# The effect of Botulinum Toxin A on keloid scarring

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## List of abbreviations

ANOVA – One-way analysis of variance test
BTA - OnabotulinumtoxinA
CINAHL - Cumulative Index to Nursing and Allied Health Literature
DoE – Directness of evidence
I/L – intralesional
H&E – hematoxylin and eosin
PICOTS – Population, intervention, comparison, outcomes, timing, study design
PRISMA – Preferred reporting items for systematic reviews and meta-analyses
PT – pressure therapy
RoB – Risk of bias
RT – radiation therapy

# Abstract

**Background:** Keloid scarring is a debilitating condition, especially when affecting the head and neck region, making it harder to hide. To date, no consensus exists on an optimal treatment modality. Surgical excision, pressure therapy, steroid injection and radiotherapy remain mainstream in the armamentarium of choices. Few studies exist comparing these treatments in a systematic fashion. However, a novel role of botulinum toxin as a new treatment modality for keloids has emerged in the literature.

**Objective:** This thesis aims to highlight various treatment modalities for auricular keloid scarring by (1) doing a systematic review and (2) characterizing the effect of Onabotulinumtoxin A on keloid scarring using an animal model.

**Methods:** For the review, eligible articles were identified through a comprehensive search of electronic databases. Using predefined inclusion criteria, published articles on auricular keloids were selected and reviewed. For the animal study, keloid scars from four patients were excised and implanted subcutaneously into 28 mice. Three small keloid tissue samples were implanted in each of the 28 mice. One week after implantation, each implant received one of three injections: botulinum toxin A (treatment drug), saline (control) or steroid injection (first line gold standard). The keloid tissue was extracted three weeks post implantation. Weight analysis, immunohistochemistry, and standard hematoxylin and eosin (H&E) pathology were performed on each extracted tissue sample.

**Results**: For the review, 18 articles, encompassing one thousand four hundred and fifteen (1415) auricular keloid patients, were identified. The mean age at diagnosis was 41, with a standard deviation of 2.5 years. The most common site of keloid formation was the earlobe (60%), and piercing was the most common cause (82%). Treatment modalities were often multimodal, with the main modality being surgical excision followed by postoperative adjuvant treatment, such as pressure therapy (64%), post-operative radiotherapy (8%), electrotherapy (6%), cryotherapy (5%) or corticosteroid injection (5%). Recurrence rates were low (13%). For the animal study, pre-post tissue weights paired t-test analysis showed that there was a statistically significant difference between the treatment and control groups ($p<0.05$). Analysis by a blinded pathologist confirmed less collagen bundles in the treatment group. Ki-67 immunohistochemistry, a marker of cell proliferation, showed that there was significantly less staining in the treatment groups.

**Conclusion:** Results show (1) that currently many treatment modalities for auricular keloid scarring are being used with no clear consensus on appropriate protocols, and (2) that OnaBotulinumtoxin A is a promising new treatment to minimize keloid scarring.

# Résumé

**Contexte:** Les cicatrices chéloïdes sont débilitants, surtout dans la région de la tête et du cou qui est plus difficile à cacher. A date, il n'existe aucun consesnsus sur le traitement optimal de cette pathologie. L'exérèse chirurgicale, la thérapie par pression, les injections de corticostéroïdes et la radiothérapie demeurent tout de même les choix principaux. Un clairsemé d'études analysent d'une façon systématique la différence entre ces traitements. De plus, le rôle émergent de la toxine botulique dans le traitement de cette pathologie reste à être clarifié.

**Objectif :** Le but de cette thèse est de souligner les divers traitements des cicatrices chéloïdes de l'oreillette à travers une revue systématique de la littérature et de caractériser le rôle de la toxine botulique à l'aide d'une étude animale.

**Méthodes :** Les articles éligibles ont été identifiés suite à une recherche exhaustive de la base de données électronique. Les articles remplissant les critères d'inclusion ont été étudiés. Pour ce qui est de l'étude animale, des cicatrices chéloïdes ont été prélevées de quatre patients. Trois petits morceaux de tissue ont été implantés en sous-cutanée dans chacune des 28 souris. Une semaine après l'implantantion, chaque tissue à reçu un des traitements suivants : injection de toxine botulique A (médicament étudié), injection de triamcinolone (médicament de première ligne) ou injection de saline (médicament de contrôle). Les tissues chéloïdes ont ensuite été prélevés 3 semaines après l'implantation. Analyse de poids, immunohistochimie, ainsi qu'une hématoxyline et éosine (H&E) ont été effectués pour chaque spécimen.

**Résultats :** Dix-huit (18) articles ont été sélectionnés, comprenant mille quatre cent quinze patients (1415) souffrant de chéloïdes auriculaires. L'âge moyen au diagnostic était de 41, avec une déviation standard de 2.5 ans. La région anatomique la plus impliquée était le lobe d'oreille (60%), avec le piercing impliqué dans la majorité des cas (82%). Les traitements sont multimodales, avec l'exérèse chirurgicale suivi d'une deuxième modalité l'option la plus fréquentés, soit la thérapie de pression (64%), la radiothérapie (8%), l'électrothérapie (6%), la cryothérapie (5%) ou les injection de corticostéroïdes (5%). Le taux de récidives était relativement bas à 13%. En ce qui concerne l'étude animale, une analyse de type paired t-test des poids avant/après des tissus a démontré une différence statistiquement significative entre les groupes de traitement et les contrôles ($p<0.05$). Analyse par une pathologiste en aveugle a confirmé moins de faisceaux de collagène dans le groupe de traitement. Immunohistochimie

Ki-67, marqueur de la prolifération cellulaire, a révélé moins de coloration pour les groupes de traitement.

**Conclusion :** Les résultats de ces projets de recherche démontrent que, à date, de multiples traitements pour les cicatrices chéloïdes auriculaires existent et sont utilisées sans lignes directrices claires. L'Onatoxinebotulique A a été démontré comme étant un choix prometteur pour le traitement des cicatrices chéloïdes.

# Preface

## Contributions of authors

Dr. Amanda Fanous performed the literature review, animal experiments, data collection, and the analysis of both manuscripts included in this thesis. Dr. Sam J. Daniel, Dr. Derin Caglar and Dr. Amanda Fanous jointly came up with the design of the animal experiments. Dr. Amanda Fanous, Aren Bezdjian, John Kaoumi and Joseph Sayegh contributed to the writing of the third manuscript. Dr. Derin Caglar conducted the histological evaluation of the pathology specimens used in this thesis. The animal study was conducted at the McGill Otolaryngology Sciences Laboratory located at the Research Institute of the Montreal Children's Hospital of the McGill University Health Centre (Sam J. Daniel is Funded by CIHR, FRQs, MUHCRI, MCHRI) and was funded by a FRQS Master's training grant to Dr. Amanda Fanous. Result interpretation and final approval of the manuscript were done by Dr. Amanda Fanous and Dr. Sam J. Daniel.

## Claims of originality

This thesis yielded the following new knowledge: (1) A systematic review showed that there is no preferred treatment for auricular keloid scars and (2) Onabotulinumtoxin A treatment minimized keloid scarring in an animal model.

## Acknowledgements

I would like to express my sincere gratitude to my supervisor and mentor, Dr. Sam Daniel. His sustained guidance is what made my research projects possible. I am forever grateful to my basic science research supervisor, Dr. Derin Caglar. Her love for pathology and basic science is contagious. I thank Dr. Bernard Segal for chairing my thesis committee meeting and for his critical review throughout my time as a master's student in the department of Otolaryngology at McGill University. I am also extremely grateful to my friend and colleague Aren Bezdjian who has made significant contributions to the work leading to this thesis.

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# Disclosure

Part of the experiments presented in this thesis was presented at the Quebec Otolaryngology meeting, held in Quebec City in October 2015, wining first prize at the resident contest, and at the Canadian Society of Otolaryngology meeting in June 2016 in Charlottetown. The first manuscript (Chapter 3) has been submitted for publication to the Laryngoscope journal in April 2021. The second manuscript (Chapter 4) was published in the Plastic and Reconstructive Surgery Journal issue: 143(3) pages 760-767 in March 2019.

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# CHAPTER ONE: Introduction

## 1.1 Rationale

Recent advances in Facial Plastic and Reconstructive Surgery have made disfigurement from cancer or trauma exceedingly rare[1]. From novel skin grafting techniques to free flap microvascular reconstruction, head and neck reconstruction has drastically evolved over the last few decades. Keloid scarring however remains a blatant exception[2]. An incision is inevitable in surgery, and when the healing process of that very incision is the cause of the disfigurement, surgeons and their armamentarium have met their match.

In the developed world, an estimated 100 million people develop scars each year as a result of elective operations or trauma[3]. This does not include other insults to the deep dermis, which may also lead to aberrant scar formation, including piercings, vaccinations, abrasions and burns. The incidence, prevention and treatment of excessive scarring, including keloids, is therefore a major concern.

Excessive scarring was first described in the Smith Papyrus in about 1700 BC[4]. Mancini in 1962 and Peacock in 1970 further elaborated on this concept many years later by dividing the excessive scarring mechanism into hypertrophic scars and keloid scars [5-6]. Excessive scarring, by definition, is raised above skin level. Whereas hypertrophic scars remain within the edges of the incision, keloid scars extend beyond those original wound margins. Often disfiguring, keloid scarring can dramatically affect a patient's quality of life, both physically and mentally.

Keloid scarring is estimated to occur in about 6 to 16% of the African population[7]. In the head and neck area, there is predilection for the earlobes and cheeks. Sex distribution is equal, with peak incidence in the second and third decades. Unlike hypertrophic scarring, spontaneous regression is rare and recurrence following surgical excision is high, making efficacious treatment modalities very sought after. Abundant literature exists on various treatment modalities individually. However, very little literature exists that compares these treatment options in a systematic fashion.

Botulinum toxin has emerged in the literature as a potential promising treatment modality for keloid scars[8]. Botulinum toxin is a neurotoxin produced by the bacterium Clostridium botulinum[9]. C. botulinum produces eight distinguishable exotoxins (A, B, C1, C2, D, E, F and G). In 1980, Scott first demonstrated the effectiveness of botulinum

toxin A injection into extraocular muscles as an alternative to strabismus surgery[10]. Since then, the applications of botulinum toxin A in medicine have vastly expanded[11]. It is thought that botulinum toxin could affect keloid scarring by inhibiting the production of collagen from fibroblasts[12].

In view of the above, there is a great need to compare these various treatment modalities, and their combinations, in a standardized and systematic fashion. Furthermore, a careful experimental study using an animal model could evaluate the utility of botulinum toxin A as a treatment for keloid scarring. This need formed the rationale of this thesis. The resulting objectives of this thesis are given in the next section (1.2).

## 1.2 Objectives & hypothesis

The overall objective of this thesis is to review current treatments of auricular keloid scarring and to elucidate the effect of Onabotulinumtoxin A on keloid scarring in an in vivo animal model.

Specifically, study 1 is an up to date systematic review of all treatment modalities for auricular keloid scarring. The objective and rationale of this study is clearly stated in Chapter 3 of this thesis. Study 2 examines the effect of botulinum toxin A on keloid scarring. The objectives of this study are listed in Chapter 4 of this thesis.

Because of its nature, the first study does not have a null hypothesis ($N_0$). The null hypothesis for the second study is that injection of the keloid scars with botulinum toxin A in an animal model would show equal or lesser effect than compared to the controls (placebo and triamcinolone injection).

# CHAPTER TWO: Background

## 2.1 Anatomy & Physiology of Human Skin

The experiments performed in the second manuscript evoke tissue scarring. Therefore, an overview of the anatomy and physiology of human skin will be presented in this chapter.

### 2.1.1 The skin envelope

The skin is the largest organ of the body. It serves as a protection from the outside world, to regulate body temperature and to allow the sensation of pain, pressure and temperature. The skin is broadly divided into three layers: epidermis, dermis, subcutaneous tissue. Figure 1 shows the different layers human skin.

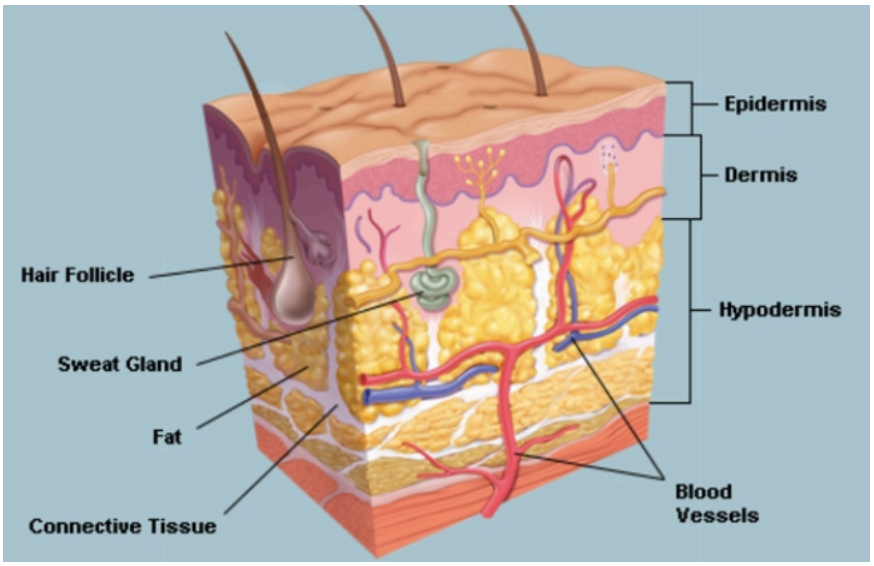


**Figure 1.** Layers of human skin. © 2014 WebMD

### 2.1.2 Epidermis

The epidermis is the outermost layer of the skin acting as a barrier that provides protection from the environment. It is devoid of blood vessels and is therefore dependent on the dermis for supply. Depending on the body region, the thickness can vary from roughly 5mm to 1.5cm. The epidermis is composed of 5 layers: stratum corneum, stratum lucidem, stratum granulosum, stratum spinosum and stratum basale which is composed of basal cells. The cellular composition of the epidermis is as follows: 90% keratinocytes, along with melanocytes, Langerhans cells and Merkle cells.

### 2.1.3 Dermis

The dermis is the layer directly underlying the epidermis. It is made up of fibrous and elastic tissue and is the thickest layer of the skin, measuring between 1 and 4mm. The dermis houses nerves, blood vessels, lymphatics and cutaneous appendages (pilosebaceous units, eccrine and apocrine sweat glands). The dermis is composed of two layers: the papillary dermis and the reticular dermis. The papillary dermis is the more superficial layer and is made up of loose connective tissue. The reticular dermis is deeper,

thicker and composed of dense connective tissue. Importantly for the purpose of this thesis, the collagen fibers in the papillary layer are loose and disorganized, as opposed to the reticular layer where they are densely packed and arranged in parallel layers.

### 2.1.4 Subcutaneous tissue

The subcutaneous tissue, also called hypodermis, is the third and deepest layer of the skin. It is directly underlying the dermis and serves to connect the skin to the underlying fascia of muscles and bones. Compared to the dermis, it contains larger nerves and blood vessels. The thickness ranges from roughly 2mm to 25mm and is directly correlated with the body mass index.

## 2.2 Keloid scarring

The principle topic of this thesis revolves around the treatment of keloid scars. Therefore, the following chapter will briefly discuss the general principles of wound healing and the aberrant pathophysiology behind excessive scarring, mainly hypertrophic scars and keloids scars.

### 2.2.1 Principles of wound healing

Wound healing is an important function of skin and occurs through four regulated processes: hemostasis, inflammation, proliferation and remodeling[13-14]. The first stage of wound healing is hemostasis, aimed at stopping the bleeding. This begins seconds to minutes after the injury. The coagulation cascade is activated leading to platelet aggregation and the activation of fibrin, ultimately resulting in the formation of a clot to plug the injured vessel and stop the bleeding. During inflammation, the second phase, the main process is vasodilatation, allowing for infiltration of leukocytes, neutrophils and monocytes. The later differentiate into macrophages allowing for phagocytosis, where debris and pathogens are engulfed to clear them. The third stage is termed proliferation and serves as a repair mechanism. Angiogenesis, collagen deposition, granulation tissue formation, epithelialization and wound contraction occur. Vascular endothelial cells form new blood cells, fibroblasts produce collagen, and epithelial cells multiply and spread across the healing tissue bed. Finally, during the fourth and last phase termed remodeling or maturation, collagen fibers are realigned and organized.

Factors affecting wound healing can be divided into local and systemic factors[13]. Local factors include oxygenation, infection, foreign body and venous sufficiency. In

hypoxic or superinfected environments, wound healing is impaired and delayed. Systemic factors include age, gender and sex hormones. Indeed, increased age is a major factor in impaired wound healing, involving both a temporal delay and degradation in the quality of wound healing. Aged males have been shown to have delayed healing of wounds compared to aged females. Other important systemic factors affecting wound healing include stress, chronic diseases such as diabetes or jaundice, obesity, medications such as steroids, chemotherapy or non-steroidal anti-inflammatories, alcoholism and smoking, immunocompromised states and nutritional status. Protein is one the most important nutritional factors affecting wound healing. Protein deficiency can impair capillary formation and fibroblast proliferation.

### 2.2.2 Collagen production

Collagen is the main protein of extracellular matrix. Over 30 types of collagen have been described and identified in the human body[15-16]. The four main types of collagen are 1,2,3 and 4. Type 1 makes up 90% of human collagen and is found in skin, tendons, vasculature, organs and bone. Type 2 is cartilage, type 3 is termed reticulate and type 4 forms the basal lamina of the basement membrane. Collagen type 1 comprises 70% of collagen found in human skin, type 3 10%, with trace amounts of type 4.

During wound healing, collagen is synthesized by fibroblasts, which are activated to proliferate, migrate into the wound site and produce collagen by various cytokines and growth factors. Collagen type 3 is the first to be produced in the initial phases of wound healing, followed by type 1 collagen. The ratio of type 1/3 remains lower in scar tissue as compared to normal skin (more type 3 collagen) but gradually remodels to a more baseline ratio as the site matures. This occurs during the fourth and final phase of wound healing termed remodeling.

### 2.2.3 Hypertrophic and keloid scars

Keloids and hypertrophic scars are defined as pathologic scars, characterized by excess formation of collagen[17]. They are caused by aberrations of physiologic wound healing and may arise following any sort of injury to the skin, whether it be from a surgical incision, a burn, a piercing or even a minor scratch.

Although there are several similarities between keloids and hypertrophic scars, some important differences exist[18]. Clinically, hypertrophic scars are limited to the incision edges whereas keloid scars extend beyond the initial wound margins. Pathologically, hypertrophic scarring consists of primarily fine, well-organized wavy type 3 collagen oriented parallel to the epidermis surface with abundant nodules containing myofibroblasts and acidic mucopolysaccharide. Furthermore, proliferating cell nuclear antigen (PCNA) expression is low. In contrast, keloid scars consist of large, thick and disorganized type 1 and 3 collagen bundles with no nodules or myofibroblasts. Poor vascularization exists with widely scattered blood vessels. Expression of PCNA is high.

A plethora of prevention and treatment options have been explored in the literature, although none with convincing results. These treatment options range from conservative options such as pressure therapy, to local administration of medication such as steroid injection, or to more aggressive options such as surgery or radiotherapy[19].

# CHAPTER THREE: Manuscript 1

# A Systematic Review Evaluating Treatment Modalities for Auricular Keloid Scars

*Submitted to the Journal of Otology and Neurotology*

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## 3.1 Abstract

*Objective:* The aim of this study is to conduct a systematic review to evaluate various treatment modalities for auricular keloids reported in the current literature.

*Methods:* Eligible articles were identified through a comprehensive search of electronic databases. Using predefined inclusion criteria, published articles on auricular keloids were selected and reviewed.

*Results:* Eighteen (18) articles encompassing one thousand four hundred and fifteen (1415) auricular keloid patients were identified. The mean age at diagnosis was 41, with a standard deviation of 2.5 years. The most common site of keloid formation was the earlobe (60%), and piercing was the most common cause (82%). Treatment modalities are traditionally multimodal, with the main modality being surgical excision followed by postoperative adjuvant treatment, such as pressure therapy (64%), post-operative radiotherapy (8%), electrotherapy (6%), cryotherapy (5%) or corticosteroid injection (5%). Recurrence rates were low (13%).

*Conclusion:* A multimodal therapeutic regimen approach including an individualized combination of surgical excision, intralesional corticosteroid injection, and pressure therapy may be an effective treatment and reduce the recurrence rate of auricular keloids.

## 3.2 Introduction

A keloid is a benign growth of dense fibrous tissue developing from an abnormal healing response to a cutaneous injury. Keloid scars, in contrast to hypertrophic scars, extend beyond the borders of the wound and are generally raised. They can develop following any trauma to the skin, whether it is surgery, flame burn, piercing, a mundane scratch, a chemical peel, etc. [20]. Furthermore, keloid scarring has an incidence of about 6 to 16% in the African population [20,21]. Areas more commonly affected are the anterior chest, shoulders, flexor surfaces of extremities and the ears [20]. Often disfiguring, this condition can have devastating psychosocial consequences.

Auricular keloids, most often located on the lobule, have become increasingly common following the popularity of ear piercing. These have important cosmetic implications that significantly affect quality of life. The incidence of keloids is reported to be 2.5% of all ear piercings [22]. It has been determined that auricular keloids are more likely to develop when ears are pierced after age 11 [23]. Besides age, the avascular cartilage bearing portion of the ear is more likely to form keloids and keloids are more

frequent in cases of prolonged auricular wound healing caused by infection [24]. Interestingly, Van Wijk et al. conducted a histologic study to determine the extent of damage to ear cartilage using different piercing techniques and found that all piercing methods give the same extent of damage to cartilage and perichondrium [25]. Each method evaluated carries the same risk for developing perichondritis, thus all display the same chances of keloid formation.

To date, there is no consensus as to which treatment modality is optimal for auricular keloid scarring. Topical agents including steroid creams, silicone creams and silicone sheets have been shown to have little, if any, benefit. Excision and revision of the scar is an option, but a high rate of recurrence exists [26]. Scar excision and revision followed by radiation therapy have been successful, however, often unfavored due to small doses of radiation that are unavoidably delivered to healthy surrounding tissues, thereby putting the patient at risk for long-term secondary malignancy. However, recent evidence supports that this risk is reduced with the optimization of treatment strategy taking dose, fractions, and intervals into consideration [27]. Steroid injection is today considered the primary standard first line treatment for most keloid scars [26]. However, these injections carry the risk of skin hypopigmentation and telangiectasia. Steroid injections, like most other keloid treatment modalities, is at most 70% effective [26].

Due to the variability of treatment approaches and the high rate of recurrence, the present study aims to convey the clinical presentations and documented managements of cases of auricular keloids reported in the literature to consider the best possible treatment options and ultimately avoid unnecessary interventions. The primary goals while planning a treatment protocol should be a low recurrence rate, significant aesthetic and symptomatic improvement, and minimal adverse effects.

## 3.3 Methods

### 3.3.1 Search strategy

A medical librarian from the McConnell Resource Centre of the McGill University Health Center identified eligible articles through a comprehensive search of three electronic databases: Medline, Embase, and CINAHL. The search strategy included

medical subject headings, subheadings, and text words such as “keloid”, “ear”, “auricular”, “hypertrophic”. A thorough search of the reference lists from relevant studies was also performed.

### 3.3.2 Inclusion and exclusion criteria

Articles presenting treatment options for auricular keloid appearing in humans were chosen. Studies presenting animal and in vitro experiments were excluded. Furthermore, articles presenting other types of tumors were excluded. Keloid tumors elsewhere than the ear region were not included. Finally, letters, commentaries, and reviews were not eligible for evaluation.

### 3.3.3 Study selection

This systematic review was conducted following the PRISMA guidelines [28]. Two authors (AF and AB) independently reviewed the titles and abstracts retrieved by the electronic search concordant with the criteria for study eligibility. The lists of articles from each author were jointly reviewed and a common list created. All relevant articles for second-stage review were reviewed as full texts; initially independently and later jointly by the two authors. All divergence among reviewers was resolved by consensus or by a third author.

### 3.3.4 Quality Assessment

All eligible articles underwent critical appraisal for Directness of Evidence (DoE) and Risk of Bias (RoB) performed by two authors using predefined criteria. DoE was assessed using 6 criteria: indication for treatment (diagnosis), demographic data (age at treatment), treatment approach (treatment characteristic, dose, and administration route), efficacy assessment, safety assessment and follow-up time.

RoB was assessed using 6 criteria: randomization, blinding, standardization of treatment, standardization of outcomes, standardization of follow up, and missing data. Table 1a presents the quality assessment results and Table 1b describes the criteria per item for the critical appraisal.

The DoE assessment was scored as high when scores were at least 5 out of a possible 6, as moderate when scores were 4 or 4.5, and as low with scores below 4. The RoB assessment based on the Cochrane Collaboration’s tool for assessing RoB was scored as low when scores were at least 5 out of a possible 6, as moderate when scores were 4 or 4.5, and as high with scores lower than 4. Articles included for data extraction

scored: (1) high for DoE and low for RoB; (2) moderate for DoE and low for RoB; or (3) high for DoE and moderate for RoB.

| | | Directness of evidence (DoE) | | | | | | | Risk of bias (RoB) | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Authors** | **Publication year** | **Study design** | **Indication for treatment** | **Demographic data** | **Treatment approach** | **Efficacy outcome measures** | **Follow-up** | **DoE score** | **Randomization** | **Blinding** | **Standardization (T)** | **Standardization (O)** | **Standardization (FU)** | **Missing data** | **RoB score** |
| **Chaudry M.R., Sajjad A.** | 1994 | RCT | ● | □ | ● | ● | ● | **H** | ○ | ○ | ● | ◑ | ● | ● | **M** |
| **Kim D.Y. *et al.*** | 2004 | RCS | ● | □ | ● | ● | ● | **H** | ○ | ○ | ● | ◑ | ● | ● | **M** |
| **Saha S.S. *et al.*** | 2004 | RCS | ● | ◑ | ● | ● | ● | **H** | ○ | ○ | ◑ | ● | ● | ● | **M** |
| **Chrisostomidis C. *et al.*** | 2007 | RCT | ● | □ | ● | ● | ● | **H** | ○ | ○ | ◑ | ● | ● | ● | **M** |
| **Rosen D.J. *et al.*** | 2007 | RCS | ● | ◑ | ● | ● | ● | **H** | ○ | ○ | ● | ◑ | ● | ● | **M** |
| **Jung J.Y. *et al.*** | 2008 | RCS | ● | ● | ● | ● | □ | **H** | ○ | ○ | ● | ◑ | ◑ | ● | **M** |
| **Kadouch D.J. *et al.*** | 2010 | RCS | ● | ◑ | ● | ● | ● | **H** | ○ | ○ | ● | ◑ | ● | ● | **M** |
| **Stahl S. *et al.*** | 2010 | RCS | ● | □ | ● | ● | ● | **H** | ○ | ○ | ● | ◑ | ● | ● | **M** |
| **Chi S.G. *et al.*** | 2011 | RCS | ● | □ | ● | ● | ● | **H** | ○ | ○ | ● | ◑ | ● | ◑ | **M** |
| **Park T.H. *et al.*** | 2011 | PCS | ● | □ | ● | ● | ● | **H** | ○ | ○ | ● | ◑ | ● | ● | **M** |
| **Park T.H. *et al.*** | 2012 | PCS | ● | □ | ● | ● | ● | **H** | ○ | ○ | ● | ◑ | ● | ● | **M** |
| **Yossi S. *et al.*** | 2012 | RCS | ● | □ | ● | ● | ● | **H** | ○ | ○ | ◑ | ◑ | ● | ● | **M** |
| **Kim K., Son D., Kim J.** | 2015 | RCS | ● | ◑ | ● | ● | ● | **H** | ○ | ○ | ◑ | ● | ● | ● | **M** |
| **Litrowski N. *et al.*** | 2013 | RCS | ● | □ | ● | ● | ● | **H** | ○ | ○ | ● | ◑ | ● | ● | **M** |
| **Lyu A., Xu E., Qang Q.** | 2019 | RCS | ● | □ | ● | ● | ● | **H** | ○ | ○ | ◑ | ◑ | ● | ● | **M** |
| **Ramesh B.A. Mohan J.** | 2018 | RCT | ● | □ | ● | ● | ● | **H** | ○ | ○ | ● | ◑ | ● | ● | **M** |
| **Khalid *et al.*[50]** | 2018 | RCT | ● | □ | ● | ● | ● | **H** | ● | ○ | □ | □ | □ | ● | **M** |
| **Walliczek *et al.*** | 2015 | CT | ● | □ | ● | ● | ● | **H** | ○ | ○ | ● | □ | □ | ● | **M** |
| **Carvalhaes *et al.*** | 2015 | RCS | ● | ○ | ● | ● | ● | **M** | ○ | ○ | ◑ | □ | ● | ● | **M** |
| **Stern J.C., Lucente F.E.** | 1989 | PCS | ● | □ | ● | ● | ◑ | **M** | ○ | ○ | □ | ◑ | ◑ | ● | **H** |
| **Lawrence W.T.** | 1996 | RCS | ● | □ | ● | ● | ◑ | **M** | ○ | ○ | □ | ◑ | ◑ | ● | **H** |
| **Russell R., Horlock N., Gault D.** | 2001 | RCS | ● | ○ | ● | ● | ● | **M** | ○ | ○ | □ | ● | ● | ● | **M** |
| **Akoz T.,** | 2002 | RCT | ● | □ | ● | ● | ◑ | **M** | ○ | ○ | ◑ | ◑ | ◑ | ◑ | **H** |

| | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Gideroglu K., Akan M.** | | | | | | | | | | | | | | | |
| **Hassel J.C. *et al.*** | 2007 | RCS | ● | □ | ● | ● | □ | **M** | ○ | ○ | ○ | ○ | ○ | ● | **H** |
| **Sand M. *et al.*** | 2007 | RCS | ● | ● | ● | ● | ● | **H** | ○ | ○ | ○ | ○ | ○ | ● | **H** |
| **Bermueller C., Rettinger G., Keck T.** | 2009 | RCS | ● | □ | ● | ● | ● | **H** | ○ | ○ | □ | ◑ | ● | ◑ | **H** |
| **Gupta M., Narang T.** | 2011 | PCS/RCT | ● | □ | ● | ● | ◑ | **M** | ● | ○ | ● | ● | ◑ | ● | **L** |
| **Brown N.A., Ortega F.R.** | 2010 | RCS | ● | □ | ● | ● | ◑ | **M** | ○ | ○ | ● | ● | ◑ | ● | **M** |
| **Hassel J.C. *et al.*** | 2011 | RCS | ● | □ | ● | ● | ◑ | **M** | ○ | ○ | ● | ◑ | ◑ | ● | **M** |
| **Carvalho B. *et al.*** | 2012 | PCS/RCS | ● | □ | ● | ● | ◑ | **M** | ○ | ○ | ◑ | ◑ | ○ | ○ | **H** |
| **Sunohara M. *et al.*** | 2012 | RCS | ● | ◑ | ● | ● | ○ | **M** | ○ | ○ | □ | ● | ● | ● | **M** |
| **Ogawa R. *et al.*** | 2014 | RCS | ● | ○ | ● | ● | ● | **M** | ○ | ○ | □ | ◑ | ● | ● | **M** |
| **Cheng *et al.*** [35] | 1972 | RCT | ● | □ | ● | ● | □ | **M** | ○ | ○ | □ | □ | □ | ○ | **H** |
| **Di Stadio *et al.*** [41] | 2016 | RCT | ● | □ | ● | ● | □ | **M** | ○ | ○ | ● | ● | ● | ● | **L** |
| **Defty *et al.*** [45] | 2016 | RCT | ● | □ | ● | ● | □ | **M** | ○ | ○ | ● | ● | ● | ● | **L** |
| **Jones *et al.*** [31] | 2017 | RCT | ● | □ | ● | ○ | ● | **M** | ○ | ○ | ● | ○ | ● | ● | **M** |
| **Kassab *et al.*** [43] | 2012 | RCT | ● | □ | ● | ● | □ | **M** | ○ | ○ | □ | □ | ● | ● | **M** |
| **Ragoowansi *et al.*** [48] | 2001 | RCT | ● | □ | ● | ● | ● | **H** | ○ | ○ | □ | □ | □ | ● | **H** |
| **Thierauf *et al.*** [51] | 2017 | RCT | ● | □ | ● | ● | ● | **H** | ○ | ○ | □ | □ | □ | ● | **H** |
| **Yang *et al.*** [46] | 2012 | RCT | ● | □ | ● | ● | ● | **H** | ○ | ○ | □ | □ | □ | ● | **H** |
| **Yang *et al.*** | 2018 | RCT | ● | □ | ● | ● | ● | **M** | ○ | ○ | ● | ● | ● | ● | **L** |

**Table 1a.** Critical appraisal of selected studies reporting patients treated for auricular keloids

Assessment per item for critical appraisal of selected studies

| | **Grading (● = 1 point, ◑ = 0.5 point, ○ = 0 point)** |
|---|---|
| **Directness of Evidence (DoE)** | |
| Study design | CT, clinical trial<br>PCS, prospective case series<br>RCS, retrospective case series<br>RCT, randomized control trial |
| Indication for treatment<br>*Diagnosis* | clearly reported, ●<br>not clearly reported, ○ |
| Demographic data<br>*Age at treatment*<br>*Ethnicity* | age individually reported and ethnicity reported, ●<br>age individually reported/means reported or ethnicity reported, ◑<br>not reported, ○ |
| Treatment approach<br>*NF used, dosage, route of administration* | reported, ●<br>not reported, ○ |
| Efficacy outcome measures<br>*Pre and post treatment assessment* | reported, ●<br>not reported, ○ |
| Follow-up (FU)<br>*Duration of follow-up at the end of treatment for all tested individuals* | FU ≥ 12 months, ●<br>12 months > FU ≥ 6 months, ◑<br>FU < 6 months, ○ |
| Overall DoE score | High, ≥ 4.5 points<br>Moderate, between 3.5-4.5 points<br>Low, < 3.5 |
| **Risk of Bias (RoB)** | |
| Randomization | randomized or concealed, ●<br>not randomized or concealed, ○ |
| Blinding | blinding of patient, researcher, observer, ●<br>single blind, ◑<br>no blinding, ○ |
| Standardization of treatment | all patients received the same therapy, ●<br>different types of NFs or dosage used, ◑<br>dosage modified throughout trial, ○ |
| Standardization of outcome measures | identical outcome reports, ●<br>reported however not standardized, ◑<br>not reported, ○ |
| Standardization of follow up | identical follow up for all patients, ●<br>reported however not standardized, ◑<br>not reported, ○ |
| Missing data | no missing data; missing data mentioned/quantified and method of handling described, ●<br>missing data mentioned in study but method of handling not described, ◑<br>missing data not reported, ○ |
| Overall RoB score | Low, ≥ 4 points |

| | |
|---|---|
| | Moderate, between 3-4 points<br>High, < 3 |

**Table 1b.** Legend critical appraisal

## 3.4 Results

### 3.4.1 Search results and quality assessment

A total of 519 articles were identified by the electronic databases search after duplicates were removed as well as articles not published in French or English. Following independent then joint review of titles and abstracts, 41 articles were selected for full review and critical appraisal of the 41 articles was performed. Out of these studies, 18 were included for data extraction (see Figure 1 for flow chart), based on the DoE and RoB scores as described above. The descriptive characteristics of selected studies are shown in Tables 1a and 1b.

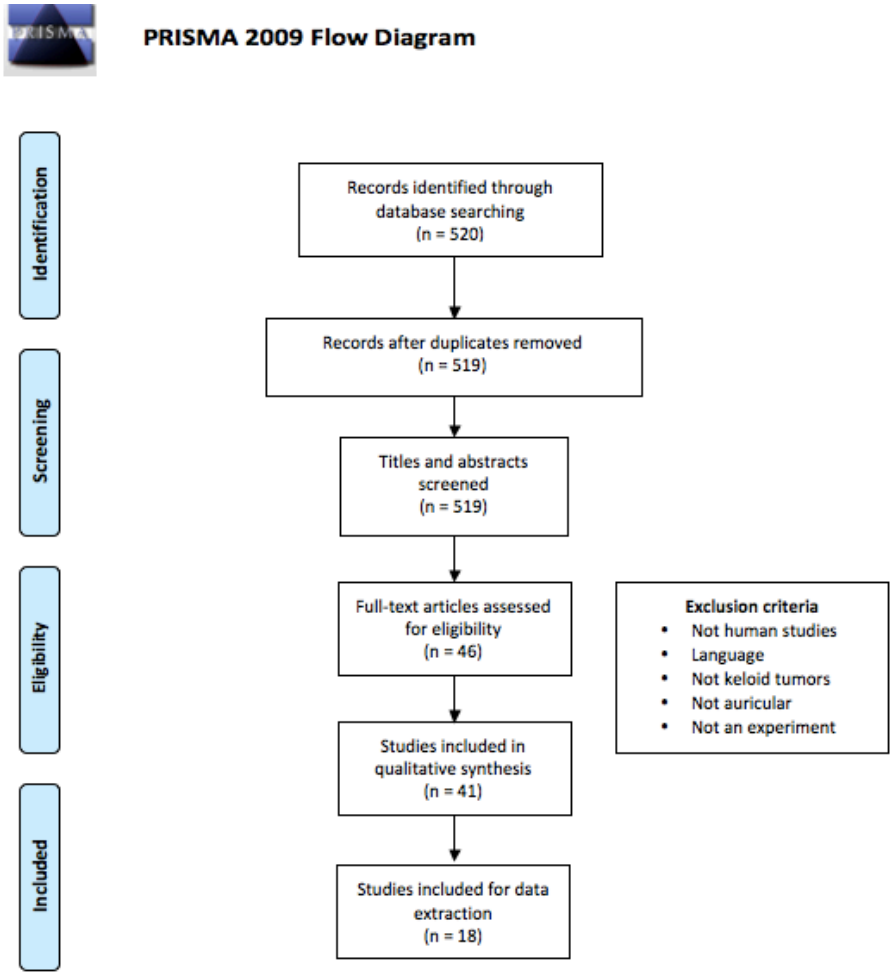

**Figure 1:** PRISMA flow diagram

### 3.4.2 Characteristics of auricular keloids

Demographic and clinical data for included patients are summarized in table 2. A total of 1,415 patients were managed for auricular keloids. The mean age at diagnosis was 41, with a standard deviation of 2.5 years. The majority of patients (82%) were female. Surprisingly, most papers do not comment on patient ethnicity (86%). As expected, the most mentioned ethnicities were Caucasian (6%) and black (6%). The earlobe was the most common site for keloid formation (60%), followed by the helix (14%). Piercing was the most common cause for the condition (82%). Surgery and infection were other etiologies.

| | Patients[1], n (%) |
|---|---|
| Age at diagnosis | |
| Mean ± SD | 41.5 ± 2.5 y |
| Range | 3 to 80 y |
| Sex | |
| Male | 217 (18) |
| Female | 1198 (82) |
| Not applicable | 0 (0) |
| Ethnicity/Race | |
| Asian | 16 (1) |
| Caucasian | 90 (6) |
| Black | 88 (6) |
| Hispanic | 7 (<1) |
| Not applicable | 1214 (86) |
| Location of keloid tumor | |
| Earlobe | 849 (60) |
| Helix | 197 (14) |
| Not applicable | 365 (26) |
| Etiology | |
| Piercing | 1162 (82) |
| Surgery | 63 (4) |
| Infection | 4 (<1) |
| Other trauma | 25 (2) |
| Not applicable | 157 (11) |

**Table 2**. Demographic and Clinical Data of Patients with Keloid Tumors in the Head and Neck from Selected Studies. [1] N = 1411. Values presented in n (%) except for age at diagnosis.

### 3.4.3 Keloid scars treatment modalities

As previously mentioned, many treatment modalities have been described in the treatment of keloid scars. Treatment modalities are traditionally multimodal, with the main modality being surgical excision followed by postoperative adjuvant treatment, such as pressure therapy (64%), post-operative radiotherapy (8%), electrotherapy (6%), cryotherapy (5%) or corticosteroid injection (5%). Other rare adjuvant treatments include mitomycin and topical silicone gel.

### 3.4.4 Keloid scars treatment outcomes

Treatment and outcomes are summarized in table 3. Most patients responded relatively well to multimodality treatment with low rates of recurrence (87%). Most recurrences followed excision and pressure therapy (8%), while the highest percentage of recurrences occurred in the excision followed by radiation therapy (38%) and the excision followed by triamcinolone injection (37.5%) groups.

| | Patients[1], n (%)<br>Total n=1411 |
|---|---|
| Surgical intervention | |
| Excision + PT using magnets | 898 (64) |
| Excision + post-operative RT | 108 (8) |
| Excision + electrotherapy | 87 (6) |
| Excision + cryotherapy | 66 (5) |
| Excision + intra-operative and post-operative TA injection | 64 (5) |
| **Excision + I/L TA injections** | 30 (2) |
| Excision + TA injections + PT | 29 (2) |
| Excision + PT using methacrylate stent | 23 (1) |
| Excision + pre-operative and post-operative RT | 23 (1) |
| Excision + PT + topical liquid silicone gel | 22 (1) |
| Excision + pre-operative and post-operative TA injections | 15 (1) |
| Excision + split-thickness skin graft | 15 (1) |
| Excision + mitomycin application | 11 (1) |
| Excision + PT | 10 (1) |
| Excision + brachytherapy | 8 (0.5) |
| Excision | 1 (0.5) |
| Excision + PT + I/L CS | 1 (0.07) |
| Prognosis | |
| No recurrence | 1221 (87) |
| Recurrence | 190 (13) |
| Recurrence | |
| Post excision + PT | 108 (12) |
| Post excision + RT | 41 (38) |
| Post excision + TA | 24 (37.5) |
| Post excision + cryosurgery | 14 (21) |
| Post excision + TA + PT | 3 (10) |

**Table 3.** Keloid scars treatment outcomes.
Abbreviations: PT, pressure therapy; I/L, intralesional, RT, radiotherapy; CS, corticosteroid; TA, triamcinolone acetonide;
[1] *N* = X. Values presented in n (%) except for age at diagnosis.

### 3.4.5 Picots table

Please refer to the PICOTS table (table 4) for a comprehensive summary.

| | |
|---|---|
| **Population(s)** | • Patients with at least one auricular keloid tumor from different age groups, genders, and ethnicities<br>• Mean age at diagnosis was 41 (standard deviation of 2.5 years)<br>• Majority of patients were female (82%)<br>• Most papers do not comment on ethnicity (86%)<br>• Most commonly mentioned ethnicities were caucasian (6%) and black (6%) |
| **Interventions** | • Surgical excision of the keloid tumor alone (0.5%)<br><br>Multimodality interventions:<br>• Surgical excision with post-operative pressure therapy (PT) using magnets or a stent (66%)<br>• Surgical excision with PT and a topical liquid silicone gel (1%)<br>• Surgical excision with PT and intralesional corticosteroid injection (0.07%)<br>• Surgical excision with post-operative electrotherapy, cryotherapy, or brachytherapy (11.5%)<br>• Surgical excision with post-operative radiotherapy (RT) (8%)<br>• Surgical excision with pre-operative and post-operative RT (1%)<br>• Surgical excision with intra-operative and post-operative triamcinolone acetonide (TA) injections (5%)<br>• Surgical excision with intralesional TA injections (2%)<br>• Surgical excision with TA injections and PT (2%)<br>• Surgical excision with pre-operative and post-operative TA injections (1%)<br>• Surgical excision with a split-thickness skin graft (1%)<br>• Surgical excision with mitomycin application (1%) |
| **Comparisons** | • Most recurrences followed excision with PT (8%), while excision with RT had the second highest recurrence rate (3%) |
| **Outcomes** | • Low rates of recurrence following multimodality treatment (13%)<br>• Rates of recurrence following excision with PT, RT, TA injections, excision with cryosurgery and excision with both TA and PT were respectively 12%, 38%, 37.5%, 21% and 10% |
| **Timing** | • Some patients had follow-ups for 4 months post-op while others were followed for up to 10 years<br>• Most studies (78%) had a follow-up period of at least 12 months for all their patients |
| **Study design** | • Letters, commentaries, and reviews were excluded |

**Table 4**. PICOTS Table

## 3.5 Discussion

Often caused by ear piercing, auricular keloids are most commonly located on the earlobe and helix. To date, there is no optimal treatment modality for keloids. Because of its high recurrence, the treatment of auricular keloids can be challenging.

The pathologic mechanisms for keloid formation have not been fully elucidated; therefore, effective targeted therapies are lacking. Moreover, there is no known correlation between clinicopathologic findings and auricular keloid recurrence rates [29]. Given these factors and its high recurrence rate, management of the condition is therapeutically challenging [30,31]. In the current study, auricular keloid therapies were reviewed.

Surgical resection of auricular keloids can be challenging because of the need to preserve the underlying three-dimensional framework of the external ear and the absence of surrounding tissue laxity [32].

Surgical intervention followed by pressure therapy remains by far the most used, probably because of its simplicity to administer. Pressure therapy has gained attraction because of convenience of use and lack of adverse effects. Nason et al. reported that the recurrence rate of keloid scar decreased from 67% to 18% after using pressure therapy[33]. However, it is important to note that pressure therapy should be instituted early post-operatively and pressure should be maintained between 1.33 and 3.3 kPa, for more than 8 hours per day for 1 year.

Intralesional injection of corticosteroids has been shown to effectively reduce keloid scar formation [34-37,]. This method has shown effectiveness in alleviating pruritus and pain and improving the appearance of the external ear by lessening inflammation. Intralesional steroids lessen scar formation by inhibiting fibroblast proliferation and collagen deposition and synthesis [38-40] . Thus, corticosteroids can abort the inflammatory process. The use of corticosteroids as an adjunct to excision has a low morbidity, is cost-effective, is easy to administer, and provides reliable and durable results [36]. Drawbacks of the injection approach include the risk of skin atrophy, telangiectasia, necrosis, ulceration, wound dehiscence, and hypopigmentation, although these risks are rare in the ear.

Radiotherapy seems to be a great post-operative adjuvant treatment option. According to Yang et al [41], radiotherapy should be performed as soon as possible after surgery as it may play a crucial role in halting recurrence by controlling collagen synthesis through the inhibition of fibroblasts [42]. Drawbacks of radiation therapy include

access to a tertiary care center and the risk of secondary malignancy, although this risk is low in the ear area since healthy tissues can more easily be protected [42].

The seemingly better reported outcomes found in the included studies is the multimodal approach for managing auricular keloids. The relatively higher proportion of recurrence following excision and radiotherapy or triamcinolone injection could possibly be explained by a selection bias, given that more aggressive keloids will likely be treated with one of these modalities. Personalized therapeutic approach for managing auricular keloids contributed to improvement in outcome. Yong-Hong used different surgical techniques according to preoperative assessment of the size, location, and extent of invasion as well as the relation between the helix and adjacent structures[30].

## 3.5 Conclusion

In conclusion, a personalized surgical approach based on the characteristics of auricular keloids in each patient and a multimodal therapeutic regimen including surgical excision, intralesional corticosteroid injection, and pressure therapy may be an effective treatment and reduce the recurrence rate of auricular keloids.

## 3.6 Funding Sources

The authors have no funding, financial relationships, or conflicts of interest to disclose.

## 3.7 Linking Statement

The above study suggests that, although a wide array of treatments for auricular keloids exists, no treatment has proven overly efficacious to emerge as the undisputed gold standard. In fact, multimodality therapy seems to be the optimal option. Further studies are needed to identify a better treatment that can be used on its own, or in combination with other treatment modalities, to improve outcomes. The following study will be exploring just that: the use of botulinum toxin as a novel treatment modality for keloid scars.

# CHAPTER FOUR: Manuscript 2

## Treatment of Keloid Scars with Botulinum Toxin A Versus Triamcinolone in an Athymic Nude Mouse Model

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## 4.1 Abstract

**Background:** Keloid scarring is a serious condition that mostly affects patients of African or Asian descent. Often disfiguring, this condition can have devastating psychosocial consequences. To date, no treatment modality has been proven ideal. Our objectives were 1. To determine the efficacy of botulin toxin A injection for the treatment of keloid scars compared to steroid injection and to control saline injection. This was achieved through a basic science animal model using athymic nude mice and implanted human keloid tissue. 2. To analyze the histopathological changes that occur in an organized keloid scar following botulinum toxin A injection as compared to steroid and saline injections.

**Methods:** Keloid scars from four patients were excised and implanted subcutaneously into 28 mice. Three small keloid tissue samples were implanted in each of the 28 mice. One week after implantation, each implant received one of three injections: botulinum toxin A (treatment drug), saline (control) or steroid injection (first line gold standard). The keloid tissue was extracted three weeks post implantation. Weight analysis, immunohistochemistry, and standard hematoxylin and eosin (H&E) pathology were performed on each extracted tissue sample.

**Results:** Pre-post tissue weights paired t-test analysis revealed a statistically significant difference between the treatment and control groups ($p<0.05$). Analysis by a blinded pathologist confirmed less collagen bundles in the treatment group. Ki-67 immunohistochemistry, a marker of cell proliferation, revealed significantly less staining for the treatment groups.

**Conclusion:** Botulinum toxin A could be an effective treatment for keloid scars.

## 4.2 Introduction

Keloid/hypertrophic scarring is a serious condition that mostly affects patients of African, Indo-Pakistani, Asian or Mediterranean descent. Hypertrophic scarring is defined as an overgrowth of dense collagen tissue, often reddish in appearance, at the site of a skin trauma or incision. Unlike keloid scars, hypertrophic scars are confined to the site of injury and most often regress with medical treatment. A keloid scar is defined as an elevated, irregular shaped and progressively enlarging scar due to excessive collagen formation in the dermis following trauma or incision. Keloid scars by definition extend beyond the site of injury. These scars can develop following any type of trauma to the skin, whether it is surgery, ear piercing, a simple mundane scratch, a chemical peel, etc. [43]. It is estimated that the prevalence of hypertrophic scarring varies from 40% to 70% following surgery to up to 91% following burn injury, depending on the depth of the wound [44,45]. Furthermore, keloid scarring has an incidence of about 6 to 16% in the African population [43,46]. Often disfiguring, this condition can have devastating psychosocial consequences.

To date, no treatment modality has been proven ideal for keloid scarring. Topical agents including steroid creams, silicone creams and silicone sheets have been shown to have little, if any, benefit. Excision and revision of the scar is an option, but a high rate of recurrence exists [47]. Scar excision and revision followed by radiation therapy has been shown to give good results. However, a small dose of radiation is unavoidably delivered to healthy surrounding tissues, thereby putting the patient somewhat at risk for long-term malignancy. This risk of secondary malignancy is of particular concern in the pediatric population or when the scar is located in high-risk areas such as the neck, the breast, or the abdomen [48-50]. Steroid injection is today considered the primary standard first line treatment for keloid scars [47]. However, these injections have multiple disadvantages. Firstly, they carry the risk of skin hypopigmentation and telangiectasia, two potentially permanent complications that are very challenging conditions to treat. Furthermore, corticosteroids cause atrophy of surrounding tissues, in particular subcutaneous fat, which can lead to skin surface irregularities. Lastly, steroid injections have been reported to be at most 70% effective in the treatment of keloid scars, with a mean of 60% [47].

It is therefore clear that a simple, safe and effective treatment for keloid scars is greatly needed. Very little literature exists on the role of botulinum toxin A (Botox ©) in the treatment of keloid scars. Botulinum toxin A is composed of enzymes that are produced by the bacterium "Clostridium botulinum" and are potent neurotoxins. Botulinum toxin A interferes with the ability of neurons to release acetylcholine at nerve-muscle junctures, thereby inducing muscle paralysis. Initially approved by the FDA in 2002 for the treatment of moderate to severe glabellar lines in both men and women [51], its indications in medicine have greatly expanded. The use of botulinum toxin has extended to the fields of neurology, laryngology, urology, ophthalmology, etc [52-58]. It has been shown to be a safe treatment with rare side effects. Toxicity related to its use is extremely rare, occurring only if the maximal dose of 500 to 3000 units is exceeded (the average dose for the treatment of fine wrinkle is 5 to 10 units per muscle, therefore an average total dose of 25-100 units). A dose of 500 units will cause botulism and a dose of 3000 units will cause death [59]. Botulinum toxin A is very easy to administer, requiring only a simple injection. Compared to steroid injections, there is no risk of hypopigmentation or telangiectasia. Therefore, if proven to be effective, botulinum toxin A would be a good first line treatment alternative for keloid scars.

Our objectives were 1. to determine the efficacy of botulin toxin A for the treatment of keloid scars compared to steroid injection and to a control saline injection. This was achieved through a basic science animal model using athymic nude mice and implanted human keloid tissue and 2. to analyze the histopathological changes that occur in an organized keloid scar following botulinum toxin A injection as compared to steroid and saline injections.

## 4.3 Materials & Methods

### 4.3.1 Animal model and animal conditions

Both the protocol for obtaining the human keloid samples as well as the ensuing animal research received ethics approval by the Institutional Review Board (IRB) of McGill University. As previously stated, the athymic nude mouse animal model was utilized. Twenty-eight homozygous (*nu/*nu) male athymic nude mice were procured from Charles River Laboratories (Wilmington, Mass), seven at a time. All animals were kept at

one of the designated McGill University Health Care Center (MUHC) animal research sites. Standard animal care protocols were implemented. As well, the mice were kept in pre-sterilized cages and placed in a laminar flow environment, with each mouse having its own cage. Sterilized mouse food and water was utilized.

### 4.3.2 Patient population

Four patients suffering from keloid scarring of the head and neck were recruited from October 1st 2014 to May 1st 2015, from McGill University Health Center Facial Plastics and Reconstructive Surgery specialized clinics. Inclusion criteria were as follows: adults 18-45 years of age with keloid scarring in the head or neck area measuring larger than $3cm^3$. Exclusion criteria included medical co-morbidities such as diabetes mellitus, HIV/AIDS, hepatitis B or C, granulomatous or vasculitic diseases, co-existing cutaneous inflammatory/infectious conditions (eczema, psoriasis, acne, pre-malignant changes such as actinic keratosis or Bowen's disease, etc.), cutaneous malignancies such as melanoma, squamous cell carcinoma or basal cell carcinoma, or any malignancy diagnosed and treated within the last 5 years. Furthermore, patients having undergone previous radiation therapy to the head and neck area or patients having received non-topical treatments for their keloid scarring (such as steroid injections) were also excluded. Standard thorough verbal and dated written consent was received from each patient prior to participation.

### 4.3.3 Overall study design

Tissue extracted from each of the four patients was transplanted into seven mice, giving twenty-eight mice total. In other words, seven mice at a time were transplanted with tissue from the same human donor. Each mouse received three implants: one on the upper back, one and the lower back and the last on the abdomen. It ensues from this that all three implants in a given mouse came from the same human donor. There were three general groups corresponding to the three treatments: botulinum Toxin A, steroid and saline. Per group of seven, three mice were randomized to the botulinum toxin A group, two to the steroid group and two to the saline group. In other words, the treatment group consisted of twelve mice total, and the control and placebo groups of eight mice each.

In the treatment group, two of the three implants were treated with botulinum toxin and the third was treated with saline as an internal control. The same principal was applied for the steroid group, two implants were injected with steroid and the third with

saline as an internal control. Finally, in the saline group, all three implants were injected with saline.

### 4.3.4 Keloid excision from human donor

Each patient underwent keloid excision and reconstruction with post-operative radiation as per our institution's protocol. A sample of the keloid scar was stored in formalin and sent to the hospital's pathology department for formal analysis. The remainder of the tissue was stored fresh in a mix of physiologic medium (medium 199, with Earle's salts, L-glutamine, and sodium bicarbonate, Sigma-Aldrich no. M4530) and antibiotic solution (penicillin/streptomycin 100x, Sigma-Aldrich, no. P4333) and transferred to the Research Laboratory on the same day personally by the first author (Fanous A.).

### 4.3.5 Implantation of keloid tissue into the athymic nude mice

Each mouse underwent general inhalational anesthesia using the McGill University Animal Facility's standard protocol. The keloid specimen excised from the human donor earlier that day was manually divided into smaller specimens measuring approximately 2-3$mm^3$ each. As stated previously, three small keloid tissue samples were implanted in each of the seven mice as follows: one on the upper back, one on the lower back and one on the abdomen. This was done by creating a small 1cm cutaneous incision using a 15 blade, dissecting a subcutaneous pocket and implanting the tissue about 1cm away from the incision. The incision was closed with rat staples. The same technique was used for each of the three sites. The keloid tissue sites were spaced at a minimum of three centimeters apart to prevent treatment diffusion. Each small keloid tissue was weighed prior to implantation using a high precision digital milligram scale.

### 4.3.6 Treatment protocol

One week after implantation, each mouse was randomized into one of three treatment groups: botulinum toxin A, steroid or saline. The injections were administered under general anesthesia using the Montreal Children's Research Laboratory's standard protocol. Given the very thin nature of athymic nude mouse skin, exact localization of the previously implanted tissue samples was straightforward with a visible tissue mound. Also, precise injection into the implanted tissue was facilitated under general anesthesia.

The doses employed were based on previous literature establishing treatment and toxicity doses in the mouse model for both botulinum toxin A and triamcinolone [18-19]. 0.5 units of botulinum toxin A using normal saline as a delivery medium was injected per site (equating to 1 unit total per mouse in that group) and 1 mg of triamcinolone was injected per site (equating to 2 mg total per mouse in that group). The volume of injection was standardized to 0.1cc per site, regardless of the injected substance. The needle size used was also standardized to 25G (0.5mm). Furthermore, the site chosen for the injection was randomized. In other words botulinum toxin A was not always given to the implanted tissue in the abdomen and not the back, etc. This was done in order to avoid the potential confounding factor of differing tensile strengths exerted on the tissues.

### 4.3.7 Excision protocol

At three weeks post implantation (two weeks post injection), the mice were sacrificed and the keloid tissues harvested. Post excision weight analysis, immunohistochemistry, and standard hematoxylin and eosin (H&E) pathology was performed on each extracted tissue sample. Results were compared between the study drug (botulinum toxin A), the first line standard (triamcinolone) and the control (normal saline). The pathologist was blinded to the treatment received by each keloid site.

### 4.3.8 Data analysis

Statistical analysis was performed using the SPSS Software (IBM version 23). Pre-post weight analysis for each of the three groups was analyzed using the paired t-test analysis. A value of $p<0.05$ was considered statistically significant. Standard H&E comparisons were performed using qualitative descriptors. Immunohistochemistry results were analyzed using a one-way analysis of variance test (ANOVA).

## 4.4 Results

### 4.4.1 Tissue distribution

As previously described, 28 mice total were implanted; 7 mice per patient with 4 patients total. 3 keloid specimens were implanted per mouse, giving 84 tissue samples total. Out of the 84 specimens, 24 were treated with botulinum toxin A, 16 with triamcinolone and 44 with saline. Out of the 44 specimens treated with saline, 24 served as external controls and 20 as internal controls. The 20 internal controls were implanted

in mice that received either botulinum toxin A or triamcinolone in the other 2 keloid tissue sites, as opposed to the external control mice that received saline injections in all 3 tissue sites. Out of the 20 internal controls, 12 served as botulinum toxin A controls and 8 as triamcinolone controls (figure 1).

A few specimens were lost during the process. 3 implants treated with saline in a control mouse, 1 implant treated with saline in a botulinum toxin A mouse and 1 implant treated with botulinum toxin A fell out of the incisions. Furthermore, 1 mouse in the saline group, 2 mice in the steroid group and 1 mouse in the botulinum toxin A group died or required early euthanasia during the experiment for causes unrelated to the injections (figure 1).

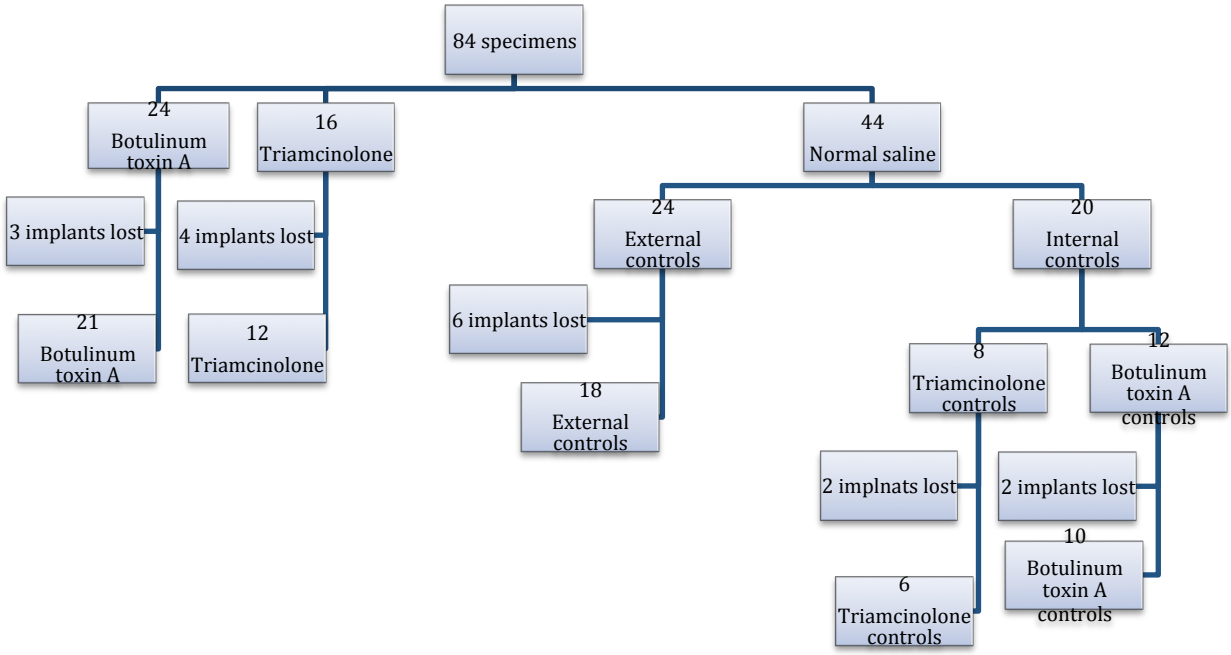


**Figure 1.** Flow diagram of keloid specimen treatments

### 4.4.2 Weight analysis

A statistically significant difference was found between pre and post weights of implants treated with botulinum toxin A, triamcinolone and saline as the internal control in the mice treated with triamcinolone (table 1).

| Treatment | Pre-treatment mean weight | Post- treatment mean weight | P value |
|---|---|---|---|
| Botulinum toxin A | 0.2319g | 0.1715g | 0.01 |
| Triamcinolone | 0.1916g | 0.1193g | 0.02 |
| Saline: all | 0.1530g | 0.1376 | >0.05 |
| Saline: external controls | 0.1466g | 0.1341g | >0.05 |
| Saline: triamcinolone controls | 0.1769 | 0.1272 | 0.03 |
| Saline: botulinum toxin controls | 0.1842 | 0.1961 | >0.05 |

**Table 1:** paired t test analysis results depicting pre and post treatment mean implant weights for each treatment category with an associated p value.

### 4.4.3 H&E pathology

All specimens were stained with standard H&E and submitted to a pathologist, who was blinded as to treatment group. Three aspects were analyzed: the amount and organization of collagen bundles; the presence of granulomatous reaction; and the presence of inflammatory markers. A Likert scale from 1 to 3 was used to further describe the collagen characteristics as follows: (1) sparse amount and architecture (2) moderate amount, organized architecture (3) abundant, disorganized architecture. Both the post-treatment botulinum toxin A and the triamcinolone groups were found to have less collagen overall, more organized collagen, absent granulomatous reaction and no inflammatory infiltrates when compared to pre-treatment specimens or saline controls (Figure 2). However, the saline internal controls in the mice treated with triamcinolone were found to have less and more organized collagen when compared to the standard saline controls.

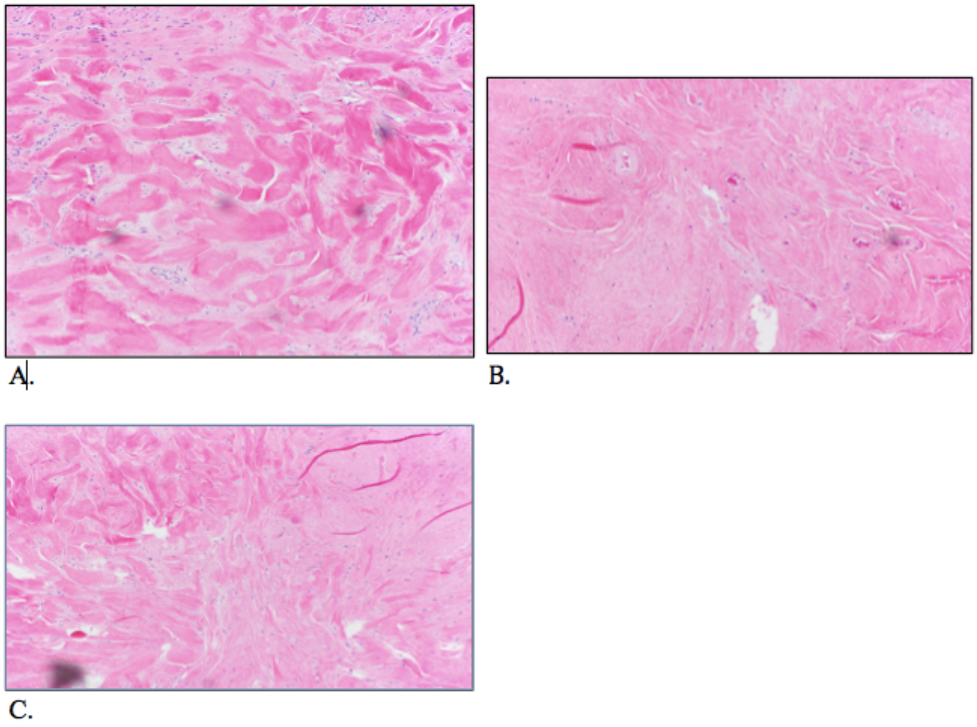


**Figure 2.** A-B-C. H&E stains depicting A. pre-treatment keloid tissue demonstrating dense and disorganized collagen bundles, B-C keloid specimens treated with botulinum toxin A (B) and triamcinolone (C) illustrating less abundant and more organized collagen.

### 4.4.4 Immunohistochemistry

Ki-67, a marker of cell proliferation and mitosis, was performed on all samples. All samples were then analyzed by the same blinded head and neck pathologist and graded on a Likert scale from 1 to 5 with 1 indicating 0-20% positivity, 2: 21-40% positivity, 3: 41-60% positivity, 4: 61-80% positivity and 5: 81-100% positivity. The higher the positivity, the more active the keloid tissue. A one-way analysis of variance (ANOVA study) showed that the effect of type of treatment (botulinum toxin A, steroid and external saline control) on Ki67 activity was significant with $F(2,46)=35.003=.000$.

## 4.5 Discussion

Our results demonstrate a statistically significant reduction in post-treatment tissue weight in both the botulinum toxin A and triamcinolone groups. Furthermore, blinded histopathological analysis revealed a subjective decrease in the amount of collagen, a more organized architecture and the absence of granulomatous or inflammatory markers in both of the above mentioned groups as compared to the

controls. The authors feel that the favorable changes observed in the internal triamcinolone control group treated with saline can be explained by the fact that triamcinolone is easily absorbed systemically, unintentionally delivering a small steroid dose to the internal saline control. Furthermore, the absence of change in the botulinum toxin A controls suggests that this effect is likely not due to diffusion of treatment from one tissue site to another.

The beneficial role of steroids in the treatment and prevention of keloid scars has been well documented in the literature [47]. However, the risks of hypopigmentation, atrophy and skin necrosis exist. If proven to be as successful if not superior to steroid treatment in future clinical trials, botulinum toxin A could offer a safe and easily administered alternative without compromising any of the beneficial effects. Furthermore, radiation therapy as a treatment for keloid scars has recently emerged as an unequivocally effective treatment modality. However, the risks of secondary malignancy, residual scarring and hypopigmentation persist. Botulinum toxin A could eventually serve as an adjunct to radiation in order to shrink the keloid prior to surgical removal, thus diminishing the scar and the size of the radiated field. Lastly, the role of botulinum toxin A as a *preventive* agent is easy to envision. Paralysis of underlying musculature reducing tensile strength on a wound combined with a potential for inhibited or reduced collagen formation could lead to a powerful addition to the surgeon's lacking armamentarium.

In 2006, Sherris et al conducted a study randomizing 31 patients with forehead lacerations to receive either botulinum toxin A or a placebo [65]. They found a significant difference based on the visual analog scale score. To date, only two animal studies have been conducted. The first, published in *Clin Exp Otorhinolaryngol* in 2009, consisted of two wounds made on fifteen rats [66]. One wound was injected with botulinum toxin A and the other with a placebo. On histopathology, the botulinum toxin A group demonstrated less collagen, less fibroblast and less inflammatory markers compared to the placebo. The second study, performed by Wang et al, involved the hypertrophic scar model in sixteen rabbits [67]. The botulinum toxin A group was found to have a smaller hypertrophic index, less fibroblasts and less collagen types I and III (the main types of collagen involved in wound healing and early and late scar formation). To our knowledge, no animal study comparing botulinum toxin A to steroids has been conducted to date.

We conducted a novel animal study in order to evaluate to efficacy of botulinum toxin A for the treatment of keloid scars. Our study has several important differences compared to previous works. Firstly, we used athymic nude mice with implanted human keloid tissue. Recent studies have found that the above-mentioned model is the one that preserves keloid tissue and mimics human conditions the best [62-64]. Secondly, we compared botulinum toxin A to not only a control, but also to steroid injections, the current standard first line treatment. Thirdly, we analyzed the histopathological characteristics of both botulinum toxin A and steroid injections as compared to a control.

Limitations of this study are mainly related to its basic science nature. Extrapolating treatment doses from the mouse model to a human clinical trial may be difficult. Furthermore, generalization and large scale response of botulinum toxin A as a treatment for keloid scars is unclear given that keloid tissue samples were extracted from four patients for the purpose of this study. Also, the preventative potential of botulinum toxin A was not explored in this animal model. Lastly, no attempt was made to isolate botulinum toxin A from its delivery medium, meaning that the positive effect witnessed may alter if the medium were to be discarded. This decision was made by the authors to approximate a realistic setting, given that, clinically, both the substance and the medium would be injected together.

## 4.6 Conclusion

This study explores a potentially improved treatment modality for keloid scars. Given the current scarcity of effective, safe and reproducible treatments, an alternative is greatly coveted. Botulinum toxin A was found to be an effective treatment for keloid scars in this animal model. Botulinum toxin A would allow for an easy, accessible and simple first line treatment alternative. Future clinical trials are needed to substantiate botulinum toxin A as a novel therapy to shift the current medical dilemma surrounding keloid scar treatment.

## 4.7 Funding Sources

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# CHAPTER FIVE: Summary

## 5.1 Overall Discussion

The present thesis presents a thorough exploration of current treatment options for auricular keloids, as well as a preliminary analysis of the novel use of botulinum toxin A, specifically Onabotulinumtoxin A, as a treatment option for keloid scars using an animal model. Pathologic scar formation due to excessive collagen production has frustrated clinicians for many years due to its aggressive nature, cosmetic and functional impairment, notoriously poor outcome with high recurrence rates and a lack of highly effective treatment options.

The first study presented in Chapter 3 explored current options for the treatment of auricular keloid scars. It showed that a personalized surgical approach, based on the characteristics of auricular keloids in each individual patient with a tailored multimodal therapeutic regimen including a combination of surgical excision, intralesional corticosteroid injection, and pressure therapy, may be an effective treatment, and it may reduce the recurrence rate of auricular keloids.

The second study discussed in Chapter 4 examined the promising effect of botulinum toxin A on keloid scars using an animal model, as compared to triamcinolone injection which was shown in Chapter 3 to be the most utilized medication to date. Botulinum toxin A was found to be an effective treatment for keloid scars in this animal model, shown to be equal or possibly superior to triamcinolone injection. Botulinum toxin A would allow for an easy, accessible and simple first line treatment alternative.

As with many treatments, there are many possible pitfalls to using Onabotulinumtoxin A. First, side effects are rare, but could include paralysis of surrounding muscles depending on injection site (eyelid droop, facial weakness including crooked smile, etc)[68]. Second, allergic reactions are also rare, but they do occur. Third, at high doses, systemic toxicity is a concern. Symptoms of systemic toxicity can range from fatigue, to seizures to death. The dose of injection (number of units) also needs to be carefully titrated. Nonetheless, Onobotulinumtoxin A remains a promising treatment options for keloids scars.

# CHAPTER SIX: Conclusion and future directions

## 6.1 Overall Conclusion

This thesis yielded the following new knowledge: (1) A systematic review showed that there is no preferred treatment for auricular keloid scars and (2) Onabotulinumtoxin A treatment minimized keloid scarring in an animal model.

## 6.2 Future Studies

The outcomes of this thesis shed knowledge on the current treatment options for auricular keloids and the basic science theory behind the effect of botulinum toxin A. The experiments described in this thesis were conducted in animal models. There is a need to translate the outcomes of this thesis into human clinical trials. Clinicians should be aware of this promising application of botulinum toxin A to treat keloid scars. Given the already widespread use of botulinum toxin to treat various medical pathologies and its excellent safety profile, a swift transition to clinical trials is easily conceivable. Botulinum Toxin A could serve as an excellent alternative to triamcinolone or could be used in conjunction with current treatment modalities to improve outcomes.

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