Risk Factors of Salivary Gland Dysfunction in Radioiodine Treated Thyroid Cancer Patients and Automation of SPECT/CT Imaging Analysis of Mouse Thyroid

DISSEPTION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

Brynn Anne Hollingsworth

Graduate Program in Biomedical Sciences

The Ohio State University

2017

Dissertation Committee:

Sissy M. Jhiang, PhD, Advisor
Scott Harper, PhD
Kun Huang, PhD
Joseph Travers, PhD
Abstract

Radioactive iodine-131 is an effective treatment of follicular-cell derived thyroid cancer due to maintained Na⁺/I⁻ symporter (NIS) expression in well-differentiated thyroid cancer. In normal thyroid tissue, NIS facilitates uptake of iodide into the thyroid for thyroid hormone production and this is exploited in thyroid cancer treatment. NIS is also expressed in the salivary glands leading to transient or even chronic salivary gland damage and dysfunction in some ¹³¹I-treated thyroid cancer patients. Because thyroid cancer’s five year survival rate is over 95%, quality of life is particularly important in these patients and effective means of predicting who will develop ¹³¹I-induced salivary gland damage and how to prevent it have not been found. Pre-clinical microSPECT/CT imaging is used to quantitate radioiodine accumulation and is instrumental for studies identifying strategies to modulate NIS expression both in the salivary glands and in thyroid tumor models of poorly or undifferentiated thyroid cancer that have decreased or no NIS expression. Optimization of image acquisition and analysis would improve these studies.

Sialadenitis and xerostomia are major adverse effects of ¹³¹I therapy in thyroid cancer patients. The risk factors for these adverse effects, other than administered activity of ¹³¹I, had not previously been investigated. In an initial study of symptom questionnaires from 216 thyroid cancer patients and a validation study search of 1507 thyroid cancer patients’ medical records for ICD9/10 codes for sialadenitis, xerostomia,
and autoimmune diseases associated with Sjogren’s syndrome (AID-SS) were performed to identify clinical and demographic risk factors of $^{131}$I-induced sialadenitis and xerostomia. We confirmed that $^{131}$I treatment associated with higher incidence of xerostomia and sialadenitis. Additionally, we found patients with xerostomia had significantly higher mean cumulative and first administered $^{131}$I activity and that increased age associated with higher incidence of xerostomia. Female gender and a history of sialadenitis associated with higher incidence of sialadenitis after $^{131}$I administration. AID-SS associated with higher incidence of both xerostomia and sialadenitis among $^{131}$I-treated patients. We conclude that risk factors for $^{131}$I-induced salivary gland damage include administered $^{131}$I activity, age, gender, history of sialadenitis before $^{131}$I treatment, and AID-SS diagnosis.

The ability of thyroid follicular cells to take up and retain iodine enables the use of radioactive iodine (RAI) for imaging and targeted killing of RAI-avid thyroid cancer following thyroidectomy. To preclinically identify novel strategies to improve $^{131}$I therapeutic efficacy for patients with non-RAI-avid disease or with poor response to $^{131}$I therapy, it is desired to optimize the workflow of imaging acquisition and enhance the capability of imaging analysis for preclinical mouse models of thyroid tumor. We implemented the use of a customized mouse cradle to facilitate consistent tissue configuration across images and developed an in-house CTViewer software to streamline imaging analysis. Consistent mouse tissue configuration allowed for rigid body registration of microSPECT/CT images acquired 1 hour (t1) and 24 hours (t24) after $^{123}$I injection. Because the thyroid retains iodine while the salivary glands do not, this
alignment allowed automatically threshold-based thyroid volumes of interest (VOI) segmented in the t24 image to be superimposed on the corresponding aligned t1 image to distinguish the thyroid from adjacent salivary glands in t1 images. Furthermore, the extent of heterogeneity in $^{123}$I accumulation within thyroid VOIs can be visualized by 3D display of voxel-based $^{123}$I gamma photon intensity. These advances will greatly facilitate preclinical mouse studies to uncover novel strategies to improve $^{131}$I therapeutic efficacy for patients with advanced thyroid cancer.

Administration of $^{131}$I is a common and effective means to treat follicular-cell derived thyroid cancer; however it can be further improved to minimize side effects and increase efficacy in patients with advanced disease. Our retrospective studies of $^{131}$I-induced salivary gland damage indicate administered $^{131}$I activity, age, gender, history of sialadenitis before $^{131}$I treatment, and AID-SS diagnosis are risk factors of $^{131}$I-induced salivary gland damage. Additionally, we report methods that have eliminated user subjectivity in analysis of $^{123}$I microSPECT/CT imaging where images were taken at t1 and t24 and a method to minimize user subjectivity in studies where only a t1 image is available. This optimization of pre-clinical microSPECT/CT imaging acquisition and analysis will assist in studies to identify novel strategies to increase radioisotope accumulation in thyroid cancer.
Dedication

To my amazingly supportive family and friends, especially my husband Christian Buettner who walked this road with me.
Acknowledgments

First I would like to thank my advisor, Dr. Sissy M. Jhiang. Your passion for teaching and training graduate students is incredible and I am very grateful for all the time you spent poring over data with me. Thank you for teaching me how to plan experiments, analyze data, interpret results, write papers and grants, as well as the art of collaboration that not every graduate student is privy to learn during their education. Thank you for pushing me to be my very best. I also want to thank my committee members, Dr. Scott Harper, Dr. Kun Huang, and Dr. Joseph Travers for your time, career guidance and research questions and suggestions. I would like to thank all the Jhiang lab members, past and present for your insight and humor through this journey. Thank you to Mike Brandt, Daniel Scarberry, Omar Ezzedin, and Dr. Zhao for your help and insight with microSPECT/CT analysis, to Aparna Lakshmanan for sharing your knowledge of thyroid cancer, and Jill Green for your career knowledge. Thank you to Zach Hurst for tag-teaming saliva sample pick-up and your passion and curiosity about science, which remind me why I decided to pursue a career in science. An enormous thank you to Dr. Peng Cheng for your patience working with me to design CTViewer and your promptness in writing the code and addressing any issues along the way. Thank you to the rest of the Menq lab for your assistance in everything ranging from poster printing to CAD designs of mouse cradles. Thank you to Sean Smart for sharing the original mouse
cradle design. Thank you to Kevin Wolf for not only being incredibly helpful 3D printing the mouse cradle, but also for your friendship and humor. My day was always brighter after a visit to the machine shop or an entertaining email from you. Thank you to Dr. Kimerly Powell, Dr. Krista LaPerle, and Dr. Tod Drost for your knowledge and insight of small animal imaging and mouse anatomy. Thank you to Jessica Pyles, Anna Bratasz, and Michelle Williams for your training in small animal imaging as well as your company in the Biomedical Research Tower basement. A huge thank you to Rebecca Nagy. You were instrumental in getting the saliva study up and running and were always prompt getting the data to us. Thank you to Leigha Senter for picking up the project after Becky and juggling it with your already full load. Thank you to Pam Brock for jumping into research so quickly, sharing your interesting research questions, and working with me to determine the best way to pull requested patient data. Thank you to Ilene Lattimer for enrolling patients in the Endocrine Neoplasia Repository as well as the salivary gland study and collecting patient saliva samples. Thank you so much to our biostatisticians, especially Dr. Xiaoli Zhang, Dr. Guy Brock, and Dr. Kevin Coombes, for your hard work performing statistical analyses for our data, your patience explaining statistical concepts and tests, and your knowledge and experience. Thank you to Dr. Matt Ringel and Dr. Jen Sipos for your support in and out of the clinic. Thank you to Dr. Wael Jarjour, Dr. Ricardo Carrau, and Dr. Richard Kloos for your insight and experience. To my graduate school friends and their families, Josh Wise, Dr. Daphne Guinn, Dr. Andrew Sharits and the whole Sharits family, Caroline Winters, and Laura Zdon, thank you so much for your friendship. I truly would not have been able to get this far without it. I look forward to
seeing where all our paths take us. To my synchronized swimming teammates and coaches Angela Gephart, Dr. Teresa Kouri-Kissel, Dr. Colleen Pema, Kristen Ferraro, Anna Farmer, Ali Brunton, and Robin Pirik, thank you for giving me a fun stress reliever and continuing to teach me new things about my favorite sport. To my incredible high school biology teacher and friend Mrs. Sherry Annee, thank you for your enthusiasm about science and teaching and your interest in me. I would not be the scientist I am today without you. To the Morris family, thank you for the laboratory experience opportunities and support over the years. To my family, thank you so much for all your support and love every day of my life. A special thank you to my mom for always asking about my research to push me to communicate it better to someone outside of science and for all the tips for IRB submissions. Thank you to my husband and fellow graduate student, Christian Buettner. Through the highs and the lows, we kept each other going. I’m grateful we never wanted to quit at the same time. And to all the thyroid cancer patients who participated in this research, thank you so much for your time, support, and trust in our research at The Ohio State University Wexner Medical Center.
Vita

May 12, 1988 .........................................Born, Indianapolis, IN

May 2010 ..................................................B.S. Biology, The College of William and Mary

July 2011 to present .................................Graduate Research Associate, Biomedical Science Graduate Program, The Ohio State University

Publications


Fields of Study

Major Field: Biomedical Sciences Graduate Program
# Table of Contents

Abstract .................................................................................................................. ii

Dedication ............................................................................................................... v

Acknowledgments ................................................................................................. vi

Vita ......................................................................................................................... ix

Publications ........................................................................................................... ix

Fields of Study ....................................................................................................... x

Table of Contents .................................................................................................. xi

List of Tables ......................................................................................................... xiv

List of Figures ....................................................................................................... xv

List of Abbreviations .............................................................................................. xix

Chapter 1: Introduction .......................................................................................... 1

1.1 Thyroid Cancer .............................................................................................. 1

1.2 Na+/I- Symporter Expression and Function ..................................................... 1

1.3 Radioiodine Therapy and Adverse Effects in Thyroid Cancer Patients .......... 3

1.4 Single Photon Emission Computed Tomography/X-ray Computed Tomography Imaging ............................................................................................................. 4
Chapter 2: Risk Factors of $^{131}$I-induced Salivary Gland Damage in Thyroid Cancer

2.1 Abstract ...................................................................................................................... 9
2.2 Introduction .............................................................................................................. 10
2.3 Materials and Methods ....................................................................................... 11
2.4 Results ..................................................................................................................... 16
2.5 Discussion ............................................................................................................... 21
2.6 Conclusions ........................................................................................................... 24

Chapter 3: Automated microSPECT/CT imaging analysis of the mouse thyroid gland. 34

3.1 Abstract .................................................................................................................. 34
3.2 Introduction ............................................................................................................ 35
3.3 Materials and Methods ........................................................................................ 37
3.4 Results .................................................................................................................... 40
3.5 Discussion .............................................................................................................. 43
3.6 Conclusions ........................................................................................................... 46

Chapter 4: Anatomical Markers for segmentation of the thyroid in SPECT/CT imaging 53

4.1 Introduction ............................................................................................................ 53
4.2 Materials and Methods ........................................................................................ 54
4.3 Results .................................................................................................................... 56
4.4 Discussion .............................................................................................................. 57

Chapter 5: Effect of $^{131}$I on saliva flow rate and saliva protein composition .......... 65

5.1 Introduction ............................................................................................................ 65
5.2 Materials/Methods ............................................................................................... 65
5.3 Results ................................................................. 68
5.4 Discussion ............................................................. 72

Chapter 6: Summary and Future Directions ........................................ 88

References ............................................................................. 104
List of Tables

Table 2.1. Patient demographics and clinical data for initial and validation study cohorts ................................................................................................................................................................................................. 26

Table 2.2. Symptom and AID-SS ICD9/10 codes searched for in the electronic medical record .......................................................................................................................................................................................................................................................... 32

Table 2.3. Clinical data of Initial Study $^{131}$I-treated patients diagnosed with AID-SS .... 33

Table 3.1. Mouse positioning consistency between serial images of the same mice ....... 49

Table 4.1. Image slices to expand out from each anatomical marker-based boundary to encompass thyroids of FVB/N, TgBRAF$^{V600E}$, and TRβ$^{PV/PV}$/Akt2KO mice ............... 62

Table 5.1. Demographics and clinical data for patients with saliva collected ............... 77

.............................................................................................................................................................................................................................................................................. 86
List of Figures

Figure 1.1 Na⁺/I⁻ Symporter Expression and Function in the Thyroid .......................... 7

Figure 1.2. Ducts of the Salivary Gland ...................................................................... 8

Figure 2.1. Patients treated with ¹³¹I had significantly higher incidence of xerostomia and sialadenitis in both the initial study (A, B) and the validation study (C, D) ...................... 27

Figure 2.2. Patients with xerostomia, but not patients with sialadenitis, had 46 mCi higher mean cumulative ¹³¹I activity in the validation study (C, D) but not in the initial study (A, B) ........................................................................................................ 28

Figure 2.3. Patients with xerostomia, but not patients with sialadenitis, had 21 mCi higher mean first administered ¹³¹I activity in the validation study (C, D) but not in the initial study (A, B) ........................................................................................................ 29

Figure 2.4. Patients who had sialadenitis before ¹³¹I therapy had higher sialadenitis incidence after ¹³¹I therapy ........................................................................................................ 30

Figure 2.5. Among ¹³¹I-treated patients, patients diagnosed with AID-SS had increased incidence of xerostomia and sialadenitis in the validation study (C, D) but less significant in the initial study (A, B) ........................................................................................................ 31

Figure 3.1. Cradle design and X-ray computed tomography image of mouse in cradle .. 47

Figure 3.2. Tissue configuration variation in head and neck region is greatly reduced with the use of cradle during image acquisition. ................................................................. 48
Figure 3.3. Automatic segmentation of thyroid volume of interest (VOI) on t1 image via superimposing thyroid VOI from t24 SPECT image ................................................................. 50

Figure 3.4. Automatic segmentation of thyroid volume of interest (VOI) on t1 and t24 SPECT images: Coronal plane view ............................................................................................................. 51

Figure 3.5. Coronal view of 3D voxel-based gamma photon intensity of a FVB/N mouse thyroid (A) and a TgBRAF<sup>V600E</sup> mouse thyroid tumor (B) ................................................................. 52

Figure 4.1. Anatomical markers to set boundaries of volume encompassing thyroid for confined threshold-based thyroid volume of interest segmentation ........................................ 60

Figure 4.2. Expansion past anatomical marker-based boundaries to encompass tumor thyroids in mouse models of thyroid cancer .................................................................................. 61

Figure 4.3. Thyroid volume of interest segmented using T24 overlay method, confined adaptive threshold-based segmentation from within anatomical marker-based volume, or manual segmentation ......................................................................................................................... 63

Figure 4.4. 123<sup>I</sup> percent injected activity in thyroid volumes of interest segmented using T24 overlay method, confined adaptive threshold-based segmentation from within anatomical marker-based volume, or manual segmentation .......................................................... 64

Figure 5.1. Cumulative and first administered 131<sup>I</sup> activity significantly correlates with stimulated saliva flow rate (B, D), but not basal saliva flow rate (A, C) .................................................. 78

Figure 5.2. Cumulative and first administered 131<sup>I</sup> activity did not associate with xerostomia scores or sialadenitis incidence ................................................................. 79
Figure 5.3. Age has a significant negative correlation with basal saliva flow rate but not stimulated saliva flow rate, and there was no significant difference in saliva flow rate between males and females.

Figure 5.4. Neither xerostomia score nor sialadenitis incidence associated with age or gender.

Figure 5.5. Subjective xerostomia had significant negative correlation with stimulated saliva flow rate but not basal saliva flow rate, and saliva flow rate between patients who reported sialadenitis and those who did not was not significantly different.

Figure 5.6. $^{131}$I-treated patients who experienced sialadenitis do not have significantly different xerostomia scores than patients who did not experience sialadenitis.

Figure 5.7. Matrix metalloproteinase-1, matrix metalloproteinase-7, and angiogenin are decreased in saliva of patients who experienced sialadenitis.

Figure 5.8. Six proteins had strong significant positive correlation with xerostomia score.

Figure 6.1. SSA-Ro and SSB-La are not over-represented in $^{131}$I-treated patients with salivary gland symptoms.

Figure 6.2. Anatomical markers for defining salivary gland volume of interest.

Figure 6.3. Saliva flow rate prior to compared to after $^{131}$I treatment.
Figure 6.4. Unilateral retroductal injection of the mouse submandibular gland can be achieved such that the mouse may serve as its own control ........................................... 100

Figure 6.5. Microbubble-facilitated gene transfer to the mouse salivary glands .......... 101

Figure 6.6. Retroductal injection of microbubbles followed by ultrasonication leads to mouse salivary gland damage and retention of radioisotope ............................................ 102

Figure 6.7. Retroductal injection of nanoparticles with siRNA results in decrease in radioisotope accumulation ........................................................................................................... 103
List of Abbreviations

$\Gamma$ .................................................Iodide

$\text{Na}^+$ ..................................................sodium ion

$^{131}\text{I}$ ..................................................Radioactive iodine-131

$^{123}\text{I}$ ..................................................Radioactive iodine-123

RAI ..........................................................radioactive iodine

NIS .........................................................Sodium iodide symporter

ICD9/10 ....................................................International classification of disease $9^{th}$ and $10^{th}$ revisions

AID-SS .....................................................Autoimmune disease associated with Sjogren’s Syndrome

SPECT ......................................................Single photon emission computed tomography

CT ............................................................X-ray computed tomography

VOI ..........................................................Volume of interest

T1 ...........................................................1 hour following radioisotope injection

T24 ...........................................................24 hours following radioisotope injection

T3 ...........................................................Triiodothyronine

T4 ...........................................................Thyroxine

TSH .........................................................Thyroid stimulating hormone

Tg ............................................................Thyroglobulin
K⁺ .............................................. Potassium ion
Cl⁻ .............................................. Chloride
HCO₃⁻ ........................................ Bicarbonate
OSUWMC........................................ The Ohio State University Wexner Medical Center
ENR.............................................. Endocrine Neoplasia Repository
IHIS.............................................. Integrated Health Information Systems
“N”.............................................. Never experienced sialadenitis
“A”.............................................. Experienced sialadenitis only after $^{131}$I
“BA”.............................................. Experienced sialadenitis before and after $^{131}$I
“B”.............................................. Experienced sialadenitis only before $^{131}$I
EBRT.............................................. External beam radiation therapy
CI.............................................. Confidence interval
HR.............................................. Hazards ratio
OR.............................................. Odds ratio
Dx.............................................. Diagnosis
SS.............................................. Sjogren’s Syndrome
SLE.............................................. Systemic lupus erythematosus
CTD.............................................. Connective tissue disease
RA.............................................. Rheumatoid Arthritis
PET.............................................. Positron emission tomography
3D.............................................. 3 dimensional
MFC.............................................. Microsoft Foundation Class
%ID .......................................................... Percent injected dose activity
T .......................................................... Tumor
N .......................................................... Nodes
M .......................................................... Metastases
VOI ......................................................... Volume of interest
MEK ........................................................ Mitogen-activated protein kinase kinase
CA .......................................................... Confined adaptive
CAT ........................................................ Confined adaptive threshold
MAP ........................................................ Multi-analyte panel
SFR .......................................................... Saliva flow rate
MMP-7 ....................................................... Matrix metalloproteinase-7
GROα ......................................................... Growth-related alpha protein
CXCL1 ..................................................... C-X-C motif ligand 1
siRNA ....................................................... Short interfering ribonucleic acid
Chapter 1: Introduction

1.1 Thyroid Cancer

There were an estimated 637,115 people living with thyroid cancer in the United States in 2013 and an estimated 64,300 new cases of thyroid cancer in 2016, approximately 75% of which were women\(^1\). Approximately 95% of thyroid cancers are follicular-cell derived including papillary thyroid cancer (80-85% of all cases) and follicular thyroid cancer (10-15% of all cases)\(^2\). The standard of care for these patients is complete thyroidectomy followed by administration of radioactive iodine-131 (\(^{131}\)I), which is taken up by both cancerous and normal thyroid cells expressing the Na\(^+\)/I\(^-\) symporter (NIS). The β radiation emitted by \(^{131}\)I effectively ablates these cells resulting in excellent prognosis for patients with radioiodine avid disease. Most thyroid cancers are well-differentiated and express NIS such that \(^{131}\)I therapy is effective; however, a subset of thyroid cancer patients have poorly or undifferentiated thyroid cancer such that NIS expression is decreased or lost. Effort is being made to determine means to increase NIS expression and function in these patients such that they may also benefit from \(^{131}\)I therapy.

1.2 Na\(^+\)/I- Symporter Expression and Function

Iodine is an important component of thyroid hormones triiodothyronine (T3) and thyroxine (T4), which regulate metabolism. These hormones are produced in the thyroid
folicles and released into the bloodstream as stimulated by thyroid stimulating hormone (TSH), produced by the anterior pituitary gland. NIS is expressed on the basolateral membrane of thyroid follicular cells towards the blood vessels in order to facilitate transport of iodide into the thyroid follicular cells against its concentration gradient resulting in iodide concentration 20-40 times that in the bloodstream (Fig 1.1)\(^3\). TSH stimulation increases NIS expression in thyroid cells. Because of this, it is recommended that patients cease thyroid hormone supplementation temporarily to increase TSH levels or are administered recombinant human TSH prior to \(^{131}\text{I}\) therapy in order to maximize uptake and therefore increase cell killing effect of \(^{131}\text{I}\)\(^4\). After transport into the follicular cell via NIS, thyroperoxidase oxidizes iodide ions to form \(\text{I}_2\). \(\text{I}_2\) is then transported across the apical membrane of the follicular cell into the follicle lumen, where it iodinates tyrosine residues on thyroglobulin (Tg). Upon TSH stimulation, Tg is endocytosed back into the follicular cell, and the endosome fuses with a lysosome. The digestive enzymes in the lysosome digest Tg such that the thyroid hormones T3 and T4 are released and diffuse into the bloodstream. A certain level of Tg circulates throughout the bloodstream. Most follicular-derived thyroid cancers retain the ability to produce Tg such that Tg level in the bloodstream is used to monitor thyroid cancer burden following complete thyroidectomy\(^4\). Anti-Tg autoantibodies may disrupt the Tg test yielding falsely low Tg levels, thus presence for anti-Tg autoantibodies must also be assessed.

NIS is also expressed in the gastric mucosa, the lactating mammary glands, and the salivary glands\(^3\). NIS is expressed in the ductal cells of the lactating breast to facilitate
transport of iodine into breast milk as iodine is essential for thyroid hormone synthesis and thyroid hormone is essential for development and metabolism; however the role of iodine in other extrathyroidal tissues is unclear. Iodine has been shown to have antioxidant effects and iodine deficient diet has been correlated with increased dental caries. NIS is expressed in the striated ductal cells of the salivary glands (Fig 1.2), which are responsible for modifying the concentrations of various ions, including Na\(^+\), Cl\(^-\), K\(^+\), and HCO\(_3\)^-, in salivary fluid. Unlike NIS expression in the thyroid, NIS expression in the salivary glands is not affected by TSH as salivary ductal cells do not express TSH receptor.

1.3 Radioiodine Therapy and Adverse Effects in Thyroid Cancer Patients

\(^{131}\)I is an effective treatment for follicular and papillary thyroid cancer and has been used for over half a century. Following total thyroidectomy, it is recommended that 30 mCi of \(^{131}\)I be used for remnant ablation to kill any remaining normal tissue such that recurrent disease can be detected by Tg measurement and/or whole body radioiodine scans. When residual or persistent disease is suspected or discovered, up to 150 mCi of \(^{131}\)I is recommended to improve disease-free survival. Of the patients with follicular-cell derived thyroid cancer enrolled in the Ohio State University Endocrine Neoplasia Repository, approximately 70% were treated with \(^{131}\)I.

\(^{131}\)I is not prescribed to pregnant or nursing women, thus NIS expression in the lactating mammary gland is not a cause for concern. NIS expression in the gastric mucosa can lead to temporary side effects after \(^{131}\)I administration such as nausea or
It has long been known that $^{131}$I also accumulates in the salivary glands, leading to damage and dysfunction in up to 40% of treated patients. NIS expression facilitates $^{131}$I uptake in the salivary ducts, and this radiation exposure can lead to ductal damage, stenosis and/or mucus plugs causing sialadenitis. Sialadenitis can lead to permanent xerostomia. With an estimated 637,115 people living with thyroid cancer in the United States, there are potentially thousands of thyroid cancer survivors living with sialadenitis, difficulty eating, oral infections, gum inflammation and even loose teeth or tooth loss. Very little is known about $^{131}$I-induced salivary gland damage and dysfunction, and current methods to stimulate saliva flow in order to minimize $^{131}$I exposure and related side effects are ineffective. With an approximate 98% 5-year survival rate for thyroid cancer patients, minimizing chronic side effects of $^{131}$I therapy is of particular importance. We anticipate our studies will provide informative risk factors for $^{131}$I-induced salivary gland damage and shed light on possible prevention strategies.

1.4 Single Photon Emission Computed Tomography/X-ray Computed Tomography Imaging

Single photon emission computed tomography (SPECT) imaging detects gamma radiation via rotating gamma cameras on a gantry to visualize gamma ray emitting radioisotope accumulation throughout the body in three dimensions. SPECT imaging is normally combined with X-ray computed tomography (CT) to allow superimposition of the functional SPECT image with an anatomical CT image. Pre-therapy diagnostic SPECT/CT scans after administration of ~5 mCi of $^{123}$I are recommended to determine
thyroid remnant radioiodine uptake and the presence of residual locoregional and/or
distant metastatic radioiodine-avid disease, which may impact decision making for $^{131}$I
treatment$^4$. Because therapeutic administered radioiodine activity is much higher than
diagnostic administered radioiodine activity, SPECT/CT imaging or two-dimensional
planar scintigraphy is conducted approximately one week following $^{131}$I to determine if
there is any additional lesions with radioiodine uptake not seen in the diagnostic scan$^4$.
Planar imaging does not provide anatomic location, thus SPECT/CT imaging is preferred.

Pre-clinically, microSPECT/CT imaging is used to assess radioisotope accumulation in animal models. MicroSPECT/CT can be used to quantify radioiodine accumulation in the thyroid and salivary glands$^{21,22}$ and has been instrumental in identifying novel strategies to increase thyroidal radioiodine accumulation in thyroid tumor mouse models$^{23,24}$. In our studies reported here, we acquired microSPECT/CT images from FVB/N and Balb/c mice with normal thyroid, TgBRAF$^{V600E}$ mice, and TRβ$^{PV/PV}$ mice crossed with Akt2 knockout mice. The TgBRAF$^{V600E}$ mouse model was used as these mice have dynamic NIS expression and thus variable radioisotope accumulation. Additionally, the BRAF$^{V600E}$ mutation is found in 40-50% of papillary thyroid carcinomas$^{25}$ and it can be a predictor of poorer prognosis$^{26}$. In the TgBRAF$^{V600E}$ mouse model, BRAF$^{V600E}$ is expressed in the thyroid under the Tg promoter, but because BRAF$^{V600E}$ expression leads to de-differentiation resulting in lower Tg expression, BRAF$^{V600E}$ expression is dynamic. This dynamic BRAF$^{V600E}$ expression also results in dynamic NIS expression as de-differentiation results in decreased NIS expression. The
TRβ<sup>PV/PV</sup>/Akt2 knockout mice were used as they have large tumors with high NIS expression due to thyroid hormone resistance leading to elevated TSH levels. We hope our studies to streamline microSPECT/CT image acquisition and automate image analysis will be of use to future pre-clinical and clinical studies modulating NIS expression and function in the thyroid and salivary glands.
Na\textsuperscript{+}/I\textsuperscript{-} symporter expression and function in the thyroid

Na\textsuperscript{+}/I\textsuperscript{-} symporter (NIS) is expressed on the basolateral membrane of thyroid follicular cells in order to facilitate transport of iodide into the thyroid follicular cells against its concentration gradient. The sodium concentration gradient maintained by Na\textsuperscript{+}/K\textsuperscript{+} ATPase drives iodide transport such that one iodide ion is transported along with two sodium ions. Thyroperoxidase oxidizes iodide ions to form I\textsubscript{2}. I\textsubscript{2} and I\textsuperscript{-} move into the lumen and iodinate thyroglobulin (TG). Upon thyroid-stimulating hormone stimulation, TG is endocytosed into the follicular cells and the endosome fuses with a lysosome. The enzymes in the lysosome digest TG such that the thyroid hormones triiodothyronine (T3) and thyroxine (T4) are released and diffuse into the bloodstream. (Figure adapted from Dr. Krista La-Perle (La-Perle 2002)
Figure 1.2. Ducts of the Salivary Gland

The human salivary gland is made up of acini (A), intercalated ducts (I), striated ducts (S), and excretory ducts (E). Acinar cells in the acini take up water from the bloodstream in a Cl⁻ gradient-driven manner via aquaporins, and this water, along with proteins secreted by the acinar cells and ions, make up the pre-saliva secreted into the salivary ducts. This pre-saliva moves through the salivary ducts, where it is modified until excreted from the excretory ducts into the oral cavity. Na⁺/I⁻ symporter is expressed in the striated ducts. Figure adapted from La-Perle et al 2013.
Chapter 2: Risk Factors of $^{131}$I-induced Salivary Gland Damage in Thyroid Cancer Patients

2.1 Abstract

**Context:** Sialadenitis and xerostomia are major adverse effects of $^{131}$I therapy in thyroid cancer patients. The risk factors for these adverse effects, other than administered activity of $^{131}$I, have not been investigated.

**Objective:** The aim of this study is to identify risk factors for $^{131}$I-induced salivary gland damage among follicular cell-derived thyroid cancer patients.

**Design:** We enrolled 216 thyroid cancer patients who visited The Ohio State University Wexner Medical Center between April 2013 and April 2014. Symptoms of xerostomia and sialadenitis were identified via questionnaire and medical record search. To validate the findings in a large cohort, we retrospectively searched for ICD9/10 codes for sialadenitis, xerostomia, and autoimmune diseases associated with Sjogren’s syndrome (AID-SS) in our existing database (N=1507). Demographic and clinical information were extracted from medical records. Multivariate analyses were performed to identify independent predictors for salivary gland damage.

**Results:** $^{131}$I treatment associated with higher incidence of xerostomia and sialadenitis. Patients with xerostomia had 46 mCi higher mean cumulative $^{131}$I activity and 21 mCi higher mean first administered $^{131}$I activity than patients without xerostomia. Increased age associated with higher incidence of xerostomia, and females had higher incidence of
sialadenitis. Patients who experienced sialadenitis before $^{131}$I therapy had higher sialadenitis incidence after $^{131}$I therapy. $^{131}$I-treated patients diagnosed with AID-SS, whether before or after $^{131}$I treatment, had a higher incidence of xerostomia and sialadenitis among $^{131}$I-treated patients.

**Conclusion:** Risk factors for $^{131}$I-induced salivary gland damage include administered $^{131}$I activity, age, gender, history of sialadenitis before $^{131}$I treatment, and AID-SS diagnosis.

2.2 Introduction

Radioiodine ($^{131}$I) is often administered to patients following total thyroidectomy to treat well-differentiated follicular cell-derived thyroid cancer $^{4,28}$. In addition to the thyroid, $^{131}$I accumulates in the salivary glands, giving rise to transient or permanent salivary gland damage. This injury can clinically manifest as acute or chronic sialadenitis and/or xerostomia $^{29,30}$. While the incidence and severity of sialadenitis and xerostomia are believed to be associated with higher cumulative administered $^{131}$I activities, patients who were administered 30-50 mCi of $^{131}$I have been reported to suffer from salivary gland dysfunction following their $^{131}$I treatment $^{31,32}$. To our knowledge, risk factors for $^{131}$I-induced salivary gland damage other than $^{131}$I activity have not been identified. In this study, we reviewed clinical data and questionnaires addressing symptoms of xerostomia and sialadenitis to identify additional risk factors of salivary gland dysfunction following $^{131}$I therapy.
2.3 Materials and Methods

This study was approved by the Institutional Review Board at The Ohio State University. All subjects gave written informed consent to medical record review. The vast majority of patients who receive $^{131}$I therapy at The Ohio State University Wexner Medical Center (OSUWMC) are prepared using recombinant human thyrotropin.

Initial Study

*Patient enrollment:* Patients who had been diagnosed with follicular cell-derived thyroid cancer and visited Endocrinology Clinic at OSUWMC between April 2013 and April 2014 were enrolled in the Endocrine Neoplasia Repository (ENR) (n=216). Relevant demographic and clinical data are shown in Table 2.1. Questionnaires for xerostomia and sialadenitis as well as autoimmune disease associated with Sjogren’s syndrome (AID-SS) were served by a registered nurse during patients’ office visit. Mean time between patients’ thyroid cancer diagnosis and questionnaire completion was 6.18 years (median=3.91 years, range 2 weeks to 62 years). Mean time between $^{131}$I-treated patients’ first $^{131}$I administration and questionnaire completion was 6.29 years (median=4.16 years, range 2 weeks to 35 years).

*Clinical questionnaire for xerostomia, sialadenitis, and autoimmunity:* Patients’ subjective feelings of dry mouth (xerostomia) were reported on a scale of 0-4 in terms of “frequency of difficulty talking due to dryness”, “frequency of mouth and throat dryness when not eating”, “frequency of sipping liquids to aid swallowing food”, and “frequency
of sipping liquids for oral comfort”. These questions were modified from the questionnaire validated by Daly et al.\(^33\) in patients with external beam radiation therapy. Among enrolled patients, 207/216 patients completed all xerostomia score questions. Additionally, the questionnaire asked patients if they experienced salivary gland swelling and/or pain (sialadenitis) prior to and/or after \(^{131}\text{I}\) therapy. All patients were well informed that salivary gland damage is a major adverse effect of \(^{131}\text{I}\) therapy prior to their treatment. Patients’ answers were grouped by whether they had never experienced sialadenitis (“N”), had experienced sialadenitis only after \(^{131}\text{I}\) therapy (“A”), experienced sialadenitis before and after \(^{131}\text{I}\) therapy (“BA”), or had experienced sialadenitis only before \(^{131}\text{I}\) therapy (“B”). Among enrolled patients, 216/216 patients completed the sialadenitis questions. Since patients with AID-SS can develop symptoms that overlap with \(^{131}\text{I}\)-induced salivary gland damage\(^34\text{-}37\), patients were also asked if they had ever been diagnosed with an autoimmune disease and if so, to name the corresponding diagnosis. Sjögren’s syndrome, rheumatoid arthritis, and lupus were provided as examples of autoimmune diseases in the question. Among enrolled patients, 211/216 patients completed the autoimmune disease question.

**Medical record review for sialadenitis and autoimmune disease:** In December 2015, electronic patient medical records (EPIC system, Madison, WI) were searched for the terms “sialadenitis”, and “salivary gland obstruction”. The free-text search identified search terms in various sections of the record including provider notes. When the terms were identified, the location of the term was investigated to determine the context of its
inclusion. The concordance between the questionnaire and medical record search was 78.7% (170/216). Only 2.8% (6/216) of patients had reported no sialadenitis via questionnaire but had sialadenitis noted in their medical record, suggesting that recall of sialadenitis is high. However, 18.5% (40/216) of patients who reported sialadenitis via questionnaire did not have sialadenitis noted in their medical record, most likely because patients did not report the symptom to their clinicians or the symptom was not entered into the medical record.

Medical records were also searched for the terms “Sjogren”, “lupus”, “rheumatoid arthritis”, “scleroderma” and “connective tissue disease”, which are well known AID-SS. Excluding the 3 patients who had AID-SS diagnosed after questionnaire completion, the concordance between the questionnaire and medical record search among $^{131}$I-treated patients was 93.9% (153/163). Only 4/163 (2.5%) were identified by questionnaire but not medical record search and 6/163 (3.7%) were identified in medical record search but not by questionnaire.

**Validation Study**

*Patient enrollment:* To validate the findings of the initial study in a large cohort, 1507 patients (cohort excluded patients in the initial study) with follicular cell-derived thyroid cancer enrolled in the ENR had their medical records retrospectively searched for salivary gland damage symptoms and AID-SS diagnoses. Relevant demographic and clinical data are shown in Table 1. Cumulative and first administered activity of $^{131}$I was
available for 1101/1165 and 1106/1165 $^{131}$I-treated patients, respectively. Among $^{131}$I-treated patients, 14/1165 received external beam radiation therapy (EBRT). Among patients not treated with $^{131}$I, 5/342 received EBRT. None of these EBRT-treated patients had sialadenitis and 1/14 $^{131}$I/EBRT-treated patients had xerostomia.

**ICD9/10 codes search for symptoms and AID-SS:** The Information Warehouse core facility at OSUWMC performed a medical record search for the international classification of disease (ICD) version 9 and 10 codes for sialadenitis, xerostomia, and AID-SS including Sjogren’s syndrome, rheumatoid arthritis, systemic lupus erythematosus, discoid lupus, mixed connective tissue disease, undifferentiated connective tissue disease, systemic sclerosis, scleroderma, and juvenile arthritis (Supplementary Table 1). The last follow-up date was defined as the latest measurement date of serum thyroglobulin level by medical record search and was available for 1385/1507 patients. Mean time between patients’ thyroid cancer diagnosis and search of electronic medical record was 9.24 years (median= 7.58 years, range 3.6 months to 49 years). Mean time between $^{131}$I-treated patients’ first $^{131}$I administration and search of electronic medical record was 9.67 years (median= 8.72 years, range 3.8 months to 49 years).

**Statistical analysis**

For the initial study, ANOVA or Spearman correlations were first used to evaluate the individual effect of $^{131}$I treatment, cumulative $^{131}$I activity, first administered
$^{131}$I activity, or being diagnosed with AID-SS on xerostomia scores. If the univariate model or the correlation test was significant, multivariate regression analysis was then conducted to determine if any of the above risk factors were independent predictors after controlling for age, gender, and other relevant variables. Chi-square or Fisher’s exact tests were used to examine whether $^{131}$I treatment, AID-SS, or a history of sialadenitis before $^{131}$I therapy had an impact on the incidence of sialadenitis after $^{131}$I therapy. ANOVA was used to investigate whether cumulative or first administered activity of $^{131}$I correlated with sialadenitis incidence. Similar to the xerostomia score analysis, if the univariate test was significant, multivariate logistic regression or multivariate linear regression was then performed to investigate if any risk factors were independent predictors after controlling for age, gender, and other relevant variables among $^{131}$I-treated patients.

For the validation study, associations between $^{131}$I therapy/AID-SS and occurrence of sialadenitis/ xerostomia were modeled using both logistic regression and Cox regression models. Cox regression models were included since the entry dates of ICD9/10 codes were available to take into account the timing of events. Since sialadenitis can be recurrent, we modeled time to first occurrence of sialadenitis in each patient. As the risk for sialadenitis may not be constant over time, we checked the proportional hazards assumption in each case using the cox.zph function in the survival package in R, which is based on the scaled Schoenfeld residuals (note: there was no significant evidence of violations with p-value >0.5 in every case). Date of thyroid cancer
diagnosis was considered study start time, and any events (AID-SS, sialadenitis, xerostomia) occurring prior to cancer diagnosis were considered left-truncated. All models were adjusted for age and gender of the patient. Estimated hazard ratios (HR) for Cox models and odds ratios (OR) for logistic regression models along with 95% confidence intervals (CI) and p-values were reported for each model. Differences in cumulative/first administered $^{131}$I activity between patients experiencing/not experiencing sialadenitis or xerostomia were modeled using linear regression, again adjusting for age and gender.

2.4 Results

Patients treated with $^{131}$I had significantly higher incidence of xerostomia and sialadenitis.

To determine if $^{131}$I therapy contributed to dry mouth and/or sialadenitis, mean xerostomia scores and sialadenitis incidence were compared between $^{131}$I-treated patients and patients who had not received $^{131}$I therapy. The mean xerostomia scores of $^{131}$I-treated patients were significantly higher than those of patients who had not received $^{131}$I therapy by 0.81 with p=0.002 (Fig 1A). Among $^{131}$I-treated patients who had no history of sialadenitis before $^{131}$I therapy, 25.9% (37/143) developed sialadenitis after $^{131}$I therapy. In contrast, only 8.5% (4/47) of patients who had not received $^{131}$I experienced sialadenitis (Fig 1B). This difference was significant after controlling for age, gender, and AID-SS (OR=5.49, 95% CI 1.50-20.08, p=0.010). Gender had no effect on mean xerostomia scores or sialadenitis incidence. Age had a significant effect on mean
xerostomia scores, with an increase in mean xerostomia score of 0.22 per decade increase in age (95% CI 0.08-0.35, p=0.002), but had no significant effect on sialadenitis incidence.

In the validation study with the large cohort, logistic regression analysis (Fig 1C), but not Cox regression analysis, indicated that $^{131}$I treatment increased the incidence of xerostomia with OR of 4.01 (95% CI 1.81-8.90, p<0.001) and HR of 2.01 (95% CI 0.98-4.13, p=0.058), respectively. $^{131}$I treatment increased the incidence of sialadenitis (Fig 1D) with HR of 7.43 (95% CI 1.67-33.01, p=0.008). Females were found to have an increased risk of sialadenitis with HR of 3.13 (95% CI 1.09-9.09, p=0.040). Age was associated with an increase in xerostomia incidence with an HR of 1.36 per decade increase in age (95% CI 1.17-1.59, p<0.001). The incidence of sialadenitis by ICD9/10 codes search (3.1%) was lower than that by questionnaire (25.9%) in $^{131}$I-treated patients and there was zero incidence of sialadenitis in patients who had not received $^{131}$I therapy. This indicates that sialadenitis was often not reported by patients and/or not recorded by the physician, particularly before $^{131}$I therapy.

*Patients with xerostomia, but not patients with sialadenitis, received 46 mCi higher mean cumulative $^{131}$I activity.*

To determine if higher cumulative $^{131}$I activity resulted in higher xerostomia scores and/or sialadenitis incidence, correlation between cumulative $^{131}$I activity and mean xerostomia scores or sialadenitis incidence was examined. In the initial study,
cumulative $^{131}$I activity did not correlate with xerostomia scores (Fig 2A). As shown in Fig 2B, there was no significant difference in cumulative $^{131}$I activities among patients who never experienced sialadenitis (N), those who had sialadenitis after $^{131}$I treatment (A), those who had sialadenitis before and after $^{131}$I treatment (BA), and those had sialadenitis before $^{131}$I treatment (B). Even when the $^{131}$I activities were normalized for body mass index, cumulative $^{131}$I activity did not correlate with xerostomia scores or sialadenitis incidence. Additionally, time since last $^{131}$I administration had no effect on xerostomia scores or sialadenitis incidence.

In the validation study, patients who had xerostomia received significantly higher cumulative $^{131}$I activity than patients who did not have xerostomia ($p=0.003$) with a mean difference of 46 mCi (95% CI 15-77 mCi) (Fig 2C). There was no significant difference in cumulative $^{131}$I activity between patients who developed sialadenitis after $^{131}$I therapy and those who did not develop sialadenitis (Fig 2D). Males received 27 mCi (95% CI 10-44 mCi) higher mean cumulative $^{131}$I activity than females ($p=0.002$).

*Patients with xerostomia, but not patients with sialadenitis, received 21 mCi higher mean first administered $^{131}$I activity.*

We examined if the first administered $^{131}$I activity correlated with xerostomia score and/or sialadenitis incidence (Fig 3A and Fig 3B). First administered $^{131}$I activity did not correlate with either xerostomia scores or sialadenitis incidence after controlling
for age, gender, and AID-SS. Males received 33 mCi (95% CI 13-52) higher mean first administered $^{131}$I activity than females (p=0.001).

In the validation study, patients who had xerostomia received 21 mCi (95% CI 6-35 mCi) higher mean first administered $^{131}$I activity than patients who did not have xerostomia (p=0.005) (Fig 3C). There was no significant difference in first administered $^{131}$I activity between patients who had sialadenitis after $^{131}$I therapy and those who did not experience sialadenitis (Fig 2D). First administered $^{131}$I activity was not associated with age at diagnosis, but males received 18 mCi (95% CI 9-26) higher mean first $^{131}$I activity than females (p<0.001).

Patients who had sialadenitis before $^{131}$I therapy had higher sialadenitis incidence after $^{131}$I therapy.

We investigated if patients who experienced sialadenitis before $^{131}$I treatment had a higher incidence of sialadenitis after $^{131}$I treatment. The incidence of sialadenitis that occurred after $^{131}$I treatment was compared between patients who had sialadenitis before $^{131}$I treatment and patients who had no sialadenitis before $^{131}$I treatment. As shown in Figure 4, 57.7% (15/26) of patients who had sialadenitis before $^{131}$I treatment suffered from sialadenitis after $^{131}$I treatment, compared to 25.9% (37/143) of patients who did not have sialadenitis before $^{131}$I treatment (OR=4.10, 95% CI 1.59-10.54, p=0.003) after controlling for age, gender and AID-SS. Of note, there was no significant difference in cumulative or first administered activities of $^{131}$I between patients who had sialadenitis
after $^{131}$I treatment (A) and patients who had sialadenitis before and after $^{131}$I treatment (BA). A validation study was not conducted to verify this finding as patients rarely reported or were asked about sialadenitis prior to $^{131}$I treatment in our ENR cohort.

Among $^{131}$I-treated patients, patients diagnosed with AID-SS had increased incidence of xerostomia and sialadenitis.

Most patients with Sjogren’s syndrome suffer from xerostomia and some suffer from sialadenitis. It has been reported that 4-48% of patients with autoimmune disease develop symptoms of Sjogren’s syndrome \(^{35}\), and that patients with autoimmune diseases are at higher risk of developing thyroid cancer \(^{38-41}\). We investigated if xerostomia scores and/or sialadenitis incidence were higher among patients diagnosed with AID-SS (see supplementary Table 2). After controlling for age and gender, the association between AID-SS and xerostomia scores (Fig 5A) and sialadenitis incidence (Fig 5B) achieved borderline significance, with $p=0.051$ and $p=0.046$, respectively. In this analysis, we excluded patients who had sialadenitis before $^{131}$I treatment to focus on sialadenitis occurrence after $^{131}$I treatment.

In the validation study, the incidence of xerostomia among $^{131}$I-treated patients was significantly higher in patients diagnosed with AID-SS by both logistic regression analysis (OR=4.50, 95% CI 2.12-9.59, $p<0.001$) and Cox regression analysis (HR=7.17, 95% CI 3.27-15.70, $p<0.001$). The incidence of sialadenitis among $^{131}$I-treated patients was also significantly higher in patients diagnosed with AID-SS by both logistic
regression analysis (OR=4.06, 95% CI 1.49-11.07, p=0.006) and Cox regression analysis (HR=5.46, 95% CI 1.23-24.28, p=0.030).

Several papers reported that patients were diagnosed with AID-SS after $^{131}$I treatment $^{42-44}$. Among our $^{131}$I-treated patients with known dates for $^{131}$I therapy and AID-SS diagnosis, 7/11 (63.6%) and 38/43 (88.4%) patients had their AID-SS diagnosed after their first $^{131}$I treatment in our initial study and validation study, respectively. However, Cox regression analysis taking time of occurrence into consideration showed that there was no association between AID-SS diagnosis and $^{131}$I treatment (HR=1.92, 95% CI 0.34-10.77, p=0.460) using the date of thyroid cancer diagnosis as time zero.

2.5 Discussion

This study confirmed that $^{131}$I-treated patients had significantly higher incidence of xerostomia and sialadenitis than patients who did not receive $^{131}$I. Despite higher mean cumulative and mean first administered $^{131}$I activity in patients with xerostomia, $^{131}$I activity alone cannot serve as a predictor for $^{131}$I-induced xerostomia for individual patients as $^{131}$I activity widely overlapped between patients with and without xerostomia (Fig 2A and Fig 3A). Increase in age associated with higher incidence of xerostomia, and females had higher incidence of sialadenitis. Patients’ experience of sialadenitis before $^{131}$I therapy may predict sialadenitis incidence after $^{131}$I therapy, which warrants further validation. Among $^{131}$I-treated patients, patients diagnosed with AID-SS had higher incidence of xerostomia and sialadenitis, suggesting a role for assessing for symptoms of AID-SS prior to administering $^{131}$I therapy to help define risk of complications.
Accurate assessment of $^{131}\text{I}$-induced salivary gland damage is instrumental to identify risk factors for $^{131}\text{I}$-induced xerostomia and/or sialadenitis in thyroid cancer patients. Both sialadenitis and xerostomia can be evaluated by subjective questionnaire. $^{131}\text{I}$ therapy is a major cause of sialadenitis and can cause xerostomia; however, many medications and/or unrelated clinical issues can also lead to xerostomia making it a complex clinical endpoint. Radioiodine uptake in the salivary glands is mediated by the sodium iodide symporter with expression limited to the salivary gland ductal cells $^{7,8}$. Accordingly, $^{131}\text{I}$ causes ductal cell damage that leads to ductal obstruction, which clinically manifests as sialadenitis. Sialadenitis may contribute to transient xerostomia and may ultimately lead to permanent xerostomia. Xerostomia following $^{131}\text{I}$ therapy that is not preceded by sialadenitis may or may not be caused by $^{131}\text{I}$ treatment. In the initial study, the incidence of subjective sialadenitis after $^{131}\text{I}$ treatment is 25.9% (37/143) via questionnaire when excluding patients with a history of sialadenitis prior to $^{131}\text{I}$ treatment. This is in agreement with other studies that report sialadenitis incidence ranging from 20% to 35% $^{17,31,32,42}$. In comparison, the incidence of subjective xerostomia is more variable among different studies ranging from 4% to 43% $^{16,17,42}$. Taken together, subjective sialadenitis appears to be more specific to $^{131}\text{I}$-induced salivary gland damage than subjective xerostomia.

The most common way to objectively assess $^{131}\text{I}$-induced salivary gland dysfunction is via salivary gland scintigraphy, which shows reduced uptake and/or reduced excretion of $^{99m}\text{TcO}_4^-$ $^{16,18,42,45}$. $^{131}\text{I}$-induced salivary gland damage can be
divided into three phases. First, ductal damage can be detected via salivary gland scintigraphy showing reduced uptake and reduced excretion of $^{99m}$TcO$_4^-$, and at that time, patients may be asymptomatic $^{45}$. Second, patients become symptomatic when ductal obstruction progresses to sialadenitis, which can be acute or chronic. Third, severe sialadenitis leads to permanent xerostomia. The incidence of salivary gland dysfunction via salivary gland scintigraphy was reported to be 44%-73% and correlated with $^{131}$I activity $^{16,18,42}$. Solans et al $^{42}$ reported the majority of salivary gland dysfunction that occurred within one year post-$^{131}$I treatment was not persistent, and that salivary gland dysfunction may occur years after $^{131}$I treatment. Jeong et al $^{16}$ showed that patients with abnormal salivary gland scintigraphy prior to $^{131}$I treatment had higher incidence of worsening salivary gland dysfunction after $^{131}$I treatment. Our finding that patients who had a history of sialadenitis before $^{131}$I treatment had higher incidence of sialadenitis after $^{131}$I treatment was in agreement with Jeong et al $^{16}$. However, it remains uncertain if the increase of sialadenitis incidence after $^{131}$I is related to $^{131}$I treatment, as sialadenitis can be chronic and/or recurrent.

Our finding that patients diagnosed with AID-SS had a higher incidence of xerostomia and sialadenitis among $^{131}$I-treated patients is of clinical interest. Since both $^{131}$I treatment and AID-SS could lead to salivary gland damage, it is possible that $^{131}$I treatment and AID-SS in combination would lead to symptoms in some people who otherwise would have remained asymptomatic if only affected by AID-SS or $^{131}$I treatment alone. The aforementioned interpretation along with the fact that 90% of
patients with Sjogren’s syndrome are female may account for the higher risk of sialadenitis in $^{131}$I-treated females.

It is possible that $^{131}$I not only directly causes salivary gland damage but may also trigger or accelerate the clinical manifestation of AID-SS, which leads to occurrence of xerostomia and sialadenitis. $^{131}$I accumulation in the salivary glands may lead to tissue injury exposing autoantigens to elicit an autoimmune response in patients susceptible to developing AID-SS. However, Cox analysis did not show significant association between receiving $^{131}$I treatment and AID-SS. Considering that most AID-SS have a preclinical stage that can last 9-10 years or more, we cannot completely exclude the possibility that $^{131}$I treatment may trigger clinical manifestation of AID-SS. For our validation study, the time between $^{131}$I therapy and medical search for ICD9/10 codes ranged from 3.8 months to 49 years with a mean of 9.67 years and a median of 8.72 years.

2.6 Conclusions

Many $^{131}$I-treated thyroid cancer survivors suffer from life-long morbidity of $^{131}$I-induced salivary gland dysfunction. $^{131}$I activity alone is a poor predictor for $^{131}$I-induced xerostomia and/or sialadenitis for individual patients as patients may be susceptible or resistant to $^{131}$I-induced salivary gland damage. This study showed that increased age, female gender, a history of sialadenitis before $^{131}$I therapy, and AID-SS diagnosis might serve as risk factors for $^{131}$I-induced salivary gland damage in thyroid cancer patients. However, these findings must be validated by prospective studies. In addition, objective
assessment of salivary dysfunction along with biomarker profiling prior to and multiple times after $^{131}$I therapy is necessary to identify biomarkers predicting individual patients’ risk and pathological progression of $^{131}$I-induced salivary gland damage.
Table 2.1. Patient demographics and clinical data for initial and validation study cohorts.

<table>
<thead>
<tr>
<th></th>
<th>Initial Study (n=216)</th>
<th>Validation Study (n=1507)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gender</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>171</td>
<td>1121</td>
</tr>
<tr>
<td>Male</td>
<td>45</td>
<td>386</td>
</tr>
<tr>
<td><strong>$^{131}$I Treatment</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>169</td>
<td>1165</td>
</tr>
<tr>
<td>No</td>
<td>47</td>
<td>342</td>
</tr>
<tr>
<td><strong>Cumulative $^{131}$I activity (mCi)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean/Median</td>
<td>136/ 125</td>
<td>150/ 125</td>
</tr>
<tr>
<td>Range&lt;sup&gt;#&lt;/sup&gt;</td>
<td>25-850</td>
<td>20-1162</td>
</tr>
<tr>
<td><strong>First $^{131}$I activity (mCi)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean/Median</td>
<td>111/ 100</td>
<td>111/ 100</td>
</tr>
<tr>
<td>Range&lt;sup&gt;#&lt;/sup&gt;</td>
<td>25-230</td>
<td>10-700</td>
</tr>
<tr>
<td><strong>Histology</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Papillary</td>
<td>194</td>
<td>1315</td>
</tr>
<tr>
<td>Follicular</td>
<td>19</td>
<td>183</td>
</tr>
<tr>
<td>Anaplastic</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>Poorly Differentiated</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td><strong>T Stage</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T0</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>T1</td>
<td>119</td>
<td>731</td>
</tr>
<tr>
<td>T2</td>
<td>27</td>
<td>373</td>
</tr>
<tr>
<td>T3</td>
<td>50</td>
<td>303</td>
</tr>
<tr>
<td>T4</td>
<td>14</td>
<td>67</td>
</tr>
<tr>
<td>unknown</td>
<td>6</td>
<td>30</td>
</tr>
<tr>
<td><strong>N Stage</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N0</td>
<td>116</td>
<td>984</td>
</tr>
<tr>
<td>N1</td>
<td>87</td>
<td>477</td>
</tr>
<tr>
<td>N2</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>NX</td>
<td>9</td>
<td>25</td>
</tr>
<tr>
<td>unknown</td>
<td>4</td>
<td>19</td>
</tr>
<tr>
<td><strong>M Stage</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M0</td>
<td>206</td>
<td>1432</td>
</tr>
<tr>
<td>M1</td>
<td>6</td>
<td>53</td>
</tr>
<tr>
<td>MX</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>unknown</td>
<td>4</td>
<td>18</td>
</tr>
</tbody>
</table>

*: Age at questionnaire (initial study) or at time of information warehouse search (validation study)

#: Only 7/1165 $^{131}$I-treated patients received <30 mCi for their first administered activity, and 5 of these 7 patients received <30 mCi cumulative activity.
Figure 2.1. Patients treated with $^{131}$I had significantly higher incidence of xerostomia and sialadenitis in both the initial study (A, B) and the validation study (C, D).

(A) A multivariate linear regression model showed that $^{131}$I treatment significantly increased the mean xerostomia score by 0.81 after controlling for age, gender, and AID-SS (p=0.002). (B) A multivariate logistic regression model showed that $^{131}$I-treated patients had significantly higher sialadenitis incidence after controlling for age, gender, and AID-SS (OR=5.49, 95% 1.50-20.08, p=0.010). $^{131}$I-treated patients who reported sialadenitis before $^{131}$I treatment (n=26) were excluded from analysis. (C) Logistic regression analysis showed $^{131}$I-treated patients had a higher incidence of xerostomia after controlling for age, gender, and AID-SS diagnosis (OR=4.01, 95% CI 1.81-8.90, p<0.001). (D) Fisher’s exact test showed $^{131}$I-treated patients had a higher incidence of sialadenitis with an infinite unadjusted OR (95% CI 3.05-infinity, p<0.001). Adjusted OR could not be calculated, as there was no sialadenitis incidence in patients without $^{131}$I treatment. One patient who experienced sialadenitis before $^{131}$I therapy and two patients with unknown $^{131}$I dates were excluded from analysis. Figure from Hollingsworth et al, 2016$^{46}$. 

![Graph A](image1.png)  ![Graph B](image2.png)  ![Graph C](image3.png)  ![Graph D](image4.png)
Figure 2.2. Patients with xerostomia, but not patients with sialadenitis, had 46 mCi higher mean cumulative $^{131}$I activity in the validation study (C, D) but not in the initial study (A, B).

(A) Pearson correlation showed no significant correlation between cumulative $^{131}$I activity and mean xerostomia scores (p=0.732). (B) Multivariate linear regression showed no significant difference in the cumulative $^{131}$I activities among patients who never had sialadenitis (“N”), patients who had sialadenitis only after $^{131}$I treatment (“A”), patients who had sialadenitis before and after $^{131}$I treatment (“BA”), and patients who had sialadenitis only before $^{131}$I treatment (“B”) after controlling for gender, age, and AID-SS (p=0.549). Patients who failed to complete all xerostomia questions were excluded from this analysis. (C) A multivariate linear model showed patients who experienced xerostomia had 46 mCi (95% CI 15-77 mCi) higher mean cumulative $^{131}$I activity than patients who did not experience xerostomia after controlling for age and gender (p=0.003). (D) A multivariate linear model showed patients who experienced sialadenitis had no significant difference in cumulative $^{131}$I activity than patients who did not experience sialadenitis after controlling for age and gender (p=0.770). One patient who experienced sialadenitis before $^{131}$I therapy and two patients with unknown $^{131}$I dates were excluded from analysis. Figure from Hollingsworth et al, 2016.
Figure 2.3. Patients with xerostomia, but not patients with sialadenitis, had 21 mCi higher mean first administered $^{131}$I activity in the validation study (C, D) but not in the initial study (A, B).

(A) Pearson correlation showed no significant correlation between first $^{131}$I administered activity and mean xerostomia scores ($p=0.320$). (B) Multivariate linear regression showed no significant difference in the first $^{131}$I activities among patients who never had sialadenitis (“N”), patients who had sialadenitis only after $^{131}$I treatment (“A”), patients who had sialadenitis before and after $^{131}$I treatment (“BA”), and patients who had sialadenitis only before $^{131}$I treatment (“B”) after controlling for gender, age, and AID-SS status ($p=0.053$). Patients who failed to complete all xerostomia questions were excluded from this analysis. (C) A multivariate linear model showed patients who experienced xerostomia had 21 mCi (95% CI 6-35 mCi) higher mean first $^{131}$I activity than patients who did not experience xerostomia after controlling for age and gender ($p=0.005$). (D) A multivariate linear model showed patients who experienced sialadenitis had no significant difference in first $^{131}$I activity than patients who did not experience sialadenitis after controlling for age and gender ($p=0.580$). One patient who experienced sialadenitis before $^{131}$I therapy and two patients with unknown $^{131}$I dates were excluded from analysis. Figure from Hollingsworth et al, 201646.
Figure 2.4. Patients who had sialadenitis before $^{131}\text{I}$ therapy had higher sialadenitis incidence after $^{131}\text{I}$ therapy.

Multivariate logistic regression controlling for age, gender, and AID-SS status showed sialadenitis before $^{131}\text{I}$ therapy correlated with increased sialadenitis incidence after $^{131}\text{I}$ therapy (OR=4.10, 95% CI 1.59-10.54, p=0.003). Patients who had sialadenitis prior to and after $^{131}\text{I}$ therapy are abbreviated “BA”. Patients who had sialadenitis only prior to $^{131}\text{I}$ therapy are abbreviated “B”. Patients who had sialadenitis only after $^{131}\text{I}$ therapy are abbreviated “A”. Patients who never had sialadenitis are abbreviated “N”. Figure from Hollingsworth et al, 2016$^{46}$.
Figure 2.5. Among ¹³¹I-treated patients, patients diagnosed with AID-SS had increased incidence of xerostomia and sialadenitis in the validation study (C, D) but less significant in the initial study (A, B).

(A) Multivariate analysis using MANOVA did not show a significant difference in xerostomia scores between patients with AID-SS (“AID-SS +”) and patients not diagnosed with AID-SS (“AID-SS –”) after controlling for age and gender (p=0.051). (B) Multivariate logistic regression showed “AID-SS +” patients had a higher sialadenitis incidence after ¹³¹I treatment than “AID-SS –” patients (OR=3.70, 95% CI 1.03-13.16, p=0.046) after controlling for age and gender. Patients who had sialadenitis before ¹³¹I therapy (“B” and “BA” patients) were excluded from analysis. (C) Logistic regression analysis showed that, among ¹³¹I-treated patients, “AID-SS +” patients had a higher incidence of xerostomia (OR=4.50, 95% CI 2.12-9.59, p<0.001) after controlling for age and gender. (D) Logistic regression analysis showed that, among ¹³¹I-treated patients, “AID-SS +” patients had a higher incidence of sialadenitis (OR=4.06, 95% CI 1.49-11.07, p=0.006) after controlling for age and gender. One patient who experienced sialadenitis before ¹³¹I therapy and two patients with unknown ¹³¹I dates were excluded from analysis. Figure from Hollingsworth et al, 2016.
Table 2.2. Symptom and AID-SS ICD9/10 codes searched for in the electronic medical record.

<table>
<thead>
<tr>
<th>Term</th>
<th>ICD9/10 Codes</th>
<th>Total</th>
<th>131\textsuperscript{I} Yes</th>
<th>Code entered before 131\textsuperscript{I}</th>
<th>Code entered after 131\textsuperscript{I}</th>
<th>unknown 131\textsuperscript{I} date</th>
<th>131\textsuperscript{I} No</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xerostomia</td>
<td>527.7, K11.7</td>
<td>84</td>
<td>3</td>
<td>71</td>
<td>3</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Sialadenitis</td>
<td>527.2, K11.20, K11.21, K11.22, K11.23</td>
<td>39</td>
<td>1</td>
<td>36</td>
<td>2</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Undifferentiated connective tissue disease</td>
<td>710.9, M35.9</td>
<td>7</td>
<td>0</td>
<td>3</td>
<td>0</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Systemic sclerosis</td>
<td>710.1, M34.0, M34.81, M34.82, M34.83, M34.89, M34.9</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Sjogren's syndrome/Sicca syndrome</td>
<td>710.2, M35.00, M35.01, M35.02, M35.03, M35.04, M35.09</td>
<td>25</td>
<td>1</td>
<td>18</td>
<td>0</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Scleroderma</td>
<td>701.0, L94.0, L94.1</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Rheumatoid arthritis</td>
<td>714.0, M05.89, M05.9, M06.9</td>
<td>19</td>
<td>5</td>
<td>11</td>
<td>0</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Mixed connective tissue disease</td>
<td>710.8, M35.1, M35.5, M35.8</td>
<td>5</td>
<td>0</td>
<td>4</td>
<td>0</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Lupus (SLE)</td>
<td>710.0, M32.10, M32.19, M32.8, M32.9</td>
<td>9</td>
<td>0</td>
<td>6</td>
<td>0</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Juvenile arthritis</td>
<td>714.30, M08.00</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Discoid lupus</td>
<td>695.4, L93.0, L93.1, L93.2</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>
Table 2.3. Clinical data of Initial Study $^{131}$I-treated patients diagnosed with AID-SS.

<table>
<thead>
<tr>
<th>Gender</th>
<th>Age</th>
<th>First $^{131}$I Activity (mCi)</th>
<th>Cumulative $^{131}$I Activity (mCi)</th>
<th>Sialadenitis</th>
<th>AID-SS Dx</th>
<th>Time between First $^{131}$I and AID-SS Dx (yrs)</th>
<th>Mean XQ Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>F</td>
<td>39</td>
<td>150</td>
<td>150</td>
<td>A</td>
<td>SLE and CTD</td>
<td>+0.32</td>
<td>0.5</td>
</tr>
<tr>
<td>F</td>
<td>54</td>
<td>100</td>
<td>100</td>
<td>B</td>
<td>RA</td>
<td>+1.35</td>
<td>ND</td>
</tr>
<tr>
<td>F</td>
<td>52</td>
<td>50</td>
<td>50</td>
<td>B</td>
<td>RA</td>
<td>+1.73</td>
<td>0.5</td>
</tr>
<tr>
<td>M</td>
<td>46</td>
<td>150</td>
<td>150</td>
<td>BA</td>
<td>CTD</td>
<td>+3.02</td>
<td>1.8</td>
</tr>
<tr>
<td>F</td>
<td>74</td>
<td>150</td>
<td>150</td>
<td>N</td>
<td>RA</td>
<td>+4.33</td>
<td>3.3</td>
</tr>
<tr>
<td>F</td>
<td>59</td>
<td>30</td>
<td>30</td>
<td>N</td>
<td>CTD</td>
<td>+6.70</td>
<td>3</td>
</tr>
<tr>
<td>F</td>
<td>43</td>
<td>100</td>
<td>457.5</td>
<td>N</td>
<td>CTD</td>
<td>+14.21</td>
<td>3.3</td>
</tr>
<tr>
<td>F</td>
<td>37</td>
<td>150</td>
<td>150</td>
<td>A</td>
<td>RA</td>
<td>-0.54</td>
<td>1.8</td>
</tr>
<tr>
<td>F</td>
<td>81</td>
<td>200</td>
<td>200</td>
<td>B</td>
<td>RA</td>
<td>-10.53</td>
<td>3.3</td>
</tr>
<tr>
<td>F</td>
<td>48</td>
<td>50</td>
<td>50</td>
<td>N</td>
<td>SLE</td>
<td>-20.25</td>
<td>0.5</td>
</tr>
<tr>
<td>F</td>
<td>58</td>
<td>100</td>
<td>100</td>
<td>N</td>
<td>RA</td>
<td>-4.52</td>
<td>2</td>
</tr>
<tr>
<td>F</td>
<td>55</td>
<td>100</td>
<td>260</td>
<td>A</td>
<td>RA</td>
<td>?</td>
<td>0.8</td>
</tr>
<tr>
<td>F</td>
<td>38</td>
<td>150</td>
<td>150</td>
<td>A</td>
<td>RA</td>
<td>?</td>
<td>4</td>
</tr>
<tr>
<td>F</td>
<td>51</td>
<td>150</td>
<td>150</td>
<td>A</td>
<td>SLE and CTD</td>
<td>?</td>
<td>3.3</td>
</tr>
<tr>
<td>F</td>
<td>48</td>
<td>130</td>
<td>130</td>
<td>A</td>
<td>SLE and RA</td>
<td>?</td>
<td>4</td>
</tr>
<tr>
<td>M</td>
<td>71</td>
<td>175</td>
<td>175</td>
<td>BA</td>
<td>RA</td>
<td>?</td>
<td>ND</td>
</tr>
</tbody>
</table>

“ND”: not determined; “?”: unknown.
“N”: never had sialadenitis; “A”: sialadenitis only after $^{131}$I therapy; “BA”: had sialadenitis before and after $^{131}$I therapy; “B”: had sialadenitis only prior to $^{131}$I therapy.
AID-SS Dx: autoimmune diagnosis; SLE: systemic lupus erythematosus; CTD: connective tissue disease; RA: rheumatoid arthritis.
Time between First $^{131}$I and AID-SS Dx“+” before number: patient was diagnosed with AID-SS after $^{131}$I; “-“ before number: patient was diagnosed with AID-SS before $^{131}$I.
Chapter 3: Automated microSPECT/CT imaging analysis of the mouse thyroid gland.

3.1 Abstract

The ability of thyroid follicular cells to take up and retain iodine enables the use of radioactive iodine (RAI) for imaging and targeted killing of RAI-avid thyroid cancer following thyroidectomy. To facilitate identifying novel strategies to improve $^{131}$I therapeutic efficacy for patients with non-RAI-avid disease or with poor response to $^{131}$I therapy, it is desired to optimize image acquisition and analysis for preclinical mouse models of thyroid cancer. **Methods:** A customized mouse cradle was designed and used for microSPECT/CT image acquisition at 1 hour and 24 hours post-injection of $^{123}$I, which reflect RAI influx/efflux equilibrium and RAI retention in the thyroid, respectively. FVB/N mice with normal thyroid glands and TgBRAF$^{V600E}$ mice as well as TRβ$^{PV/PV}$/Akt2 knockout mice with thyroid tumors were imaged. In-house CTViewer software was developed to streamline image analysis with new capabilities along with display of 3D voxel-based $^{123}$I gamma photon intensity in MATLAB. **Results:** Our customized mouse cradle facilitates consistent tissue configuration such that rigid body registration can be applied to align serial images of the same mouse via our in-house CTViewer software package. Automatic segmentation of thyroid volumes of interest (VOI) from adjacent salivary glands in t1 images is enabled by superimposing the thyroid
VOI from the t24 image onto the corresponding aligned t1 image. The extent of heterogeneity in $^{123}$I accumulation within thyroid VOIs can be visualized by 3D display of voxel-based $^{123}$I gamma photon intensity. **Conclusion:** These advances will greatly facilitate preclinical mouse studies to uncover novel strategies to improve $^{131}$I therapeutic efficacy for patients with advanced thyroid cancer. Furthermore, our approach of superimposing thyroid VOIs from t24 images to select thyroid VOIs on corresponding aligned t1 images can be applied to studies in which target tissue has differential radiotracer retention from surrounding tissues.

3.2 Introduction

The ability of thyroid follicular cells to take up and retain iodine enables the use of radioactive iodine (RAI) for imaging and targeted killing of RAI-avid residual and metastatic thyroid cancer following thyroidectomy. The gamma ray emitting $^{123}$I and the positron emitting $^{124}$I are used for diagnostic single photon emission computed tomography (SPECT) and positron emission tomography (PET) imaging to identify RAI-avid lesions, respectively. After confirming RAI-avid disease by diagnostic scan, $^{131}$I is administered to patients, as the beta-particles emitted from $^{131}$I selectively kill RAI-avid cells and nearby cells. The concomitantly emitted gamma rays from $^{131}$I allow imaging of RAI-avid lesions with inferior resolution to $^{123}$I.

$^{131}$I therapy is the standard of care and has proven to be effective in improving overall survival$^{28,47}$, especially for patients with RAI-avid micrometastases. However, patients with structurally discrete RAI avid metastatic disease often require multiple $^{131}$I
treatments, yet the patients may still have persistent disease. Furthermore, patients with non-RAI-avid disease do not benefit from $^{131}$I therapy as their tumors have lost the ability to take up $^{131}$I. These patients are in need of novel treatments as their tumors are generally resistant to conventional chemotherapy$^{48}$. To date, no novel treatment has shown improved overall survival for patients with progressive non-RAI-avid metastatic disease, despite improved progression-free survival with manageable adverse effects in some patients treated with emerging small molecule inhibitor therapy$^{49-51}$. Much effort has been focused on restoring and increasing RAI uptake in non-RAI-avid lesions and nuclear imaging of preclinical thyroid tumor mouse models$^{23,24}$ was instrumental in identifying a mitogen-activated protein kinase kinase (MEK) inhibitor that restored/enhanced RAI uptake in some patients with non-RAI-avid lesions$^{52}$.

To identify novel strategies to further improve $^{131}$I therapeutic efficacy for patients with non-RAI-avid disease or with poor response to $^{131}$I therapy, it is desired to further optimize the workflow of image acquisition and enhance the capability of image analysis for thyroid cancer mouse models. In this study, we developed a mouse cradle to achieve consistent tissue configuration of the head and neck region during image acquisition, which greatly facilitated subsequent image analysis. In-house CTViewer software was developed with the objective to streamline image analysis with new capabilities. We are able to conduct automatic segmentation of thyroid volumes of interest (VOI) in SPECT/X-ray computed tomography (CT) images acquired 1 hour (t1) and 24 hours (t24) post-injection of $^{123}$I, which reflect radioiodine influx/efflux.
equilibrium and radioiodine retention, respectively. In addition, we are able to identify xyz coordinates of areas with differential gamma photon intensity from $^{123}$I accumulation such that the extent of heterogeneity of $^{123}$I accumulation within thyroid VOI can be quantitatively evaluated.

3.3 Materials and Methods

**Animals**

All studies were approved by The Ohio State University’s Institutional Animal Care and Use Committee, which oversees the responsible use of animals in university research and instructional activities. All research activities conformed to the statutes of the Animal Welfare Act and the guidelines of the Public Health Service as issued in the Guide for the Care and Use of Laboratory Animals. Mice used for microSPECT/CT imaging in this study include FVB/N mice, TgBRAF$^{V600E}$ mice carrying BRAF$^{V600E}$-driven thyroid tumors$^{53}$, and $\text{Tg}^{\text{PV/PV}}$ mice$^{54}$ crossed with Akt2-knockout (KO) mice.

**Cradle design and mouse placement in cradle**

To facilitate consistent tissue configuration and positioning of the mouse head and neck where the thyroid is located among image acquisitions, a cradle comprised of a jig with a groove for a gas anesthesia tube, a tooth bar, two cheek bars, and a cover with a holder for an Eppendorf tube containing known radioactivity for decay control was designed (Fig. 3.1A). The cradle components were printed using RDG720 material with
an Objet Prime 30 3D printer (Stratasys, Eden Prairie, MN, USA) at .0006 inch resolution, and were assembled and attached to the mouse bed in the Flex-XO SPECT/CT system (Trifoil Imaging, Inc., Chatworth, CA) using nylon screws and fasteners so as not to distort the X-ray CT image. The jig serves as a base for all cradle parts, and the tooth bar holds the mouse’s top incisors to maintain the head position in the cranial/caudal direction. The cheek bars are placed on either side of the mouse’s head to maintain the head position in the right/left direction. The cover traps gas anesthesia inside of the cradle to ensure the mouse remains unconscious and holds an Eppendorf tube containing known radioactivity for decay control. The components of the cradle relative to the mouse being imaged are shown in Figure 3.1B-D.

For mouse placement in the cradle, the mouse first had its top incisors positioned in the hole of tooth bar. The limbs were placed on the outside of the cradle and the body was positioned symmetrically in the right/left direction. The tail was gently pulled back to straighten the spine, the cheek bars were placed against the mouse, and the cover was placed on the jig (Supplementary Video 1).

MicroSPECT/CT image acquisition

All mice subjected to microSPECT/CT imaging were intraperitoneally injected with 400–450 μCi $^{123}$I or 185-250 μCi $^{99m}$TcO$_4^-$ in 200 μL saline prepared by the nuclear pharmacy at our institute. Radioactivity was measured by a dose calibrator (CRC-12; Capintec) and normalized for radioactive decay. The mouse was anesthetized with 4%
isoflurane mixed with O₂ at a flow rate of 1.5 L/min for 3 minutes and then placed in the cradle with 1.5-2% isoflurane mixed with O₂ at the same flow rate. A 0.25-mL Eppendorf tube containing 10-30 μCi ¹²³I was included as a decay control. Image acquisition was conducted at 1 hour (t1) and 24 hours (t24) post-¹²³I injection or 1 hour post-⁹⁹ᵐTcO₄⁻ injection. Mice imaged with ⁹⁹ᵐTcO₄⁻ were imaged multiple times, and these images were used for mouse rotation and translation analyses. SPECT images were acquired with two gamma cameras equipped with 1 mm single-pinhole collimators (75 mm focal distance) using the following settings: 180-degree rotation per camera, 32 projections per camera, 30-second projection duration, and 30-mm radius of rotation. CT images were acquired with a single X-ray detector camera at the following settings: X-ray tube set to 75 kVp, 360 degree rotation, 1024 projections, 1 frame per projection, and 2.5X magnification. SPECT image is 80×80×80 pixels with a resolution of 0.52 mm, while the CT image is 512×512×512 pixels with a resolution of 0.075 mm.

Image analysis with in-house CTViewer software

A Microsoft foundation class (MFC)-based CT and SPECT image analysis software, CTViewer, was designed in-house, in which all the algorithms for image processing and data analysis were written in C++. The capabilities of CTViewer include 1) loading RAW data sets of SPECT and CT images, 2) co-registering SPECT and CT images based on the pre-calibrated coordinate transformation, 3) displaying the SPECT and CT images in three orthogonal planes, whether separately or in fusion mode, 4)
automatically selecting VOIs in a threshold-based manner, and 5) automatically calculating percentage of injected radioisotope in thyroid VOIs. In addition to the aforementioned capabilities, a two-step rigid body registration was developed to align images of the same mice taken at different times. First, CTViewer coarsely aligns the target CT image to the reference CT image based on the skull (the rigid body) using principle component analysis and iteratively adjusting VOIs. In the second step, the two CT images are finely aligned using 3D image correlation in 3 iterations. Consequently, the computation time is greatly reduced. The same coordinate transformation matrix is then applied to align the corresponding two SPECT images. The features of CTViewer and the time required for the whole process are shown in the Supplementary Video 2.

3.4 Results

_Cradle enabling pose control leads to consistent tissue configuration_

Due to the flexible body of a living mouse, the spatial relationship between target tissues of interest and surrounding anatomic landmarks varies significantly among images acquired on a standard CT imaging bed. As shown in Figure 3.2, consistency of tissue configuration within the head and neck region is greatly improved when the cradle is employed. To evaluate the effectiveness of our cradle in achieving pose control, we calculated translation and rotation required to align two images acquired at different times, such as t1 and t24, for each individual mouse. This process was applied to 4 FVB/N mice, 6 TgBRAF<sup>V600E</sup> mice (3 by user 1 and 3 by user 2), and 4 TR<sup>PV/PV</sup> Akt2KO mice. The sagittal (x), coronal (y), and transaxial (z) translation and
the rotation around x, y, and z axes of these 14 mice were summarized in Table 3.1. Regardless of the difference in age (4.8 to 16.7 months), body weight (19.9 to 45 g), mouse model, and user (n=two users), the x, y, and z translations for all mice are less than 1 mm. The rotation around the x and y axes for all mice are less than 5 degrees. The rotation around the z axis has the greatest variability, yet is less than 11 degrees.

**Automatic segmentation of thyroid VOIs on t1 and t24 SPECT images**

At 1 hour post-$^{123}$I injection, $^{123}$I not only accumulates in the thyroid gland but also in the salivary glands as both glands express $\text{Na}^+/$I$^{-}$ symporter, which mediates $^{123}$I influx against its concentration gradient. The mouse thyroid and salivary glands are in close proximity and the boundaries of soft tissues are not discernable via our anatomical CT image. It is, therefore, difficult to separate $^{123}$I accumulation in the thyroid gland from that in adjacent salivary glands in t1 SPECT images. At 24 hours post-$^{123}$I injection, $^{123}$I has been organified into thyroglobulin and retained in the thyroid follicles, whereas $^{123}$I in the salivary gland has been excreted. Consequently, $^{123}$I accumulation in the thyroid gland is readily discernable in t24 SPECT images and the thyroid VOI can be automatically segmented in a threshold-based manner. After t1 and t24 images are aligned, the thyroid VOI from the t24 image can be superimposed onto the t1 image to enable automatic segmentation of t1 thyroid VOI. The procedure involved in thyroid VOI segmentation on t24 and t1 SPECT images is shown in Figure 3.3. The CT images of t1 and t24 prior to and after alignment are shown in Fig 3.3A versus 3.3C (sagittal view),
3.3B versus 3.3D (transaxial view). Automatic segmentation of the thyroid VOI in the t24 image is shown in Figure 3.3E and 3.3F, wherein the perimeter of VOI is indicated with red curve. The perimeter of t24 thyroid VOI is then superimposed onto the aligned t1 image as shown in Figure 3.3G and 3.3H. It can be seen that the red curve perfectly encloses the region with highest gamma photon intensity from $^{123}$I, the anticipated thyroid VOI of the t1 image. This demonstrates the effectiveness of pose control using cradle and the accuracy of image alignment. The coronal view of the aforementioned process is shown in Figure 3.4. Taken together, our cradle along with our in-house CTViewer software allows us to automatically define thyroid VOI in t1 and t24 SPECT images without user subjectivity.

_Evaluating the extent of $^{123}$I uptake heterogeneity within the thyroid via 3D voxel-based $^{123}$I gamma photon intensity_

MicroSPECT/CT imaging is used to locate tissues accumulating radioisotope tracer and quantify their radioisotope tracer uptake. Heterogeneity of radioisotope tracer uptake within target tissues of interest is rarely quantitatively evaluated. It is known that thyroid cancer patients with RAI-avid lesions of larger size are less responsive to $^{131}$I therapy, yet the underlying mechanism(s) has not been investigated. One could assume that $^{131}$I uptake in larger metastatic lesions would have a greater extent of heterogeneity in $^{131}$I uptake such that areas with insufficient $^{131}$I uptake would escape from $^{131}$I therapeutic effect. To investigate the effects of heterogeneity on $^{131}$I therapeutic efficacy,
we need to be able to quantitatively evaluate the extent of $^{131}$I uptake heterogeneity within lesions of interest. Our current CTViewer does not have the capability of 3D display. The 3D datasets of the thyroid VOI in the SPECT image were exported and loaded into MATLAB to display 3D voxel-based $^{123}$I gamma photon intensity. Accordingly, the spatial distribution of areas with different levels of gamma photon intensity can be visualized and analyzed (Fig. 3.5 and Supplementary Videos 3 and 4). In normal thyroid VOI from FVB/N mice, the center of both thyroid lobes had the highest level of $^{123}$I accumulation. In thyroid tumor VOI from Tg-BRAF$^{V600E}$ mice, the spatial distribution of heterogeneity in $^{123}$I accumulation is asymmetric between thyroid lobes and had many areas with low $^{123}$I accumulation. As shown in Figure 3.5, normal thyroid VOI from the FVB/N mouse is smaller than thyroid tumor VOI from the TgBRAF$^{V600E}$ mouse, and 2% of normal thyroid voxels have a gamma photon intensity $\geq$32, while 2% of thyroid tumor voxels are $\geq$18. This indicates outgrowth of and decreased RAI uptake in the thyroid tumor of the TgBRAF$^{V600E}$ mouse. Identifying the location and quantifying the volume of areas with high or low gamma photon intensity will help us to uncover mechanism(s) underlying differential response to $^{131}$I therapy among $^{131}$I-avid lesions.

3.5 Discussion

In this study, we utilized a mouse cradle during image acquisition to achieve consistent tissue configuration within the head and neck region. This allows us to apply rigid body registration to align serial images taken at different times of the same subject.
mice with accuracy. Automatic segmentation of thyroid VOIs in t1 images is enabled by superimposing thyroid VOIs from the t24 images onto their corresponding aligned t1 images. Based on 3D voxel-based $^{123}$I gamma photon intensity, we are able to quantitatively evaluate the extent of heterogeneity of $^{123}$I uptake within thyroid VOIs in terms of spatial distance and volume of areas with high or low gamma photon intensity. These advances will greatly facilitate preclinical mouse studies to uncover novel strategies to improve $^{131}$I therapeutic efficacy for patients with advanced thyroid cancer.

Cradles have been routinely used to facilitate the accuracy of cranial irradiation in small animals\textsuperscript{55-58} or co-registration of multi-modality and longitudinal images\textsuperscript{59-62}. Among various cradles for the mouse head and neck region, the tooth bar anchoring upper incisors is essential. Ear bars\textsuperscript{57,62} are effective but difficult to use leading to the use of alternatives such as a Styrofoam neck collar\textsuperscript{55} or cheek bars in our cradle. With 3D printing technology, cradles can be easily customized and produced at low cost. For studies requiring consistency in tissue configuration, use of a cradle during image acquisition is necessary. Consistent tissue configuration allows rigid body registration among serial images of the same mice to easily correct any difference in position and orientation post image acquisition. We hope that our studies will encourage cradles to be used widely among investigators.

VOI selection for targets with discrete signal in functional images is not problematic. VOI selection for multiple targets with close physical proximity in functional images requires co-registration of anatomic images that show discernable
boundaries between targets. The boundaries of soft tissues are usually not discernable without contrast agent in CT images. While MR imaging is superior to CT imaging in visualizing the boundaries of soft tissues, MRI is time consuming, at high cost, and not widely available. Alternatively, the 3D MOBY digital mouse phantom\textsuperscript{63} has been used as a common reference to facilitate VOI selection in functional images\textsuperscript{64,65}. However, this approach is less accurate as the proportion of the target tissues to the rest of the mouse body may be different between subject mice and the mouse phantom. Most importantly, this approach cannot be applied to tumors with varying size and shape.

The fact that thyroid tissue has discrete signal at t24 image due to its unique ability to retain radioiodine allows us to conduct automatic segmentation of the thyroid VOI in the t24 image in a threshold-based manner. The thyroid VOI from the t24 image can then be superimposed onto its corresponding aligned t1 image to automatically select the thyroid VOI in the t1 image. In this study, consistent tissue configuration facilitated by the use of our cradle during image acquisition allows us to treat the head and neck region as a rigid body such that anatomic landmarks can be used for alignment of serial images by rigid body registration. While deformable registration is a powerful avenue to align images\textsuperscript{64,66}, the need to compensate difference in tissue configuration between images may lead to changed voxel-based geometry of target tissues. Taken together, our approach can be applied for studies in which target tissue has differential radiotracer retention from surrounding tissues.
The overall response to $^{131}$I therapy is likely limited by the volume of areas with insufficient $^{131}$I accumulation\textsuperscript{67}. We reason that insufficient $^{131}$I accumulation in microfoci would have less impact than macro-foci on overall therapeutic outcome due to possible bystander effects of $^{131}$I. The 3D dataset of voxel-based gamma photon intensity allows us to quantitatively measure the volume of areas with low $^{131}$I accumulation and their spatial distance from areas with high $^{131}$I accumulation. This capability will facilitate the investigation of the impact of volume and spatial distance of areas with low $^{131}$I accumulation on the overall outcome of $^{131}$I therapy in patients with advanced thyroid cancer.

3.6 Conclusions

In summary, our customized mouse cradle facilitates consistent tissue configuration such that rigid body registration can be applied to align serial images of the same mouse via our in-house CTViewer software. Our approach of superimposing thyroid VOIs from t24 images to select the thyroid VOI on corresponding aligned t1 images can be applied to studies in which target tissue has differential radiotracer retention from surrounding tissues. The ability to identify location with xyz coordinates and to quantitatively measure the volume of areas with low $^{131}$I accumulation allows investigators to predict response to $^{131}$I therapy and to uncover novel strategies to improve $^{131}$I therapeutic efficacy.
Figure 3.1. Cradle design and X-ray computed tomography image of mouse in cradle.

(A) Computer-aided design of the mouse cradle components: a jig with groove for a gas anesthesia tube, a tooth bar with a hole to anchor the mouse’s top incisors, two cheek bars, and a cover with holder for an Eppendorf tube containing decay control of known radioactivity. (B-D) X-ray computed tomography image of a mouse in the cradle in sagittal view (B), coronal view (C), and transaxial view (D).
Figure 3.2. Tissue configuration variation in head and neck region is greatly reduced with the use of cradle during image acquisition.

The top 2 rows show the same sagittal, coronal and transaxial views of X-ray computed tomography (CT) images of the same mouse acquired on separate days without the cradle. The bottom 2 rows show the same sagittal, coronal, and transaxial views of CT images of the same mouse acquired on separate days with the cradle.
Table 3.1. Mouse positioning consistency between serial images of the same mice

<table>
<thead>
<tr>
<th></th>
<th>FVB/N (n=4)</th>
<th>TRβ&lt;sup&gt;PV/PV&lt;/sup&gt;/Akt2KO (n=4)</th>
<th>TgBRAF&lt;sup&gt;V600E&lt;/sup&gt; (n=3)</th>
<th>*TgBRAF&lt;sup&gt;V600E&lt;/sup&gt; (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>X-axis rotation</td>
<td>1.33 +/- 0.68</td>
<td>1.43 +/- 0.74</td>
<td>1.05 +/- 0.80</td>
<td>2.60 +/- 1.34</td>
</tr>
<tr>
<td></td>
<td>(degrees)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Y-axis rotation</td>
<td>1.71 +/- 1.74</td>
<td>1.26 +/- 1.43</td>
<td>1.68 +/- 1.63</td>
<td>1.00 +/- 1.05</td>
</tr>
<tr>
<td></td>
<td>(degrees)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Z-axis rotation</td>
<td>6.30 +/- 3.15</td>
<td>3.74 +/- 2.55</td>
<td>1.42 +/- 0.22</td>
<td>2.21 +/- 2.64</td>
</tr>
<tr>
<td></td>
<td>(degrees)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>X translation (mm)</td>
<td>0.22 +/- 0.24</td>
<td>0.17 +/- 0.11</td>
<td>0.36 +/- 0.33</td>
<td>0.19 +/- 0.24</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Y translation (mm)</td>
<td>0.37 +/- 0.21</td>
<td>0.16 +/- 0.11</td>
<td>0.21 +/- 0.15</td>
<td>0.58 +/- 0.19</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Z translation (mm)</td>
<td>0.14 +/- 0.11</td>
<td>0.23 +/- 0.05</td>
<td>0.09 +/- 0.06</td>
<td>0.46 +/- 0.41</td>
</tr>
</tbody>
</table>

The translation and rotation between images for each mouse model are presented as Mean +/- Standard Deviation. *Mouse placement in the cradle and subsequent imaging conducted by a second user.
Figure 3.3. Automatic segmentation of thyroid volume of interest (VOI) on t1 image via superimposing thyroid VOI from t24 SPECT image.

MicroSPECT/CT images are acquired at 1 hour (t1) and 24 hours (t24) post 123I injection. The sagittal (A, C) and transaxial (B, D) views of a t1 CT image (red) and a t24 CT image (white) prior to (A, B) and after (C, D) alignment are shown. (E, F): the sagittal (E) and transaxial (F) views of the thyroid VOI on the t24 SPECT image. (G, H): the sagittal (G) and transaxial (H) views of the thyroid VOI from the t24 image superimposed on the t1 SPECT image. The location of decay control is included in C, E and G.
Figure 3.4. Automatic segmentation of thyroid volume of interest (VOI) on t1 and t24 SPECT images: Coronal plane view.

MicroSPECT/CT images are acquired at 1 hour (t1) and 24 hours (t24) post 123I injection. The coronal (A, B) views of a t1 CT image (red) and a t24 CT image (white) prior to (A) and after (B) alignment are shown. (C): the coronal view of the thyroid VOI on the t24 SPECT image. (D): the coronal view of the thyroid VOI from the t24 image superimposed on the t1 SPECT image.
Figure 3.5. Coronal view of 3D voxel-based gamma photon intensity of a FVB/N mouse thyroid (A) and a TgBRAF<sup>V600E</sup> mouse thyroid tumor (B)

The 3D contour of the thyroid VOI was segmented using a threshold of 4. Spatial heterogeneity of gamma photon intensity within the thyroid VOI is shown by further segmentation of regions with threshold values corresponding to 20%, 2%, and 0.2% of the VOI voxels.
4.1 Introduction

We are able to automatically segment the thyroid volume of interest in a t24 SPECT image and apply it to the corresponding aligned t1 image to distinguish the thyroid from the salivary glands in the t1 image, but this method requires a t24 image. In some cases, only a t1 image is available as is the case in SPECT/CT imaging with $^{99m}$TcO$_4^-$, which is a substrate of Na$^+$/I$^-$ symporter (NIS), which facilitates iodide uptake into thyroid follicular cells, but not organified and retained in the thyroid. Because $^{99m}$TcO$_4^-$ has a half-life of 6 hours, compared to $^{123}$I half-life of 13 hours, and is not organified and retained in the thyroid and, $^{99m}$TcO$_4^-$ decays and is cleared from the mouse body more quickly than $^{123}$I allowing more frequent imaging time points to assess the equilibrium between influx and efflux or radioisotope in the thyroid rather than retention. This is useful for studies requiring frequent SPECT/CT imaging timepoints, such as examining temporal dynamics of a drug’s effect. However, because both the salivary glands and thyroid have $^{99m}$TcO$_4^-$ accumulation and are in close proximity to each other, delineating the boundary between the thyroid and salivary gland via functional imaging is difficult, particularly distinguishing the ventral boundary of the thyroid from the dorsal boundary of the salivary gland. As the thyroid and salivary glands are soft tissues, their
boundary is difficult to delineate even in a CT image as soft tissue has low contrast in CT images. In this study, we examine different thyroid volume of interest segmentation methods in the t1 image to achieve accurate thyroid volume of interest segmentation with minimal user variation, using the automated t24 volume of interest overlay method as a gold standard for comparison.

4.2 Materials and Methods

Animals

All studies were approved by The Ohio State University’s Institutional Animal Care and Use Committee, which oversees the responsible use of animals in university research and instructional activities. All research activities conformed to the statutes of the Animal Welfare Act and the guidelines of the Public Health Service as issued in the Guide for the Care and Use of Laboratory Animals. Mice used for microSPECT/CT imaging in this study include FVB/N mice, TgBRAF^{V600E} mice carrying BRAF^{V600E}-driven thyroid tumors, and TRβ^{PV/PV} mice crossed with Akt2-knockout mice. MicroSPECT/CT image acquisition was as described in chapter 3.

Thyroid volume of interest segmentation methods

All imaging analysis was performed using our in-house CTViewer software. Prior to volume of interest (VOI) segmentation, images taken 1 hour after ^{123}I injection (t1) were transformed to re-position the image such that the trachea was vertical and the base of the skull was horizontal. This was to facilitate consistent positioning of mice for
anatomical marker selection. Images taken at 24 hours after $^{123}$I injection (t24) were aligned to the transformed t1 images.

Threshold-based VOIs were segmented in the aligned t24 images using a threshold of 2 such that connected voxels ≥2 would be selected. This “t24 VOI” was then overlaid onto the transformed t1 image to which the t24 image was aligned. Anatomical marker-based volumes from which we segmented the “confined adaptive” (CA) thyroid VOI in a threshold-based manner were set around the by selecting right, left, dorsal, ventral, cranial, and caudal boundaries based on pre-determined anatomical markers visible in the CT image and known to have a spatial relationship with the thyroid. These anatomical markers are shown in Figure 4.1. As the thyroids of TgBRAF$^{V600E}$ mice and TRβ$^{PV/PV}$/Akt2-knockout mice have tumors, the boundaries to encompass the thyroid for later CA VOI segmentation needed to be expanded for these mouse models of thyroid cancer (Fig 4.2). The number of slices to expand out from the anatomical marker-based boundaries to encompass the largest thyroid signal for each mouse model was determined (Table 4.1). This expansion out from the anatomical markers was determined using images from 3 TgBRAF$^{V600E}$ mice, 1 female and 2 males, and 4 male TRβ$^{PV/PV}$/Akt2-knockout mice. CA VOIs were segmented in a threshold-based manner from within the anatomical marker box in the t1 image using a range of thresholds. The CA VOIs’ % injected dose activity (%ID) were compared to the %ID of the t24 VOI overlaid onto the t1 image and manually segmented VOI segmented by one user one time.
4.3 Results

Comparison of thyroid volume selected and %ID of T24 overlay thyroid VOI segmentation, confined adaptive threshold-based thyroid VOI segmentation from within anatomical marker-based volume, and manual thyroid VOI segmentation.

We compared the volume selected and the %ID in the thyroid VOI when it was segmented using T24 overlay, CA threshold-based segmentation from within anatomical marker-based volume, or manual segmentation methods. We used a range of thresholds for the CA threshold-based segmentation and determined the threshold that yielded a VOI with the closest %ID to the %ID of the t24 overlay VOI. The t24 overlay, CA threshold-based segmentation with %ID closest to the %ID of the t24 overlay, and manual segmentation of representative images from male FVB/N, TRβPV/PV/Akt2-knockout, and TgBRAFV600E mice are shown in Figure 4.3. While the area with highest signal intensity is included in VOIs segmented using each method, the volume segmented by each method is slightly different for all images.

Graphs of the %ID calculated using the various VOI segmentation methods are shown in Figure 4.4 for FVB/N mice images (Fig 4.4A), TRβPV/PV/Akt2-knockout mice images (Fig 4.4B), and TgBRAFV600E mice images (Fig 4.4C). Thresholds for CA VOI segmentation (CAT) that resulted in the %ID closest to the %ID of the t24 overlay VOI were between 7 and 11 for the images from 4 FVB/N mice (Fig 4.4A), between 9 and 12 for the images from 4 TRβPV/PV/Akt2-knockout mice (Table 4.4B), and between 9 and 10 for the images from 3 TgBRAFV600E mice (Fig 4.4C). While the CAT that resulted in %ID
closest to the %ID of the t24 overlay VOI varied across mice, the trend when comparing mice of the same mouse model was the same for FVB/N and TRβ\textsuperscript{PV/PV}/Akt2-knockout mice, but not TgBRAF\textsuperscript{V600E} mice. TgBRAF\textsuperscript{V600E} male 2 had the smaller thyroid volume by t24 VOI overlay and manual segmentation than TgBRAF\textsuperscript{V600E} male 1 and female 1 (data not shown). This resulted in more salivary gland being included in the anatomical marker-based volume than TgBRAF\textsuperscript{V600E} male 1 and female 1 such that at lower thresholds, salivary gland signal was included in the CA threshold-based VOI (data not shown).

4.4 Discussion

In this study we compared confined adaptive threshold-based thyroid VOI segmentation from within anatomical marker-based volume to t24 overlay and manual thyroid VOI segmentation methods. Our objective was to determine a method to accurately segment the thyroid VOI with minimal user variation in mouse models of thyroid cancer when only a t1 image is available. We found that while the area with highest signal intensity is included in VOIs segmented using each method, the volume segmented by each method is different even when the %ID is very similar. However, the trend of %ID when comparing mice of the same mouse model was similar for the VOI segmentation methods evaluated in FVB/N and TRβ\textsuperscript{PV/PV}/Akt2-knockout mice, but not TgBRAF\textsuperscript{V600E} mice.
Different mouse models of thyroid cancer may have different NIS expression and thus different radioisotope uptake. In this study, we analyzed SPECT/CT images from normal FVB/N mice, TRβ<sub>PV/PV</sub>/Akt2-knockout mice, and TgBRAF<sup>V600E</sup> mice. TRβ<sub>PV/PV</sub>/Akt2-knockout mice have an inserted mutation in the thyroid hormone receptor β gene leading to thyroid hormone resistance<sup>54</sup>. This thyroid hormone resistance results in thyroid hyperplasia to account for a perceived deficiency in thyroid hormone. While the TRβ<sub>PV/PV</sub>/Akt2-knockout mice have thyroid tumors, NIS expression is not decreased in them and thus radioisotope is readily taken up and retained in the thyroids of these mice. Conversely, expression of the BRAF<sup>V600E</sup> oncogene under the thyroglobulin (Tg) promoter in the thyroids of TgBRAF<sup>V600E</sup> mice leads to de-differentiation and subsequently decreased NIS expression and thus decreased radioisotope uptake<sup>53</sup>. However, de-differentiation leads to decreased Tg promoter activity and thus decreased expression of BRAF<sup>V600E</sup> oncogene. Because of this dynamic, NIS expression and thus radioisotope accumulation varies within the thyroid of the same mouse and among different mice of this mouse model. The heterogeneity of radioisotope accumulation in these thyroids is a complicating factor when applying threshold-based VOI segmentation as some areas of thyroid tissue may have very low signal and would thus not be included in the VOI. Because BRAF<sup>V600E</sup> mutations are present in 40-50% of human papillary thyroid carcinomas, the most common type of thyroid cancer,<sup>25</sup> optimized imaging analysis of SPECT images from TgBRAF<sup>V600E</sup> mice is of particular importance.
In summary, CA threshold-based VOI segmentation using the range of thresholds we evaluated resulted in the same trend of %ID as seen with the t24 overlay VOI when comparing SPECT images from FVB/N mice with normal thyroids and TRβPV/PV/Akt2-knockout mice, but CA threshold-based VOI segmentation in SPECT images from TgBRAFV600E mice resulted in different trends depending on the threshold used. Heterogeneity of radioisotope accumulation in the thyroids of TgBRAFV600E mice is a complicating factor in accurate, minimally user subjective segmentation of the thyroid VOI in t1 images that we have not yet overcome.
Figure 4.1. Anatomical markers to set boundaries of volume encompassing thyroid for confined threshold-based thyroid volume of interest segmentation.

To set image volume from which a threshold-based thyroid volume of interest is selected, the cranial and caudal boundaries are set to the most caudal point of the mandible bone (A) and the base of the skull (B), respectively. The right and left boundaries are set to the point where the tympanic bullae touch cranial boundary (C and D), as the tympanic bullae are symmetrical. The dorsal and ventral boundaries are set to the point where the spinal column appears to come to a point (E) and the point where the trachea completely disappears (F), respectively.
Figure 4.2. Expansion past anatomical marker-based boundaries to encompass tumor thyroids in mouse models of thyroid cancer.

Transaxial (top) and sagittal (bottom) views of 2D summation SPECT images overlaid onto respective CT images are shown for a male FVB/N mouse with normal thyroid (A-B), a male TRβPV/PV/Akt2-knockout mouse with thyroid tumors (C-D), and a male TgBRAFV600E mouse with BRAFV600E–driven thyroid tumors (E-F). Images shown were acquired 1 hour after 123I injection (t1). Anatomical marker-based boundaries for normal thyroids are shown in orange and expanded anatomical marker-based boundaries to account for largest TRβPV/PV/Akt2-knockout thyroid (C-D) and TgBRAFV600E thyroid (E-F) are shown in red.
Table 4.1. Image slices to expand out from each anatomical marker-based boundary to encompass thyroids of FVB/N, TgBRAF\textsuperscript{V600E}, and TRβ\textsuperscript{PV/PV}/Akt2KO mice.

<table>
<thead>
<tr>
<th>Anatomical Marker</th>
<th>FVB/N</th>
<th>TgBRAF\textsuperscript{V600E}</th>
<th>TRβ\textsuperscript{PV/PV}/Akt2KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caudal anatomical</td>
<td>None</td>
<td>None</td>
<td>-60 slices</td>
</tr>
<tr>
<td>marker</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cranial anatomical</td>
<td>None</td>
<td>None</td>
<td>+30 slices</td>
</tr>
<tr>
<td>marker</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Right anatomical</td>
<td>+10 slices</td>
<td>+20 slices</td>
<td>+40 slices</td>
</tr>
<tr>
<td>marker</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Left anatomical</td>
<td>-10 slices</td>
<td>-20 slices</td>
<td>-40 slices</td>
</tr>
<tr>
<td>marker</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ventral anatomical</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>marker</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dorsal anatomical</td>
<td>+5 slices</td>
<td>+15 slices</td>
<td>+20 slices</td>
</tr>
<tr>
<td>marker</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Each slice is equal to 0.075 mm

Slices to expand based on largest thyroid signal for each mouse model (FVB/N n=4, TgBRAF\textsuperscript{V600E} n=3, and TRβ\textsuperscript{PV/PV}/Akt2KO n=4)
Figure 4.3. Thyroid volume of interest segmented using T24 overlay method, confined adaptive threshold-based segmentation from within anatomical marker-based volume, or manual segmentation.

Transaxial slice views of SPECT/CT image acquired 1 hour after $^{123}$I injection (t1) with overlaid volumes of interest (VOIs) segmented via three methods. Slice with largest thyroid area is displayed. SPECT/CT image slice from male FVB/N mouse with normal thyroid shown in A-C. SPECT/CT image slice from male TRβPV/PV/Akt2-knockout mouse with thyroid tumors shown in D-F. SPECT/CT image slice from male TgBRAFV600E mouse with BRAFV600E-driven thyroid tumors are shown in G-I. VOIs from SPECT image acquired 24 hours after $^{123}$I injection overlaid on t1 image is shown in red in A, D, G. Conflined adaptive threshold-based VOIs with % injected activity closest to the t24 overlay VOI (red) and the anatomical marker-based volumes from which they were segmented (orange) are shown in B, E, H. Manually segmented VOIs are shown in C, F, I.
Figure 4.4. $^{123}$I percent injected activity in thyroid volumes of interest segmented using T24 overlay method, confined adaptive threshold-based segmentation from within anatomical marker-based volume, or manual segmentation.

Percent injected activity (%ID) were calculated from volumes of interest (VOI) segmented by overlay of the VOI from the image taken 24 hours after $^{123}$I injection (red), segmented manually (green), or segmented by confined adaptive threshold-based segmentation from within the anatomical marker-based volume using various thresholds (CAT). These %ID are shown for images from FVB/N (A), TRβ$^{PV/PV}$/Akt2-knockout (B), and TgBRAF$^{V600E}$ (C) mice.
Chapter 5: Effect of $^{131}\text{I}$ on saliva flow rate and saliva protein composition.

5.1 Introduction

Many studies have been performed showing decreased salivary gland function post-$^{131}\text{I}$ in thyroid cancer patients\textsuperscript{15-18}, but only one retrospective study comparing $^{131}\text{I}$-treated thyroid cancer patients’ saliva to untreated thyroid cancer patients’ saliva\textsuperscript{68} and one prospective study comparing saliva prior to and after $^{131}\text{I}$ administration\textsuperscript{69} have been reported. Both studies assessed a limited number of saliva components, including total protein concentration, amylase, albumin, lactate dehydrogenase, superoxide dismutase, and peroxidase\textsuperscript{68,69}. More extensive protein profiling of saliva from $^{131}\text{I}$-treated thyroid cancer patients has not been reported. Here we investigated the association between saliva flow rate, $^{131}\text{I}$ administered activity, and self-reported symptoms as well as investigated saliva protein composition associated with symptoms in a retrospective study.

5.2 Materials/Methods

This study was approved by the Institutional Review Board at The Ohio State University. All subjects gave written informed consent to medical record review. The vast majority of patients who receive $^{131}\text{I}$ therapy at The Ohio State University Wexner Medical Center (OSUWMC) are prepared using recombinant human thyrotropin.

Patient enrollment:
Patients diagnosed with follicular cell-derived thyroid cancer who had previously received $^{131}$I therapy were enrolled in the study (n=58). Patient demographics including age, gender, histology, TNM stage, and mean cumulative and first administered $^{131}$I activity are given in Table 5.1. Patients were served questionnaires for xerostomia and sialadenitis and had basal and stimulated saliva samples collected by a registered nurse during patients’ office visits at the Endocrinology Clinic at OSUWMC. A total of 72 basal and 72 stimulated saliva samples were collected from 58 patients. Saliva samples were collected twice from 10 patients and three times from two patients. Complete xerostomia score questionnaire on the day of saliva collection was available for 47 saliva collections from 40 patients. Complete sialadenitis questionnaire data was available for 47 patients (55 saliva collections). Cumulative and first administered activity of $^{131}$I was available for 56/58 and 57/58 $^{131}$I-treated patients, respectively. Of the 58 enrolled patients, 16 had received more than one $^{131}$I treatment. Mean time between patients’ last $^{131}$I administration and saliva sample collection was 4.6 years (median= 3.11 years, range 10 days-16.5 years).

*Saliva collection and processing:*

Saliva was collected at patients clinic visits between the hours of 8AM and 5PM. Patients were asked to refrain from eating and/or performing oral hygiene for at least one hour prior to collection. Each patient was asked to sit quietly for 2 minutes prior to saliva collection and asked to swallow all saliva during that time. Unstimulated whole saliva
was collected for 5 minutes in a 50 mL conical tube on ice. Following the unstimulated whole saliva collection, patients were given a 5x5cm square paraffin strip and asked to chew on it for 2 minutes and swallow all saliva during that time. After the 2 minutes, whole stimulated saliva was collected in a second 50 mL conical tube on ice for 5 minute while still chewing the paraffin strip. All saliva samples were kept on ice until brought to the lab where they were spun at 2600 x g for 15 minutes at 4°C to remove cells and debris. Cell-free whole saliva volume was then aliquoted and treated with 20% ethanol by volume, a method previously shown to preserve protein. Processing was completed within 3 hours of saliva collection. Aliquots were stored at -80°C until proteome profiling was performed.

Protein analysis by multiplex immunoassay

We submitted 33 basal saliva samples collected after 131I treatment from 28 patients and 35 stimulated saliva samples from 30 patients (5 patients had saliva samples from two collections after 131I) for proteome profiling by multiplex immunoassay (Myriad RBM, Austin, TX), a luminex multibead platform. Sufficient volume was available to analyze 25 basal saliva samples from 22 patients and 31 stimulated saliva samples from 27 patients using the Explorer multi-analyte panel (MAP), which determines the concentration of 141 analytes involved in inflammatory/immune response, cell/tissue generation, coagulation, and other processes via multiplex bead immunoassay. The other 8 basal saliva samples from 6 patients and 4 stimulated saliva samples from 3
patients did not have sufficient volume for the ExplorerMAP and were analyzed using the InflammationMAP, which requires less volume but determines the concentration of only 46 analytes. All InflammationMAP analytes are also included in the ExplorerMAP. Samples with analyte concentration outside the linear range of the test had the respective analyte levels set to the lower limit of quantitation for analysis.

**Statistical analysis**

Pearson and Spearman correlations were used to evaluate the univariate association between two continuous variables including protein concentration as determined by ExplorerMAP or InflammationMAP, saliva flow rate (SFR), xerostomia score, cumulative $^{131}$I activity, first administered $^{131}$I activity, and age. Correlation coefficients ($r$) were determined for each correlation. Unpaired t tests were used to evaluate the univariate association between discrete variables including gender and whether a patient experienced sialadenitis and continuous variables. Fisher’s exact test was used to determine the impact gender had on sialadenitis incidence.

**5.3 Results**

*Cumulative and first administered $^{131}$I activity had weak negative correlation with stimulated saliva flow rate.*

To determine if cumulative or first administered $^{131}$I activity had an effect on SFR, we examined the correlation between SFR and cumulative or first administered $^{131}$I activity. Of all the patients with saliva collected, 56/58 (70 saliva collections) had known
cumulative $^{131}$I activity and 57/58 (71 saliva collections) had known first administered $^{131}$I activity. Neither cumulative nor first administered $^{131}$I activity had a significant correlation with basal SFR (Fig 5.1A and 5.1C, respectively). Stimulated SFR had a weak but significant negative correlation with cumulative $^{131}$I activity (Pearson $r= -0.27$, $p=0.02$; Spearman $r= -0.30$, $p=0.01$) and first administered $^{131}$I activity (Pearson $r= -0.27$, $p=0.02$; Spearman $r= -0.28$, $p=0.02$), shown in Figure 3.1B and 3.1D, respectively. Of note, neither cumulative nor first administered $^{131}$I activity had a significant correlation with xerostomia score and there was no significant difference in cumulative or first administered $^{131}$I activity between patients who experienced sialadenitis and patients who did not (Figure 5.2).

*Age had weak negative correlation with basal, but not stimulated saliva flow rate.*

To determine if age had an effect on SFR, Pearson and Spearman correlation between age and basal SFR/stimulated SFR was determined. Age had a weak but significant negative correlation with basal SFR (Pearson $r= -0.28$, $p=0.02$; Spearman $r= -0.23$, $p=0.049$) (Fig 5.3A), but not stimulated SFR (Fig 5.3B). There was no significant difference in basal or stimulated SFR between males and females (Fig 5.3C and 5.3D). Of note, neither age nor gender significantly associated with xerostomia score or sialadenitis incidence (Figure 5.4).
Xerostomia score had weak negative correlation with stimulated saliva flow rate, but no correlation with basal saliva flow rate. Saliva flow rate of patients with sialadenitis was not significantly different compared to patients without sialadenitis.

To determine if SFR correlated with subjective xerostomia in $^{131}$I-treated patients, correlation between xerostomia score at the time of saliva collection and basal/stimulated SFR was investigated. Xerostomia scores from a questionnaire completed at the time of saliva collection were available for 47 saliva collections from 40 $^{131}$I-treated patients. Xerostomia score had a borderline significant correlation with basal SFR (Fig 3.5A), but did have a weak significant negative correlation with stimulated SFR (Pearson $r=-0.34$, $p=0.02$; Spearman $r=-0.37$, $p=0.01$) (Fig 3.5B).

To determine if sialadenitis had an effect on SFR, we compared the SFR of 17 saliva collections from 16 patients who had reported experiencing sialadenitis to the SFRs of 38 saliva collections from 31 patients who never reported experiencing sialadenitis. There was no significant difference in basal or stimulated SFR (Fig 3.5C and 3.5D) between patients who had experienced sialadenitis and those who had not. Of the patients who had experienced sialadenitis, 11 had experienced sialadenitis only after $^{131}$I treatment, 4 experienced sialadenitis before and after $^{131}$I, and one reported experiencing sialadenitis only before $^{131}$I. There was no significant difference in mean xerostomia score between patients who experienced sialadenitis and patients who did not (Figure 5.6).
MMP-1, MMP-7, and Angiogenin mean concentrations are lower in stimulated saliva of patients who experienced sialadenitis.

To determine if any proteins associated with sialadenitis, we compared the proteins’ concentration in saliva of patients who experienced sialadenitis to that in saliva of patients who had never experienced sialadenitis. Unpaired t-test showed concentrations of matrix metalloproteinase (MMP)-1 and MMP-7 were significantly decreased in the basal and stimulated saliva of patients who experienced sialadenitis compared to saliva of patients who did not experience sialadenitis (Fig 5.7A-D). Unpaired t-test also showed concentration of angiogenin was lower in the stimulated, but not the basal, saliva of patients who experienced sialadenitis compared to saliva of patients who did not experience sialadenitis (Fig 5.7E).

Xerostomia score positively correlated with GRO-α, fibulin-1C, osteoprotegerin, GC-SF, TRAIL-R3, and EN-RAGE in basal saliva.

To determine which proteins associated with xerostomia, we determined the Pearson and Spearman correlation between proteins’ concentrations and xerostomia score. A correlation was considered significant if both Pearson and Spearman correlations had $p<0.05$. Pearson and Spearman correlation showed strong significant positive correlation between xerostomia score at saliva collection and basal concentrations of growth-regulated alpha (GRO-α) (Pearson $r=0.61$, $p=0.001$; Spearman $r=0.68$, $p=0.0002$), fibulin-1c (Pearson $r=0.59$, $p=0.002$; Spearman $r=0.59$, $p=0.002$),
osteoprotegerin (Pearson r=0.55, p=0.004; Spearman r=0.56, p=0.004), granulocyte colony stimulating factor (GC-SF) (Pearson r=0.54, p=0.005; Spearman r=0.57, p=0.003), tumor necrosis factor related apoptosis inducing ligand receptor 3 (TRAIL-R3) (Pearson r=0.54, p=0.006; Spearman r=0.52, p=0.008), and EN-RAGE (Pearson r=0.53, p=0.007; Spearman r=0.51, p=0.009) protein concentration in 25 basal saliva samples from 22 patients (Fig 5.8). Additionally, Pearson correlation showed a strong negative correlation between basal saliva flow rate and GC-SF (Pearson r= -0.52, p=-.008), TRAIL-R3 (Pearson r= -0.51, p=0.01), and EN-RAGE (Pearson r= -0.64, p=0.0006) protein concentration in 25 basal saliva samples from 22 patients.

5.4 Discussion

In this study, we examined the correlation between saliva flow rate, an objective measure of salivary gland function, and $^{131}$I activity, subjective xerostomia, and sialadenitis, as well as association between biomarker concentration and sialadenitis or xerostomia score. We report that increased cumulative and first administered $^{131}$I activity correlated with decreased stimulated saliva flow rate, and increase in age correlated with decreased basal saliva flow rate. Xerostomia score had a weak but significant correlation with stimulated saliva flow rate, but patients who experienced sialadenitis did not have significantly different saliva flow rates compared to patients who did not experience sialadenitis. Additionally, we found that MMP-1, MMP-7 and angiogenin were significantly lower in stimulated saliva of patients who experienced sialadenitis compared to those who did not experience sialadenitis, and increase in basal saliva GRO-α, fibulin-
1C, osteoprotegerin, GC-SF, TRAIL-R3, and EN-RAGE concentration significantly correlated with increase in xerostomia score.

Our finding that cumulative and $^{131}$I activity significantly correlate with stimulated saliva flow rate, but not basal saliva flow rate is consistent with the observation that the parotid salivary glands are more susceptible to $^{131}$I-induced damage when assessed via scintigraphy$^{71-73}$, as parotid glands produce the majority of the stimulated saliva. Though the correlation is significant, it is weak and many patients had low stimulated saliva flow rate even if they received ≤50 mCi $^{131}$I. Decreased saliva flow rate has many causes other than $^{131}$I treatment. It is possible that low $^{131}$I activity can lead to decreased saliva flow rate, that other factors are affecting saliva flow rate, or both.

It has been reported that only one in six people who have either xerostomia or salivary gland hypofunction, defined as <0.1 mL/min basal saliva flow rate or <0.5 mL/min stimulated saliva flow rate, will have both$^{74}$. While the correlation between xerostomia and stimulated saliva flow rate was significant and the correlation between xerostomia score and basal saliva flow rate was borderline significant, the correlation for each was weak indicating patients even with higher saliva flow rate can experience xerostomia. Taken together, these data suggest there may be a saliva composition component to xerostomia. Ish-Shalom et al reported while mean basal saliva flow rate was not significantly different between $^{131}$I-treated and untreated thyroid cancer patients, total protein and superoxide dismutase were significantly decreased in $^{131}$I-treated patients’ saliva; however, these composition differences could not be correlated with
complaints of dry mouth probably due to low n number. Hesselink et al also reported that total protein and amylase concentration in saliva was decreased 5 months after treatment in a prospective study, but changes of other specific proteins were not investigated.

Upon examination of saliva concentration of 141 proteins via multiplex bead-based immunoassay, we observed decreased MMP-1, MMP-7 (also known as matrilysin), and angiogenin concentration in saliva of patients who experienced sialadenitis compared to patients who did not. MMP-1 and MMP-7 are members of the matrix metalloproteinase family of proteases responsible for extracellular matrix and other tissue remodeling. Angiogenin’s interaction with actin activates tissue plasminogen activator to generate plasmin, which destroys the basement membrane. Decreased MMP-1 has been found in fibrosis, possibly indicating importance in wound healing. MMP-7 can activate components of the innate immune system against bacterial infection. MMP-7 knockout mice are both highly susceptible to intestinal bacterial infections and have the most severe wound healing impairment of the seven MMP knockout mouse lines, possibly due to the fact that it not only remodels tissue but also activates MMP-2 and MMP-9. Perhaps the decrease in MMP-1, MMP-7, and angiogenin in the saliva of patients who experienced sialadenitis indicate impaired wound healing capability of the salivary ductal cells, an impaired innate immune system, or both. Interestingly, Delaleu et al found MMP-1 and MMP-7 to be significantly increased in the saliva of Sjogren’s Syndrome patients compared to patients without Sjogren’s Syndrome. This may be due to constant
tissue remodeling and repairs in the face of autoimmune injury in the salivary glands of Sjogren’s Syndrome patients. The role MMP-1, MMP-7, and angiogenin play in $^{131}$I-induced salivary gland injury will need to be further investigated.

GRO-α, fibulin-1C, osteoprotegerin, GC-SF, TRAIL-R3, and EN-RAGE concentration in basal saliva samples had strong positive correlation with xerostomia score. GRO-α recruits neutrophils, GC-SF stimulates survival, proliferation, and function or neutrophils, and EN-RAGE is expressed on and secreted by neutrophils. All three are associated with immune and inflammatory conditions. GRO-α was found to have increased expression in salivary gland epithelial cell biopsies from Sjogren’s Syndrome patients compared to controls. Additionally, EN-RAGE was found to be increased in the saliva 24 hours after total body irradiation. TRAIL-R3 is a decoy receptor for tumor necrosis factor related apoptosis inducing ligand (TRAIL) acting to protect cells from TRAIL’s effect thus preventing apoptosis. Osteoprotegerin promotes bone resorption in periodontitis, but may also be a decoy receptor for TRAIL. Fibulin-1C is a glycoprotein tightly connected to the basement membrane and is involved in cell growth, differentiation, and angiogenesis. Taken together, these proteins’ correlations with xerostomia score may suggest inflammation of the salivary glands contributes to the sensation of dry mouth and that protection of cells against apoptosis is occurring in some patients possibly to preserve tissue during inflammation.

In summary, our data is in agreement with previous studies showing $^{131}$I treatment affects the parotid glands more than the submandibular glands. Patients who experienced
sialadenitis did not have a significant difference in saliva flow rate compared to patients who did not experience sialadenitis, but we did find a significant decrease in MMP-1, MMP-7, and angiogenin concentration in the stimulated saliva of patients who experienced sialadenitis by univariate analysis, suggesting extra-cellular matrix remodeling may be important in preventing sialadenitis. The correlation between xerostomia score and saliva flow rate was weak reflecting previously reported studies that xerostomia and salivary gland hypofunction do not often overlap. We did find GRO-α, fibulin-1C, osteoprotegerin, GC-SF, TRAIL-R3, and EN-RAGE concentrations in basal saliva had a strong significant positive correlation with xerostomia score by univariate analysis, possibly suggesting inflammatory and immune response, as well as protection against apoptosis, may be occurring in the salivary glands of patients with xerostomia. These findings would need to be validated in a larger prospective study.
### Table 5.1. Demographics and clinical data for patients with saliva collected

**131I-treated thyroid cancer patients**  
(n=58)

<table>
<thead>
<tr>
<th>Age* (years)</th>
<th>Mean/Median</th>
<th>51/51</th>
<th>Range</th>
<th>20-89</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td>Female</td>
<td>40</td>
<td>Male</td>
<td>18</td>
</tr>
<tr>
<td>Cumulative $^{131}$I activity (mCi)</td>
<td>Mean/Median</td>
<td>151/125</td>
<td>Range$^a$</td>
<td>25-645</td>
</tr>
<tr>
<td>First $^{131}$I activity (mCi)</td>
<td>Mean/Median</td>
<td>117/103</td>
<td>Range$^a$</td>
<td>25-201</td>
</tr>
<tr>
<td>Histology</td>
<td>Papillary</td>
<td>49</td>
<td>Follicular</td>
<td>8</td>
</tr>
<tr>
<td>T Stage</td>
<td>T1</td>
<td>14</td>
<td>T2</td>
<td>15</td>
</tr>
<tr>
<td>N Stage</td>
<td>N0</td>
<td>26</td>
<td>N1</td>
<td>31</td>
</tr>
<tr>
<td>M Stage</td>
<td>M0</td>
<td>52</td>
<td>M1</td>
<td>4</td>
</tr>
</tbody>
</table>

*: Age at questionnaire (initial study) or at time of information warehouse search (validation study)  
*: Only 1/58 $^{131}$I-treated patients received $<$30 mCi for their first administered activity and it was their only treatment.
Figure 5.1. Cumulative and first administered $^{131}$I activity significantly correlates with stimulated saliva flow rate (B, D), but not basal saliva flow rate (A, C).

Pearson and Spearman correlations between cumulative/first $^{131}$I activity and basal or stimulated saliva flow rate of 70 saliva collections from 56 patients with known cumulative $^{131}$I activity and 71 saliva collections from 57 patients with known first $^{131}$I activity were determined. There was no significant correlation between basal saliva flow rate and cumulative $^{131}$I activity (A) or first $^{131}$I activity (C). There was a significant negative correlation between stimulated saliva flow rate and cumulative $^{131}$I activity (Pearson correlation coefficient = -0.269, p=0.02; Spearman correlation coefficient = -0.30, p=0.01) (B) as well as first $^{131}$I activity (Pearson correlation coefficient = -0.27, p=0.02; Spearman correlation coefficient = -0.28, p=0.02) (D).
Figure 5.2. Cumulative and first administered $^{131}$I activity did not associate with xerostomia scores or sialadenitis incidence.

Pearson and Spearman correlation between xerostomia scores and cumulative or first administered $^{131}$I activity was determined (n=46 xerostomia scores from time of saliva collection from 39 patients). There was no significant correlation between xerostomia and cumulative $^{131}$I activity (A) or first administered $^{131}$I activity (B). Cumulative and first administered $^{131}$I activity was compared between patients who experienced sialadenitis (n=15 patients) and patients who did not have sialadenitis (n=31 patients). Unpaired t-test showed no difference in and cumulative $^{131}$I activity (C) or first administered $^{131}$I activity (D) between patients who experienced sialadenitis and those who did not.
Figure 5.3. Age has a significant negative correlation with basal saliva flow rate but not stimulated saliva flow rate, and there was no significant difference in saliva flow rate between males and females.

Pearson and Spearman correlations between age on the day of saliva collection and basal or stimulated saliva flow rate of 72 saliva collections from 58 patients were determined. Age had a significant negative correlation with basal saliva flow rate (Pearson correlation coefficient= -0.28, p=0.02; Spearman correlation coefficient= -0.23, p=0.049) (A), but not stimulated SFR (B). Unpaired t-test showed no significant difference in basal saliva flow rate (C) or stimulated saliva flow rate (D) between $^{131}$I-treated females (n=47 saliva collections from 40 patients) and $^{131}$I-treated males (n=25 saliva collections from 18 patients).
Figure 5.4. Neither xerostomia score nor sialadenitis incidence associated with age or gender.

(A) Pearson and Spearman correlation between xerostomia scores and age (n=47 xerostomia scores from time of saliva collection from 40 patients) were not significant (Pearson correlation coefficient= -0.11, p=0.48; Spearman correlation coefficient= -0.10, p=0.50). (B) Unpaired t-test showed no significant difference in age between patients who experienced sialadenitis (n=16 patients) and patients who did not experience sialadenitis (n=31 patients). (C) Unpaired t-test showed no significant difference in xerostomia scores between females (n=28 xerostomia score questionnaires from 27 patients) and males (n=19 xerostomia score questionnaires from 13 patients). (D) Fisher’s exact test shows no significant difference in the percent of patients who experienced sialadenitis between females (12/32 female patients experienced sialadenitis) and males (4/15 male patients experienced sialadenitis).
Figure 5.5. Subjective xerostomia had significant negative correlation with stimulated saliva flow rate but not basal saliva flow rate, and saliva flow rate between patients who reported sialadenitis and those who did not was not significantly different.

Pearson and Spearman correlations between xerostomia score on the day of saliva collection and basal or stimulated saliva flow rate of 47 saliva collections from 40 patients were determined. Correlation between xerostomia score at time of collection and basal saliva flow rate in $^{131}$I-treated patients was not significant by Pearson or Spearman correlation (A). Xerostomia score has a significant negative correlation with stimulated saliva flow rate (Pearson correlation coefficient= -0.34, p=0.02; Spearman correlation = -0.37, p=0.01) (B). Unpaired t-test showed no significant difference in basal saliva flow rate (C) or stimulated saliva flow rate (D) between $^{131}$I-treated patients who had experienced sialadenitis (n=17 saliva collections from 16 patients) and $^{131}$I-treated patients who had not experienced sialadenitis (n=38 saliva collections from 31 patients).
Figure 5.6. $^{131}$I-treated patients who experienced sialadenitis do not have significantly different xerostomia scores than patients who did not experience sialadenitis

Unpaired t-test showed no significant difference in mean xerostomia scores between patients who experienced sialadenitis (16 xerostomia scores from day of saliva collection from 15 patients) and patients who did not have sialadenitis (35 xerostomia scores from day of saliva collection from 29 patients).
Figure 5.7. Matrix metalloproteinase-1, matrix metalloproteinase-7, and angiogenin are decreased in saliva of patients who experienced sialadenitis.

Unpaired t-test showed that Matrix metalloproteinase (MMP)-1 (A-B) and MMP-7 (C-D) concentrations were significantly lower in both the basal (A, C) and stimulated (B, D) saliva of patients who experienced sialadenitis compared to patients who did not experience sialadenitis. Unpaired t-test also showed angiogenin was decreased in the stimulated saliva of patients who experienced sialadenitis compared to patients who did not experience sialadenitis (E). For basal saliva analyses, 7 basal saliva samples from 7 patients who experienced sialadenitis were compared with 18 basal saliva samples from 15 patients who did not experience sialadenitis. For stimulated saliva analyses, 10 stimulated saliva samples from 10 patients who experienced sialadenitis were compared to 21 basal saliva samples from 17 patients who did not experience sialadenitis.
Figure 5.7 continued

E

Stim Angiogenin

Sialadenitis (n=10) No Sialadenitis (n=21)

(LLOQ = 0.054 ng/mL)

p = 0.009
Figure 5.8. Six proteins had strong significant positive correlation with xerostomia score.

Pearson and Spearman correlation showed strong significant positive correlation between xerostomia score at saliva collection and basal concentrations of (A) growth-regulated alpha (GRO-alpha) (Pearson r=0.61, p=0.001; Spearman r=0.68, p=0.0002), (B) fibulin-1c (Pearson r=0.59, p=0.002; Spearman r=0.59, p=0.002), (C) osteoprotegerin (Pearson r=0.55, p=0.004; Spearman r=0.56, p=0.004), (D) granulocyte colony stimulating factor (GC-SF) (Pearson r=0.54, p=0.005; Spearman r=0.57, p=0.003), (E) tumor necrosis factor related apoptosis inducing ligand receptor 3 (TRAIL-R3) (Pearson r=0.54, p=0.006; Spearman r=0.52, p=0.008), and (F) EN-RAGE (Pearson r=0.53, p=0.007; Spearman r=0.51, p=0.009) protein concentration in 25 basal saliva samples from 22 patients.
Figure 5.8 continued
Administration of $^{131}$I is a common and effective means to ablate remnant tissue and metastases of well-differentiated follicular-cell derived thyroid cancer; however it is not without side effects and some patients are unable to benefit from $^{131}$I therapy. We investigated and reported on risk factors of $^{131}$I-induced salivary gland damage and saliva composition associated with xerostomia and sialadenitis in $^{131}$I-treated patients in retrospective studies. Additionally, optimization of pre-clinical microSPECT/CT imaging acquisition and analysis will assist in studies to identify novel strategies to increase radioisotope accumulation in thyroid cancer. We report methods that have eliminated user subjectivity in analysis of $^{123}$I microSPECT/CT imaging where images were taken at t1 and t24 and a method to minimize user subjectivity in studies where only a t1 image is available.

In chapter 2, we confirmed that $^{131}$I treatment is associated with higher incidence of xerostomia and sialadenitis and also that administered $^{131}$I activity, age, gender, history of sialadenitis before $^{131}$I treatment, and AID-SS diagnosis are risk factors for $^{131}$I-induced salivary gland damage. Female gender and history of sialadenitis correlated with increased incidence of sialadenitis while age and $^{131}$I activity correlated with increased xerostomia. AID-SS correlated with both xerostomia and sialadenitis incidence.
As AID-SS correlated with sialadenitis and xerostomia following $^{131}$I treatment and anti-nuclear autoantibodies SSA-Ro and SSB-La are prevalent in 62% and 42% of patients with systemic lupus erythematosus with secondary Sjogren’s, respectively, we investigated if these anti-nuclear autoantibodies are over-represented in patients with these symptoms. For comparison, $^{131}$I-treated patients who had no history of autoimmunity, no sialadenitis, and mean xerostomia questionnaire scores ≤1 also were screened for the autoantibodies (n=13). Screening was conducted using a Bioplex 2200 multiplex platform (Bio-Rad Clinical Diagnostics, Hercules, CA) at The Ohio State University Wexner Medical Center Clinical Laboratory. As shown in Figure 6.1, the incidence of SSA-Ro and SSB-La autoantibodies in these selected $^{131}$I-treated patients was 3.2% (3/95). The incidence of SSA-Ro and SSB-La autoantibodies was 10.3% (3/29) among patients who had sialadenitis and xerostomia scores ≥2. This is within the reported range (0.5-15%) of SSA-Ro and SSB-La autoantibodies incidence in healthy people, which can vary depending on the test used, the age of patients, and the ethnicity of population. In comparison, SSA-Ro and SSB-La autoantibodies were not detected in the group of patients with no sialadenitis, no history of autoimmunity, and mean xerostomia scores ≤1 (n=13). None of the $^{131}$I-treated patients tested were diagnosed with primary Sjogren’s syndrome and only 4 were diagnosed with systemic lupus erythematosus, which has a secondary Sjogren’s syndrome incidence of 6.5-19%. The most common AID-SS in our patient cohort was rheumatoid arthritis and it has been reported that only ~5% of patients with rheumatoid arthritis with secondary Sjogren’s
were positive for SSA-Ro and/or SSB-La autoantibodies. This may account for the low prevalence of SSA-Ro and SSB-La autoantibodies. Therefore, SSA-Ro and SSB-La autoantibodies cannot be used as predictive markers of \(^{131}\)I-induced salivary gland damage. However, it is important to give special consideration to \(^{131}\)I treatment of thyroid cancer patients also diagnosed with AID-SS.

In chapter 3 and 4, we report methods to minimize user subjectivity and error in analysis of both t1 and t24 microSPECT/CT images. With our capability of 3D voxel-based visualization of gamma photon intensity, we hope to correlate voxel-based gamma photon intensity with tumor response to determine the activity required for tissue ablation as well as identify particularly radio-resistant tumors in mice and retrospectively in a human study. Additionally, we hope to align 3D voxel-based VOIs with thyroid histology to determine gene expression changes in areas with low \(^{131}\)I uptake or radio-resistance. We have also determined anatomical markers for salivary gland volume of interest segmentation with minimal user subjectivity (Fig 6.2).

In chapter 5, we reported proteins differentially expressed in patients who experienced sialadenitis and that correlated with xerostomia score. Matrix metalloproteinase-7, matrix metalloproteinase -1, and angiogenin were decreased in patients who experienced sialadenitis, suggesting a possible role for these proteins and extra-cellular matrix remodeling in prevention of \(^{131}\)I-induced sialadenitis. EN-RAGE, granulocyte colony-stimulating factor (GC-SF), TNF-related apoptosis inducing ligand receptor 3 (TRAIL-R3), growth regulated α protein (GROα), fibulin-1C, and
osteoprotegerin all had strong positive correlations (r>0.5) with xerostomia score.
Xerostomia score’s correlation with EN-RAGE, GC-SF, and GROα suggest an
inflammation may play a role in $^{131}$I-induced xerostomia, and xerostomia score’s
correlation with TRAIL-R3 and osteoprotegerin suggest that protection against apoptosis
may be occurring in some patients with xerostomia. Further validation of these findings
and a more comprehensive proteome profiling, such as mass spectrometry, is required for
better understanding of the pathology of $^{131}$I-induced salivary gland damage. In addition
to shedding light on the pathology of $^{131}$I-induced salivary gland damage, we hypothesize
that differences in saliva composition prior to $^{131}$I may also indicate risk of $^{131}$I-induced
salivary gland damage. To investigate this hypothesis, we are currently enrolling newly
diagnosed thyroid cancer patients prescribed $^{131}$I treatment in a prospective study. To
date, we have collected saliva prior to $^{131}$I treatment from 19 patients with saliva
collected after $^{131}$I treatment from 11/19 patients. We have collected saliva two times
after $^{131}$I treatment from 4/11 patients. The mean $^{131}$I activity administered to the 12
patients who have received $^{131}$I treatment thus far is 104 mCi (range 30-200mCi). Patients
had a mean xerostomia score of 0.9 out of 4 (range 0-2.75) prior to $^{131}$I treatment and
none reported sialadenitis prior to $^{131}$I treatment. Of the 7 patients with questionnaires
after $^{131}$I therapy, 2 reported experiencing sialadenitis following $^{131}$I treatment. The basal
and stimulated saliva flow rate of these patients prior to and after $^{131}$I is shown in Figure
6.3. No clear trend in change in saliva flow rate after $^{131}$I treatment is apparent in our
cohort at this time; however, our n is small. Upon enrolling sufficient number of patients,
we will submit saliva samples for proteome profiling by mass spectrometry to identify biomarkers predictive of salivary gland damage as well as changes in saliva protein composition over time to further elucidate the pathways involved in $^{131}$I-induced salivary gland damage and subsequent healing.

While identifying risk factors for susceptibility to $^{131}$I-induced salivary gland damage and understanding the mechanisms involved in pathological progression of that damage are important in identifying which patients will most likely suffer from $^{131}$I-induced salivary gland damage, prevention of this damage is the main objective. Previously, all methods to minimize or prevent salivary gland damage focused on excreting $^{131}$I from the salivary glands by stimulating saliva flow during $^{131}$I therapy. Results varied from study to study, from slight decrease of chronic xerostomia incidence, to an increase in xerostomia incidence$^{19,20}$. To date, no effective means of preventing $^{131}$I-induced salivary gland damage exists. Case studies show that patients with loss of function NIS mutations do not have iodide accumulation in the salivary gland$^{93}$. Additionally, several groups have accomplished gene transfer localized to the salivary glands via direct injection into the saliva duct, also known as retroductal injection, in preclinical models$^{94-101}$, and Baum et al showed successful retroductal gene transfer to salivary glands in humans in a clinical trial$^{97}$. We hypothesized that salivary gland targeted NIS knockdown will prevent $^{131}$I accumulation in the salivary glands without decreasing $^{131}$I accumulation in remnant thyroid tissue and/or thyroid cancer lesions.
To pre-clinically test this hypothesis, we were able to retroductally deliver agents to one mouse submandibular gland with no evidence of leakage to the other submandibular gland (Fig 6.4) such that each mouse could serve as its own control. Passineau et al had reported successful gene transfer of a pCMV-GL3 luciferase-expressing plasmid by facilitating plasmid uptake with microbubbles followed by ultrasonication to disturb the cell membrane such that plasmids may enter the cell through these disturbances in the cell membrane. We were able to recapitulate plasmid gene transfer method in our own laboratory (Figure 6.5). However, retroductal injection of NIS-siRNA in conjunction with microbubbles followed by ultrasonication to facilitate uptake of siRNA lead to salivary gland damage such that radioisotope could not be excreted even upon pilocarpine-induced salivation resulting in higher radioisotope accumulation in the treated submandibular gland compared to the untreated submandibular gland (Figure 6.6). Retroductal injection with PBS alone resulted in no difference in radioisotope accumulation or histological damage (data not shown). Arany et al utilized nanoparticles to facilitate cellular uptake of siRNA to knock down Pkcδ in order to prevent radiation-induced apoptosis in irradiated salivary glands. We collaborated with this group and indeed saw decreased radioisotope accumulation in salivary glands treated with nanoparticles complexed with NIS-siRNA, but also saw this same decreased radioisotope accumulation in salivary glands treated with nanoparticles complexed with non-targeting siRNA (Figure 6.7), with radioisotope accumulation recovery by 10 days post-retroductal injection. NIS expression in the salivary glands is
decreased in the presence of inflammation, and indeed, histology showed acinar cell damage and neutrophil infiltration in submandibular glands retroductally injected with nanoparticles complexed with siRNA (data not shown).

In the future, we hope to induce limited acute inflammation or identify other factors that suppress NIS expression such that NIS expression can be decreased in the salivary glands in a temporary targeted manner with minimal side effects. Targeted delivery of NIS siRNA via retroductal injection to the salivary glands is invasive and uncomfortable and should only apply to thyroid cancer patients who are at high risk of developing \textsuperscript{131}I-induced salivary gland damage. Our studies reported in chapter 2 and chapter 5 and our ongoing prospective study stated above are important to determine clinical factors and biomarkers predictive of risk of \textsuperscript{131}I-induced salivary gland damage in order to identify these high risk patients for intervention. Preventing \textsuperscript{131}I-induced damage would make sialadenitis and xerostomia and subsequent oral diseases a problem of the past, improving quality of life for thyroid cancer patients.

A subset of thyroid cancer patients have disease that takes up radioiodine, but does not respond to \textsuperscript{131}I therapy. To investigate the factors associated with this radio-resistance, we performed an electronic medical record search to update and expand upon data captured at the time of enrollment in the ENR (n=2647 patients). Data captured initially in the ENR included gender, race, diagnosis date, age at diagnosis, histology, initial TNM staging, thyroidectomy and lymphadenectomy dates, and \textsuperscript{131}I treatments administered before or within 6 months of enrollment. The Ohio State University
Medical Center initiated electronic medical record usage via IHIS in 2008 with departments beginning to use it at different times thereafter. Thus discrete searchable data fields were only able to be searched back to 2008 or later for some data, but a free text search of the clinical notes could yield information regarding treatment or tests performed before 2008. Searchable data newly added to the ENR data included Tg, Tg auto-antibody, TSH, and T4 test dates and levels dating back to February 2008, recombinant human TSH administrations dating back to July 2009, and vital status, as well as a free-text search for “Positive for the BRAF V600E mutation” and “Negative for the BRAF V600E mutation”. Surgeries related to thyroid cancer dating back to October 2011 and diagnostic radioiodine scans and $^{131}\text{I}$ administrations dating back to November 2014 were searched to update the data initially captured in the ENR. A free text search for “mCi” and “millicurie” to capture $^{131}\text{I}$ administrations prior to November 2014 and a free text search for “cGy” to capture any external beam radiation therapy administered were also performed. This data is currently being analyzed and expanded upon to determine indicators of radio-resistance in the medical record. Additionally, Dr. de la Chapelle has genotyping data from 1352 of these thyroid cancer patients for germline DNA variants (deCODE Genetics, Iceland). We plan to analyze germline DNA variants associated with radio-resistance.

Taken together, we hope that our studies investigating the risk factors and biomarkers associated with $^{131}\text{I}$-induced salivary gland damage provide insight into preventing this adverse event and that our efforts to automate SPECT/CT imaging
analysis will aid in future pre-clinical and clinical studies of radioiodine accumulation in the thyroid and salivary gland to improve $^{131}$I efficacy while decreasing adverse effects.
Figure 6.1. SSA-Ro and SSB-La are not over-represented in $^{131}$I-treated patients with salivary gland symptoms.

Plasma screening for SSA-Ro and SSB-La was conducted using a Bioplex 2200 multiplex platform. Patients who received $^{131}$I therapy and were diagnosed with autoimmune diseases (“AID”), had sialadenitis, and/or had mean xerostomia scores ≥2 (“XQ Score ≥2”) were screened (total n=95). Among the 95 patients tested, 2 patients tested positive for SSA-Ro and one patient tested positive for SSB-La. The incidence of SSA-Ro and SSB-La was 10.3% (3/29) among patients who had sialadenitis and xerostomia scores ≥2. Patients who received $^{131}$I therapy and had no history of autoimmunity, no sialadenitis, and mean xerostomia questionnaire scores ≤1 (n=13) were also screened and none were positive for SSA-Ro or SSB-La.
Figure 6.2. Anatomical markers for defining salivary gland volume of interest.

For the salivary glands volume of interest (VOI), the cranial and caudal boundaries are set to the most caudal point where the mandible bones are completely visible (A) and the point where the proximal tip of both clavicles are visible (B), respectively. The right and left boundaries are set to the point 1.50 mm past where the ear canal becomes visible (C and D). The dorsal and ventral boundaries are set to the point where the teeth and mandible meet (E) and 0.45 mm past where the skin of the mouse is no longer visible (F), respectively.
Figure 6.3. Saliva flow rate prior to compared to after $^{131}$I treatment.

The basal (A) and stimulated (B) saliva flow rates prior to and after $^{131}$I treatment of 18 enrolled study patients are shown with time 0 set as the date of $^{131}$I administration. Ten patients had saliva collected both before and after $^{131}$I treatment. Color indicates the $^{131}$I activity the patient received: green indicates <100 mCi, blue indicates 100-150 mCi, orange indicates 151-200 mCi, black indicates patient hasn’t received $^{131}$I yet. Solid points indicate the patient is male while open points indicate the patient is female. Red point indicates the patient reported experiencing sialadenitis in questionnaire from that collection as seen after $^{131}$I treatment in patients 005-VB and 023-JP.
Figure 6.4. Unilateral retroductal injection of the mouse submandibular gland can be achieved such that the mouse may serve as its own control.

(A) The mouse Wharton’s duct is the main excretory duct of the submandibular glands and is located at the base of the oral cavity under the tongue. Each submandibular gland has its own excretory duct, which can be cannulated such that agent can be delivered unilaterally. (B) 50 µL of 4% toluene blue in phosphate-buffered saline was retroductally injected to one of the mouse’s submandibular glands, and the glands were harvested to show perfusion of the retroductally injected gland without leakage to the non-injected gland.
Figure 6.5. Microbubble-facilitated gene transfer to the mouse salivary glands.

Mice were retroductally injected with 50uL of 15% Definity microbubbles in PBS either alone or with 25 µg pBABE-CMV-luciferase plasmid. IVIS imaging 24 hours after retroductal injection shows luminescence in the mouse salivary gland retroductally injected with 25 µg pBABE-CMV-luciferase plasmid (A), but not the mouse salivary gland retroductally injected with PBS alone (B). By 72 hours after retroductal injection, the mouse salivary gland no longer exhibits luciferase activity (C). All images were taken 10 minutes after intraperitoneal injection of 10µL/g body weight of 15 mg/mL filter-sterilized luciferin.
Figure 6.6. Retroductal injection of microbubbles followed by ultrasonication leads to mouse salivary gland damage and retention of radioisotope.

(A) Percent injected activity of $^{99m}$TcO$_4^-$ in the retroductally injected or uninjected submandibular glands of three Balb/c mice retroductally injected with 50 µL 15% microbubble solution with PBS alone, with 25 µg NIS-siRNA, or with 25 µg NIS-siRNA. Mice were imaged 1 hour after $^{99m}$TcO$_4^-$ intraperitoneal injection 24 hours, 48 hours, 72 hours, and 96 hours after retroductal injection. (B) Transaxial slice view of dynamic microSPECT/CT image of mouse submandibular gland retroductally injected with 15% microbubble solution in PBS and the uninjected submandibular gland. Image was acquired 90 minutes after $^{99m}$TcO$_4^-$ intraperitoneal injection and 40 minutes after pilocarpine administration to induce salivation. (C) Histology of mouse submandibular gland harvested 5 days after retroductal injection of 50 µL 15% microbubbles in PBS. (D) Histology of untreated submandibular gland from same mouse as C.
Figure 6.7. Retroductal injection of nanoparticles with siRNA results in decrease in radioisotope accumulation.

Mice retroductally injected with 25 µL containing 7µg non-targeting siRNA (A) or NIS-siRNA (B) complexed with nanoparticles to facilitate cellular uptake. Summation projection of microSPECT/CT images taken 50 minutes after $^{99m}$TcO$_4^-$ intraperitoneal injection, 96 hours after retroductal injection are shown. The un.injected submandibular glands are circled in green and the submandibular glands retroductally injected with nanoparticles+siRNA are circled in red.
References


