AWARD NUMBER: W81XWH-19-1-0439

TITLE: The Survival Factor Renalase and Pancreatic Cancer

PRINCIPAL INVESTIGATOR: Fred Sanford Gorelick

CONTRACTING ORGANIZATION: Yale University, New Haven CT

REPORT DATE: DECEMBER 2021

TYPE OF REPORT: FINAL

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
DECEMBER 2021	FINAL	9/1/2019 - 8/31/2021

4. TITLE AND SUBTITLE

The Survival Factor Renalase and Pancreatic Cancer

5a. CONTRACT NUMBER
W81XWH-19-1-0439

5b. GRANT NUMBER
CA180514

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Yale University
105 Wall Street
New Haven, CT 06511

8. PERFORMING ORGANIZATION REPORT NUMBER
GFEBS001114085600001

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

This proposal relates to the FY18 CDMRP topic area of Pancreatic Cancer. The project explores the role of a novel survival factor, the secretory protein renalase. We examine its potential value as a disease biomarker as well as its function to promote pancreatic cancer growth. We reported that renalase levels are increased in some pancreatic cancer tissue and that the levels correspond inversely with survival. Clinically, we propose to use human samples to examine whether *plasma* levels of renalase will also correspond to survival and whether tissue renalase levels are increased in pre-malignant conditions of the pancreas such as chronic pancreatitis and intrapancreatic mucinous neoplasms. Mechanistically, we will study whether renalase directly affects human pancreatic cancer cell growth by examining human cancer organoids. Using this new cancer model, we will examine the effects of added renalase or an inhibitory antibody we developed. If our predictions are correct, these studies could lead to a new biomarker that could guide pancreatic cancer therapy, including surgery, and could lead to a new form of therapy that uses the inhibition of renalase to reduce pancreatic cancer growth.

15. SUBJECT TERMS

NONE LISTED

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRDC
Unclassified	Unclassified	Unclassified	Unclassified	44	19b. TELEPHONE NUMBER *(include area code)*

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1. INTRODUCTION

Our work relates to the FY18 PRCPR topic area of Pancreatic Cancer (Pancreatic Ductal Adenocarcinoma: PDAC) "Idea Award with Special Focus". PDAC has become one of the most common causes of cancer death in the United States and one of the most resistant to therapy. Identification of the factors that promote the development and growth of PDAC could guide therapy, including the appropriateness of surgery and the use of specific drugs. Identifying such factors could also reveal new therapeutic targets. We have shown that the secretory protein renalase (RNLS) is a potent prosurvival factor for a broad range of cancer and non-cancer cells. We also reported that high tissue levels of PDAC inversely relate to patient survival and that antibody inhibition of renalase reduces PDAC growth in an experimental murine model and cultured human PDAC cells. As planned in Aim 1 of our proposed work, we examined whether plasma levels of renalase in humans could be a useful PDAC biomarker and guide such therapeutic interventions such as surgery. We found and published that the RNLS can be a biomarker for both. We also examined whether RNLS is present in precancerous pancreatic lesions and found it often was (Aim 2). This finding suggests that increases in tissue RNLS could drive cancer development in addition to growth and has been published. We are also examining if renalase is required to grow human PDAC organoids (Aim 2). These cultured cancer tissues are obtained from individuals with PDAC as part of a diagnostic or surgical procedure and grown to recapitulate many of the features of intact cancers. To meet our research goals, we have previously developed additional humanized antibodies to RNLS that inhibit the growth of experimental PDAC in mice. The effectiveness of the antibodies to RNLS for inhibiting growth is being examined in PDAC organoids. Our work represents a new and innovative topic in PDAC research. It holds the promise of developing a new and valuable PDAC biomarker (plasma renalase levels and forms) and providing data in a more pathologically relevant system (human PDAC organoids) that help develop renalase inhibition, a new therapy for this deadly disease.

2. KEYWORDS

Pancreatic cancer, renalase, biomarker, cancer organoids, prognosis, cancer growth, growth factor, precancerous lesions, antibody, ELISA

Abbreviations: pancreatic ductal adenocarcinoma: PDAC; renalase: RNLS

3. ACCOMPLISHMENTS

What were the major goals (Specific Aims) of the Project?

Aim 1: Examine whether the high molecular or low weight forms of renalase (RNLS) in human serum mediate its signaling and growth effects in cultured cancer cells and human pancreatic cancer (PDAC) organoids and changes in serum RNLS in PDAC.

a. Fractionation of RNLS in human serum: Progress made but in progress (delayed by COVID19)
b. Effects of plasma RNLS on PDAC cell growth: Progress made but in progress
c. Correlation of RNLS plasma peaks with Elisa: In progress
d. Inhibition of PDAC growth by anti-RNLS antibody: In progress: several studies performed showing linear growth of human PDAC organoids; preliminary data shows inhibition of organoid growth when inhibitory antibodies to RNLS were added.

Correlate plasma RNLS levels with PDAC clinical stage: Study completed, and manuscript published (see Appendices: Gao et al. renalase is a novel tissue and serological biomarker in pancreatic ductal adenocarcinoma. PLOS ONE. 2021: PMC**8480607)**

Aim 2: Determine the relationship between cancer grade and type and RNLS content and what forms of RNLS are secreted from cultured tumor cells and human PDAC organoids.

a. Examine the levels and distribution of RNLS in PDAC and precancerous lesions using resected human tissues. Study completed and published. Abstract presented at the American Pancreatic

Association annual meeting (November 2020). Manuscript published (see Appendices: PLOS ONE. 2021: PMC**8480607)**

b. Investigate the types of RNLS made by PDAC organoids and cell lines. In progress

What was accomplished under Aim 1 (also see figures that follow):

a. With relevance to Aim 1d, we have grown organoids from 6 human pancreatic cancer and have linear growth (tested up to two weeks). The example shown in Fig 1A demonstrates that these maintain cancer morphology and show renalase immunoreactivity in cancer cells. We only completed 4 experiments before being closed for COVID19, examining the effects of an inhibitor renalase antibody on PDAC organoid growth. As shown in Fig 1B, the antibody inhibited growth (more significant number by Glo-assay =more growth; values in photos/second/cm2/) in 3 of 4 studies (see avg values: controls (yellow shading) are higher than treated with antibodies (red shading). We are expanding Aim 1d to include examining the effects of the different isoforms of renalase on PDAC cell and organoid growth. In that context, Ms. C. Shugrue has cloned and expressed the major forms of human renalase to use in these studies. We are also examining the effects of both RNLS inhibitory antibodies and the RNLS peptide (PR220) that stimulates cell growth on murine pancreatic cancer organoid growth from the KPC mouse model used in Dr. Joshi's laboratory (not show).

b. With relevance to 1e, we examined plasma renalase (RNLS) levels in pancreatic cancer (PDAC) patients who were prospectively followed at Yale School of Medicine. Critical data from our published manuscript is shown below. The study focused on those with locally advanced/borderline resectable disease because this represents a population in which decisions about therapies, including surgery, can be difficult. Using a de-identified but robust database at Yale that includes access to coded plasma samples, we assayed plasma renalase levels in over 100 patients with PDAC at all stages and 75 with borderline resectable disease. As shown in Fig 2, plasma renalase levels (only acid-bound, not free) correlated negatively with patient survival (Fig 2A) and predicted resectability (Fig. 2B). It had much greater sensitivity and specificity than the often-used plasma CA19-9 (Fig 2B). These findings are now published (see Appendices; PLOS ONE. 2021: PMC**8480607)**

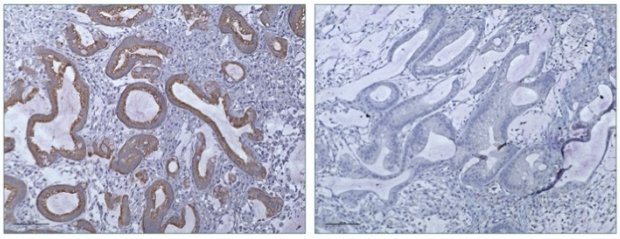
What was accomplished under Aim 2 (also see figures that follow):

a. With relevance to Aim 2a, we have examined the renalase content of pancreatic cancers and precancerous lesions. Fig 3 provides an example of the labeling in PDAC and two precancerous lesions (chronic pancreatitis and intrapancreatic epithelial neoplasms. We are analyzing >50 samples with our pathology expert, Prof Marie Robert, to determine the distribution and intensity of labeling. Our preliminary assessment is that renalase may be expressed in a precancerous stage (See publication by Gao et al in Appendices). We have examined whether plasma RNLS levels in all PDAC patients are-resectable plus non-resectable and find that the acid-sensitive form correlates inversely with survival (See publication by Gao et al in Appendices).

b. The existence of RNLS splice variants has been predicted, but virtually nothing has been done relating to their expression. In examining the variants expressed by cancer cells, we were surprised to find that they often express a form that appears to be rarely found in normal tissues. This splice variant may delete a site on the RNLS thought to modulate cellular metabolism, specifically NADH-oxidation. If so, the expression of this RNLS variant could have a profound effect on cancer cell metabolism. We are now cataloging the RNLS variants expressed by multiple types of human pancreatic cancer cells in culture, human pancreatic cancer, and normal tissues. We are also expressing RNLS in recombinant systems and plan to test the enzymatic activities of the different variants. A new grant proposal based on these findings is planned.

Figure.1 Renalase is present in organoids and antibodies to renalase may inhibit grown. 1A) Human pancreatic cancer (PDAC) organoids can be grown and show renalase immunoreactivity using antibody M28 (representative of 4 organoids.). **B)** An inhibitory antibody (M28) to renalase tends to reduce pancreatic cancer organoid growth. Relates to Aim 1d and A 2b.

A.



Human PDAC organoids make renalase. Left panel shows renalase immunoreactivity (arrow; brown stain-left panel); this labeling is eliminated by co-incubation with the antigen used to generate this antibody (m28: rabbit monoclonal IgG-generated by G. Desir, Yale). Representative of 4 different patient organoids.

B.

replicates

	Column 4	Column 5	Column 6	Averages
Exp 1	2.78E+07	1.02E+08	2.87E+07	5.28E+07
		2.79E+07	2.12E+07	2.45E+07
Exp 2	8.11E+07	9.99E+07	8.50E+07	8.87E+07
		1.01E+08	1.34E+08	1.18E+08
	Column 4	Column 5	Column 6	
Exp 3	6.02E+07	4.76E+07	4.76E+07	5.18E+07
		6.82E+07	4.97E+07	5.89E+07
Exp 4	5.05E+07	1.28E+08	8.83E+07	8.89E+07
		1.59E+08	9.69E+07	1.28E+08

Antibodies to renalase inhibit human PDAC organoid growth in 3/4 studies. 7 Days in culture. Pink: plus antibody m28; yellow-no antibody addition. CellTiter Glo-3D assay

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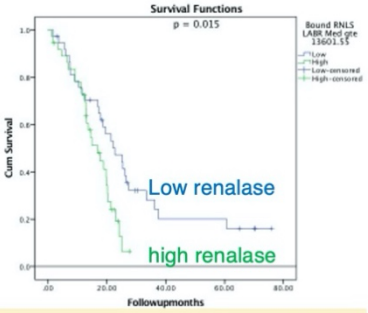
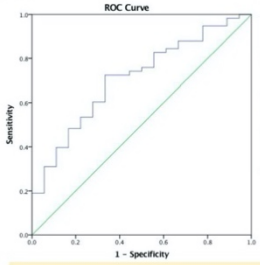
Figure. 2. In patients with locally advanced/borderline resectable pancreatic cancer, total plasma renalase levels at the time of diagnosis correspond inversely with A) *survival* and B) *resectability*. High renalase 37 patients; low renalase: 38 patients. Plasma RNLS ROC is a much better predictor of resectability that CA19-9, a standard PDAC biomarker. Total, but not free RNLS, correlated with survival (not shown). Relates to Aim 1e.

A. survival



Plasma renalase predicts survival

B. resectability



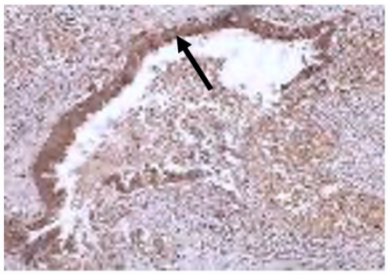
ROC: plasma renalase predicts resection

	Sensitivity (95% CI)	Specificity (95% CI)
RNLS (Median)	43.10% (20.16%-56.77%)	27.78% (9.69%-53.48%)
RNLS (AUC)	70.69% (57.27%-81.91%)	66.67% (40.99%-86.66%)
CA19-9	0.00% (0.00%-45.93%)	50.00% (24.65%-75.35%)

plasma renalase predicts resection

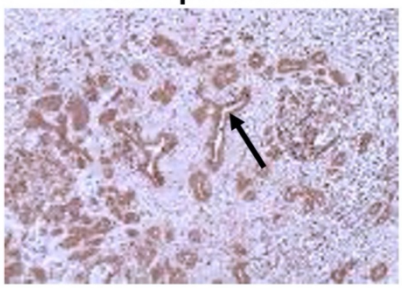
Figure. 3. Renalase immunoreactivity in increased in pancreatic cancer and precancerous conditions. Tissues from pancreatic cancer and premalignant conditions (chronic pancreatitis and PanIN [pancreatic intraepithelial neoplasm high grade]) all demonstrate renalase immunoreactivity (m28 antibody-brown color/arrows) predominantly on glandular structures. Specificity of labeling confirmed by competition with antigen. Images representative of 3-10 other patient tissues. Relates to Aim 2a

Cancer



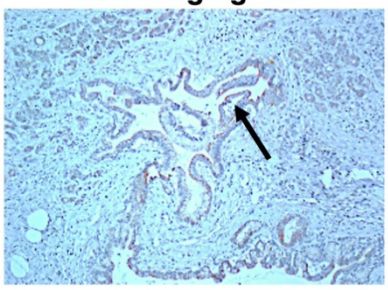
M28 1:1000
Mag 400x

Chronic pancreatitis



M28 1:1000
Mag 100x

PanIN- high grade



M28 1:1000
Mag 100x

structure-function

What opportunities for training and professional development has the Project provided?
We have involved three students in this research; all have presented related abstracts. Yasheen Gao is a Yale graduate who plans to matriculate in medical school. She worked in our laboratory during college and has continued during a post-Bac year. She has labeled human pancreatic cancer tissue for renalase and is a co-first author on a related manuscript (see appendices). Yaseen is now working on the effects of RNLS on human PDAC organoid growth. Govind Nair is a 3rd year Yale medical student who did the initial work on the human cancer organoids who generated the data showing growth inhibition by antibodies to renalase in the organoids-this was the last experiment before our labs were closed in March 2020. Thomas Pointer graduated from Yale in 2021 and has worked in our laboratory on RNLS signaling since 2020. He authored a review on renalase signaling (see appendicies) and continues his studies in our laboratory during a post-Bac year. Dr. Melinda Wang graduated from Yale Medical School in 2021and had been supported to do one year of research by an NIH student fellowship while a Yale Medical Student. She co-first-authored a PDAC RNLS publication (appendicies) and began her internal medicine residency at UCSF in June 2021. She plans to pursue a career as a physician-scientist in the field of gastroenterology. I think this award has played a central role in encouraging these students to pursue careers as physician-scientists; all have interests in pancreatic cancer and pursuing careers as physician-scientists.

How were the results disseminated to communities of interest?
A manuscript that included major components of the work in our DOD proposal has been published (Guo et al. PLOS ONE. 2021: PMC**8480607)** as was a review on the biologic properties of RNLS (Pointer et al. Cells. PMC**8391834)**. These are included in the Appendicies.

What do you plan to do during the next reporting period to accomplish these goals?
Although this award ended on August 31, 2021, we have continued work on this topic and plan to submit a grant proposal in 2022. We do have monetary support for continuing this work through an endowment to the PI. The areas we are pursuing in the short term are summarized below:

Specific Aim1: *Examine whether the high molecular or low weight forms of renalase (RNLS) in human serum mediate its signaling and growth effects in cultured cancer cells and human pancreatic cancer (PDAC) organoids and changes in serum RNLS in PDAC.*

Confirmatory studies are underway to demonstrate how the RNLS forms detected by our published ELISA correspond to the forms detected in human plasma by column chromatography (gel filtration and ion exchange).

We are now regularly examining the effects of our humanized anti-renalase antibodies on the growth of human PDAC organoids. Our preliminary data suggest that such inhibition of RNLS dramatically slows organoid growth. It also appears to affect the morphologic phenotype of organoids. We are performing RNAseq analysis to determine how RNLS inhibition is changing the gene expression in organoids. Such findings may provide insights into how RNLS drives tumor growth and possible tumor differentiation. Overall, the 5 months of the closing of the Joshi lab due to COVID-19 has greatly impeded work on both the organoids and pancreatic cancer culture lines. However, we are continuing this work with the plan to submit a grant proposal on the topic in 2022.

Specific Aim 2: *Determine the relationship between cancer grade and the type and RNLS content and what forms of RNLS are secreted from cultured tumor cells and human PDAC organoids and cancer cells and human pancreatic cancer (PDAC) organoids and changes in serum RNLS in PDAC.*

Although we had a 5-month delay due to the COVID-19 pandemic that primarily affected our organoid studies, we made substantial progress on other parts of the proposed work (see PLOS ONE publication in Appendices) and have made progress on organoid studies as shown (Fig 1).

4. **Impact**

a. Impact on the development of the principal discipline of the Project

This year, we refined and reconstructed our humanized anti-RNLS antibodies and now have generated a probe with an estimated inhibitory efficiency that at least 10-times that of our lead antibody a year ago (m28). We are also studying whether the Fc component of antibodies is needed for this inhibition and collaborating to develop nanobodies (a smaller version of Fab that can also be engineered to couple to an Fc antibody segment). Funding for continuing these studies is coming from Yale.

b. Impact on other disciplines

We have reported the development of the unique ELISA and its detection of two plasma forms of renalase (Chang et al. Kidney Int Rep 5: 362-368, 2020). We are pursuing follow-up studies in the context of renal failure and the use of plasma renalase as a biomarker. We also published a preliminary study showing that plasma renalase levels were directly related to disease severity and morbidity in COVID-19 patients (Wang et al. medRxiv https://doi.org/10.1101/2020.06.02.20120865 June 2020).

c. Impact on technology transfer: nothing to report

d. Impact on society beyond science and technology: nothing to report

5. Changes/Problems

a. Due to the COVID-19 pandemic, we had to stop PDAC organoid studies but continued to characterize forms of renalase in the blood. The PI's lab at the West Haven VA and Dr. Joshi's at Yale were closed for over 5 months, a period representing almost half of our first year of support. We also lost the services of a post-doctoral fellow we had hired and a medical student to perform these studies. However, working with Dr. Robert, Yasheen Gao, and Melinda Wang, we made significant progress on the clinical aspects of the proposal during COVID and published a manuscript on this topic. As mentioned above, we continued work on anti-RNLS antibodies during COVID and have generated a more effective humanized antibody. Although the lab closings have greatly impeded our progress for both aims, we have shifted our study priorities, made considerable progress on select topics, and published two relevant manuscripts (see Appendices).

b. We shifted our emphasis in year 1 to complete Aim 1e because our laboratory performing renalase ELISA continued because it was also doing COVID-19 research. Soon after activating this award, we published a study characterizing our renalase ELISA (J. Chang et al. Kidney International Reports, 5: 358-372, 2020). We used knowledge of the ELISA to assay plasma levels of renalase in patients who were being assessed to undergo PDAC resection and a larger group of patients. We also had access to patient charts during the outbreak; the ELISA data enabled us to complete Aim 1e and submit a manuscript (see attached).

c. Labeling of human cancer specimens for renalase immunoreactivity was far along during lab closings in March 2020, and we completed the studies this Spring (2021). We also completed a more general survey of plasma renalase levels in PDAC patients during that period. Thus, much of Aim 2 has been completed, as shown in the attached manuscript and published.

6. Products

a. Publications (manuscripts and abstracts)

DIRECTLY RELATED TO THIS AWARD

Manuscripts

1. Pointer, TC, **Gorelick FS,** Desir, GV. **Renalase: A Multi-Functional Signaling Molecule with Roles in Gastrointestinal Disease**. Cells. 2021 Aug 6;10(8):2006.doi: 10.3390/cells10082006. PMID: **34440775** PMCID: PMC8391834
   a. Support acknowledged: **YES**
2. Yaseen Gao, Melinda Wang, Xiaojia Guo, Joanna Hu, Tian-min Chen, Sade´ M.B. Finn, Jill Lacy, John W. Kunstman, Charles H. Cha, Melena D. Bellin, Marie E. Robert , Gary V. Desir, and **Fred S. Gorelick**. **Renalase is a novel tissue and serological biomarker in pancreatic ductal adenocarcinoma.** PLoS One . 2021 Sep 29;16(9):e0250539. DOI: 10.1371/journal.pone.0250539
   a. Support acknowledged: **YES**

Abstracts:

1. G Nair et al. **Organoid Based Preclinical Models Recapitulate Renalase Signaling in Pancreatic Ductal Adenocarcinoma.** Presented virtually Digestive Diseases Week/DDW June 2020
2. M Wang et al. **Utility of Renalase as a Novel Biomarker to Stratify Pancreatic Adenocarcinoma Candidates for Surgery.** American Pancreatic Assoc annual meeting Nov 2020
3. Y. Gao et al. **renalase may Play a Role in the Development of Pancreatic Cancer and in the Survival of Pancreatic Cancer Patients.** American Pancreatic Assoc annual meeting Nov 2020

NOT DIRECTLY OR LIMITED RELATIONSHIP TO THIS AWARD:

Manuscripts:

1. M Wang et al. **Decreased plasma levels of the survival factor renalase are associated with worse outcomes in COVID-19** medRxiv https://doi.org/10.1101/2020.06.02.20120865 June 2020 PMID: 32577678
2. M Wang et al. **Post-pancreatitis Diabetes Confers Higher Risk for Pancreatic Cancer Than T2D** Diabetes Practice July 2020 (https://www.practiceupdate.com/content/postpancreatitis-diabetes-confers-higher-risk-for-pancreatic-cancer-than-t2d/103161/65/8/1)
   Support acknowledged: **YES**
3. J Williams et al. **American Pancreatic Association Frank Brooks Symposium: Fifty Years of Pancreatic Cell Biology**. Pancreas May/June 2020 49: 604-611
4. F Gorelick and M Nathanson. **TRPV4 helps Piezo1 put the squeeze on pancreatic acinar cells**. J Clin Invest 2020 130: 2199-2201
5. J Chang et al. **Identification of two forms of human plasma renalase and their association with all-cause mortality**. Kidney Int Rep 2019 16: 362-368
6. SR Malla at al. **Early trypsin activation develops independently of autophagy in caerulein-induced pancreatitis in mice**. Cell Mol Life Sci 2020 77: 1811—1825
7. Melinda Wang et al. **Zinc: Roles in Pancreatic Physiology and Disease.** Pancreatology. 2020 Oct;20(7):1413-1420. DOI: 10.1016/j.pan.2020.08.016. PMID: 32917512
8. **Gorelick, FS**. **Protein Lysine Acetylation: An Unexpected Mediator in Pancreatitis.** Cell Mol Gastroenterol Hepatol. 2020 Dec 3:S2352-345X(20)30189-2. DOI: 10.1016/j.jcmgh.2020.11.010. PMID: 33279460
9. Wang, M and Gorelick FS**. Ketamine and Xylazine Effects in Murine Model of Acute Pancreatitis.** Am J Physiol Gastrointest Liver Physiol. 2021 April 21. DOI: 10.1152/ajpgi.00023.2021. PMID: 33881355

10. Olga A. Mareninova et al. **Dysregulation of mannose-6-phosphate dependent cholesterol homeostasis in acinar cells mediates pancreatitis.** J Clin Invest. https://doi.org/10.1172

11. Wang, M, **Gorelick, F,** Bhargava A. **Sex Differences in the Exocrine Pancreas and Associated Diseases**. Cell Mol Gastroenterol Hepatol. DOI: 10.1016/j.jcmgh.2021.04.005. PMID: 33895424

Abstracts:

1. T Kolodecik et al. **Recovery from acute experimental pancreatitis is delayed in mice with chronic kidney disease**. American Pancreatic Assoc annual meeting Nov 2020
2. T Kolodecik et al. **Cerulein induced acute pancreatitis causes a transient expansion of the lymphatic system of the pancreas; enhancement by renalase (RNLS) deletion**. American Pancreatic Assoc annual meeting Nov 2020

b. Books or other non-periodical, one-time publications: **nothing to report**

c. Other publications: **nothing to report**

d. Website: **nothing to report**

e. Technology or techniques

1. ELISA for renalase: J Chang et al. **Identification of two forms of human plasma renalase and their association with all-cause mortality**. Kidney Int Rep 2019 16: 362-368

f. Inventions, patent applications, and/or licenses: **nothing to report**

g. Other products: **nothing to report**

7. Participants & Other Collaborating Organizations

a. What individuals have worked on the Project?*

Name:	**Xiaojia Guo, PhD**
Project role	Research Scientist
Research Identifier	Orcid: 0000-0002-2749-7956
Nearest person month worked	5
Contribution to Project	Established renalase ELISA and performed all on patient plasma. statistical analysis
Funding support	DOD; Yale endowment

Name:	**Pelli Wang, PhD**
Project role	Associate Research Scientist
Research Identifier	None
Nearest person month worked	5
Contribution to Project	Generation of renalase humanized antibodies and purified renalase and renalase agonist
Funding support	DOD

Name:	**Marie Robert, MD**
Project role	Professor of Pathology
Research Identifier	Orcid: 0000-0002-6385-4578
Nearest person month worked	2
Contribution to Project	Direct interpretation of pathologic samples; obtained samples for analysis
Funding support	DOD

Name:	Christine Shugrue
Project role	Senior Research Associate
Research Identifier	None
Nearest person month worked	4
Contribution to Project	Generation of variants of recombinant renalase; deve of PCR for renalase isoforms
Funding support	DOD and VA Merit

Name:	Amanda Hutchins
Project role	Research Associate
Research Identifier	None
Nearest person month worked	5
Contribution to Project	Maintain pancreatic cancer organoids
Funding support	DOD, NCI

***Not paid by this award but substantially contributing were Yasheen Gao and Thomas Pointer, two Yale University, under-graduate students who pursued their senior thesis with work on renalase and topics related to the DOD aims. Both have recently published first-author studies (see appendices) from this work.**

b. **Change in PI support: n**othing to report

c. What organizations were involved as partners?

Organizations:

Veterans Administration: VA Healthcare CT West Haven, CT
In-kind support: instrumentation, computers
Facilities: laboratory space

Yale University School of Medicine
In-kind support: instrumentation, computers
Facilities: laboratory space

Personnel exchanges:
Between the PI's lab and Dr. Nik Joshi's

8. Special Reporting Requirements

Collaborative Awards: **nothing to report**

Quad charts: **nothing to report**

9. Appendices

Form DD0882: Invention statement signed by University Official

Letter by PI: confirming statement of DD0882

Reprints (directly related to this award)**:**

1. **Renalase is a novel tissue and serological biomarker in pancreatic ductal adenocarcinoma.** PLoS ONE: 2021 Sep 29;16(9):e0250539. DOI: 10.1371/journal.pone.0250539
2. **Renalase: A Multi-Functional Signaling Molecule with Roles in Gastrointestinal Disease**. Cells. 2021 Aug 6;10(8):2006.doi: 10.3390/cells10082006. PMID: **34440775** PMCID: PMC8391834

REPORT OF INVENTIONS AND SUBCONTRACTS

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Yale University	W81XWH1910439	Yale University	W81XWH1910439	a. INTERIM [ ] b. FINAL [X]

b. ADDRESS *(Include ZIP Code)*	d. AWARD DATE *(YYYYMMDD)*	b. ADDRESS *(Include ZIP Code)*	d. AWARD DATE *(YYYYMMDD)*	4. REPORTING PERIOD *(YYYYMMDD)*
P.O. Box 208327 New Haven CT 06520-8327	2019/09/01	P.O. Box 208327 New Haven CT 06520-8327	2019/09/01	a. FROM 2019/09/01 b. TO 2021/08/31

SECTION I - SUBJECT INVENTIONS

5. "SUBJECT INVENTIONS" REQUIRED TO BE REPORTED BY CONTRACTOR/SUBCONTRACTOR *(If "None," so state)*

NAME(S) OF INVENTOR(S) *(Last, First, Middle Initial)* a.	TITLE OF INVENTION(S) b.	DISCLOSURE NUMBER, PATENT APPLICATION SERIAL NUMBER OR PATENT NUMBER c.	ELECTION TO FILE PATENT APPLICATIONS *(X)* d.				CONFIRMATORY INSTRUMENT OR ASSIGNMENT FORWARDED TO CONTRACTING OFFICER *(X)* e.	
			(1) UNITED STATES		(2) FOREIGN			
			(a) YES	(b) NO	(a) YES	(b) NO	(a) YES	(b) NO
None	None	None						

f. EMPLOYER OF INVENTOR(S) NOT EMPLOYED BY CONTRACTOR/SUBCONTRACTOR		g. ELECTED FOREIGN COUNTRIES IN WHICH A PATENT APPLICATION WILL BE FILED	
(1) (a) NAME OF INVENTOR *(Last, First, Middle Initial)*	(2) (a) NAME OF INVENTOR *(Last, First, Middle Initial)*	(1) TITLE OF INVENTION	(2) FOREIGN COUNTRIES OF PATENT APPLICATION
None	None		
(b) NAME OF EMPLOYER	(b) NAME OF EMPLOYER		
None	None		
(c) ADDRESS OF EMPLOYER *(Include ZIP Code)*	(c) ADDRESS OF EMPLOYER *(Include ZIP Code)*		
None	None		

SECTION II - SUBCONTRACTS *(Containing a "Patent Rights" clause)*

6. SUBCONTRACTS AWARDED BY CONTRACTOR/SUBCONTRACTOR *(If "None," so state)*

NAME OF SUBCONTRACTOR(S) a.	ADDRESS *(Include ZIP Code)* b.	SUBCONTRACT NUMBER(S) c.	FAR "PATENT RIGHTS" d.		DESCRIPTION OF WORK TO BE PERFORMED UNDER SUBCONTRACT(S) e.	SUBCONTRACT DATES *(YYYYMMDD)* f.	
			(1) CLAUSE NUMBER	(2) DATE *(YYYYMM)*		(1) AWARD	(2) ESTIMATED COMPLETION
None	None	None	None	None	None	None	None

SECTION III - CERTIFICATION

7. CERTIFICATION OF REPORT BY CONTRACTOR/SUBCONTRACTOR *(Not required if: (X as appropriate))* [ ] SMALL BUSINESS or [X] NONPROFIT ORGANIZATION

I certify that the reporting party has procedures for prompt identification and timely disclosure of "Subject Inventions," that such procedures have been followed and that all "Subject Inventions" have been reported.

a. NAME OF AUTHORIZED CONTRACTOR/SUBCONTRACTOR OFFICIAL *(Last, First, Middle Initial)*	b. TITLE	c. SIGNATURE	d. DATE SIGNED
Eileen M. Joyce	Award Closeout Manager	Eileen Joyce Digitally signed by Eileen Joyce Date: 2021.11.22 16:29:02 -05'00'	

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December 10, 2021

Re: Final statements for award CA180514 (Contract W81XWH-19-1-0439)

To Whom It May Concern:

This letter serves as our official response for final reporting requirements on the referenced award.

(4) List of equipment purchased with award funds.
Response: No equipment was purchased with funds from this award.

(5) List of residual inventories of unused supplies exceeding in value.
Response: There is no residual inventory of unused supplies exceeding

(6) Transition plan or other documents as specified in the agreement document
Response: Not applicable to this award.

Thank you very much for your support of this research.

Sincerely,

Sincerely,

Fred Gorelick
Professor of Medicine (Digestive Diseases) and Cell Biology

PLOS ONE

RESEARCH ARTICLE

Renalase is a novel tissue and serological biomarker in pancreatic ductal adenocarcinoma

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Citation: Gao Y, Wang M, Guo X, Hu J, Chen T-m, Finn SMB, et al. (2021) Renalase is a novel tissue and serological biomarker in pancreatic ductal adenocarcinoma. PLoS ONE 16(9): e0250539. https://doi.org/10.1371/journal.pone.0250539

Editor: Gloria H. Su, Columbia University, UNITED STATES

Received: March 20, 2021

Accepted: August 10, 2021

Published: September 29, 2021

Data Availability Statement: All original data files are available from the OSF database (DOI: 10.17605/OSF.IO/GT4V9).

Funding: This study was financially supported by the U.S. Department of Defense (DOD) Cancer Program 2019 (CA180514) to FG and GD for salary support, laboratory analyses/equipment and materials necessary for this study, U.S. Veterans Administration Merit Award (BX00325) to FG for salary support and an NIH-NIDDK medical student fellowship award (DK007107) to MW for a stipend.

Abstract

Dysregulated expression of the secretory protein renalase can promote pancreatic ductal adenocarcinoma (PDAC) growth in animal models. We characterized renalase expression in premalignant and malignant PDAC tissue and investigated whether plasma renalase levels corresponded to clinical PDAC characteristics. Renalase immunohistochemistry was used to determine the presence and distribution of renalase in normal pancreas, chronic pancreatitis, PDAC precursor lesions, and PDAC tissues. Associations between pretreatment plasma renalase and PDAC clinical status were assessed in patients with varied clinical stages of PDAC and included tumor characteristics, surgical resection in locally advanced/borderline resectable PDAC, and overall survival. Data were retrospectively obtained and correlated using non-parametric analysis. Little to no renalase was detected by histochemistry in the normal pancreatic head in the absence of abdominal trauma. In chronic pancreatitis, renalase immunoreactivity localized to peri-acinar spindle-shaped cells in some samples. It was also widely present in PDAC precursor lesions and PDAC tissue. Among 240 patients with PDAC, elevated plasma renalase levels were associated with worse tumor characteristics, including greater angiolymphatic invasion (80.0% vs. 58.1%, $p = 0.012$) and greater node positive disease (76.5% vs. 56.5%, $p = 0.024$). Overall survival was worse in patients with high plasma renalase levels with median follow-up of 27.70 months vs. 65.03 months ($p < 0.001$). Renalase levels also predicted whether patients with locally advanced/borderline resectable PDAC underwent resection (AUC 0.674; 95%CI 0.42–0.82, $p = 0.04$). Overall tissue renalase was increased in both premalignant and

Funding to FG from the Henry J. and Joan W Binder endowment also supported this work.

Competing interests: G V Desir is a named inventor on several issued patents related to the discovery and therapeutic use of renalase. Renalase is licensed to Bessor Pharma, and G V Desir holds an equity position in Bessor and its subsidiary Personal Therapeutics. This does not alter our adherence to PLOS ONE policies on sharing data and materials and the authors will fully comply with the guidelines.

malignant PDAC tissues compared to normal pancreas. Elevated plasma renalase levels were associated with advanced tumor characteristics, decreased overall survival, and reduced resectability in patients with locally advanced/borderline resectable PDAC. These studies show that renalase levels are increased in premalignant pancreatic tissues and that its levels in plasma correspond to the clinical behavior of PDAC.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is the seventh leading cause of cancer death worldwide with an estimated 430,000 annual deaths and an overall survival rate of less than 10% [1, 2]. PDAC is often not detected until after it has disseminated systemically, is resistant to drug therapies, and is recognized as an urgent unmet medical need [3, 4]. A better understanding of the biology and requirements for PDAC growth is crucial to improving the diagnostic and therapeutic approaches to this disease.

Renalase (RNLS) is a novel secretory protein [5–7] that can be highly expressed in PDAC tissue [8]. Recombinant RNLS can promote the growth of human PDAC cell lines, and its inhibition leads to experimental tumor cell death in both in vitro and in vivo [8]. Overall PDAC patient survival correlates inversely with RNLS tissue levels in tumor samples [8]. These studies suggest that RNLS could be an important factor in maintaining PDAC growth and influence patient outcomes. Whether RNLS activity is associated with PDAC development, including in precursor lesions, has not been explored.

The risk of developing PDAC is increased in several premalignant lesions including chronic pancreatitis [13], pancreatic intraepithelial neoplasia (PanIN), and pancreatic cystic neoplasms. PanIN [9], the most common of these, progresses step-wise from non-invasive neoplasms (grades 1–3) to invasive PDAC with KRAS mutations being one of the earliest genetic events [10–12]. Pancreatic cystic neoplasms, comprising intraductal papillary mucinous neoplasms (IPMNs) and less frequently, mucinous cystic neoplasms (MCNs), can also progress to PDAC [13].

Since RNLS is a secretory protein, changes in its tissue levels could be reflected in its plasma levels. As seen with other tumors and biomarkers (e.g., CA19.9, CEA and PSA), it is possible that plasma RNLS levels could parallel tissue levels of RNLS in PDAC and serve as a new biomarker in PDAC.

Here we show that RNLS is present in premalignant (chronic pancreatitis, PanINs, and mucinous pancreatic cystic neoplasms) and malignant PDAC tissue suggesting a potential role for pancreatic RNLS in PDAC development. We also observed that elevations of a distinct form of plasma RNLS appear to correspond to PDAC prognosis in a subset of pancreas cancer patients with locally advanced disease at presentation.

Methods

Tissue specimens

Human pancreatic tissue samples from resection specimens of normal pancreas, chronic pancreatitis, PDAC precursor lesions (PanIN, IPMN, and MCN), and PDAC, were obtained from Yale Surgical Pathology (Yale HIC approval number: 2000021579). Additional chronic pancreatitis tissue samples were obtained from University of Minnesota Medical Center (IRB Approval Number: 0609M91887). Studies were given exceptions by the Veterans

Administration HIC and Research committees under protocols FG0011-2020 and FSG0012-2020 (Oct 28, 2020)

Immunohistochemistry protocol

Immunohistochemistry was performed as described [8]. Sections (5-μm) from formalin-fixed paraffin-embedded tissues were mounted on slides and de-paraffinized and hydrated. After antigen retrieval in a pressure cooker containing citrate buffer (1g NaOH, 2.1 g citric acid in 1 L H2O pH 6), sections were blocked with DAKO Dual endogenous enzyme block (Agilent, Santa Clara, CA, USA) for 10 min and 2.5% normal horse serum for 1 hr followed by incubation with primary antibody and isotype control IgG overnight at 4˚C. The m28-RNLS monoclonal antibody was diluted at 1μg/mL in buffer (TBS/1% Tween with 300 mM NaCl, pH = 7.4). ImmPRESS peroxidase-anti-rabbit IgG (Vector Laboratories, Burlingame, CA, USA) was used to detect primary antibodies. The color was developed using a Vector DAB substrate kit and tissue was counterstained with hematoxylin (Vector Laboratories). Hematoxylin and eosin stained and RNLS IHC stains were examined at the light microscope to confirm the histologic diagnosis and document the distribution of RNLS immunopositivity by a pancreaticobiliary pathologist (MER). RNLS IHC staining was categorized as present or absent in all components of the pancreas (benign exocrine, endocrine, pancreatic ducts and stroma; neoplastic tissues, either in-situ or invasive). The specificity of labeling on each tissue was confirmed by either labeling in tissue sections in the absence of the m28-RNLS primary antibody or2 using m28-RNLS antibody that had been pre-incubated with the peptide antigen (RP-220) [14].

Immunofluorescence protocol

The slides were deparaffinized and processed as above for immunofluorescence microscopy. After antigen retrieval, sections were blocked with TBS/0.3% TritonX-100/10% goat serum) for 1 hr and incubated with a cocktail of m28-RNLS (1:100) plus α-smooth-muscle actin (αSMA) mouse monoclonal (1:400 Sigma-Aldrich, St. Louis, MI, USA) at 4˚C overnight. Alexa 488-conjugated goat anti-rabbit (1:2000, Invitrogen Corporation, Carlsbad, CA) and Alexa 594-conjugated goat anti-mouse (1:2000, Invitrogen Corporation, Carlsbad, CA) were used to detect the primary antibodies. Tissue autofluorescence was quenched using a commercial preparation (Vector TrueVIEW Autofluorescence Quenching Kit, Vector Laboratories, Burlingame, CA, USA). The specificity of labeling on each tissue was confirmed by labeling tissue sections in the absence of the m28-RNLS and αSMA primary antibodies.

Measurement of plasma RNLS and CA-19.9 levels

Plasma RNLS levels were determined by assaying the denaturation acid-sensitive pool and non-acid treated pool by ELISA as described [15]. Here we refer to the acid-treated value as RNLS and the untreated value as non-acid treated RNLS. The median value was used to separate low and high RNLS levels Serum CA19-9 levels were obtained from medical records before the first date of PDAC treatment.

Because of potentially spurious CA19-9 values in patients with biliary obstruction, patients with total bilirubin $>$ 2.0 mg/dL were excluded from the CA19-9 analysis. CA19-9 levels of $<$ 10 U/mL were also excluded because artifactually low CA19-9 levels can be seen in patients, especially those who have Lewis a-b-blood group antigen [16]. A threshold CA19-9 value of 300 U/mL was designated elevated based on studies showing that pre-operative CA19-9 values above 300 U/mL suggest advanced disease and unresectable cancer [17].

Clinical data collection

Prospectively collected plasma samples were obtained from consented patients with pathologically confirmed PDAC prior to initiation of treatment as part of the intake procedure for the Yale Gastrointestinal Tumor Biorepository from April 2012 to March 2019 (YGTB; Yale HIC #1203009817). Following definitive treatment, clinical and pathologic information were used to retrospectively annotate the plasma samples. Sociodemographic and clinicopathologic data were extracted from oncology visit notes, operation reports, and surgical pathology reports. Management was determined by the treating oncology team. Patients with locally advanced/borderline resectable (LA/BR) PDAC were determined at the time of diagnosis by cross-sectional imaging using a dedicated pancreatic contrast administration protocol and reviewed by a multidisciplinary PDAC management team that included an experienced pancreatic surgeon. Specifically, definitions of "resectable", "borderline resectable", and "locally advanced" were ascribed to patients by treating surgeons in accordance with the published National Comprehensive Cancer Network (NCCN) guidelines germane to the year of diagnosis [18] For the purposes of this study, patients who were identified as LA/BR at diagnosis who underwent resection with curative intent were considered "resectable" versus those who did not undergo surgery with curative intent. Any patients undergoing surgery for palliative purposes were not considered resectable.

Statistical analyses

Nonparametric statistical analyses were performed using SPSS Version 24 (IBM Statistics; Armonck, NY). Bivariate nonparametric analyses of sociodemographic and treatment-related variables were performed using the calculated cut-off value for RNLS. Multivariate analyses were performed for statistically significant variables within the bivariate analysis. Sensitivity and specificity values were calculated using the calculated cut-off value for RNLS and predetermined cut-off value for CA19-9. Kaplan-Meier survival analyses were performed using log rank analysis to calculate statistical significance to calculate overall survival and resectability of patients. All quantitative data were reported as median (range) or mean when median was unavailable and a p-value < 0.05 was used to determine statistical significance.

Results

RNLS immunoreactivity in human pancreatic tissue

We examined the presence and distribution of RNLS in normal human pancreas (n = 11), chronic pancreatitis (n = 32), PDAC precursor lesions (PanIN: n = 5, IPMN: n = 6, MCN: n = 4), and PDAC (n = 9) by immunohistochemistry. Sociodemographic and labeling characteristics for the patients examined are summarized in **Tables 1** and **2**.

Among normal human pancreatic tissue from patients who had undergone Whipple procedure for duodenal adenoma (n = 7), there was little to no RNLS labeling (**Fig 1A**). In patients who had undergone distal pancreatectomy associated with trauma-related splenectomy (n = 4) there was granular RNLS labeling in the apical cytoplasm of pancreatic acinar cells as well as diffuse RNLS labeling in pancreatic islets and ducts (**Fig 1B**). One of these tissues showed RNLS labeling in spindle-shaped cells surrounding pancreatic acinar cells, a cellular distribution more often seen in chronic pancreatitis. Normal tissue from the pancreatic body or tail from non-abdominal trauma cases were not available for analysis. Representative images showing the spectrum of RNLS labeling in normal pancreas cases are presented in **S1 Fig.**

Chronic pancreatitis tissues were obtained from patients with the following etiologies: alcoholic chronic pancreatitis (n = 5), genetic chronic pancreatitis (n = 6), idiopathic chronic

Table 1. Sociodemographic characteristics for RNLS immunohistochemistry patients.

	Histological variant					
Characteristics	**Normal Pancreas (n = 11)[a]**	**Chronic Pancreatitis (n = 32)[b]**	**PanIN (n = 5)**	**IPMN (n = 6)**	**MCN (n = 4)**	**PDAC (n = 9)**
Age[c], years	70 (23–94)	56.8 (27–85)	71 (62–82)	70 (44–78)	52 (26–74)	67 (51–80)
Male[d]	4 (36.4)	13 (40.6)	2 (40.0)	4 (66.7)	0	5 (55.6)
White[d]	7 (63.6)	28 (87.6)	5 (100)	5 (83.3)	4 (80)	8 (88.9)
BMI[c], kg/m^2	28.0 (25.8–30.1)	24.3 (13.3–37.1)	26.0 (18.9–28.7)	28.4 (14.9–39.6)	22.6 (13.3–34.1)	30.4 (23.3–34.9)
Current or past smoker[d]	4 (36.4)	17 (53.1)	3 (60)	4 (66.7)	2 (40)	5 (55.6)
Alcohol use[d]	0	5 (15.6)	0	0	1 (20)	1 (11.1)
Hypertension[d]	3 (27.3)	13 (40.6)	2 (40)	2 (33.3)	1 (20)	3 (33.3)
Cardiovascular Disease[d]	0	3 (8.4)	1 (20)	0	0	1 (11.1)
Chronic Kidney Disease[d]	0	2 (6.3)	0	0	1 (20)	1 (11.1)
Diabetes[d]	0	9 (28.1)	3 (60)	2 (33.3)	2 (40)	2 (22.2)

[a] Seven normal pancreas cases were taken from patients who had undergone Whipple surgery for duodenal adenoma. Four normal pancreas cases were taken from patients who had undergone splenectomy associated with trauma.

[b] Five cases were taken from patients who had alcohol induced chronic pancreatitis. Four cases were taken from patients with idiopathic chronic pancreatitis. Six cases were taken from patients who had genetic chronic pancreatitis. One case came from a patient who had chronic pancreatitis due to pancreatic divisum. One case was taken from a patient who had chronic pancreatitis due to Sphincter of Oddi dysfunction. Two cases were taken from patients who had chronic pancreatitis associated with benign inflammatory pseudocyst. Thirteen cases were taken from patients with PDAC precursor lesions and PDAC.

[c] Data are given as median (range).

[d] Data are given as number (percentage).

https://doi.org/10.1371/journal.pone.0250539.t001

pancreatitis (n = 4), chronic pancreatitis due to pancreatic divisum (n = 1), chronic pancreatitis due to Sphincter of Oddi dysfunction (n = 1), chronic pancreatitis adjacent to a benign inflammatory pseudocyst (n = 2), chronic pancreatitis associated with precursor lesions (n = 9), and chronic pancreatitis associated with PDAC (n = 4).

Overall, RNLS labeling in chronic pancreatitis was present, but variable, as noted below and in Table 2 and S2 Fig. RNLS immunoreactivity was found in spindle-shaped cells surrounding pancreatic acinar cells in six of the nineteen non-tumor associated chronic pancreatitis cases (Fig 2A) and two of the thirteen tumor-associated chronic pancreatitis cases (Fig 2B). These included one case each of the following etiologies: 1) Mutations in the cystic fibrosis transmembrane conductance regulator (CTFR), 2) Mutation in chymotrypsin C (CTRC), 3) Idiopathic chronic pancreatitis, 4) Alcoholic chronic pancreatitis, 5) Pancreatic divisum, 6) Associated with a pseudocyst, 7) Associated with MCN and 8) Chronic pancreatitis associated with PDAC. To determine the identity of the spindle-shaped containing RNLS immunoreactivity, we performed double-label (RNLS and αSMA- smooth muscle actin)

Table 2. Labeling characteristics for RNLS immunohistochemistry patients.

Pancreatic tissue type	**n**	**Benign ductal epithelium**	**Acinar cell**	**Islets**	**Neoplastic cells**	**Stellate cells**
Normal pancreas (trauma)	4	4/4 (100)	4/4 (100)	4/4 (100)	N/A	1/4 (25)
Normal pancreas (duodenal adenoma)	7	1/7 (14)	3/7 (43)	1/7 (14)	N/A	0/7 (0)
Chronic pancreatitis (Non-tumor associated)	19	6/19 (32)	8/19 (42)	6/19 (32)	N/A	6/19 (32)
Chronic pancreatitis (adjacent to pancreatic precursor lesion/PDAC)	13	12/13 (92)	13/13 (100)	12/13 (92)	N/A	2/13 (15)
Precursor Lesion (PanIN)	5	5/5 (100)	5/5 (100)	5/5 (100)	5/5 (100)	0/5 (0)
Precursor Lesion (IPMN)	6	6/6 (100)	6/6 (100)	5/6 (83.3)	6/6 (100)	0/6 (0)
Precursor Lesion (MCN)	4	4/4 (100)	4/4 (100)	4/4 (100)	4/4 (100)	0/4 (0)
PDAC	9	4/9 (44)	4/9 (44)	5/9 (55)	9/9 (100)	0/9 (11)

https://doi.org/10.1371/journal.pone.0250539.t002

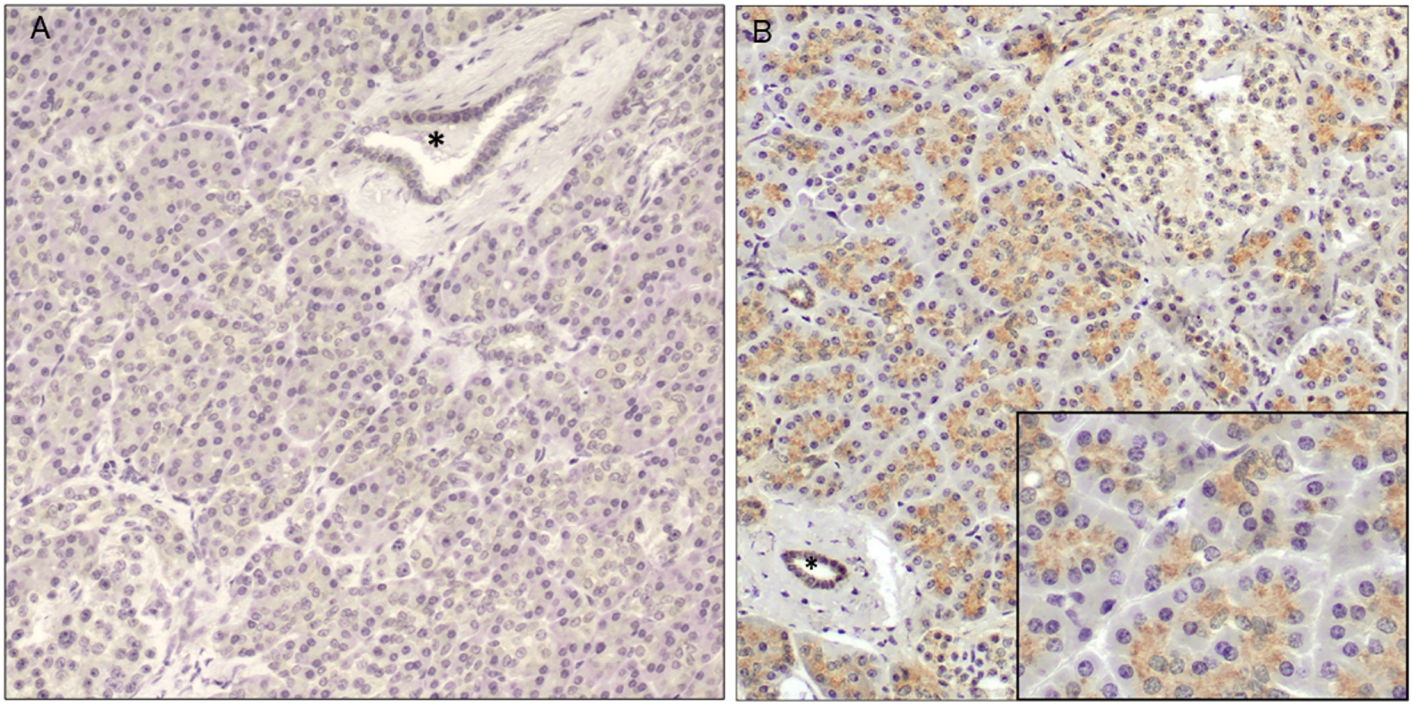

Fig 1. RNLS labeling in normal human pancreas. Representative images of RNLS labeling detected by immunohistochemistry using the m28-RNLS antibody in the normal human pancreas from (A) a patient who had undergone Whipple procedure for duodenal adenoma with essentially no signal and (B) a patient who had undergone splenectomy for trauma, showing granular cytoplasmic staining in acinar cells. The asterisk indicates a normal pancreatic duct. Original magnification 200x; inset 400x.

https://doi.org/10.1371/journal.pone.0250539.g001

immunofluorescence on six non-tumor associated and three tumor associated chronic pancreatitis cases. In a chronic pancreatitis case (associated CTRC mutation) we observed co-localization of RNLS and αSMA (Fig 2C–2E), suggesting that RNLS may be present in stellate cells. In the remainder of the cases the labeling was faint, and it was unclear whether the markers co-distributed. In non-tumor associated chronic pancreatitis samples, RNLS immunoreactivity was also observed in acinar cells (8/19 samples), pancreatic ducts (6/19) and islet cells (6/19). (Table 2). Granular cytoplasmic RNLS labeling of acinar cells, pancreatic ducts and islet cells was also present in regions of chronic pancreatitis associated with neoplastic conditions, including PanIN (n = 2), IPMN (n = 4), MCN (n = 3), and PDAC (n = 5). Finally, RNLS labeling was noted in stromal cells, including mononuclear inflammatory cells and fibroblasts, in regions of chronic pancreatitis associated with neoplastic precursor lesions in four samples.

We labeled for RNLS immunoreactivity in PDAC precursor lesions (n = 15) including PanIN (n = 5), IPMN (n = 6), and MCN (n = 4), and in PDAC tumors (n = 9). In all premalignant and malignant PDAC tissues there was diffuse RNLS labeling in in-situ and invasive neoplastic epithelium, adjacent benign duct cells, acinar cells, and islets (Fig 3A–3D). In six of the fifteen precursor lesion cases and three of the nine PDAC cases, RNLS labeling was noted in stromal cells (comprising fibroblasts, endothelial cells and inflammatory cells) (Fig 3E). Additional representative images of RNLS labeling in PDAC precursor lesions and PDAC are presented in S3 Fig and S4 Fig**.** In-situ and invasive pancreatic ductal neoplasms and precursor lesions showed increased RNLS staining compared to benign samples (normal pancreas and most cases of pancreatitis). Specifically, in precursor and PDAC samples, RNLS was present in most cell types, including all benign elements. The intensity of labeling often appeared greater in neoplastic than in benign epithelium but was not quantified because of limitations of the labeling technique.

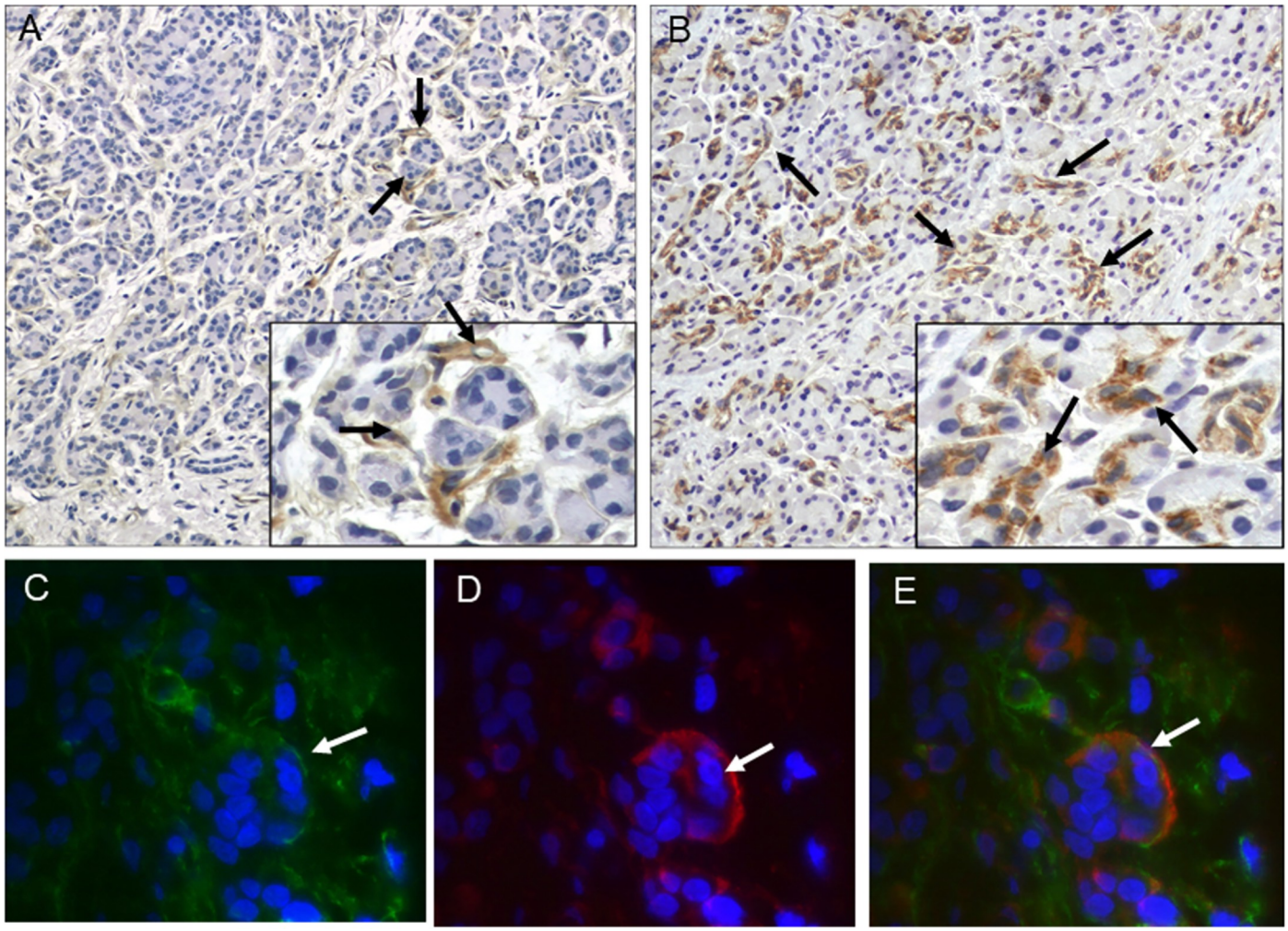

Fig 2. RNLS is present in spindle-shaped cells in human chronic pancreatitis. Representative images of RNLS immunoreactivity using the m28-RNLS antibody in (A) genetic chronic pancreatitis (CTRC mutation) and (B) PDAC-associated chronic pancreatitis. (C-E) Immunofluorescence labeling of RNLS with m28-RNLS antibody (green) and alpha-smooth muscle actin (red) in a patient with genetic chronic pancreatitis (CTRC mutation). The white arrows point to cells positive for both proteins (stellate cells). Original magnification A and B: 200x, insets 400x. Original magnification C-E: 10000x.

https://doi.org/10.1371/journal.pone.0250539.g002

Plasma RNLS in patients with PDAC

Three hundred and forty-seven patients with biopsy confirmed PDAC were identified. Of those, 240 patients had both plasma RNLS levels drawn prior to any PDAC treatment and medical records available for review. Median acid-treated RNLS level was 13291.15 ng/ml for all patients. Among patients with high or low acid-treated plasma RNLS, the median was 17337.52 vs 10135.56 ng/ml, respectively. The difference between these two groups was statistically different ($p < 0.001$). *Median non-acid treated RNLS level was 1774.30 ng/ml. Median high non-acid treated RNLS level was 2824.05 ng/ml and median low non-acid treated RNLS level was 647.03 ng/ml ($p < 0.001$)*. High acid-treated plasma RNLS was associated with younger age at diagnosis but not associated with *race/ethnicity*, gender, or BMI. Patients with higher serum protein levels at diagnosis also had higher plasma RNLS levels at diagnosis (7.30 vs. 7.00, $p = 0.004$). Patients with high acid-treated RNLS levels also exhibited worse PDAC disease attributes (**Table 3**). This included higher angiolymphatic invasion (80.0% vs. 58.1%, $p = 0.012$), and greater node positive disease (76.5% vs. 56.5%, $p = 0.024$) particularly hepatic

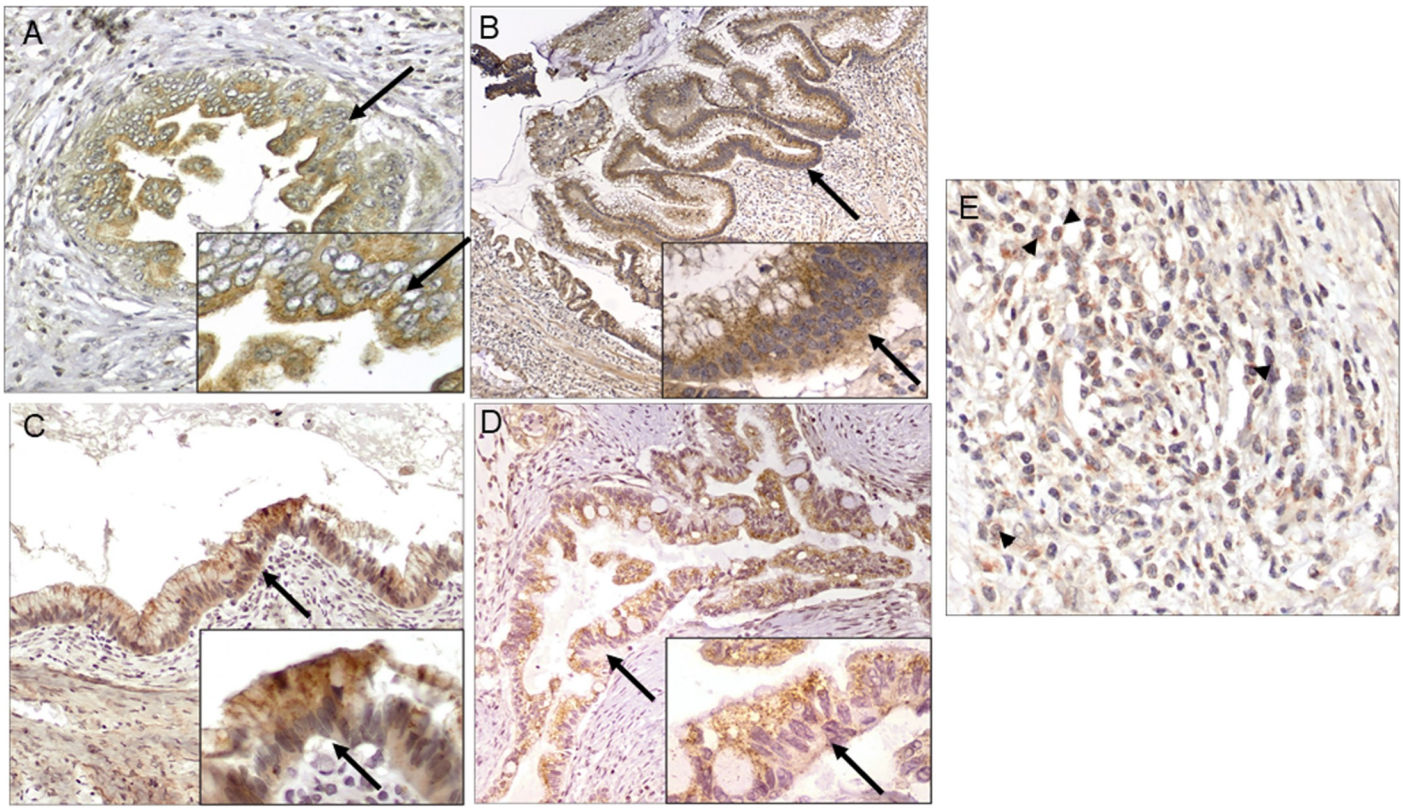

Fig 3. RNLS distribution in human precursor and PDAC tissue. Representative images of RNLS labeling detected by immunohistochemistry using the m28-RNLS antibody in: (A) PanIN, (B) IPMN, (C) MCN, and (D-E) PDAC. Intense granular cytoplasmic staining is present in the majority neoplastic epithelial cells, and in a subset of mononuclear inflammation near tumor. The black arrows point to ductal cells in (A) PanIN, (B) IPMN, (C) MCN, and (D) PDAC. The black arrowheads point to mononuclear inflammatory cells in the PDAC stroma. Original magnification: A, E 400x, inset 600x; B 100x, inset 600x; C, D 200x, insets 400x.

https://doi.org/10.1371/journal.pone.0250539.g003

artery lymph node positivity (26.7% vs. 6.7%, p = 0.022). Patients with higher plasma acid-treated RNLS levels did not exhibit larger tumor size, or greater rates of margin positivity (Table 3). In concordance with worse disease features, patients with higher plasma acid-treated RNLS levels were less likely to be deemed clinically resectable at diagnosis (39.2% vs. 58.0%, p = 0.004) and were more likely to have disseminated disease (distant metastasis) at diagnosis (25.8% vs. 13.4%, p = 0.022). Higher plasma acid-treated RNLS levels at diagnosis were associated with decreased survival with a median of 27.70 months vs. 65.03 months ($p < 0.001$; Fig 4A). The survival difference persisted when acid-treated RNLS levels were divided into tertiles (n = 80 per group; Bottom tertile = 19.13 months, Middle tertile = 38.47 months, Top tertile = 65.03 months; $p < 0.001$; Fig 4B) and into quartiles (n = 60 per group; Bottom quartile = 19.13 months, Bottom middle quartile = 38.47 months, Top middle quartile = 70.43 months, Top quartile = 65.03 months, $p < 0.001$; Fig 4C). There was no correlation between non-acid treated RNLS levels and either tumor characteristics or survival (S1 Table, S5 Fig).

Plasma RNLS in patients with locally advanced/borderline resectable PDAC

Among patients with PDAC and available plasma RNLS levels, 76 patients presented with LA/BR PDAC according to the treating oncology team at diagnosis. Median acid-treated RNLS

Table 3. Sociodemographic and clinicopathologic features among patients with PDAC with high vs. low plasma RNLS.

Factor	Low RNLS	High RNLS	Total	p-value
Sociodemographic Characteristics				
Age at Diagnosis	68.00 (9.83)	66.00 (9.62)	68.00 (9.80)	0.018
Race/ethnicity				0.236
White	114/120 (95.0%)	107/120 (89.2%)	221/240 (92.1%)	
Black	5/120 (4.2%)	10/120 (8.3%)	15/240 (6.3%)	
Other	1/120 (0.8%)	3/120 (2.5%)	4/240 (1.7%)	
Male	71/120 (59.2%)	75/120 (62.5%)	146/240 (60.8%)	0.692
BMI at Diagnosis	26.37 (5.29)	26.34 (6.92)	26.29 (6.04)	0.719
Albumin	4.00 (0.50)	4.00 (0.50)	4.00 (0.49)	0.237
Total Protein	7.00 (0.71)	7.30 (0.69)	7.10 (0.73)	**0.004**
Clinicopathologic Characteristics				
Perineural Invasion	61/75 (81.3%)	46/50 (92.0%)	107/125 (85.6%)	0.122
Angiolymphatic Invasion	43/74 (58.1%)	40/50 (80.0%)	83/124 (66.9%)	**0.012**
Differentiation				0.734
Well	3/75 (4.0%)	1/50 (2.0%)	4/125 (3.2%)	
Moderate	37/75 (49.3%)	23/50 (46.0%)	50/125 (48.0%)	
Poor	35/75 (46.7%)	26/50 (52.0%)	61/125 (48.8%)	
Node Positive	43/76 (56.6%)	39/51 (76.5%)	82/127 (64.6%)	**0.024**
HALN Positive	3/45 (6.7%)	8/30 (26.7%)	11/75 (14.7%)	**0.022**
Tumor Size $\geq$ 2cm	58/73 (79.5%)	44/49 (89.8%)	102/122 (83.6%)	0.144
Any Margin	15 (19.7)	15 (29.4)	30 (23.6)	0.237
Whipple Characteristics	**Low (n = 53)**	**High (n = 36)**	**Total (n = 89)**	
Neck Margin	1 (1.9)	2 (5.6)	3 (3.4)	0.563
Bile Duct Margin	0	0	0	–
Anterior Circumferential Margin	0	0	0	–
Posterior Circumferential Margin	11 (20.8)	6 (16.7)	17 (19.1)	**0.785**
Vascular Groove	2 (3.8)	0 (0.0)	2 (2.2)	**0.513**
Uncinate Process	10 (18.9)	6 (16.7)	16 (18.0)	1.000
Duodenal	0 (0.0)	1 (2.8)	1 (1.1)	0.404
Invades Duodenum	25 (472)	19 (52.8)	44 (49.4)	0.669
Gastric	0	0	0	–
Pancreatic Duct	13 (24.5)	13 (36.1)	26 (29.2)	0.248
CBD	12 (22.6)	9 (25.7)	21 (23.9)	0.801
Peripancreatic soft tissue	43 (81.1)	32 (88.9)	75 (84.3)	0.386
Any margin	12 (22.6)	9 (25.0)	21 (23.6)	0.805
Whipple	53 (68.8)	36 (70.6)	89 (69.5)	1.000
Distal Pancreatectomy Characteristics	**Low (n = 24)**	**High (n = 15)**	**Total (n = 39)**	
Peripancreatic soft tissue	16 (69.6)	13 (86.7)	29 (76.3)	0.273
Pancreatic	0	0	0	–
Anterior	1 (4.3)	1 (6.7)	2 (5.3)	1.000
Posterior	2 (8.7)	4 (26.7)	6 (15.8)	0.188
Spleen	0 (0.0)	1 (6.7)	1 (2.6)	0.395
Any Margin	3 (13.0)	6 (40.0)	9 (23.7)	0.115

Low RNLS is defined as RNLS $\leq$ 13291.15ng/ml (median of data), high RNLS defined as $>$ 13291.15ng/ml (median of data). Total is defined as data for all patients included in the dataset. P-value column includes p-value between low and high RNLS groups.

https://doi.org/10.1371/journal.pone.0250539.t003

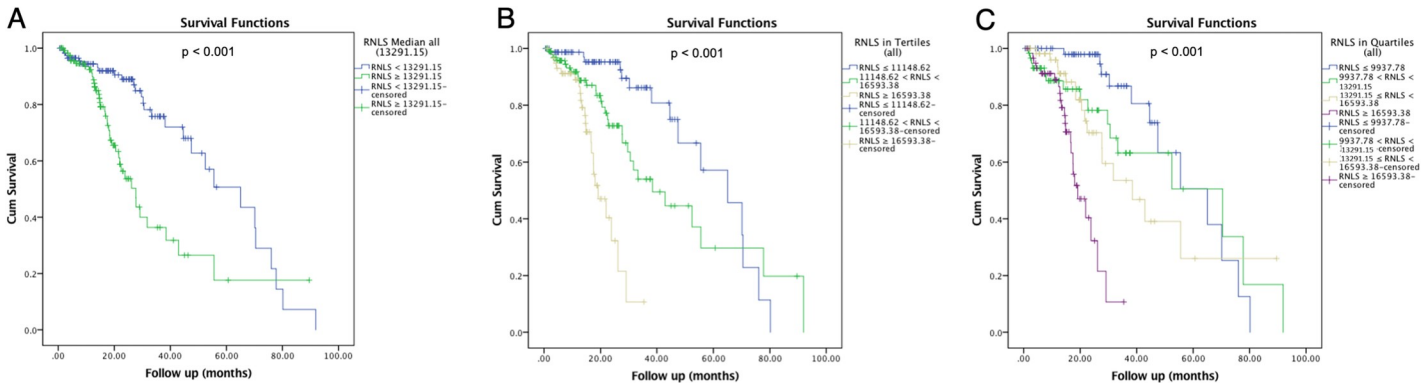

Fig 4. High plasma RNLS levels are associated with worse survival in patients with PDAC. (A) When RNLS is cut by median, (B) by tertiles, and (C) by quartiles.

https://doi.org/10.1371/journal.pone.0250539.g004

level among all LA/BR PDAC patients was 13601.55 ng/ml. Plasma acid-treated RNLS levels did not correlate with age, race/ethnicity, gender, BMI, serum albumin, or total blood protein at diagnosis (Table 4). High acid-treated RNLS level, defined as values above the median, did not correlate with high CA19-9 levels (Table 4). Of note, only 22 of the total 76 patients had CA19-9 values at diagnosis that met the exclusion criteria. Additionally, acid-treated RNLS levels were not associated with the reported tumor size, tumor location, or vessel encasement on imaging at initial presentation (Table 4). Among LA/BR patients, high acid-treated RNLS

Table 4. Patient characteristics for low vs. high plasma RNLS levels in patients with locally advanced/borderline resectable (LA/BR) pancreatic adenocarcinoma (PDAC).

Factor	Low RNLS (n = 38)	High RNLS (n = 38)	p-value
Age at Diagnosis	67.0 (46–90)	63.5 (37–86)	0.651
Race/ethnicity			0.744
White	35 (92.1%)	33 (86.8%)	
Black	2 (5.3%)	3 (7.9%)	
Other	1 (2.6%)	2 (5.3%)	
Gender			0.818
Male	20 (52.6%)	22 (57.9%)	
Female	18 (47.4%)	16 (42.1%)	
Chronic Kidney Disease	1 (2.6%)	2 (5.3%)	1.000
Hypertension	12 (31.6%)	14 (36.8%)	0.809
Heart Failure	1 (2.6%)	0 (0.0%)	1.000
Coronary Artery Disease	4 (10.5%)	2 (5.3%)	0.674
BMI at Diagnosis	25.45 (17.81–40.04)	26.34 (16.46–68.11)	0.875
Albumin at Diagnosis	4.10 (2.50–4.70)	4.00 (3.20–4.70)	0.232
Total Protein at Diagnosis	7.10 (5.60–8.20)	7.15 (5.70–8.30)	0.555
High CA19-9 at Diagnosis [a]	6 (50.0%)	2 (20.0%)	0.204
Large Vessel Encasement	10 (26.3%)	12 (31.6%)	0.801
PDAC Location			0.377
Head	31 (81.6%)	28 (73.7%)	
Body	6 (15.8%)	6 (15.8%)	
Tail	1 (2.6%)	4 (10.5%)	
PDAC Size by imaging	30.50 (15.00–90.00)	31.50 (18.00–92.00)	0.705

[a] Only includes patients with total bilirubin < 2.0 and CA19-9 >10 (n = 22).

https://doi.org/10.1371/journal.pone.0250539.t004

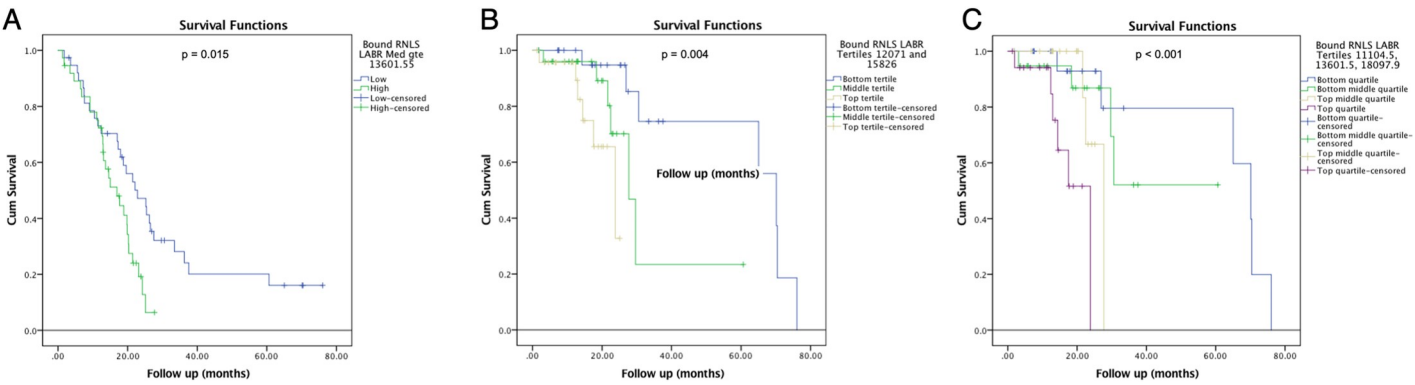

Fig 5. High plasma RNLS levels are associated with worse survival in patients with LA/BR PDAC. (A) When RNLS is cut by median, (B) by tertiles, and (C) by quartiles.

https://doi.org/10.1371/journal.pone.0250539.g005

levels were associated with worse overall survival than were low RNLS levels (p = 0.015, Fig 5A). At a median follow-up of 19.60 months, median overall survival was 22.83 months among patients with low RNLS and 16.93 months among those with high RNLS levels. Survival correlations were especially discriminatory when RNLS levels were analyzed using tertiles (n = 25 per group; Bottom tertile = 70.13 months, Middle tertile = 27.70 months, Top tertile = 23.83 months; p = 0.004, Fig 5B) and quartiles (n = 19 per group; Bottom quartile = 60.63 months, Bottom middle quartile = 43.67 months, Top middle quartile = 25.81 months, Top quartile = 18.62 months; $p < 0.001$, Fig 5C). As a continuous variable, RNLS level was also associated with survival (p = 0.002). CA19-9 levels were not as predictive of survival. Median survival among those with low vs. high CA19-9 levels was 21.47 vs. 19.60 months (p = 0.548). There was no significant difference in survival when compared to values for non-acid treated RNLS (S6 Fig).

Of the 76 patients with LA/BR PDAC, 18 (23.7%) underwent successful resection. Reasons for no eventual surgery in 58 patients were persistent local disease in 37 (48.7%), metastatic disease in 7 (9.2%), poor surgical candidacy in 4 (5.3%), and death in 3 (3.9%). The remaining 7 (9.2%) underwent exploratory laparoscopy and were subsequently found to have either unresectable local (5) or metastatic (2) disease upon surgical examination. Low RNLS levels were associated with greater adjusted odds ration (aOR) of undergoing resection (aOR = 0.29 (0.09–0.93, p = 0.036, Table 5). Of patients with low RNLS levels, 34.2% underwent resection compared to 13.2% patients with high RNLS levels, a > 2-fold difference. On the other hand, serum CA19-9 levels showed no relationship to resection status (aOR = 0.33, p = 0.272, Table 5).

Table 5. Treatments for high vs. low RNLS levels in patients with locally advanced/borderline resectable pancreatic adenocarcinoma as compared to CA19-9.

RNLS				
Factor	**Low (n = 38)**	**High (n = 38)**	**aOR (95%CI)**	**p-value**
Resection	13 (34.2%)	5 (13.2%)	0.29 (0.09–0.93)	**0.036**
Chemotherapy	36 (94.7%)	36 (97.3%)	2.00 (0.17–23.06)	0.578
Radiotherapy	18 (48.6%)	21 (58.3%)	1.48 (0.59–3.73)	0.408
CA19-9				
Factor	**Low (n = 14)**	**High (n = 8)**	**aOR (95%CI)**	**p-value**
Resection	3 (42.9%)	3 (20.0%)	0.33 (0.05–2.37)	0.272
Chemotherapy	6 (85.7%)	15 (100.0%)	–	1.000
Radiotherapy	5 (71.4%)	7 (46.7%)	0.35 (0.05–2.41)	0.286

https://doi.org/10.1371/journal.pone.0250539.t005

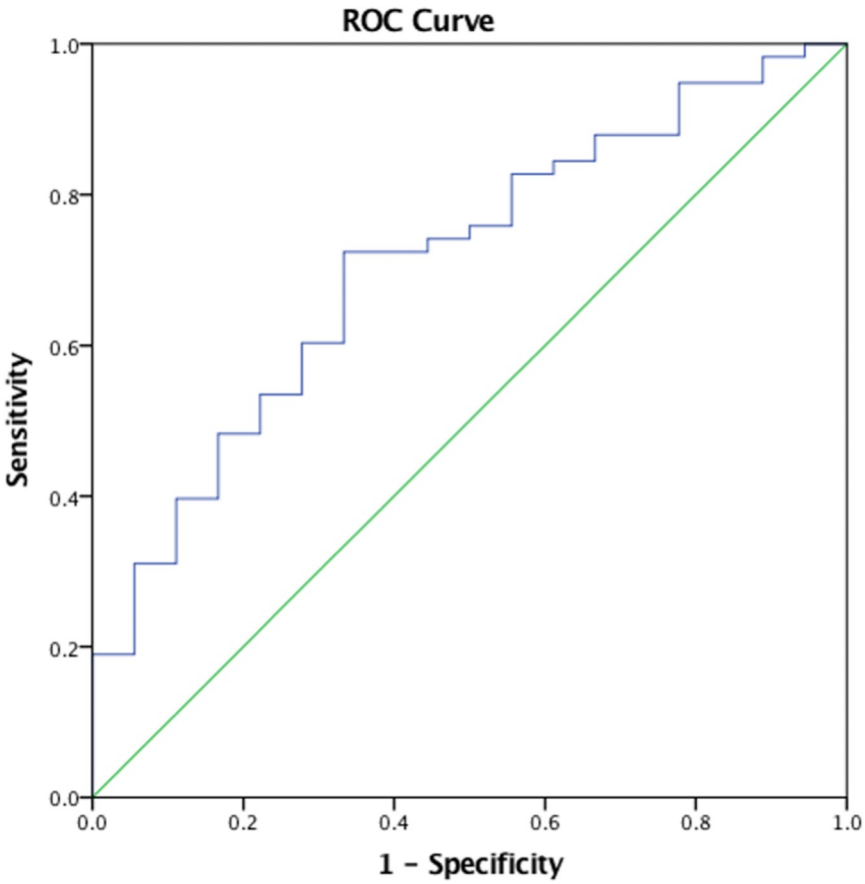

Fig 6. Plasma RNLS levels predict patients which will undergo resection in patients with locally advanced/ borderline resectable pancreatic adenocarcinoma in ROC Curve. AUC (area under curve) = 0.674.

https://doi.org/10.1371/journal.pone.0250539.g006

Further, receiver operating characteristic (ROC) analysis shows that lower plasma RNLS levels at diagnosis predicted resection among patients with LA/BR disease (AUC (area under curve) = 0.674; 95% CI 0.53–0.82; p = 0.037, Fig 6. The cut-off value with the highest sensitivity and specificity as determined by ROC analysis, 12548.46 ng/mL, was similar to the median of 13601.55 ng/mL and used as the cut-off for the designation of low vs high RNLS status. When using a cut-off value specified by AUC analysis, low RNLS was associated with resection with a sensitivity of 70.69% and a specificity of 66.67% (Table 6). Low CA19-9 levels showed lower sensitivity and lower specificity when compared to RNLS levels at AUC or median cut-off.

Discussion

Our data show that little to no RNLS immunoreactive protein was present in normal pancreatic head tissues but that RNLS was detectable to varying degrees in premalignant PDAC and

Table 6. Sensitivity and specificity tests of CA19-9 and RNLS levels to predict resection in patients with locally advanced/borderline resectable pancreatic adenocarcinoma.

	Sensitivity (95% CI)	Specificity (95% CI)
RNLS (Median)	43.10% (20.16%-56.77%)	27.78% (9.69%-53.48%)
RNLS (AUC)	70.69% (57.27%-81.91%)	66.67% (40.99%-86.66%)
CA19-9	0.00% (0.00%-45.93%)	50.00% (24.65%-75.35%)

https://doi.org/10.1371/journal.pone.0250539.t006

in PDAC tissues. The finding suggests a potential role for RNLS in the development of PDAC. Additionally, we found that high plasma RNLS levels correspond to worse PDAC tumor characteristics, worse overall survival, and less resectability in a subset of patients with LA/BR PDAC.

In a subset of samples primarily represented by chronic pancreatitis, RNLS was also present in stromal cells, including stellate cells with a myofibroblastic phenotype and other unidentified cell types. Stellate cells have important roles in the deposition of the extracellular matrix (stellate cells) and signaling to PDAC cells. The presence of RNLS in stellate cells shown in at least one chronic pancreatitis patient suggests that this protein could have a role in stellate cell function. Previous studies have shown that tumor microenvironment may exert selective pressures, including host immune response, proliferative or survival ability of cancer cells, or physiological restraints, which lead to the predominance of highly malignant PDAC cells [19, 20]. We have previously shown that RNLS is present in tumor-associated macrophages adjacent to melanoma, which can promote tumor growth through a STAT3-mediated mechanism [21]. Therefore, these findings suggest that RNLS could play a role in the tumor microenvironment during PDAC development. Future studies will determine the identity of the RNLS-labeled stromal cells that do not correspond to activated stellate cells and if RNLS levels can distinguish activated versus non-activated stellate cells.

Though essentially absent in other normal pancreatic head tissues taken from patients undergoing a Whipple procedure for duodenal adenomas, RNLS labeling was present in pancreatic tissue of patients who had undergone splenectomy with distal pancreatectomy for abdominal trauma. Normal pancreatic tissues from the pancreatic body or tail obtained in the absence of abdominal trauma were not available for analysis. Whether the RNLS immunoreactivity in the pancreatic tail reflects a local increase as an acute phase reactant [22, 23] in response to trauma or true differences in the RNLS content between distinct regions of the pancreas remains unclear.

A major finding of our study is that plasma levels of acid-treated RNLS correspond to PDAC prognosis. High plasma acid-treated RNLS levels were also associated with worse overall survival, angiolymphatic invasion, node positive disease, including sentinel hepatic artery lymph node positive disease, and metastasis. Plasma acid-treated RNLS levels were not associated with tumor size or surgical margin status among patients treated with Whipple or distal pancreatectomy surgery. It may be relevant that node positivity and metastasis reflect the clinically prognostic finding of tumor spread outside of the pancreas but that the effect of tumor size and margin status on prognosis in PDAC is controversial [24, 25]. Overall, these findings suggest that plasma acid-treated RNLS levels correlate with poor prognostic histopathologic features in PDAC. On the other hand, non-acid treated RNLS levels did not correspond with PDAC prognosis. Previous studies have found that non-acid treated plasma RNLS, considered its free form, is associated with mortality in patients with chronic kidney disease [15]. Our findings suggest that acid treated RNLS, hypothesized to represent a RNLS form in which its antigenic epitopes for RNLS antibody are hidden prior to acid treatment, however, may be associated with PDAC. The functional significance of this difference requires further evaluation.

Previous studies have suggested that tissue expression of RNLS is higher in select cancers than in benign tissues, including PDAC [26–30]. Tissue RNLS has been also described as a survival factor during ischemic or toxic injury and as a cytokine that activates several pathways, including PI3K/AKT, MAPK, p-ERK1/2, protein kinase B, and JAK/STAT pathways [28–30]. In PDAC tissue studies, high RNLS tumor expression was associated with worse overall survival [8]. Conditions that block RNLS can inhibit both in vitro and in vivo PDAC tumor growth [8]. Further studies would benefit from understanding the mechanism of elevated

plasma RNLS levels in patients with PDAC, whether different forms of RNLS have distinct tumor effects, if tissue RNLS contributes the levels of plasma RNLS or whether the changes in plasma levels represents host responses to the tumor. Knowledge of whether plasma RNLS levels change with PDAC resection and can predict recurrence could also be clinically useful.

When considering patients who presented with LA/BC PDAC, our data show that reduced plasma RNLS, but not CA19-9, can predict both whether LA/BR PDAC patients will be subject to resection and their overall survival; CA-19 blood levels were not found to be predictive of these outcomes. Specifically, low plasma RNLS levels at diagnosis were associated with eventual eligibility for PDAC resection and with increased overall survival. There was a more than 2-fold increase in the rate of resection in patients with low plasma RNLS levels compared to high plasma RNLS levels. Previous studies have found survival improvements with resection in appropriately selected patients with locally advanced/borderline resectable PDAC [31]. Though our study includes a limited number of patients, we found a non-significant trend towards improved survival in patients undergoing resection. Future studies should prospectively assess the role of RNLS in resection clinical decision making and survival outcomes. Additionally, whether plasma RNLS levels can predict chemoresistance should be considered in future studies to determine the potential utility of plasma RNLS in tailoring chemotherapeutic strategies. Together with tumor characteristic findings in resected PDAC samples, these findings suggest that plasma RNLS levels may reflect a tumor biology that predict outcome independent of radiologic tumor size, location, or vessel encasement at presentation. Plasma RNLS levels could also reflect characteristics that were not analyzed such as genetic, epigenetic or stromal microenvironment characteristics that portend worse outcomes. Future studies should also consider measuring RNLS levels in patients throughout the development of PDAC from normal controls to chronic pancreatitis to develop a broader understanding of how RNLS may change throughout PDAC development.

Our study has a few limitations. First, the small sample size of each tissue type and immunohistochemical protocol limits quantitative comparisons of the levels of RNLS in pancreatic tissue between each group. Future studies using greater numbers of tissue samples and an automated immunohistochemistry platform are needed to quantitively compare tissue levels of RNLS across tissues. For plasma RNLS studies, the retrospective cohort study design limited the ability to accurately assess survival over long periods of time to predict resectability without bias from independent treatment team decisions. We were also limited in analyzing factors that may play a role in determining a patient's candidacy for surgery such as comorbidities, fitness, and anatomical limitations that were not uniformly present in chart review. Without serial plasma RNLS measurements or concurrent tissue expression of RNLS on plasma RNLS samples, we cannot correlate plasma RNLS with underlying PDAC tissue expression. The small sample size of this study also limited power. A larger sample size may have yielded a potential relationship between CA19-9 and RNLS that was not seen here among LA/BR patients. Additionally, for example, we found the median survival among patients who underwent resection versus no resection was 29.67 months vs. 30.57 months ($p = 0.060$). This small sample size makes it difficult to evaluate this parameter in this cohort. Finally, there may have been selection bias associated with the type of patients who obtained care at our institution and subsequently were included in the biobank.

In conclusion, a key finding of our study is that RNLS is increased in both premalignant and malignant PDAC tissue compared to normal pancreas, suggesting a potential role for RNLS in the early development of PDAC. In addition, we found that the relationship between plasma RNLS levels and clinical outcomes of patients with PDAC complements published data that correlated tissue RNLS expression and PDAC survival. These findings suggest that plasma RNLS could be used as a predictive biomarker in patients with PDAC and guide therapies

such as resectability in LA/BR PDAC. The RNLS levels in tissue and plasma suggest a potential pathophysiological mechanism of RNLS for the development of PDAC and severe PDAC disease. Further studies should explore the potential mechanism of action of RNLS in pre-malignant pancreatic tissue and its expression in stromal cells including stellate cells. Additionally, further studies should also explore disease progression patterns, plasma RNLS levels and tumor RNLS expression after resection, and the origin of plasma RNLS compared to tumor expression of RNLS. To further assess the ability of plasma RNLS to predict resectability in LA/BR pancreatic disease, larger prospective studies are needed that examine changes in plasma RNLS levels following neoadjuvant treatment. If plasma and tissue RNLS indeed reflects tumor biology and pathophysiology, it holds promise as a guide to surgical interventions and potential therapies that inhibit the pro-survival effects of RNLS in PDAC.

Supporting information

S1 Fig. RNLS labeling in normal pancreas.
(TIFF)

S2 Fig. RNLS labeling in chronic pancreatitis.
(TIFF)

S3 Fig. RNLS labeling in PanIN and MCN lesions.
(TIFF)

S4 Fig. RNLS labeling in IPMN and PDAC lesions.
(TIFF)

S5 Fig. Association between high and low RNLS levels with PDAC survival.
(TIFF)

S6 Fig. Association between high and low RNLS levels with survival in locally advanced/borderline resectable PDAC.
(TIFF)

S7 Fig. Acid-treated vs non-treated plasma RNLS in PDAC.
(TIFF)

S1 Table. Demographics of PDAC patients.
(TIFF)

Acknowledgments

We would like to acknowledge the staff members of the Yale Gastrointestinal Tumor Biorepository for providing access to patient samples for this study. The authors also wish to thank Christine Shugrue and Thomas Kolodecik for providing technical and constructive comments.

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Review

Renalase: A Multi-Functional Signaling Molecule with Roles in Gastrointestinal Disease

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Abstract: The survival factor renalase (RNLS) is a recently discovered secretory protein with potent prosurvival and anti-inflammatory effects. Several evolutionarily conserved RNLS domains are critical to its function. These include a 20 aa site that encodes for its prosurvival effects. Its prosurvival effects are shown in GI disease models including acute cerulein pancreatitis. In rodent models of pancreatic cancer and human cancer tissues, increased RNLS expression promotes cancer cell survival but shortens life expectancy. This 37 kD protein can regulate cell signaling as an extracellular molecule and probably also at intracellular sites. Extracellular RNLS signals through a specific plasma membrane calcium export transporter; this interaction appears most relevant to acute injury and cancer. Preliminary studies using RNLS agonists and antagonists, as well as various preclinical disease models, suggest that the immunologic and prosurvival effects of RNLS will be relevant to diverse pathologies that include acute organ injuries and select cancers. Future studies should define the roles of RNLS in intestinal diseases, characterizing the RNLS-activated pathways linked to cell survival and developing therapeutic agents that can increase or decrease RNLS in relevant clinical settings.

Keywords: cell survival; renalase; PMCA4b; signaling; protein kinase; inflammation; pancreatitis; pancreatic cancer

Citation: Pointer, T.C.; Gorelick, F.S.; Desir, G.V. Renalase: A Multi-Functional Signaling Molecule with Roles in Gastrointestinal Disease. *Cells* **2021**, *10*, 2006. https://doi.org/10.3390/cells10082006

Academic Editor: Marc Basson

Received: 17 June 2021
Accepted: 28 July 2021
Published: 6 August 2021

1. Introduction

Renalase (RNLS) was first identified through the Mammalian Gene Collection Project [1]. Early work on RNLS focused on the proximal renal tubule of the kidney as a major source of circulating RNLS. Its tissue distribution and its enzymatic activity led to the name renalase. The reduced plasma renalase levels in chronic kidney disease (CKD) led to the posit that it might mediate complications of this condition. Its potential enzymatic functions, specifically its effects on catecholamine regulation and blood pressure, provided potential links between RNLS and its reduced levels with renal insufficiency, as well as CRF complications [1–4]. Subsequent studies have shown how RNLS can modulate the severity of acute injuries in experimental disease models that include the pancreas, liver, kidney, and heart. RNLS plasma and tissue levels were also reported to be dynamically regulated by environmental changes and injury. Given its prosurvival effects in acute injury settings, it is not surprising that RNLS was a molecular driver for select cancers. In preclinical studies, increasing extracellular RNLS levels has been shown to reduce a range of acute injuries while reducing RNLS levels has been shown to slow cancer growth. Recent studies find that extracellular RNLS signals through a specific plasma-membrane protein and elicits distinct intracellular signals. Although RNLS signaling is known in some systems, how the signals link to biologic responses remains unclear. Here, we review some of the foundational preclinical studies on this protein and then focus on its interesting structural and evolutionary features as well as its regulation and mechanisms of action.

2. Renalase Has Potential Roles in Disease: Findings from Preclinical Studies

2.1. Effects on Acute Injury

Initial studies of RNLS function examined whether it could reduce the effects of CRF using a murine model of acute kidney injury (AKI) that was treated with exogenous recombinant RNLS (rRNLS) [5]. The rRNLS treatment reduced plasma catecholamine levels and dramatically decreased ischemic renal injury, including inflammation. rRNLS was also protective in cellular models of oxidative injury and an in vivo murine model of acute ischemic renal injury. The protective effects of rRNLS we observed in the kidney prompted our examination of RNLS in another preclinical model of acute injury, acute pancreatitis.

Murine models of acute pancreatitis, using both isolated cells and in vivo preparations and treatments with cholecystokinin orthologue, cerulein, are often used for preclinical studies [6]. In the in vivo cerulein model, injury first occurs in the acinar cell, and later phases are mediated by inflammation. In both isolated acinar cells and in vivo, rRNLS reduced cerulein-induced injury [7]. Notably, the benefits of rRNLS in vivo were observed when administered well after the induction of pancreatitis, suggesting that RNLS can reduce inflammatory responses independent of its effects on the acinar cell in this model. The effects on inflammation are likely mediated in part by RNLS's suppression of macrophage-dependent IL6 release, a pathway important to many forms of acute cellular injury. An unexpected finding in the pancreatitis study was that the plasma levels of renalase were dramatically reduced early in the course of acute pancreatitis but rebounded far above basal levels during recovery. This suggests that plasma RNLS levels are dynamically regulated in both the acute and recovery phases of injury.

Subsequent studies by others have suggested that RNLS may have a role in mediating other forms of gastrointestinal injury. RNLS was shown to reduce oxidative liver injury in cellular and an in vivo murine model of ischemia/reperfusion injury that was superimposed on models of fatty liver disease [8]. Similar to acute pancreatitis effects, exogenous rRNLS reduced injury in intact liver and related cellular disease models. Although its protective mechanism is not fully understood, RNLS reduced mitochondrial injury in liver cells. A high fat diet reduced both hepatic RNLS gene expression and plasma RNLS levels, underscoring the potential importance of environmental regulation of tissue RNLS expression. It also suggests that plasma levels may be relevant to acute injury responses. Environmental oxidative stress changed RNLS expression in small intestinal crypts and cultured transformed intestinal cells [9]. Similar to the liver, increasing RNLS levels (by transfection) reduced oxidative injury in cultured intestinal cells. Together, these studies suggest that environmental factors could modulate tissue and plasma levels of RNLS. It also suggests that RNLS could have essential roles in modulating injury in the pancreas, liver, and small intestine. Together, these studies underscore the potential for using RNLS agonists as therapeutic agents to modulate the acute injury severity.

2.2. Effects on Cancer

The prominent prosurvival effects of RNLS on multiple cell types raised the possibility that it might have a similar role in cancer. RNLS mRNA levels were increased in PDAC and melanoma and other cancers. Our first study examined a potential role for RNLS in pancreatic cancer (PDAC) using human tissues, cultured cells, and a murine model [10]. Tissues levels of RNLS in PDAC corresponded to patient survival inversely; the higher RNLS levels the shorter the survival. Inhibition of RNLS using siRNA or an inhibitory monoclonal antibody to RNLS reduced PDAC growth in cultured cells and in vivo. Similar studies in patients with melanoma showed that RNLS tissue levels are inversely related to survival and required for experimental melanoma growth [11]. Using immune-localization to determine the identity of cancer cells associated with RNLS, researchers found that much of the RNLS immunoreactivity was in tumor-associated macrophages as well as melanoma cells. The localization of RNLS in cancer and immune cells suggests that more than one cell type might mediate the effects of RNLS on cancer cell growth. Together, these promising findings suggest that agents that inhibit the effects of RNLS in select cancers could be

attractive new therapeutic tools and should also encourage investigations of RNLS in other cancer types.

Collectively, these models of acute injury and cancer show prosurvival roles for RNLS. Depending on the disease context, RNLS could either be of benefit (in acute injury) or detriment (in cancer) to the organism.

2.3. The Cell Growth and Anti-Inflammatory Activities of RNLS Localize to a Specific Site

The biologic activities have been examined by using sequence homology analysis and studies specific regions of the RNLS protein. Most relevant to this review, its cell growth and anti-inflammatory activities are found in a 20 amino acid peptide (aa 220 to 239 of human RNLS), which is referred to as the RP-220 peptide [12]. The critical role of the RP-220 is underscored by the fact that it has been used to identity the cellular RNLS receptor (Section 6.2.1) and also as the target for generating a humanized monoclonal antibody (m28) that greatly reduces pancreatic cancer and melanoma growth in cells and in vivo (Guo and Hollander).

Together, these studies provided compelling evidence of key regulatory roles for RNLS as a prosurvival factor and modulatory of inflammatory responses in preclinical models of acute tissue injury as well as in cancer. To understand RNLS's mechanism and as a critical step in developing agents that could modify or mimic its biologic activities, we have begun to study its structure and complex effects on cells including its links to distinct intracellular signals. This will be the subject of the remainder of this review.

3. RNLS Forms, Conservation, Structure, and Expression

3.1. RNLS Isoforms

The human RNLS (hRNLS) gene contains approximately 300,000 nucleotides across 11 exons and is located on chromosome 10 [13]. Two major isoforms, RNLS1 and RNLS2, are found in humans (Figure 1). RNLS1 has 1477 nucleotides, 7 exons, 342 residues, and a theoretical molecular mass of 37.85 kD. RNLS2 has 2107 nucleotides, 5 exons, 315 residues, and a theoretical molecular mass of 34.95 kD [14]. Potential distinct splice variants were also identified in humans [13,15,16]. The other variants, including much the much shorter RNLS 3 and 4 (RNLS3 and RNLS4), would lack oxidase funct
potential existence of these variants complicates the characteriz
responses. Further, RNLS1 antibodies have been shown to recc
that effects previously attributed to RNLS1 could be RNLS2 o
the isoforms [14].

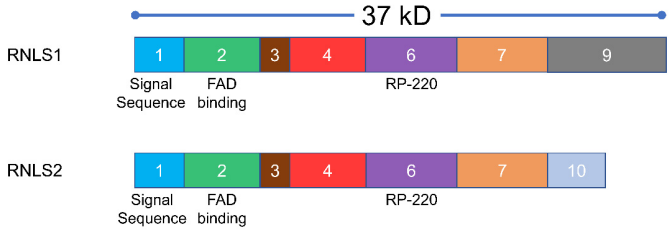

Figure 1. Retinaldehyde domains are conserved between RNLS1 and RNLS2, which only differ in the C-terminal site because of differences in splicing. The RP-220 site has prosurvival activity; it is also target for an inhibitory RNLS antibody.

Figure 1. Retinaldehyde domains are conserved between RNLS1 and RNLS2, which only differ in the terminal site because of differences in splicing. The RP-220 site has prosurvival activity; it is also target for an inhibitory RNLS antibody.

3.2. RNLS Sequence Conservation

Soon after the discovery of RNLS in 2005, investigations into the evolutionary history of the protein yielded surprising findings. One study of cyanobacteria-originated metazoan/fungi proteins (COG4) developed a hypothesis that RNLS originated from ancient horizontal gene transfer [17]. This study places the horizontal gene transfer event that included RNLS around 700 million years ago between the first eukaryote naissance (1200 million years ago) and the first metazoan appearance (570 million years ago) [18,19]. Further, the gene would have diverged to produce the lineages and genes that we see today with an accumulation of changes. The conservation of RNLS sequence domains is supported by more recent work that found overall sequence similarity between plant gene products and mammalian RNLS [13]. Through evolution, it is likely that the function of this protein has evolved and may be reflected in changes in sequence. However, the existence of the protein dating so far back in the tree of life suggests integral, but potentially changing, function(s).

We have examined segments of the RNLS sequence that encode for predicted or known activities to provide insights into their relative functional importance [20]. We included alignment of the hRNLS amino acid sequence to RNLS genes found in *Pan troglo-*

In contrast to the two RNLS isoforms expressed in humans, only one form is found in murine tissues; the murine form is most similar to human RNLS1 (hRNLS). hRNLS1 and hRNLS2 only differ in their C-terminal domains, and the key functional domains are conserved between the isoforms (Figure 1). This includes N-terminal signal sequences and a FAD-binding domain and the RP-220 site in exon 6 (see Section 3.3). The enzymatic FAD oxidase of RNLS is in the N-terminal region and a putative catecholamine-binding

motif is found in the center of the molecule. Details of RNLS structure are discussed below (see Section 3.3).

3.2. RNLS Sequence Conservation

Soon after the discovery of RNLS in 2005, investigations into the evolutionary history of the protein yielded surprising findings. One study of cyanobacteria-originated metazoan/fungi proteins (COPs) developed a hypothesis that RNLS originated from nuclear-localized plastid-like DNA (nupDNA) fragments from cyanobacteria [17]. This study places the horizontal gene transfer event that included RNLS around 700 million years ago between the first eukaryote naissance (1200 million years ago) and the first metazoan appearance (570 million years ago) [18,19]. After this transfer, the gene would have diverged to produce the lineages and genes that we see today with an accumulation of changes. The conservation of RNLS sequence domains is supported by more recent work that found overall sequence similarity between plant gene products and mammalian RNLS [13]. Through evolution, it is likely that the function of this protein has evolved and may be reflected in changes in sequence. However, the existence of the protein dating so far back in the tree of life suggests integral, but potentially changing, function(s).

We have examined segments of the RNLS sequence that encode for predicted or known activities to provide insights into their relative functional importance [20]. We included alignment of the hRNLS amino acid sequence to RNLS genes found in *Pan troglodytes* (chimpanzee) and *Rattus norvegicus* (rat). The RNLS N-terminus is thought to be necessary for FAD-binding and some of the oxidase activity of RNLS [21,22]. Of the first 15 amino acids, 11 are identical and 3 more have been substituted by conserved residues. The RP-220 region that has effects on cell survival and inflammation (discussed below) is also highly conserved. Of the 20 residues in the region (aa 220-239 of hRNLS 1 and 2), 16 are identical, and 1 has been substituted by a residue with similar properties among these three species.

We have also analyzed sequences from non-vertebrates, such as *Actinia tenebrosa* (Australian red waratah sea anemone). In the 15 residues that aligned with the sequence of the N-terminal of hRNLS, 9 are identical and 4 have been substituted by residues with similar properties. The identical residues include the GXGXXG motif of a Rossman-fold, a necessary component for the binding of FAD to the protein [21,23]. For the RP-220 region, nine residues are identical and five more have been substituted by residues with conserved properties. When comparing the sequences, research found that other regions show high conservation. For example, the residues 190–200 are identical throughout all four sequences, suggesting that it has an important but yet to be identified biological function. This site is also predicted to be exposed on the surface of RNLS in a potential binding region [24].

3.3. RNLS Structure

The three-dimensional RNLS1 crystal structure has been determined using purified recombinant soluble human renalase expressed in E. coli bacteria [24,25]. A prominent feature of the structure showed a FAD-binding domain (Rossmann fold) [24]. This three-dimensional structure is similar to MAO-A, MAO-B, and polyamine oxidase, but lacks some of the residues predicted to be essential for amine oxidation catalysis [15,26,27]. Of note, analysis of RNLS1's crystal structure revealed no disulfide bonds. This was unexpected since the protein contains 12 cysteine residues [15]. Unless specifically engineered to do so, bacteria lack the machinery needed to properly align disulfide residues. This makes recombinant proteins made in bacteria susceptible to misfolding and could produce misleading results relating to protein structure and function.

The potential of RNLS to metabolize amines was identified as a possible function based on its sequence homology with other flavoproteins. More recently, RNLS was noted to lack the helical domain found in other flavin-dependent amine oxidases (FAO) superfamily members. In other FAO proteins, this helical domain appears to be important in substrate recognition/binding and membrane interactions [28]. Interestingly, the RNLS NAD(P)H oxidase activity increased after mutations that inserted more positively charged

residues in the active site [29]. The association with FAD with RNLS is thought to be non-covalent [21]. It has since been shown that the large hydrophilic cavity of RNLS seems appropriate for a charged nicotinamide substrate [28]. The RNLS cleft that associates with nicotinamide dinucleotides does not appear to exhibit hydrogen bonds with ribose; instead, it interacts with the pyrophosphate moiety [30]. Whether RNLS binds PPi and the potential role of this as a signaling moiety in such processes as glucose metabolism deserve further consideration [31]. Overall, the structure of RNLS1 has been well characterized, especially those regions involved in FAD and NAD(P)H binding; the importance of RNLS interactions with FAD and NAD are discussed below (see Section 4). Other three-dimensional regions (including RP-220 that contains important biologic activities) are not as well understood and deserve further investigation, perhaps using recombinant proteins engineered to ensure proper folding.

4. RNLS Cell Biology

4.1. Cellular Distribution/Secretion

RNLS has an n-terminal signal sequence that appears to be cleaved in secreted forms [1,16,32–36]. RNLS1 and RNLS2 have been shown to contain the FAD-binding moiety and N-terminal peptide before secretion. One group detected RNLS in the urine and found that it lacked this N-terminal signal peptide [21]. The FAD-binding region forms an alpha helix and a beta-strand critical to the formation of the Rossman fold, according to in silico studies [21]. Those authors suggest that this truncated RNLS is incapable of binding FAD, meaning any effects of this truncated RNLS are FAD-independent [22]. It may be relevant that our unpublished data suggest that RNLS might be secreted, at least in part, through a non-canonical secretory pathway and may also retain its signal peptide that does not exhibit truncation of the putative FAD binding domain. Additional studies are needed to define the mechanism(s) of RNLS secretion, how secretion is stimulated, and the processing of its signal peptide. The intracellular itinerary of RNLS may vary in an isoform and tissue-dependent manner and be affected by local and systemic environmental factors.

The intracellular distribution of RNLS has not been fully defined; in some cells, it has been localized to distinct intracellular structures and in others at or near the plasma membrane.

4.2. RNLS Cellular Expression Is Regulated

Tissue levels of RNLS can be regulated and this response

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Several factors (Figure 2) have been shown to upregulate RNLS gene expression including STAT3, Sp1, ZBP89, NF-κB, HIF-1α, and TNF-α in various tissues [5,37]. Suggested with positive feedback loop with RNLS has been suggested with STAT3 enhancing RNLS production and RNLS, in turn, increasing STAT3 activation [11,37].

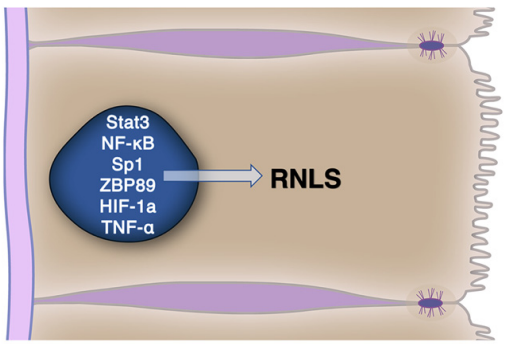

Figure 2. Renalase gene expression is regulated by a range of transcription factors, several of which are linked to proinflammatory responses.

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To investigate these pathways as well as RNLS function, our laboratory generated a rabbit monoclonal antibody (m28) to the human RNLS aa220-239 RP-220 peptide that encodes for its prosurvival activity. The antibody inhibited the growth of cultured pancreatic cancer cells as well as in vivo tumors growth in a murine model of pancreatic cancer. This was associated with a significant reduction in both RNLS expression and STAT3 (both phosphorylated and total), also supporting the presence of a positive feedback loop. The inhibition of RNLS and reduced STAT3 signaling were associated with decreased cell proliferation and increased apoptosis [11]. Similar RNLS-dependent STAT3 signaling was found in Panc1 pancreatic cancer cells and the human kidney HK-2 cells [10]. A link between RNLS expression and STAT3 was also observed in a model of fatty liver disease [8].

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Another potentially important RNLS regulatory pathway that may be relevant in the context of both ischemic injury and cancer relates to the four potential HIF-1α-binding motifs identified within the promoter region of RNLS. These are responsible for HIF-1α-induced increases in RNLS expression. In HIF-1 α knockdown mice, the genetic deletion worsened experimental ischemia/reperfusion (I/R) cardiac injury compared to wild-type (WT) mice. Injection of RNLS reversed the effects of HIF-1α deletion on the excess deterioration of cardiac function [39]. This indicates a significant role for RNLS in mediating the protective effects of HIF-1 α, at least in the context of I/R cardiac injury. Together, the involvement of these factors suggests complex, multi-faceted roles for RNLS action and regulation of its levels that will likely affect divergent and distinct signaling pathways.

5. Renalase Signaling

5.1. Enzymatic Activities of RNLS

The potential enzymatic functions of RNLS1 were the focus of the original studies of the protein [1,4,26,27]. Although RNLS's in catecholamine metabolism remains unclear (although direct or indirect effects are very likely), interests in the enzymatic properties of RNLS, which may be most relevant to the function of an intracellular pool, has shifted to the oxidative metabolism of NAD(P)H [30,40,41]. Though the scope of studies has since broadened to include other functions of the protein (specifically its signaling capabilities), it is still critical to consider the enzymatic properties when determining the role of RNLS1 in disease and recovery.

5.1.1. Enzymatic Activities and NAD

Our laboratory has predicted and shown evidence that RNLS functions as a NADH oxidase [42–44]. Other studies showed that RNLS oxidizes and epimerizes α-NAD(P)H molecules [24,45,46]. Subsequent studies showed that this oxidation takes place at the 2- or 6-position of the nicotinamide ring, rather than the metabolically active 4-position. This supports a role as a "scavenging" enzyme that prevents the buildup of unusable substrates [41]. That RNLS can convert metabolically inert 2 or 6 NADH to active 4-NAD+ may have biologic relevance since a reduction of NAD/NADH is observed in the myocardium of RNLS knockout mice [47]. NAD metabolism is tightly coupled to cellular energy metabolism [48]. That cellular ATP levels are also dramatically reduced in RNLS knockout mice (unpublished data) suggests that RNLS, particularly an intracellular pool that modulates NAD metabolism, could have profound effects on cellular energy production. Overall, this function is compatible with the larger theme of RNLS as a prosurvival factor, and this functionality might apply to both its intracellular and extracellular functions.

5.1.2. Other Potential Enzymatic Activities

Findings related to the potential catecholamine-metabolizing activity of RNLS vary widely [1,3,13,25,42,46,49]. One study claimed that although RNLS might bind epinephrine, this binding is distant from the oxidase site and does not affect NADPH binding or the flavin environment [46]. Whether RNLS directly regulates catecholamine levels enzymatically remains unclear. However, it does appear to have a prominent effect on plasma catecholamine levels with plasma epinephrine levels dramatically increasing in RNLS

knockout mice [16,47]. In the context of its enzymatic activities, the full-length recombinant human RNLS (rhRNLS) used for published assays have used proteins made in yeast that may not have been properly folded and could provide misleading information relating to its enzymatic activities. In addition, RNLS may interact with other molecules that either modify its activities or serve as biologic targets. These functions of RNLS have likely to have changed significantly over time and across lineages. Some functions may be remnants of ancient forms of the protein that are now redundant with other proteins.

6. Signaling by Extracellular RNLS

6.1. The Growth and Modulatory Effects of RNLS Are Found in a Specific RNLS Site

As mentioned above, we have used models of acute renal injury and truncated rRNLS constructs, to identify the prosurvival and anti-inflammatory effects of RNLS and localized the activity to a 20 amino acid peptide (aa 220 to 239 of human RNLS referred to as RP-220) [12]. The site is located far from the region of the molecule that contains enzymatic activity. The Rp-220 peptide replicates many of the biologic effects and signaling pathways activated by full-length RNLS [50,51]. It also has one of the most highly conserved amino acid sequences through evolution in the molecule (discussed elsewhere). The importance of the RP-220 sequence to the function of RNLS is underscored by the finding that monoclonal antibodies to the RP-220 peptide can be potent inhibitors of cancer growth [10,11].

6.2. Extracellular RNLS Stimulates Intracellular Signaling

Although the enzymatic function of RNLS was the initial focus of our laboratory, in later structure–function studies, we discovered that RNLS also has independent functions as an extracellular signaling factor that plays an important role in modulating inflammation and recovery from acute injury [7]. We find that many of RNLS's prosurvival functions are independent of the oxidase properties of RNLS [12].

6.2.1. Identification of a Plasma Membrane Receptor for RNLS

Since treatments adding rRNLS or RP-220 to the extracellular compartment resulted in biologic responses, we anticipated that the molecules were likely acted by binding to a protein on the cell surface. To identify the protein, we covalently linked the RP-220 peptide to membrane proteins. The bound proteins identified by mass spectroscopy included plasma-membrane calcium export pump, PMCA4b (plasma-membrane calcium ATPase 4b), as a leading candidate. Its interaction with RNLS is also shown by co-immunoprecipitation. PMCA4b is concentrated on the basolateral membrane of acinar cells, suggesting that it responds to extracellular RNLS. This was confirmed by functional studies that included showing that PMCA4b downregulation by siRNA or its inhibition by caloxin 1b1 blocked the prosurvival effects of RNLS [7,50]. Since PMCA4b is a widely distributed plasma-membrane transporter, it may mediate the effects of extracellular RNLS in other tissues. Further details relating to PMCA4b signaling are provided in Section 6.2.2.

Limited studies suggest that RNLS and PMCA4b may interact with other cell surface receptors, such as the G-protein-sensitive estrogen receptor (GPER), which can downregulate PMCA4b when activated with estradiol (Section 6.2.2). Likely, the signals elicited by extracellular RNLS described in the following section can be modified by other yet unknown signaling mechanisms.

6.2.2. RNLS Stimulates Distinct Intracellular Signals

We have examined the effects of extracellular RNLS on intracellular-regulated protein kinase pathways. These include activation of AKT, ERK, p38, B cell lymphoma 2, and inhibition of c-Jun K-terminal kinase (Figure 3) [12]. The RP-220 peptide sequence and related peptides common to all known forms of RNLS have been used in most of our studies. The RP-220 site (RNLS1 aa 220-239) has no known enzymatic activities, but it has demonstrated a prosurvival effect after acute injury to kidney, heart, and corneal epithelial cells [12,51,52]. In select systems, the signals induced by full-length RNLS have been

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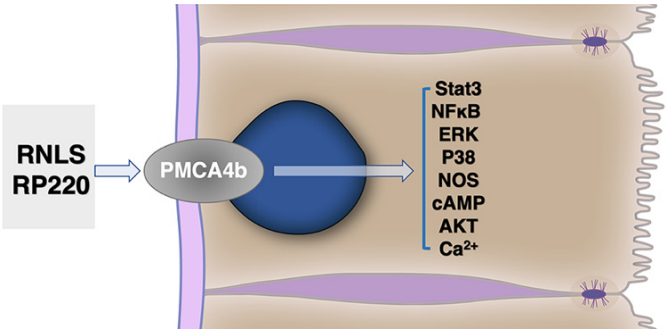

Figure 3. Renalase signaling through the plasma-membrane calcium ATPase (PMCA4b) is linked to the stimulation of multiple downstream signals.

Some of the same signaling pathways activated by RNLS and RP-220 have also been linked to PMCA4b signaling. These include the Ras/Raf/MEK/ERK, p38, NOS, NF-κB, cAMP, and AKT pathways [12,53–55]. One study described an interesting potential feedback loop, finding that p38 (which is upregulated with RNLS treatment) induces degradation and downregulation of PMCA4b [55]. When treated with a p38 inhibitor, PMCA4b degradation was blocked and calcium clearance from the cell increased. This p38-dependent response could be therapeutically important in certain types of injury, such as acute pancreatitis, where calcium efflux is a key modulator of injury and recovery [56]. It has also been suggested that inhibition of PMCA4b could increase NO accumulation [57]. This suggests the possibility that if RNLS activates PMCA4b, NO accumulation could be reduced, leading to less severe injury or more rapid recovery. NF-κB-regulated RNLS expression has also been shown to cause increased phosphorylation of AKT through a PMCA4b-dependent mechanism [58]. In B16-F10 cells, PMCA4b was also found to have a key role in RNLS-dependent ERK and STAT3 phosphorylation [11]. Together, these studies demonstrate that RNLS can affect several distinct cell signaling pathways in a PMCA4b-dependent manner.

PMCA4b has a PDZ domain, a protein motif that often links transmembrane proteins or proteins that concentrate near membranes. In the case of PMCA4b, it can link the calcium exporter to other signaling pathways [59]. For example, PDZ-dependent signaling of PMCA4 was found to be a negative regulator of NOS-I [60]. PDZ domains interact with other proteins that often have scaffolding functions and that can anchor multiple protein signaling cassettes and do so in a tissue-dependent manner [61–63]. The interactions of the PDZ domain have been found in a variety of cell types including neuronal cells, smooth muscle cells, and cardiomyocytes [60,61,64,65]. This model is potentially crucial to understanding the role of PMCA4b and the PDZ domain and might provide one mechanism to relate cellular Ca^{2+} levels to NO production and other cells signals [53] More recently, it was determined that constitutive ERK activation is PDZ-dependent, while activation by a specific agonist, G-1, is PDZ-independent [66]. This variety in signaling could help promote signal specificity and have distinct outcomes in a tissue-specific manner.

The PMCA4b PDZ domain appears to enable the membrane protein GPER (G-protein estrogen receptor) to be coupled in a regulatory manner to PMCA4b through the linker protein, PSD95 (post-synaptic density 95). Activation of GPER by estradiol results in the tyrosine phosphorylation of PMCA4b and downregulation of its calcium-export activity [57,67]. This observation establishes an important precedent for PMCA4b, and hence RNLS-signaling, to be modified by another tightly coupled receptor. In the case of GPER, its activation could in theory counteract the effects of RNLS and reduce the prosurvival and anti-inflammatory effects of RNLS. It will be important to consider how PMCA4b activity

might be modulated by PDZ-linked proteins as well as how PMCA4b itself might change the function of linked proteins.

7. RNLS Presence in Human Intestine

Though discovered and primarily studied in the kidney, RNLS gene expression can be found in virtually every organ including those of the gastrointestinal tract. Our preliminary studies have also detected RNLS in select gastrointestinal tissues using immunocytochemistry. For example, we used the m28 antibody to detect immunoreactivity (protocol Appendix A) in colonic mucosal cells (Figure 4). This tissue shows RNLS labeling (dark brown stain not seen in the control lacking the primary antibody) can be seen in human colonic cells surface epithelial cells throughout the crypt and scattered throughout the submucosa. The presence of RNLS in cells at baseline suggests that it may have a role in tissue homeostasis. A model characterizing the role of RNLS in one or more of these tissues may apply to other tissues. Other proteins essential for RNLS signaling, such as the isoform 4b of plasma membrane calcium ATPase (PMCA4b), have also been identified in these tissues [68–70]. A model describing the role of RNLS in reducing injury and modulating inflammation across these systems would be of great importance. The potential role of RNLS as a modulator of inflammation may be best studied in the intestine due t
pro-inflammatory state. Given the strong moderating effect of RNLS on the
and immune response, we speculate that RNLS could contribute to gut ho
modulating the response to foreign molecules that activate innate immune r

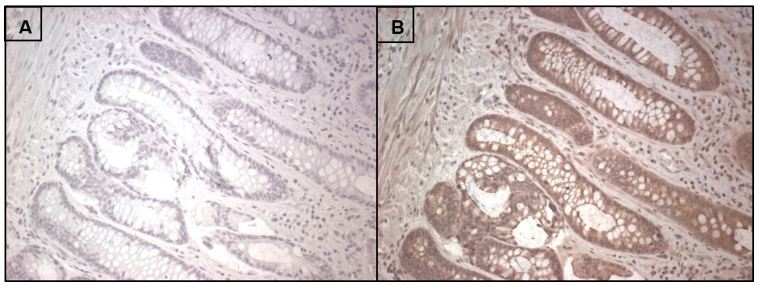

Figure 4. Renalase immunoreactivity in normal human colonic mucosa labeled with anti-m28-RNLS or 2.5% horse serum using an immunoperoxidase method labeled with (**A**) no primary antibody as a negative control; (**B**) anti-renalase m28 antibody. The 2.5% horse serum (**B**) an immunoperoxidase method labeled with (**A**) no primary antibody as a negative control; (**B**) anti-renalase m28 antibody. The specific brown RNLS labeling (**B**) is most prominent on the epithelial cells lining the crypts of the intestinal glands. Tissue samples were de-identified and acquired through a Yale HIC-approved protocol by Dr. Marie Robert.

8. Cell and Tissue-Specific RNLS Responses

8.1. Macrophages

There is growing evidence that immune cells are an important target for RNLS signaling [51,71,72]. Macrophages are often referred to as having characteristics of an M1 (proinflammatory) or M2 (anti-inflammatory) phenotype. Exposure of macrophages to RNLS has been shown to increase RNLS production and to drive M2 phenotype [11]. It has also been suggested that dysregulated RNLS signaling could influence macrophages toward a tumor-promoting, M2-like phenotype [51]. Exposure of activated (M1) macrophages to RNLS has been shown to block inflammasome activation and IL1β production [51]. Although macrophages have been shown to express PMCA4 [73], whether RNLS signals through this transporter remains to be explored.

8.2. Others

In a study in the liver, a marker of mature macrophages, Adgre1, was found to be significantly higher in RNLS KO mice during the progression of nonalcoholic steatohepatitis (NASH). It is suggested that this hepatocyte dysfunction and hepatic fibrosis progression could be connected to the absence of RNLS. Additionally, the absence of RNLS may cause an increase in oxidative stress and bring on macrophage infiltration in these tissues [58].

9. Conclusions/Future Studies

It has been established that RNLS has a potent prosurvival role in several different systems. The distinct enzymatic and signaling properties of RNLS have potentially profound effects on injury and recovery. Since RNLS is abundantly found in many non-diseased tissues, it seems certain that it will also have a role in tissue homeostasis. That tissue

8.3. Cancer

As discussed in Section 2.2, RNLS1 promotes cell survival in most cancer cells as shown for pancreatic cancer and melanoma. In PDAC cells, siRNA and anti-RNLS1 antibodies decreased the survival of PDAC cells [10]. The correlation between cancer and RNLS1 levels in the kidney has led some to suggest that tissue RNLS1 could be a biomarker for renal tumors [74]. Elevated RNLS1 levels are also found to be associated with breast cancer [75]. A systematic evaluation of RNLS in gastrointestinal and non-GI tumors is needed.

9. Conclusions/Future Studies

It has been established that RNLS has a potent prosurvival role in several different systems. The distinct enzymatic and signaling properties of RNLS have potentially profound effects on injury and recovery. Since RNLS is abundantly found in many non-diseased tissues, it seems certain that it will also have a role in tissue homeostasis. That tissue RNLS levels can be modified by environmental factors such as a high-fat diet suggests such changes could modify injury responses. The RNLS properties that modulate acute injury responses likely have key roles in the malignant behavior of some cancers. Although direct effects of RNLS on parenchymal cells (e.g., pancreatic acinar cells) and cancer cells (pancreatic and melanoma) have been observed, it is likely that RNLS acts on other cell types that modulate disease responses. Thus, though the immunologic effects of RNLS are still being described, they likely have a role in modulating injury responses and establishing and maintaining tumor niches.

Our studies of RNLS as a potential therapeutic tool for use in the treatment of acute injury as well in select cancers have focused on the extracellular RNLS pool. Delivery of intact RNLS has been shown to decrease the severity of a range of acute injuries. RNLS inhibition using a specific monoclonal antibody has been shown to reduce the growth of select cancers. Although these findings represent attractive avenues to therapy, a more refined understanding of RNLS function may allow for the creation of more effective and specific therapeutic tools. This includes understanding details of the RNLS signaling pathways and structure–function relationships of the molecule.

In closing, RNLS is a multi-functional protein with activities that are broadly important for maintaining tissue homeostasis and for resolving acute tissue injury. It is also a driver for select cancers. Developing agents that are RNLS agonists or antagonists might have broad therapeutic applications.

Author Contributions: Conceptualization, T.C.P., F.S.G. and G.V.D.; methodology, T.C.P.; resources, F.S.G. and G.V.D.; writing—original draft preparation, T.C.P.; writing—review and editing, F.S.G. and G.V.D.; visualization, F.S.G.; supervision, G.V.D.; project administration, G.V.D.; funding acquisition, F.S.G. and G.V.D. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by NIH R01 CA216846 (to G.V.D.: CoPI:), Veterans Administration Merit Award (BX003250) to F.S.G., a DOD Cancer Award (CA180514) to F.S.G. and G.V.D., and the Henry and Joan Binder Endowment and Chair (to F.S.G.).

Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and qualified for an exemption by the Institutional Review Board.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: F. Gorelick has no conflict of interest with this publication. G. Desir is a named inventor on several issued patents related to the discovery and therapeutic use of renalase. Renalase is licensed to Bessor Pharma, and G. Desir holds an equity position in Bessor and its subsidiary Personal Therapeutics. Bessor Pharma has had no involvement in the preparation of this manuscript or influenced its content.

Appendix A

Appendix A.1. Tissue Specimens

De-identified human samples of normal stomach, duodenum, jejunum, ileum, and colon, as well as a pancreatic cancer specimen, were obtained from Yale Surgical Pathology and approved for exemption by the VA Connecticut Healthcare Human Investigation Committee.

Appendix A.2. Immunohistochemistry

Specimens (5 µm) were embedded in paraffin and attached to glass slides. The sections were deparaffinized and hydrated followed by antigen retrieval by steaming and a citrate buffer for 20 m. Samples were then blocked in DAKO Dual endogenous enzyme block (Agilent, Santa Clara, CA, USA) for 10 min followed by blocking in normal horse serum, 2.5% (Vector Laboratories, Burlingame, CA, USA) for 1 h before incubation with m28 anti-RNLS (1:500 dilution) in TBS/1% Tween20 overnight, or 2.5% normal horse serum overnight. ImmPRESS peroxidase-anti-rabbit IgG (Vector Laboratories, Burlingame, CA, USA) detected the primary antibodies. A Vector DAB substrate kit was used to develop the brown color. The slides were finally stained with hematoxylin (Sigma Aldrich, St. Louis, MO, USA) and rehydrated. Coverslips were fixed to the slides using Vectamount (Vector Laboratories) and observed via light microscopy.

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