

Biofilm–Host Immune Crosstalk at the Diabetic Foot Ulcer Interface: Molecular Mechanisms, Immune Evasion, and Next-Generation Anti-Biofilm Strategies

Yu Han¹, Ye Yang²

¹Department of Vascular and Thyroid Surgery, The First Hospital of China Medical University, Shenyang, Liaoning Province, 110001, People's Republic of China; ²Department of Rehabilitation, The First Hospital of China Medical University, Shenyang, Liaoning Province, 110001, People's Republic of China

Correspondence: Ye Yang, Department of Rehabilitation, The First Hospital of China Medical University, No. 155 Nanjing North Street, Heping District, Shenyang, Liaoning Province, People's Republic of China, Email yangyeyy1996@163.com

Abstract: Diabetic foot ulcers (DFUs) are chronic wounds in which microbial persistence and defective host defense interact to impair healing. This review examines DFU through the biofilm–host immune interface rather than viewing biofilm as a purely microbiological problem. We summarize how the diabetic wound milieu, including hyperglycemia, impaired perfusion, neuropathy, and polymicrobial community structure, favors persistent biofilm infection, and how DFU-relevant biofilms evade clearance through matrix shielding, altered innate recognition, virulence-associated host modulation, and intracellular *Staphylococcus aureus* persistence. We further highlight two major immune-dysregulation axes: excessive neutrophil extracellular trap formation with NLRP3-centered inflammatory amplification, and perforin-2 suppression linked to AIM2-mediated pyroptotic injury. We also appraise emerging immune-aware antibiofilm strategies, particularly quorum-sensing interference, enzymatic matrix disruption, phage therapy, and selected immune-directed interventions. Overall, current evidence supports a model in which non-healing DFU reflects failed host–pathogen resolution at the biofilm–immune interface, with important implications for mechanism-guided therapeutic development.

Keywords: diabetic foot ulcer, biofilm, innate immunity, immune evasion, neutrophil extracellular traps, pyroptosis, quorum sensing, phage therapy, inflammasome

Introduction

Diabetes mellitus remains a major global public health challenge, and diabetic foot ulcer (DFU) is one of its most serious complications. Approximately 19% to 34% of individuals with diabetes will develop a foot ulcer during their lifetime, and these ulcers precede most diabetes-related lower-extremity amputations and are a major contributor to non-traumatic limb loss.^{1,2} People with a diabetic foot ulcer have an approximately 30% 5-year mortality, rising to more than 70% after major amputation. The direct costs of DFU care in the United States alone are estimated at \$9–13 billion annually, underscoring the substantial economic burden associated with hospitalization, limb-threatening infection, and amputation-related care.¹

Diabetic foot infections are commonly polymicrobial, with *Staphylococcus aureus* remaining a frequent pathogen, but community composition varies substantially with wound depth, chronicity, and host context.^{3,4} Importantly, wound microorganisms are often organized, at least in part, within biofilm-structured communities, a mode of growth biologically linked to persistence, impaired clearance, and delayed healing in chronic wounds.^{5,6}

Clinically, microbial colonization, overt infection, and biofilm presence in DFU should not be regarded as interchangeable states.^{4,7} Chronic wounds often harbor complex microbial communities without fulfilling criteria for clinical infection, and detection of biofilm reflects an organized mode of microbial persistence rather than, by itself, definitive



evidence of clinically relevant pathogenic activity.^{6,7} The relevance of the biofilm–host immune interface therefore lies in clarifying when persistent host–microbe interactions shift a wound toward non-resolving inflammation, tissue injury, and impaired healing.⁸

The impact of biofilms on DFU chronicity therefore extends far beyond simple antimicrobial resistance. Biofilms actively engage the host immune system through a sophisticated repertoire of immune evasion strategies, subverting rather than merely resisting host defenses. The extracellular polymeric substance (EPS) matrix physically restricts leukocyte penetration, while biofilm-associated virulence factors directly modulate immune cell function.⁸ Recent work has shown that intracellular *S. aureus* can accumulate within DFU keratinocytes due to suppression of the antimicrobial effector molecule perforin-2, triggering absent in melanoma 2 (AIM2) inflammasome-mediated pyroptosis and contributing to prolonged inflammation and impaired healing.⁹ In parallel, excessive neutrophil extracellular traps (NET) formation has emerged as a pathologic feature of diabetic wounds and DFUs, with experimental and translational studies linking dysregulated NET responses to impaired healing and persistent inflammation. Mechanistic work further suggests that NET-derived extracellular DNA and associated mediators can reinforce NOD-like receptor family pyrin domain containing 3 (NLRP3) inflammasome activation, establishing a feed-forward inflammatory loop.^{10–12} However, the extent to which NET material is structurally incorporated into mature DFU biofilms in vivo remains incompletely defined.

Although the importance of biofilm in DFU has been increasingly recognized, most DFU-focused reviews have primarily emphasized microbial composition, biofilm formation, diagnosis, clinical implications, or antibiofilm management. Immune-centered discussion does exist, but is often brief within DFU reviews or is developed in broader chronic-wound reviews rather than in a DFU-specific framework. Accordingly, a DFU-focused synthesis that integrates biofilm immune evasion, dysregulated host responses, and immune-informed therapeutic strategies still appears limited.^{4,8} This review addresses that gap by asking four related questions: how the diabetic wound microenvironment promotes persistent biofilm infection; how DFU-relevant biofilms evade host clearance; which immune-dysregulation pathways most strongly sustain non-resolving inflammation and impaired healing; and how these mechanisms may inform next-generation antibiofilm and immune-aware therapeutic strategies. Framed in this way, the biofilm–host immune interface is clinically relevant not only for explaining chronic wound persistence, but also for clarifying why some DFUs fail to resolve despite standard care and where mechanism-guided intervention may be most effective.

The Diabetic Wound Microenvironment: A Permissive Niche for Biofilm Establishment

Hyperglycemia as a Driver of Bacterial Persistence and Virulence

Among the metabolic abnormalities of the diabetic wound microenvironment, hyperglycemia is more firmly supported as an amplifier of staphylococcal persistence and virulence than as a definitively proven direct inducer of biofilm formation in human DFU tissue. In diabetic skin-infection models, excess glucose enhances *agr*-dependent virulence output and worsens infection severity, while hyperglycemia has also been linked to increased aureolysin-associated protease activity. Taken together, these findings support a mechanistic framework in which the diabetic milieu not only weakens host defense but also augments pathogen programs relevant to persistence; however, direct confirmation that glucose alone drives clinically meaningful biofilm expansion in human DFU remains limited.^{13,14}

Microbial Heterogeneity, Functional Pathogroups, and Polymicrobial Interactions

Culture-independent profiling shows that DFU microbiomes are heterogeneous rather than compositionally fixed. Although *S. aureus* is commonly detected, deeper, longer-duration, or non-healing wounds often show greater diversity, including increased representation of anaerobes and Proteobacteria.^{3,15} Longitudinal work further suggests that non-healing wounds may differ not only in composition but also in strain-level variation and biofilm- or virulence-associated functional signatures.^{15,16} Importantly, these observations support a functional rather than purely taxonomic view of DFU pathogenicity: wound behavior is shaped not simply by the presence of individual species, but by the collective properties of microbial communities.

Within this framework, polymicrobial interactions can further reshape persistence and treatment response, although they should not be assumed to be uniformly synergistic across all DFU communities. The best-supported example is *S. aureus*–*P. aeruginosa* coexistence, which in experimental wound-related systems can drive persistence phenotypes such as small-colony-variant-like states and increased tolerance to eradication.^{17,18} For the present review, the main importance of polymicrobiality is therefore not that every interspecies interaction is mechanistically central, but that community-level interactions can complicate clearance and help sustain immune-dysregulated biofilm states.

Vascular and Neuropathic Contributions to Biofilm Persistence

These microbial-community features operate within a host environment that already favors persistence. Beyond metabolic dysregulation, vascular insufficiency and neuropathy create a poorly perfused, repeatedly traumatized, and immunologically disadvantaged wound bed. Reduced oxygen delivery, impaired clearance, and recurrent tissue injury together increase the likelihood that biofilm persistence becomes chronic rather than transient.^{2,19} In this sense, DFU chronicity reflects not only microbial complexity, but also a wound microenvironment that repeatedly fails to eliminate and resolve biofilm-associated infection.

Biofilm-Mediated Immune Evasion: Molecular Mechanisms at the Host-Pathogen Interface

The capacity of biofilms to persist in DFU wounds despite the presence of a functional (albeit impaired) immune system reflects a sophisticated array of immune evasion mechanisms that operate at multiple levels. These mechanisms can be conceptualized as a multi-layered defense system, with each layer targeting distinct components of the host immune response.

The EPS Matrix as a Physical and Chemical Barrier

The EPS matrix constitutes the first barrier to effective immune access. By organizing bacteria within a hydrated three-dimensional scaffold, the matrix spatially restricts efficient phagocyte engagement and favors persistent, low-efficiency inflammatory confrontation rather than sterilizing clearance. The biological consequence is sustained leukocyte activation with collateral oxidative and proteolytic tissue injury, helping to stabilize a non-resolving wound environment.^{6,20}

The physical barrier function of EPS is complemented by chemical properties that further diminish antimicrobial efficacy. The EPS matrix can retard antibiotic penetration in a drug- and matrix-dependent manner rather than uniformly blocking diffusion.^{21,22} For positively charged antibiotics such as aminoglycosides, extracellular DNA and other anionic matrix components can sequester the drug and thereby further delay effective delivery to biofilm-embedded cells.²³ Moreover, oxygen and nutrient gradients generate metabolically heterogeneous biofilm subpopulations, including slow-growing or dormant cells, thereby reducing susceptibility to antibiotics that preferentially target active bacterial processes.^{22,24}

Pathogen-Associated Molecular Pattern Masking and Pattern Recognition Receptor Evasion

Beyond physical restriction of immune cell access, biofilms employ a subtler strategy of immune evasion by masking pathogen-associated molecular patterns (PAMP) that would otherwise activate pattern recognition receptors (PRR) on host immune cells. Biofilm growth can attenuate innate immune recognition of canonical PRR ligands by both shielding surface-exposed microbial structures within the extracellular matrix and altering the expression of selected immunostimulatory determinants; in *P. aeruginosa* biofilms, for example, classical PAMPs are less exposed to the immune system and flagellar expression may be reduced.²⁵ In chronic wounds, the biological consequence is not immune silence but persistent, low-grade inflammatory activation coupled to inefficient clearance. Accordingly, the biofilm–host interface is better understood as a state of dysregulated innate sensing in which ongoing microbial and host-derived danger signaling sustains non-resolving inflammation without generating effective microbicidal eradication.⁸

In parallel with PAMP masking, *S. aureus* biofilms also deploy humoral immune-evasion strategies that further reduce opsonophagocytic clearance, even though these mechanisms are conceptually distinct from direct PRR escape. By binding the Fc region of IgG and interfering with IgG hexamer formation, SpA reduces C1q recruitment, complement activation, and opsonophagocytic killing.²⁶ SpA can also be released from the bacterial surface into the extracellular milieu.²⁷ However, direct evidence specifically establishing this as a DFU biofilm-matrix mechanism remains limited. Accordingly, this pathway is better interpreted as humoral immune evasion than as direct PRR escape.²⁶

Virulence Factors as Immune Modulators

In parallel with physical shielding and PAMP masking, biofilm-associated bacteria continue to express and secrete virulence factors that actively modulate host immune function. In *S. aureus*, these include alpha-toxin, a pore-forming cytotoxin capable of activating NLRP3 inflammasome signaling in experimental infection models;²⁸ phenol-soluble modulins (PSM), a family of amphipathic peptides with neutrophil-lytic and biofilm-remodeling activities;²⁹ and surface adhesins such as ClfA, ClfB, and SdrC, which mediate attachment to host proteins and participate in biofilm organization.³⁰

A critical finding with direct relevance to DFU is that the hyperglycemic wound environment can amplify bacterial virulence programs rather than merely weaken host defense. Elevated glucose has been shown to increase *S. aureus* aureolysin expression and activity; because aureolysin sits upstream of the extracellular protease cascade that includes SspA/V8 protease, these data are consistent with enhanced protease-mediated tissue injury under hyperglycemic conditions.¹³ More broadly, diabetic skin-infection models show that excess glucose enhances *agr*-dependent virulence output and worsens infection severity.¹⁴ Together, these findings support a feed-forward pathobiological loop in which metabolic dysregulation simultaneously weakens host defense and amplifies the virulence program of wound-colonizing *S. aureus*.

P. aeruginosa adds further immune-disruptive pressure to polymicrobial DFU biofilms through secreted effectors, quorum-linked host-active signals, and redox-active metabolites that impair host-cell function.^{31,32} In the polymicrobial setting, these mechanisms likely broaden tissue-damaging and immune-modulatory pressure, although their integrated effect in human DFU remains insufficiently quantified.⁶

Perforin-2 Suppression and Intracellular *Staphylococcus aureus* Accumulation

A DFU-specific study by Pastar et al showed intracellular accumulation of *S. aureus* within the epidermis of clinically uninfected DFUs, together with marked suppression of perforin-2 (P-2/MPEG1), a pore-forming innate effector involved in intracellular antibacterial defense. Using qPCR, single-cell analyses, immunohistochemistry, and confocal microscopy, the study further showed substantially reduced P-2 expression in DFU tissue compared with location-matched non-ulcerated plantar foot-skin controls.⁹

Functionally, P-2 suppression is consistent with impaired intracellular clearance of internalized *S. aureus*, thereby favoring persistence of an epidermal intracellular reservoir. Rather than being categorically inaccessible to therapy, intracellular *S. aureus* is better regarded as a state associated with reduced susceptibility to antibiotic killing and enhanced persistence, which may contribute to recurrence and delayed resolution.³³ This interpretation is supported by P-2-deficient mice, in which epicutaneous *S. aureus* challenge was associated with impaired bacterial control and enhanced dissemination, consistent with a critical role for P-2 in cutaneous intracellular antibacterial defense.⁹

The AIM2 Inflammasome–Caspase-1–Gasdermin D Pyroptotic Cascade

The intracellular accumulation of *S. aureus* associated with P-2 suppression is linked to activation of AIM2 inflammasome signaling rather than remaining immunologically silent. AIM2 is a cytosolic dsDNA sensor that recruits ASC and activates caspase-1, leading to IL-1 β maturation; downstream, caspase-1 cleaves gasdermin D (GSDMD), and the liberated N-terminal fragment oligomerizes in the plasma membrane to form pores that execute pyroptosis.^{34,35}

GSDMD pore formation drives pyroptotic membrane permeabilization and release of intracellular contents, thereby amplifying local inflammatory injury. In a four-week longitudinal clinical study, Pastar et al showed that AIM2 levels, ASC pyroptosome assembly, and cleaved GSDMD persisted or increased in nonhealing DFUs, whereas these pyroptosis-related readouts decreased during healing.⁹ These data support pyroptosis as a DFU-relevant mechanism linking

Biofilm–host immune crosstalk in diabetic foot ulcers

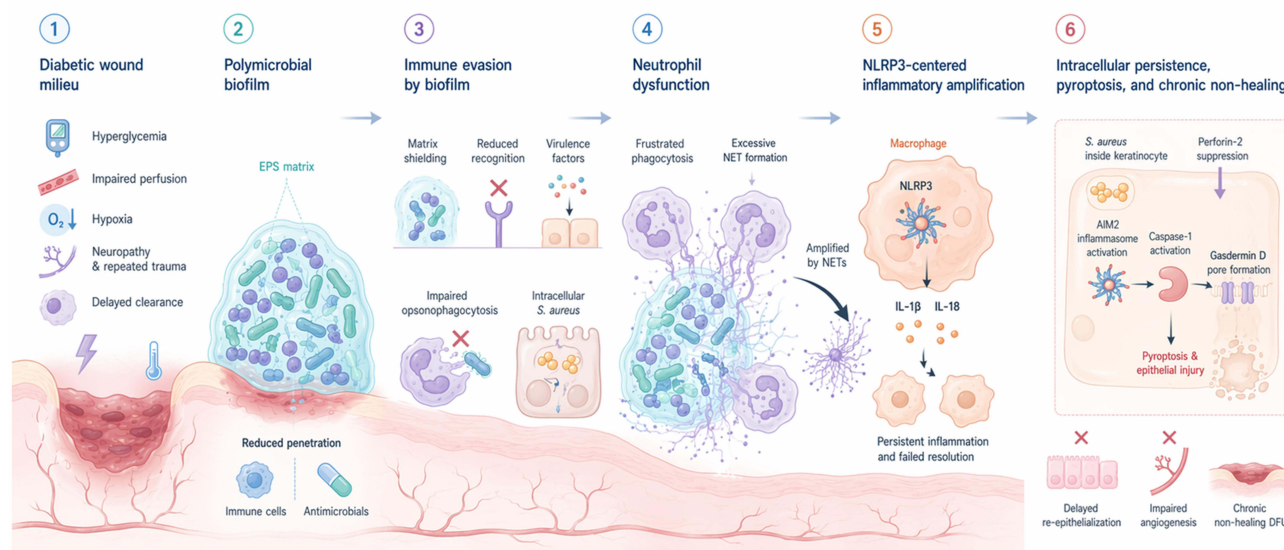


Figure 1 Biofilm–host immune crosstalk in diabetic foot ulcers. The diabetic wound milieu, characterized by hyperglycemia, impaired perfusion, hypoxia, neuropathy, repeated trauma, and delayed clearance, favors establishment of polymicrobial biofilms. Biofilm structure and virulence programs promote matrix shielding, reduced innate recognition, impaired opsonophagocytosis, neutrophil dysfunction, and excessive neutrophil extracellular trap (NET) formation. NET-associated signals amplify macrophage NLRP3 activation and IL-1 β /IL-18-centered inflammatory responses. In parallel, intracellular *Staphylococcus aureus* persistence in keratinocytes, together with perforin-2 suppression, is linked to AIM2 inflammasome activation, caspase-1 signaling, gasdermin D pore formation, pyroptotic injury, and chronic non-healing. Overall, biofilm persistence and dysregulated host responses reinforce one another to sustain delayed re-epithelialization, impaired angiogenesis, and chronic diabetic foot ulceration.

intracellular *S. aureus* persistence to unresolved inflammation and impaired healing. Released intracellular contents from pyroptotic cells may further amplify local inflammatory signaling; however, a fully autonomous feed-forward loop independent of ongoing biofilm infection has not been directly demonstrated in human DFU tissue.

Collectively, these findings identify the P-2–AIM2–GSDMD axis as a DFU-relevant pathway linking intracellular *S. aureus* persistence with unresolved inflammation and impaired healing, while also suggesting—at a hypothesis-generating level—that restoration of P-2 activity or modulation of pyroptotic signaling may merit future therapeutic investigation.

Taken together, these immune-evasion strategies do not merely permit microbial persistence; they also reshape the host response into a chronically activated yet poorly resolving inflammatory state (Figure 1).

Host Innate Immune Responses to Biofilm and Their Dysregulation in DFU Neutrophils: From First Responders to Inadvertent Accomplices

Neutrophils are among the earliest immune cells recruited to the wound bed and are central to early antimicrobial defense. In biofilm-associated DFU, however, neutrophil responses become functionally dysregulated and can contribute to persistent inflammation, tissue injury, and wound chronicity.⁸

The systemic effects of diabetes on neutrophil function are well documented. Overall, the balance of evidence supports reduced neutrophil chemotactic responsiveness and impaired phagocytic handling in diabetes, although these defects are not uniform across all assays and stimuli. In parallel, intracellular reactive oxygen species (ROS) generation required for efficient microbicidal killing is frequently diminished, whereas extracellular ROS release and other pro-inflammatory neutrophil outputs may be preserved or increased.³⁶ Within diabetic wounds, these abnormalities are superimposed on a hypoxic and persistently inflammatory microenvironment that further hinders effective bacterial clearance.⁸

When neutrophils encounter biofilm aggregates, effective phagocytic clearance is limited, and sustained extracellular release of oxidants and proteases contributes more to tissue injury than to bacterial eradication.

Neutrophil Extracellular Traps: Defense Mechanism Turned Pathological Amplifier

Among the dysregulated neutrophil responses implicated at the DFU biofilm–host interface, one of the most consequential is the formation of NETs. NETs are extracellular chromatin structures decorated with antimicrobial proteins—including neutrophil elastase, myeloperoxidase, and cathelicidin (LL-37)—released by activated neutrophils. Although often discussed in the context of NETosis, NET release should not be framed as being restricted to a single cell-death program.³⁷ NETs can immobilize microbes, but their effects are highly context-dependent; in experimental *Staphylococcus aureus* biofilms, biofilm growth can actively skew neutrophils toward NET formation while remaining poorly cleared by NET-mediated antimicrobial activity.³⁸

Experimental evidence supports a pathological contribution of excessive NET formation to impaired diabetic wound repair. Wong et al showed that diabetes primes neutrophils for PAD4-dependent NET formation and that genetic or enzymatic disruption of NETs accelerates healing in diabetic wound models.¹⁰ Extending these observations to human DFU, Yang et al reported that circulating NET markers were increased in patients with DFU relative to diabetic patients without DFU and healthy controls, correlated positively with the Diabetic Ulcer Severity Score (DUSS) and Wound, Ischemia, and foot Infection (WIFI) severity indices, and identified citrullinated histone H3 (citH3) as a marker associated with impaired 12-month healing and higher amputation risk; patients in the highest citH3 quartile had significantly worse healing outcomes than those in the lower three quartiles.³⁹ A complementary translational study by Sawaya et al further identified the FOXM1–TREM1 network as a regulator of ROS-dependent NET formation in DFU and showed that triggering receptor expressed on myeloid cells 1 (TREM1) expression correlated with clinical healing outcomes, reinforcing the clinical relevance of dysregulated neutrophil/NET biology in non-healing ulcers.¹¹ Collectively, current human data support an association between increased NET burden and adverse DFU outcomes, but the available clinical evidence remains limited and should not be overinterpreted as establishing causality.

Mechanistic studies further suggest that NETs can impair repair programs beyond microbial trapping. In experimental diabetic wound systems, NET exposure has been linked to endothelial dysfunction, impaired angiogenic responses, and delayed closure, providing one plausible route by which excessive NETosis may worsen healing.⁴⁰

The NLRP3 Inflammasome–NET Feed-Forward Loop

A mechanistically important aspect of NET biology in diabetic wounds is the amplification loop between excessive NET formation and macrophage NLRP3 inflammasome activation. This loop appears to be superimposed on a broader diabetic wound environment in which macrophage inflammasome activity is already persistently elevated.⁴¹ Liu et al showed that NETs generated in diabetic wounds can provide both a priming signal, through TLR-4/TLR-9/NF- κ B-dependent upregulation of NLRP3 and pro-IL-1 β , and an activation signal, through ROS-dependent promotion of TXNIP–NLRP3 association in macrophages.¹² This mechanism was associated with enhanced IL-1 β release and sustained inflammatory injury; however, its causal weight in biofilm-positive human DFUs has not yet been directly quantified.

Huang et al further used MFG-E8 as an experimental modulator of this axis. In *Mfge8*-deficient diabetic mice, wounds showed enhanced NET deposition, increased NLRP3 inflammasome activity with higher IL-1 β /IL-18 output, impaired angiogenesis, and delayed closure.⁴² Recombinant MFG-E8 suppressed IL-18-primed NETosis and dampened NET/mCRAMP-induced inflammasome activation in macrophages; the authors further linked this effect to an integrin β 3-restricted P2X7 signaling mechanism.⁴² These data support MFG-E8 as an endogenous negative regulator of the NLRP3–NET loop in experimental diabetic wound healing, rather than establishing it as a validated therapeutic target in human DFU.

In the same experimental line, DNase I-mediated NET digestion reduced NLRP3 inflammasome activation, modulated inflammatory-cell infiltration, and accelerated healing in a diabetic rat wound model. This finding supports proof-of-concept interruption of the NET–inflammasome axis, but remains preclinical.¹² Taken together, current evidence supports the NLRP3–NET axis as a plausible inflammatory amplification loop in diabetic wounds and DFU-related experimental systems. Its quantitative contribution specifically in biofilm-positive human DFUs, however, remains to be defined by direct clinical studies.

At the biofilm interface, NETs also behave paradoxically: they may spatially constrain microbial spread yet still fail to eradicate established biofilm communities. In experimental systems, this interface is associated more with persistent inflammatory injury than with sterilizing clearance, whereas evidence that NET-derived DNA is routinely incorporated into mature DFU biofilms *in vivo* remains limited.³⁸

Macrophage Polarization Failure and Biofilm Persistence

Macrophages play a pivotal role in coordinating the transition from inflammation to tissue repair during wound healing; operationally, this is often summarized as a shift from an early inflammatory M1-like program toward pro-resolving and pro-reparative M2-like states. In diabetic wounds, this transition is impaired, contributing to persistent inflammation and defective repair.^{43,44}

In the setting of biofilm-associated infection, persistent microbial stimulation is more defensibly viewed as reinforcing a non-resolving inflammatory macrophage program and delaying timely transition toward reparative states; however, current high-quality evidence in DFU does not justify specifying NF- κ B/STAT1 dominance or EPS-mediated loss of scavenger- or mannose-receptor engagement as established mechanisms of macrophage polarization failure.⁸ The resulting macrophage compartment is better described as persistently inflammatory and poorly pro-resolving, with increased expression of cytokines such as IL-1 β , TNF- α , and IL-6 and reduced expression of healing-associated mediators including IL-10, IGF-1, and TGF- β . This non-resolving phenotype is consistent with sustained macrophage inflammasome activity and IL-1 β -centered inflammatory circuitry documented in diabetic human and murine wounds.^{41,44}

Furthermore, persistent NLRP3 inflammasome activity in wound macrophages represents an additional mechanism that may reinforce non-resolving inflammation in diabetic wounds. Available evidence supports associations with sustained IL-1 β -centered inflammatory signaling, impaired healing, and experimental amplification by NET-derived stimuli; however, whether NLRP3 operates in biofilm-positive DFU as a discrete checkpoint governing the transition from inflammatory to reparative macrophage states remains unresolved.^{12,41}

Quorum Sensing as a Regulatory Bridge at the Biofilm–Immune Interface

Quorum sensing (QS) is best understood here as a regulatory layer rather than a stand-alone endpoint. In DFU-relevant pathogens, QS coordinates biofilm maturation, dispersal, and virulence deployment; in *P. aeruginosa* this network is classically organized around *las*, *rhl*, and *PQS* signaling, whereas in *S. aureus* the *agr* system regulates the shift from surface-associated colonization toward secreted toxins, proteases, and phenol-soluble modulins.^{45,46}

The key relevance of QS at the DFU biofilm–immune interface is that it links microbial coordination to host-active outputs. Experimental studies indicate that *S. aureus agr* signaling can suppress keratinocyte repair-associated programs and delay wound healing, while selected *P. aeruginosa* QS molecules can engage mammalian sensing pathways and reshape inflammatory or barrier responses. Direct DFU clinical validation, however, remains limited. Accordingly, QS should be viewed as a biologically plausible bridge between biofilm behavior and host dysfunction, whereas its therapeutic exploitation is considered separately below.

Next-Generation Anti-Biofilm Strategies: From Immune Evasion Disruption to Immune Restoration

The growing recognition that biofilm-mediated immune evasion, together with biofilm-associated antimicrobial tolerance and broader host–microbe dysregulation, is a major driver of DFU chronicity supports a reorientation of therapeutic strategy beyond conventional bactericidal approaches alone. Accordingly, next-generation approaches increasingly target the biofilm–immune interface, aiming either to weaken biofilm-associated immune evasion and structural protection or to restore effective host clearance and wound repair.⁶ The principal pathological axes discussed in this review and the major mechanism-guided therapeutic strategies are summarized in [Figure 2](#).

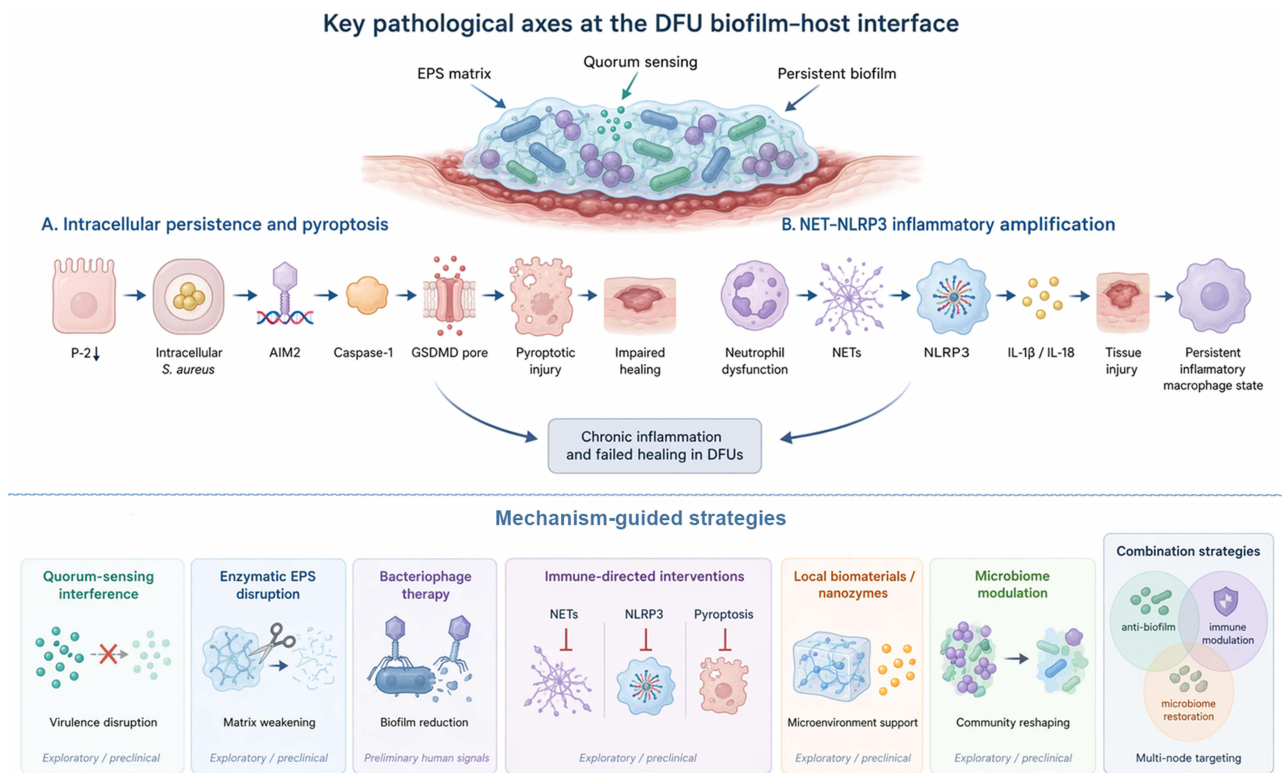


Figure 2 Key pathological axes at the DFU biofilm–host interface and mechanism-guided strategies. The upper panel summarizes two major innate immune pathobiological axes in diabetic foot ulcers: intracellular *Staphylococcus aureus* persistence with AIM2–caspase-1–gasdermin D-mediated pyroptosis, and NET-associated NLRP3 inflammatory amplification that sustains tissue injury and a persistent inflammatory macrophage state, converging on chronic inflammation and failed healing. The lower panel maps emerging intervention concepts to these mechanisms, including quorum-sensing interference, enzymatic EPS disruption, bacteriophage therapy, immune-directed interventions, local biomaterials/nanozymes, microbiome modulation, and multi-node combination strategies. Most approaches remain exploratory or preclinical, whereas bacteriophage therapy currently provides the most tangible preliminary human signal.

Quorum Sensing Inhibitors

Quorum sensing inhibitors (QSI) represent a conceptually attractive anti-virulence strategy because they disrupt communication networks that regulate virulence expression and biofilm-associated phenotypes without necessarily relying on direct bactericidal activity. Mechanistically, QS interference can be achieved through inhibition of signal synthesis, enzymatic degradation of signal molecules, antagonism of signal receptors, or interruption of downstream quorum-responsive transcriptional programs.⁴⁷ By attenuating QS-regulated phenotypes, these agents may enhance biofilm vulnerability to host clearance⁴⁸ or to concomitant antibiotics in selected experimental systems,⁴⁹ but such effects remain context-dependent and cannot yet be generalized across pathogens or infection settings.

Selected QS-disruption strategies have shown preclinical efficacy in wound-related infection models, but the evidence remains heterogeneous and largely non-DFU. At present, the strongest rationale for QS interference in DFU is mechanistic rather than clinical: quorum-sensing systems coordinate virulence deployment, biofilm behavior, and selected host-active outputs, making them plausible adjunctive targets rather than established therapies.^{31,45–47,50} Taken together, current data support QS disruption as a biologically plausible strategy for weakening persistence, but not yet as a clinically validated DFU therapy.

Despite these encouraging preclinical findings, translation of QSIs to DFU remains challenging. Because DFU biofilms are polymicrobial and may involve heterogeneous quorum-signaling architectures—single-pathway inhibition may provide incomplete coverage unless paired with species-matched or combination strategies.^{45–47}

Enzymatic EPS Disruption Strategies

Direct enzymatic disruption of the EPS matrix remains one of the most mechanistically coherent strategies for converting an immune-excluded biofilm into an exposed, therapeutically accessible structure. DNase I targets extracellular DNA (eDNA), a widespread biofilm-matrix component whose degradation can destabilize biofilms and increase antimicrobial susceptibility.^{24,51} Dispersin B hydrolyzes PNAG/PIA and is therefore best framed as a composition-matched strategy for PNAG/PIA-rich staphylococcal biofilms rather than a universal enzyme for all staphylococcal matrices.⁵² Rather than functioning primarily as stand-alone bactericidal agents, matrix-degrading enzymes are better viewed as adjunctive biofilm-disruption tools. Current evidence most strongly supports their use in combination regimens, in which matrix weakening is paired with antibiotics or antiseptics to increase access to previously shielded bacteria, whereas direct restoration of host immune clearance remains less firmly established.^{24,53,54}

Bacteriophage Therapy

Bacteriophage (phage) therapy is an investigational antibiofilm strategy with strong biological plausibility and a small but growing DFU-specific human evidence base; however, the available clinical data remain limited in scale and are insufficient to establish efficacy.^{55,56} Phages are bacterial viruses that selectively infect susceptible bacterial hosts. Their proposed relevance to biofilm infection lies in self-amplification where target bacteria are present, the capacity of some phages or phage-derived enzymes to disrupt matrix-associated barriers, and antibacterial activity that is mechanistically distinct from conventional antibiotics; however, activity remains isolate-specific and depends on phage susceptibility testing rather than being uniformly preserved across all antibiotic-resistant strains.^{56,57}

Across preclinical studies and early translational reports, phage therapy in DFU has mainly focused on pathogen-matched treatment and phage–antibiotic combination strategies designed to broaden coverage and reduce resistance escape; however, superiority over optimized single-phage or standard-care regimens has not been established in DFU patients.^{55–57}

Among peer-reviewed human datasets in DFU, the randomized double-blind TP-102 study is currently the most informative: 19 participants with infected or non-infected DFUs were randomized after susceptibility testing, 13 received TP-102, no treatment-related adverse events were reported, and microbiological reduction and wound-closure trends numerically favored TP-102; however, the study was explicitly underpowered to establish superiority.⁵⁵ Accordingly, phage therapy in DFU is best regarded at present as a promising investigational adjunct rather than an established treatment modality, and further progress will require larger efficacy-defining trials together with standardized susceptibility testing, manufacturing, and delivery workflows.⁵⁸

From an immunological perspective, phage-mediated reduction of bacterial burden and biofilm biomass could, in principle, lessen biofilm-driven inflammatory stimulation and thereby improve conditions for host clearance; however, this remains inferential rather than directly demonstrated in DFU patients.⁵⁶ Current human DFU studies have assessed safety, microbiology, and healing outcomes rather than directly demonstrating reversal of NET- or inflammasome-centered inflammatory circuits; therefore, immune re-engagement should be framed as a mechanistically plausible but unproven translational hypothesis.^{55,56}

Exploratory Local Adjunctive Platforms

Nanozyme-based platforms, peptide-enabled local biomaterials, and other natural-product-derived local approaches remain exploratory adjunctive strategies at the biofilm–immune interface.⁵⁹ Across preclinical diabetic wound models, these systems have shown combinations of antibacterial, antibiofilm, ROS-modulating, and wound-repair-promoting effects; however, DFU-specific human evidence remains limited, and none can yet be regarded as an established therapy.^{60,61} Their current significance is therefore conceptual rather than practice-defining: they suggest that future local treatment platforms may need to combine bacterial control with microenvironmental or immune modulation. At present, these approaches are better viewed as future-oriented components of combination therapy than as standalone evidence-based DFU treatments.

Immune-Directed Interventions: Targeting NETosis and Pyroptosis

Given the increasingly recognized pathological roles of excessive NET formation and pyroptosis in diabetic wounds and DFU-related experimental systems, direct targeting of these processes represents a mechanistically rational therapeutic strategy. Several agents have shown preclinical proof-of-concept activity, although human efficacy data remain limited.

Disulfiram, a drug originally developed for alcohol use disorder, has been repurposed as a GSDMD-targeting agent; in DFU-related experimental systems, it suppressed NET formation and accelerated wound healing by inhibiting the NLRP3/caspase-1/GSDMD axis.⁶² In parallel, PAD4 inhibition—either pharmacologic or genetic—has reduced NET burden and improved diabetic wound healing in preclinical models.^{10,63}

Recombinant MFG-E8, as discussed earlier, functioned as an experimental negative regulator of the NLRP3–NET inflammatory loop and promoted angiogenesis and wound closure in diabetic mice. In that study, it suppressed IL-18-primed NETosis and attenuated NET/mCRAMP-induced inflammasome activation in macrophages.⁴²

DNase I represents a mechanistically straightforward NET-directed intervention. By digesting extracellular NET material, DNase I attenuated NET-driven NLRP3 inflammasome activation, modulated inflammatory-cell infiltration, and accelerated healing in diabetic wound models.¹² Its therapeutic rationale lies in interrupting the NET–inflammasome amplification loop; notably, this rationale does not depend on assuming that NET-derived DNA is routinely incorporated into mature DFU biofilms *in vivo*, a point that remains unresolved.

Microbiome Modulation and Probiotic Approaches

Microbiome-directed intervention remains an exploratory but mechanistically appealing direction in DFU. Its rationale arises from growing recognition that wound behavior is shaped by community-level microbial functions rather than by single taxa alone, suggesting that future interventions may seek to reshape local ecology rather than merely suppress individual pathogens.^{6,15} At present, however, therapeutic evidence for probiotics or related microbiome-modulating strategies in DFU remains preclinical, heterogeneous, and insufficient to support efficacy claims in patients.

Methodological Advances in Studying the Biofilm–Immune Interface

Three-Dimensional Host–Biofilm Models

The study of biofilm–immune interactions has historically been limited by the reductionism of conventional *in vitro* models, which fail to recapitulate the three-dimensional architecture, cellular heterogeneity, and microenvironmental complexity of DFU wounds. Recent advances in three-dimensional tissue engineering have begun to address this limitation. Three-dimensional wound models are valuable not simply because they are more complex than conventional culture systems, but because they create an experimental bridge between microbial architecture and host cell state. Their greatest future value will likely lie in combining biofilm challenge with single-cell and spatial readouts, enabling direct mapping of how specific microbial structures correspond to epithelial stress states and, in future immune-competent versions, to macrophage polarization states and zones of failed repair.

Current three-dimensional epidermal biofilm platforms are best understood as tissue-engineered host–biofilm models: within a stratified human epidermal architecture, they can incorporate wounded-skin context and defined biofilm challenge, thereby enabling host–microbe interactions that are not accessible in conventional monolayer culture. More importantly, a human epidermis organoid model has been shown to support structured methicillin-resistant *Staphylococcus aureus* (MRSA) and *P. aeruginosa* biofilms while retaining measurable epithelial host readouts, including cytotoxicity and pro-inflammatory cytokine readouts in wounded-skin settings.⁶⁴ In an MRSA-biofilm setting, transcriptomic analysis further showed impaired barrier function, extracellular-matrix remodelling, and activation of IL-17-, IL-12-family, and IL-6-family-associated inflammatory programs, making this platform experimentally tractable for linking biofilm architecture to epithelial injury and response to antibiofilm candidates.⁶⁵ The next methodological step will be to extend current epidermal biofilm models toward immune-cell incorporation, spatially resolved host-response profiling, and patient-derived materials, rather than treating them simply as higher-complexity static culture systems.

Shotgun Metagenomics, Metatranscriptomics, and Spatially Resolved Profiling

While 16S rRNA amplicon sequencing has underpinned much of DFU microbiome characterization, it is primarily suited to inference of community composition and does not directly resolve strain-level gene content or transcriptional activity.⁷ By contrast, shotgun metagenomic sequencing extends DFU analysis from community composition to species- or strain-resolved functional potential, enabling direct profiling of antibiotic-resistance determinants together with biofilm- and virulence-associated gene repertoires from wound specimens.¹⁶ When further integrated with metatranscriptomic or dual-RNA-seq data, this framework moves beyond gene presence toward gene activity, helping prioritize transcriptionally active microbial populations and host–microbe response programs over taxa detected only at the DNA level. In DFU, however, such approaches are better viewed as emerging tools for biological stratification than as definitive clinical discriminators between colonization and pathogenicity.⁶⁶

Spatially resolved platforms such as spatial transcriptomics and imaging mass cytometry are especially promising for dissecting the biofilm–immune interface. Spatial transcriptomics localizes wound-region-specific transcriptional states, whereas imaging mass cytometry resolves multiplex immune and stromal phenotypes together with their cellular neighborhoods at single-cell resolution. In DFU, spatial transcriptomics has already begun to identify region-specific fibroblast and macrophage states, but published work has thus far emphasized host tissue architecture rather than direct spatial co-mapping of microbial biofilm organization.⁶⁷ Experience from other inflammatory skin disorders further suggests that these technologies can uncover disease-relevant cellular neighborhoods and signaling circuits that remain obscured in dissociative sequencing datasets.⁶⁸

Challenges, Knowledge Gaps, and Future Directions

The Translational Gap: From *in vitro* Models to Clinical Complexity

Despite the considerable advances reviewed herein, a substantial translational gap persists between mechanistic insights obtained *in vitro* or in animal models and their application to clinical DFU management. *In vitro* biofilm models, even sophisticated three-dimensional systems, cannot fully recapitulate the polymicrobial complexity, oxygen gradients, immune cell dynamics, and chronic metabolic derangements of the human DFU wound.⁶⁹ Commonly used diabetic wound animal models reproduce impaired healing and selected metabolic abnormalities, but no current preclinical model fully captures the combined neuropathic, vascular, infectious, immunological, and chronic features of human DFU.⁷⁰ These limitations constrain the predictive validity of preclinical findings and underscore the need for clinical validation of mechanistic hypotheses. Recent DFU-focused literature further suggests that this translational challenge also includes the practical identification and management of wound biofilm in routine care.⁷¹ A recent review highlighted persistent limitations in clinically identifying biofilm in diabetic foot infections, whereas subsequent randomized clinical work has begun to evaluate biofilm-detection-based wound management in DFU.⁷² Together, these developments reinforce the need to connect mechanistic insight with clinically feasible diagnostic and therapeutic frameworks.

The Challenge of Polymicrobial Complexity

The polymicrobial nature of DFU biofilms represents a formidable challenge for targeted therapeutic strategies. Much of the mechanistic literature and preclinical therapeutic testing still relies on simplified systems centered on dominant pathogens such as *S. aureus* or *P. aeruginosa*, whereas clinical DFU wounds harbor more heterogeneous microbial communities and interspecies interactions.¹⁶ Cooperative and competitive interactions within polymicrobial biofilms can reshape collective phenotypes—including virulence, persistence, and treatment tolerance—such that findings from monospecies systems should not be assumed to translate directly to polymicrobial DFU biofilms.⁴ Future research must prioritize the development and validation of therapeutics in polymicrobial biofilm models that more faithfully represent clinical DFU microbiology.

Longitudinal, Stratified, and Clinically Actionable Human Studies

The current human evidence base remains largely cross-sectional, whereas the biofilm–immune interface is dynamic and patient-specific. Future progress depends on longitudinal serial-sampling studies that integrate microbial composition and function with host inflammatory phenotypes, NET- and inflammasome-related readouts, treatment exposure, and standardized healing outcomes within the same patient.^{7,16,66} This approach is needed not only to support biologically

informed stratification, but also to identify clinically actionable windows for intervention and to determine which patients may plausibly benefit from precision antibiofilm strategies. Fungi, the wound virome, and adaptive immune pathways—including T-cell- and antibody-mediated responses—remain important but less mature lines of investigation, and their contribution to the broader biofilm–immune landscape in DFU requires more direct clarification.

Priority Research Directions

Based on the synthesis presented in this review, several research directions merit priority attention. First, the P-2 suppression–intracellular *Staphylococcus aureus*–AIM2–GSDMD axis identified by Pastar et al represents a high-priority target for mechanistic validation and therapeutic exploration. However, specific approaches to restore P-2 expression or activity in DFU keratinocytes remain unvalidated, and any therapeutic benefit should currently be regarded as hypothesis-generating rather than established.⁹ Second, because biofilm persistence is multifactorial, multi-targeted combination strategies that simultaneously weaken matrix protection, reduce bacterial burden, and temper maladaptive inflammation are biologically plausible and warrant systematic evaluation in polymicrobial and immune-relevant preclinical models. Third, the field needs validated biomarker panels that capture both microbial organization and host inflammatory state. Candidate platforms may include integrated host–microbe transcriptomic signatures and microbiologically informed molecular profiling, but no biomarker framework is yet established for DFU biofilm stratification. Fourth, future clinical trials of phage therapy and other biofilm-directed interventions in DFU should clearly distinguish early safety/tolerability studies from efficacy-defining trials and should incorporate standardized microbiologic or biofilm-centered endpoints; inclusion of mechanistically informative immune-related secondary outcomes would further strengthen trial interpretation. Recent systematic synthesis of the field likewise indicates that evidence on DFU biofilm prevalence, diagnostics, and treatment remains fragmented and that validated biofilm endpoints are still needed.⁷³ Fifth, applying single-cell RNA sequencing and spatial transcriptomics to microbiologically annotated, ideally biofilm-positive, DFU tissue could move the field beyond descriptive microbiology by defining spatially resolved host response states. This would support a more precise framework for classifying DFUs according to dominant inflammatory circuitry, reparative failure, and microbial organization. However, direct host–microbe spatial co-mapping in DFU remains limited.

Conclusion

Non-healing DFU is best understood as a state of failed host–pathogen resolution in which biofilm persistence and innate immune dysregulation reinforce one another. Rather than acting solely as a structural barrier to antibiotics, biofilm helps sustain a chronically inflammatory, poorly resolving wound environment through matrix-mediated immune exclusion, intracellular *Staphylococcus aureus* persistence linked to inflammasome activation, excessive NET/NLRP3-driven inflammatory amplification, and delayed transition toward reparative macrophage states. These interlocking processes help explain why microbial persistence and tissue injury can coexist over prolonged periods instead of progressing toward effective clearance and healing. Therapeutically, this framework supports a shift beyond purely bactericidal logic toward combination strategies that weaken biofilm protection while restoring effective immune resolution. At present, phage therapy provides one of the most tangible preliminary human signals, whereas most other approaches—including quorum-sensing interference, enzymatic matrix disruption, and immune-directed interventions—remain preclinical or hypothesis-generating. Future progress will depend on longitudinal human studies, polymicrobial and immune-relevant models, and biomarker-guided translational strategies that can more precisely link microbial organization with host inflammatory state.

Highlights

- Non-healing DFU reflects failed biofilm–host immune resolution.
- Direct DFU evidence centers on perforin-2 suppression and intracellular *Staphylococcus aureus* persistence.
- Excessive NET formation and inflammasome signaling amplify tissue injury.
- Phage therapy provides one of the most tangible preliminary human signals, whereas most other approaches remain preclinical.

Declaration of Generative AI Use

Authors declare no AI use during the preparation of this work.

Abbreviations

AHL, acyl-homoserine lactone; AIM2, absent in melanoma 2; AIP, autoinducing peptide; DUSS, Diabetic Ulcer Severity Score; DFU, diabetic foot ulcer; eDNA, extracellular DNA; EPS, extracellular polymeric substances; GSDMD, gasdermin D; MFG-E8, milk fat globule-EGF factor 8; MRSA, methicillin-resistant *Staphylococcus aureus*; NETs, neutrophil extracellular traps; NLRP3, NOD-like receptor family pyrin domain containing 3; P-2, perforin-2; PAD4, peptidylarginine deiminase 4; PAMPs, pathogen-associated molecular patterns; PIA, polysaccharide intercellular adhesin; PNAG, poly-N-acetylglucosamine; PRRs, pattern recognition receptors; PQS, Pseudomonas quinolone signal; QS, quorum sensing; QSIs, quorum sensing inhibitors; ROS, reactive oxygen species; TREM1, triggering receptor expressed on myeloid cells 1; TXNIP, thioredoxin-interacting protein; WIfI, Wound, Ischemia, and foot Infection.

Data Sharing Statement

The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author.

Author Contributions

Yu Han: Conceptualization; Formal analysis; Investigation; Writing – original draft.

Ye Yang: Conceptualization; Supervision; Validation; Writing – review and editing.

All authors gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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