Histological and Transcriptional Expression differences between Diabetic Foot and Pressure Ulcers

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Abstract

Decubitus and diabetic foot ulcers remain as important clinical challenges with significant socioeconomic impact. Both are individual forms of chronic wounds with diverse proximal ethiopathogenic triggers. This study aimed to characterize and compare the main histological features as the transcriptional expression profile of a set of wound-healing relevant genes of the ulcers' granulation tissue. Following patients' consent, biopsies were collected from sacral pressure ulcer patients (N=5, stage IV) and diabetic foot ulcer patients (N=9, both of neuropathic and ischemic origin) with clean, non-infected granulation tissue. Biopsies fragments were processed for histological analysis and for RNA extraction and subsequent transcriptional expression characterization via RT-PCR. The group of targeted genes included cell proliferation control, extracellular matrix, glucose metabolism, anabolism-survival, as anti-hypoxia and anti-oxidant defense. Gene expression was determined, normalized with an internal housekeeping gene, and statistically compared. Each class of chronic ulcer granulation tissue: decubitus, and diabetics' ischemic and neuropathic proved to develop a particular histological pattern thus establishing individual differences. Moreover, diabetes appeared to significantly reduce the expression of numerous genes irrespective to their biological significance. Most importantly, we found that diabetic granulation tissue cells exhibit a sort of "genetic or epigenetic imprinting" for the expression of glucose-metabolism related genes which are deeply involved in type-2 diabetes pathophysiology. Our data indicate that in addition to a protracted inflammation and abnormal angiogenesis, diabetic granulation tissue cells are affected by gene expression failures that may lead to a negative pro-anabolic and energetic balance.

Keywords: Diabetes; Diabetic ulcer; Decubitus ulcer; Wound healing; Chronic ulcer

Abbreviations: PU: Pressure Ulcer; DFU: Diabetic Foot Ulcer; MMP2: Matrix Metalloproteinase 2; MMP9: Matrix Metalloproteinase 9; HIF-1α or HIF1A: Hypoxia Inducible Factor 1, Alpha Subunit; VEGF: Vascular Endothelial Growth Factor; HSP: Heat Shock Protein; ROS: Reactive Oxygen Species; AGE: Advanced Glycation End Products; RAGE: Receptor for Advanced Glycation End Products; FOXO: Forkhead Box Protein O; yrs: years; H/E: Hematoxylin/Eosin; RNA: Ribonucleic Acid; EGFR: Receptor for Epidermic Growth Factor; AKT1: Human v-akt Murine Thymoma Viral Oncogene Homolog 1; PIK3CA: Phosphatidylinositol-4,5-bisphosphate 3-Kinase, Catalytic Subunit Alpha; PTEN: Phosphatase and Tension Homolog; MTOR: Mechanistic Target of Rapamycin; CCND1: Cyclin D1; CDK4: Cyclin-dependent Kinase 4; PCNA: Proliferating Cell Nuclear Antigen; RB1: Retinoblastoma 1; E2F1: E2F Transcription Factor 1; MYC: v-myc Avian Myelocytomatosis Viral Oncogene Homolog; TP53: Tumor Protein p53; COL1A1: Collagen, Type I, Alpha 1; COL3A1: Collagen, Type III, Alpha 1; TGFBI: Transforming Growth Factor, Beta 1; ILK: Integrin-Linked Kinase; INSR: Insulin Receptor; SLC2A1: Solute Carrier Family 2 (facilitated glucose transporter), Member 1; HK1: Hexokinase 1; HK2: Hexokinase 2; PFKPH: Phosphofructokinase, Platelet; PKM: Pyruvate Kinase, Muscle; PDHA1: Pyruvate Dehydrogenase (lipoamide) Alpha 1; PDK4: Pyruvate Dehydrogenase Kinase, Isozyme 4; PPARG: Peroxisome Proliferator-Activated Receptor Gamma; PPARGCI1: Peroxisome Proliferator-Activated Receptor Gamma, Coactivator 1 Alpha; SIRT1: Sirtuin 1; SOD2: Superoxide Dismutase 2, Mitochondrial; TNF: Tumor Necrosis Factor; RNA18S1: RNA, 18S Ribosomal 1

Introduction

Pressure or decubitus ulcers (PU) and diabetic foot ulcers (DFU) are among the most common chronic wounds representing individual clinical entities but sharing a significant socioeconomic impact [1]. These chronic ulcers translate in systemic repercussion as they may act as pro-inflammatory and pro-oxidant organs super-imposed to a host that could evolve to a chronic low-grade inflammatory response [2].

Pressure ulcers continue to be a worldwide common health problem, particularly among the neurologically impaired or bedridden elderly. Its medical history dates back to the renaissance when the French surgeon Ambroise Paré enlisted; nutrition, pain relief and debridement as key factors to heal these ulcers. Surprisingly, it is not quite different than the present modality to some extent [3]. To date, a large percent of grades 3 and 4 pressure ulcers evolve to chronification and lead the patient to death due to ulcer complications such as sepsis or osteomyelitis [4].

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Received September 02, 2013; Accepted October 07, 2013; Published October 12, 2013

Citation: Mendoza-Mari Y, Valdés-Pérez C, Rodríguez-Corrales E, Suárez-Alba J, García-Ojalvo A, et al. (2013) Histological and Transcriptional Expression differences between Diabetic Foot and Pressure Ulcers. J Diabetes Metab 4: 296. doi:10.4172/2155-6156.1000296

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From the ethiopathogenic perspective, cutaneous ischemia appears to be the proximal trigger of a downstream cascade of molecular events that converge to impose a chronic evolution. These include the over-activation of molecular regulators toward a pro-apoptotic program [5], imbalance in matrix metalloproteinases regulation (MMP2 and MMP9) [6], and adhesion molecules overexpression [7]. At the experimental level, a reduction of the cutaneous cells constitutive expression of endogenous cytoprotective molecules such as hypoxia-inducible factor-1 alpha (HIF-1α), VEGF, HSP 70 and 90 and hemeoxigenase-1 [8] have been demonstrated. Despite the medical and social problem that the pressure ulcer represents, still remains a paucity of data aimed to characterize its molecular pathophysiology in clinical samples.

Within the diabetic context, hyperglycemia stands as the proximal ethiopathogenic trigger for the onset and progression of biochemical disturbances that steers the systemic complications, in which ulcer healing failure and amputation are included. The vicious circle between wound chronicity and deficient local infection control, determines that 85% of all non-traumatic lower extremity amputations are rendered by diabetic subjects [9]. At the molecular level, this high glucose burden unleashes acute and chronic self-perpetuating loops, which include ROS-lipid peroxidation, hyperinflammation/disimmunity, AGE-RAGE toxicity, mitochondrial dysfunction, nytrosilation end-products accumulation, FOXO genes deregulation and a perspicuous reduction of growth factors physiology. The concerted action of these elements enforces granulation tissue-producing cells to senescence, arrest and apoptosis. Furthermore, the failure of the agonistic stimulation of the insulin axis and other tyrosine kinase receptors, negatively impacts on the biology of diabetic wound cells [10].

This study intends to be a modest contribution to the characterization of the chronic wound microenvironment, by characterizing and comparing granulation tissue samples histology, as the gene expression profiles in these two common and devastating types of human chronic wounds.

**Experimental Procedures**

**Population and study enrollment criteria**

The enrollment of patients was based on the following criteria addressed as “clean” as possible the experimental substrate for a neat constitutive gene expression profile. Diabetic co-morbidities and pressure ulcer-affected patients with concurrent clinical conditions, requiring medication that we understood as potentially gene expression modifiers were not enrolled in the study. Thus, for diabetics, only those with oral hypoglycemic treatment were sampled. Insulin-dependent and uncompensated patients were not included. Furthermore, patients with clinical depression, heart failure, renal insufficiency, unstable angina, asthma, angiotensin-converting-enzyme inhibitors-medicated, and subjects with auto-immune processes were excluded. As all the pressure ulcer samples population had a spinal injury (lumbar segment) as the primary process, the only subjects included were those free of lower limbs hyperspastic episodes, simply receiving physical therapy/postural rotation and no pharmacological intervention. Obviously, patients with malignant diseases and history of chemotherapy were discarded. No pregnant or breast feeding women were conceived to be included.

All the patients were consulted about the experimental procedure and approved to offer a biopsy fragment for the study. For the chronic wounds, the granulation tissue fragment was obtained from a productive area, with no macroscopic evidence of infection, during a routine wound debridement and cleansing. The investigation material was collected from: (1) sacroiliac pressure ulcers (stage IV) from non-necrotic patients with history of spinal trauma [11], and (2) diabetic foot ulcers of both neuropathic and ischemic origin from reasonably compensated in-hospital patients [12]. As control of an acute wound we used a fragment derived from granulation tissue, obtained from a healthy female donor (20-yrs old) who experienced wound dehiscence following esthetic breast surgery. The sample was collected during the final surgical wound closure when the exuberant granulation was trimmed.

**Tissue processing**

Six-millimeter diameter punch biopsies were collected days after a series of surgical debridements and ulcers bed preparation, as part of the standard wound care procedures; using disposable sterile biotomes (Acuderm Inc., USA), washed in normal saline to remove blood and immediately hemisectioned along its longitudinal axis. A hemisection was fixed in 10% buffered formalin, horizontally paraffin-embedded, processed, and 5-µm slides stained with H/E and Mallory trichrome for a better definition of collagen accumulation. The other hemisection was preserved in RNA later solution (Ambion, Life Technologies, USA) and processed for gene expression analysis.

**Gene expression analyses by semi-quantitative RT-PCR**

Total RNA was purified according to TRI Reagent standard procedure (Sigma, USA), followed digestion with RQI DNase 1 (Promega, USA) to remove contaminating genomic DNA. Afterward, 500 nanograms of DNA-free RNA were reverse transcribed using a commercial available kit (OmniScript RT, Qiagen, Germany) with oligo-dT primer. The RT reaction was performed at 42°C for 60 min. PCR mixtures contained 2 µL cDNA, 1 µL of each primer (10 µM), 12.5 µL 2x Taq MasterMix (Qiagen, Germany) in a final volume of 25 µL. Specific sense and antisense primers, annealing temperatures and number of repeating cycles are referred in Table 1. Amplifying conditions were performed as followed: a first step of 95°C for 5 minutes, thereafter repeating cycles comprised of 95°C for 30 seconds, specific annealing temperature for 30 seconds and 72°C for 30 seconds, and a final extension step of 5 minutes at 72°C. PCR bands (8 µL of PCR product with 2 µL of gel loading buffer) were resolved on a 1.5% (w/v) agarose gel electrophoresis and visualized under ultraviolet light subsequent to being stained with ethidium bromide. PCR products were quantified using the Kodak ID 3.6 software package (Kodak Inc, USA). Ribosomal subunit 18S was used as housekeeping gene for normalization. Despite the broad spectrum of biological functions of some of the genes studied, for practical reasons we have grouped them as follows:

1. Genes involved in signaling pathways: EGFR, AKT1, PIK3CA, PTEN, MTOR
2. Genes involved in cell proliferation control: CCND1, CDK4, PCNA, RB1, E2F1, MYC, TP53
3. Genes involved in extracellular matrix biology: COL1A1, COL3A1, TGFβ1, ILK
4. Genes involved in glucose metabolic pathways: INS, SLC2A1, HK1, HK2, PFKP, PKM1, PKM2, PDHA1, PDK4
5. Genes involved in survival and anabolism: FOXO1, FOXO3, PPARG, PPARGC1, SIRT1
6. Anti-oxidant and anti-hypoxia defense: SOD2, HIF1A
7. Pro-inflammatory: TNF
8. Housekeeping gene: RNA18S1
| Gene   | Approved symbol | Gene Bank accession number | Primer sequence | Tm (°C) | No. cycles | Product length (bp) |
|--------|----------------|---------------------------|----------------|---------|------------|-------------------|
| EGFR   | EGFR           | NM_005228.3               | sense          | 58      | 35         | 213               |
|        |                |                           | antisense      |         |            |                   |
| Akt    | AKT1           | NM_005163.2               | sense          | 62      | 35         | 189               |
|        |                |                           | antisense      |         |            |                   |
| PI3K   | PIK3CA         | NM_006218.2               | sense          | 54      | 35         | 151               |
|        |                |                           | antisense      |         |            |                   |
| PTEN   | PTEN           | NM_000314.4               | sense          | 54      | 35         | 226               |
|        |                |                           | antisense      |         |            |                   |
| mTOR   | MTOR           | NM_004958.3               | sense          | 56      | 35         | 249               |
|        |                |                           | antisense      |         |            |                   |
| Cyclin | CCND1          | NM_053056.2               | sense          | 60      | 35         | 186               |
|        |                |                           | antisense      |         |            |                   |
| CDK4   | CDK4           | NM_000075.3               | sense          | 55      | 35         | 188               |
|        |                |                           | antisense      |         |            |                   |
| PCNA   | PCNA           | NM_002592.2               | sense          | 55      | 35         | 228               |
|        |                |                           | antisense      |         |            |                   |
| RB     | RB1            | NM_000321.2               | sense          | 62      | 35         | 246               |
|        |                |                           | antisense      |         |            |                   |
| E2F1   | E2F1           | NM_005225.2               | sense          | 58      | 35         | 187               |
|        |                |                           | antisense      |         |            |                   |
| c-myc  | MYC            | NM_002467.4               | sense          | 58      | 35         | 151               |
|        |                |                           | antisense      |         |            |                   |
| p53    | TPS3           | NM_000546.4               | sense          | 56      | 35         | 203               |
|        |                |                           | antisense      |         |            |                   |
| Collagen I | COL1A1        | NM_000088.3               | sense          | 60      | 35         | 218               |
|        |                |                           | antisense      |         |            |                   |
| Collagen II | COL3A1        | NM_00090.3               | sense          | 56      | 35         | 205               |
|        |                |                           | antisense      |         |            |                   |
| TGF β1 | TGFBI          | NM_000660.4               | sense          | 58      | 35         | 196               |
|        |                |                           | antisense      |         |            |                   |
| ILK    | ILK            | NM_000546.4               | sense          | 56      | 35         | 193               |
|        |                |                           | antisense      |         |            |                   |
| Glut1  | SLC2A1         | NM_006516.2               | sense          | 58      | 35         | 156               |
|        |                |                           | antisense      |         |            |                   |
| Hexokinase I | HK1          | NM_000188.2               | sense          | 58      | 35         | 218               |
|        |                |                           | antisense      |         |            |                   |
| Hexokinase II | HK2          | NM_000189.4               | sense          | 58      | 35         | 159               |
|        |                |                           | antisense      |         |            |                   |
| PFK    | PFK            | NM_002627.4               | sense          | 58      | 35         | 203               |
|        |                |                           | antisense      |         |            |                   |
| PKM1   | PKM            | NM_182470.2               | sense          | 58      | 35         | 227               |
|        |                |                           | antisense      |         |            |                   |
| PKM2   | PKM            | NM_002654.4               | sense          | 58      | 35         | 150               |
|        |                |                           | antisense      |         |            |                   |
| PDH    | PDHA1          | NM_000284.3               | sense          | 56      | 35         | 248               |
|        |                |                           | antisense      |         |            |                   |
| PDK4   | PDK4           | NM_002612.3               | sense          | 56      | 35         | 199               |
|        |                |                           | antisense      |         |            |                   |
| FOXO1  | FOXO1          | NM_002015.3               | sense          | 56      | 35         | 159               |
|        |                |                           | antisense      |         |            |                   |
| FOXO3  | FOXO3          | NM_001455.3               | sense          | 56      | 35         | 215               |
|        |                |                           | antisense      |         |            |                   |
| PPAR γ | PPAR           | NM_138712.3               | sense          | 56      | 35         | 207               |
|        | γ              |                           | antisense      |         |            |                   |
| PGC1 α | PPARGC1A       | NM_013261.3               | sense          | 56      | 35         | 237               |
|        | α              |                           | antisense      |         |            |                   |
| SIRT1  | SIRT1          | NM_001142498.1            | sense          | 56      | 35         | 153               |
|        |                |                           | antisense      |         |            |                   |
| Mn-SOD | SOD2           | X14322.1                  | sense          | 56      | 35         | 160               |
|        |                |                           | antisense      |         |            |                   |
| HIF-1 α| HIF1A          | NM_001530.3               | sense          | 54      | 35         | 179               |
|        |                |                           | antisense      |         |            |                   |
| TNF-α  | TNF            | NM_000594.3               | sense          | 58      | 35         | 155               |
|        |                |                           | antisense      |         |            |                   |
| 18S    | RNA18S1        | M10098.1                  | sense          | 62      | 30         | 150               |
|        |                |                           | antisense      |         |            |                   |

Table 1: Genes investigated and amplification data.
Stage C: Non-infected, ischemic wound.

Stage A: Non-infected and non-ischemic wound.

Grade 3: Wound penetrating to bone or joint.

Grade 2: Wound penetrating to tendon or capsule.

University of Texas Classification for DFUs:

HbA1c: Glycated Hemoglobin; ND: Not Determined.

NPAUP: National Pressure Ulcer Advisory Panel (United States of America). Stage IV: Full thickness wound with bone, tendon or muscle exposed/damaged.

Table 2: Demographic characteristic of the chronic ulcers-affected patients.

| Variable | Diabetic foot ulcers (DFU) | Pressure ulcers (PU) |
|----------|---------------------------|---------------------|
| Age (years; mean ± SD) | 60.1 ± 15.4 | 46.2 ± 14.6 |
| Male | 6 (66.7%) | 6 (100%) |
| Female | 3 (33.3%) | - |
| Ethnicity | All mixed Afro-Cuban | All white Latin/Hispanic |
| Basic disease duration (yrs, mean; range) | 15.8 (0.1 - 30) | 5.9 (0.6 - 19) |
| Glycemia level | 7.3 ± 1.17 mmol/L ulcer sampling time HbA1c: 7.1 ± 1.55% | 3.11 ± 0.91 mmol/L ulcer sampling time HbA1c: ND |
| Ulcer duration (in days) | (mean; range) | 50 (36 – 195) | 180 (60 – 540) |
| Ethopathogenic classification | 5 ischemics 4 neuropathics | Not applicable |
| Classification (University of Texas DFU scale and NPUAP* staging, respectively) | Grade 3. Stage C: 3 patients Grade 2. Stage C: 2 patients | Stage IV |
| Grade 2. Stage A: 3 patients Grade 3. Stage A: 1 patient |

Statistical analysis

Statistical analysis of normalized values was carried out using GraphPad Prism 6 for Windows, version 6.01. For gene expression data, normal distribution (Kolmogorov-Smirnov) and variance homogeneity (Brown-Forsythe) tests were performed. Once normality was demonstrated, differences between pressure and diabetic foot ulcers for each gene were analyzed using two-tailed unpaired Student’s t-test. In all cases, p-values <0.05 were considered statistically significant. The values shown represent mean ± S.D. (error bars).

Results

Demographic characterization of the study population

As shown in Table 2, diabetic foot granulation tissue donors included both genders; in contrast, pressure ulcers samples were solely from male patients who had suffered spine compressive traumas. Although the study population is small, the racial profile is representative of the Cuban ethnicity and for the case of diabetics; the basic disease duration almost tripled the one detected for pressure ulcers. Thus, we succeeded in confronting a substrate of chronicity for the basic disease as for the wounds evolution age. As for the later, all the wounds studied met clinical criteria of non-infection, chronicity and for the case of diabetics; the basic disease duration almost tripled the one detected for pressure ulcers. As opposed to ischemic ulcers, these wounds also exhibited an abnormal, or peripheral fibrin cuffs suggesting hyperpermeability. As noted for ischemic ulcers, these wounds also exhibited an abnormal, mixed inflammatory infiltration.

Histological imprinting in pressure and diabetic foot ulcers

Pressure ulcers, ischemic and neuropathic diabetic foot ulcers show particular histological aspects that seem to distinguish each of these lesions. The granulation tissue sections from the group of pressure ulcers appeared characterized by the presence of thick, densely packed, wavy, and Mallory positive collagen fibers. Scattered fat vacuoles embedded within the granulation tissue were seen along with an outsized hyperplasic epidermal layer at the wound edge indicating epithelial edge migration abortion. Moreover two main qualitative findings appeared notorious: (1) the limited amount of immunoinflammatory cells infiltrating the granulation tissue as compared to other forms of chronic wounds, and (2) the relative scarcity of fibroblasts in relation to the amount and density of the collagen deposited. The fibroblasts population appeared polarized within the field of evolving collagen bundles. In other microscopic fields, closer to the wound surface, irreversible nuclear changes in fibroblasts were observed. As opposed to ischemic ulcers, a large number of small capillaries were observed, often with endothelial hypertrophy and/or peripheral fibrin cuffs suggesting hyperpermeability. As noted for ischemic diabetic ulcers, these wounds also exhibited an abnormal, mixed inflammatory infiltration.
Gene expression differences between pressure and diabetic ulcers

Differences in gene transcriptional expression were detected between pressure ulcers and diabetic foot ulcers samples. Table 3 shows only those genes with significant different expression.

EGFR and downstream signaling targets: A marginally lower although not significant difference (p=0.07) of the EGFR gene expression was detected in the diabetic patients' samples as referred to pressure ulcers counterparts. A similar observation is applicable to AKT1 gene transcription (p=0.06). Surprisingly, the expression levels of PIK3CA and its antagonistic regulator PTEN were found very similar between diabetic and pressure ulcers (p=0.12 and 0.13 respectively). The metabolic master switch MTOR appeared far more underexpressed in diabetic ulcers than in pressure ulcers (p=0.001) which is expected given the theoretical anabolic depression of the cells in diabetes (Figure 2A).

Cell proliferation control: CCND1 expression appeared statistically similar between the two groups of wounds (p=0.17). However the expression levels of other cell proliferation-promoting genes as CDK4, PCNA, and E2F1 appeared significantly depressed in diabetic ulcers as compared to pressure ulcers (all p<0.05) (Figures 2B-D). Furthermore, c-myc which is a well-known cell proliferation-committed transcription factor also exhibited a marginal reduction (p=0.06) in diabetic foot ulcers. Canonic cell cycle inhibitors as RB1 (p=0.55) and TP53 (p=0.177) showed no transcriptional differences between pressure and diabetic ulcers.

Extracellular matrix biology: Pressure ulcers exhibited far more gene transcriptional activity for COL1A1, COL3A1 and TGFβ1 than diabetic ulcers (all p ≤ 0.04) (Figure 3). No statistical differences were observed between the two ulcer groups for ILK gene expression (p=0.12).

Glucose metabolic pathways: A significant reduction in the expression of genes involved in glucose metabolic regulation in diabetic foot ulcers was detected in granulation tissue cells. Accordingly, INSR, SLC2A1, and enzymes as HK1 and HK2 isoforms, PFKP, PKM isoforms 1 and 2, and PDHA1 appeared significantly underexpressed in diabetic ulcers (all p ≤ 0.03) in relation to pressure ulcers. In biological line with the latest finding, PDK4 gene which downregulates PDH, showed higher expression in diabetic ulcers than in pressure ulcers tissue (p=0.017) (Figure 4).

Survival and anabolism: No statistical differences were detected in FOXO1 gene transcription between diabetic and pressure ulcers (p=0.6). However it is notorious that FOXO3 gene appeared significantly underexpressed in diabetic granulation tissue as compared to pressure ulcers tissue (p=0.031) (Figure 5A). Again, in close biological correspondence, other metabolic regulator gene as SIRT1 exhibited significantly less expression in diabetic ulcers than in non-diabetic counterparts (p=0.042) (Figure 5B). Finally, no significant differences were noted for PPARG (p=0.88) nor PPARGC1 (p=0.088) gene expression between the two types of ulcers.

Anti-oxidant and anti-hypoxia defenses: SOD2 enzyme isoform...
| Gene                        | Parameters                                                                 | Diabetic Foot Ulcers (n=9) | Pressure Ulcers (n=3) | Acute wound | Diff. between means (DFU-PU) | CI 95% | CI 99% | p value |
|-----------------------------|-----------------------------------------------------------------------------|-----------------------------|-----------------------|-------------|-----------------------------|--------|--------|---------|
| MTOR                        | Serine/threonine protein kinase that regulates cell growth, cell proliferation, cell motility, cell survival, protein synthesis, and transcription | Mean ± SD 0.574 ± 0.393 1.422 ± 0.288 1.154 | Median (25%; 75% percentile) 0.517 (0.169; 0.964) 1.266 (1.193; 1.728) | - | -0.848 ± 0.202 -1.287 to -0.409 -1.464 to -0.232 | 0.0012** |
| E2F1                        | Transcription factor. Plays a crucial role in the control of cell cycle and action of tumor suppressor proteins | Mean ± SD 0.353 ± 0.381 0.831 ± 0.187 0.774 | Median (25%; 75% percentile) 0.304 (0.033; 0.720) 0.728 (0.724; 0.988) | - | -0.478 ± 0.165 -0.838 to -0.117 -0.983 to 0.028 | 0.0136* |
| PCNA                        | Colfactor of DNA polymerase delta. Helps increase the processivity of leading strand synthesis during DNA synthesis and repair | Mean ± SD 1.264 ± 0.341 1.737 ± 0.496 1.880 | Median (25%; 75% percentile) 1.257 (0.938; 1.510) 1.732 (1.532; 1.944) | - | -0.472 ± 0.172 -0.850 to -0.094 -1.005 to 0.061 | 0.019** |
| CDK4                        | Catalytic subunit of the protein kinase complex that leads G1 to S phase progression during cell cycle | Mean ± SD 1.027 ± 0.377 1.412 ± 0.150 1.566 | Median (25%; 75% percentile) 1.145 (0.816; 1.298) 1.446 (1.286; 1.521) | - | -0.384 ± 0.178 -0.773 to -0.004 -0.930 to 0.160 | 0.05* |
| COL1A                       | Fibrillar collagen found in most connective tissues that strengthens and supports many tissues in the body, including cartilage, bone, tendon, skin and sciera | Mean ± SD 0.966 ± 0.474 1.448 ± 0.117 1.432 | Median (25%; 75% percentile) 0.940 (0.692; 1.336) 1.377 (1.359; 1.572) | - | -0.482 ± 0.166 -0.855 to -0.109 -1.015 to 0.050 | 0.016* |
| COL3A                       | Collagen of granulation tissue, it is produced quickly by young fibroblasts before the tougher type I collagen is synthesized. Reticular fiber. Also found in artery walls, skin, intestines and the uterus | Mean ± SD 0.937 ± 0.392 1.498 ± 0.117 1.421 | Median (25%; 75% percentile) 1.138 (0.568; 1.286) 1.451 (1.410; 1.610) | - | -0.5606 ± 0.1409 -0.873 to -0.247 -1.005 to -0.116 | 0.00255** |
| TGFBI                       | Multifunctional cytokine that regulates proliferation, differentiation, adhesion, migration, and other functions in many cell types. TGF-beta is a key regulator of ECM assembly and remodeling. It inhibits the synthesis of extracellular proteases while upregulating the production of their inhibitors and that of structural ECM components, such as collagen | Mean ± SD 1.266 ± 0.524 1.838 ± 0.183 2.065 | Median (25%; 75% percentile) 1.118 (0.924; 1.782) 1.825 (1.667; 2.015) | - | -0.572 ± 0.246 -1.107 to -0.036 -1.323 to 0.179 | 0.038* |
| INSR                        | Transmembrane receptor that mediates the biological effects of insulin | Mean ± SD 0.626 ± 0.375 1.242 ± 0.176 1.282 | Median (25%; 75% percentile) 0.643 (0.258; 0.976) 1.184 (1.110; 1.405) | - | -0.617 ± 0.180 -1.009 to -0.225 -1.167 to -0.067 | 0.0055** |
| SLC2A1                      | Glucose transporter responsible for the low-level of basal glucose uptake required to sustain respiration in all cells | Mean ± SD 0.684 ± 0.420 1.658 ± 0.662 1.172 | Median (25%; 75% percentile) 0.714 (0.230; 1.077) 1.935 (0.979; 2.200) | - | -0.974 ± 0.286 -1.598 to -0.350 -1.849 to -0.099 | 0.0053** |
| HK1                         | Enzyme that localizes to the outer membrane of mitochondria, mediates phosphorylation of glucose to glucose-6-phosphate, the first step in glucose metabolism pathway | Mean ± SD 0.933 ± 0.489 1.996 ± 0.385 1.105 | Median (25%; 75% percentile) 0.933 (0.640; 1.346) 2.079 (1.679; 2.273) | - | -1.064 ± 0.255 -1.619 to -0.509 -1.842 to -0.286 | 0.0013** |
| HK2                         | Predominant form found in skeletal muscle. Expression studies suggest that it is involved in the increased rate of glycogen seen in rapidly growing cancer cells | Mean ± SD 0.945 ± 0.457 1.685 ± 0.502 0.926 | Median (25%; 75% percentile) 0.916 (0.536; 1.413) 1.702 (1.203; 2.149) | - | -0.739 ± 0.288 -1.382 to -0.097 -1.653 to 0.174 | 0.028* |
| PFKP                        | Catalyzes the phosphorylation of fructose-6-phosphate to fructose-1,6-bisphosphate | Mean ± SD 1.069 ± 0.283 1.426 ± 0.227 1.564 | Median (25%; 75% percentile) 1.139 (0.849; 1.259) 1.491 (1.231; 1.588) | - | -0.357 ± 0.148 -0.678 to -0.035 -0.809 to 0.095 | 0.032* |
relative gene expression in diabetic foot ulcers, pressure ulcers and acute wound

Table 3: Relative gene expression in diabetic foot ulcers, pressure ulcers and acute wound

| Gene      | Diabetic Foot Ulcers | Pressure Ulcers | Acute Wound |
|-----------|----------------------|----------------|-------------|
| PKM1      | Mean ± SD            | Median (25%; 75% percentile) | Median (25%; 75% percentile) | Median (25%; 75% percentile) |
|           | 1.520 ± 0.773        | 1.804 (1.014; 2.149) | 3.071 (2.220; 3.533) | -1.395 ± 0.423 |
|           | (min; max)           | (0.001; 2.390) | (1.792; 3.633) | -2.317 to -0.474 |
| PKM2      | Mean ± SD            | Median (25%; 75% percentile) | Median (25%; 75% percentile) | Median (25%; 75% percentile) |
|           | 1.085 ± 0.417        | 0.910 (0.803; 1.486) | 2.321 (1.781; 2.566) | -1.108 ± 0.236 |
|           | (min; max)           | (0.325; 1.621) | (1.770; 2.282) | -1.622 to -0.595 |
| PDHA      | Mean ± SD            | Median (25%; 75% percentile) | Median (25%; 75% percentile) | Median (25%; 75% percentile) |
|           | 1.149 ± 0.381        | 1.220 (0.944; 1.411) | 2.016 (1.793; 2.281) | -0.876 ± 0.189 |
|           | (min; max)           | (0.325; 1.621) | (1.770; 2.282) | -1.288 to -0.463 |
| PDK4      | Mean ± SD            | Median (25%; 75% percentile) | Median (25%; 75% percentile) | Median (25%; 75% percentile) |
|           | 1.050 ± 0.397        | 0.796 (0.722; 1.209) | 0.520 (0.208; 0.625) | -0.538 ± 0.195 |
|           | (min; max)           | (0.437; 1.770) | (0.202; 0.727) | -0.113 to 0.962 |
| FOXO3     | Mean ± SD            | Median (25%; 75% percentile) | Median (25%; 75% percentile) | Median (25%; 75% percentile) |
|           | 1.156 ± 0.404        | 1.172 (1.013; 1.452) | 1.515 (1.417; 1.616) | -0.360 ± 0.144 |
|           | (min; max)           | (0.243; 1.633) | (1.340; 1.628) | -0.681 to -0.039 |
| SIRT1     | Mean ± SD            | Median (25%; 75% percentile) | Median (25%; 75% percentile) | Median (25%; 75% percentile) |
|           | 0.373 ± 0.152        | 0.339 (0.250; 0.482) | 0.836 (0.527; 1.188) | -0.478 ± 0.173 |
|           | (min; max)           | (0.168; 0.643) | (0.513; 1.425) | -0.931 to -0.026 |
| SOD2      | Mean ± SD            | Median (25%; 75% percentile) | Median (25%; 75% percentile) | Median (25%; 75% percentile) |
|           | 1.382 ± 0.232        | 1.294 (1.188; 1.565) | 1.750 (1.510; 1.829) | -0.304 ± 0.120 |
|           | (min; max)           | (1.070; 1.782) | (1.430; 1.875) | -0.565 to -0.043 |

Gene expression was assessed by semi-quantitative RT-PCR and normalized to ribosomal subunit 18S mRNA. SD: Standard Deviation; min: Minimum; max: Maximum; df: Difference; CI: Confidence Interval; *p < 0.05, **p < 0.01, ***p < 0.001.

The factors that “wipe away” from the cells the intrinsic mechanisms for a physiological repair and consequently lead to wound chronification remain unclear. In an attempt to gain further insights into the biology of chronic wounds, we have characterized the histology and compared the transcriptional activity of various wound healing-relevant genes, between diabetic and pressure ulcers as clinical paradigms of pernicious ulcers. The major limitation of this work resides in the reduced number of samples of chronic ulcers studied. Yet, the donor patients were rigorously selected so that they were representative of elemental features as ethnic homogeneity, long data of basic disease, wound chronicity and metabolic control for the case of diabetics. An additional limitation we confronted is the lack of “control” granulation tissue samples from acute, full-thickness wounds derived from non-diabetic, age-matched healthy donors. This has therefore restricted our views on the transcriptional activity of the genes studied in acute wounds from healthy subjects and excluded the possibility of statistical comparisons as a single acute wound sample was obtained. Thus, this control wound sample included is used solely as a limited reference.

As previously mentioned, we found that although a stagnant granulation process is a common factor for diabetic and pressure ulcers, each type of ulcer shows a particular morphological imprinting, thus establishing differences between pressure ulcers, diabetic-ischemic and diabetic-neuropathic; so that the two later classes appear as histological unrelated entities. The early onset of dense fibrotic matrix made up by thick collagen fibers had been previously described in pressure ulcers.
However, the driving forces behind this morphological appearance does not seem to be clarified. Whether these microstructural changes are a consequence of constant/repeated cutaneous pressure [14]; a sort of “lasting mechanical memory” could then be hypothesized as pressure-relief does not acutely restores the physiological healing trajectory [15]. An intriguing qualitative finding is that this fibroplastic induration appeared inversely correlated with the local fibroblast population, suggesting an imbalance between a consolidated fibrotic matrix and the matrix-producing cells. The fact that pressure ulcers-cultured fibroblasts exhibit a short replicative life becoming prematurely senescent [16] and that these cells are “homed” into a pro-apoptotogenic environment [5] incite to speculate that these wounds become “stalled” within the fibrogenic phase with no subsequent turnover or remodeling by a deficit of healthy effector cells. Globally speaking, fibroblasts dysfunction and apoptosis appear as pivotal factors toward wound chronification [17]. Although still persists scarcity of literature focusing on the histopathology of pressure ulcers [18], previous descriptions include an intense immune-inflammatory infiltrate [13]. Contradictorily, we...
rarely detected inflammatory infiltration across the microscopic fields. This discrepancy may be related to the characteristics of our study population: solely male, relatively younger, and non-emaciated subjects with spinal cord trauma as a basic condition, versus other studies based on elderly/debilitated patients [19].

Although persistent hyperglycemia is the common and proximal factor that disrupts fibroblasts, pericytes, keratinocytes and endothelial cells physiology thus hindering wound healing in diabetics [20,21]; it became clear that foot ulcer ethiopathogenic component (ischemic or neuropathic) is associated to a privative granulation tissue histopathological image. This finding may be a contribution of this work. To the best of our knowledge only Loots et al. [22] and Piaggessi et al. [23] had conducted histopathological studies of diabetic ulcers describing only the neuropathic lesions as “frozen” in a chronic low-

Figure 4: Relative gene expression in diabetic foot ulcers (solid bar), pressure ulcers (grey bar) and acute wound (open bar) of INSR (A), SLC2A1 (B), HK1 (C), HK2 (D), PFKP (E), PKM1 (F), PKM2 (G), PDHA1 (H) and PDK4 (I). Gene expression was assessed by semi-quantitative RT-PCR and normalized to ribosomal subunit 18S mRNA. Data are presented as mean ± standard deviation.

*p< 0.05, **p < 0.01, ***p< 0.001.

Figure 5: Relative gene expression in diabetic foot ulcers (solid bar), pressure ulcers (grey bar) and acute wound (open bar) of FOXO3 (A), SIRT1 (B) and SOD2 (C). Gene expression was assessed by semi-quantitative RT-PCR and normalized to ribosomal subunit 18S mRNA. Data are presented as mean ± standard deviation.

*p< 0.05.
grade inflammatory state associated to a scarce provisional extracellular matrix. These studies did not include ischemics-derived samples. The mechanisms mediating the differences of each type of extracellular matrix, angiogenic response, its organization and density; and even in fibroblasts cytology appear far to be elucidated. Similarly, it remains to be explained how and why, long-term evolution events like diabetic microvascular disease (i.e., media thickening or neointimal hypertrophy) can be so rapidly recreated by a growing neovessel within an incipient granulation tissue. Since the most aberrant morphological changes we found emerged from ischemic granulation tissue samples; hypoxia and its downstream biochemical disturbances could be raised as an instrumental ethiopathogenic ingredient for these vascular aberrancies.

The data derived from statistical comparisons described above indicate that the expression of different functional classes of genes, appear far more affected in diabetic patients ulcer cells rather than in decubitus ulcers counterparts. EGFR and some of its downstream phosphorylation targets were selected as paradigms of tyrosine kinase signaling pathways which may be disrupted by hyperglycemia [24]. Globally speaking our evidences support the notion that these pathways are dysfunctional in diabetic wound cells as compared to decubitus ulcers. This alteration has been attributed to glucose toxicity, receptors’ non-enzymatic glycation or by the local inflammation [10,25,26] all of which appear to reduce kinase receptors phosphorylation activation. Furthermore, although we found no significant differences in the expression of two major cell proliferation cycle inhibitors (TP53 and RB1) between diabetic and pressure ulcers; critical mitosis promoters as CDK4, PCNA and E2F1 appeared underexpressed by diabetic wound cells. CDK4 and PCNA expression failure have been previously invoked as molecular markers of chronic wounds fibroblasts proliferation reluctance [27,28]. In line with these findings is that TGF-β1 appears significantly down-regulated in diabetic ulcers as compared to pressure ulcers. This growth factor is a well-reputed actor for fibroblasts proliferation, migration and secretion, which offers theoretical explanation for the remarkable transcriptional reduction in collagen I and III as for the fibroblasts proliferation promoters, observed in the diabetic wound samples [29]. As curious note with no major biological translation, the expression of all the above mentioned genes was arithmetically similar between pressure ulcers and the single acute-wound granulation tissue sample.

Perhaps, the most relevant contribution of this work with no previous anticipation in the field; is that we found that diabetic granulation tissue cells, although “de novo” cells exhibit a sort of “genetic or epigenetic imprinting” for the deranged expression of glucose-metabolism related genes which have been largely implicated in type-2 diabetes pathophysiology [30]. As compared to decubitus ulcers, diabetic wound cells express far less insulin receptor, hexokinase isoforms 1 and 2, phosphofructokinase, pyruvate kinase isoforms 1 and 2, pyruvate dehydrogenase, and significantly more of its inhibitor enzyme pyruvate dehydrogenase kinase isoform 4. Interestingly, all of them have been considered as molecular markers of insulin resistance, glucose intolerance and predictors for the onset of type-2 diabetes [30]. Most remarkable is the fact that all of these and other genes deregulations had solely been described in the liver, skeletal muscle and adipose tissue as the most important glucose consumers and major insulin targets [30–32]. Thus, for the first time it is shown that a transient, de novo multicellular material, as the diabetic granulation tissue, somewhat “inherits” and reflects the same molecular derangements detected in the most important glucose clearance tissues/organs. In other words, granulation tissue, despite its early age, is also a diabetic tissue. Again, and as a curious comment, all these genes exhibit similar expression between decubitus granulation tissue and the single-acute granulation tissue control sample; suggesting that the above described derangements are diabetics’ privative traits.

Although we found no differences between pressure and diabetic ulcers in critical genes involved in energy homeostasis and cytoprotection as PPARG and PPARGC1; a complementary finding around the metabolic disorders that prevail in diabetic granulation tissue is the significant underexpression of other important genes such as FOXO3 and SIRT1. The observation that SOD2 expression is significantly lower in diabetic granulation tissue, may contribute to explain the well-described pathogenic role of the pro-oxidant environment in diabetic complications [33]. The fact that FOXO transcription factors reduce the level of oxidative stress by the transcriptional induction of SOD [34] may assist us to explain why SOD2 appeared underexpressed in diabetic wound samples. A meaningful finding to suggest that diabetic granulation tissue cells may be under a negative pro-anabolic and energetic balance is supported by the complementary underexpression of SIRT1. SIRT1 is known to regulate metabolism, to stimulate mitochondrial protein synthesis and activity, as to selectively regulate FOXO to certain targets genes involved in cells self-defense [35]. Taken together these data incite to suggest that diabetic foot ulcer cells may be endowed with a myriad of molecular disorders that may encompass from insulin receptor underexpression to mitochondrial functional failure.

This study, has allowed for a more comprehensive idea of the intrinsic histological aspects of different types of chronic ulcers, has offered a broader notion of the transcriptional profile of wound healing and metabolically-relevant genes, and has further strengthened our hypothesis that wound chronification is a distal destiny reachable from different or alternative roads. It has the merit to show for the first time that not only the major glucose-clearance organs, but the young-aged granulation tissue cells also exhibit a molecular “imprinting” toward glucose homeostasis failure. Moreover, we still miss however to gain a major enlightening on the core of pressure ulcers pathobiology.

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