Supporting Information

Characterization of the Blister Fluid Proteome for Pediatric Burn Classification

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In-gel digestion for 2-D spots and LC-MS/MS analysis

In brief, the excised gel spots were reduced at 56 °C for 30 minutes with 10 mM dithiothreitol (DTT) (Roche Life Science, Castle Hill, NSW, Australia) in 100 mM NH_4HCO_3 and alkylated with 55 mM iodoacetamide (IAA) (Merck Pty Ltd, Bayswater, Victoria, Australia) in 100 mM NH_4HCO_3 at room temperature for 20 minutes. The gel pieces were then digested by incubating with 1200 ng of sequencing grade trypsin (Roche Diagnostics) in 10% acetonitrile with 10 mM NH_4HCO_3 overnight at 37 °C. The extracted peptides were resuspended in 1% formic acid (FA) and 2% acetonitrile (ACN) for LC-MS/MS analysis.

LC-MS/MS: Aliquots of 12 µL of tryptic digest peptides were injected onto a C18 reverse phase trap column (Agilent Zorbax 300SB C18 5 µm 5 × 0.3 mm) using a Shimadzu Nexera nano HP LC at a flow rate of 30 µL / min. Peptides were desalted in 0.1% FA for 4 minutes. Peptides then were resolved using a C18 nano-LC resolving column (Vydac Everest C18 300 Å, 5 µm reverse phase, 150 mm x 75 µm) over 30 minutes with a multi-step gradient utilizing 0.1% FA and 0.1% trifluoroacetic acid (TFA) in ACN as follows: 0-40% TFA solution over 30 minutes, 50-80% TFA solution over 5 minutes, 80% TFA solution for 5 minutes and re-equilibration to 0% TFA solution. The MS measurements were performed using a nanospray III ion source and a Quadrupole-time of flight (QqTOF) MS/MS (SCIEX, Mulgrave, VIC, Australia).

Mass spectrometry data were analysed using ProteinPilot (Sciex). The raw data were searched against the Human SwissProt/UniProt database (February 2012) using trypsin specificity, cysteine carbamidomethylation and appropriate instrument settings. In addition, all biological modifications contained within the Unimod database (www.unimod.org) and common amino acid substitutions were also considered in the analyses.

The results of the ProteinPilot search were confirmed by analysis of the same spectral data using a SpectraSt (Version 4.0; Institute for Systems Biology, Seattle, USA) search of the NIST Library of Peptide Ion Fragmentation Spectra (QqTOF: Human; SpectraST format; www.peptideatlas.org/speclib) with peptides shorter than 7 amino acids excluded.

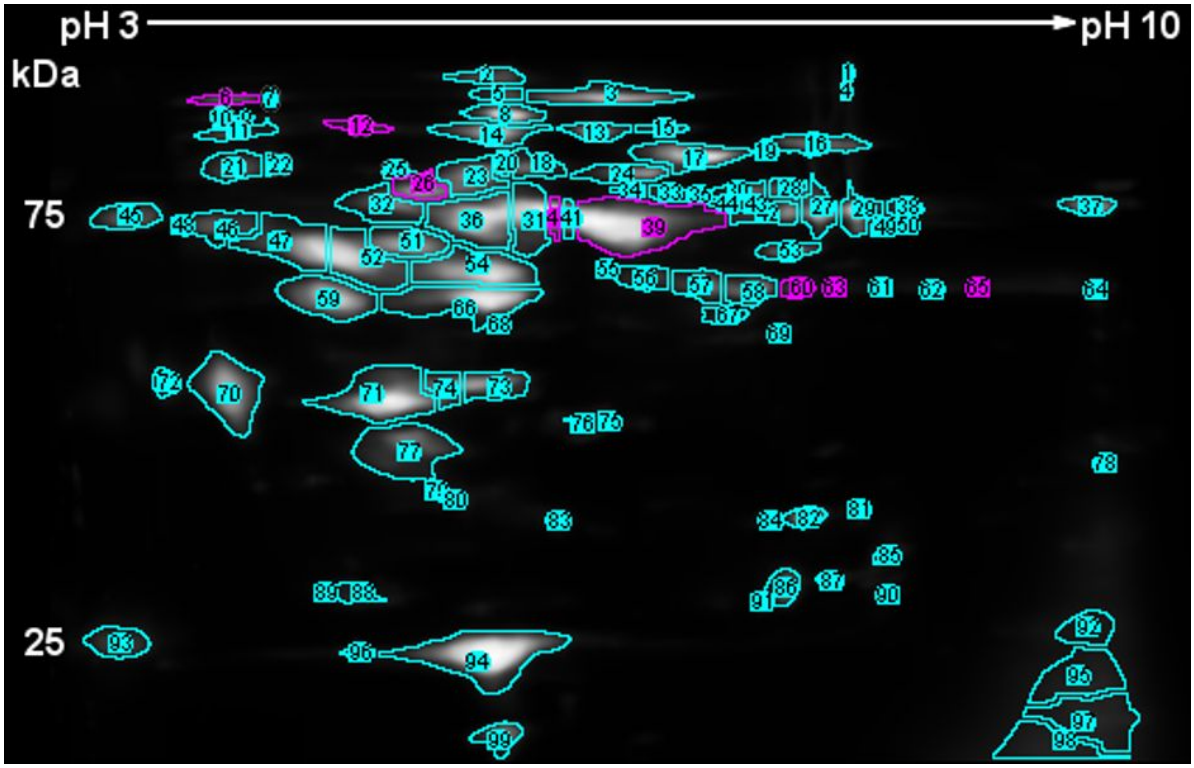

Figure S1. Eight protein spots identified as different between more severe or less severe burns. Using ImageJ software, 12 different 2-D SDS PAGE gels (6 for more severe and 6 for less severe) were analyzed and 99 distinct spots were identified from the warped and stacked images. The eight spots outlined in pink were significantly different between cohorts ($p<0.05$) and were subsequently excised for identification by LC-MS/MS. Spot 94 was also excised and digested as a quality control for the in-gel digestion protocol. Spot 6 = alpha-2-macroglobulin; 12 = keratin (which would be expected in a human skin wound sample or could possibly indicate contamination) and trypsin (which was used in the digestion protocol) and was therefore classified as non-identified; 26 = prothrombin; 39 = apolipoprotein A-1; 40 = hemopexin; 60 = fibrinogen beta chain; 63 = fibrinogen beta chain; 65 = fibrinogen beta chain; 94 = apolipoprotein A-1.

Table S1. In gel digestion protein identifications and basic metrics

Spot #	Accession Number	Proteins	Apparent Molecular Weight (kDa)	Apparent pI	Molecular Weight (kDa)	pI range (SWISS-2DPAGE) Human plasma	Number of matching peptides with 95% confidence		Percent sequence coverage
							Protein Pilot	*SpectraST*	
6	P01023	Alpha-2-macroglobulin	130	4.0	163.62	5.33-5.62	9	*11*	18.2
26	P00734	Prothrombin	80	5.3	70.17	4.95-5.09	10	*7*	34.1
39	P02647	Apolipoprotein A-1	70	6.7	30.83	4.79-7.27	4	*9*	44.9
40	P02790	Hemopexin	75	6.2	51.77	4.48-5.59	9	*10*	45.9
60	P02675	Fibrinogen beta chain	50	7.9	56.04	6.10-6.55	13	*6*	44.4
63	P02675	Fibrinogen beta chain	50	8.2	56.04	6.10-6.55	6	*5*	37.3
65	P02675	Fibrinogen beta chain	50	9.1	56.04	6.10-6.55	4	*3*	15.3
94	P02647	Apolipoprotein A-1	24	5.8	30.83	4.79-7.27	32	*12*	80.5

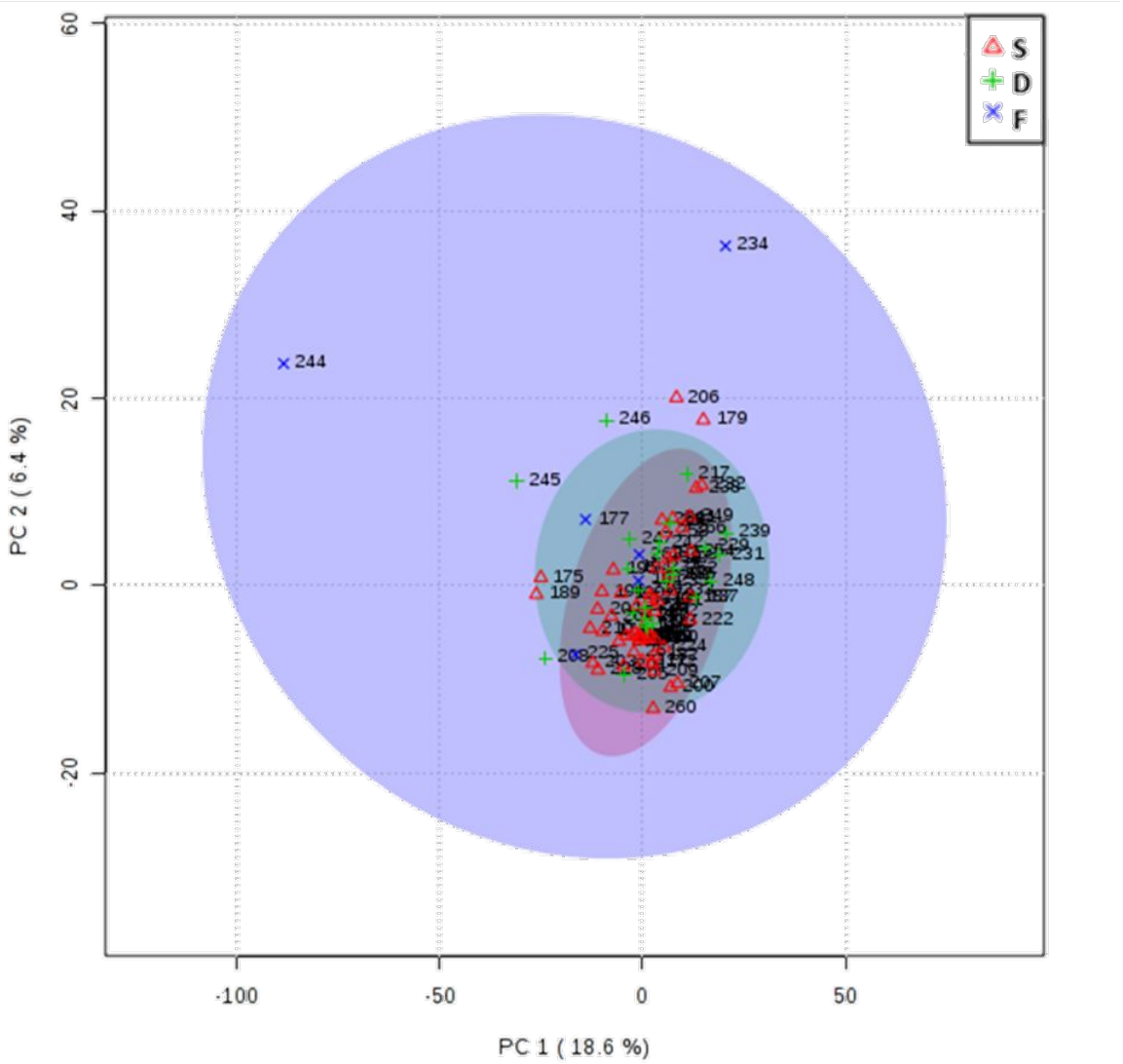

Figure S2. Score plot of PCA, with samples classified according to burn depth.
Burn depth is indicated where S = Superficial partial thickness burn; D = Deep partial thickness burn; F = Full thickness burn.

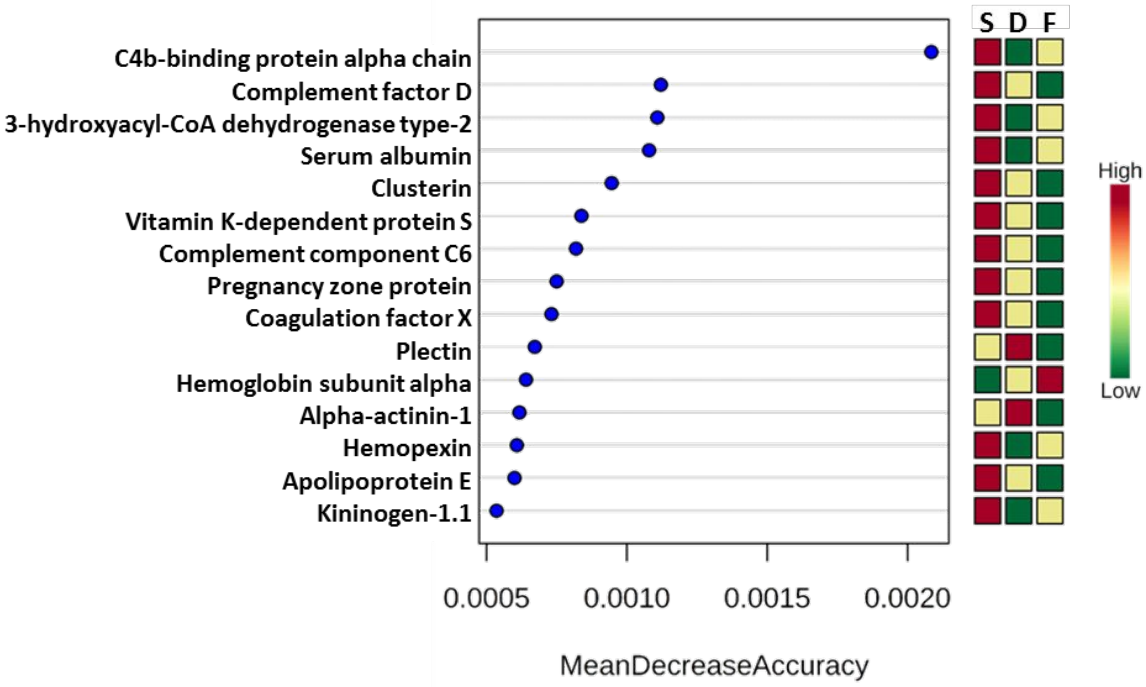

Figure S3. Random Forest analysis indicating the top 15 proteins with the highest mean decrease accuracy, which indicates the most important features in burn depth classification prediction.

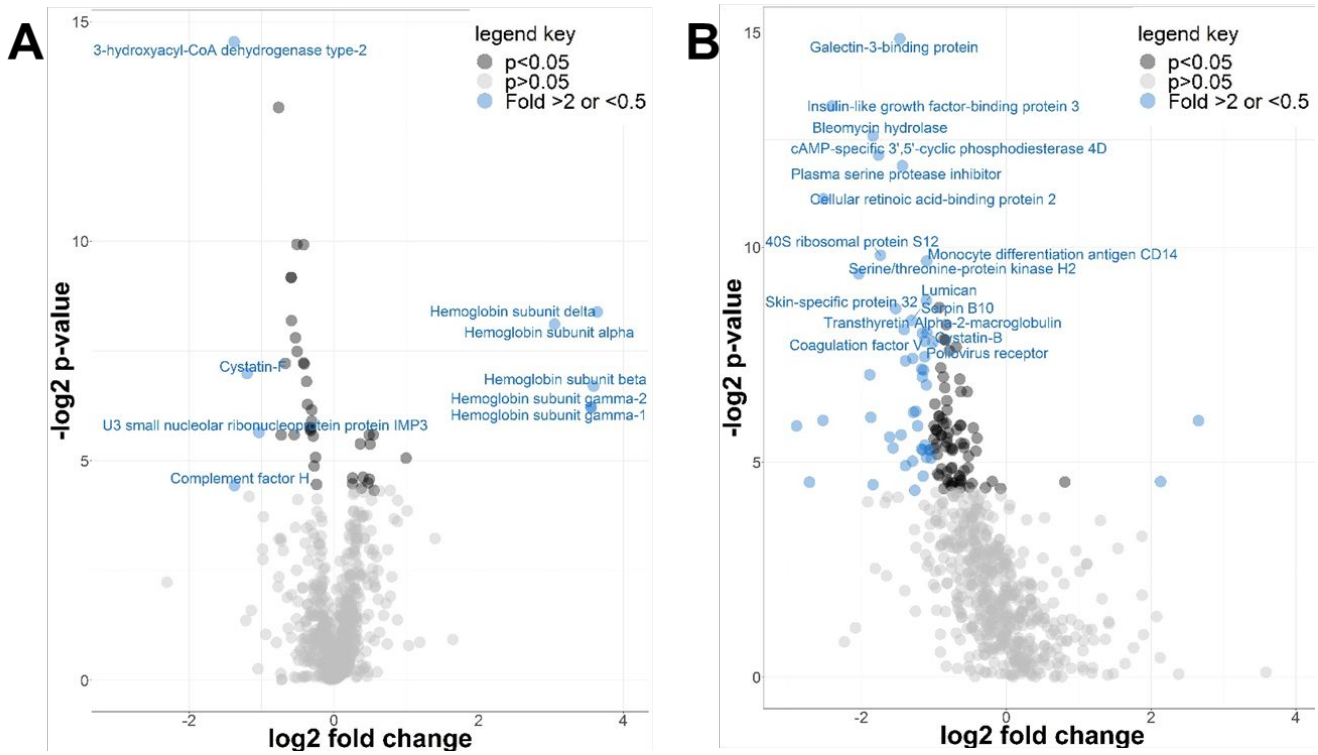

Figure S4. Volcano plots of the comparisons between (A) superficial partial and deep partial thickness and (B) deep partial and full thickness depth cohorts. The x-axis represents log2 transformed protein abundance fold change, and y-axis represents log2 transformed p values. The color indicates the significance level, grey = $p > 0.05$, black = $p < 0.05$, blue = $p < 0.05$ & fold change ≥ 2. The significant proteins ($p < 0.05$ in (A) and $p < 0.005$ in (B)) with a more than two-fold change in abundance were labelled.

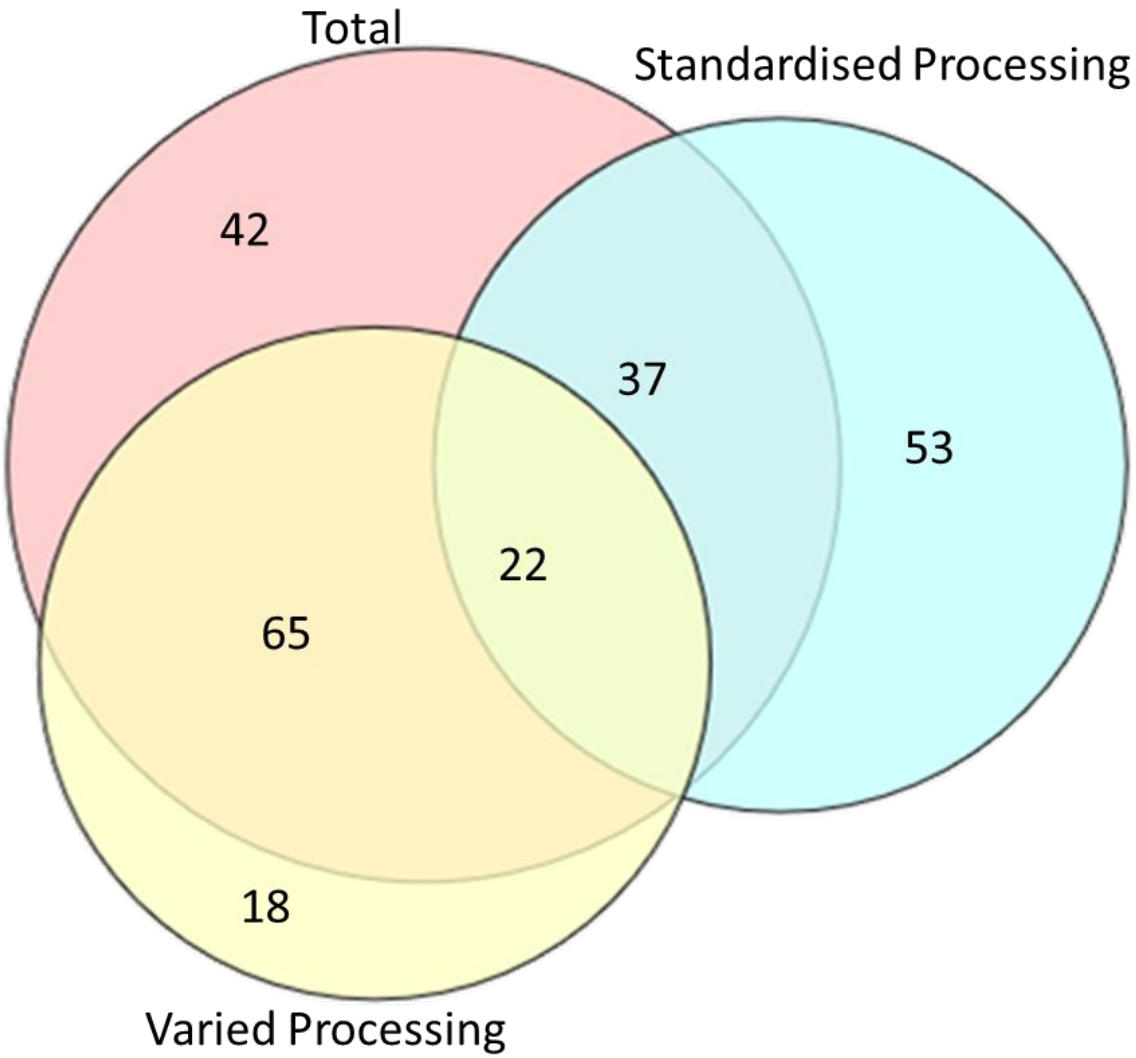

Figure S5. Venn diagram of significant proteins from the 87 total samples, the 56 “standardized processing” sample cohort and the 31 “varied processing” sample cohort.

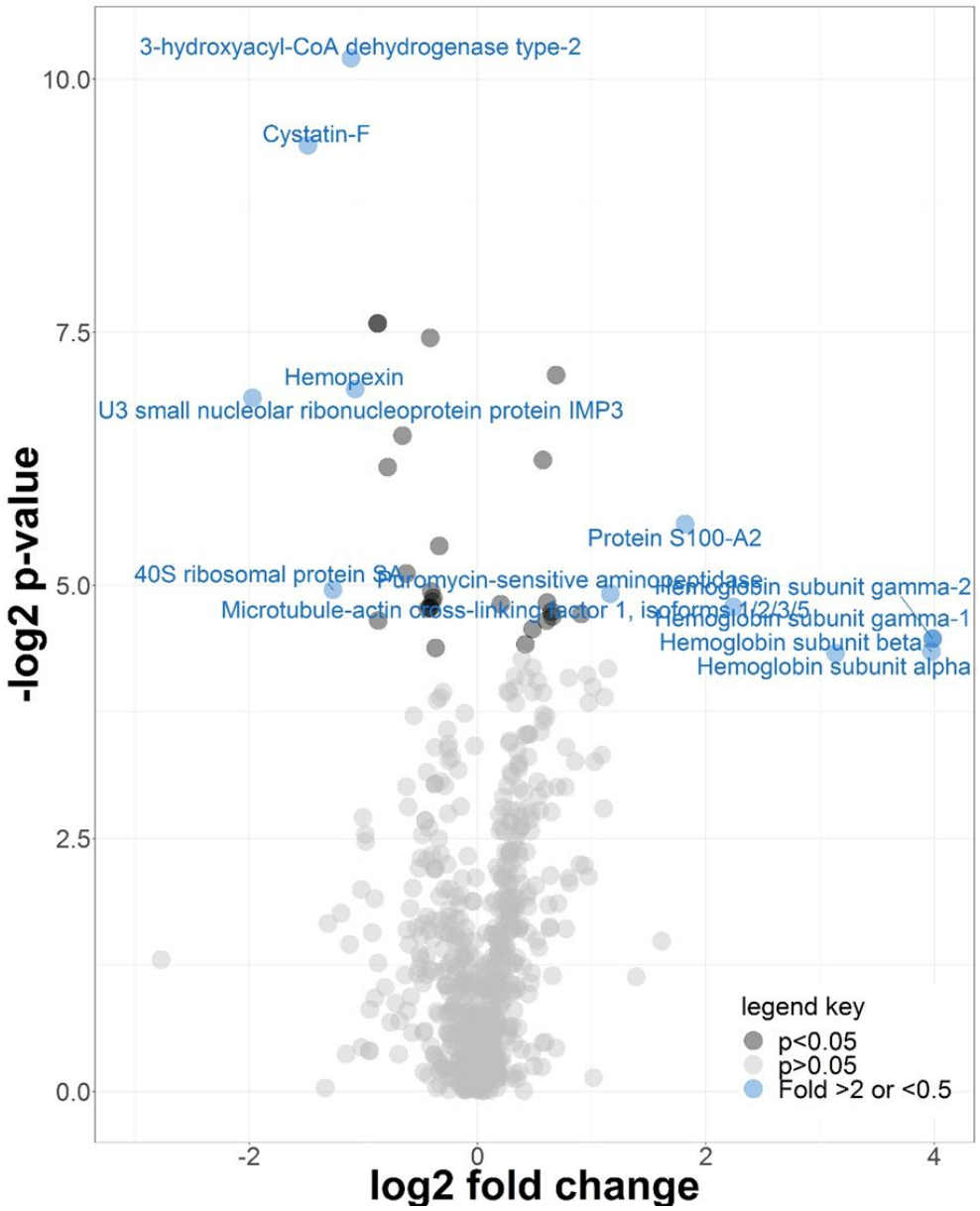

Figure S6. Volcano plot of the comparison between superficial partial and deep partial thickness burns from the "standardized processing" cohort. The x-axis represents log2 transformed protein abundance fold change, and y-axis represents log2 transformed p values. The color indicates the significance level, grey = $p > 0.05$, black = $p < 0.05$, blue = $p < 0.05$ & fold change ≥ 2. The significant proteins ($p < 0.05$) with a more than two-fold change in abundance were labelled

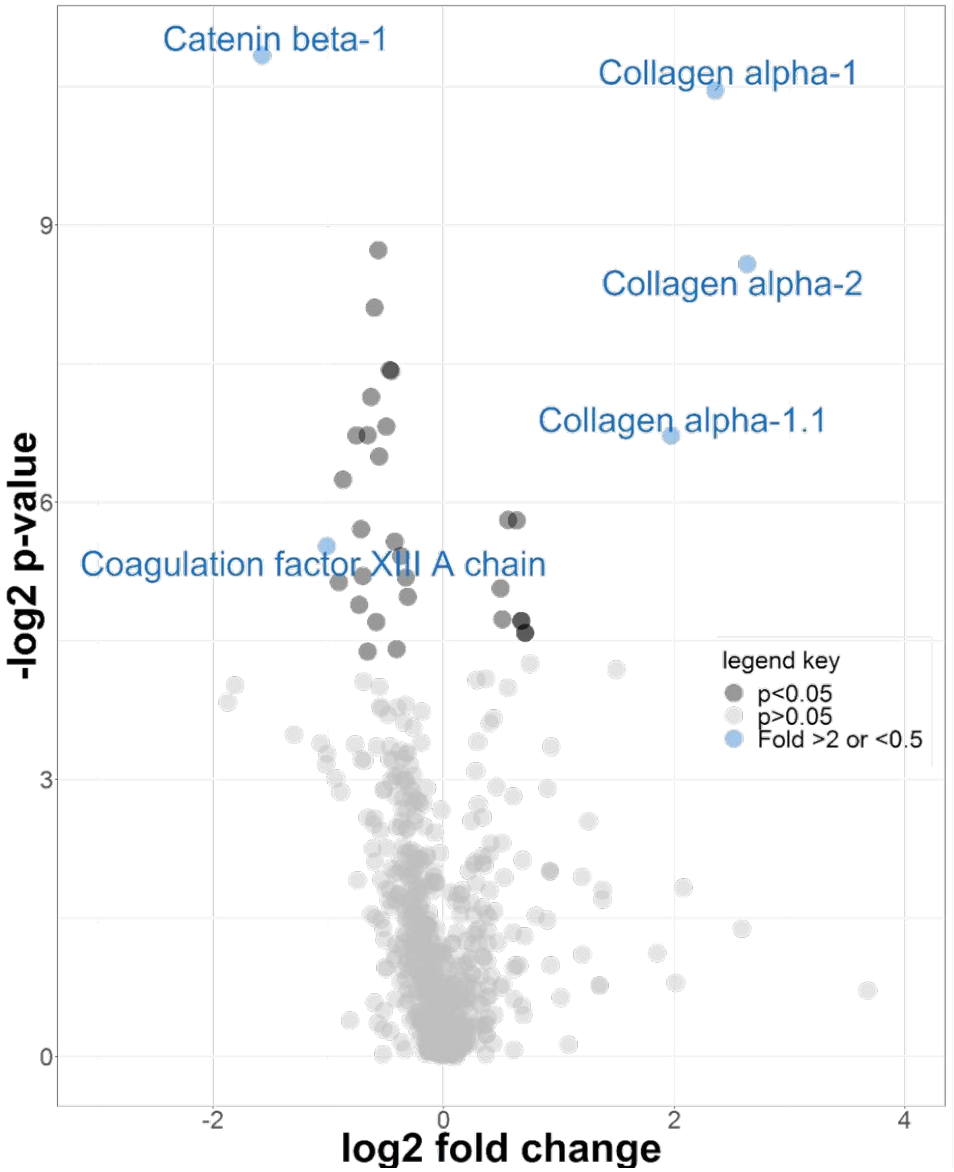

Figure S7. Volcano plot comparison of blister fluid proteins derived from burns which re-epithelialized within 21 days compared to those that took longer than 21 days to re-epithelialize. The x-axis represents log2 transformed protein abundance fold change, and y-axis represents log2 transformed p values. The color indicates the significance level, grey = $p > 0.05$, black = $p < 0.05$, blue = $p < 0.05$ & fold change ≥ 2. The significant proteins ($p < 0.05$) with a more than two-fold change in abundance were labelled.

Table S2. The top ten most over-represented Biological Processes (BPs) in Gene Ontology (GO) enrichment analysis using significant proteins identified by Student's t-test and PLS-DA analysis of burn depth classification.

	Biological Process	corrected p-value	Proteins in test set
T-test	response to wounding	2.05E-08	P02649|P68871|P02768|P69892|P69891|A0A0A0MRJ7|P04075|P01034|P07225|P10909
	regulation of body fluid levels	1.15E-07	P68871|P02768|P12830|P69892|P69891|A0A0A0MRJ7|P04075|P07225|P10909
	blood coagulation	2.60E-07	P68871|P02768|P69892|P69891|A0A0A0MRJ7|P04075|P07225|P10909
	coagulation	2.60E-07	P68871|P02768|P69892|P69891|A0A0A0MRJ7|P04075|P07225|P10909
	hemostasis	2.60E-07	P68871|P02768|P69892|P69891|A0A0A0MRJ7|P04075|P07225|P10909
	oxygen transport	6.82E-07	P68871|P69892|P69891|P69905
	wound healing	9.75E-07	P68871|P02768|P69892|P69891|A0A0A0MRJ7|P04075|P07225|P10909
	gas transport	9.75E-07	P68871|P69892|P69891|P69905
	platelet activation	1.49E-06	P68871|P02768|A0A0A0MRJ7|P04075|P07225|P10909
	regulation of proteolysis	2.57E-06	P13671|P02649|P06681|C9JV77|P12830|P20742|P01034|P07225|P10909
PLS-DA	oxygen transport	7.83E-10	P02042|P68871|P69892|P69891|P69905
	gas transport	8.91E-10	P02042|P68871|P69892|P69891|P69905
	blood coagulation	3.00E-07	P02671|P01042|P02042|P68871|P05160|P69892|P69891
	coagulation	3.00E-07	P02671|P01042|P02042|P68871|P05160|P69892|P69891
	hemostasis	3.00E-07	P02671|P01042|P02042|P68871|P05160|P69892|P69891
	regulation of body fluid levels	9.94E-07	P02671|P01042|P02042|P68871|P05160|P69892|P69891
	wound healing	9.94E-07	P02671|P01042|P02042|P68871|P05160|P69892|P69891
	response to wounding	1.98E-06	P02671|P01042|P02042|P68871|P05160|P69892|P69891
	response to stress	1.19E-03	P02671|P01042|P02042|P68871|O75874|P05160|P08294|P69892|P69891|P69905
	response to inorganic substance	1.39E-03	P02671|P68871|P08294|P69905

Table S3. The top ten most over-represented Molecular Functions (MFs) in Gene Ontology (GO) enrichment analysis using significant proteins identified by Student's t-test and PLS-DA analysis of burn depth classification.

	Molecular Function	corrected p-value	Proteins in test set
T-test	oxygen transporter activity	3.09E-07	P68871|P69892|P69891|P69905
	oxygen binding	1.29E-05	P68871|P69892|P69891|P69905
	endopeptidase inhibitor activity	6.77E-04	C9JV77|P20742|P01034|P07225
	endopeptidase regulator activity	6.77E-04	C9JV77|P20742|P01034|P07225
	peptidase inhibitor activity	6.77E-04	C9JV77|P20742|P01034|P07225
	heme binding	7.93E-04	P68871|P69892|P69891|P69905
	peptidase regulator activity	7.93E-04	C9JV77|P20742|P01034|P07225
	tetrapyrrole binding	7.93E-04	P68871|P69892|P69891|P69905
	iron ion binding	1.02E-03	P68871|P69892|P69891|P69905
	enzyme inhibitor activity	4.14E-03	C9JV77|P20742|P01034|P07225
PLS-DA	oxygen transporter activity	8.28E-11	P02042|P68871|P69892|P69891|P69905
	oxygen binding	1.14E-08	P02042|P68871|P69892|P69891|P69905
	heme binding	6.04E-06	P02042|P68871|P69892|P69891|P69905
	tetrapyrrole binding	6.04E-06	P02042|P68871|P69892|P69891|P69905
	iron ion binding	7.78E-06	P02042|P68871|P69892|P69891|P69905
	transition metal ion binding	3.32E-04	P01042|P02042|P68871|P08294|P22894|P69892|P69891|P69905
	cholate 7-alpha-dehydrogenase activity	5.35E-03	Q99714
	testosterone dehydrogenase [NAD(P)] activity	5.35E-03	Q99714
	3-hydroxy-2-methylbutyryl-CoA dehydrogenase activity	5.35E-03	Q99714
	cysteine-type endopeptidase inhibitor activity	5.39E-03	O76096|P01042

Table S4. The top ten most over-represented Cellular Components (CCs) in Gene Ontology (GO) enrichment analysis using significant proteins identified by Student's t-test and PLS-DA analysis of burn depth classification.

	Cellular Component	corrected p-value	Proteins in test set
T-test	blood microparticle	1.84E-15	P02790|P02649|P68871|P02768|P69892|P69905|P20742|P01861|P07225|P10909
	cytoplasmic membrane-bounded vesicle lumen	3.27E-14	P02790|P02649|P68871|P02768|P04075|P69905|P07225|P10909
	vesicle lumen	3.27E-14	P02790|P02649|P68871|P02768|P04075|P69905|P07225|P10909
	extracellular region	4.92E-14	P02790|Q9BUN1|P62805|P40926|O75874|P12830|P69892|Q96NZ9|P13671|P02649|P68871|P06681|C9JV77|P02768|P04075|P69905|P02533|P20742|P01861|P01034|P07225|P10909
	extracellular exosome	8.81E-14	P02790|P62805|P40926|O75874|P12830|P13671|P02649|P68871|P06681|P02768|P04075|P69905|P02533|P20742|P01861|P01034|P07225|P10909
	extracellular membrane-bounded organelle	8.81E-14	P02790|P62805|P40926|O75874|P12830|P13671|P02649|P68871|P06681|P02768|P04075|P69905|P02533|P20742|P01861|P01034|P07225|P10909
	extracellular vesicle	8.81E-14	P02790|P62805|P40926|O75874|P12830|P13671|P02649|P68871|P06681|P02768|P04075|P69905|P02533|P20742|P01861|P01034|P07225|P10909
	extracellular organelle	8.81E-14	P02790|P62805|P40926|O75874|P12830|P13671|P02649|P68871|P06681|P02768|P04075|P69905|P02533|P20742|P01861|P01034|P07225|P10909
	extracellular region part	1.50E-13	P02790|P62805|P40926|O75874|P12830|P69892|P13671|P02649|P68871|P06681|C9JV77|P02768|P04075|P69905|P02533|P20742|P01861|P01034|P07225|P10909
	extracellular space	6.13E-12	P02790|P69892|P02649|P68871|P06681|C9JV77|P02768|P04075|P69905|P20742|P01861|P01034|P07225|P10909
PLS-DA	hemoglobin complex	1.61E-11	P02042|P68871|P69892|P69891|P69905
	blood microparticle	1.12E-08	P02671|P01042|P02042|P68871|P69892|P69905
	cytoplasmic membrane-bounded vesicle lumen	2.37E-06	P02671|P01042|P68871|P69905
	vesicle lumen	2.37E-06	P02671|P01042|P68871|P69905
	cytosolic part	3.60E-06	P02042|P68871|P69892|P69891|P69905
	extracellular region	3.60E-06	P02671|P01042|P02042|Q9BUN1|O75874|P69892|Q96NZ9|O76096|P68871|P05160|P08294|P22894|P69905
	extracellular space	1.06E-05	P02671|P01042|P02042|P68871|P08294|P22894|P69892|P69905

	haptoglobin-hemoglobin complex	1.06E-05	P68871|P69905
	endocytic vesicle lumen	3.77E-04	P68871|P69905
	extracellular region part	1.32E-03	P02671|P01042|P02042|P68871|O75874|P08294|P22894|P69892|P69905

Table S5. The top ten most over-represented Biological Processes (BPs) in Gene Ontology (GO) enrichment analysis using significant proteins identified by Student's t-test and PLS-DA analysis of time to re-epithelialization classification.

	Biological Process	corrected p-value	Proteins in test set
T-test	peptide cross-linking	2.37E-07	P02461|P00488|A6PVK5|P07996|Q08188
	platelet activation	1.54E-05	P02452|P02461|P00488|P07996|P01023|P07225
	response to reactive oxygen species	6.09E-05	P02452|P02649|P06727|Q06830|P07996
	response to oxygen-containing compound	7.48E-05	P02452|Q14126|P02461|P02649|P06727|P13646|Q06830|P10720|P07996
	negative regulation of endopeptidase activity	7.48E-05	P0C0L5|P03973|P20742|P07996|P01023|P07225
	negative regulation of peptidase activity	7.74E-05	P0C0L5|P03973|P20742|P07996|P01023|P07225
	platelet degranulation	7.74E-05	P00488|P07996|P01023|P07225
	regulation of cellular component movement	9.58E-05	P02452|Q14126|P02461|P02649|P10720|P31146|P07996
	cell activation	1.01E-04	P02452|P02461|P00488|P31146|P07996|P01023|P07225
	response to wounding	1.01E-04	P02452|P02461|P02649|P00488|P07996|P01023|P07225
PLS-DA	platelet activation	4.64E-08	P02452|P60709|P00488|P07996|P01023|P07225|P10909
	response to wounding	4.64E-08	P02452|P60709|P04070|P02649|P00488|P07996|P01023|P07225|P10909
	blood coagulation	4.65E-08	P02452|P60709|P04070|P00488|P07996|P01023|P07225|P10909
	coagulation	4.65E-08	P02452|P60709|P04070|P00488|P07996|P01023|P07225|P10909
	hemostasis	4.65E-08	P02452|P60709|P04070|P00488|P07996|P01023|P07225|P10909
	platelet degranulation	1.60E-07	P00488|P07996|P01023|P07225|P10909
	regulation of body fluid levels	1.68E-07	P02452|P60709|P04070|P00488|P07996|P01023|P07225|P10909
	wound healing	1.68E-07	P02452|P60709|P04070|P00488|P07996|P01023|P07225|P10909

	regulation of biological quality	1.36E-06	P02452|P60709|P29373|P06727|Q6S8J3|P30101|P04070|P02649|P00488|P07996|P01023|P07225|P10909
	vesicle-mediated transport	6.35E-06	P02452|P02760|P60709|P02649|P01615|P00488|P07996|P01023|P07225|P10909

Table S6. The top ten most over-represented Molecular Functions (MFs) in Gene Ontology (GO) enrichment analysis using significant proteins identified by Student's t-test and PLS-DA analysis of time to re-epithelialization classification.

	Molecular Function	corrected p-value	Proteins in test set
T-test	endopeptidase inhibitor activity	6.30E-05	P0C0L5|P03973|P20742|P01023|P07225
	endopeptidase regulator activity	6.30E-05	P0C0L5|P03973|P20742|P01023|P07225
	peptidase inhibitor activity	6.30E-05	P0C0L5|P03973|P20742|P01023|P07225
	peptidase regulator activity	9.91E-05	P0C0L5|P03973|P20742|P01023|P07225
	growth factor binding	1.38E-04	P02452|P02461|P07996|P01023
	phosphatidylcholine-sterol O-acyltransferase activator activity	2.59E-04	P02649|P06727
	identical protein binding	4.66E-04	P02452|P13716|P02649|P06727|Q06830|P31146|P07996
	platelet-derived growth factor binding	4.87E-04	P02452|P02461
	enzyme inhibitor activity	4.87E-04	P0C0L5|P03973|P20742|P01023|P07225
	enzyme regulator activity	7.38E-04	P0C0L5|P03973|P02649|P06727|P20742|P01023|P07225
PLS-DA	phosphatidylcholine-sterol O-acyltransferase activator activity	1.19E-03	P02649|P06727
	identical protein binding	2.94E-03	P02452|P02760|P60709|P02649|P06727|P07996
	alcohol binding	2.94E-03	P29373|P02649|P06727
	lipoprotein particle binding	2.94E-03	P02649|P07996
	protein-lipid complex binding	2.94E-03	P02649|P07996
	growth factor binding	2.94E-03	P02452|P07996|P01023
	cholesterol transporter activity	7.53E-03	P02649|P06727
	sterol transporter activity	7.53E-03	P02649|P06727
	endopeptidase inhibitor activity	7.53E-03	P02760|P01023|P07225
	endopeptidase regulator activity	7.53E-03	P02760|P01023|P07225

Table S7. The top ten most over-represented Cellular Components (CCs) in Gene Ontology (GO) enrichment analysis using significant proteins identified by Student's t-test and PLS-DA analysis of time to re-epithelialization classification.

	Cellular Component	corrected p-value	Proteins in test set
T-test	extracellular region	8.52E-16	P02452|Q14126|P49862|O15143|P13646|P10412|Q08188|P0C0L5|P02649|P01615|P00488|P07996|P02461|P03973|P13716|P06727|Q06830|P10720|P31146|P00915|P36980|Q9NZP8|P20742|P01023|P07225
	extracellular region part	1.81E-15	P02452|Q14126|P02461|P03973|P13716|O15143|P06727|P13646|Q06830|P10412|P10720|P31146|Q08188|P0C0L5|P00915|P02649|P01615|P00488|Q9NZP8|P20742|P07996|P01023|P07225
	membrane-bounded vesicle	6.42E-15	P02452|Q14126|P03973|P49862|P13716|O15143|P06727|P13646|Q06830|P10412|P31146|Q08188|P0C0L5|P00915|P02649|P01615|P00488|Q9NZP8|P20742|P07996|P01023|P07225
	vesicle	9.50E-15	P02452|Q14126|P03973|P49862|P13716|O15143|P06727|P13646|Q06830|P10412|P31146|Q08188|P0C0L5|P00915|P02649|P01615|P00488|Q9NZP8|P20742|P07996|P01023|P07225
	extracellular exosome	7.79E-14	Q14126|P03973|P13716|O15143|P06727|P13646|Q06830|P10412|P31146|Q08188|P0C0L5|P00915|P02649|P01615|Q9NZP8|P20742|P07996|P01023|P07225
	extracellular membrane-bounded organelle	7.79E-14	Q14126|P03973|P13716|O15143|P06727|P13646|Q06830|P10412|P31146|Q08188|P0C0L5|P00915|P02649|P01615|Q9NZP8|P20742|P07996|P01023|P07225
	extracellular vesicle	7.79E-14	Q14126|P03973|P13716|O15143|P06727|P13646|Q06830|P10412|P31146|Q08188|P0C0L5|P00915|P02649|P01615|Q9NZP8|P20742|P07996|P01023|P07225
	extracellular organelle	7.79E-14	Q14126|P03973|P13716|O15143|P06727|P13646|Q06830|P10412|P31146|Q08188|P0C0L5|P00915|P02649|P01615|Q9NZP8|P20742|P07996|P01023|P07225
	blood microparticle	8.10E-10	P0C0L5|P02649|P06727|P00488|P20742|P01023|P07225
	extracellular space	9.10E-10	P02452|P02461|P06727|Q06830|P10720|P0C0L5|P02649|P00488|Q9NZP8|P20742|P07996|P01023|P07225
PLS-DA	blood microparticle	6.41E-14	P02760|P60709|P02649|P06727|P00488|Q6S8J3|P01023|P07225|P10909
	cytoplasmic membrane-bounded vesicle lumen	5.13E-10	P02649|P00488|P07996|P01023|P07225|P10909
	vesicle lumen	5.13E-10	P02649|P00488|P07996|P01023|P07225|P10909

platelet alpha granule lumen	4.62E-09	P00488|P07996|P01023|P07225|P10909
membrane-bounded vesicle	9.84E-09	P02452|P02760|P60709|P29373|P06727|Q6S8J3|P30101|P02649|P01615|P00488|P67936|P07996|P01023|P07225|P10909
platelet alpha granule	9.84E-09	P00488|P07996|P01023|P07225|P10909
secretory granule lumen	9.84E-09	P00488|P07996|P01023|P07225|P10909
vesicle	9.84E-09	P02452|P02760|P60709|P29373|P06727|Q6S8J3|P30101|P02649|P01615|P00488|P67936|P07996|P01023|P07225|P10909
extracellular space	1.24E-08	P02452|P02760|P60709|P02649|P06727|P00488|Q6S8J3|P07996|P01023|P07225|P10909
extracellular region part	1.51E-08	P02452|P02760|P60709|P29373|P06727|Q6S8J3|P30101|P02649|P01615|P00488|P67936|P07996|P01023|P07225|P10909