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**14. ABSTRACT**

**Scope**. Biofilms represent a significant barrier to healing of battlefield wounds. *Acinetobacter baumannii* (ACIN) and *Pseudomonas aeruginosa* (PSEUD) are among the most significant bacterial species associated with infection of battlefield related wounds. **Purpose.** The present study tested the efficacy of OligoG, a novel guluronate-rich alginate oligomer, in preventing biofilm formation in pre-clinical full thickness burn wounds infected with biofilm. **Findings**. A multispecies biofilm (ACIN/PSEUD) was established in porcine burn wounds. Scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM) confirmed the presence of multispecies biofilms in these wounds. Impairment in barrier function as measured by trans-epidermal water loss (TEWL) was noted in biofilm-infected wounds. The infected burn wounds were treated with topical application of either 15% OligoG or placebo formulation. SEM and CLSM clearly showed that wounds treated with OligoG exhibited a marked reduction and disruption in bacterial biofilm formation in the burn wounds. Furthermore, OligoG treatment also significantly improved healing by restoring the barrier function at the burn site as determined by TEWL.
**Significance**. These studies indicate that OligoG when used topically at the time of wounding could provide an effective therapeutic strategy in preventing microbial and subsequent biofilm colonization of battlefield wounds, and facilitating wound healing.

**15. SUBJECT TERMS**
Wound infection, biofilm, TEWL, inflammation, OligoG, burn wounds

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# INTRODUCTION

Biofilms pose significant threat to military wounds [1,2]. According to the Center for Disease Control 60% of all chronic infections in the United States are related to biofilms, which are difficult to eradicate. New treatments with topical agents that inhibit biofilm formation or promote their detachment and reduce wound infections would have a significant impact not only for military medicine, but also for civilian hospitals, wound care centers, and trauma units worldwide. Lack of pre-clinical models poses a serious impediment to biofilm research. The aims of the project were to develop, i) a novel porcine pre-clinical model of multispecies biofilm on burn wounds and study its role in impaired healing, and ii) determine the efficacy of the alginate oligomer OligoG on eradicating biofilms in this porcine burn wound model.

# BODY

As outlined in the Statement of Work the project addressed the following hypotheses:
1. Biofilm is formed on pre-clinical full-thickness porcine burn wounds and impairs healing.
2.OligoG inhibits biofilm formation and potentiates antibiotic activity against gram negative infected wounds.

## 1. Biofilm is formed on pre-clinical full-thickness porcine burn wounds and impairs healing.

### *1.1 Acinetobacter spp. and Pseudomonas spp. form biofilm on full thickness experimental burns on preclinical porcine model.*

There is no prior report of any large animal pre-clinical model for biofilms. Development of such pre-clinical large animal model would enable screening of therapeutics and facilitate our understanding of underlying impaired healing mechanisms. The porcine model has been favored over other animals for studying burn injuries due to: anatomical, biochemical and physiological considerations [3,4,5]. To standardize the approach for inducing a burn injury in the pig, we constructed a microprocessor controlled electrically heated burning device that was able to create uniform, full-thickness thermal injuries of a defined size and depth. The design of this apparatus was critical in establishing a robust and reproducible model of burn wounds, while avoiding operator variations in applying pressure, burn time, wound temperature and depth (**Figure 1**).

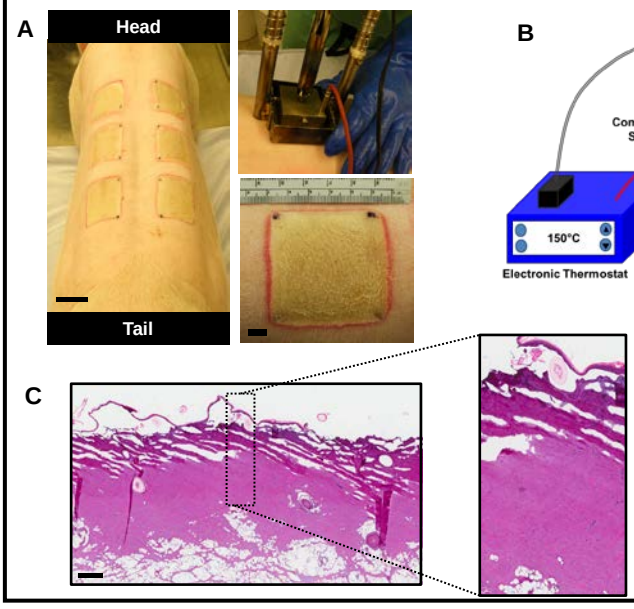


**Figure 1. Establishment of full-thickness preclinical porcine burn model. A.** Six 2 x 2 sq inch size burn wounds were created with a burning device shown in B**. B.** Microprocessor controlled electrically heated burning device that can uniformly create full-thickness thermal injuries (burns) shown in A. To control the amount of pressure applied to the skin, engineering modifications were done to allows application of uniform pressure regardless of the operator thus, creating more uniform burn wounds. **C**. Histological evidence that 50 sec of contact was sufficient to create a full-thickness burn wound.

We also established a standardized approach for inoculating the bacteria into the burn wounds. Topical application versus intra-dermal injection and low bacterial load ($5x10^3$) versus high bacterial load ($5x10^7$) were tested. Sampling of these wounds was done at various time points after inoculation. Visual inspection of the wounds revealed yellowish green discoloration with discharge that increased with time (**Figure 2A**). Histological analysis using gram staining and microbiological analysis confirmed the presence of bacteria in the wound bed. Infected animals maintained localized infections in the wound-site while no evidence of systemic infection was observed.

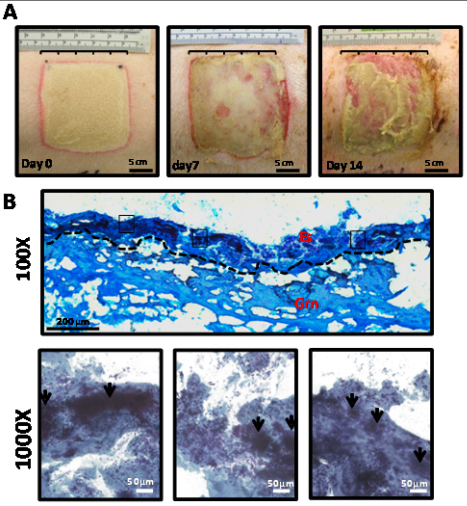


**Figure 2. Co-infection of burn wounds was performed by co-inoculation of Pseudomonas (PAO1) and Acinetobacter baumanii bacterial suspensions.** The co-infections of burn wounds was performed 3 days post burn. **A.** Representative images of post inoculated (PI) wounds on days 0, 7 and 14. **B.** Gram stained images of inoculated wounds shows presence of bacterial aggregates *upper panel*, lower, magnification, scale bar, 200 μM; *lower panel*, higher magnification, scale bar, 50 μM. Es = eschar; Grn, granulation.

Military casualties are known to be at high risk of acquiring MDR infections and increasing numbers of cases of MDR septicemia, urinary tract infections, and ventilator-associated pneumonia have been reported [6,7,8]. Multidrug resistant *Acinetobacter spp*. (ACETB) and *Pseudomonas aeruginosa* (PSEUD) are among the most significant bacterial species that are associated with battle wound infections [8,9,10]. Therefore, a multispecies infection approach was used, combining *Acinetobacter baumannii* and *Pseudomonas aeruginosa* to co-infect the burn wounds and establish a clinically relevant biofilm infection.

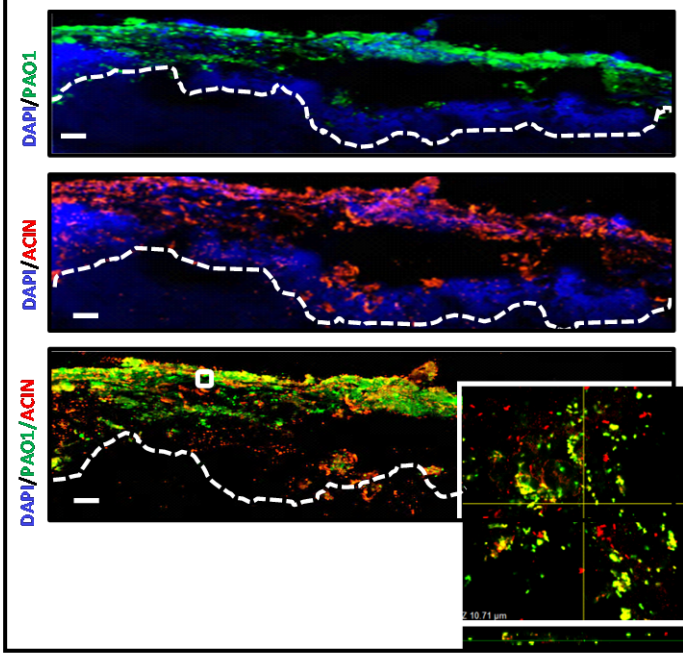


**Figure 3. Immuno-histochemical staining of Pseudomonas and Acinetobacter burn wounds inoculated with both strains.** **A.** Visualization of both bacterial strains was done using anti-pseudomonas (green) and anti-acinetobactor (red) antibody and immunofluorescence microscopy. Anti-acinetobactor antibody was developed in Dr. Sen's laboratory as a part of the current award; *Inset*: day 7 post inoculation burn wound visualized using a confocal laser scanning microscopy (CLSM) images showing co-localized bacterial colonies in Z plane.

Following infection, visualization of the bacterial species was essential to determine localization and overall burden of biofilm in wounds. We already had already access to an anti-*Pseudomonas* antibody, but had to develop our own antibody specific for *Acinetobacter baumannii*. Immunohistochemical (IHC) optimizations using anti-*Pseudomonas* and anti-*Acinetobacter* antibodies were performed. Visualization of *Acinetobacter* and *Pseudomonas* were done by imaging with a confocal laser scanning microscope (CLSM). The CLSM images confirmed the presence of both

organisms in burn wounds 7 days post inoculation (**Figure 3**), which also showed areas of co-localization of both bacterial strains suggesting that this was a true mixed species biofilm infection (**Figure 3**).

The biofilm was further characterized according to standard criteria [11]:

*i)* *adherence to a surface*
*ii)* *aggregates of bacteria in EPS*
*iii)* *persistent and localized infection*
*iv)* *anti-microbial drug resistance*

The data presented in Figures 4 and 5, demonstrated that the co-infection in the burn wound model was effective in establishing a biofilm.

***i).Adherence to a surface***. To test adherence of biofilm to wound surface, a flush technique was used. This technique removed free (planktonic) bacteria from the burn wounds before biopsy so that further analysis of the tissue biopsies will only determine the bacteria that are adherent to the wound surface. Washing was performed with double opened end sterile plastic tubes; 3 times with sterile saline and 1 time with a detergent (4% Tween in dd$H_2O$). Microbiology analysis showed that bacterial counts did not significantly change before or after flush, suggesting strong bacterial adherence to the wound.

*ii). Aggregates of bacteria in EPS*. Imaging of burn wound biopsies with scanning electron microscope (SEM) showed aggregates of *P. aeruginosa* attached to the surface of the burn wounds and embedded in an EPS indicating formation of a biofilm matrix (**Figure 4A**).

*iii). Persistent and localized infection.* The multi species bacterial infection was present in the burn wounds until

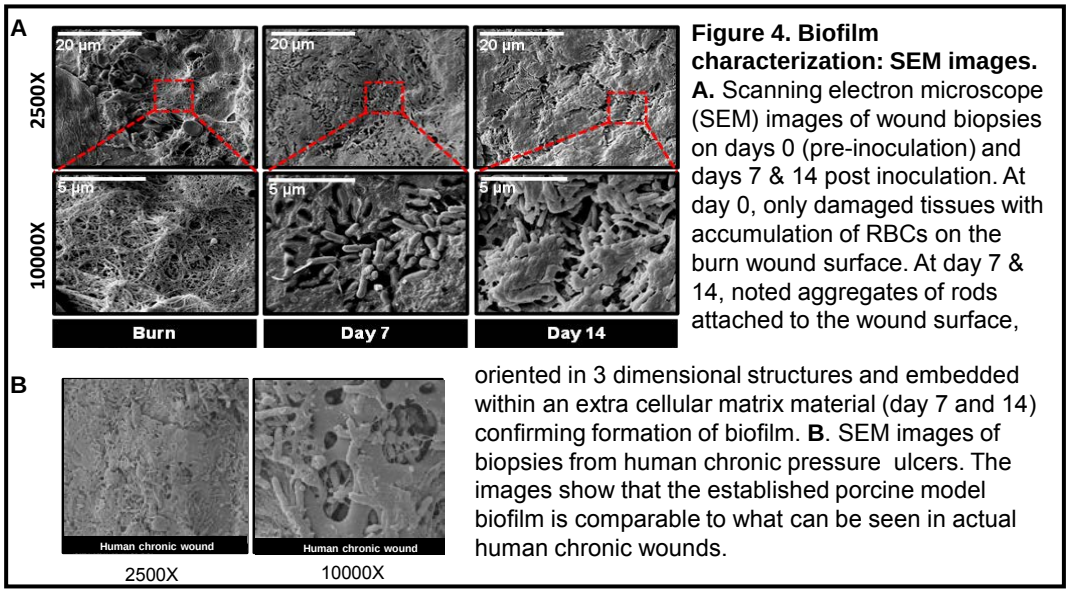


**Figure 4. Biofilm characterization: SEM images.** **A.** Scanning electron microscope (SEM) images of wound biopsies on days 0 (pre-inoculation) and days 7 & 14 post inoculation. At day 0, only damaged tissues with accumulation of RBCs on the burn wound surface. At day 7 & 14, noted aggregates of rods attached to the wound surface, oriented in 3 dimensional structures and embedded within an extra cellular matrix material (day 7 and 14) confirming formation of biofilm. **B**. SEM images of biopsies from human chronic pressure ulcers. The images show that the established porcine model biofilm is comparable to what can be seen in actual human chronic wounds.

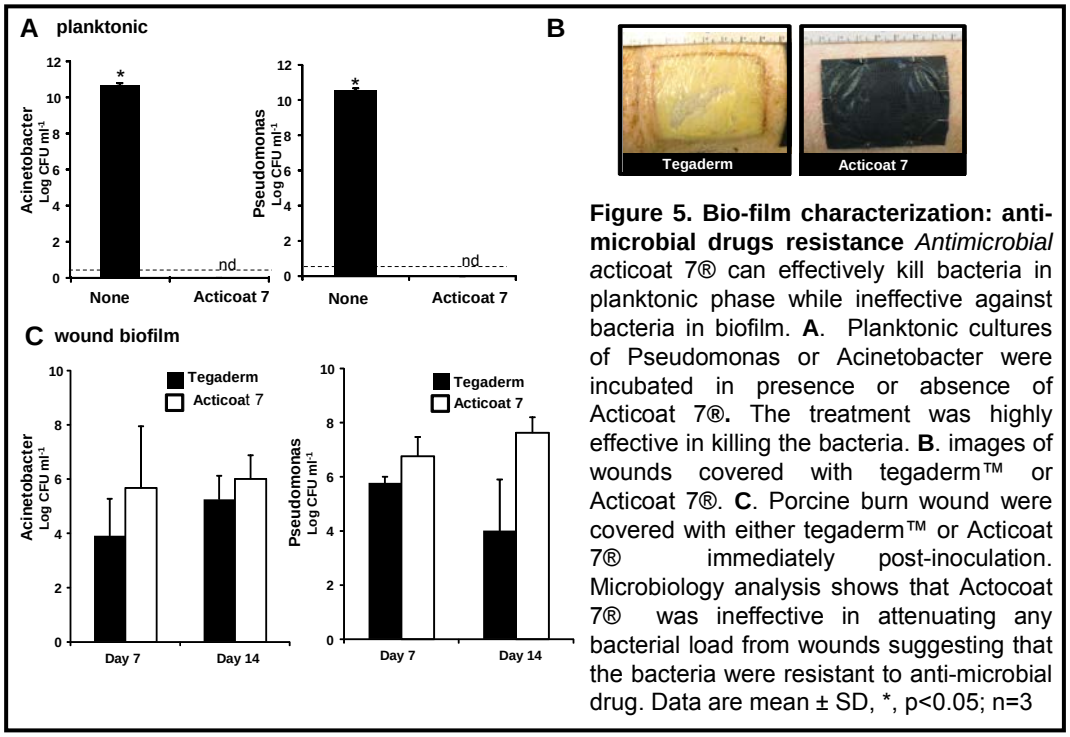


**Figure 5. Bio-film characterization: anti-microbial drugs resistance** *Antimicrobial* acticoat 7® can effectively kill bacteria in planktonic phase while ineffective against bacteria in biofilm. **A**. Planktonic cultures of Pseudomonas or Acinetobacter were incubated in presence or absence of Acticoat 7®**.** The treatment was highly effective in killing the bacteria. **B**. images of wounds covered with tegaderm™ or Acticoat 7®. **C**. Porcine burn wound were covered with either tegaderm™ or Acticoat 7® immediately post-inoculation. Microbiology analysis shows that Actocoat 7® was ineffective in attenuating any bacterial load from wounds suggesting that the bacteria were resistant to anti-microbial drug. Data are mean ± SD, *, $p<0.05$; n=3

day 35 post inoculation, indicating a persistent infection. Nevertheless, blood cultures from the pigs did not show bacterial growth and no systemic signs of infection were observed in the animals suggesting the infection was localized.

*iv). Anti-microbial drugs resistance.* Silver is the most prevalent topical antimicrobial used to prevent and treat bacterial infection and prevent wound sepsis *[12,13,14,15].* While it is effective in killing planktonic infection, biofilms are known to be resistant. Acticoat 7, a routinely used standard of care dressing for burn wounds in patients was used to demonstrate this resistance in the infected pig wound model. Results showed that Acticoat 7 was ineffective in reducing the bacterial burden in the infected pig wounds (**Figure 5**).

### *1.2. Immunosuppression, as also noted in battlefield situation, did not facilitate biofilm development and persistence in burn wound model.*

Immunosuppression of animals was performed using the long acting steroid (Depo-medrol 240 mg, IM). The studies indicated that there was no significant difference in the bacterial numbers in pigs treated with Depo-medrol (24h before establishing burn wounds) compared to pigs not treated with Depo-medrol. We reproduced immune-suppression in pigs by doubling the dose of Depo-medrol, 24h before establishing burn wounds and 14 days later. However, there was no significant difference in the bacterial numbers in those pigs treated with Depo-medrol compared to pigs not treated with Depo-medrol. Although immunosuppression in this model did not appear to influence biofilm development, biofilm still persisted in the non-immune suppressed animals.

### *1.3. Wound debridement does not completely eradicate biofilms in experimental burn wounds.*

Debridement of wounds represents a key tool used to disrupt biofilms and facilitate healing. However, there is debate on whether such disruption is sufficient to eradicate the biofilm or if it provides only temporary relief, which if not adequately addressed would allow the biofilm to re-establish over time. Non-vital wound tissue is a “reservoir” for bacterial growth which facilitates the rapid re-establishment

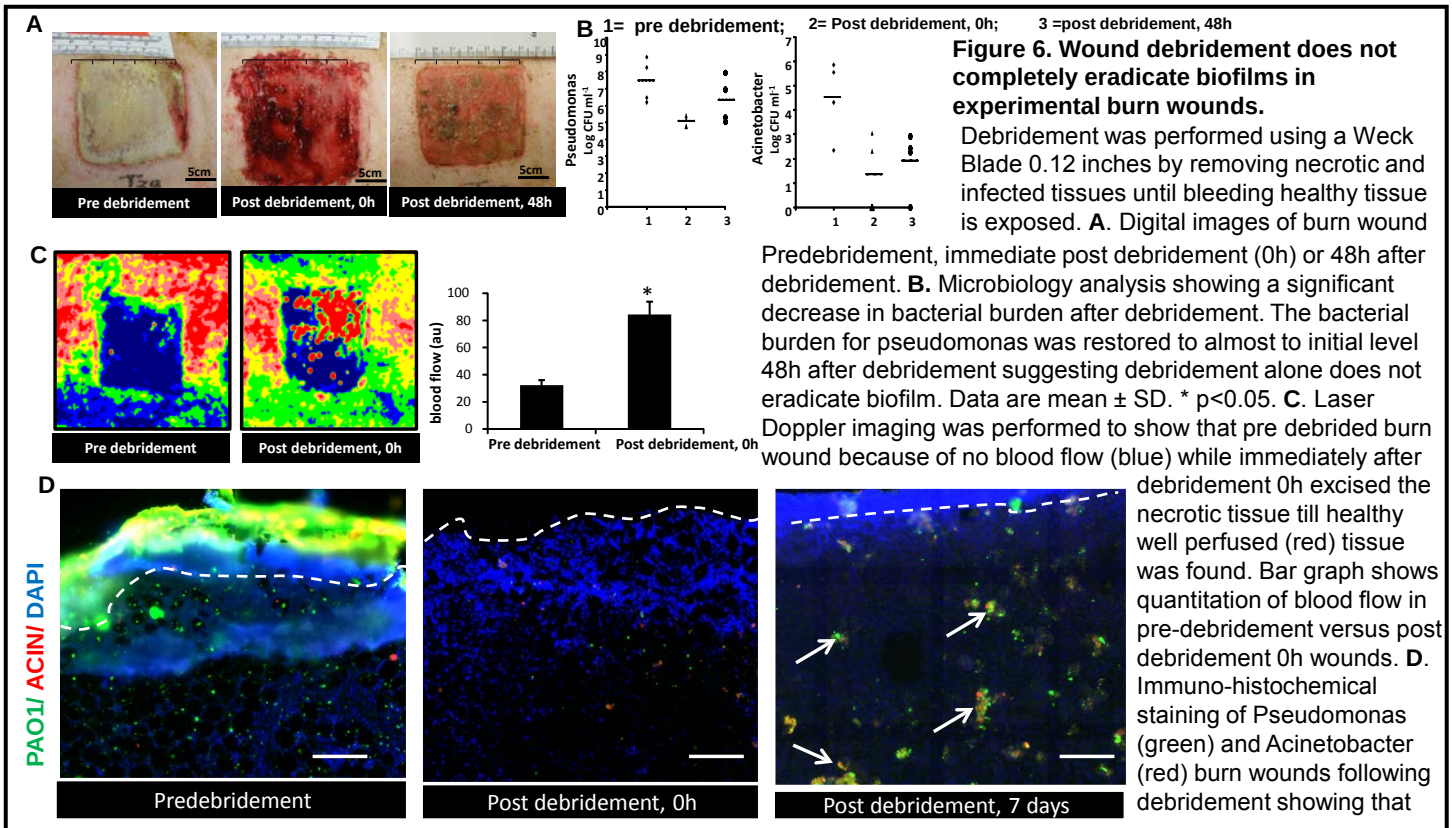


**Figure 6. Wound debridement does not completely eradicate biofilms in experimental burn wounds.** Debridement was performed using a Weck Blade 0.12 inches by removing necrotic and infected tissues until bleeding healthy tissue is exposed. **A**. Digital images of burn wound Predebridement, immediate post debridement (0h) or 48h after debridement. **B.** Microbiology analysis showing a significant decrease in bacterial burden after debridement. The bacterial burden for pseudomonas was restored to almost to initial level 48h after debridement suggesting debridement alone does not eradicate biofilm. Data are mean ± SD. * $p<0.05$. **C**. Laser Doppler imaging was performed to show that pre debrided burn wound because of no blood flow (blue) while immediately after debridement 0h excised the necrotic tissue till healthy well perfused (red) tissue was found. Bar graph shows quantitation of blood flow in pre-debridement versus post debridement 0h wounds. **D**. Immuno-histochemical staining of Pseudomonas (green) and Acinetobacter (red) burn wounds following debridement showing that the bacterial biofilm in form of micro colonies are restored in deeper layers of the wounds in day 7 post debridement. White arrows indicate the bacterial colonies established following debridement. White dashed line shows the line of debridement.

of wound microflora and wound biofilm. “Persister” bacteria are known to remain deep in the tissues and are able to re-colonize the wound within 48 hours [9].
Wound debridement was performed to confirm whether this clinical feature was also reflected in the pig model. A Weck knife was used to remove necrotic and infected tissues until bleeding healthy tissue was exposed. In control wounds (spontaneously colonized from skin flora), debridement was effective in preventing infection and keeping the total bacterial burden below $10^3$, which is considered to be the initial colonization burden in the wounds.
Debridement of wounds with established multispecies biofilm infection showed that bacterial burden was significantly lower immediately after debridement, but after 48h the bacterial counts returned almost to the pre-debrided levels (**Figure 6**). Furthermore, micro-colonies appeared to be more deeply embedded in the wound following debridement.

***1.4. Biofilms impair the healing of full-thickness burn wounds in a pre-clinical porcine model.***
Wound repair is a highly coordinated and complex dynamic series of events including clotting, inflammation, granulation tissue formation, epithelialization, neovascularization, collagen synthesis, tissue remodeling and wound contraction [16,17,18]. When a wound becomes infected it delays the resolution of inflammation and closure is delayed [1,19,20,21]. From a pathophysiological standpoint, infection places a massive oxygen demand on the wound tissue. A leukocyte respiratory burst consumes copious amounts of oxygen to fight infection [22]. This results in an increase in the severity of wound hypoxia stifling oxidative metabolism. Also, persistent infection dysregulates the inflammatory system impairing resolution [23,24]. Furthermore, components of infectious agents, such as lipopolysaccharide (LPS), are known to stall cell proliferation and migration which are necessary for wound healing [25,26].

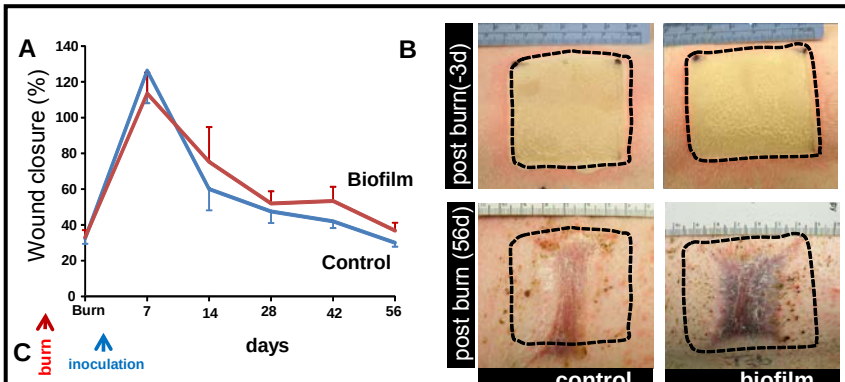


**Figure 7. Closure and histological characterization of burn wound infected with biofilm in a pre-clinical porcine model. A**. Wound surface area was calculated using Image J software. Data presented as means ± SD (n = 4). **B**. Digital photos of full thickness burn wounds at day of burn and day 56 post-inoculation. Black dashed squares represent the original wound size after thermal injury application to porcine skin. **C**. Wound-edge tissues were collected at the indicated time points after wounding.
Formalin fixed paraffin embedded wound biopsies tissue day 35 post inoculation were sectioned (5 μm) and stained using Masson trichrome procedure. This results in blue-black nuclei; blue collagen and cytoplasm. Epidermal cells appear reddish. The mosaic images of whole wounds were collected under 20X magnification guided by MosaiX software (Zeiss) and a motorized stage. Each mosaic image was generated by combining 12-15 images. The images show greater inflammatory cell infiltration in the biofilm infected wounds suggesting persistent inflammation even 35 days post inoculation.

In the pig wound model the overall closure of the biofilm infected wounds were not significantly different compared to control wounds (**Figure 7A-B**). Although no differences in closure were observed, the histological characterization using Masson trichrome staining revealed a greater infiltration of inflammatory cells in the biofilm infected wounds compared to control wounds (**Figure 7C** and **Figure 8**), indicating that there was an inflammatory response in these wounds.

When skin is damaged, its barrier function is impaired resulting in higher water loss. The measurement of trans-epidermal water loss (TEWL) is an established method to assess the integrity of the skin barrier in vivo [27]. Therefore in addition to visual measurement of wound closure, a TEWL assay was performed in the pig model to quantitatively determine the re-establishment of barrier function. Interestingly, a marked impairment in the restoration of TEWL was noted in biofilm-infected wounds compared to control wounds that received sterile saline (**Figure 9A**). The barrier function of the skin is maintained by integrity of epithelial cells. This is mediated by adhesive interaction at the epithelial apical junction complex comprising mainly the tight

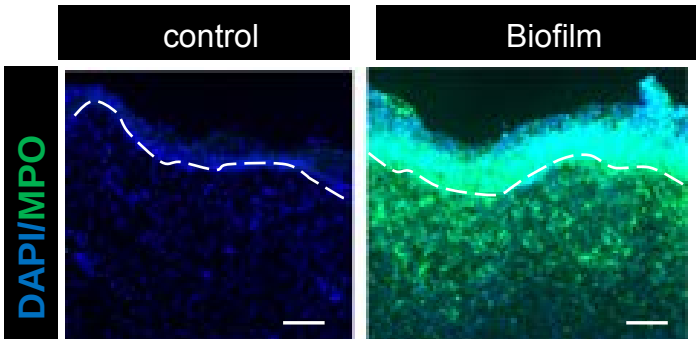


**Figure 8. Biofilm infection in burn wounds results in chronic persistent inflammation. A.** Representative day 35 immunohistochemical images of neutrophils (green) from control or biofilm infected burn wounds. The neutrophils were immunostained with myeloperoxidase (MPO, green) followed by counterstaining with DAPI (nuclear, blue). Increased MPO staining was noted in biofilm infected wounds compared to control wounds suggesting persistent inflammation in these wounds. Dashed line indicates the top of the burn wound.

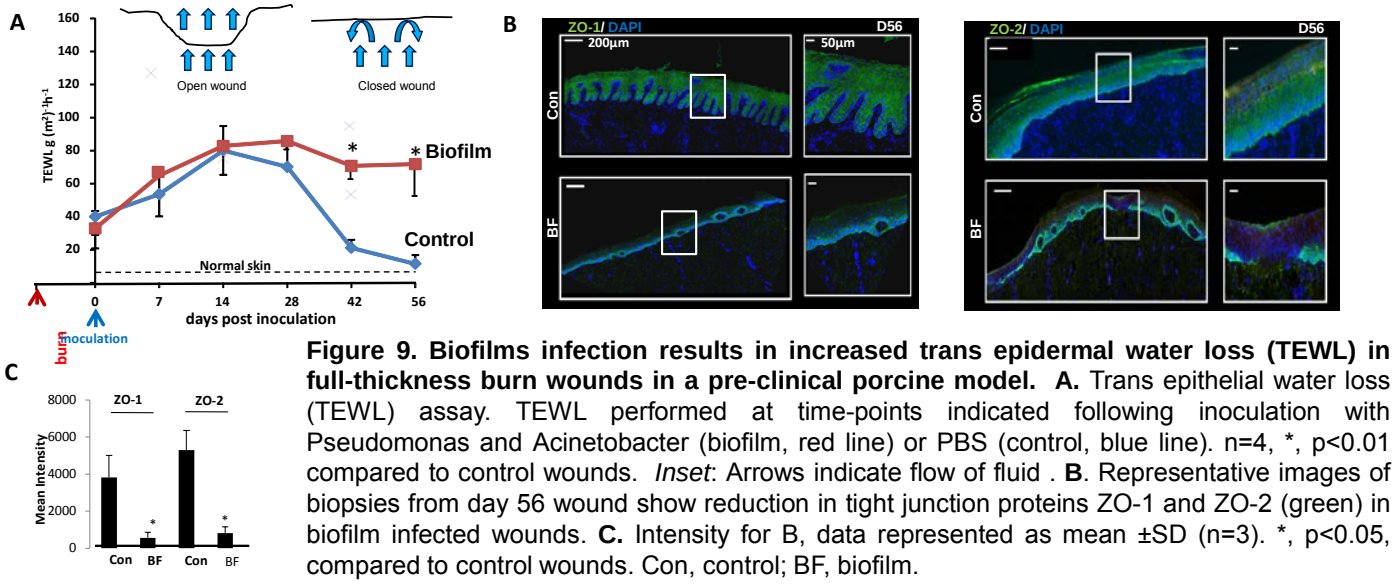


**Figure 9. Biofilms infection results in increased trans epidermal water loss (TEWL) in full-thickness burn wounds in a pre-clinical porcine model. A.** Trans epithelial water loss (TEWL) assay. TEWL performed at time-points indicated following inoculation with Pseudomonas and Acinetobacter (biofilm, red line) or PBS (control, blue line). n=4, *, $p<0.01$ compared to control wounds. *Inset*: Arrows indicate flow of fluid . **B**. Representative images of biopsies from day 56 wound show reduction in tight junction proteins ZO-1 and ZO-2 (green) in biofilm infected wounds. **C.** Intensity for B, data represented as mean ±SD (n=3). *, $p<0.05$, compared to control wounds. Con, control; BF, biofilm.

junctions, adherens junctions, desmosomes and gap junction proteins. Zona Occludens-1 (ZO-1) and Zona Occludens-2 (ZO-2), two key tight junction proteins were found to be significantly down regulated in biofilm infected burn wounds (**Figure 9B-C**). ZO proteins belong to the large family of membrane-associated guanylate kinase (MAGUK)-like proteins, they provide a link between the integral membrane proteins and the filamentous cytoskeleton. ZO proteins are scaffolding proteins, they recruit various types of proteins to the cytoplasmic surface of the tight junction [28]. Epithelial cells lacking ZO-1 and ZO-2 do not form tight junctions [29]. Besides their structural function at cell-cell contacts, ZO proteins have been reported to participate in the regulation of cell growth and proliferation. ZO-1 and ZO-2 knock-out has been reported to be embryonically lethal [30,31]. ZO-1 and ZO-2 interacts with a range of other tight junction proteins like Junction Adhesion Molecule 1 (JAM-1), Occludin and others. Bacterial infections have been reported to disrupt epithelial tight junctions and this can be mediated via disrupting ZO proteins [32]. Based on these observations, TEWL was used as one of the primary outcomes in determining the efficacy of the novel sodium alginate oligomer (OligoG) treatment in reducing the bacterial burden and disrupting biofilm formation in burn wounds.

## 2. OligoG inhibits biofilm formation and potentiates antibiotic activity against gram negative infected wounds.

***2.1 OligoG treatment is effective in reducing the bacterial burden and inhibiting biofilm formation on burn wounds.*** Studies in part 1 showed TEWL is an indicator of barrier function of the skin. A loss in TEWL was noted in biofilm infected wounds suggesting that bacterial biofilms impaired the wound epithelial barrier function. To determine if OligoG treatment was effective in wound epithelial barrier function loss, a TEWL assay was performed in biofilm-infected treated or untreated wounds. In addition biopsies were taken at the day 7 timepoint to evaluate histological and molecular responses. A placebo formulation omitting the OligoG was used as a control. Two series of experiments were performed to investigate OligoG activity in i) preventing the establishment of biofilms, and ii) disrupting existing biofilms.

In the first series OligoG (15% salve formulation) or placebo was applied to the burn wounds immediately following bacterial inoculation OligoG treatment was reapplied every 7 days up to day 49. Although there were no differences in visual wound closure, significant effects were observed over the course of treatment for OligoG treated wounds in the restoration of barrier function as measured by TEWL (**Figure 10**)

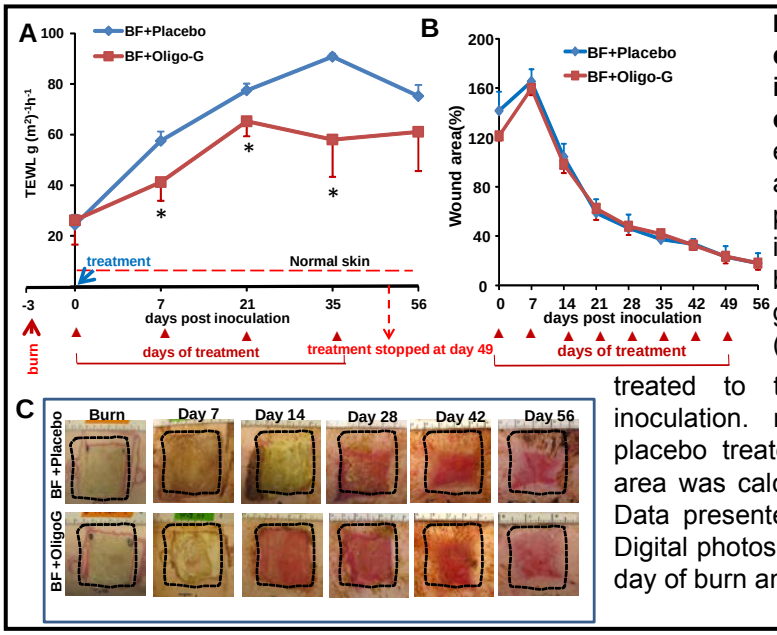


**Figure 10. Oligo G treatment effectively restores biofilm infection mediated impairment of TEWL function. A.** Trans epithelial water loss (TEWL) assay. TEWL performed at time-points indicated following inoculation with mixed species biofilm. Oligo G (15%, 2.5 g/wound; red line) or placebo (2.5 g/wound; blue line) were treated to the wounds at the day of inoculation. n=4, *, $p<0.05$ compared to placebo treated wounds. **B**. Wound surface area was calculated using Image J software. Data presented as means ± SD (n = 4). **C**. Digital photos of full thickness burn wounds at day of burn and day 7-56 post-inoculation.

In the second series OligoG (15% salve formulation) or placebo was applied to the burn wounds only at day 7 after the biofilm had been established. OligoG treatment was reapplied thereafter every 7 days up to day 49. Although a similar TEWL response was not observed for the OligoG treated wounds (**Figure 11A**), a potentiating effect was observed in combination with antibiotics (**Figure 11B**).

### *2.2. Does OligoG effectively potentiate antibiotic action leading to eradication of biofilms in experimental burn wounds?*

OligoG has previously been shown to inhibit cell adhesion and potentiate the abilities of selected antibiotics in eradicating established biofilms [33]. This potentiating effect of OligoG was tested in the biofilm infected wound model in combination with the antibiotic Azithromycin, a sub-class of the macrolide antibiotics.

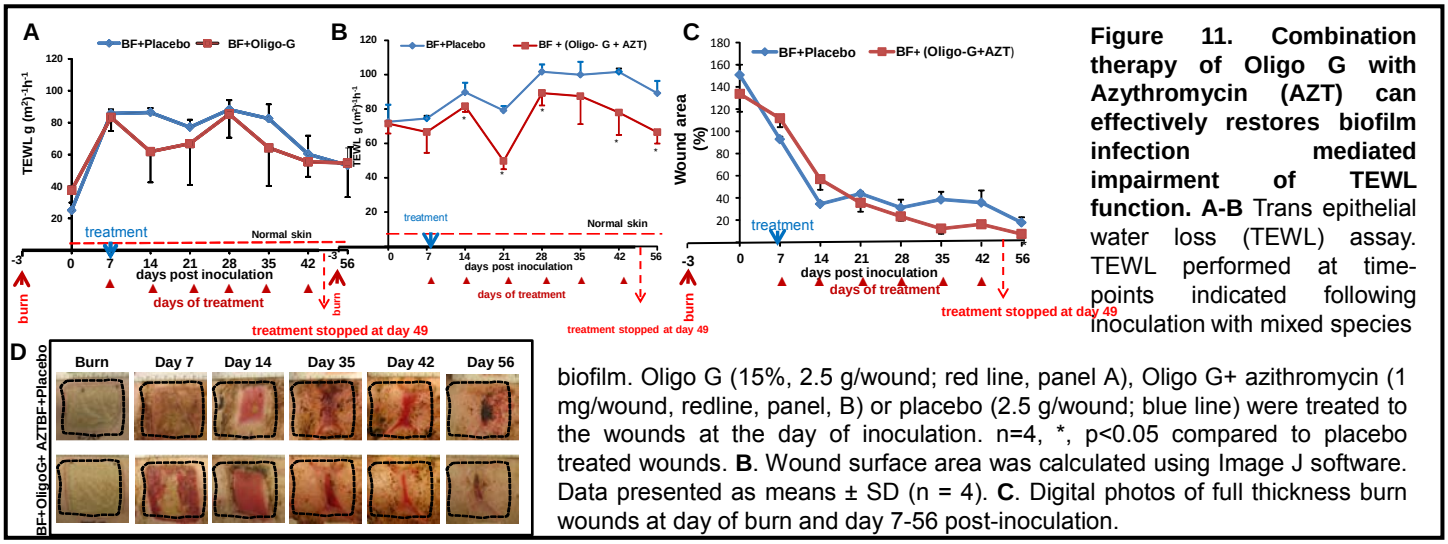


**Figure 11. Combination therapy of Oligo G with Azythromycin (AZT) can effectively restores biofilm infection mediated impairment of TEWL function. A-B** Trans epithelial water loss (TEWL) assay. TEWL performed at time-points indicated following inoculation with mixed species biofilm. Oligo G (15%, 2.5 g/wound; red line, panel A), Oligo G+ azithromycin (1 mg/wound, redline, panel, B) or placebo (2.5 g/wound; blue line) were treated to the wounds at the day of inoculation. n=4, *, p<0.05 compared to placebo treated wounds. **B**. Wound surface area was calculated using Image J software. Data presented as means ± SD (n = 4). **C**. Digital photos of full thickness burn wounds at day of burn and day 7-56 post-inoculation.

Azithromycin together with OligoG (15% salve formulation) or placebo was applied to the burn wounds at day 7 after the biofilm had been established. Azithromycin and OligoG or placebo treatment was reapplied thereafter every 7 days up to day 49. This combination treatment of Oligo G with Azithromycin showed a significant improvement in restoration of the barrier function as measured by TEWL when compared to the placebo (**Figure 11B**). In addition there appeared to be some minor improvement in wound closure (**Figure 11C**).

### *2.3 Histological and molecular biological characterization of the efficacy of treatment modalities.*

Based on the primary TEWL outcome, the efficacy of OligoG in preventing wound biofilm formation was investigated by SEM and immunohistochemistry/ CLSM. In addition a range of inflammatory

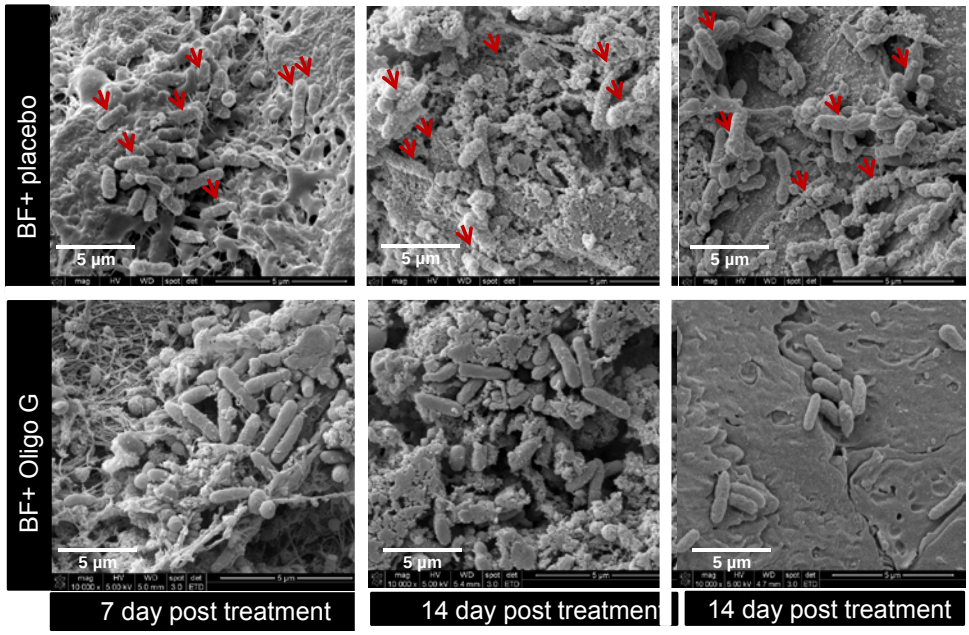


**Figure 12. Scanning electron microscopy (SEM) images showing dispersion of biofilm matrix in mixed biofilm infected wounds treated with OligoG at the time of bacterial inoculation.** SEM images of biopsies at days 7, 14 and 35 post inoculation from wounds treated with Oligo G or placebo at the time of wound inoculation. Oligo-G treated wounds show less abundance of biofilm matrix and decreased number of bacteria embedded in extra polymeric substance (EPS) compared to placebo treated ones.

markers were evaluated by expression analysis in homogenates of wound tissue biopsies.

SEM imaging of the wound surface showed clear attachment of microbial aggregates. The presence of these aggregates were markedly reduced after 14 days treatment with OligoG alone. Moreover the

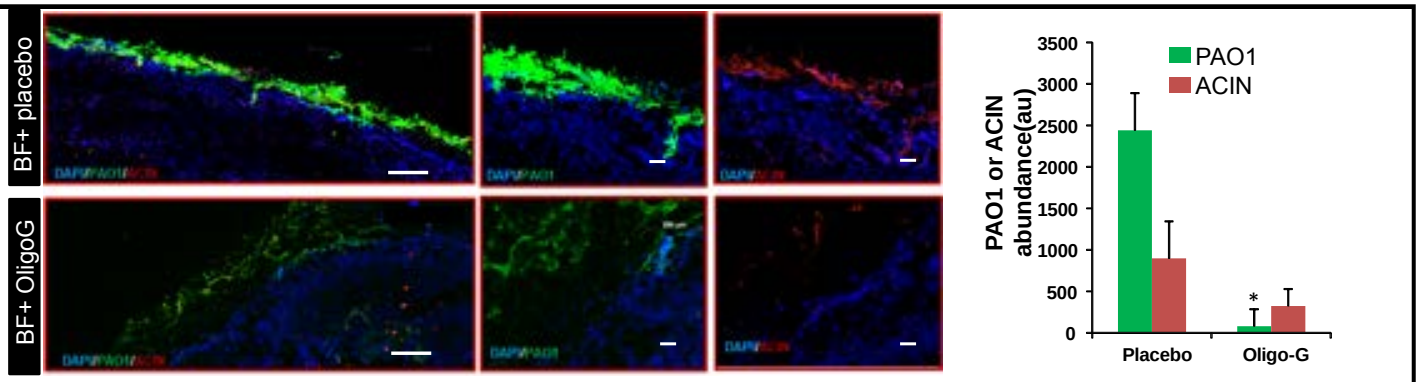


**Figure 13. Immuno-histochemical staining of Pseudomonas and Acenitobacter in biofilm infected burn wounds treated with Oligo-G or placebo in pre-clinical porcine model.** Representative images of biopsies from wound treated with OligoG at the time of bacterial inoculation on day 3 post burn. Images show a significant reduction in Pseudomonas abundance in Oligo-G treated wounds compared to placebo treated wounds. Bacteria stained with anti-Pseudomonas (PAO, green) and anti-Acenitobacter (ACIN, red). Data represented as means ±SD (n=3). *p =0.06 compared to placebo treated wounds.

presence of EPS, indicative of biofilm formation, was also far less abundant when compared with the placebo control. The phenotypic character of the microbial cell surface showed a less granular appearance after OligoG treatment alone, which suggests a direct action on the bacteria in preventing the formation and/or eradicating the presence of biofilm in burn wounds (**Figure 12**).

Immunohistochemical (IHC) staining using anti-*Pseudomonas* and anti-*Acinetobacter* antibodies confirmed the SEM findings: The same biofilm wounds (as demonstrated by SEM) showed heavy staining with both fluorescent labeled antibodies. However, there was a statistically significant reduction in biofilm formation in those wounds treated with OligoG alone (**Figure 13**).

Protein and mRNA expression analysis was performed on tissue biopsies for IL-1beta and Toll-like receptor 4 (TLR4) expression at the wound site. Interleukin IL-1 is a

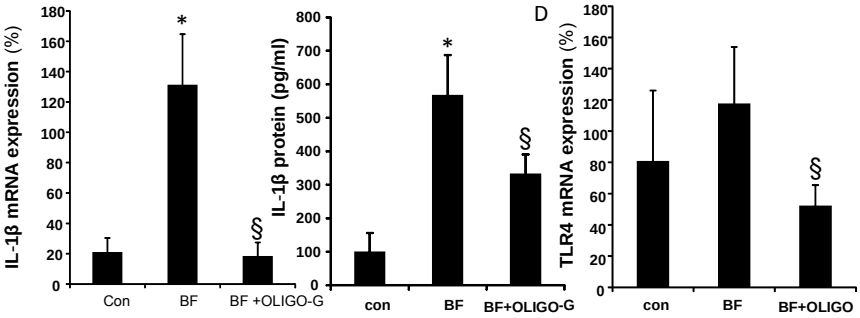


**Figure 14. Oligo G prevents biofilm infection mediated persistent inflammation in porcine burn wounds**. Wound tissues were collected on day 7 after inoculation and treatment with Oligo G or placebo. Non-infected wounds were used as controls (con). RNA or protein were extracted. The levels of IL-1β and TLR-4 mRNA was determined using real-time PCR. The protein levels for IL-1β in wound tissue was determined using ELISA. Expression of mRNA was normalized to β-2 Globulin. All data represented as mean ±SD (n=3). *, p<0.05 compared to control wounds , §, p<0.05 compared to BF infected wounds. Con, control, BF, biofilm.

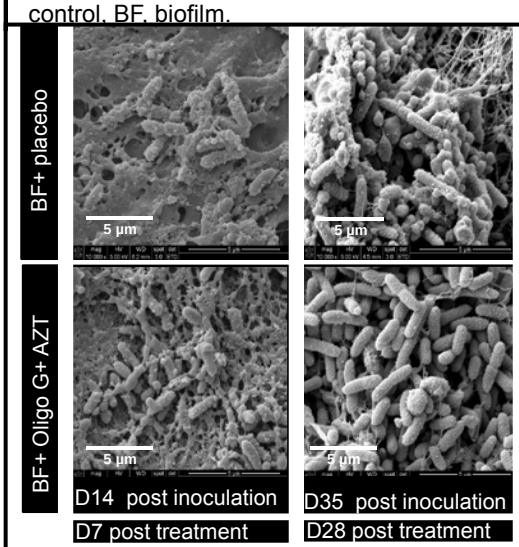


**Figure 15. Scanning electron microscopy (SEM) images showing dispersion of biofilm matrix in mixed biofilm infected wounds treated with OligoG + azithromycin 7d after establishment of biofilm.** SEM images of biopsies at days 14 and 35 post inoculation that corresponds to days 7 and 28 post therapy, respectively. The wounds treated with combination therapy show less abundance of biofilm matrix and decreased number of bacteria embedded in extra polymeric substance (EPS) compared to placebo treated ones.BF, biofilm; AZT, azithromycin.

key player in the regulation of inflammatory processes [36] and an increase in IL-1beta mRNA and protein has previously been observed in wounds infected with biofilms. TLR4 is involved in the activation of the innate immune system, particularly in response to gram-negative bacteria and plays an important role in pathogen recognition.

Both the protein and mRNA expression data showed significantly reduced levels of IL-1beta and TLR4 expression at the wound site for OligoG treated samples compared to placebo [**Figure 14**] These findings are consistent with the hypothesis that OligoG is actively reducing the microbial burden at the wound site, which in turn reflects a reduction in inflammation leading to wound closure and healing. SEM images of those wounds treated with combination therapy appeared to show less abundance of biofilm matrix and decreased number of bacteria embedded in the extra polymeric substance (EPS) compared to placebo treated wounds (**Figure 15**). The SEM imaging data was verified using immunohistochemical/CLSM approach. The data show that wounds treated with combination therapy is effective in decreasing overall bacterial load in well-established mixed biofilm infected wounds (**Figure 16**). This data further supports parallel findings of OligoG potentiating activity in eradicating *Pseudomonas aeruginosa* biofilm infections in mouse lungs [34].

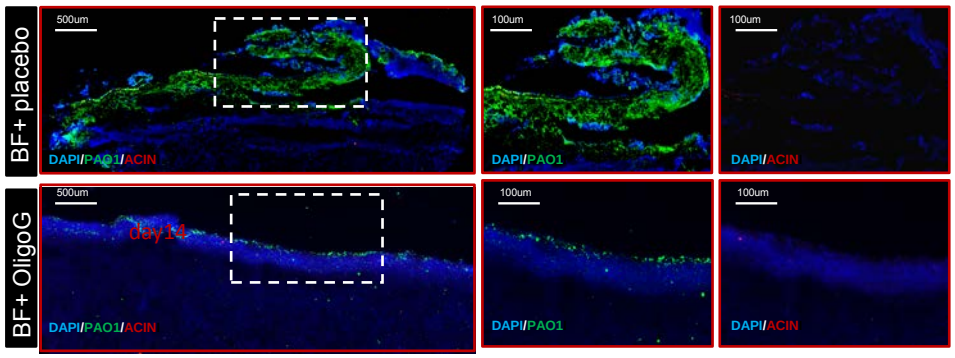


**Figure 16. Immuno-histochemical staining of Pseudomonas and Acenitobacter in biofilm infected burn wounds treated with combination of Oligo-G +azythromycin or placebo in pre-clinical porcine model.** Representative images of biopsies from wound treated with OligoG + Azithromycin treatment on 7 days post after bacterial inoculation (established biofilm. Day 14 post inoculation ( day 7 post treatment) Images show a significant reduction in Pseudomonas abundance in wounds treated with Oligo-G and antibiotics compared to placebo treated wounds. Bacteria stained with anti-Pseudomonas (PAO, green) and anti-Acinetobacter (ACIN, red). Data represented as means ±SD (n=3). *p<0.05 compared to placebo treated wounds.

## KEY RESEARCH ACCOMPLISHMENTS

- Developed a custom device for producing standardized uniform burn injuries on porcine skin.
- Establishment and characterization of a robust and reliable porcine burn wound biofilm infection model.
- Establishment of the first pre-clinical large animal multi-species biofilm burn injury model to facilitate testing of therapeutics.
- A novel anti-Acinetobacter monoclonal antibody has been developed. This reagent will enable detection Acinetobacter in infected wounds including military wounds.
- Demonstration of ineffectiveness of debridement or anti-microbial silver containing dressing in eradication of biofilm from infected wounds.
- Novel observation that biofilm infection results in impaired restoration of barrier function of epidermis (shown using TEWL assay) providing evidence that biofilm infection impairs wound healing.
- The novel anti-microbial agent OligoG has been shown to have efficacy in disrupting and preventing multispecies biofilm in a relevant *in vivo* model.

## REPORTABLE OUTCOMES

1. ***Manuscripts, abstracts, presentations***

**Manuscripts in preparation**.

1. S. Roy, H. Elgharably, M. Sinha, K. Ganesh, Sarah Chaney, C. Miller, S. Khanna, G. M. Gordillo, V. Bergdall, D. Wozniak, C.K. Sen. Host response disruption of epidermal Barrier function by mixed infection biofilm in a preclinical model of full thickness thermal injury. *Manuscript in preparation.*

2. K. Ganesh, S. Roy, M. Sinha, C. Miller, Sarah Chaney, V. Bergdall, D. Wozniak, P. Rye, E. Onsoyen, C.K. Sen Prevention and disruption of multispecies biofilm formation and improved healing outcome using OligoG in a reproducible porcine burn wound model. *Manuscript in preparation*.

**Poster and invited oral presentations.**

1. Elgharably, H., Chaney, S., Mann, E., Roy, S., Dickerson, J., Powell, H., Gordillo, G., Bergdall, V., Wozniak, D., Sen, CK. **Multispecies Biofilm Impairs Wound Closure in a Full Thickness Swine Burn Model.** Presented at: 23nd Annual Meeting of the Wound Healing Society; Denver, CO (2013, May)-**Invited talk**
2. Elgharably, H., Chaney, S., Mann, E., Roy, S., Dickerson, J., Powell, H., Gordillo, G., Bergdall, V., Wozniak, D., Sen, CK. **Development of a Pre-clinical Model to Study Biofilm in Full-thickness Cutaneous Swine Burn Wound**. Presented at: 22nd Annual Meeting of the Wound Healing Society; Atlanta, GA **(2012, April, oral presentation)**

**Poster Presentation**

2. Christina Miller, Haytham Elgharably, MD, Kasturi Ganesh, MD, *Ethan Mann, PhD, Sashwati Roy, PhD, *Danial Wozniak, PhD, Chandan K. Sen, PhD **(March 2013). First preclinical model for a multispecies biofilm in chronic wound infections.** Poster presented at: 6th Annual Translational to Clinical (T2C)Regenerative Medicine Wound Care Conference; Columbus, OH.

3. Kasturi Ganesh Barki, MD ,Haytham Elgharably, MD, Sashwati Roy, PhD, Sarah Chaney, DVM, *Ethan Mann, PhD, , Jennifer Dickerson RVT, Heather Powell, PhD, Gayle Gordillo, MD, Valerie Bergdall, DVM, Kareem H Mohammed, MD, Christina Miller RVT, *Daniel Wozniak, PhD, Chandan K. Sen, PhD**.(October 2012) Development of a Preclinical Model to Study Biofilm in Full Thickness Cutaneous Swine Burn Wound.** Poster presented at: Davis Heart and Lung Research Institute Annual Research Day, Columbus, OH.

4. Elgharably, H., Chaney, S., Mann, E., Roy, S., Dickerson, J., Powell, H., Gordillo, G., Bergdall, V., Wozniak, D., Sen, CK (2012, March). **Development of a Pre-clinical Model to Study Biofilm in Full Thickness Burn Wound.** Poster presented at: 5th Annual Translational to Clinical (T2C) Regenerative Medicine Wound Care Conference; Columbus, OH.

5. S.Roy1, K. Ganesh1, C. Miller1, S. Chaney, H. Elgharably, V. Bergdall, D. Wozniak, P. Rye, E. Onsoyen and C. K. Sen1 Prevention and disruption of multispecies biofilm formation and improved healing outcome using OligoG in a reproducible porcine burn wound model. Abstract submission to MHSRS, August 2013 (Fort Lauderdale)

6. Late breaking abstract planned for ICAAC, September 2013 (Denver)

- ***Development of cell lines, tissue or serum repositories***

7. Development of antibody specific against Acinetobacter.
   - ***Informatics such as databases and animal models, etc***

Establishment and characterization of a pre-clinical large animal multi-species biofilm burn injury model to facilitate the study of specific interventions.

- ***Employment or research opportunities applied for and/or received based on experience/training supported by this award***

The studies were integral component for post-doctoral training of Dr. Haytham Elgharably, MD, Dr. Kasturi Ganesh, MD and Dr. Mithun Sinha, PhD.

## CONCLUSION

The porcine model established in this study represents a unique, robust and reproducible tool in investigation of future therapeutic strategies in the treatment and management of biofilm infected wounds. Using this model, the findings have clearly established that OligoG therapy alone is effective in preventing biofilm formation. Imaging studies also showed that wounds treated with OligoG exhibited a marked disruption in bacterial biofilm in burn wounds. Furthermore, OligoG treatment also significantly improved healing by an improved restoration of the barrier function at the burn site as determined by TEWL. OligoG was also effective in disrupting established biofilms when used in combination with Azithromycin in this study.

These studies indicate that OligoG when used topically at the time of burn could provide an effective therapeutic strategy in preventing microbial and subsequent biofilm colonization of battlefield wounds, and facilitating wound healing. A spray formulation, based on the aqueous preparation already used in current human cystic fibrosis phase II trials, is being developed for potential application in a Role 2/Role 3 hospital setting. This represents a novel anti-microbial therapeutic which could have significant impact on wound management and recovery from surgical trauma. These findings warrant future support for clinical evaluation to test the efficacy of the novel sodium alginate oligomer, OligoG, in humans.

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**APPENDIX**.

**Supplemental images**

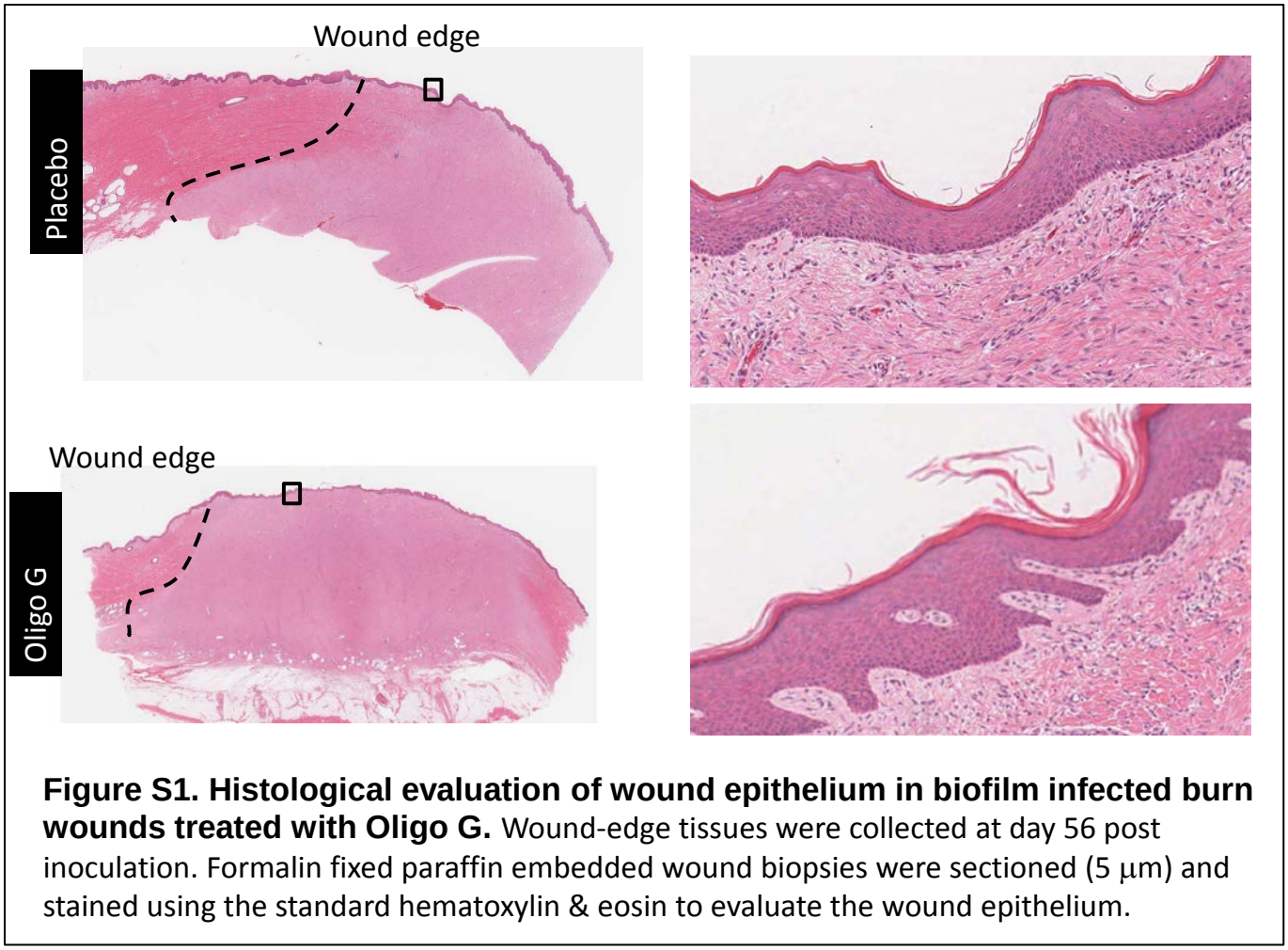


**Figure S1. Histological evaluation of wound epithelium in biofilm infected burn wounds treated with Oligo G.** Wound-edge tissues were collected at day 56 post inoculation. Formalin fixed paraffin embedded wound biopsies were sectioned (5 μm) and stained using the standard hematoxylin & eosin to evaluate the wound epithelium.

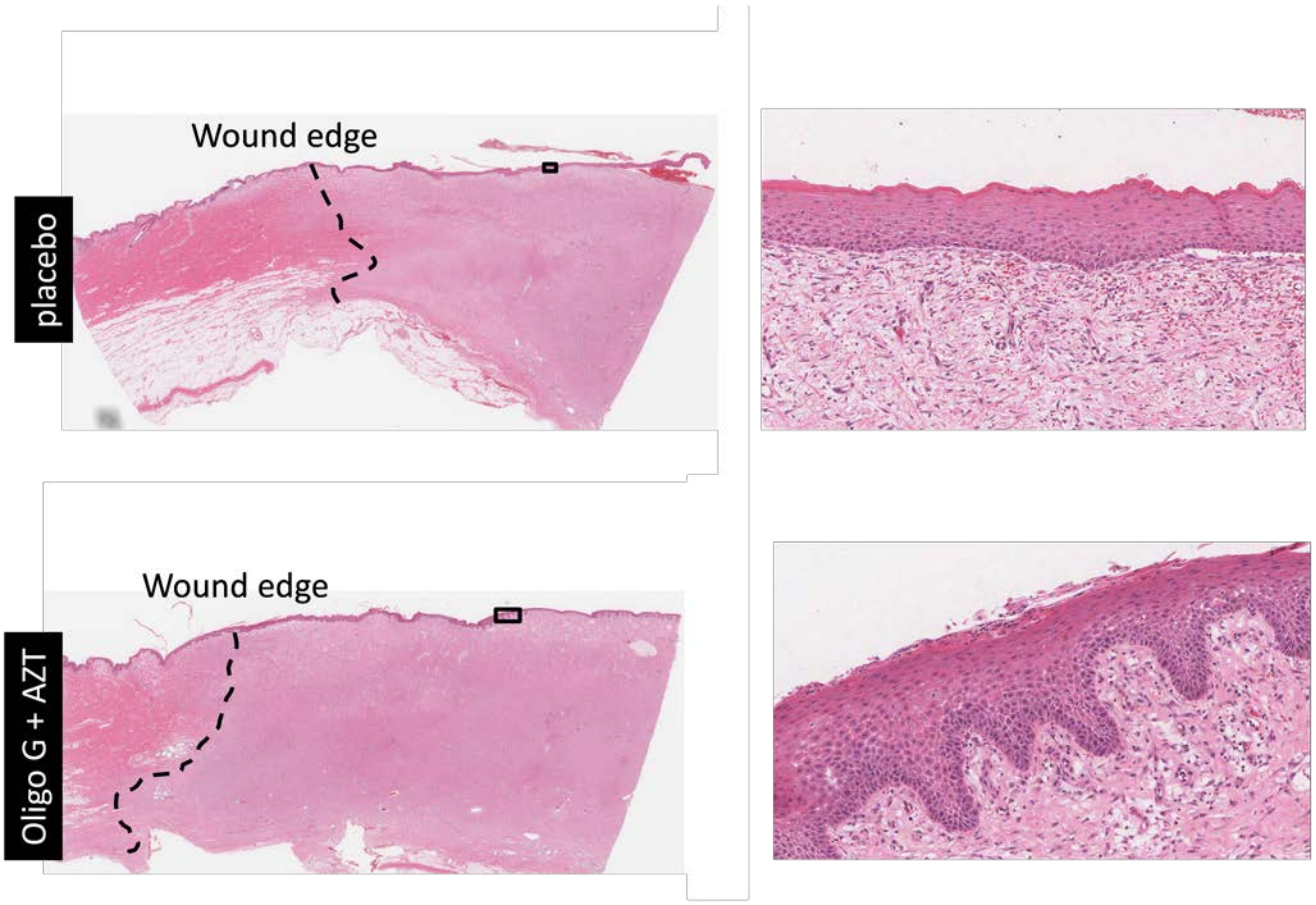


**Figure S2. Histological evaluation of wound epithelium in biofilm infected burn wounds treated with a combination therapy of Oligo G and Azithromycin**. Wound edge tissue was collected at day 56 post inoculation. Formalin fixed paraffin embedded wound biopsies were sectioned and stained using standard hematoxylin & Eosin to evaluate wound epithelium.

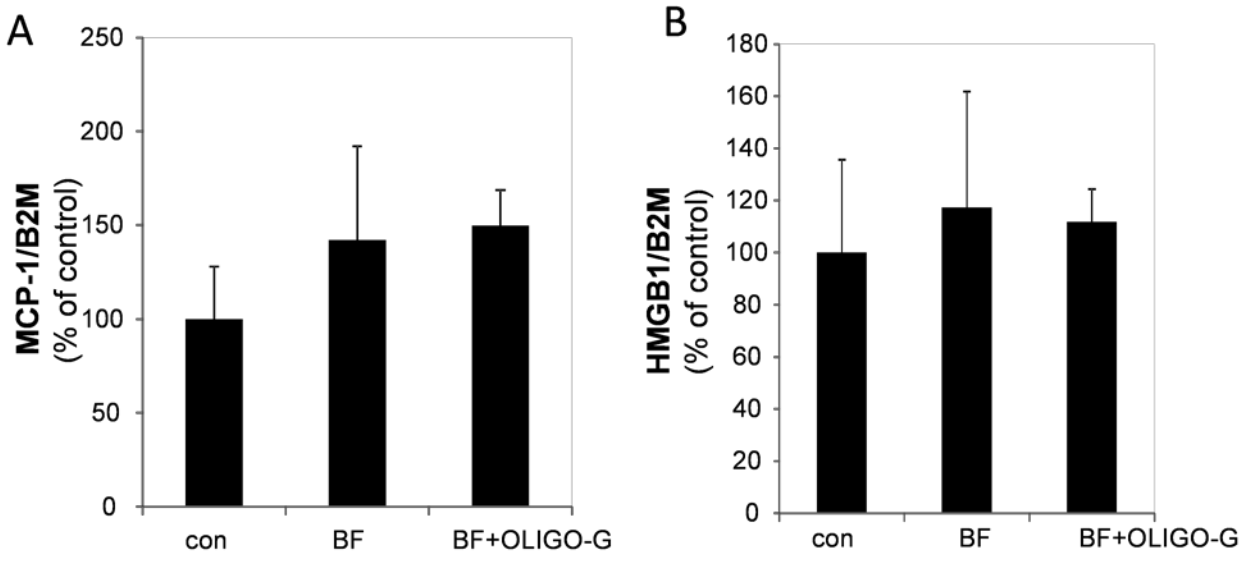


**Figure S3. Efect of Oligo G on genes associated with wound inflammation**. Wound tissues were collected on day 7 after inoculation and treatment with Oligo G or placebo. Non-infected wounds were used as controls (con). RNA were extracted. The levels of MCP-1 and HMGB1 mRNA was determined using real-time PCR. Expression of mRNA was normalized to β-2 Globulin. All data represented as mean ±SD (n=3).