Original Research

(+)-Alpha-Lipoic Acid Regulates Lipid Metabolism Gene Expression and Lipidic Profile in a Cellular Model of Fatty Acid Overload

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Abstract

Background: Nonalcoholic fatty liver disease (NAFLD) is a prevalent condition characterized by hepatic fat accumulation, often progressing to severe liver injury, for which approved treatments are currently lacking. This study explores the potential therapeutic impact of alpha-lipoic acid (ALA), a natural compound crucial in lipid metabolism, on NAFLD using an in vitro model. Methods: HepG2 cells were treated with a palmitic acid:oleic acid (PA:OA) mixture, representing a cellular model of steatosis. Subsequent treatment with ALA at concentrations of 1 µM and 5 µM aimed to evaluate its effects on lipid content and metabolism. Real-time polymerase chain reaction (PCR), BODIPY staining, cytofluorimetric analysis, and lipidomics were used to assess gene expression, lipid droplet accumulation, and fatty acid profiles. Results: Our results showed that ALA significantly reduced lipid droplets in PA:OA-treated HepG2 cells, with a concentration-dependent effect. Analysis of fatty acid profiles demonstrated a decrease in palmitic acid levels with ALA treatment, while oleic acid reduction was observed only at the higher concentration. Moreover, ALA modulated the expression of genes involved in cholesterol biosynthesis and low-density lipoprotein (LDL) metabolism, indicating a potential role in lipid homeostasis. Further insights into molecular mechanisms revealed that ALA modulated peroxisome proliferator activated receptors (PPARs), specifically PPAR-alpha and PPAR-gamma, involved in fatty acid metabolism and insulin sensitivity. Finally, ALA counteracted the overexpression of thermogenic genes induced by exogenous fatty acids, suggesting a regulatory role in energy dissipation pathways. Conclusion: In conclusion, this study highlights ALA as a therapeutic agent in mitigating lipid accumulation and dysregulation in NAFLD.

Keywords: lipoic acid; liver steatosis; fatty acid; lipid metabolism

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is a condition characterized by the accumulation of fat in the liver, which can progress to more severe liver injury [1]. The liver is a central organ in lipid metabolism, responsible for processing dietary fats, synthesizing lipoproteins, and regulating lipid storage and release. Despite numerous clinical trials exploring responses to various medications and supplements, no approved treatment has yet been suggested for managing NAFLD.

Lipoic acid, also known as alpha-lipoic acid (ALA), is a natural compound that plays a crucial role in lipid metabolism and various other biological processes [2]. It has been investigated for its potential to improve NAFLD by promoting the breakdown of triglycerides (a type of lipid) in liver cells [3]. Additionally, lipoic acid exhibits antioxidant properties that can reduce oxidative stress in the liver, a factor in the development and progression of NAFLD [4–6]. The pharmacological effects of ALA are multifaceted, as it influences several aspects of lipid metabolism, including energy production, antioxidant defense, fatty acid oxidation, and insulin sensitivity regulation [7].

Previous research showed that ALA serves as a co-factor for various enzymes involved in mitochondrial energy production, particularly in the citric acid cycle (Krebs cycle) and oxidative phosphorylation [2]. These processes are critical for breaking down macronutrients, including
lips. Furthermore, ALA has demonstrated potential benefits in improving insulin sensitivity, a factor associated with abnormal lipid metabolism and elevated circulating lipids (hyperlipidemia) in conditions such as type 2 diabetes and obesity [7]. In particular, previous studies showed that ALA exhibited similar effects to metformin, significantly upregulating the glycolytic enzymes glucokinase (GCK), hesokinase-1 (HK-1) and pyruvate kinase (PK), and the glycogen synthesis enzyme GS, and downregulating the gluconeogenic enzymes PEPCK and G6Pase, thus decreased glucose production, and promoted glycogen synthesis and glucose utilization in liver. Moreover, ALA markedly increased PKB/Akt and GSK3β phosphorylation, and nuclear carbohydrate response element binding protein (ChREBP) expression in liver [8]. Research also suggests that ALA may directly modulate cholesterol metabolism by regulating the expression of genes involved in cholesterol synthesis and transport, contributing to lipid homeostasis [9]. Although the molecular mechanisms responsible for the effects of ALA on lipid metabolism are not fully understood, previous reports indicated its interactions with peroxisome proliferator-activated receptors (PPARs) [10]. It was demonstrated that in rats and mice, activation of PPAR-γ and PPAR-α prevented hyperinsulinemia, hypertriglyceridemia, high fat diet-induced insulin resistance and hepatic steatosis [11–13]. These nuclear receptor proteins play a significant role in regulating various aspects of lipid metabolism. Specifically, lipoic acid may activate PPAR-alpha and PPAR-gamma, responsible for regulating fatty acid metabolism and insulin sensitivity, respectively [10]. This interaction may enhance fatty acid oxidation, leading to improved lipid profiles and reduced lipid accumulation in the liver. However, it is worth noting that the effects of ALA on PPARs can vary based on factors such as dosage, duration of supplementation, and specific tissue or cell types, potentially accounting for inconsistencies in published results under various experimental conditions. Given the increasing global prevalence of NAFLD and the absence of approved treatment options, coupled with the potential benefits of ALA supplementation and its positive metabolic effects, this study aims to investigate the impact of ALA on lipid content in an in vitro model of NAFLD and its impact on intracellular fatty acid content and metabolism.

2. Materials and Methods

2.1 Cell Culture and Pharmacological Treatments

HepG2 cells (American Type Culture Collection, Manassas, VA, USA; ATCC HB-8065™) were a kind gift from Prof. Maurizio Parola of the University of Turin. Briefly, low-passage cells were grown in DMEM (Sigma-Aldrich, Milan, Italy) supplemented with 10% FBS (EuroClone, Milan, Italy), 100 U/mL penicillin (Life Technologies, Milan, Italy), and 100 µg/mL streptomycin (Life Technologies) at 37 °C in a humidified incubator in an atmosphere of 95% air and 5% CO₂ [14]. Upon reaching 80–90% confluency, to induce steatosis, HepG2 cells were pretreated for 24 h as follows: HepG2 + vehicle (bovine serum albumin (BSA) 5%); HepG2 + PA:OA (BSA 5% + palmitic acid 250 µM and oleic acid 500 µM). Following 24 h of PA:OA treatment, cells were treated as follows: HepG2 + PA:OA + α-lipoic acid (BSA 5% + palmitic acid 250 µM and oleic acid 500 µM + α-lipoic acid 1–5 µM).

The choice of mixture (PA:OA - 250 µM:500 µM) was based on a previous study that showed that the FFA mixture containing a low proportion of palmitic acid (palmitate/oleate 1:2 ratio) is associated with minor toxic and apoptotic effects, thus representing a cellular model of steatosis [15]. The choice of ALA concentration was based on a dose-response curve, which showed that the concentration of 1 and 5 µM is not toxic to cells, and furthermore, it is in a clinically relevant range. Cell culture was tested for possible mycoplasma contamination before all the experimental procedures with a (PlasmoTest™, rep-pt1, Invivogen, San Diego, CA, USA) according to manufacturer’s protocol. Additional STR profiling is available at the following link: https://www.atcc.org/products/hb-8065.

2.2 Real-Time PCR for Gene Expression Analysis

HepG2 cells were pretreated with PA:OA to induce steatosis and, in the following 6 h, treated with ALA. RNA was extracted using Trizol® reagent (Invitrogen, Carlsbad, CA, USA). First-strand complementary DNA (cDNA) was then synthesized with a reverse transcription reagent from Applied Biosystems (Foster City, CA, USA). Quantitative real-time PCR (qRT-PCR) was performed in a StepOne Fast Real-Time PCR System (Applied Biosystems) using the SYBR Green PCR MasterMix (Life Technologies, Monza, Italy). The specific PCR products were detected with SYBR green fluorescence. The relative messenger RNA (mRNA) expression level was calculated by the threshold cycle (Ct) value of each PCR product and normalized with that of actin using a comparative 2−ΔΔCt method [16]. The sequences of the primers used are presented in Table 1.

2.3 BODIPY Staining and Cytofluorimetric Analysis

BODIPY staining and cytofluorimetric analysis were used for lipid droplet measurement. Briefly, HepG2 cells were seeded in 24-well plates and treated with PA:OA and ALA. Following 24 h of ALA treatment, cells were washed using PBS to remove media/serum and incubated with BODIPY staining solution in the dark for 15 min at 37 °C. Cells were washed with PBS to remove staining solution and then trypsinized to generate a single cell suspension. This was then centrifuged at 250 ×g, 5 min, 4 °C and the supernatant was aspirated. Cells were washed using PBS and centrifuged at 250 ×g, 5 min, at 4 °C. The pellet was resuspended in 300 µL of PBS and then flow cytometry analysis was performed. Data were analyzed as mean fluorescence.
Table 1. Genes of interest.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward 5' → 3'</th>
<th>Reverse 5' → 3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>COX1</td>
<td>CGCCAGTGAATCCCTGTTGTT</td>
<td>AAGGTGGGATGGACAAACTCC</td>
</tr>
<tr>
<td>COX2</td>
<td>CTGGCGCTCAGCCATACAG</td>
<td>CGCACTTAACTGGTGCAATCC</td>
</tr>
<tr>
<td>HMGCR</td>
<td>TTTGGGTGATCTTCATGGAQA</td>
<td>TGTAGGGTGAATGAGGGGC</td>
</tr>
<tr>
<td>DHCR7</td>
<td>GCTGCAAATCCGACCCAAA</td>
<td>GCTGCACTGGAAACCCAGT</td>
</tr>
<tr>
<td>CYP51A1</td>
<td>GAAAGCGCACGCTGCTCAGA</td>
<td>ACGGCGATCCGATCTGAGC</td>
</tr>
<tr>
<td>SREBF2</td>
<td>CCTGGGAGACATCGACGAGAT</td>
<td>TGAATGACCGTTGCACTGAG</td>
</tr>
<tr>
<td>PCSK9</td>
<td>AGAAGCGGCTCTTCGCAGT</td>
<td>GGAGTCCTGAGCTGTAGTC</td>
</tr>
<tr>
<td>LDLR</td>
<td>GAGAGCTGATCTTGCGAGAC</td>
<td>CGGAGCATTGATGCTGCAATC</td>
</tr>
<tr>
<td>PPARa</td>
<td>AAGAGCTTGGAGCTCGGAC</td>
<td>TGAAAGCCTGGCTGATGAG</td>
</tr>
<tr>
<td>PPARD</td>
<td>CAGAGGATGGATGCGGGAC</td>
<td>TGAACACCGTAGTGGAAGCC</td>
</tr>
<tr>
<td>UCP1</td>
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<td>GCCAGGTGATGGCAACAGT</td>
</tr>
<tr>
<td>UCP2</td>
<td>TGCTCTACATGGGCTGGTTT</td>
<td>GAGCATGTGAAAGCGCAAGT</td>
</tr>
<tr>
<td>β-Actin</td>
<td>CCTTGGCCGATCCGGCCG</td>
<td>AACATGTCTGGGTACATC</td>
</tr>
</tbody>
</table>

2.4 Lipidomic Analysis

Liquid chromatography coupled with mass spectrometry (HPLC-MS/MS) (Waters Alliance 2695/Waters Micromass Quattro Micro API) was used to analyze the fatty acid content. The separation was performed using water:methanol as mobile phase with a gradient elution as follows: 0–8 min, 50:50 (v/v); 8–12 min, 2:98 (v/v); and 12–15 min, 50:50 (v/v). The elution rate was 300 µL/min for 15 min on a C18 column (Atlantis Waters) 3 µm C18, 150 × 2.1 mm, with an injection volume of 20 µL. To conduct a thorough evaluation of the analyzed spectrum, the utilization of ESI-MS/MS with negative polarity was employed for a detailed spectrum analysis, with the following equipment settings: collision energy (CE) 45 eV, cone voltage (CV) 7 V, cone gas 60 L/h, desolvation gas 800 L/h, temperature 300 °C, and column temperature 40 °C. To quantify the fatty acid content, they were properly diluted in methanol, and the calibration curve was constructed in the range of 0.5 µg/mL–100.00 µg/mL. All solvents (methanol LC/MS grade, water LC/MS grade) and chemical standards (oleic acid, palmitoleic acid, myristic acid, arachidonic acid, docosahexaenoic acid, and ALA) were purchased from Merck (Milan, Italy). In this study, the method created by Waters (confidential information) was adapted with slight modifications to measure the levels of free fatty acids (FFA) and released fatty acids. The decision to employ the HPLC-MS method was based on the better suitability of using this equipment in comparison with GC-MS. The application of HPLC-MS avoids the derivatization into fatty acid methyl ester (FAME), thus facilitating the sample preparation process, shortening the analysis times, and obviating the potential for erroneous reaction products resulting from molecular reorganization during the derivatization procedure.

2.5 Statistical Analysis

Statistical analysis was performed using GraphPad Prism software, version 9.0 (GraphPad Software Inc., San Diego, CA, USA, RRID: rid_000081). For comparison of n ≥ 3 groups, one-way analysis of variance (ANOVA) with the Holm–Sidak post hoc test for multiple comparisons was used. Data are expressed as mean ± SD, unless otherwise stated, and p-values < 0.05 were considered statistically significant.

3. Results

3.1 ALA Reduces Lipid Droplet in PA:OA-Treated HepG2 Cells

In order to measure the accumulation of fatty acids within hepatocytes treated with PA:PO, we measured lipid droplets through staining with BODIPY. Our data show that PA:PO significantly increased the lipid content in HepG2 cells compared to controls and that a 1 µm dose of ALA increased this fat accumulation. Different results were obtained with the dose of 5 µm of ALA, this concentration was able to reduce the accumulation of fats inside the cells previously treated with PA:OA. No effects were found on cells treated with ALA alone at both concentrations (Fig. 1A,B).

3.2 Free Fatty Acid Profile in PA:OA-Treated HepG2 Cells

To analyze fatty acid profiles in cell content, we performed HPLC to measure the concentration of different fatty acids. Our results show that palmitic acid was increased in PA:OA-Treated HepG2 cells, confirming the accumulation of exogen addition while both ALA concentrations were able to decrease PA after PA:OA treatment. Interestingly, Fig. 2 shows that oleic acid induces no significant differences in PA:OA cells with respect to control treatment. Moreover, ALA at both concentrations reduced OA cell content after PA:OA treatment. In our investigation we also analyzed the cell content of linoleic acid and arachidonic acid (Fig. 2C,D). Our results showed that, increasing linoleic acid concentrations were found in PA:OA treated HepG2 cells while only 1 µm of ALA was able to decrease this accumulation with respect to the control treatment. Moreover, both doses of ALA alone were able to
increase the content of this fatty acid. Inversely, the concentration of arachidonic acid decreased in all treatments with only 1 µm ALA showing no significant change with respect the control treatment.

3.3 ALA Reduces Cyclooxygenase Gene Expression

In order to investigate the impact of cyclooxygenases on lipid profile, we analyzed the expression of COX1 and COX2 (Fig. 3A,B). Both genes were overexpressed in the treatment with exogenous fatty acids while both concentrations of ALA were able to reduce them to similar values to the PA:OA treatment.

3.4 ALA Reduces the Expression of Genes Related to Cholesterol Biosynthesis and LDL Intake

In order to investigate cholesterol biosynthesis, we analyzed the gene expression of its enzyme pathway. Fig. 4 shows gene expression of SREBF2, HMGCR, DHCR7 and CYP51A1. Our data show that all these genes were significantly increased after PA:OA treatment and that both doses of ALA were able to decrease this gene pathway. In accordance with a previous study, we also investigated the expression of PCSK9 and low-density lipoprotein (LDL) receptor genes (Fig. 4E,F). We found PCSK9 and LDLR both increased after PA:OA treatment while both doses of ALA decreased their expression. ALA treatments alone decreased PCSK9 gene expression, confirming its role as a systemic hypolipidemic agent.

### 3.5 PA:OA Induce an Increase in the Expression of Genes Related to Thermogenesis and Lipid Metabolism

As shown in Fig. 5, PPARa and PPARδ gene expression were increased in PA:OA-treated HepG2 cells while both doses of ALA were able to restore this gene’s overexpression to control values (Fig. 5A,B).

Furthermore, in order to investigate the thermogenic pathway that dissipates excess energy, we analyzed the expression of uncoupling proteins (UCPs) 1 and 2. Both UCP genes were overexpressed in the treatment with exogenous fatty acids while both concentrations of ALA were able to reduce it to similar values to the control treatment (Fig. 5C,D).

### 4. Discussion

NAFLD is a persistent disorder associated with liver damage and closely linked to obesity, insulin resistance, and metabolic syndrome. We recently showed a significant association between steatosis and alterations in mitochondrial dynamics, impacting the metabolic function of liver cells and contributing to intracellular lipid accumulation [4]. However, the direct effects of ALA on hepatic liver metabolism have not been investigated. This study investigates the impact of ALA on gene expression within the primary lipid regulatory pathways using a model of hepatic steatosis induced by exogenous fatty acids [15]. In our experimental model, we observed an accumulation of lipid droplets in HepG2 cells.
Fig. 2. PA:OA treatment in HepG2 cells modulates free fatty acid profile. HPLC analysis of free fatty acid in HepG2 cells treated with PA:OA and/or ALA. (A) Palmitic Acid. (B) Oleic Acid. (C) Linoleic Acid. (D) Arachidonic Acid. Data are presented as mean ± SD of four independent experiments. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. 
droplets in cells exposed to the PA:OA mixture. There was an increase in free palmitic acid levels, while oleic acid concentrations decreased. This is consistent with previous studies indicating that oleic acid induces more effective steatogenic effects and reduces cytoplasmic oleic acid concentrations [17]. The accumulation of cytoplasmic palmitic acid has been linked to increased apoptosis through PPARα and PPARδ activation, resulting in enhanced beta-oxidation and oxidative stress [18]. PPARα and PPARδ belong to the superfamily of nuclear receptors. PPARs are transcription factors activated by various fatty acids and their derivatives [19]. Three isotypes of PPAR exist, PPARα, PPARγ (expressed principally in adipose tissue and regulate adipogenesis and insulin sensitivity) and PPARδ. PPARδ stimulates temporary or more severe fatty acid accumulation in mice and potentially in the human liver [20]. The lipogenic activity of PPARδ increases the production of mono-unsaturated fatty acid (MUFAs), which are activators of PPARδ and may protect the liver from FFA-mediated lipotoxicity and inflammatory response [21]. In addition, it was demonstrated that human PPARδ in the mouse liver promoted hepatic steatosis that was associated with a significant loss of fat mass, suggesting extensive adipose tissue lipolysis and consequently an influx of fatty acids into the liver. This effect required PPARα signaling, with PPARα working downstream of PPARδ [22]. Our study, through the administration of free fatty acids to liver cells, mimics prolonged food deprivation, including the release of large quantities of fatty acids from adipose tissue, followed by their oxidation in the liver. It has been demonstrated that PPARα is involved in the transcriptional response to fasting, it is induced during fasting in wild-type mice, indicating that PPARα plays a critical role in managing energy stores during fasting. By modulating gene expression, PPARα stimulates the oxidation of hepatic fatty acids to provide substrates that can be metabolized by other tissues [23].

Furthermore, previous studies have indicated that PPARα activation up-regulates UCP2 expression in hepatocytes by increased transcription involving unknown proteins [24]. Our data show PPARα and PPARδ activation pathways due to PA/OA. In particular, we highlighted the correlation with UCPs. PPARs, together with UCPs, stimulate thermogenesis as well as beta oxidation to dissipate the energy contained in the reduced carbon chains of fatty acids. The administration of ALA after treatment with PA:OA was able to decrease the lipid content, the free palmitic acid and the expression of PPARα and PPARδ by regulating lipid metabolism.

To this regard, we found that ALA mitigates the overexpression of UCPs induced by PA:OA treatment. While UCPs activate thermogenesis pathways to catabolize excess long-chain fatty acids, the administration of ALA was able to reduce the expression of UCPs, suggesting a potential role in modulating lipid metabolism.
Fig. 4. The cholesterol biosynthesis and LDL intake were reduced by ALA treatment. (A) mRNA expression levels of HMGCR. (B) mRNA expression levels of DHCR7. (C) mRNA expression levels of CYP51A1. (D) mRNA expression levels of SREBF2. (E) mRNA expression levels of PCSK9. (F) mRNA expression levels of LDLR. Data are presented as mean ± SD of four independent experiments. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.
Fig. 5. PPARs and thermogenesis genes were induced by PA:OA treatment. (A) mRNA expression levels of PPARα. (B) mRNA expression levels of PPARδ. (C) mRNA expression levels of UCP1. (D) mRNA expression levels of UCP2. Data are presented as mean ± SD of four independent experiments. * \( p < 0.05; ** \( p < 0.01; *** \( p < 0.001; **** \( p < 0.0001. \)
fatty acids, overexpression, particularly of UCP2, may contribute to compromised mitochondrial physiology and liver diseases [25,26]. Finally, in order to analyze the genetic pathway of cholesterol biosynthesis, our results suggest that the PA:OA model was able to upregulate the gene expression of the main target enzymes of the biosynthetic pathway such as SREBF2, HMGCR, DHCR7 and CYP51A1 and that ALA restores this upregulation confirming a regression in cholesterol biosynthesis.

Furthermore, the expression of the LDL receptor and PCSK9 genes were analyzed. LDLR mediate LDL clearance and increased LDLR expression improves LDL hepatic absorption and decreases plasma LDL. Conversely, PCSK9 functions as a chaperone, guiding LDLR to internal degradation and preventing its recycling to the cell surface [27]. Our results showed an overexpression of both genes in PA:OA treated cells and a decrease mediated by ALA treatment. Elevated HMGCR activity and PCSK9 are associated with diminished LDLR expression and LDL uptake in the liver. Although the expected expression of PCSK9 and LDLR should be inversely proportional, the increase in LDLR could be the effect mediated by PPARs which allows the influx of fatty acids into the hepatocyte or a secondary mechanism affected by the consequence of feedback.

Our data showed other free fatty acid profiles present in the cytoplasm such as linoleic acid and arachidonic acid. Both fatty acids are responsible for activating the prostaglandin pathway through the action of cyclooxygenases. PA:OA treatment highlighted an accumulation of linoleic acid while arachidonic acid concentration was significantly downregulated compared to control cells. Interestingly, 1 µm ALA showed a reduction in free linoleic acid concentration. Therefore, in order to investigate cyclooxygenases, we measured the gene expression of COX1 and COX2. Both genes were overexpressed following PA:PO treatment and ALA administration significantly downregulated their expression. To this regard, COX-1 and COX-2 are heme proteins, and such a prosthetic group is crucial for the expression of catalytic activity [28]. Recent studies demonstrated that ALA reduces lipotoxicity and induces the heme oxygenase system, the enzymes responsible for regulating intracellular heme content, in acute lung injury and an in vitro model of steatosis [29,30]. Consistently with this hypothesis, previous reports showed that prostaglandin synthesis reduces in response to treatments that upregulate the expression of heme oxygenase (HO)-1 [31,32]. Our results reveal that ALA acts by inhibiting cyclooxygenases, playing a role in the inflammation exhibited in hepatic steatosis.

5. Conclusion

In conclusion, our results demonstrated a direct effect of ALA on hepatic liver metabolism and lipid profile that combined with the other systemic effects of improved insulin resistance, oxidative stress and systemic inflammation may represent a strong rationale for the use of ALA in metabolic syndrome treatment or NAFLD. Since ALA is commercially available for several clinical purposes, its use could be easily and highly recommended for patients with NAFLD.

Availability of Data and Materials

The datasets used and/or analysed in this study are reported within the manuscript and are available from the corresponding authors.

Author Contributions

LL, DT, TZ, GLV and IAB designed the research study. LL, ELS, TZ, GZ and SR performed the research. AN, AMA, AS, ET and SR contributed to formal analysis, data interpretation and in editing the final version of the manuscript. LL, FMS, MA, WC and FG analyzed the data. GLV and IAB wrote the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate
Not applicable.

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Conflict of Interest

Given their role as Guest Editor, GLV had no involvement in the peer-review of this article and have no access to information regarding its peer review. Full responsibility for the editorial process for this article was delegated to Margaret O. James. The authors declare no other conflict of interest.

References


