



# The effect of high maternal linoleic acid on endocannabinoid signalling in rodent hearts

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## Brief Report

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## Abstract

The endocannabinoid system (ECS), modulated by metabolites of linoleic acid (LA), is important in regulating cardiovascular function. In pregnancy, LA is vital for foetal development. We investigated the effects of elevated LA in H9c2 cardiomyoblasts *in vitro* and of a high linoleic acid (HLA, 6.21%) or low linoleic acid (LLA, 1.44%) diet during pregnancy in maternal and offspring hearts. H9c2 cell viability was reduced following LA exposure at concentrations between 300 and 1000  $\mu$ M. HLA diet decreased cannabinoid receptor type 2 (CB<sub>2</sub>) mRNA expression in foetal hearts from both sexes. However, HLA diet increased CB<sub>2</sub> expression in maternal hearts. The mRNA expression of fatty acid amide hydrolase (FAAH) in foetal hearts was higher in females than in males irrespective of diet and N-acyl phosphatidylethanolamine-specific phospholipase D (NAPE-PLD) mRNA expression showed an interaction between diet and sex. Data indicate that a high LA diet alters cell viability and CB<sub>2</sub> expression, potentially influencing cardiac function during pregnancy and development of the offspring's heart.

## Introduction

Long-chain polyunsaturated omega 6 (n-6) fatty acids (FAs) are obtained from our diet through vegetable oils (such as corn and soybean), chicken, eggs and processed foods. Studies reveal relative over-consumption of n-6 compared to omega-3 (n-3) FAs in Western diets, at a ratio ranging from ~10:1 to 25:1.<sup>1</sup> Linoleic acid (LA) is the major n-6 in our diet, and its primary role is incorporation into cell membranes to maintain normal membrane fluidity, structure and function.<sup>2</sup> Elevated concentrations of LA are also known to be pro-inflammatory and pro-oxidative.<sup>2</sup> In Australia, LA consumption has increased to three times the recommended daily intake between 1991 and 2009.<sup>3</sup> This increase mirrors other Western societies, with LA availability in the USA diet increasing by ~160% over the same period.<sup>3</sup> Women of childbearing age are also consuming increasing levels of LA before and during pregnancy. While LA is vital for foetal and postnatal development,<sup>2</sup> we have demonstrated that elevated LA decreases placental cell viability,<sup>4</sup> suggesting that in addition to LA's role in maintaining normal cellular function, elevated concentrations may be detrimental.

LA is transported by syncytiotrophoblast brush-border membranes of the placenta from mother to the foetus.<sup>5</sup> Disruption in normal cellular function in the placenta is associated with pregnancy complication and affects foetal development.<sup>6</sup> Research suggests that an optimal ratio of n-6 to n-3 of 1:1 or 2:1 is required for human health.<sup>7</sup> In humans, plasma concentrations of LA vary and estimated to be 280–5000  $\mu$ M in one Western population.<sup>8</sup> Maternal plasma LA concentrations of ~600–1200  $\mu$ M<sup>9,10</sup> have been identified during pregnancy. The concentration of n-6 in pregnancy is critical, as imbalance between n-6 and n-3 FA in maternal diet impairs neocortical development in offspring.<sup>11</sup>

Among potential mechanisms, LA modulates the endocannabinoid system (ECS), which modifies physiological functions and processes during early foetal development.<sup>12</sup> The ECS comprises the endogenous ligands 2-arachidonyl glycerol (2-AG) and anandamide (AEA), which can be generated via LA metabolism and act predominantly via the cannabinoid receptor 1 (CB<sub>1</sub>) and cannabinoid receptor 2 (CB<sub>2</sub>).<sup>13</sup> The ligands 2-AG and AEA are modified by synthesising and degradation enzymes.<sup>2</sup> ECS signalling controls a variety of physiological processes, and abnormal ECS function has been identified in a variety of diseases.<sup>14,15</sup> Emerging research also suggests an important role for CB<sub>2</sub> in protecting cardiomyocytes during disease; knockout of CB<sub>2</sub> exaggerates cardiac apoptosis, inflammation and dysfunction during ischemia–reperfusion.<sup>16</sup>

Emerging research suggests a role for ECS in development.<sup>17</sup> The ECS may control development via CB<sub>1</sub> and CB<sub>2</sub>, although our recent study *in vivo* suggests that elevated maternal LA may

also control growth and development via leptin.<sup>3</sup> In our rodent model of elevated maternal LA, circulating leptin concentrations in pregnancy are reduced, potentially via downregulation of mRNA expression in adipose tissue.<sup>3</sup> As leptin is important for organ development,<sup>18</sup> elevated maternal LA may have developmental consequences when LA and its metabolites are in excess. Further, we have previously demonstrated that elevated LA reduces placental cell viability,<sup>4</sup> suggesting that LA may control cell organ development through the reduction in cell number, and thereby affecting growth.

Currently, we have a limited understanding of the developmental mechanisms modulated by ECS overactivity and the downstream targets involved.<sup>12</sup> Compounding this, there are little data concerning the effects of prenatal exposure to elevated concentrations of the endogenous ECS ligands. We have identified in rodents that maternal consumption of elevated LA prior to and during pregnancy significantly elevates foetal plasma arachidonic acid (AA).<sup>3</sup> LA is metabolised into AA, which is processed by enzymes including N-acyl phosphatidylethanolamine-specific phospholipase D (NAPE-PLD) into the ECS ligand AEA.<sup>2</sup> Conversely, AA is processed by diacylglycerol lipase (DAGL) to produce 2-AG. AEA and 2-AG modulate a number of targets including the CB<sub>1</sub>, CB<sub>2</sub>, GPR55 and GPR18 cannabinoid receptors.<sup>19</sup> The termination of AEA and 2-AG's signalling capacity is mediated by fatty acid amide hydrolase (FAAH) activity.<sup>20</sup> Cannabinoid receptors interact with additional targets that may either inhibit inflammation (including leptin<sup>21</sup>) or promote inflammation (including tumour necrosis factor alpha (TNF $\alpha$ ).<sup>22</sup>

Although the ECS influences development and plays a role in regulating cardiovascular function,<sup>23</sup> there is a paucity of data concerning the effects of high LA exposure on cardiomyocyte viability and development. Based on prior studies, we therefore predict that 1) exposure to elevated LA will alter cardiomyocyte viability and 2) consumption of elevated concentrations of LA upregulates LA metabolic by-products,<sup>3</sup> which leads to altered expression of LA metabolic enzymes and increased downstream pro-inflammatory mediators.

## Methods

### *In vitro* study

Rat H9c2 cardiomyoblasts were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin and maintained in a humidified incubator at 37 °C with 5% CO<sub>2</sub>. LA and bovine serum albumin (BSA) complex was prepared in a 5:1 ratio. BSA is required to allow the cells to effectively take up LA. Cells were seeded at an initial density of 10,000 cells/well in 96-well plates. After reaching 80% confluence, cells were incubated in serum-free media for 2 h before treatment with various concentrations of LA-BSA complex (12.5, 25, 100, 200, 300, 400, 500 and 1000  $\mu$ M) for 24 h. Cell viability was measured using a 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma Aldrich, St. Louis, MO, USA) similar to our previous study.<sup>4</sup> A positive control (15% DMSO) was used as an indicator of induced cell death.

### *In vivo* study

Wistar Kyoto rats (8 weeks of age) were purchased from the Australian Resource Centre (ARC, WA, Australia) and

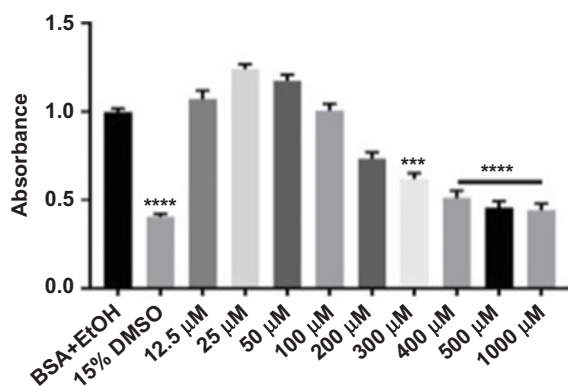
acclimatised in accordance with the Australian Code of Practice for Care and Use of Animals for Scientific Purpose after ethical approval was granted by the Griffith University Animal Ethics Committee (NSC/01/17/AEC). After 1 week of acclimatisation, rats were divided into low linoleic acid (LLA;  $n = 8$ ) and high LA (HLA;  $n = 10$ ) dietary groups, as previously described.<sup>3</sup> Briefly, female rats were exposed to either LLA or HLA diet for 10 weeks before mating. The LLA diet contains ~1.44% LA of energy, and the HLA diet contains ~6.21% LA of energy (which is the average daily LA consumption in Australia, representative of Western society).<sup>3</sup> The LLA (SF17-109) and HLA (SF17-110) diets were custom prepared by Specialty Feeds (WA, Australia) based on the AIN-93G diet. The major source of LA in HLA was safflower oil. Respective diets were provided throughout during pregnancy. The gestation period for a rat is 22 days. Pregnant rats were sacrificed at day 20 (E20) as previously described.<sup>3</sup> Left ventricular myocardium from maternal hearts and whole foetal hearts were harvested, immediately snap-frozen in liquid nitrogen and stored at -80 °C for RNA extraction. For the analysis, one foetal heart from each sex was used per litter.

### *Sex determination of foetus*

DNA was extracted from the tail of each foetus. Sex determination was undertaken as described previously<sup>3</sup> using q polymerase chain reaction (PCR) amplification of the SRY (sex-determining region Y) gene using a commercially available hydrolysis probe (Rn04224592\_ul; NM\_012772.1; Applied Biosystems).

### *Quantitative polymerase chain reaction*

Total RNA was extracted from E20 maternal and foetal heart tissues, as described previously with minor modifications.<sup>3</sup> Briefly, maternal cardiac tissue was homogenised in lysis buffer supplemented with proteinase K and incubated at 57 °C for 10 min prior to the extraction of the RNA. The RNA was extracted using RNeasy mini kit (Qiagen) following manufacturer's guidelines. Then, the RNA was synthesised to complementary DNA using an iScript gDNA clear cDNA synthesis kit (Bio-rad) according to manufacturer's guidelines. Real-time quantitative polymerase chain reaction (qPCR) was performed using QuantiNova SYBR<sup>®</sup> green master mix (Qiagen) following manufacturer's guidelines, in line with Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines.<sup>24</sup> Initial holding stage was set at 95 °C for 10 min to fully denature the DNA. qPCR reactions were set for 40 cycles. The denature stage was set for 15 seconds at 95 °C, and extension set at 60 °C for 1 min using StepOne<sup>™</sup> real-time PCR systems (Applied Biosystems). The  $2^{-\Delta\Delta C_q}$  method was used to quantify gene expression, which was normalised to the geometric mean of  $\beta$ -actin and  $\beta$ -2 microglobulin as reference genes, as previously prescribed.<sup>3</sup> KiCqStart<sup>™</sup> pre-designed primers from Sigma Aldrich were used for qPCR: Leptin (*Lep*; NM\_013076), Leptin receptor (*Lepr*; NM\_012596),  $\beta$ -actin (*Actb*; NM\_031144),  $\beta$ -2 microglobulin (*B2m*; NM\_012512), GPR18 (*Gpr18*; NM\_001079710), GPR55 (*Gpr55*; XM\_001063474), FAAH (*Faah*; NM\_024132), IL-6 (*Il6*; NM\_012589), DAGL- $\alpha$  (*Dagla*; NM\_001005886), DAGL- $\beta$  (*Daglb*; NM\_001107120), CB1 (*Cnr1*; NM\_012784), CB2 (*Cnr2*; NM\_020543), TNF- $\alpha$  (*Tnfai3*; NM\_001107387) and NAPE-PLD (*Napepld*; NM\_199381). PCR products were validated by sequencing using the Applied



**Fig. 1.** LA decreases cardiomyocyte viability. Cardiomyocyte metabolic activity after 24 hr experimental treatments assessed via MTT assay in cells seeded at 80% confluence. Data presented as means  $\pm$  SEM,  $n = 6$  for all treatment conditions. \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$  compared to vehicle control (BSA + EtOH).

Biosystems 3130 $\times$ 1 Genetic Analyzer, following clean-up using ExoSAP-IT, performed at the Griffith University DNA sequencing facility.

### Statistical analysis

Data are expressed as the mean  $\pm$  standard error of the mean (SEM). A  $p$  value less than 0.05 was considered indicative of statistical significance. Multiple comparisons among groups were performed using one- and two-way ANOVA (maternal diet and sex as 2 factors) or Mann-Whitney U test followed by Dunnett post hoc test using GraphPad Prism 7.

## Results

### LA decreases viability of H9c2 cells

Exposure of H9c2 cells with LA (between 300  $\mu$ M and 1000  $\mu$ M) significantly decreased their viability compared to cells exposed to vehicle control alone (Fig. 1).

### Effect of high maternal LA on mRNA expression of genes in foetal heart

As described previously, there was no change in the body or heart weight in E20 foetuses from mothers consuming a high LA diet.<sup>3</sup> However, mRNA expression of  $CB_2$  was significantly decreased in both male and female foetuses of HLA mothers ( $p < 0.05$ , Fig. 2a). There was no change in mRNA expression of *GPR18* in foetal cardiac tissue (Fig. 2b), while mRNA for *FAAH* was significantly decreased in hearts of male compared with female foetuses ( $p < 0.05$ , Fig. 2c). Cardiac *NAPE-PLD* mRNA showed an interaction between diet and sex (interaction  $p < 0.05$ , Fig. 2d). There were no significant changes in mRNAs for *DAGL- $\alpha$* , *DAGL- $\beta$* , leptin and *TNF- $\alpha$*  (Fig. 2e–2h) in foetal hearts. The mRNA expressions of *IL-6*, *CB\_1*, *GPR55* and *LepR* were below the levels of detection in foetal heart (data not shown).

### Effect of high LA on mRNA expression of genes in maternal heart

There was a significant increase in maternal  $CB_2$  mRNA expression in the hearts of rats fed the HLA vs. LLA diet ( $p < 0.05$ , Fig. 2i). There was no difference in maternal mRNA expression for

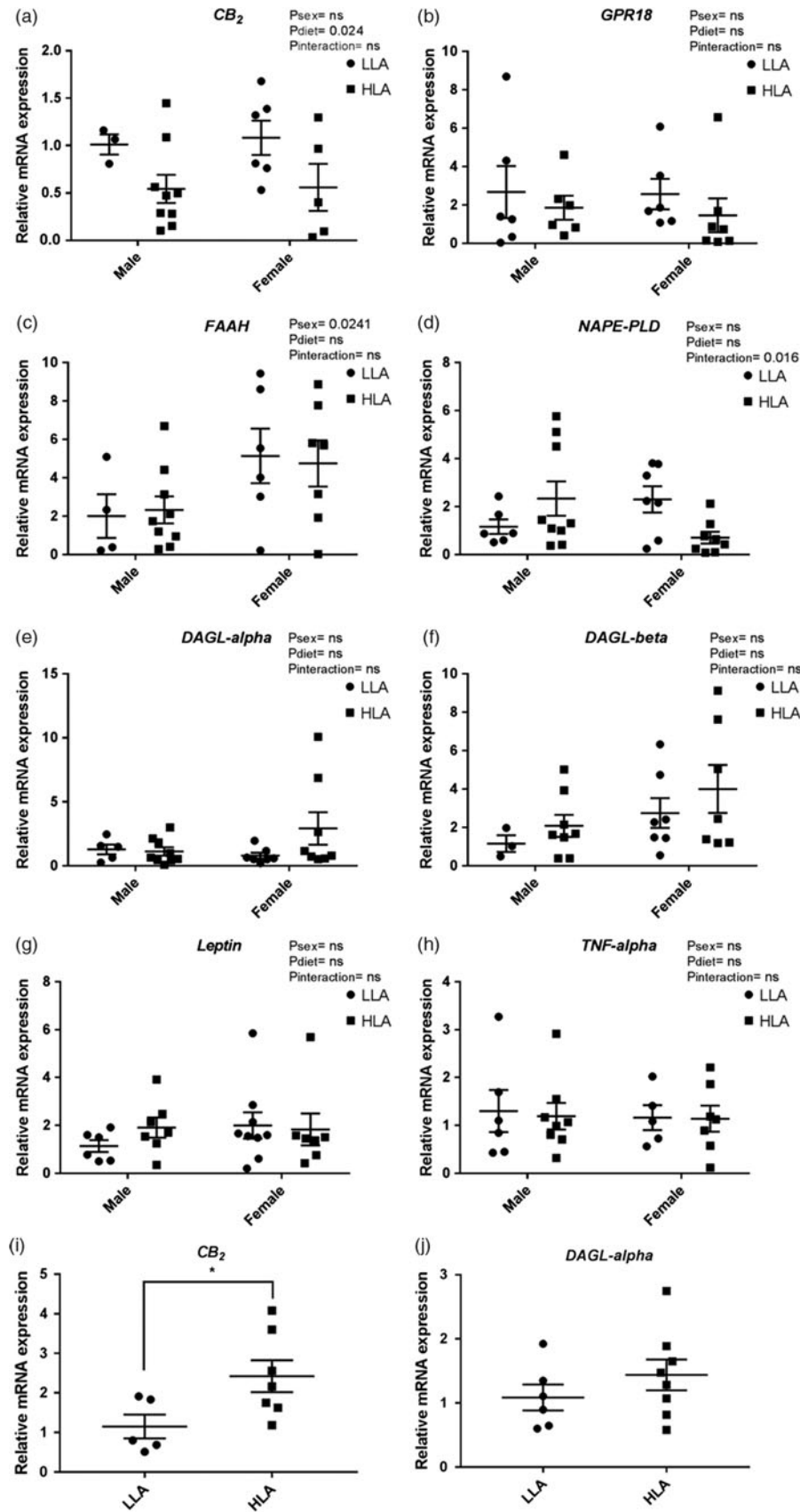
*GPR18*, *DAGL- $\alpha$*  and *DAGL- $\beta$* , *NAPE-PLD*, *LEPR* or *TNF- $\alpha$*  (Fig. 2j–2n). The mRNA expressions of *IL-6*, *CB\_1*, leptin, *GPR55*, *GPR18* and *FAAH* were below the level of detection in maternal heart (no data shown).

## Discussion

LA is a precursor for AA and the ECS ligands and may be deleterious to growth and development. This is the first study to investigate the effects of LA on cardiomyocyte viability, revealing a significant reduction in H9c2 cell viability when exposed to  $\geq 300$   $\mu$ M LA. Previous studies demonstrate that H9c2 cells exhibit hypertrophic responses, similar to primary cardiomyocytes, rendering them suitable for *in vitro* analysis of cardiomyocyte function.<sup>25</sup> We have previously demonstrated that elevated LA reduces viability of trophoblast cells,<sup>4</sup> suggesting the LA modulates molecular pathways that are important in different cell types. There is prior evidence of FA modulation of cell survival, for example, 50–500  $\mu$ M palmitate reduces cell viability, an effect countered by n-3 polyunsaturated fatty acid.<sup>26</sup> However, our earlier observation that elevated maternal LA does not alter heart weight<sup>3</sup> suggests that *in vivo* effects of LA on the heart may be independent of cell viability. Future research should investigate if apoptosis of the heart tissue occurs in response to elevated LA.

This study identified altered ECS gene expression in cardiac tissue of mothers and offspring with maternal consumption of elevated concentrations of LA. The mRNA expression of *FAAH* was increased in female foetal heart irrespective of diet. As *FAAH* terminates AEA and 2-AG's signalling capacity,<sup>20</sup> this suggests potentially augmented ECS activity in male but not female hearts. Downregulation of *FAAH* promotes reactive oxygen species generation in the liver tissue,<sup>27</sup> which could additionally affect the development and function in the male offspring. *NAPE-PLD* mRNA exhibited a sex dependence, with a decrease in females compared with an increase in males in HLA group. This suggests potentially augmented AEA generation in male but not the female foetuses. A key outcome from this study is evidence that sex-specific differences in the programming effects of LA<sup>3</sup> may reflect in part sex-specific differences in the ECS, with a bias towards augmented AEA generation and AEA/2-AG signalling in male vs. female offspring, although cardioprotective  $CB_2$  was significantly decreased by maternal LA in both sexes. Mechanistically, this sexual dimorphism may be influenced by hormones, as  $CB_2$  activity in females has been demonstrated to involve estrogen, which also protects against inflammation.<sup>28</sup>

Of particular interest is a significant change in  $CB_2$  expression in maternal rats fed a high LA diet during pregnancy.  $CB_2$  receptor expression in non-pregnant females has been demonstrated to be cardioprotective, with up-regulation decreasing risk of cardiovascular diseases.<sup>29</sup> In further support of a beneficial role for  $CB_2$ , the activation of the receptor increases the production of anti-inflammatory proteins.<sup>30</sup> Although we have not investigated cardiovascular function in mothers consuming elevated LA diets and circulating concentrations of pro-inflammatory cytokines appear unaltered,<sup>3</sup> the current data suggest that elevated LA during pregnancy may modify maternal cardiovascular function.



**Fig. 2.** Elevated maternal LA consumption alters endocannabinoid signalling in maternal and offspring hearts. Foetal mRNA expression (a to h) and maternal mRNA expression (i to n) of endocannabinoid targets are altered in response to elevated maternal LA. For offspring hearts,  $n = 3-6$  (LLA) and  $n = 6-8$  (HLA). In maternal hearts,  $n = 5-6$  (LLA) and  $n = 7-8$  (HLA). Data are presented as means  $\pm$  SEM, \* $p < 0.05$ .

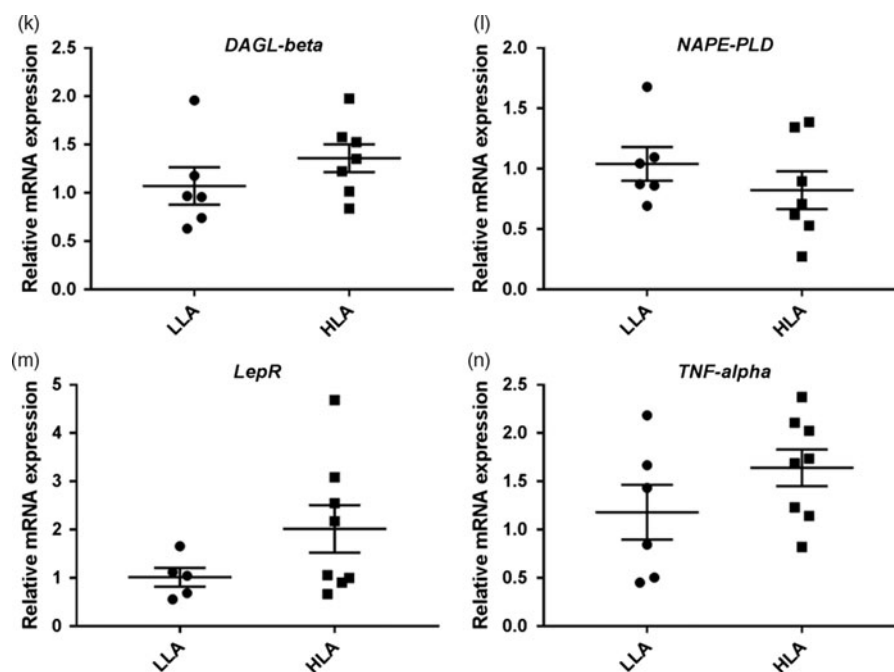


Fig. 2. (Continue)

This study provides further insight into the importance of LA during pregnancy in modulating key signalling pathways that influence cell viability and cardiovascular function. Our highly novel data show a potential mechanistic pathway that links maternal diet in pregnancy and developmental consequences in her offspring. Importantly, altered ECS gene expression suggests LA consumption may modify foetal heart development, potentially leading to detrimental effect on cardiac function in later life. This preliminary evidence that elevated maternal LA consumption may be deleterious for a developing baby's cardiovascular health highlights the importance of intake levels prior to and during pregnancy.

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**Conflicts of Interest.** The authors declare that there is no conflict of interests regarding publication of this article.

**Ethical Standards.** Ethical approval was obtained from the Griffith University Animal Ethics Committee (NSC/01/17/AEC).

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