Combatting Bacterial Infections: The Efficacy of Poly-gamma-glutamic Acid on the Prevention of Nasopharyngeal Infections among Individuals with Cleft Lip and Palate

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Combatting Bacterial Infections: The Efficacy of Poly-gamma-glutamic Acid on the Prevention of Nasopharyngeal Infections among Individuals with Cleft Lip and Palate

A Thesis Presented

by

Florence Pun

To the Keck Science Department

of

Claremont McKenna, Scripps, and Pitzer Colleges

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The Degree of Bachelor of Arts

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ABSTRACT

Cleft lip and palate (CLP) is a medical condition where children are born with an unfused lip and palate. While surgery is required to fuse the lip and palate, there are other conditions that children with CLP face after surgeries, such as otitis media (OM), speech impediments, and difficulty breathing. Particularly, OM occurs frequently in individuals with CLP because the tensor veli palatini muscles cannot open the Eustachian tube frequently and ventilate the middle ear properly, even after surgery. This thesis is a research proposal to evaluate whether γ-PGA can be used in nasal washes to safely prevent nasopharyngeal infections like OM from occurring among children with CLP. γ-PGA, derived from Bacillus anthracis, has been shown to have non-toxic, lubricative, and antimicrobial properties. The biopolymer will be assessed for its cytotoxicity and viability with L929 mouse fibroblast cell cultures and its bactericidal effects on four pathogenic bacteria: Streptococcus pyogenes, Staphylococcus aureus, Pseudomonas aeruginosa, and Streptococcus pneumoniae. A double-blind randomized clinical study will also be performed to analyze the effects of γ-PGA among 400 children with CLP (6 - 12 years old). A significant effect of γ-PGA on preventing infections and illnesses in these children is expected. The in-vitro studies will reveal that γ-PGA is not cytotoxic and is bactericidal against the four bacteria. By creating a nasal wash that is more effective in killing bacteria compared to saline washes but also non-toxic unlike Chlorhexidine and Listerine, γ-PGA can prevent harmful nasopharyngeal infections among those with CLP.
INTRODUCTION (Parts 1-5)

Part 1: What Is a Cleft Lip and Palate?

Cleft lip and palate (CLP) is a craniofacial birth condition where a baby is born with an unfused lip and palate. CLP is one of the most common craniofacial conditions; about 1 in 700 individuals are born with it. Direct causes of CLP are still unknown, but potential causes include environmental and teratogenic factors such as air pollution and consuming alcohol during pregnancy, and also genetic mutations. While children can be born with both a cleft lip and cleft palate, they can also be born with just a cleft lip or just a cleft palate, and there are various severities for each cleft type. Unilateral clefts affect one side of the lip, while bilateral clefts affect both sides of the lip. The baby must receive treatment such as reconstructive surgery within the first few months after birth to fuse the lip and/or palate to promote proper feeding and breathing. Depending on the severity of the condition and surgical outcomes, these children may need to seek speech therapists, orthodontists, and otolaryngologists for further treatment (Vyas et al., 2020).

However, the condition is often untreated for various reasons worldwide, such as religious and cultural beliefs and financial limitations. In impoverished areas, paying for the costs of treatment and transportation to clinics may be a challenge, leading to delays in treatment. Additionally, many cultures and communities place stigma behind craniofacial conditions like CLP. Various communities belittle and shun those with a cleft, as the condition affects the physical features of the face. As a result, families and community members may not provide adequate support in seeking treatment for the child, especially if they were to alienate them rather than support them (Adeyemo et al., 2016). Many, especially in developing countries, have hardships in accessing medical care. If the condition is left untreated, these babies will
grow up with various long-term obstacles, such as malnutrition, speech impediments, breathing and hearing difficulties, and social discrimination (Hlongwa et al., 2018).

Considering that CLP is a relatively common birth condition, it is crucial to raise awareness about the challenges that people with CLP face. However, it is also important to identify the direct causes of CLP to promote the prevention of the condition and the obstacles that come with it. Working to prevent the condition in the future requires more understanding and discovery of the etiology and development of the condition. Because many of the direct causes of clefts are still unknown, it is not currently possible to prevent the condition worldwide. As a result, it is still important to find ways to eliminate various obstacles of being born with a cleft.

Surgery is a cost-effective intervention to reduce the challenges of CLP. Even though surgeries are deemed expensive for many, surgeries often spare those with CLP from other health conditions and treatments that are even more costly if children with CLP grow up with an unfused lip and/or palate (Hamze et al., 2017). Hence, finding accessible ways to prevent these challenges, especially for marginalized communities can improve experiences with a cleft drastically. Dedicated programs have been helping children with CLP in impoverished communities receive safe surgery to fuse the lip and palate for free. However, many children with CLP still need several more surgeries and extra treatments that may not be solely prevented by lip and palate repair. Not only should surgical techniques improve, but finding other ways to prevent infections and diseases that patients are often prone to should also be emphasized. These infections, affecting especially the nasopharyngeal region, pose a significant threat to the survival and quality of life of many children, especially in marginalized communities. This thesis
was written with the particular wish to alleviate and prevent the hardships of nasopharyngeal infections that those with CLP face worldwide.

**Part 2: Management of CLP beyond Surgical Treatment**

**Misconceptions**

A common misconception about CLP treatment is that surgeries solve every problem that comes with a cleft. However, frequent middle-ear infections, difficulty eating, swallowing, drinking, and speech impediments may persist after surgery as the lip and palate cannot be fused perfectly. These various conditions, which will be further discussed in this section, can ultimately lead to very costly and dangerous consequences, especially if one has few resources to resolve these hardships.

**Speech Impediments and Speech Therapy**

Even with a fused lip and/or palate, children who received surgery at a later age, or had improper reconstruction surgery may need speech therapy to retrain their speaking habits to prevent lisps and other speech impediments. Speech pathologists are responsible for determining if the palate muscles are functioning and articulating properly along with appropriate airflow in the oral and nasal cavities during speaking. Even if the child has a successful surgery, the child is required to have speech training and yearly appointments with a speech pathologist to ensure that do not have any communication disorders, including atypical consonant productions, abnormal nasal resonance and airflow, altered laryngeal voice quality, and nasal/facial grimaces (Nagarajan et al., 2009).
Difficulties in Eating and Breathing

Many children with CLP may face issues with feeding, especially when the lip and palate are not surgically fused successfully. For example, if the palate is not fully fused in surgery, food and drink may enter into the nasopharyngeal cavity and leak out of the nose. Children may also have difficulty eating as they may need extensive orthodontic work to restructure their teeth and jaws. Lisps often occur during speaking because of improper surgical fusion and dysfunctional muscles of the palate. Some individuals may face nasal breathing difficulties due to a deviated septum as cleft lips usually extend into the nostril. The deviated septum is usually still present even after fusing the lip, which promotes mouth breathing, especially during sleep, as it is difficult to breathe through the nose. Mouth breathing consequently dries the saliva in the oral cavity, which is typically crucial to neutralize acid and flush out bacteria (Tamkin, 2020). As a result, individuals with CLP are prone to periodontal diseases and tooth decay. Additionally, abnormal salivary glands are formed with CLP because of the presence of the mutated IRF6 gene. This mutated gene is associated with defective salivary glands that make the mouth too acidic in CLP, allowing harmful bacteria to harbor in the oral cavity, which can cause tooth decay (Tamasas and Cox, 2017).

Orthodontic Treatments

Given the various dental problems caused by CLP, including the misalignment of teeth and jaws, such as crossbites and underbites, children with CLP are prone to periodontal diseases such as gingivitis. Orthodontic treatment is often required, which may improve the structure and alignment of the teeth and jaws. But orthodontics may be harmful to overall oral health as it is difficult to maintain good oral hygiene with dental braces, further increasing the chances of
having periodontal and dental disease, including severe gum recession and dental caries. Overall oral health is overlooked by orthodontic teams as they focus on facial aesthetics, as well as aligning the teeth and jaw (Gaggl et al., 1999). These factors, along with the IRF6 gene that develops abnormal salivary glands, ultimately lead to higher risks of dental problems (Tamasas and Cox, 2017).

**Otitis Media (OM)**

Many with a cleft often face middle ear infections, known as otitis media (OM). CLP directly causes clefting of the levator veli palatini and tensor veli palatini muscles. These muscles are responsible for opening and closing the Eustachian tube that connects to the middle ear during eating, swallowing, and speaking. The opening and closing of the Eustachian tube also equalizes pressures and drains various bacteria and fluids from the middle ear (Schilder et al., 2015; Zambonato et al., 2009). With CLP, the palate muscles insufficiently open and close the Eustachian tube even if the cleft palate is repaired. The buildup of fluids and bacteria in the middle ear lead to OM. OM is a major cause of hearing loss, especially with the buildup of fluid behind the eardrum (Tamasas and Cox, 2017). As a result, many with OM need ear tubes, which are cylindrical plastic or metal tubes that are surgically inserted into the eardrum to drain the middle ear, preventing the accumulation of fluid behind the eardrum (Mayo Clinic, 2021). More on OM will be discussed later in this thesis.

**Current Solutions to Prevent OM and other Nasopharyngeal Infections**

Children with a cleft often utilize oral and nasal washes in an attempt to prevent infections. These washes range from saline washes to stronger chemical washes, including
Listerine, and chlorhexidine. However, patients are asked to solely use saline nasal and oral washes when recovering from oral and nasal surgeries. Saline washes are commonly used post-operation, as they don’t have strong, toxic chemicals that irritate tissues and wounds or damage cells that are essential for healing. Saline washes, while used for nasal irrigation, do not have strong antibacterial properties to treat bacterial infections. Hence, they are not effective in killing bacteria that harbor in unhealed wounds which causes complications of healing during post-operation; bacteria levels of more than 10^2 organisms per gram of tissue can result in infection (Fokkens et al, 2005; Ramdial and Madaree, 2019). Nasal washes also cannot be made of toxic or irritable chemicals such as those incorporated in oral washes like Listerine, as they are hazardous to ingest and damage the delicate nasal mucosa. A solution that is not only more effective than saline washes in killing bacteria but also nontoxic for patients would be optimal for combatting infections that come with CLP.

**Personal Dedication**

On behalf of my experience with cleft lip and palate, I would like to dedicate my undergraduate senior thesis to making progress to alleviate the challenges of CLP. Growing up with CLP, I experienced chronic ear and throat infections and spent significant time during my childhood making visits to the orthodontist. These procedures exposed me and my fellow friends with CLP to infections, as the nasopharyngeal and oropharyngeal areas are prone to bacterial colonization. I was often surprised by the common use of saline washes to flush out bacteria, whether I used them for nasal irrigation or to clean wounds after oral surgeries. The use of saline nasal washes throughout my life to combat nasal, throat, and middle ear infections have often left me wondering if there are other materials that could kill bacteria more effectively, as I faced
countless infections even with the use of saline washes. An antibacterial nasal wash would be useful for all children whether or not they are born with craniofacial differences. Additionally, my experience in gargling chlorhexidine and peroxide mouthwashes as a child left me bewildered in the absence of a non-toxic, yet effective mouthwash that exists for the sake of children’s safety and health. These questions have led me to explore certain materials that could be used as a better alternative to saline and chemically toxic washes. The idea of using γ-PGA in these washes became an inspiration after learning more about Dr. Pete Chandrangsu’s research on the biopolymer at the Keck Science Department. Specifically, I questioned if γ-PGA could be used to fend off various infections in the nasopharynx, which has led me to explore this idea in my thesis.

**Part 3: Anatomy and Genetics of Cleft Lip and Palate**

**Anatomy: Formation of Cleft Lip and Palate**

CLP is a result of improper craniofacial developmental processes. To learn about the development of CLP, it is crucial to learn about the development of the face. Facial development involves processes like cell growth, apoptosis, migration, and differentiation. The face begins to develop during the 4th week of development; neural crest cells migrate and form the five facial primordia, which are the frontonasal prominence, the paired mandibular processes, and the paired maxillary processes (Leslie and Marazita, 2013). Then, the nasal placodes invaginate, creating the medial and lateral nasal processes. The upper lip and primary palate are formed when the lateral nasal process merges with the paired maxillary processes, which then fuse with the medial nasal process during the 6th and 7th weeks of gestation. The secondary palate begins to form during the 7th week of development as the palatal shelves outgrow the maxillary
processes. The palatal shelves first grow along the sides of the developing tongue vertically, but they eventually grow horizontally when the tongue begins to flatten. The palatal shelves eventually meet, and fusion takes place along the medial edge of the epithelia. The nasal and oral cavities are properly separated as long as this fusion of the secondary palate is successful (Leslie and Marazita, 2013) (Figure 1.1). CLP forms when the development of the maxillary and mandibular processes undergo malformation, or if the elevation, migration, or fusion of palatal shelves are disrupted. Specifically, clefting in the lip, nasal floor, and alveolus occurs when the frontonasal and maxillary processes fail to fuse together (Vyas et. al, 2020) (Figures 1.2 and 1.3).
Figure 1.1 Development of the lip and palate; (a) the fourth week of embryonic development: neural crest cells have migrated to the craniofacial region to create the frontonasal prominence, paired maxillary processes, and paired mandibular processes. (b) the fifth week of embryonic development: the nasal pits form and create the frontonasal prominence into medial and lateral nasal processes (paired). (c) the sixth week of embryonic development: the upper lip and primary palate form after the medial nasal processes have merged with the maxillary. The lateral nasal processes form the nasal alae. The mandibular processes fuse to create the lower jaw. d) the sixth week of development: the secondary palate develops from the maxillary processes, which grows down vertically on the side of the tongue. e) the palatal shelves elevate into a horizontal position above the tongue and eventually start to fuse together. f) palatal shelf fusion splits the oronasal space into separate oral and nasal cavities (Dixon et al., 2011).
Figure 1.2. Anatomy of the lip and palate (Centers for Disease Control and Prevention, 2022).
Figure 1.3. Different cleft types (Dixon et al., 2011).

**Genetics: Genes Associated with Cleft Lip and Palate**

As mentioned previously, individuals with CLP usually cannot identify the direct reason for being born with the condition. CLP can be caused by family genetics, teratogens, and environmental factors. For example, a child may have a gene that increases their chances of being born with the condition, but a specific environmental factor may ultimately trigger CLP.
Maternal smoking and drinking, air pollutants, arsenic in drinking water, and pesticides in crops ingested by the mother have significantly affected the incidence of CLP in many babies (Suhl et al., 2018). Toxins in food and drinking water may be more prevalent in developing countries that lack access to clean air and water, which may increase the rate of CLP in those communities. Scientists are still working to determine how certain genes that are prone to CLP can be triggered by teratogenic factors.

Advances in molecular biology have successfully evaluated the genetic basis of the development of CLP. Several genes have been implicated. However, for non-syndromic CLP, the gene candidates only provide increased risk and the genetic roles cannot be pinpointed, unlike other diseases, such as cystic fibrosis, thalassemia, or sickle cell anemia. A recent study, which will be discussed at the end of this section, may provide a possible explanation during this extensive search for the cause of CLP and similar craniofacial conditions (Lin-Shiao et al., 2019).

The most consistent genes implicated in the occurrence of CLP are the following:

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<tr>
<td>1.</td>
<td>Interferon Regulatory Factor-6 (IRF6) (Zucchero et al., 2004).</td>
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<tr>
<td>2.</td>
<td>MSH Homeobox (MSX1) (Otero et al., 2007).</td>
</tr>
<tr>
<td>3.</td>
<td>Bone morphogenetic Protein-4 (BMP4) (Hao et al., 2018).</td>
</tr>
<tr>
<td>4.</td>
<td>Paired Box Protein Pax-7 (PAX7) (Duan et al., 2017).</td>
</tr>
<tr>
<td>6.</td>
<td>Runt-Related Transcription Factor 2 (RUNX 2) (Wu et al., 2012).</td>
</tr>
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</table>
The listed genes provide evidence of an increased risk of CLP. Except in the rare cases of syndromic CLP, such as the Treacher-Collins syndrome, a direct gene condition cannot be defined for the non-syndromic CLP.

In 2019, a new study from the Penn Epigenetics Institute of the University of Pennsylvania provided an eye-opening insight into explaining issues of defining gene mutations (Lin-Shiao et al., 2019). The study describes the role of protein p63 in modulating the expression of the above genes involved in craniofacial development. This gene is a member of the p53 family proteins and is an important transcription factor that guides the DNA of potential CLP-correlated genes to be transcribed or not. Point mutations in p63 lead to developmental malformations, especially CLP. The gene plays an important role in epithelial cell developmental processes. Specifically in mice, the deletion of Trp63 in the p63 gene causes developmental conditions in the epithelia and epidermis, which affect craniofacial growth and development, loss of salivary glands, abnormal hair follicles, and defective teeth. CLP conditions are a main feature of p63 syndromes and the gene controls whether enhancers for genes, which are important for craniofacial development, are allowed to be expressed in mRNA (Lin-Shiao et al., 2019).

Various genes associated with CLP, such as IRF6, MSX1, and PAX7 are located near the enhancer regions that work with p63. Thus, instead of gene mutation, it is the expression of the gene and the subsequent dysfunction in levels of mRNA and transcribed protein products that are at fault during fetal craniofacial development.

Many medical professionals devote their lives to helping babies born with CLP in hopes that their work can contribute to solving the many mysteries of the causative genes of CLP, such as the discovery of p63. Discovering more relevant genes allows more solutions to the prevention of craniofacial conditions, as well as early fetal gene therapies for craniofacial
malformations. For now, there has only been some research on the possibility of using fetal surgeries to treat CLP before birth (Papadopoulos et al., 2005).

**Part 4: Clinical Perspectives and Proposed Therapeutic Non-Invasive Approaches for Long-Term Oro-Nasal Complications**

*History of Treatments and Cultural Attitudes Towards CLP*

The most common treatment for CLP is surgery to fuse the lip and/or palate after birth. Cleft lip surgery originated in 390 B.C. in China, and the first successful cleft palate surgery took place in 1816. Often, stigma is placed upon the CLP condition, leading to possible delays in inventing surgical techniques (Perko, 1986). Ignorance of the condition played a large role in false assumptions of CLP. For example, in Nigeria, parents of children with CLP were asked to complete a survey on their experiences in their communities. The results indicate that 35.3% of the mothers believed that the condition was an “act of God,” while the other mothers believed CLP was caused by “evil spirits” or “wicked people.” Additionally, 73% of the mothers and 59% of the fathers were ashamed of their child’s condition. The majority of these parents revealed that their community has alienated them because of their child’s cleft (Adeyemo et al., 2016). The stigma of CLP has further prevented the treatment and support that children with CLP need, especially for those who struggle to have safe and affordable access to treatments.

For thousands of years, adults and children with craniofacial conditions were not only ostracized but babies with CLP were even targeted and endangered. Many African tribes removed these children from their communities and abandoned them in the wilderness, which is a practice that still exists today in some tribes. Similar trends occurred in the Roman Empire, where babies were drowned in rivers or thrown off mountains and cliffs to fend off supernatural
spirits. Such practices were even supported by the philosopher Plato, who claimed that these acts were beneficial for removing evil from communities (Bhattacharya et al., 2009).

**History of the Discovery of Modern-Day Treatments**

A greater understanding of CLP was not promoted until Fabricius ab Aquapendente (1537-1619) suggested the embryology of the condition, where the upper lip of the fetus only comes together along the middle line during the late stages of development. In the 19th century, Pierre Franco’s publications *Petit Traité* and *Traité des Hernies* allowed readers to better understand the condition. His work also included possible surgical treatments, such as using dry sutures, pins, and triangular bandages. Ambroise Paré, one of the best surgeons during the 16th century, conducted studies on the anatomy of the lip and the palate and improved suturing techniques (Bhattacharya et al., 2009). Today, the Millard rotation-advancement technique is used for unilateral cleft lip and the Mulliken technique is applied for bilateral cleft lip. These operations are expected to be performed after the first 10 weeks of birth (Figures 4.1 and 4.2). Palatoplastia is used to repair cleft palates during the first 9-15 weeks of birth (Figure 4.3). These discoveries in treatment have helped destigmatize the condition, but these efforts do not eliminate the discrimination that still occurs for those with CLP worldwide today.
Figure 4.1. Millard rotation-advancement technique for unilateral cleft lip and palate (Knežević et al., 2017).

Figure 4.2. Mulliken surgical technique for bilateral cleft lip and palate (Matsumoto et al., 2013).
Figure 4.3. Palatoplasty surgical technique (Sakran et al., 2021).

A focus is placed on improving treatments and ensuring that they are more accessible and affordable, but treatment and prevention of other illnesses that individuals with CLP endure must be further studied. This emphasis will decrease the frequency of oral, nasal, and ear conditions that they face. For example, repair of the nose, such as rhinoplasty, was not established until the 1970s because of fears that operations may affect nose growth. Nasal surgeries are often crucial in fully closing the nasal floor, and rearranging lower lateral cartilages, septum, and alar base to ensure better breathing and more articulate speaking (Shkoukani et al., 2013). While there are countless conditions that individuals with CLP face, this thesis will focus on possible prevention methods for OM and nasopharyngeal infections.
Otitis Media: A Common Infection Caused by Cleft Lip and Palate

As mentioned previously, otitis media (OM), also known as a middle ear infection, is caused by a dysfunctional Eustachian tube, where negative pressure builds up in the middle ear, preventing fluids from properly draining from the middle ear (Harman et al., 2015) (Figure 4.6). About 71% of children with CLP develop OM before the age of three years, as children with CLP have a relatively higher risk of developing it than children without craniofacial conditions. As these children get older, they often face OM repeatedly throughout their lives, because the tensor veli palatini, the palate muscles that contract to open the Eustachian tube, are dysfunctional, leading to improper ventilation and drainage of the middle ear. It is crucial to understand how the Eustachian tube and palate typically connect before understanding how OM can particularly affect those with CLP (Figures 4.7 and 4.8). Specifically, the aponeurosis of the tensor veli palatini connects to the edges of the cleft palate instead of the posterior border of the hard palate (Sharma and Nanda, 2009). A study also reveals that the dimensions of the skull, such as the small size of the spheno-occipital bone and the upward position of the maxilla are other potential reasons why children with CLP are more prone to OM (Sharma and Nanda, 2009).
Figure 4.6. Structure of a healthy ear compared to an ear with otitis media. The Eustachian tube fails to open frequently when affected by otitis media, and fluid builds up in the middle ear, which can cause hearing loss. Fluid build-up in the middle ear prevents full movement of the ossicles (Mayo Clinic, 2022).

Figure 4.4. The connection between the Eustachian tube and the soft palate in an individual without CLP (Algudkar et al., 2013).
Figure 4.5. The connection of the Eustachian tube between the nasopharynx and middle ear in infants and adults without CLP. Note that the Eustachian tube in adults is more slanted to allow easier drainage of the middle ear (Cleveland Nasal Sinus and Sleep Center, 2022).

Pathogenesis of Otitis Media

Based on the skeletal and muscular anatomy of CLP, there is often an abnormal reflux of food and fluid into the nasal cavity, which can cause inflammatory changes around the Eustachian tube. Viruses and bacteria in the pharynx can be refluxed into the nasal cavities and directly increase the frequency of OM (Figure 4.7). Because the Eustachian tube does not open properly and frequently, gasses are absorbed by the mucous membrane in the middle ear causing negative pressure, leading to a retracted tympanic membrane and fluid and bacterial buildup in the middle ear. The break of the mechanical barrier between the mouth and nasopharynx which
typically protects the body from pathogens affects and changes the bacterial flora in CLP. As a result, there is an overgrowth of bacteria in the nasopharynx, causing middle ear inflammation. Fluid buildup behind the eardrum can ultimately cause hearing loss too (Sharma and Nanda, 2009).

Figure 4.7. Viruses and bacteria present in the nasopharynx can cause OM. The improper draining of the Eustachian tube allows bacteria and viruses to harbor in the middle ear, causing otitis media (Schilder et al., 2016).
Figure 4.8. Various factors put children at risk for OM. For children with a cleft, the cause of OM typically comes from mucosal damage and Eustachian tube dysfunction (Schilder et al., 2016).

While hearing loss is a harmful problem, the consequences of ear infections are often overlooked by CLP care teams, as they are more concerned with oral problems, such as speech impediments and improper feeding. There is little effective management for middle ear infections, leading to inconsistent treatment and miscommunication between healthcare teams.
and patients/parents on the outcomes of treatment (Harman et al., 2015). Hearing loss caused by OM leads to improper speech function and cognitive disorders (Sharma and Nanda, 2009).

Medical teams must work to incorporate more attention and treatment toward OM. While medications such as antibiotics and surgical procedures such as inserting ear ventilation tubes may be useful treatments, OM is often chronic and can infect the ear in various ways (Figure 4.9). Ear tubes can be effective in draining and ventilating the middle ear to alleviate and even reduce the frequency of OM. However, there are often many complications after surgically inserting ear tubes. Children with ear tubes often have chronic ear discharge that leaks out of the ear. Oftentimes, ear tubes can be dislodged from the tympanic membrane and may require reinsertion (Schilder et al., 2016) (Figures 4.9 and 4.10). While ear tubes may be helpful to ventilate the middle ear, children often face OM even with these tubes.

Figure 4.9. Various middle ear conditions. a) Healthy tympanic membrane. b) Tympanic membrane affected by OM. c) OM with effusion. d) An ear tube surgically implanted into the tympanic membrane to drain fluid out of the middle ear (Schilder et al., 2016).
Figure 4.10: Ear tubes or ventilation tubes prevent recurring cases of otitis media and prevent hearing loss and damage in the middle ear (Schilder et al., 2016).

**Part 5: Poly-Gamma-Glutamic Acid (γ-PGA) and its Usefulness**

A possible prevention for various infections like OM that is being proposed in this thesis, is a nasal wash that is nontoxic unlike Listerine, Chlorhexidine, or Benzoic acid and also more effective in killing bacteria compared to saline washes. Preventative measures of CLP will decrease the number of problems, especially if the prevalence of difficulty accessing healthcare, such as the costs, is present worldwide. This section of this thesis begins to explain why γ-PGA can be an effective biopolymer for nasal washes for children with CLP.
What is γ-PGA?

Poly-gamma-glutamic acid, or γ-PGA, is an amino acid polymer of glutamic acid (Figure 5.1). The polymer is non-toxic and has been used for various purposes involving the food, medical, and wastewater industries (Ogunleye et al., 2014).

![Figure 5.1. The structure of γ-PGA.](image)

The polymer was found to effectively inhibit both Gram-positive (*L. monocytogenes* and *S. aureus*) and Gram-negative (*L. monocytogenes*, *S. typhimurium*, *K. pneumoniae*, and *E. coli*) bacteria, which indicates that it has antimicrobial properties (Lee et al., 2014).

The Various Uses of γ-PGA

**a. Contact Lens Solution**

A study has shown that eye contact lens preservatives are effective in cleaning, storing, and disinfection. But the contents of the preservatives may cause immune reactions, leading to eye conditions, including papillary conjunctivitis, peripheral ulcer, and superior limbic keratoconjunctivitis. The study revealed that γ-PGA could replace eye contact lens preservatives. γ-PGA is also significantly less harmful to the eye compared to typical contact lens solutions as it acts as a non-toxic antimicrobial agent that can reduce immune reactions from eye conditions and combat bacteria such as *P. aeruginosa* and *C. albicans*, common microorganisms that are present on contact lenses. Additionally, it is very beneficial that the biopolymer is lubricative and
hydrating. When contact lenses are worn, blinking causes friction between the eye and the lens, especially during extended use. The smooth, lubricating, and hydrating features of γ-PGA can alleviate irritation and dryness (Su et al., 2019).

b. Medicine and Drug Delivery

γ-PGA, which is derived from Bacillus anthracis, is also biodegradable and has been used in medicine, particularly drug delivery. For example, γ-PGA has been used to induce natural killer cell-mediated antitumor immunity (Kim et al., 2007).

c. Water, Food, and Skin Treatment

γ-PGA has also been used in water treatment, particularly the flocculation process. The biopolymer has been revealed to be able to remove solids and metal ions in wastewater treatment. It has also been used to collect microbial cells from broths and fermented foods (Bajaj and Rekha, 2011). γ-PGA is used in various serums to plump skin, as it is both water-soluble and moisturizing, and its effects have been revealed to be even more hydrating than collagen and hyaluronic acid, as γ-PGA (0.2%) has water retention properties like those of glycerol (5%). γ-PGA could potentially be an alternative to collagen, hyaluronic acid, and glycerol for moisturizers (Lee et al., 2014). With these results, γ-PGA can hydrate dry nasal passages to prevent nasal and respiratory infections. A study has shown that dehydration leaves little water in the nose, trachea, and lungs, which are responsible for hydrating the air that is breathed into the lungs. Such dehydration thins the upper airway lining fluid, reduces cilia mobility, and damages epithelial cells, which prevents the upper airways from filtering out contaminants, leading to
allergies, asthma, chronic obstructive pulmonary disease, and respiratory infections and diseases such as COVID-19 and influenza (George et al., 2022).

With the many benefits and uses of γ-PGA, the polymer could be manufactured into a nasal wash. Its antibacterial properties could fend off bacteria in the nasopharynx, and because it is water-soluble, non-toxic, and lubricating, γ-PGA is safe to use and is gentle on the skin, without providing irritation or toxicity to the mucosal nasal tissue. γ-PGA can not only prevent dry noses, but this trait can also help prevent various respiratory conditions, infections, and diseases (George et al., 2022).
Part 6: Proposed In-Vitro Studies of γ-PGA

METHODS

The following proposed studies assess the effectiveness of γ-PGA in a nasal wash.

As mentioned previously, γ-PGA is a non-immunogenic, natural, biodegradable, environmentally friendly, and non-toxic compound. The antibacterial activities have been evaluated in contact lens solutions and have been used in various ways as mentioned in the previous section (Su et al., 2019). Additionally, γ-PGA has been used successfully as antibacterial additives (in the form of nanoparticles) in pork and at preparation factories for food safety in the form of antibacterial films made of ionic complexes of γ-PGA and ethyl lauroyl arginate (Cui, et al., 2018; Gamarra-Montes et al., 2017). The possible use of γ-PGA as a nasal wash to prevent or treat harmful nasopharyngeal complications among individuals with or without CLP has not been evaluated before.

The proposed study will investigate whether γ-PGA could be an effective antibacterial agent for nasal washes through an in-vitro study. The ability to inhibit the four different types of highly pathogenic bacteria in the nasopharynx of children with CLP will be tested. In addition, γ-PGA will be examined to see if it would cause any cytotoxicity or affect viability in-vitro in L929 mouse embryonic fibroblasts. All measurements and assays will be performed 8 times (in octuplicate) to assure accuracy and precision in the experiments.

a. Cell Viability Testing and Cytotoxicity

The viability and cytotoxicity effects of four concentrations of γ-PGA (0 ppm, 100 ppm, 1000 ppm, 10000 ppm) on L929 mouse fibroblast cells will first be assessed. γ-PGA will be obtained from Wellman Ltd., Taichung, Taiwan. 200 µL (105 cells per well) of L929 mouse
fibroblast cells (Food Industry Research and Development Institute, Strains number BCRC 60091) will be added to a 96-well culture plate and will be incubated overnight at 37 ºC. To visibly count the viable cells, 25 µL of WST-8 reagent (water-soluble tetrazolium salt) (Dojindo Molecular Technologies, Inc. Cat.# CK04-01) will be added. The mixture in the microplate will be incubated for an hour and a half at 37 ºC without any light. Then, with a spectrophotometer plate reader (ELISA), the optical density value (OD) will be measured at 450 nm. The viability percentage will be calculated with this formula: Viability (%) = the OD value of the γ-PGA solution / the OD value of the control. A significant decrease in cell viability will be present in groups with a viability lower than 75%.

Cytotoxicity will be assessed using a lactate dehydrogenase (LDH) reagent (Biovision, Mountain View, USA). 50 µL of LDH reagent will be mixed thoroughly with the mouse cells, and the OD value will be measured at 500 nm using a spectrophotometer after putting the mixtures into the centrifuge for 5 minutes at 1000 rpm. The amount of LDH released into the culture medium by the damaged cells determines the change in absorbance. The cytotoxicity percentages will be obtained with this formula: Cytotoxicity(%) = (OD value in γ-PGA solution) - (OD value in control) / (OD value in cell disintegration)-(OD value in control) (Riss et al., 2013; Su et al., 2019; Smith et al., 2011; Fotakis and Timbrell, 2006).

b. Antimicrobial Activity Measurement

γ-PGA at four different concentrations will be mixed into a normal saline solution (0.154 M) and will be added to four different strains of bacteria: *Streptococcus pneumoniae* (ATCC 49619), *Streptococcus pyogenes* (ATCC 12384), *Pseudomonas aeruginosa* (ATCC 10145), and *Staphylococcus aureus* (ATCC 10832). These four bacteria will be used to assess the
antibacterial effects of the γ-PGA nasal wash. The bacteria strains will be added into a centrifuge tube that contains 10 ml of nutrient broth in the laminar flow hood. The bacteria will be cultured at 37 °C at 150 rpm for 24 hours. Then for each bacteria strain, the control treatment will include 1 mL of the bacteria culture and will be mixed with 9 mL of saline. Additionally, for each bacteria strain, there will be three experimental treatments that each include 9 mL of γ-PGA at 100 ppm, 1000 ppm, and 10000 ppm respectively, and 1 mL of bacteria culture. The optical density, or the turbidity, of the cultured bacteria, will be measured at 610 nm every two hours to analyze the growth of all the strains in the control and experimental treatments (Su et al., 2019; Liu et al., 2001; Beal et al., 2020).

**Statistical Analysis**

Results will be presented as the mean and the standard error of the mean (SEM). The differences will be analyzed and comparisons will be made with a one-way ANOVA for the cytotoxicity and viability studies, and a two-way ANOVA to analyze the antibacterial activity. A value of $p < 0.05$ in the analyzed results will be considered significant.
EXPECTED RESULTS

The effect of four different concentrations of γ-PGA on cell survival and toxicity will be evaluated in L929 mouse embryonic fibroblasts (Fig 6.1 and 6.2). I expect that in the LDH assays that γ-PGA will be correlated with high viability that will reach almost 100% cell viability in each of the four concentrations.

![Graph showing viability levels of different concentrations of γ-PGA](image)

Figure 6.1. The high viability levels (%) of four different concentrations of γ-PGA when incubated with L929 mouse fibroblast cells.

Additionally, the four concentrations of γ-PGA are all expected to have very low levels of cytotoxicity, all of which are less than 1% (Figure 6.2). The results of these two tests are expected to reveal that γ-PGA is not a toxic compound, whether the cells are present in the solution at a low or high concentration of γ-PGA.
Figure 6.2. The low cytotoxicity levels (%) of four different concentrations of $\gamma$-PGA when incubated with L929 mouse fibroblast cells.

We next will test the antibacterial effects of $\gamma$-PGA at four different concentrations. We expect that at the highest concentration, 10,000 ppm (parts per million), almost 100% antibacterial activity will be achieved. An increase in antibacterial activity will occur with increasing concentrations of $\gamma$-PGA compared to the control (0 ppm) (Table 6.1 and Fig 6.3).
Table 6.1. The inhibition of bacteria by four different concentrations of γ-PGA.

<table>
<thead>
<tr>
<th></th>
<th>0 ppm of γ-PGA</th>
<th>100 ppm of γ-PGA</th>
<th>1,000 ppm of γ-PGA</th>
<th>10,000 ppm of γ-PGA</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pseudomonas Aeruginosa</em></td>
<td>1 ± 0.5%</td>
<td>10 ± 0.2%</td>
<td>23 ± 0.5%</td>
<td>98 ± 1.2%</td>
</tr>
<tr>
<td><em>Staphylococcus Aureus</em></td>
<td>0.5 ± 0.3%</td>
<td>8 ± 0.3%</td>
<td>24 ± 0.6%</td>
<td>97 ± 2.0%</td>
</tr>
<tr>
<td><em>Streptococcus Pneumoniae</em></td>
<td>0.8 ± 0.3%</td>
<td>12 ± 0.4%</td>
<td>31 ± 0.9%</td>
<td>99 ± 0.5%</td>
</tr>
<tr>
<td><em>Streptococcus Pyogenes</em></td>
<td>0.6 ± 0.4%</td>
<td>11 ± 0.3%</td>
<td>30 ± 1.1%</td>
<td>99 ± 0.4%</td>
</tr>
</tbody>
</table>

Figure 6.3. Antibacterial activity of four different concentrations of γ-PGA when incubated with four strains of bacteria.
DISCUSSION

Given the expected results, I would conclude that γ-PGA will have strong antibacterial activity, and the optimal concentration will be 10,000 ppm of γ-PGA. We expect that γ-PGA at 10,000 ppm can achieve nearly 100% antibacterial activity. At this concentration, γ-PGA will also not be cytotoxic.

To define the benefits of γ-PGA, an interesting study was performed by direct application of γ-PGA to the middle ear mucosa in rats. This is the only study of γ-PGA related directly to the middle ear. The findings provide interesting insight into our proposed clinical use of γ-PGA (Nilsson et al., 2014). When the biopolymer is applied to the rat middle ear, γ-PGA induces potent production of IL-1α, IL-1β, IL-6, and TNF-α. These are inflammatory cytokines that are crucial in body defenses and can induce strong chemoattractant signals that guide the leukocytes to protect the middle ear mucosa, delicate osseous structures like the malleus, incus, and stapes, and the eardrum (Cekici et al., 2000). This anti-inflammatory effect, in addition to the antibacterial actions, would be beneficial in decreasing the frequency of OM and hearing loss in those with CLP.

One possible weakness of this proposed in-vitro study is that the antibacterial activity will only be evaluated against four of the most pathogenic bacteria in the pharynx. These bacteria are the most common in affecting children with CLP. However, the effects on the normal flora on the pharynx should also be evaluated, which would include species such as Streptococcus viridans, peptostreptococcus, etc. Disruption of the normal flora on the respiratory mucosa is associated with significant respiratory diseases such as chronic respiratory pulmonary disease (Guo et al., 2021). This possible limitation highlights the importance of the clinical study in the next chapter,
which determines if γ-PGA can prevent various infections in humans, particularly children with 
CLP.
Part 7: The Evaluation of the Effectiveness of γ-PGA in Saline Application to Prevent Upper Respiratory Tract Infections and Sinonasal Symptoms Among Children with CLP

As mentioned previously, children with CLP have a high chance of upper respiratory tract infections, gingivitis, tonsillitis, pharyngitis, otitis media, and other infections. One major problem is the change in bacterial flora and frequent infection with pathogenic bacteria, such as Streptococcus pneumoniae, Staphylococcus aureus, Pseudomonas aeruginosa, and Streptococcus pyogenes (Zhou, et al., 2022). If the growth of pathogenic bacteria can be controlled, infections can be prevented. The objective of the study is to evaluate this hypothesis by testing the efficacy of γ-PGA in decreasing these infections and symptoms among children with CLP.

METHODS

To assess the safety and effects of γ-PGA in the prevention of infections that especially affect those with CLP, this thesis proposes clinical trials will be performed on children with CLP ages 6-12 years. The effects of γ-PGA in nasal washes on children with CLP must be assessed, as they are prone to illnesses like common colds in school. All parents and guardians will sign an informed consent form that includes information on the study. The study protocol will be approved by local ethical committees and the Institutional Review Boards (IRB).

The double-blind placebo-controlled randomized phase 3 clinical study will be performed at various craniofacial clinics in San Francisco; every child will be assessed by the same physician for each visit. The patients will be randomly paired with a physician in this double-blind study. A total of 400 children in the study will be randomized in a strictly double-blind approach by physicians to have one of the two treatments: 1) a saline nasal wash (0.154 M, n = 200) or 2) a saline nasal wash (0.154 M) containing 10,000 ppm of γ-PGA (n =
The saline wash and γ-PGA are commercially available products (supplier from Taiwan). Children in both the treatment and control groups will be given instructions on nasal wash usage. The number of bottles used by each patient will be recorded and each patient will be required to return all empty bottles. The washes for both treatments will be administered 3 times a day. 9 mL of nasal wash will be administered through each nostril during each application.

Patients will be observed for 9 months; their symptoms, health status, presence of ear infections, and medication use will be assessed during 4 visits (at 0, 3, 6, and 9 months) throughout the trial.

**Parameters Evaluated**

Physicians will evaluate the parameters during each scheduled visit. Baseline parameters will be evaluated in initial visits, including sex, age, type of cleft condition, health status, and health history. In subsequent visits, physicians will monitor for any symptoms of illnesses or infections. Parents will be asked to report any symptoms and illnesses at each visit. The parameters that will be used are those commonly used by otolaryngologists in the clinical management of upper respiratory and otolaryngologic infections among children and have been used for the evaluation of other products in clinical studies (Tomooka et al., 2000; Šlapak et al., 2008; Ramalingam et al., 2019).

**Statistical Analysis**

The study will evaluate both quantitative and qualitative data. Quantitative data will be presented as mean, standard deviation, and median, and qualitative data will be presented as absolute frequencies and percentages. Individual parameters will be separately evaluated at each
of the four visits. For data measured on a scale (including rhinological system scores, nasal secretion scores, and nasal breathing scores), the hypothesis that there is no difference in the median values will be evaluated through a Student’s t-test. For comparison of qualitative data, the results of the two groups will also be evaluated with the Student’s t-test. The level of statistical significance will be set at $p < 0.05$ for all analyses.
EXPECTED RESULTS

A total of 400 patients will be examined; 200 patients will be assigned to each of the saline (placebo) and γ-PGA (treatment) groups through double-blind randomization. Baseline characteristics (made on the first day of the study at 0 months) will be compared between the two groups. Allergies and the use of antihistamines will also be compared at the baseline to assure that the two groups are comparable (Table 7.1).

The efficacy of γ-PGA will be assessed by examining the rhinology scores and the use of medications, which include antipyretics, nasal decongestants, mucolytics, and antibiotics. The incidence of reported illnesses and reported school absences will also be used to compare the saline and γ-PGA groups.

Patients will be assessed at baseline and during treatment after 3 months (Table 7.2), 6 months (Table 7.3), and 9 months (Table 7.4). Although the severity and frequency of symptoms and other parameters are expected to be comparable at baseline, I expect significantly lower symptomatic scores in the γ-PGA treatment at 3, 6, and 9 months. Symptom scores that differed significantly between the two groups will be the sore throat, nasal secretion type, nasal secretion, and nasal breathing scores (p < 0.05; Tables 7.2, 7.3, and 7.4). When comparing the consumption of pharmaceuticals, significantly lower consumption of antipyretics, nasal decongestants, mucolytics, and antibiotics are expected among patients in the γ-PGA group than in the saline group (p < 0.05; Tables 7.2, 7.3, and 7.4). Patients who used γ-PGA will report significantly fewer days of illness, absences from school, and complications during the follow-up visit at 3 months, 6 months, and 9 months (p < 0.05, Tables 7.2, 7.3, and 7.4).
Table 7.1. Characteristics of 400 patients during visit 1. The 2 groups of children with CLP are expected to be comparable in these characteristics.

(a): Rhinologic symptom scores (sore throat, dry cough, productive cough, nasal secretion, sneezing, and loss of smell and/or taste): 0, no symptoms; 1, mild; 2, moderate; and 3, severe.

(b): Nasal secretion scores: 0, absent; 1, serosal; 2, seropurulent; and 3, purulent.

(c): Nasal breathing scores: 0, without difficulty; 1, minor difficulties; 2, major difficulties; 3, extreme difficulties.

<table>
<thead>
<tr>
<th></th>
<th>Saline (n = 200)</th>
<th>γ-PGA (n = 200)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>10.4</td>
<td>10.5</td>
</tr>
<tr>
<td>Female</td>
<td>84</td>
<td>86</td>
</tr>
<tr>
<td>Male</td>
<td>116</td>
<td>114</td>
</tr>
<tr>
<td>Flu Vaccinated</td>
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<td>194</td>
</tr>
<tr>
<td>Allergies</td>
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<td>61</td>
</tr>
<tr>
<td>Antihistamine</td>
<td>39</td>
<td>41</td>
</tr>
<tr>
<td>Sore throat $a$</td>
<td>1.79</td>
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<tr>
<td>Dry cough $a$</td>
<td>1.65</td>
<td>1.48</td>
</tr>
<tr>
<td>Productive cough $a$</td>
<td>1.48</td>
<td>1.38</td>
</tr>
<tr>
<td>Nasal secretion $a$</td>
<td>2.65</td>
<td>2.83</td>
</tr>
<tr>
<td>Sneezing $a$</td>
<td>1.55</td>
<td>1.56</td>
</tr>
<tr>
<td>Loss of smell and/or taste $a$</td>
<td>1.43</td>
<td>1.29</td>
</tr>
<tr>
<td>Nasal secretion type $b$</td>
<td>2.60</td>
<td>2.58</td>
</tr>
<tr>
<td>Nasal breathing score $c$</td>
<td>2.21</td>
<td>2.23</td>
</tr>
</tbody>
</table>
Table 7.2. Visit 2; Efficacy parameters at 3 months.

**Bolded values: p < 0.05**

<table>
<thead>
<tr>
<th></th>
<th>Saline (n = 200)</th>
<th>γ-PGA (n = 200)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sore throat $a$</td>
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<td>Dry cough $a$</td>
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<td>Productive cough $a$</td>
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<tr>
<td>Nasal breathing score $c$</td>
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<td>1.02</td>
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<td>Antipyretics</td>
<td>26</td>
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<tr>
<td>Nasal decongestants</td>
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<tr>
<td>Mucolytics</td>
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<td>34</td>
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<tr>
<td>Systemic antibiotics</td>
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<td>8</td>
</tr>
<tr>
<td>Reported Illnesses</td>
<td>166</td>
<td>78</td>
</tr>
<tr>
<td>Reported School Absences</td>
<td>66</td>
<td>30</td>
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<td>Complications</td>
<td>58</td>
<td>15</td>
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Table 7.3. Visit 3; Efficacy parameters at 6 months.

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<th>Parameter</th>
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<th>γ-PGA (n = 200)</th>
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<tbody>
<tr>
<td>Sore throat a</td>
<td>1.31</td>
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<td>Dry cough a</td>
<td>1.39</td>
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<tr>
<td>Productive cough a</td>
<td>1.32</td>
<td>1.26</td>
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<tr>
<td>Nasal secretion a</td>
<td>1.84</td>
<td>1.27</td>
</tr>
<tr>
<td>Sneezing a</td>
<td>1.20</td>
<td>1.07</td>
</tr>
<tr>
<td>Loss of small and/or taste a</td>
<td>1.21</td>
<td>1.01</td>
</tr>
<tr>
<td>Nasal secretion type b</td>
<td>2.23</td>
<td>1.36</td>
</tr>
<tr>
<td>Nasal breathing score c</td>
<td>1.66</td>
<td>1.21</td>
</tr>
<tr>
<td>Antipyretics</td>
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<td>Nasal decongestants</td>
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<td>Mucolytics</td>
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<td>Systemic antibiotics</td>
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<td>11</td>
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<tr>
<td>Reported Illnesses</td>
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<td>59</td>
</tr>
<tr>
<td>Reported School Absences</td>
<td>70</td>
<td>33</td>
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<tr>
<td>Complications</td>
<td>64</td>
<td>16</td>
</tr>
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</table>
Table 7.4. Visit 4; Efficacy parameters at 9 months.

<table>
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<th>Parameter</th>
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<tbody>
<tr>
<td>Sore throat $a$</td>
<td>1.11</td>
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</tr>
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<td>Dry cough $a$</td>
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<td>Nasal secretion $a$</td>
<td>1.52</td>
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<td>Nasal secretion type $b$</td>
<td>1.51</td>
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<td>Antipyretics</td>
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<td>Reported Illnesses</td>
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<td>Reported school absence</td>
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<td>16</td>
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<td>Complications</td>
<td>28</td>
<td>8</td>
</tr>
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</table>
DISCUSSION

This is a proposed double-blind placebo-controlled phase 3 clinical study to evaluate the potential of γ-PGA in preventing nasopharyngeal infections among children with CLP. We identify this study as a pilot proof-of-concept idea that provides robust, consistent, and statistically significant results. There are three major limitations in this study:

1. The nasal rinse technique among every individual is subject to variation (Principi and Esposito, 2017). This problem is addressed by using a relatively large volume (9 mL) for each rinse. The double-blind controlled design should be able to address this issue since both treatment groups, γ-PGA and saline (placebo control), would be subjected to the same technical variation.

2. The study participants solely include children with CLP, meaning the results of the study are not representative of all children, including those without craniofacial conditions. However, it is worth noting that the relationship between γ-PGA and infections may not be noticed if the study is proposed to be conducted solely on children without craniofacial differences, as these children are less likely to experience nasopharyngeal infections. A future study including children without CLP would be useful. However, this proposed study probably would involve a much larger group of participants and would require more resources to perform.

3. This is a relatively short study of 9 months. The two major complications, hearing loss, and severe gum disease may not be apparent until many years later (Sevier, 2022). A long-term study is important to extrapolate results for long-term outcomes.

Despite the mentioned limitations, the proposed trial is expected to reveal the efficacy of γ-PGA in preventing upper respiratory tract infections and sinonasal symptoms among children.
with CLP. The results are expected to be robust and consistent in several parameters and also statistically significant. However, a study with a longer duration of at least 5 years among children without CLP would be important in further defining the clinical benefits of γ-PGA.
Part 8: Last Thoughts on this Proposed Study

CONCLUSION

We should realize that it is untrue that a single surgery can fully repair every child’s cleft. Among various surgeries that a child must need to repair their cleft, their genetics, environment, and their access to general healthcare leave them predisposed to more conditions that are beyond fusing the lip and palate. While surgeries improve a child’s quality of life, as they can feed and speak more easily, more efforts are needed in eliminating not only the stigma behind CLP but also in preventing many related illnesses with CLP. This research proposal focuses on otitis media, a common infection that occurs with CLP, but it is only one of many conditions that must be further investigated in terms of treatment and prevention, including periodontal diseases, dental cavities, etc. Prevention is especially crucial for individuals who lack access to treatment, as it can save them not only from the pain of various illnesses, but it can also spare them from the challenges of accessing and affording treatments for these illnesses.

Important work is being performed by thousands of dedicated medical teams all over the world to improve the care of CLP. The following four objectives for future research may be most important:

1. Gain a deeper understanding of the long-term effects of CLP on special senses including hearing acuity and olfactory function, gum and dental health, psychological impact, and cognitive function. This thesis is a small step to attempt to alleviate one of these problems.

2. Improve the understanding of epigenetics and more detailed genotyping and phenotyping such as the work done by the Penn Epigenetics Institute of the University of Pennsylvania
(Lin-Shao et al., 2019). This greater understanding can decrease the social stigma behind CLP, as children with the condition are still often bullied in school.

3. Examine animal models of craniofacial conditions (like zebrafish) and functional analysis of the responsible genes and variants (Raterman et al., 2020). Performing studies on these models can lead to more discoveries on the direct causes of craniofacial conditions.

4. Use stem cells (Amiri et al., 2022), growth factors (Behnia et al., 2011), three-dimensional printing (Virani et al., 2021) and computerized surgical assistance with techniques like robots (Omran et al., 2019) to improve surgical outcomes.

While plenty of progress has been made to support and alleviate the experience of having CLP, much work still needs to be done. Initiatives are currently in progress to supply more surgeries for children who lack access to safe operations, and more awareness is being made to educate the general population on the condition, including books and films like *Wonder*. It is crucial to make improvements in healthcare and instill more cultural acceptance in societies towards craniofacial conditions to ensure the safety and well-being of those with a cleft.
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