Effects of aspalathin on insulin resistance and mitochondrial dysfunction in cultured skeletal muscle cells - potential implications for type 2 diabetes mellitus therapy

Sithandiwe E. Mazibuko-Mbeje[1], Sinenhlanhla X.H. Mthembu[1,2], Christo J.F. Muller[2,3,4], Khanyisani Ziqubu [1], Ndivhuwo Muvhulawa[1], Reneilwe V. Modibedi[1], Luca Tiano[5], Phiwayinkosi V. Dludla[2]

[1]Department of Biochemistry, North-West University, Mafikeng Campus, Mmabatho 2735, South Africa.

[2]Biomedical Research and Innovation Platform, South African Medical Research Council, Tygerberg 7505, South Africa.

[3]Division of Medical Physiology, Stellenbosch University, Tygerberg 7505, South Africa.

[4]Department of Biochemistry and Microbiology, University of Zululand, KwaDlangezwa 3886, South Africa.

[5]Department of Life and Environmental Sciences, Polytechnic University of Marche, Ancona 60131, Italy.

Correspondence:

Professor Sithandiwe E. Mazibuko-Mbeje, PhD

Department of Biochemistry, Faculty of Natural and Agricultural Sciences, North-West University, Mafikeng Campus, Private Bag X 2046, Mmabatho 2735, South Africa. Email: 36588296@nwu.ac.za. Tel.:018 399 2354

Abstract

Natural compounds may bare promising therapeutic benefits against metabolic diseases such as type 2 diabetes mellitus (T2DM), which are characterized by a state of insulin resistance and mitochondrial dysfunction. Here, we examined the cellular mechanisms by which aspalathin, a dihydrochalcone *C*-glucoside unique to rooibos, may ameliorate palmitate-induced insulin resistance and mitochondrial dysfunction in cultured C2C12 myotubules. This current study demonstrated that aspalathin remains effective in improving glucose uptake in insulin-resistant skeletal muscle cells, supported by the upregulation of insulin-dependent signaling that involves the activation of insulin receptor (IR) and direct phosphorylation of protein kinase B (AKT). Interestingly, aspalathin also improved mitochondrial respiration and function, which was evident by an increased expression of carnitine palmitoyltransferase 1 (*Cpt1*), fatty acid transport protein 1 (*Fatp1*), sirtuin 1 (*Sirt1*), nuclear respiratory factor 1 (*Nrf1*), and transcription factor A, mitochondrial (*Tfam*). Importantly, our results showed that aspalathin treatment was effective in ameliorating the devastating outcomes of insulin resistance and mitochondrial dysfunction that are linked with an undesired pro-inflammatory response, by reducing the levels of well-known pro-inflammatory markers such as interleukin-6 (*IL-6*), tumor necrosis factor-alpha (*TNF-α*), and protein kinase C-theta (PKC-θ). Thus, beyond improving glucose uptake and insulin signaling, the current study brings a new perspective in the therapeutic benefits of aspalathin in improving mitochondrial respiration and blocking inflammation to attenuate the detrimental effect of palmitate in skeletal muscle cells.

Keywords:

Type 2 diabetes mellitus; skeletal muscle insulin resistance; aspalathin; insulin signaling; mitochondrial dysfunction and inflammation.

1. Introduction

The rapid increase in the prevalence of diabetes mellitus, particularly type 2 diabetes mellitus (T2DM) [1] is of major concern, due to its significant contribution to the overall increase in global deaths [2]. Modifiable risk factors such as excessive nutrient intake, which are usually coupled with a sedentary lifestyle, are acknowledged to be the main instigators in the development of diverse metabolic diseases including T2DM [3]. In fact, excessive body fat accumulation in a state of obesity is responsible for the development of insulin resistance, a major characteristic feature of T2DM [3]. Indeed, impairments in insulin signaling, mainly accompanied by suppressed muscle glucose homeostasis or derangements in free fatty acid oxidation within the skeletal muscle are recognized as established pathological mechanisms unique to T2DM [4]. This also explains the significance of the skeletal muscle in maintaining essential physiological processes, especially its role in regulating energy metabolism, including the uptake and utilization of major substrates like glucose and free fatty acids [5].

Notably, well-known interventions like physical activity are acknowledged to alleviate some metabolic complications by improving skeletal muscle glucose utilization, or even by modulating molecular mechanisms involved in energy metabolism and insulin signaling [6, 7]. Regarding the latter, prime examples include effective regulation of insulin-dependent mechanisms like the phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT) pathway. Briefly, through binding to the insulin receptor (IR), insulin can directly stimulate PI3K, which leads to the activation of protein kinases such as AKT that promote glucose uptake within the skeletal muscle [8]. Interestingly, accumulative research has indicated that diverse pharmacological compounds, including established antidiabetic drugs like insulin and metformin, mainly function through activation of such molecular mechanisms to reverse insulin resistance or alleviate complications linked with T2DM [9-11].

Likewise, available research has indicated that herbal medicine has the potential to ameliorate metabolic abnormalities linked with insulin resistance by improving insulin sensitivity, in part though effective regulation of PI3K/AKT pathways [12-14]. Particular interest has also been placed on characterizing and determining the relative levels of bioactive compounds found in herbal medicines, as the major therapeutic effects of these plants are attributed to these exceptional compounds. In fact, due to its envisaged health capabilities, there has been a general interest in understanding the therapeutic potential of bioactive compounds found in rooibos (*Aspalathus linearis*) in ameliorating metabolic diseases and improving human health [15-19]. As such, our research group has progressively explored the ameliorative effects of aspalathin, the major dihydrochalcone unique to rooibos, against metabolic complications linked with insulin resistance and T2DM in various preclinical models. Available evidence suggests that beyond controlling substrate metabolism, including regulating the utilization of glucose and free fatty acids [20-22], aspalathin or extracts rich in this bioactive compound can modulate essential cellular mechanisms involved in cell survival, such as the PI3K/AKT signaling, mitochondrial respiration, and even autophagy to hinder cellular damage in various experimental models of diabetes [23-28]. Notably, there is not much information that has been reported on the direct impact of aspalathin in salvaging palmitate-induced insulin resistance, mitochondrial dysfunction, or suppressing pro-inflammatory markers in cultured C2C12 myotubes. Thus, beyond enhancing our current understanding of the therapeutic effects of

aspalathin, the current study completes a global perspective on the link between insulin resistance and mitochondrial dysfunction. Lastly this study also demonstrated the potential protective effect of aspalathin has gained palmitate induce inflammation, which is also known as risk factor of T2DM.

2. Materials and methods

2.1. Reagents

Murine C2C12 skeletal muscle cells (Catalog No. CRL-1772) were obtained from the American Type Culture Collection (Manassas, VA, USA). Aspalathin (≥ 98% purity, batch SZ1-356-54) was acquired from High Force Research LTD (Durham, UK). Dulbecco's Modified Eagle's Medium (DMEM), Dulbecco's phosphate-buffered saline (DPBS, pH 7.4 with calcium and magnesium), penicillin-streptomycin and luminescent ATP kit were obtained from Lonza (Walkersville, MD, USA). Horse serum (HS) and fetal calf serum (FCS) were from Biochrom (Berlin, Germany), and PrestoBlue™ cell viability reagent was purchased from Invitrogen (Karlsruhe, Germany). 2-Deoxy-[^{3}H]-D-glucose was from American Radiolabelled Chemicals (St Louis, MO, USA). Bradford protein assay kits were from Bio-Rad Laboratories (Hercules, CA, USA). Cell Signalling Technology (Beverly, MA, USA) supplied primary antibodies, including protein kinase C-theta (PKC θ), p/PKC θ $^{(Ser\ 643/676)}$, protein kinase B (AKT), p-AKT (Ser473) (Ser ^{473}), as well as insulin receptor (IR). The reference control, beta (β)-actin, and the secondary antibodies; goat-anti-mouse and goat-anti rabbit IgG – horseradish peroxidase (HRP) were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Quant-iT™ PicoGreen™ dsDNA quantitation assay kits, TRIzol reagent, Turbo DNase Kit, and all PCR probes were supplied by ThermoFisher Scientific, Inc. (Waltham, MA, USA). Taqman Master Mix, QIAzol, QuantiTect Reverse Transcription Kit were obtained from Qiagen (Valencia, CA, USA). Reagents for Seahorse experiments such as oligomycin, carbonyl cyanide p-triflouromethoxyphenylhydrazone (FCCP), rotenone, Antimycin A were purchased from Agilent (Santa Clara, CA, USA). Palmitic acid (C18:0) and all other chemicals were obtained from Sigma-Aldrich (St Louis, MO, USA), except when specified.

2.2. Cell culture treatments

An established experimental model was used to culture and differentiate skeletal (C2C12) muscle cells [29]. Briefly, cells were cultured and sub-cultured in DMEM containing 4.5g/L glucose and 20 mM HEPES, supplemented with 10% (v/v) FCS, 100 U/mL penicillin, and 100 µg/mL penicillin-streptomycin for 3 days or until 90-100 % confluent. Upon confluency, these myoblasts were differentiated by switching to low serum DMEM containing 2% HS to induce myotube formation. Then, C2C12 myotubules were seeded into cell bind 24-well plates (25 000 cells/well), 96-well plates (5 000 cells/well) or 6-well plates (75 000 cells/well); and for seahorse, 96 well XF-96 microplate plates (12 000 cells/well) were used. Media was replaced every 48 h, on day 5 cells were fully differentiated into myotubules, and then further experiments were performed.

2.3. Induction of insulin resistance and treatment with aspalathin

Upon confluence, insulin resistance was induced by culturing C2C12 myotubules in DMEM containing 2% BSA, 5.5 mM glucose and 0.75 mM palmitate for 16 h, as previously described [29]. On the other hand, experimental control cells were cultured in DMEM without palmitate. After induction of insulin resistance, cells were serum and glucose starved before treatment with aspalathin, which was prepared as previously described [21]. For treatment, the cells were exposed for 1 h in either 1 µM insulin (used as a positive control) or 10 µM aspalathin that was diluted in 2% BSA DMEM culture media with or without palmitate. Choice of dose and treatment time for aspalathin was based on previously published research [21].

2.4. Determination of cell viability and metabolic activity

The PrestoBlue™ dye was used as a measure of cell viability, whilst the luminescent ATP kit was used to evaluate metabolic activity. Briefly, after treatment 10 µL PrestoBlue™ was added to each well and incubated for 40 min at room temperature and absorbance (570 nm) was measured after 10 min using PHERAstar® plate reader (BMG LABTECH, Germany). To measure the level of ATP within the cell, the luminescent ATP kit was used, as described by the manufacturer. ATP luminescence was read using a BioTek FLx800 plate reader and Gen 5 software for data acquisition (BioTek Instruments Inc., Winooski, VT, USA).

2.5. Determination of glucose uptake

Briefly, the glucose uptake test was performed using radiolabelled 2-Deoxy-[^{3}H]-D-glucose, as previously described [20, 21]. Briefly, after 16 h of incubation with palmitate and treatment with or without aspalathin for 1h, 0.5 µCi/mL 2-Deoxy-[^{3}H]-D-glucose was added to each well and incubated at 37°C in 5% CO_2 and humidified air for 15 min. Thereafter 2-Deoxy-[^{3}H]-D-glucose as assessed by liquid scintillation using (2220 CA, Packard Tri-Carb series, PerkinElmer, Downers Crove, IL, USA).

2.6. mRNA expression analysis

Total RNA was extracted from treated C2C12 myotubules using Qiazol buffer according to the manufacturer's instructions, thereafter RNA concentration was determined using NanoDrop spectrophotometer (Thermo Scientific, USA). To remove contaminating genomic DNA from RNA, samples were DNase treated using QuantiTect Reverse Transcription Kit, and total RNA was reverse transcribed into cDNA according to the manufacturer's recommendations. Differential gene expression was analysed by quantitative RT-PCR using QuantStudio™ 6 (Applied Biosystems, CA, USA). Gene expression, for interleukin (*Il*)-*6,* tumor necrosis factor alpha (*Tnf-α*)*,* fatty acid transport protein 1 (*Fatp1*)*,* carnitine palmitoyltransferase 1 (*Cpt1*)*,* uncoupling protein 2 (*Ucp2*)*,* NAD-dependent deacetylase sirtuin 1 (*Sirt1*)*,* nuclear respiratory factor 1 (*Nrf1*) and transcription factor

A, mitochondrial (*Tfam*) was calculated as delta Ct using Actin beta (Act b) for normalization and relative to the untreated (experimental) control group normalized to a value of 100%, stated as "relative mRNA expression" in the y axis label. The list of gene expression probes used in this study as supplied in Table 1.

Table 1. The list of mRNA probes used in the study.

Probe	Role	Assay ID
Interleukin 6 (*Il6*)	Inflammation	Mm00446190_m1
Tumor necrosis factor alpha (*Tnf-α*),	Inflammation	Mm00443258_m1
fatty acid transport protein 1 (*Fatp1*),	Lipid metabolism	Mm00444340_m1
carnitine palmitoyltransferase 1 (*Cpt1*)	Lipid metabolism	Mm00487191_g1
Uncoupling Protein 2 (*Ucp2*)	Mitochondrial bioenergetics	Mm00627599_ml
Sirtuin (*Sirt1*)	Mitochondrial biogenesis	Mm01168521_m1
Nuclear Respiratory Factor 1 (*Nrf1*)	Mitochondrial biogenesis	Mm01135606_g1
transcription factor A, mitochondrial (*Tfam*)	Mitochondrial bioenergetics	Mm00447485_m1
Actin beta (*Act b)*	Housekeeping	Mm02619580_g1

2.7. Cellular bioenergetics

An established protocol was used to assess cellular bioenergenitics [22, 30]. Briefly, before the measurements of cellular respiration, treated cells were removed from the CO_2 incubator and the media were changed to XF Assay Medium containing 8 mM glucose with or without palmitate, aspalathin or etomoxir (1 mM), then equilibrated for another hr at 37 °C without CO_2. Thereafter, the XF9-6 plate was transferred to a Seahorse XF96 extracellular flux analyser (Agilent Technologies, Chicopee, MA 01022) and basal respiration/glycolysis values determined in three assay cycles (1 min mixing, 2 min waiting, 3 min measuring). Then, oligomycin (2 µg/ mL) was injected in port A, followed by FCCP (2.5 µM) injection in port B, rotenone and antimycin A (2.5 µM) port C and 2-Deoxy-[^{3}H]-D-glucose (100 µM) in port D. Results were normalised to DNA using PicoGreen® dsDNA quantitation assay kit according to manufactures recommendations.

2.8. Western blot analysis

Western blot analysis was performed using a standardised protocol, as previouldy described [20, 29]. Briefly, protein was extracted using RIPA buffer, 40 µg for cells, was extracted using Tissue Lysis buffer (Qiagen) thereafter, heat denatured. Protein was separated on a 10 or 12% SDS-PAGE gel and transferred to a PVDF-P membrane. Successful transfer was determined using Ponceau stain, membrane was blocked with 5% (w/v) low-fat milk powder in Tris-buffered saline with Tween 20 (TBST) at room temperature for 2 h. Membranes were then labelled overnight at 4°C with the relevant primary antibody (PKC θ, p/PKC θ $^{(Ser\ 643/676)}$, pAKT, tAKT, and IR)

and HRP conjugated secondary antibodies applied for 1½ h the following day. Proteins were detected and quantified by chemiluminescence using a Chemidoc-XRS imager and Quantity One 1-D software (Biorad Laboratories, Hercules, CA, USA), respectively. Here, β-actin was used as the reference control.

2.9. Statistical analysis

All results are expressed as ± standard error of the mean (SEM) of three independent biological experiments. The significant difference between groups was determined using one-way of variance (ANOVA) followed by Tukey's post hoc test using GraphPad Prism version 8.0.1 (GraphPad Software Inc., San Diego, CA, USA). Results were considered significant at $p \leq 0.05$. For PCR results, comparisons between groups were performed using one-way multivariate ANOVA, followed by unpaired Student *t*-test, and a p-value of ≤ 0.05 was deemed as statistically significant.

3. Results

3.1. Aspalathin restored cell viability and ATP production in palmitate-induced insulin-resistant C2C12 myotubules

The effects of palmitate and aspalathin on cell viability and metabolic activity were investigated in differentiated C2C12 myotubules. The results showed that insulin has an ability to improve cell viability and ATP production in normal cells (not exposed to palmitate) ($p < 0.05$ and $p < 0.001$, respectively) (Figure 1A, B). Interestingly, 0.75 mM palmitate significantly reduced cell viability ($p < 0.01$) and ATP production ($p < 0.001$) when compared to experimental (normal) controls (Figure 1A, B). The addition of aspalathin, either as a monotherapy or in combination with insulin, significantly improved cell viability ($p < 0.001$ and $p < 0.01$, respectively) and ATP production ($p < 0.001$) in cells exposed to palmitate (Figure 1A, B). However, insulin, as a positive control did not have a significant effect in improving cell viability and ATP production in comparison in cells exposed to palmitate (Figure 1A, B).

Figure 1. Effect of aspalathin on cell viability (A) and energy production (ATP) (B) in insulin resistant skeletal muscle (C2C12) cells. *Briefly, C2C12 myotubules were treated with or without 0.75 mM palmitate for 16 h, thereafter cultured with aspalathin (10 µM) for 1 h, while insulin (1 µM) was added in the last 15 min as a positive control. As an endpoint measurement, cell viability was assessed by prestoBlue dye and energy production (ATP) by luminescent ATP kit. Results are expressed as the mean of three independent experiments relative to the control at 100% ± SEM. *p < 0.05, **p< 0.01, ***p< 0.001 versus experimental control: ##p < 0.01, ###p < 0.001 versus palmitate control.*

3.2. Aspalathin improved glucose uptake and the expression levels of proteins involved insulin signaling in palmitate-induced insulin resistant C2C12 myotubules

Determining the levels of cellular glucose uptake, including the modulation of protein markers involved in insulin signaling like AKT/IR remains essential to evaluate the capability of treatment compounds to ameliorate insulin resistance. Insulin as comparative control, significantly improved glucose uptake ($p < 0.001$) and the phosphorylation of AKT in the normal cells ($p < 0.001$) (Figure 2A, B and C). However, exposure of C2C12 myotubules to 0.75 mM palmitate significantly suppressed glucose uptake ($p < 0.01$), including protein expression levels of IR (not statistically significant) and p/AKT ($p < 0.001$) when compared to an experimental control (Figure 2A, B, and C). Nonetheless, treatment with aspalathin, as monotherapy or in combination with insulin, improved glucose uptake ($p < 0.001$), IR protein expression ($p < 0.01$ and $p < 0.001$), and the phosphorylation of AKT ($p < 0.001$) (Figure 2A, B and C). Notably, insulin as a comparative control, showed similar results to aspalathin relevant to glucose uptake ($p < 0.01$) and p/AKT ($p < 0.001$) in palmitate exposed cells (Figure 2).

Figure 2. Effect of aspalathin on glucose uptake (A), and protein expression levels of insulin receptor (IR; B), and phosphorylated protein kinase B (p/AKT; C) in insulin resistant skeletal muscle (C2C12) cells*. Briefly, C2C12 myotubules were treated with or without 0.75 mM palmitate for 16 h, thereafter cultured with aspalathin (10 µM) for 1 h, while insulin (1 µM) was added in the last 15 min as a positive control. Treated cells were lysed and subjected to Western blot analysis. Results are expressed as the mean of three independent experiments relative to control set at 100% ± SEM **p< 0.01, ***p< 0.001 versus experimental control: ##p < 0.01, ###p < 0.001 versus palmitate control.*

3.3. Aspalathin improved gene expression levels of some molecular markers of fatty acid transport and inflammation in palmitate-induced insulin resistant C2C12 myotubules

Briefly, aberrant cellular fatty acid transportation, which may lead to the downstream detrimental effects of inflammation, remains an important aspect to determine, as it is also linked with the exacerbation of insulin resistance. Accordingly, exposure of differentiated C2C12 myotubules to 0.75 mM palmitate resulted in markedly increased mRNA expression levels of fatty acid transporters like *Fatp1* ($p < 0.001$) and *Cpt1* ($p < 0.001$), including prominent markers of inflammation such as *Il-6* ($p < 0.001$), *Tnf-α* ($p < 0.001$), and PKC-θ ($p < 0.001$) in comparison to the experimental control (Figure 3A-E). Treatment with aspalathin, either as a monotherapy or in combination with insulin, showed comparative results in significantly reducing these fatty acid transport and inflammation makers. Notably, insulin as a positive control, did not significantly affect the mRNA expression levels of *Fatp1* and *Cpt1*, or pro-inflammatory markers (*Il-6*, *Tnf-α* and PKC-θ) in comparison to palmitate only exposed cells (Figure 3).

Figure 3. Effect of aspalathin on the regulation of mRNA expression levels of genes involved in beta-oxidation like fatty acid transport protein 1 (Fatp1; A) carnitine palmitoyltransferase 1 (Cpt1; B), and genes involved in inflammation such as intereleukin-6 (Il6; C) and tumor necrosis factor alpha (TNF-α; D), as well as protein expression of protein kinase C theta (PKC-ϑ; E) in insulin resistant skeletal muscle (C2C12) cells*. Briefly, C2C12 myotubules were treated with or without 0.75 mM palmitate for 16 h, thereafter cultured with aspalathin (10 µM) for 1 h, while insulin (1 µM) was added in the last 15 min as a positive control. Treated cells were lysed and quantified by RT-PCR and protein analysis. Results are expressed as the mean of three independent experiments relative to control set at 100% ± SEM. ***p< 0.001 versus experimental control: ##p < 0.01, ###p < 0.001 versus palmitate control.*

3.4. Aspalathin improved cellular bioenergetics in palmitate-induced insulin resistant C2C12 myotubules

To characterize the effect of aspalathin on the process of mitochondrial bioenergetics, we measured the OCR (Figure 5A) and ECAR (Figure 5B) in palmitate-induced insulin resistant C2C12 myotubules using Agilent Seahorse XF Technology. After induction of insulin resistance with palmitate for 16 h, muscle cells displayed significantly suppressed basal OCR ($p < 0.01$), ATP production ($p < 0.01$), maximal respiration ($p < 0.001$), and spare respiratory capacity ($p < 0.05$) (Figure 4C-F). Treatment with aspalathin showed comparative results to its combination with insulin in improving all markers of mitochondrial bioenergetics (Figure 4C-F). The use of insulin, as a comparative control, failed to show any effect (except for increasing maximal respiration, $p < 0.001$) in improving mitochondrial bioenergetics using the current experimental model of C2C12 myotubules exposed to elevated concentrations of palmitate.

Figure 4. Effect of aspalathin on the regulation of mitochondrial bioenergetics, including basal oxygen consumption rate (OCR; A) and extracellular acidification rates (ECAR; B) of all treatments, before independent analysis of basal OCR (C), adenosine triphosphate (ATP) production (D), maximal respiration (E), and spare respiratory capacity (F) in insulin resistant skeletal muscle (C2C12) cells*. Briefly, C2C12 myotubules were treated with or without 0.75 mM palmitate for 16 h, thereafter cultured with aspalathin (10 µM) for 1 h, while insulin (1 µM) was added in the last 15 min as a positive control. The OCR and ECAR were determined using Seahorse XF-96 Metabolic Flux Analyzer. Groups (A, B, C, D, E and F) show data of changes in OCR, ECAR, in response to the sequential administration (arrows) of oligomycin (2 µg/ mL) port A, Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) (2.5 µM) port B, antimycin and Rotenone (2.5 µM) port C. Results are expressed as mean ± SEM of 3 independent experiments. *p ≤0.05, **p< 0.001 ***p< 0.001 versus experimental control; # p < 0.05, ## p < 0.05### p < 0.001 versus palmitate control.*

3.5. Aspalathin improved the expression levels of genes involved in mitochondrial energetics in palmitate-induced insulin resistant C2C12 myotubules

We further investigated the effect of aspalathin on the expression levels of genes involved in mitochondrial energetics in palmitate induced C2C12 myotubules. Here, exposure of differentiated C2C12 myotubules to 0.75

mM palmitate significantly reduced the mRNA expression levels of *Ucp2* ($p < 0.001$), *Sirt1* ($p < 0.001$), *Nrf1* ($p < 0.001$), and *Tfam* ($p < 0.001$) (Figure 5A-D). Interestingly, also consistent with Seahorse data (Figure 4), treatment with aspalathin, either as a monotherapy or in combination with insulin, significantly improved the expression levels of all analyzed genes ($p < 0.001$) (Figure 5A-D). The use of insulin, as a comparative control, showed some effect in enhancing the expression *Ucp2* ($p < 0.001$), *Sirt1* ($p < 0.001$), *Nrf1* ($p < 0.001$), and *Tfam* ($p < 0.001$) in comparison to palmitate control (Figure 5).

Figure 5. Effect of aspalathin on the regulation of mRNA expression levels of genes involved mitochondrial function, including uncoupling protein 2 (Ucp2; A), silent mating type information regulation 2 homolog (Sirt1; B), nuclear respiratory factor 1 (Nrf1; C) and transcription factor A, mitochondrial (Tfam; D) in insulin resistant skeletal muscle (C2C12) cells*. Briefly, C2C12 myotubules were treated with or without 0.75 mM palmitate for 16 h, thereafter cultured with aspalathin (10 µM) for 1 h, while insulin (1 µM) was added in the last 15 min as a positive control. Treated cells were lysed and quantified by RT-PCR analysis. Results are expressed as the mean of three independent experiments relative to control set at 100% ± SEM. ***p< 0.001 versus experimental control: ###p < 0.001 versus palmitate control.*

4. Discussion

Pathological features, insulin resistance and mitochondrial dysfunction, are known hallmarks of T2DM, and are widely studied through the application of diverse preclinical models that mimic human disease [31-33]. Research from our group has explored an in vitro model of exposing cultured cells to elevated concentrations of palmitate as a feasible strategy to study pathological mechanisms of T2DM, including the development of insulin resistance and mitochondrial dysfunction [21, 29, 34, 35]. In fact, there has been a growing interest in understanding the cause-effect relationship between insulin resistance and mitochondrial dysfunction during the pathogenesis of T2DM, especially within the skeletal muscle [31-33]. As such, beyond inducing alterations in substrate metabolism which may be characterized by suppressed glucose metabolism, exposing cultured cells to elevated concentrations of palmitate (0.75 mM) for 16 h has been shown to significantly hinder the process of mitochondrial respiration, including reducing the expression of major genes involved insulin signaling and mitochondrial function [22, 36]. Other studies have reported that oxidative stress, which is often exacerbated by mitochondrial dysfunction, might also accelerate cellular apoptosis, a consequence that has been explored in experimental models of metabolic disease [37, 38]. Indeed, previous reviews have critically discussed the pathological role of apoptosis in the development of T2DM, especially its implication in the destruction of pancreatic β- islet cells [39, 40]. Further, highlighting the importance of understanding the pathological features of T2DM, specifically mitochondrial dysfunction in order to protect against destructive processes involving apoptosis or accelerated cell death.

Notably, palmitate exposure, as an experimental model to mimic the toxic effects of lipid overload, has been increasingly studied [41]. In fact, the current study showed that exposing skeletal muscle (C2C12) myotubules to elevated concentrations of palmitate could effectively reduce glucose uptake and cell viability, and this was consistent with suppressing mitochondrial respiration, including genes involved in this process, such as *Ucp2*, *Sirt1*, *Nrf1*, and *Tfam*. Interestingly, also to further characterize the classical features T2DM, the current study also showed that exposing C2C12 myotubules to elevated concentrations of palmitate resulted in enhanced levels of molecular makers indicating an abnormal inflammatory response, such as the mRNA levels of *Il-6*, *Tnf-α*, and phosphorylation of PKC-θ. This certainly affirms the relevance of using this in vitro model, of exposing C2C12 cells to elevated concentrations of palmitate, to assess the therapeutic effects of aspalathin against T2DM-related complications, including insulin resistance, mitochondrial dysfunction and an abnormal inflammatory response.

Intriguingly, research has progressively explored the use of plant-derived sources for their ameliorative effects against skeletal muscle insulin resistance, through the application of in vitro techniques. For example, the blueberry leaf extract was shown to attenuate TNF-α-induced insulin resistance by promoting glucose uptake and improving insulin signaling via upregulating AKT phosphorylation in cultured skeletal (C2C12) myotubules [42]. Alternatively, the well-known phytochemicals like quercetin, rutin and gallic acid, found in rooibos and other food sources, have been shown to hinder skeletal muscle atrophy and block cell apoptosis, in part by effectively scavenging for free radical species and improving mitochondrial function in cultured in C2C12 myotubules [43, 44]. Here, the major results showed that aspalathin treatment could alleviate palmitate-induced skeletal muscle insulin resistance by enhancing glucose uptake and mitochondrial respiration, blocking FFA-transport, as well as improving insulin signaling as partly demonstrated through increased phosphorylation of AKT in cultured C2C12 myotubules (Figure 6). Interestingly, such findings are consistent with findings from other cellular models, mimicking complications of T2DM, reporting on the bioactive properties from rooibos, including aspalathin [22, 35, 36]. Furthermore, it has been reported that inflammation also plays a major role in the development of T2DM [45]. For example, macrophages and other immunocompetent cells can modulate an immune response and subsequently an inflammatory state through their secretion of cytokines such as TNF-α and IL-6, further taking part in different immune responses and protection against pathogens and diseases [46, 47]. However, excessive immune activation, or exacerbated secretion of these pro-inflammatory cytokines has been implicated in the deterioration of metabolic complications linked with T2DM [46, 48]. Similarly, there was increased mRNA expression of *TNF-α* and *IL-6* upon the exposure of cells to palmitate in the current study. Such findings promote an important aspect entailing on the anti-inflammatory properties of this dihydrochalcone, as it effectively blocked PKC-θ phosphorylation in addition to reducing the mRNA levels of *Il-6* and *Tnf-α* in these palmitate-exposed skeletal muscle cells. In fact, this is validating the hypothesis that beyond its free radical scavenging properties [49], aspalathin presents with enhanced ameliorative effects against inflammation [50].

Figure 6: An overview of therapeutic mechanisms by which aspalathin ameliorates palmitate-induced insulin resistance in skeletal muscle (C2C12) myotubules. Briefly, the major results in this study showed that aspalathin treatment could alleviate palmitate-induced skeletal muscle insulin resistance by enhancing glucose uptake and mitochondrial respiration blocking free fatty acid (FFA)-transport, as well as improving insulin signaling as partly demonstrated through increased expression insulin receptor (IR)/phosphorylation of protein kinase B (AKT) in cultured C2C12 myotubules. This bioactive compound could effectively attenuate inflammation by reducing the expression of markers such as interleukin-6 (*IL-6*), tumor necrosis factor-alpha (*TNF-α*), and protein kinase C-theta (PKC-θ). Moreover, markers of mitochondrial function included, included uncoupling protein 2 (*Ucp2*), NAD-dependent deacetylase sirtuin 1 (*Sirt1*), nuclear respiratory factor 1 (*Nrf1*) and transcription factor A, mitochondrial (*Tfam*).

5. Conclusions

Through the use of in vitro model of skeletal muscle insulin resistance, the current results support the notion that aspalathin presents with an enhanced therapeutic capacity to ameliorate diverse complications of metabolic syndrome, as previously reviewed [19]. Although such information remains relevant and could enhance the therapeutic development of aspalathin as a potential nutraceutical, the current study is not without limitations. Firstly, it remains essential to confirm these results using an established in vivo model of T2DM. Importantly, the in vivo model of T2DM could be used for a complete analysis of molecular mechanisms, linking both insulin resistance and mitochondrial dysfunction to better understand the therapeutic benefits of aspalathin and its potential synergetic effect with insulin (especially making use of in vivo models of T2DM). Notably, although insulin can be used as an effective comparative control [36, 51], other reference drugs, especially those specific for T2DM like metformin will add value to the understanding of therapeutic benefits of aspalathin in future studies. Lastly, future studies should also look the therapeutic effects of aspalathin against oxidative stress-related cell death within the skeletal muscle, including the implications of inflammation during the development of T2DM.

Abbreviations

AKT, protein kinase B; **Cpt1**, carnitine palmitoyltransferase 1; **ECAR**, extracellular acidification rate; **Fatp1**, fatty acid transport protein 1; **FFA**, free fatty acids; **IL-6**, interleukin-6; **IR**, insulin receptor; **Nrf1**, nuclear respiratory factor 1; **OCR**, mitochondrial oxygen consumption rate; **PI3K**, phosphoinositide 3-kinase; **PKC-θ**, protein kinase C-theta; **Sirt1**, NAD-dependent deacetylase sirtuin 1; **T2DM**, Type 2 diabetes mellitus; **Tfam**, transcription factor A; **TNF-α**, tumor necrosis factor-alpha; **Ucp2**, uncoupling protein 2

Funding statement

This work was funded by the National Research Foundation (NRF) of South Africa Thuthuka Programme grant 128296 and NRF support for rated scientist 113674 to S.E. Mazibuko-Mbeje. Baseline funding from the Biomedical Research and Innovation Platform of the South African Medical Research Council (SAMRC) and Northwest University is also acknowledged. Grant holders acknowledge that opinions, findings and conclusions or recommendations expressed in any publication generated by the NRF or SAMRC supported research are those of the authors, and that the NRF or SAMRC accepts no liability whatsoever in this regard.

Acknowledgements

The authors, S.X.H. Mthembu and K. Ziqubu, are funded by the SAMRC through its Division of Research Capacity Development under the internship scholarship program from funding received from the South African National Treasury. Grant holders acknowledge that opinions, findings and conclusions or recommendations expressed in any publication generated by the SAMRC supported research are those of the authors, and that the SAMRC accepts no liability whatsoever in this regard.

Declaration of competing interest

The authors declare no conflict of interest.

Authors' contributions

S.E. Mazibuko-Mbeje, C.J.F. Muller, and P.V. Dludla - concept and original draft; S.E. Mazibuko-Mbeje, S.X.H. Mthembu, K. Ziqubu, N. Muvhulawa, and R.V. Modibedi - performed the experiments and data analysis; S.E. Mazibuko-Mbeje- funding and resources; S.E. Mazibuko-Mbeje, C.J.F. Muller, S.X.H. Mthembu, K. Ziqubu, N. Muvhulawa, R.V. Modibedi, L. Tiano, P.V. Dludla - manuscript writing and approval of the final draft.

Data availability statement

Data related to search strategy, study selection and extraction items will be made available upon request after the manuscript is published.

References

1. International Diabetes Federation, IDF Diabetes Atlas 2021. Available at: https://diabetesatlas.org/. Accessed 02 January 2022.

2. World Health Organization (WHO), The top ten leading causes of death. Available at: https://www.who.int/news-room/fact-sheets/detail/the-top-10-causes-of-death. Accessed 03 January 2022.

3. Grundy SM. Metabolic syndrome update. Trends Cardiovasc Med 2016;26(4):364-73.

4. DeFronzo RA, and Tripathy D. Skeletal muscle insulin resistance is the primary defect in type 2 diabetes. Diabetes Care 2009;32 Suppl 2(Suppl 2):S157-63.

5. Hargreaves M, and Spriet LL.Skeletal muscle energy metabolism during exercise. Nat Metab 2020;2(9):817-828.

6. Wojtaszewski JF, and Richter EA. Glucose utilization during exercise: influence of endurance training. Acta Physiol Scand 1998;162(3):351-8.

7. Evans PL, McMillin SL, Weyrauch LA, and Witczak CA. Regulation of skeletal muscle glucose transport and glucose metabolism by exercise training. *Nutrients* 2019;*11*(10):2432.

8. Chang L, Chiang SH, Saltiel AR. Insulin signaling and the regulation of glucose transport. Molecular medicine 2004;10(7):65-71.

9. Rena G, Hardie DG, Pearson ER. The mechanisms of action of metformin. Diabetologia 2017; 60(9):1577-85.

10. Foretz M, Guigas B, Bertrand L, Pollak M, Viollet B. Metformin: from mechanisms of action to therapies. Cell metabolism 2014;20(6):953-66.

11. Petersen MC, Shulman GI. Mechanisms of insulin action and insulin resistance. Physiological reviews 2018;98(4):2133-223..

12. Li J, Bai L, Wei F, Zhao J, Wang D, Xiao Y, Yan W, Wei J. Therapeutic mechanisms of herbal medicines against insulin resistance: a review. Frontiers in Pharmacology 2019 ;10:661.

13. Cui X, Qian DW, Jiang S, Shang EX, Zhu ZH, Duan JA. Scutellariae radix and coptidis rhizoma improve glucose and lipid metabolism in T2DM rats via regulation of the metabolic profiling and MAPK/PI3K/Akt signaling pathway. International journal of molecular sciences 2018;19(11):3634.

14. Kuai M, Li Y, Sun X, Ma Z, Lin C, Jing Y, Lu Y, Chen Q, Wu X, Kong X, Bian H. A novel formula Sang-Tong-Jian improves glycometabolism and ameliorates insulin resistance by activating PI3K/AKT pathway in type 2 diabetic KKAy mice. Biomedicine & Pharmacotherapy 2016;84:1585-94.

15. Joubert ED, de Beer D. Rooibos (Aspalathus linearis) beyond the farm gate: From herbal tea to potential phytopharmaceutical. South African Journal of Botany 2011;77(4):869-86.

16. Muller CJ, Malherbe CJ, Chellan N, Yagasaki K, Miura Y, Joubert E. Potential of rooibos, its major C-glucosyl flavonoids, and Z-2-(β-D-glucopyranosyloxy)-3-phenylpropenoic acid in prevention of metabolic syndrome. Critical Reviews in Food Science and Nutrition 2018;58(2):227-46.

17. Dludla PV, Joubert E, Muller CJ, Louw J, Johnson R. Hyperglycemia-induced oxidative stress and heart disease-cardioprotective effects of rooibos flavonoids and phenylpyruvic acid-2-O-β-D-glucoside. Nutrition & metabolism 2017;14(1):1-8.

18. Abdul NS, Marnewick JL. Rooibos, a supportive role to play during the COVID-19 pandemic?. Journal of Functional Foods 2021;86:104684.

19. Johnson R, de Beer D, Dludla PV, Ferreira D, Muller CJ, Joubert E. Aspalathin from rooibos (Aspalathus linearis): a bioactive C-glucosyl dihydrochalcone with potential to target the metabolic syndrome. Planta medica 2018;84(09/10):568-83.

20. Johnson R, Dludla P, Joubert E, February F, Mazibuko S, Ghoor S, Muller C, Louw J. Aspalathin, a dihydrochalcone C-glucoside, protects H9c2 cardiomyocytes against high glucose induced shifts in substrate preference and apoptosis. Molecular nutrition & food research 2016;60(4):922-34.

21. Mazibuko SE, Joubert E, Johnson R, Louw J, Opoku AR, Muller CJ. Aspalathin improves glucose and lipid metabolism in 3T3-L1 adipocytes exposed to palmitate. Molecular nutrition & food research 2015;59(11):2199-208.

22. Mazibuko-Mbeje SE, Dludla PV, Johnson R, Joubert E, Louw J, Ziqubu K, Tiano L, Silvestri S, Orlando P, Opoku AR, Muller CJ. Aspalathin, a natural product with the potential to reverse hepatic insulin resistance by improving energy metabolism and mitochondrial respiration. PloS one 2019 ;14(5):e0216172.

23. Kawano A, Nakamura H, Hata SI, Minakawa M, Miura Y, Yagasaki K. Hypoglycemic effect of aspalathin, a rooibos tea component from Aspalathus linearis, in type 2 diabetic model db/db mice. Phytomedicine 2009;16(5):437-43.

24. Ku SK, Kwak S, Kim Y, Bae JS. Aspalathin and nothofagin from rooibos (Aspalathus linearis) inhibits high glucose-induced inflammation in vitro and in vivo. Inflammation 2015;38(1):445-55.

25. Smit SE, Johnson R, Van Vuuren MA, Huisamen B. Myocardial glucose clearance by aspalathin treatment in young, mature, and obese insulin-resistant rats. Planta Medica 2018;84(02):75-82.

26. Muller CJ, Joubert E, De Beer D, Sanderson M, Malherbe CJ, Fey SJ, Louw J. Acute assessment of an aspalathin-enriched green rooibos (Aspalathus linearis) extract with hypoglycemic potential. Phytomedicine 2012;20(1):32-9.

27. Mazibuko-Mbeje SE, Dludla PV, Roux C, Johnson R, Ghoor S, Joubert E, Louw J, Opoku AR, Muller CJ. Aspalathin-enriched green rooibos extract reduces hepatic insulin resistance by modulating PI3K/AKT and AMPK pathways. International journal of molecular sciences 2019;20(3):633.

28. Johnson R, Shabalala S, Louw J, Kappo AP, Muller CJ. Aspalathin reverts doxorubicin-induced cardiotoxicity through increased autophagy and decreased expression of p53/mTOR/p62 signaling. Molecules 2017;22(10):1589.

29. Mazibuko SE, Muller CJ, Joubert E, De Beer D, Johnson R, Opoku AR, Louw J. Amelioration of palmitate-induced insulin resistance in C2C12 muscle cells by rooibos (Aspalathus linearis). Phytomedicine 2013;20(10):813-9.

30. Mazibuko-Mbeje SE, Mthembu SX, Dludla PV, Madoroba E, Chellan N, Kappo AP, Muller CJ. Antimycin A-induced mitochondrial dysfunction is consistent with impaired insulin signaling in cultured skeletal muscle cells. Toxicology in Vitro 2021;76:105224.

31. Das M, Sauceda C, Webster NJ. Mitochondrial dysfunction in obesity and reproduction. Endocrinology 2021;162(1):bqaa158.

32. Mthembu SX, Dludla PV, Nyambuya TM, Kappo AP, Madoroba E, Ziqubu K, Nyawo TA, Nkambule BB, Silvestri S, Muller CJ, Mazibuko-Mbeje SE. Experimental models of lipid overload and their relevance in understanding skeletal muscle insulin resistance and pathological changes in mitochondrial oxidative capacity. Biochimie 2021 .

33. Sergi D, Naumovski N, Heilbronn LK, Abeywardena M, O'Callaghan N, Lionetti L, Luscombe-Marsh N. Mitochondrial (Dys) function and insulin resistance: from pathophysiological molecular mechanisms to the impact of diet. Frontiers in physiology 2019;10:532.

34. Dludla PV, Silvestri S, Orlando P, Mazibuko-Mbeje SE, Johnson R, Marcheggiani F, Cirilli I, Muller CJ, Louw J, Chellan N, Obonye N. Palmitate-induced toxicity is associated with impaired mitochondrial respiration and accelerated oxidative stress in cultured cardiomyocytes: The critical role of coenzyme Q9/10. Toxicology in Vitro 2020;68:104948.

35. Mazibuko-Mbeje SE, Ziqubu K, Dludla PV, Tiano L, Silvestri S, Orlando P, Nyawo TA, Louw J, Kappo AP, Muller CJ. Isoorientin ameliorates lipid accumulation by regulating fat browning in palmitate-exposed 3T3-L1 adipocytes. Metabolism Open 2020;6:100037.

36. Mazibuko-Mbeje SE, Mthembu SX, Tshiitamune A, Muvhulawa N, Mthiyane FT, Ziqubu K, Muller CJ, Dludla PV. Orientin Improves Substrate Utilization and the Expression of Major Genes Involved in Insulin Signaling and Energy Regulation in Cultured Insulin-Resistant Liver Cells. Molecules 2021;26(20):6154.

37. M Victor V, Rocha M, Herance R, Hernandez-Mijares A. Oxidative stress and mitochondrial dysfunction in type 2 diabetes. Current pharmaceutical design 2011;17(36):3947-58.

38. Dludla PV, Jack B, Viraragavan A, Pheiffer C, Johnson R, Louw J, Muller CJ. A dose-dependent effect of dimethyl sulfoxide on lipid content, cell viability and oxidative stress in 3T3-L1 adipocytes. Toxicology reports 2018;5:1014-20.

39. Tomita T. Apoptosis of pancreatic β-cells in Type 1 diabetes. Bosnian journal of basic medical sciences 2017;17(3):183.

40. Kilanowska A, Ziółkowska A. Apoptosis in Type 2 Diabetes: Can It Be Prevented? Hippo Pathway Prospects. International Journal of Molecular Sciences 2022;23(2):636.

41. Gunaratnam K, Vidal C, Boadle R, Thekkedam C, Duque G. Mechanisms of palmitate-induced cell death in human osteoblasts. Biology open 2013;2(12):1382-9.

42. Yamasaki M, Hamada K, Fujii K, Nishiyama K, Yamasaki Y, Tari H, Araki K, Arakawa T. Vaccinium ashei leaves extract alleviates insulin resistance via AMPK independent pathway in C2C12 myotube model. Biochemistry and biophysics reports 2018;14:182-7.

43. Chen C, Yang JS, Lu CC, Chiu YJ, Chen HC, Chung MI, Wu YT, Chen FA. Effect of Quercetin on Dexamethasone-Induced C2C12 Skeletal Muscle Cell Injury. Molecules 2020;25(14):3267.

44. Chang WT, Huang SC, Cheng HL, Chen SC, Hsu CL. Rutin and gallic acid regulates mitochondrial functions via the SIRT1 pathway in C2C12 myotubes. Antioxidants 2021;10(2):286.

45. Tsalamandris S, Antonopoulos AS, Oikonomou E, Papamikroulis GA, Vogiatzi G, Papaioannou S, Deftereos S, Tousoulis D. The role of inflammation in diabetes: current concepts and future perspectives. European cardiology review 2019;14(1):50.

46. Bu L, Cao X, Zhang Z, Wu H, Guo R, Ma M. Decreased secretion of tumor necrosis factor-α attenuates macrophages-induced insulin resistance in skeletal muscle. Life Sciences 2020;244:117304.

47. Juhas U, Ryba-Stanisławowska M, Szargiej P, Myśliwska J. Different pathways of macrophage activation and polarization. Advances in Hygiene & Experimental Medicine/Postepy Higieny i Medycyny Doswiadczalnej 2015;69.

48. Mahlangu T, Dludla PV, Nyambuya TM, Mxinwa V, Mazibuko-Mbeje SE, Cirilli I, Marcheggiani F, Tiano L, Louw J, Nkambule BB. A systematic review on the functional role of Th1/Th2 cytokines in type 2 diabetes and related metabolic complications. Cytokine 2020;126:154892.

49. Snijman PW, Joubert E, Ferreira D, Li XC, Ding Y, Green IR, Gelderblom WC. Antioxidant activity of the dihydrochalcones aspalathin and nothofagin and their corresponding flavones in relation to other rooibos (Aspalathus linearis) flavonoids, epigallocatechin gallate, and Trolox. Journal of agricultural and food chemistry 2009;57(15):6678-84.

50. Lee W, Bae JS. Anti-inflammatory effects of aspalathin and nothofagin from rooibos (Aspalathus linearis) in vitro and in vivo. Inflammation 2015;38(4):1502-16.

51. Sanvee GM, Bouitbir J, Krähenbühl S. Insulin prevents and reverts simvastatin-induced toxicity in C2C12 skeletal muscle cells. Scientific reports 2019;9(1):1-0.

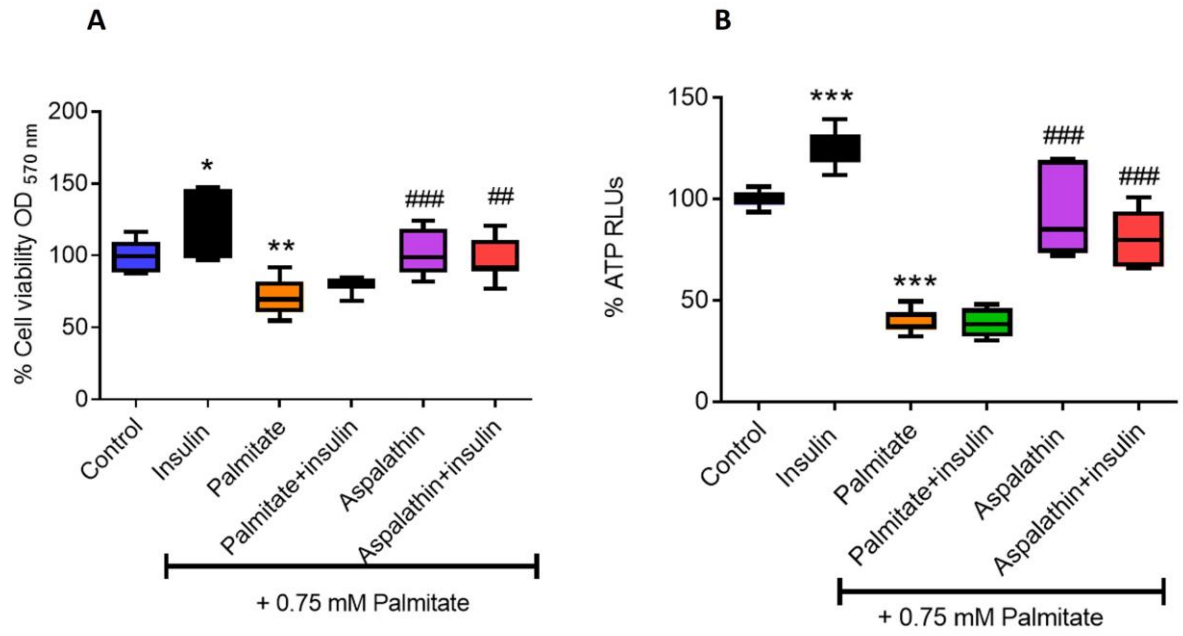


Figure 1. Effect of aspalathin on cell viability (A) and energy production (ATP) (B) in insulin resistant skeletal muscle (C2C12) cells. *Briefly, C2C12 myotubules were treated with or without 0.75 mM palmitate for 16 h, thereafter cultured with aspalathin (10 µM) for 1 h, while insulin (1 µM) was added in the last 15 min as a positive control. As an endpoint measurement, cell viability was assessed by prestoBlue dye and energy production (ATP) by luminescent ATP kit. Results are expressed as the mean of three independent experiments relative to the control at 100% ± SEM. *p < 0.05, **p< 0.01, ***p< 0.001 versus experimental control: ##p < 0.01, ###p < 0.001 versus palmitate control.*

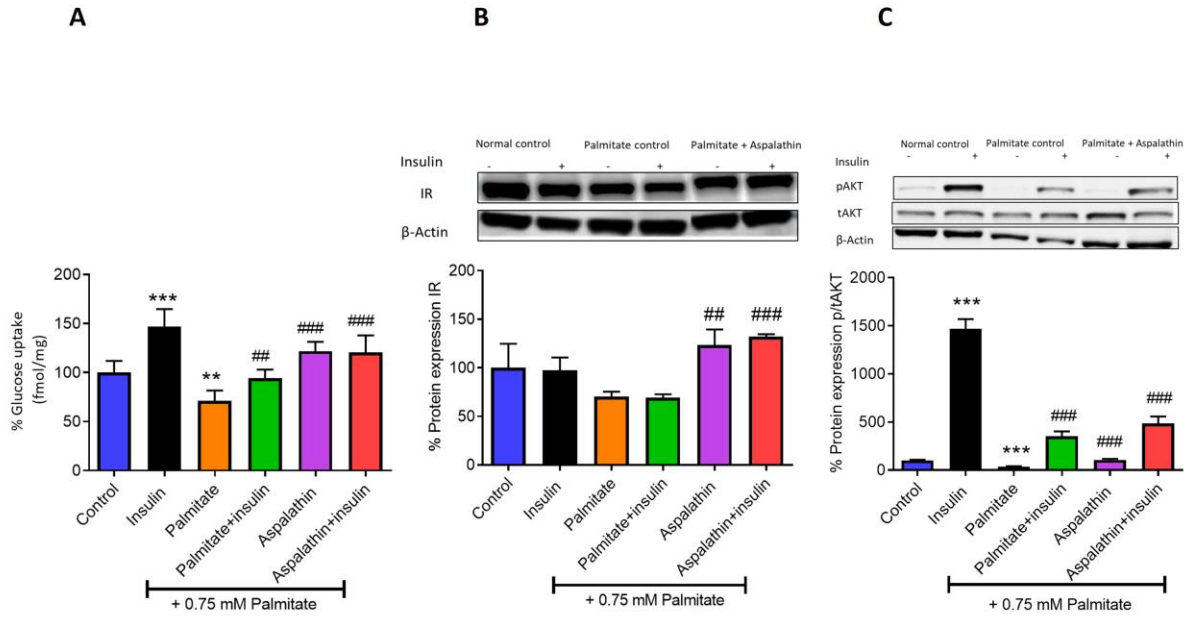


Figure 2. Effect of aspalathin on glucose uptake (A), and protein expression levels of insulin receptor (IR; B), and phosphorylated protein kinase B (p/AKT; C) in insulin resistant skeletal muscle (C2C12) cells. *Briefly, C2C12 myotubules were treated with or without 0.75 mM palmitate for 16 h, thereafter cultured with aspalathin (10 µM) for 1 h, while insulin (1 µM) was added in the last 15 min as a positive control. Treated cells were lysed and subjected to Western blot analysis. Results are expressed as the mean of three independent experiments relative to control set at 100% ± SEM **p< 0.01, ***p< 0.001 versus experimental control: ##p < 0.01, ###p < 0.001 versus palmitate control.*

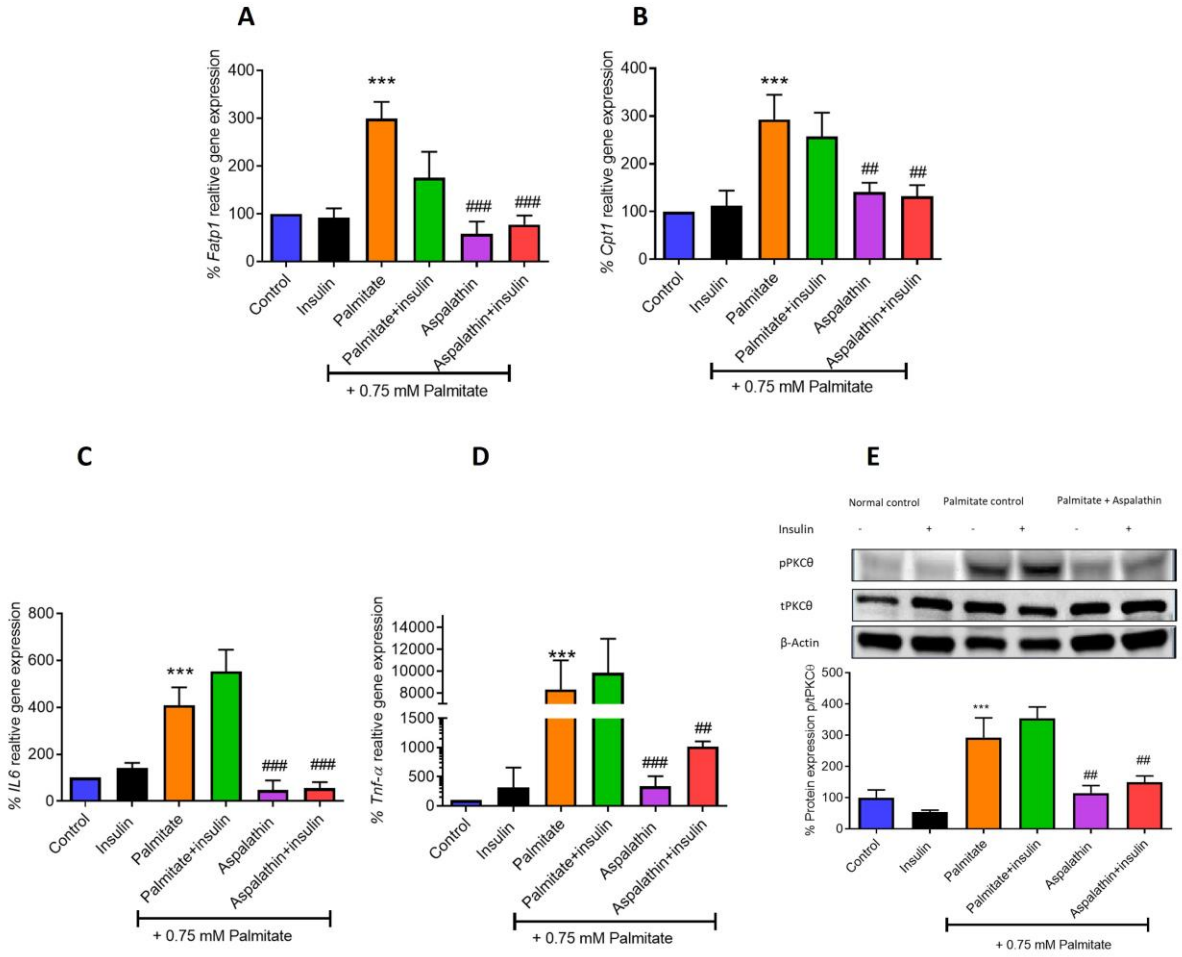


Figure 3. Effect of aspalathin on the regulation of mRNA expression levels of genes involved in beta-oxidation like fatty acid transport protein 1 (Fatp1; A) carnitine palmitoyltransferase 1 (Cpt1; B), and genes involved in inflammation such as intereleukin-6 (Il6; C) and tumor necrosis factor alpha (TNF-α; D), as well as protein expression of protein kinase C theta (PKC-ϑ; E) in insulin resistant skeletal muscle (C2C12) cells*. Briefly, C2C12 myotubules were treated with or without 0.75 mM palmitate for 16 h, thereafter cultured with aspalathin (10 µM) for 1 h, while insulin (1 µM) was added in the last 15 min as a positive control. Treated cells were lysed and quantified by RT-PCR and protein analysis. Results are expressed as the mean of three independent experiments relative to control set at 100% ± SEM. ***p< 0.001 versus experimental control: $^{##}p < 0.01$, $^{###}p < 0.001$ versus palmitate control.*

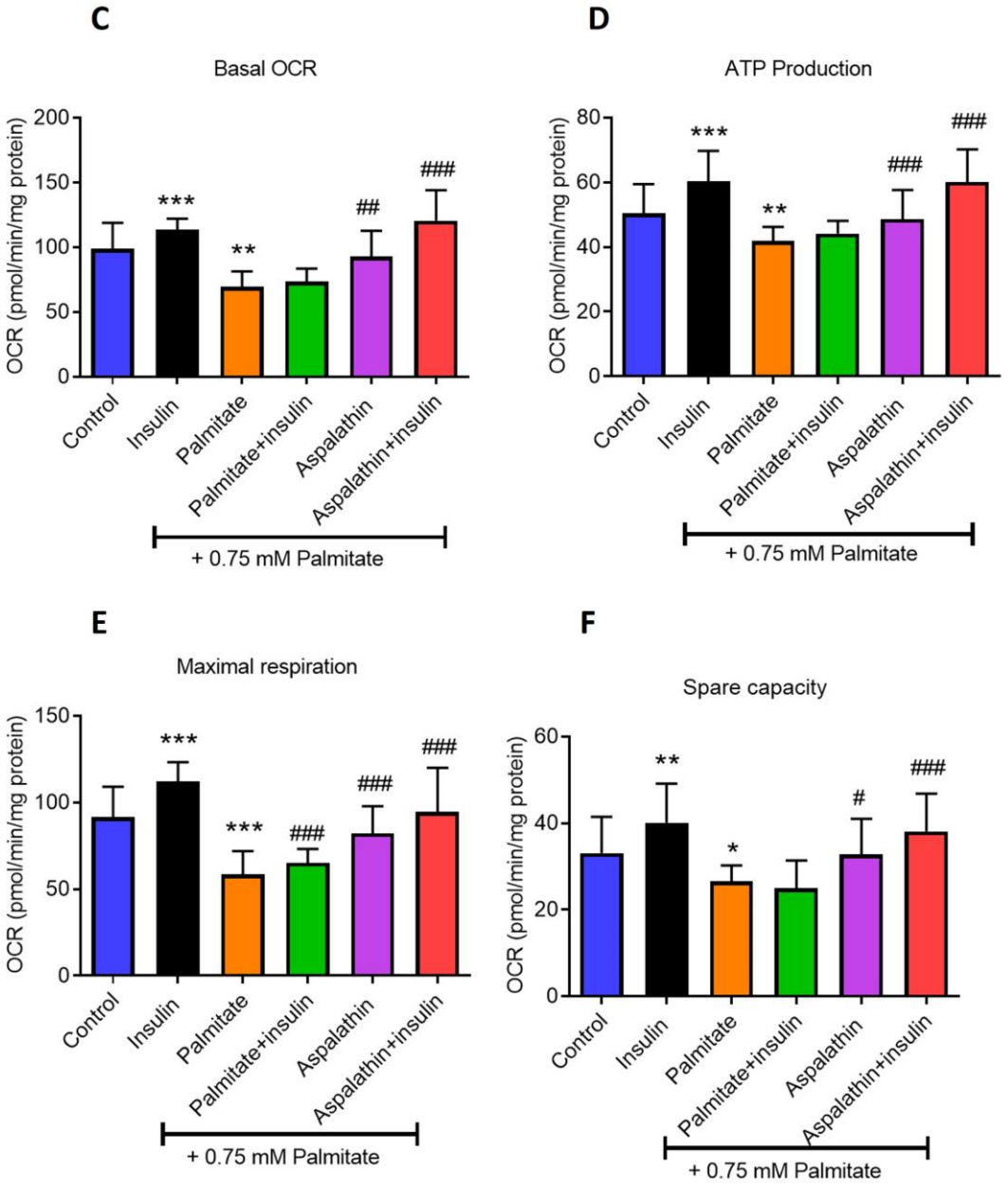


Figure 4. Effect of aspalathin on the regulation of mitochondrial bioenergetics, including basal oxygen consumption rate (OCR; A) and extracellular acidification rates (ECAR; B) of all treatments, before independent analysis of basal OCR (C), adenosine triphosphate (ATP) production (D), maximal respiration (E), and spare respiratory capacity (F) in insulin resistant skeletal muscle (C2C12) cells. *Briefly, C2C12 myotubules were treated with or without 0.75 mM palmitate for 16 h, thereafter cultured with aspalathin (10 µM) for 1 h, while insulin (1 µM) was added in the last 15 min as a positive control. The OCR and ECAR were determined using Seahorse XF-96 Metabolic Flux Analyzer. Groups (A, B, C, D, E and F) show data of changes in OCR, ECAR, in response to the sequential administration (arrows) of oligomycin (2 µg/ mL) port A, Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) (2.5 µM) port B, antimycin and Rotenone (2.5 µM) port C. Results are expressed as mean ± SEM of 3 independent experiments. *p ≤0.05, **p< 0.001 ***p< 0.001 versus experimental control; # p < 0.05, ## p < 0.05### p < 0.001 versus palmitate control.*

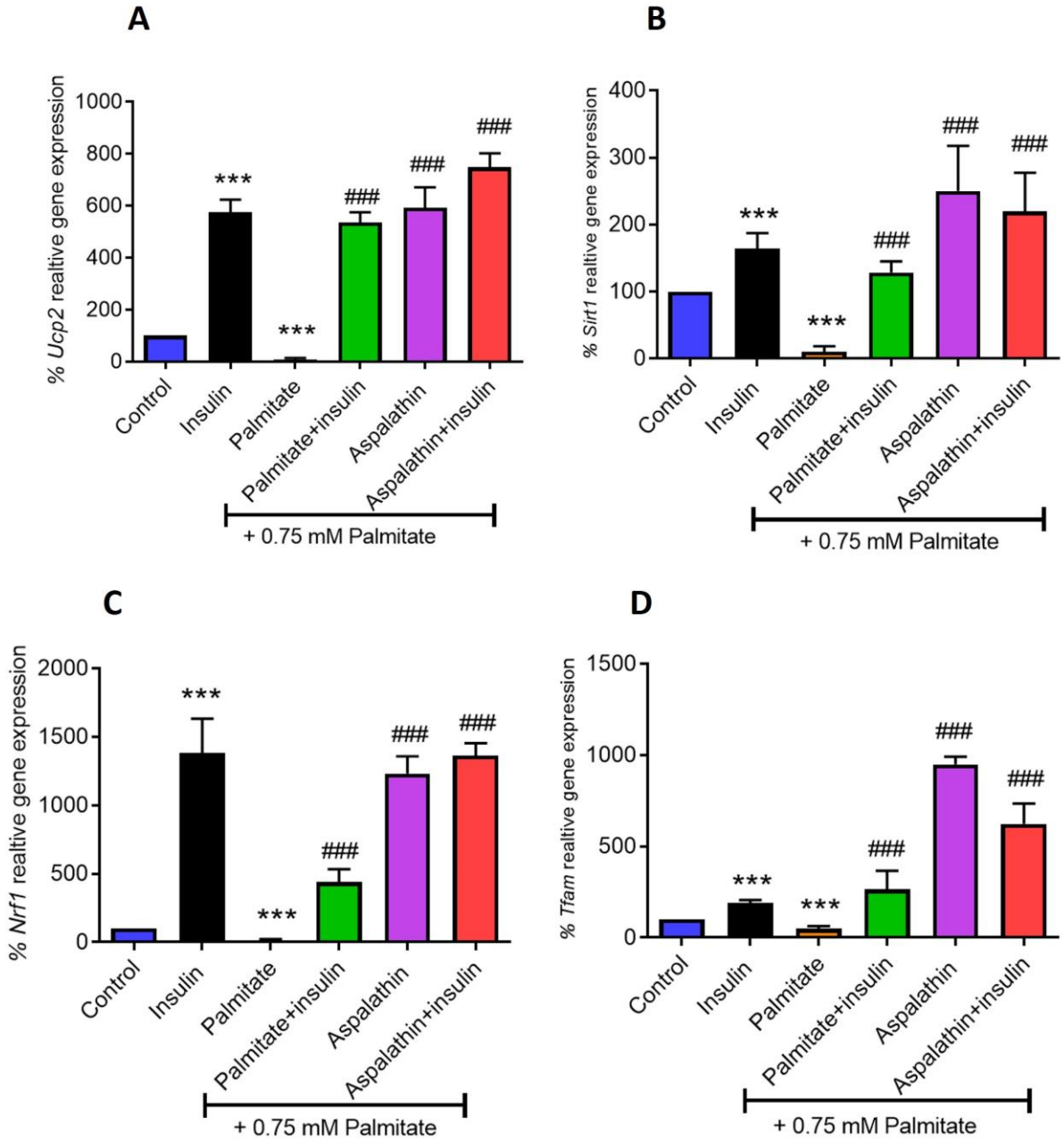
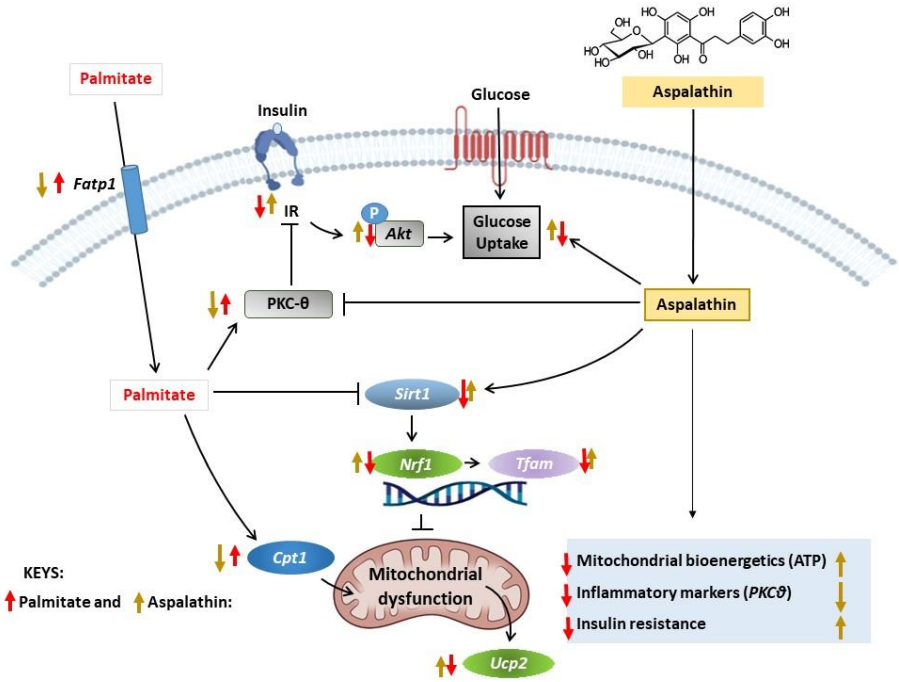


Figure 5. Effect of aspalathin on the regulation of mRNA expression levels of genes involved mitochondrial function, including uncoupling protein 2 (Ucp2; A), silent mating type information regulation 2 homolog (Sirt1; B), nuclear respiratory factor 1 (Nrf1; C) and transcription factor A, mitochondrial (Tfam; D) in insulin resistant skeletal muscle (C2C12) cells. *Briefly, C2C12 myotubules were treated with or without 0.75 mM palmitate for 16 h, thereafter cultured with aspalathin (10 µM) for 1 h, while insulin (1 µM) was added in the last 15 min as a positive control. Treated cells were lysed and quantified by RT-PCR analysis. Results are expressed as the mean of three independent experiments relative to control set at 100% ± SEM. ***p< 0.001 versus experimental control: ###p < 0.001 versus palmitate control.*



583

584 ***Figure 6:*** An overview of therapeutic mechanisms by which aspalathin ameliorates palmitate-induced insulin resistance in
585 skeletal muscle (C2C12) myotubules. Briefly, the major results in this study showed that aspalathin treatment could alleviate
586 palmitate-induced skeletal muscle insulin resistance by enhancing glucose uptake and mitochondrial respiration blocking
587 free fatty acid (FFA)-transport, as well as improving insulin signaling as partly demonstrated through increased expression
588 insulin receptor (IR)/phosphorylation of protein kinase B (AKT) in cultured C2C12 myotubules. This bioactive compound could
589 effectively attenuate inflammation by reducing the expression of markers such as interleukin-6 (*IL-6*), tumor necrosis factor-
590 alpha (*TNF-α*), and protein kinase C-theta (PKC-θ). Moreover, markers of mitochondrial function included, included
591 uncoupling protein 2 (*Ucp2*), NAD-dependent deacetylase sirtuin 1 (*Sirt1*), nuclear respiratory factor 1 (*Nrf1*) and
592 transcription factor A, mitochondrial (*Tfam*).