Clinical Investigation Plan	
Clinical Investigation Plan/Study Title	A Double-blind, Multi-Center, Prospective, Randomized Placebo-controlled Pilot Study Evaluating Intra-articular Injection of Axolotl Ambient™ Compared to Saline in Subjects with Ankle Osteoarthritis
Short Study Title	Phoenix Study
Study Product Name	Axolotl Ambient™
Sponsor	Axolotl Biologix, Inc. 2201 N Gemini Dr, Ste 140 Flagstaff, AZ 86001
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Phoenix Study

A Double-blind, Multi-Center, Prospective, Randomized Placebo-controlled Pilot Study Evaluating Intra-articular Injection of Axolotl Ambient™ Compared to Saline in Subjects with Ankle Osteoarthritis

Protocol Signature Page

I have read this protocol and agree it contains all necessary details for carrying out the described study. I will conduct this study as outlined herein and in compliance with all applicable local regulatory requirements. I will make a reasonable effort to complete this study within the designated time frame. I will provide copies of this protocol and all information provided by Axolotl Biologix to all sub-investigators and other study personnel under my supervision. I will discuss this material with them to ensure that they are fully informed about the investigational product and conduct of the study.

Investigational products for this study will be sent to:

Name of Institution: _____
Attention/Title: _____
Address: _____
Telephone: _____
Fax: _____
Email: _____

I, and any sub-investigator(s) participating in this study under my supervision as Principal Investigator (PI), will conduct this study in compliance with this protocol, all applicable regulatory requirements, and the conditions of approval imposed by the IRB/EC. Follow-up procedures will be performed at the institution listed below:

Name of Institution: _____
Address: _____
_____

_____	_____	____/____/_____
Principal Investigator (Name)	Principal Investigator (Signature)	Day/Month/Year

Abbreviations

AOS - Ankle Osteoarthritis Scale
ADR – Adverse Drug Reaction
AE – Adverse Event
BLA – Biologics Licensing Application
BMAC – Bone Marrow Aspirate Concentrate
BMI – Body Mass Index
CCS – Composite Clinical Success
CEC – Clinical Events Committee
CFR – Code of Federal Regulations
CRF – Case Report Form
eCRF – Electronic Case Report Form
EDC – Electronic Data Capture
EQ-5D – EuroQol-5 Dimension
EU – European Union
FAOS - Foot and Ankle Outcomes Score
FDA – Food and Drug Administration
GCP – Good Clinical Practice
HCT/P - Human Cells, Tissues, and Cellular and Tissue-Based Products
ICF – Informed Consent Form
IND – Investigational New Drug
IP – Investigational Product
IPMS – Investigational Product Management System
IRB/EC – Institutional Review Board/Ethics Committee
ITT - Intention-To-Treat
LOCF - Last-Observation-Carried-Forward
MCID – Minimum Clinically Important Differences
MRI – Magnetic Resonance Imaging
OA - Osteoarthritis
PRP – Platelet-Rich Plasma
SAE – Serious Adverse Event
SAR – Suspected Adverse Reaction
SSAR – Serious Suspected Adverse Reaction
SUSAR – Suspected, Unexpected, Serious Adverse Reaction
SDV – Source Data Verification
UAE – Unexpected Adverse Event
USAR – Unexpected Suspected Adverse Reaction
VAS – Visual Analog Scale

Table of Contents

1 Protocol Summary 7
2 Introduction 15
2.1 Background and Study Purpose 15
2.2 Investigational Product Description 15
3 Study Design and Population 16
3.1 Study Design Overview 16
3.2 Study Design Rationale 16
3.3 Comparator 16
3.4 Determination of Study Eligibility 17
3.5 Inclusion Criteria 17
3.6 Exclusion Criteria 18
3.7 Subject Screening Exceptions 19
3.8 Number of Subjects and Investigational Sites 19
4 Investigational Procedure 19
4.1 Investigator Training 19
4.2 Screening and Pre-procedure 19
4.2.1 Informed Consent 19
4.2.2 Medical History & Demographic Data 20
4.3 General Procedures 21
4.3.1 Randomization, Treatment Allocation, and Concealment 21
4.3.2 Staggered Enrolment 21
4.3.3 Blinding 21
4.3.4 Intra-articular Injection 22
4.4 Post-procedure Follow-up Assessments 22
4.4.1 4 Weeks Post-Treatment (± 7 days) 22
4.4.2 3 Months Post-Treatment (± 14 days) 22
4.4.3 6 Months Post-Treatment (± 30 days) 23
4.4.4 12 Months Post-Treatment (± 60 days) 23
4.4.5 Unscheduled Visit 23
4.5 Cross-Over 23
5 Study Endpoints 24
5.1 Study Hypothesis 24
5.2 Primary Outcomes 24
5.3 Secondary Outcomes 25
6 Early Withdrawal of Subjects 26
7 Investigational Product Receiving, Storage, Dispensing, and Return 26
7.1 Storage of Investigational Products 26
7.2 Dispensing of the Investigational Product 27
7.3 Return of Investigational Product 27
8 Benefits and Risks 27
8.1 Benefits 27
8.2 Risks 27
9 Clinical Adverse Event Reporting 28
9.1 Adverse Events 28
9.2 Adverse Event Classification 29
9.3 Reporting of Adverse Events 30
9.4 Subject Death 31
9.5 Stopping Rules 31
9.5.1 Study Stopping Rules 31
9.5.2 Subject Stopping Rules 31

10 Statistical Considerations and Analysis Plan 31
10.1 Sample Size 32
10.2 Primary Analyses to Assess Treatment Effect and Variance 32
10.3 Secondary Analysis of Non-primary Outcomes and Intergroup Comparisons 33
10.4 Sub-group analyses 33
10.5 Missing data and sensitivity analyses 33
10.6 Adverse Events 33
10.7 Interim Analysis 34
11 Clinical Events Committee (CEC) 34
12 Quality Control and Data Monitoring 34
12.1 Organizational Preparations 34
12.2 Training 34
12.3 Monitoring 35
12.4 Source Document Requests 35
12.5 Source Data Verification 35
12.6 Data Management 36
12.7 Protocol Amendments/Modifications & Protocol Deviations 36
13 Ethics and Regulatory Requirements 37
13.1 Code of Conduct 37
13.2 Institutional Review Board/Ethics Committee (IRB/EC) 37
13.3 Subject Informed Consent 38
13.4 Subject Confidentiality 38
14 Data Reporting 38
14.1 Case Report Forms 38
14.2 Final Report 39
14.3 Retention of Records by the Investigator 39
14.4 Investigator Final Report 39
15 Termination of an Institution by the Sponsor 39
16 Use of Information and Publication 39
17 References 39
18 Summary of Changes 42
Appendix A: Schedule of Events 43

1 Protocol Summary

Title	A Double-blind, Multi-Center, Prospective, Randomized Placebo-controlled Pilot Study Evaluating Intra-articular Injection of Axolotl Ambient™ Compared to Saline in Subjects with Ankle Osteoarthritis
Short Study Title	Phoenix Study
Protocol Number	CA-2021-01
Clinical Phase	Pilot study to estimate treatment effect size
Study Design	Multi-center, prospective, placebo-controlled, randomized, double-blind (patient and investigator/assessor)
Investigational Product	Axolotl Ambient
Sponsor	Axolotl Biologix, Inc. 2201 N Gemini Dr, Ste 140 Flagstaff, AZ 86001
Anticipated Number of Study Sites	Up to 3, based on the current estimates of sample size
Indication under investigation	Intra-articular injection for ankle osteoarthritis (OA) to alleviate the symptoms of pain and associated dysfunction or disability.
Objective	Establish estimates for the treatment effect size and variability of the investigational product compared to control in the primary endpoint as well as the proportion of patients achieving treatment success in the investigational vs. control groups.
Study Hypothesis	Treatment of symptomatic ankle OA with one intra-articular injection of Axolotl Ambient is superior to a placebo (saline) injection in the alleviation of pain and restoration of function at 6 months post-injection. The current study is designed to evaluate the treatment effect size, which will inform the design of a pivotal study to test this hypothesis. The current study is not designed or powered to test this hypothesis.
Investigational Group	Eligible patients with symptomatic ankle OA will receive one intra-articular injection of Axolotl Ambient
Control Group	Eligible patients with symptomatic ankle OA will receive one intra-articular injection of sterile, normal saline
Study Duration	Up to 12 months of recruitment, plus an additional 12 months of follow-up for a total of up to 24 months

Primary Endpoints	This study is designed to estimate the treatment effect size and variability in the following outcomes at 6 months follow-up:
	• Ankle Osteoarthritis Scale (AOS; 48 hr recall) Pain and Function subscales
	• Foot and Ankle Outcomes Score (FAOS; 48 hr recall) subscales:
	  o Pain
	  o Activities of Daily Living (ADL) (i.e., Function)
	• Proportion of patients achieving composite clinical success (CCS), with CCS defined as a patient meeting all of the three following criteria:
	  1. Improvement in AOS (Pain and Function) or FAOS scores (Pain and ADL) of at least 20% from baseline
	  2. No surgical intervention or prescription pain medications for ankle OA
	  3. No treatment or procedure-related serious adverse events

Secondary Outcomes	Secondary outcome measures will include:
	• Incidence of adverse events through 6 months and 12 months
	• Remaining FAOS subscales:
	  o Quality of Life (QoL)
	  o Symptoms
	  o Sports and Recreation
	• Visual analog scale (VAS) for ankle pain (average during last 48 hours)
	• VAS ankle pain (worst during last 48 hours)
	• EuroQOL-5 dimension-5 level (EQ-5D-5L) quality of life scale
	• Working status
	• K-L Grade from weightbearing X-ray at baseline and 6 months
	• Pain catastrophizing scale

Treatment Allocation	Subjects will be randomized in a 1:1 ratio (Investigational:Control) at the time of enrollment
Blinding	Patients, investigators, and outcome assessors will be blinded to the treatment allocation until completion of the 6-month follow-up visit
Cross-Over	Subjects randomized to the saline control group will be offered an injection of Ambient after unblinding at 6 months or up to the 12-month timepoint. Cross-over subjects will be followed for an additional 12 months at the same visit schedule for safety evaluation.
Sample Size	The target sample size will be 40 patients (20 subjects per group) in order to estimate the standard deviation of the primary outcome variables (AOS and FAOS scores) and proportions of CCS responders with sufficient precision. Therefore, target enrollment will be 48 subjects to allow for 15% loss to follow-up:

	Enrollment in Control group: 24 subjects Enrollment in Investigational group: 24 subjects
Staggered Enrollment	Enrollment in this study will be staggered to allow for safety evaluation of the first six (6) subjects. After the first six subjects are enrolled, further enrollment will be paused until after the sixth subject completes the 4 week follow-up visit. At that point, the independent Clinical Events Committee (CEC) will evaluate the safety data for the first six subjects through 4 weeks and make a recommendation to the Sponsor for continuing enrollment. Enrollment will then continue per the same randomization scheme for the remaining 42 subjects.
Inclusion/Exclusion Criteria	**Inclusion** 1. Signed written informed consent 2. Willing to be randomized to either the investigational or control group 3. Male or female at least 18 years of age and less than 80 4. Clinical diagnosis of symptomatic tibiotalar (ankle) OA (primary idiopathic OA or post-traumatic OA), modified K-L grade 1-3a 5. One ankle is the most symptomatic lower extremity joint and the only lower extremity joint currently planned for treatment 6. Willing and able to comply with all study requirements including all postoperative clinical and radiographic evaluations 7. For women of childbearing potential (not post-menopausal for 12 months or surgically sterile), a urine pregnancy test with a negative result must be obtained at screening and on the day of procedure, prior to injection. Male and female trial participants must commit to adequate birth control (e.g., surgically sterilized, oral contraceptive, two methods of barrier birth control, or abstinence) through the final follow-up 8. Chronic ankle pain for ≥ 6 months 9. AOS Pain score ≥ 30 and ≤ 80 and AOS Function score ≥ 30 and ≤ 80 10. Willing to discontinue all prescription medications and over-the-counter pain medications being used for ankle OA symptoms for the duration of the study, except for acetaminophen, which is not allowed for the 3 days preceding each scheduled follow-up visit and the day of the follow-up visit. **Exclusion**

	1. Previously received intra-articular injections in the index joint of Axolotl Ambient, bone marrow aspirate concentrate (BMAC), platelet-rich plasma (PRP), or other biologic-based products within the last 12 months. 2. Modified K-L Grade 3b or 4 osteoarthritis of the index joint 3. Diagnosis of ipsilateral peri-articular arthritis (e.g. subtalar joint) that the investigator determines may significantly confound the study results 4. Received intra-articular hyaluronic acid injection in the index joint within the last 6 months 5. Received intra-articular steroid injection in the index joint within the last 3 months 6. Prior major surgical repair or reconstruction of the ankle that the investigator determines may significantly confound the study results 7. Baseline pain catastrophizing score ≥ 23 8. Any surgery of the index ankle within the last 12 months 9. Planned arthroscopy or other procedure on the ankle during the study 10. Bilateral ankle OA requiring treatment of both ankles 11. Pregnancy, breastfeeding, or woman of child-bearing potential not using adequate contraception 12. Bone deficit 13. Charcot foot disease 14. Radiographic evidence of open epiphyses 15. Systemic inflammatory condition 16. Substantial vascular insufficiency 17. Current treatment with anticoagulants 18. Deformity in coronal, axial or sagittal plane greater the 30° 19. Patients weighing less than 47 kg. 20. BMI greater than 45 kg/m^2 21. Documented medical history of, or radiographic evidence of, a bone disease (e.g. severe osteoporosis or avascular necrosis) or other condition (e.g., osteolysis) 22. Comorbidity that would limit the ability to administer any functional measurements 23. Has at the time of injection, a systemic or local infection at the site of injection 24. Medical condition, serious intercurrent illness, or extenuating circumstance that, in the opinion of the Investigator, would preclude participation in the study or potentially decrease survival or interfere with ambulation or rehabilitation (e.g.,

	history of transient ischemic attack, stroke or liver disease) 25. Known hypersensitivity to penicillin 26. Known allergy to phenol red 27. Currently receiving treatment with a drug known to interfere with bone or cartilage metabolism [e.g., systemic steroid therapy (topical steroid therapy is permissible), methotrexate] 28. Medical condition requiring radiation, chemotherapy or immunosuppression 29. Have an active history of malignancy (except for basal cell carcinoma of the skin) 30. Has a history of autoimmune disease known to affect bone or cartilage. Examples include spondyloarthropathies (e.g., ankylosing spondylitis, Crohn's disease, and ulcerative colitis), Juvenile Arthritis, rheumatoid arthritis, Grave's disease, and Hashimoto's thyroiditis 31. Has obvious or documented alcohol or illicit drug addictions 32. Is a prisoner in a correctional institution/facility 33. Actively involved in litigation or workman's compensation 34. Has participated in clinical studies evaluating investigational devices, pharmaceuticals or biologics within 6 months of randomization 35. Bedridden, confined to a wheelchair, or unable to walk 50 m without the help of a walker, crutches, or cane 36. Is currently taking prescription pain medication for any conditions other than ankle osteoarthritis.
Study Procedures and Assessments	Data will be collected at baseline and at 4 weeks, 3 and 6 months post-injection, with 6 months being the primary endpoint for analysis and the timepoint for unblinding of subjects and investigators. Following the 6-month endpoint, investigators may treat subjects, as necessary, within their standard clinical practice (e.g. hyaluronic acid injection; not to include the investigational product) for any subject requiring additional treatment related to persistent or recurring symptoms. All subjects will be observed for an additional 6 months, monitoring for adverse events and collecting outcomes at a planned 12-month follow-up. The Sponsor may cover the cost of one Ambient injection for subjects that were randomized to the Control group and require additional treatment for persistent or recurring symptoms during the 6-12 months observational follow up period. Any subject originally randomized to Control, but later received Ambient, will have an additional 12 months of follow-up and data collection. **<u>One (1) injection on Day 0 of either:</u>** • Axolotl Ambient (2 mL)

- Saline control (2 mL)

Injection technique: intra-articular injection into the tibiotalar joint via the anteromedial portal (0.5-1 cm below the Tibial plafond and between the Tibialis Anterior tendon and the medial malleolus) of the ankle.

Time course:

- Screening, including X-ray (AP, Lateral, 20° Mortise)
- Baseline data collection, randomization and injection
- Follow-up visit 1 (4 weeks ± 7 days)
- Follow-up visit 2 (3 months ± 14 days)
- Follow-up visit 3 (6 months ± 30 days)
- Follow-up visit 4 (12 months ± 60 days)

Baseline data collection:

- Patient characteristics
    - Age
    - Gender
    - Ethnicity
    - Education
    - Body mass index (BMI)
    - Laterality (Left/Right)
    - Modified Kellgren-Lawrence Grade
    - Talar tilt
    - Symptom duration
    - OA etiology (post-traumatic or idiopathic/non-traumatic)
    - Presence of ipsilateral peri-articular arthritis
    - Previous surgeries on the ankle
    - Previous intra-articular injections
    - Current non-operative treatments
        i. Prescription or over-the-counter pain medications being discontinued (e.g. opioids)
        ii. Over-the-counter medications being used
        iii. Other interventions (e.g. Rocker Bottom shoe wear, orthotics, braces, physical therapy, ambulation assistance devices)
- Patient-reported outcomes
    - AOS score (48-hour recall)
    - FAOS scores (48-hour recall)
    - VAS pain (average and worst in last 48 hours)
    - EQ-5D-5L
    - Pain catastrophizing score
- Working status

	<u>Follow-up visit data collection (4 weeks, 3, 6, and 12 months):</u>
	• X-ray (6 months only)
	  ○ AP
	  ○ Lateral
	  ○ 20° Mortise
	• Patient-reported outcomes
	  ○ AOS score (48-hour recall)
	  ○ FAOS scores (48-hour recall)
	  ○ VAS pain (average and worst in last 48 hours)
	  ○ EQ-5D-5L
	• Working status
	• Adverse events
	• Co-interventions that would constitute treatment failure (surgical, injections, or prescription medications/therapies)
	• Use of allowed rescue medications (acetaminophen)
	• New or modification of other non-operative treatments (e.g. Rocker Bottom shoe wear, orthotics, braces, physical therapy, ambulation assistance devices)

Safety Assessments	The incidence, severity, and product- or injection-relatedness of all AEs will be monitored throughout the course of the study. An independent clinical events committee (CEC) will adjudicate relatedness and severity.
Statistical Analysis Plan	All analyses of this pilot trial are focused on quantification of the effect size and variance associated with each outcome to inform the design of a subsequent pivotal trial. These analyses are not powered or intended to yield definitive assessments for decision-making related to regulatory approval or proceeding to larger trials of similar or different design. Descriptive statistics such as mean, median, standard deviation, and confidence intervals from 80%-95% will be calculated for each outcome. Normality of the distributions will be assessed using the D'Agostino-Pearson test. Changes from baseline to each follow-up timepoint will be calculated for each outcome metric and statistical significance of changes will be assessed through repeated measures ANOVA. If missing data exists at some time points, a mixed effects model may be fit using the Restricted Maximum Likelihood method. Intergroup comparisons in continuous outcome scores (AOS and FAOS) will be conducted using repeated measures ANOVA with corrections for multiple comparisons (e.g., Sidak's test). Intergroup comparisons of proportions (e.g., CCS) will be conducted using Fisher's exact test.

	Efficacy comparisons between the investigational and control groups will be completed using a modified intention-to-treat (mITT) population, which includes all subjects who received an injection. The as-treated population will be used for sensitivity analyses on efficacy outcomes and for safety analyses.

2 Introduction

2.1 Background and Study Purpose

Symptomatic osteoarthritis (OA) is defined as the radiographic presence of joint space narrowing, osteophytes, sclerosis, and/or deformity, combined with clinical symptoms such as pain, stiffness, and decreased function. OA in the ankle most commonly develops secondary to trauma (post-traumatic OA), which accounts for about 80% of the cases, followed by OA secondary to disease (e.g. rheumatoid arthritis; 12%), and then primary idiopathic OA (8%) (Valderrabano et al. 2009). The prevalence of symptomatic ankle OA in the general population has not been established. An estimate of community-dwelling adults suggests a prevalence of approximately 3.4% in that population (Murray et al. 2018). Unlike primary OA, post-traumatic OA can develop in young people (< 30 years) (Valderrabano et al. 2009), posing significant limitations for the rest of their lives. End-stage ankle OA has been characterized as highly debilitating and at least as severe as hip OA (Glazebrook et al. 2008).

For end-stage ankle OA, arthrodesis has traditionally been the gold standard, but newer designs and more recent evidence for total ankle arthroplasty have suggested that the two approaches may be comparable overall, with various tradeoffs that should be considered on a patient-specific basis (Kim et al. 2017, Lawton et al. 2017). However, patients with mild to moderate forms of symptomatic ankle OA are not yet candidates for surgical intervention. In these patient populations, non-operative therapies are essential to help alleviate symptoms and, if possible, slow or halt further degeneration of the joint to delay or avoid the need for surgical intervention. However, there are currently no evidence-based guidelines for non-operative therapies of ankle OA in the U.S. (Witteveen et al. 2015). Currently, non-operative care is typically comprised of shoe wear modifications (e.g. rocker bottom, cushioned soles), orthotics, braces, physiotherapy, weight reduction, activity modification, ambulatory assistance devices, or oral analgesics. Other non-operative treatments include the use of intra-articular injections, such as corticosteroids, hyaluronic acid, or platelet-rich plasma (PRP) (Witteveen et al. 2015, Vannabouathong et al. 2018). Recent systematic reviews found the current evidence to be inconclusive for the efficacy of hyaluronic acid and PRP in the ankle and no studies were available for corticosteroid injections (Witteveen et al. 2015, Vannabouathong et al. 2018). Therefore, continued research and development is essential to establish evidence-based non-operative therapies for symptomatic ankle OA.

The goal of this pilot study and subsequent pivotal trial is to evaluate the safety and effectiveness of Axolotl Ambient intra-articular injection for ameliorating the pain and dysfunction associated with ankle OA. The pilot study will be conducted in a limited population to allow an estimate of the effect size and variability of treatment with Axolotl Ambient compared to saline for key clinical outcomes. The results of this pilot study will then be used to establish appropriate scientific methods and sample sizes for a subsequent pivotal trial, which is expected to support a BLA application to the FDA.

2.2 Investigational Product Description

Axolotl Ambient is an allogeneic amniotic intra-articular injection therapy consisting of a growth factor and cytokine-rich fluid derived from human amnion cells. Axolotl Ambient is manufactured using a proprietary BioSym™ process through *in vitro* culture of human amniotic cells extracted from human amniotic tissues. During culture, the media is conditioned with the secreted factors from the amnion cells, which is comprised of multiple growth factors and cytokines that are key mediators in tissue

healing, regeneration, and anti-inflammatory pathways. The conditioned media is collected, frozen and terminally irradiated to ensure no viable cells are included in the final product. The fluid can be stored at ambient temperature (15-30°C, 59-86°F) with a shelf life of 18 months from the date of manufacture.

3 Study Design and Population

3.1 Study Design Overview

This pilot study will be conducted in a limited number of patients to gain preliminary data that can be used to inform the primary hypothesis and study design of a subsequent pivotal trial. The pilot study design will be a double-blind (investigator/assessor and patient), multi-center, prospective, randomized, placebo-controlled trial. Upon randomization, patients will receive 1 intra-articular injection of the investigational product or control fluid. Follow-up will be conducted at 4 weeks, 3 and 6 months, with 6 months serving as the primary endpoint. The primary outcomes will consist of patient-reported outcomes of pain and function, specific to the foot and ankle. Subjects will be monitored for an additional 6 months after completion of the primary endpoint and unblinding to establish safety over a 1-year follow-up.

3.2 Study Design Rationale

Currently, there are no evidence-based guidelines for non-operative therapies of ankle OA in the U.S. (Witteveen et al. 2015). Non-operative care is typically comprised of shoe wear modifications (e.g. rocker bottom, cushioned soles), orthotics, braces, physiotherapy, weight reduction, activity modification, ambulatory assistance devices, or oral analgesics. Recent systematic reviews have found the current evidence to be inconclusive for the efficacy of intra-articular injections, including hyaluronic acid and PRP in the ankle, and no studies are available for corticosteroid injections (Witteveen et al. 2015, Vannabouathong et al. 2018).

Axolotl Ambient is comprised of the secretome of amnion-derived cells, which includes growth factors and cytokines that are key mediators in tissue healing, regeneration, and anti-inflammatory pathways (Insausti et al. 2014, Parolini et al. 2014, Dabrowski et al. 2017). These factors are hypothesized to contribute to the alleviation of pain and inflammation in the osteoarthritic ankle. Initial safety of Axolotl Ambient has been confirmed through laboratory assays, which ensure the absence of viral and other microbial contaminants and a pre-clinical study in rats to evaluate the potential local or systemic toxicology of the product. Through marketing under Section 361 HCT/P regulations, there have been no reported adverse reactions attributed to transmission of communicable disease. Based on the safety of Axolotl Ambient and the potential benefits to patients with ankle osteoarthritis, this study is justified and important in advancing the healthcare of this patient population.

3.3 Comparator

The comparator will be an intra-articular injection of 0.9% normal sterile saline. The same volume (2 mL) and technique of injection will be used in both the Control and Investigational groups.

3.4 Determination of Study Eligibility

Skeletally mature patients with a clinical diagnosis of symptomatic ankle osteoarthritis will be screened for enrollment. All subjects will be informed on the nature of the study, and once all questions have been answered, will voluntarily sign the Institutional Review Board (IRB/EC)-approved informed consent form and will also need to fulfill the necessary eligibility criteria as described in this document to be enrolled in the study.

Where a patient fails to fulfil any element of the inclusion and exclusion criteria, this will be documented and the signed consent form and completed inclusion/exclusion criteria retained by the Principal Investigator. The patient will not be advanced any further into this clinical investigation.

Subjects meeting all inclusion criteria and no exclusion criteria will be randomized to intra-articular injection in the index ankle with either the investigational product (Axolotl Ambient) or control (saline).

3.5 Inclusion Criteria

Subjects must meet all of the following criteria to be eligible for treatment in the study:

1. Signed written informed consent
2. Willing to be randomized to either the investigational or control group
3. Male or female at least 18 years of age and less than 80
4. Clinical diagnosis of symptomatic tibiotalar (ankle) OA (primary idiopathic OA or post-traumatic OA), modified K-L grade 1-3a
5. One ankle is the most symptomatic lower extremity joint and the only lower extremity joint currently planned for treatment
6. Willing and able to comply with all study requirements including all postoperative clinical and radiographic evaluations
7. For women of childbearing potential (not post-menopausal for 12 months or surgically sterile), a urine pregnancy test with a negative result must be obtained at screening and on the day of procedure, prior to injection. Male and female trial participants must commit to adequate birth control (e.g., surgically sterilized, oral contraceptive, two methods of barrier birth control, or abstinence) through the final follow-up
8. Chronic ankle pain for ≥ 6 months
9. AOS Pain score ≥ 30 and ≤ 80 and AOS Function score ≥ 30 and ≤ 80
10. Willing to discontinue all prescription and over-the-counter pain medications being used for ankle OA symptoms for the duration of the study, except for acetaminophen, which is not allowed for the 3 days preceding each scheduled follow-up visit and the day of the follow-up visit.

3.6 Exclusion Criteria

Subjects will be excluded from the study if any of the following criteria are met:

1. Previously received intra-articular injections in the index joint of Axolotl Ambient, bone marrow aspirate concentrate (BMAC), platelet-rich plasma (PRP), or other biologic-based products within the last 12 months.
2. Modified K-L Grade 3b or 4 osteoarthritis of the index joint
3. Diagnosis of ipsilateral peri-articular arthritis (e.g. subtalar joint) that the investigator determines may significantly confound the study results
4. Received intra-articular hyaluronic acid injection in the index joint within the last 6 months
5. Received intra-articular steroid injection in the index joint within the last 3 months
6. Prior major surgical repair or reconstruction of the ankle that the investigator determines may significantly confound the study results
7. Baseline pain catastrophizing score ≥ 23
8. Any surgery of the index ankle within the last 12 months
9. Planned arthroscopy or other procedure on the ankle during the study
10. Bilateral ankle OA requiring treatment of both ankles
11. Pregnancy, breastfeeding, or woman of child-bearing potential not using adequate contraception
12. Bone deficit
13. Charcot foot disease
14. Radiographic evidence of open epiphyses
15. Systemic inflammatory condition
16. Substantial vascular insufficiency
17. Current treatment with anticoagulants
18. Deformity in coronal, axial or sagittal plane greater the 30°
19. Patients weighing less than 47 kg.
20. BMI greater than 45 kg/m^2
21. Documented medical history of, or radiographic evidence of, a bone disease (e.g. severe osteoporosis or avascular necrosis) or other condition (e.g., osteolysis)
22. Comorbidity that would limit the ability to administer any functional measurements
23. Has at the time of injection, a systemic or local infection at the site of injection
24. Medical condition, serious intercurrent illness, or extenuating circumstance that, in the opinion of the Investigator, would preclude participation in the study or potentially decrease survival or interfere with ambulation or rehabilitation (e.g., history of transient ischemic attack, stroke or liver disease)
25. Known hypersensitivity to penicillin
26. Known allergy to phenol red
27. Currently receiving treatment with a drug known to interfere with bone or cartilage metabolism [e.g., systemic steroid therapy (topical steroid therapy is permissible), methotrexate]

28. Medical condition requiring radiation, chemotherapy or immunosuppression
29. Have an active history of malignancy (except for basal cell carcinoma of the skin)
30. Has a history of autoimmune disease known to affect bone or cartilage. Examples include spondyloarthropathies (e.g., ankylosing spondylitis, Crohn's disease, and ulcerative colitis), Juvenile Arthritis, rheumatoid arthritis, Grave's disease, and Hashimoto's thyroiditis
31. Has obvious or documented alcohol or illicit drug addictions
32. Is a prisoner in a correctional institution/facility
33. Actively involved in litigation or workman's compensation
34. Has participated in clinical studies evaluating investigational devices, pharmaceuticals or biologics within 6 months of randomization
35. Bedridden, confined to a wheelchair, or unable to walk 50 m without the help of a walker, crutches, or cane
36. Is currently taking prescription pain medication for any conditions other than ankle osteoarthritis.

3.7 Subject Screening Exceptions

There will be no screening exceptions for inclusion or exclusion criteria granted for this study.

3.8 Number of Subjects and Investigational Sites

The study population will include 48 subjects (24 in the investigational group and 24 in the control group). Up to 3 investigational sites will participate in the study.

4 Investigational Procedure

4.1 Investigator Training

No investigational study site will participate in this study without investigators who have adequate training and familiarity with the interventional techniques required for intra-articular injection into the ankle, as well as the requirements established in this Protocol, and applicable GCP regulations established in the U.S. Code of Federal Regulations (21 CFR Part 11, Part 50, Part 54, Part 56, Part 812) and ISO 14155 (current revision).

4.2 Screening and Pre-procedure

4.2.1 Informed Consent

Signed IRB/EC-approved informed consent must be obtained (along with other locally required documents, if applicable) from each subject prior to the start of any procedures specific to the protocol and documented according to the principles of informed consent in the current version of the Declaration of Helsinki.

Prior to obtaining informed consent, information must be given in a language and at a level of

complexity understandable to the subject in both oral and written form by the investigator or designee. Subjects must not be coerced, persuaded or unduly influenced to participate, or continue to participate, in the study. Subject must be given ample time and opportunity to inquire about details of the study. All questions about the study must be answered to the satisfaction of the subject. Prior to the performance of any study-specific related activity or procedure, the written informed consent form must be signed and dated by the subject, and by the person who conducted the informed consent discussion (investigator or designee). If the subject is unable to read the consent form, a witness must be present during the entire informed consent discussion. After the informed consent is read to the subject and signed by the subject the witness must also sign the consent form, attesting that the informed consent was freely given by the subject.

The subject must receive a copy of the signed and dated informed consent form. The consent form must be updated or amended whenever new information becomes available that may be relevant to the subject.

Once a subject has signed the approved informed consent form, the subject is assigned a study identification number. The study identification number consists of the site number and subject number at the site (in sequential order). If a subject signs the informed consent form but is not eligible for the study due to not meeting the inclusion and exclusion criteria, the site will document the reason on the screening log. Once a subject number is assigned, it cannot be re-used.

4.2.2 Medical History & Demographic Data

Subjects will be evaluated for potential inclusion in the study. The investigator will verify the subject's eligibility according to all general inclusion and exclusion criteria.

Informed consent must be obtained for all subjects who are potential study candidates prior to the procedure. The subject must sign the study informed consent form prior to any study-specific procedures being performed. It is possible to combine Screening and Baseline activities into one visit if all required activities at these visits can be accomplished. The subject should stop the use of any pain medications (including acetaminophen) at least three days prior to completing outcomes questionnaires.

The following baseline data must be collected for all subjects within 90 days prior to the study procedure, or at the onset of the study procedure:

1. Sign and date current IRB/EC approved study informed consent form
2. Inclusion & Exclusion
3. Patient characteristics
    - Age
    - Gender
    - Ethnicity
    - Education
    - Body mass index (BMI)
    - Laterality of index ankle (Left/Right)
    - X-Rays: AP, Later, 20° Mortise

- Modified Kellgren-Lawrence Grade
- Talar Tilt

4. Medical history
   - Symptom duration
   - OA etiology (post-traumatic or idiopathic/non-traumatic)
   - Presence of ipsilateral peri-articular arthritis
   - Previous surgeries on the ankle
   - Previous intra-articular injections
5. Concomitant medications and other therapies (e.g. orthotics, braces)
   - Current non-operative treatments
   - Prescription or over-the-counter pain medications being discontinued (e.g. opioids, NSAIDs)
   - Over-the-counter medications being used
   - Other therapies (e.g. Rocker Bottom shoe wear, orthotics, braces, physical therapy, ambulation assistance devices)
6. Physical exam
7. Working status
8. Weightbearing X-rays
9. AOS (48-hour recall)
10. FAOS (48-hour recall)
11. EQ-5D-5L
12. VAS for average ankle pain during last 48 hours
13. VAS for worst ankle main during last 48 hours

4.3 General Procedures

4.3.1 Randomization, Treatment Allocation, and Concealment

Permuted block randomization will be performed within the EDC and stratified by OA etiology (non-traumatic vs. post-traumatic). Randomization will be performed through the EDC at the study site at the time of injection. Treatment allocation will remain blinded within the EDC to the subject, investigator and any outcomes assessor through the 6-month primary endpoint.

4.3.2 Staggered Enrolment

Enrollment in this study will be staggered to allow for safety evaluation of the first six (6) subjects. After the first six subjects are enrolled, further enrollment will be paused until after the sixth subject completes the 4 weeks follow-up visit. At that point, the independent Clinical Events Committee (CEC) will evaluate the safety data for the first six subjects through 4 weeks and make a recommendation to the Sponsor for continuing enrollment. Enrollment will then continue per the same randomization scheme for the remaining 42 subjects.

4.3.3 Blinding

A study assistant will draw 2 mL of the allocated treatment fluid from the respective vial into an amber syringe out of sight of the patient, investigator, and outcome assessors. After ensuring no residual fluid is visible, the syringe will be passed to the investigator to perform the injection.

Unblinding prior to the 6-month endpoint will only be performed if necessary, for the investigator to evaluate and treat an adverse event. In the event of unblinding, the Sponsor and IRB will be notified with the reason for the unblinding.

4.3.4 Intra-articular Injection

The tibiotalar (ankle) joint will be injected with 2 mL of the investigational or control fluid, as allocated upon randomization. Injection will occur via the anteromedial portal (0.5-1 cm below the Tibial plafond and between the Tibialis Anterior tendon and the medial malleolus) of the ankle.

4.4 Post-procedure Follow-up Assessments

Follow-up assessments will be performed according to the Schedule of Events described in Appendix A: Schedule of Events. During each specified evaluation, the corresponding electronic Case Report Form (eCRF) must be completed per the instructions provided in the eCRF Completion Guidelines. The subject should stop the use of any pain medications (including acetaminophen) at least three days prior to completing outcomes questionnaires.

4.4.1 4 Weeks Post-Treatment (± 7 days)

1. Office visit
2. Patient-reported outcomes surveys (AOS, FAOS, EQ-5D-5L, VAS)
3. Working status
4. Record any co-interventions (surgery, injections, over-the-counter pain medications, or prescription medications)
5. Record use of rescue medications (acetaminophen)
6. Record new or modified use of other non-operative treatments (e.g. orthotics or braces)
7. Record all adverse events
8. Record all protocol deviations

4.4.2 3 Months Post-Treatment (± 14 days)

1. Office visit
2. Patient-reported outcomes surveys (AOS, FAOS, EQ-5D-5L, VAS)
3. Working status
4. Record any co-interventions (surgery, injections, over-the-counter pain medications, or prescription medications)
5. Record use of rescue medications (acetaminophen)
6. Record new or modified use of other non-operative treatments (e.g. orthotics or braces)
7. Record all adverse events
8. Record all protocol deviations

4.4.3 6 Months Post-Treatment (± 30 days)

1. Office visit
2. Weight-bearing X-rays (AP, Lateral, 20° Mortise)
3. Patient-reported outcomes surveys (AOS, FAOS, EQ-5D-5L, VAS)
4. Working status
5. Record any co-interventions (surgery, injections, over-the-counter pain medications, or prescription medications)
6. Record use of rescue medications (acetaminophen)
7. Record new or modified use of other non-operative treatments (e.g. orthotics or braces)
8. Record all adverse events
9. Record all protocol deviations

4.4.4 12 Months Post-Treatment (± 60 days)

1. Office visit
2. Patient-reported outcomes surveys (AOS, FAOS, EQ-5D-5L, VAS)
3. Working status
4. Record any co-interventions (surgery, injections, over-the-counter pain medications, or prescription medications)
5. Record use of rescue medications (acetaminophen)
6. Record new or modified use of other non-operative treatments (e.g. orthotics or braces)
7. Record all adverse events
8. Record all protocol deviations

4.4.5 Unscheduled Visit

1. Office visit
2. Patient-reported outcomes surveys (AOS, FAOS, EQ-5D-5L, VAS)
3. Working status
4. Record any co-interventions (surgery, injections, over-the-counter pain medications, or prescription medications)
5. Record use of rescue medications (acetaminophen)
6. Record new or modified use of other non-operative treatments (e.g. orthotics or braces)
7. Record all adverse events
8. Record all protocol deviations

4.5 Cross-Over

Subjects randomized to the saline control group will be offered an injection of Ambient after unblinding at 6 months or up to the 12-month timepoint. Cross-over subjects will be followed for an additional 12 months at the same visit schedule for safety evaluation.

5 Study Endpoints

5.1 Study Hypothesis

Treatment of symptomatic ankle OA with one intra-articular injection of Axolotl Ambient is superior to a placebo (saline) injection in the alleviation of pain and restoration of function at 6 months post-injection. The current study is designed to evaluate the treatment effect size, which will inform the design of a pivotal study to test this hypothesis. The current study is not designed or powered to test this hypothesis.

5.2 Primary Outcomes

The primary outcome metrics will be patient-reported pain and function:

- Ankle Osteoarthritis Scale (AOS; 48-hour recall): Pain and Function subscales
- The Pain and Activities of Daily Living (ADL) subscales of the Foot and Ankle Outcome Score (FAOS; 48-hour recall)

The Ankle Osteoarthritis Scale (AOS) is an 18-question scale of pain and disability that was developed and validated specifically for ankle osteoarthritis (Domsic and Saltzman 1998) and has commonly been used in prospective studies of intra-articular injections (Cohen et al. 2008, Witteveen et al. 2008, DeGroot et al. 2012). The pain and function sections of the AOS are separate and can be scored independently. The AAS scale is an abbreviated 8 question variant of the AOS scale, which eliminates duplicates and less relevant questions to yield a more efficient outcome measure for pain and disability (Wing et al. 2017). The AAS may be scored out of the AOS for post hoc, exploratory analyses. For this study, subjects will be asked to recall the last 48 hours for the questionnaire.

The FAOS score was developed to reflect a foot and ankle version of the Knee Osteoarthritis Outcomes Score (KOOS), which has been used and validated extensively in knee osteoarthritis research. The FAOS score includes pain and function (ADL) subdomains. It has been used in a prospective study of hyaluronic acid injection in the ankle (Murphy et al. 2017) and extensively evaluated for its utility in end-stage ankle osteoarthritis (Coster et al. 2012, Mani et al. 2015). Mani et al. and Coster et al. both reported that the Pain and ADL subscales are among the most responsive and most useful, while the Symptoms and Sports & Recreation subscales are less responsive (Coster et al. 2012, Mani et al. 2015). For this study, subjects will be asked to recall the last 48 hours for the questionnaire.

In addition to the two primary outcome measures above, a responder analysis will be conducted based on the following 2 sets of composite outcome data for defining treatment success (must achieve all 3 criteria):

- Improvement in AOS score in each of the two subscales (Pain and Function) of at least 20% from baseline; co-primary endpoints are represented by this assessment of improvement in both pain and function, independently measured through the separate subscales
- No surgical intervention or prescription pain medications for ankle OA
- No treatment-related serious adverse events

OR

- Improvement in each primary FAOS subscale (Pain and ADL) of at least 20% from baseline; co-primary endpoints are represented by this assessment of improvement in both pain and function, independently measured through the separate subscales
- No surgical intervention or prescription pain medications for ankle OA
- No treatment-related serious adverse events

Typically, a minimum clinically important difference (MCID) is used to define the threshold for improvement in pain or function outcomes. However, there are no established MCIDs for any patient-reported outcomes related to non-operative treatment of ankle arthritis. In such cases, Roos recommends that a 20% improvement is reasonable to reflect a moderate effect in a responder analysis of low-risk non-operative interventions (Roos 2018).

5.3 Secondary Outcomes

Secondary outcome measures will include:

- Incidence of adverse events through 6 months and 12 months
- The Symptoms, Quality of Life and Sports and Recreation subscales of the FAOS
- Visual analog scale (VAS) for average ankle pain during the last 48 hours
- Visual analog scale (VAS) for the worst ankle pain during the last 48 hours
- EQ-5D (5 level) as a standardized assessment of health-related quality of life (Barton et al. 2009) (Obradovic et al. 2013)
- Pain catastrophizing score to exclude high catastrophizing subjects from the study (Linton et al. 2011)
- Working status (not working, unrelated to ankle; not working because of ankle; working with major limitations related to ankle; working with minor limitations related to ankle; working without limitations related to ankle)
- Modified K-L Grade from weightbearing X-rays at baseline and 6 months
- Co-interventions (surgical, injections, over-the-counter pain medications, or prescription medications)
- New or modification of other non-operative treatments (e.g. shoe wear, orthotics, braces) or over-the-counter medications

The following patient characteristics data will be collected at baseline:

- Age
- Gender
- Ethnicity
- Education
- Body mass index (BMI)
- Laterality (Left/Right)
- Modified Kellgren-Lawrence Grade (Holzer et al. 2015)
- Talar tilt
- Symptom duration
- Osteoarthritis etiology (post-traumatic or idiopathic)
- Previous surgeries on the ankle
- Previous intra-articular injections
- Presence of ipsilateral periarticular arthritis

- Current non-operative treatments
    - Prescription or over-the-counter pain medications for ankle OA symptoms being discontinued for the study (e.g. opioids, NSAIDs)
    - Over-the-counter medications for ankle OA symptoms
    - Other (e.g. shoe wear, orthotics, braces, physiotherapy, ambulation assistance devices)
- All adverse events (AEs) or serious adverse events (SAEs)

6 Early Withdrawal of Subjects

A subject can cease study participation by withdrawing consent at any time or when no longer able to participate in the study. However, the reason for discontinuation must be recorded in the source documentation and on the appropriate eCRF.

Withdrawing consent will not result in any penalty whatsoever for the subject. Further treatment will be performed according to local practice.

The data of the subject who withdraws his or her informed consent will not be discarded and will be used in the final analysis of the study, unless the subject specifically requests that data not be used.

Possible reasons for premature discontinuation may include, but are not limited to, the following:

- Withdrawal of Consent: Subject decides to withdraw from study
- Adverse Events: subject must be followed until the adverse experience resolves or until a stable clinical endpoint is reached or until death occurs; or
- Lost to Follow-up: after 3 attempts to reach the subject by telephone have failed over the course of at least one year, a certified letter will be sent to the subject. The subject will be considered lost to follow-up if these communications are unsuccessful.

7 Investigational Product Receiving, Storage, Dispensing, and Return

The investigator is responsible for Axolotl Ambient product accountability at the study site.

The investigator may assign some of the investigator's duties for product accountability at the study site to an appropriate staff member. This staff member must be listed on the study personnel authorization form (Delegation of Authority Log).

The investigator should maintain a record that documents receipt of Axolotl Ambient at the study site, the inventory at the site, administration to each subject, and return of investigational product(s) to Axolotl Biologix. These records include dates, quantities, lot numbers, expiration dates, and the unique code numbers assigned to the investigational product and study subject(s).

7.1 Storage of Investigational Products

All Axolotl Ambient product must be stored in a secure, temperature-controlled area within the study site in accordance with the product Instructions for Use. A High/Low thermometer will be provided to the study site, and daily monitoring shall be recorded on a provided log. Only the investigator, or

designated staff member(s) shall have access to the investigational product.

7.2 Dispensing of the Investigational Product

The investigator is responsible for Axolotl Ambient product accountability at the study site. The investigator may assign some of the duties for product accountability at the site to an appropriate staff member. The investigator agrees to not supply the investigational product to any person(s) except those named as sub-investigators on the delegation of authority log. The investigator must ensure that the investigational product is only used in accordance with the IFU and only for this study protocol.

7.3 Return of Investigational Product

Unused investigational product returned as excess inventory following study completion or due to expiration, damage, or defect will be returned to Axolotl Biologix with a completed clinical product return form. The investigator must notify the Sponsor prior to shipment and return of the product(s) to the address printed on the clinical product return form.

In case of Axolotl Ambient malfunctions or complaints, product(s) should be kept on-site until Axolotl Biologix provides instructions for return or disposal of the product(s). The investigator must notify Axolotl Biologix of malfunctions or complaints within 24 hours of such knowledge.

8 Benefits and Risks

Axolotl Biologix has conducted a risk analysis for Axolotl Ambient and concluded that, from a technology, constituent, application and design perspective, intolerable risks were either not inherent to the design of the product or were successfully mitigated.

8.1 Benefits

Anticipated benefits include relief of osteoarthritis symptoms including pain and inflammation.

Moreover, study subjects are expected to have more frequent medical follow-up during the study visits than other patients with this medical condition, which can support beneficial outcomes.

8.2 Risks

Anticipated AEs associated with Axolotl Ambient:

- Allergic reaction to penicillin
- Allergic reaction to phenol red
- Infection
- Persistent or recurrent ankle pain, or discomfort
- Reduced tissue healing
- Adhesions
- Catching, locking, stiffness, or crepitation (crackling sound) of the ankle joint
- Tissue hypertrophy
- Heterotopic ossification

- Arthritis development or progression
- Unplanned medical intervention
- Unplanned surgical intervention

Anticipated AEs associated with intra-articular injection into the ankle:

- Arthralgia
- Arthritis development or progression
- Cartilage injury
- Catching, locking, stiffness, or crepitation (crackling sound) of the ankle joint
- Damage to joint tissues
- Delayed healing of injection site
- Distention of the joint space
- Effusion
- Foreign body in joint (needle tip)
- Hemarthrosis
- Hematoma (clotted blood) in the ankle joint
- Infection
- Inflammation or swelling of joint or peri-joint tissues
- Local irritation
- Pain at the injection site
- Persistent or recurrent ankle pain, or discomfort
- Procedural pain
- Reduced tissue healing
- Swelling, inflammation, or allergic reaction at injection site
- Unplanned medical intervention
- Unplanned surgical intervention

A complete list of current anticipated adverse events and information can be found in the current version of the Investigator's Brochure.

9 Clinical Adverse Event Reporting

9.1 Adverse Events

An AE is any untoward medical occurrence associated with the use of a drug in humans, whether or not considered drug related (21 CFR 312.32). AE information will be collected throughout the study beginning at the time of the index procedure and will be documented in the subject's medical record. All AEs occurring up to 12 months post-injection, whether or not associated with the investigational product or procedure, will be recorded on the appropriate eCRF.

Event, date of onset, seriousness, severity, duration, and relationship to the investigational product will be recorded on the appropriate eCRF. AEs will be followed until symptoms are resolved, or, in case of permanent impairment, until symptoms stabilize. AEs will be reported to the IRB/EC by the principal investigator as required by the institution or IRB/EC guidelines. The sponsor will be responsible for reporting AEs, as appropriate, per local regulations to the regulatory authorities.

9.2 Adverse Event Classification

An **Adverse Event (AE)** is any untoward medical occurrence, unintended disease or injury, or untoward clinical signs (including abnormal laboratory findings) in study subjects whether or not considered related to the investigational product. This definition includes events related to the investigational product or the comparator and events related to the procedures involved.

A pre-existing condition should not be reported as an AE unless the condition worsens or episodes increase in frequency during the AE reporting period.

A **Suspected Adverse Reaction (SAR)** is any adverse event for which there is a reasonable possibility that the drug caused the adverse event. For the purposes of safety reporting, "reasonable possibility" means there is evidence to suggest a causal relationship between the drug and the adverse event. Suspected adverse reaction implies a lesser degree of certainty about causality than adverse drug reaction, which means any adverse event caused by a drug.

An **Adverse Drug Reaction (ADR)** is an AE related to the use of a study product. All noxious and unintended responses to a medicinal product related to any dose should be considered adverse drug reactions.
A **Serious Adverse Event (SAE)** or **Serious Suspected Adverse Reaction (SSAR)** is an adverse event or suspected adverse reaction that is considered "serious" if, in the view of either the investigator or sponsor, it results in any of the following outcomes:

- Death
- Life-threatening adverse event
- Inpatient hospitalization or prolongation of existing hospitalization
- A persistent or significant incapacity or substantial disruption of the ability to conduct normal life functions
- A congenital anomaly/birth defect

Important medical events that may not result in death, be life-threatening, or require hospitalization may be considered serious when, based upon appropriate medical judgment, they may jeopardize the patient or subject and may require medical or surgical intervention to prevent one of the outcomes listed in this definition.

SAEs may or may not be related to the investigational product. Planned hospitalization for pre-existing condition(s), or a procedure required by the study protocol, without serious deterioration in health, is not considered a serious adverse event.

Note: The term “life-threatening” in the definition of “serious” refers to an event in which the subject was at risk of death at the time of the event; it does not refer to an event, which hypothetically might have caused death if it were more severe.

A **life-threatening adverse event** or **life-threatening suspected adverse reaction** is an adverse event or suspected adverse reaction that is considered "life-threatening" if, in the view of either the investigator or sponsor, its occurrence places the patient or subject at immediate risk of death. It does not include an adverse event or suspected adverse reaction that, had it occurred in a more severe form, might have caused death.

An **Unexpected Adverse Event (UAE)** or **Unexpected Suspected Adverse Reaction (USAR)** is an adverse event or suspected adverse reaction that is considered "unexpected" if it is not listed in the investigator brochure or is not listed at the specificity or severity that has been observed; or, if an investigator brochure is not required or available, is not consistent with the risk information described in the general investigational plan or elsewhere in the current application, as amended.

A **Serious and Unexpected Suspected Adverse Reaction (SUSAR)** is a suspected adverse reaction that is both serious and unexpected.

All AEs, SARs, ADRs, SAEs, SSARs, UAEs, USARs, and SUSARs must be recorded, as applicable, on the eCRFs provided.

Adverse Event Severity:
The following AE severity grades are to be used:

- Grade 1 - Mild; asymptomatic or mild symptoms; clinical or diagnostic observations only; intervention not indicated.
- Grade 2 - Moderate; minimal, local or noninvasive intervention indicated; limiting age appropriate instrumental ADL*
- Grade 3 - Severe or medically significant but not immediately life-threatening; hospitalization or prolongation of hospitalization indicated; disabling; limiting self care ADL**
- Grade 4 - Life-threatening consequences; urgent intervention indicated.
- Grade 5 - Death related to AE
  *Instrumental ADL refer to preparing meals, shopping for groceries or clothes, using the telephone, managing money, etc.
  **Self care ADL refer to bathing, dressing and undressing, feeding self, using the toilet, taking medications, and not bedridden.

Action Taken as a Result of the Event:
The treatment provided will be recorded as none, medication given, hospitalization, or other (which must then be specified).

Outcome Assessment:
The outcome of the event will be assessed as resolved, resolved with sequelae, recovering, unknown, ongoing, or death (including date of death and cause).

9.3 Reporting of Adverse Events

The AE reporting period for this study begins at Day 0 (defined as the time when the product enters the body) and ends at the 12-month follow-up assessment. All reported AEs occurring in subjects during this period must be reported to Axolotl Biologix, whether the event is considered product or procedure-related. Each subject will be questioned about AEs on the day of the injection procedure and at 4 weeks, 3 months, 6 months, and 12 months post-injection.

The procedure for reporting any AE (whether product-related or not) is as follows:

- If an AE occurs, complete all sections of the appropriate AE form(s);
- Each AE will be classified by the investigator as serious or non-serious. This classification of

the event determines the reporting procedures to be followed;

- Any SAR, ADR, SAE, SSAR, UAE, USAR, or SUSAR must be reported to Axolotl Biologix within 24 hours of knowledge by entering the data into the eCRF or via email (sday@telospartnersllc.com or atabor@axobio.com);
- Use separate AE form(s) to document each AE.
- All AEs should be followed until they are resolved, or a stable clinical end point is reached, or the subject's participation in the study ends;
- It is the responsibility of the investigator to inform their IRB/EC of AEs as required by local and country-specific regulations.

9.4 Subject Death

Subject death during the investigation must be reported to Axolotl Biologix within 24 hours of investigator's knowledge of the death. Notification of death must include a brief statement of pertinent details and be signed by the investigator or co-investigator. A copy of the de-identified death records, death certificates, and an autopsy report (if performed) must be provided to the Sponsor.

9.5 Stopping Rules

9.5.1 Study Stopping Rules

The following stopping rules will apply at a study level:

1) Any patient death related to an adverse event
2) Any adverse event with Grade 3 or higher severity regardless of relationship to the product.
3) Any Joint Infection in the injected joint confirmed requiring joint aspiration or surgical intervention.

Stopping rules will be triggered from either initial reporting of an Adverse Event as assessed by the investigator or following severity adjudication by an independent Clinical Events Committee (CEC), whichever comes first. Upon triggering of study stopping rules, enrollment and injection of subjects will stop until evaluated the CEC. The CEC will then make recommendations to the Sponsor regarding continuation of the study.

9.5.2 Subject Stopping Rules

The following stopping rules will apply at the individual study subject level:

1) Subject withdraws from the study
2) Subject is lost to follow-up

Any adverse events related to the subject withdrawal will be documented with attempts made to follow the subject until the adverse event resolves or a stable endpoint is reached.

10 Statistical Considerations and Analysis Plan

This is a prospective, placebo-controlled, randomized, double blinded (patient and investigator/ assessor) study designed to estimate the treatment effect size and variance associated with intra-

articular injection of Axolotl Ambient vs. saline (placebo control) for the treatment of symptomatic ankle osteoarthritis. Below are the justifications for the sample size as well as the statistical analysis plan.

Efficacy comparisons between the Investigational and Control groups will be completed using the modified intention-to-treat (mITT) population. The mITT population will include all enrolled, randomized subjects who received an injection. The as-treated population will be used for sensitivity analyses on efficacy outcomes and for safety analyses.

10.1 Sample Size

The goal of this study is to estimate, with sufficient precision, the treatment effect size and variability in the primary outcome measures and the proportion of responders to definitions of treatment success related to those primary outcome measures. For continuous variables, such as AOS and FAOS scores, a 20% greater improvement in the investigational group compared to control would constitute a moderate improvement that is clinically justifiable for a low risk therapy (Roos 2018), such as an injection of Axolotl Ambient. Based on an anticipated 20% greater improvement, and the variability in AOS scores at baseline from 2 randomized controlled trials (Cohen et al. 2008, DeGroot et al. 2012), an estimated effect size for the pivotal trial hypothesis would be 0.5 (medium effect). Based on the work of Whitehead et al., and using the non-central t-distribution as a basis, a sample size of 12 subjects per group should provide sufficient precision to confirm the variability in the primary outcome measures to inform a pivotal trial based on 90% power, 5% type I error, and an anticipated effect size of 0.5 (Whitehead et al. 2016). However, since a responder analysis for composite clinical success is a likely possibility in the pivotal trial, this pilot should also provide sufficient estimates of responder rates. There are marked gains in precision of a proportional estimate when increasing the sample size from 10 to 20 subjects per group. The width of the 95% confidence interval narrows by 7.5% between 10 and 15 subjects and by another 5% from 15-20 subjects. Between 20-25 subjects, the width of the 95% confidence interval only narrows by 3.6%, suggesting that gains in precision are more marginal with additional subjects. Therefore, the target sample size of this pilot study will be 20 subjects per group to provide sufficient precision in the estimates of the primary outcomes and responder analyses. To allow for 15% of subjects lost to follow-up, a target of 24 subjects will be enrolled per group.

10.2 Primary Analyses to Assess Treatment Effect and Variance

The primary goal of this pilot study is to inform the primary hypothesis of a subsequent pivotal study through estimates of the means, mean differences, and variance of the primary outcome variables as well as estimates of the proportion of patients achieving composite clinical success (CCS).

Descriptive statistics such as mean, median, interquartile ranges, standard deviation, and confidence intervals from 80%-95% will be calculated for each outcome. Non-parametric analysis methods will be used where available and necessary if assumptions for parametric tests are not valid.

Changes from baseline to each follow-up timepoint (4 weeks, 3 and 6 months) will be calculated for each outcome metric and statistical significance of changes will be assessed through repeated measures ANOVA or a mixed effects model to accommodate missing data points.

Two options (based on either AOS or FAOS scores) for CCS will be defined as:

- Improvement in AOS (Pain and Function, independently) *or* FAOS (Pain and ADL, independently) subdomain scores of at least 20% from baseline
  AND
- No medical or surgical intervention beyond allowed rescue medication
  AND
- Absence of treatment-related SAEs

The primary hypothesis and CCS criteria of the pivotal trial will not necessarily reflect the criteria that is prospectively described herein.

10.3 Secondary Analysis of Non-primary Outcomes and Intergroup Comparisons

Intergroup comparisons in continuous outcome scores (e.g. AOS and FAOS) will be conducted using repeated measures ANOVA or a mixed effects model to accommodate missing data points, with appropriate corrections for multiple comparisons. Intergroup comparisons of proportions will be conducted using Fisher's exact test.

This pilot study is not intended to be powered to observe statistically significant differences, but analyses will be conducted to assess trends that will be informative for the primary hypothesis of the subsequent pivotal trial. These analyses are not intended to yield definitive assessments of effectiveness for decisions related to regulatory approval or proceeding to larger trials of similar or different design.

10.4 Sub-group analyses

For the above analyses, sub-groups may be explored based on patient characteristics (e.g., age, gender, OA etiology) to determine if any potential trends exist that should be explored further in the pivotal study.

10.5 Missing data and sensitivity analyses

Sensitivity analyses, such as tipping point analysis and last-observation-carried-forward (LOCF), will be performed for missing data to better understand how variations in the missing data may affect the observed trends. A sensitivity analysis will also be performed for the threshold of AOS or FAOS score improvement. Roos et al. recommends reporting both 20% and 50% improvements for responder analyses, so the success criteria will also be evaluated with a 50% improvement threshold (Roos 2018).

10.6 Adverse Events

Site reported AEs and SAEs will be summarized as subject-based and event-based counts and subject-based percentages by MedDRA system organ class and preferred term. In addition, subject listings will also be provided for the serious and unanticipated adverse reactions.

10.7 Interim Analysis

There will be no formal interim analyses or adaptation based on the primary endpoint.

11 Clinical Events Committee (CEC)

The CEC is an independent group of 3 physicians with pertinent experience to review safety event data from the ongoing clinical study. The CEC will approve specific criteria, which will be used for the categorization of clinical events and clinical study endpoints. At the onset of the study, the CEC will approve explicit rules outlining the minimum amount of data required, and the algorithm followed in order to classify an event. The CEC will also review and validate adverse events that occur over the course of the study and subsequent classification of these adverse events as related to the investigational product or procedure and severity. If two of the adjudicators agree on an assessment of an adverse event, the event will be considered adjudicated. In the interest of ensuring consistency with regard to the classification of adverse events identified within the study, the CEC classification will serve as the final word on events adjudicated by the CEC.
The CEC will also be responsible for evaluating the 4-week safety data from the first six subjects and making a recommendation to the Sponsor on continuing enrollment.
The CEC will also be responsible for the triggering of study stopping rules if an event is upgraded in severity from initial investigator assessment. The CEC will evaluate events that trigger stopping rules and make recommendations to the Sponsor on continuing study enrollment.
The CEC will meet at least quarterly during the entire study period. Ad hoc meetings will also occur within 48 hours for any Serious AEs and/or any AEs that trigger stopping rules.

12 Quality Control and Data Monitoring

12.1 Organizational Preparations

A site visit will be performed by the Sponsor, prior to the start of the study to review this study protocol in detail, as well as Good Clinical Practices (GCP) procedures. This protocol will be conducted under applicable GCP Regulations (21 CFR Part 11, Part 50, Part 54, Part 56, and Part 312), ISO 14155(current revision), and the principles described in the Declaration of Helsinki.

12.2 Training

The primary consideration in investigator selection for this study is adequate experience in intra-articular ankle injection, commitment to safety, and consistency in adherence to this clinical protocol and GCP standards. Risk mitigation will include careful subject selection and selection of investigators who are, by virtue of their experience and training, accomplished in treating osteoarthritis of the ankle.

Training of study site research personnel to the study protocol and EDC system will be the responsibility of the Sponsor. To ensure uniform data collection and protocol compliance, personnel from the Sponsor will review the techniques for the identification of eligible subjects, activities related to data collection, and schedules for follow-up with the research coordinators. Data completion guidelines will be provided to the sites in order to assist with use of the EDC system.

12.3 Monitoring

Ongoing site monitoring will be conducted per the Monitoring Plan in order to ensure protocol compliance is met, enrollment commitments are achieved, eCRFs are accurate, and product accountability is accurate. The purpose of monitoring the study is to verify that the rights and well-being of subjects are protected, that the reported study data are accurate, complete, and verifiable from source documents and that the conduct of the study is in compliance with the current approved protocol/amendment(s), GCP, and in conformance with all applicable local laws and regulations.

The investigator must allow regular inspection of all study records including eCRFs, source documents and the site trial master file (including regulatory documents) during the study by the Sponsor. This measure is to ensure that the study is carried out and documented in accordance with federal regulations and the terms of this protocol. The investigator also agrees to allow inspections by FDA or other regulatory agencies before, during, or after the study has concluded, if such inspections are requested.

Criteria for the frequency of monitoring visits at each site will depend in part on the number of subjects enrolled, compliance to this study protocol, and change in study personnel; however, on-site monitoring visits should be conducted for each site within eight weeks after the first subject has received the index treatment, and subsequent visits will take place at least annually.

12.4 Source Document Requests

Source documentation for this study will be maintained to document the treatment and study course of a subject and to substantiate the integrity of the study data submitted for review to the FDA. Source documentation will include, but not be limited to, worksheets, hospital and/or clinic or office records documenting subject visits including study and other treatments or procedures, medical history and physical examination information, laboratory and special assessments results, pharmacy records, product accountability records, and medical consultations (as applicable).

12.5 Source Data Verification

Source data verification (SDV) is an essential element to ensure accuracy and credibility of the data and conclusions derived from clinical investigations. Entries in the eCRFs are verified using the original documents of SDV such as subject files, informed consent forms signed by subjects, product accountability forms, and original recordings from automated instruments, MRI films, laboratory notes, etc. whereby subject privacy is to be respected.

All eCRFs will be reviewed for completeness and clarity upon receipt by the Sponsor. Missing or unclear data will be queried by either the data manager or the monitor, and the sites shall provide missing data or clarify responses as necessary throughout the study. The Sponsor may request further documentation such as physician and/or procedure notes when AEs are observed and reported.

Primary data collection based on source-documented hospital chart reviews will be performed by research coordinators at each study site. The Sponsor will provide clinical monitoring, including review of eCRFs and parity checks with the source documentation including worksheets retained with eCRF documentation and hospital charts.

12.6 Data Management

Study data will be recorded in a limited access, secure EDC system (DataTrak). Access to the EDC system will be provided by the Sponsor to the study sites prior to study start. The database is designed to meet regulatory compliance for deployment as part of a validated system compliant with laws and regulations applicable to the conduct of clinical studies pertaining to the use of electronic records and signatures. Database backups will be performed regularly.

The development of the eCRFs in this database will be performed by the Sponsor. The Sponsor will also be responsible for auditing the database and confirming the overall integrity of the data. Automatic, validated edit checks will be built into the system prior to start-up and throughout the investigation to minimize entry errors.

An instruction for entering data into the electronic system will be provided in the eCRF-Completion Guidelines, and training will be provided to the applicable person(s) of the study team before they can access the system.

The investigator or a delegated representative will enter study data into the database in a timely manner once the information of the respective subject visit becomes available. Subjects will only be identified by using the subject-ID number, which is a combination of a three-digit site number and a sequentially-assigned subject number (xxx-xxx). All data entered into the EDC system will be source document verified by the monitor during routine monitoring visits (monitoring is described elsewhere in this protocol). All data queries issued by the monitor or the database manager will be resolved with the assistance of monitoring staff.

If a correction is required for an eCRF, the system tracks the person's name entering or updating the new information, together with a rationale for the change, and data and time stamp, in the audit trail. The investigator provides his/her electronic signature on the appropriate eCRFs in compliance with local regulations. Once all the subject data has been collected, monitored, and signed by the investigator, the analysis and reporting may be conducted.

12.7 Protocol Amendments/Modifications & Protocol Deviations

All items in this protocol are to be followed. All proposed changes/amendments to the protocol must be reviewed by Axolotl Biologix and all changes must be approved in writing by Axolotl Biologix and the IRB/EC, consistent with U.S. GCP Regulations. Each investigator must sign the protocol amendment before implementing the change at his/her site.

All amendments will be distributed to all protocol recipients with instructions. Documentation of all correspondence regarding protocol amendment study activity should be forwarded to the Sponsor.

In situations requiring departure from the protocol, the investigator or other physician in attendance will contact the Sponsor. If possible, this contact will be made before implementing any departure from the protocol. In all cases, contact with the Sponsor must be made as soon as possible in order to discuss the situation and agree on an appropriate course of action. The eCRF and source documentation will describe any departure from the protocol and the circumstances requiring it.

It is the responsibility of investigators to inform their IRB/EC of all protocol amendments/modifications

as required by their IRB/EC and country regulations.

Protocol Deviation/Violation

A protocol deviation is an incident where the investigator, study site personnel, or subject did not conduct or participate in the study according to the clinical protocol or the investigator agreement.

Protocol Violation: Any deviation from subject inclusion and exclusion criteria or subject informed consent procedures. Protocol Violations should be reported to the Sponsor and Clinical Study Manager within 5 business days of site awareness of the event via the Protocol Deviation Form and must be documented appropriately.

Protocol Deviation: Deviation from the clinical protocol requirement such as incomplete/inadequate subject testing procedures, non-compliance with study follow-up windows, etc.

The investigator should make all the necessary efforts to comply with the protocol requirements. When the investigator becomes aware of protocol deviations, the event is to be recorded on a protocol deviation eCRF.

Protocol deviations/violations should be reported to the IRB/EC per local requirements.

13 Ethics and Regulatory Requirements

13.1 Code of Conduct

The investigator will ensure that the clinical study is conducted in accordance with GCP and all applicable regulatory and institutional requirements, including the Declaration of Helsinki, 21 CFR Part 11, Part 50, Part 54, Part 56, Part 312, and ISO 14155(current revision).

13.2 Institutional Review Board/Ethics Committee (IRB/EC)

The investigator must obtain appropriate IRB/EC approval before the study can be initiated. A copy of the written approval from the IRB/EC and a copy of the approved informed consent form must be sent to the Sponsor for filing. A list of the IRB/EC members, or a statement from the IRB/EC specifying that the membership complies with applicable regulations must be provided to the Sponsor.

If the investigator advertises for subjects, whether in a professional or consumer publication, radio, television or community notice, all advertising must receive prior approval by the Sponsor and the IRB/EC.

All patient-facing materials, including subject questionnaires, must be approved by the IRB/EC prior to use.

The investigator must forward to the IRB/EC any written safety reports, changes to the Investigator's Brochure or updates from the Sponsor.

The investigator must keep the IRB/EC informed of the progress of the study as required by the IRB/EC, but at least annually.

13.3 Subject Informed Consent

The investigator must observe the requirements of the appropriate regulatory body by obtaining written subject informed consent. The Sponsor or designee will supply a sample subject informed consent form. Whether or not the sample informed consent form is used or adapted, the site will submit the proposed informed consent form to the Sponsor or designee for review prior to submission to the IRB/EC. The informed consent form must be approved by the IRB/EC. Copies of the informed consent form used in the study must contain the IRB/EC approval stamp (if applicable) and version date.

Subjects will be informed of new information learned during the study, which may affect they subject's decision to continue participation in the study.

The study informed consent form must be obtained prior to the initiation of any study procedures. The subject must be allowed sufficient time to thoroughly read the informed consent form. The investigator must answer any questions that the subject might have. If the subject agrees to participate in the study, the subject must sign the informed consent form. One copy of the informed consent form must be given to the subject. The original signed consent form should be filed with the study documents. The study staff should adequately and accurately document the sequence of actions in the consenting process as well as the date and time of the subject's signature on the informed consent form in the subject's records in order to document that informed consent was obtained prior to initiating any study procedures.

13.4 Subject Confidentiality

The Sponsor will maintain the confidentiality of the identity of subjects enrolled in the study and the information contained in their study records. The records will be made available as required for review by FDA and the reviewing IRB/EC, however to the extent possible; the subject's identity will not be disclosed. All data used in the analysis and reporting of the evaluation will be used in a manner without identifiable reference to the subject.

14 Data Reporting

14.1 Case Report Forms

Data for this clinical study will be collected and documented on the subject eCRFs. Authorized study personnel will complete eCRFs only. eCRFs must be reviewed and electronically signed by the principal investigator or co-investigator listed in the Delegation of Authority Log.

Since there is the potential for errors, inaccuracies, and misinterpretation in transcribing data onto the eCRFs, originals or copies of all relevant records and reports, and copies of test results must be available at all times for inspection and comparison to the eCRFs by the study monitor.

eCRFs will be used to collect all subject data during the study. Detailed description of the eCRF components and eCRF completion guidelines will be provided. eCRFs must be fully completed for each subject, and available for review by appointed monitors in a timely manner after the event they document.

14.2 Final Report

Following completion of this study, final reports will be issued as required.

14.3 Retention of Records by the Investigator

Investigators will retain records for a period of 2 years following the date a marketing application is approved for the product for the indication for which it is being investigated or as per local requirements, whichever is longer in duration. If no application is filed or if the application is not approved for this indication, records are to be retained until 2 years after the investigation is discontinued and, if required, the appropriate regulatory agency is notified.

14.4 Investigator Final Report

Upon completion or termination of the study, a final report will be prepared. This report will contain an evaluation of all data collected during the course of the study at each institution. The report will be provided to the IRB/EC as required.

15 Termination of an Institution by the Sponsor

The Sponsor may terminate an institution from the study for any of the following reasons:

- Repeated failure to complete eCRFs
- Failure to obtain subject informed consent
- Repeated failure to report SAEs within 24 hours of such knowledge
- Loss of or unaccounted investigational product inventory
- Repeated protocol deviations/violations

16 Use of Information and Publication

Any publication or other public presentation of the investigational product or results from this study requires prior review by Axolotl Biologix. No sub-group data or site data will be published or presented until the publication of the overall study data. Draft abstracts, manuscripts, and materials for presentation at scientific meetings should be provided to Axolotl Biologix at least thirty (30) working days prior to abstract or other relevant submission deadlines.

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18 Summary of Changes

Version	Effective Date	Summary of Changes	Change Author
Draft 1.0	12-May-2021	Draft Protocol for Initial IND Application	Patrick Reischling
1.0	14-July-2021	Final Protocol for release	Patrick Reischling
2.0	13-Apr-2022	Updated Telos Information	Cheyanne Floyd

Appendix A: Schedule of Events

Action	Screening Evaluations	Pre-treatment (Baseline)	Treatment	4 weeks post-treatment	3 months post-treatment	6 months post-treatment	12 months post-treatment
Obtain written Informed Consent	X						
Screening Visit, Inclusion & Exclusion, Demographics, Medical History	X						
Complete Physical Exam	X						
X-rays		X				X	
Pain catastrophizing score		X					
AOS		X		X	X	X	X
FAOS		X		X	X	X	X
VAS		X		X	X	X	X
EQ-5D		X		X	X	X	X
Working Status		X		X	X	X	X
Randomization			X				
Index Procedure			X				
Concomitant Treatments			X	X	X	X	X
Adverse Events			X	X	X	X	X
Protocol Deviations			X	X	X	X	X