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Systemic immune response alteration in patients with severe pressure ulcers

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Wound healing is a dynamic process involving tissue formation, debris removal and ultimately remodeling to restore skin integrity. Although wound healing is generally successful, this process can eventually fail, leading to chronic wounds like pressure ulcers (PUs), whose presence/absence has been considered by WHO as good indicator of patient's wellbeing and care quality. PUs are stratified into grades I to IV grades based on their severity, however, the existence of systemic markers predicting their clinical progression remains unexplored. Here, we performed a serum proteomic and transcriptomic profiling of 54 patients with PUs ranging from grade II to grade III-IV. Unsupervised clustering identified a distinctive immune-related proteomic and transcriptomic blood profile in high-grade PUs. Specifically, pathways controlled by inflammatory-linked genes such as *IER3*, *TSLP*, and *TNFAIP6* (*TSG-6*) were found to be upregulated in high-grade PUs, together with a reduction in the levels of potent immunomodulators such as IL-10, IFNγ, MCP-2/CCL8, and CXCL-10 in serum from grade III-IV PUs patients. All together, indicating an altered inflammatory state in advanced PUs. This study provides novel insights regarding the use of omic approaches to find potential systemic biomarkers for the prediction of severity in PUs and could help to understand the molecular mechanisms underlying the chronic progression of this pathology.

Keywords Wound healing, Pressure ulcers, Biomarkers, Transcriptomic, Proteomic, Systemic inflammation

Wound healing is a concept encompassing both the physiological processes of new tissue formation and debris removal. This process is often divided into four overlapping and continuous phases, lasting approximately three months including haemostasis, inflammation, proliferation and remodeling[1]. The healing process is mediated by the action of a plethora of molecules including cytokines, chemokines and growth factors, with direct steps leading to the restoration of the skin's physiological barrier function[2]. Combined with impaired cellular and systemic host responses to stress, local tissue hypoxia, repetitive trauma and heavy bacterial burden, have been described to effectively disrupt the wound healing process, causing tissue damage and ultimately promoting a persistent inflammatory state[1].

The term 'chronic wounds' includes a heterogeneous group of skin lesions that do not follow the normal course of healing, and includes diabetic foot ulcers, venous and arterial ulcers and pressure ulcers (PUs), among others[3]. In this work we will focus on PUs. PUs are clinically defined as localized damage affecting the skin and/or the underlying tissue, as a consequence of pressure with or without the combination of shear[4]. PUs are usually developed on bony prominences, however, their occurrence can also be related due to an overuse of medical devices[4].

WHO considers the presence of PUs as a good indicator of the quality of care received by the patient. PUs significantly affect both patient's quality of life and healthcare resource utilization[5,6]. The reported worldwide prevalence of PUs, according to WHO, ranges from 5 to 12%, however, their true incidence is difficult to determine, as many cases are treated at home and go unreported. PUs are mostly caused by external pressure

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applied to an area of the body, mainly bony prominences, leading to blockage of capillaries and causing ischemia, hypoxia, edema, inflammation and ultimately necrosis and ulcer formation. PUs are generally classified into grades I-IV based on the international National Pressure Ulcer Advisory Pane/European Pressure Ulcer Advisory Panel (NPUAP/EPUAP) system, established in 2019[4]. Grades I-II PUs include incipient ulcers that can easily regenerate if the antipathogenic factors are alleviated. In case underlying causes persist, the natural progression leads to ulcers becoming more severe and potentially irreversible, with involvement of injured subcutaneous tissues including in some cases muscle or bone (Grades III-IV PUs)[7].

Patients suffering from chronic ulcers are heterogeneous cohorts, usually composed of elderly people with multiple comorbid diseases, and under diverse treatments[8]. Thus, this clinical situation makes extremely needed the identification of systemic molecular biomarkers, which could easily predict disease severity outcomes, with a future aim of improving patient's guidance for the selection of better and more effective interventions[23]. From the molecular perspective, normal wound repair usually involves several processes such as cytokine release, rearrangement of adhesion molecules/cytoskeletal components, as well as the alteration in the expression of tissue remodeling molecules such as matrix metalloproteases (MMPs). Particularly, non-healing wounds commonly manifest the presence of a prolonged inflammation status, deregulation of protease levels, a reduced growth factor activity, stem cell dysfunction and cellular senescence[9–12]. In addition, some cellular events have been also associated with the wound healing process, such as the release of tumor necrosis factor alpha (TNF-α) and different growth factors, involving platelets, neutrophils, macrophages and fibroblasts[12]. To clarify the complex role exerted by all these mediators in this process, the combination of omic approaches such as transcriptomics and proteomics can provide a holistic understanding of cellular mechanisms at a molecular level. This multi-omic approach could enable a personalized medicine with the ability to validate biomarkers for diagnosis and ultimately help to describe potential therapeutic targets to prevent wound healing worsening[12].

In this work, we propose that PUs progression to irreversible stages (Grades III-IV) lies in a systemic and chronic inflammatory process, elucidated by specific transcriptomic and proteomic profiling changes. Considering most of the research conducted on ulceration uses skin/tissue-derived samples, this work also aimed to identify non-invasive and easy to detect systemic markers able to classify patients with grade I-II and III/IV (irreversible) PUs, lately shedding a light into the understanding of the molecular/cellular mechanisms governing the chronic progression of this pathology.

Results

Patient phenotypes and characteristics

Patient information was thoroughly analyzed to ensure that significant differences in the transcriptomic and proteomic analyses were solely and exclusively due to ulcer-related factors.

Information was collected on the medical pathologies related to risk factors (sex; age; hospitalizations; admission to ICU; diabetes mellitus; hypercholesterolemia; vascular, neurological and cardiac pathology; antiplatelet, anticoagulant or antibiotic treatment) and on ulceration in question (ulcer location, grade and group) of the 54 patients selected (Supplementary Table 1).

Samples from all the patients in the cohort were used for the proteomic study. For transcriptomics, 10 patients from each group were used. Statistical analysis showed that no significant differences between patients with pressure ulcer grade II and patients with pressure ulcer grade III-IV were found for any of the clinical variables analyzed (Supplementary Table 2). Likewise, we didn't observe significant statistical differences for any of the clinical variables considering the patients included for the RNA sequencing (Supplementary Table 3).

Our results conclude that cohort of study display homogeneous clinical parameters. Therefore, we continue to analyze the proteomic and transcriptomic profile of these patients.

Patients with grade III-IV Pressure Ulcers display an altered inflammatory response

Next, we analyze the proteomic profile in serum samples from all enrolled patients (Supplementary Table 1) with Olink® Target 96 Inflammation panel (Uppsala, Sweden). Anti-inflammatory Interleukin 10 (IL-10), macrophage activation factor gamma Interferon (IFNg), Monocyte Chemotactic Protein (MCP-2 or CCL8) and C-X-C motif chemokine ligand 10 (CXCL10) showed significant differences between patients with grade II PUs and patients with grade III-IV PUs (Fig. 1). The four proteins show a significant decrease in the Grade III-IV group.

These results demonstrate a systemic dysregulation of immune mediators related to the inflammatory response taking place during pression ulcers.

Identification of differentially expressed genes between patients with grade II versus grade III/IV pressure ulcers

Additionally, we perform a transcriptomic analysis of the PBMCs (peripheral blood mononuclear cells) to figure out which immune mediators are contributing to the development of the inflammatory response in these patients. This was carried out by RNA sequencing to determine the different transcriptomic profiles of patients with grade II PUs and patients with grade III-IV PUs. In each case, 10 patients were selected to perform RNA sequencing (Supplementary Table 1).

Figure 2 shows distribution of patients with grade II PUs and patients with grade III-IV PUs according to a Principal Component Analysis (PCA) model. From the 44,714 transcripts sequences obtained, 1081 transcripts were identified as significantly differentially expressed between the experimental groups. Differential expression analysis (grade III-IV PUs patients vs. grade II PUs patients) considered as significant transcripts those with p-values < 0.05 and changes in expression Log_2 (Fold Change) greater that 2 or lesser than −2. A total of 117 differentially expressed genes were identified. From all of them, 48 genes were downregulated (Table 1 upside) and 69 were upregulated (Table 1 downside) in patients with grade III-IV PUs vs. grade II PUs. Among overexpressed genes, *IER3*, *TSLP*, *CD177*, *DCSTAMP*, *IGLV3-6*, and *C2CD4A* were directly related to

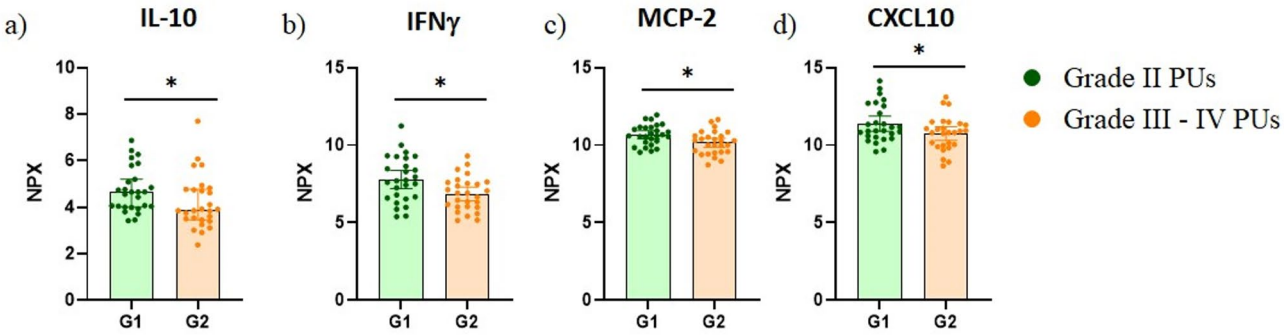

Fig. 1. IL-10, IFNg, MCP-2 and CXCL10 relative concentration (NPX) comparing patients with grade II PUs (Group 1, G1) and patients with grade III-IV PUs (Group 2, G2) subjects. Mann-Whitney *U*-test was performed for IL-10, IFNg and MCP-2 and Unpaired T-test for CXCL10. Median with interquartile range was presented. (**a**) IL-10 (p-value 0.0272); (**b**) IFNg (p-value 0.0178); (**c**) MCP-2 (p-value 0.0125); (**d**) CXCL10 (p-value 0.0494). *: p-value < 0.05.

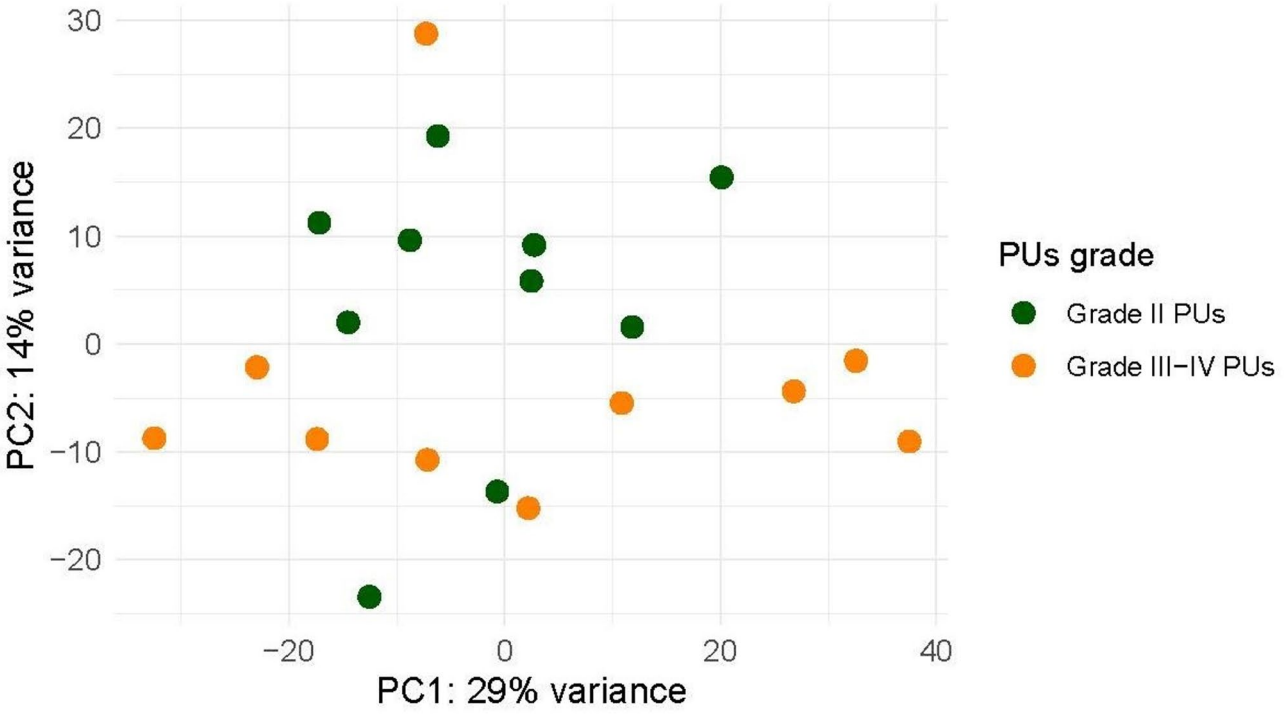

Fig. 2. Principal Component Analysis (PCA) plot of the mRNA expression profiles from patients with grade II PUs (G1, green dots) and patients with grade III-IV PUs (G2, orange dots).

inflammation and immune-linked processes, and *MMP27*, *NSG2* related with tissue remodeling. Among the downregulated genes, we noticed changes in the expression of genes encoding for receptor of cytokines with a widely described role in tissue healing (*IL22RA1* and *IL17RD*) or the proteasome formation (*CHGB*).

A hierarchical clustering of Z-score normalized expression values was performed with the differentially expressed genes (Fig. 3). Thus, we observed differential gene expression, with genes such as *TRAV38-1*, *FAM106A*, *IL11*, *MIR6797*, and *TRAJ22* showing higher expression levels in patients with grade II ulcers (genes related with T cell receptors and immune response), whereas genes such as *EPCAM*, *MMP27*, *MIR31B*, and *CHIT1* exhibited increased expression in patients with grade III–IV ulcers (genes related with matrix extracellular remodeling).

Our results demonstrate that different stages of PU progression are associated with a specific transcriptomic fingerprint of PBMCs, suggesting a systemic inflammatory response in these patients linked to the progression of the ulcer.

Downregulated	Log_2FoldChange	*p*-value
LOC107984850	− 3.327	0.0243
CYP26B1	− 3.156	0.0265
AOX3P-AOX2P	− 3.129	0.0143
TRAJ22	− 3.118	0.0006
BMPR1AP1	− 3.075	0.0322
LINC02764	− 3.028	0.0014
LOC107984551	− 3.021	0.0024
SLC5A4	− 2.874	0.0456
LOC105375905	− 2.814	0.0286
LOC112267900	− 2.813	0.0165
CEACAM22P	− 2.703	0.0448
NGEF	− 2.698	0.0451
IL11	− 2.636	0.0026
LINC00622	− 2.634	0.0044
VTI1BP1	− 2.626	0.0327
DPP10	− 2.615	0.0116
P2RX3	− 2.606	0.0334
LOC105371372	− 2.603	0.0089
DYNC1LI2– DT	− 2.556	0.0099
LOC105379091	− 2.513	0.0275
LOC150051	− 2.513	0.0362
LOC107984211	− 2.504	0.0213
IL22RA1	− 2.503	0.0336
CYP11B1	− 2.428	0.0317
LOC105370596	− 2.415	0.0132
SOWAHA	− 2.371	0.0480
IL17RD	− 2.345	0.0431
FOXQ1	− 2.318	0.0143
REEP1	− 2.311	0.0083
PNMA2	− 2.298	0.0008
MTHFD2P1	− 2.288	0.0286
IGF2BP1	− 2.269	0.0489
LOC107987084	− 2.268	0.0465
LOC107986167	− 2.257	0.0216
TPSB2	− 2.243	0.0191
LINGO4	− 2.230	0.0095
LOC112268218	− 2.218	0.0204
LOC105369322	− 2.205	0.0352
CHGB	− 2.170	0.0194
SCGB3A1	− 2.138	0.0045
LOC102723408	− 2.112	0.0378
MIR6797	− 2.107	0.0422
RPL4P2	− 2.107	0.0218
MIR6730	− 2.099	0.0238
TRAV38– 1	− 2.095	$0.3E^{-5}$
NOTUM	− 2.064	0.0273
CKS1BP4	− 2.030	0.0228
FAM106A	− 2.002	0.0068
Upregulated	**Log_2FoldChange**	***p*-value**
IER3	5.868	0.0474
MTRNR2L1	5.222	0.0003
CTB-3M24.3	3.775	0.0237
LINC01954	3.567	0.0189
CSNK1A1P1	3.373	0.0252
LINC01811	3.352	0.0011
LOC101927691	3.345	0.0084
Continued		

Upregulated	Log_2FoldChange	*p*-value
TSLP	3.308	0.0030
LOC107985269	3.293	0.0097
MMP27	3.246	0.0045
RBM12B-DT	3.223	0.0254
OLAH	3.182	0.0125
LINC00908	3.096	0.0031
LINC01991	3.021	0.0199
LOC107984564	2.921	0.0089
PGA5	2.887	0.0499
TRL-CAA5-1	2.868	0.0106
LIX1	2.748	0.0016
MIR371B	2.721	0.0165
CD177	2.700	0.0013
NSG2	2.684	0.0131
HSPA8P11	2.622	0.0334
DCSTAMP	2.585	0.0162
LOC105376411	2.560	0.0284
LOC389895	2.558	0.0366
AP3B2	2.557	0.0002
CA12	2.557	0.0172
LOC401312	2.546	0.0251
IGLV3-6	2.523	0.0408
RPS6P3	2.521	0.0199
CBX1P4	2.519	0.0367
NPBWR1	2.507	0.0232
GPR17	2.489	0.0071
ADAMTS2	2.461	0.0277
MAFA-AS1	2.451	0.0271
SNHG31	2.426	0.0357
KLHDC7A	2.407	0.0053
RPL5P9	2.403	0.0105
LINC01725	2.384	0.0386
LOC403312	2.381	0.0347
LOC105373475	2.359	0.0472
SNORA37	2.358	0.0431
LOC105372633	2.356	0.0079
EPCAM	2.355	0.0317
RPSAP6	2.328	0.0373
HLA-DOB	2.326	0.0403
C2CD4A	2.321	0.0242
LOC105374545	2.290	0.0015
LINC01118	2.289	0.0328
NUTF2P7	2.284	0.0385
SCN2A	2.271	0.0047
TNFAIP6 TSG6	2.266	0.0018
LOC107986984	2.255	0.0383
LOC107985888	2.253	0.0231
GNMT	2.245	0.0359
LINC02605	2.243	0.0316
LOC107985895	2.240	0.0299
LOC103908605	2.186	0.0264
LOC105376103	2.147	0.0113
LOC284600	2.133	0.0267
LOC107984195	2.122	0.0416
LOC107985073	2.116	0.0403
SUMO1P1	2.078	0.0011
Continued		

Upregulated	Log_2FoldChange	*p*-value
COL19A1	2.060	0.0009
LOC107985308	2.055	0.0227
LOC107984118	2.054	0.0297
LOC101929555	2.053	0.0425
CNTNAP3C	2.028	0.0455
CHIT1	2.018	0.0081

Table 1. Differentially expressed genes in group 2 patients compared to group 1 patients ordered by Log2FoldChange.

PUs progression involves immunological pathways

Gene Set Enrichment Analysis (GSEA) was then performed using all genes to further investigate their biological relevance. Notably, GSEA results indicated that signatures related to inflammation and altered immune responses are significantly enriched in patients with grade III-IV PUs (Group 2) in comparison to grade II PUs patients (Group 1) (Tables 2 and 3; Fig. 4). Concretely, most of the enriched pathways when comparing Groups 2 and 1 of PUs patients indicate immunological alterations, involving a plethora of cellular entities such as T cells, B cells and monocytes/macrophages. Some examples are HAY_BONE_MARROW_FOLLICULAR_B_CELL(FDR q-val < 1.0E-4); GSE22886_NAIVE_BCELL_VS_NEUTROPHIL_DN (FDR q-val < 1.0E-4); GSE22886_ TCELL_VS_BCELL_NAIVE_DN (FDR q-val 5.877E-4); GSE3982_MEMORY_ CD4_TCELL_VS_BCELL_DN (FDR q-val 0.002); GSE25123_WT_VS_ PPARG_KO_MACROPHAGE_UP (FDR q-val 0.002); GSE29618_ MONOCYTE_VS_MDC_UP (FDR q-val 0.002); HAY_BONE_MARROW_NEUTROPHIL (FDR q-val 0.002), which are enriched in Group 2; and to a lesser extend some examples also enriched in Group 1 of less severe PUs patients such as GOCC_T_CELL_RECEPTOR_COMPLEX (FDR q-val 0.0); GSE10325_CD4_ TCELL_ VS_MYELOID_UP (FDR q-val 0.02); GSE11057_CD4_EFF_MEM_ VS_PBMC_UP (FDR q-val 0.02).

Finally, an experimental validation in the expression of selected differentially expressed genes assessed by transcriptomics (*AP3B2*, *CD177*, *COL19A1*, *CYP26B1*, *IER3*, *LIX-1*, *MMP27*, *NEGF*, *OLAH*, *PGA5*, *PNMA2*, *SCN2A*,*SUMO1P1*, *TNFAIP6*, *TSLP*) was examined by RT-qPCR. When compared with RNA-seq data, up to 95% of the selected transcripts showed the same expression pattern, thus reinforcing the reliability of the observed changes (Fig. 5). Supplementary Table 5 contains the genes, and the primers sequences used to validate RNA-seq data.

Discussion

Pressure Ulcers (PUs) is a complex and multifactorial injury which is becoming a significant and concerning healthcare problem worldwide, especially after COVID pandemic[13]. As mentioned before, PUs usually affects patients older than 70 years, being developed in more than 80% of hospitalized individuals within the first 5 days of inpatient hospital stay[14]. PUs progression and chronicity is a dynamic process clinically involving since skin rubbing to blood discharge, and sometimes if become so deep (i.e. stage IV) with profound damage affecting tendons, joints and even the muscle and bone, resulting in life-threatening complications such as infections, malnutrition and anemia[15]. In this sense, several specialists agreed that prognostic intervention is nowadays the best treatment of PUs, implying the early detection of the injury. Chronic development of PUs are affected by several factors including the advancing age, nutritional status, presence of other chronic comorbidities complicating their detection before they are visually, clinically -and molecularly- irreversible[16,17]. Majority of PUs are preventable and the availability of biomarkers capable of predicting their onset and progression would represent a significant economic benefit for healthcare systems (e.g., bed availability, treatment duration), while also enhancing patient care and overall wellbeing. The complexity and clinical stratification (grades) of PUs necessitate large-scale studies (omics) to identify biomarkers, enabling big molecular analysis of changes at protein levels and function, as well as describing gene expression alterations to elucidate the cellular events behind their development and chronicity[18].

Most of the research performed at describing the development of PUs commonly used skin samples -collected from different depths and location-, with the aim to identify local biomarkers that could help at guiding the selection for the best treatment and predict outcomes[19–22]. However, tissue obtained from the surroundings of non-healing wounds (as observed in advanced PU stages) exhibits a hyperproliferative epidermal phenotype, varying levels of fibrosis, and heightened cellular infiltration (mainly leukocytes, macrophages, neutrophils)[19,23]. In addition, skin sample collection is extremely invasive and painful for patients, involving heterogeneous samples (varying in depth, width, layers of study, etc.), and other less aggressive alternatives need to be considered for routine sampling. Regarding the latter, we wanted here to gain insight into the systemic (instead of local) mechanisms underlying the defective wound healing observed in patients with different stages of PUs, using a multi-omic approach (proteomic and transcriptomic) with the aim to describe blood biomarkers that could help to predict irreversible wound healing in elderly patients. Concretely, we have identified serum alterations related to immune regulation and inflammation when comparing patient samples with low-grade (II) and high-grade (III-IV) PUs. Notably, systemic inflammation has been usually discarded to predict damage for other types of ulceration (e.g. diabetic foot ulcers (DFUs)) due to the insufficient elevation of this signature, mainly influenced by the etiology of the disease (e.g. DFUs are not caused because of immobility but as a result of diabetes complications). We proposed here that a systemic alteration of the inflammatory/immune status is a

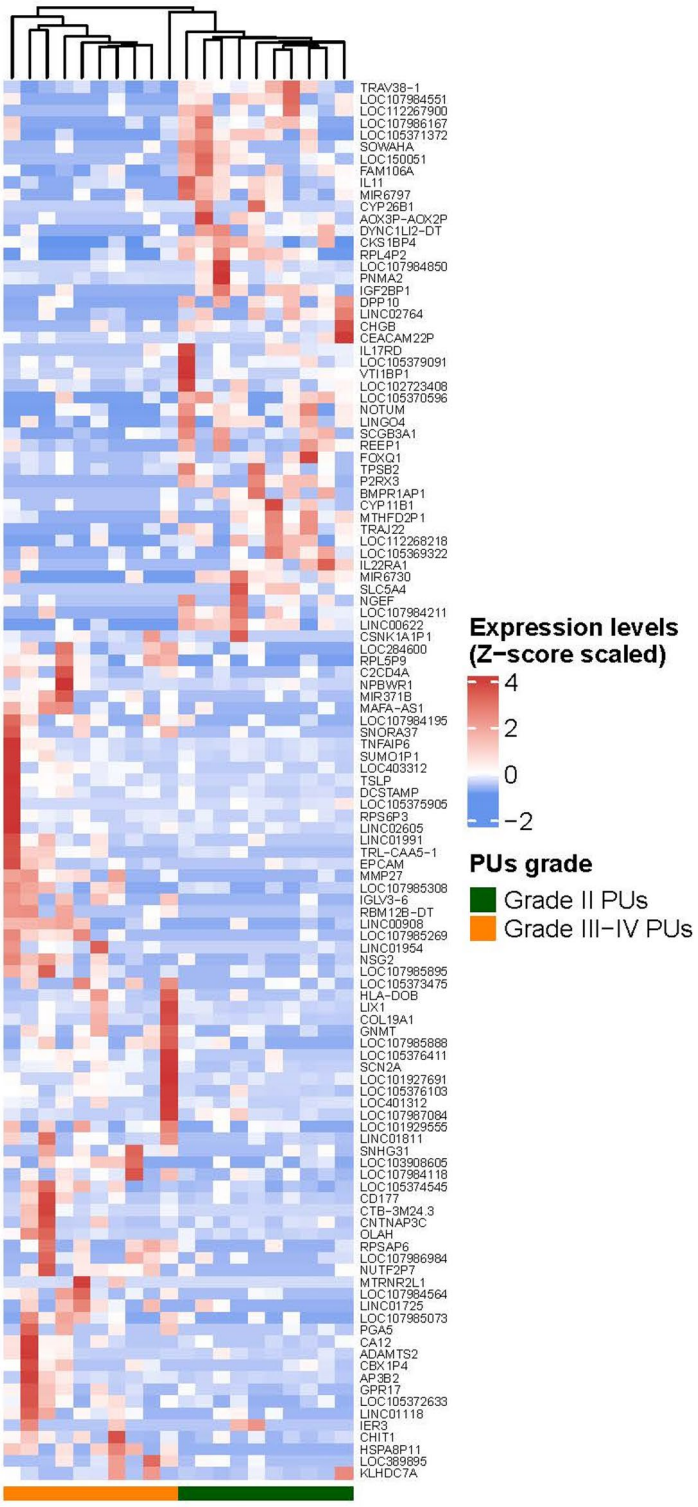

Fig. 3. 2D hierarchical clustering heatmap of the differentially expressed genes, using Z-score normalized RNA-seq data from patients with grade II PUs (Group 1) compared with patients with grade III-IV PUs (Group 2). Blue bands indicate low gene expression values; red bands indicate high gene expression values.

molecular signature that may help to distinguish severity PUs grades. Transcriptomics have been previously utilized to identify differentially expressed genes at specific stages of wound healing process[24–26] and mostly focused in DFUs[27–30]. Here, a multi-omic approach was performed and transcriptomics were complemented by a proteomic analysis, where we found altered blood levels (decrease in patients with high-grade (III-IV) PUs) of four important immunoregulatory molecules such as IL-10, IFNγ, CXCL10 and MCP-2/CCL8.

Pathway	FDR q-val	NES	*n*
HAY_BONE_MARROW_FOLLICULAR_B_CELL	0.000	2.47	141
GSE10325_CD4_TCELL_VS_BCELL_DN	0.000	2.39	188
GSE10325_LUPUS_CD4_TCELL_VS_LUPUS_BCELL_DN	0.000	2.35	191
GSE22886_NAIVE_BCELL_VS_NEUTROPHIL_DN	0.000	2.32	198
REACTOME_CD22_MEDIATED_BCR_REGULATION	0.000	2.20	47
GOCC_IMMUNOGLOBULIN_COMPLEX	0.000	2.20	109
GSE22886_TCELL_VS_BCELL_NAIVE_DN	$5.88E^{-04}$	2.12	186
GSE29618_BCELL_VS_PDC_UP	$5.46E^{-04}$	2.12	194
AIZARANI_LIVER_C34_MHC_II_POS_B_CELLS	$6.83E^{-04}$	2.10	131
REACTOME_ANTIGEN_ACTIVATES_B_CELL_RECEPTOR_BCR_LEADING_TO_GENERATION_OF_SECOND_MESSENGERS	$6.43E^{-04}$	2.10	72
GSE3982_NEUTROPHIL_VS_EFF_MEMORY_CD4_TCELL_UP	0.0012	2.08	193
GSE3982_MEMORY_CD4_TCELL_VS_BCELL_DN	0.0018	2.07	192
GSE9988_ANTI_TREM1_VS_ANTI_TREM1_AND_LPS_MONOCYTE_DN	0.0018	2.06	192
GSE25123_WT_VS_PPARG_KO_MACROPHAGE_UP	0.0018	2.05	189
GSE36888_UNTREATED_VS_IL2_TREATED_TCELL_17H_DN	0.0018	2.05	196
REACTOME_CREATION_OF_C4_AND_C2_ACTIVATORS	0.0022	2.04	54
GSE29618_MONOCYTE_VS_MDC_UP	0.0023	2.04	198
HAY_BONE_MARROW_NEUTROPHIL	0.0025	2.03	440
GSE9988_ANTI_TREM1_VS_LPS_MONOCYTE_DN	0.0029	2.02	196
GSE9988_ANTI_TREM1_VS_LOW_LPS_MONOCYTE_DN	0.0033	2.015	196
GSE9988_ANTI_TREM1_AND_LPS_VS_VEHICLE_TREATED_MONOCYTES_UP	0.0033	2.014	187
REACTOME_FCGR_ACTIVATION	0.0037	2.009	56
GSE29618_BCELL_VS_MONOCYTE_UP	0.0044	1.99	188
GSE22886_CD8_TCELL_VS_BCELL_NAIVE_DN	0.0049	1.99	183
GSE9006_HEALTHY_VS_TYPE_1_DIABETES_PBMC_AT_DX_DN	0.0057	1.98	190
GOCC_SPECIFIC_GRANULE	0.0056	1.98	155
REACTOME_INITIAL_TRIGGERING_OF_COMPLEMENT	0.0057	1.98	61

Table 2. GSEA top 27 significantly enriched Pathways in Group 2 compared to Group 1 ordered by FDR q-value.

Pathway	FDR q-val	NES	*n*
GOCC_T_CELL_RECEPTOR_COMPLEX	0.000	− 2.42	112
CHR14Q11	0.0016	− 2.24	229
GSE10325_LUPUS_CD4_TCELL_VS_LUPUS_BCELL_UP	0.0197	− 2.11	199
GSE11057_PBMC_VS_MEM_CD4_TCELL_DN	0.0231	− 2.08	193
GSE10325_CD4_TCELL_VS_MYELOID_UP	0.0232	− 2.07	199
GSE11057_CD4_EFF_MEM_VS_PBMC_UP	0.0208	− 2.07	198
GSE10325_LUPUS_CD4_TCELL_VS_LUPUS_MYELOID_UP	0.0221	− 2.05	198

Table 3. GSEA top 7 significantly enriched pathways in group 1 compared to group 2 ordered by FDR q-value.

Concretely, IL-10 is a widely described immune mediator that can be secreted by T regulatory cells, B regulatory cells and macrophages[31], and its alteration has been previously described in the context of pressure ulceration (e.g. DFUs and venous ulcerates)[32,33]. IL-10 is a pleiotropic factor, controlling inflammation and other varied processes such as M2 polarization of macrophages and wound healing[34–36]. An M2 phenotype downregulation has been previously reported in diabetic foot ulceration[32,37–39] and could explain here the perpetuation of the inflammatory stage observed in grade III-IV PUs.

Regarding the tissue repairing capabilities of IL-10, decrease levels of this cytokine perfectly correlated with the observed downregulation of *IL22RA1* and *IL17RD* in PBMCs of more graded PUs, encoding receptors of cytokines such as IL-22 and IL-17 with a potent and widely described role in promoting wound healing[39,40]. Despite not being detected among our altered set of molecules, it is worth mentioning that IL-17 secreted by endothelial cells has been already proposed -using transcriptomics- as a promising candidate for the therapeutic treatment of severely developed DFUs[41]. If these observations can be effectively transferred to other clinically non-related contexts such as altered wound healing occurring at PUs, and together with IL-10, being considered a potential therapy for PUs will require further validation. In addition, research conducted on fluids collected from wounds revealed that non-healing wounds showed alteration of several structural components such

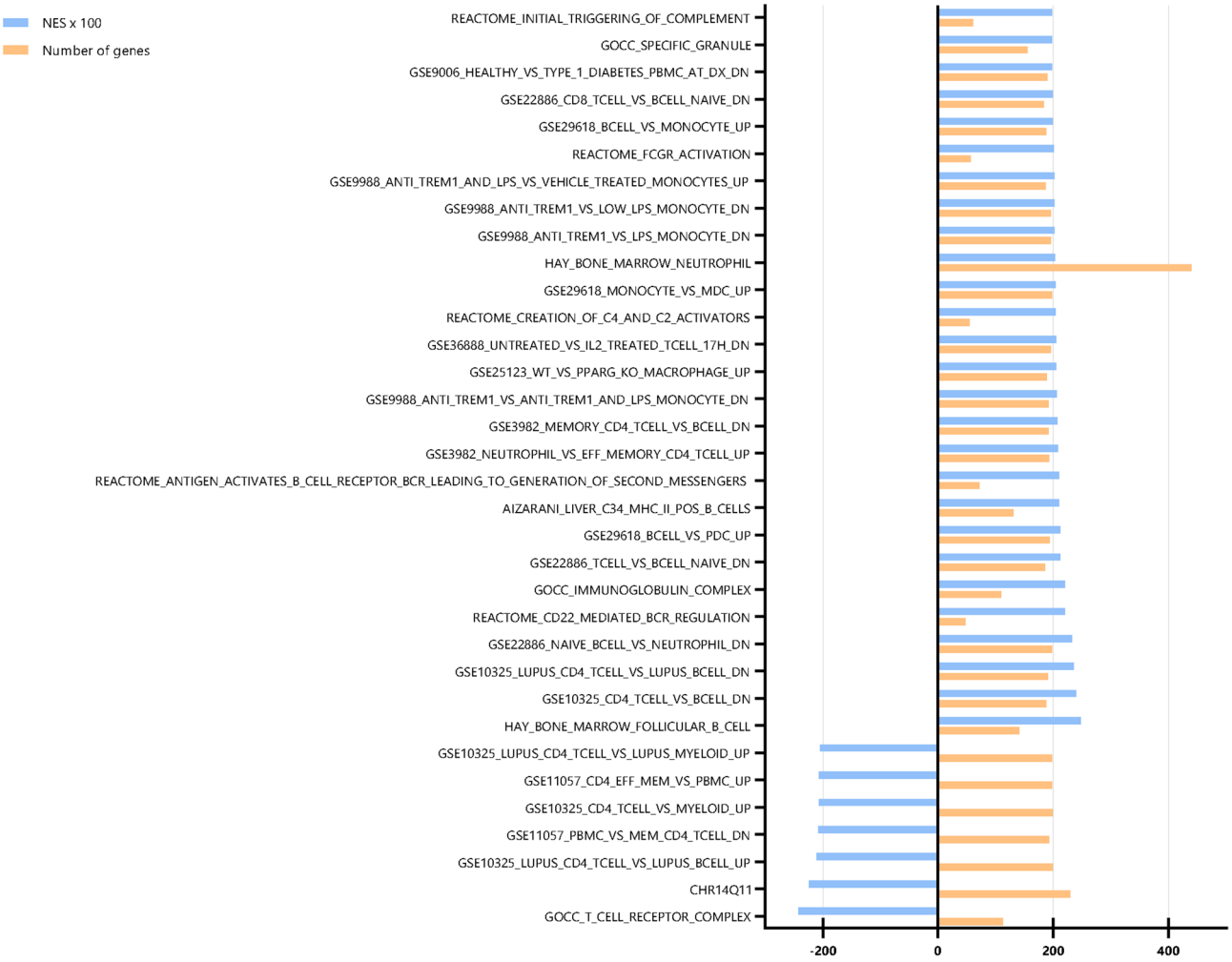

Fig. 4. Top 32 enriched gene sets from GSEA analysis (grade III-IV PUs -Group 2- vs. grade II PUs patients -Group 1-). Normalized Enrichment Score (NES) and number of genes inside the gene set are represented for each gene set.

as matrix metalloproteinases (MMPs)[42]. In our research, the only gene encoding for MMPs is *MMP27* and resulted overexpressed in patients with grade III-IV PUs, however no reports about its role in ulceration has been published up to date. As observed in other transcriptomic studies of DFUs[42], other unexplored genes related to the modulation of keratinocyte migration and wound healing such as *ADAMTS2* (encoding for the Metallopeptidase With Thrombospondin Type 1 Motif 2) were also found here to be upregulated in grade III-IV PUs patients, but its importance as a biomarker and contribution to the pathogenesis of chronic PUs wounds will require further exploration.

In addition to IL-10, our proteomic analysis yielded a significant decrease in IFN-γ blood levels in patients with deeper ulcers (grade III-IV PUs), supporting the systemic inflammatory signature described in this study. Taking together the proteomic and transcriptomic data, an alteration of IL-10 and IFN-γ, together with CXCL10 and *IL11* is a common feature observed in other inflammatory contexts affecting elderly patient cohorts -usually affected by PUs during hospitalizations- such as COVID19 infections[43–45]. Supporting the systemic inflammation context of grade III-IV PUs, we found that *TSLP* – which encodes for the alarmin TSLP (Thymic stromal lymphopoietin) –. TSLP is a well-known pro-inflammatory epithelial cell-derived cytokine, which in the context of our observations has been described as a potent inhibitor of IL-10 secretion by T regulatory cells[46,47] and key negative regulator of tissue remodeling -via collagen release by fibroblasts- not only in the airways but also in the skin[48]. Considering collagen deposition is fundamental to the development and resolution of normal wound healing, TSLP levels could play a crucial role in ulcers progression that should not be lightly dismissed and being considered as target of future research in this regard. Finally, regarding the lack of M2 polarization observed in other ulceration processes[32,36–38], IFN-γ can also influence macrophage polarization towards an M2 phenotype in certain contexts and its decrease levels together with IL-10, supports again the creation of an immune environment inclined to a non-polarization of M2 macrophages. Future studies targeting macrophage function in chronic wound healing will help to clarify this observation.

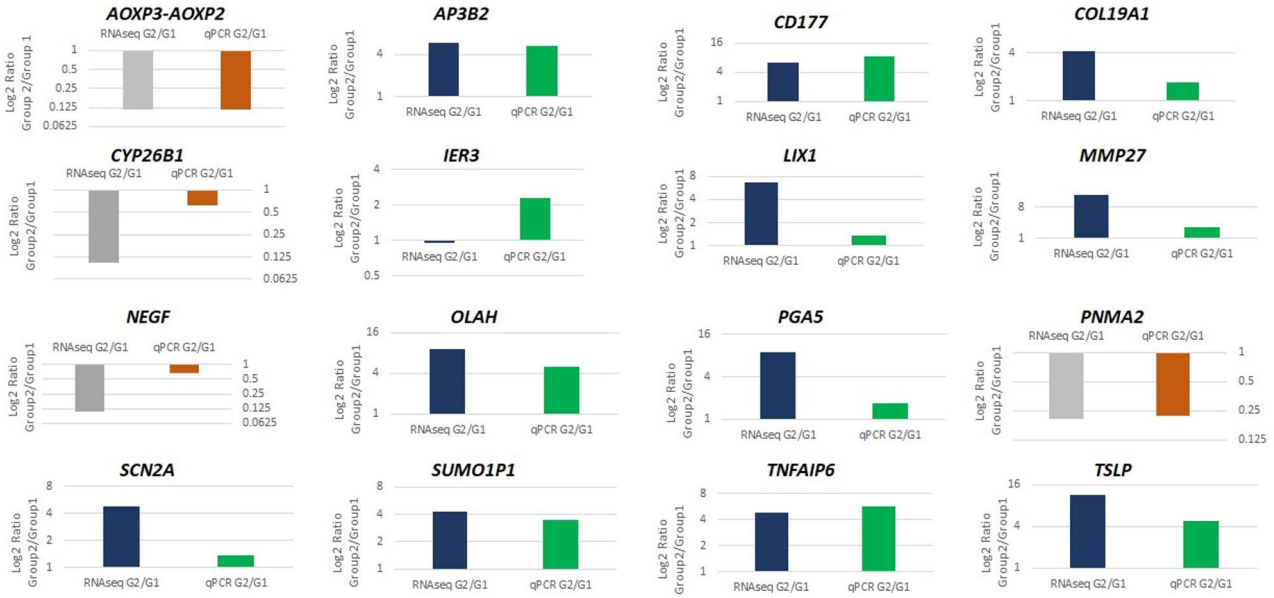

Fig. 5. Validation of RNA-seq data by RT-qPCR and $2^{-\Delta\Delta CT}$ normalization method. RNA-seq (grey or dark blue) compared to RT-qPCR results (dark orange or green). RNA-seq and qPCR results are represented by Log_2(FC Group2/Group1; G2/G1). G1: Group 1, corresponding to patients with grade II PUs; G2: Group 2, corresponding to patients with grade III-IV PUs.

In connection with systemic inflammation, two genes such as *CD177* and *TNFAIP6* linked with immune infiltration appeared upregulated in grade III-IV PUs. *CD177* is a marker of activated neutrophils which is usually expressed on the surface of these immune cells before migrating to inflamed areas and has been previously described to be also upregulated in invasive *S. aureus*-infected DFUs[49,50]. *TNFAIP6* (encoding for the protein TSG-6) also modulates neutrophil, and monocyte recruitment wound inflammation, and its gene expression levels has been also described to upregulated in fibroblasts isolated from skin ulcers non-healing DFUs[28]. In terms of monocyte/macrophage cell infiltration, MCP-2/CCL-8 levels appeared decreased in patients with severe PUs and has been also described as relevant predictor of wound healing in patients with non-healing DFUs[51].

Proteomic and transcriptomic data in this study not only intend to help to understand, at a molecular level, how aberrant wound healing processes take place in these ulcers, but also aim to find potential systemic cell-derived biomarkers that could help to predict their severity endpoints. In this sense, a set of less well-known genes and proteins were found to be significantly altered in grade III-IV PUs and could be potentially used to predict their development and pathogenesis. These genes include *IER3*, *DCSTAMP* and *C2CD4A*, and proteins such as MCP-2/CCL8. Concretely, *IER3* has a relevant role in immune regulation (via NFκB activation), alteration of blood pressure control, genome stability and more importantly in the context of severe PUs, osteogenic differentiation[52,53]. *IER3* gene alteration was accompanied by an upregulation of genes already described to modulate bone resorption such as *DCSTAMP* (expressed by dendritic cells and a therapeutic target of inflammatory arthritis)[54,55]. These observations are of great relevance for the context deeper (grade III-IV/severe) PUs, which can affect not only the skin but also the muscles and bones of the patients. To our knowledge, *IER3* and *DCSTAMP* have not been previously reported to be altered while phenotyping patients the context of ulceration. *C2CD4A* is a gene involved in the regulation of vascular permeability and, although not been described yet, if related with vessels rupture as observed in deeper ulcers, requires further investigation[56].

In conclusion, in this work we have performed a serum analysis and found relevant proteomic and transcriptomic alterations occurring when comparing patients with grade II and III/VI pressure ulcers. The combination of these omics approaches have allowed us to delve deeper into the molecular mechanisms underlying the wound healing of pressure ulcers. This data supports the hypothesis that PUs development to irreversible stages could be associated with altered inflammatory processes. Despite its limitations, this exploratory work we intended to propose a panel of transcripts (e.g. *IL11*, *TSLP*, *CD177*, *DCSTAMP*, *TNFAIP6*, among others) and proteinic (IL-10, IFNγ, CXCL10, MCP-2) blood markers which could be associated with the abnormal development of wound healing in severe ulcers, including PUs. Our data suggests a blockade of the wound healing process in these patients, mainly governed by uncontrolled inflammatory status. This study focused on PUs could open the window of new investigations targeting more specific interventions to treat patients with stage III-IV ulcers (and other types such as DFUs) (e.g., through the application of IL-10 to the wound once developed) or anticipating the development of irreversible ulcers by carrying out a gene study in at-risk patients with ulcers that are still incipient.

Methods

Study design

This project employs a cross-sectional research design and utilizes non-probabilistic, consecutive sampling. All subjects provided written informed consent. The protocol was approved by the Committee of Research and Ethics from Getafe University Hospital (Ref.: CEIM 19/21). All research included here was performed in accordance with relevant guidelines/regulations and informed consent was obtained and signed by all patients. All participants were patients from any medical service evaluated by the plastic surgery service of the Getafe University Hospital during a period of 18 months -starting on 1 July 2019- who attended either on an outpatient basis (emergency or outpatient) or during their hospital stay (admission or discharge).

A total of 54 patients (aged 44–99 years) were recruited and divided into two groups: Group 1 ($n = 27$ patients with grade II PUs) and Group 2 ($n = 27$ patients with grade III-IV PUs). Group 1 includes patients with mild ulcers characterized by partial-thickness skin loss with exposed dermis, presenting as a shallow open ulcer with a viable, pink wound bed. Group 2 includes patients with more advanced ulcers, characterized by full-thickness skin loss, with visible subcutaneous fat, and frequently presenting with granulation tissue (epibole) in grade III PUs. For grade IV PUs, the damage may extend to muscle and/or supporting structures such as fascia, tendons, ligaments, cartilage, or bone. All patients included in this study were analyzed by proteomics but only 10 of each group were included in RNA sequencing.

The inclusion criteria required that the patients had the capacity to give informed consent, or, where applicable, a legal representative instead. Following NPUAP/EPUAP guidelines, the patients needed to have developed grade II, III or IV PUs of, at least, 2 cm diameter size, and serum albumin levels > 2 g/dl. Likewise, all individuals with ulcers showing signs of local infection (erythema, heat, purulent discharge or bad odor), active tumour processes, or SARS-CoV2 positive patients were excluded.

Sample collection and processing

Whole blood was collected in BD Vacutainer SST™ II tubes and K3 EDTA BD Vacutainer™ tubes to obtain serum and plasma, respectively. Samples were centrifuged at 2000 g for 10 min to avoid serum recovery. Serum samples were stored at -80ºC until further proteomic analyses. Ficoll-Paque (GE Healthcare™, Chicago, Illinois, USA) density gradient centrifugation gradient was used to separate PBMCs, which were then lysed in Buffer RLT and immediately stored at -80ºC until transcriptomic analyses.

Proteomic study

Protein profiling was obtained by using 1 µl of serum sample at the Proximity Extension Assay (PEA; Olink®, Uppsala, Sweeden) as previously described[57,58]. The samples were analyzed with the Olink® Target 96 Inflammation panel including 92 proteins[59]. All samples passed quality control and were randomized prior to analysis on a 96 well plate. Protein levels are reported as Normalized Protein eXpression (NPX) values, a relative quantification unit which is logarithmically related to protein concentration[60].

RNA sequencing analysis

RNA was extracted from lysed PBMCs using RNeasy® Mini Kit (Qiagen, Hilden, Germany) with DNase treatment following manufacturer's recommendations. RNA concentration was determined using a NanoDrop™ 2000/2000c Spectrophotometer, and its quality was assessed with Experion RNA StdSens analysis kit (RNA quality Indicator; Bio-Rad Laboratories Inc., Hercules, CA, USA), establishing RQI as an indicator of quality. Samples performing an RQI ≥ 8 were selected for the RNA sequencing analysis.

RNA sequencing (RNA-seq) was carried out at the Genomics Unit from CNIC. RNA-seq was performed on DNase I-treated RNA samples (200 ng in 50 ml RNase–free water) with a RIN > 8 (RNA Integrity Number; Agilent; Assessed with the Agilent 2100 Bioanalyzer System; Agilent Technologies, Santa Clara, USA). RNA-seq libraries and sequences were created using the New England BioLabs Next® Ultra II Directional RNA Library Prep Kit at the Illumina HiSeq 2500. All samples were indexed, and multiplex sequencing was conducted on the HiSeq to generate a dataset (minimum of 8 M reads per sample) at 50 nucleotides read length in single-end format (1 × 50). The quality and integrity of sequencing results were monitored for each data collection and sample.

Analysis of RNA-Seq data

FASTQ files obtained after sequencing (GSE230161 on GEO database) were pre-processed first by removing rRNA sequences using SortMeRNA 2.1 and then trimming adapters and low-quality sequences using BBMap version 38.92 and Cutadapt 1.15[61]. Reads were aligned to GRCh38.p13 (NCBI) using STAR 2.7.10b software[62]. Samtools 1.13 was used to transform the alignment file into a bam format file and HTSeq 0.6.1p1 (with option -m intersection-nonempty) was used to obtain the reads' raw counts for each feature (transcript)[63,64].

Data normalization, PCA (considering the 500 top genes with the highest variance) and differential expression analysis were performed using DESeq2 package[65]. Genes with fewer than 10 counts in the samples were discarded. Finally, genes with a p-value less than 0.05 and a Log_2 FC (fold change) greater than 2 (absolute value) were considered as differentially expressed genes.

We used the R package ComplexHeatmap to perform a hierarchical classification of samples and build a heatmap (using Z-scores measurements, "completeness" and "Euclidean distance")[66–68].

GSEA Preranked was used to perform gene set enrichment analysis on a pre-ranked gene list, establishing 1000 gene set permutations (enrichment statistic: weighted). Only gene sets with significant enrichment levels were considered (FDR q-value < 0.05 according to GSEA recommendations)[69].

Gene expression validation by RT-qPCR

RT-qPCR analysis/validation was performed only for candidate genes previously catalogued as differentially expressed genes ($Log_2FC \geq 2$ and $\leq$ -2; p-value < 0.05) when comparing individuals with grades II ($n = 10$) and III-IV ($n = 10$) PUs. Briefly, RNA samples (1 µg) were reversely transcribed into a final volume of 20 µl using the High-Capacity RNA to cDNA Kit (Applied Biosystems, Foster City, California, USA). Primers were designed by using OligoArchitect™ (Sigma-Aldrich, San Luis, Missouri, USA), and RT-qPCR was performed using SYBR Green master mix (Takara Kusatsu, Japan) in the equipment Real Time HT 7900 (Applied Biosystems). Reactions were run in triplicates. Expression data were normalized using the $2^{-\Delta\Delta CT}$ method[70], using as housekeeping genes GAPDH and HPRT1.

Statistics

Clinical characteristics were compared between patients with grade II PUs and patients with grade III-IV PUs to determine if there were significant differences in clinical variables. Quantitative variables were analyzed by Shapiro-Wilk test for assessing normality of the data. For normally distributed data, the unpaired Student's T-test was applied. Otherwise, the non-parametric Mann-Whitney *U*-test was used. For qualitative variables, Fisher's exact test was applied. A p-value of 0.05 was considered as a threshold of significance.

For the proteomic approach we compared continuous variables using unpaired Student's T-test or Mann–Whitney *U*-test (when data were not normally distributed) between 27 patients with grade II PUs (Group 1) and 27 patients with grade III-IV PUs (Group 2). Data were presented as median with interquartile range (GraphPad Prism 9.2.0). A p-value < 0.05 was considered statistically significant.

Data availability

Raw sequencing data are available in GEO database under accession numbers: [geo] GEO Submission (GSE230161) [NCBI tracking system #23839902]. Please, contact the corresponding author (PFM) for further information if necessary.

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Author contributions
LTP, PFM, AEP, RAS, LSZM, OGC and CTC analyzed and interpreted laboratory data. AEP, OGC and CTL analyzed the RNA sequencing data. LTP, JBL and XSH had selected and included the patients. LTP, AEP, JCLR and PFM were major contributors in the writing of the manuscript. MME and TCL contributed on the discussion, correction and writing of the manuscript. All authors read and approved the final manuscript.

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Declarations

Ethics approval and consent to participate
The Ethics local Committee of Research and Ethics from Hospital Universitario de Getafe of Madrid, Spain (approval reference: CEIm19/21) approved this study.

Consent for publication
All subjects provided written informed consent.

Competing interests
The authors declare no competing interests.

Informed consent
All research included here was performed in accordance with relevant guidelines/regulations and informed consent was obtained and signed by all patients.

Additional information
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