COMPREHENSIVE INVITED REVIEW

Advanced Wound Diagnostics: Toward Transforming Wound Care into Precision Medicine

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Significance: Nonhealing wounds are an ever-growing global pandemic, with mortality rates and management costs exceeding many common cancers. Although our understanding of the molecular and cellular factors driving wound healing continues to grow, standards for diagnosing and evaluating wounds remain largely subjective and experiential, whereas therapeutic strategies fail to consistently achieve closure and clinicians are challenged to deliver individualized care protocols. There is a need to apply precision medicine practices to wound care by developing evidence-based approaches, which are predictive, prescriptive, and personalized.

Recent Advances: Recent developments in "advanced" wound diagnostics, namely biomarkers (proteases, acute phase reactants, volatile emissions, and more) and imaging systems (ultrasound, autofluorescence, spectral imaging, and optical coherence tomography), have begun to revolutionize our understanding of the molecular wound landscape and usher in a modern age of therapeutic strategies. Herein, biomarkers and imaging systems with the greatest evidence to support their potential clinical utility are reviewed.

Critical Issues: Although many potential biomarkers have been identified and several imaging systems have been or are being developed, more high-quality randomized controlled trials are necessary to elucidate the currently questionable role that these tools are playing in altering healing dynamics or predicting wound closure within the clinical setting.

Future Directions: The literature supports the need for the development of effective point-of-care wound assessment tools, such as a platform diagnostic array that is capable of measuring multiple biomarkers at once. These, along with advances in telemedicine, synthetic biology, and "smart" wearables, will pave the way for the transformation of wound care into a precision medicine.

Clinical Trial Registration number: NCT03148977.

Keywords: wound healing, diagnostics, biomarkers, imaging, smart dressings, synthetic biology

SCOPE AND SIGNIFICANCE

Nonhealing wounds are a global pandemic, with management costs and mortality rates exceeding those of many common cancers. Existing standards for the diagnosis and management of nonhealing wounds remain subjective and experiential,

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whereas available treatments fail to consistently achieve wound closure. There is a great need for advanced wound diagnostics capable of elucidating the nebulous microenvironment of nonhealing wounds; such tools would transform wound healing into a precision medicine by allowing clinicians to deliver personalized therapeutic regimens. The most promising biomarkers and imaging modalities with the potential to achieve this vision are reviewed here.

TRANSLATIONAL RELEVANCE

Although many possible wound-healing biomarkers have been identified, their translation into simple, cost-effective clinical assays remains challenging. Many biomarkers of interest, such as genes and transcription factors, are not easily quantifiable at the point-of-care. This highlights the need for a diagnostic platform tool that can quantitatively and simultaneously measure many biomarkers, for example, proteases, other small molecules, genes, and beyond. In addition, imaging systems for wounds are promising but their utility remains uncertain. Continued research on the cellular and biochemical mechanisms underlying wound healing will be invaluable to coalesce the role of imaging and biomarkers in the future of wound healing.

CLINICAL RELEVANCE

Nonhealing wounds are challenging for clinicians, owing to the high degree of difficulty, cost, and time required to adequately assess wound status. Such wounds often fail to respond to established standards of care and advanced therapies. Currently, available advanced wound diagnostic tools may be challenging to implement in a clinical setting due to unclear utility or incomplete validation. By herein outlining the strengths and limitations of currently available biomarkers and imaging systems, the authors provide clinicians with guidance for their clinical implementation while also highlighting important areas for future clinical research.

DISCUSSION

Nonhealing wounds: a clinical conundrum and call to action

Nonhealing wounds represent an immense and ever-growing global pandemic, with incidence and mortality rates exceeding those of many common cancers (Fig. 1).[1–10] Despite their staggering international cost burden (Table 1),[4,11–13] the "silent epidemic" of nonhealing wounds has historically suffered from lack of public awareness and policy support due to being overshadowed by other medical comorbidities such as diabetes and obesity.[1] Lack of formal wound care training in U.S. medical schools and the fact that no single medical specialty is responsible for wounds have also contributed to the underappreciation of nonhealing wounds as an international pandemic.[4]

Despite the fact that modern advances in wound research have increased our understanding of the molecular and cellular factors driving impaired wound healing, very little has been successfully translated into the clinical setting.[14] Standards for the diagnosis and stratification of wounds remain surprisingly subjective and experiential, sometimes lacking rigor or precision. Algorithms using clinical signs to detect wound characteristics, for example, "NERDS" for superficial infection (Nonhealing, Exudative, Red/bleeding surface, Debris [yellow/black necrotic tissue], Smell) and "STONEES" for deep infection (Size increase, Temperature increase, Os probe to/probe to bone, New or satellite areas of breakdown, Exudate/Erythema/Edema, Smell),[15] have been developed; whether or not such tools are accurate predictors of the characteristics they attempt to measure remains controversial,[16,17] and accordingly none have been widely adopted.

Available therapeutic strategies fail to consistently convert nonhealing wounds into those capable of closure, whereas practitioners remain unable to deliver individualized care.[14] The U.S. Wound Registry (USWR) suggests that healing rates exceeding 40% are not currently possible for nonhealing wounds in real-world settings.[18] Such phenomena are apparent across the continuum of wound care, whether in civilian settings or on the front lines of battle. It has been estimated that nearly 24% of modern combat deaths could be preventable with smart diagnostics for triage or to help guide the timing of wound closure.[19]

There remains a tremendous and ever-growing need to change subjective, qualitative, and often ineffective wound care into quantitative, evidence-based approaches that are predictive, prescriptive, and personalized. The establishment of objective global standards for wound assessment and the development of algorithms to determine which wounds will benefit from specific interventions will allow for the invention of individualized treatment strategies for those in need.[14]

Nonhealing wounds remain a significant management challenge owing to the high degree of difficulty, cost, and time required to adequately assess wound status, which is typically nonquantitative or

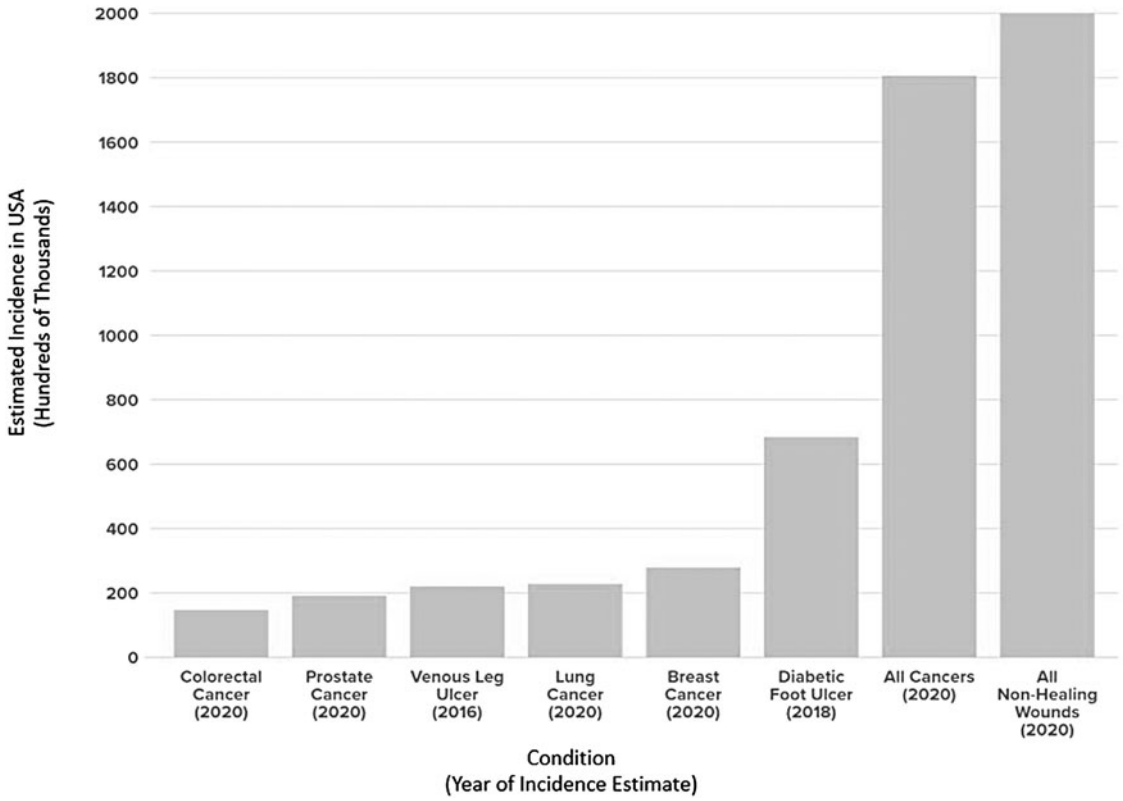

Figure 1. Estimated incidence of various subtypes of cancer versus nonhealing wounds in the United States. Some types of nonhealing wounds have higher incidence than some of the most common cancers, and nonhealing wounds overall are estimated to occur at a higher rate than cancer in general.[2,5–10]

merely experiential. Accurate appraisal of wounds requires years of specialized training and even so is limited by subjectivity and significant intra- and interobserver variability. Some indicators of wound status such as temperature, perfusion, and bacterial colonization are not readily appreciable to the naked eye. The slow delivery of lab reports and monitoring that requires multiple follow-up visits hinder the delivery of appropriate treatment.

Although there is no universally agreed-upon definition of a nonhealing wound, it is typically considered to be any wound which "fail[s] to proceed through an orderly and timely process to produce anatomic and functional integrity."[20] Wounds classified as recalcitrant, complex, chronic, hard-to-heal, or that result in amputation are included under the umbrella of nonhealing wounds.[21] Nonhealing wounds as discussed within the scope of this article will include primarily venous leg ulcers (VLUs), diabetic foot ulcers (DFUs), and pressure ulcers (PUs). Other acute wounds, for example, combat wounds will also be considered. Note that although these wounds are commonly considered together within the category of nonhealing wounds, their pathophysiology is different and this may affect the diagnostic and therapeutic modalities that are best suited for each.

Table 1. *Approximate annual cost of wound care by region*

Country/Region	*Estimated Cost of Nonhealing Wounds (Year)*
United States	US $28–98.6 billion (2014)[a,4]
United Kingdom	GBP £5 billion (2012)[11]
Australia	AU $2.85 billion (annually)[12]
Denmark	DKK 244 million (2020, projected)[13]

[a]Medicare alone.[4,11–13]

Nonhealing wounds suffer from a lack of consistent standards of care, with varying guidelines for classification, diagnosis, and management, and there are a few tools to aid practitioners with these tasks. There is no single objective system for wound evaluation or prognostic factors, and technical approaches vary from provider to provider.[14] Nonhealing wounds often fail to respond appropriately to established standards of care, and there is a paucity of reliable metrics to predict this response.[14] A multitude of advanced therapies are available (Table 2),[22–29] but these also achieve inconsistent results and there are no algorithms to determine which patients will benefit from which interventions or to aid in the development of personalized, hierarchical treatment schedules for a given patient.[14]

Table 2. *Overview of current approaches to major chronic wound subtypes*

Wound Type	*Healing Status Metric*	*Standard of Care*	*Select Advanced Treatments + Level of Evidence (or FDA Approval?)*
Venous leg ulcers	30% size reduction in 4 weeks[5]	• Compression[5] • Leg elevation[5]	• Oasis[24] • Apligraf[24] • Epifix[24] • Dermagraft (FDA)[26]
Diabetic foot ulcers	50% size reduction in 4 weeks[22]	• Offloading[25] • Vascular optimization[25] • Infection control[25]	• HBOT[a] • Becaplermin (FDA)[29] • Oasis[24] • Integra[24] • Apligraf[24] • Epifix[24] • Grafix[24] • Omnigraft (FDA)[26] • Dermagraft (FDA)[26] • EZ Derm (FDA)[26] • Allopatch (FDA)[26]
Pressure ulcers	PUSH Tool[27]: • Size • Exudate amount • Tissue type	• Off-loading[22] • Nutritional optimization[22] • Reduce moisture, shear, friction[22]	• EZ Derm (FDA)[26]

Tools for evaluating wound healing status remain subjective and experiential, and often contended. Despite established standards of care, and the existence of many advanced treatments for most wound subtypes, clinicians remain unable to consistently achieve wound closure. FDA—Product is FDA-approved for the stated indication.

[a]Evidence supporting the efficacy of HBOT in improving healing for diabetic foot ulcers remains conflicting. Alloderm—donated acellular human dermal allograft; Allopatch—donated acellular human dermal allograft; Apligraf—Bilayered living cellular construct; Dermagraft—cryopreserved cellular human fibroblast-derived dermal substitute; Epifix—dehydrated human amnion/chorion membrane; EZ Derm—porcine-derived dermal xenograft; Grafix—cryopreserved placental membrane; Oasis—porcine-derived acellular dermal matrix.

FDA, Food and Drug Administration; HBOT, hyperbaric oxygen therapy; PUSH, Pressure Ulcer Scale for Healing.

Food and Drug Administration (FDA)-approved treatments for nonhealing wounds have been underwhelming; for example, Becaplermin (recombinant human platelet-derived growth factor) was approved for the treatment of DFUs in 1997.[30] Despite the FDA approval, Becaplermin's effectiveness has not translated well into the clinical setting, which, along with its high cost, has led to its use being limited.[31] The same is true for other modalities such as hyperbaric oxygen therapy and the various novel cell- and tissue-based products (CTPs). This may be due to a lack of generalizability of many of the randomized-controlled trials (RCTs) that were carried out to get FDA approval for these products. Indeed, it has been suggested that many pivotal trials for CTPs were carried out on patients with less severe wounds than average and excluded many common and serious comorbidities shared by more than 50% of patients with nonhealing wounds.[32]

The global need to transform wound care into a precision medicine is clear and has been answered by worldwide efforts to bring together outstanding practitioners and experts in wound healing (*e.g.*, Symposium on Advanced Wound Care, Wound Healing Society, Alliance of Wound Care Stakeholders, U.S. Wound Registry, Diabetic Foot Consortium). Biomarkers for wound healing hold promise for the standardization of wound diagnosis, monitoring, and treatment by providing a "molecular barcode" for wounds.[3,14,33,34] The continued development of imaging systems and other sensing devices (*e.g.*, smart bandages or "wearables") to provide real-time measurement of various wound milieu characteristics has been major advances toward the development of smart point-of-care tools for the clinician's arsenal in the war on wounds.[14] There is a need for a platform diagnostic that can monitor biomarkers associated with healing. Such a platform would revolutionize the diagnosis of nonhealing wounds, catapult our understanding of the molecular and cellular mechanisms behind impaired wound healing to new heights, and usher in a modern age of therapeutic strategies for wound care.

This article will review the advanced wound diagnostic modalities and burgeoning technologies (biomarkers and analysis of such with advanced computational models, and sensing devices such as imaging systems and electronic noses) with the most robust evidence to support their potential to transform the wound care landscape; their suggested roles, underlying physiology, and possible therapeutic strategies to target them.

Biomarkers and the wound microenvironment: molecular barcoding of wounds

Whether healing appropriately, infected, or otherwise complicated, the wound microenvironment

is characterized by a lush landscape of biomarkers with functional, and potentially diagnostic and therapeutic, significance. Biomarkers are quantitatively measurable substances that signal the presence of an underlying physiologic or pathologic process, or they allow for the determination of treatment response.[3] Processes of interest for wounds include stage of healing, bacterial colonization/infection, degree and character of inflammation, fibroblast senescence, and keratinocyte activation, to name a few.[3,20,30]

Biomarkers for wound healing continue to garner increasing attention within the wound care community, as the prevalence of Pubmed articles related to "wound biomarkers" has increased by 558% from the years 2000 to 2017. Modern high-throughput "omics" approaches (*e.g.*, genomics, proteomics, lipidomics, metabolomics) have allowed for an increasingly detailed evaluation of the wound-healing environment at a molecular level.[3,33] Analysis of human wound biomaterials has led to the identification of hundreds of compounds with potential diagnostic, prognostic, or indicative value.[3,33,35–37] The potential benefits of biomarkers for wound healing include the development of novel and personalized treatment modalities, earlier identification of wounds requiring intervention, and their use as measurable endpoints or inclusion/exclusion criteria for clinical trials.[3,33] We suggest that each wound has a unique biochemical footprint, and that each patient would benefit from receiving treatment tailored to their wound's individual genetic, proteomic, and metabolomic makeup—this is the same principle on which today's advanced cancer treatments are based.[30]

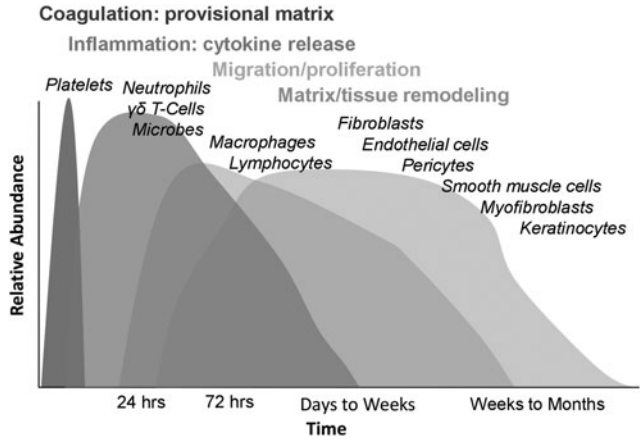

Figure 2. Acute wound-healing responses—phases and cellular effectors. Normal wound healing proceeds through multiple distinct yet overlapping phases: coagulation, inflammation, migration/proliferation, and remodeling.[38]

The cellular and molecular mechanisms underlying tissue repair and impaired wound healing remain incompletely understood.[38] Normal acute wound healing progresses through four distinct yet overlapping phases requiring intricate conversation between cellular constituents and extracellular matrix (ECM) components: hemostasis, inflammation, proliferation, and maturation (Fig. 2).[38] A derangement in any of these phases or the processes that comprise them can result in impaired wound healing (Fig. 3).[38] This may be further exacerbated by systemic factors such as age, obesity, diabetes, smoking, nutritional status, immune status, and others.[38,39]

Inflammation is a key player in the pathogenesis of wounds—the inflammatory profiles of acute and nonhealing wounds are markedly different, and nonhealing wounds exhibit markedly altered genomic and transcriptomic profiles, especially at the wound edge (Fig. 4).[14,40–47] In regular wound healing, pro-inflammatory cytokine release by the clot and surrounding wound tissue leads to the sequential infiltration of neutrophils, macrophages, and lymphocytes.[39] Under normal conditions, this inflammation is a self-limiting process; on the other hand, nonhealing wounds are characterized by a persistent inflammation that is less effective to support progression of healing.[40] For example, neutrophils are typically absent by 72 h in acute wounds, but persist in nonhealing wounds,[40] whereas recent observations show overall deregulated recruitment of inflammatory

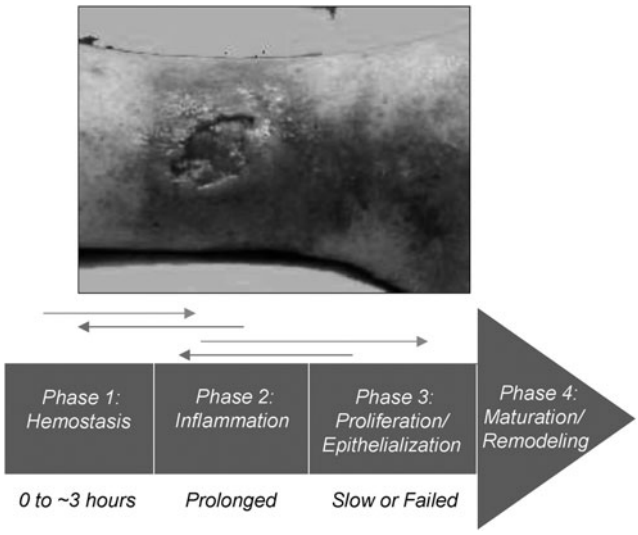

Figure 3. Nonsequential progression toward wound closure; stalled, chronically inflamed wound bed in a venous ulcer. A derangement in any of the normal phases of wound healing can result in a nonhealing wound. Nonhealing wounds are frequently characterized by prolonged, deleterious inflammation, dysfunctional proliferation, and/or failed epithelialization.[38]

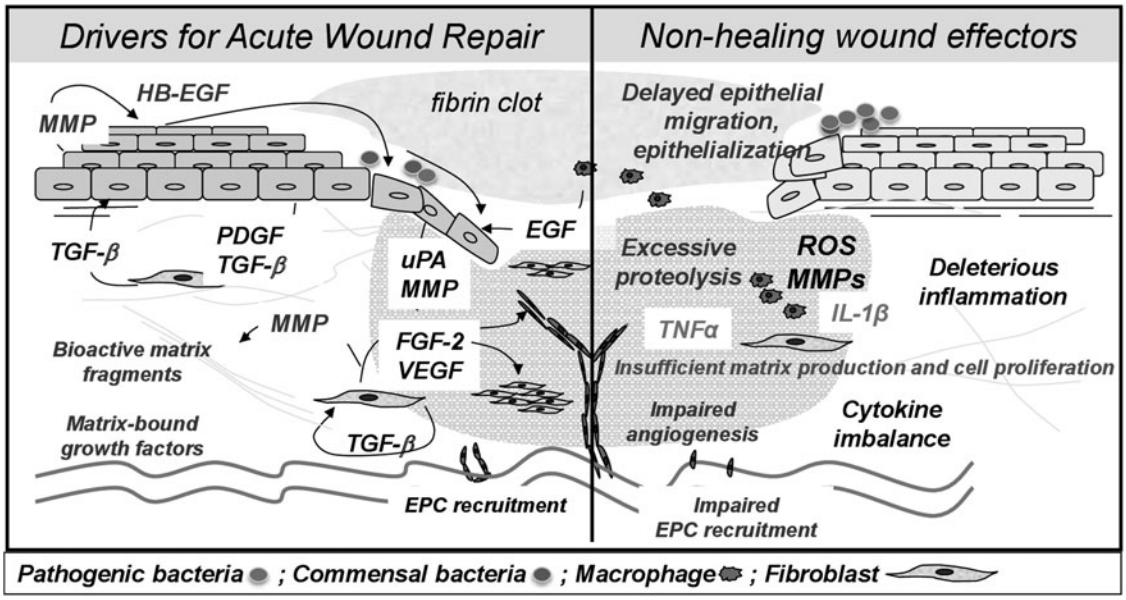

Figure 4. The chronic wound microenvironment. Chronic wounds are characterized by a prolonged, deleterious inflammatory state. Imbalance in pro-inflammatory cytokines, excessive proteolysis by MMPs, and various other cellular derangements prevent the timely and orderly restoration of the skin barrier. ECM, extracellular matrix; EGF, epidermal growth factor; EPC, endothelial progenitor cell; FGF, fibroblast growth factor; IL-1β, interleukin one beta; MMPs, matrix metalloproteases; PDGF, platelet-derived growth factor; ROS, reactive oxygen species; TNFα, tumor necrosis factor alpha; TGF-β, transforming growth factor beta; VEGF, vascular endothelial growth factor.[14,41–43]

cells in DFUs.[45] Accordingly, nonhealing wounds are characterized by a persistence of both pro-inflammatory mediators, such as tumor necrosis factor alpha and interleukin one beta (IL-1β),[38,48–50] and anti-inflammatory signals[48–50]; such dysregulation and mixed signals that lack spatiotemporal control result in ineffective inflammation. In addition, these factors lead to elevations in matrix metalloproteases (MMPs), which degrade ECM and growth factors, thus inhibiting cell migration, angiogenesis, collagen synthesis, mitogenic activity, and other processes that are instrumental for repair.[38,40] A break in the skin also allows bacteria to access the underlying tissue, which further increases inflammation and MMP levels via endotoxin release and host production of antimicrobial factors.[39] Other factors known to play a role in impaired wound healing include depletion of local stem cell populations and increases in cellular senescence.[40,51,52]

Thus, the wound-healing system comprised many highly organized layers of scale, with a delicate interplay between the various cell types, intercellular messengers, synthetic products, and enzymes.[40] This interplay may broadly be affected by variations in immune response, metabolism, neuroendocrine signaling, oxygenation, pH, and other factors, all of which are linked to complex changes in genome expression.[39,40] In nonhealing wounds, the cellular processes necessary for repair are inhibited in ways that are likely unique to each wound for each given patient. This explains, at least in part, why available treatment modalities for nonhealing wounds are unable to achieve a consistent response for a particular wound type and underscores challenges in standardizing patients for clinical trials.[14]

Despite the fact that many possible biomarkers for wound healing have been identified, their translation into simple, cost-effective, and predictive clinical assays remains challenging.[3,35] No biomarkers have yet been widely adopted for diagnostic or monitoring purposes. Recently, National Institutes of Health (NIH), under the leadership of the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), formed Diabetic Foot Consortium, a research network that aims at testing and validating multiple predictive biomarkers (tissue based or transepidermal water loss measurements) that can predict either the clinical outcomes or recurrence of previously healed chronic wounds. The establishment of standardized molecular analyses for determining wound healing status will allow for more effective identification and stratification of nonhealing wounds based on their predicted response to various treatment modalities, and it will also aid in the quest to uncover novel therapeutic strategies for individualized wound care.

Many potential biomarkers that have been identified are genes, transcription factors, micro-RNAs, and other molecules requiring either cumbersome techniques (*e.g.*, polymerase chain reaction [PCR], immunohistochemistry) to evaluate or need to undergo wide clinical validation, which requires time and resources. As such, we have chosen to focus on the few biomarkers with the most robust evidence to support their ability to modulate and/or provide insight into the wound microenvironment, and to help transform wound care into a personalized, predictive, and precision medicine.

Proteases. Proteases are the currently the best-studied biomarkers. Elevated protease activity (EPA) in nonhealing wounds has been known for some time to be associated with impaired wound healing.[53,54] MMPs, human neutrophil elastase (HNE), and cathepsin G (CG) are the best studied.[3,55] MMPs are a family of zinc-dependent endopeptidases with critical importance for normal physiological wound healing.[3] By modulating the release of cytokines, growth factors, and other biologically active molecules, MMPs regulate key wound-healing processes, including cell–cell and cell–matrix interactions, cell migration and death, ECM remodeling, and angiogenesis.[54,56–58]

Dysregulation of MMPs and their inhibitors (tissue inhibitors of metalloproteases [TIMPs]) is a significant contributor to the failure of wound healing; in chronic wounds, MMP expression is altered at the mRNA and protein levels.[58,59] Deleteriously elevated levels of MMPs in chronic wounds result in unintended cleavage of essential growth factors and cytokines, tipping the balance of ECM turnover to favor net destruction.[58] This increased proteolytic activity is chiefly due to the significant influx of inflammatory cells (*i.e.*, neutrophils and macrophages) as well as modulation by invading bacteria—for example, *Pseudomonas aeruginosa* secretes thermolysin, which activates MMPs 1, 8 and 9.[58]

More than 24 MMPs have been identified with diverse functions.[58] Factors affecting the activity of MMPs are extremely complex; they can exert opposite effects on the same biological process by virtue of nuances in the wound microenvironment.[56] Overexpression of various MMPs, most significantly MMP-2 and MMP-9, and increased MMP/TIMP ratios have been demonstrated in wound fluid, wound tissue, and serum of patients with nonhealing wounds[3,54,59,60] Shifting pH down to six reduces the activity of most chronic wound-associated MMPs by 40–90%, which is part of the rationale linking wound acidification to improved healing.[61] Wound fluid MMP expression profile varies depending on the stage of healing as well as the age of the wound.[54] MMP expression may also be useful in assessing the infection status of a wound; for example, as previously stated, *P. aeruginosa* is known to activate MMPs 1, 2, 8, and 9.[58,62] On the other hand, a case-control study of 129 patients with VLUs found that infected ulcers demonstrated high levels of MMPs 1 and 8, whereas noninfected ulcers were MMP-2 and -9 predominant.[63] This highlights the complexity inherent in the relationship between MMPs, wound healing trajectory, and infection status, as well as the limited clinical utility of a single measurement of protease activity.

Further research is needed to develop the utility of protease levels as an effective biomarker for nonhealing wounds. MMP overexpression varies by wound etiology and within subgroups of similar wounds due to variations between patients, making the establishment of global threshold values a challenging prospect.[64] Various studies have been carried out to this end, all resulting in different suggested cut-off values to indicate impaired healing in populations with DFUs or wounds of varied aetiologies.[53,59,60,64]

For example, MMP-9 levels >0.38 pg/ug predicted identified nonhealing DFUs with sensitivity of 81.8% and specificity of 64.6%.[59] Another team determined that wound fluid total MMP levels greater than 962.2 pg/mL accurately discriminated between healing and nonhealing VLUs with sensitivity of 92% and specificity of 61%.[60] Finally, a study of 290 wounds of varied etiologies suggested values of 5.5 mU/μL for HNE (sensitivity 67%, specificity 86%) and 13 mU/μL for total MMP (sensitivity 81%, specificity 71%) to distinguish healing status.[53] Widely accepted, quantitative cut-off values that allow for the consistent determination of wound healing status have yet to be discovered.[53]

WoundChek Protease (WCP; WoundChek™ Laboratories)is a qualitative, immunochromatographic test for the assessment of EPA in wounds currently available for purchase in the United Kingdom.[65] The WCP system measures the combined activity of proteases, primarily MMP-8, MMP-9, and HNE, and provides a binary measure of protease activity—elevated (EPA) versus nonelevated (NEPA).[66] This assay was found to have a sensitivity and specificity of 28% and 90%, respectively, for identifying nonhealing wounds.[64,67] Limiting factors of this study included small sample size and retrospectivity.

WCP may also be a useful tool to study how EPA affects wound healing. One study used WCP to

assess EPA in 35 patients with DFUs who underwent dermal grafting with Integra® or Hyalomatrix®. Graft integration rate at 1 month was found to be significantly lower in the group with EPA compared with the group without it; every patient in whom the graft failed was found to have EPA before graft application, whereas 36% of patients with EPA experienced successful grafting.[66] Although the sample size was small, this study highlights the potential usefulness of EPA in predicting clinical response to treatment. Further research could shed light on whether graft success can be improved with protease-modulating therapies. Indeed, a clinical trial is ongoing to determine the utility of a quantitative, point-of-care MMP assay to predict graft success in patients undergoing cutaneous autografting for acute burn injury.

Some therapies with protease modulation capabilities exist, and others continue to be developed. Doxycycline is a broad-spectrum MMP inhibitor (MMPI) with well-characterized anti-inflammatory properties, and it has been observed to reduce protease activity in DFUs.[68] A recent study found microneedling to be effective in achieving intradermal delivery of doxycycline in an *in vitro* human skin model, with a resulting decrease in MMP activity.[69] Novel collagen-based dressings (*e.g.*, oxidized regenerated cellulose/collagen; Promogran®) have the ability to absorb, retain and inactivate inflammatory proteases, which has been demonstrated both *in vitro* and in human chronic wounds.[70] The potency of these effects is related to the amount of intact collagen fiber in the dressing.[70] The mechanism of protease modulation by collagen dressings may be due to acting as a competitive inhibitor or "sacrificial substrate" for aberrantly expressed proteases.[70] Negative-pressure wound therapy (NPWT) may also be an effective treatment for wounds with elevated proteases, as chronic wounds treated with NPWT exhibit lower levels of pro-MMP-9 and decreased MMP-9/TIMP-1 ratios.[71]

Significant efforts have been made to develop pharmaceutical MMPIs for applications, such as wound healing and cancer.[72] No broad-range MMPIs were successful in cancer clinical trials due to the fact that MMPs as a broad group exert opposing effects on various pathophysiological processes, for example, tumorigenesis.[72] To our knowledge, MMPIs have not been tested in wound-healing trials. Increasingly high-resolution elucidation of MMP structure based on modern protein engineering techniques has spurred renewed efforts to design MMPIs.[72] Several new protein-based inhibitors, customized TIMPs, and anti-MMP antibodies are currently being tested.[72]

pH. pH may be the overarching taskmaster of the wound microenvironment and the biomarkers therein. Skin is acidic, with normal pH ranging from 4 to 6.[61] On wounding, a temporary physiologic acidosis occurs due to local production of organic acids and increased pCO_2 due to stasis of tissue perfusion.[61] In unimpeded acute wound healing, wound pH progresses from neutral to acidic as epithelialization occurs.[73] Alkaline acute wounds have been noted to heal more slowly than those with neutral pH.[73,74] Similarly, chronic wounds are characterized by a persistent alkaline wound environment.[73,74] Further, most pathogenic bacteria require a pH above 6, and bacterial growth is inhibited at values lower than this.[61]

Thus, it is generally accepted that wound healing occurs best at low pH.[73] The pH of chronic wounds has been observed to be generally between 7.15 and 8.9, with values as high as 9.25 being reported.[74] Studies of VLUs have suggested pH ranges of 7.3–8.9 or 7.5–7.9, with decreases in wound surface pH occurring concurrently with healing and re-epithelialization.[61,75] One group found that the pH of PUs increases with worsening ulcer grade, with average pH values of 5.7, 6.9, and 7.6 for stage I, II, and III PUs, respectively.[76]

A plethora of cellular processes depend on pH, such as cell cycle progression and enzymatic activity.[73] Figure 5 demonstrates the dynamic relationship between pH, proteases, and wound-healing physiology.[77,78] MMP-2, neutrophil elastase, and CG have highest activity at pH 7–8, which fits in with our understanding of the increased protease activity seen in chronic wounds.[74,79] Acidification of wounds promotes proliferation of fibroblasts, keratinocytes, and blood vessels and increases oxygen dissociation from hemoglobin in tissues.[74,80] Low pH increases the antimicrobial activity of silver dressings, and hypochlorite kills bacteria twice as quickly at pH 6 compared with pH 8.[74,81,82]

Existing therapies can acidify the environment of wounds: for example, manuka honey,[83] occlusive dressings (due to retention of acidic wound exudate),[61] and even a pH-switchable antimicrobial nanofiber dressing.[84] These dressings have other properties that improve wound healing and there are not yet any good randomized controlled trials to suggest that acidifying therapies are superior to the rest of the field, though they are commonly employed by wound care practitioners.

The role of measuring pH directly in chronic wounds remains unclear. Dissemond and team measured wound fluid pH in 39 patients with various wound types for 12 months and found that pH can vary by up to 1.73 over time in a given pa-

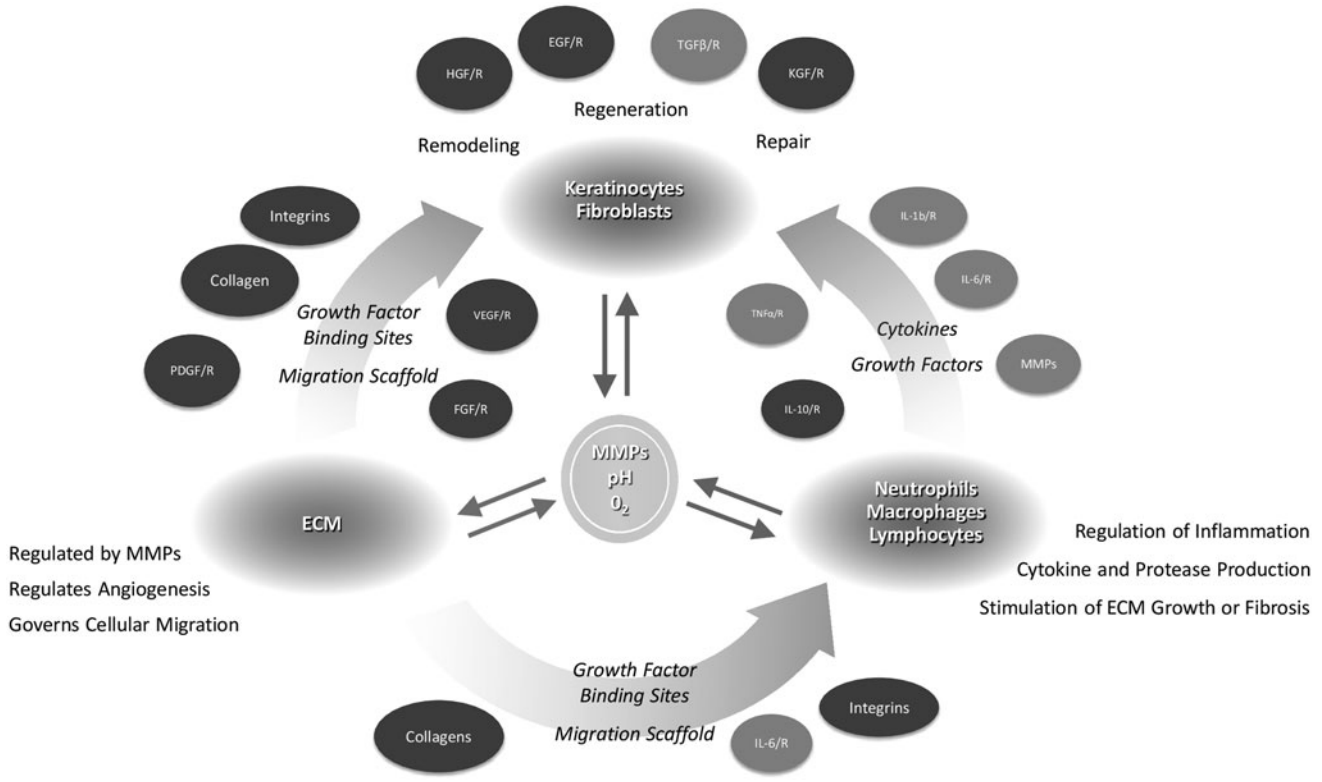

Figure 5. Relationship of biomarkers to physiologic wound healing. Overview of the role of pH and MMPs within the complex wound microenvironment. Some, but not all, important wound-healing cytokines and substrates are shown—those in *orange*, although necessary for physiologic healing, are commonly overexpressed in nonhealing wounds. MMPs and pH interplay intricately within the wound microenvironment in a phenomenon known as "dynamic reciprocity." pH affects ECM synthesis, enzyme activity, cell cycle progression, oxygen delivery, fibroblast activity, and bacterial growth. The effects of MMPs are also manifold, as they are crucial for ECM remodeling and angiogenesis, cell migration, and cytokine regulation, though their overexpression results in poor wound healing. FGFR, fibroblast growth factor; HGF, hepatocyte growth factor; IL-X, interleukin X; KGF, keratinocyte growth factor; /R, receptor.[77,78]

tient.[85] Although pH increases with bacterial colonization, this further complicates interpretation since acidic or basic pH could be considered normal based on the infection status and healing stage of the wound. Thus, pH measurements would be best considered not in a vacuum but rather in concert with clinical signs and other biomarkers.

The understanding of wound fluid pH has until recently been limited by measurement techniques. Litmus paper has low resolution and is subject to inaccurate interpretation by the measurer.[74] Glass pH electrodes have issues with sterility and discomfort for the patient, and both litmus paper and glass electrodes require the wound dressing to be removed.[74] The recent invention of smart dressings with continuous pH-monitoring capabilities opens the door to further characterizing pH as a biomarker of interest.[86] The ability to observe granular pH trends over time will shed light on the potential ability of pH, either alone or in tandem with other markers, to predict or detect infection, or to determine whether the wound is responding appropriately to treatment.

In addition to standing on its own as a potential biomarker, pH modulates and interacts with other biomarkers that exert effects on the wound microenvironment, such as nitric oxide (NO) and uric acid (UA). NO plays a key role in various processes that are necessary for normal tissue repair, including angiogenesis, granulation tissue formation, keratinocyte migration, collagen production, and the activation and upregulation of growth factors.[87] The utility of wound fluid NO as a biomarker for human wounds remains unclear, with the few existing studies being limited by small sample sizes and cumbersome methods of detection such as spectrophotometry.[87] Nonetheless, wound fluid NO measurements were found to discriminate between healing and nonhealing wounds of various etiologies with an area under the curve (AUC) of 0.81.[87] The development of more elegant, high-fidelity NO measurement techniques (*e.g.*, field effect transistors)[88] may aid in future research and is ongoing.[88]

UA is another biomarker whose production is linked to pH. The hypoxic environment of VLUs results in the depletion of ATP and an accumula-

tion of purine metabolites such as adenosine and inosine, which are then broken down into UA in a process that occurs more abundantly at alkaline pH.[89] UA is believed to inhibit healing by directly promoting inflammation,[90] whereas its synthesis contributes to tissue damage via production of damaging superoxide radicals.[89] Several tools have been developed for point-of-care monitoring of UA in wound fluid, namely: paper-based smart bandages,[91,92] inkjet-printed carbon nanotubes,[93] gauze with embroidered electrochemical sensors,[94] and a wearable enzymatic biosensor.[95] However, it remains unclear as to how the elucidation of UA levels will guide management decisions for nonhealing wounds. Further research could make use of novel UA biosensing technologies to further characterize the relationship between UA, wound-healing outcomes, and response to treatment.

Biomarkers for infection detection

The prevention and identification of wound infections remains a clinical conundrum.[34] Cardinal signs of infection (increased pain, erythema, edema, heat, and purulent exudate) may be diminished by physiological phenomena commonly seen in patients with chronic wounds, namely: neuropathy, vasculopathy, venous disease, and impaired leukocyte function secondary to diabetes.[34] Clinical signs are still seen as controversial in diagnosing wound infection,[34] having consistently exhibited poor correlation with the current gold standard of microbiological tissue culture.[16,96] For example, in the case of DFUs, it is generally agreed upon that infection should be diagnosed based upon clinical signs as set forth by the Infectious Diseases Society of America (IDSA) (purulent exudate OR two of warmth, tenderness, pain, induration, or erythema being diagnostic of infection).[97,98]

However, research suggests that IDSA signs alone or together are not significant predictors of wound infection, and in fact performed no better than chance in identifying tissue-culture-positive infections in a cohort of 64 patients with DFUs.[99] It may be that so-called "secondary" signs of infection, unique to wound healing by secondary intention (serous exudate + inflammation, delayed healing, discoloration, friable granulation tissue, foul odor, and wound breakdown), are more effective at identifying infection than the classic signs.[96] Superficial wound swabbing is an alternative that suffers from lack of technique standardization and poor efficacy; a systematic review of eight randomized controlled trials found superficial swabs to be only 49% sensitive and 62% specific when compared with deep tissue cultures.[100]

Although tissue culture is considered the gold standard for wound infection detection, it is far from a holy grail. It is invasive, requires anesthesia, and is often foregone over concerns about harming the patient or worsening the wound bed.[16,34] It may be contraindicated in certain cases, such as severe peripheral arterial disease.[16] Qualitative cultures, although inexpensive, have low sensitivity and require 1–3 days turnaround for results.[101] Quantitative cultures, although more accurate, are expensive, labor intensive and require specialized facilities.[101] These delays allow the bacterial burden to change character by the time results are received.[102] In addition, false negative results are often seen in patients already taking antibiotics, a situation commonly encountered in clinical practice.[98]

Most importantly, recent advances in microbiological metagenomics have changed the way we look at the wound microbiome and revealed significant limitations in conventional culture techniques. The advent of next-generation sequencing techniques, such as 16S RNA pyrosequencing, allows for the identification of the complete bacterial genome of a wound.[101] In doing so, we have realized that the chronic wound microbiome is much more complex than was previously appreciated[101]; indeed, less than 2% of known bacterial species can routinely grown in culture, most notably anaerobes that are known to be present in most chronic wounds.[103] Metagenomic sequencing may be difficult to interpret, as it also identifies dead or commensal organisms that might be a source of confusion.[102] In addition, these techniques are highly expensive, require specialized facilities and personnel, and are largely used thus far in research settings, placing them far from regular bedside application.[101]

Further, in recent years, it has become increasingly recognized that most bacteria are found in wound biofilms.[104] Biofilms are three-dimensional (3D) collections of bacteria composed of 80–85% extracellular polymer and 15–20% organism, by mass.[104] These biofilms protect bacteria from antimicrobials and the host immune response.[98] They inhibit wound healing by creating a physical barrier to re-epithelialization and opsonization, and they promote a chronic inflammatory environment through the constant release of waste products.[105]

It has been observed that biofilms complicate 60% of chronic wounds, compared with only 6% of acute wounds.[106] Repeating patterns of co-aggregated species termed "functional equivalent pathogroups" have been observed to synergistically form pathological biofilms, further highlighting the

importance considering the entire wound microenvironment when characterizing the nature of infections.[104] Biofilms can develop in chronic wounds as quickly as within 10 h, and they tend to recur even 2 days after being removed by surgical debridement.[104] They are invisible to the naked eye, detectable only by scanning electron microscopy. Hence the importance of developing strategies to easily identify and treat biofilms cannot be overstated, and many wound care products have been developed to target them (Table 3).[107–130]

To further complicate matters, recent research has demonstrated that bacteria that classically occupy the extracellular space (such as *Staphylococcus aureus*) can hide and persist intracellularly in skin cells, including within professional (macrophages, dendritic cells, B cells) and nonprofessional (keratinocytes, fibroblasts, endothelial cells) antigen-presenting cells, thus contributing to the recalcitrant nature of skin wounds.[131,132] In the case of *S. aureus*, this is achieved by phenotype switching of the bacteria into a "small-colony-variant" (SCV) that exhibits slower growth and metabolism, downregulated virulence genes, low cytotoxicity, and increased antibiotic resistance.[132] By virtue of these traits, SCV bacteria can evade the host immune response and may remain dormant intracellularly for years; this may be why wound infections are sometimes observed to reappear after apparently successful antimicrobial treatment.[132] The SCV phenotype is upregulated by the acidic environment of phagolysosomes, can hijack them, and can revert into the classical or "aggressive" cytotoxic phenotype on exposure to neutral pH. Targeting intracellular pathogens is difficult, as conventional antibiotics tend to remain in the extracellular space, or do not remain long enough or maintain high enough concentrations intracellularly for effective bacterial killing.[132]

With this in mind, efforts are ongoing to imagine novel therapeutic strategies for these stealthy pathogens, including: lysosomal alkalizing agents (*e.g.*, chloroquine combined with other antibiotics)[132]; peptide antibiotics that can cross eukaryotic cell membranes[132]; thymol-loaded wound dressings[133]; and hyaluronan-based nanogels,[134] to name a few. Further, the critical role of Perforin-2, a membrane-bound protein present in endosomal vesicles, in cells' innate immune response against intracellular pathogens has recently come to light.[131] This represents a valuable weapon in the battle against intracellular bacteria as efforts are ongoing to leverage activation of this protein to fight persistent wound infections.

Overall, the development of novel biomarkers for the assessment of wound infection status would aid in untangling the complex web of the wound microbiome. It is clear that three facets of wound microbiota should be considered: total bacterial load, diversity, and the presence of biofilms.[3] Recent research on acute phase reactants, neutrophilic enzymes, and volatile organic compounds (VOCs) may improve our ability to robustly characterize the microbial status of wounds.

Table 3. *Anti-biofilm agents for wound care*

Product	*Mechanism of Anti-Biofilm Action*	*Anti-biofilm Evidence*	*Healing Outcomes Evidence*
Polyhexanide (PHMB)	Disruption and increased permeability of bacterial cell membranes	Level VI[103]	Level IV[104]
Poloxamer-based surfactants (Plurogel®; Medline Industries, Inc., Northfield, IL)	Inhibition of bacterial surface adherence Reduces cohesion of constituent biofilm molecules	Level VI[105]	Level IV[106] Level IV[107]
Benzalkonium chloride-based surfactant (BlastX™; Next Science, St. Paul, MN)	Dissolves the extracellular polysaccharide matrix, exposing encapsulated bacteria for removal Osmotic lysis of cell wall	Level VI[108]	Level I[109] Level I[110]
Cadexomer iodine (IODOSORB™; Smith and Nephew, London, United Kingdom)	Directly destroys biofilms Collapses bacterial glycocalyx Traps bacteria within beads.	Level VI[111] Level VI[112] Level IV[113]	Level I[114]
Honey	High osmolarity Low pH Peroxide produced by breakdown of glucose	Level VI[115]	Level I[116]
Hypochlorous acid	Chemical inactivation of various cellular processes, including amino acid modification and protein synthesis	Level VI[117]	Low-to-strong based on wound type[118]
Lasers and phototherapy	Induction of oxidative stress Impaired polysaccharide production	Level VI[119]	None
Low-frequency ultrasound	Microstreaming and cavitational effects	Level VI[120]	Varies[121]
Electroceuticals	Disruption of electrostatic adhesion forces Superoxide production Bacterial membrane enzyme disruption	Level VI[122] Level VI[123] Level VI[124]	Level VI[124] Level III[125]

A list of available anti-biofilm wound care products currently available on the market, their mechanisms of action, and levels of evidence to support their ability to destroy biofilms and improve wound-healing outcomes. Adapted with permission from Weigelt *et al.*[107]

PHMB, polyhexamethylene biguanide.

Acute phase reactants. Inflammatory markers such as C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), and procalcitonin may aid in the diagnosis of infection in nonhealing wounds. These biomarkers have been studied predominantly in DFUs, with conflicting results. The utility of inflammatory markers in differentiating infected DFU (IDFU) from noninfected DFU (NIDFU) remains unclear. Some authors found CRP to be significantly elevated in IDFUs, with varying sensitivity and specificity,[135–137] whereas others found no association.[138] Similar results have been described for ESR[138–141] and procalcitonin.[135–140] In general, it is agreed that the measurement of multiple markers provides the most utility[135,136,138–140]; for example, a composite algorithm using CRP and procalcitonin was developed with an AUC of 0.947 to identify IDFUs.[135]

There are limitations of these studies that make interpretation of the results challenging. Some studies looked at hospitalized patients whereas some took place in an outpatient setting. Pretest probabilities of infection are higher in hospitalized patients, which upwardly skews the positive-predictive value of a test. Further, some authors included patients with osteomyelitis and other high-grade IDFUs whereas others looked only at mildly infected (IDSA grade II) versus uninfected (IDSA grade I). This is relevant as it affects the levels of inflammatory markers. For example, procalcitonin is higher in higher-grade IDFUs[139] and has been observed to increase very little in localized infections without systemic manifestations.[135] CRP, on the other hand, has been shown to increase significantly in response to local infection.[135] We suggest that the most utility would be gained by studying mildly IDFU versus NIDFU in the outpatient setting, as did Ingram *et al.*, since these patients frequently pose the most significant clinical challenge when considering whether or not to prescribe antibiotics.[136]

Most notably, the use of clinical grading as the comparator in these studies is a significant limitation due to its uncertain validity. The IDSA recommends that the diagnosis of infected DFUs be based primarily on clinical assessment, namely: the presence of either purulent secretion or two of warmth, tenderness, pain, induration, or erythema.[97] However, clinical signs of infection are often absent in patients with diabetes.[138] Future research should use tissue biopsy as a reference standard, although as previously mentioned microbiological results can also be misleading. This highlights the need for highly sensitive tools to identify infection in patients lacking clinical signs. Inflammatory markers do not currently offer this ideal, objective information; as such, they are best interpreted by experienced providers in the context of both clinical signs and microbiological analysis.

Despite this, CRP may be an effective tool to monitor treatment response in chronic wounds as has previously been demonstrated with CRP and ESR in osteomyelitis.[142] Goodfield found that hospitalized VLU patients with elevated CRP demonstrated improved wound-healing outcomes and an according decrease in CRP when treated with antibiotics, whereas patients without elevations in CRP did not respond to antibiotic therapy.[143] Further, Liu *et al.* found decreasing wound fluid CRP levels in correlation to positive wound outcomes in a cohort of 20 patients with chronic trauma-related wounds who received treatment with debridement, antibiotics, and NPWT.[144] Efforts are ongoing to develop devices that are capable of high-resolution measurement of CRP in wound fluid,[80] which stands to further characterize the utility of CRP as a wound-healing biomarker.

Myeloperoxidase and lysozyme. Myeloperoxidase (MPO) and lysozyme (LYS) are enzymes secreted by host neutrophils in response to infection.[145] Spectrophotometric analysis of MPO and LYS activities in wound fluid has revealed them to be significantly elevated in clinically infected wounds of various etiologies when compared with noninfected wounds.[146–148] These studies also served as proof-of-viability for various enzyme detection systems: chitosan-[148] and peptidoglycan[146]-based colorimetric assays for LYS and a similar concept for MPO.[147] Schiffer *et al.* developed a test strip with covalently immobilized MPO substrate, which demonstrated a significant change in color when exposed to fluid from clinically infected wounds, as compared with noninfected wound fluid.[149] These studies were all limited by their use of clinical judgment as the comparator for determination of infection.

Others have studied the correlation between wound fluid MPO/LYS and quantitative superficial wound culture. Blockhuis *et al.* developed a 30-min colorimetric assay to measure the activity of four enzymes in wound fluid (HNE, CatG, MPO, and LYS) and compared the results with clinical judgment and quantitative superficial wound culture.[145] They found that three models of interpretation for their test were superior to clinical judgement in predicting infection as diagnosed by wound culture (AUC ranging from 0.67 to 0.74).[145] Although the model requiring only one positive enzyme was the most sensitive, models involving multiple enzymes were more specific, highlighting the importance of measuring multiple enzymes.

Schiffer *et al.* measured HNE, MPO, and LYS levels spectrophotometrically and colorimetrically, and they compared them with clinical judgment and quantitative culture results.[150] Measured HNE and LYS levels were significantly elevated in wounds determined to be infected or critical by microbiologic analysis, whereas MPO failed to reach statistical significance on its own.[150] The authors indicate that combined assessment of all three enzyme activities by both color changes and spectrophotometric analysis could have successfully identified 85% of culture-positive samples that were judged to be uninfected clinically.[150]

A screen-printed amperomatric sensor for fast detection of MPO in wound fluid has been developed by Hajnsek and team.[151] They found a significant difference in MPO activity in infected and critical wounds compared with uninfected wounds based on quantitative wound fluid culture.[151] Such technology could set the stage for continued research by allowing immediate high-resolution measurement of enzyme levels in wound fluid.

Although MPO and LYS have some promise as biomarkers for wound infection, existing research has been limited by the use of clinical judgment and superficial swab/fluid cultures as standards of comparison. Future studies should use the gold standard deep tissue culture as the comparator.

Volatile organic compounds. VOC is a generic term referring to a wide range of molecules with a boiling point less than 300°C, for example, alcohols, aldehydes, ketones, isocyanates, sulfides, and hydrocarbons.[152] The human body contains and emits many such molecules as part of normal physiologic processes, and they can thus be detected from tissue (such as skin) and bodily fluids.[152] VOCs change in their relationship to metabolic state, hormonal changes, and ingestion of certain foods, and they are also emitted by bacteria present on the skin.[152] VOCs have been identified as possible biomarkers for various malignancies and other medical conditions such as asthma, inflammatory bowel disease, and diabetes.[153] Recent research has highlighted the potential to use VOCs to discriminate between infected and noninfected wounds and even to identify specific bacterial strains based on unique VOC profiles. The VOC profile of a wound reflects the compounds carried to and from the wound by the blood, metabolites produced by underlying cells and bacterial flora, and VOCs from the environment.[152]

The most common methods to evaluate VOCs in wounds are mass spectrometry and "electronic noses" (e-noses). E-noses are sensors or sensor arrays that can detect, identify, and discriminate between chemicals and compounds from a given sample of air ("headspace").[154] Such sensors swell upon adsorption of a gas, thereby increasing their electrical resistance. This pattern of resistance is transmitted to a computer for analysis to identify the compound.[153,154]

One of the earliest applications of e-nose technology for wounds was by Greenwood at al., who in 1997 used a 20-strong array of polymer sensors to evaluate VOC profiles in 15 patients with chronic ulcers of venous, arterial, or mixed etiology. Thirteen out of 15 patients demonstrated a definite change in the computed aroma profile as the ulcers progressed to healing.[155] More recently, Tian and team used an e-nose comprising seven gas sensors (six metal oxide and one electrochemical) coupled with a probabilistic neural network computational model to accurately identify 100% of monospecies and 94.9% of two-species bacterial cultures *in vitro*.[156] Moving into the clinical domain, a study of 10 patients with severe burns revealed different VOC profiles between infected and uninfected wounds as determined by cultures of wound swabs and dressings.[157]

Although e-noses are powerful tools that are capable of providing rapid results, they are subject to some limitations in their current form. Many factors can have a bearing on the VOC readings, including wound etiology and duration, types of dressings, and topical treatment agents used.[158] Electrical VOC sensors are quite susceptible to temperature, humidity, and external interference from environmental compounds; the strict control of experimental conditions and procedures required to mitigate this phenomenon are difficult to achieve in a clinical setting.[158]

Elucidation of which specific VOCs should be targeted by e-nose systems will allow for the continued development of more advanced tools, since e-noses can only detect VOC patterns they have been preprogrammed to detect.[153] Mass spectrometry has been used to this end due to its high sensitivity. Ashrafi and team recently used gas chromatography mass spectrometry to identify VOC profiles for both planktonic and biofilm bacteria, in *in vitro* and *ex vivo* cutaneous human wound models. VOC production was found to be affected by biofilm development and by the skin model used. Further, they identified VOCs specific for either planktonic or biofilm growth and others with significantly different relative abundances between the two bacterial states. Finally, some VOCs were observed to correlate directly with biofilm metabolic activity and mass.[159]

In a subsequent experiment, they used electrical stimulation, ciprofloxacin, or both to disrupt biofilms (measured by fluorescence staining, enumeration, metabolic assays, and biomass quantification). They observed a significant variation in VOCs after biofilm disruption, for example butanedione and acetic acid ethyl ester were identified as possible breakdown products of methicillin-resistant *Staphylococcus Aureus* biofilms.[160] This highlights the possibility of using VOCs to track treatment response as part of a biofilm-based wound care approach. Though a valuable tool for research, direct clinical application of mass spectrometry is limited by significant time requirements and high cost.[152,153]

In general, the analysis of sensor output data from complex mixtures of molecules is difficult but can theoretically be achieved by using modern data analysis methods.[158] Optimization and maturation of sensor equipment, guided by further identification of VOCs of interest, will be instrumental in bringing this technology into the clinic.[152] VOC sampling technology has the potential to provide painless, rapid, noninvasive diagnostic information for nonhealing wounds.[153] VOC analysis may allow for the assessment of the wound-healing trajectory as well as the determination of treatment bioavailability and bacterial antibiotic susceptibility.[152] Valuable directions for future research have been suggested, including: developing a VOC library to develop fingerprints for given bacteria or wound states; determining the change in VOC profile that occurs over time based on the wound-healing trajectory; and the effects of ingested medications, if any, on VOC profiles.[152]

Wounded warriors: biomarkers for combat wound triage

War has historically been a significant driver of medical practice and innovation.[161] Blast injuries make up more than half of all combat wounds in modern warfare[162]; this, along with the advent of body armor, has led to a shift in combat wound patterns from central (trunk, head) to the periphery (extremities).[161] These blast-related injuries have massive zones of effect that can result in severe multisystem trauma affecting soft tissue, bone, and neurovascular structures with significant potential for gross bacterial colonization.[163,164] Although the proportion of survivable combat traumas has increased, up to 24% of combat deaths may be preventable—most of these deaths are due to hemorrhage and occur before reaching surgical care, highlighting the need for markers to guide triage.[19]

Large wound beds due to penetrating blast injuries are difficult to manage.[165] Standard of care is to leave these wounds open and treat them with negative pressure dressings alongside serial debridements and washouts with eventual primary closure.[161] However, the question of when to initiate primary closure is a challenging conundrum for even the most experienced military surgeons.[166] Premature closure of a wound is more likely to lead to dehiscence, resulting in unnecessary procedures and increased morbidity for the patient.[166] On the other hand, late closure of wounds results in prolonged hospital stays, delayed rehabilitation, increased risk of hospital-acquired infections, and other complications.[166] The fact remains that up to 25% of war wounds dehisce fully or partially after closure.[167]

Conventional visual assessment (*i.e.*, the "4 Cs": color, consistency, contractility when stimulated, capacity to bleed when incised) has not been found to correlate reliably with wound outcomes.[166] Factors that may affect a surgeon's decision to initiate primary closure may include wound appearance, location, perfusion, presence of local or systemic infection, nutritional status, white blood cell count, ESR, and CRP; however, clinical algorithms based on these factors have also been poorly performed.[167] A wound that one surgeon might deem ready for closure, another would disagree with—as such, there is a need for objective biomarkers and clinical decision-making algorithms to guide appropriate timing of closure for both combat casualties and complex civilian trauma.[161]

As such, much research has been conducted to shed light on the molecular landscape of combat wounds. Much like chronic wounds, failed (dehisced) combat wounds are characterized by a dysregulated immune response, leading to an aberrant, prolonged inflammatory state that promotes cellular infiltration and tissue breakdown.[163] It is suspected that the immune dysregulation in the local wound environment is affected by the systemic pro-inflammatory response to massive injury.[168] Various prospective studies on service members with penetrating blast injuries to the extremities highlighted possible serum, tissue, and wound effluent biomarkers of wound failure, heterotopic ossification, and critical colonization using Luminex bead array assays.[163,165,168–170]

Other methods have been used to characterize prognostic biomarkers for combat casualties. Luczek *et al.* used NMR spectroscopy to study plasma metabolites in 78 injured fighters with blast injuries and gunshot wounds. They found that hypoxanthine and the heme precursor

5-aminolevulinic acid outperformed lactate as markers of traumatic injury. Further, succinate outperformed lactate as a marker of mortality.[19] Thus, these molecules may represent biomarkers to improve triaging of combat wounds, although the development of effective point-of-care tests is paramount as NMR spectroscopy is not an agile modality.

Janak and team sought to determine the relationship between urinary biomarkers and injury severity according to the Injury Severity Score (ISS). They found four biomarkers that were significantly higher in severe (ISS >24) versus nonsevere combat injuries: kidney injury molecule 1 (Kim-1), cystatin C, neutrophil gelatinase-associated lipocalin, and liver-type fatty acid binding protein (LFABP). Notably, Kim-1 plays a role in renal recovery and LFABP is a marker for renal hypoxia and vulnerability, thus the elevation of these markers may suggest the need to optimize the patient for renal recovery. Interestingly, LFABP levels drawn at admission were found to predict a subsequent diagnosis of severe abdominal injury with 80% sensitivity and 75% specificity.[171] The authors used a 22-patient cohort of civilian burn patients as surrogates to test generalizability and found good concordance in biomarker trends between both groups.

Modern computational techniques have allowed for even greater predictive insight. Forsberg *et al.* used machine learning to develop highly predictive algorithms to help surgeons decide when to close wounds. They prospectively enrolled four patients with a minimum 75 cm^2 combat wound and 18 civilian trauma patients to assess validity and reliability. Serum, tissue, and wound effluent biomarkers were measured by using Luminex bead assay, whereas quantitative transcriptomic PCR was used to characterized gene expression. A Random Forest (a type of machine-learning algorithm) model designed to select the 10 most important decision criteria was found to have an AUC of 0.79 for predicting wound failure, and these results were generalizable to the civilian population.[166] Using the same cohort of patients, Dente *et al.* created a predictive model for blood stream infections (BSI) and pneumonia (PNA) in critically injured people by using Bayesian belief networks (a probabilistic model). A model incorporating volume of blood products received, presence of critical colonization in the wound, elevated serum IL-2 receptor, and serum Monokine Induced by Gamma Interferon was found to have an AUC of 0.832 (sensitivity of 0.5, specificity of 0.912) for predicting BSI. For PNA, a model using serum IL-7, head ISS, chest ISS, and critical colonization achieved an AUC of 0.856 (sensitivity 0.556, specificity 0.953) for predicting PNA in the same cohort.[172] Small samples sizes in these analyses may have increased the risk of overfitting the models and overestimating their accuracies, although computational steps were taken to minimize these risks.[166,172]

Although much light has been shed on the ability of biomarkers to guide the care of wounded service members, the relationships between these biomarkers and wound outcomes would benefit from further validation in larger patient cohorts. In addition, generalizability comes into question when considering the relatively homogenous population of service members, that is, predominantly young, otherwise healthy males.[166] Global efforts continue to improve these diagnostic tools and invent new ones; for example, the Combat Wound Initiative Program is a multidisciplinary, collaborative, interservice translational research program seeking to provide state-of-the-art tailored wound care tools.[167] Continued research efforts in this field will hopefully provide objective measures for guiding combat wound closure as well as for aiding in the management of complex civilian trauma.[168]

Illuminating the chronic wound microenvironment

There is a great need for diagnostic tools that can provide objective measurement criteria and thus guide evidence-based treatment decisions to the benefit of patients with wounds.[173] Imaging systems have the potential to fulfill this need and revolutionize wound care.[173] Many imaging modalities and commercial devices have emerged with the ability to measure wound parameters, including tissue oxygenation, burn depth, and bioburden. Such technologies are advantageous due to their noninvasive nature, and most function in the optical range of the electromagnetic spectrum, which presents minimal risk to the patient.[174] The ideal imaging system should be able to be handled by users with varying levels of education and experience as well as fit into challenging clinical environments.[174] Here, we present the technologies with the greatest clinical evidence for their potential to help transform wound care into a precision medicine.

Ultrasound. Ultrasound (US) is a frequently used diagnostic tool in medicine due to its low cost, low risk, and ability to provide feedback in real time.[175] It involves the use of a probe to propagate sound waves through the skin, wherein they are

reflected, scattered, or absorbed by the underlying tissues and create an image dependent on the amplitude of the returning echoes.[176] The US waves with higher frequencies have lower tissue penetrance but produce higher-resolution images; the opposite is true for lower frequencies.[176] When it comes to skin imaging, high-frequency ultrasound (HFUS) scanners of 15 mHz or greater are generally considered optimal to provide sufficient detail.[176] Such devices have been used to measure skin characteristics in a wide range of dermatological conditions, including skin tumors, fibrosing disorders, psoriasis, and wound healing.[176]

It has been suggested that wound epithelialization alone is not an absolute indicator of wound healing status: The new epithelium may lack barrier function or the underlying dermis may still exhibit derangements in molecular healing processes.[177] Indeed, nonhealing wounds are characterized by functional healing deficiencies that are likely unique to each wound. The US has demonstrated the ability to measure tissue properties, which may shed light on the true functional status of wounded skin tissue. HFUS has demonstrated comparable ability to histology in evaluating key characteristics of the wound-healing process, such as depth, collagen accumulation, and granulation tissue formation in porcine wound models and human cadaveric tissue sections.[178] The same characteristics have been measured via US in human DFUs.[179]

Mukai *et al.* used three-dimensionally reconstructed HFUS images to track microvascular growth in a murine full-thickness wound healing model.[180] HFUS has also been used to quantitatively assess the healing of artificially induced PUs on guinea pigs by monitoring structural changes in deep tissue layers,[181] as well as to characterize the sonographic morphology of various cellulose-derived artificial skin products.[182] Gnyawali *et al.* used US elastography, which measures tissue stiffness by quantifying distortion in response to mechanical stress,[177] to demonstrate that diabetic wounds in mice are characterized by a persistent early inflammatory phase with delayed recovery of elasticity.[183]

The ability of HFUS to study wound healing has been further examined in clinical settings. Kuhn and Agern used HFUS on 20 patients with 22 nonhealing leg ulcers of varying etiology (predominately VLUs), 18 of whom were treated with grafting of a BLCC, and compared US assessment with conventional visual assessment for the determination of healing. They found that most patients exhibited significant derangements in elastic and collagen fibers in the underlying dermis at the time of "clinical healing" as determined by visual assessment of complete re-epithelialization.[184] The follow-up period was not long enough to determine whether these findings were associated with an increased risk of wound recurrence. The authors suggested that such wounds, if identified, could be intervened upon with adjunctive therapies such as extracorporeal shockwave therapy. Even so, it is not certain that this phenomenon is an abnormal wound-healing process.

Dyson *et al.* performed HFUS analysis of human punch biopsies and found significant variation in wound diameter over time at the point halfway between the base of the wound and the surface; they theorized that this was due to narrowing of the wound base from myofibroblast contraction and of the wound surface due to scab formation and epithelialization.[185] Thus, the fact that epithelialization occurs before the underlying tissue is fully repaired is not necessarily a deleterious process, and it certainly makes sense from an evolutionary perspective as restoration of the epidermis is perhaps the most time-sensitive process required to prevent further insults, for example, pathogen invasion.

With all this in mind, it remains unclear what actionable interventions are made possible by US assessment: What specific therapies should be employed to target the US-measurable tissue characteristics discussed thus far, if intervention is even necessary, and the resulting effects on wound healing outcomes. This represents a potential area for future research. At the very least, HFUS could be used to compare treatment modalities in their ability to improve the speed and strength of dermal healing.[184]

The most promising clinical application for US in the wound healing space is the early detection and evaluation of deep-tissue pressure injuries (DTPIs) and PUs. The National Pressure Injury Advisory Panel defines a DTPI as a localized area of nonblanchable deep red, maroon, or purple discoloration, or epidermal separation that reveals a dark wound bed or blood-filled blister.[186] Many PUs begin at the skin surface due to friction and shearing forces, whereas some PUs begin as DTPIs that form at the bone–muscle interface far below the skin surface.[186]

The prevalence of DTPIs is believed to be underestimated, as they may remain invisible until they reach advanced stages; deep tissue damage at the bone level can take up to a week to appear on the skin.[186,187] There is no way to visually assess

the degree of skin damage below the surface of stage 1 PUs, pressure-related intact discolored areas of skin, or even normal-appearing skin; as such, patients who present to hospitals for care may already have incurred significant and irreversible vascular or skin damage, and underlying tissue may already be dead or dying.[187] Early detection of such lesions would facilitate prompt intervention to benefit the patients. Further, if these patients later develop a DTI or PU, it may be labeled as hospital-acquired despite having been present yet undetectable or in early stages of progression on admission, which has significant legal and cost implications for the hospital.[187]

To this end, Scheiner *et al.* used US (2.5 to 12 MHz) to scan 13 common PU sites in 33 patients at high risk for PUs who were admitted to the emergency department at an academic hospital center (23 patients completed the study). The US scans were able to identify pressure necrosis at the levels of the subcutaneous fat and in the deep muscularis at the level of bone. Patients were examined for PUs every day for 7 days after the initial scan, which was conducted on admission. Twenty percent of patients were found to have subsurface DTIs that later appeared as PUs on the skin. The US identified these sites with a sensitivity of 100% and a negative-predictive value of 100%.[187] Another group used HFUS to scan the heels of 130 patients at high risk for PUs who were admitted to the hospital for a period of 1 year. They noted that 89.8% of patients had abnormal scans (fluid accumulation beneath the dermis at the bony prominence) of at least one heel, but too few patients developed PUs to determine any predictive pattern or capability of the HFUS scans.[188]

Other groups have suggested US patterns that may predict progression of DTIs into PUs. Matsumoto *et al.* evaluated 11 patients with visible DTPIs at a Japanese university hospital by using 18 MHz US and found that those exhibiting a "cloud-like" echogenicity pattern exhibited progression of injury, whereas those exhibiting a "cobblestone-like" pattern did not get worse.[186] Another small study using 10 MHz US identified key ultrasonographic signs unique to DTPI as well as some specific characteristics that may predict progression. Both of these studies were limited by small sample size and a short follow-up period.[189] Future studies using US to detect subclinical PUs/DTPIs and to determine algorithms predicting progression in larger cohorts will be valuable.

Autofluorescence imaging. Autofluorescence (AF) occurs when biological substrates emit light in the UV-visible to near-infrared spectral range ($\sim$200–1100 nm) when excited by a specific wavelength of incumbent light.[190] Subcellular labeling techniques and computer-based image composition software have allowed for the characterization and study of endogenous chromophores in human cells.[190] The AF signals from the cell cytoplasm arise mainly from the reduced form of NADPH and from flavins—the spectral emissions of these compounds are directly related to their redox state, which is a reflection of energy metabolism, oxidative defense, biosynthesis, and signal transduction in the cell.[190]

Other compounds with discernible spectral signals that can be evaluated by using AF include collagen, elastin, free fatty acids, vitamin A, lipofuscin-like pigments, and porphyrins.[190] Indeed, laser-induced AF has been used to quantify endogenous collagen in burn wound biopsies.[191] Mehrvar *et al.* used AF to quantify NADH/FAD (redox ratio) in wounds of diabetic and nondiabetic mice and found the ratio to be decreased in diabetic wounds, reflecting higher production of reactive oxygen species and greater oxidative stress.[192] This is an example of how AF allows for real-time determination of the metabolic state of wounds.

The ability of AF to noninvasively characterize various tissue compounds has been exploited to the benefit of patients with wounds. As previously mentioned, clinical signs and symptoms and classic swabbing techniques have significant limitations for the characterization of wound bioburden. One AF imaging device (LID, MolecuLight i:X, MolecuLight Inc., Toronto, ON, Canada) has been developed for fast characterization of wound bioburden. The device allows for the visualization of bacteria at loads $>10^4$ CFUs/g by using violet light to target bacteria-specific porphyrins that fluoresce red (*S. aureus* porphyrins) or cyan (Pseudomonal pyoverdine) in a composite image generated on the high-resolution LCD display, whereas background endogenous tissues (*e.g.*, collagen) show up as green.[193] By facilitating easy visualization of bacteria within the wound, LID has the potential to allow for more efficient targeted debridement and evaluation of debridement success.[193]

The LID has demonstrated promising results in clinical trials. A study on 33 patients with wounds of varying etiology found LID to have a positive predictive value (PPV) and negative predictive value (NPV) of 95.4% and 100%, respectively, compared with microbiological swab for the identification of clinically significant bacterial load in wounds.[194] The LID allowed for targeted sampling

of the wound bed with swabs, which may have otherwise resulted in false-negative readings.[194] A nonrandomized single blind trial of 60 patients with chronic wounds found a PPV of 100% compared with both quantitative PCR and semi-quantitative curettage culture for the identification of clinically significant bioburden.[195]

Another trial of 31 patients with DFUs found that LID more accurately identified the presence of moderate or heavy bacterial loads compared with the standard Levine swabbing technique (78% accuracy for AF vs. 52% with swabbing), and furthermore maximized the effectiveness of bacterial load swab sampling with no detrimental impact on clinical workflow.[196] Similar results were noted in other small cohorts of chronic wounds (sensitivity 72%, accuracy 74% when combined with clinical signs and symptoms) and pediatric burns.[197,198] Finally, the utility of LID to guide targeted debridement was observed in a study of 22 DFUs in which fluorescence imaging demonstrated significant remaining bioburden after initial debridement in each wound.[173]

Conversely, one group did not observe a clear benefit of LID in 14 patients with burn wounds, in which they observed a sensitivity and specificity of 78% and 64%, respectively, for AF-guided swabs compared with standard swabs. They did, however, note 100% sensitivity and NPV for *Pseudomonas*, highlighting the potential strength of LID in ruling out *Pseudomonas* infection in burns.[199] Their study may have been limited by their definition of a positive bacterial swab, as they considered any bacterial load to be positive whereas the LID does not detect loads below 10^4 CFUs/g. The exact level of bacterial bioburden that inhibits wound healing remains controversial.

DaCosta *et al.* used a different AF imaging device, operating on the same mechanism as LID, to assess 28 patients with wounds—primarily DFUs with various other etiologies. They found that AF identified clinically significant bacterial load in 85% of wound peripheries that were missed by conventional swab methods. Conventional visual assessment missed 50% of wounds with high bacterial load as identified by AF. During the longitudinal follow-up phase of 12 patients, it was observed that 90% of patients would have been sent home at least once with false-negative high bacterial loads that were detected successfully by AF. This represents missed opportunities to treat the wound early, resulting in increased chronicity and morbidity for the patient.[194,200]

The most promising utility of AF imaging for wound care appears to be the mitigation of false-negative results, that is, the identification of bacterial load that has yet to manifest clinical signs and symptoms or that may be missed by using conventional swabbing techniques.[200] Limitations of using AF for wound evaluation include the need for a dark room during imaging, which is not always easy or feasible. However, a disposable "DarkDrape" is available to create a portable dark environment in settings where lights can't be turned off. Some authors consider active bleeding or visual vascularized tissue to be a relative contraindication for AF modalities, as these characteristics show up black and may confound image interpretation.[194] Silver dressings have been noted to impart a similar effect.[194] In addition, the 405 nm wavelength blue light used only penetrates to a depth of 1–1.5 mm, meaning that deeper bacteria evade quantification.[200]

AF cannot differentiate between many commensal and pathogenic bacteria due to the similarities in endogenous chromophores across species.[200] Finally, since visualization of bacteria depends on their metabolic activity, bacteria in biofilm may not be visualized due to the decreased metabolic activity associated with the sessile bacterial phenotype.[193] Nonetheless, AF imaging is a promising modality to improve the care of patients with wounds by providing fast, simple, and effective feedback on bacterial bioburden, which merits further exploration in large clinical trials with homogenous wound etiologies.

Hyperspectral and multispectral imaging. Both multispectral imaging and hyperspectral imaging (HSI) (spectral imaging [SI]) are spectrophotometric approaches wherein data are collected at multiple points simultaneously from a wound area.[174] They involve the reconstruction of a reflectance spectrum of light in the visible to near-infrared wavelengths ($\sim$400–1100 nm) for every pixel in image by recording serial images of biologic tissue in multiple narrow spectral bands.[201] These data are processed into a 3D "data cube" from which two-dimensional (2D) maps of tissue parameters, most commonly oxygenation, are generated.[202] Although these modalities are quite similar, HSI typically has more spectral channels and better spectral resolution, with the trade-off being slower image acquisition times.

SI has most commonly been used to quantify tissue oxygenation of DFUs via the measurement of oxy- and deoxy-hemoglobin.[203] Two studies have observed an association between oxygenation of peri-wound skin and healing of DFUs, where increased peri-wound oxygenation was as-

sociated with an increased probability of healing at 6 months.[203,204] These studies then retrospectively derived "healing indices" based on SI oxygenation measurements to predict DFU healing, which offered higher NPV and PPV than the commonly used metric of wound size change in 4 weeks. The sensitivity and specificity for predicting healing were found to be 93% and 86%, respectively, in the first study, and 80% and 74%, respectively, in the second study.[203,204] Thus, SI may allow for the early identification of at-risk DFUs and spur more expeditious preventative measures.[204] These studies were limited by small sample size and poorly defined healing indices, and their results have not been confirmed in prospective studies.[205]

Building on these data, a commercial SI device (DeepView®; SpectralMD Inc., Dallas, TX) designed for advanced burn and wound care management demonstrated a sensitivity and specificity of 98.8% and 93.9%, respectively, when combined with a machine-learning algorithm in predicting which areas of a DFU would heal at 28 days.[206] Conversely, a more recent study using novel computational software for SI analysis of 43 patients with DFUs found a negative association between baseline oxygenation and healing at 12 weeks.[205] The authors attribute this to a possible inverse relationship between oxygenation of hemoglobin and extravascular tissue oxygenation in patients with microvascular disease or neuropathy (*i.e.*, microvascular shunting).[205] Further prospective studies are necessary to establish the utility of HSI for predicting DFU healing.

Yudovsky *et al.* retrospectively analyzed HSI data collected from 21 patients with DFUs and compared them with those from ulcer-free diabetics to try and predict the risk of ulcer formation.[207] Using images that were collected an average of 58 days before ulceration was clinically apparent, they found that SI predicted DFU formation with a sensitivity and specificity of 95% and 80%, respectively.[207] These claims have not yet been confirmed in prospective studies.

Spectral imaging techniques have historically been limited by their application in darker skin types, where melanin has been found to affect accurate determination of hypervascularization and metabolism, and to under-estimate hemoglobin oxygenation; however, these limitations may be diminished by utilization of more advanced SI techniques such as polarization-sensitive HSI.[208]

Optical coherence tomography. Optical coherence tomography (OCT) uses low coherence interferometry from optical laser beam scattering to provide structural information about the skin.[209] After projecting a focused laser beam into the skin, backscattered light from subsurface cutaneous structures enters the scanning system and interacts with a reference laser, providing measurements of the scattering intensity at each tissue depth.[210] This allows for visualization of skin microstructures to a depth of ~ 2 mm at a resolution of 3–5 μm.[209] It has been likened to "optical US" as it provides 2D cross-sectional images but uses IR light instead of sound waves.[211] Functional extensions of OCT have become increasingly popular and allow for the determination of a wide variety of parameters (*e.g.*, OCT angiography or doppler OCT for quantification of blood flow, spectroscopic OCT for molecular properties).[212] For these reasons, OCT is becoming increasingly accepted as a tool to diagnose basal cell carcinoma and other skin conditions.[210]

The OCT holds promise as a tool to allow for more effective wound research. Li *et al.* used phase-variance OCT to track regeneration of the vascular network in diabetic mice who received treatment with a pro-angiogenic ointment, demonstrating the ointment's efficacy.[213] OCT has also demonstrated significant value as a tool for translational research, as one group used it to evaluate epithelialization in an *ex vivo* human skin wound model. OCT accurately identified epidermis, papillary and reticular dermis, and migrating keratinocytes in the wound bed. It was, thus, able to demonstrate the efficacy of allogeneic adipose stem cells in increasing re-epithelialization speed. The advantages of OCT compared with histopathological analysis for such research include faster turnaround, avoidance of the deleterious effects of biopsy, and the ability to visualize the entire wound at once instead of small biopsy sections.[214]

OCT offers a way to noninvasively assess cutaneous wound-healing stages without the deleterious effects of biopsy. Studies have found OCT assessment to correlate well with histopathologically defined stages of healing in acute wounds in human subjects.[215] Some groups have used OCT to analyze chronic wounds. OCT was able to detect partial epidermal loss, vasoconstriction, vasodilation, and epithelialization in six patients with VLUs, pyoderma gangrenosum, and vasculitis.[216] Assessment of 15 patients with chronic lower extremity wounds (VLUs and mixed arteriovenous ulcers) revealed a unique abnormal peri-wound vessel morphology (glomerulus-like, believed to be associated with tissue growth) that differs from more distant peri-wound skin, or skin from the

contralateral extremity.[210] Thus, OCT has the potential to aid in the determination of wound etiology and assessment of wound healing, though its exact clinical utility has yet to be proven. A potential limitation of OCT is difficulty in image interpretation, although this may be assuaged by the continued development of machine-learning-based image interpretation approaches.

Future directions

Synthetic biology. Given the incredible complexity of the wound microenvironment and the delicate interplay of many processes required for normal tissue repair, it is a significant challenge to create diagnostics capable of evaluating the entire wound landscape and providing accurate spatiotemporal analytics to better inform treatment decisions. Synthetic biology is an exciting field with great potential to meet this challenge.

Synthetic biology seeks to engineer delicate control over cellular behavior and biological processes.[217] Synthetic biologists have developed tools that allow for rational engineering of biologic systems with specific, customizable functions.[218,219] This holds great promise for molecular diagnostics and has been used to develop portable, point-of-care biosensors.[220] One example, the "toehold switch," allows for cell-free translational expression of a target gene (encoding a reporter protein) to be activated by specific target molecules, providing a colorimetric readout.[220] Initially created with the ability to detect RNAs, recent technical refinements allow for a single toehold-switch sensor to respond to multiple targets, including RNA, DNA, proteins, and other small molecules.[220] Assays can be freeze-dried on paper or other porous material to facilitate transport and storage at room temperature.[219]

Paper-based synthetic biology platforms based on the toehold switch principle have been devised with the capability of accurately identifying and quantifying species-specific mRNA and clinically relevant host biomarkers (*e.g.*, calcitonin) from the gut.[221] One team recently combined cell-free biosensors with a nanostructural electrochemical interface to achieve expanded, multiplexed characterization of multiple antibiotic resistance genes in parallel.[219] Such coupling of gene-circuit based sensors with electrochemical systems allows for data to be shared easily with computational tools.[219] Limitations of the toehold switch include its very high cost and laboriousness, as time-heavy sequence optimization is required for each target of interest and subsequently the risk of design failure is high.[220] Efforts to mitigate the latter point using a multi-step transduction process are ongoing.[220]

The potential applications of diagnostics rooted in synthetic biology for wound care are limited only by the imagination. Combining the measurement of biofilm markers, polymicrobial metabolites, and antibiotic resistance genes could facilitate rapid and reliable infection detection as well as appropriate and prompt treatment choice. Other systems might combine intrinsic wound factors such as metabolic activity, inflammatory profile, and protease activity to further guide the delivery of therapy and allow for high-resolution tracking of healing response with subsequent fine-tuning of the treatment strategy. Synthetic networks designed to evaluate dysfunctional signaling pathways would provide insight into propensity of the wound to respond to a given treatment, for example, angiogenic growth factors for wounds defective in angiogenesis.[222] Multiplexing many such assays onto a single strip of paper as described earlier holds great promise for the evolving landscape of wound healing by providing instant feedback on wound status and the characteristics of the wound microenvironment (Fig. 6).

In addition, as care shifts to increasingly take place in the home and/or via telemedicine (TM) such a diagnostic platform could be shipped directly to patients or included in the tool kit of visiting care providers, helping to guide therapy and determine the need for in-person clinic visits. In the future, skin grafts or existing CTPs used for wound care might be genetically modified in such a manner so as to provide information about the microenvironment of the wound—for example, markers of "take," infection, or other factors. Genetically altered bacteria, that is, synthetic probiotics could also be created to respond to specific components of the wound microenvironment.[218]

Synthetic biology has the potential to create systems that are capable of intelligent and autonomous therapy delivery. Toehold-switch-based hydrogel templates have been engineered to allow for active burst release of chemotherapeutic drugs in response to cancer-specific DNA sequences.[223] Current gene and engineered-cell therapies, which involve modifying a cell's genetic programming to induce therapeutically beneficial behavior, have been limited by lack of fine control over the timing, strength, and cellular context of therapeutic delivery.[217] Building complex synthetic gene circuits into existing gene and engineered-cell therapies has the potential to refine their therapeutic execution by programming the cells to respond to microenvironmental queues in a sophisticated manner.[217] For wounds, this could be a genetically engineered skin substitute whose cells can detect

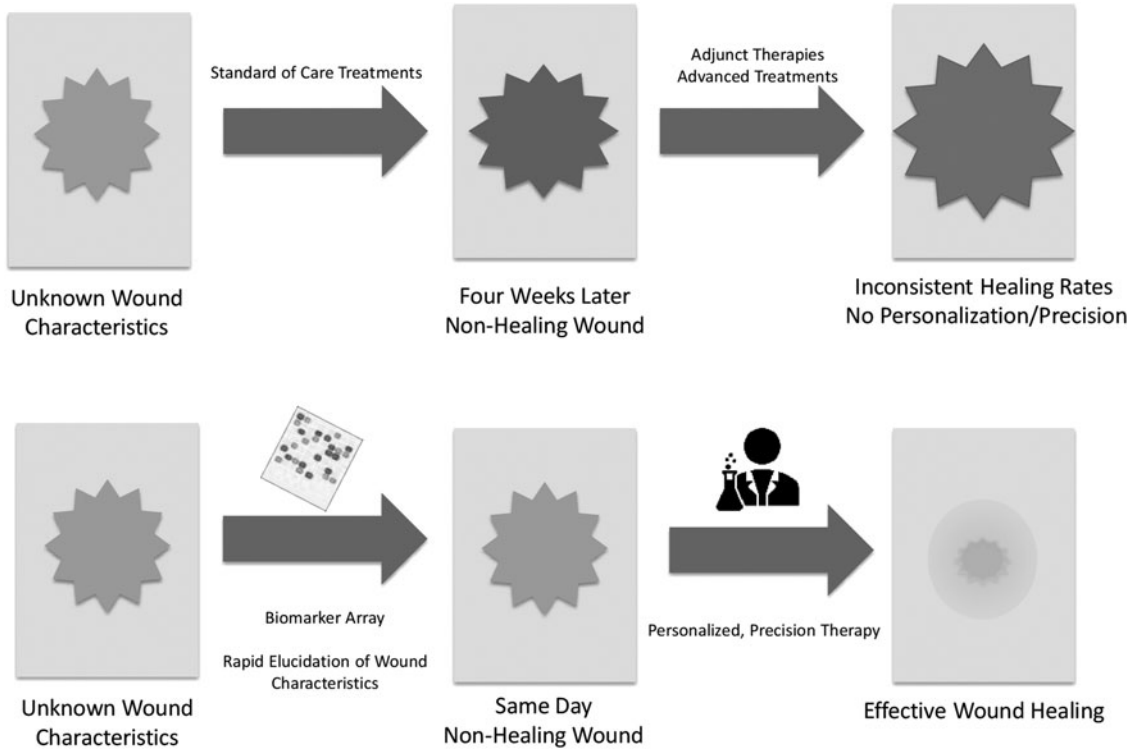

Figure 6. A theoretical diagnostic platform for wound biomarkers. The current approach to wound appraisal and treatment is limited by the time cost of determining healing status, as well as the inability of available treatments to achieve consistent wound closure and provide personalized/precision therapies. A diagnostic platform with an array of wound biomarkers would theoretically allow for instant determination of wound status and facilitate tailoring of specific, individualized wound therapies.[14]

antimicrobial resistance genes and respond by producing appropriate antimicrobial molecules or releasing pro-angiogenic factors in response to a deficiency of the same. Fibroblasts could be engineered to produce certain ECM molecules in specific amounts in response to the ECM composition of the wound, or cells might alter their contractility in response to the stiffness of the ECM. Biofilm-responsive elements could produce biofilm-degrading enzymes. These capabilities could be

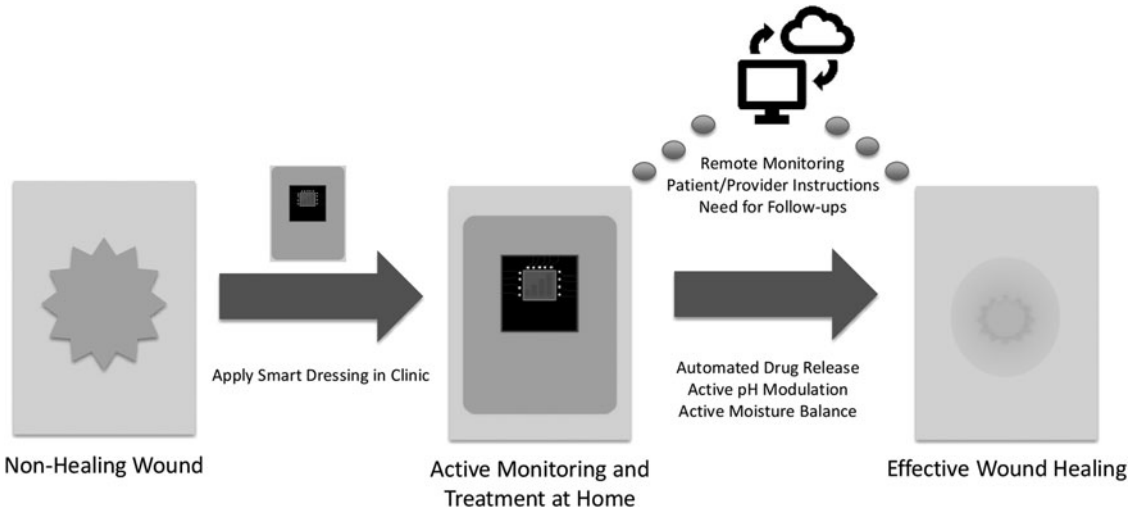

Figure 7. Connected "smart" dressings for wound care. As the wound care landscape continues to shift toward telemedicine, smart dressings have great potential to facilitate remote wound status monitoring. This could guide at-home wound care by providing instructions to patients or visiting clinicians, and determine the need for follow-up appointments. Smart dressings are also being engineered to deliver therapy in an automated fashion, which might include drug delivery (*e.g.*, antimicrobials), pH modulation, and moisture balance.[14,224,233,234]

combined into a single "smart" engineered tissue that is capable of measuring and treating multiple aspects of wound chronicity. A chief limitation of such an approach is the fact that cell-based approaches are quite laborious in nature, as they require that all processes are encoded inside a living organism.[219]

Although promising, difficulties inherent in synthetic biology approaches currently limit their development and clinical application. It remains difficult to engineer gene circuits that behave exactly as intended, often requiring many iterations to be built and tested at no small cost before a given goal is achieved.[217] A common reason for this is the fact that engineered circuits may fail to accurately capture the dynamic, heterogenous *in vivo* environment in which the circuit must operate—this is evident when considering, for example, the differences between cultured cell lines and primary cells. Two seemingly identical circuits often behave quite differently *in vitro* than *in vivo,* owing to often unaccounted-for contextual effects such as resource competition and other dynamic qualities of the cellular microenvironment.

Continued development of advanced biological circuit simulation software, as well as "organs-on-a-chip" or organ buds grown three-dimensionally *in vitro* may be helpful to overcome these challenges. Finally, as with any medical therapy the issue of ensuring safe and effective delivery to the patient's body is a potential hurdle. Immunogenicity is a potential complicating factor as either the delivery vector or delivered molecule may cause T cell sensitization or produce anti-"drug" antibodies.[217]

Nonetheless, as we continue to identify pathways that are deficient or altered in nonhealing wounds, our ability to engineer synthetic biologic networks as advanced diagnostic and therapeutic tools targeting the factors of wound chronicity will continue to grow. As such, the realm of synthetic biology holds significant promise for transforming wound care into a precision medicine.

The changing landscape of care: telemedecine and "smart" wearables. The increasing popularity of TM stands to change the clinical practice of wound care, especially for patients located in remote geographical areas.[224] The implementation of TM, optimally via real-time synchronous video conferencing, may enable better distribution of health services, promote equality of care, save patients' time and travel costs, and save room space for health care organizations.[225] Studies evaluating the clinical outcomes of TM are scarce, and those pertaining specifically to wound care are rarer still.[226] The results of these studies are mixed, and RCTs and comparative studies are lacking.[227] One RCT demonstrated greater size reductions for PUs at 12 weeks for TM compared with face-to-face visits,[228] whereas another RCT failed to reveal any changes in clinical outcomes due to TM.[229] One study showed that TM resulted in improved healing times and decreased amputation rates for DFUs,[230] whereas a different RCT on DFUs failed to recapitulate these results.[231] Nonetheless, these studies and studies on populations with nonhealing wounds of various etiologies have generally demonstrated noninferiority of TM approaches in achieving beneficial wound-healing outcomes.[225–227]

TM may reduce costs to patients and the health care system; studies have suggested savings of €4,583 euros per patient over 9 months for wounds of varying etiologies,[226] and savings of €2,039 euros per patient over 6 months in those with DFUs.[231] These results suggest that TM is a safe option to remotely manage wounds that may reduce time and money costs for patients and providers.[227] TM is limited by providers' inability to physically sense the wound and the inability to carry out manual procedures such as debridement, which require the physical presence of an experienced wound care specialist.[225] Further studies evaluating the quality of life follow-up, satisfaction of patients and staff, success in treatment implementation, cost reduction, and causes of hospitalization and mortality are necessary to coalesce the role of TM in the future of wound care.[225]

The use of TM for wound care will continue to increase. Even today, for most patients the management and treatment of wounds is conducted largely in the community.[232] This highlights the need for tools to facilitate the rapid, accurate, point-of-care evaluation of wound conditions by practitioners with various levels of training, expertise, and experience.[232] Given that certain clinical criteria or scoring algorithms (*e.g.*, NERDS/STONEES[15]) cannot be used in TM settings given the constraints of existing technologies, the ability to quantitatively monitor wound status at a distance and without removing the dressing would be of immense value.[232] The development of intelligent, centralized technologies is necessary to fulfill this need and would stand to reduce treatment times, minimize complications, and reduce costs.[232] "Smart" systems (*e.g.*, smart dressings, smart bandages) with the ability to measure, report, and even respond to wound conditions hold great potential to solve many of the aforementioned

challenges (Fig. 7).[224] Indeed, smart dressings have been engineered to measure wound parameters, including pH, temperature, oxygen, moisture, enzyme levels, and mechanical and electrical properties of skin.[224] Others have active drug delivery systems that release therapy in response to a specific external stimulus. For example, hydrogel-based dressings can release antibiotics in response to supposed markers of wound infection. These dressings respond to temperature or pH by using heat or light (UV or near-infrared) to release drugs from the hydrogel carrier into the wound.[233,234]

The key challenge with designing these dressings is identifying which parameters to measure and which pathophysiological processes to target.[224] Indeed, temperature and pH have not yet been characterized as reliable markers of wound infection. As such, there is a gap between the advanced platforms developed by researchers and the products that are used in current clinical practice.[224] There is a need for dressings that can detect more specific markers and perhaps release multiple drugs, and the development of such dressings will be facilitated by the ongoing refinement and characterization of accurate biomarkers for wound healing.[224]

CONCLUSION

The literature supports both the need for and the potential clinical utility of, a platform diagnostic that can monitor biomarkers associated with the healing process. Such a platform would stand to revolutionize the diagnosis of nonhealing wounds, catapult our understanding of the molecular and cellular mechanisms behind impaired wound healing to new heights, and usher in a modern age of therapeutic strategies for wound care. There is still much we do not know about the cellular and molecular mechanisms underlying impaired wound healing, and we still struggle to translate our basic science knowledge into effective point-of-care tools. As described in this review, the discovery and translation of many biomarkers and imaging systems, at various stages of their characterization and many with yet uncoalesced significance, is ongoing. Difficulty in designing high-quality randomized controlled trials is a key factor hindering the clinical implementation of these technologies Further work is necessary to ascertain the exact significance of these biomarkers as well as to engineer treatments that target them, thus transforming wound care into a precision medicine. Synthetic biology may offer an attractive solution to these challenges by allowing for the creation of advanced cell-based materials with the ability to dynamically respond to changes in the wound environment. The increasing popularity of TM and inception of smart "wearables" that are capable of remotely monitoring and even responding to wound conditions stand to improve global access to care and reduce time and money costs for patients and practitioners.

SUMMARY

Nonhealing wounds are a global pandemic with mortality rates and management costs exceeding many common cancers. Although our understanding of the molecular and cellular factors driving wound healing has grown, clinicians remain unable to consistently achieve wound closure. Standards for the diagnosis and evaluation of wounds remain subjective and imprecise, and nonhealing wounds often resist treatment with available therapies. There is a need to transform wound healing into a precision medicine; advanced wound diagnostic modalities (namely biomarkers and imaging systems) have the potential to achieve this goal by granting clinicians the ability to tailor individualized therapeutic regimens based on the unique molecular signature of a given wound.

Although many potential biomarkers have been identified, their translation into fast, effective, cost-efficient point-of-care tools remains challenging. MMPs and pH are the biomarkers with the best evidence to support their potential utility as prognostic indicators, and both have a multitude of ways by which they can be modulated. Other promising modalities include acute phase reactants, volatile emissions, and the use of artificial intelligence algorithms. A variety of other biomarkers are still in the early stages of investigation, and all require further translational and clinical research before they earn a spot in the clinician's arsenal. This highlights the need for a platform diagnostic that can quantify multiple diverse biomarkers simultaneously, which would be a valuable tool for both clinicians and researchers alike.

Imaging systems are similarly positioned; promising applications for AF, spectral imaging, and OCT in the realm of wound infection detection, wound healing research, and more have been identified, though further clinical validation of these systems is necessary. Finally, the future of wound healing as a precision medicine lies within the realms of synthetic biology, TM, and smart dressings as care delivery continues to shift into the home rather than the clinic.

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All authors made significant contributions to the conception, writing, and reviewing of the article, revision of intellectual and technical content; they gave final approval of the version to be published. All authors assume responsibility and accountability for the information contained herein.

AUTHOR DISCLOSURE AND GHOSTWRITING

M.A.W., H.A.L.-T., M.T. -C., and R.S.K.: None to disclose. W.D.L. is a founding member and current Chief Scientific Officer of Precision Healing Inc., as well as Chief Executive Officer of Precision Healing, Inc. R.W. is the Director of Research and Development for both Precision Healing Inc., and Precision Healing, Inc. D.S. is currently Vice President of Research and Innovation for both Precision Healing Inc., and Precision Healing, Inc. I.M.H. is currently the Senior Director of Biological Sciences at Precision Healing, Inc. No ghostwriters were employed in the writing of this article.

TAKE-HOME MESSAGES

- Nonhealing wounds represent an immense and ever-growing global pandemic, with incidence and mortality rates exceeding those of many common cancers.
- A nonhealing wound is typically considered to be any wound that "fail[s] to proceed through an orderly and timely process to produce anatomic and functional integrity."
- Existing standards for diagnosis and evaluation of nonhealing wounds are subjective and experiential, and such wounds frequently resist treatment with available therapies, making them a significant challenge for clinicians.
- There is a need to transform wound healing into a precision medicine wherein clinicians can deliver personalized therapeutic strategies based on the molecular characteristics of a given wound. Advanced wound diagnostics, specifically biomarkers and imaging systems, have the potential to achieve this vision.
- Although many possible biomarkers for wound healing have been identified, their translation into simple, cost-effective, and predictive clinical assays remains challenging. MMPs and pH have the best evidence to support their potential utility; however, these and the many other biomarkers thus far identified require further translational research and clinical validation before their role in the future of wound healing becomes clear.
- This highlights the need for a diagnostic platform tool that is capable of quantifying multiple, diverse biomarkers simultaneously in an array to facilitate high-quality studies across the research continuum.
- Similarly, advanced imaging systems may be useful in various domains of wound healing, such as infection quantification, early detection, and burn evaluation, but these would also benefit from further validation in large RCTs before they become a fixture in the clinician's arsenal.
- The future of wound care as a precision medicine lies in synthetic biology, TM, and smart dressings as the landscape of care continues to shift toward the home.

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Abbreviations and Acronyms

2D = two-dimensional
3D = three-dimensional
AF = autofluorescence
AUC = area under the curve
BSI = blood stream infections
CG = cathepsin G
CRP = C-reactive protein
CTPs = cell- and tissue-based products
DFU = diabetic foot ulcer
DTPIs = deep-tissue pressure injuries
ECM = extracellular matrix
e-nose = electronic nose
EPA = elevated protease activity
ESR = erythrocyte sedimentation rate
FDA = Food and Drug Administration
HBOT = hyperbaric oxygen therapy
HFUS = high frequency ultrasound
HNE = human neutrophil elastase
HSI = hyperspectral imaging
IDFU = infected DFU
IDSA = Infectious Diseases Society of America
IL = interleukin
ISS = Injury Severity Score
Kim-1 = kidney injury molecule 1
LFABP = liver-type fatty acid binding protein
LID = autofluorescence imaging device (MolecuLight i:X, MolecuLight Inc., Toronto, ON, Canada)
LYS = lysozyme
MMP = matrix metalloprotease
MMPI = matrix metalloprotease inhibitor
MPO = myeloperoxidase
MSI = multispectral imaging
NEPA = nonelevated protease activity
NERDS = Nonhealing, Exudative, Red/bleeding surface, Debris [yellow/black necrotic tissue], Smell
NIDDK = National Institute of Diabetes and Digestive and Kidney Diseases
NIDFU = noninfected DFU
NIH = National Institutes of Health
NO = nitric oxide
NPV = negative predictive value
NPWT = negative-pressure wound therapy
OCT = optical coherence tomography
PCR = polymerase chain reaction
PNA = pneumonia
PPV = positive predictive value
PU = pressure ulcer
RCT = randomized-controlled trial
ROS = reactive oxygen species
SCV = small-colony-variant
SI = spectral imaging
STONEES = Size increase, Temperature increase, Os probe to/probe to bone, New or satellite areas of breakdown, Exudate/Erythema/Edema, Smell
TIMP = tissue inhibitors of metalloprotease
TM = telemedicine
TNFα = tumor necrosis factor alpha
UA = uric acid
US = ultrasound
USWR = U.S. Wound Registry
VLU = venous leg ulcer
VOC = volatile organic compound
WCP = WoundChek Protease