Characterization of the Blister Fluid Proteome for Pediatric Burn Classification

Tuo Zang, ‡, §, † Leila Cuttle, ‡, || Daniel A. Broszczak, †, ¶ James A. Broadbent, ‡, § Catherine Tanzer, †, §, || and Tony J. Parker ‡, †, ¶

† Tissue Repair and Translational Physiology Program, Institute of Health and Biomedical Innovation, Queensland University of Technology, Kelvin Grove, Queensland 4059, Australia
‡ School of Biomedical Sciences, Faculty of Health, Queensland University of Technology, Brisbane, Queensland 4000, Australia
§ Wound Management Innovation Co-operative Research Centre, Brisbane, Queensland 4000, Australia
∥ Centre for Children’s Burns and Trauma Research, Queensland University of Technology, Institute of Health and Biomedical Innovation at the Centre for Children’s Health Research, South Brisbane, Queensland 4101, Australia
¶ School of Science, Faculty of Health Sciences, Australian Catholic University, Brisbane, Queensland 4014, Australia

Supporting Information

ABSTRACT: Blister fluid (BF) is a novel and viable research matrix for burn injury study, which can reflect both systemic and local microenvironmental responses. The protein abundance in BF from different burn severities were initially observed using a 2D SDS-PAGE approach. Subsequently, a quantitative data independent acquisition (DIA) method, SWATH, was employed to characterize the proteome of pediatric burn blister fluid. More than 600 proteins were quantitatively profiled in 87 BF samples from different pediatric burn patients. These data were correlated with clinically assessed burn depth and time until complete wound re-epithelialization through several different statistical analyses. Several proteins from these analyses exhibited significant abundance change between different burn depth or re-epithelialization groups, and can be considered as potential biomarker candidates. Further gene ontology (GO) enrichment analysis of the significant proteins revealed the most significant burn related biological processes (BP) that are altered with burn depth, including homeostasis and oxygen transport. However, for wounds with re-epithelialization times more or less than 21 days, the significant GO annotations were related to enzyme activity. This quantitative proteomics investigation of burn BF may enable objective classification of burn wound severity and assist with clinical decision-making. Data are available via ProteomeXchange with identifier PXD011102.

KEYWORDS: blister fluid, burn injury, proteomics, SWATH, burn depth, time to re-epithelialization

INTRODUCTION

Over the past decade, advancements in burn wound management and clinical intervention have resulted in a reduction in mortality rates; however, burn injury remains associated with significant morbidity.1 Consequently, recent studies have focused on pain reduction, promotion of wound healing, and improvements to quality of life.2−10 Improvements to quality of life are especially valuable in pediatric populations, as scar tissue formed from severe burn injuries lacks the ability to expand with the growing child.10 Contractures, which are caused by scar formation across joints, require long-term follow-up reconstructive surgeries until the cessation of the child’s growth to maintain full joint movement and permit unrestricted bone development.9,10 In addition, and contrary to adults, children suffer a longer postinjury period, which negatively impacts on their psychosocial well-being, rehabilitation, and their family.11

The scar formation and outcome of burn injuries are dependent on various factors, including burn size, burn depth, and time to re-epithelialization.12,13 The burn size is estimated by the percentage of total body surface area (% TBSA) and is based on clinical rules, such as the “Rule of Palm”, “Rule of Nines”, and the “Lund−Browder Chart”, and is relatively easy to diagnose by observation.14−17 Burn depth can be classified as superficial, superficial partial thickness, deep-partial thickness, and full thickness on the basis of the relative damage to the epidermis, dermis, and subcutaneous tissue.17,18 Ongoing tissue necrosis within the so-called “stasis zone” of a burn wound will result in an increase in burn depth within the first few hours postinjury.19 During this time, a patient’s burn can, for example, progress from superficial partial to deep-partial thickness. Adequate acute treatment, first aid, and immediate medical attention can limit this progression and subsequently influence the time to re-epithelialization and scar tissue deposition.

Received: May 21, 2018
Published: December 6, 2018
Currently, the diagnosis of burn depth is predominantly based on the treating clinician’s experience, and it can take up to 2 weeks to establish an accurate diagnosis.20,21 Both burn size and burn depth influence re-epithelialization time postinjury,22 which is an important predictor of severity and risk of hypertrophic scarring.23–25 Re-epithelialization of the wound within 21 days is also an important predictor of the need for skin grafting.23–25 The evaluation of both burn depth and time to spontaneous re-epithelialization are time-consuming and subjective, especially the assessment of intermediate depth burns, which can sometimes be inaccurate and unreliable.26,27 Thus, objective and timely quantitative measurements that can reflect the physiological state of a burn wound could assist with clinical diagnosis. Burn injury elicits a complex cascade of molecular responses that result in the presence of a diverse complement of proteins at the wound site.28–32 The resulting burn blister fluid or wound exudate is a useful liquid tissue that reflects both systemic and local microenvironmental responses.33

To determine if burn blister fluid could be used to quantitatively classify burns according to burn depth or time to re-epithelialization, we analyzed the proteome of burn blister fluid from a pediatric population to profile the biochemical differences. Analysis of the quantitative data using multiple statistical and bioinformatics approaches revealed key proteins that were associated with burn depth and time to re-epithelialization. This analysis may have identified some burns, which were incorrectly diagnosed (e.g., burn depth) by the clinical team.

### Materials and Methods

**Patient Recruitment and Sample Collection**

Ethical approval for the collection of blister fluid and clinical data from pediatric burn patients presenting to the Royal Children’s or Lady Cilento Children’s Hospitals (Brisbane, Queensland, Australia) was provided by the Children’s Health Queensland Human Research Ethics Committee (HREC/14/QRCH/10) and the Queensland University of Technology Human Research Ethics Committee (#HREC/140000551).

Blister fluid samples were collected, as previously described,34 during routine blister de-roofing procedures. Briefly, the blisters were punctured by a needle or scissors and the effluent fluid was aspirated with a syringe or collected using 200 μL ringcaps capillary pipettes (Hirschmann Laborgeräte, Eberstadt, Germany). The fluid was centrifuged at 855 X g to remove cellular debris prior to storage of aliquots at −80 °C in Lo-bind Micro tubes (Eppendorf; North Ryde, New South Wales, Australia). Patient clinical data were collected from patient medical records.

A total of 101 samples from 89 patients were collected, with some samples collected from different anatomical regions of the body on the same patient. The protein concentrations of the blister fluid samples were estimated using a Pierce BCA protein assay kit (Thermo Fisher Scientific, Scoresby, Victoria, Australia).

**Two-Dimensional SDS-PAGE and In-Gel Digestion**

Highly abundant proteins, such as albumin, Immunoglobulin (Ig)G, IgA, transferrin, haptoglobin, and antitrypsin, constitute approximately 85% to 90% of total protein in serum and may mask the detection of less abundant proteins.35 Consequently, 0.45 mL Human-6 Multiple Affinity Removal Spin (MARS) cartridges (Agilent Technologies, Santa Clara, USA) were used according to the manufacturer’s instructions to remove these highly abundant proteins from a subset of 12 samples (superficial partial thickness n = 6, deep partial thickness n = 3, and full thickness n = 3). Following depletion, 10 μg of each depleted sample was subjected to two-dimensional gel electrophoresis (2D-GE) analysis using a standard protocol.36 The gels were stained using Krypton Infrared Protein stain (Thermo Fisher Scientific) and scanned at 700 nm with a laser intensity of 4.0 using a Li-Cor Odyssey Infrared Scanner (Li-Cor Biosciences, Lincoln, USA) for quantitative analyses. Protein spot analysis using ImageJ (National Institutes of Health, Bethesda, USA) plugins BUnwarps and watershed was performed as previously described.37 The intensity of individual spots was normalized to the combined intensity of all spots on individual gels and significant differences between cohorts were determined by Student’s t test using R statistical software (Version 2.8.1, 12/2008; www.R-project.org).38 From 2D sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) gels, eight statistically significant protein spots (Figure S1, outlined in pink) were excised. In addition, one darkly stained 2D spot (Figure S1, spot 94) was used as a quality control for the in-gel digestion protocol.39 Further LC–MS/MS measurements were utilized to identify the proteins in these spots. The sample preparation and LC–MS/MS analysis process is described in more detail in the Supporting Information.

**Sample Preparation for SWATH**

Large-scale SWATH MS measurement was performed on all 101 collected samples to generate a more comprehensive biochemical profile of burn blister fluid. An aliquot containing 60 μg of total protein from each unprocessed BF sample was diluted with 50 mM ammonium bicarbonate (Sigma-Aldrich, Castle Hill, New South Wales, Australia) to a final volume of 18 μL. The proteins were reduced by the addition of 2 μL of 200 mM dithiothreitol (DTT) (Roche Life Science, Castle Hill, NSW, Australia) and incubated at 56 °C in an oven for 45 min, prior to alkylation with 2 μL of 400 mM iodoacetamide (IAA) (Merck Pty Ltd., Bayswater, Victoria, Australia) and incubation in the dark at room temperature for 20 min. Sequencing-grade modified trypsin (Promega, Alexandria, New South Wales, Australia) was then added to each sample to achieve an enzyme to protein ratio of 1:50 (w/w) prior to incubation in an oven at 37 °C overnight. The resulting tryptic peptides were desalted and concentrated using custom-made Stage-Tips prepared as described elsewhere.40 The eluted peptides were dried in a rotary evaporator and resuspended in 0.1% formic acid (FA) and 2% acetonitrile (ACN) prior to mass spectrometry analysis.

**Mass Spectrometry Analysis**

A liquid chromatography tandem mass spectrometry (LC–MS/MS) system consisting of an Eksigent 400 nanoLC system and a TripleTOF 5600+ mass spectrometer (SCIEX) was used to perform both data dependent and independent acquisitions (DDA and DIA, respectively). The peptides in each sample were separated by a 65 min gradient using a C18 nano-LC resolving column (Eksigent Chrom XP C18 CL-120, 3 μm particle size, 120 Å pore size, 75 μm × 150 mm) with a flow rate of 300 nL/min as previously described.41 The spectra were acquired in DIA mode with SWATH variable window widths, which were calculated using the AB Sciex SWATH Variable Window Assay Calculator Version 1.0 (June 26, 2014), from

---

*Journal of Proteome Research*
300 m/z to 1250 m/z with 100 window targets and a 1 Da window overlap.

**Ion Library Generation and SWATH Data Extraction**

The MS data from 2D SDS-PAGE was searched against the Human SwissProt/UniProt database (February 2012) using ProteinPilot (SCIEX) software and the Paragon Algorithm.

The peptide spectral ion library was developed using the DDA results as described previously, which included 645 proteins identified by at least one peptide and 14691 peptides at 95% confidence.

SWATH-MS data analysis was performed using the SWATH MicroApp (v2.0) plug-in for PeakView (v2.2) (SCIEX). The protein identifications were accepted as positive where five peptides were detected per protein, with five transitions per peptide, 95% peptide confidence score, 1% false discovery rate (FDR), four min peak detection window, and 70 ppm XIC extraction window. Prior to ion extraction, retention time (RT) recalibration was performed using five highly abundant albumin peptides spread across the gradient and detected across all samples. This resulted in a final detection of all the 645 high confidence multipeptide proteins in the ion library.

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the data set identifier PXD011102.

**Statistical Analysis**

Replicate samples (n = 12) obtained from different blisters on the same patient, a single blood blister sample (n = 1), and a significant outlier sample (very low chromatographic intensity in two LC–MS/MS detections, possibly due to faulty sample preparation) (n = 1) were excluded from further data analysis. Thus, there were 87 samples (Table 1) measured by SWATH LC–MS/MS that were subjected to additional statistical analysis. Furthermore, the data derived from an additional subset of samples that included those collected by negative pressure devices instead of directly from a blister (n = 4), samples with protease inhibitor added (n = 10), and samples that had not been initially centrifuged (n = 17) were also removed. Removal of these “varied processing” samples (n = 31) from the total samples (n = 87) left a “standardized processing” cohort of n = 56 samples (Table 2). As the sample aliquots were based on total protein amount, the relative abundance of every protein from each individual sample were normalized by the sum of abundance. To reduce the differences in the magnitude of abundance between different proteins, the quantitative data were logarithm transformed and subjected to mean-centered scaling.

The original 87 individual samples (and then the n = 56 “standardized processing” cohort) were grouped according to clinically determined burn depth (superficial partial, deep partial, or full thickness burns), which was diagnosed by the treating clinician. SWATH data were normalized in MarkerView (SCIEX) using the Area Sums technique, where any missing values were automatically replaced by naught. Subsequently, the data were log transformation and mean-center scaled using the default settings in MetaboAnalyst, which converted naught values to a small numerical value. Principle component analysis (PCA), partial least-squares-discriminant analysis (PLS-DA), ANOVA/Student’s t test, random forest (RF) analysis (number of trees = 5000, number of predictors = 10, randomness = fix current random seed), and correlation heat maps were produced using MetaboAnalyst 3.0 (http://www.metaboanalyst.ca/) June 2017). Volcano plots were generated using R, based on fold change and the p-values derived from MetaboAnalyst 3.0.

As re-epithelialization of the wounds within 21 days is the predominant predictor of whether or not skin grafting is required, the 56 samples in the “standardized processing” cohort were reclassified by time to re-epithelialization, as per each patient’s medical records and grouped into two cohorts (≤21 days and >21 days). The data were then processed as described above.

Hereafter, gene ontology (GO) enrichment analyses were performed on the proteins that exhibited the most significant abundance change according to Student’s t test (p < 0.05) and PLS-DA (the 25 most important proteins) in both burn depth

### Table 2. Patient Demographics and Burn Wound Characteristics for the “Standardized Processing” Cohort

|              | standardized cohort | Patients | N |
|--------------|---------------------|----------|---|
|              | range               | mean     | SD |
| Age (months) | 0.6–191.3           | 67.97    | 53.49 |
| Total body surface area (%) | 0.25–35 | 4.25 | 6.75 |
| Days post injury | 0–6 | 2.14 | 1.59 |
| Days to re-epithelialization | 4–115 | 22.21 | 21.97 |
| Protein concentration (mg/mL) | 13.60–90.81 | 42.97 | 17.41 |
| Gender | | |
| Male | 38 | 76.86 |
| Female | 18 | 32.14 |
| Depth (by clinical judgment) | | |
| Superficial partial thickness | 36 | 64.28 |
| Deep partial thickness | 18 | 32.14 |
| Full thickness | 2 | 3.57 |
| Mechanism of injury | | |
| Scald | 26 | 46.43 |
| Contact | 19 | 33.93 |
| Flame | 7 | 12.50 |
| Other (e.g., sunburn, chemical) | 4 | 7.14 |
and time to re-epithelialization classification, respectively. The GO analysis was performed using a Hypergeometric test and Benjamini–Hochberg FDR correction, with respect to biological process (BP), molecular function (MF), and cellular component (CC).

## RESULTS

### 2D-PAGE

Patient samples were initially classified into two groups: more severe (deep partial and full thickness burns with TBSA \(\geq 5\%\)) and less severe (superficial partial thickness burns with TBSA \(\leq 5\%\)). Following 2D SDS-PAGE, imaging, and image analysis using ImageJ, 99 individual spots were compared between the two groups, and eight were found to have significantly different intensity between the more severe and less severe burn cohorts \((p < 0.05)\) (Figure S1.). The relevant spots were identified following excision, in-gel digestion, and subsequent LC–MS/MS. The proteins identified included alpha-2-macroglobulin (A2M), prothrombin, apolipoprotein A-1 (ApoA1) (two spots), hemopexin, and three fibrinogen isoforms. Combined with the spot intensity measured from 2D-PAGE, A2M, prothrombin, ApoA1, and hemopexin exhibited higher intensity in the more severe burn group; however, fibrinogen exhibited lower intensity in more severe burns.

### SWATH MS

The 2D-PAGE LC–MS/MS analysis was an initial investigation of burn BF within a small sample size, and indicated the potential proteomic differences between different burn severities. A more extensive MS based approach (SWATH) was then utilized for quantitative analysis of a large number of samples. The 645 proteins included in the peptide ion library were quantitatively extracted in each individual sample. For robustness and reproducibility, proteins were filtered such that only those with \(<1\%\) FDR were accepted for further analysis. The samples were then classified by burn depth and time to re-epithelialization.
Burn Depth

Initially, all of the 87 samples were used in the analyses that were classified by burn depth. PCA was initially utilized for variable dimension reduction to examine the classification of burn depth based on protein abundance. The sample distribution from different clinically determined burn depths substantially overlapped and there was no clear boundary detected between the three burn depths (Figure S2). Subsequently, a supervised PLS-DA was conducted, which revealed a clearer distinction between the three clinical groups (cross validation of 3 components: $R^2 = 0.848$ and $Q^2 = 0.231$) (Figure 1A). A variable importance in projection (VIP) analysis arising from the PLS-DA revealed the top 15 proteins that most contributed to the separation of samples according to burn depth classification (Figure 1B). These included hemoglobin (Hb)-α, β, γ, and δ subunits; U3 small nuclear ribonucleoprotein protein IMP3, actin-related protein 2/3 complex subunit 1B, neutrophil collagenase (MMP8), myeloblastin, 60S ribosomal protein L12, 40S ribosomal protein SA, N-myc downstream-regulated gene 1 protein (NDRG1), myristoylated alanine-rich C-kinase substrate, macrophage-capping protein, and cellular retinoic acid-binding protein 2 (Figure 1B). To develop a more focused list of targets that show significant change between different burn depths, a machine learning—Random Forest algorithm was also used to provide a random variable classification that utilizes only the protein abundance. The top 15 proteins with highest mean decrease accuracy included C4-binding protein α-chain, complement factor D, 3-hydroxyacyl-CoA dehydrogenase type-2, serum albumin, clusterin, vitamin K-dependent protein S, complement component C6, pregnancy zone protein, coagulation factor X, plectin, Hb α subunit, α-actinin-1, hemopexin, apolipoprotein E, and kininogen-1.1 (Figure S3).

To more closely examine the difference in abundance of individual proteins between burn depths, pairwise comparisons of the degree of significance and fold change were conducted (Figure S4A,B). Hb subunits exhibited a higher abundance in deep partial thickness burns, compared to superficial partial thickness burns ($p < 0.05$) (Figure S4A). However, galectin 3-binding protein, insulin-like growth factor-binding protein 3 (IGFBP-3), and A2M, among others, exhibited significantly lower abundance in full thickness burns relative to deep partial thickness burns ($p < 0.005$) (Figure S4B). An analysis of variance (ANOVA), identified that there were 55 significant proteins (adjusted p-value (FDR) < 0.05 based on post hoc using Fisher’s least significant difference) that exhibited differential abundance levels between superficial partial, deep partial and full thickness burn groups. The relative abundances of the 15 most significant proteins across all depths in each individual sample were subjected to cluster analysis (Figure 1C). Hemoglobin subunits (HBA1, HBG2, HBG1, HBB, and HBD) exhibited a significant increase in abundance predominantly in full thickness burns. Conversely, IGFBP-3 and A2M exhibited a significant decrease in abundance in full thickness burns. From the sample clustering observed in the heat map, some of the samples from burns clinically classified as superficial partial and deep partial thickness burns by the treating clinician were clustered among the full thickness burn samples (Figure 1C red arrows). The clinical data for these particular burns indicate that while samples 173, 175, 217, 242, and 246 were derived from clinician-classified superficial partial and deep partial thickness burns, they took longer than 21 days to re-epithelialize, suggesting these burns may have actually been deeper than their initial clinical diagnosis (Table 3). Indeed, the burns from which samples 173, 217, and 242 were obtained were subjected to skin graft operations, which suggests they were more likely to be full thickness burns. Similarly, samples 189, 217, and 242 were derived from larger burns (>10% TBSA) and this may have contributed to the reason these samples displayed the biochemical profile of more severe injuries and were clustered with full thickness burns. However, there were two samples that were clustered with the full thickness burns that, according to the available clinical parameters, we were not able to discern a possible reason for the exhibited clustering.

Interestingly, there were a number of proteins that exhibited a significant difference in abundance and were highly ranked in multiple analytical approaches utilized (PLS-DA, ANOVA, volcano plot, and RF). The data for nine of these proteins was selected for closer examination, with particular reference to those previously identified by our 2D SDS-PAGE analysis and our previously published findings with regards to blister fluid Hb.34,46 Proteins that exhibited significant difference in abundance but which had multiple zero data values were excluded. In general, the abundance of hemoglobin subunits increased as the burn depth increased ($p < 0.05$) (Figure 2A,B,C). In contrast, 3-hydroxyacyl-CoA dehydrogenase type-2, c4b-binding protein alpha chain and hemopexin exhibited a higher abundance in superficial partial thickness burns ($p < 0.005$), but displayed similar abundance in deep partial and full thickness burns ($p > 0.05$) (Figure 2D,E,F). In addition, 60S ribosomal protein L12, IGFBP-3 and A2M exhibited lower abundance in full thickness burns compared to superficial partial and deep partial thickness burns ($p < 0.01$) (Figure 2G,H,I).

To account for any effects due to different sample processing conditions, the samples were further subdivided into “varied processing” ($n = 31$) and “standardized processing” ($n = 56$) cohorts. The significant proteins ($p < 0.05$) from ANOVA for each cohort were compared (Figure S5). Although there were 59 common proteins between the “total” cohort and the “standardized processing” cohort, there were still 107 and 53 unique proteins in the “total” and “standardized processing” cohorts, respectively. In addition, there remained a large proportion of proteins that exhibited a significant difference in abundance that were derived from the “varied processing” conditions.
Therefore, the analyses of burn depth classification and further classification based on time to complete wound re-epithelialization were performed on the “standardized processing” cohort only.

As there were only two samples from full thickness burns in the “standardized processing” cohort, the analysis could only be conducted between superficial partial and deep partial thickness burns (Figure 3). PLS-DA revealed a clearer distinction between these two burn depths compared to the total sample cohort (cross validation of 3 components: $R^2 = 0.954$ and $Q^2 = 0.114$) (Figure 3A). The 15 most important features from PLS-DA exhibited some of the same proteins identified in the analysis of the total cohort, such as U3 small nucleolar ribonucleoprotein protein IMP3 and Hb subunits $\alpha$, $\beta$, $\gamma$ and $\delta$ (Figure 3B). Similar to the results for the total cohort, the abundances for 3-hydroxyacyl-CoA dehydrogenase type-2; Cystatin-F; Hemopexin; and U3 small nucleolar ribonucleoprotein protein IMP3 were significantly decreased.
in deep partial thickness burns, while Protein S100-A2 and Hb subunits were increased ($p < 0.05$) (Figure S6). A cluster analysis indicated the relative abundance distribution of the 10 most significant proteins between each sample (Figure 3C).

Interestingly, two samples from the superficial partial thickness burns (samples 268 and 249) were clustered with the deep partial thickness burn samples (Figure 3C, red arrows) and two samples from deep partial thickness burns (samples 237 and 247) were clustered with superficial partial thickness burns (Figure 3C, blue arrows). The burn wound from which sample 268 was collected had a TBSA of 12% and re-epithelialization time of 47 days post injury (Table 4), which suggests it may have been clinically misclassified and was actually a more severe burn. Conversely, sample 247 re-epithelialized in 12 days, so it may not have been as deep as initially classified (diagnosed as deep partial thickness). The reason for the mis-clustering of the other two samples was unclear based on available clinical parameters.

**Receiver Operating Characteristic (ROC) Analysis**

Receiver Operating Characteristic (ROC) curves were generated from the abundance of single proteins or as ratios between the abundance of pairs of proteins identified from the “standardized processing” cohort, to determine their sensitivity and specificity in the classification of burn depth. The most accurate single protein predictor of superficial or deep partial burn depth was 3-hydroxyacyl-CoA dehydrogenase type-2, with an Area Under the Curve (AUC) of 0.747 (Figure 4A). As the ratio of abundance values between two proteins can increase the overall classification power, MetaboAnalyst was used to compute ratios of all possible protein pairs. The top ranked ratios were included in the data for a further multiple-feature ROC curve. The ratio between 3-hydroxyacyl-CoA dehydrogenase type-2 and C4b-binding protein alpha chain produced the greatest AUC of 0.867 (Figure 4B).

---

**Table 4. Clinical Parameters for the Samples That Were Classified by the Treating Clinician as Superficial Partial (S) Thickness Burns, but Were Clustered with Deep Partial Thickness (D) Burns (Red Arrows in Figure 3C), and Deep Partial Thickness Samples That Were Clustered with Superficial Partial Thickness Samples (Blue Arrows in Figure 3C)**

| sample | classified depth | time to re-epithelialization | % TBSA | arrow color |
|--------|-----------------|-----------------------------|--------|-------------|
| 268    | S               | 47                          | 12     | red         |
| 249    | S               | 12                          | 1      | red         |
| 237    | D               | 25                          | 7      | blue        |
| 247    | D               | 12                          | 5      | blue        |
ratios that produced high AUCs above 0.8 included 3-hydroxyacyl-CoA dehydrogenase type-2 and proline-rich acidic protein 1 (AUC = 0.855) (Figure 4C); 3-hydroxyacyl-CoA dehydrogenase type-2 and Serine/threonine-protein kinase H2.

Figure 4. ROC curves of specific protein and protein ratios can discriminate between superficial partial and deep partial burns in the "standardized processing" sample cohort. ROC curve and distribution of (A) 3-hydroxyacyl-CoA dehydrogenase type-2. ROC curve and distribution of the ratio between (B) C4b-binding protein alpha chain/3-hydroxyacyl-CoA dehydrogenase type-2. (C) 3-Hydroxyacyl-CoA dehydrogenase type-2/proline-rich acidic protein 1. (D) 3-Hydroxyacyl-CoA dehydrogenase type-2/Serine/threonine-protein kinase H2. (E) Galectin-3/3-hydroxyacyl-CoA dehydrogenase type-2. (F) cDNA FLJ55673 (highly similar to Complement factor B)/proline-rich acidic protein 1. (G) Multiple features ROC curve using linear SVM based on the ratios of B, C, D, E, and F. S = Superficial partial thickness burn; D = Deep partial thickness burn.
AUC = 0.836) (Figure 4D); galectin-3 and 3-hydroxyacyl-CoA dehydrogenase type-2 (AUC = 0.849) (Figure 4E); and cDNA FLJ55673 (highly similar to Complement factor B) and proline-rich acidic protein 1 (AUC = 0.849) (Figure 4F). A ROC analysis was then performed with these five protein ratios using a linear Support Vector Machine (SVM) classification algorithm that resulted in an AUC of 0.968 (Figure 4G). These data suggest that to achieve sufficient burn depth classification power it may be necessary to develop quantitative methods and validation of multiple proteins.

**Time to Re-epithelialization**

Clinically, a burn that re-epithelializes in less than 21 days has a better prognosis, with decreased risk of hypertrophic scar formation.23−25 Thus, we utilized a PLS-DA approach to determine if the protein profiles from the “standardized processing” cohort samples could discriminate between burns.
that took more or less than 21 days to re-epithelialize. We found that PLS-DA analysis revealed a clear demarcation between the ≤21 days and >21 days cohorts (cross validation of 3 components: $R^2 = 0.949$ and $Q^2 = 0.093$ (Figure 5A)). A subsequent VIP scores plot revealed that Catenin beta-1, U3 small nucleolar ribonucleoprotein protein IMP3 and various collagens were the most important proteins responsible for the observed group separation by PLS-DA (Figure 5B). Interestingly, collagen alpha chains exhibited a higher abundance in burns that took more than 21 days to re-epithelialize (Figure S7). However, collagen alpha chains exhibited a decrease in samples obtained from burns that took more than 21 days to re-epithelialize (Figure S7). In contrast to the previous cluster analysis, the cluster analysis based on time to re-epithelialization employed the 25 most significant proteins, as it showed better clustering relationships than when only 10 or 15 proteins were included (Figure 5C). There were some samples that were clustered within the >21 days group based on the blister fluid proteome but actually re-epithelialized in less than 21 days (Figure 5C, red arrows). Sample 189 came from a patient with 10% TBSA, which may influence the biochemistry such that the wound looks like it should take longer than 21 days to heal. However, it was also classified as a superficial burn. Sample 261 took 20 days, which was very close to 21 days and is thus more like a severe burn. Sample 214, 220, and 245 were diagnosed as deep partial thickness burns but had a small burn size, this may be the reason these burns re-epithelialized faster than expected from the wound proteome aspect (Table S5). However, the reason for the potential missclassification of sample 260 was not clear based on our current analysis.

| sample | depth | time to re-epithelialization | % TBSA | graft |
|--------|-------|-----------------------------|--------|-------|
| 189    | S     | 12                          | 10     | N     |
| 214    | D     | 9                           | <1     | N     |
| 220    | D     | 8                           | 1.5    | N     |
| 245    | D     | 17                          | 1.5    | N     |
| 260    | S     | 8                           | 3      | N     |
| 261    | S     | 20                          | 4      | N     |

Similar to the burn depth analysis, a series of ROC analyses of the time to re-epithelialization data were conducted. These showed that the ratio of protein abundance values between Complement C1r subcomponent-like protein and Thrombospondin-1 generated the highest AUC, 0.863 (Figure 6A). Additional ratios with a high AUC included Anthithrombin-III and Collagen alpha-1(I) chain (AUC = 0.854) (Figure 6B); Thrombospondin-1 and 6-phosphoglucuronate dehydrogenase, decarboxylating (AUC = 0.854) (Figure 6C); Collagen alpha-1(I) chain and Pregnancy zone protein (AUC = 0.846) (Figure 6D); Alpha-2-macroglobulin and Collagen alpha-1(I) chain (AUC = 0.833) (Figure 6E); Complement C1r subcomponent-like protein; and Ig kappa chain V-III region FR (AUC = 0.831) (Figure 6F). Again, similar to the analysis performed for the burn depth classification, a multiratio ROC analysis was performed with the above six ratios using a SVM classification algorithm that could discriminate between burns that re-epithelialized in more or less than 21 days with an AUC of 0.947 (Figure 6G). This demonstrated that an improved accuracy to predict time to re-epithelialization requires the quantitative measurements of multiple proteins.

A series of univariate, multivariate, and biomarker analysis (Student’s t test, PLS-DA, RF, and ROC) were performed on the proteome between different burn depth and time to re-epithelialization groups using the standardized processing cohort. We found that four proteins exhibited significant abundance change between different burn depth and time to re-epithelialization in three or more of the analytical approaches used (Table 6).

### Gene Ontology (GO) Enrichment Analysis of Significant Proteins

To examine the underlying biological responses that may be associated with burn depth and/or time to re-epithelialization, the proteins that exhibited significant difference in abundance between the various groups of the “standardized processing” cohort were analyzed using GO enrichment analysis.

For the burn depth classification, the proteins that exhibited a significant abundance difference ($p < 0.05$) ($n = 38$) from the Student’s t test and the top 25 most important proteins from the PLS-DA were analyzed respectively (Table S1). The top ten over-represented Biological Processes (BPs) included eight terms typically associated with cutaneous injury such as response to wounding; blood coagulation; coagulation; hemostasis; regulation of body fluid levels; oxygen transport; wound healing; and gas transport (Table S1). Interestingly, while terms associated with inflammation and immune responses were over represented in the broader analysis, they were not included in the top ten BP GO terms for either the Student’s t test or PLS-DA. There were two unique BP GO terms associated with each of the analytical approaches, platelet activation and regulation of proteolysis for the Student’s t test approach and response to stress and response to inorganic substance for the PLS-DA (Table S1).

Correspondingly, the Molecular Function (MF) GO terms, oxygen transporter activity oxygen binding, heme binding, tetrapyrrole binding, and iron ion binding were included in the top ten over-represented terms in both the Student’s t test and the PLS-DA results, clearly reflecting the high abundance of the hemoglobin subunits described in the analysis above (Table S2). Similarly, transition metal ion binding was also included in the top ten MF GO terms in the PLS-DA. Interestingly, the top ten Student’s t test results uniquely included the MF GO terms endopeptidase inhibitor activity; endopeptidase regulator activity; peptidase inhibitor activity; peptidase regulator activity; and enzyme inhibitor activity (Table S2). The proteins associated with these GO terms included alpha-2-HS-glycoprotein, pregnancy zone protein, cystatin-C and vitamin K-dependent protein S, whereas the top ten PLS-DA MF GO terms uniquely included cholate 7-alpha-dehydrogenase activity; testosterone dehydrogenase [NAD(P)] activity; 3-hydroxy-2-methylbutyryl-CoA dehydrogenase activity; and cysteine-type endopeptidase inhibitor activity (Table S2).

The three former were due to a single protein: 3-hydroxyacyl-CoA dehydrogenase type-2 and the later to two protease inhibitors, Cystatin-F and Kininogen-1.

With regard to the top ten Cellular Component (CC) GO terms associated with burn depth, blood microparticle, cytoplasmic membrane-bounded vesicle lumen, vesicle lumen, extracellular region, extracellular region part, and extracellular space were all over represented in both the Student’s t test and PLS-DA (Table S3). Extracellular exosome; extracellular membrane-bounded organelle; extracellular vesicle; and extrac-
Figure 6. AUC ROC curves of specific protein ratios can discriminate between burns that re-epithelialized in more or less than 21 days in the “standardized processing” sample cohort. ROC curve and distribution of the ratios between (A) Complement C1r subcomponent-like protein and Thrombospondin-1; (B) Antithrombin-III and Collagen alpha-1(I) chain; (C) Thrombospondin-1 and 6-phosphogluconate dehydrogenase, decarboxylating; (D) Collagen alpha-1(I) chain and Pregnancy zone protein; (E) Collagen alpha-1(I) chain and Alpha-2-macroglobulin; (F) Complement C1r subcomponent-like protein and Ig kappa chain V–II region FR; (G) Multiple features ROC curve using linear SVM based on the ratios of A, B, C, D, E, and F.
**DISCUSSION**

The results of this investigation demonstrated that the abundances of specific proteins in burn blister fluid change significantly with burn depth and time to re-epithelialization (healing). In relation to burn depth classification, the distinction between superficial partial thickness and deep partial thickness burns can often be clinically difficult to diagnose and both depths are often present in a burn injury concurrently, particularly for scald or flame burns.\(^7\) This may be reflected in the overlap between the proteome profiles of superficial partial and deep partial blister fluids observed in the PLS-DA (Figure 1A and B). In terms of clinical diagnosis, full thickness burns are relatively easier to identify from the other burn depths and, in this study, the protein profile of the full thickness group was the most distinct. This is perhaps unsurprising, as full thickness burns would likely produce the most significant changes in protein abundance due to the largest amount of cutaneous damage.\(^8\) Burn wound depth can change within a few hours or days postinjury, due to necrosis of the wound center and progressing damage to the surrounding area.\(^9\) This will largely delay the diagnosis of burn depth regardless of the clinicians’ experience or the use of more advanced photoacoustic or laser Doppler imaging of the burn, as these techniques are all based on the phenotypic changes at the wound site. Thus, the blister fluid proteome could possibly predict the final burn depth at an earlier stage of the injury response.

There were several individual blister fluid proteins identified by the statistical methods, which significantly contribute to the classification of burn depth or were involved in both burn depth and time to re-epithelialization. The abundance of hemoglobin subunits was found to increase with burn depth, which is consistent with previous Western blot and ELISA results published by our group.\(^46\) Our proteomics study on burn blister fluid demonstrates a release of all hemoglobin subunits at the wound bed and the abundance level was related to burn depth. Previously reported evidence indicates that the release of reactive oxygen species into the blood after a burn injury\(^48\) can cause erythrocyte fragility and decrease their half-life.\(^49,50\) Therefore, enhanced erythrocyte lysis potentially led to the detection of increased free hemoglobin in the blister fluid of deeper or more severe wounds. This is also consistent with other previous findings in plasma.\(^46,51\) Thus, the increase in erythrocyte lysis with increased burn depth may be caused

---

Table 6. Summary Table of Biomarker Candidates To Classify Different Burn Depths and Time to Re-epithelialization

| Clinical Parameter | Analytical Method | PLS | RF | t Test | ROC |
|--------------------|-------------------|-----|----|--------|-----|
| Depth              |                   |     |    |        |     |
| 3-Hydroxyacyl-CoA dehydrogenase-type 2 | Y       | Y   | Y  | Y      |     |
| C4b-binding protein alpha chain | Y       | Y   | Y  | Y      |     |
| Time to re-epithelialization |                   |     |    |        |     |
| Catenin beta-1 | Y       | Y   | Y  | –      |     |
| Collagen alpha-1(I) chain | Y       | Y   | Y  | Y      |     |

Cellular organelles were all uniquely overrepresented in the top ten CC GO terms from the Student’s t test. In contrast, hemoglobin complex, haptoglobin–hemoglobin complex, endocytic vesicle lumen, and cytosolic part were all uniquely over represented in the top ten CC GO terms from the PLS-DA and all due to the hemoglobin subunits included in the test set (Table S3).

The same analyses were conducted for the time to re-epithelialization groups (≤21 days and >21 days). Interestingly, unlike the GO terms associated with burn depth, there was less cohesion between the results for the Student’s t test and PLS-DA of the test set for time to re-epithelialization (Table S4–S6). The common BP GO terms between the two analytical approaches included platelet activation, platelet degranulation and response to wounding. The proteins in the test set associated with these GO terms included collagen alpha-1(I) chain (COL1A1) and collagen alpha-1(III) chain (COL3A1), and coagulation cascade proteins (Table S4). The unique BP GO terms over represented in the top ten Student’s t test results included peptide cross-linking; response to reactive oxygen species; response to oxygen-containing compound; negative regulation of endopeptidase activity; negative regulation of peptidase activity; regulation of cellular component movement; and cell activation, with COL1A1 and/or COL3A1 featured in most of these GO terms (Table S4). Interestingly, COL1A1 was over represented in almost all of the top ten BP GO terms from the PLS-DA except platelet degranulation. The unique BP GO terms over represented in the top ten terms from the PLS-DA included blood coagulation; coagulation; hemostasis; regulation of body fluid levels; wound healing; regulation of biological quality; and vesicle-mediated transport, with most of these represented by the same proteins from the test set (Table S4).

With regard to the top ten MF GO terms, five were common to both the Student’s t test and PLS-DA including endopeptidase inhibitor activity; endopeptidase regulator activity; growth factor binding; phosphatidylylcholine-sterol O-acyltransferase activator activity; and identical protein binding (Table S5). These were largely due to proteins associated with regulation of coagulation. Of the unique MF GO terms from the top ten Student’s t test results, peptidase inhibitor activity, peptidase regular activity, enzyme inhibitor activity, and enzyme regulator activity all exhibited the same over-represented proteins, which included complement C4–B, pregnancy zone protein, antileukoproteinase, α-2-macroglobulin, and vitamin K-dependent protein S. Interestingly, only COL1A1 and COL3A1 were over represented in the MF GO term platelet-derived growth factor binding, which was unique to the top 10 results from the Student’s t test analysis. There were five unique MF GO terms produced in the PLS-DA, including alcohol binding,
by both more severe thermal damage and/or increased exposure to reactive oxygen species.

C4-binding protein (C4BP) alpha chain abundance was found to be the most important protein for the classification of burn depth in random forest clustering (Figure S3) and its ratio with 3-hydroxyacyl-CoA dehydrogenase type-2 had the highest AUC of ROC analysis between superficial partial and deep partial burns (Figure 4A). C4b is an opsonin that can covalently bind to microbes to activate the complement system.52,53 In the classic complement pathway, C4BP acts to hydrolyze the complement fragment C4b and interferes with the assembly of the membrane-bound C3 convertase.52,53 Thus, it can inhibit the innate immune system through the inhibition of complement activation. *Staphylococcus aureus* can evade the immunological responses through recruitment of C4BP to its surface where it functions to inhibit C4 complement.54 It is possible that the decrease of C4BP alpha chain with the increase of burn depth might indicate that complement activation is enhanced in burns of increased severity.

Importantly, A2M exhibited one of the most significant abundance differences between burn depth classifications, with lower abundance in full thickness burns compared to superficial partial and deep partial thickness burns (Figure 1). This was interesting because the results from our pilot 2D-PAGE analysis suggested that there was a higher protein abundance for A2M in more severe burns compared to less severe burns (Figure S1). This discrepancy was possibly due to the low numbers of samples used to generate the pilot data (n = 6 per group); differences in the initial sample classification criteria as “more or less severe” rather than specific clinically diagnosed depth; and the sensitivity and robustness of the SWATH proteomics approach.

Among the three complement activation pathways, the classical and the lectin pathways require the binding of mannann-binding lectin (MBL) or C1q activator structures, to activate MBL associated serine proteases (MASPs).55,56 Interestingly, A2M can block the proteolytic activity of MASPs.55,56 Thus, it can inhibit the innate immune response through the inhibition of two complement activation pathways. Therefore, a decrease of both C4BP and A2M may indicate reduced inhibition of the innate immune system and an increased level of immunological response in more severe burns. Furthermore, a previous study has reported that the concomitant binding of C4BP and A2M can down-regulate complement under the influence of MBL.56 This may help to explain why C4BP and A2M both have lower abundance in more severe burn depths.

Interestingly, IGFBP-3 exhibited a significant decrease in full thickness burns compared with superficial partial and deep partial burns (Figure 4B). The functions of IGFBP-3 include the transportation of insulin-like growth factor I (IGF-I) in plasma, thus protecting IGF-I from degradation, and modulation of IGF-I binding with its receptors.57,58 Therefore, it may be reasonable to observe an increase in free IGFBP-3 in blister fluid from burns of greater depth, due to the severe tissue damage and perfusion of serum into the wound. However, IGF-I interaction with the extracellular matrix protein vitronectin is known to be mediated through binding with IGFBP-3, which dramatically promotes the proliferation and migration of primary skin keratinocytes and enhances keratinocyte protein synthesis.59–61 Thus, the observed decrease in IGFBP-3 levels in full thickness burn samples may be due to IGFBP-3 binding with vitronectin and IGF-I in the wound bed, resulting in depletion of free IGFBP-3 from the blister fluid. Alternatively, the observed IGFBP-3 decrease may be due to proteolytic degradation and subsequent release of IGF-I, possibly increasing IGF-I bioavailability in the wound site.62 This hypothesis is further supported by early research findings, which suggested that intact IGFBP-3 decreases granulation tissue synthesis.53 Interestingly, however, previous work by Abribat et al. (2000), demonstrated that proteolysis of serum IGFBP-3 also occurred in burn patients between 4 days to 16 days postinjury, which led to a decrease of serum IGF-I levels.64 Thus, it is possible that the reduced IGFBP-3 levels in deeper burns may be due to reduced levels of intact IGFBP-3 in the serum that has infiltrated the burn following severe burn injuries. Given the reported importance of the IGF axis on wound healing, further examination of the mechanisms underlying the stability of IGFBP-3 following thermal insult is warranted.

An analysis was performed on all samples as well as a “standardized processing” cohort to limit the potential variability in quantification of the blister fluid proteome due to sample collection or processing techniques. Statistical analysis indicated that the proteins quantified in the “standardized processing” cohort exhibited improved correlation with the clinical parameters. A limitation of this analysis was that there were relatively few full thickness samples collected that met the “standard processing” criteria as full thickness burns are not as common as burns of other depths for pediatric patients. Moreover, a large proportion of full thickness burns do not form blisters and therefore representative blister fluid cannot be collected from them.

Other biomarker studies have demonstrated that ratios of RNA, metabolites and proteins enabled an improved predictive performance compared to individual biomarkers.65–69 In this study, the protein abundance ratios were used in the ROC analysis to discover predictors of burn depth and time to re-epithelialization. The ROC curve based on multiple features using an SVM algorithm led to a dramatic improvement in AUC compared with the best performing single protein or the various ratios of two proteins (Figure 4 and 6). Importantly, linear SVM classification has been utilized for biomarker discovery, diagnostics with multiple biomarkers and genomics studies previously.70–74 Thus, linear SVM is a feasible algorithm for the discovery of multiple biomarkers. However, ROC and AUC analysis is only one way to report the prognostic value of biomarkers and other measures may be more appropriate to determine accurate risk classification for clinical burn outcomes and biomarker performance evaluation.

Re-epithelialization within 21 days is an important predictor of hypertrophic scar formation, which is influenced by both burn depth and burn size (TBSA).22,24 This study showed that the abundance of specific proteins could differentiate burns based on this re-epithelialization threshold. Although the presence and abundance of the relative proteins are not able to provide a quantitative probability of hypertrophic scar formation, they may provide an additional diagnostic tool for assisting clinicians in their decisions regarding wound management and grafting. The most important proteins in the PLS-DA, volcano plot, and Student’s t test analyses for time to re-epithelialization classification were catenin beta-1, COL1A1 and collagen alpha-2(1) chain (COL1A2). The blister fluid derived from the patients that re-epithelialized in more than 21 days exhibited a significant increase in the abundance of both
COL1A1 and COL1A2. Both of these collagen subunits are derived from type I collagen (fibril forming collagen) found in most connective tissue but enriched in the dermis,77 and involved in type I collagen triple helix formation.76 Previous evidence indicates that COL1A1 and COL1A2 are downregulated rapidly after thermal injury and then upregulated during the healing process.77 It is known that fetal skin contains a higher proportion of type III collagen, which is deposited in neat, well-organized reticular patterns and is able to achieve skin regeneration without scar formation following wounding.78–80 However, in postnatal wounding, the extracellular matrix contains increased levels of type I collagen, which is deposited in thick disorganized patterns and forms scar tissue more readily.78–80 Although the deposition patterns of collagen were not detected in this study, the higher abundance of COL1A1 and COL1A2 in wounds that re-epithelialized in longer than 21 days may be an indicator of increased risk of scar formation.81–83

The abundance of catenin beta-1 (Beta-catenin 1) was significantly decreased in the blister fluid from burns that re-epithelialized in more than 21 days. Beta-catenin 1 is a dual function protein, which is a component of cell adherent junctions and a key downstream component of the canonical Wnt signaling pathway.81 Beta-catenin 1 links cadherin to the cytoskeleton and has been used as a biomarker of epithelial-mesenchymal transitions (EMT).82 It has been reported that beta-catenin 1 is a key component of the cell–cell adhesion-dependent regulation of EMT and beta-catenin 1 signaling can strengthen the TGF-β1-induced activation of the α-smooth muscle actin promoter and protein expression in EMT.83 Beta-catenin 1 has been found to be involved in tissue fibrosis and regulation of COL1A1/A2 synthesis.84 In addition, beta-catenin 1 is essential for full thickness skin wound healing in mice through the regulation of motility and adhesion of macrophage cells.85 Moreover, it has been demonstrated that there is an increased level of beta-catenin 1 in hypertrophic scar and keloid tissues compared with atrophic scar tissues.86,87 However, how beta-catenin 1 affects time to re-epithelialization is unclear and cannot be clarified until further investigation.

Gene ontology enrichment analysis revealed that the proteins involved in the significantly enriched annotations oxygen transport, oxygen transporter activity, oxygen binding, and heme binding largely encompassed the hemoglobin subunits α, β, γ, and δ. It was then not surprising that the enriched BPs response to wounding, coagulation, and processes associated with hemostasis were also observed. Burn injured children suffer significant fluid loss;88 therefore, the over-representation of tissue hemostasis processes was also somewhat expected (Table S1–S3). These over-represented annotations were more or less recapitulated in the time to re-epithelialization comparison, with additional emphasis on regulation of enzyme activity (Table S4). The over representation of collagen fragments in the blister fluid and the abundance of protease inhibitors potentially suggests a high degree of protein turn over in the burn wounds prior to medical treatment and that regulation of this may be important in the early burn. Indeed, Younan et al. (2010) found that mice deficient in either of the secretory granule mast cell serine proteases chymase or elastase, were resistant to scald burn wound injury, which in wild type mice consisted of epidermal/dermal junction breakdown, disruption of tight junctions, edema, ulceration and dermal collagen disruption. Treatment of the protease deficient mice with recombinant forms of the enzymes following burn injury produced epidermal coagulation necrosis, damage to hair follicles, edema, and collagen denaturation.89 Thus, regulation of the proteolytic environment in the nascent burn wounds may be a critical factor determining the longer term injury response. Regarding the CC aspect of GO enrichment analysis in either the burn depth classification or time to re-epithelialization, the most over-represented annotations were related to vesicle and extracellular region. This is consistent with the sample type—blister fluid, which is the infiltration of body fluid to the wound bed and is comprised of large amounts of exosomes and secreted proteins.34,89

The current limitations associated with this study include the limited number of samples from full thickness burns. Furthermore, since burn wounds can deepen in the days following injury, the content of the blister fluids may also change with respect to the time from injury to the time to collection, possibly due to protein degradation,90,91 which was not taken into account in this study. There are other clinical variables that also may have contributed to the protein presence and abundance, such as burn size, first aid treatment, time from injury to collection, and mechanism of injury. Ideally, a complex multivariable analysis should be performed, where all factors that may influence healing and protein content are included. In future planned analyses, variables including the time until sample collection, burn depth, TBSA, time to re-epithelialization, mechanism of injury, and first aid treatment will be assessed to determine the extent that they influence protein presence and abundance, and models will be constructed to allow for the variables to be included as covariates in analyses. Finally, the potential biomarker candidates described in this discovery phase project are not yet validated in larger cohorts of burn samples; however, this is also the subject of planned future studies.

■ CONCLUSION

This investigation quantitatively profiled the protein complement of blister fluid samples from pediatric burns and demonstrated that the blister fluid proteome can be used to classify pediatric burn wounds according to burn depth and time to re-epithelialization. Moreover, the clustering analysis based on the burn blister fluid proteome revealed several burns that were potentially clinically misclassified. The high accuracy of this proteome-based measurement of select proteins in blister fluid, compared with the time spent on the diagnosis using clinical experience (days to weeks), may assist with clinical decision making at an earlier stage than typically performed. Potential biomarker candidates, such as Hb subunit α, β, γ, and δ, IGFBP 3, and 3-hydroxacyl-CoA dehydrogenase type-2, exhibited significant abundance differences across various depths. Beta-catenin 1 and collagen alpha-1(I) and alpha-2(I) chains were discovered as potential biomarker candidates to predict time for burn wound re-epithelialization. In addition, a multiple features ROC curve of a suite of protein ratios produced high AUC values, indicating that a multiplex diagnostic approach would be useful for clinical diagnosis in this field.
The authors declare the following competing financial interest(s): JB was employed by SCIEX Australia Pty Ltd.; however, the company had no involvement in the study design, analysis, or publication. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties at the time of publication.

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the data set identifier PXD011102.

ACKNOWLEDGMENTS

The authors would like to acknowledge the support of the Australian Government’s Cooperative Research Centers Program, specifically under the auspices of the Wound Management Innovation Cooperative Research Centre (WMICRC) for their research support and TZ’S scholarship. The authors also would like to acknowledge the generous donation of blister fluids from pediatric patients and the assistance of the sample collection by clinical staff at the Royal Children’s Hospital and Lady Cilento Children’s hospital. The authors also acknowledge Dr. Pawel Sadowski and Dr. Rajesh Gupta from the Central Analytical Research Facility (CARF) at the Queensland University of Technology for their assistance with the mass spectrometry. LC was supported by a National Health and Medical Research Council Fellowship (APP1130862).

ABBREVIATIONS

BF, blister fluid; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; MS, mass spectrometry; LC–MS/MS, liquid chromatography–tandem mass spectrometry; DIA, data independent acquisition; SWATH, sequential window acquisition of all theoretical fragment ion spectra; FDR, false discovery rate; PCA, principle component analysis; PLS-DA, partial least-squares–discriminant analysis; ROC, receiver operating characteristic; GO, gene ontology; BP, biological process; MF, molecular function; CC, cellular component.

REFERENCES

(1) Ryan, C. M.; Schoenfeld, D. A.; Thorpe, W. P.; Sheridan, R. L.; Cassem, E. H.; Tompkins, R. G. Objective estimates of the probability of death from burn injuries. N. Engl. J. Med. 1998, 338 (6), 362–6.
(2) Desai, C.; Wood, F. M.; Schug, S. A.; Parsons, R. W.; Fridlender, C.; Sunderland, V. B. Effectiveness of a topical local anaesthetic spray as analgesia for dressing changes: a double-blinded randomised pilot trial comparing an emulsion with an aqueous lidocaine formulation. Burns 2014, 40 (1), 106–12.
(3) Lim, J.; Liew, S.; Chan, H.; Jackson, T.; Burrows, S.; Edgar, D. W.; Wood, F. M. Is the length of time in acute burn surgery associated with poorer outcomes? Burns 2014, 40 (2), 235–40.
(4) Honardar, S.; Kordestani, S. S.; Daliri, M.; NayebHabib, F. The effect of chitosan-based gel on second degree burn wound. J. Wound Care 2016, 25 (8), 488–94.
(5) Baartmans, M. G.; de Jong, A. E.; van Baar, M. E.; Beethuizen, G. I.; van Loey, N. E.; Tibboel, D.; Nieuwenhuis, M. K. Early management in children with burns: Cooling, wound care and pain management. Burns 2016, 42 (4), 777–82.
(6) Hsu, K. C.; Chen, L. F.; Hsiep, P. H. Effect of music intervention on burn patients’ pain and anxiety during dressing changes. Burns 2016, 42 (8), 1789–1796.
(7) Lee, J. O.; Herndon, D. N. The pediatric burned patient. In Total Burn Care, 3rd ed.; Herndon, D. N., Ed.; W. B. Saunders: Edinburgh, 2007; Chapter 36, pp 485–495.
(8) Holland, A. J. Pediatric burns: the forgotten trauma of childhood. Can. J. Surg. 2006, 49 (4), 272–7.
(9) Schneider, J. C.; Holavanahalli, R.; Helm, P.; Goldstein, R.; Kowalske, K. Contractures in burn injury: defining the problem. J. Burn Care Res. 2006, 27 (4), 508–14.
(10) Young, R. C.; Burd, A. Paediatric upper limb contracture release following burn injury. Burns 2004, 30 (7), 723–8.
(11) Ahn, C. S.; Maitz, P. K. The true cost of burn. Burns 2012, 38 (7), 967–74.
(12) Gangemi, E. N.; Gregori, D.; Berchialla, P.; Zingarelli, E.; Cairo, M.; Bollero, D.; Ganem, J.; Capocelli, R.; Cuccuru, F.; Cassano, P.; Risso, D.; Stella, M. Epidemiology and risk factors for pathologic scarring after burn wounds. Arch. Facial Plast. Surg. 2008, 10 (2), 93–102.
(13) van der Wal, M. B.; Vloemans, J. F.; Tuinebreijer, W. E.; van de Ven, P.; van Unen, E.; van Zuijlen, P. P.; Middelkoop, E. Outcome after burns: an observational study on burn scar maturation and predictors for severe scarring. Wound Repair Regen. 2012, 20 (5), 676–87.
(14) Rossiter, N. D.; Chapman, P.; Haywood, I. A. How big is a hand? Burns 1996, 22 (3), 230–1.
(15) Knaysi, G. A.; Crikelar, G. F.; Cosman, B. The role of nines: its history and accuracy. Plast Reconstr Surg 1960, 41 (6), 560–3.
(16) Giretzlehner, M.; Dirnberger, J.; Owen, R.; Haller, H. L.; Risso, D.; Stella, M. Epidemiology and risk factors for pathologic scarring after burn wounds. Arch. Facial Plast. Surg. 2008, 10 (2), 93–102.
(17) Lloyd, E. C.; Rodgers, B. C.; Michener, M.; Williams, M. S. Outpatient burns: prevention and care. Am. Fam. Physician 2012, 85 (1), 25–32.
(18) Shakespeare, P. G. Standards and quality in burn treatment. Burns 2001, 27 (8), 791–2.
(19) Jackson, D. M. The diagnosis of the depth of burning. Br. J. Surg. 1953, 40 (164), 588–96.
(20) Devgan, L.; Bhat, S.; Aylward, S.; Spence, R. J. Modalities for the assessment of burn wound depth. J. Burns Wounds 2006, 5, e2.
(21) Heimbach, D. M.; Afromowitz, M. A.; Engrav, L. H.; Marvin, J. A.; Perry, B. Burn depth estimation–man or machine. J. Trauma 1984, 24 (5), 373–8.
(22) Brown, N. J.; Kimble, R. M.; Gramotnev, G.; Rodger, S.; Cuttle, L. Predictors of re-epithelialization in pediatric burn. Burns 2014, 40 (4), 751–8.
(23) Deitch, E. A.; Wheelahan, T. M.; Rose, M. P.; Clothier, J.; Cotter, J. Hypertrophic burn scars: analysis of variables. J. Trauma 1983, 23 (10), 895–8.
(24) Cubison, T. C.; Pape, S. A.; Parkhouse, N. Evidence for the link between healing time and the development of hypertrophic scars (HTS) in paediatric burns due to scald injury. Burns 2006, 32 (8), 992–9.
(25) Hawkins, H. K.; Finnerty, C. C. Pathophysiology of the burn scar. In Total Burn Care, 4th ed.; Herndon, D. N., Ed.; W. B. Saunders: London, 2012; Chapter 46, pp S07–S16.

(26) Heimbach, D.; Engrav, L.; Grube, B.; Marvin, J. Burn depth: a review. World J. Surg. 1992, 16 (1), 10–5.

(27) Monstrey, S.; Hoeksema, H.; Verbelen, J.; Pirayesh, A.; Blondeel, P. Assessment of burn depth and burn wound healing potential. Burns 2008, 34 (6), 761–9.

(28) Zhang, T.; Broszczak, D. A.; Broadbent, J. A.; Cuttle, L.; Lu, H.; Parker, T. J. The biochemistry of blister fluid from pediatric burn injuries: proteomics and metabolomics aspects. Expert Rev. Proteomics 2016, 13 (1), 35–53.

(29) Zhang, T.; Broszczak, D. A.; Cuttle, L.; Broadbent, J. A.; Tanzer, C.; Parker, T. J. The blister fluid proteome of paediatric burns. J. Proteomics 2016, 146, 122–132.

(30) Pan, S. C.; Wu, L. W.; Chen, C. L.; Shieh, S. J.; Chiu, H. Y. Angiogenin expression in burn blister fluid: implications for its role in burn wound neovascularization. Wound Repair and Regeneration 2012, 20 (5), 731–9.

(31) Nissen, N. N.; Gamelli, R. L.; Polverini, P. J.; DiPietro, L. A. Differential angiogenic and proliferative activity of surgical and wound fluids. J. Trauma 2003, 54 (6), 1205–10. Discussion, 1211.

(32) Dehne, M. G.; Sablotzki, A.; Hoffmann, A.; Muhling, J.; Dietrich, F. E.; Hempelmann, G. Alterations of acute phase reaction and cytokine production in patients following severe burn injury. Burns 2002, 28 (6), 535–42.

(33) Zhang, Y.; Bai, X.; Wang, Y.; Li, N.; Li, X.; Han, F.; Su, L.; Hu, D. Role for heat shock protein 90alpha in the proliferation and migration of HaCaT cells and in the deep second-degree burn wound healing in mice. PLoS One 2014, 9 (8), e103723.

(34) Zhang, T.; Broszczak, D. A.; Cuttle, L.; Broadbent, J. A.; Tanzer, C.; Parker, T. J. The blister fluid proteome of paediatric burns. J. Proteomics 2016, 146, 122–32.

(35) Mozozinski, P.; Zolotarjova, N.; Chen, H. Agilent Multiple Affinity Removal Spin Cartridges for the Depletion of High-Abundant Proteins from Human Proteomic Samples. Agilent Technologies, 2005.

(36) Carrette, O.; Burkhard, P. R.; Sanchez, J. C.; Hochstrasser, D. F. State-of-the-art two-dimensional gel electrophoresis: a key tool of proteomics research. Nat. Protoc. 2006, 1 (2), 812–23.

(37) Natale, M.; Maresca, B.; Abrescia, P.; Bucci, E. M. Image Analysis Workflow for 2D Electrophoresis Gels Based on ImageJ. Proteomics Insights 2011, 4, 37–49.

(38) Chang, J.; Remmen, H. V.; Ward, W. F.; Regnier, F. E.; Richardson, A.; Cornell, J. Processing of Data Generated by 2-Dimensional Gel Electrophoresis for Statistical Analysis: Missing Data, Normalization, and Statistics. J. Proteome Res. 2004, 3 (6), 1210–1218.

(39) Shevchenko, A.; Tomas, H.; Havlis, J.; Olsen, J. V.; Mann, M. In-gel digestion for mass spectrometric characterization of proteins and proteomes. Nat. Protoc. 2006, 1 (6), 2856–60.

(40) Rappsilber, J.; Mann, M.; Ishihama, Y. Protocol for micro-purification, enrichment, pre-fractionation and storage of peptides for proteomics using StageTips. Nat. Protoc. 2007, 2 (8), 1896–906.

(41) Zhang, T.; Broszczak, D. A.; Cuttle, L.; Broadbent, J. A.; Tanzer, C.; Parker, T. J. Mass spectrometry based data of the blister fluid proteome of paediatric burn patients. Data Brief 2016, 8, 1099–110. DOI: 10.1016/j.dib.2016.06.012.

(42) Viscaínco, J. A.; Csordas, A.; del-Toro, N.; Dianes, J. A.; Griss, J.; Lavidas, I.; Mayer, G.; Perez-Riverol, Y.; Reisinger, F.; Ternet, T.; Xu, Q. W.; Wang, R.; Hermjakob, H. 2016 update of the PRIDE database and its related tools. Nucleic Acids Res. 2016, 44 (D1), D447–56.

(43) Deutsch, E. W.; Csordas, A.; Sun, Z.; Jarnuczak, A.; Perez-Riverol, Y.; Ternet, T.; Campbell, D. S.; Bernal-Llanares, M.; Okuda, S.; Kawano, S.; Moritz, R. L.; Carver, J. J.; Wang, M.; Ishihama, Y.; Bandeira, N.; Hermjakob, H.; Viscaínco, J. A. The ProteomeXchange consortium in 2017: supporting the cultural change in proteomics public data deposition. Nucleic Acids Res. 2017, 45 (D1), D1100–D1106.
protease activity in the course of wound healing. *Wound Repair and Regeneration* 1996, 4 (2), 252–8.

(63) Blat, C.; Villaudy, J.; Binoux, M. In vivo proteolysis of serum insulin-like growth factor (IGF) binding protein-3 results in increased availability of IGF to target cells. *J. Clin. Invest.* 1994, 93 (5), 2868–90.

(64) Abrimat, T.; Nedelec, B.; Jobin, N.; Garrel, D. R. Decreased serum insulin-like growth factor-1 in burn patients: relationship with serum insulin-like growth factor binding protein-3 proteolysis and the influence of lipid composition in nutritional support. *Crit. Care Med.* 2000, 28 (7), 2366–72.

(65) Kaeser, L.; Wenzl, R.; Proestling, K.; Balendran, S.; Pateisky, P.; Yotova, S.; Yerlikaya, G.; Streubel, B.; Husslein, H. Soluble VCAM-1/soluble ICAM-1 ratio is a promising biomarker for diagnosing endometriosis. *Hum. Reprod.* 2017, 32 (4), 770–779.

(66) Fukuda, A.; Wickman, L. T.; Venkatreddy, M. P.; Wang, S. Q.; Chowdhury, M. A.; Wiggins, J. E.; Shedden, K. A.; Wiggins, R. C. Urine podocin: nephrin mRNA ratio (PNR) as a podocyte stress biomarker. *Nephrol., Dial., Transplant.* 2005, 32 (4), 1780–85.

(67) Shen, Z.; Zhou, S.; Wang, Y.; Li, R. L.; Zhong, C.; Liang, C.; Sun, Y. Higher intratumoral infiltrated Foxp3+ Treg numbers and Foxp3+/CD8+ ratio are associated with adverse prognosis in resectable gastric cancer. *J. Cancer Res. Clin. Oncol.* 2010, 136 (10), 1585–95.

(68) Kamiguchi, H.; Murabayashi, M.; Mori, I.; Horinouchi, A.; Higaki, K. Biomarker discovery for drug-induced phospholipidosis: phenylacetylglucine to hippocid acid ratio in urine and plasma as potential markers. *Biomarkers* 2017, 22 (2), 178–188.

(69) Dominguez-Rodriguez, A.; Abreu-Gonzalez, P.; Garcia-Gonzalez, M. J.; Kaski, J. C. Soluble CD40 ligand:interleukin-10 ratio predicts in-hospital adverse events in patients with ST-segment elevation myocardial infarction. *Thromb. Res.* 2007, 121 (3), 293–9.

(70) Guyon, I.; Weston, J.; Barnhill, S.; Vapnik, V. Gene selection for cancer classification using support vector machines. *Machine learning* 2002, 46 (1), 389–422.

(71) Nanni, L.; Brahnam, S.; Lumin, A. Combining multiple approaches for gene microarray classification. *Bioinformatics* 2012, 28 (8), 1151–7.

(72) Zhang, F.; Kaufman, H. L.; Deng, Y.; Drabier, R. Recursive SVM biomarker selection for early detection of breast cancer in peripheral blood. *BMC Med. Genomics* 2013, 6 (Suppl 1), 54.

(73) Furey, T. S.; Cristianini, N.; Duffy, N.; Bednarski, D. W.; Schummer, M.; Haussler, D. Support vector machine classification and validation of cancer tissue samples using microarray expression data. *Bioinformatics* 2000, 16 (10), 906–14.

(74) Wang, M. C.; Li, S. ROC analysis for multiple markers with tree-based classification. *Lifetime Data Anal* 2013, 19 (2), 257–77.

(75) Trojanowska, M.; Carville LeRoy, E.; Eckes, B.; Krieg, T. Pathogenesis of fibrosis: type 1 collagen and the skin. *J. Mol. Med.* 1998, 76 (3), 266–274.

(76) Dzobo, K.; Leamer, V. D.; Parker, M. I. Absence of feedback regulation in the synthesis of COL1A1. *Life Sci.* 2014, 103 (1), 25–33.

(77) Liu, J.; Luo, C.; Yin, Z.; Li, P.; Wang, S.; Chen, J.; He, Q.; Zhou, J. Downregulation of let-7b promotes COL1A1 and COL1A2 expression in dermis and skin fibroblasts during heat wound repair. *Mol. Med. Rep.* 2016, 13 (3), 2683–8.

(78) Cuttle, L.; Nataatmadja, M.; Fraser, J. F.; Kempf, M.; Kimble, R. M.; Hayes, M. T. Collagen in the scarless fetal skin wound: detection with picrosirius-polarization. *Wound Repair and Regeneration* 2005, 13 (2), 198–204.

(79) Zgheib, C.; Xu, J.; Liechty, K. W. Targeting Inflammatory Cytokines and Extracellular Matrix Composition to Promote Wound Regeneration. *Adv. Wound Care (New Rochelle)* 2014, 3 (4), 344–355.

(80) Colwell, A. S.; Longaker, M. T.; Lorenz, H. P. Fetal wound healing. *Front. Biosci., Landmark Ed.* 2003, 8, s1240–8.

(81) Bienen, M. beta-Catenin: a pivot between cell adhesion and Wnt signalling. *Curr. Biol.* 2005, 15 (2), R64–7.

(82) Zeisberg, M.; Neilson, E. G. Biomarkers for epithelial-mesenchymal transitions. *J. Clin. Invest.* 2009, 119 (6), 1429–37.

(83) Masszi, A.; Fan, L.; Rosival, L.; McCulloch, C. A.; Rotstein, O. D.; Mucsi, I.; Kapus, A. Integrity of cell-cell contacts is a critical regulator of TGF-beta 1-induced epithelial-to-myoﬁbroblast transition: role for beta-catenin. *Ann. J. Pathol.* 2004, 165 (6), 1955–67.

(84) Rob, M. R.; Kumar, R.; Rajadurai, A.; Njauw, C.; Ryooy, U. H.; Chung, K. Y.; Tsao, H. Beta-catenin causes ﬁbrotic changes in the extracellular matrix via upregulation of collagen I transcription. *Br. J. Dermatol.* 2017, 177 (1), 312–315.

(85) Amini-Nik, S.; Cambridge, E.; Yu, W.; Guo, A.; Whetstone, H.; Nadesan, P.; Poon, R.; Hinz, B.; Alman, B. A. beta-Catenin-regulated myeloid cell adhesion and migration determine wound healing. *J. Clin. Invest.* 2014, 124 (6), 2599–610.

(86) Sato, M. Upregulation of the Wnt/beta-catenin pathway induced by transforming growth factor-beta in hypertrophic scars and keloids. *Acta Derm Venereol* 2006, 86 (4), 300–7.

(87) Lee, J. O.; Norbury, W. B.; Herndon, D. N. Special considerations of age: The pediatric burned patient. In *Total Burn Care*, 4th ed.; Herndon, D. N., Ed.; W. B. Saunders: London, 2012; Chapter 35, pp 405–414.

(88) Younan, G.; Suber, F.; Xing, W.; Shi, T.; Kunori, Y.; Abrink, M.; Pejler, G.; Schlenker, S. M.; Rodewald, H. R.; Moore, F. D., Jr.; Stevens, R. L.; Adachi, R.; Austen, K. F.; Gurish, M. F. The inflammatory response after an epidermal burn depends on the activities of mouse mast cell proteases 4 and 5. *J. Immunol.* 2010, 185 (12), 7681–90.

(89) Widgerow, A. D.; King, K.; Tocco-Tussardi, I.; Banyard, D. A.; Chiang, R.; Awad, A.; Afzel, H.; Bhatnager, S.; Melkumyan, S.; Wirth, G.; Evans, G. R. The burn wound exudate—an under-utilized resource. *Burns* 2015, 41 (1), 11–7.

(90) Jambunathan, K.; Galande, A. K. Sample collection in clinical proteomics—proteolytic activity profile of serum and plasma. *Proteomics: Clin. Appl.* 2014, 8 (5−6), 299–307.

(91) Pesek, J.; Kruger, T.; Krieg, N.; Schiel, M.; Norgauer, J.; Grosskreutz, J.; Rhode, H. Native chromatographic sample preparation of serum, plasma and cerebrospinal fluid does not comprise a risk for proteolytic biomarker loss. *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.* 2013, 922−924, 102−9.