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Quantification of Fibrosis and Infiltrates in the Salivary Gland

Nikhita Kumar

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“Quantification of Fibrosis and Infiltrates in the Salivary Gland”

An honors thesis presented to the
Department of Biological Sciences
University at Albany
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In partial fulfillment for
graduation with Honors in Biological Sciences
and
graduation from The Honors College.

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Research Mentors: Jennifer Morrissey & Deirdre Nelson
Research Advisor: Dr. Melinda Larsen
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Abstract

Fibrosis is characterized by the excessive buildup of extracellular matrix components (ECM) in tissues and organs in response to injury, chronic inflammation, auto immune disorders, and genetic alterations. Non-obese diabetic mice (NOD) are a model for Sjögren’s Syndrome. We hypothesize that NOD mice, which start to develop Sjögren’s-like disease at approximately 12 weeks of age are suffering from hyposalivation at least in part due to fibrosis of the submandibular salivary glands (SMGs). Fibrosis refers to the over-production of extracellular matrix proteins in the connective tissue compartment. To test this hypothesis 16-week-old mice were treated with either the fibrosis inhibitor Nintedanib (treatment) or vehicle (non-treatment) as a negative control for 4 or 8 weeks and compared to an untreated C57BL/6 background strain mouse. The SMGs were then removed and cryopreserved. Histological, serial sections were created to allow for characterization of the entire gland. Masson’s Trichrome staining was performed on cryopreserved sections of the left salivary gland of non-obese diabetic (NOD) mice and control mice to characterize where collagen is deposited as trichrome staining detects fibrillar collagens and elastin. These were compared with salivary glands of NOD mice treated with or without Nintedanib to determine whether there was a reduction in fibrosis. In this study, H&E staining was used to determine where infiltrates were found in the WT, NOD, and Nintedanib-treated mouse tissue sections from the salivary glands. These stained sections were imaged using a brightfield microscope and the number of infiltrates were quantified to determine if Nintedanib decreased infiltrates in the ECM in the salivary glands of NOD compared to the controls. These studies will provide insights into the contribution of ECM deposition on salivary gland hypofunction and inform development of therapeutics for salivary hypofunction.
Acknowledgments

I would like to thank my mentor, Dr. Melinda Larsen, for providing the opportunity to conduct research in her lab and the continued support she provided over the years. I would also like to thank the lab members especially Jennifer Morrissey, Dr. Deirdre Nelson, and Amber Altrieth for guiding me in the lab. Lastly, I would like to thank my parents and my brother for cheering me on from afar throughout this process.
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**Introduction**

Sjögren’s Syndrome is an autoimmune disorder that is characterized by hyposalivation. This syndrome leads to chronic, severe dry mouth and dry eyes. Between 400,000 to 3.1 million people are affected by Sjögren’s Syndrome in the United States. On average females are nine-times more likely to develop Sjögren’s Syndrome than males. This disease is divided into two subtypes: primary and secondary. Primary Sjögren’s Syndrome has no associated connective tissue disease. Secondary Sjögren’s Syndrome has an association with underlying rheumatic disease (either systemic lupus erythematosus or rheumatoid arthritis) (Sullivan et al, 2018). In the submandibular salivary gland (SMG), the acinar epithelial cells fail to produce sufficient saliva (Salivary Gland, 2021). In this disease, excessive inflammation occurs as well as fibrosis, which is a buildup of extracellular matrix (ECM proteins), such as fibrillar collagens. Excess ECM is a contributing factor to decline in the function of acinar cells, which produce saliva. As fibrosis increases, less saliva is secreted by those with Sjögren’s Syndrome. Xerostomia and halitosis follow hyposalivation. Multiple tests are necessary for a diagnosis of Sjögren’s Syndrome as there is no single diagnostic test available (Kuklinski et al, 2017).

Currently there is no cure for Sjögren’s Syndrome. Palliative treatments are available to treat symptoms as they emerge. Often artificial saliva and tears are used to keep the mouth and eyes moist, respectively, as the salivary and lacrimal glands are underproducing (Yoo et al, 2014). Corticosteroids can be used in the short-term as they decrease inflammation and can manage flares. NSAIDS like Advil, Motrin, and Alieve can be used to reduce pain and inflammation (Vitali et al, 2010). Although these treatments may abate symptoms, they are only...
temporary and may have systemic or negative side effects. If we can determine what causes the hyposalivation from Sjögren’s Syndrome, we can identify a cure. In order to do this, we need to better understand the disease.

The Larsen lab uses mouse models to study causative factors for hyposalivation. We use surgical models where partial gland resection (O’Keefe et al, 2019) and ductal ligation are performed (Woods et al, 2015). Transgenic mice and inbred mouse strains like Non-Obese Diabetic (NOD) are used currently to model secondary Sjögren’s Syndrome. Research previously performed in the Larsen lab indicates that fibrosis may contribute to hyposalivation (O’Keefe et al, 2019). For the data in this paper, samples from C57BL/6 mice which were subjected to ductal ligation surgery were used. This induces a fibrotic response similar to Sjögren’s Syndrome but does not include the autoimmune component.

Fibrosis in salivary glands is present in Sjögren’s Syndrome patients and patients that present with hyposalivation. In work previously performed by the Larsen lab (Figure 1), fibrosis was found in and around acinar regions of the labial salivary gland. It often replaces the epithelial tissue of the glands. Oftentimes inflammation and necrosis are also seen with fibrosis. Chronic cases may show fibrosis as regions of acellular tissue. In these cases, the affected gland or lobule may be small (Kooistra et al.). Therefore, glands with high amounts of fibrosis will typically underproduce saliva, which is due to a decrease in functional acinar cells. Nintedanib was also used in this study and showed that fibrosis was inhibited with the addition of this drug.
Figure 1. Trichrome Staining in Patients with Salivary Gland Hypofunction. Left: Representative minor salivary gland biopsies from normal, non-Sjögren’s Syndrome, and Sjögren’s Syndrome samples. Right: Using FIJI to quantify the amount of cyan blue color in images from each patient sample, these values were expressed relative to the total area of the tissue. Images from n = 7 samples were graphed and averages are shown. ANOVA was used to compare the differences between samples with Tukey’s post hoc test used to compare samples. * p < 0.05. Tissues were provided by Janicke Jenssen, University of Oslo (O’Keefe, et al. unpublished data).

The Larsen lab previously compared ECM protein deposition from minor salivary gland biopsies from normal, non-Sjögren’s Syndrome, and Sjögren’s Syndrome samples. Figure 1 shows representative trichrome staining of minor salivary gland biopsies from normal, non-Sjögren’s Syndrome, and Sjögren’s Syndrome samples. Both the non-Sjögren’s and Sjögren’s syndrome samples appear to have more cyan blue staining than the control. However, these images were also quantitively analyzed. On the right of the images, a graph with the area of trichrome is constructed where n=7. The graph shows quantification of trichrome staining in multiple patient samples. Quantification shows that there is more ECM deposition in all patients suffering from salivary hypofunction than in normal patients, regardless of the cause.

Sjögren’s Syndrome is an autoimmune disease that has variable lymphocytic infiltration in the affected organs such as salivary glands. Minor salivary gland lesions are mainly composed of B and T cells. The amount of T cells and B lymphocytes correlate to the severity of the lesions (Christodoulou et al., 2010). This is how the immune response can be tracked by observing the B and T immune cells in NOD mouse tissue, a model for Sjögren’s Syndrome. By tracking these
infiltrates, the predicted severity of the symptoms of Sjögren’s Syndrome can be monitored. This is particularly useful when considering the ability of Nintedanib as a treatment to reverse or lessen lesions (and therefore, B and T cells). It is important to note that not all cells related to the immune system will correlate with lesion severity. In Christodoulou et al., T cells, CD4+ T cells, and B lymphocytes were found to be significantly different in minor salivary gland tissues between mild, intermediate, and severe inflammatory lesions. This is contrasted with CD8+ T cells, follicular dendritic cells, and natural killer cells where no correlation with lesion severity was found. In this study, we will use hematoxylin and eosin (H&E) staining to detect lymphocytes in general.

Fibrosis inhibitors are designed to reduce growth factors that are implicated in driving fibrosis. Fibrosis in tissues is associated with an upregulated production of growth factors. It promotes proliferation of immune cells and fibroblasts. One particular growth factor, TGF-β, is known as the “master” modulator in fibrogenesis. TGF-β leads to the transcription of collagen I and III genes which further leads to accumulation of collagen fibers in the extracellular matrix. (Meng et al., 2016). There are both intracellular and extracellular factors that affect fibrosis development. Extracellular enzymes like matrix metalloproteinases (MMPs) can prevent excessive buildup by degrading extracellular matrix including collagens and fibronectin. Tissue inhibitor of metalloproteinases (TIMPs) inhibit MMPs. The balance of MMPs and TIMPs regulate the amount of ECM accumulated with fibrosis development. Intracellular factors like several kinases propagate the cell signaling received externally. A few intracellular factors include TGF-α, TGF-β, and epidermal growth factors (Meng et al., 2016).

Many drugs have been developed to reduce fibrotic tissue formation and inflammation often associated with fibrosis. Three classes of drugs are categorized as single-component drugs
targeting extracellular factors, single-component drugs targeting intracellular components, and multi-component drugs. As the names imply, each of these classes have different pathways and factors that inhibit fibrosis. Nintedanib is the fibrosis inhibitor that this research is concerned with. Nintedanib is a single-component drug that targets extracellular factors, specifically signaling through vascular endothelial growth factor receptor (VEGFR) platelet-derived growth factor receptor (PDGRF), and Fibroblast growth factor receptors (FGFR). This drug works as an antagonist. Antagonists inhibit signaling by targeting cell membrane receptors to dampen downstream signaling (Meng et al., 2016). Nintedanib affects VEGFR, FGFR, and PDGFR on the cell surface by binding to the intracellular ATP binding domains so that the ligands are unable to bind to their respective receptors (Wollin et al., 2015). This blocks the signal transduction cascade from continuing within the cell and reducing fibrosis signaling.

The goals for this thesis project were to determine how to quantify the amount of fibrosis and infiltrates present in NOD vehicle, Nintedanib treated, and control tissues to determine the effects of Nintedanib treatment on the salivary glands of NOD mice as a model for Sjögren Syndrome. I performed H&E staining and Masson’s Trichrome staining, image tissue sections using the Leica and Nanozoomer microscopes, and used programs including Photoshop and FIJI Image J to quantify the data, and Prism to perform statistical analysis. H&E staining was used to determine if Nintedanib reduced infiltrates associated with inflammation. This goal was achieved by staining a series of different times for staining with hematoxylin to determine the best time of incubation in the stain. Then the new protocol was used to stain NOD vehicle, Nintedanib treated, and control tissues. The infiltrates were quantified by counting the number of loci of each tissue section at 5x magnification on the Zeiss microscope. Fibrosis was quantified by using
Masson’s Trichrome staining and using masks created in FIJI Image J to select for collagen deposition using color thresholding.
Materials and Methods

Nintedanib treatment of NOD mice

All animal husbandry, surgical procedures, and tissue collection were performed in accordance with protocols approved by the University at Albany, SUNY IACUC committee. Mice were housed in 12-hour light/dark cycle with access to water and dry food in the Life Science Research Building Animal Facility at University at Albany, SUNY. NOD/ShiLtJ mice were purchased from The Jackson Laboratory (Strain #:00197). Animals were assigned a unique identifier prior to experimental manipulations so that all manipulations were performed blind. 10 NOD mice were used per treatment. Treatment started at 16 weeks, at the onset of disease. Animals were treated via oral gavage every other day for 4 weeks with either Nintedanib at a dose of dose 60mg/ml or vehicle control, 0.5% Hydroxyethyl cellulose. The volume of vehicle control given was equal to the volume of treatment that would have been given. Health status and images were taken weekly for all animals. Saliva was collected at 15 weeks as a baseline prior to the start of treatment and every week after Figure 2. At 20 weeks animals were euthanized and both SMGs were harvested.

Sample preparation

The samples were cryopreserved before any staining was applied. The slides were frozen by incubating the slides in a 4% paraformaldehyde solution for 2 hours at 4°C. This was followed by 3 washes with phosphate buffered saline. Next, an incremental increase of sucrose solution were applied to the samples from 5%, 10%, and 15% sucrose. These were applied for 1 hour each at 4°C. A 30% sucrose solution was left overnight with the slides in 4°C. Another overnight incubation was given to let the samples sit in a 15% sucrose and 50% OCT solution overnight at
4°C. The samples were then frozen indirectly with liquid nitrogen. The liquid nitrogen was filled right above the pipette tip holder so the holder could float. The samples were placed on top of the pipette tip holder. After 5 minutes, the samples were frozen solid. The samples were then moved to -80°C until they were cut. Then the samples were sectioned in 10μm sections using the cryostat machine. Cryosectioning was performed by Ph.D. candidate, Jennifer Morrissey.

**Masson’s Trichrome Stain**

Slides which have already been prepared with tissues sections and placed in -80°C freezer were used to perform Masson’s Trichrome staining. The slides were allowed to sit warm up to room temperature. Then they were fixed using Bouin’s fixative for 20 minutes. The slides were rinsed under cold tap water until the yellow color was removed (approximately 5 minutes). Stain was added using Weigert’s Iron hematoxylin working solution for 5 seconds at room temperature then rinsed for 2 minutes in cold tap water. Scarlet-acid Fuchsin solution was used for 15 seconds as a stain. The slide was rinsed with DI water until the water ran clear after coming off the slide. 400 μL of phosphotungstic/phosphomolybdic acid was pipetted directly onto the tissue sections. After sitting for 10 minutes, the solution was drained into a Kimwipe. Aniline Blue was used for 30 seconds to stain. The slide was then rinsed under DI water 3 times for 30 seconds each. Then 500 μL of 1% acetic acid was directly pipetted onto the tissue section for 1 minute. The slide was rinsed once again. The tissue section was rinsed in 95% ethanol for 2 minutes, 100% ethanol for 2 minutes, and xylene for 2 minutes. Lastly, the tissue section was mounted with 45 μL of Permount.
**Hematoxylin and Eosin (H&E) Staining**

Slides were removed from the -80°C freezer and put in 1x PBS for 10 minutes to rehydrate the tissue. The slides were washed for 1 minute under running tap water. The slides were incubated in hematoxylin 7211 for 2.5 minutes (this time was variable during the optimization of H&E experiment). Under running tap water, the slides were washed for 1 minute and 30 seconds. Then, they were incubated in eosin-Y Alcoholic for 3 minutes. Next, the slides were washed again under running tap water for 1 minute. Lastly, the slides were incubated in 100% ethanol for 3 minutes and mounted with about 20-40 ml of Permount mounting media.

**Imaging**

Images were captured using a Leica DM4000 B LED fluorescence microscope 689 equipped with a Leica DFC310 FX camera, a Nikon Eclipse, a Zeiss AxioObserver microscope, and a Hamamatsu NanoZoomer 2.0 RS (Dr. Paul Higgins laboratory, Albany Medical College). The brightfield setting was used for the microscopes. A magnification of 4x and 10x were used respectively to image the tissue sections. The light settings were not changed during imaging to preserve differences in staining per section. For imaging with the Nikon Eclipse, images were collected with a Nikon Digital Rebel digital SLR camera. Overlapping images were collected, which later were manually tiled. The number of images for each tissue section depended on the size of the tissue and magnification. However, between 2 and 10 images containing 20-30% overlap with consecutive photos were required for the program to recognize the images as continuous tissue. For the images collected on the Nanozoomer, the images were automatically tiled and stitched together. The Zeiss microscope has a camera that can only produce images in black and white.
Adobe Photoshop

Once images overlapping by 25-30% were acquired using the Nikon camera, the photos were stitched together to show the complete slice of tissue on the slide. This was repeated for each tissue section. In Adobe Photoshop, select File, then Automate, then Photo Merge was performed after browsing for images that included all the small sections of the tissue slice so that they could be merged together. Photoshop finds the overlapping parts of the images to stitch them together correctly. Then select Layer and Flatten Image were used to flatten all the layers into one image. This reduces the file size and makes it possible to complete the next step where the images will be quantified.

FIJI Image J

To quantify the stained sections, FIJI Image J was used. The correct full image was dragged into FIJI Image J. Then the color threshold setting was used to separate the stained blue fibrotic tissue. Select Image then Adjust then Color Threshold to achieve this. The parameters for Hue were set to 127/168, Saturation was set to 30/255, and Brightness was set to 50/255 as default parameters and were optimized during the course of the experiment. These parameters can be changed to accurately selected the blue fibrotic tissue; however, the same parameters are used for images taken on the same day for consistency. A mask was created to compare the selected region from the whole tissue section. Using the Color Threshold tab, the Select button was pressed. Then Edit, Selection, and Create Mask were pressed in that order to create a mask. The masks were saved with appropriate labels before continuing. The measure function was used to
calculate the total area of the gland. Percent trichrome stained area was calculated by dividing the trichrome stained area by the total area and multiplying by 100.

**Foci quantification**

In H&E staining, many blue dots in one section show aggregation of infiltrates. These clusters were identified as foci if they were large enough to be viewed in 5x magnification on the Zeiss microscope. A few test samples were reviewed by two lab members to agree on how big the clusters must be to be identified as foci. Any aggregation of blue dots in the sublingual gland were excluded so that only the infiltrates in the submandibular gland were counted. This quantification was created from live counting on the Zeiss microscope and paneling the image until the entire tissue section was covered. The counts were performed in a blind setting so the individuals counting were not aware of the type of tissue they were quantifying during the process. The counts of loci were validated by having two different individuals count the same tissue sections (NK and DN).

**Saliva Collection Methods**

CD1 mice were anesthetized, and saliva was stimulated with pilocarpine. Animals were placed in a conical tube and oriented with their head and neck lower than their chest to reduce the possibility of asphyxia. A SalivaBio sponge was cut into 4 pieces and placed into a 0.75 ml microcentrifuge tube. The 0.75 ml tube was punctured at the conical bottom using a 18G needle, and then placed inside a preweighed 2 ml microcentrifuge tube. The assembly was weighed. The sponge was removed from the assembly and placed into the animal’s mouth. Saliva collection began 5 minutes after the pilocarpine injection and continued for 15 minutes. The sponge was
removed, placed in the preweighed tubes, and weighed. The weight of saliva collected was taken by subtracting the final weight from the initial weight. The assembly was placed in a centrifuge and run at 7,000 rpm for 5 minutes. Volume was then measured via pipetting. Our lab adapted a previously established survival, saliva collection method for mice. Analysis of saliva output was used to assess the functional recovery of the gland response to Nintedanib. Following previously published protocols, mice were anesthetized, and saliva was stimulated with pilocarpine. Pilocarpine is an alkaloid pharmacologic used in treating xerostomia resulting from radiation exposure, Sjögren syndrome, and glaucoma. By collecting saliva and analyzing it we can detect molecular differences between healthy vs. disease animals. By performing this reproducibly we can monitor the efficacy of treatments in real time.

**Measurement of Saliva Collections**

The saliva which was collected was measured by both weight and volume. The weight was measured on an analytical balance. The initial weight was subtracted from the final weight of the tube and sponge. The saliva measurements were then normalized to the mouse weight by dividing the collected measurement by the animal weight. The volume was estimated by adjusting a Ranin pipettor to collect all of the saliva present in the microcentrifuge tube to measure the volume. This number was recorded. All saliva samples which were collected were frozen and stored at -80°C.

**Results**

**Chapter 1- Optimizing Hematoxylin (in H&E) Staining and Looking for Infiltrates in Control, Vehicle, and Nintedanib-Treated Samples**
Optimization of H&E staining needed to be performed in order to accurately detect the number of infiltrates in each tissue section. The original protocol called for slides to be immersed in the Hematoxylin (blue) stain for 10 minutes. However, this proved to be too long in previous experiments. The blue stain was too strong in the tissue sections. In this experiment, the submandibular salivary glands of NOD mice were harvested and sectioned to create 10 mm cryosections. H&E staining was applied to distinguish the fibrillar collagen and elastin buildup from the rest of the tissue. The areas with collagen buildup show where fibrosis has occurred. In Figure 2, images of two tissue sections from each time point in Hematoxylin stain were taken from the Leica microscope. The time points progressed with an incremental increase from 1 to 2.5 to 5 to 7.5 minutes.
Figure 2: Optimization of Hematoxylin Stain on Control Tissue with Times: 1 min, 2.5 min, 5 min, and 7.5 min

Two tissue sections of mouse salivary glands in hematoxylin and eosin stain are shown at each time point of hematoxylin stain. Images A-C were in the Hematoxylin stain for 1 minute. Images D-F were stained in hematoxylin for 2.5 minutes. Images G-I were stained for 5 minutes. Images J-L were stained for 7.5 minutes. Images A, B, D, E, G, H, J, and K are all full glands that were stitched together from tiled overlapping images. Images C, F, I, and L are all enlarged sections from the stitched images to better show the increase in blue nuclear staining from the increased times spent in the hematoxylin stain.

Figure 2 shows that 2.5 minutes in the hematoxylin stain is the most effective time point. The 1-minute samples appear to have far too little staining and exclude some cell nuclei from the blue staining. The samples with 5-minute and 7.5-minute hematoxylin stains appear to have too much
staining which can blur where the infiltrates are specifically located. These images were all taken on the Nikon Eclipse camera attached to the Leica microscope. The nuclei were much more clearly seen directly through the microscope instead of what is represented in the captured images.

The protocol was adjusted to account for the slides remaining in the hematoxylin stain for 2.5 minutes instead of the original 10 minutes. Then this adjusted protocol was used to observe the infiltrates in three tissue types: control, NOD vehicle-treated, and NOD nintedanib-treated samples. The control is a C57BL/6 mouse with no interventions. The NOD tissue represents tissue similar to Sjögren’s Syndrome patients. The treated tissue is taken from NOD mice which were introduced to nintedanib at 18 weeks as a possible treatment for Sjögren’s Syndrome.
**Figure 3:** H&E Staining of Control, NOD, and Nintedanib-Treated Samples

H&E staining of salivary glands of each tissue type: control, NOD, and treated. Images A and B are the control. Images C and D are the NOD vehicle-treated mouse tissue. Image E and F are the NOD mouse treated with nintedanib. Images A, C, E are stitched together tiled images of the 4x images. Images B, D, F were collected with 4x magnification on the Leica microscope.

Figure 3 shows that the control has no infiltrates, and the NOD model has infiltrates. The nintedanib-treated samples appear to have some aggregation of infiltrates as well. Once
optimized parameters were determined, additional samples were examined to determine the effects of nintedanib on the NOD mice.

The Leica microscope was not effective at imaging the tissue sections due to uneven lighting and insufficient clarity. The images in this figure are particularly dark and uneven, even though the same settings on the camera were used. This issue was likely due to setting changes in Photoshop where the brightness and contrast levels may have been changed between the days of processes each batch of images. In an attempt to gain clearer images where the individual nuclei of the infiltrates are visible, the Zeiss microscope was used next. However, the Zeiss microscope only has a black and white camera attached to it. The black and white images of the same slides as Figure 3 are shown below (Figure 4).

**Figure 4 Detection of infiltrates in NOD, NOD-vehicle and Nintedanib-treated salivary glands.** The presence or lack of infiltrates in the three different tissue types: control, NOD, and NOD nintedanib-treated using the same samples of salivary glands stained with H&E stain as in Figure 3. Images A, C, and E are at 10x magnification on the Zeiss microscope. Images B, D, and F are at 20x magnification. The red arrows in Images D and E highlight the areas where many infiltrates are observed, which are detectable as dark spots.
The Zeiss microscope shows the stained samples more clearly. However, the images still do not accurately represent what can be seen through the microscope lenses without the camera. Through the microscope, the individual nuclei can be seen, and these images were used for quantification.

**Chapter 2- Comparing Number of Loci in Vehicle vs Nintedanib-Treated Salivary Glands**

The optimized H&E staining times were used to stain more slides of vehicle and treated tissue sections in an attempt to quantify the number of infiltrates (as defined by foci). A focus is defined by a group of infiltrates that is easily identifiable by the eye at 5x magnification on the Zeiss microscope. This may appear to be a vague definition; however, the two individuals who counted the loci agreed upon the definition beforehand. Controls were not imaged because no foci were identified among those tissue samples.

**Figure 5: Images of Vehicle and Nintedanib-Treated Tissues Stained with H&E**

The foci of infiltrates of the vehicle- versus Nintedanib-treated salivary gland tissues. Images A-C are sections from NOD vehicle-treated mice. Image A is section A1 from slide 660. Image B is section B2 from slide 934. Image C is section B2 from slide 974. Images D-F are sections from NOD salivary gland tissues treated with Nintedanib. Image D is section B2 from slide 612. Image E is section B3 from slide 688. Image F is section A2 from slide 729.
In images A-C and images D-F, foci were identified with red arrows pointing to example viewed in each of the tissue sections. No foci were identified in the control sections and the controls were not imaged.

Seven slides (one per mouse) were quantified by counting the number of foci. The graphs are shown below in Figure 6, 7, and 8. Figure 6 shows the points, error bars, and average bars of each tissue type.

![Graph showing comparison of foci counts between vehicle and treated groups](image)

**Figure 6 Average number of Foci in Vehicle vs Nintedanib-Treated SMGs.** Counts of foci between the NOD vehicle and NOD treated with Nintedanib between two different people counting (NK and DN). Each bar represents the average number of loci per tissue type and counter. The dots represent the average number of loci counted from each slide within the tissue type: vehicle or treated. Error bars are present in each bar. Results of a student’s ANOVA test comparing vehicle and treatment for each scorer revealed no difference between vehicle and treatment.

There is no significant difference between the vehicle and treated in the counts from each individual. There is also no significant difference in number of foci of the vehicle from NK vs
DN nor treated from NK vs DN. The error bars overlap, and the p-values are greater than 0.05. This suggests that Nintedanib treatment does not significantly reduce the number of infiltrates in NOD tissue.

**Figure 7: Average Number of Foci of Vehicle vs Nintedanib-Treated SMGs**

Left: The orange bar on the left represents foci averaged from the vehicle tissues. Right: The red bar on the right represents the average number of foci from the Nintedanib-treated salivary glands.

A t-test was performed on this data and a p-value of 0.0182 was assigned to it. This p-value is less than 0.05 which identifies this data as significant. The values of all of tissue sections of each tissue type averaged together are significantly different from each other which means that the Nintedanib treatment does reduce the amount of infiltrates in the SMG of the mouse model.

The average number of foci are shown in Figure 7. The counts from each slide are included in the bar graphs in Figures 8 and 9 to represent the foci from the vehicle tissue and treated tissue respectively. Each slide was randomly selected to represent one of seven mice.
Figure 8: Average number of foci per animal of from salivary glands of Vehicle-treated animals.

The number of infiltrates in each slide was manually counted by two different counters (NK and DN) and shown graphically by averaging the number of foci from each tissue section on the slides. The counts from each individual are compared side by side with NK in blue and DN in orange. These counts were taken from using the 5x magnification on the Zeiss microscope, paneling through each section, and recording the number of foci counted into an excel sheet.

Figure 8 shows there is a slight difference between the counts from the two individuals; however, this difference is not significant. The p-value from the t-test greater 0.05. Only slide 550 appears to have a great difference. This difference could be due to difference in what qualifies as a focus.

DN counts are regularly greater than NK counts likely due to counting smaller clusters of infiltrates as loci.

The same technique as in Figure 8 was used to determine the number of foci in the treated tissues as seen in Figure 9.
Figure 9: Average number of foci per animal of Nintedanib-Treated Tissue.

The average number of foci from each slide counted by two individuals is shown, as reported for vehicle-treated slides in fig. 8. The counts from NK are in blue while the counts from DN are in orange.

There is a slight difference between the counts from the two individuals; however, this difference is not significant. The p-value from the t-test is greater than 0.05.

**Chapter 3- Quantifying the Fibrosis by Creating Masks After Masson’s Trichrome Staining**

Infiltrates show the immune response in the Sjögren’s disease mouse model. However, the fibrosis is another key factor to examine. Fibrosis is characterized by a buildup of collagen and other extracellular matrix proteins in the stromal compartment of the cell. Those collagen
and other ECM proteins were stained and quantified to measure the differences in the control, vehicle, and treated mouse models of salivary glands.

The submandibular salivary glands of NOD mice were harvested and sectioned. Trichrome staining was applied to distinguish the collagen buildup in blue from the rest of the tissue. The areas with blue collagen buildup show where fibrosis has occurred. Two different microscopes were used to captures these images. Then Fiji Image J was used to create a mask and calculate the area of trichrome staining present. In Figure 10 the quantifications of the images taken from the Leica microscope are shown. The percent trichrome stained area for each image taken from the Leica microscope shown is shown in Figure 11.

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<tr>
<td>C57 R2 Slide 28 S1</td>
<td>953.325</td>
<td></td>
</tr>
</tbody>
</table>
Figure 10. Table of Trichrome Staining Quantification (10 samples). The total area was calculated by FIJI Image J. Second, only the area within a selected region (encompassing the whole tissue) was calculated. Masks with hue 127/168, saturation 50/255, and brightness 30/255 were created. The percent trichrome from the Leica microscope and masks with these specific settings yielded results ranging from 52.10% to 80.25%. However, on average the percent trichrome was 71.57%. The masks appeared to accurately distinguish the blue regions in the images from the remaining tissue. Third, the percent trichrome was calculated by dividing the selected area by the total area and multiplying 100 for the percent area.

![Percent Trichrome Calculated from Leica Scope](image)

Figure 11: Graph of Average Percent Trichrome of the Images from Leica Microscope
Using this graph, the percent trichrome can be observed to be similar between many of the images but not the same. This was a blinded experiment so the experimental and control tissue samples could not be distinguished while completing the quantification to avoid bias. However, the samples are still not identified as a control or experimental image because more quantification must be done.

The second microscope used was the NanoZoomer. The NanoZoomer is very precise and easy-to-use microscope. It would be preferred because of the clean images it produced. Figure 12 presents samples of data collected from quantifying the NanoZoomer.
Figure 12: Trichrome and area measurements captured from the NanoZoomer Slide Scanner. These samples are the same as those in the Leica microscope table (Figure 10). Only the microscope and camera used to image these samples changed. However, a significant decrease in trichrome area across all samples were observed. This is likely due to incorrect color thresholds used to create the masks. Different color thresholds for hue, saturation, and brightness must be used for the NanoZoomer because it is a different microscope.
Figure 13: Optimization of color thresholding for H&E-stained images. Different color thresholds to optimize settings to detect the most blue, indicative of collagen/elastin detection. A. H&E-stained image of a SMG. Masks produced with FIJI were collected with different threshold settings. B. Hue 127/168, Saturation 50/255, and Brightness 30/255. C. Hue 147/168. Image D hue 147/168 and brightness 90/255. Image E hue 147/178. Image F hue 144/174 saturation 50/255 and brightness 30/255).

Figure 13, one sample image (C57 L1 081720 Slide 22 Sec 1 5X300) was used to create multiple masks with various color settings to maximize the amount of blue staining displayed. Mainly the hue was adjusted to observe significant changes in a positive direction. The brightness was also
adjusted (as seen in Image D). The change in brightness did not help reach a more accurate mask. **Image A** shows the original image that is uploaded to FIJI Image J without any color changes. **Image B** is the mask that was used in the quantifications in Figure 4. Image B has settings of hue 127/168, saturation 50/255, and brightness 30/255. These settings worked well with the Leica microscope but not the NanoZoomer. **Image C** demonstrates the first attempt to change the settings where the Hue 147/168 was increased to a darker blue closer to purple than green. This seemed to still pick up on some blue without significant change. **Image D** uses color settings hue 147/168 and brightness 90/255. The change in brightness with the hue change only slightly changed the mask. **Image E** is where a large amount of color is picked up on in the mask. The settings of hue 147/178 increased the range of blue color that was selected. **Image F** is what looked the most accurate to my eyes out of all the masks above. The color settings were set at hue 144/174 with the other two setting at the “normal” setting (saturation 50/255 and brightness 30/255). This appeared to pick up more of the blue staining without also selecting for the red/purple colors.

**Chapter 4- Optimizing Masks of Masson’s Trichrome Staining in Fiji Image J for Nanozoomer Images**

Masson’s trichrome staining is a three-color staining procedure which distinguishes collagen (blue), nuclei (brown), and cytoplasm (pink). Using this stain, the amount of collagen buildup is able to be visualized and measured. Because fibrosis is characterized by excess collagen in the extracellular matrix, this stain is a good method of measurement of fibrosis.

The submandibular salivary glands of NOD mice were harvested and sectioned. Trichrome staining was applied to distinguish the fibrillar collagen and elastin buildup in blue
from the rest of the tissue. The areas with blue collagen buildup show where fibrosis has occurred. Two different microscopes were used to captures these images. In Figure 14 an example images taken from the Leica microscope and NanoZoomer are shown in Image A and B respectively.

![Figure 14](image)

**Figure 14: Comparison of Stitched Trichrome Images Captured on Two different microscopes.** Images after being captured and computationally stitched together. **Image A** was taken by the Leica microscope. Using the Leica microscope requires several images to be taken and complied. This is a lengthy process that can take about 20 minutes per image. **Image B** was taken by the NanoZoomer microscope. The NanoZoomer can image the whole section in about 2 minutes. The shorter time of capturing tissue sections is the main advantage of using the NanoZoomer. The NanoZoomer also provides clearer images which can provide more accurate quantification of fibrosis.

Then Fiji Image J was used to create a mask and calculate the area of trichrome staining present. In Figure 3 the original image and mask of the image taken from the Leica microscope are shown.
**Figure 15: Comparison of H&E image with Mask**

**Image A** shows the original image captured by the Leica microscope. Using FIJI Image J color thresholds were placed on the image to isolate the blue staining caused by excess collagen deposition in the extracellular matrix of the tissue. **Image B** is a mask created with hue 127/168, saturation 50/255, and brightness 30/255. These settings appeared to accurately distinguish the blue regions in the images from the remaining tissue in the Leica microscope.

The second microscope used was the NanoZoomer. The NanoZoomer is a very precise and easy to use microscope. It would be preferred because of the clean images it produced and the ease in which the images can be acquired. Figure 16 presents a sample of data collected from quantifying the NanoZoomer using the settings that were identified to be optimal for the images captured using the Leica microscope. Settings were adjusted using FIJI to identify the settings that are most critical for detecting the blue color (See Figure 16).
Figure 16: Comparison of H&E Image with Two Different Mask Settings.

Image A is the original image captured by the NanoZoomer. A mask was created with the settings used for the Leica microscope shown in Image B. The settings were hue 127/168, saturation 50/255, and brightness 30/255. Very little blue staining was selected for with these settings. Different color thresholds for the hue, saturation, and brightness must be used for the NanoZoomer because it is a different microscope. Image C shows the mask created from using the adjusted settings specialized for the NanoZoomer. These settings are hue 127/175, saturation 15/255, and brightness 30/255.

The adjusted settings appear to accurately select for the tissue. It is important to note that the settings were customized to select for collagen deposition in only the submandibular gland. The sublingual gland is present in some samples and requires different settings. The sublingual gland was excluded from the quantification to maintain more accurate data.
Figure 17: Optimization of color thresholds
One sample image (C57 L1 081720 Slide 22 Sec 1 5X300) was used to create multiple masks with various color settings to maximize the amount of blue staining displayed. Image A shows the original image that is uploaded to FIJI Image J without any color changes. Image B demonstrates the change to the settings where the hue 147/168 was increased to a darker blue closer to purple than green. This seemed to still pick up on some blue without significant change. Image C showed an increase in brightness. The only change from image B to C was brightness increased to 90/255. The brightness changing did not affect the mask very much. Image D shows how changing the saturation to 30/255 increases the amount of blue staining selected for. Image E was created by selecting hue 127/172, saturation 15/255, and brightness 30/255. This is very close to the final settings selected for. This is where a large amount of color is picked up on in the mask. The hue and saturation were only adjusted here. The brightness was kept at the same setting as used for the Leica microscope. Image F only included a slight change to the hue compared to Image E. This resulted in hue 127/175, saturation 15/255, and brightness 30/255.
After additional manipulations were performed in FIJI, optimal settings were identified to quantify fibrotic staining in the SMG images captured using the NanoZoomer. Mainly the hue was adjusted to observe significant changes in a positive direction. The saturation was also adjusted (as seen in Image D). A change in brightness did not help reach a more accurate mask. The settings used to create Image F looked the most accurate to my eyes out of all the masks above. This appeared to pick up more of the blue staining without also selecting for the red/purple colors.

**Discussion**

The quantification of H&E staining of the submandibular gland can be used to examine the extent of lymphocyte infiltration. The optimization of the H&E staining and the observed qualitative difference in the control, NOD vehicle, and NOD-nintedanib-treated slides have made possible the evaluation of the effect of this drug on infiltrates.

Using the infiltrates as a marker, the treatment with nintedanib appears to reduce the inflammatory infiltrates seen in a mouse model of Sjögren’s Syndrome. It is important to note that not all cells related to the immune system will correlate with lesion severity. CD8+ T cells, follicular dendritic cells, and natural killer cells have no correlation with lesion severity was found. However, B and T lymphocytes do correlate with disease severity. This is how infiltrates were able to be measured.

The quantification of trichrome staining on the SMG of NOD mice can be accurately performed as a result of this work. Future studies to be performed by the laboratory will compare the trichrome levels in the SMGs that were subjected to nintedanib treatment or vehicle control treatment to determine if nintedanib has any efficacy in improving fibrosis in this mouse model.
of Sjogren’s Disease. If results are positive, nintedanib could be further explored as a novel treatment for Sjogren’s Disease.

When using FIJI to correct color threshold settings must be used specific to each microscope and camera. This is limiting in that for images collected from different microscopes, different settings must be used. In the future, the quantification of fibrosis may be possible using artificial intelligence (AI) technologies that are sample agnostic. With these methods, an algorithm can be trained by the user to recognized patterns in images that can be detected in images regardless of the source. Such methods will be useful in future work in the laboratory.

Brightfield images are difficult to quantify because all colors are present. In fluorescent imaging, only one color is present at a time. This makes it easy for a computer to calculate the area that is fluorescent. However, computers have difficulty differentiating colors in brightfield colorimetric images. Using FIJI Image J, images with multiple colors can be quantified. The color settings must be specified for this differentiation. With AI-based image quantification methods, the ability to distinguish different colors may be improved.

As nintedanib is still in clinical trials for idiopathic fibrosis in the lung, it remains of interest as a therapeutic for other fibrotic diseases. Nintedanib is not an ideal therapeutic as it does have side effects. Alternative methods of interfering with fibrosis may be more effective and have fewer side effects. For example, an inhibitor of Smad3 signaling was recently shown to have efficacy in treating fibrosis in the salivary gland ductal ligation mouse model. If Nintedanib can be proven to reduce the number of infiltrates and fibrosis in salivary glands of Sjögren’s Syndrome model mice, then Nintedanib could go on to further testing which could lead to patients with Sjögren’s Syndrome potentially having a treatment available to them. This would be the first treatment that would be able to stop the production of fibrosis in salivary
glands instead of simply treating the symptoms of the disease. Nintedanib has a potential to give relief to patients who have never had this option before.


