Molecular analysis of Idiopathic Subglottic Stenosis for *Mycobacterium* species: A North American Airway Collaborative (NoAAC) TS-04 study

Alexander Gelbard, MD3, Nicolas-George Katsantonis, MD1, Masanobu Mizuta, MD1, Dawn Newcomb, PhD1, Joseph Rotsinger, MS2, Bernard Rousseau, PhD, CCC-SLP3, James J. Daniero, MD1, Eric S. Edell, MD4, Dale C. Ekborn, MD5, Jan L. Kasperbauer, MD6, Alexander T. Hillel, MD6, Liying Yang, MD7, C. Gaelyn Garrett, MD8, James L. Netterville, MD1, Christopher T. Wootten, MD1, David O. Francis, MD1, Charles Stratton, MD1, Kevin Jenkins, MD9, Tracy L. McGregor, MD9, Jennifer A. Gaddy, PhD10, Timothy S. Blackwell, MD3,11, and Wonder P. Drake, MD2,11

1Dept. of Otolaryngology, Vanderbilt University, Nashville, Tennessee 2Dept. of Medicine, Division of Pulmonary & Critical Care, Vanderbilt University, Nashville, Tennessee 3Dept. of Medicine, Division of Infectious Disease, Vanderbilt University, Nashville, Tennessee 4Dept. of Otolaryngology, University of Virginia Health System, Charlottesville, Virginia 5Dept. of Medicine, Division of Pulmonary & Critical Care, Mayo Clinic, Rochester, Minnesota 6Dept. of Otolaryngology, Mayo Clinic, Rochester, Minnesota 7Dept. of Otolaryngology, Johns Hopkins, Baltimore, Maryland 8Dept. of Medicine, New York University School of Medicine, New York, New York 9Dept. of Pathology, Microbiology and Immunology 10Dept. of Pediatrics, Division of Medical Genetics, Vanderbilt University, Nashville, Tennessee 11Veterans Affairs Tennessee Valley Healthcare Services, Nashville, Tennessee

Abstract

**Rationale**—Idiopathic subglottic stenosis (iSGS) is an unexplained obstruction involving the lower laryngeal and upper tracheal airway. Persistent mucosal inflammation is a hallmark of the disease. Epithelial microbiota dysbiosis is found in other chronic inflammatory mucosal diseases; however, the relationship between tracheal microbiota composition and iSGS is unknown.

**Objectives**—Given the critical role for host defense at mucosal barriers, we analyzed tissue specimens from iSGS patients for the presence of microbial pathogens.

Corresponding Author: Alexander Gelbard, M.D., (alexander.gelbard@vanderbilt.edu), Assistant Professor, Department of Otolaryngology, Vanderbilt School of Medicine, Medical Center East, S. Tower. 1215 21st Ave. South, Suite 7302, Nashville, TN 37232-8783.

Conflicts of Interest: None to disclose

Author Contributions: AG designed & performed experiments, analyzed data, & wrote the paper, NK analyzed data, MM designed & performed experiments, DN designed & performed experiments, BR aided in experimental design, JD, EE, DE, JK, AH analyzed data, preformed critical scientific review, GG aided in experimental design, LY analyzed data, JR conducted experiments, JN aided in experimental design, CW aided in experimental design, DF aided in experimental design, statistical analysis, CS conducted experiments, KJ conducted experiments, TM aided in experimental design, data analysis, review of manuscript, TB aided in experimental design, experiments, data analysis, review of manuscript, JG conducted experiments, WD aided in experimental design, experiments, data analysis, review of manuscript.
Methods—Utilizing 20 human iSGS, 20 intubation-related tracheal stenosis (iLTS) and 10 healthy control specimens we applied molecular, immunohistochemical, electron microscopic, immunologic and Sanger™ sequencing techniques.

Main Results—With unbiased culture-independent nucleic acid, protein, and immunologic approaches, we demonstrate that Mycobacterium species are uniquely associated with iSGS. Phylogenetic analysis of the mycobacterial virulence factor rpoB suggests that rather than Mycobacterium Tuberculosis (Mtbc), a variant member of the Mycobacterium Tuberculosis Complex (Mtbc), or a closely related novel mycobacterium is present in iSGS specimens.

Conclusions—These studies identify a novel pathogenic role for established large airway bacteria, and provide new targets for future therapeutic intervention.

Level of Evidence—NA.

Keywords
Mycobacterium; Mtbc; Idiopathic Subglottis Stenosis; Tracheal Stenosis; Laryngotracheal Stenosis; iSGS; ISS; Microbiome

Introduction
Idiopathic subglottic stenosis (iSGS) is a debilitating extrathoracic obstruction involving the lower laryngeal and upper tracheal airway. It arises without known antecedent injury or associated disease. Emerging study has demonstrated affected patients possess tightly conserved clinical demographics, histopathologic findings, anatomic injury, and physiologic impairment. Despite description of iSGS more than four decades ago, only recently has the inflammatory fibrosing phenotype been characterized at the molecular level. Data show highly upregulated activation of the inflammatory IL-17A/IL-23 pathway in the mucosal scar in iSGS, yet the mechanisms responsible for the characteristic demarcated airway inflammation are unknown.

In alternate pulmonary pathologies, both structural and functional changes in the lung epithelium appear to be integral to fibrotic remodeling, occurring in the setting of chronic airway inflammation. Epithelial microbiota dysbiosis with subsequent sustained host inflammation is found in other chronic inflammatory mucosal diseases. Although the trachea is lined with respiratory epithelia, which readily supports colonization by a diverse microbiome at other upper respiratory sites such as the oropharynx to date nothing is known of the composition of the resident microbiome of the large airway or its contribution to airway remodeling in idiopathic subglottic stenosis. Microbiological studies that rely on culture-based techniques underestimate the diversity of species present, and offer limited detection of intracellular pathogens. The application of culture-independent approaches offers the opportunity to both provide a broader picture of tracheal microbiome composition and identify discrete pathogenic species associated with disease states.

Previously work has demonstrated activation of the canonical IL-23/IL-17A pathway in the tracheal mucosa of iSGS patients, and identified γδ T cells as the primary cellular source of IL-17A. Given the established role of γδ T cell IL-17A in host defense at mucosal
barriers, we analyzed tissue specimens from iSGS patients for the presence of microbial pathogens. Our unbiased molecular interrogation of the tracheal microbiota of iSGS patients provides detailed nucleic acid, protein, and immunologic evidence to demonstrate *Mycobacterium* species within tracheal scar. Together with our previous work, these studies offer new insights into the pathogenesis of iSGS. They suggest that human tracheal mucosal health is highly dependent on the composition of the resident microbiota, identify a novel pathogenic role for established large airway bacteria, and offer targets for future therapeutic interventions.

**Materials and Methods**

This study was performed in accordance with the Declaration of Helsinki, Good Clinical Practice, and was approved by the Institutional Review Board at Vanderbilt University Medical Center (IRB#: 140429).

**Patients**

In all, 20 iSGS, 20 intubation-related tracheal stenosis (iLTS), and 10 normal control patients were utilized for experiments (Figure S1.). Each iSGS & iLTS diagnosis was confirmed using previously described clinical and serologic criteria. The control population consisted of patients without known tracheal pathology, malignancy, or systemic infection. Tracheal scar or freshly isolated peripheral blood mononuclear cells (PBMC) was the source of all specimens from the iSGS, and iLTS patients, and normal trachea or PBMC was the source for the control patients.

**Culture independent qPCR profiling of respiratory microbime**

*DNA Isolation:* Genomic DNA was extracted with the Qiagen DNAeasy extraction kit (Qiagen, Valencia, CA) according to the manufacturer's instructions with slight modification as previously described. The gDNA concentration and quality were confirmed using the Bioanalyzer 2100 system (Agilent, CA, USA). Human respiratory pathogen qPCR array (Qiagen, Vallencia, CA) was performed as per manufacture's instructions in a StepOnePlus™ instrument (Applied Biosystems®, USA) Expression analysis was performed using PCR array analysis software (Qiagen, Valencia, CA; https://www.qiagen.com)

**In situ Hybridization for Mycobacterial gene product GyraseA**

Paraffin embedded iSGS and iLTS airway stenosis tissues and healthy controls (US Biomax Inc. product# RS321), were pretreated and probed for Gyrase A (Advanced Cell Diagnostics #436701) following a modified RNAscope® 2.0 Assay's HD Detection Kit (Red) protocol. Tissue was digested with proteinase-K (1:100 dilution) (Sigma) in 20 mM Tris-Cl (p.H. 8.0) for 5 minutes at room temperature. Experimental controls run in parallel included bacterial gene DapB as a negative control to assess background signal and Homo sapiens HS-PP1B to assess positive signals and protocol efficacy.

**Sanger Sequencing of Mycobacterial Species**

*Molecular subtyping of Mycobacterial Species*—Nested PCR analysis was performed as previously described for Mycobacterial *rpoB* (with conditions and primers...
listed in supplemental data). Negative and positive controls were run in parallel. Genomic DNA extracted from M. tuberculosis strain H37rv served as a positive control (Vircell Technologies, Granada, Spain), while PCR master mix inoculated with 5 μL of sterile water, and PCR master mix alone were used as negative controls.

**Determination of DNA Sequence of Amplified Products**

*rpoB* gene products were run on a 2% gel and purified the 360bp band using the Qiagen QIAquick® Gel Extraction kit (Qiagen, Vallencia, CA) and sequenced directly on both strands in the Vanderbilt Cancer Center Core Sequencing Laboratory. Alignments of the *rpoB* sequences were performed with Sequencher software (v5.3, Gene Codes Corporation, Ann Arbor, MI).

**Immunogold labeling**

Human tracheal mucosal biopsies were obtained in the operating theater and immediately fixed with chilled buffer (50 mM sodium cacodylate [pH 7.4]) containing 2.5% glutaraldehyde and 2.0% paraformaldehyde and placed in 4°C overnight. The samples were then prepared as previously described27. Briefly, samples were blocked with 0.1% coldwater fish skin gelatin in 50 mM sodium cacodylate buffer and stained with rabbit polyclonal anti-Mtb antibodies (LS-C72966, LSBio, Inc.), followed by goat anti-rabbit IgG conjugated to 20 nm gold particles (Electron Microscopy Sciences). Samples were washed three times with phosphate buffered saline containing 0.1% Tween 20 (PBS-T) and analyzed with an FEI T-12 transmission electron microscope equipped with a side-mounted digital camera. A total of 30-35 individual cells in each group were imaged to analyze subcellular architecture and presence of bacteria.

**Elispot**

Preparation of PBMC and ESAT-6 peptides23, and Elispot™ assay were preformed as described previously28. The number of specific gamma interferon-secreting T cells was calculated by subtracting the mean negative-control value from the mean spot-forming-cell (SFC) count for duplicate wells inoculated with peptide. Negative controls always had <50 SFC per 10^6 input cells. A positive response was defined as a concentration of at least 50 SFC/10^6 PBMC that is at least three times higher than the background level. Research assistants were blind to the clinical diagnoses of the study participants throughout the analysis.

**Statistical Analysis**

Statistical significance was set at p value less than 0.05 and a mean difference equal to or greater than 2-fold change in expression levels. Normal distribution of the variables was tested using the Shapiro-Wilk test. Differences between x and y groups were determined using the Kruskal Wallis and Mann Whitney tests for normal and non-normal distributions, respectively. Data were expressed as median ± SD for non-normal distributed variables. All statistical analyses were performed with Prism version 6.0 software.
Results

Culture-Independent Profiling of Respiratory Microbial Flora

Given the role of γδ T cell IL-17A in host defense against pathogens at epithelial and mucosal barriers, we analyzed tissue specimens from iSGS patients for the presence of microbial species. All iSGS patients (10/10) demonstrated PCR positivity to Mycobacterium species (Fig. 1A), while only 2 of 10 iLTS patients were positive by PCR (p<0.001). In contrast, among iLTS patients, 10/10 showed PCR positivity to Acinetobacter baumannii (an established ICU pathogen\(^{29}\)), while only 1 of 10 iSGS patients showed a positive signal for this pathogen (p<0.001).

Further confirmatory testing was performed on an additional 10 iSGS, 10 iLTS, and 10 healthy controls with \textit{in situ} hybridization probing RNA expression of the specific mycobacterial virulence factor DNA gyrase subunit A (\textit{gryA})\(^{30}\). 7 of 10 iSGS specimens tested positive, predominantly in the tracheal epithelium, while only 1 of 10 iLTS specimens and 0 of 10 healthy control samples showing detectable signal (p=0.03) (Fig. 1B).

Localization of \textit{Mycobacterium} Species Within iSGS Tracheal Scar

To further investigate for \textit{Mycobacterium} species within tracheal scar tissue, we utilized immunogold labeling and high-resolution transmission electron microscopy. This analysis revealed multiple structures with associated labels that exhibit typical size (500 nm-2 μm) and shape (coccolid or bacillus) of \textit{Mycobacterium} species within the extracellular matrix (Fig. 1E-H), while controls using secondary antibody alone (Fig. 1C) or an unrelated antibody to \textit{Haemophilus influenzae} (Fig. 1D) were negative. Digital quantification of gold labels per bacterial cell by computerized algorithm confirmed visual analysis of anti-\textit{Mycobacterium tuberculosis} complex (\textit{MtbC}) antibody binding in iSGS tissues (p<0.005) (Fig. S2).

Systemic Immunologic Response to \textit{Mycobacterium} Antigens in iSGS

After detection of nucleic and amino acid signal for mycobacteria within iSGS, we sought to assess the systemic immunologic response in iSGS. Utilizing Elispot, we analyzed antigen specific responses of peripheral leukocytes from iSGS patients to the mycobacterial 6-kDa early secreted antigenic protein (ESAT-6). We chose ESAT-6 peptides due to prior reports of systemic cellular immune responses to these conserved \textit{MtbC} virulence factors in Sarcoidosis\(^{28,31}\). PBMC from iSGS patients showed a mean IFN-γ spot-forming-cell (SFC) count of 165.9 (SEM +/- 42.4) compared with 27.4 (SEM +/- 18.1) in normal controls (p<0.0076) (Fig. 1I.). This IFN-γ response suggests systemic immunologic memory to \textit{MtbC} exposure and is consistent with a pathological role for \textit{MtbC} in iSGS.

\textit{Mycobacterium} Species Subtyping via Sanger Sequencing

It was not possible to subtype the \textit{Mycobacterium} species based on the initial primers in our discovery assay, thus we utilized Sanger sequencing\(^{26}\) to further classify the \textit{Mycobacterium} species in a subset of samples based on the rpoB gene sequence. rpoB PCR yielded a product of 360 bp, which Sanger sequence analysis identified as \textit{Mycobacterium Tuberculosis} Complex (\textit{MtbC}) in 8 iSGS samples. 7 of the 8 positive iSGS specimens

\textit{Laryngoscope}. Author manuscript; available in PMC 2018 January 01.
showed consistent polymorphisms in the same locations (at 2312 & 2313 base pairs) (Fig. 2A). While the predicted protein coding sequence from iSGS specimens was homologous to Mtb reference sequences (Fig. 2B), phylogenetic analysis of the amino acid sequencing suggests that rather than Mycobacterium Tuberculosis (Mtb), a variant member of the MtbC, or a closely related novel mycobacterium (Fig. 2C), is present in iSGS specimens.

Discussion

We demonstrate through multiple distinct approaches the presence of Mycobacterium within tracheal scar of iSGS patients. Our prior findings suggest a major role for γδ T cells in the IL-17A dependent tissue inflammation and fibrotic remodeling seen in the airways of iSGS patients. Given the established role for γδ T cells in the early production of IL-17A in MtbC infection, and critical role for IL-17A in host clearance of pulmonary MtbC, our prior results are consistent with our current work demonstrating Mycobacterial species within the airway scar of iSGS patients.

An early report describing iSGS as a clinical entity could not isolate bacterial species in routine microbiologic culture. Similarly, all iSGS patients included in our study were culture negative. However, since this publication in 1993, culture independent techniques have become an established alternate methodology for identification of infectious agents. PCR was used to identify the etiologic agents of bacillary angiomatosis (Bartonella henselae) and Whipple's disease (Tropheryma whippelii).

The use of antigen-specific immune responses to microbial antigens has also been utilized to identify novel infectious agents, including Sin Nombre virus in hantavirus pulmonary syndrome, as well as a previously unknown coronavirus in severe acute respiratory syndrome, and Mycobacterium in sarcoidosis. PBMCs from iSGS patients stimulated ex-vivo with mycobacterial virulence factor ESAT-6 demonstrate a pronounced IFNγ response. This finding suggests that despite negative culture results from iSGS specimens, mycobacterial antigens induce T-cell-specific responses in the blood of iSGS patients at similar frequencies to those of tuberculosis subjects. The observation of a pronounced cellular immune response to Mycobacterium EAST-6 antigens in all 10 iSGS patients tested strongly supports the results from our molecular and protein analysis of iSGS scar.

The inability to identify mycobacterial microorganisms by routine histologic staining or to culture microorganisms from pathologic tissues provides caution to the establishment of a causative role for infectious agents in iSGS pathogenesis. However, based upon prior microbiological experience with fastidious mycobacteria, there are several explanations for the failure to detect microbial species in iSGS in the initial reports of the disease. The bacteria may be present in quantities below the detection of histologic staining. Alternatively, the agent may have an ultra-slow growth pattern that necessitates incubation periods much longer than the standard 6 weeks that cultures are held for isolation of Mycobacterium tuberculosis, similar to the time needed for isolation of M. ulcerans. Conversely, iSGS pathogenesis may reflect an immune response to infectious antigens, and
might not be dependent upon actively replicating organisms, similar to the hypersensitivity pneumonitis induced by *Mycobacterium avium*\(^3\).

An association between *Mycobacterium* and iSGS immunopathogenesis is supported by detection of mycobacterial proteins and nucleic acids in iSGS scar, as well as local and peripheral cellular immune responses to mycobacterial antigens in iSGS subjects. However, it remains unresolved if the identified mycobacterial constituents drive disease or whether inflammation per se creates a niche for the outgrowth of specific bacteria. It should be noted however, that tracheal stenosis arising after intubation (iLTS; which also possess an inflammatory tissue phenotype in the airway), appears in our cohort to have a much lower percentage of patients with detected *Mycobacterium*. Given the disease rarity, these results will require confirmation in larger cohorts pooled from multiple institutions.

The presence of *Mycobacterium* within iSGS scar is particularly striking in light of proven association of *Mycobacterium* with otherwise healthy, older white females (the “Lady Windermere” syndrome)\(^4\). The characteristics of these patients (women without immunocompromise or underlying chronic lung disease and proven pulmonary *Mycobacterium* infection) closely mirrors the iSGS population. Lady Windermere patients are predominantly Caucasian (86%) women (81%), presenting in their mid 60s. The dramatic demographic similarities of the 2 diseases (NTM pulmonary infection/Lady Windermere Syndrome & iSGS) offers clinical precedent for a pathogenic role for *Mycobacterium* in the development or progression of iSGS.

Although our results demonstrate *Mycobacterium* species within the tracheal scar of iSGS patients, the role of host genetics to iSGS pathogenesis has not yet been explored. Interestingly, strong alternate evidence links host genotype to mycobacterial susceptibility via the IL-23/IL-17A axis. Molecular analysis of patients suffering from Mendelian susceptibility to mycobacterial disease has implicated polymorphisms in both the ligand (IL-12B)\(^45,46\) and receptor (IL-12R\(\beta1\))\(^47,48\) responsible for IL-17A activation. Similarly, although iSGS affects women nearly exclusively, the influence of estrogen on disease initiation and recurrence is unknown. Estrogen has been shown to directly drive IL-23/IL-23R signaling and increase IL-17A production in severe asthma\(^49\). The role of estrogen in promoting Mycobacterial colonization/infection, or its role in accelerating the host response to pathogen are questions meriting future study.

**Conclusion**

Although iSGS has long been considered strictly an anatomic abnormality, requiring a surgical remedy, we offer the first evidence that the disease may represent a manifestation of altered local microbial flora coupled to a pathologic host inflammatory response. We demonstrate through multiple distinct approaches, a unique association of mycobacterial species and iSGS airway mucosa. Together with prior reports demonstrating significantly upregulated local IL-17A, evidence of *Mycobacterium* species within tracheal scar offers new avenues for therapeutic intervention in iSGS patients. Several established reagents are available to inhibit the IL-17A pathway\(^50,51\). Alternatively, multiple drugs are available targeting *Mycobacterium* species. Interestingly, limited cases series supports a clinical
benefit for one of these reagents in iSGS patients. The benefit of IL-17A inhibition in the absence of pathogen control is unclear, thus future clinical trials could test the clinical response of immunomodulation in combination with antibacterial therapy. Thus, the implications this work may extend beyond the confines of iSGS to other disease arising at the interface of pathogen and host inflammatory response.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This was a North American Airway Collaborative (NoAAC) Study.

Financial Disclosure/Funding: Research in NoAAC is made possible by infrastructure supported by the Patient-Centered Outcomes Research Institute under award number 1409-22214. We would also like to acknowledge the Vanderbilt the genomics core laboratory VANTAGE (Vanderbilt Technologies for Advanced Genomics) supported by an ARRA funded NIH award as well as the Translational Pathology Shared Resource supported by NCI/NIH Cancer Center Support Grant 2P30 CA068485-14. The content is solely the responsibility of the authors.

References


Laryngoscope. Author manuscript; available in PMC 2018 January 01.


Figure 1. Mycobacterium Species in iSGS Patients
qPCR results for panel of respiratory pathogens from 10 iSGS, and 10 iLTS patients. Yellow indicates positive PCR products, blue indicates negative result. 10/10 iSGS patients had detectable PCR products for Mycobacterium tuberculosis complex (MtbC), compared with 2/10 iLTS patients (Two-tailed, Chi-squared test with continuity correction; p<0.001) (A.).

Representative images from In situ hybridization for RNA of Mycobacterium gene product gyraseA (arrows depicting positive signal in iSGS specimen), accompanying summary graph depicting 7/10 iSGS patients with detectable in situ hybridization signal, compared with
1/10 iLTS, and 0/10 controls, (Two-tailed, Chi-squared test; asterisk denotes p<0.001) (B.). Immunogold labeling with an anti-MtbC antibody and high-resolution transmission electron microscopy analyses revealed multiple structures with associated labels that exhibit typical size (500 nm-2 μm) and shape (coccoid or bacillus) of *Mycobacterium* spp. Treatment with secondary antibody alone (not shown) or an unrelated antibody to *Haemophilus influenzae* (Fig. C) revealed sparse labeling that was significantly less than the labeling achieved with the anti-Mtb treatment (Fig. D, E). Distribution of IFN-γ production from ESAT-6 stimulated PBMC isolated from the peripheral blood of iSGS patients (red, n=10) or healthy controls (green, n=10). Bars represent the median (50th percentile), asterisk denotes significance (Two-tailed, Mann Whitney test; p<0.005) (F.).
Figure 2. Sanger Sequencing of Mycobacterium Species

Sanger sequencing of the *mycobacterium rpoB* gene in iSGS scar demonstrating 99% positional identity with *MtbC* in 8 of 20 iSGS samples. 7 of the 8 positive samples demonstrated 2 identical synomonous substitution at positions 2312 & 2313 (A.). Predicted identical rpoB amino acid sequence in MtbC and iSGS specimens (B.). Analysis of rpoB DNA sequences from 29 Mycobacterium species and from patients with iSGS showing distinct clustering of iSGS samples. Phylograms based on nucleotide alignments were generated with HKY85 distances matrices using Paup 4.0b10 (Sinauer Associates, Sunderland MA). Bootstrap values >50 (based on 500 replicates) are represented at each node, and the branch length index is represented below the phylogram (C.).