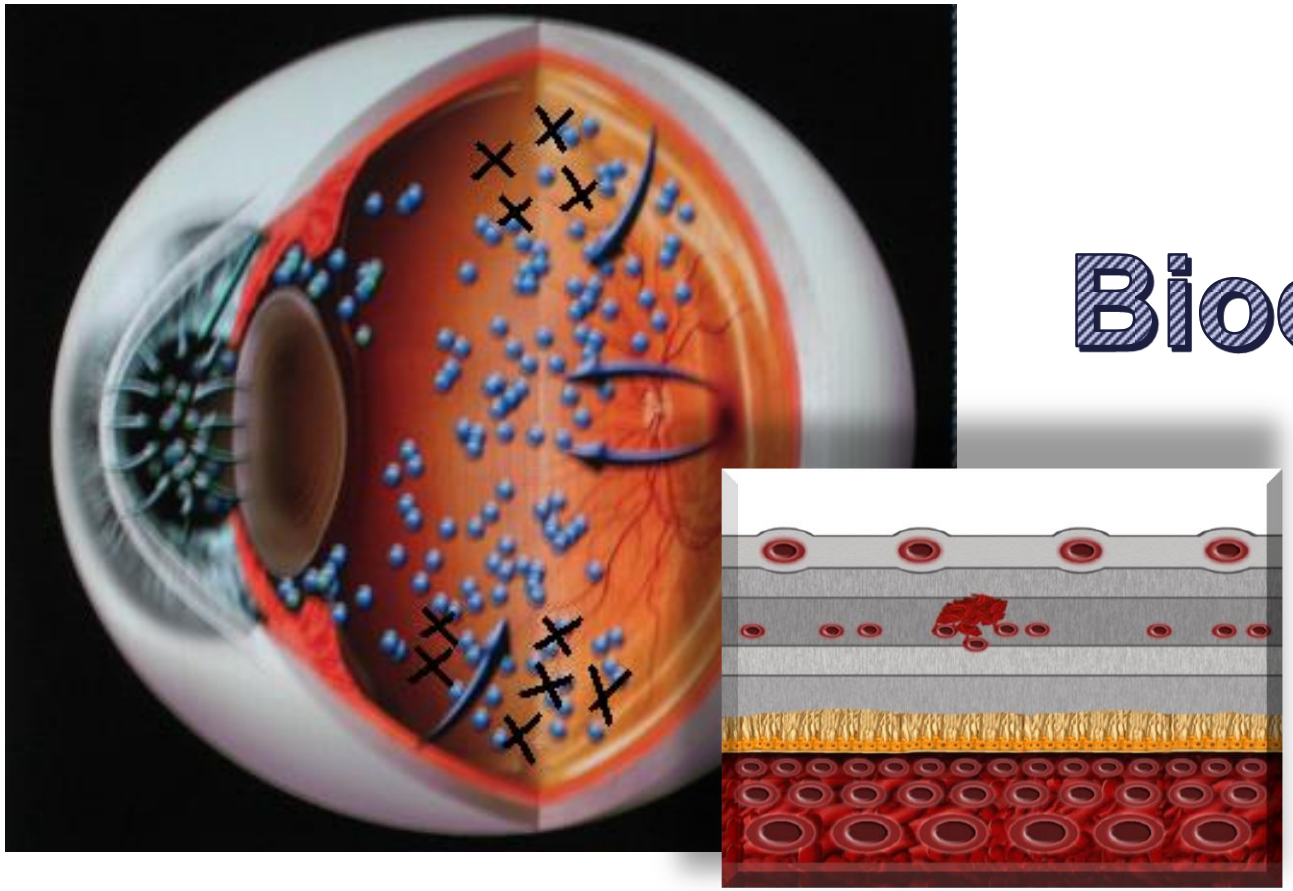
Diabetic Retinopathy (DR)



Age-related Macular Degeneration (AMD)



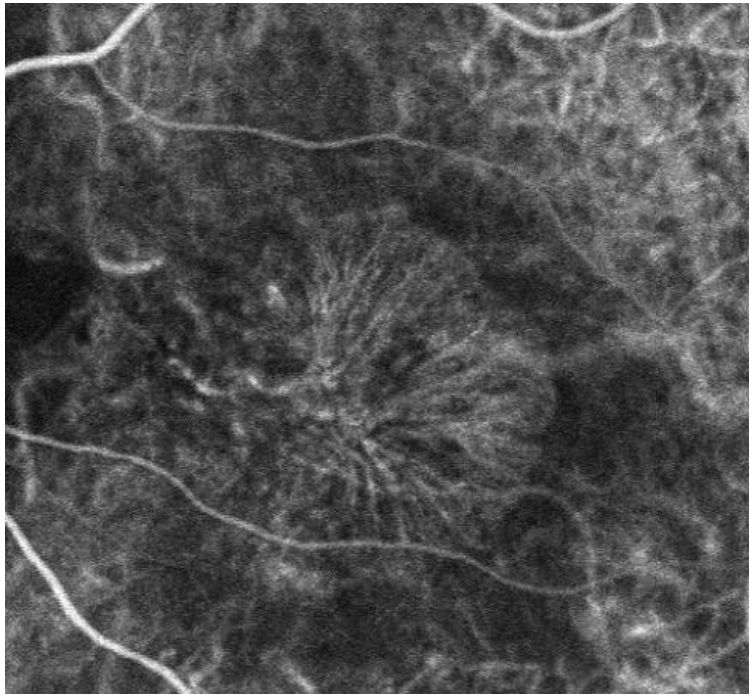
Mgmt by Morphology: Good/Bad



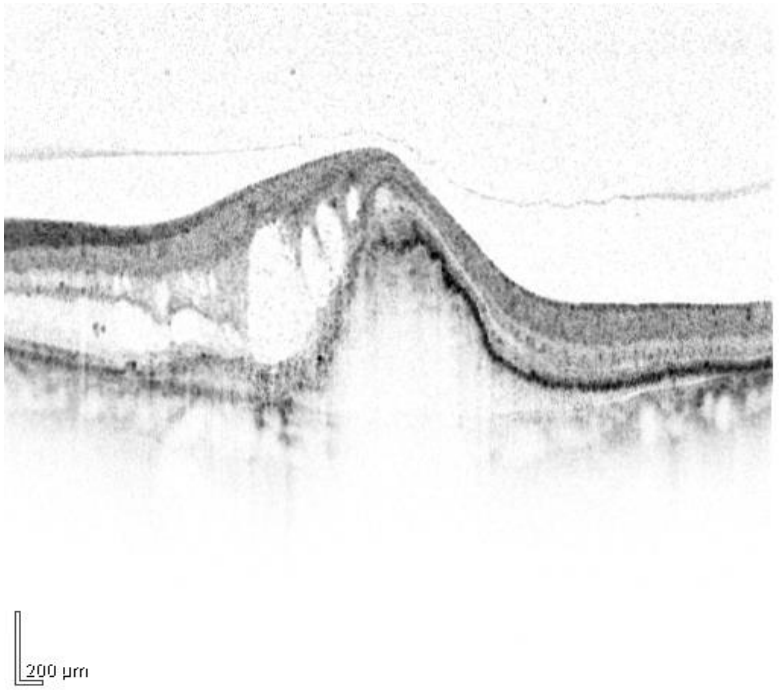
Biochem ?

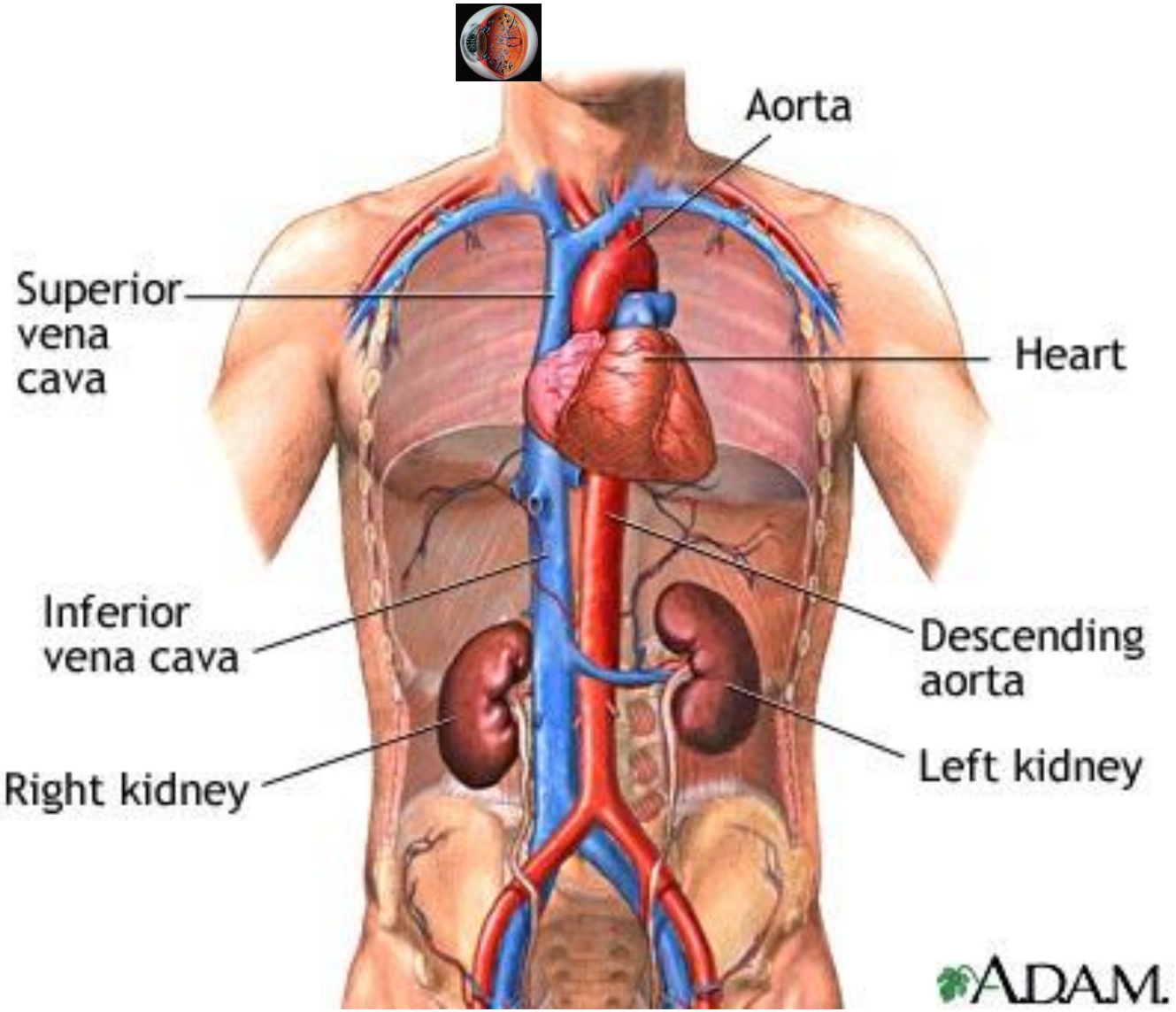
MORPHOLOGY

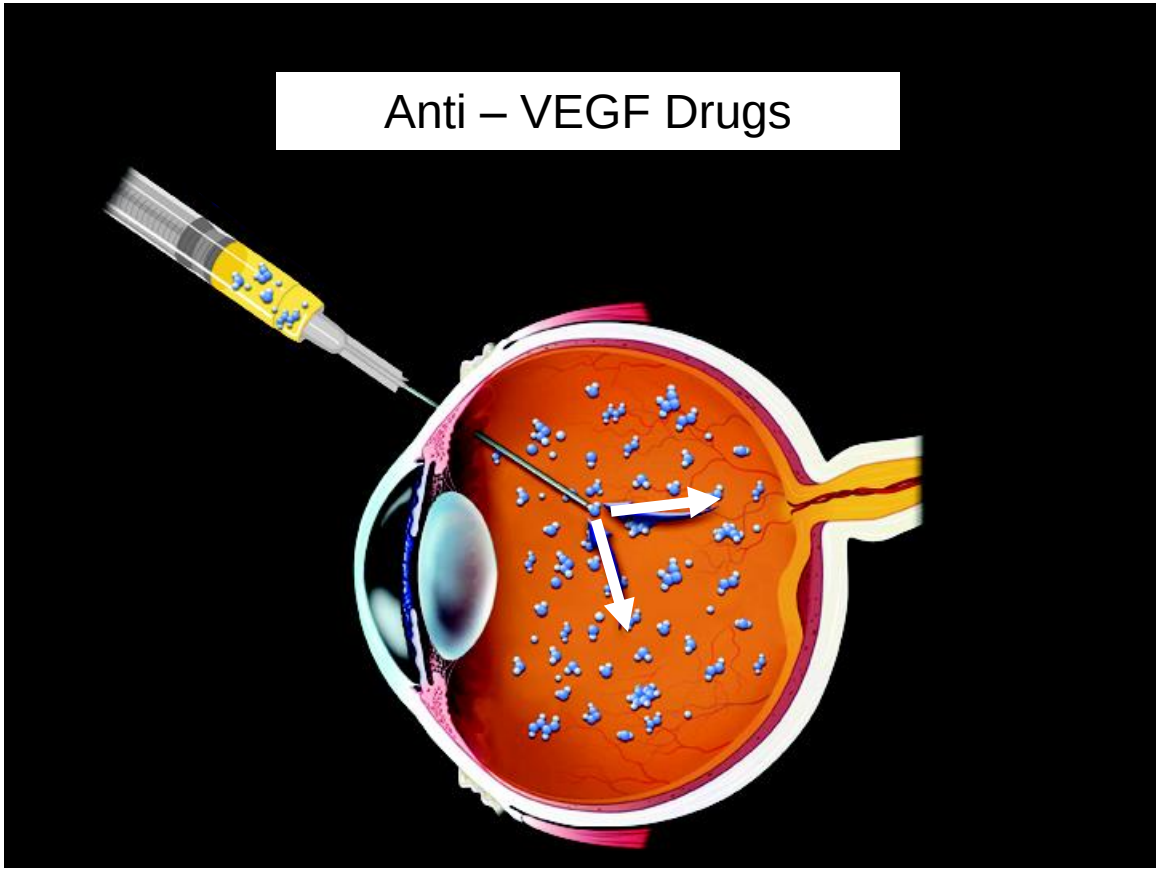
Optical Coherence Tomography



Fluorescein Angiogram



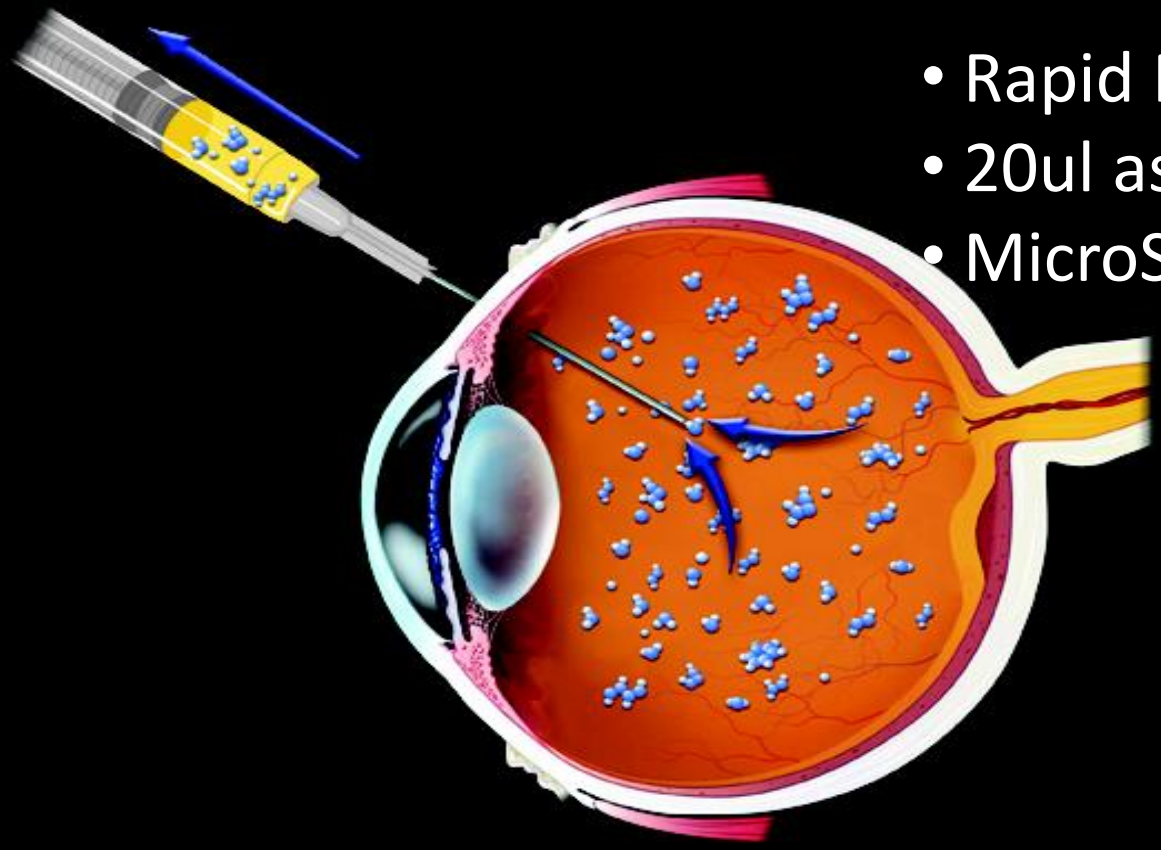

Aorta
Superior vena cava
Heart
Inferior vena cava
Descending aorta
Right kidney
Left kidney
A.D.A.M.


Anti – VEGF Drugs

What can In-office Vitreous Samples Tell Us?

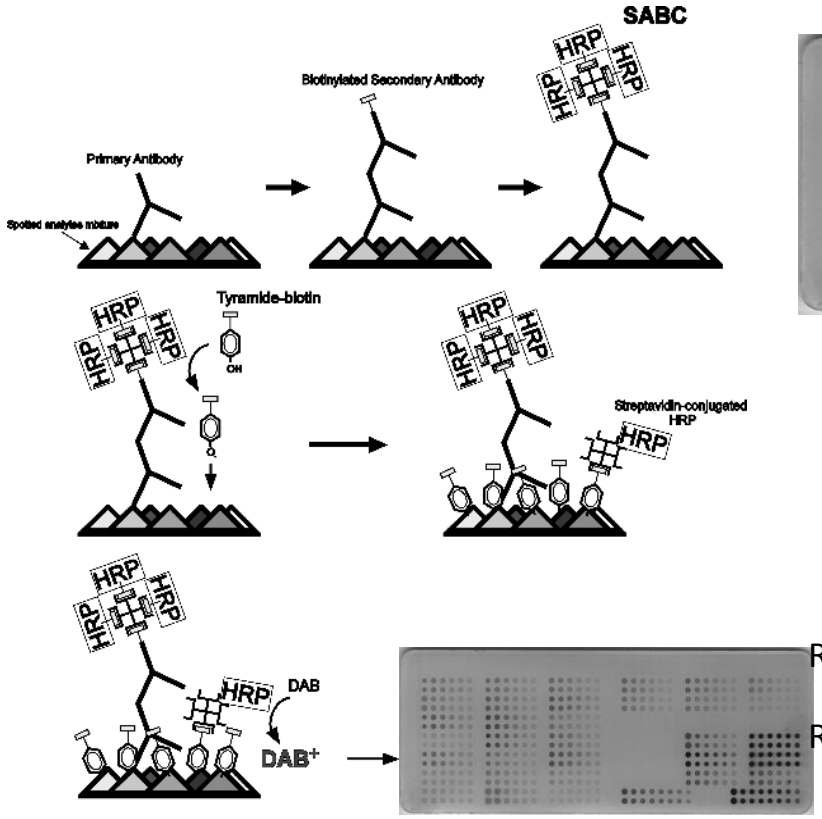
Vitreous Proteomics Biomarker Discovery

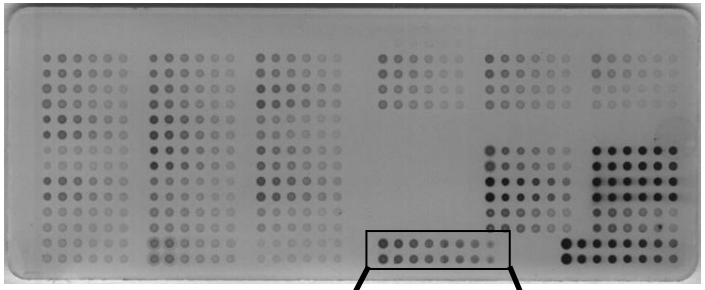
Technologic Advances

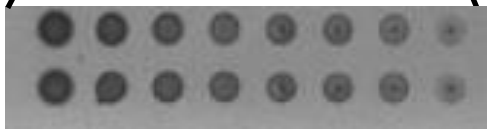


- Rapid Microarray
- 20ul assay
- MicroSampler

Reverse Phase Protein Microarray







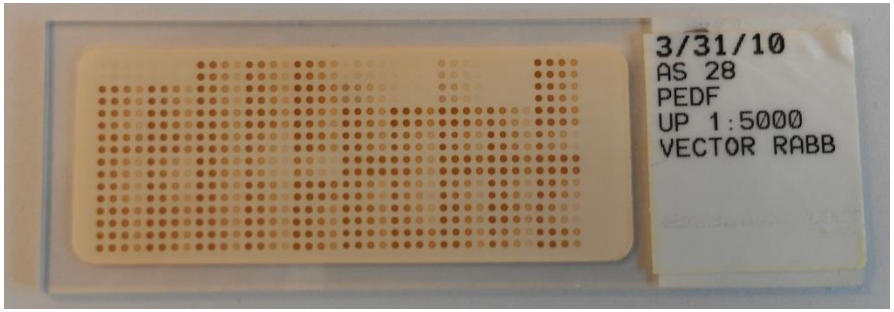
Undil.,1:2,1:4,1:8,1:16,1:32,1:64,1:128

Control sample, duplicate spots

Reverse Phase Protein Microarray Technology

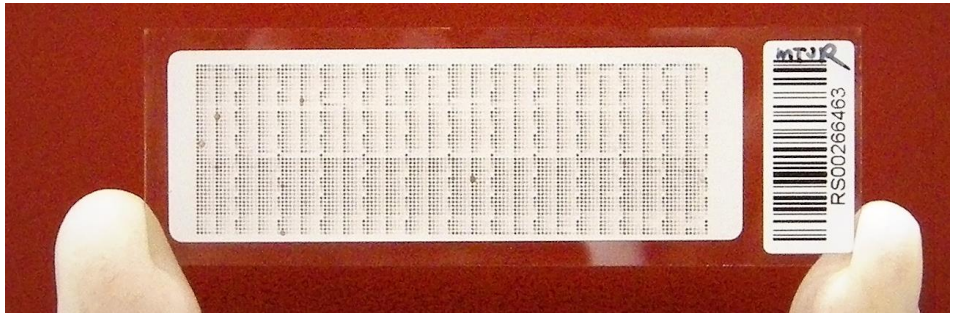
Current Ocular Proteomics System- 350µm Pins

Each slide accommodates 72 samples in 4 point dilution curves
Each slides accommodates additional 8, 8point dilution control spots
Each sample and control printed in duplicate
Each slide probed for one antibody out of a 48 protein panel

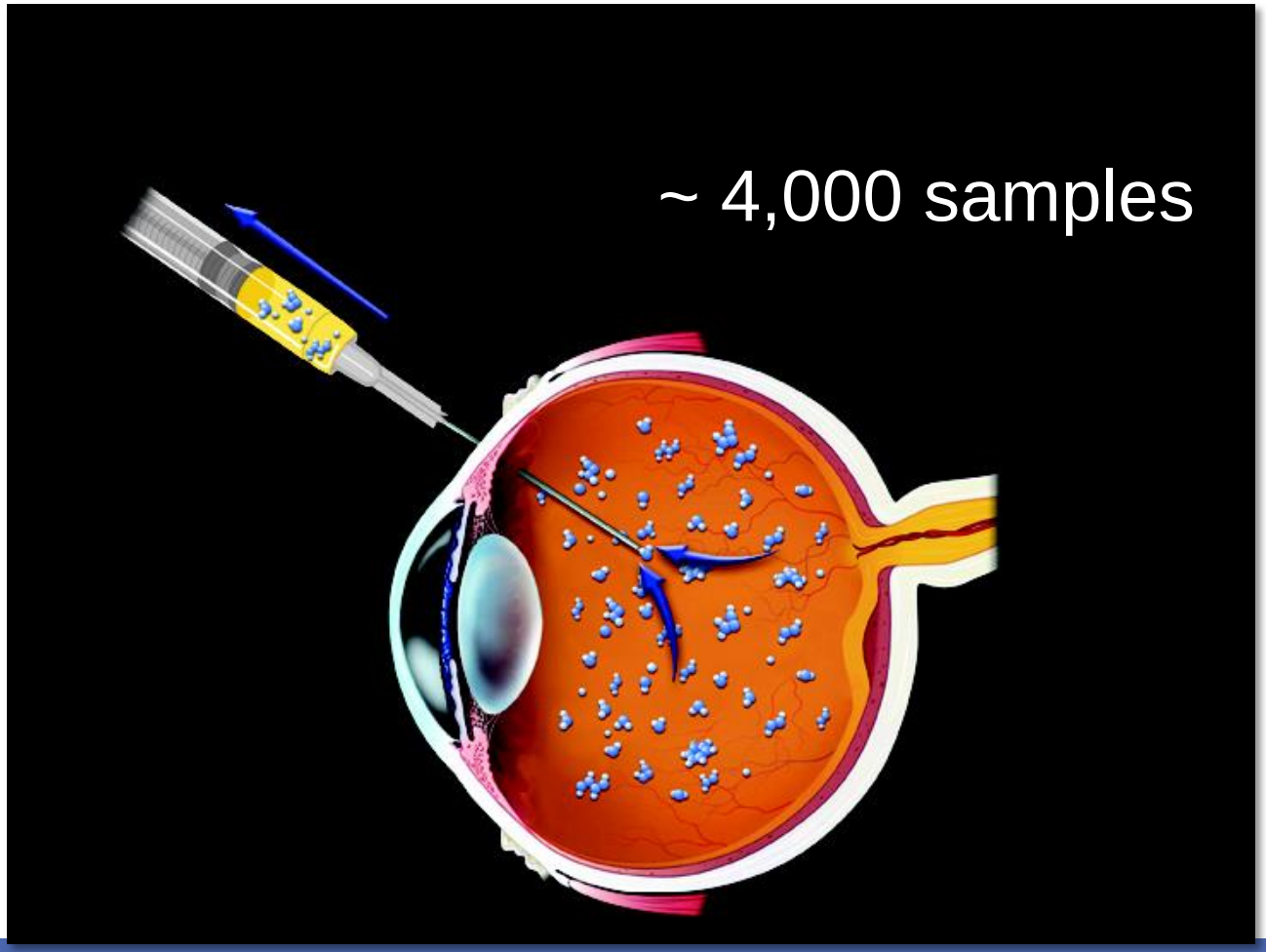


Upcoming Upgrade to Ocular Proteomics System-185µm pins

Each slide accommodates **1056** samples in 5pt dilution curves
Each slides accommodates additional 528 replicated control spots
Each sample and control printed in duplicate
Each slide probed for one antibody out of a 48 protein panel



Diagnostic Vitreous Sampling



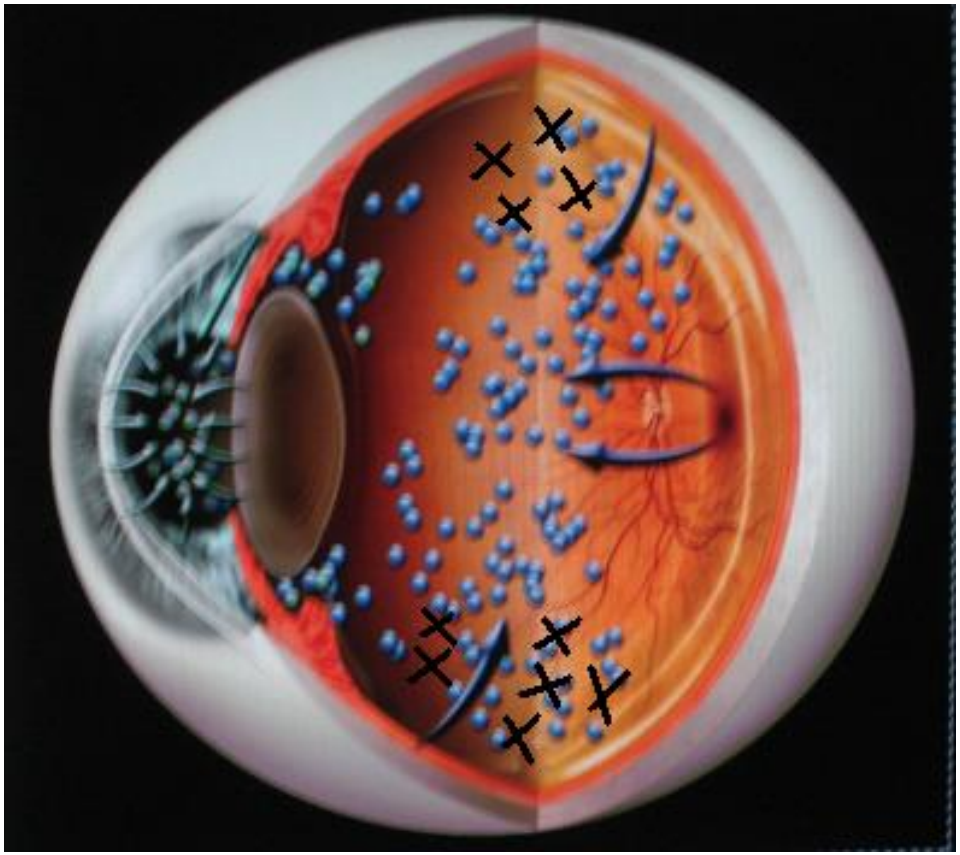
Antibody	
αβ crystallin	IL-10
AKT T308	Il-12
AMPK α1 S485	IL-8
BAD S112	IL-6
BCL2 T56	Integrin α5β1
cABL T735	MMP-14
CF-C3	MMP-2
CF-C5	MMP-9
CF-C9	Musashi
CF-H	PDGFRβ Y716
cKIT Y703	PDGFRβ Y751
cKIT Y719	PEDF
COX-2	TIMP2
eNOS S1177	TGF-Beta
FGF-R	TNF-α
Fibronectin	VEGF-A
Heme Oxygenase 1	VEGFR2 Y1175
IL-1β	VEGFR2 Y951
	VEGFR2 Y996

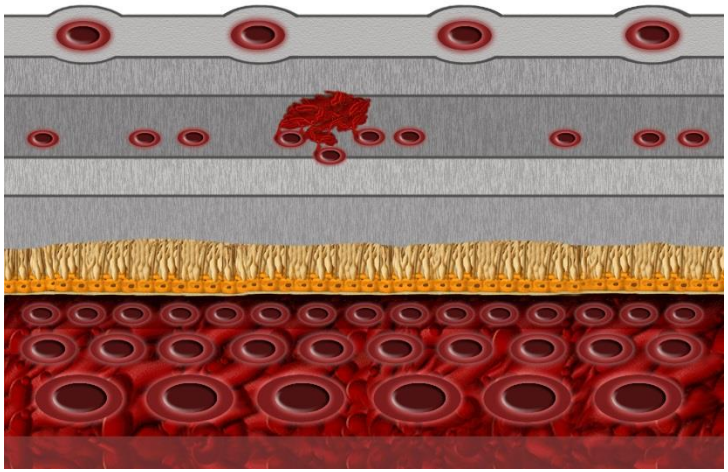
NIH FUNDED

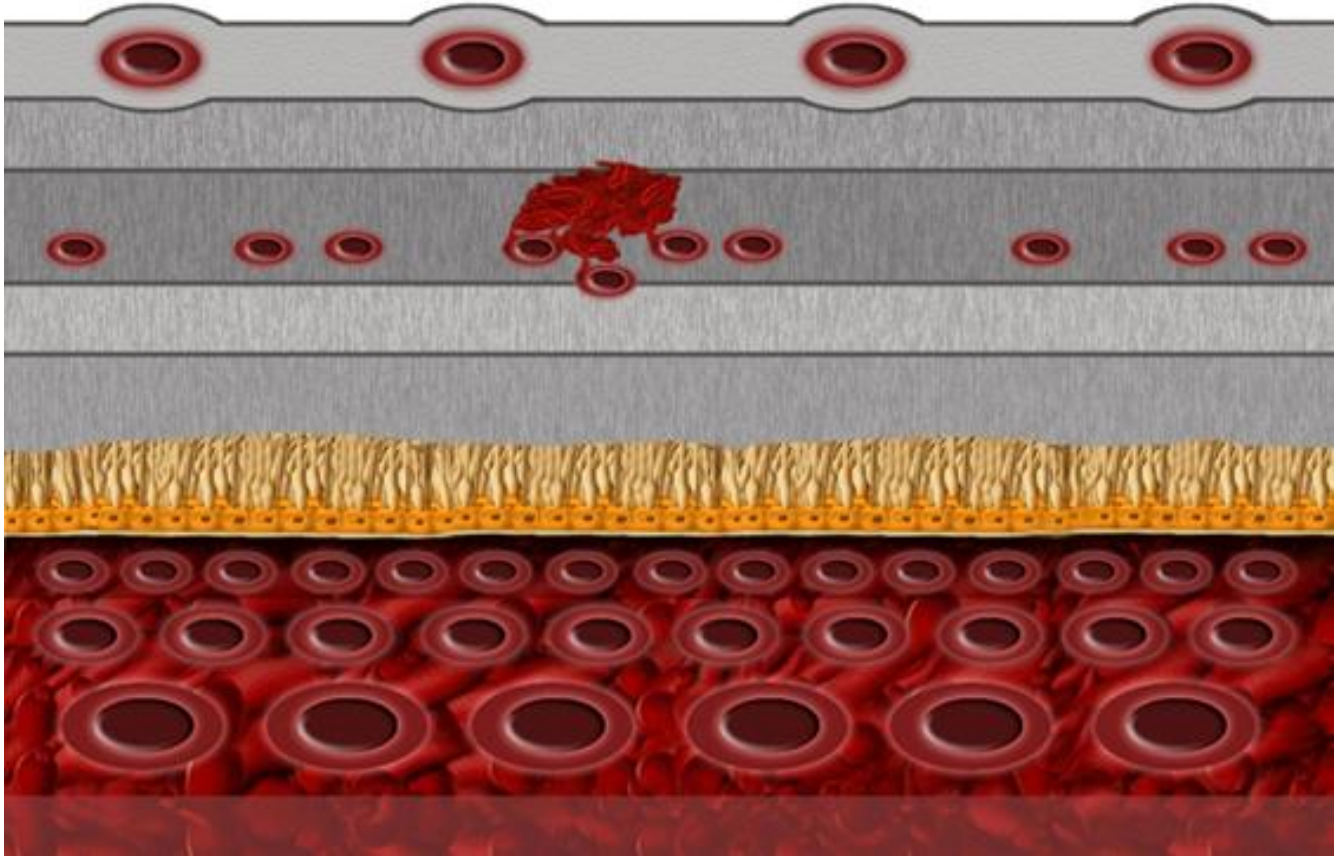
IRB APPROVED

- Western IRB: 1075302/20060001

AMD & DR Activity Sites







Studies

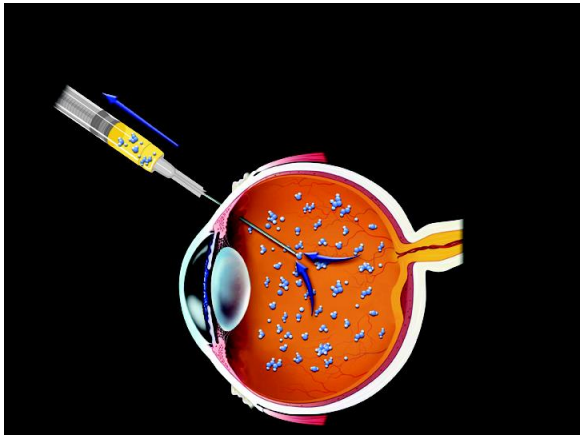
- Aid Management of Disease
- Characterization of Disease
- Staging of Disease

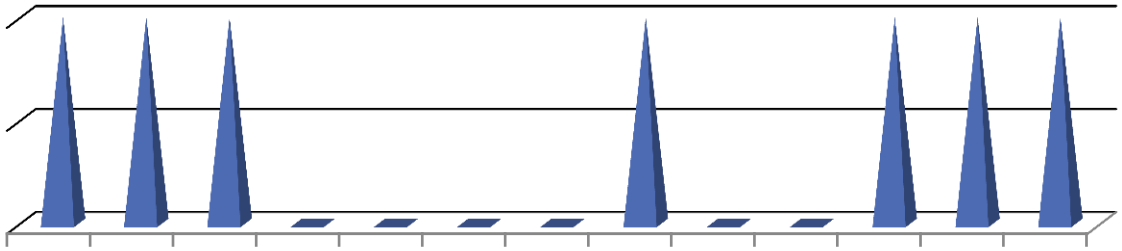
Studies

- Aid Management of Disease
- Characterization of Disease
- Staging of Disease

Bevacizumab Treat & Extend Regimen

- OCT macula thickening
- Decreased VA from previous
- New or persistent heme
- FA leakage
- Increase lesion size on FA





Treat and Extend Rx

- Individualize treatment interval

- How is interval optimized?
  - Current: Trial & Error Ø
  - OPL approach: Vitreous Proteome

Antibody	
αβ crystallin	IL-10
AKT T308	Il-12
AMPK α1 S485	IL-8
BAD S112	IL-6
BCL2 T56	Integrin α5β1
cABL T735	MMP-14
CF-C3	MMP-2
CF-C5	MMP-9
CF-C9	Musashi
CF-H	PDGFRβ Y716
cKIT Y703	PDGFRβ Y751
cKIT Y719	PEDF
COX-2	TIMP2
eNOS S1177	TGF-Beta
FGF-R	TNF-α
Fibronectin	VEGF-A
Heme Oxygenase 1	VEGFR2 Y1175
IL-1β	VEGFR2 Y951
	VEGFR2 Y996

Predicting Responders to TER Therapy

Demographics	**VA Stable Patients (n=12)**	**VA Worsening Patients (n=9)**
Mean Age	79	82
Sex	66.6% Female	66.6% Female
Extended Treatment Follow-up (days)		
Mean	78.3	66.25
Range	51-180	47-97

Results

Patients with Stable VA
During Treatment Extension Period

Stable BCVA

Vitreous Biomarkers Compared Before & After Extension Period

Antibody	
αβ crystallin	IL-10
AKT T308	Il-12
AMPK α1 S485	IL-8
BAD S112	IL-6
BCL2 T56	Integrin α5β1
cABL T735	MMP-14
CF-C3	MMP-2
CF-C5	MMP-9
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FGF-R	TNF-α
Fibronectin	VEGF-A
Heme Oxygenase 1	VEGFR2 Y1175
IL-1β	VEGFR2 Y951
	VEGFR2 Y996

Significant Wilcoxon Matched Pairs

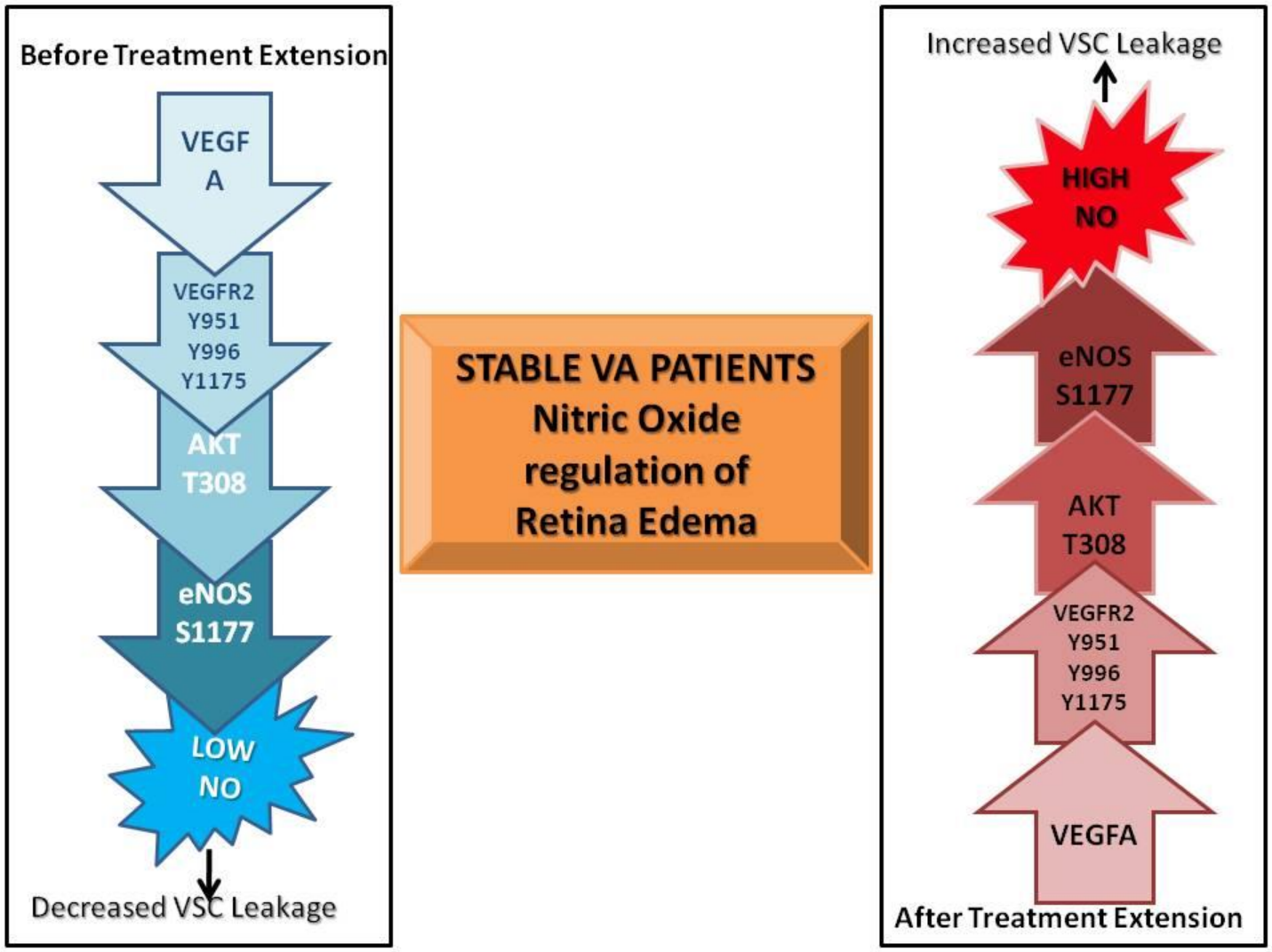
t-test Results for Stable VA Patients

Protein	**Fold Change After Extended Follow-up Interval**	**P-Value**
AKT T308	41.3% Higher After Extended Follow-up Interval	P= 0.0322
eNOS S1177	47.8% Higher After Extended Follow-up Interval	P= 0.0288
MMP-9	60.1% Higher After Extended Follow-up Interval	P= 0.0210
Musashi	43.5% Higher After Extended Follow-up Interval	P= 0.0186
PDGFRβ Y716	54.8% Higher After Extended Follow-up Interval	P= 0.0210
PDGFRβ Y751	42% Higher After Extended Follow-up Interval	P= 0.0425
TIMP2	59.5% Higher After Extended Follow-up Interval	P= 0.0068
VEGFR2 Y996	46.8% Higher After Extended Follow-up Interval	P= 0.0425

Possible Regulation of Retinal Edema Through eNOS → Nitric Oxide Signaling

eNOS → Nitric Oxide Signaling Pathway Proteins

Antibody	Stable VA Patients Before Treatment Extension Period	Stable VA Patients After Treatment Extension Period	**P-value**
VEGFA	0.969	1.281	0.3394
VEGFR2 Y951	1.142	1.774	0.2661
VEGFR2 Y996*	0.7768	1.459	0.0425
VEGFR2 Y1175	1.205	1.473	.9097
AKT T308*	1.044	1.778	0.0322
eNOS S1177*	0.5458	2.005	0.0288


Before Treatment Extension
VEGF A
VEGFR2 Y951 Y996 Y1175
AKT T308
eNOS S1177
LOW NO
Decreased VSC Leakage
STABLE VA PATIENTS
Nitric Oxide regulation of Retina Edema
Increased VSC Leakage
HIGH NO
eNOS S1177
AKT T308
VEGFR2 Y951 Y996 Y1175
VEGFA
After Treatment Extension

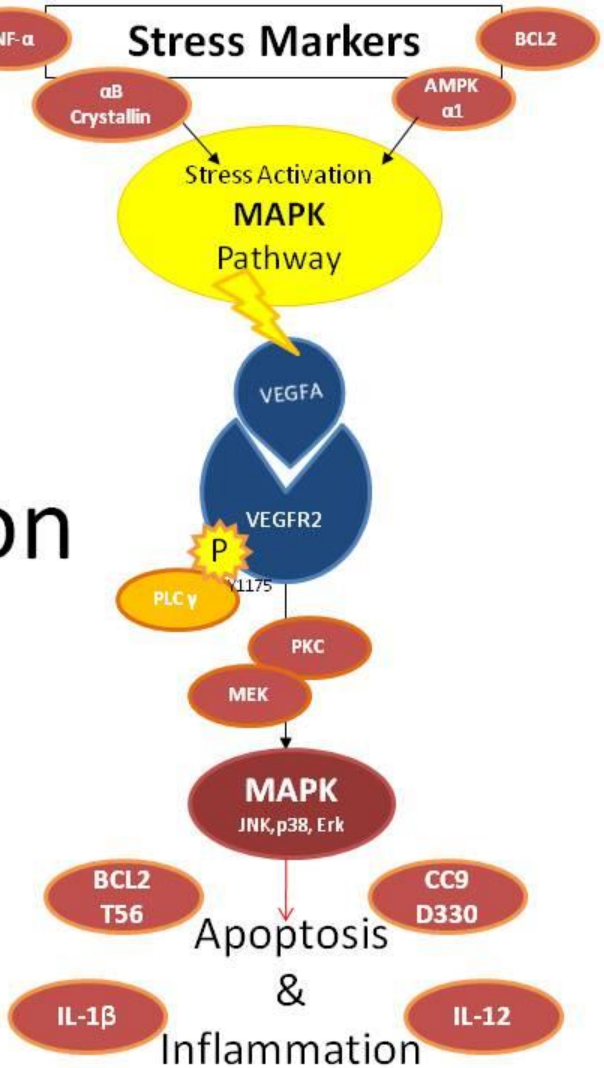
Results

Patients with Unstable VA
During Treatment Extension period

No protein expression changes

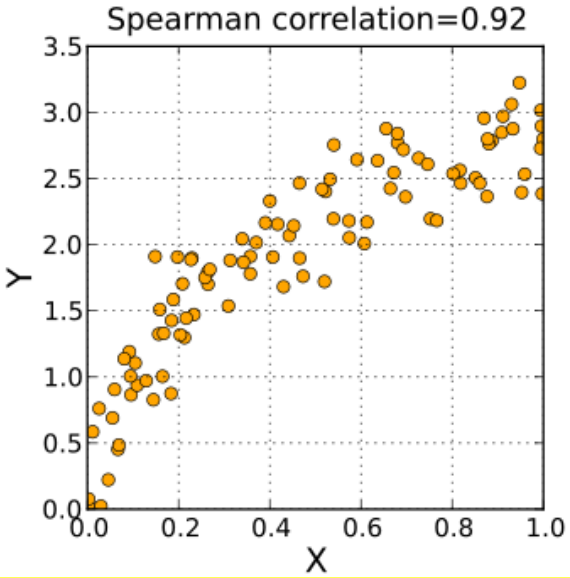
Instead:
Protein Interaction Changes
Suggesting MAPK Regulation

MAPK Regulation



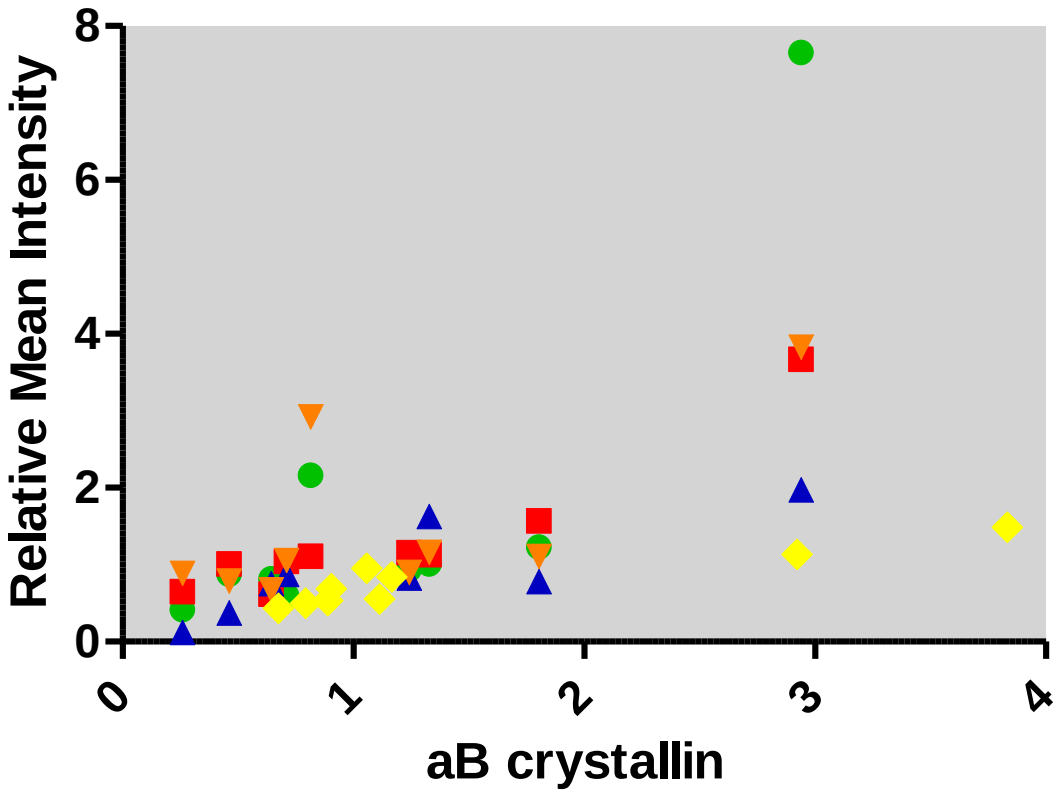
Spearman's Rho Correlation

- Determines if 2 variables work as a function of each other



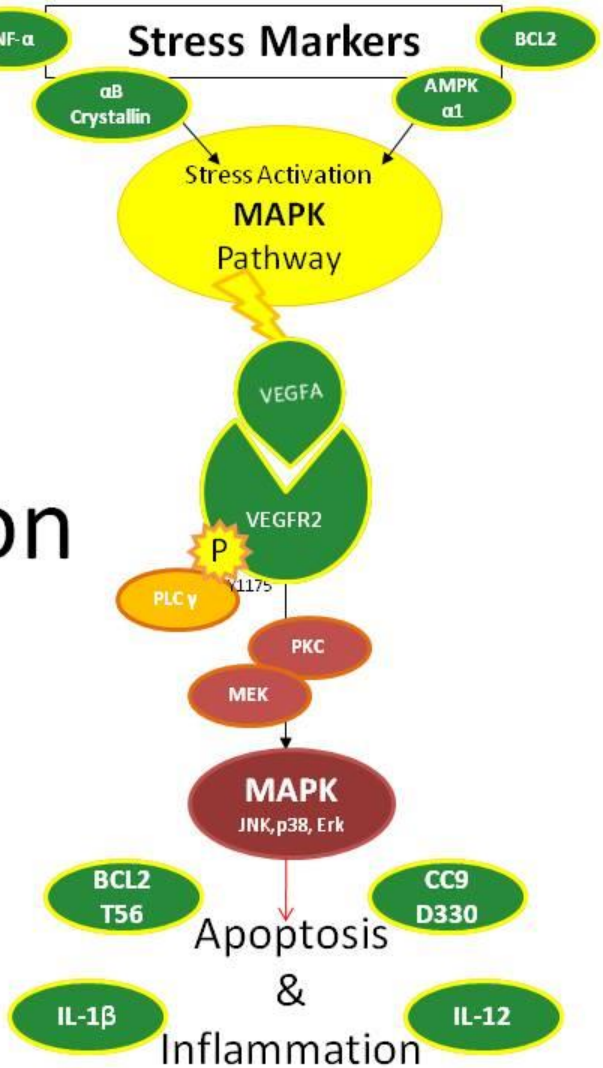
- Used to Determine:
  - If Two Proteins Interact with each other
    - Suggests Biological connectivity
    - Possible Signaling Pathway Interaction

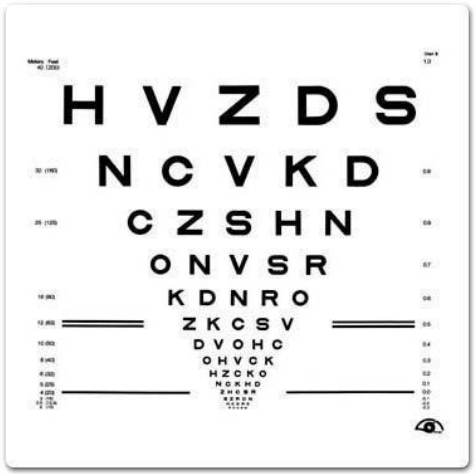
Correlation Analysis: Strong protein interactions with αB Crystallin Suggests MAPK Regulation

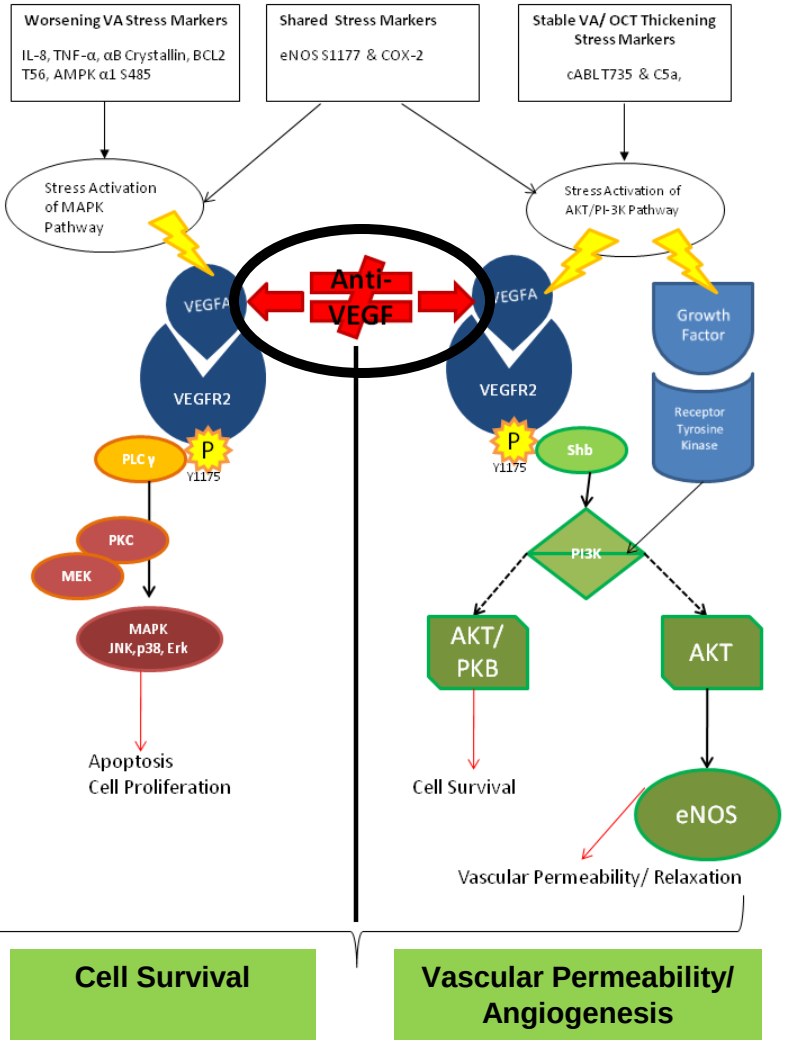


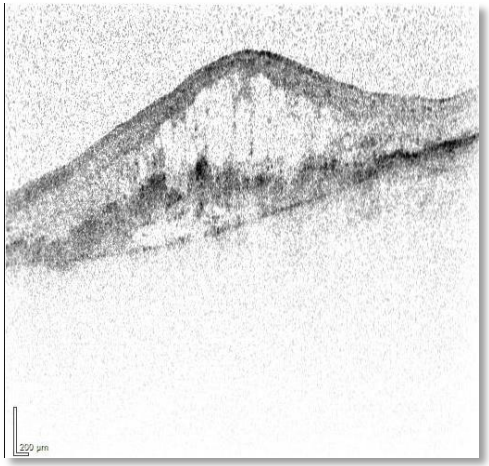
- IL-12
  ρ= 0.917, P<0.001
- BCL2 T56
  ρ=0.833, P<0.01
- CC9 D330
  ρ=0.933, P<0.0001
- IL-1B
  ρ= 0.833, P<0.01
- AMPK α1 S485
  ρ= 0.783, P<0.01

MAPK Regulation

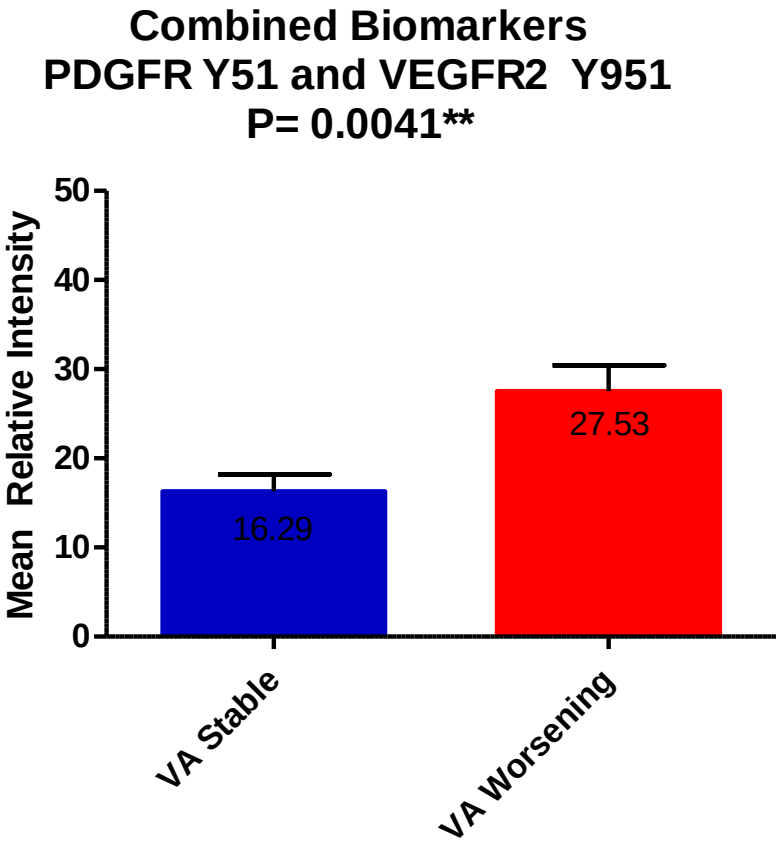


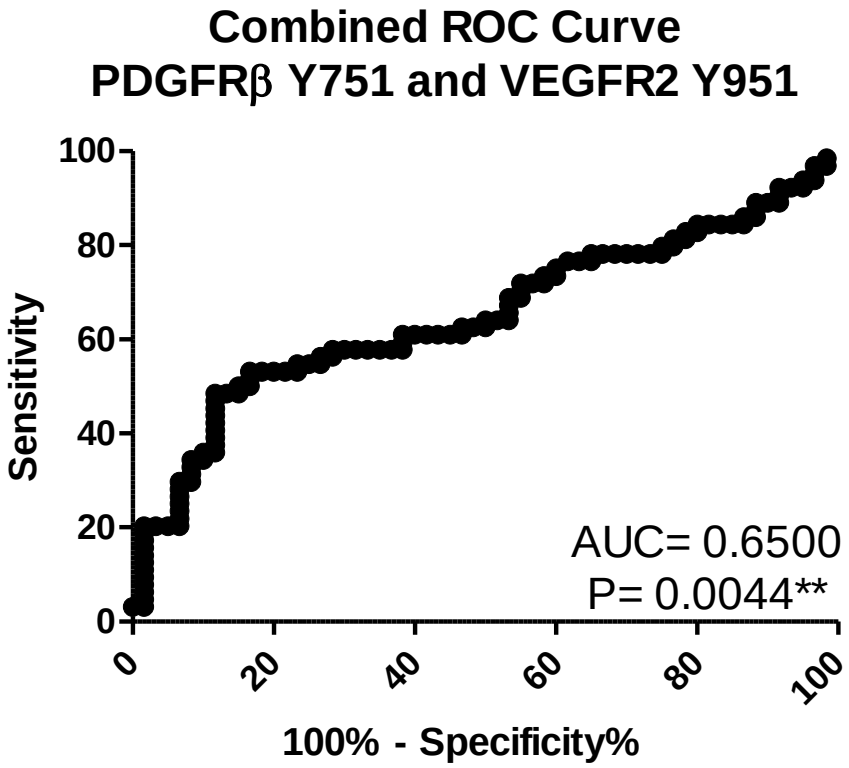

H V Z D S
N C V K D
C Z S H N
O N V S R
K D N R O
Z K C S V
D V O H C
O H V C K
H Z O K O

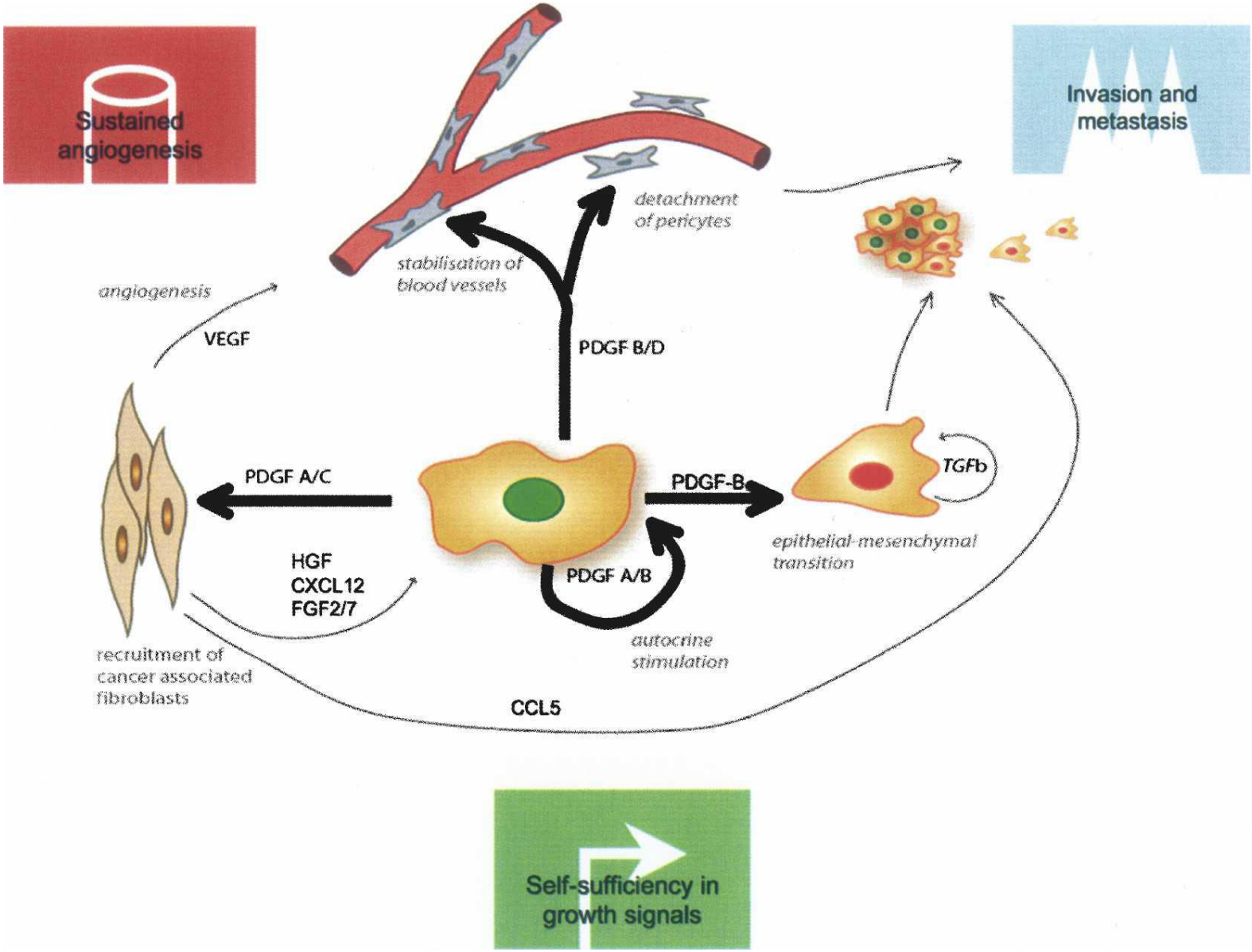

Worsening VA Stress Markers
IL-8, TNF-α, αB Crystallin, BCL2 T56, AMPK α1 S485
Shared Stress Markers
eNOS S1177 & COX-2
Stable VA/ OCT Thickening Stress Markers
cABL T735 & C5a,
Stress Activation of MAPK Pathway
Stress Activation of AKT/PI-3K Pathway
Anti-VEGF
VEGFA
VEGFA
VEGFR2
VEGFR2
Growth Factor
Receptor Tyrosine Kinase
PLCγ
P
Y1175
P
Y1175
Shb
PKC
MEK
PI3K
MAPK JNK,p38, Erk
AKT/ PKB
AKT
Apoptosis
Cell Proliferation
Cell Survival
eNOS
Vascular Permeability/ Relaxation
Cell Survival
Vascular Permeability/ Angiogenesis



Who can be extended?







Andrae, J., Gallini, R., & Betsholtz, C. (2008). Role of platelet-derived growth factors in physiology and medicine. *Genes and Development*, *22*, 1276–1312. doi:10.1101/gad.1653708.revealing

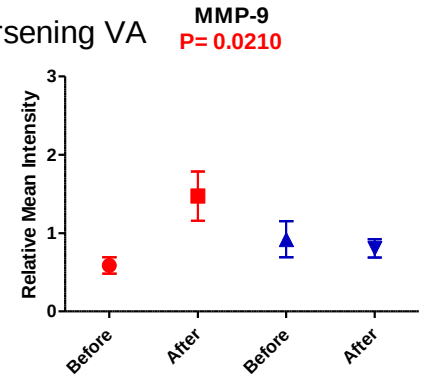
When do we retreat?

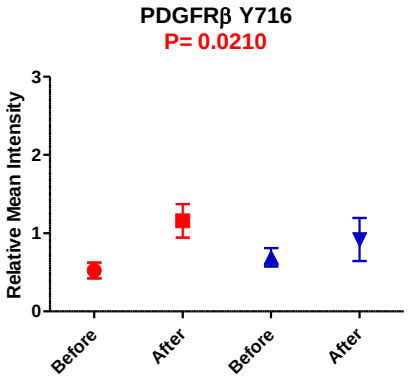
- Current
  - Signs of damage
    - Decreased VA
    - Increased macular edema or heme
- Future
  - Vitreous Proteome

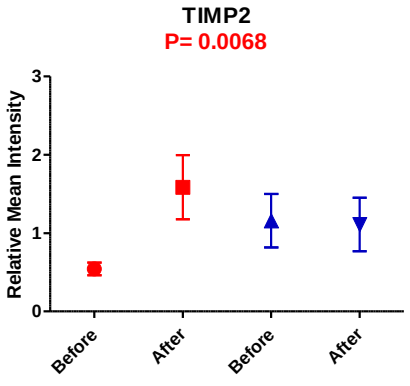
When do we retreat?

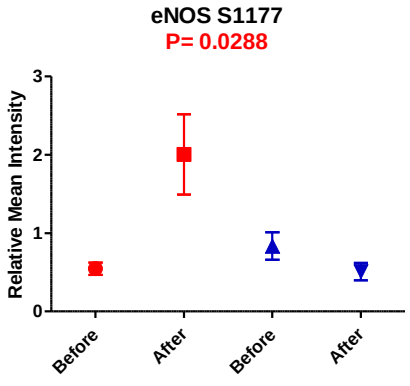
Stable VA

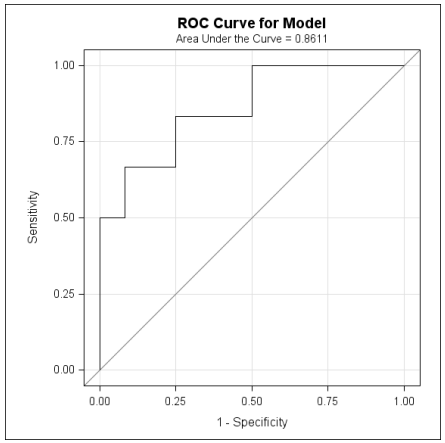
Worsening VA

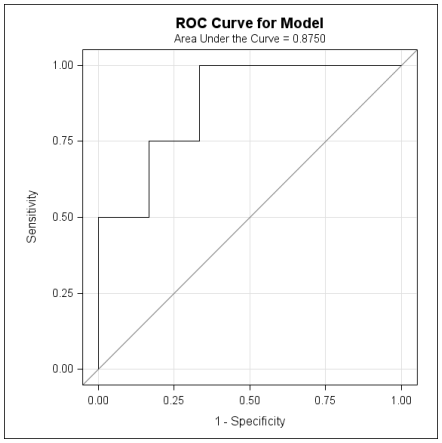


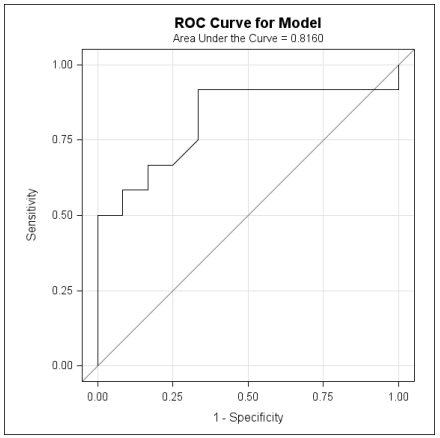


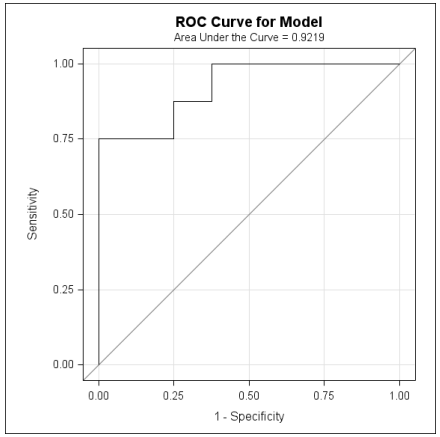








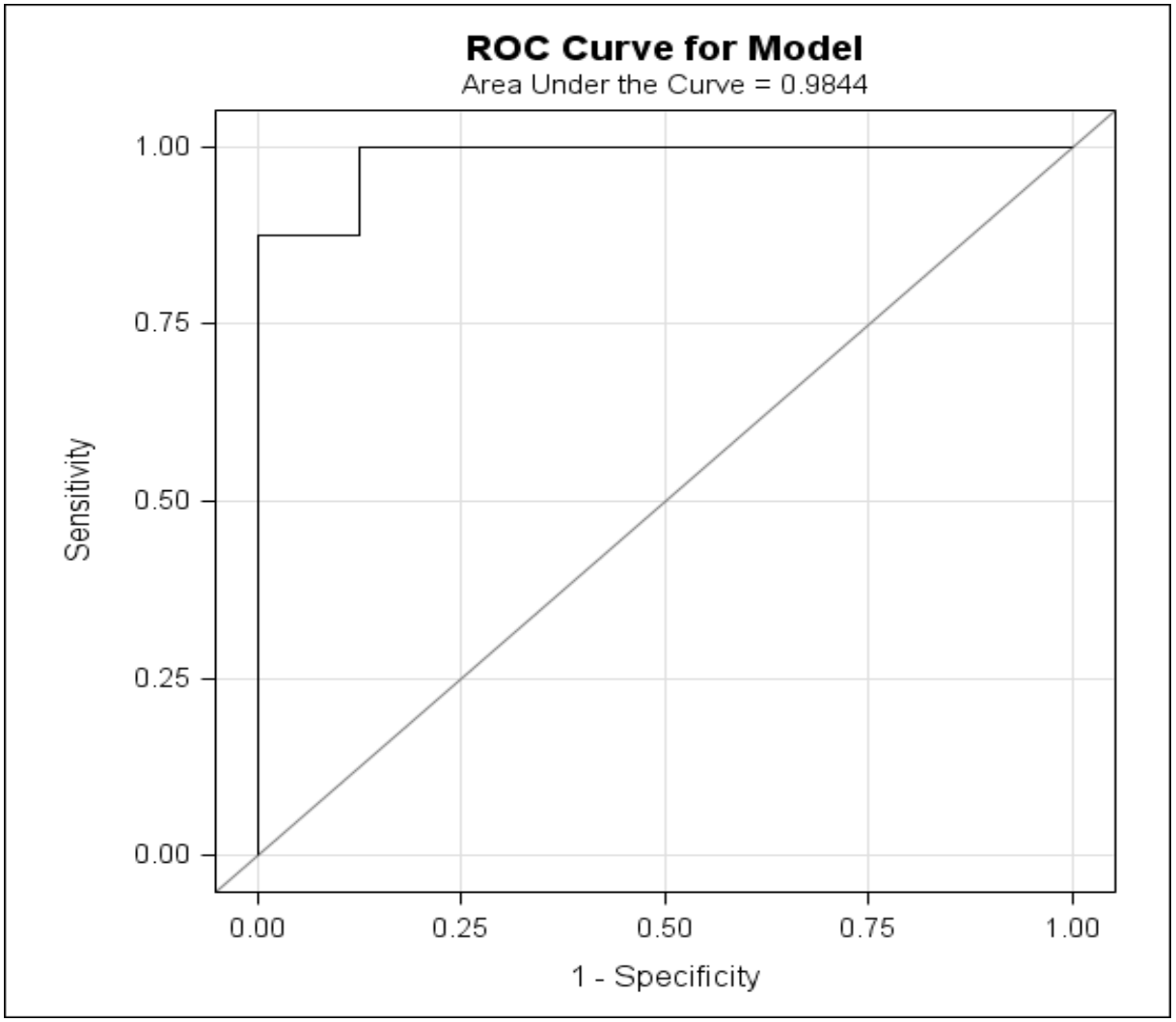




When do we retreat?

Biomarker Panel: MMP-9, PDGFR Y716, and eNOS S1177

AUC= 0.9844 P<0.0001



Studies

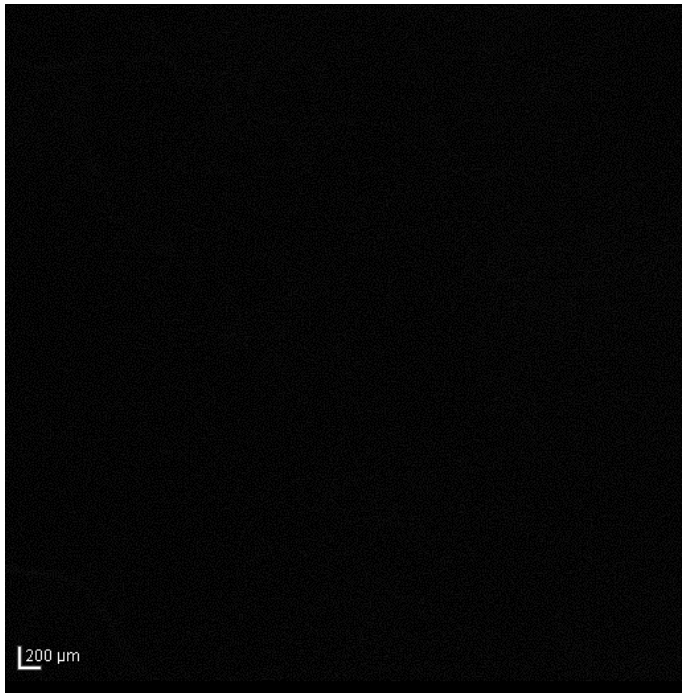
- Aid Management of Disease
- Characterization of Disease
- Staging of Disease

AMD

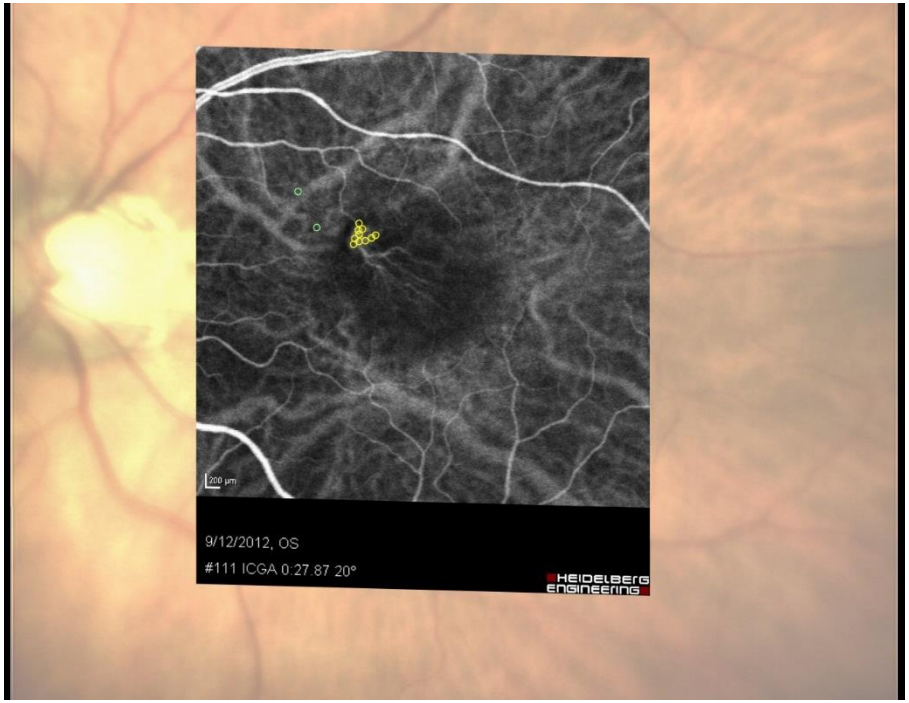
- Heterogeneous
- Multiple Diseases

RAP + CNV

PRE - ICG



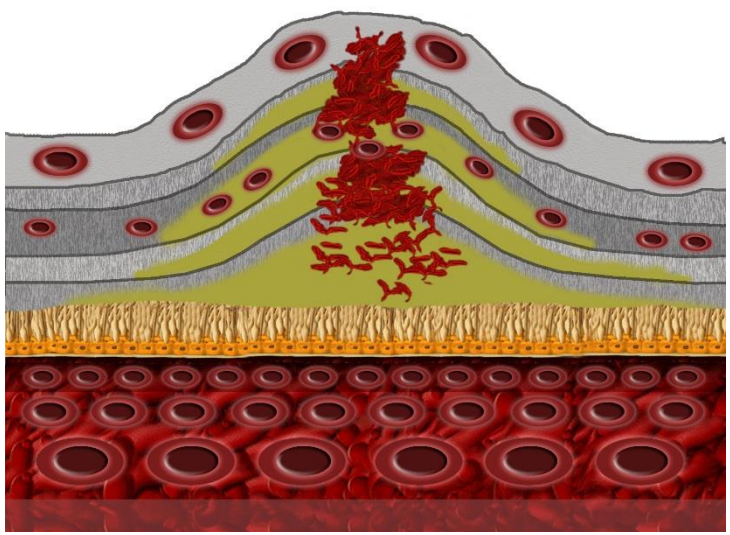
NAVILAS PLAN

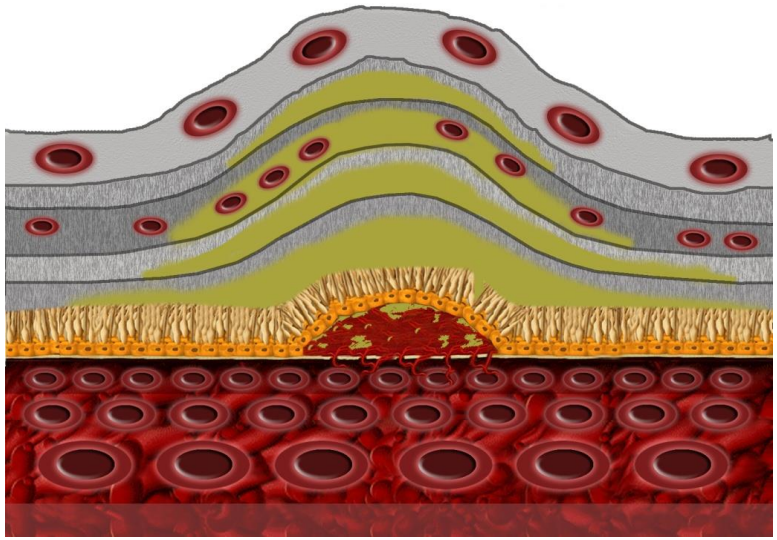


RAP

?

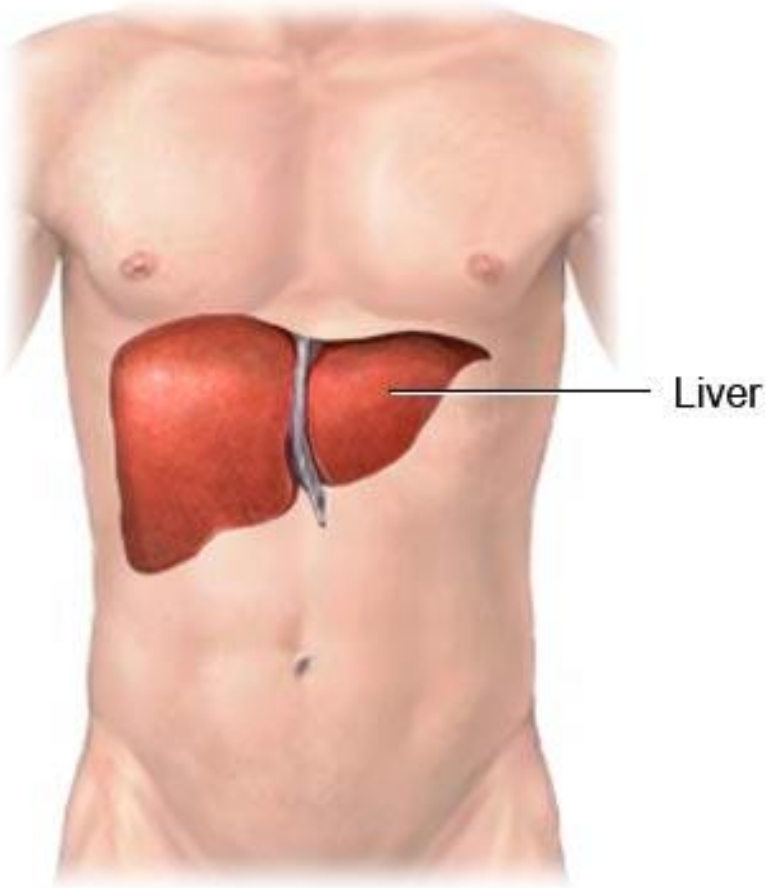
CNV



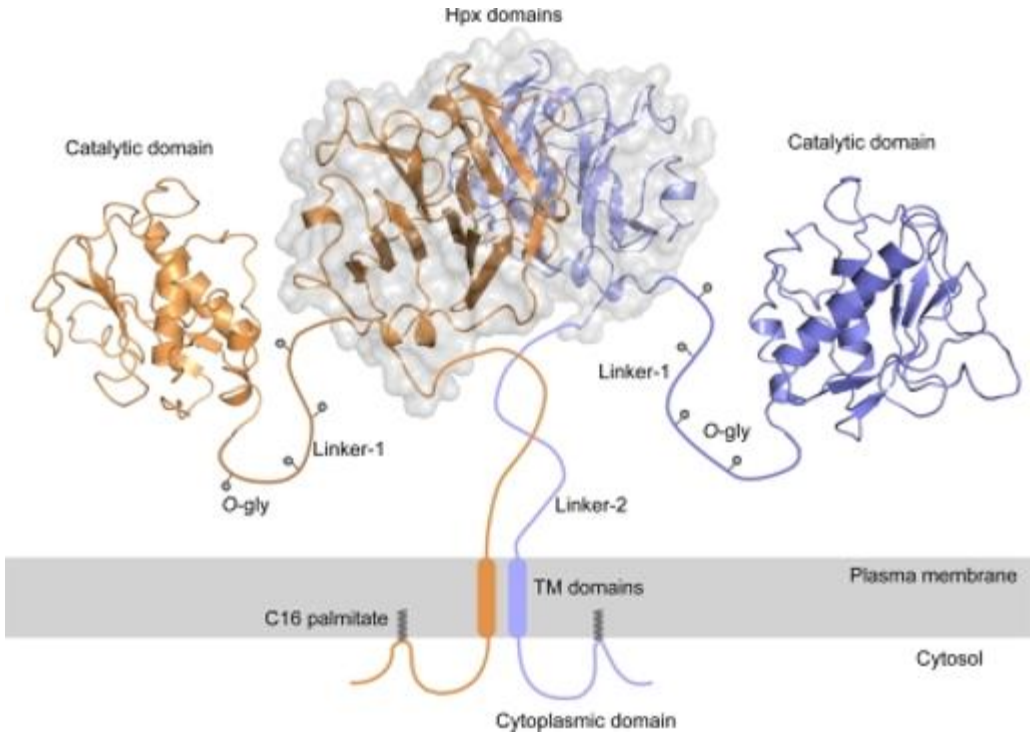


Vitreous Proteome: Differentiates CNV vs. RAP

Morphology



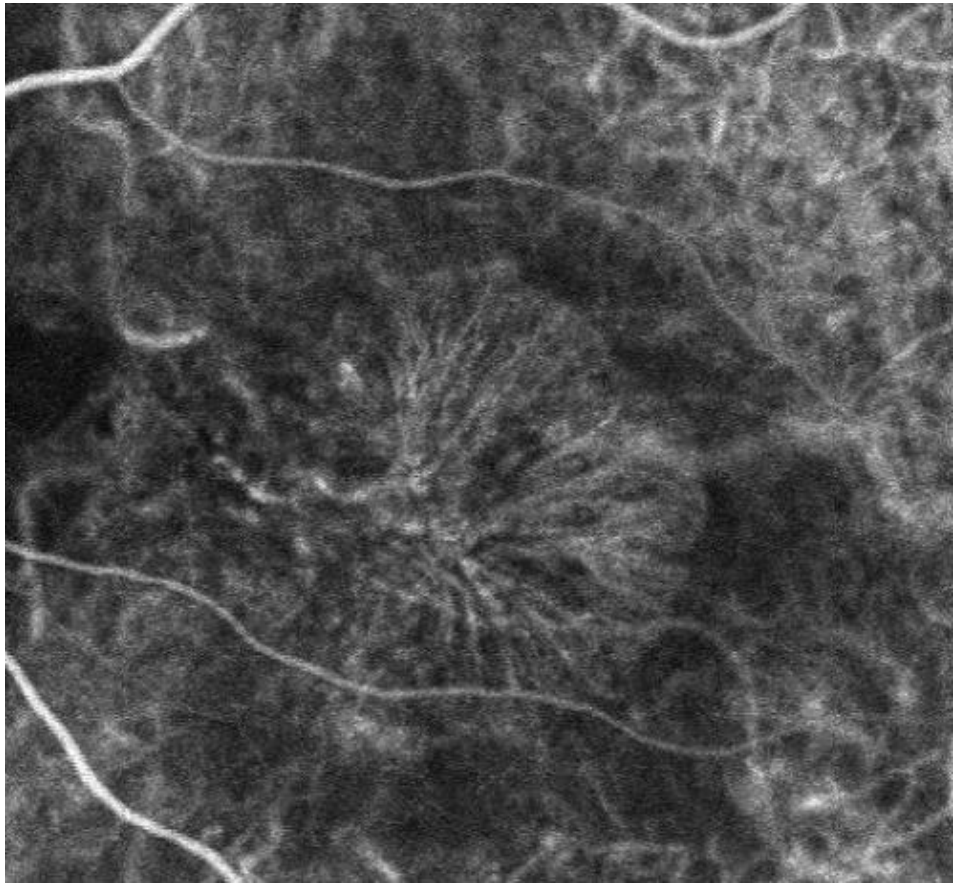
Biochemistry



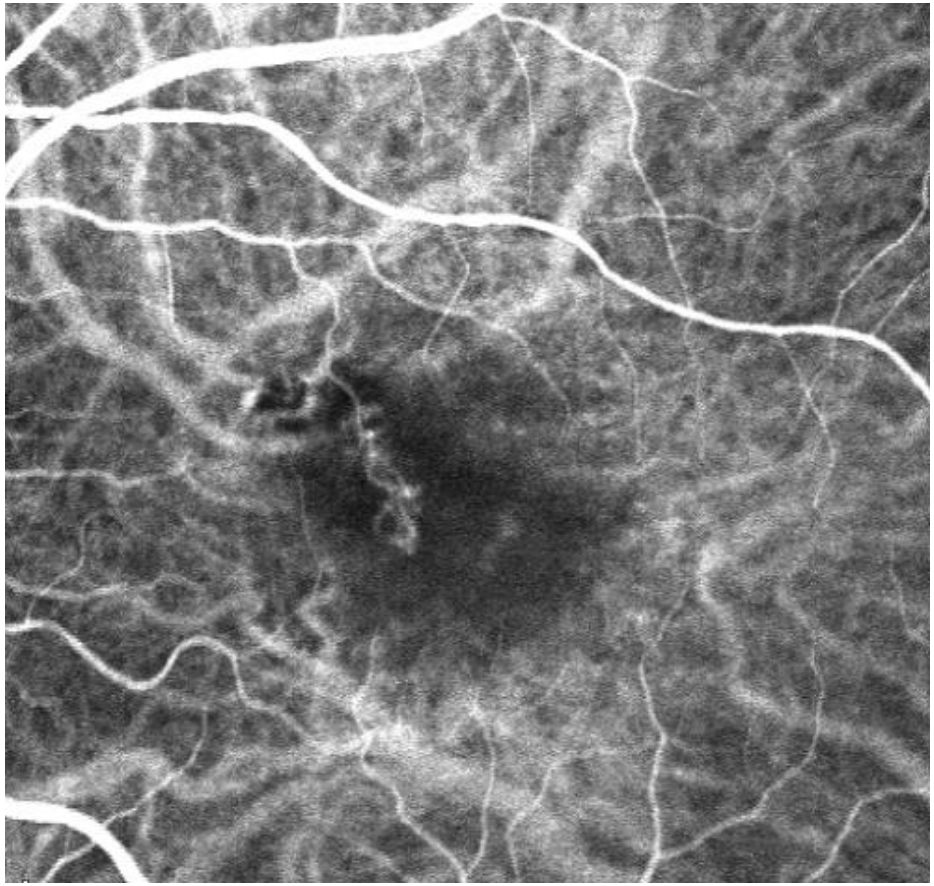

AKT
eNOS
PDGFRβ
TGF-β
Integrin
FGFR
IL-1β
TNF-2
IL-6
IL-10
BCL-2
TIMP2
BAD
MMP-2
C3a
VEGF
MMP-14
C5a
CF-H
MMP-9

Biochemistry of In-Office Vitreous Aspirates

CNV



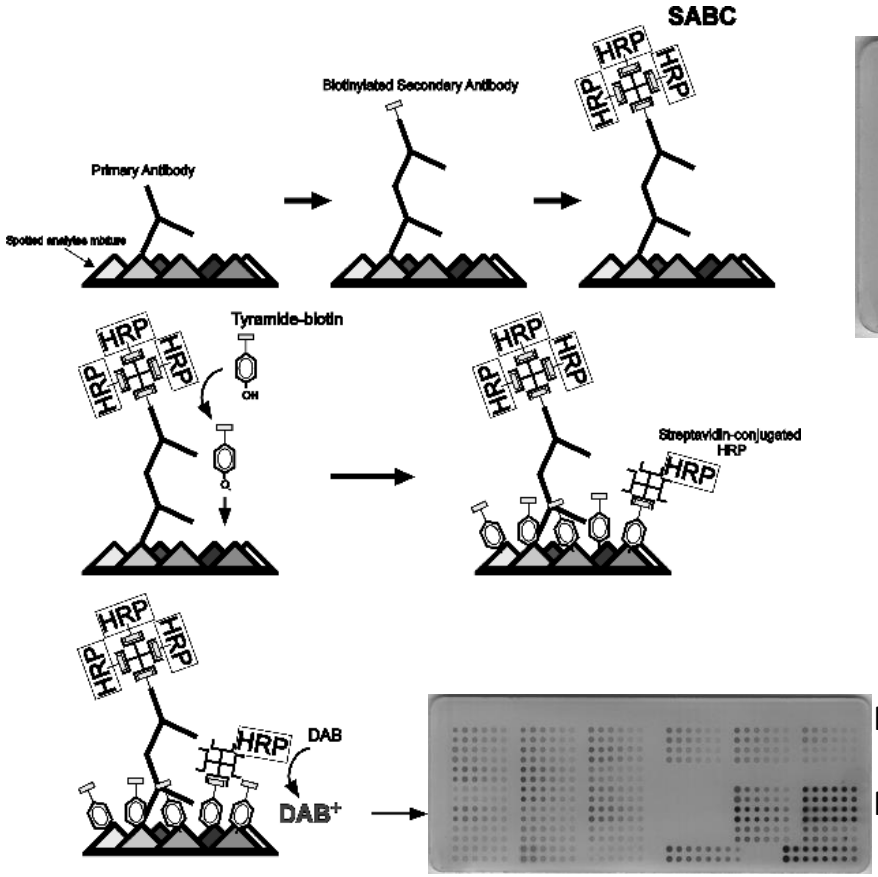
RAP

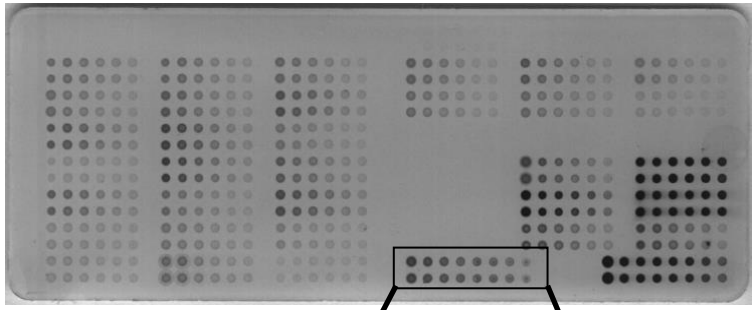


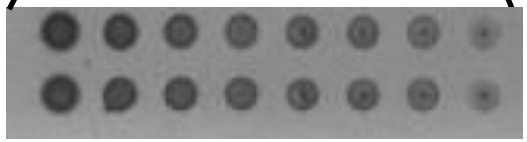
Study Patient Information

Demographic	CNV	RAP	Controls
Total Number of Eyes	**50**	**56**	**24**
Total Number of Patients	36	41	24
Average Age	77 (56-89)	75 (41-88)	65(31-89)
Percent Female	61%	73%	50%

Reverse Phase Protein Microarray







Replicate 1

Replicate 2

Undil.,1:2,1:4,1:8,1:16,1:32,1:64,1:128

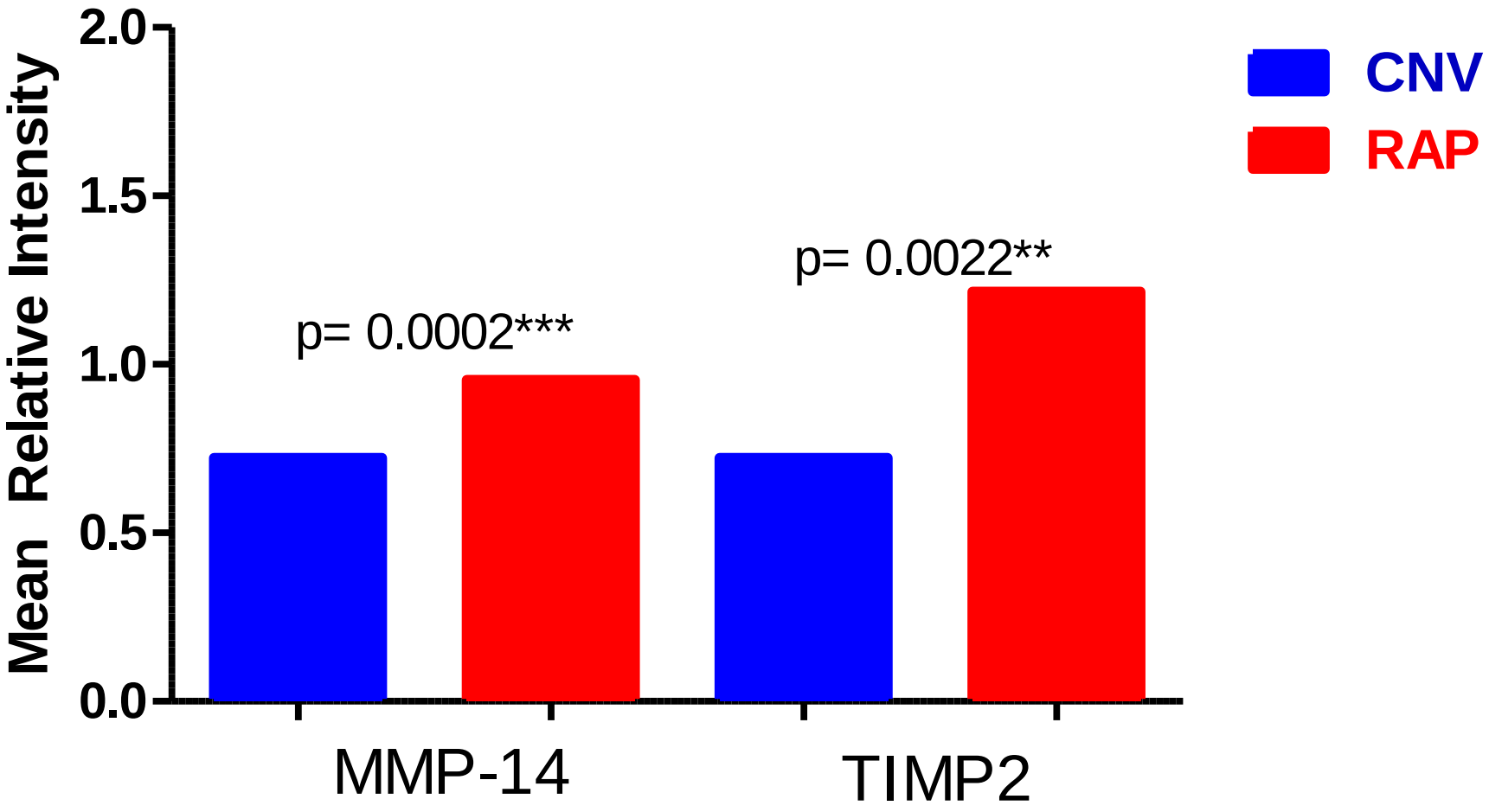
Control sample, duplicate spots

Antibody	
αβ crystallin	IL-10
AKT T308	Il-12
AMPK α1 S485	IL-8
BAD S112	IL-6
BCL2 T56	Integrin α5β1
cABL T735	MMP-14
CF-C3	MMP-2
CF-C5	MMP-9
CF-C9	Musashi
CF-H	PDGFRβ Y716
cKIT Y703	PDGFRβ Y751
cKIT Y719	PEDF
COX-2	TIMP2
eNOS S1177	TGF-Beta
FGF-R	TNF-α
Fibronectin	VEGF-A
Heme Oxygenase 1	VEGFR2 Y1175
IL-1β	VEGFR2 Y951
	VEGFR2 Y996

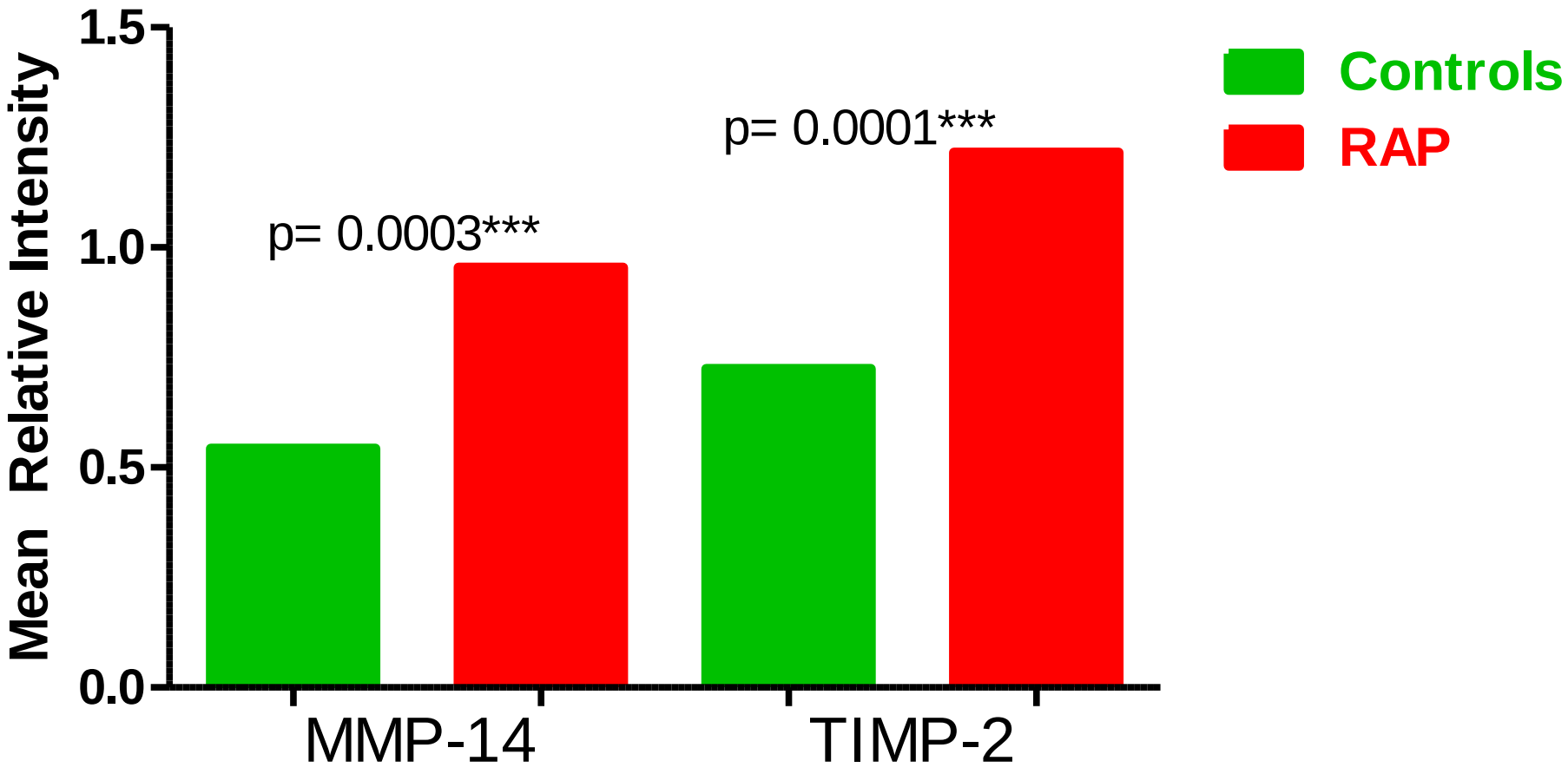
Vitreous Proteome Results

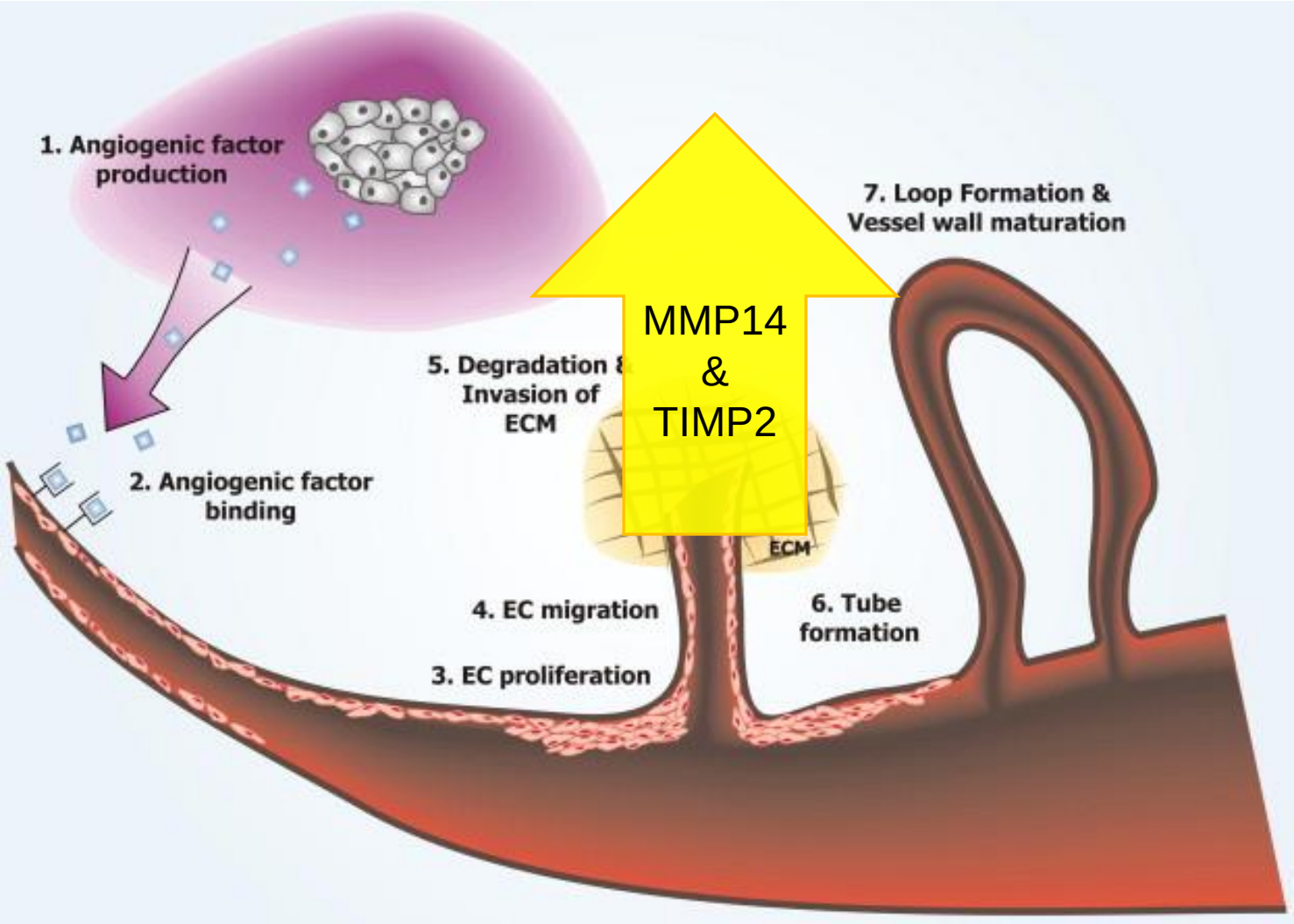
Protein	CNV Mean RI	RAP Lesion Mean RI	P-Value
MMP-14	0.7233	1.042	0.0002
TIMP-2	0.7228	1.216	0.0022
Complement C5a	1.387	1.657	0.0101
Complement C9	1.016	1.400	0.0449
CF-H	0.912	1.405	0.0442

Matrix Metalloprotinase Family



Matrix Metalloprotinase Family

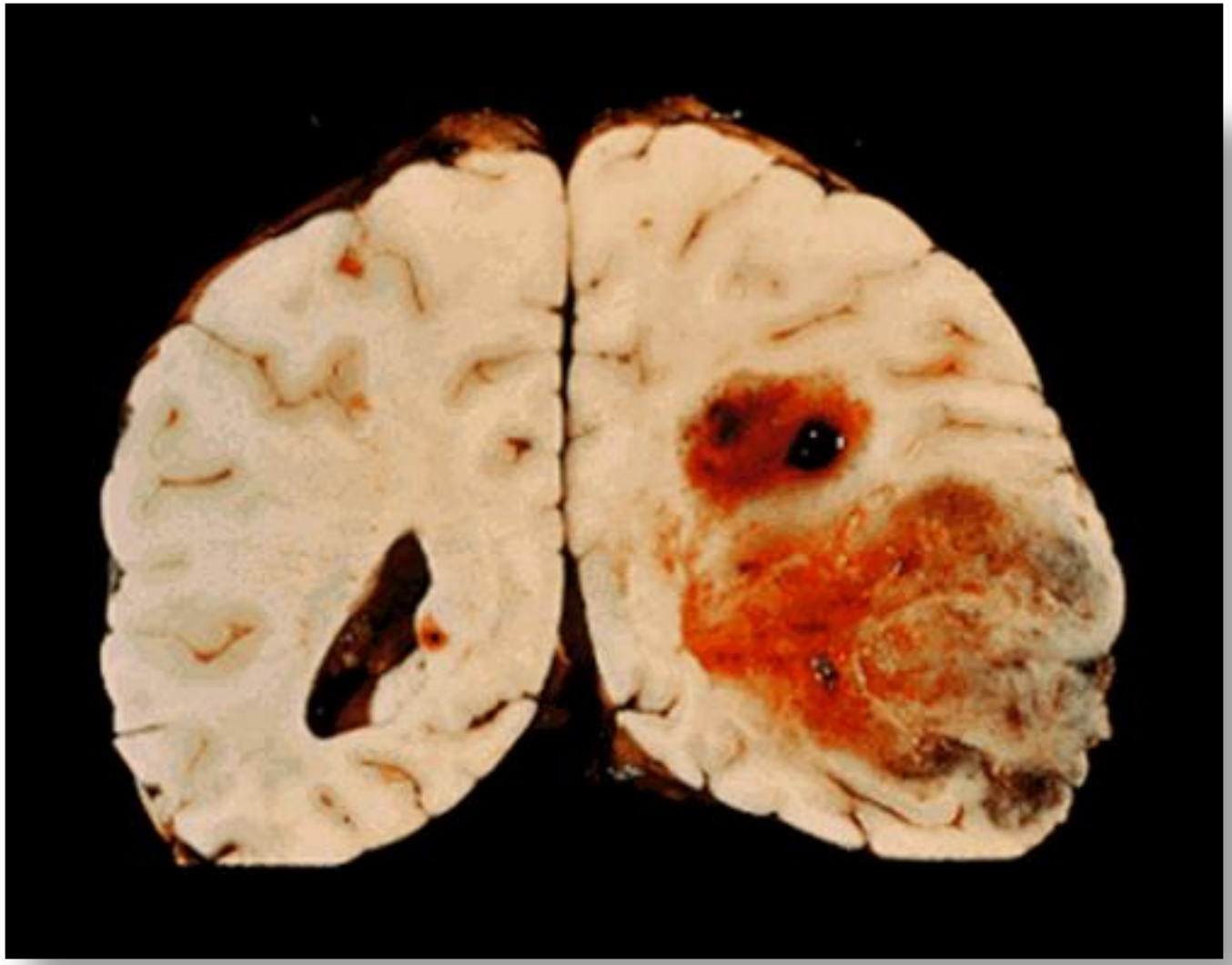




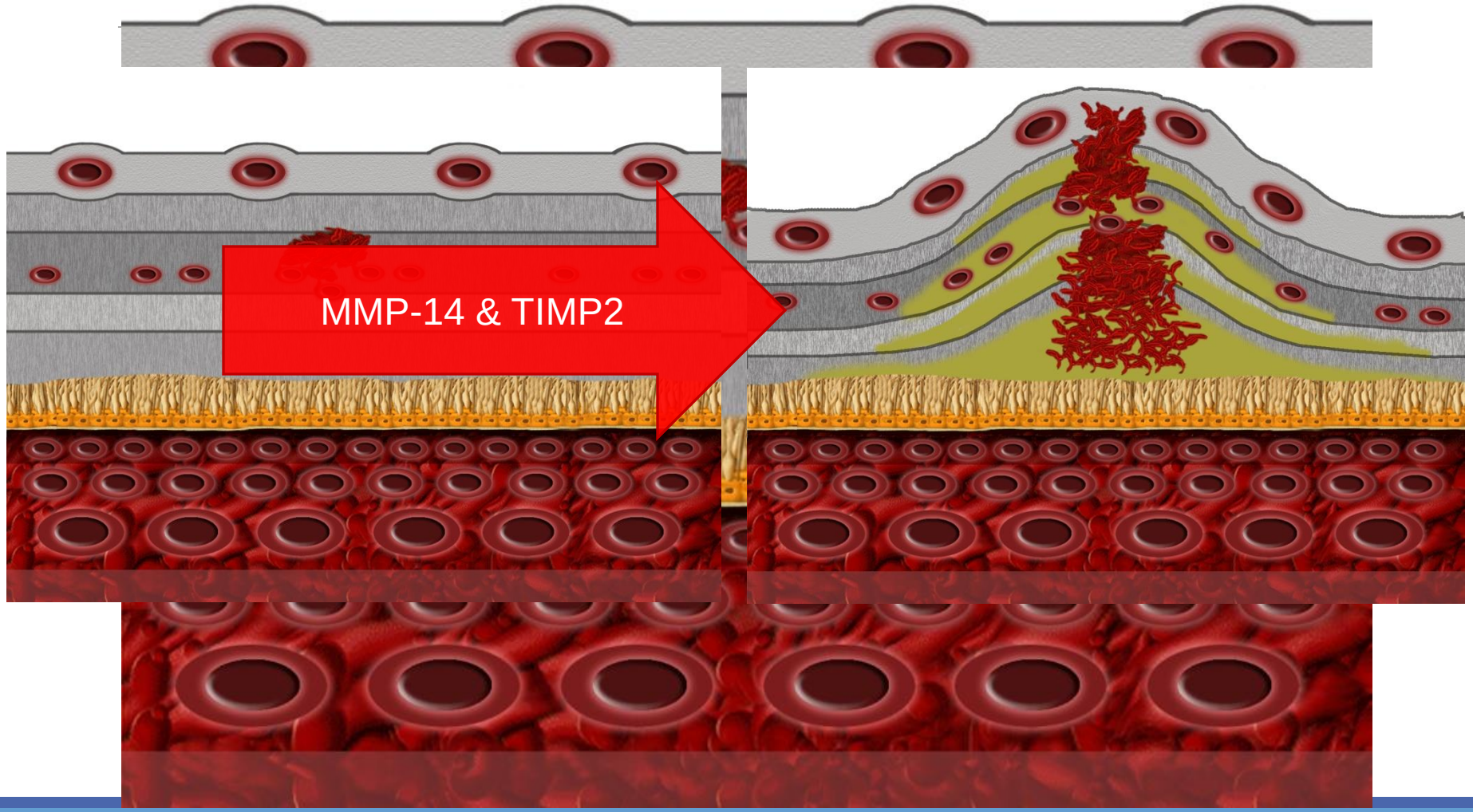
Wong et.al. Tumour angiogenesis: its mechanism and therapeutic implications in malignant gliomas
J Clin Neurosci. 2009 Sep;16(9):1119-30.

Glioblastoma

MMP-14
&
TIMP2

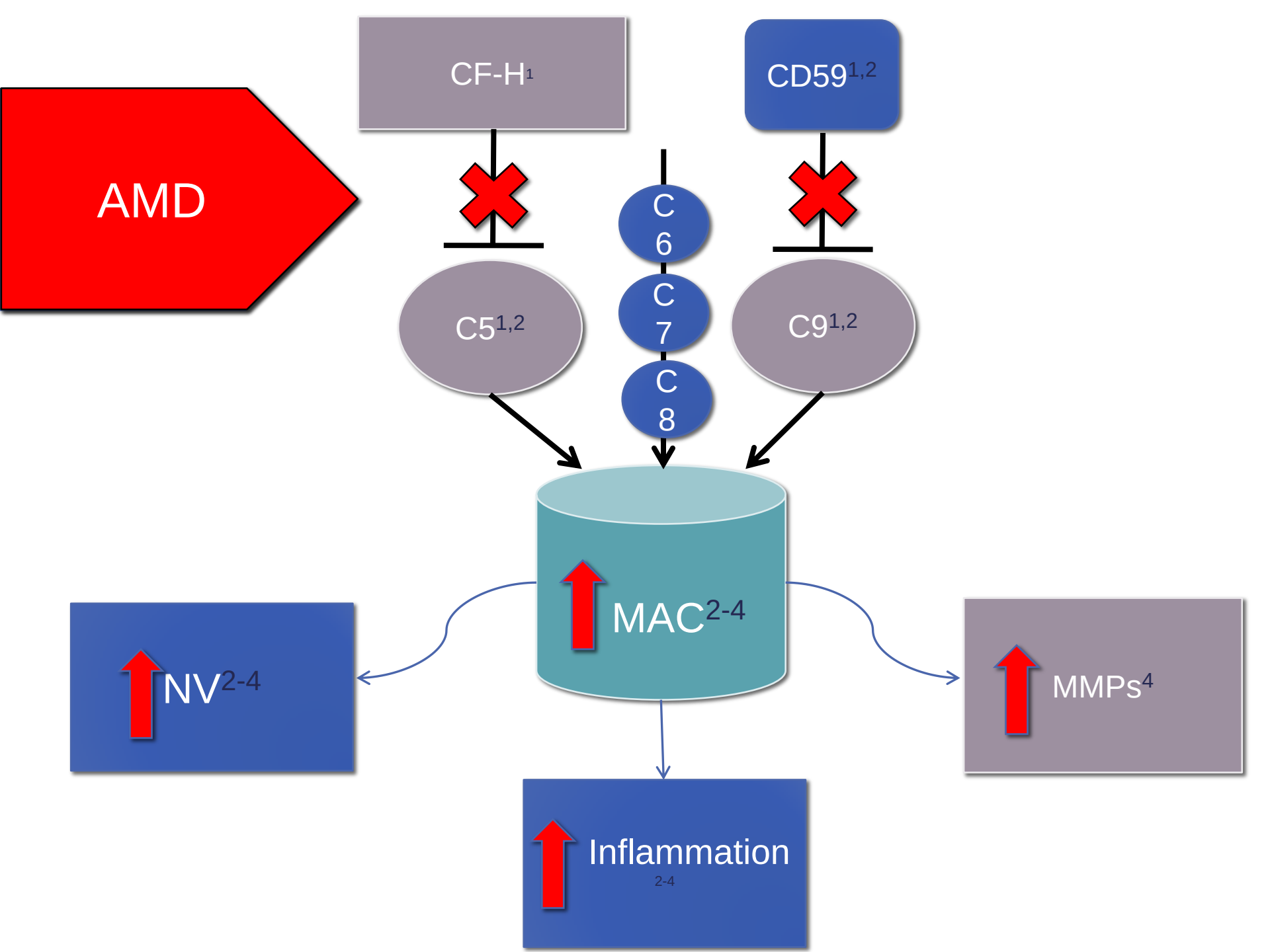


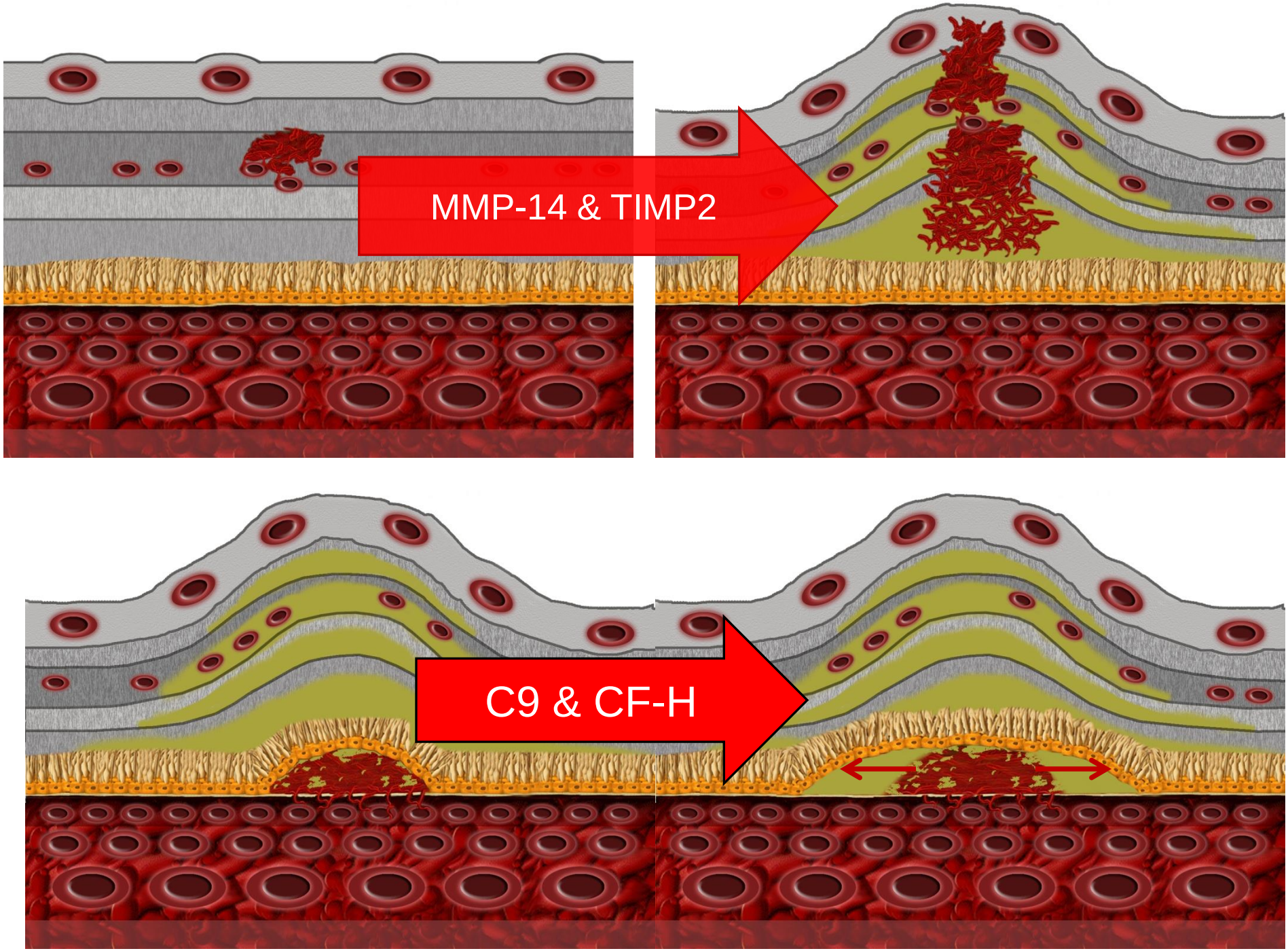
RAP



CNV vs. Controls Results

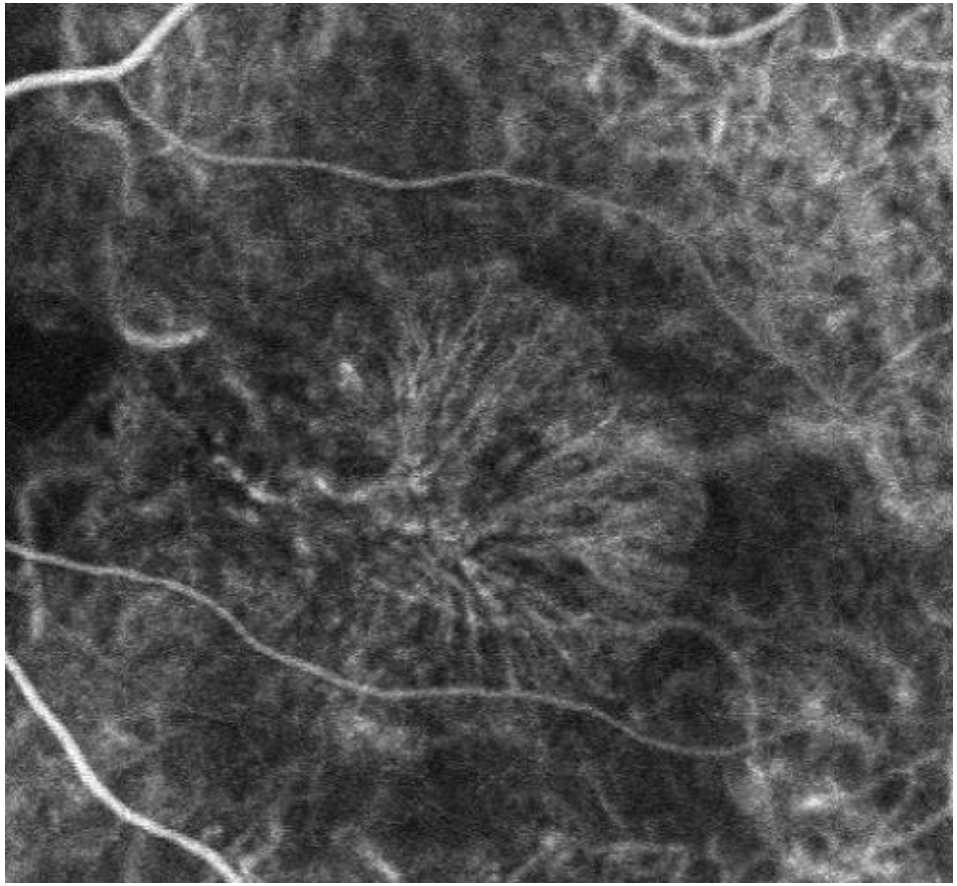
Protein	CNV Mean RI	Control Mean RI	P-Value
MMP-14	0.7233	0.5425	0.1950
TIMP-2	0.7228	0.7239	0.5564
Complement C5a	1.387	1.512	0.4116
Complement C9	1.016	0.7774	0.0914
CF-H	0.912	0.4966	0.0127*


AMD
CF-H[1]
CD59[1,2]
C5[1,2]
C6
C7
C8
C9[1,2]
MAC[2-4]
NV[2-4]
MMPs[4]
Inflammation
[2-4]

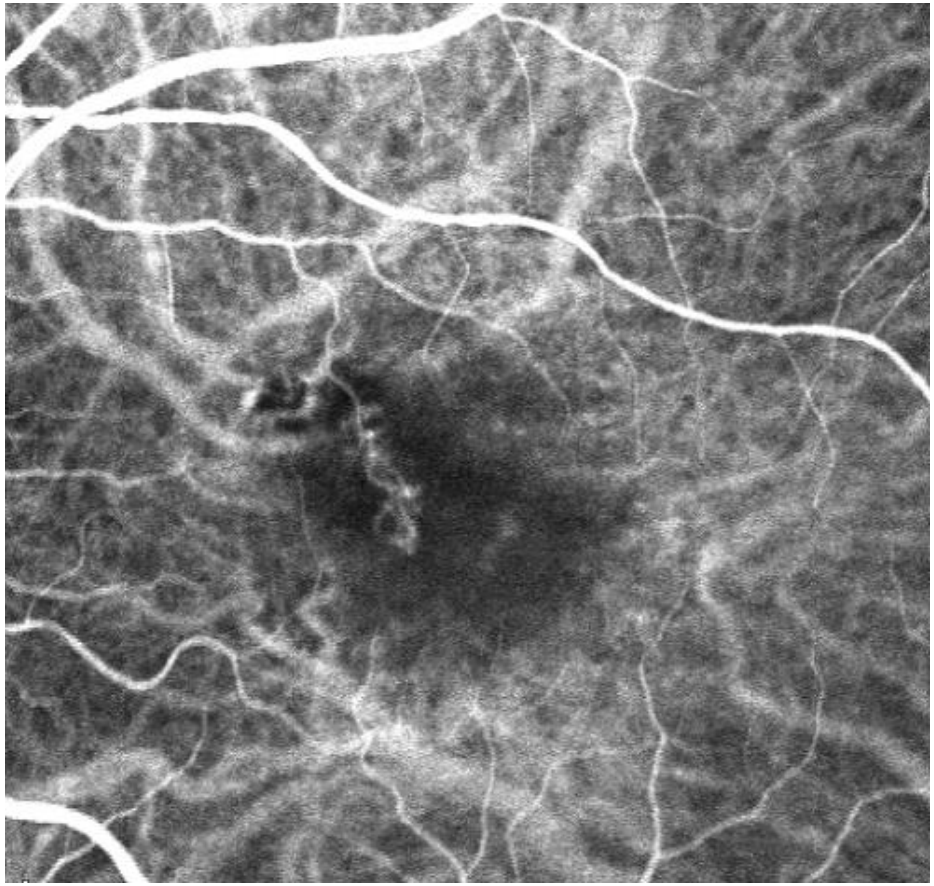

MMP-14 & TIMP2
C9 & CF-H

Biochemistry of In-Office Vitreous Aspirates

CNV



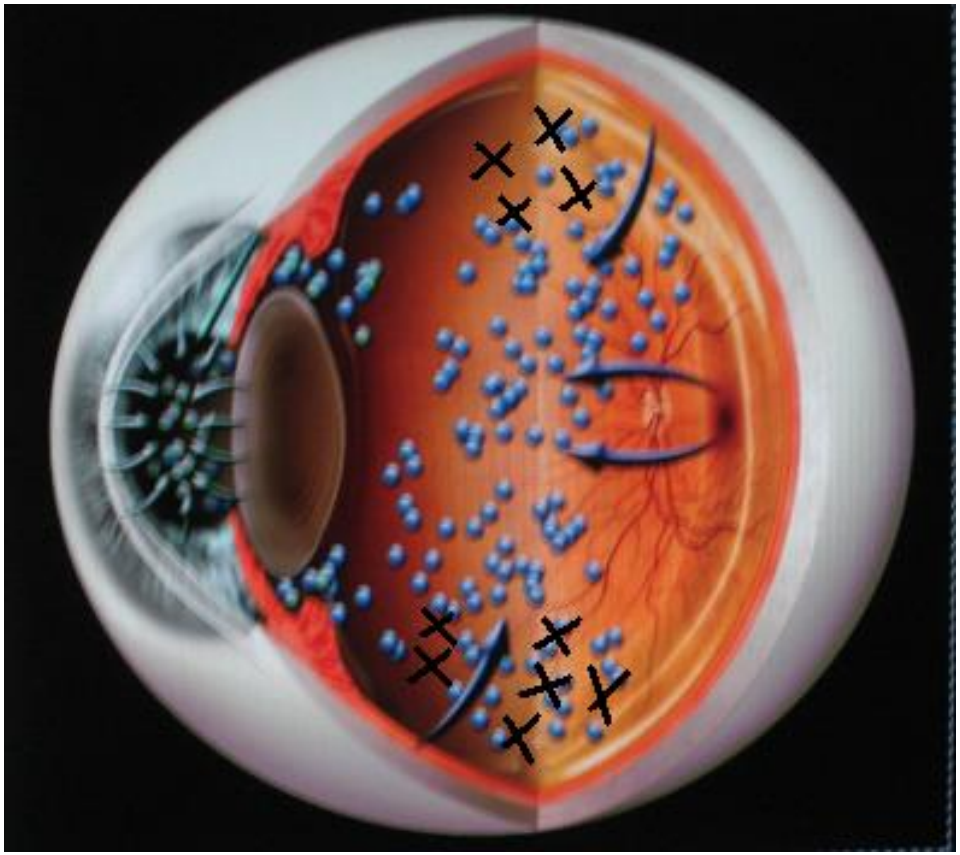
RAP

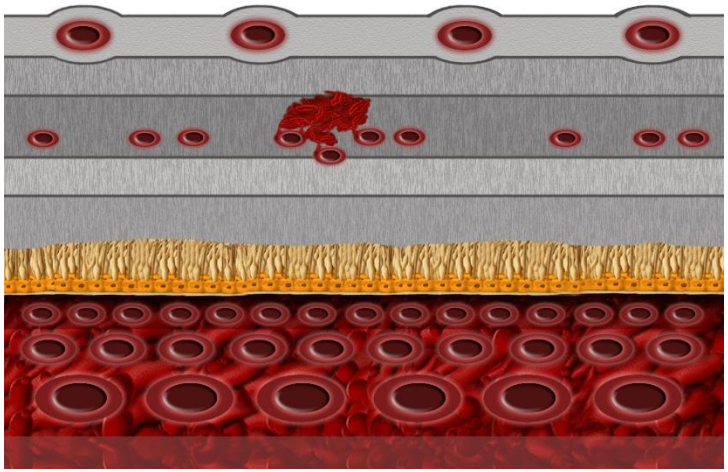


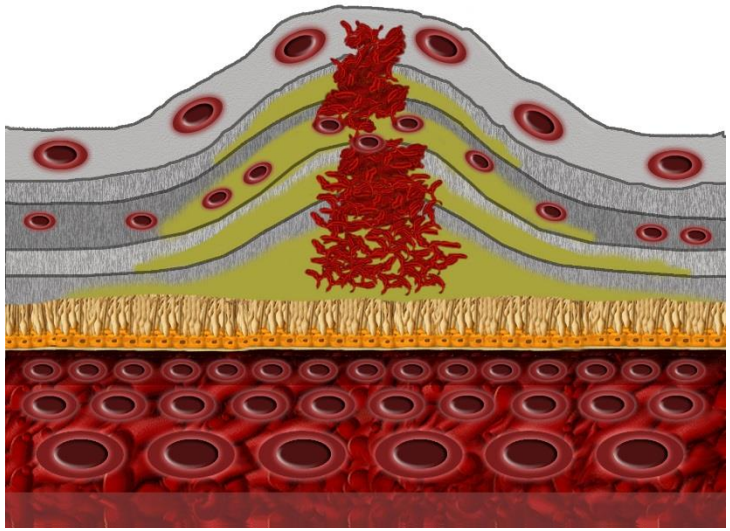
Studies

- Aid Management of Disease
- Characterization of Disease
- Staging of Disease

NPDR vs. PDR

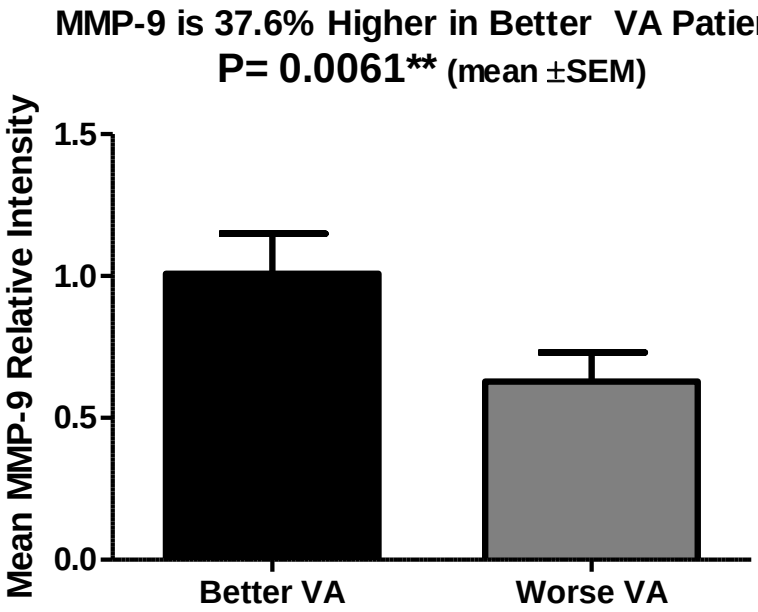


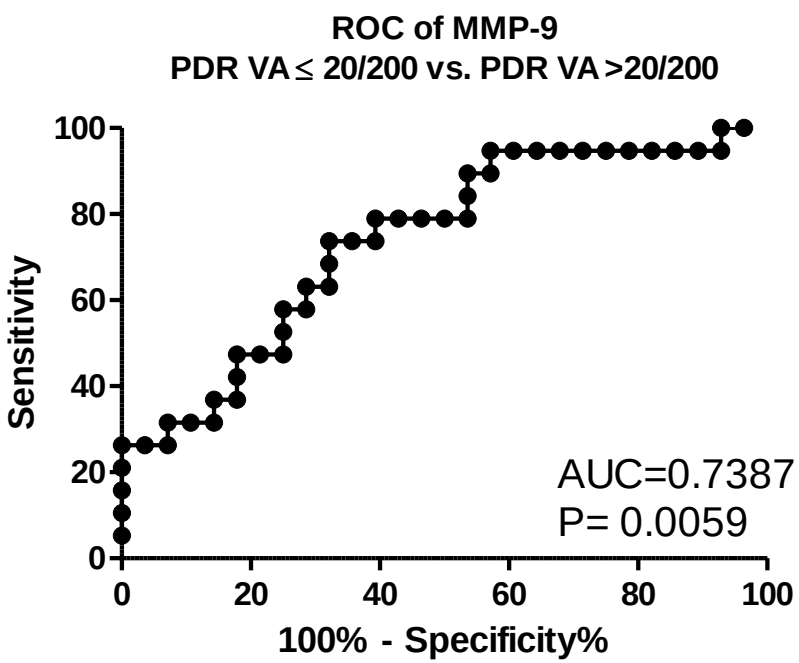




Levels of MMP-9 Correlate with The Visual Outcomes in Proliferative Diabetic Retinopathy

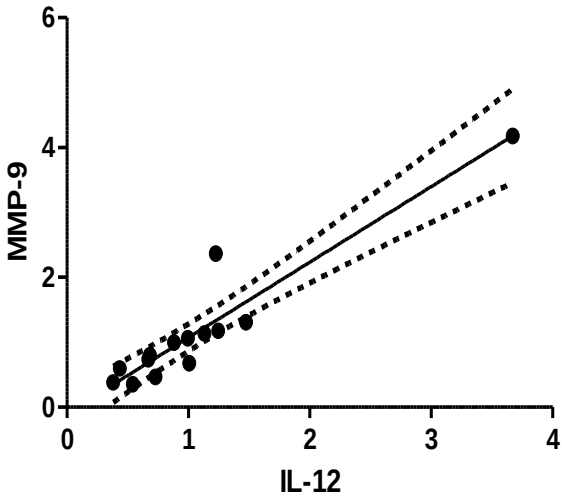
Patient Demographics			
	All PDR Patients	**VA >20/200**	**VA ≤ 20/200**
No. of Patients	42	28	19
Mean Age	58.1	56.7	58.52
Age Range	28-82	28-78	28-82
Sex	54.8% female	53.6% female	42.1% female



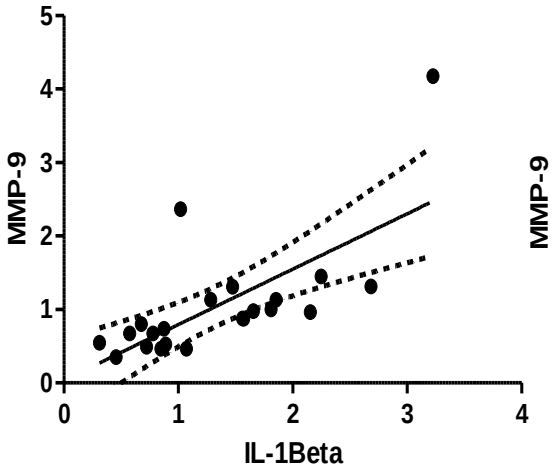


In Patients with VA Better than 20/200 MMP-9 Has strong correlations with Pro-Inflammatory Cytokines

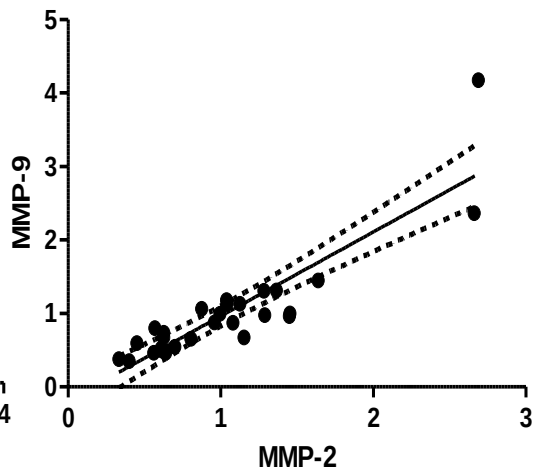
IL-12 (ρ= 0.891, P<0.0001)



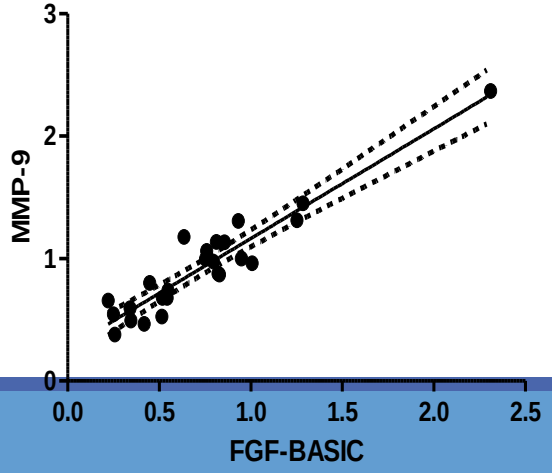
IL-1Beta (ρ= 0.890, P<0.0001)



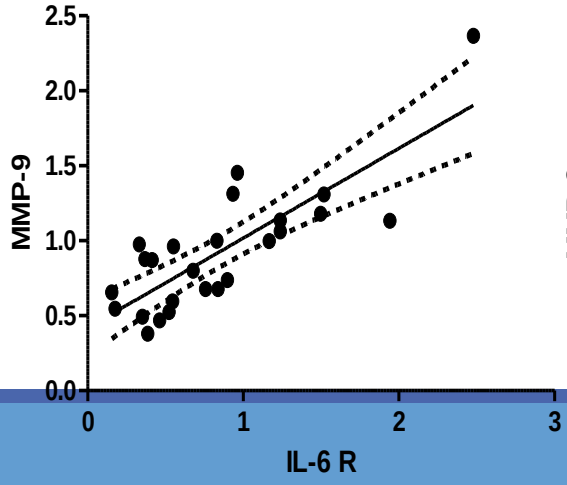
MMP-2 (ρ= 0.0842, P<0.0001)



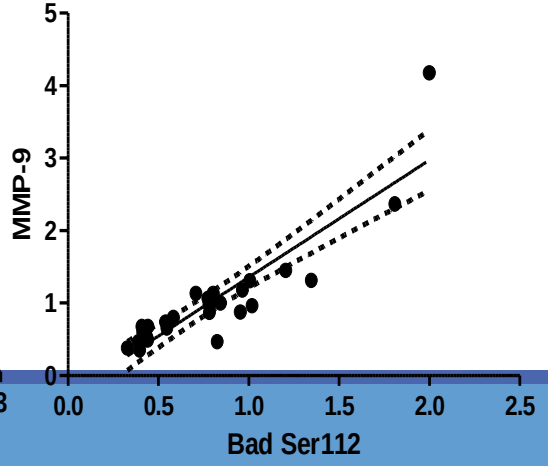
FGF Basic (ρ= 0.858, P<0.0001)



IL-6 Receptor (ρ= 0.763, P<0.0001)

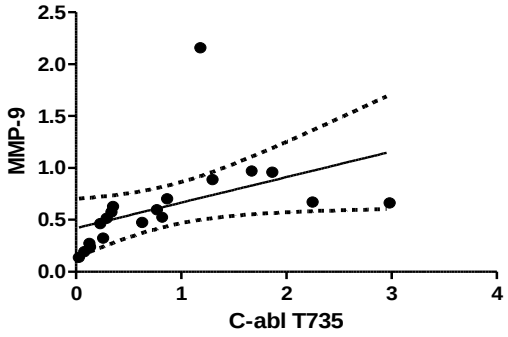


BAD Ser112 (ρ= 0.846, P<0.0001)

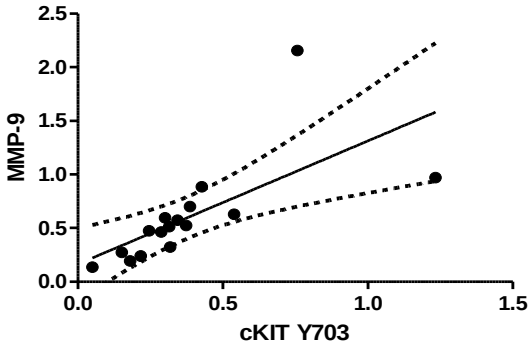


In Patients with VA 20/200 or worse MMP-9 has strong correlations with Pro-Angiogenic Cytokines

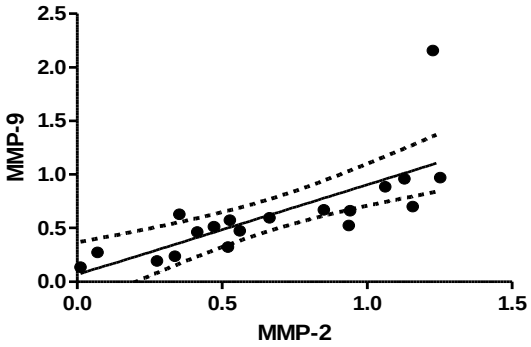
cABL Thr 735 (ρ= 0.893, P<0.0001)



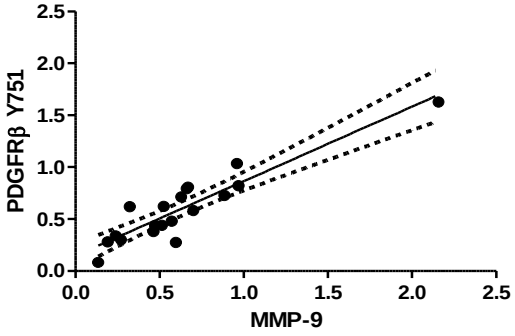
cKIT Y703 (ρ= 0.921, P0.0001)



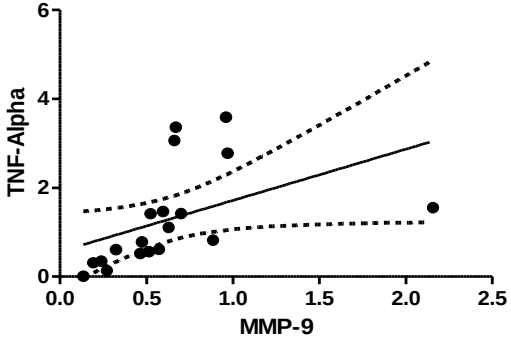
MMP-2 (ρ=0.909, P<0.0001)



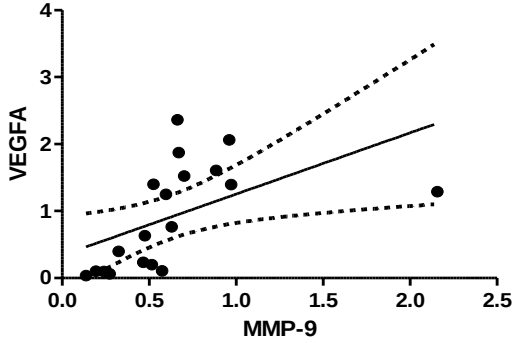
PDGFRβ Y751 (ρ= 0.849, P<0.0001)



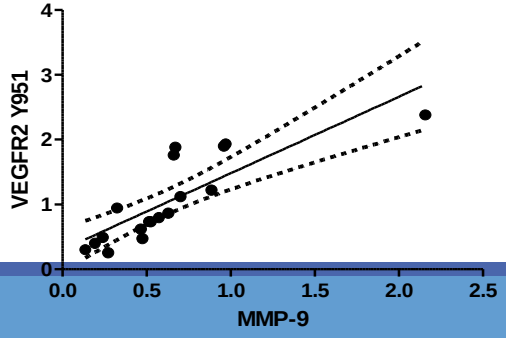
TNF-Alpha (ρ= 0.879, P<0.0001)



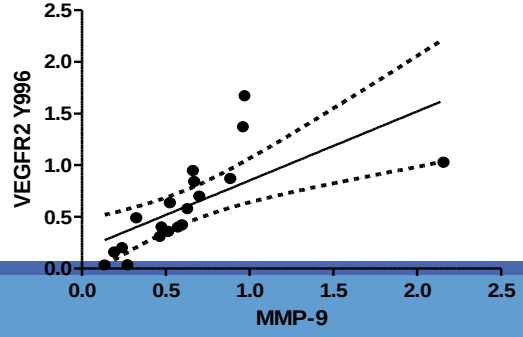
VEGFA (ρ= 0.843, P<0.0001)



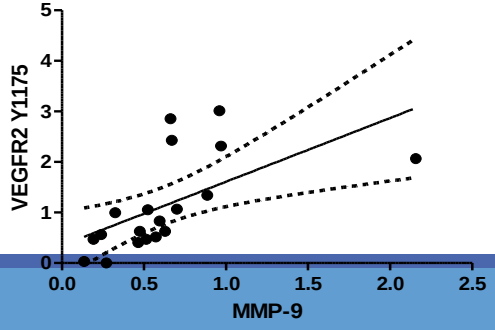
VEGFR2 Y951 (ρ= 0.915, P<0.0001)



VEGFR2 Y996 (ρ= 0.930, P<0.0001)



VEGFR2 Y1175 (ρ= 0.875, P<0.0001)



Future Studies:

NEI supported Multi-Center Trial

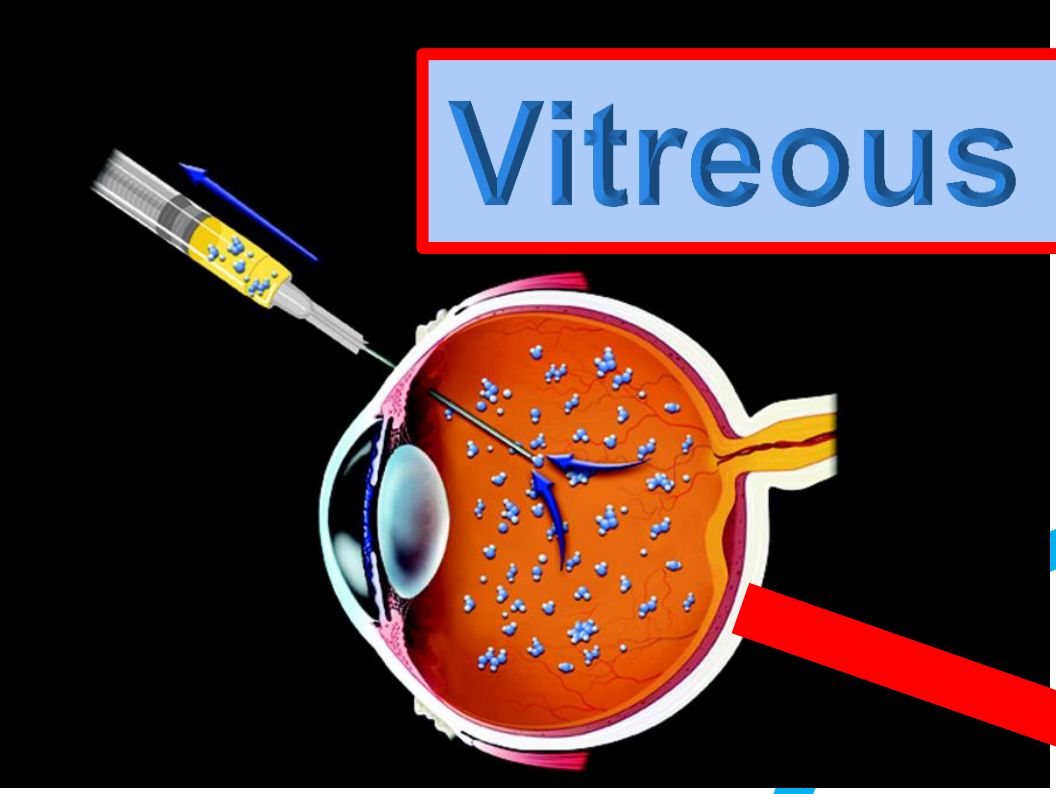
National Retina Institute
PI: Bert Glaser, MD

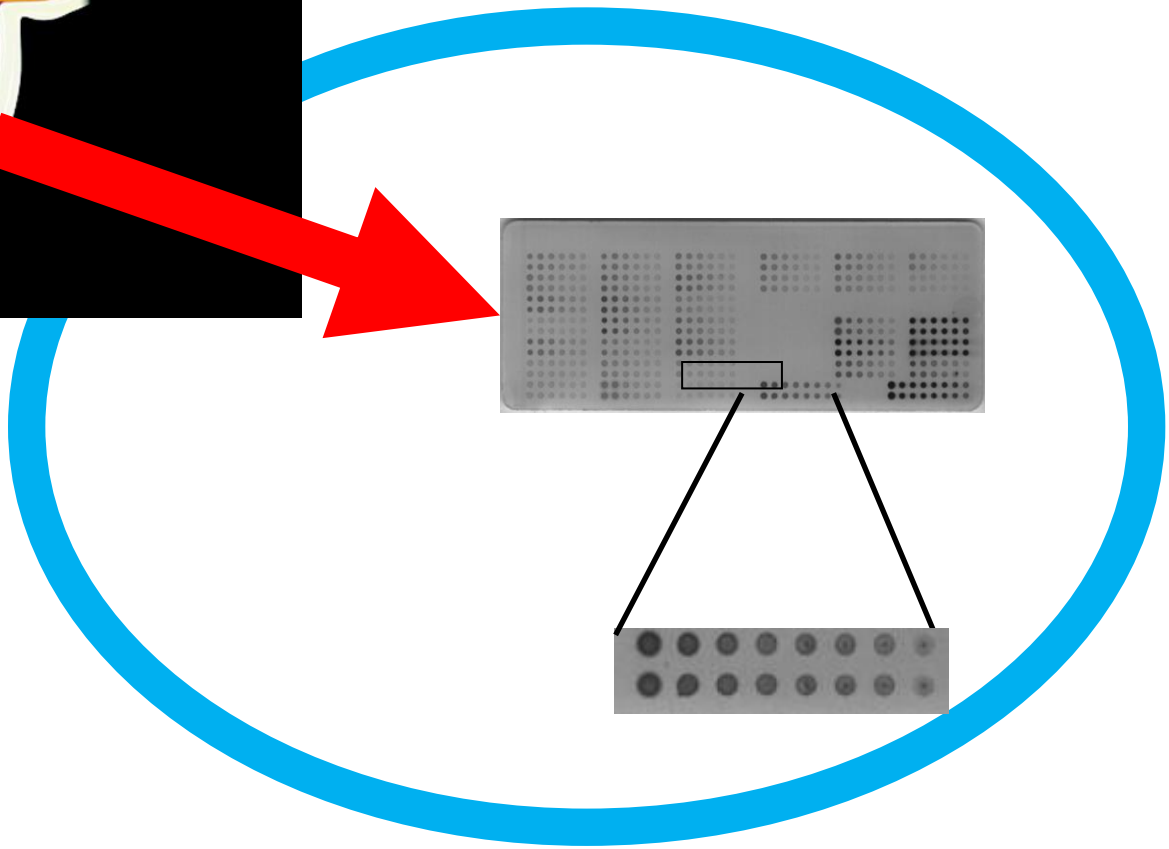
Retina Associates of Cleveland
PI: Larry Singerman, M.D.

Illinois Retina Associates
PI: Kirk Packo, M.D.

National Eye Institute
PI: Frederick Ferris, MD

Vitreous Proteomics



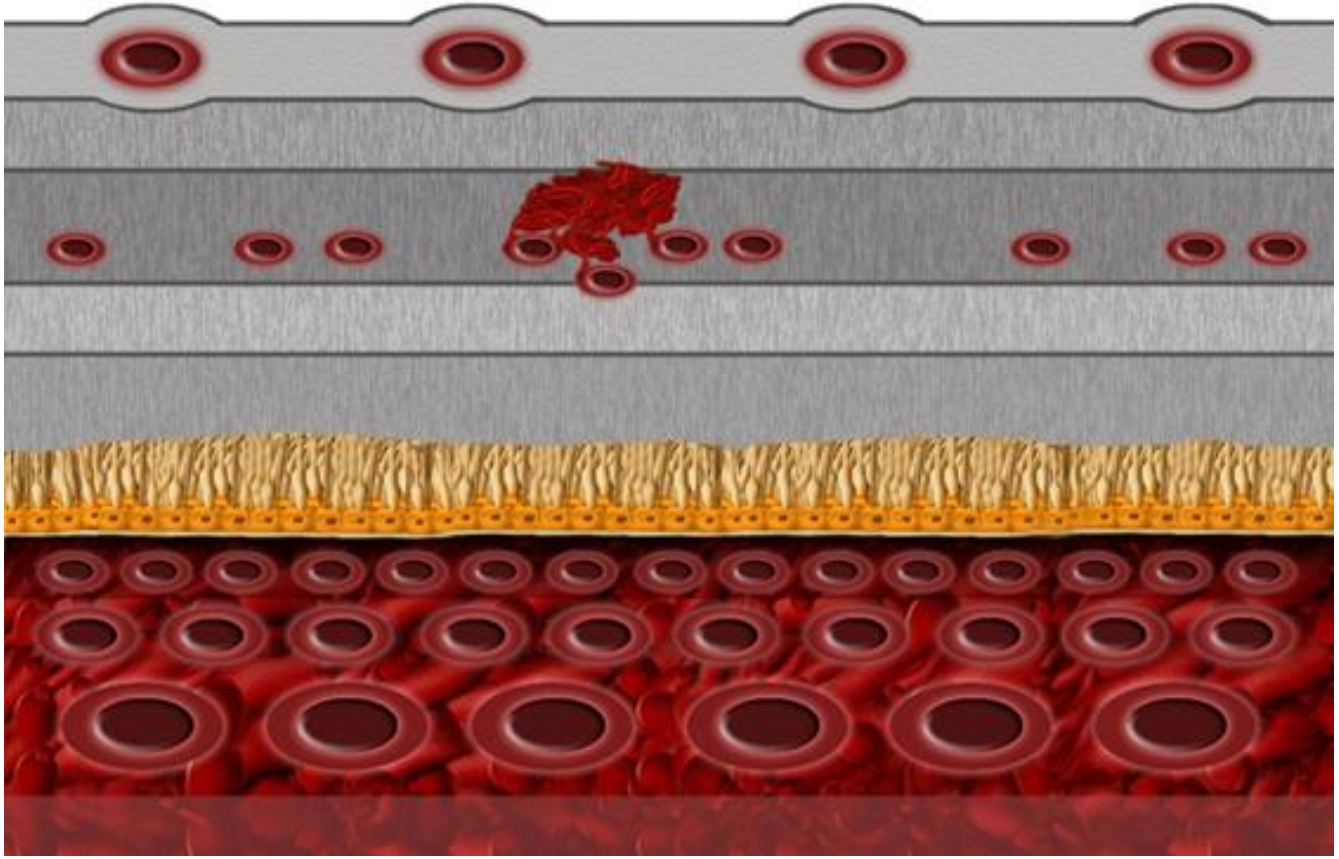


Ocular Proteomics

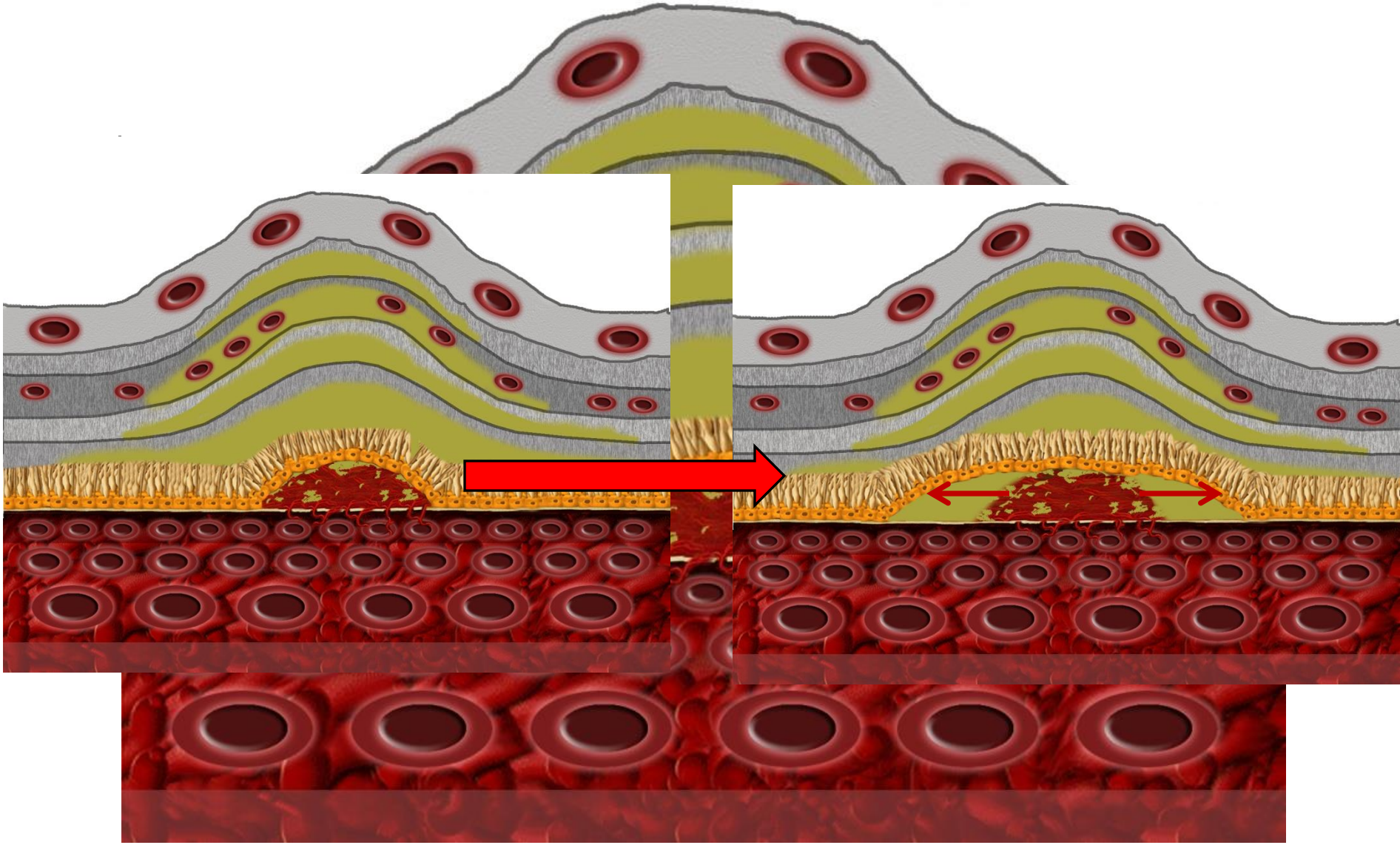
- Aid Management of Disease
- Characterization of Disease
- Staging of Disease

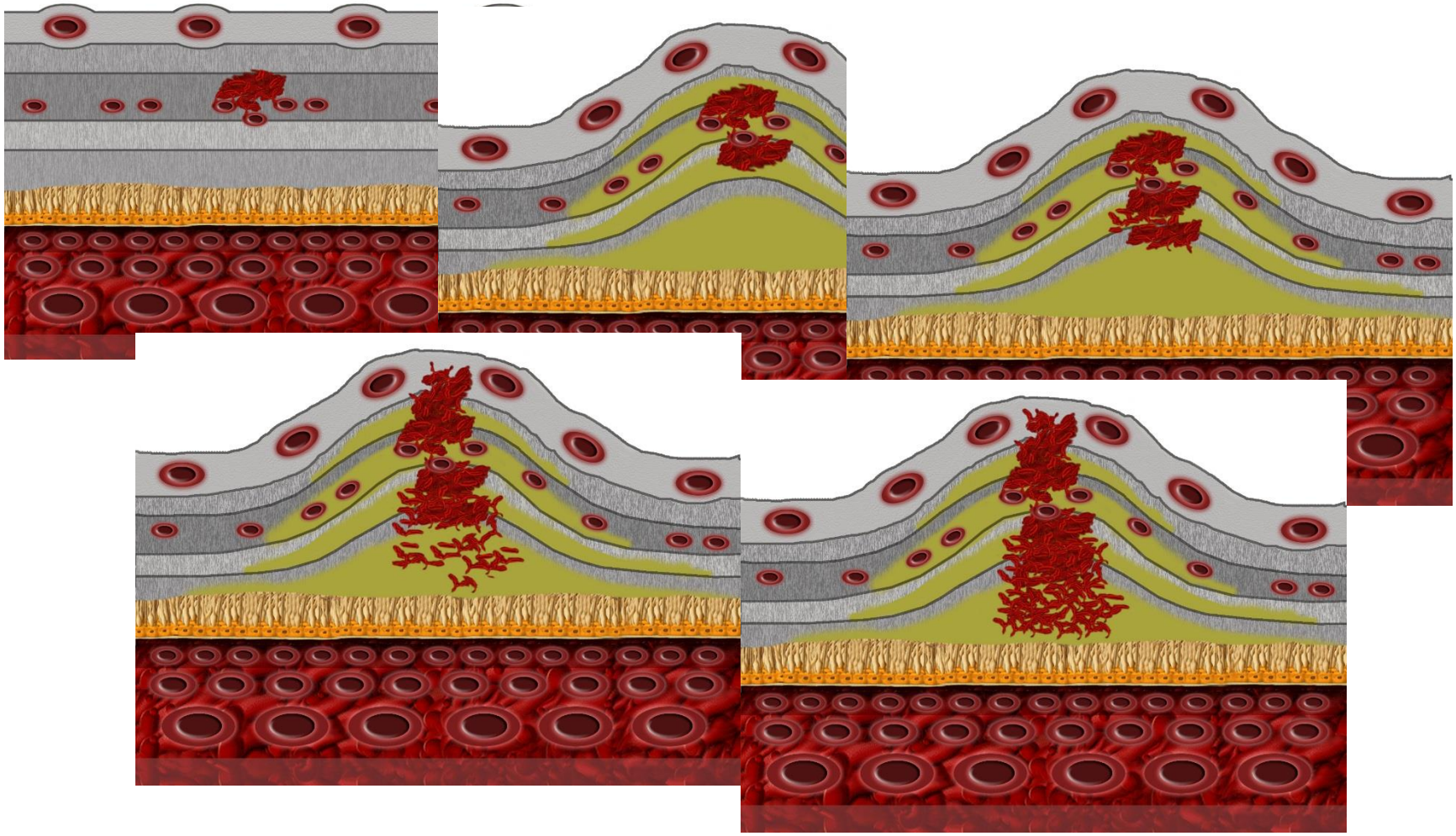
NRI Innovations

- Characterizing AMD
  - RAP vs CNV
- Treat and Extend
  - Who
  - When

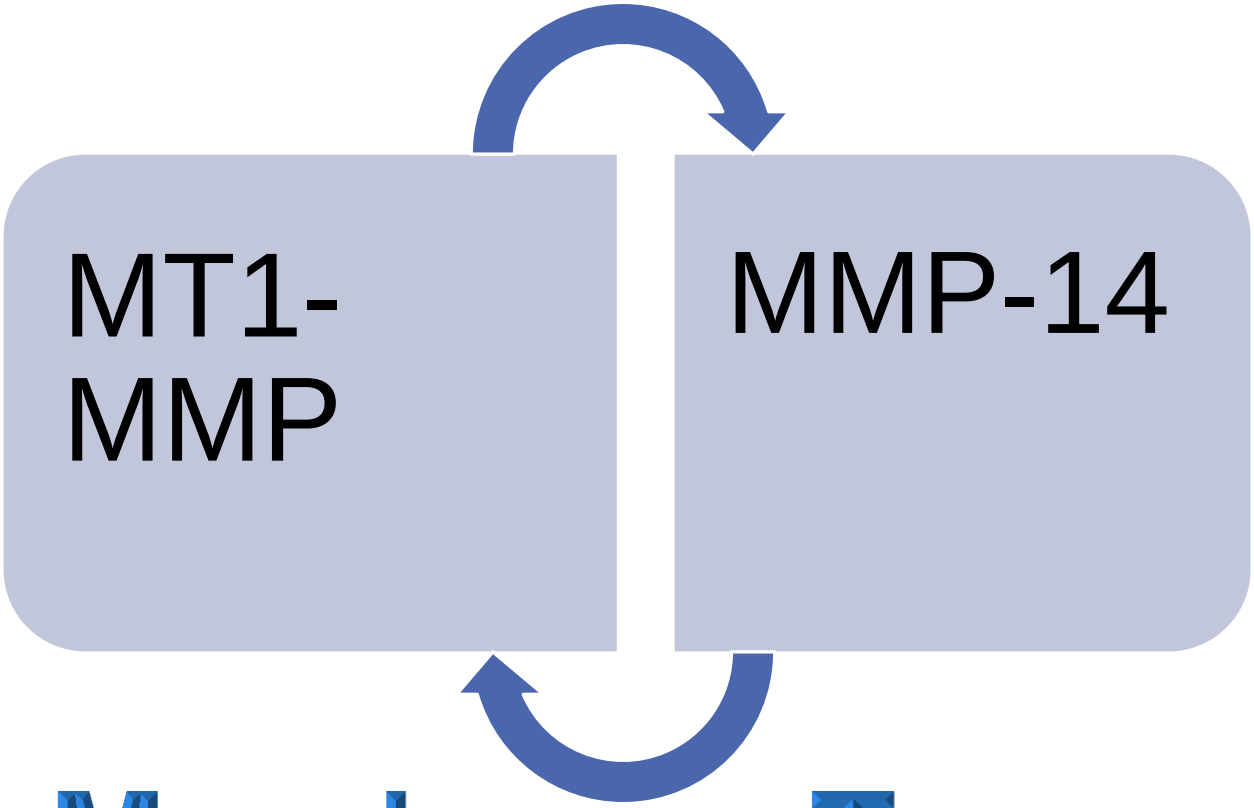


CNV

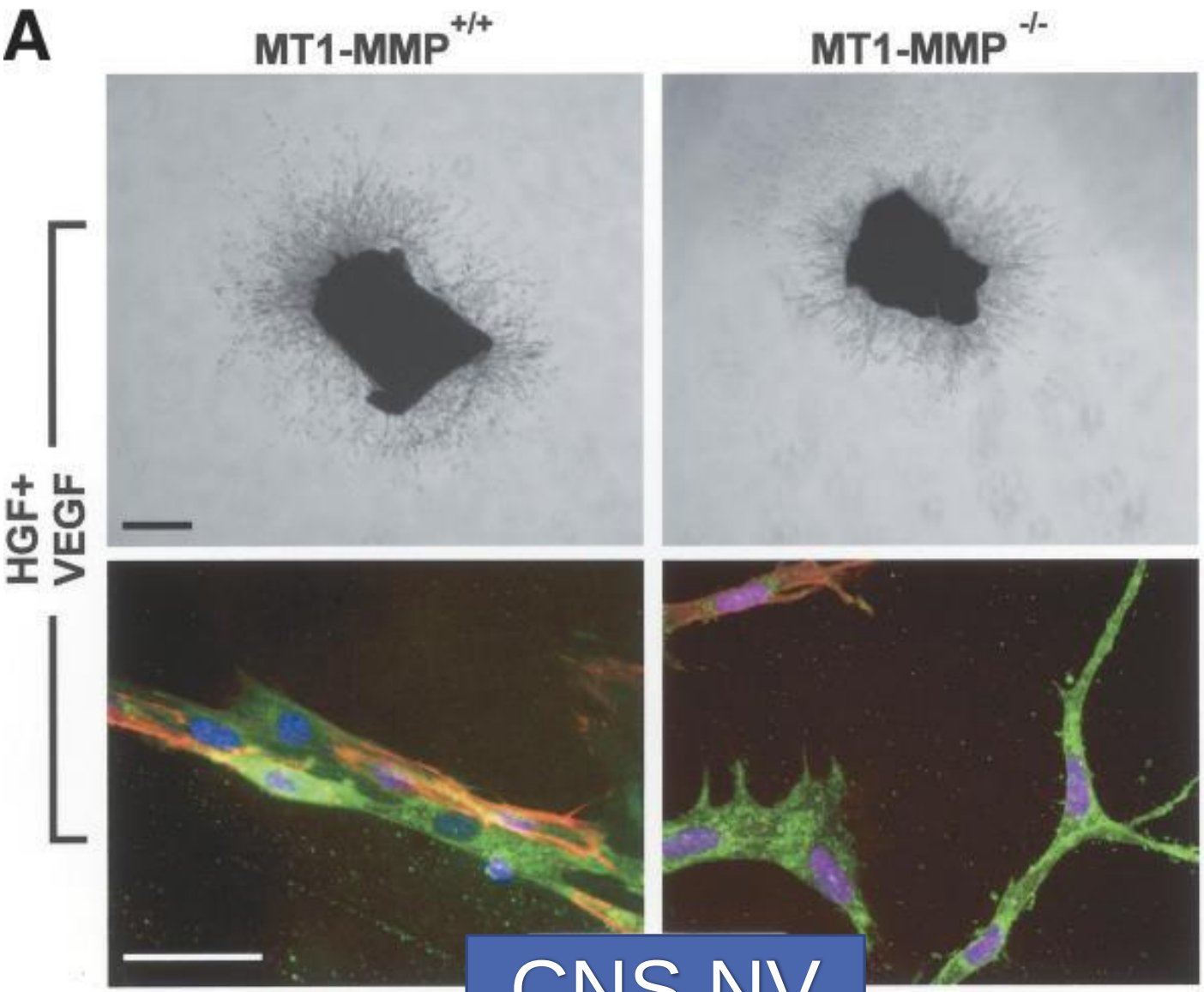




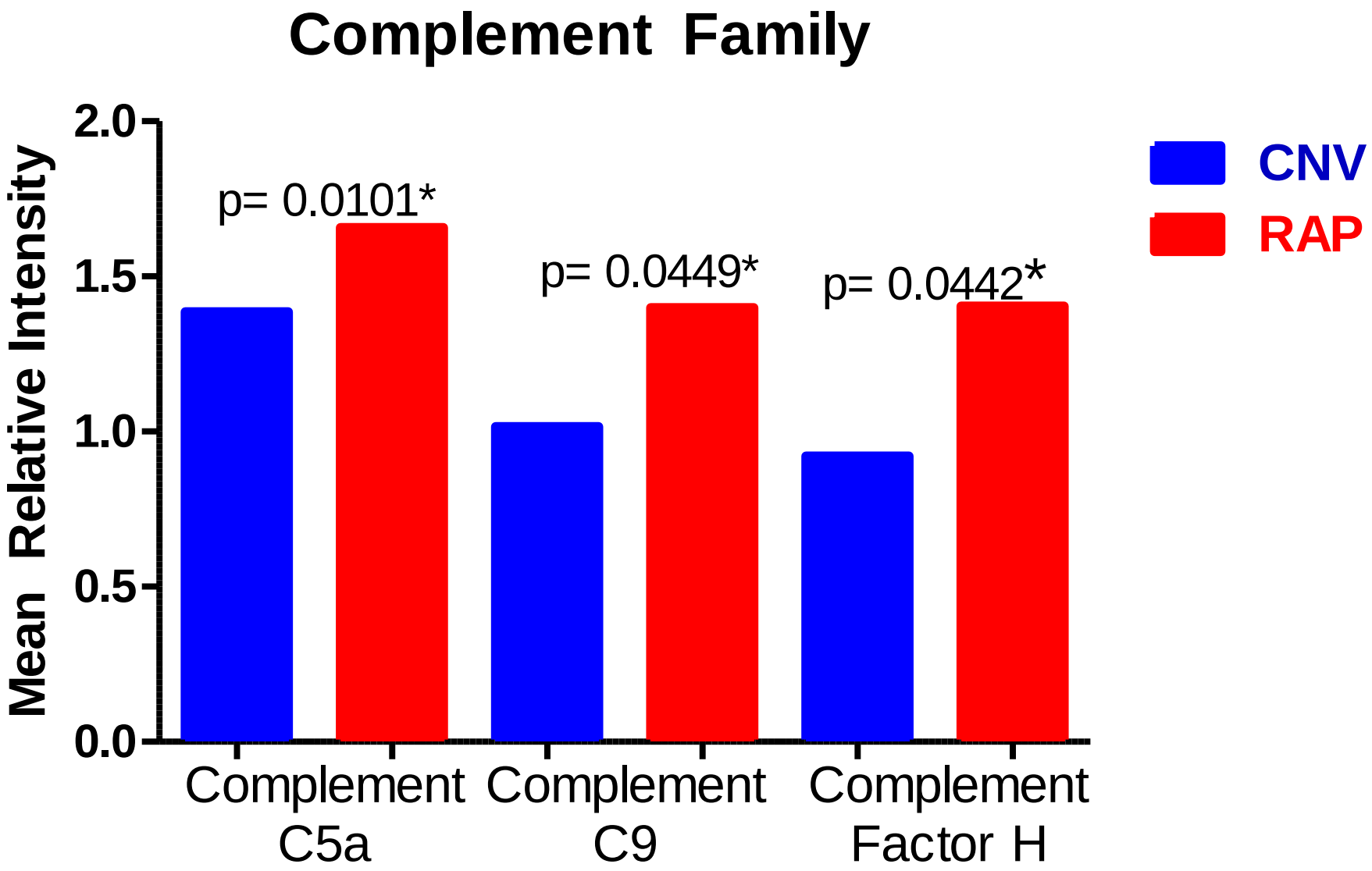
Matrix MetaloProteinase



MT = Membrane Type



CNS NV


Complement Family
Mean Relative Intensity
2.0
1.5
1.0
0.5
0.0
p= 0.0101*
p= 0.0449*
p= 0.0442*
CNV
RAP
Complement C5a
Complement C9
Complement Factor H

Complement Family

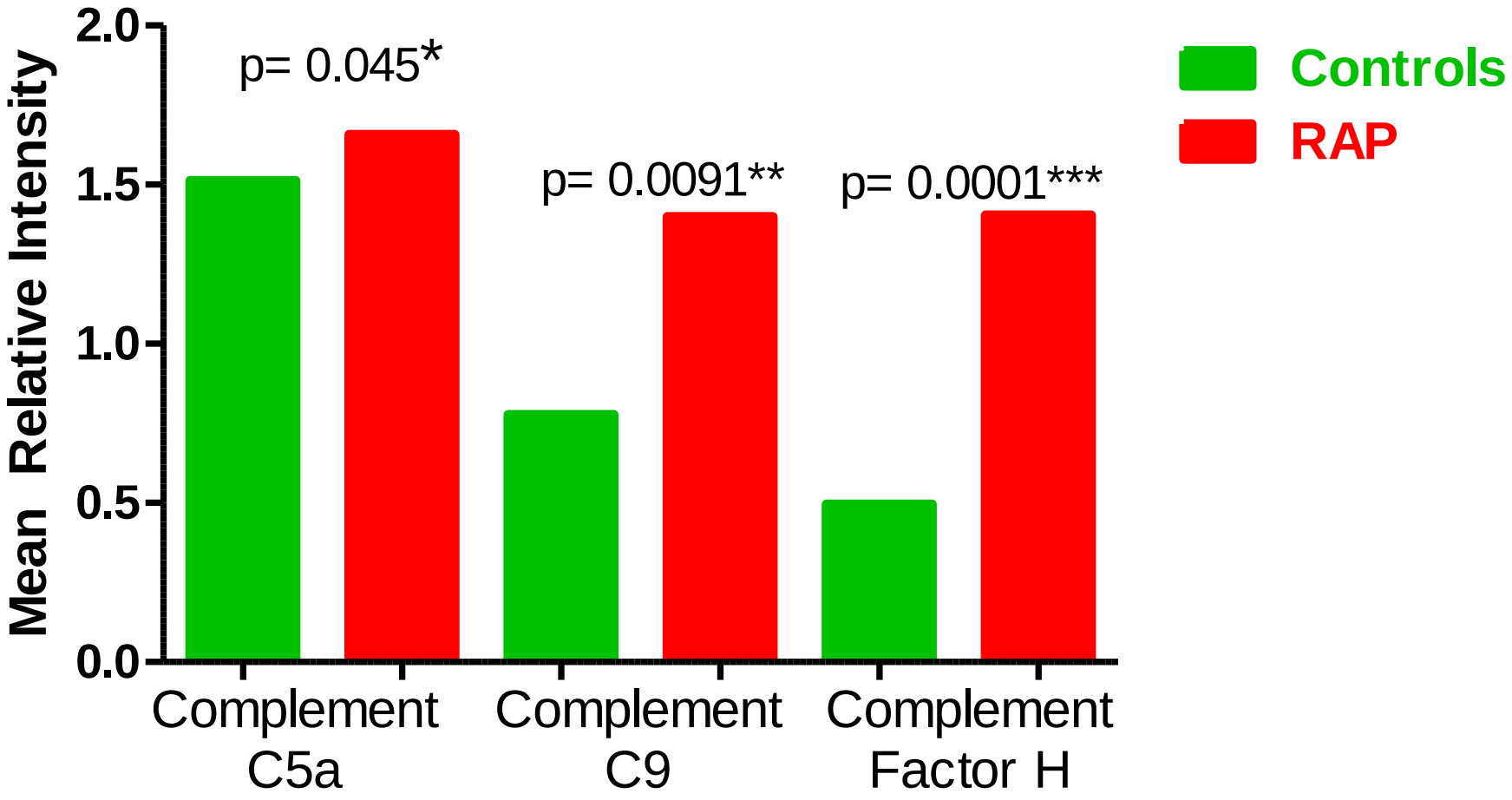


Figure References

1. Mullins, R. F., Dewald, A. D., Streb, L. M., Wang, K., Kuehn, M. H., & Stone, M. (2011). Elevated Membrane Attack Complex in Human Choroid With High Risk Complement Factor H Genotypes. *Experimental eye research*, *93*(4), 565–567. doi:10.1016/j.exer.2011.06.015.

2. Lueck, K., Wasmuth, S., Williams, J., Hughes, T. R., Morgan, B. P., Lommatzsch, a, Greenwood, J., et al. (2011). Sub-lytic C5b-9 induces functional changes in retinal pigment epithelial cells consistent with age-related macular degeneration. *Eye (London, England)*, *25*(8), 1074–82. doi:10.1038/eye.2011.109

3. Bora, N. S., Jha, P., & Bora, P. S. (2008). The role of complement in ocular pathology. *Seminars in Immunopathology*, *30*(2).

4. Liu, F., Qin, A., Zhang, L., & Qin, X. (2011). *The Role of Complement in the Pathogenesis of Artery Aneurysms, Etiology, Pathogenesis and Pathophysiology of Aortic Aneurysms and Aneurysm Rupture*. doi:10.5772/19727

Supporting Work on Complements and MMPs

Mechanistic studies have shown that inflammation, complement activation, extracellular matrix (ECM) turnover, growth factor imbalance, and oxidative stress are fundamental components AMD. (Bandyopadhyay et al IOVS 2012, *53*(4), 1953–61)

reported results link AMD pathogenesis; oxidative stress; complement activation; VEGF/PEDF ratio; and MMP activity. (Bandyopadhyay et al IOVS 2012, *53*(4), 1953–61)

MMP14

degrades various components of the ECM

Implicated in pathological angiogenesis

TIMP-2

- Can Inhibit or Activate MMP-2 when in complex with MMP-14
- Noda et. al. 2003 suggest that MMP2 and MMP14 when activated with TIMP2 may be involved in pathogenesis of PDR

Vitreous Proteome Results

Protein	CNV Mean RI	RAP Lesion Mean RI	P-Value
MMP-14	0.7233	1.042	0.0002
TIMP-2	0.7228	1.216	0.0022
Complement C5a	1.387	1.657	0.0101
Complement C9	1.016	1.400	0.0449
CF-H	0.912	1.405	0.0442

Complement Pathway

C5a is formed during the activation of the complement cascade-causes chemotactic and PROINFLAMMATORY effects

Activation of Complement system is a major aspect of chronic INFLAMMATORY Diseases

C9 is integral to the formation of the Membrane attack complex (MAC) which is the result of complement activation.

“Interestingly, MAC deposition has been shown to be the highest in the macula, and MAC staining intensity appears to be correlated with AMD severity and the loss of RPE cells.” (Bandyopadhyay et al IOVS 2012, *53*(4), 1953–61)

Disclosure:
Ocular Proteomics Lab - BMG Pres.

- Mission: Delineate ***novel biomarkers*** of ocular disease.

- Products: None

Many Promising Approaches

Genetics; Biochemistry; Electrophysiology; Blood Flow; Auto fluorescence ……