“The effect of high maternal linoleic acid on cardiometabolic risk in rat mothers and their offspring”

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Submitted in fulfilment of the requirements for the degree of Master of Medical Research

School of Medical Science
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25th November 2019
Keywords

Cardiomyocytes, Contractility, Coronary, Development, Diet, Endocannabinoids, Fatty acid, Gene expression, Gut microbiome, Heart, Linoleic acid, Maternal, Mitochondrial respiration, Pregnancy, Polyunsaturated fatty acid
Abstract

In pregnancy, linoleic acid (LA), obtained from the maternal diet, is vital for fetal development. LA is metabolised into a number of fatty acid modulators that regulate the endocannabinoid system (ECS), and are key for normal metabolic homeostasis. We are currently consuming elevated levels of LA in Western societies, including during pregnancy. We investigated if elevated maternal linoleic acid is detrimental to the mother and her fetus during pregnancy and early life, and the mechanism for this. The primary aims of this study were to investigate if elevated maternal LA consumption during pregnancy alters: 1) the maternal microbiome; 2) the maternal cardiac ECS; 3) the fetal cardiac ECS at embryo (E) day 20; and 4) cardiovascular development, function and the cardiac ECS in offspring at postnatal (PN) day 40. Effects of LA exposure on an in vitro H9c2 cardiomyoblast model (viability, respiration, glycolytic metabolism) were also assessed. A secondary aim was to test whether any of the latter postnatal effects are reversed with a low LA diet.

Female Wistar Kyoto rats were fed a diet high in LA (HLA - 6.21% of energy intake) or a control LA diet (LLA - 1.44% of energy) for 10 weeks prior to mating and throughout pregnancy. At E0, E10 and E20, maternal faeces were collected, and the microbiome analysed by 16S sequencing. At E20 and PN40 maternal and offspring rats were sacrificed, body and organ weights recorded, and mRNA extracted from heart tissue to assess transcription of endocannabinoid signalling targets using real time PCR. Hearts from PN40 rats were also assessed for coronary reactivity, and ventricular diastolic and systolic pressure-volume relationships. In addition, effects of elevated LA exposure on cell viability and mitochondrial respiration were assessed in isolated cardiomyocytes.

The current results showed that throughout pregnancy there was an increased abundance of *Cyanobacteria* and *Lentisphaerae*, and decrease *Verrucomicrobia* in the
microbiome of HLA mothers. Over time, HLA and LLA diets decreased the abundance of *Actinobacteria* and *Tenericutes*. There was an interaction effect for *Verrucomicrobia* in which pregnancy and diet interacted in influencing its abundance.

In terms of effects of LA in H9c2 cardiomyoblasts, LA exposure reduced cell viability at concentrations between 300 and 1000 µM. However, there were no effects of an acute exposure to LA on mitochondrial respiration.

Just prior to birth, an elevated maternal LA diet increased CB$_2$ expression in maternal hearts whereas in her E20 offspring cardiac CB$_2$ expression declined in both sexes. FAAH mRNA expression was unaltered by diet, and appeared higher in female vs. male hearts. There were no effects of a high maternal LA diet on mRNA expression of endocannabinoid receptor GPR18, DAGL alpha and DAGL beta in E20 offspring hearts.

We further assessed cardiovascular development/function in adolescent rats. Data demonstrated that there were no changes in heart mass or heart: body weight ratios. Within the heart, no changes in resting or baseline coronary flow rate, or peak coronary reactivity were detected. However, there was a decline in coronary flow repayment during reactive hyperaemia in female hearts that was significant for the LLA maternal / offspring HLA (LHF) compared with LLA maternal/offspring (LLF) group. A trend of declining coronary flow repayment (after 30 s occlusion) was also observed in HLA maternal/offspring LLA (HLM) and HLA maternal/offspring HLA (HHM) compared to LLM and LHM hearts, though this trend did not achieve statistical significance. In terms of left ventricular diastolic mechanics, there was evidence of an increased diastolic stiffness in HHF hearts (an effect not evident in males), together with an unexpected increase in diastolic compliance in HLM/HHM hearts (counter to changes in HHF vs. other groups). Thus, there is evidence of sexually dimorphic effects of LA on coronary and diastolic function in developing hearts. Systolic pressure development was subtly modified, with a trend to reduced contractility (declining slopes of pressure-volume.
relationships, increased volume required to generate 100 mmHg pressure) in HL and HH diets in both sexes, though this also failed to achieve statistical significance.

Molecular analysis of PN40 hearts demonstrated CB$_2$, LepR, Notch1, NPPA and TGFB1A mRNAs were all expressed in higher levels in HLM vs. LHM groups (with NPPA also elevated vs. LLM). Expression changes in these mRNAs were not evident in females. Collectively, these changes provide further evidence of a sexually dimorphic effect of maternal diet on myocardial phenotype of offspring. In conclusion, a maternal diet high in linoleic acid alters the maternal microbiome and cardiac ECS signalling. This is associated with distinct ECS signalling changes, and alterations in cardiac metabolism and functionality at embryonic and early postnatal stages in offspring hearts. Male and female offspring demonstrated different pathophysiology, with data at these early life stages suggesting that female offspring of mothers consuming a diet high in LA may in particular exhibit altered cardiovascular makeup and function, and potentially greater risk developing cardiovascular disease later in life.
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<th>Description</th>
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<tbody>
<tr>
<td>2-AG</td>
<td>2-arachidonyl glycerol</td>
</tr>
<tr>
<td>20-HETE</td>
<td>20-hydroxy-5,8,11,14-eicosatetraenoic acid</td>
</tr>
<tr>
<td>9-HODE</td>
<td>9-hydroxy-octadecadienoic</td>
</tr>
<tr>
<td>13-HODE</td>
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<tr>
<td>AA</td>
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</tr>
<tr>
<td>AEA</td>
<td>anandamide</td>
</tr>
<tr>
<td>ALA</td>
<td>α-linolenic acid</td>
</tr>
<tr>
<td>BMI</td>
<td>body mass index (kg/m²)</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>COX2</td>
<td>cyclooxygenase-2</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>CVD</td>
<td>cardiovascular disease</td>
</tr>
<tr>
<td>DAGL</td>
<td>diacylglycerol lipase</td>
</tr>
<tr>
<td>DHA</td>
<td>docosahexaenoic acid</td>
</tr>
<tr>
<td>EI</td>
<td>energy intake</td>
</tr>
<tr>
<td>ECAR</td>
<td>extracellular acidification rate</td>
</tr>
<tr>
<td>EPA</td>
<td>eicosapentaenoic acid</td>
</tr>
<tr>
<td>FA</td>
<td>fatty acid</td>
</tr>
<tr>
<td>FAAH</td>
<td>fatty acid amide hydrolase</td>
</tr>
<tr>
<td>FAS</td>
<td>fatty acid synthase</td>
</tr>
<tr>
<td>FAT/CD36</td>
<td>fatty acid translocase cluster of differentiation 36</td>
</tr>
<tr>
<td>IL-1β</td>
<td>interleukin 1β</td>
</tr>
<tr>
<td>IL-6</td>
<td>interleukin 6</td>
</tr>
<tr>
<td>LA</td>
<td>linoleic acid</td>
</tr>
<tr>
<td>LV</td>
<td>left ventricle</td>
</tr>
<tr>
<td>LVH</td>
<td>left ventricular hypertrophy</td>
</tr>
<tr>
<td>MUFA</td>
<td>monounsaturated fatty acid</td>
</tr>
<tr>
<td>NAPE-PLD</td>
<td>N-acyl phosphatidylethanolamine-specific phospholipase D</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>OA</td>
<td>oleic acid</td>
</tr>
<tr>
<td>OCR</td>
<td>oxygen consumption rate</td>
</tr>
<tr>
<td>OXLAMs</td>
<td>oxidised linoleic acid metabolites</td>
</tr>
<tr>
<td>PPARα</td>
<td>peroxisome proliferator-activated receptor γ</td>
</tr>
<tr>
<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>PUFA</td>
<td>polyunsaturated fatty acid</td>
</tr>
<tr>
<td>SFA</td>
<td>saturated fatty acid</td>
</tr>
<tr>
<td>T2DM</td>
<td>type 2 diabetes mellitus</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumour necrosis factor-α</td>
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Statement of Original Authorship

The work contained in this thesis has not been previously submitted to meet requirements for an award at this or any other higher education institution. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made.

Signature: __________________________

Date: __25/11/2019______________
Acknowledgements

This work would not have been possible without the support of the Allen Foundation. I am especially indebted to Dr. Deanne Hryciw, Deputy Dean, Learning and Teaching (Sciences), Senior Lecturer, School of Environment and Science, Senior Fellow of The Higher Education Academy, and National Secretary-Australian Physiological Society, and Professor John Headrick Deputy Head of School (Research), who have been supportive of my career goals and who worked actively to provide me with the protected academic time to pursue those goals. Dr. Deanne Skelly, as my primary supervisor, she has taught me more than I could ever give her credit for here. She has shown me, by her example, what a pro-active, driven for success a researcher (and person) should be in professional and personal life. Words could never express how grateful I am for her assistance. I am grateful to Dr. Olivia Holland for her expertise, genuine support for me to do this work and her assistance to complete this thesis successfully. I am deeply grateful to Nirajan Shrestha (PhD student School of Medicine) aka “lab husband” for his guidance and helping me build the necessary skills to complete my project. It has been an amazing opportunity to work alongside Nirajan during this and other related projects and I thank him for his friendship, empathy, and great sense of humor.

I would like to acknowledge Professor Anthony Perkins who has provided me personal and professional guidance and taught me a great deal about both scientific research and life in general. I would especially like to thank Dr Ian Cassady Master of Medical Research Coordinator for believing in me to achieve my goals in this project. I am very grateful to Program Director Associate Professor Jason Peart for giving me the opportunity to do research and providing invaluable guidance throughout this research. Finally, my
thanks go to all the people who have supported me to complete the research work directly or indirectly, but especially to Tessa, Josh, Dan, Lucy, Saba, Tia, and Pierre.

Nobody has been more important to me in the pursuit of this project than the members of my family. I would like to thank my mother Faye, her partner Geoff and acknowledge my late father John, whose love and guidance are with me in whatever I pursue. They are the ultimate role models. Most importantly, I wish to thank my loving and supportive partner, Ben, and my four wonderful children (inherited an extra 3 children), Bayley, Tyler and Brianna (daughter to me) and McKenzie, who provide unending inspiration and kept the fun going while times were tough. Their love, prayers, care and sacrifices are acknowledged for allowing me to continue my education and help prepare me for the future in my pursuit for research in the health industry.

Simone Sleep
Chapter 1: Introduction

1. LITERATURE REVIEW

1.1 SUMMARY

Omega-6 (n-6) and omega-3 (n-3) polyunsaturated fatty acids (PUFAs) are essential for normal growth and development. PUFAs are only obtained through our diets and not generated by cellular metabolism. n-6 PUFAs are obtained primarily through foods such as corn and soybean oils, chicken, eggs and processed foods (Meyer et al., 2003), while n-3 PUFAs are found in foods such as flaxseeds, walnuts, fatty fish and their respective oil supplements (Gebauer, Psota, Harris, & Kris-Etherton, 2006). Studies have shown that change to a so-called Western diet has led to the overconsumption of n-6 compared to n-3 PUFAs, at a ratio of ~10:1 to 25:1 (Simopoulos, 2003). The recommended n-6:n-3 ratio in our diet is 2:1. This low ratio is recommended for cardiovascular health and the prevention of cardiovascular disease (CVD) (Simopoulos, 2003). Linoleic acid (LA) is the main n-6 in our diet and plays an important role in maintaining normal membrane fluidity, structure and function (Naughton, Mathai, Hryciw, & McAinch, 2016). LA and α-linolenic acid (a-LA) are essential fatty acids that are pro- or anti-inflammatory, respectively. Both LA and a-LA, are metabolised by the same enzymes, Δ-5 desaturase (FADS1) and Δ-6 desaturase (FADS2) (Simopoulos, 2016). Overconsumption of LA reduces the processing of a-LA by about 40% in healthy individuals (Naughton et al., 2016).

Conflicting evidence has been published concerning the link between n-6 PUFA and CVD risk. In the past, the American Heart Association has recommended substitutions of saturated fat with n-6 in our diets to decrease low-density lipoprotein (LDL) cholesterol levels (Gomez Candela, Bermejo Lopez, & Loria Kohen, 2011). However, controversy persists
regarding benefits versus harm, as elevated n-6 also leads to an increase in circulating inflammatory cytokines (Johnson & Fritsche, 2012). One study demonstrated increased risk of CVD, with increased obesity due to the obesogenic properties of LA, which causes weight gain, inflammation and mitochondrial dysfunction (Bournat & Brown, 2010).

Emerging research has also identified a role for n-3 PUFA in fetal development. n-3 PUFA is essential for neurodevelopment during pregnancy. Further, LA is an essential fatty acid for the normal fetal development of vital organs (Innis, 2005). At this time, we have a clearer understanding about the minimum concentration of LA required for development, however despite Australians consuming elevated LA, we have little understanding about the impact of LA overconsumption during critical periods of fetal development.

One of the key pathways by which LA influences biology is modulation of the endocannabinoid system (ECS), which controls normal physiological functions and early fetal development (Fride et al., 2009). The ECS is comprised of 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 (CB₁) and cannabinoid receptor 2 (CB₂) (Pertwee, 2008). The 2-AG and AEA ligands are modified by synthesising and degradation enzymes (Fride et al., 2009). Emerging research supports a role for the ECS in development (Shrestha, Cuffe, Holland, Bulmer, et al., 2019), potentially via CB₁ and CB₂ modulation. Moreover, our recent in vivo study suggests that elevated maternal LA may also control growth and development via the adipokine leptin (Sonnweber, Pizzini, Nairz, Weiss, & Tancevski, 2018). We have developed a rodent model, where mothers consume elevated LA before and during pregnancy. In these mothers, circulating leptin concentrations in pregnancy are reduced (Shrestha, Cuffe, Holland, Bulmer, et al., 2019). As leptin is important for organ development (Briffa, McAinch, Romano, Włodek, & Hryciw, 2015), maternal LA may have developmental consequences when it and its metabolites are in excess. Despite this, we
currently have a limited understanding of developmental mechanisms modulated by ECS overactivity and the downstream targets involved (Fride et al., 2009). LA also has the capacity to modulate normal cardiovascular function, although there is a paucity of data concerning the effects of high LA exposure on cardiomyocyte development, and whether elevated LA during the prenatal period alters cardiometabolic disease risk.

Cardiometabolic disease involves CVD in association with obesity and type 2 diabetes (Hansen, Gøbel, Hansen, & Pedersen, 2015). Combination of the 3 pathologies (multimorbidity) is highly detrimental. Mechanistically, more recent studies have identified an important link between cardiometabolic disease and the gut microbiome. Microbes play an important metabolic role in fermenting plant proteins (Liu et al., 2019) and bio hydrogenated LA into stearic acid to produce nutrients necessary for growth and maintenance (Alvheim et al., 2013). Changes in bacterial composition and function can lead to a state of chronic disequilibrium or dysbiosis (Cani & Delzenne, 2007). Further studies show changes in microbiota diversity can be linked to a variety of health issues, including metabolic dysfunction and inflammatory disorders (Gohir, Ratcliffe, & Sloboda, 2015). Diversity of microbiota is an indicator of overall health, is influenced by environmental factors including levels of types of nutrition, obesity during pregnancy and fetal gut development (Zhu, Du, & Ford, 2014), that regulate energy levels and metabolism (Gohir, Ratcliffe, et al., 2015). Elevated consumption of before and during pregnancy may alter the diversity of gut microbiota (Ma et al., 2014) with gut microbiota during pregnancy potentially linking maternal LA consumption and cardiometabolic health. However, the details and relevance of such a link requires further investigation.
### 1.2 LA METABOLISM

LA is metabolised into downstream FA which can be essential for human health. LA is metabolised to arachidonic acid (AA), which is processed by enzymes including N-acyl phosphatidylethanolamine-specific phospholipase D (NAPE-PLD) into the endocannabinoid AEA (Naughton et al., 2016) (Figure 1).

**Figure 1: Linoleic acid (LA) is the precursor of arachidonic acid.**

Metabolism of LA is mediated via enzyme systems that direct the pathway to endocannabinoid receptors, and multiple downstream products via lipoxygenase (LOX), non-enzymatic auto-oxidation (NE), prostaglandin (PG), and thromboxane TXB inflammatory pathways.

δ-5 desaturase (D5D) and δ-6 desaturase (D6D) are encoded by fatty acid desaturase genes FADS1 and FADS2, and are key enzymes converting LA to arachidonic acid (Lankinen et al., 2019). Increased endogenous conversion of LA to AA is of concern due to increased production of pro-inflammatory and pro-thrombotic AA and eicosanoid metabolites.

AEA derived from LA, together with 2-AG (the 2 endogenous ECs ligands), modulate a number of targets including both CB1 and CB2 receptors (Pertwee, 2008), and G-protein coupled receptor (GPR) 55 and 18 (GPR55 and GPR18) (Ramirez-Orozco et al., 2019).
Termination of the signalling function occurs via fatty acid amide hydrolase (FAAH) (Dainese et al., 2014).

Figure 2: The endocannabinoid pathway

Endogenous ECS ligands, the "endocannabinoids", include AEA and 2-AG which have more molecular targets than just CB\textsubscript{1}R and CB\textsubscript{2}R (Cairns, Baldridge, & Kelly, 2016). 2-AG and AEA are formed from arachidonic acid-containing phospholipids. 2-AG is formed from DAG by DGL\textalpha or DGL\textbeta and is metabolized either via COX-2 to form prostaglandin glyceryl esters or by ABHD6 or MAG-L to form arachidonic acid. The production of AEA occurs through conversion of NAPE by either a NAPE-PLD dependent or independent pathway and broken down by FAAH to form AA (Cairns et al., 2016).

The CB receptors interact with additional signalling targets that modulate inflammation including leptin (Shrestha, Cuffe, Holland, Bulmer, et al., 2019) and the inflammatory...
modulator tumour necrosis factor-α (TNFα) (Nagarkatti, Pandey, Rieder, Hegde, & Nagarkatti, 2009). Thus, the ECS and its ligands 2-AG and AEA can modulate a variety of cell signalling in target tissues by controlling various downstream targets that control cell growth and development (Fride et al., 2009). The degradation of lipid endocannabinoids, in particular 2-AG and AEA, is further influenced by diacylglycerol lipase (DAGL), which increases the ability to bind to CB receptors and further degradation of enzymes towards inflammatory process (Fride et al., 2009). CB₁ and CB₂ have specific localisations within the body, with CB₁ primarily expressed in the nervous system, and CB₂ in the periphery (Freundt-Revilla, Kegler, Baumgartner, & Tipold, 2017). The main area of CB₂ expression in the body is within the immune system.

1.3 THE INFLUENCES OF LA ON CARDIOVASCULAR HEALTH

Normal heart function is essential for human health. Mechanistically the heart can respond to long term stress or hemodynamic overload through hypertrophic remodelling. Normal contractile function is maintained short term, however chronic changes may lead to heart failure (Nabeebaccus et al., 2017). Cardiomyocyte hypertrophy, abnormal excitation-contraction coupling, and contractile dysfunction are just a few of the structural and functional perturbations characterised as left ventricular hypertrophy (LVH) (Nabeebaccus et al., 2017). A healthy heart preferentially employs fatty acid oxidation for energy generation, however in pathological LVH a switch in substrate use from fatty acids to carbohydrates is evident (Nabeebaccus et al., 2017), suggesting a therapeutic approach for LVH and heart failure by improving cardiac efficiency for oxygen consumption. As described previously, PUFAs have been recommended to improve heart health for over 30 years as a substitute for consuming saturated fat (Zhao & Schooling, 2019). This dietary shift towards using polyunsaturated seed oils has been shown to lower serum cholesterol and diabetes risk. Importantly, a diet high in
LA does not prevent the risk of ischaemic heart disease (Zhao & Schooling, 2019). Mechanistically, studies show LA can decrease LDL with no effect on high density lipoprotein, while increasing oxidation derivatives (Ramsden et al., 2016). Furthermore, LA oxidized derivatives alter non-cholesterol lipid mediators, including eicosanoids, endocannabinoids, hydroperoxyl and hydroxy-octadecadienoic acids, promoting pathogenesis of multiple conditions including ischaemic heart disease (Ramsden et al., 2016). Oxidative metabolism and mitochondrial respiration is essential for adenosine triphosphate (ATP) production, energy supply and cell function and viability (Bertram, Gram Pedersen, Luciani, & Sherman, 2006). Cardiomyocytes are complex and energetic cells, requiring large and continuous supplies of energy for repetitive contraction-relaxation, and ionic homeostasis (Pennanen et al., 2014). Normal cardiac function thus relies on tight coupling ATP production and myocardial contraction (Kolwicz, Purohit, & Tian, 2013). Recent studies are targeting metabolism as a therapeutic approach in different pathological conditions (Kolwicz et al., 2013). For example, a recent study showed trophoblast cell exposure to LA alters expression of genes involved in fatty acid transportation, respiration and mitochondrial function (Shrestha, Cuffe, Holland, Perkins, et al., 2019). Therefore, excessively elevated LA may adversely affect cardiometabolic function independent of potential reductions in cholesterol and LDL. As already highlighted, LA modulates the ECS, and CB2 has been investigated as a potential therapeutic in several organ pathologies, including CVD. CB2 has shown to play a key role in protecting cardiomyocytes from external stressors (O'Sullivan, Kendall, & Kendall, 2012), and activity in the initial phase of ischemic/reperfusion may decrease apoptosis, reduce inflammation and maintain ventricular function (Duerr et al., 2014). On the other hand, excessively elevated LA may adversely affect heart function via the ECS.
1.4 ROLE OF LA IN NORMAL PREGNANCY

Fetal development is entirely dependent on the maternal supply of essential fatty acids (EFA), and deficiencies in maternal FA intake may have negative impacts on birth weight, and length of neonates (E. Lee, Kim, Kim, Ha, & Chang, 2018). Emerging research has identified a direct role for LA in fetal organ development, and effects on function and associated risks to later stage development in the offspring. Since LA is an essential nutrient that can only be obtained from the diet (and is vital for human organ development, energy and cell membrane maintenance) (Naughton et al., 2016), the fetus completely relies on maternal dietary LA (Bobinski & Mikulska, 2015). The optimum dietary concentration of LA in pregnancy is approximately 1.4%, and deficiency in LA is detrimental to fetal development leading to abnormal function of vital organs (Grieger & Clifton, 2014). However, whether elevated LA has detrimental effects on development of the fetus and later health, or disease risk is unknown. It is nonetheless clear that a woman’s health status before and during pregnancy is important for the outcome of her pregnancy. The importance of nutrition in early life, as a contributing factor to growth, metabolism and disease risk in the later life of the offspring, is relatively well researched (Patel, Srinivasan, & Laychock, 2009). Nonetheless, much remains to be detailed in terms of the roles and effects of essential factors such as PUFAs.

1.4.1 Role for LA in cardiovascular development

Research has revealed relationships between the intrauterine environment and adult disease risks and shown that the prenatal period is key in governing normal growth and fetal organ development (Patel et al., 2009). During embryogenesis 4 weeks after fertilization the cardiovascular system is first developed (Stock & Vacanti, 2001). Maturity follows after 4 months and cardiac development is completed after 5 months in humans (Stock & Vacanti, 2001). During the lactation period the mother’s breast milk contains essential nutrients which
not only improve immunity but is additionally important for infant organ development through to adolescent maturation (Ballard & Morrow, 2013). Maternal diet is an important determinant of the fatty acid composition of breast milk, ensuring the health of developing and functioning organs (Ballard & Morrow, 2013). However, findings are sometimes controversial. One study found a diet elevated in PUFAs and reduced in total saturated fats did not support current recommendations for cardiovascular health (Chowdhury et al., 2014). A study of newborn rats has demonstrated chronic hyperinsulinemia and adult-onset obesity after exposure to increased carbohydrate intake (Patel et al., 2009). These female rats passed on an obesity phenotype to their offspring, inducing a trans-generational outcome (Patel et al., 2009). Research on immature myocardium of fetal hearts shows limited functional ability for cardiac output in utero, while cardiac output increases after birth with a vital role for prenatal thyroid hormone myocardial maturation and postnatal development (Teitel & Rudolph, 1985). Recent research shows elevated LA during pregnancy increases maternal inflammation and effects development of offspring. In addition, elevated inflammation is likely to result in increased disease risk in the mother. Chronic low-grade inflammation and associated obesity in pregnant mothers is a common factor that increases the risk of later metabolic disorders in adult offspring (Segovia, Vickers, & Reynolds, 2017). Reports are now showing maternal obesity has long-term effects on the health of offspring throughout their adult life, including obesity, metabolic (glucose/insulin) and vascular dysfunction, and hypertension.

Our group’s recent work demonstrates that maternal consumption of elevated LA prior to and during pregnancy in rats increases plasma AA - a LA metabolite and ECS ligand precursor - in the offspring just before birth (Shrestha, Cuffe, Holland, Perkins, et al., 2019). However, whether elevated maternal LA alters offspring growth and development is unclear. Recent research shows elevated LA during pregnancy increases inflammation, which can affect development of offspring (Shrestha, Cuffe, Holland, Perkins, et al., 2019). In addition, our
recent study demonstrated that elevated maternal LA significantly decreased circulating leptin (Shrestha, Cuffe, Holland, Bulmer, et al., 2019). Leptin, in addition to its role as an adipokine, modulates organogenesis. During development and maturation leptin is vital for cardiovascular system, brain, kidney and pancreas development. Altered leptin levels can thus perturb offspring organogenesis and development, and thus adult disease risk and onset, including cardiometabolic and renal disorders (Briffa et al., 2015). This suggests elevated maternal LA may have significant impacts on offspring organ development and disease risk. At this time, we do not know if elevated maternal LA and reduced maternal leptin has adverse effects on organ development. Compounded with this, unlike humans, organ development is not completed in utero in rodents. Notably, development of the cardiomyocyte commences prior to birth in rodents, with a full complement of binucleated cardiomyocytes present at birth in humans (Paradis, Gay, & Zhang, 2014). Following this period of hyperplasia, postnatal cardiomyocytes undergo hypertrophy in humans (Kreipke & Birren, 2015). Therefore two critical windows of development of the heart occur both before and after birth. The staggered development of the heart in rodents provides an opportunity to investigate if any adverse outcomes associated with maternal perturbations could be therapeutically countered postnatally. In rodents, there is a critical period in adolescence, between 5 and 9 weeks of life (Asif et al., 2018). During this period, positive lifestyle modifications such as exercise have the capacity to reverse potentially adverse effects of the maternal in utero environment. Previous researchers have utilized this to investigate the effects of an obesogenic diet on offspring development, and potential benefits in the adolescent period (Loche et al., 2018). Postnatal lifestyle and dietary modifications may be beneficial for heart development following an adverse maternal environment (Dickinson et al., 2016).
1.5 METABOLISM OF LA IN THE GUT

The gut microbiota can regulate energy homeostasis and whole-body metabolism (Gohir, Ratcliffe, et al., 2015). Mechanistically, this involves the digestion of polysaccharides to produce essential nutrients (Chow, Lee, Shen, Khosravi, & Mazmanian, 2010). Bacterial diversity is important for the metabolism of diverse nutrients. LA is bio hydrogenated by microbes into the saturated fatty acid stearic acid (Jenkins, Wallace, Moate, & Mosley, 2008), with a number of intermediates or bioactive metabolites (Druart et al., 2015). Biohydrogenation occurs in the rumen and plays a key role in fatty acid breakdown of meat and dairy products (Devillard, McIntosh, Duncan, & Wallace, 2007). Diet has a strong influence over the host’s ecosystem, involving metabolic, immune and microbiome interactions and crosstalk with the host and its overall physiology (Boulange, Neves, Chilloux, Nicholson, & Dumas, 2016). Therefore, maintenance of a health pregnancy may in part involve maintenance of a healthy gut microbiome. The diversity of gut microbiota is associated to a range of host mechanisms influencing health, including lipid levels and forms, and inflammatory processes (H. C. Lee et al., 2019). Studies show Roseburia species in human gram-positive intestinal bacteria to be the most active in metabolising LA (Devillard et al., 2007). In a recent study, diets high in PUFA produced PUFA-derived metabolites, and is congruent with previous studies (Druart et al., 2015). The human colon metabolises LA via conjugated LA to vaccenic acid (both beneficial to health), and then to stearic acid (Devillard et al., 2007). A recent study showed changes in gut microbiota controls metabolic endotoxemia, inflammation and gut permeability providing further understanding the causative of diseases and strategies to control gut microbiota. Mice fed on a high LA diet exhibited increased levels of metabolic endotoxemia and inflammation (Cani & Delzenne, 2007). Emerging research has also demonstrated that pregnancy can impact the diversity of gut microbiota (Gohir, Whelan, et al., 2015). In pregnancy, hormonal
alterations modulate the maternal metabolic environment to ensure appropriate fetal nutrition. This places the mother in a state of metabolic dysfunction that becomes more overt as the pregnancy advances. This state of metabolic dysfunction can be further impacted by diet and contributes to pregnancy disorders that occur when physiological metabolic dysfunction progresses to pathology. Current hypotheses suggest that changes to the gut microbiota, under the influence of pregnancy specific hormones, contribute to pregnancy associated metabolic changes (Gomez-Arango et al., 2016). Further, alterations in the maternal gut microbiota during pregnancy can alter the microbiome and immune system of offspring later in life (Nyangahu et al., 2018). However, at present we do not know if elevated maternal LA consumption alters the maternal microbiome during pregnancy, which may in turn influence offspring development.

Sexual dimorphism can occur in the development and function of the cardiovascular system in animals, where sex specific differences occur between male and females via altered gene expression on sex chromosomes in response to perturbations in physiology (Isensee et al., 2008). For example, one study showed estrogen to be cardioprotective post-menopausal and testosterone to be deleterious to cardiac function, and that cardiac functioning is dependent on sex based differences (Prabhavathi, Selvi, Poornima, & Sarvanan, 2014).

1.6 HYPOTHESIS AND AIMS

The general hypothesis of this study is that elevated maternal LA before and during pregnancy is deleterious to maternal cardiometabolic health, and adversely affects offspring cardiovascular development and function, an effect that may be reversed postnatally. This work and testing this hypothesis requires development of a model in which a maternal diet high in LA alters pro-inflammatory mediators and leptin, and modifies metabolic health including the
microbiome. This adverse prenatal environment may lead to adverse organ development, which can be reversed by postnatal modification of dietary LA.

1.7 PURPOSE

The purpose of this project is to determine the relationships between elevated LA consumption during pregnancy, maternal health, and cardiac development and function in offspring.

1.8 OBJECTIVES

The specific objectives of this study are thus to investigate if elevated maternal LA consumption during pregnancy alters:

1) the maternal microbiome
2) cardiomyocyte viability and respiration, and the maternal cardiac ECS
3) the ECS in embryonic E20 hearts
4) cardiovascular development and function, and gene expression in postnatal PN40 offspring, in a manner that may be reversible with a low LA diet

1.9 SIGNIFICANCE AND SCOPE

Currently, the long-term health consequences of excessive maternal LA consumption on the cardiac development of her offspring are unknown. The findings of this project will determine if the consumption of a high LA diet negatively impacts the development of the heart in her offspring, which may have long-term consequences on cardiovascular disease risk. This study may assist in developing a message for good health for the general public, and lead to the formulation of guidelines for daily intake of LA in pregnant women. This novel project will be the first to establish if increased maternal LA intakes has negative effects on fetal
growth, with a specific focus on the development and function of the heart, and whether these effects are different between males and females.
Chapter 2: Materials and Methods

IN-VIVO STUDY

2.1 ANIMAL MODEL

Wistar Kyoto rats (8 weeks of age) were purchased from the Australian Resource Centre (ARC, WA, Australia) and separated into low linoleic acid (LLA) and high linoleic acid (HLA) dietary groups. Rats were acclimatized in accordance to the Australian Code of Practice for Care and Use of Animals for Scientific Purpose after ethical approval being granted by the Griffith University Animal Ethics Committee (NSC/01/17/AEC).

<table>
<thead>
<tr>
<th>Energy (%) derived from</th>
<th>LLA</th>
<th>HLA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbohydrate</td>
<td>56.8</td>
<td>56.8</td>
</tr>
<tr>
<td>Protein</td>
<td>19.4</td>
<td>19.4</td>
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<tr>
<td>Crude fibre</td>
<td>4.7</td>
<td>4.7</td>
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<tr>
<td>AD fibre</td>
<td>4.7</td>
<td>4.7</td>
</tr>
<tr>
<td>Others</td>
<td>4.5</td>
<td>4.5</td>
</tr>
<tr>
<td>Fat</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>SFA</td>
<td>1.36</td>
<td>1.04</td>
</tr>
<tr>
<td>MUFA</td>
<td>5.82</td>
<td>1.72</td>
</tr>
<tr>
<td>18:2n-6 (LA)</td>
<td>1.44</td>
<td>6.21</td>
</tr>
<tr>
<td>18:3n-3 (ALA)</td>
<td>0.36</td>
<td>0.3</td>
</tr>
<tr>
<td><strong>n-6/n-3</strong></td>
<td><strong>4.0</strong></td>
<td><strong>20.7</strong></td>
</tr>
</tbody>
</table>

Table 1. Composition (% energy) of experimental diets.

LLA: low linoleic acid; HLA: high linoleic acid; SFA: saturated fatty acid; MUFA: monounsaturated fatty acid; LA: linoleic acid; ALA: alpha-linolenic acid (Shrestha, Cuffe, Holland, Perkins, et al., 2019).

After one week of acclimatization, the housing and specialised feeding of rats was followed in accordance with previous work (Shrestha, Cuffe, Holland, Bulmer, et al., 2019). The control group consumed a low linoleic acid diet (LLA: ~2% energy) while the experimental group...
consumed a high linoleic acid diet (HLA: ~6% energy, the average daily LA consumed in Australia). Composition (% energy) of the diets is shown in Table 1. In our preliminary experiments, these diets are significantly different in their concentration of LA and ALA as described in (Nirajan’s paper JPhysio).

The E20 pregnant female rats and their offspring were sacrificed (E20 to investigate embryonic development and harvest placenta used in another study). A second cohort were allowed to wean at PN25, and randomly allocated to feed on the LLA or HLA diets. These offspring were sacrificed at PN40 (PN40 was harvested because the cardiovascular development was fully completed), following 2 weeks consuming the post weaning diet (Figure 3). Both groups of rats were sacrificed as previously described (Shrestha, Cufte, Holland, Bulmer, et al., 2019). The left ventricle of maternal hearts and whole hearts of fetal rats were harvested and snap frozen immediately in liquid N₂ before storage at -80°C until RNA extraction and analysis.

**Figure 3: Rat Model.**
Mothers consume either low LA (LLA) or high LA (HLA) for 10 weeks prior to mating and during pregnancy. At weaning, offspring are randomly allocated to either an LLA or HLA diet, giving rise to the following groups: LL, LH, HL and HH, with the first letter representing the mothers diet and the second letter representing the weaning diet. Groups: Mothers E20 (2 groups), Offspring E20 (2 groups) and Offspring PN40 (4 groups).

2.2 SEX DETERMINATION OF FETUS AT E20

DNA were extracted from the tail of each fetus. Sex determination was undertaken as described previously (Shrestha, Cuffe, Holland, Bulmer, et al., 2019), using qPCR amplification of SRY (sex-determining region Y) gene with a commercially available hydrolysis probe (Rn04224592_ul; NM_012772.1; Applied Biosystems).

2.3 QUANTITATIVE POLYMERASE CHAIN REACTION (QPCR)

Total RNA were extracted from E20 maternal and whole fetal heart tissue, following previously published methods (Shrestha, Cuffe, Holland, Bulmer, et al., 2019). Briefly, maternal heart tissue were homogenised in lysis buffer supplemented with Proteinase K and incubated at 57°C for 10 min. The RNA was synthesised to reversed transcription complementary DNA using iScript gDNA clear cDNA synthesis kit (Bio-rad) following manufacturer’s guidelines. Real time qPCR was performed using QuantiNova SYBR® green master mix (Qiagen) following manufacturer’s guidelines, in line with Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin et al., 2009). Initial holding stage were set at 95°C for 10 min and recommended prior to PCR cycling to fully denature the DNA. qPCR reaction runs were set for 40 cycles. Denature stage were set for 15 seconds at 95°C and extension were set at 60°C for 1 minute using StepOne™ real-time PCR systems (Applied Biosystems). The $2^{-\Delta\Delta C_q}$ method were used to quantify gene expression and normalised to geometric mean of β-actin and β-2 microglobulin as reference.
genes as previously prescribed (Shrestha, Cuffe, Holland, Bulmer, et al., 2019). KiCqStart™ pre-designed primers from Sigma- Aldrich as shown in PCR products were validated by sequencing the above primers using the Applied Biosystems 3130x1 Genetic Analyzer, following clean-up using ExoSAP-IT, performed at the Griffith University DNA sequencing facility.

Table 2. List of Primer Targets and KiCqStart Primer Numbers used for QPCR

<table>
<thead>
<tr>
<th>Primer Targets</th>
<th>KiCqStart™ primer numbers</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Housekeepers</strong></td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td>NM_031144</td>
</tr>
<tr>
<td>β-2 microglobulin</td>
<td>NM_012512</td>
</tr>
<tr>
<td><strong>Endocannabinoid signalling</strong></td>
<td></td>
</tr>
<tr>
<td>Leptin</td>
<td>NM_013076</td>
</tr>
<tr>
<td>Leptin receptor</td>
<td>NM_012596</td>
</tr>
<tr>
<td>GPR18</td>
<td>NM_001079710</td>
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<tr>
<td>GPR55</td>
<td>XM_001063474</td>
</tr>
<tr>
<td>FAAH</td>
<td>NM_024132</td>
</tr>
<tr>
<td>IL-6</td>
<td>NM_012589</td>
</tr>
<tr>
<td>DAGL-α</td>
<td>NM_001005886</td>
</tr>
<tr>
<td>DAGL-β</td>
<td>NM_001107120</td>
</tr>
<tr>
<td>CB1</td>
<td>NM_012784</td>
</tr>
<tr>
<td>CB2</td>
<td>NM_020543</td>
</tr>
<tr>
<td>TNF-α</td>
<td>NM_001107387</td>
</tr>
<tr>
<td>NAPE-PLD</td>
<td>NM_199381</td>
</tr>
</tbody>
</table>
### Cardiac Dysfunction

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOTCH1</td>
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</tr>
<tr>
<td>HIF1A</td>
<td>NM_024359</td>
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<tr>
<td>WNT2</td>
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<td>TGFB1</td>
<td>NM_021578</td>
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<tr>
<td>COL1A1</td>
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</tr>
<tr>
<td>COL3A1</td>
<td>NM_032085</td>
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<tr>
<td>NPPA</td>
<td>NM_012612</td>
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<tr>
<td>NPPB</td>
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<tr>
<td>ATP2A2</td>
<td>NM_001110139</td>
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<tr>
<td>MYH6</td>
<td>NM_017239</td>
</tr>
<tr>
<td>MYH7</td>
<td>NM_017240</td>
</tr>
</tbody>
</table>

### 2.4 LANGENDORFF HEART MODEL

After measurement of body weight PN40 rats were injected with pentobarbitone sodium (Pentobarbital®; 60mg/kg IP) to induce a surgical plane of anaesthesia (assessed from no pedal withdrawal response, response to tail pinch), before excision of the hearts and their perfusion in a Langendorff mode, as detailed previously (Reichelt, Willems, Hack, Peart, & Headrick, 2009; See Hoe et al., 2014). Hearts were excised into ice-cold saline, the aorta cannulated and heart perfused at a coronary pressure of 80 mmHg with modified Krebs-Henseleit buffer containing (in mM): NaCl, 119; NaHCO₃, 22; KCl, 4.7, MgCl₂, 1.2; KH₂PO₄, 1.2; EDTA, 0.5; CaCl₂, 1.85; D-glucose, 11; and Na- pyruvate, 2 (all from Sigma Aldrich, St. Louis, MO, USA), bubbled with 95% O₂/5% CO₂ to maintain pH at 7.4 at 37°C. Contractile function was measured via a fluid-filled plastic film balloon inserted in top the left ventricle and connected to a pressure transducer, with coronary flow measured via an in-line Doppler flow probe.
(Transonic Systems Inc., Clifton, NJ, USA). Functional data were continuously recorded on a 4-channel MacLab system (ADInstruments Pty Ltd.; Bella Vista, Australia) (Reichelt et al., 2009; See Hoe et al., 2014). After preparation, hearts were immersed in a 5-mL water jacketed chamber (37°C), with perfusion fluid and chamber temperatures monitored via needle thermistors. Hearts were paced at 360 beats/min via silver wire pacing leads connected to a Grass stimulator (SD9 stimulator; Grass Instruments, Quincy, MA).

2.5 REACTIVE HYPERAEMIA PROTOCOL

To assess coronary function and reactivity hearts were stabilised for a 20 min period before being subjected to 20 and 30 s occlusions (separated by a minimum of 5 min) followed by 3 min of reperfusion, as detailed previously (Kaakinen et al., 2017; Zatta, Matherne, & Headrick, 2006). Baseline and peak flows were determined, with % coronary flow repayment calculated over the initial 3 min of reperfusion (% repayment = 100 x [post-occlusion flow - pre-occlusion flow] / occlusion flow debt).

2.6 VENTRICULAR PRESSURE-VOLUME RELATIONSHIPS (PVRs)

After assessment of hyperaemic responses, systolic and diastolic PVRS were acquired in each heart, as outlined previously (Kaakinen et al., 2017). Briefly, the ventricular balloon volume was adjusted to yield a systolic pressure of zero over a 10 min stabilisation period. Left ventricular balloon volume was then incrementally increased in 2.65 µl steps using a 500 µl threaded syringe (Hamilton; Reno, NV, USA). Contractile function was assessed after 2 min at each LV volume. Experiments were terminated when end-diastolic pressure exceeded 30 mmHg.

2.7 DNA EXTRACTION OF MATERNAL RAT FAECES

Maternal faecal samples were collected in the morning between 10:00-11:00 am, weighed, and immediately frozen at -20°C. Following a similar study design (Bustin et al.,
2009) to extract DNA from stool a QIAamp DNA Stool Mini Kit (Qiagen) will be used for 1gm of frozen stool. Briefly, the ASL buffer helps lysis of bacterial cells in the stool, InhibitEX reagent destroys impurities and use of spin column for purification process of DNA. Total volume of eluted DNA of 200 µL is stored -20°C. If exploring different microbial strains, the methodology would be similar to applied stool samples.

2.8 STATISTICAL ANALYSIS OF IN VIVO STUDIES

Values are mean ± SEM, and Grubbs’ test was used to identify outliers. Data was analysed for normality. Parametric tests were done if normal and non-parametric if not normal. An F-test was used to compare variances. Paired/unpaired t-tests, Mann–Whitney U tests (for unequal variances in unpaired samples) or Kruskal–Wallis test (for unequal variances in unpaired multi-group samples) were used for comparisons. Differences between interventions and groups were compared by two-way ANOVA with repeated measures followed by Sidak’s post-hoc testing was employed. In all tests a P≤0.05 was considered significant, with all analyses performed using Prism 7 (GraphPad Software, Inc; San Diego, CA, USA).

IN-VITRO STUDY

2.9 CELL CULTURE AND TREATMENT WITH LA

The cardiac myoblast H9c2 cell line (ATCC® CRL-1446™) was cultured in DMEM supplemented with 10% FBS and 1% penicillin and streptomycin. Shrestha et al. detailed the isolation and characterization of the Swan71 cell line (Shrestha, Cuffe, Holland, Perkins, et al., 2019). Cells (at 30-40 passage) were cultured in humidified incubator at 37°C with 5% CO₂. LA and BSA complex was prepared in the molar ratio of 5:1 and incubated at 37°C for 15 min. Cells were transferred into serum free media for 2 hours after they were ~70% confluent. After 2 hours, cells were treated with vehicle control (BSA + 0.1% ethanol) or different
concentrations of LA-BSA complex (12.5, 25, 100, 200, 300, 400, 500 and 1000 µM) for 24 hours. Physiological LA concentration is approximately 100 µM, however concentrations up to 1000 µM have been quantified (see Nirajan’s paper).

2.10 MTT ASSAY

H9c2 cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin and streptomycin and maintained in a humidified incubator at 37°C with 5% CO₂. Preparation of LA and bovine serum albumin (BSA) complex were in a ratio of 5:1. BSA was used as a transporter for the uptake of LA for the cells. 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 (lacking LA) for 2 hours before treatment with increasing concentrations of LA-BSA complex (12.5, 25, 100, 200, 300, 400, 500 and 1000 µM) for 24 hours, similar to our previous study (Shrestha, Cuffe, Holland, Perkins, et al., 2019). Cells were incubated in 1 mg/ml of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) (Sigma Aldrich, St. Louis, MO, USA) for 2-4 hours. The medium with MTT were removed and 130 µL of dimethylsulfoxide (DMSO) is added in each well and incubated for 30 min to dissolve the formazan compound. Absorbance were measured at 540 nm using Infinite M Plex microplate reader (TECAN, Switzerland) and relative cell viability normalized to control viability.

2.11 SEAHORSE OXYGRAPH

H9c2 cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin and streptomycin, maintained in a humidified incubator at 37°C with 5% CO₂. The Seahorse XF analyser was turned on the day before analyses. A MXFp FluxPak (Part # 103022-100) was used according to manufacturer’s instructions, containing Sensor Cartridge, utility plate,
cartridge lid, XFp Miniplate with lid and XF Calibrant (100 mL). H9c2 cells were harvested at 70-80% confluency and plated at 7x10^3 on the XFp Miniplate filling wells from B to F (A and G left empty as controls) in DMSO medium and left overnight in a CO2 incubator to allow cells to attach. The sensor cartridges plate was prepared and hydrated. The conical tube of calibrant were removed, and each well of the utility plate filled with 200 µL of pre-warmed XF Calibrant (400 µL for outside wells). The sensor cartridge was replaced onto the utility plate to submerge the sensors in the calibrant. A container was lined with paper and small amount of water and the sensor cartridge placed into container. The sensor cartridge was placed in a non-CO2 37°C incubator overnight prior to loading the injection ports of the sensor cartridge.

Seahorse XF DMEM medium is supplemented with 200 µL (x100 dil:200 mM glutamine stock) and warmed in 37°C 20 min prior to use. While keeping the medium warm, 1.0 N NaOH was added for pH adjustment, 15 µL at a time until reaching an optimal 7.4 pH. The medium was filtered using a 0.22 µM filter for sterilisation. Media was aspirated off cells, which were then washed with phosphate buffered saline (PBS). Wells B to D were subjected to LA treatment (12.5 µM), and wells E to G received the control BSA treatment for 2 hours. Treatment solutions were then aspirated off and cells equilibrated with the Seahorse XF medium (180 µL per well) for 45 min at 37°C in a CO2-free incubator. Compounds from the glycolysis test kit containing glucose 30 nmol, oligomycin 14.4 nmol and 2-DG 150 nmol were reconstituted with XF Seahorse media and loaded in the XFe sensor cartridge following manufacturers guidelines. The Xfe sensor cartridge was loaded into the Seahorse analyser for calibration and the cell miniplate then transferred to the XF analyser. The protein was quantified by the BSA protocol and samples were standardised for protein content.
2.12 STATISTICAL ANALYSIS FOR IN VITRO STUDIES

Data from MTT assay are expressed as mean ± standard error (SEM). Multiple comparisons among groups was performed by one-way ANOVA followed by a Dunnett post-hoc test using GraphPad software (GraphPad, San Diego, CA). P values <0.05 were considered statistically significant.

Data from the Seahorse XF assay were reviewed and statistical analysis was carried out using GraphPad software. LA treated H9c2 cells was analysed via non-parametric Kruskal-Wallis test comparing different treatment samples followed by Dunn’s multiple comparison post-hoc test. P values <0.05 were considered indicative of significance and values reported as mean ± standard error of the mean (SEM).

Chapter 3: The Effect of LA on The Maternal Microbiome

The aim of this Chapter was to test whether elevated maternal LA consumption prior to and during pregnancy alters maternal microbiome diversity. Female rat faecal samples were collected following 10 weeks of nutritional intervention and DNA extracted as described in section 2.7. The Australian Genome Research Facility (AGRF) performed 16s sequencing of the V1-V3 region of the 16s rRNA gene using forward primer AGAGTTTGATCMTGGCTCAG and reverse primer GWATTACCAGGGCKGCTG, and amplified the 27F-519R target as previously described.
3.1 RESULTS

*Gut microbiota phylum in response to an HLA diet.*

Microbiota profiles are presented at the phylum level. There was an effect of gestational age for *Actinobacteria*, with a peak of expression at E10 (Figure 4A, p<0.04) and *Tenericutes* with a peak of expression at E20 (Figure 4H, p<0.016). Diet had an effect for *Cyanobacteria* (Figure 4C, p<0.02) and *Lentisphaerae* (Figure 4E, p<0.0003) which were elevated in HLA mothers. *Verrucomicrobia* showed an effect of diet, where it was decreased in HLA mothers (Figure 4I, p<0.004). Further the abundance of *Verrucomicroia* was affected by pregnancy (Figure 4I, p<0.0257).
Figure 4: Gut microbiota phyla abundances in response to an HLA diet and gestational age.

Microbiota profiles are presented at the phylum level. There was an effect of gestational age for Actinobacteria, with a peak of expression at E10 (Figure 4A, \( p<0.04 \)) and Tenericutes with a peak of expression at E20 (Figure 4H, \( p<0.016 \)). Diet had an effect for Cyanobacteria (Figure 4C, \( p<0.02 \)) and Lentisphaerae (Figure 4E, \( p<0.0003 \)) which were elevated in HLA mothers. Verrucomicrobia showed an effect of diet, where it was decreased in HLA mothers (Figure 4I, \( p<0.004 \)). Further the abundance of Verrucomicrobia was affected by pregnancy (Figure 4I, \( p<0.0257 \)). Data are expressed as Mean±SEM.
Chapter 4: The Effects of LA on Cardiomyocyte Cell Viability and Mitochondrial Respiration and E20 Maternal ECS

The aim of this chapter was to i) investigate if elevated maternal LA consumption during pregnancy alters maternal and offspring cardiac ECS (in-vivo) ii) characterise cardiomyocyte viability and respiration (in-vitro). To investigate the effects of elevated LA on cardiomyocyte function: in vitro H9c2 cells were exposed for 2 hours to elevated LA and BSA according to methods in section 2.9; maternal hearts and whole fetal hearts were used for QPCR to analyse cardiac ECS as outlined in section 2.3; and cardiomyocyte viability was analysed using MTT assay on LA treated cells according to section 2.10. Maternal hearts were also isolated at E20 and RNA extracted as described in section 2.3.

Please note that Figures 5 and 8 have been published (Sleep et al., 2019 Journal of Developmental Origins of Health and Disease; Appendix B).

4.1 RESULTS

Effect of high LA acute treatment on H9c2 cardiomyocyte metabolic activity, respiration and glycolytic metabolism.

Cell viability (or metabolic activity) in H9c2 cardiomyocytes - assessed using an MTT assay - showed a significant decline following 24-hour exposure to 300-1000 µM LA, (Figure 5). Examining metabolic effects of 2 hour LA exposure in H9c2 cells using the Seahorse system, data reveal no significant difference in non-glycolytic acidification, glycolysis, glycolytic capacity, OR glycolytic reserve (absolute or %) (Figure 6). The representative OCR
and ECAR results (Figure 6) demonstrate that both mitochondrial respiration and glycolytic activity, respectively, were unaltered in response to acute LA exposure.

**Effect of high LA on maternal heart mRNA expression profiles**

There was a significant reduction in maternal CB₂ mRNA expression in the hearts of rats fed the HLA vs. LLA diet (p<0.05, Figure 7A). There was no difference in maternal mRNA expression for GPR18, DAGL-α and DAGL-β, NAPE-PLD, LEPR or TNF-α (Figure 8B–8F). Maternal mRNA levels for IL-6, CB₁, leptin, GPR55, GPR18 and FAAH were below the level of detection (no data shown).

![Figure 5. Effects of LA on H9c2 cardiomyocyte metabolic activity (MTT assay).](image)

Cardiomyocyte metabolic activity after 24 hr experimental treatments assessed via MTT assay in cells seeded at 80% confluence. Data expressed as Mean±SEM (n 6 for all treatment conditions). ***p < 0.001, ****p < 0.0001 compared to vehicle control (BSA+EtOH).
Figure 6: Effects of LA acute treatment on H9c2 cardiomyocyte respiratory and glycolytic activities.

Cardiomyocyte glycolytic stress test and metabolic activity after 2 hr experimental treatments assessed via Seahorse assay in cells seeded at 7x10^4. No significant difference compared to vehicle control (BSA+EtOH). Data expressed as Mean±SEM, n = 5 for treatment conditions.
Figure 7: Elevated maternal LA consumption alters endocannabinoid signalling in maternal hearts.

mRNA expression of CB2 is significantly increased in HLA vs. LLA (panel A). The following genes showed no significant change; DAGL-α, DAGL-β, NAPE-PLD, LepR, and TNF-α (panels B to F). n=6 (LLA) and n=8 (HLA). Data are expressed as Mean ±SEM, *p<0.05.
Chapter 5: The Effects of LA on Offspring E20 Rat Hearts

Chapters 3 and 4 describe evidence that an elevated maternal LA consumption significantly affects cardiometabolic status and the ECS within the mother. This leads to the hypothesis that an elevated maternal LA diet may alter offspring embryonic E20 hearts via shifts in the ECS. Offspring rats were generated as described in section 2.1. At E20, offspring hearts were removed, and RNA extracted as described in section 2.3.

5.1 RESULTS

Effect of high maternal LA on fetal hearts and mRNA expression

In a previous study we showed there was no change in body or heart weight in offspring from mothers consuming a high LA diet (Shrestha, Cuffe, Holland, Bulmer, et al., 2019). Cardiac expression of CB$_2$ mRNA was significantly decreased in both male and female offspring of mothers consuming an HLA diet (Figure 8). There was no change in cardiac mRNA expression of GPR18 in male or female offspring, while cardiac mRNA for FAAH was significantly decreased in male compared with female offspring. Cardiac NAPE-PLD mRNA was significantly decreased in female offspring while significantly increased in male offspring of mothers consuming HLA. There were no significant changes in mRNAs for DAGL-α, DAGL-β, leptin or TNF-α in offspring hearts (Figure 8). Levels of mRNA for IL-6, CB$_1$, GPR55 and LepR were below the limit of detection (data not shown).
Figure 8: Elevated maternal LA consumption during pregnancy alters endocannabinoid signalling in offspring hearts. Cardiac mRNA expression of CB2 was significantly decrease in HLA fed males vs. females (Figure A, ≤P0.05), and FAAH was significantly decreased in males vs. females (Figure C, ≤P0.05). The was no significant change in GPR18, NAPE-PLD, DAGL-α, DAGL-β, Leptin and TNF-α (Figure B, D-H). Offspring hearts, n=6 (LLA) and n=6 (HLA). Data are expressed as Mean±SEM, *p< 0.05 as published.
Chapter 6: The Effects of Dietary LA on PN40 Rat Hearts

Chapter 5 describes how elevated maternal LA consumption alters the ECS in offspring hearts in a sex-specific manner. This leads to the hypothesis that elevated maternal LA consumption may alter postnatal cardiovascular development and function via alterations to the ECS, an effect potentially reversed with a low LA diet. Offspring rodents were generated as described in section 2.1. At PN25, offspring were allocated to an LLA or HLA diet group as described in section 2.1. At PN40, heart tissue was excised as previously described and functional analyses carried out as described in section 2.3 and 2.4. Cardiac perfusions described in sections 2.5 and 2.6 were undertaken working with Prof. John Headrick, with the candidate assisting with the animal handling, anaesthesia and tissue isolation, data collection and analysis and interpretation of all data. Myocardial RNA was extracted as described in section 2.3.

6.1 RESULTS

Coronary Function & Reactivity

Coronary flow and reactivity were measured in hearts performing minimal work (uninflated ventricular balloon), with vasodilatory responses to 20 and 30 second occlusions assessed (reactive hyperaemia) (Figure 9). High maternal linoleic acid diet did alter baseline coronary flow for interaction and were detected in female 20 s and 30 s which was assessed prior to both 20 and 30 sec coronary occlusions (Figure 10). This was consistent with no differences in baseline ventricular pressure work (discussed further below). In females there was a trend to lower hyperaemic flows in HHF vs. other dietary groups, while LHF hearts
exhibit slightly elevated hyperaemic flows (Figure 9). Differences in hyperaemic responses were less evident in male hearts. Peak hyperaemic flow rates (with 20 or 30 s occlusions) did differ between interaction and maternal dietary female groups (Figure 11). However, the % debt recovery was significantly reduced in the LHF compared to LLF group and differences in maternal and offspring effects (Figure 12).

**Cardiac Function**

To assess cardiac mechanical properties in hearts of PN40 rats, left ventricular pressure volume relationships (PVRs) were acquired, with data reported for end diastolic and systolic function (Figure 13). In females, the EDP PVR exhibited a clear leftward shift in the HHF vs. other group, reflecting increased diastolic stiffness (Figure 13). This was confirmed by a decline in the ventricular volume yielding 15 mmHg EDP in the HHF vs other groups and a change in interaction effect. No clear differences in EDP PVRs were evident in males.

Initial and final slopes for the EDP PVRs for PN40 hearts are shown in Figure 14. The extracellular matrix has negligible influence on passive tension over normal cardiac stretch or loads (initial slope): intracellular elements govern diastolic stiffness (Linke, Popov, & Pollack, 1994) with myofibrillar proteins and specifically titin the dominant determinants. Stiffness at higher loads is largely influenced by the extracellular matrix (final slope). The initial slopes of males showed a high maternal linoleic acid diet had significant increase effect in the LHM group compared to the HLM and HHM groups and a change effect on the maternal diet. (Figure 14). There was no effect for initial and final slopes of female diastolic PVRs, or final slopes in males (Figure 14). However, initial slope of the EDP PVR was reduced in HHM and HLM vs. LHM hearts, supporting an increased cardiac compliance (Figure 14).

Full systolic PVRs are shown in (Figure 13), with the initial linear slopes (from 0-20 µL LV volume) and the LV volume yielding 100 mmHg systolic pressure shown in (Figure
15). These data reflect the inotropic or contractile status of the heart (i.e. rise in systolic force relative to ventricular volume). A high maternal linoleic acid diet had no effect on the slopes of the systolic PVRs for hearts from either male or female PN40 rats. LV volume required to generate 100 mmHg systolic pressure was also not significantly altered (Figure 15).

In terms of body and heart weights, a significant reduction in body weight was observed in LHF compared with HHF female rats and a change effect for interaction. There was no change in body weights among male groups. The heart weight showed no significant change for both female and male groups, and the ratio of heart to body weight showed no changes across groups (Figure 16).

**mRNA Expression in heart perfused PN40 rats**

Analysis of offspring hearts exposed to a high maternal linoleic acid consumption, demonstrated that DAGL-α expression decreased in the LHM compared to HLM group and significant increase in HLM compared to HHM group. The LepR mRNA expression had decreased in the LHM compared to HLM group. There was no further significant change observed with genes associated with the ECS for either male or female groups (Figure 17).

There was an apparent effect of a high maternal linoleic acid diet on hypertrophy related mRNA expression in male (not female) PN40 hearts. Notch1 mRNA expression was increased in HLM compared with LHM hearts, NPPA mRNA expression was increased in HLM compared to LLM and HLM hearts, and COL1A, COL3A and ATP2A mRNA had a maternal diet effect for male (Figure 18). The NPPB mRNA expression had an offspring diet effect for female rats (Figure 18). NPPA mRNA expression had a significant increase LLM compared to HHM group and decreased LHM compared to HLM (Figure 18).
Figure 9: Effect of maternal LA consumption on coronary flow and reactive hyperaemia in perfused hearts of PN40 rats. Coronary reactive hyperaemic responses to 20 and 30 s occlusions are shown for female hearts (n = 6) in (Figures A and B); and male hearts (n=6) in (Figures C and D). Data are expressed as Mean±SEM.
Figure 10: Effect of maternal LA consumption on baseline coronary flow for Langendorff perfused hearts from PN 40 rats. Baseline coronary flow rates were determined prior to 20 and 30 s occlusions. Interaction changes were detected at female 20 s (Figure B, ≤P0.02) and 30 s (Figure D, ≤P0.03) occlusions for female rats. There was no significant change for 20 s and 30 s male groups (Figures A-C). Data are expressed as Mean±SEM.
**Figure 11:** Effect of maternal LA consumption on peak hyperaemic coronary flows in Langendorff perfused hearts from PN 40 rats. Peak flows were determined following 20 and 30 second occlusions. There was significant interaction differences detected for 20 s occlusion female group (Figure B; ≤P0.01). There was no significant changes for the male group (Figure A). There was significant changes detected for 30 s occlusion female group in LHF vs. HHF groups (Figure D, ≤P0.01) and there was change in maternal diet (Figure D; ≤P0.04) and interaction (Figure D; ≤P0.04). There was no significant differences detected for 30 s male rats (Figure C). Data are expressed as Mean±SEM (females n=6 males n=3-6).
Figure 12: Effect of maternal LA consumption on % debt repayment during reactive hyperaemia in perfused hearts from PN40 Rats. % debt repayment was measured over 3 min of reperfusion following 20 or 30 second occlusions in female and male hearts. A trend to greatest repayment in LLF and LLM vs other groups was evident, though only achieved statistical significance for LLF compared to HLF groups in the 20 s experiment. There was significant change in female maternal diet (Figure B, ≤P0.04) and interaction (Figure B, ≤P0.03) and no differences for 20 s male rats and 30 s female and male rats. Data are expressed as Mean±SEM (females n=6 males n=3-6)
Figure 13: Effect of maternal LA consumption on LV end-diastolic and systolic pressure-volume relationships in perfused hearts from PN40 Rats. Top figures (A-D) show diastolic (left) and systolic PVRs (right) for males and females. The female ventricular volumes yielding a 15 mmHg EDP (V15) show a significant change in HLF vs. HHF (Figure E; ≤P0.04) and there was change in interaction (Figure E; ≤P0.01). There was no change to the male group.
(Figures F) showing the ventricular volumes yielding a 15 mmHg EDP (V15). Data are expressed as Mean±SEM.
**Figure 14: Effect of maternal LA consumption on slopes of end-diastolic pressure-volume relationships in perfused hearts from PN40 rats.** There was significant difference of LHM compared to HLM groups and HLM compared to HHM groups (Figure B; ≤ P0.03). There was change for male offspring diet rats (Figure B; ≤ P0.001). There was no significant change for female rats (Figure A). Final slopes of EDP PVRs were determined for males and females (Figures C-D). The EDP PVR exhibits a biphasic response (Figure 13 and lower panel here). The initial and final slopes (determined as shown in Figure E) predominantly reflect influences of the passive compliance of intracellular elements (initial) and of the extracellular matrix (late), respectively. Data are expressed as Mean±SEM.
Figure 15: Effect of maternal LA consumption on slopes of systolic PVRs (0-20 µL volume), and the LV volume yielding 100 mmHg systolic pressure in hearts from PN40 rats. The linear slope of the systolic PVRs (shown in Figure 13) were determined from 0 to 20µL LV volumes and are shown in (Figures A-B). LV volume yielding 100 mmHg systolic pressure is shown in (Figures C-D). Data are expressed as Mean±SEM.
Figure 16: Effect of maternal LA consumption on heart and body weights in PN40 rats.

Body weight data for female rats showed a significant change in LHF vs. HHF (Figure B, ≤P0.01) and interaction difference (Figure B, ≤P0.003) and no significant change for the male group (Figure A). There was no significant change to heart weight for both groups (Figures
C-D). The ratio of heart to body weight data showed no significant change for both groups (Figures E-F). Data are expressed as Mean±SEM.
Figure 17: The effects of maternal LA consumption on metabolic and inflammatory gene expression in hearts from PN 40 rats. mRNA expression for multiple metabolic and inflammatory genes was determined in hearts of PN40 rats. mRNA expression for DAGL-α in males exhibited a significant decrease in LHM vs. HLM hearts (Figure C; ≤P0.007) and significant increase in HLM vs. HHM hearts (Figure C; ≤P0.04) and significant change in maternal diet (Figure C; ≤P0.03) and offspring diet (Figure C; ≤P0.007). There was no significant change for females (Figure D). LepR mRNA expression was significantly increased in LHM vs. HLM hearts (Figure G; ≤P0.03) and there was change in offspring diet (Figure G; ≤P0.005). No significant change for LepR was detected in females (Figure H). mRNA expression for CB2 (Figures A-B), DAGL-β (Figures E-F), IL-6 (Figures I-J), GPR18 (Figures K-L), FAAH (Figures M-N), TNF-α (Figures O-P) and NAPE (Figures Q-R) was unchanged in either males or females. Data are expressed as Mean±SEM.
A. COL1A Male

B. COL1A Female

C. COL3A Male

D. COL3A Female

E. ATP2A Male

F. ATP2A Female
**HIF1A Male**

- **Maternal diet**
  - Low
  - High

  - **P**  maternal diet = ns
  - **P**  offspring diet = ns
  - **P**  interaction = ns

**Relative mRNA Expression**

- LOW
- HIGH

---

**HIF1A Female**

- **Maternal diet**
  - Low
  - High

  - **P**  maternal diet = ns
  - **P**  offspring diet = ns
  - **P**  interaction = ns

**Relative mRNA Expression**

- LOW
- HIGH

---

**MYH6 Male**

- **Maternal diet**
  - Low
  - High

  - **P**  maternal diet = ns
  - **P**  offspring diet = ns
  - **P**  interaction = ns

**Relative mRNA Expression**

- LOW
- HIGH

---

**MYH6 Female**

- **Maternal diet**
  - Low
  - High

  - **P**  maternal diet = ns
  - **P**  offspring diet = ns
  - **P**  interaction = ns

**Relative mRNA Expression**

- LOW
- HIGH

---

**MYH7 Male**

- **Maternal diet**
  - Low
  - High

  - **P**  maternal diet = ns
  - **P**  offspring diet = ns
  - **P**  interaction = ns

**Relative mRNA Expression**

- LOW
- HIGH

---

**MYH7 Female**

- **Maternal diet**
  - Low
  - High

  - **P**  maternal diet = ns
  - **P**  offspring diet = ns
  - **P**  interaction = ns

**Relative mRNA Expression**

- LOW
- HIGH
Figure 18: The effect of maternal LA consumption on hypertrophy/remodelling related gene expression in hearts from PN40 rats. mRNA expression for multiple hypertrophy/remodelling related genes was determined in male and female hearts. Data show COL1A expression of male maternal diet significantly change (Figure A; ≤P0.04) and no significant change for female rats (Figure B). COL3A show expression of male maternal diet significantly change (Figure C; ≤P0.02) and no change for female rats (Figure D). ATP2A show expression of male maternal diet significantly change (Figure E; ≤P0.03) and no change for female rats (Figure F). NOTCH1 shows decreased expression of LHM compared to HLM (Figure M; ≤P0.008) and maternal diet significantly change (Figure M; ≤P0.01) and offspring diet change (Figure M; ≤P0.02). Data show no change for female rats (Figure N). NPPA mRNA
expression was significant change in LLM compared to HHM (Figure O; ≤P0.02), and LHM compared to HLM hearts (Figure O; ≤P0.01), change to offspring diet (Figure O; ≤P0.04) and interaction change (Figure O; ≤P0.04). There was no change for the female group (Figure P). NPPB mRNA expression show change in female offspring diet (Figure R, ≤P0.03), and was unaltered across male groups (Figure Q). mRNA expression from the following genes was unaltered: HIF1A (Figures G-H), MYH6 (Figures I-J), MYH7 (Figures K-L), TGFB1A (Figures S-T) and WNT2 (Figures U-V). Data are expressed as Mean±SEM.
Chapter 7: Discussion

7.1 Introduction

Pregnancy is a critical time for both mother and offspring, where maternal physiology is modified and impacts the growth and development of offspring, in turn effecting early and later life function and disease risks. The major findings from this study are that elevated maternal LA consumption: 1) alters the diversity of the maternal microbiome; 2) reduces cardiomyocyte viability without altering mitochondrial respiration; 3) modifies the maternal cardiac ECS; 4) indices distinct changes in the ECS in embryonic (E20) hearts; and 5) alters cardiovascular development and function in PN40 offspring in a sex-specific manner. These novel findings demonstrate that elevated maternal LA during pregnancy does significantly influence maternal physiology and her offspring’s growth and development in a highly sex-specific manner. Fetal development is impacted directly from maternal nutrition and effects the health for the next generation (Andraweera et al., 2016). Altered programming outcomes may affect male and female rats differently - for example, evidence indicates males may continue to grow when subjected to maternal stress, whereas females may cease growth (Dickinson et al., 2016).

7.2 Gut Microbiota

Western diets have increased (and also reduced) concentrations of a number of key nutrients, however the specific health effects of elevated LA consumption, which is pro-inflammatory, are poorly understood. Furthermore, the influence of elevated LA consumption during pregnancy on fetal development is unknown (Shrestha, Cuffe, Holland, Bulmer, et al., 2019). This study thus investigated the relationships between elevated LA consumption
during pregnancy, maternal health and ECS elements, and cardiac function, development and molecular profiles in offspring.

Elevated LA has been demonstrated to affect homeostasis and promote inflammation, which can affect metabolic function, including within the gut microbiome (David et al., 2014). Novel data presented here demonstrate that elevated LA prior to and during pregnancy alters microbiota diversity, with increased abundances of *Cyanobacteria* and *Lentisphaerae*, and a reduced abundance of *Verrucomicrobia* with HLA vs. LLA diet. Microbiota composition of the gut is well established as sensitive to dietary makeup and intake, and there is prior evidence a diet high in LA may change biome diversity (David et al., 2014). High diversity of microbiota is associated with enhanced health outcomes, and bacterial makeup influences key processes, including metabolism of lipids and production of inflammatory mediators (H. C. Lee et al., 2019). Other research has demonstrated that intake of a high fat/high sugar diet (also reflecting Western dietary changes), alters bacterial groups within 3 days, however these shifts in gut microbiota are reversible. In rats on this diet the dominant phyla were *Firmicutes*, *Verrucomicrobia* and *Bacteroidetes* (Carmody et al., 2015). Our study suggests that elevated LA consumption increases abundance of *Cyanobacteria* and *Lentisphaerae*, and reduces abundance of *Verrucomicrobia* throughout pregnancy. This suggests that the former phyla may be involved in the processing of LA. Further, prior to pregnancy, at E0, there was no difference in *Actinobacteria*, *Bacterocides*, *Firmicutes*, *Proteobacteria*, *TM7* and *Tenericutes*. This suggests that these phyla may not effectively metabolise LA in these rats. The relevance of the specific species changes is unclear. The reduced *Verrucomicrobia* have been linked to negative effects on lipid metabolism in humans (Zhang et al., 2019). Conversely, elevations are reportedly associated with improved lipid handling (Nie, Zhang, Zhao, & Du, 2019) and anti-diabetic intervention in obese animals (Zheng et al., 2020). Interestingly, and distinct from current findings for the n-6 PUFA LA,
reduced maternal n-3 PUFA intake has been associated with reduced *Verrucomicrobia* abundance (Robertson et al., 2018). Increased *Cyanobacteria* have been linked to beneficial effects on metabolism and oxidative stress (Henning et al., 2017) and lipid handling (Nie et al., 2019). However, such findings are associative and at this time there is limited research mechanistically linking specific phyla and health - future research is needed to link specific phyla changes in pregnancy with the growth and development of offspring.

7.3 **The effect of LA on cardiomyocyte survival and mitochondrial respiration**

Based upon our previous work in the placenta, it is possible LA may affect homeostasis via alteration of cell metabolic activity/viability and respiratory capacity (Shrestha, Cuffe, Holland, Perkins, et al., 2019). Energy homeostasis is dependent upon mitochondrial respiration which is measured by the OCR in oxygraphy or the Seahorse instrument employed here (Nicholls et al., 2010). ATP is generated via glycolysis to a limited extent, providing anaerobic ATP, and is predominantly generated via oxidative phosphorylation (oxygen independent). The ECAR can also be measured, this acidification reflecting glycolytic production of lactate. The ECAR measurement in the Seahorse assay thus indicates glycolytic function or stress in cardiomyocytes. Combining both OCR and ECAR provides overall mitochondrial metabolic function (Nicholls et al., 2010). In our study, LA did not alter mitochondrial respiration in the short term (2 hours). Previous research in which cardiomyocytes are exposed to palmitate (which inhibits glycolysis and can induce insulin-resistance) demonstrates that newborn cells are relatively resilient to this stimulus (Mdaki, Larsen, Weaver, & Baack, 2016), which may reflect differing glycolysis vs. fatty acid β-oxidation in neonates (Cao et al., 2019). The current study is the first to investigate the effects of LA on cardiomyocyte viability, with data indicating that H9c2 cells are sensitive to LA at ≥ 300 µM (cell reductive capacity or metabolic viability assessed from MTT assay declining
significantly). There is prior evidence PUFAs (or ECS modulation) can influence viability, for example demonstrating that reduced cell viability with 50-500 µM palmitate is countered by n-3 PUFA (Cetrullo et al., 2012).

7.4 Maternal and offspring ECS signalling

LA is metabolised into a number of downstream products that have the capacity to modulate the ECS. Our study shows that elevated maternal LA alters different ECS targets in mothers and their offspring prior and after birth. Data also demonstrate sex-specific changes to ECS targets in the offspring prior to birth and during adolescence, in response to elevated maternal LA. LA can be metabolised into 2-AG and AEA, which interact with the ECS - specifically CB₁ and CB₂ - in a cell specific manner (Nagarkatti et al., 2009). In the current study, only CB₂ was altered in response to elevated maternal LA. This may be because CB₁ is primarily localised to the central nervous system (Kendall & Yudowski, 2016). Curiously, differing changes in CB₂ expression are evident in maternal hearts (up-regulated) vs. E20 hearts (down-regulated in males and females) and PN40 hearts (up-regulated in males only).

Prior to birth, this study shows that ECS gene expression is altered by maternal consumption of elevated LA in both mothers and offspring at E20. We demonstrate that elevated maternal LA consumption does not modify cardiac FAAH mRNA, though there is evidence for higher FAAH mRNA levels in females vs. males. NAPE-PLD mRNA also exhibited sex-dependence, with an insignificant trend to a decrease in female vs. increase in male offspring of rats on HLA vs. LLA diet. On the other hand, CB₂ was significantly decreased in both male and female offspring. Elevated maternal LA consumption is thus shown to alter offspring ECS signaling in a sex-specific manner. Consistent with sexual dimorphism, prior work has shown sex-specific changes in ECS signaling in male offspring exposed to a high fat diet, including increased expression of DAGL-α and decreased FAAH in adipose tissue.
(Engeli et al., 2014), changes not seen in females. Further, activation of CB2 in females may involve the hormone estrogen, which protects against inflammation, further supporting sex-specific modulation of the ECS (Cao et al., 2019). These findings indicate that, prior to birth, elevated maternal LA consumption depresses CB2 transcription (thus potentially cannabinoid signaling), while other elements of the ECS are expressed in a sex-specific manner. These expression patterns may have functional consequences later in life. In contrast to these changes, there was a significant elevation in CB2 mRNA expression in maternal rats fed high LA diet during pregnancy. The CB2 receptor has been demonstrated to be cardioprotective in non-pregnant females, with up-regulation decreasing risk of cardiovascular diseases (Carmody et al., 2015). In further support of a beneficial role for CB2, activation of the receptor increases production of anti-inflammatory proteins (Cetrullo et al., 2012). The increase in mothers may thus be of value, however the decline in CB2 mRNA in E20 offspring may be detrimental. Whether the select up-regulation in PN40 males vs. females might lead to sexually dimorphic outcomes in later life awaits further study.

7.5 **Offspring cardiovascular changes in response to a maternal LA consumption.**

Maternal LA consumption significantly affected offspring cardiovascular function in adolescence. The extent of physiological differences also varies between male and female rats, again highlighting the importance of sex. Stress alters cardiomyocytes causing metabolic and intracellular signal transduction changes (Lakatta, Sollott, & Pepe, 2001). Changes to cardiomyocytes can be characterised by size/structure, membrane composition, reduced homeostasis and altered function (Pepe & McLennan, 2002). To assess heart physiology and function the Langendorff model was employed. The balloon method of LV pressure assessment is a widely employed technique for measurement of isovolumetric LV performance. The balloon is inflated to a physiologically relevant diastolic pressure, and LV pressures are recorded via a pressure transducer for continual assessment of ventricular performance
(Reichelt et al., 2009). Age of rats studied here is carefully controlled and effects of sex specifically addressed, reducing variances in contractility and stress responses of the heart (Reichelt et al., 2009). The model also permits assessment of coronary function.

**Coronary function.** Coronary hyperaemic responses to brief occlusion were studied, with peak hyperaemic flow (post occlusion), and percentage of debt repayment (relative to pre-occlusion flow rate) assessed as measures of vascular function or reactivity. The current study showed there were significant interaction changes in peak hyperaemic flow following 20 and maternal and interaction changes in the 30 second occlusions with LA diet in female groups. The results for percentage debt repayment suggest greatest repayments in LL vs. other groups, which is statistically significant for LLF vs. LHF (after 20 second occlusion) and an effect for maternal and offspring diet. This data suggests impaired flow repayment in both female and male rats fed on high LA diet, to the point where there is essentially zero in LLF groups. The multigroup comparisons and variance suggest further experimentation (increased n value) may improve statistical power to expose these effects. Prior work supports reductions in coronary reflow with ageing that may promote dysfunction and injury (Reichelt et al., 2009; Willems, Zatta, Holmgren, Ashton, & Headrick, 2005). There are no prior studies of maternal PUFA intake on offspring coronary phenotype. However, maternal fatty acid intake have been shown to influence the PUFA profile of cardiac tissue in different species (Lamontagne-Kam, Chalil, Aristizabal Henao, Hogenhout, & Stark, 2018). Concentrations of docosahexaenoic acid are reduced in maternal liver, adipose, and heart in rats fed high-fat diets without docosahexaenoic acid throughout pregnancy, which may have functional relevance (Nettleton & Salem, 2019).

**Cardiac contractile function.** Interestingly, we observed a left shift in the ventricular EDP PVR in the HHF group, supporting higher passive stiffness in this group. The basis of such changes is likely directly myocardial in origin - for example LV EDP shifts do not appear
related to vascular dysfunction or reflow (Gupta, Ratcliffe, Fallert, Edmunds, & Bogen, 1994). Ventricular remodelling could contribute, although data indicate that at this age there are no obvious signs of hypertrophy (heart and heart: body weights unchanged). Nonetheless, data suggests there may be a pattern of remodelling emerging (with relevant mRNA changes at PN40), in conjunction with a trend for diastolic stiffness and decreased contractility with elevated maternal dietary LA. Remodelling includes changes in the organization of membrane components, and LA is known to influence these responses, for example via phospholipases disruptive to membrane function (Mann & Bersten, 1987). Further, increased activation of $K^+$ channels contribute to coronary vasodilation leading to cardiac ischemia (Berwick et al., 2010). Exposure to LA has shown to increase stimulatory adenosine receptors modulating adenosine and coronary reactive hyperaemia function (Murphy & Byczko, 1992). Production of nitric oxide (NO) is vital for regulating cardiac function via vascular-dependent and independent outcomes (Massion, Feron, Dessy, & Balligand, 2003). NO also has direct effects on contractility, excitation-contraction coupling, mitochondrial respiration and signalling (Massion et al., 2003). Inflamed cardiomyocytes delivering excess NO leads to heart failure and dysfunction in cardiac output (House et al., 2015).

The shift in EDP PVR is supported by a lower ventricular volume generating a 15 mmHg diastolic pressure in HHF hearts, consistent with impaired compliance in female hearts. However, the slope of the EDP PVR also suggests an improved diastolic compliance in HLM and HHM hearts. Thus, sex is an important factor, with distinct (potentially opposing) changes in females and males. With respect to systolic function, no parameters were statistically different between groups. However, a consistent trend to declining slope of the systolic PVR with increased LA, coupled with a trend towards increasing volume required to generate 100 mmHg systolic pressure support a negative influence of LA diet on cardiac contractility. While the considerable variances demand further analyses and animals to boost statistical power, data
support a trend to worsened performance with elevations in LA. Advanced ageing is associated with decreased LV systolic with delayed relaxation and increased stiffness in diastolic in male rats (Pacher et al., 2004). Young aged rat hearts are more resilient and here we observe a notable change occurring at a very early age. Ageing of the arterial system and structure with changes of the LV myocyte size, decreased production of myocytes, remodelling, fibrosis and diastolic filling is reduced (Pepe & Lakatta, 2005). While not a factor in our studies, as the rats have not been aged, the changes resemble a pro-aging effect (Reichelt et al., 2009).

In our study, at PN40 we thus observe a significant effect of maternal LA consumption intake on cardiovascular function that is sex-specific. Previous studies have demonstrated that as female rats lose their protective action of oestrogen at menopause, there is an increased risk to cardiovascular disease (Reichelt et al., 2009). Female rats indicated myocardial hypertrophy with the presence of estrogen in both normotensive and hypertensive groups (Wallen, Cserti, Belanger, & Wittnich, 2000). Research for impaired coronary vascular reserve in hypertensive rats, found an association with ventricular dysfunction (Alfaro, Schaible, Malhotra, Yipintsoi, & Scheuer, 1983). Further, it resulted in lower coronary flows, effluent lactate/pyruvate ratios, increased LVEDP (ml) levels and limited ATPase activity (Alfaro et al., 1983). Our study shows the LHF group increased the hyperaemic response of coronary flow at both 20 and 30 second occlusions. Prior observations in obese mothers showed cardiac systolic and diastolic dysfunction in her offspring despite the offspring eating a low-fat diet to counteract cardiovascular disease (Blackmore et al., 2014). Today’s Western diet is contributing to cardiovascular dysfunction and shown to decrease the protective mechanisms of female sex on cardiovascular function (Druart et al., 2015). The Langendorff model reveals changes in intrinsic vascular and cardiac function in PN40 hearts as a result of maternal intake of LA.
Changes in coronary and contractile function are associated with changes in cardiac gene expression. Observations in the hearts of PN40 rats for metabolic and inflammatory genes showed DAGL-α and LepR mRNA expression increased in HLM compared to LHM hearts. The adipokine leptin is linked with cardiovascular dysfunction causing hypertension and cardiac complications (Hall, Harmancey, & Stec, 2015). On the other hand, LepR activity is protective in terms of lipid accumulation in cardiomyocytes (Hall, Maready, Hall, & Stec, 2014). The current study identified an increased expression of LepR in HLM hearts, which suggests that leptin signalling may be modified, although whether circulating leptin concentrations in these rats is altered (eg. a fall leading to LepR upregulation) is not known. Nonetheless, this may contribute to altered cardiovascular phenotype observed. This warrants further investigation.

Mechanistically, changes in cardiovascular function observed here could also be influenced to some degree by shifts in hypertrophy/remodelling genes in PN40 hearts. In our study, COL1A, COL3A, NOTCH1, and NPPA were increased in HLM compared with LHM rats. NPPB female showed an offspring effect. A clear limitation is that the study is insufficiently powered, however despite this, there are unlikely to be changes in the genes of interest that can provide a mechanism for the changes in function observed. Endothelial NOTCH1 signalling has a major role regulating fatty acid transportation via endothelium to reduce angiogenesis in the ageing heart and inhibition of this receptor would create vascular remodelling and poor heart development (Jabs et al., 2018). Recent research showed that inhibition of endothelial NOTCH1 in mice fed on high fat diets, is associated with vascular inflammation and atherosclerosis (Briot et al., 2015). Other genes changes support this, with an increased mRNA expression of NPPA indicating enhanced inflammation in cardiomyocytes, increased cytokines and oxidative stress (Geng et al., 2019). These early gene changes, and emerging shifts in cardiovascular functionality with high maternal LA
consumption (including sex-specific changes) may well evolve over time, with future studies of more mature animals suggested to identify the later consequences of these early changes.
Chapter 8: Summary

This thesis summarises comprehensive information characterising molecular to phenotypic effects of a high LA maternal diet in both the mother and in terms of fetal and offspring development and cardiac function. LA is vital for fetal heart development. LA is metabolised into a number of fatty acid modulators that regulate the endocannabinoid system (ECS) and are key for normal metabolic homeostasis. Exposure to high levels of LA in vitro and in vivo revealed significant cardiovascular changes. The aims of this study were to investigate if elevated maternal LA consumption during pregnancy alters: 1) the maternal microbiome; 2) maternal cardiomyocyte viability, respiration and the cardiac ECS; 3) the fetal cardiac ECS at embryo (E) day 20; and 4) cardiovascular development, function and the cardiac ECS in offspring at postnatal (PN) day 40. Overall, the study showed that there was a change in the maternal microbiome with LA intake, and that an elevated maternal LA consumption increased CB2 expression in maternal hearts. In her offspring, quite different ECS changes were apparent in a sex-specific manner. We further investigated cardiovascular development/function in adolescent rats. Maternal LA consumption led to a decline in coronary flow repayment during reactive hyperaemia in female hearts (LHF compared with LLF group), and a trend to declining coronary flow repayment in HLM and HHM groups. These data support sex-specific impairment of coronary reactivity with a high maternal LA consumption. Altered coronary reactivity may predispose to coronary dysfunction and disease in later life. An increased myocardial diastolic stiffness was also observed in HHF hearts (an effect not evident in males) vs. increased diastolic compliance in HLM and HHM (vs. LLM and LHM) hearts. Thus, there is evidence of sexually dimorphic effects of LA on cardiovascular function in developing hearts, with changes suggesting a potentially greater impact and cardiometabolic risk in female vs male offspring. Further, a high maternal LA consumption altered genes
responsible for endocannabinoid and cardiac function in HLM compared to LHM group. No changes in genes were observed in the females. Collectively, these changes provide further evidence of a sexually dimorphic effect between the males and females. In brief, the maternal diet high in LA, reflecting changes in Western diets, significantly alters the maternal microbiome and cardiac ECS signalling, and induces differing effects on cardiovascular function and gene expression in male vs. female offspring. The more overt changes in females, and their nature, suggest that female offspring born of mothers consuming a diet high in LA may be at a greater risk of developing cardiovascular dysfunction or disease later in life than male offspring, though this will require further study across the lifespan of such offspring. Either way, data highlight the potential importance of maternal LA consumption in governing offspring cardiovascular phenotype and health.
Chapter 9: Future Directions

Given the increasing prevalence of cardiovascular disease in children exposed to a Western diet, we need to better understand the specific effects of a high LA diet across the lifespan, so that sound research findings can inform advice and our understanding regarding the risk of such diet during pregnancy. Women of childbearing age have increased their consumption of foods high in LA and there is evidence of association between abnormalities such as diabetes, cardiovascular disease and hypertension with maternal nutrition. While not directly investigated here, but the importance of the ratio of n-6 and n-3 prior to conception is a potentially important factor and area for research. Optimising fetal development outcomes and creating a positive impact for perinatal health is imperative.

An Australian national household survey showed 35% of pregnancies involve mistimed, unintended and unwanted children (Yeates, Elder, & Grover, 2019). Therefore, there is a significant proportion of women of child bearing age who may potentially have been exposed to poor maternal diets, including excessive LA, and are unaware of the effects on their child. Informing women of child bearing age, prior to pregnancy, of the importance of healthy eating should be encouraged to maintain the health of both mother and offspring. Understanding the mechanistic elements of dietary determination of offspring cardiovascular phenotype and function is important in limiting cardiovascular disease.

We have demonstrated there are gene expression changes in male offspring (between LH and HL groups), and these genetic changes as in response to the pregnancy and post-weaning diets in males. This suggests the genes are influenced in utero and ex utero, and in some cases a combination effect. E20 showed significant altered gene expression of ECS in our publication (Appendix B) and a future goal is to investigate the PN180 (adult rat) to
ascertain how early changes reported here evolve over time to influence cardiovascular phenotype and health at later stages. At this time, investigations to characterise the cardiovascular phenotype and the expression of endocannabinoid and cardiac function genes, can test if changes continue into later life.

A key research endeavour is further investigation into shifts in circulating fatty acid, cytokine and leptin, similar to our previous study (Shrestha, Cuffe, Holland, Bulmer, et al., 2019). This will confirm whether the changes observed in the mothers and her offspring at E20 are maintained at PN40, and whether the change in diet (i.e. HL and LH vs LL and HH), resolves any potential deficits.

Consumption of HLA diet prior to pregnancy alters gut microbiota composition, as does pregnancy in rats consuming a control LLA diet. A reduction in microbiome biodiversity is associated with poor physiological outcomes. Further investigation is needed to mechanistically understand microbiome functions in the context of this model. There is fragmentary understanding on the effects of changes in microbiome diversity and its direct actions on the host, with this research in its infancy (Fricker, Podlesny, & Fricke, 2019).

Interesting and distinct changes observed in female and male offspring warrant further investigation to understand underlying mechanisms that affect heart function in a sexually dimorphic manner. Our collaborators have also demonstrated that during adolescence, exercise reverses programmed cardiac disease risk in a different model (Wadley, Chen, Lip, Fisher, & Aldred, 2016). Therefore, further studies to investigate the effects of exercise or other lifestyle intervention in reversing cardiovascular risk and abnormalities is also warranted.

Our study has a number of limitations. First, the maternal rats experienced stress, and through their protective behaviour (early pregnancy: PN1 to PN7) they consumed offspring which delayed the progress of this study. Consequently, some analyses are underpowered. The
influences of this type of maternal stress itself are also worthy of study. The time limitation of
the Masters program itself also provides constraints in terms of the practical study of pregnancy
and offspring development. Further rats in the PN40 cohort are required to generate sufficient
data for publication. Finally, we have identified the need for further studies in humans to
establish a link between adolescent heart disease and maternal LA consumption prior to and
during pregnancy.
References


Appendices

Appendix A

Conference Publications and Contributions


2. 3 Minute Thesis presentation 23rd July 2019 (2nd place) School of Medicine, Griffith University Gold Coast.


Publications

1. Nirajan Shrestha, Simone Sleep, James SM Cuffe, Olivia J Holland, Andrew J McAinch, Marloes D Nitert, Deanne H Hryciw. Pregnancy and diet-related changes in the maternal gut microbiota following exposure to an elevated linoleic acid diet. Am J Physiol Endocrinol Metab. [Published]


3. Nirajan Shrestha, Simone L Sleep, James SM Cuffe, Olivia J Holland, Anthony V Perkins1, Suk Yu Yau3,4, Andrew J McAinch, Deanne H Hryciw. Role of Omega-6 Fatty Acids In Fetal Programming. Clinical and Experimental Pharmacology and Physiology. [Published]
Appendix B

Publication

Journal of Developmental Origins of Health and Disease

www.cambridge.org/doh

Brief Report

Cite this article: Siege L, Shrestha K, Cuffe JMH, Holland GI, Headrick JP, McKinlay A, and Hryckow DJ. The effect of high maternal linoleic acid on endocannabinoid signalling in rodent hearts. Journal of Developmental Origins of Health and Disease doi:10.1017/S2040114413000013

Received: 22 July 2013
Revised: 28 October 2013
Accepted: 23 October 2013

Keywords:
Endocannabinoids; heart; linoleic acid

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

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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 fetal development. We investigated the effects of elevated LA in Hpc2 cardiomyoblasts in vitro and of a high linoleic acid (HLaA, 6.21%) diet during pregnancy in maternal and offspring hearts. Hpc2 cell viability was reduced following LA exposure at concentrations between 300 and 1000 μM. HLaA diet decreased cannabinoid receptor type 2 (CB2) mRNA expression in fetal hearts from both sexes. However, HLaA diet increased CB1 expression in maternal hearts. The mRNA expression of fatty acid oxidase (FAO) in fetal hearts was higher in females than in males irrespective of diet and N-acetyl 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 CB1 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 sunflower), 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 25:1.1 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.2 Elevated concentrations of LA are also known to be pro-inflammatory and pro-oxidative.3-5 In Australia, LA consumption has increased to three times the recommended daily intake between 1991 and 2009.6 This increase mirrors other Western societies, with LA availability in the USA diet increasing by ~160% over the same period.7 Women of childbearing age are also consuming increasing levels of LA before and during pregnancy. While LA is vital for foetal and postnatal development,8,9 we have demonstrated that elevated LA decreases placental cell viability, suggesting that in addition to LA’s role in maintaining normal cellular function, elevated concentrations may be detrimental.36

LA is transported by synaptotrophin-rich brush-border membranes of the placenta from mother to the foetus.10 Disruption in normal cellular function in the placenta is associated with pregnancy complications and affects foetal development.11 Research suggests that an optimal ratio of n-6 to n-3 of 1:1 or 2:1 is required for human health.12 In humans, plasma concentrations of LA vary and estimated to be 280–500 μM in one Western population.13 Maternal plasma LA concentrations of ~600–1200 μM14 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 impacts necrocardial development in offspring.15

Among potential mechanisms, LA modulates the endocannabinoid system (ECS), which modifies physiological functions and processes during early foetal development.16 The ECS comprises the endogenous ligands 2-arachidonoyl glycerol (2-AG) and anandamide (AEA), which can be generated via LA metabolism and act predominantly via the cannabinoid receptor 1 (CB1) and cannabinoid receptor 2 (CB2).17 The ligands 2-AG and AEA are metabolised by synthesizing and degradation enzymes.18 ECS signalling controls a variety of physiological processes, and abnormal ECS function has been identified in a variety of diseases,19,20 emerging research also suggests an important role for CB1 in protecting cardiomyocytes during disease; knock-out of CB1 exaggerates cardiac apoptosis, inflammation and dysfunction during ischemia-reperfusion.21 Emerging research suggests a role for ECS in development.22 The ECS may control development via CB1 and CB2, although our recent study in vivo suggests that elevated maternal LA may...
acclimated 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 (SSC01/17/ASC). After 1 week of acclima-
sation, rats were divided into low linoleic acid (LLA; n = 8) and high LA (HLA; n = 10) dietary groups, as previously described. Briefly, female rats were exposed to either LLA or HLA diet for 10 weeks before mating. The LLA diet contains =1.14% LA of energy, and the HLA diet contains =6.2% LA of energy (which is the average daily LA consumption in Australia, representative of Western society).1 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 through-
out during pregnancy. The gestation period for a rat is 12 to 13 days. Pregnant rats were sacrificed at day 20 (E20) as previously described.1 Left ventricular myocardiurn from mater-
nal hearts and whole fetal 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.

**Methods**

In vitro study

Rat H9C2 cardiomyoblasts were cultured in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin and maintained in a humidified incubator at 37°C with 5% CO2. LA and bovine serum albumin (BSA) complex was prepared in a 1:1 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 24 h before treatment with various concentrations of LA. BSA-RSA complex (12.5, 25, 100, 200, 400, 500, 1000 µg/mL) 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. A positive control (1% 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

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112 Not a typo; it seems to be a placeholder or an error. The correct number should be 112, not 111.
**Results**

LA decreases viability of HSC2 cells

Exposure of HSC2 cells to LA (between 300 µM and 1000 µM) 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 of heart weight in E20 fetuses from mothers consuming a high LA diet. However, mRNA expression of CbR, was significantly decreased in both male and female fetuses of HLA mothers (p < 0.05, Fig. 2a).

There were no changes in mRNA expression of GPR18, DAGL-α and DAGL-β, NAPE-PLD, EP2R or TNF-α in foetal hearts (Fig. 2a-2c). The mRNA expression of IL-6, IL-1, IL-2, IL-6, IFN-γ, IL-2, 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 HSC2 cell viability when exposed to 200 µM LA. Previous studies demonstrate that HSC2 cells exhibit hypertrophic responses, similar to primary cardiomyocytes, rendering them suitable for in vitro analysis of cardiomyocyte function. We have previously demonstrated that elevated LA induces reduction of trophoblast cells, 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, 30–500 µM palmitate reduces cell viability, an effect countered by n-3 polyunsaturated fatty acid. However, our earlier observation that elevated maternal LA does not alter heart weight suggests that its 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, a signalling capacity, this suggests potentially augmented ECS activity in male but not female hearts. Downregulation of FAAH promotes reactive oxygen species generation in the liver tissue, 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 the HLA group. This suggests potentially augmented AEA generation in male but not the female fetuses. A key outcome from this study is the need for sex-specific differences in the programming effects of LA, which may reflect in part sex-specific differences in the ECS, with a bias towards augmented AEA generation and 2-AG/AR signalling in males vs. female offspring, although cardiovascular CB1 was significantly increased by maternal LA in both sexes. Mechanistically, this sexual dimorphism may be influenced by hormones, as CB1 activity in females has been demonstrated to involve estrogen, which also protects against inflammation.

Of particular interest is a significant change in CB1 expression in maternal rats fed a high LA diet during pregnancy. CB1 receptor expression in non-pregnant females has been demonstrated to be cardio-protective, with up-regulation decreasing the risk of cardiovascular diseases. In further support of a beneficial role for CB1, the activation of the receptor increases the production of anti-inflammatory proteins. Although we have not investigated cardiovascular function in mothers consuming elevated LA diets and circulating concentrations of pro-inflammatory cytokines appear unaffected, the current data suggest that elevated LA during pregnancy may modify maternal cardiovascular function.
Fig. 3. Elevated maternal LA consumption alters endocannabinoid signaling in maternal and offspring hearts. Fetal 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 = 3–5 (LA) and n = 6–8 (HUE). In maternal hearts, n = 5–6 (LA) and n = 6–8 (HUE). Data are presented as means ± SEM. *p < 0.05.
This study provides further insight into the importance of LA during pregnancy in modulating key signaling 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 fetal 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.

Acknowledgments. This work was supported by the Allen Foundation (DAF II A.J.M.) and through the Australian Government’s Collaborative Research Network (CRN) program (A.J.M.). Scholarship funding is provided by Griffith University International Postgraduate Research Scholarship (GIPRS-N.S.) and Griffith University Postgraduate Research Scholarship (GIPRS-N.S.) and Griffith Medical Top Up Scholarship (N.S.).

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

References
