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Ana Regina Barros Caldas

Overexpression of pyruvate dehydrogenase kinase discloses DCA as a possible cutaneous melanoma therapeutics

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Overexpression of pyruvate dehydrogenase kinase discloses DCA as a possible cutaneous melanoma therapeutics

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Overexpression of pyruvate dehydrogenase kinase supports DCA as a candidate for cutaneous melanoma therapy

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Key words: DCA, melanoma, metabolism, mTOR, PDK.

Abstract

We aimed to verify if there is evidence to consider DCA, which inhibits the pyruvate dehydrogenase kinase (PDK) and reverts the metabolic shift of cancer cells from glycolysis to oxidative phosphorylation, as a promising drug for therapy of cutaneous melanoma (CM) patients. We assessed the expression profile of PDK 1, 2 and 3 in a series of melanoma samples, to verify if melanoma tumours express the DCA targets, if this expression correlates with the activation of important signalling cascades for melanomagenesis and also with the prognosis of melanoma patients. We also established the sensitivity of melanoma cell lines to DCA treatment, by assessing their metabolic alterations, proliferation and survival.

We observed that both PDK 1 and 2 isoforms are overexpressed in CM compared to nevi, being this expression associated with the expression of the mTOR pathway effectors and independent of the BRAF mutational status. Melanoma cell lines treated with DCA showed a shift in metabolism, i.e. a decrease in glucose consumption and lactate production, downregulation of proliferation, an increase of apoptosis and a decrease in mTOR pathway activation.

Our results suggest that PDK expression may play a role in melanoma development and that DCA can be useful for CM therapy, alone or in combination with mTOR inhibitors.

1. Introduction

Cutaneous melanoma (CM) is a very aggressive malignancy, and despite being the least common type of skin cancer, it is responsible for the majority of skin cancer-related deaths. As the incidence of CM is rising, it is at present the most probable invasive cancer to develop before the age of 50 years in male gender [1, 2]. Exposure to UV radiation is considered the main risk factor for melanomagenesis [2]. CM can be classified in different histological subtypes, being the most common the superficial spreading melanoma (SSM), followed by nodular (NM), lentigo maligna (LMM) and acral lentiginous (ALM) melanoma. SSM and NM arise in skin with intermittent sun exposure, whereas LMM occurs in chronic sun-damaged skin and ALM is restricted to skin with no sun exposure. This histological classification has no prognostic value [3, 4]. Staging of CM takes into account tumour thickness, ulceration, mitotic rate, node involvement and the presence of metastasis [5]. Fortunately, most cases of CM are diagnosed in an early stage, with a 5-year survival rate reaching 98% [1]. Nevertheless, for patients with metastatic melanoma the median survival is only 8-9 months [5].

CM is a very heterogeneous tumour and many cell signalling pathways are deregulated in melanomagenesis [6, 7]. The MAPK pathway is constitutively activated in the majority of CMs. NRAS mutations were reported in 10-20% of CMs, being $NRAS^{Q61K/R}$ the most common. BRAF mutations, and in particular the $BRAF^{V600E}$ mutation, were identified in 40-60% of cases. Our group observed an association between the presence of BRAF mutations and the recently described TERT promoter mutations in CM [8]. $BRAF^{V600E}$, and to a lesser extent, $NRAS^{Q61K/R}$ mutations were also reported in almost 80% of nevi (benign melanocytic lesions that are considered as precursors of CM in 25% of the cases), indicating that the activation of the MAPK pathway may be necessary but not sufficient for melanoma development [7, 9, 10]. In fact, $BRAF^{V600E}$

has been related to oncogene-induced senescence and therefore may lead, in many nevi, to a growth-arrest condition [11]. Activation of the PI3K-AKT-mTOR pathway may overcome this senescence phenotype and boost tumour progression [12]. Our group has confirmed the importance of the PI3K-AKT-mTOR pathway in CM aggressiveness by associating its activation with the presence of BRAF mutations and worse prognostic features. Indeed, overexpression of mTOR pathway effectors associated with higher mitotic rate, higher Clark level, increased tumour thickness and presence of skin ulceration [13].

Before 2011, treatment options for patients with advanced CM relied mainly on conventional chemotherapy, which had low response rates and minor effects in patients' overall survival (OS). Ever since, five new drugs were approved for stage IV CM patients [14]. Ipilimumab, an anti-CTLA4 antibody, revealed marked improvements in OS of the patients, but its adverse effects turned it not suitable for all patients [15]. Vemurafenib and dabrafenib, selective inhibitors of $BRAF^{V600E}$, and trametinib, a MEK inhibitor, all target the MAPK pathway and were reported to achieve response rates greater than 50%, even better than those for ipilimumab. However, their improvements in OS do not exceed 7-8 months due to the development of resistance [16-18]. Recently, another immune modulator, pembrolizumab, an antibody against the programmed death receptor-1 (PD-1), was approved for the treatment of patients with unresectable or metastatic melanoma and disease progression following ipilimumab or, if $BRAF^{V600}$ mutation positive, a BRAF inhibitor [19]. Clinical trials with drugs targeting the PI3K-AKT-mTOR pathway are underway [4, 14], yet new therapeutic approaches are warranted.

Cancer cell metabolism differs from that of normal cells. In normal cells, depending on O2 availability, glucose can be partially metabolized to lactate through glycolysis in

hypoxic conditions, or it can be fully oxidized to CO_2 in the presence of O_2, through mitochondrial oxidative phosphorylation, a more efficient energetic process. On the other hand, cancer cells metabolize most glucose to lactate, regardless of their O_2 supply, the so called "Warburg effect" [20, 21]. The acquisition of this glycolytic phenotype in cancer cells is not fully understood, still an essential role has been recognised for the transcription factor hypoxia-inducible factor 1α (HIF1-α) [22]. HIF1-α protein is known to be stabilized under hypoxia, but oncogenic pathways, such as the MAPK and the mTOR pathways, also seem to mediate its activation in cancer (Fig. 1) [22, 23]. Strong evidence indicates that CM acquires this glycolytic phenotype, which can be confirmed by FDG-PET scanning of patients [24-27]. In fact, HIF1-α appears to be overexpressed in CM, because the skin is a mild-hypoxic environment and the production of melanin indirectly stimulates HIF1-α expression by ROS production [28-30]. Yet, cytoplasmic glycolysis must be uncoupled from the mitochondrial oxidative phosphorylation, so that most pyruvate can be converted to lactate. This last process is driven by pyruvate dehydrogenase kinase (PDK), an enzyme upregulated by HIF1-α (Fig. 1) [31, 32].

PDK is an element of the mitochondrial pyruvate dehydrogenase complex (PDC). PDC comprises the pyruvate dehydrogenase (PDH) enzyme and its regulatory proteins: PDK that by phosphorylating PDH acts as an inhibitor, and pyruvate dehydrogenase phosphatase that activates PDH by dephosphorylation [33]. Dephosphorylated PDH catalyses the oxidative decarboxylation of pyruvate into acetyl-CoA, CO_2 and NADH (H^+), thereby linking the glycolytic pathway to the oxidative pathway of tricarboxylic acid cycle (Fig. 1) [33]. There are four isoforms of PDK (1, 2, 3, 4) which differ in their intrinsic activity, tissue distribution and sensitivity to their selective inhibitor drug, dichloroacetate (DCA) [33-35]. Although PDK1 is the only one able to bind to all PDH

phosphorylation sites, PDK3 appears to be the most active isoform [33]. PDK2 is the most sensitive to inhibition by DCA and is ubiquitously expressed in different human tissues, while the others are more tissue specific [33, 34]. PDK4 is essentially related to physiological metabolic flexibility and it is the only isoform not upregulated by HIF1-α [32, 36]. PDK isoforms expression has been evaluated in various types of cancer, namely head and neck squamous cell carcinoma (HNSCC) [37], colon cancer [38], renal cell carcinoma [39] and gastric cancer [40].

DCA has been used in the treatment of conditions associated with lactic acidosis in the setting of mitochondrial dysfunction, such as congenital mitochondrial diseases [35]. In non-small-cell lung, glioblastoma and breast cancer cells, Bonnet *et al.* reported that DCA induces apoptosis and decreases cell growth, by promoting glucose oxidation, mitochondrial membrane depolarization and ROS production [41]. These effects have been replicated in different cancer models and seem to be selective in cancer cells [35, 41, 42]. Moreover, DCA is also able to suppress angiogenesis through indirect HIF1-α inhibition [43]. All these data combined with the well-known safety profile in humans, turn DCA into a promising drug for cancer therapy. A clinical trial to evaluate the effect of DCA in glioblastoma patients was already performed, reporting increased apoptosis and decreased angiogenesis, with promising results in four of five patients [44].

In the present study, PDK 1-3 isoforms expression in CM was assessed and its association with the expression of major signalling pathway cascades for melanomagenesis and with the prognosis of melanoma patients was evaluated. Also, *in vitro* studies were performed to assess the effect of DCA treatment in metabolism, proliferation and survival of melanoma cell lines with different genetic profiles.

2. Materials and Methods

2.1 Sample selection, clinical-pathological and prognostic parameters

Formalin-fixed, paraffin-embedded tissues from 120 CM cases and from 22 melanocytic nevi (12 compound nevi and 10 Spitz nevi) were retrieved from the Service of Anatomic Pathology of the Hospital S. João, Porto, and of Hospital S. Marcos, Braga. Clinical-pathological (Table 1) and follow-up data were obtained from the patients' records and the Oncology Registries of Hospital S. João and of Hospital S. Marcos, and from RORENO (Oncology Registry of North Region). All cases were revised and staged according to the 7th edition of AJCC [5]. Follow-up data includes the recurrences and metastases (DFS) (n=108) and the number of deaths due to melanoma (disease-specific mortality; OS) (n=118). The mean follow-up time of the patients for DFS was 51 months (SE±3.59, range 1–195) and for OS was 55 months (SE±3.48, range 1–207). This work was approved by the Local Ethical Committee and was in accordance to the National Ethical rules.

2.2 Immunohistochemical analysis

Staining for the proteins analysed was performed on 3-µm paraffin sections of representative tumour areas, and mounted on poly-L-lysine-coated slides, with the Envision G/2 System/AP (K5355; Dako, Denmark). Sections were deparaffinised and rehydrated, followed by a microwave antigen retrieval procedure with 10 mM sodium citrate buffer pH 6.0 with 1 mM EDTA pH 9.0 (PDK2), or EDTA buffer pH 9.0 (PDK1 and PDK3). The sections were incubated overnight at 4°C in a humidified chamber with the primary antibodies PDK1 (polyclonal, rabbit, 1:50), PDK2 (polyclonal, rabbit, 1:150), PDK3 (monoclonal, mouse, 1:500), all from Sigma-Aldrich Co. (St. Louis, Missouri, USA). The detection was obtained with the alkaline phosphatase anti-

phosphatase method, and the colour was developed with permanent red chromogen. The slides were counterstained with haematoxylin, and then mounted using a water-miscible mounting medium. The alkaline phosphatase anti-phosphatase method was performed to avoid melanin pigmentation interference with immunohistochemistry analysis. Human stomach tissue was used as negative (omission of primary antibody) and positive control.

2.3 Immunohistochemical evaluation

Three observers (J.M.L., H.P. and R.C.) evaluated tumour cell immunoreactivity without the knowledge of any clinical data of the cases. Adjacent non-tumour tissue immunoreactivity was used as an internal control. An IHC score was settled for PDK1-3, and results from the multiplication of the intensity score of the staining (negative=0, weak=1, moderate=2 and strong=3) and the extension score of the immunoreactivity of tumour cells (0-5%=0, 6-25%=1, 26-50%=2, 51-75%=3, 76-100%=4). IHC scores were then classified as negative/low (score value $\leq$4) and moderate/high (score value >4). Analysis of the MAPK and the PI3K-AKT-mTOR cascades in part of the series has been previously done [13] and the results were used in this study.

2.4 DNA extraction and mutation analysis

Extraction of DNA from tumours smaller than 5mm was performed after microdissection with PALM MicroLaser Systems (PALM, Germany) and using the Quiamp DNA micro kit (Quiagen, Hilden). In tumours larger than 5mm, DNA extraction was done by manual dissection of 10μm whole sections of paraffin-embedded tissue using the Invisorb spin tissue mini kit (Invitek, Berlin). BRAF exon 15 and NRAS exon 2 fragments were amplified by polymerase chain reaction (PCR) using

previously described primers [45]. Genomic DNA (25-100 ng) was amplified by PCR using the following cycling conditions: 35s at 94°C, 40s at 58°C for BRAF and 57°C for NRAS, and 45s at 72°C for 40 cycles.

All PCR products were purified and directed sequenced on an ABI Prism 3130 xl Automatic sequencer (Perkin-Elmer, Foster City, CA) using the ABI Prism Dye Terminator Cycle sequencing Kit (Perkin-Elmer). The sequencing reaction was performed in the forward direction, and an independent PCR amplification, in the forward and reverse direction, was performed in samples suspected to carrier mutations. Again, BRAF and NRAS mutational analysis in part of the series has been previously done [13] and the results were used in this study.

2.5 Cell lines and culture conditions

Two cell lines were used in this work, A375 skin melanoma cell line, which harbours $BRAF^{V600E}$ and Mewo skin melanoma cell line that harbours $BRAF^{wt}$. Both cell lines were tested for the presence of mycoplasma.

A375 was maintained in RPMI medium (Gibco/BRL – Invitrogen) and Mewo was maintained in DMEM medium (Gibco/BRL – Invitrogen). All media were supplemented with 10% of fetal bovine serum, 100U/mL Penicillin and 100ug/mL Streptomycin. Cell lines were maintained in a humidified atmosphere (5% CO2) at 37ºC.

2.6 Treatment of melanoma cell lines with DCA

Sodium dichloroacetate, purchased from Sigma-Aldrich (St. Louis, MO, EUA), was dissolved in dH_2O and added to the culture medium and used for 24 and 48h treatment.

Melanoma cells incubated with culture medium supplemented with dH2O served as control.

2.7 Cell viability assay

The effects of DCA in melanoma cell lines growth was analysed by PrestoBlue (PB) assay. Cells were seeded in 96-well plates at a density of $5x10^3$ in 200 µl medium. After 24h, the medium was replaced by a medium containing 5, 20, 40, 60 mM of DCA. Cells were incubated for 24 and 48h, washed with PBS (pH 7.4) and assayed for cell growth using PB according to the manufacturer's instructions. During incubation with the cells, PB reagent is modified by the reducing environment of the viable cells and becomes highly fluorescent. Fluorescence was measured using a microplate reader (Synergy HT Multi-Mode Microplate Reader, BioTek Instruments Inc., Winooski, VT, USA) at excitation and emission wavelengths of 560 and 590nm, respectively. The absorbance of the wells containing culture medium and tumour cells was used as control and each experimental condition was evaluated with triplicates and repeated in duplicate. By comparing the measured fluorescence/absorbance of the wells containing DCA with the measurements of the wells containing untreated cells, it was possible to generate dose-response profiles and determine IC_{50} (the concentration that inhibits survival in 50%) values, using GraphPadPrism5.0 (GraphPad Software, Inc., La Jolla, CA).

2.8 Glucose and lactate quantification

For glucose and lactate levels measurements, melanoma cells were plated in 6-well plates at a final density of 2×10^5 cells/well and incubated at 37 °C for 24 h. Cells were then treated with 35mM of DCA. As controls, cells were incubated with the vehicle compound (dH_2O). Following 24 and 48h of treatment, culture medium was collected.

Glucose levels present in the conditioned medium in culture were quantified using Glucose GOD/PAP Kit (Roche Applied Sciences) and subtracted to the initial levels (0 hours). Lactate was quantified in a similar manner, using the LO-POD enzymatic colorimetric assay (Spinreact, Sant Esteve de Bas, Spain).

2.9 Cell cycle and apoptosis analysis

For cell cycle profile and apoptosis analysis, melanoma cells were plated in 6-well plates at a final density of 1×10^5 cells/well and incubated at 37 °C for 24 h. Cells were then treated with DCA at 35mM for 24 and 48h of treatment. As controls, cells were incubated with the vehicle compound (dH2O). For cell cycle analysis, cells were then harvested and fixed overnight in ice-cold 70% ethanol. Afterwards cells were resuspended in PBS with 0.1 mg/mL RNase A and 5 μg/mL propidium iodide, before analysis. For apoptosis measurements, cells were harvested and the levels of apoptosis were analysed by flow cytometry using the Annexin-V FITC Apoptosis Kit (Clontech Laboratories, Inc., Saint-Germain-en-Laye, France) according to the manufacturer's instructions. Flow cytometry analysis of cellular DNA content and phosphatidylserine externalization was performed with a flow cytometer (BD Accuri C6), plotting at least 20000 events *per* sample. The data was analysed using the FlowJo 7.6.5 software (Tree Star, Inc., Ashland, USA).

2.10 Western blot analysis and antibodies

Cells were lysed for 15 min at 4°C using RIPA buffer (1% NP-40 in 150 mM NaCl, 50 mM Tris (pH 7.5), 2 mM EDTA) containing phosphatase and protease inhibitors. Proteins were quantified using a modified Bradford assay (Biorad). Protein samples (50 μg) were separated in 10% SDS/PAGE gels and electroblotted to Hybond ECL

membrane (Amersham Biosciences). We used the following primary antibodies: PDH and pPDH Ser293, from Abcam; PDK1 (Sigma-Aldrich), PDK2 (Sigma-Aldrich), HIF1-α (Transduction Laboratories), and the mTOR pathway effectors phospho-S6 Ser235/236, s6, phospho-4E-BP1 Thr37/46, 4EBP1, all from Cell Signaling Technology. Secondary antibodies were conjugated with peroxidase (Santa Cruz Biotechnology) and visualized by the ECL detection solution. Membranes were re-stained with a goat polyclonal anti-actin (Santa Cruz Biotechnology) antibody for loading protein control. All experiments and quantifications (using Bio-Rad Quantity One 1-D Analysis software (4.6.6 version)) were performed in triplicate.

2.11 Statistical analysis

Statistical analyses were performed using STAT VIEW-J 5.0 (SAS Institute, Inc., Cary, NC). The relationship between the average expression level (score) of the immunohistochemistry markers and clinical-pathological parameters was evaluated by ANOVA. When appropriate, multiple comparison corrections were performed using the post hoc Bonferroni and Tamhane tests. The correlation between the immunoreactivity score of the different markers was assessed using the Fisher's exact test. The data from the cell lines experiments was analysed by the two-tailed unpaired Student's t-test. The Kaplan-Meier method and log-rank test were used to evaluate the melanoma survival data. Univariate and multivariate analyses were performed to determine the prognostic value of covariates regarding OS and DFS using the Cox regression model. OS and DFS were calculated from the time of diagnosis until death due to disease or metastasis, respectively, or censored at the time of the latest follow-up or death unrelated to the disease. A p value <0.05 was considered statistically significant.

3. Results

3.1 Expression of PDKs in CM and nevi

Both in CM and nevi lesions, PDK 1, 2 and 3 isoforms were expressed not only in melanocytes/melanoma cells, but also in keratinocytes and in the cells of sebaceous glands and hair follicles. The expression levels of all PDK isoforms analysed were positively correlated between them, suggesting a concomitant expression of these proteins (Table 2).

3.1.1 PDK1 immunohistochemical expression

Cytoplasmic PDK1 staining was observed in 88% of CM and 76% of nevi (Fig. 2A). Negative/low staining score was observed in 52% and 76% and moderate/high staining score was observed in 48% and 24% of melanomas and nevi, respectively. Therefore, cutaneous melanoma displayed significantly higher mean levels of PDK1 expression than nevi ($p=0.03$) (Fig. 2B). Regarding the histological subtypes of CM, LMM displayed greater PDK1expression levels than NM ($p=0.04$) and ALM ($p=0.04$). No association was found between the expression of PDK1 and type of sun exposure. With regard to the prognostic factors, PDK1 was more expressed in tumours $\leq$ 1 mm thick ($p=0.02$). Higher PDK1 expression levels were associated with lower tumour stages ($p=0.04$), but not with overall and disease-free survival of the patients. We also evaluated if the expression of PDK isoforms was related to the MAPK and the PI3K-AKT-mTOR pathways. No association was found between the expression of PDK1 and BRAF/NRAS mutations or with the activation of the MAPK pathway (evaluated by pERK expression). PDK1 expression was positively correlated with the expression of proteins of the PI3K-AKT-mTOR cascade, namely mTOR and 4E-BP1 ($p=0.02$ and $p=0.04$, respectively).

3.1.2 PDK2 immunohistochemical expression

PDK2 displayed both nuclear and cytoplasmic expression in 87% of CM and 83% of nevi (Fig. 2A). Negative/low staining score was observed in 58% and 89% and moderate/high staining score was observed in 42% and 11% of melanomas and nevi, respectively. The mean expression level of PDK2 was higher in CM compared to nevi ($p<0.01$) (Fig. 2B). With regard to the histological subtypes of CM, SSM exhibited higher levels of PDK2 expression than ALM ($p=0.02$). We did not find any association between type of sun exposure and the expression of PDK2. Lower tumour stages showed significantly higher levels of PDK2 ($p=0.02$), although no association was found between the expression of this isoform and the main prognostic factors of CM, neither with overall and disease-free survival of the patients. PDK2 expression was not associated with BRAF/NRAS mutational status or with the activation of the MAPK pathway. A significant positive correlation was found between PDK2 expression and AKT expression from the PI3K-AKT-mTOR cascade ($p=0.03$).

3.1.3 PDK3 immunohistochemical expression

PDK3 displayed cytoplasmic expression in all CM and nevi. Negative/low staining score was observed in 28% and 22% and moderate/high staining score was observed in 72% and 78% of CM and nevi, respectively. The level of expression of PDK3 isoform was similar between CM and nevi (Fig. 2B). There were no significant differences when comparing the mean level of PDK3 expression between CM subtypes and the type of sun exposure. Tumours with thickness ≤ 1 mm and those with lower stages displayed higher levels of PDK3 ($p=0.01$ and $p<0.01$, respectively). Ulceration, mitotic rate and overall and disease-free survival of the patients were not significantly associated with PDK3 expression. No association was found between the expression of PDK3 and BRAF/NRAS mutations nor the activation of the MAPK cascade. Regarding the PI3K-

AKT-mTOR pathway, PDK3 expression was positively correlated with total AKT, mTOR and 4E-BP1 expression ($p<0.01$, $p<0.01$ and $p=0.04$, respectively). Due to the general expression of PDK3 in every CM and nevi, the subsequent *in vitro* analysis did not include this PDK isoform.

3.2 The effect of DCA treatment in melanoma cell lines viability

A375 and Mewo melanoma cell lines were exposed to increasing concentrations of DCA to establish the effect on cell viability, using the PrestoBlue assay. DCA reduced the viability of both cell lines in a dose-dependent manner after 24 and 48h treatment, although A375 was slightly more sensitive to DCA treatment, with a higher growth inhibition when compared to Mewo cell line (Fig. 3). The effect of DCA on A375 cell viability was observed after treatment with 5mM and in Mewo cell line was more intense after 20mM treatment, with inhibition values ranging between 3 and 87% in A375 and 5 to 65% in Mewo. The IC_{50} values were estimated as 33±5.5mM for A375 and 39.9±2.2mM for Mewo cell line, after 48h of DCA treatment (Fig. 3).

3.3 The effect of DCA treatment in glucose consumption and lactate production levels of melanoma cell lines

To evaluate the effect of DCA in the metabolism of melanoma cell lines, glucose and lactate levels were quantified in the culture medium after 24h and 48h treatment with 35mM of DCA, a concentration near the IC_{50} for both cell lines. The consumption of glucose and the production of lactate decreased in both melanoma cell lines after treatment with DCA (Fig. 4). A375 and Mewo cell lines displayed a significant decrease in the glucose consumption level both after 24h and 48h of DCA treatment ($p=0.01$ and $p<0.01$, respectively). The lactate production level was also significantly decreased after

24h and 48h in A375 ($p=0.01$ and $p<0.01$, respectively) and in Mewo ($p<0.01$) cell lines.

3.4 The effect of DCA treatment in PDK and mTOR pathway effectors, and in HIF1-α expression in melanoma cell lines

The efficacy of DCA treatment in inhibiting the PDK activity was evaluated by analysing the expression of the phosphorylated downstream effector PDH, by western blot. At 24h after DCA treatment, significantly inhibition of the phosphorylation of PDH in both cell lines was observed ($p<0.01$) (Fig. 5). PDK1 and PDK2 expression was not altered by DCA treatment (Fig. 5), as expected. The expression of the two downstream effectors and readouts of mTOR pathway activation, S6 and 4EBP1 was also evaluated. Although DCA treatment did not alter S6 and 4EBP1 expression, the expression level of the phosphorylate forms of both proteins was decreased in A375 ($p=0.03$ and not significant, respectively) and in Mewo ($p<0.01$ and $p=0.01$, respectively) (Fig. 5). As DCA may affect HIF1-α levels, the expression of this protein was also evaluated, and no change was observed after DCA treatment (Fig. 5). Similar results were obtained at 48h after DCA treatment, which significantly inhibits phosphorylation of PDH in both cell lines ($p<0.01$). The expression level of the phosphorylated forms of S6 and 4EBP1 was significantly decreased in A375 ($p=0.01$ and $p=0.02$, respectively) and in Mewo ($p=0.01$ and not significant, respectively). The expression of PDK1, PDK2 and HIF1-α was not altered (data not shown).

3.5 The effect of DCA treatment in cell cycle and apoptosis of melanoma cell lines

To clarify the mechanism of action of DCA, cell cycle analysis and apoptosis measurements were performed in both cell lines, after 24 and 48h of treatment with

35mM of DCA. The cell cycle was analysed with propidium iodide. After 48h of DCA treatment, A375 cell line displayed a significant increase in the percentage of cells in the G0/G1 phase of the cell cycle (from 65.0±4.9% observed in non-treated cells to 85.2±10.2% in DCA treated cells; p=0.04), a significant decrease in G2/M (from 11.3±1.3% observed in non-treated cells to 5.2±2.2% in DCA treated cells; p=0.01) and a decrease, not reaching the threshold of statistical significance, in S phase (from 19.7±1.8% observed in non-treated cells to 9.1±6.7% in DCA treated cells; p=0.06) (Fig. 6). The same tendency was observed in Mewo cell line at 48h after DCA treatment (increase in the percentage of cells in Go/G1, and decrease in S and G2/M) although not reaching statistical significance (Fig. 6). Flow cytometry analysis was carried out following Annexin V/Propidium iodide staining. A significant increase in the number of apoptotic cells in A375 cell line after 48h of DCA treatment was observed (3.0±0.5% in non-treated cells to 16.9±4.6% in DCA treated cells; $p<0.01$) (Fig. 7). Non-significant increases in the percentage of apoptotic cells in A375 cell line after 24h and in Mewo cell line after 24h and 48h treatment with DCA were observed.

4. Discussion

In this work we established, for the first time, that cutaneous melanoma overexpresses PDK1 and PDK2 proteins. PDKs are key proteins, regulated by HIF1-α, driving malignant cells into the aerobic glycolysis phenotype, a hallmark of cancer [20, 22, 31]. The Warburg effect is considered a resistance factor to conventional chemotherapies [21]. The overexpression of PDK found in CM, lead us to evaluate the effect of DCA treatment in melanomas cell lines. We observed that DCA treatment decreases glucose consumption and lactate production in melanoma cell lines, consistent with the effect of

DCA in the shifting from aerobic glycolysis to oxidative phosphorylation previously reported in cancer cells [46]. This shift in the glucose metabolism probably results from the observed significant decrease in the expression of the phosphorylated form of PDH, which also confirms the efficacy of DCA treatment. It is relevant that the correct readout of DCA effect is the level of phosphorylated PDH, since by inhibiting PDK activity and not its expression, DCA stimulates PDH dephosphorylation (Fig. 1).

We observed a dose-dependent decrease in cell viability after DCA treatment in both melanoma cell lines. Also, a downregulation of proliferation, through G0/G1 arrest, along with an increase in apoptosis was observed in both cell lines, but only statistically significant in A375 cell line. The fact that the concentration of DCA selected in the present study (35 mM) is slight below the IC_{50} for Mewo cell line may explain the less pronounced effect observed in those cells.

The relationship between DCA and HIF-1α in cancer is not completely understood, as DCA increases tissue oxygen consumption and ROS production, which should lead to HIF-1α up-regulation or stabilization, respectively [47, 48]. Conversely, some authors reported that DCA leads to a decrease in HIF1-α expression [25, 49, 50]. In our work, no alteration was observed in HIF1-α level after DCA treatment of melanoma cell lines, concordant with Shahrzad *et al.* who reported that HIF1-α expression only decreases after DCA treatment in hypoxic conditions [50].

To overcome the mutant BRAF-induced senescence and to progress to more aggressive phenotypes, CM probably activates other signalling cascades, such as the mTOR pathway [12]. This pathway contributes to the Warburg effect by promoting HIF1-α activity, which in turn can increase PDK expression [23, 31]. We have previously reported a complete activation of this pathway in CM [13]. In the present study we observed a concomitant expression of PDK 1, 2 and 3 and of the mTOR pathway

effectors in cutaneous melanomas. The expression of the PDK isoforms in CM correlated positively with the expression of mTOR, as well as with upstream and downstream effectors of this pathway: AKT and 4E-BP1. Moreover, we observed a significant decrease in pS6 and p4EBP1 in A375 and Mewo cell lines after DCA treatment.

Nevi share some genetic alterations with CM, as both present a high frequency of BRAF mutations [10]. Although in a much lower level, PDKs expression was also observed in nevi. We evaluated whether the BRAF mutational status and the downstream activation of ERK could be associated with a higher expression of PDK. We did not find any association in nevi nor in CM. A375 cell line harbour the $BRAF^{V600E}$ mutation and Mewo cell line is wild type for this gene and the response of both cells lines to DCA treatment was similar, despite with variable intensity. In fact, it was already reported that the BRAF mutational status does not alter the sensibility to DCA treatment [51]. These results contrast with our previous findings using mTOR pathways inhibitors in several melanoma-derived cell lines, where we found higher sensitivity to RAD001 treatment in the CM cell lines harbouring a $BRAF^{V600E}$ mutation [52].

PDK isoforms were expressed in the cytoplasm of the melanoma cells, thus supporting the well-known mitochondrial function of such isoforms [33]. Regarding PDK2, a nuclear expression was observed in the majority of the CM cases. This nuclear location has also been reported for PDK1 in HNSCC [37]. As far as we know, it remains to be clarified the role of PDK in the nucleus but, recently, a nuclear function was advanced for PDH, the PDK downstream effector. Sutendra *et al*. reported that PDH is involved in the nuclear formation of acetyl-CoA [53]. In response to growth factor stimulation, PDH and its phosphatase translocate from the mitochondria, where it is inhibited by

PDK, to the nucleus, but a nuclear location for PDK was not identified [53]. Yet, there is evidence that some glycolytic enzymes are engaged in other cellular functions besides the glycolysis, namely in transcriptional regulation [54]. Additional studies are necessary to clarify whether PDK plays also a nuclear function.

In renal cell carcinoma, PDK1 expression was reported to decrease along tumour progression [39]. Concordantly, in our work, PDK was found to be more expressed in lower tumour stages, without influencing the overall and the disease-free survival of the melanoma patients. At variance, PDK overexpression was identified as a marker of tumour progression, poor prognosis and recurrence, in HNSCC and gastric cancer [37, 40, 55]. Although our aim was not to explore the prognostic role of PDK in CM, our results suggest that PDKs in CM may play a more active role in melanoma development than in melanoma progression. We are aware that our series is mainly composed by primary CM, and that further studies in advanced (metastatic) melanoma will be necessary in order to clarify this issue.

Our aim in the present study was to explore biomarkers for new therapeutic approaches in CM patients. Our results suggest a potential usefulness of DCA in the management of CM patients, in line with Abildgaard *et al.* who reported a decrease in ATP levels and melanoma growth using DCA treatment [51]. These authors also reported a synergistic combination between DCA and a BRAF inhibitor, vemurafenib [51]. Furthermore, our results show a concomitant expression of PDK and mTOR pathway effectors in CM and downregulation of mTOR pathway activity by DCA treatment. Considering the results of Hong *et al*. who reported a synergistic effect of the combination of DCA with a S6K1 (mTOR pathway effector) inhibitor [56], it is tempting to speculate that a synergistic therapeutic effect may be achieved in CM using the combination of DCA with a direct mTOR inhibitor.

5. Conclusions

We report for the first time the overexpression of PDK1 and 2 in CM in comparison to nevi. The expression of PDKs was found to be associated with the expression of mTOR pathway effectors and not related with the BRAF mutational status. DCA treatment leads to a shift in metabolism, downregulation of proliferation, increased apoptosis and decreased mTOR pathway activation in melanoma cell lines. Taking all these results together, we conclude that PDK expression may play a role in melanoma development and that its inhibition by DCA alone or in combination with direct mTOR inhibitors may benefit CM patients.

Declaration of interest: We are grateful to all of the patients who participated in this study as well as the physicians that provided clinical, pathological and follow-up information. We thank Dr. Madalena Pinto, from CEQUIMED, Faculty of Pharmacy, University of Porto, Portugal that kindly provided us A375 skin melanoma cell line, and Dr. Marc Mareel, from the Department of Radiotherapy and Nuclear Medicine, Ghent University Hospital, Belgium that kindly provided us Mewo skin melanoma cell line. We thank Gabriela Almeida for the helpful technical advices regarding the PB assay. We also thank Prof Manuel Sobrinho Simões for the critical reading of this manuscript. This study was supported by the Portuguese Foundation for Science and Technology through Post-Doc grant to HP (Ref.: SFRH/BPD/85249/2012). Further funding was obtained from the project "Microenvironment, metabolism and cancer" that was partially supported by Programa Operacional Regional do Norte (ON.2 – O Novo Norte) under the Quadro de Referência Estratégico Nacional (QREN) and the Fundo Europeu de Desenvolvimento Regional (FEDER). IPATIMUP integrates the i3S

Research Unit, which is partially supported by FCT, the Portuguese Foundation for Science and Technology. This work is funded by FEDER funds through the Operational Programme for Competitiveness Factors - COMPETE and National Funds through the FCT, under the projects "PEst-C/SAU/LA0003/2013". The authors disclose no potential conflicts of interest.

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Table 1 – Clinical-pathological features of cutaneous melanomas.

Clinical-pathological features	
Number of cases (n)	120
Median age (±SD)	61.5±17.0
Gender [n (%)]	
Female	68 (56.7)
Male	52 (43.3)
Sun exposure	
Absent	27 (22.7)
Intermittent	72 (60.5)
Chronic	20 (16.8)
Histological subtype [n (%)]	
LMM	16 (13.3)
ALM	24 (20.0)
NM	19 (15.8)
SSM	61 (50.8)
Median thickness [mm]	3.7 (0-70)
Epidermal ulceration [n (%)]	
absent	79 (65.8)
present	41 (34.2)
Mitotic rate [n (%)]	
$< 1/mm^2$	45 (37.5)
$\geq 1/mm^2$	75 (62.5)
pT [n (%)]	
≤ pT2	62 (51.7)
> pT2	58 (48.3)

Table 2 – Mean pyruvate dehydrogenase kinases (PDKs) expression and positive p value correlation found between all PDK isoforms in cutaneous melanomas tumours.

Protein	Mean expression level (±SD)	Correlation (p value)
PDK1	4.8±3.5	<0.01 (PDK1–PDK2); <0.01 (PDK1–PDK3)
PDK2	4.6±3.6	<0.01 (PDK2–PDK3)
PDK3	7.0±3.2	

Figure 1 – Schematic representation of pyruvate dehydrogenase kinase (PDK) interactions. PDK can be upregulated by HIF1-α, which in turn can be activated by mTOR and BRAF. PDK phosphorylates and inactivates pyruvate dehydrogenase enzyme (PDH) activity, whereas pyruvate dehydrogenase phosphatase (PDP) catalyses the reverse reaction. Activated PDH converts pyruvate in acetil-CoA and promotes tricarboxylic acid (TCA) cycle. By inhibiting PDK, DCA stimulates PDH and therefore increases the flux of pyruvate into the mitochondria, promoting glucose oxidation over glycolysis.

Figure 2 – A) Representative microphotographs of PDK1 (a, b) and PDK2 (c, d) expression observed in cutaneous melanomas (a, c) and nevi (b, d). APAP×200 (a-d). B) Graphic representation of the mean expression levels of PDK1, 2 and 3 observed in cutaneous melanoma and nevi. The data are presented as mean±SD. * refers to significant ($p<0.05$) difference when comparing the protein expression level observed in cutaneous melanomas with the expression observed in nevi.

Figure 3 – A) Graphic representation of the percentage of viable cells of A375 and Mewo melanoma cell lines treated with various concentrations of DCA for 24 and 48h, determined by Presto Blue assay. The grey line marks the IC_{50} values obtained that were estimated as 53±2.8mM for A375 after 24h of DCA treatment, and 33±5.5mM for A375 and 39.9±2.2mM for Mewo, after 48h of DCA treatment. In Mewo cell line, growth inhibition was observed after 24h of DCA treatment, although it did not reach an IC_{50} value. B, C) Column graphic representation of the percentage of viable cells of A375 (B) and Mewo (C) melanoma cell lines treated with various concentrations of DCA for 24 and 48h. The data are presented as mean±SD. * refers to significant ($p<0.05$)

difference when comparing cells treated with DCA for 24h or 48h compared to non-treated cells.

Figure 4 – Graphic representation of the glucose consumption (A, B) and lactate production (C, D) evaluation in A375 (A, C) and Mewo (B, D) cell lines in the medium of cells non-treated or treated with 35mM of DCA for 24 and 48h. The data are presented as mean±SD. * refers to significant ($p<0.05$) difference when comparing cells treated with 35mM of DCA for 24h or 48h compared to non-treated cells.

Figure 5 – A) Representative western blot analysis of pPDH, PDH, PDK1, PDK2, HIF 1-α, pS6, S6, p4EBP1 and 4EBP1 expression observed in the melanoma cell lines after 35mM of DCA treatment for 24h compared to non-treated cells. B) Graphic representation of the mean fold change of activated protein expression observed. The data are presented as mean±SD. * refers to significant ($p<0.05$) difference when comparing cells treated with 35mM of DCA for 24h compared to non-treated cells.

Figure 6 – Graphic representation of the cell cycle analysis of A375 (A, B) and Mewo (C, D) cells non-treated or treated with 35mM of DCA for 24h (A, C) and 48h (B, D), determined by Flow cytometry. The data are presented as mean±SD. * refers to significant ($p<0.05$) difference when comparing cells treated with 35mM of DCA for 24h or 48h compared to non-treated cells.

Figure 7 – Graphic representation of the percentage of apoptotic A375 (A) and Mewo (B) cells non-treated or treated with 35mM of DCA for 24 and 48h. The data are

presented as mean±SD. * refers to significant ($p<0.05$) difference when comparing cells treated with 35mM of DCA for 24h or 48h compared to non-treated cells.

Figure 1

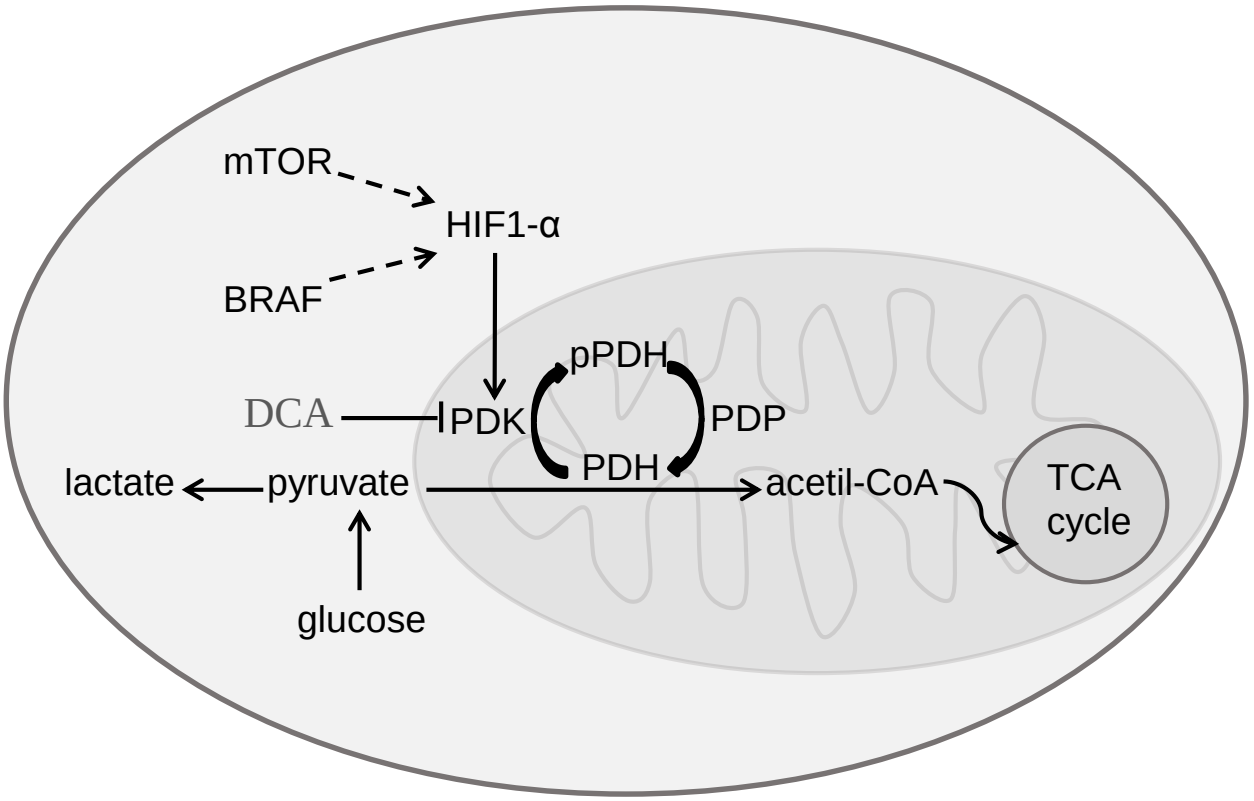
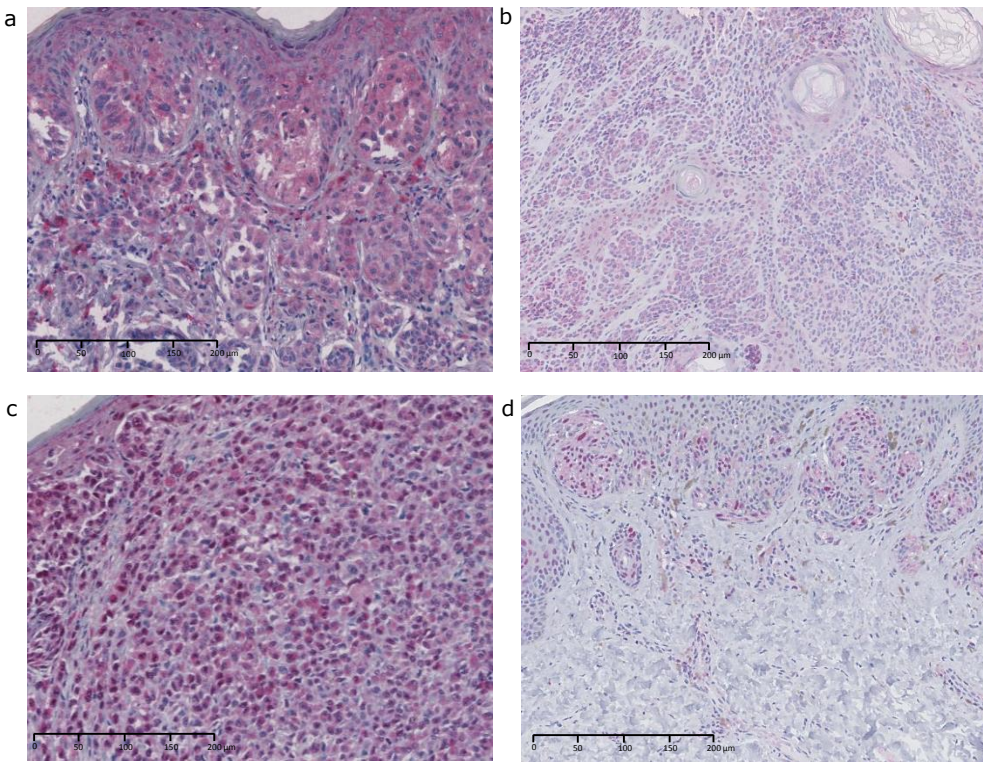

mTOR
HIF1-α
BRAF
pPDH
DCA
PDK
PDP
PDH
lactate
pyruvate
acetil-CoA
TCA
cycle
glucose

Figure 2

A


a
b
c
d

B

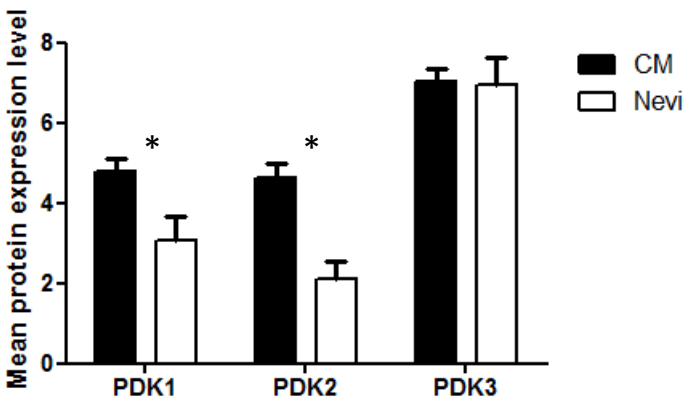
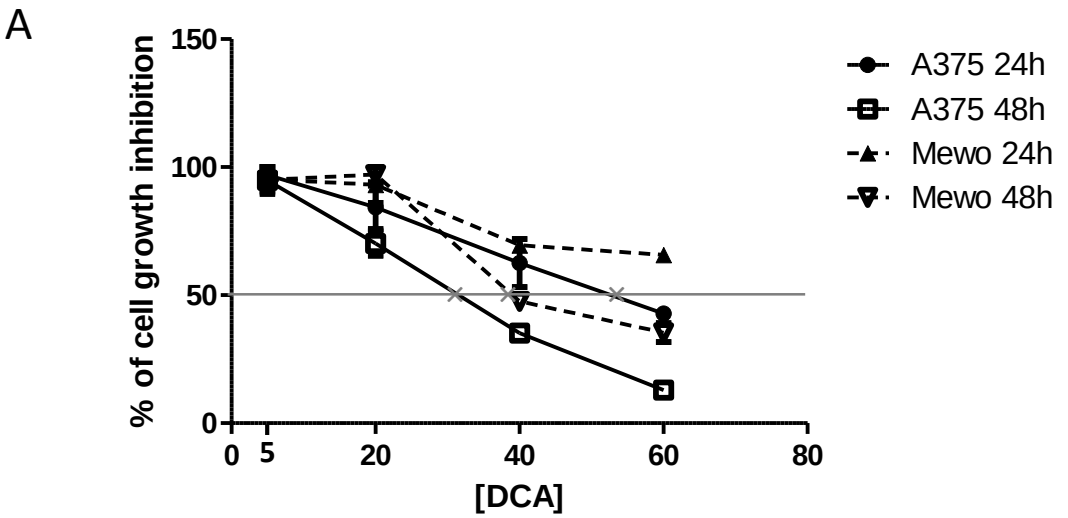
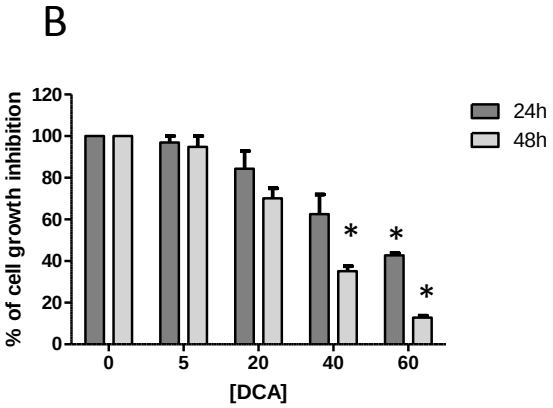

Mean protein expression level
8
6
4
2
0
*
*
PDK1
PDK2
PDK3
CM
Nevi

Figure 3


A
% of cell growth inhibition
150
100
50
0
0 5 20 40 60 80
[DCA]
A375 24h
A375 48h
Mewo 24h
Mewo 48h


B
% of cell growth inhibition
120
100
80
60
40
20
0
0 5 20 40 60
[DCA]
24h
48h
*
*
*

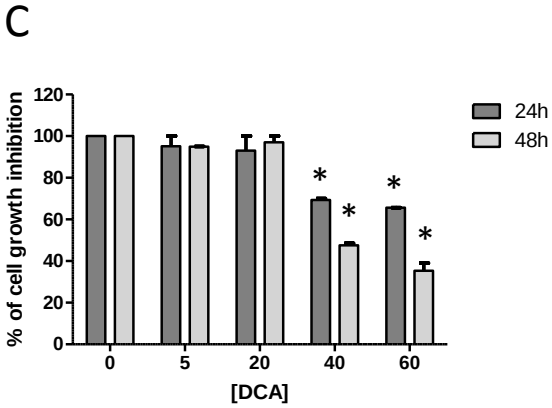

C
% of cell growth inhibition
120
100
80
60
40
20
0
0 5 20 40 60
[DCA]
24h
48h
*
*
*
*

Figure 4

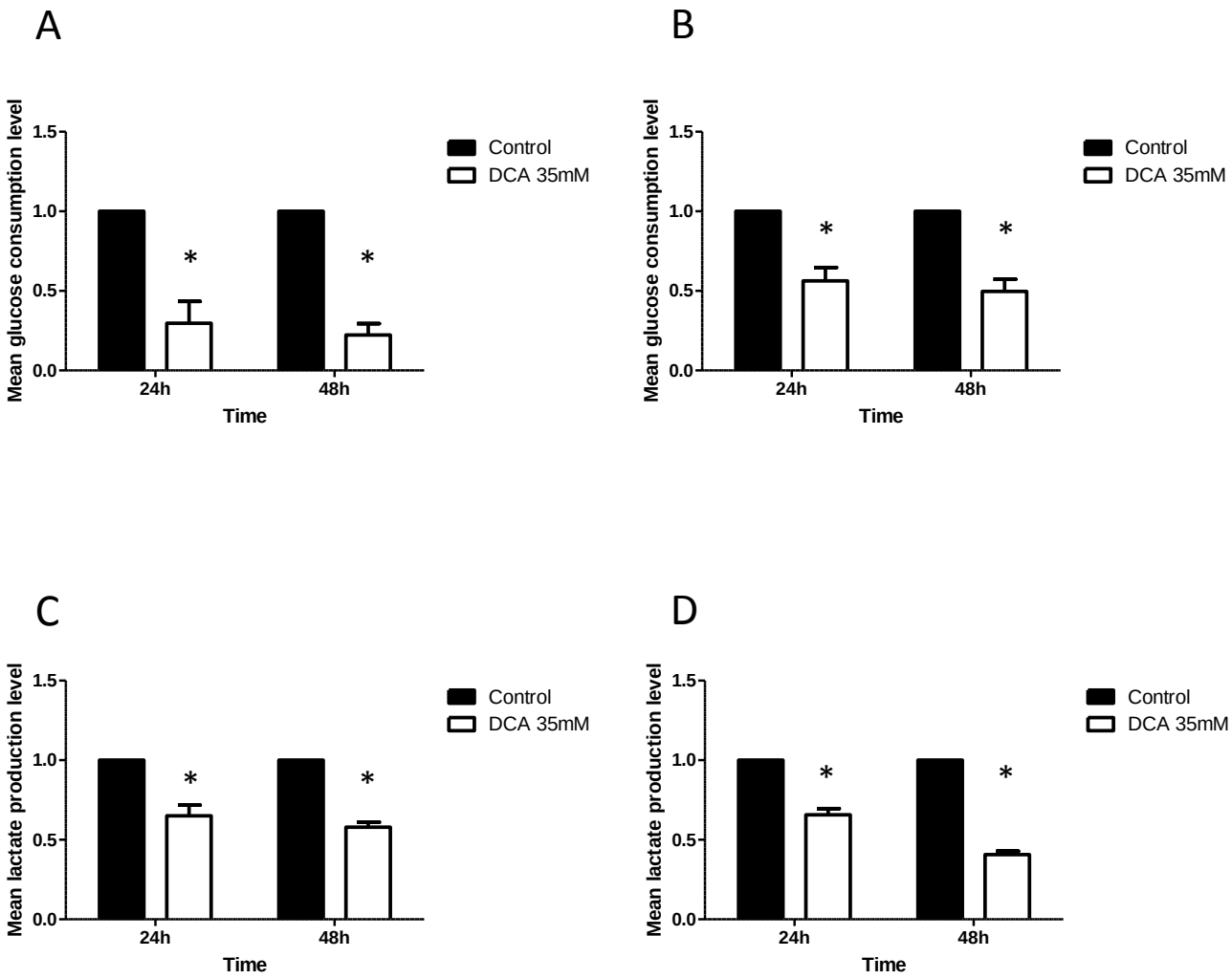
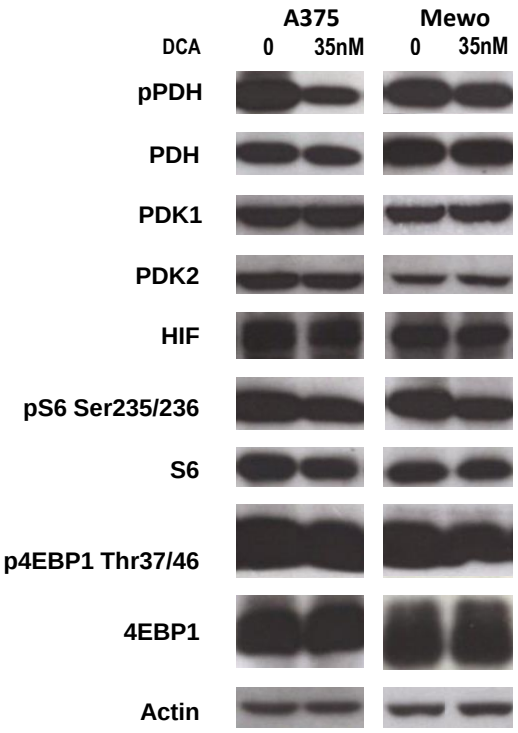

A
Mean glucose consumption level
1.5
1.0
0.5
0.0
*
*
24h
48h
Time
Control
DCA 35mM
B
Mean glucose consumption level
1.5
1.0
0.5
0.0
*
*
24h
48h
Time
Control
DCA 35mM
C
Mean lactate production level
1.5
1.0
0.5
0.0
*
*
24h
48h
Time
Control
DCA 35mM
D
Mean lactate production level
1.5
1.0
0.5
0.0
*
*
24h
48h
Time
Control
DCA 35mM

Figure 5

A


A375
Mewo
DCA
0
35nM
0
35nM
pPDH
PDH
PDK1
PDK2
HIF
pS6 Ser235/236
S6
p4EBP1 Thr37/46
4EBP1
Actin

B

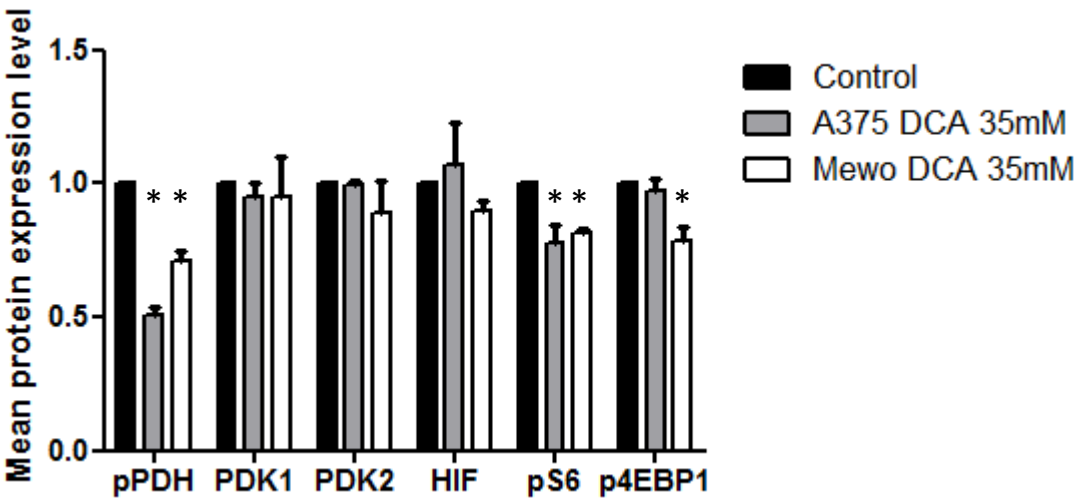

Mean protein expression level
1.5
1.0
0.5
0.0
pPDH
PDK1
PDK2
HIF
pS6
p4EBP1
Control
A375 DCA 35mM
Mewo DCA 35mM

Figure 6

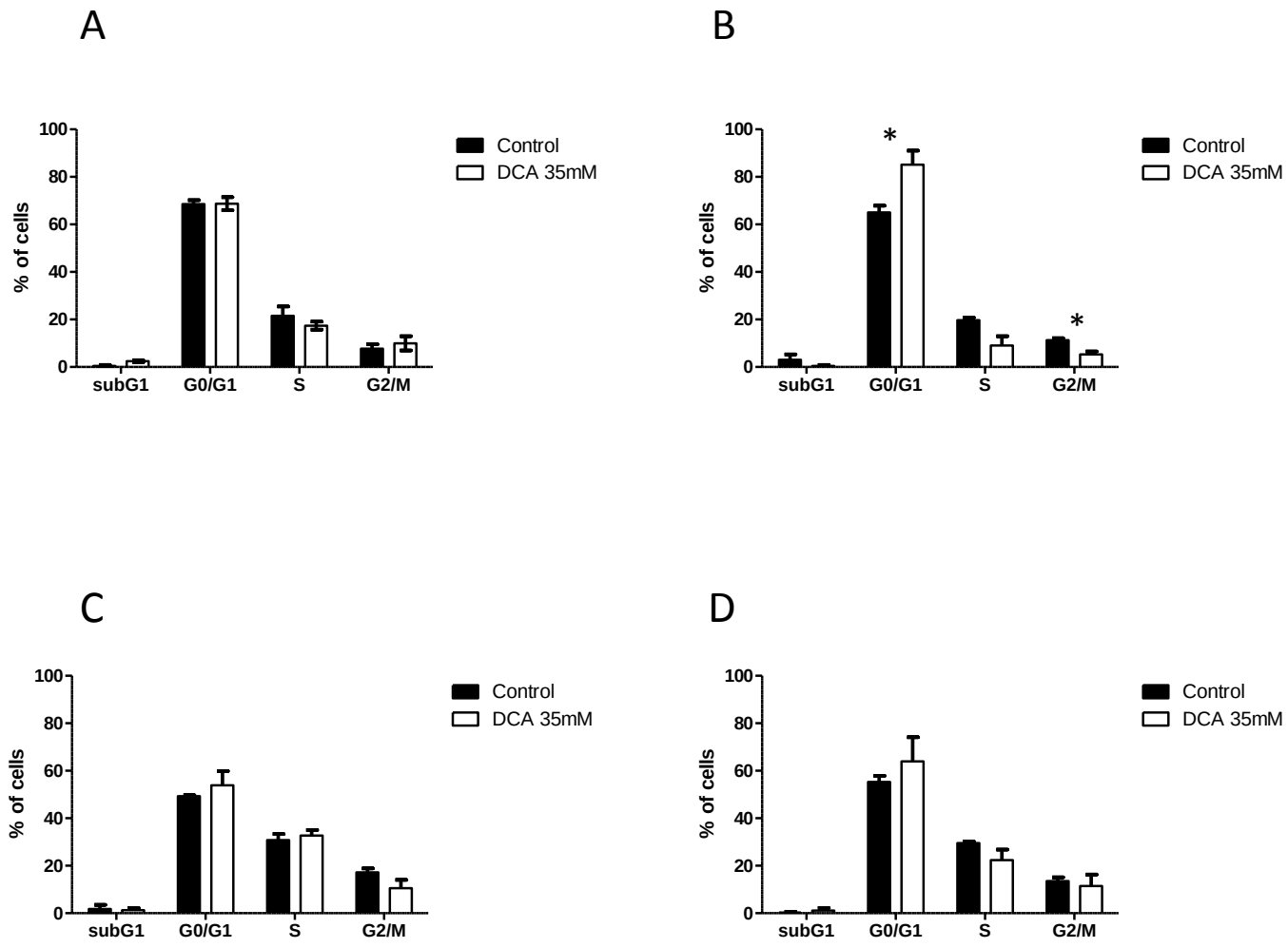
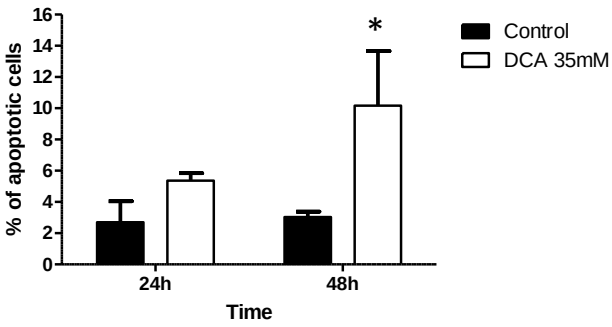

A
B
C
D
% of cells
100
80
60
40
20
0
subG1
G0/G1
S
G2/M
Control
DCA 35mM
*
*

Figure 7

A


16
14
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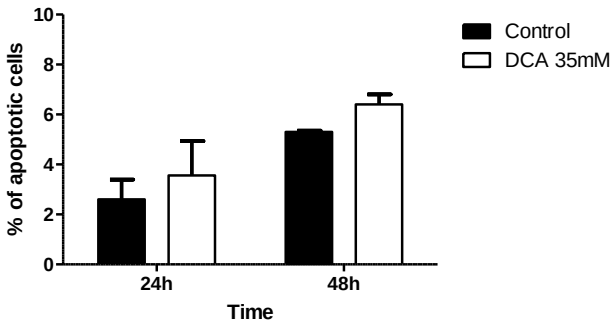
% of apoptotic cells
*
24h
48h
Time
Control
DCA 35mM

B


10
8
6
4
2
0
% of apoptotic cells
24h
48h
Time
Control
DCA 35mM

Agradecimentos

Agradeço à Professora Paula Soares, pela orientação ao longo destes dois anos e por sempre ter mostrado disponibilidade e preocupação em discutir comigo os mais variados aspetos da tese.

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Agradeço aos meus pais e ao resto da minha família, pelo incrível apoio, por me manterem sempre focada no mais importante e por compreenderem as minhas ausências.

Anexos

Expert Opinion on Therapeutic Targets: Guidelines for Original Research

1. Overview

Expert Opinion on Therapeutic Targets (EOTT) is the premier journal dedicated to in-depth reports and reviews of the latest developments in the field of drug target discovery and validation. It is distinguished from other publications by its high quality authorship and expertly drafted articles, which are structured to incorporate the author's own expertise on the subject. The following document details the requirements for 'Original Research' submissions.

1.1 Audience

The audience consists of scientists, managers and decision makers in the pharmaceutical industry, academic researchers working in the field of molecular medicine and others closely involved in R&D.

1.2 Scope

In addition to review papers, technology evaluations and editorials, EOTT is also a medium for the publication of original research on recently identified novel molecular drug targets.

Contributions are welcomed in the form of:

- Target selection and validation studies
- Basic mechanism of action studies for investigative and marketed drugs
- In vivo and in vitro studies, animal models, screening, assays, knockout studies, gene expression/function studies

Supplements are published and actively encouraged. These include both traditional free-standing and in-journal supplements based on symposia proceedings (or sponsored workshops and meetings), and specific areas. Enquiries from potential guest editors, meetings organizers and sponsors are welcomed.

In addition, EOTT has a Correspondence section. We welcome the submission of any correspondence piece or letters regarding any article published in the journal since its launch. The Correspondence section of the journal presents an open arena for objective commentary, debate and lively discussion.

For more information regarding a Supplement or for the submission of a Correspondence piece, please send an email to claire.attwood@informa.com

1.3 Rapid Publication

Publication in EOTT is driven entirely by editorial considerations and independent authoritative peer review. As part of the journal's responsive approach to the publication of clinical evidence, we offer two prioritised modes of rapid publication and a third non-prioritised mode:

• FastTrack: This offers the most highly prioritised service, with a submission to online publication timeline of 5–7 weeks (subject to 1–2 week author revision following initial peer-review and prompt turnaround of proofs). There is a publication support fee for this, based on a charge of £550 / €625 / $850 per published page. This charge supports the ultra-swift processing of material and 20 downloads of the article via access-tokens.

• Rapid Track: This prioritised service offers submission to online publication in 10–12 weeks and is subject to a publication support fee of £260 / €300 / $400 per page; 10 downloads of the article via access-tokens are provided for this service.

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All material should be prepared as detailed below. Please refer to the Submission Checklist at the end of this document and ensure that all criteria are met before submission. This will ensure timely publication of your article.

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II .D.1. – Peer review
II. E.1. – Patients and study participants
II.F – Protection of human subjects in research
IV.A.3 – Conflict of interest notification

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All manuscripts must be accompanied by a covering letter and an electronic version of the Disclosure Form signed by the principal author(s). The covering letter should include all of the following information:

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- A statement that the manuscript has been read and approved by all the authors, and that all the conditions as previously stated by the ICMJE have been met.
- The body providing explicit ethical approval of the work reported should also be stated.
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- The authors' preference for publication with either English (UK) or US spellings.

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4. Manuscript content

4.1 General

Manuscripts submitted should be in English and written for an international, general medicine readership. Where national or regional issues are discussed, the international context should also be considered. When a licensed drug or device is being discussed outside its licensed indication, this must be made clear to the reader.

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4.2 Every article must contain:

- **Title page:** All articles should have a concise, informative title that contains no brand names (no more than 50 words). In addition to the title itself, the title page should also have the name(s) and initials of all the authors and their institutional affiliation(s). The name of the corresponding author and their mailing address should be given in full, including email, telephone and fax numbers. Should any of the material contained in the paper have been previously presented at a meeting, the full name, location and inclusive dates of the meeting should also appear on the title page. Authors are to avoid using Trade names in their titles, and are encouraged to have non-promotional titles for their articles.

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- **Body of the article:** This should include the following sections: introduction; patients and methods; results; discussion; conclusions (see section 5 for organization of content).

- **Acknowledgements:** This must include any declaration of interest by the authors (see also Section 3: Correspondence), including grants, fellowships or any commercial assistance or financial sponsorship received. It should also list any affiliation(s), organization(s) or entity(ies) that are relevant to the work reported. Some or all of this information may be published at the discretion of the Editor. Any contributions to the research, data analysis or assistance in manuscript preparation must also be acknowledged in this section. Finally, if any trial registration information and/or a trial registration number are available for the study, this should also be mentioned in the acknowledgements section.

- **References:** Full references to relevant material cited in the text should be provided (see Reference style in Section 9 below). References should be comprehensive, accurate and up-to-date: wherever possible, please use primary references, and as far as possible, avoid the use of unpublished references (such as 'Data on file' or 'Poster').

5. Organization of content

5.1 General

Keep all formatting to a minimum and avoid assigning 'styles' to headings, extracts or paragraphs. Make sure that the 'normal' style is used throughout the text. Turn off the automatic hyphenation feature. Please use double-line spacing throughout the manuscript. Headings and sub-headings should be used to provide structure to the text with numbers (Arabic numerals) to indicate a hierarchy of the headings/sub-headings (i.e., 1.0, 1.1, 2.0, 2.1, 2.1.1, 2.1.2 and so on).

5.2 Units and symbols

Use SI units where possible. Non-SI units should be accompanied by SI equivalents, for example, LDL-C < 70 mg/dl (1.8 mmol/l). To indicate atom positions in a molecule, use the convention C-1, C-2 and so on.

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Manuscripts may be submitted in either US or UK English and will be published using the version of English used in the manuscript submitted to the Editor. All accepted manuscripts will be copyedited in house upon acceptance, but authors are advised to check their work for English spelling and grammar prior to submission. **The Editorial Office can arrange an English language edit prior to submission if required - please contact the editorial office for details of available services and costs.**

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Abbreviations should be defined on their first appearance both in the Abstract and in the text; commonly used abbreviations need not be defined. Authors are encouraged to submit a list of abbreviations used to the Editorial Office alongside the manuscript. Only underline or italicize words and letters that are required to appear in italics.

5.5 Introduction

This should state the clinical relevance and background to the study, its rationale and purpose.

5.6 Patients (where appropriate) and experimental methods

Authors should be as concise as possible about the experimental methods used. Should this concern human studies, this should contain details of the study population and setting, subject selection (inclusion/exclusion criteria), methods of randomization and blinding, and efficacy and safety measures. The study design and statistical methodology should be described, with justification for the choice of analysis and sample size given; CONSORT guidelines (www.consort-statement.org) should be considered where appropriate. All materials should be identified precisely, with drugs referred to by their generic names (proprietary names, if required, should be given in parentheses along with the company name and country of the manufacturer), and with dose and routes of administration. The ethical approval procedure followed and the name of the ethics committee should be stated. Indicate how adverse events were determined (and by whom) and indicate if/how compliance was measured. (Authors are reminded to ensure that they follow the ICMJE requirements when dealing with privacy and informed consent from patients – see Section II.E.1.of the ICMJE requirements document http://www.icmje.org/#privacy).

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Use should be made of tables and figures to help with the clear presentation of results data. The sample size of each data point should be shown, with p-values and confidence intervals quoted for significant findings. Any data not included in the analysis (including patients withdrawn from the study) should be detailed. Details of data on efficacy and adverse events should be provided in a balanced fashion.

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This will essentially be a discussion of the results and experimental data collected. The section should include implications of the findings and their limitations, with reference to all other relevant studies and the possibilities these suggest for future research. In addition, the discussion affords authors the opportunity to discuss the developments that are likely to be important in the future and the avenues of research likely to become exciting as further studies yield more detailed results.

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This section must summarize the paper, with a concise statement of the clinical implications of the study results. Ensure that extrapolations are reasonable and that conclusions are justified by the data presented. Indicate if the study design can be generalized to a broader patient population.

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Each table (with a brief, comprehensive title/header) should be presented in cellular format (rather than a simple tabbed form) on a separate page, at the end of the text. Tables should be numbered with Arabic numerals (Table 1, Table 2….) and each table should be followed by a footnote where necessary. Define in the footnotes all abbreviations that are used in the table. Be sure that each table is cited in the text (but not within any section headings) and number tables consecutively in the order of their first citation in the text. In case data from another published or unpublished source is being used, please obtain permission and acknowledge the source(s).

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Do include illustrations (figures/diagrams/structures) as appropriate. Please ensure that the following recommendations are adhered to as closely as possible:

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- Please note that we cannot improve resolution beyond that of the file submitted.

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Books:

2. Gottman J. Time Series Analysis. Cambridge: CUP, 1981

Working party reports and similar:

3. Clinical Disputes Forum Working Party. Pre-action protocol for the resolution of clinical disputes. London: Clinical Disputes Forum, 1998

Pre-publication articles assigned DOI numbers:

4. de Lau LM, Koudstaal PJ, Hofman A, Breteler MM. Subjective complaints precede Parkinson disease: the Rotterdam study. Arch Neurol 2006: published online 9 January 2006, doi:10.1001/archneur.63.3.noc50312

Internet articles and website information:

5. Suicidality in adults being treated with antidepressant medications. FDA Public Health Advisory. Washington, DC: FDA/Center for Drug Evaluation and Research, 2005. Available at: www.fda.gov/cder/drug/advisory/SSRI200507.htm [Last accessed 3 January 2006]

Patents:

6. Basf AG. Means and methods for preventing and treating caries. WO2006027265 (2006)

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   b) A statement that the contribution represents original work, has not been previously published or simultaneously submitted for publication, that the manuscript has been read and approved by all authors, and that all the conditions as previously stated by the ICMJE have been met.
   c) A statement of financial or other relationships of a declarable nature.

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4. Four to six keywords are provided.
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