# BIOSIMULATION OF VOCAL FOLD INFLAMMATION AND WOUND HEALING

by

Nicole Yee-Key Li

Bachelor of Science (Speech & Hearing Sciences), The University of Hong Kong, 2000

Master of Philosophy (Voice Physiology), The University of Hong Kong, 2003

Submitted to the Graduate Faculty of

School of Health and Rehabilitation Sciences in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

University of Pittsburgh

2009

UNIVERSITY OF PITTSBURGH

SCHOOL OF HEALTH AND REHABILITATION SCIENCES

This thesis was presented

by

Nicole Yee-Key Li

It was defended on

March 30, 2009

and approved by

Katherine Verdolini, PhD, Professor

John Durrant, PhD, Professor

Patricia A. Hebda, PhD, Associate Professor

Susan Shaiman, PhD, Associate Professor

Yoram Vodovotz, PhD, Professor

Thesis Advisor: Katherine Verdolini, PhD, Professor

**BIOSIMULATION OF VOCAL FOLD INFLAMMATION AND WOUND HEALING**

Nicole Yee-Key Li, PhD

University of Pittsburgh, 2009

**BIOSIMULATION OF VOCAL FOLD INFLAMMATION AND WOUND HEALING**

Nicole Yee-Key Li, PhD

University of Pittsburgh, 2009

Personalized, pre-emptive and predictive medicine is the capstone of contemporary medical care. The central aim of this dissertation is to address clinical challenges in prescribing personalized therapy to patients with acute phonotrauma. Inflammation and healing, which are innate tissue responses to mechanical stress/ trauma, are regulated by a complex dynamic system. A systems biology approach, which combines empirical, mathematical and computational tools, was taken to study the biological complexity of this dynamic system in vocal fold injury.

Computational agent-based models (ABMs) were developed to quantitatively characterize multiple cellular and molecular interactions around inflammation and healing. The models allowed for tests of various hypothetical effects of motion-based treatments in individuals with acute phonotrauma. A phonotrauma ABM was calibrated and verified with empirical data of a panel of inflammatory mediators, obtained from laryngeal secretions in individuals following experimentally induced phonotrauma and a randomly assigned motion-based treatment. A supplementary ABM of surgically induced vocal fold trauma was developed and subsequently calibrated and verified with empirical data of inflammatory mediators and extracellular matrix substances from rat studies, for the purpose of gaining insight into the "net effect" of cellular and molecular responses at the tissue level.

ABM simulations reproduced and predicted trajectories of inflammatory mediators and extracellular matrix as seen in empirical data of phonotrauma and surgical vocal fold trauma.

The simulation results illustrated a spectrum of inflammatory responses to phonotrauma, surgical trauma and motion-based treatments. The results suggested that resonant voice exercise may optimize the combination of para- and anti-inflammatory responses to accelerate healing. Moreover, the ABMs suggested that hyaluronan fragments might be an early molecular index of tissue damage that is sensitive to varying stress levels – from relatively low phonatory stress to high surgical stress.

We propose that this translational application of biosimulation can be used to quantitatively chart individual healing trajectories, test the effects of different treatment options and most importantly provide new understanding of laryngeal health and healing. By placing biology on a firm mathematical foundation, this line of research has potential to influence the contour of scientific thinking and clinical care of vocal fold injury.

**TABLE OF CONTENTS**

## LIST OF TABLES

## LIST OF FIGURES

# PREFACE

- I would like to thank my advisor Katherine Verdolini for her unwavering support and patience to this fledgling scientist. Thank you for staying behind me, in front of me and beside me in this journey. I never thank you enough.
- I would like to thank Nathan Welham and Xinhong Lim for the animal data.
- I would like to thank Kevin Kim for statistical discussions.
- I would also like thank my thesis committee for many useful discussions.
- Finally, I would like to thank my family for keeping me in Pittsburgh.

## 1.0 STATEMENT OF THE PROBLEM, SIGNIFICANCE AND SPECIFIC AIMS

*Phono*trauma involves vocal fold injury associated with *phon*ation (i.e., voice production). When people speak or sing, the vocal folds oscillate at a relatively high frequency. Typically, the vocal folds make contact at some point during the vibratory cycle. The associated motions generate various types of biomechanical forces within the oscillating vocal fold tissues (Gunter, 2003, 2004; Jiang, Diaz, & Hanson, 1998; I.R. Titze, 1994). Although microinjury likely occurs within the tissue across the time course of any given day involving phonation, injury may or may not result in clinically appreciable entities, depending on the conditions of voice use as well as the host. Specifically, under conditions of typical voice use, the tissues are generally competent to withstand the biomechanical forces physiologically, probably with relatively minor and transient inflammatory responses locally. In such cases, the levels of tissue injury are sufficiently microscopic to be regenerated "intrinsically," without a full scale inflammatory or wound healing response. On the other hand, when phonation conditions are frankly phonotraumatic, the associated biomechanical forces are sufficiently large to cause substantial cell death and macroscopic tissue damage. If such conditions persist without adequate rest, the damaged tissue does not have time needed to repair (I. R. Titze, Svec, & Popolo, 2003). As a result, benign lesions at the mid-membranous vocal folds may develop, leading to dysphonia and voice-related changes in quality of life (Fulljames & Harris, 2006; B. H. Jacobson et al., 1997; Ma & Yiu,

2001; Rosen, Murry, Zinn, Zullo, & Sonbolian, 2000; E. Smith et al., 1996; Thomas, de Jong, Cremers, & Kooijman, 2006).

Therapeutic management of phonotrauma may involve medical or sometimes surgical intervention. However, behavioral voice therapy is generally considered central to any treatment program, and is usually preferred as the first-line approach (Boone & McFarlane, 1994; Colton & Casper, 1996; Johns, 2003). Unfortunately, strong scientific evidence is still lacking regarding the effectiveness and efficacy of voice therapy for phonotrauma.

*Evidence-based medicine* (EBM), which aims to illuminate the relative benefits of various approaches to treatment of health problems in general, has been endorsed enthusiastically in clinical practice and also within the research realm. EBM emphasizes the importance of basing clinical practice on the results of scientific inquiry. Relevant research findings are ideally derived from randomized controlled trials, which are regarded as the highest level of evidence (Sackett, Richardson, Rosenberg, & Haynes, 1997). Although outcome data from a handful of randomized clinical studies indicate voice therapy can produce some benefits for patients with phonotrauma (Bassiouny, 1998; Behrman, Rutledge, Hembree, & Sheridan, 2008; Gillivan-Murphy, Drinnan, O'Dwyer, Ridha, & Carding, 2006; MacKenzie, Millar, Wilson, Sellars, & Deary, 2001; Rattenbury, Carding, & Finn, 2004; Roy et al., 2001; Roy et al., 2003; Roy et al., 2002; Sellars, Carding, Deary, MacKenzie, & Wilson, 2002; Simberg, Sala, Tuomainen, Sellman, & Ronnemaa, 2006; Verdolini-Marston, Burke, Lessac, Glaze, & Caldwell, 1995), the typical mode of treatment remains reactive and fails to elucidate preferred treatment options for individual patients, potentially increasing the costs of treatment. In reality, patients do not seek medical attention until an acute phonotraumatic event has occurred or more commonly, a chronic condition requires them to do so. In fact, when patients come to seek

medical attention, phonotraumatic tissue changes are typically advanced in their development and may require phonosurgical excision in addition to behavioral treatment. Also, even if published data suggest that some individuals may be more genetically susceptible to vocal fold injury than others (Gray, Hirano, & Sato, 1993; Gray, Pignatari, & Harding, 1994), no predictive tools for phonotraumatic risk are available to permit early detection and prevention of phonotrauma. Most importantly, humans are known to differ remarkably along a variety of relevant parameters, such as genetic polymorphism. Thus, a "one-size-fits-all" approach to treatment is not necessarily the most rational one. The same treatment may help one patient and either harm or have no effect for another. Further, even if information were available about the particular dosage of a treatment that can heal a patient, increasing the treatment dosage does not necessarily increase the treatment benefit. Experimental data have already suggested that the benefits of behavioral voice exercises are patient-specific and dose-dependent, at least in the case of acute phonotrauma (Verdolini et al., in preparation). However, data from existing clinical studies are insufficient to inform us how to select treatments based on individual patient profiles. If clinicians could identify which patients are likely to benefit from which treatment, patients would be spared unnecessary and costly treatments and their potentially harmful side effects.

To move forward from the current state, the vision of voice care must be revolutionized towards seeking a predictive, pre-emptive and personalized approach to treatment and prevention of voice problems (Hood, Heath, Phelps, & Lin, 2004). Research within this domain would benefit from gearing towards developing clinical tools that can: (1) identify molecular signatures or biomarkers that are *predictive* of phonotrauma or treatment outcome; (2) characterize individual's probabilistic future health history or risk to *pre-empt* the development or exacerbation of phonotraumatic tissue changes; and (3) prescribe *personalized* treatment regimes

for individual patients with existing phonotraumatic tissue changes depending on their unique probabilistic future health histories.

We argue that this vision is beyond the capacity of EBM as it is generally conceived currently. First, the "evidence" collected under the rubric of EBM typically only informs us of an "average" value for outcomes in groups of patients, especially where randomized clinical studies are involved. However, we need outcomes that inform clinicians and patients about *individual* outcomes. In other words, even if we have evidence-based treatments, we still need to know *whom* those evidence-based treatments may benefit. Although more adequately powered, prospective, randomized controlled trials could arguably help to identify patient-specific treatments, the number of such trials would be prohibitively large to consider all biological, temporal and contextual factors that are relevant to the question. Moreover, typical approaches to statistical management of the data are generally inadequate to identify the roles of numerous variables for individual outcomes, especially given the likely interactive, non-linear nature of those variables. Second, and following from the foregoing point, randomized controlled trials are typically reductionistic in essence and only work to explain simple systems at best. However, phonotrauma, with its accompanying inflammation and healing, is a highly complex process induced by a variety of stimuli and modulated by numerous cells and their products. Reductionism is conceptually poorly equipped to appreciate non-linear dynamics and evolution in health and disease relevant to voice and other phenomena (De Simone, 2006a, 2006b; Jacobson, Edwards, Granier, & Butler, 1997; Jenicek, 2006; Little, 2003). We argue that a more systems-oriented tactic should be sought to decipher the biological complexity in health and disease, including disease affecting the voice.

A *systems biology* approach is emerging that may ultimately overwhelm typical evidence-based approaches to medicine. Modeling and simulation, which comprise a working methodology used within systems biology, have the capability to (1) handle a large number of system components and interactions within complex processes simultaneously; (2) quantify interrelationships (organization or structure) and interactions (dynamics or behavior) of genes, proteins and metabolites; and (3) integrate this information into visible working models that can provide predictive hypotheses to elucidate emergent phenomena behind complex processes (Hood, 2003; Hood et al., 2004; Vodovotz, 2006; Vodovotz, Clermont, Chow, & An, 2004; Vodovotz, Csete, Bartels, Chang, & An, 2008). With the integration of rapidly accumulating and high-throughput genetic and molecular data within the domain of voice science, systems biology has the capability to bridge a critical gap between benchside and bedside, in a novel way. We argue that systems biology with its predictive power can accelerate the advent of predictive, pre-emptive and personalized care of voice.

Work from our laboratory has made good early progress in translating a systems biology approach to the encoding of cellular and molecular responses to the case of phonotrauma, and in reproducing the tissue level effects of these elements *in silico*. Specifically, we have developed a series of systems-oriented biological models, including Agent-Based Models (ABMs) and Ordinary Differential Equations Models (ODMs) that predict expected time-varying pro-inflammatory and anti-inflammatory responses to physical insult to vocal fold tissue as a function of individual-specific inflammatory profiles (N. Y. Li et al., 2008; N. Y. K. Li et al., 2007; N. Y. K. Li, Verdolini, Clermont, Mi, Hebda et al., 2006; N. Y. K. Li, Verdolini, Clermont, Mi, Vodovotz et al., 2006; N.Y.K. Li, Verdolini, Qi, Vodovotz, & Hebda, 2005). We have calibrated the models using baseline and early follow-up inflammatory mediator data from

the laryngeal secretions of individuals subjected to experimentally induced acute phonotrauma and subsequent behavioral treatment modalities (voice rest, resonant voice exercise and spontaneous speech) (Verdolini et al., in preparation). *Molecular* outputs from these models have shown good correspondence with experimental human data for the same subjects at a 24-hr post-baseline time point. However, these models are currently limited in terms of (1) the number of inflammatory mediators and extracellular matrix substance represented and (2) the validation of the model's *tissue-level* outputs with experimental data. This thesis addressed the foregoing gaps by targeting the following aims.

The central aim of this thesis is to address clinical challenges in prescribing personalized therapy to patients with acute phonotrauma. A systems biology approach was applied to study the inflammatory and healing response to mechanical stress in vocal folds. Computational models, ABMs, were developed to produce elaborated route maps of cellular and molecular interactions around inflammation and healing, with the capability of allowing *in silico* (in computer) testing of various hypothetical effects of motion-based treatments in individuals following acute phonotrauma **(Main study: Specific Aim 1)**. A secondary aim of this thesis is to gain insight into the "net effect" of cellular and molecular responses at the tissue level in vocal fold inflammation and healing. For this purpose, we developed a supplementary ABM of surgically induced vocal fold trauma and attempted experimental cross-scale (molecule-tissue) model validation **(Supplementary study: Specific Aim 2)**. The relevant specific aims are:

**MAIN STUDY**

**Specific Aim 1:**

**(1a)** Expand the existing agent-based model (ABM) of phonotraumatic vocal fold inflammation and healing to compute temporal trajectories of (i) cytokines (interleukin [IL]-1β,

IL-6, IL-8, IL-10 and tumor necrosis factor [TNF]-α), (ii) a collagenase (matrix metalloproteinase [MMP]-8), (iii) growth factors (transforming growth factor [TGF]-β1 and basic fibroblast growth factor [bFGF]) and (iv) extracellular matrix (ECM) substances (collagen type I, elastin and hyaluronan [HA]) across time following injury.

**(1b)** Specify the model's parameters to reproduce individual-specific inflammatory and healing responses to acute phonotrauma and subsequent motion-based treatments, using cytokine and collagenase data empirically obtained from human laryngeal secretions at baseline, immediate post vocal loading and post a 4-hr treatment.

**(1c)** Assess the expanded model's prediction accuracy for cytokines and the collagenase at a 24-hr post baseline time point by comparing model outputs to the empirical data set.

**(1d)** Compare the adequacy of the original and expanded models, with specific reference to whether the elements added in the expanded model increase the model's prediction accuracy or not.

**SUPPLEMENTARY STUDY**

**Specific Aim 2:**

**(2a)** Specify the ABM's parameters (from **1a**) to reproduce the inflammatory and healing response to surgically induced vocal fold trauma, using published rat messenger ribonucleic acid (mRNA) data of (i) cytokines (IL-1β and TNF-α), (ii) a growth factor (TGF-β1) and (iii) matrix markers (procollagen subtype I, elastin synthase and hyaluronan synthase-2 (HAS-2)) at baseline, 1-hr, 4-hr, 8-hr, 16-hr and 24-hr post-injury time points.

**(2b)** Assess the models' prediction accuracy for the same set of markers **(2a)** at a 72-hr post-injury time point by comparing model outputs to the empirical data set.

**(2c)** Examine data generated from both models developed in this series **(1b and 2a)** to determine whether they point to new understanding in wound healing in general and phonotrauma, surgical trauma, and motion-based treatments in particular.

The thesis is organized as follows. Chapter 2 provides discussion of principles of evidence-based medicine and existing evidence around voice therapy for phonotrauma. Chapter 3 presents a discussion of the biological complexity involved in inflammation and healing along with the motivation to use a systems biology approach to unravel this complexity. Focus then turns to a promising computational modeling tool that has been used to study the dynamics of biological complexity in other tissues, namely, agent-based modeling (ABM). Chapter 4 presents our preliminary studies on the biosimulation of vocal fold inflammation and healing in acute phonotrauma, followed by discussion of limitations found in existing ABMs. Chapter 5 presents the methodologies used to develop ABMs to simulate the trajectories of inflammation and healing in acute phonotrauma and surgically induced vocal fold trauma. In Chapter 6, the ABM simulation results are presented. In Chapter 7, the significant findings are discussed as are the implications for understanding the complex system of inflammation and healing. The thesis concludes with discussion of both challenges and future directions in applying biosimulation in voice research. A comprehensive literature review relating to this thesis is provided in Appendix A – C for the readers' interest.

# 2.0 BACKGROUND

*Phono*trauma is a source of vocal fold injury due to *phon*ation. When people speak or sing, nearly continuous biomechanical stresses are exerted upon and within the vocal fold mucosal tissues. These phonation-related forces include tensile, contractile, aerodynamic, inertial, impact (collision) and shear stresses. Depending on the vocal style, phonation-related forces can induce physiological or pathological changes in the extracellular matrix of the vocal fold tissues (Gunter, 2003, 2004; J. J. Jiang, Diaz, & Hanson, 1998; I.R. Titze, 1994). The cells in the vocal fold tissues are responsible for maintaining the fine balance between the synthesis and degradation of extracellular matrix in response to the biomechanical environment. These load-sensitive cells continually produce mediators and extracellular matrices in an effort to maintain tissue homeostasis. Under typical vocal styles, the vocal fold cells and tissues are competent to adapt to the biomechanical forces by adjusting physiological parameters, probably with very minor and transient inflammatory response to repair the stressed or damaged tissues. In this case, the inherent mucosal repair process is effective to restore healthy tissue structure and function in a scar-free fashion, without inducing a full-scale inflammatory or wound healing process.

In contrast, when a vocal style is phonotraumatic, the biomechanical forces, especially impact and shear stresses, are abnormally high and can cause substantial cell death and macroscopic tissue damage. In that case, the sensitive balance between extracellular matrix synthesis and degradation are perturbed, and pathological states can result that impair the quality

of healing. If the phonotraumatic conditions persist without adequate rest, the stressed or damaged tissues do not have the time required to repair properly (Gray, 1991; Gray & Titze, 1988; Gray, Titze, & Lusk, 1987). Frank benign lesions, which are characterized by excessive and poorly organized matrices, may be deposited within the vocal folds, typically at the midpoint of the vibratory margin of the membranous folds. The deposition of phonotraumatic lesions destroys normal vocal fold tissue architecture and compromises proper vocal fold vibratory functions, leading to clinical dysphonia and related quality-of-life changes (B. H. Jacobson et al., 1997; Ma & Yiu, 2001; Raaijmakers, Dekker, & Dejonckere, 1998; E. Smith et al., 1996; Verdolini, Rosen, & Branski, 2006).

Alongside medical (pharmaceutical or surgical) treatments, behavioral voice therapy is also prescribed to treat phonotrauma and is typically the first-line treatment approach (Boone & McFarlane, 1994; Colton & Casper, 1996; Johns, 2003). Unfortunately, extremely few well-designed, prospective, controlled studies have been conducted on the treatment of phonotrauma to date. Therefore, investigations are still ongoing in search of scientific evidence of the benefits of voice therapy in treating phonotrauma. Studies using an evidence-based medicine model have become a recent focus of intervention reports in voice care.

## 2.1 EVIDENCE-BASED MEDICINE: TENETS AND LIMITATIONS

Evidence-based medicine (EBM) promotes the idea that clinical decision making should be based on rational analysis of evidence. Evidence in this case is derived from scientific research with the objective of determining “best practice.” Randomized controlled trials (RCTs) are

considered the “gold-standard” in research methodology in this regard. The five basic tenets of EBM have been suggested to be the following (Davidoff, Haynes, Sackett, & Smith, 1995):

1. Clinical decisions should be based on the best available scientific evidence.
2. The clinical problem determines the evidence to be sought.
3. Identifying the best evidence involves epidemiological and biostatistical thinking. Conclusions based on the available evidence are useful only if put into action for individual patients or for population health care decisions.
4. Clinical performance should be constantly evaluated.

EBM has been enthusiastically endorsed in medical practice and clinical research, and represents a clear advance over previous practice, which often failed to take into consideration evidence beyond personal observation (Bernstein, 2004; Cook & Levy, 1998; Davidoff et al., 1995; Dickenson & Vineis, 2002; Haynes, 2002; L. D. Jacobson et al., 1997; Sackett et al., 1997). The fundamental problem with the anecdotal approach is that it is drenched in idiosyncrasy and as such fails to elucidate *principles* of treatment that may be generalizable.

As such, EBM’s tenets are, in principle, generally sound. However, problems are encountered in the way that EBM is typically played out, and that is, through RCTs that are considered the “summit” of EBM research. Major criticisms of RCTs include but are not restricted to (1) their limited usefulness for individual patients, (2) their cost-ineffectiveness and (3) their characteristic reductionism (Cohen, Stavri, & Hersh, 2004; De Simone, 2006a, 2006b; Feinstein & Horwitz, 1997; L. D. Jacobson et al., 1997; Little, 2003; Miles, Bentley, Polychronis, Grey, & Price, 1998; Miles et al., 2000; Miles, Grey, Polychronis, Price, & Melchiorri, 2003, 2004; Miles, Polychronis, & Grey, 2006; Parker, 2001; Summerton, 1997).

Considerations around these notions are as follows. First, results from typical RCTs are limited by their direct applicability only to an “average” randomized patient who fulfilled the

criteria for enrollment in the trial. The methodology does not allow for predictions about treatment options for patients who do not fit the specific profiles of the patients who participated in the study. Moreover, in reality, every individual is unlike the "average" person and thus the results of RCTs often fail to predict an individual's response even if the individual is similar to study participants. Information about an individual's current clinical state and previous response to treatment are essential to adjust treatment dosages to optimize outcome for any given person. Unfortunately, results from RCTs typically do not have the capability to take this information into account.

Second, RCTs are characteristically expensive and time-intensive. Adequately powered prospective, randomized controlled trials may help to identify a few standard therapeutic interventions based on numerical evidence. However, impressively large clinical trials would be needed to establish optimized patient-specific treatment interventions that consider all relevant biological, temporal and contextual factors. Therefore, reliance on this purely empirical approach is actually not always realistic towards the end of gathering "the best available" evidence for personalized and predictive treatment.

Last, RCTs are overwhelmingly linked to a reductionist discipline, which takes complex phenomena and breaks them down into constituent parts in order to see how they work. Stated differently, RCTs and by extension EBM, as it is generally conceived, implies a simplistic and mechanistic world view in which cause and effect can be easily distinguished. Unfortunately, health and disease are complex in nature. Constituent parts of these phenomena work in an interconnected, interacting and interdependent fashion. Therefore, information about the relationship among parts may be lost in what is considered the highest level of evidence within EBM.

In contrast to this approach, some pockets of contemporary thinking are now turning towards a predictive, pre-emptive and personalized model of medical practice. General aims of the emerging approach are to (1) identify molecular signatures or biomarkers that are *predictive* of disease or treatment outcome; (2) characterize individual's probabilistic future health history for a range of diseases and design *pre-emptive* treatment program for the highly probable disease; and (3) prescribe *personalized* treatment regimes for individual patients depending on their unique probabilistic future health histories. A biomarker usually refers to a candidate molecule that contributes to a disease, i.e., vocal fold pathology herein. The definition of a biomarker can be read as a 'characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention' (Voit, 2009). Speculations have been made that predictive, preventive and personalized medicine may extend the normal life span by 10 to 30 years and improve quality of life (Hood, 2003; Hood et al., 2004). This vision is largely beyond the capacity of EBM as it is currently conceived and practiced. To address this problem, systems thinking, which conceptualizes the complex system as a whole, is needed to improve the current paradigm of clinical research. We now proceed to review existing evidence around behavioral treatments for phonotrauma and we then discuss the application of systems biology in clinical research.

## 2.2 CURRENT EVIDENCE OF BEHAVIOURAL INTERVENTIONS FOR PHONOTRAUMA

The primary goal of voice therapy is to restore the affected individual's vocal function by behavioral means that target an approach to voice production that generates the best possible

sounds and helps to reverse or prevent phonotraumatic injury and resultant voice disorders. Two primary orientations to voice therapy for phonotrauma include voice rest and voice production exercise. In many cases, these two approaches may be founded in opposite belief systems. Proponents of voice rest hold that elimination of voice use allows the tissue to heal and minimizes the aggravation or addition of trauma to vocal fold mucosal tissue. In contrast, proponents of voice production exercises hold that vocal training can improve coordination within and across voice production sub-systems (respiration, phonation and resonance), generally understood in some non-specific way as targeting "better" voice performance. Additionally, novel thinking is that vocal fold tissue mobilization involved in voice production exercise may have the ability to actively improve mucosal healing of the stressed or traumatized vocal folds (R. C. Branski et al., 2006; Verdolini et al., in preparation).

### 2.2.1 Evidence for voice rest

Published data on the efficacy of voice rest are limited and scattered across animal surgical trauma, human surgical trauma, vocal loading, and chronic phonotrauma studies (Behrman & Sulica, 2003; Cho, Kim, Lee, Kim, & Park, 2000; Hoover, Sataloff, Lyons, & Hawkshaw, 2001; Koufman & Blalock, 1989; van der Merwe, 2004; Vintturi et al., 2001). The primary goal of voice rest for phonotrauma is intuitive and sensible on the surface. If voice use is the cause of the problem (phonotrauma), removing the cause (voice use) should reverse the problem (phonotrauma). When it is used, absolute voice rest (abstinence from any phonation) is usually prescribed for periods ranging from a few days to a week in cases of acute vocal fold trauma (Emerich, Spiegel, & Sataloff, 2000; Hoover et al., 2001; R. T. Sataloff, 2001). Partial voice rest, i.e., reducing the amount of voice use, is usually recommended for conditions involving chronic

vocal pathology and vocal fatigue (Colton & Casper, 1996; R.T. Sataloff, 1997). The use of voice rest for the treatment of phonotrauma is largely anecdotal rather than evidence-based (Behrman & Sulica, 2003; Koufman & Blalock, 1989; R.T. Sataloff, 1997). Limited evidence suggests either confirmation or rejection of the idea that voice rest may benefit either surgical or phonotrauma (Behrman & Sulica, 2003; Hoover et al., 2001; Koufman & Blalock, 1989; R.T. Sataloff, 1997). The benefits of voice rest may partly depend on whether acute or chronic trauma is involved, and the severity of the condition.

### 2.2.2 Evidence for voice production work

Scientific evidence generally supports the idea that targeting voice production work (with or without a so-called indirect therapy or "vocal hygiene" component) can be effective for a spectrum of voice disorders (Bassiouny, 1998; S. H. Chen, Hsiao, Hsiao, Chung, & Chiang, 2006; Gillivan-Murphy et al., 2006; Kotby, El-Sady, Basiouny, Abou-Rass, & Hegazi, 1991; MacKenzie et al., 2001; Rattenbury et al., 2004; Roy et al., 2001; Roy et al., 2003; Sellars et al., 2002; Simberg et al., 2006; R. Speyer et al., 2002; R. Speyer, Wieneke, & Dejonckere, 2004a, 2004b; R. Speyer, Wieneke, van Wijck-Warnaar, & Dejonckere, 2003; Wingate, Brown, Shrivastav, Davenport, & Sapienza, 2006). Evidence has been clearest for some specific benign vocal fold pathologies in particular, namely, contact granuloma and nodules (Behrman et al., 2008; Holmberg, Hillman, Hammarberg, Sodersten, & Doyle, 2001; McCrory, 2001; Murry & Woodson, 1992; Verdolini-Marston et al., 1995; Ylitalo & Hammarberg, 2000).

Nine randomized controlled studies thus far have investigated the effectiveness of voice production work for phonotrauma (Bassiouny, 1998; Behrman et al., 2008; Gillivan-Murphy et al., 2006; MacKenzie et al., 2001; Roy et al., 2001; Roy et al., 2003; Sellars et al., 2002; Simberg

et al., 2006; Verdolini-Marston et al., 1995). These studies concurrently show that significant pre-post treatment improvements in psychosocial and physiological measures (quality-of-life, auditory perceptual voice quality, acoustic and aerodynamic) were found only in treatment groups focusing on voice production but not in control groups. For instance, Roy et al. (2001) conducted a randomized control study comparing results from instructions in "vocal function exercises" (Stemple, Lee, D'Amico, & Pickup, 1994) to those from vocal hygiene instructions and a no-treatment control condition. Subjects were 58 teachers with self-reported current and/ or previous voice problems. The vocal function exercise treatment group showed improvement in both *Voice Handicap Index* scores and self-reported voice severity ratings, whereas the vocal hygiene group and the control group did not have significant improvements in either of these measures. Three other studies pointed to benefits from resonant voice therapy over vocal hygiene training. Verdolini et al. (1995) compared results from resonant voice therapy (N=3) to those from confidential voice therapy (N=5) and those from a voice hygiene control group (N=5). Results showed that the resonant voice therapy and confidential voice therapy groups had a greater probability of improvement in voice quality, laryngeal appearance and phonatory effort as compared to the vocal hygiene group. Similarly, Roy et al. (2001) conducted a study comparing the results from resonant voice therapy to those from respiratory muscle training and amplification (a specific vocal hygiene technique) in 64 teachers with self-reported current and/ or previous voice problems. Results showed that both resonant voice therapy and amplification groups had significant improvements in *Voice Handicap Index* (VHI) scores and self-reported voice severity ratings after training. In contrast, these gains were not found for the respiratory muscle training group. Recently, Behrman et al. (2003) conducted a study comparing the results from resonant voice therapy (N=31) to those from a hygiene control group (N=31) in females

with phonotrauma. Results showed that only resonant voice therapy had significant improvements in VHI scores after training.

Compared to the aforementioned benefits of voice production work on psychosocial and physiological aspects of vocal function, the benefits of voice production work on improving laryngeal appearance is less obvious. The ideal outcome of voice therapy would, of course, involve the regeneration of damaged vocal folds. However, complete resolution of phonotraumatic lesions is rarely reported following voice therapy targeting voice production, in carefully documented reports. In fact, only four randomized controlled of which we are aware, group studies thus far, even include laryngeal appearance as an outcome measure (Bassiouny, 1998; MacKenzie et al., 2001; Simberg et al., 2006; Verdolini-Marston et al., 1995). Reduction in phonotraumatic lesion size or overall severity after voice production work is reported in two out of the four studies (Bassiouny, 1998; Verdolini-Marston et al., 1995). However, only one of the studies documents complete resolution of phonotraumatic lesions in four of nine subjects (MacKenzie et al., 2001). Besides these randomized controlled studies, data from one within-subjects study in which all subjects received different treatment *foci*, randomly ordered during treatment, suggested that significant improvements were seen in size and surrounding edema of the vocal folds in nine of eleven subjects with bilateral vocal nodules after voice therapy targeting voice work (one subject had incomplete laryngeal images and one subject did not complete the therapy) (Holmberg et al., 2001)  In sum, the data around the benefits of voice production work for phonotrauma are sparse. Currently available data do not allow for confident support or refutation of potential regenerative benefits of voice production exercise for phonotrauma. The results are encouraging at psychosocial and physiological levels. However, they seem to indicate benefits of voice production work seem tilted to more compensatory rather

than wholly regenerative effects, based on psychosocial and physiological versus lesion data respectively.

Having said as much, considering the data that do exist as well as logic, first, the benefits of voice production exercises may depend on the chronicity of the lesion. Unfortunately, information about chronicity is commonly lacking in published studies. In fact, an obstacle to such reporting is that no clinical assessment tools are available to determine the "age" of a phonotraumatic lesion, although it may be grossly estimated based on subject reports of symptom onset. Second, vocal fold status is assessed variably in the literature. Different imaging techniques (rigid versus flexible endoscopy with or without stroboscopy) and rating scales are used across the studies. A specific imaging technique and scaling method may have effects on the judgment of vocal fold appearance but no studies have yet systematically tested these potential effects. Thus, the diverse imaging techniques and rating scales make data comparison difficult across studies. Third, data around the inter- and intra-judge reliability of vocal fold appearance ratings—which are indeed perceptual—are not commonly reported. Therefore, the quality of data associated with rating vocal fold status is in question. Last, only two studies on voice production work of which we are aware have reported results from delayed follow-up after the completion of treatment (Simberg, 2004; Verdolini-Marston et al., 1995). Morphological changes in phonotraumatic lesions may take time to be visually identified. To conclude, evidence exists to support the idea that some types of voice exercises may be effective for improving psychosocial and physiological functions in individuals with phonotrauma. However, current literature is insufficient to elucidate the direct effects of voice production exercises on tissue healing for phonotrauma, specifically. Targeted experimental study of this issue is warranted.

### 2.2.3 Evidence from *in vitro* and *in vivo* experiments

At the time of this writing, no experimental studies using biological data have been completed on the effects of tissue mobilization for chronic phonotrauma. Only two experimental studies to date examined the effects of tissue mobilization for acute vocal fold inflammation (R. C. Branski et al., 2006; Verdolini et al., in preparation). The first study used an *in vitro* model to evaluate the effects of cyclic equibiaxial tensile strain (CTS) on rabbit vocal fold fibroblast cultures in the presence or absence of interleukin (IL)-1β-induced (IL-1β) inflammation (R. C. Branski et al., 2006). IL-1β is a key pro-inflammatory cytokine and induces numerous pro-inflammatory mediators, such as inducible-nitric oxide synthase (iNOS), nitric oxide (NO), cyclooxygenase-2 (COX-2), prostaglandin E2 (PGE2) and matrix metalloproteases (MMPs). Excessive synthesis of pro-inflammatory markers generally leads to unfavorable healing outcomes (Dinarello, 1997, 2000; Guirao & Lowry, 1996). In this *in vitro* study, CTS was manipulated in the time domain (4 -36 continuous hrs), in the magnitude domain (0 - 18%) and in the frequency domain (static – 0.5 Hz) in cell cultures in the presence or absence of IL-1β. Results showed that when the rabbit vocal fold fibroblast cultures were inflamed with IL-1β and stimulated with CTS for four hours, iNOS mRNA expression was reduced most under a specific CTS regimen: low magnitude (6%) and relatively high frequency (0.5 Hz). This CTS regimen was then used in two subsequent experiments. The first experiment tested the effects of CTS on the synthesis of COX-2, MMP-1, $PGE_2$ and NO in the fibroblast cultures. Results showed that low magnitude CTS blocked COX-2, MMP-1 and $PGE_2$ synthesis up to 24 hours and NO up to 36 hours in the IL-1β-induced inflamed cultures, compared to the absence of CTS condition. The second experiment tested the effects of CTS on the synthesis of matrix proteins, namely, procollagen type I. IL-1β, which is known to inhibit the synthesis of procollagen type I, as for example, in dermal fibroblasts

(Mauviel et al., 1991), lung fibroblasts (Diaz, Munoz, Johnston, Korn, & Jimenez, 1993) and osteoblastic cells (Harrison, Vargas, Petersen, Lorenzo, & Kream, 1990). Counter-intuitively, the results from these two experiments showed that CTS up-regulated procollagen type I synthesis after 24 or 48 hours of CTS exposure in the IL-1β-induced inflamed vocal-fold fibroblast cultures.

The second study investigated the biological effects of voice rest versus two different forms of tissue mobilization (resonant voice exercises and spontaneous speech) for experimentally-induced acute vocal fold inflammation in human subjects (Verdolini et al., in preparation). The main obstacle for this line of research, initially, was the lack of a technology to quantify vocal fold inflammatory in human subjects. Recently, a laboratory at the University of Pittsburgh developed an approach that addresses the relevant technological gap. That approach indicated that vocal fold inflammatory and healing state could be assessed by biochemically assaying vocal fold surface secretions (Branski, Rosen, Hebda, & Verdolini, 2003; Branski, Rosen, Verdolini, & Hebda, 2005b; Branski, Verdolini, Rosen, & Hebda, 2004; Verdolini et al., in preparation; Verdolini, Rosen, Branski, & Hebda, 2003). A concern can be voiced about the extent to which information in laryngeal secretions reflects information within the tissue. The concern is a valid one, which we are pursuing empirically in our laboratories. Preliminary data indicate a correspondence between inflammatory mediator concentrations in secretions and those in tissue in the rabbit subglottis, albeit with relatively small lag in time and magnitude (Sandulache et al., 2007). Other human surgical data are currently under analysis (Rosen, Li, Hebda, & Verdolini, in preparation). Pending the outcome of those analyses, existing evidence increases confidence in the ability of mediator concentrations in laryngeal secretions to reasonably quantitatively reflect concentrations in tissue. At that level, a methodology has

become available to pursue the novel hypothesis that certain forms of tissue mobilization may have value for the modulation of inflammatory and healing states in human vocal folds.

Voice rest, resonant voice exercises and spontaneous speech can be considered on a continuum of tissue mobilization and especially vocal fold impact stress magnitude: none (voice rest), normal- to large-amplitude vocal fold oscillations and low impact stress (resonant voice exercises) and normal-to large-amplitude oscillations but potentially larger impact stress, depending on the speaker (spontaneous speech). Specifically, compared to pressed and breathy phonation modes, resonant voice involves comparatively large-amplitude but low-impact vocal fold vibrations (Berry et al., 2001; Peterson, Verdolini-Marston, Barkmeir, & Hoffman, 1994; Verdolini, 2000; Verdolini, Druker, Palmer, & Samawi, 1998). The vocal fold vibratory characteristics associated with spontaneous speech vary, but are thought to tend towards pressed speech in many speakers. In Verdolini et al.'s study, nine vocally health human participants were subjected to a vocal loading task involving 45 minutes of loud voice phonation (75-90 dB SPL at 15 cm microphone-to-mouth distance) during a 1-hr period. Then, the participants were randomly assigned to one of three treatment groups: voice rest, resonant voice exercises or spontaneous speech as monitored for 4 hours in the clinic. The resonant voice condition involved cycles of 4 minutes of resonant voice exercises followed by 16 minutes of voice rest, whereas the spontaneous speech condition involved 16 minutes of conversational speech followed by 4 minutes of silence. After a 4-hour treatment period, participants were discharged to home with instructions to continue to follow their corresponding treatment condition (with slight temporal modifications for the resonant voice and spontaneous speech groups). The next morning, participants were required to observe complete voice rest between awakening and their arrival at the clinic in the morning. Laryngeal secretions were sampled from the vocal fold surfaces at the

following time points: pre-loading (baseline), immediately post-loading, 4 hr post-treatment onset, and 24 hr post baseline. Enzyme-Linked ImmunoSorbent Assays (ELISA) were then run to measure the concentrations of a panel of biomarkers in the secretions.

Biomarkers concentration results showed high subject variability. Most importantly, the secretion data suggested that some of the participants had "pre-inflamed" vocal folds that *post hoc* violated the inclusion criteria of the study (denial or evidence of a current voice problem). Also, some subjects did not respond to the vocal loading task and failed to demonstrate an inflammatory response after loading. As a result, data from only one subject from each treatment condition could be used for further analysis in the most rigorous sense. For those subjects, results showed that the concentrations of pro-inflammatory mediators (IL-1β, IL-6 and MMP-8) were lowest following resonant voice exercises and highest following the spontaneous speech condition at the 24-hour post baseline time point. At the same time, the concentration of the anti-inflammatory marker IL-10 showed something of an opposite trend at the 24-hour time point, i.e., concentrations for this marker were highest following resonant voice exercises and lowest following voice rest. These preliminary findings suggest that large-amplitude, low-impact vocal fold tissue mobilization, as reported for resonant voice exercises, may relatively optimize the quality of the healing response for acute phonotrauma by attenuating pro-inflammatory and stimulating anti-inflammatory response responses. Although at first glance it may appear that a confound in the data involved the differing durations of voice use across resonant voice and spontaneous speech conditions (4 versus 16 minutes in every 20-minute period for 4 hr), a further factor limits concern in this regard. If treatment benefits were inversely attributable to amount of voice use alone, the voice rest condition would have produced the best results, followed by resonant voice and then spontaneous speech. However, results were not graded in

this fashion. Rather, resonant voice generated benefits that were even greater than those for voice rest, suggesting there may be something therapeutic about this particular form of voice production.

Initial results from both human and *in vitro* data in our laboratory provide optimism that some – but not all forms of tissue mobilization may indeed enhance both the speed and final level of recovery from phonotraumatic inflammation, at least in the short term. However, further data are needed. With unlimited resources, the issues could be pursued with additional human clinical trials. However, such clinical trials are invasive and the dollars to subject ratio is prohibitive for the large subject cohort that would be needed. An appealing alternative is systems-oriented modeling-simulation of complex biological systems, which we will pursue within the current study.

# 3.0 DECIPHERING THE COMPLEXITY OF INFLAMMATORY AND HEALING IN PHONOTRAUMA: FROM EVIDENCE-BASED TO SYSTEMS BIOLOGY

Personalized, predictive and pre-emptive medicine is a central goal of modern medicine (Zerbouni, 2008). To approach this goal, the complexity of biological processes must be tamed. Even as demand for evidence-based medicine reaches a crescendo, this purely empirical approach is deemed to be unattractive due to the aforementioned concerns of its (1) limited applicability of research findings derived from randomized controlled trials, (2) cost-ineffectiveness and (3) typical reductionism. Although evidence-based medicine is only now in its infancy in the field of speech-language pathology, we propose that the paradigm of voice care may benefit from a shift in vision of what constitutes "good evidence".

Systems-oriented modeling-simulation of complex systems is a possible avenue to pursue in this light. Such modeling has recently shown promise as an approach by which to tame the seemingly unpredictable behavior of complex biological phenomena and account for the plethora of known and unknown interactions among biologic pathways (Kitano, 2002a, 2002b; Vodovotz, 2006). A modeling approach can yield testable predictions and establish new paradigms. Moreover, a systems biology approach that involves mathematical modeling may prove useful in settings such as phonotrauma, in which it is difficult to obtain statistically sufficient sample sizes for the types of questions asked (Whitcomb, Aoun, Vodovotz, Clermont, & Barmada, 2005). Investigators within our research group have recently coined the term "translational systems

biology" to refer to the process of creating, calibrating, and validating computational simulations in settings of complex diseases, simulations that are designed *a priori* for the purpose of modifying clinical treatment, carrying out *in silico* clinical trials, generating novel therapies, and refining diagnosis (Vodovotz et al., 2008). Those investigators have pioneered the use of translational systems biology in the settings of sepsis and trauma (An, Hunt, Clermont, Neugebauer, & Vodovotz, 2007), and now we extend this approach to phonotrauma. A detailed background of the application of computational modeling for clinical research and different approaches in simulation modeling is in Appendix C. This chapter is focused on the discussion around biological complexity and the use of biosimulation to unravel the complexity underlying inflammation and healing.

### 3.1.1 Biological complexity in inflammation and healing

Vocal fold biomechanical stresses generated during phonation are widely acknowledged as the primary cause of phonotrauma (Gray, 1997; Gray & Titze, 1988; Gray, Titze, Alipour, & Hammond, 2000; Gray et al., 1987; Hunter, Titze, & Alipour, 2004; J. J. Jiang et al., 1998). Phonotrauma, like all other forms of trauma, is a highly complex process induced by a variety of stimuli, modulated by numerous cells and their molecular products, and affecting different tissues in diverse ways. At the heart of the response to phonotrauma is the intertwined process of inflammation and wound healing (Calvin, 1998; Clark, 1988; R. A. F. Clark, 1998; R.A.F. Clark, 1998; Gillitzer & Goebeler, 2001; Mast & Schultz, 1996; Werner & Grose, 2003).

Inflammation is a complex, dynamic and multi-scale biologic process that distinguishes itself by definition from linearity and reductionism (Vodovotz et al., 2008). Inflammation is the earliest and necessary response for subsequent wound healing (Hardy, 1989). Inflammation can

be understood in terms of an information system that processes and controls the biochemical signals induced by injury and/ or infection. These signals include: (1) “Go” signals to initiate the inflammatory reactions, (2) “Stop” signals to temper the inflammation, and (3) “Switch” signals to convert a hostile damaging tissue response mode to a synthetic healing mode (Nathan, 2002). Also, these signals are actively monitored and regulated by a number of checkpoints within the system. If any of the aforementioned signals is missing or if the checkpoints malfunctions, the normal inflammatory process is disturbed possibly resulting in persistent inflammation, abnormal healing and tissue distortion. A prompt transition from the inflammatory phase to the healing phase is a key determinant of good wound healing, which involves the replacement of traumatized tissue by living tissue (Walter & Israel, 1987).

Two possible healing processes can occur, namely, *reparative regeneration* and *constructive repair*. Reparative regeneration is *scarless* wound healing, in which traumatized tissue is completely restored in normal architecture and function, by proliferation of surrounding undamaged specialized cells and replacement of specialized structures. *Reparative regeneration* usually occurs when (1) the damaging stimuli are cleared quickly, (2) no necrosis (death of cells and tissues) occurs, (3) there is minimal inflammatory cell infiltrate, and (3) the tissue framework has remained intact. On the other hand, *constructive repair* results from injuries involving necrosis and a destroyed tissue framework. The lost tissue is replaced by granulation tissue which matures into a scar. Depending on the amount of scarring, scarred tissue does not usually restore the architecture and perform the normal function of the original tissue.

Typically, the vocal folds are capable of withstanding phonatory stresses and have the reparative capability to resolve microscopically phonotraumatic damage incurred during daily phonation. Biomechanical loadings have been suggested to play roles in regulating cellular

functions, including gene expression, protein synthesis, cell growth and differentiation (J. H. Wang & Thampatty, 2006). Especially in load-sensitive cells, such as fibroblasts, mechanical forces are important in regulating the balance of cellular synthesis and degradation of matrix components for tissue homeostasis. However, when mechanical forces are excessive, the equilibrium may tilt from cellular anabolism to catabolism. Also, abnormal mechanical forces would cause macroscopic cell death and tissue damage. In that case, inflammation becomes clinically evident and constructive repair is involved. Consequently, tissue pathophysiological conditions result, as seen in benign vocal fold lesions with accompanying prolonged dysfunction in vocal fold vibratory functions (Catten, Gray, Hammond, Zhou, & Hammond, 1998; Gray, 2000).

Contemporary therapeutic interventions in phonotrauma are oriented towards modulating the inflammatory and healing processes to promote reparative healing of the traumatized vocal folds. A plausible approach is to both blunt the inflammatory response and activate the healing program. Upon mechanical challenges, the acute inflammatory cascade is immediately activated in damaged or stressed (exercised) tissues (Butterfield, Best, & Merrick, 2006; Toumi, F'Guyer, & Best, 2006). The inflammatory cells infiltrate into the area of injury to remove damaged and dead cells and tissue debris. This inflammatory reaction brings out additional tissue damage and cell death, which exacerbates the initial tissue damage and amplifies the signals for scarring. Theoretically, inflammation-blocking interventions may reduce the “secondary” tissue damage and the possibility for fibrosis and scarring. At the same time, this approach may potentially reduce the supply of growth factors and cytokines from the inflammatory cells to facilitate tissue repair. Thus, a therapeutic balance between the need to limit inflammation from causing tissue damage and the need for inflammation to initiate tissue repair is important to optimize the quality

of healing outcomes and the recovery of physiological functions (Butterfield et al., 2006; Eming, Krieg, & Davidson, 2007; Stramer, Mori, & Martin, 2007; Tidball, 1995; Toumi et al., 2006). In the literature related to sports injury, evidence suggests that modified mobilization exercise may blunt the cells' pro-inflammatory responses but enhance their anti-inflammatory responses, depending on the time and dose of the exercise (Agarwal et al., 2004; Keylock et al., 2008; J. M. Peake et al., 2005; Petersen & Pedersen, 2005; J. A. Smith & Pyne, 1997).

This assertion provides a good testing platform to uncover the potential therapeutic effects of vocal exercises in treating phonotrauma. The relevant research program starts with studying the dynamics of inflammation and healing at cellular and molecular levels and the responses of these agents to various biomechanical loading conditions, i.e. loading magnitude, and frequency and duration of various types of voice production. Also, the interaction between mechanical forces and soluble factors, including growth factors and cytokines should be investigated to discern how changes in mechanical forces are associated with the development and remodeling of vocal fold tissues as well as the functional disorders that ensue from them. To date, vocal fold inflammatory and healing responses have mainly been studied in animal models of vocal fold surgical trauma (Hansen & Thibeault, 2006; Lim, Tateya, Tateya, Munoz-Del-Rio, & Bless, 2006; I. Tateya, Tateya, Lim, Sohn, & Bless, 2006; T. Tateya, Tateya, Sohn, & Bless, 2006; S.L. Thibeault, Gray, Li et al., 2002; S. L. Thibeault, Rousseau, Welham, Hirano, & Bless, 2004; Welham, Lim, Tateya, & Bless, 2008). However, specific information about how multiple cells and molecules respond to phonotrauma and more clinically relevant – how inflammation can be systematically controlled in phonotrauma by voice production exercises – is not clear.

As discussed previously, two studies have been carried out to investigate how tissue mobilization involved in voice production exercise may modify inflammation following acute

phonotrauma. The recent *in vitro* and human data derived from concentrations of inflammatory mediators in vocal fold surface secretions suggest that some forms of vocal fold tissue mobilization may help to control inflammation and promote healing following acute phonotrauma (R.C. Branski, 2005; Verdolini et al., in preparation). Equally important, both *in vitro* and human data suggest that the benefits of tissue mobilization for acute vocal fold inflammation are dose-dependent and subject-specific (R.C. Branski, 2005; Verdolini et al., in preparation). These observations suggest a commonality across tissue types in the response to injury, given that active rehabilitation is now used to treat many types of injuries (Burroughs & Dahners, 1990; Eiff, Smith, & Smith, 1994; Kerkhoffs et al., 2002; Kerkhoffs et al., 2003; Kvist, 2004; Mulligan, 1995; Paungmali, O'Leary, Sowvlis, & Vicenzino, 2003; Pijnenburg, Van Dijk, Bossuyt, & Marti, 2000; Salter, 1994, 1996; Thornton, Shrive, & Frank, 2003; Threlkeld, 1992; Visser et al., 1998; Williams, Moran, Thonar, & Salter, 1994). However, details are lacking about mobilization doses that may optimize healing in the vocal folds, and how optimized doses may interact with the specific initial inflammatory status of the tissue.

One might argue that in order to identify vocal fold mobilization doses that can optimize tissue healing post-traumatically, randomized clinical trials would be the most straightforward and sufficient approach. That is, one can experimentally correlate the change of inflammatory mediator concentrations with a variety of vocal dose intensities across a large subject cohort, and then prescribe the dose that has the highest effectiveness. However, as already indicated, the problem with this approach is that the time-evolution of inflammation depends on the combination of initial conditions and the specific vocal dose used in mobilization. Also, the cumbersome nature of data collection complicates the potential for biologically oriented clinical trials on the value of therapeutic interventions for phonotrauma in humans. To overcome the

prohibitively large number of experiments needed to characterize the multitude of possible combinations, we propose to adopt a systems biology approach to elucidate apparently contradictory and unpredictable behavior emerging from the plethora of interactions among biologic pathways involved in the acute inflammatory response (Chow et al., 2005; Day et al., 2006; Prince et al., 2006).

### 3.1.2 Promise of systems biology in deciphering complexity in inflammation and healing

Physiologic systems, whether in their healthy or pathologic states, exhibit a remarkable variability in structural patterns and temporal behaviors (Bassingthwaighte, Liebovitch, & West, 1994; Goldberger, 1996, 2006; Goldberger et al., 2002; Goldberger, Bhargava, West, & Mandell, 1986; Goldberger & West, 1987; Goldenfeld & Kadanoff, 1999). Classical concepts of homeostasis, linear constructs and reductionist methodologies are inadequate to understand such physiological complexity (Goldberger, 2006). Specifically, the reductionist approach fails to illuminate the behavior of whole systems in which system behavior is different from the simple sum of reactions in isolated system parts. The understanding of such complex processes requires an understanding of inter-relationships and non-linear patterns of interactions among component parts over time.

Systems biology is an emerging approach that addresses this issue by studying the behavior of biological systems as unities (K. D. Smith & Bolouri, 2005). Systems biology is used to (1) handle a large number of system components and interactions within complex processes; (2) quantify interrelationships (organization or structure) and interactions (dynamics or behavior) of genes, proteins and metabolites; and (3) integrate this information into visible working models that can provide predictive hypotheses to elucidate emergent phenomena behind complex

processes (Hood et al., 2004; Kitano, 2002a, 2002b; Wolkenhauer, 2001). Mathematical and computational modeling-simulation are the essential tools for system analysis in clinical research, especially in knowledge discovery (data-mining) and simulation-based analysis (Kitano, 2002a, 2002b). Modeling's role is to transfer real-world system components and interactions into an abstracted representation. For knowledge discovery, model abstraction is useful to conceptualize the "hidden order/pattern" behind the observed phenomena (e.g., inflammatory response) from huge volumes of experimental data (e.g., genomic and proteomic data). For instance, knowledge discovery has been extensively applied to predict protein structure from genetic sequence (Baldi & Brunak, 2001).

On the other hand, simulation-based analysis is used to test hypotheses with computer (*in silico*) experiments and then to provide predictions to be tested by *in vivo/in vitro* experiments (Kitano, 2002a, 2002b). In clinical research, simulation-based analysis can sharpen our understanding and intuition of a particular (patho-) physiological process of interest. Simulations from computer-executable models predict a range of scenarios relevant to the properties of systems. For instance, systems-level modeling can be used to simulate and predict the responses of a whole inflammatory/wound healing system, rather than the response of particular inflammatory mediators alone. Through modeling and simulation, the dynamic outcome of interactions and feedback loops among the component system parts at multi-scale levels (signal pathway, molecules, cell, organ, and organism) can be illustrated effectively (An, 2004, 2005; Kumar, Clermont, Vodovotz, & Chow, 2004; Vodovotz et al., 2004).

The application of systems-oriented modeling-simulation to clinical practice is acknowledged in drug discovery and gene therapy to pursue the goal of predictive, preventive and personalized clinical care (Hood, 2003; Hood et al., 2004; Kitano, 2002a, 2002b). Also,

system-level analysis has begun to be applied to study pathogenesis in various diseases such as tumor growth, malaria, and systemic inflammatory response syndrome (An, 2004, 2005; Kumar et al., 2004; Vodovotz et al., 2004; Vodovotz et al., 2008). For instance, in systemic inflammatory responses, the interaction between pro-inflammatory (e.g., tumor necrosis factor (TNF)-α) and anti-inflammatory (e.g., interleukin (IL)-10) mediators has been shown to be time- and context-dependent in the establishment of a long-term equilibrium state of a system (An, 2005; Smallwood, Holcombe, & Walker, 2004; Vodovotz et al., 2004; Walker, Hill, Wood, Smallwood, & Southgate, 2004). This equilibrium state is achieved by multiple positive and negative feedback loops among the inflammatory mediators. System-level analysis has successfully shown that the pathologic state of the inflammatory response (e.g., sepsis) results from a disruption of the interaction among the aforementioned mediators, rather than the deficiency of any individual mediator.

In summary, the robust systems-approach with its predictive power can overcome the problems of carrying out prohibitively large number of experiments needed to (1) collect comprehensive data in considering all known relevant temporal and contextual factors and (2) extract and integrate useful information and patterns from huge quantities of data. By direct modeling/ simulating of a complex system, one can observe, manipulate and understand the behavior of the whole system in an efficient way. As such, systems-oriented modeling-simulation improves both biological sight and insight into mechanisms within complex systems (Goldenfeld & Kadanoff, 1999; Kitano, 2002a, 2002b; Vicsek, 2002).

### 3.1.3 Agent-based modeling in inflammation and healing

The systems-level modeling of interest for the present study involves Agent-Based Models (ABMs), which are a relatively new approach for system modeling and simulation to study the macroscopic world through defining a system at a microscopic level. Agent-based modeling is stochastic modeling that simulates the behavior of real-world systems under random conditions (Gilbert & Bankes, 2002). In such models, individual components of a given complex system interact based on rules whose outcomes are partially based on stochastic processes (Ermentrout & Edelstein-Keshet, 1993). More specifically, ABM involves discrete event simulation to study the behavior of complex systems. "Agents" in ABM represent the component parts of the system that contribute to the system's behavior. The rules can involve mathematical equations or "If…Then" conditional statements. On the basis of these rules, a simulated environment is created to allow agents to respond and interact, and to allow for quantitative outputs of the simulation. The relative importance of various rules is dictated by model parameters.

The ABM is particularly suitable for modeling complex adaptive systems, e.g., biological systems (Holland, 1992, 1995; Mitchell, 2003). Complex adaptive systems are composed of diverse entities that interact nonlinearly and dynamically. These systems display self-organization and adaptation to produce emergent structures and behaviors, which cannot be easily predicted (Cilliers, 2005). In biology, the interactions are often context-dependent and time-dependent, which makes biological systems adaptive. Also, biological systems are complex in terms of their structures, functional processes and evolutionary processes (Mitchell, 2003). Agent-based modeling provides a robust and flexible framework to handle these three complexities.

ABMs have been used to simulate the temporal evolution of complex systems and to encode complicated time-dependent cellular and molecular events that occur during inflammation and wound healing (An, 2001; Mi, Riviere, Clermont, Steed, & Vodovotz, 2007; Smallwood, Holcombe, & Walker, 2004; Vodovotz et al., 2004; Walker, Hill, Wood, Smallwood, & Southgate, 2004). We previously developed a preliminary ABM that simulates the biological dynamics of vocal fold inflammation and wound healing (N. Y. Li et al., 2008). In brief, the model currently has generally good ability to predict clinically expected time-varying consequences for a limited panel of inflammatory mediators in the vocal folds (IL-1β, TNF-α, IL-10, transforming growth factor-beta [TGF-β]) up to 24 hr post-baseline, following intervening induction of phonotrauma. Details around that model are provided shortly.

# 4.0 DEVELOPMENT OF PRELIMINARY BIOLOGICAL MODELS OF VOCAL FOLD INFLAMMATION

We have developed a patient-specific ABM for acute vocal fold inflammation, with the ultimate goal of identifying individually optimized treatments (N. Y. Li et al., 2008). The freeware *Netlogo* (Center for Connected Learning and Computer-Based Modeling, Northwestern University, Evanston, IL) was used as the platform for model building and simulation. An overview of our model building and simulation is provided in the next paragraphs, followed by more detailed discussion.

First, detailed literature on inflammation and wound healing from the skin setting was reviewed to identify the essential *components* and *rules* for building the *structure* (or framework) of the model (Cockbill, 2002; P. Martin, 1997; Robson, Steed, & Franz, 2001; Witte & Barbul, 1997). Skin literature was used to glean information about the general cellular and mediator processes for model development because (1) inflammation and healing have been comprehensively studied in the skin domain, (2) knowledge of vocal fold wound healing was limited at the time of the original work, and (3) cellular and molecular processes are believed to involve similar mechanisms in wound healing across tissue domains, although some differences may exist in magnitude and timing of responses across tissue types (Robson et al., 2001).

Once basic wound healing rules were established, the model's *parameter* values for the cellular and molecular responses to insults had to be estimated numerically. We iteratively

calibrated these values by verifying the model's simulation outputs against (1) generally recognized patterns of cellular and molecular responses reported in the wound healing literature up to a 2-week time point (Cockbill, 2002; P. Martin, 1997; Robson et al., 2001; Witte & Barbul, 1997), adjusting parameter values accordingly as needed to obtain a general match between model output and those patterns (qualitative verification-calibration), and subsequently (2) experimental measures of inflammatory mediators in human laryngeal secretions from an acute phonotrauma study, up to a 4-hr post injury time point (Verdolini et al., in preparation). The model's parameters were then again iteratively adjusted until the patterns of simulation outputs and the empirical data were well matched, subjectively (quantitative verification-calibration).

Last, we input subject-specific initial inflammatory profiles into the model and ran simulations to evaluate the model's accuracy in predicting the levels of inflammatory mediators at a 24-hr time point following baseline, which had been followed by vocal loading and behavioral treatments. The details of the aforementioned process (model structure, model verification-calibration and model evaluation) are described shortly.

## 4.1 OVERVIEW OF MODEL STRUCTURE

The ABM of phonotrauma represents processes thought to occur in the vocal fold mucosal tissue and aims to simulate the mucosal repair response to biomechanical damage during phonation. For each simulation, the user can define the initial levels of three inflammatory mediators (IL-1β, TNF-α and IL-10), add a phonotraumatic event, and then a 4-hr treatment event (voice rest, resonant voice exercises or spontaneous speech). In the model, one step of simulated time represents 0.1 day (2.4 hr).

The model consists of platelets, inflammatory cells (neutrophils and macrophages) and fibroblasts, mediators that regulate inflammation and wound healing (IL-1β, TNF-α, IL-10, and TGF-β1), a representative component of the extracellular matrix (collagen type I), and, perhaps most important, a tissue damage function functionally analogous to alarm/ danger signals (Matzinger, 2002) that produces positive feedback to induce further inflammation (Vodovotz, 2006) (Figure 1). The model assumes that biomechanical stress during phonation causes mucosal tissue damage and activates platelets, neutrophils and macrophages. Platelets release TGF-β1, which chemoattracts both neutrophils and macrophages. Activated neutrophils and macrophages secrete pro-inflammatory mediators, which in turn induce anti-inflammatory mediator release. Pro-inflammatory mediators also induce neutrophils and macrophages to produce free radicals that damage tissue. In our model, the activity of free radicals was subsumed in the actions of TNF-α. Anti-inflammatory mediators contribute to fibroblast activation. Activated fibroblasts secrete an extracellular matrix molecule, collagen, which mediates tissue repair. In the model, collagen accumulation is considered as the surrogate for healing outcome following phonotrauma. Collagen is an important extracellular matrix protein involving both structural and biomechanical functions in the vocal folds (Gray & Titze, 1988; Gray et al., 2000). The changes in temporal concentration of inflammatory cells, mediators, tissue damage and collagen were plotted and refolded into the model at each time step. The details of the ABM codes are available in the published paper (N. Y. Li et al., 2008).

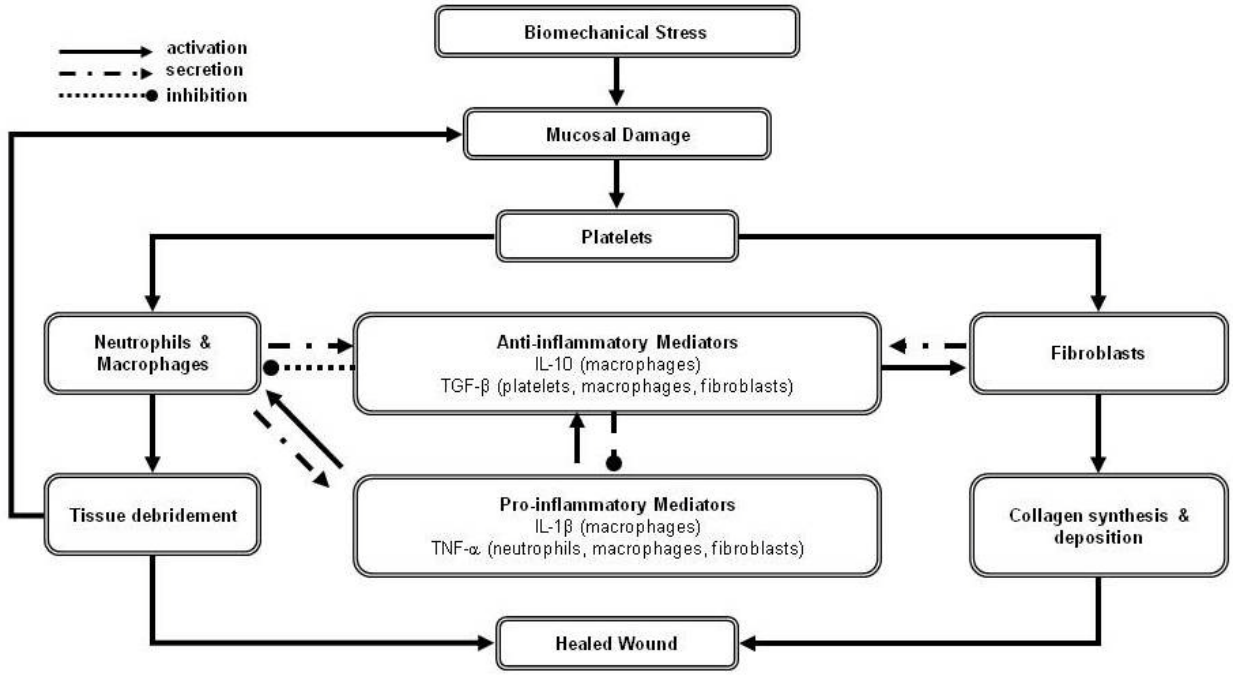


**Figure 1. An overview of the model structure. The model assumes that biomechanical stress during phonation causes mucosal damage and activates platelets, neutrophils and macrophages. Platelets produce TGF-β1, which chemoattracts both neutrophils and macrophages. Activated neutrophils and macrophages secrete pro-inflammatory mediators, which in turn induce anti-inflammatory mediator release. Pro-inflammatory mediators also induce neutrophils and macrophages to produce free radicals that damage tissue. In our model, the activity of free radicals was subsumed in the actions of TNF-*α*. Anti-inflammatory mediators contribute to fibroblast activation. Activated fibroblasts secrete collagen that mediates tissue repair. In the model, collagen accumulation is considered as the surrogate for healing outcome following phonotrauma. Collagen is an important ECM protein involving both structural and biomechanical functions in the vocal folds (Gray & Titze, 1988; Gray et al., 2000). (Reprint from Li, N. Y., Verdolini, K., Clermont, G., Mi, Q., Rubinstein, E. N., Hebda, P. A., et al. (2008). A patient-specific in silico model of inflammation and healing tested in acute vocal fold injury. *PLoS ONE, 3*(7), e2789. Permission not required.)**

## 4.2 PARAMETER ESTIMATTION THROUGH ITERATIVE VERIFICATION AND CALIBRATION

Pattern-oriented analysis (Grimm et al., 2005; Railback, 2001) was used to estimate the model's *parameters* for the cellular and molecular responses to insults through an iterative validation and calibration process (Figure 2). Using this approach, we compared the patterns of simulation-generated data curves with (1) the patterns of inflammatory and wound healing reported in the wound healing literature across a roughly 2-week period (qualitative verification-calibration) and (2) the empirical data of inflammatory mediators in laryngeal secretions from an acute phonotrauma study following insult (quantitative verification-calibration). If the model-predicted and empirical curves failed to match according to subjective evaluation, the model would be calibrated to minimize differences between model predictions. Of note, not the *structure* of the model (i.e., components and rules) but only the values of the *parameters* were adjusted during the calibration process. Finally, an ABM was developed having a single set of parameters (i.e., a single model) that simulated patient-specific treatment response following acute phonotrauma.

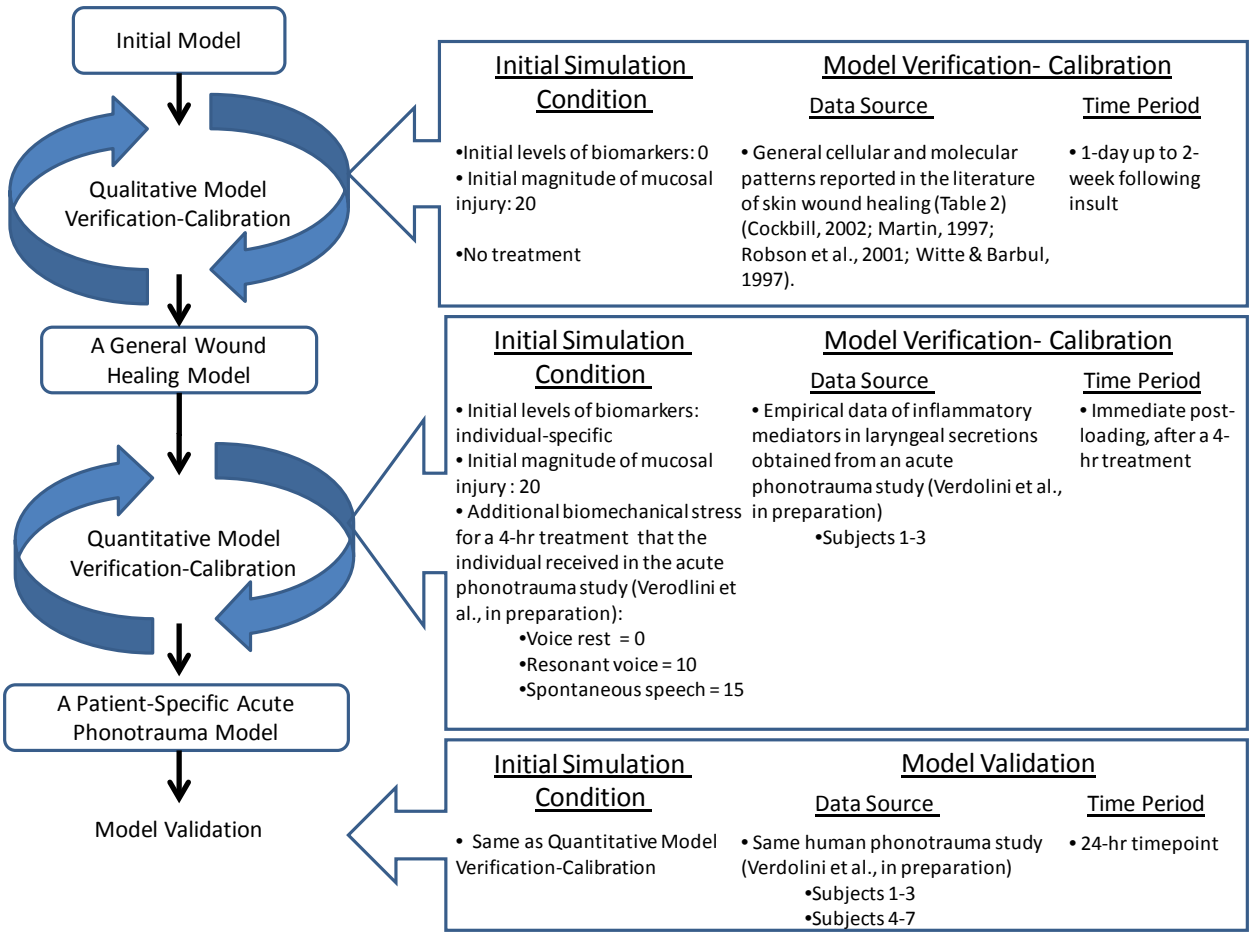


**Figure 2. Iterative verification-calibration process in the existing ABM.**

### 4.2.1 Qualitative verification-calibration of the model using literature data

In greater detail, first, a qualitative verification was carried out to test whether the model reproduces the generally-accepted patterns of cellular and molecular responses reported in the wound healing literature (Cockbill, 2002; P. Martin, 1997; Robson et al., 2001; Witte & Barbul, 1997) (Table 1). The user-defined initial magnitude of mucosal injury (range 0 – 40 in arbitrary units of damage) was first set at a value of 20 and the initial pre-injury values of IL-1β, TNF-α and IL-10 were set as zero. We then ran simulations to determine model outcome in the case of an acute phonotraumatic event and compared the model's outputs with pre-specified patterns reported in the wound healing literature up to a 2-wk time point (Cockbill, 2002; P. Martin, 1997; Robson et al., 2001; Witte & Barbul, 1997) (Table 1). If the model-predicted and empirical curves failed to match subjectively, the model's parameters were calibrated iteratively to produce a better qualitative match.

**Table 1. Patterns Used for the Existing ABM in the "Comparison Condition", i.e., the Condition with Intermediate Magnitude of Initial Mucosal Injury Input.**

| Patterns of Inflammation and Healing | Reference |
|---|---|
| Neutrophils arrive in wound site in the first few hours | (Cockbill, 2002; P. Martin, 1997; Robson et al., 2001; Witte & Barbul, 1997) |
| Neutrophil number is at maximum by 24 hours | (Cockbill, 2002; P. Martin, 1997; Robson et al., 2001; Witte & Barbul, 1997) |
| Neutrophil number decreases rapidly on Day 3 | (Cockbill, 2002; P. Martin, 1997; Robson et al., 2001; Witte & Barbul, 1997) |
| Macrophage number is at maximum by 24-48 hours | (Cockbill, 2002; P. Martin, 1997; Robson et al., 2001; Witte & Barbul, 1997) |
| Fibroblast number is at maximum by Day 5-7 | (Cockbill, 2002; P. Martin, 1997; Robson et al., 2001; Witte & Barbul, 1997) |
| Fibroblast number decreases gradually on Day 7 | (Cockbill, 2002; P. Martin, 1997; Robson et al., 2001; Witte & Barbul, 1997) |
| Collagen curve is sigmoid-shaped | (Robson et al., 2001; Witte & Barbul, 1997) |

### 4.2.2 Quantitative verification-calibration of the model using empirical laryngeal data

When qualitative behavior of the simulation appeared satisfactory, we proceeded to *quantitative verification-calibration* of the model by adjusting parameter values not found in the literature to fit the quantity and time-course of measured vocal fold mediators. The user-defined initial

magnitude of mucosal injury, which represented the phonotraumatic event, was set at a value of 20. The initial values of IL-1β, TNF-α and IL-10 and the simulated treatment event (voice rest, resonant voice exercises or spontaneous speech) were subject-specific, based on values obtained in the acute phonotrauma study (Verdolini et al., in preparation). Specifically, we ran simulations for three subjects (Subjects 1 – 3), using their baseline mediator levels in laryngeal fluid as initial inputs for the model, added a phonotraumatic event and then a 4-hr treatment event (Subject 1: voice rest, Subject 2: resonant voice and Subject 3: spontaneous speech). The treatment event was modeled by introducing additional mechanical stress to the simulated mucosal tissue. The magnitude of mechanical stress for the treatment was arbitrarily set as 0 for voice rest, 10 for resonant voice and 15 for spontaneous speech.

Subsequently, we compared each subject's model outputs for IL-1β, TNF-α and IL-10 with those from the empirical data, immediately after phonotrauma induction, and following a 4-hr treatment (Verdolini et al., in preparation). If the model-predicted and empirical data did not match subjectively, the parameter values were calibrated iteratively to optimize the model's prediction of vocal fold mediators. The quantitative verification-calibration iterative process continued until it eventually yielded a satisfactory model, based on subjective judgment.

### 4.2.3 Evaluating the model's prediction accuracy of inflammatory mediator outputs

Last, we input individual-specific inflammatory profiles (IL-1β, TNF-α and IL-10) at baseline and the category of treatment conditions (voice rest, resonant voice exercises or spontaneous speech) into the model and ran simulations up to 5 days. Then, we evaluated the ABM by comparing the predicted mediator levels with the empirical mediator levels at 24 hr (1) for Subjects 1, 2 and 3 whose data from early time points had been used for model calibration, as

well as (2) for Subjects 4 – 7 whose data had never been used for the model but only their individual inflammatory profiles at baseline were used as input to the model for simulation. Individual Z-tests using the *p-value* approach to testing statistical significance were carried out to compare the human empirical data to the data predicted by the model for IL-1β, TNF-α and IL-10 at the 24-hr time point for (1) the base cohort (Subjects 1 – 3), and (2) the remaining subjects (Subjects 4 – 7). Z-tests were carried out for each mediator for each subject.

## 4.3 THE AGENT-BASED MODEL OF PHONOTRAUMA: SIMULATION RESULTS

In general, the ABM reproduced and predicted subject-specific trajectories of inflammatory mediators as seen in our human data. Results are shown in Figures 3 – 4. For the base cohort (Subjects 1 – 3), the ABM predicted empirically obtained mediator values at 24 hr for 80% of mediators (12/15; $p < 0.05$). In Subjects 4 – 7, the ABM predicted 24-hr mediator values 67% (2/3; $p < 0.05$) of cases for markers that were categorized "valid" and 44% (4/9; $p < 0.05$) of the instances that were categorized as "pre-inflamed and/or non-responsive". Predicted levels of pro-inflammatory marker IL-1β for spontaneous speech were significantly higher than for either voice rest or resonant voice conditions ($p < 0.05$, both comparisons). In contrast, levels of anti-inflammatory marker IL-10 for voice rest were significantly lower than for either resonant voice or spontaneous speech ($p < 0.05$, both comparisons). This work, although preliminary and small-scale, demonstrates a concrete realization of the synergistic power of modeling iterating with clinical data to provide a new understanding of the process of acute phonotrauma, with real implications for therapy.

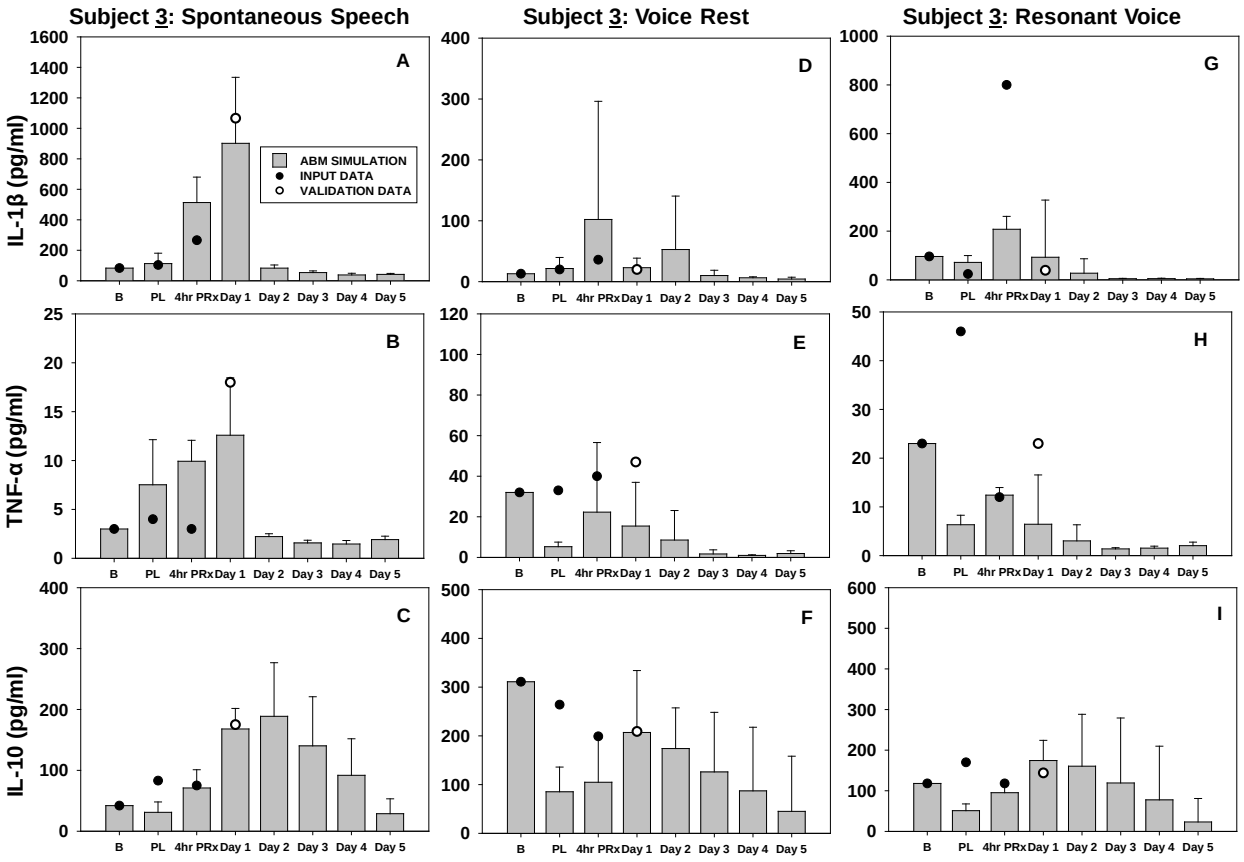


**Figure 3. Empirical and model-predicted inflammatory and wound healing responses to acute phonotrauma in a single human subject (Subject 3) following spontaneous speech (Panels A-C), voice rest (Panels D-F) and resonant voice treatment conditions (Panels G-I). Panels A, D and G display empirical and predicted trajectories of IL-1β. Panels B, E and H show empirical and predicted trajectories of TNF-α. Panels C, F and I show empirical and predicted trajectories of IL-10. Inflammatory marker concentrations are in pg/ml. The grey bars represent the mean of the simulated data, and the error bars represent standard deviations in the simulated data. The dark circles represent the input data for the first three time points (baseline, post-loading, 4-hr post treatment onset), obtained from human laryngeal secretion data. The empty circles represent the validation data at the 24-hr time point from the human laryngeal secretion data. B: baseline; PL: post vocal loading; 4hrPRx: following a 4-hr treatment. Note that human validation data for Days 2-5 have not yet been generated. (Reprint from Li, N. Y., Verdolini, K., Clermont, G., Mi, Q., Rubinstein, E. N., Hebda, P. A., et al. (2008). A patient-specific in silico model of inflammation and healing tested in acute vocal fold injury. *PLoS ONE, 3*(7), e2789. Permission not required.)**

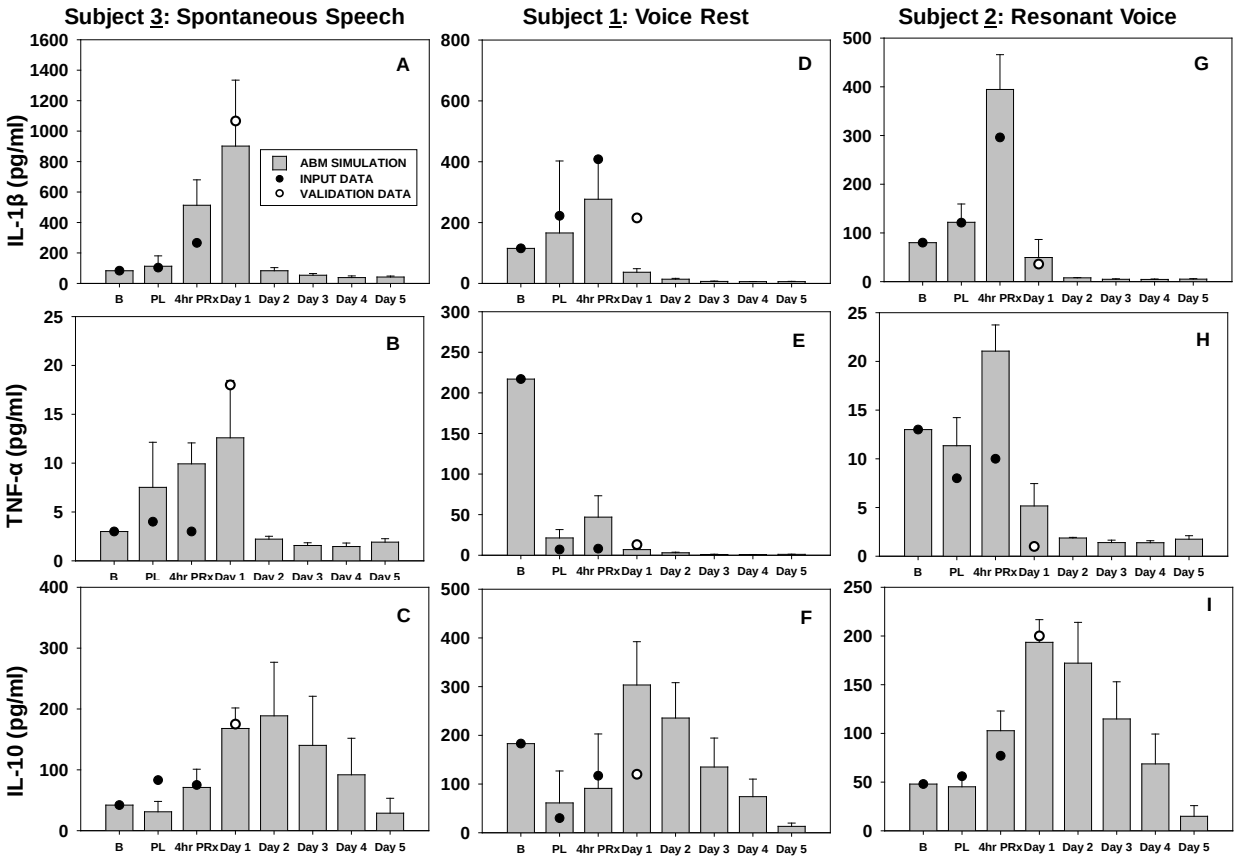


**Figure 4. Empirical and model-predicted inflammatory and wound healing responses to acute phonotrauma in three subjects following spontaneous speech (Subject 3; Panels A-C), voice rest (Subject 1; Panels D-F) and resonant voice treatment conditions (Subject 2; Panels G-I). Panels A, D and G display empirical and predicted trajectories of IL-1β. Panels B, E and H show empirical and predicted trajectories of TNF-α. Panels C, F and I show empirical and predicted trajectories of IL-10. Inflammatory marker concentrations are in pg/ml. The grey bars represent the means from the simulated data, and the error bars represent the standard deviation from the simulated data. The dark circles represent the input data for the first three time points (baseline, post-loading, 4-hr post treatment onset) from the human laryngeal secretion data. The empty circles represent the validation data at the 24-hr time point from the human laryngeal secretion data. B: baseline; PL: post vocal loading; 4hrPRx: following a 4-hr treatment. Note that human validation data for Days 2-5 have not yet been generated. (Reprint from Li, N. Y., Verdolini, K., Clermont, G., Mi, Q., Rubinstein, E. N., Hebda, P. A., et al. (2008). A patient-specific in silico model of inflammation and healing tested in acute vocal fold injury. *PLoS ONE, 3*(7), e2789. Permission not required.**

## 4.4 ORDINARY DIFFERENTIAL EQUATION MODEL OF PHONOTRAUMA

In addition to the development of a patient-specific agent-based model of vocal fold inflammation, the parallel development of an ordinary differential equation model was pursued in the interest of cross-platform comparison of results (N. Y. K. Li, Verdolini, Clermont, Mi, Hebda et al., 2006). To test the validity of our agent-based model, "model docking" was used. "Model docking" is a well-vetted validation strategy based on a comparison of predictions from different models across an array of user input data. The finding of similar predictions in agent-based and equation-based models would increase confidence in the underlying assumptions made in the current agent-based model.

In the comparison study, the agent-based and equation-based models predicted similar cellular and molecular patterns for inflammatory and wound healing responses under small initial damage (Figure 5). However, the models' results diverged in their predictions of inflammatory and wound healing responses for a large initial insult (Figure 5). Stated differently, with small initial damage, both models seemed to be robust to structural differences and limitations. However, beyond a given threshold of input damage, the models did not "dock." It is unclear whether this threshold is beyond commonly observed intensities of phonotraumatic insult. Encouragingly, the agent-based and equation-based models both anticipated that net collagen deposition peaks on Day 9 post injury. This predictive pattern helps to generate a hypothesis for a "wet-lab" experiment designed to identify putative mediators or enzymes correlated with the predicted collagen curves.

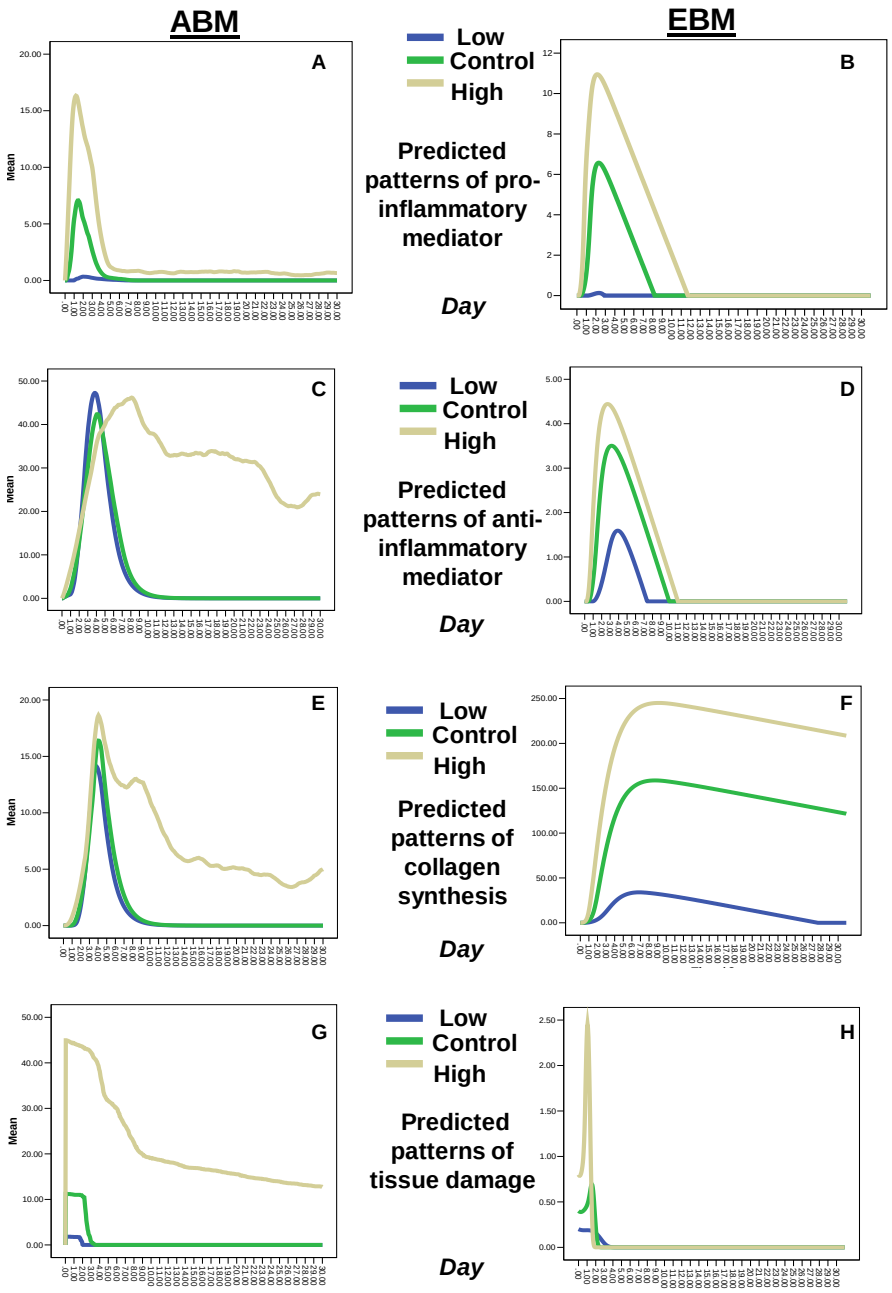


**Figure 5. The time course of cellular and molecular tissue abundances in wound healing predicted by both agent-based models (ABM) and equation-based models (EBM) under low, control and high initial magnitudes of mucosal damage (N. Y. K. Li, Verdolini, Clermont, Mi, Hebda et al., 2006). Panels A-B are the predications of a pro-inflammatory mediator. Panels C-D are the predictions of an anti-inflammatory mediator. Panels E-F and G-H are the predictions of collagen synthesis and tissue damage respectively.**

## 4.5 LIMITATIONS OF THE PRELIMINARY AGENT-BASED MODELS OF ACUTE PHONOTRAUMA THAT WILL BE ADDRESSED IN THIS STUDY

The previous work is encouraging in terms of the potential translational utility of ABM in the setting of vocal fold inflammation. However, at least three major limitations are noted and are addressed in the current project.

### 4.5.1 Underrepresentation of vocal fold extracellular matrix and its biomechanical roles in mediating inflammation and healing

The published ABM used collagen type I as the sole extracellular matrix (ECM) representative for the virtual vocal folds. In real vocal folds, elastin and hyaluronan (HA) in addition to collagen are also the main ECM substances composing vocal fold tissues and have equally important biomechanical effects in controlling vocal fold vibratory properties (Chan, Fu, Young, & Tirunagari, 2007; Chan, Gray, & Titze, 2001; Chan & Titze, 2006; Gray et al., 2000; Gray, Titze, Chan, & Hammond, 1999). Thus, elastin and HA were added in the new ABM in order to make it a more realistic replication of the ECM environment specific to the vocal folds.

Other than the importance of *biomechanical* roles in vocal folds, a growing literature supports the idea that ECM components have *biochemical* roles in regulating the wound healing process. Studies to date have shown that aberrant scarring/ fibrosis is at least partly due to the response of fibroblasts in the wound to both inflammatory mediators and ECM components (Hirschi, Gray, & Thibeault, 2002; S.L. Thibeault, Bless, & Gray, 2003; S.L. Thibeault, Gray, Bless, Chan, & Ford, 2002; S.L. Thibeault, Gray, Li et al., 2002; I. R. Titze et al., 2004). In particular, fragments of some ECM substances are known to constitute alarm/ danger signals that

influence cellular responses in the wound environment (Gallucci & Matzinger, 2001; Girish & Kemparaju, 2007; D. Jiang, Liang, & Noble, 2007; Lee & Spicer, 2000; Matzinger, 2002; Noble, 2002; O'Reilly, Gaggar, & Blalock, 2008; Stern, Asari, & Sugahara, 2006). The inflammation and healing process in the existing ABM were primarily mediated by interactions between inflammatory mediators and cells. The new ABM was augmented to explicitly represent biochemical roles of ECM fragments ensuing from initial mechanical injury or collateral damage from inflammation throughout the wound healing process.

### 4.5.2 Underrepresentation of the interplay among inflammatory mediators, growth factors and ECM substances across different phases of inflammation and healing as well as their response to tissue mobilization

The published ABM simulated (1) inflammation, (2) proliferation and (3) collagen formation (N. Y. Li et al., 2008). The model did not account for a final phase of the wound healing process, which involves extracellular matrix (ECM) reorganization. According to the literature on dermal wound healing, ECM reorganization is initiated once neo-matrix such as collagen is deposited at the wound site (Cockbill, 2002; P. Martin, 1997; Witte & Barbul, 1997). Collagen is indeed a core component of the ECM, and undergoes remodeling that is dependent on both continued collagen synthesis and compensatory collagen degradation. The degradation of wound collagen is controlled by a variety of *collagenase enzymes*, and the net increase in wound collagen is determined by the balance of these opposing mechanisms. Currently, no *in vivo* measurement of collagen remodeling in human vocal folds is available. At the same time, we may be able to capture the collagen dynamics indirectly by including *collagenase enzymes* in the model.

Matrix metallopeptidase-8 (MMP-8), which is a neutrophil collagenase, has been reported for its changes in vocal fold tissues following acute phonotrauma (Verdolini et al., in preparation). MMP-8 is known to preferentially cleave collagen type I and subsequently collagen fragments are generated. Besides MMP-8, several growth factors also influence ECM dynamics throughout wound healing. In particular, basic fibroblast growth factor (bFGF) has been documented to affect not only collagen but also elastin and HA productions in vocal fold fibroblasts. This growth factor has also been suggested for its therapeutic potential for tissue regeneration of injured vocal folds (Hirano, Bless, del Rio, Connor, & Ford, 2004; Hirano, Bless, Heisey, & Ford, 2003; Hirano et al., 2005; Luo, Kobler, Zeitels, & Langer, 2006). The new ABM was augmented with the rules around the interplay among growth factors, collagenase, cells and ECM components in order to yield more precise predictions of ECM dynamics throughout inflammation and healing.

Two additional cytokine mediators, IL-6 and IL-8, were incorporated in the new model as well. Delayed or aberrant inflammatory and healing response was reported in IL-6- or IL-8-deficient animals (Devalaraja et al., 2000; Grose & Werner, 2004; Hang, Frendeus, Godaly, & Svanborg, 2000; Lin, Kondo, Ishida, Takayasu, & Mukaida, 2003). Changes in IL-6 and IL-8 levels following vocal loading were observed in the laryngeal secretion data set from the study of acute phonotrauma (Verdolini et al., in preparation). Also, expressions of IL-6 and IL-8 in anatomical sites adjacent to the vocal folds are widely reported. Up-regulations of IL-6 and IL-8 are found in various respiratory diseases, such as bronchial asthma, acute lung injury, pulmonary fibrosis, and pneumonia (Coraux, Hajj, Lesimple, & Puchelle, 2005; Kammouni, Figarella, Marchand, & Merten, 1997; Kolb, Margetts, Anthony, Pitossi, & Gauldie, 2001; Losa Garcia et al., 1999; Lynch, Standiford, Rolfe, Kunkel, & Strieter, 1992; Martinet, Menard, Vaillant,

Vignaud, & Martinet, 1996; Maus, Rosseau, Knies, Seeger, & Lohmeyer, 1998; Nakamura et al., 1995; Rom, 1991; Takizawa, 1998, 2005; Takizawa, Ohtoshi, Yamashita, Oka, & Ito, 1996; Takizawa et al., 1997; Vaillant, Menard, Vignaud, Martinet, & Martinet, 1996; van den Berg et al., 2005; C. H. Wang et al., 2005; Xaubet et al., 1998). These investigations pointed to IL-6 and IL-8 as possible active mediators in inflammation and repair within the respiratory system, at the very least.

Furthermore, IL-6 and IL-8 were found sensitive to mechanical stimulation in muscle contraction during exercise work. A marked increase in both mRNA and protein levels of IL-6 and IL-8 following exercise without indicated tissue damage is consistently reported in literature (Akerstrom et al., 2005; Frydelund-Larsen et al., 2007; Nielsen & Pedersen, 2007; Pedersen & Hoffman-Goetz, 2000; Pedersen et al., 2003; Pedersen et al., 2001; Petersen & Pedersen, 2006; Steensberg, Fischer, Keller, Moller, & Pedersen, 2003). In fact, IL-6 is labeled as the "exercise factor" that mediates the health beneficial effects of exercise by suppressing pro-inflammatory response and amplifying anti-inflammatory response (Nielsen & Pedersen, 2007; Pedersen & Hoffman-Goetz, 2000; Pedersen et al., 2003; Pedersen et al., 2001). Another mechano-sensitive cytokine is IL-8, which is a well-studied mediator of its roles in attracting neutrophils into an area of tissue injury. Exercise-induced IL-8 is suggested to play a local role in stimulating angiogenesis through the activation of cell surface receptors (CXCR2) present in the capillary (Frydelund-Larsen et al., 2007). The new ABM with the addition of these two cytokines might improve the representation of the biological effects of motion-based treatments in stressed vocal fold tissues following vocal loading.

### 4.5.3 Limited validation of the model's tissue-level outputs

A third limitation in the existing work is that only the model's molecular outputs were calibrated with cytokine data from human laryngeal secretions (Verdolini et al., in preparation), whereas the model's tissue-level outputs/ predictions were yet to be experimentally validated. The integration of cellular and molecular responses and their manifestations at the higher level of tissue-wide and physiological changes is necessary to (1) obtain a fuller understanding of the pathophysiology of phonotrauma and (2) provide insights on how to manipulate or regenerate the stressed or traumatized tissues.

In the vocal folds, *tissue status* (damage, scar) is typically characterized by the change of *extracellular matrix substances* following injury. For example, excessive and disorganized collagen deposition, and decrease or total loss of elastin and HA within the lamina propria are reported in both human and/ or animal scarred vocal folds (R. C. Branski, Rosen, Verdolini, & Hebda, 2005a; Hansen & Thibeault, 2006; Hirano, Bless, Rousseau et al., 2003; Hirano et al., 2008; Rousseau et al., 2004; Rousseau et al., 2003; I. Tateya et al., 2006; T. Tateya, Tateya, Sohn, & Bless, 2005; T. Tateya, I. Tateya, J. H. Sohn et al., 2006; S.L. Thibeault et al., 2003; S.L. Thibeault, Gray, Bless et al., 2002; S. L. Thibeault et al., 2004). In the past, animal surgical models have focused on the changes in extracellular matrix substance in injured vocal folds (R. C. Branski et al., 2005a; Hansen & Thibeault, 2006; Hirano, Bless, Rousseau et al., 2003; Rousseau et al., 2004; Rousseau et al., 2003; I. Tateya et al., 2006; T. Tateya et al., 2005; T. Tateya, I. Tateya, J. H. Sohn et al., 2006; S.L. Thibeault et al., 2003; S.L. Thibeault, Gray, Bless et al., 2002; S. L. Thibeault et al., 2004). Until recently, three reports have been published to describe the inflammatory mediator profile in injured vocal folds, using animal surgical models (R. C. Branski, Rosen, Verdolini, & Hebda, 2005b; Lim et al., 2006; Welham et al., 2008). These

animal surgical data seem to provide an excellent opportunity for encoding the inflammatory mediator response according to its tissue level effects in injured vocal folds. Also, the time points, which have been reported in animal surgical models so far, are more wide-spread and abundant, compared to available human phonotrauma data. For instance, in rat models, the reported time points have spanned from points as early as 1 hr to as late as 3 months following injury (Lim et al., 2006; I. Tateya et al., 2006; T. Tateya et al., 2005; T. Tateya, I. Tateya, J. H. Sohn et al., 2006; Welham et al., 2008). From the perspective of systems biology, a large number of time points in a series helps to improve a models' accuracy.

In the second part of this dissertation project, an animal surgical ABM was generated in parallel to the augmented human phonotrauma ABMs in the main study. Studies of rats were used because the data are the most comprehensive among the animal species (Lim et al., 2006; I. Tateya et al., 2006; T. Tateya et al., 2005; T. Tateya, I. Tateya, J. H. Sohn et al., 2006; Welham et al., 2008). In the human phonotrauma ABMs, we were only able to calibrate and validate the model using inflammatory mediators at best. However, with this supplementary animal ABM, we were not only able to conduct modeling for the same inflammatory mediators, but we were also able to model ECM substances. This work is valuable because the complex interactions among the inflammatory mediators make it necessary to evaluate the "net effect" of these mediators at the *tissue* level. Hopefully, this work led us to predict actual vocal fold *tissue status,* at some integrated level, following injury.

## 4.6 SUMMARY

Predictive, pre-emptive and personalized mode of intervention is a central goal of modern medicine. To approach this goal, the complexity of biological processes must be tamed. We suggest that a systems biology approach that involves modeling/ simulation is integral to sorting through the perplexing array of factors that dictate success or failure of clinical trials for complex diseases (Whitcomb et al., 2005). Our ultimate, long-term goal is to generate *in silico* models that can be queried to identify biomechanical treatments that will optimize the wound healing process in the vocal folds, as a function of patient-specific inflammatory profiles. Although our long-term interests include both acute and chronic phonotrauma, the present study focuses on the acute case. Currently, biologically-based ABMs of acute phonotrauma have been developed relevant to this goal. However, several gaps are noted as mentioned in Section 4.5. These gaps were addressed in the current study and specific experimental hypotheses are provided shortly.

# 5.0 RESEARCH DESIGN AND METHODS

The present dissertation project involved the development of agent-based models (ABMs) in the context of (1) human phonotrauma and (2) animal surgical trauma to the vocal fold mucosa. The particular objective of this work is to construct a quantitative framework for evaluating biological effects of motion-based therapies for patients with acute phonotrauma. Ultimately, computational tools will be available that empower clinicians to (1) characterize an individual's probabilistic future health risk for the development or exacerbation of phonotraumatic vocal fold tissue changes and (2) prescribe personalized treatment regimes for individual patients with traumatized vocal folds depending on patients' current laryngeal status and unique probabilistic future health risks.

The overarching experimental questions for the current project are: (1) Can an ABM offer a personalized prediction of biological events within the vocal folds presumed to be closely related to tissue status? (2) If so, will the simulated wound healing response vary specifically as a function of the initial settings of biomarker level and treatment prescribed? Positive answers to these experimental questions would strengthen confidence in the eventual utility of mathematical modeling in general, and of ABM in particular, for understanding the complex vocal fold wound healing system and for predicting the healing outcome following vocal fold injury and varied treatment types.

### 5.1.1 EXPERIMENT 1 (SPECIFIC AIM 1)

#### 5.1.1.1 Purpose

The purpose of Experiment 1 was to generate subject-specific agent-based models (ABMs) of vocal fold inflammation due to acute phonotrauma to compute variables of (1) empirically derived cytokines and a collagenase (interleukin [IL]-1β, IL-6, IL-8, IL-10, tumor necrosis factor [TNF]-α and matrix metalloproteinase [MMP]-8), (2) growth factors (transforming growth factor [TGF]-β1 and basic fibroblast growth factor [bFGF]) and (3) extracellular matrix substances (collagen, elastin and hyaluronan [HA]). The models' prediction accuracy for the cytokines and the collagenase was subsequently evaluated, using empirical data generated from our treatment study of acute phonotrauma (Verdolini et al., in preparation).

#### 5.1.1.2 Hypotheses

**$H_0$**: The empirical inflammatory marker data (IL-1β, IL-6, IL-8, IL-10, TNF-α, and MMP-8) will not be within the 95% confidence interval for the simulated population mean at the 24-hr follow-up time point, given subject-specific cytokine and collagenase inputs to the model's baseline, immediate post loading and 4-hr post treatment onset time points.

**$H_1$**: The empirical inflammatory marker data (IL-1β, IL-6, IL-8, IL-10, TNF-α, and MMP-8) will be within the 95% confidence interval for the simulated population mean at the 24-hr follow-up time, given subject-specific cytokine and collagenase inputs to the model's baseline, immediate post loading and 4-hr post treatment onset time points.

#### 5.1.1.3 Equipment and software

The freeware *Netlogo* 4.0.3 (Center for Connected Learning and Computer-Based Modeling, Northwestern University, Evanston, IL) were used as the platform for model building and simulation. Statistical software, Statistical Package for the Social Sciences (SPSS) 15.0 (SPSS Inc., Chicago, IL, USA), was used for statistical inspection of the empirical data and statistical evaluation of the ABMs' prediction accuracy.

#### 5.1.1.4 Experimental protocol of acute phonotrauma in different treatment modalities

The treatment study of acute phonotrauma (Verdolini et al., in preparation), which provided the necessary empirical data for the current modeling study, was approved by the Institutional Review Board at the University of Pittsburgh. A total of nine subjects participated in the study; six females (21-46 years) and three males (21-29 years). Nine subjects participated in a between-subjects study design, which involved exposure to a randomly assigned "treatment" condition (voice rest, resonant voice exercises or spontaneous speech) following a vocal loading task. One of the female subjects (Subject 3) also participated in a within-subjects design, which involved exposure to all three "treatment" conditions. For Subject 3, treatments were randomly allocated without replacement on different pairs of days separated by intervals ranging from 1-6 months.

Prior to subjects' participation in the experimental part of the protocol, written informed consent was first obtained by an investigator or research coordinator and then subjects received a clinical screening to assess for gag response and nasal patency. Exclusion criteria included subject report of gagging with tooth-brushing or other history or evidence of exaggerated gag reflex, deviated septum (based on otolaryngology exam), report of current or recent voice problems (within the past year) or any history of speech or language deficits, use of drugs that

may influence the voice (e.g., diuretics, decongestants), or report of allergy to local anesthetics (especially lidocaine).

In this experimental protocol, the vocal loading task aimed to induce acute phonotrauma. The protocol for vocal loading entailed three consecutive cycles, each involving 15 minutes of loud phonation (~75 – 90 dB @ 15 cm microphone-to-mouth distance) followed by 5 minutes of silence, for a total 60 minutes. Then, subjects were randomly assigned to one of three treatment groups: voice rest, resonant voice exercises or spontaneous speech for 4 hours in the clinic, under the careful supervision of a voice trainer, who — for the majority of subjects — was blinded to the experimental hypotheses. These three treatment modalities can be thought of on a two-dimensional continuum involving vibratory stress (none — voice rest; intermediate — spontaneous speech and resonant voice) and impact stress (none — voice rest; low — resonant voice; or intermediate — spontaneous speech). The prescription of resonant voice exercises involved cycles of 4 minutes of exercise followed by 16 minutes of rest, whereas the spontaneous speech treatment involved cycles of 16 minutes of conversational speech followed by 4 minutes of silence. In the voice rest condition, subjects did not phonate at all during the 4-hr period. After the 4-hour treatment, participants were discharged to home with instructions to continue to follow their corresponding treatment condition, with slight modifications for the resonant voice and spontaneous speech groups. The next morning, all subjects were required to observe complete voice rest until their arrival at the clinic around 7:00 a.m. or shortly thereafter.

#### 5.1.1.5 Laryngeal secretion procedure, assessment of inflammatory analytes and data used for modeling

A total of 4 secretion specimens were collected from each subject at 4 different time points: baseline, immediately post vocal loading, following the start of the 4-hr treatment and 24

hour post baseline. For the initial secretion collection, an otolaryngologist first examined the subject's oral cavity, oropharynx, and nasal cavity and placed a cotton pledget (a flat absorbent pad) soaked with lidocaine and decongestant into the subject's more patent nasal cavity. Cetacaine® was sprayed in the oropharynx. Rigid laryngeal stroboscopy was performed to obtain a baseline stroboscopic evaluation on the patient. Then 4% lidocaine was dripped into the endolarynx through the working channel of the previously noted chip-tip flexible laryngoscope. After approximately 5 minutes, subsequent to verification of vocal fold anesthesia to light touch, a 1 millimeter plastic cannula was passed through the working channel of the scope and guided down to the free edge and superior surface of the vocal folds while suction was applied to the catheter. That procedure allowed for the collection of a small amount of vocal fold secretions bilaterally (about 100 μl total), while minimizing contact of the scope with the vocal folds. Secretions were captured in a modified sinus trap and then transferred into a 0.2 ml microfuge tube using a 1cc syringe. The tubes were labeled using codes that could not be traced to the subject or the subject's condition—except by way of a secret list retained by one investigator who was not involved with secretion data analysis--and the tubes were placed on dry ice. Tubes were then stored at -80 $^{o}$C until analysis.

All secretion analyses were carried out by an investigator who was blinded to subjects' conditions (time point and treatment condition). For the analyses, a known volume was aliquoted for analysis and served as the dilution factor. The appropriate volume of sterile saline was added to the tube to bring the total volume up to 2.0 ml. Standard Enzyme-Linked ImmunoSorbent Assays (ELISAs) were performed for IL-1β, IL-6, IL-8, IL-10, TNF-α and MMP-8 utilizing the manufacturer's recommended protocol (R&D Systems, Minneapolis, MN). All samples were run on the same kit to avoid inter-kit variability in the data.

Inspection of the resulting secretion data was carried out in order to derive the cleanest data for the development of ABM. The data were sorted into three main categories across subjects and inflammatory markers: (1) data showing high baseline concentrations of pro-inflammatory markers ($\geq$ 1 standard deviation relative to the average for the set as a whole; designated as "pre-inflamed" data); (2) data showing normal baseline concentrations of markers (< 1 standard deviation from average), but paradoxically decreasing post loading ("non-responsive" data); and (3) data showing normal baseline concentrations of markers (< 1 standard deviation from average) and increase after loading ("responsive" data) (Verdolini et al., in preparation). In addition, all data from two subjects (Subject 8 and Subject 9) were considered as invalid due to thick secretions that compromised interpretation of ELISA results. Therefore, none of their data were used in the initial ABM development (N. Y. Li et al., 2008) or in the current study.

The upshot was that a "base dataset" was identified involving data from three subjects (Subjects 1, 2 and 3), whose data were considered "responsive" and "not pre-inflamed," according to the schematic used. The first three time points of the base data set (baseline, immediate post loading, and 4-hr post treatment initiation) were used for the ABM calibration (N. Y. Li et al., 2008). Subject 1 and Subject 2, who were the between-group subjects, received voice rest and resonant voice exercises respectively. Data from these two subjects were used to calibrate parameters relative to voice rest and resonant voice exercises for immediate post-loading and 4-hr post treatment initiation time points. For Subject 3, who was the within-group subject, all data from the subject were used to calibrate the parameters relative to voice rest, resonant voice exercises and spontaneous speech in the ABM for immediate post-loading and 4-hr post treatment initiation time points. Model validation primarily involved comparison of data

from Subjects 1 – 3 at the 24-hr time point vis-a-vis 24-hr data predicted by the model, individually for each subject. The details of how empirical data were used for verification-calibration and validation procedures of the previously reported ABM (N. Y. Li et al., 2008) are described in Section 4.2. The same procedures were replicated for the new ABM with minor modifications that are described shortly in Sections 5.1.1.9 – 5.1.1.10.

#### 5.1.1.6 New model components and rules

A single ABM with expanded components was developed for simulating both human phonotrauma **(Experiment 1)** and animal surgical trauma **(Experiment 2)**. In these two experiments, the initial magnitude of mucosal injury and the empirical data set used for model verification-calibration and evaluation were different, as described in Sections 5.1.1.9 – 5.1.1.12 for human phonotrauma simulation and Sections 5.1.2.5 – 5.1.2.7 for animal surgical simulation. Otherwise, both experiments used the same general model with identical model structure in terms of agent components and rules, as described below.

The ABM of acute vocal fold inflammation and healing represented biological processes that are known to occur within the vocal fold mucosal tissue and simulated the mucosal repair response to mechanical injuries. The previously reported model (N. Y. Li et al., 2008) was specified to the setting of phonotraumatic injury through iterative verification and calibration procedures with vocal fold secretion data from a human phonotrauma study (Verdolini et al., in preparation). The model was composed of (1) platelets, (2) cells (neutrophils, macrophages and fibroblasts), (3) a growth factor (TGF-β1) and three cytokines (IL-1β, TNF-α, IL-10) involved in inflammation and wound healing, (4) a matrix substance (collagen type I) and (5) a tissue damage function analogous to alarm/danger signals constituting positive feedback to induce additional inflammation (Matzinger, 2002). In the present study, additional mediators, growth

factors and matrix substances were introduced to the previously developed ABM (N. Y. Li et al., 2008) Specifically, in addition to existing model components, six new elements were added into the model: two cytokines (IL-6 and IL-8), a collagenase (MMP-8), a growth factor (bFGF) and two extracellular matrix substances (elastin and HA). The model was then specified for either the human phonotrauma setting **(Experiment 1)** or the animal surgical trauma setting **(Experiment 2)**, by calibrating the values of model parameters using the respective empirical data (See Section 5.1.11 for human phonotrauma and Section 5.1.2.6 for animal surgical trauma).

Detailed literature on inflammation and healing was reviewed to identify rules for the further development of the ABMs in the present context (Armstrong & Jude, 2002; Broughton, Janis, & Attinger, 2006; Calvin, 1998; Cockbill, 2002; Croce et al., 2001; Diegelmann & Evans, 2004; Gillitzer & Goebeler, 2001; Hackham & Ford, 2002; Henry & Garner, 2003; Kuang et al., 2007; Leask & Abraham, 2004; P. Martin, 1997; Matsumoto, Okazaki, & Nakamura, 1992; Moulin, 1995; O'Reilly et al., 2008; Page-McCaw, Ewald, & Werb, 2007; Palmon et al., 2001; Parks, 1999; Parks, Wilson, & Lopez-Boado, 2004; Robson et al., 2001; Schiller, Javelaud, & Mauviel, 2004; Weng, Mohan, Li, & Wilson, 1997; Werner & Grose, 2003; Witte & Barbul, 1997). Further, relevant literature on the vocal folds (Hirano, Bless, Heisey et al., 2003; Hirano, Bless, Nagai et al., 2004; Luo et al., 2006) was used to specify the model to the setting of vocal fold injury. The cell source and biological functions of the existing and augmented (in *italics)* models are summarized in Table 2.

**Table 2. Summary of the Components Involved in the ABM. The Items in *Italics* Represent the Extension of the Existing ABM.**

| Substances | Cell Sources | Biological Functions in Wound Healing used in ABM |
|---|---|---|
| TGF-β1 | Platelets<br>Macrophages<br>Fibroblasts | Chemotactic to neutrophils, macrophages and fibroblasts<br>Inhibit expression of TNF-α in neutrophils, macrophages and fibroblasts<br>*Inhibit expression of MMP-8 in neutrophils*<br>Inhibit expression of IL-1β in macrophages (minimal effect)<br>Stimulate resting fibroblasts to activated fibroblasts<br>Mitogenic to fibroblasts (proliferation)<br>Stimulate collagen synthesis in fibroblasts<br>*Stimulate elastin synthesis in fibroblasts*<br>*Stimulate native hyaluronan synthesis in fibroblasts* |
| *bFGF* | *Macrophages*<br>*Fibroblasts* | *Chemotactic to neutrophils and macrophages*<br>*Mitogenic to fibroblasts (proliferation)*<br>*Stimulate fibroblast migration*<br>*Inhibit collagen synthesis in fibroblasts*<br>*Inhibit elastin synthesis in fibroblasts*<br>*Stimulate native hyaluronan synthesis in fibroblasts* |
| TNF-α | Neutrophils<br>Macrophages<br>Fibroblasts | Chemotactic to neutrophils and macrophages<br>Activate neutrophils and macrophages<br>*Stimulate expression of MMP-8 in neutrophils*<br>Stimulate expressions of TNF-α, IL-1β, *IL-6 and IL-8* in macrophages<br>Stimulate expression of TGF-β in macrophages and fibroblasts<br>Mitogenic to fibroblasts (proliferation)<br>*Stimulate expression of IL-6 in fibroblasts*<br>*Inhibit elastin synthesis in fibroblasts*<br>*Stimulate native hyaluronan synthesis in fibroblasts*<br>Induce tissue damage |
| IL-1β | Platelets<br>Macrophages | Chemotactic to neutrophils and macrophages<br>Activate macrophages<br>Stimulate expressions of TNF-α, IL-1β, *IL-6 and IL-8* in macrophages<br>Mitogenic to fibroblasts (proliferation)<br>Inhibit collagen synthesis in fibroblasts<br>*Inhibit elastin synthesis in fibroblasts*<br>*Stimulate native hyaluronan synthesis in fibroblasts* |
| *IL-6* | *Macrophages*<br>*Fibroblasts* | *Chemotactic to neutrophils*<br>*Stimulate collagen synthesis in fibroblasts* |
| *IL-8* | *Macrophages*<br>*Fibroblasts* | *Chemotactic to neutrophils*<br>*Inhibit collagen synthesis in fibroblasts* |
| IL-10 | Macrophages | Inhibit expression of TNF-α in neutrophils, macrophages and fibroblasts<br>Inhibit expression of IL-1β in macrophages<br>*Inhibit expressions of IL-6 and IL-8 in macrophages and fibroblasts*<br>Stimulate expression of TGF-β in macrophages and fibroblasts<br>Stimulate expression of IL-10 in macrophages<br>Inhibit activated neutrophil survival<br>Inhibit activation of neutrophils and macrophages |

*(Cont'd on next page)*

| Substances | Cell Sources | Biological Functions in Wound Healing used in ABM |
|---|---|---|
| *MMP-8* | *Platelets*<br>*Neutrophils* | *Stimulate collagen degradation* |
| *Collagen (type I)* | *Fibroblasts* | Native collagen repairs tissue damage<br>*Collagen fragments are chemotactic to neutrophils and macrophages* |
| *Elastin* | *Fibroblasts* | Native elastin repairs tissue damage<br>*Elastin fragments are chemotactic to macrophages* |
| *Hyaluronan* | *Fibroblasts* | *Native HA repairs tissue damage*<br>*Native HA inhibits expression of TNF-α and IL-8 in fibroblasts*<br>*Native HA inhibits collagen synthesis in fibroblasts*<br>*HA fragments stimulate expressions of TNF-α, IL-1β and IL-8 in macrophage*<br>*HA fragments are mitogenic to fibroblasts (proliferation)*<br>*HA fragments stimulate collagen synthesis in fibroblasts* |

#### 5.1.1.7 Overview of ABM structures: regions, agents and patches

A typical ABM is composed of three elements: region, agent and patch. The *region* is composed of small *patches* on which *agents* move and operate. *Agents* are "alive" objects that follow the rules programmed in ABM, whereas *patches* are immobile components that characterize the physical-spatial environment.

##### *ABM Structure: Regions*

In the present study, the ABM's virtual "world" was a square grid, in the dimension of 120 x 120 units. As shown in Figure 6, four *regions* were created to simulate (1) lumen, (2) epithelium, (3) capillaries and (4) the mucosal tissue itself. Specifically, the "world" represented the cross-sectional schematic view of a typical vocal fold at its midpoint. The two right-most regions of the world were lumen (height: 120 units; width: 20 units) and epithelium (height: 120 units; width: 10 units). Next to the epithelium was the mucosal tissue region (height: 120 units; width: 90 units).

In this model, four-capillary regions with a diameter of 9 units were created within the mucosal tissue region (yellow region in Figure 6). Platelets and inflammatory cells (neutrophils and macrophages) circulated within capillaries and migrated to the wounded mucosal tissue region upon injury. The mucosal tissue region was populated with sparse resident cells (macrophages and fibroblasts). The mucosal tissue region was the site where phonotraumatic injury occurred (native ECM breakdown) and was subsequently repaired by fibroblasts (neo-ECM deposition). This specific ABM typology was consistent with existing research findings in vocal fold microarchitecture (see Appendix A for literature review).

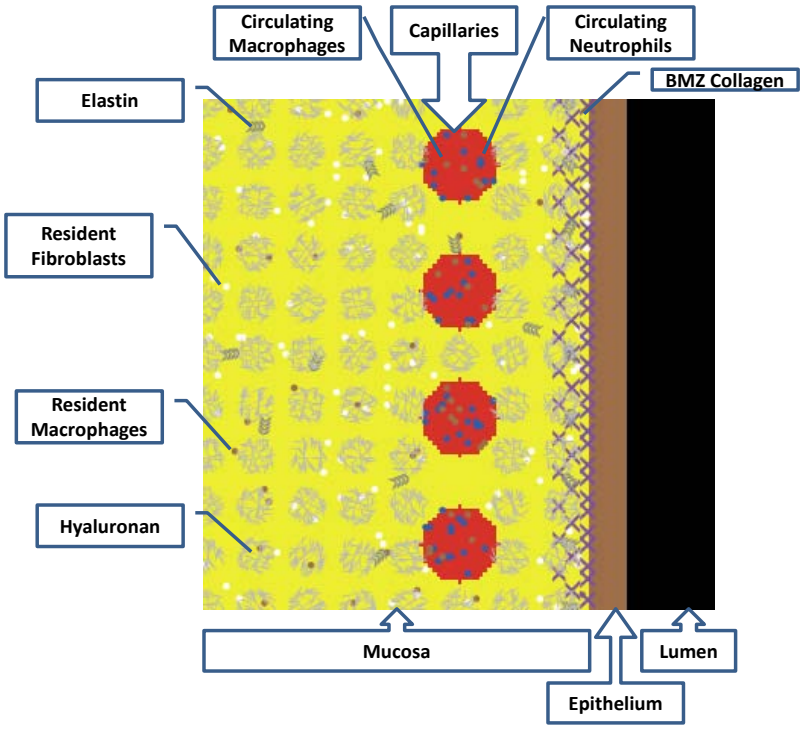


**Figure 6. The world of the new vocal fold ABM. Four compartments were designed in the model: (1) lumen (black); (2) epithelium (brown); (3) blood capillaries (red); and (4) mucosa (yellow). Inflammatory cells, namely, neutrophils (blue circles) and macrophages (brown circles), circulated within capillaries. Resident cells, including tissue macrophages (brown circles) and fibroblasts (white circles) were populated sparsely within the mucosal region. Native hyaluronan (light gray circles) was abundantly distributed throughout the mucosal area, whereas elastin (dark gray leaf shape) was randomly distributed in the mucosal area. A thin layer of collagen network (purple crosses) was attached immediately below the basement membrane zone (BMZ) between epithelium and mucosa.**

### *ABM Structure: Agents*

Within the ABM framework, only the class of *agents* is mobile. In the expanded ABM, agent variables were used to represent (1) platelets, (2) cells (neutrophils, macrophages and fibroblasts), (3) native ECM substances (collagen type I, elastin and HA), (4) the ECM fragments induced by injury and inflammatory responses and (5) tissue damage. Platelets and cells (neutrophils, macrophages and fibroblasts) were represented as agents because (1) cells are mobile in biological nature, (2) they could be organized based on common behavioral rules, and (3) the response of a particular cell type to various mediators is readily characterized in the literature (An, 2004). In the ABM, cells had three states: resting, activated or dead. Depending on the cell type, the cellular responses included rest, activation, migration, proliferation, death, secreting inflammatory mediators and generating ECM substance (Table 2).

Another class of agents was ECM substances, which are the major structural proteins in the vocal folds and their content and organization are prone to disturbance following phonotrauma or surgical trauma (Courey, Shohet, Scott, & Ossoff, 1996; Gray, Hammond, & Hanson, 1995; Hahn, Kobler, Zeitels, & Langer, 2006; Madruga de Melo et al., 2003; Neves, Neto, & Pontes, 2004; T. Tateya, Tateya, & Bless, 2006). The native form of matrices (collagen type I, elastin and HA) were modeled as agents. Each matrix substance had unique distribution and orientation within the vocal folds in the model, which were consistent with those reported in the vocal fold literature (Figure 6; Appendix A for relevant literature review). When the native form of matrices was degraded as fragments upon tissue injury or inflammation, these ECM fragments were modeled as another class of agents.

ECM fragments have been reported to interact with various cell types at the site of inflammation and thus modulated inflammatory and healing processes (Gallucci & Matzinger,

2001; Girish & Kemparaju, 2007; D. Jiang et al., 2007; Lee & Spicer, 2000; Matzinger, 2002; Noble, 2002; O'Reilly et al., 2008; Stern et al., 2006). In the current ABM, the initial mechanical injury and the subsequent inflammatory response of the pro-inflammatory mediators and the collagenase broke down the native matrices into fragments, causing more tissue damage – yet another class of agents. Tissue damage itself acted as a stimulus for further inflammation in the model.

### *ABM Structure: Patches*

In this ABM, *patch* variables were used to represent (1) tissue status (healthy, damaged, and healed); (2) inflammatory mediators (IL-1β, IL-6, IL-8, IL-10, TNF-α and MMP-8), and (3) growth factors (TGF-β1 and bFGF). Each patch was characterized by its instantaneous tissue status, as well as by the concentration of mediators and growth factors. When an agent moved onto a particular patch, all attributes of that patch would be accessed by the agent and the behavior of this agent would change according to algorithms (agent rules) programmed in the model. The complete rules of the ABM with explanations are described in Appendices E (Experiment 1: human acute phonotrauma ABM) and F (Experiment 2: animal surgical vocal fold trauma).

### 5.1.1.8 General logic flow of simulating acute phonotrauma

The overall flowchart of the new ABM is depicted in Figure 7. In the model, the assumption was that one step of simulated time represented 0.5 hours. The changes in temporal concentration of platelets, cells, mediators, tissue damage and extracellular matrices were plotted and refolded into the model at each time step.

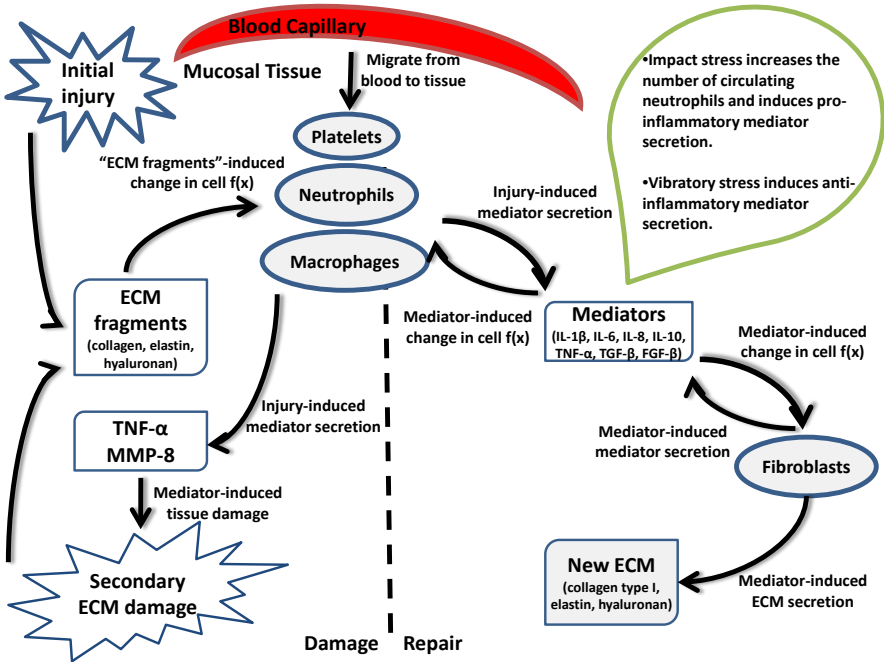


**Figure 7. Schematic depiction of (1) the theoretical modeling framework of acute vocal fold injury and repair, as well as (2) proposed effects of tissue mobilization (applied to Experiment 1 only) within this modeling framework. In general, initial phonotrauma or surgical injury leads to extracellular matrix (ECM) damage and activates platelets, neutrophils, macrophages and fibroblasts. Activated cells secrete an array of mediators (such as cytokines, growth factors and proteases), which in turn modulate cell functions (f(x)), including migration, proliferation, death, secretion of mediators and ECM substances. The specific cell source and functions of each mediator in the current ABM are described in Table 2. Tissue mobilization in phonation, namely, impact and vibratory stress, is proposed to perpetuate the injury and repair cascade by modulating circulating neutrophil counts in blood vessels and mediator expression in cells. Details about the effects of tissue mobilization are described in the text.**

In the initial setting of the model, circulatory neutrophils, circulatory macrophages and platelets were in the blood capillary region, whereas resident macrophages and fibroblasts were present with a random distribution within the tissue region. An initial mucosal injury (with magnitude as 20 for phonotrauma and 40 for surgical trauma) was simulated to traumatize the mucosal tissue in the middle of region, triggering platelet degranulation and native matrix substance degradation. Shortly afterwards, a chemoattractant gradient was created that stimulated the infiltration and activation of neutrophils and macrophages from blood capillaries. Shortly thereafter, resident macrophages and fibroblasts were activated by mediators and "alarm/ damage signals" (also known as "Damage-Associated Molecular Patterns" [DAMPs]) composed of ECM fragments. Activated neutrophils and macrophages secreted collagenase (MMP-8) and a pro-inflammatory mediator (TNF-α) that further degraded the matrices, causing secondary tissue damage. Fibroblasts secreted extracellular matrices to repair both the initial and the inflammation-induced damage. This modeling wiring was applied for both human phonotrauma **(Experiment 1)** and animal surgical trauma **(Experiment 2)**, except the initial setting of mucosal injury was different in magnitude between phonotrauma (magnitude = 20) and surgical trauma (magnitude = 40).

In order to represent the acute phonotrauma study (Verdolini et al., in preparation), additional algorithms regarding biological effects of motion-based treatments were implemented in the human phonotrauma ABM. For each simulation, the user defined the initial levels of inflammatory mediators, added a phonotraumatic event and then a 4-hr motion-based treatment event (voice rest, resonant voice exercises or spontaneous speech). The user-defined initial magnitude of mucosal injury, which represented the phonotraumatic event, was set at a value of

20. The treatment event was represented by additional mechanical stress, specifically, impact stress and vibratory stress, applied to the traumatized mucosal tissue.

The magnitude of mechanical stress for the treatment event was arbitrarily set as: no additional vibratory and impact stress for voice rest; intermediate vibratory stress and low impact stress for resonant voice; intermediate vibratory stress and intermediate impact stress for spontaneous speech (Table 3).

**Table 3. Magnitude of ABM-Simulated Phonatory Stress (range 0 – 10 in arbitrary units).**

| Phonation Type | Magnitude of Simulated Vibratory Stress (in arbitrary unit) | Magnitude of Simulated Impact Stress (in arbitrary unit) |
|---|---|---|
| **Voice Rest** | 0 | 0 |
| **Resonant Voice** | 10 | 5 |
| **Spontaneous Speech** | 10 | 10 |

In the current ABM, phonation-induced impact stress was constructed as "damaging stress". This construct was gleaned from the literature in voice science and exercise physiology. First, impact stress between the vocal folds during phonation has been suggested to be destructive to vocal fold mucosal tissue and is regarded as a major causative factor of phonotraumatic lesions (F.G. Dikkers & Nikkels, 1995; Gray, 1989, 1997; Gray et al., 1995; Gunter, 2003, 2004; Jiang et al., 1998; I.R. Titze, 1994). Second, intense muscle loading from exercise has been found to induce an immediate "para-inflammatory" response, without necessary overt tissue injury. Circulating neutrophil counts and pro-inflammatory mediator levels have been found to increase after the onset of exercise and return to baseline quickly after exercise (Butterfield et al., 2006; Peake, Nosaka, & Suzuki, 2005; Toumi et al., 2006). In that light, the component of impact stress from the 4-hr treatment event was constructed in a similar way. Within the specific 4-hr treatment window, circulatory neutrophil counts and pro-

inflammatory mediator (IL-1β and TNF-α) levels increased in different amounts, depending on the magnitude of impact stress generated from a particular treatment (Table 3).

On the other hand, vibratory stress was considered stress arising from tissue stretching, which would cause vocal fold cell deformation. Vibratory stress was constructed as "healing stress", which was linked to the functions of anti-inflammatory responses in the model. As for impact stress described above, this construct was based on the literature in voice science and exercise physiology. First, the effects of cyclic equibiaxial tensile strain (CTS) on rabbit vocal fold fibroblast cultures have been evaluated, in the presence or absence of IL-1β-induced inflammation (R. C. Branski et al., 2006). That *in vitro* study concluded that low magnitude and high frequency mobilization (6% CTS & 0.5 Hz) might assist in healing for acute vocal fold inflammation by attenuating pro-inflammatory mediator expressions. Second, researchers in exercise physiology have looked for an "exercise factor" that mediates the beneficial health effects of exercise. Among other cytokines (e.g., IL-1β, TNF-α etc.), IL-6 was identified as the "exercise factor" in muscle (Pedersen et al., 2003). Essentially, the level of IL-6 in circulation was zero during rest but had a rapid increase (about 100-fold) in response to exercise and decreased rapidly in the post-exercise period (Keller et al., 2001; Pedersen & Hoffman-Goetz, 2000; Pedersen et al., 2003). Also, exercise-induced IL-6 was shown to have inhibitory effects on the expression of pro-inflammatory mediator IL-1β and TNF-α, while having stimulatory effects on the expression of anti-inflammatory IL-10 in exercised tissue (Gleeson, 2007; J. Peake et al., 2005; Petersen & Pedersen, 2005, 2006; Toumi et al., 2006; Woods, Vieira, & Keylock, 2006). In that light, IL-6 was chosen as the "exercise mediator" of focus in the current ABM. The algorithm for the component of vibratory stress from the 4-hr tissue mobilization treatment was constructed in a way that IL-6 level increased in different amounts, depending on the

magnitude of simulated vibratory stress generated from a particular treatment (Table 3). Also, IL-6 was included in the inhibitory term for the functions of secreting pro-inflammatory mediator IL-1β and TNF-α, as well as in the stimulatory term for the functions of secreting anti-inflammatory mediator IL-10, in order to mimic IL-6 functions of suppressing pro-inflammation and amplifying anti-inflammation reported in the exercise physiology literature (see Appendix E for the relevant ABM rules).

#### 5.1.1.9 Parameter estimation: iterative verification and calibration process

Procedures for estimating the values of the model parameters generally replicated those from the published ABM (Section 4.2), with minor modifications (Figure 8). The modifications were involved in *qualitative verification and calibration* (describe shortly): (1) the initial magnitude of mucosal injury was set as 40 to represent surgical setting and (2) data were included from the literature on vocal fold wound healing following surgical trauma.

Pattern-oriented analysis (Grimm et al., 2005; Railback, 2001) was used to estimate the conformity of simulation-generated data curves with empirical data. The model's *parameters* were then calibrated iteratively until a match was generated between predicted and empirically obtained outcomes, as specified shortly. Two tiers of parameter estimation were involved. First, *qualitative verification-calibration* was carried out to compare the simulation curves with the general inflammatory and wound healing patterns reported in the literature for skin and vocal fold tissue across a roughly 2-week period. Then, *quantitative verification calibration* was carried out to compare the simulation curves with the empirical data of inflammatory mediators in laryngeal secretions from an acute phonotrauma study across a 4-hr period. Of note, not the *structure* of the model (i.e., components and rules) but only the values of the *parameters* were adjusted during the calibration process.

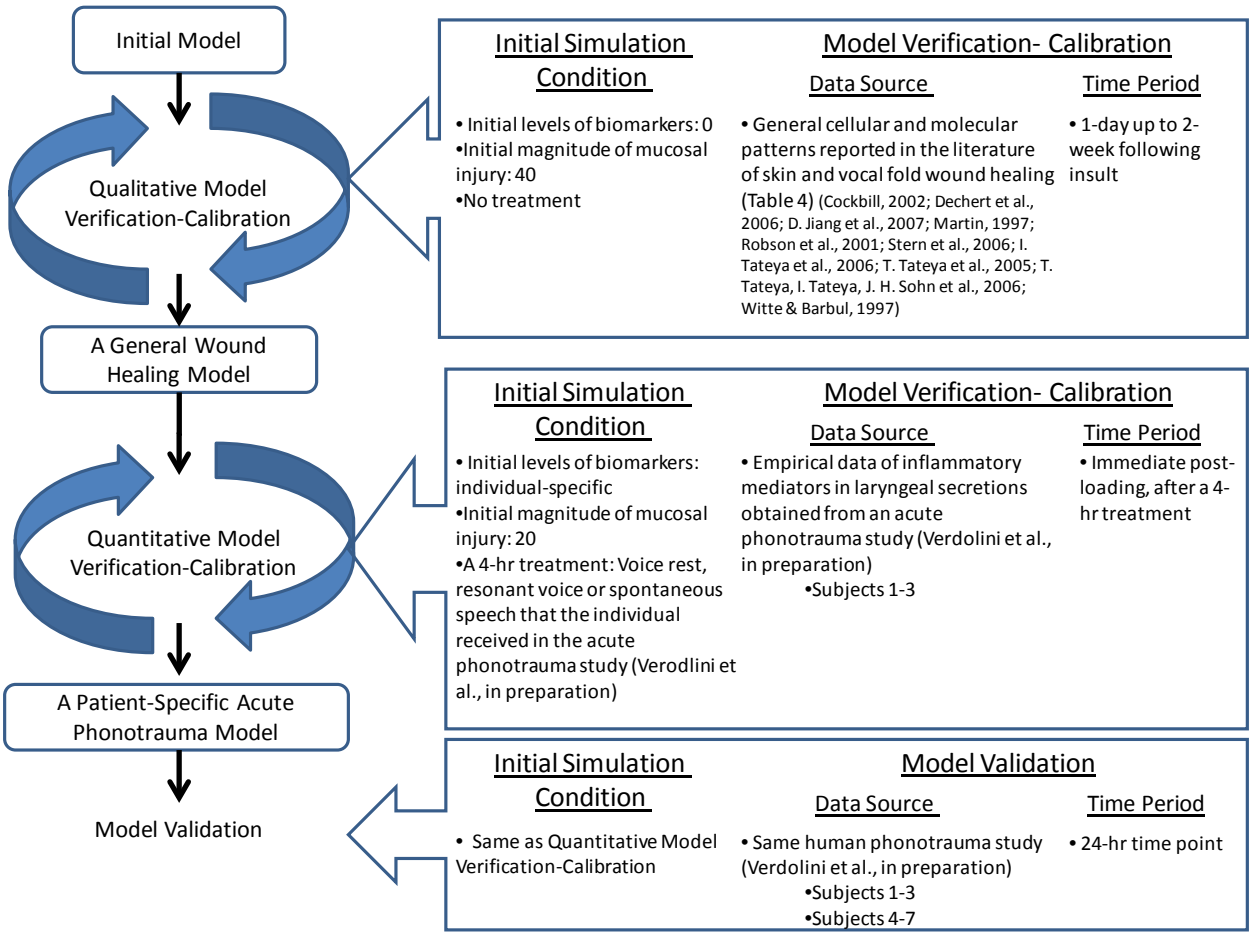


**Figure 8. Iterative verification-calibration process in the new ABM.**

#### 5.1.1.10 Qualitative verification-calibration of the model using literature data

First, a qualitative verification was carried out to test whether the model reproduced the generally-accepted patterns of cellular and molecular responses according to the literature in surgical skin wound healing (Cockbill, 2002; Dechert, Ducale, Ward, & Yager, 2006; D. Jiang et al., 2007; P. Martin, 1997; Robson et al., 2001; Stern et al., 2006; Witte & Barbul, 1997) as well as in surgical vocal fold wound healing (I. Tateya et al., 2006; T. Tateya et al., 2005; T. Tateya, I. Tateya, J. H. Sohn et al., 2006) (Table 4). The user-defined initial magnitude of mucosal injury

was first set at a value of 40 (range 0 – 40 in arbitrary units of damage), because that setting represented realistic predictions of massive mucosal damage and healing when compared with the general consensus around surgical wound healing documented in the literature. The pre-traumatic values of inflammatory markers (IL-1β, IL-6, IL-8, IL-10, TNF-α and MMP-8) were set to zero. We then ran simulations to determine model outcomes and compared the model's outputs with pre-specified patterns reported in the skin and vocal folds wound healing literature (Cockbill, 2002; Dechert et al., 2006; D. Jiang et al., 2007; P. Martin, 1997; Robson et al., 2001; Stern et al., 2006; I. Tateya et al., 2006; T. Tateya et al., 2005; T. Tateya, I. Tateya, J. H. Sohn et al., 2006; Witte & Barbul, 1997) (Table 4). If the model-generated and empirical curves failed to match, the model's parameters were adjusted iteratively to produce a better qualitative match.

**Table 4. Patterns Used for the Human Phonotrauma ABM in the "Comparison Condition", i.e., the Condition with High Magnitude of Initial Mucosal Injury Input.**

| Validation Patterns | Source |
|---|---|
| Neutrophils arrive at the wound site in the first few hours | (Cockbill, 2002; P. Martin, 1997; Robson et al., 2001; Witte & Barbul, 1997) |
| Neutrophil number is at maximum by Day 1-2 | (Cockbill, 2002; P. Martin, 1997; Robson et al., 2001; Witte & Barbul, 1997) |
| Neutrophil number decreases rapidly around Day 3-4 | (Cockbill, 2002; P. Martin, 1997; Robson et al., 2001; Witte & Barbul, 1997) |
| Macrophage number is at maximum by Day 2-4 | (Cockbill, 2002; P. Martin, 1997; Robson et al., 2001; Witte & Barbul, 1997) |
| Fibroblasts start proliferation at Day 1. | (I. Tateya et al., 2006) |
| Fibroblast number decreases significantly at Day 7 and stays low until Day 14. | (Cockbill, 2002; P. Martin, 1997; Robson et al., 2001; I. Tateya et al., 2006; Witte & Barbul, 1997) |
| Hyaluronan is first seen on Day 3 and peaks at Day 5, starts to drop significantly at Day 7 and then remains at a low level until Day 14. | (Dechert et al., 2006; D. Jiang et al., 2007; I. Tateya et al., 2006; T. Tateya et al., 2005) |

*(Cont'd on next page)*

| Validation Patterns | Source |
|---|---|
| The peak of accumulated hyaluronan content occurs at the same time as the peak of inflammatory cells (neutrophils and macrophages) | (D. Jiang et al., 2007; Stern et al., 2006) |
| Hyaluronan level is generally lower than for uninjured vocal folds following injury throughout the healing period. | (I. Tateya et al., 2006; T. Tateya et al., 2005) |
| Collagen type I curve is sigmoid-shaped | (Robson et al., 2001; Witte & Barbul, 1997) |
| Collagen type I is first seen on Day 3, peaks at Day 5. | (I. Tateya et al., 2006; T. Tateya et al., 2005) |
| Collagen type I level was generally higher than for uninjured vocal folds following injury throughout the healing period. | (I. Tateya et al., 2006; T. Tateya et al., 2005) |

#### 5.1.1.11 Quantitative verification-calibration of the model using empirical inflammatory mediator data from laryngeal secretions

When the qualitative behavior of the simulation appeared satisfactory, *quantitative verification-calibration* of the model was carried out by adjusting parameter values not found in the literature to fit the quantity and time-course of measured vocal fold mediators in laryngeal secretions. The user-defined initial magnitude of mucosal injury decreased from 40 to 20, because that setting represented smaller mucosal damage and healing in phonotrauma when compared with the setting of surgical trauma as 40 used in *qualitative verification-calibration*. Also, the magnitude of mechanical stress ensued from the vocal loading task in the acute

phonotrauma study (Verdolini et al., in preparation) was expected not as vigorous as those loud voice use in real life, such as cheering in football games. The initial inflammatory marker profile (IL-1β, IL-6, IL-8, IL-10, TNF-α and MMP-8) and the simulated treatment event (voice rest, resonant voice exercises or spontaneous speech) were individual-specific, based on values obtained in the acute phonotrauma study (Verdolini et al., in preparation).

Specifically, we ran simulations for three subjects (Subjects 1 – 3), using their baseline inflammatory marker levels in laryngeal fluid as initial inputs for the model, added a phonotraumatic event and then a 4-hr treatment event (Subject 1: voice rest, Subject 2: resonant voice; Subject 3: voice rest, resonant voice and spontaneous speech). Subsequently, simulation outputs for each inflammatory marker (IL-1β, IL-6, IL-8, IL-10, TNF-α and MMP-8) for each subject were compared with those of the empirical data at two time points: immediately after phonotrauma induction and following a 4-hr treatment (Verdolini et al., in preparation). If the model-predicted and empirical data did not match, the parameter values were calibrated iteratively to optimize the model's prediction of inflammatory markers. The quantitative verification-calibration iterative process was continued until the model eventually yielded a satisfactory match between simulation data and empirical data, based on subjective judgment.

#### 5.1.1.12 Model validation: evaluating the model's prediction accuracy of inflammatory marker outputs

Following both qualitative and quantitative verification-calibration, the model was tested for its accuracy in predicting individual-specific biomarker levels at a 24-hr time point. The user input each individual's baseline biomarker levels (IL-1β, IL-6, IL-8, IL-10, TNF-α and MMP-8), added a phonotraumatic event (initial magnitude of mucosal injury = 20), and then a 4-hr

treatment event (voice rest, resonant voice exercises or spontaneous speech), as previously. Due to the inherent stochasticity of the ABM framework, the ABM was run 100 times for each subject of the full subject cohort (Subjects 1 – 7), i.e., 900 runs (+700 runs from 7 between-group subjects, each with 100 runs; + 300 runs from 1 within-group subject – Subject 3 – who received each of the treatments ; -100 runs to account for double-counting the "spontaneous" treatment for Subject 3 in both between- and within-group measurements; thus 900 different runs in total) in order to generate representative population data for the following statistical analysis (Kim, personal communication).

The ABM was statistically evaluated by comparing the predicted inflammatory marker levels (IL-1β, IL-6, IL-8, IL-10, TNF-α and MMP-8) with the empirical inflammatory marker levels at 24 hr (1) for Subjects 1, 2 and 3 – in which the first three time points of inflammatory marker data were used for model calibration as well as (2) the remaining Subjects 4 – 7 – none of whose the inflammatory marker data were used for model calibration. Specifically, the ABM for Subjects 1 – 3 was run to determine how well the model predicted results for other individuals, i.e., Subjects 4 – 7 by inputting individual baseline biomarker levels and mode of treatment. A 95% confidence interval was computed as follows: for *each inflammatory marker* for *each subject* at the 24-hr time point from the simulation runs, i.e., 54 confidence intervals were generated (+42 mediator measurements from 7 between-group subjects, each with one of the 3 different treatments; +18 mediator measurements from one within-group subject—Subject 3 – who received each of the treatments; -6 mediator measurements to account for double-counting the "spontaneous speech" treatment for Subject 3 in both between- and within-group measurements; hence 54 different mediator measurements in total).

$$\text{Confidence interval} = \overline{X} \pm z * \left( \frac{ó}{\sqrt{n}} \right)$$

$\overline{X}$ = Mean of a predicted inflammatory marker level

z = The value on the standard normal curve with area (1-α) between –z and z

σ = Standard deviation of a predicted inflammatory marker level

n = Number of simulation run

If the empirical result for a given marker fell within the 95% confidence interval (α = 5% = 0.05; *z* = 1.96) from the simulation runs, the conclusion was that the model was generally adequate to predict the empirically obtained levels of the inflammatory marker. We expected that the model would have better accuracy in predicting the inflammatory marker levels for Subjects 1 – 3 than for Subjects 4 – 7 in general. We did not adjust for alpha inflation in this series because we opted to protect from Type II (β) error more than Type I (α) error, at this early stage of inquiry in attempting to predict patient-specific biological responses following phonotrauma (Kim, personal communication).

### 5.1.2 EXPERIMENT 2 (SPECIFIC AIM 2)

#### 5.1.2.1 Purposes

The purpose of Experiment 2 was to generate an animal surgical trauma agent-based model (ABM) in parallel to the human phonotrauma ABM from **Specific Aim 1**, using published rat mRNA data, and assess the models' prediction accuracy for the inflammatory markers (IL-1β, TNF-α, TGF-β1) and the matrix markers (procollagen subtype I, elastin synthase and hyaluronan synthase-2 (HAS-2)) at a 72-hr post injury time point. The success of this work would aid in encoding the inflammatory mediator response for its tissue-level manifestations in injured tissue, allowing predictions of acute vocal fold tissue status *in silico* and ultimately advancing understanding of inflammation and healing in a clinically useful way.

#### 5.1.2.2 Hypotheses

**$H_0$**: The empirical inflammatory marker data (IL-1β, TNF-α, TGF-β1) and the matrix marker data (procollagen subtype I, elastin synthase and HAS-2) will not be within the 95% confidence interval for the simulated population mean at the 72-hr post surgery baseline time point.

**$H_1$**: The empirical inflammatory marker data (IL-1β, TNF-α, TGF-β1) and the matrix marker data (procollagen subtype I, elastin synthase and HAS-2) will be within the 95% confidence interval for the simulated population mean at the 72-hr post surgery baseline time point.

#### 5.1.2.3 Equipment and software

The same equipment and software was used as for Experiment 1. To reiterate, the freeware *Netlogo* 4.0.3 (Center for Connected Learning and Computer-Based Modeling,

Northwestern University, Evanston, IL) was used as the platform for model building and simulation. Statistical software, SPSS 15.0 (SPSS Inc., Chicago, IL, USAU), was used for statistical inspection of the empirical data and statistical evaluation of the ABMs' prediction accuracy.

#### 5.1.2.4 Literature on animal surgical trauma in vocal fold mucosa

Studies of rat vocal folds were used for model calibration and validation because these data were the most comprehensive among the animal species in terms of (1) the wide spread of time points following injury and (2) the relatively complete profiles of the changes in inflammatory mediators and ECM substances following injury (Lim et al., 2006; I. Tateya et al., 2006; T. Tateya et al., 2005; T. Tateya, I. Tateya, J. H. Sohn et al., 2006; Welham et al., 2008). Table 5 summarizes the methods and the major findings from these studies and Figure 9 summarizes the changes in the extracellular matrix components in rats from the histological studies (T. Tateya et al., 2005; T. Tateya, I. Tateya, J. H. Sohn et al., 2006). It turned out that the animal surgical protocols were nearly identical across the studies. In brief, Sprague-Dawley male rats (4 to 6 months old) were used in all studies. Except for the single cellular study (I. Tateya et al., 2006) which made an incision in the mid-third portion of membranous vocal folds in the transverse direction, all other studies using the same approach to create vocal fold injuries. Specifically, injuries were induced using a 25-gauge needle and microforceps to strip the vocal folds until the thyroarytenoid muscle was exposed. All laryngeal specimens were harvested and stored in the same manner following injury. Real-time reverse transcription-polymerase chain reactions were used to measure *in vivo* messenger RNA (mRNA) for the expressions of inflammatory mediators and ECM substances.

**Table 5. Summary of Rat Vocal Fold Scarring Studies. H&E = hematocylin and eosin; RT-PCR = reverse transcription-polymerase chain reaction; mRNA = messenger ribonucleic acid; IL = interleukin; IFN-γ = interferon gamma, TNF-α = tumor necrosis factor alpha; NF-κβ = nuclear factor kappa beta; TGF-β1= transforming growth factor beta isoform 1; COX-2 = cyclooxygenase 2; HAS= hyaluronic acid synthase; HA= hyaluronan.**

| Studies | Animals | Methods | Biological Analysis | Major Findings |
|---|---|---|---|---|
| (T. Tateya et al., 2005) | 30 Sprague-Dawley male rats (4 to 6 months old) | • Injuries were performed by vocal fold stripping with a 25-gauge needle and microforceps.<br>• Larynges were harvested at 4 time points post injury: 2, 4, 8 and 12 weeks.<br>• Vocal folds were sectioned at 10 μm in the axial or coronal plane with a cryostat. | • Hyaluronidase digestion technique followed by alcian blue staining was performed for HA<br>• Masson's trichrome staining was performed for collagen<br>• Immunohistochemical staining was performed for (1) collagen type I, (2) collagen type III and (3) fibronectin. | • Collagen type III expression was constantly high for the 12 weeks, whereas collagen type I expression reduced from Week 2 until 8 and then stabilized until Week 12.<br>• The total collagen level (type I plus type III) peaked at Weeks 2 and 4 and then declined and became stable from Weeks 8 to 12.<br>• HA density remained significantly lower than control levels at all time points in the experiment.<br>• The level of fibronectin peaked at Week 2, remained high at Week 4, and started to decrease from Week 8 until Week 12. At the end point of the study (i.e., Week 12), the concentration of fibronectin was slightly control concentrations. |
| (T. Tateya, I. Tateya, J. H. Sohn et al., 2006) | 27 Sprague-Dawley male rats (4 to 6 months old) | • Injuries were performed by vocal fold stripping with a 25-gauge needle and microforceps.<br>• Larynges were harvested at 5 time points post injury: 1, 3, 5, 7 and 14 days.<br>• Vocal folds were sectioned at 10 μm in the axial or coronal plane with a cryostat. | • Hyaluronidase digestion technique followed by alcian blue staining was performed for HA.<br>• Masson's trichrome staining was performed for collagen.<br>• Immunohistochemical staining was performed for (1) collagen type I, (2) collagen type III and (3) fibronectin. | • Collagen type I was present on Day 3, peaked at Day 5, decreased significantly from Day 5 to Day 7 and was then stabilized until Day 14.<br>• Collagen type III expression was present on Day 1 and increased and remained intense from Day 3 to 14.<br>• HA was first seen on Day 3, peaked at Day 5, dropped significantly at Day 7 and then remained at a low level until Day 14.<br>• Fibronectin deposition was first seen on day 1 and remained at high levels until Day 14. |

*(Cont'd on next page)*

| Studies | Animals | Methods | Biological Analysis | Major Findings |
|---|---|---|---|---|
| (I. Tateya et al., 2006) | 24 Sprague-Dawley male rats (4 to 6 months old) | • Injuries were performed by making a transverse incision on the vocal fold at the mid-third of the epithelium and lamina propria down to the thyroarytenoid muscle. | • Immunohistochemical staining was performed for (1) vimentin, a marker for fibroblasts; (2) alpha-smooth muscle actin, a marker for myofibroblasts; (3) CD68, a marker for macrophages and (4) 5-bromo-2-deoxyuridine, a marker for newly proliferated cells at four time points: Day 1, 3, 5 and 14 postoperatively. | • On Day 1 post injury, epithelization and fibroblast proliferation started.<br>• On Day 3 post injury, the proliferation of fibroblasts in the lamina propria was at peak.<br>• On Day 7, the total number of cells decreased about 33-fold and stayed low until the endpoint of the experiment at Day 14 post injury.<br>• Myofibroblasts and macrophages were found to minimally proliferate at all time points. |
| (Lim et al., 2006) | 30 Sprague-Dawley male rats (4 to 6 months old) | • Injuries were performed by vocal fold stripping with a 25-gauge needle and microforceps.<br>• Larynges were harvested at 5 time points post injury: 4, 8, 16, 24 and 72 hours.<br>• Vocal folds were sectioned at 60 μm in the axial plane for mRNA analysis with a cryostat.<br>• Lamina propria was dissected from each section using 30-gauge needles. | • Real time RT-PCR was used for mRNA analysis for IL-1β, IFN-γ, TNF-α, NF-κβ, TGF-β1, COX-2, HAS-1, HAS2, procollagen type I, procollagen type 3, elastin, and β2-microglobuin (as housekeeping gene). | • Compared to the control vocal fold, IL-1β, NF-κB, TNF-α and HAS-1 had peak expressions at 4 and 8 hours. From 8 to 16 hours, procollagen type III expression decreased. At 16 hours, HAS-2 peaked. At 24 hours, IFN-γ expression decreased. At 72 hours, TGF-β, HAS-2, procollagen I and procollagen III expressions were all at peak.<br>• For all time points, COX-2 expressions were significantly higher than for control tissue, whereas elastin expressions were similar to the expression levels of the controls. |

*(Cont'd on next page)*

| Studies | Animals | Methods | Biological Analysis | Major Findings |
|---|---|---|---|---|
| (Welham et al., 2008) | 11 Sprague-Dawley male rates (4 to 6 months old) | • Injuries were performed by vocal fold stripping with a 25-gauge needle and microforceps.<br>• Larynges were harvested 1-hr post injury<br>• Vocal folds were sectioned (1) at 60 μm in the axial plane (for mRNA analysis) or (2) at 5 μm in the coronal plane (for histological analysis) with a cryostat.<br>• Lamina propria was dissected from each section using 30-gauge needles. | • H&E staining was used for histological analysis.<br>• Real time RT-PCR was used for mRNA analysis of IL-1β, IFN-γ, TNF-α, NF-κβ, TGF-β1, COX-2, HAS-1, HAS2, procollagen type I, procollagen type 3, elastin, and β2-microglobuin (as housekeeping gene). | • Subepithelial bleeding was seen throughout the injured vocal folds.<br>• mRNA expressions of IL-1β, TNF-α, COX-2, HAS-1 in the injured vocal folds were significantly up-regulated at 1-hr post injury, compared to the uninjured control tissue. |

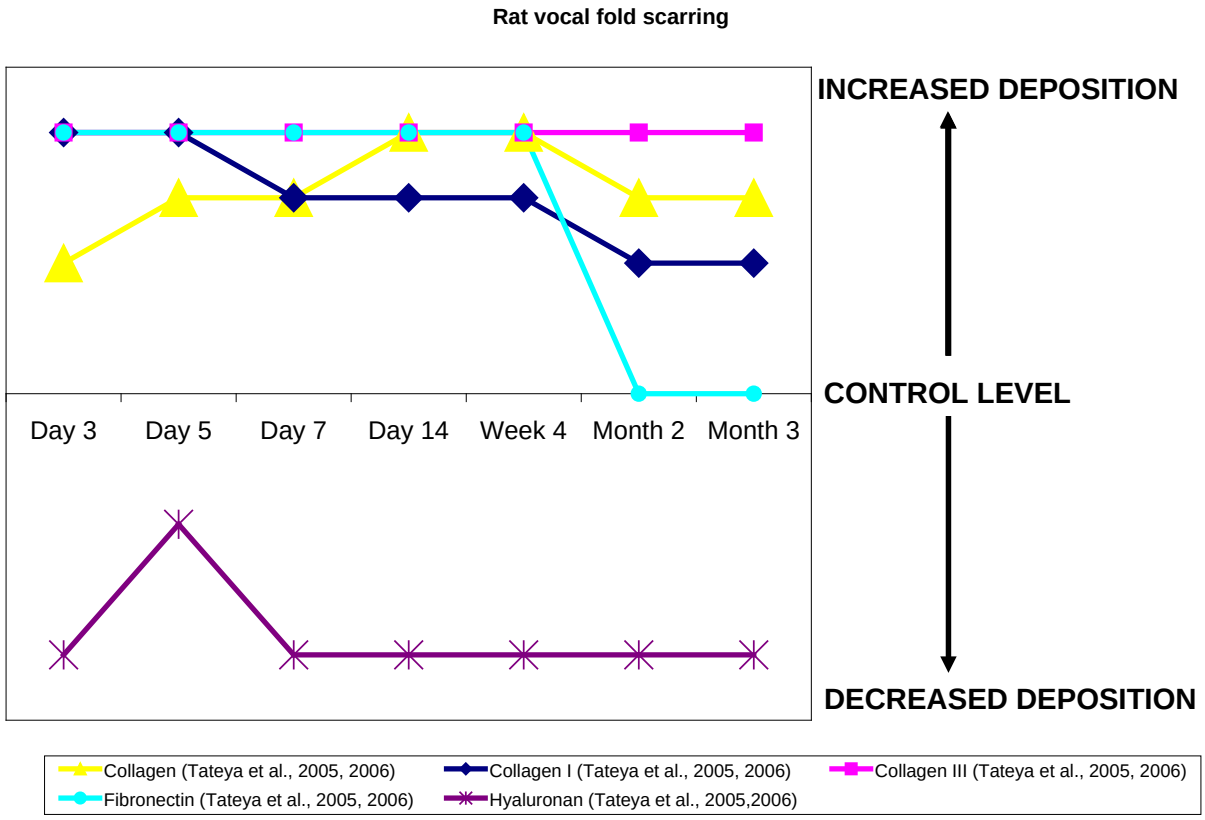


**Figure 9. Schematic representation of collagen, collagen type I, collagen type III, fibronectin and hyaluronan in injured rat vocal folds.**

### 5.1.2.5 Empirical rat mRNA tissue data used for animal surgical trauma model

Empirical mRNA tissue data from two published rat vocal fold injury papers (Lim et al., 2006; Welham et al., 2008) were used for model calibration and evaluation in this experiment. Research protocols of these two papers are summarized in Table 5. In brief, the materials and methods used in terms of animal surgical procedures, tissue preparation, mRNA analysis and statistical analysis, were reported as identical in these two papers.

In these two rat papers, the mRNA levels were expressed as the ratio of target gene concentration to housekeeping gene β-2MG in a natural logarithmic (ln) scale. Mathematically, the ln scale can only be defined for positive real numbers or non-zero complex numbers. However, from the practical consideration of modeling, we could not exclude the case that zero values would be predicted by the ABM, i.e., no mRNA expression for a particular marker. In that case, an error output would be returned if a natural logarithmic scale was used in the model. Thus, the first authors (Lim and Welham) from the two rat vocal fold papers were contacted. The authors graciously agreed to provide individual ln-transformed data and thus we could "un-transform" the ln data to actual data for our modeling purposes.

Following data "un-transformation", inspection of the empirical rat mRNA data was carried out to derive the cleanest data for the development of the animal surgical ABM. Inspection was carried out using the SPSS 15.0 statistical program for each marker at each time point. Individual data showing more than 3 times the interquartile range (i.e., the difference between the $75^{th}$ percentile and the $25^{th}$ percentile) were regarded as "extremes". "Extremes" were excluded from the data pool for subsequent model calibration and evaluation.

In this experiment, the ABM was based on the animal mRNA data. One might question the correspondence of the interested studied biomarkers in mRNA levels and protein levels. We acknowledged that from the detection of mRNA to the inference of how that mRNA eventually leads to a functional protein, several steps are involved, namely, mRNA transcription, mRNA stability, mRNA translation and finally protein stability. The question would be answered easily if the correspondence between mRNA levels and protein levels for our target biomarkers in vocal folds was known in the literature. Unfortunately, after an exhaustive literature search, only one published report (S.L. Thibeault, Gray, Li et al., 2002) and one conference paper (I. Tateya,

Tateya, & Bless, 2004) existed that linked the mRNA data to the corresponding protein data in the setting of the vocal folds.

Thibeault and her group used reverse transcriptase-polymerase chain reaction amplification (RT-PCR) to measure mRNA expressions and used Western blot analysis to study protein expressions for two matrix markers, fibronectin and collagen type I, in five samples of vocal fold polyps. The researchers concluded that the changes in protein levels were parallel to those in their mRNA levels for these two matrix markers. In Tateya et al.'s conference paper (2004), 38 male rats' unilateral vocal folds were injured by using the mucosal stripping method. The researchers used RT-PCR to study mRNA expressions for two HASs (HAS-1 and HAS-2), which are the enzyme of HA synthesis. Also, histological analysis was used to detect the presence of HA in the vocal folds. Both HASs and HA in the new granulation tissue were notably present at Day 3 after surgery. These results suggested that the induction of HASs gene and HA protein were temporally consistent in injured rat vocal folds. Although the existing data of mRNA and protein correspondence in the vocal fold setting are not ample, the results from these two studies seemed to point to a direction indicating mRNA expressions for matrix markers were quite consistent with their corresponding protein levels or functional activities in the environment of the vocal folds.

Given these observations, we assumed that the protein expression would follow immediately after the mRNA expression in the current model. Specifically, mRNAs of procollagen subtype I (precursor of collagen type I), elastin synthase (enzyme for elastin synthesis) and HAS-2 (enzyme for HA synthesis) would led to functional proteins of collagen type I, elastin and HA respectively. Of note, mRNA expression profiles of two HASs, HAS-1 and HAS-2, were studied in the rat papers (Lim et al., 2006; Welham et al., 2008). These two

enzymes were suggested to share similar induction pathways in rat vocal fold fibroblast cultures (Lim, Bless, Munoz-Del-Rio, & Welham, 2008). At the same time, only HAS-2 showed consistent temporal mRNA profiles, namely, peak expressions at Day 3 post surgical injury, across two independent *in vivo* rat vocal fold studies (I. Tateya et al., 2004; Welham et al., 2008). Therefore, HAS-2 was selected as the mRNA marker for HA in the animal surgical ABM herein.

#### 5.1.2.6 Model building and quantitative verification-calibration

The animal surgical ABM had the identical model structure and components as the human phonotrauma ABM developed in **Experiment 1**. The method of model verification-calibration and evaluation was essentially the same as in **Experiment 1** but the data used for calibration and evaluation were changed from human secretion data to rat tissue data. As *qualitative verification-calibration* was already completed in **Experiment 1**, we proceeded to *quantitatively* calibrate the model by comparing the model outputs with experimental data (Lim et al., 2006; Welham et al., 2008). Then, the model's accuracy in predicting the empirical inflammatory markers and matrix markers at 72 hr following surgery was evaluated.

First, we calibrated the model's behavior by adjusting parameter values to fit the quantity and time-course of measured mRNA levels of vocal fold mediators and ECM products in the surgery-traumatic tissue (Lim et al., 2006; Welham et al., 2008). Apart from individual-specific simulations in **Experiment 1,** average trends for the rat population were of interest in **Experiment 2.** We then ran simulations for the rat population up to 24 hr following surgery -- input the average baseline mRNA levels of mediators (IL-1β, TNF-α, TGF-β1) and matrices (procollagen subtype I, elastin synthase and HAS-2s) in rat laryngeal tissue (Lim et al., 2006; Welham et al., 2008) and then add a surgical trauma event.

The initial magnitude of mucosal injury, which denoted the surgical trauma event, was set at a value of 40 (range 0 – 40 in arbitrary units of damage), which represented a realistic prediction of mucosal damage and healing of high magnitude of surgical trauma. Simulation outputs for each inflammatory marker (IL-1β, TNF-α, TGF-β1) and matrix (procollagen subtype I, elastin synthase and HAS-2) in laryngeal tissue for the rat population were compared with those of the empirical data across 5 time points: 1hr, 4hr, 8hr, 16hr and 24hr following surgery (Lim et al., 2006; Welham et al., 2008). The model parameter values were iteratively adjusted to achieve optimal fit to the empirical laryngeal mRNA data. The quantitative verification-calibration iterative process was continued until the model eventually yielded a satisfactory match between simulation data and empirical data, based on subjective judgment.

#### 5.1.2.7 Model evaluation

Following quantitative verification-calibration, the calibrated ABM was tested for its accuracy in predicting population-trend mRNA levels of mediators and matrices at the 72-hr time point (Welham et al., 2008). The method of model evaluation was the same as for **Experiment 1** but the context was surgical trauma.

The user input the population's baseline levels of IL-1β, TNF-α, TGF-β1, procollagen subtype I, elastin synthase and HAS-2, and then added a surgical trauma event (the initial magnitude of mucosal injury = 40). The ABM was run 100 times up to five simulated days following surgery, in order to generate a representative data pool for the following statistical analysis (Kim, personal communication).

Subsequently, the ABM was statistically evaluated by comparing the predicted the levels of each inflammatory marker and matrix marker with the corresponding marker levels at 72 hr

for the rat population as a whole. A 95% confidence interval was computed for *each marker*, i.e., 6 confidence intervals in total (6 markers: IL-1β, TNF-α, TGF-β1, procollagen subtype I, elastin synthase and HAS-2) at the 72-hr time point from the simulation runs:

$$\text{Confidence interval} = \overline{X} \pm z * \left( \frac{\acute{o}}{\sqrt{n}} \right)$$

$\overline{X}$ = Mean of a predicted biomarker level

z = The value on the standard normal curve with area (1-α) between –z and z

σ = Standard deviation of a predicted marker level

n = Number of simulation runs

If the empirical result for a given marker fell within the 95% confidence interval from the simulation runs, the model was considered adequate to predict the levels of markers seen in the empirical experiment. As for **Experiment 1,** unadjusted alpha levels were used because we opted to protect from Type II (β) error more than Type I (α) error at this early stage of inquiry in predicting biological responses following surgical trauma to rat vocal folds.

# 6.0 RESULTS

## 6.1 QUALITATIVE VERIFICATION OF THE ABM

The new ABM was evaluated to determine if it reproduced generally recognized patterns of cellular and molecular responses according to the literature in surgical skin wound healing (Cockbill, 2002; Dechert et al., 2006; D. Jiang et al., 2007; P. Martin, 1997; Robson et al., 2001; Stern et al., 2006; Witte & Barbul, 1997) as well as in surgical vocal fold wound healing (I. Tateya et al., 2006; T. Tateya et al., 2005; T. Tateya, I. Tateya, J. H. Sohn et al., 2006) (Table 5 in Section 5.1.2.4). Simulations under high magnitude of initial injury input (setting = 40) were run up to 14 simulated days. Figures 10 – 12 show representative ABM simulations of cells, HA and collagen after qualitative verification-calibration. The simulated cellular and ECM dynamics from this general wound healing model showed good concordance with wound healing patterns reported in the literature (Table 4 in Section 5.1.1.10). This *qualitatively* calibrated ABM was then specified to the human phonotrauma ABM (Experiment 1) and animal surgical ABM (Experiment 2) through the process of *quantitative* model verification-calibration using laryngeal secretion data and tissue mRNA data respectively (Figure 8 in Section 5.1.1.9).

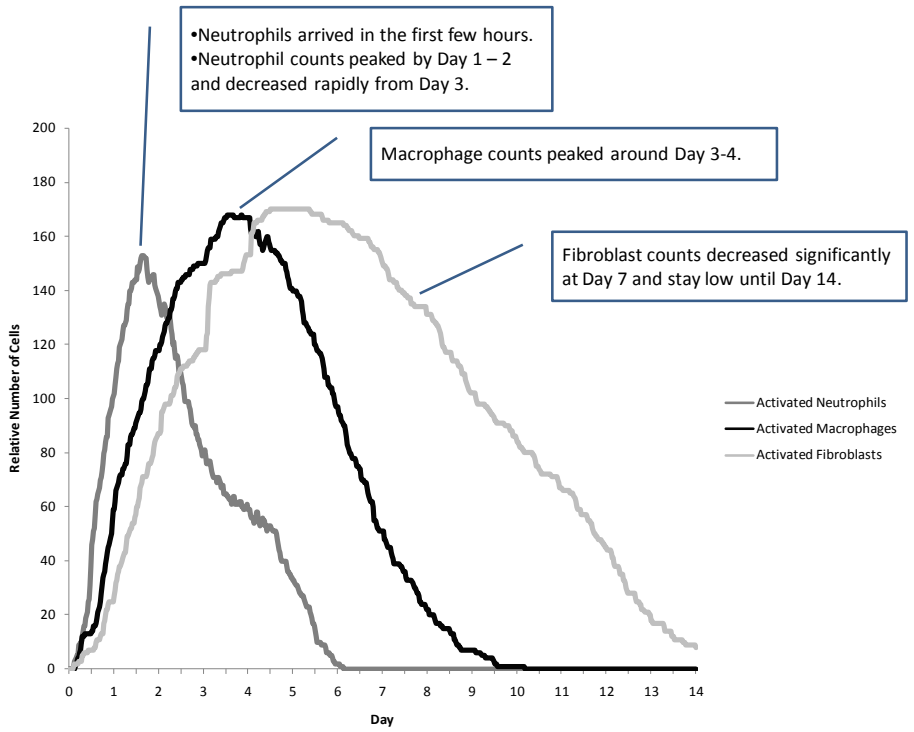


**Figure 10. Representative ABM simulation results after qualitative verification-calibration of the model. The dynamics of activated cells (neutrophils, macrophages and fibroblasts) were consistent with the literature on surgical skin and vocal fold wound healing (in boxes; refer to Table 4 in Section 5.1.1.10 for the complete list of validation patterns) up to 14 simulated days.**

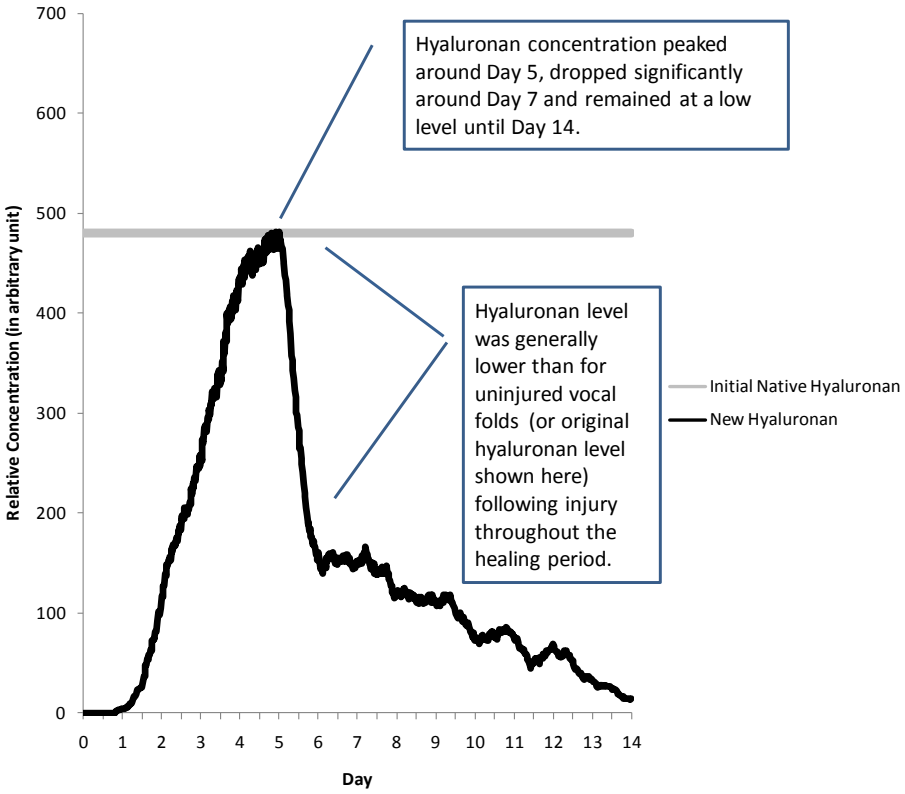


**Figure 11. Representative ABM simulation results after qualitative verification-calibration of the model. The dynamics for hyaluronan were in concordance with the literature on surgical skin and vocal fold wound healing (in boxes; refer to Table 4 in Section 5.1.1.10 for the complete list of validation patterns) up to 14 simulated days.**

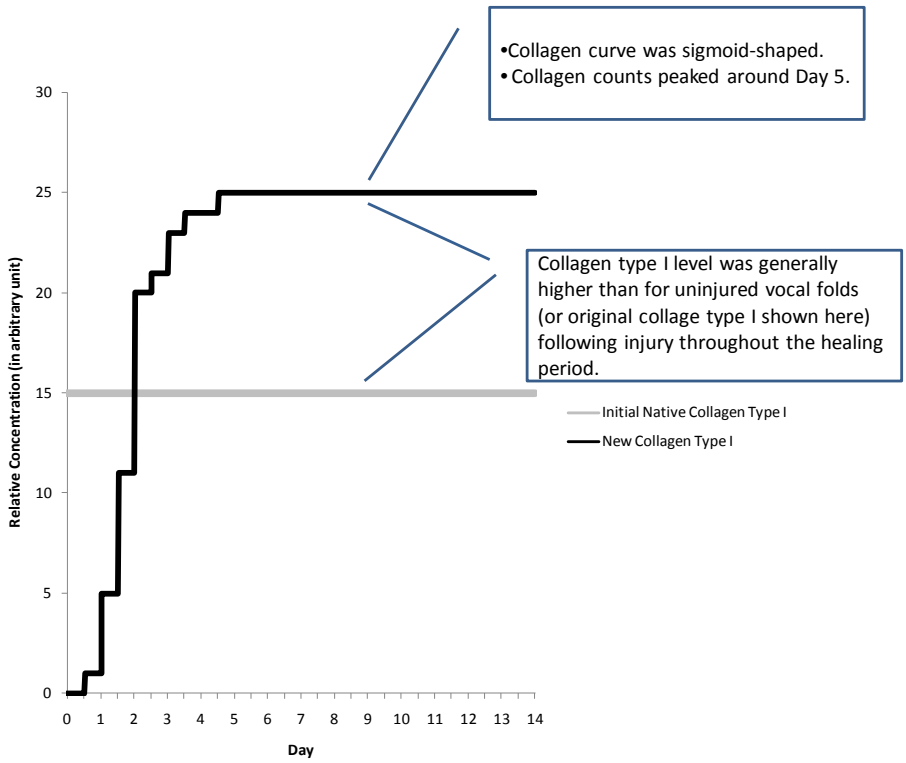


**Figure 12. Representative ABM simulation results after qualitative verification-calibration of the model. The dynamics for collagen were in concordance with the literature on surgical skin and vocal fold wound healing (in boxes; refer to Table 4 in Section 5.1.1.10 for the complete list of validation patterns) up to 14 simulated days**

## 6.2 EXPERIMENT 1: QUANTITATIVE CALIBRATION OF THE ABM PREDICTING HEALING OUTCOMES FOLLOWING HUMAN PHONOTRAUMA

Following qualitative verification-calibration of the model, the ABM was quantitatively calibrated using mediator levels in laryngeal mucosal secretion at baseline, immediately after phonotrauma induction, and following a 4-hr treatment (voice rest, resonant voice exercises, or spontaneous speech) from Subjects 1 – 3 (Figures 13 – 17, dark circles) (Verdolini et al., in preparation). Of note, Subject 3 was the within-group subject who had participated in all three treatments. The calibrated ABM was run 100 times for each subject to generate individual-specific predictions of mediator level for the full cohort of 7 subjects up to three days following phonotrauma and treatment. The ABM for Subjects 1 – 3 was run to determine how well the model predicted results for Subjects 4 – 7 by inputting individual-specific baseline biomarker levels and mode of treatments. For each mediator, the model's prediction accuracy was evaluated against the criterion of whether the empirical mediator level at 24 hrs fell within a 95% confidence interval for the mean of model predictions, in Subjects 1 – 3 (Figures 13 – 17, empty circles) and Subjects 4 – 7 (Table 6).

Although the same ABM for Subjects 1 – 3 was used to predict results for Subjects 4 – 7, the model inputs, i.e., the baselines of inflammatory mediators, were individual-specific. That is, empirical data from Subjects 4 – 7 were used for model input (baseline) for the simulation of Subjects 4 – 7. Also, the empirical data for validating the model's outputs at 24-hr time point were individual-specific. That is, empirical data for Subjects 1 – 7 were used to compare the model's predicted outputs for Subjects 1 – 7 respectively. The only difference between the simulations of Subjects 1 – 3 and Subjects 4 –7 was at the level of model calibration. Empirical data at immediate post-loading and after a 4-hr treatment from Subjects 1 – 3 were used for

estimating parameter values during model calibration, whereas none of the empirical data from Subjects 4 – 6 were used in this regard (c.f. Figure 8 in Section 5.1.1.9 for the procedures of the iterative verification-calibration process).

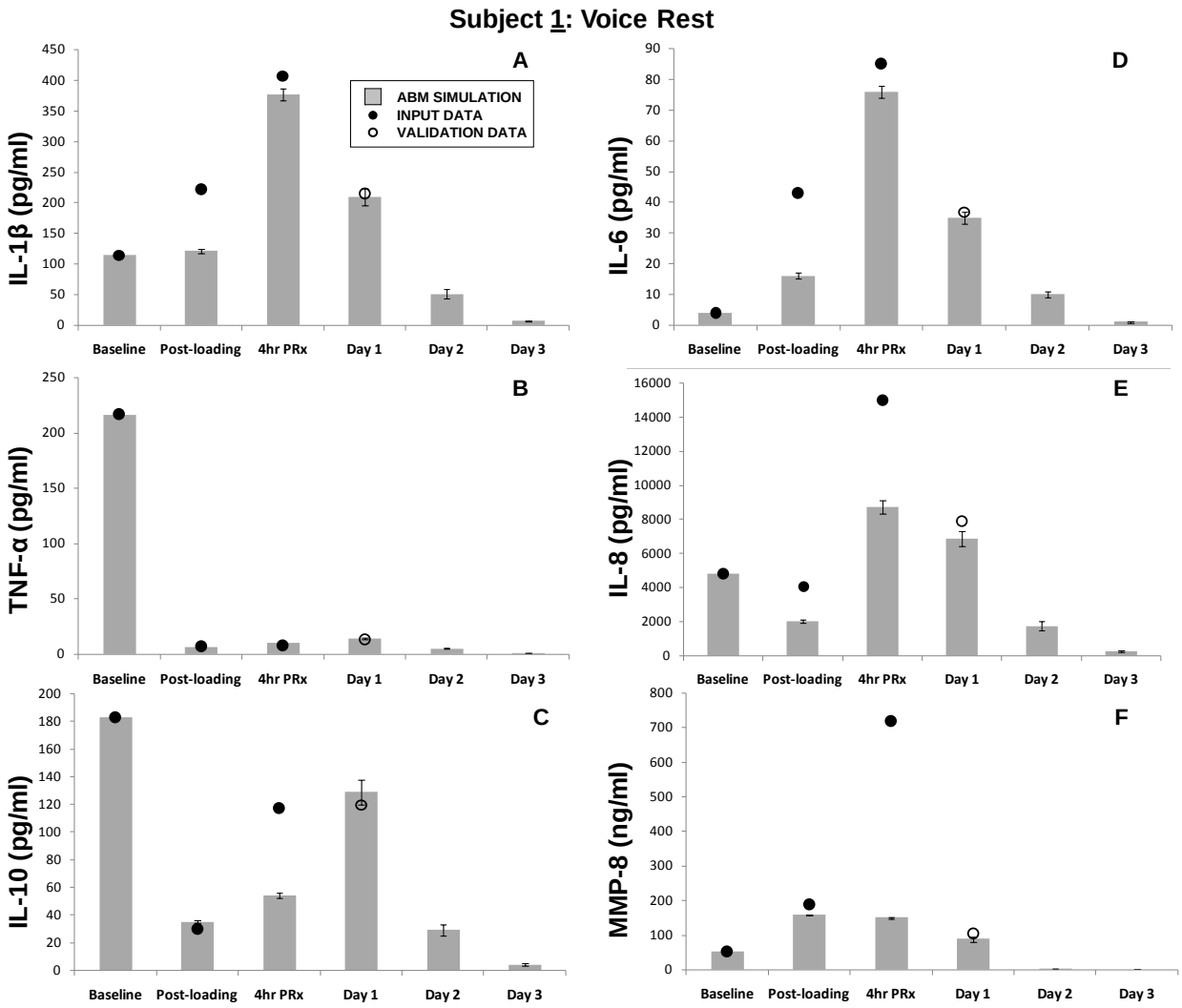


**Figure 13. Predictions of inflammatory and wound healing responses to acute phonotrauma in the between-group Subject 1 following a 4-hr voice rest treatment. Panels A – C are the predicted mediator trajectories for IL-1β, TNF-α and IL-10, which were included in the published ABM (N. Y. Li et al., 2008). Panels D – F are the predicted mediator trajectories for IL-6, IL-8 and MMP-8, which were the additional mediators in the current study. Inflammatory marker concentrations are in pg/ml. The grey bars represent the mean of the simulated data, and the error bars represent 95% confidence intervals in the simulated data. The dark circles represent the input data for the first three time points (baseline, post-loading, 4-hr post treatment), obtained from human laryngeal secretion data. The empty circles represent the validation data at the 24-hr time point from the human laryngeal secretion data. B: baseline; PL: post vocal loading; 4hrPRx: following a 4-hr treatment. Note that human validation data for Days 2 – 3 have not yet been generated.**

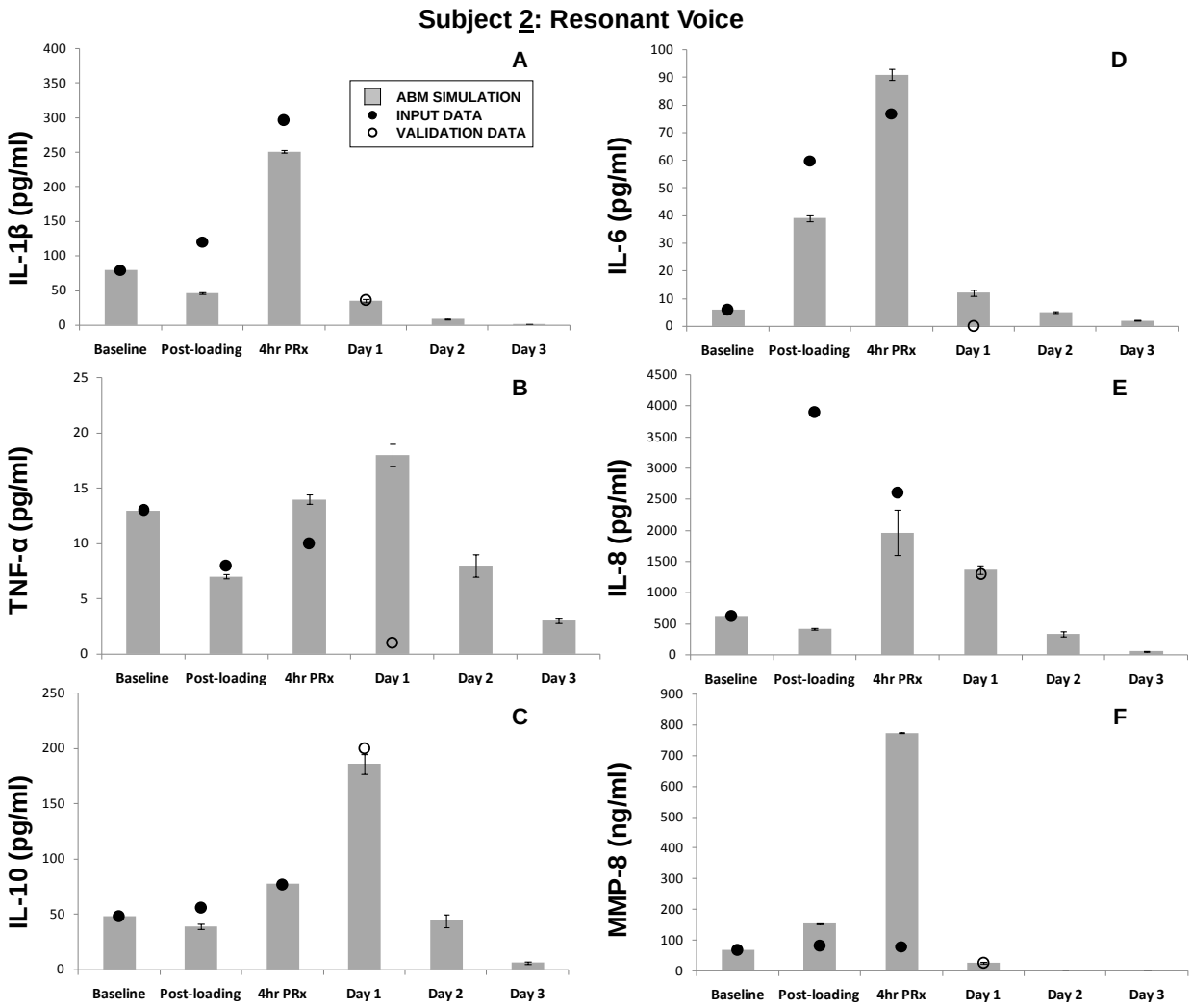


**Figure 14. Predictions of inflammatory and wound healing responses to acute phonotrauma in the between-group Subject 2 following a 4-hr resonant voice treatment. Panels A – C are the predicted mediator trajectories for IL-1β, TNF-α and IL-10, which were included in the published ABM (N. Y. Li et al., 2008). Panels D – F are the predicted mediator trajectories for IL-6, IL-8 and MMP-8, which were the additional mediators in the current study. Inflammatory marker concentrations are in pg/ml. The grey bars represent the mean of the simulated data, and the error bars represent 95% confidence intervals in the simulated data. The dark circles represent the input data for the first three time points (baseline, post-loading, 4-hr post treatment), obtained from human laryngeal secretion data. The empty circles represent the validation data at the 24-hr time point from the human laryngeal secretion data. B: baseline; PL: post vocal loading; 4hrPRx: following a 4-hr treatment. Note that human validation data for Days 2 – 3 have not yet been generated.**

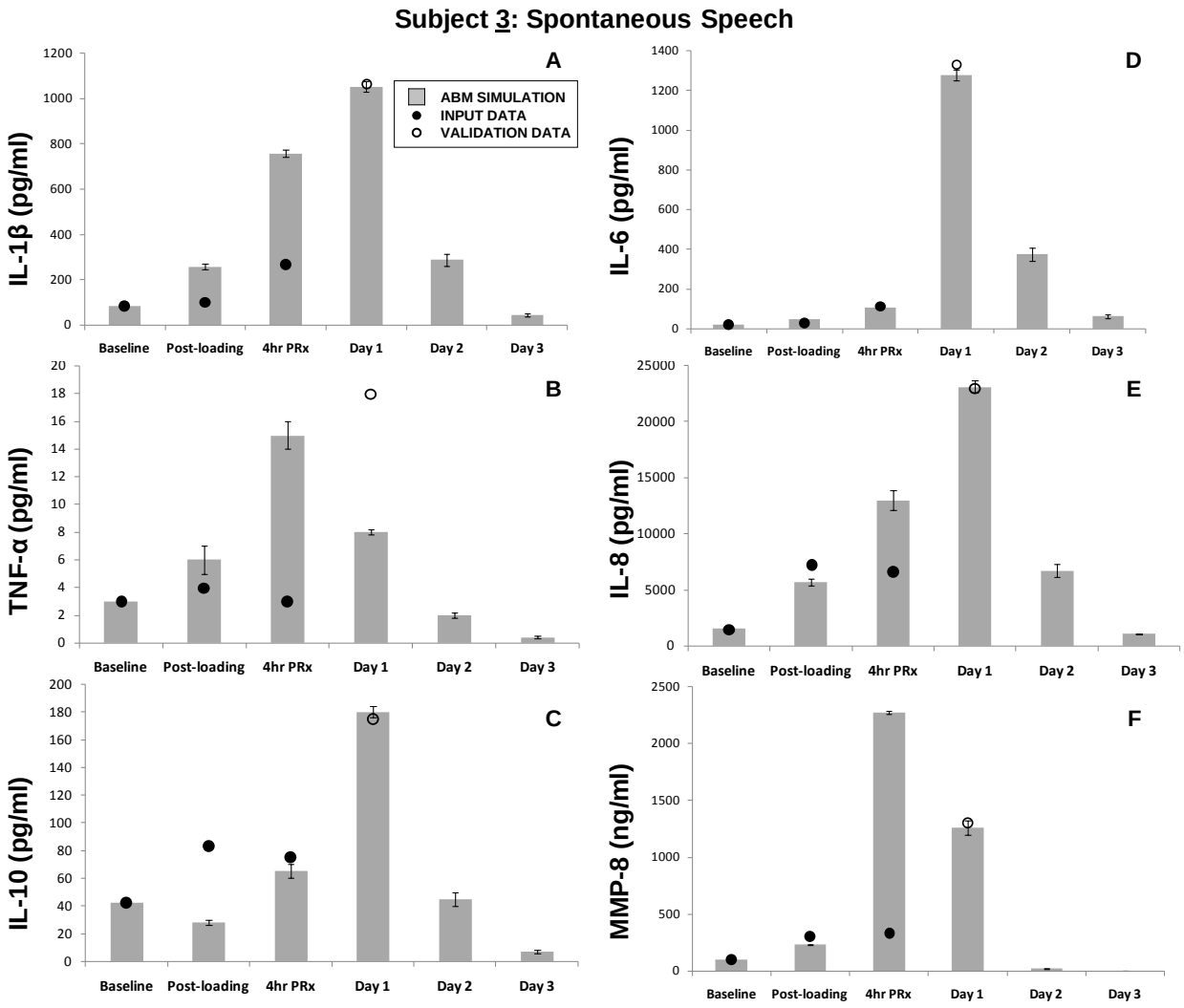


**Figure 15. Predictions of inflammatory and wound healing responses to acute phonotrauma in the single within-group Subject 3 following a 4-hr spontaneous speech treatment. Panels A – C are the predicted mediator trajectories for IL-1β, TNF-α and IL-10, which were included in the published ABM (N. Y. Li et al., 2008). Panels D – F are the predicted mediator trajectories for IL-6, IL-8 and MMP-8, which were the additional mediators in the current study. Inflammatory marker concentrations are in pg/ml. The grey bars represent the mean of the simulated data, and the error bars represent 95% confidence intervals in the simulated data. The dark circles represent the input data for the first three time points (baseline, post-loading, 4-hr post treatment), obtained from human laryngeal secretion data. The empty circles represent the validation data at the 24-hr time point from the human laryngeal secretion data. B: baseline; PL: post vocal loading; 4hrPRx: following a 4-hr treatment. Note that human validation data for Days 2 – 3 have not yet been generated.**

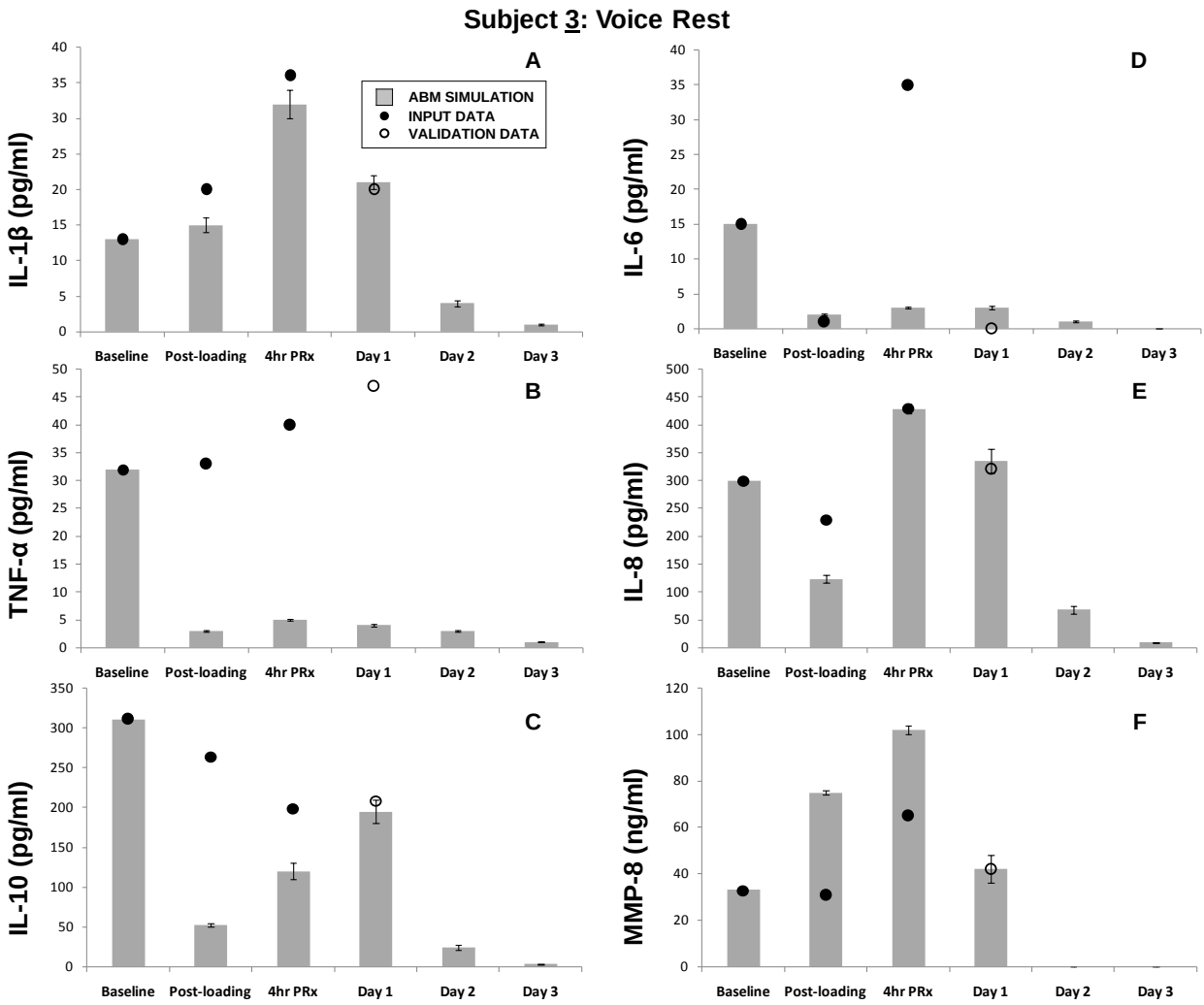


**Figure 16. Predictions of inflammatory and wound healing responses to acute phonotrauma in the single within-group Subject 3 following a 4-hr voice rest treatment. Panels A – C are the predicted mediator trajectories for IL-1β, TNF-α and IL-10, which were included in the published ABM (N. Y. Li et al., 2008). Panels D – F are the predicted mediator trajectories of IL-6, IL-8 and MMP-8, which were the additional mediators in the current study. Inflammatory marker concentrations are in pg/ml. The grey bars represent the mean of the simulated data, and the error bars represent 95% confidence intervals in the simulated data. The dark circles represent the input data of the first three time points (baseline, post-loading, 4-hr post treatment), obtained from human laryngeal secretion data. The empty circles represent the validation data at the 24-hr time point from the human laryngeal secretion data. B: baseline; PL: post vocal loading; 4hrPRx: following a 4-hr treatment. Note that human validation data for Days 2 – 3 have not yet been generated.**

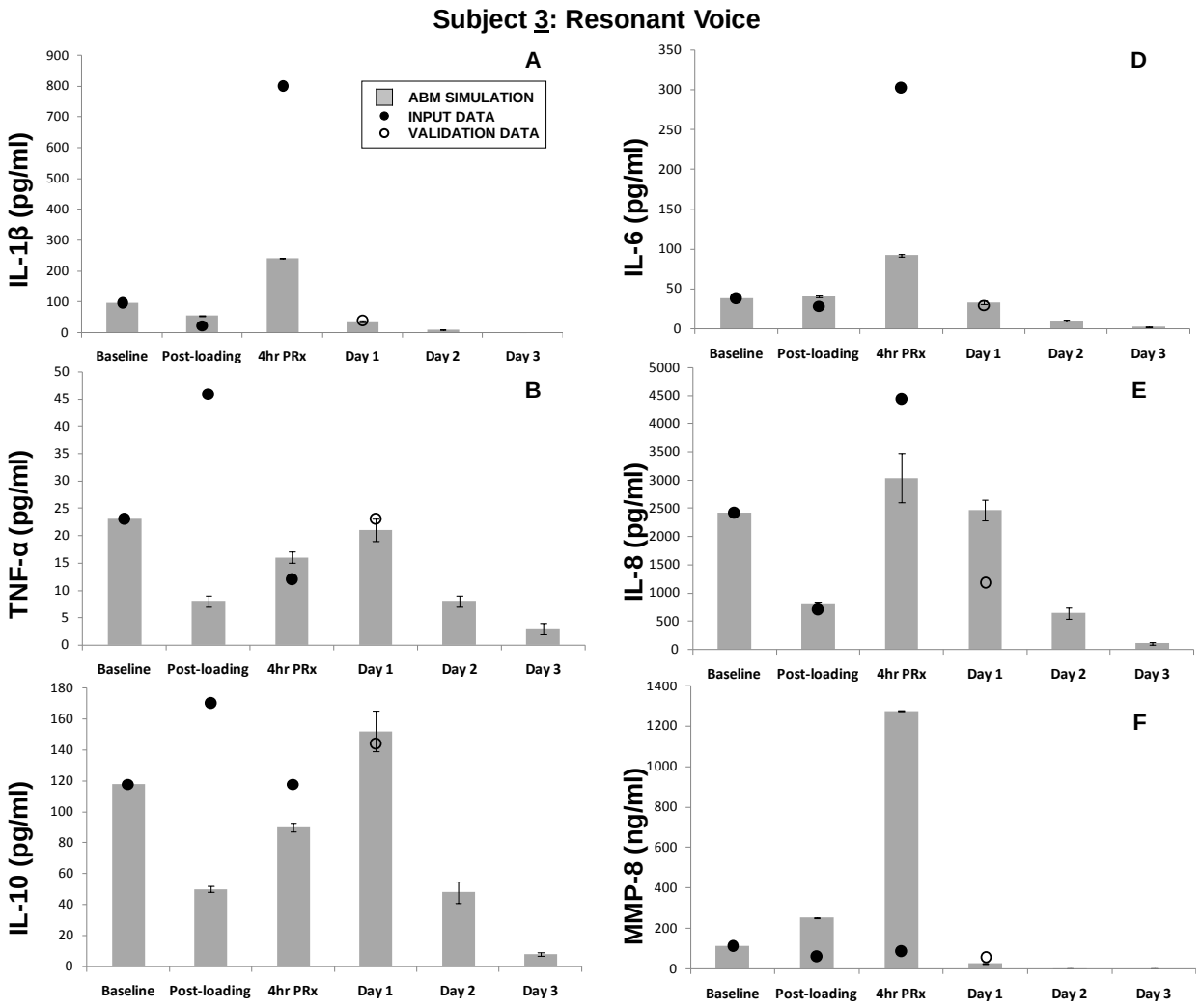


**Figure 17. Predictions of inflammatory and wound healing responses to acute phonotrauma in the single within-group Subject 3 following a 4-hr resonant voice treatment. Panels A – C are the predicted mediator trajectories for IL-1β, TNF-α and IL-10, which were included in the published ABM (N. Y. Li et al., 2008). Panels D – F are the predicted mediator trajectories of IL-6, IL-8 and MMP-8, which were the additional mediators in the current study. Inflammatory marker concentrations are in pg/ml. The grey bars represent the mean of the simulated data, and the error bars represent 95% confidence intervals in the simulated data. The dark circles represent the input data of the first three time points (baseline, post-loading, 4-hr post treatment), obtained from human laryngeal secretion data. The empty circles represent the validation data at the 24-hr time point from the human laryngeal secretion data. B: baseline; PL: post vocal loading; 4hrPRx: following a 4-hr treatment. Note that human validation data for Days 2 – 3 have not yet been generated.**

**Table 6. ABM Predictions of Mediator Levels for Subjects 4 – 7 at the 24-hr Time Point (Human Laryngeal Secretion Data). Predictions Indicate 95% Confidence Intervals. Parenthesis Indicate the Empirical Laryngeal Secretion Data From the Acute Phonotrauma Study. Checkmarks "√" Indicate (*Empirical*) Data that Fell within the 95% Confidence Interval.**

| | Subject (sex) | IL-1β (pg/ml) | IL-6 (pg/ml) | IL-8 (pg/ml) | TNF-α (pg/ml) | MMP-8 (ng/ml) | IL-10 (pg/ml) |
|---|---|---|---|---|---|---|---|
| **Spontaneous Speech** | **6 (F)** | 1015-1150 | 853-962 | 9260-10420 | 29-34 √ | 275-346 √ | 335-380 |
| | | *(466)* | *(98)* | *(1,443)* | *(32)* | *(276)* | *(202)* |
| **Voice Rest** | **5 (F)** | 1696-1757 | 223-237 √ | 5409-5600 | 37-40 √ | 86-95 | 255-314 √ |
| | | *(39)* | *(236)* | *(411)* | *(39)* | *(32)* | *(300)* |
| | **7 (F)** | 1154-1298 | 207-229 | 4861-5441 | 20-23 | 28-36 √ | 100-121 √ |
| | | *(30)* | *(15)* | *(744)* | *(49)* | *(28)* | *(120)* |
| **Resonant Voice** | **4 (M)** | 12-14 | 104-118 | 1633-1865 | 33-38 √ | 14-19 √ | 350-398 |
| | | *(34)* | *(371)* | *(701)* | *(34)* | *(18)* | *(213)* |

### 6.2.1 Predicted trajectories of inflammatory mediators for human phonotrauma simulations

The ABM reproduced and predicted most subject-specific mediator trajectories in Subjects 1 – 3 following phonotrauma and treatment (Figures 13 – 17). Specifically, the ABM predicted 24-hr mediator values in 73% of cases (22/30 were within the 95% confidence intervals: +18 mediator measurements from 3 between-group subjects, each with one of the 3 different treatments; +18 mediator measurements from one within-group subject – Subject 3 – who received each of the treatments; -6 mediator measurements to account for double-counting the "spontaneous speech" treatment for Subject 3 in both between- and within-group measurements; hence 30 different mediator measurements in total). Further post hoc analyses were as follows. Markers were broken down into two groups: (1) IL-1β, TNF-α and IL-10 as "old" markers that were propagated from the published model to the current model and (2) IL-6, IL-8 and MMP-8 as "new" markers that were added in the current model. The reason for this breakdown was to assess whether the addition of these markers affected the models' accuracy, and if so, in which direction.

For the "old" markers (IL-1β, TNF-α and IL-10), the expanded ABM predicted empirically obtained 24-hr mediator value in 80% of cases (12/15 cases were within the 95% of confidence interval). Interestingly, the exact same result was obtained for these markers in the earlier published ABM (N. Y. Li et al., 2008). For the "new" markers (IL-6, IL-8 and MMP-8), the ABM predicted 24-hr mediator values in 67% of cases (10/15 mediators were within the 95% of confidence interval).

For Subjects 4 – 7, none of whose empirical data were used for model calibration except their baseline mediator inputs, the ABM predicted 24-hr mediator values (1) in 42% of cases for

the “old” markers (i.e. 5/12 cases were within the 95% confidence interval for markers), and (2) in 33% of cases for the “new” markers (4/12 cases were within the 95% confidence interval) (Table 6). Thus, the prediction accuracy for “old” markers is higher than the “new” markers (44%; 4/9) in this expanded ABM. However, the prediction accuracy for these “old” markers slightly decreased in the expanded ABM, compared to the published ABM (50% ; 6/12) (N. Y. Li et al., 2008).

In sum, the introduction of additional components to the model reduced its prediction accuracy overall. Moreover, the ABM showed poorer prediction accuracy for Subjects 4 – 7 than for Subjects 1 – 3 whose data were used in calibration. This model breakdown revealed the interesting and important finding that patient-specific models that did not produce accurate population-general predictions. Thus, the models indeed speak to personalized medicine rather than generalized findings.

For the single within-group subject (Subject 3; Figures 15 – 17), both empirical data and simulation results showed that concentrations of inflammatory mediators had distinctive temporal and quantitative expression patterns across treatment assignment. For the spontaneous speech condition, the ABM predicted that the inflammatory response would be escalated, i.e., would involve massive secretion of both pro- and anti-inflammatory mediators following the 4-hr treatment. Specifically, concentrations of pro-inflammatory mediators (IL-1β, TNF-α and IL-8) and collagenase (MMP-8) reached their peaks at Day 1 post injury and resolved to baseline concentrations around Day 2 – 3 post injury. One anti-inflammatory mediator, IL-10, was also predicted to be secreted in great quantities by wound macrophages. The concentration of the “exercise factor”, IL-6, was increased after the 4-hr spontaneous speech treatment and remained elevated up to Day 1 post injury.

In contrast, under conditions of voice rest and resonant voice exercise, concentrations of pro-inflammatory mediators (IL-1β and IL-8) and collagenase (MMP-8) dropped rapidly after the 4-hour treatment and then remained low through the end of simulation, i.e., Day 3. IL-1β was particularly down-regulated following resonant voice exercise. The anti-inflammatory mediator IL-10 was predicted to be secreted rapidly after the 4-hr treatment and remained at a high level at Day 1 post injury. The concentration of "exercise factor", IL-6, was predicted to be strongly secreted following the 4-hr treatment and drop rapidly to a minimal level at Day 1 post injury. The degree of drop in IL-6 was lower following resonant voice compared to the voice rest condition. Similar mediator patterns were found in the larger dataset for the between-group subjects (Figures 13 – 15).

### 6.2.2 Predicted trajectories of cells, ECM synthesis and tissue damage for human phonotrauma simulations

For simulations of acute phonotrauma, the ABM predicted massive platelet infiltration and remarkable resident fibroblast activation during the first 12-hr period post injury, without the intervention of resonant voice or spontaneous speech (Figure 18a). Inflammatory cells (neutrophils and macrophages) were predicted to arrive at relatively later time points – between 12 hr and Day 1 post injury. Fibroblasts were the dominant cell type throughout the simulation period. HA was predicted to be secreted in large quantities by activated fibroblasts during the acute phase of healing (Figure 18b). This prediction is consistent with the findings in the general healing literature indicating HA accumulates during the earliest phase of inflammation and this accumulation is considered one of the cardinal signs of acute inflammation – edema formation (swelling) (D. Jiang et al., 2007; Stern et al., 2006). In fact, in a previous report, vocal fold

edema was visually observed in a subject immediately following 1 hr of loud phonation (74 – 121 dB @ 12 inches) (Verdolini, Rosen, Branski, & Hebda, 2003), suggesting a similar mechanism of rapid HA accumulation and edema in the vocal folds as in other tissues. In contrast, the ABM predicted minimal accumulation of collagen and elastin compared to HA following phonotrauma. This prediction suggested that microscopic tissue injury from a single event of phonotrauma – at least phonotrauma of the magnitude studied – is unlikely to lead to substantial loss of structural proteins (collagen and elastin), in which case fibroblasts would need to secrete massive collagen and elastin to repair the loss. In fact, abnormal collagen and elastin accumulation has rarely been reported in acute phonotrauma but has been commonly reported in benign vocal fold lesions (repeated phonotrauma) and in vocal fold scar (surgical trauma) (Hansen & Thibeault, 2006).

Tissue damage in the current model is considered as a general index of tissue health (Figure 18b). Tissue damage in this model was unitless. In the current ABM, the initial mechanical injury and the subsequent inflammatory response of collagenase (MMP-8) and reactive oxygen species (subsumed as TNF-α actions) broke down the native matrices into fragments, causing more tissue damage. Damage was predicted to spike immediately following injury and to resolve within the first 24 hours post injury (Figures 18b and 19). Within this time window, damage was predicted to be slightly lower following phonotrauma in subjects for whom voice rest and resonant voice exercise were prescribed, compared to the spontaneous speech condition (Figure 19; the shaded area). Also, the earliest possible time of complete tissue recovery was around 12-hr post injury in both voice rest and resonant voice conditions and around 18-hr post injury in spontaneous speech (Figure 19; the lower boundary of the shaded area). Compared to the voice rest condition, the range of tissue damage in the resonant voice

condition was generally larger from 5-hr to 12-hr post injury but smaller from 13-hr to 24-hr post injury. Thus, based on the current simulated tissue damage data, confidence around the absolute benefits of voice rest and voice exercise is somewhat reduced.

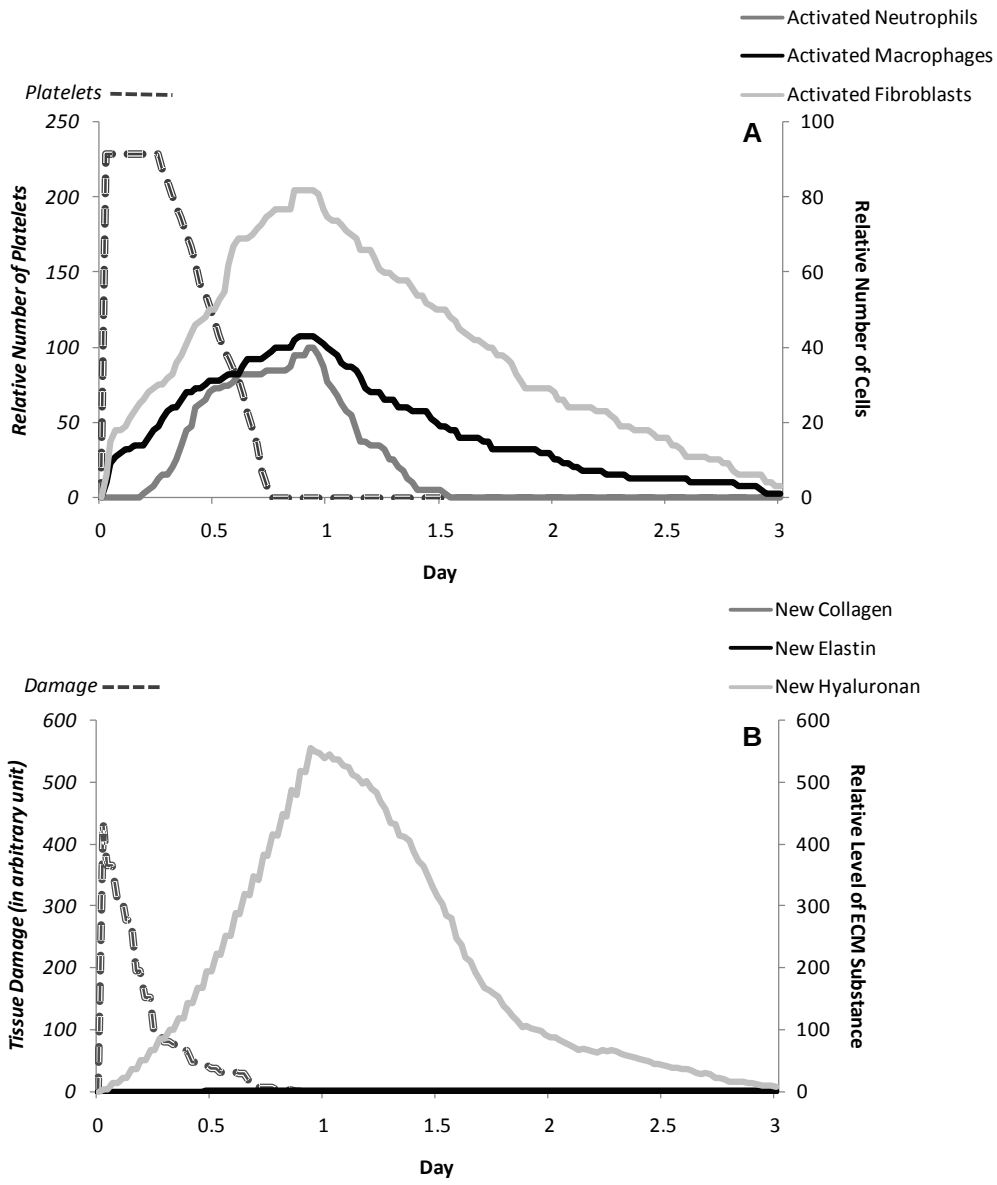


**Figure 18. Representative ABM predictions of cell counts, extracellular matrix (ECM) synthesis and amount of tissue damage in human acute phonotrauma up to 3 simulated days following injury. No tissue mobilization treatments (resonant voice or spontaneous speech) were applied in this prediction. Panel A is predicted cell trajectories for platelets, activated neutrophils, activated macrophages and activated fibroblasts. Panel B is predicted ECM trajectories for new collagen, new elastin and new hyaluronan as well as extent of tissue damage. Amount of tissue damage is in arbitrary units.**

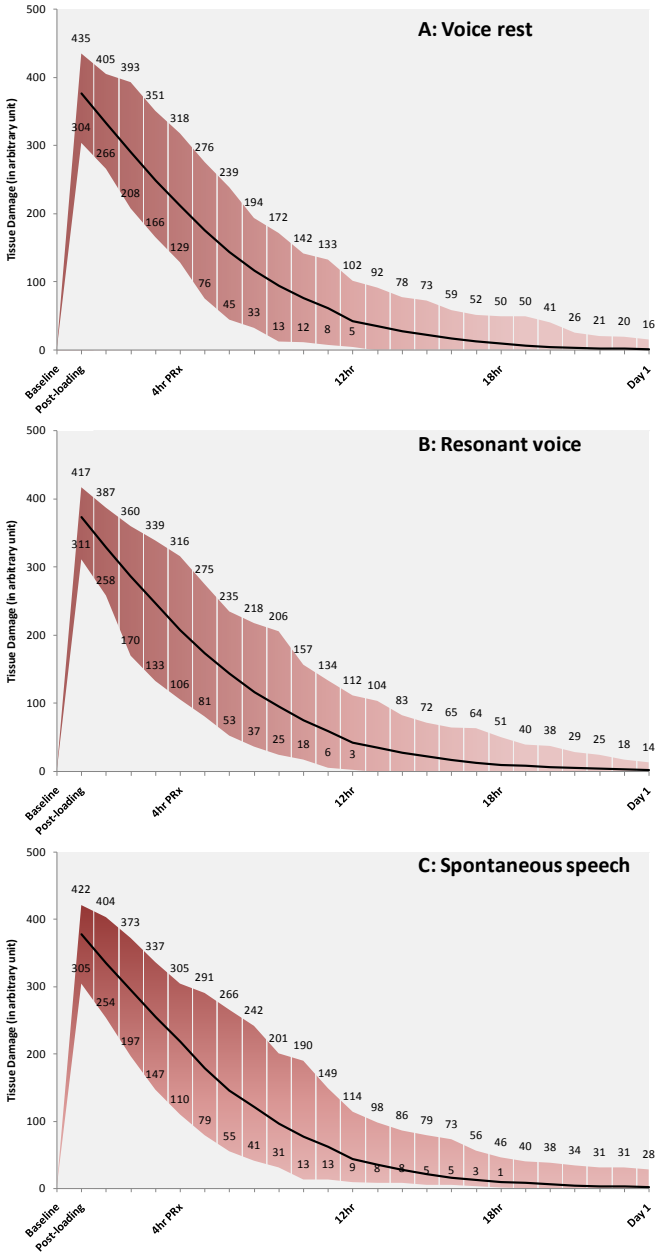


**Figure 19. ABM predictions of tissue damage following acute phonotrauma and a 4-hr motion-based treatment. Panel A is predicted tissue damage in Subject 1 following a voice rest treatment. Panel B is predicted tissue damage in Subject 2 following a resonant voice treatment. Panel C is predicted tissue damage in Subject 3 following a spontaneous speech treatment. The shaded area represents the range (maximum-minimum) of simulated tissue damage for each subject. The numbers on the upper and lower boundaries of the shaded area denote the maximum and the minimum amounts of predicted tissue damage respectively. The solid lines represent the mean of the simulated data. PRx: following a 4-hr treatment. Amount of tissue damage is in arbitrary units.**

## 6.3 EXPERIMENT 2: PREDICTION ACCURACY OF THE ANIMAL SURGICAL TRAUMA ABM

The animal surgical ABM was quantitatively calibrated using mean mRNA mediator levels in rat laryngeal tissues at baseline, 1hr, 4hr, 8hr, 16hr and 24hr following surgery (Lim et al., 2006; Welham et al., 2008). Individual data that categorized as "extremes" were excluded for model calibration and subsequent evaluation. The distribution of "extremes" was: 1 from IL-1β at 4 hours and 8 hours each, 1 from TNF-α at 8 hours, 1 from TGF-β1 at 72 hours each and, 16 hours, 24 hours and 72 hours each, 1 from procollagen subtype I at baseline, 1 hour and 24 hours each, 1 from elastin synthase at baseline and 24 hours each and 1 from HAS-2 at 1 hour. Table 7 lists the means and standard deviations of each marker at each time point after removing the extremes from the data pool.

**Table 7. Means (and standard deviations) of mRNA Expression Levels for Inflammatory Markers and Matrix Markers after Removal of "Extremes". "Extremes" were Values that were More than 3 Times the Interquartile Range, Defined by SPSS. This Data Set Corresponded to the Published Papers of Lim et al. (2006) and Welham et al. (2008) and was Graciously Provided by the First Authors (Lim and Welham) of these Two Papers.**

| Time | IL-1β | TNF-α | TGF-β1 | Procollagen subtype I | Elastin synthase | Hyaluronan synthase-2 |
|---|---|---|---|---|---|---|
| **Baseline** | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.0 |
| | (0.1) | (0.1) | (0.0) | (0.0) | (0.1) | (0.1) |
| **1 hour** | 652.1 | 7.7 | 0.5 | 0.0 | 0.2 | 0.0 |
| | (305.0) | (4.7) | (0.3) | (0.0) | (0.2) | (0.0) |
| **4 hours** | 5009.0 | 1.1 | 0.3 | 0.2 | 0.1 | 0.1 |
| | (2766.0) | (1.3) | (0.2) | (0.1) | (0.0) | (0.1) |
| **8 hours** | 6448.6 | 10.4 | 0.3 | 0.0 | 0.1 | 0.1 |
| | (4795.3) | (19.3) | (0.1) | (0.0) | (0.0) | (0.1) |
| **16 hours** | 707.8 | 0.1 | 0.3 | 0.1 | 0.1 | 1.0 |
| | (872.7) | (0.1) | (0.2) | (0.1) | (0.1) | (1.2) |
| **24 hours** | 268.3 | 1.3 | 0.4 | 0.0 | 0.0 | 0.1 |
| | (141.1) | (0.4) | (0.2) | (0.0) | (0.0) | (0.1) |
| **72 hours** | 80.4 | 0.3 | 0.6 | 1.6 | 0.1 | 0.5 |
| | (65.6) | (0.1) | (0.2) | (1.0) | (0.0) | (0.2) |

The calibrated ABM was run 100 times to generate population-trend predictions of mediator and ECM marker levels for the rat population up to five days following surgery. The model's prediction accuracy was evaluated against the criterion of whether the empirical mediator and ECM levels at the 72-hr time point fell within the 95% confidence interval for the mean of model predictions.

### 6.3.1 Predicted trajectories of inflammatory mediators and ECM markers for animal surgical vocal fold trauma simulations

The ABM generally reproduced and predicted population-trend inflammatory mediator and ECM marker trajectories in the rat population following surgical trauma (Figure 20). Specifically, the

ABM predicted 72-hr mediator values in 83% of cases (5/6 cases were within the 95% confidence interval). Overall, compared to the acute phonotrauma ABMs (published and Experiment 1), the current animal surgical ABM showed better predictive value possibly due to the calibration of more time points (5 calibration data points in the surgical ABM vs 2 calibration data points in the phonotrauma ABMs).

Both empirical and simulation results showed time-dependent changes in mRNA gene expression of mediators and ECM markers from rat vocal folds after injury. IL-1β was expressed massively and immediately following injury and remained elevated up to 8-hr following injury. IL-1β expression decreased notably 16 hr following injury and remained at a low level until the end of simulation, i.e., Day 5. TNF-α expression showed relative fluctuation across the simulation period. TGF-β1 expression increased progressively following injury up to Day 3, and was predicted to progressively drop through the end of the simulation.

Regarding the ECM markers, elastin synthase showed early expression immediately following injury and remained at a notable level until the end of simulation. Compared to elastin synthase, expressions of procollagen subtype I and HAS-2 revealed a more protracted course. Both procollagen subtype I and HAS-2 were predicted to be expressed in considerable amounts starting in Day 1 and to reach at a high level until Day 3 post injury.

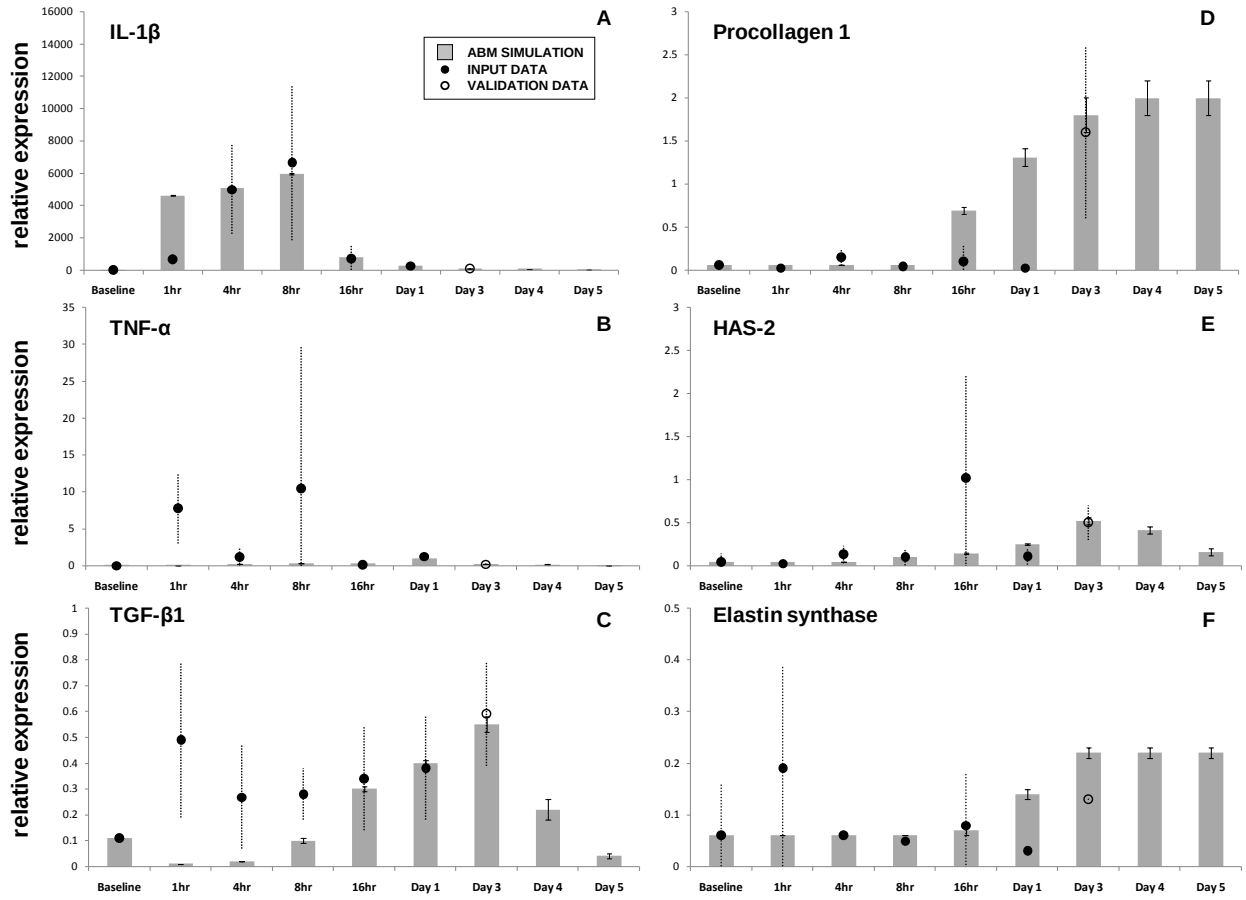


**Figure 20. Predictions of inflammatory and wound healing responses to surgical vocal fold trauma in the rat population. Panels A – C are the predicted mediator marker trajectories for IL-1β, TNF-α and TGF-β1. Panels D – F are the predicted ECM marker trajectories for procollagen 1, HAS-2 and elastin synthase. Marker concentrations are in relative mRNA expression. The grey bars represent the mean of the simulated data, and the error bars represent 95% confidence intervals in the simulated data. The dark circles represent the input data for the first six time points (baseline, 1-hr, 4-hr, 8-hr, 16-hr, Day 1 following surgery), obtained from rat laryngeal tissue data. The empty circles represent the validation data at the 72-hr time point from the rat laryngeal tissue data. The dashed lines represent the standard deviations of the empirical rat mRNA tissue data. Note that animal validation data for Days 4 – 5 have not yet been generated.**

### 6.3.2 Predicted trajectories of cells, ECM synthesis and tissue damage for animal surgical vocal fold trauma simulations

Triggered by the high-magnitude of tissue damage from surgical trauma, a large inflammatory response in the form of massive inflammatory cell infiltration was predicted. In particular, a large degree of platelets and neutrophil infiltration were predicted for the first 12-hr post-injury period (Figure 21a). Macrophages and fibroblasts were predicted to start accumulating in the wound area slightly after neutrophils – between 12-hr and Day 1 post injury. Neutrophil and macrophage counts declined from Day 3 post injury in the simulation, whereas fibroblasts remained at high levels until the end of simulation at Day 5. Compared to predictions for ECM levels for acute phonotrauma (Figure 18b), all ECM products, especially collage type I, were predicted to be secreted in greater quantities by activated fibroblasts following surgical trauma (Figure 21b) The first elevation of collagen was predicted to occur between approximately Days 1 and 3 post injury. In fact, collagen type I accumulation has been well described in the literature on scarred vocal folds following vocal fold "stripping" in different animal models (Hansen & Thibeault, 2006).

The predicted trajectory of tissue damage in surgical trauma (Figure 21b) was very different from that seen for acute phonotrauma in voice rest condition (Figure 18b). Damage was predicted to be higher and more prolonged following surgical trauma than in phonotrauma. Damage slowly subsided until Day 3, which temporally corresponded to the decline of inflammatory cell counts (neutrophils and macrophages). A small rebound "bump" was seen between Days 0.5 and 1 post injury. Of note, the timing of the "bump" in the damage curve was not programmed into the ABM code but rather emerged as a property of the system. This bump might be attributed to a positive feedback loop involved in the function of tissue damage.

Inflammatory cells produced positive feedback to induce further inflammation due to their collateral ECM damage (Figure 7 in Section 5.1.1.8), thereby amplifying the pro-inflammatory response and delaying the tissue healing process.

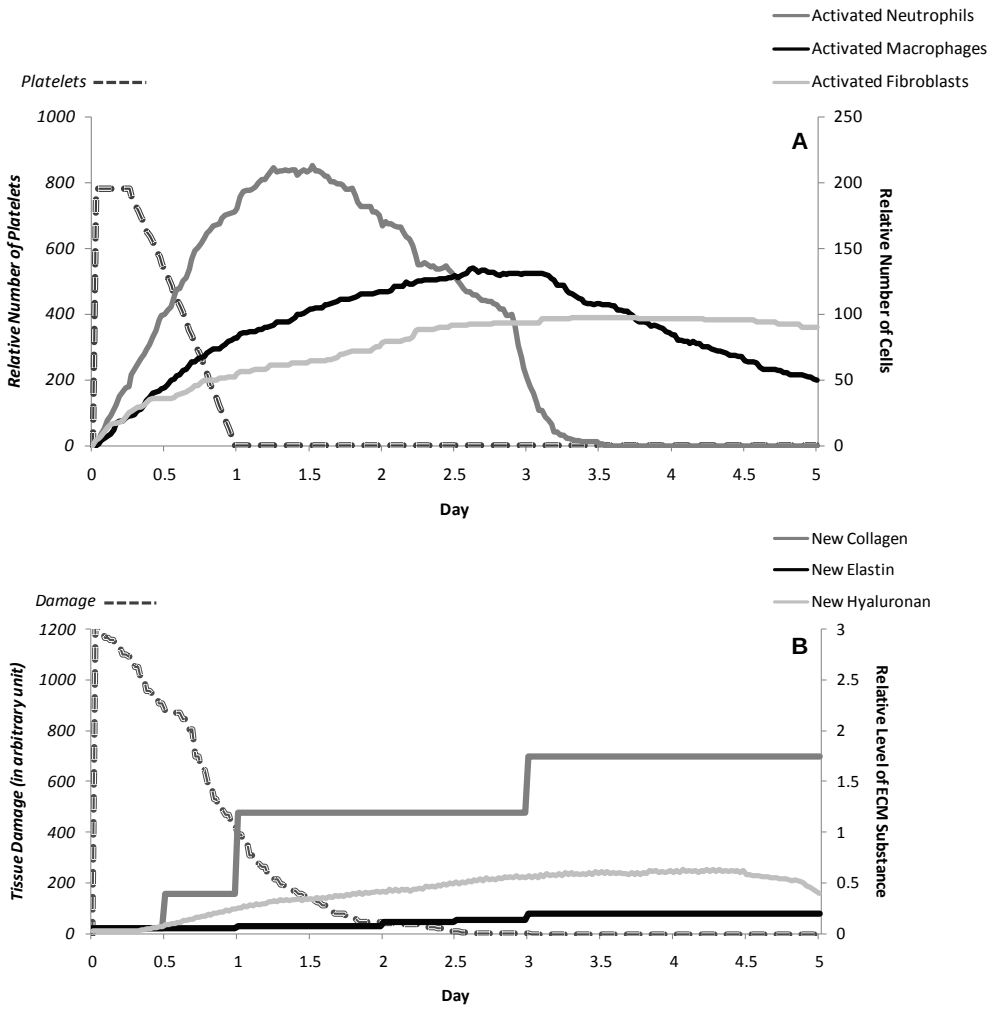


**Figure 21. Representative ABM predictions of cell counts, extracellular matrix (ECM) substance and amount of tissue damage in animal vocal fold surgical trauma up to 5 simulated days following injury. Panel A shows predicted cell trajectories for platelets, activated neutrophils, activated macrophages and activated fibroblasts. Panel B shows predicted ECM trajectories for new collagen, new elastin and new hyaluronan as well as extent of tissue damage. Amount of tissue damage is in arbitrary units.**

# 7.0 DISCUSSION

The published ABM (N. Y. Li et al., 2008) was deficient in: (1) the representation of vocal fold extracellular matrix (ECM) and its biochemical roles in mediating inflammation and healing, (2) the representation of inflammatory mediators and growth factors and their responses to tissue mobilization, and (3) the validation of the model's tissue-level outputs. This thesis project set out to address these shortcomings as follows.

First, the panel of ECM substances (collagen type I, elastin and hyaluronan [HA]) was now expanded in the current ABMs to create a more realistic replication of the ECM environment specific to the vocal folds. Also, fragments of the ECM substances ensuing from injury or inflammation were now involved in the function of "tissue damage" in the ABM, aiming to mimic alarm/ danger signals postulated in Matzinger's model of immunity (Gallucci & Matzinger, 2001; Matzinger, 2002).

Second, the profile of inflammatory mediators and growth factors (interleukin [IL]-1β, IL-6, IL-8, IL-10, tumor necrosis factor [TNF]-α, matrix metalloproteinase [MMP]-8, transforming growth factor [TGF]-β1 and basic fibroblast growth factor [bFGF]) was now expanded in the current ABMs. Each of these signaling molecules is known to be distinctively involved in the regulation of cell behavior as well as ECM production and remodeling during different phases of inflammation and healing. This model expansion would provide fuller coverage of the interplay among cells, inflammatory mediators, growth factors and ECM

substances in the progression of vocal fold healing following acute injury. In addition, IL-6, the identified "exercise factor" to which exercise benefits in muscle are attributed, was of particular interest in the current ABM. The noted biological effects of exercise-induced IL-6 were now abstracted and implemented under "treatment" simulations in the human phonotrauma ABM. With this addition, the ABM would have greater ability to elucidate cellular and molecular responses to varying magnitudes of biomechanical stresses (impact stress and vibratory stress) from each motion-based treatment in vocal fold healing following acute phonotrauma.

Finally, the protocol of model calibration-validation was now extended from molecular levels (using inflammatory mediator and growth factor data) to tissue levels (using ECM data) in the animal surgical trauma ABM. A biological system is organized in multiple levels from small (low) to large (high): molecules → cells → tissues → organs → organisms → populations. To understand the pathogenesis of vocal fold lesions, of particular interest in the current extended project, we need to know which lower biological levels of molecular events give rise to effects at higher levels of biological organization, to damage cells and tissues. Although the cross-scale calibration and validation of the ABM was exploratory in this study, the work will hopefully shed a ray of light on understanding the "net effect" of the complex interactions among inflammatory mediators at the tissue level in order to tease out certain molecular factors in the pathogenesis of vocal fold lesions.

Discussion now turns to detailed consideration of the major findings and potential implications from the augmented ABMs. Discussion concludes with the consideration of challenges and future research directions for ABM in the field of voice care.

## 7.1 ABMS PROVIDED A PLATFORM TO INTEGRATE, FORMULATE AND EXECUTE EMPIRICAL ASSUMPTIONS AROUND INFLAMMATION AND HEALING

This project demonstrated that modeling is a useful methodology to provide a quantitative framework for understanding how the many pieces of the biology puzzle fit together. Vast biological data relating to vocal fold healing behavior have been accumulated across several laboratories. Still, only a small fraction of the system behavior in vocal fold inflammation and healing is represented in a clinically useful way. To obtain a more complete understanding of biological systems, a unified representation of the system is required to integrate different scales of data or postulations drawn from the data. Computational biological models, such as ABMs in this study, are used to integrate various biological events and represent biological reality as an abstraction with clinical relevance.

Computational models are ideally built on available empirical data but they may also be based on some educated assumptions derived from theories or anecdotal evidence. To check the correctness of the assumptions in the model, we can execute the models and then compare the model's behavior with laboratory-based observation. If the biological model's behavior is consistent with empirical data, confidence increases in the hypothetical mechanisms or assumptions underlying the model's behavior in the current understanding of the system.

Current ABMs (Experiment 1: phonotrauma ABM; Experiment 2: surgical vocal fold trauma ABM) are based on three major assumptions: (1) platelets are the major cell source of mediator secretion during the earliest phase of healing, (2) the initiation of injury-induced inflammation in phonotrauma and surgical trauma is centered around the release of "alarm/ danger" signals from stressed or damaged tissues, and (3) the mode of the tissue's adaptive response to disturbance is related to the magnitude of mechanical stress through the induction of

an array of “alarm/ danger” signals as well as downstream pro- and anti-inflammatory mediators. The current ABMs’ outputs were compared with empirical data around inflammation and healing obtained from laryngeal surface secretions in humans (Experiment 1) or laryngeal tissues in rats (Experiment 2). Both models generally reproduced empirically expected trajectories of inflammatory mediators and ECM substances following vocal fold injury, suggesting the plausibility of the aforementioned postulations underlying the system of vocal fold wound healing.

### 7.1.1 Current ABMs confirmed the cell source of early inflammatory mediator secretion following vocal fold injury

The presence of platelets has not been reported in any studies of vocal fold wound healing. However, Welham et al. (2008) postulated that the early release of inflammatory mediators into wounded vocal folds was highly dependent on platelets and probably some resident cells following surgical trauma. In the current study, parameters of the surgical ABM were calibrated with empirical data up to the first 24 hrs post injury. Within this time window, the adjustment of platelet and activated macrophage parameters was noted to be the most effective one to improve a match between model outputs and experiment data. This observation was consistent with Welham’s assertion that platelets and possibly some resident cells may be involved in mediator secretions immediately following surgical vocal fold trauma.

On the other hand, parameters for the phonotrauma ABM were calibrated with empirical data only up to the first 4 hrs post injury (i.e., up to the empirical time point of 4-hr post treatment onset). Given this time window, the adjustment of platelet parameters was noted to be the most effective one to improve the matching of simulated outputs and experimental data.

Varying parameters of inflammatory cells (activated neutrophils and macrophages) did not markedly improve the match for this early time window, even though these two cell types produced some overlap of inflammatory mediators (IL-1β, TGF-β1 and MMP-8) with platelets.

In wound healing, this early time window corresponds to the platelet-dominant hemostasis response and probably the transition to a neutrophil/ macrophage-dominant inflammatory phase depending on the extent of injury. During hemostasis, platelets aggregate to the wound site and a fibrin clot is formed to stop the bleeding at the site. Platelets also release multiple mediators to activate the subsequent inflammation cascade. Hemostasis is the first important line of defense to control the extent of injury, especially in the case of extensive trauma following surgery. The current phonotrauma ABM predicted that platelets were markedly present and seemed to be the major source of mediators shortly after injury (Figure 18a). Although the magnitude of injury in phonotrauma was relatively smaller than injury in surgical trauma, this ABM's prediction suggested that vocal loading could probably change properties of blood capillaries followed by platelet aggregation.

### 7.1.2 The current ABMs applied the "alarm/ danger" theory to simulate injury-induced inflammation and healing

The current ABMs incorporated the concept of Matzinger's "alarm/ danger" model to simulate the vocal fold inflammatory response following mechanical injuries: phonotrauma or surgical trauma (Gallucci & Matzinger, 2001; Matzinger, 2002). Specifically, inflammation is activated by the innate immune system in response to noxious stimuli. The classical immunology model of "self-non-self" discrimination stipulates that the immune system does not react against the self but reacts against the non-self or foreign agents such as pathogenic bacteria, viruses and parasites

(C. Janeway, 1989; C. A. Janeway, Jr., 1992). Matzinger proposed a competing "alarm/ danger" model (Gallucci & Matzinger, 2001; Matzinger, 2002), suggesting that the innate immune response is induced as a result of "danger" detection, rather than the discrimination between "self" and "non-self" antigens.

The central difference between the "self-non-self" model and the "alarm/ danger" model lies with the way in which an innate immune response is triggered. According to the "self-non-self" discrimination model, an organism classifies its own cells as self and everything else as non-self by recognizing proteins found on the surface of foreign cells, i.e., antigens. The surface proteins of foreign cells are different in structure and shape from the host cell's surface proteins, i.e., self-antigens. The "self-non-self" model assumes that the detection of a foreign agent is sufficient to initiate the innate immune response and direct the adaptive response to external entities, i.e., non-self antigens. However, the "self-non-self" model has limited ability to explain many scenarios where "self-non-self" classification fallacies occur. For instance, no immune reaction is triggered to the bacteria in our intestinal tracts or to the food we eat or to the air we breathe that are classified as "non-self". Also, in auto-immune diseases, the immune system is triggered to attack certain host's cells that are classified as "self".

The "alarm/ danger" model offers a potential explanation for the scenarios the "self-non-self" model missed. The "alarm/ danger" model proposes that the innate immune response is provoked by the presence of antigens in the context of tissue damage. The body does not need to attack everything that is foreign, unless there is good evidence that cells are injured, stressed or destroyed by necrosis. In other words, the body is more alarmed by disruption of homeostasis or damage than with foreignness. The "alarm/ danger" theory postulates that an immune response is triggered when innate immune cells recognize "signs" of cell or tissue distress. These signs,

termed as “alarm/ danger signals”, are molecules that are found exclusively inside cells normally but are released into the extracellular space from injured or stressed cells subsequent to exposure to pathogens, toxins, or mechanical stimuli.

Inflammation is an innate immune response that can be triggered by infection or injury. In the vocal folds, inflammation caused by phonotrauma and surgical trauma is primarily induced by mechanical injury. Bacterial infections are rarely associated with phonotrauma or surgical vocal fold trauma. Hence, in these two examples of vocal fold injuries, the principle purveyor of the innate immune response to activate injury-induced inflammation is essentially from endogenous products of distressed tissues, rather than not from foreign invaders. For example, ECM fragments generated from sterile injury such as exercise and surgery constitute the endogenous signals of inflammation. The fragments function as “alarm/ danger signals” that alert the immune system to cell or tissue damage in the absence of infection. Also, the fragments provide a ready means to recruit innate inflammatory cells and elicit an inflammatory response and subsequent wound healing cascade to damaged tissues (Matzinger, 2002; Rubartelli & Lotze, 2007). In that regard, Matzinger’s “alarm/ danger” model is more appropriate than the “self-non-self” model as a central concept in the current construction of ABMs, in which the driving force for the inflammatory response is not from the recognition of foreign bodies but rather from cells or tissues undergoing injury or stress.

The “alarm/ danger” theory fit nicely fitted with the current ABMs. ECM fragments, including collagen type I, elastin and HA, were coded as “alarm/ danger signals” in the algorithm of “tissue damage” that initiated, sustained or amplified inflammation. Native ECM was degraded into fragments due to initial mechanical stimuli and subsequent inflammatory responses, where degradation occurred by way of collagenase MMP-8 as well as oxidative stress

subsumed in the actions of TNF-α. The functions of ECM fragments are two-fold: pro-inflammatory (such as recruitment of inflammatory cells and stimulation of inflammatory mediator secretion) and anti-inflammatory (such as recruitment of repair cells and stimulation of collagen production) (Table 2 in Section 5.1.1.6). Therefore, a balance between pro-inflammatory signals and anti-inflammatory signals determined the final healing outcome of tissue damage.

The trajectories of simulated tissue damage were predicted to have distinctive temporal and magnitude patterns across phonotrauma and surgical trauma. After phonotrauma, simulated tissue damage was predicted to be low and to resolve promptly within 24 hr post injury (Figures 18b and 19). On the other hand, simulated tissue damage was predicted to be high and persist for at least three days following surgical trauma (Figure 21b). Also, a small rebound "bump" in tissue damage was predicted between Days 0.5 and 1 post injury exclusively in surgical trauma. This damage rebound might be attributed to the following cellular and molecular events. Large magnitude of surgical trauma resulted in extensive ECM disruption and generated large amounts of ECM fragments. These fragments constituted strong "alarm/ danger signals" and triggered an exaggerated inflammatory response. Excessive inflammatory cells were recruited and activated. The inflammatory cells secreted collagenase and reactive oxygen species (subsumed as TNF-α actions in current ABMs) in the wound site, leading to collateral tissue damage. The "alarm/ danger" signals in the form of ECM fragments were further amplified, whereas the anti-inflammatory response was comparatively small and sufficiently prompt to counteract excessive collateral tissue damage. As a result, the inflammatory response following surgical trauma was prolonged and the transition to the healing phase was delayed.

Of note, the cellular and molecular events involved in injury-induced inflammation are not as well studied as those of infection-induced inflammation (Medzhitov, 2008). There is uncertainty about the extent to which knowledge gained about infection-induced inflammation may apply to injury-induced inflammation. For instance, the way that an immune response is initiated is markedly different across these two types of inflammation. Nevertheless, current work confirmed the proposition suggested by the "alarm/ danger" theory that the immune system reacts to situations that are potentially harmful to the body even in the absence of foreign substances. The "alarm/ danger signals" were made realistic within computer models *in silico*. Current ABMs yielded a clear view on the generation and flow of the "alarm/ danger signals" within the system of inflammation and healing, and such signals can be potential targets for manipulation to optimize healing outcomes following tissue damage or stress.

### 7.1.3 The current ABMs illustrated a spectrum of inflammatory responses to phonotrauma, surgical trauma and motion-based treatments

Current ABMs aimed to simulate (1) the inflammatory and repair response within vocal fold tissues following phonotrauma and surgical trauma, as well as (2) effects of tissue mobilization in modulating the inflammatory responses following phonotrauma. The ABM simulation results essentially illustrated the central idea around inflammation described by Medzhitov (2008) – inflammation is a *spectrum* of *adaptive* response to noxious stimulus. Depending on the type and magnitude of disturbance, the state of the tissue can be altered from basal, to stressed (or malfunctioning), to damaged. Consequently, stress response and tissue adaptation with varying magnitudes are induced: from homeostasis, to para-inflammation, to inflammation (Figure 22) (Medzhitov, 2008).

In Medzhitov's framework, resident-tissue macrophages and fibroblasts maintain normal tissue turnover by a variety of homeostatic control mechanisms in the basal state. When the tissue state is disturbed to an extent that the homeostatic mechanisms cannot handle, different types of stress response and tissue adaptation are initiated. At the highest end of the spectrum of the response is the classic inflammation, which is triggered following tissue damage. This full-scale inflammatory response is characterized by massive leukocyte influx and scar formation. Medzhitov (2008) proposed that an intermediate stress response between homeostatic control and a full-scale inflammation, which is termed as "para-inflammation", is induced by mild tissue stress without significant tissue injury. Resident cells are engaged in para-inflammation to assist tissues in adapting to the stressful condition and restoring tissue functionality and homeostasis. Additional resident cells or leukocytes may be recruited if the para-inflammatory response is close to the inflammatory state. Unlike having scar as a distinctive trait of the classic inflammation, the outcome of stress adaptation in para-inflammation has not yet been characterized (Medzhitov, 2008). Discussion now turns to how Medzhitov's framework explains (1) differentiated inflammatory and healing responses between phonotrauma and surgical trauma as well as (2) differences in biological effects following three types of motion-based treatments.

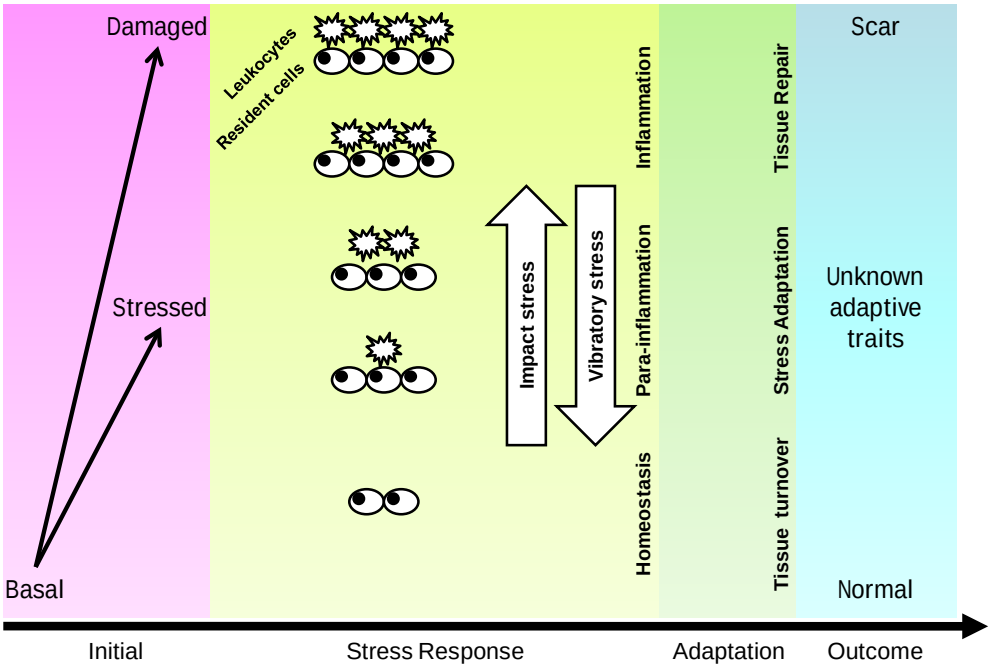


**Figure 22. Schematic representation of (1) three modes of stress response and tissue adaptation in the setting of the vocal folds based on Medzhitov (2008) and (2) proposed mechanisms of phonatory stress in modulating the stress response following phonotrauma. The state of a tissue is graded, ranging from basal, to stressed, to damaged. Depending on the initial tissue state, tissue responses are varied by engaging different cell types. In basal conditions, tissues are maintained in a homeostatic state primarily by resident cells. Tissue damage from surgical vocal fold trauma induces a full-blown inflammatory response. Resident cells and leukocytes are recruited and activated in the wound site. Consequently, a tissue repair response is initiated and a scarred tissue is likely formed. In contrast, no overt tissue destruction but rather only mild tissue stress is expected following phonotrauma. An intermediate para-inflammatory response ensues and resident cells are engaged for tissue adaptation to the noxious stimuli. Depending on the extent of the problem, a small-scale recruitment of leukocytes or additional recruitment of resident cells might be involved in para-inflammation. The outcomes of stress adaptation remain to be characterized clinically. Biomechanical stress in phonation, namely vibratory stress and impact stress, are hypothesized to modulate the inflammatory response within stressed tissues (relevant discussion is in Section 7.1.3.2). Impact stress is regarded as a "damaging force", which signals recruitment of leukocytes and pro-inflammatory responses in cells and thus escalates stress response. Vibratory stress is regarded as a "healing force", which signals anti-inflammatory actions in cells through the induction of IL-6 and thus attenuates the stress response.**

#### 7.1.3.1 Differentiated inflammatory and healing responses under phonotrauma and surgical trauma

The current ABMs generally reproduced and predicted differentiated inflammatory and healing patterns across phonotrauma and surgical trauma. Tissue fibroblasts were predicted as dominant in phonotrauma-induced inflammation. Also, minimal depositions of collagen type I and elastin but relatively large quantities of HA deposition were expected throughout the healing process following phonotrauma. On the other hand, surgical trauma was expected to induce massive inflammatory cell infiltration concurrent with activation of tissue fibroblasts and a related tissue repair program. Collagen type I was expected to be secreted in relatively greater quantities than those for elastin and HA to repair the surgical wound. Aforementioned inflammatory patterns from ABM simulations appeared to conform to Medzhitov's hypothesized spectrum of tissue stress adaptation to different tissue states (2008) (Figure 22).

According to Medzhitov's framework, relatively mild stress occurring with phonotrauma induced intermediate para-inflammatory responses followed by tissue stress adaptation. Phonotrauma due to 1 hr of vocal loading did not necessarily cause extensive tissue damage but simply stressed the tissues sufficiently to call resident fibroblasts into action. Massive inflammatory cell infiltration and scarring were not expected by the simulation of acute phonotrauma, which is consistent with the anticipated response and outcome associated with para-inflammation in Medzhitov's framework (2008).

On the other hand, high magnitude stress as occurs in surgical trauma induced a full-scale inflammatory response followed by tissue repair with scar formation. The massive tissue damage resulting from surgical trauma may exceed the level that para-inflammatory responses can handle. The strong surgical stress broke down a substantial area of ECM within mucosal tissues,

subsequently generated strong “alarm/ danger” signals, attracted large amounts of neutrophils and macrophages accompanied by collateral tissue damage and then further amplified “alarm/ danger” signals in the system. Meanwhile, fibroblasts were activated to secrete ECMs in an attempt to “patch” the wound. Damaged tissues would be repaired with excessive collagen deposition as predicted by the ABM, a finding that concords with the anticipated outcome of full-scale inflammation in Medzhitov’s framework (2008). In fact, increased collagen content in scarred vocal folds relative to normal vocal folds following laryngeal surgery has been commonly reported empirically and clinically (Hansen & Thibeault, 2006).

Of note, the differences in the inflammatory response between phonotrauma and surgical trauma were not “programmed” in the ABMs but rather emerged by themselves. For instance, not a single line of code was written in the model saying that only neutrophils were recruited in surgical trauma but not in phonotrauma. The wiring of the phonotrauma and surgical trauma ABMs was identical, except for differences in parameter values and initial inputs of injury magnitude. We postulated that the initial magnitude of stress or injury (ranging from 0 – 40; phonotrauma as 20; surgical trauma as 40) was the main factor to generate the inflammatory response. In other words, the magnitude of the immune response reflects the extent of injury. Depending on the magnitude of initial stress, affected tissues generate varying amounts of ECM fragments, which act as “alarm/ danger” signals to vary the magnitude of stress response across homeostasis, para-inflammation and full-blown inflammation. Nevertheless, current ABM results echoed Matzinger’s “alarm/ danger” concept of initial immune regulation as well as Medzhitov’s suggestions regarding a spectrum of inflammatory responses.

#### 7.1.3.2 Biological effects of motion-based treatments in acute phonotrauma

In Experiment 1, the phonotrauma ABM had the capability of testing three motion-based treatment regimes *in silico*. Voice rest, resonant voice exercise and spontaneous speech, which were the three motion-based treatment modalities, can be considered on a continuum of vocal fold mobilization magnitude and vocal fold impact stress magnitude: none to minimal mobilization and impact stress (voice rest), normal to large amplitude vocal fold oscillations and low impact stress (resonant voice exercises) and normal to large amplitude oscillations but potentially larger impact stress (spontaneous speech), depending on the speaker (Peterson et al., 1994; Verdolini et al., 1998).

The current work successfully incorporated the effects of mechanical stresses in the construct of the biologically-based model (Figure 22). Impact stress from phonation was constructed as the "damaging stress", which increased the number of circulating neutrophils in capillary compartments. Due to the critical role of neutrophils in most inflammatory events, an increase in circulating neutrophils counts could escalate the inflammatory response. On the other hand, vibratory stress, another stress component during phonation, was construed as a "healing stress". Vibratory stress was linked to the anti-inflammatory response through the induction of IL-6 by macrophages and fibroblasts. IL-6 was noted to be mechano-sensitive during muscle stretching in exercise work (Nielsen & Pedersen, 2007; Pedersen et al., 2003; Pedersen et al., 2001; Petersen & Pedersen, 2006; Woods et al., 2006). The mechano-sensitive property of IL-6 was also suggested by the empirical data set in the acute phonotrauma study (Verdolini et al., in preparation). Following a 4-hr treatment of resonant voice exercise or spontaneous speech, robust and immediate up-regulations of IL-6 were noted with a rapid decrease during the post-treatment period, i.e., at the 24-hr post baseline time point.

A novel debate that has emerged from our empirical data set is whether voice rest or resonant voice exercise is a better intervention for patients with acute phonotrauma. Traditional wisdom suggests that voice rest is the ideal approach. However, the current phonotrauma ABM suggested that biomechanical signals generated from resonant voice exercises might optimally assist stressed tissues to restore their homeostasis by regulating an array of pro-inflammatory and anti-inflammatory mediators, based on the readouts of mediator profile. First, the induction of IL-6 was enhanced immediately following the resonant voice treatment (i.e., the time point following a 4-hr treatment). Exercise-induced IL-6 has been suggested to stimulate the production of IL-10 and inhibit the synthesis of TNF-α and IL-1β in exercised tissues (Gleeson, 2007; J. Peake et al., 2005; Petersen & Pedersen, 2005, 2006; Toumi et al., 2006; Woods et al., 2006). Both empirical and simulation data in our series showed that during the post-treatment period (i.e., the 24-hr post baseline time point), although IL-10 was on an upward trajectory in both voice rest and resonant voice exercise, the trajectory was shaper following resonant voice exercise. In other words, although an anti-inflammatory process may be already programmed physiologically (as indicated in the voice rest condition), vibratory stress from resonant voice enhanced the pre-existing anti-inflammatory process by augmenting the anti-inflammatory effects of IL-10 through the induction of IL-6. Also, marked decrease of pro-inflammatory mediators (TNF-α and IL-1β) were noted following resonant voice exercises at the 24-hr post-baseline time point. Those observations suggest that TNF-α/ IL-1β-induced destruction events might be optimally attenuated following resonant voice exercise, at least for some individuals and some conditions of resonant voice exercise.

On the other hand, one might dispute this suggestion and argue that voice rest is better than resonant voice exercise because “damaging stress” (i.e., impact stress) is absent in voice

rest. However, a counterargument is that paradoxically, the magnitude of para-inflammation caused by impact stress in resonant voice may not be all bad for healing. In fact, arguably impact stresses associated with some forms of resonant voice may actually have some redeeming qualities. The argument is as follows. In the embryo, perfect tissue regeneration has been reported in the absence of inflammation (Broker & Reiter, 1994; Dang, Ting, Soo, Longaker, & Lorenz, 2003; Hantash, Zhao, Knowles, & Lorenz, 2008; Robson et al., 2001; Singer & Clark, 1999). However, embryonic tissues are much simpler and the cells actively undergo morphogenesis and migrations during their developmental program with minimal inflammation. Adult wound healing may need inflammation to kick-start cell migration and other functions needed for tissue repair. As a matter of fact, a near-total blockage of inflammation, as with immunosuppressive treatment, has been reported as not very helpful in clinical therapy for inflammation-related diseases (Serhan, Chiang, & Van Dyke, 2008; Stramer et al., 2007). At the same time, too much inflammation, possibly in the range of magnitude of "damaging stress" from spontaneous speech, could lead to unfavorable collateral tissue damage. Thus, a therapeutic balance between the need for inflammation to initiate tissue repair and the need to keep inflammation from causing collateral tissue damage appears important to optimize healing outcomes. The current work suggests that although the pro-inflammatory markers IL-1β and TNF-α showed an immediate increase following resonant voice exercise, their levels decreased rapidly to the 24-hr time point. This result suggests that resonant voice exercise might generate an optimal level of mixed para-inflammatory and anti-inflammatory response to accelerate the transition from inflammation to healing and protect tissues from collateral damage in the process of restoring tissue homeostasis.

## 7.2 INSIGHTS FROM THE PREDICTED TISSUE DAMAGE TRAJECTORIES OF THE HUMAN PHONOTRAUNA AND ANIMAL SURGICAL ABMS

In Experiment 2, the surgical trauma ABM was calibrated with experimental data across two biological organization levels, i.e., molecules (inflammatory mediators) and tissues (ECM substances) with the long-term goal of reconstructing the link between molecular factors and their tissue-level manifestation. If we succeed, we will have a fuller understanding of the pathophysiology of vocal fold pathology and potentially develop a clinical tool to measure the moment that damage is initiated and the subsequent temporal progression of damage due to mechanical stress.

Thus far, research into vocal fold pathology has mainly addressed damage at the tissue level. For example, vocal fold scar owing to surgical trauma has been characterized in terms of altered ECM structure and distribution, rather by a profile of genetic or protein markers. Clinically, instrumental measures, such as phonation threshold pressure and an array of other aerodynamic and acoustic measures, are used to capture physiological signs of vocal fold tissue changes. These measures may capture accumulated damage but fail to effectively capture the early signs of tissue stress/ damage. In order to select the optimal treatment in a given clinical scenario, it is important for us to clearly define and detect the earliest signs of tissue stress/ damage in molecular terms before histopathologic changes of vocal fold tissues are indicated.

While the initiating pathophysiologic event involved in surgical trauma occurs primarily at the tissue level, numerous cellular and molecular cascades are immediately set in motion by it. A previous animal study was carried out to assess *in vivo* tissue damage resulting from compression stress in porcine liver, ureter and small bowel as a function of the magnitude and duration of mechanical stress (De, Rosen, Dagan, & Hannaford, 2007). The researchers excised a

portion of stressed tissue and carried out histological and image analysis to quantitatively measure acute indicators of tissue response to mechanical damage: percent apoptotic cell area (cellular death) and neutrophils counts (inflammation). Their data suggested that these two indicators were sensitive to varying levels of mechanically induced damage in liver and small bowel. De et al.'s study (2007) provided an excellent model for measuring tissue damage in animals and probably a similar methodology can be applied in the field of voice research. However, if our ultimate goal is to develop a non-invasive (i.e., without tissue excision) clinical tool allowing near real-time assessment and multiple measures of vocal fold status in living humans, at present the assay of biomarkers in laryngeal secretions appears to be the only non-invasive and fast tool to capture the progression of inflammation and healing.

### 7.2.1 The ABM-simulated tissue damage trajectories are in good correspondence with empirical observations

The trajectories of tissue damage generated from both phonotrauma and surgical ABMs were not prospectively calibrated with any empirical data in this study. Surprisingly, these trajectories were found to be in good correspondence with recent empirical reports by other authors.

Specifically, a human study was carried out to chart 86 teachers' voice recovery trajectories after a 2-hr vocal loading task for using loudness comparable to loudness in teaching (Hunter & Titze, in press). Following the loading task, subjects were then sent home with audio and written instructions and rating forms. Three perceptual rating scales were used as indices of voice recovery in the study. Subjects were required to rate their (1) current speaking effort level, (2) inability to produce soft voice, and (3) laryngeal discomfort. All ratings were performed every two hours on the remainder of the day of loading, one day after loading and two days after

loading, beginning at the time subjects woke up until going bed. No teaching duties yet no specific control of voice use were required for these subjects during the recovery days.

The data indicated that, according to these measures, full vocal recovery occurred within 12 – 18 hr post loading. This result is strikingly consistent with the trajectory of tissue damage predicted by the phonotrauma ABM in the current study, in the analogous condition of spontaneous speech. Specifically, in the model, tissue damage was predicted to be resolved around 18 hrs from vocal loading at the earliest, under the simulation of a 1-hr vocal loading task followed by a 4-hr spontaneous speech treatment (Figure 19c). The congruence of tissue damage results across these two independent studies is exciting, making a case for cross-validation for the perceptual rating data of vocal recovery and the ABM predictions of tissue damage.

In the current ABM, simulated tissue damage is expressed in *molecular* terms. On the other hand, ratings of vocal recovery pertained to a *perceptual* level of tissue damage as described in Hunter and Titze's study. From the foregoing findings, if we consider that the earliest possible vocal recovery time is 18 hrs and 12 hrs as suggested by the molecular index and the perceptual index of tissue damage respectively, a hypothetical correspondence of tissue damage at molecular and perceptual levels in phonotrauma is generated herein (Figure 23). A threshold factor may be operative – molecular changes in damaged tissues may need to accumulated to exceed some critical threshold before the changes are perceived. Stated differently, when subjects feel their voice is affected following vocal loading, tissue damage may be already operative and accumulated at the molecular level. When subjects start to feel that their voice is recovered, some residual damage or "alarm/ damage signals" may be still lie hidden deeply in the structure and regulation of inflammation and healing. The hypothetical correspondence of tissue damage between the molecular and the perceptual levels as depicted in

Figure 23 is worthy of further investigation. Nonetheless, the current work presents an example of how biosimulation can be used as a working platform for hypothesis testing as well as hypothesis generation for empirical experiments to improve scientific thinking around inflammation and healing.

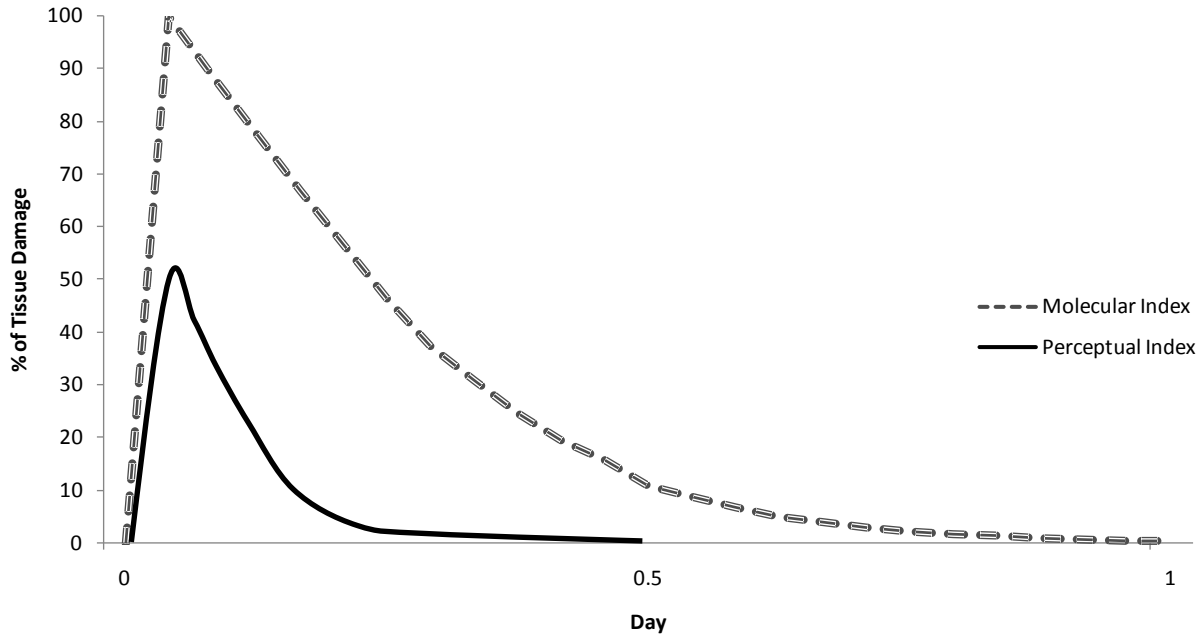


**Figure 23. Hypothetical trajectories of tissue stress/ damage following vocal loading. The dashed line is predicted by the ABM (c.f. Figure 19c), which represents (average) tissue stress/ damage at the molecular level. The solid line is deduced from data by Hunter & Titze (in press), which represents hypothetical tissue stress/ damage at the perceptual level.**

A similar case is found for the surgical ABM. A rat vocal fold study showed that epithelization and fibroblast proliferation started on Day 1 post mucosal stripping surgery and fibroblasts were at peak number on Day 3 post surgery (T. Tateya, I. Tateya, J. H. Sohn et al., 2006). These observations suggested that the degree of ultimate tissue damage might be contained with the initiation of a fibroblast-dominant repair program starting on Day 1 following

injury. Also, the damaged tissues were expected to be replaced by neo-matrix from Day 3 post injury, at the time when fibroblasts were the most abundant in the wound site. Encouragingly, in Experiment 2 of the current work (i.e., the rat surgical ABM), the predicted trajectory of tissue damage showed a reasonably good correspondence with the afore noted empirical observations – simulated damage was in a down-slope from Day 1 post injury and was almost resolved from Day 3 post injury (Figure 21b).

### 7.2.2 The current ABMs shed lights in identifying the surrogate of tissue damage

The surrogate of vocal fold tissue damage at the molecular level remains to be empirically verified. However, we may be able to rationalize the surrogate of tissue damage from the literature and current simulation work. In the current ABMs, the mathematical function of simulated tissue damage was basically constructed to be analogous to "alarm/ danger" signals. These signals are endogenous inducers of inflammation, which can be constitutive or inducible, intracellular or secreted, or even a part of the ECM (Matzinger, 2007; Medzhitov, 2008; Rubartelli & Lotze, 2007). Some "alarm/ danger signals" have been empirically identified, including heat-shock proteins, chromatin-associated protein high-mobility group box 1 (HMGB1), adenosine-5'-triphosphate (ATP), uric acid, free DNA, IL-1α, IL-18 and degraded matrix components (Behrens et al., 2008; W. Chen, Syldath, Bellmann, Burkart, & Kolb, 1999; Davies et al., 2006; Farkas, Kilgore, & Lotze, 2007; Heath & Carbone, 2003; Panayi, Corrigall, & Henderson, 2004; Prohaszka & Fust, 2004; Scheibner et al., 2006; Shi, Evans, & Rock, 2003; Stern et al., 2006; Taylor et al., 2004). The relevant "alarm/ danger" signals in the current ABMs were ECM degradation products from native collagen type I, elastin and HA.

We had attempted to empirically measure hydroxyproline, a component of collagen, in laryngeal secretions from the subject pool that participated in the acute human phonotrauma study (Verdolini et al., in preparation). Unfortunately, no conclusive results were obtained from the hydroxyproline experiment (unpublished data). We had several speculations: (1) hydroxyproline was too big to migrate across the epithelial barrier in order to be detected in secretions, (2) the time points that we measured were too early (up to 24 hr post baseline) for the initiation of collagen synthesis or (3) the vocal loading task did not induce any collagen disruption at all. However, we do not exclude the possibility of detecting collagen fragments in secretions following surgical trauma, in which the superficial lamina propria would be exposed.

Ideally, we would like to identify a marker that is sensitive to varying stress levels (from low phonatory stress to high surgical stress) and indicates the earliest sign of tissue damage, well before conventional indicators or overt histopathologic changes of vocal fold damage can be seen. Among all ECM candidates in the current ABMs, HA fragments or low molecular weight HA seemed to be the potential surrogate of tissue damage. Collagen and elastin are both structural proteins in the vocal folds and are sparsely found in the vocal fold superficial lamina propria. These structural proteins may be more resistant to destruction than other ECM proteins in the vocal folds, at least within the range of physiological stresses typical of phonatory stresses examined in the present series. All in all, the chance of detecting collagen and elastin fragments in laryngeal surface secretions following phonotrauma may be slight at best.

On the other hand, HA is abundant in the superficial lamina propria and has high turn-over rates (Ward, Thiebault, & Gray, 2002). Thus, we speculate that HA would not be as “inert” as its matrix counterparts and may be more promptly degraded even under low levels of mechanical stress in phonation, compared to its counterparts in surgical stress. Besides the

current ECM markers in our ABMs, other potential candidates exist for assessing tissue damage empirically. Further discussion of identifying surrogates for tissue damage is entertained in Section 7.4 on “Future Directions and Conclusions”.

In brief, future work will not only help assess tissue damage from surgical stress; it should also be especially worthwhile for the case of repetitive phonatory stress. Nowadays, when a patient comes to the clinic with an existing vocal fold lesion, it is impossible for clinicians to accurately estimate the degree of tissue damage by looking at the appearance of the vocal folds. We assume that the ideal prescription dose for motion-based treatments will vary with tissue status. Once an *in vivo* measuring tool of tissue damage is available, clinicians will be able to increase their diagnostic accuracy and prescribe a tailored intervention for patients based on current tissue status.

## 7.3 CHALLENGES FOR THE CURRENT WORK

The results from current work are encouraging in terms of the potential translational utility of ABM in the setting of vocal fold inflammation. That said, at least two shortcomings are noted, (1) challenges in making predictions for subjects with a pre-inflamed mediator profile in Experiment 1 and (2) challenges in simulating protein on mRNA data in Experiment 2.

### 7.3.1 Challenges in predicting results for subjects with pre-inflamed mediator profiles

The phonotrauma ABM showed a higher accuracy in predicting inflammatory mediator level for Subjects 1 – 3, compared to Subjects 4 – 7 whose data were not used for model

calibration. As indicated by biomarker profiles, despite subject reports and clinical observations that both voice and larynx were healthy, biological data indicated that Subjects 4 – 7 had pre-inflamed vocal folds on the day of the experiment. The current phonotrauma ABM primarily relied on data and assumptions derived from experimental and clinical studies in "pristine" larynges, i.e., without history of traumatization or other forms of noxious exposures. Thus, the current phonotrauma ABM may not be applicable for the simulation of populations with laryngeal inflammation that existed prior to a discrete phonotraumatic event. This observation as well as logic suggest that additional factors are required to augment the model to simulate a more diverse patient population. For example, genetic variation and epigenetic factors have been suggested to influence the evolving inflammatory state (Lowry & Calvano, 2008). Genetic variability in inflammation and wound healing is typically mediated via single-nucleotide gene polymorphisms (SNPs) of various inflammatory mediators (Clermont et al., 2004; De Maio, Torres, & Reeves, 2005; Imahara & O'Keefe, 2004). SNPs are variations in short deoxyribonucleic acid (DNA) sequences. These genetic variants are suggested to be linked to the presence of particular diseases, such as, type 2 diabetes, age-related macular degeneration, and cardiovascular disease (Jakobsdottir, Gorin, Conley, Ferrell, & Weeks, 2009). Epigenetic factors, such as age, gender and ethnicity, may also affect individual's course and outcome of inflammation (G. S. Martin, Mannino, Eaton, & Moss, 2003; van Eijk, Dorresteijn, & Pikkers, 2008; van Eijk et al., 2007). Future model development may need to include not only inflammatory history but also genetic and epigenetic factors in order to simulate various patients' behaviors in reality.

### 7.3.2 Challenges in simulating protein basing on animal mRNA data

In Experiment 2, the animal surgical ABM was calibrated with mRNA data from a panel of inflammatory mediators and ECM substances and then was applied to predict their translated protein products. The rationale for using animal mRNA data is presented in Section 5.1.2.5 and is not repeated here.

First, we recognized the concern about whether the mRNA data were adequate to predict corresponding protein levels. In the example of TGF-β1, its mRNA stability, translation and/or post-translational processing have been reported to be altered by tumor, cytosolic factors or other growth factors (Fowlis, Flanders, Duffie, Balmain, & Akhurst, 1992; Fraser, Wakefield, & Phillips, 2002; Roberts, 1998; Romeo, Park, Roberts, Sporn, & Kim, 1993). In other words, the correspondence of mRNA level to protein level in TGF-β1 is not necessarily 1:1 – an induction of TGF-β1 mRNA expression might not result in a stimulation of protein synthesis. Thus, we must remain cautious in extrapolating the mRNA results to the predicted protein response in Experiment 2. Future work is required to investigate the correspondence between mRNA and protein expression in biomarkers related inflammation and healing.

Second, the other issue is whether animal data can be assumed to be predictive of human outcomes. This issue has been a long-standing controversy in biomedical research. Proponents have argued that animal models are reasonably predictive of human outcomes in biomedical research aimed at preventing, developing cures or alleviating human diseases. Supporting arguments for using laboratory animals include at minimum the following considerations: (1) animals allow for more controlled environmental and genetic manipulation than humans, (2) animal studies often provide unique insights into the pathophysiology and etiology of disease as well as novel directions for treatments and (3) animal studies offer a relatively cost-effective way

of testing a new drug before testing it in humans (Hackam, 2007). That said, cautions are still warranted in the extrapolation of findings from animal research to the case of humans.

The major concern is that the use of laboratory animals may not adequately model human pathophysiology *per se*. One example is that chimpanzees are regarded as the closest living relatives to humans. According to Darwin's theory of evolution, humans are descended from chimpanzee species. Chimpanzees have thus been assumed to be the best available models for accurately predicting human outcomes in biomedical research. The genomes between chimpanzees and humans differ by only 1 – 2%. However, 80% of chimpanzee proteins are different from human proteins, leading to remarkable phenotypic differences between chimpanzees and humans (Glazko, Veeramachaneni, Nei, & Makalowski, 2005). Other laboratory animal species are expected to have even less genetic and phenotypic similarity to humans, making people suspicious of extrapolating findings from animal research for understanding human diseases.

Relative to the setting of the vocal folds, besides anatomical differences between vocal folds and larynx, phonatory patterns in terms of voice acoustics and amount of voice use are obviously distinct between animals and humans. Also such differences may influence vocal fold inflammatory and healing responses. Hence, the use of laboratory animals may inadequately mimic vocal fold pathophysiology in humans. At the same time, due to existing technology and ethical dilemmas, we cannot subject living humans to multiple vocal fold injuries for the sake of research purposes. We can only interpret animal data with caution. Although the road in translating research findings from animals to humans is full of hurdles, each small step we are taking now has potential to pave the way for critical improvements in the clinical success in voice care.

## 7.4 FUTURE DIRECTIONS AND CONCLUSIONS

Systems biology is a fast advancing field that combines empirical, mathematical and computational techniques to enhance understanding of complex biological and physiological phenomena. The more that genomics, proteomic, bioinformatics and many other growing fields reveal about life, the more apparent it becomes that the current understanding of health and disease is not up to the task. Systems biology places biology on a solid mathematical foundation to make the task of understanding biological complexity (or understanding "life") more tractable. The work presented here successfully demonstrate the even simple computational models (ABMs) can be of practical use in voice care and has profound implications for future studies of unraveling the complexity of inflammation and wound healing following vocal fold trauma.

First, the correspondence of mRNA expression and protein expression for the same panel of inflammatory and ECM markers will be investigated in the vocal folds during the course of inflammation and healing. At present, most of biological data around vocal fold inflammation and healing are mRNA-based. If we pursue to make use of the existing mRNA data for improving the ABMs, we will need to formulate a correction factor of converting mRNA values to protein values. We expect that at least a time lag is likely present between mRNA expression and protein expression of biomarkers. The ABM can then be calibrated with correction factors accordingly for the ultimate prediction of functional protein expressions.

Second, the surrogate of tissue damage for vocal folds will be verified prospectively. The current dissertation work implicated that HA fragments may be one of the possible surrogates of tissue damage, particularly in the case of vocal fold injury. HA fragments are generated during the moment of tissue damage and the subsequent inflammatory response and then are removed by activated macrophages in the wound site during the transition of inflammation to healing. In

fact, HA fragments, due to its hydrophilic properties, was reported to accumulate in inflamed tissues and attribute to edema formation, one of the cardinal signs of inflammation and wound repair (D. Jiang et al., 2007; Stern et al., 2006). Failure to remove HA fragments was suggested to lead to persistent inflammation and probably scar formation (Gao et al., 2008). Thus, the level of HA fragments seem to be sensitively associated with the state of tissues. Assuming HA fragments are small enough to pass through the epithelial barrier for secretion samplings, we hypothesize that the level of HA fragments in laryngeal secretion could be the indicator of very early vocal fold stress/ damage and might be in a good concordance with the evolution of underneath tissue state following injury.

Apart from HA fragments, other possible indicators of tissue damage include but not limit to: (1) free deoxyribonucleic acid (DNA) released by apoptotic cells, (2) high mobility group box 1 (HMGB1; a nuclear chromosomal protein) released by inflammatory cells, as well as (3) lactate dehydrogenase (LDH) released by various cell types. Free DNA and HMGB1 are only be secreted when tissue damage is indicated. The expression of LDH, which is the enzyme constitutively expressed in cells for inter-conversion of pyruvate and lactate, is remarkably up-regulated in pathologic conditions. All these three candidates have been consistently documented for their presence in a serum of body fluids in other organ injuries and were used as clinical measures of tissue damage (Atamaniuk et al., 2008; Brancaccio, Maffulli, Buonauro, & Limongelli, 2008; Foell, Wittkowski, & Roth, 2007; Klune, Dhupar, Cardinal, Billiar, & Tsung, 2008; Palumbo et al., 2004; H. Wang, Yang, & Tracey, 2004; Yang & Tracey, 2005; Yun et al., 2008). Although speculative, these substances may exist in laryngeal surface secretions (another form of body fluids) following vocal fold stress/ injury. Once the biomarker of tissue damage is

identified, the data can be used to calibrate and validate the parameter of simulated tissue damage in ABMs and improve the model's prediction accuracy of tissue state.

In conclusion, this thesis showed significant improvement over previous ABMs and satisfactory agreements with theoretical framework of inflammation and healing in sorts of ways. ABM represents a powerful computational technique in biological simulation, which provides a real framework to put all the pieces of puzzle fit together and produces detailed route maps of biological networks. We believe that ABM simulation results can be applied to chart individualized healing trajectories and help clinicians to determine when, where, and how to intervene disease. The success of this research program will reduce animal testing, have better targeted clinical trials and make a reality of personalized medicine.

# APPENDIX A

## VOCAL FOLD MICROARCHITECTURE

The purpose of this chapter is to describe the microarchitecture of the vocal folds in the order of (1) epithelium, (2) basement membrane zone, (3) lamina propria and (4) macula flava. This review is primarily based on human adult models. However, data from animal studies may also use as supplementary information when appropriate.

The vocal folds, which are housed in larynx, have distinctive geometry, histology and viscoelasticity for efficient oscillation during phonation. Figure A1 describes the schematic multi-layer structure of the vocal folds (Gray, 2000; Gray, Hirano, & Sato, 1993; Gray, Pignatari, & Harding, 1994; Gray & Titze, 1988; M. Hirano, 1977, 1981; M. Hirano & Kakita, 1985; M. Hirano, Kurita, & Nakashima, 1981; M. Hirano & Sato, 1993). Traditionally, the human vocal folds are described as three anatomically distinctive layers: epithelium (0.05 - 0.1 mm thick), lamina propria (1.5 - 2.5 mm thick at the middle of the vocal folds) and vocalis muscle (7-8 mm thick) (M. Hirano, 1977, 1981; M. Hirano et al., 1981). Later, a very thin layer of the basement membrane is identified that connects between vocal fold epithelium and lamina propria (Gray, 1989; Gray, Pignatari, & Harding, 1994). Each epithelium, basement membrane and lamina

propria each has its own special layered structure. For example, the lamina propria can be sub-divided into: superficial (Reinke's space), intermediate and deep lamina propria.

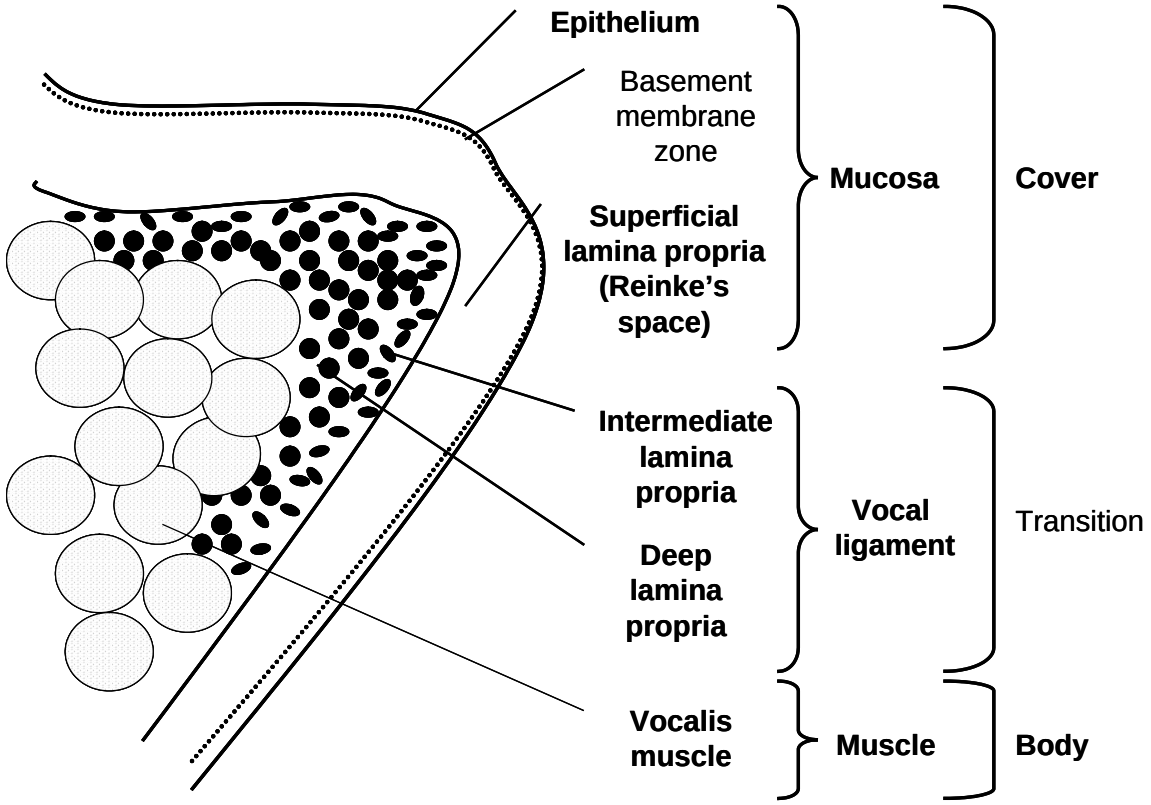


**Figure A1.** Schematic presentation of a coronal view of the left human vocal fold (Gray, Hirano, & Sato, 1993; M. Hirano, 1981).

Vocal fold structure can also be described in different schemas (Figure A1). One schema is that the epithelium and the superficial lamina propria are grouped as mucosa, whereas the intermediate and deep lamina propria are grouped as vocal ligament (M. Hirano, Kurita, & Nakashima, 1981). The other schema is based on the cover-body theory of vocal fold vibration (M. Hirano & Kakita, 1985). According to the cover-body theory, the human vocal folds can be divided into the *cover*, the *transition zone* and the *body* based on the mechanical property of the

vocal folds. The cover is composed of the epithelium and the superficial lamina propria, which is pliable, elastic and non-contractile. The body is composed of the vocalis muscle, which is contractile to adjust the stiffness and concentration of the mass of the vocal folds. The intermediate and deep layers of lamina propria are the transition layer between the cover and the body.

That said, the consensus of the exact boundary of the cover and the body related to the anatomical structure of the vocal folds has not been reached yet. The boundary of the transition zone is especially in debate. For instance, Dikkers, Hammond et al. and Titze (F. G. Dikkers, 1994; Hammond, Gray, Butler, Zhou, & Hammond, 1998; I. R. Titze, 1994) do not have the transition zone in their cover-body models. Dikkers defines the cover as the composition of the epithelium and the superficial lamina propria, with the body as conus elasticus and vocalis muscle. On the other hand, Hammond et al. and Titze define the cover as the composition of the epithelium, the superficial and intermediate lamina propria. At the same time, Titze defines the body as the composition of the deep lamina propria and the vocalis muscle, whereas Hammond et al. include these two composites plus the intermediate lamina propria for their definition of the body.

Defining the anatomical boundary of the cover and the body within the lamina propria is difficult. The amount of tissue in vibration varies with the mode of phonation, age and gender, which affects the involvement of the intermediate lamina propria in the cover (Gray, Titze, Alipour, & Hammond, 2000; Hammond, Gray, Butler, Zhou, & Hammond, 1998a; Hammond et al., 1998b; Hammond, Gray, & Butler, 2000; I. R. Titze, 1994). Nevertheless, the main idea of cover-body theory is that during phonation, the mucosal wave primarily occurs on the pliable superficial layer of the vocal folds moving over a more rigid deeper layer. The integrity of the

cover is especially critical for proper vocal fold vibration to generate proper voice sound. Injury of the cover causes a significant loss of vocal fold volume and geometry as well as vocal fold mucosal pliability, leading to voice disturbance (Gray, 1991; Gray, Hammond, & Hanson, 1995; Gray & Titze, 1988; Hertegard et al., 2006; S. Hirano et al., 2003; Rousseau et al., 2003; Thibeault, Gray, Bless, Chan, & Ford, 2002).

## A.1 EPITHELIUM

The vocal fold epithelium can be described as a three-layer structure (from superior to inferior): the squamous cell layer, the basal cell layer and the basement membrane. The whole epithelium of the vocal folds is from 0.05 to 0.10 mm thick (M. Hirano, 1977). The surfaces of the epithelium appear corrugated with the presence of microvilli in canine vocal folds under electron microscopy (Gray, Titze, & Lusk, 1987). Microvilli are also found on the squamous cell layer in human vocal folds (M. Hirano, 1977). The microvilli have three proposed functions: (1) trapping laryngeal mucus through the increased surface area of the grooves, (2) providing a better traction for vocal fold contact during vibration and (3) holding and spreading the mucous droplets in an even layer on the vocal fold surface (Gray, 2000; Gray & Titze, 1988).

Two different populations of squamous cells are found within the epithelium of the vocal folds: respiratory epithelium (pseudostratified squamous) on the superior and inferior aspects of the vocal folds and (2) non-keratinizing squamous epithelium on the medial contact surface (Gray, 2000). The basal layer, which is right underneath the bottom layer of squamous cells, is lined by cubodial cells. The basal layer is attached to the basement membrane through hemidesmosomes.

Desmosomes are strong attachment structures that connect the epithelial cells as indicated in an canine vocal fold model (Gray et al., 1987). Desmosomes provide strength and cohesiveness to the surface of the cover of the vocal folds. Interestingly, desmosomes are absent on the superficial layers of the epithelium but are abundant in the deeper layers of epithelium in canine vocal folds (Gray & Titze, 1988). This unique feature allows normal exfoliation of surface squamous cells while provides necessary resistance in the deeper layers against mechanical damage during vocal fold oscillation.

## A.2 BASEMENT MEMBRANE ZONE

The basement membrane zone (BMZ) of the vocal folds is a very thin layer of glycoprotein between the epithelium and the superficial lamina propria (Figure A2). The BMZ is composed of three layers: lamina lucida, lamina densa and sub-lamina densa. These three layers in conjunction with the anchoring filaments and anchoring fibres form an anchoring complex of joining two different tissue substrates, the cellular epithelium and the acellular lamina propria, together (Courey, Shohet, Scott, & Ossoff, 1996; Gray, 2000; Gray et al., 1994; S. Hirano, Bless, Heisey, & Ford, 2003; S. Hirano, Bless, Rousseau et al., 2003). Within the epithelium, basal cells attach to each other by desmosomes. However, the underneath superficial lamina propria, which is non-cellular, has no desmosome-like attachment structures. Due to heterogeneous composition, direct and firm attachment between the basal cells of the epithelium and the lamina propria is almost impossible. Therefore, the BMZ exists as an anchoring layer for epithelium-lamina propria attachment.

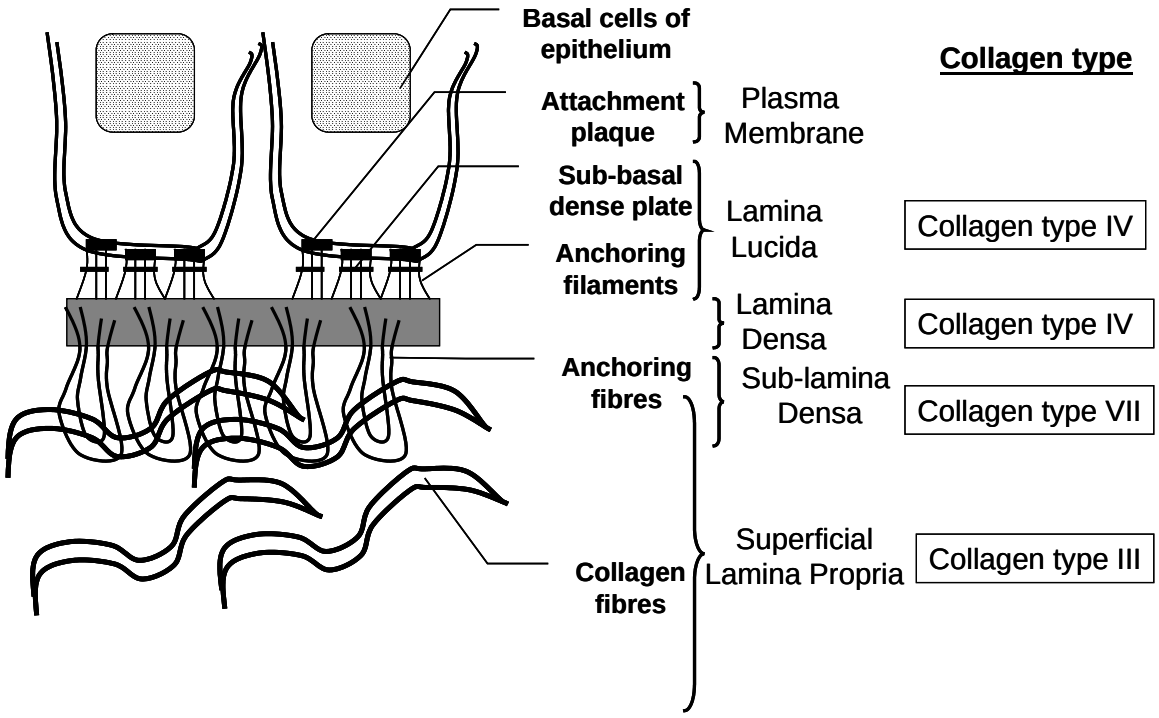


**Figure A2.** Schematic representation of the layered structure and the identified collagen types of the basement membrane zone (Courey, Shohet, Scott, & Ossoff, 1996; Gray, 2000; Gray et al., 1994; S. Hirano, Bless, Heisey, & Ford, 2003; S. Hirano, Bless, Rousseau et al., 2003).

Anchoring filaments and anchoring fibres, which are in similar collagenous structures, are particularly important components for the anchoring function of BMZ. Anchoring filaments from the basal cells hold the hemidesmosomes into the lamina densa and the lamina lucida. On the other hand, anchoring fibres from the lamina densa loop into the superficial lamina propria and then attach back to the lamina densa (Figure A2) (Gray et al., 1994). Further, collagen type III fibres and reticular fibres in the superficial lamina propria do not directly attach to the lamina densa but pass through the loops of the anchoring fibres in normal human vocal folds (Gray et

al., 1993; Gray et al., 1994; Sato, 1998). This unique attachment framework enhances the flexibility of the tissue to glide and move (Gray et al., 1993).

The special collagen network of BMZ-superficial lamina propria is critical to the integrity of the vocal fold cover during vibration. Collagen III fibres of the superior lamina propria form a chain, which attaches firmly to the vocal fold epithelium. Collagen IV, which is found in lamina densa and lamina lucida, provides strength and elasticity of the BMZ. Collagen VII is the main component of anchoring fibres and anchoring filaments of the BMZ. Other than collagens, laminin and fibronectin are also identified in the BMZ. The functions of these two proteins in BMZ are not elucidated yet.

Repetitive and intense phonation may cause BMZ disruption as indicated in the histological examination of phonotraumatic vocal nodules (Gray, 1989, 1991; Gray et al., 1995). Some people may be anatomically predisposed to BMZ injury due to the lack of anchoring fibres. The density of anchoring fibres are suggested to be genetically linked (Gray et al., 1994). In human normal vocal folds, about 80-120 fibres per unit area of BMZ are estimated (Gray et al., 1994). If an individual has a recessive form of that gene, the individual may have fewer or no anchoring fibres and becomes more at risk of epithelium-lamina propria separation during phonation.

## A.3 LAMINA PROPRIA

The lamina propria is a hypocellular composite of various extracellular matrix (ECM) molecules (proteoglycans, collagen, elastin and hyaluronic acid). Lamina propria provides essential tissue support and maintains proper viscoelasticity of the vocal folds (Chan, Gray, &

Titze, 2001; Gray, 2000; Gray et al., 1993; Gray, Titze, Alipour, & Hammond, 2000; Gray, Titze, Chan, & Hammond, 1999; M. Hirano et al., 1981; Thibeault, Bless, & Gray, 2003). Cells, such as myofibroblasts, macrophages and fibroblasts, distribute sparsely in various locations throughout the lamina propria (Boseley & Hartnick, 2006; Catten, Gray, Hammond, Zhou, & Hammond, 1998; M. Hirano, Sato, & Nakashima, 1999a, 2000; Jecker, Ptok, Pabst, & Westermann, 1996; Pawlak, Hammond, Hammond, & Gray, 1996).

The lamina propria is a three-layered structure: superficial, intermediate and deep layers (Figure A1). This definition is based on the distribution of elastin and collagen fibres under the examination of light microscopy (Hammond et al., 1998; Hammond, Gray, & Butler, 2000; M. Hirano, 1977, 1981; M. Hirano et al., 1981) and electron microscopy (Ishii, Yamashita, Akita, & Hirose, 2000). The superficial lamina propria contains sparse elastin and collagen fibres, which make this layer pliable for mucosal oscillation. The intermediate lamina propria contains more elastin and collagen fibres. The deep lamina propria has less elastin but more collagen fibres. The intermediate and deep lamina propria constitute vocal ligament, which provide elasticity and stiffness for the vocal folds (Figure A1).

The thickness of each layer of the lamina propria at the mid-portion of the human normal vocal folds were reported (M. Hirano et al., 1981). The superficial, intermediate and deep lamina propria are about 0.4 -0.5 mm, 0.45 – 0.55 mm and 0.2 – 0.3 mm thick respectively. These numbers are in a similar magnitude of order with a later report on the regional depth of the lamina propria (Gray, Titze et al., 2000; Hammond et al., 2000). The superficial lamina propria consists about 25% – 35% of the total depth of the lamina propria in adults. The intermediate and the deep lamina propria consist about 45% - 55% and 20% of the total depth of the lamina propria respectively (Gray, Titze et al., 2000).

On the other hand, a debate exists whether the layered structure of the lamina propria should be defined by the distribution of fibrous protein composition or that of cellular composition. This argument is particularly prominent when describing the maturation process of paediatric vocal fold lamina propria. In the newborn vocal folds, a layered structure of lamina propria and vocal ligament is not yet developed (Boseley & Hartnick, 2006; M. Hirano et al., 1981; M. Hirano, Kurita, & Nakashima, 1983; Ishii et al., 2000; Sato & Hirano, 1995a; Sato, Hirano, & Nakashima, 2001a). Conclusions about the time of the formation of a layered structure of lamina propria during maturation are differentiated. The discrepancy may be related to if the investigators define the vocal fold layers based on their cellular composition or fibrous protein composition.

One study used light microscopy to measure the depth of cell layer for a group of paediatric vocal fold specimens (Boseley & Hartnick, 2006). Results suggest that the superficial lamina propria is formed as early as 7 years of age. On the other hand, a study used electron microscope to examine the changes in collagen and elastic fibres in newborn vocal folds (Ishii et al., 2000). Their results suggest that the superficial lamina propria is formed until 12 years of age.

Furthermore, a study examined both the cellular and fibrous protein compositions of a group of paediatric vocal fold specimens (Hartnick, Rehbar, & Prasad, 2005). A clear demarcation between the intermediate and deep lamina propria was seen by 7 years of age in terms of differential cell density and population. At the same time, the lamina propria with layered and organized composition of collagen and elastin fibres were seen until 13 years of age (Hartnick et al., 2005).

Therefore, findings from these three studies leave an argument that at which time-point of the vocal folds can be regarded as "having a mature structure" during vocal fold development. Alternative, a more appropriate question may be "*when do the vocal folds possess the same biomechanical property as in adult vocal folds during development?*". Future research is promising to compare the biomechanical properties of the vocal folds in relation to its structure from the populations of 7 year-old and 13 year-old specimens.

The microarchitecture of the lamina propria from the aspects of vasculature, cellularity and extracellular matrix are dissected in the following sections.

### A.3.1 Vasculature of lamina propria

Ten studies have been carried out to study the vasculature and blood supply of the vocal folds (Arnstein, Berke, Trapp, Bell, & Natividad, 1990; Arnstein, Berke, Trapp, & Natividad, 1989; Franz & Aharinejad, 1994; Frenzel & Kleinsasser, 1982; Matsuo et al., 1987; Mihashi et al., 1981; Nakai, Masutani, Moriguchi, Matsunaga, & Sugita, 1991; Sato & Hirano, 1997; Stec, Hertegard, & Juto, 2007; Tomita, Matsuo, Maehara, Umezaki, & Shin, 1988). The vocal fold vascular structure and blood supply are specialized to withstand the high magnitude and frequency of mechanical stresses within the vocal fold tissues during phonation. Figure A3 shows the schematic representation of the vasculature of the normal human vocal folds (Mihashi et al., 1981).

The vascular architecture of the vocal folds has been studied using light and electron microscopes (Mihashi et al., 1981; Nakai et al., 1991). The blood vessels of the superficial lamina propria are separated from the vocalis muscle in the human normal vocal folds. On the mucosal cover, the blood vessels near the free edge run along the longitudinal axis of the vocal

folds and arise from the anterior and posterior ends of the vocal folds (Figure A3-1a). This parallel distribution minimizes the circulatory disturbance to the vocal fold movement and also allows for smooth blood flow from the anterior end of the vocal folds (Nakai, Masutani, Moriguchi, Matsunaga, & Sugita, 1991). At the same time, the blood vessels, which are away from the free edge, run upward and medially from deep inside the layer (Figure A3-1b). The separation of the vascular network permits optimal flexibility for mucosal oscillation over the body of the vocal folds.

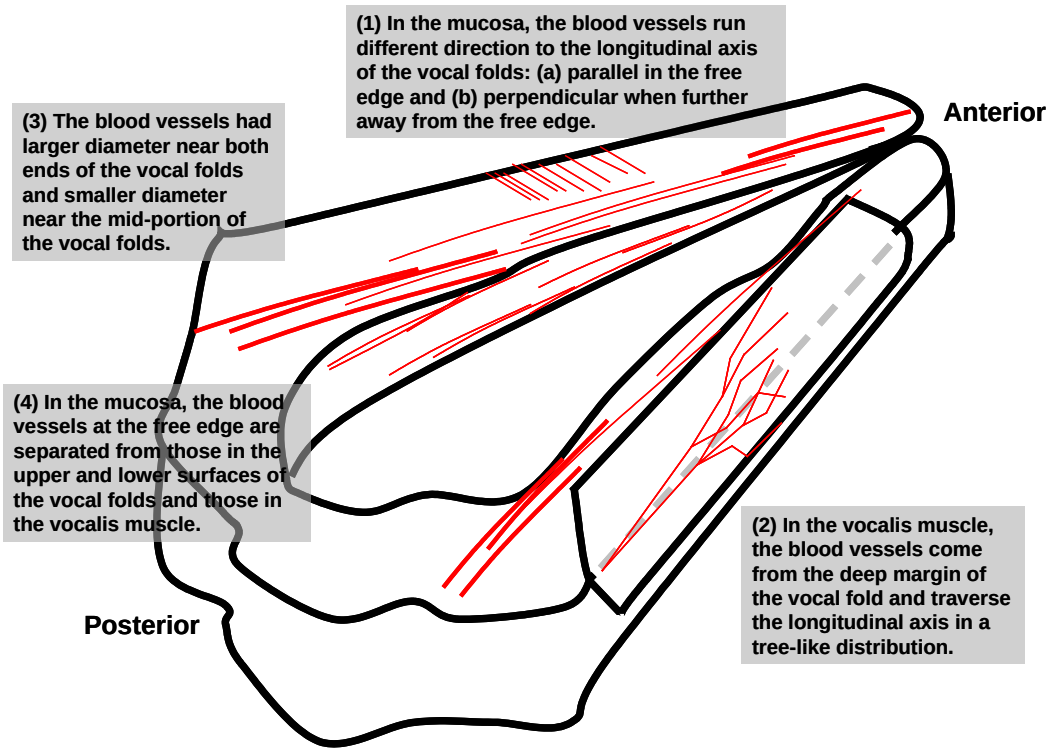


**Figure A3.** Schematic representation of vascular network of the human normal vocal folds (Mihashi et al., 1981).

The vocal fold vascular network is characterised by (1) direct anastomosis between arterioles and venules and (2) undulating distribution of blood vessels in both animal and human vocal folds (Franz & Aharinejad, 1994; Mihashi et al., 1981). The arteriorvenous anatomosis suggests local regulation of blood flow within the vocal folds, which ensures stable and sufficient blood supply for the vocal folds. This feature is crucial because ischemic changes may occur owing to vocal fold vibration or tissue damage (Franz & Aharinejad, 1994; Mihashi et al., 1981; Nakai et al., 1991). Furthermore, the endothelium of blood vessels are mechanically supported and protected by myocytes, the filaments of endothelial cells, and the basal lamina of pericytes. This structural lattice protects the blood vessels from mechanical shearing during vocal fold movement (Frenzel & Kleinsasser, 1982; Sato & Hirano, 1997b).

Regarding the blood supply of the vocal folds, a canine study (Mihashi et al., 1981) shows that the blood supply for the mucosal layer are from three peripheral branches of three arteries: the superior laryngeal, cricothyroid and interior laryngeal arteries. On the other hand, the blood supply for vocalis muscle is from one vessel only: branches of the cricothyroid arteries.

The blood supply in the vocal folds may be affected by the vibratory movement of the vocal folds. A canine study (Mihashi et al., 1981) was set out to estimate the blood flow to the vocal folds by measuring local tissue oxygen pressure ($Pt0_2$) at rest and during phonation. The canines were induced to phonate by various stimuli. Results indicated that $Pt0_2$ decreased in both mucosal cover and vocalis muscle of the vocal folds during phonation, compared to the rest condition. Such ischemic response was less prominent that of the mucosal cover ($3.5 \pm 1.1$ mm Hg) than that of the vocalis muscle ($15.4 \pm 5.8$ mm Hg).

In another canine study, the dogs were induced to phonate by stimulating their recurrent laryngeal nerves (Tomita, Matsuo, Maehara, Umezaki, & Shin, 1988). $Pt0_2$ is found to decrease

in the vocalis muscle with the increase in the frequency of nerve stimulation (10 Hz, 30 Hz and 60 Hz). On the other hand, $Pt0_2$ of the mucosal cover is slightly higher under low-frequency stimulation (10 and 30 Hz) and is slightly lower under high-frequency stimulation (60 Hz) of recurrent laryngeal nerve. The investigators conclude that: (1) the decrease of $Pt0_2$ in vocalis muscle is due to muscle contraction; (2) the increase of $Pt0_2$ in mucosal cover under low-frequency stimulation is related to an increase in blood flow by the pumping action of perfused blood and (3) the decrease of $Pt0_2$ in mucosal cover under high-frequency stimulation is related to a decrease in blood flow by other unknown factors. The rapid acceleration and deceleration of vocal fold tissue during vibration can be one of the factors that disturb the blood flow in the vocal folds (Svec & Sram, 2001; I.R. Titze, 1994).

However, using $Pt0_2$ as an estimate the blood flow may be confounded in these two canine studies. $Pt0_2$ is a factor of both blood flow and arterial oxygenation. Many variables can affect oxygenation, such as, metabolic rate. Therefore, the decrease in $Pt0_2$ may be related to the increase in oxygen consumption during phonation, rather than a change in blood flow (Arnstein et al., 1990; Arnstein et al., 1989).

A new technology is using laser Doppler flowmetry to measure superficial blood flow by positioning the laser bean from the cranial direction to the vocal fold tissues (Stec, Hertegard, & Juto, 2007). Researchers reported that from their 53 human subjects with normal vocal folds, the velocity of moving blood cells (a measure of blood flow) was significantly lower at midmembranous position of the vocal folds than that of at the 2 mm behind midmembranous position. Also, male subjects showed significantly higher velocity than that of female subjects. Furthermore, smokers also showed significantly higher velocity than that of non-smoker subjects at both midmembranous position and 2 mm behind midmembranous position of the vocal folds.

Researchers suggested that vasoconstrictions of the vocal folds may be accompanied with smoking, leading to an increase of blood flow.

In any case, no research has been done in human so far to study the particular effects of blood flow and oxygenation to the general blood supply for the vocal folds at rest and during phonation. This line of research will provide important information how vocal fold vibration can influence vasculature of the vocal folds, resulting possible inflammatory response and fluid leakage.

To sum, the vasculature for the mucosal cover is distinctive from those for the vocalis muscle. The mucosal cover is the striking site of vocal fold oscillation. Thus, the blood vessels in the mucosa need special structures and blood supply to prevent blood vessel rupture and the disturbance of blood circulation owing to phonation.

### A.3.2 Cellularity of lamina propria

Myofibroblasts, macrophages and fibroblasts are the three dominant cell populations in human lamina propria (Boseley & Hartnick, 2006; Catten et al., 1998; M. Hirano et al., 1999a, 2000; Jecker et al., 1996; Pawlak et al., 1996). Table A1 summarizes the distributions and major functions of macrophages, myofibroblasts and fibroblasts in human normal vocal folds.

On the other hand, side population cells were present in a population of about 0.2% of the total number of cells in human vocal folds. Side population cells are suggested to be enriched with stem cells and may have important roles in tissue regeneration. These cells were found in the epithelium, Reineke's space and the anterior and posterior maculae flavae of the vocal folds (Yamashita et al., 2007).

Further, the numbers of other immunocompetent cells, such as mast cells, dendritic cells, natural killer cells and T and B lymphocytes are very limited at the mucosa level of the normal vocal folds in rats and pigs, compared to the supraglottic and subglottic regions of the laryngeal mucosa (Ishida, Yoshida, Iwae, & Amatsu, 2005; Jecker et al., 1996). Research in verifying these animal findings with human vocal folds is warranted.

**Table A1**. Summary of cellularity and extracelluar matrix distribution and their major functions of the adult human vocal fold lamina propria. Annotation of distribution: "++" = most abundant; "+" = present; "-" = minimal/absent.

| | Main Functions | Superficial Lamina Propria | Vocal Ligament | |
|---|---|---|---|---|
| | | | Intermediate Lamina Propria | Deep Lamina Propria |
| **Cells** | | | | |
| Macrophages | -Immune response to mucosal irritants;<br>-regulate the inflammatory response by cytokine and growth factor secretion;<br>-hyaluronic acid synthesis | ++ [1, 2] | + [1, 2] | + [1, 2] |
| Myo-fibroblasts | -Reparative repair of injury due to normal use of vocal folds;<br>-repair collagen and elastin;<br>-wound contraction | ++ [1, 2] | + [1, 2] | + [1, 2] |
| Fibroblasts | -General maintenance role: deposition, degradation and rearrangement of extracellular matrix;<br>-synthesize hyaluronic acid with hyaluronan synthetase;<br>- repair of vocal fold injury | + [1, 3, 4] | + [1] | ++ [1] |
| **Extracellular Matrix** | | | | |
| Fibrous protein | | | | |
| Collagen I | -provide tensile strength around the basement membrane and deep lamina propria to withstand vibratory forces | + [5, 6, 7] (around the basement membrane) | - [7]<br>+ [5] | ++ [5, 6, 7] |
| Collagen III | -maintain the structure of the lamina propria;<br>-provide flexibility and elasticity to the lamina propria | + [5, 6, 7] | + [5, 7]<br>++ [6] | + [7]<br>++ [5, 6] |
| Oxytalan and elaunin | -unknown function in the lamina propria | ++ [8,9] (around the basement membrane) | + [8]<br>- [9] | + [8]<br>- [9] |
| Mature Elastin fibres | -provide elasticity to the lamina propria | + [8] (around the basement membrane)<br>- [9] | ++ [8,9] | + [8,9] |
| **Interstitial element** | | | | |
| Fibronectin | -not well-documented in the vocal folds, probably maintain and assemble the proteins and cells in the extracellular matrix. | + [10] | No information | No information |
| Decorin | -may bind the collagen fibres and reduce their size in the superficial lamina propria | ++ [11] (around the basement membrane)<br>++ 11, 12 (in the superficial lamina propria) | + [11, 12] | + [11, 12] |
| Fibromodulin | -may affect the vocal ligament performance by binding to the collagen fibres there | - [12] | ++ [12] | ++ [12] |
| Hyaluronan | -act as osmotic regular, tissue-damper and shock-absorber for the vocal fold tissue;<br>-regulate the viscoelasticity property of the vocal folds;<br>-contribute to the repair of damaged vocal fold tissue | + [8, 13]<br>Not assessed [14] | ++ [8,13, 14, 15] | + [8, 13,15]<br>Not assessed [14] |

[1](Boseley & Hartnick, 2006); [2](Catten, Gray, Hammond, Zhou, & Hammond, 1998); [3](M. Hirano, Sato, & Nakashima, 1999a); [4](M. Hirano, Sato, & Nakashima, 2000); [5](Hahn, Kobler, Zeitels, & Langer, 2006); [6](Madruga de Melo et al., 2003); [7](T. Tateya, Tateya, & Bless, 2006); [8](Hahn, Kobler, Starcher et al., 2006); [9](Hammond et al., 1998); [10](Courey, Shohet, Scott, & Ossoff, 1996); [11] (Hahn, Kobler, Zeitels, & Langer, 2005); [12](Pawlak, Hammond, Hammond, & Gray, 1996); [13](Butler, Hammond, & Gray, 2001); [14](Hammond, Zhou, Hammond, Pawlak, & Gray, 1997); [15] (Lebl, Martins, Nader, Simoes Mde, & De Biase, 2007).

### A.3.3 Macrophages

Macrophages play important roles in both innate and adaptive immune response. Macrophages (1) ingest the microbes by phagocytosis, (2) act directly as the antigen-presenting cells and (3) secrete various cytokines and growth factor to regulate the inflammatory process. The location of macrophages were found mainly in the superficial lamina propria for both adult and children (7 – 14 years old) vocal fold specimen (Boseley & Hartnick, 2006; Catten et al., 1998). This specific location suggests that macrophages may assist in combating inflammatory agents, such as bacteria, viruses and environmental irritants, crossing the epithelium of the respiratory tract. Interestingly, the density of macrophages in the vocal folds may be related to the history of the exposure of mucosal irritants. Studies showed that macrophages were absent in the 2-day-old and 2-month-old paediatric vocal fold specimens (Boseley & Hartnick, 2006). Even for adults, macrophages were only found in one-third of the vocal fold specimens (Catten et al., 1998). Other than the involvement of the inflammatory response, a study using cytoplasmic staining suggest that macrophages may involve in the synthesis of hyaluronic acid for superficial lamina propria as well (Pawlak et al., 1996).

### A.3.4 Myofibroblasts

Myofibroblasts are differentiated from fibroblasts. Myofibroblasts are specialized for tissue repair and are present only when injury occurs. Myofibroblasts have contractile property, which make the cells capable of contracting the wound during the remodeling phase (Chaponnier, Desmouliere, & Gabbiani, 2006). Similar to macrophages, the density of myofibroblasts were the highest in the superficial lamina propria for both adult and children (7 –

14 years old) vocal fold specimens (Boseley & Hartnick, 2006; Catten et al., 1998). In vocal folds, myofibroblasts are suggested to play roles in (1) reparative repair for the microscopic injuries related to daily voice use in normal tissues and (2) constructive repair in pathological tissues (Boseley & Hartnick, 2006; Catten et al., 1998; Gray, 2000; Pawlak et al., 1996). Particularly, myofibroblasts may involve in the synthesis of extracellular matrix (collagen and elastin) and wound contraction for vocal fold tissue repair.

### A.3.5 Fibroblasts

Fibroblasts are the most abundant cells in the lamina propria of the vocal folds. The cells exist in all layers of the lamina propria and have the highest density in the deepest layer of the lamina propria in adult vocal folds (Catten et al., 1998). Fibroblasts in the lamina propria contribute to both the maintenance of the extracellular matrix and the repair for vocal fold injury for all levels of the vocal folds (Catten et al., 1998; Gray, 2000). Most of the proteins in the basement membrane zone, namely, microfibrils, are synthesized and assembled by the fibroblasts (Gray et al., 1993).

Besides residing within the lamina propria, fibroblasts are also found in the macula flava (Gray et al., 1993; M. Hirano et al., 1999a, 2000). The fibroblasts in these two locations exhibit different properties. Under transmission electron microscopy, fibroblasts in the superficial lamina propria and those in the macula flava are spindles and stellate in shape respectively (M. Hirano et al., 1999). Also, the adult vocal fold fibroblasts in the superficial lamina propria have higher nucleus/cytoplasm ratio and less developed rough endoplasmic reticulum and Golgi apparatus than those in the macula flava (M. Hirano et al., 1999a). The degree of the development of rough endoplasmic reticulum and Golgi apparatus is positively related to the

activity of fibroblasts in the synthesis of elastic and collagenous fibres. Therefore, the fibroblasts in the superficial lamina propria are suggested to be dormant unless vocal fold injury is indicated. At the same time, fibroblasts in the macula flava are believed for active synthesis and maintenance of the vocal ligaments. Further research is needed to identify the biochemical signalling pathways that may lead to differentiated functioning of these two types of fibroblasts.

The activity of fibroblast is probably age-specific (M. Hirano et al., 1999a, 2000). For the fibroblasts in the superficial lamina propria, the percentage of well-developed rough endoplasmic reticulum and Golgi apparatus increased with age-population: new-borns 0%; adults: 10% and geriatrics: 20%. Investigators suggest that fibroblasts are in their proliferative phase for the new-borns and so have less developed rough endoplasmic reticulum and Golgi apparatus. For the geriatrics, their fibroblasts are relatively active in the superficial lamina propria than those in the macula flava, which may account for the increased thickness of the superficial lamina propria of geriatric vocal folds.

On the other hand, for the fibroblasts in the macula flava, the percentage of well-developed rough endoplasmic reticulum and Golgi apparatus were the highest in adults (66%), intermediate in new-borns (40%) and the least in geriatrics (4%). The active macula flava in new-borns may involve in the synthesis of collagenous and elastic fibres for the development of the vocal ligament. At the same time, for the geriatric population, the decreased activity of the macula flava fibroblasts may account for the atrophy of elastic fibres and deterioration of collagenous fibre arrangements in the vocal ligament during the aging process. These findings suggest that the aging of human vocal ligament may be related to the inappropriate activity of fibroblasts. Specifically, the maintenance role of the vocal folds may be switched from the fibroblasts in the macula flava to the fibroblasts in the superficial lamina propria during aging.

### A.3.6 Extracellular Matrix

As mentioned earlier, the lamina propria of the human vocal folds are mostly non-cellular (Chan et al., 2001; Gray, 2000; Gray et al., 1993; Gray, Titze et al., 2000; Gray et al., 1999; M. Hirano et al., 1981; Thibeault et al., 2003). This hypocellular layer is a matrix of fibrous proteins (collagens, reticular fibres and elastins) and interstitial elements (proteoglycans, glycoproteins and glycoaminoglycan), which determines the physical properties of the vocal folds. The fibrous proteins provide shape and form for the lamina propria, whereas interstitial elements regulate tissue viscosity, water content, tissue size and shock absorption (Gray, Titze et al., 2000; Gray et al., 1999). Interstitial elements are situated in the spaces among the fibrous proteins, whereas fibrous proteins runs through the spaces (Sato, 1998). Extracellular matrix is important to maintain and modulate the viscoelastic properties of the vocal fold lamina propria, which subsequently affects the vocal fold vibratory behaviour. Table A1 summarizes the distributions and main functions for each extracellular matrix component identified in the human vocal fold lamina propria.

### A.3.7 Fibrous proteins (collagen, reticular fibres and elastin)

Collagen and elastin are the structural proteins for extracellular matrix in the vocal fold lamina propria. The distribution of collagen and elastin defines the three layers of the lamina propria (M. Hirano et al., 1981) and also determines the mechanical property of the lamina propria that in turn affects vocal fold vibratory behaviour. The superficial lamina propria consists of sparse and loose fibrous proteins. The intermediate and deep lamina propria are mainy composed of elastic fibres and collagenous fibres respectively (Gray et al., 1993).

Numerous reports have been published recently to advance our understanding of the ultrastructure and distribution of fibrous proteins within the lamina propria of the vocal folds (Gray, Chan, & Turner, 2000; Gray, Titze et al., 2000; Hahn, Kobler, Starcher, Zeitels, & Langer, 2006; Hahn, Kobler, Zeitels, & Langer, 2006; Hammond et al., 1998b; Hammond et al., 2000; Hammond, Zhou, Hammond, Pawlak, & Gray, 1997; Madruga de Melo et al., 2003; T. Tateya, Tateya, & Bless, 2006, 2007). Research about quantification of fibrous proteins, identification of fibrous protein subtypes and their distributions across the lamina propria are of particular interest.

### *Collagen and reticular fibres.*

Studying collagen in human vocal folds has been a challenge. Collagen was thought to lose their antigenicity quickly, which made collagen-specific studies unreliable (Gray, Titze et al., 2000). However, recent studies indicate that the antigenicity of collagen is not a problem at all (T. Tateya, I. Tateya, & D. M. Bless, 2006; T. Tateya et al., 2007). These studies successfully identified the collagen subtypes by using immunohistochemistry. The investigators suggest that the success of immunohistochemistry depends on the quality of antibodies and the method of tissue preparation. As long as the tissue specimen is obtained within 24 hours post-mortem, the reliability of collagen immunolabelling is guaranteed.

Initial observations suggest that most of the collagen is present in the deep layer of the lamina propria (Hammond et al., 2000; M. Hirano et al., 1981). The collagen distributions across the lamina propria were quantified by two studies (Hahn, Kobler, Zeitels et al., 2006; Hammond et al., 2000). Hammond et al. (2000) reported the relative density of collagen in human lamina propria with respect to age and gender. Results showed that collagen was found in the region immediately below epithelium and the vocalis muscle. Also, the collagen density was more

gender-related than age-related. The adult male vocal folds had about 131.5% of the collagen found in female adult vocal folds. On the other hand, Hahn et al. (2006) quantified the collagen levels by measuring the absolute amount of tissue hydroxyproline (mg collagen per mg total protein) across the lamina propria. Their results showed that collagen made up 43.4% ± 2.6% of total protein in human lamina propria. Adult males had approximately 130% of the adult female total collagen content in the lamina propria, which generally agree with Hammond et al.'s results. Furthermore, the collagen levels in the superficial third, intermediate third and deep third regions of the lamina propria (defined by a Mat-lab program) were approximately 0.27, 0.32 and 0.71 mg per milligram of total protein respectively, which again agreed with the previous Hammond et al.'s report.

Regarding the distribution of collagen subtypes in human vocal folds, collagen types I and III are identified in the lamina propria (Gray et al., 1993; Hahn, Kobler, Zeitels et al., 2006; Madruga de Melo et al., 2003; T. Tateya, I. Tateya, & D. M. Bless, 2006; T. Tateya et al., 2007). Collagen types I and III are the fibrillar collagen, which are made up of tropocollagen units. These collagens are nonelastic, i.e., they do not stretch well. Also, collagen types I and III are in coil-like shape, which give expansibility and resilience to the lamina propria. Also, these collagens are oriented longitudinally from anterior to posterior. This orientation may allow the collagens to bear stress and resist deformation while withstanding vibratory forces during phonation (Gray, Titze et al., 2000).

The distribution of collagen types I and III in human vocal folds remain controversial. A study used a picrosirius polarization method to examine the distribution and orientation of collagen type I and collagen type III in human vocal folds (Madruga de Melo et al., 2003). Their results suggest that collagen type I and type III form an organized "wicker-basket-like" network

immediately below the epithelium and also a high density network in the deep lamina propria. In contrary, a loose and delicate network of collagen type III fibres was indicated in the intermediate lamina propria. The investigators conclude that the network of collagen type I and type III allows the lamina propria to be stabilized during vibration but also provides flexibility to the structure to deform without stretching the collagens. Furthermore, the investigators claim that collagen type I fibres are thick and strong, whereas collagen type III fibres are thin and weak in the lamina propria.

However, several studies suggest that collagen type III is the predominant type of collagen and appear to make up the thick collagenous fibres in the lamina propria (Gray et al., 1993; Hahn, Kobler, Zeitels et al., 2006; T. Tateya, I. Tateya, & D. M. Bless, 2006). Tateya et al. (T. Tateya, I. Tateya, & D. M. Bless, 2006; T. Tateya et al., 2007) used immunohistochmistry and immno-scanning electron microscope to examine the collagen subtypes in the vocal folds. Their results show that collagen type I is relatively thin in structure (T. Tateya, I. Tateya, & D. M. Bless, 2006; T. Tateya et al., 2007) and is located in the basement membrane and the deep lamina propria (Hahn, Kobler, Zeitels et al., 2006; T. Tateya, I. Tateya, & D. M. Bless, 2006; T. Tateya et al., 2007). The distribution of collagen type I indicate that the collagen may contribute to the shape of the lamina propria and the tensile strength for the basement membrane and the deep lamina propria when subjected to vibratory forces. Most importantly, their results indicate that the large bundles of collagen fibres in the vocal fold lamina propria are not collagen type I but collagen type III.

Collagen type III is distributed throughout the lamina propria (Hahn, Kobler, Zeitels et al., 2006; T. Tateya, I. Tateya, & D. M. Bless, 2006; T. Tateya et al., 2007) and is organized in both thin fibres and wavy and thick fibres (T. Tateya, I. Tateya, & D. M. Bless, 2006). Both thin

and thick collagen type III fibres were speculated as reticular fibres and collagenous fibres in vocal fold lamina propria respectively (Sato, 1998; T. Tateya, I. Tateya, & D. M. Bless, 2006). A later study using immuno-scanning electron microscopy confirms that reticular fibres are composed of both collagen type I and type III, whereas the collagenous fibres are composed of collagen type I (T. Tateya, Tateya, & Bless, 2007). Reticular fibres are reported to locate in the superficial and intermediate layers of the lamina propria of the vocal fold mucosa, especially around the vocal fold edge (Sato, 1998). The spaces of the reticular fibres are filled with interstitial elements and elastic fibres running through the spaces (Sato, 1998). The network of the reticular fibres, interstitial elements and elastic fibres contributes to the biomechanical properties of the vocal fold lamina propria. Nevertheless, both thick collagen type III and thin collagen type III are important to give the structure and flexibility to the lamina propria.

### *Elastin.*

Elastin is the other important structural protein of the vocal fold lamina propria. Recall that collagen mainly gives strength and structure to the lamina propria, elastin contributes to the elasticity and resilience of the vocal folds. Elasticity and resilience enable the vocal fold tissue to be deformed and to return to the original shape when subjected to the vibratory force. Elastin materials can be classified into 3 types depending on their elastin-to-fibril ratio: (1) oxytalan (no elastin), (2) elaunin (intermediate elastin-to-microfibril ratio) and (3) mature elastic/elastin fibres (high elastin-to-microfibril ratio) (Kielty, Sherratt, & Shuttleworth, 2002). Oxytalan is solely composed of microfibrils with 10 - 12 nm in diameter. Elaunin has microfibrils and a small elastin amorphous component. Mature elastin fibres have high elastin amorphous component as a core surrounding by the microfibrils. The high ratio of the elastin component makes the mature elastin fibres the most elastic (Kielty et al., 2002). In the vocal fold lamina propria, the elastin

fibres are able to be stretched to about two times of their original length and then return to the original length after removal of the stretching force. This elastic property is known to be important for the proper vocal fold vibration (Gray et al., 1993; Gray, Titze et al., 2000).

The distribution of elastin materials (all types) in the human lamina propria are described in several histological studies (Hahn, Kobler, Starcher et al., 2006; Hammond et al., 1998b; Hammond et al., 2000; Hammond et al., 1997; T. Tateya, I. Tateya, & D. M. Bless, 2006), whereas the structure and orientation of elastin are examined by electron miscroscopy (Hammond et al., 1997; Ishii et al., 2000; Sato & Hirano, 1997a). A general notion is that elastin materials are present throughout all layers of the lamina propria but each type of elastin materials has different density across the lamina propria (Gray, Chan et al., 2000; Gray, Titze et al., 2000; Hahn, Kobler, Starcher et al., 2006; Hammond et al., 1998b; Hammond et al., 2000; Hammond et al., 1997).

Regarding the distribution of elastin materials (all types) in the lamina propria, fluorescent elastin materials are found throughout the lamina propria with intense amount in the superficial lamina propria immediately below the basement membrane (Gray, Titze et al., 2000). This observation is substantiated by another study using immunohistochemistry (T. Tateya, I. Tateya, & D. M. Bless, 2006). Elastin materials are found in the superficial lamina propria around the basement membrane and in the intermediate and the deep lamina propria as well as macula flava. One study quantified the concentration of elastin materials by measuring the amino acid desmosine level in the human lamina propria (Hahn, Kobler, Starcher et al., 2006). The desmosine levels in the superficial third, the intermediate third and the deep third of the lamina propria were approximately 1.1, 2.9 and 4.1 picomoles per mg total protein respectively. Also,

the elastin materials constituted approximately 8.5% ± 2.1%, 6.0% ± 0.7%, and 7.5% ± 1.2% of human lamina propria total protein.

The distribution of elastin subtypes have been studied using elastin-van Gieson staining and electron microscopy methods (Hammond et al., 1998; Hammond et al., 1997). Results suggest that oxytalan and elaunin are dominant in the superficial lamina propria, whereas mature elastin fibres are intense in the intermediate and deeper layers of the lamina propria. The population density of mature elastin fibres in the lamina propria may be age-related. Infant vocal folds have about 23% of the elastin found in adults and geriatric vocal folds have about 879% of the elastin found in adults (Hammond et al., 1998).

The identification of oxytalan and elaunin has been a challenge. Only mature elastin fibre is stained well and displays as fibre form with elastin-van Gieson (EVG) staining. To identify oxytalan and elaunin, investigators can only make speculation about their presence if a specimen shows EVG staining but the staining pattern is not in fibre form under the examination of electron microscopy (Gray, Titze et al., 2000; Hammond et al., 1998).

An alternative method is to use fibrillin-1 staining together with elastin staining (Hahn, Kobler, Starcher et al., 2006) to differentiate the oxytalan from the elaunin and mature elastin fibres. Relatively strong fibrillin-1 staining but weak elastin staining suggests oxytalan (no elastin component). In contrary, relatively weak fibrillin-1 staining but strong elastin staining suggest the elaunin or mature elastin fibres (relatively high elastin component). Hahn et al. (2006) reported that the superficial lamina propria, especially the region immediately below the basement membrane, had high fibrillin-1 staining relative to that of elastin. This observation indicates the presence of oxytalan in the superficial lamina propria. At the same time, the intermediate lamina propria had relatively weak fibrillin-1 staining but strong elastin staining,

which indicates the presence of elaunin or mature elastic fibres. However, the investigators did not explain how to differentiate elaunin from the mature elastic fibres in their protocol.

In sum, the localization of the elastin materials is layer-specific. The least-elastic forms of elastin (oxytalan and elaunin) are present in the matrix of the superficial lamina propria. The mature elastin fibres are intensely present in the intermediate lamina propria.

### A.3.8 Interstitial elements (glycosaminoglycan, proteoglycans and glycoproteins)

Glycoaminoglycan (hyaluronic acid), glycoprotein (fibronectin) and proteoglycan (decorin and fibromodulin) are the well-studied interstitial elements in the human vocal fold lamina propria. (Barbosa et al., 2008; Gray, 2000; Gray et al., 1993; Hahn, Jao, Faquin, & Grande-Allen, 2008; Hahn et al., 2005; Hirschi, Gray, & Thibeault, 2002; Lebl et al., 2007; Pawlak et al., 1996; Ward, Thiebault, & Gray, 2002)These elements constitute the extracellular matrix and modulate the viscosity of the vocal folds. Interstitial elements are closely associated with fibrous proteins (collagen and elastin). The elements may bind fibrous proteins to alter the mechanics of collagen and elastin fibres (Pawlak et al., 1996). For instance, decorin and hyaluronic acid have influence over collagen fibril formation to alter the viscoelastic properties of the vocal fold lamina propria (Gray, Chan, & Turner, 2000). Table A1 summarizes the distribution and major functions of the interstitial elements in the human vocal fold lamina propria.

#### *Glycoproteins: Fibronectin.*

In human normal vocal folds, fibronectin is abundant in amount (Gray et al., 1995). A microarray study reported that the genes of fibronectin are up-regulated over four-fold in the true

vocal folds than those in the false vocal folds (Hirschi, Gray, & Thibeault, 2002). Another study using immunohistochemical staining reported that fibronectin is particularly abundant in the superficial lamina propria of the normal vocal folds (Courey et al., 1996).

Fibronectin is an adhesive glycoprotein that has binding sites for fibrin (collagens), glycosaminoglycan (hyaluronic acid) and cells (fibroblasts and endothelial cells). Fibronectin play roles in: (1) extracellular matrix formation and organization, (2) mediating cell-cell and cell-matrix interactions and (3) being chemoattractant for inflammatory cells and fibroblasts (Couchman, Austria, & Woods, 1990; Grinnell, 1984; Grinnell, Toda, & Takashima, 1988). A study suggest that fibronectin may contribute more to assemble and maintain the proteins and cells in the extracellular matrix, rather than shear-bearing for the vocal fold mucosa (Chan & Titze, 1999). Fibronectin also plays an important role in wound healing. After tissue injury, fibronectin is deposited on the damaged tissue and form a scaffold, which enhances fibroblast migration and collagen deposition (Grinnell, 1984; Grinnell et al., 1988). Benign vocal fold lesions are reported to have an increased level of fibronectin within the lamina propria (Courey et al., 1996; Gray et al., 1995; Thibeault, Gray, Li et al., 2002).

### *Proteoglycans: Decorin and Fibromodulin.*

Decorin and fibromodulin are the small chain proteoglycans identified in the human vocal fold lamina propria (Gray et al., 1999; Pawlak et al., 1996). These small chain proteoglycans are composed of a leucine-rich protein core and a glycosaminoglycan chain. Decorin and fibromodulin bind to and interact with collagen types I and II fibrils with different binding sites in other tissue domains (Goetnick, 1991; Hardingham & Fosang, 1992; Oldberg, 1993). The presence of decorin and fibromodulin in the extracellular matrix may delay collagen fibril formation and yield thinner and smaller collagen fibrils (Hardingham & Fosang, 1992).

Although decorin and fibromodulin have similar structure and function, their locations in the vocal fold lamina propria are different. Decorin is intensely present in the superficial lamina propria, whereas fibromodulin is primarily present in the intermediate and the deep lamina propria (Gray et al., 1999; Hahn et al., 2005; Pawlak et al., 1996). Table A1 summarizes the locations and major functions of decorin and fibromodulin in human normal vocal fold lamina propria.

### *Decorin.*

Decorin was first identified in the superficial lamina propria in a human study using immunohistochemical methods (Pawlak et al., 1996). This finding is substantiated by a later study (Hahn et al., 2005). Decorin is found intensely in both the superficial and deep lamina propria, whereas the decorin stain is more intense in the superficial lamina propria of human vocal folds. At the same time, decorin is minimally present in the intermediate lamina propria.

The functions of decorin in the vocal folds remain speculative. In other tissues, decorin binds to the collagen type I and II fibrils and change their kinetics of formation and assembly, resulting the reduction of the collagen fibril size (Goetnick, 1991; Hardingham & Fosang, 1992; Oldberg, 1993). Interestingly, within the vocal fold superficial lamina propria, intense decorin is present (Gray et al., 1999; Hahn et al., 2005; Pawlak et al., 1996) with sparse and thing collagen fibres (Sato, 1998). Speculatively, decorin may inhibit the collagen fibre formation in the superficial lamina propria resulting the sparse population of collagen fibres in that layer (Gray et al., 1999).

### *Fibromodulin.*

Fibromodulin is present in the intermediate and the deep layers of lamina propria. Specifically, the staining pattern of fibromodulin demarcates an area that correlates with the

vocal ligament (Gray et al., 1999; Pawlak et al., 1996). The identification of fibromodulin in the vocal folds has been a challenge. First, fibromodulin analysis requires fresh-frozen tissue or the antigenicity is lost. Second, the distribution of fibromodulin is highly variable across individuals (Hahn et al., 2005). Hahn et al. (2005) used the antibodies of keratin sulphate, which is the glycosaminoglycan attachment of fibromodulin, to locate the fibromodulin in the vocal fold lamina propria (Hahn et al., 2005). The results of the intensity and location of keratin sulphate stain are inconclusive due to the extreme variations across their human tissue specimens. Interestingly, these findings are against the earlier results of Pawlak et al.'s (1996) study, in which the keratin sulphate stain are consistently found in the vocal ligament across their human tissue specimens (Pawlak et al., 1996). The contradiction may be related to the age and gender of the human specimens between these two studies. Unfortunately, Pawlak et al. (1996) did not report the information of their specimens and thus the factors of age and gender on the distribution of fibromodulin remain speculative.

Regarding the functions of fibromodulin in the vocal folds, fibromodulin may be closely associated with the surrounding collagen and elastin to maintain the tensile strength and support the architecture of the vocal ligament. Further, fibromodulin may lubricate the collagen and elastin fibres of the vocal ligament to promote tissue ligament performance for various vocal functions (Gray et al., 1999; Pawlak et al., 1996).

### *Glycosaminoglycan: Hyaluronan.*

Hyaluronan or hyaluronic acid is a non-antigenic, negatively-charged, hydrophilic, and high-molecular mass (about 100 – 5,000 kilodaton) glycosaminoglycans or mucopolysaccharides identified in the extracellular matrix of the vocal fold lamina propria (Butler, Hammond, & Gray, 2001; Hahn, Kobler, Starcher et al., 2006; Laurent & Fraser, 1992; Pawlak et al., 1996). In the

vocal folds, the stellate cells in the macula flava as well as the fibroblasts and macrophages in the superficial lamina propria have been suggested to produce hyaluronan acid (Hallen, Dahlqvist, & Laurent, 1998). One study investigated a cell surface receptor for hyaluronan, CD44, and its relationship to the distribution of hyaluronan in human vocal folds (Sato, Sakamoto, & Nakashima, 2006). Results show that many of the stellate cells in the macula flava are stained with CD44, whereas sparse fibroblasts in adult Reinke's spaces are stained with CD-44. Also, abundant hyaluronan is around the stellate cells in the macula flava, which suggests that the stellate cells can be the main cell source of producing hyaluronan in human vocal folds. Another study also indicated HA was concentrated in the anterior and posterior macula flava, esp in the middle and deep layers, in a male individual (Barbosa et al., 2008). CD-44 receptors were mainly accumulated in the vocal fold epithelium but very few in Reinke's space.

The half-life of hyaluronan is short, which is about 3-5 days in rabbit vocal folds (Hallen et al., 1998). Hyaluronan appears as globular, porous and loosely folded (Laurent & Fraser, 1992). Also, hyaluronan is able to form highly polarized chains in the extracellular matrix to attract and regulate water content of the surrounding tissues (Ward, Thiebault, & Gray, 2002).

Hyaluronan has multiple functions. First, hyaluronan regulates cell proliferation and differentiation, molecular transport, inflammation, angiogenesis and wound healing in the surrounding tissues. Second, hyaluronan acts as interstitial "filler" materials that surround the fibrous component (collagen and elastin fibres) within the extracellular matrix of the vocal folds. Third, hyaluronan is negative-charged, random-coiled and osmotic-active macromolecule that attracts water and cations. Therefore, hyaluronan has the capacity to regulate tissue osmosis, tissue viscosity and tissue flow in order to alter the volume of tissue it surrounds. The space filling as well as osmotic and viscosity regulation functions are particularly crucial for the vocal

folds because these functions directly influence the thickness and viscoelasticity of the vocal fold mucosa and subsequently the vocal fold performance (Butler et al., 2001; Chan et al., 2001). Further, these capacities allow hyaluronan to act as a shock absorber to resist compression as well as a tissue damper to dissipate stress during phonation for vocal fold tissue. Shock absorption and stress dampening are important for protecting the vocal fold edges from phonotrauma (Butler et al., 2001; Gray et al., 1999; Laurent, Laurent, & Fraser, 1995).

In addition to the roles of shock absorber and tissue damper, hyaluronan has been shown to have biochemical roles in the regulation of the vocal folds' viscoelasticity (viscosity and elasticity) (Chan et al., 2001; Chan & Titze, 1999). Optimal viscosity and elasticity of the vocal folds are necessary to initiate and sustain phonation (Chan et al., 2001; Chan & Titze, 1999). The effects of hyaluronan on vocal fold viscosity and elasticity were quantified by measuring the biomechanical properties of the vocal folds with and without hyaluronan (Chan & Titze, 1999). After removing hyaluronan from the lamina propria of human vocal folds *in vitro*, the stiffness of the vocal fold cover decreased by 35% on average, whereas the dynamic viscosity of the vocal fold cover at high frequencies (>1 Hz) increased by 70% on average. These results indicate that hyaluronan likely contributes to maintain optimal conditions necessary for phonation. Also, hyaluronan may contribute to the regulation of tissue stiffness that is essential for vocal fundamental frequency control. Generally speaking, a higher concentration of hyaluronan in the tissue increases tissue viscosity and gives the tissue more dampening and shocking absorbing properties (Chan et al., 2001; Chan & Titze, 1999).

Regarding the distribution, hyaluronan is present throughout the extracellular matrix of the lamina propria and is slightly more concentrated in the intermediate layer (Butler et al., 2001; Hahn, Kobler, Starcher, Zeitels, & Langer, 2006; Hammond et al., 1997; Lebl et al., 2007). An

immunohistochemical study used Enzyme-Linked ImmunoSorbeny Assay (ELISA) to quantify the hyaluronan concentrations across the lamina propria of human vocal folds (Hahn, Kobler, Starcher et al., 2006). The hyaluronan concentrations in the superior-third, intermediate-third and deep-third of the depths of the lamina propria were estimated as 7.4, 8.7 and 8.5 μg/mg total protein respectively. A follow-up study using flurophore-assisted carbohydrate electrophoresis showed that normal human vocal folds contained approximately hyaluronan 6.4 +/- 4.1 μg of hyaluronan per milligram of total proteins within lamina propria. (Hahn, Jao, Faquin, & Grande-Allen, 2008).

In addition to the layer-specific distribution pattern, the concentration of hyaluronan in the lamina propria of the normal human vocal fold is also gender-dependent. One study quantified the amount of hyaluronan in the lamina propria in 18 normal adult human vocal folds (10 males, 8 females; age range 20-60 year-old) using the acid mucopolysaccharide staining method (Hammond et al., 1997). Only the midsections of the membranous vocal folds were stained for hyaluronan and the slides were compared using computer imaging software. Despite of moderate individual variability in the hyaluronan content per area, male subjects had approximately three-fold higher hyaluronan than the female subjects. The individual variability may be due to the fact that only the midsections of the vocal folds were analysed.

Another study used the same staining method to quantify hyaluronan throughout the complete depth of the lamina propria at the vocal fold's leading edge and to examine the age- and gender-related effects (Butler et al., 2001). In general, the distribution of hyaluronan is more gender-related than age-related. Female subjects had relatively less hyaluronan in the more superficial areas (0%-15% depth of the total lamina propria) and had more hyaluronan in the deeper areas (40%-100% depth of the total lamina propria). At the same time, male subjects

showed a relatively uniform distribution pattern throughout the depth of the lamina propria. Furthermore, the male subjects had approximately two-fold higher hyaluronan content than the female subjects had in the superficial lamina propria, which corroborates with Hammond et al.'s (1997) findings. The noted gender difference in hyaluronan distribution may suggest the male-female differences in the occurrence of phonotrauma. If the concentration of hyaluronan is higher in tissue, the viscosity of the tissue is higher and gives more dampening and shock absorbing properties for the tissue. The less hyaluronan in the more superficial areas of the female vocal fold may lead to less shock-absorbing and tissue-dampening as well as wound healing capability of the tissue against phonotrauma. This finding may explain why phonotrauma, clinically, is more common in women than in men (Miller & Verdolini, 1995; Roy et al., 2004; Smith, Kirchner, Taylor, Hoffman, & Lemke, 1998).

Interestingly, another study, which used hyaluronan-binding proteins (HABP) from bovine cartilage to detect hyaluronan in human adult vocal folds, reported contradictory results (Lebl et al., 2007). Women vocal folds showed approximately two-time higher hyaluronan concentrations than that in male vocal folds overall. In studies of Butler et al. and Hammond et al., indirect histological method was used to measure HA concentrations, i.e., compare the different of staining intensities between tissue samples treated and without treated with hyaluronidase. This method did not directly measure HA concentrations in tissue samples and may be interference by other GAG proteins. Lebl et al. suggested that HABP used in their study provided specific binding to hyaluronan and eliminated the interference of other GAG proteins. Further, the researchers acclaimed that the high concentration of HA in female vocal folds may act as a protective factor for increased impact-absorption capacity against the production of high-frequency sounds.

## A.4 MACULA FLAVA

The macula flava are situated at the anterior and posterior ends of the intermediate lamina propria of the human vocal fold (Sato & Hirano, 1995a, 1995b; K. Sato, M. Hirano, & T Nakashima, 2003a). The anterior macular flava forms an oval mass at the anterior end of the intermediate lamina propria, which is connected to the anterior commissure of the thyroid cartilage. The posterior macula flava is located at the posterior end of the intermediate lamina propria, which is connected to the vocal process of the arytenoid cartilage (M. Hirano & Sato, 1993; Sato & Hirano, 1995). The macula flava form the visible mucosal bulges as seen as whitish yellow masses. The macula flava are about 1.5 x 1.5 x 1.0mm in size (Sato & Hirano, 1995). The functions of the macula flava in human vocal folds are unclear. The macula flava may play roles in (1) shock-absorption during phonation, (2) producing elastic and collagenous fibres for develop and maintain vocal ligament, (3) regulating the metabolism of extracellular matrix for growth, development and aging of the vocal fold mucosa; and (4) inducing inflammatory and wound healing responses for the injured vocal folds (Fuja, Probst-Fuja, & Titze, 2005a, 2005b; M. Hirano et al., 1999a, 2000; Sato & Hirano, 1995a, 1995b; Sato, Hirano, & Nakashima, 2001b; Sato et al., 2003a; K. Sato, M. Hirano, & T. Nakashima, 2003b; Sato & Nakashima, 2005; Sato et al., 2006).

The light and electron microscopic examinations indicate that the macula flava are composed of dense fibrous tissue (elastic and collagenous fibres) and ground substance (M. Hirano & Sato, 1993; Sato & Hirano, 1995). Three-dimensional imaging supports this observation in which numerous elastic, collagenous and reticular fibres are found to run in various directions within the human macula flava and to extend to the lamina propria of the vocal folds (K. Sato, M. Hirano, & T Nakashima, 2003a). Also, abundant hyaluronic acid is located

among the spaces of the fibrous tissue, which enhances shock absorption during phonation (Sato et al., 2003a; Sato et al., 2006).

Regarding the cellularity, Sato et al. (K. Sato, M. Hirano, & T. Nakashima, 2003b) discovered a novel cell type in the macula flava, namely, “vocal fold stellate cells”. These cells are not identified in the lamina propria but are abundantly present in the macula flava. Vocal fold stellate cells compose 82% of the total cells in adult macula flava (M. Hirano et al., 1999a). The vocal fold stellate cells have a fibroblast-like structure but have different morphology and functions from the fibroblasts in the superficial lamina propria.

First, the vocal fold stellate cells have smaller ratio of nucleus and cytoplasm and well-developed intracellular organelles (rough endoplasmic reticulum and Golgi apparatus), compared to the fibroblasts in the superficial lamina propria (M. Hirano, Sato, & Nakashima, 1999b; Sato, Hirano, & Nakashima, 2001).

Second, vitamin A-containing lipid droplets are found in the cytoplasm of the vocal fold stellate cells (Sato et al., 2003b). Vitamin A in the stellate cells: (1) can be a morphogen that controls the cell differentiation and morphogenesis, (2) involve in the induction of transforming growth factor-β that stimulates the synthesis of extracellular matrix substances, and (3) strongly affects the activity of adenosine triphosphase sulphurylase that related to the synthesis of glycosaminoglycan, i.e., hyaluronan in the vocal fold lamina propria (Fuja et al., 2005a, 2005b; Glick, Flanders, Danielpour, Yuspa, & Sporn, 1989; Sato et al., 2003a; Sato & Nakashima, 2005).

Lastly, vocal fold stellate cells are surrounded by dense reticular fibres and hyaluronan (Sato et al., 2001a; Sato & Nakashima, 2005). This observation suggests that stellate cells actively and constantly synthesize the extracellular matrix substances, including elastin,

collagenous and reticular fibres and hyaluronan under normal conditions of the vocal folds (M. Hirano et al., 1999a).

On the other hand, vocal fold stellate cells have a dual-phenotype (Amir & Kishon-Rabin, 2004; Fuja et al., 2005a, 2005b). In their resting state, the cells maintain vitamin A in the intracellular lipid droplets and have stellate morphology. After a cultured-induced process of activation or transdifferentiation, vocal fold stellate cells increase their proliferation, lose vitamin A and lipid droplets and express the activated marker of α-smooth muscle actin. Subsequently, the activated vocal fold stellate cells express significantly high levels of collagen type 1α1 and type III, decorin, laminin-β, matrix metalloproteinase-2 and -14, tissue inhibitors of matrix metalloproteinase-1 and -3, connective tissue growth factor, transforming growth factor-β1, platelet derived growth factor receptor-β, hyaluronic acid synthase 2, hyaluronidase II, plasminogen activator inhibitor I, and peroxisome proliferator-activated receptors γ and β/δ, compared to the control fibroblasts from trachea. Also, vocal fold stellate cells show a well-organized actin cytoskeleton as myofibroblast-like phenotype after culture-induced activation (Fuja, Probst-Fuja, & Titze, 2005). Collectively speaking, in addition to the role of extracellular matrix synthesis under normal conditions, activated vocal fold stellate cells may also contribute to the regulation of the vocal fold extracellular matrix under pathological conditions.

# APPENDIX B

# BIOLOGY OF VOCAL FOLD WOUND HEALING

Mechanical injury associated with phonation or phonosurgery is the common origin of vocal fold tissue damage. The purposes of this chapter are to (1) review the biological changes in vocal fold tissue after phonotrauma or phonosurgery, (2) postulate the possible healing mechanisms in traumatized vocal fold tissue, and (3) discuss the roles of tissue mobilization in tissue healing.

## B.1 PHONATORY BIOMECHANICAL INJURY: PHONOTRAUMA

Phonotrauma is induced by biomechanical stress during phonation (Gunter, 2003, 2004; Jiang et al., 1998; I.R. Titze, 1994). The human vocal folds vibrate naturally at frequencies of 100-1000 Hz and amplitudes of about 1 mm (Titze, 1989). A study reports that teachers turn their voices on and off about 1,800 times an hour at work and accumulate up to about 20,000 times a day (Titze, Hunter, & Svec, 2007). Various mechanical stresses, namely, tensile, contractile, aerodynamic, inertia, impact and shear stresses, act on the mucosa and/or muscles of the vocal folds during phonation (Gunter, 2003, 2004; Jiang et al., 1998; I.R. Titze, 1994). Pertinent data

suggest that phonatory stresses may (1) alter the tissue's physical structure by disrupting intracellular adhesion and cellular structure (Gray & Titze, 1988; Gray et al., 1987), and/or (2) elicit the tissue's biological responses by altering gene expression and spatial distribution of cells and extracellular matrix substances (Branski et al., 2006; Ding & Gray, 2001; Thibeault et al., 2003; Titze et al., 2004; Verdolini et al., 2003).

The injury mechanism of phonatory stresses to the vocal fold tissue is not detailed out yet. However, speculations can be made from the physical models of vocal fold vibration that describe the magnitude and distribution of the phonatory stresses in the tissue (Gunter, 2003, 2004; Jiang et al., 1998; I.R. Titze, 1994). Among the aforesaid stresses, tensile stress associated with tissue stretching has the greatest value (~ 1.0 MPa). Contractile stress induced by the thyroarytenoid and cricothyroid muscle contraction is smaller in magnitude (~ 100 kPa), and aerodynamic stresses induced by subglottal pressure is the smallest (~1-10 kPa).

Interestingly, inertia (~1-2 kPa) and impact stresses (~0.5-5.0 kPa), which are intermediate in magnitude among the stresses, have been suggested to play a significant role in vocal fold injury (Gunter, 2003, 2004; Jiang et al., 1998; I.R. Titze, 1994). Inertial stress can be understood as the acceleration and deceleration of the vocal folds without collision. Rapid acceleration and deceleration of vocal fold tissue may disturb blood circulation and subsequently affect oxygen supply to the tissue (Svec & Sram, 2001; I.R. Titze, 1994; Tomita et al., 1988). In fact, the acceleration from vocal fold vibration may build up intravascular pressure by moving fluid toward the mid-point of the vocal folds (Czerwonka, Jiang, & Tao, 2008). Physical models predicted that vocal fold intravascular pressure rised significantly during vocal fold vibration, close to the range of magnitudes causing capillaries damage and the leakage of erythrocytes

(Czerwonka et al., 2008). The intravascular pressure was predicted over 20 $cmH_2O$ in speaking frequency and intensity, whereas the pressure could reach higher levels in singing and speaking.

On the other hand, impact stress is the compressive force delivered perpendicularly to plane of vocal fold contact. Titze (1994) proposes that vocal ligament is structurally more accommodated for tensile stress but not for impact stress. The exposed soft tissues of the vocal folds absorb most of the impact stress during phonation, which poses a risk of tissue failure. Despite of inertia and impact stresses, vertical shear stress (~0.8 kPa), which is the force parallel to the plane of contact in the vertical direction, may also involve in vocal fold injury. A three-dimensional finite element model of vocal fold vibration predicted an increase in both impact and vertical shear stresses at the centre of the vocal fold edge during vocal fold collision (Gunter, 2003, 2004). This vocal fold location is exactly the injury-prone site where phonotraumatic lesions normally develop.

The vocal folds are believed to undergo repetitive microtrauma owing to phonation throughout the life. A post-mortem study of 266 adult autopsies reported that 36.5% and 64% of the vocal folds were considered as normal and as presenting microscopic vocal fold lesion respectively (Salge, Peres, Reis, Teixeira Vde, & Castro, 2006). None of the autopsy cases exhibited clinically significant phonotraumatic lesions. These data suggest that the vocal folds can generally sustain phonatory injury and do not necessarily develop a macroscopic vocal fold lesion throughout the life. The vocal folds may be structurally capable to withstand phonatory stresses and/or may have the reparative capabilities to resolve the microscopically phonotraumatic damage. However, when the injury culminates in a threshold episode, inflammation becomes evident and may eventually develop macroscopic vocal fold lesions. Phonotrauma can result from a single traumatic force of relatively large magnitude or from

repeated forces of relatively small magnitude. When a single vocal loading (e.g., screaming at a football game) produces traumatic injury to vocal fold tissue, the injury is known as *acute phonotrauma*. When repeated or chronic vocal loadings over a period of time produce an injury, the injury is known as *chronic phonotrauma*. The following sections discuss the biological and microarchitectural changes of the vocal folds after acute and chronic phonotrauma.

### B.1.1 Acute phonotrauma

Acute phonotrauma may cause abrasion of epithelium, separation of the surface epithelial cells from the beneath lamina propria and disruption of vascular network, which lead to vocal fold inflammation. In two similar animal studies, two groups of anesthetized canines were subjected to artificially phonate for two and four hours at high phonatory intensity (60-90 dB at 6 ft.) (Gray & Titze, 1988; Gray et al., 1987). Electron-microscopic examination indicated disruption of the surface microvillae of the epithelial cells and desquamation of the surface epithelial cells after two hours of phonation. However, the epithelium geminating layer, the basement membrane zone (BMZ) and the lamina propria were relatively preserved. After four hours of phonation, injuries extended to the BMZ. Greater area of destruction of epithelium, total desquamation of epithelial cells, presence of fluid among epithelial cells and destruction of organelles of the remaining epithelial cells were observed in the canine vocal folds. Also, interstitial fluid was present (1) between the basal cells of the epithelium and the BMZ and/or (2) between the BMZ and the superficial lamina propria among the canines. The collection of interstitial fluid may be probably due to the damage of anchoring fibres and detachment of collagen fibres in the superficial lamina propria from the BMZ. No vocal fold haemorrhage was indicated after four hours of intensive phonation.

Beyond cellular rupture and separation of tissue elements, vascular network may also be affected following acute phonotrauma. A physical model of vocal fold vibration predicted that the magnitude of intravascular pressure built-up during singing and screaming might lead to capillaries damage and the trigger of inflammatory response (Czerwonka et al., 2008). A human study reported that a female individual developed subtle vocal fold edema along the vocal fold free edges after continuous loud vocal loading for an hour (Verdolini, Rosen, Branski, & Hebda, 2003). This individual showed marked increase in the concentrations of three pro-inflammatory cytokines: interleukin-1beta, tumour necrosis factor-alpha, and matrix metalloproteinase-8, from baseline to the 10-minute post-loading time point. Acute edema may be the outcome of increased intravascular pressure, submucosal capillary rupture, vasodilation, leakage of blood plasma into extravascular compartment and the inflammatory cytokine release (Courey et al., 1996; Czerwonka et al., 2008) (Courey et al., 1996). Clinically, vocal fold haemorrhage or acute laryngitis is manifested after acute phonotrauma.

Molecular response to experimentally induced phonation was investigated in a rabbit study (Rousseau, Ge, French et al., 2008). Results showed a significant increased gene expression of MMP-1 and nonsignificant increases in MMP-9 and IL-1β following a prolonged (3-hour) phonation within a physiological range of human voice production (77 ± 3 dB; 429 ± 141 Hz) and a 1-hour recovery. The upregulation of MMP-1 gene expression following prolonged phonation may be related to the homestatic control of collagen in response to phonation-induced injury.

### B.1.2 Chronic phonotrauma

Various types of non-epithelial abnormalities are observed in benign vocal fold lesions associated with phonotrauma. These lesions are not operatively defined and their diagnoses are typically based on subjective evaluation of macroscopic appearance of the lesions. Also, consensus is lacking around diagnostic criteria for the lesions themselves (Rosen & Murry, 2000). As a result, data comparison across studies becomes confounded. To avoid further complication of defining vocal fold lesions, this section adheres to the nomenclature in *The Classification Manual of Voice Disorders – I* (CMVD-I) (Verdolini, Rosen, & Branski, 2006). To keep in mind, the studies discussed below may not use exactly the same set of diagnostic criteria in CMVD-I.

According to CMVD-I, vocal fold nodules, polyps, cysts (sub-epithelial and ligament) and Reinke's edema are the benign vocal fold lesions, owing to chronic phonotrauma. Although the injury and healing mechanism for each type of lesion is not elucidated yet, the literature generally agrees that repeated phonotrauma disrupts cell functions in creating and maintaining a healthy tissue state and thus lead to pathological tissue changes. Subsequently, the change of tissue composition develops into a macroscopic vocal fold lesion, which affects vocal fold vibratory properties. Tables B1-5 summarize the histological characteristics of the vasculature, epithelium, the basement membrane zone (BMZ) and the superficial lamina propria as well as the expression of genes and cytokines for vocal fold nodules, polyps, cysts and Reinke's edema.

**Table B1.** Summary of histological changes of the vasculature in benign vocal fold lesions.

| | Vocal Fold Nodules | Vocal Fold Polyp(s) | Vocal Fold Cyst | Reinke's Edema |
|---|---|---|---|---|
| (De Biase & Pontes, 2008) | Absence of observable vessels. | Presence of visible vessels with longtitudinal and transverse orientation, abrupt reduction, and dilatation. | Presence of visible vessels with longtitudinal, transverse or tangled orientation, abrupt reduction, dilatation and tortuosities. | Not included in the study. |
| (Courey et al., 1996) | No increase in vascularity. | Large clusters of angiomatous-appearing blood vessels are frequently found in the lamina propria. | Some may have interrupted fibronectin distribution due to increased vasculature. | Some may have interrupted fibronectin distribution due to increased vasculature. |
| (F.G. Dikkers & Nikkels, 1995) | Absence of haemorrhage and edematous lake. Less frequent pathologic changes in submucosal blood vessels. | Recent bleeding, depositions of iron and fibrin and thrombosis. | Not included in the study. | Edematous lakes, extravascular erythrocytes and increased thickness of submucosal blood vessels. Little fibrin, iron and thrombosis. |
| (F. G. Dikkers & Nikkels, 1999) | Minimal hyaluronan is accumulated around the blood vessels.<br>Collagen deposition around the vessels is not specific to the lesion. | Hyaluronan is accumulated around the blood vessels.<br>Collagen deposition around the vessels is not specific to the lesion. | Not included in the study. | Minimal hyaluronan is accumulated around the blood vessels.<br>Collagen deposition around the vessels is not specific to the lesion. |
| (Jovanovic et al., 2007) | Not included in the study. | Not included in the study. | Not included in the study. | Dilated blood vessels in loops and branching networks.<br>Longitudinal blood vessles in different diameters.<br>Anastomoses.<br>Thin blood vessels walls.<br>Discontinusous or turbulent type of blood flow.<br>Occational "slow motion" of blood elements.<br>Vascular varicosities and erythrocyte accumulations in the inner wall of blood vessels. |
| (Jovanovic et al., 2008) | Not included in the study. | No mucosal "blue lines" seen. | No included in the study. | Longitudinal arranged mucosal "blue lines" were commonly seen in subepithelial well-developed hollow spaces. |
| (Kotby, Nassar, Seif, Helal, & Saleh, 1988) | Sparse vascularization.<br>Normal endothelial lining of the blood vessels.<br>Hyaline degeneration in the stroma is seen. | Abundant vascularization.<br>Normal endothelial lining of the blood vessels.<br>No hyaline degeneration in the stroma. | Not included in the study. | Not included in the study. |
| (Loire, Bouchayer, Cornut, & Bastian, 1988) | Capillaries in the lamina propria are either normal of with hyaline deposition (15%). | Not specifically described in the paper. | Not specifically described in the paper. | Not specifically described in the paper. |

| | | | | |
|---|---|---|---|---|
| (Sato, Hirano, & Nakashima, 1999) | Not included in the study. | Not included in the study. | Not included in the study. | Subepithelial vascularization and vessel dilation. Thin endothelium with small holes and vesicles and a thickened basement membrane of the vessels. Endothelial cells and pericytes around the vessels are degenerated and few in number to support the vessels. Fibroblasts and/or inflammatory cells secrete vascular endothelial growth factor, which may increase the capillary permeability. |
| (Sone et al., 2006) | No hypervascularity is present with no remarkably high blood flows. | Capillary rupture and dilation are seen. Hypervascularity is present with high blood flows. | Not specifically described in the paper. | Not included in the study. |
| (Wallis, Jackson-Menaldi, Holland, & Giraldo, 2004) | Smaller areas of telangiectasias. | Larger areas of telangiectasias. | Not included in the study. | Not included in the study. |

**Table B2.** Summary of histological changes of the epithelium in benign vocal fold lesions.

| | Vocal Fold Nodules | Vocal Fold Polyp(s) | Vocal Fold Cyst | Reinke's Edema |
|---|---|---|---|---|
| (Carriero et al., 2000) | Not included in the study. | Normal epithelium with normal cell size and distribution. | Not included in the study. | Homogeneous epithelium. Cell nuclei are larger in size and have increased nucleus-to-cytoplasm ratio. |
| (F. G. Dikkers, Hulstaert, Oosterbaan, & Cervera-paz, 1993) | Variable degeneration signs are observed for the epithelial cells, including cytoplasmic vacuoles, attenuated cell junctions and distorted desmosomal junctions. | The degenerative signs of the epithelial cells are occasionally occurred in a few restricted areas. | Not specifically described in the paper. | Not specifically described in the paper. |
| (Kotby et al., 1988) | Thickened epithelium (80.85μm) with different degree of keratinisation. Extensive disruption of the intercellular junctions between the epithelial cells. Desmosomal junctions are sparse. | Less thickened epithelium than nodules (66μm). Normal intercellular junctions of epithelial cells. Desmosomal junctions are more reserved. | Not included in the study. | Not included in the study. |
| (Loire et al., 1988) | Thickened epithelium with incomplete keratinisation. | Atrophy or acantosis of the epithelium is occasionally seen. | Mucus retention cyst: glandular epithelium. Epidermoid cyst: between 10 to 30 cellular layers. Some with keratinisation. | Irregular thickening of the epithelium; some regions may acanthotic or parakeratotic. Duplicate epithelium. |
| (Marcotullio et al., 2002) | Normal epithelium or keratosis is commonly seen. | Normal epithelium or keratosis is commonly seen. | Not included in the study. | Normal epithelium or keratosis is commonly seen. Dysplasia is also seen in Reinke's edema but not in nodules and polyps. |
| (Neves, Neto, & Pontes, 2004) | Epithelium abnormality is non-specific to nodules, polyps and Reinke's edema | | | |
| (Sakae et al., 2008) | Not included in the study. | Not included in the study. | Not included in the study. | Collagen fiber arrangements was preserved underneath epithelium. |
| (Tillmann, Rudert, Schunke, & Werner, 1995) | Not included in the study. | Not included in the study. | Not included in the study. | No pathological changes in the vocal fold epithelium under light or electron microscopy. |
| (van der Velden et al., 1996) | Atrophic epithelium | Keratinizing and hyperplastic epithelium | Not included in the study. | Not included in the study. |
| (Volic, Kirincic, & Markob, 1996) | Not included in the study. | Not included in the study. | Not included in the study. | From normal to hyperplastic to hyperkeratotic to parakeratotic epithelium |

**Table B3.** Summary of histological changes of the basement membrane zone (BMZ) in benign vocal fold lesions.

| | Vocal Fold Nodules | Vocal Fold Polyp(s) | Vocal Fold Cyst | Reinke's Edema |
|---|---|---|---|---|
| (Courey et al., 1996) | An average BMZ thickness of 1.88μm (range 0.5 to 3.0 μm) on collagen IV staining and 1.63 μ m (range 0.5 to 2.0 μm) on fibronectin staining. Thickened BMZ and dense fibronectin deposition. | An average BMZ thickness of 0.84 μm (range 0.5 to 2.0 μm) on collagen IV staining and 1.21 μm (range 0.5 to 5.0 μm) on fibronectin staining. Unaltered BMZ width except fibronectin deposition clustered around the neovasculatiry. | An average BMZ thickness of 1.04 μm on collagen IV staining and 1.14 μm on fibronectin staining. BMC thickness is between polyps and nodules. | An average BMZ thickness of 0.75 μm on both collagen IV and fibronectin staining. |
| (F. G. Dikkers et al., 1993) | Some parts of BMZ are lacking and some parts are thickened. A near absence of normal hemidesmosomes and anchoring fibres. | Not specifically described in the paper. | Not specifically described in the paper. | Not specifically described in the paper. |
| (F.G. Dikkers & Nikkels, 1995) | Thickened BMZ is commonly seen. | Thickened BMZ is not commonly seen. | Not included in the study. | Thickened BMZ is commonly seen. |
| (F. G. Dikkers & Nikkels, 1999) | Collagen deposition in the BMZ is not specific to the lesion. | Collagen deposition in the BMZ is not specific to the lesion. | Not included in the study. | Collagen deposition in the BMZ is not specific to the lesion. |
| (Gray et al., 1995) | BMZ injury as indicated by thick collagen type IV bands. | Rare BMZ injury. | Not included in the study. | Rare BMZ injury. |
| (Loire et al., 1988) | Irregular thickened BMZ | Thinning of the BMZ. | Mucus retention cyst: quite thin BMZ. | Normal or thickened BMZ are seen. |
| (Neves et al., 2004) | Thickened BMZ with increased collagen type IV and laminin. | BMZ is not significantly thickened. | Not included in the study. | Reinke's edema was not differentiated from nodules and polyps under histological and immunohistochemical analysis. |
| (Volic et al., 1996) | Not included in the study. | Not included in the study. | Not included in the study. | Thickened BMZ. |

**Table B4.** Summary of histological changes of the lamina propria in benign vocal fold lesions.

| | Vocal Fold Nodules | Vocal Fold Polyp(s) | Vocal Fold Cyst | Reinke's Edema |
|---|---|---|---|---|
| (Courey et al., 1996) | Fibronectin lost the normal laminar pattern. | Fibronectin generally maintained its general laminar distribution. | Fibronectin generally maintained its general laminar distribution. | Fibronectin generally maintained its general laminar distribution. |
| (F. G. Dikkers & Nikkels, 1999) | Unusual perpendicular orientation of elastic fibre to the BM is commonly seen. Minimal hyaluronan is accumulated in connective tissues. | Unusual perpendicular orientation of elastic fibre to the basement membrane is occasionally present. Minimal hyaluronan is accumulated in connective tissues | Not included in the study. | Unusual perpendicular orientation of elastic fibre to the basement membrane is occasionally present. Minimal hyaluronan is accumulated in connective tissues |
| (Gray et al., 1995) | Intense fibronectin deposition. | Little fibronectin deposition. Few structural proteins,collagen and elastin. | Not included in the study. | Little fibronectin deposition. Few structural proteins, e.g., collagen and elastin. |
| (Kotby et al., 1988) | No inflammatory cell infiltration. Abundant collagen deposition. | No inflammatory cell infiltration. Scattered collagen fibres. | Not included in the study. | Not included in the study. |
| (Loire et al., 1988) | Moderate cellular infiltration with some fibroblasts and few lymphocytes. Edema is not common. | Fibrinous exudates were organized in a loose network or in cluminuous clumps. Probably attached to the connective tissue or endothelial cells. Diffuse edema and cellular infiltration with lymphocytes and few fibroblasts are present. | Mucus retention cyst: edema or fibrosis with some lymphocytes. Epidermiod cyst: commonly fibrous than edematous. Filled with detached parakeratotic cells or desquamated keratin. | Marked edema with pseudovascularity. Increased number of blood vessels. The vessels are dilated and packed with red blood cells. Infrequent cell infiltration with lymphocytes and collagen deposition. |
| (Marcotullio et al., 2002) | Edematous is commonly seen. | Edematous-angiomatous is commonly seen. | Not included in the study. | Edematous is commonly seen. |
| (Sakae et al., 2008) | Not included in the study. | Not included in the study. | Not included in the study. | Collagen fibers were fragmented, loosely arranged and intermixed with myxoid stroma in the deeper region of the superficial layer of the lamina propria. |
| (Sato et al., 1999) | Not included in the study. | Not included in the study. | Not included in the study. | Plasma is accumulated in Reinke's space. |
| (Tillmann et al., 1995) | Not included in the study. | Not included in the study. | Not included in the study. | Protein-rich fluid accumulates in the highly ramified fissured area in Reinke's space. Fibroblasts have their cytoplasmic extensions overlapped in two to three layers. |
| (Volic et al., 1996) | Not included in the study. | Not included in the study. | Not included in the study. | Loose and edematous lamina propria with short and completely disorganized connective fibres. |

**Table B5.** Summary of genetic-related and other changes in benign vocal fold lesions.

| | Vocal Fold Nodules | Vocal Fold Polyp(s) | Vocal Fold Cyst | Reinke's Edema |
|---|---|---|---|---|
| **Genetics** | | | | |
| (Duflo, Thibeault, Li, Shu, & Prestwich, 2006) | Not included in the study. | Genes related to extracellular matrix remodeling, cell growth, repair proliferation, negative cell progression are overexpressed. No genes related to protect against oxidative stress are expressed. | Not included in the study. | Genes related to protection against oxidative stress, apoptosis as well as control of cell growth and differentiation are expressed. |
| (Thibeault, Gray, Li et al., 2002) | Not included in the study. | Upregulated mRNA: procollagen-I, haluronic acid synthase 2, decorin and fibronectin.<br>Downregulated mRNA: MMP-1, MMP-12 and fibromodulin.<br>High gene activity. | Not included in the study. | Upregulared mRNA: procollagen-I, haluronic acid synthase 2, decorin and fibromodulin.<br>Downregulated mRNA:<br>MMP-1, MMP-12, fibronectin.<br>Low gene activity. |
| **Others** | | | | |
| (Kang, Hsiung, & Wang, 2005) | iNOS (inducible form of nitric oxid synthase) and 3-NT (3-nitrotyrosine) are less accumulated in nodules, compared to polyps. | iNOS and 3-NT are significantly higher in polyps than in nodules. That may suggest the increase in peroxynitrite production may have a pathogenic role in polyps. | Not included in the study. | Not included in the study. |
| (Karahan, Baspinar, Yariktas, & Kapucuoglu, 2009) | Not included in the study. | MMP-2, MMP-9 and COX-2 are significantly higher in stromal spindle cells and vascular wall of polyps than t the normal vocal folds. | Not included in the study. | Not included study. |
| (Verdolini et al., 2003) | Not included in the study. | PGE-2 is dominantly present in the laryngeal secretions. | PGE-2 is dominantly present in the laryngeal secretions. | Not included in the study. |
| (Wallis et al., 2004) | Lesion size less than 0.3 cm. | Lesion size larger than 0.3 cm. | Not included in the study. | Not included in the study. |

Two issues are still under debate in the literature of benign vocal fold lesions. The first one is if the epithelium layer of the vocal folds is affected in chronic phonotraumatic lesions. The second one is whether vocal fold nodules, polyps, cysts and Reinke's edema can be differentiated by chronicity, i.e., the age of the lesions.

Regarding the first issue of the epithelial changes in chronic phonotraumatic lesions, hyperproliferative responses of the epithelium have been suggested to be absent in most of phonotraumatic lesions (Gray, 1997; Zeitels & Healy, 2003). A study used contact endoscopy with methylene blue staining (Carriero et al., 2000) to visualize the superficial layer of vocal fold epithelium. Although the epithelial cells of Reinke's edema had higher nuclear density, most of the polyps and Reinke's edema in their specimens exhibited homogenous and normal epithelium.

In contrary, epithelial changes of phonotraumatic lesions are documented in other studies. The epithelial changes may be related to the hyperactivity and high turnover rate of the basal cells of the vocal fold epithelium. Dikkers et al. (F.G. Dikkers & Nikkels, 1995) reported that the basal cells of their lesion specimens displayed the signs of decrease in the amount of condensed chromatin and increase in the sizes of their nucleoli, vesicles and mitochondria. These cellular responses together with the thickened epithelium may be the typical reactions to phonotrauma in all benign vocal fold lesions (nodules, polyps, cyst, Reinke's edema, granuloma and broad-based thickening). Various epithelial abnormalities are also reported for benign vocal fold lesions: atrophy, hyperplasia, keratosis, parakeratosis, and dyskeratosis. A study (Loire et al., 1988) using optical microscopy reported that vocal nodules exhibited a range of epithelial abnormalities across their patients, in which dyskeratosis (82%) and parakeratosis (66%) were the most commonly observed. Atrophic epithelium and irregular thickness of epithelium were observed in some of the polyps and Reinke's edema respectively but in a less occurrence. Another study

(Kotby et al., 1988) using electron microscopy also reported similar observation. Vocal nodules showed greater thickness and keratinization of epithelium than vocal polyps. Paradoxically, atrophic epithelium in nodules and epithelial hyperplasia in polyps were reported in another study (van der Velden et al., 1996). The discussion is further complicated by another histological study of nodules, polyps and Reinke's edema (Marcotullio, Magliulo, Pietrunti, & Suriano, 2002). The investigators classified the epithelial changes into five categories: normal epithelium, basal hyperplasia, hyperkeratosis, keratosis-hyperplasia and dysplasia. Nodules, polyps and Reinke's edema exhibited as either normal epithelium (nodules: 39.79%; polyps: 40.65%; Reinke's edema: 44.12%) or hyperkeratosis (nodules: 47.95%; polyps: 41.75%; Reinke's edema: 30.88%) in their specimens. Lastly, two studies (F.G. Dikkers & Nikkels, 1995; Neves et al., 2004) both reported that diverse types of epithelial abnormalities were present among the phonotraumatic lesions. In sum, benign vocal fold lesions do not necessarily have epithelial changes in response to chronic phonotrauma. Also, even epithelial abnormality exist among some patients with benign vocal fold lesions, the isolated abnormality is not specific to the lesion type.

The second issue is about the chronicity (age) of the vocal fold lesions. Marcotullio et al. (Marcotullio et al., 2002) propose that nodules and Reinke's edema are younger lesions than polyps. The investigators categorized the changes in the lamina propria into five phases of maturation: edematous (the least mature), edematous angiomatous, angiomatous, angiomatous-hyaline and hyaline (the most mature). Their histological results suggest that vocal nodules and Reinke's edema are in the earlier edematous stage, whereas vocal polyps are in the more advanced stage of edematous-angiomatous. Another study by Wallies et al. (Wallis et al., 2004) subjected both videostroboscopic images and histological pictures to blinded otolaryngologist

and voice pathologist for clinical diagnosis. The investigators conclude that no definitive histological difference can be made between nodules and polyps. The only significant difference is the size of the lesion, which polyps (> 0.3 cm) are bigger than nodules (< 0.3 cm). The investigators further propose that nodules and polyps are on the same pathological process in a continuum. That is, if nodules are left alone and with repeated phonotrauma, the nodules may transform into a more advanced polyps.

Contrary to the conjecture that polyps are the more advanced lesions than nodules, Kotby et al. (Kotby et al., 1988) claimed that polyps are younger than nodules. The investigators reported that polyps had a thinner epithelium than nodules using light microscopy in their study, which suggested a less long-standing nature of polyps. This claim is supported by Dikkers et al. (F. G. Dikkers & Nikkels, 1999). The investigators reasoned that polyps are the youngest, nodules are older, and Reinke's edema is the mostly advanced lesion based on the clinical management of these lesions. Patients with vocal polyps tend to seek medical consultation at the very early stage, whereas patients with Reinke's edema tend to delay from months to years before getting diagnosed and surgically excised. Having said that, Dikkers et al. did not consider that nodules, polyps and Reinke's edema represent the same pathological continuum, which is against Wallis et al.'s proposal (Wallis et al., 2004). Dikker et al.'s exposition is that these lesions are distinctive in histological and stroboscopic features. Also, patients seldom exhibit such lesion transformation, for instance, polyps will develop into nodules over time.

### B.1.3 Vocal fold nodules

Vocal fold nodules are macroscopically described as round, sessile, white/opague, bilaterally symmetric lesions (M. Hirano, 1981; Johns, 2003; Pontes, Kyrillos, Behlau, De Biase,

& Pontes, 2002). Vocal fold nodules usually locate on the free margin of the anterior two-thirds of the vocal folds (M. Hirano, 1981; Johns, 2003). The size of nodules is suggested to be less than 0.3 cm (Wallis et al., 2004).

An array of histological features in relation to vocal fold nodule is reported. The appearance of nodules varies from edema to fibrosis (M. Hirano, 1981; Pontes et al., 2002). Also, the vasculature of nodules varies from normal blood flow and absence of haemorrhage (Courey et al., 1996; F.G. Dikkers & Nikkels, 1995; Loire et al., 1988; Pontes et al., 2002; Sone, Sato, Hayashi, Fujimoto, & Nakashima, 2006), microstromal haemorrhages and vascular proliferation (Kotby et al., 1988; Wallis et al., 2004) and hyaline deposition (Loire et al., 1988). Inflammatory cell infiltration and edematous lake had been reported as absent (F.G. Dikkers & Nikkels, 1995; Kotby et al., 1988) but are later rejected by another study (Wallis et al., 2004). Furthermore, the epithelium varies from atrophy (van der Velden et al., 1996) to different degrees of keratinisation (Kotby et al., 1988; Loire et al., 1988). Although vocal nodules present great diversity of the aforementioned histology, disruption of basement membrane zone (BMZ) in vocal nodules is commonly reported.

First, the BMZ architecture of vocal nodules appears disorganized in the form of: reduplication of the lamina densa, disorientation of the anchoring fibres, disorganization of the attachment plaque, separation of the epithelium from the beneath superficial lamina propria (Gray, 1989, 1991, 1997; Gray et al., 1995; Gray & Titze, 1988; Gray et al., 1987), irregular thickened with zones of blurring (Loire et al., 1988), gapping of the intercellular junctions, absence of basal lamina, and perpendicular arrangement of elastic fibres to the BMZ (F. G. Dikkers & Nikkels, 1999). Second, abnormal increase in protein depositions are observed in the BMZ, which include laminin (Neves et al., 2004), collagen IV (Courey et al., 1996; Kotby et al.,

1988) and fibronectin (Courey et al., 1996). The average thickness on collagen type IV and fibronectin staining in the BMZ is estimated as 1.88 μm (range from 0.3 to 3.0 μm) and 1.63 μm (range from 0.5 to 2.0 μm) respectively (Courey et al., 1996). The abnormal deposition of these BMZ components result a thickened epithelium (80.85 μm) of vocal nodules (Kotby et al., 1988) in general. Lastly, epithelial cell degeneration is reported in the form of cytoplasmic vacuoles and disrupted cell-cell (desmosomal) junctions (F. G. Dikkers et al., 1993).

The BMZ is the injury-prone location along the vocal folds in response to phonotrauma. BMZ bears strong shearing and impact stresses from the epidermis to the thyroarytenoid muscle during vocal fold vibration (Gray et al., 1995). These stresses may induce tissue inflammatory response of the vocal folds. A study reported heavy immunoreactivity of iNOS (inducible form of nitric oxide synthase) on the epithelium and relatively faint 3-NT accumulation (3-nitrotyrosine) in the tissue samples of vocal nodules using immunohistochemistry (Kang et al., 2005). The investigators speculate that the production of inflammatory cytokines, NO and other toxic metabolites are ensued from the stress-induced iNOS. This inflammatory response may exacerbate the healing process, leading to the formation of vocal nodules.

Other than inflammation-induced injury, phonatory stresses may also contribute to the structural disruption of the BMZ architecture. These stresses may damage the anchoring fibres in the BMZ and result the separation between the epithelium and the lamina propria. This separation may create a pocket for interstitial fluid resulting edema. The increased accumulation of adhesion molecules (collagen IV, laminin, and fibronectin) in the BMZ may correspond to the healing response of “patching” the torn tissues (Courey et al., 1996; Gray et al., 1995). However, this healing process may be disrupted by repetitive injury, re-separation of the epithelium and the superficial lamina propria and reattachment of epithelium/BMZ complex during continuous

phonation (Gray, 1991). As a result, the vocal folds are healed in a disorganized way with a thickened BMZ and excessive deposition of adhesion molecules. Also, the deposition of fibronectin is relatively permanent and leads to increased stiffness of the vocal folds, which in turn affects vocal fold vibration (Gray, 1991). Further, the disorganized BMZ may predispose the individuals at risk of exacerbating the existing lesion. As a result, complete resolution of vocal nodules in response to voice therapy is very rare as seen in clinic.

### B.1.4 Vocal fold polyp(s)

Vocal fold polyps have heterogeneous appearance: sessile or pedunculated, translucent to red, and unilateral or bilateral lesions (F. G. Dikkers & Nikkels, 1999; Johns, 2003). Vocal fold polyps usually locate on the middle musculo-membranous region of the vocal folds (F. G. Dikkers & Nikkels, 1999). The size of polyps is suggested to be bigger than 0.3 cm (Wallis et al., 2004).

In vocal polyps, changes in the structure of epithelium, the size of epithelial cells and distribution of epithelial cells are not commonly reported (Carriero et al., 2000). Sometimes, keratinisation (Kotby et al., 1988) or hyperplasia (van der Velden et al., 1996) with irregularities may occur in the epithelium of polyps. The thickness of epithelium in polyps is estimated as about 66 μm, compared to 80.85 μm that of nodules (Kotby et al., 1988). Alternation of the BMZ n polyps is not commonly reported as well. Abnormal deposition of collagen type IV and fibronectin are not commonly observed in the BMZ of polyps (Courey et al., 1996; F. G. Dikkers et al., 1993). The average thickness on collagen type IV and fibronectin staining in the BMZ is estimated as 0.84 μm (range from 0.5 to 2.0 μm) and 1.21 μm (range from 0.5 to 5.0 μm) respectively (Courey et al., 1996). Also, collagen type IV is relatively less in amount in the BMZ

than those in the lamina propria, which may predispose a risk for the separation of the BMZ from superficial lamina propria.

Vascularization is within the superficial lamina propria is commonly reported in polyps. Various forms of vascularization are observed: ranging from vascular to fibrotic to mixoid (Johns, 2003; Kotby et al., 1988; Sone et al., 2006). The vessels were commonly in longtitudinal or transver orientation with abrupt reductions and diltations in calibre of blood vessels (De Biase & Pontes, 2008). Increased hyaluronic acid (F.G. Dikkers & Nikkels, 1995; F. G. Dikkers & Nikkels, 1999) and fibronectin (Courey et al., 1996) may accumulate around the neovasculature. The presence of recent bleeding, deposition of iron and fibrin, and thrombosis confirm the diagnosis of polyps from nodules (F.G. Dikkers & Nikkels, 1995). A speculation is that phonotrauma may create a haemorrhagic response of capillary damage and increase in vascular permeability (Gray et al., 1995; Sone et al., 2006). After prolonged irritation, vocal polyps may form larger areas of telangiectasias (enlarged blood vessels), associated with haemorrhage and fibrin formation (Wallis et al., 2004). Another speculation is, rather than the direct mechanical damage to blood vessels from phonotrauma, the inflammatory process at the lamina propria or the histologic alterations (e,g, fibrous protein depositions) in the development of polyps, might modify the orientation of the vessels (De Biase & Pontes, 2008).

Two studied used laser Doppler flowmetry to compare the blood flow in benign vocal fold lesions with normal vocal folds. In general, vocal fold polyps had significantly higher blood flow than normal vocal folds (Stec et al., 2007). A higher mean ratio of blood flow (72%) in polyps was reported, compared to 21.8% in nodules (Sone, Sato, Hayashi, Fujimoto, & Nakashima, 2006). Capillary damage may lead to edema, bleeding and leakage of fibrin (F. G. Dikkers & Nikkels, 1999) and subsequently the formation of fibrinous exudates, which makes up

the major volume of the polyps (Loire et al., 1988). These exudates may appear as a loose network or in capacious clumps, which are connected by endothelial cells or connective tissues (Loire et al., 1988).

Besides abundant vascularity, the extracellular matrix components of the lamina propria are less disturbed in polyps (F.G. Dikkers & Nikkels, 1995). Some polyps exhibit abnormally perpendicular orientation of elastic fibres to the basement membrane, which is probably induced by the phonatory forces (F.G. Dikkers & Nikkels, 1995). A study reported the changes in messenger ribonucleic acid (mRNA) expression of a number of extracellular matrix components in polyps (Thibeault, Gray, Li et al., 2002). Results indicated an upregulation of the gene for procollagen -1, which is a marker for neo-collagen synthesis. Matrix metalloproteinases -1 and -12 were downregulated, which are responsible for the breakdown of collagen and elastin respectively. Hyaluronic acid synthase 2 were also downregulated, which is an important enzyme for hyaluronic synthesis. These changes may alter the viscoelastic property of the superficial lamina propria, and predispose a risk of vocal fold scarring. Also, upregulation of fibronectin and downregulation of fibromodulin were also noted in polyps, which indicate an increase in stiffness of the vocal folds. Overall, the total gene expression scores were high in polyps. This high gene activity may account for the acute condition of polyps. This speculation is substantiated by another study using complementary deoxyribonucleic (cDNA) microarray analysis (Duflo et al., 2006). The investigators identified 65 genes to differentiate polyps from Reinke's edema. In polyps, SPARC, SPARCL1 and Col6A3 were the three significantly overexpressed genes, which are responsible for extracellular remodeling, cell growth and repair proliferation. Col1A2, Col3A1, Col5A2, PRCAM1 and ITGB2 were also overexpressed in polyps, which are responsible for fibroblast proliferation and inflammatory processes. Therefore,

polyps are likely in an inflammatory condition with active fibroblasts for healing (Duflo et al., 2006).

Lastly, the role of inflammation in the pathophysiology of vocal polyps remains to be elucidated. Vocal polyps showed significanylg higher levels of metalloproteinase (MMP)-2, MMP-9 and cyclooxygenase (COX)-2 in stromal spindle cells, vascular endothelial cells and inflammatory cells, compared to normal vocal folds. These three markers are associated with microvascular permeability, tissue remodeling and angiogenesis, which these three processes may play an important role in the development and growth of polyps (Karahan et al., 2009).

Besides the aforesaid inflammatory processes, inflammatory cell infiltration has been reported as either absent (Kotby et al., 1988) or present (Loire et al., 1988). Nitric oxide (NO) is an important inflammatory mediator during inflammatory process. NO is generated by iNOS (inducible form of nitric oxide synthase) and 3-NT (3-nitrotyrosine) is a biological footprint of the toxic NO metabolite perosynitrite (Beckman, Beckman, Chen, Marshall, & Freeman, 1990; Fukuto, 1995). A study using immunohistochemistry reported that both iNOS (inducible form of nitric oxide synthase) and 3-NT (3-nitrotyrosine) were more intense on the epithelium and parachyma in their tissue samples of vocal polyps, compared to those of vocal nodules (Kang et al., 2005). The investigators suggest that the increased NO may damage the vocal fold tissue by its own toxic actions and/or through the production of 3-NT. This iNOS/NO action may contribute to the formation of polyps (Kang et al., 2005). Shear stress during phonation may account for the expression of iNOS as suggested by studies from other tissue domains (Cai, Xin, Pollock, & Pollock, 2000; Gosgnach, Messika-Zeitoun, Gonzalez, Philipe, & Michel, 2000; Kang et al., 2005). On the other hand, inflammatory cytokines, interleukin-1 beta (IL-1β) and prostaglandin E2 (PGE2), may also take part of the iNOS expression for polyps. In an

intraoperative human study, PGE2 was more dominant than IL-1β in the laryngeal secretion samples collected from subjects with polyps and cysts (Branski, Verdolini, Rosen, & Hebda, 2004), whereas IL-1β was more dominantly expressed in subjects with epithelial-based vocal fold lesions (vocal fold cancer and recurrent respiratory papilloma). A speculation is that PGE2 may contribute to the upregulation of iNOS expression in polyps.

At the same time, the involvement of iNOS/NO in polyps is questioned by the results of a cDNA microarray study of vocal polyps and Reinke's edema (Duflo et al., 2006). NO is known to induce oxidative stress, which reacts with superoxide anion ($O2^-$) to yield peroxynitrite ($ONOO^-$) (Beckman, Beckman, Chen, Marshall, & Freeman, 1990). If iNOS/NO have an important pathogenic role in polyps, a cellular defence mechanism of providing protection against oxidative stress should be turned on. However, genes that involve in such defence mechanism, such as MAP2K3, SOD1, GPX2 and GTSA2, were only overexpressed in Reinke's edema but not in polyps. This finding indicates that polyps may not be induced by oxidative stress, which is in contrary to Kang et al.'s (2005) speculation. Further research is appreciated to investigate the importance of iNOS/NO in the injury and healing mechanism for polyps and other benign vocal fold lesions.

### B.1.5 Vocal fold cyst

Vocal fold cysts can be a massive growth or a discrete and whitish lesion (Milutinovic & Bojic, 1996). Cysts are either classified as mucous retention cysts or epidermoid inclusion cysts and each of them can locate in either Reinke's space (sub-epithelial) or near the vocal ligament (Verdolini et al., 2006). Mucous retention cysts consist of a very thin wall with a cavity collecting translucent mucous (Johns, 2003; Milutinovic & Bojic, 1996). Mucous retention cysts

are probably associated with an obstructed mucous gland duct or phonotrauma (Johns, 2003; Milutinovic & Bojic, 1996; Verdolini et al., 2006). Epidermoid (or squamous) inclusion cysts usually have a thickened wall and appear as fusiform masses with desquamated keratin accumulation in the subepithelial layer (Johns, 2003; Milutinovic & Bojic, 1996). Epidermoid cysts are believed to be related to phonotrauma or to congenital defects (Verdolini et al., 2006).

In vocal fold cysts, changes in vasculature are varied. Some cysts may have interrupted fibronectin distribution due to increased vasculature (Courey et al., 1996). Some may present vessels with longtitudinal, transverse of tangled orientation. Changes in calibre and tortuosity of blood vessels, specifically, diltations, abrupt reduction, or tortuosities, were also reported (De Biase & Pontes, 2008).

Regarding the epithelial changes, cyst lesion may be lined with either columnar or squamous epithelium, in which having about 10 to 30 cellular layers (Loire et al., 1988; Shvero et al., 2000). Especially for the epidermoid inclusion cyst, keratinisation is progressively occurred (Loire et al., 1988). The cells with viable nuclei are observed to be desquamated into the cavity of the cyst (Loire et al., 1988).

The thickness of the basement membrane zone (BMZ) for both mucous retention and epidermoid inclusion cysts is similar. The average thickness of the BMZ is estimated about 1.04 μm on collagen type IV staining and 1.14 μm on fibronectin staining, which is in between the thickness of nodules and polyps (Courey et al., 1996). Abnormal deposition and distribution of collagen type IV and fibronectin are not commonly observed throughout the BMZ and the superficial lamina propria (Courey et al., 1996). For the mucosa layer, cysts are reported more commonly as fibrous than edematous (Loire et al., 1988). The injury and healing mechanism of cysts are not clear. Some insights may be able to obtain from an intraoperative human study of

biomarkers in surface laryngeal secretions from patients with laryngeal pathology (Branski et al., 2004). Prostaglandin E2 (PGE-2) was more dominant than interleukin-1 beta (IL-1β) in the laryngeal secretion samples collected from subjects with cysts. PGE-2 is a mediator that involves in ubiquitous stages of wound healing, whereas IL-1β is a marker of acute inflammatory stage. This finding suggests that cysts are not likely in an acute inflammation condition.

### B.1.6 Reinke's edema

Reinke's edema, which is edematous and swollen in the superficial lamina propria, is also known as polypoid vocal fold, polypoid degeneration, chronic polypoid corditis and chronic edematous hypertrophy (Sato, Hirano, & Nakashima, 1999; Verdolini et al., 2006). Reinke's edema appears as a unilateral or bilateral, pale-white and fluid-filled swelling. Reinke's edema can be sessile or mobile during phonation (F.G. Dikkers & Nikkels, 1995). The histological differentiation of Reinke's edema from nodules and polyps is difficult (Neves et al., 2004).

The epithelium has been reported as normal (Tillmann et al., 1995) or irregularly thickened along the lesion: varying from thinner, acanthotic, parakeratotic to duplicate epithelium (Loire et al., 1988). Minimal collagen disarrangement of Reinke's edema was reported in the region underneath vocal fold epithelium using picrosirious polarization method (Sakae et al., 2008).

The basement membrane zone (BMZ) for Reinke's edema is reported as normal without any pathological alternations in some studies (Gray et al., 1995; Loire et al., 1988). Sparse collagen type IV and fibronectin deposition is noted in the BMZ (Courey et al., 1996). The average thickness of BMZ is reported as 0.75 μm on both collagen type IV and fibronectin staining, which is the thinnest among nodules, polyps and cysts (Courey et al., 1996). At the

same time, some studies report significantly thickened BMZ for Reinke's edema (F.G. Dikkers & Nikkels, 1995; Volic et al., 1996). Hyperplastic, hyperkeratotic and parakeratotic BMZ may occur in some patients with Reinke's edema (Volic et al., 1996).

The vasculature in the superficial lamina propria (Reinke's space) is remarkably disrupted in Reinke's edema. Hypervascularization and dilated blood vessels with packed red blood cells are reported in the Reinke's space (F.G. Dikkers & Nikkels, 1995; Gray et al., 1995; Loire et al., 1988; Sato et al., 1999). Compared to the parallel direction of blood vessels in normal vocal fold mucosa, pathological blood vessel networks in loop or branch forms for Reine's edema were observed under contact telescopy (Jovanovic et al., 2007). Also, mucosal "blue lines" were observed in subepithelial well-developed hollow spaces. The "blue lines" may be formed by the decreased blood flow by mechanically compressed surgace layers in chronic traumatic edema (Jovanovic et al., 2008). The blood vessels have thin endothelium (Jovanovic et al., 2007) with many small pores (fenestrae) and vesicles (Sato et al., 1999). The basement membrane of the blood vessel walls is thickened by the increased depositions of collagen type IV and fibronectin surrounded (Courey et al., 1996; F.G. Dikkers & Nikkels, 1995). As a result, the blood vessels increase their permeability and subsequently plasma leaks into surrounding space through the small pores of the blood vessels (Sato et al., 1999). That may explain why vascular lake and increased fibrin deposition are observed in the specimens of Reinke's edema under electron microscopy (Gray et al., 1995; Sato et al., 1999). Furthermore, some pericytes and endothelial cell are degenerated and lose their functions of maintaining the vascular walls, resulting vessel occlusion and fragile blood vessels (Sato et al., 1999). Vascular endothelial growth factor is present in cytoplasm of inflammatory cells and fibroblasts within Reinke's space (Sato, 1999). This growth factor may contribute to the increase in vessel permeability and

fragility. These changes in the vasculature may lead to accumulation of edema in the extracellular matrix of the superficial lamina propria (F.G. Dikkers & Nikkels, 1995; Sato et al., 1999) and may exacerbate the disrupted organization of connective fibres in the lamina propria (Volic et al., 1996).

Phonotrauma may account for the disorganized matrix of lamina propria in Reinke's edema (F.G. Dikkers & Nikkels, 1995). The connective tissues in the superficial lamina propria for Reinke's edema are described as highly ramified fissured spaces (Tillmann et al., 1995) with short, torn, scattered connective fibres (elastin and collagen) (Gray et al., 1995; Volic et al., 1996) and minimal fibronectin (Gray et al., 1995). Some of the Reinke's edema may have unusually perpendicular orientation of elastic fibres to the basement membrane zone (BMZ) in Reinke's edema (F.G. Dikkers & Nikkels, 1995). In a study using picrosirius polarization method, both collagen type I and III fibers were fragmented, loosely arranged and intermized with different amounts of myxoid stroma in the deeper regions of the superficial lamina propria (Sakae et al., 2008). The lack of structure fibrous proteins and glycoproteins in the lamina propria may cause the vocal folds with excessive propensity for deformability and so prone to injury (Gray et al., 1995).

The healing mechanism of Reinke's edema may not be fibroblast-dominant because excess collagen deposition is not commonly reported in Reinke's edema (Gray et al., 1995). A speculation is that Reinke's edema get looped in the inflammatory stage and fails to transit to the later healing phase (Thibeault, Gray, Li et al., 2002). A study reported the changes in messenger ribonucleic acid (mRNA) expression of a number of extracellular matrix components in Reinke's edema (Thibeault, Gray, Li et al., 2002). Similar to polyps, an upregulation of the gene for procollagen-1, downregulations of matrix metalloproteinases 1 and 12 and hyaluronic acid

synthase 2 are indicated in Reinke's edema. These changes may alter the viscoelastic property of the superficial lamina propria and predispose a risk of vocal fold scarring. In contrast to polyps, fibronectin is downregulated and fibromodulin is upregulated in Reinke's edema, which may contribute to the less stiffness phenotype of Reinke's edema. Overall, the total gene expression scores are lower in Reinke's edema than that in polyps. The high gene activity of polyps may be related to its chronic inflammatory condition (Thibeault, Gray, Li et al., 2002). Further, an complementary deoxyribonucleic (cDNA) microarray study (Duflo et al., 2006) elucidated the injury and healing mechanisms of Reinke's edema. In Reinke's edema, MAP2K3, SOD1, GPX2, and GTSA2 were the four significantly overexpressed genes, which are responsible for the protection against oxidative stress. Also, gene CASP9, CCNG2 and SP100 were overexpressed, which are responsible for cellular regulation and protection to prevent lesion progression and/or cell differentiation. Therefore, Reinke's edema is likely induced by oxidative stress. Also, a cellular defence mechanism is turned on to protect the tissue against the oxidative injury. This defence mechanism is important for the system to avoid tumour growth and develop cancer.

### B.1.7 Tissue mobilization therapy for phonotrauma

The aims of tissue mobilization therapy for acute and chronic phonotrauma have different orientations. For acute phonotrauma, tissue mobilization aims to alter mechanical signalling in order to suppress inflammation by attenuating the pro-inflammatory responses and escalating the anti-inflammatory signals (Branski et al., 2006). This effect is important to limit the detrimental events during acute inflammation, such as oxidative damage and debridement actions of inflammatory cells to the mucosal tissue. Second, mucous secretions are usually accumulated during initial inflammatory stage of healing. The oscillatory movement of the mucosa during the

phonation may assist in moving mucous secretions off the vocal folds (Behrman & Sulica, 2003). The absence of phonatory mucosal wave may result globus sensation in larynx, which induce coughing and throat-clear to exacerbate tissue damage. Lastly, the oscillation of the mucosal wave associated with mobilization may increase capillary perfusion and migration of inflammatory cells (Behrman & Sulica, 2003). These changes may help to bypass a prolonged inflammatory phase and avoid a full-scale of healing response. As such, the probability of scar formation is minimized in long term.

For chronic phonotraumat, tissue mobilization targets to resolve the established disorganization of matrix proteins in the mucosa. For example, vocal fold nodules (F. G. Dikkers & Nikkels, 1999) are characterized by abnormal arrangement of elastic fibres, which are lined perpendicularly to the basement membrane (F. G. Dikkers & Nikkels, 1999). Also, intense fibronectin and collagen depositions are accumulated in the lamina propria (Gray et al., 1995; Kotby et al., 1988). Fibronectin deposition is a relatively permanent event, which increase the stiffness of the vocal folds (Gray et al., 1995). Tissue mobilization may (1) improve the orientation of provisional matrix parallel to the stress lines of the normal fibres, (2) stimulate the expression of matrix metalloproteinases (MMPs) for regulating matrix turn-over during the remodeling phase of healing and (3) prevent the tissue atrophy by immobilization (Huang & Ingber, 1999; Keller, 2006; Kim et al., 1999; Martin & Wood, 2002; Moore et al., 2005; Noorlander et al., 2002; Seliktar et al., 2003; Wang & Thampatty, 2006). The absence of dynamic shear force and medial impact force may exacerbate disarrangement of these proteins and result in a permanent lesion (Behrman & Sulica, 2003).

Among all types of vocal exercise, resonant voice exercise is the one that implements the concepts of tissue mobilization in healing phonotrauma. Resonant voice is defined as “easy

phonation involving anterior oral vibrations and comparatively large-amplitude and low-impact vocal fold vibrations" (Berry et al., 2001; Peterson et al., 1994; Verdolini, 2000; Verdolini et al., 1998; Verdolini et al., in preparation). The exercise primarily involves the production of easy sustained /m/ sounds with a focus on anterior oral vibrations in a comfortable pitch range and then extends to the higher pitch ranges. Details regarding resonant voice exercises are reported in the manual of the Lessac-Madsen Resonant Voice Therapy Program (Verdolini, 2000). The benefits of resonant voice exercise are two-fold: optimizing vocal fold physiological functioning and mucosal healing. The laryngeal configuration in resonant voice involves barely ab/adducted vocal folds, which has been shown to yield the greatest acoustic outputs with the least amount of inter-vocal fold impact stress during phonation (Berry et al., 2001; Peterson et al., 1994; Verdolini et al., 1998). Regarding the wound healing benefits, the large-amplitude vibrations induce certain forms of vocal fold tissue mobilization, which may cause cell deformation and subsequently induce therapeutic mechanical signalling for wound healing. Also, the low-impact vibratory property of resonant voice minimizes further vocal fold injury or inflammatory response.

Another similar vocal exercise is Vocal Function Exercise (Roy et al., 2001; Sabol et al., 1995; Stemple et al., 1994). Vocal Function Exercise basically involves the practice of maximally sustained vowels and pitch glides. The primary principle is to coordinate among respiratory, laryngeal and vocal tract functions in order to optimize vocal performance. Although Vocal Function Exercise is initially contrived to improve vocal fold physiology, the exercise itself may also have potential wound healing benefits. The maximally sustained phonation associated with Vocal Function Exercise involves relatively large mid-lateral vocal fold movement yet barley ab/adducted vocal folds to optimize the glottic closure for pulmonary

preservation. At the same time, the pitch glides involve some forms of tissue stretching, which may induce therapeutic mechanical signalling for mucosal healing.

## B.2 NON-PHONATORY BIOMECHANICAL INJURY: PHONOSURGERY

According to Classification Manual for Voice Disorders-I (CMVD-I) (Verdolini et al., 2006), vocal fold scar is defined as "a permanent change to the microarchitecture of the lamina propria, consisting of a loss of the viscoelastic properties of the tissue". Vocal fold scarring can be induced by inflammation, or more commonly following micro-phonosurgery of vocal fold lesions (S. Hirano, 2005). Numerous studies employ animal models to characterize the histological changes in both acute and chronic vocal fold scarring from surgical trauma.

Vocal fold scar is regarded as a fibrous tissue resulting from a complete wound healing process (S. Hirano, 2005; Thibeault, 2005). Animal vocal fold scar is characterized by fibrosis (increase in collagen or procollagen during the early stages of healing) or a disorganized collagen scaffolding (loss of regular 3-dimensional collagen structure at the end of healing) (Thibeault, 2005). Also, numerous extracellular matrix components are altered during vocal fold scarring as seen in animals. Specifically, decreased elastin, increased fibronectin, decreased decorin and decreased fibromodulin are reported, although variations across animal species are noted (Thibeault, 2005). This alteration of the fibrous and interstitial proteins affects the vocal fold cover-body relationship and the propagation of normal mucosal wave, which severely affects one's voice quality (Thibeault, 2005). Figures B1 to B4 summarize the changes of the extracellular matrix components in rats, rabbits, canines and pigs respectively in the literature. Detailed descriptions of the changes for each component are given shortly.

The translation from animal findings to human is a challenge. Results from a histological study of human scarred vocal folds were barely consistent with the animal counterparts (S. Hirano et al., 2008). A wide invidual differences in the extracellular matrix deposition of human scarred vocal folds were observed. Inconclusive results were drawn about the deposition of elastin, fibronectin and hyaluronan in human scarred vocal folds. Less decorin deposition in the superficial lamina propria and excessive collagen deposition in the form of thick bundles throughout the lamina propria were observed following deep cordectomy, compared to shallow cordectomy.

The difference in the vocal fold architecture and the amount of vocal use between animal and human is acknowledged in the literature (Benninger et al., 1996). Another major concern of using animal surgical model to mimic the situation in human following phonosurgery is that the initial tissue state between these two models is fundamentally different. In current animal models, the surgical procedure is done on healthy vocal fold tissue, whereas in human the surgical procedure is done on pre-inflamed or pathologic vocal fold tissues. Further research can inflame or injure the animal vocal folds before performing the surgical procedure and examine if the healing outcomes are different from those without pre-inflamed or pre-injured animal vocal folds.

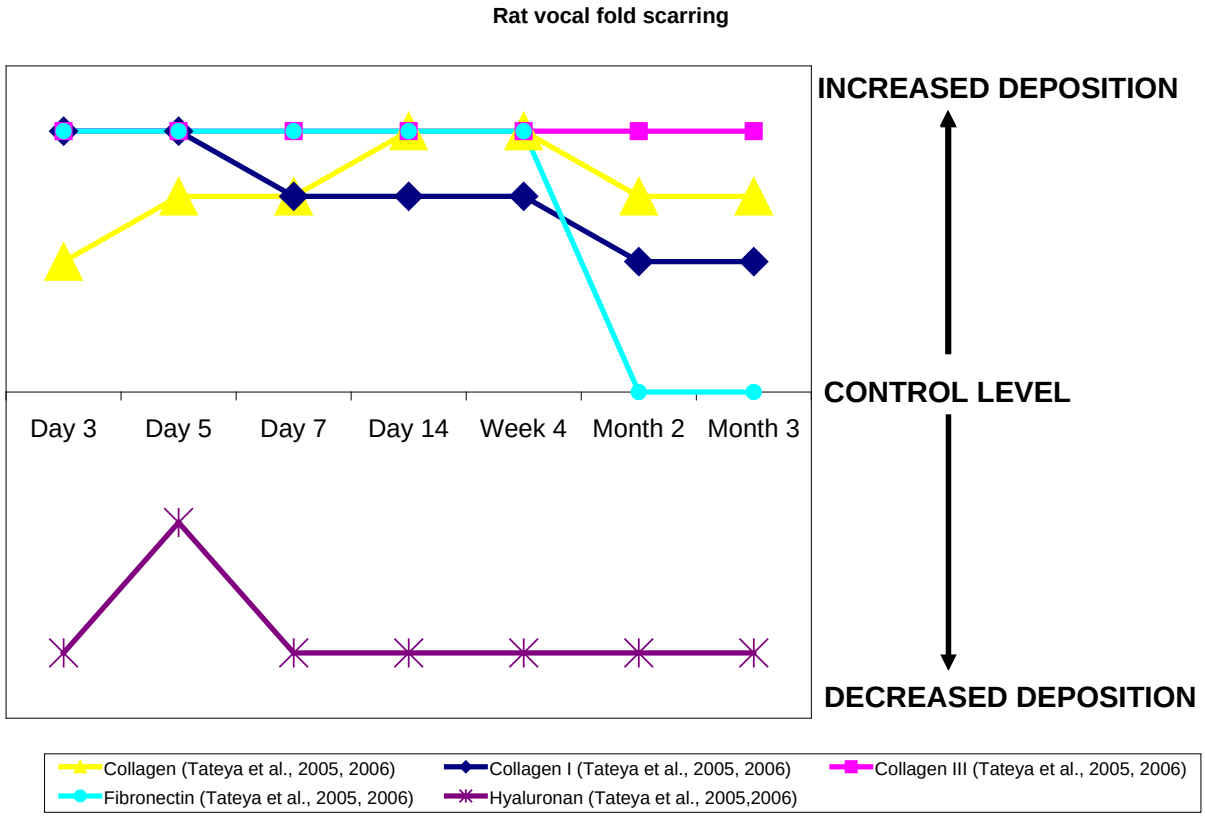


**Figure B1.** Schematic representation of collagen, collagen type I, collagen type III, fibronectin and hyaluronan in injured rat vocal folds.

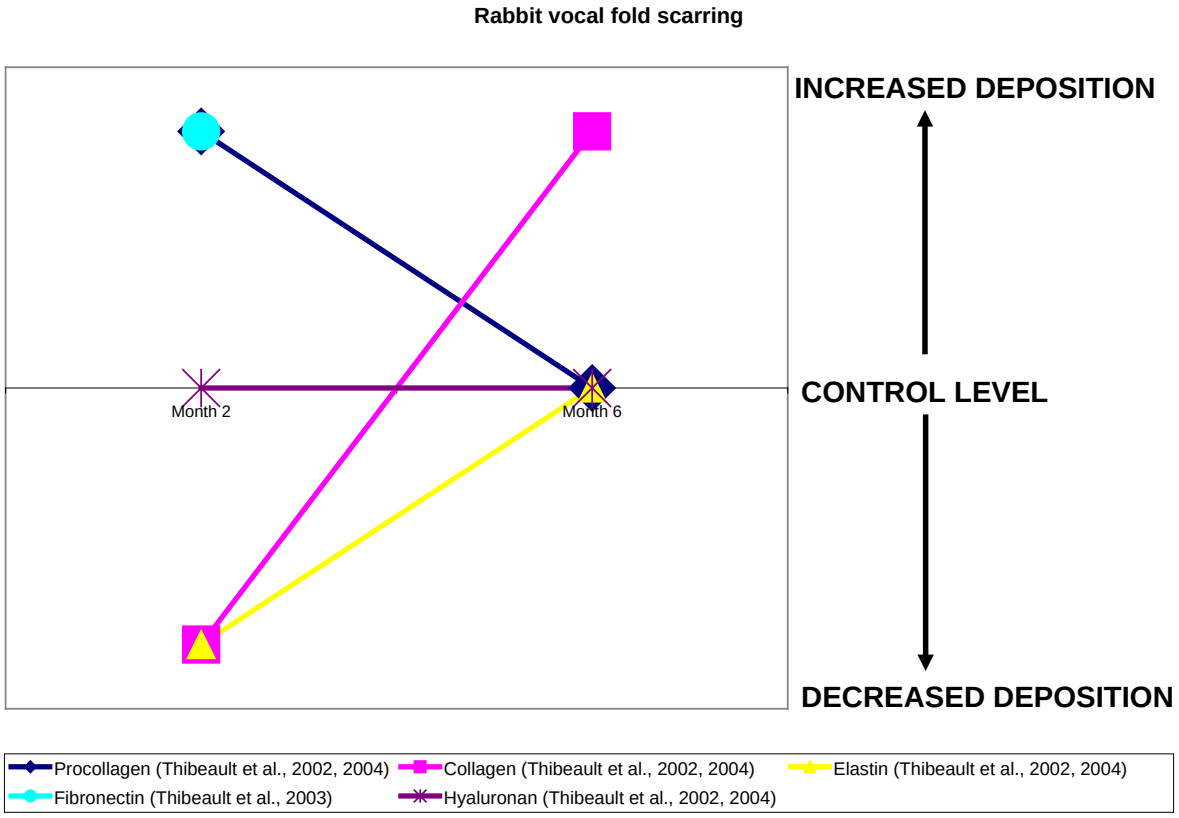


**Figure B2.** Schematic representation of procollagen, collagen, elastin, fibronectin and hyaluronan in injured rabbits vocal folds.

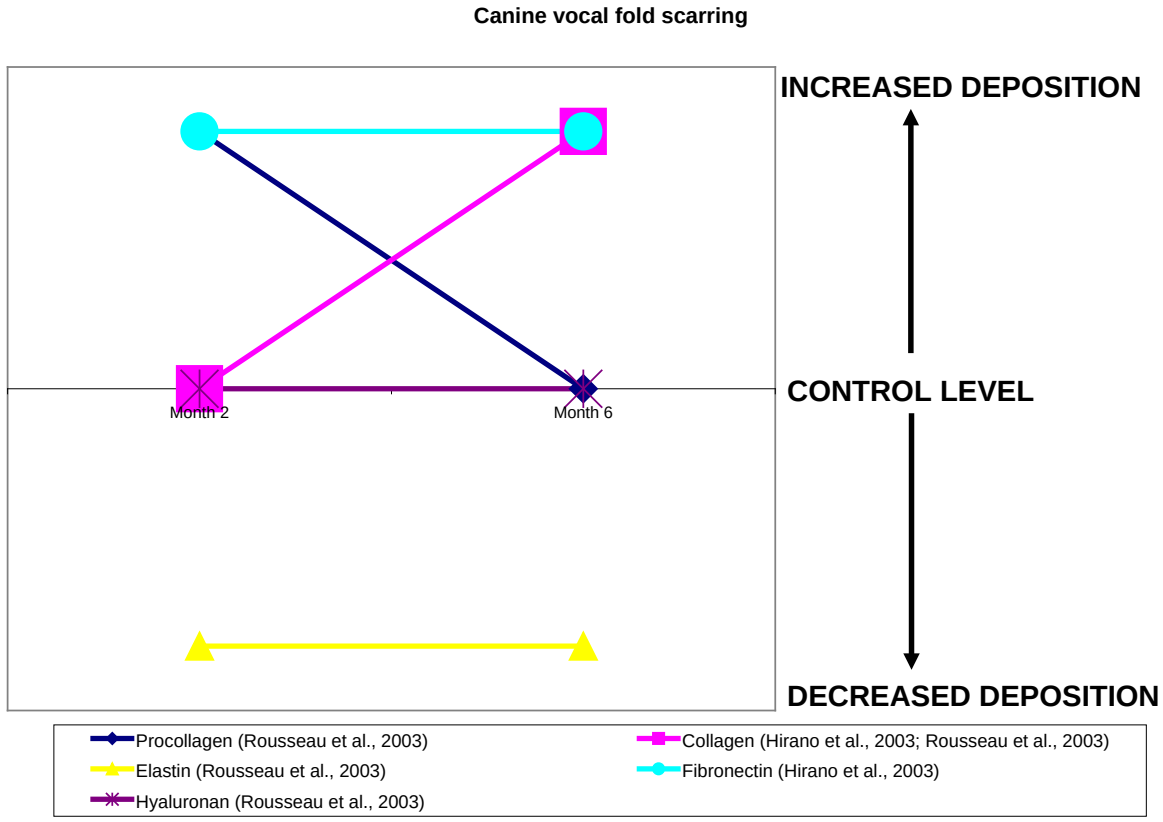


**Figure B3.** Schematic representation of procollagen, collagen, elastin fibronectin and hyaluronan in injured canine vocal folds.

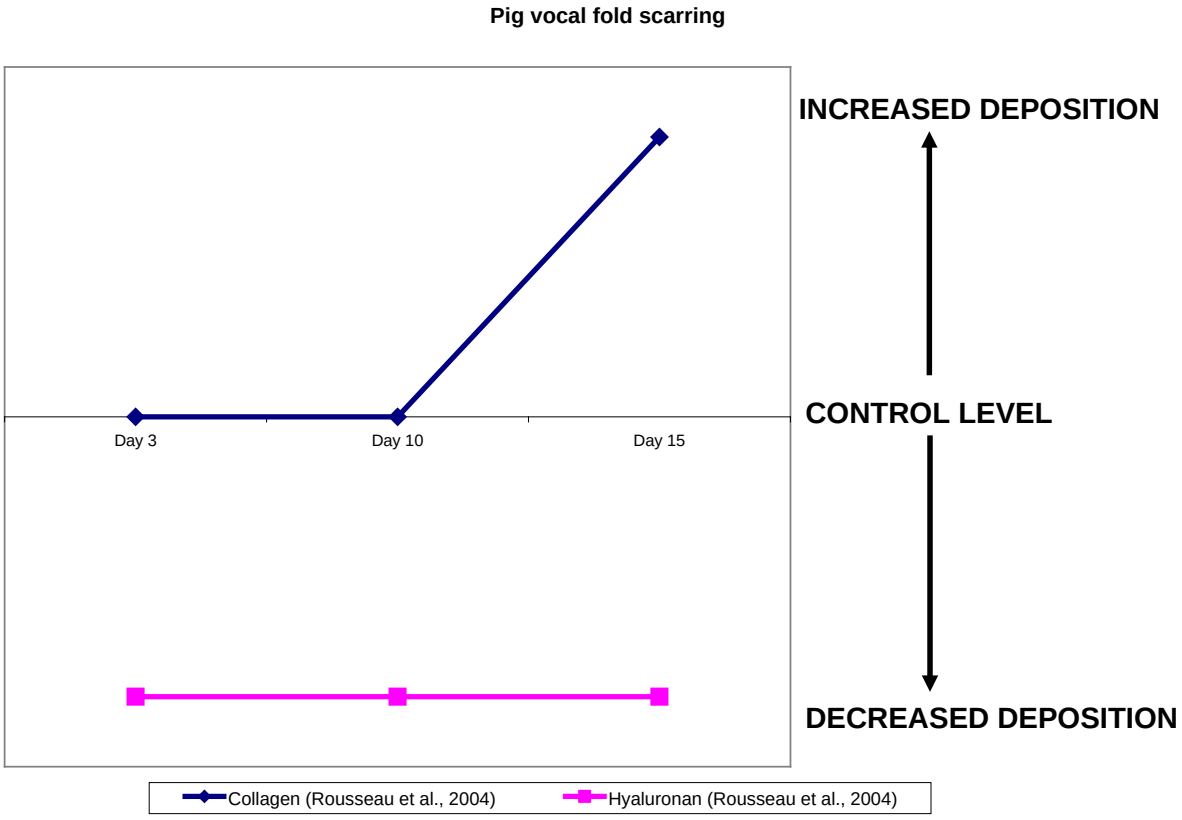


**Figure B4.** Schematic representation of collagen and hyaluronan in injured pig vocal folds.

### B.2.1 Cellularity

A report by Tateya et al. (I. Tateya, Tateya, Lim, Sohn, & Bless, 2006) is the only published study that investigate cell proliferation during acute vocal fold scarring. In a rat model, immunohistochemical staining were performed for (1) vimentin, a marker for fibroblasts; (2) alpha-smooth muscle actin, a marker for myofibroblasts; (3) CD68, a marker for macrophages and (4) 5-bromo-2-deoxyuridine, a marker for newly proliferated cells at four time points: Day 1, 3, 5 and 14 following vocal fold mucosal stripping. At day 1 post injury, epithelization and fibroblast proliferation started. At day 3 post injury, the proliferation of fibroblasts in the lamina propria was at peak. Of note, only the fibroblasts in the lamina propria actively proliferated in

this study. Fibroblasts in the macula flava (vocal fold stellate cells) did not proliferate actively in response to injury. This observation suggest that the vocal fold stellate cells in the macula flava have different functions from those in lamina propria in response to vocal fold injury and repair. At day 7, the total number of cells decreased about 33 folds and stayed low by the endpoint of the experiment at Day 14 post injury. Myofibroblasts and macrophages were found to minimally proliferate at all time points. These finding suggests that extracellular matrix deposition may start from Day 3 in injured rat vocal folds. Another study (Branski, Rosen, Verdolini, & Hebda, 2005a) also reported massive infiltration of cells and neo-lamina propria at day 3 following mucosal stripping in a rabbit model, although specific cell types were not identified in that study.

### B.2.2 Cytokines and growth factors

Four studies investigate the profiles of various inflammatory mediators in rat and rabbit following vocal fold stripping (Branski, Rosen, Verdolini, & Hebda, 2005b; Lim, Tateya, Tateya, Munoz-Del-Rio, & Bless, 2006; Rousseau, Ge, Ohno, French, & Thibeault, 2008; Welham, Lim, Tateya, & Bless, 2008). Except the study of Branski et al. focusing on protein levels, the other three studies were looking at the messenger RNA (mRNA) expressions of inflammatory mediators following vocal fold injury.

A rat study (Welham et al., 2008) used real-time reverse transcription-polymerase chain reaction to measure *in vivo* mRNA for a panel of inflammatory mediators, compared to the control side of vocal folds, focusing on the time window of first hour following surgery. Compared to the injured controls, gene expressions of cyclooxygenase 2 (COX-2), interleukin-1beta (IL-1β), tumour necrosis factor-alpha (TNF-α) and hyaluronan synthase 1 (HAS-1) were significantly up-regulated. Researchers suggested that platelets, resident cells and some early

arriving immune cells were probably the cell source for these mediators at such early time window of inflammation and healing.

Another related rat study (Lim et al., 2006) used a similar protocol described above by focusing on a later time window of inflammation: 4hr, 8hr, 16hr, 24 hr and 72 hr postoperatively. Results indicate a time-dependent expression profile of the inflammatory mediators. Nuclear factor-kappa B (NF-κB), IL-1β, TNF-α, and HAS-1 had peak expressions at 4 and 8 hours. From 8 to 16 hours, procollagen III expression decreased. At 16 hours, hyaluronan synthase 2 (HAS-2) was peak. At 24 hours, interferon gamma (IFN-γ) expression decreased. At 72 hours, transforming growth factor beta (TGF-β), HAS-2, procollagen I and procollagen III expressions were all at peak. For all time points, COX-2 expressions were significantly higher than the controls, whereas elastin expressions were similar to expression levels of the controls.

The investigators suggested that the simultaneous peaks of IL-1β, NF-κB and TNF-alpha expressions during the first 8 hours post injury induced the expression of HAS-1 and HAS-2 for hyaluronan production at the later time points. The decrease of procollagen III expression during 8 to 16 hours post injury might be related to the upregulation of collagenase by the actions of IL-1β and TNF-α. Also, at 16 hr time-point, the expression profiles were the most various. This time-point is speculated as an induction point of various cytokine signalling pathways in order to build up the mediator levels to a physiological level for later actions (Lim et al., 2006). At 72 hours, the upregulation of TGF-β expression might induce extracellular matrix deposition associated with HAS-2 and procollagen I and III. Elastin level was not significantly expressed from the starting point till the end point of the study at 72 hours post-injury. This finding suggests that elastin does not involve in acute inflammation.

The expression profile described in Lim et al.'s rat study is generally matched with the cell progression in Tateya et al.'s rat study (I. Tateya et al., 2006). Tateya et al. reported that the proliferation of fibroblasts in the lamina propria was peak at Day 3 (72 hours) post injury. At the same time, Lim et al. reported that TGF-β, HAS-2, procollagen I and procollagen III expressions were all at peak at Day 3 (72 hours). Although previous study (Sato et al., 2006) suggests that the stellate cells in the macula flava rather than the fibroblasts in the lamina propria are the main cell source of hyaluronan and collagen production, stellate cells are minimally proliferated in injured vocal folds (I. Tateya et al., 2006). Combining the findings of these two studies suggest that fibroblasts in the lamina propria are activated by tissue injury for wound repair, whereas the fibroblasts in the macula flava contribute to the maintenance of normal tissue. Also, the activated fibroblasts in the lamina propria may secrete a significant amount of growth factors and matrix proteins for repair around Day 3 post injury.

A rabbit vocal fold scarring study (Rousseau, Ge, Ohno et al., 2008) investigated the termporal mRNA profiles of MMP-1, MMP-9, fibronectin as well as collagen types I and III up to 72 hours following vocal fold stripping. Compared to the control speciments, time-dependent changes of these mRNAs were reported. MMP-1 and MMP-9 expression was significantly up-regulated at 4, 7, 24, 48, and 72 hours post-injury. Fibronectic expression was significantly up-regulated during 24-72 hours after injury, which may act as the provisional matrix for coming inflammatory and healing cell adhesion and migration. Collagen types I and III were significantly up-regulated at 72 hours post-injury, which is consistent with the fibroblast proliferative phase in injured rabbit vocal folds (Branski et al., 2005a).

Another rabbit scarring study (Branski et al., 2005b) investigated the temporal expressions of two inflammatory mediators: IL-1β and prostaglandin 2 (PGE-2) extending up to

21 days following vocal fold stripping. The corresponding mediator expression was measured from the concentrations of these two mediators in the surface laryngeal secretions. IL-1β had peak concentration at Day 1 and returned to baseline by Day 7 post injury. At the same time, PGE-2 was peak at Day 7 and remained elevated by the end point of the experiment, i.e., Day 21 following injury.

Although the validity of laryngeal secretion analysis for the vocal folds is awaiting for empirical confirmation, two other studies (Lim et al., 2006; Sandulache et al., 2007) may provide some indirect data to support the use of laryngeal secretion analysis. First, Lim et al.'s rat vocal fold study reported that IL-1β messenger ribonucleic acid (mRNA) expression at the tissue level was peak at 4 and 8 hrs post injury, whereas Branski et al.'s (Branski et al., 2005a) rabbit study reported that the same mediator at the secreted protein level (laryngeal secretion) was peak at Day 1 post injury. The time lag for the IL-1β concentration between that of tissue and of secretion can be explained by the process from gene translation to extracellular secretion of IL-1β. Second, one rabbit upper airway mucosal wound healing study evaluated directly the correspondence of IL-1β and PGE-2 from both subglottic airway secretions and the underlying subglottic tissue (Sandulache et al., 2007). Their results indicate that the up-regulation of inflammatory mediators in tissue mRNA levels is in parallel with that in secretion protein levels, although the changes in the tissue levels are greater that of the secretion levels after injury. The difference can be explained by the fact that mucosal secretions represent only one of several compartments into which inflammatory and wound healing mediators are partitioned and significant portions are being retained within the tissue. Although Lim et al.'s study (2006) used a different species (rat) and Sandulache et al.'s study used a different location of mucosal injury

(subglottic) compared to Branski et al.'s rabbit vocal fold study, these two studies seem to support the validity of laryngeal secretion analysis as a window of airway inflammation.

### B.2.3 Collagen

Collagen fibres are the important structural protein of the vocal folds. Collagen levels in scarred vocal folds have been studied in rats (T. Tateya, Tateya, Sohn, & Bless, 2005, 2006), rabbits (Branski et al., 2005a; Rousseau, Hirano et al., 2004; Thibeault, Gray, Bless et al., 2002), pigs (Rousseau, Sohn, Montequin, Tateya, & Bless, 2004) and canines (S. Hirano, Bless, Rousseau et al., 2003; Rousseau et al., 2003). Different forms of collagens are studied, namely, procollagen (Rousseau, Hirano et al., 2004; Rousseau et al., 2003; Thibeault, Gray, Bless et al., 2002), collagen (S. Hirano, Bless, Rousseau et al., 2003; Rousseau, Hirano et al., 2004; Rousseau et al., 2003; Rousseau, Sohn et al., 2004; Thibeault, Gray, Bless et al., 2002) and collagen types I and III (T. Tateya et al., 2005; T. Tateya, I. Tateya, J. H. Sohn et al., 2006).

In a rat study (T. Tateya, Tateya, Sohn, & Bless, 2006), the expression patterns of collagen types I and III were examined at 5 time points, i.e., Day 1, 3, 5, 7, and 14 following vocal fold mucosa stripping. Epithelization and granulation tissue was first observed at Day 3. The epithelium became thinner and approached to a single epithelial cell layer as observed in the controls at Day 7. The epithelization was complete with a single epithelial cell layer at the wound sites by Day 14. Collagen type I expression was strong and widespread throughout the lamina propria. Collagen type I was first indicated at Day 2 and then is peak at Day 5. Subsequently, the level of collagen type I decreases significantly from Day 5 to Day 7 and became stabilized until the endpoint of the experiment, i.e., Day 14.

Collagen type III expression was also strong and widespread, which was first indicated at Day 1. Then, the level of collagen type III increased and remained intense from Day 3 to 14. The investigators speculate that the collagen type III may contribute to the scaffold of the granulation tissue and collagen type I may enhance the strength for the new tissue. Further, the remodeling phase may start around day 7, in which collagen type III begins to replace collagen type I.

The collagen dynamics during the later remodeling phase of vocal fold scarring is described in a rat study (T. Tateya, Tateya, Sohn, & Bless, 2005). The collagen accumulation was examined at four time points, i.e., Week 2, 4, 8 and 12 following operation. Overall, collagen types I and III were more intense in the scarred vocal folds than that of controls at all time points. Collagen type III expression was constantly high for the 12 weeks, whereas collagen type I expression reduced from Week 2 until 8 and then stabilized until Week 12. Also, the total collagen level (type I plus type III) was peak at Week 2 and 4 and then declined and became stable during Week 8 and 12. Combining to the results of this study with the previously mentioned study on acute rat vocal fold scarring (T. Tateya et al., 2005; T. Tateya, I. Tateya, J. H. Sohn et al., 2006), the remodeling phase in vocal fold wounds can be characterize as: Day 7 - the start of remodeling; Week 2 and 4 - active remodeling phase and Week 8 - stable remodeling phase.

Collagen production seems to start relatively late in other larger animals. A rabbit study (Thibeault et al., 2002) showed that procollagen I (a marker of new collagen synthesis) was abundant in the superficial lamina propria, whereas collagen was sparse at 2 months postoperatively. Also, the collagen scaffold appeared disorganized than that of the control vocal folds. This observation suggests that the wound is still undergoing matrix deposition and does

not enter into the remodeling phase at 2 months following injury. The disorganized collagen scaffolding may account for the increased stiffness and viscosity of the scarred tissue. Subsequently, another rabbit study (Rousseau, Hirano et al., 2004) reported that procollagen was declined to the control level, whereas collagen was significantly increased at 6 months following vocal fold mucosa stripping. Also, collagen was found throughout the lamina propria and appeared thick and organized in the scarred vocal folds. The findings from these two rabbits suggest that the remodeling phase may not begin until 6 months postoperatively and may take up to 1 year. However, in another rabbit study (Branski et al., 2005a), mature collagen fibres were seen as early as Day 7, which suggests the initiation of remodeling phase.

One conjecture for this discrepancy is related to the use of trichrome stain for collagen detection. Compared to the immunohistochemistry method, trichrome stain is less sensitive and less specific if the collagen is in small deposits, thin and immature (T. Tateya, I. Tateya, J. H. Sohn et al., 2006). Therefore, the use of trichrome stain may underestimate the amount of collagen in the wounds during the first 6 months of Thibeault and Rousseau et al.'s rabbit studies because the collagen fibres were still immature and thin during that time. However, this conjecture can not apply to Branski et al.'s study (2005) because trichome stain was also used. However, mature collagen fibres were observed at a very early time point, i.e., Day 7 after injury. Further research is needed to examine the effects of various staining methods in relation to different forms of collagen.

In two canine studies (S. Hirano, Bless, Rousseau et al., 2003; Rousseau et al., 2003), collagen levels were similar to the control vocal folds at 2 months postoperatively. Collagen was primarily present in the extracellular matrix and minimally around the walls of the blood vessels of the lamina propria (S. Hirano et al., 2003). At the same time, procollagen was abundant in the

scarred tissues at 2 months but declined to the control level at 6 months (Rousseau et al., 2003). On the other hand, collagen density was significantly increased and the collagen fibres formed thick yet disorganized bundles (S. Hirano, Bless, Rousseau et al., 2003; Rousseau et al., 2003) at 6 months postoperatively. Lastly, a pig study (Rousseau et al., 2004) reported that the collagen levels were stable during the first 10 days and then started to increase at Day 15 following vocal fold mucosa stripping.

### B.2.4 Elastin

Elastin is a structural protein that is responsible for vocal fold recoil. The expression of elastin for vocal folds scarring are studied in rabbits (Rousseau, Hirano et al., 2004; Thibeault, Gray, Bless et al., 2002) and canines (Rousseau et al., 2003).

At 2 months following surgery, both rabbits and canines indicated decreases in the elastin levels in the lamina propria (Rousseau et al., 2003; Thibeault et al., 2002). Also, the elastin fibres appeared as shorter, tangled and disorganized. At 6 months following surgery, the elastin density was similar to the control sample in rabbits (Rousseau, Hirano et al., 2004) but was significantly less than the controls in canines (Rousseau et al., 2003). In both cases, elastin fibres were still disorganized throughout the entire scarred lamina propria following 6 months of surgery.

### B.2.5 Fibronectin

Fibronectin is an important adhesion protein, which provides a scaffold for cell migration during wound healing and relates to fibrosis at the site of injury. The expressions of fibronectin for

scarred vocal folds are studied in rats (T. Tateya et al., 2005; T. Tateya, I. Tateya, J. H. Sohn et al., 2006), rabbits (Thibeault et al., 2003), and canines (S. Hirano et al., 2003).

In an acute rat model (T. Tateya, I. Tateya, J. H. Sohn et al., 2006), fibronectin deposition was first indicated by day 1, which began earlier than epithelization and granulation. Fibronectin remained at high levels up to the end point of the experiment, i.e., Day 14. The strong expression of fibronectin was concomitantly with collagen deposition and collagen remodeling during Day 3 to 14 (Week 2) in this rat study. Another rat study (T. Tateya et al., 2005) reported the expression of fibronectin from Week 2 to 12 postoperatively. The level of fibronectin was peak at Week 2 and remained significantly high at Week 4. Then, the concentration of fibronectin started to decrease from Week 8 until Week 12. At the end point of the study (i.e., Week 12), the concentration of fibronectin was slightly higher that those of the controls. Combining these two rat studies (T. Tateya et al., 2005; T. Tateya, I. Tateya, J. H. Sohn et al., 2006), fibronectin was peak in the first 4 weeks post injury, which indicates the acute phase of wound healing. By then, tissue remodeling became slow and the scar was mature approximately two months (8 weeks) following vocal fold injury.

In a rabbit model (Thibeault et al., 2003), immunohistological examination showed significantly increase in fibronectin content than the controls at two months postoperatively. In contrary to the aforementioned rat model, increased fibronectin was found to be occurred with low collagen level in another rabbit study (Thibeault et al., 2002). The investigators speculated that the two-month-old scar is regarded as immature in rabbits after surgical trauma. This speculation is substantiated by a canine study of vocal fold scarring (S. Hirano et al., 2003). That study used immunohistochemical and image analyses to examine the expressions of fibronectin and several other adhesion molecules (cadeherin, syndecan-1 and syndecan-4) on the canine

vocal folds at both 2 and 6 months following vocal fold stripping. A significant increase in fibronectin and syndecan-4 were found in the superficial lamina propria at both 2 and 6 months, whereas co-deposition of collagen with fibronectin was only reported at 6 months post injury. Other adhesion proteins did not significantly increase after vocal fold injury. The increase of fibronectin and syndecan-4 may contribute to the increased viscoelasticity of the scarred vocal folds. Further, the characteristics of scar at 2 and 6 months were different, which suggest that the 2-month scar are not yet mature and stable.

### B.2.6 Decorin and fibromodulin

Decorin is a proteoglycan that is abundant in the superficial lamina propria of the vocal folds. Decorin plays a contributing role in collagen binding and the kinetics of collagen fibril formation. Fibromodulin is also a proteoglycan that helps in regulating collagen synthesis. In a rabbit vocal fold scarring study (Thibeault et al., 2003), immunohistological examination indicate that decorin and fibromodulin were both significantly decreased 60 days following surgery. Decrease in decorin level may change collagen organization, leading to abnormal vocal fold architecture. Also, decrease in fibromodulin may promote more collagen synthesis, leading to scar formation.

### B.2.7 Hyaluronan

Hyaluronan is a glycosaminoglycan that is abundant in the superficial lamina propria of the vocal folds. Hyaluronan plays an important role for shock absorption and maintaining optimal viscoelasticity of the vocal folds. The expressions of hyaluronan in scarred vocal folds are

studied in rats (T. Tateya et al., 2005; T. Tateya, I. Tateya, J. H. Sohn et al., 2006), rabbits (Rousseau, Hirano et al., 2004; Thibeault, Gray, Bless et al., 2002; Thibeault, Rousseau, Welham, Hirano, & Bless, 2004), canines (Rousseau et al., 2003) and pigs (Rousseau, Sohn et al., 2004).

In a rat model (T. Tateya, I. Tateya, J. H. Sohn et al., 2006), hyaluronan was first indicated by Day 3, peak at Day 5 and then started to decline until the end point of the experiment, i.e., Day 14. This trend is similar to a rabbit model (Thibeault, Rousseau, Welham, Hirano, & Bless, 2004), in which the level of HA was peak at Day 5 following vocal fold stripping. Also, another pig study (Rousseau, Sohn et al., 2004) reported that hyaluronan was significantly reduced in the injured vocal folds than that of the controls at all three time points of the study, i.e., Day 3, 10 and 15 following vocal fold mucosa stripping. Compared to the rat and rabbit models, the first hyaluronan accumulation was fairly late in pigs. Hyaluronan was not indicated until Day 15 post injury in pig vocal folds. The investigators (Rousseau, Sohn et al., 2004) suggest that hyaluronan is particularly important during the first few days during inflammatory and proliferative phases of wound healing. The absence and minimally expressed hyaluronan during the acute phase of vocal fold scarring may affect the vocal fold ultrastructure and contribute to the increased phonation threshold pressure and the decreased vocal economy in long term.

For the studies with longer observation time, a rat study (T. Tateya et al., 2005) reported that hyaluronan density remained significantly lower than that of the control levels at all time points of the experiment, i.e., Week 2, 4, 8 and 12 following surgery. This finding is similar to a rabbit study and a canine study, which hyaluronan levels were similar to the control levels at 2 months (Rousseau et al., 2003; Thibeault et al., 2002) and 6 months (Rousseau et al., 2003;

Rousseau, Sohn et al., 2004) post injury. The low level of hyaluronan may associate with the increased stiffness of the scarred vocal folds (Rousseau, Sohn et al., 2004). Lastly, the distribution of hyaluronan in scarred vocal folds is reported to be different from that of normal vocal folds. In scarred vocal folds, hyaluronan distribute throughout all layers of the lamina propria, whereas hyaluronan is primarily located in the deep lamina propria of normal vocal folds in the pig study (Rousseau, Sohn et al., 2004).

# APPENDIX C

# MATHEMATICAL AND COMPUTATIONAL MODELS IN MEDICINE

The purpose of this chapter is to (1) discuss system modeling and simulation for clinical research; (2) discuss two approaches in simulation modeling: equation based and agent based; and (3) overview current mathematical and computational models for a range of clinical diseases.

## C.1 NEW TECHNOLOGIES ARE NEEDED TO DEVELOP PREDICTIVE, PREVENTIVE AND PERSONALIZED MEDICINE

Current treatment paradigms for clinical diseases are reactive. Both surgical and behavioural approaches focus on treating pre-existing tissue changes, which are typically late in their development. Also, some genes may predispose to the susceptibility to vocal fold injury and so an individual may be more prone to a particular disease. Furthermore, human differ remarkably with each other relating to genetic polymorphisms. Health care is beginning revolutionized towards a predictive, preventive and personalized mode (Hood, 2003; Hood, Heath, Phelps, & Lin, 2004; Latterich, 2005; Vodovotz, 2006; Vodovotz, Clermont, Chow, & An, 2004). A long-range goal is to generate a technology that can: (1) identify molecular

signatures or biomarkers that are *predictive* of disease or treatment outcome; (2) characterize individual's probabilistic future health history for a range of diseases concerned and design *preventive* treatment program for the highly probable disease; and (3) prescribe *personalized* treatment regimes for individual patients depending on their unique probabilistic future health histories. Predictive, preventive and personalized medicine may extend the normal life spans by 10 to 30 years. Modeling-simulation, a working methodology of systems biology, is required in this endeavour (Hood, 2003; Hood et al., 2004; Vodovotz, 2006; Vodovotz et al., 2004).

## C.2 PURPOSES OF MODELING AND SIMULATION

### C.2.1 Modeling

Modeling is a typical research practice and theory testing method to solve problems when the structures or processes underlying a real-world system are difficult to observe and measure directly or controlled experimentation is infeasible or too expensive.

A model is an abstraction of a real-world system. A model aims to represent a real-world system to a certain degree that establishes correct quantitative relationship between the real-world system and the model of this real system. If the model is formulated as a computer program, it is known as a computer model. Models can be characterized by various dimensions (Table C1). The choice of dimension depends on the problems of interest and practicability. For example, for a model whose analytical solution does not exist or may be very difficult to find, simulation modeling rather than analytical modeling may be applied.

**Table C1.** Dimensions of a model and the related descriptions and examples.

| Model Dimensions | Description | Example |
|---|---|---|
| Formal vs Judgmental | A formal model is formed by equations and formulas.<br>A judgemental model is formed by the deductions and assessments from an individual's experience or verbal description. | Formal: a mathematical expression<br>Judgmental: expert opinion |
| Causal vs correlation | A causal model reflects cause-effect relationship. A correlational model does not reveal such causal relationship. | Causal: almost not exist<br>Correlational: weather forecasting |
| Deterministic vs Stochastic | A deterministic model generates the output to a given input by one fixed law. A stochastic model generates the output from a set of possible responses based on random or a fixed probability distribution. | Deterministic: differential equations<br>Stochastic: agent based |
| Dynamic vs Static | A dynamic model describes the time-spread behaviour in a system. A static model describes the system at a given instant of time and in an assumed state of equilibrium. | Dynamic: a cartoon; differential equations<br>Static: a photo |
| Analytical vs Numerical (simulation) | An analytic model is formed by explicit equations that permit a solution. A numerical (simulation) model is that the solution is obtained by experimentating the model rather than by an explicit solution algorithm. | Analytical: any mathematical models that can be solved<br>Numerical/Simulation: most models written in partial differential equations |

Modeling is an iterative process of abstraction, model building, model analysis, model optimization and model implementation. First, theoretical assumptions of system structures and processes are abstracted to build a model. During model building, a model is parameterized via a set of parameter settings. These settings allow the model to be adapted to different situations of the same system. Then, the modeller uses the information from the target system to set or estimate the parameters in order to optimize the model performance. Lastly, the model is

implemented and the model output is compared with real-world data for validation purpose. Some discrepancy between the model outputs and the real-world outcomes are common, which is known as *model error* (Edmonds, 2005).

Models are used in three ways: (1) for explanatory purpose, (2) for predictive purpose and (3) for simulation purpose (MacFarlane, 1986 ). The following paragraphs first discuss the distinction between explanatory models and predictive models. The next section discusses simulation models. Simply speaking, an explanatory model answers "how" and "why" questions, whereas a predictive model answers "what" questions.

An explanatory model aims to provide an explanation of how structures and mechanisms underlying a target system contribute to the observed behaviour of the system. To provide such explanation, a model requires the information about the initial conditions and the observed data from the target system. An explanatory model can give a candidate explanation of *how* and *why* the outcomes in the target system come from its initial conditions. The validity of explanatory model can be tested with known empirical data. A common testing method is to divide the data into in-sample and out-of-sample sections, then models are calibrated with the in-sample data and models are evaluated to see if they match the out-of-sample section to a sufficient degree (Edmonds, 2005; MacFarlane, 1986 ).

A predictive model is used in order to make inference about *what* is going to happen in the future based on the current knowledge of the system. The predictions do not need to have 100% accuracy but simply accurately enough for the modeller's purpose. Compared to explanatory models, predictive models require much more information for model building. If the prediction is beyond a certain time limit or the systems exhibit chaotic behaviour, impossibly large amount of information is required to predict the systems' future behaviour. As a matter of

fact, the real world has finite amounts of useful information and this explains why our predictive ability to future is so limited (Edmonds, 2005; MacFarlane, 1986 ).

### C.2.2 Simulation

Simulation is the process of model implementation or execution. A simulation model is whose solution is obtained by executing the model, instead of solving by an explicit algorithm. A set of rules or mathematical equations defines how a simulation model changes over time, given its current state. To execute the model, a computer program is required to take the model through time and to update the state and event variables in the model simultaneously. The time steps can be discrete or continuous.

Traditionally, a formal model is built by mathematical equations in order to find analytical solutions for explaining or predicting the behaviour of the target system from a set of parameters and initial conditions. If the model can be solved analytically, simulation approach is not needed. However, there are many mathematical equations that simply do not have analytical solutions. This problem is not rare, especially when (1) the model is very complex with many interacting components; (2) the interactions of the components are nonlinear; (3) time dynamics is important; and (4) the model contains random variables. For these situations, a complete numeration of all possible states is impossible. Simulation is applied to generate a sample of representative scenarios for a model. Therefore, simulation is very useful to explore complex models and to establish possible processes and outcomes by manipulating the parameters of the models (Edmonds, 2005).

## C.3 GENERAL FRAMEWORK OF MODELING-SIMULATION IN SYSTEMS BIOLOGY

The focus of systems biology is to capture all components and interactions of a target functional system and its underlying dynamics. To achieve these goals, systems biology has to tackle a large volume of data and a level of complexity that cannot be modelled with simple graphics alone. In systems biology, computer-aided modeling and simulation is indispensable. Computers not only help to store and compile data in an efficient way but also help to integrate data into network models for simulation purposes.

In clinical research, a model is to provide an abstract representation of the information obtained from experimental observations on the structure and function of a specific biological functional element. This functional element can have different levels of complexity, ranging from simple enzyme reactions to signal transduction pathways to cell mitosis to organ functions or even to whole human being. Modeling-simulation assists system-level analysis of biological systems in three aspects. First, models enable to describe the structure of the interactions that govern system behaviour. Second, models integrate and summarize the current knowledge that can facilitate cross-discipline communication. Third, models enable to simulate system responses given specified perturbation and to give quantitatively accurate predictions that can be experimentally verified (Butcher, 2007; Butcher, Berg, & Kunkel, 2004; Ideker, Galitski, & Hood, 2001).

System modeling and simulation involves four major steps: (1) component identification and modeling, (2) system perturbation and monitoring; (3) model refining and (4) model testing. The idea of this framework is to transfer a descriptive and qualitative model to a statistical or probabilistic model (Ideker et al., 2001).

1. Component identification and model building. The first step is to define all the components of the target system to formulate an initial mathematical model. Ideally, using existing biochemical and genetic knowledge for component identification is the best because no prior assumptions are intervened in the model. However, when prior knowledge about the target system is limited, hypothetical interactions among the components can be involved. In that way, the initial model becomes descriptive.
2. Experimental perturbation. The second step is to systematically perturb and monitor the components of the target systems. Perturbation can be genetic, such as, gene deletions, gene overexpressions or undirected mutations. Perturbation can also be environmental, such as, changes in temperature, growth conditions or growth factors stimulation. High-throughput technologies are needed to measure the corresponding responses to each perturbation and to integrate the data from multiple experiments. The measures should be taken at multiple levels of the system, such as, messenger ribonucleic acid (mRNA) expression, protein expression, and protein function. The observed data are then integrated into the initial mathematical model (Step 1).
3. Model calibration and optimization. The third step is to refine the model until model predictions are closely matched with experimental observations. A goodness-of-fit measure is commonly used to evaluate agreements between predictions and observations. If disagreement exists between the predicted and observed responses, alternative hypotheses are proposed to refine the model structure or function in order to maximize the goodness of fit. However, the proposed refinements may result multiple models whose predictions are in good agreement with the known empirical data but with different underlying hypotheses. To differentiate these models, Step 4 is required.
4. Iterative model testing. The last step is to design and perform new perturbation experiments that can distinguish among multiple model hypotheses. During model refinement (Step 3), multiple models with competing hypotheses may be resulted. Current data set (Step 2) are insufficient to distinguish these models and thus new perturbations and measurements are required to discriminate the models. New perturbations should be able to elicit differentiated system responses among the models. After a set of new perturbations are identified, steps 2 to 4 are repeated to expand and optimize the model continually over successive refinements. The goal is to bring

experimental data and model predictions into close agreement by iterative testing, so that model predictions can accurately represent biological reality.

## C.4 PROMISES OF SYSTEM MODELING AND SIMULATION IN CLINICAL RESEARCH

Physiologic systems, whatever in their healthy and pathologic states, exhibit a remarkable variability of structural patterns and temporal behaviours. Classical homeostasis, linear constructs and reductionist methodologies are inadequate to understand such physiological complexity (Goldberger, 2006). For example, reductionist strategy contributes in identification of numerous biomarkers that characterize an array of diseases. These biomarkers may have beneficial and/or adverse effects on the body, depending on the amount and timing of their production. With these functional dependencies, reductionist approach is limited to differentiate the roles of biomarkers -- are the markers merely signatures of immunologic response or are the markers actually involved in the pathophysiology of disease (Neugebauer, Willy, & Sauerland, 2001; Tjardes & Neugebauer, 2002)? To answer these questions, prohibitively large number of animal or *in vitro* experiments is anticipated (1) to collect comprehensive data in considering all temporal and contextual factors and (2) to extract useful information and pattern from such huge quantities of data. In order to overcome the limitations of animal and *in vitro* experiments, systems biology is an alternative approach in studying complex processes, like in pathophysiology. Systems biology helps to (1) handle a plethora of components and interactions within complex processes simultaneously; (2) quantify interrelationships (organization or structure) and interactions (dynamics or behaviour) of genes, proteins and metabolites; and (3) to

integrate this information into visible working models that can provide predictive hypotheses to elucidate emergent phenomena behind complex processes (Hood et al., 2004; Kitano, 2002a, 2002b; Wolkenhauer, 2001). For a detailed discussion of system-level analysis, please refer to Chapter 5, Section 5.5.2.

Mathematical and computational modeling-simulation are the essential tools for system analysis in clinical research, especially in knowledge discovery (data-mining) and simulation-based analysis (Kitano, 2002a, 2002b). Modeling is used to transfer real-world components and interactions into an abstracted representation. For knowledge discovery, model abstraction is useful to conceptualize the "hidden order/pattern" behind the observed phenomena (e.g., immunologic response) from huge volume of experimental data (e.g., proteomic data). Building a conceptual framework is helpful to explain unknown relationship, to make predictions, to formulate hypotheses for designing experiments and to suggest which variables to measure and the rationale (Wolkenhauer, 2001). For instance, knowledge discovery has been extensively applied to predict protein structure from genetic sequence, for instance (Baldi & Brunak, 2001).

On the other hand, simulation-based analysis is to test hypotheses with computer (*in silico*) experiments and then to provide predictions to be tested by *in vivo/in vitro* experiments (Kitano, 2002a, 2002b). In clinical research, simulation-based analysis can sharpen our understanding and intuition of a particular (patho-) physiological process. Simulations from computer-executable models predict a range of scenarios relevant to the properties of systems. These simulation outputs are compared with known empirical data to evaluate the validity of the underlying assumption of systems. Inconsistent findings reflect that the validity of the underlying assumption of the models or the prior knowledge to the target system is in question. In that case, hypotheses of the models have to be revised until they pass the validation process. A validated

model can then be used to provide predictions to be tested by *in vivo and in vitro* experiments as well as to explore problems that are not feasible to experimental inquiry. Modeling-simulation approach has improved our understanding of various biological phenomena, such as, bifurcation analysis of cell cycles (Borisuk & Tyson, 1998; Chen et al., 2000; Lovrics et al., 2006) and metabolic analysis (Covert, Knight, Reed, Herrgard, & Palsson, 2004; Edwards, Ibarra, & Palsson, 2001). The application of modeling-simulation to clinical practice is acknowledged in drug discovery and gene therapy to pursue the goal of predictive, preventive and personalized clinical care (Hood, 2003; Hood et al., 2004; Kitano, 2002a, 2002b).

To sum, by direct modeling-simulating a complex system, one can observe, manipulate and understand the behaviour of the whole system in a more efficient way. As such, modeling-simulation can improve both of biological sight and insight into mechanisms within complex systems (Goldenfeld & Kadanoff, 1999; Kitano, 2002a, 2002b; Vicsek, 2002).

## C.5 CHALLENGES OF MODELING-SIMULATION IN CLINICAL RESEARCH

Application of modeling-simulation is still in its infancy in clinical research. Challenges are described as follows. First, building a full-scale human model or even a whole-cell model is difficult at the current stage of technology (Kitano, 2002a, 2002b). Existing models focus on relatively specific processes at particular scales, such as, at the metabolic pathway level. Simulation of a full-scale model has to integrate multiple models, which are heterogeneous in terms of scales, resolutions and modalities. For example, some biochemical processes may take place in the order of millisecond, whereas some are in the order of hours or days; some biological phenomena is best modelled by stochastic computation or differential equations,

whereas some biological processes have to use a combination of both methods. Although forming integrative models across scales and modalities seems impossible at the time of this writing, profiling data from high-throughput assays may help to incorporate biological complexity at multiple levels: multiple interacting molecular pathways, multiple interacting cell types and multiple different environments (Butcher, 2007; Butcher et al., 2004). These data provide a solid foundation for cross-scale model building.

Second, a question that is always asked during model building is "*what the level of details should be*". Incorporating every single known interaction into a model is demanding. Subsequently, parameter estimations for models can also be complicated with the number of parameters. Estimates may need to come from diverse experiments, which may give dramatically different values for parameters. Moreover, model calibration with known biological or clinical data becomes even more challenging. Models may be able to show high-level resemblances between the emergent properties of models and real-world phenomena. However, calibrating models that produce confirmable regularities of real-world systems is challenging.

Lastly, the modeling-simulation approach has been challenged for its over-interpretation of model outputs (Edmonds, 2005; Latterich, 2005). Confusion between explanatory and predictive types of model may happen in systems biology. The origin of concern is that biological systems are regarded as complex adaptive systems (Bradbury, 2002). Behaviour of these systems is regarded as *unpredictable* owing to their extreme sensitivity to initial conditions as well as adaptation and self-organization of component parts (Bradbury, 2002). Therefore, the models of complex adaptive systems are explanatory at best but not predictive in strict sense. One can run the models to generate a distribution of scenarios and use the outputs to understand the underlying process of the target systems (Rand et al., 2003). However, the model outputs

cannot be considered to conclude as predictions about the target systems. Even though one may find a good correlation between model outputs and experimental data, correlation alone is not a strong scientific proof *per se* (Latterich, 2005). Furthermore, if the models are constructed as predictive, the models should be validated against unknown data to the modeller. However, these "predictive" models sometimes are erroneously validated as explanatory models – using in-sample and out-of-sample data set from a same system (Edmonds, 2005). As such, researchers have to be very cautious when making justification and interpretation of model outcomes.

## C.6 MODELING TECHNIQUES: EQUATION-BASED AND AGENT-BASED MODELING

### C.6.1 General Overview

Equation-based modeling and agent-based modeling are the widely accepted simulation techniques in studying biological complexity. The basic structures of these two models are distinctive. Each of the models has its own strengths and limitations and are considered complementary (Vodovotz et al., 2004). Table C2 summarizes the principles, properties, strengths and limitations of equation-based and agent-based modeling.

Equation-based and agent-based modeling have a common. Both models realize the real world consisting of two entities, which are *individuals* and *observables* (van Dyke Parunak, Savit, & Riolo, 1998). Each individual is characterized by a unique set of traits. Individuals interact with each other through their behaviour and affect the values of observables. For instance, individuals can be understood as "people in a city", who "do things" over time.

Observables can be understood as “the economy of the city”, which are “measurable characteristics” of interest. Equation-based modeling and agent-based modeling treat *individuals* and *observables* differently in the way of modeling their relationship among entities and the level of focus.

The first fundamental difference between equation-based and agent-based modeling is how to model the relationship among entities. Equation-based modeling begins with a set of mathematical equations that express relationship among *observables*. These equations may be algebraic, or they may capture system dynamics over time (ordinary differential equations) or over time and space (partial differential equations). The interacting behaviours of the individuals are not explicitly represented in equation-based models. On the other hand, agent-based modeling begins with the behaviours that express the interactions among *individuals*. These behaviours may involve multiple individuals directly (e.g., men marry women) or indirectly through a shared environment (e.g., men and women compete for a job). Individuals affect the values of observables by their actions. However, the direct relationships among observables are not informed but rather emerge in agent-based models (van Dyke Parunak et al., 1998).

The second fundamental difference between equation-based and agent-based modeling is the level at which the model focuses on. A system is composed of interacting individuals. Some of the observables can only be expressed at the system level (e.g., the economy of a city), while some may be defined at the individual level (e.g., the monthly salary of people in a city). Equation-based modeling primarily uses observables at the system level to drive the model’s dynamics. On the other hand, agent-based modeling is not driven by system-level information. Agent-based modeling maps its building block “*agents”* to the *individuals* of the target systems.

Then, agent-based modeling use observables at the individual level to define agent behaviours (van Dyke Parunak et al., 1998).

**Table C2.** Comparison of equation-based and agent-based modeling (Bonabeau, 2002; Neugebauer & Tjarders, 2004; van Dyke Parunak et al., 1998).

| | **Equation-based modeling** | **Agent-based modeling** |
|---|---|---|
| **Principle** | The dynamics of the system is modelled as a collection of differential equations and constitutes the mathematical foundations of Newtonian determinism. The response of the system is determined by solving the differential equations. | Objects ("agents") are created by abstraction from the real world objects. Different classes of agents are created to represent their individual properties. Rules of interaction are then fed into the system. The behaviour of the system components can be observed by running simulation. |
| **Properties** | | |
| Building Block | Feedback loop connecting behavioural variables (observables). | Individual agents connected by feedback loop. |
| Unit of Analysis | Structure, which is fixed over time | Rules, which can be adaptive over time. |
| Perspective | Top-down: infer from structure to systems behaviour. | Bottom-up: infer from individual agent's behaviour to systems behaviour. |
| Handling of Time | Continuous simulation. | Discrete or continuous simulation. |
| Mathematical Formulation | Integral equations. | Logic. |
| Handling of Randomness | Deterministic: no random elements. | Stochastic: contains random (probabilistic) elements. |
| **Strengths** | Amenable to mathematical systems analysis, e.g., bifurcation theory.<br>Requires good experimental evidence to derive valid coefficients.<br>No need to have an exact definition of the states of physiological systems. | "One-to-one modeling": "Agents" can be applied directly to its "real-world" counterparts.<br>Models are explicitly based on the formulation of the investigated mechanisms.<br>Suitable for mapping the spatial structure of processes.<br><br>The model computation is parallel rather than sequential, which is closer to reality. |
| **Limitations** | Difficult to separate mathematical phenomena from physiological reality.<br>Difficult to implement spatial structure of the real world into the model.<br><br>Analytical approach will be failed if the model is composed of partial differential equations.<br>Numerical method has to be applied to analyze the model.<br>The construction of adequate differential equation is very demanding because the corresponding coefficients have to be determined with a high degree of precision. | Technical limitations of agent complexity and number of agents.<br>Analysis is limited to spatial events. The model has to be transferred into differential equations for a mathematical analysis. |

### C.6.2 Equation-based Modeling

Equation-based model is composed of a series of (usually nonlinear) differential equations that describe the change of the states of the variables with time. Equation-based modeling is formal and rigorously quantitative to simulate a system. Equation-based modeling is continuous, which does not model discrete events. The structure of an equation-based model is fixed and is organized by interacting feedback loops. Equation-based modeling is particularly suitable for the human physiology when the laws of nature (e.g., Newton's law of gravitation, Newton's three laws of motion, the ideal gas laws, Mendel's laws, the laws of supply and demand, etc.) apply. Circulatory system and respiratory system are the good exemplars for equation-based modeling (Neugebauer & Tjarders, 2004; Vodovotz et al., 2004).

Constructing an equation-based model with adequate differential equations that can accurately represent human physiology is a non-trivial task. To approach a target system in equation-based modeling, one has to quantitatively formulate system behaviour as a number of interacting feedback loops, balancing or reinforcing, and delay structures (Borshchev & Filippov, 2004). A typical feedback-rich equation-based model is composed of several dozens to several hundreds of equations (Borshchev & Filippov, 2004). The model has to go through an iterative process of expansions and reductions until a minimal feedback structure in terms of number of differential equations is identified to adequately simulate a predefined reference mode of the target system (Scholl, 2001).

Usually, the differential equations in equation-based models have to be derived from known and hypothesized kinetics of the components of biological systems (Vodovotz et al., 2004). The parameters of the equations usually represent the average concentrations of various components in the systems, such as, cell types or inflammatory mediators. Also, the coefficients

of the equations, which specify a model to its target system, have to be determined with high precision, either by experiments, statistical methods or expert knowledge (Neugebauer & Tjarders, 2004; Vodovotz et al., 2004). Ordinary differential equations are usually applied to characterize time-dependent dynamics of systems. When spatial dynamics is an issue as well, partial differential equations have to be applied instead. If a model is composed of complex ordinary differential equations or partial differential equations, analytical approach is usually failed to solve the equations. In that case, numerical approach (i.e., simulation), which is commonly with the aid of computers, has to be applied to solve the equations. The other approach is to employ the methods from nonlinear dynamics analysis, such as, bifurcation analysis, to explore the dynamics of the systems without solving the equations (Kitano, 2002a, 2002b; Vodovotz et al., 2004). The mathematical structure and tools for analysis of equation-based models are discussed in the following paragraphs.

### C.6.3 Mathematical structure and tools for analysis for equation-based modeling

A classical example of equation-based modeling is the problem of population growth. The question of interest is what the population will be in the coming years. A simplified model for this problem is an exponential model. That is, the rate of change of the population is proportional to the existing population. The equation is written as:

$$\frac{dP}{dt} = kP$$

Rate of change of the population — Existing population

P is the population. t is time. k is a rate constant. The initial condition population is $P_0$. The solution of this equation is $P_t = P_0 e^{kt}$.

A more complex biological model is given herein. For the ease of understanding, Figure C1 presents a “stock-flow” diagram of neutrophil dynamics in an equation-based model. Briefly, the diagram has two basic variables: (1) “stocks”, representing the *states* of the system, and (2) “flows,” representing an activity that changes the stock magnitude. The “flow” connects the “stocks/ states” in the model. The flow is not a constant but is the *rate of change* in the stock at any *instant of time*. In calculus terminology, that *instantaneous rate of change* of the system is the *derivative* of stock with respect to time *t.*

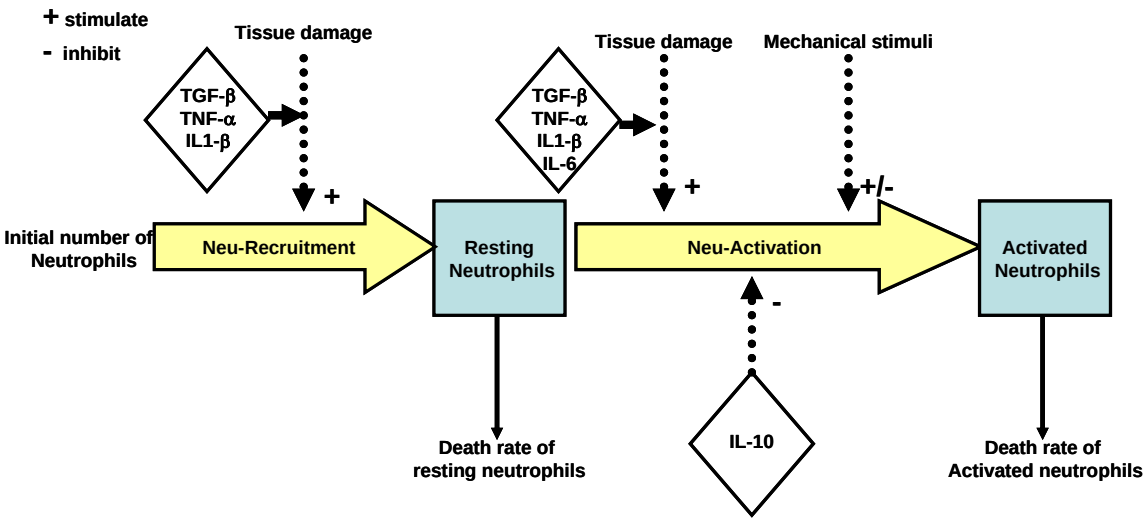


**Figure C1.** A schematic dynamics of neutrophils in an equation-based model. The rectangular boxes represented the two "stocks/ states" of neutrophils over time: resting and activated states. The broad arrows are "flows/rates". The flows include: (1) "Neu-recruitment" -- the process at which neutrophils are recruited to the wound site, and (2) "Neu-activation" -- the process at which resting neutrophils are converted to activated neutrophils. These two flows can be affected by systematic variables: (1) tissue damage and (2) mechanical stimuli induced by tissue mobilization. The *diamond boxes* (e.g., TGF-β) are auxiliary variables. They can inhibit (e.g., IL-10) or stimulate (e.g., TGF-β) the neutrophil recruitment and activation processes directly (e.g., IL-10) or indirectly (e.g., TGF-β). Lastly, both resting and activated neutrophils die at their respective death rate.

To model biological systems, *states* of the system is usually specified by the concentrations of all cellular and biochemical components involved at any *instant of time* (Tyson, Chen, & Novak, 2001; Tyson, Csikasz-Nagy, & Novak, 2002). The *rate of change* of the concentration for each component is formulated as differential equations in equation-based modeling. The equations inform how much each concentration will change in the next small interval of time. To know the temporal progression of each component, the rate constants have to be specified and the differential equations have to be solved by integration. However, most of the

time, the rate constants are not measurable and alternative methods are required to find the solution of the equations. Tools from system dynamics provide an alternative solution for equation-based modeling analysis. Bifurcation analysis is one of the tools, which has been widely used in biological modeling (Day et al., 2006; Reynolds et al., 2006; Tyson et al., 2001; Tyson et al., 2002).

Simply put, bifurcation theory is the analysis of a dynamical system of ordinary differential equations under parameter variation. Bifurcation analysis is powerful to describe how a system behaves over time with its parameter(s), because it predicts what kind of behaviour (systems in equilibrium or in oscillation) occurs where in parameter space. Bifurcation analysis traces time-dependent changes in the *state* of the system in a multidimensional space. For a biological system, each dimension can be realized as concentration of a particular cell type or protein involved. The bifurcation *solution* to an equation-based model is the topological features (e.g., the number of stationary points or periodic orbits) of the system over time under various conditions. “Steady states” and “oscillations” are the two critical solutions from bifurcation analysis. Biological interpretation of “steady state” is that the rates of change in the concentrations of cell or proteins involved are all identically zero. In other words, at a steady state, cell or protein concentrations are unchanged in time. On the other hand, the biological interpretation of “oscillations” is that the concentrations of cell or protein change in time and repeat themselves after a certain time-point (Tyson et al., 2002).

Both “steady state” and “oscillatory” solutions can be either stable or unstable. Stable means that any perturbations has little temporal effect to drive the system away from its original state (steady or oscillatory) and the system return to its original state very quickly. Unstable means that some perturbations grow larger with time and the system leaves its original state

(steady or oscillatory). Stable solutions represent physiologically observable states of the system, whereas unstable solutions make the existence of the state to be known only indirectly (Tyson et al., 2002).

The bifurcation solutions of a dynamical system depend on the parameters in the equation-based models. Parameter variation may lead to quantitatively or qualitative different behaviour of the system. The qualitative change of the solutions of a dynamical system is known as “bifurcations”, which is of interest for researchers. For example, a stable oscillatory state may lose its stability or even disappear, and a steady solution may kick into existence. “Bifurcation points” is the particular values of parameter(s) at which bifurcations occur (Tyson et al., 2002). Scientists are interested in searching for the bifurcation points in system behaviour and see how to control the physiological properties of a system by manipulating the involved parameters.

### C.6.4 Strength and limitations of equation-based modeling

The advantage of equation-based modeling over agent-based modeling is its suitability for rigorous mathematical analysis. Equation-based modeling allows characterization of system behaviour using standard techniques, such as bifurcation analysis. However, equation-based models are rigid in structure and lacks the capability to modify them, which is one of the strengths of agent-based modeling (Scholl, 2001). Also, equation-based modeling lies on the assumption of homogeneous mixing of individuals and the application of mean-field approximation. That is, individuals are identical or can at least be represented by some “average” type. However, recent notion suggests that system dynamics are more realistic to be modelled by treating individuals in a heterogeneous way (van Dyke Parunak et al., 1998). In that sense, agent-based modeling provides a perfect alternative in that avenue.

### C.6.5 Agent-based Modeling

Agent-based modeling or individual-based modeling is a relatively new approach for system modeling and simulation, which studies macro-level world through defining micro-level of a system. Agent-based modeling is stochastic modeling that simulates the behaviour of real-world systems under random conditions (Gilbert & Bankes, 2002). "Agents" are the building blocks in agent-based models. Agents represent the component parts of a system that contribute to the system's behaviour. Agent behaviour is defined by a set of rules, which is implemented during the simulation process. The rules can involve mathematical equations or "If…Then" conditional statements. On the basis of these rules, a virtual environment is created to allow agents to respond and interact, and to allow for the quantitative visualization of the emergent behaviour.

### C.6.6 Suitability of agent-based modeling in clinical research

Agent-based modeling is particularly suitable for modeling complex adaptive systems, e.g., biological systems (Holland, 1992, 1995; Mitchell, 2003). Complex adaptive systems are composed of diverse entities that interact nonlinearly and dynamically. These systems display self-organization and adaptation to produce emergent structures and behaviours, which cannot be easily predicted (Cilliers, 2005). In biology, the interactions are often context-dependent and time-dependent, which makes biological systems adaptive. Also, biological systems are described as "complex" from three aspects: constitutive complexity (complex structure), dynamic complexity (complex functional processes) and evolved complexity (complex evolutionary processes) (Mitchell, 2003). Agent-based modeling provides a robust and flexible framework to encompass these three complexities.

For constitutive complexity, agent-based modeling assumes heterogeneous mixing of individuals and acknowledges the difference between the basic units in the target systems. Also, agent-based models are flexible in its structure, which is opposition to equation-based modeling. Equation-based modeling has to make hypotheses about the global structure of the system *a prior* and then try to validate with a top-down procedure. Agent-based modeling allows specifying model structures both by posing constraints on the model as a whole (top-down) or by specifying the organization of agents individually (bottom-up). Moreover, agent-based models are inherently compositional/ modular in structure. Multiple models or multiple scales in physiologic organization can be integrated in the same agent-based models, without rebuilding a new model. The assumption of heterogeneous mixing and the structural flexibility enriches agent-based models to generate diverse structure patterns for modeling-simulation purpose (Mitchell, 2003; Neugebauer & Tjarders, 2004).

For dynamic complexity, agents' rules are adaptive. The rule-based simulation allows modeling the context- and time-dependent nature of biological functions. The rules can be formulated in a way that agents can participate in multiple pathways and processes at different time or in different environments. Thus, agents are able to perform complex interactions with others (Griffin, 2006; Mitchell, 2003).

For evolved complexity, agent-based modeling is able to encode history-dependent events. Each agent has unique entities and their history-dependent behaviours can change and adapt each others to produce new sets of behaviour. The history of an agent forms part of the model's history and in turn feedback to the model to determine the agent's current behaviour. The recursive agent-agent and agent-environment interactions create the continuously evolving processes as observed in biological systems (Griffin, 2006; Mitchell, 2003).

Last but not least, the robustness and flexibility of agent-based models allow for the addition of new components in existing models. This advantage is important to accommodate the quickly changing landscape of knowledge in clinical research. Agent-based modeling has been applied to study various fields of medical science, for instance, immunology (An, 2001, 2004; Day et al., 2006; Gammack, Ganguli, Marino, Segovia-Juarez, & Kirschner, 2005; Melby, 2004; G. Muller, Grebaut, & Gouteux, 2004; J. Muller, Kretzschmar, & Dietz, 2000; Reynolds et al., 2006; Robbins & Garrett, 2005; Tay & Jhavar, 2005; Vodovotz et al., 2006; Vodovotz et al., 2004), tumour growth (C. Athale, Mansury, & Deisboeck, 2005; C. A. Athale & Deisboeck, 2006; Mansury & Deisboeck, 2004a, 2004b; Mansury, Kimura, Lobo, & Deisboeck, 2002; Zhang, Athale, & Deisboeck, 2007), vasculature (Peirce, Van Gieson, & Skalak, 2004) and stem cells (d'Inverno & Prophet, 2005). Therefore, agent-based modeling appears to be the most direct initial approach to simulate the mechanism of biological systems, and to encode complicated history-dependent internal cellular and molecular events (An, 2005; Smallwood, Holcombe, & Walker, 2004; Vodovotz et al., 2004; Walker, Hill, Wood, Smallwood, & Southgate, 2004).

### C.6.7 General principles of agent-based modeling

The principle of agent-based modeling is that agents are created by abstraction from the real-world entities of interest. An agent-based model contains a system of numerous yet heterogeneous agents (ranging from tens to thousands) and the relationships between them. Different classes of agents are characterized by their function and behaviour. The rules of interaction (computer algorithms) among the agents are fed into the system. The rules are usually derived from theory or empirical data. If simulation is run, the behaviour of the system components can be observed. Beyond examining the component behaviour, the modeller is able

to explore possible underlying mechanisms that synthesize the observed emergent phenomena. For instance, system behaviour can be explored by varying the initial conditions of the models, or by imposing some restraints on the models, or by changing the rules of interactions (An, 2001; Bonabeau, 2002; Griffin, 2006; Holland, 1995; Neugebauer & Tjarders, 2004; Srbljinovic & Skunca, 2003; Troisi, Wong, & Ratner, 2005).

The idea behind agent-based modeling is that decision making is distributed among agents. Agents can operate individually but can also compete and cooperate with other agents and their environment. Both micro-level patterns (e.g., local behaviour and interaction between the agents) and macro-level phenomena (e.g., collective behaviour emerging from agents' individual characteristics) are observable in agent-based models. Agent-based modeling creates a virtual environment for artificial agents to inhabit. Agents perceive the state of the environment, and then operate in respect to the information they acquire. Agents may change themselves or change others' in their environment accordingly. Agents' actions are goal-oriented and they evolve. Agents have intentions to actively change the state of the environment in order to achieve their goals according to their internalized decision plan. Except being goal-directed, agents may also have cognitive properties (such as adaptation, memory and learning) to seek information or communicate intentions. The aforementioned characteristics permit the agents to participate in complex interactions and thus synthesize unanticipated behaviours at the global level (An, 2001; Bonabeau, 2002; Griffin, 2006; Holland, 1995; Neugebauer & Tjarders, 2004; Srbljinovic & Skunca, 2003; Troisi et al., 2005).

The rules in agent-based models are considered as "generative rules", which are not exactly the same as the rules/laws of traditional science. Generative rules determine how the agents behave in their virtual environment over time. Generative rules use feedback and learning

algorithms to permit agents adapting to their environment over time. As agents' information exchanges and interactions take place during simulation, agents and their governing rules evolve and produce complex biological processes that may have some resemblance to the real world (An, 2001; Bonabeau, 2002; Griffin, 2006; Holland, 1995; Neugebauer & Tjarders, 2004; Srbljinovic & Skunca, 2003; Troisi et al., 2005). Finding a set of generative rules that can mimic real-world behaviour is the main interest of scientists for system control and prediction.

Bonabeau (2002) explicitly describes a set of conditions when to use agent-based modeling at best.

1. When the interactions among the agents are complex, nonlinear, discontinuous, discrete and can be characterized by thresholds, if-then rules or nonlinear coupling.
2. When spatial aspect is important and the agents' positions are not fixed.
3. When the population and the agent' interactions are heterogeneous and when averages do not work.
4. When the agents exhibit complex behaviour, including memory, path-dependence, hysteresis, non-markovian behaviour, temporal correlations (learning and adaptation).
5. When stochasticity applies to the agents' behaviour.

For biological systems, these five conditions are satisfied in essence. The questions now become how to translate the entities and relationships from biological knowledge into the framework of agent-based modeling.

### C.6.8 Modeling agents and rules in biological systems

The first step in building an agent-based model is to identify the biological entities that can be modelled by agents (Griffin, 2006; Mitchell, 2003). The definition of a biological entity depends on the model itself. Not all biological entities need to be modelled by agents. Entities can be differentiated as passive or active. Passive entities have no behaviour of their own. Any

changes in their states are due to other components of the model, such as, other entities and global changes. Passive entities can be modelled by object type, instead by agent type. On the other hand, active entities have both state and a defined behaviour. Active entities act on their environment, such as objects and other agents. Active entities can be modelled by agents. Object type and agent type are specified differently in terms of state variables and state transition rules in agent-based modeling. For example, in a wounded environment, inflammatory cells (neutrophils and macrophage) and repair cells (fibroblasts) can be modelled by agents because these cells have state (rest/ activated) and they secrete biochemical substrates to affect other agents and the environment. On the other hand, the secreted biochemical substrates can be regarded as the objects in agent-based modeling.

Agents are sensitive and are able to change their environment. Rules determine agents' interactions with their environment and which part of the model compose agents' environment. An environment is defined relative to an agent and can be dynamic. The environment of an agent include: objects, other agents and state variables.

Interactions between agents and their environment can be direct or indirect. *Mediated interactions* are those indirect interactions between agents and the environment. For example, when an agent communicates with another agent, a set of conditions have to be met for the information to be transmitted. An intermediate system component, namely, "*mediator*", exists to ensure that the communication take place when the conditions are met. A biological example is that molecule A is the catalyst for a reaction between molecules B and C. In agent-based modeling, molecule A only performs the role of catalysis when molecule A encounters molecules B and C at the same time. In other times, molecule A performs other roles according

to its own sets of rules, which are not related to the reaction between B and C. For this case, A is the "mediator" for the interaction between B and C.

Agent-based modeling offers a means for coping with structural complexity of biological systems. Agent-based modeling allows different aspects of biological entities to be expressed and enables them to have multiple roles at the same time. For example, molecule A (an entity) can act as an inhibitor for molecular B-C reaction and can also act as a stimulator for molecular D-E reaction at the same time. Also, different reactions and signalling pathways can be defined as different groups or sub-models in agent-based modeling. These groups can interact with each other and molecules (entities) can participate in different groups at the same time. Therefore, agent-based modeling is suitable to model the interdependent and concurrent relationship of different sub-systems in physiology. Lastly, agent-based modeling captures the physical-spatial aspects of entity and system behaviour. Agent behaviour can be expressed in physical-spatial terms, making the simulation of spatio-temporal dynamics more tractable in agent-based models.

### C.6.9 Strengths of agent-based modeling

Agent-based modeling is a useful tool for the development of new theories and the formulation of existing theories. Agent-based modeling is regarded as more explanatory than predictive. By performing *in silico* experiments, agent-based modeling improves human intuition of the modelled phenomena. Agent-based modeling has also been suggested to have the following advantages (An, 2001; Neugebauer et al., 2001; Pogson, Smallwood, Qwarnstrom, & Holcombe, 2006; Troisi et al., 2005).

First, the programming languages are relatively intuitive and concrete in agent-based modeling. The rules identified in basic science are easier to translate into the rules in agent-based modeling than the mathematical equations in equation-based modeling. Emergent phenomena can be modelled by simple rules governing the behaviour of the agents. Second, agent-based modeling is modular in structure, which is lacking in equation-based modeling. This structural flexibility enables agent-based models to be constructed as various sub-systems to model different scales of physiologic organization. For example, a cellular model can encapsulate another lower-order molecular model. Third, agent-based modeling allows simulating heterogeneous components, whereas is difficult to implement in equation-based modeling. Fourth, agent-based modeling is basically a bottom-up approach, which permits model construction in the absence of knowledge at the global, aggregate level. Since agent-based modeling does not have top-down assumption in the programme, new information about the agents can be coded into existing models without the need to rebuild the simulation. Lastly, agent-based modeling has built-in "randomness" feature through the use of random number generators in the model. Thus, agent-based modeling can produce stochastic behaviour, which acknowledges the issue of variability in population dynamics as observed in the real world.

### C.6.10 Limitations of agent-based modeling

The first limitation is that agent-based modeling is computationally intensive. Agent-based models are built by numerous local agents and thus simulating the behaviour of all of the agents can be extremely computation intensive and time demanding. Therefore, agent-based modeling may encounter computational problem when modeling large systems. Second, agent-based modeling does not allow for formal mathematical analysis of the model, such as

bifurcation analysis and sensitivity analysis. Therefore, system dynamics and emergent properties are only discovered through simulation. Lastly, agent-based modeling usually generates a massive amount of data for each parameter configuration and so agent-based modeling is relatively difficult to validate and calibrate with real-world data.

## C.7 MODELING-SIMULATION IN DISEASE AND TREATMENT

Systems biology simulations begin with molecular and cellular levels, such as, feedback circuit for bacteria chemotaxis (Alon, Surette, Barkai, & Leibler, 1999; Mello & Tu, 2003; Yi, Huang, Simon, & Doyle, 2000), heart-beat rhythm (Gonze, Halloy, Leloup, & Goldbeter, 2003; Gunawan & Doyle, 2007; Leloup & Goldbeter, 1998, 2004; Leloup, Gonze, & Goldbeter, 1999), signal-transduction pathway (Bhalla, 2002; Bhalla & Iyengar, 1999, 2001; Cho, Johansson, & Wolkenhauer, 2005; Eungdamrong & Iyengar, 2004; Schoeberl, Eichler-Jonsson, Gilles, & Muller, 2002), cell cycles (Chen et al., 2000; Ciliberto, Novak, & Tyson, 2003; Csikasz-Nagy, Kapuy, Gyorffy, Tyson, & Novak, 2007; Lovrics et al., 2006; Novak, Csikasz-Nagy, Gyorffy, Chen, & Tyson, 1998) and red blood cells (Jamshidi, Edwards, Fahland, Church, & Palsson, 2001; Ni & Savageau, 1996a, 1996b). Currently, research begins towards large-scale simulations to integrate multiple scales of models from genetics to physiology. For example, attempts are undertaking to create a virtual heart that represents its essential features *in silico* (computer-generated) (Bassingthwaighte, 2000; Bassingthwaighte, Qian, & Li, 1999; Noble, 2002; Rudy, 2000). Most recently, simulation models for various diseases are developed for predicting disease development and designing novel therapy. This section reviews selected simulation

models for infectious disease, inflammatory responses, and wound healing to elucidate the application of system modeling in clinical research.

### C.7.1 Infectious Disease: influenza virus infection

Influenza virus infections have been simulated by equation-based modeling (Baccam, Beauchemin, Macken, Hayden, & Perelson, 2006; Bocharov & Romanyukha, 1994; Hancioglu, Swigon, & Clermont, 2007). Bocharov et al. (1994) proposed an exhaustive ordinary differential equation model of the infection to influenza virus infection, which focused on the control of the infection by the innate, cellular and humoral immune response. The model consists of 10 equations, which represent 12 different cell populations with more than 60 parameters. The simulation results expected that at the peak of infection, approximately 30% to 50% of the epithelial cells in the upper respiratory tract would be damaged due to viral infection. Moreover, sensitivity analysis of the model indicates that the parameters representing virus-epithelial cells relationship have more control on the disease progression rather than the effects of antiviral immune (innate, cellular and humoral) response.

Later, a study (Baccam et al., 2006) presented another ordinary differential equation model of influenza virus infection. Three models are developed in that study: (1) a target cell-limited model, (2) a target cell-limited model with delayed virus production and (3) a target cell limited models with delayed virus production and innate interferon response. “Target cell-limited” refers when influenza virus infection is limited by the availability of susceptible target epithelial cells more than the control of immune response. Model validation was carried out by fitting the data from an experimental report of H1N1 influenza A/Hong Kong/123/77 infection. Their simulation results expected that the inclusion of delayed virus production (i.e.,

delay from time of infection to viral production) is crucial for building viral dynamic model. Also, their simulation anticipated that a single viral-infected cell could spread the infections rapidly by infecting about 22 new cells. The infection slowed down when target epithelial cells were consumed. During the peak of infection at days 2 to 3, approximately 33% to 67% of target epithelial cells would be destroyed. This finding suggests that influenza infection can be cell-limiting. Furthermore, the model reproduced the presence of two virus titer peaks versus time as seen in some patients. This bimodal behaviour can be explained by the model with innate interferon response. The peak of interferon levels occurred after the first viral titer peak due to the delay in interferon synthesis from infected cells. As the infection diminished due to target cell limitation, the levels of infected cells and interferon dropped as well. The drop in interferon levels allowed the remaining infected cells to increase their viral production, leading to the second viral titer peak.

Most recently, another study presented a model of immune response to influenza virus infection, which aimed to explore the effect of initial viral load to the progression of disease (Hancioglu et al., 2007). Same as Bocharov et al.'s model (1994), Hancioglu's model also focuses on the control of the infection by innate, cellular and humoral immunity. This model has 10 ordinary differential equations with two parameters. Their simulation results suggest that (1) small initial viral load results asymptomatic disease; (2) intermediate viral load leads to typical disease progression and (3) high viral load results severe disease progression. Furthermore, the absence of humoral (virus-specific antibodies) immunity results a recurrence of infection followed by a transition to a chronic state with nontrivial persistent viral load.

### C.7.2 Acute Inflammation

Acute inflammatory responses are induced from various forms of stresses, such as bacterial infection, viral infection, mechanical trauma or autoimmunity (Nathan, 2002). Inflammatory process often leads to healing and recovery from infections but can also results organ dysfunction and fatality (Vodovotz, 2006). Acute inflammation has been simulated by equation-based modeling (Chow et al., 2005; Day et al., 2006; Kumar, Clermont, Vodovotz, & Chow, 2004; Reynolds et al., 2006; Vodovotz et al., 2006) and agent-based modeling (An, 2004). Two studies demonstrate the *proof of concept* of the potential use of *in silico* clinical trials for sepsis (An, 2004; Clermont et al., 2004).

Kumar et al. (Kumar et al., 2004) reported a three-variable ordinary differential equation model of acute inflammation. The model consists of a pathogen and two inflammatory mediators. In the model, an infectious agent triggered an early inflammatory mediator to kill the pathogen and later another inflammatory mediator was triggered to further exacerbate inflammation. The model was able to reproduce the health state and various negative states as observed in septic patients. Their simulation results also point out that the clinical presentations of sepsis arise from diverse underlying physiological states and thus different therapeutic approaches are required.

Later, a study (Reynolds et al., 2006) reported a four-variable model of acute inflammation. This model is expanded from Kumar et al.'s (2004) model by incorporating an anti-inflammatory mediator in the system. The anti-inflammatory mediator in the model has the functions of (1) inhibiting the build-up of pro-inflammatory mediator and the tissue damage, and (2) promoting further production of anti-inflammatory mediator. Their simulation results suggest

that the rates of dynamic anti-inflammatory response may be modified therapeutically to yield optimal healing outcome after pathogenic infection.

Furthermore, another study used the same four-variable model of acute inflammatory response (Day et al., 2006) to explore the effects of repeated administration of endotoxin (bacterial lipopolysaccharide) on the system. Their model was capable to display the clinical scenarios of endotoxin tolerance and potentiation from a single parameter set with repeated endotoxin challenges. This finding suggests that various biological responses (endotoxin tolerance and potentiation) to repeated bacterial infection are dynamic manifestations of a unified acute inflammatory response.

In the line of this research, a study reported the work of calibrating an acute inflammatory model to experimental data in mice with endotoxemia, surgical trauma or surgical trauma followed by haemorrhage (Chow et al., 2005). The model was capable to predict a dose range of endotoxin leading to death of mice, despite being calibrated on data from non-lethal inflammatory insults. The most current model was calibrated to simulate acute inflammation in mice, swine and human in order to scale up the model to the whole-patient level (Vodovotz, 2006).

Lastly, two papers demonstrate the application of inflammation modeling for *in silico* clinical trails. An equation-based model (Clermont et al., 2004) was set up to create a population of 1,000 simulated “patients” with different clinical profiles. Then, these “patients” were submitted to one of three types of anti-sepsis therapy (anti-tumour necrosis factor neutralizing antibodies). Their simulation results suggest that anti-tumour necrosis factor therapy can be effective and harmful to the patients, depending on the dose and duration of treatment as well as the patient’s clinical profiles. Another published work used agent-based model (An, 2001) to

simulate acute inflammatory responses to infectious and non-infectious tissue injury. The model focuses at cellular level with the interface between endothelial cells, blood-borne inflammatory cells and mediators. This model was able to reproduce the dynamics of inflammatory response as reported in the literature. Also, the model was able to reproduce qualitative patterns of the adverse outcomes of administrating anti-inflammatory therapy to acute inflammation as reported in the literature.

### C.7.3 Wound Healing

Two agent-based models are reported that extend the simulation window from acute inflammatory response to later healing response in the context of diabetic foot ulcers (Mi, Riviere, Clermont, Steed, & Vodovotz, 2007) and acute vocal fold phonotrauma (N. Y. Li et al., 2008).

### C.7.4 Diabetic foot ulcer

An agent-based model was developed to examine the genesis of diabetic foot ulcer (DFU) and to test *in silico* possible therapies (a neutralizing anti-tumour necrosis factor antibody, latent transforming growth factor-β1 and a mediator that increase the activation of endogenous latent transforming growth factor-β1) for DFU (Mi et al., 2007). Regarding models for skin wound healing, several papers have reported to use partial differential equations (Sherratt & Murray, 1990) or agent-based model (Walker et al., 2004) to simulate the wound closure of epithelial cell monolayer. Also, equation-based model based on mechanical theory is reported to simulate dermal wound contraction (Tranquillo & Murray, 1992, 1993). Subsequently, models

for skin wound healing with the expansion of cell types and extracellular matrix are reported as well (Dallon, Sherratt, & Maini, 2001; Olsen, Sherratt, & Maini, 1995) However, no models have been developed to investigate the relationship between inflammation and wound healing in the context of DFU.

Mi et al.'s DFU model includes cells (neutrophils, macrophages, fibroblasts), inflammatory cytokines (interleukin-1β, tumour necrosis factor-α, transforming growth factor-β1, interleukin-10), extracellular matrix (collagen), and a tissue damage function functionally analogous to alarm/danger signal. The model was calibrated using data on the skin would healing literature. The DFU model was able to reproduce the basic cellular and molecular responses in wound healing. Also, the simulation results suggested that reduced expression of transforming growth factor-β1 would lead to the aberrant skin healing of DFU. Furthermore, an interesting finding from this study is that all DFU therapies would have similar treatment effects of curing DFU as indicated in their *in silico* clinical trials.

### C.7.5 Vocal folds in phonotrauma

A patient-specific agent-based model was developed for vocal fold inflammation, with the goal of identifying individually optimized treatments (N. Y. Li et al., 2008). Specifically, an agent-based model of generic vocal fold injury was developed using data from the wound healing literature and from experimental measures of inflammatory cytokines in human laryngeal secretions (Clark, 1998; Verdolini et al., in preparation; Verdolini et al., 2003). Similar to Mi et. al.'s DFU model (Mi et al., 2007), the phonotrauma model includes cells (neutrophils, macrophages, fibroblasts), inflammatory cytokines (interleukin-1β, tumour necrosis factor-α,

transforming growth factor-β1, interleukin-10), extracellular matrix (collagen), and a tissue damage function. The model was calibrated using data from baseline human cytokine levels in laryngeal fluid, immediately after phonotrauma induction, and following a 4-hr treatment (voice rest, "tissue mobilization exercises," or spontaneous speech) (Verdolini et al., in preparation). Multiple runs of the model were carried out. The model reproduced subject-specific cytokine trajectories. Six of nine times, the model predicted empirically obtained cytokine values—not used for model calibration—at 24 hr. Predicted cytokine and tissue damage levels for spontaneous speech were significantly worse than for either voice rest or tissue mobilization exercises ($p < 0.001$), which were equivalent. These results demonstrate that the complex inflammatory/healing response is amenable to model-guided prediction and individualized therapy. Also, this translational science has potential for high impact in health care, especially as a model for future extension to other domains.

In addition to the development of a patient-specific agent-based model of vocal fold inflammation, a parallel development of an ordinary differential equation model has been pursued in the interest of cross-platform comparison of results (N. Y. K. Li et al., 2006). To test the validity in our agent-based model, "model docking" was used. "Model docking" is a well-vetted validation strategy based on a comparison of predictions of different models across an array of user input data. The finding of similar predictions in agent-based and equation-based models would increase confidence in the underlying assumptions made in the current agent-based model. In that study, the agent-based and equation-based models predicted similar cellular and molecular patterns of the inflammatory and wound healing responses under low initial damage. However, the models' results diverged in their predictions of inflammatory and wound healing responses for a high initial insult. Stated differently, with low initial damage, both

models seemed to be robust to structural differences and limitations. However, beyond a given threshold of the input damage, the models did not "dock." It is unclear whether this threshold is beyond commonly observed intensities of phonotrauma. Encouragingly, the agent-based and equation-based models both anticipated that net collagen deposition is peak on Day 9 post-injury. This predictive pattern helps to generate a hypothesis for a "wet-lab" experiment designed to identify putative mediators or enzymes correlated with the predicted collagen curves.

Currently, first-generation agent-based model of acute phonotrauma is developed to predict expected time-varying pro- and anti-inflammatory responses to physical insult to vocal fold tissue as a function of initial inflammatory profile. However, this biological model is currently limited in terms of the number of inflammatory mediators and extracellular matrix substances represented and empirical support for longer-term outcomes in humans (e.g. 3 week follow-up). As important, the model lacks the ability to receive input from physical models of phonation (e.g., finite-element models of vocal fold vibration) because data about the quantitative links between physical output (mechanical stress distributions on vocal folds) and biological consequences is lacking. Future direction is (1) to establish the quantitative links between physical output and biological consequences empirically for augmenting the current model and (2) to generate a large database of biological responses in phonotrauma for model validation purposes.

## C.8 CONCERNS OF CURRENT MATHEMATICAL MODELS IN CLINICAL RESEARCH

The aforementioned system models in clinical research are excellent examples of how much we still need to learn about health and diseases. This research also indicates that the strategy of combining *in vivo/ in vitro* and *in silico* tools will prove a useful tool in this quest. At the same time, mathematical models in clinical applications are commonly criticized that either the models perform poorly when compared to experimental data or too simplistic to capture the dynamics of interest. One of the major challenges for existing models is the lack of high quality human clinical data for model calibration and validation. The iterative process of calibration-validation is extremely important to improve model validity and predictability. Otherwise, the models become explanatory rather than predictive at best. Moreover, research on how to evaluate a model is sparse. The study of comparing agent-based and equation-based model for acute phonotrauma demonstrates that cross-platform analysis is a useful tool to evaluate the underlying assumption of models and drives hypotheses for testing empirically (N. Y. K. Li et al., 2006).

## APPENDIX D

## REFERENCES FOR APPENDIX CHAPTERS


Alon, U., Surette, M. G., Barkai, N., & Leibler, S. (1999). Robustness in bacterial chemotaxis. *Nature, 397*(6715), 168-171.

An, G. (2001). Agent-based computer simulation and sirs: building a bridge between basic science and clinical trials. *Shock, 16*(4), 266-273.

An, G. (2004). In silico experiments of existing and hypothetical cytokine-directed clinical trials using agent-based modeling. *Critical Care Medicine, 32*(10), 2050-2060.

An, G. (2005). Mathematical modeling in medicine: a means, not an end. *Critical Care Medicine, 33*(1), 253-254.

Athale, C., Mansury, Y., & Deisboeck, T. S. (2005). Simulating the impact of a molecular 'decision-process' on cellular phenotype and multicellular patterns in brain tumors. *J Theor Biol, 233*(4), 469-481.

Athale, C. A., & Deisboeck, T. S. (2006). The effects of EGF-receptor density on multiscale tumor growth patterns. *J Theor Biol, 238*(4), 771-779.

Baccam, P., Beauchemin, C., Macken, C. A., Hayden, F. G., & Perelson, A. S. (2006). Kinetics of influenza A virus infection in humans. *J Virol, 80*(15), 7590-7599.

Baldi, P., & Brunak, S. (Eds.). (2001). *Bioinformatics: The Machine LEarning Approach* (2nd ed.). Cambridge, M.A.: MIT Press.

Barbosa, L. H., Ramos, H. V., Neves, L. R., Biase, N. G., Oshima, C., Pedroso, J. E., et al. (2008). Detection of hyaluronic acid receptor in human vocal folds by immunohistochemistry. *Braz J Otorhinolaryngol, 74*(2), 201-206.

Bassingthwaighte, J. B. (2000). Strategies for the physiome project. *Ann Biomed Eng, 28*(8), 1043-1058.

Bassingthwaighte, J. B., Qian, H., & Li, Z. (1999). The Cardiome Project. An integrated view of cardiac metabolism and regional mechanical function. *Adv Exp Med Biol, 471*, 541-553.

Beckman, J. S., Beckman, T. W., Chen, J., Marshall, P. A., & Freeman, B. A. (1990). Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. *Proc Natl Acad Sci U S A, 87*(4), 1620-1624.

Behrman, A., & Sulica, L. (2003). Voice rest after microlaryngoscopy: current opinion and practice. *Laryngoscope, 113*(12), 2182-2186.

Benninger, M. S., Alessi, D., Archer, S., Bastian, R., Ford, C., Koufman, J., et al. (1996). Vocal fold scarring: Current concepts and management. *Otolaryngology-Head and Neck Surgery, 115*(5), 474-482.

Bhalla, U. S. (2002). Mechanisms for temporal tuning and filtering by postsynaptic signaling pathways. *Biophys J, 83*(2), 740-752.

Bhalla, U. S., & Iyengar, R. (1999). Emergent properties of networks of biological signaling pathways. *Science, 283*(5400), 381-387.

Bhalla, U. S., & Iyengar, R. (2001). Functional modules in biological signalling networks. *Novartis Found Symp, 239*, 4-13; discussion 13-15, 45-51.

Bocharov, G. A., & Romanyukha, A. A. (1994). Mathematical model of antiviral immune response. III. Influenza A virus infection. *J Theor Biol, 167*(4), 323-360.

Bonabeau, E. (2002). Agent-based modeling: methods and techniques for simulating human systems. *Proc Natl Acad Sci U S A, 99 Suppl 3*, 7280-7287.

Borisuk, M. T., & Tyson, J. J. (1998). Bifurcation analysis of a model of mitotic control in frog eggs. *J Theor Biol, 195*(1), 69-85.

Borshchev, A., & Filippov, A. (2004). *From System Dynamics and Discrete Event to Practical Agent Based Modeling: Reasons, Techniques, Tools*. St. Petersburgh: St. Petersburgh Technical University.

Boseley, M. E., & Hartnick, C. J. (2006). Development of the human true vocal fold: depth of cell layers and quantifying cell types within the lamina propria. *Ann Otol Rhinol Laryngol, 115*(10), 784-788.

Bradbury, R. (2002). Futures, predictions and other foolishness. . In M. A. Janssen (Ed.), *Complexity and ecosystem management: The theory and practice of multi-agent systems*. U.K., Cheltenham: Edward Elgar.

Branski, R. C., Perera, P., Verdolini, K., Rosen, C. A., Hebda, P. A., & Agarwal, S. (2006). Dynamic Biomechanical Strain Inhibits IL-1beta-induced Inflammation in Vocal Fold Fibroblasts. *J Voice*.

Branski, R. C., Rosen, C. A., Verdolini, K., & Hebda, P. A. (2005a). Acute vocal fold wound healing in a rabbit model. *Ann Otol Rhinol Laryngol, 114*(1 Pt 1), 19-24.

Branski, R. C., Rosen, C. A., Verdolini, K., & Hebda, P. A. (2005b). Biochemical markers associated with acute vocal fold wound healing: a rabbit model. *J Voice, 19*(2), 283-289.

Branski, R. C., Verdolini, K., Rosen, C. A., & Hebda, P. A. (2004). Markers of wound healing in vocal fold secretions from patients with laryngeal pathology. *Annals of Otology, Rhinology, and Laryngology, 113*(1), 23-29.

Butcher, E. C. (2007). Can cell systems biology rescue drug discovery? *Ernst Schering Res Found Workshop*(61), 153-172.

Butcher, E. C., Berg, E. L., & Kunkel, E. J. (2004). Systems biology in drug discovery. *Nat Biotechnol, 22*(10), 1253-1259.

Butler, J. E., Hammond, T. H., & Gray, S. D. (2001). Gender-related differences of hyaluronic acid distribution in the human vocal fold. *Laryngoscope, 111*(5), 907-911.

Carriero, E., Galli, J., Fadda, G., Di Girolamo, S., Ottaviani, F., & Paludetti, G. (2000). Preliminary experiences with contact endoscopy of the larynx. *Eur Arch Otorhinolaryngol, 257*(2), 68-71.

Catten, M., Gray, S. D., Hammond, T. H., Zhou, R., & Hammond, E. H. (1998). Analysis of cellular location and concentration in vocal fold lamina propria. *Otolaryngology Head and Neck Surgery, 118*(5), 663-667.

Chan, R. W., Gray, S. D., & Titze, I. R. (2001). The importance of hyaluronic acid in vocal fold biomechanics. *Otolaryngol Head Neck Surg, 124*(6), 607-614.

Chan, R. W., & Titze, I. (1999). Hyaluronic acid (with fibronectin) as a bioimplant for the vocal fold mucosa. *Laryngoscope, 109*, 1142-1149.

Chaponnier, C., Desmouliere, A., & Gabbiani, G. (2006). *Tissue Repair, Contration and the Myofibroblast*. New York: Spring Science + Business Media.

Chen, K. C., Csikasz-Nagy, A., Gyorffy, B., Val, J., Novak, B., & Tyson, J. J. (2000). Kinetic analysis of a molecular model of the budding yeast cell cycle. *Mol Biol Cell, 11*(1), 369-391.

Cho, K. H., Johansson, K. H., & Wolkenhauer, O. (2005). A hybrid systems framework for cellular processes. *Biosystems, 80*(3), 273-282.

Chow, C. C., Clermont, G., Kumar, R., Lagoa, C., Tawadrous, Z., Gallo, D., et al. (2005). The acute inflammatory response in diverse shock states. *Shock, 24*(1), 74-84.

Ciliberto, A., Novak, B., & Tyson, J. J. (2003). Mathematical model of the morphogenesis checkpoint in budding yeast. *J Cell Biol, 163*(6), 1243-1254.

Cilliers, P. (2005). *Complexity and Postmodernism: Understanding Complex Systems*. London: Routledge.

Clark, R. A. F. (Ed.). (1998). *The Molecular and Cellular Biology of Wound Repair*. New York: Plenum Press.

Clermont, G., Bartels, J., Kumar, R., Constantine, G., Vodovotz, Y., & Chow, C. (2004). In silico design of clinical trials: a method coming of age. *Crit Care Med, 32*(10), 2061-2070.

Courey, M. S., Shohet, J. A., Scott, M. A., & Ossoff, R. H. (1996). Immunohistochemical characterization of benign laryngeal lesions. *Ann Otol Rhinol Laryngol, 195*(7), 525-531.

Covert, M. W., Knight, E. M., Reed, J. L., Herrgard, M. J., & Palsson, B. O. (2004). Integrating high-throughput and computational data elucidates bacterial networks. *Nature, 429*(6987), 92-96.

Csikasz-Nagy, A., Kapuy, O., Gyorffy, B., Tyson, J. J., & Novak, B. (2007). Modeling the septation initiation network (SIN) in fission yeast cells. *Curr Genet, 51*(4), 245-255.

Czerwonka, L., Jiang, J. J., & Tao, C. (2008). Vocal nodules and edema may be due to vibration-induced rises in capillary pressure. *Laryngoscope, 118*(4), 748-752.

d'Inverno, M., & Prophet, J. (2005). Multidisciplinary investigation into adult stem cell behaviour. Unpublished Lect. Notes Comput. Sc.

Dallon, J. C., Sherratt, J. A., & Maini, P. K. (2001). Modeling the effects of transforming growth factor-beta on extracellular matrix alignment in dermal wound repair. *Wound Repair Regen, 9*(4), 278-286.

Day, J., Rubin, J., Vodovotz, Y., Chow, C. C., Reynolds, A., & Clermont, G. (2006). A reduced mathematical model of the acute inflammatory response II. Capturing scenarios of repeated endotoxin administration. *J Theor Biol*.

De Biase, N. G., & Pontes, P. A. (2008). Blood vessels of vocal folds: a videolaryngoscopic study. *Arch Otolaryngol Head Neck Surg, 134*(7), 720-724.

Dikkers, F. G. (1994). *Benign Lesions of the Vocal Folds: Clinical and Histopathological Aspects*. Groningen: Rijksuniversitsti Groningen.

Dikkers, F. G., Hulstaert, C. E., Oosterbaan, J. A., & Cervera-paz, F. J. (1993). Ultrastructural changes of the basement membrane zone in benign lesions of the vocal folds. *Acta Otolaryngol (Stockh), 113*, 98-101.

Dikkers, F. G., & Nikkels, P. G. (1995). Benign lesions of the vocal folds: histopathology and phonotrauma. *Ann Otol Rhinol Laryngol, 104*, 698-703.

Dikkers, F. G., & Nikkels, P. G. (1999). Lamina propria of the mucosa of benign lesions of the vocal folds. *Laryngoscope, 109*(10), 1684-1689.

Duflo, S., Thibeault, S. L., Li, W., Shu, X. Z., & Prestwich, G. D. (2006). Vocal Fold Tissue Repair in Vivo Using a Synthetic Extracellular Matrix. *Tissue Eng*.

Edmonds, B. (2005). Simulation and complexity - how they can relate. In V. Feldmann & K. Muhlfeld (Eds.), *Virtual Worlds of Precisoin - Computer-Based Simulations in the Sciences and Social Sciences* (pp. 5-43). Munster: LIT Verlag.

Edwards, J. S., Ibarra, R. U., & Palsson, B. O. (2001). In silico predictions of Escherichia coli metabolic capabilities are consistent with experimental data. *Nat Biotechnol, 19*(2), 125-130.

Eungdamrong, N. J., & Iyengar, R. (2004). Modeling cell signaling networks. *Biol Cell, 96*(5), 355-362.

Fuja, T. J., Probst-Fuja, M. N., & Titze, I. R. (2005). Changes in expression of extracellular matrix genes, fibrogenic factors, and actin cytoskeletal organization in retinol treated and untreated vocal fold stellate cells. *Matrix Biol*.

Gammack, D., Ganguli, S., Marino, S., Segovia-Juarez, J., & Kirschner, D. E. (2005). Understanding the immune response in tuberculosis using different mathematical models and biological scales. *Multiscale Model Sim., 3*(2), 312-345.

Gilbert, N., & Bankes, S. (2002). Platforms and methods for agent-based modeling. *Proc Natl Acad Sci U S A, 99 Suppl 3*, 7197-7198.

Goldberger, A. L. (2006). Complex systems. *Proc Am Thorac Soc, 3*, 467-472.

Goldenfeld, N., & Kadanoff, L. P. (1999). Simple lessons from complexity. *Science, 284*(5411), 87-89.

Gonze, D., Halloy, J., Leloup, J. C., & Goldbeter, A. (2003). Stochastic models for circadian rhythms: effect of molecular noise on periodic and chaotic behaviour. *C R Biol, 326*(2), 189-203.

Gray, S. D. (1989). *Basement membrane zone injury in vocal nodules*. Sand Diego: Singular Publishing Group.

Gray, S. D. (1991). *Basement membrane zone injury in vocal nodules*. San Diego: Singular Press.

Gray, S. D. (2000). Cellular Physiology of the Vocal Folds. In C. A. Rosen & T. Murry (Eds.), *The Otolaryngologic Clinics of North America*. Philadelphia: W.B. Saunders Company.

Gray, S. D., Chan, K. J., & Turner, B. (2000). Dissection plane of the human vocal fold lamina propria and elastin fibre concentration. *Acta Otolaryngol, 120*(1), 87-91.

Gray, S. D., Hammond, E. H., & Hanson, D. (1995). Benign pathologic response of the larynx. *Ann Otol Rhinol Laryngol, 104*, 8-13.

Gray, S. D., Hirano, M., & Sato, K. (1993). Molecular and Cellular Structure of Vocal Fold Tissue. In I. R. Titze (Ed.), *Vocal Fold Physiology*. San Diego: Singular Publishing Group, Inc.

Gray, S. D., Pignatari, S. S., & Harding, P. (1994). Morphologic ultrastructure of anchoring fibers in normal vocal fold basement membrane zone. *Journal of Voice, 8*(1), 48-52.

Gray, S. D., & Titze, I. R. (1988). Histologic investigation of hyperphonated canine vocal cords. *Ann Otol Rhinol Laryngol, 97*(4), 381-388.

Gray, S. D., Titze, I. R., Alipour, F., & Hammond, T. H. (2000). Biomechanical and histologic observations of vocal fold fibrous proteins. *Ann Otol Rhinol Laryngol, 109*, 77-85.

Gray, S. D., Titze, I. R., Chan, R. W., & Hammond, T. H. (1999). Vocal fold proteoglycans and their influence on biomechanics. *Laryngoscope, 109*, 845-854.

Gray, S. D., Titze, I. R., & Lusk, R. P. (1987). Electron microscopy of hyperphonated canine vocal cords. *Journal of Voice, 1*(1), 109-115.

Griffin, W. A. (2006). Agent-based modeling for the theoretical biologist. *Journal of Biological Theory, 1*(4), 404 - 409.

Gunawan, R., & Doyle, F. J., 3rd. (2007). Phase sensitivity analysis of circadian rhythm entrainment. *J Biol Rhythms, 22*(2), 180-194.

Gunter, H. E. (2003). A mechanical model of vocal-fold collision with high spatial and temporal resolution. *J. Acoust. Soc. Am., 113*(2), 994-1000.

Gunter, H. E. (2004). Modeling mechanical stresses as a factor in the etiology of benign vocal fold lesions. *Journal of Biomechanics, 37*, 1119-1124.

Hahn, M. S., Kobler, J. B., Zeitels, S. M., & Langer, R. (2005). Midmembranous vocal fold lamina propria proteoglycans across selected species. *Ann Otol Rhinol Laryngol, 114*(6), 451-462.

Hahn, M. S., Kobler, J. B., Zeitels, S. M., & Langer, R. (2006). Quantitative and comparative studies of the vocal fold extracellular matrix II: collagen. *Ann Otol Rhinol Laryngol, 115*(3), 225-232.

Hallen, L., Dahlqvist, A., & Laurent, C. (1998). Dextranomeres in hyaluronan (DiHA): a promising substance in treating vocal cord insufficiency. *Laryngoscope, 108*(3), 393-397.

Hammond, T. H., Gray, S. D., Butler, J., Zhou, R., & Hammond, E. (1998). A study of age and gender related elastin distribution changes in human vocal folds. *Otolaryngology-Head and Neck Surgery, 119*, 314-322.

Hammond, T. H., Zhou, R., Hammond, E. H., Pawlak, A., & Gray, S. D. (1997). The intermediate layer: A morphologic study of elastin and hyaluronic acid constituents of normal human vocal folds. *Journal of Voice, 11*(1), 59-66.

Hancioglu, B., Swigon, D., & Clermont, G. (2007). A dynamical model of human immune response to influenza A virus infection. *J Theor Biol, 246*(1), 70-86.

Hardingham, T. E., & Fosang, A. J. (1992). Proteoglycans: many forms and many functions. *FASEB J, 6*, 861-870.

Hartnick, C. J., Rehbar, R., & Prasad, V. (2005). Development and maturation of the pediatric human vocal fold lamina propria. *Laryngoscope, 115*(1), 4-15.

Hertegard, S., Cedervall, J., Svensson, B., Forsberg, K., Maurer, F. H. J., Vidovska, D., et al. (2006). Viscoelastic and histologic properties in scarred rabbit vocal folds after mesenchymal stem cell injection *The Laryngoscope, 116*(1248-1254).

Hirano, M. (1977). Structure and vibratory behavior of the vocal folds. In M. Sawashima & S. C. Fankin (Eds.), *Dynamic Aspects of Speech Production* (pp. 13-30). Tokyo: University of Tokyo Press.

Hirano, M. (1981). *Structure of the vocal fold in normal and disease states anatomical and physical studies.* Paper presented at the Assessement of Vocal Pathology, Rockville, MD.

Hirano, M., & Kakita, Y. (1985). Cover-body theory of vocal fold vibration. In Daniloff (Ed.), *Speech Science*. San Diego: College Hill Press.

Hirano, M., Kurita, S., & Nakashima, T. (1981). The structure of the vocal folds. In K. N. Stevens & M. Hirano (Eds.), *Vocal Fold Physiology*. Tokyo: University of Tokyo Press.

Hirano, M., & Sato, K. (1993). *Histological color atlas of the human larynx*. San Diego: Singular Publishing Group.
Hirano, M., Sato, K., & Nakashima, T. (1999a). Fibroblasts in human vocal fold mucosa. *Acta Otolaryngol, 119*, 271-276.
Hirano, M., Sato, K., & Nakashima, T. (1999b). Fibroblasts in the human vocal fold mucosa. *Acta Otolaryngol, 119*, 271-276.
Hirano, M., Sato, K., & Nakashima, T. (2000). Fibroblasts in geriatric vocal fold mucosa. *Acta Otolaryngol, 120*, 336-340.
Hirano, S., Bless, D. M., Rousseau, B., Welham, N., Scheidt, T., & Ford, C. N. (2003). Fibronectin and adhesion molecules on canine scarred vocal folds. *Laryngoscope, 113*(6), 966-972.
Hirano, S., Minamiguchi, S., Yamashita, M., Ohno, T., Kanemaru, S. I., & Kitamura, M. (2008). Histologic Characterization of Human Scarred Vocal Folds. *J Voice*.
Hirschi, S. D., Gray, S. D., & Thibeault, S. L. (2002). Fibronectin:an interesting vocal fold protein. *J Voice, 16*(3), 310-316.
Holland, J. H. (1992). Complex adaptive systems. *Daedalus, 121*(1), 17-30.
Holland, J. H. (Ed.). (1995). *Hidden Order: How Adaptation Builds Complexity*. Reading, M. A.: Addison-Wesley.
Hood, L. (2003). Systems biology: integrating technology, biology, and computation. *Mech Ageing Dev, 124*(1), 9-16.
Hood, L., Heath, J. R., Phelps, M. E., & Lin, B. (2004). Systems biology and new technologies enable predictive and preventative medicine. *Science, 306*(5696), 640-643.
Ideker, T., Galitski, T., & Hood, L. (2001). A new approach to decoding life: systems biology. *Annu Rev Genomics Hum Genet, 2*, 343-372.
Jamshidi, N., Edwards, J. S., Fahland, T., Church, G. M., & Palsson, B. O. (2001). Dynamic simulation of the human red blood cell metabolic network. *Bioinformatics, 17*(3), 286-287.
Jovanovic, M. B., Milutinovic, Z., Perovic, J., Grubor, A., Milenkovic, S., & Malobabic, S. (2008). Vocal Fold Mucosa "Blue Lines" Observed on Contact Telescopy at Reinke's Edema. *J Voice*.
Jovanovic, M. B., Mulutinovic, Z., Perovic, J., Grubor, A., Milenkovic, S., & Malobabic, S. (2007). Contact telescopy reveals blood vessel alterations of vocal fold mucosa in Reinke's edema. *J Voice, 21*(3), 355-360.
Kang, B. H., Hsiung, M. W., & Wang, H. W. (2005). Possible involvement of nitric oxide and peroxynitrite in the pathogenesis of human vocal polyps and nodules. *Eur Arch Otorhinolaryngol, 262*(1), 72-76.
Karahan, N., Baspinar, S., Yariktas, M., & Kapucuoglu, N. (2009). Matrix Metalloproteinases (MMP-2 and MMP-9) and cyclooxygenase-2 (COX-2) expressions in vocal fold polyps. *J Voice, 23*(1), 29-33.
Kielty, C. M., Sherratt, M. J., & Shuttleworth, C. A. (2002). Elastic fibres. *J Cell Sci, 115*(Pt 14), 2817-2828.
Kitano, H. (2002a). Computational systems biology. *Nature, 420*(6912), 206-210.
Kitano, H. (2002b). Systems biology: a brief overview. *Science, 295*(5560), 1662-1664.
Kotby, M. N., Nassar, A. M., Seif, E. I., Helal, E. H., & Saleh, M. M. (1988). Ultrastructural features of vocal fold nodules and polyps. *Acta Otolaryngol, 105*, 477-482.

Kumar, R., Clermont, G., Vodovotz, Y., & Chow, C. C. (2004). The dynamics of acute inflammation. *J Theor Biol, 230*(2), 145-155.

Latterich, M. (2005). Molecular systems biology at the crossroads: to know less about more, or to know more about less? *Proteome Sci, 3*, 8.

Laurent, T. C., & Fraser, J. R. (1992). Hyaluronan. *Faseb J, 6*(7), 2397-2404.

Lebl, M. D., Martins, J. R., Nader, H. B., Simoes Mde, J., & De Biase, N. (2007). Concentration and distribution of hyaluronic acid in human vocal folds. *Laryngoscope, 117*(4), 595-599.

Leloup, J. C., & Goldbeter, A. (1998). A model for circadian rhythms in Drosophila incorporating the formation of a complex between the PER and TIM proteins. *J Biol Rhythms, 13*(1), 70-87.

Leloup, J. C., & Goldbeter, A. (2004). Modeling the mammalian circadian clock: sensitivity analysis and multiplicity of oscillatory mechanisms. *J Theor Biol, 230*(4), 541-562.

Leloup, J. C., Gonze, D., & Goldbeter, A. (1999). Limit cycle models for circadian rhythms based on transcriptional regulation in Drosophila and Neurospora. *J Biol Rhythms, 14*(6), 433-448.

Li, N. Y., Verdolini, K., Clermont, G., Mi, Q., Rubinstein, E. N., Hebda, P. A., et al. (2008). A patient-specific in silico model of inflammation and healing tested in acute vocal fold injury. *PLoS ONE, 3*(7), e2789.

Li, N. Y. K., Verdolini, K., Clermont, G., Mi, Q., Hebda, P. A., & Vodovotz, Y. (2006). *Simulating acute phonotrauma: a comparison of agent-based and equation-based models.* Paper presented at the 5th International Conference on Voice Physiology and Biomechanics, Tokyo, Japan.

Lim, X., Tateya, I., Tateya, T., Munoz-Del-Rio, A., & Bless, D. M. (2006). Immediate inflammatory response and scar formation in wounded vocal folds. *Ann Otol Rhinol Laryngol, 115*(12), 921-929.

Loire, R., Bouchayer, M., Cornut, G., & Bastian, R. W. (1988). Pathology of benign vocal fold lesions. *Ear Nose Throat J, 67*(5), 357-358, 360-352.

Lovrics, A., Csikasz-Nagy, A., Zsely, I. G., Zador, J., Turanyi, T., & Novak, B. (2006). Time scale and dimension analysis of a budding yeast cell cycle model. *BMC Bioinformatics, 7*, 494.

MacFarlane, A. G. J. (1986 Sept., 8). *Discussion on the Second Day's Papers.* Paper presented at the Royal Society of London. Series A. Mathematical and PHysical Sciences.

Madruga de Melo, E. C., Lemos, M., Aragao Ximenes Filho, J., Sennes, L. U., Nascimento Saldiva, P. H., & Tsuji, D. H. (2003). Distribution of collagen in the lamina propria of the human vocal fold. *Laryngoscope, 113*(12), 2187-2191.

Mansury, Y., & Deisboeck, T. S. (2004a). Simulating "structure-function" patterns of malignant brain tumors. *Physica, A331*(1-2), 219-232.

Mansury, Y., & Deisboeck, T. S. (2004b). Simulating the time series of a selected gene expression profile in an agent-based tumor model. *Physica, D 196*(1-2), 193-204.

Mansury, Y., Kimura, M., Lobo, J., & Deisboeck, T. S. (2002). Emerging patterns in tumor systems: simulating the dynamics of multicellular clusters with an agent-based spatial agglomeration model. *J Theor Biol, 219*(3), 343-370.

Melby, N. J. (2004). *Comparative relative strength artificial immune systems: System wellness.* Paper presented at the IEEE Southeastcon.

Mello, B. A., & Tu, Y. (2003). Perfect and near-perfect adaptation in a model of bacterial chemotaxis. *Biophys J, 84*(5), 2943-2956.

Mi, Q., Riviere, B., Clermont, G., Steed, D. L., & Vodovotz, Y. (2007). Agent-based modeling of inflammation and wound healing: insights into diabetic foot ulcer pathology and the role of transforming growth factor-beta 1. *Wound Repair and Regeneration, 15*(5), 671-682.

Mihashi, S., Okada, M., JKurita, S., Nagata, K., Oda, M., Hirano, M., et al. (1981). Vascular network of the vocal fold. In K. N. Stevens & M. Hirano (Eds.), *Vocal Fold Physiology*. Tokyo: University of Tokyo Press.

Milutinovic, Z., & Bojic, P. (1996). Functional trauma of the vocal folds: classification and management strategies. *Folia Phoniatr Logop, 48*(2), 78-85.

Mitchell, S. D. (Ed.). (2003). *Biological Complexity and Integrative Pluralism*. Cambridge: Cambridge University Press.

Muller, G., Grebaut, P., & Gouteux, J. P. (2004). An agent-based model of sleeping sickness: simulation trials of a forest focus in southern Cameroon. *C R Biol, 327*(1), 1-11.

Muller, J., Kretzschmar, M., & Dietz, K. (2000). Contact tracing in stochastic and deterministic epidemic models. *Math Biosci, 164*(1), 39-64.

Nakai, Y., Masutani, H., Moriguchi, M., Matsunaga, K., & Sugita, M. (1991). Microvascular structure of the larynx. A scanning electron microscopic study of microcorrosion casts. *Acta Otolaryngol Suppl, 486*, 254-263.

Nathan, C. (2002). Points of control in inflammation. *Nature, 420*(6917), 846-852.

Neugebauer, E. A., & Tjarders, T. (2004). New approaches to shock and trauma research: learning from multidisciplinary exchange. *Journal of Trauma Injury, Infection and Critical Care, 56*, 1156-1165.

Neugebauer, E. A., Willy, C., & Sauerland, S. (2001). Complexity and non-linearity in shock research: reductionism or synthesis? *Shock, 16*(4), 252-258.

Neves, B. M. J., Neto, J. G., & Pontes, P. (2004). Histopathological and immunohistochemical differentiation of epithelial alterations in vocal nodule comparing to polyps and to laryngeal edema. *Brazillian Journal of Otolaryngology, 70*(4), 439-448.

Ni, T. C., & Savageau, M. A. (1996a). Application of biochemical systems theory to metabolism in human red blood cells. Signal propagation and accuracy of representation. *J Biol Chem, 271*(14), 7927-7941.

Ni, T. C., & Savageau, M. A. (1996b). Model assessment and refinement using strategies from biochemical systems theory: application to metabolism in human red blood cells. *J Theor Biol, 179*(4), 329-368.

Noble, D. (2002). Modeling the heart--from genes to cells to the whole organ. *Science, 295*(5560), 1678-1682.

Novak, B., Csikasz-Nagy, A., Gyorffy, B., Chen, K., & Tyson, J. J. (1998). Mathematical model of the fission yeast cell cycle with checkpoint controls at the G1/S, G2/M and metaphase/anaphase transitions. *Biophys Chem, 72*(1-2), 185-200.

Olsen, L., Sherratt, J. A., & Maini, P. K. (1995). A mechanochemical model for adult dermal wound contraction and the permanence of the contracted tissue displacement profile. *J Theor Biol, 177*(2), 113-128.

Pawlak, A. S., Hammond, T., Hammond, E., & Gray, S. D. (1996). Immunocytochemical study of proteoglycans in vocal folds. *Ann Otol Rhinol Laryngol, 105*, 6-11.

Peirce, S. M., Van Gieson, E. J., & Skalak, T. C. (2004). Multicellular simulation predicts microvascular patterning and in silico tissue assembly. *Faseb J, 18*(6), 731-733.

Pogson, M., Smallwood, R., Qwarnstrom, E., & Holcombe, M. (2006). Formal agent-based modeling of intracellular chemical interactions. *Biosystems, 85*(1), 37-45.

Rand, W., Brown, D. G., Page, S. E., Riolo, R., Fernandez, L. E., & Zellner, M. (2003). *Statistical validation of spatial patterns in agent-based models.* Paper presented at the Agent-based Simulation, Montpellier, France.

Reynolds, A., Rubin, J., Clermont, G., Day, J., Vodovotz, Y., & Bard Ermentrout, G. (2006). A reduced mathematical model of the acute inflammatory response: I. Derivation of model and analysis of anti-inflammation. *J Theor Biol*.

Robbins, M. J., & Garrett, S. M. (2005). *Evaluating theories of immunological memory using large-scale simulations.* Paper presented at the ICARIS LNCS.

Rosen, C. A., & Murry, T. (2000). Nomenclature of Voice Disorders and Vocal Pathology. In C. A. Rosen & T. Murry (Eds.), *The Otolaryngologic Clinics of North America*. Philadelphia: W.B. Saunders Company.

Rousseau, B., Ge, P., French, L. C., Zealear, D. L., Thibeault, S. L., & Ossoff, R. H. (2008). Experimentally induced phonation increases matrix metalloproteinase-1 gene expression in normal rabbit vocal fold. *Otolaryngol Head Neck Surg, 138*(1), 62-68.

Rousseau, B., Ge, P. J., Ohno, T., French, L. C., & Thibeault, S. L. (2008). Extracellular matrix gene expression after vocal fold injury in a rabbit model. *Ann Otol Rhinol Laryngol, 117*(8), 598-603.

Rousseau, B., Hirano, S., Chan, R. W., Welham, N. V., Thibeault, S. L., Ford, C. N., et al. (2004). Characterization of chronic vocal fold scarring in a rabbit model. *J Voice, 18*(1), 116-124.

Rousseau, B., Hirano, S., Scheidt, T. D., Welham, N. V., Thibeault, S. L., Chan, R. W., et al. (2003). Characterization of vocal fold scarring in a canine model. *Laryngoscope, 113*, 620-627.

Rousseau, B., Sohn, J., Montequin, D. W., Tateya, I., & Bless, D. M. (2004). Functional outcomes of reduced hyaluronan in acute vocal fold scar. *Ann Otol Rhinol Laryngol, 113*(10), 767-776.

Rudy, Y. (2000). From genome to physiome: integrative models of cardiac excitation. *Ann Biomed Eng, 28*(8), 945-950.

Sakae, F. A., Imamura, R., Sennes, L. U., Mauad, T., Saldiva, P. H., & Tsuji, D. H. (2008). Disarrangement of collagen fibers in Reinke's edema. *Laryngoscope, 118*(8), 1500-1503.

Salge, A. K., Peres, L. C., Reis, M. A., Teixeira Vde, P., & Castro, E. C. (2006). Histopathologic changes in human true vocal folds: a postmortem study. *Ann Diagn Pathol, 10*(5), 274-278.

Sandulache, V. C., Chafin, J. B., Li-Korotky, H. S., Otteson, T. D., Dohar, J. E., & Hebda, P. A. (2007). Elucidating the role of interleukin 1beta and prostaglandin E2 in upper airway mucosal wound healing. *Arch Otolaryngol Head Neck Surg, 133*(4), 365-374.

Sato, K. (1998). Reticular fibers in the vocal fold mucosa. *Ann Otol Rhinol Laryngol, 107*(12), 1023-1028.

Sato, K., & Hirano, M. (1995). Histologic investigation of the macula flava of the human vocal fold. *Ann Otol Rhinol Laryngol, 104*(2), 138-143.

Sato, K., Hirano, M., & Nakashima, T. (1999). Electron microscopic and immunohistochemical investigation of Reinke's edema. *Ann Otol Rhinol Laryngol, 108*(11 Pt 1), 1068-1072.

Sato, K., Hirano, M., & Nakashima, T. (2001). Stellate cells in the human vocal fold. *Ann Otol Rhinol Laryngol, 110*(4), 319-325.

Sato, K., Hirano, M., & Nakashima, T. (2003a). 3D structure of the macula flava in the human vocal fold. *Acta Otolaryngol, 123*, 269-273.

Sato, K., Hirano, M., & Nakashima, T. (2003b). Vitamin A-storing stellate cells in the human vocal fold. *Acta Otolaryngol, 123*, 106-110.

Schoeberl, B., Eichler-Jonsson, C., Gilles, E. D., & Muller, G. (2002). Computational modeling of the dynamics of the MAP kinase cascade activated by surface and internalized EGF receptors. *Nat Biotechnol, 20*(4), 370-375.

Scholl, H. (2001). *Agent Based and System Dynamics Modeling: A Call for Cross Study and Joint Research* Paper presented at the Proceedings of the 34th Annual Hawaii International Conference on System Sciences.

Sherratt, J. A., & Murray, J. D. (1990). Models of epidermal wound healing. *Proc Biol Sci, 241*(1300), 29-36.

Smallwood, R. H., Holcombe, W. M. L., & Walker, D. C. (2004). Development and validation of computational models of cellular interaction. *Journal of Molecular Histology, 35*, 659-665.

Sone, M., Sato, E., Hayashi, H., Fujimoto, Y., & Nakashima, T. (2006). Vascular evaluation in laryngeal diseases: comparison between contact endoscopy and laser Doppler flowmetry. *Arch Otolaryngol Head Neck Surg, 132*(12), 1371-1374.

Srbljinovic, A., & Skunca, O. (2003). An introduction to agent-based modeling and simulation of social processes. *Interdisciplinary Description of Complex Systems, 1*(1), 1-8.

Stec, I., Hertegard, S., & Juto, J. E. (2007). Laser Doppler measurements of the vocal fold blood micro-circulation. *J Voice, 21*(3), 345-354.

Svec, J. G., & Sram, F. (2001). *Voilet-reddening of the vocal folds and the vocal fatigue*. Paper presented at the 25th World Congress of the International Association of Logopedics and Phoniatrics.

Tateya, I., Tateya, T., Lim, X., Sohn, J. H., & Bless, D. M. (2006). Cell production in injured vocal folds: a rat study. *Ann Otol Rhinol Laryngol, 115*(2), 135-143.

Tateya, T., Tateya, I., & Bless, D. M. (2006). Collagen subtypes in human vocal folds. *Ann Otol Rhinol Laryngol, 115*(6), 469-476.

Tateya, T., Tateya, I., & Bless, D. M. (2007). Immuno-scanning electron microscopy of collagen types I and III in human vocal fold lamina propria. *Ann Otol Rhinol Laryngol, 116*(2), 156-159.

Tateya, T., Tateya, I., Sohn, J. H., & Bless, D. M. (2005). Histologic characterization of rat vocal fold scarring. *Ann Otol Rhinol Laryngol, 114*(3), 183-191.

Tateya, T., Tateya, I., Sohn, J. H., & Bless, D. M. (2006). Histological study of acute vocal fold injury in a rat model. *Ann Otol Rhinol Laryngol, 115*(4), 285-292.

Tay, J. C., & Jhavar, A. (2005). *A complex adaptive framework for immune system simulation.* Paper presented at the ACM Symposium of Applied Computing.

Thibeault, S. L. (2005). Advances in our understanding of the Reinke space. *Curr Opin Otolaryngol Head Neck Surg, 13*, 148-151.

Thibeault, S. L., Bless, D. M., & Gray, S. D. (2003). Interstitial protein alterations in rabbit vocal fold with scar. *J Voice, 17*(3), 377-383.

Thibeault, S. L., Gray, S. D., Bless, D. M., Chan, R. W., & Ford, C. N. (2002). Histologic and Rheologic characterization of vocal fold scarring. *Journal of Voice, 16*(1), 96-104.

Thibeault, S. L., Rousseau, B., Welham, N. V., Hirano, S., & Bless, D. M. (2004). Hyaluronan levels in acute vocal fold scar. *Laryngoscope, 114*(4), 760-764.

Tillmann, B., Rudert, H., Schunke, M., & Werner, J. A. (1995). Morphological studies on the pathogenesis of Reinke's edema. *Eur Arch Otorhinolaryngol, 252*(8), 469-474.

Titze, I. R. (1989). On the relation between subglottal pressure and fundamental frequency in phonation. *J Acoust Soc Am, 85*, 901-906.

Titze, I. R. (1994). Mechanical stress in phonation. *J Voice, 8*(2), 99-105.

Titze, I. R. (1994). *Principles of voice production*. Englewood Cliffs, NJ: Prentice-Hall.

Titze, I. R., Hunter, E. J., & Svec, J. G. (2007). Voicing and silence periods in daily and weekly vocalizations of teachers. *J Acoust Soc Am, 121*(1), 469-478.

Tjardes, T., & Neugebauer, E. (2002). Sepsis research in the next millennium: concentrate on the software rather than the hardware. *Shock, 17*(1), 1-8.

Tomita, M., Matsuo, K., Maehara, N., Umezaki, T., & Shin, T. (1988). Measurements of oxygen pressure in the vocal fold during laryngeal nerve stimulation. *Arch Otolaryngol Head Neck Surg, 114*(3), 308-312.

Tranquillo, R. T., & Murray, J. D. (1992). Continuum model of fibroblast-driven wound contraction: inflammation-mediation. *J Theor Biol, 158*(2), 135-172.

Tranquillo, R. T., & Murray, J. D. (1993). Mechanistic model of wound contraction. *J Surg Res, 55*(2), 233-247.

Troisi, A., Wong, V., & Ratner, M. A. (2005). An agent-based approach for modeling molecular self-organization. *Proc Natl Acad Sci U S A, 102*(2), 255-260.

Tyson, J. J., Chen, K., & Novak, B. (2001). Network dynamics and cell physiology. *Nat Rev Mol Cell Biol, 2*(12), 908-916.

Tyson, J. J., Csikasz-Nagy, A., & Novak, B. (2002). The dynamics of cell cycle regulation. *Bioessays, 24*(12), 1095-1109.

van der Velden, L. A., Schaffsma, H. W., Manni, J. J., Link, M., Ruiter, D. J., Ramaekers, F. C. S., et al. (1996). Ctyokeratin and vimenin expression in normal epithelium and benign lesions of the vocal cords. *Acta Otolaryngol (Stockh), 116*(325-331), 325.

van Dyke Parunak, H., Savit, R., & Riolo, R. L. (1998). *Agent-based modeling vs equation-based modeling: a case study and users' guide.* Paper presented at the Multi-agent Systems and Agent-based Simulation.

Verdolini, K. (2000). Resonant Voice Therapy. In J. C. Stemple (Ed.), *Voice Therapy: Clinical Studies* (pp. 46-62). San Deigo: Singular Publishing Group.

Verdolini, K., Li, N. Y. K., Branski, R. C., Rosen, C. A., Urban, E. G., & Hebda, P. A. (in preparation). The effect of targeted vocal exercise on recovery from acute inflammation.

Verdolini, K., Rosen, C. A., & Branski, R. C. (2006). *Classification Manual for Voice Disorders-I*. New Jersey: Lawrence Erlbaum Associates, Inc. .

Verdolini, K., Rosen, C. A., Branski, R. C., & Hebda, P. A. (2003). Shifts in biochemical markers associated with wound healing in laryngeal secretions following phonotrauma: A preliminary study. *Annals of Otology, Rhinology, & Laryngology, 112*(12), 1021-1025.

Vicsek, T. (2002). The bigger picture. *Nature, 418*, 131.

Vodovotz, Y. (2006). Deciphering the complexity of acute inflammation using mathematical models. *Immunol Res, 36*(1-3), 237-245.

Vodovotz, Y., Chow, C. C., Bartels, J., Lagoa, C., Prince, J. M., Levy, R. M., et al. (2006). In silico models of acute inflammation in animals. *Shock, 26*(3), 235-244.

Vodovotz, Y., Clermont, G., Chow, C., & An, G. (2004). Mathematical models of the acute inflammatory response. *Current Opinion in Critical Care, 10*(5), 383-390.

Volic, S. V., Kirincic, N., & Markob, D. (1996). Some morphological, histological, cytological and histochemical aspects of Reinke's oedema. *Acta Otolaryngol (Stockh), 116*(322-324), 322.

Walker, D. C., Hill, G., Wood, S. M., Smallwood, R. H., & Southgate, J. (2004). Agent-based computational modeling of wounded epiethelial cell monolayers. *IEEE Transactions on Nonobioscience, 3*(3), 153-163.

Wallis, L., Jackson-Menaldi, C., Holland, W., & Giraldo, A. (2004). Vocal fold nodule vs. vocal fold polyp: answer from surgical pathologist and voice pathologist point of view. *J Voice, 18*(1), 125-129.

Ward, P. D., Thiebault, S. L., & Gray, S. D. (2002). Hyaluronic acid: Its role in voice. *Journal of Voice, 16*(3), 303-309.

Welham, N. V., Lim, X., Tateya, I., & Bless, D. M. (2008). Inflammatory factor profiles one hour following vocal fold injury. *Ann Otol Rhinol Laryngol, 117*(2), 145-152.

Wolkenhauer, O. (2001). Systems biology: the reincarnation of systems theory applied in biology? *Brief Bioinform, 2*(3), 258-270.

Yamashita, M., Hirano, S., Kanemaru, S., Tsuji, S., Suehiro, A., & Ito, J. (2007). Side population cells in the human vocal fold. *Ann Otol Rhinol Laryngol, 116*(11), 847-852.

Yi, T. M., Huang, Y., Simon, M. I., & Doyle, J. (2000). Robust perfect adaptation in bacterial chemotaxis through integral feedback control. *Proc Natl Acad Sci U S A, 97*(9), 4649-4653.

Zhang, L., Athale, C. A., & Deisboeck, T. S. (2007). Development of a three-dimensional multiscale agent-based tumor model: simulating gene-protein interaction profiles, cell phenotypes and multicellular patterns in brain cancer. *J Theor Biol, 244*(1), 96-107.

## APPENDIX E

## [ABM RULES OF THE HUMAN PONOTRAUMA MODEL]

| Parameter Descriptions | Rules |
|---|---|
| Extent of mucosal damage created by user-defined magnitude | Initial damage = ( Magnitude $^{1.5}$ ) * ( 2 + a random integer greater than or equal to 0, but strictly less than 5) |
| Extent of ECM fragmentation caused by initial damage | Native ECM (collagen, elastin and hyaluronan) that is 2 units around the damage will be degraded. |
| Extent of ECM degradation induced by TNF-$\alpha$ | If TNF-$\alpha$>10, native ECM (collagen, elastin and hyaluronan) will be degraded to ECM fragments. |
| Extent of collagen degradation induced by MMP-8 | If MMP-8>10, native collagen will be degraded to collagen fragments. |
| ECM fragments serve as danger signals | Each ECM fragment (collagen, elastin and hyaluronan) creates a damage signal. |
| Magnitude of impact stress from resonant voice exercise (RVIS) | RVIS = 5 |
| Magnitude of impact stress from spontaneous speech (SSIS) | SSIS = 10 |
| Magnitude of vibratory stress from resonant voice exercise (RVVS) | RVVS = 10 |

| Parameter Descriptions | Rules |
|---|---|
| Magnitude of vibratory stress from spontaneous speech (SSVS) | SSVS = 10 |
| Effect of the 4-hr resonant voice exercise on mucosal tissue | If the time step is between 5 and 13, create a number of RVIS * 20 platelets in damaged area.<br>If the time step is between 5 and 13, create a number of RVSS neutrophils in blood capillary. |
| Effect of the 4-hr spontaneous speech on mucosal tissue | If the time step is between 5 and 13, create a number of SSIS * 20 platelets in damaged area.<br>If the time step is between 5 and 13, create a number of SSSS neutrophils in blood capillary. |
| Number of platelets created by initial mucosal damage | Number of platelets = Extent of mucosal damage created by user-defined magnitude |
| TGF-β1 secreted by platelets | TGF-β1 = TGF-β1 + 0.1 |
| IL-1β secreted by platelets | IL-1β = IL-1β + (baseline-IL-1β * 0.1) |
| MMP-8 secreted by platelets | MMP-8 = IL-1β + (baseline-MMP-8 * 0.1) |
| Platelet lifespan | 0.5 to 1 day |
| Inflammatory mediator diffusion speed | IL-1β = 0.5 unit/step<br>IL-6 = 0.5 unit/step<br>IL-8 = 0.5 unit/step<br>IL-10 = 0.5 unit/step<br>TNF-α = 0.5 unit/step<br>TGF-β1 = 0.5 unit/step<br>FGF = 0.5 unit/step<br>MMP-8 = 0.5 unit/step |
| Inflammatory mediator degradation speed | IL-1β = 0.02 unit/step<br>IL-6 = 0.02 unit/step<br>IL-8 = 0.02 unit/step<br>IL-10 = 0.5 unit/step<br>TNF-α = 0.02 unit/step<br>TGF-β1 = 0.02 unit/step<br>FGF = 0.02 unit/step<br>MMP-8 = 0.02 unit/step |
| New hyaluronan lifespan | 0.5 to 1 day |

| Parameter Descriptions | Rules |
|---|---|
| New collagen repairs tissue damage | Damage on the patch and the eight surrounding patches of the new deposited collagen will be repaired. |
| New elastin repairs tissue damage | Damage on the patch and the eight surrounding patches of the new deposited elastin will be repaired. |
| New hyaluronan repairs tissue damage | Damage on the patch and the eight surrounding patches of the new deposited hyaluronan will be repaired. |
| Initial number of circulated neutrophils | 50 |
| Number of neutrophils circulating in the blood | If no damage, the number of neutrophils in the blood will be added by 8 every two hour. |
| Number of neutrophils circulating in the blood relating to damage | If total-damage > 0, the number of neutrophils in the blood will be added by (8 + total-damage * 0.01) every two hour. |
| Chemoattractant factors for neutrophils (Neu-chemo) | Neu-chemo = IL-1β + TNF-α + TGF-β1 + FGF + IL-6 + IL-8 + collagen fragments |
| Neutrophils are attracted by chemoattractants | Neutrophils will move one step towards the patch with the highest concentration of neu-chemo. |
| TNF-α (and IL-10) stimulates (and inhibits) activation of neutrophils | If total-damage > 0 and TNF-α > = IL-10 * 0.1, 100% of the change that the neutrophil is activated.<br>If total-damage > 0 and TNF-α > 0 but TNF < IL-10, 25% of the chance that the neutrophil is activated.<br>If total-damage > 0 but TNF = 0, 10% of the chance that the neutrophil is activated. |
| Inflammatory mediators secreted by activated neutrophils under voice rest condition | TNF-α = TNF-α + 1 * ( 1 / ( TGF-β1 + IL-10 ) )<br>MMP-8 = MMP-8 + 1 * ( 250 + TNF-α ) * ( 1 / ( 1 + TGF-β1) |
| Inflammatory mediators secreted by activated neutrophils under resonant voice condition | TNF-α = TNF-α + 10 * ( 1 / ( TGF-β1 + IL-10 ) )<br>MMP-8 = MMP-8 + 1 * ( 10 + TNF-α * 2 ) * ( 1 / ( 1 + TGF-β1 * 0.5 ) |

| Parameter Descriptions | Rules |
|---|---|
| Inflammatory mediators secreted by activated neutrophils under spontaneous speech condition | TNF-α = TNF-α + 1 * ( 1 / ( TGF-β1 + IL-10 ) )<br>MMP-8 = MMP-8 + 15 * ( 100 + TNF-α * 3 ) * ( 1 / ( 1 + TGF-β1) |
| Activated neutrophils clear ECM fragments | Activated neutrophils will clear ECM fragments on the patches that the cells are on. |
| Neutrophil lifespan | 0.5 days |
| Activated neutrophil lifespan | 1 day |
| IL-10 inhibits activated neutrophil survival | Activated neutrophil age – 0.01 * IL-10 |
| Apoptosis of activated neutrophils | If all damage is cleared, the chance of activated neutrophil apoptosis is 10%. |
| Initial number of circulated macrophages | 50 |
| Initial number of residential macrophages | 50 |
| Number of macrophages circulating in the blood | If no damage, the number of macrophages in the blood will be added by 1 every two hour. |
| Number of macrophages circulating in the blood relating to damage | If total-damage > 0, the number of macrophages in the blood will be added by (1 + total-damage * 0.01) every two hour. |
| Number of tissue macrophages recruited relating to damage | ( 1 + total-dam * 0.01) * 2 every 6 hour |
| Chemoattractant factors for macrophages (mac-chemo) | mac-chemo = IL-1β + TNF-α + TGF-β1 + FGF + collagen fragments + elastin fragments |
| Macrophages are attracted by chemoattractants | Macrophages will move one step towards the patch with the highest concentration of mac-chemo. |

| Parameter Descriptions | Rules |
|---|---|
| IL-1β,TNF-α (and IL-10) stimulates (and inhibits) activation of macrophages | If total-damage > 0 and (TNF-α + IL-1β - IL-10 * 0.1 > 0), 100% of the change that the macrophage is activated.<br>If total-damage > 0 and (TNF-α + IL-1β) > 0 but (TNF-α + IL-1β - IL-10 * 0.1 < 0), 25% of the chance that the macrophage is activated.<br>If total-damage > 0 but (TNF + IL-1β) = 0, 10% of the chance that the macrophage is activated. |
| Inflammatory mediators secreted by activated macrophages under voice rest condition | TNF-α = TNF-α + baseline-TNF-α * 0.05 + 1 * (1 / (1 + TGF-β1 + IL-10)) * (1 + TNF-α+ IL-1β + number of surrounding hyaluronan fragments)<br>IL-1β = IL-1β + baseline-IL-1β * 2 + 1 * (1 / (1 + TGF-β1 + IL-10)) * (15 + TNF-α + IL-1β * 15 + number of surrounding hyaluronan fragments)<br>IL-10 = IL-10 + baseline-IL-10 * 0.01 + (1 + IL-10 * 0.1)<br>IL-6 = IL-6 + baseline-IL-6 * 0.01 + 1 * (1 / (1 + IL-10)) * (1 + TNF-α+ IL-1β)<br>IL-8 = IL-8 + baseline-IL-8 * 2 + 1 * (1 / (1 + IL-10)) * (10 + TNF-α * 5 + IL-1β * 5 + number of surrounding hyaluronan fragments)<br>TGF-β1 = TGF-β1 + (1+ IL-10 + TNF-α)<br>FGF = FGF + 1 |
| Inflammatory mediators secreted by activated macrophages under resonant voice condition | TNF-α = TNF-α + baseline-TNF-α * 0.5 + 2 * (1 / (1 + TGF-β1 + IL-10 + IL-6 * 0.1)) * (1 + RVIS * 0.1 + TNF-α+ IL-1β + number of surrounding hyaluronan fragments)<br>IL-1β = IL-1β + baseline-IL-1β * 0.5 + 1 * (1 / (1 + TGF-β1 + IL-10 + IL-6)) * (1 + RVIS + TNF-α * 0.5 + IL-1β + number of surrounding hyaluronan fragments)<br>IL-10 = IL-10 + baseline-IL-10 * 0.01 + 1 * (4 + IL-10 * 0.001 + IL-6 * 0.001)<br>If steps <= 14, IL-6 = IL-6 + 0.5 * (1 + RVVS * 10)<br>If steps > 14, IL-6 = IL-6 + baseline-IL-6 + 0.5 * (1 / (1 + IL-10)) * (1 + TNF-α+ IL-1β)<br>IL-8 = IL-8 + baseline-IL-8 + 1 * (1 / (1 + IL-10 * 0.5)) * (100 + RVIS * 0.1 + TNF-α + IL-1β * 100 + number of surrounding hyaluronan fragments)<br>TGF-β1 = TGF-β1 + (1+ IL-10 + TNF-α)<br>FGF = FGF + 1 |

| Parameter Descriptions | Rules |
|---|---|
| Inflammatory mediators secreted by activated macrophages under spontaneous speech condition | TNF-α = TNF-α + baseline-TNF-α * 1 + 1 * (1 / (1 + TGF-β1 + IL-10 + IL-6)) * (1 + SSIS * 0.1 + TNF-α+ IL-1β * 5 + number of surrounding hyaluronan fragments)<br>IL-1β = IL-1β + baseline-IL-1β * 13 + 1 * (1 / (1 + TGF-β1 + IL-10 + IL-6)) * (1 + SSIS + TNF-α + IL-1β + number of surrounding hyaluronan fragments)<br>IL-10 = IL-10 + baseline-IL-10 * 0.05 + 1 * (1 + IL-10 * 0.0005 + IL-6 * 0.0005)<br>If steps <= 14, IL-6 = IL-6 + 0.5 * (1 + SSVS * 10)<br>If steps > 14, IL-6 = IL-6 + baseline-IL-6 + 1 * (1 / (1 + IL-10 * 0.5)) * (1 + TNF-α+ IL-1β * 4)<br>IL-8 = IL-8 + baseline-IL-8 + 10 * (1 / (1 + IL-10 * 0.5)) * (1 + SSIS * 0.1 + TNF-α + IL-1β * 7 + number of surrounding hyaluronan fragments)<br>TGF-β1 = TGF-β1 + (1+ IL-10 + TNF-α)<br>FGF = FGF + 1 |
| Activated macrophages clear ECM fragments | Activated macrophages will clear ECM fragments on the patches that the cells are on. |
| Activated macrophages become quiescence | If all damage is cleared, the chance of macrophages back to quiescence is 3%. |
| Circulating macrophage lifespan | 8hrs to 3 days |
| Resident macrophage lifespan | 60 to 120 days |
| Activated macrophage lifespan | 2 to 4 days |
| Initial number of residential fibroblasts | 100 |
| Magnitude of damage to recruit tissue fibroblasts | If damage > magnitude * 1.2, tissue fibroblasts will be recruited. |
| Number of tissue fibroblasts recruited relating to damage | ( 1 + total-dam * 0.01) * 2 every 6 hour |
| Chemoattractant factors for fibroblasts (fib-chemo) | fib-chemo = TGF-β1 |
| Fibroblasts are attracted by chemoattractants and its migration is stimulated by FGF | Fibroblasts will move (one + mean concentration of surrounding FGF) step towards the patch with the highest concentration of fib-chemo. |

| Parameter Descriptions | Rules |
|---|---|
| Tissue fibroblasts differentiate to activated fibroblasts | If total-damage > 0 and TGF-β1 <= 10, 100% of the change that the fibroblast is activated.<br>If total-damage > 0 and TGF-β1 > 10, 50% of the chance that the fibroblast is activated.<br>If total-damage > 0 but TGF-β1 = 0, 25% of the chance that the fibroblast is activated. |
| Fibroblast proliferation are stimulated by IL-1β, TNF-α, FGF, low-concentration TGF-β1 and hyaluronan fragments | Under low concentration of TGF-β1 (between 0 to 10), the % of chance that activated fibroblasts will proliferate is ( 25 + log (1 + TGF-β1 + FGF + TNF-α + IL-1β + number of surrounding hyaluronan fragments) )<br><br>Under high concentration of TGF-β1 (greater than 10), the % of chance that activated fibroblasts will proliferate is ( 25 + log (1 - TGF-β1 + FGF + TNF-α + IL-1β + number of surrounding hyaluronan fragments) ) |
| Inflammatory mediators secreted by activated fibroblasts under voice rest | TNF-α = TNF-α + 10 * (1 / (1 + TGF-β1 + IL-10 * 2 + number of surrounding new hyaluronan))<br>IL-6 = IL-6 + 0.01 * (1 / (1 + IL-10)) * (1 + TNF-α)<br>IL-8 = IL-8 + 1 * (1 / (1 + IL-10 + number of surrounding new hyaluronan))<br>TGF-β1 = TGF-β1 + 10 + 0.5 * (1 + TNF-α + IL-10)<br>FGF = FGF + 5 |
| Inflammatory mediators secreted by activated fibroblasts under resonant voice | TNF-α = TNF-α + 20 * (1 / (1 + TGF-β1 + IL-10 + IL-6 + number of surrounding new hyaluronan))<br>If steps < = 14, IL-6 = IL-6 + 1 * ( 1 + RVVS * 10 )<br>If steps > 14, IL-6 = IL-6 + 0.5 * (1 / (1 + IL-10)) * (1 + TNF-α)<br>IL-8 = IL-8 + 0.05 * (1 / (1 + IL-10 + number of surrounding new hyaluronan))<br>TGF-β1 = TGF-β1 + 10 + 0.5 * (1 + TNF-α + IL-10)<br>FGF = FGF + 5 |
| Inflammatory mediators secreted by activated fibroblasts under spontaneous speech | TNF-α = TNF-α + 1 * (1 / (1 + TGF-β1 + IL-10 + IL-6 + number of surrounding new hyaluronan))<br>If steps < = 14, IL-6 = IL-6 + 1 * (1 + SSVS * 10)<br>If steps > 14, IL-6 = IL-6 + 10 * (1 / (1 + IL-10)) * (1 + TNF-α)<br>IL-8 = IL-8 + 5 * (1 / (1 + IL-10 + number of surrounding new hyaluronan))<br>TGF-β1 = TGF-β1 + 10 + 0.5 * (1 + TNF-α + IL-10)<br>FGF = FGF + 5 |

| Parameter Descriptions | Rules |
|---|---|
| Collagen stimulation factor | Collagen stimulation factor = log ( (1 + mean of surrounding TGF-β1 + mean of surrounding IL-6) / ( 1 + mean of surrounding FGF + mean of surrounding IL-1β + mean of surrounding IL-8) ) |
| Collagen secreted by activated fibroblasts every 6 hours | If there is damage surrounded the fibroblast and the number of hyaluronan fragments is higher than that of new hyaluronan, the % of chance that activated fibroblasts will secrete collagen is (50 + collagen stimulation factor).<br>If there is damage surrounded the fibroblast and the number of hyaluronan fragments is equal to that of new hyaluronan, the % of chance that activated fibroblasts will secrete collagen is (25 + collagen stimulation factor).<br>If there is damage surrounded the fibroblast and the number of hyaluronan fragments is lower than that of new hyaluronan, the % of chance that activated fibroblasts will secrete collagen is (10 + collagen stimulation factor). |
| Elastin stimulation factor | Elastin stimulation factor = log ( (1 + mean of surrounding TGF-β) / ( 1 + mean of surrounding FGF + mean of surrounding IL-1β + mean of surrounding TNF-α) ) |
| Elastin secreted by activated fibroblasts every 6 hours | If there is damage surrounded the fibroblast, the % of chance that activated fibroblasts will secrete elastin is (25 + elastin stimulation factor). |
| Hyaluronan stimulation factor | Hyaluronan stimulation factor = log ( (1 + mean of surrounding TGF-β1 + mean of surrounding FGF + mean of surrounding IL-1β + mean of surrounding TNF-α) ) |
| Hyaluronan secreted by activated fibroblasts every hour | If there is damage surrounded the fibroblast, the % of chance that activated fibroblasts will secrete hyaluronan is (50 + hyaluronan stimulation factor).<br>If there is no damage surrounded the fibroblast, the % of chance that activated fibroblasts will secrete hyaluronan is (5 + collagen stimulation factor). |
| Fibroblast lifespan | 5 to 12 days |

## APPENDIX F

## [ABM RULES OF THE ANIMAL SURGICAL VOCAL FOLD TRAUMA MODEL]

Rules in shaded are different from those of the acute phonotrauma model.

| Parameter Descriptions | Rules |
|---|---|
| Extent of mucosal damage created by user-defined magnitude | Initial damage = ( Magnitude $^{1.5}$ ) * ( 2 + a random integer greater than or equal to 0, but strictly less than *5*) |
| Extent of cell death caused by initial damage | Cells (neutrophils, macrophages and fibroblasts) that are 2 units around the damage will die. |
| Extent of ECM fragmentation caused by initial damage | Native ECM (collagen, elastin and hyaluronan) that is 2 units around the damage will be degraded. |
| Extent of ECM degradation induced by TNF-$\alpha$ | If TNF-$\alpha$>10, native ECM (collagen, elastin and hyaluronan) will be degraded to ECM fragments. |
| Extent of collagen degradation induced by MMP-8 | If MMP-8>10, native collagen will be degraded to collagen fragments. |
| ECM fragments serve as danger signals | Each ECM fragment (collagen, elastin and hyaluronan) creates a damage signal. |
| Number of platelets created by initial mucosal damage | Number of platelets = Extent of mucosal damage created by user-defined magnitude |
| TGF-$\beta$1 secreted by platelets | TGF-$\beta$1 = TGF-$\beta$1 + 0.000001 |
| IL-1$\beta$ secreted by platelets | IL-1$\beta$ = IL-1$\beta$ + 300 + (baseline-IL-1$\beta$ * 0.002) |
| MMP-8 secreted by platelets | MMP-8 = IL-1$\beta$ + (baseline-MMP-8 * 0.002) |

| Parameter Descriptions | Rules |
|---|---|
| Platelet lifespan | 0.5 to 1 day |
| Inflammatory mediator diffusion speed | IL-1β = 0.5 unit/step<br>IL-6 = 0.5 unit/step<br>IL-8 = 0.5 unit/step<br>IL-10 = 0.5 unit/step<br>TNF-α = 0.5 unit/step<br>TGF-β1 = 0.5 unit/step<br>FGF = 0.5 unit/step<br>MMP-8 = 0.5 unit/step |
| Inflammatory mediator degradation speed | IL-1β = 0.02 unit/step<br>IL-6 = 0.02 unit/step<br>IL-8 = 0.02 unit/step<br>IL-10 = 0.5 unit/step<br>TNF-α = 0.02 unit/step<br>TGF-β1 = 0.02 unit/step<br>FGF = 0.02 unit/step<br>MMP-8 = 0.02 unit/step |
| New hyaluronan lifespan | 0.5 to 1 day |
| New collagen repairs tissue damage | Damage on the patch and the eight surrounding patches of the new deposited collagen will be repaired. |
| New elastin repairs tissue damage | Damage on the patch and the eight surrounding patches of the new deposited elastin will be repaired. |
| New hyaluronan repairs tissue damage | Damage on the patch and the eight surrounding patches of the new deposited hyaluronan will be repaired. |
| Initial number of circulated neutrophils | 50 |
| Number of neutrophils circulating in the blood | If no damage, the number of neutrophils in the blood will be added by 8 every two hour. |
| Number of neutrophils circulating in the blood relating to damage | If total-damage > 0, the number of neutrophils in the blood will be added by (8 + total-damage * 0.01) every two hour. |
| Chemoattractant factors for neutrophils (Neu-chemo) | Neu-chemo = IL-1β + TNF-α + TGF-β1 + FGF + IL-6 + IL-8 + collagen fragments |

| Parameter Descriptions | Rules |
|---|---|
| Neutrophils are attracted by chemoattractants | Neutrophils will move one step towards the patch with the highest concentration of neu-chemo. |
| TNF-α (and IL-10) stimulates (and inhibits) activation of neutrophils | If total-damage > 0 and TNF-α > = IL-10 * 0.1, 100% of the change that the neutrophil is activated.<br>If total-damage > 0 and TNF-α > 0 but TNF < IL-10, 25% of the chance that the neutrophil is activated.<br>If total-damage > 0 but TNF = 0, 10% of the chance that the neutrophil is activated. |
| Inflammatory mediators secreted by activated neutrophils | TNF-α = TNF-α + 8 * ( 1 / ( TGF-β1 + IL-10 ) )<br>MMP-8 = MMP-8 + 1 * ( 1 + TNF-α ) * ( 1 / ( 1 + TGF-β1) |
| Activated neutrophils clear ECM fragments | Activated neutrophils will clear ECM fragments on the patches that the cells are on. |
| Neutrophil lifespan | 0.5 days |
| Activated neutrophil lifespan | 1 day |
| IL-10 inhibits activated neutrophil survival | Activated neutrophil age – 0.01 * IL-10 |
| Apoptosis of activated neutrophils | If all damage is cleared, the chance of activated neutrophil apoptosis is 10%. |
| Initial number of circulated macrophages | 50 |
| Initial number of residential macrophages | 50 |
| Number of macrophages circulating in the blood | If no damage, the number of macrophages in the blood will be added by 1 every two hour. |
| Number of macrophages circulating in the blood relating to damage | If total-damage > 0, the number of macrophages in the blood will be added by (1 + total-damage * 0.01) every two hour. |
| Number of tissue macrophages recruited relating to damage | ( 1 + total-dam * 0.01) * 2 every 6 hour |
| Chemoattractant factors for macrophages (mac-chemo) | mac-chemo = IL-1β + TNF-α + TGF-β1 + FGF + collagen fragments + elastin fragments |

| Parameter Descriptions | Rules |
|---|---|
| Macrophages are attracted by chemoattractants | Macrophages will move one step towards the patch with the highest concentration of mac-chemo. |
| IL-1β,TNF-α (and IL-10) stimulates (and inhibits) activation of macrophages | If total-damage > 0 and (TNF-α + IL-1β - IL-10 * 0.1 > 0), 100% of the change that the macrophage is activated.<br>If total-damage > 0 and (TNF-α + IL-1β) > 0 but (TNF-α + IL-1β - IL-10 * 0.1 < 0), 25% of the chance that the macrophage is activated.<br>If total-damage > 0 but (TNF + IL-1β) = 0, 10% of the chance that the macrophage is activated. |
| Inflammatory mediators secreted by activated macrophages | TNF-α = TNF-α + 0.01 * (1 / (1 + TGF-β1 + IL-10)) * (1 + TNF-α+ IL-1β + number of surrounding hyaluronan fragments)<br>IL-1β = IL-1β + 10 * (1 / (1 + TGF-β1 + IL-10)) * (35 + TNF-α + IL-1β + number of surrounding hyaluronan fragments)<br>IL-10 = IL-10 + 2 * (1 + IL-10 * 0.01)<br>IL-6 = IL-6 + 1 * (1 / (1 + IL-10)) * (1 + TNF-α+ IL-1β)<br>IL-8 = IL-8 + 1 * (1 / (1 + IL-10)) * (1 + TNF-α + IL-1β + number of surrounding hyaluronan fragments)<br>TGF-β1 = TGF-β1 + 5 * (1+ IL-10 + TNF-α)<br>FGF = FGF + 1 |
| Activated macrophages clear ECM fragments | Activated macrophages will clear ECM fragments on the patches that the cells are on. |
| Activated macrophages become quiescence | If all damage is cleared, the chance of macrophages back to quiescence is 3%. |
| Circulating macrophage lifespan | 8hrs to 3 days |
| Resident macrophage lifespan | 60 to 120 days |
| Activated macrophage lifespan | 2 to 4 days |
| Initial number of residential fibroblasts | 100 |
| Magnitude of damage to recruit tissue fibroblasts | If damage > magnitude * 1.2, tissue fibroblasts will be recruited. |
| Number of tissue fibroblasts recruited relating to damage | ( 1 + total-dam * 0.01) * 2 every 6 hour |

| Parameter Descriptions | Rules |
|---|---|
| Chemoattractant factors for fibroblasts (fib-chemo) | fib-chemo = TGF-β1 |
| Fibroblasts are attracted by chemoattractants and its migration is stimulated by FGF | Fibroblasts will move (one + mean concentration of surrounding FGF) step towards the patch with the highest concentration of fib-chemo. |
| Tissue fibroblasts differentiate to activated fibroblasts | If total-damage > 0 and TGF-β1 <= 10, 100% of the change that the fibroblast is activated.<br>If total-damage > 0 and TGF-β1 > 10, 50% of the chance that the fibroblast is activated.<br>If total-damage > 0 but TGF-β1 = 0, 25% of the chance that the fibroblast is activated. |
| Fibroblast proliferation are stimulated by IL-1β, TNF-α, FGF, low-concentration TGF-β1 and hyaluronan fragments | Under low concentration of TGF-β1 (between 0 to 10), the % of chance that activated fibroblasts will proliferate is ( 25 + log (1 + TGF-β1 + FGF + TNF-α + IL-1β + number of surrounding hyaluronan fragments) )<br><br>Under high concentration of TGF-β1 (greater than 10), the % of chance that activated fibroblasts will proliferate is ( 25 + log (1 - TGF-β1 + FGF + TNF-α + IL-1β + number of surrounding hyaluronan fragments) ) |
| Inflammatory mediators secreted by activated fibroblasts | TNF-α = TNF-α + 0.01 * (1 / (1 + TGF-β1 + IL-10 + number of surrounding new hyaluronan))<br>IL-6 = IL-6 + 1 * (1 / (1 + IL-10)) * (1 + TNF-α)<br>IL-8 = IL-8 + 1 * (1 / (1 + IL-10 + number of surrounding new hyaluronan))<br>TGF-β1 = TGF-β1 + 1 * (1 + TNF-α + IL-10)<br>FGF = FGF + 1 |
| Collagen stimulation factor | Collagen stimulation factor = log ( (1 + mean of surrounding TGF-β1 + mean of surrounding IL-6) / ( 1 + mean of surrounding FGF + mean of surrounding IL-1β + mean of surrounding IL-8) ) |

| Parameter Descriptions | Rules |
|---|---|
| Collagen secreted by activated fibroblasts every 6 hours | If there is damage surrounded the fibroblast and the number of hyaluronan fragments is higher than that of new hyaluronan, the % of chance that activated fibroblasts will secrete collagen is (50 + collagen stimulation factor).<br>If there is damage surrounded the fibroblast and the number of hyaluronan fragments is equal to that of new hyaluronan, the % of chance that activated fibroblasts will secrete collagen is (25 + collagen stimulation factor).<br>If there is damage surrounded the fibroblast and the number of hyaluronan fragments is lower than that of new hyaluronan, the % of chance that activated fibroblasts will secrete collagen is (10 + collagen stimulation factor). |
| Elastin stimulation factor | Elastin stimulation factor = log ( (1 + mean of surrounding TGF-β) / ( 1 + mean of surrounding FGF + mean of surrounding IL-1β + mean of surrounding TNF-α) ) |
| Elastin secreted by activated fibroblasts every 6 hours | If there is damage surrounded the fibroblast, the % of chance that activated fibroblasts will secrete elastin is (25 + elastin stimulation factor). |
| Hyaluronan stimulation factor | Hyaluronan stimulation factor = log ( (1 + mean of surrounding TGF-β1 + mean of surrounding FGF + mean of surrounding IL-1β + mean of surrounding TNF-α) ) |
| Hyaluronan secreted by activated fibroblasts every hour | If there is damage surrounded the fibroblast, the % of chance that activated fibroblasts will secrete hyaluronan is (50 + hyaluronan stimulation factor).<br>If there is no damage surrounded the fibroblast, the % of chance that activated fibroblasts will secrete hyaluronan is (5 + collagen stimulation factor). |
| Fibroblast lifespan | 5 to 12 days |

## REFERENCES


Agarwal, S., Deschner, J., Long, P., Verma, A., Hofman, C., Evans, C. H., et al. (2004). Role of NF-kappaB transcription factors in antiinflammatory and proinflammatory actions of mechanical signals. *Arthritis Rheum, 50*(11), 3541-3548.

Akerstrom, T., Steensberg, A., Keller, P., Keller, C., Penkowa, M., & Pedersen, B. K. (2005). Exercise induces interleukin-8 expression in human skeletal muscle. *J Physiol, 563*(Pt 2), 507-516.

An, G. (2001). Agent-based computer simulation and sirs: building a bridge between basic science and clinical trials. *Shock, 16*(4), 266-273.

An, G. (2004). In silico experiments of existing and hypothetical cytokine-directed clinical trials using agent-based modeling. *Critical Care Medicine, 32*(10), 2050-2060.

An, G. (2005). Mathematical modeling in medicine: a means, not an end. *Critical Care Medicine, 33*(1), 253-254.

An, G., Hunt, C. A., Clermont, G., Neugebauer, E., & Vodovotz, Y. (2007). Challenges and rewards on the road to translational systems biology in acute illness: four case reports from interdisciplinary teams. *J Crit Care, 22*(2), 169-175.

Armstrong, D. G., & Jude, E. B. (2002). The role of matrix metalloproteinases in wound healing. *J Am Podiatr Med Assoc, 92*(1), 12-18.

Atamaniuk, J., Stuhlmeier, K. M., Vidotto, C., Tschan, H., Dossenbach-Glaninger, A., & Mueller, M. M. (2008). Effects of ultra-marathon on circulating DNA and mRNA expression of pro- and anti-apoptotic genes in mononuclear cells. *Eur J Appl Physiol, 104*(4), 711-717.

Baldi, P., & Brunak, S. (Eds.). (2001). *Bioinformatics: The Machine LEarning Approach* (2nd ed.). Cambridge, M.A.: MIT Press.

Bassingthwaighte, J. B., Liebovitch, L. S., & West, B. J. (1994). *Fractal physiology*. Oxford, U.K.: University Press.

Bassiouny, S. (1998). Efficacy of the accent method of voice therapy. *Folia Phoniatr Logop, 50*(3), 146-164.

Behrens, M. D., Wagner, W. M., Krco, C. J., Erskine, C. L., Kalli, K. R., Krempski, J., et al. (2008). The endogenous danger signal, crystalline uric acid, signals for enhanced antibody immunity. *Blood, 111*(3), 1472-1479.

Behrman, A., Rutledge, J., Hembree, A., & Sheridan, S. (2008). Vocal hygiene education, voice production therapy, and the role of patient adherence: a treatment effectiveness study in women with phonotrauma. *J Speech Lang Hear Res, 51*(2), 350-366.

Behrman, A., & Sulica, L. (2003). Voice rest after microlaryngoscopy: current opinion and practice. *Laryngoscope, 113*(12), 2182-2186.

Bernstein, J. (2004). Evidence-based medicine. *J Am Acad Orthop Surg, 12*(2), 80-88.
Berry, D. A., Verdolini, K., Montequin, D. W., Hess, M. M., Chan, R. W., & Titze, I. R. (2001). A quantitative output-cost ration in voice production. *J Speech Lang Hear Res, 44*(1), 29-37.
Boone, D. R., & McFarlane, S. C. (1994). *The Voice and Voice Therapy* (5th ed.). Englewood Cliffs: Prentice-Hall, Inc.
Brancaccio, P., Maffulli, N., Buonauro, R., & Limongelli, F. M. (2008). Serum enzyme monitoring in sports medicine. *Clin Sports Med, 27*(1), 1-18, vii.
Branski, R. C. (2005). *Vocal fold fibroblast response to mechanical stress.* Unpublished doctoral dissertation, University of Pittsburgh, Pittsburgh.
Branski, R. C., Perera, P., Verdolini, K., Rosen, C. A., Hebda, P. A., & Agarwal, S. (2006). Dynamic Biomechanical Strain Inhibits IL-1beta-induced Inflammation in Vocal Fold Fibroblasts. *J Voice*.
Branski, R. C., Rosen, C. A., Verdolini, K., & Hebda, P. A. (2005a). Acute vocal fold wound healing in a rabbit model. *Ann Otol Rhinol Laryngol, 114*(1 Pt 1), 19-24.
Branski, R. C., Rosen, C. A., Verdolini, K., & Hebda, P. A. (2005b). Biochemical markers associated with acute vocal fold wound healing: a rabbit model. *J Voice, 19*(2), 283-289.
Broker, B. J., & Reiter, D. (1994). Fetal wound healing. *Otolaryngol Head Neck Surg, 110*(6), 547-549.
Broughton, G., 2nd, Janis, J. E., & Attinger, C. E. (2006). The basic science of wound healing. *Plast Reconstr Surg, 117*(7 Suppl), 12S-34S.
Burroughs, P., & Dahners, L. E. (1990). The effect of enforced exercise on the healing of ligament injuries. *Am J Sports Med, 18*(4), 376-378.
Butterfield, T. A., Best, T. M., & Merrick, M. A. (2006). The dual roles of neutrophils and macrophages in inflammation: a critical balance between tissue damage and repair. *J Athl Train, 41*(4), 457-465.
Calvin, M. (1998). Cutaneous wound repair. *Wounds, 10*(1), 12-32.
Catten, M., Gray, S. D., Hammond, T. H., Zhou, R., & Hammond, E. H. (1998). Analysis of cellular location and concentration in vocal fold lamina propria. *Otolaryngology Head and Neck Surgery, 118*(5), 663-667.
Chan, R. W., Fu, M., Young, L., & Tirunagari, N. (2007). Relative contributions of collagen and elastin to elasticity of the vocal fold under tension. *Ann Biomed Eng, 35*(8), 1471-1483.
Chan, R. W., Gray, S. D., & Titze, I. R. (2001). The importance of hyaluronic acid in vocal fold biomechanics. *Otolaryngol Head Neck Surg, 124*(6), 607-614.
Chan, R. W., & Titze, I. R. (2006). Dependence of phonation threshold pressure on vocal tract acoustics and vocal fold tissue mechanics. *J Acoust Soc Am, 119*(4), 2351-2362.
Chen, S. H., Hsiao, T. Y., Hsiao, L. C., Chung, Y. M., & Chiang, S. C. (2006). Outcome of Resonant Voice Therapy for Female Teachers With Voice Disorders: Perceptual, Physiological, Acoustic, Aerodynamic, and Functional Measurements. *J Voice*.
Chen, W., Syldath, U., Bellmann, K., Burkart, V., & Kolb, H. (1999). Human 60-kDa heat-shock protein: a danger signal to the innate immune system. *J Immunol, 162*(6), 3212-3219.
Cho, S. H., Kim, H. T., Lee, I. J., Kim, M. S., & Park, H. J. (2000). Influence of phonation on basement membrane zone recovery after phonomicrosurgery: A canine model. *Ann Otol Rhinol Laryngol, 109*, 658-666.
Chow, C. C., Clermont, G., Kumar, R., Lagoa, C., Tawadrous, Z., Gallo, D., et al. (2005). The acute inflammatory response in diverse shock states. *Shock, 24*(1), 74-84.

Cilliers, P. (2005). *Complexity and Postmodernism: Understanding Complex Systems*. London: Routledge.

Clark, R. A. F. (1988). Cutaneous wound repair: Molecular and cellular controls. *Prog Dermatol, 22*, 1-12.

Clark, R. A. F. (1998). Wound Repair. Overview and General Considerations. In R. A. F. Clark (Ed.), *The Molecular and Cellular Biology of Wound Repair*. New York: Plenum Press.

Clark, R. A. F. (Ed.). (1998). *The Molecular and Cellular Biology of Wound Repair*. New York: Plenum Press.

Clermont, G., Bartels, J., Kumar, R., Constantine, G., Vodovotz, Y., & Chow, C. (2004). In silico design of clinical trials: a method coming of age. *Crit Care Med, 32*(10), 2061-2070.

Cockbill, S. (2002). Wound: the healing process. *Hospital Pharmacist, 9*, 255-260.

Cohen, A. M., Stavri, P. Z., & Hersh, W. R. (2004). A categorization and analysis of the criticisms of Evidence-Based Medicine. *Int J Med Inform, 73*(1), 35-43.

Colton, R., & Casper, J. K. (1996). *Understanding Voice Problems: A Physiological Perspective for Diagnosis and Treatment* (2nd ed.). Baltimore: Williams & Wilkins.

Cook, D. J., & Levy, M. M. (1998). Evidence-based medicine. A tool for enhancing critical care practice. *Crit Care Clin, 14*(3), 353-358.

Coraux, C., Hajj, R., Lesimple, P., & Puchelle, E. (2005). [Repair and regeneration of the airway epithelium]. *Med Sci (Paris), 21*(12), 1063-1069.

Courey, M. S., Shohet, J. A., Scott, M. A., & Ossoff, R. H. (1996). Immunohistochemical characterization of benign laryngeal lesions. *Ann Otol Rhinol Laryngol, 195*(7), 525-531.

Croce, M. A., Dyne, K., Boraldi, F., Quaglino, D., Jr., Cetta, G., Tiozzo, R., et al. (2001). Hyaluronan affects protein and collagen synthesis by in vitro human skin fibroblasts. *Tissue Cell, 33*(4), 326-331.

Dang, C., Ting, K., Soo, C., Longaker, M. T., & Lorenz, H. P. (2003). Fetal wound healing current perspectives. *Clin Plast Surg, 30*(1), 13-23.

Davidoff, F., Haynes, B., Sackett, D., & Smith, R. (1995). Evidence based medicine. *Bmj, 310*(6987), 1085-1086.

Davies, E. L., Bacelar, M. M., Marshall, M. J., Johnson, E., Wardle, T. D., Andrew, S. M., et al. (2006). Heat shock proteins form part of a danger signal cascade in response to lipopolysaccharide and GroEL. *Clin Exp Immunol, 145*(1), 183-189.

Day, J., Rubin, J., Vodovotz, Y., Chow, C. C., Reynolds, A., & Clermont, G. (2006). A reduced mathematical model of the acute inflammatory response II. Capturing scenarios of repeated endotoxin administration. *J Theor Biol*.

De Maio, A., Torres, M. B., & Reeves, R. H. (2005). Genetic determinants influencing the response to injury, inflammation, and sepsis. *Shock, 23*(1), 11-17.

De, S., Rosen, J., Dagan, A., & Hannaford, B. (2007). Assessment of tissue damage due to mechanical stresses. *The International Journal of Robotics Research, 26*(11-12), 1159-1171.

De Simone, J. (2006a). Beyond 'faith-based medicine' and EBM. *J Eval Clin Pract, 12*(4), 438-444.

De Simone, J. (2006b). Reductionist inference-based medicine, i.e. EBM. *J Eval Clin Pract, 12*(4), 445-449.

Dechert, T. A., Ducale, A. E., Ward, S. I., & Yager, D. R. (2006). Hyaluronan in human acute and chronic dermal wounds. *Wound Repair Regen, 14*(3), 252-258.

Devalaraja, R. M., Nanney, L. B., Du, J., Qian, Q., Yu, Y., Devalaraja, M. N., et al. (2000). Delayed wound healing in CXCR2 knockout mice. *J Invest Dermatol, 115*(2), 234-244.

Dickenson, D., & Vineis, P. (2002). Evidence-based medicine and quality of care. *Health Care Anal, 10*(3), 243-259.

Diegelmann, R. F., & Evans, M. C. (2004). Wound healing: an overview of acute, fibrotic and delayed healing. *Front Biosci, 9*, 283-289.

Dinarello, C. A. (1997). Interleukin-1. *Cytokine & Growth Factor Reviews, 8*(4), 253-265.

Dinarello, C. A. (2000). Proinflammatory Cytokines. *Chest, 118*(2), 503-508.

Eiff, M. P., Smith, A. T., & Smith, G. E. (1994). Early mobilization versus immobilization in the treatment of lateral ankle sprains. *Am J Sports Med, 22*(1), 83-88.

Emerich, K. A., Spiegel, J. R., & Sataloff, R. T. (2000). Phonomicrosurgery III: pre- and postoperative care. *Otolaryngol Clin North Am, 33*(5), 1071-1080.

Eming, S. A., Krieg, T., & Davidson, J. M. (2007). Inflammation in wound repair: molecular and cellular mechanisms. *J Invest Dermatol, 127*(3), 514-525.

Ermentrout, G. B., & Edelstein-Keshet, L. (1993). Cellular automata approaches to biological modeling. *J Theor Biol, 160*(1), 97-133.

Farkas, A. M., Kilgore, T. M., & Lotze, M. T. (2007). Detecting DNA: getting and begetting cancer. *Curr Opin Investig Drugs, 8*(12), 981-986.

Feinstein, A. R., & Horwitz, R. I. (1997). Problems in the "evidence" of "evidence-based medicine". *Am J Med, 103*(6), 529-535.

Foell, D., Wittkowski, H., & Roth, J. (2007). Mechanisms of disease: a 'DAMP' view of inflammatory arthritis. *Nat Clin Pract Rheumatol, 3*(7), 382-390.

Fowlis, D. J., Flanders, K. C., Duffie, E., Balmain, A., & Akhurst, R. J. (1992). Discordant transforming growth factor beta 1 RNA and protein localization during chemical carcinogenesis of the skin. *Cell Growth Differ, 3*(2), 81-91.

Fraser, D., Wakefield, L., & Phillips, A. (2002). Independent regulation of transforming growth factor-beta1 transcription and translation by glucose and platelet-derived growth factor. *Am J Pathol, 161*(3), 1039-1049.

Frydelund-Larsen, L., Penkowa, M., Akerstrom, T., Zankari, A., Nielsen, S., & Pedersen, B. K. (2007). Exercise induces interleukin-8 receptor (CXCR2) expression in human skeletal muscle. *Exp Physiol, 92*(1), 233-240.

Gallucci, S., & Matzinger, P. (2001). Danger signals: SOS to the immune system. *Curr Opin Immunol, 13*(1), 114-119.

Gao, F., Koenitzer, J. R., Tobolewski, J. M., Jiang, D., Liang, J., Noble, P. W., et al. (2008). Extracellular superoxide dismutase inhibits inflammation by preventing oxidative fragmentation of hyaluronan. *J Biol Chem, 283*(10), 6058-6066.

Gilbert, N., & Bankes, S. (2002). Platforms and methods for agent-based modeling. *Proc Natl Acad Sci U S A, 99 Suppl 3*, 7197-7198.

Gillitzer, R., & Goebeler, M. (2001). Chemokines in cutaneous wound healing. *J Leukoc Biol, 69*(4), 513-521.

Gillivan-Murphy, P., Drinnan, M. J., O'Dwyer, T. P., Ridha, H., & Carding, P. (2006). The effectiveness of a voice treatment approach for teachers with self-reported voice problems. *J Voice, 20*(3), 423-431.

Girish, K. S., & Kemparaju, K. (2007). The magic glue hyaluronan and its eraser hyaluronidase: a biological overview. *Life Sci, 80*(21), 1921-1943.

Glazko, G., Veeramachaneni, V., Nei, M., & Makalowski, W. (2005). Eighty percent of proteins are different between humans and chimpanzees. *Gene, 346*, 215-219.
Gleeson, M. (2007). Immune function in sport and exercise. *J Appl Physiol, 103*(2), 693-699.
Goldberger, A. L. (1996). Non-linear dynamics for clinicians: chaos theory, fractals, and complexity at the bedside. *Lancet, 347*(9011), 1312-1314.
Goldberger, A. L. (2006). Complex systems. *Proc Am Thorac Soc, 3*, 467-472.
Goldberger, A. L., Amaral, L. A., Hausdorff, J. M., Ivanov, P., Peng, C. K., & Stanley, H. E. (2002). Fractal dynamics in physiology: alterations with disease and aging. *Proc Natl Acad Sci U S A, 99 Suppl 1*, 2466-2472.
Goldberger, A. L., Bhargava, V., West, B. J., & Mandell, A. J. (1986). Some observations on the question: is ventricular fibrillation "chaos"? *Physica*, 282 - 289.
Goldberger, A. L., & West, B. J. (1987). Fractals in physiology and medicine. *Yale J Biol Med, 60*(5), 421-435.
Goldenfeld, N., & Kadanoff, L. P. (1999). Simple lessons from complexity. *Science, 284*(5411), 87-89.
Gray, S. D. (1991). *Basement membrane zone injury in vocal nodules*. San Diego: Singular Press.
Gray, S. D. (1997). Benign Pathologic Responses of the Larynx. *NCVS Status and Progress Report, 11*, 135-148.
Gray, S. D. (2000). Cellular Physiology of the Vocal Folds. In C. A. Rosen & T. Murry (Eds.), *The Otolaryngologic Clinics of North America*. Philadelphia: W.B. Saunders Company.
Gray, S. D., Hammond, E. H., & Hanson, D. (1995). Benign pathologic response of the larynx. *Ann Otol Rhinol Laryngol, 104*, 8-13.
Gray, S. D., Hirano, M., & Sato, K. (1993). Molecular and Cellular Structure of Vocal Fold Tissue. In I. R. Titze (Ed.), *Vocal Fold Physiology*. San Diego: Singular Publishing Group, Inc.
Gray, S. D., Pignatari, S. S., & Harding, P. (1994). Morphologic ultrastructure of anchoring fibers in normal vocal fold basement membrane zone. *Journal of Voice, 8*(1), 48-52.
Gray, S. D., & Titze, I. R. (1988). Histologic investigation of hyperphonated canine vocal cords. *Ann Otol Rhinol Laryngol, 97*(4), 381-388.
Gray, S. D., Titze, I. R., Alipour, F., & Hammond, T. H. (2000). Biomechanical and histologic observations of vocal fold fibrous proteins. *Ann Otol Rhinol Laryngol, 109*, 77-85.
Gray, S. D., Titze, I. R., Chan, R. W., & Hammond, T. H. (1999). Vocal fold proteoglycans and their influence on biomechanics. *Laryngoscope, 109*, 845-854.
Gray, S. D., Titze, I. R., & Lusk, R. P. (1987). Electron microscopy of hyperphonated canine vocal cords. *Journal of Voice, 1*(1), 109-115.
Grimm, V., Revilla, E., Berger, U., Jeltsch, F., Mooij, W. M., Railsback, S. F., et al. (2005). Pattern-oriented modeling of agent-based complex systems: lessons from ecology. *Science, 310*(5750), 987-991.
Grose, R., & Werner, S. (2004). Wound-healing studies in transgenic and knockout mice. *Mol Biotechnol, 28*(2), 147-166.
Guirao, X., & Lowry, S. F. (1996). Biologic control of injury and inflammation: much more than too little or too late. *World J. Surg, 20*, 437-446.
Gunter, H. E. (2003). A mechanical model of vocal-fold collision with high spatial and temporal resolution. *J. Acoust. Soc. Am., 113*(2), 994-1000.

Gunter, H. E. (2004). Modeling mechanical stresses as a factor in the etiology of benign vocal fold lesions. *Journal of Biomechanics, 37*, 1119-1124.
Hackam, D. G. (2007). Translating animal research into clinical benefit. *Bmj, 334*(7586), 163-164.
Hackham, D. J., & Ford, H. R. (2002). Cellular, Biochemical, and Clinical Aspects of Wound Healing. *Surgical Infections, 3*(Supplement.1), S23-S35.
Hahn, M. S., Kobler, J. B., Zeitels, S. M., & Langer, R. (2006). Quantitative and comparative studies of the vocal fold extracellular matrix II: collagen. *Ann Otol Rhinol Laryngol, 115*(3), 225-232.
Hang, L., Frendeus, B., Godaly, G., & Svanborg, C. (2000). Interleukin-8 receptor knockout mice have subepithelial neutrophil entrapment and renal scarring following acute pyelonephritis. *J Infect Dis, 182*(6), 1738-1748.
Hansen, J. K., & Thibeault, S. L. (2006). Current understanding and review of the literature: vocal fold scarring. *J Voice, 20*(1), 110-120.
Hantash, B. M., Zhao, L., Knowles, J. A., & Lorenz, H. P. (2008). Adult and fetal wound healing. *Front Biosci, 13*, 51-61.
Hardy, M. A. (1989). The biology of scar formation. *Phys Ther, 69*(12), 1014-1024.
Harrison, J. R., Vargas, S. J., Petersen, D. N., Lorenzo, J. A., & Kream, B. E. (1990). Interleukin-1 alpha and phorbol ester inhibit collagen synthesis in osteoblastic MC3T3-E1 cells by a transcriptional mechanism. *Mol Endocrinol, 4*(2), 184-190.
Haynes, R. B. (2002). What kind of evidence is it that Evidence-Based Medicine advocates want health care providers and consumers to pay attention to? *BMC Health Serv Res, 2*(1), 3.
Heath, W. R., & Carbone, F. R. (2003). Immunology: dangerous liaisons. *Nature, 425*(6957), 460-461.
Henry, G., & Garner, W. L. (2003). Inflammatory mediators in wound healing. *Surg Clin North Am, 83*(3), 483-507.
Hirano, S., Bless, D. M., del Rio, A. M., Connor, N. P., & Ford, C. N. (2004). Therapeutic potential of growth factors for aging voice. *Laryngoscope, 114*(12), 2161-2167.
Hirano, S., Bless, D. M., Heisey, D., & Ford, C. N. (2003). Effect of growth factors on hyaluronan production by canine vocal fold fibroblasts. *Ann Otol Rhinol Laryngol, 112*(7), 617-624.
Hirano, S., Bless, D. M., Nagai, H., Rousseau, B., Welham, N. V., Montequin, D. W., et al. (2004). Growth factor therapy for vocal fold scarring in a canine model. *Ann Otol Rhinol Laryngol, 113*(10), 777-785.
Hirano, S., Bless, D. M., Rousseau, B., Welham, N., Scheidt, T., & Ford, C. N. (2003). Fibronectin and adhesion molecules on canine scarred vocal folds. *Laryngoscope, 113*(6), 966-972.
Hirano, S., Minamiguchi, S., Yamashita, M., Ohno, T., Kanemaru, S. I., & Kitamura, M. (2008). Histologic Characterization of Human Scarred Vocal Folds. *J Voice*.
Hirano, S., Nagai, H., Tateya, I., Tateya, T., Ford, C. N., & Bless, D. M. (2005). Regeneration of aged vocal folds with basic fibroblast growth factor in a rat model: a preliminary report. *Ann Otol Rhinol Laryngol, 114*(4), 304-308.
Hirschi, S. D., Gray, S. D., & Thibeault, S. L. (2002). Fibronectin:an interesting vocal fold protein. *J Voice, 16*(3), 310-316.
Holland, J. H. (1992). Complex adaptive systems. *Daedalus, 121*(1), 17-30.

Holland, J. H. (Ed.). (1995). *Hidden Order: How Adaptation Builds Complexity*. Reading, M. A.: Addison-Wesley.

Holmberg, E. B., Hillman, R. E., Hammarberg, B., Sodersten, M., & Doyle, P. (2001). Efficacy of a behaviorally based voice therapy protocol for vocal nodules. *Journal of Voice, 15*(3), 395-412.

Hood, L. (2003). Systems biology: integrating technology, biology, and computation. *Mech Ageing Dev, 124*(1), 9-16.

Hood, L., Heath, J. R., Phelps, M. E., & Lin, B. (2004). Systems biology and new technologies enable predictive and preventative medicine. *Science, 306*(5696), 640-643.

Hoover, C. A., Sataloff, R. T., Lyons, K. M., & Hawkshaw, M. (2001). Vocal fold mucosal tears: maintaining a high clinical index of suspicion. *J Voice, 15*(3), 451-455.

Hunter, E. J., & Titze, I. R. (in press). Quantifying vocal foatigue recovery: dynamic vocal recovery trajectories after a vocal loading exercise. *Ann Otol Rhinol Laryngol*.

Hunter, E. J., Titze, I. R., & Alipour, F. (2004). A three-dimensional model of vocal fold abduction/adduction. *J Acoust Soc Am, 115*(4), 1747-1759.

Imahara, S. D., & O'Keefe, G. E. (2004). Genetic determinants of the inflammatory response. *Curr Opin Crit Care, 10*(5), 318-324.

Jacobson, B. H., Johnson, A., Grywalski, C., Silbergleit, A., Jacobson, G., Benninger, M. S., et al. (1997). The voice handicap index (VHI): Development and validation. *American Journal of Speech-Language Pathology, 6*, 66-70.

Jacobson, L. D., Edwards, A. G., Granier, S. K., & Butler, C. C. (1997). Evidence-based medicine and general practice. *Br J Gen Pract, 47*(420), 449-452.

Jakobsdottir, J., Gorin, M. B., Conley, Y. P., Ferrell, R. E., & Weeks, D. E. (2009). Interpretation of genetic association studies: markers with replicated highly significant odds ratios may be poor classifiers. *PLoS Genet, 5*(2), e1000337.

Janeway, C. (1989). Immunogenicity signals 1,2,3 ... and 0. *Immunol Today, 10*(9), 283-286.

Janeway, C. A., Jr. (1992). The immune system evolved to discriminate infectious nonself from noninfectious self. *Immunol Today, 13*(1), 11-16.

Jenicek, M. (2006). The hard art of soft science: Evidence-Based Medicine, Reasoned Medicine or both? *J Eval Clin Pract, 12*(4), 410-419.

Jiang, D., Liang, J., & Noble, P. W. (2007). Hyaluronan in tissue injury and repair. *Annu Rev Cell Dev Biol, 23*, 435-461.

Jiang, J. J., Diaz, C. E., & Hanson, D. G. (1998). Finite modeling of vocal fold vibration in normal phonation and hyperfunctional dysphonia: implications for the pathogenesis of vocal nodules. *Ann Otol Rhinol Laryngol, 107*, 603-610.

Johns, M. M. (2003). Update on the etiology, diagnosis, and treatment of vocal fold nodules, polyps, and cysts. *Curr Opin Otolaryngol Head Neck Surg, 11*(6), 456-461.

Kammouni, W., Figarella, C., Marchand, S., & Merten, M. (1997). Altered cytokine production by cystic fibrosis tracheal gland serous cells. *Infect Immun, 65*(12), 5176-5183.

Keller, C., Steensberg, A., Pilegaard, H., Osada, T., Saltin, B., Pedersen, B. K., et al. (2001). Transcriptional activation of the IL-6 gene in human contracting skeletal muscle: influence of muscle glycogen content. *Faseb J, 15*(14), 2748-2750.

Kerkhoffs, G. M., Struijs, P. A., Marti, R. K., Assendelft, W. J., Blankevoort, L., & van Dijk, C. N. (2002). Different functional treatment strategies for acute lateral ankle ligament injuries in adults. *Cochrane Database Syst Rev*(3), CD002938.

Kerkhoffs, G. M., Struijs, P. A., Marti, R. K., Blankevoort, L., Assendelft, W. J., & van Dijk, C. N. (2003). Functional treatments for acute ruptures of the lateral ankle ligament: a systematic review. *Acta Orthop Scand, 74*(1), 69-77.

Keylock, K. T., Vieira, V. J., Wallig, M. A., DiPietro, L. A., Schrementi, M., & Woods, J. A. (2008). Exercise accelerates cutaneous wound healing and decreases wound inflammation in aged mice. *Am J Physiol Regul Integr Comp Physiol, 294*(1), R179-184.

Kitano, H. (2002a). Computational systems biology. *Nature, 420*(6912), 206-210.

Kitano, H. (2002b). Systems biology: a brief overview. *Science, 295*(5560), 1662-1664.

Klune, J. R., Dhupar, R., Cardinal, J., Billiar, T. R., & Tsung, A. (2008). HMGB1: endogenous danger signaling. *Mol Med, 14*(7-8), 476-484.

Kolb, M., Margetts, P. J., Anthony, D. C., Pitossi, F., & Gauldie, J. (2001). Transient expression of IL-1beta induces acute lung injury and chronic repair leading to pulmonary fibrosis. *J Clin Invest, 107*(12), 1529-1536.

Kotby, M. N., El-Sady, S. R., Basiouny, S. E., Abou-Rass, Y. A., & Hegazi, M. A. (1991). Efficacy of the accent method of voice therapy. *Journal of Voice, 5*, 316-320.

Koufman, J. A., & Blalock, P. D. (1989). Is voice rest never indicated? *J. Voice, 3*(1), 87-91.

Kuang, P. P., Zhang, X. H., Rich, C. B., Foster, J. A., Subramanian, M., & Goldstein, R. H. (2007). Activation of elastin transcription by transforming growth factor-beta in human lung fibroblasts. *Am J Physiol Lung Cell Mol Physiol, 292*(4), L944-952.

Kumar, R., Clermont, G., Vodovotz, Y., & Chow, C. C. (2004). The dynamics of acute inflammation. *J Theor Biol, 230*(2), 145-155.

Kvist, J. (2004). Rehabilitation following anterior cruciate ligament injury: current recommendations for sports participation. *Sports Med, 34*(4), 269-280.

Leask, A., & Abraham, D. J. (2004). TGF-beta signaling and the fibrotic response. *Faseb J, 18*(7), 816-827.

Lee, J. Y., & Spicer, A. P. (2000). Hyaluronan: a multifunctional, megaDalton, stealth molecule. *Curr Opin Cell Biol, 12*(5), 581-586.

Li, N. Y., Verdolini, K., Clermont, G., Mi, Q., Rubinstein, E. N., Hebda, P. A., et al. (2008). A patient-specific in silico model of inflammation and healing tested in acute vocal fold injury. *PLoS ONE, 3*(7), e2789.

Li, N. Y. K., Verdolini, K., Clermont, G., Mi, Q., Hebda, P., & Vodovotz, Y. (2007). *A patient-specific in silico model of inflammation and healing tested in acute vocal fold injury*. Paper presented at the The 8th International Conference on Systems Biology.

Li, N. Y. K., Verdolini, K., Clermont, G., Mi, Q., Hebda, P. A., & Vodovotz, Y. (2006). *Simulating acute phonotrauma: a comparison of agent-based and equation-based models.* Paper presented at the 5th International Conference on Voice Physiology and Biomechanics, Tokyo, Japan.

Li, N. Y. K., Verdolini, K., Clermont, G., Mi, Q., Vodovotz, Y., & Hebda, P. A. (2006). Agent-based simulation of acute phonotrauma in cases with various cytokine profiles. *Journal of Critical Care, 21*(4), 347.

Li, N. Y. K., Verdolini, K., Qi, Y., Vodovotz, Y., & Hebda, P. (2005). *An Agent-Based Model of Vocal Fold Inflammation and Wound Healing*. Paper presented at the Voice Foundation 34th Annual Symposium.

Lim, X., Bless, D. M., Munoz-Del-Rio, A., & Welham, N. V. (2008). Changes in cytokine signaling and extracellular matrix production induced by inflammatory factors in cultured vocal fold fibroblasts. *Ann Otol Rhinol Laryngol, 117*(3), 227-238.

Lim, X., Tateya, I., Tateya, T., Munoz-Del-Rio, A., & Bless, D. M. (2006). Immediate inflammatory response and scar formation in wounded vocal folds. *Ann Otol Rhinol Laryngol, 115*(12), 921-929.

Lin, Z. Q., Kondo, T., Ishida, Y., Takayasu, T., & Mukaida, N. (2003). Essential involvement of IL-6 in the skin wound-healing process as evidenced by delayed wound healing in IL-6-deficient mice. *J Leukoc Biol, 73*(6), 713-721.

Little, M. (2003). 'Better than numbers...' A gentle critique of evidence-based medicine. *ANZ J Surg, 73*(4), 177-182; discussion 182.

Losa Garcia, J. E., Rodriguez, F. M., Martin de Cabo, M. R., Garcia Salgado, M. J., Losada, J. P., Villaron, L. G., et al. (1999). Evaluation of inflammatory cytokine secretion by human alveolar macrophages. *Mediators Inflamm, 8*(1), 43-51.

Lowry, S. F., & Calvano, S. E. (2008). Challenges for modeling and interpreting the complex biology of severe injury and inflammation. *J Leukoc Biol, 83*(3), 553-557.

Luo, Y., Kobler, J. B., Zeitels, S. M., & Langer, R. (2006). Effects of growth factors on extracellular matrix production by vocal fold fibroblasts in 3-dimensional culture. *Tissue Eng, 12*(12), 3365-3374.

Lynch, J. P., 3rd, Standiford, T. J., Rolfe, M. W., Kunkel, S. L., & Strieter, R. M. (1992). Neutrophilic alveolitis in idiopathic pulmonary fibrosis. The role of interleukin-8. *Am Rev Respir Dis, 145*(6), 1433-1439.

Ma, E., & Yiu, E. (2001). Voice activity and participation profile: Assessing the impact of voice disorders on daily activities. *Journal of Speech, Language, and Hearing Research, 44*, 511-524.

MacKenzie, K., Millar, A., Wilson, J. A., Sellars, C., & Deary, I. J. (2001). Is voice therapy an effective treatment for dysphonia? A randomised controlled study. *BMJ, 323*, 1-6.

Madruga de Melo, E. C., Lemos, M., Aragao Ximenes Filho, J., Sennes, L. U., Nascimento Saldiva, P. H., & Tsuji, D. H. (2003). Distribution of collagen in the lamina propria of the human vocal fold. *Laryngoscope, 113*(12), 2187-2191.

Martin, G. S., Mannino, D. M., Eaton, S., & Moss, M. (2003). The epidemiology of sepsis in the United States from 1979 through 2000. *N Engl J Med, 348*(16), 1546-1554.

Martin, P. (1997). Wound Healing-aiming for perfect skin regeneration. *Science, 276*(5309), 75-81.

Martinet, Y., Menard, O., Vaillant, P., Vignaud, J. M., & Martinet, N. (1996). Cytokines in human lung fibrosis. *Arch Toxicol Suppl, 18*, 127-139.

Mast, B. A., & Schultz, G. S. (1996). Interactions of cytokines, growth factors, and proteases in acute and chronic wounds. *Wound Repair Regen, 4*(4), 411-420.

Matsumoto, K., Okazaki, H., & Nakamura, T. (1992). Up-regulation of hepatocyte growth factor gene expression by interleukin-1 in human skin fibroblasts. *Biochem Biophys Res Commun, 188*(1), 235-243.

Matzinger, P. (2002). The danger model: a renewed sense of self. *Science, 296*(5566), 301-305.

Matzinger, P. (2007). Friendly and dangerous signals: is the tissue in control? *Nat Immunol, 8*(1), 11-13.

Maus, U., Rosseau, S., Knies, U., Seeger, W., & Lohmeyer, J. (1998). Expression of pro-inflammatory cytokines by flow-sorted alveolar macrophages in severe pneumonia. *Eur Respir J, 11*(3), 534-541.

McCrory, E. (2001). Voice therapy outcomes in vocal fold nodules: a retrospective audit. *Int J Lang Commun Disord, 36 Suppl*, 19-24.

Medzhitov, R. (2008). Origin and physiological roles of inflammation. *Nature, 454*(7203), 428-435.

Mi, Q., Riviere, B., Clermont, G., Steed, D. L., & Vodovotz, Y. (2007). Agent-based modeling of inflammation and wound healing: insights into diabetic foot ulcer pathology and the role of transforming growth factor-beta 1. *Wound Repair and Regeneration, 15*(5), 671-682.

Miles, A., Bentley, P., Polychronis, A., Grey, J., & Price, N. (1998). Recent progress in health services research: on the need for evidence-based debate. *J Eval Clin Pract, 4*(4), 257-265.

Miles, A., Charlton, B., Bentley, P., Polychronis, A., Grey, J., & Price, N. (2000). New perspectives in the evidence-based healthcare debate. *J Eval Clin Pract, 6*(2), 77-84.

Miles, A., Grey, J. E., Polychronis, A., Price, N., & Melchiorri, C. (2003). Current thinking in the evidence-based health care debate. *J Eval Clin Pract, 9*(2), 95-109.

Miles, A., Grey, J. E., Polychronis, A., Price, N., & Melchiorri, C. (2004). Developments in the evidence-based health care debate - 2004. *J Eval Clin Pract, 10*(2), 129-142.

Miles, A., Polychronis, A., & Grey, J. E. (2006). The evidence-based health care debate - 2006. Where are we now? *J Eval Clin Pract, 12*(3), 239-247.

Mitchell, S. D. (Ed.). (2003). *Biological Complexity and Integrative Pluralism*. Cambridge: Cambridge University Press.

Moulin, V. (1995). Growth factors in skin wound healing. *Eur J Cell Biol, 68*(1), 1-7.

Mulligan, B. (1995). *Manual Therapy "NAGS," "SNAGS," "MWMSs" etc* (3rd ed.). Wellington, New Zealand: Plane View Service.

Murry, T., & Woodson, G. E. (1992). A comparison of three methods for the management of vocal fold nodules. *Journal of Voice, 6*(3), 271-276.

Nakamura, H., Fujishima, S., Waki, Y., Urano, T., Sayama, K., Sakamaki, F., et al. (1995). Priming of alveolar macrophages for interleukin-8 production in patients with idiopathic pulmonary fibrosis. *Am J Respir Crit Care Med, 152*(5 Pt 1), 1579-1586.

Nathan, C. (2002). Points of control in inflammation. *Nature, 420*(6917), 846-852.

Neves, B. M. J., Neto, J. G., & Pontes, P. (2004). Histopathological and immunohistochemical differentiation of epithelial alterations in vocal nodule comparing to polyps and to laryngeal edema. *Brazillian Journal of Otolaryngology, 70*(4), 439-448.

Nielsen, A. R., & Pedersen, B. K. (2007). The biological roles of exercise-induced cytokines: IL-6, IL-8, and IL-15. *Appl Physiol Nutr Metab, 32*(5), 833-839.

Noble, P. W. (2002). Hyaluronan and its catabolic products in tissue injury and repair. *Matrix Biol, 21*(1), 25-29.

O'Reilly, P. J., Gaggar, A., & Blalock, J. E. (2008). Interfering with extracellular matrix degradation to blunt inflammation. *Curr Opin Pharmacol*.

Page-McCaw, A., Ewald, A. J., & Werb, Z. (2007). Matrix metalloproteinases and the regulation of tissue remodelling. *Nat Rev Mol Cell Biol, 8*(3), 221-233.

Palmon, A., Roos, H., Reichenberg, E., Grosskop, A., Bar Kana, I., Pitaru, S., et al. (2001). Basic fibroblast growth factor suppresses tropoelastin gene expression in cultured human periodontal fibroblasts. *J Periodontal Res, 36*(2), 65-70.

Palumbo, R., Sampaolesi, M., De Marchis, F., Tonlorenzi, R., Colombetti, S., Mondino, A., et al. (2004). Extracellular HMGB1, a signal of tissue damage, induces mesoangioblast migration and proliferation. *J Cell Biol, 164*(3), 441-449.

Panayi, G. S., Corrigall, V. M., & Henderson, B. (2004). Stress cytokines: pivotal proteins in immune regulatory networks; Opinion. *Curr Opin Immunol, 16*(4), 531-534.

Parker, M. (2001). The ethics of evidence-based patient choice. *Health Expect, 4*(2), 87-91.

Parks, W. C. (1999). Matrix metalloproteinases in repair. *Wound Repair Regen, 7*(6), 423-432.

Parks, W. C., Wilson, C. L., & Lopez-Boado, Y. S. (2004). Matrix metalloproteinases as modulators of inflammation and innate immunity. *Nat Rev Immunol, 4*(8), 617-629.

Paungmali, A., O'Leary, S., Sowvlis, T., & Vicenzino, B. (2003). Hypoalgesic and sympathoexcitatory effects of mobilization with movement for lateral epicodylalgia. *Physical Therapy, 83*(4), 374-383.

Peake, J., Nosaka, K., & Suzuki, K. (2005). Characterization of inflammatory responses to eccentric exercise in humans. *Exerc Immunol Rev, 11*, 64-85.

Peake, J. M., Suzuki, K., Hordern, M., Wilson, G., Nosaka, K., & Coombes, J. S. (2005). Plasma cytokine changes in relation to exercise intensity and muscle damage. *Eur J Appl Physiol, 95*(5-6), 514-521.

Pedersen, B. K., & Hoffman-Goetz, L. (2000). Exercise and the immune system: regulation, integration, and adaptation. *Physiol Rev, 80*(3), 1055-1081.

Pedersen, B. K., Steensberg, A., Fischer, C., Keller, C., Keller, P., Plomgaard, P., et al. (2003). Searching for the exercise factor: is IL-6 a candidate? *J Muscle Res Cell Motil, 24*(2-3), 113-119.

Pedersen, B. K., Steensberg, A., Fischer, C., Keller, C., Ostrowski, K., & Schjerling, P. (2001). Exercise and cytokines with particular focus on muscle-derived IL-6. *Exerc Immunol Rev, 7*, 18-31.

Petersen, A. M., & Pedersen, B. K. (2005). The anti-inflammatory effect of exercise. *J Appl Physiol, 98*(4), 1154-1162.

Petersen, A. M., & Pedersen, B. K. (2006). The role of IL-6 in mediating the anti-inflammatory effects of exercise. *J Physiol Pharmacol, 57 Suppl 10*, 43-51.

Peterson, K. L., Verdolini-Marston, K., Barkmeir, J. M., & Hoffman, H. T. (1994). Comparison of aerodynamic and electroglottographic parameters in evaluating clinically relevant voicing patterns. *Ann Otol Rhinol Laryngol, 103*(5 Pt 1), 335-346.

Pijnenburg, A. C., Van Dijk, C. N., Bossuyt, P. M., & Marti, R. K. (2000). Treatment of ruptures of the lateral ankle ligaments: a meta-analysis. *J Bone Joint Surg Am, 82*(6), 761-773.

Prince, J. M., Levy, R. M., Bartels, J., Baratt, A., Kane, J. M., 3rd, Lagoa, C., et al. (2006). In silico and in vivo approach to elucidate the inflammatory complexity of CD14-deficient mice. *Mol Med, 12*(4-6), 88-96.

Prohaszka, Z., & Fust, G. (2004). Immunological aspects of heat-shock proteins-the optimum stress of life. *Mol Immunol, 41*(1), 29-44.

Raaijmakers, M. F., Dekker, J., & Dejonckere, P. H. (1998). Diagnostic assessment and treatment goals in logopedics: impairments, disabilities and handicaps *Folia Phoniatr Logop, 50*(2), 71-79.

Railback, S. F. (2001). Getting "results": the pattern-oriented approach to analyze natural systems with individual-based models. . *Natural Resource Modeling, 14*, 465-474.

Rattenbury, H. J., Carding, P. N., & Finn, P. (2004). Evaluating the effectiveness and efficiency of voice therapy using transnasal flexible laryngoscopy: a randomized controlled trial. *J Voice, 18*(4), 522-533.

Roberts, A. B. (1998). Molecular and cell biology of TGF-beta. *Miner Electrolyte Metab, 24*(2-3), 111-119.

Robson, M. C., Steed, D. L., & Franz, M. G. (2001). Wound healing: biologic features and approaches to maximize healing trajectories. *Curr Probl Surg, 38*(2), 72-140.

Rom, W. N. (1991). Relationship of inflammatory cell cytokines to disease severity in individuals with occupational inorganic dust exposure. *Am J Ind Med, 19*(1), 15-27.

Romeo, D. S., Park, K., Roberts, A. B., Sporn, M. B., & Kim, S. J. (1993). An element of the transforming growth factor-beta 1 5'-untranslated region represses translation and specifically binds a cytosolic factor. *Mol Endocrinol, 7*(6), 759-766.

Rosen, C. A., Li, N. Y. K., Hebda, P. A., & Verdolini, K. (in preparation). Correspondence of inflammatory mediators in laryngeal secretions and laryngeal tissue.

Rousseau, B., Hirano, S., Chan, R. W., Welham, N. V., Thibeault, S. L., Ford, C. N., et al. (2004). Characterization of chronic vocal fold scarring in a rabbit model. *J Voice, 18*(1), 116-124.

Rousseau, B., Hirano, S., Scheidt, T. D., Welham, N. V., Thibeault, S. L., Chan, R. W., et al. (2003). Characterization of vocal fold scarring in a canine model. *Laryngoscope, 113*, 620-627.

Roy, N., Gray, S. D., Simon, M., Dove, H., Corbin-Lewis, K., & Stemple, J. C. (2001). An evaluation of the effects of two treatment approaches for teachers with voice disorders: A prospective randomized clinical trial. *Journal of Speech, Language, and Hearing Research, 44*, 286-296.

Roy, N., Weinrich, B., Gray, S. D., Tanner, K., Stemple, J. C., & Sapienza, C. M. (2003). Three treatments for teachers with voice disorders: a randomized clinical trial. *J Speech Lang Hear Res, 46*(3), 670-688.

Roy, N., Weinrich, B., Gray, S. D., Tanner, K., Toledo, S. W., Dove, H., et al. (2002). Voice amplification versus vocal hygiene instruction for teachers with voice disorders: A treatment outcomes study. *Journal of Speech, Language, and Hearing Research, 45*, 625-638.

Rubartelli, A., & Lotze, M. T. (2007). Inside, outside, upside down: damage-associated molecular-pattern molecules (DAMPs) and redox. *Trends Immunol, 28*(10), 429-436.

Sackett, D. L., Richardson, W. S., Rosenberg, W., & Haynes, R. B. (1997). *Evidence-Based Medicine: How to Practice and Teach EBM*. New York: Churchill Livingstone.

Salter, R. B. (1994). The physiologic basis of continuous passive motion for articular cartilage healing and regeneration. *Hand Clinics, 10*, 211.

Salter, R. B. (1996). History of rest and motion and the scientific basis for early continuous passive motion. *Hand Clinics, 12*, 1-11.

Sandulache, V. C., Chafin, J. B., Li-Korotky, H. S., Otteson, T. D., Dohar, J. E., & Hebda, P. A. (2007). Elucidating the role of interleukin 1beta and prostaglandin E2 in upper airway mucosal wound healing. *Arch Otolaryngol Head Neck Surg, 133*(4), 365-374.

Sataloff, R. T. (1997). *Voice rest* (2nd ed.). San Diego: Singular Publishing Group, Inc.

Sataloff, R. T. (2001). Vocal health. from http://www.voiceofundation.org/vfSATALOFFVocalHlt.htm

Scheibner, K. A., Lutz, M. A., Boodoo, S., Fenton, M. J., Powell, J. D., & Horton, M. R. (2006). Hyaluronan fragments act as an endogenous danger signal by engaging TLR2. *J Immunol, 177*(2), 1272-1281.

Schiller, M., Javelaud, D., & Mauviel, A. (2004). TGF-beta-induced SMAD signaling and gene regulation: consequences for extracellular matrix remodeling and wound healing. *J Dermatol Sci, 35*(2), 83-92.

Sellars, C., Carding, P. N., Deary, I. J., MacKenzie, K., & Wilson, J. A. (2002). Characterization of effective primary voice therapy for dysphonia. *The Journal of Laryngology & Otology, 116*, 1014-1018.

Serhan, C. N., Chiang, N., & Van Dyke, T. E. (2008). Resolving inflammation: dual anti-inflammatory and pro-resolution lipid mediators. *Nat Rev Immunol, 8*(5), 349-361.

Shi, Y., Evans, J. E., & Rock, K. L. (2003). Molecular identification of a danger signal that alerts the immune system to dying cells. *Nature, 425*(6957), 516-521.

Simberg, S. (2004). *Prevalence of vocal symptoms and voice disorders among teacher students and teachers and a model of early intervention.* Unpublished Dissertation, University of Helsinki, Helsinki, Finland.

Simberg, S., Sala, E., Tuomainen, J., Sellman, J., & Ronnemaa, A. M. (2006). The effectiveness of group therapy for students with mild voice disorders: a controlled clinical trial. *J Voice, 20*(1), 97-109.

Singer, A. J., & Clark, R. A. (1999). Cutaneous wound healing. *N Engl J Med, 341*(10), 738-746.

Smallwood, R. H., Holcombe, W. M. L., & Walker, D. C. (2004). Development and validation of computational models of cellular interaction. *Journal of Molecular Histology, 35*, 659-665.

Smith, E., Verdolini, K., Gray, S., Nichols, S., Lemke, J., Barkmeier, J., et al. (1996). Effects of voice disorders on quality of life. *Journal of Medical Speech-Language Pathology, 4*, 223-244.

Smith, J. A., & Pyne, D. B. (1997). Exercise, training, and neutrophil function. *Exerc Immunol Rev, 3*, 96-116.

Smith, K. D., & Bolouri, H. (2005). Dissecting innate immune responses with the tools of systems biology. *Curr Opin Immunol, 17*(1), 49-54.

Speyer, R., Weinke, G., Hosseini, E. G., Kempen, E. G., Kersing, W., & Dejonckere, P. H. (2002). Effects of voice therapy as objectively evaluated by digitized laryngeal stroboscopic imaging. *Ann Otol Rhinol Laryngol, 111*(10), 902-908.

Speyer, R., Wieneke, G. H., & Dejonckere, P. H. (2004a). Documentation of progress in voice therapy: perceptual, acoustic, and laryngostroboscopic findings pretherapy and posttherapy. *J Voice, 18*(3), 325-340.

Speyer, R., Wieneke, G. H., & Dejonckere, P. H. (2004b). Self-assessment of voice therapy for chronic dysphonia. *Clin Otolaryngol Allied Sci, 29*(1), 66-74.

Speyer, R., Wieneke, G. H., van Wijck-Warnaar, I., & Dejonckere, P. H. (2003). Effects of voice therapy on the voice range profiles of dysphonic patients. *J Voice, 17*(4), 544-556.

Steensberg, A., Fischer, C. P., Keller, C., Moller, K., & Pedersen, B. K. (2003). IL-6 enhances plasma IL-1ra, IL-10, and cortisol in humans. *Am J Physiol Endocrinol Metab, 285*(2), E433-437.

Stemple, J. C., Lee, L., D'Amico, B., & Pickup, B. (1994). Efficacy of vocal function exercises as a method of improving voice production. *Journal of Voice, 8*(3), 271-278.

Stern, R., Asari, A. A., & Sugahara, K. N. (2006). Hyaluronan fragments: an information-rich system. *Eur J Cell Biol, 85*(8), 699-715.

Stramer, B. M., Mori, R., & Martin, P. (2007). The inflammation-fibrosis link? A Jekyll and Hyde role for blood cells during wound repair. *J Invest Dermatol, 127*(5), 1009-1017.

Summerton, N. (1997). Evidence-based medicine. Literacy criticism. *Health Serv J, 107*(5572), 27.

Takizawa, H. (1998). Airway epithelial cells as regulators of airway inflammation (Review). *Int J Mol Med, 1*(2), 367-378.

Takizawa, H. (2005). Bronchial epithelial cells in allergic reactions. *Curr Drug Targets Inflamm Allergy, 4*(3), 305-311.

Takizawa, H., Ohtoshi, T., Yamashita, N., Oka, T., & Ito, K. (1996). Interleukin 6-receptor expression on human bronchial epithelial cells: regulation by IL-1 and IL-6. *Am J Physiol, 270*(3 Pt 1), L346-352.

Takizawa, H., Satoh, M., Okazaki, H., Matsuzaki, G., Suzuki, N., Ishii, A., et al. (1997). Increased IL-6 and IL-8 in bronchoalveolar lavage fluids (BALF) from patients with sarcoidosis: correlation with the clinical parameters. *Clin Exp Immunol, 107*(1), 175-181.

Tateya, I., Tateya, T., & Bless, D. M. (2004). *Homeostasis of hyaluronic acid in scarred rat vocal folds*. Paper presented at the International Conference on voice Physiology and Biomechanics.

Tateya, I., Tateya, T., Lim, X., Sohn, J. H., & Bless, D. M. (2006). Cell production in injured vocal folds: a rat study. *Ann Otol Rhinol Laryngol, 115*(2), 135-143.

Tateya, T., Tateya, I., & Bless, D. M. (2006). Collagen subtypes in human vocal folds. *Ann Otol Rhinol Laryngol, 115*(6), 469-476.

Tateya, T., Tateya, I., Sohn, J. H., & Bless, D. M. (2005). Histologic characterization of rat vocal fold scarring. *Ann Otol Rhinol Laryngol, 114*(3), 183-191.

Tateya, T., Tateya, I., Sohn, J. H., & Bless, D. M. (2006). Histological study of acute vocal fold injury in a rat model. *Ann Otol Rhinol Laryngol, 115*(4), 285-292.

Taylor, K. R., Trowbridge, J. M., Rudisill, J. A., Termeer, C. C., Simon, J. C., & Gallo, R. L. (2004). Hyaluronan fragments stimulate endothelial recognition of injury through TLR4. *J Biol Chem, 279*(17), 17079-17084.

Thibeault, S. L., Bless, D. M., & Gray, S. D. (2003). Interstitial protein alterations in rabbit vocal fold with scar. *J Voice, 17*(3), 377-383.

Thibeault, S. L., Gray, S. D., Bless, D. M., Chan, R. W., & Ford, C. N. (2002). Histologic and Rheologic characterization of vocal fold scarring. *Journal of Voice, 16*(1), 96-104.

Thibeault, S. L., Gray, S. D., Li, W., Ford, C. N., Smith, M. E., & Davis, R. K. (2002). Genotypic and phenotypic expression of vocal fold polyps and Reinke's edema: a preliminary study. *Ann Otol Rhinol Laryngol, 111*(4), 302-309.

Thibeault, S. L., Rousseau, B., Welham, N. V., Hirano, S., & Bless, D. M. (2004). Hyaluronan levels in acute vocal fold scar. *Laryngoscope, 114*(4), 760-764.

Thornton, G. M., Shrive, N. G., & Frank, C. B. (2003). Healing ligaments have decreased cyclic modulus compared to normal ligaments and immobilization further compromises healing ligament response to cyclic loading. *Journal of Orthopaedic Research, 21*, 716-722.

Threlkeld, A. J. (1992). Manual Therapy: An American Physical Therapy Association Monograph. *Physical Therapy*, 59-68.

Tidball, J. G. (1995). Inflammatory cell response to acute muscle injury. *Med Sci Sports Exerc, 27*(7), 1022-1032.

Titze, I. R. (1994). Mechanical stress in phonation. *J Voice, 8*(2), 99-105.

Titze, I. R., Hitchcock, R. W., Broadhead, K., Webb, K., Li, W., Gray, S. D., et al. (2004). Design and validation of a bioreactor for engineering vocal fold tissues under combined tensile and vibrational stresses. *J Biomech, 37*(10), 1521-1529.

Titze, I. R., Svec, J. G., & Popolo, P. S. (2003). Vocal dose measures: quantifying accumulated vibration exposure in vocal fold tissues. *J Speech Lang Hear Res, 46*(4), 919-932.

Toumi, H., F'Guyer, S., & Best, T. M. (2006). The role of neutrophils in injury and repair following muscle stretch. *J Anat, 208*(4), 459-470.

Vaillant, P., Menard, O., Vignaud, J. M., Martinet, N., & Martinet, Y. (1996). The role of cytokines in human lung fibrosis. *Monaldi Arch Chest Dis, 51*(2), 145-152.

van den Berg, A., Kuiper, M., Snoek, M., Timens, W., Postma, D. S., Jansen, H. M., et al. (2005). Interleukin-17 induces hyperresponsive interleukin-8 and interleukin-6 production to tumor necrosis factor-alpha in structural lung cells. *Am J Respir Cell Mol Biol, 33*(1), 97-104.

van der Merwe, A. (2004). The Voice Use Reduction Program. *American Journal of Speech-Language Pathology, 13*, 208-218.

van Eijk, L. T., Dorresteijn, M. J., & Pikkers, P. (2008). Gender influences in vivo human responses to endotoxin. *Shock, 29*(3), 422; author reply 422-423.

van Eijk, L. T., Dorresteijn, M. J., Smits, P., van der Hoeven, J. G., Netea, M. G., & Pickkers, P. (2007). Gender differences in the innate immune response and vascular reactivity following the administration of endotoxin to human volunteers. *Crit Care Med, 35*(6), 1464-1469.

Verdolini-Marston, K., Burke, M. K., Lessac, A., Glaze, L., & Caldwell, E. (1995). Preliminary study of two methods of treatment for laryngeal nodules. *Journal of Voice, 9*(1), 74-85.

Verdolini, K. (2000). Resonant Voice Therapy. In J. C. Stemple (Ed.), *Voice Therapy: Clinical Studies* (pp. 46-62). San Deigo: Singular Publishing Group.

Verdolini, K., Druker, D. G., Palmer, P. M., & Samawi, H. (1998). Laryngeal adduction in resonant voice. *J Voice, 12*(3), 315-327.

Verdolini, K., Li, N. Y. K., Branski, R. C., Rosen, C. A., Urban, E. G., & Hebda, P. A. (in preparation). The effect of targeted vocal exercise on recovery from acute inflammation.

Verdolini, K., Rosen, C. A., & Branski, R. C. (2006). *Classification Manual for Voice Disorders-I*. New Jersey: Lawrence Erlbaum Associates, Inc. .

Verdolini, K., Rosen, C. A., Branski, R. C., & Hebda, P. A. (2003). Shifts in biochemical markers associated with wound healing in laryngeal secretions following phonotrauma: A preliminary study. *Annals of Otology, Rhinology, & Laryngology, 112*(12), 1021-1025.

Vicsek, T. (2002). The bigger picture. *Nature, 418*, 131.

Vintturi, J., Alku, P., Lauri, E. R., Sala, E., Sihvo, M., & Vilkman, E. (2001). The effects of post-loading rest on acoustic parameters with special reference to gender and ergonomic factors. *Folia Phoniatr Logop, 53*(6), 338-350.

Visser, N. A., de Koning, M. H., Lammi, M. J., Hakkinen, T., Tammi, M., & van Kampen, G. P. (1998). Increase of decorin content in articular cartilage following running. *Connect Tissue Res, 37*(3-4), 295-302.

Vodovotz, Y. (2006). Deciphering the complexity of acute inflammation using mathematical models. *Immunol Res, 36*(1-3), 237-245.

Vodovotz, Y., Clermont, G., Chow, C., & An, G. (2004). Mathematical models of the acute inflammatory response. *Current Opinion in Critical Care, 10*(5), 383-390.

Vodovotz, Y., Csete, M., Bartels, J., Chang, S., & An, G. (2008). Translational systems biology of inflammation. *PLoS Comput Biol, 4*(4), e1000014.

Voit, E. O. (2009). A systems-theoretical framework for health and disease: Inflammation and preconditioning from an abstract modeling point of view. *Math Biosci, 217*(1), 11-18.

Walker, D. C., Hill, G., Wood, S. M., Smallwood, R. H., & Southgate, J. (2004). Agent-based computational modeling of wounded epiethelial cell monolayers. *IEEE Transactions on Nonobioscience, 3*(3), 153-163.

Walter, J. B., & Israel, M. S. (Eds.). (1987). *General Pathology*. Churchill: Livingstone.

Wang, C. H., Liu, C. Y., Wan, Y. L., Chou, C. L., Huang, K. H., Lin, H. C., et al. (2005). Persistence of lung inflammation and lung cytokines with high-resolution CT abnormalities during recovery from SARS. *Respir Res, 6*, 42.

Wang, H., Yang, H., & Tracey, K. J. (2004). Extracellular role of HMGB1 in inflammation and sepsis. *J Intern Med, 255*(3), 320-331.

Wang, J. H., & Thampatty, B. P. (2006). An introductory review of cell mechanobiology. *Biomech Model Mechanobiol, 5*(1), 1-16.

Ward, P. D., Thiebault, S. L., & Gray, S. D. (2002). Hyaluronic acid: Its role in voice. *Journal of Voice, 16*(3), 303-309.

Welham, N. V., Lim, X., Tateya, I., & Bless, D. M. (2008). Inflammatory factor profiles one hour following vocal fold injury. *Ann Otol Rhinol Laryngol, 117*(2), 145-152.

Weng, J., Mohan, R. R., Li, Q., & Wilson, S. E. (1997). IL-1 upregulates keratinocyte growth factor and hepatocyte growth factor mRNA and protein production by cultured stromal fibroblast cells: interleukin-1 beta expression in the cornea. *Cornea, 16*(4), 465-471.

Werner, S., & Grose, R. (2003). Regulation of wound healing by growth factors and cytokines. *Physiol Rev, 83*(3), 835-870.

Whitcomb, D. C., Aoun, E., Vodovotz, Y., Clermont, G., & Barmada, M. M. (2005). Evaluating disorders with a complex genetics basis. the future roles of meta-analysis and systems biology. *Dig Dis Sci, 50*(12), 2195-2202.

Williams, J. M., Moran, M., Thonar, E. J., & Salter, R. B. (1994). Continuous passive motion stimulates repair of rabbit knee articular cartilage after matrix proteoglycan loss. *Clin Orthop, 304*, 252-262.

Wingate, J. M., Brown, W. S., Shrivastav, R., Davenport, P., & Sapienza, C. M. (2006). Treatment Outcomes for Professional Voice Users. *J Voice*.

Witte, M. B., & Barbul, A. (1997). General principles of wound healing. *Surgical Clinics of North America, 77*(3), 509-528.

Wolkenhauer, O. (2001). Systems biology: the reincarnation of systems theory applied in biology? *Brief Bioinform, 2*(3), 258-270.

Woods, J. A., Vieira, V. J., & Keylock, K. T. (2006). Exercise, inflammation, and innate immunity. *Neurol Clin, 24*(3), 585-599.

Xaubet, A., Agusti, C., Luburich, P., Barbera, J. A., Carrion, M., Ayuso, M. C., et al. (1998). Interleukin-8 expression in bronchoalveolar lavage cells in the evaluation of alveolitis in idiopathic pulmonary fibrosis. *Respir Med, 92*(2), 338-344.

Yang, H., & Tracey, K. J. (2005). High mobility group box 1 (HMGB1). *Crit Care Med, 33*(12 Suppl), S472-474.

Ylitalo, R., & Hammarberg, B. (2000). Voice characteristics, effects of voice therapy, and long-term follow-up of contact granuloma patients. *J Voice, 14*(4), 557-566.

Yun, S. J., Choi, M. S., Piao, M. S., Lee, J. B., Kim, S. J., Won, Y. H., et al. (2008). Serum lactate dehydrogenase is a novel marker for the evaluation of disease severity in the early stage of toxic epidermal necrolysis. *Dermatology, 217*(3), 254-259.

Zerbouni, E. A. (2008). Strategic vision for the future: from curative to preemptive medicine. from http://www.nih.gov/strategicvision.htm