

# **Purified Tissue Dissociation Enzymes: Past, Present, Future**

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# Brief chronology collagenase: biochemistry & applications

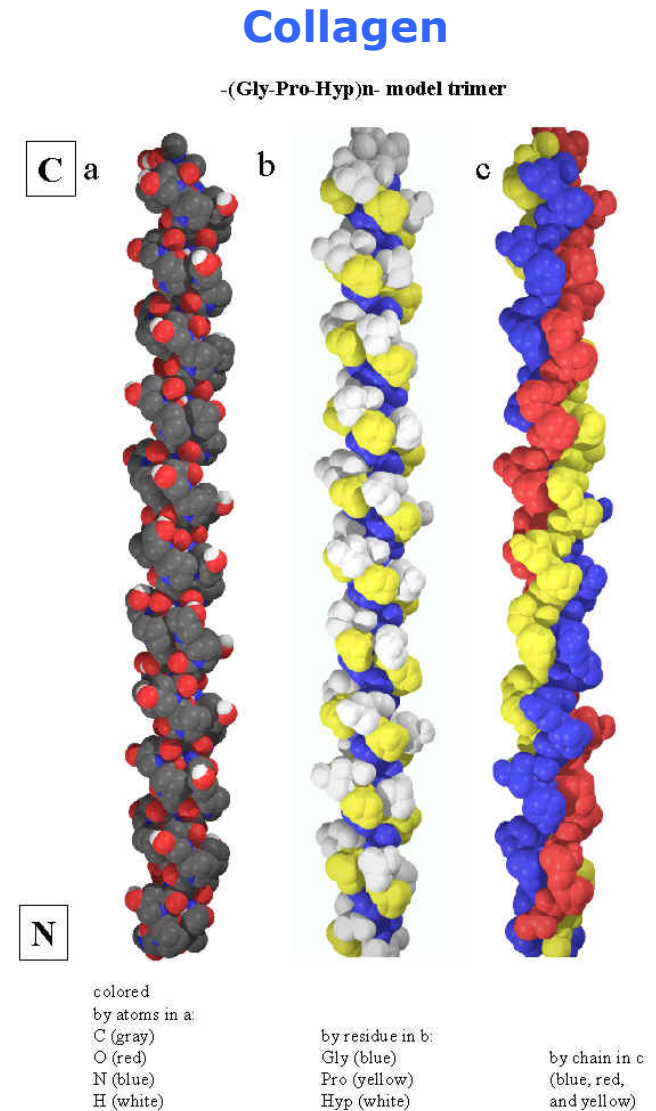
- ⊕ *C. histolyticum* primary organism used for generating collagenase.
- ⊕ Collagenase used initially in wound debridement for burn victims, led to early efforts to purify these enzymes
- ⊕ Worthington Biochemicals rapidly became primary source for collagenase
- ⊕ Applications of collagenase extend into tissue dissociation of mammalian cells & tissue for academic research
- ⊕ Ricordi semi-automated islet isolation method developed and rapidly adopted to isolate islets from higher mammals
- ⊕ Boehringer Mannheim encouraged by Ricordi to solve “collagenase problem”
- ⊕ Success of Edmonton led to expansion of islet transplantation programs
  - ITN trials
  - Islet resource centers

## Key Dates: *C. histolyticum* collagenase

- 1953 1<sup>st</sup> report for isolation & characterization of enzyme
- 1961 Worthington Biochemicals offers enzyme
- 1965 isolation of guinea pig islets
- 1967 isolation of rodent hepatocytes
- 1974 1<sup>st</sup> clinical islet allotransplant at Minnesota
- 1981, intraductal delivery of enzyme to increase islet yield
- 1988 Ricordi method
- 1994 Launch of Liberase HI
- 2000 Edmonton Protocol

# Biochemical characterization of the collagenase enzymes from *C. histolyticum*

- ⊕ Collagenase unique, only enzyme that cleaves native collagen triple helical structure, resistant to other proteases
- ⊕ Extensive research by Van Wart et al, showed isolation of seven electrophoretic variants, 68 - 130 kD
- ⊕ Two isoforms, class I (CI) and class II (CII), differ in enzyme specificity
  - CII has > 10x specific activity for synthetic peptides than CI
- ⊕ Studies with rec collagenase confirmed substrate specificity of isoforms
  - Single copy gene, intact mRNA molecule
  - CI & CII mw 116 kd
  - Domain structure: catalytic and binding domains at opposite ends of protein, intact molecule required for native collagen degradation
  - CI has 2 collagen binding domains, CII has one domain



# Commercial collagenase products

- ⊕ Most commonly used product is crude collagenase: steps in preparation
  - *C histolyticum* cultured anaerobically at 30 or 37° C in media containing or supplemented with collagen
  - Recover supernatants after centrifugation
  - Often concentrate supernatant then precipitate with ammonium sulfate
  - Resuspend pellet, lyophilize
- ⊕ Disadvantages of current product
  - Contaminated with pigment, other proteases, and endotoxin
  - Lot composition dependent on culture conditions, every lot unique
- ⊕ Lot qualification programs
  - Led suppliers to develop lot qualification/selection programs where customer tests sample prior to purchase

## Purified Collagenase

- Van Wart commented that the paucity of effort to study enzymes “has been the extreme difficulty of purifying individual *C. histolyticum* collagenase to homogeneity”.
- Purified collagenase alone inefficient in tissue dissociation as noted in a number of reports analyzing cells from different tissues
- Boehringer Mannheim was first company to develop purified enzyme product tailored to isolate a specific cell type

# Boehringer Mannheim & islet community: Genesis of Liberase™ Purified Enzyme Blend Products



Activity	Collagenase	
	BM Type P	Sigma Type XI
Wunsch	1.5-3.5	0.5-1.4
Protease	Variable Low	Variable High

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TRANSPLANTATION  
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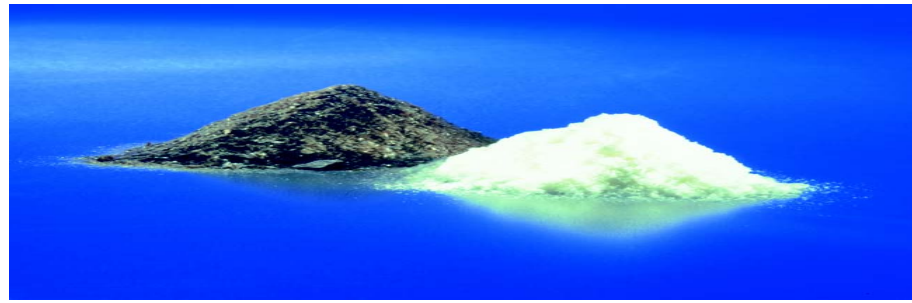
Vol. 53, 407-414, No. 2, February 1992  
Printed in U.S.A.

## HUMAN ISLET ISOLATION AND ALLOTRANSPLANTATION IN 22 CONSECUTIVE CASES<sup>1,2</sup>

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ANTHONY J. DEMETRIS, DANIEL H. MINTZ, AND THOMAS E. STARZL

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## Compare the old with the new



	<b>Crude collagenase</b>	<b>Liberase enzymes</b>
Enzyme activities	>15	3
Endotoxin units / mg	300-7000	<50
Lot-to-lot precision	Low: Affected by conditions of fermentation	High: Formulation of purified enzymes
How to get best results	Screen different lots	Design optimal formulation: DOE
Cell transplantation impact	Dependent on availability of "magic lot": reproducibility?	Produce reproducible reagents, leads to systematic improvement

# Retrospective assessment of Liberase products, 12 years after introduction

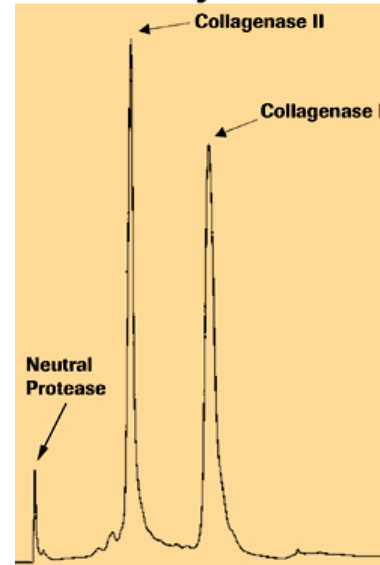
## Plus/Benefits

- ⊕ Purification led to identification of key enzymes responsible for tissue dissociation & problems associated with endotoxin contamination
- ⊕ Increased reproducibility of manufacture of product
- ⊕ Readily modify enzyme composition to address customer needs

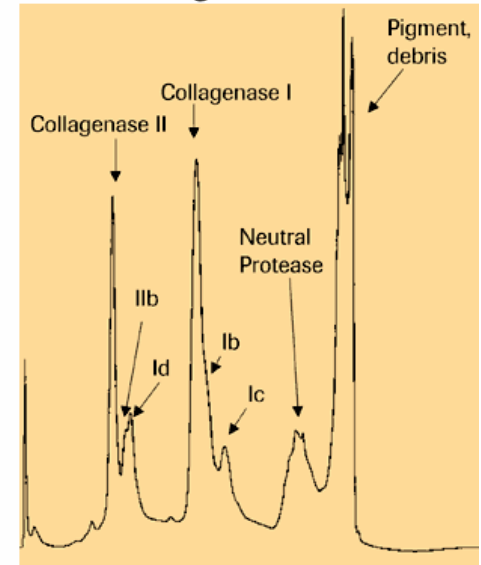
## Minus/Needs Improvement

- ⊕ QC assays suboptimal:
  - Peptide substrate CII >> CI
  - Peptide substrates measure intact and degraded enzyme
  - No assay for CI activity
- ⊕ Assays used to assess enzymes must reflect use of reagent in final application, tissue dissociation

Liberase Enzyme HI



Crude Collagenase



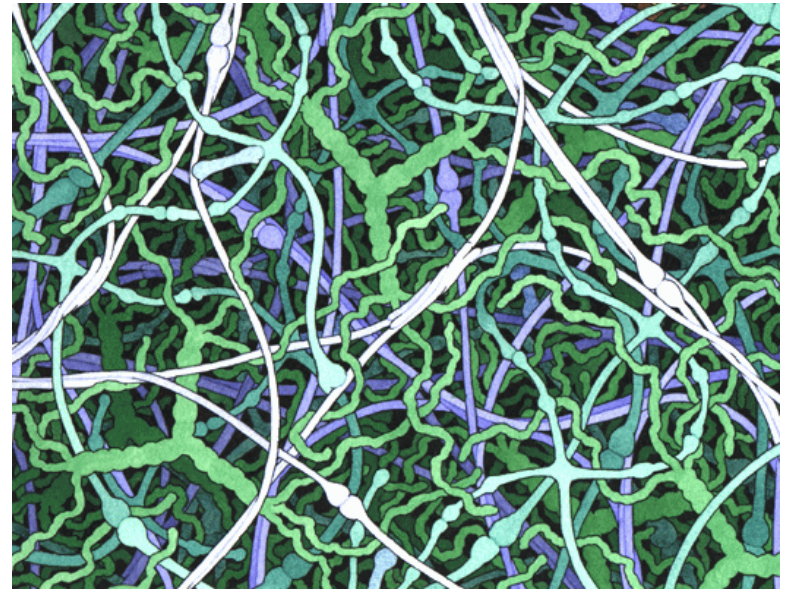
*Low yields and poor reproducibility are the consequences of our lack of understanding the actual principles of the enzymatic dissociation process.*

*Van Schilfgaarde, et al 1994*



# VitaCyte will take a systematic approach to develop basic knowledge of the tissue dissociation process

- ⊕ Many factors influence islet quality and yield, among them are:
  - Donor characteristics
  - Tissue dissociation enzymes (TDEs)
  - Endogenous protease
  - Histochemical composition
  - Experience of isolation team
- ⊕ VitaCyte's goal is to systematically analyze factors that affect islet yield
- ⊕ Focus on collagenase, critical for successful cell isolation: only enzyme that initiate collagen degradation
- ⊕ Focus is to improve enzymes by using superior analytical assays
  - Does assay result correlate with enzyme function?
  - What is correlation of assay result to islet yield?



## **Schematic basement membrane**

Type IV collagen in light blue  
Laminin: blue green  
Proteoglycans: green

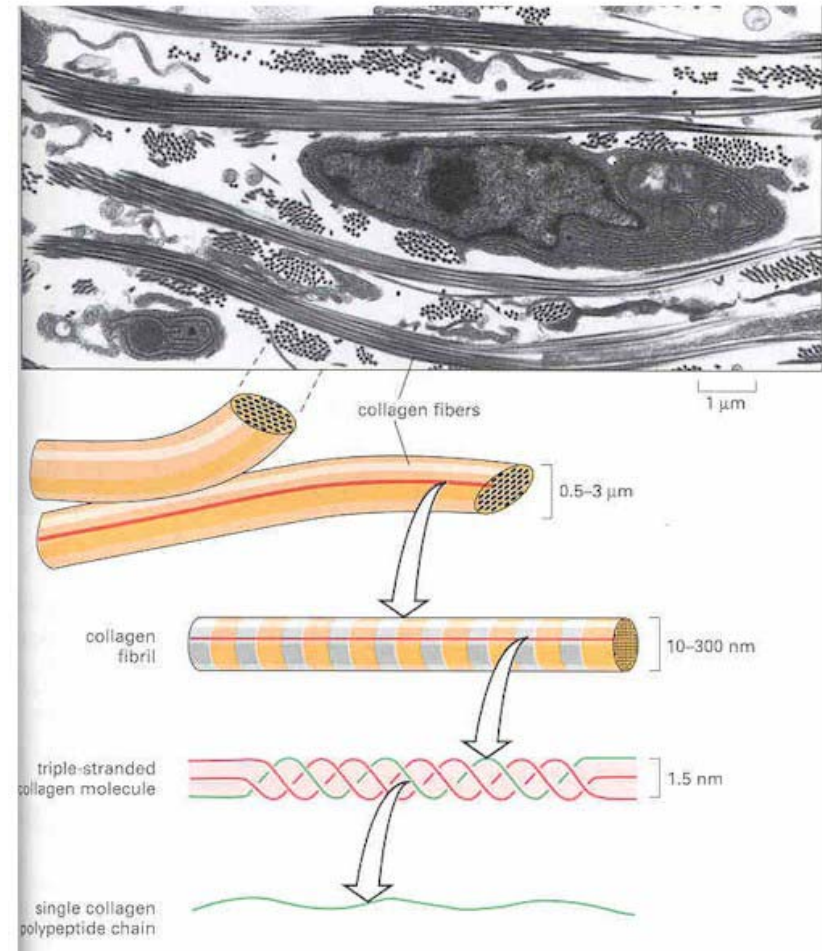


# Comparison of biochemical assays for collagenase

<b>Assay</b> CDA = collagen degradation assay	<b>Investigator</b>	<b>Substrate</b>	<b>Bias</b> CI = Class I CII = Class II	<b>Molecular Species Detected</b>	<b>Turn Around Time Hours</b>
<b>Peptide Substrate</b>	<b>Wunsch Van Wart</b>	<b>PZ peptide FALGPA</b>	<b>CII &gt; CI</b>	<b>Intact Degraded</b>	<b>0.5 to 2.0</b>
<b>CDA</b>	<b>Mandl Peterkofsky</b>	<b>Insoluble fibers</b>	<b>CI <math>\approx</math> CII</b>	<b>Intact</b>	<b>2-7</b>
<b>CDA</b> (Van Wart)	<b>Van Wart</b>	<b>Tritiated soluble fibrils</b>	<b>CI <math>\approx</math> CII</b>	<b>Intact Degraded</b>	<b>2</b>
<b>CDA using azo dye- fibers</b>	<b>Kosma- Penkova</b>	<b>Active- orange-GT insoluble fibers</b>	<b>CI &gt; CII</b>	<b>?</b>	<b>1</b>
<b>CDA using FITC coupled substrate</b>	<b>VitaCyte</b>	<b>FITC- soluble fibrils</b> $A_{494}$ or $E_{530}$	<b>CI &gt; CII</b>	<b>Intact</b>	<b>1-2</b>
<b>CDA using FITC coupled substrate</b>	<b>VitaCyte</b>	<b>FITC- insoluble fibers <math>A_{494}</math></b>	<b>CI &gt; CII</b>	<b>Intact</b>	<b>2</b>

# CDA is the probably the best surrogate assay

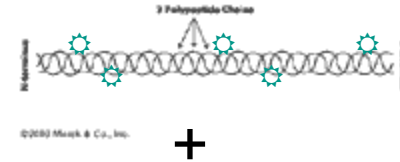
- ⊕ **Collagen is most predominant protein in the body**
  - Functions as the major structural protein
  - Vast, resilient sheets support internal organs
- ⊕ **Triple helical structure is resistant to degradation by nearly all proteases**
- ⊕ **Collagenases are exception, bacterial collagenase (e.g., *C. histolyticum*) has broadest specificity**
- ⊕ **Specificity dependent on conformation and form of protein, temperature critical**
  - Heat denatured collagen (gelatin) is degraded by many different protease
  - Temperature lability monomers>fibrils>fibers



# FITC-collagen fibrils found to be effective substrate for spectrophotometric & fluorometric CDAs

## ⊕ Advantages of FITC

- High molar extinction coefficient
- Commonly used fluorescent dye
- Readily couples to protein



## ⊕ Conjugated to soluble collagen fibrils and insoluble fibers

- Fibrils provide superior assay, easier to manipulate, improved precision
- Challenge is to conjugate FITC to collagen without compromising conformation or solubility of molecule

## ⊕ Developed FITC fibril assay in two different formats

- Spectrophotometric assay (SP)
- Fluorometric microplate assay (FL)

## ⊕ Both assays specific for collagenase:

- SP assay: specific activity rec trypsin 0.29% of CI & 1.6% of CII
- FL assay: specific activity rec trypsin 0.002% of CI & 0.004% of CII

Collagenase

100 mM Tris 10mM CaCl<sub>2</sub>  
pH 7.5 , 35°C

**SP** **FL**

60 min

90 min read

@ 5 min  
intervals

Precipitate  
Collagen

Ex 485/20 nm  
Em 528/20 nm

Centrifuge

Read A494  
supernatant  
at pH 8.0

# Relevance of assay is to determine what is best functional assay to assess quality of purified TDEs

⊕ Publication last year by Lakey's lab and his presentation at last year's ICR meeting highlights problem of assessing quality of purified TDEs

⊕ Striking observation from these data were

- Temporal change CI peak as detection by analytical anion exchange chromatography
- Correlated change with loss of CI enzyme activity
- Correlated change with suboptimal human islet yields

## Quantitative Assessment of Collagenase Blends for Human Islet Isolation

Matthew J. Barnett,<sup>1</sup> Xiaojun (Wendy) Zhai,<sup>1</sup> Donald F. LeGatt,<sup>2</sup> Siew Bang Cheng,<sup>2</sup> A. M. James Shapiro,<sup>3</sup> and Jonathan R. T. Lakey<sup>1,4</sup>

**Background.** The variability in collagenase blends has been speculated as the single most important determinant of the success or failure in isolated islet yields in clinical islet transplantation. Examination of the formulation and potency of the widely used Liberase HI enzyme blend will uncover possible sources of imprecision.

**Methods.** High performance liquid chromatography (HPLC) and kinetic measurements of collagenase and protease activity were used to assess potency. Between four and nine clinical lots were assessed for various parameters such as relative formulation of collagenase isoforms, and recovered collagenase and protease potencies postreconstitution.

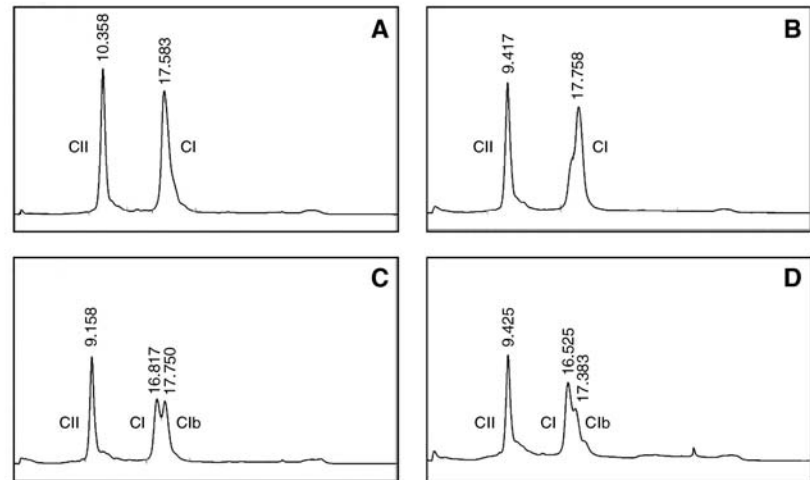
**Results.** Six vials from a single typical lot had a mean enzyme content of  $489 \pm 62.5$  mg (mean  $\pm$  SEM; range 398–610 mg). The mean recovered collagenase activity was  $2235 \pm 310$  Wünsch units (WU)/vial (range 1794–2968 WU/vial). The percent coefficients of variation for collagenase and protease activity in these vials were 17.4%, and 13.4%, respectively. The increase in the presence of the collagenase Ib (CIb) isoform detected by HPLC analysis was related to the chronological order of the date of manufacture. The CIb isoform was found to have a reduced specific activity compared to intact collagenase I (CI) ( $3.8 \pm 1.2$  WU/mg vs.  $2.1 \pm 0.7$  WU/mg,  $P < 0.05$ ). The presence of CIb was related to reduced islet yields in twelve human isolations studied.

**Conclusions.** Variation in potency was observed between, and within lots of Liberase HI in this study. Differences in relative collagenase isoform composition may also affect the stability and potency characteristics of these blends.

**Keywords:** Islet isolation, Collagenase blends, Enzyme analytics, Thermolysin, Islet transplantation.

(*Transplantation* 2005;80: 723–728)

### Formulation Analysis by HPLC



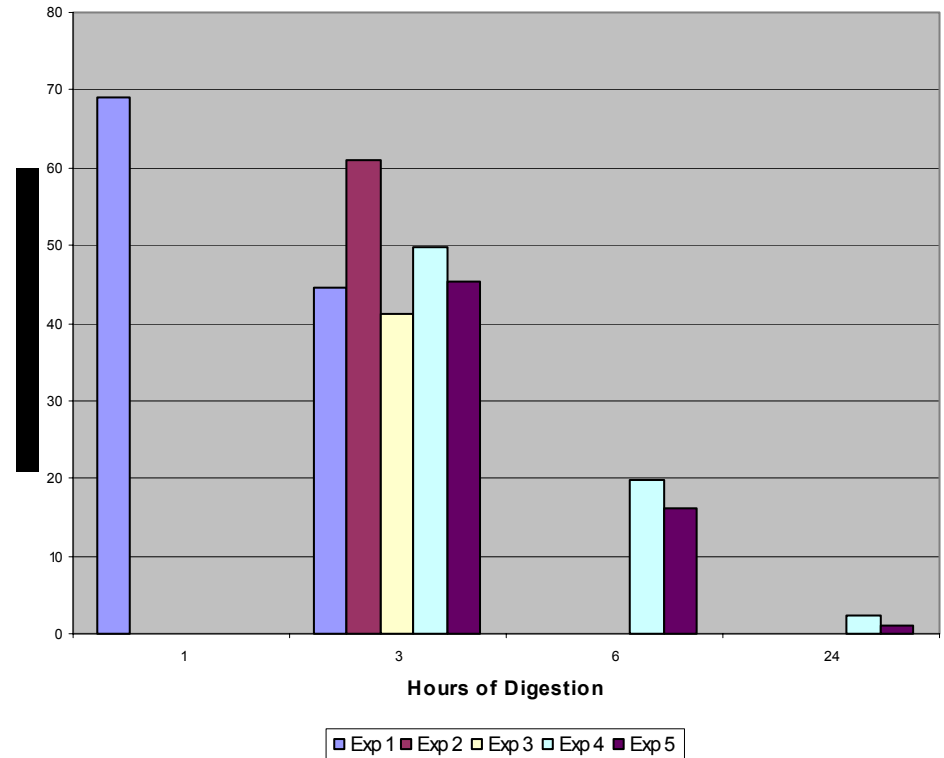
# Slide from Lakey's presentation at last years ICR meeting

## Comparative Isolation Outcomes based on CI Activity (Where successful >300,000 IE Yield)

Unsuccessful N=5			Successful N=5	
	CI Activity CDU/ug	IE Yield	CI Activity CDU/ug	IE Yield
Mean	1.64 <sup>†</sup>	177,764 IE	2.98 <sup>†</sup>	488,075 IE
SEM	+/-0.2	+/-73,687 IE	+/-1.1	+/-122,769 IE
t-test	$p = 0.0447^{\dagger}$ one-tailed, paired: $p < 0.05$			

# Performed several experiments to assess effect of protease on CI activity using SP CDA

- ⊕ Tested several proteases for their ability to degrade collagenase
- ⊕ Found chymotrypsin superior to other proteases for degrading CI as measured by decrease in specific activity using SP CDA
- ⊕ Results show that CI loses collagen degrading activity when digest 1 mg/mL of CI with 2% (w/w) chymotrypsin at 37° C
- ⊕ Degradation by proteases more complex in tissue dissociation mixture
- ⊕ These data indicate that CDAs may be an effective surrogate assay to assess quality of TDE products



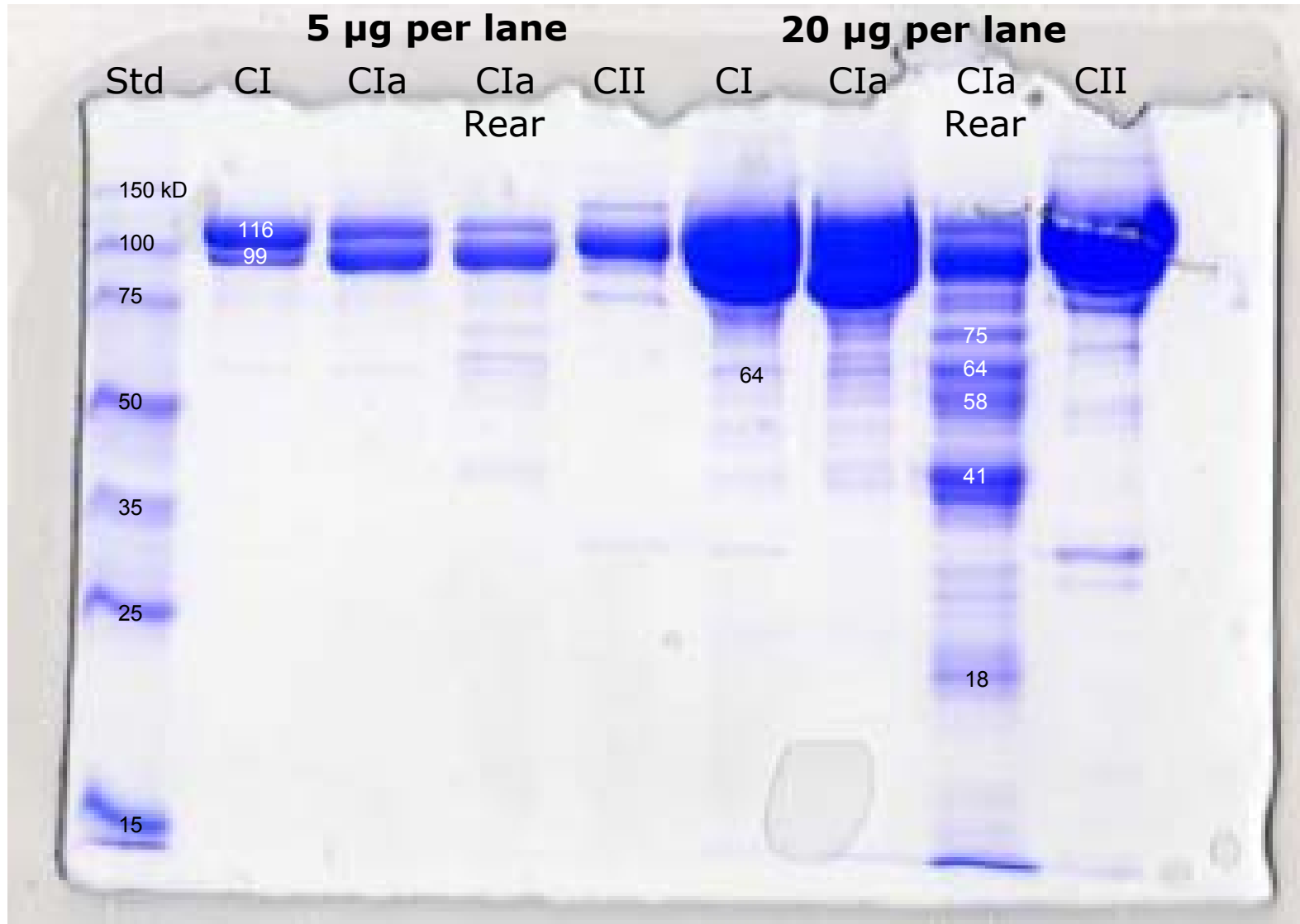
# **FL CDA appears to be more sensitive than SP CDA assay in detecting degraded collagenase**

- ⊕ **Improper thawing of one portion of raw material created greater amount of degraded CI**
- ⊕ **Divided CI peak into 3 pools: CI, CIa, CIa rear and analyzed samples by SDS PAGE and SP or FL CDA**
- ⊕ **Results show that FL CDA more sensitive than SP CDA in detecting variant collagenase forms**
- ⊕ **Result confirmed by analysis of these materials by SDS-PAGE with increase in 99 kD band in CI rear sample (next slide)**
  - **116 kD band is intact CI**
  - **99 kD band in CI reflects loss of the C terminal collagen binding domain**

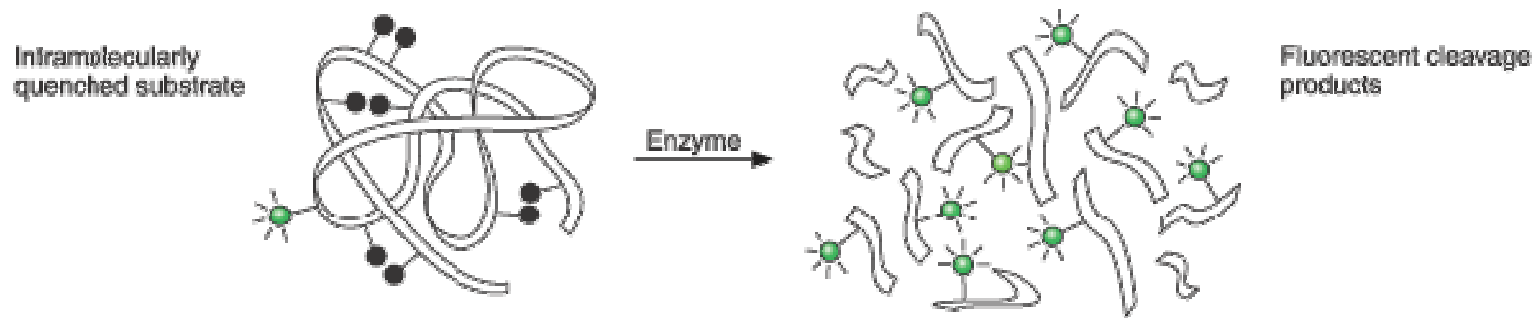
	SP CDA		FL CDA	
	Specific Activity	%	Specific Activity	%
CI	1937	100	42483	100
CIa	1883	97.2	21947	51.7
CIa rear	1483	76.6	11783	27.7
CII	160	8.3	2352	5.5



# 7.5% SDS-PAGE analysis of CI, CIa, CIa rear, CII



# Potential explanation for different results explained by FITC quenching



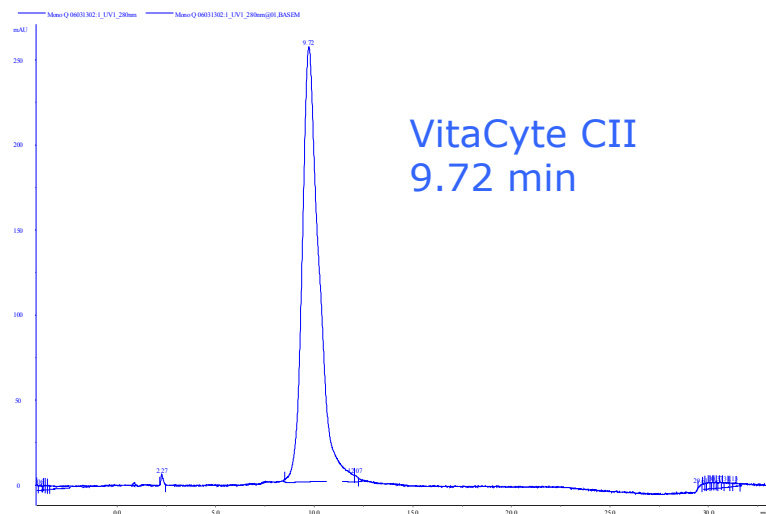
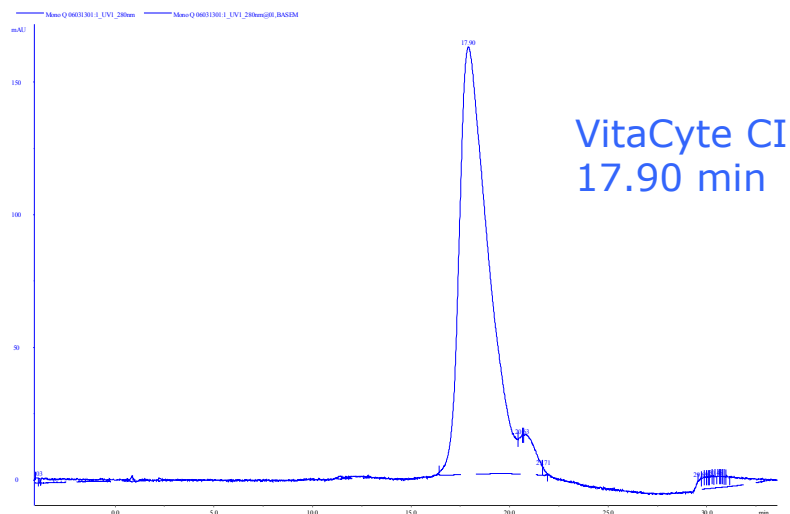
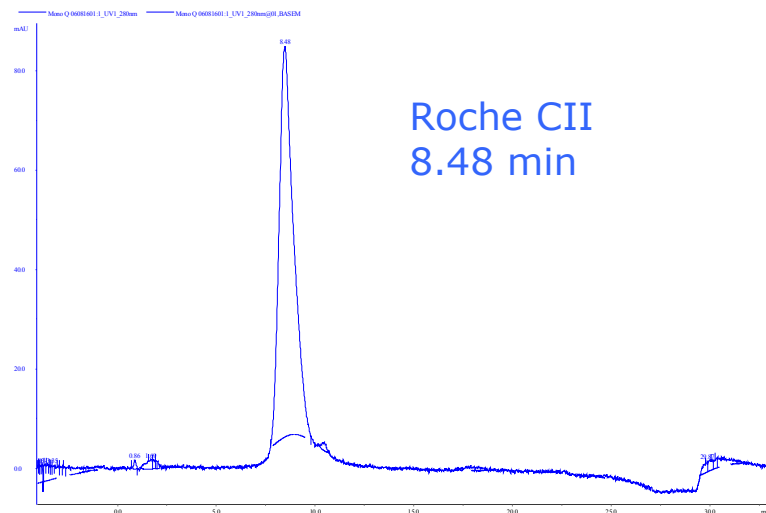
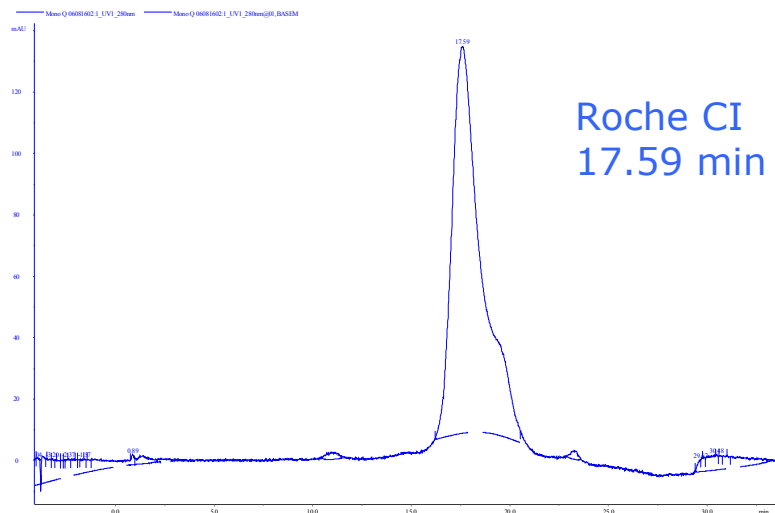
- ⊕ **Close proximity of FITC in the folded protein structure leads to quenching: emitted fluorescence is absorbed by neighboring FITC**
- ⊕ **Once the protein is degraded, the FITCs separate, leading to increase in fluorescence over time**
- ⊕ **In SP CDA, must use precipitating agent and all that is measured is light absorbed in supernatant at 494 nm, proximity of FITC irrelevant**
- ⊕ **Current hypothesis is FL CDA can detect loss of one CI collagen binding domain based on the following facts:**
  - **Predominant band in CIa Rear is at 99 kD, a decrease of 17 kD from the full length enzyme (116 kD)**
  - **This loss reflects  $\approx$  150 amino acid residues: C terminal collagen binding domain accounts for last 108 amino acid residues of the CI enzyme**
- ⊕ **Loss of one CI collagen binding domain makes the variant CI behave like CII in FL CDA**

# Comparison of collagenase activity in VitaCyte & Liberase purified collagenase preparations

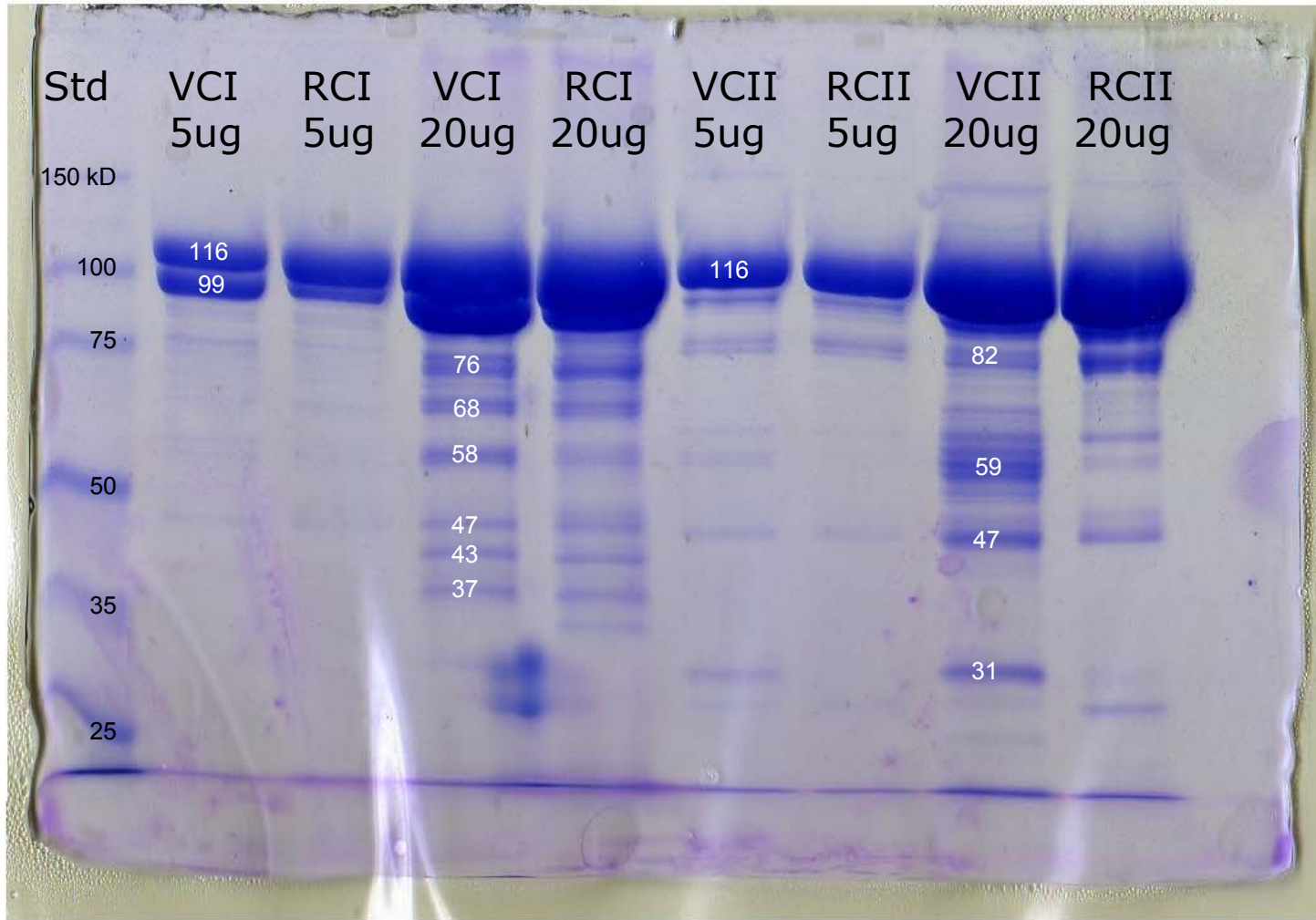
Assay	CI Specific Activity		CII Specific Activity	
	Roche	VitaCyte	Roche	VitaCyte
Wunsch	0.245	0.230	10.98	10.40
SP CDA	390.01	415.8	43.06	23.05
FL CDA	41978	41708	8119	2432

Samples of purified CI and CII used in Liberase HI provided by J. Lakey, University of Alberta

# Physicochemical characterization of Roche and VitaCyte purified collagenases



# SDS-PAGE analysis using 7.5% acrylamide gel comparing Roche (R) and VitaCyte (V) CI & CII



# Incorporated VitaCyte's purified collagenase into purified TDE mixture for porcine islet isolation

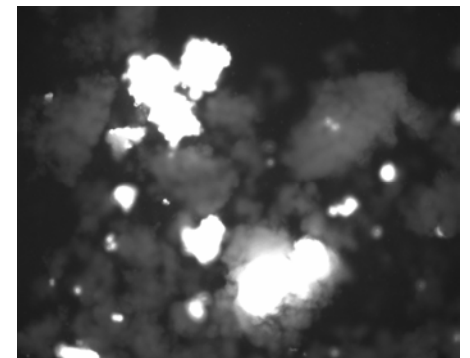
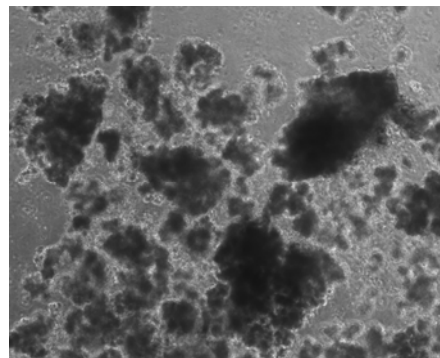
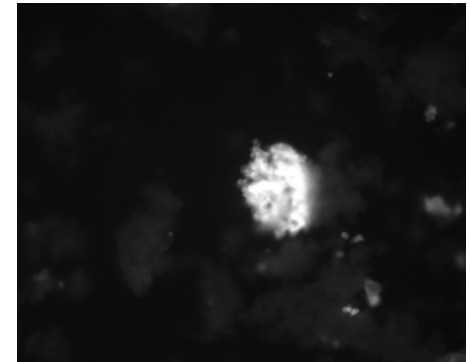
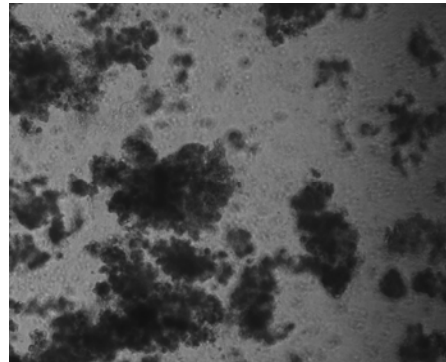
- ⊕ **Isolating islets in collaboration with Dick Sidner in Mark Pescovitz's lab at Indiana University School of Medicine**
- ⊕ **Recover pancreas from freshly slaughtered pig from farmer with uniform herd**
- ⊕ **Digest using 0.5 g of purified collagenase (60% CI, 40% CII) and 6 mg of purified dispase**

	Digest Time Min	Pre-Ficoll EIN/organ (EIN/g)	Post Ficl EIN/organ (EIN/g)
#1	15	761,333 (7613)	348,222 (3482)
#2	15	502,000 (5175)	211,000 (2175)
#3	17	832,000 (11,556)	288,500 (4007)

Pre-ficoll preparation, images captured using QICAM FAST 1394 monochrome camera

Bright Field

TSQ  
Fluorescence



# New directions for purified tissue dissociation enzymes

- ⊕ Liberase is a first generation product: overcame problems using crude collagenase but additional research required to optimize reagent
- ⊕ Current suppliers are manufacturers, focused on manufacturing product
- ⊕ VitaCyte's R&D will be grown organically by first understanding structure-functional aspects of the collagenase enzymes: development of superb biochemical analysis critical
- ⊕ Use this knowledge to understand how enzymes function in their end use: tissue dissociation mixtures

## *Open Questions for Further R&D*

- What is the optimal formulation for an islet specific, tissue dissociation enzyme blend?
- What is the influence of composition of the extracellular matrix on success of islet isolation?
- What is the impact of endogenous protease activity on success of islet transplant?
- What factors in the isolation procedure are detrimental to islet engraftment?



# Acknowledgments

- ⊕ Small Business Innovation Research grants (SBIRs) responsible for this R&D
- ⊕ SBIR/STTR awards offer a path to do leading edge commercial R&D, unlikely to be supported by established suppliers
- ⊕ VitaCyte has freedom to take novel approach to problem, ultimately leading to development of more robust commercial products
- ⊕ Grant support critical
  - NIDDK grants R43 DK065467, R43 DK070402, and
  - SBIR matching grants from the Indiana 21<sup>st</sup> Century Research & Technology Fund

## **VitaCyte Staff**

- Christopher Dwulet
- Beth Spurlin
- Francis Dwulet

## **IU School of Medicine**

- Dick Sidner
- Sherly Jules
- Ashesh Shah
- Mark Pescovitz