Diabetic Complications Consortium

Application Title: Cortisol synthesis Enzyme CYP11 B1 as Tissue Biomarker for Diabetic foot ulcers

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1. Project Accomplishments:

In this project we evaluated individual components of the cutaneous cortisol synthesis pathway, CYP11B1, HSD11B1/2 and pGR as potential predictive biomarkers for diabetic foot ulcers (DFU) healing outcome. We have developed a statistically rigorous study plan, operational protocol and analytical methods for the clinical validation of these proteins as clinical biomarkers with predictive and diagnostic value. Successful completion of this project resulted in:

- Optimization of the quality assessment of DFU tissue specimens among multiple clinical sites.
- Optimization of staining protocols, standardization of controls, and authentication of antibodies which were tested for lot-to-lot and source variations.
- Establishment of automated biomarker quantification process. Quantification of nuclear presence of pGR was accomplished by open source QuPath imaging software, blinded to the clinical outcome.
- Due to low expression levels of CYP11β1 and HSD11β1/2 enzymes in both DFUs tissue and control foot skin this proteins were not considered for predictive or diagnostic markers for DFU.
- p-GR was selected as biomarkers due to feasibility, robustness of measurement, reproducibility and scalability.
- Baseline nuclei staining of pGR was found to be highly predictive of non-healing ($p<0.004$, 95% CI 0.00023-0.0668).

Data generated from this study supported application for the NIDDK Diabetic Foot Consortium (DFC) Biomarker study application. Further analysis and validation of pGR as predicative and diagnostic biomarker through the consortium will allow for center based effects (from all DFC clinical research units).

2. Specific Aims:

Specific Aim 1. To create an administrative core, which will develop the team, clinical and laboratory methodologies, investigative approach and logistics in preparation for a large clinical multi-center study of biomarkers for prediction of responsiveness to treatment of neuropathic and neuroischemic DFUs.

Results: As proposed we have streamlined the process of already collected diabetic foot ulcer tissue shipment and processing from multiple clinical sites (University of Miami-UM, University

of Manchester and NYU). We fully optimized operational protocol that is currently implemented in multiple aspects of the ongoing NIDDK DFC biomarker validation study. We have also optimized and standardized quality assurance and assessment of the received specimens as well as data acquisition and analyses. During this study international team was meeting monthly via Skype and also in person twice annually which allowed successful data analyses and application for the NIDDK DFC biomarker study.
In summary the goal of this aim was achieved as we established and successfully tested the operational protocol and team of experts for maximum efficiency including confirming, optimizing and validating best tissue procurement and shipment methods for the biomarker analyses and quantification.

Specific Aim 2. To optimize methodology and confirm reproducibility of the biomarker, established from previous collaborative work in patients receiving standard care.

During this study we have fully optimized tissue biomarker analysis protocols and confirmed reliability and reproducibility of the p-GR as DFU biomarker.
Results: This study provided collection of the thirty two additional DFU samples from 3 indented clinical sites (New York University, University of Manchester, and University of Miami) confirmed by H&E staining for the quality and pathology assessments, which together with previous studies allowed biomarker analyses on fifty eight tissue samples. As proposed we analyzed individual components of the cutaneous cortisol synthesis pathway as potential predictive biomarkers for DFU healing outcome. Our initial studies have focused on validation of cortisol synthesis enzyme CYP11B1, enzymes responsible interconversion HSD11B1 /HSD11B2 and downstream target p-GR. Prior to biomarker assessment each tissue was first analyzed by histopathology for presence of epidermis and dermis. Once approved for biomarker testing, sectioned tissue was immunostained using specific anti-human p-GR, CYP11B1, HSD11B1 and HSD11B2 antibodies. We consistently found in both DFUs tissue and control foot skin, often below the level of detection, resulting in a "NO GO" decision for CYP11β1 and HSD11β1/2 molecules as either predictive or diagnostic biomarkers (**Fig 1**).

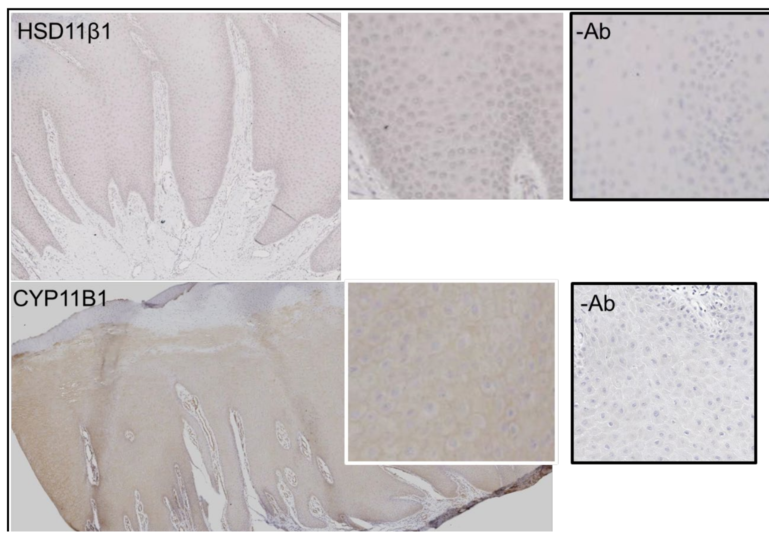

Fig 1. CYP11β1 and HSD11β1/2 enzymes in DFU tissue were determined as low and resulted in "No Go" decision for biomarker validation.

In line with our observation that local cortisol synthesis is increased in DFU tissue, prominent glucocorticoid receptor (GR) pathway activation was detected in DFU tissue, measured by nuclear presence of activated phospho-Ser211-GR (p-GR), in particular in non-healing DFUs. Initially, we developed protocol for immunofluorescence staining (**Fig 2**). High nuclear presence was easily observable in non-healing tissues. Similarly, low nuclear presence was found in healing tissues, suggesting its potential as biomarker. However, automatization of quantification revealed a challenge in quantifying immunofluorescence. Therefore, we optimized immunoperoxidase based staining method for p-GR instead of initially used immunofluorescence, which allowed automated quantification of nuclear p-GR staining. The reproducibility of using p-GR immunoperoxidase staining protocol was tested on samples collected from multiple clinical sites and it was confirmed to be high. Furthermore, specific positive controls and all staining quality assurances are in place. We also tested multiple lots of p-GR antibody and found the antibody very reliable. To date we analyzed tissues from 58 DFU samples. For the high quality, reproducible, objective analysis of biomarker quantification and reliable clinical correlation we have optimized the use of newly designed image analysis software QuPath (https://qupath.github.io; (Bankhead P, Sci Rep. 2017). We have developed a script in QuPath open source software that automates the counting and measuring of positively stained nuclei using peroxidase staining (**Fig 3**). First, a polygon object is drawn to represent the region of interest to be analyzed in order to remove cores which are unsuitable for analysis. This is followed by stain separation by color deconvolution and number of positive cells/mm^2 of tissue is automatically counted using a fast peak-finding algorithm which first estimates the full extent of each cell based upon a constrained expansion of the nucleus region, and calculates up to 33 measurements of intensity and morphology, including nucleus area, circularity, staining intensity for hematoxylin and DAB. Importantly, this method of quantification can be easily implemented at different sites participating in DFC. Quantification of nuclear presence of p-GR was accomplished blinded to the clinical outcome (which was independently

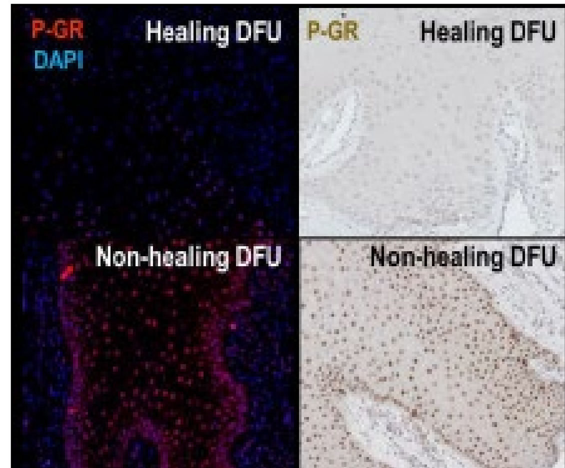

Fig 2. Increased nuclear p-GR presence in non-healing DFUs. Left panel – immunofluorescence; right – immunoperoxidase.

Table 1. pGR as predictive biomarker for DFU healing outcomes.

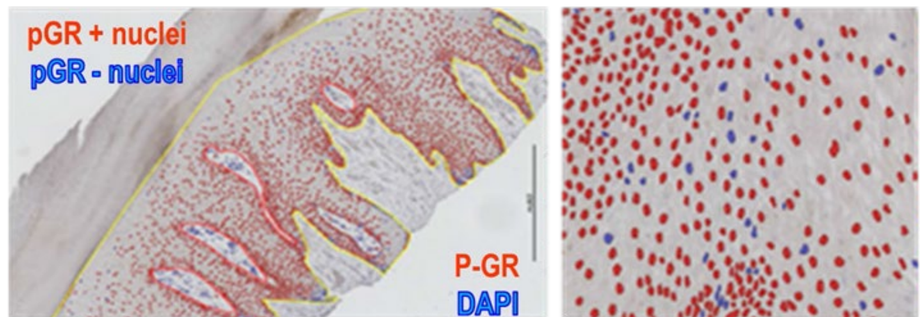

Fig 3. p-GR quantification using open source software QuPath. Red selects p-GR positive nuclei, whereas blue selects p-GR negative nuclei.

recorded by clinical sites). Upon performing automatic quantifications data were subjected to statistical analyses. To validate p-GR as a biomarker, percent of nuclei positive for p-GR was quantified in tissue obtained from 58 patients, 24 were predicted healers and 34 non-healers. Clinical outcomes data and biomarker quantification data were sent to Dr Margolis at UPenn to perform statistical analyses. We found that baseline nuclei staining of pGR is highly predictive of non-healing ($p<0.004$, 95% CI 0.00023-0.0668) based on linear regression results (Table 1).

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This compares the healer outcome (Healer==1) to the continuous measurement of
percentage of pGR and nuclei at week 0
. logistic diout pGRCnuc
Logistic regression                               Number of obs     =         58
                                                  LR chi2(1)        =      21.15
                                                  Prob > chi2       =     0.0000
Log likelihood = -28.763211                       Pseudo R2         =     0.2688
------------------------------------------------------------------------------
       diout | Odds Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
     pGRCnuc |   .0038913   .0056436    -3.83   0.000      .0002268    .0667731
       _cons |   7.171037   4.725237     2.99   0.003      1.971046    26.08959
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Note: _cons estimates baseline odds.
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From this pilot study we conclude that that quantification of nuclear pGR provides is an excellent predictor of DFU healing outcomes at the initial visit, confirming that pGR is a very strong candidate for predictive tissue biomarker for DFUs. Based on the data generated during this Pilot Study we proposed to validate pGR as a predicative and diagnostic biomarker in a multicenter clinical trial by the clinical research units of the recently formed NIDDK Diabetic Foot Consortium.

3. <u>Publications:</u>

The manuscript summarizing data generated in this study on p-GR as predictive and diagnostic tissue biomarker for DFU is in preparation. In addition we have published the review with guidelines on DFU tissue collection and validation:

Pastar I, Wong LL, Egger AN, Tomic-Canic M. Descriptive vs mechanistic scientific approach to study wound healing and its inhibition: Is there a value of translational research involving human subjects? Exp Dermatol. 2018 May;27(5):551-562.