Diabetology & Metabolic Syndrome

RESEARCH Open Access

Vildagliptin promotes diabetic foot ulcer healing through autophagy modulation

Erik Biros[1,2*], Venkat Vangaveti[1,2] and Usman Malabu[1,3]

Abstract
The study aimed to investigate the molecular mechanisms underlying the effects of Vildagliptin on the healing of diabetic foot ulcers (DFUs). The research compared patients who received 12 weeks of Vildagliptin treatment to those who did not. Various molecular markers associated with wound healing were measured. Wound fluid samples were collected from DFUs using a filter paper absorption technique, and total RNA was extracted for quantitative real-time PCR (qPCR). The results showed that the autophagy marker *NUP62* was significantly downregulated in the Vildagliptin group at week 12 compared to baseline (median expression 0.57 vs. 1.28; $P=0.0234$). No significant change was observed in the placebo group (median expression 1.61 vs. 1.48; $P=0.9102$). Both groups showed substantial downregulation of *RIPK3*, a necroptosis marker, at week 12 compared to their respective baselines. In addition to its effects on blood sugar levels, Vildagliptin may promote DFU healing by reducing autophagy in patients with diabetes.

Keywords Diabetic foot ulcer, Vildagliptin, Wound fluid, Wound healing, Autophagy

Introduction

Diabetic foot ulcer (DFU) is a life-altering condition characterized by a non-healing wound on a foot, serving as a constant reminder of the diabetes complications. Diabetes affects around 530 million adults worldwide [1], and approximately a quarter of these individuals will develop DFU, which accounts for roughly 85% of lower limb amputations, leading to a dramatic decrease in quality of life and increased mortality [2, 3]. The current standard of care for DFU primarily involves topical treatments that require frequent clinic visits and mechanical wound handling, often resulting in pain and a higher risk of infections [4]. These challenges underscore the urgent need for more effective and less invasive therapeutic strategies. In this context, oral medications simultaneously controlling blood sugar levels and promoting wound healing are particularly intriguing. Such treatments could improve patient outcomes and reduce the medication burden for diabetic patients, who are already at a higher risk of polypharmacy due to associated comorbidities [5].

Recent research has indicated that medications targeting the body's natural incretin hormones, which stimulate insulin release, might also play a role in wound healing [6]. Vildagliptin, in particular, is a potent and selective inhibitor of the dipeptidyl peptidase-4 (DPP-4) enzyme (DPP4i) [7]. DPP-4 rapidly degrades the gut's incretin hormones glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic peptide (GIP), which are responsible for insulin secretion upon glucose intake [8]. Notably, our recent observations suggest that DPP4i

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Vildagliptin not only aids in glycemic control but also improves DFU healing [6]. We hypothesize that Vildagliptin's benefits extend beyond blood sugar regulation, influencing cellular processes vital for wound healing. Our analysis of wound fluid from DFU patients treated with Vildagliptin revealed significant changes in autophagy, a lysosome-dependent process that breaks down and recycles damaged cell components [9]. This study sheds light on the molecular mechanisms underlying Vildagliptin's effectiveness in promoting DFU healing, providing a solid rationale for further investigation into its therapeutic potential.

Materials and methods

Ethics

This study adhered to national and international guidelines, including the Guidance on Good Clinical Practice [CPMP/GCP/135/95] and the annotated version with Therapeutic Goods Administration (TGA) comments [DSEB, July 2000]. Additionally, it followed the NHMRC National Statement on Ethical Conduct in Human Research (2007) and complied with all other applicable Australian Commonwealth, State, or Territory laws or guidelines of Regulatory Authorities. The study also upheld ethical principles derived from the Declaration of Helsinki. Furthermore, the study protocol received approval from an independent ethics committee (IEC), specifically HREC/13/QTHS/65, and is registered under Trial Registration ACTRN12613000418774. After fully explaining the purpose and nature of all procedures, written consent was obtained from each patient or subject.

Patients and wound fluid sample collection

This study used samples from a large randomized, double-blind, placebo-controlled clinical trial described elsewhere [6]. The initial study included 50 participants, 25 randomly assigned to the placebo group and 25 to the treatment group. The inclusion criteria stated that the participants had to be adult males or females with type 2 diabetes who managed their condition through diet alone or non-DPP4i medication. All patients had a diabetes index foot ulcer graded at A1 or higher, according to the University of Texas Wound Classification System of Diabetic Foot Ulcers [10]. The primary exclusion criteria included type 1 diabetes and current index foot ulcer of any non-diabetic origin [6]. The trial compared the effectiveness of taking 100 mg of Vildagliptin per day, split into two 50 mg doses—one in the morning and one in the evening—along with the standard of care (SOC), with taking a placebo along with SOC. The treatment lasted 12 weeks, during which participants regularly visited their podiatry clinics. The duration and dosage used in the trial align with previous research [11]. The wound fluid samples were collected using filter paper absorption during the patient's first and last visits to the podiatry clinic. The peri-wound area was gently cleaned with sterile saline to minimize contamination. A sterile filter paper disc of appropriate size was then gently applied to the wound bed, ensuring it was in contact with the wound fluid but not surrounding tissue/debris. The filter paper was left in place for approximately 1 min to allow sufficient absorption of the wound fluid. After removal, the filter paper was placed into a sterile 1.5 ml Eppendorf tube and immediately snap-froze in liquid nitrogen. The frozen samples were then stored at -80 °C until they could be assayed.

Molecular markers

We assessed a range of molecular markers to investigate various aspects of cellular processes. The following markers were evaluated:

(a) Proliferation: *MKI67* (marker of proliferation Ki-67) and *MCM2* (minichromosome maintenance complex component 2) (b) Senescence: *GLB1* (galactosidase beta 1) and *CDKN1A* (cyclin-dependent kinase inhibitor 1 A) (c) Apoptosis: *BAX* (BCL2 associated X, apoptosis regulator) and *BCL2* (BCL2 apoptosis regulator) (d) Necroptosis: *RIPK3* (receptor-interacting serine/threonine kinase 3) and *MLKL* (mixed lineage kinase domain-like pseudokinase) (e) Energy metabolism: *SIRT1* (sirtuin 1) and *MT-CO3* (mitochondrially encoded cytochrome c oxidase III) (f) Autophagy: *ATG7* (autophagy-related 7), NUP62 nucleoporin 62, also known as p62 (g): Mitophagy: *PINK1* PTEN-induced kinase 1, *PRKN* parkin RBR E3 ubiquitin protein ligase (h): Four Yamanaka Factors related to pluripotency regulation: *POU5F1* (OCT3/4), *SOX2* (SRY-box transcription factor 2), *KLF4* (KLF transcription factor 4), *MYC* (MYC proto-oncogene, bHLH transcription factor).

Gene expression

Quantitative real-time reverse transcription PCR (qPCR) assays were performed to assess the differential expression of selected markers in wound fluids of diabetic patients with DFU with and without Vildagliptin treatment. Total RNA was extracted using QIAzol lysis reagent (cat. no. 79306, Qiagen) and purified using the RNeasy Mini Kit (cat. no. 74104, Qiagen) following the manufacturer's instructions. The relative expression of a gene in each sample was calculated using the concentration-Ct-standard curve method and normalized using the average expression of the ribosomal protein S13 (*RPS13*) gene using the Rotor-Gene Q operating software version 2.0.24 (Qiagen). The one-step QuantiTect SYBR Green RT-PCR Kit (cat. no. 204243, Qiagen) was combined with the QuantiTect Primer Assays (Qiagen) following the manufacturer's instructions with ten nanograms of total RNA as a template. The QuantiTect Primer

Assays (Qiagen) were used for *RPS13* (QT00224539), *MKI67* (QT00014203), *MCM2* (QT00070812), *GLB1* (QT00066206), *CDKN1A* (QT00062090), *BAX* (QT00031192), *RIPK3* (QT00046102), *MLKL* (QT00495117), *SIRT1* (QT00051261), *ATG7* (QT00008974), *NUP62* (QT00064414), *PINK1* (QT00056630), *POU5F1* (QT00210840), *SOX2* (QT00237601), and *KLF4* (QT00061033). The SYBR Green PCR sense (5′-ATCCGTATTACTCGCATC-3′) and anti-sense (5′-TACTCTGAGGCTTGTAGG-3′) primers were designed for *MT-CO3* (reference sequence NC_012920.1:9207–9990), *BCL2* (sense 5′-TAACTCC TCTTCTTTCTC-3′ and anti-sense 5′-TACTTCATC ACTATCTCC-3′; reference sequence NM_000633.3), *PRKN* (sense 5′-GACACCAGCATCTTCCAG-3′ and anti-sense 5′-GCACAGTCCAGTCATTCC-3′; reference sequence NM_004562.3), and *MYC* (sense 5′-ACACAT CAGCACAACTACG-3′ and anti-sense 5′-CGCCTCT TGACATTCTCC-3′; reference sequence NM_002467.6) using the AlleleID software (PREMIER Biosoft). These primer pairs were manufactured and purchased from Merck. All reactions were independently repeated in duplicate to assess the repeatability of the results. The mean of the two raw values for each sample was used for analyses.

Statistical analysis

Data were analyzed using Stata/MP 16.0 (StataCorp LP, USA), and summary statistics are provided as a median (bold horizontal line) and interquartile range (whiskers). The Wilcoxon signed-rank test was used to compare gene expression between the first and last visit in patients with and without Vildagliptin treatment. The statistical significance was assumed at the conventional 5% level. All data points were graphed for the best visual inspection using GraphPad Prism 9 (GraphPad Software, USA).

Results

Patients characteristics

Eight patients with Vildagliptin and nine receiving placebo from a larger clinical trial of 50 patients (25/group) were included in this analysis. The inclusion was based solely on the availability of the paired wound fluid samples at the beginning and end of the study. These patients had similar characteristics across all measured parameters, ensuring a comparable baseline for analysis (Table 1).

Differential changes in DFU surface area

DFU surface area, our crucial measure of wound size, significantly decreased in patients receiving Vildagliptin treatment. Compared to the baseline measurement, the average DFU surface area decreased by ~ 23% after twelve weeks of treatment (348 mm^2 vs. 265 mm^2, $P=0.0047$;

Table 1 Patients' characteristics at the first visit to the podiatric clinic

Characteristic	Vildagliptin group	Control group	*P*
Number of patients	8	9	-
Sex (M/F)	7/1	8/1	> 0.999
Mean age in years (SD)	65.1 ± 11.8	66.6 ± 8.8	0.416
Median BMI in kg/m^2 (IQR)	35 (33.5–39.5)	32 (28.8–35.8)	0.111
The median duration of diabetes in years (IQR)	23.5 (18.5–33.5)	24.0 (20.0-25.3)	0.962
Mean Systolic blood pressure in mmHg (SD)	140 ± 10	144 ± 24	0.677
Dyslipidemia (%)	7 (87.5)	9 (100)	0.471
Retinopathy (%)	4 (50.0)	7 (77.8)	0.335
Nephropathy (%)	5 (62.5)	4 (44.4)	0.637
CAD (%)	3 (37.5)	3 (33.3)	> 0.999
CVA/TIA (%)	2 (25.0)	0 (0.0)	0.206
PVD (%)	3 (37.5)	4 (44.4)	> 0.999
PVD vascular surgery (%)	3 (37.5)	1 (11.1)	0.294
Amputation (non-traumatic) (%)	5 (62.5)	4 (44.4)	0.637
Microvascular complications (%)	7 (87.5)	7 (77.8)	> 0.999
Neuropathy (%)	7 (87.5)	9 (100.0)	0.471
Alcohol (%)	4 (50.0)	3 (33.3)	0.637
Smokers (%)	1 (12.5)	2 (22.1)	> 0.999
Insulin (%)	6 (75.0)	6 (66.7)	> 0.999
OHGs (%)	5 (62.5)	8 (88.9)	0.294
Mean Hemoglobin A1c (HbA1c) in % (SD)	9.09 ± 1.59	10.21 ± 1.80	0.195

Data are means ± SD or medians with IQR or counts (%). BMI, body mass index; CAD, coronary artery disease; CVA/TIA, cerebrovascular accident/transient ischemic attack; PVD, peripheral vascular disease; OHG, oral hypoglycaemic drugs; HbA1c, a normal level is below 5.7%; P, two-sided P-value by Student's t-test or Mann–Whitney U test or Fisher's exact test depending on samples distribution; SD, standard deviation; IQR, interquartile range

Table 2 DFU size at the start and end of the study

Group	First visit (week 1)				Last visit (week 12)				*P*
	Mean (mm^2)	SD	Min	Max	Mean (mm^2)	SD	Min	Max	
Vildagliptin ($n=8$)	348	505	25	1575	265	510	15	1505	0.0047
Placebo ($n=9$)	147	110	12	375	172	217	2	625	0.7855

P, two-sided P-value by Paired t-test; SD, standard deviation; Min, minimum value; Max, maximum value

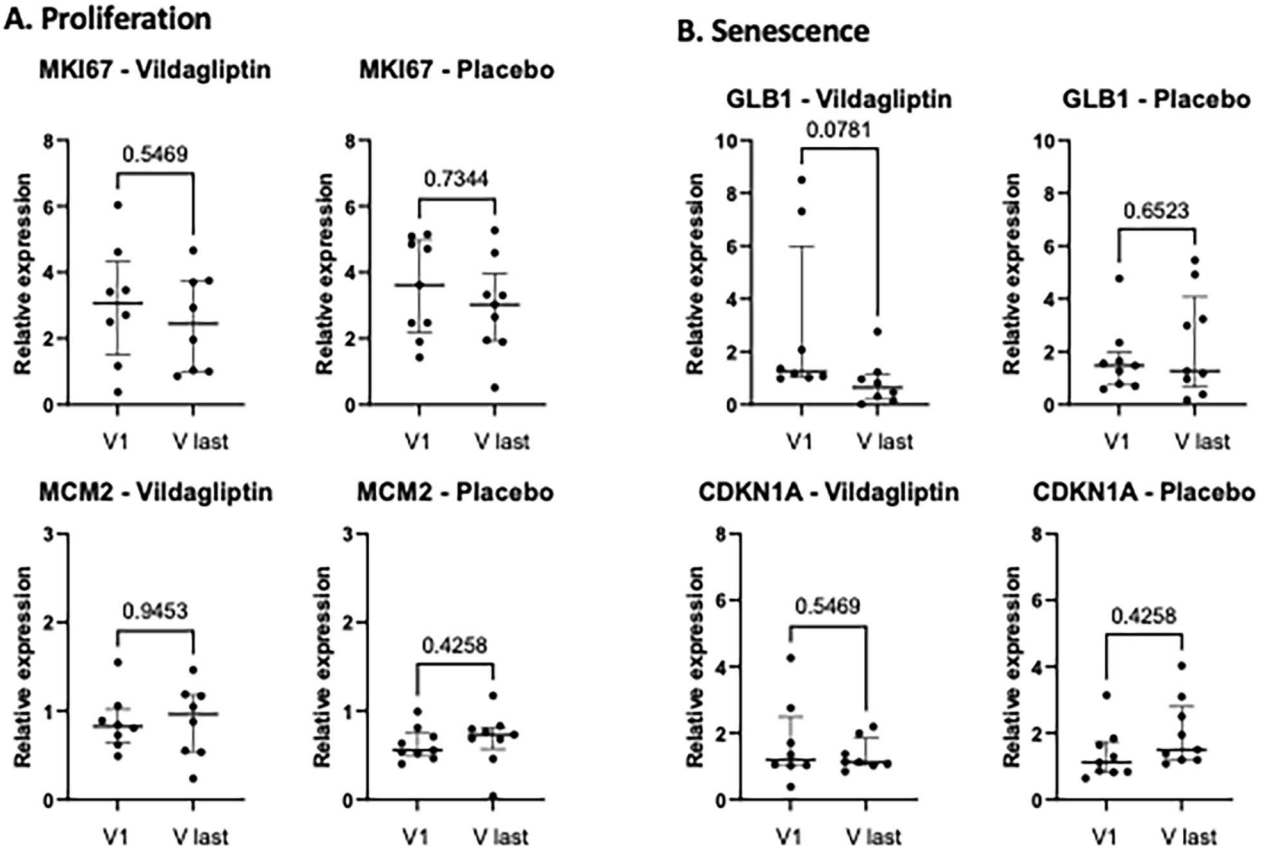

Fig. 1 Expression of proliferation and senescence markers in patients with and without Vildagliptin treatment. Data show a similar expression of *MKI67* and *MCM2* proliferation markers **(A)** and *GLB1* and *CDKN1A* senescence markers **(B)** in both groups. The median (bold horizontal line) and interquartile range (whiskers) are shown. Statistical significance was determined using the paired Wilcoxon signed rank. *MKI67*, marker of proliferation Ki-67; *MCM2*, minichromosome maintenance complex component 2; (b) *GLB1*, galactosidase beta 1 and *CDKN1A*, cyclin dependent kinase inhibitor 1 A; V1, first visit (baseline); V last, last visit (week 12)

Table 2). Notably, all patients in the Vildagliptin group exhibited decreased DFU surface area at the end of the study (Supplementary Table 1).

In contrast, the placebo group did not experience a statistically significant change in average DFU surface area at the end of the study (147 mm^2 vs. 172 mm^2, $P=0.7855$; see Table 2). One patient did not show any change, and the other two patients in the placebo group demonstrated an increased DFU surface area at the end of the study (refer to Supplementary Table 1).

Expression of cell proliferation and senescence markers

We assessed the differential gene expression of two cell proliferation markers, *MKI67* and *MCM2*. Both genes were similarly expressed in the Vildagliptin and placebo groups at the first and last visits ($P>0.05$; Fig. 1A).

Similarly, there was no difference in the expression of two cell senescence markers, *GLB1* and *CDKN1A*, between the first and last visit in both Vildagliptin and placebo groups ($P>0.05$; Fig. 1B).

Expression of apoptosis, necroptosis, and cell energy metabolism markers

We assessed the differential expression of two genes critically involved in cell apoptosis, *BAX* and *BCL2*. The results are presented as *BAX* to *BCL2* ratio and showed no statistically significant difference between the first

and last visits in both Vildagliptin and placebo groups ($P>0.05$; Fig. 2A).

However, when we assessed cell necroptosis markers, we found that the expression of the *RIPK3* gene was significantly reduced at the last visit compared to the first visit in the Vildagliptin group (median expression 1.14 vs. 1.97, $P=0.0156$; Fig. 2B) and placebo group (median expression 0.64 vs. 1.06, $P=0.0078$; Fig. 2B). The second marker of necroptosis, *MLKL*, was similarly expressed at both groups' first and last visits ($P>0.05$; Fig. 2B).

We also assessed two genes, *SIRT1* and mitochondrially encoded *MT-CO3*, which are crucial in cells' energy metabolism. There were no differences between the first and last visits in both groups' expression of these two genes ($P>0.05$; Fig. 2C).

Expression of autophagy end mitophagy markers

We assessed the differential gene expression of two autophagy markers, *ATG7* and *NUP62*. The ATG7 gene was similarly expressed in the Vildagliptin and placebo groups at the first and last visits ($P>0.05$; Fig. 3A). However, when we assessed the second autophagy marker *NUP62*, we found that this gene was significantly downregulated at the last visit compared to the first visit in the Vildagliptin group (median expression 0.57 vs. 1.28, $P=0.0234$; Fig. 3A), representing ~ 55% reduction in the relative expression. However, this reduction at last compared with the first visit was not found in the placebo group (median expression 1.61 vs. 1.48, $P=0.9102$; Fig. 3A).

When we assessed the differential gene expression of two cell mitophagy markers, *PINK1* and *PRKN*, both genes were similarly expressed in the Vildagliptin and placebo groups at the first and last visits ($P>0.05$; Fig. 3B).

Expression of Yamanaka factors

We assessed the differential gene expression of four Yamanaka pluripotency factors, which play a critical role in cellular plasticity. All transcription factors were similarly expressed in the Vildagliptin and placebo groups at the first and last visits ($P>0.05$; Fig. 4).

Discussion

DFUs are often challenging to treat, and these wounds can become stagnant even if the best available treatment is provided. Previous research has shown that medications primarily used to control blood sugar levels, such as DPP4i(s), may help heal DFUs [12]. Consistent with these findings, our last study of diabetic patients with

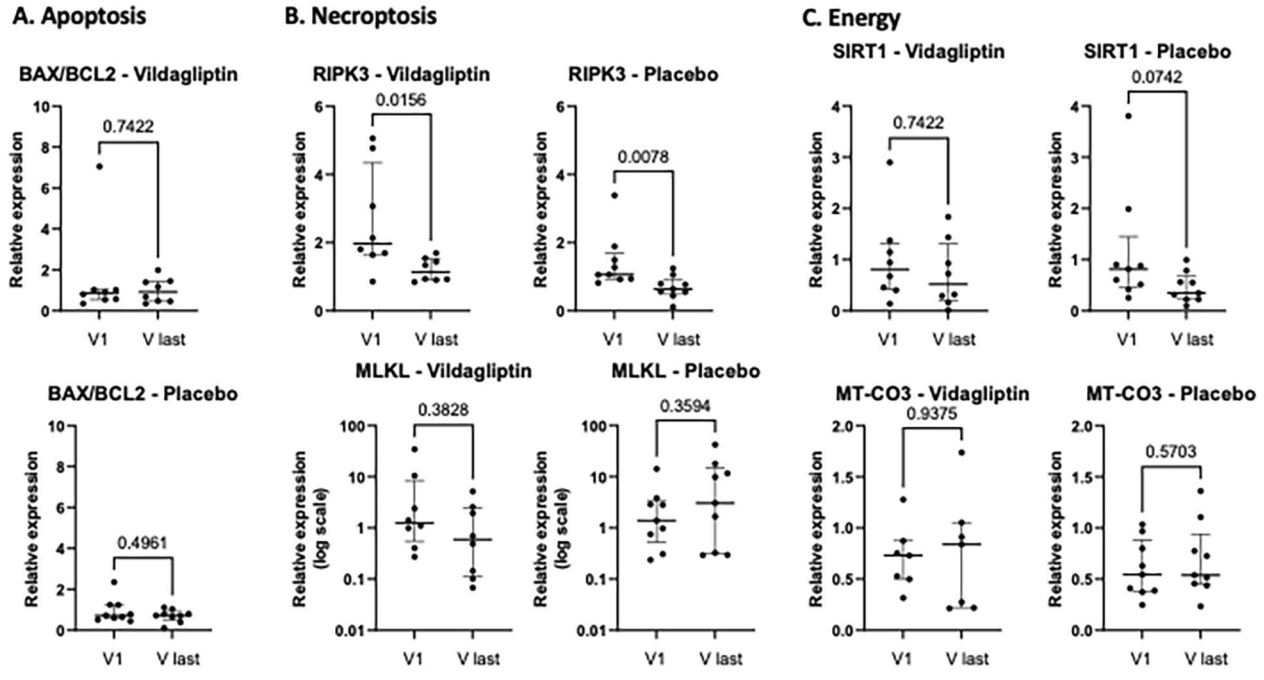

Fig. 2 Expression of apoptosis, necroptosis, and energy metabolism markers in patients with and without Vildagliptin treatment. Data show a similar ratio of apoptosis markers *BAX/BCL2* in both groups **(A)**, downregulation of necroptosis marker *RIKK3* in both groups **(B)**, and similar expression of energy metabolism genes *SIRT1* and *MT-CO3* in both groups **(C)**. The median (bold horizontal line) and interquartile range (whiskers) are shown. Statistical significance was determined using the paired Wilcoxon signed rank. *BAX*, BCL2 associated X, apoptosis regulator; *BCL2*, BCL2 apoptosis regulator; *RIPK3*, receptor interacting serine/threonine kinase 3; *MLKL*, mixed lineage kinase domain like pseudokinase; *SIRT1*, sirtuin 1; *MT-CO3*, mitochondrially encoded cytochrome c oxidase III; V1, first visit (baseline); V last, last visit (week 12)

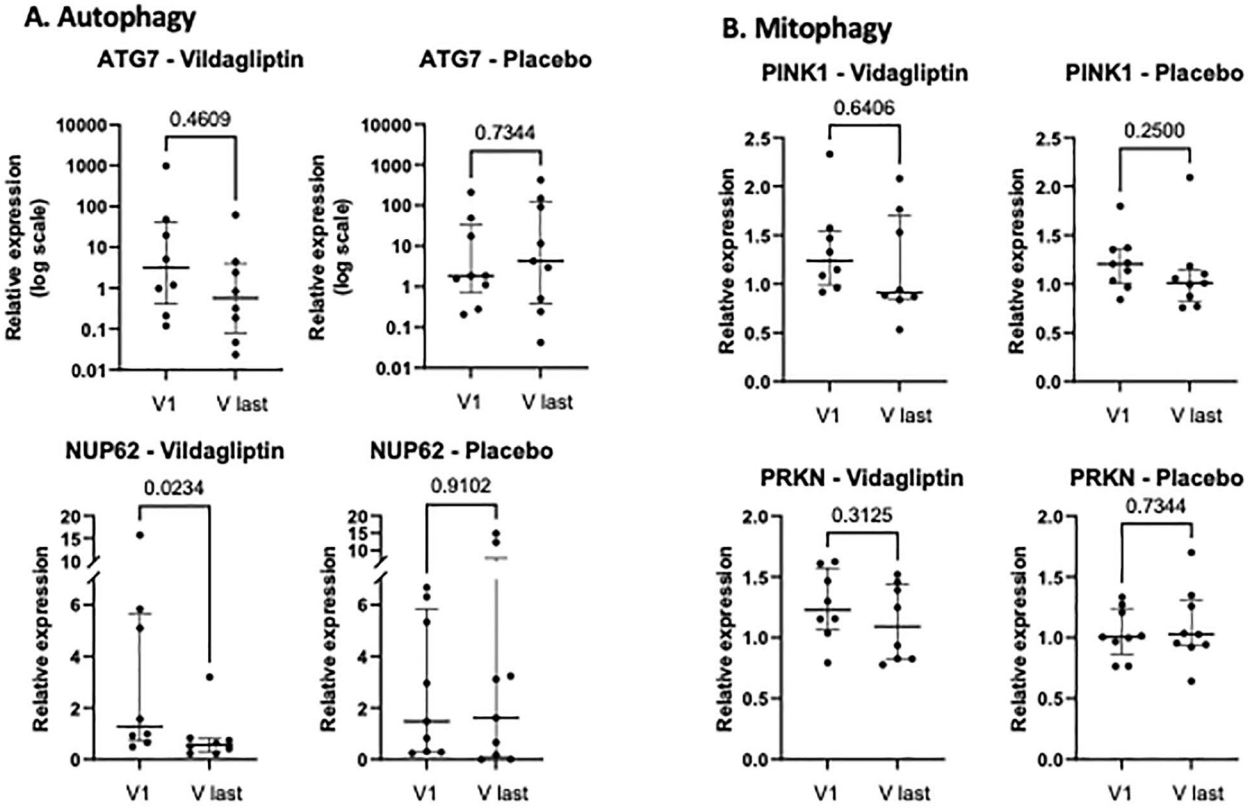

Fig. 3 Expression of autophagy and mitophagy markers in patients with and without Vildagliptin treatment. Data show significant downregulation of the autophagy marker *NUP62* in Vildagliptin but not in the placebo group **(A)** and similar expression of mitophagy markers *PINK1* and *PRKN* in both groups **(B)**. The median (bold horizontal line) and interquartile range (whiskers) are shown. Statistical significance was determined using the paired Wilcoxon signed rank. *ATG7*, autophagy related 7; *NUP62*, nucleoporin 62; *PINK1*, PTEN induced kinase 1; *PRKN*, parkin RBR E3 ubiquitin protein ligase; V1, first visit (baseline); V last, last visit (week 12)

DFU found that DPP4i Vildagliptin improved healing by approximately 35% compared to placebo [6]. Therefore, we investigated the molecular mechanisms by which Vildagliptin might promote wound healing.

Diabetes mellitus may lead to various microangiopathies, including DFUs, often due to undergoing endothelial cell (EC) dysfunction [13, 14]. Interestingly, DPP-4i(s), including Vildagliptin, was found to protect from EC dysfunction [15]. Perhaps, even more importantly, the protective effect is seen even in a normoglycemic context where Vildagliptin attenuates EC dysfunction in a non-diabetic mouse model [16]. This significant finding suggests that Vildagliptin may also affect cellular processes beyond its canonical inhibition of DPP-4. To further elucidate these findings, we assessed the differential expression of selected genes critically involved in essential cellular functions, some of which were evaluated previously and sometimes in different contexts. For example, Pujadas and colleagues previously reported that another DPP4i, Teneligliptin, increases the proliferation of human umbilical vein endothelial cells (HUVEC) exposed to hyperglycemia [17]. However, we did not find any effect of DPP-4i Vildagliptin on levels of cell proliferation markers in wound fluid obtained from diabetic patients with DFU. Likewise, it is indicative that DPP4i(s) might attenuate EC senescence in vitro and animal models [18, 19]. Still, we did not find a similar effect of Vildagliptin on the expression of senescence markers in DFU wound fluid in our patients.

Nevertheless, Zhao and colleagues reported that the DPP4 enzyme promotes EC apoptosis and autophagy [20]. Although vildagliptin did not affect the expression of apoptotic markers in our context, our findings suggest that it attenuates autophagy. This is consistent with a study by Zhao and colleagues [20]. Our results showed that Vildagliptin treatment for 12 weeks resulted in a more than twofold reduction in the mRNA levels of the autophagy marker nucleoporin 62 (*NUP62* or *p62*) in the DFU wound fluid. Autophagy is an essential physiological cell self-renewal process; however, if in excess, it can trigger so-called autophagic cell death due to excessive degradation of cellular content [21, 22]. The effect of vildagliptin on NUP62/p62 mRNA levels may be due to its ability to control blood sugar levels, which indirectly

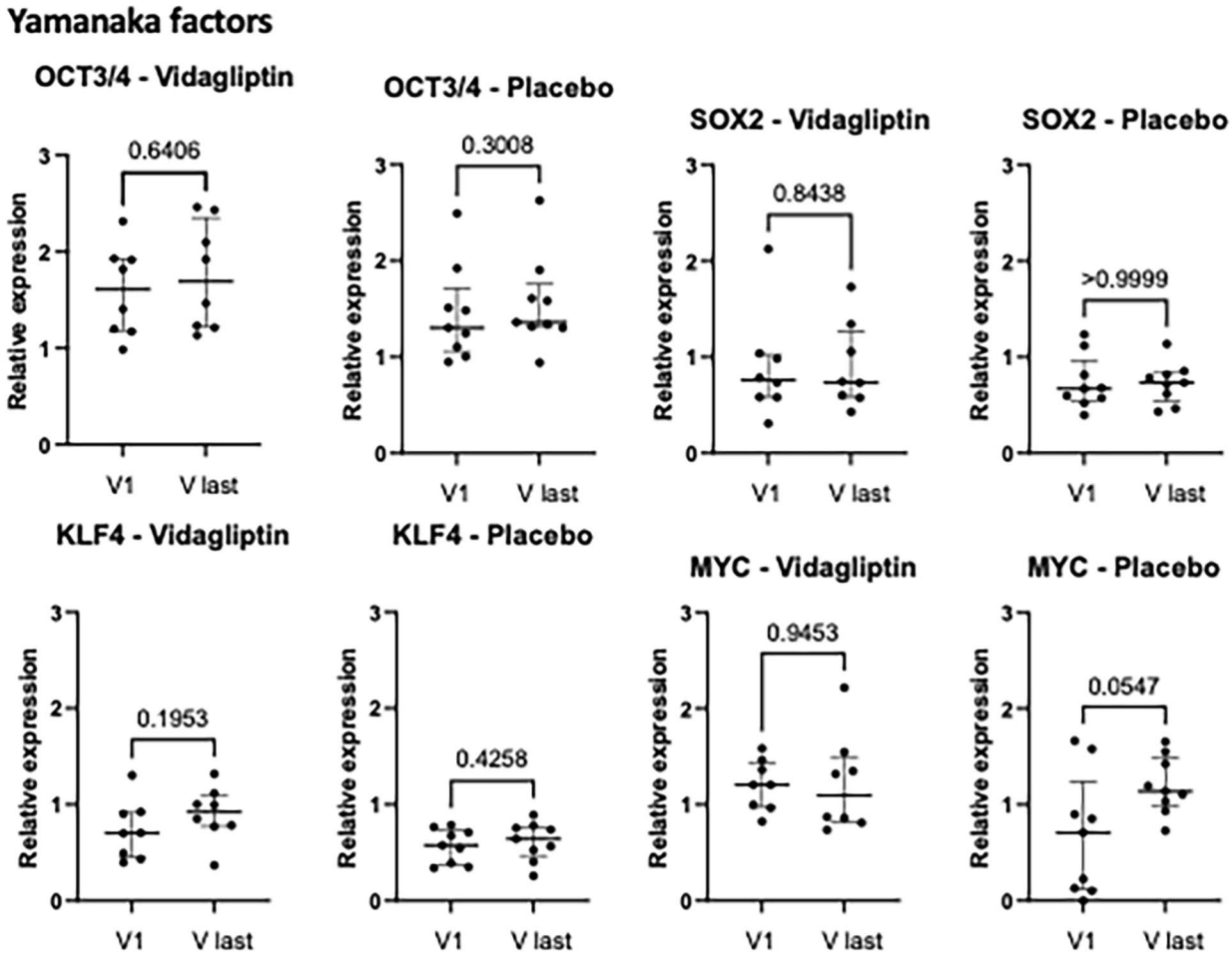

Fig. 4 Expression of pluripotency Yamanaka factors in patients with and without Vildagliptin treatment. Data show similar expressions of all four Yamanaka factors in both Vildagliptin and placebo groups. The median (bold horizontal line) and interquartile range (whiskers) are shown. Statistical significance was determined using the paired Wilcoxon signed rank. *POU5F1* (*OCT3/4*), POU class 5 homeobox 1; *SOX2*, SRY-box transcription factor 2; *KLF4*, KLF transcription factor 4; *MYC*, MYC proto-oncogene, bHLH transcription factor. V1, first visit (baseline); V last, last visit (week 12)

affects autophagy. When cells are exposed to a high concentration of glucose, the level of O-linked N-acetylglucosamine (O-GlcNAc) modification of the p62 nucleoporin increases [23]. Nucleoporins, including p62, are constitutively O-GlcNAcylated [24]. These modifications protect them from ubiquitination, thus proteasomal degradation [24], a hallmark of autophagy [9]. Indeed, p62 is a receptor for intracellular cargo to be degraded by autophagy, including ubiquitinated proteins [25, 26]. Hence, p62 is used as an autophagy marker.

An intriguing and somewhat unexpected finding emerged regarding the necroptosis marker *RIPK3*. After twelve weeks, *RIPK3* levels significantly decreased in the wound fluid of DFU patients from both the Vildagliptin and placebo arms. Necroptosis is often seen as harmful to wound healing because of its pro-inflammatory nature [27]. However, the decrease in a necroptosis marker in both groups, although not completely understood, may suggest reduced inflammation within DFU, possibly creating a more favorable environment for healing. It is worth noting that there was a slightly higher rate of DFU improvement in the Vildagliptin group (8 out of 8 patients) compared to the placebo group (6 out of 9 patients) at the end of the study. This finding warrants further investigation into the interplay between *RIPK3* and Vildagliptin's mechanism of action in wound healing.

Furthermore, this study establishes the utility of filter paper absorption for collecting wound fluid samples to monitor multiple healing biomarkers within DFU. This minimally invasive method provides biological material for detecting local changes that might not be reflected in the circulation [6].

Our study has limitations. Due to the primary clinical use of DFU wound fluid, we could only collect samples from a relatively small group of patients ($N=17$) who completed the 12-week treatment. While filter paper

absorption is a patient-friendly method, it yields a lower sample volume than aspiration techniques. This limited volume necessitated qPCR, a highly sensitive method ideal for small samples. However, this approach focuses on gene expression and may not capture protein levels exactly. Despite these limitations, we could comprehensively evaluate 18 genes related to six critical cellular processes. Future studies with larger sample sizes could be more focused now and further substantiate our findings by incorporating protein testing methods. Second, given the filter paper absorption sampling, we expected more subtle differences in molecular marker expression within the collected samples because the DFUs had only partially closed by week 12. Finally, it is essential to note that wound fluid composition is complex and includes genetic material from various resident cell types, not just endothelial cells. Despite these limitations, our data provide valuable molecular insights into the ongoing processes within DFUs during the systemic administration of Vildagliptin.

Conclusions

Building upon previous evidence of Vildagliptin's effectiveness in glycemic control and DFU healing, our study sheds light on a potential mechanism - autophagy modulation. We observed that Vildagliptin treatment influenced autophagy-related gene expression in DFU wound fluid, suggesting a novel pathway for its wound healing properties. While these findings are promising, further research is required to substantiate and expand these results.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13098-024-01444-3.

Supplementary Material 1

Acknowledgements
Not applicable.

Author contributions
E.B., V.V., and U.M. conceptualized the work. E.B. performed gene expression analysis. E.B. and V.V. performed formal statistical analysis. E.B. wrote the main manuscript text. All authors reviewed the manuscript.

Funding
We acknowledge the financial contribution of Novartis Australia for this study.

Data availability
Data is provided within the manuscript.

Declarations

Ethical approval
The study protocol, informed consent form, and case report form (CRF) have been approved by an independent human research ethics committee (HREC) located at the Townsville Hospital and Health Service (THHS) under the registration number HREC/13/QTHS/65.

Competing interests
The authors declare no competing interests.

Received: 21 June 2024 / Accepted: 9 August 2024
Published online: 22 August 2024

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