Biology and Biomarkers for Wound Healing

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Abstract

Background—As the population grows older, the incidence and prevalence of conditions which lead to a predisposition for poor wound healing also increases. Ultimately, this increase in non-healing wounds has led to significant morbidity and mortality with subsequent huge economic ramifications. Therefore, understanding specific molecular mechanisms underlying aberrant wound healing is of great importance. It has, and will continue to be the leading pathway to the discovery of therapeutic targets as well as diagnostic molecular biomarkers. Biomarkers may help identify and stratify subsets of non-healing patients for whom biomarker-guided approaches may aid in healing.

Methods—A series of literature searches were performed using Medline, PubMed, Cochrane Library, and Internet searches.

Results—Currently, biomarkers are being identified using biomaterials sourced locally, from human wounds and/or systemically using systematic high-throughput "omics" modalities (genomic, proteomic, lipidomic, metabolomic analysis). In this review we highlight the current status of clinically applicable biomarkers and propose multiple steps in validation and implementation spectrum including those measured in tissue specimens e.g. β-catenin and c-myc, wound fluid e.g. MMP's and interleukins, swabs e.g. wound microbiota and serum e.g. procalcitonin and MMP's.

Conclusions—Identification of numerous potential biomarkers utilizing different avenues of sample collection and molecular approaches is currently underway. A focus on simplicity, and

consistent implementation of these biomarkers as well as an emphasis on efficacious follow-up therapeutics is necessary for transition of this technology to clinically feasible point-of-care applications.

Introduction

Wound healing is a multifaceted process governed by sequential, yet overlapping phases including: hemostasis, inflammation, proliferation and remodeling. This process is executed through communication between the various local skin compartments, supporting extracellular matrix (ECM) and systemic contributors. Under physiological conditions in a healthy individual the process of cutaneous epidermal repair is highly efficient; however, when this process stalls tissue fails to regain structural and functional integrity resulting in chronic wounds. In the context of an increasing elderly population with a myriad of progressively prevalent disease states such as diabetes, vascular diseases, and obesity, clinicians, particularly those in surgical specialties and physicians treating healing disorders daily encounter chronic non-healing wounds including diabetic foot ulcers (DFUs), venous leg ulcers (VLUs) and pressure ulcers (PUs).

It is estimated that the number of people with chronic wounds in the US is over 6.5 million and continues to increase, as population ages and incidence of diseases predisposing to poor wound healing escalate[1,2]. The economic impact of the cost of care for such wounds is significant, with projections in the billions in the US alone[3]. In addition to morbidity, chronic wounds pose significant mortality, with a 5 year rate for DFUs and ischemic ulcers being higher than commonly encountered cancers such as breast and prostate[4–6]. Furthermore, in cases in which amputation is necessary treatment approach the 5-year mortality rate is nearly 50%[6].

Despite an obvious alarming clinical need and extensive studies conducted in recent years, basic science and clinical research targeted at understanding and improving delayed healing is still lagging due to multifactorial reasons. Patients with chronic wounds usually consist of elderly people with multiple comorbid diseases in addition to poly-pharmacologic effects that make patient-to-patient variability a significant challenge. This variability further underscores the need for large-scale studies and personalized approaches. Furthermore, such complexity of the disease presents a challenge in development of suitable pre-clinical animal models to study chronic wounds and potential treatments, further limiting translation from pre-clinical trials to clinic[7].

Tools that can be used to better direct therapy, predict healing outcomes, monitor disease progression and measure response to treatment are in great need. Here we focus on the pathophysiology of chronic wounds and describe how knowledge gained is utilized in a journey of discovery of molecular biomarkers that may, in the near future, allow for personalized, efficacious and cost effective treatment.

Biology of Wound Healing

Physiologic wound healing initiated after tissue injury jumpstarts a signaling network between different cell types and compartments of the skin. Upon injury, hemostasis results

in the formation of a fibrin clot whose constituents serve both as a scaffold and a source of growth factors and chemokines that recruit a gambit of cells, including inflammatory cells, to migrate into the wound bed[8–10]. Changes in the expression of numerous adhesion molecules on endothelial cells allow for the recruitment and extravasation of neutrophils and macrophages from the circulation to clear potential infection. These inflammatory cells release growth factors and cytokines including interleukins (IL1, IL6), tumor necrosis factor-α (TNFα), platelet-derived growth factor (PDGF), and fibroblast growth factor-2 (FGF2)[2]. Fibroblasts then proliferate and lay down provisional matrix over which keratinocytes migrate from the wound margins. In response to injury, keratinocytes at the wound edge release cytokines to communicate that the barrier has been compromised[2]. Together with simultaneous rearrangement of integrins and cytoskeletal components and the expression of proteases such as matrix metalloproteinases (MMPs), keratinocytes are able clear a path for migration along the interface between the fibrin clot and the underlying dermis. This process deemed re-epithelization is followed by keratinocyte proliferation at the wound margin thus reestablishing stratified epidermis[11]. The integrity of the epithelial barrier is then restored and the remodeling of the underlying matrix resumes.

Aberrant execution of normal repair mechanisms can result in healing impairment and development of chronic wounds. Chronic wounds are defined by inadequate progress through a timely sequence of anatomic and functional restoration[12]. They can broadly be categorized as fitting into one of 3 major etiologic groups: vascular insufficiency (e.g. VLUs), diabetes mellitus (e.g. DFUs) and local-pressure effects (e.g. PUs). Additionally, various systemic factors can impair healing[2]. However, at the heart of non-healing wounds are shared characteristics irrespective of distinct wound etiologies: presence of prolonged and sub-optimal inflammation and concurrent infection, deregulation of proteases, reduced growth factor activity, stem cell dysfunction, and cellular senescence[13–15].

Prolonged sub-optimal inflammation in the wound microenvironment, elevated levels of MMPs and reduced levels of their inhibitors, all contribute to inhibition of cell migration and healing[13,16–18]. Perpetuating this inflammatory state is polymicrobial infection of the wound; often with microbes capable of forming biofilms that together sustain the influx of proinflammatory cells while respectively hampering the "host response" to infection[17,19–22]. Impaired angiogenesis and vasculogenesis, through aberrant regulation and cleavage of growth factors, and their receptors leads to insufficient oxygenation and inadequate delivery of nutrients to the wound[23,24]. Epidermal stem cell dysfunction due to constant cycling and subsequent depletion together with deregulated chemokines necessary to recruit bone marrow and endothelial progenitors leads to inadequate healing[11,25]. Furthermore, prematurely senescent fibroblasts and keratinocytes secrete elevated levels of MMPs, antiangiogenic factors and ILs[26], leading to de-regulation of their target genes subsequently contributing to a delay in healing. Any number of these de-regulated molecules and cellular processes can be potentially utilized for diagnostic purposes and monitoring of healing progression.

Biomarkers and Their Use

A biomarker is an objectively quantifiable substance that can be assessed as an indicator of a normal physiological or pathological process, or a pharmacologic response to a therapeutic intervention[27]. As such, although not mutually exclusive, biomarkers can be categorized based on the type of information they provide as predictive, diagnostic and indicative (Figure 1). *Predictive biomarkers* can be used to predict outcomes or provide likelihood of benefit from treatment. They can serve as a powerful tool in tailoring treatment modalities for specific subsets of patient populations. *Diagnostic biomarkers* can be utilized to identify the presence of a single or multiple factors that have the potential to influence the clinical outcome. An *indicative biomarker* can be utilized to monitor disease progression and/or response to therapy in real time.

The development of biomarkers that are clinically applicable first involves the discovery process of finding molecules that inhibit or regulate healing followed by their validation in patients (Figure 2). Multiple high-throughput "omics" modalities using human wounds as a source of biomaterials are being employed to understand the mechanisms of impairment, thus facilitating the discovery of potential biomarkers[11,13,17,28–31]. It is hoped that such biomarkers will allow personalized assessment, provide predictions of patients' healing status and insights into therapeutic approaches.

In order to assess the presence or follow dynamics of a biomarker during wound healing, specific molecular/cellular biology approaches are utilized to analyze biomaterials. Potential sources of biomaterials include wound fluid and swabs, tissue specimens and patient serum (Figure 2) each contributing different information. Wound fluid can be collected for protein and lipid analyses and for culturing of wound-associated inflammatory cells[32–34]. Wound swabs provide biomaterial that can be used for semi-quantitative and qualitative microbiology, global "omics" and selective proteomic evaluation, including specific proteases and their regulators. Histopathology, biomarker diagnostics, generation of primary cell cultures, quantitative and qualitative microbiology and profiling "omics" can all be ascertained using wound tissue specimens, highlighting the versatility of this type of biomaterial. On the other end of the biomarker spectrum, systemic markers of wound healing can be assessed using blood or serum from patients alone or in conjunction with the aforementioned sources. One can speculate that in the near future, change and/or presence of an associated biomarker will be used as one of the endpoints in clinical trials. However, evidence based data including extensive characterization and validation in multicenter clinical trials will likely be necessary, prior to transitioning to point-of-care.

Current Status of Biomarkers in Chronic Wounds

Tissue Biomarkers

Tissue specimens are an excellent source to obtain information regarding diagnosis, directing therapy and predicting outcomes, once molecular markers are identified and validated. Histology, in conjunction with different molecular markers, has long been established as a diagnostic approach for disease states such as vasculitis, kidney disease and

transplant rejection as well as its most well-known application in cancer and pre-cancerous lesions[35–37].

In the last decade, knowledge gained from patients' tissue specimens revealed the first molecular markers associated with healing impairment. Tissue from the edge of non-healing wounds demonstrates hyperproliferative epidermis, various degrees of fibrosis and increased cellular infiltrate[2,29]. Our laboratory has described multiple tissue-based molecular markers downstream of the Wnt signaling pathway: nuclear β-catenin and c-myc, to be clinically associated with healing impairment[29]. The Wnt pathway is important for skin development and homeostasis in early stages of development during patterning and morphogenesis, contributing to the post-natal control of hair cycling, maintenance/control of stem cells and cellular fate in the epidermal compartments[38,39]. Intriguingly, studies in our laboratory have found activation of β-catenin and its downstream target, oncogene c-myc, in the non-healing edge of chronic wounds, resulting in thickened, hyperproliferative and parakeratotic epidermis indicating aberrant proliferation and inappropriate differentiation[28]. Nuclear presence of these biomarkers in tissue specimens is quantifiable by use of immunohistochemistry (Figure 1, 3), highlighting their clinical feasibility and utility as a tissue biomarker. Currently, these two biomarkers are being further evaluated and encouraging preliminary data from our laboratory suggest that these two biomarkers may potentially have significant predictive and diagnostic power and their clinical validation is undergoing.

Additional studies regarding molecular and cellular pathophysiology of chronic wounds may also serve as potential diagnostic molecular tools and they are summarized in Table 1. However, small sample sizes utilized in these studies may mitigate potential clinical implications and large multicenter prospective trials are clearly needed to confirm these data and allow transition to the point of care for clinical use. Additional challenges for tissue-based biomarkers originate from standardization of specimen retrieval[40], logistics of service-based diagnostics (similar to pathology service) and incorporation of this approach into standard care and clinical trial protocols. Nevertheless, this approach may prove to be very valuable in early diagnostics that would facilitate more personalized treatment protocols.

Wound Fluid Biomarkers

The MMPs are metalloproteinase enzymes and their regulators, tissue inhibitor of matrix metalloproteinase (TIMPs), play important role in wound healing. Metalloproteinases are a group of endopeptidases that can be broadly categorized depending on their primary catalytic substrate: collagenases, gelatinases, stromelysins, matrilysins, and membrane-type MMPs[41–46]. MMPs mediate critical steps in almost every phase of the wound healing[47–49]. MMPs are required for a wound to heal properly at an appropriate level, in the correct location and for a precise duration of time. Thus, it is not surprising that chronically elevated levels of MMPs and reduced levels of TIMPS, or aberrations in their ratio have been correlated with non-healing in chronic wounds[15,50–57,58, 59,60]. Studies have shown that therapeutic interventions which lower MMP activity such as MMP absorbent collagen/oxidized regenerated cellulose dressings, promote healing of stalled wounds and that decreasing MMP-2 tissue levels are reflective of wound healing itself. These findings

implicate MMPs as potential diagnostic, predictive and indicative biomarkers for wound healing[61–64].

Quantitative assessments of MMPs as diagnostic biomarkers to predict wounds with poor healing potential are currently being tested in clinical trials[65]. In one study authors utilized wound fluid collected from PUs to assess MMP-9/MMP-1 ratio[55]. They show that the average MMP-9/MMP-1 ratio decreased significantly in healing wounds and that the ratio was significantly lower, at the time of collection, in wounds that healed over 36 days suggesting that MMP-9/MMP-1 ratio may serve as a predictive biomarker for PU healing[55]. Effect of topical negative pressure therapy was assessed by measurement of MMP9/TIMP-1 wound fluid ratio and MMP-9/TIMP-1 ratio was significantly lower after 10 days of treatment indicating biomarker utilization in the assessment of the effect of therapeutic modalities[66]. These findings have facilitated the development of technology that allows for point-of-care measurement of elevated protease activity (EPA) (including MMP-8 and MMP-9 levels) in the clinical setting using a swabbing technique[67]. Ideally, this point-of-care measurement would be used early to better direct care for those patients who would normally fail standard of care (SOC) therapy. It remains to be seen if patients identified to have EPA will benefit from employing protease reducing therapies at the initiation of treatment[68]. Currently, results are pending for two randomized controlled clinical trials evaluating the healing outcome of patients with DFUs or VLUs whose wounds were identified to have EPA. In the future it will also be important to more completely understand the subset of patients that show positive EPA and the underlying mechanisms leading to increased protease levels in order to develop molecular-based therapies.

Additionally, levels of multiple cytokines and growth factors have been found to be significantly higher in wound fluid of healing impaired VLUs[69,70] and may also serve as prognostic biomarkers for healing outcomes and are summarized in Table 1.

Overall collection of wound biomaterials for diagnostic purposes shows great promise and is under clinical development. Ideally, each of the diagnostic biomarkers would be coupled with specific guidelines of each particular clinical protocol. For example, if a predictive biomarker at the patients's initial visit shows that a wound is healing, one should continue using standard of care. If, however, the predictive biomarker indicates that the wound is non-healing one should implement a more aggressive treatment protocol (Figure 3). In spite of multiple challenges for clinical validation, one can envision their utilization and incorporation into standard clinical protocols in the near future. In the future, determination of specific targeted interventions that would benefit patients identified with such biomarkers is needed.

Systemic Biomarkers Associated with Wound Healing Impairment

It should come as no surprise that cross-talk between the body as a whole and local tissue impairment can be recognized on the molecular level. Therefore, along with local markers of non-healing, there have been studies showing the presence of systemic, healing-impairment-associated markers.

For instance, using serum from patients with acute combat wounds, systemic biomarkers indicative of non-healing have been identified[71,72]. In one such study patients were followed for 6 weeks and 24 cytokines and chemokines were quantified revealing an independent association between increased serum levels of IL-3 and IL-12p70 with heterotopic ossification and increased serum procalcitonin with failure to heal[73]. Another study evaluating serum levels of MMPs in acute combat wounds found elevated levels of MMP-2 and MMP-7 in patients who demonstrated impaired wound healing, defined as delayed wound closure >21 days past the injury or wound dehiscence[71].

Recently, Thom et al. showed that the number of CD34+/CD45-dim stem/progenitor cells (SPC) in a patient's serum and the cellular content of hypoxia-inducible factors can serve as predictors of healing outcome in DFU patients[74]. Results of this study show that more SPC entered the bloodstream in patients who healed than in those who did not[74]. One can further speculate how such approaches may be utilized to predict response to therapies, such as hyperbaric oxygen, or systemic cell therapies. In another study, plasma miRNA signatures in DFU patients displayed altered miRNA-200b or miRNA-191 levels compared to diabetic patients without ulcers. These miRNAs modulate cellular migration and angiogenesis via paracrine regulation of zonula occludens-1 to delay ulcer healing[75] and may be used to predict the risk of DFU development (Table 2).

Both blood and serum can easily be obtained from peripheral venous blood samples and are routinely collected for various clinical applications making serum biomarkers a feasible method for predicting healing outcomes if larger clinical trials validate biomarkers described above.

Wound Microbiota as Diagnostic Biomarkers

The human microbiome is the ecological community of commensal, symbiotic, and pathogenic microorganisms that quite literally share our body space. The skin serves as an important barrier for the body and is in direct contact with the outside world resulting in its colonization with a complex and dynamic microflora in a spatiotemporal manner[76,77]. Injury resulting in the compromise of the barrier leads to microbial influx and colonization of a wound; in response to which the host cells release anti-microbial peptides in an effort to maintain the bacterial load at a manageable level[4,22,78–81]. Chronic wounds, often harbor a high burden of polymicrobials prone to forming biofilms. The composition of biofilms differs according to wound etiology, location and clinical context but is dominated by *Staphylococcus, Pseudomonas*, and *Corynebacterium* generas including other anaerobic species[78,82,83]. Persistent polymicrobial infection in wounds has been suggested to contribute to poor healing by provoking a prolonged inflammatory phase secondary to sustained recruitment of pro-inflammatory cells[20,84]. Moreover, studies in animal models demonstrate that polymicrobial infection delays epithelialization[19].

Although high bioburden is known to contribute to non-healing, it can be clinically challenging to identify wounds harboring problematic microbes due to lack of clinical signs of wound infection, formation of biofilms and culture methods that underrepresent many bacterial strains[78,85,86]. Historically, the gold standard for extracting this information has been through culture based methods, however, numerous studies using molecular techniques

have now demonstrated that standard culture methods select for those microbes that survive best under the experimental laboratory conditions and do not reflect the true diversity of chronic wound flora especially with regard to anaerobic microbes[76,85,87,88]. When considering the impact of wound bioburden on healing outcomes three distinct facets of the wound microbiota need to be considered: total microbial load, microbial diversity, and the presence of pathogenic microbes, for most of which standard culture techniques are inadequate[78,82,86,89,90]. However, technological advances in DNA-sequencing now allow for the identification and quantification of microbial communities to such a degree that all of these facets can be addressed[91].

The next-generation sequencing methods utilize the 16S ribosomal RNA (rRNA) gene to evaluate bacterial phylogeny and taxonomy[91]. Using this technology wound microbiota "footprints" can be generated and while most studies to date have focused on only generating footprints, a few studies have assessed the correlation of these footprints with clinical measures of wound healing[17,85,92]. Gardner at al[85] found that DFU duration and depth positively correlated with relative abundance of anaerobic bacteria, while conversely finding a negative association with *Staphylococcus* abundance. In contrast, the most recent study focusing on DFU osteomyelitis identified *Staphylococcus* in all of the affected bone samples[17]. These findings highlight the potential of using wound microbiota footprints as biomarkers to predict the clinical outcome of wounds, although many other factors shown to influence wound microbiota must be taken into consideration. Therefore using personalized targeted topical anti-microbials, as already shown in retrospective studies, highlights the economic benefit of treating microbial infections identified with molecular techniques, claiming a 68% reduction in the total cost of care with better clinical outcomes as compared to SOC[93]. Sample collection with the use of a minimally invasive swabbing technique has been shown to provide similar representation of wound bioburden when compared to more invasive punch biopsies[17,91,94,95]. However, this approach does not come without challenges: technically questions remain about methods for sample collection as well as interpretation of data because molecular techniques do not discern between live and dead microbiome. Furthermore, longitudinal studies will be needed to properly stratify patients and extrapolate relationships between microbiome and healing outcomes. Although this technology is far from the bedside, looking forward, the future generation of microbiome-based biomarker profiles would help clinicians to better differentiate benign wound colonization from a pathogenic state of bioburden and guide better management and treatment[91].

The future: "omics"- based diagnostics

Traditionally, genomics, proteomics, lipidomics, metalobomics, and other "omics" have been utilized for research purposes[11,13,17,28–31]. However, one can envision their use as personalized diagnostics. As computational-based analyses are being developed for large data sets, better known as "big data", these personalized profiles may be coupled with other clinical diagnostics in electronic medical records to further identify specific disease pattern that can be followed by a specific, more customized, treatment plan. Although currently prohibitively expensive, technology developments are underway to decrease the costs of these analyses, making this approach feasible in the future.

Closing Remarks

Although the clinical field is desperate for better diagnostic tools, their development is slow. At the core of identifying clinically relevant biomarkers is the motivation for early recognition of patients that would both fail SOC therapy before starting treatment and would benefit from early therapeutic interventions tailored to their specific healing prognosis, ultimately resulting in improved and more cost effective outcomes. Many promising biomarkers utilizing different avenues of sample collection are currently in development. The transition to point-of-care clinically applicable technology will necessitate a focus on simplicity, economy and consistent implementation of these biomarkers as well as an emphasis on efficacious follow-up therapeutics.

Acknowledgments

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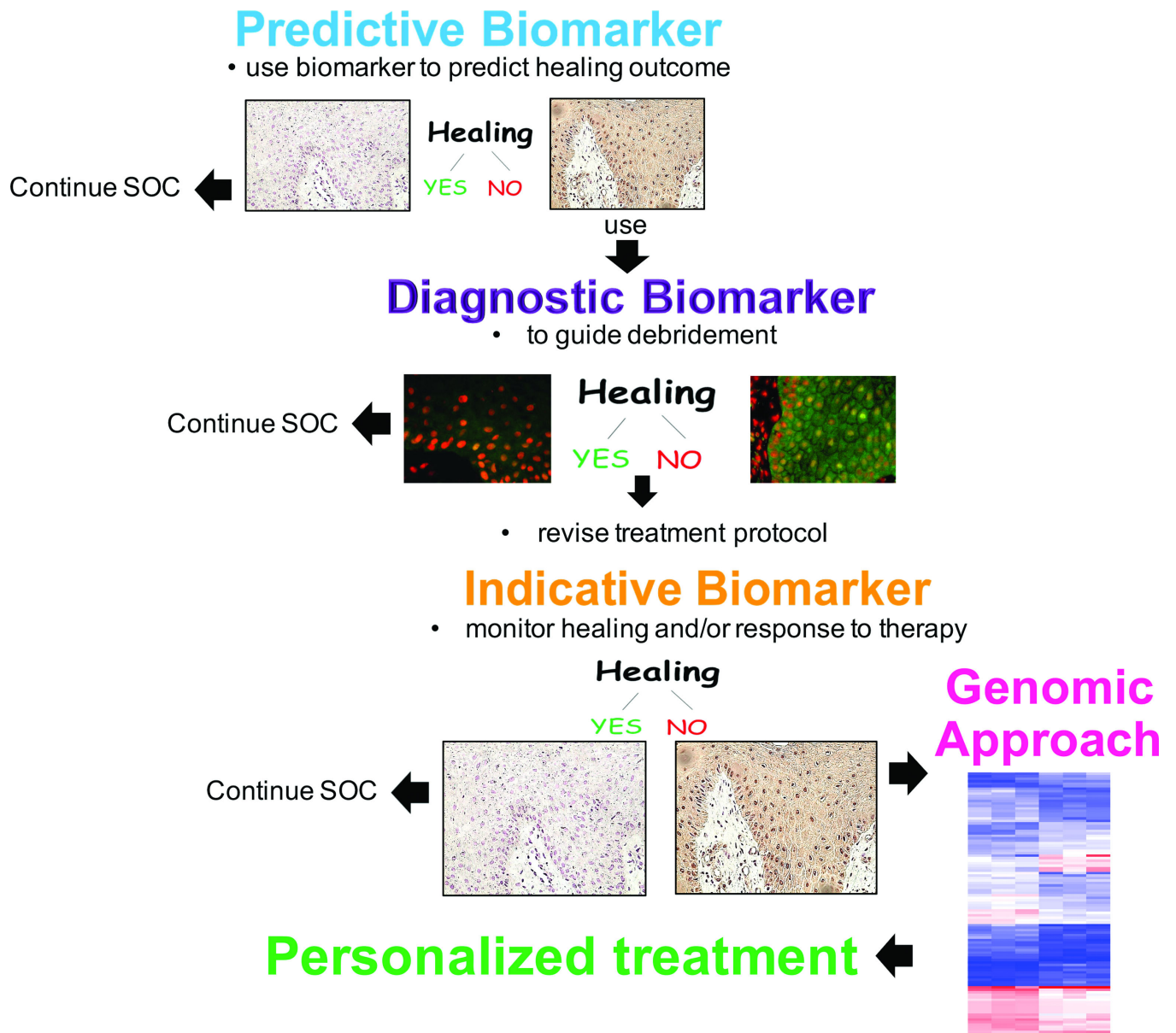
Figure 1. The future

The flow chart shows potential use of various types of biomarkers to personalize treatment approach in patients with chronic wounds.
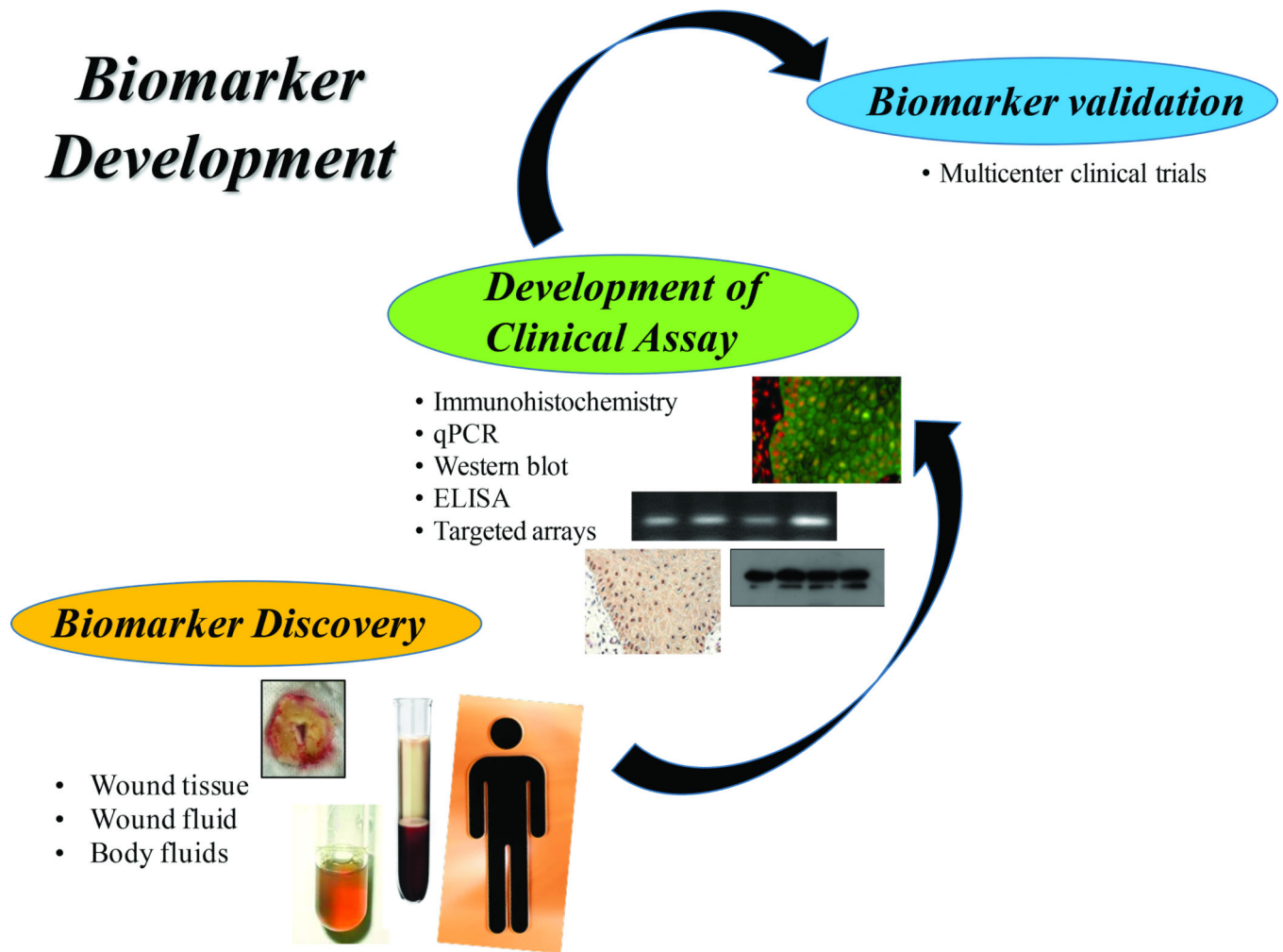
Figure 2. The long road of discovery and development of biomarkers
Steps in development of biomarker start from biomarker discovery, development of specific assay to be used in clinic and a final biomarker validation in multicenter clinical trials before it becomes available for use as a guiding tool by physicians in clinics.
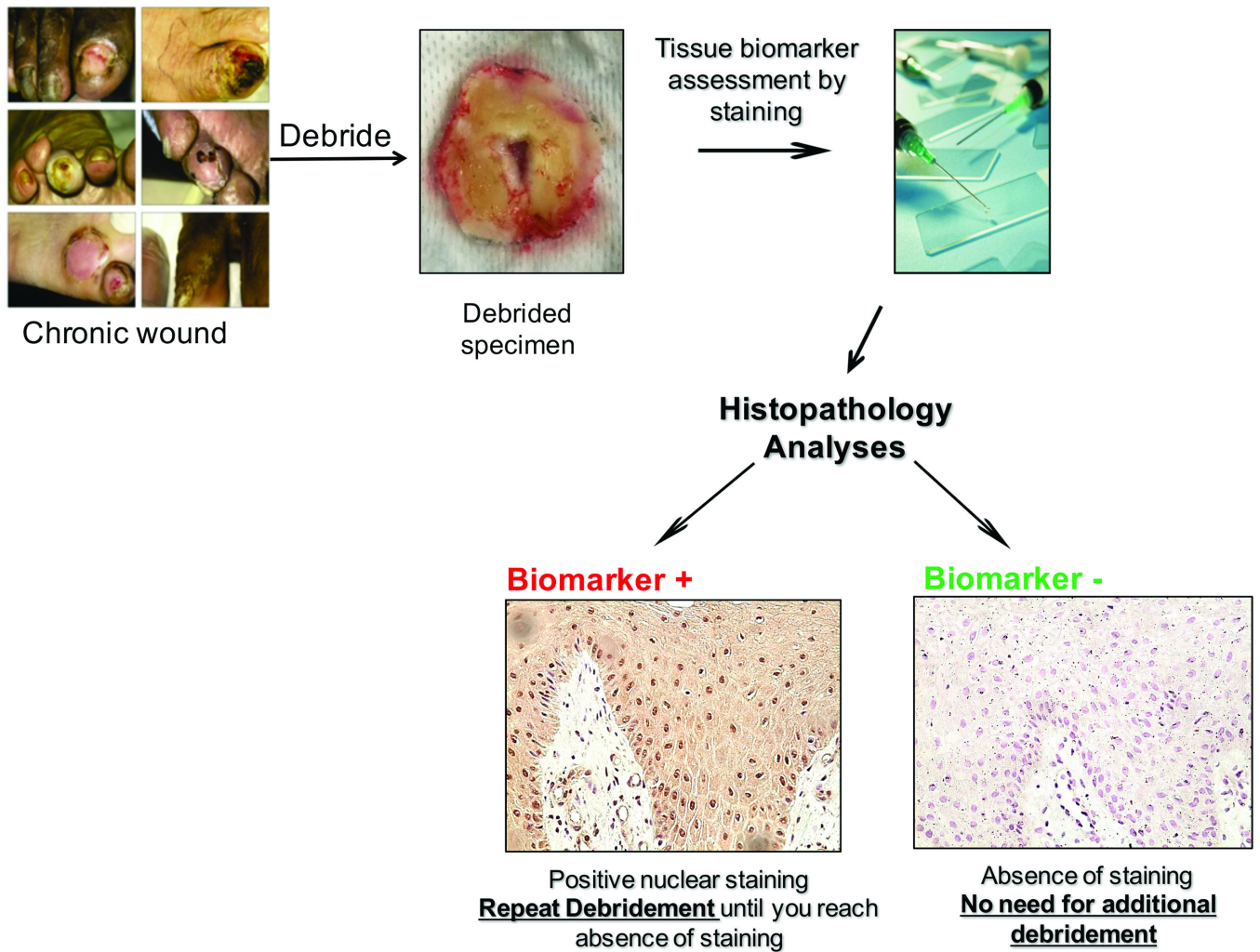
Figure 3. Biomarker-based guidance to debridement

Possible use of biomarkers as an indicator for a debridement margin e.g. how far wound care practitioner should debride a chronic wound.

Table 1

Healing Impairment Associated Tissue and Wound Biomarkers

Quantified Measure of Non-healing		Biomaterial/Method	Reference
Tissue Markers:			
↑ **β-catenin** ↑ **c-myc**	↑nuclear/cytoplasmic staining	_Biomaterial:_ debrided tissue _Method:_ immunostaining (IHC/IF)	14,28,29
↓ **BMPR** ↓ **LRIG1** ↓ **GATA3** ↓ **IDR2,4** ↓ **K15**	↓ gene expression ↓ gene expression ↓ nuclear staining ↓membranous staining	_Biomaterial:_ debrided tissue _Methods:_ microarray, q-PCR, IHC/IF	14
↑ **ADAM12**	↑ gene expression ↑nuclear/cytoplasmic staining	_Biomaterial:_ debrided tissue _Methods:_ microarray, q-PCR, IHC/IF	96
EGFR	↑ cytoplasmic staining	_Biomaterial:_ debrided tissue _Method:_ IHC/IF	3
↓ **cathelicidin**	↓ staining wound edge, wound bed infiltrate and exudate	_Biomaterial:_ debrided tissue _Method:_ IHC/IF	97
tPA, uPA, PAI-1	variable expression	_Biomaterial:_ debrided tissue _Methods:_ mRNA in situ hybridization, IHC/IF	98
↓ **TGFβI,II,III ligands**	↓ gene expression ↓ staining	_Biomaterial:_ debrided tissue _Methods:_ qPCR, IHC/IF	99
↓ **phospho-smad2**	↓ nuclear staining	_Biomaterial:_ debrided tissue _Methods:_ IHC/IF	100
↑ **miR-16, -20a, -21, -106a, -130a, -203**	↑ expression	_Biomaterial:_ debrided tissue _Methods:_ q-PCR, in situ hybridization	80,101
Wound Fluid Markers:			
↑ **MMP1, 2, 3, 7, 8, 9, 10, 11, 13**	↑ protein expression	_Biomaterial:_ collected using swabbing technique or wound vac _Methods:_ ELISA; Point-of-care qualitative measurement device available in select countries for clinical use (WOUNDCHECK™)	15,50–54,57,58, 70
↓ **TIMP1**	↓ protein expression	_Biomaterial:_ wound fluid _Methods:_ ELISA, quantitative gelatin zymography	56
↑ **MMP9: TIMP1**	↑ protein expression MMP9 ↓ protein expression TIMP1	_Biomaterial:_ collected using porous, inert hydrophilic dextranomer beads applied to the wound _Methods:_ ELISA, quantitative gelatin zymography	55
↑ **IL1** ↑ **IL6**	↑ protein expression	_Biomaterial:_ collected directly from wound bed _Methods:_ ELISA	69,70
↓**albumin** ↓ **total protein**	< 20g/l ↓ protein expression	_Biomaterial:_ collected using transparent occlusive dressing _Methods:_ Bayer Axon clinical chemistry analyzer	70

Table 2

Systemic Biomarkers of Non-healing

Quantified Measure	Biomaterial/Method	Reference
↑ procalcitonin	*Biomaterial:* serum *Methods*: Beadlyte1 Human 22-Plex Multi-Cytokine Detection System (Upstate/Millipore, Billerica, MA, USA)	[73]
↑ MMP3 ↑ MMP2	*Biomaterial:* serum *Methods* Luminex multiplex system; (Upstate/Millipore, Billerica, MA, USA)	[71]
↓ CD34+/CD45-dim circulating cells	*Biomaterial:* plasma, debrided tissue *Methods*: Flow Cytometry, Immunohistochemistry	[74]
↓ HIF1-2: HIF 3	*Biomaterial:* plasma, debrided tissue *Methods*: Flow Cytometry, Immunohistochemistry	[74]
↓ miRNA-200b ↓miRNA -191	*Biomaterial:* plasma *Methods*: Plasma-derived microRNA (miRNA) array, qPCR	[75]